ITALIAN JOURNAL OF FOOD SCIENCE

Rivista italiana di scienza degli alimenti

IJFS

Volume XXIX
Number 4
2017

CHIRIOTTI EDITORI

ITALIAN JOURNAL OF FOOD SCIENCE

(RIVISTA ITALIANA DI SCIENZA DEGLI ALIMENTI) 2nd series

Founded By Paolo Fantozzi under the aegis of the University of Perugia
Official Journal of the Italian Society of Food Science and Technology
Società Italiana di Scienze e Tecnologie Alimentari (S.I.S.T.Al)
Initially supported in part by the Italian Research Council (CNR) - Rome - Italy
Recognised as a "Journal of High Cultural Level"
by the Ministry of Cultural Heritage - Rome - Italy

Publisher:
Alberto Chiriotti - Chiriotti Editori srl, Viale Rimembranza 60, I-10064 Pinerolo, Italy - Tel. +39 0121 393127 - Fax +39 0121 794480 e-mail: alberto@chiriottieditori.it - URL: www.chiriottieditori.it

Aim:

The Italian Journal of Food Science is an international journal publishing original, basic and applied papers, reviews, short communications, surveys and opinions on food science and technology with specific reference to the Mediterranean Region. Its expanded scope includes food production, food engineering, food management, food quality, shelf-life, consumer acceptance of foodstuffs. Food safety and nutrition, and environmental aspects of food processing.
Reviews and surveys on specific topics relevant to the advance of the Mediterranean food industry are particularly welcome.
Upon request and free of charge, announcements of congresses, presentations of research institutes, books and proceedings may also be published in a special "News" section.

Review Policy:

The Co-Editors with the Editor-in-Chief will select submitted manuscripts in relationship to their innovative and original content. Referees will be selected from the Advisory Board and/or qualified Italian or foreign scientists. Acceptance of a paper rests with the referees.

Frequency:

Quarterly - One volume in four issues. Guide for Authors is published in each number and annual indices are published in number 4 of each volume.

Impact Factor:

Impact Factor: 0.556 published in 2016 Journal of Citation Reports, Scopus CiteScore 2016: 0.84.
IJFS is abstracted/indexed in: Chemical Abstracts Service (USA); Foods Adlibra Publ. (USA);
Gialine - Ensia (F); Institut Information Sci. Acad. Sciences (Russia); Institute for Scientific Information; CurrentContents®/AB&ES; SciSearch® (USA-GB); Int. Food Information Service - IFIS (D); Int. Food Information Service - IFIS (UK); EBSCO Publishing; Index Copernicus Journal Master List (PL).

IJFS has a publication charge of € 350.00 each article.
Subscription Rate: IJFS is now an Open Access Journal and can be read and downloaded free of charge at http://www.ijfs.eu
Journal sponsorship is € 1,2010.00

REVIEW

THE ITALIAN ACADEMIC RESEARCH ON BEER: PAST, PRESENT and FUTURE

P. FANTOZZI
Department of Agricultural, Food and Environmental Sciences, University of Perugia, Via S. Costanzo, Perugia, Italy
*Corresponding author. Tel.: +39 0755857910; fax: +39 0755857939-5857943
E-mail addresses: paolo.fantozzi@ijfs.eu, paolo.fantozzi@unipg.it

ABSTRACT

In this review, the Author wants to share his experience and opinions after 45 years of academic research on beer. The first part of this review is dedicated to the over time situation of the Italian beer firms. The main core of the paper deals with the historical aspects of the Italian academic research on beer and its relationship with beer industries. The importance of the Italian research is also compared with the European situation. The University Teaching on this matter is also discussed. Finally it is suggested how to further upgrade the quality of the existing University research centres and laboratories.
Up to 194 references are presented.

Keywords: beer, malt, hop, *Humulus lupulus,* breweries, research

1. THE REFERENCE FRAMEWORK

The scenario of barley, malt and beer production in Italy has changed in a very important way in the last 20 years.
Italian farmers were always involved for producing barley basically for animal feeding (*Hordeum vulgare*) whith high content of starch and protein and in limited quantities as *Hordeum disticum* for selling to the Malting Industries (Agroalimentare Sud and Saplo) (1-2) for their utilization in malt production. In this case it was requested a limited presence of nitrogen (<1,6%) and beta-glucans.
The Italian average year production is ca. 1,2 million of Tons (= ca.300.000 Ha).
This reality did not changed until Craft Breweries started to enter in the market, often asking for special malts. The Malting Industries could not easily provide such malts in limited quantities because the size of their producing plants, obviously not satisfying all the different and small requests of Craft Brewers.
While many small Craft Brewers finally accepted to produce beer with the standard malts available on the Italian market, some other new born Craft Brewers were then obliged to search their malt with particular specific characteristics outside Italy, were the elasticity of some Malt Producers was more present. (3)
In force of a further increasing need, few large Craft Brewers decided to build their own Malting Plants (e.g.: Mastri Birrai Umbri (PG), Cobi (AN), La vallescura (PC) (4-6), for their internal utilization and also for specific external customers.
In the last 5 years this fact convinced several farmers to modify their crop rotation introducing barley for beer, indirectly answering to the increasing and continuous requests of Craft Brewers. This particular type of barley, in some particular Region, was also considered by the Regional Agricultural Officials as a real and possible alternative utilization to some traditional crops that appears to be not economical anymore.
In addition to that, the cultivation of hop *(Humulus lupulus* is also emerging, even if in small dimension.
While beer was traditionally considered an industrial product (so then under the control and jurisdiction of the Italian Ministry of Industry), these changes were finally recognized by the Italian Ministry of Agriculture. With its Ministerial Decree 212/2010 Beer was recognized to be an agricultural good, allowing all the products of the processing chain to have also access to Italian and EU agricultural advantages (PAC, FEASR, Horizon 2020, etc.).

2. THE ITALIAN BEER INDUSTRY SITUATION

Even if the presence of Beer Industry in Italy really started in 1829 (Wührer), in 1766 Mr. Lenz requested H.M. Empress of Austria authorization to produce Theresianer Beer in Trieste and in 1789 Mr. Ketter obtained from the Savoy State permission of producing beer in at that time Italian town of Nice (7).
Many important changes have occurred in the last 20 years, modifying deeply the Italian Beer Firms scenario. At the beginning, the majority of Italian beer Firms were owned by Italian families:

1829. Birra Wührer (fam. Wührer, Brescia)
1835. Birra Venezia (fam. Biliotti, Venezia)
1837. Birra Zimmermann (fam. Zimmermann, Aosta)
1837. Birrificio Rienza- Birra di Dobbiaco (fam. Reichholf, Dobbiaco)

1840. Birra Chiavenna (fam. G.Ritter, Chiavenna)
1845. Birra Bosio & Caratsch (famm. Bosio e Caratsch, Torino)
1846. Birra Peroni (fam. Peroni, Vigevano)
1846. Birra Menabrea,fam. G. Menabrea, Biella)
1848. Birra Perla Crova - Metzger (fam. Metzger,Torino)
1857. Birra Forst (fam. Wallnöfer e Franz Tappeiner, (Lagundo)
1858. Birra Bonino (fam. G. Bonino, Cuneo)
1859. Birra Moretti (fam. Moretti, Udine)
1865. Birra Dreher (fam. P- Revoltella, Trieste)
1877. Birrificio Angelo Poretti (fam. Poretti, Vedano Olona)
1879. Birra Orobia (fam. Von Wunster, Bergamo)
1893. Birra Livorno (famm. Del Moro e Di Giacomo, Chiavenna)
1897. Birra Pedavena (fam. Luciani, Canale dìAgurdo)
1905. Birra Busalla (famm. Ricchini e Poggi, Savignone)
1912. Birra Ichnusa (fam. Amsicora Capra, Cagliari)
1995. Birra Castello (Gruppo E.Verardo, S. Giorgio di Nogaro)
2000. Birra Theresianer (fam. M. Zanetti, Nervesa della Battaglia)

In addition, up to 1959, were existing and operating almost other 80 small Craft Breweries (8).
Historically, the first multinational corporation entrance in Italy happens in 1959, when Heineken acquired 5-10% of Dreher. In 1974 Dreher was then fully absorbed by Heineken.
Between 1982 and 2002, Carlberg progressively acquired the Poretti Group.
Today the opposite is true: all the industrial Breweries belong to Multinational Corporations (Heineken, Anheuser-Bush Inbev, Carlsberg, Asahi, SAB, etc.), absorbing almost all of the old family Firms, while only three of them still remain family owned (Castello, Forst, Theresianer).
In the mean time, we observed a massive growth of small-medium craft Breweries, almost reaching about a thousand, with a very large variability on their product quality and quantity. Their almost exhaustive list could be found in the Web Sites of Assobirra and Unionbirrai (9) (10).

3. THE ITALIAN ACADEMIC RESEARCH ON BEER

As far as I can go back in time, beer research was started and carried on by prof. C. Cantarelli, University of Perugia, in occasion of the 1965 Beer Monde Selection. In this occasion, the Institute od Food Science, University of Perugia, was part of the analytical selection of participating beers and started the interest on beer.
In 1970, was published the first academic paper on beer (11).
According to Scopus (12) the papers published in Italy by academic scientists on beer from 1971 up to 2017 were 194, divided as follows:

From 1971 to 1989
Only 2 papers published, 1 on alcohol risk on beverages, including beer, ad 1 on hop botanical characteristics.

From 1990 to 1999 :
22 papers, 8 of which in beer technology.

From 2000 to 2009:
64 papers, 26 of which in beer technology.

From 2010 to 2017.
106 paper on different topic, 93 of them on beer (Table 1).

For a more complete information, I listed in the Addenda A all the found references since 1971) (13).

Table 1. Papers published on beer in the Italian Universities (years 2010-2017).

University	# of Paper(*)
Perugia	34
Viterbo (Tuscia)	11
Piacenza (Cattolica)	11
Ancona (Marche)	7
Sassari	7
Salerno	5
Milan	4
Udine	4
Naples	3
Rome	3
Florence	2
Turin	1
Trieste	1
Total	**93**

(*) some papers may be contemporarily listed in different Universities (Co-Authors).

All these data do not take into account oral communications or posters in national or international Congresses, as suggested by the ANVUR-VQR (Italian Evaluation of Quality in Research) (14) because of their low scientific impact.
This 35 years analysis show a progressive increase in papers, but only in the last decade it has been seen an important amount of interests inside the Italian Universities.
Furthermore, if we compare papers from the top 20 publishing European Countries (only Universities), we may observe on Table 2 the last 8-years situation.
From this table, Italy appears to be third in ranking, indicating how the interest of Italian Academic world increased in the last 8 years on this matter.
An important point that needs be further explored, and not still done, should be the comparison between the sum of "citations" received worldwide by the Italian papers with the sum of "citations" received by other Country papers. In fact, these data will potentially show the true interest (… and indirectly the quality) of the Italian scientific research on beer.

Table 2. Distribution of paper on beer published in European Universities in the 2010-2017 period.

Country	# of papers	%
Germany	280	13,2
United Kingdom	202	9,6
Italy	**194**	**9,2**
Spain	191	9,0
France	98	4,6
Belgium	183	8,7
Czech Republic	188	8,9
Netherlands	131	6,2
Poland	31	1,5
Denmark	120	5,7
Sweden	115	5,4
Portugal	87	4,1
Ireland	73	3,4
Finland	54	2,6
Russian Federation	37	1,8
Switzerland	42	2,0
Romania	23	1,1
Austria	21	1,0
Bulgaria	34	1,5
Greece	10	0,5
Total	**2116**	**100,0**

4. THE ACADEMIC RELATIONSHIP WITH BREWERIES

Before 1995, only direct consultancies and assignments existed between single University scientists and Breweries. The quantity or quality of them is generally covered by privacy.
The first known public Italian academic joint research with Italian Breweries started in 1995, when Assobirra, the Italian Association of Malsters and Brewers, financed a strategic project on "Micronutrients on Beer", having two main consultants (University of Perugia and INN Rome, the Italian Institute of Nutrition). This Project ended in 1998.
From 1999 up to 2002, Assobirra was the Lead Partner of the MIUR-PNR Project on "Nutritional Characteristics of fermented beverages and Product innovation", totally devoted to demonstrate the antioxidant capacity of beer. This Project was carried on by the Consortium "BirraViva" formed by Peroni, Forst, Heineken, Poretti, Castello and Menabrea. Several Italian Research Centres and Scientists participated as Consultants (Universities of Milan, Perugia, Rome and INN, Rome).
Considering the outstanding obtained results of the project, Assobirra started a deep scientific cooperation with the University of Perugia that finally gave birth in 2003 to CERB (Centro di Eccellenza per la Ricerca sulla Birra - Italian Brewing Research Centre) which I directed from the foundation to my retirement in 2015.
During this period CERB had the unique possibility to observe all the research (and the related researchers) carried on in Italy in the beer sector. Moreover, my membership in the

EBC Brewing Science Group still provide also the possibility to compare the Italian and the European situation on academic research.
Within respect to the other Countries, I may observe that today in Italy there is a general lack of public founding for basic and applied public research and it is almost absent a fundamental research partnership with big beer Industries.
In fact, international Beer Corporations posses their own large research laboratories and, when there is a need of external assistance, they are used to discuss and get quick answers (no waiting for preliminary studies on the requested matter) with well-established and proactive European academic realities (e.g.: Notthingham, Ghent, Copenaghen, Louvain, Wheinstephan, Berlin) rather than emerging although qualified Italian realities (Perugia, Piacenza, Viterbo, Ancona, Sassari and Udine)
As a matter of fact, only the following three important Projects:
A) on Mycotoxins (A-MICO) (15),
B) on Cereals (CERSUOM) - both financed by the Ministry of Agriculture in the years 2008-2011 (16),
C) on Supply Chain GMO Traceability, financed by Assobirra - still outgoing- (17), were financed during the last 10 years.
Meanwhile, and mainly at regional level (Directive 2014/23/EU for financing POR-FESR 20114_20120 -PSR- Regional Developing Plans), small projects and consultancies are active between several University Departments and small Craft Breweries on different specific topics.

5. THE ITALIAN ACADEMIC TEACHING ON BEER

"Teaching" is an important part of the academic duties.
To my knowledge, while "Beer" is partially present in the Courses in Food Technology in many Italian Universities, only two Universities have official regular Courses on beer: Perugia and Udine.
During my stay at the University of Udine, I activated in 1981 the first Course in "Beer Technology" inside the Degree of Food Science and Technology at the local Department of Food Science and Technology.
At the beginning, this Course was exceptionally taugh by Dr. Zangrando and successively by Dr. Collavo, at that time respectively Plant Managers of Birra Moretti and Birra Pedavena.
Today the Course is under the responsibility of Prof. Buiatti, University of Udine.
In Perugia the official teaching of beer started on 2007 and the situation was progressively upgraded during the last 15 years.
In 2007 CERB started a 5 day "preparatory" internal Course ("How to Become a Brewmaster") that contributed effectively to the large increase in number, and often in quality, of Italian Craft Breweries.
This course reached this year the 20th edition and, in 8 years, received more than 350 students.
To enlarge the floor and to raise quality, the Department of Agricultural, Food and Environmental Sciences of University of Perugia created in 2009 a First Level Master Degree on "Brewing Technology" and in 2014 we enlarged the previous University Degree Course in Agro-Food Sciences and Technologies with an additional 3 years Curriculum in "Brewing Technologies". During my Professor tenure in Perugia I considered fundamental, as I did previously in Udine, to call for seminars every year specialists from the Italian Beer Companies in order to enrich, with their expertise, the students, allowing them to have a true contact with the industrial external reality, in addition to the internal

academic experience. In 2013 together with Assobirra and the Director of our Department in Perugia we formally agreed to have, every year, additional distance-learning Courses given by specialists from Beer Companies on applied beer technology. When I retired, I left this important tool to the Professors and Researchers of my Group, hoping they would understand how a continuous scientific exchange between industrial and academic people should be kept alive and not abandoned.

5. CONCLUSIONS

How to allow all "young" Italian academic Research Centres or Laboratories to upgrade and to reach the level of others well-established European labs? A brief list of priorities includes:
First: increasing their internal scientific force by hiring permanent and multitasking skilled expert scientists, in order to be contemporarily proactive for incoming requests,
Then: publishing qualified and peer-reviewed scientific researches in high level Journals rather than communications or posters in national or international Congresses,
Finally: being principal rather than side-Partners in international Projects and being ready in solving industrial needs.
To accomplish these priorities and to obtain a real upgrade in quality and external credibility, the Italian University Research Centres compulsory need to have availability of money, granted by European or Italian public Organizations or from the Beer Industries.
Consequently, the Universities must be professionally dedicated in Fund Raising.
They should not count on money coming from Teaching. In fact, while "Teaching" is an important mission and an academic duty because is a fundamental preparation of students for their future external position and careers, the real impact on research fund raising is minimal.
The net income from student taxes cannot allow its utilization for research project or contracts, being limited in quantity and in availability. The only limited positive point is a utilization of students as a partial support in the research projects when already in progress in the labs.
It is obvious that the major responsibility for a scientific growth falls to the involved Scientists, because funding comes only from their scientific credibility and from their international well-established reliability.
Of course, another alternative or parallel route for found rising is to focus on Craft Breweries, which continuously need a lot of help and advice from University Scientists. This choice can allow a safe but slower growth of the Italian University research on Beer.

ACKNOWLEDGEMENTS

During my long academic tenure as Professor at the University of Perugia, many top Executives and Managers of Assobirra, together with skilled Technologists of the Beer Industry (Carlsberg, Heineken, Inbev, Peroni, Saplo, Theresianer), participated as Teachers in my Courses and as Special Guests in particular Events, believing all in the idea of an Italian Academic School on Beer. Hoping I did not miss someone, I want to thank all of them for that. They are: Andrea Bagnolini, Flavio Boero, Michele Cason, Roberto Cavalli, Francesco Collavo, Leonardo Di Stefano, Leonardo Di Vincenzo, Pietro Fontana, Alberto Frausin, Harald Fuchs, Vittorio Gorza, Antonio Marra, Gennaro Marturano, Lorenzo Morena, Mauro Niada, Paolo Papetti, Rodolfo Peroni, Piero Perron, Daniele Rossi,

Raffaele Sbuelz, Fabio Scappaticci, Alessandro Senia, Luigi Serino, Filippo Terzaghi, Tullio Zangrando, Agata Zani, Giorgio Zasio.
Finally, from the Academic side, I want to sincerely thank Prof. Luigi Montanari, University of Sassari. He believed and helped me from the beginning, thereby allowing our Italian University "Beer Dream" to become a reality.

REFERENCES

1. http://italmalt.com/
2. http://saplo.it/
3. https://www.weyermann.de/
4. http://www.mastribirraiumbri.com/
5. http://cobibirragricola.it/
6. http://www.agrivallescura.it/
7. Zangrando T. 2017, personal communication
8. Zasio G. 2017, personal communications
9. http://www.assobirra.it/gli-associati/
10. http://unionbirrai.it/associati-unionbirrai/
11. Fantozzi P. 1970. Determinazione dell'acido citramalico nella birra. Birra e Malto 6:217.
12. Scopus.2017. https://www.scopus.com/search/form.uri?display=basic (Key Words utilized: "Beer", "Malt", "Hop (*Humulus lupulus)"*.
13. Addenda A. 2017 Italian Universities References on beer.
14. MIUR ANVUR-VQR. 2017: http://www.anvur.org/index.php?lang=it
15. MIPAAF. 2008-2011. Project: A-MICO. Studio pilota sul possibile impiego di anticorpi policlonali fissati a matrice inerte come mezzo innocuo di decontaminazione da presenza occasionale di MICOtossine nelle fasi preliminari dei processi di preparazione di bevande fermentate.
16. MIPAAF. 2012-2015. Project: CERSUOM - CEReali al SERvizio dell'UOMo: Funzionalità integrata dei prodotti e sostenibilità di filiera.
17. ASSOBIRRA. 2003. Ricerca e sperimentazione per la rintracciabilità di materie prime geneticamente modificate (semola di mais) nella filiera produttiva della birra in Italia (Audit OGM).

ADDENDA A. Italian Universities 194 references on beer from 1980 up to 2017

Accorsi C.A., Bandini M.M. and Forlani L. 1984. Societá botanica Italiana 80° Congresso sociale: Atlante farplacopalinologico - Contributi n. 2: "*Huhulus Lupulus*" L. Giornale Botanico Italiano 118:195.

Addolorato G., Leggio L., Ferrulli A., Gasbarrini G. and Gasbarrini A. 2008. Beer affects oxidative stress due to ethanol: A preclinical and clinical study. Beer in Health and Disease Prevention 1:459.

Addolorato G., Leggio L., Ojetti V., Capristo E., Gasbarrini G. and Gasbarrini A. 2008. Effects of short-term moderate alcohol administration on oxidative stress and nutritional status in healthy males. Appetite 50:50.

Alaniya M.D., Kavtaradze N.S., Skhirtladze A.V., Pizza C. and Piacente S. 2010. Flavonol glycoside from *humulus lupulus*. Chemistry of Natural Compounds 46:641.

Albanese L., Ciriminna R., Meneguzzo F. and Pagliaro M. 2017. Beer-brewing powered by controlled hydrodynamic cavitation: Theory and real-scale experiments. Journal of Cleaner Production 142:1457.

Allamani A., Pepe P., Baccini M., Massini G. and Voller F. 2014. Europe. An analysis of changes in the consumption of alcoholic beverages: The interaction among consumption, related harms, contextual factors and alcoholic beverage control policies, Substance Use and Misuse 49:1692.

Allamani A., Voller F., Pepe P., Baccini M., Massini G. and Cipriani F. Italy between drinking culture and control policies for alcoholic beverages. 2014. Substance Use and Misuse 49:1646.

Altieri A., Bosetti C., Gallus S., Franceschi S., Dal Maso L., Talamini R., Levi F., Negri E., Rodriguez T. and La Vecchia C. 2004. Wine, beer and spirits and risk of oral and pharyngeal cancer: A case-control study from Italy and Switzerland. Oral Oncology 40:904.

Andreotti G., Birmann B., De Roos A.J., Spinelli J., Cozen W., Camp N.J., Moysich K., Chiu B., Steplowski E., Krzystan J., Boffetta P., Benhaim-Luzon V., Brennan P., De Sanjosé S., Costas L., Costantini A.S., Miligi L., Cocco P., Becker N., Foretová L., Maynadié M., Nieters A., Staines A., Tricot G., Milliken K., Weisenburger D., Zheng T., Baris D. and Mark P. 2013. A pooled analysis of alcohol consumption and risk of multiple myeloma in the international multiple myeloma consortium. Cancer Epidemiology Biomarkers and Prevention 22:1620.

Andrisano-Ruggieri R., Crescenzo P., Ambrosio R., Pinto G. and Grieco F. 2016. Building therapeutic relationship in schizophrenic and alcohol-related disorder cases. Psychodynamic Practice 22:236.

Arfelli G. and Sartini E. Characterisation of brewpub beer carbohydrates using high performance anion exchange chromatography coupled with pulsed amperometric detection. 2014. Food Chemistry 142:152.

Asciutto R., Lugo A., Pacifici R., Colombo P., Rota M., La Vecchia C. and Gallus S. 2016. The particular story of Italians' relation with alcohol: Trends in individuals' consumption by age and beverage type. Alcohol and Alcoholism 51:347.

Azzini E., Intorre F., Venneria E., Foddai M.S., Toti E., Catasta G., Nardini M., Palomba L., Rossi L., Marconi O., Peretti G., Fantozzi P. and Maiani G. 2014. Effects of beer and lettuce (*Lactuca sativa*) consumption on antioxidant status in healthy volunteers: A small-scale crossover trial. Current Nutrition and Food Science 10:163.

Baiano A., Conte A. and Del Nobile M.A. Beer: Advances in processing and preservation. 2012. Recent Patents on Engineering 6:83.

Baiano A., Viggiani I., Terracone C., Romaniello R. and Del Nobile M.A. 2015. Phenolic content, physical and sensory properties of breads made with different types of barley wort. Journal of the Science of Food and Agriculture 95:2736.

Battistutta F., Buiatti S., Zenarola C. and Zironi R. 1994. Rapid analysis of free medium-chain fatty acids and related ethyl esters in beer using SPE and HRGC. Journal of High Resolution Chromatography 17:662.

Bedini S., Flamini G., Cosci F., Ascrizzi R., Benelli G. and Conti B. 2016. Cannabis sativa and Humulus lupulus essential oils as novel control tools against the invasive mosquito Aedes albopictus and fresh water snail Physella acuta. Industrial Crops and Products 85:318.

Bedini S., Flamini G., Girardi J., Cosci F. and Conti B. 2015. Not just for beer: evaluation of spent hops (*Humulus lupulus* L.) as a source of eco-friendly repellents for insect pests of stored foods. Journal of Pest Science 88:583.

Benedetti P., Salvi S., Giomo A., Van Deun R., Boselli E. and Frega N.G. 2016. Taste-active Components of Beers from Emmer Wheat (*Triticum dicoccum*) Malt. Scientia Agriculturae Bohemica 47:82.

Bertuzzi T., Rastelli S., Mulazzi A., Donadini G. and Pietri A. 2011. Mycotoxin occurrence in beer produced in several European countries. Food Control 22:2059.

Bevilacqua A., Petruzzi L., Casanova F.P. and Corbo M.R. 2015. Viability and Acidification by Promising Yeasts Intended as Potential Starter Cultures for Rice-based Beverages. Advance Journal of Food Science and Technology 9:326.

Biagi G., Fusaro I., Pezzi P. and Formigoni A. 2007. Effect of dietary supplementation with malt extracts on milk production. Italian Journal of Animal Science 6 SUPPL. 1:260.

Bosetti C., La Vecchia C., Negri E. and Franceschi S. 2000. Wine and other types of alcoholic beverages and the risk of esophageal cancer. European Journal of Clinical Nutrition 54:918.

Brandolini A., Landi P., Monfredini G. and Tano F. 2000. Variation among Andean races of maize for cold tolerance during heterotrophic and early autotrophic growth. Euphytica 111:33.

Bravi E., Benedetti P., Marconi O. and Perretti G. 2014. Determination of free fatty acids in beer wort. Food Chemistry 151:374.

Bravi E., Marconi O., Perretti G. and Fantozzi P. 2012. Influence of barley variety and malting process on lipid content of malt. Food Chemistry 135:1112.

Bravi E., Marconi O., Sileoni V. and Perretti G. 2017. Determination of free fatty acids in beer. Food Chemistry 215:341.

Bravi E., Perretti G., Buzzini P., Sera R.D. and Fantozzi P. 2009. Technological steps and yeast biomass as factors affecting the lipid content of beer during the brewin a process. Journal of Agricultural and Food Chemistry 57:6279.

Bravi E., Perretti G., Montanari L., Favati F. and Fantozzi P. 2007. Supercritical fluid extraction for quality control in beer industry. Journal of Supercritical Fluids 42 3 SPEC. ISS. 342.

Buiatti S. and Zironi R. 1997. Technological innovation and improved beer quality. Alcologia 9:55.

Buiatti S. Beer composition: An overview. 2008. Beer in Health and Disease Prevention 17:213.

Buiatti S., Boschelle O., Mozzon M. and Battistutta F. 1995. Determination of biogenic amines in alcoholic and non-alcoholic beers by HPLC. Food Chemistry 52:199.

Canonico L., Comitini F. and Ciani M. 2014. Dominance and influence of selected Saccharomyces cerevisiae strains on the analytical profile of craft beer refermentation. Journal of the Institute of Brewing 120:262.

Canonico L., Comitini F. and Ciani M. 2017. Torulaspora delbrueckii contribution in mixed brewing fermentations with different Saccharomyces cerevisiae strains. International Journal of Food Microbiology 259:7.

Caporale G. and Monteleone E. 2004. Influence of information about manufacturing process on beer acceptability. Food Quality and Preference 15:271.

Cappa C. and Alamprese C. 2017. Brewer's spent grain valorization in fiber-enriched fresh egg pasta production: Modelling and optimization study. LWT - Food Science and Technology 82:464.

Carlucci G., Mazzeo P., Del Governatore S., Di Giacomo G. and Del Re G. 2001. Liquid chromatographic method for the analysis of tocopherols in malt sprouts with supercritical fluid extraction. Journal of Chromatography A 935:87.

Castellari M., Arfelli G., Riponi C., Carpi G. and Amati A. 2000. High hydrostatic pressure treatments for beer stabilization. Journal of Food Science 65:974.

Cataldi T.R.I., Campa C., Casella I.G. and Bufo S.A. 1999. Determination of maltitol, isomaltitol, and lactitol by high-pH anion- exchange chromatography with pulsed amperometric detection. Journal of Agricultural and Food Chemistry 47:157.

Cecchini F., Manzano M., Mandabi Y., Perelman E. and Marks R.S. 2012. Chemiluminescent DNA optical fibre sensor for Brettanomyces bruxellensis detection. Journal of Biotechnology 157:25.

Ceppi E.L.M. and Brenna O.V. 2010. Brewing with rice malt - a gluten-free alternative. Journal of the Institute of Brewing 116:275.

Ceppi E.L.M. and Brenna O.V. 2010. Experimental studies to obtain rice malt. Journal of Agricultural and Food Chemistry 58:7701.

Chiapparino E., Donini P., Reeves J., Tuberosa R. and O'Sullivan D.M. 2006. Distribution of β-amylase I haplotypes among European cultivated barleys. Molecular Breeding 18:341.

Cimini A. and Moresi M. 2014. Beer Clarification Using Ceramic Tubular Membranes. Food and Bioprocess Technology 7:2694.

Cimini A. and Moresi M. 2015. Novel cold sterilization and stabilization process applied to a pale lager. Journal of Food Engineering 145:1.

Cimini A. and Moresi M. 2015. Pale Lager Clarification Using Novel Ceramic Hollow-Fiber Membranes and CO2 Backflush Program. Food and Bioprocess Technology 8:2212.

Cimini A. and Moresi M. 2016. Assessment of the optimal operating conditions for pale lager clarification using novel ceramic hollow-fiber membranes. Journal of Food Engineering 173:132.

Cimini A. and Moresi M. 2016. Carbon footprint of a pale lager packed in different formats: Assessment and sensitivity analysis based on transparent data. Journal of Cleaner Production 112:4196.

Cimini A., De Francesco G. and Perretti G. 2017. Effect of crossflow microfiltration on the clarification and stability of beer from 100% low-β-glucan barley or malt. LWT - Food Science and Technology 86:61.

Cimini A., Marconi O. and Moresi M. 2013. Rough beer clarification by crossflow microfiltration in combination with enzymatic and/or centrifugal pretreatments. Chemical Engineering Transactions 32:1729.

Cimini A., Marconi O., Perretti G. and Moresi M. 2014. Novel Procedure for Lager Beer Clarification and Stabilization Using Sequential Enzymatic, Centrifugal, Regenerable PVPP and Crossflow Microfiltration Processing. Food and Bioprocess Technology 7:3156.

Cimini A., Pallottino F., Menesatti P. and Moresi M. 2016. A Low-Cost Image Analysis System to Upgrade the Rudin Beer Foam Head Retention Meter. Food and Bioprocess Technology 9:1587.

Coda R., Rizzello C.G., Trani A. and Gobbetti M. 2011. Manufacture and characterization of functional emmer beverages fermented by selected lactic acid bacteria. Food Microbiology 28:526.

Colussi I., Cortesi A., Gallo V. and Vitanza R. 2016. Biomethanization of Brewer's spent grain evaluated by application of the Anaerobic Digestion Model No.1. Environmental Progress and Sustainable Energy 35:1055.

Costanzo S., Di Castelnuovo A., Donati M.B., Iacoviello L. and De Gaetano G. 2011. Wine, beer or spirit drinking in relation to fatal and non-fatal cardiovascular events: A meta-analysis. European Journal of Epidemiology 26:833.

Crispo A., Talamini R., Gallus S., Negri E., Gallo A., Bosetti C., La Vecchia C., Dal Maso L. and Montella M. 2004. Alcohol and the risk of prostate cancer and benign prostatic hyperplasia. Urology 64:717

Curioni A., Pressi G., Furegon L. and Peruffo A.D.B. 1995. Major Proteins of Beer and Their Precursors in Barley: Electrophoretic and Immunological Studies. Journal of Agricultural and Food Chemistry 43:2620.

Curioni A., Santucci B., Cristaudo A., Canistraci C., Pietravalle M., Simonato B. and Giannattasio M. 1999. Urticaria from beer: An immediate hypersensitivity reaction due to a 10- kDa protein derived from barley. Clinical and Experimental Allergy 29:407.

De Francesco G., Freeman G., Lee E., Marconi O. and Perretti G. 2014. Effects of operating conditions during low-alcohol beer production by osmotic distillation. Journal of Agricultural and Food Chemistry 62:3279.

De Francesco G., Sileoni V., Marconi O. and Perretti G. 2015. Pilot plant production of low-alcohol beer by osmotic distillation. Journal of the American Society of Brewing Chemists 73:41.

De Francesco G., Turchetti B., Sileoni V., Marconi O. and Perretti G. 2015. Screening of new strains of Saccharomycodes ludwigii and Zygosaccharomyces rouxii to produce low-alcohol beer. Journal of the Institute of Brewing 121:113.

De Gaetano G., Di Castelnuovo A., Rotondo S., Iacoviello L. and Donati M.B. 2002. A meta-analysis of studies on wine and beer and cardiovascular disease. Pathophysiology of Haemostasis and Thrombosis 32 5-6:353.

de Meo B., Freeman G., Marconi O., Booer C., Perretti G. and Fantozzi P. 2011. Behaviour of malted cereals and pseudo-cereals for gluten-free beer production. Journal of the Institute of Brewing 117:541.

De Stefano A. and Montanari L. 1996. Minor components of beer: A review. Alcologia 8:43.

Di Castelnuovo A., Costanzo S., di Giuseppe R., de Gaetano G. and Iacoviello L. 2009. Alcohol consumption and cardiovascular risk: Mechanisms of action and epidemiologic perspectives. Future Cardiology 5:467.

Di Castelnuovo A., Costanzo S., Donati M.B., Iacoviello L. and de Gaetano G. 2007. Alcohol consumption and cardiovascular risk: An epidemiological perspective. Nutrition, Metabolism and Cardiovascular Diseases 17:561.

Di Castelnuovo A., Rotondo S., Iacoviello L., Donati M.B. and De Gaetano G. 2002. Meta-analysis of wine and beer consumption in relation to vascular risk. Circulation 105:2836.

Di Ghionno L., Marconi O., Lee E.G., Rice C.J., Sileoni V. and Perretti G. 2017. Gluten-Free Sources of Fermentable Extract: Effect of Temperature and Germination Time on Quality Attributes of Teff [Eragrostis tef (zucc.) Trotter] Malt and Wort. Journal of Agricultural and Food Chemistry 65:4777.

Di Ghionno L., Marconi O., Sileoni V., De Francesco G. and Perretti G. 2017. Brewing with prolyl endopeptidase from *Aspergillus niger*: the impact of enzymatic treatment on gluten levels, quality attributes and sensory profile. International Journal of Food Science and Technology 52:1367

Di Ghionno L., Sileoni V., Marconi O., De Francesco G. and Perretti G. 2017. Comparative study on quality attributes of gluten-free beer from malted and unmalted teff [Eragrostis tef (zucc.) trotter]. LWT - Food Science and Technology 84:746.

Di Giuseppe R., De Lorgeril M., Salen P., Laporte F., Di Castelnuovo A., Krogh V., Siani A., Arnout J., Cappuccio F.P., Van Dongen M., Donati M.B., De Gaetano G. and Iacoviello L. 2009. Alcohol consumption and n-3 polyunsaturated fatty acids in healthy men and women from 3 European populations. American Journal of Clinical Nutrition 89:354.

Donadini G. and Fumi M.D. 2010. Sensory mapping of beers on sale in the Italian market. Journal of Sensory Studies 25:19.

Donadini G. and Porretta S. 2017. Uncovering patterns of consumers' interest for beer: A case study with craft beers. Food Research International 91:183.

Donadini G., Fumi M.D. and de Faveri D.M. 2011. Sensory characteristics of Romanian, Polish, Albanian and former yugoslavian beers. Journal of the Institute of Brewing 117:507

Donadini G., Fumi M.D. and de Faveri M.D. 2011. How foam appearance influences the Italian Consumer's beer perception and preference. Journal of the Institute of Brewing 117:523.

Donadini G., Fumi M.D. and Lambri M. 2013. A preliminary study investigating consumer preference for cheese and beer pairings. Food Quality and Preference 30:217.

Donadini G., Fumi M.D. and Newby-Clark I.R. 2014. Consumers' preference and sensory profile of bottom fermented red beers of the Italian market. Food Research International 58:69.

Donadini G., Fumi M.D. and Newby-Clark I.R. 2015. An investigation of matches of bottom fermented red beers with cheeses. Food Research International 67:376.

Donadini G., Fumi M.D., Kordialik-Bogacka E., Maggi L., Lambri M. and Sckokai P. 2016. Consumer interest in specialty beers in three European markets. Food Research International 85:301.

Donadini G., Spalla S. and Beone G.M. 2008. Arsenic, cadmium and lead in beers from the Italian market. Journal of the Institute of Brewing 114. 283.

Donadini G., Spigno G., Fumi M.D. and Pastori R. 2008. Evaluation of ideal everyday Italian food and beer pairings with regular consumers and food and beverage experts. Journal of the Institute of Brewing 114:329.

Donato F., Monarca S., Chiesa R., Feretti D., Modolo M.A. and Nardi G. 1995. Patterns and covariates of alcohol drinking among high school students in 10 towns in Italy: a cross-sectional study. Drug and Alcohol Dependence 37:59.

Donato F., Pasquale L., Monarca S., Bonetti F., Chiesa R. and Nardi G. 1993. Alcohol drinking among adolescents from town and mountainous areas in North Italy. European Journal of Epidemiology 9:40.

Erzetti M., Marconi O., Bravi E., Perretti G., Montanari L. and Fantozzi P. 2009. Haccp in the malting and brewing production chain: Mycotoxin, nitrosamine and biogenic amine risks. Italian Journal of Food Science 21:211.

Fantozzi P., Montanari L., Mancini F., Gasbarrini A., Addolorato G., Simoncini M., Nardini M., Ghiselli A. and Scaccini C. 1998. *In vitro* antioxidant capacity from wort to beer. LWT - Food Science and Technology 31:221.

Ferrari P., Jenab M., Norat T., Moskal A., Slimani N., Olsen A., Tjønneland A., Overvad K., Jensen M.K., Boutron-Ruault M.-C., Clavel-Chapelon F., Morois S., Rohrmann S., Linseisen J., Boeing H., Bergmann M., Kontopoulou D., Trichopoulou A., Kassapa C., Masala G., Krogh V., Vineis P., Panico S., Tumino R., Van Gils C.H., Peeters P., Bueno-de-Mesquita H.B., Ocké M.C., Skeie G., Lund E., Agudo A., Ardanaz E., López D.C., Sanchez M.-J., Quirós J.R., Amiano P., Berglund G., Manjer J., Palmqvist R., Van Guelpen B., Allen N., Key T., Bingham S., Mazuir M., Boffetta P., Kaaks R. and Riboli E. 2007. Lifetime and baseline alcohol intake and risk of colon and rectal cancers in the European Prospective Investigation into Cancer and Nutrition (EPIC). International Journal of Cancer 121:2065.

Ferraroni M., Decarli A., Franceschi S. and La Vecchia C. 1998. Alcohol consumption and risk of breast cancer: A multicentre Italian case-control study. European Journal of Cancer 34:1403.

Ferraroni M., Decarli A., Franceschi S., La Vecchia C., Enard L., Negri E., Parpinel M. and Salvini S. 1996. Validity and reproducibility of alcohol consumption in Italy. International Journal of Epidemiology 25:775.

Fiotti N., Tubaro F., Altamura N., Grassi G., Moretti M., Dapas B., Farra R., Mizzau M., Guarnieri G., Buiatti S. and Giansante C. 2008. Alcohol reduces MMP-2 in humans and isolated smooth muscle cells. Alcohol 42:389.

Floridi S., Miniati E., Montanari L. and Fantozzi P. 2001. Carbohydrate determination in wort and beer by HPLC-ELSD. Monatsschrift fur Brauwissenschaft 54:209.

Floridi S., Montanari L., Marconi O. and Fantozzi P. 2003. Determination of free phenolic acids in wort and beer by coulometric array detection. Journal of Agricultural and Food Chemistry 51:1548.

Fontana M. and Buiatti S. 2008. Amino acids in beer. Beer in Health and Disease Prevention 3:273.

Fumi M.D., Galli R., Lambri M., Donadini G. and De Faveri D.M. 2011. Effect of full-scale brewing process on polyphenols in Italian all-malt and maize adjunct lager beers. Journal of Food Composition and Analysis 24:568.

Fumi M.D., Galli R., Lambri M., Donadini G. and de Faveri D.M. 2009. Impact of full-scale brewing processes on lager beer nitrogen compounds. European Food Research and Technology 230:209.

Galeone C., Malerba S., Rota M., Bagnardi V., Negri E., Scotti L., Bellocco R., Corrao G., Boffetta P., La Vecchia C. and Pelucchi C. 2013. A meta-analysis of alcohol consumption and the risk of brain tumours. Annals of Oncology 24:514.

Gallus S., Scotti L., Talamini R., Franceschi S., Dal Maso L., Negri E. and La Vecchia C. 2007. Reply to: Alcohol consumption and ovarian cancer risk in a population-based case-control study by Peterson et al. [1]. International Journal of Cancer 121:2578.

Garavello W., Bosetti C., Gallus S., Dal Maso L., Negri E., Franceschi S. and La Vecchia C. 2006. Type of alcoholic beverage and the risk of laryngeal cancer. European Journal of Cancer Prevention 15:69.

Garofalo C., Osimani A., Milanović V., Taccari M., Aquilanti L. and Clementi F. 2015. The occurrence of beer spoilage lactic acid bacteria in craft beer production. Journal of Food Science 80:M2845.

Gasbarrini A., Addolorato G., Simoncini M., Gasbarrini G., Fantozzi P., Mancini F., Montanari L., Nardini M., Ghiselli A. and Scaccini C. 1998. Beer affects oxidative stress due to ethanol in rats. Digestive Diseases and Sciences 43:1332.

Ghiselli A. and Serafini M. 1998. Diet and total plasma antioxidant capacity in humans. Medecine Biologie Environnement 26:171.

Ghiselli A., Natella F., Guidi A., Montanari L., Fantozzi P. and Scaccini C. 2000. Beer increases plasma antioxidant capacity in humans. Journal of Nutritional Biochemistry 11:76.

Ghiselli L., Tallarico R., Romagnoli S., De Acutis L. and Benedettelli S. 2015. Antioxidant and mineral element characterization in spontaneous hop (*Humulus lupulus* L.) in central Italy. Agrochimica 59:319.

Girotti S., Bolelli L., Fini F., Budini R. and Arfelli G. 2002. Chemiluminescent determination of antioxidant capacity of beverages. Italian Journal of Food Science 14:113.

Giusto C., Iacumin L., Comi G., Buiatti S. and Manzano M. 2006. PCR-TTGE and RAPD-PCR techniques to analyze *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis* isolated from craft beers. Journal of the Institute of Brewing 112:340.

Granieri L., Del Pino A.M., Mazzoni M., Mancinelli L., Proietti P., Perretti G. and Palmerini C.A. 2017. Chelating properties of beer: Implications on calcium homeostasis in PE/CA-PJ15 cells. Journal of Nutrition and Intermediary Metabolism 7:7.

La Vecchia C., Negri E., Franceschi S., Parazzini F., Gentile A. and Fasoli M. 1992. Alcohol and epithelial ovarian cancer. Journal of Clinical Epidemiology 45:1025.

Laido G., Barabaschi D., Tondelli A., Gianinetti A., Stanca A.M., Li Destri Nicosia O., Fonzo N.D.I., Francia E. and Pecchioni N. 2009. QTL alleles from a winter feed type can improve malting quality in barley. Plant Breeding 128:598.

Leonardi M., Skomra U., Agacka M., Stochmal A., Ambryszewska K.E., Oleszek W., Flamini G. and Pistelli L. 2013. Characterisation of four popular Polish hop cultivars. International Journal of Food Science and Technology 48:1770.

Liguori L., De Francesco G., Russo P., Albanese D., Perretti G. and Di Matteo M. 2015. Quality improvement of low alcohol craft beer produced by evaporative pertraction. Chemical Engineering Transactions 43:13.

Liguori L., De Francesco G., Russo P., Perretti G., Albanese D. and Di Matteo M. 2015. Production and characterization of alcohol-free beer by membrane process. Food and Bioproducts Processing 94. 158.

Liguori L., De Francesco G., Russo P., Perretti G., Albanese D. and Di Matteo M. 2016. Quality Attributes of Low-Alcohol Top-Fermented Beers Produced by Membrane Contactor. Food and Bioprocess Technology 9:191.

Lupinacci E., Meijerink J., Vincken J.-P., Gabriele B., Gruppen H. and Witkamp R.F. 2009. Xanthohumol from hop (*Humulus lupulus* L.) Is an efficient inhibitor of monocyte chemoattractant protein-1 and tumor necrosis factor-α release in LPS-Stimulated RAW 264.7 mouse macrophages and U937 human monocytes. Journal of Agricultural and Food Chemistry 57:7274.

Mack W.J., Preston-Martin S., Dal Maso L., Galanti R., Xiang M., Franceschi S., Hallquist A., Jin F., Kolonel L., La Vecchia C., Levi F., Linos A., Lund E., McTiernan A., Mabuchi K., Negri E., Wingren G. and Ron E. 2003. A pooled analysis of case-control studies of thyroid cancer: Cigarette smoking and consumption of alcohol, coffee, and tea. Cancer Causes and Control 14:773.

Maifreni M., Frigo F., Bartolomeoli I., Buiatti S., Picon S. and Marino M. 2015. Bacterial biofilm as a possible source of contamination in the microbrewery environment. Food Control 50:809.

Mancini F., Miniati E. and Montanari L. 2000. Determination of organic acid anions in Italian beers by a new HPLC method. Italian Journal of Food Science 12:443.

Manzano M., Cristina C., Bartolomeoli I., Buiatti S. and Comi G. 2005. Microbiological analyses of dry and slurry yeasts for brewing. Journal of the Institute of Brewing 111:203.

Manzano M., Iacumin L., Vendrame M., Cecchini F., Comi G. and Buiatti S. 2011. Craft beer microflora identification before and after a cleaning process. Journal of the Institute of Brewing 117:343.

Marconi O., Mayer H., Chiacchieroni F., Ricci E., Perretti G. and Fantozzi P. 2013. The influence of glumes on malting and brewing of hulled wheats. Journal of the American Society of Brewing Chemists 71:,41.

Marconi O., Rossi S., Galgano F., Sileoni V. and Perretti G. 2016. Influence of yeast strain, priming solution and temperature on beer bottle conditioning. Journal of the Science of Food and Agriculture 96:4106

Marconi O., Sileoni V., Sensidoni M., Amigo Rubio J.M., Perretti G. and Fantozzi P. 2011. Influence of barley variety, timing of nitrogen fertilisation and sunn pest infestation on malting and brewing. Journal of the Science of Food and Agriculture 91. 820.

Marconi O., Tomasi I., Dionisio L., Perretti G. and Fantozzi P. 2014. Effects of malting on molecular weight distribution and content of water-extractable β-glucans in barley. Food Research International 64:677.

Marongiu A., Zara G., Legras J.-L., Del Caro A., Mascia I., Fadda C. and Budroni M. 2015. Novel starters for old processes: use of Saccharomyces cerevisiae strains isolated from artisanal sourdough for craft beer production at a brewery scale. Journal of Industrial Microbiology and Biotechnology 42:85.

Marron M., Boffetta P., Møller H., Ahrens W., Pohlabeln H., Benhamou S., Bouchardy C., Lagiou P., Lagiou A., Slámová A., Schejbalová M., Merletti F., Richiardi L., Kjaerheim K., Agudo A., Castellsague X., Macfarlane T.V., Macfarlane G.J., Talamini R., Barzan L., Canova C., Simonato L., Biggs A.-M., Thomson P., Conway D.I., McKinney P.A., Znaor A., Healy C.M., McCartan B.E., Brennan P. and Hashibe M. 2012. Risk of upper aerodigestive tract cancer and type of alcoholic beverage: A European multicenter case-control study. European Journal of Epidemiology 27:499.

Marte L., Belloni P., Genorini E., Sileoni V., Perretti G., Montanari L. and Marconi O. 2009. Near-infrared reflectance models for the rapid prediction of quality of brewing raw materials. Journal of Agricultural and Food Chemistry 57:326.
Marte L., Floridi S., Marconi O. and Perretti G. 2009. Moisture content determination of colored malts. Italian Journal of Food Science 21. 529.

Martínez-Tomé M., Murcia M.A., Frega N., Ruggieri S., Jiménez A.M., Roses F. and Parras P. 2004. Evaluation of antioxidant capacity of cereal brans. Journal of Agricultural and Food Chemistry 52:4690.

Mascia I., Fadda C., Dostálek P., Karabín M., Zara G., Budroni M. and Del Caro A. 2015. Is it possible to create an innovative craft durum wheat beer with sourdough yeasts? A case study. Journal of the Institute of Brewing 121:283.

Mascia I., Fadda C., Dostálek P., Olšovská J. and Del Caro A. 2014. Preliminary characterization of an Italian craft durum wheat beer. Journal of the Institute of Brewing 120:495.

Mascia I., Fadda C., Karabín M., Dostálek P. and Del Caro A. 2016. Aging of craft durum wheat beer fermented with sourdough yeasts. LWT - Food Science and Technology 65:487.

Masotti P., Campisi B. and Bogoni P. 2016. Carbon footprint evaluation of an Italian micro-brewery. Procedia Environmental Science, Engineering and Management 3:119.

Matrai S., Casajuana C., Allamani A., Baccini M., Pepe P., Massini G. and Gual A. 2014. The relationships between the impact of alcoholic beverage control policies, selected contextual determinants, and alcohol drinking in Spain. Substance Use and Misuse 49:1665.

Mayer H., Ceccaroni D., Marconi O., Sileoni V., Perretti G. and Fantozzi P. 2016. Development of an all rice malt beer: A gluten free alternative. LWT - Food Science and Technology 67:67.

Mayer H., Marconi O., Floridi S., Montanari L. and Fantozzi P. 2003. Determination of Cu(II) in Beer by Derivative Potentiometric Stripping Analysis. Journal of the Institute of Brewing 109:332.

Mayer H., Marconi O., Perretti G., Sensidoni M. and Fantozzi P. 2011. Investigation of the suitability of hulled wheats for malting and brewing. Journal of the American Society of Brewing Chemists 69:116.

Mayer H., Marconi O., Regnicoli G.F., Perretti G. and Fantozzi P. 2014. Production of a saccharifying rice malt for brewing using different rice varieties and malting parameters. Journal of Agricultural and Food Chemistry 62:5369.

Mol H.G.J., MacDonald S.J., Anagnostopoulos C., Spanjer M., Bertuzzi T. and Pietri A. 2016. European survey on sterigmatocystin in cereals, cereals-based products, beer and nuts. World Mycotoxin journal 9:633.

Monarca S., Modolo M.A., Spiazzi R., Pasquale L., Nardi G., Donato F. and Brunelli L. 1991. Drinking habits among high school students in Perugia, Italy, in 1981 and 1988: Time trends and correlates. International Journal of the Addictions 26:1107.

Mongelli A., Rodolfi M., Ganino T., Marieschi M., Caligiani A., Dall'Asta C. and Bruni R. 2016. Are *Humulus lupulus* L. ecotypes and cultivars suitable for the cultivation of aromatic hop in Italy? A phytochemical approach. Industrial Crops and Products 83:693.

Mongelli A., Rodolfi M., Ganino T., Marieschi M., Dall'Asta C. and Bruni R. 2015. Italian hop germplasm: Characterization of wild *Humulus lupulus* L. genotypes from Northern Italy by means of phytochemical, morphological traits and multivariate data analysis. Industrial Crops and Products 70:16.

Montanari L., Floridi S., Marconi O., Tironzelli M. and Fantozzi P. 2005. Effect of mashing procedures on brewing. European Food Research and Technology 221 1-2:175.

Montanari L., Marconi O., Mayer H. and Fantozzi P. Production of alcohol-free beer. 2008. Beer in Health and Disease Prevention 8: 61.

Montanari L., Mayer H., Marconi O. and Fantozzi P. Minerals in beer. 2008. Beer in Health and Disease Prevention 3:359.

Mozzon M., Boselli E., Obiedziński M.W. and Frega N.G. 2015. Occurrence of biogenic amines in beers produced with malted organic Emmer wheat (*Triticum dicoccum*). Food Additives and Contaminants - Part A Chemistry and Analysis and Control and Exposure and Risk Assessment 32:756.

Nardini M. and Ghiselli A. 2004. Determination of free and bound phenolic acids in beer. Food Chemistry 84:137.

Nardini M., Natella F., Ghiselli A. and Scaccini C. 2008. The absorption and metabolism of phenolic acids from beer in man. Beer in Health and Disease Prevention 1:491.

Nardini M., Natella F., Scaccini C. and Ghiselli A. 2006. Phenolic acids from beer are absorbed and extensively metabolized in humans. Journal of Nutritional Biochemistry 17:14.

Passaghe P., Bertoli S., Tubaro F. and Buiatti S. 2015. Monitoring of some selected heavy metals throughout the brewing process of craft beers by inductively coupled plasma mass spectrometry. European Food Research and Technology 241:199.

Pavsler A. and Buiatti S. 2008. Lager beer. Beer in Health and Disease Prevention 31:43.

Pavsler A. and Buiatti S. 2008. Non-lager beer. Beer in Health and Disease Prevention 17:4.

Pelucchi C., Negri E., Franceschi S., Talamini R. and La Vecchia C. 2002. Alcohol drinking and bladder cancer. Journal of Clinical Epidemiology 55:637.

Perretti G., Floridi S., Turchetti B., Marconi O. and Fantozzi P. 2011. Quality control of malt: Turbidity problems of standard worts given by the presence of microbial cells. Journal of the Institute of Brewing 117:212.

Pezzica P., Rossi A. and Conti L. 1996. A study on the relationship between alcohol abuse and affective/anxious disorders. Alcoholism 32:155.

Picariello G., Bonomi F., Iametti S., Rasmussen P., Pepe C., Lilla S. and Ferranti P. 2011. Proteomic and peptidomic characterisation of beer: Immunological and technological implications. Food Chemistry 124:1718.

Picariello G., Mamone G., Cutignano A., Fontana A., Zurlo L., Addeo F. and Ferranti P. Proteomics and Peptidomics and and Immunogenic Potential of Wheat Beer (Weissbier). 2015. Journal of Agricultural and Food Chemistry 63:3579.

Picariello G., Mamone G., Nitride C., Addeo F., Camarca A., Vocca I., Gianfrani C. and Ferranti P. 2012. Shotgun proteome analysis of beer and the immunogenic potential of beer polypeptides. Journal of Proteomics 75:5872.

Picariello G., Nitride C., Mamone G., Iimure T., and Addeo F., and Ferranti P. 2013. Beer proteomics. Proteomics in Foods: Principles and Applications 399:4614.

Pietri A., Bertuzzi T., Agosti B. and Donadini G. 2010. Transfer of aflatoxin B1 and fumonisin B1 from naturally contaminated raw materials to beer during an industrial brewing process. Food Additives and Contaminants - Part A Chemistry and Analysis and Control and Exposure and Risk Assessment 27:1431.

Porretta S. and Donadini G. 2008. A preference study for no alcohol beer in Italy using quantitative concept analysis. Journal of the Institute of Brewing 114:315.

Pošćić F., Mattiello A., Fellet G., Miceli F. and Marchiol L. 2016. Effects of cerium and titanium oxide nanoparticles in soil on the nutrient composition of barley (*Hordeum vulgare* L.) kernels. International Journal of Environmental Research and Public Health 13:577.

Press G., Curioni A., Peruffo A.D.B. and Zamorani A. 1993. Effectiveness of the electroendosmotic preparative electrophoresis for the purification of all proteins and polypeptides from beer. Journal of the Institute of Brewing 99:63.

Ragozzino E., Faggioli F. and Cirillo A. 2008. Wild hop (*Humulus lupulus* L.) in Campania Region (southern Italy) is free of hop stunt viroid. Acta Horticulturae 780:71.

Ragozzino E., Faggioli F. and Cirillo A. 2008. Wild hop (*Humulus lupulus* L.) is free of any viroid in Campania Region (Southern Italy). Acta Horticulturae 781:547.

Rainieri S. 2008. The brewer's yeast genome: From its origins to our current knowledge. Beer in Health and Disease Prevention 89:101.

Reschiglian P., Zattoni A., Casolari S., Krumlova A., Budinska M. and Chmelík J. 2002. Size characterization of barley starch granules by gravitational field-flow fractionation: A rapid and low-cost method to assess the brewing capability of different strains. Annali di Chimica 92:457.

Rohrmann S., Linseisen J., Vrieling A., Boffetta P., Stolzenberg-Solomon R.Z., Lowenfels A.B., Jensen M.K., Overvad K., Olsen A., Tjonneland A., Boutron-Ruault M.-C., Clavel-Chapelon F., Fagherazzi G., Misirli G., Lagiou P., Trichopoulou A., Kaaks R., Bergmann M.M., Boeing H., Bingham S., Khaw K.-T., Allen N., Roddam A., Palli D., Pala V., Panico S., Tumino R., Vineis P., Peeters P.H.M., Hjartåker A., Lund E., Cornejo M.L.R., Agudo A., Arriola L., Sánchez M.-J., Tormo M.-J., Barricarte Gurrea A., Lindkvist B., Manjer J., Johansson I., Ye W., Slimani N., Duell E.J., Jenab M., Michaud D.S., Mouw T., Riboli E. and Bueno-De-Mesquita H.B. 2009. Ethanol intake and the risk of pancreatic cancer in the European prospective investigation into cancer and nutrition (EPIC). Cancer Causes and Control 20:785.

Rossi S., Sileoni V., Perretti G. and Marconi O. 2014. Characterization of the volatile profiles of beer using headspace solid-phase microextraction and gas chromatography-mass spectrometry. Journal of the Science of Food and Agriculture 94:919.

Rossini F., Loreti P., Provenzano M.E., De Santis D. and Ruggeri R. 2016. Agronomic performance and beer quality assessment of twenty hop cultivars grown in central Italy. Italian Journal of Agronomy 11:180.

Russo P., Liguori L., Albanese D., Crescitelli A. and Di Matteo M. 2013. Investigation of osmotic distillation technique for beer dealcoholization. Chemical Engineering Transactions 32:1735.

Santucci B., Cristaudo A., Cannistraci C., Curioni A. and Furegon L. and Giannattasio M. 1996. Urticaria from beer in 3 patients. Contact Dermatitis 34:368.

Sensidoni M., Marconi O., Perretti G., Freeman G. and Fantozzi P. 2011. Monitoring of beer filtration using photon correlation spectroscopy (PCS). Journal of the Institute of Brewing 117:639.

Sileoni V., Marconi O. and Perretti G. 2015. Near-infrared Spectroscopy in the Brewing Industry. Critical Reviews in Food Science and Nutrition 55:1771.

Sileoni V., Marconi O., Perretti G. and Fantozzi P. 2013. Evaluation of different validation strategies and long term effects in NIR calibration models. Food Chemistry 141:2639.

Sileoni V., Perretti G., Marte L., Marconi O. and Fantozzi P. 2010. Near-infrared spectroscopy for proficient quality evaluation of the malt and maize used for beer production. Journal of the Institute of Brewing 116:134.

Sileoni V., Van Den Berg F., Marconi O., Perretti G. and Fantozzi P. 2011. Internal and external validation strategies for the evaluation of long-term effects in NIR calibration models. Journal of Agricultural and Food Chemistry 59:1541.

Sluik D., Jankovic N., Hughes M., O'doherty M.G., Schöttker B., Drygas W., Rolandsson O., Männistö S., Ordóñez-Mena J.M., Ferrieres J., Bamia C., De Gaetano G., Kiefte-De Jong J.C., Franco O.H., Sluijs I., Spijkerman A.M.W., Sans S., Eriksson S., Kromhout D., Trichopoulou A., Wilsgaard T., Brenner H., Kuulasmaa K., Laatikainen T., Söderberg S., Iacoviello L., Boffetta P., Kee F. and Feskens E.J.M. 2017. Alcoholic beverage preference and diabetes incidence across Europe: The Consortium on Health and Ageing Network of Cohorts in Europe and the United States (CHANCES) project. European Journal of Clinical Nutrition 71:659.

Sovrano S., Buiatti S. and Anese M. 2006. Influence of malt browning degree on lipoxygenase activity. Food Chemistry 99:711.

Sovrano S., Buiatti S., Cossi S. and Anese M. 2010. Corrigendum to "Influence of malt browning degree on lipoxygenase activity", Food Chemistry 118:436.

Spence C. and Gallace A. Tasting shapes and words. 2011. Food Quality and Preference 22:290.

Stafisso A., Marconi O., Perretti G. and Fantozzi P. 2011. Determination of dimethyl sulphide in brewery samples by headspace gas chromatography mass spectrometry (HS-GC/MS). Italian Journal of Food Science 23:19.

Tavani A., Bertuzzi M., Negri E., Sorbara L. and La Vecchia C. 2002. Alcohol and smoking and coffee and risk of non-fatal acute myocardial infarction in Italy. European Journal of Epidemiology 17:1131.

Tavani A., Gallus S., Dal Maso L., Franceschi S., Montella M., Conti E. and La Vecchia C. 2001. Coffee and alcohol intake and risk of ovarian cancer: An Italian case-control study. Nutrition and Cancer 39:29.

Tavani A., Gallus S., La Vecchia C. and Franceschi S. 2001. Alcohol drinking and risk of non-Hodgkin's lymphoma. European Journal of Clinical Nutrition 55:824.

Tavani A., Negri E. and Vecchia C.L. 1994. Alcohol Consumption and Risk of Prostate Cancer. Nutrition and Cancer 21:25.

Tavani A., Pregnolato A., Negri E. and La Vecchia C. 1997. Alcohol consumption and risk of pancreatic cancer. Nutrition and Cancer 27:157.

Terracini B., Merletti F., Boffetta P., Ciccone G. and Mashberg A. 1989. Role of Tobacco and Alcoholic Beverages in the Etiology of Cancer of the Oral Cavity/Oropharynx in Torino and Italy. Cancer Research 49:4919.

Tomasi I., Marconi O., Sileoni V. and Perretti G. 2017. Validation of a high-performance size-exclusion chromatography method to determine and characterize β-glucans in beer wort using a triple-detector array. Food Chemistry 214:176.

Turati F., Gallus S., Tavani A., Tramacere I., Polesel J., Talamini R., Montella M., Scotti L., Franceshi S. and La Vecchia C. Alcohol and endometrial cancer risk: A case-control study and a meta-analysis. 2010. Cancer Causes and Control 21:1285.

Turati F., Garavello W., Tramacere I., Pelucchi C., Galeone C., Bagnardi V., Corrao G., Islami F., Fedirko V., Boffetta P., La Vecchia C. and Negri E. 2013. A meta-analysis of alcohol drinking and oral and pharyngeal cancers: Results from subgroup analyses. 2013. Alcohol and Alcoholism 48:107.

Vecchia C.L., Negri E., Franceschi S. and D'Avanzo B. 1993. Moderate Beer Consumption and the Risk of Colorectal Cancer. Nutrition and Cancer 19:303.

Vendrame M., Manzano M., Comi G., Bertrand J. and Iacumin L. 2014. Use of propidium monoazide for the enumeration of viable Brettanomyces bruxellensis in wine and beer by quantitative PCR. Food Microbiology 42:196.

Vitanza R., Cortesi A., Gallo V., Colussi I. and De Arana-Sarabia M.E. 2016. Biovalorization of brewery waste by applying anaerobic digestion. Chemical and Biochemical Engineering Quarterly 30:351.

Voller F., Kubicka L., Bloomfield K. and Allamani A. 2000. Drinking cultures and the position of women in nine European countries. Substance Abuse 21:231.

Woffenden H.M., Ames J.M., Chandra S., Anese M., Nicoli M.C. 2002. Effect of kilning on the antioxidant and prooxidant activities of pale malts. Journal of Agricultural and Food Chemistry 50:4925.

Zanoli P. and Zavatti M. 2008. Neuropharmacological activity of *Humulus Lupulus* L. Beer in Health and Disease Prevention 549:556.

PAPER

PRELIMINARY DATA ON VOLATILE COMPOSITION OF OLIVE FRUITS OF CV. "SIMONA" AND POSSIBLE RELATIONSHIP TO RESISTANCE TO FLY OVIPOSITION

M. BONONI* and F. TATEO
Department of Agricultural and Environmental Science, University of Milan, Via Celoria, 2, Milan, Italy
*Corresponding author. Tel.: Tel: +39 0250316538
E-mail address: monica.bononi@unimi.it

ABSTRACT

By characterizing the volatile compounds of the olive cultivar "Simona" from southern Italy (Apulia region), we identified a way to analyze characteristics possibly linked to this olive's well-known resistance to fly oviposition. The pool of volatile compounds in the unripe and ripe fruits was identified, and even if the relative amounts of these compounds tended to vary with ripening, the fly repellent action appeared to be related to the sesquiterpenes such as α-copaene, cycloisosativene, α-muurolene, β-cubebene and hydrocarbons such as (*E*)-2-dodecene, undecane, tridecane, and 3-methyl undecane.
In agreement with the concept that the collective pool of volatile substances can enhance olfactory pleasure/repulsion more than would the effects of a single compound, the pool of volatile compounds identified in this paper may be among the possible characteristic mixtures with repellent action against *Bactrocera oleae*.
The selection of volatile compounds made by the cuticle and responsible of the headspace quality surrounding the whole olive fruit is also demonstrated to be markedly different from the headspace volatile compounds produced by the extracted oil.

Keywords: cv. Simona, olive fruits, olive oil, HS-SPME-GC/MS, *Bactrocera oleae*

1. INTRODUCTION

Olive growing in the Apulia region (southern Italy) has led to a specific varietal population selected through centuries. About 50 varieties have been developed, most of low agronomic interest. The major crops are limited to no more than about 15 widely grown cultivars, whereas some were abandoned for low productivity, despite their specific sensory and nutritional properties. Today, the promotion of local products is a common marketing trend, and the defined sensory characteristics represent the greatest attraction to consumers.

In recent years, some authors have noted that temperature can influence the acidic composition of olive oils in each cultivar and that various cultivars respond differently to different environmental conditions (FIORINO *et al*., 2003a; FIORINO *et al*., 2003b; PANNELLI, 2006).

Recently, a very old cultivar, "Simona," long grown in southern Italy, in the Apulia region, and particularly in the southeast of the province of Bari, in the territories of the towns of Castellana Grotte, Conversano, Monopoli, and Polignano, has been reconsidered for quality characteristics. GODINI *et al*. (2002) described the essential phenological and morphological traits of this "Simona" cultivar, which produces yellow oil and is slightly fruited, with fruits that are small, ellipsoidal, pruinose, and black violet at maturity. The origin is unknown, but presumably it is native to the Apulia region.

The main defect of this olive is the low oil output (GODINI *et al*., 2002): farmers identify the mean oil extraction value at 12%, *i.e*., about 20% less than the other cultivars. However, until 1960, the cv. "Simona" represented about 33% of the olive cultivation in the region, probably because the resulting oil preserves its sensory characteristics for more than one year (TATEO and BONONI, 2016).

The cv. "Simona" has not been well described. LOMBARDO *et al*. (2008), studying the triacylglycerol composition of 188 Italian cultivars from 2001 to 2005, observed an extreme variability of percentage of each fatty acid, making each cultivar peculiar in its triacylglycerol composition. For example, in the same year 2003, the percentage of oleic acid varied from a minimum of 49.8% (cv. "Orbetana") to 78.2% (cv. "Simona"), while the percentage of linoleic acid ranged from 3.9% (cv. "Simona") to 23.9% (cv. "Racioppella"). The paper confirmed the great sensitivity of olives to climatic changes.

The "Simona" cultivar shows good resistance to the fly *Bactrocera oleae*. Studies on the influence of oviposition have been carried out for some species of pest flies (FOSTER and HARRIS, 1997), but for "Simona" cultivars, no studies have addressed how the olive fruit affects susceptibility to *B. oleae*. The "Simona" cultivar is particularly resistant to *B. oleae* and its oil is of lower acidity than other oils produced in the same growing area from different cultivars (for example, "Oliastra", "Pasola"). Therefore, these characteristics justify the studies of the "Simona" olive fruit to discover the parameters that influence its resistance to *B. oleae* attack.

For plants, volatile organic compounds are largely recognized as having roles in reproduction, tritrophic interactions, below ground defense, and abiotic stress (DUDAREVA *et al*., 2006; PARÈ and TUMLINSON, 1999; TAMIRU *et al*., 2011; UNSICKER *et al*., 2009; WU and BALDWIN, 2010). Moreover, *B. oleae* appears to demonstrate cultivar preference in attacking specific olive cultivars (BURRACK and ZALOM, 2008; GONÇALVES *et al*., 2012; IANNOTTA *et al*., 2007; NAVROZIDIS *et al*., 2007). Other authors believe that oviposition is conditioned from host selection based on chemical, physical, and molecular features (CORRADO *et al*., 2012; KOMBARGI *et al*., 1998; IMPERATO *et al*., 2012; MALHEIRO *et al*., 2016; NEUENSCHWANDER *et al*., 1985; RIZZO *et al*., 2012; SPADAFORA *et al*., 2008). The amount of sesquiterpenes has been correlated to growth of olives; for example, in the case of cv. "Serrana," SPME-GC-MS analyses have

indicated an increase in α-copaene while the green fruit grows followed by a decrease related to fruit ripening (DE ALFONSO *et al*., 2014). On the other hand, a cultivar effect for sesquiterpenes and terpenes has been observed; for example, in cv. "Cobrançosa", the relative proportion of these compounds increased during olive maturation (MALHEIRO *et al*., 2015). The same authors cited the cv. "Verdeal Transmontana" as the most susceptible and the cv. "Cobrançosa" as the least susceptible to olive fly oviposition and affirmed that the susceptibility differences could be ascribed to olive volatile content and composition. Thus, the volatile composition of olives may depend on the olive cultivar and be influenced by olive maturation. Other authors (BURRACK and ZALOM, 2008; GONÇALVES *et al*., 2012) also support the view that a combination of several factors may affect the ovipositional preference of *B. oleae*.
Considering that cv. "Simona" shows a particularly resistance to *B. oleae,* the aim of this work was the determination of volatile compounds released from the whole unripe and ripe olives harvested in an area of Castellana Grotte (Bari, Italy). Moreover, the determination of volatile compounds in the oil obtained has been carried out in order to distinguish the selection of volatiles effected by the cuticle of whole fruits.
We determined that the volatile compounds surrounding the whole olive fruits were represented by sesquiterpenes and hydrocarbons, while the volatile compound composition of the olive oil was characterized by aldehydes, alcohols, esters.
The volatile compounds selected by the cuticle may represent a possible natural adaptation useful to reduce the susceptibility to olive fly attack.
This preliminary study was focused on the headspace characterization of whole olive fruit of cv. Simona which shows a particularly high resistant to fly attach.

2. MATERIALS AND METHODS

2.1 Description of the geographical area

We evaluated the volatile compounds found in whole unripe and ripe olives harvested in an area of Castellana Grotte (latitude and longitude 40.86907314710559 and 17.1785811334848, respectively). In this area (2.4 ha), owned by the "Agriculture" foundation, 170 "Simona" olives are grown with other cultivars (e.g., "Oliastra", "Coratina", and "Pasola") in lower quantity. The olive production period covered in this study was October 2015 to January 2016.

2.2. Evaluation of fly infestation level and maturity index

Following MALHEIRO *et al.* (2016), to assess the fly infestation level of cv. "Simona" in comparison with two other cv. "Pasola" and "Oliastra" growing in the same area, 20 random hand-picked fruits were collected in early November 2015 from five olive trees per cultivar, and the resulting 100 fruits per cultivar were inspected for signs of infestation in a stereo microscope SMZ-168 TH – 1:6.7 Zoom Ratio (Motic-Italy) equipped with a PrimoCam 5 HD Zeiss digital camera (TiEsseLab-Italy).
For calculation of the maturation index, we followed the method described by HERMOSO *et al*. (2001), separating 100 olive fruits (20 fruits per tree) in levels from 0 to 7, and examining the epidermis and pulp color. If the epidermis was green, the fruit was classified as "0". Fruits showing epidermis of yellowish-green were classified as "1". Fruits showing red spots on less than half fruit were classified as "2"; epidermis red or purple on more than half the fruit led to classification "3", and fruits with black epidermis and white pulp were classified as "4". The level "5" was due to fruits with black epidermis

and purple on less than half the pulp and the level "6" was due to fruits with black epidermis and purple on more than half the pulp but not reaching the stone. The level "7" is due to fruits with black epidermis and whole pulp purple, reaching the stone. Using the letters a, b, etc., to identify the number of fruits and the seven levels cited before, the maturation index is calculated as follows: MI = (a x 0 + b x 1 + c x 2 + d x 3 + e x 4 + f x 5 + g x 6 + h x 7)/100.

2.3. Oil extraction process

Within 12 h of fruit harvest, oil was extracted using an "Oliomatic 150" system, equipped with a hammer crusher, a vertical malaxator, and a two phase decanter (Enoagricola Rossi - Perugia), operated at 25 °C and with 3% of water. The oil was not filtered after the extraction.

2.4. Olive oil samples

Oil was produced by olives of cv. "Simona" at a maturation index between 3.5 and 5.5 and stored in a 5 litre dark glass bottle at 12 - 15 °C for 4 days before the HS-SPME analysis.

2.5 HS-SPME analysis of whole olive fruits and oil and GC-MS conditions

For olive fruits, three aliquots of 80 ripe or unripe whole olive fruits were placed in a Pyrex 250 mL round media bottle sealed with a screwcap equipped with a septum.
For olive oil samples of cv. "Simona", three 100 g aliquots were weighed and placed into the same type of media bottle.
For whole fruit and oil, the sealed bottle was placed in a thermostatic bath at 40 °C, with a magnetic stirrer for the oil aliquots. After 1 h, an SPME fiber covered with 2 cm of DVB/CAR/PDMS, 50/30 mm (divinylbenzene/carboxen/polydimethylsiloxane) (Supelco, Milan, Italy) was inserted through the septum and left in the headspace for 5 h. With respect to the repeatability of the results, the headspace equilibrium, from to the cuticle of whole olive fruit, was reached in the time adopted.
The volatiles absorbed by the fiber were thermally desorbed in the injection port of a GC/MS (gas chromatography/mass spectrometer) for 10 min at 220 °C in a gas chromatograph with a split/splitless injection port. The injector was operated in split mode (1:5). Before sampling, the fiber was reconditioned for 15 min in the GC injection port at 240 °C, and blank runs were carried out before every analysis. The efficiency of the fiber was periodically verified by monitoring the signal of the internal standard.
Gas chromatography/mass spectrometry (GC/MS) analyses were performed in a Shimadzu 2010 gas chromatograph coupled to a Shimadzu QP-2010 MSD quadrupole mass spectrometer (Shimadzu, Milan, Italy). The gas chromatograph was equipped with a Supelcowax -10 30 m × 0.25 mm column, 0.25 mm film thickness (Supelco – Italy). The operating conditions for the GC/MS were: helium flow 1.0 ml min^{-1}, and oven temperature 40 °C for 5 min, increased to 240 °C at a rate of 3 °C min^{-1}. The temperature of the ion source was 200 °C, the electron energy was 70 eV, and the interface temperature was 240 °C. Mass spectra were acquired over the mass range 40–300 a.m.u.

2.6. Volatile compound identification and quantification

Volatile compounds in the headspace of the whole olive fruits and the olive oil were identified by matching their mass spectra with the reference mass spectra of a private library (Tateo-Bononi Oils) and the NIST 147 library.

To verify that the fly repellent action produced by headspace of whole olive fruits was maintained by the same pool of volatile compounds identified in the unripe and ripe stage, we compared data expressed as normalized area counts from whole unripe and ripe olive fruits. These data were compared to the volatile composition of the oil extracted from cv. "Simona", expressed as normalized area counts. All data were normalized to the most represented volatile compound (a-copaene in unripe whole olive fruits).

2.7. Statistical analysis

All analyses were performed in triplicate and the results are reported as mean values. The significance of the differences between data of whole olive fruits and olive oil was confirmed using Student's *t* test. A p-value < 0.05 was set as a statistical threshold for significance.

3. RESULTS

In the year 2015, the fly infestation measured with the method described in 2.2, and concerning our samples collected in the limited area described in 2.1, was not particularly high: the fruits of cv. "Simona" showed no oviposition sites or exit holes, while the two other cv. showed 25% ("Oliastra") and 18% ("Pasola") *B. oleae* infestation. These data represent the mean of three replicate measurements. So, we can affirm that the values reported in this paper correspond to a meaningfully different behavior of cv. "Simona" with respect of other cultivars considered for comparison from the same area.
Considering the headspace for whole olive fruit samples at two maturation index values 0.0 and 5.2, as shown in Fig. 1 and in Table 1, we deduced that the fly repellent action was maintained by the same pool of volatile compounds, even though their amounts were naturally modified by ripening. These compounds are sesquiterpenes and hydrocarbons, the two most highly represented classes of volatiles in the headspace of olive whole fruits. Data reported also showed the substantially different composition between the headspace produced by whole olive fruits (ripe or unripe) and by olive oil. Indeed, the olive oil headspace contained a very important fraction of more volatile oxygen-containing compounds such as alcohols ((*Z*)-3-hexenol, (*E*)-2-hexenol, octanol), aldehydes (hexanal, (*Z*)-3-hexenal, (*E*)-2-hexenal, nonanal), and some other typical compounds such as 5-ethyl-2(5*H*)-furanone. In contrast, the volatile compounds released through the cuticle of the whole unripe olive fruits were almost exclusively represented by sesquiterpenes (a-copaene, cycloisosativene, α-muurolene, and β-cubebene, in decreasing order of representation) and hydrocarbons ((*E*)-2-dodecene, undecane, tridecane, and 3-methyl undecane, in decreasing order). This result suggests that the effective fly repellent action is related to the pool of these volatile compounds.
The data obtained demonstrate that GC/MS identification of the volatiles starting from whole fruits is fundamental for evaluating volatile compounds responsible for the fly repelling activity.
However, the volatile composition reported in Fig. 1 and derived from whole fruit of cv. "Simona" appeared to be specific and not comparable to volatile composition of the olive oil, and the characterized pool of sesquiterpenes and hydrocarbons could be identified as repellent to *B. oleae*.

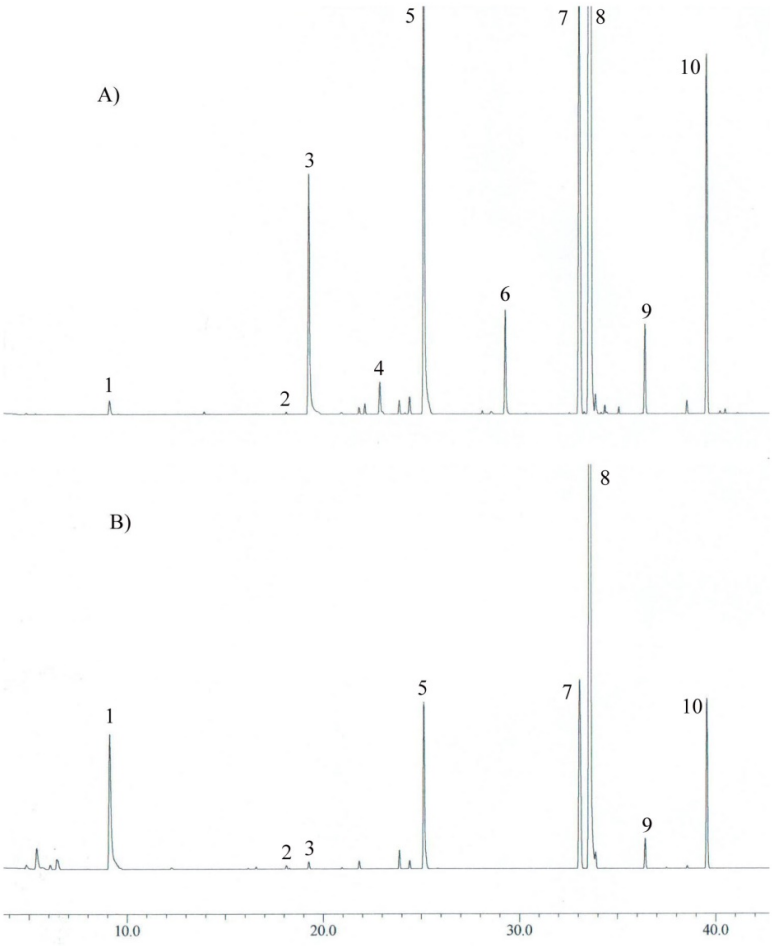


Figure 1. HS-SPME-GC/MS trace of unripe A) and ripe B) whole olive fruits cv. "Simona." Peak identification: 1) ethyl-2-methyl butyrate; 2) (*Z*)-3-hexenyl acetate; 3) undecane; 4) 3-methyl undecane; 5) (*E*)-2-dodecene; 6) tridecane; 7) cycloisosativene; 8) α-copaene; 9) β-cubebene; 10) α-muurolene.

Table 1. Volatile compound identification and comparison of normalized for whole unripe (M.I. = 0.0) and ripe (M.I. = 5.2) olive fruits and olive oil (M.I. 3.5 - 5.5). Values are normalized to the most represented volatile compound (α-copaene in unripe whole olive fruits). Data reported demonstrate the substantially different composition between the headspace produced by whole olive fruits (ripe or unripe) and by olive oil.

cv. "Simona"	unripe fruits	CV%	ripe fruits	CV%	oil	CV%
hexanal					0.2	0.01
(*Z*)-3-hexenal					2.8	0.03
(*E*)-2-hexenal					33.6	0.48
(*Z*)-3-hexenol					7.0	0.34
(*E*)-2-hexenol					2.9	0.03
(*Z*)-3-hexenyl acetate			0.1	0.04	10.3	0.42
D^3carene					1.8	0.02
5-ethyl-2(5*H*)-furanone					2.3	0.03
ethyl-2-methyl butyrate	0.4	0.02	5.3	0.62		
octanol					0.2	0.01
nonanal					5.0	0.28
3-methyl undecane *(H)*	0.7	0.05				
undecane *(H)*	6.7	0.92	0.2	0.07		
dodecane *(H)*					4.6	0.25
(*E*)-2-dodecene *(H)*	15.9	0.74	4.3	0.73	26.3	0.51
tridecane *(H)*	2.5	0.03				
phenyl ethyl alcohol					1.2	0.02
2,2-dimethyl-3-heptanone					6.7	0.13
cycloisosativene *(S)*	15.0	0.81	6.7	0.74	5.3	0.20
α-copaene *(S)*	100.0	1.20	47.0	0.82	45.8	0.40
α-muurolene *(S)*	8.8	0.58	4.1	0.05	17.0	0.32
α-farnesene *(S)*					46.4	0.42
β-cubebene *(S)*	2.1	0.07	0.7	0.08		
Total	152.1		68.4		122.4	
Σ sesquiterpenes *(S)*	125.9		58.5		114.5	
Σ hydrocarbons *(H)*	25.8		4.5		30.9	

(S)= sesquiterpenes
(H) = hydrocarbons

4. CONCLUSIONS

The resistance activity shown by cv. "Simona" to fly oviposition was evidenced in a well-defined growing area of 2.4 ha as described in 2.1 where other cultivars were present but did not show the same repellent activity against *B. oleae*.
Considering that as of today the factors associated with repelling a fly attack have not been identified with certainty, we think that a concrete approach to investigate the fly repellent action could be the identification of the pool of active specific volatile compounds starting from whole fruit of other cultivars showing resistance to fly attach, following the method suggested in this paper. The analysis of whole fruit versus oil of cv. "Simona" yielded two very different patterns of volatiles: the data demonstrate that the

whole fruit does not convey into the headspace the same qualitative and quantitative abundance of volatile compounds produced in the pulp, and by selective permeation, only selected volatile substances that are truly active in repelling the flies are conveyed into the headspace.
This identification of a mixture of substances that can reduce the negative consequences of *B. oleae* attack is in accord with a fundamental principle of flavoring science and technology: no single volatile compound can always produce the best olfactory response, and often, various volatile substances collectively produce a more effective olfactory stimulus. Thus, it is reasonable to suggest that a synergistic effect of a mix of various flavorings might underlie the repellence of *B. oleae*, and in the case of cv. "Simona," the active substances able to prevent the fly attack seem to be a pool of sesquiterpenes and hydrocarbons and no other volatile oxygen compounds.
This proposed line of research is compatible with the accepted conclusion of many authors that fly repellence is related to the specificity of the cultivar.
This analytical approach, based on the evidence of selective permeation of volatile compounds through the cuticle of the whole olive fruit, could be used to evaluate the composition of headspace surrounding the whole olive fruits for other cultivars traditionally recognized as not being susceptible to infection or as being more resistant to fly attack.

ACKNOWLEDGEMENTS

The authors are grateful to "Foundation Agriculture Onlus" (Castellana-Bari) for providing us the olive fruits and oil from cv. "Simona" used in this study.

REFERENCES

Burrack H.J. and Zalom F.G. 2008. Olive fruit fly (Diptera:Tephritidae) ovipositional preference and larval performance in several commercially important olive varieties in California. Ecol. Behav. 101:750-758.

Corrado G., Alagna F., Rocco M., Renzone G., Varricchio P., Coppola V., Coppola M., Garonna A., Baldoni L., Scaloni A. and Rao R. 2012. Molecular interactions between the olive and the fruit fly *Bactrocera oleae*. BMC Plant. Biol. 12:1-17.

de Alfonso I., Vacas S. and Primo J. 2014. Role of α-copaene in the susceptibility of olive fruits to *Bactrocera olea* (Rossi). J. Agric. Food Chem. 62:11976-11979.

Dudareva N., Negre F., Nagegowda D.A. and Orlova L. 2006. Plant volatiles: recent advances and future perspective. Crit. Rev. Plant. Sci. 25:417-440.

Fiorino P. and Ottanelli A. 2003a. Growth and evolution of oil accumulation in olive fruits (*Olea europaea* L.) cv Frantoio in inland Tuscany. International Symposium on the Olive Tree and Environment - Chania, Crete, 1-3 October, 122-126.

Fiorino P. and Ottanelli A. 2003b. Crescita ed inolizione dei frutti di cv di olivo (*Olea europaea*) nella Toscana interna e possibili influenze dell'ambiente nella determinazione dei trigliceridi. National Congress "Germoplasma olivicolo e tipicità dell'olio" - Perugia, Italy, 5 December, 158-164.

Foster S.P. and Harris M.O. 1997. Behavioral manipulation methods for insect pest-management. Annu. Rev. Entomol. 42:123-146.

Godini A., Mariani R., Pacifico A. and Palasciano M. 2002. Repeatedly reported but hitherto undescribed olive cultivars native to southern Italy. Acta Hort. 586:201-204.

Gonçalves M.F., Malheiro R., Casal S., Torres L. and Pereira J. A. 2012. Influence of fruit traits on oviposition preference of the olive fly, *Bactrocera oleae* (Rossi) (Diptera:Tephritidae), on three Portuguese olive varieties (Cobrançosa, Madural and Verdeal Transmontana). Sci. Hortic. 145:127-135.

Hermoso M., Uceda M., Frias L. and Beltran G. 2001. In: Barranco, D., Fernández-Escobar, R. Rallo, L., Editors. El cultivo de olivo. Madrid; Ediciones Mundi-Prensa 2001. Pp. 153-170.

Kombargi W.S., Michelakis S.E. and Petrakis C.A. 1998. Effect of olive surface waxes on oviposition by *Bactrocera oleae* (Diptera:Tephritidae). J. Econ. Entomol. 91:993-998.

Iannotta N., Noce, M.E., Ripa V., Scalercio S. and Vizzarri V. 2007. Assessment of susceptibility of olive cultivars to the *Bactrocera oleae* (Gmelin, 1790) and *Camarosporium dalmaticum* (Thüm.) Zachos & Tzav.-Klon. attacks in Calabria (Southern Italy). J. Environ. Sci. Health. Part B 42:789-793.

Imperato A., Corrado G., Alagna F., Varricchio P., Baldoni L. and Rao R. 2012. Olive molecular response to attack of *Bactrocera oleae*: identification of up-regulated genes in infested olive fruits. Acta Hortic. 929:125-128.

Lombardo N., Marone E., Alessandrino M., Godino G., Madeo A. and Fiorino P. 2008. Influence of growing season temperatures in the fatty acids (FAs) of triacilglycerols (TAGs) composition in Italian cultivars of *Olea europaea*. Adv. Hort. Sci. 22:49-53.

Malheiro R., Casal S., Cunha S.C., Baptista P. and Pereira J.A. 2015. Olive volatiles from Portuguese cultivars Cobrançosa, madural and Verdeal Transmontana:role in oviposition preference of Bactrocera oleae (Rossi) (Diptera:Tephritidae). Plos One 10:1-15.

Malheiro R., Casal S., Cunha S.C., Baptista P. and Pereira J.A. 2016. Identification of leaf volatiles from olive (*Olea europaea*) and their possible role in the ovipositional preferences of olive fly, *Bactrocera oleae* (Rossi) (Diptera:Tephritidae). Phytochemistry 121:11-19.

Navrozidis E., Zartaloudis Z., Thomidis T., Karagiannidis N., Roubos K. and Michailides Z. 2007. Effect of soil plowing and fertilization on the susceptibility of four olive cultivars to the insect *Bactrocera oleae* and the fungi *Sphaeropsis dalmatica* and *Spilocaea oleagina*. Phytopatology 35:429-432.

Neuenschwander P., Michelakis S., Holloway P. and Berchtol W. 1985. Factors affecting the susceptibility of different olive varieties to attack by *Dacus oleae* (Gmel.) (Dipt. Tephritidae). J. Appl. Entomol. 100:174-188.

Pannelli G. 2006. Variabilità genetica ed ambientale nella composizione in acidi grassi, steroli ed alcoli in olio di germoplasma ovicolo umbro. Italus Hortus 13:293-297.

Paré P.W. and Tumlinson J.H. 1999. Plant volatiles as a defense against insect herbivores. Plant Physiol. 121:325-331.

Rizzo R., Caleca V. and Lombardo A. 2012. Relation of fruit color, elongation, hardness, and volume to the infestation of olive cultivars by the olive fruit fly, *Bactrocera oleae*. Entomol. Exp. Appl.145:15-22.

Spadafora A., Mazzuca S., Chiappetta F.F., Parise A. and Innocenti A.M. 2008. Oleuropein-specific-b-glucosidase activity marks the early response of olive fruits (*Olea europaea*) to mimed insect attack. Agric. Sci. China 7:703-712.

Tamiru A., Bruce J.A.T., Woodcock C.M., Caulfield J.C., Midega C.A.O., Ogol C.K.P.O., Mayon P., Birkett M.A., Pickett J.A. and Khan Z.R. 2011. Maize landraces recruit egg and larval parasitoids in response to egg deposition by a herbivore. Ecol. Lett. 14:1075-1083.

Tateo F. and Bononi M. 2016. L'olivo "Simona" e la sperimentazione congiunta fra i "Laboratori di Ricerche Analitiche e Tecnologiche su Alimenti e Ambiente – UniMi" e la "Fondazione Agriculture Onlus" di Castellana Grotte. URL (http://www.foodchem.it/Home/Index/2425/#/l-olivo-simona-e-la-sperimentazione-congiunta-fra-i-laboratori-di-ricerche-analitiche-e-tecnologiche-sugli-alimenti-unimi-e-la-fondazione-agriculture-) (most recent access date 02 May 2017).

Unsicker S.B., Kunert G. and Gershenson J. 2009. Protective perfumers:the role of vegetative volatiles in plant defense against herbivores. Curr. Opin. Plant Biol. 12:479-485.

Wu J. and Baldwin I.T. 2010. New insight into plant responses to the attack from insect herbivores. Annu. Rev. Genet. 44:1-24.

Paper Received March 2, 2017 Accepted June 6, 2017

PAPER

THE CONTAMINATION RATE OF AFLATOXINS IN GROUND RED PEPPERS, DRIED FIGS, WALNUTS WITHOUT SHELL AND SEEDLESS BLACK RAISINS COMMERCIALIZED IN SAKARYA CITY CENTER, TURKEY

S. ÖZTÜRK YILMAZ
Sakarya University, Faculty of Engineering, Department of Food Engineering, TR-54187 Sakarya, Turkey
Tel.: +90 264 2955832
E-mail address: suzanyilmaz@sakarya.edu.tr

ABSTRACT

The investigation of this study is concerned with the occurrence of aflatoxin total (AFT) and aflatoxin B1 (AFB1) in 120 specimens randomly bought from retail stores, bazaars, supermarkets and regional stores in Sakarya City center. Immune affinity (IAC) clean-up with high-performance liquid chromatography (HPLC) and fluorescence detection (FID) methods were used to investigate the specimens to determine the incidence of aflatoxin (B1, B2, G1, G2) contamination. The findings indicate that the percentages of ground red peppers, dried figs, walnuts without shell and seedless black raisins contaminated with AFT are about 72%, 51%, 64% and 64%, respectively. One ground red peppers (18.68-10.49 μg/kg) and one specimen of walnuts without shell (10.26-5.06 μg/kg) exhibit the maximum contamination levels of AFT and AFB1, respectively. Of all the contaminated specimens, two ground red peppers and one dried fig specimens (2.5%) exceed the recommended AFB1 (5 μg/kg) limit defined by the Turkish Food Codex (TFC) regulations. This study presents the details of the first inspection regarding the presence of aflatoxins in seedless black raisin specimens in Sakarya City center of Turkey.

Keywords: aflatoxin, HPLC-FLD, pepper, fig, walnut, raisins

1. INTRODUCTION

As secondary metabolites, mycotoxins are generated by micro fungi which result in vertebrates contracting illnesses ((LUTTFULLAH and HUSSAIN, 2011). Aflatoxins (AFs) from mycotoxin family are immensely deleterious secondary metabolic compounds of *Aspergillus flavus, A. parasiticus and A. nomius* (SET and ERKMEN, 2010). They can cause cancer, mutations and physiological abnormalities in animals and humans (ERKMEN and BOZOGLU, 2008). AFs are impossible to avoid because they are found in nature as contaminants (IARC 1993). External circumstances such as climate, moisture and the span of precipitation during tilling and crop yield seasons affect the contamination (DİNİ *et al*., 2013). Such products as cereals, oil seeds, nuts, beans and spices are affected the most (REDDY *et al*., 2011). As a toxic chemical, AFB1 is one of the most potent substance which causes cancer and is categorized group I by International Agency for Research on Cancer (IARC 1993). The scale of toxicity, AFB1>AFG1>AFB2>AFG2, indicates that the fatal furan moiety of AFB1 is the significant point to detect the stage of bioactivity of this group of mycotoxins (COLAK *et al*., 2006). Numerous researches demonstrate that gastrointestinal diseases, hepatic neoplasms and hepatocellular carcinoma observed in humans in Africa, Philippines and China are related to aflatoxins (LUTTFULLAH and HUSSAIN, 2011).
Additionally, it has been manifested that feeding on products polluted by aflatoxin has led to some aflatoxicosis-related epidemics (REDDY and RAGHAVENDER, 2007). Aflatoxicosis-related epidemics were observed to affect a large geographical area and result in the demise of 123 people in Kenya (CDC, 2004).
It is of great importance to safety not only the well-being of people but also the interests of producers by applying an array of action to decrease the aflatoxin contamination to the minimum. If this is not achieved, the situation can have debilitating repercussions for producers in terms of their economic perpetuity and can also debar people from a substantial food source (DİNİ *et al*., 2013). Some studies and investigative research have been conducted in many countries to acquire a prevalent paradigm as regards foods contaminated with aflatoxin (REDDY *et al*., 2011). Owing to the fact that AFs are toxic and observed quite often, many countries, including Turkey, have outlined protocols and tolerance margins for aflatoxins. The Turkish Food Codex (TFC) states that the maximum levels for AFB1 and AFT in dried fruits are 8 and 10 μg/kg, in nuts or peppers 5 and 10 μg/kg, respectively (TFC, 2011).
Owing to the considerable risks of health complications caused by aflatoxins in foods, it is of utter importance to collect data regarding the prevalence of these toxic substances in foods in Sakarya (Turkey). Despite a lot of studies on aflatoxin rates in a variety of food which are present in the diet of people in Turkey, the research on aflatoxins in ground red peppers, figs and walnuts in Sakarya is scarce. Therefore, the aim of this study is to specify the AFT (AFB1, AFB2, AFG1, and AFG2) and AFB1 contamination rates in ground red peppers, walnuts without shell, dried figs and seedless black raisins, to show how contamination is a risk to the public health and to compare the findings to the highest aflatoxin tolerance margins defined by the TFC. This is also first survey to determine the incidence of AFs contamination in seedless black raisins in Sakarya city.

2. MATERIALS AND METHODS

2.1. Specimen preparation

120 food specimens were purchased in 2014 and 2015 from different retail shops, bazaars, supermarkets and local markets in Sakarya City center, Turkey. While a few of the

specimens were foreign origins, the majority of the specimens were from Turkey. They have been produced traditionally and locally consumed in generally. Most of the specimens sold in bulk were purchased unpackaged except for a few. The selected commodity groups are 25 ground red peppers and dried fruits including 45 dried figs, 25 walnuts without shell, 25 seedless black raisins (each about 500g). A subsample divider was used to divide the specimens. A 200 g subsample was ground and placed in plastic bags and kept at -20ºC until they were analyzed and put away to avoid sunlight. Two specimens were selected and two separate analyses were carried out for each specimen (SET and ERKMEN, 2010). Mean results of the four analyses were presented. All the testing and fluxing agents were of LC grade delivered from Merck (Darmstadt, Germany) and Sigma (St. Louis, USA). The aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2 (AFB1, AFB2, AFG1, AFG2) mix standard were obtained from Supelco (Sincer, Turkey). Standard stock solutions (2600 ng/mL) of mixed aflatoxin were produced using methanol, covered with aluminium foil so that aflatoxins would steadily dissolve under ultraviolet light and be kept for up to 3 months. The stock solutions were thinned to the rates of 0.3 and 1.0 μg/mL (standard solutions) using methanol and kept at -20°C (SET and ERKMEN, 2010).

2.2. Aflatoxin analyses in specimens by HPLC

This study was carried out making a few adjustments using HPLC method as described by AOAC (2000) and STROKA *et al*. (2000). Briefly, 5 g of NaCl was put in 50 g of walnuts without shell and afterwards mixed with 200 mL of methanol/water (80:20) and 200 mL of cyclohexane for 3 min. following the parting of the two phases, cyclohexane was done away with. For dried figs, ground red peppers and raisins, specimens were removed only with 200 mL of methanol / water (80:20). Extracts were filtrated using a Whatman filter paper No. 4 with a pore size of 30 um (LUTTFULLAH and HUSSAIN, 2011). 10 mL filtrate was thinned using 40 mL Phosphate buffer saline (PBS) from Sigma (St. Louis, MO, USA). 10 mL of filtrate was sent through the immune-affinity column (AflaPrep, R-Biopharm Rhone, Scotland) at a speed of 2-3 mL/min. The column was cleaned using 20 mL distilled water. Finally, bounded aflatoxins were eluted slowly using 1 mL methanol and air was pushed through the column to collect the last drops of eluate and finally thinned using 1 mL water (ÖZKAN *et al*., 2015). The extract was moved to a 1.8 mL vial for the injection (SET and ERKMEN, 2010).

The mobile phase was water-methanol-acetonitrile (5:2:3, v/v/v). The final concentration of the mobile phase was set to be 88.23 mg/L and 120 mg/L by nitric acid (LC grade, Sigma-Aldrich, Germany) and potassium bromide (LC grade, Merck, Germany) respectively. The mobile phase was filtered through a disposable filter unit (0.45μm). The presence of aflatoxins was observed by HPLC (Shimadzu, Tokyo, Japan) using a post-column derivatization electrochemically generated bromine (Kobra cell) and a fluorescence detector (RF 20A) at 362 nm (excitation) and 450 nm (emission) (SET and ERKMEN, 2010). The Intersil ODS-3 (25 cm - 4.6 mm ID, 5 μm, Tokyo, Japan) column was connected as HPLC column and the flow rate was 1 mL/min. 100 μL specimen was injected to HPLC automatically. The peaks were then compared with the actual specimen peaks obtained with that of aflatoxin standards.

The recovery studies were carried out by spiking to uncontaminated spicemens with two concentration levels of each toxin (AFT and AFB1) at least 1 hour prior to analysis. The recovery rate of aflatoxins was determined at a rate of 5.2 μg/kg and 2.6 μg/kg in all specimens in triplicate. The fortified spicemens were extracted and analyzed. The limits of detection (LOD) and the limits of quantification (LOQ) were defined according to signal to noise ratio; S/N=3/1 and S/N=10/1, respectively.

3. RESULTS

This study examined AFT and AFB1 contamination in 25 ground red peppers, 25 walnuts without shell, 25 seedless black raisins and 40 dried fig specimens. The findings were assessed in line with the legal limits for AFT and AFB1 specified by the Turkish Food Codex (TFC 2011).

The following recovery rates were obtained as μg/kg in ground red peppers and dried figs respectively: AFB1, 99.8%-106.3%; AFB2, 100.6%-100.1%; AFG1, 99.0%-98.2% and AFG2, 92.3%-102.2%. The limits of detection were as follows: AFB1, 0.05 μg/kg; AFB2, 0.02 μg/kg; AFG1, 0.04 μg/kg and AFG2, 0.05 μg/kg. The following recovery rates were calculated as μg/kg in walnuts without shell and seedless black raisins respectively: AFB1, 77.4%-84.0%; AFB2, 85.1%-107.0%; AFG1, 99.0-77.7% and AFG2, 75.9%-108.2% (Table 1).

Table 1. Recoveries of aflatoxins in the fortified specimens (%).

Commodities	Mean recovery[a] ± RSD[b] (%)			
	AFB1	AFG1	AFB2	AFG2
Ground red peppers	99.8±1.5	99.0±4.2	100.6±2.1	92.3±3.4
Walnuts without shell	77.4±3.7	99.0±4.5	85.1±2.0	75.9±2.7
Seedless black raisins	84.0±1.8	77.7±5.3	107±3.7	108.2±1.8
Dried figs	106.3±2.1	98.2±5.3	100.1±3.4	102.2±4.1

[a]Number of replicates: N= 3; [b]Relative standard deviation.

Table 2 presents the summary of the results of 120 specimens for AFT. The results clearly show that 18 ground red peppers, 23 dried fig specimens, 16 walnuts without shell and 16 seedless black raisins are contaminated with aflatoxins. Of all the specimens, ground red peppers have the highest contamination rates. The mean values of ground red peppers, walnuts without shell, dried figs and seedless black raisins are as 2.30 μg/kg, 1.68 μg/kg, 0.40 μg/kg, 1.78 μg/kg, respectively. AFT was observed in 73 specimens (68%) and 69 specimens had contamination below rates varying between 0.02 and 3.47 μg/kg; while 3 specimens had contamination varying between 5.30 and 18.68 μg/kg, exceeding the recommended limit for AFT.

Table 2. Total aflatoxins in the specimens.

Commodities	Contaminated specimens with AFT (μg/kg)				
	Number of specimens analyzed /positive	Frequency (%)	Below limit (<10 μg/kg)	Above limit (>10 μg/kg)	Mean value[a] (μg/kg)
Ground red peppers	25/18	72	23 (0.04-3.47)[b]	2(12.09-18.68)	2.30
Walnuts without shell	25/16	64	15(0.66-2.62)	1(10.26)	1.68
Seedless black raisins	25/16	64	16(0.02-2.07)	-	0.40
Dried figs	45/23	51	22 (0.16-5.20)	-	1.78
Total	**120/73**	**68**	**69(0.02-3.47)**	**3(5.30-18.68)**	

[a]Average contamination on positive samples that higher than the LOD; [b]Total aflatoxin range.

Table 3 demonstrates the results of the analysis of AFB1 where there are 34 contaminated specimens at a rate <5 μg/kg, 3 specimens varying between 5 and 10.49 μg/kg. As one can see in Table 3, the mean values of ground red peppers, walnuts without shell, dried figs and seedless black raisins are 1.38 μg/kg, 0.86 μg/kg, 0.11 μg/kg, 1.08 μg/kg, respectively. AFB1 was observed only in 37 specimens (38%), in addition to 34 not exceeding the established limit and 3 specimens above the established limit.

Table 3. Aflatoxin B1 in the specimens.

Commodities	Contaminated specimens with AFB1 (μg/kg)				Mean value[a] (μg/kg)
	Number of specimens analyzed /positive	Frequency (%)	Below limit (<5-8*μg/kg)	Above limit (>5-8*μg/kg)	
Ground red peppers	25/8	32	6(0.08-1.96)[b]	2(7.41-10.49)	1.38
Walnuts without shell	25/5	20	5(0.66-2.62)	1(5.06)	0.86
Seedless black raisins	25/16	64	16(0.02-0.26)	-	0.11
Dried figs	40/8	20	7(0.29-3.60)	-	1.08
Total	**120/37**	**38**	**34(0.02-3.60)**	**3(5.06-10.49)**	

[a]Average contamination on positive samples that higher than the LOD; [b]Aflatoxin B1 range.
*Aflatoxin B1 maximum limits for dried fruits in Turkey

Two ground red pepper specimens (12.09-18.68 μg/kg) exceed the established limit (10 μg/kg) specified by the Turkish regulations for AFT (TFC 2011), whereas 18 specimens vary between 0.085 and 3.47 μg/kg with aflatoxins below the established limit. The presence of AFs in ground red peppers has been documented by numerous researchers from various countries (JUAN *et al*., 2008). In Turkey, ground red peppers contaminated with AFB1 are 5 to 25 μg/kg in Bursa and 0.025 to 40.9 μg/kg in Istanbul, respectively (DOKUZLU *et al*., 2001). Red pepper specimens contaminated with AFB1 are 1.48 to 70.05 μg/kg in Kayseri (REDDY *et al*., 2001). 18.2% of ground red peppers contaminated with total aflatoxins (AFT) varies between 1.1 and 97.5 μg/kg in Şanlıurfa (ERDOGAN, 2004). SET and ERKMEN (2010) report the contamination rates of AFT and AFB1 ranging from 0.13 to 57.3 μg/kg and from 0.07 to 55.90 μg/kg, respectively, in unpacked ground red peppers and from 0.08 to 2.06 μg/kg and from 0.08 to 1.95 μg/kg, respectively, in packed specimens. ÖZKAN *et al*., (2015) state the content of AFB1 exceeds the legal limit in 49 of 180 red chilli pepper specimens and AFs exceeds the legal limit in 37 specimens. FAZEKAS *et al*., (2005) point to the presence of AFB1 in 25.7% (18/70) of ground red peppers in Hungary. ABDULKADAR *et al*., (2000) also suggest that 66.7% (4/6) chilli pepper powder contains AFT from 5.60 to 69.28 μg/kg in Qatar. ROMAGNOLI *et al*., (2007) observe the AFT contamination in 45.5% red pepper specimens varying between 0.57 and 30.7 μg/kg in Italy. REDDY (2001) also reports AFB1 contamination of 39.5% of pepper specimens in India ranging from 10 to 99 μg/kg. The present data indicate that the quality of peppers is better in comparison with the previous studies.
Among the 25 walnuts without shell, only one specimen is contaminated with aflatoxins rate of 10.26 μg/kg AFT and 5.06 μg/kg AFB1, which is above the legal limit. Various investigators also have reported similar results. For example LUTFULLAH and HUSSAIN, (2011) report that three in walnuts without shell contain concentrations varying between 6.0 and 10.8 μg/kg, which exceeds the recommended limit for AFT. GÜRSES, (2006) detected that 6 of 24 walnuts are positive within the contamination range 3-28 μg/kg. In a

study Mekkah determined quantitative AFB1 and AFT in walnuts 0-17.4 μg/kg and 0.9-36.6 μg/kg, respectively (EL TAWILA *et al*., 2013). ASGHAR *et al*., (2017) state about 37% walnut specimens showing AFs contamination, ranged from 0.68−6.66 μg/kg. In another study the contamination levels in walnut specimens vary between 0.56 and 2500 μg/kg for AFB1 and between 1.24 and 4320 μg/kg for AFT (JUAN *et al*., 2008). These values exceed the results presented in this paper.

The AFT rate in the contaminated specimens of dried figs varies between 0.16 and 5.20 μg/kg, which is below the recommended legal limit. The findings indicate that the AFs contamination of the specimens of the dried figs is low according to many studies as follows. Of late, the aflatoxin contamination level of the dried figs in Turkey has varied between 117.9 to 471.9 μg/kg (KARACA and NAS, 2006). KAYA and TOSUN, (2013) state that the aflatoxin rates in dried figs varied between 0 and 10.47 μg/kg in 2013**.** BIRCAN (2009) reports that 34% of the specimens has noticeable levels of AFT (0.20-208.75 μg/kg) in 7326 dried figs in Aydin province. LUTFULLAH and HUSSAIN, (2011) also point out that the contamination level of AFT in dried fig specimens varies between 0.8 and 12.5 μg/kg. The values in this study are below these findings. On the other hand, ASGHAR *et al*., (2017) indicate about 30% samples were contaminated with AFs, ranged from 0.69−3.44 μg/kg. The results from current study are similar with these previous findings.

In seedless black raisins, all contaminated specimens have lower limits than the maximum rate set by the Turkish regulations (TFC 2011). AFB1 rates detected in all dried figs and the seedless black raisins do not exceed the Turkish acceptable maximum rates (<8 μg/kg). Despite the low rates of AFB1 observed in most specimens, it still brings with it detrimental risks of health conditions to people who consume those contaminated foods (REDDY *et al*., 2011). LUTFULLAH and HUSSAIN (2011) report that one of the two contaminated specimens exceeds the recommended rate of aflatoxin while the other is below the recommended rate. 16% of the dried grapes in Brazil was found to contain AFB1 and AFB2 (IAMANAKA *et al*., 2007).

Several studies have been stated that dried raisins do not exhibit the satisfactory surface or environment for Aflatoxins production. However, ASGHAR *et al*., (2017) determined that 12 (13%) samples out of 90 tested specimens were determined positive with AFs. The concentrations ranged from 0.24-4.86 μg/kg. ALGHALIBI et *al*., (2008) also report that the aflatoxin contamination range of 3 out of 7 dried grape specimens in Sana'a City, Republic of Yemen is between 2678.66 and 11556.88 ng/kg. Whereas in the present study, small quantity of AFT and AFB1 was described in dried seedless black raisins. The concentrations ranged from 0.02-2.07 μg/kg and 0.02-0.26 μg/kg respectively. The results from current study indicate that the AFB1 and AFT rates of seedless black raisins from Sakarya city does not exceed the recommended limit by TFC (TFC 2011). In present research the lowest AFB1 and AFT mean level detected in seedless black raisins was 0.11-0.40 μg/kg respectively. In contrast, ground red peppers exhibited equivalent to 1.30-2.30 μg/kg for AFB1 and AFT, respectively.

Due to serious toxicity concerned with afaltoxins, various countries established guidelines for the acceptance level of AFs in dry fruits and nuts. For example, the European commission has set the maximum legal limits 4-10 μg/kg for AFT in dry fruits and nuts, respectively. European maximum limits are 5 and 2μg/kg for AFB1 in hazelnuts and dried figs for AFB1, respectively. (EC 2010). Additionally Turkish regulations for aflatoxins consider different limits for unprocessed products and product intended for direct human consumption, as well as for different commodities. For instance TFC has set the maximum legal limits 5-10 μg/kg in nuts and spices; 8-10 μg/kg in dried fruits; 2-4 μg/kg in cereals for AFB1 and AFT, respectively (TFC 2011). In both Turkey (TFC 2011) and the European Commission (EC 2010), the maximum legal limits for ground red pepper are 5 μg/kg for AFB1 and 10 μg/kg for AFT. As a result, the determination of AFs showed that there is a

powerful need for further research, routine analysis and to establish a monitoring program or project as per food quality control standard and procedures. Furthermore to improve and implement some food quality control procedures and standards such as good manufacturing practices (GMP) and the hazard analysis and critical control point (HACCP) system to minimize the risk and finally prevent the formation of AFs.

4. CONCLUSIONS

The goals of this study are to specify the AFT and AFB1 contamination rates in ground red peppers, dried figs, walnuts without shell and seedless black raisins, and to draw attention to the effects of contamination in these foods on public health. The results of this study provide insight regarding the danger of AFs contained various types of foods. The analysis of all the findings indicates that 68% and 38% of the specimens are contaminated by AFT and AFB1, respectively. Of all the contaminated specimens, 2.5% of specimens exceed the recommended AFB1 (5 μg/kg) limit defined by the Turkish Food Codex (TFC) regulations. Consequently, aflatoxins entail a health risk for those who consume contaminated foods. Cases of AFs contamination show that constant surveillance and a better food safety system should be implemented so that the content of AFs in foods can be kept at the minimum level (LEONG *et al*., 2010). On the other hand, more comprehensive studies should be carried out on a wide range of foods consumed in Turkey in order to design appropriate executive projects and to bring forth regulations on AFs encompassing all sustenance consumed. This research will pave the way for new studies on AFs in order to establish a comprehensive food safety system, which will protect humans' health. This study is the primary documentation, which addresses the occurrence of commercial seedless black raisins contaminated with aflatoxin in Sakarya.

ACKNOWLEDGEMENTS

This work was supported by Research Fund of The Sakarya University (Project number: 201-01-16-011).

REFERENCES

Abdulkadar A.H.W., Al-Ali A. and Al-Jedah J. 2000. Aflatoxin contamination in edible nuts imported in Qatar. Food Control 11:157-160.

Asghar M.A., Ahmed A., Zahir E., Asgher M. A., Iqbal J. and Walker G. 2017. Incidence of aflatoxins contamination in dry fruits and edible nuts collected from Pakistan, Food Control, doi: 10.1016/j.foodcont.2017.02.058

AOAC (Association of Official Analytical Chemists). 2000. Natural toxins, 17th ed. Chapter: 9 Gaithersburg, Maryland.

Bircan C. 2009. Comparison of homogenization techniques and incidence of aflatoxin contamination in dried figs for export. Food additives contaminants part b-surveillance 2:171-177.

CDC (Centres for Disease Control and Prevention). 2004. Outbreak of aflatoxin poisoning-eastern and central provinces, Kenya, January-July 2004. Morbidity and Mortality Weekly Report 3:790-793.

Colak H., Bingöl E.B., Hampikyan H., and Nazlı B. 2006. Determination of Aflatoxin contamination in red-scaled, red and black pepper by ELISA and HPLC. J. of Food and Drug Analysis 14(3):292-296.

Dini A., Khazaeli P., Roohbakhsh A. and Madadlou A. 2013. Aflatoxin contamination level Iran's pistachio nuts during years 2009-2011. Food Control 30:540-544.

Dokuzlu C. 2001. Aflatoxin in red pepper. Uludag University Veterinary Faculty Journal 20:1-2.

Doster M.A. and Michailides T.J. 1994. Aspergillus molds and aflatoxin in pistachio nuts in California. Phytopathology 84:583-590.

EC. European Commission, 2010. Regulation No: 165/2010 of 26 February 2010 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union 2010, L 50/8-12.

El Tawila M.M, Neamatallah A. and Serdar S.A. 2013. Incidence of aflatoxin in commercial nuts in the holy city Mekkah. Food Control 29:121-124.

Erdogan A. 2004. The aflatoxin contamination of some pepper types sold in Turkey. Chemosphere 56:321-325.

Erkmen O. and Bozoglu T.F. 2008. Food Microbiology 1: Microorganisms in foods, microbial growth, foodborne diseases and detection of microorganisms and their toxins. Ilke Publishing Company, Ankara.

Fazekas B., Tar A. and Kovacs M. 2005. Aflatoxin and ochratoxin a content of spices in Hungary. Food Additives and Contaminants 22:856-863.

Gürses M. 2006. Mycoflora and aflatoxin content of hazelnuts, walnuts, peanuts, almonds and roasted chickpeas leblebi sold in Turkey. International Journal of Food Properties 9:395-399.

Iamanaka B.T., Menezes C.H., Vicente E., Leite R.S.F. and Taniwaki M.H. 2007. Aflatoxigenic fungi and aflatoxins occurrence in sultanas and dried figs commercialized in Brazil. Food Control 18:454-457.

IARC (International Agency for Research on Cancer). 1993. Some naturally occurring substances: food items and constituents, heterocyclicaromatic amines and mycotoxins. IARC monographs on the evaluation of carcinogenic risks to humans. 56:245-395, Lyon, France.

Juan C., Zinedane A., Molto J.C., Idrissi L. and Manes J. 2008. Aflatoxin levels in dried fruits and nuts from Rabat-Sale area, Morocco. Food Control 19:849-853.

Karaca H., and Nas S. 2006. Aflatoxins, patulin and ergosterol contents of dried figs in Turkey. Food Additives and Contaminants, 23, 502-508.

Kaya S.B. and Tosun H. 2013. Occurence of total aflatoxin, ochratoxin a and fumonisin in some organic foods. Journal of Pure And Applied Microbiology 7:2925-2932.

Leong Y.H., Ismail N., Latif AA. and Ahmad R., 2010. Aflatoxin occurrence in nuts and commercial nutty products in Malaysia. Food Control 21:334-338.

Lutfullah G. and Hussain A. 2011. Studies on contamination level of aflatoxins in some dried fruits and nuts of Pakistan. Food Control 22:426-429.

Ozkan A., Bindak R. and Erkmen O. 2015. Aflatoxin B-1 and aflatoxins in ground red chilli pepper after drying. Food additives contaminants part b-surveıllance 8:227-233.

Reddy B.N. and Raghavender C.R. 2007. Outbreaks of aflatoxicoses in India. The African Journal of Food, Agriculture, Nutrition and Development 7:1-15

Reddy D.V.R. 2001. Aflatoxins B1 in different grades of chillies (*Capsicum annum* L.) in India as determined by indirect competitive-ELISA. Food Addit Contam 18:553-558.

Reddy K.R.N., Farhana K.I. and Salleh B. 2011. Occurence of Aspergillus spp. and aflatoxin B1 in Malaysian foods used for human consumption. J. of Food Science 76:99-104.

Romagnoli B., Menna V., Gruppioni N. and Bergamini C. 2007. Aflatoxins in spices, aromatic herbs, herb-teas and medicinal plants marketed in Italy. Food Control 18:697-701.

Set E. and Erkmen O. 2010. The aflatoxin contamination of ground red pepper and pistachio nuts sold in Turkey. Food and Chemical Toxicology 48:2532-2537.

Stroka J., Anklam E., Jorissen U. and Gilbert J. 2000. Immunoaffinity column cleanup with liquid chromatography using post-column bromination for determination of aflatoxins in peanut butter, pistachio paste, fig paste, and paprika powder: Collaborative study. Journal of AOAC International 83:320-340.

TFC. Turkish Food Codex, 2011. Regulation No. 2011/28157, the maximum allowed level of food contaminants. Official Gazette of Publication: 29.12.2011-28157, Ankara.

Paper Received November 5, 2016 Accepted June 12, 2017

PAPER

PHYSICOCHEMICAL AND SENSORY PROPERTIES OF SAVORY CRACKERS INCORPORATING GREEN GRAM FLOUR TO PARTIALLY OR WHOLLY REPLACE WHEAT FLOUR

K. VENKATACHALAM[1]* and M. NAGARAJAN[2]

[1]Department of Food Technology, Faculty of Science and Industrial Technology, Prince of Songkla University, Makhamtia, Muang, Surat Thani 84000, Thailand

[2]Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Kho-Hong, Hatyai, Songkhla 90112, Thailand

*Corresponding author. Tel.: +66 77 355040 ext. 8605; fax: +66 77 355453

E-mail address: karthikeyan.v@psu.ac.th; drkkvfood@gmail.com

ABSTRACT

Green gram flour (GGF) has numerous health benefits and it is an excellent raw material for baking. However, knowledge of its use as a baking material is limited. The present study was aimed to develop savory crackers using GGF. The crackers were prepared at various ratios of wheat flour (WF) to green gram flour (GGF) (T1 100:0; T2 80:20; T3 60:40; T4 40:60; T5 20:80; T6 0:100). The physicochemical and ultrastructural properties, antioxidant activities, and sensory attributes of savory crackers were studied. Weight, height, thickness, moisture, a_w (water activity) and texture tended to increase with GGF application in the enriched crackers (T4-T6). Similarly, the proximate composition of crackers was improved by GGF. The color characteristics were excellent with a high content of WF because GGF content increased a^* and ΔE^* color coordinates and decreased the color coordinates of L^* and b^*. The antioxidant potential was higher in the GGF enriched crackers. In terms of ultrastructure, the GGF enriched crackers had smoother surfaces. The GGF enriched crackers showed various improvements and a high content of WF gave better sensory scores, while consumer acceptability of the GGF enriched crackers suffered slightly.

Keywords: green gram flour, crackers, nutrition, antioxidant ability, ultrastructure, sensory attributes

1. INTRODUCTION

Novel functional snack foods, especially bakery products from the food industry (with potential health benefits) are in high demand by consumers. Numerous studies have assessed the production of bakery products (bread, biscuits, cookies and crackers) with health promoting ingredients, such as pulses, grains, and tubers (JULIANTI *et al.,* 2017; AHMED and ABOZED, 2015; DOKIĆ *et al.,* 2015; SEDEJ *et al.,* 2011). In Thailand, the baked snack food market value was estimated to be 7 million Euro in 2012, accounting for about 4% of the total snack sales. The baked snack products include cookies, crackers, and biscuits. Crackers are a versatile thin and crispy food that can appeal to a variety of consumer expectations. They serve as a quick snack with low contents of moisture, sugar, and fat. Crackers can be divided into three broad categories, namely; soda crackers, snack crackers, and savory crackers (AHMED and ABOZED, 2015). Snack crackers with sugar and/or desiccated coconut sprinkled on top are widely sold in Thailand. The main ingredient in crackers is wheat flour, whose consumption may trigger celiac disease in a significant fraction (1:100-1:200) of the global population. Gluten is the major component in wheat flour that generates this inflammatory disease response. Therefore, the need for food manufacturers to provide alternative gluten-free bakery products. While gluten free alternatives for bakery products such as bread, cakes, and biscuits are widely available, alternatives to crackers are not common, especially in Thailand. Additionally, only a few studies have been done on gluten-free crackers from alternative flours, such as pulse flour and buckwheat flour (SEDEJ *et al.,* 2011).

The flour used as raw materials play a crucial role in the bakery industry, and these are obtained from a wide range of plants. Flours from legumes have been put to diverse use in the bakery industries and can be used in bread, tortillas, chips, doughnuts, spreads and extruded snacks (GRAHAM and VANCE, 2003). Green gram (*Vigna radiata* (L.) Wilczek) is a legume widely grown and consumed in South East Asia. It is an excellent source of digestible protein, with a higher lysine content than any other legume, and is free from factors that cause flatulence. Normally, green gram seeds are consumed as whole or split grains, sprouts, immature seeds, or as flour (ADSULE *et al.,* 1986). Green gram flour is used in various Thai desserts. CHANDRA *et al.* (2015) demonstrated that composite flours containing green gram have excellent functional and rheological properties. Moreover, green gram flour is gluten free. Despite these positive aspects, only a few scientific studies have addressed the production of crackers with green gram flour and to the best of our knowledge, bakery products prepared from mixed wheat flour (WF) and whole green gram flour (GGF) do not currently exist. Therefore, the present study aimed at producing savory crackers with various fractions of green gram flour in mixtures with wheat flour, and evaluate their physicochemical and sensory characteristics.

2. MATERIALS AND METHODS

2.1. Materials

Commercially available raw materials such as flours (refined wheat flour (WF) and whole green gram flour (GGF), sugar, vegetable oil, chemical leavening agent, honey and fresh garlic for savory flavor were purchased from the local supermarkets in Muang, Surat and Thani in Thailand. Then, prior to processing, the flours were blended in the following WF:GGF (dry weight) ratios: 100:0 (T1), 80:20 (T2), 60:40 (T3), 40:60 (T4), 20:80 (T5) and 0:100 (T6). The flour blends were stored separately in airtight containers, in darkness at

room temperature until further use. All the chemicals and reagents used in this study were of analytical grade.

2.2. Cracker preparation

Crackers made from WF, GGF and the actual mixtures of WF and GGF, were produced on a laboratory scale by a straight dough process, using a commercial cracker recipe with slight modifications. The ingredients, namely flour/blend, sugar (2%), salt (2%), chemical leavening agent (sodium bicarbonate, 2%), vegetable oil (10%) and water (15%) were thoroughly mixed together to form a cohesive dough using an electric mixer (RBSFOODMIXERPRO, Cuizimate, Thailand) at low (80 rpm, 1 min) and moderate speeds (100 rpm, 10 min). The dough was wrapped in polyethylene film and was allowed to sit for 10 min under refrigerated temperature and then for 20 min at room temperature. After leaving to sit, the dough was sheeted to 5 mm thickness using a pasta roller, and cut into 4 cm squares with a mold. These molded crackers were baked in a double deck infrared conventional oven at 180ºC for 11min and were pulled out of the oven to apply the flavoring agent. For savory flavor, finely chopped garlic was mixed with honey and brushed on the crackers, after which the baking continued for another 4 min at 180ºC, before cooling down to room temperature. Whole baked crackers were subjected to physical, ultrastructural and sensorial analyses, while coarsely crushed crackers were used in chemical and antioxidant analyses.

2.3. Quality analyses of crackers

2.3.1. Physical characteristics

Physical characteristics such as color, texture, stack weight and thickness were determined for the crackers (15 replicate crackers per experimental point). The color coordinates L^*, a^* and b^* were observed with a colorimeter (MiniScan EZ 4000S, HunterLab, Reston, Virginia). The total color difference (ΔE^*) to the baseline case T1, was measured as reported by AHMED and ABOZED (2015). The texture was analyzed by the peak force (N) test using a texture machine (CT3 Texture Analyzer, Brookfield, Stuttgart, Germany) equipped with a blade probe. Stack weight was measured using an electronic weighing balance and the results are expressed in grams (g). Cracker thickness was measured using the Vernier caliper, while crackers were stacked on top of each other, and an average value was recorded.

2.3.2. Chemical analyses

The crackers were analyzed for proximate composition in terms of moisture, fiber, fat, ash, and protein, using methods 934.01, 985.29, 983.23, 945.38, and 920.87, respectively (AOAC, 2000). A protein conversion factor of 5.7 was applied to determine the presence of protein in the samples. Carbohydrate content in the crackers was estimated by the subtraction of other components. The mineral contents of K, Ca, Mg, Fe, and Zn were measured using an individually coupled plasma optical emission spectrophotometer (ICP-OES, Vista Pro, Varian Inc., Mulgrave VIC, Australia). The pH of the crackers was measured using a handheld digital pH meter (pH30 Tester, CLEAN, Shanghai, China). Water activity (a_w) of crackers was measured using an a_w analyzer (Series 4TEV, Aqua Lab, WA, USA).

2.3.3. Antioxidant activities

Extracts from crackers for determining antioxidant properties were prepared using 10 g samples mixed with acidified methanol (50 mL) in an amber reagent bottle. The suspension was mixed thoroughly and placed in a water bath with an electric shaker, for 8 hr of extraction at 30 ºC. Thereafter, the extracted samples were centrifuged at 7800 x *g* for 15 min, after which the supernatant was collected and stored in a dark container at -4°C until analysis. The antioxidant properties were measured as follows. The total chlorophyll content (TCC) of the crackers was measured with method 940.03 (AOAC, 2000). The results are represented as mg chlorophyll/g. Total phenolic content (TPC) was measured with the method of SINGLETON *et al.* (1999). The results are shown as mg equivalents of gallic acid/g (mg Eq GA/g). Total flavonoid content (TFC) in the samples was determined with the method of ZHISHEN *et al.* (1999). The results are expressed as mg equivalents of catechin/g (mg Eq CE/g). The radical scavenging capacity of the crackers for DPPH (2, 2-diphenyl-1-picrylhydrazyl) radicals and DPPH-RSC, were determined using the method of SÁNCHEZ-MORENO *et al.* (1999). The results are expressed as percentages. The ferric reducing antioxidant power (FRAP) of crackers was measured according to the method of BENZIE and STRAIN (1996). The results are expressed as percentages. The metal chelating activities (MCA) of the crackers were measured according to the method of AKTUMSEK *et al.* (2013). The results are expressed as mg equivalents of EDTA/g (mg Eq EDTA/g).

2.3.4. Ultrastructural properties

Crackers were broken into tiny pieces to observe the surface ultrastructure. The pieces were mounted on aluminium stubs with adhesive tape, sputter coated with gold-palladium to render them thermo electrically conductive, and then observed with a JSM 5800 LV scanning electron microscope (JEOL, Akishima, Japan).

2.3.5. Sensory analysis

Sensory attributes of the crackers, namely color, taste, crispness, odor, appearance and overall acceptability, were determined by a panel of sixty consumers (18-60 years of age; equal proportions of males and females). Candidate panelists were screened for their frequency of cracker snack consumption, accepting to be among the panelist for those consuming the cracker snack at least once a month. Each panelist evaluated the various formulations of savory green gram crackers using a 9-point hedonic scale, in which the lowest value (1) stands for extreme dislike and the highest value (9) represents an extreme liking. During the sensory evaluation, crackers of each experimental type were placed separately on plastic plates that were labelled randomly with three-digit codes. Prior to each tasting a sample, lemon water was served to the panelists to neutralize their mouth feel, in order to maintain comparable testing over a sequence of samples.

2.4. Statistical analyses

All the experiments were conducted with six replications, except for the physical quality determinations that had 15 replications, and the data are expressed as a mean±standard deviation. The significance of differences between the cases was determined by analysis of variance (ANOVA) with significance threshold $P<0.05$. The means were compared by Duncan's multiple range test. Statistical analysis was performed using SPSS for Windows (version 8, SPSS Inc., Chicago, IL, USA).

3. RESULTS AND DISCUSSIONS

The physical characteristics of the composite crackers made of WF:GGF blends are shown in Table 1. The stack weight ranged from 69.45 to 87.90 g. The crackers with high GGF content were slightly heavier than those having more WF. The thickness of crackers did not differ much between the experimental cases ($P<0.05$), but as might be expected from cracker height of the WF rich crackers, these were slightly thicker than those with high GGF content. The moisture content differed significantly between the samples, ranging from 4.46 to 6.59%. The crackers with highest GGF levels (T4-T6) had higher moisture contents than the rest (T1-T3), but samples in the middle (T2-T5) did not show any significant differences. Moisture plays a very vital role in baked products affecting their texture, crispness and shelf life in storage. Generally, moisture tends to degrade the finished product's functionality. However, the crackers in the present study had significantly lower moisture levels than composite crackers in various prior studies (AHMED and ABOZED, 2015; DOKIĆ *et al.*, 2015; SEDEJ *et al.*, 2011). Similarly, the water activity (a_w) was comparatively high in the crackers with a high proportion of GGF, ranging from 0.39 to 0.48. In addition, the tested crackers were slightly alkaline in this study, with the pH varying from 7.20 to 7.96 ($P<0.05$). While the GGF rich crackers tended to have slightly decreased pH relative to others. The texture of crackers was significantly affected by the WF:GGF blends proportions. High WF level in the crackers gave a crispier structure, while the GGF rich crackers had a slightly hardened texture.

The color parameters of crackers are shown in Fig. 1A-D. Lightness (L^*) was significantly affected by the mixture proportions of WF:GGF. Increased level of WF in the crackers caused a considerably lighter surface (Fig. 1A). This could be due to the effects of chlorophyll reducing lightness on the cracker surface. The redness (a^*) was significantly affected in this study, with GGF in the crackers increasing a^* value; *this* coordinate indicates the level of browning in a baked product and should be high for brown surfaces (Fig. 1B). This suggests that GGF in the crackers could increase protein availability to Maillard reactions during baking. On the other hand, yellowness (b^*) was not significantly affected and slightly decreased with GGF content (Fig. 1C). The total color difference (ΔE^*) to T1 baseline crackers was significantly affected (Fig. 1D) in a consistent pattern that correlates with the L^* values. AHMED and ABOZED (2015) reported that changes in ΔE^* represented the darkness/lightness variation in crackers, matching well this present study despite the fact that the raw materials are different.

The proximate composition of crackers made of WF:GGF blends are shown in Fig. 2A. It was observed that GG has higher protein and mineral contents than wheat. SEDEJ *et al.* (2011) reported similar results for buckwheat crackers when compared to wheat crackers. The fat content of crackers was unaffected, as the natural fat content in WF and GGF is very similar. The total dietary fiber content did not vary significantly, because its trend decreased with GGF content. Previous studies have reported reduced levels of dietary fiber in novel crackers (AHMED and ABOZED, 2015; SEDEJ *et al.*, 2011). The ash content in the crackers increased with GGF level. The contents of protein, carbohydrate, fat and fiber contents were converted to food energy using a water general factor system according to FAO recommendations (AHMED and ABOZED, 2015; FAO, 2003). The estimated energy of crackers was not significantly different in all the treatments. On the other hand, the contents of K, Ca, Mg (Fig. 2B), Fe and Zn (Fig. 2C) minerals slightly increased with the proportion of GGF in the crackers. Based on these results, the use of GGF in crackers may provide a nutritional benefit to the consumers.

Table 1. Characteristics of savory crackers made with various blend proportions of WF and GGF.

End product characterization	Flour blend					
	T1	T2	T3	T4	T5	T6
Stack Weight (g)*	69.45±0.48^{c}	73.65±0.23^{c}	79.95±0.40^{b}	85.20±0.44^{ab}	87.30±0.43^{a}	87.90±0.66^{a}
Thickness (mm)**	6.94±0.12^{a}	6.83±0.22^{b}	6.60±0.18^{c}	6.39±0.27^{d}	6.38±0.14^{d}	6.34±0.09^{d}
Moisture (%)	4.46±0.40^{c}	5.98±0.11^{b}	6.01±0.01^{b}	6.06±0.24^{b}	6.13±0.22^{b}	6.59±0.21^{a}
Water activity (a_w)	0.393±0.01^{f}	0.406±0.00^{e}	0.415±0.00^{d}	0.436±0.00^{c}	0.452±0.00^{b}	0.482±0.01^{a}
pH	7.96±0.02^{a}	7.62±0.07^{b}	7.48±0.02^{c}	7.45±0.02^{c}	7.33±0.03^{d}	7.20±0.02^{e}
Texture (g)	2742.33±259.76^{c}	2790.83±360.05^{bc}	3113.75±649.25^{b}	3483.75±462.53^{a}	3493.67±282.60^{a}	3798.50±171.92^{a}

T1, T2, T3, T4, T5 and T6 represent WF:GGF flour's different blend ratios for making crackers (100:0, 80:20, 60:40, 40:60, 20:80 and 0:100).
Note: The data shown in mean±SD.
Different letters in the same row indicate significant differences ($P \leq 0.05$) among samples.
*Stack of 15 crackers
**Average value in a stack of 15 crackers

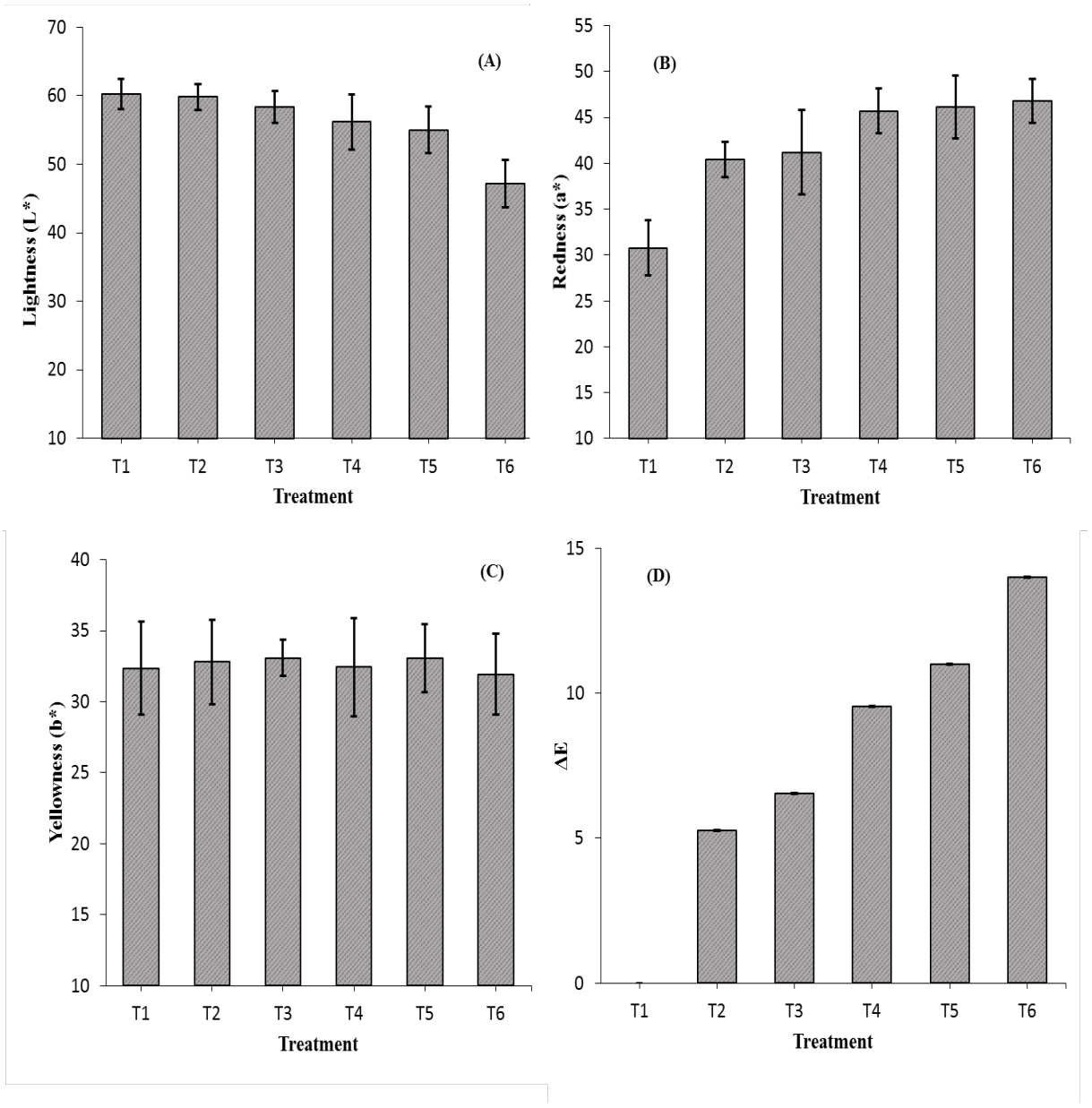


Figure 1. CIE-LAB color coordinates L^* (A), a^* (B), b^* (C), and ΔE^* (D) difference to baseline case T1, for savory crackers made with various WF:GGF blend proportions. T1, T2, T3, T4, T5 and T6 represent WF:GGF flour's different blend ratios for making crackers (100:0, 80:20, 60:40, 40:60, 20:80 and 0:100).

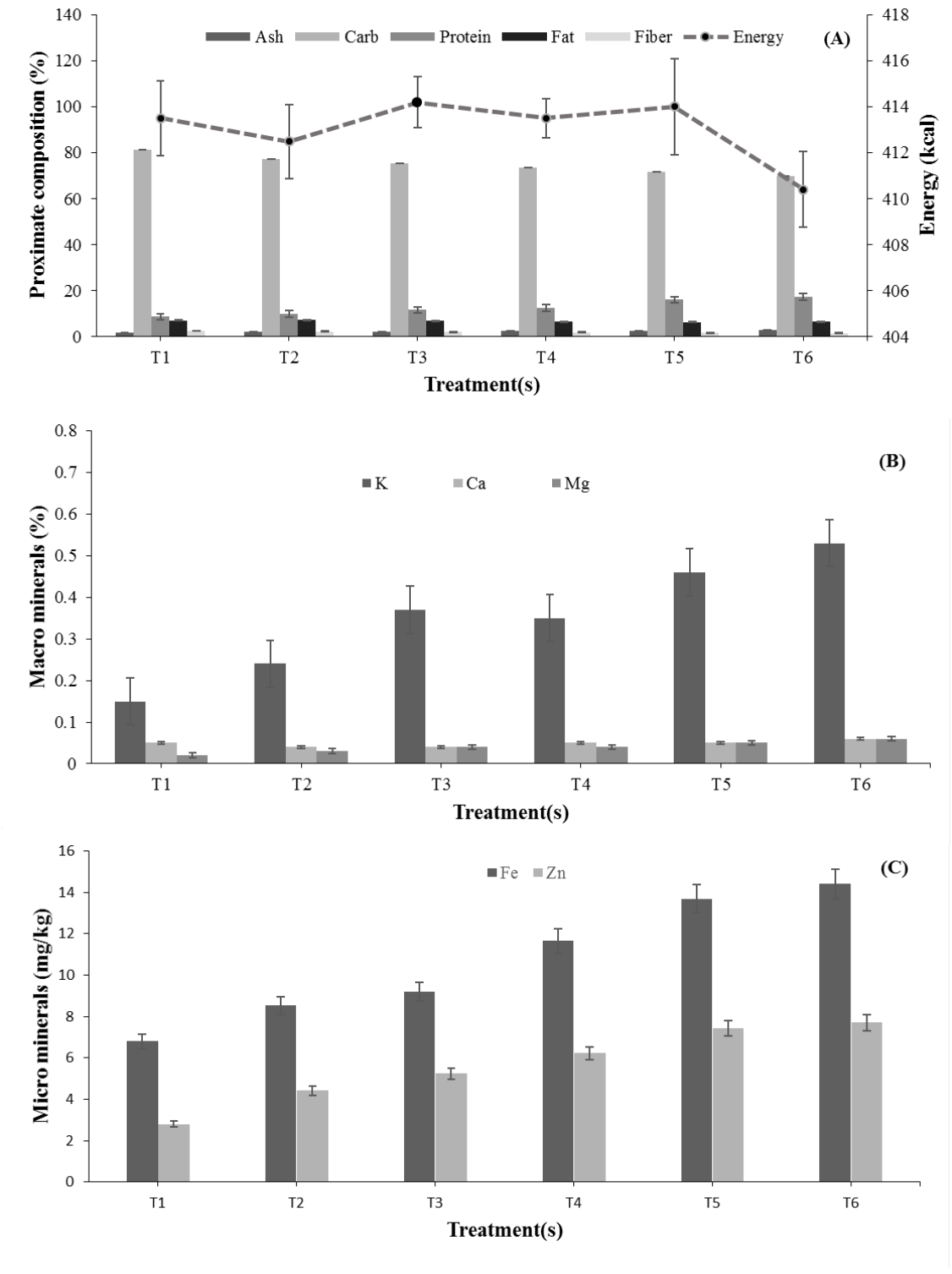


Figure 2. Proximate composition (A), energy (A) and mineral contents (B& C) of the various savory crackers. T1, T2, T3, T4, T5 and T6 represent WF:GGF flour's different blend ratios for making crackers (100:0, 80:20, 60:40, 40:60, 20:80 and 0:100).

Antioxidant properties of the crackers with various WF:GGF blend ratios are presented in Fig. 3. The antioxidant abilities of the crackers are directly linked to their ingredients, and the predominant contribution is from the GGF ingredient. The phytochemical content was highest in the GGF rich crackers, and TCC increased with GGF content (Fig. 3A), being maximal in case T6 made with only GGF. FERRUZZI *et al.* (2002) reported that natural pigments such as chlorophyll exhibited antioxidant capabilities along with the other phytochemicals, such as polyphenols in plants. Similar patterns were found for TPC and TFC (Fig. 3A), both increasing gradually with the content of GGF in the dough mixture, which is in agreement with the study by KARTHIGA and JAGANATHAN (2013).

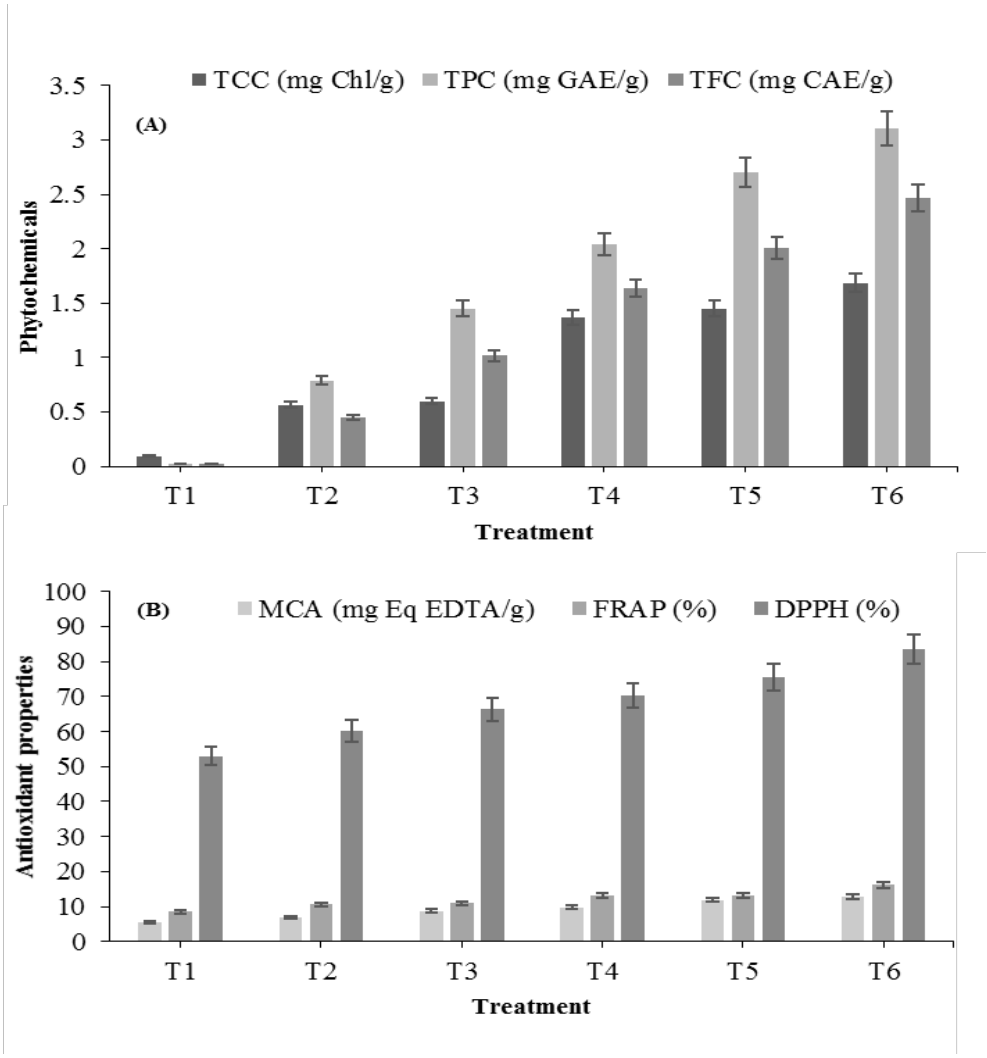


Figure 3. Phytochemical contents (A) and antioxidant properties (B) of the various savory crackers. T1, T2, T3, T4, T5 and T6 represent WF:GGF flour's different blend ratios for making crackers (100:0, 80:20, 60:40, 40:60, 20:80 and 0:100). TCC: Total chlorophyll content; TPC: Total phenolic content; TFC: Total flavonoid content; MCA: Metal chelating activity; FRAP: Ferric reducing antioxidant power; DPPH: 2,2-Diphenyl-1-picrylhydrazyl radical scavenging capacity.

Normally, whole green gram legumes contain more phytochemicals than processed green gram, such as split grains or flour, and generally, GGF contains more phytochemicals than WF. This was also very distinct in the present study, as TPC and TFC increased with GGF content. While for the GGF made crackers rich in phytochemicals, uncooked GGF would be superior in this respect. SHARMA and GUJRAL (2014) reported that a continuous baking process could modify the chemical structure of a phenolic compound, particularly by polymerization, and consequently reduce extractable polyphenols in baked products. The antioxidant properties of crackers were evaluated in terms of DPPH radical scavenging activity and a FRAP assay (Fig. 3B). The GGF in crackers increased the radical scavenging ability from 50.92 to 83.57%, even though the GGF enriched crackers (T5-T6) had improved radical scavenging ability (RSA) and also the WF rich crackers had a reasonable RSA. Similarly, the ferric reducing potential was improved by GGF in the crackers, probably because of the phytochemicals, namely polyphenols. In addition, the added honey and garlic, and the chemical reactions during baking may have contributed to the antioxidant potential of the end product.

Surface ultrastructure for crackers made of WF:GGF flour mixes is shown in Fig. 4. The mixing proportions significantly affected the ultrastructure of the cracker surface. The high content of WF in the composite flour (T1-T3) induced continuous aggregation with few fissures on the cracker surface, while the GGF rich crackers had smoother surfaces with reduced or no aggregation. The samples T5 and T6 with a high proportion of GGF had small discontinuous patches on the surfaces. The smoother compact cell surfaces of GGF rich crackers might be the results of complete gelatinization during baking, whereas, WF rich crackers have rough surface which could be the cause of incomplete gelatinization and leaving intact or partially embedded sugars and lipids in the protein matrix (CHEVALLIER *et al.,* 2000; FILIPČEV *et al.,* 2014). DACHANA *et al.* (2010) reported that incomplete gelatinization of starch in the crackers could be caused by insufficient water in the matrix. Such lack of water in the WF rich crackers could be caused by tight bonding of water to gluten during dough making (JAMES and RINTOUL, 1982). With less gluten, the crackers might leave more water for starch gelatinization, which could improve the surface smoothness of the crackers (AHMED and ABOZED, 2015).

The sensory attributes of the savory crackers made with WF:GGF flour blends are presented in Table 2. The sensory scores were better for crackers with a high proportion of WF and correspondingly low proportion of GGF. The blend composition significantly affected color (7.23 for WF vs 5.17 for GGF), odor (7.67 for WF vs 5.17 for GGF), taste (8.63 vs 6.23, similarly), texture (7.27 vs 6.17), crispness (7.70 vs 5.57), appearance (7.80 vs 6.30) and overall acceptability (7.88 vs 6.17). All traits were negatively affected by GGF content in the crackers. GGF does have its specific color and aroma, which the consumers are not used to. Furthermore, DOKIĆ *et al.* (2015) reported that increasing the level of dietary fiber in crackers could degrade texture and crispness due to weak rheological behavior and poor uniformity of structure. The acceptability of food products always depend on texture, and crispness is of particular importance when making crackers or other crispy snack products (ROUDAUT *et al.,* 1998). In the present study, texture and crispness were affected, nevertheless not excessively, by the GGF content; crispness is a very important trait for acceptable crackers (SIAW *et al.,* 1985). The relatively high fiber content of GGF, as compared to WF, is evident from the proximate composition of crackers (Fig. 2A). While the GGF enriched composite crackers had slightly lower sensory scores than the unblended WF, still, acceptable mean scores greater than 4.5 were found: indicating neither like nor dislike. Of relevance is that samples T4, T5 and T6 always had similar grades. Therefore, despite their slightly inferior sensory scores and considering the superior nutritional and health benefits, the T6 crackers could be proposed as a possible alternative to bread wheat crackers.

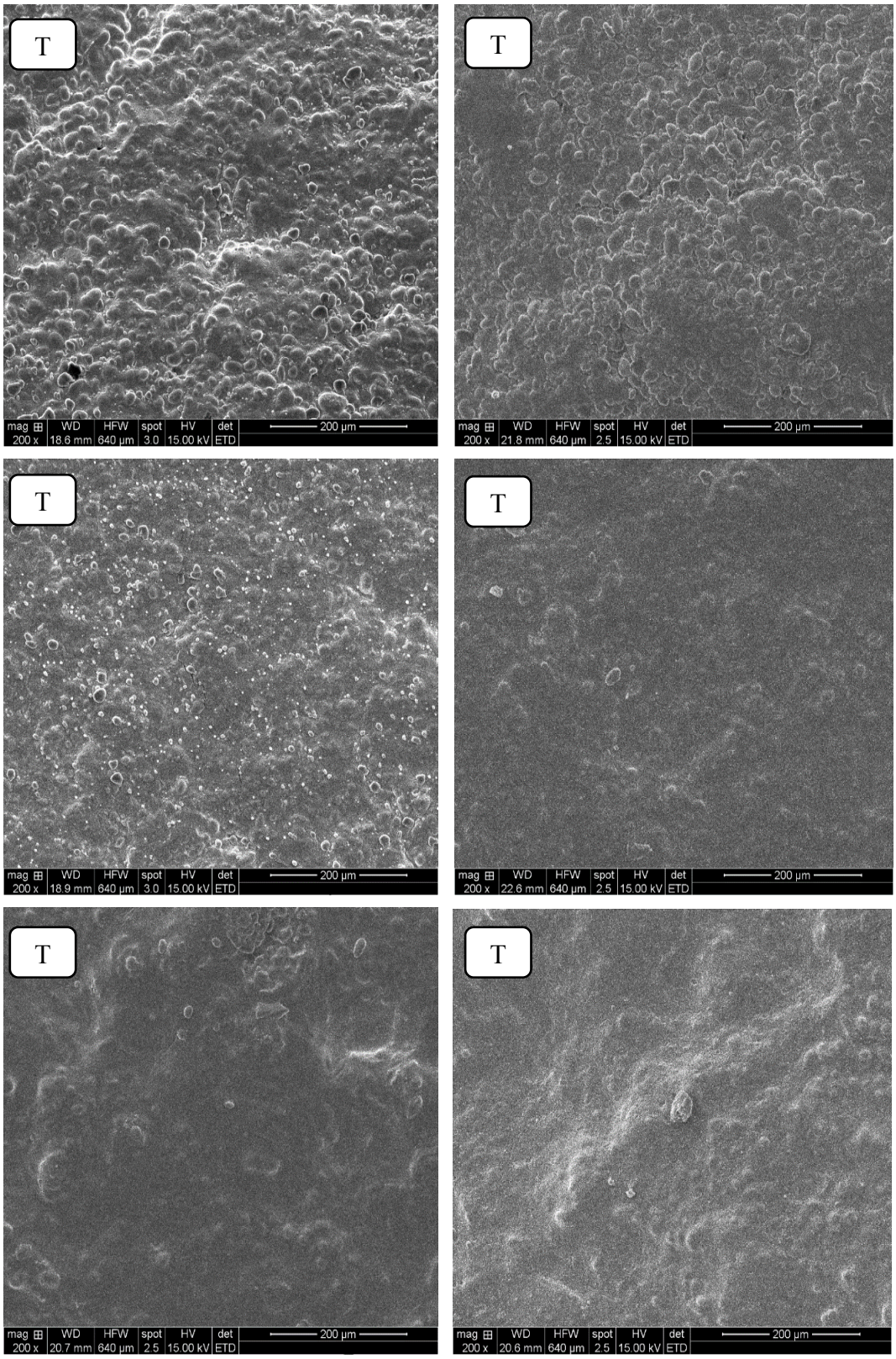


Figure 4. Surface ultrastructure images of the different savory crackers. T1, T2, T3, T4, T5 and T6 represent WF:GGF flour's different blend ratios for making crackers (100:0, 80:20, 60:40, 40:60, 20:80 and 0:100).

Table 2. Sensory scores of savory crackers made with various blend proportions of WF and GGF.

Sensory attribute	Flour blend					
	T1	T2	T3	T4	T5	T6
Color	7.23±1.17^{a}	6.20±1.49^{b}	5.67±1.92^{bc}	5.50±1.25^{bc}	5.70±1.49^{bc}	5.17±1.82^{c}
Odor	7.67±1.54^{a}	7.70±1.66^{a}	7.37±1.61^{ab}	6.17±1.60^{b}	5.87±1.49^{bc}	5.60±1.69^{bc}
Taste	8.63±1.47^{a}	7.70±2.00^{ab}	7.03±1.77^{bc}	6.87±2.06^{bc}	6.23±2.18^{bc}	6.37±1.77^{bc}
Texture	7.27±1.76^{a}	7.63±1.63^{ab}	6.63±1.96^{c}	6.53±1.96^{c}	6.17±1.82^{c}	6.50±1.59^{c}
Crispness	7.70±2.10^{a}	7.63±1.90^{a}	6.83±2.42^{ab}	5.83±1.90^{b}	5.83±2.18^{b}	5.57±1.96^{b}
Appearance	7.80±1.63^{a}	7.00±1.58^{b}	6.53±1.68^{bc}	6.93±1.51^{bc}	6.37±1.75^{bc}	6.30±1.40^{bc}
Overall acceptability	7.88±1.11^{a}	7.20±1.30^{b}	6.40±1.65^{c}	6.83±1.60^{c}	6.17±1.66^{c}	6.23±1.50^{c}

T1, T2, T3, T4, T5 and T6 represent WF:GGF flour's different blend ratios for making crackers (100:0, 80:20, 60:40, 40:60, 20:80 and 0:100).
Note: The data are shown as mean±SD.
Different letters in the same row indicate significant differences ($P \leq 0.05$) among samples.

4. CONCLUSIONS

The present study assessed key effects of replacing wheat flour (WF) with green gram flour (GGF) in savory crackers. The results indicate GGF as a potential alternative to WF in bakery products, especially in crackers. The GGF enriched crackers exhibited improved nutritive and functional properties, with the elevated antioxidant activities possibly providing health benefits to consumers, and perhaps extending shelf life. However, in sensory acceptance testing, the GGF enriched crackers received slightly poorer scores than the baseline WF based crackers. Overall, GGF appears to be a good alternative to WF, especially for use in gluten-free bakery products. Other types of bakery products other than crackers may be assessed in further studies, for the suitability of GGF as raw material.

ACKNOWLEDGEMENTS

This research was financially supported by the Prince of Songkla University, Surat Thani Campus, Collaborative Research Fund, and by additional funding from the Prince of Songkla University, Surat Thani Campus, in 2016. The authors would like to thank Assoc. Prof. Dr. Seppo Karrila, Mr. Asrof Kareena and Ms. Hatairad Phetsang for their support and contributions to this work. Authors would also like to acknowledge Food Innovation and Product Development laboratory for the provided research space and equipment support.

REFERENCES

Adsule R.N., Kadam S.S. and Salunkhe D.K. 1986. Chemistry and technology of green gram (*Vigna radiata* [L.] Wilczek). Crit. Rev. Food Sci. Nut. 25:73.

Ahmed Z.S. and Abozed S.S. 2015. Functional and antioxidant properties of novel snack crackers incorporated with Hibiscus sabdariffa by-product. J. Adv. Res. 6:79.

Aktumsek A., Zengin G., Guler G.O, Cakmak Y.S. and Duran A. 2013. Antioxidant potentials and anticholinesterase activities of methanolic and aqueous extracts of three endemic *Centaurea* L. species. Food Chem. Toxic.55:290.

AOAC. 2000. Official methods of analysis of AOAC international (17th Ed.). MD, Gaithersburg: Association of Official Analytical Chemists.

Benzie I.F. and Strain J.J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. An. Biochem. 239:70.

Chandra S., Singh S. and Kumari D. 2015. Evaluation of functional properties of composite flours and sensorial attributes of composite flour biscuits. J. Food Sci. Technol. 52:3681.

Chevallier S., Colonna P., Valle D.G. and Lourdin D. 2000. Contribution of major ingredients during baking of biscuit dough systems. J. Cereal Sci. 31:241.

Dachana K.B., Rajiv J., Indrani D. and Prakash J. 2010. Effect of dried moringa (*Moringaoleifera Lam*) leaves on rheological, microstructural, nutritional, textural and organoleptic characteristics of cookies. J. Food Quality. 33:660.

Dokić L., Pajin B., Fišteš A., Šereš Z., Simović D.Š. and Krstonošić V. 2015.Rheological and textural properties of cracker dough with the addition of pea dietary fiber. Acta Periodic. Technol. 46:29.

FAO. 2003. Food energy-methods of analysis and conversion factors (Food and Nutrition Paper 77). Rome, Italy: Food and Agriculture Organization of the United Nation.

Ferruzzi M.G, Böhm V., Courtney P.D. and Schwartz S.J. 2002. Antioxidant and antimutagenic activity of dietary chlorophyll derivatives determined by radical scavenging and bacterial reverse mutagenesis assays. J. Food Sci. 67:2589.

Filipčev B., Šimurina O. and Bodroža-Solarov M. 2014. Quality of gingernut type biscuits as affected by varying fat content and partial replacement of honey with molasses. J. Food Sci. Technol. 51:3163.

Graham P. H. and Vance C. P. 2003. Legumes: Importance and Constraints to Greater Use. Plant Physiol. 131:872.

Julianti E., Rusmarilin H., Ridwansyah. and Yusraini, E. 2017. Functional and rehological properties of composite flour from sweet potato, maize, soybean and xanthan gum. J. Saudi Soc. Agric. Sci. 16:171.

James D. and Rintoul L. 1982. Protein-water interactions in solution: The water-gelatin-electrolyte system. Aus. J. Chem. 35:1157.

Karthiga S. and Jaganathan D. 2013. Total antioxidant capacity and total phenol content of pulses and root vegetables commonly used in India. Int. J. Food Nut. Sci. 2:24.

Roudaut G., Dacremont C. and Meste M.L. 1998. Influence of water on the crispness of cereal-based foods: acoustic, mechanical, and sensory studies. J. Texture Stud. 29:199-213.

Sánchez-Moreno C., Larrauri J.A., and Saura-Calixto, F. 1999. Free radical scavenging capacity and inhibition of lipid oxidation of wines, grape juices and related polyphenolic constituents. Food Res. Int. 32:407.

Sedej I., Sakač M., Mandić A., Mišan A., Pestorić M., Šimurina O. and Čanadanović-Brunet J. 2011. Quality assessment of gluten-free crackers based on buckwheat flour. LWT-Food Sci. Technol. 44:694.

Sharma P. and Gujral H.S. 2014. Anti-staling effects of beta-glucan and barley flour in wheat flour chapatti. Food Chem. 145:102.

Siaw C.L., Idrus A.Z. and Yu S.Y. 1985. Intermediate technology for fish cracker ('keropok') production. Int. J. Food Sci. Technol. 20:17.

Singleton V.L., Orthofer R. and Lamuela-Raventós R.M. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-ciocalteu reagent. Method. Enzymol. 299:152.

Zhishen J., Mengcheng T. and Jianming W. 1999. The determination of flavonoid contents in mulberry and their scavenging effects on super oxide radicals. Food Chem. 64:555.

Paper Received March 22, 2017 Accepted June 26, 2017

PAPER

MODELING OF COOKING AND COOLING PROCESSES OF TUNISIAN SALAMI

J. MEJRI*[a], M.N. MELKI[a] and M. MEJRI[b]

[a]Département Génie Mécanique et Agro-Industriel, Ecole Supérieure des Ingénieurs de Medjez el Bab, ESIM, 9070 Medjez el Bab, Tunisia

[b]Unité de recherche: Les énergies renouvelables en agriculture et agro-industrie, Ecole Supérieure des Ingénieurs de Medjez el Bab, ESIM, 9070 Medjez el Bab, Tunisia

*Corresponding author. Tel.: +216 97 577532; fax: + 216 78561700

E-mail address: jmejri2001@yahoo.fr

ABSTRACT

The aim of this paper is to develop and validate the heat transfer model in the cooking and cooling process of sausage products to analyze its effectiveness and performance. The modeling of the heat transfer was followed by a numerical simulation to predict the temperature evolution as a function of time. Solving the heat equation was carried out using the finite difference method. Discretizing the equation was carried out in space and in time. Then, a program developed on Matlab was used to solve the discrete equation. The cooked meat was in cylindrical shape (D = 5.6 cm x L = 24 cm) and with a weight of 565 g ± 5 g. The predictions from the model are compared with the experimental results with good agreement (R^2 = 0.98 for cooking and R^2 = 0.99 for cooling process). The maximum deviation between measured and calculated temperatures was within 2.59°C for the cooking, within 1.49°C for the cooling by immersion water and within 0.19°C for the air cooling processes. Percentage differences between predicted and experimental temperatures for the cooking, cooling by immersion water and air cooling processes were around 1.58%, 1.72% and 0.66%, respectively. Thus, the developed model can be considered as a valuable tool to assess the effectiveness and the performance of cooking and cooling processes.

Keywords: cooked meat, salami, heat transfer, cooking, cooling, processes, modeling

1. INTRODUCTION

Thermal processing has remained the technology of choice to extend the shelf-life, to improve the eating quality and safety of food products. The meat industry is one of the most important food industries. In the cooked meat industry, rapid cooling is required to cool the meat after finishing the cooking process to safe storage temperature in order to minimize the growth of surviving microorganisms. Guidelines for the control of this cooling process for cooked meats have been issued and recommended by many European countries (SUN & WANG; 2006).
After removal from the cooking process, it is recommended that meat joints should be chilled from 74°C to 10°C within 2.5 h (SUN and WANG, 2006). If the meat products are cooled properly immediately after cooking, potential germination and outgrowth of spore-forming pathogenic microorganisms that are able to survive the heat treatment applied during cooking can be prevented.
In the last decade, a number of heat and mass transfer models for simulating cooling of ready-to-eat (RTE) meat products have been developed (CHEN *et al*., 1999; HU and SUN, 2000; WANG and SUN, 2002a; DELGADO and SUN, 2003; WANG and SUN, 2004; AMÉZQUITA *et al*., 2005; DRUMMOND and SUN, 2008; ; RINALDI *et al*., 2011; CEPEDA *et al*., 2013; COGNÉ *et al*., 2013; KONDJOYAN *et al*., 2013; RINALDI *et al*., 2014; CHAPWANYA and MISRA, 2015).
However, existing models are subject to simplifications that limit their applicability in the meat industry. Some of the most common simplifications include (1) The process of slaughtering animals: ritual slaughter to which depends the obtained meat, (2) The microbiological quality of the meat used in the preparation of hams, (3) The operating parameters of the processing methods used in each country and (4) Consumption patterns of each population, are made. These four parameters have an effect on the quality of salami.
In Tunisia, the numerical modeling of the meat industry processes has not been studied. Therefore, validated computer models to simulate cooking and cooling of cooked meat products are needed. So, in order to simulate and optimize the cooking and cooling process for cooked meats, in the current study, a heat transfer model is developed. The main objective of this research is to define a model that describes heat transfer during cooking and cooling of Tunisian salami and to develop and solve model in proprietary software (MATLAB). The model will be validated under meat processing conditions and adapted to provide accurate simulations based on parameters that can be easily provided by a meat processor. Latest aim is to make the model available to the meat industry.

2. MATERIALS AND METHODS

2.1. Samples

Salami samples were realized at a Tunisian producer (SAVIMO, Boumhel Ben Arous, Tunisia). Salami was made from crushed (minced) meat and emulsified fats. Raw product is filled in a cylindrically shaped gut. Sample dimensions were 5.6 x 24 (diameter x length) and mean weight was 565 g ± 5 g.

2.2. Cooking and cooling treatments

The product has been cooked in steam oven with a volume of 2 x 1 x 1.5 = 3 m^3 until at least 75°C core temperature was reached. Cooking time was 95 min. The steam allows an

excellent heat exchange with the product and ensures good homogeneity of temperature. For the study of the heat transfer and model validation, cooking and cooling trials were carried out in quadruplicate. The oven temperature was monitored with wire thermocouples (E-type; Ni/Al-Ni/Cu). After cooking, cooling is very important step to avoid microbial development. The cooling was carried out in two phases: immersion water and air cooling processes. The first one consists on pulverization of water at 19-20°C during 50 min. For the second step samples were treated with air at temperature of 2-4°C. Temperature data were collected in an Excel (Microsoft product) worksheet.

3. MODEL DEVELOPMENT

3.1. Heat transfer equation

In the cooked meat industry, meat joints are formed by small pieces of boned-out legs and the joints are injected with brine solution. Therefore there is abundance of macro-pores among the meat pieces. For developing the model, the cooked meat sample is assumed to be in a cylindrical shape (Fig. 1).
The heat transfer equation for the cooked meat is heat conduction in a homogeneous, isotropic body without inner heat generation. Initially, the temperature is evenly distributed in the product.

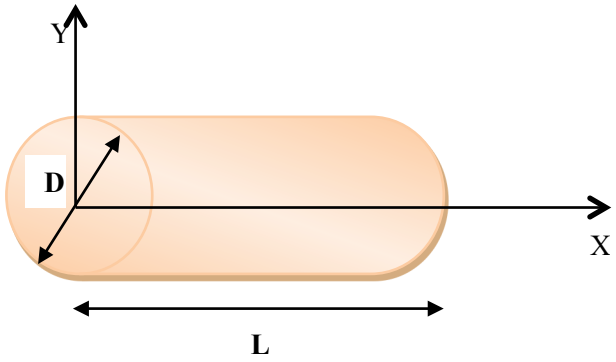


Figure 1. Shape of the cooked meat sample.

$$\rho . Cp . \frac{\partial \mathrm{T}}{\partial \mathrm{t}} = k \nabla^2 T \qquad (1)$$

In a cylindrical coordinate system, the equation of the heat conduction can be written in the following form:

$$\rho . Cp . \frac{\partial \mathrm{T}}{\partial \mathrm{t}} = k \, . \frac{1}{r} \, . \frac{\partial}{\partial r} \left(r . \frac{\partial T}{\partial r} \right) + \frac{\partial^2 T}{\partial z^2} \qquad (2)$$

Or else:

$$\rho . Cp . \frac{\partial \mathrm{T}}{\partial \mathrm{t}} = k . \frac{1}{r} \, . \left(\frac{\partial T}{\partial r} + r \frac{\partial^2 T}{\partial r^2} \right) + \frac{\partial^2 T}{\partial z^2} \qquad (3)$$

The initial condition is as follows: It is considered that the temperature distribution is uniform in the product:

$$t = 0, \quad T(r,t) = T(r,0) = T_0 \tag{4}$$

$$t = 0, \quad T(z,t) = T(z,0) = T_0 \tag{5}$$

The boundary conditions were: Product is maintained at the temperature of the oven during cooking and the temperature of cold room during cooling:

$$T(r,t) = T(z,t) = T_a \tag{6}$$

The physical characteristics of salami (CHEN *et al.*, 1999) are shown in the Table 1.

Table 1. Physical characteristics of salami.

Symbol	Definition	Value	Units
C_p	Heat capacity	3530	J/kg K
K	Thermal conductivity	0.412	W/m K
P	Density	1100	kg/m^3

3.2. Meshing and Discretization of differential equations

It performs a discretization of the domain validation of differential equations: the temperature should be calculated by a finite number of points. To simplify, the selected mesh is a grid of step:

$$\Delta r = \Delta z = p \text{ (Fig. 2).}$$

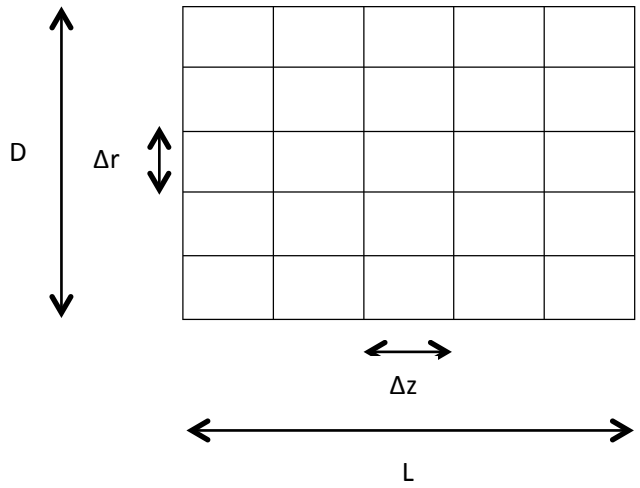


Figure 2. Domain mesh system.

In the system of validating domain of differential equations, any point M is located on a coordinate node (r_i, z_j, t_k), with:

r_i= i x Δr; i between 0 and n,
z_j = j x Δz; j between 0 and m,
t_k= k x Δt; k between 0 and q.

The finite difference approximations for derivatives are one of the simplest and of the oldest methods to solve differential equations. The advent of finite difference techniques in numerical applications began in the early 1950s and their development was stimulated by the emergence of computers that offered a convenient framework for dealing with complex problems of science and technology.
The principle of finite difference methods is close to the numerical schemes used to solve ordinary differential equations. It consists in approximating the differential operator by replacing the derivatives in the equation using differential quotients. The domain is partitioned in space and in time and approximations of the solution are computed at the space or time points. The error between the numerical solution and the exact solution is determined by the error that is committed by going from a differential operator to a difference operator.
The first derivative and the second derivative of the differential equation can be represented in different ways, in the form of finite difference, using the Taylor series development.
The approximation of $\frac{\partial T}{\partial t}$ is obtained by the Taylor expansion in the first order of the function T(r, t).

$$\frac{\partial T}{\partial t} = \frac{T(r,t+dt)-T(r,t)}{dt} \quad (7)$$

The same for the approximation of $\frac{\partial T}{\partial r}$:

$$\frac{\partial T}{\partial r} = \frac{T(r+dr,t)-T(r,t)}{dr} \quad (8)$$

The approximation of $\frac{\partial^2 T}{\partial r^2}$ is obtained by the Taylor expansion in the second order:

$$T(r+dr,t) = T(r,t) + \frac{\partial T}{\partial r}(r,t)dr + \frac{1}{2}\frac{\partial^2 T}{\partial r^2}(r,t)dr^2 \quad (9)$$

$$T(r-dr,t) = T(r,t) - \frac{\partial T}{\partial r}(r,t)dr + \frac{1}{2}\frac{\partial^2 T}{\partial r^2}(r,t)dr^2 \quad (10)$$

$$T(r+dr,t) + (T(r-dr,t) = 2T(r,t) + \frac{\partial^2 T}{\partial r^2}(r,t)dr^2 \quad (11)$$

$$\frac{\partial^2 T}{\partial r^2}(r,t) = \frac{T(r+dr,t)+T(r-dr,t)-2T(r,t)}{dr^2} \quad (12)$$

And similarly for the approximation of $\frac{\partial^2 T}{\partial z^2}$, we obtain:

$$\frac{\partial^2 T}{\partial z^2}(z,t) = \frac{T(z+dz,t)+T(z-dz,t)-2T(z,t)}{dz^2} \quad (13)$$

Hence, the differential equation (3) is approximated as follow:

$$\rho.Cp.\frac{\partial \mathrm{T}}{\partial \mathrm{t}} = k.\frac{1}{r}.\left(\frac{\partial T}{\partial r} + r\frac{\partial^2 T}{\partial r^2}\right) + \frac{\partial^2 T}{\partial z^2}$$

So:

$$\rho.Cp.\frac{T(r,t+dt)-T(r,t)}{dt} = k.\frac{1}{r}.\left(\frac{T(r+dr,t)-T(r,t)}{dr} + r\frac{T(r+dr,t)+T(r-dr,t)-2T(r,t)}{dr^2}\right)+$$

$$+\frac{T(z+dz,t)+T(z-dz,t)-2T(z,t)}{dz^2} \quad (14)$$

3.3. Evaluation of model performance

Model performance evaluation was carried out by comparing the deviation between the observed and predicted temperatures in different (r, z) locations over time. It was estimated by calculating the root mean square error (RMSE) per each validation test (Equation 15).

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}(T_{observed}-T_{predicted})^2}{n}} \quad (15)$$

Where n represents the number of observations.
The mean RMSE among the validation tests ± standard deviation was used to report the overall performance model.
In addition, the assessment of the prediction accuracy of the numerical simulation was made by comparison of the numerically calculated temperature with the experimentally measured temperature. The percentage differences were calculated from (DELGADO and SUN, 2003):

$$Diff(\%) = \frac{Predicted\ temperature - Experimental\ temperature}{Experimental\ temperature} x100 \quad (16)$$

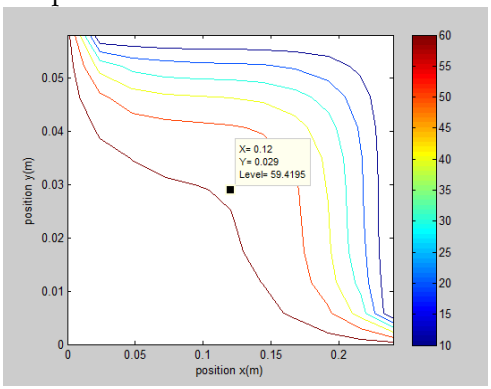
4. RESULTS AND DISCUSSIONS

4.1. Temperature distribution

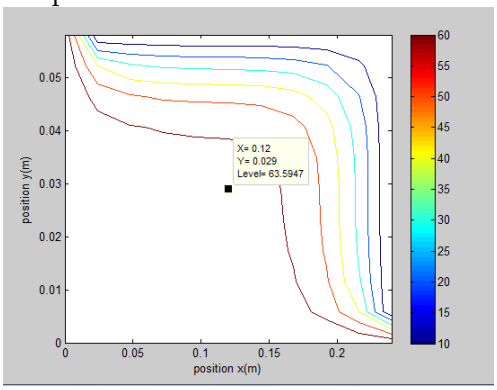
During cooking, it is assumed that the initial temperature of the cooked meats is homogeneous. Initially, the temperature of the salami is 15°C and the oven temperature is

20°C. However, we measured the temperature after one hour (3600 s), since we cannot insert probe thermometer at the beginning of cooking as the initial state of salami is pasty.
The results of the numerical solution of the discrete equation are shown in Fig. 3. We observe that as the heat flows by conduction described by the Fourier equation in our model, the interior temperature rises slowly at both heating ends. Also, we note that the temperature equilibrates in the interior of the salami as time proceeds.
It may be noted that this typical profile of the heat transfer will be obtained irrespective of a 1-D or 2-D approach, given the temperature profile is evaluated at the central axis of the salami. However, when considering the spatial temperature profile, the edges of the salami sample are open to the ambient environment, which causes a heat loss. This is in agreement with the experimental results reported by CHAPWANYA and MISRA (2015).
Finally, we also observe from Fig. 3 that the temperature at the core/geometric center of the salami of diameter 5.6 cm reaches a temperature of 59.41°C in 60 min. Then, the temperature continues to rise, reaching 75.21°C after 35 min of heating. Conduction heating of salami involves the transfer of kinetic energy between non-isothermal adjacent layers, without mass transfer. TOM *et al*. (2013) employed Kelvin equation and Halsey equation to determine the average pore size of beef. The authors report that the pores enlarge with increase in moisture levels and sorption temperature. It should be noted that the porosity refers to volume fraction of solvent. We can suppose, in our case, that the porosity of artificial gut and moisture follow this relation and also support the observations made by TOM *et al*. (2013).
RINALDI *et al.* (2011) studied simultaneous heat and mass transfer for cooking process of Mortadella Bologna PGI. The authors developed mathematical model to estimate thermal and mass properties by means of direct finite differences method. Weight loss was significantly lower at 80°C (4.56%) in comparison with those obtained at 90 and 100°C (5.46 and 5.44%), while no significant differences were found between the latter. Water content decreased at the surface and half-radius of the samples as oven temperature increased, as expected, due to the evaporation. In contrast, water content did not show significant differences at Mortadella center among the three temperatures (80, 90 and 100°C), probably due to the low water diffusion rate within this section. This aspect didn't carry out in our work. It may be studied in the future. Thermal processing involves not only simultaneous heat and mass transfer, but also physicochemical reactions, such as protein denaturation. The thermal properties of salami change during processing.
Temperature distributions for cooling process (water immersion) are shown in Fig. 4. We also observe from that the temperature at the core/geometric center of the salami is 5.6 cm in diameter reaches a 30.58°C in 50 min of cooling. Predicted temperature distributions for cooling process (air) are shown in Fig. 5. We also observe from that the temperature at the core/geometric center of the salami is 5.6 cm in diameter reaches a 10.45°C in 40 min of cooling. For the two cooling phases, cooling time to reach 10.45°C at the salami center was 90 min.
An integrated model of heat transfer and dynamic growth of *C. perfringens* in cured salami was developed and validated for three cooling scenarios (AMÉZQUITA *et al*., 2005). Results showed good agreement between predicted and experimental data. So, effective integration of engineering and microbial modeling offer a useful quantitative tool to support several food safety and microbiological risk assessment.
In a first time, the agreement between the predicted and the experimental kinetics allows us to validate the model hypothesis and has ensured us a quite good accuracy even if we are aware of the fact that this model is quite simplified in the heat transfer on a homogeneous and isotropic medium. In a second time, to face this challenge and improve the efficiency of our model, the next steps of this work will be to implement the composition of salami and its various ingredients.

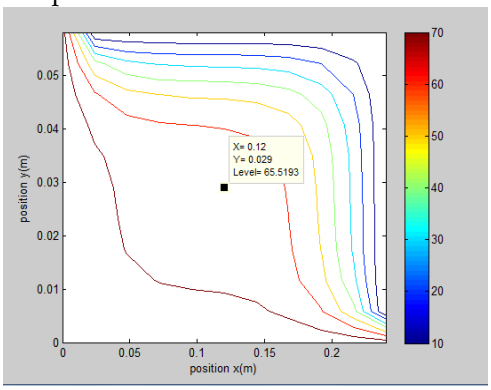
Temperature distribution for t = 3600 s



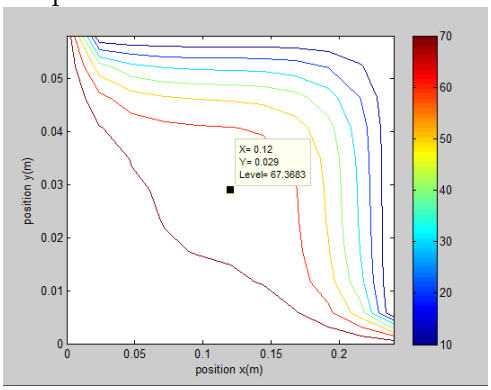
Temperature distribution for t = 3900 s



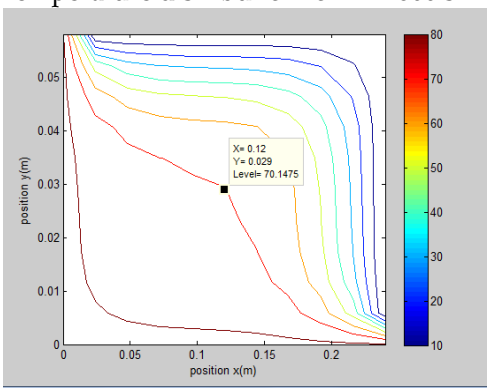
Temperature distribution for t = 4200 s



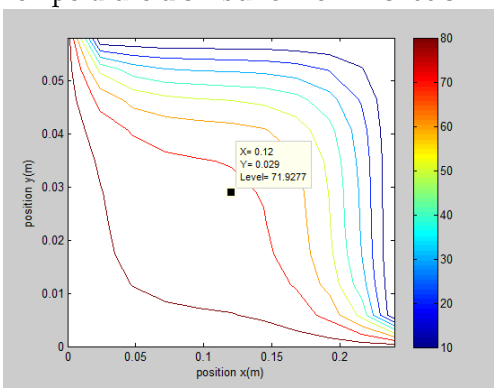
Temperature distribution for t = 4500 s



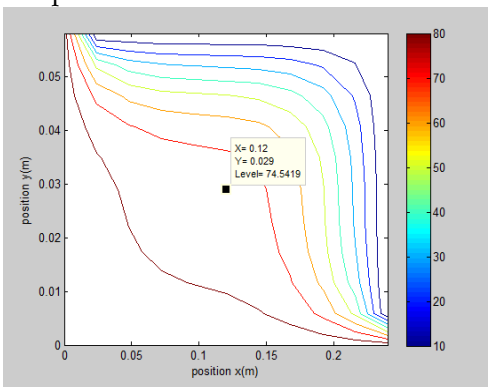
Temperature distribution for t = 4800 s



Temperature distribution for t = 5100 s



Temperature distribution for t = 5400 s



Temperature distribution for t = 5700 s

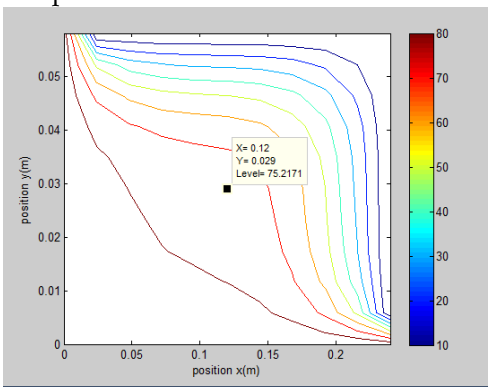
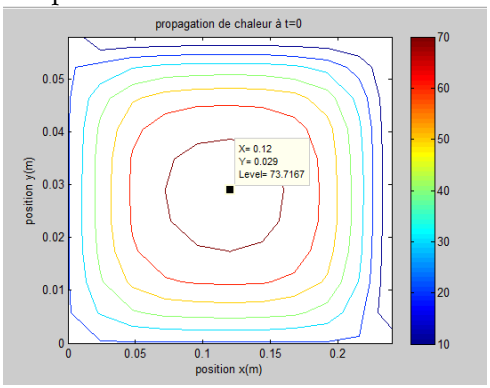
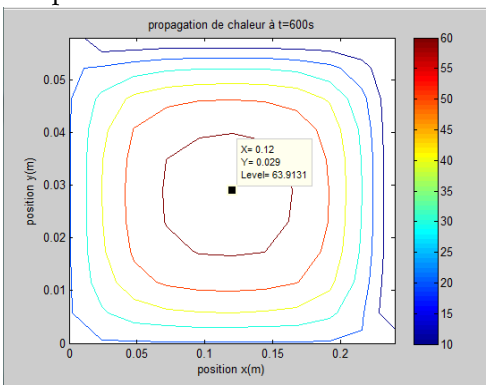


Figure 3. Predicted temperature (°C) distributions for cooking process.

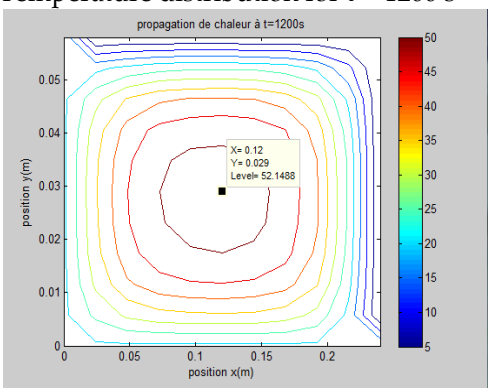
Temperature distribution for t = 0 s



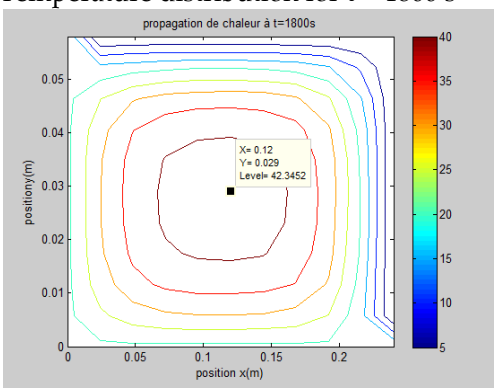
Temperature distribution for t = 600 s



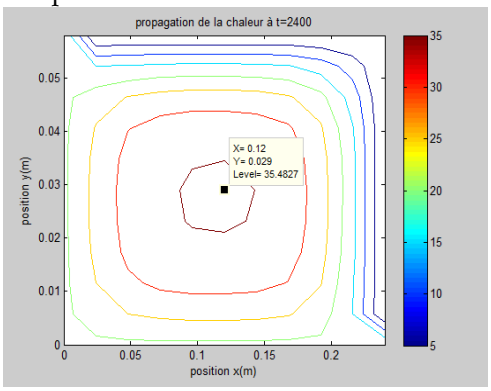
Temperature distribution for t = 1200 s



Temperature distribution for t = 1800 s



Temperature distribution for t = 2400 s



Temperature distribution for t = 3000 s

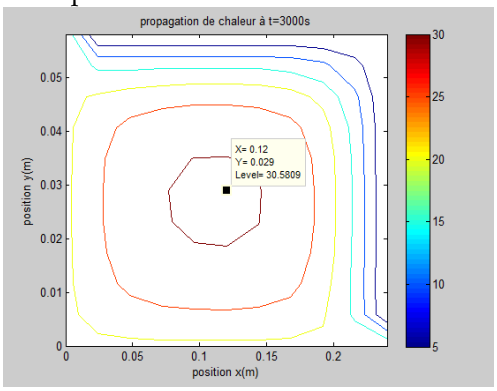


Figure 4. Predicted temperature (°C) distributions for cooling process (water immersion).

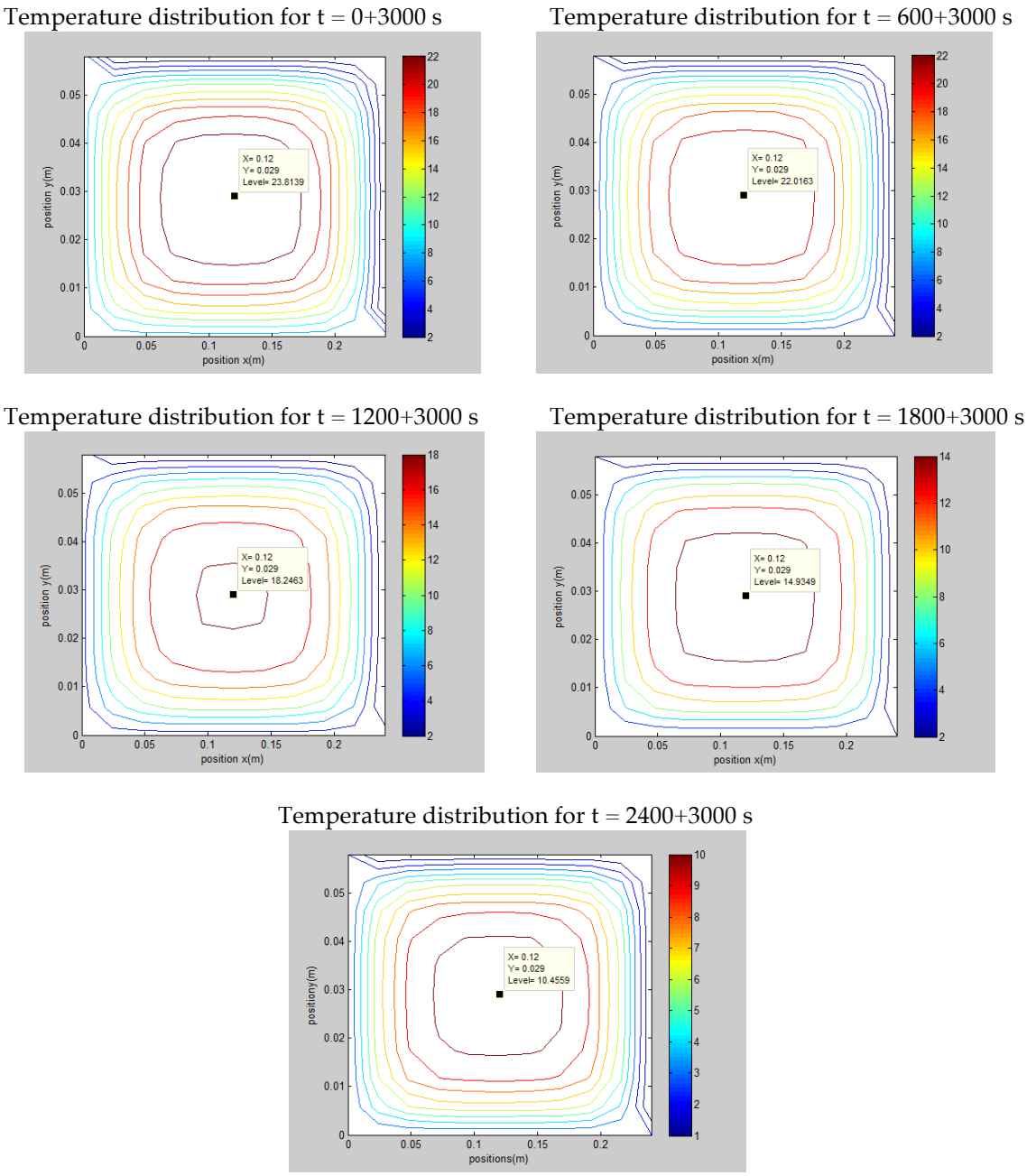


Figure 5. Predicted temperature (°C) distributions for cooling process (air).

The heat released from the product surface to the cooling medium (water) by convection, depends on its properties and parameters, such as its viscosity, density, turbulence and so on, as well as on surface roughness and on the geometry of the salami. Comparing the two cooling processes, we note that the kinetics of the process using water immersion (0.87 °C / min) is faster than that of the cooling air process (0.33 °C / min). Ayadi and colleagues (2009) shows that the variation of the heat penetration is attributed to raw material and formulation. Materiel composition affects the hardness and the elasticity of cooked meat during heating. Thus, textural and sensorial characteristics of salami change.

In previous work, mathematical modelling of heat transfer in Mortadella Bologna PGI during evaporative pre-cooling was studied (RINALDI *et al.*, 2014). Mortadella evaporative pre-cooling process from 70 to 50°C at core was investigated. The effects of ventilation and water spraying with different intervals (0, 5, 10 and 15 min) were tested at core and surface temperatures and cooling times were compared. Both ventilation and water spraying increased the cooling rate.
The slowest and the fastest cooling rates were obtained under no ventilation and no water spraying condition, and with ventilation and water spraying every 5min, respectively. In this last condition, about half time was required to reach 50 °C at the product thermal core compared to first one sample. Compared to these results we can conclude that two phases cooling processes (immersion water + air) is the fastest than the combined one (ventilation + spraying).

4.2. Model performance

In order to validate the model, we set-up the parameters for numerical simulations as per those of experiments. Fig. 6 presents the evolution of both experimental and simulated salami temperature profiles. Firstly, we observe that the temperature of the geometric center of the salami shows a continuous increase and decrease respectively for cooking and cooling processes.
To evaluate the accuracy of the models, we employ the statistical criterion of the Root Mean Squared Error (RMSE). The proposed model was in good agreement with the experimental values when the simulations were carried out including initial conditions, boundary conditions and physical characteristics of salami. However, the best prediction was obtained when both thermal conductivity and specific heat were modeled as state-dependent functions in the simulation (CHEN *et al.*, 1999).

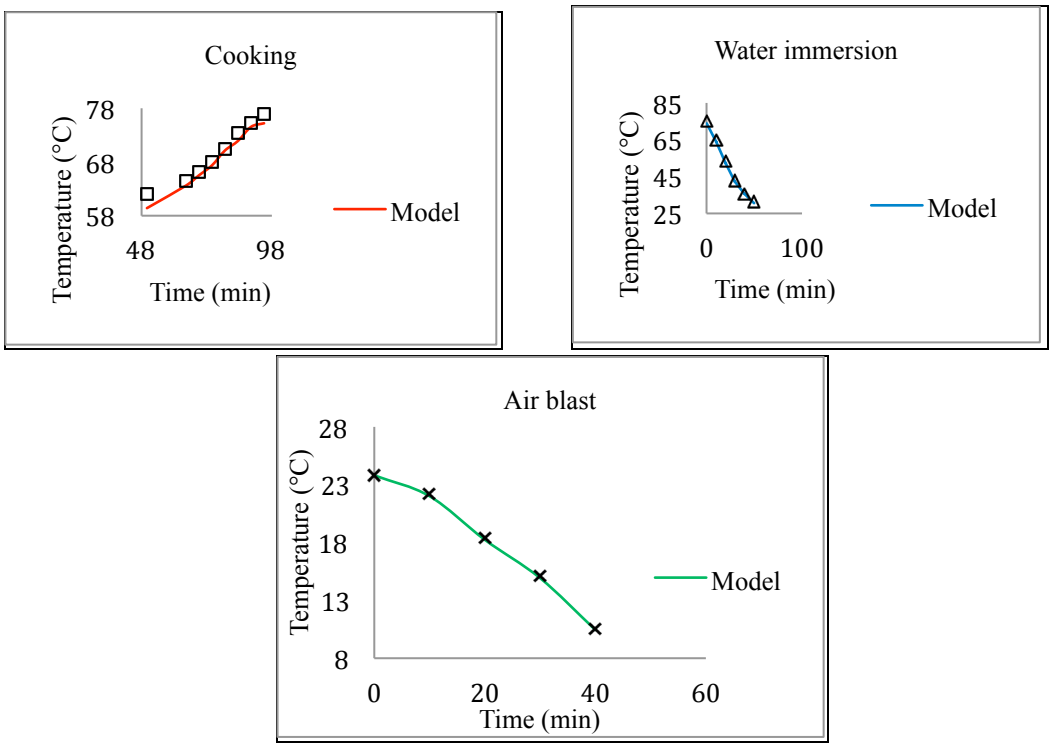


Figure 6. Evolution of experimental (open symbols) and simulated temperature (solid line) at the core of salami.

Calculated RMSE and percentage differences values were presented in Table 2.

Table 2. Evaluation of model performance at core of product.

Processes	RMSE (°C)	Diff (%)
Cooking	1.09 ± 0.72	1.58
Cooling (water immersion)	0.87 ± 0.45	1.72
Cooling (air blast)	0.116 ± 0.087	0.66

In theory, the accuracy of the predictions can be improved when a method for getting better estimations of initial temperatures within the product prior to cooling, and temperature distribution of the air surrounding the products are available. This difference could be due to several reasons (PAN *et al*., 2000). The primary cause for this difference may be the slight dislocation of the thermocouple junction, placed initially at the center of the salami. Although the thermocouple location was verified at the end of the experiment, the non-rigid state of the cooked salami may result in some movement of the thermocouple. The thermal properties were estimated for use in the model. A certain level of uncertainty in predicted values is due to the estimated values of properties. Regardless of these uncertainties, the predicted values are in good agreement with the experimental data as seen in Figure 6. Future research is needed to determine the variation of thermal properties with temperature for salami cooking.
In previous work, transient temperature distributions inside the chicken patties were predicted (CHEN *et al*., 1999). Samples were cooked in a convection oven for model validation. The predicted transient center temperature had an error of 3.8 ± 5.7 °C, as compared to experimental data. However, the best prediction was obtained when both thermal conductivity and specific heat were modeled as state-dependent functions in the simulation (CHEN *et al*., 1999).
A similar approach was applied for simulation and experimental validation of simultaneous heat and mass transfer for cooking process of Mortadella Bologna PGI (RINALDI *et al*., 2011). Obtained errors (RMSE) were higher compared to this work. This result is confirmed by RINALDI *et al*. (2014), it is probably due to both phenomena summed together by coupling mass end heat transfer.

5. CONCLUSIONS

The cooking and cooling processes of Tunisian salami (cooked meat) are modeled. Two cooling processes are carried out: cooling air blast and cooling water immersion. The finite difference numerical method is used to solve the model. The model demonstrated good predictive capabilities for core temperature of the salami. The model has some issues in predicting temperatures for some hams that needs to be addressed (type of gut, composition, formulation).
The mathematical method developed has successfully described the temperature evolution in the salami. Experimentally measured and calculated results are in good agreement, especially during the first (water immersion) and second (air blast) stages of cooling. The maximum deviation between measured and calculated temperatures was within 2.59°C for the cooking, within 1.49°C for the cooling by immersion water and within 0.19°C for the air blast cooling processes. Percentage differences between predicted

and experimental temperatures for the cooking, cooling by immersion water and air blast cooling processes were around 1.58%, 1.72% and 0.66%, respectively.
Although we considered the case of salami heating to demonstrate the validity of the model, the modification of boundary conditions for other cases heating with flipping, oven roasting or frying should be straightforward. The opening of the oven while cooking the salami may be incorporated with appropriate functional relationship of the temperature determined experimentally with time. Furthermore, with incorporation of equations for microbial inactivation, this model could also be used to predict microbial safety.

ACKNOWLEDGEMENTS

Author gratefully acknowledges Mr Sahbi BOUAZIZI (English teacher at Ecole Supérieure des Ingénieurs de l'Equipement Rural, ESIER, Medjez el Bab, Tunisia) for reviewing the English of this paper.

NOMENCLATURE

ρ	Density (kg/m^3)
Cp	Heat capacity (J/kg K)
k	Thermal conductivity (W/m K)
T	Temperature (°C)
t	Time (s)
∇	Divergency operator
r	Radius (m)
D	Diameter (m)
L	Length (m)

REFERENCES

Amézquita A., Weller C.L., Wang L., Thippareddi H. and Burson D.E. 2005. Development of an integrated model for heat transfer and dynamic growth of *Clostridium perfringens* during the cooling of cooked boneless ham. International Journal of Food Microbiology 101:123-144.

Ayadi M.A., Makni I. and Attia H. 2009. Thermal diffusivities and influence of cooking time on textural, microbiological and sensory characteristics of turkey meat prepared products. Food and Bioproducts Processing 87:327-333.

Cepeda J.F., Weller C.L., Thippareddi H., Negahban M. and Subbiah J. 2013. Modeling cooling of ready-to-eat meats by 3D finite element analysis: Validation in meat processing facilities. Journal of Food Engineering 116:450-461.

Chapwanya M. and Misra N.N. 2015. A mathematical model of meat cooking based on polymer–solvent analogy. Applied Mathematical Modelling 39:4033-4043.

Chen H., Marks B.P. and Murphy R.Y. 1999. Modeling coupled heat and mass transfer for convection cooking of chicken patties. Journal of Food Engineering 42:139 - 146.

Cogné C., Nguyen, P.U., Lanoisellé J.L., Van Hecke E. and Clausse D. 2013. Modeling heat and mass transfer during vacuum freezing of puree droplet. International Journal of Refrigeration 36:1319-1326.

Delgado A.E. and Sun D.W. 2003. One-dimensional finite difference modeling of heat and mass transfer during thawing of cooked cured meat. Journal of Food Engineering 57:383-389.

Drummond L. and Sun D.W. 2008. Temperature evolution and mass losses during immersion vacuum cooling of cooked beef joints – A finite difference model. Meat Science 80:885-891.

Holm E.S, Adamsen A.P.S., Feilberg A., Schäfer A., Løkke M.M. and Petersen M.A. 2013. Quality changes during storage of cooked and sliced meat products measured with PTR-MS and HS-GC–MS. Meat Science 95:302-310.

Hu Z. and Sun D.W. 2000. CFD simulation of heat and moisture transfer for predicting cooling rate and weight loss of cooked ham during air-blast chilling process. Journal of Food Engineering 46:189-197.

Kondjoyan A., Oillic S., Portanguen S. and Gros J.B. 2013. Combined heat transfer and kinetic models to predict cooking loss during heat treatment of beef meat. Meat Science 95:336-344.

Pan Z., Singh R.P. and Rumsey T.R. 2000. Predictive modeling of contact-heating process for cooking a hamburger patty. Journal of Food Engineering, 46:9-19.

Rinaldi M., Chiavaro E., Gozzi E. and Massini R. 2011. Simulation and experimental validation of simultaneous heat and mass transfer for cooking process of Mortadella Bologna PGI. International Journal of Food Science and Technology 46:586-593.

Rinaldi M., Chiavar, E. and Massini R. 2014. Mathematical Modelling of Heat Transfer in Mortadella Bologna PGI during Evaporative Pre-Cooling. International Journal of Food Engineering 10:233-241.

Sun D.W. and Wang L. 2006. Development of a mathematical model for vacuum cooling of cooked meats. Journal of Food Engineerin, 77 379-385.

Tom A., Bruneau D., Alexis K. and Aregba A.W. 2013. Desorption isotherms for fresh beef : an experimental and modeling approach. Meat Science, 96:1417-1424.

Wang L. and Sun D.W. 2002. Evaluation of performance of slow air, air blast and water immersion cooling methods in the cooked meat industry by the finite element method. Journal of Food Engineering, 51:329-340.

Wang L. and Sun D.W. 2004. Effect of operating conditions of a vacuum cooler on cooling performance for large cooked meat joints. Journal of Food Engineering, 61:231-240.

Paper Received November 5, 2016 Accepted June 25, 2017

PAPER

BIOLOGICALLY ACTIVE COMPOUNDS AND ANTIOXIDANT CAPACITY OF *CICHORIUM INTYBUS* L. LEAVES FROM MONTENEGRO

D. JANCIC*[1], V. TODOROVIC[2], H. SIRCELJ[3], M. DODEVSKA[4], B. BELJKAS[1], D. ZNIDARCIC[3] and S. SOBAJIC[2]

[1]LLC Center for Ecotoxicological Research Podgorica, Bulevar Sarla de Gola 2, Podgorica, Montenegro

[2]University of Belgrade, Faculty of Pharmacy, Department of Bromatology, Vojvode Stepe 450, Belgrade, Serbia

[3]Agronomy Department, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, Ljubljana, Slovenia

[4]Center for Food Analysis Ltd, Zmaja od Nocaja 11, Belgrade, Serbia

*E-mail address: dejan.jancic@ceti.co.me

ABSTRACT

The aim of this study was to determine biologically active substances (BAS) in the samples of *Cichorium intybus* L. leaves from different sources (wild and cultivated) in Montenegro and to investigate the potential influence of location and origin on the BAS. Fiber and fatty acid composition, amount of pigments, total phenols and flavonoids and some phenolic acids were analyzed. Antioxidant activity was also determined by three methods (DPPH, FRAP and ABTS) and the results obtained from all tests were used to calculate the antioxidant potency composite index (ACI). The dietary fiber profile confirmed chicory leaves as an important source of fiber. The majority of fats in chicory leaves consist-of unsaturated fatty acids, while saturated fatty acids were represented mainly by palmitic acid. Chlorophyll a and b, lutein and β-carotene were the main pigments in chicory leaves. ACI index had a good correlation with the total phenolic and total flavonoid content. All these features reinforced the interest of including chicory in modern diet as a healthy alternative to the variety of commonly used vegetables.

Keywords: antioxidant activity, chicory, fibres, fatty acids, pigments

1. INTRODUCTION

Chicory plant (*Cichorium intybus* L.) is a member of the *Asteraceae* family. It is an erect, glandular, biennial herb with a tuberous taproot and a rosette of 30-70 leaves, which grows up to 90 cm in height. The leaves of several *Cichorium* species have been used for centuries as part of traditional diet in the Mediterranean countries (as salads or cooked vegetable, and in meat dishes), while the roots (var. *sativum*) are baked, ground, and used as a substitute for coffee and inulin source. The bitter taste of chicory leaves is very well appreciated in certain Mediterranean cuisines (in Italy, Spain, Greece, Turkey, and so on). In the Montenegrin part of the Adriatic coast, especially in Boka Bay, wild chicory leaves are used in traditional diets, whereas, the cultivation of this vegetable crop just recently began to expand in certain areas of Montenegro. Most of the information available on plant chemical composition refers to the root and seed, while both the leaves and the differences between wild and cultivated plants have seldom been investigated (JAN *et al*., 2011; YING and GUI, 2012). Although, data exist on the differences in composition of some BAS in chicory leaves (SAHAN *et al*., 2017; D'ACUNZO *et al*., 2017), only data on nutrient composition and its difference between wild and cultivated plants from Montenegro is available (JANCIC *et al*., 2016). BAS include a group of nutritive components, which are naturally present in plants, fruits, and vegetables. Conducting a research on BAS is important due to their numerous health benefits. Biologically Active Substances can reduce the risks of vascular and renal diseases, lower glycemic index in diabetics, reduce risks of cancer and increase bifidobacteria population in the colon (GUHR and LaCHANCE, 1997; HASLER, 1998).
The aim of this study was to estimate the profile of biologically active ingredients in chicory leaves, growing in different locations throughout Montenegro. Within the category of BAS, the profile of dietary fiber (total, soluble and insoluble dietary fibers, hemicelluloses, cellulose, lignin, and fructan content), essential fatty acid profile, pigments and major antioxidant compounds were determined. This study is the first comprehensive study on the above named compounds in chicory leaves that are growing in Montenegro.

2. MATERIALS AND METHODS

2.1 Sample collection

Fresh materials (leaves) of the same chicory variety were collected from different locations in Montenegro. Out of the nine samples examined, seven were samples of wild plants (Zoganje, Risan, Podgor, Tivat, Pricelje, Plavnica and Pljevlja locations) and two were samples of greenhouse cultivated plants (Komani and Susanj). All plant leaves (ca. 2 kg) were sampled in their vegetative stage (before flowering) of growth between May and July, 2015. The leaves were collected in the morning.

2.2. Sample preparation

Fresh leaves were separated and all dirt was removed. A part of the leaves were immediately wrapped in aluminum foil to avoid degradation of pigments by light.

One part of the fresh plant material (ca. 1 kg) was milled using an electric grinder (IKA A11, Staufen, Germany) and stored in well-labeled air tight polyethylene bottles at -18 °C until the time for chemical testing in the laboratory. The remaining samples (for the purpose of polyphenol, pigment, and antioxidative capacity determinations) were lyophilized (Alpha 1-4LD, Christ, Germany) and grounded to a fine powder using a planetary ball mill (S100, Retsch, Germany) and stored at room temperature in tightly closed humidity-proof plastic containers until analysis. Before and after lyophilisation, samples were weighed in order to recalculate the data obtained from dry weight (DW) to fresh weight (FW).

2.3. Determination of content of total, soluble and insoluble dietary fibers, hemicelluloses, cellulose, lignin and fructan

For the determination of total (TDF), soluble (SDF) and insoluble (IDF) fractions of dietary fibers, samples were analyzed in accordance with AOAC 991.43 following the enzymatic-gravimetric procedure (AOAC, 1995) as described by LEE *et al*. (1992).
Determination of neutral detergent fibers (NDF), acid detergent fibers (ADF) and acid detergent lignin (ADL) was performed in order to obtain the content of hemicellulose, cellulose and lignin. NDF was determined gravimetrically as a fibrous residue (primarily cell wall components of plants as cellulose, hemicellulose, and lignin), which was formed after refluxing with a neutral detergent solution and heat-stable amylase. ADF was determined gravimetrically as the residue of cellulose, lignin, and heat damaged protein and a portion of cell wall protein and minerals (ash) remaining after extraction with an acidified quaternary detergent solution. ADL was determined gravimetrically upon treatment with an acid detergent solution, which includes cooking, filtering and drying. Hemicellulose, cellulose and lignin content were calculated as follow: a) Hemicellulose=NDF-ADF; b) Lignin=ADL; c) Cellulose=NDF-Hemicellulose-Lignin (GOERING and VAN SOEST, 1970; VAN SOEST *et al*., 1991; AOAC, 1990).
Fructan content was measured in accordance with the enzymatic/spectrophotometric AOAC 999.03 and AACC 32.32 methods using the enzyme assay kit K-FRUC (Megazyme, Bray, Ireland) (McCLEARY and BLAKENEY, 1999; AOAC, 2002; AACC INTERNATIONAL, 2000). In the process of fructan analysis, raffinose oligosaccharides were removed with the α-galactosidase treatment (Megazyme, Bray, Ireland) before degradation of starch, maltosaccharides and sucrose, as described in the kit.

2.4. Determination of fatty acids profile

The method recommended by the Association of Official Analytical Chemists AOAC 930.09 was used for the determination of crude lipid content (AOAC, 1990).
Fatty acids were determined by gas chromatography (GC) after the following trans-esterification procedure: fatty acids in crude fat with added hexane were methylated by shaking for 20 s with 5 mL of 2 M KOH. Sample was heated for 60 s on water bath (60°C) and additionally shaken up for 20 s. After addition of 10 mL of 1 N HCl, the mixture was shaken up well again and another portion of hexane was added. In the separated phases, fatty acid methyl esters (FAME) were in the upper hexane layer. FAMEs were analyzed using gas chromatograph with FID detector (Shimadzu GC-17A, Japan) and Supelco SP-2560 Fused Silica Capillary Column (100 m, id 0.25 mm, deb.

phase: 0.20 μm, Sigma-Aldrich, Germany). Fatty acid identification was performed by comparing the relative retention times of FAME peaks from samples with the standards. FAME standard (Supelco 37 Component FAME Mix, Sigma Co, St Louis, MO, USA) was used for the identification process. All solvents and chemicals were of analytical grade.

2.5. Determination of pigments

Extraction of pigments was carried out according to ZNIDARCIC *et al*. (2011): 100 mg of the dry leaf powder with 5 mL of ice-cold acetone on an ice bath, using T-25 Ultra-Turrax (Ika-Labortechnik, Staufen, Germany) homogenizer for 25 s. All extraction procedures were performed in dim light. Acetone extracts were filtered through 0.2 μm Minisart SRP 15 filter (Sartorius Stedim Biotech GmbH, Goettingen, Germany) and then subjected to HPLC gradient analysis (a Spherisorb S5 ODS-2 250x4.6 mm column with an S5 ODS-2 50x4.6 mm precolumn, Alltech Associaties, Inc., Deerfield, USA), using the following solvents: solvent A: acetonitrile/methanol/water (100/10/5, v/v/v); solvent B: acetone/ethylacetate (2/1, v/v), at a flow rate of 1 mL/min, employing linear gradient from 10% solvent B to 70% solvent B in 18 min, with a run time of 30 min, and photometric detection at 440 nm. The HPLC analysis was performed on a Spectra-Physics HPLC system with Spectra Focus UV-VIS detector (Fremont, USA). Pigments were quantified by determining peak areas under the curve in the high-performance liquid chromatograms calibrated against known amounts of standards. Each peak was confirmed by the retention time and characteristic spectra of the standards. The following standards were used for the determination of photosynthetic pigments: neoxanthin, violaxanthin, antheraxanthin, zeaxanthin, lutein, chlorophyll a and b, pheophytin a and b, and α-, β-carotene, all from DHI LAB products (Hoersholm, Denmark). All standards were highly purified. The solvents acetone, ethylacetate, methanol, and acetonitrile were from Merck and HPLC grade.

2.6. Determination of total polyphenol, total flavonoid, chlorogenic and caffeic acid content

Plant extracts were prepared as described by WEN *et al*., 2005. One gram of each lyophilized sample was taken in a measuring flask and dissolved in methanol/water/trifluoroacetic acid (50/50/0.1, v/v/v) mixed solvent, and then the volume of the turbid fluid was adjusted to 10 mL accurately. The mixture was sonicated for 30 min at room temperature, centrifuged at 3000 rpm for 5 min, and finally filtered through a 0.45 μm nylon filter.

Total polyphenol content (TPC) of plant extracts was determined spectrophotometrically according to the Follin-Ciocalteau method as described by TODOROVIC *et al*. (2015). Briefly, to a 0.5 mL aliquot of samples, 2.5 mL Folin-Ciocalteu's reagent, 30 mL distilled water and 7.5 mL of 20% Na_2CO_3 were added and filled up to 50 mL with distilled water. The absorbance of blue coloration was measured at 765 nm against a blank sample, after 2 h storage in the dark. Gallic acid (Sigma Aldrich, Germany) was used as the standard and the results were expressed as mg Gallic Acid Equivalents (GAE) per gram of fresh sample. A calibration curve was developed for the working solutions of gallic acid in the concentration range of 0-80 mg/mL and it showed good linearity.

For the purpose of determination of total flavonoid content (TFC), the following procedure was followed (TODOROVIC *et al*., 2015): 0.5 mL of each extract was transferred into a 5 mL volumetric flask and 0.15 mL of the 5% $NaNO_2$ was added for 6 min and evenly mixed. After that 0.15 mL of the 10% $AlCl_3$ solution was added and shaken up. Six minutes later 1 mL of the 1 M NaOH solution was added. The mixture was diluted with distilled water up to 5 mL. Absorbance was measured on a UV-VIS spectrophotometer (J.P. Selecta, Barcelona, Spain) at 510 nm and compared to the blank solution. A standard curve was prepared using a 1000 mM solution of catechin (Sigma Aldrich, Germany) at intervals of 200 mM catechin concentration. The results were expressed as μmol Catechin Equivalents (CE)/g FW.

The separation of chlorogenic and caffeic acid was performed using LC-MS/MS Quattro Micro™ API tandem quadrupole mass spectrometer (Waters, Milford, MA, USA) equipped with SunFire C18 column (3.5 μm; 3.0 x 100.0 mm, Waters, Milford, MA, USA). The elution was carried out at a flow rate of 0.5 mL/min with 0.1% formic acid in water (eluent A) and acetonitrile (eluent B). The gradient started with 5% B, reached 31% B in 28 minutes, and 71% B after 35 minutes; while 76% B was kept for 2 minutes. The temperature of column was controlled at 40°C. Injection volume was 5 μL.

The components were detected by negative electrospray ionization (ESI-): capillary voltage, 3.2 kV; ion source temperature, 120°C; desolvation gas temperature, 450°C; desolvation gas flow rate, 600 L/h; and cone gas flow rate, 50 L/h. The multiple reactions monitoring (MRM) was used to confirm the detected substances. Characteristic masses for component identification and their retention times (MRM parameters) are presented in Table 1. Calibration curves were developed using standard solution prepared from chlorogenic (≥95%, Sigma Aldrich, Germany) and caffeic acid (≥98%, Sigma Aldrich, Germany).

Table 1. Characteristic mass for chlorogenic and caffeic acid identification and their retention times (MRM parameters).

Component	Parent ion (m/z)	Daughter ion (m/z)	Dwell (s)	Cone voltage (V)	Collision energy (V)
Chlorogenic acid	353.00	191.00	0.2	25	22
Caffeic acid	179.00	135.00	0.2	18	19

2.7. Antioxidant activity determination

Antioxidant capacity of extracts was determined by running 3 tests.

In DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay, every diluted sample (0.2 mL) was added to the DPPH working solution (2.8 mL). DPPH solution was prepared as a mixture of 1.86×10^{-4} mol/L DPPH in ethanol and 0.1 M acetate buffer (pH 4.3) in volume ratio 2:1. The free radical scavenging capacity was evaluated at room temperature by measuring the absorbance at 525 nm after 1 h of reaction in the dark. Calibration curve, in the range of 0.2–0.7 mmol Trolox l^{-1} was used for the quantification of antioxidant activity. The results are expressed as μM Trolox Equivalents (TE)/g FW (BRAND-WILLIAMS *et al*., 1995).

In FRAP (ferric ion reducing antioxidant power) assay, stock solutions were prepared by mixing 300 mM of acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \times 6H_2O$ solution. The fresh working solution was made using 25 mL of acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3 \times 6H_2O$ solution and then warmed at 37°C before using. Methanol/water/trifluoroacetic acid diluted samples (300 μL) were allowed to react with 3 mL of the FRAP solution for 40 minutes under dark conditions. Readings of the colored product (ferrous tripyridyltriazine complex) were then taken at 593 nm. The antioxidant activity was calculated from the calibration curve using the range 0.1-0.8 mmol Trolox l^{-1}. Results are expressed in μM Trolox Equivalents (TE)/g of FW (BENZIE and STRAIN, 1996).
The Trolox equivalent antioxidant capacity (TEAC or ABTS assay) of chicory extracts was estimated by the ABTS radical action decolorization assay. Stock solutions of ABTS (14 mM) and potassium peroxodisulfate (4.9 mM) in phosphate buffer (pH 7.4) were prepared, and mixed together in equal volumes. The mixture was left to react overnight (12-16 h) in the dark, at room temperature. On the day of analysis, the ABTS radical solution was diluted with phosphate buffer to an absorbance of 0.70 ($\pm$0.02) at 734 nm. Exactly 30 μL of aliquoted samples were added to 3.0 mL of the ABTS radical solution, and after 6 min at 30°C the absorbance readings were taken. Instead of the sample, the reagent blank used was 30 μL of phosphate buffer. Calibration curve was developed using a range of 0.2-1.5 mmol Trolox l^{-1}. The results were expressed as μM Trolox Equivalents (TE)/g of fresh weight (RE *et al*., 1999).
An overall antioxidant potency composite index was determined according to SEERAM *et al*. (2008). An index value of 100 was assigned to the best score for each test and an index score was calculated for all other samples within the test as follows: Antioxidant index score = [(sample score/best score) × 100]. The overall mean index value was determined by dividing the sum of the individual index by the number of tests (three assays in total: DPPH, FRAP and ABTS).

2.8. Statistical analyses

Analyses were performed in triplicate. Results are expressed as mean values with the corresponding standard deviation (SD).
Statistical difference between means of the two groups (wild and cultivated plants) was determined using Student's t-test, *two sample assuming unequal variances*, and a p value <0.05 was considered statistically significant.
Analysis of variance, one-way ANOVA was performed to test the significance of the observed differences between wild plants locations. When the observed differences were significant ($p<0.05$) the mean values were compared by the Least Significant Difference (LSD) Post hoc multiple comparison test.
Correlation analysis was performed using Pearson's. All statistical analyses were performed using the software SPSS ver. 19 (BRYMAN and CRAMER, 2012).

3. RESULTS AND DISCUSSIONS

3.1. Fiber profile

The results for total, soluble and insoluble dietary fibers, hemicelluloses, cellulose, lignin and fructan in analyzed samples are presented in Table 2. The results are expressed on fresh weight (FW) basis.

Table 2. Fiber profile in leaves of wild and cultivated chicory*.

Location	Content (g/100 g)						
	Total fiber	Insoluble fiber	Soluble fiber	Hemicelluloses	Lignin	Cellulose	Fructan
Wild chicory (*Cichorium intybus* L.)							
Zoganje	5.0±0.1^{b}	3.7±0.1^{a}	1.26±0.15^{b}	1.14±0.11^{bc}	0.24±0.10	1.60±0.25^{a}	0.23±0.05^{a}
Risan	4.3±0.2^{c}	3.3±0.3^{b}	1.10±0.09^{c}	1.10±0.20^{bc}	0.20±0.12	1.49±0.17^{a}	0.06±0.03^{cf}
Podgor	4.1±0.2^{c}	2.9±0.2^{c}	1.17±0.14^{bc}	1.31±0.16^{ac}	0.17±0.08	1.31±0.22^{ab}	0.15±0.07^{b}
Tivat	3.0±0.2^{e}	2.1±0.2^{d}	0.93±0.13^{d}	1.07±0.18^{bc}	0.09±0.14	0.77±0.26^{cd}	0.07±0.02^{cd}
Pricelje	3.4±0.1^{d}	2.8±0.2^{c}	0.64±0.12^{e}	1.51±0.19^{a}	0.11±0.06	1.06±0.38^{bc}	0.12±0.04^{bc}
Plavnica	3.3±0.1^{de}	2.1±0.2^{d}	1.14±0.12^{bc}	1.34±0.22^{ab}	0.19±0.06	0.41±0.14^{d}	0.07±0.^{01ce}
Pljevlja	6.2±0.2^{a}	3.8±0.3^{a}	2.35±0.11^{a}	1.56±0.19^{a}	0.21±0.08	1.56±0.25^{a}	0.07±0.02^{bdef}
Average	4.2±0.1	3.0±0.1	1.23±0.02	1.29±0.04	0.17±0.03	1.17±0.08	0.11±0.02
Cultivated chicory (*Cichorium intybus* L.)							
Komani	4.3±0.2	3.2±0.2	1.08±0.10	0.80±0.16	0.19±0.08	1.11±0.19	0.10±0.03
Susanj	2.9±0.1	2.2±0.1	0.67±0.08	0.58±0.12	0.18±0.11	1.30±0.16	0.06±0.02
Average	3.6±0.1	2.7±0.1	0.88±0.01	0.69±0.03$^{\#}$	0.19±0.02	1.21±0.02	0.08±0.01

*data are expressed on 100 g fresh weight and presented as mean±SD of three independent determinations.

$^{\#}$statistically significant difference between wild and cultivated chicory, $p<0.05$.
Data sharing the same letter (a, b, c, d, e, f) in the same column are not significantly different, $p>0.05$.

Determined average values for total fiber were 4.2 g/100 g for the leaves of wild plants and 3.6 g/100 g of the cultivated plant leaves. Results indicate that the amount of soluble fiber was 1.6 to 4.4 times higher than the content of soluble fiber (DODEVSKA *et al*., 2015). Hemicellulose and cellulose were the main insoluble fiber in chicory leaves (average values of 1% and 1.19%, respectively), while lignin was present in small amounts (ca. 0.2%). Fructan is an important fiber in chicory plant, but its content in leaves is quite low (on average around 0.1%). Fructan is a fructose polymer that stores carbohydrate in a large number of plant species. The content of inulin and other fructan in chicory root is 15-20% and these compounds comprise more than 70% of total carbohydrates in fresh chicory roots (GUPTA *et al*., 2003). Chicory fructan is known as inulin and the chicory root, together with Jerusalem artichoke root, are the most important sources for industrial inulin production. Since fructan is not digested in the small intestine because of the b (2-1) bonds between fructose molecules, it belongs to the

soluble dietary fiber fraction. There are several known positive nutritional effects of fructan and inulin: when consumed in adequate quantities they increase stool frequency, have beneficial effects on blood lipids, and have prebiotic effects (stimulate growth of "good" bifidobacteria in the intestine) (ROBERFORID, 1999). Unfortunately, although the root of chicory plants is rich in fructan, the leaves are very poor in this prebiotic carbohydrate and the result of our investigation showed that fructans represented only ca. 4% of insoluble and ca. 2% of total fiber fraction. MILALA *et al*. (2009) also detected low amounts of fructan in chicory leaves, although their results were slightly higher.

Results for dietary fiber profile showed that wild plants had higher amounts of almost all fiber fractions in comparison to cultivated plants, but significant difference was identified only in the hemicellulose content ($p<0.05$). The location influenced the total fiber content both in wild and cultivated plants, which was obvious from the wide range of results (3.0-6.2% of total fiber in wild leaf samples, and 2.9-4.3% in cultivated ones). After statistical analysis, it was seen from the results obtained that there was a significant statistical difference in the content of TDF, IDF, SDF, cellulose, and fructan depending on the wild plants' sampling locations.

In comparison with other leafy vegetables, chicory leaves are significantly better fiber sources. Our results for cellulose content were about twenty times higher than results published for chard and even forty times higher than those published for lettuce. As far as hemicellulose content is concerned, wild chicory was twice richer than spinach, 3.5 times than chard, and 7.5 times richer than lettuce (HERRANZ *et al*., 1981).

3.2. Total fat content and fatty acid composition

Composition of fatty acids (FA) and total fat content of analyzed samples of wild and cultivated chicory are presented in Table 3. The results for total fat content are presented on FW basis, while results for FA composition are expressed as percentage of total fatty acids.

All studied samples showed fat content to be lower than 0.5 g/100 g, ranging between 0.22-0.49g/100 g, with no difference between cultivated and wild plants. Low level of fat is common in leafy vegetables.

Ten different fatty acids were identified and quantified. The main fatty acids found were α-linolenic (C18:3n-3), linoleic (C18:2n-6c), and palmitic acid (C16:0) and they comprised more than 95% of all fatty acids. These results are in good correlation with the data obtained for cultivated chicory leaves in Slovenia and Holland (SINKOVIC *et al*., 2015(a); WARNER *et al*., 2010). Alpha-linolenic acid was the most abundant fatty acid in chicory leaves and its content varied between 60.0 and 75.3% for the wild plants leaves, while the average content in cultivated plants was 75.8%. The linoleic acid content ranged from 11.0% in the samples from Komani to 17.4% in the samples from Podgor. Oleic acid (C18:1n-9c) was the only determined unsaturated fatty acid in all chicory samples. The difference between the contents of linoleic acid and α-linolenic acid in the leaves of wild and cultivated plants was significant ($p<0.05$). Wild plants were richer in linoleic, while cultivated ones had higher content of α-linolenic acid. Furthermore, obvious differences in the content of palmitic, linoleic and α-linolenic acid was noticed between wild plants sampled from different locations.

Table 3. Total fat content* and fatty acid composition** in wild and cultivated chicory leaves.

	Wild chicory (*Cichorium intybus* L.)								Cultivated chicory (*Cichorium intybus* L.)		
%	**Zoganje**	**Risan**	**Podgor**	**Tivat**	**Pricelje**	**Plavnica**	**Pljevlja**	***Average***	**Komani**	**Susanj**	***Average***
C14:0	0.5±0.1	0.2±0.1	0.4±0.1	0.2±0.1	0.3±0.1	0.3±0.1	0.2±0.1	0.3±0.1	0.4±0.1	0.2±0.1	0.3±0.1
C15:0	nd	nd	0.2±0.1	nd	nd	0.2±0.1	nd	-	nd	nd	-
C16:0	17.5±0.6^{a}	9.6±0.5^{d}	13.6±0.7^{b}	10.0±0.3^{d}	12.6±0.4^{c}	12.2±0.7^{c}	10.2±0.4^{d}	12.2±0.5	10.2±0.5	10.4±0.1	10.3±0.3
C18:0	2.0±0.1	0.7±0.1	1.0±0.1	0.8±0.2	0.8±0.2	1.1±0.2	1.4±0.3	1.1±0.2	0.9±0.2	0.6±0.2	0.8±0.2
C18:1n-9c	2.8±0.2	0.8±0.2	1.1±0.2	0.7±0.2	1.4±0.4	1.1±0.2	1.2±0.4	1.3±0.3	0.7±0.1	0.6±0.1	0.7±0.1
C18:2n-6c	15.7±0.4^{b}	12.1±0.6^{d}	17.4±0.8^{a}	14.4±0.5^{c}	15.5±0.8^{b}	15.5±0.5^{b}	13.8±0.8^{c}	14.9±0.6	11.0±0.6	11.2±0.8	11.1±0.7$^{\#}$
C18:3n-3	60.0±0.8^{e}	75.3±0.9^{c}	64.8±1.0^{d}	72.5±1.1^{b}	68.1±1.2^{c}	67.7±0.9^{c}	71.9±0.8^{b}	68.6±1.0	75.5±1.0	76.0±1.2	75.8±1.1$^{\#}$
C20:0	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1
C22:0	0.5±0.1	0.4±0.1	0.5±0.1	0.5±0.1	0.5±0.1	0.7±0.2	0.3±0.1	0.5±0.1	0.5±0.1	0.3±0.1	0.4±0.1
C24:0	0.8±0.2	0.7±0.2	0.8±0.2	0.7±0.1	0.7±0.1	0.9±0.1	0.8±0.2	0.8±0.1	0.6±0.1	0.5±0.1	0.6±0.1
Total fat	**0.45±0.09**	**0.49±0.10**	**0.35±0.05**	**0.41±0.11**	**0.45±0.10**	**0.33±0.14**	**0.22±0.07**	**0.39±0.09**	**0.48±0.06**	**0.39±0.10**	**0.44±0.06**

*data are expressed on 100 g fresh weight and presented as mean±SD of three independent determinations.
**expressed as relative percentage of total fatty acids.

$^{\#}$statistically significant difference between wild and cultivated chicory, $p<0.05$.
Data sharing the same letter (a, b, c, d) in the same row are not significantly different, $p>0.05$.
myristic acid (C14:0); pentadecanoic acid (C15:0); palmitic acid (C16:0); stearic acid (C18:0); oleic acid (C18:1n-9c); linoleic acid (C18:2n-6c); α-linolenic acid (C18:3n-3); arachidic acid (C20:0); behenic acid (C22:0); lignoceric acid (C24:0); nd: not detected.

The ratio of unsaturated and saturated acids in wild plants was 6:1, while in cultivated ones it was 7:1. Alpha-linolenic acid as a ω-3 polyunsaturated fatty acid has many nutritional and health benefits (it contributes to lowering the level of LDL cholesterol, reducing triglyceride levels and platelet aggregation, vasoconstriction and ventricular arrhythmia) and increasing its intake in diet have been widely suggested (BRADBERRY and HILLEMAN, 2013). It is not unusual that plant leaf lipids contain significant amounts of C18:3n-3, which is a component of chloroplast membrane lipids. Through history, it has been observed that wild plants are very important sources of this essential omega-3 fatty acid (SIMOPOULOS, 2004).
Compared to data from literature on fatty acid composition in the most-commonly consumed leafy vegetable species, chicory from Montenegro was richer in *α*-linolenic acid than lettuce by almost 20% (VIDRIH *et al*., 2009) and by 40% than spinach (NARSING RAO *et al*., 2015).

3.3. Pigments

Three classes of pigments namely: xanthophylls, chlorophylls and carotenes were identified and quantified (Table 4). The results are presented on FW basis.

Table 4. Pigments in wild and cultivated chicory leaves*.

Location	Content (mg/100 g)				
	Lutein	Violaxanthin	Antheraxanthin	VAZ**	Neoxanthin
			Wild chicory (*Cichorium intybus* L.)		
Zoganje	7.0±0.5[f]	3.0±0.4[c]	0.25±0.04[a]	3.2±0.2[b]	3.3±0.2[d]
Risan	10.7±0.9[a]	5.2±0.5[b]	0.14±0.02[bc]	5.4±0.4[b]	5.4±0.5[a]
Podgor	9.5±0.6[bd]	3.1±0.4[c]	0.11±0.02[bc]	3.2±0.3[b]	3.5±0.2[cd]
Tivat	10.3±0.6[ab]	3.5±0.3[c]	0.10±0.02[c]	3.6±0.2[b]	4.9±0.4[a]
Pricelje	9.6±0.6[bc]	6.5±0.6[a]	0.29±0.05[a]	6.8±0.4[a]	4.0±0.3[bc]
Plavnica	8.9±0.6[cde]	6.3±0.6[a]	0.15±0.04[bc]	6.5±0.4[a]	4.0±0.3[bc]
Pljevlja	9.5±0.6[b,e]	5.5±0.5[b]	0.16±0.05[b]	5.7±0.3[b]	4.2±0.4[b]
Average	9.4±0.6	4.7±0.5	0.17±0.03	4.9±0.3	4.2±0.3
			Cultivated chicory (*Cichorium intybus* L.)		
Komani	13.1±0.2	5.8±0.5	0.05±0.01	5.8±0.3	6.6±0.6
Susanj	11.9±0.2	3.3±0.3	0.06±0.02	3.3±0.2	6.5±0.7
Average	12.5±0.2	4.6±0.4	0.05±0.01[#]	4.6±0.2	6.6±0.7[#]
Location	**Content (mg/100 g)**				
	Chlorophyll a	**Chlorophyll b**	**Pheophytin a**	**Pheophytin b**	***β*-carotene**
			Wild chicory (*Cichorium intybus* L.)		
Zoganje	45.0±0.9[f]	13.7±0.9[f]	1.6±0.2[c]	20.1±0.4[b]	3.7±0.5[c]
Risan	97.2±1.3[a]	30.2±1.0[a]	2.1±0.1[b]	13.4±0.3[c]	6.1±0.7[a]
Podgor	74.0±1.1[e]	23.4±1.0[de]	2.1±0.2[b]	19.8±0.2[b]	6.2±0.6[a]
Tivat	77.6±1.0[d]	28.2±1.0[b]	2.4±0.4[a]	22.9±0.4[a]	6.1±0.7[a]
Pricelje	92.5±1.2[b]	25.5±1.0[c]	1.7±0.1[c]	10.4±0.2[d]	5.8±0.4[ab]
Plavnica	84.3±1.0[c]	24.1±1.0[ce]	0.1±0.1[d]	8.7±0.2[e]	6.2±0.5[a]
Pljevlja	77.9±1.2[d]	24.2±1.1[cd]	1.7±0.2[c]	10.4±0.3[d]	4.8±0.5[b]
Average	78.4±0.1	24.2±1.0	1.7±0.2	15.1±0.3	5.6±0.6
			Cultivated chicory (*Cichorium intybus* L.)		
Komani	115.0±1.3	36.9±0.9	2.2±0.2	13.9±0.9	8.2±0.5
Susanj	107.9±1.5	36.0±0.9	0.6±0.1	3.5±0.7	6.5±0.6
Average	111.5±1.4[#]	36.4±0.9[#]	1.4±0.2	8.7±0.8	7.4±0.6

*data are expressed on the original weight basis and presented as mean±SD of three independent determinations.
**Content of xanthophyll cycle pigments (violaxanthin, antheraxanthin, zeaxanthin).
[#]statistically significant difference between wild and cultivated chicory, $p<0.05$.
Data sharing the same letter (a, b, c, d, e, f) in the same column are not significantly different, $p>0.05$.

Four xanthophyll pigments – lutein, violaxanthin, antheraxanthin, and neoxanthin were identified and quantified in the leaves of chicory. Zeaxantin was not found in any of the samples. Based on concentration, the major xanthophyll was lutein representing on average 50% of the total xanthophylls, which is in agreement with the results of ZNIDARCIC *et al*. (2011). The lowest lutein content was measured in chicory from Zoganje (7.0 mg/100 g), while the highest was measured in chicory from Komani (13.1 mg/100 g). Lutein was confirmed as the main xanthophyll in the edible portion of wild and cultivated chicory varieties grown in the South of Italy as supported by MONTEFUSCO *et al*. (2015). Although, the reported concentrations in the tissues were much lower (0.8-3.0 mg/100 g FW). The content of xanthophyll cycle pigments - VAZ (violaxanthin, antheraxanthin and zeaxanthin) in analyzed chicory varied at intervals 3.2-6.8 mg/100 g FW. The major cycle pigment was violaxanthin, with antheraxanthin representing only 0.9-7.8% of the VAZ pool. The difference between wild and cultivated chicory was significant in the case of antheraxanthin ($p<0.05$). The average values for the only non-VAZ pigment neoxantin content in chicory leaves were 4.2 mg/100 g in wild plants and 6.6 mg/100 g FW in cultivated plants, and there was a significant difference between them ($p<0.05$). A significant difference for all pigments in wild samples from different locations was noticed.

Dietary carotenoids, especially xanthophylls, enjoyed significant scientific attention because of their characteristic biological activities, including anti-allergic, anti-cancer, and anti-obese actions. Lutein is one of the major xanthophylls present in green leafy vegetables and it is known to selectively accumulate in the macula of the human retina (KOTAKE-NARA and NAGAO, 2011). As an antioxidant (MILLER *et al*., 1996; DI MASCIO *et al*., 1989) and as a blue light filter (JUNGHANS *et al*., 2001), lutein can protect the eyes from oxidative stress, of which a non-protectioncan lead to age-related macular degeneration and cataracts. When compared with data from literature, our results for lutein content in chicory leaves were twice higher than in spinach and even sixty times higher than in lettuce (PERRY *et al*., 2009).

The chlorophyll a/b ratio was found to be similar in all analyzed samples, although the chlorophyll a and b contents varied greatly (chlorophyll a 45.0-115.0 mg/100 g and chlorophyll b 13.7–36.9 mg/100 g). There was a statistical difference in chlorophyll a and b contents between leaves of cultivated and wild plants, with cultivated ones being richer than the wild ones. The results also indicated that significant amounts of chlorophyll a and b were converted into pheophytin a and b, probably during the lyophilization process. Chlorophyll gives a characteristic coloration to the green leafy plants and its content correlate with the photosynthetic potential, giving some indication of the plant physiological status. There are indications that chlorophyll can play an important role in the prevention of various diseases associated with oxidative stress and certain environmental contaminants, such as cancer, cardiovascular diseases and other chronic diseases (GAMON and SURFUS, 1999; SANGEETHA and BASKARAN, 2010). The mechanism underlying the supposed chlorophyll suppression of *in vitro* mutagenicity of certain environmental contaminants could be the trapping of the carcinogenic molecules (SARKAR *et al*., 1994). In comparison with lettuce, chicory contained about 7 times more chlorophyll a and chlorophyll b. The obtained results confirm the similarity between chicory and spinach in chlorophyll a and chlorophyll b content (DUMA *et al*., 2014).

Beta-carotene was found in all samples (3.7–8.2 mg/100 g), while α-carotene was below detection limit. These results were similar to the results obtained by MONTEFUSCO *et al*. (2015). Compared to spinach and chard, chicory leaves contained 10-30% and 500%, respectively, more β-carotene (PERRY *et al*., 2009; RAJU *et al*., 2007). An increased intake of β-carotene rich food in daily diet may be one of the strategies for improving vitamin A status instead of synthetic vitamin A (GOPALAN, 1992). Obtained results for β-carotene

showed that consumption of 100 g of chicory leaves satisfied up to 135% of the referenced daily intake values (RDI) of vitamin A.

3.4. Total polyphenols, total flavonoids, chlorogenic and caffeic acid

The total amount of polyphenols and flavonoids in different chicory samples ranged between 0.65 and 3.73 mg GAE/g FW and 1.57-4.42 μmol CE/g FW, respectively (Table 5). The flavonoids content followed the content of total polyphenols in all samples. Our results showed that plants grown on different locations had different TPC and TFC.

Table 5. Content of polyphenols (TPC), flavonoids (TFC), chlorogenic and caffeic acid in wild and cultivated chicory leaves*.

Location	TPC (mg GAE/g)	TFC (μM CE/g)	Chlorogenic acid (μg/100 g)	Caffeic acid (μg/100 g)
Wild chicory (*Cichorium intybus* L.)				
Zoganje	3.73±0.04^{a}	4.42±0.01^{a}	1034±18^{a}	7.0±0.6^{a}
Risan	1.17±0.02^{d}	2.54±0.12^{e}	251±9^{f}	3.2±0.1^{d}
Podgor	2.90±0.08^{b}	3.81±0.01^{b}	908±14^{b}	6.6±0.1^{a}
Tivat	1.45±0.02^{c}	2.67±0.03^{d}	437±9^{d}	4.6±0.8^{bc}
Pricelje	1.45±0.03^{c}	3.00±0.09^{c}	440±10^{d}	4.4±0.6^{bc}
Plavnica	1.15±0.02^{d}	2.50±0.02^{e}	378±10^{e}	3.1±0.8^{d}
Pljevlja	1.05±0.02^{e}	2.29±0.04^{f}	526±8^{c}	3.8±0.5^{cd}
Average	1.84±0.03	3.03±0.05	568±4	4.7±0.3
Cultivated chicory (*Cichorium intybus* L.)				
Komani	0.82±0.01	2.01±0.03	104±4	2.1±0.5
Susanj	0.65±0.01	1.57±0.02	104±6	1.4±0.6
Average	0.74±0.01$^{\#}$	1.79±0.01$^{\#}$	104±1$^{\#}$	1.7±0.5$^{\#}$

*data are expressed on the original weight basis and presented as mean±SD of three independent determinations.

$^{\#}$statistically significant difference between wild and cultivated chicory, $p<0.05$.

Data sharing the same letter (a, b, c, d, e, f) in the same column are not significantly different, $p>0.05$.

TPC - Total Polyphenol Content; TFC - Total Flavonoid Content; GAE - Galic Acid Equivalents; CE - Catechin Equivalent.

Similar range of TPC was obtained for "Catalogna" landraces (*C. intybus*) in the study carried out by D'ACUNZO *et al*. (2017). The average value for content of total polyphenols was lower than in the work of SAHAN *et al*. (2017) and MILALA *et al*. (2009), but at the same time was significantly higher than in all chicory cultivars analyzed in the study of SINKOVIC *et al*. (2014) Also, our results for TFC were higher than those obtained by MONTEFUSCO *et al*. (2015).

The most important phenolic compounds in chicory leaves are hydroxycinnamic acid derivatives, such as chlorogenic and chicoric acids (MILALA *et al*., 2009; SINKOVIC *et al*., 2015(b)). The chlorogenic acid content (sum of isomers) in our study was the highest in the sample from Zoganje (1034 μg/100 g FW) and the lowest in those samples cultivated in Komani and Susanj (104 μg/100 g FW). Overall range for caffeic acid content was 1.4-7.0 μg/100 g FW.

Difference in TPC, TFC and analyzed polyphenol acids between wild and cultivated plants, as well as between wild samples from different locations, was significant ($p<0.05$). The wild plants were 2.5 times richer in TPC and 1.7 times in TFC than the cultivated ones.

3.5. Antioxidant capacity

Antioxidant activities of obtained extracts from chicory leaves, their abilities to scavenge the synthetic DPPH and ABTS radicals, as well as their power to reduce ferric (FRAP) ions were examined. The analysis were performed in triplicates and expressed as μM TE/g of fresh weight (Table 6).
The FRAP assay is quick and simple to perform, the reaction is reproducible and the reducing power that is measured in this assay is linearly related to the molar concentration of the antioxidants (MÜLLER *et al.*, 2010). Therefore, total phenolic content represents a reliable indicator of the antioxidant activity of analyzed plant. The antioxidant activity measured with FRAP test and total polyphenol content was almost 3 times lower in cultivated than in wild chicory samples.

Table 6. Antioxidant capacities of wild and cultivated chicory leaves*.

Location	FRAP (μM TE/ 1g)	DPPH (μM TE/1 g)	ABTS (μM TE/ 1g)
Wild chicory (*Cichorium intybus* L.)			
Zoganje	46.90±1.08^{a}	11.66±0.02^{a}	32.81±0.15^{a}
Risan	13.31±0.42^{e}	6.85±0.26^{e}	16.82±0.09^{c}
Podgor	36.45±0.56^{b}	10.29±0.03^{b}	26.32±0.15^{b}
Tivat	19.44±0.36^{c}	7.64±0.08^{d}	16.20±0.11^{d}
Pricelje	17.43±0.50^{d}	8.41±0.27^{c}	15.12±0.17^{e}
Plavnica	13.65±0.09^{e}	6.90±0.29^{e}	10.61±0.08^{f}
Pljevlja	13.33±0.27^{e}	6.19±0.22^{f}	10.80±0.09^{f}
Average	22.93±0.47	8.28±0.17	18.38±0.12
Cultivated chicory (*Cichorium intybus* L.)			
Komani	8.80±0.52	5.05±0.27	11.07±0.07
Susanj	6.75±0.13	3.76±0.17	10.46±0.09
Average	7.77±0.33$^{\#}$	4.40±0.22$^{\#}$	10.77±0.08

*data are expressed on the original weight basis and presented as mean±SD of three independent determinations.
$^{\#}$statistically significant difference between wild and cultivated chicory, $p<0.05$.
Data sharing the same letter (a, b, c, d, e, f) in the same column are not significantly different, $p>0.05$.
DPPH - 2,2-diphenyl-1-picrylhydrazyl; FRAP - Ferric ion Reducing Antioxidant Power; ABTS (TEAC) - Trolox Equivalent Antioxidant Capacity; TE - Trolox Equivalent.

For evaluation of free radical scavenging properties of the extracts, we used two assays: the DPPH radical and the ABTS radical cation assay.
The relatively stable organic radical DPPH has been widely used in the determination of antioxidant activity of different plant extracts (COSTA *et al.*, 2009). The free radical scavenging ability varied between 3.76 μM TE/g in cultivated sample from Susanj and 11.66 μM TE/g in wild sample from Zoganje, using the DPPH antioxidant method.

In accordance with the obtained data for the ABTS radical cation, it is interesting to note that the reduced antioxidant activity of cultivated samples is in agreement with the lower total flavonoid content of the same samples compared to the wild ones (40% lower on average). The reason for this pattern could be that flavonoids are the phenol class that specifically reflect and are better indicators of the segment of antioxidant activity obtained in ABTS test.

Obtained results for all three assays have shown that the antioxidant capacity of analyzed extracts, as presumed, followed the pattern of TP and TF contents. FRAP and DPPH assays showed significantly different results for wild and cultivated plants ($p<0.05$), as well as for wild plants sampled from different locations. Correlations among results obtained with all three antioxidant assays were positively high as well as correlation between antioxidant activity with TP and TF contents ($r\sim0.99$, $p<0.05$).

When compared with data of SAHAN *et al*. (2017), the obtained results for ABTS and DPPH assay were lower.

The overall antioxidant activity of analyzed samples is expressed as antioxidant composite index (ACI). ACI value is followed by one statistical-mathematical model, which is a modern ranking tool for the representation of antioxidant activity of various plants. One basic advantage of using ACI is the value being expressed in percent, which covers all segments of antioxidant action obtained in different antioxidant assays. Another advantage of using ACI index instead of three assays is because it simplifies the process of comparison between antioxidant properties of different foods. The antioxidant composite index (ACI) of chicory samples (Table 7) was in the following decreasing order: Zoganje > Podgor > Tivat > Pricelje > Risan > Plavnica > Pljevlja > Komani > Susanj. All samples of wild plants had higher ACI than the cultivated plants.

Table 7. Antioxidant composite index (ACI) of wild and cultivated chicory leaves.

Location	FRAP index	DPPH index	ABTS index	ACI (%)
Wild chicory (*Cichorium intybus* L.)				
Zoganje	100.0	100.0	100.0	100.0
Risan	28.4	58.7	51.3	46.1
Podgor	77.7	88.2	80.2	82.1
Tivat	41.4	65.5	49.2	52.1
Pricelje	37.2	72.1	46.1	51.8
Plavnica	29.1	59.2	32.3	40.2
Pljevlja	28.4	53.1	32.9	38.1
Cultivated chicory (*Cichorium intybus* L.)				
Komani	18.8	43.3	33.7	31.9
Susanj	14.4	32.2	31.9	26.2

FRAP - ferric ion reducing antioxidant power; DPPH - 2,2-diphenyl-1-picrylhydrazyl; ABTS (TEAC) - Trolox equivalent antioxidant capacity; ACI - Antioxidant composite index.

4. CONCLUSIONS

This study is the first comprehensive study offering detailed information on fibers, fatty acids, pigments, phenolics, flavonoids, and antioxidant activity of chicory (*Cichorium intybus* L.) leaves grown in Montenegro. Chicory leaves are rich sources of fiber, polyphenols, flavonoids, and almost all analyzed pigments. The lipid content in chicory

leaves is quite low, but they have high nutritional value due to their favorable balance of fatty acids with the omega-3 fatty acids as the dominant ones. The antioxidant potential of chicory leaves extract was positively correlated with their phenolic and flavonoid content. Accordingly, this composition of biologically active substances offer a number of different ways of using chicory leaves in the field of nutrition and production of healthy food. Wild plant samples had higher content of the majority of analyzed BAS in comparison with the cultivated ones.

REFERENCES

AACC International. 2000. Approved Methods of Analysis, 10th ed. St. Paul, MN, U.S.A.

AOAC. 1990. "Official Methods of Analysis" 15th ed. Association of Official Analytical Chemists, Washington, DC.

AOAC. 1995. "Official Methods of Analysis" 16th ed. Association of Official Analytical Chemists, Arlington, VA.

AOAC. 2002. "Official Methods of Analysis" 17th ed. Association of Official Analytical Chemists, Gaithersburg, MD.

Benzie I.F. and Strain J.J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay. Anal. Biochem. 239:70.

Bradberry J.C. and Hilleman D.E. 2013. Overview of omega-3 fatty acid therapies. P T. 38:681.

Brand-Williams W., Cuvelier M.E. and Berset C. 1995. Use of a free radical method to evaluate antioxidant activity. Lebensm. Wiss. Technol. 28:25.

Bryman A. and Cramer D. 2012. Quantitative Data Analysis with IBM SPSS 17, 18 & 19:A Guide for Social Scientists. Routledge.

Costa R.M., Magalhaes A.S., Pereira J.A., Valentao P., Carvalho M. and Silva B.M. 2009. Evaluation of free radical-scavenging and antihemolytic activities of quince (*Cydonia oblonga*) leaf: A comparative study with green tea (*Camellia sinensis*). Food Chem.Toxicol. 4:860.

D'Acunzo F., Giannino D., Longo V., Ciardi M., Testone G., Mele G., Nicolodi C., Gonnella M., Renna M., Arnesi G., Schiappa A. and Ursini O. 2017. Influence of cultivation sites on sterol, nitrate, total phenolic contents and antioxidant activity in endive and stem chicory edible products. Int J Food Sci Nutr. 68 (1):52.

Di Mascio P., Kaiser S. and Sies H. 1989. Lycopene as the most efficient biological carotenoid singlet oxygen quencher. Arch Biochem Biophys. 274:532.

Dodevska M., Sobajic S. and Djordjevic B. 2015. Fibre and polyphenols of selected fruits, nuts and green leafy vegetables used in Serbian diet. J. Serb. Chem. Soc. 80 (1):21.

Duma M., Alsina I., Zeipina S., Lepse L. and Dubova L. 2014. Leaf vegetables as source of phytochemicals. In:9th Baltic Conference on Food Science and Technology "Food for Consumer Well-Being" FOODBALT 2014 Conference Proceedings. Jelgava, LLU. p 262.

Gamon J.A. and Surfus J.S. 1999. Assessing leaf pigment content with a reflectometer. New Phytologist. 43:105.

Goering H.K. and Van Soest P.J. 1970. Forage Fiber Analysis (apparatus, reagents, prosedures and some applications). USDA Agricultural Handbook No. 379.

Gopalan C. 1992. New dimensions of 'old problem'. In Nutrition in development transition in South-East Asia, p. 34. New Delhi:World Health Organization, Regional Office for Southeast Asia.

Guhr G. and LaChance P.A. 1997. Role of phytochemicals in chronic disease prevention. Chapter 32, In Nutraceuticals: Designer foods III:Garlic, soy, licorice. Food nutrition Press. p. 311.

Gupta A.K., Kaur N. and Kaur N. 2003. Preparation of inulin from chicory roots. J. Sci. Industrial Res. 62:916.

Hasler C.M. 1998. Functional foods: Their role in disease prevention and health promotion. Food Technol. 52:63.

Herranz J., Vidal-Valverde C. and Rojas-Higaldo E. 1981. Cellulose, Hemicellulose and lignin content of raw and cooked Spanish vegetables. J. Food Sci. 46:1927.

Jan G., Kahan M., Ahmad M., Iqbal Y., Afzal A., Afzal M., Shah G.M., Majid A., Fiaz M., Zafar M., Waheed A. and Gul F. 2011. Nutritional analysis, micronutrients and chlorophyll contents of *Cichorium intybus* L. J. Med. Plants Res. 5 (12):2452.

Jancic D., Todorovic V., Basic Z. and Sobajic S. 2016. Chemical composition and nutritive potential of *Cichorium intybus* L. leaves from Montenegro, J. Serb. Chem. Soc. 81 (10):1141.

Junghans A., Sies H. and Stahl W. 2001. Macular pigments lutein and zeaxanthin as blue light filters studied in liposomes. Arch Biochem Biophys. 391:160.

Kotake-Nara E. and Nagao A. 2011. Absorption and Metabolism of Xanthophylls. Mar Drugs. 9 (6):1024.

Lee S.C., Prosky L. and DeVries J.W. 1992. Determination of total, soluble, and insoluble, dietary fiber in foods–enzymatic gravimetric method, MES-TRIS buffer: Collaborative study. J. Assoc. Off. Ana. Chem. 75:395.

McCleary B.V. and Blakeney A.B. 1999. Measurement of inulin and oligofructan. Cereal Foods World. 44:398.

Milala J., Grzelak K., Król B., Juśkiewicz J. and Zduńczyk Z. 2009. Composition and properties of chicory extracts rich in fructans and polyphenols. Pol. J. Food Nutr. Sci. 59 (1):35.

Miller N.J., Sampson J., Candeias L.P., Bramley P.M. and Rice-Evans C.A. 1996. Antioxidant activities of carotenes and xanthophylls. FEBS Lett. 384:240.

Montefusco A., Semitaio G., Marrese P.P., Iurlaro A., De Caroli M., Piro G., Dalessandro G., Lenucci M.S. 2015. Antioxidants in Varieties of Chicory (*Cichorium intybus* L.) and Wild Poppy (*Papaver rhoeas* L.) of Southern Italy. Journal of Chemistry. Article ID 923142, 8 pages.

Müller L., Gnoyke S., Popken A.M. and Bohm V. 2010. Antioxidant capacity and related parameters of different fruit formulations. LWT-Food Sci. Technol. 6:992.

Narsing Rao G., Prabhakara Rao P.G., Sulochanamma G. and Satyanarayana A. 2015. Physico-chemical Amino acid composition, fatty acid profile, functional and antioxidant properties of *Spinacia oleracea* L. leaf. J. Food Pharm. Sci. 3:27.

Perry A., Rasmussen H. and Johnson E.J. 2009. Xanthophyll (lutein, zeaxanthin) content in fruits, vegetables and corn and egg products. J. Food Comp. Anal. 22:9.

Raju M., Varakumar S., Lakshminarayana R., Krishnakantha T.P. and Baskaran V. 2007. Carotenoid composition and vitamin A activity of medicinally important green leafy vegetables. Food Chem. 101:1598.

Re R., Pellegrini N., Proteggente A., Pannala A., Yang M. and Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic. Bio. Med. 26:1231.

Roberforid M.B. 1999. Concepts in functional foods: the case of inulin and oligofructose. J. Nutr. 129:1398S.

Sahan Y., Gurbuz O., Guldas M., Degirmencioglu N. and Begenirbas A. 2017. Phenolics, antioxidant capacity and bioaccessibility of chicory varieties (*Cichorium* spp.) grown in Turkey. Food Chem. 217:483.

Sangeetha R.K. and Baskaran V. 2010. Carotenoid composition and retinol equivalent in plants of nutritional and medicinal importance. Efficacy of *β*-carotene from *Chenopodium album* in retinol-deficient rats. Food Chem. 119:1584.

Sarkar D., Sharma A. and Talukder G. 1994. Chlorophyll and chlorophyllin as modifiers of genotoxic effects. Mutation Res. 318:239.

Seeram N.P., Aviram M., Zhang Y., Henning S.M., Feng L., Dreher M. and Heber D. 2008. Comparison of antioxidant potency of commonly consumed polyphenol-rich beverages in the United States. J. Agric. Food Chem. 56:1415.

Simopoulos A.P. 2004. Omega-3 fatty acids and antioxidants in edible wild plant plants. Biol Res. 37 (2):263.

Sinkovic L., Hribar J. and Vidrih R. 2014. Influence of cultivar and storage of chicory (*Cichorium intybus* L) plants on polyphenol composition and antioxidative potential. Czech J Food Sci. 32 (1):10.

Sinkovic L., Demsar L., Znidarcic D., Vidrih R., Hribar J. and Treuttler D. 2015(a). Phenolic profiles in leaves of chicory cultivars (*Cichorium intybus* L.) as influenced by organic and mineral fertilizers. Food Chem. 166:507.

Sinkovic L., Hribar J., Vidrih R., Ilin Z. M. and Znidarcic D. 2015(b). Fatty acid composition of leaves of forced chicory (*Cichorium intybus* L.). Arch. Biol. Sci. 67 (2):647.

Todorovic V., Radojcic Redovnikovic I., Todorovic Z., Jankovic G., Dodevska M. and Sobajic S. 2015. Polyphenols, methylxanthines, and antioxidant capacity of chocolates produced in Serbia. J. Food Compos. Anal. 41:137.

Van Soest P.J., Robertson J. B. and Lewis B. A. 1991. Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583.

Vidrih R., Filip S. and Hribar J. 2009. Content of higher fatty acids in green vegetables. Czech J. Food Sci. 27:S125.

Ying G.V. and Gui L.J. 2012. Chicory seeds: a potential source of nutrition for food and feed. J. Anim. Plant Sci. 13 (2):1736.

Warner D., Jensen S.K., Cone J.W. and Elgersma A. 2010. Fatty acid composition of forage herb species. In Proceedings of the 23rd General Meeting of the European Grassland Federation, Kiel, Germany, Grassland in a changing world, Grassland Science in Europe, Kiel, p. 491.

Wen D., Li C., Di H., Liao Y. and Liu H. 2005. A Universal HPLC Method for the Determination of Phenolic Acids in Compound Herbal Medicines. J. Agric. Food Chem. 53:6624.

Znidarcic D., Ban D. and Sircelj H. 2011. Carotenoid and chlorophyll composition of commonly consumed leafy vegetables in Mediterranean countries. Food Chem. 129:1164.

Paper Received March 2, 2017 Accepted June 25, 2017

PAPER

THE EFFECT OF PLANT EXTRACTS ON PORK QUALITY DURING STORAGE

A.M. KACZMAREK*[a], M. MUZOLF-PANEK[a], M. RUDZIŃSKA[b], T. SZABLEWSKI[a] and R. CEGIELSKA-RADZIEJEWSKA[a]

[a]Department of Food Quality Management, Faculty of Food Science and Nutrition, Poznań University of Life Sciences Wojska Polskiego 31, 60-642 Poznań, Poland

[b]Department of Food Chemistry and Instrumental Analysis, Faculty of Food Science and Nutrition, Poznań University of Life Sciences Wojska Polskiego 31, 60-642 Poznań, Poland

*Corresponding author. Tel.: +48 8487363

E-mail address: ak@up.poznan.pl

ABSTRACT

This study investigated the effects of the natural antioxidants contained in onion, garlic and marjoram on the oxidant and microbial stability of raw minced pork during refrigerated storage. The most antioxidant active plant extract was marjoram. The highest inhibition of secondary oxidation products was observed in the samples with garlic, next was onion and finally, marjoram. GC analysis showed that the lowest rates of fatty acids degradation were recorded in the samples with garlic. The highest bacteriostatic effect was noted in meat samples with fresh onion added. These results suggest that the addition of these plants enhances the quality of the raw minced pork meat during refrigerated storage.

Keywords: antimicrobial, antioxidant, meat, extract, plant, storage

1. INTRODUCTION

Consumer concerns on the quality and safety of meat have greatly increased during the past decades. Lipid and microbial stabilities are important parameters that influence the quality and acceptance of meat, particularly minced meat. Minced meat has its cell structure disrupted, leading to increased exposure of lipids to oxygen, enzymes, heme pigments, and metal ions. Oxidative products, which are the results of lipid oxidation and/or microbiological changes in the meat, negatively affect the quality and safety of the product. For consumers this means the undesirable rancidity of meat. The heme pigment content, in conjunction with catalase activity, determines the lipid peroxidation potential in raw meat. In cooked meat, the major determinant for lipid peroxidation is the content of PUFA (MIN and AHN, 2005). The application of suitable ingredients possessing both antimicrobial and antioxidant properties can be useful for extending meat's shelf life and thus preventing economic losses. In recent years, naturally occurring compounds exhibiting antioxidant and antimicrobial properties have been preferred for use in meat products because of their potential health benefits and safety, especially when compared with synthetic derivatives, which were routinely used in processed foods. Studies associated with natural antioxidants for the inhibition of microbial spoilage and lipid oxidation have been extensively conducted (FALOWO *et al*., 2014; KARRE *et al*., 2013; SHAH *et al*., 2014). Onion (*Allium cepa* L.), garlic (*Allium sativum* L.) and marjoram (*Origanum majorana* L.) possess both antioxidant and antimicrobial activity (BUSATTA *et al*., 2008; ROBY *et al*., 2013; SALLAM *et al*., 2004; YE *et al*., 2013). Onion and garlic bulbs, as well as dried marjoram, are three major meat additives widely used in food preparation. Park and Chin (2010) used onion extracts to control the development of lipid oxidation in fresh pork patties. EL-ALIM *et al*. (1999) investigated the use of ground marjoram as an antioxidant in raw ground chicken. CAO *et al*. (2013) proved the antimicrobial and antioxidant properties of onion and garlic for extending the shelf life of stewed pork during refrigerated storage. Moreover, PARK *et al*. (2008) demonstrated the antimicrobial and antioxidant activity of garlic and onion powder in fresh pork belly and loin during storage. Fresh garlic blended with paprika was also successfully used as an antioxidant in dry sausages (AGUIRREZÁBAL *et al*., 2000). However, due to the fact that the rate and extent of lipid oxidation are influenced by a number of factors, which include iron content, distribution of unsaturated fatty acids, pH, the form of natural additives (fresh, dried or extract) and various processing technologies, further research is still needed, particularly with reference to the meat system.

The aim of the present study was to evaluate the effect of natural antioxidants contained in onion, garlic and marjoram on the oxidant and microbial stability of refrigerated minced pork blade meat during refrigerated storage.

2. MATERIALS AND METHODS

2.1. Samples preparation

Samples of dried marjoram leaves, fresh garlic and onion bulbs were purchased in a local market. All the products were produced in Poland. Three pork blades (each from different animals) were obtained from a local market (where the process of cutting and deboning is conducted locally) and transported to the laboratory under chilled conditions in iceboxes. Blade muscles containing approximately 4% intramuscular fat were used. The blades were chopped and minced separately in a meat grinder, after the removal of excessive fat and connective tissue. Dried marjoram leaves and freshly crushed garlic and onion bulbs were

mixed with the meat. The following four samples were prepared from each blade: 1 control (meat without any addition) and 3 treatments, namely, with garlic 0.1% (m/m), onion 0.5% (m/m) and marjoram 0.5% (m/m). The mixing process for each sample was conducted separately for each blade. The concentrations of the additives were established during preliminary studies and were accepted by a sensory panel (data not presented). Meat samples were placed in plastic foam meat trays, wrapped with polyethylene and kept at 4°C for 9 days. A strict sanitation procedure was followed during the preparation of the meat samples to avoid microbial contamination.

2.2. Antioxidant activity of plant extract

The antioxidant activities of marjoram leaves, garlic and onion were determined by methods, which reflected the various mechanisms of antioxidant action, such as the scavenging of free radicals or chelation of transition metal ions (methods such as DPPH and TEAC or FRAP, respectively). Each method has its advantages and limitations, thus a combination of these methods was implemented for assessing antioxidant activity to get more insight into the antioxidative potential of the extracts studied.
To investigate the antioxidant activity of the plant material, extracts were prepared in methanol. For the extraction, 2 g of dried marjoram leaves, onion or garlic were mixed with 30 mL of pure ethanol. The procedure was conducted in the dark at room temperature. After 30 min of extraction, each extract was put through a filter (type 388) and the solutions of the corresponding concentrations were prepared in methanol for antioxidant activity measurements.

2.3. DPPH method

The antioxidant activity of marjoram leaves, garlic and onion were evaluated by the DPPH method according to the procedure described by Sánchez-Moreno *et al*. (1998) with some modifications. Briefly, 10 μL of the plant extract samples was added to 990 μL DPPH in methanol (giving a final concentration 0.1 mM) and mixed in a vortex. The reaction mixtures were incubated in the dark at room temperature for 30 min, and the decrease in absorbance caused by the plant extract was measured at 515 nm using a Cary 1E spectrophotometer (Varian, Berlose, Australia). For each sample, three separate determinations were conducted. The corresponding solvent blank readings were also recorded (methanol). The DPPH· radical scavenging activities of the plant extracts were expressed in TEAC(DPPH) values - Trolox Equivalents Antioxidant Capacity (mM of Trolox/g of dry weight). TEAC(DPPH) values were calculated as the ratio of the slope of the linear plot for the scavenging of DPPH· radicals by the plant extract tested to the slope of the plot for DPPH· radicals scavenging by the water-soluble vitamin E analogue Trolox, used as an antioxidant standard.

2.4. TEAC assay

The TEAC (Trolox Equivalent Antioxidant Capacity) assay is based on the inhibition of the absorbance of the blue-green coloured ABTS·+ radical cation by antioxidants (Miller *et al.* 1993). In the study, the ABTS·+ radical cation was generated with potassium disulphate according to the modifications of Re *et al*. (1999). Briefly, ABTS was dissolved in water to a 7 mM concentration and mixed with 2.45 mM potassium disulphate at a ratio of 2:1 (stoichiometrical reaction). To produce ABTS·+ radical cation, the mixture was allowed to stand in the dark at room temperature for 12-16 h. The radical was stable in this form for more than two days (i.e. stored in the dark at room temperature). The ABTS·+ solution was

diluted with PBS (phosphate buffer saline), pH 7.4 to an absorbance of 0.7 (±0.02). 10 μL of plant extract was added to 990 μL $ABTS^{\cdot+}$ solution in PBS, pH 7.4 and the decrease in the absorbance of the mixture after 6 min of incubation in the dark was monitored spectrophotometrically at 734 nm. The corresponding solvent blank readings were run (ABTS in PBS, pH 7.4 with methanol). All determinations were conducted in triplicate, and the results were expressed as TEAC values. The TEAC value represents the ratio of the angle of the plot for the scavenging of $ABTS^{\cdot+}$ by the particular extract under investigation to the slope of the plot for $ABTS^{\cdot+}$ scavenging by Trolox, used as an antioxidant standard (MILLER *et al.*, 1993). The TEAC value is expressed in milimolar concentrations (mM), according to the definition of the TEAC value introduced by MILLER *et al*. (1993). The TEAC value is defined as the concentration of Trolox solution with an equivalent antioxidant potential to a 1 mM concentration of the compound under investigation (MILLER *et al*., 1993).

2.5. FRAP assay

The FRAP (Ferric Reducing Antioxidant Power) assay directly measures the ability of antioxidants to reduce a ferric tripyridyltriazine complex (Fe+3-TPTZ) to a ferrous complex (Fe+2-TPTZ) at a low pH, with an intense blue color and an absorption of 593 nm (BENZIE and STRAIN 1996). The FRAP solution was prepared by mixing an acetate buffer (300 mM, pH 3.6), 10mM TPTZ (2,4,6-tripyridyl-s-triazine) and $FeCl_3 \bullet 6H_2O$ (20 mM in 40 mM HCl) at a ratio of 10:1:1. The mixture was allowed to stand in the dark at 30°C for 30 min directly before use. Then, 50 μL of an increasing concentration of plant extract was added to the FRAP solution in a 1mL total volume and allowed to react for 10 min in the dark. Readings were taken at 593 nm and results were expressed as mM Trolox equivalent (TE) per g of dry weight. All determinations were conducted in triplicate.

2.6. Total polyphenol content of plant extracts

The Total Polyphenol Content (TPC) of the plant materials was determined using the Folin- Ciocalteu reagent (FCR) as described by Singleton and Rossi (1995). An aliquot of 20 μl of plant extract (onion, garlic or marjoram), prepared in the same way as for antioxidant activity measurements, was added to 100 μl of FCR, mixed and incubated for 3 min at room temperature in a dark place. Then, 300 μl of sodium carbonate (20% m/v) was added and filled to 2 mL with distilled water. After 2h of incubation in the dark at room temperature the absorbance was read at 765 nm against the blank sample (solvent instead of extract). The results were presented as mg Gallic Acid Equivalent (GAE) per g of dry weight (DW). All determinations were conducted in triplicate.

2.7. Total flavonoid content

The total flavonoid content was determined by using the aluminum chloride colorimetric method as described by MEDAA *et al*. (2005). The basis of this assay is that aluminium chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, it also forms acid labile complexes with the ortho-dihydroxyl groups in the A or B ring of flavonoids. 100 μL of plant extracts were mixed with aluminium chloride in methanol (2% m/v) to a final volume of 1 mL. The mixtures were incubated for 15 min in the dark at room temperature. Then, the absorbance was monitored at 410 nm. The results were expressed in mg of quercetin per g of dry weight.

2.8. Measurement of lipid oxidation

The extent of lipid oxidation was monitored by the formation of Thiobarbituric Acid-Reactive Substances (TBARS). The TBARS index was determined, in triplicate samples, by the extraction method of SØRENSEN and JØRGENSEN (1996) with some modification. For extraction, 10 g meat was homogenized (15,000 rpm, 30 s, 20°C) together with 30 mL of a 7.5% aqueous solution of trichloroacetic acid (TCA). After filtration and centrifugation, (5 min, 5000 rpm), a 5.0 mL extract was mixed with 5.0 mL of 0.02 mol l^{-1} aqueous thiobarbituric acid (TBA) in a stoppered test tube. The samples were incubated at 100C for 35 min in a water-bath and subsequently cooled for 10 min in cold water. Absorbance was measured at 532nm by a Carry 1E UV/VIS spectrophotometer against a blank containing 5mL distilled water and 5mL TBA reagent. The results, expressed as milligrams malondialdehyde per kg meat, were calculated from the standard curve of TEP (1,1,3,3-tetraethyoxypropane) standards. The antioxidant potential, which is expressed in terms of the percentage of antioxidant activity (%AOA), were calculated using the following equation (WIJEWICKREME and KITTS 1998):

$$\%AOA = ((X \text{ value of the control} - X \text{ value of the test sample}) \times 100) / X \text{ value of the control}$$

where X is the TBARS value.

2.9. Fatty acids composition analysis

The meat samples were minced in a blender. Lipids were extracted from the samples with chloroform:methanol (2:1 v/v) according to FOLCH *et al*. (1957). The extracts were dried in a vacuum on a rotary evaporator and under a nitrogen flow. The fatty acid content was determined as Fatty Acid Methyl Ester (FAME) derivatives in a HP 5980 series II gas chromatographer (Hewlett Packard, Oalo Alto, USA) using the AOCS Official Method Ce 1k-07. The GC system consisted of an Innowax capillary column (30 m x 0.2 mm x 0.2 mm) and FID detector. Hydrogen was used as the carrier gas, at a flow rate of 1.5 mL/min. The temperature of both the injector and detector was set at 240°C. The column temperature was maintained at 220°C isotherm. Peak identification was based on the fatty acid ester standards, and fatty acid compositions were estimated from the chromatogram peak areas and were expressed as mg/1 g meat sample.

2.10. Microbial analysis

An amount of 10 g of meat was collected from prepared samples and introduced, under sterile conditions, to a flask containing 90 mL of 0.1% sterile peptone diluents (Bacteriological Peptone, Oxoid, England). The samples were homogenized using an Ultra-Turrax T25 homogeniser (IKA, Germany), producing an initial suspension of 1:10.
The microbiological analyses were conducted in line with ISO reference methods (according to the Polish Standard of PN-ISO). Bacterial counts were given in CFU g^{-1}. The total bacterial count was determined on a Standard Plate Count Agar (CM 463, Oxoid, England). Incubation was run at 30°C for 72 h. For counts of the *Enterobacteriaceae* rods, a 1 mL sample was inoculated into 15 mL of a molten selective VRBG medium (P-0256, BTL, Poland). After setting, a 10 mL overlay of molten medium was added and incubation was conducted at 37°C for 24-48 h. The counts of *Pseudomonas* were determined on a solid Pseudomonas Agar medium (CM 0559, Oxoid, England) supplemented with Pseudomonas CFC Selective Agar Suplement (SR 0103, Oxoid, England) after incubation

at 30°C for 48 h. De Man, Rogosa and Sharpe (MRS) Agar (CM 0361, Oxoid, England) was used for determining lactic acid bacteria counts. MRS agar was also overlayed with a molten medium and incubation was conducted at 30°C for 48-72 h. An oxidase test was used to confirm lactic acid bacteria (MBO 266, Oxoid, England). Dichloran Rose Bengal Chloramphenicol DRBC Agar (CM 0727B, Oxoid, England) was used in the determination of moulds and yeasts. Incubation was conducted at 20°C for 5 days. Total Viable aerobic bacteria Counts (TVC) were conducted as an indicator of microbial spoilage in the pork meat samples. The samples (10 g) were homogenized with 90 mL with sterile peptone water (1g/l) using an Ultra-Turrax T25 homogenizer (IKA, Germany). Serial decimal dilution was performed and plated onto a Standard Plate Count Agar (CM 463, Oxoid, Basingstoke, England). Incubation was performed at 30°C for 72 h. Bacterial counts were enumerated and expressed as $\log_{10}$ cfu/g.

2.11. Statistical analysis

All tests were run in triplicate. The statistical package Statistic 13.1 was used for analysis of covariance (ANCOVA). The influence of various plant additions on oxidative and microbial raw meat stability was assessed using analysis of covariance. This analysis procedure is applied when looking at group effects on a continuous outcome (TBARS value, fatty acid content and microbial count) when another continuous explanatory variable (storage time) may also have an effect on the outcome. Comparison of the treatment means was based on Tukey's Honest Significant Difference (HSD) test. This test is the most useful for multiple comparisons and avoids Type II errors. In addition, Dunnett's T-test was performed for a comparison of the treatments to the control. To compare the rates (slope of regression equation) of fatty acids' degradation, a linear regression analysis between the storage time and the fatty acid content was calculated. To compare the regression coefficients (slope) of the control with the other treatments, the null hypothesis H0: $B_c=B_t$, (where B_c is the regression coefficient for the control and B_t is the regression coefficient of the sample with additives) was tested. The comparisons between the coefficients were performed introducing two dummy variables as predictors to regression analysis. The first dummy variable was coded "0" for the control and "1" for the meat sample with plant additive, and the second, which was the product of the first dummy variable and storage time. The significant differences between the regression coefficients were based on the result of the *T*-test ($P \leq 0.05$) for the second dummy variable. Bacterial counts were converted to their equivalent log cfu for data uniformity. Differences were considered significant at the $P \leq 0.05$ level.

3. RESULTS AND DISCUSSIONS

3.1. Antioxidant activity and total polyphenol content of the plant extracts

All results of the antioxidant activity and phenolic content (total polyphenol content TPC and total flavonoid content TFC) measurements are presented in Table 1. According to radical scavenging methods like DPPH and ABTS and ion chelating methods such as FRAP, the most antioxidant active plant extract was marjoram. There were no statistically significant differences between the antioxidant activities of garlic and onion. GORINSTEIN *et al*. (2008) showed higher antioxidant activity of garlic in comparison to onion in the DPPH method but significantly lower antioxidant activity of garlic than onion in the ABTS and FRAP methods. MARIUTTI *et al*. (2008) reported that marjoram is the most antioxidant active among the tested extracts in the DPPH and ABTS methods, which

is in accordance with the results presented in this paper. However, the authors showed that garlic is more antioxidant active than onion as a DPPH• and ABTS•+ radical scavenger

Table 1. Phenolic content and the antioxidant activities of marjoram, garlic and onion.

	Plant extract		
	Marjoram	**Garlic**	**Onion**
Phenolic content	*Mean±SD*	*Mean±SD*	*Mean±SD*
TPC (mg GAE/g)	24.48 ± 2.66 [a]	0.79 ± 0.23 [b]	2.74 ± 0.65 [b]
TFC(mg QE/g)	15.72 ± 1.26 [a]	0.22 ± 0.00 [b]	0.27 ± 0.01 [b]
Antioxidant activity			
TEAC(DPPH)(mM TE/g)	175.11 ± 11.43 [a]	3.05 ± 0.46[b]	3.77 ± 0.51[b]
TEAC(ABTS)(mM TE/g)	261.84 ± 15.04 [a]	7.68 ± 0.25[b]	19.09 ± 1.15[b]
FRAP(mM TE/g)	186.9 ± 10.60[a]	4.16 ± 0.06[b]	3.86 ± 0.19[b]

[(a-b)]Means with the same superscript within the same row are not different ($P > 0.05$).

In this study, the TPC and TFC values correspond to the antioxidant activity (Table 1). r-Pearson coefficients for the linear correlations between the antioxidant activity and TPC or TFC values are equal to 0.99. The higher the TPC (and TFC) value, the higher the antioxidant activity is. In literature, data on the TPC values of onion, garlic and marjoram vary. Generally, it was shown that onion possesses a higher TPC value compared to garlic (GORINSTEIN *et al*. 2008; NUUTILA, *et al*. 2002). It is also hard to compare the TPC values of marjoram to reference results since various modifications to the methods and units were implemented by the authors.

3.2. Measurement of lipid oxidation

The lipid oxidation in the fresh minced pork meat subjected to refrigerated storage was determined using the TBARS method. This method is widely used to estimate the degree of lipid oxidation because of its relatively simple measurement and good correlation with the sensory quality of foods (MOTTRAM 1998). TBARS are formed through the second stage of lipid oxidation, during which peroxides are oxidized to aldehydes and ketones such as malondialdehyde (MDA). Fig. 1 presents the TBARS values of the samples under study. The results showed that both the storage period and the type of additive affected the TBARS values (with $P < 0.05$). The TBARS values of all the tested samples (control and treatment) increase constantly with the time of storage (Fig. 1). This increase was the most pronounced for the control sample, whose TBARS values rose from 0.11 mg MDA/kg meat on the first day of analysis to 0.352 mg MDA/kg meat at the end of storage (9th day). The TBARS values in the minced meat were influenced by onion, garlic and marjoram, resulting in significantly lower values than those without treatment. As shown in Fig. 1, the most effective treatments in inhibiting the lipid oxidation were the onion and garlic ones. There were no statistically significant differences between these two samples ($P >0.05$). Samples treated with onion and garlic reached TBARS values of 0.22 and 0.186 mg MDA/kg respectively on the last day of storage, compared to the control samples which exhibited a TBARS value equal to 3.5 MDA/kg. Significant differences in the TBARS were found between the treated samples and the control from day 3 to the end of storage. YIN and CHENG (1998) found that garlic had a stronger antioxidant effect than onion in their

liposome model system. In this study, due to sensory acceptability, different levels of additives were tested (0.15 m/m for garlic and 0.5% m/m for onion), which may have caused the lack of difference between the onion and garlic inhibition of lipid oxidation. A statistically significant protective effect for lipid oxidation in meat was also noted for samples with the addition of marjoram. The TBARS value for the treatment with marjoram at the end of storage was 0.28 mg MDA/kg and was significantly lower than the control one. However, this effect was less pronounced when compared to the samples with garlic and onion. In conclusion, the highest inhibition of secondary oxidation products was observed in the samples with garlic (47%). In the samples with onion and marjoram, the percentage of inhibition was 37% and 25% respectively. These results were consistent with those of previous studies in which fresh onion and garlic bulbs or their extracts showed antioxidant effects in the meat matrix (CAO *et al*., 2013; KIM *et al*., 2010; PARK *et al*., 2008; SALLAM *et al*., 2004).

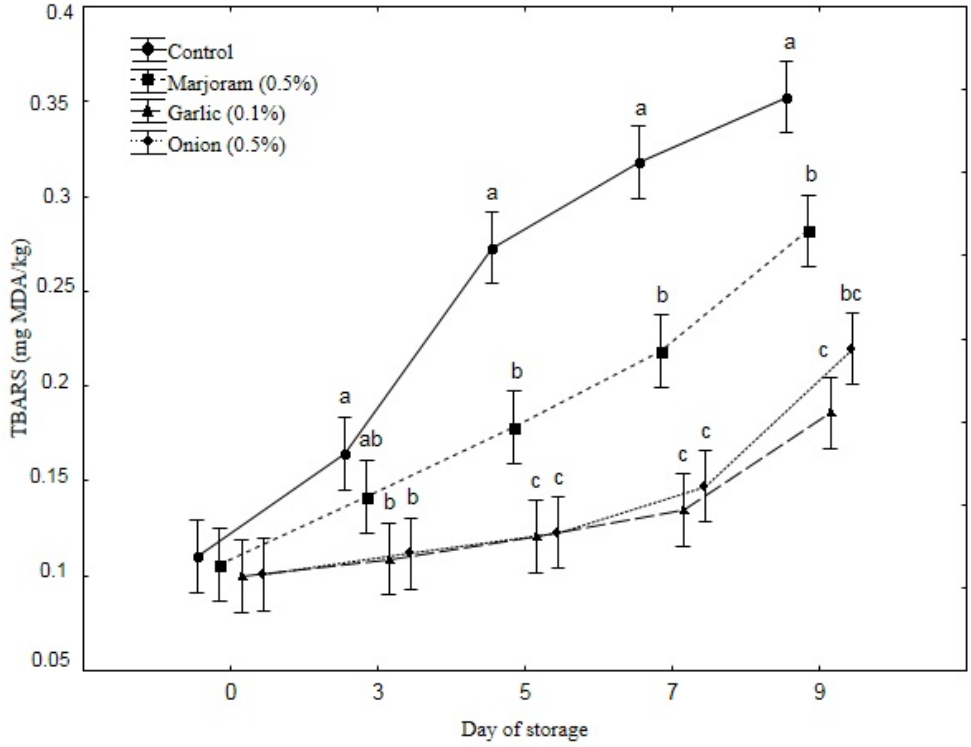


Figure 1. Changes in the TBARS values of fresh minced pork with different treatments during refrigerated storage at 4°C.

3.3. Fatty acid composition analysis

The fatty acid composition of minced pork muscle fat is shown in Table 2. Both the type of additive and storage time influenced the fatty acid composition. The predominant fatty acids in all meat samples were oleic (C18:1n-9), palmitic (C16:0), stearic (18:0) and linoleic (C18:2n-6) acids, in order of the increasing concentration in the samples. The amount of these acids accounted for more than 91% of the total fatty acid contents in all samples. PARK *et al*. (2008) obtained similar results in fresh pork belly and loin. MUFAs were the major part followed by SFAs. Generally, a decrease in the fatty acid content in the

intramuscular lipid was noted during storage. This effect was expected because, due to cellular structure damage and exposure to the oxygen, minced muscle is very prone to lipid and protein oxidation (MIN and AHN, 2005). Therefore, to evaluate whether the fatty acid contents were equal in the groups being studied (type of additive) analysis of covariance was performed. This analysis allows for comparisons of the effects of additives and control the influence of storage time simultaneously. Comparison of the additives showed that a stronger inhibition of lipid oxidation was exhibited by garlic (Table 2). Statistically significantly higher contents of stearic (56.82 mg/g) palmitileic (15.28 mg/g), linoleic (41.1 mg/g), cis-10-heptadanoic (1.62 mg/g) and arachidonic acids (5.14 mg/g) were observed in this treatment when compared to the control. The lowest content of fatty acids was noted in the samples with the addition of fresh onion, which makes it the least effective in the retardation of lipid oxidation. The marjoram additive was the most effective in inhibiting the decomposition of linoleic (but only until the 7th day of storage) and cis-10-heptadanoic acids. Furthermore, to compare the effect of the added natural ingredients on the change rates of fatty acids, linear regression analysis was performed.

Table 2. Effect of onion, garlic and marjoram on the fatty acid profile of fresh minced pork during storage at 4°C (mg/g).

Fatty acid	Treatment				
	Control	Onion	Garlic	Marjoram	SE
C14:0	4.90^{a}	4.62^{a}	6.08^{a}	5.01^{a}	0.39
C16:0	84.31^{ab}	79.95^{a}	106.26^{b}	89.22^{ab}	6.86
C18:0	41.41^{a}	39.89^{a}	56.82^{b}*	47.02^{ab}	3.40
C16:1	12.42^{a}	11.56^{a}	15.28^{b}*	12.64^{a}	0.62
C17:1	1.26^{a}	1.18^{a}	1.62^{b}*	1.85^{c}*	0.05
C18:1	176.59^{ab}	166.92^{a}	198.84^{b}	159.50^{a}	12.06
C20:1	3.35^{a}	3.33^{a}	3.20^{a}	2.74^{a}	0.15
C18:2	35.53^{ab}	33.27^{a}	41.10^{bc}*	42.75^{c}*	1.79
C20:2	1.52^{a}	1.52^{a}	1.47^{a}	1.52^{a}	0.08
C18:3	4.91^{a}	4.56^{a}	2.38^{b}	2.52^{b}	0.21
C20:4	3.33^{a}	3.31^{a}	5.14^{b}*	3.92^{a}*	0.17
SFA	130.63^{ab}	124.46^{a}	169.15^{b}*	141.26^{ab}	10.62
MUFA	193.62^{ab}	182.99^{a}	218.95^{b}	176.73^{a}	8.94
PUFA	45.29^{ab}	42.66^{a}	50.08^{ab}	50.70^{b}	3.19

$^{a-c}$Means with the same superscript within the same row are not different ($P > 0.05$). (*) Paired comparison (control compared with other natural additives) significantly greater than control at the $P < 0.05$ level using Dunnett's-*T* test.

Results showing the regression coefficients from the linear models of the decay of fatty acids are presented in Table 3. The linear model was statistically significant in almost all cases ($P \leq 0.05$). Only for saturated fatty acids in the meat samples with garlic and marjoram, was the linear model not significant ($P > 0.05$). In the samples with garlic, the level of saturated fatty acids was stable during storage. However, in the samples with marjoram the level of saturated fatty acids increased till the 7th day of storage and then rapidly decreased, reaching the lowest values of all the meat samples. The values of the determination coefficients ranged from 74% to 96%.

Table 3. Effect of onion, garlic and marjoram on the fatty acids content changes of fresh minced pork during storage at 4°C (expressed as slope (mg/g/day)).

Fatty acid	Treatment			
	Control	Onion	Garlic	Marjoram
C14:0	-0.568	-0.472	-0.057^{N}	-0.340^{N}
C16:0	-0.500	-7.801	-1.282^{N}	-6.130^{N}
C18:0	-4.357	-3.713	-0.831^{N}	-3.040^{N}
C16:1	-1.391	-1.298	-0.762*	-1.865
C17:1	-0.123	-0.098	-0.076*	-0.173
C18:1	-19.89	-18.97	-9.381*	-23.94
C20:1	-0.401	-0.422	-0.144*	-0.346
C18:2	-3.299	-3.169	-1.613*	-6.089*
C20:2	-0.162	-0.148	-0.068*	-0.133
C18:3	-0.652	-0531	-0.214*	-0.256
C20:4	-0.282	-0.200	-0.165*	-0.621*
SFA	-14.42	-11.99	-2.165*	-9.512
MUFA	-21.81	-20.79	-10.36*	-26.32
PUFA	-4.396	-4.051	-2.060*	-7.101*

$^{(N)}$Regression coefficients are not significant ($P > 0.05$). (*) Paired comparison (control compared with other natural additives) were significantly different to the control at $P < 0.05$.

All the slopes have a negative value indicating the inverse relationship between the storage time and fatty acid content. The lowest rate of fatty acid degradation was recorded in the samples with the fresh garlic addition. This dependency was noted for all fatty acids, which confirms the highest inhibiting effect of garlic on lipid degradation. In addition, discriminant analysis corroborates these outcomes. As shown in Fig. 2, the first discriminant function extracts the sample of meat with garlic, while the second extracts the meat samples with marjoram. The meat samples with onion exhibit the same fatty acid composition during storage as the meat samples without any addition (the points are located in the same place of the graph). The LDA model allows meat samples enriched with garlic and marjoram to be distinguished with 100% accuracy. The highest decomposition rate was noted in the meat samples with marjoram, namely, arachidonic and linoleic acid. The contents of these acids decreased from 6.24 to 1.63 and from 60.2 to 9.2 mg/g respectively. The level of linoleic acid in the samples with marjoram was significantly the lowest among the tested samples at the end of storage.

3.4. Microbial changes

The bacteria selected in this study such as *Pseudomonas* and *Enterobacteriaceae* belong to the genera of bacteria constituting microflora typical for spoilage processes in meat during storage. To assess the effect of the addition of the plants on bacterial growth in meat samples covariance analysis was performed.
Throughout the storage period, the inhibition effect of the plant additives on bacterial growth was observed. Only for lactic acid bacteria was this effect not statistically significant.

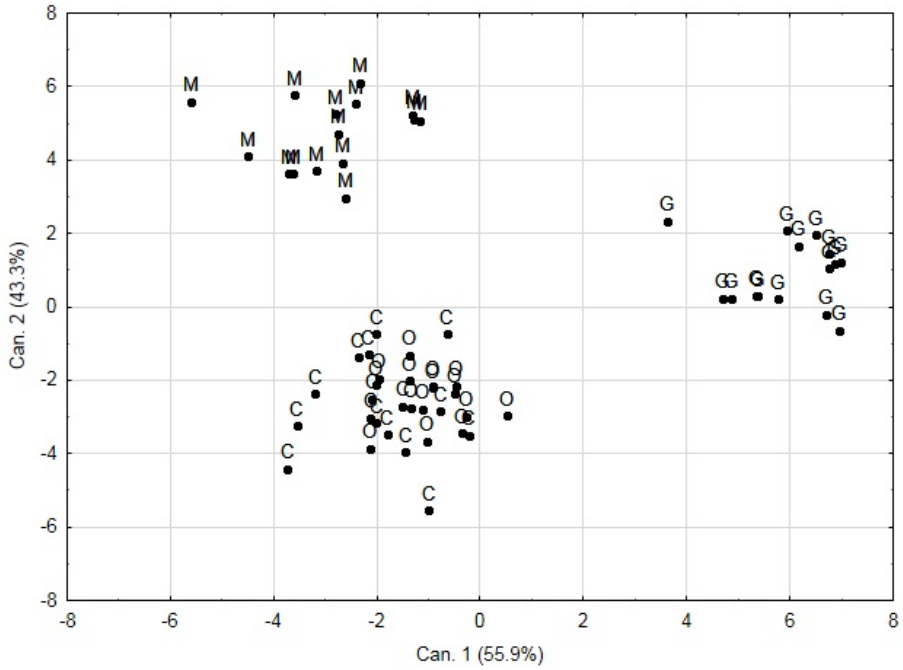


Figure 2. Discrimination of minced meat samples based on the fatty acid composition during cold storage (4°C); C-control, G-meat with garlic (0,1%), M- meat with marjoram (0.5%) and O- meat with onion (0.5%).

As shown in Table 4, the highest numbers of all the tested bacteria were noted in the control samples. The results showed that the addition of onion and marjoram to fresh meat exerted a statistically significant bacteriostatic effect against TVC, *Enterobacteriaceae* and *Pseudomonas* bacteria. The highest inhibitory effect against *Enterobacteriaceae* and *Pseudomonas* bacteria was noted in the meat samples with the addition of fresh onion. The results of our study supported the findings of other authors. GROHS *et al*., (2000) reported that spice mixtures might delay bacteria growth in fresh pork and beef. PARK *et al*., (2008) showed that the addition of garlic and onion powder to pork loin and belly inhibited the growth of the total amount of bacteria and *Enterobacteriaceae*. SALLAM *et al*., (2004) noted that fresh garlic and garlic powder, through their antioxidant and antimicrobial effects, are potentially useful in preserving meat products. However, MURRAY (1997) claimed that only fresh garlic preparations provide the full range of beneficial compounds.

Table 4. Effect of onion, garlic and marjoram additions on the microbial changes (cfu/g) of fresh minced pork stored at 4°C.

Type of bacteria	Treatment			
	Control	Onion	Garlic	Marjoram
TVC	8.02^{a}	7.54^{c*}	7.92^{ab}	7.8^{b*}
Enterobacteriaceae	6.16^{a}	5.65^{b*}	6.09^{a}	5.70^{b*}
Pseudomonas	8.00^{a}	7.52^{c*}	7.84^{ba}	7.74^{bc*}
Lactic acid bacteria	2.84	2.64	2.71	2.65

$^{(a-c)}$Means with the same superscript within the same row are not different ($P > 0.05$).
(*) Paired comparison (control compared with other natural additives) is significant at the $P < 0.05$ level using Dunnett's *T*- test.

4. CONCLUSIONS

Due to the natural potential health benefits and safety of the plant species when compared to synthetic derivatives, natural species have gained appreciable interest among the research and industry community. The highest antioxidant activity is demonstrated by fresh garlic, whereas fresh onion in minced pork meat reveals the highest antibacterial activity. The synergistic behaviour of these plants would require further research. Although marjoram was characterised by the highest antioxidant activity, its protective effect on lipids in meat was less significant than other treatments. This result confirms the necessity to examine the plant antioxidant effect in particular food products. Our data indicated that the addition of fresh garlic and onion, as well as dried marjoram, enhances raw minced meat safety and shelf life. In addition to their economical and health promoting benefits, the application of plants, as natural and safe bio-preservatives, could be highly recommended for the improvement in the quality of ground pork.

ACKNOWLEDGEMENTS

This research project was partly supported by the Polish State Committee for Scientific Research (No. 2014/15/D/NZ9/04261).

REFERENCES

Aguirrezábal M., Mateo J., Domínguez M. and Zumalacárregui J. 2000. The effect of paprika, garlic and salt on rancidity in dry sausages. Meat Sci. 54:77-81.

American Oil Chemist's society (AOCS) Official Method Ce 1k-07 (2007) Direct methylation of Lipids for the Determination of Total Fat, Saturated, cis-Monosaturated, cis-Polyunsaturated and trans Fatty Acids by Chromatography.

Busatta C., Vidal R.S., Popiolski A.S., Mossi A.J., Dariva C., Rodrigues and M.R. Cansian R.L. 2008. Application of Origanum majorana L. essential oil as an antimicrobial agent in sausage. Food Microbiol. 25:207-211.

Cao Y., Gu W., Zhang J., Chu Y., Ye X., Hu Y. and Chen J. 2013. Effects of chitosan, aqueous extract of ginger, onion and garlic on quality and shelf life of stewed-pork during refrigerated storage. Food Chem. 141:1655-1660.

El-Alim S.S.L.A., Lugasi A., Hóvári J. an Dworschák E. 1999. Culinary herbs inhibit lipid oxidation in raw and cooked minced meat patties during storage. J. Sci. Food Agr. 79:277-285.

Falowo A.B., Fayemi P.O. and Muchenje V. 2014. Natural antioxidants against lipid-protein oxidative deterioration in meat and meat products: A review. Food Res. Int. 64:171-181.

Folch J., Lees M. and Sloane-Stanley G.R. 1957. A simple method for the isolation and purification of total lipids from animal tissue. J. Biol. Chem. 226:497-509.

Gorinstein S.. Leontowicz H.. Leontowicz M.. Namiesnik J.. Najman K.. Drzewiecki J. and Trakhtenberg S. 2008. Comparison of the main bioactive compounds and antioxidant activities in garlic and white and red onions after treatment protocols. J. Agr. Food Chem. 56:4418-4426.

Karre L., Lopez K. and Getty K.J.K. 2013. Natural antioxidants in meat and poultry products. Meat Sci. 94:220-227.

Kim Y.J., Jin S.K., Park W.Y., Kim B.W., Joo S.T. and Yang H.S. 2010. The effect of garlic or onion marinade on the lipid oxidation and meat quality of pork during cold storage. J. Food Quality 33(SUPPL. 1):171-185.

Mariutti L.R.B., Barreto G.P.D.M., Bragagnolo N. and Mercadante A. Z. 2008. Free radical scavenging activity of ethanolic extracts from herbs and spices commercialized in Brazil. Braz. Arch. Biol. Techn. 51:1225-1232.

Min B. and Ahn D.U. 2005. Mechanism of Lipid Peroxidation in Meat and Meat Products -A Review. Food Sci. Biotechnol. 14:152-163.

Mottram D.S. 1998. Flavour formation in meat and meat products : a review. Food Chem. 62:415-424.

Murray M. 1997. Which is better. Aged versus fresh garlic; glucosamine sulfate versus chondroitin sulfate. Am. J. Med. 4:5-8.

Nuutila A.M., Kammiovirta K. and Oksman-Caldentey K.M. 2002. Comparison of methods for the hydrolysis of flavonoids and phenolic acids from onion and spinach for HPLC analysis. Food Chem. 76:519-525.

Park S.Y. and Chin K.B. 2010. Effects of onion on physicochemical properties, lipid oxidation and microbial growth of fresh pork patties. Int. J Food Sci. Tech. 45:1153-1160.

Park S.Y., Yoo S.S., Shim J.H. and Chin KB 2008. Physicochemical properties, and antioxidant and antimicrobial effects of garlic and onion powder in fresh pork belly and loin during refrigerated storage. J. Food Sci. 73:577-584.

Roby M.H.H., Sarhan M.A., Selim K.A.H. and Khalel K.I. 2013 Evaluation of antioxidant activity, total phenols and phenolic compounds in thyme (*Thymus vulgaris* L.), sage (*Salvia officinalis* L.), and marjoram (*Origanum majorana* L.) extracts. Ind. Crop Prod. 43:827-831.

Sallam K.I., Ishioroshi M. and Samejima K. 2004. Antioxidant and antimicrobial effects of garlic in chicken sausage. Food Sci Technol-LEB 37:849-855.

Sánchez-Moreno C., Larrauri J.A. and Saura-Calixto F. 1998. A procedure to measure the antiradical efficienc y of polyphenols. J. Sci. Food Agr. 270:270-276.

Shah M.A., Bosco S.J.D. and Mir S.A. 2014. Plant extracts as natural antioxidants in meat and meat products. Meat Sci 98:21-33.

Singleton V.L. Rossi J.A. 1965. Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. Am. J. Enol. Viticult. 16:144-158.

Sørensen G. and Jørgensen S.S. (1996). A critical examination of some experimantal variables in the 2thiobarbituric acid (TBARS) test for lipid oxidation in meat products. Z Lebensm Unters For 202:205-210.

Wijewickreme A.N. nd Kitts D.D. 1998. Modulation of metal-induced genotoxicity by Maillard reaction products isolated from coffee. Food Chem. Toxicol. 36:543-553.

Ye C.L. Dai D.H. and Hu W.L. 2013. Antimicrobial and antioxidant activities of the essential oil from onion (*Allium cepa* L.). Food Control 30:48-53.

Yin M.C. and Cheng W.S. 1998. Antioxidant activity of several Allium members. J Agric Food Chem 46:4097-4101.

Paper Received March 24, 2017 Accepted July 10, 2017

PAPER

FATTY ACID COMPOSITION OF DIFFERENT ADIPOSE TISSUES IN HEAVY PIGS

A. DAZA[a], A. OLIVARES*[b], M.A. LATORRE[c], A.I. REY[b], A. CALLEJO[a] and C.J. LÓPEZ BOTE[b]

[a]Departamento de Producción Animal, ETSI Agrónomos, Universidad Politécnica. 28040, Madrid, Spain

[b]Departamento de Producción Animal, Facultad de Veterinaria, Universidad Complutense. 28040, Madrid, Spain

[c]Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Universidad de Zaragoza. 50013, Zaragoza, Spain

*E-mail address: alolivares@vet.ucm.es

ABSTRACT

Forty-seven castrated male Duroc x (Landrace x Large White) pigs were used to determine fatty acids compositions from different adipose tissues. The outer subcutaneous backfat layer had a lower proportion of saturated and higher monounsaturated and polyunsaturated fatty acids than the inner layer. Liver fat had the highest proportion of polyunsaturated fatty acids. Intramuscular fat followed by subcutaneous backfat had the highest monounsaturation indexes. Moreover, omental and hepatic fat had the highest amount of n-3 fatty acids. In conclusion, the fatty acid profile was depended on fat location, with intramuscular and outer backfat the most beneficial from the point of view of nutrition and health.

Keywords: fatty acid profile, fatty tissues, heavy pigs

1. INTRODUCTION

The fatty acid profile of adipose tissue in pigs is especially important since it relates to quality aspects in the process of preparing dry-cured products such as consistency, lipid oxidation, salt and water migration, curing duration, etc. (LOPEZ-BOTE, 2000; LOPEZ BOTE *et al.,* 2004) and the production of volatile compounds that are responsible for odor and flavor (BELITZ and GROSCH, 1997). Moreover, much attention is currently being paid to the fatty acid profile in animal products because of its association with consumer health (WOOD and ENSER, 1997; FAO, 2012). Hence a correlation has been found between saturated fatty acid consumption and cardiovascular diseases (FAO, 2012), whereas polyunsaturated fatty acids (mainly n-3 fatty acids) have been found to improve the status of the cardiovascular system and the immune response (WOOD and ENSER, 1997). However, high proportions of n-3 fatty acids in tissues reduce oxidative stability of meat (REY *et al*., 2001).
In pigs, fatty acid composition of fat tissue is influenced by a wide range of factors such as genetics, sex, weight and age at slaughter, livestock production system, feed, environmental conditions, pre-slaughter management, etc. (MOUROT and HERMIER 2001; DE SMET *et al.,* 2004; LOPEZ-BOTE *et al.,* 2004). The effect of adipose tissue location on fatty acid profile has recently been studied in the Celta pig breed, which is a rustic breed from Spain (DOMINGUEZ *et al.* 2014; DOMINGUEZ & LORENZO 2014); however, there is a lack of informations in pigs of improved genotypes at heavy weights. Therefore, given the importance of the topic, the aim of this study was to investigate the fatty acid profile of fat from different locations (hepatic, intramuscular and omental) in heavy pigs.

2. MATERIALS AND METHODS

2.1 Animals, diet and sampling

All the experimental procedures used in the study were in compliance with the Spanish guidelines for the care and use of animals in research (BOLETÍN OFICIAL DEL ESTADO, 2007).
Forty-seven castrated male Duroc x (Landrace x Large White) pigs were used. All the animals received the same diet during the finishing period and before the beginning of the experiment all pigs were subjected to the same management and feeding conditions. The feedstuff consisted of a commercial diet based on barley, wheat and various vegetable protein sources (soybean, rapeseed, sunflower) that was administered *ad libitum*. The calculated energy (FEDNA, 2010) and composition (AOAC, 2000) of the diet are shown in Table 1. The pigs were slaughtered at 126±2.8 kg of live weight. On each carcass, fat thickness was measured by means of a graduated rule at the level of the *Gluteus medius* muscle. Individual samples of inner and outer subcutaneous backfat layers and *Longissimus dorsi* muscle at the level of the last rib from each left loin, and omental and hepatic fat samples were taken. Samples were vacuum-packaged in individual bags and stored at -20ºC for 3 weeks until subsequent analysis.

2.2 Fatty acid analysis of diet and fat

Fatty acids of the diet were extracted and quantified using the one-step procedure described by SUKHIJA and PALMQUIST (1988) in lyophilized samples. Pentadecenoic acid (C15:1) (Sigma, Alcobendas, Madrid, Spain) was used as internal standard. Previously, methylated fatty acids samples were identified according to REY *et al.* (1997)

using a gas chromatograph (Model HP6890; Hewlett Packard Co., Avondale, PA, USA) and a 30 m x 0.32 mm x 0.25 μm cross-linked polyethylene glycol capillary column (Hewlett Packard Innowax). A temperature program of 170ºC to 245ºC was used. The injector and detector were maintained at 250 ºC. The carrier gas (helium) flow rate was 3 ml/min.
Lipids from subcutaneous and omental fat were extracted using the procedure proposed by BLIGH and DYER (1959), whereas lipids from *Longissimus dorsi* and liver fat samples were extracted according to method developed by MARMER and MAXWELL (1981). Fat extracts were methylated in the presence of sulphuric acid and analysed as described above. Fatty acid percentages of the saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) were calculated. The indexes used to estimate thioesterase (Zhang et al., 2007) and elongase enzyme activity were C16:0/C14:0 and C18:0/C16:0 respectively. The Δ-9 desaturase activity was estimated by means of the following ratios: C16:1n-7/C16:0, C18:1n-9/C18:0 and MUFA/SFA.

Table 1. Calculated and determined analyses of the diet**.**

Calculated analysis	**g/kg**
Digestible energy (MJ/kg)	13.62
Determined analysis [(1)]	886.2
Crude protein (N x 6.25)	145.2
Crude fat	56.2
Crude fibre	35.1
Fatty acids (%)	
C14:0	0.29
C16:0	4.71
C18:0	1.61
C18:1 n-9	5.68
C18:2 n-6	16.23
C18:3 n-3	3.49

The ingredients composition of the diet in g/kg was: barley 430, wheat 301, Full fat soybean toasted 65.2, Rapeseed meal 84.0, Sunflower meal 54.2, Fat (lard) 31.0, Calcium carbonate 11.0, Sepiolite, 10.0, L - lysine 50% 5.5, Sodium chloride 4.1, Vitamin and mineral premix 4.0.
[(1)]According to Association of Official Analytical Chemists (2000).

2.3 Statistical analysis

The pig was the experimental unit for statistical analysis. Data were analyzed by means of variance analysis that included the adipose tissue location as the main effect. The Newman-Keuls test was used to assess differences between means. The relations between C18:2n-6 and C16:0 proportions in subcutaneous backfat layers were calculated by simple linear regression, comparing intercepts and slopes using "t" Student test. Correlation coefficients between *Gluteus medius* fat thickness and major fatty acid proportions from subcutaneous backfat, and correlation coefficients between intramuscular fat percentage from *Longissimus dorsi* and major fatty acid proportions of such tissue were calculated. All analyses were carried out using the GLM and CORR procedures of SAS (1999).

3. RESULTS

The omental fat had the highest proportion of SFA followed by the liver fat due to the high C18:0 proportion (Table 2). The outer subcutaneous backfat layer showed a lower saturation and higher mono and polyunsaturation than the inner layer. The C16:0 and C18:0 proportions were lower (P<0.0001) in the outer subcutaneous backfat layer than in the inner layer, whereas C16:1n-7, C18:1n-9, C18:1n-7, C18:2n-6 and C18:3n-3 were higher (P<0.0001) (Table 2).

Table 2. Major fatty acid composition (%) of different adipose tissues from heavy pigs.

Tissue	SFOL	SFIL	LDIF	OF	HF	sem	P <
n	47	47	47	47	46		
C16:0	23.78^{c}	24.84^{b}	24.43^{b}	27.38^{a}	21.97^{d}	0.22	0.0001
C16:1 n-7	1.86^{c}	1.67^{d}	3.49^{a}	1.58^{d}	2.43^{b}	0.061	0.0001
C18:0	14.56^{d}	16.74^{c}	13.29^{e}	20.37^{b}	23.18^{a}	0.45	0.0001
C18:1 n-9	41.84^{ab}	40.53^{b}	43.05^{a}	35.72^{c}	30.72^{d}	0.48	0.0001
C18:1 n-7	2.41^{b}	2.16^{c}	3.54^{a}	1.50^{d}	-	0.036	0.0001
C18:2 n-6	10.56^{a}	9.36^{b}	6.70^{c}	8.98^{b}	11.16^{a}	0.24	0.0001
C18:3 n-3	0.79^{a}	0.70^{b}	0.044^{d}	0.72^{b}	0.44^{c}	0.020	0.0001
C20:4 n-6	0.63^{b}	0.58^{b}	0.14^{b}	0.42^{b}	5.96^{a}	0.18	0.0001
SFA	40.06^{d}	43.26^{c}	39.64^{d}	49.66^{a}	47.03^{b}	0.37	0.0001
MUFA	47.81^{b}	45.96^{c}	51.57^{a}	40.07^{d}	34.14^{e}	0.050	0.0001
PUFA	12.13^{b}	10.77^{c}	8.77^{d}	10.27^{c}	18.83^{a}	0.47	0.0001
MUFA+PUFA	59.94^{a}	56.73^{b}	60.34^{a}	50.34^{d}	52.97^{c}	0.47	0.0001
n-6	11.19^{b}	9.93^{c}	6.84^{d}	9.41^{c}	16.84^{a}	0.54	0.0001
n-3	0.93^{b}	0.83^{b}	0.37^{c}	0.86^{b}	1.74^{a}	0.42	0.0001

n = number of observations, SFOL= subcutaneous backfat outer layer, SFIL = subcutaneous backfat inner layer, LDIF = *Longissimus dorsi* intramuscular fat, OF = omental fat, HF = hepatic fat. sem = error standard of the mean. SFA, MUFA, PUFA, n-6, n-3 = sum of all saturated (SFA), monounsaturated (MUFA), polyunsaturated (PUFA), n-6 and n-3 fatty acids.

In the current experiment, the relations obtained between C18:2n-6 and C16:0 proportions in the outer and inner subcutaneous backfat layers were described by the following simple regression equations:

Outer layer: C18:2n-6 (%) = (28.40^{a}±2.58) – (0.750^{b}±0.11) C16:0 (%)
R^2 = 0.53, RSD = 0.98, P <0.0001 n = 47.

Inner layer: C18:2n-6 (%) = (29.76^{a}±2.48) – (0.823^{a}±0.10) C16:0 (%)
R^2 = 0.62, RSD = 0.88, P <0.0001, n = 47.

Table 2 also shows that C16:1n-7, C18:1n-9, C18:1n-7 and MUFA proportions were higher in the intramuscular fat of *Longissimus dorsi* muscle than in the other adipose tissues (P<0.0001).

The omental fat had lower monounsaturated and polyunsaturated fatty acids than subcutaneous backfat. The 18:2n-6, C20:4n-6, n-6, n-3 and PUFA proportions were higher in hepatic fat than in the other adipose tissues.
In this study, the highest unsaturation ranges (sum of MUFA and PUFA fatty acids) were obtained in intramuscular fat from *Longissimus dorsi* muscle and outer subcutaneous backfat layer followed by inner subcutaneous backfat layer, liver fat and omental fat.
The enzymatic activity indexes of different adipose tissues are shown in Table 3. The thioesterase index (C16:0/C14:0), that indicates catalysis of C16:0 synthesis from C14:0, and the elongase index, as an indicator of C18:0 synthesis from C16:0 (C18:0/C16:0), were higher ($P<0.0001$) in liver fat than in the other adipose tissues. The C16:0/C14:0 and C18:0/C16:0 indexes in omental fat were higher than those in longissimus dorsi intramuscular fat ($P<0.0001$). However, C16:0/C14:0 indexes were of similar magnitude in omental fat and inner backfat layer. Both indexes were higher in subcutaneous backfat than in intramuscular fat. The highest monounsaturation indexes C18:1 n-9/C18:0 and MUFA/SFA were found in intramuscular fat followed by outer the subcutaneous backfat layer, inner subcutaneous backfat layer, omental fat and liver fat. However, the C16:1 n-7/C16:0 index was higher in hepatic fat than in subcutaneous and omental fat.
The highest PUFA/SFA ratios were found in hepatic fat and the outer subcutaneous backfat layer and the lowest in intramuscular and omental fat. The lowest n-6/n-3 ratios were found in hepatic and omental fat and the highest in intramuscular fat from *Longissimus dorsi* muscle. The highest MUFA/SFA ratio was observed in intramuscular fat.
The subcutaneous backfat thickness at the level of ham *Gluteus medius* muscle was 23.5±5.93 mm. This variable was positively correlated with C16:0 and SFA and negatively correlated with C18:1n-9, MUFA, C18:2n-6 and PUFA proportions of the outer and inner subcutaneous backfat layers (Table 4). The intramuscular fat percentage from *Longissimus dorsi* muscle was 4.02±0.90%. This variable was positively correlated with C16:0 ($r = 0.47$ $p<0.001$) and SFA ($r = 0.30$ $p<0.01$), and negatively correlated with C18:2n-6 ($r = -0.38$, $p<0.01$) and PUFA ($r = –0.39$ $p<0.01$) proportions found in *Longissimus dorsi* intramuscular fat. However, correlations were not found between *Longissimus dorsi* intramuscular fat and C18:1n-9 and MUFA proportions.

Table 3. Saturation and monounsaturation and quality index of different tissues from heavy pigs.

Tissue	SFOL	SFIL	LDIF	OF	HF	sem	P <
n	47	47	47	47	46		
C16:0/C14:0	20.15^{c}	22.04^{b}	16.59^{d}	21.38^{bc}	27.26^{a}	0.57	0.0001
C18:0/C16:0	0.61^{c}	0.68^{bc}	0.54^{d}	0.74^{b}	1.08^{a}	0.025	0.0001
C16:1 n-7/C16:0	0.078^{c}	0.067^{d}	0.14^{a}	0.057^{e}	0.11^{b}	0.0024	0.0001
C18:1n-/C18:0	2.92^{b}	2.41^{c}	3.28^{a}	1.77^{d}	1.51^{e}	0.072	0.0001
MUFA/SFA	1.20^{b}	1.07^{c}	1.31^{a}	0.81^{d}	0.75^{e}	0.019	0.0001
PUFA/SFA	0.31^{b}	0.25^{c}	0.22^{cd}	0.21^{d}	0.41^{a}	0.011	0.0001
n-6/n-3	12.52^{b}	12.03^{bc}	19.08^{a}	11.00^{cd}	10.32^{d}	0.44	0.0001

n = number of observations, SFOL= subcutaneous backfat outer layer, SFIL = subcutaneous backfat inner layer, LDIF = *Longissimus dorsi* intramuscular fat, OF = omental fat, HF = hepatic fat. sem = error standard of the mean. SFA, MUFA, PUFA, n-6, n-3 = sum of all saturated (SFA), monounsaturated (MUFA), polyunsaturated (PUFA), n-6 and n-3 fatty acids.

Table 4. Correlation coefficients (r) between fat thickness (mm), at the level of *Gluteus medius* muscle, and major fatty acid proportions (%) in outer and inner backfat layers.

Fatty acid (outer layer)	r	Fatty acid (inner layer)	r
C16:0	0.64****	C16:0	0.55***
SFA	0.61****	SFA	0.58***
C18:1 n-9	- 0.41**	C18:1 n-9	- 0.40**
MUFA	- 0.44**	MUFA	- 0.45**
C18:2 n-6	- 0.53***	C18:2 n-6	-0.52***
PUFA	- 0.54***	PUFA	-0.52***

n = 47, ** p<0.01, ¨ p<0.001, ¨¨ p<0.0001. SFA, MUFA, PUFA = sum of all saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids.

4. DISCUSSIONS

The fact that omental and liver fat were more saturated than subcutaneous and intramuscular fat, is explained by the activity of lipogenic enzymes involved in *de novo* synthesis, which vary with tissue location (NARVAEZ-RIVAS *et al.*, 2009). Thus, in our experiment, the highest elongase index values (C18:0/C16:0) were detected in liver and omental fat whereas the thioesterase index (C16:0/C14:0) was higher in the liver, omental and inner subcutaneous backfat layer compared to the other fatty tissues. These results are in agreement with DOMINGUEZ *et al.* (2014), who observed in the Celta breed pigs higher values of elongase and thioesterase indexes in internal perirenal fat than in subcutaneous and intramuscular fat. According to BEE (2001), lipogenic activity of malic enzyme was higher in omental fat than inner and outer subcutaneous backfat layers, but fatty acid synthase activity was similar in these tissues, for different dietary fat types. On the other hand, the activities of both enzymes were similar in the inner and outer subcutaneous backfat layers when pigs received the same energy and fat content in feed as in this experiment. Moreover, BENITEZ *et al.* (2012) found in Iberian pigs fed saturated (5% hydrogenated lard), monounsaturated (5% high oleic sunflower) or polyunsaturated (5% sunflower) enriched diets that liver fat was more saturated than inner and outer subcutaneous backfat layers and *Longissimus dorsi* intramuscular fat. In our study, the outer subcutaneous backfat layer was more monounsaturated and polyunsaturated and less saturated than the inner layer, which is consistent with the results observed by IRIE and SAKIMOTO (1992), BEE *et al.* (2002), DAZA *et al.* (2007) and MONZIOLS *et al.* (2007). In a later study, BEE *et al.*, (2002) observed that the activity of the different lipogenic enzymes was not uniform in the outer and inner subcutaneous backfat layers. Hence, these authors reported that the activity of malic enzyme was lower in the inner than outer subcutaneous backfat layer, the activity of glucose 6-phosphate dehydrogenase was similar in both layers, whereas the fatty acid synthase enzyme activity was higher in the inner than in the outer layer.

The metabolic principles, on which the deposition of linoleic acid is higher in the outer than inner subcutaneous backfat layers in pigs, have not been sufficiently explained (MONZIOLS *et al.*, 2007). The higher *de novo* synthesis of SFA (especially C16:0) in the inner subcutaneous backfat layer resulted in a higher dilution of C18:2n-6 in the inner than outer subcutaneous backfat layer, in agreement with the results of the regression equations obtained between the proportions of C18:2n-6 and C16:0. The slope of the regression equation corresponding to the inner subcutaneous backfat layer was higher

than in the outer layer (0.823 *vs* 0.750, p<0.01). This means that as the C16:0 proportion increased in subcutaneous backfat, the decrease in the proportion of C18:2n-6 was higher in the inner than in the outer layer.
These results are in agreement with those obtained by CHRISTIE *et al.* (1972) and MONZIOLS *et al.* (2007). According to the earlier study of THOMPSON and ALLEN (1968), the degree of unsaturation of the fatty tissue in swine decreases from external to internal tissues, so that the high-to-low unsaturation gradation follows the series: outer subcutaneous layer, middle subcutaneous layer, inner subcutaneous layer, intramuscular fat and internal fat (omental, perirenal, etc.) (VILLEGAS *et al.*, 1973; MONZIOLS *et al.*, 2007). DEAN and HILDITCH (1933) reported that this gradation can be explained by a possible adaptation of adipose tissue to temperature in order to maintain adequate physical fluidity of lipids in different adipose tissues, so that the melting point of the fat would increase from subcutaneous fat to internal locations.
In this study, the unsaturation of *Longissimus dorsi* intramuscular fat was similar to the outer subcutaneous backfat layer and higher than the internal and omental fat, due to high proportions of C16:1n-7, C18:1n-9 and C18:1n-7 in intramuscular fat. Unsaturation indexes such as C16:1n-7/C16:0, C18:1n-9/C18: 0 and MUFA/SAT, which estimate the activity of the enzyme delta-9 desaturase (responsible for the formation of C18:1n-9 from C18:0), were higher in the *Longissimus dorsi* intramuscular fat than subcutaneous backfat, omental and hepatic fat. Also, LOPEZ-BOTE *et al.* (2002), BEE (2001), BEE *et al.* (2002) and BEE *et al.* (2008) in improved white pigs, DAZA *et al.* (2014) in Iberian pigs and DOMINGUEZ *et al.* (2014) and DOMINGUEZ and LORENZO (2014) in Celta breed pigs found that intramuscular fat was more monounsaturated than outer and inner subcutaneous backfat and omental fat. However, LLUCH *et al.* (1993) found that the intramuscular fat was less monounsaturated than subcutaneous fat, although this result was obtained from male and female heterogeneous samples. FRANCO *et al.* (2006) did not detect significant differences between the ratio of MUFA/SFA in intramuscular fat and subcutaneous backfat in Celta breed pigs, but observed lower concentrations of C18:1n-9 in intramuscular fat than subcutaneous backfat. MONZIOLS *et al.* (2007) found higher proportions of C18:1n-9 and MUFA in subcutaneous backfat than intermuscular fat from the loin, but these authors did not provide data on fatty acid profile of intramuscular fat.
Omental fat showed lower (p <0.05) proportions of C18:1n-9 and MUFA fatty acids than outer and inner subcutaneous backfat layers and intramuscular fat of *Longissimus dorsi.* These results agree with those obtained by BEE (2001) and BEE *et al.* (2002). Liver fat was the most polyunsaturated because the fatty acid profile in liver is largely influenced by diet. Pig liver has little ability to synthesize fatty acids, hence fatty acids used in the synthesis of lipids are mainly derived from circulating fatty acids provided by adipose tissue or feed (mainly C18:2n-6). Most triglycerides synthesized in the liver are esterified or stored, so that the composition is a reflection of the composition of plasma fatty acids, mainly, from the feed consumed (OTTEN *et al.* 1993). Also, DOMINGUEZ *et al.* (2014) found in Celta breed pigs, that liver fat was the most polyunsaturated when compared with the dorsal and ventral subcutaneous fat and *Longissimus dorsi* intramuscular fat.
The nutritional value of adipose tissues is related to high values of PUFA/SAT, MUFA/SAT ratios and low values for n-6/n-3 ratio. PUFA/SAT and n-6/n-3 values of the present study were similar to those obtained by DOMINGUEZ and LORENZO (2014) and DOMINGUEZ *et al.* (2014) in Celta breed pigs**.** In turn, in agreement with the present study, DOMINGUEZ *et al.* (2014) observed the highest value of PUFA/SFA in liver fat and the lowest in *Longissimus dorsi* intramuscular fat. WOOD and ENSER (1997), recommended values for PUFA/SAT higher than 0.45 and n-6:n-3 lower than 4.0 in contrast to the results found in the present study. The high n-6/n-3 fatty acid ratio of the present study is explained by the fact that pigs were fed a concentrate rich in

carbohydrates and C18:2 n-6. According to the results of the present study, the highest n-3 fatty acid proportion was found in omental and hepatic fat. These tissues would have lower lipid stability than the others (REY *et al.* 2001) and this may lead to more production of undesirable substances for consumers than potential benefits. Regarding the curing process of meat and in order to obtain adequate fat quality, MOUROT and HERMIER (2001) recommended that pig adipose tissue should contain no more than 12% of C18:2n-6 and exceed 12% of C18:0. All adipose tissues studied in the present research were within those ranges.

In the present study, we found positive correlations between backfat thickness and C16:0 and SFA proportions, whereas correlations were negative between thickness and C18:1n-9, MUFA, C18:2n-6 and PUFA proportions. MONZIOLS *et al.* (2007) found positive and negative correlation coefficients among PUFA and SFA ratios and muscle percentage of carcass, which is negatively correlated with backfat thickness at *Gluteus medius* level (IRTA, 2013). According to MOUROT and HERMIER (2001), a reduction of backfat thickness in pigs leads to an increase in C18:2n-6 proportion and a decrease in endogenous synthesis. In the present experiment, saturated and polyunsaturated fatty acids proportions and *Longissimus dorsi* intramuscular fat percentages were positively and negatively correlated, respectively. These results explain that *de novo* synthesis of fatty acids is reduced in the leanest pigs and, consequently, C18:2n-6 is less diluted which is related to a higher content of C18:2n -6 in adipose tissue.

5. CONCLUSIONS

Fatty acid profile varies according to fat tissue location probably due to variability of enzyme activity. In heavy pigs fed conventional feed, the omental and liver fats are the most saturated followed by the inner, outer subcutaneous backfat and intramuscular fat. The highest unsaturation corresponded to intramuscular fat, due to increased activity of the delta-9 desaturase enzyme, followed by outer, inner subcutaneous backfat, omental and liver. Negative correlations were detected between the backfat thickness and the percentage of intramuscular fat with respect to the proportion of PUFA, which is important given the effect of PUFA content on susceptibility to oxidation. The fatty acid profile of adipose tissue from heavy pigs is suitable for the preparation of cured products, and intramuscular and outer backfat are the most suitable from the point of view of nutrition and health.

ACKNOWLEDGEMENTS

This research was supported by INIA (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Spain) project (PET-2007-08-C11-04).

REFERENCES

AOAC. 2000. Official methods of analysis. AOAC , Arlington, VA.

Belitz H.D. and Grosch W. 1997. *Química de los alimentos*. Ed Acribia, Zaragoza, España.

Bee G. 2001. Dietary conjugated linoleic acids affect tissue lipid composition but not de novo lipogenesis in finishing pigs. Animal Research 50:383-399.

Bee G., Gebert S. and Messikommer R. 2002. Effect of dietary energy supply and fat source on the fatty acid pattern of adipose and lean tissues and lipogenesis in the pig. Journal of Animal Science 80:1564-1574.

Bee G., Jacot S., Guex G. and Biolley C. 2008. Effects of two supplementation levels of linseed combined with CLA or tallow won meat quality traits and fatty acid profile of adipose and different muscle tisúes in slaughter pigs. Animal 2:800-811.

Benítez R., Núñez Y., Fernández A., Alves E., Martín Palomino P., Rodrigáñez J., Fernández A.I., Rodríguez C., López Bote C., Silió L. and Oviló C. 2012. Influencia de la composición de la dieta sobre el perfil de ácidos grasos y la expresión génica en tejidos adiposos, muscular y hepático de cerdos ibéricos. Revista Complutense de Ciencias Veterinarias 6:25-29.

Bligh E.G. and Dyer W.J. 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37:911-917.

BOE 2007. Ley 32/2007 de 7 de noviembre para el cuidado de los animales en su explotación, transporte, experimentación y sacrificio, vol. 268 BOE Madrid, Spain, pp. 45914-45920.

Christie W.W., Jenkinson D.M. and Moore J.H. 1972. Variation in lipid composition through the skin and subcutaneous adipose tissue of pigs. Journal of the Science of Food and Agriculture 23:1125-1129.

Daza A., Mateos A., Rey A.I., Ovejero I. and López Bote C.J. 2007. Effect of duration of feeding under free-range conditions on production results and carcass and fat quality in Iberian pigs. Meat Science76:411-416.

Dean H.K. and Hilditch T.P. 1933. The body fats of the pigs III. The influence of body temperature on the composition of depot fats. Biochemical Journal 27:1950-1961.

De Smet S., Raes, K. and Demeyer, D. 2004. Meat fatty acid composition as affected by fatness and genetic factors:a review. Animal Research 53:81-98.

Domínguez R., Martinez S., Carballo J. and Franco I. 2014. Fatty acid profile and cholesterol and retinol contents in different locations of Celta pig bred. Grasas y Aceites, 65:e036. doi: http://dx.doi.org/10.3989/gya.0115141.

Domínguez R. and Lorenzo J.M. 2014. Effect of genotype on fatty acid composition of intramuscular and subcutaneous fat of Celta pig bred. Grasas y Aceites, 65:e037.doi:http://dx.doi.org/10.3989/gya.0234141.

FAO. 2012. Grasas y ácidos grasos en nutrición humana. Consulta de expertos. Spain, FAO y FINUT.

FEDNA. 2010. Tablas FEDNA de composición y valor nutritivo de alimentos para la fabricación de piensos compuestos. ETS de Ingenieros Agrónomos, Universidad Politécnica de Madrid. Spain, FEDNA.

Franco I., Escamilla M.C., García, J., García M.C. and Carballo J. 2006. Fatty acid profile of the fat from Celta pig breed fattened using a traditional feed: Effect of the location in the carcass. Journal of Food Composition and Analysis 19**:**792-799.

Irie M. and Sakimoto M.. 1992. Fat characteristics of pigs fed fish on containing eicosapentaenoic and docosahexaenoic acids. Journal of Animal Science 70:470-477.

López Bote C.J. 2000. Dietary treatment and quality characteristics of Mediterranean meat products. In: Decker E., Faustman C. and López Bote C.J. (Eds. Antioxidants in muscle foods: nutritional strategies to improve quality. New York, Wiley Interscience 345-366.

López Bote C., Isabel B. and Daza, A. 2002. Partial replacement of poly-with monounsaturated fatty acids and vitamin E supplementation in pigs diets:effect on fatty acid composition of subcutaneous and intramuscular fat and on fat and lean firmness. Animal Science 75**:**349-358.

López Bote C.J., Rey A.I. and Menoyo D. 2004. Efecto de la alimentación en la composición y características de la grasa. Porci 84:34-58.

Lluch M.C., Pascual J., Parcerisa J., Guardiola F., Codony R., Rafecas M. and Boatella J. 1993. Contenidos de isómeros trans de los ácidos grasos en productos cárnicos. (II)Tejido adiposo y grasa intramuscular del cerdo. Grasas y Aceites. 44 Fasc. 2, 97-100.

Marmer W.N. and Maxwell R.J. 1981. Dry column method for the quantitative extraction and simultaneous class separation of lipids from muscle tissue. Lipids 16:365-371.

Monziols M., Bonneau M., Davenel A. and Kouba M. 2007. Comparison of the lipid content and fatty acid composition of intermuscular and subcutaneous adipose tisúes in pig carcasses. Mear Science 76:54-60.

Mourot J. and Hermier D. 2001. Lipid in monogastric animal meat. Reproduction Nutrition Development 41:109-118.

Narváez-Rivas M., León-Camacho M. and Vicario I.M. 2009. Fatty acid triacylglycerol composition of the subcutaneous fat from Iberian pigs fattened on the traditional feed:"Montanera" effect of anatomical location and length of feeding. Grasas y Aceites 60:238-247.

Otten W., Wirth C., Itazzo P.A. and Eichinger H.M. 1993. A high omega 3 fatty acid diet alters fatty acid composition of Herat, liver, kidney, adipose tissue and skeletal muscle in swine. Annals of Nutrition and Metabolism 37:134-141.

Rey A.I., López Bote C.J. and Arias R. 1997. Effect of extensive feeding on alpha-tocopherol concentration and oxidative stability of muscle microsomes from Iberian pigs. Animal Science 65:515-520.

Rey A.I., Kerry J.P., Lynch P.B. López-Bote C.J., Buckley D.J. and Morrissey P.A. 2001. Effect of dietary oils and alpha-tocopheryl acetate supplementation on lipid (TBARS) and cholesterol oxidation in cooked pork. Journal of Animal Science 79:1201-1208.

SAS. 1990). SAS user´s guide: statistics. Statistical Análisis Systems Institute Inc, Cary, NC.

Sukhija P.S. and Palmquist D.L. 1988. Rapid method for determination of total fatty acid content and composition of feedstuffs and feces. Journal of Agricultural and Food Chemistry 36:1202-1206.

Thompson E.H. and Allen E. 1969. Relationship between stearic acid desaturase and fatty acid composition. Journal of Animal Science 29:127-128.

Villegas F.J., Hedrick H.B., Deum T.L., Mc Fate K.L. and Bailey M.E. 1973. Effect of diet and breed on fatty acid composition of porcine adipose tissue. Journal of Animal Science 36:663-668.

Wood J.D. and Enser M. 1997. Factors influencing fatty acids in meat and the role of antioxidants in improving meat quality. British Journal of Nutrition 78:S49-S60.

Zhang S., Knight T.J., Stalder K.J., Goodwin R.N., Lonergan S.M. and Beitzt,D.C. 2007. Effects of breed, sex, and halothane genotype on fatty acid composition of pork longissimus muscle. Journal of Animal Science 85:583-591.

Paper Received November 13, 2016 Accepted June 6, 2017

PAPER

DIETARY BEHAVIOURS AND AWARENESS OF SEASONAL FOOD AMONG COLLEGE STUDENTS IN CENTRAL ITALY

M. TAMBURRO[1], G. RIPABELLI*[1], M.B. FORLEO[2] and M.L. SAMMARCO[1]

[1]Department of Medicine and Health Sciences "V. Tiberio", University of Molise, Campobasso, Italy

[2]Department of Economics, Management, Society and Institutions, University of Molise, Campobasso, Italy

*Corresponding author. Tel. +39 0874 404961

E-mail address: ripab@unimol.it

ABSTRACT

In this study, eating behaviours and knowledge of food seasonality among college students who will become teachers were investigated. A questionnaire was administered to students to collect data on dietary habits, physical activity, and awareness of food season. Two-hundred eighty students participated (95.4% female, 26±6 years old); 14% was overweight/obese, but 68% never practiced physical activity. Almost 76% were aware of differences between consumption of fruit in and out of season, but a poor knowledge of foodstuffs seasonality emerged. These findings underline the need of improving awareness on proper diet and food seasonality because of the societal role of these students.

Keywords: university students, future teachers, Mediterranean Diet, eating habits, seasonal food

1. INTRODUCTION

The Mediterranean Diet (MedDiet) has been identified one of the best models for health prevention as part of a "Mediterranean-lifestyle" that include both physical and social activity (DEL BALZO *et al*., 2012). This diet has beneficial effects due to the synergistic and interactive nutrients combination (DEL CHIERICO *et al*., 2014), mitigates against overweight and obesity (EGUARAS *et al*., 2015), and provides dose-dependent protection against chronic diseases, such as cancers and cardiovascular diseases (GROSSO *et al*., 2014; DAVIS *et al*., 2015). However, since economic, social and demographic changes have taken place, Southern European countries, including Italy, have deviated from this pattern toward food choices typical of a "Western" diet, rich in animal-derived saturated fats, processed meat, eggs, sugars, and poor in legumes, whole cereals, fruit and vegetables (BLOOMFIELD *et al*., 2015; MORENO *et al*., 2002). Therefore, overweight and obesity have increased in young adults and in the areas commonly characterized by healthy diet patterns (MORENO *et al*., 2002; DE PIERO *et al*., 2015).

An additional advantage of MedDiet is the consumption of locally produced foodstuffs, as well as their availability during harvest season, the nutritional variety, the sustainability and environmental impact because of reduced "food miles" (EDWARDS-JONES *et al*., 2008; EDWARDS-JONES, 2010). The food seasonality is referred as the time-period when the harvest or flavour of a food is at its peak, usually corresponding when a food item is cheapest and freshest on the market. The best consequence of eating seasonally is that food has the best tasting carrying benefits to health, and while the costs fall down, the whole quality drives up.

Since food choices have been identified as key elements in diseases pathogenesis and prevention (DAVIS *et al*., 2015), as well as for public health promotion (SAMMARCO *et al*., 1997), the assessment of eating habits can support the design and improvement of measures for reducing the negative effects of unhealthy food patterns (DE PIERO *et al*., 2015).

The start of adult life is a critical period because of multiple physiological and psychological changes determining health-related habits in later adulthood. The college students can be considered as an index group reflecting general changes in lifestyles, because most at risk of poor nutrition and unsuitable dietary habits (RIPABELLI *et al*., 2001), due to a reduced food variety and scarce consumption of fruit or vegetables (RODRÍGUEZ *et al*., 2013; SHIVE and MORRIS, 2006). For students, the years spent at the university represent a critical period influencing the quality of lifestyle, being characterized by freedom and independence, and referred to the first time for assuming responsibilities for food choices and preparation (TELEMAN *et al*., 2015).

To date, studies on dietary patterns amongst Italian college students with respect of MedDiet recommendations are still scarce, and previous investigations were only conducted in some metropolitan areas (STEPTOE *et al*., 2002; BALDINI *et al*., 2009; LUPI *et al*., 2015; TELEMAN *et al*., 2015). Furthermore, no researches were performed to investigate knowledge on harvest season of locally produced food among college students who are studying to become teachers, and would be responsible for teaching healthy lifestyles in children attending the primary school (EGEDA *et al*., 2014).

Indeed, the aims of this study were to assess food choices and eating-related behaviours among college students attending the Faculty of Primary Education Sciences at University of Molise, Central Italy, and to evaluate their awareness and knowledge on harvest season of locally produced food.

2. METHODS

2.1. Study design

This survey was conducted enrolling college students at University of Molise that represents the only public higher education institution in Molise Region. The University counting 7,304 students during 2013-2014 academic year (http://statistica.miur.it/scripts/IU/vIU1.asp) is mostly characterized by students from Molise Region (more than 50%) and from the neighbouring Southern Regions of Puglia and Campania.

2.2. Participants and recruitment

All the students attending the degree course of Primary Education Sciences during 2013-2014 academic year were recruited by sending an invitation at their institutional email address. At time of the study, 304 students were attending this academic course, and were mostly females (n=281, 92.4%) (internal data referred to 2013-2014 academic year for the degree course of Primary Education Sciences provided by University administrative offices). The enrolment was voluntary and anonymous. An informed signed consent was obtained from each student who agreed to participate to the survey, receiving verbal information by a trained interviewer.

2.3. Data collection and procedures

Participants completed a newly developed questionnaire, which was validated in a pilot study on a small sample of randomly selected students (unpublished data). Particularly, the questionnaire included 48 items ordered in four sections: a) socio-demographic characteristics; b) lifestyles, food habits and dietary intake assessment; c) knowledge and understanding of harvest season with respect to Italian food production, focusing on seasonality and freshness of some fruit and seafood; d) sources of information on food quality. Self-reported weight and height were used to calculate Body Mass Index (BMI) for each student (DE WAURE *et al*., 2015). A dietary index of the nutrition status was also identified, based on the MedDietScore (MDS) as previously described (PANAGIOTAKOS *et al*., 2007; PANAGIOTAKOS *et al*., 2009), with some modifications in the list of food, which included: non-refined cereals; fruit; vegetables; legumes; potatoes; fish and seafood; red meat and derived products; white meat; milk and dairy products; and olive oil. Based on the reported intake, rates between 0 and 5 were used to score the food frequency for each student. For food suggested on a daily basis or more than three portions per week, the score 0 was assigned when no consumption was reported, while the scores from 1 to 5 were applied to proportional consumption rate. For food consumption deviating from MedDiet pattern, a reverse scale was applied, with the score of 5 for rare or no consumption, and the score 0 for daily consumption.

2.4. Statistical analysis

Questionnaire data were analyzed using the Statistical Package for Social Sciences software (IBM SPSS Statistics for Windows, Armonk, NY: IBM Corp) version 22.0. Qualitative variables were reported as absolute frequencies and percentages, whilst quantitative variables expressed as means ± standard deviation (SD), median and range. Differences among categorical variables were analyzed by Chi-square test. P-values were calculated based on a two-tailed test, and compared to 0.05 significance level.

3. RESULTS

3.1. Socio-demographic and anthropometric characteristics of enrolled students

Two-hundred and eighty out of 304 students agreed to participate to the study (response rate 92.1%). The sample had mean age of 25.9±6.2 years (median 23 years, range 19-50 years) and was mostly characterized by female students (n=267, 95.4%). The 77.8% (n=218) was single, and 11.8% (n=33) had children. At time of study, 32% (n=89) was already graduated, and the remaining has attended secondary high school. The 44.3% (n=124) of students lived away from parental home. Particularly, 57.1% were residents in Molise Region, and an important proportion was from the neighbouring Regions, such as Puglia (19.6%), Campania (16.8%), Abruzzo and Lazio (both 3.2%). The 57.8% (n=162) of students stated to have a paid employment, but none was food handler. Familial history of diseases was as follows: hypertension (36.4%), diabetes (23.6%), high cholesterol (21.1%), cardiovascular diseases (15.4%), obesity (3.2%), and thyroid disorder (2.5%).
According to BMI (mean 21.4±3.0, min 15.4, max 30.1), 71.1% (n=199) was classified within the normal range, followed by 15.0% (n=42) as underweight, and 13.9% (n=39) as overweight/obese. Physical activity was only practiced by 32.1% (n=90) with a frequency of three-four times per week, mainly going to the gym, running, walking, cycling and swimming. Significant differences between overweight/obese students and normal/underweight ones were found for education level (secondary school *vs* degree p=0.001). A greater proportion of underweight (11.1% *vs* 3.9%) and overweight/obese (12.9% *vs* 1.1%) students have attended secondary school compared to graduation. Conversely, no significant differences between normal weight and overweight/obese or underweight students were observed in relation to gender, living away from home, practicing sport, and familial diseases history.

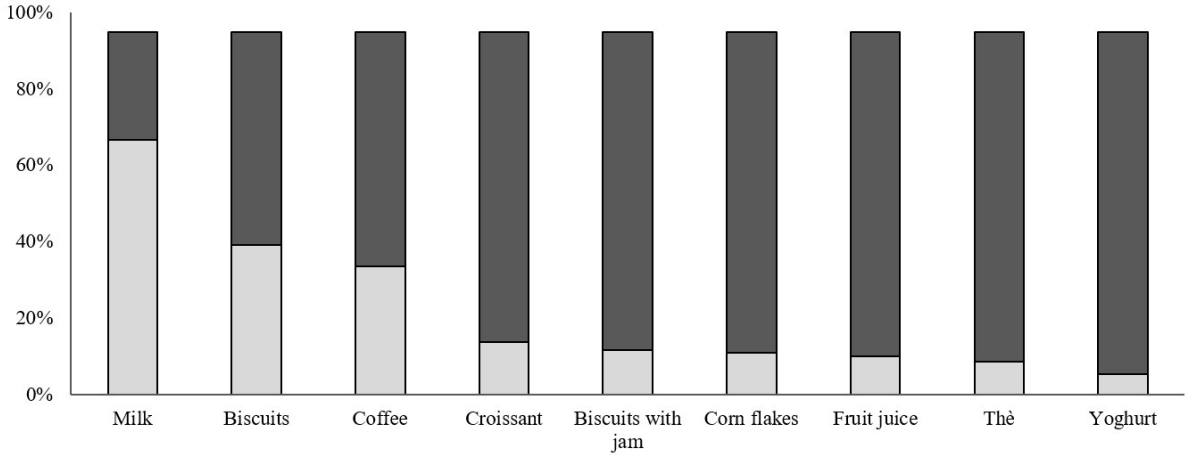
3.2. Dietary patterns and behaviours

Concerning questionnaire items on food-related behaviours and habits, the possible answers were reported as never; sometimes or 1-4 times/week; often or 5-8 times/week; very often or 8-10 times/week; always or daily; the results are shown in Table 1.
The analysis of eating habits and behaviours revealed that 61.8% students eat sometimes outside home (i.e. bars, take-away, restaurants, etc.), and 84.0% and 46.0% have both a daily breakfast and a snack before lunch or in the afternoon, respectively (Table 1). Among students having breakfast daily or 1-4 times/week, the consumption of milk, biscuits and coffee was highly reported (Fig. 1), while fruit, confectionery products/biscuits and fruit juice were the food items mostly consumed for snack before lunch or in the afternoon (Fig. 2).
About 50.0% (n=146) of students stated drinking at least 8 glasses (about 1.5-2L) of water per day, as recommended (EFSA Publication 2010). Only 9.3% (n=26) indicated to drink wine during meals, with an average of 125 ml (min 50 ml, max 750 ml). For food preparation and cooking, about 60.0% of students reported to use always extra virgin olive oil, followed by 17.9% who used it often and 16.8% very often (Table 1); however, 53.2% reported to use butter sometimes. Salty (i.e. pretzels, chips, popcorn, fast food burgers, sandwiches, mixed nuts, etc.) and whole foodstuffs (i.e. whole-wheat and grains) were mainly consumed sometimes (38.0% and 35.0%, respectively) and half of the students do not eat these food.

Table 1. Eating habits and behaviours.

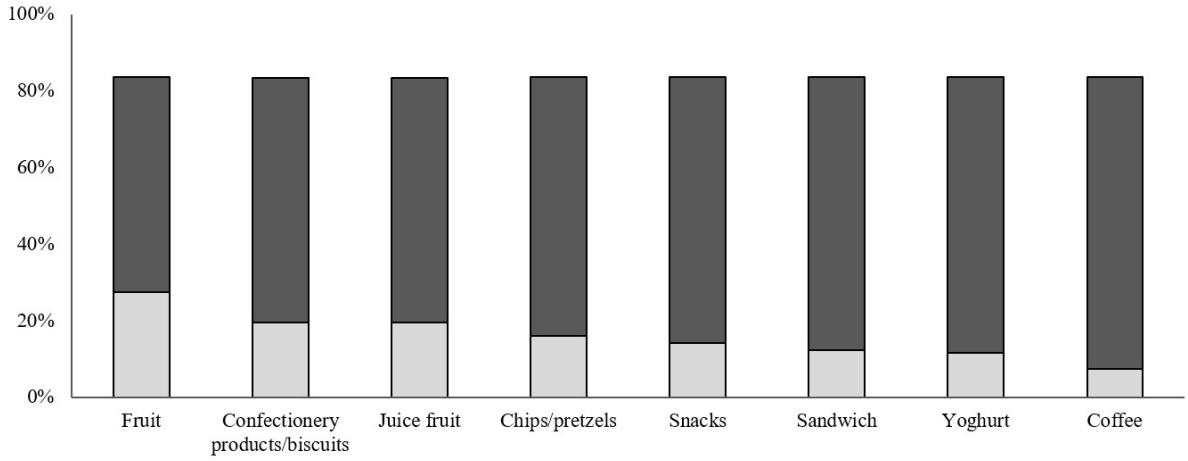
	Frequency per week				
	Never	**Sometimes (1-4 times/week)**	**Often (5-8 times/week)**	**Very often (8-10 times/week)**	**Always or Daily**
	n (%)	**n (%)**	**n (%)**	**n (%)**	**n (%)**
Eating outside home	2 (0.7)	173 (61.8)	60 (21.4)	35 (12.5)	10 (3.6)
Breakfast	14 (5.0)	32 (11.4)			234 (83.6)
Snack	46 (16.4)	105 (37.5)			129 (46.0)
Use of extra virgin olive oil	2 (0.7)	14 (5.0)	50 (17.9)	47 (16.8)	167 (59.6)
Use of butter	115 (41.1)	149 (53.2)	10 (3.6)	4 (1.4)	2 (0.7)
Salty food consumption	141 (50.4)	106 (37.9)			33 (11.8)
Whole food consumption	138 (49.3)	99 (35.4)	33 (11.8)	9 (3.2)	1 (0.4)
Fried food consumption	52 (18.6)	186 (66.0)	33 (11.8)	7 (2.5)	2 (0.7)
Watching television during meals	29 (10.4)	55 (19.6)			196 (70.0)

Fig. 1. Food items consumed at breakfast by the students.



The grey bar corresponds to the percentage of students consuming daily or sometimes specific food at breakfast; the black bar indicates the proportion of students who did not consume these foods at breakfast.

Fig. 2. Food items consumed as snacks by the students.



The grey bar corresponds to the percentage of students consuming daily or sometimes specific food at snack time; the black bar indicates the proportion of students who did not consume these food at snack time.

Approximately 70% of students reported to eat fried food (i.e. chips, fish, chicken) sometimes and to watch television during meals (Table 1). No significant differences between normal weight and overweight/obese or underweight students were found for water consumption, extra virgin olive oil and butter use, consumption of whole and fried food, and watching television, while differences were significant for salty food consumption ($p=0.04$). Particularly, 36.8% of normal weight students did not consume salty food compared to 3.2% and 7.9% in the underweight and overweight/obese categories, respectively.
Furthermore, students declared to achieve most of the information on food quality from food labels (n=173, 62.0%), television programs on health topics (47.1%) and food preparation/cooking (35.4%), farmers and/or shopkeeper (19.6%).

3.3. Food intake assessment

The consumption frequency of selected food in terms of servings per week is reported in Table 2.
Briefly, the consumption of both read meat and salami reported by students was very high; 60.0% and 36.0% reported 2-3 servings/week for red meat and salami, respectively; furthermore, 13.6% and 29.6% stated 4-5 servings/week for the same food. The frequency of white meat was commonly reported as 2-3 servings/week (64.3%) and less than 1 serving/week (19.6%). Moreover, dairy products and cheese were largely consumed by 48.9% and 43.6% as 2-3 servings/week, respectively, and 25.7% and 21.4% as 4-5 servings/week, while milk was never consumed by 16.0% of the students. Pasta and bread consumption was generally reported on daily frequency (43.2% and 62.9%, respectively), compared with rice mainly consumed as less than 1 serving/week. Students reported a highly different consumption of vegetables, and a similar proportion (30.0%) was reported for 2-3 and 4-5 servings/week, whilst only 24.6% consumed daily these food items. A poor intake of legumes was also observed, as most (48.2%) students reported 1-2 servings/week, as well as for fish/shellfish, which were mostly reported as 1-2 servings/week (49.3%) and less than 1 serving/week (39.3%).
Fruit consumption was properly reported as 16-21 servings/week only by 42.5% of students, while about 4.0% do never consume them and 9.0% less than 1 serving/week (Table 2).

Table 2. Frequency of weekly consumption in terms of servings per week of certain food reported by students.

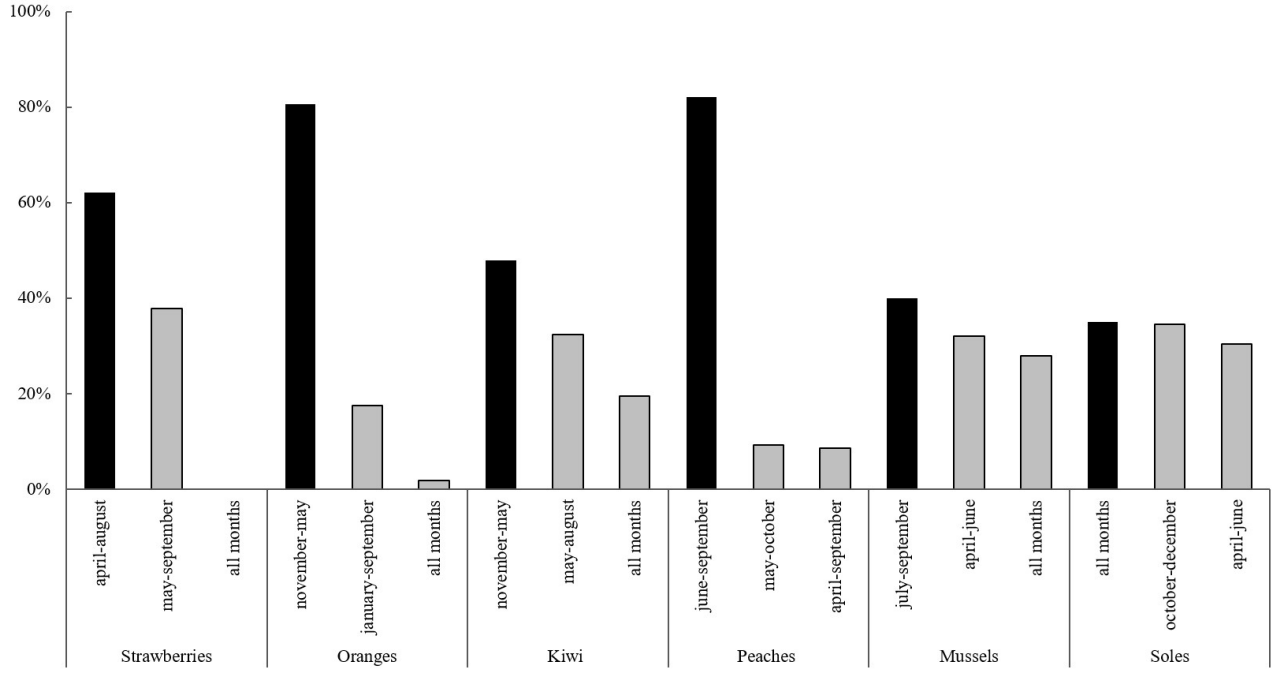
Food	Frequency of consumption reported by students				
	Never	**≤1 serving/week**	**2-3 servings/week**	**4-5 servings/week**	**6-7 servings/week**
		n (%)	**n (%)**	**n (%)**	**n (%)**
White meat	10 (3.6)	55 (19.6)	180 (64.3)	35 (12.5)	
Red meat	11 (3.9)	62 (22.1)	169 (60.4)	38 (13.6)	
Salami	15 (5.4)	74 (26.4)	101 (36.1)	83 (29.6)	7 (2.5)
Eggs	12 (4.3)	126 (45.0)	119 (42.5)	19 (6.8)	4 (1.4)
Milk	46 (16.4)	27 (9.6)	33 (11.8)	37 (13.2)	137 (48.9)
Dairy products	11 (3.9)	37 (13.2)	137 (48.9)	72 (25.7)	23 (8.2)
Cheese	26 (9.3)	55 (19.6)	122 (43.6)	60 (21.4)	17 (6.1)
Bread	2 (0.7)	23 (8.2)	20 (7.1)	59 (21.1)	176 (62.9)
Rice	12 (4.3)	141 (50.4)	86 (30.7)	33 (11.8)	8 (2.9)
Pasta	8 (2.9)	20 (7.1)	40 (14.3)	91 (32.5)	121 (43.2)
Pizza	2 (0.7)	104 (37.1)	138 (49.3)	29 (10.4)	7 (2.5)
Vegetables	11 (3.9)	32 (11.4)	83 (29.6)	85 (30.4)	69 (24.6)
Confectionery products	11 (3.9)	54 (19.3)	95 (33.9)	65 (23.2)	55 (19.6)
Chocolate	22 (7.9)	86 (30.7)	74 (26.4)	57 (20.4)	41 (14.6)
Coffee	54 (19.3)	20 (7.1)	25 (8.9)	21 (7.5)	160 (57.1)
Fruit juice	38 (13.6)	86 (30.7)	75 (26.8)	53 (18.9)	28 (10.0)
Sparkling processed beverages	83 (29.6)	89 (31.8)	64 (22.9)	27 (9.6)	17 (6.1)
	Never	**<1**	**1-2**	**3-4**	**5-6**
Fish/shellfish	16 (5.7)	110 (39.3)	138 (49.3)	16 (5.7)	
Legumes	14 (5.0)	78 (27.9)	135 (48.2)	45 (16.1)	8 (2.9)
	Never	**1-4**	**5-8**	**9-12**	**13-18**
Potatoes	3 (1.1)	89 (31.8)	150 (53.6)	34 (12.1)	4 (1.4)
	Never	**1-4**	**5-8**	**9-15**	**16-21**
Fruit	11 (3.9)	26 (9.3)	51 (18.2)	73 (26.1)	119 (42.5)

The calculated MDS was on average 29.4±3.9 (median 30, ranging between 18 and 38) compared to 50 maximum score. Four MDS categories were identified: score 18-22 related to the lowest adherence (5% of study population), score 23-27 to low adherence (25.7%), score 28-32 to intermediate adherence (47.1%), score 33-38 to moderate/acceptable compliance (22.1%). The majority (49.2%) of normal weight students had intermediate-moderate score compared to underweight (10.7%) and overweight/obese (9.3%) students, but differences were not significant.

3.4. Awareness and knowledge on seasonality of local food

Almost 44.0% (n=124) of students reported that both consumption and transportation of food out of season could produce high impact on the ecosystem, followed by an intermediate and low impact for 48.6% and 7.1%, respectively. Most of students (n=214, 76.4%) stated that there are remarkable differences between consumption of fruit in season and out of the harvest period, while 16.4% (n=46) did not recognize any differences. The main reasons reported by students for choosing fruit in season were limited treatment with pesticides (49.1%), great freshness (45.8%), low cost (42.5%), enhanced nutritional intake (39.2%), high quality in terms of nutritional properties (33.2%), guarantee of safety of origin and cultivation/production practice (32.7%), reduced impact on environment (29.4%), and scarce additive/preservative content (23.3%).
Knowledge on seasonality of selected fruit was inadequate. Only 62.0% (n=174) and 48.0% (n=134) could correctly identify the seasonal period of strawberries (April-August - as its best) and kiwi fruit (November-May), and differences between students who correctly and inaccurately identified these periods were significant ($p<0.001$). Conversely, awareness of the harvest period of oranges (November-May) and peaches (June-September) resulted more satisfactory, being correctly identified by 80.7% and 82.1% (Fig. 3), but differences were not significant.

Fig. 3. Proportion of students aware of harvest period of selected food.



The black bar indicates the correct answer on the natural harvest season of local food.

More than half students (n=154, 55.0%) reported to have knowledge of seasonal freshness of fish and seafood, which is closely related to their reproductive period, whereas 34.3% (n=96) did not know it, and 10.7% (n=30) said that there is no a specific period. The main reasons reported by students to prefer the consumption of fish and seafood at the natural reproductive period were great freshness (65.0%), enhanced nutritional intake (41.8%), healthy nutrition (26.4%), low cost (11.8%), and adequate size of products (10.0%). However, only 40.0% (n=112) and 35.0% (n=98) could recognize the natural period of mussels and sole, respectively (Fig. 3), and differences between students who properly and incorrectly identified these periods were significant ($p<0.05$).

4. DISCUSSION

This study aimed to evaluate eating habits and adherence to MedDiet model, as well as to investigate knowledge on the seasonal period of locally produced food among college students who were studying to become teachers. The survey revealed that the majority of study population had BMI within the normal range, and only 14% was classified as overweight/obese, in agreement to a recent study conducted amongst Italian university students (TELEMAN *et al*., 2015). Our findings differed from other studies conducted in Europe and Italy, in which different percentages of overweight/obese have been reported (PELTZER and PENGPID, 2015; ORGANIZATION FOR ECONOMIC COOPERATION AND DEVELOPMENT, 2014; OSSERVATORIO NAZIONALE SULLA SALUTE NELLE REGIONI ITALIANE, 2014; EPICENTRO, 2013); however, data are difficult to compare because of the different target population, and because the percentages were pooled and stratified by specific age groups.

In our study, 68% of students reported to never practice physical activity, which is consistent with other results (RODRÍGUEZ *et al*., 2013; OSSERVATORIO NAZIONALE SULLA SALUTE NELLE REGIONI ITALIANE, 2014), indicating high prevalence of inactivity in females who represented more than 95% of whole sample. Prevalence of physical inactivity was estimated of 21% in individuals aged ≥15 years from 76 countries (DUMITH *et al*., 2011) worldwide, whereas in Italy percentages were of 27.2%, 27.8% and 34.8% among 18-19, 20-24 and 25-34 years old individuals, respectively (OSSERVATORIO NAZIONALE SULLA SALUTE NELLE REGIONI ITALIANE, 2014), and a similar trend was reported in individuals aged 18-34 years (EPICENTRO, 2013). These findings are of serious concerns since the World Health Organization recommends that adults should practice at least 150 minutes of moderate-intensity physical activity or 75 minutes of vigorous-intensity through the week. It is also well known that physical activity is associated to better anthropometric measures (ZACCAGNI *et al*., 2014), whilst the inactivity is related to several health risk factors, such as smoking, unhealthy diet, and chronic diseases (MORENO-GÓMEZ *et al*., 2012). Hence, because of great benefits regardless of changes in anthropometric outcomes, especially in preventing weight gain (CONN *et al*., 2014), college education should focus on health promotion encouraging students in regular physical activity (STANFORD *et al*., 2014).

Our study also revealed that 16% of students never or not every day have breakfast, which could affect performance during the rest of the day, causing fatigue and poor attention among students, as previously reported (ACKUAKU-DOGBE and ABAIDOO, 2014). Breakfast consumption is associated with a better nutritional profile and a lower risk of overweight/obesity (COOPER *et al*., 2011). Indeed, irregular breakfast habits are associated with poor nutrition, and skipping this meal could be an indicator of unhealthy eating habits in a population, being also linked with low vegetable intake (LAZZERI *et al*., 2013).

Most college students further did not meet dietary and physical activity guidelines, suggesting the need of preventive interventions and increased understanding on overweight-

related risk, in agreement with previous reports (HUANG *et al*., 2003; SÁNCHEZ SOCARRÁS and AGUILAR MARTÍNEZ, 2014). Hence, specific strategies involving a combination of physical activity, nutritional, and educational interventions instead of single component-based programs (SHIRLEY *et al*., 2015) should be improved to yield better obesity-related outcomes.

In our target population, the main nutritional deviations were related to low intake of vegetables, fruit and legumes, which represent the main components of a balanced diet together with cereal grains and derived products (GARCÍA-MESEGUER *et al*., 2014). A prevalent consumption of red meat and products compared to white meat was found, in agreement with other European countries (DE PIERO *et al*., 2015). The low consumption of fruit and vegetables was reported in other studies conducted in Western countries and USA (KING *et al*., 2007; KELLER *et al*., 2008; DODD *et al*., 2010), showing that university students did not follow the recommended consumption because of their taste and price, which represent the most critical barriers (SHIVE and NEYMAN, 2003). Conversely, studies conducted in South East Asia reported a consumption of fruit and vegetables of five servings per day among female university students (SAKAMAKI *et al*., 2005; YAHIA *et al*., 2008; PERERA and MADHUJITH, 2012).

Significant dietary changes can occur since starting university, and students living away from home are more likely to develop unfavourable eating habits by decreasing weekly consumption of fruit, vegetables, seafood, olive oil, and increasing sugar, alcohol and fast food intake (FIORE *et al*., 2015). In our survey, the majority of students were unmarried, and 44% were living outside from family home, suggesting that the unsatisfactory food intake could be probably related to poor education on proper food choices, limited budget and/or to lack of skills to prepare a basic healthy meal.

The MDS revealed a poor adherence to MedDiet, in agreement with other studies among university population (CERVERA BURRIEL *et al*., 2014; GARCÍA-MESEGUER *et al*., 2014). The unsatisfactory adherence to MedDiet was mostly associated with low consumption of food groups suggested on a daily basis or more than three portions per week, and with high intake of food suggested on rare consumption.

Our survey also investigated awareness on seasonal period of local food. Students showed a lack of knowledge on the harvest period of many locally produced foodstuffs, indicating misperception and significant understanding gaps, as previously reported among university students and consumers (WILKINS *et al*., 2002; BROOKS *et al*., 2011). Most students associated the quality of seasonal food to inappropriate attributes, by referring their preference due to limited chemical treatment or content, guarantee of origin production and for environmental reasons. Fruit have proper nutritional and sensory quality at natural harvest season, providing a good source of health-promoting compounds (VOĆA *et al*., 2014). For example, in addition to beneficial compounds, the attributes of sweetness, acidity, colour and flavour are the most important characteristics of strawberries, affecting either quality, marketability, or choice of consumers. Indeed, the ripening process and maturity strongly affect their nutritional composition and contribute to typical taste, indicating that strawberries should be consumed at stage of full maturity (VOĆA *et al*., 2014). Similar findings can be associated with seafood since - in the context of human health - the season could influence their compositions of both amino acids and fatty acids (ÇAĞLAK and KARSL, 2017). Moreover, sensory properties and nutritional value are two sets of characteristics that, together with freshness, are accountable for fish quality, and are affected by many factors including seasonal changes (PETROVIĆ *et al*., 2015).

The debate on MedDiet with emphasis on plant-based foodstuffs consumption and recommendations for seasonal local food consumption are key topics of global framework of sustainable eating (FORLEO *et al*., 2015). Nevertheless, sustainability of seasonal food in term of environmental performance may be controversial and depends on the definition of

seasonal characteristics and environmental impact. Some studies have assessed that seasonality is unlikely to deliver large environmental benefits, except for water footprint (FOSTER *et al*., 2014). Reduction in greenhouse gas emissions from eating seasonal vegetables is also limited, representing a minor proportion of the total emissions from food consumption (RÖÖS and KARLSSON, 2013). Indeed, the multifaceted concept of seasonality should be considered under a nutritional-health perspective and a global approach, including environmental, socio-cultural and economic pillars of sustainability (AKHATOU and FERNÁNDEZ-RECAMALES, 2014).
The study has some limitations: sample size might not be representative of the whole university population of Central Italy; being the sample represented mostly by females, the results could not be directly generalizable to male subjects; anthropometric measurements, as well as dietary intake were self-reported and assessed by short questions with possible under or over estimation; there were no opportunities for follow-up and verification. Despite this, our survey was performed in a short data collection time and involved a priority young-adult population that could be targeted for strengthening public health strategies for eating behaviours improvement. Our findings have important implications for research and practice, and provide hitherto invaluable information on a sample of Italian college students recruited in a non-metropolitan area never investigated before, and could be useful in systematic reviews and meta-analysis studies.

5. CONCLUSIONS

The survey highlights the need to extend policies among college students, providing a proper nutritional education because of their future societal role as educators, as well as for their own health.
Education should focus on specific strategies aimed at improving awareness on quality and regularity of breakfast, physical activity, and on the importance of fruit, vegetables and legumes intake, as well as on the consumption of seasonal food.

ACKNOWLEDGEMENTS

The Authors acknowledge Dr. Jim McLauchlin, Public Health England, Colindale (London, UK) for the helpful comments.

REFERENCES

Ackuaku-Dogbe E.M. and Abaidoo B. 2014. Breakfast eating habits among medical students. Ghana Med. J. 48(2):66-70.

Akhatou I. and Fernández-Recamales A. 2014. Nutritional and nutraceutical quality of strawberries in relation to harvest time and crop conditions. J. Agric. Food Chem. 62(25):5749-5760.

Baldini M., Pasqui F., Bordoni A. and Maranesi M. 2009. Is the Mediterranean lifestyle still a reality? Evaluation of food consumption and energy expenditure in Italian and Spanish university students. Public Health Nutr. 12(2):148-155.

Bloomfield H., Kane R., Koeller E., Greer N., MacDonald R. and Wilt T.J. 2015. Benefits and harms of the Mediterranean diet compared to other diets. VA ESP Project #09-009.

Brooks M., Foster C., Holmes M. and Wiltshire J. 2011. Does consuming seasonal foods benefit the environment? Insights from recent research. Nutrition Bulletin. 36(4):449-445.

Çağlak E. and Karsli B. 2017. Seasonal variation of fatty acid and amino acid compositions in the muscle tissue of zander (Sander Lucioperca Linnaeus, 1758) and the evaluation of important indexes related to human health. Ital. J. Food Sci. 29(2):266-275.

Cervera Burriel F., Serrano Urrea R., Daouas T., Delicado Soria A. and García Meseguer M.J. 2014. Food habits and nutritional assessment in a Tunisian university population. Nutr. Hosp. 30(6):1350-1358.

Conn V.S., Hafdahl A., Phillips L.J., Ruppar T.M. and Chase J.A. 2014. Impact of physical activity interventions on anthropometric outcomes: systematic review and meta-analysis. J. Prim. Prev. 35(4):203-215.

Cooper S.B., Bandelow S. and Nevill M.E. 2011. Breakfast consumption and cognitive function in adolescent schoolchildren. Physiol. Behav. 103(5):431-439.

Davis C., Bryan J., Hodgson J. and Murphy K. 2015. Definition of the Mediterranean Diet; a Literature Review. Nutrients. 7(11):9139-9153.

De Piero A., Bassett N., Rossi A. and Sammán N. 2015. Trends in food consumption of university students. Nutr. Hosp. 31(4):1824-1831.

de Waure C., Poscia A., Virdis A., Di Pietro M.L. and Ricciardi W. 2015. Study population, questionnaire, data management and sample description. Ann. Ist. Super. Sanità. 51(2):96-98.

del Balzo V., Diolordi L., Pinto A., Giusti A.M., Vitiello Cannella C., Dernini S., Donini L.M. and Berry E.M. 2012. Mediterranean diet pyramids: towards the Italian model. Ann. Ig. 24(5):443-447.

Del Chierico F., Vernocchi P., Dallapiccola B. and Putignani L. 2014. Mediterranean diet and health: food effects on gut microbiota and disease control. Int. J. Mol. Sci. 15(7):11678-11699.

Dodd L.J., Al-Nakeeb Y., Nevillm A. and Forshaw M.J. 2010. Lifestyle risk factors of students: a cluster analytical approach. Prev. Med. 51(1):73-77.

Dumith S.C., Hallal P.C., Reis R.S. and Kohl H.W. 2011. Worldwide prevalence of physical inactivity and its association with human development index in 76 countries. Prev. Med. 53(1-2):24-28.

Edwards-Jones G. 2010. Does eating local food reduce the environmental impact of food production and enhance consumer health?. Proceedings of Nutrition Society. 69:582-591.

Edwards-Jones G., Milà i Canalsb L., Hounsomec N., Truningerd M., Koerbera G., Hounsomee B., Crossa P., Yorka E.H., Hospidob A. and Plassmann K. 2008. Testing the assertion that 'local food is best': the challenges of an evidence-based approach. Trends of Food Sciences Technology. 19(5):265-274.

EFSA Publication. 2010. EFSA Panel on Dietetic Products, Nutrition, and Allergies (NDA); Scientific Opinion on establishing Food-Based Dietary Guidelines. Parma, Italy: European Food Safety Authority. The EFSA Journal. doi:10.2903/j.efsa.2010.146

Egeda Manzanera J.M. and Rodrigo Vega M. 2014. Adherence to the Mediterranean diet of future teachers. Nutr. Hosp. 30(2):343-350.

Eguaras S., Toledo E., Hernández-Hernández A., Cervantes S. and Martínez-González M.A. 2015. Better adherence to the Mediterranean diet could mitigate the adverse consequences of obesity on cardiovascular disease: the SUN prospective cohort. Nutrients. 7(11):9154-9162.

Epicentro. 2013. Rapporto nazionale Passi 2013: attività fisica.
Available from:http://www.epicentro.iss.it/passi/rapporto2013/AttivitaFisica.asp.

Fiore M., Ledda C., Rapisarda V., Sentina E., Mauceri C., D'Agati P., Oliveri Conti G., Serra-Majem L. and Ferrante M. 2015. Medical school fails to improve Mediterranean diet adherence among medical students. Eur. J. Public Health. 25(6):1019-1023.

Forleo M.B., De Boni A., Di Cesare C., Roma R. and Salvatori G. 2015. Food styles in a multidimensional perspective of sustainability. Some methodological insight from SAFEBIO Project. In the proceedings of the 52nd SIDEA Annual Conference "The value of food: internationalization, competition and local development in agro-food systems".

Foster C., Guében C., Holmes M., Wiltshireb J. and Wynnb S. 2014. The environmental effects of seasonal food purchase: a raspberry case study. J. Clean. Prod. 73:269-274.

García-Meseguer M.J., Burriel F.C., García C.V. and Serrano-Urrea R. 2014. Adherence to Mediterranean diet in a Spanish university population. Appetite. 78:156-64.

Grosso G., Pajak A., Mistretta A., Marventano S., Raciti T., Buscemi S., Drago F., Scalfi L. and Galvano F. 2014. Protective role of the Mediterranean diet on several cardiovascular risk factors: evidence from Sicily, southern Italy. Nutr. Metab. Cardiovasc. Dis. 24(4):370-377.

Huang T.T., Harris K.J., Lee R.E., Nazir N., Born W. and Kaur H. 2003. Assessing overweight, obesity, diet, and physical activity in college students. J. Am. Coll. Health. 52(2):83-86.

Keller S., Maddock J.E., Hannöver W., Thyrian J.R. and Basler H.D. 2008. Multiple health risk behaviors in German first year university students. Prev. Med. 46(3):189-195.

King K.A., Mohl K. and Bernard A.L. 2007. Does involvement in healthy eating among university students differ based on exercise status and reasons for exercise?. Calif. J. Health. Promot. 5(3):106-119.

Lazzeri G., Pammolli A., Azzolini E., Simi R., Meoni V., de Wet D.R. and Giacchi M.V. 2013. Association between fruits and vegetables intake and frequency of breakfast and snacks consumption: a cross-sectional study. Nutr. J. 12:123.

Lupi S., Bagordo F., Stefanati A., Grassi T., Piccinni L., Bergamini M. and De Donno A. 2015. Assessment of lifestyle and eating habits among undergraduate students in northern Italy. Ann. Ist. Super. Sanità 51(2):154-161.

Moreno L.A., Sarría A. and Popkin B.M. 2002. The nutrition transition in Spain: a European Mediterranean country. Eur. J. Clin. Nutr. 56(10):992-1003.

Moreno-Gómez C., Romaguera-Bosch D., Tauler-Riera P., Bennasar-Veny M., Pericas-Beltran J., Martinez-Andreu S. and Aguilo-Pons A. 2012. Clustering of lifestyle factors in Spanish university students: the relationship between smoking, alcohol consumption, physical activity and diet quality. Public Health Nutr. 15(11):2131-2139.

Organization for Economic Cooperation and Development. 2014. Health at a glance: Europe 2014. OECD Publishing.

Osservatorio Nazionale sulla Salute nelle Regioni Italiane. 2014. Rapporto Osservasalute 2014.

Panagiotakos D.B., Kalogeropoulos N., Pitsavos C., Roussinoua G., Pallioub K., Chrysohooub C. and Stefanadis C. 2009. Validation of the MedDietScore via the determination of plasma fatty acids. Int. J. Food. Sci. Nutr. 60(5):168-180.

Panagiotakos D.B., Pitsavos C., Arvaniti F. and Stefanadis C. 2007. Adherence to the Mediterranean food pattern predicts the prevalence of hypertension, hypercholesterolemia, diabetes and obesity, among healthy adults; the accuracy of the MedDietScore. Prev. Med. 44(4):335-340.

Peltzer K. and Pengpid S. 2015. Correlates of healthy fruit and vegetable diet in students in low, middle and high-income countries. Int. J. Public Health. 60(1):79-90.

Perera T. and Madhujith T. 2012. The pattern of consumption of fruits and vegetables by undergraduate students: a case study. Trop. Agric. Res. 23(3):261-271.

Petrović M., Krešić G., Zrnčić S., Oraić D., Džafić N. and Pleadin J. 2015. Influence of season and farming location on the quality parameters of sea bass (Dicentrarchus labrax) and sea bream (*Sparus aurata*). Ital. J. Food Sci. 27(2):151-159.

Ripabelli G., Sammarco M.L., Carano Q., Manfredi Selvaggi T., Quaranta A. and Grasso G.M. 2001. Eating behavior of children and parents and patterns of hygienic safety: study of elementary schools in Campobasso. Ann. Ig. 13(5):427-439.

Rodríguez F., Palma X., Romo A., Escobar D., Aragú B., Espinoza L., McMillan N. and Gálvez J. 2013. Eating habits, physical activity and socioeconomic level in university students of Chile. Nutr. Hosp. 28(2):447-455.

Röös E. and Karlsson H. 2013. Effect of eating seasonal on the carbon footprint of Swedish vegetable consumption. J. Clean. Prod. 59:63-72.

Sakamaki R., Toyama K., Amamoto R., Liu C.J. and Shinfuku N. 2005. Nutritional knowledge, food habits and health attitude of Chinese university students: a cross sectional study. Nutr. J. 4(4).

Sammarco M.L., Ripabelli G. and Grasso G.M. 1997. Consumer attitude and awareness towards food-related hygienic hazards. Ital. J. Food Saf. 17(4):215-221.

Sánchez Socarrás V. and Aguilar Martínez A. 2014. Food habits and health-related behaviors in a university population. Nutr. Hosp. 31(1):449-457.

Shirley K., Rutfield R., Hall N., Fedor N., McCaughey V.K. and Zajac K. 2015. Combinations of obesity prevention strategies in US elementary schools: a critical review. J. Prim. Prev. 36(1):1-20.

Shive S.E. and Morris M.N. 2006. Evaluation of the energize your life! Social marketing campaign pilot study to increase fruit intake among community college students. J. Am. Coll. Health. 55(1):33-39.

Shive S.E. and Neyman P. 2003. Prevalence and predictors of fruit intake among community college women in the extended opportunity program and services. Calif. J. Health Prom. 1(1):91-102.

Stanford F.C., Durkin M.W., Stallworth J.R., Powell C.K., Poston M.B. and Blair S.N. 2014. Factors that influence physicians' and medical students' confidence in counselling patients about physical activity. J. Prim. Prev. 35(3):193-201.

Steptoe A., Wardle J., Cui W., Bellisle F., Zotti A.M., Baranyai R. and Sanderman R. 2002. Trends in smoking, diet, physical exercise, and attitudes toward health in European university students from 13 countries, 1990-2000. Prev. Med. 35(2):97-104.

Teleman A.A., de Waure C., Soffiani V., Poscia A. and Di Pietro M.L. 2015. Nutritional habits in Italian university students. Ann. Ist. Super. Sanità. 51(2):99-105.

Voća S., Zlabur J.S., Dobričević N., Jakobek L., Seruga M., Galić A. and Pliestić S. 2014. Variation in the bioactive compound content at three ripening stages of strawberry fruit. Molecules. 19(7):10370-10385.

Wilkins J.L., Bowdish E. and Sobal J. 2002. Consumer perceptions of seasonal and local foods: A study in a U.S. community. Ecol. Food Nutr. 41(5):415-439.

Yahia N., Achkar A., Abdallah A. and Rizk S. 2008. Eating habits and obesity among Lebanese university students. Nutr. J. 7:32.

Zaccagni L., Barbieri D. and Gualdi-Russo E. 2014. Body composition and physical activity in Italian university students. J. Transl. Med. 12:120.

Paper Received July 21, 2017 Accepted August 21, 2017

PAPER

EFFECTS OF BASIL (*OCIMUM BASILICUM* L.) SEED MUCILAGE SUBSTITUTED FOR FAT SOURCE IN SPONGE CAKE: PHYSICOCHEMICAL, STRUCTURAL, AND RETROGRADATION PROPERTIES

K.Y. SONG, H. O, K.Y. JOUNG, S.Y. SHIN and Y.S. KIM*
Department of Food and Nutrition, Korea University, Seoul 02841, Republic of Korea
*Corresponding author. Tel.: +82 232905638; fax: +82 29217207
E-mail address: kteresa@korea.ac.kr

ABSTRACT

Seeds of the herb plant basil (*Ocimum basilicum* L.) are a source of dietary fiber that are used in desserts and beverages in some parts of Asia. The mucilage extracted from basil seeds contains polysaccharide, mainly composed of glucomannan and xylan. It has high non-gelling and shear-thinning properties and can be used abundantly in foods. The purpose of this study was to investigate the possibility and effects of substituting basil seed mucilage (BSM) extracted by hot water for fat. In sponge cake, BSM was added as a substitute for fat (butter). 1 g of BSM replaced 15 g of butter, resulting in a 75% reduction in total fat content after addition of 1% BSM. Therefore, BSM is considered preferable to use as substitute for fat in foods, which can improve the quality and health properties of manufactured foods.

Keywords: basil seed mucilage (BSM), sponge cake, fat, substitute

1. INTRODUCTION

The fat content of cake is generally between 15% and 25% of dough weight (RODRÍGUEZ-GARCÍA *et al*., 2012). Fat is an important ingredient in cakes, as it assists in the entrapment of air during the creaming process, resulting in the creation of a more tender product. Fat is dispersed in the dough or batter during mixing, which softens the structure and obstructs starch and protein from forming a continuous network (RODRÍGUEZ-GARCÍA *et al*., 2012). Fat generates a desirable flavor and softer texture in the cake (LAKSHMINARAYAN *et al*., 2006). In addition, fat can delay starch gelatinization by retarding water transfer into starch granules, thereby improving tenderness, moisture content, and flavor, while extending the shelf-life of the product (BENT *et al*., 2013; PIZARRO *et al*., 2013).

Owing to consumer demand, the baking industry is currently focused on developing low-calorie or reduced-fat products that have at least one-third fewer calories compared to traditional products; however, the reduced-fat product can be also be considered nutritious food (Sanchez *et al*., 1995). Fat is associated with several health hazards, including cardiovascular disease, atherosclerosis, diabetes, and obesity (HASSEL, 1993). However, several problems, including lower volume, denser crumbs, firmer texture, and reduced flavor are encountered when making reduced-fat products and cakes as compared to normal cakes (HASSEL, 1993). Accordingly, it is necessary to replace the role of fat in baked products (CAUVAIN and Young, 2006; FELISBERTO *et al*., 2015). Reduction of fat content can be made possible throughout the use of fat substitutes, which produce lower fat content in baked products (BRENNAN and TUDORICA, 2008). Substitutes can be classified as carbohydrate-, lipid-, or protein-based materials (SANCHEZ *et al*., 1995). Carbohydrate-based substitutes combined with water form a gel-type structure, resulting in desirable texture (Hassel, 1993). In particular, dietary fibers, that are carbohydrate-based materials can act as a thickener and stabilizer to provide an effective fat substitute in foods (BRENNAN and TUDORICA, 2008).

Hydrocolloids are used as thickening, texturizing, stabilizing, and gelling agents. Hydrocolloids are hydrophilic and generally not considered to be an effective surfactant compared to proteins that have strong hydrophilic and hydrophobic regions (Huang *et al*., 2001). However, as protein residues were found in the hydrophilic phase of hydrocolloids, their role as emulsifiers has emerged (AKHTAR *et al*., 2002). Hydrocolloids like arabic gum, guar gum, locust bean gum, pectin, and galactomannans are able to act as emulsifying and foaming agents (NAJI-TABASI and RAZAVI, 2016). The hydrophilic and hydrophobic properties of hydrocolloids promote surfactant and emulsifier effects as active agents (GARTI and LESER, 2001).

Basil (*Ocimum basilicum* L.) is an herb that is produced primarily in warm regions of India, Iran, and Africa (Fekri *et al*., 2008). Basil seeds have traditionally been used as natural remedies for the treatment of dyspepsia, ulcers, diarrhea, sore throat, and kidney disease. In some Asian regions, basil is used in desserts and beverages as a source of dietary fiber (SIMON *et al*., 1999). The polysaccharides from basil seeds consist mainly of glucomannans (43%) and 1,4-linked xylan (24.3%) (ANJANEYALU and GOWDA, 1979). Mucilage extracted from basil seeds has high non-gelling and high shear-thinning properties and can be used not only as a fat substitute but also as a surfactant and emulsifier in foods (HOSSEINI-PARVAR *et al*., 2010).

Sponge cake is made up according with the AACC method (2000), by denaturalization of the egg protein to foam dough as the base of the cake. By using whole eggs product resulting is characterized by the pores, creamy bubbles, and a moist shiny texture. Recently, to improve the quality of sponge cake, additions such as grains, vegetables, and

fruits have been used rather than flour; however, few studies have evaluated the production of reduced-fat sponge cakes.
In this study, we aimed to determine the effects of basil seed mucilage (BSM), a type of dietary fiber, as a fat substitute in cake.

2. MATERIALS AND METHODS

2.1. Materials

Basil seed (Herbalkart, India) and cake flour (CJ Cheiljedang Co., Ltd, Incheon, Korea) were used for this study. Butter (Seoul Milk, Co., Ltd, Ansan, Korea), fresh eggs (Pulmuone Co., Ltd, Seoul, Korea), sugar (CJ Cheiljedang Co., Ltd, Incheon, Korea), and salt (CJ Cheiljedang Co., Ltd, Incheon, Korea) were purchased from local markets.

2.2. BSM extraction

Mucilage was extracted from basil seeds according to the method published by RAZAVI *et al*. (2009). Basil seeds were washed and soaked in water for 20 min at 50°C under the optimized condition (water: seeds = 50:1 (w/w)) and processed for 2 hr at 68°C using an extractor. After extraction, the mucilage was collected with a rough plate and filtered through cheesecloth. The mucilage obtained was freeze-dried (FD8508, Ilshinbiobase Co., Ltd, Dongducheon, Korea), ground, packed, and stored at -20°C (KF-600F, Lassele Co., Ltd, Dongducheon, Korea) (Fig. 1).

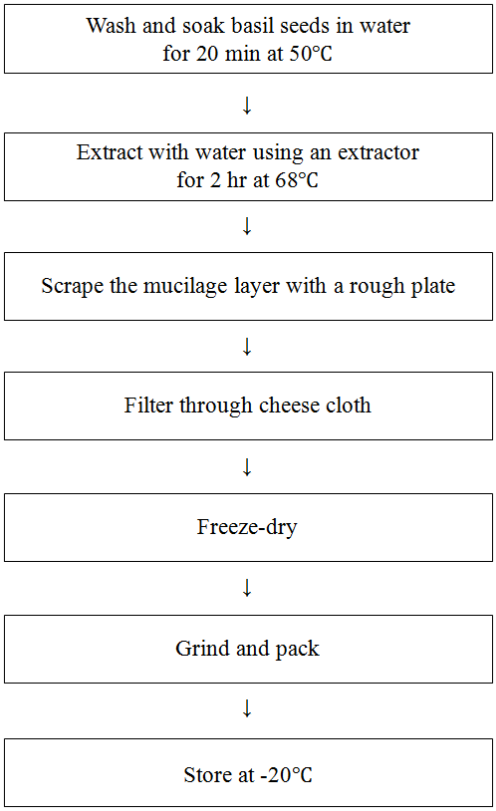


Figure 1. Flow chart of extraction procedure of mucilage from basil seeds.

2.3. Sponge cake preparation

The recipes for sponge cakes are shown in Table 1. A whole egg was poured into a bowl, beaten by a table mixer (KMC010, Kenwood, Havant, England) at 40 rpm for 30 s, and then mixed at 79 rpm for 5 min with sugar and salt.

Table 1. Formula for sponge cake prepared containing different levels of butter and gums.

Samples	Ingredients (g)						
	Cake flour	Butter	Guar gum	Basil seed mucilage (BSM)	Egg	Sugar	Salt
Control	100	20	0	0	180	120	1
Guar-0	100	0	1	0	180	120	1
Guar-25	100	5	1	0	180	120	1
Guar-50	100	10	1	0	180	120	1
Gaur-75	100	15	1	0	180	120	1
BSM-0	100	0	0	1	180	120	1
BSM-25	100	5	0	1	180	120	1
BSM-50	100	10	0	1	180	120	1
BSM-75	100	15	0	1	180	120	1

Control: Without added gum. Guar-0: Addition of 0% of butter with 1 g of guar gum. Guar-25: Addition of 25% of butter with 1 g of guar gum. Guar-50: Addition of 50% of butter with 1 g of guar gum. Guar-75: Addition of 75% of butter with 1 g of guar gum. BSM-0: Addition of 0% of butter with 1 g of basil seed mucilage. BSM-25: Addition of 25% of butter with 1 g of basil seed mucilage. BSM-50: Addition of 50% of butter with 1 g of basil seed mucilage. BSM-75: Addition of 75% of butter with 1 g of basil seed mucilage.

The sample was further mixed at 79 rpm for an additional 5 min followed by 40 rpm for 30 s. Cake flour was passed through a sieve three times, added to a bowl with gum and butter, and mixed. The sponge cake batter (350 g) was deposited into a round cake pan (8 inches in diameter). Then, the cake was baked at 170°C for 20 min in a microwave oven (MC366GAAW5A, Youngreem electron, Seoul, Korea). The sponge cakes were allowed to cool for 1 hr and were then removed from the pans. The cooled sponge cakes were packed in polypropylene bags at room temperature to prevent the cake from drying out (Fig. 2).

2.4. Specific gravity, dough yield, and baking loss of sponge cakes

The specific gravity, dough yield, and baking loss of sponge cakes were calculated using the following formulas, according to the methods described by the AACC (2000):

Specific gravity = Weight of cake dough / Weight of water
Dough yield (%) = (Weight of cake / Weight of dough) × 100
Baking loss (%) = (Weight of dough - Weight of cake) × 100

Figure 2. Photograph of sponge cake prepared containing different levels of butter and gums.
Control: Without added gum. Guar-0: Addition of 0% of butter with 1 g of guar gum. Guar-25: Addition of 25% of butter with 1 g of guar gum. Guar-50: Addition of 50% of butter with 1 g of guar gum. Guar-75: Addition of 75% of butter with 1 g of guar gum. BSM-0: Addition of 0% of butter with 1 g of basil seed mucilage. BSM-25: Addition of 25% of butter with 1 g of basil seed mucilage. BSM-50: Addition of 50% of butter with 1 g of basil seed mucilage. BSM-75: Addition of 75% of butter with 1 g of basil seed mucilage.

2.5. Volume and symmetry indices of sponge cakes

The volume and symmetry indices of the sponge cakes were measured after cutting through the midsection of the cake, according to the methods described by the AACC (2000). The calculation method is shown in Fig. 3.

Volume index = A + B + C
Symmetry index = 2B – A – C

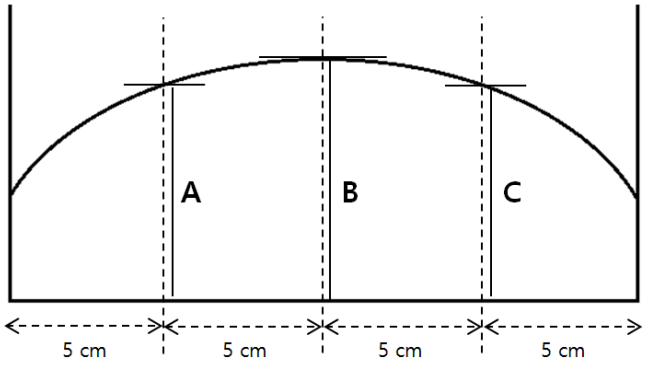


Figure 3. Measurement of volume index and symmetry index of sponge cake prepared with different levels of butter and gums.

2.6. pH of the sponge cakes

For pH determination, 10 g of crumb was mixed with 90 mL distilled water and shaken using a homogenizer (Ingenieurburo CATM. Zipperer, Staufen, Germany) for 1 min by ZHANG *et al*. (2015). pH was measured with a pH meter (SP-701, Suntex, New Taipei City, Taiwan) and the experiments were carried out in triplicate.

2.7. Moisture content of sponge cakes

5 g of sample was cut to 1 cm thickness and incubated at 105°C. When the weight of the sample ceased to change, an infrared moisture analyzer (MB35, OHAUS, Zurich, Switzerland) was used to measure the moisture content.

2.8. Color of sponge cakes

The color of the sponge cake was quantified from the midsection of the crust and crumbs by SONG *et al*. (2015). The L^* (lightness), a^* (redness), and b^* (yellowness) of the color were determined by using a color-measuring spectrophotometer (CR-400; Minolta, Tokyo, Japan) set to Hunter's value. The total color difference (ΔE) compared to the control was calculated. The results of the L^*, a^*, b^*, and ΔE were averaged from triplicate measurements. For the standard white plates used in this study, the L^*, a^*, and b^* values were 95.74, 0.34, and 1.87, respectively.

$$\Delta E = \sqrt{(L_{sample} - L_{standard})^2 + (a_{sample} - a_{standard})^2 + (b_{sample} - b_{standard})^2}$$

ΔE was used to determine the total color difference discernible by the human eyes, as follows:

$\Delta E < 1$ – color differences were not obvious to the human eye
$1 < \Delta E < 3$ – color was not appreciably different to the human eye
$\Delta E > 3$ – color differences were obvious to the human eye

2.9. Textural profile analysis (TPA) of sponge cakes

The texture profile analysis of sponge cakes was carried out by using a rheometer (Compac-100II; Sun Scientific Co., Ltd, Tokyo, Japan) by ZHANG *et al*. (2015). Samples were excised from the center of each sponge cake (30 × 30 × 30 mm). The texture profile was determined by means of a two-bite compression test with a cylindrical probe (20 mm in diameter). The texture parameters recorded in this study were hardness, springiness, cohesiveness, gumminess, and chewiness. Texture values were averaged from triplicate measurements (Table 5).

2.10. Retrogradation rate of sponge cakes during storage

The retrogradation rates of sponge cakes during storage were measured by using a rheometer (Compac-100II; Sun Scientific Co., Ltd, Tokyo, Japan). The Avrami exponent (n) and time constant (1/k) were determined using the Avrami equation (AVRAMI, 1939; CHUNG *et al*., 2003), as follows:

$$\theta = (T_\infty - T_t)/(T_\infty - T_0) = \exp(-kt^n) \quad (1)$$

$$T_\infty - T_t = (T_\infty - T_0) \exp(-kt^n)$$

$$T_t = (T_\infty - T_0) \exp(-kt^n) \quad (2)$$

where θ is the remaining amorphous portion after time (t), k is the rate constant, n is the Avrami exponent, t is the storage time, T_0 is the hardness at the initial time (t = 0), T_t is the hardness after time (t), and T_∞ is the highest hardness that can theoretically be reached.

2.11. Scanning electron microscopy (SEM)

Samples (2 × 2 × 2 mm) were freeze-dried using a freeze-dryer (FD8508; Ilshinbiobase Co., Ltd, Dongducheon, Korea). The surface of a freeze-dried sample was mounted on a specimen holder and coated with gold palladium alloy under vacuum for 120 s with an automatic magnetron sputter coater (JSM670-1F; JEOL Co., Ltd, Tokyo, Japan). Morphology of samples was investigated in random order using scanning electron microscopy (JSM-6701F; JEOL Co., Ltd, Tokyo, Japan). Images were obtained at an accelerating voltage of 15 kV with 200× magnification.

2.12. Statistical analysis

All data are shown as the means of triplicate experiments with their standard deviations. Data analysis was carried out using one-way ANOVA. Duncan's multiple range test was also used to identify significant differences between the means at significance level $p<0.05$ using SPSS 12.0 (SPSS Inc., Chicago, Quarry Bay, Hong Kong) statistical software.

3. RESULTS AND DISCUSSION

3.1. Specific gravity, dough yield, and baking loss of sponge cakes

The physical properties of reduced-fat dough are shown in Table 2. The specific gravity of dough ranged from 0.42 to 0.58. Lower values for specific gravity indicate that the dough contains a lot of air, whereas higher values for specific gravity indicate that the bubble content of the dough is lower, resulting in reduced cake volume (PARK *et al*., 2009). If the specific gravity of the dough is high, the dough becomes heavy due to a lack of air, and the pores are small and dense, making the texture hard (KIM *et al*., 2014). Unlike bread, sponge cake quality varies depending on the amount of air contained during kneading. In general, the appropriate specific gravity of sponge cake prepared using common methods is 0.5 ± 0.05 (KIM *et al*., 2014). This specific gravity is consistent with that of the dough in our experiment. Dough yield was 90.37%-93.09%. Guar-75 showed the highest yield (93.09%), and this value was significantly different from those of other samples. Baking loss increased as the fat content increased, ranging from 7.71% to 9.63%. The lowest baking loss was observed for BSM-25.

Table 2. Specific gravity, dough yield, baking loss, volume index, symmetry index and pH of sponge cake prepared containing different levels of butter and gums.

Samples	Specific gravity	Dough yield (%)	Baking loss (%)	Volume index	Symmetry index	pH
Control	0.47±0.01[1)f]	91.99±0.14[d]	8.01±0.14[d]	12.90±0.17[ab]	0.50±0.17[a]	8.22±0.06[bc]
Gaur-0	0.47±0.01[f]	91.23±0.01[e]	8.77±0.01[c]	8.47±0.51[f]	0.43±0.06[a]	8.21±0.08[bc]
Guar-25	0.45±0.01[g]	91.99±0.01[d]	8.01±0.01[d]	12.23±1.64[bc]	0.67±0.46[a]	8.32±0.07[b]
Guar-50	0.52±0.01[d]	90.97±0.01[f]	9.03±0.01[b]	11.10±0.10[cde]	0.70±0.26[a]	8.05±0.03[d]
Guar-75	0.42±0.00[h]	93.09±0.01[a]	6.91±0.01[g]	13.50±0.44[a]	0.50±0.10[a]	8.68±0.08[a]
BSM-0	0.49±0.01[e]	92.18±0.01[c]	7.82±0.01[e]	8.33±0.55[f]	0.77±0.32[a]	7.69±0.02[d]
BSM-25	0.53±0.01[c]	92.29±0.02[b]	7.71±0.02[f]	12.07±0.38[bcd]	0.53±0.23[a]	8.28±0.06[bc]
BSM-50	0.58±0.00[a]	91.99±0.01[d]	8.01±0.01[d]	10.77±0.06[d]	0.53±0.12[a]	8.18±0.05[c]
BSM-75	0.57±0.01[b]	90.37±0.01[g]	9.63±0.01[a]	10.97±0.15[de]	0.63±0.25[a]	8.05±0.08[d]

[1)]The data are mean±SD in triplicates.

[a–g]Different letters within the same column are significantly different by Duncan's multiple range test ($p<0.05$).

3.2. Volume and symmetry indices of sponge cakes

Table 2 shows the volume indices of sponge cakes. The Guar-0 and BSM-0 cakes showed the lowest values, ranging from 8.33 to 8.47, whereas the Guar-75 and BSM-25 cakes showed the highest values (13.50 and 12.07, respectively). The volume index of the cakes increased as the amount of fat substitute was reduced, and this effect was related to the type and amount of fat or fat substitute. The symmetry index did not differ significantly among samples (range: 0.43-0.77). These results were similar to those reported by FELISBERTO *et al*. (2015), who evaluated fat percentages in pound cakes and showed that 25% fat substitute resulted in the highest specific volume. Moreover, in the present study, replacement with vegetable fat substitute, chia, or chia mucilage gel (CMG) did not cause differences greater than 50% in specific volume as compared to that with other formulations (BEDOYA-PERALES and STEEL, 2014; FELISBERTO *et al*., 2015; PIZARRO *et al*., 2013).

3.3. pH of sponge cakes

The pH of control (8.22) was similar to that of Guar-0 (8.21); however, that of BSM-0 was lower (7.69; Table 2). Groups with added BSM showed significantly lower pH than other samples. The pH of 1% guar solution was 6.39, and that of 1% BSM solution was 7.58. The pH of the cake was determined by the main ingredients and the shape of swelling. Additionally, sugar, shortening, and baking powder did not affect pH (KWON and LEE, 2015). The degree of coloring of the cake has also been reported to be related to pH (LEE *et al*., 2002). pH affects the color and texture of the final product. When the pH is nearly alkaline, the pores become rough, the crust becomes thicker, and the volume increases; in contrast, when the pH is more acidic, the pores become small, the crust becomes soft, and the volume decreases (LEE and LEE, 2013).

3.4. Moisture content of sponge cakes

As shown in Table 3, moisture content was not significantly different among samples except for Guar-75, which had the highest moisture content of 38.87%. This could be related to the combination of fat and guar gum in this sample. Moisture content in foods is important for achieving desirable sensory properties, particularly softness, which is critical for cakes (DADKHAH *et al*., 2012). Moreover, the moisture content did not differ as the amount of added fat substitute (guar gum and xanthan gum) increased (ZAMBRANO *et al*., 2004). Retention of water in foods is caused by interactions between moisture content and dietary fiber (MORAES *et al*., 2010).

3.5. Colors of sponge cakes

The colors of sponge cakes are shown in Table 4. Color is an important property affecting the appearance of the cake, and the color of the crust is influenced by the Maillard reaction. Additionally, the color of crumbs is affected by the ingredients in the formula (AKESOWAN, 2007). The crust of sponge cakes showed a decrease in L, a, and b values as the fat level increased; however, the total difference was increased. In crumbs, L and b values were generally decreased as the fat level increased, although the values showed an overall increase. This could be explained by the color values of butter ($L^* = 85.93$, $a^* = -5.89$, $b^* = 31.53$), guar ($L^* = 88.62$, $a^* = -1.10$, $b^* = 9.63$), and BSM ($L^* = 67.70$, $a^* = 0.83$, $b^* = 7.86$). The low L^* values of BSM affected the L^* values of cakes to a greater degree compared with those of guar and butter. FELISBERTO *et al*. (2015) observed a reduction in b^* with the addition of CMG. Similarly, CMG showed a low L^* of 66.41 compared with that of

vegetable margarine (81.81); this affected the L* color in cakes, resulting in a darker appearance (IDRIS *et al*., 1996). For ΔE, the crust showed values of 51.03-54.66, without significant differences among the samples. In crumbs, ΔE tended to be higher in the BSM groups than in the control and guar gum additive groups (range: 25.48-32.64).

3.6. Textural properties of sponge cakes

As shown in Table 6, the hardness of sponge cake was high in the Guar-0 and BSM-0 samples (11.98 and 14.60 N, respectively). However, the Guar-75 and BSM-25 samples showed low values (3.84 and 6.95 N, respectively) compared with the control (4.87 N). Springiness did not differ significantly among groups, except for that of BSM-0 one. Guar-0 and Guar-75 showed the highest significantly cohesiveness values at 0.77 and 0.76, respectively. Gumminess in Guar-0 and BSM-0 was also high (9.17 and 10.49 N, respectively). The gumminess of control was 3.55 N. Chewiness was significantly higher in the Guar-0 and BSM-0 samples (125.69 and 126.62 N·mm). Hydrocolloids, except for alginate acid, locust bean gum, and hydroxypropyl methylcellulose (HPMC), generally increase the hardness of crumbs as the amount of additive is increased, and the hardness of guar gum cake increased up to 140% compared with that of the control (GOMEZ *et al*., 2007). However, hardness varied depending on the type of hydrocolloids added, and in the case of carrageenan, pectin, and guar gum, the hardness tended to increase further after storage for 48 hr (GOMEZ *et al*., 2007). Xanthan gum decreased the hardness by 40% relative to the control because the chemical interactions between hydrocolloids and starches differed, resulting in changes to moisture content or hardness (GOMEZ *et al*., 2007).

Hardness is also related to the amount of hydrocolloids added. For example, when the amount added is less than 1%, the volume of the cake increases, and the hardness tends to decrease (Gularte *et al*., 2012). However, when the amount of hydrocolloids added is 20%, the volume of the cake decreases, and the hardness tends to increase (GULARTE *et al*., 2012). Chewiness and gumminess are dependent on the results of hardness and therefore showed results similar to those for hardness. GOMEZ *et al*. (2007) also showed that there were no significant differences in springiness with the addition of hydrocolloids, except for alginate acid.

Table 3. Moisture content of sponge cake prepared containing different levels of butter and gums for 72 hr.

Samples	Storage time (hr)			
	0	24	48	72
Control	32.28±0.72[1)b]	33.72±0.58[ab]	34.18±0.33[ab]	34.31±0.33[NS]
Gaur-0	33.07±1.59[b]	35.44±1.71[a]	34.45±0.26[ab]	34.80±0.61
Guar-25	31.61±3.41[b]	34.38±0.78[ab]	34.65±0.14[ab]	34.57±0.07
Guar-50	32.39±0.99[b]	33.47±0.91[b]	34.34±0.56[ab]	34.90±0.25
Guar-75	38.87±0.75[a]	33.42±0.46[b]	33.77±0.35[b]	34.85±0.87
BSM-0	32.90±1.80[b]	34.46±0.90[ab]	34.66±0.77[ab]	34.13±0.29
BSM-25	33.54±2.05[b]	34.63±0.67[ab]	34.62±0.53[ab]	34.14±0.72
BSM-50	33.55±1.30[b]	34.31±0.56[ab]	34.69±0.49[a]	34.72±0.13
BSM-75	33.94±0.69[b]	34.76±1.59[ab]	34.06±0.38[ab]	34.70±0.48

[1)]The data are mean±SD in triplicates.

[ab]Different letters within the same column are significantly different by Duncan's multiple range test ($p<0.05$).

[NS]Not significant.

Table 4. Color values of sponge cake prepared with different levels of butter and gums.

Samples	Crust				Crumb			
	L*[2)]	a*	b*	ΔE	L*	a*	b*	ΔE
Control	47.04±3.06[1)ab]	9.29±0.66[abc]	12.84±1.13[b]	51.98±2.99[ab]	73.89±0.91[b]	-4.90±0.10[d]	17.95±0.29[c]	28.52±0.60[b]
Gaur-0	48.32±1.46[a]	9.58±0.20[a]	13.90±0.48[ab]	51.03±1.28[b]	83.80±0.83[a]	-5.22±0.04[e]	21.22±0.12[a]	23.95±0.52[c]
Guar-25	45.10±0.27[ab]	9.43±0.16[ab]	12.98±0.55[b]	53.88±0.11[ab]	73.91±0.56[b]	-4.50±0.09[b]	16.59±0.41[d]	27.68±0.41[b]
Guar-50	44.05±2.35[b]	8.76±0.37[c]	10.85±0.53[c]	54.39±2.33[a]	71.12±1.53[c]	-4.73±0.12[cd]	18.50±0.50[b]	31.07±0.98[a]
Guar-75	44.21±1.22[b]	9.15±0.50[abc]	12.77±1.37[b]	54.66±0.83[a]	74.04±2.84[b]	-4.69±0.21[bc]	17.86±0.43[c]	28.35±2.15[b]
BSM-0	47.92±1.95[a]	9.24±0.06[abc]	15.07±0.22[a]	51.64±1.89[ab]	75.69±1.20[b]	-2.74±0.03[a]	15.66±0.32[e]	25.48±0.91[c]
BSM-25	47.46±1.77[a]	9.11±0.19[abc]	13.33±0.60[b]	51.64±1.64[ab]	68.24±0.84[d]	-2.79±0.09[a]	14.48±0.03[f]	31.46±0.76[a]
BSM-50	47.99±0.84[a]	8.91±0.20[bc]	13.80±1.01[ab]	51.20±0.76[b]	67.09±1.44[d]	-2.80±0.09[a]	14.79±0.12[f]	32.64±1.29[a]
BSM-75	44.04±1.01[b]	8.78±0.17[c]	11.36±0.21[c]	54.49±1.01[a]	67.39±1.35[d]	-2.66±0.17[a]	14.54±0.12[f]	32.26±1.20[a]

[1)]The data are mean±SD in triplicates.

[2)]L*: lightness (0 black↔white +100), a*: redness (-80 green↔red +100), b*: yellowness (-80 blue↔yellow +70), ΔE: total color difference.

[a-f]Different letters within the same column are significantly different by Duncan's multiple range test ($p<0.05$).

Table 5. The operating conditions of rheometer for texture of sponge cake.

Parameters	Operating conditions
Probe	20 mm cylinder probe
Head speed	120 mm/min
Strain	33.33%
Trigger force	10 kg
Option	TPA (texture profile analysis)
Distance	1 mm

Table 6. Textural properties of sponge cake prepared with different levels of butter and gums.

Samples	Hardness (N)	Springiness (mm)	Cohesiveness	Gumminess (N)	Chewiness (N·mm)
Control	4.87±0.62[1)de]	13.57±0.15[a]	0.73±0.01[b]	3.55±0.45[de]	48.18±6.46[c]
Gaur-0	11.98±0.73[b]	13.65±2.30[a]	0.77±0.04[a]	9.17±0.88[b]	126.62±34.50[a]
Guar-25	5.55±1.91[d]	13.62±0.06[a]	0.74±0.01[b]	4.09±1.39[d]	55.73±18.86[bc]
Guar-50	7.47±0.81[c]	13.63±0.09[a]	0.72±0.00[b]	5.39±0.57[c]	73.51±7.65[b]
Guar-75	3.84±0.43[e]	13.61±0.10[a]	0.76±0.00[a]	2.92±0.31[e]	39.70±4.22[c]
BSM-0	14.60±1.79[a]	11.98±0.22[b]	0.72±0.01[b]	10.49±1.24[a]	125.69±14.73[a]
BSM-25	6.95±0.23[c]	13.67±0.10[a]	0.74±0.00[b]	5.12±0.16[c]	70.00±1.86[b]
BSM-50	7.75±0.91[c]	13.66±0.09[a]	0.72±0.00[b]	5.54±0.62[c]	75.62±8.20[b]
BSM-75	7.18±0.22[c]	13.63±0.10[a]	0.72±0.01[b]	5.18±0.16[c]	70.61±2.11[b]

[1)]The data are mean±SD in triplicates.

[a–e]Different letters within the same column are significantly different by Duncan's multiple range test (p<0.05).

3.7. Avrami exponent (n) of sponge cakes during storage

The sponge cakes were stored at 4°C and the hardness was measured after 0, 24, 36, and 72 hr, as shown in Table 7. The Avrami equation was used to analyze the retrogradation rate of sponge cakes, as shown in Table 8. The Avrami exponent (n) indicates the crystallization form according to formation time and rate; values of 1-2 indicate that the crystallization is constant, whereas values of 3-4 indicate that the crystallization progresses until the crystal nucleation becomes sporadic and the saturation is complete (Korea Food Research Institute, 1997). The Avrami exponent (n) of the control was 0.0655, and those of Guar-25 and Guar-50 samples were 0.0149 and 0.0647, respectively. For the BSM-additive group, the Avrami exponents ranged from 0.0221 to 0.0449, indicating that retrogradation was inhibited compared to the control. The time constant (1/k) is the reciprocal of the rate constant (k), which represents the rate of progression of retrogradation; smaller rate constants indicate a greater time constant (1/k), reflecting the inhibition of retrogradation (KIM and CHUNG, 2010). The time constant (1/k) for Guar-50 was 285.71, which was higher than that of the control (100.00), and in the BSM-additive group, this value ranged from 270.27 to 588.24, indicating that BSM inhibited retrogradation. In particular, BSM-25 exhibited the highest time constant (1/k), indicating that this sample showed the greatest inhibition of retrogradation. This is because the water content or hardness was changed due to the chemical interaction between the hydrocolloids and starch, and the difference in the hardness could be due to water retention in the sample (GOMEZ *et al*., 2007). When

carboxymethyl cellulose (CMC) and k-carrageenan were added to bread, the increase in hardness was lower than that of the control (LEE *et al*., 2008).

Table 7. Hardness of sponge cakes prepared with different levels of butter and gums for 72 hr.

Samples	Storage time (hr)			
	0	24	48	72
Control	4.95±0.28[1)d]	3.17±0.47[cd]	3.64±0.26[b]	4.20±0.30[c]
Gaur-0	12.16±0.37[b]	7.09±0.81[a]	5.91±0.10[a]	8.82±0.35[a]
Guar-25	5.24±0.99[d]	3.83±0.05[c]	3.86±0.17[b]	3.13±0.09[e]
Guar-50	7.77±0.64[c]	2.40±0.18[de]	2.90±0.40[c]	3.24±0.49[de]
Guar-75	3.76±0.36[e]	3.45±0.15[c]	3.82±0.35[b]	3.72±0.25[cd]
BSM-0	14.91±0.91[a]	4.90±1.09[b]	5.79±0.23[a]	5.90±0.50[b]
BSM-25	6.90±0.10[c]	2.27±0.24[de]	2.05±0.10[e]	2.25±0.03[f]
BSM-50	7.78±0.67[c]	1.95±0.15[e]	2.50±0.25[d]	2.84±0.28[c]
BSM-75	7.25±0.06[c]	2.38±0.07[de]	2.32±0.07[de]	2.68±0.30[ef]

[1)]The data are mean±SD in triplicates.
[a–f]Different letters within the same column are significantly different by Duncan's multiple range test (p<0.05).

Table 8. Avrami exponent (n), rate constant (k) and time constant (1/k) of sponge cakes prepared with different levels of butter and gums.

Avrami equation analysis	Avrami exponent (n)[1)]	Rate constant (k)[2)]	Time constant (hr) (1/k)
Control	0.0653	1.00×10^{-2}	100.00
Gaur-0	0.4793	2.58×10^{-2}	38.76
Guar-25	0.0149	1.50×10^{-2}	66.67
Guar-50	0.0647	0.35×10^{-2}	285.71
Guar-75	0.1887	12.38×10^{-2}	8.08
BSM-0	0.0291	0.37×10^{-2}	270.27
BSM-25	0.0221	0.17×10^{-2}	588.24
BSM-50	0.0318	0.33×10^{-2}	303.03
BSM-75	0.0449	0.33×10^{-2}	303.03

[1)]Values obtained from slop of plot $\log\{-\ln(T_\infty-T_t)/(T_\infty-T_0)\}$ vs log t.
[2)]Values obtained from slop of plot $\ln(T_\infty-T_t)$ vs time.

3.8. Micrographs of sponge cakes

Fig. 4 shows micrographs of sponge cakes prepared with different amounts of butter and gum. Fig. 4A shows a micrograph of crumbs from the control. Starch granules were observed around gluten protein. The microscopic analysis of starch granules allows visualization of the gluten protein matrix, with thin and irregularly shaped sheets (ASHWINI *et al*., 2009). However, the starch granules lose their shape following gelatinization (SOWMYA *et al*., 2009). Pores and fractions appeared as the content of the gums and butter varied. The presence of many pores and fractions indicated that cakes

were more porous. Fig. 4B and 4F show micrographs of the crumbs from cakes prepared without fat. Fig. 4F, showing samples containing 1% BSM, demonstrated the presence of medium size pores. As the amount of fat substitute increased, the crumb matrix became irregular. Fat in baked food acts as a lubricant, interconnecting and forming a continuous matrix with all ingredients (RODRÍGUEZ-GARCÍA *et al*., 2012). Additionally, fat substitutes, such as gums, can limit hydration of gluten and starch, causing the formation of a less developed structure (RODRÍGUEZ-GARCÍA *et al*., 2012).

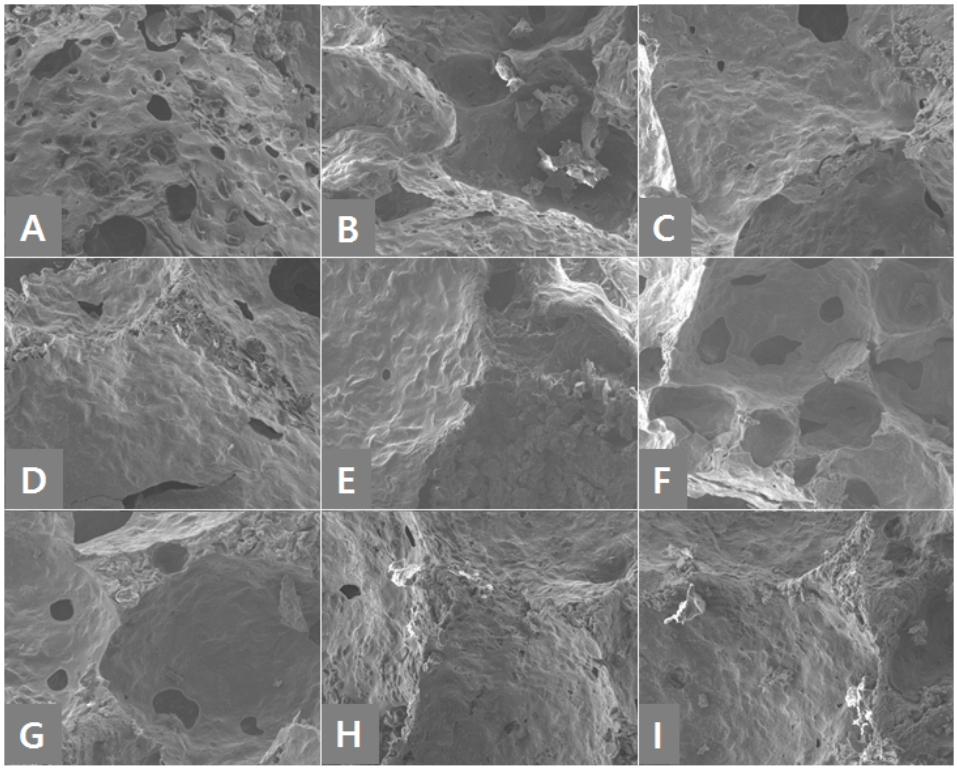


Figure 4. Micrographs of sponge cake prepared with different levels of butter and gums.
A: Control, B: Guar-0, C: Guar-25, D: Guar-50, E: Guar-75, F: BSM-0, G: BSM-25, H: BSM-50, I: BSM-75.

4. CONCLUSIONS

For sponge cakes, the physicochemical properties of the dough, including specific gravity, dough yield, baking loss, volume index, and symmetry index, were improved in Guar-25, Guar-50, and BSM-25 samples compared to those in other samples. The Guar-75 sample showed the highest moisture content, and groups with added hydrocolloids showed increased in hardness compared with the control. However, the hardness of samples prepared with added hydrocolloids tended to decrease compared with the control during storage. We also confirmed that the Guar-50 and BSM-25 samples inhibited retrogradation, as determined using the Avrami equation. Therefore, we found that the quality characteristics of the cakes were different according to the type of hydrocolloid. Based on these findings, BSM, which can inhibit retrogradation and reduce 75% of the fat content by substituting only 1% BSM, was considered the most effective fat substitute.

-

REFERENCES

AACC. 2000. Approved methods of the American Association of Cereal Chemists 10th ed. pp. 10–91. St. Paul, MN:American Association of Cereal Chemists, Inc.

Akesowan A. 2007. Effect of a konjac flour/soy protein isolate mixture on reduced-fat, added water chiffon cakes. Assumption University Journal of Technology. 11(1):23-27.

Akhtar M, Dickinson E, Mazoyer J. and Langendorff V. 2002. Emulsion stabilizing properties of depolymerized pectin. Food Hydrocoll. 16(3):249-256.

Anjaneyalu Y.V, and Gowda D.C. 1979. Structural studies of an acidic polysaccharide from *Ocimum basilicum* seeds. Carbohydrate Research. 75:251-256.

Ashwini A, Jyotsna R. and Indrani D. 2009. Effect of hydrocolloids and emulsifiers on the rheological, microstructural and quality characteristics of eggless cake. Food Hydrocoll. 23(3):700-707.

Avrami M. 1939. Kinetics of phase change. I General theory. J Chem Phys. 7(12):1103-1112.

Bedoya-Perales N.S., and Steel C.J. 2014. Effect of the concentrations of maltogenic α-amylase and fat on the technological and sensory quality of cakes. Food Sci. Technol. (Campinas). 34(4):760-766.

Bent A.J, Bennion E.B. and Bamford,G. 2013. The Technology of Cake Making:Springer Science and Business Media.

Brennan C.S. and Tudorica C.M. 2008. Carbohydrate-based fat replacers in the modification of the rheological, textural and sensory quality of yoghurt: comparative study of the utilisation of barley beta-glucan, guar gum and inulin. Int J Food Sci Technol. 43(5):824-833.

Cauvain S, and Young L. 2006. Ingredients and their influences. Medical innovation: Science, Technology and Practice. pp. 72-98. Oxford, Blackwell Publishing.

Chung M.S, Lee, J.K. Hur N.Y, Kim D.S. and Baik M.Y. 2003. Textural analysis for bread staling. Food Sci. Biotechnol. 12(6):727-736.

Coorey R., Tjoe A. and Jayasena V. 2014. Gelling properties of chia seed and flour. J Food Sci. 79(5):859-866.

Dadkhah A., Hashemiravan M. and Seyedain-Ardebili M. 2012. Effect of shortening replacement with nutrim oat bran on chemical and physical properties of shortened cakes. Ann Biol Res. 3(6):2682-2687.

Fekri, N., Khayami, M., Heidari, R. and Jamee R. 2008. Chemical analysis of flaxseed, sweet basil, dragon head and quince seed mucilages. Res J Biol Sci. 3(2):166-170.

Felisberto M.H.F., Wahanik A.L, Gomes-Ruffi C.R, Clerici M.T.P.S, Chang Y.K. and Steel C.J. 2015. Use of chia (*Salvia hispanica* L.) mucilage gel to reduce fat in pound cakes. LWT-Food Sci. Technol. 63(2):1049-1055.

Garti N. and Leser M.E. 2001. Emulsification properties of hydrocolloids. Polym. Adv. Technol. 12(1-2):123-135.

Gomez M, Ronda F, Caballero P.A, Blanco C.A. and Rosell C. M. 2007. Functionality of different hydrocolloids on the quality and shelf-life of yellow layer cakes. Food Hydrocoll. 21(2):167-173.

Gularte M.A., de la Hera E., Gómez M. and Rosell C.M. 2012. Effect of different fibers on batter and gluten-free layer cake properties. LWT-Food Sci. Technol. 48(2):209-214.

Hassel C. 1993. Nutritional implications of fat substitutes. Cereal Foods World. 38(3):142.

Hosseini-Parvar S., Matia-Merino L., Goh K., Razavi S., and Mortazavi S. 2010. Steady shear flow behavior of gum extracted from *Ocimum basilicum* L. seed: Effect of concentration and temperature. J Food Eng. 101(3):236-243.

Huang X., Kakuda Y., and Cui W. 2001. Hydrocolloids in emulsions: particle size distribution and interfacial activity. Food Hydrocoll. 15(4):533-542.

Idris, N. A, Tang, T, and Chong, C. 1996. Chemical composition and physical properties of soft (tub) margarines sold in Malaysia. J. Am. Oil Chem. Soc. 73(8):995-1001.

Korea Food Research Institute. 1997. Development of automated single equipment for rice cake production. pp. 45-46. Ministry of Agriculture, Food and Rural Affairs, Sejong, Korea.

Kim M.K, Lee E.J. and Kim K.H. 2014. Effects of Helianthus tuberosus powder on the quality characteristics and antioxidant activity of rice sponge cakes. J. Korean Soc. Food Culture. 29(2):195-204.

Kim S.S and Chung H.Y. 2010. Retarding retrogradation of Korean rice cakes (Karedduk) with a mixture of trehalose and modified starch analyzed by Avrami kinetics. Korean J. Food Nutr. 23(1):39-44.

Kwon M. and Lee M. 2015. Quality characteristics of sponge cake added with rice bran powder. Korean J Culinary Res. 21(3):168-180.

Lakshminarayan S.M, Rathinam V. and KrishnaRau L. 2006. Effect of maltodextrin and emulsifiers on the viscosity of cake batter and on the quality of cakes. Sci. Food Agric. 86(5):706-712.

Lee K., Lee Y. and Yang J. 2002. Effects of irradiated egg white on the quality of angel food cake. Korean J. Food Cookery Sci. 18:30-33.

Lee S.E. and Lee J.H. 2013. Quality and antioxidant properties of sponge cakes incorporated with pine leaf powder. Korean J. Food Sci. Technol. 45(1):53-58.

Lee S.J., Cho S.K. and Lee S.J. 2008. Study on the texture and staling of breads with addition of various hydrocolloids. Korean J Food Cookery Sci. 24(5):636-644.

Mathews S, Singhal R. and Kulkarni P. 1993. *Ocimum basilicum*:a new non-conventional source of fibre. Food Chem. 47(4):399-401.

Moraes É.A, Dantas M.I.D.S, Morais D.D.C, Silva C.O.D, Castro F.A.F.D, Martino H.S.D. and Ribeiro S.M.R. 2010. Sensory evaluation and nutritional value of cakes prepared with whole flaxseed flour. Food Sci. Technol. (Campinas). 30(4):974-979.

Naji-Tabasi S. and Razavi S.M.A. 2016. New studies on basil (*Ocimum bacilicum* L.) seed gum: Part-Emulsifying and foaming characterization. Carbohydrate Polymers. 149:140-150.

Park J.E, Jeong, H.D. and Jang M.S. 2009. Optimization of ingredient mixing ratio for preparation of sponge cake with bamboo (Pseudosasa japonica Makino) leaves powder. Korean J Food Cookery Sci. 25(3):317-329.

Pizarro P.L., Almeida E.L, Sammán N.C and Chang Y.K. 2013. Evaluation of whole chia (Salvia hispanica L.) flour and hydrogenated vegetable fat in pound cake. LWT-Food Sci Technol. 54(1):73-79.

Razavi S., Mortazavi S.A., Matia-Merino L., Hosseini-Parvar S.H., Motamedzadegan A., and Khanipour E. 2009. Optimisation study of gum extraction from Basil (*Ocimum bacilicum* L.) seeds. Int. J. Food Sci. Technol. 44(9):1755-1762.

Rodríguez-García J., Puig A., Salvador A. and Hernando I. 2012. Optimization of a sponge cake formulation with inulin as fat replacer: structure, physicochemical, and sensory properties. J. Food Sci. 77(2):189-197.

Sanchez C., Klopfenstein C. and Walker C. 1995. Use of carbohydrate-based fat substitutes and emulsifying agents in reduced-fat shortbread cookies. Cereal Chem. 72(1):25-29.

Simon J.E., Morales M.R., Phippen W.B., Vieira R.F., and Hao Z. 1999. Basil: a source of aroma compounds and a popular culinary and ornamental herb. Perspectives on New Crops and New Uses. 499-505.

Song, K.Y., O H., Zhang Y.Y., Joung K.Y. and Kim Y.S. 2016. Effects of pumpkin (*Cucurbita moschata* Duch.) leaf powder on quality characteristics, antioxidant activities, and retarding retrogradation by shelf-life of sponge cake. Korean J. Food Nutr. 29(6):930-937.

Sowmya M., Jeyarani T., Jyotsna R. and Indrani D. 2009. Effect of replacement of fat with sesame oil and additives on rheological, microstructural, quality characteristics and fatty acid profile of cakes. Food Hydrocoll. 23(7):1827-1836.

Zambrano F., Despinoy P., Ormenese R. and Faria E. 2004. The use of guar and xanthan gums in the production of 'light' low fat cakes. Int. J. Food Sci. Technol. 39(9):959-966.

Zhang Y.Y, Song K.Y, O H., Choi B.B and Kim Y.S. 2015. Quality and antioxidant characteristics of sponge cake with asparagus (*Asparagus officinals* L.) powder. Korean J. Food Cookery Sci. 31(5):642-651.

Paper Received February 27, 2017 Accepted July 12, 2017

PAPER

YOGHURT DRINK FOR LACTOSE AND GALACTOSE INTOLERANT PATIENTS

İ.E. TONGUÇ and C. KARAGÖZLÜ*
Ege University Faculty of Agriculture Dairy Technology Department, Bornova, Izmir, Turkey
*Corresponding author. Tel.: +90 2323112902; fax: +90 2323881864
E-mail address: cem.karagozlu@ege.edu.tr

ABSTRACT

In this study, yoghurt drink with lactose free and low galactose content for patients with galactose intolerance were produced using a 1:1 mixture of lactose free milk and two different types of infant formula, fortified with strawberry flavor. The results indicated that galactose content of yoghurt drinks produced from lactose free raw materials were declined to a level that is suitable for the diets of patients with galactosemia. Chemical, microbiological and sensory properties of these products were found to match the common quality characteristics of a commercial fermented dairy product.

Keywords: yoghurt drink, Food allergies, galactosemia, lactose intolerance

1. INTRODUCTION

Classic galactosemia is an autosomal recessive disorder of carbohydrate metabolism (OMIM 230400), due to a severe deficiency of the enzyme, galactose-1-phosphate uridyltransferase (GALT, EC 2.7.7.12), which catalyzes the conversion of galactose-1-phosphate and uridine diphosphate glucose (UDPglucose) to uridine diphosphate galactose (UDPgalactose) and glucose-1-phosphate. Upon consumption of lactose in the neonatal period, the affected infants develop a potentially lethal disease process with multiorgan involvement. However, since the advent of newborn screening (NBS) for galactosemia, we rarely encounter such overwhelmingly ill newborns. The following case report illustrates the acute neonatal toxicity that may be seen in infants with severe GALT deficiency, marked elevation of galactose-1-phosphate in target tissues and severe hypergalactosemia due to lactose ingestion (RIEDEL *et al*., 2005; BERRY, 2012). Its estimated incidence is 1/40,000 - 60,000 live births. This form is called the classical galactosemia. Patients with GALT deficiency appear normal at birth but soon develop severe hepatic, renal and gastro-intestinal manifestations that, if not treated, mostly lead to death. Removal of dietary lactose and galactose is essential as this will prevent or decrease the severity of the initial metabolic crisis in the neonate (KERCKHOVE *et al*., 2015).
Individuals with galactosemia are intolerant of dietary lactose and galactose, primarily found in milk and milk products. If untreated, the disorder can cause liver failure, kidney dysfunction, sepsis, and death. If it is diagnosed soon after birth and treated by removal of lactose and galactose from the diet, the symptoms will resolve and many of the long-term complications, including cataracts and mental retardation, can be prevented (GRANGE, 2004).
Individuals suffering from galactosemia cannot consume products containing galactose, which means that they cannot consume dairy products, which have a critical role in healthy growth and nutrition. A strategy, similar to the introduction of the gluten free products to the diet of individuals with celiac disease, may be adopted for galactosemia suffering individuals by developing galactose free dairy products.
Previous studies have reported the following galactose consumption limit values that were verified by doctors and dieticians based on many years of experience: babies 50 (–200 mg), infants 150 (–200 mg), schoolchildren 200 (–300 mg), youth 250 (–400 mg), adults 300 (-500 mg) galactose/day (VARGA *et al*., 2006, SCHWEITZER *et al*., 1998). Depending on these limit values, the galactose content of the yoghurt-like product developed by SZIGETI and KRÁSZ (1992) was found to be higher than the required for children suffering from galactosemia, where VARGA *et al*., (2006) found the galactose levels of kefir-like products suitable for galactosemia patients from all ages.
Yoghurt is a fermented dairy product commonly produced in the world, produced by the fermentation of milk by *Lb. bulgaricus* and *S. thermophilus* bacteria. Yoghurt drink also carries the health and nutritional value of yoghurt and it is produced by the addition of water to yoghurt. It is called in different ways in different regions such as ayran in Turkey, dough in Iran, tan in Armenia, laban in Syria and Lebanon. As well its many health benefits, yoghurt drink has positive effects on health including the regulation of lactose intolerance and supporting the immune system (ERKAYA *et al*., 2015).
The aim of this study was to develop yoghurt drink for individuals with galactosemia and/or lactose intolerance by full hydrolization of lactose content and lowering the galactose levels suitable for the safe consumption of these products by galactosemic individuals. Accordingly, yoghurt drink with galactose levels lower than 200 mg/100 c m^3 for galactosemic patients from all ages by using by using a 1:1 mixture of lactose free milk and two different types of infant formula, fortified with strawberry flavor.

2. MATERIALS AND METHODS

2.1. Milk samples and fermented dairy production, experimental design

UHT cow's milk and lactose hydrolyzed UHT cow's milk used in the studies were obtained from Pinar Sut Co. (Izmir, TURKEY). In order to lower the galactose content before the fermentation, lactose hydrolyzed UHT cow's milk was mixed with galactose free infant formulas. The ratios in the mixtures were one part of lactose hydrolyzed milk and one part of galactose free infant formula (1:1). Two different galactose free infant formulas were used as supplements of lactose hydrolyzed milk: Neocate, a maltose based, galactose free infant formula (Milupa/Numico, Netherlands) and Galactomin 19, fructose based, galactose free complete infant formula (SHS, UK). The sensory properties of the two formulas were different, possibly influencing the sensory properties of both raw material mixtures and fermented products. UHT cow's milk as the control group, lactose hydrolyzed milk and the two types of mixtures were inoculated with yoghurt drink cultures respectively (Table 1).

Table 1. Raw material properties of fermented dairy drinks for the individuals with galactosemia.

Raw Material	Dry Matter (%)	Fat (%)	Protein (%)	pH	Lactose ($mg/100cm^3$)	Galactose ($mg/100cm^3$)
C	10.31±0.50	1.50±0.06	3.10±0.00	6.7±0.03	4208.35±23.35	0.00±0.00
L	10.19±0.18	1.45±0.05	3.10±0.04	6.6±0.11	0.00±0.00	2160.40±34.21
LN	10.54±0.24	2.50±0.07	2.97±0.02	6.4±0.14	0.00±0.00	1068.11±12.30
LG19	10.42±0.08	2.80±0.01	2.98±0.06	6.5±0.08	0.00±0.00	1080.07±14.10

C: Conventional UHT milk, L: Lactose-free UHT milk,
LN: Lactose-free UHT milk + Neocate, LG19: Lactose-free UHT milk + Galactomin 19.

Commercial freeze-dried yoghurt drink starter culture LB340, containing *Lactobacillus delbrueckii spp. bulgaricus* and *Streptococcus thermophilus*, were obtained from Ezal (Texel, France). The strawberry sauce and flavor used for enhancing the sensory properties of the products were obtained from Aromsa Co. (Kocaeli, Turkey). Skim milk powder used for the preparation of starter cultures were obtained from Pinar Sut Co. (Pinarbasi, Izmir).
500 mL of reconstituted skim milk with 12 % non-fat dry matter were inoculated with freeze-dried yoghurt drink (2 %, in 42° C) cultures. The inoculations ended when the pH levels of the inocula dropped to 4.6. Raw materials prepared for the production of fermented drinks were inoculated with 3.25 % culture in all cases. Incubation parameters for the products were, 3 hours in 42° C. Fermentation were run in duplicate and repeated twice in bottles containing 500 mL of raw materials and 3.25 % inoculum. In order to enhance the sensory properties of products, fermented drinks were fortified with galactose free strawberry sauce (1.8 %) and strawberry aroma (0.1 %). Manufacture of the products were run in duplicate and repeated twice in all cases.
4 different raw materials were coded as follows;

CAY: Control Yoghurt Drink,
LAY: Lactose free milk Yoghurt Drink,
LNAY: Lactose free milk + Neocate Yoghurt Drink,
LG19AY: Lactose free milk + Galactomin 19 Yoghurt Drink.

2.2. Chemical, microbiological and sensory analyses

The pH was determined using a pH meter (Hanna pH 211 Microprocessor, Portugal). Dry matter, protein and fat contents were determined according to A.O.A.C (2006). Tyrosine levels were measured according to HULL (1947). Acetaldehyde contents of the samples were determined using spectrophotometric method according to ROBINSON *et al*., (1977). For the determination of lactose and galactose levels, Megazyme K-LACGAR 12/05 enzymatic kit obtained from Megazyme International Ireland Limited (Co.Wicklow, Ireland) was used.
Bacterial enumerations were carried out at the 1st, 10th, 20th, 30th days of the storage period. Samples (1 mL) were diluted with ringer solution (9 mL). Serial dilutions were carried out, and bacteria were counted, applying the pour plate method. *L. bulgaricus* counts in yoghurt drink samples were enumerated in MRS agar (pH 5.8) (Merck / 1.10660, Darmstadt, Germany) anaerobically at 42°C for 48 h, whereas *S. thermophiles* in yoghurt drink samples were counted in M17 agar (pH 6.9) aerobically at 37°C for 48 h (BRACQUART, 1981).
Samples were evaluated for their sensory properties (taste-aroma, consistency, overall). The evaluation cards were prepared according to BODYFELT *et al*., (1998). The evaluation was performed by the academicians from the Dairy Technology Department of Ege University.

2.3. Statistical analyses

The experiments were performed in two repetitons with three parallels. The mean value of the six values for each sample was calculated (n=6). The obtained data was statistically analyzed by one-way ANOVA using the general linear model. The constant effects (different production process and storage period and the effects of the interactions between these effects were analyzed by analysis of variance (ANOVA) using SPSS© v.15.00 (SPSS Inc., Chicago, Illınois USA). The significant data as a result of ANOVA were tested according to the Duncan multiple comparison test at $p < 0.05$ level.

3. RESULTS AND DISCUSSIONS

3.1. Chemical properties

In terms of obtaining the desired structural and sensory properties of yoghurt drink dry matter content and its properties are the important basic parameters. Many studies have reported that dry matter contents had a direct effect on the structural, microbiological and sensory properties of the products. In the production of fermented dairy products, it is required to comply with the legally prescribed minimum dry matter levels. Dry matter, fat and protein contents of yoghurt drink samples were analyzed on the 1st day of the storage (Table 2). The results showed that dry matter, fat and protein contents of all yoghurt drink and kefir samples were in accordance with the nutrient contents specified in Fermented Dairy Products Communiqué (Communiqué No: 2009/25) in Turkish Food Codex (2009).
In accordance with the aim of our study, lactose-free milk and milk-formula mixtures were used in productions. Additionally, no lactose hydrolization process was done in control group that is conventional semi-skimmed UHT drinking milk. Therefore, lactose was not detected in the study, except the control samples coded as CAY (Table 2).

Table 2. The results for the compositional analysis of yoghurt drinks for the individuals with galactosemia.

	Dry Matter (%)	Fat (%)	Protein (%)	Acetaldehyde (ppm)	Lactose (mg / 100 cm^3)	Galactose (mg / 100 cm^3)
CAY	10.94±0.08^b	1.50±0.00^a	2.71±0.19^b	6,97±0,01^c	2129.20±23.81	120.67±.072^b
LAY	10.82±0.47^b	1.55±0.00^b	3.01±0.36^c	7,04±0,01^c	≤0.01±0.00	225.51±2.78^d
LNAY	11.05±0.50^c	2.56±0.00^c	2.69±0.10^a	6,00 ±0,01^a	≤0.01±0.00	153.79±2.05^c
LG19AY	10.62±0.21^a	2.76±0.02^d	2.66±0.84^a	6,30±0,01^b	≤0.01±0.00	63.70±6.39^a

$^{a, b, c, d}$: Values with different lower-case letters in the same column differ significantly ($P < 0.05$).

Examining the galactose levels of other three lactose free samples, it was found that galactose level in LAS sample was higher than the level reported by VARGA *et al*., (2006), with 212.46 mg/100 cm^3. Different raw material contents had a significant effect on the galactose contents in all kefir samples ($p<0,05$). It was found that LKF, LNKF and LG19KF samples contained lower galactose than the threshold reported by VARGA *et al*., (2006), with 161.95, 132.74 and 106.54 mg/100 cm^3, respectively. VARGA *et al*., (2006), in their study, determined the galactose level of kefir sample pre-determined as the control sample produced from lactose free milk as 270 mg/100 cm^3, the galactose level of kefir produced from milk-formula mixture containing Pregomin as 169 mg/100 cm^3, and the galactose level of kefir produced from milk-formula mixture containing Nutrilon as 171.5 mg/100 cm^3. In their study, the researchers determined the milk-formula ratio as 2 parts milk and 1 part formula (2:1). Comparing those results with our study, the galactose levels obtained in our study appear to be lower than those by VARGA *et al*., (2006). The most likely reason for this difference is the 1:1 milk-formula ratio we adopted for our method.
In all samples pH decreased during storage (Fig. 1).

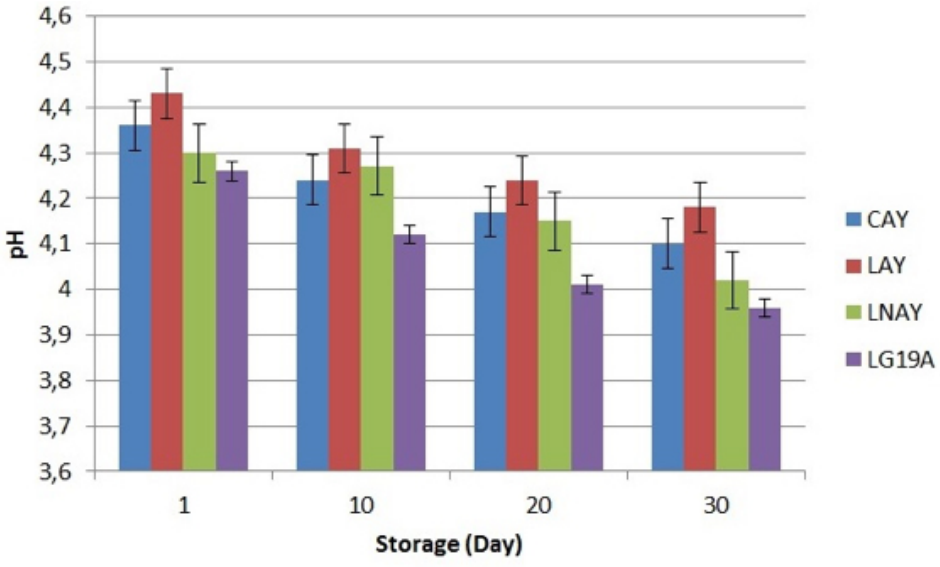


Figure 1. pH values of yoghurt drinks for the individuals with galactosemia.

The differences between the pH values in acidophilus milk samples at the 10th, 20th and 30th day of the storage were found to be statistically not significant ($p>0.05$). In yoghurt

drink samples, different raw material content had no effect on the pH values at the 1st and the 20th days of the storage ($p<0.05$). pH values of yoghurt drink samples in our study were similar to those in similar studies by OZER *et al*., (2005), MARTINI *et al*., (1991), TONGUC *et al*., (2013), YERLIKAYA *et al*., (2013) and ERKAYA *et al*., (2015). In fermented dairy products, as a result of the hydrolization of lactose by culture bacteria and the formation of lactic acid during incubation, pH reaches to a certain level and coagulates and maintains the gel formation. During ripening and storage, acidity increases and the decrease in pH value continues. The type of the bacteria used in the incubation is largely responsible for the speed of the decrease in pH.

It was reported that the amount of acetaldehyde required for the formation of characteristic aroma in fermented dairy products varied between 13 and 48 ppm (SAHAN *et al*., 2008; CHENG, 2010). Different raw material compositions had a statistically significant effect on the acetaldehyde contents in all samples ($p<0.05$). This result was supported by the panelists' comments in taste-aroma evaluations in sensory analyses reporting that they perceived acetaldehyde aroma in products.

Tyrosine values of yoghurt drink samples and the changes in these values during storage are given in Fig. 2. The differences between tyrosine values of yoghurt drink samples were statistically significant ($p<0.05$). Also, storage had a statistically significant effect on the tyrosine values of the samples ($p<0.05$). Tyrosine levels obtained in our study was compatible with those reported in studies on various fermented dairy products (OZER *et al*., 2005, YERLIKAYA *et al*., 2013). However, tyrosine levels of sample LN were higher than those values. In this perspective, tyrosine values of all the samples and the scores obtained in sensory evaluations were substantially parallel. High tyrosine values determined in sample LNAY supports the claim by De MANN (2013) that bitter taste forms as tyrosine content increases. Sample LNAY received the lowest taste-flavor scores in sensory evaluations throughout the whole storage. Panelists reported "bitter taste-flavor formations" in the sensory evaluation sheets many times throughout the sensory evaluation process.

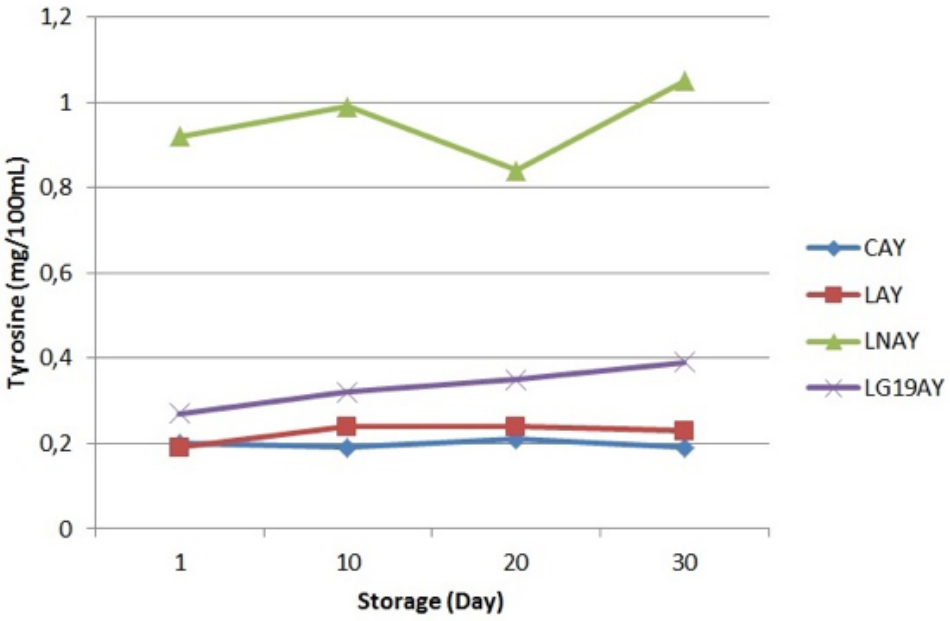


Figure 2. Tyrosine values of yoghurt drinks.

3.2. Microbiological properties

Examining the microbiological properties of yoghurt drink samples, it was found that different raw material contents had a significant effect on the *L. bulgaricus* counts in yoghurt drink samples at the 10th and 20th day of the storage (Table 3). The effect of storage period on the *L. bulgaricus* counts in all yoghurt drink samples was significant ($p<0.05$). Using different raw materials had a significant effect on the *S. thermophilus* counts of yoghurt drink samples ($p<0.05$). In addition, the effect of storage on *S. thermophilus* counts of yoghurt drink samples was statistically significant ($p<0.05$). *S. thermophilus* counts of yoghurt drink samples were above log 7 cfu/ml and the lowest value was determined in LAY with log 6.97 cfu/ml on the 10th day of the storage (Table 4). *Lb. bulgaricus* and *S. thermophilus* counts in LNAY and LG19AY samples maintained their viability during 30-day storage period. *Lb. bulgaricus* and *S. thermophilus* counts in our samples were higher than the results found in previous studies by AKALIN and UNAL (2010) and were similar to those obtained by VARGA *et al*., (2003), YERLIKAYA *et al*., (2012), TONGUC *et al*., (2013) and ERKAYA *et al*., (2015).

Table 3. Microbiological contents of yoghurt drinks for the individuals with galactosemia.

Helv	Storage (Day)			
	1st	10th	20th	30th
***Lb. bulgaricus* (log cfu/ml)**				
CAY	8.07±0.26Y^{b}	8.12±0.10Y^{b}	7.42±0.04X^{a}	7.57±0.08X
LAY	7.85±0.34XY^{ab}	8.02±0.01Y^{b}	7.58±0.07XY^{b}	7.40±0.01X
LNAY	7.50±0.07X^{a}	8.03± 0.06W^{b}	7.61±0.01XY^{b}	7.71±0.02Y
LG19AY	7.46 ±0.03XY^{a}	7.79±0.01Y^{a}	7.32±0.07X^{a}	7.54±0.25XY
***S. thermophilus* (log cfu/ml)**				
CAY	7.37±0.04Y^{a}	7.12±0.10X^{b}	7.63±0.03W^{b}	7.16±0.01X^{a}
LAY	7.48±0.01W^{ab}	6.97±0.04X^{a}	7.85±0.04Z^{b}	7.22±0.02Y^{a}
LNAY	7.41±0.00Y^{ab}	7.30±0.01X^{c}	7.39±0.00Y^{a}	7.53±0.03W^{b}
LG19AY	7.54±0.08Y^{b}	7.27±0.02X^{bc}	7.66±0.07Y^{b}	7.66±0.08Y^{b}

$^{a, b, c}$: Values with different lower-case letters in the same column differ significantly ($p<0.05$).
X, Y, W, Z: Values with different capital letters in the same row for each analysis differ significantly ($P<0.05$).

3.3. Sensory properties

In yoghurt drink samples, using different raw material formulations had a significant effect on the taste-aroma properties on the 1st, 10th and the 20th day of the storage ($p<0,05$). LNAY received considerably lower points compared to the other samples (Table 4). The difference between taste-aroma scores of the samples was found to be statistically not significant on the 30th day of the storage ($p>0.05$). Also, it was found that storage had no significant effect on the taste-aroma properties of the samples ($p>0.05$). The panelists reported that taste-aroma characteristics of yoghurt drink samples, including LNAY sample, which received the lowest points, were very stable throughout the storage, and no negative developments occurred such as increase in acidity or souring. As a result of ANOVA and Duncan tests, it was found that different raw material composition of the yoghurt drink samples had a significant effect on their consistency properties ($p<0.05$).

Among the yoghurt drink samples, LG19AY received the highest consistency points and much better and more tasteful product compared to the control sample. The difference between general scores of the yoghurt drink samples was found to be statistically not significant at the 30th day of the storage ($p>0.05$). CAY, LAY and LG19AY samples had no significant differences in terms of general sensory analysis scores at the 1st, 10th and 20th days of the storage ($p>0.05$), and LNAY was statistically different than the other three yoghurt drink samples during the storage ($p<0.05$).

Table 4. Sensory evaluation of yoghurt drinks for the individuals with galactosemia.

	Storage (Day)			
	1st	10th	20th	30th
Taste- Aroma				
CAY	6.87±0.17^{b}	6.75±1.06^{ab}	6.80±0.28^{ab}	7.40±0.84^{b}
LAY	6.65±0.21^{b}	7.55±0.07^{b}	6.65±0.49^{ab}	7.01±0.55^{b}
LNAY	4.10±0.14^{a}	5.15±0.49^{a}	4.57±1.80^{a}	5.80±0.28^{a}
LG19AY	7.10±0.14^{b}	8.00±0.00^{b}	7.51±0.12^{b}	7.30±0.98^{b}
Consistency				
CAY	7.02±1.10^{ab}	7.50±0.70^{ab}	7.37±0.32^{b}	7.50±0.14^{b}
LAY	7.35±0.77^{b}	7.95±0.77^{b}	7.64±0.50^{b}	7.60±0.00^{b}
LNAY	4.72±0.67^{a}	6.10±0.14^{a}	5.22±0.88^{a}	5.47±0.38^{a}
LG19AY	7.87±0.17^{b}	8.35±0.21^{b}	8.00±0.56^{b}	8.10±0.14^{b}
General				
CAY	6.75±0.35^{b}	7.05±0.63^{b}	6.80±0.28^{b}	7.50±0.70^{b}
LAY	6.87±0.17^{b}	7.45±0.07^{b}	6.94±0.48^{b}	7.01±0.55^{b}
LNAY	4.52±0.38^{a}	5.60±0.56^{a}	4.71±1.00^{a}	5.88±0.68^{a}
LG19AY	7.20±0.28^{b}	8.25±0.35^{b}	7.80±0.28^{b}	7.76±0.90^{b}

$^{a, b, c}$: Values with different lower-case letters in the same column differ significantly ($p < 0.05$).

4. CONCLUSIONS

Consumption of dairy products by patients with galactosemia in their daily diet leads to consequences physiologically far more different and serious than those in lactose intolerance cases. Therefore, galactosemia patients have to eliminate dairy products from their daily diet completely in order not to experience these serious adverse effects and physiological damages. In our study, it was found that galactose levels in yoghurt drink produced from lactose free milk and infant formula mixtures for patients with galactosemia were lower than the galactose threshold values reported in the referred studies (VARGA *et al*., 2006) Yoghurt drink samples exhibited good acidity development, microbiological content, and the stability of these contents. In our study, LG19AY sample was possibly the most successful sample among the yoghurt drinks fulfilling the aim and purpose of our study. Strawberry flavor fortification, comparing with the previous studies, improved the sensory properties and positive results were obtained in the sensory analysis. However, it is necessary to confirm these results with further studies and subsequently these products can be presented to the consumption of the patients.

ACKNOWLEDGEMENTS

The authors thank the Ege University Scientific Research Fund (Project No: 2009-ZRF-018) Council for financial support to this study. The authors also would like to thank Pinar Sut Inc. and Aromsa Inc. for their lactose free milk and flavor procurements.

REFERENCES

Akalin A. S. and Unal G. 2010. The influence of milk supplementation on the microbiological stability and textural characteristics of fermented milk. Milchwissenschaft 65: 291- 294.

AOAC. 2006. Official Methods of Analysis of AOAC International, 18th ed. Association of Official Analytical Chemists, Arlington, Virginia. USA.

Berry, G. T. 2012. Galactosemia: When is it a newborn screening emergency? Molecular Genetics and Metabolism 106: 7-11).

Bodyfelt F.W., Tıbias J. and Trout G. 1988. The sensory evaluation of dairy products, Van Nostrand Reinhold, New York, USA.

Bracquart P. 1981. An agar medium for the differential enumeration of *Streptococcus thermophilus* and Lactobacillus bulgaricus, Journal of Applied Bacteriology 51:303–305.

Cheng H.F. 2010. Volatile flavor compounds in yogurt: A review. Critical Review Food Science. Nutrition, 50: 938-950.

De Mann J.M. 2013. Principles of Food Chemistry. Springer Inc. ISBN 978-1-4614-6390-0. New York, USA.

Erkaya T., Başlar M., Şengül M. and Ertugay M.F. 2015. Effect of thermosonication on physicochemical, microbiological and sensorial characteristics of ayran during storage, Ultrasonics Sonochemistry 23:406-412.

Grange D.K. 2004. Galactosemia in Encyclopedia of Gastroenterology, Edited by Leonard R. Johnson, University of Tennessee College of Medicine, Memphis, Tennessee, USA.

Hull M.E. 1947, Studies on milk proteins, II. Colorimetric determination of the partial hydrolysis of the proteins in milk, Journal of Dairy Science, 30:881

Kerckhove K.V., Diels M., Vanhaesebrouck S., Luyten K., Pyck N., De Meyer A., Van Driessche M., Robert M., Corthouts K., Caris A., Duchateau E., Dassy M. and Bihet G. 2015. Consensus on the guidelines for the dietary management of classical galactosemia. Clinical Nutrition ESPEN. 10: e1-e4.

Martini C.M., Lerebours E.C., Lin W., Harlander S.D., Berrada M.N., Antoine J.M. and Savaiano D. 1991. Strains and Species of Lactic Acid Bacteria in Fermented Milks : Effect on *in vivo* Lactose Digestion. The American Journal of Clinical Nutrition 54:1041-1046.

Özer D., Akın S. and Özer, B. 2005. Effect of inulin on survival of *Lactobacillus acidophilus* LA-5 and *Bifidobacterium bifidum* BB-02 in acidophilus-bifidus yoghurt. Food Science and Technology International 11:19-24.

Ridel R.K., Leslie N.D. and Gilbert D.L. 2005. An updated review of the long-term neurological effects of galactosemia. Pediatric Neurology 33: 153-161.

Robinson R.K., Tamime A.Y. and Chubb L.W. 1977. Acetaldehyde as an indicator of flavour intensity in yoghurt. Milk Industry 79:4-6

Sahan N. Yasar, K. and Hayaloglu A.A. 2008. Physical, chemical and flavor quality of non-fat yogurt as affected by a β-glucan hydrocolloidal composite during storage. Food Hydrocolloids 22: 1291-1297.

Schweitzer S., Pryzembel H., Ullrich K. and Wendel U. 1998. Empfehlung Der Arbeitsgemeinschaft Für Pädiatrischestoffwechselstörungen (Aps) Zurbehandlung Der Galaktosämie (Recommendations Of Team Of Metabolic Disease In Childhood For Treatment Of Galactosemia). In: Thauer E. and Bake G. (Eds.) Galaktosämie. Jubileumsausgabe Elterninitiative Galaktosämie E. V. (Proceedings Of Parent Initiative Registered Association On Galactosemia), Düsseldorf, 21-24.

Szigeti J. and Krász Á. 1992. Dietetikusigényeketkielégít savanyútejkészítményekel állítása" (Production of fermented dairy products for diet). Tejipar 42:25-29.

Tonguç I.E., Kinik Ö. Kesenkaş H.and Acu M. 2013. Physicochemical, Microbiological and Sensory Characteristics of Using Different Probiotic Fermented Milk. Pakistan Journal of Nutrition 12:549-554.

Turkish Food Codex. 2009. Fermented Dairy Products Communiqué (Communiqué No: 2009/25) Ankara, Turkey.

Varga L., Szieti J. and Csengeri É. 2003. Effect of oligofructose on the microflora of an ABT-type fermented milk during refrigerated storage. Milchwissenschaft 58: 55-58.

Varga Z., Palvolgyi M., Juhasz-Roman M. and Toth-Markus M. 2006. Development of Therapeutic kefir-like products with low galactose content for patients with galactose intolerance. Acta Alimentaria 35:295-304.

Yerlikaya O., Akpınar A., Torunoglu A., Kınık O., Akbulut N. and Uysal, H. 2012. Effect of some prebiotic combination on viability of probiotic bacteria in reconstituted whey and milk beverages. AgroFOOD Industry Hi-Tech, Monographic Supplement Series: Dietary Fibers and Pre/Probiotics 23:27-29.

Yerlikaya O., Ender G., Torunoglu, A. and Akbulut, N. 2013. Production of probiotic milk drink containing *Lactobacillus acidophilus*, *Bifidobacterium animalis* ssp. *lactis* and *Lactobacillus casei*. Agro Food Industry Hi-Tech 22:49-52.

Paper Received January 9, 2017 Accepted July 10, 2017

PAPER

COMPARISON OF BLACK TEA TYPES WITH GRADES AND BLENDS

A. CIFTASLAN[a] **and A.L. INANC***[b]

[a]Department of Food Engineering, Graduate School of Natural and Applied Sciences, KSU, Kahramanmaras, Turkey

[b]Department of Food Engineering, Faculty of Engineering and Architecture., KSU, Kahramanmaras, Turkey

*Corresponding author. Fax: +90 3443002084

E-mail address: linanc@ksu.edu.tr

ABSTRACT

The chemical and sensory properties of conventional and organic black tea kinds (*Camellia sinensis var. sinensis*) regarding of blend and grade were compared. Organic teas were produced from teas harvested from Hemsin region, Rize, Turkey where has a latitude of 41°2'53.53"N and a longitude of 40°53'56.61"E. Conventional black teas were produced from teas harvested from Tirebolu region, Giresun, Turkey where has a latitude of 41°0'26.85"N and a longitude of 38°48'52.54"E. The water extract, cellulose, polyphenol, mineral, and caffeine contents; TF/TR ratio; and a sensory evaluation were used as parameters to compare conventional and organic black tea blends and grades. The polyphenol contents (12.53-8.57%) of conventional teas were higher than that (9.86-7.60%) of organic teas, and the cellulose contents (17.86-13.45%) of organic teas were higher than that (17.55-11.60%) of conventional tea samples. The highest caffeine contents were found in first grades of first blends of tea samples. The amount of caffeine in blend 1 of grade 1 of conventional tea was 2.67% as it was 1.86% for organic tea in the same blend and grade. Regarding the TF/TR ratios and the sensory evaluation scores, both the conventional and organic teas were similar. The grade and blend affected significantly the quality of black tea.

Keywords: black tea, blend, chemical component, grade, sensory

1. INTRODUCTION

Tea (*Camellia sinensis*), which is one of the oldest beverages, is the most consumed, hot or cold, manufactured drink in the world. It is the second most popular non-alcoholic beverage, after water, consumed by approximately half of the world's population. It is available for consumption in different varieties, which are mainly based on the oxidization and fermentation technique used. There are specific climatic requirements for tea crops. Tea can only be grown in tropical and subtropical climates. The tea plant requires temperatures between 10-30°C, an annual rainfall of at least 1250 mm, acidic soils, ideally 0.5-10° slopes and elevations up to 2000 meters. Thus, tea production is geographically limited to a few areas around the world, and the growing conditions are highly sensitive (CAI *et al.*, 2016; CHEN, 2016). The secondary metabolite compounds in plants serve as defense compounds and vary in the amounts depending on the parameters such as environmental conditions, agro-techniques, and producing processes. The amounts of the compounds like polyphenolic catechin compounds in tea plants vary with geographic location, cultivar, herbivory, season, shade, soil, slope, water availability, and management. It can be perceived changes in the amounts of tea functional compounds by their sensory characteristics such as astringency, bitterness or sweetness. thus, tea quality influences the purchasing decisions, farmer livelihoods, and functional benefits derived from crops (AHMED *et al.*, 2014a; AHMED *et al.*, 2014b; AHMED *et al.*, 2012; AHMED *et al.*, 2010; LIN *et al.*, 2003). In general, tea quality is determined via sensory testing and by examining the significant correlations between some of the chemical compounds and the sensory tests. The chemical compounds in tea affect the sensory properties such as color, taste, odor, and flavor in addition to the nutritional and pharmacological benefits of tea (SHEKHAR *et al.*, 2016).

Tea contains many chemical compounds but theaflavin (TF), thearubigin (TR), phenolics, caffeine and minerals are the most important compounds for tea quality. Moreover, the crude fiber content of tea is an important parameter to determine the tea quality (MARBANIANG, 2011), and the presence of water-soluble ingredients in tea is very important for the crude fiber content. The water-soluble substances in tea are flavonols, acids, caffeine, amino acids, carbohydrates and organic acids. In hot water, the low solubility substances are starches, pectins, ashes and pentoses. The insoluble substances are cellulose, lipids, some pigments and volatiles. The fresher the tea leaves are, the higher the water extract is, and this depends on the environmental conditions, ecological impacts and production procedures. Cellulose indirectly affects the tea quality and is an undesirable compound because it reduces the proportions of the other compounds in the total solid. The amount of cellulose in the tea leaves increases as the length of the sprouts increases, which occurs when a standard harvest is not performed (OZDEMIR and KARKACIER, 1997).

Tea leaves contain large quantities of polyphenols, especially catechin, and the catechin amount is related to the black tea quality (OWUOR and OBANDA, 2011). Oxidoreductase enzymes such as polyphenol oxidase (PPO) and peroxidase (PO) interact with the phenolic compounds in tea leaves and react to produce the well-known, golden-yellow color in fermented teas. Golden-yellow theaflavin, a product of the condensation reaction between two molecules of o-quinone (one derived from epicatechin (dihydroxy) and the other derived from epigallocatechin (trihydroxy), is probably generated by PPO due to the exposure of the tea leave surfaces to air. Additionally, thearubigins, which are more intensely colored products with diverse structures, form because of the reactions of o-quinones with amines, phenols, amino acids, peptides, and proteins (MAHANTA and BARUAH, 1992).

The mineral content is 4-5% for fresh tea leaves and 5-6% for processed tea. Mineral substances have an important role in plant physiology and in their chemical and biochemical functions in addition to the growth of the tea plant. Some of the minerals are absorbed by the human body from drinking tea. Minerals are essential for the proper functioning and maintenance of the human body and metabolic events (KACAR, 1997).
The amount of minerals in tea leaf shoots vary depending upon the soil type and husbandry of the bush. Additionally, the genetic characteristics, growing locations, and tea production methods contribute to the quality of black tea. Among the minerals and essential trace elements, Ca, Na, K, Mg, and Mn are present in tea leaves at g/kg levels, and Cr, Fe, Co, Ni, Cu, Zn are present at mg/kg levels (STREET *et al.*, 2006). A previous study reported that there is a wide variation in the percent transfer for the examined elements from the black tea leaves to the tea infusion. The solubilities of Ca and K are the highest among the elements studied. The extraction of trace metals, such as Mn, Zn and Al, is also relatively high. Only Fe is insoluble and remains in the solid particles during beverage preparation (DAMBIEC *et al.*, 2013).
Conventional and organic agriculture are two of the primary cultural agricultures used in the production of food. One of the clearest distinctions between organic tea and conventional tea is that organic tea is grown without the use of chemical fertilizers, pesticides, fungicides, or herbicides. These chemicals have well-documented harmful effects on the environment and farmers and consumers who may ingest the residues. Conventional tea growing methods may maximize production in the short term but with serious environmental consequences and human costs. Additionally, organic farm land is prohibited from being treated with synthetic pesticides and herbicides for at least 3 years prior to harvest (ASAMI *et al.*, 2003).
Camellia plants usually have a rapid growth rate. Typically, they will grow about 30 cm per year until mature, although this does vary depending on their variety and geographical location. When the plant is harvested for tea, the shoot and two to three leaves are harvested every 8 to 10 days. These buds/shoot and leaves are called 'flushes'. A plant will grow a new flush every 7 to 15 days during the growing season. The tea spring leaf tip is valued the most. *Camellia sinensis* usually will produce an abundant crop twice a year, once in the spring and again in the summer. Harvesting can be done every 7 to 15 days during these periods, until the plant no longer produces new growth (DAFF, 2016). In this study, the aim was to investigate effects of blend (blend of tea leave harvested in the first flush period) and grade on the quality of black tea, and to determine chemical and sensory differences among the black teas produced via organic and conventional production techniques depending on blend and grade.

2. MATERIALS AND METHODS

2.1. Materials

The first flush organic and conventional black teas (*Camellia sinensis var. sinensis*) that were harvested at the end of the first growth period during the growing season in 2013 were chosen for this study. Organic black tea was obtained from Hemsin, Rize, Turkey Tea Factory in the Caykur general directorate, and conventional black tea was obtained from the Tirebolu, Giresun, Turkey Factory in the same directorate. The climate in Hemsin (a latitude of 41°2'53.53"N and a longitude of 40°53'56.61"E) is warm and temperate. The rainfall in Hemsin is significant, with precipitation even during the driest month. According to Köppen and Geiger, this climate is classified as Cfb (Oceanic climate). The average annual temperature, rainfall and relative humidity are respectively 12.5°C, 1423

mm and 75% in Hemsin (CLIMATE-DATA 2017a; DISTANCESTO 2017a). The climate in Tirebolu (a latitude of 41°0'26.85"N and a longitude of 38°48'52.54"E) is mild, and generally warm and temperate. The rainfall in Tirebolu is significant, with precipitation even during the driest month. The climate here is classified as Cfa by the Köppen-Geiger system. The average annual temperature, rainfall and relative humidity in Tirebolu are respectively 14.5°C, 1002 mm and 67.5%. The average field slopes and altitues of both regions are 10-20% and 100-300 m (CLIMATE-DATA 2017b; DISTANCESTO 2017b).

2.2. Preparation of the black tea samples

Samples were collected over 30 days from the sorting stages, which are after the drying stage, at the production lines in the factories based on the diameter of the teas. The sorting process was performed at two levels. In the first level, the graded teas were passed through three different sieves with diameters of 1.405, 0.776 and 0.505 mm (12, 20 and 30 mesh), and the teas were coded as grade 1, grade 2 and grade 3. In the second level, the teas that did not pass through the sieves and had diameters of 2.057 and 1.676 mm (8 and 10 mesh) were passed through the sieves (12, 20 and 30 mesh). These teas were coded as grade 4, grade 5 and grade 6. The sorting process was performed five times a day. After the daily sorting process, 100 g of tea were taken for each grade, and a sample of 500 g of tea was taken for one grade in one day. The sorting process was divided into three different periods of 10 consecutive days. These three periods were called blend 1, blend 2 and blend 3. A total of 5,000 g of tea was sampled by mixing the teas of the same grade into one blend. The samples were stored in sealed glass jars in the dark at room temperature until analysis.

2.3. Analysis

All analyses of the tea samples outlined below were replicated three times.

2.3.1. Water extract

The water extract analysis was conducted using the method described by ISO (1994). Distilled boiling water (200 mL) was added to the tea leaf (2±0.001 g) in a balloon and was boiled for 1 h using a reflux condenser. Tea liquor was filtered through cotton wool, and the residue (extract) was washed with distilled water three times. The tea liquor was cooled to room temperature, and the washings were diluted to 200 mL with distilled water. The tea liquor (75 mL) was placed in a weighed evaporating dish and evaporated to dryness over a water bath. The tea residue in the dish was completely dried in a vacuum oven at 103°C for 16 h until the weight of the dish with the residue was constant. The water extract of the black tea was expressed as a percentage of the mass of the dry tea leaf.

2.3.2. Crude fiber content

The tea leaf was ground using a mill and passed through a 1 mm screen. The tea (2±0.001 g) was then weighed into a 1 L conical flask. A 0.255 N sulfuric acid solution (200 mL) was measured at room temperature, boiled, and added to the sample. A reflux condenser was inserted into the neck of the flask, and the solution was boiled gently for 30 min. A Buchner flask with a Hartley funnel and wet filter paper (Whatman No. 541) were used for filtration. After boiling, the acid digest was poured into a shallow layer of hot water in the funnel under gentle suction, and the flask was rinsed with two aliquots of approximately 50 mL of boiling water poured through the filter funnel. Using a dispenser capable of

dispensing 200 mL of hot liquid, the insoluble matter was washed from the filter paper into the original 1 L conical flask using 200 mL of a 0.313 N sodium hydroxide solution and boiled for 30 min. Using boiling water, all the insoluble matter was transferred into a sintered glass crucible (porosity no. 1, 40 mm plate diameter and 70 mL capacity) fitted to the Buchner flask via an adaptor by applying gentle suction. The residue was washed with approximately 50 mL aliquots of boiling water, HCl solution (1%; v/v) and boiling water. Finally, the residue was washed twice with ethanol (95%; v/v) and three times with acetone. The crucible and residue were heated in an oven at 103°C for 2 h. The crucible was cooled in a desiccator, weighed to the nearest 0.001 g, returned to the oven and heated again for 1 h. Finally, the crucible was cooled in a desiccator and weighed. The crude fiber content is expressed as a mass fraction, in percent, of the sample on a dry basis (ISO, 2012a).

2.3.3. Total phenolic content

The extraction tube (10 mL) containing the ground tea leaf (0.2±0.001 g) was placed in a water bath set at 70°C. Hot (70°C) 70% methanol (5 mL) was dispensed into the extraction tube, which was stoppered and mixed on the vortex mixer. The extraction tube was heated in the water bath for 10 min with vortex mixing after 5 and 10 min. The extraction tube was removed from the water bath and allowed to cool to room temperature. The stopper was removed, and the tube was placed in a centrifuge at 3500 r/min for 10 min. The supernatant was carefully decanted into a graduated tube. The extraction steps were repeated, and the extracts were combined, diluted to 10 mL with cold 70 % methanol and mixed. The leaf tea extract (1 mL) was diluted to 1/100 (v/v), transferred into a tube and 5.0 mL of dilute Folin-Ciocalteu phenol reagent was added. Within 3 to 8 min after the addition of the Folin-Ciocalteu phenol reagent, 4.0 mL of a sodium carbonate solution were pipetted into the tube, which was stoppered and mixed. The tube stood at room temperature for 60 min, and the optical densities were measured in 10 mm path length cells against water on a spectrophotometer (UV-160 Shimadzu) set at 765 nm. Gallic acid standard solutions were used for the standard curve. The total phenolic content was calculated as a mass percentage of the dry tea leaf using the following formula (gallic acid equivalent; mg GAE). The concentration of gallic acid was established in mg/ml using the calibration curve (ISO, 2005).

$$w_t=((D_{sample}-D_{intercept})*V_{sample}*d*100)/(S_{std}*m_{sample}*10000*w_{(DM,sample)})$$

where D_{sample} is the optical density obtained for the sample solution; $D_{intercept}$ is the optical density at the point; S_{std} is the slope obtained from the best-fit linear calibration; msample is the mass in grams of the sample; V_{sample} is the sample extraction in ml; d is the dilution factor used prior to the colorimetric determination; $w_{DM,\ sample}$ is the dry matter content expressed as a mass fraction percent.

2.3.4. Caffeine

The tea liquor (50 mL) obtained from the water extract analysis method was poured into a separatory funnel and 5 mL of an ammonia solution (70 g/L) and 50 mL of chloroform were added. After careful mixing, the water phase and the chloroform phase were separated. The water phase was washed twice with chloroform. The chloroform phases passed through a glass cotton filter and were collected in a volumetric flask. The phases were diluted to the mark with chloroform and mixed. Seven different caffeine standard solutions were prepared (0.5, 1, 2, 3, 4, 5 and 8 g/mL) in chloroform. The absorbance of the

samples and the standard caffeine solutions in chloroform were measured against chloroform blank at 276 nm using a UV-visible spectrometer (UV-160 Shimadzu). The caffeine content of the sample is expressed as a mass percent of the dry tea leaf (ISO, 2012b).

2.3.5. Theaflavin (TF) and thearubigin (TR)

The TF and TR analysis was conducted using the method described by Kumar *et al.* (2011). Water (125 mL) was added to 3±0.001 g of ground tea leaf and boiled for 10 min. The black tea extract was obtained by filtering the black tea through a cloth filter. After mixing the extract (10 mL) with ethyl acetate (10 mL), the mixture separated into two liquid phases, the water phase (WP) and the organic phase (OP). Five different solutions, S0, S1, S2, S3 and S4, were prepared from the WP and OP as indicated below.

S0 = OP (10 mL) + 2.5% NaHCO3 (10 mL)
S1 = OP (4 mL) + Methanol (21 mL)
S2 = WP (2 mL) + Distilled water (10 mL) + Methanol (13 mL)
S3 = WP (2 mL) + Oxalic acid (2 mL) + Distilled water (6 mL) + Methanol (15 mL)
S4 = OP (4 mL) of S0 + Methanol (21 mL)

The optical densities of solutions 1, 2, 3 and 4 (E1, E2, E3 and E4) were measured at 380 nm using a spectrophotometer (Optimum-One, Chebios, Roma, Italy). The percentages of TF and TR were calculated using the followings formulas:

$$TF\ (\%)=2.25*E3$$

$$TR\ (\%)=(1.77*E4+E1-E3)*7.06$$

$$TF/TR\ Ratio=(TF(\%))/(TR(\%))$$

2.3.6. Minerals

A standard method (NMKL, 1998) based on atomic absorption spectroscopy was used to determine the mineral content. Pure HNO3 (10 mL) was added to the ground tea leaf (0.2±0.001 g), and the mixture equilibrated for 30 min. After 30 min, the mixture was combusted in a microwave oven (Speedwave Four, BERGHOF, Eningen, Germany) at 190°C. The sample solution was then transferred to a 50 mL volumetric flask and diluted to the mark with ultra-distilled water. A standard solution was prepared for each mineral (Cu, Fe, Zn, Mn, Mg, Ca, K). The samples were analyzed using an atomic absorption spectrometer with an inserted hollow cathode lamp (GBC, Avanta P, Australia). The mineral content in the samples is expressed in g/kg.

2.3.7. Sensory test

The tea liquor used in the sensory test was prepared by infusing the tea leaf according to ISO (1980). The tea was weighed (2.8±0.05 g) and transferred to a pot. The pot was filled with approximately 140 mL of fresh, boiling water. The tea was allowed to brew for 6 min, and the liquid was poured through the serrations into a bowl to separate the liquid from the solid tea. The lid was removed and inverted, and the infused leaf was placed on the inverted lid to allow the infused tea leaf to be inspected. Black teas were assessed using sensory test method of TS EN ISO 13299 (TSE, 2016). Eight tea sensory experts (four males

and four females, aged 25-40 years) from the Sensory test and Chemical Analysis Laboratory at Caykur Fabric, Rize, Turkey served as the panel. The experts completed 200 h of sensory testing for all samples. The experts evaluated the sensory attributes of the samples by using the sensory evaluation chart from the Turkish Standard Institute (Table 1). The sensory evaluation chart includes 5 disciplines (properties) having different maximum point, totally 100 points. The experts gave a point for each discipline between 0-its maximum point. It is rated as good tea if a tea sample collects 50 points or above on the basis of the total 100 points.

Table 1. Sensory evaluation chart of Turkish black tea.

Sensory properties of black tea	Description	Point
Appearance of the dried tea leaf	should be good appearance, black or dark copper color, and no fiber and stalk	10
Color of tea liquor	should be bright dark red or reddish color. should not be dull, fuzzy and a residual or brownish color	25
Astringency and body	should be a lively puckery sensation on the tongue and gums, and also the good impression of a tea's weight in the mouth, its viscosity and mouth feel	30
Color and odor of the infused leaf	should be bright copper red color, no excess green leaf and no brownish color	15
Aroma of liquor	should be unique and pleasant for good tea	20
TOTAL		100

2.3.8 Statistical analysis

Two types of tea, three blends and six grades were compared. Differences were considered to be significant at $p \leq 0.05$. The data, collected from organic and conventional black tea samples in triplicate, were subjected to a three-way analysis of variance (ANOVA) using the SPSS software (SPSS for Win, Release 19.0, 2012). The means were compared using Duncan's multiple range test for multiple comparisons, and the "Student" T-test was applied to the two sets of data that were significantly different.

3. RESULTS AND DISCUSSION

3.1. Water soluble extract

The water-soluble extract amounts and their statistical results for the samples are presented in Fig. 1. The extract amounts for the conventional teas were in the range of 30.83-35.69% and 31.73-35.26% for the organic teas depending on the grades and blends. In the comparison of teas in the same grade and blend, the extracts amount in the conventional teas were higher than those in the organic teas. The highest values were from the first blends, and the lowest values were from the third blends in both tea blends. The extract amount decreased from grade 1 to grade 6 for all tea blends. The triplet interaction among the tea type-grade-blend was not significant ($p>0.05$), but the double interaction among them was very significant ($p\leq0.05$). The differences between grade 3 and grade 4 in

blend 2 for conventional tea and between grade 4 and grade 5 in blend 1 and grade 2 and grade 3 in blend 3 for organic tea were less.
Additionally, compared to the blends, there was no difference between grade 2 and grade 3 in blend 3 of the conventional tea, but this was not seen in the organic tea. The extract amounts for the tea leaves at the beginning of the first flush period are higher than that seen in the other periods. A difference among the tea extracts was not observed for the mid- and end-first flush periods. It was reported that the maximum extract amount for black tea produced in Turkey is from the first harvest season, and the second and third harvest season have less. Additionally, it was reported that the extract amount is higher in fresh tea leaves. Similarly, the extract amounts in the tea produced by different methods decreased from grade 1 to grade 6 (GOKALP *et al.*, 1991; KACAR, 1997).

3.2. Crude fiber content

The crude fiber contents of the conventional teas were in the range of 11.60 to 17.55% and 13.45 to 17.86% for the organic teas with respect to the grades and blends (Fig. 2). The crude fiber contents of almost all the organic teas were higher than that of the conventional teas with the same grade and blend.
The highest crude fiber contents among the blends of both tea types were found in the third blend, and the lowest values were in the first blend. The values increase from the first grade to the final grade, which is the inverse of the extract behavior. The contents of grade 2 and grade 3 teas in blend 2 of the conventional samples were very close, and the contents of grade 1 and grade 2 teas in blend 3 of the organic samples were almost the same. Otherwise, the contents of the tea types with the same grade and blend were different ($p \leq 0.05$). The crude fiber contents in the study were similar to those noted by VENKATESAN and GANAPATHY (2004) for Indian teas.

3.3. Total phenolic content and theaflavin (TF)/thearubigin (TR) ratio

The total phenolic content of the tea samples and the statistical results are given in Fig 3a. The values in the conventional teas were higher than that in the organic teas compared to the polyphenols in the two tea types with the same grade and blend.
The highest and lowest polyphenol contents among the blends in the conventional tea were in blend 1 and blend 3 and in blend 2 and blend 3 in the organic tea. Neither a negative or positive trend was found in the comparison of the grades. The contents of the second, third, fifth and sixth grade teas in blend 1 of the conventional samples and in grade 2, 3 and 5 teas in blend 1 of the organic samples were close to each other. Compared to the blends of one grade, there was very little difference between blend 1 and blend 2 teas in grade 2 of the conventional sample and between blend 1 and blend 2 teas in grade 4 of the organic tea ($p \leq 0.05$). The values (3.79-8.36%) of the tea samples reported by OZDEMIR *et al.* (2008) were lower than those in both the conventional and organic samples.
The TF/TR ratios are shown in Fig 3b. In the conventional teas, the TF/TR ratios of the blends were identified in the range from 0.037 to 0.040 and from 0.034 to 0.044 for the grades. In the organic teas, the ratios were 0.036 to 0.040 for the blends and 0.032 to 0.043 for the grades. The lowest TF/TR was found in blend 3 in the conventional teas, but the ratios in blend 1 and 2 were very close. The lowest and the highest TF/TR ratios were in blend 2 and 1 in the organic teas. An increasing or decreasing trend was not found for the TF/TR ratios of the grades from the blends for all the tea samples.

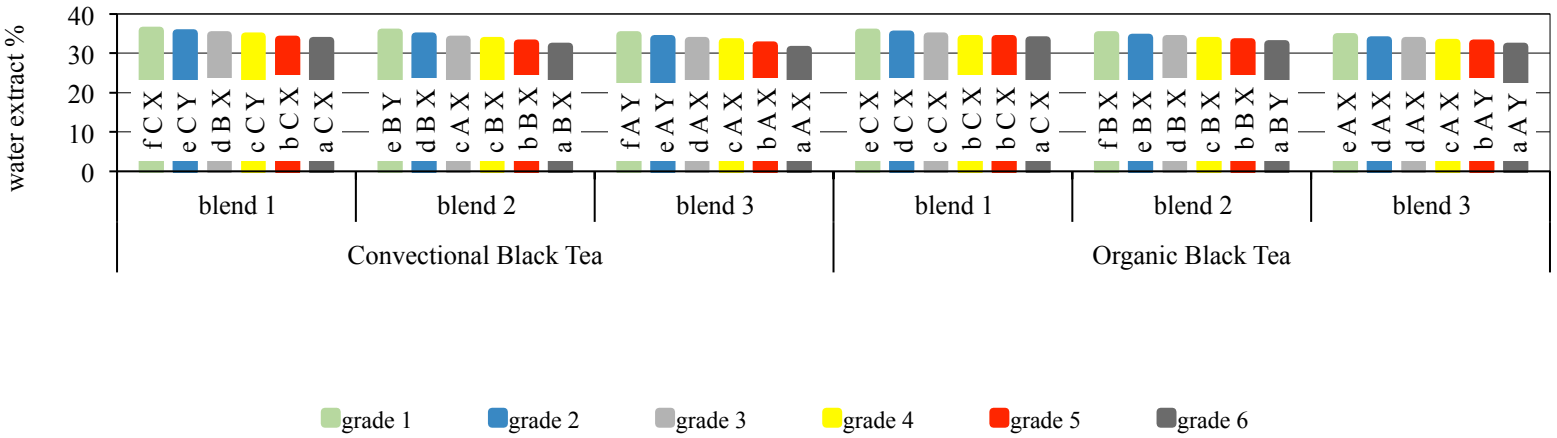


Figure 1. The water soluble extract amounts and their statistical results for the teas.
Values followed by the same letter are not significantly different at the level of 5% (series 'a-f' for grades in a blend of a tea type, series 'A-C' for the same grades in blends of a tea kinds, and series 'X-Y' for teas in the same blend and the same grade).

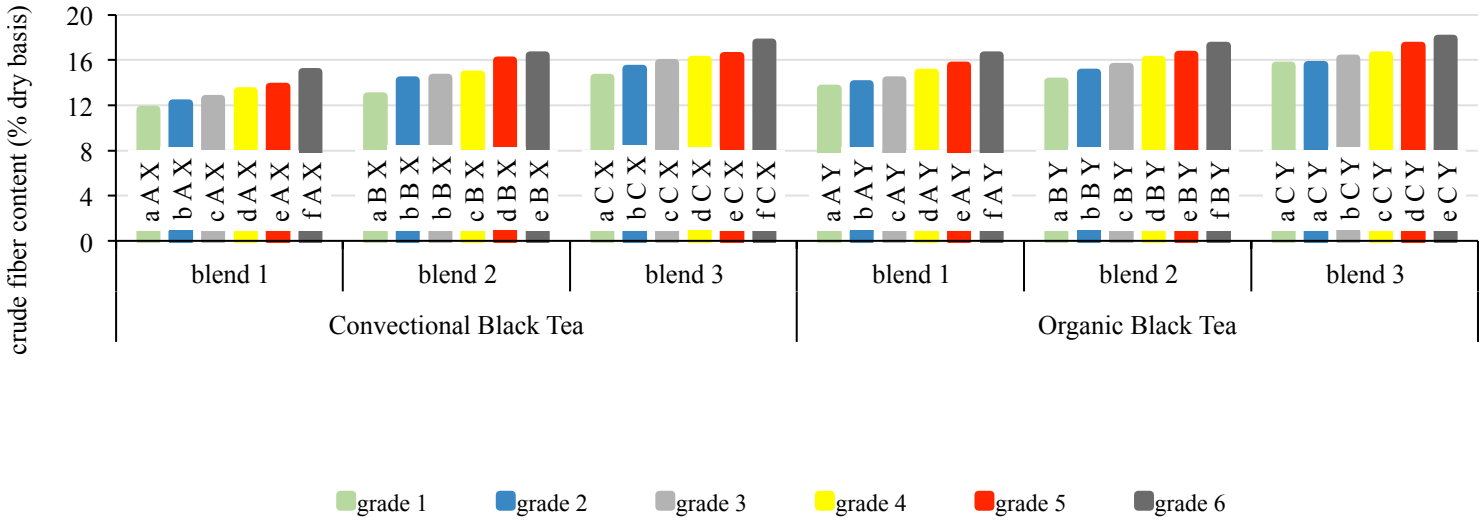


Figure 2. The crude fiber contents of the teas.
Values followed by the same letter are not significantly different at the level of 5% (series 'a-f' for grades in a blend of a tea type, series 'A-C' for the same grades in blends of a tea kinds, and series 'X-Y' for teas in the same blend and the same grade).

fig. 3a

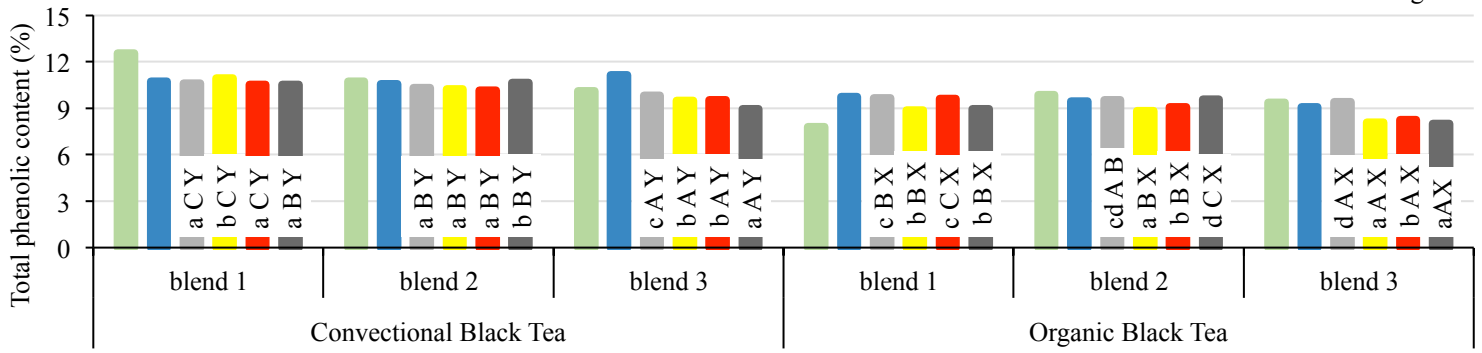


fig. 3b

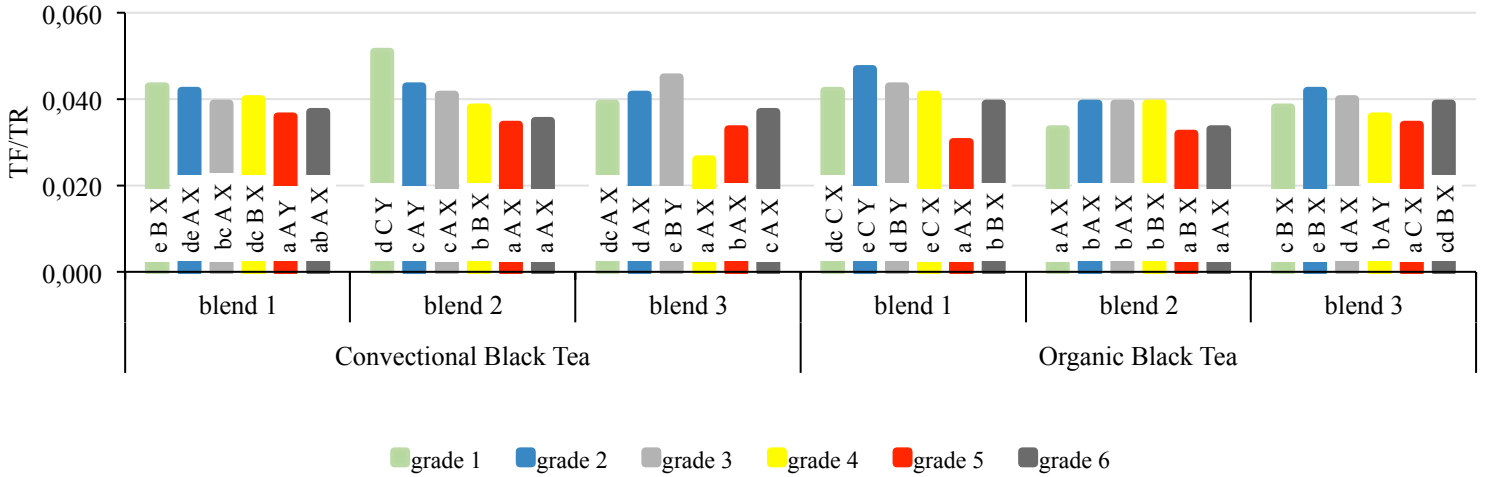


Figure 3. Total phenolic content and theaflavin (TF)/thearubigin (TR) ratio of the teas.
Values followed by the same letter are not significantly different at the level of 5% (series 'a-f' for grades in a blend of a tea type, series 'A-C' for the same grades in blends of a tea kinds, and series 'X-Y' for teas in the same blend and the same grade).

For the statistical evaluation of the tea types at the 5% level, the difference among the blends of grade 2, 5 and 6 in the conventional teas was less, and there was a similarity between blend 2 and blend 3 in grade 3 and blend 1 and blend 3 in grade 6 for the organic teas (Fig. 3b). A decrease in the TF/TR ratio was observed from the beginning of the flush period until the end. The reason for this could be the aging of the tea leaves and a decrease in the oxidative compounds as the flush period continues (GOKALP *et al.*, 1991). OZDEMIR and KARKACIER (1997) reported that black and green teas had a mean TF/TR ratio of 0.032. The TF and TR contents and their ratio are components of the tea quality index (YAO *et al.*, 2006). KUMAR *et al. (*2011) reported that the TF/TR ratio should be in the range of 1:10-1:12 to achieve a taste-water extract balance for a quality tea.

3.4. Caffeine

The ranges and statistical results for the caffeine content in dry tea samples are shown in Fig 4. The results show that the caffeine content in conventional teas was in the range of 2.31-2.67% while organic teas contained 1.50-1.89% caffeine.
The caffeine content in conventional tea was higher than that in organic tea for the same blend and grade. No relationship was found between the caffeine values of the tea types depending on the blend and grade. No similarity was found among tea types belonging to the same blend and grade ($p \leq 0.05$). One of the reasons that conventional teas contain high caffeine and water extract amounts is the usage of nitrogen fertilizer (CHEN *et al.*, 2015). OZDEMIR *et al. (*2008) reported that the caffeine content in black teas was in the range of 1-5% and 1.5-2.49% in seven different tea types within a third flush period. The results in the study are similar to the results (2.21-2.80%) reported by KHOKAR and MAGNUSDOTTIR (2002).

3.5. Mineral elements

The ranges and statistical evaluations of the total element contents in the tea samples are summarized in Table 2. The mean concentrations of the elements in both tea leaf types differed significantly with the grades and blends ($p \leq 0.05$).

Copper (Cu): The Cu concentrations in the organic teas were higher than those in the conventional teas for the same blend and grade. The lowest and highest Cu concentrations were found in blend 1 and blend 2, respectively, in the conventional teas. The lowest Cu concentration was found in blend 3 among the organic tea blends, the Cu values in blend 1 and blend 2 were very close. During the black tea manufacturing process, one of the important chemical changes in the leaves is the oxidation of polyphenols via polyphenol oxidases. A polyphenol oxidase is a tetramer that contains four atoms of copper per molecule (Sullivan, 2015). Therefore, the copper content may vary in blends. MARBANIANG *et al. (*2011) reported that the Cu content varied from 0.072 to 0.105 g/kg. However, STREET *et al. (*2006) reported that different black teas sold in the Czech Republic contained 0.103 to 0.405 g/kg Cu, and these values are similar to the values in the present work.

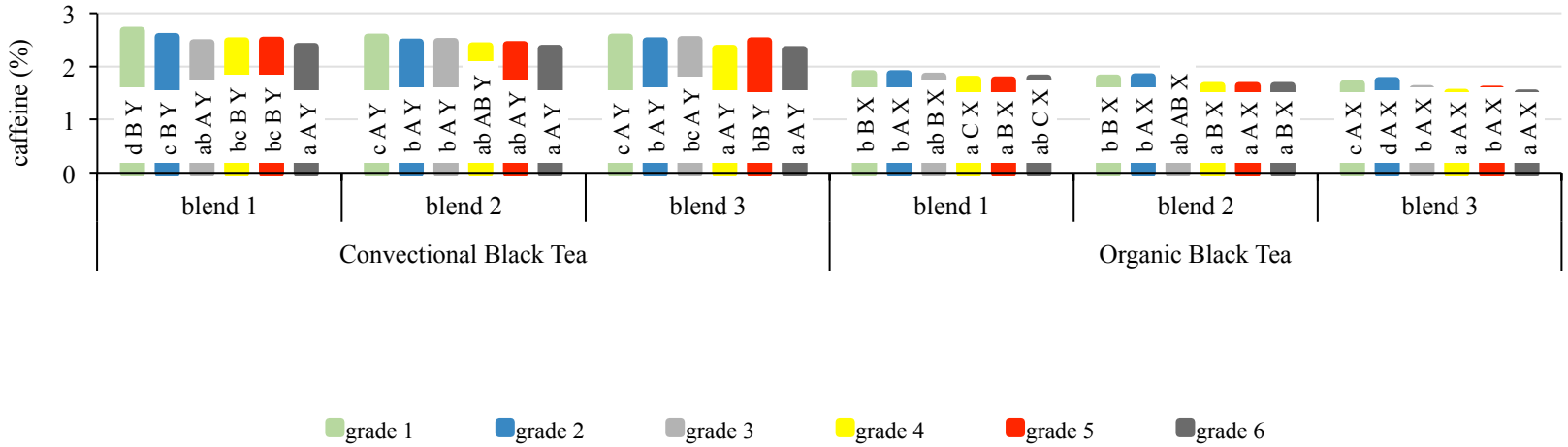


Figure 4. the caffeine content in dry tea samples
Values followed by the same letter are not significantly different at the level of 5% (series 'a-f' for grades in a blend of a tea type, series 'A-C' for the same grades in blends of a tea kinds, and series 'X-Y' for teas in the same blend and the same grade).

Iron (Fe): The Fe concentrations were in the range of 0.145-0.377 g/kg in the conventional teas and 0.049-0.223 g/kg in the organic teas. The iron amounts in the organic teas were higher than those in the conventional teas for the same blend and grade. While the lowest iron values were found in the grades of the third blend for the conventional tea types, the values for the grades of blend 2 and 3 were close. In the organic tea types, the highest values were for the grades of blend 1, and the lowest values were for the grades of blend 3.

Table 2. Mineral contents in the tea samples.

Mineral	Blend	Grade											
		1		2		3		4		5		6	
Na	C1	0.055	cBY	0.047	bBX	0.047	bBY	0.059	dCY	0.046	aBX	0.047	bAX
	C2	0.056	eBY	0.058	fCY	0.045	aAY	0.048	dAY	0.046	bCX	0.047	cAY
	C3	0.045	bAY	0.047	cAY	0.052	eCY	0.050	dBY	0.043	aAX	0.057	fBY
	O1	0.050	eCX	0.048	dCX	0.041	bCX	0.038	aBX	0.046	cAX	0.047	cBX
	O2	0.046	dBX	0.042	cBX	0.037	aAX	0.036	aAX	0.048	eBY	0.038	bAX
	O3	0.038	bAX	0.036	aAX	0.038	bBX	0.042	cCX	0.060	eCY	0.054	dCX
K	C1	10.88	fCX	10.759	eCX	10.691	dCX	10.496	cCX	10.320	aCX	10.377	bCX
	C2	10.70	fBX	9.82	cBX	9.914	dBX	9.964	eBX	9.408	aAX	9.797	bBX
	C3	9.79	eAX	9.39	cAX	9.869	fAX	9.127	bAX	9.641	dBX	8.916	aAX
	O1	14.00	fBY	13.449	eCY	13.394	dBY	13.091	bBY	13.200	cBY	13.038	aBY
	O2	14.00	eBY	13.368	bBY	13.525	cCY	13.707	dCY	13.222	aCY	14.213	fCY
	O3	12.63	fAY	12.222	dAY	11.629	bAY	12.334	eAY	11.925	cAY	11.510	aAY
Ca	C1	4.168	bAX	4.097	aAX	4.244	cAX	4.307	dBX	4.312	dBX	4.333	eAX
	C2	4.310	cBX	4.316	cBX	4.350	eBX	4.220	aAX	4.231	bAX	4.333	dAX
	C3	4.434	bCX	4.568	eCX	4.379	aCX	4.458	cCX	4.559	eCX	4.506	dBX
	O1	4.691	bAY	4.619	aBY	4.774	dBY	4.809	fBY	4.724	cAY	4.789	eAY
	O2	4.871	bcBY	4.864	bCY	4.796	aCY	4.868	bCY	4.875	cBY	4.925	dCY
	O3	4.880	dBY	4.561	aAX	4.739	bAY	4.553	aAY	4.944	eCY	4.818	bCY
Mg	C1	1.220	dAX	1.097	aAX	1.220	dAX	1.219	dAX	1.167	cAX	1.126	bAX
	C2	1.339	eBX	1.277	cBX	1.265	bBX	1.309	dBX	1.195	aBX	1.192	aBX
	C3	1.627	dCX	1.659	eCX	1.602	cCX	1.714	fCX	1.596	bCX	1.588	aCX
	O1	1.226	bAY	1.168	aAY	1.241	cAY	1.259	dAY	1.265	dAY	1.231	bAY
	O2	1.388	bBY	1.438	dBY	1.398	cBY	1.473	eBY	1.367	aBY	1.534	fBY
	O3	1.842	eCY	1.711	cCY	1.656	bCY	1.723	dCY	1.717	cdCY	1.624	aCY

Table 2. Continues.

Cu	C1	0.015	dAX	0.014	aAX	0.015	dAX	0.015	cAX	0.015	dAX	0.014	bAX
	C2	0.017	cCX	0.017	cCX	0.017	cBX	0.015	bBX	0.015	bAX	0.015	aBX
	C3	0.016	cBX	0.015	aBX	0.015	aAX	0.015	aBX	0.016	bBX	0.016	bCX
	O1	0.019	cBY	0.018	bBY	0.018	bBY	0.018	aBY	0.018	bBY	0.018	bBY
	O2	0.019	dCY	0.019	cCY	0.018	bBY	0.018	aBY	0.018	aAY	0.018	bBY
	O3	0.018	cAY	0.017	bAY	0.017	bAY	0.017	bAY	0.018	cAY	0.017	aAY
Zn	C1	0.022	bcdCY	0.024	dBY	0.023	cdBY	0.022	bcCY	0.021	bCX	0.018	aCX
	C2	0.020	cBXX	0.017	bAX	0.016	abAX	0.015	aBX	0.016	abBX	0.016	abBX
	C3	0.017	dAY	0.017	dAY	0.016	cAY	0.013	bAX	0.012	bAX	0.011	aAX
	O1	0.020	bBX	0.018	aBX	0.020	bCX	0.019	bCX	0.021	cBX	0.020	bCX
	O2	0.020	dBX	0.019	cCY	0.018	bcBY	0.018	bcBY	0.016	aAX	0.017	bBX
	O3	0.014	dAX	0.013	cAX	0.012	aAX	0.013	bAX	0.015	eAY	0.015	fAY
Fe	C1	0.327	fAY	0.230	dAY	0.236	eCY	0.209	cCY	0.203	bCY	0.177	aBY
	C2	0.362	eBY	0.308	dBY	0.206	cBY	0.182	bAY	0.179	bBY	0.161	aAY
	C3	0.377	fCY	0.236	eAY	0.167	bAY	0.202	dBY	0.145	aAY	0.177	cBY
	O1	0.166	dBX	0.122	cAX	0.163	dCX	0.091	bBX	0.0	aCX	0.125	cCX
	O2	0.223	eCX	0.137	dCX	0.093	cAX	0.062	bAX	0.0	aAX	0.060	bBX
	O3	0.155	eAX	0.130	dBX	0.098	cBX	0.098	cCX	0.0	bBX	0.049	aAX
Mn	C1	1.049	fAY	0.890	aAX	0.947	cAY	0.991	eAY	0.9	dAX	0.924	bAX
	C2	1.159	eBY	1.061	cBX	1.040	aBY	1.140	dBY	1.0	aBY	1.054	bBX
	C3	1.324	dCX	1.170	aCX	1.164	aCX	1.170	aCX	1.2	cCX	1.187	bCX
	O1111	0.986	fAX	0.908	bAY	0.900	aAX	0.978	eAX	0.9	dAY	0.922	cAX
	O2	1.093	eBX	1.086	dBY	1.012	bBX	1.057	cBX	0.9	aAX	1.126	fBY
	O3	1.346	fCY	1.246	bCY	1.258	cCY	1.294	eCY	1.2	dBY	1.193	aCY

Values followed by the same letter are not significantly different at the level of 5% (series 'a-f' for grades in a blend of a tea type, series 'A-C' for the same grades in blends of a tea kinds, and series 'X-Y' for teas in the same blend and the same grade).
O: organic blend, C: convectional blend.

When comparing the Fe values for the grades belonging to blends, a decrease in Fe from grade 1 through grade 6 was observed. The differences between grade 4 and grade 5 in blend 2 of the conventional tea, and the differences between grade 1 and grade 3 in blend 1, grade 4 and grade 6 in blend 2 and grade 3 and grade 4 in blend 3 of the organic teas were very low ($p>0.05$). No such similarity was found among the blends of the organic teas, and the difference between blend 1 and blend 3 in grade 2 and grade 6 of the conventional teas was very low. However, the tea types with the same grade and blend had significantly different values ($p\leq 0.05$) TASCIOGLU and KOK (1998) reported that

seven different teas produced in Turkey contained 0.130-0.171 g/kg Fe, and AKSUNER *et al.* (2012) found 0.235 g/kg Fe in black tea.

Zinc (Zn): The Zn concentrations of the conventional teas were higher than that in the organic teas for the same blend and grade. The highest Zn concentration among the blends was in the first blend for both tea types, and the lowest was in the third blends.
A linear increase or decrease in the Zn concentration was not seen among the grades belonging to the blends of teas. There was a small difference among the Zn concentration for grades 1, 2, 3, 4 and 5 in blend 1 of the conventional tea and for grades 1, 3, 4 and 6 in blend 1 of the organic tea. Moreover, there were small differences between blend 2 and 3 in grade 2 of the conventional tea and between blend 1 and 2 in grade 1 and blend 2 and 3 in grade 5 of the organic tea ($p \leq 0.05$). The Zn concentrations in 10 commercial Turkish black blend teas in a previous work (ARSLAN and TOGRUL, 1995) were found in the range of 0.033-0.052 g/kg. The Zn concentration in fresh cells is higher than that found in aged tea plant cells (KACAR, 1997).

Sodium (Na): The Na concentrations in conventional and organic teas were determined to be in the range of 0.043-0.060 and 0.036-0.059 g/kg, respectively. The Na concentration among grades 6, 2 and 3 in blend 1 of the conventional teas were close to each other ($p \leq 0.05$). A similar situation existed between blend 1 and blend 2 for grade 1 of the conventional teas. However, this was not found for the organic teas. The Na concentrations in teas produced in different regions of China were 0.026-0.079 g/kg (ZHANG *et al.*, 2011), and McKENZIE *et al.* (2010) reported concentration in the range of 0.011-0.86 g/kg.
Potassium (K): K was the most abundant macro-mineral in all the tea samples analyzed. The K values in conventional teas and organic teas were in the range of 8.916-10.886 g/kg and 11.510-14.213 g/kg, respectively, depending on the blends and grades. Interaction among the tea type-blend-grade was found to be significant according to the results of the Anova of the K values ($p \leq 0.05$). In organic tea, there was an important difference among the blends except for blend 1 and 2 in grade 1, but all the K values were significantly different in the blends of the conventional teas. The present results correspond to the data of McKENZIE *et al.* (2010).

Calcium (Ca): The Ca concentrations in the conventional and organic teas were in the range of 4.097-4.568 and 4.553-4.944 g/kg, respectively. The Ca concentrations in the organic teas were higher than those in the conventional teas for the same blend and grade. The highest Ca concentration among the blends in the conventional teas was in blend 3, and the lowest concentration was in blend 1. The highest and lowest Ca concentrations in the blends of organic teas were in blend 2 and blend 1, respectively ($p \leq 0.05$). The Ca values in a study by MALIK *et al.* (2008) were 4.33-6.68 g/kg, but PEREIRA *et al.* (2006) reported values in the range of 3.13-9.72 g/kg.

Manganese (Mn): The maximum and minimum values for Mn were in the third blends and first blends of the conventional and organic teas, respectively. A linear relationship between the samples was not observed when comparing the grades of the blends of the teas. The differences between the samples were significant, except for the relationship among grades 2, 3 and 4 of blend 3 in the conventional tea ($p \leq 0.05$). The Mn values in the study are similar to the values in studies by OZDEMIR *et al.* (1999), NARIN *et al.* (2004), PEREIRA *et al.* (2006), and MEHRA and BAKER (2007).

Magnesium (Mg): The magnesium concentration in all the tea samples varied in the range of 1.097-1.842 g/kg. The Mg concentrations of the organic teas were higher than those in the conventional teas for the same blend and grade. It was determined that the triple interaction among the tea samples was significant at the 5% level. The Mg values in a study (HORUZ and KORKMAZ, 2006) were found to be 3.3 g/kg for the first harvest tea, 4.7 g/kg for the second harvest tea and 3.9 g/kg for the third harvest tea.

3.6. Sensory test

Sensory evaluation of tea types is presented in Fig. 5.
The conventional teas collected 76-89 points from the panelists. In the evaluation of the conventional tea blends, the mean points for blend 1, 2 and 3 were 85, 83.16 and 81.16, respectively. The evaluation points for the grades between 1 and 6 were in the range of 87-79. Otherwise, the organic teas collected 75-90 points, and the points for blends 1, 2 and 3 of the organic teas were 86.66, 84.50 and 80.83, respectively. The points for the grades were in the range of 88.33-80.00. The evaluation points for the organic teas were higher than those of the conventional teas depending on the blends and grades. Compared to the grades of blends in both tea types, a decreasing trend in the points from grade 1 to grade 6 was observed. The triplet interaction among the tea type-grade-blend was not significant ($p>0.05$), but the two-way interactions between the tea kind-grade and grade-blend were significant ($p\leq 0.05$). there is a reverse relation between the score of overall acceptability and total catechins amounts (XU *et al.*, 2017). Besides A high amounts of minerals, especially calcium and magnesium (MOSSION *et al.*, 2008; ANANINGSIH *et al.*, 2013) such as high pH (ZHOU *et al.*, 2009) influence the extraction yield and stability of catechins and other chemicals in tea infusions. Therefore, these chemicals may change sensory quality of tea. In a study investigating the relationship between theaflavin and tea quality, it was reported that the scores should be among 18.2-78 for good quality teas and 14.4-53 for poor quality teas (WRIGHT *et al.*, 2002). The astringency of tea infusions increases with increasing Ca^{2+} ion while bitterness and umami intensity decreased (YIN et al 2014). XU *et al.* (2017) reported that total scores (overall acceptability) for different taste attributes, including bitter, astringent and umami tastes of black tea brewing with different water types were in range of 7.6-6.4. Another study reported that the sensory property of organic food was better than that of conventional food. It was stated that the shelf-life of organic food was too long, and there was a good correlation between a low nitrate level and good taste perception (OCO, 2016).

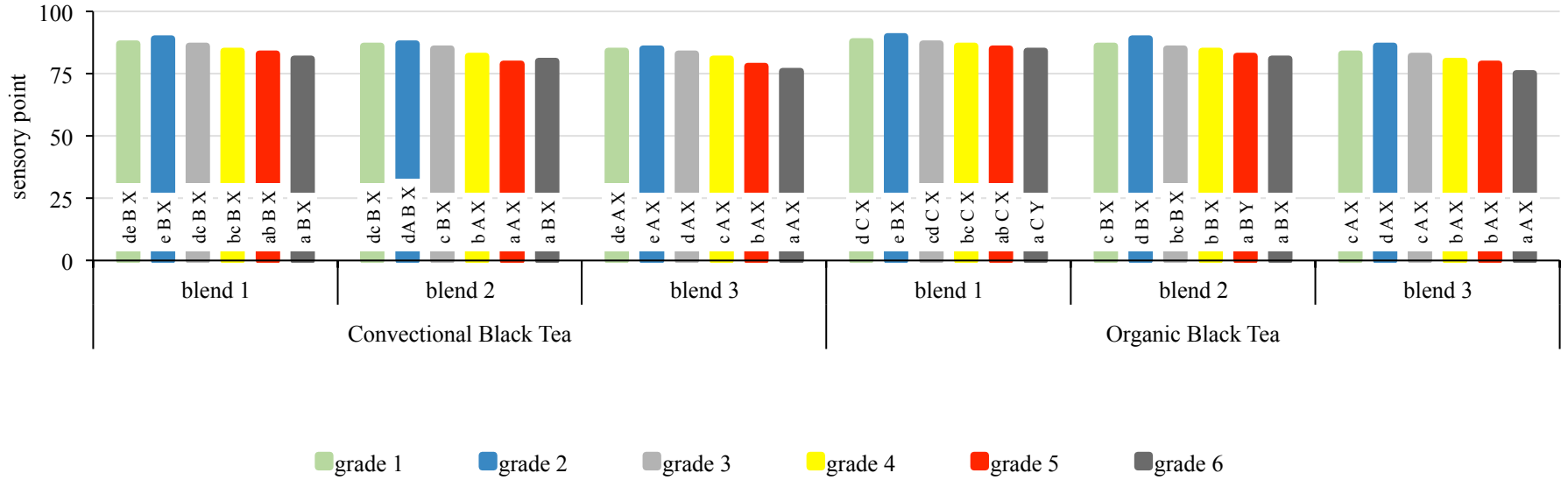


Figure 5. Sensory evaluation of tea types.
Values followed by the same letter are not significantly different at the level of 5% (series 'a-f' for grades in a blend of a tea type, series 'A-C' for the same grades in blends of a tea kinds, and series 'X-Y' for teas in the same blend and the same grade).

4. CONCLUSIONS

Some chemicals and sensory properties of conventional and organic black Turkish teas were investigated according to their grades and blends. The extract, polyphenol and caffeine contents in both teas decreased from blend 1 to blend 3, and the cellulose contents increased from blend 1 to blend 3. Similar trends were observed for the grades.
The extract and TF/TR values were close to each other among the tea types. The cellulose values in organic teas and caffeine and polyphenol values in conventional teas were higher than those of the other tea type. For the TF/TR ratios, there was not a linear increase or decrease among the blends and grades. Whereas the organic black tea was rich in Cu, K, Ca, Mn and Mg, the conventional black tea was rich in Fe, Zn and Na. In the sensory evaluation of the teas, the sensory scores of the conventional and organic black Turkish teas were close, but the scores of the blends in both tea types decreased from blend 1 through blend 3 and there was no linear relationship among the grades.

REFERENCES

Ahmed S, Orians C.M, Griffin T.S, Buckley S., Unachukwu U., Stratton A.E, Stepp J.R, Robbat A.J., Cash S. and Kennelly E. 2014a. Effects of water availability and pest pressures on tea (*camellia sinensis*) growth and functional quality. Aob Plants 6:1.

Ahmed S., Peters C.M, Chunlin L., Meyer R., Unachukwu U., Litt A., Kennelly E. and Stepp J.R. 2012. Biodiversity and phytochemical quality in indigenous and state-supported tea management systems of Yunnan, China. Conservation Letters 5 (6):28.

Ahmed S., Stepp J.R., Orians C., Griffin T., Matyas C., Robbat A., Cash S., Xue D., Long C., Unachukwu U., Buckley S., Small D. and Kennelly E. 2014b. Effects of Extreme Climate Events on Tea (*Camellia sinensis*) Functional Quality Validate Indigenous Farmer Knowledge and Sensory Preferences in Tropical China. Plos One 9 (10):1.

Ahmed S., Unachukwu U., Stepp J.R., Peters C.M., Long C. and Kennelly E. 2010. Pu-erh tea tasting in Yunnan, China:correlation of drinkers' perceptions to phytochemistry. J. Ethnopharmacol 132:176.

Aksuner N., Henden E., Aker Z., Engin E. and Satik S. 2012. Determination of essential and non-essential elements in various tea leaves and tea infusions consumed in Turkey. Food Additives and Contaminants 5:126.

Ananingsih V.K., Sharma A. and Zhou W. 2013. Green tea catechins during food processing and storage: A review on stability and detection. Food Research International 50(2):469.

Arslan N. and Togrul H. 1995. Turk caylarinda kalite parametreleri ve mineral maddelerin farkli demleme kosullarinda deme gecme miktarlari. Gida 20:179.

Asami D.K., Hong Y., Barrett D.M. and Mitchell A.E. 2003. Comparison of the total phenolic and ascorbic acid content of freeze-dried and air-dried marionberry strawberry and corn grown using conventional organic and sustainable agricultural practices. J. Agric. Food Chem. 51:1237.

Cai H., Zhu X., Peng C., Xu W., Li D., Wang Y., Fang S., Li Y., Hu S. and Wan X. 2016. Critical factors determining fluoride concentration in tea leaves produced from a hui province China. Ecotoxicology and Environmental Safety 131:14.

Chen P., Lin S., Liu C., Su Y., Cheng H., Shiau J. and Chen I. 2015. Correlation between nitrogen application to tea flushes and quality of green and black teas. Scientia Horticulturae 181:102.

Chen S., Li J., Lu D. and Zhang Y. 2016. Dual extraction based on solid phase extraction and solidified floating organic drop microextraction for speciation of arsenic and its distribution in tea leaves and tea infusion by electrothermal vaporization ICP-MS. Food Chemistry 211:741.

Climate-data 2017a. Climate: Hemşin, https://en.climate-data.org/location/30519/. Accessed June 2017.

Climate-data 2017b. Climate: Tirebolu, https://en.climate-data.org/location/8529/. Accessed June 2017.

DAFF. 2016. Black Tea Production guideline, The Department of Agriculture, Forestry and Fisheries. http://www.daff.gov.za/Daffweb3/Portals/0/Brochures%20 and %20 Production %20 guidelines/Black%20tea%20guideline.pdf. Accessed June 2017.

Dambiec M., Ska L.P. and Klink A. 2013. Levels of essential and non-essential elements in black teas commercialized in Poland and their transfer to tea infusion. J. Food Composition and Analysis 31:62.

Distancesto 2017a. Hemşin Latitude and Longitude, https://www.distancesto.com/coordinates/tr/hemsin-latitude-longitude/history/116016.html. Accessed June 2017.

Distancesto 2017b. Tirebolu Latitude and Longitude, https://www.distancesto.com/coordinates/tr/tirebolu-latitude-longitude/history/99483.html. Accessed June 2017.

Food and Agriculture Organization of the United Nations (FAO). 2016. http://faostat3.fao.org/download/Q/QC/E/. Accessed 15 06 2016.

Gokalp H.Y., Nas S. and Ozdemir F. 1991. Birinci surgun donemi caylardan Ortodoks metotla uretilen farkli sinif siyah caylarin bazi kimyasal kalitatif ozellikleri. Ekonomik ve Teknik Dergi Standard 30:18.

Horuz A. and Korkmaz A. 2006. Farklı surgun donemlerinde hasat edilen cayin verimi azot ve mineral madde kompozisyonu. OMU Ziraat Fakultesi Dergisi 21:49.

ISO. 1980. ISO 3103-Tea-Preparation of liquor for use in sensory tests method. International Organization for Standardization Geneva, Switzerland.

ISO. 1994. ISO 9768:1994-Tea-Determination of water extract method. The International Organization for Standardization, Geneva, Switzerland.

ISO. 2005. ISO14502-1-Content of total polyphenols in tea-Colorimetric method using Folin-Ciocalteu reagent method. The International Organization for Standardization, Geneva, Switzerland.

ISO. 2012a. IS16041:2012-Tea-determination of crude fibre content method. The International Organization for Standardization, Bureau of Indian Standards, New Delphi, Indian.

ISO. 2012b. IS16039-coffee-determination of caffeine content method reference method. The International Organization for Standardization, Bureau of Indian Standards, New Delphi Indian.

Kacar B. 1997. Cayin Biyokimyası ve Isleme Teknolojisi. Cay Isletmeleri Genel Mudurlugu Yayini 6:1.

Khokar S. and Magnusdottir S.G. 2002. Total phenol catechin and caffeine contents of teas commonly consumed in The United Kingdom. J. Agric. Food Chem. 50:565.

Kumar R.S.S., Muraleedharan N.N., Murugesan S., Kottur G., Anand M.P. and Nishadh A. 2011. Biochemical quality characteristics of ctc black teas of south India and their relation to organoleptic evaluation. Food Chemistry 129:117.
Lin Y, Tsai Y, Tsay J, Lin J. 2003. Factors affecting the levels of tea polyphenols and caffeine in tea leaves. J. Agric. Food Chem. 51:1864.

Mahanta P.K and Baruah H.K. 1992. Theaflavin pigment formation and polyphenol oxidase activity as criteria of fermentation in orthodox and ctc teas J. Agric. Food Chem. 40:860.

Malik J., Szakova J., Drabek O., Balik J. and Kokoska L. 2008. Determination of certain micro and macro elements in plant stimulants and their infusions. Food Chemistry 111:520.

Marbaniang D.G., Baruah P., Decruse R., Dkhar E.R., Diengdoh D.F. and Nongpiur C.L. 2011. Study of trace metal Cr Mn Fe Co Ni Cu Zn And Cd composition in tea available at Shillong Meghalaya India. Int. J. Env. Protec. 1:13.

McKenzie J.S., Jurado J.M. and Pablos F. 2010. Characterization of tea leaves according to their total mineral content by means of probabilistic neural networks. Food Chemistry 123:859.

Mehra A. and Baker C.L. 2007. Leaching and bioavailability of aluminium copper and manganese from tea camellia sinensis. Food Chemistry 100 :1456.

Mossion A., Potin-Gautier M., Delerue S., Le Hecho I. and Behra P. 2008. Effect of water composition on aluminium, calcium and organic carbon extraction in tea infusions. Food Chemistry 106:1467.

Narin I., Colak H., Turkoglu O., Soylak M. and Dogan M. 2004. Heavy metals in black tea samples produced in Turkey. Bull Environ. Contam. Toxico. 72:844.

NMKL. 1998. Metals determination by atomic absorption spectrophotometry after wet digestion in a microwave oven - method no. 161". Nordic Committee on Food Analysis, Oslo Norway.

OCO. 2016. Organic fruits and vegetables taste, The Organic Center Organization, https://www.organic-center.org/publications/do-organic-fruits-and-vegetables-taste-better-than- conventional-fruits-and-vegetables/. Accessed October 2016.

Owuor P.O. and Obanda M. 2001. Comparative responses in plain black tea quality parameters of different tea cultivars to fermentation temperature and duration. Food Chemistry 72:319.

Ozdemir F. and Karkacier M. 1997. Bazi siyah ve yesil caylarin kimyasal bilesimi ve ekstraksiyon verimi. Ekonomik ve Teknik Dergi Standard 36:86.

Ozdemir F., Sahin H., Akdogan A., Dincer C. and Topuz A. 2008. Turk siyah cayinin fenolik madde kompozisyonu uzerine rakim surgun donemi ve cay sinifinin etkisi. Turkiye 10. Gida Kongresi Erzurum.

Ozdemir F., Topuz A. and Erbas M. 1999. Mineral contents of different classes of black tea produced by orthodox and caykur methods. J. Agri. and Fores. 23:809.

Pereira F.M.V., Pereira-Filho E.R. and Bueno M.I. 2006. Development of a methodology for calcium iron potassium magnesium manganese and zinc quantification in teas using x-ray spectroscopy and multivariate calibration. J. Agri. and Food Chem. 54:5723.

Shekhar H.U., Howlader Z.H. and Kabir Y. 2016. Health Benefits of Tea: Beneficial Effects of Tea on Human Health. Ch. 5. In "Exploring the Nutrition and Health Benefits of Functional Foods". S. Mitra and S. Khandelwal (Ed.), p.99. IGI Global Publishing Co., Hershey, USA.

Street R., Szolova J., Drobek O. and Mladlova L. 2006. The status of micronutrients Cu Fe Mn Zn in tea and tea infusions in selected samples imported to the Czech Republic. J. Food Sci. 24:62.

Sullivan M.L. 2015. Beyond brown: polyphenol oxidasesas enzymes of plant specialized metabolism. J. Frontiers in Plant Sci. 5:1.

Tascioglu S. and Kok E. 1998. Temperature dependence of copper iron nickel and chromium transfers into various black and green tea ınfusions. J. Sci. Food Agri. 76:200.

TSE. 2016. TS EN ISO 13299 - Sensory analysis - Methodology - General guidance for establishing a sensory profile. Turkish Standard Institute, Ankara Turkiye.

Venkatesan S. and Ganapathy M.N.K. 2004. Impact of nitrogen and potassium fertilizer application on quality of ctc teas. Food Chemistry 84:325.

Wright L.P., Mphangwe N.I.K., Nyirenda H.E. and Apostolides Z. 2002. Analysis of the theaflavin composition in black tea camellia sinensis for predicting the quality of tea produced in central and Southern Africa. J. The Sci. of Food and Agri. 82:517.

Yao L.H., Jiang Y.M., Caffin N., D Arcy B., Datta N., Liu X., Singanusong R. and Xu Y. 2006. Phenolic compounds in tea from Australian supermarkets. Food Chemistry 96:614.

Yong-Quan X., Chun Z., Ying G., Jian-Xin C., Fang W., Gen-Sheng C. and Jun-Feng Y. 2017. Effect of the type of brewing water on the chemical composition, sensory quality and antioxidant capacity of Chinese teas. Food Chemistry 236:142.

Zhang H.Q., Ni B.F., Tian W.Z., Zhang G.Y., Huang D.H., Liu C.X., Xiao C.J., Sun H.C. and Zhao C.J. 2011. Study on essential and toxic elements intake from drinking of Chinese tea. J. Radioanal Nuclear Chem. 287:887.

Zhou, D., Chen, Y. and Ni D. 2009. Effect of water quality on the nutritional components and antioxidant activity of green tea extracts. Food Chemistry 113:110.

Paper Received July 2, 2017 Accepted July 20, 2017

PAPER

NUTRITIONAL CONTENT AND ANTIOXIDANT PROPERTIES OF SELECTED SPECIES OF *AMARANTHUS* L.

W. BIEL[1], E. JENDRZEJCZAK[2], A. JAROSZEWSKA*[3], R. WITKOWICZ[4], E. PIĄTKOWSKA[5] and A. TELESIŃSKI[6]

[1]Department of Pig Breeding, Animal Nutrition and Food, West Pomeranian University of Technology in Szczecin, Judyma 10, 71460 Szczecin, Poland

[2]Department of Botany and Ecology, University of Science and Technology in Bydgoszcz, Prof. S. Kaliskiego 7, 85796 Bydgoszcz, Poland

[3]Department of Agronomy, West Pomeranian University of Technology in Szczecin, Papieża Pawła VI 3, 71434 Szczecin, Poland

[4]Institute of Plant Production, University of Agriculture in Cracow, Mickiewicza 21, 31120 Krakow, Poland

[5]Department of Human Nutrition, University of Agriculture in Cracow, Balicka 122, 30149 Krakow, Poland

[6]Department of Plant Physiology and Biochemistry, West Pomeranian University of Technology in Szczecin, Słowackiego 17, 71434 Szczecin, Poland

*Corresponding author. Tel.: +48914996292;
E-mail address: anna.jaroszewska@zut.edu.pl

ABSTRACT

The aim was to assess the entire plant of three species of *Amaranthus* L. with regard to their chemical content, determination of the most valuable genotype, and the optimum harvest time in relation to the species' nutritional value. The amaranth harvested later was found to contain less protein and ash, but clearly more fibre, and nitrogen-free extracts. The highest content of mineral compounds was observed at the beginning of blossoming. The genotypes were characterized by very high levels of radical scavenging activity, which was dependent on harvest timing. Betanine and amaranthine concentration decreased with the delay in harvesting.

Keywords: amaranth, antioxidant activity, betacyanins, chemical composition, genotype, harvest time

1. INTRODUCTION

The effects of globalization and the observed unification of food production around the world have limited the number of cultivated plant species. To counteract this trend, new plants - previously not used on a massive scale, but with a proven nutritional value - have been introduced in commercial agriculture. This group includes one of the oldest cultivated plants, belonging to the genus *Amaranthus*. The value of amaranth depends mainly on the unique chemical composition of its seed (MOTA *et al*., 2016). The chemical composition of the foliage is also very interesting and presents significant nutritional value. The content of high quality protein in the leaves amounts to 19,4%-30% of dry matter (ANDINI *et al*., 2013; NGUGI *et al*., 2017). The entire amaranth plant contains biologically active components such as anthocyanins, flavonoids, and phenyl acids, which are responsible for the plants' pharmacological properties (LI *et al*., 2015).
Apart from the aforementioned nutritional value of the whole amaranth plant, there are other reasons justifying efforts to increase its cultivation volume. Two such economic factors are the relatively undemanding cultivation and the high yield. In order for the plants to produce valuable flowers and seeds, a relatively low crop density is recommended (from 10 to 30 plants per m^2) (YARNIA, 2010). In the case of forage crop cultivation the plants should produce delicate and thin shoots (O'BRIEN and PRICE, 2008). An increase in crop density to 140 plants per m^2 results in better parameters for green foliage (required ratio of leaf mass to shoot mass). A lower crop density results in higher shoot mass and higher fibre content. In the case of high crop density the produced shoots are narrower, and tend to drop older leaves that receive less light.
The choice of the best time for forage crop harvesting is related to the size of the harvest and its nutritional value, which could be determined in two ways: by choosing a specific day after sowing (ABBASI *et al*., 2012) or by following the plant's development phase (POSPIŠIL and POSPIŠIL, 2008). Regardless of the harvest method, all the mentioned papers report that the most beneficial period for harvest in terms of the nutritional value and digestibility of the plant is the end of its vegetative stage, and if the size of the collected yield is concerned – full bloom stage. A delay in harvest quite negatively influences the quality of the collected mass.
In this study, we attempted to assess whether the species of the genus *Amaranthus* L. differentiates chemical composition of the whole plants, and whether the nutritional value whole plants *of Amaranthus* L. is dependent on the harvest time.
Therefore, the aim of study was to assess the chemical composition of the whole plant of three species of the genus *Amaranthus* L. and to determine the most valuable genotype, as well as the optimum harvest time with regard to the plants' nutritional value.

2. MATERIALS AND METHODS

2.1. Study sites

The experiment was conducted in 2014 at the Mochełek Experimental Station near Bydgoszcz, Poland (53°12′24″N, 17°51′40″E). The experiment was conducted in a totally random system and was repeated three times. The soil in which the crops were grown is typical Haplic Luvisols, created from fluvioglacial sands on sandy loam, class IVa. The soil pH was slightly acidic (pH 5.6 in KCl). The content of silt and clay in the arable layer stood at 15%. Analysis of soil mineral content showed high levels of phosphorus (20.9 mg/100g), very low levels of magnesium (2.2 mg/100g), and low levels of potassium (8.0 mg/100g). Sowing was carried out on 15th May with a spacing of 0.45 m, 5 kg per ha, at 1-2 cm depth

by spacing seeder. The area of the harvest plot was 10 m^2. No mineral fertilization was used. The plant maintenance included mechanical weeding of the interrows. The fore crop for amaranth was spring barley.

2.2. Plant material

The first experimental factor (A) was genotype: (A1) *Amaranthus cruentus* L. 'Rawa' cultivar, (A2) *Amaranthus hypochondriacus* L. 'Aztek' cultivar, (A3) *Amaranthus caudatus* L. 'Oscar Blanco', and 'Phule Kartiki' cultivar. The second factor (B) was harvest time – the number of days after sowing: (B1) the beginning of blossoming, (B2) full bloom, and (B3) full seed development. The choice of experimental factor (harvest time) was made based on observation of the developmental stage of the majority of plants in the plot field. The term of specific agrophenophases was established *a priori* and not using the calendar. On the day prior to harvest, random plant samples were collected for chemical analysis (20 samples of each cultivar).

2.3. Climatic conditions

The atmospheric conditions for the year of the study are presented in Table 1. In comparison with the mean values of multi-year meteorological parameters (1996-2013) the humidity during sowing and germination periods was higher (the sum of precipitation was higher by 45%), but in the following months it was relatively lower. In addition, very high temperatures were registered over the entire vegetative phase. As a result, the values of Seljaninov hydrothermal indicator in June, July, and August were lower than the mean, which confirms the occurrence of drought. Despite this, based on long-term research on amaranth cultivation conducted at the same location, it could be assumed that the weather during the year 2014 was particularly beneficial for the growth and development of all the chosen amaranth species.

Table 1. Temperature, precipitation and hydrothermal index during the experiment as compared with multiyear average [1996-2013].

Years	The meteorological parameter	Decade of the month	Month				
			April	May	June	July	August
2014	The average air temperature (°C)	I	7.3	10.4	17.6	21.1	21.8
		II	8.7	12.8	15.8	20.6	16.1
		II	13.7	16.3	14.5	22.8	14.1
	Average monthly temperature		9.9	13.3	16.0	21.5	17.2
	Precipitation (mm)	I	17.6	32.1	10.2	14.4	18.9
		II	18.6	10.6	12.0	7.7	16.2
		II	4.5	23.0	22.7	33.3	22.2
	Monthly amounts of precipitation [mm]		40.7	65.7	44.9	55.4	57.3
	Hydrothermal index		1.37	1.59	0.94	0.83	1.07
1996-2013	The average air temperature (°C)		8.0	13.2	16.3	18.5	17.8
	Rainfall (mm)		28.0	60.9	53.5	88.8	67.0
	Hydrothermal index		1.17	1.49	1.09	1.55	1.21

2.4. Chemical analyses

The chemical composition of samples were determined according to procedures of the Association of Official Analytical Chemists (AOAC, 2012): dry matter was determined by drying at 105ºC to a constant weight, crude fat by Soxhlet extraction with diethyl ether, crude ash by incineration in a muffle furnace at 580ºC for 8 h. Crude protein (N × 6.25) by Kjeldahl method using a Büchi Distillation Unit B - 324 (Büchi Labortechnik AG, Switzerland). Crude fibre was determined in an ANKOM 220 fibre analyzer (ANKOM Technology, USA). Nitrogen-free extract (NFE_s) was calculated as: $NFE_s = 100 - (\text{moisture} + \text{crude protein} + \text{crude fat} + \text{crude ash} + \text{crude fibre})$. The fibre components were determined using the detergent method according to VAN SOEST *et al*. (1991) performed with the ANKOM 220 fibre analyzer. Determination of neutral detergent fibre (NDF) was conducted on an ash-free basis and included sodium dodecyl sulphate (Merc 822050). Determination of acid detergent fibre (ADF) included hexadecyl-trimethyl-ammonium bromide (Merc 102342), while acid detergent lignin (ADL) was determined by hydrolysis of ADF samples in 72% sulphuric acid. Hemicellulose content was calculated as the difference between NDF and ADF, while cellulose content as the difference between ADF and ADL.

The material for analyses of the major dietary element concentrations was subjected to mineralization in concentrated H_2SO_4 and $HClO_4$ acids, whilst the material for analyses of the micro-compound concentrations was subjected to mineralization in a mixture of HNO_3 and $HClO_4$. The concentration of phosphorus (P) were determined by colorimetric method, with ammonium molybdate, at wavelength 660 nm, using a Specol 221 apparatus. An Atomic Absorption Spectrometer apparatus (iCE 3000 Series, Thermo Fisher Scientific) was used to determine potassium (K), sodium (Na) and calcium (Ca) by means of emulsion flame spectroscopy, whilst magnesium (Mg), zinc (Zn), iron (Fe), manganese (Mn) and copper (Cu) by means of absorption flame spectroscopy.

Assessment of betacyanins was carried out using a spectrophotometer; UV-1800r. Exactly 2g of ground plant material was placed in a mortar before homogenization with 10cc demineralized water to ease extraction. The pH of the resulting solution was adjusted to 5.4 (STINTZING *et al*., 2004). Samples were diluted in a 0.05 M phosphate buffer (pH 6.5) as described by STINTZING *et al*. (2003) using the extinction coefficients of betanin (ε = 60000 $dm^3/mol/cm$; λ = 538 nm; molecular weight = 550) (WYLER and MEUER, 1979) and of amaranthine (ε = 56600 $dm^3/mol/cm$; λ = 538 nm; molecular weight = 726) (PIATTELLI *et al*., 1969).

The level of polyphenols was determined by the POLI-SWAIN and HILLIS (1959) method, using Folin-Ciocalteu reagent. The extract used for determination of polyphenols was obtained by grinding dried samples of amaranth green forage in the lab sample mill (OG 109), followed by extraction with 40 mL 0.08 M HCl in 80% methanol, at a temperature of 18-22ºC for two hours. The extract from 5g of green forage was centrifuged at 1500 g for 15 minutes and the remains were re-extracted twice with 40 mL 70% acetone for two hours. The content of polyphenols was expressed in mg of chlorogenic acid in 100g of the product. The extract's capacity to eliminate free radicals was assessed using the method described by RE *et al*. (1999) using ABTS•+. ABTS•+ free radical diluted in a solution of potassium persulfate adjusted to provide an absorbance of 0.74-075 at 734 nm. The methanol-acetone extract (0.8 mL) was topped up to 1 mL with a 1:1 mixture of acetone and methanol. Subsequently, 2 mL of ABTS•+ free radicals were added to the mixture. The extract was incubated at 3ºC for 6 minutes and the absorption measurement was subsequently carried out at 734 nm.

The capacity to eliminate free RSA (Radical Scavenging Activity) was derived from the following equation:

$$RSA = (E_1 – E_2) / E_1$$

where:
E_1 – sample absorbance before incubation,
E_2 – sample absorbance after incubation.

2.5. Statistical analysis

The data were subjected to statistical analysis using ANOVA in a randomized block design. The separate factors in the analysis were genotype and harvest time. The sum of the errors and the interaction of genotype × harvest date were used to test the significance of the main effects. The significance of differences between means shown in the form of homogeneous groups was assessed using a Duncan test at P = 0.05. Results are presented as mean±SD (standard deviation). All chemical analyses were performed in three replications.

3. RESULTS AND DISCUSSIONS

3.1. Chemical composition

Traditional vegetables provide low-cost quality nutrition for large parts of the population in both rural and urban areas. One such example is amaranth, containing more nutrients than typical leafy vegetables (VENSKUTONIS and KRAUJALIS, 2013) In this study, we found statistically significant differences were found in the dry matter content, crude ash, crude protein, crude fibre, and nitrogen-free extract (NFE) in the whole plant between the studied genotypes (Table 2). The harvest time influenced amaranth chemical composition. Similar to AMADUCCI *et al*. (2000) and FRASER *et al*. (2001), who showed that the delay in harvesting of plants caused changes in their chemical content, in our study a later harvesting time caused an increase in dry matter and crude fibre content (Table 3), but a decrease in crude protein content. Green leafy vegetables have long been recognized as the cheapest and most abundant potential source of protein because of their ability to synthesize amino acids from a wide range of virtually unlimited and readily available primary materials such as water, CO_2, and atmospheric nitrogen (ALETOR *et al*., 2002). Our results concerning crude protein in amaranth leaves do not provide a clear indication of statistical difference between the studied species, with protein content ranging between 95.9 and 101 g per kg of dry matter, similar to the results of other authors (AKUBUGWO *et al*., 2007; MODIL, 2007).
However, the results of our analysis permit the claim that *Amaranthus hypochondriacus* L. ('Aztek') tends to have a slightly higher protein level than the other studied genotypes. The highest amounts of protein were found in plants harvested at the beginning of blossoming (128 g/kg DM), being 37% and 32% higher than the samples from the two consecutive terms, respectively. Even higher crude protein levels in amaranth biomass (152 to 216·kg DM) were reported by POSPIŠIL *et al*. (2009), which depended on the cultivar and the year. Crude fat content in the whole plant differed between the studied amaranth cultivars, with the highest amount found in the 'Rawa' cultivar. Levels depended not only on the genotype but also on the harvest time.

Table 2. The tested the whole plant of amaranth composition [g/kg DM].

Specification	Dry matter	Crude ash	Crude protein	Crude fat	Crude fibre	NFEs
			Cultivar (A)*			
(A1) 'Rawa'	939±0.26	146±1.15	95.9±0.79	22.6^{a}±0.12	235±0.18	499±0.65
(A2) 'Aztek'	936±0.40	144±0.20	101.0±0.74	17.6^{ab}±0.17	240±1.27	495±0.16
(A3) 'Phule Kartiki'	941±0.75	163±1.37	98.0±0.81	11.3^{b}±0.31	219±0.34	508±2.15
(A4) 'Oscar Blanco'	938±0.38	154±0.58	97.9±0.02	12.2^{b}±0.45	249±1.00	486±0.05
			Term of the mowing - no. of days after sowing date (B)**			
(B1) - 60	934±0.12	183^{a}±0.83	128.0^{a}±0.66	15.4^{b}±0.31	203^{b}±0.90	470^{c}±0.76
(B2) - 90	942±0.25	145^{b}±0.26	80.3^{b}±0.53	11.5^{b}±0.35	268^{a}±0.86	494^{b}±0.78
(B3) - 120	939±0.41	128^{b}±0.67	86.9^{b}±0.57	20.8^{a}±0.27	236^{ab}±1.36	527^{a}±18.9

*The cultivar's means denoted by different letters differ statistically at (for all columns separately).
**The term of mowing's means denoted by different letters differ statistically at (for all columns separately).

3.2. Fibre Fractions

Recently, increasing attention has been paid to the components that are difficult to digest in the gastrointestinal tract of humans, belonging to the dietary fibre group. The source of the dietary fibre and the ratios of its fractions determine its properties and applications; the chemical and physical properties of its many structures have various effects on the physiology of the human body (MANN *et al*., 2009). In our study, the content of crude fibre in the selected species was affected only by harvest time; a delay in harvest increased the content of crude fibre in the whole plant. The levels of specific fibre fractions were also determined (Table 3) and indicated that a later harvest time caused a increase in NDF, ADF, ADL and cellulose content. HCEL are best at binding ions of heavy metals (HU *et al*., 2010). Celluloses and lignin also have these properties, but to a lesser degree and are dependent on the fraction's origin. Cellulose does not have good ion exchange properties, nor does it bind bile acids or salts (KAHLON *et al*., 2007). Cellulose fibres are virtually undigested in the gastrointestinal tract; however, they aid intestine peristalsis.

Table 3. The tested the whole plant of amaranth dietary fibre [g/kg DM].

Specification	NDF***	ADF	ADL	HCEL	CEL
		Cultivar (A)*			
(A1) 'Rawa'	440±3.02	304±1.06	51.2^{b}±1.99	136±4.08	253±0.93
(A2) 'Aztek'	440±2.57	332±0.16	63.1^{a}±2.42	109±2.73	269±2.57
(A3) 'Phule Kartiki'	415±0.29	291±3.91	54.5^{ab}±0.88	126±4.20	236±3.03
(A4) 'Oscar Blanco'	453±3.39	329±2.61	55.1^{ab}±0.16	124±5.99	274±2.76
		Term of the mowing - no. of days after sowing date (B)**			
(B1) - 60	408^{b}±1.83	288^{b}±2.20	51.8^{b}±0.30	121±0.38	236^{b}±2.50
(B2) - 90	460^{a}±0.83	347^{a}±0.01	54.1^{b}±1.05	113±0.83	293^{a}±1.05
(B3) - 120	444^{a}±0.01	307^{ab}±1.90	62.1^{a}±1.18	137±1.90	245^{b}±0.72

*The cultivar's means denoted by different letters differ statistically at (for all columns separately).
**The term of mowing's means denoted by different letters differ statistically at (for all columns separately).
***NDF, neutral detergent fibre, ADF, acid detergent fibre, ADL, acid detergent lignin, CEL, cellulose, HCEL, hemicelluloses.

In our study, the highest relative amount of cellulose was found at the second harvest time (almost 20% more in comparison to the first harvest term), which is the result of an increase in content in tissues during the development and aging of the plant. Lignin accumulates in the cell wall at the end of cell growth, after the formation of polysaccharide scaffolding of the wall is completed. The highest level of ADL fraction was present in the dry matter from the last harvest term. This fraction does not present a high capacity for binding heavy metals. However, similar to cellulose, it also aids in intestine peristalsis.
NDF had a highest share in dietary fibre, followed by ADF, which includes lignin and cellulose. A later harvest time increased the amount of these fractions in the whole amaranth plant, which was probably caused by the production of cell wall components, as well as more polyphenols as a result of temperature stress. The delay in harvest promoted lignification as well as an increase of cell wall participation in cells of plant tissue (AMADUCCI *et al*., 2000).

3.3. Macronutrients

A special role is assigned to mineral components due to their participation in numerous anti-oxidation processes. The enzyme peroxide dismutase is activated by copper, zinc, and manganese. Deficiency of the mentioned elements, or magnesium, calcium and potassium, decreases the efficiency of internal anti-oxidant mechanisms causing an increased risk of degenerative diseases (AMES, 2010; PRASHANTH *et al*., 2015). In our study, we assessed the level of selected elements in the amaranth samples and found that they were influenced by the experimental factors (Tables 4 and 5). The content of the tested mineral components in the studied cultivars of amaranth differed, which indicates various capacities to absorb and accumulate the mentioned components in the biomass by the different cultivars growing in similar conditions. The highest content of calcium and magnesium were found in the 'Aztek' cv., while the lowest calcium was detected in 'Rawa' cv., and the lowest magnesium in 'Phule Kartiki' cv. (Table 4). 'Rawa', 'Phule Kartiki' and 'Oscar Blanco' cultivars had higher phosphorus concentrations than 'Aztek' cv. (6.8 g/kg DM). The content of potassium in 'Oscar Blanco' cv. (63.7 g/kg DM) was the highest and differed from other cultivars. Present research confirmed that amaranth was a rich source of potassium. The richest in potassium was 'Oscar Blanco' cv., containing 10 g/kg more of this element than spinach (MATRASZEK *et al*., 2002), which belongs to the superfood group and is considered a rich source of potassium. 'Aztek' cultivar contained the highest level of magnesium (4.4 g/kg DM). The highest concentration of sodium was recorded for 'Rawa' cv. (111 g/kg DM) and 'Oscar Blanco' cv. (95.8 g/kg DM). Phosphorus was the only element not influenced by the time of harvest, and its mean concentration in the studied genotypes of amaranth amounted to 8.9 g/kg DM. Differences in Ca and Na content were recorded between the two harvest times. A tendency was found for less of the elements in the green mass of amaranth in the consecutive harvest terms (Table 4). The amaranth is a valuable product and could be an important source of necessary nutrients in the human and the animal diet (AKUBUGWO *et al*., 2007; ONWORDI *et al*., 2009; MLYNEKOVÁ *et al*., 2014). The application of food minerals in an organism largely depends on the ratios. The requirements regarding particular mineral components and their ratios in the animal diet depend, *inter alia*, on the species of animal, age, and physiological stage. The potassium to sodium ratio (K:Na) in humans plays an important role in the regulation of blood pressure; it should be less than one to avoid adverse effects (YUSUF *et al*., 2007). As shosphorus metabolism is linked to calcium metabolism in the system, calcium-phosphate homeostasis crucially depends on an appropriate Ca:P ratio in food (close to 1). An inappropriate Ca:P ratio decreases calcium absorption, which negatively affects the skeletal system and physiological

processes (DRIVER *et al*., 2006; YE *et al*., 2006). A ratio lower than 1:2 impairs calcium absorption and vitamin D synthesis. This results in higher levels of parathyroid hormone and accelerates bone resorption processes (KEMI *et al*., 2010). 'Rawa', 'Phule Kartiki', and 'Oscar Blanco' cultivars all fulfilled the aforementioned requirements, which confirms the results of AKUBUGWO *et al*. (2007). In addition, the 'Phule Kartiki' and 'Aztek' cultivars had a K:Na ratio close to 1, which qualifies their use in poultry feeds.

Table 4. The tested the whole plant of amaranth macronutrients [g/kg DM].

Specification	Ca	P	K	Mg	Na	Ca:P	K:Na
			Cultivar (A)*				
(A1) 'Rawa'	10.4^{b}±0.13	9.6^{a}±0.01	55.6^{b}±0.33	3.8^{b}±0.01	111.8^{a}±0.56	1.1^{b}±0.01	0.5^{c}±0.01
(A2)' Aztek'	13.7^{a}±0.07	6.8^{b}±0.05	54.3^{b}±0.41	4.4^{a}±0.01	65.4^{b}±1.83	2.0^{a}±0.01	0.8^{a}±0.02
(A3) 'Phule Kartiki'	10.7^{ab}±0.10	9.1^{ab}±0.09	54.6^{b}±0.14	3.6^{b}±0.01	60.9^{b}±1.17	1.2^{b}±0.02	0.9^{a}±0.01
(A4) 'Oscar Blanco'	10.8^{ab}±0.13	10.1^{a}±0.06	63.7^{a}±0.51	3.7^{b}±0.01	95.8^{a}±0.69	1.0^{b}±0.02	0.7^{b}±0.01
			Term of the mowing - no. of days after sowing date (B)**				
(B1) - 60	14.6^{a}±0.16	10.3±0.01	65.6^{a}±0.26	4.7^{a}±0.02	90.4^{a}±0.25	1.5^{a}±0.01	0.8^{a}±0.01
(B2) - 90	9.7^{b}±0.08	8.2±0.04	62.1^{b}±0.51	3.1^{c}±0.01	70.1^{b}±0.46	1.3^{ab}±0.01	0.9^{a}±0.01
(B3) - 120	9.9^{b}±0.07	8.3±0.13	43.5^{c}±0.27	3.8^{b}±0.01	89.5^{a}±0.79	1.2^{b}±0.02	0.5^{b}±0.01

*The cultivar's means denoted by different letters differ statistically at (for all columns separately).
**The term of mowing's means denoted by different letters differ statistically at (for all columns separately).

3.4. Micronutrients

Iron and zinc are both responsible for the proper functioning of specific and non-specific immune responses. The studied amaranth genotypes turned out to be a rich source of iron. The harvest time did not affect the content of this component (Table 5). The whole plant of amaranth was found to contain on average 236.4 mg/kg of iron, which confirms the results of KAMGA *et al*. (2013). By comparison, MATRASZEK *et al*. (2002) recorded 30% and 7% less iron in the respective dry matter of lettuce (*Lactuca sativa* L.) and spinach (*Spinacia oleracea* L.). As confirmed in the samples in the presented research, FUNKE (2011) found 40% more iron in amaranth leaves cultivated in Nigeria than in lettuce. The daily diet should also provide proper amount of zinc, since it is not accumulated in tissues, but excreted from the system. Zinc has a beneficial effect on the production, maturation and activity of leukocytes. However, excessive consumption may limit iron and copper absorption, which can lead to anaemia. In our study, both the cultivar and the harvest time had an effect on the content of Zn. 'Phule Kartiki' cv. biomass had the highest content of Zn. The later the harvest, the lower the amount of Zn in the selected amaranth cultivars. Iron metabolism also involves copper, and the highest content of copper was found in 'Aztek' cv. The highest levels of copper were also found in the amaranth at the beginning of blossoming (Table 5). Manganese is an essential trace element, necessary for development and growth of the organism. It is a component of metalloenzymes such as superoxide dismutase, arginase, and pyruvate carboxylase, and is involved in amino acid, lipid and carbohydrate metabolism. Disturbances in manganese absorption and retention may play a role in the etiopathogenesis of several diseases and disorders. There has been no specific manganese deficiency syndrome described in humans (ZABŁOCKA-SŁOWIŃSKA and GRAJETA, 2012; PANEL ON DIETETIC PRODUCTS, NUTRITION AND ALLERGIES, 2013). Manganese content in the studied plants was significantly

correlated to both cultivar type and harvest time. The highest content of manganese was reported in the 'Phule Kartiki' cv. (177.5 mg/kg DM). The mean content of manganese in the studied cultivars of amaranth stood at 150.8 mg/kg DM. The highest manganese content was reported in amaranth collected during the full bloom period. Manganese concentration in vegetable leaves, including amaranth, varies between 2.54 mg/kg DM in indian spinach (*Basella alba* L.), 5.46 mg/kg DM in bush buck (*Gongronema latifolium* L.), 6.14 mg/kg DM in roselle plant (*Hibiscus sabdariffa* L.) and 10.6 mg/kg DM in smooth amaranth (*Amaranthus hybridus* L.) (ASAOLU *et al*., 2012).

Table 5. The tested the whole plant of amaranth micronutrients [g/kg DM].

Specification	Zn	Fe	Mn	Cu
		Cultivar (A)*		
(A1)' Rawa'	37.8^{b}±0.36	231.1^{ab}±1.23	132.6^{b}±0.01	2.8^{c}±0.03
(A2) 'Aztek'	44.4^{b}±0.04	237.4^{ab}±0.17	140.4^{b}±0.20	3.7^{a}±0.30
(A3) 'Phule Kartiki'	82.4^{a}±0.06	272.3^{a}±0.14	177.5^{a}±0.10	3.5^{ab}±0.10
(A4) 'Oscar Blanco'	41.0^{b}±0.09	205.0^{c}±1.03	152.7^{ab}±0.18	3.0^{bc}±0.02
		Term of the mowing - no. of days after sowing date (B)**		
(B1) - 60	60.0^{a}±0.25	248.4±1.11	147.9^{ab}±0.02	4.3^{a}±0.20
(B2) - 90	48.6^{b}±0.04	219.7±0.47	171.9^{a}±0.22	2.5^{b}±0.01
(B3) - 120	45.6^{b}±0.04	241.1±1.07	132.7^{b}±0.12	2.8^{b}±0.09

*The cultivar's means denoted by different letters differ statistically at (for all columns separately).
**The term of mowing's means denoted by different letters differ statistically at (for all columns separately).

3.5. Polyphenol, pigments and scavenging ability

Betanins are water-soluble nitric herbal dyes and can be found in the cell fluids. Their stability within a wide pH range from 3.5 to 7.0 contributes to the fact that they are excellent food dyes and good substitutes for anthocyanins (MORENO *et al*., 2008). In a collective summary for the *Amaranthaceae* family, KHAN and GIRIDHAR (2015) found the betanin content between 7.6 and 117.0 mg/kg DM (in *Celosia* spp., *Achyrranthes* spp., *Aerva sanguinolenta* spp., *Alternanthera* spp., *Iresine herbstii* spp., *Gomphrrena globose* spp.). In the studied whole plant of amaranth, betanin levels of 11.4-17.3 mg/kg DM and amaranthine of 106.5-161.1 mg/kg in air dried mass of different cultivars of amaranth were found. In amaranth shoots, VENSKUTONIS and KRAUJALIS (2013) recorded 1.77 mg/100g of betanin. They also showed that amaranthine and isoamaranthine are the dyes found in the highest quantities, at 15.3 and 5.87 mg/100g, respectively. The presence of amaranthine and the absence of isoamaranthine were reported in *Celosia argentea* L. (SCHLIEMANN *et al*., 2001). In our study, a content of betanin in the whole plant of amaranth ranged between 1.19 and 1.54 mg/100g and depended on genotype (Table 6). The highest content was reported in the leaves of the 'Aztek' and 'Oscar Blanco' cultivars. Analysis of the effect of harvest time on betanin content showed that a delay in harvest decreased the content of this compound in the whole plant. The highest content of amaranthine was reported in dry matter of plants from the first harvest, as well as in the dry matter of the 'Aztek' and 'Oscar Blanco' cultivars. Betacyanins also display a free radical scavenging capacity, and betanin and its metabolites maintain their properties in acidic conditions (TAIRA *et al*., 2015). The free radical scavenging activity (RSA) of bethanidine is comparable to that of vitamin E (TESORIERE *et al*., 2009). The concentrations found in the

various cultivars fell within the range 2.9 mg/g to 35 mg/g DM. Free radial scavenging activity is also correlated with the content of polyphenols in raw material. The statistical analysis of the genotypes allowed their classification into two homogenous groups. The group with the highest content of polyphenols was the 'Phule Kartiki' cv. (2792.5 mg/100g DM). Such a high content of total polyphenols in the whole plant of this cultivar did not result in statistically significantly higher antioxidant activity compared to other cultivars. The high capacity to remove free radicals in the studied cultivars oscillated within a very narrow range (97.5% to 98.4%), which can be ascribed to harvest time, with RSA slightly higher in the second and third terms. Although the highest levels of polyphenols were found in the second harvest term (2884.2 mg/kg DM), and the lowest in the third term (1482.3 mg/kg DM), free radical scavenger properties were statistically unchanged. Differences in phenol compounds content between leafy vegetables, including *Amaranthus cruentus* L. and *Amaranthus hybridus* L. were shown by ADEMOYEGUN *et al*. (2013). Of these two species, it was *A. hybridus* that had higher phenol content and higher anti-radical activity. Due to the fact that these compounds play an important role both for these plants' ontogenesis, and their health promoting and sensory properties, it seems they should be considered as potential food supplements. In this regard, amaranth is more beneficial than sprouts and leaves of oat and buckwheat, commonly known for their antioxidant potential; no less important is the presence of betacyanines in amaranth (PIĄTKOWSKA *et al*., 2015; WITKOWICZ *et al*., 2015).

Table 6. Polyphenol content [mg/100g DM], pigments content [mg/kg DM] and scavenging ability RSA [%] in the tested the whole plant of amaranth.

Specification	Polyphenol	RSA	Betanine	Amaranthine
		Cultivar (A)*		
(A1) 'Rawa'	1669.7^{b}±49.3	98.0±0.22	11.9^{b}±0.31	111.3^{b}±2.96
(A2) 'Aztek'	1846.3^{b}±53.3	98.1±0.22	14.4^{a}±0.76	134.5^{a}±7.07
(A3)' Phule Kartiki'	2792.5^{a}±182.8	97.8±0.31	12.1^{b}±0.35	113.3^{b}±3.33
(A4) 'Oscar Blanco'	1768.3^{b}±61.73	98.0±0.22	15.4^{a}±0.02	143.7^{a}±0.18
		Term of the mowing - no. of days after sowing date (B)**		
(B1) - 60	1690.9^{b}±55.6	97.5^{b}±0.09	17.3^{a}±0.09	161.1^{a}±0.82
(B2) - 90	2884.2^{a}±172.2	98.1^{a}±0.09	11.7^{b}±0.13	109.5^{b}±1.30
(B3) - 120	1482.3^{b}±32.6	98.4^{a}±0.21	11.4^{b}±0.10	106.5^{b}±0.93

*The cultivar's means denoted by different letters differ statistically at (for all columns separately).
**The term of mowing's means denoted by different letters differ statistically at (for all columns separately).

4. CONCLUSIONS

The weather in the vegetative season in 2014 was particularly beneficial for the growth and development of the cultivated amaranth species. The factor that determined the nutritional value of the studied plants was the harvest time. A delay in harvest time decreased the amount of crude protein and mineral compounds (determined in ash). The amaranth from later harvests (full bloom and full seed development) had increased concentration of crude fibre and total carbohydrates. The content of mineral compounds was different, which indicated differences in the abilities to absorb and accumulate particular elements by the studied cultivars. With a delay in harvest the concentrations of the macro and micro-compounds decreased. The studied amaranth demonstrated very

high free radical scavenging activity, with the highest RSA values recorded in the two later harvest terms. The content of pigments in amaranth was correlated to both the genotype and the harvest term. The highest concentrations of pigments were observed in 'Oscar Blanco' cv. and 'Aztek' cv. This content then decreased with the delay in harvest. The amaranth is a valuable product and may be an excellent source of essential nutritional components in the human and animal diet. However, based on the presented results, it is difficult to unequivocally indicate the most valuable genotype and optimal harvest term with reference to the nutritional value.

REFERENCES

Abbasi D., Rouzbehan Y. and Rezaei J. 2012. Effect of harvest date and nitrogen fertilization rate on the nutritive value of amaranth forage (*Amaranthus hypochondriacus*). Anim Feed Sci Tech. 171(1):6-13.

Ademoyegun T.A., Akin-Idowu P.E., Ibitoye D.O. and Adewuyi G.O. 2013. Phenolic contents and free radical scavenging activity in some leafy vegetables. International Journal of Vegetable Science. 19:126-137.

Akubugwo I.E., Obasi N.A., Chinyere G.C. and Ugbogu A.E. 2007. Nutritional and chemical value of *Amaranthus hybridus* L. leaves from Afikpo, Nigeria. Afr. J. Biotechnol. 6(24):2833-2839.

Aletor O., Oshodi A.A. and Ipinmoroti K. 2002. Chemical composition of common leafy vegetables and functional properties of their leaf protein concentrates. Food Chem. 78:63-68.

Amaducci S., Amaducci M.T., Benati R. and Venturi G. 2000. Crop yield and quality parameters of four annual fibre crops (hemp, kenaf, maize and sorghum) in the North of Italy. Ind Crops Prod. 11:179-186.

Ames B.N. 2010. Prevention of mutation, cancer, and other age-associated diseases by optimizing micronutrient intake. J Nucleic Acids. 2010:1-11.

Andini R., Shigeki Y. and Ohsawa R. 2013. Variation in protein content and amino acids in the leaves of grain, vegetable and weedy types of amaranths. Agronomy. 3:391-403.

AOAC Association of Official Analytical Chemists. 2012. Official Methods of Analysis. Publisher: Association of Official Method of Analysis. Washington, DC.

Asaolu S.S., Adefemi O.S., Oyakilome I.G., Ajibulu K.E. and Asaolu M.F. 2012. Proximate and mineral composition of nigerian leafy vegetables. Food Research. 1(3):214-218.

Driver J.P., Pesti G.M., Bakalli R.I. and Edwards H.M. Jr. 2006. The effect of feeding calcium- and phosphorus-deficient diets to broiler chickens during the starting and growing-finishing phases on carcass quality. Poult. Sci. 85(11):1939-1946.

Fraser M.D., Fychan R. and Jones R. 2001. The effect of harvest date and inoculation on the yield, fermentation characteristics and feeding value of forage pea and field bean silages. Grass Forage Sci. 56:218-230.

Funke O.M. 2011. Evaluation of nutrient contents of amaranth leaves prepared using different cooking methods. Food Nutr Sci. 2:249-252.

Hu G., Huang S., Chen H. and Wang F. 2010. Binding of four heavy metals to hemicelluloses from rice bran. Food Res Int. 43:203-206.

Kahlon T.S., Chapman M.H. and Smith G.E. 2007. In vitro binding of bile acids by okra, beets, asparagus, eggplant, turnips, green beans, carrots, and cauliflower. Food Chem. 103:676-680.

Kamga T.R., Kouamé C., Atangana A.R., Chagomoka T. and Ndango R. 2013. Nutritional evaluation of five African indigenous vegetables. J. Hortic. Res. 21(1):99-106.

Kemi V.E., Kärkkäinen M.U., Rita H.J., Laaksonen M.M., Outila T.A. and Lamberg-Allardt C.J. 2010. Low calcium:phosphorus ratio in habitual diets affects serum parathyroid hormone concentration and calcium metabolism in healthy women with adequate calcium intake. Br. J. Nutr. 103(4):561-568.

Khan M.I. and Giridhar P. 2015. Plant betalains: Chemistry and biochemistry. Phytochemistry 117:267-295.

Li H., Deng Z., Liu R., Zhu H., Draves J., Marcone M., Sun Y. and Tsao R. 2015. Characterization of phenolics, betacyanins and antioxidant activities of the seed, leaf, sprout, flower and stalk extracts of three Amaranthus species. J Food Compos Anal. 37:75-81.

Mann J.I. and Cummings J.H. 2009. Possible implications for health of the different definitions of dietary fibre. Nutr. Metab. & Cardiovasc Dis. 19(3):226-229.

Matraszek R., Szymańska M. and Wróblewska M. 2002. Effect of nickel on yielding and mineral composition of the selected vegetables. Acta Sci. Pol., Hortorum Cutlus. 1(1):13-22.

Mlyneková Z., Chrenková M. and Formelová Z. 2014. Cereals and legumes in nutrition of people with celiac disease. International Journal of Celiac Disease. 2(3):105-109.

Modil A.T. 2007. Growth temperature and plant age influence on nutritional quality of Amaranthus leaves and seed germination capacity. Water SA. 33(3):369-375.

Moreno D.A., Garcia-Viguera C., Gil J.I. and Gil-Izquierdo A. 2008. Betalains in the era of global agri-food science, technology and nutritional health. Phytochem Rev. 7:261-280.

Mota C., Santos M., Mauro R., Samman N., Matos A.S., Torres D. and Castanheira I. 2016. Protein content and amino acids profile of pseudocereals. Food Chem. 193:55-61.

Ngugi C.C., Oyoo-Okoth E., Manyala J.O., Fitzsimmons K. and Kimotho A. 2017. Characterization of the nutritional quality of amaranth leaf proteinconcentrates and suitability of fish meal replacement in Nile tilapiafeeds. Aquaculture Reports. 5 62-69.

O'Brien G.K. and Price M.L. 2008. Amaranth. Grain and Vegetable. ECHO Technical Note. www.echonet.org.

Onwordi C.T., Ogungbade A.M. and Wusu A.D. 2009. The proximate and mineral composition of three leafy vegetables commonly consumed in Lagos, Nigeria. Afr. J. Pure Appl. Chem. 3(6):102-107.

Panel on Dietetic Products, Nutrition and Allergies. 2013. Scientific Opinion on Dietary Reference Values for manganese. EFSA Journal 11(11):3419.

Piattelli M., Giudici di Nicola M. and Castrogiovanni V. 1969. Photocontrol of amaranthin synthesis in Amaranthus tricolor. Phytochemistry 8:731-736.

Piątkowska E., Witkowicz R., Janeczko Z., Kopeć A., Leszczyńska T., Pisulewska E. and Suchecki Sz. 2015. Basic chemical composition and antioxidant activity leaves of selected buckwheat's varietes and tartary buckwheat. Fragm. Agron. 32(1):92-100 .

Poli-Swain T. and Hillis W.E. 1959. The phenolic constituents of *Prunus domestica*. The quantitative analysis of phenolic constituents. J. Sci. Food Agric. (10):63-68.

Pospišil A. and Pospišil M. 2008. Green mass and dry matter yield, and nutritional value of forage sorghum and amaranth at different growth stages. CLUJ-Napoca Agriculture 65(1):338.

Pospišil A., Pospišil M., Maćešić D. and Svečnjak Z. 2009. Yield and quality of forage sorghum and different amaranth species (*Amaranthus* spp.) biomass. Agric. Conspec. Sci. 74(2):85-89.

Prashanth L., Kattapagari K.K., Chitturi R.T., Ramana V., Baddam R. and Prasad L.K. 2015. A review on role of essential trace elements in health and disease. Intr. Univ. Health Sci. 4(2):75-85.

Re R., Pellegrini N., Proteggente A., Pannala A., Yang M. and Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assey. Free Radic. Biol. Med. 26(9-10):1231-1237.

Schliemann W., Cai Y., Degenkolb T., Schmidt J. and Corke H. 2001. Betalains of Celosia argentea. Phytochemistry. 58(1):159-165.

Stintzing F.C., Schieber A. and Carle R. 2003. Evaluation of color properties and chemical quality parameters of cactus juices. Eur Food Res Technol. 216:303-311.

Stintzing F.C., Kammerer D., Schieber A., Adama H., Nacoulma O.G. and Carle R. 2004. Betacyanins and phenolic compounds from *Amaranthus spinosus* L. and *Boerhaavia erecta* L. Z. Naturforsch. 59 (1-2):1-8.

Taira J., Tsuchida E., Katoh M.C., Uehara M. and Ogi T. 2015. Antioxidant capacity of betacyanins as radical scavengers for peroxyl radical and nitric oxide. Food Chem. 166:531-536.

Tesoriere L., Allegra M., Gentile C. and Livrea M.A. 2009. Betacyanins as phenol antioxidants. Chemistry and mechanistic aspects of the lipoperoxyl radical-scavenging activity in solution and liposomes. Free Radic. Res. 43(8):706-717.

Van Soest P.J., Robertson J.B. and Lewis B.A. 1991. Methods for dietary fiber, neutral detergent fiber and nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci. 74(10):3583-3597.

Venskutonis P.R. and Kraujalis P. 2013. Nutritional components of amaranth seed and vegetables:A reviev on composition, properties, and uses. Compr. Rev. Food Sci. F. 12:381-412.

Witkowicz R., Pisulewska E., Leszczyńska T., Piątkowska E. and Kidacka A. 2015. Basic chemical composition and antioxidant activity of different genotype of oat (*Avena sativa*). Żywność Nauka Technologia Jakość. 22(4):176-187.

Wyler H. and Meuer U. 1979. Zur biogenese der betacyane:Versuche mit [2-14C]-dopaxanthin. Helv Chim Acta. 62:1330-1339.

Yarnia M. 2010. Sowing dates and density evaluation of amaranth (Cv. Koniz) as a new crop. Adv Environ Biol. 4(1):41-46.

Ye C.X., Liu Y.J., Tian L.X., Mai K.S., Zhen Yu. Du., ZY Yang H.J. and Niu J. 2006. Effect of dietary calcium and phosphorus on growth, feed efficiency, mineral content and body composition of juvenile grouper, Epinephelus coioides. Aquacult. 255:263-271.

Yusuf A.A., Mofio, B.M. and Ahmed A.B. 2007. Proximate and mineral composition of *Tamarindus indica* Linn. 1753 seeds. Sci. World J. 2:1-4.

Zabłocka-Słowińska K. and Grajeta H. 2012. The role of manganese in etiopathogenesis and prevention of selected diseases. Postępy Hig Med Dosw. 66:549-553.

Paper Received January 1, 2017 Accepted July 20, 2017

PAPER

TOTAL PHENOLIC CONTENT, ANTIOXIDATIVE AND ANTIDIABETIC PROPERTIES OF COCONUT (*COCOS NUCIFERA L.*) TESTA AND SELECTED BEAN SEED COATS

K.A. ADEKOLA[a], A.B. SALLEH[a], U.H. ZAIDAN[a], A. AZLAN[b], E. CHIAVARO*[c], M. PACIULLI[c] and J.M.N. MARIKKAR[d]**

[a]Department of Biochemistry, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia
[b]Department of Nutrition and Dietetics, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia
[c]Department of Food and Drug, Parco Area delle Scienze 47/A, University of Parma, 43124 Parma, Italy
[d]International Institute for Halal Research & Training, International Islamic University Malaysia, PO Box 10, 50728 Kuala Lumpur, Malaysia
*Corresponding author. Tel.: +39 0521905888; fax: +39 0521906028
E-mail address: emma.chiavaro@unipr.it
**Corresponding author. Tel.: +603 61965619; fax: +603 61965596
E-mail address: nazrim@iium.edu.my

ABSTRACT

Natural alternatives for the treatment of diabetes mellitus have been the interest of many researchers. In this study, the brown testas of mature coconuts were compared to beans seed coats of four varieties in terms of antioxidative and anti-hyperglycaemic properties. The total phenolic and flavonoid contents, the antioxidant potentials and the α-amylase and α-glucosidase inhibitory activities of the crude extracts were studied *in vitro*. The results showed that extracts of coconut testa and red kidney bean seed coat displayed higher α-glucosidase inhibition (IC_{50}=19.90±5.67 and 4.84±1.43 μg/mL) and α-amylase inhibition (IC_{50}=120.5±15.4 and 532.8±68.0 μg/mL) than the other extracts. These two extracts showed higher antioxidant capacities owing to their high phenolic and flavonoid contents. These results suggest that red kidney bean seed coat and tender coconut testa would have higher potential as nutraceuticals and could serve as natural alternative sources of anti-diabetic remedy.

Keywords: anti-diabetic, antioxidants, polyphenolic compounds, coconut testa, bean seed coat

1. INTRODUCTION

Consumption of wholesome food is recognized as an important step to maintain optimal health. In this respect, consumption of whole grains and cereals is recommended as diet to prevent chronic diseases such as diabetes and cancer. As whole grains are rich in vitamins, minerals, and phytochemicals, they are believed to help reduce the risk factors associated with diabetes. Dry beans that include red bean, red kidney bean, white bean and black bean are recognized to have many nutritional attributes. According to XU *et al.* (2007), red bean and red kidney beans are rich in nutrients and dietary fibre and were found to display higher content of phenolics and antioxidant activity. LAYER *et al.*(1985) found that white bean *in vitro* was able to inhibit α-amylase. Recent investigations of TANKO *et al*. (2017) concluded that bran of *Beras merah* and *Beras hitam* rice varieties had high potential to be used in dietary formulations intended for diabetes.

The recent trend advocates exploring the food values of tropical fruits such as coconut for healthy living. The most important part of coconut is kernel or endosperm, which is usually found to possess moisture, fat, protein, carbohydrates, minerals and vitamins. The oil extracted from the kernel is reported to have health benefits owing to the presence of lauric, capric, caproic and caprylic acid (MARIKKAR and MADHRAPPERUMA, 2012). The defatted kernel residue left after extraction of virgin coconut oil is another valuable product. The fibrous component of the defatted kernel residue is of particular interest due to the beneficial health effects (AIN NAJWA *et al*., 2016). As a source of dietary fibre, defatted kernel residue can provide health benefits particularly in relation to chronic illnesses such as cancer and diabetes mellitus. TRINIDAD *et al*. (2003) found that the incorporation of defatted coconut flour in bakery products would lower their glycemic indices. Other than these, there are other reports, which indicated that defatted coconut flour might contribute to reduce the concentration of cholesterol in blood (SENEVIRATNE *et al*., 2009)

The brownish outer covering of coconut kernel serving as a protective layer in the fruit is an interesting by-product, traditionally known as coconut testa (SENEVIRATNE *et al*., 2009). Owing to its dark brown color, testa is usually removed before processing coconut products such as desiccated coconut, coconut cream and milk powder. The testa of coconut constitutes approximately 18% (w/w, wet basis) of the total kernel weight (MARIKKAR and MADHRAPPERUMA, 2012). In an attempt to utilize coconut testa for edible oil production, APPAIAH *et al*. (2014) came to know that the oil was composed of several short and medium chain fatty acids along with phenolics, phytosterols, tocopherols, tocotrienols etc. These studies indicated that the fruit component of coconut such as coconut kernels might have medicinal properties due to these valuable constituents (DEBMANDAL AND MANDAL, 2011). Recently, SALIL *et al*., 2011 found that coconut kernel protein has potent anti-diabetic activity through the reversal of glycogen levels and activities of carbohydrate metabolizing enzymes. To date, no report compared the anti-hyperglycaemic activity of coconut testa with those of common beans namely, red kidney bean, black-eyed pea, red bean and black beans. As the removed testa is largely wasted in processing factories without any value addition, it is timely to undertake further studies on it. The results might have various direct and indirect environmental and economic impacts reducing the disposal costs and increasing the added value of the final products, giving emphasis to explore their health benefits. If the removed testa is crushed in the dry form, it might give flour, which could be used for preparation of wholemeal bread beneficial to those who suffer from type 2 diabetes. Hence, the objective of this study was to compare brown testa of coconut with seed coats of four varieties of beans with respect to total flavonoid and phenolic contents, and antioxidative and anti-hyperglycemic properties.

2. MATERIALS AND METHODS

2.1. Sample collection and preparation

Samples of brown testa from coconut (TCO) were collected in triplicate from the coconut farm located in the university agricultural park, Serdang, Malaysia. Samples of four varieties of *Phaseolus vulgaris* (red kidney bean (RKB), black-eyed pea (BEP), red bean (RB) and black beans (BB) were collected in triplicate from individual farms located at Setiawan, Malaysia. The bean samples were dehulled manually while the testae of coconuts were removed from their shells to be dried and milled into fine particles. Samples were packed in airtight Ziploc plastic bags and stored at 4°C until further analysis.

2.2. Chemicals

Pancreatic α-amylase, α-glucosidase (*Saccharomyces cerevesiae*), 1,1-diphenyl-2-picrylhydrazine (DPPH), hydrochloric acid, p-nitrophenyl- α-d-glucopyranoside were purchased from Sigma-Aldrich. Chemicals, namely, sodium carbonate, sodium acetate buffer (pH 6), sodium potassium tartarate, and sodium hydroxide were purchased from R&M chemicals. Other chemicals such as; dinitrosalicyclic acid (DNS), potassium persulphate were purchased from Acros Organics (Private) Ltd. 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonicacid) diammonium salt (ABTS) from Amresco, Folin Ciocalteu phenol reagent and gallic acid from Cayman. 2,4,6-tripyridyl-s-triazine (TPTZ) was purchased from Affymetrix.

2.3. Preparation of ethanolic extracts of sample

Hundred (100) gram portions of the milled bean seed coats and coconut testa were extracted with 1000 mL of 70% ethanol-water for 48 h at 25 °C. The extracts were centrifuged with eppendorf 8510R centrifuge at 8300 rpm for 10 min and filtered with Whatman no. 1 filter paper. The solvent was evaporated under reduced pressure using a Büchi Model R-205 rotary evaporator (Büchi, Switzerland) and the semi-solid extracts were freeze-dried using a Virtis bench top pro-freeze dryer SJIA-10N (FD-1B-50) (Virtis, New York). They were then re-dissolved in 70% ethanol in the ratio of 10mg/mL of solvent to serve as stock solution for various analyses.

2.4. Total phenolic content (TPC)

TPC was assayed using Folin-Ciocalteu regent in a 96-well microplate according to the method described by SINGLETON *et al*. (1999). About 20 µL of the sample extract was mixed with 110 µL of freshly prepared Folin-Ciocalteu regent. Subsequently, the mixture was added with 70 µL of 20% Na_2CO_3 and allowed to incubate for 30 min at room temperature. After incubating for 30 min at 25±2°C, absorbance was read at 765 nm using a microplate reader (Synergy 2, BioTek instruments, USA). Total phenolic content of each sample was calculated from the mean of triplicates and expressed as milligrams (mg) of gallic acid equivalent (GAE) per 1g of dry sample.

2.5. Total flavonoid content (TFC)

Total flavonoid content of the extracts was determined according to the method described by ZHISHEN *et al.* (1999) with some modifications. Briefly, 25 µL of the ethanolic extract,

125 μL of distilled water and 7.5 μL of 5% $NaNO_2$ were mixed together and the mixture was allowed to stand for 5 min. After adding 15 μL of 10% $AlCl_3$, the mixture was allowed to incubate at 25°C for an additional 5 min. At the 6th min, the mixture was added with 50 μl of 1M NaOH and subsequently diluted by adding 27.5 μl of distilled water. Absorbance of the solution was measured at 515 nm using a microplate reader (Synergy 2, BioTek instruments, USA). Total flavonoid content of each sample was calculated from the mean of triplicates and expressed as milligram (mg) quercetin equivalent (QE) per 1g of dry sample.

2.6. Analysis of flavonoid and phenolic acids by HPLC

Chromatographic analysis was performed on Agilent 1100 series HPLC-DAD system (Agilent Technologies Deutschland GmbH, Germany) using a reverse phase C18 column (150 X 4.5 mm) packed with 5 μm diameter particles which was controlled at a temperature of 30°C. The flow rate was 0.7 mL/min and the injection volume was 50 μL. The mobile phase consisted of 2% acetic acid (Solvent A) and absolute methanol (Solvent B), and the composition gradient was run as follows: 13% of B until 10min and changed to obtain 20%, 30%, 50%, 70%, 20% and 10% B at 20, 30, 40, 50, 60, 70 and 80 min respectively, following the method described by IRONDI *et al*. (2014) with slight modifications. A stock standard solution of individual compound was prepared in methanol at a concentration range of 0.030-0.250 mg/mL for quercetin, kaempferol, catechin, rutin and epicatechin; and 0.050-0.450 mg/mL for caffeic, ferulic, gallic, ellagic and chlorogenic acids. Peaks of chromatograms were confirmed by comparing its retention time with those of reference standards. Gallic acid was detected at 257 nm, catechin and epicatechin at 281 nm, ferulic, chlorogenic, ellagic and caffeic acids at 325 nm and kaempferol, quercetin and rutin at 366 nm. All analyses were carried out in triplicates.

2.7. Antioxidant analyses

2.7.1. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was performed according to the method described by BENZIE and SZETO (1999) in a 96-well microplate. The working FRAP reagent was prepared by mixing 300 mM of acetate buffer (pH 3.6), 10 mM of TPTZ solution and 20 mM of $FeCl_3.6H_2O$ in a ratio of 10:1:1 and then heating to 37°C. The TPTZ solution was prepared by dissolving 10mM TPTZ in 40 mM HCL. A 20 μL sample solution was added to a mixture of 150 μL of working FRAP reagent and 30 μL of acetate buffer. The whole reaction mixture was allowed to stand for 8 min and the absorbance was recorded at 600 nm. Results were expressed as mmol of $FeSO_4$ per 100 g of the dry sample.

2.7.2. DPPH radical scavenging activity

DPPH scavenging activity was performed according to the method described by BLOIS (1958) in a 96-well microplate. A 150 μL portion of 125 μM DPPH was mixed with 50 μL of sample extract with varying concentrations ranging from 3.12-200 μg/mL in a microplate, the mixture was allowed to incubate at 25±2°C for fifteen minutes in the dark and the absorbance was measured at 517 nm using a Biotek microplate reader.

The percentage of free radical scavenging activity was calculated as follows:

$$\% \text{ DPPH Scavenging effect} = \frac{Ac - As}{Ac} x100$$

Where Ac = absorbance of control, As = absorbance of sample.

2.7.3. $ABTS^{\cdot}$ radical scavenging activity

The $ABTS^{\cdot}$ radical scavenging assay was performed according to the method described by RE *et al.* (1999) in a 96-well microplate. A stable stock solution of $ABTS^{\cdot}$ radical was obtained by mixing 7.8 mM ABTS and 2.45 mM of potassium persulfate at 37°C for 16 h in the dark. The $ABTS^{\cdot}$ radical stock solution was diluted with 80% ethanol until an absorbance of 0.7±0.02 at 734nm was achieved. 40 µL of varying concentrations of sample extracts (3.12-200 µg/mL) were added to 160 µL of diluted $ABTS^{\cdot}$ stock solution. The mixture was incubated at 25±2°C for 10 min and the absorbance was measured at 734 nm using a Biotek microplate reader. The percentage of free radical scavenging activity was calculated as follows:

$$\% \text{ ABTS Scavenging effect} = \frac{Ac - As}{Ac} x100$$

Where Ac = absorbance of control, As = absorbance of sample.

2.8. Enzyme inhibition assays

2.8.1. α-Amylase inhibition assay

The α-Amylase inhibition assay was performed according to the method described by BERNFELD (1955) with modifications in a 96-well microplate. Briefly, 50 µL of varying concentrations (12.5-200 µg/mL) of ethanolic sample extract was added to 40 µL of cooked starch (1% w/v) and 50 µL of α-amylase enzyme (0.5 mg/mL), 860 µL of 100 mM acetate buffer (pH 6.0) was added to the mixture and incubated at 40°C for 15 min. 0.5 mL of DNS (3,5-dinitrosalicyclic acid) was added after incubation and then placed in a boiling water bath for 5 min. It was then cooled in cold-water bath and the absorbance was recorded at 540 nm. The percentage inhibition of each sample was calculated using the following formula:

$$\text{Inhibition (\%)} = \frac{Ac - (As - Ab)}{Ac} x100$$

Where Ac = absorbance of control, Ab = absorbance of blank and As = absorbance of sample.

2.8.2. α-Glucosidase inhibition assay

The α-glucosidase inhibition assay was done according to the method described by MATSUI *et al.* (2001) with modifications, in a 96-well microplate. Reaction mixtures of 20 µL of 4 mM of p-nitrophenyl-α-d-glucopyranoside , 30 µL of 50 mU/mL of α-glucosidase enzyme and 40 µL of different concentrations (2.5-80 µg/mL) of sample extracts (1mg of

each of the freeze-dried sample was reconstituted in 1mL of 50mM sodium acetate buffer)were added together to give a volume of 100 μL in 50 mM sodium acetate buffer (pH 5.8). The mixtures were incubated at 37°C for 30 min after which the reaction was stopped by addition of 50 μL of 0.1M sodium carbonate. Absorbance was measured at 405 nm using a 96-well microplate reader. The inhibition of α-glucosidase in percentage was calculated using the following equation:

$$\text{Inhibition (\%)} = \frac{Ac - (As - Ab)}{Ac} x100$$

Where Ac = absorbance of control, Ab = absorbance of blank and As = absorbance of sample.

2.9. Statistical Analysis

All measurements were carried out in triplicate data (n=3). All the results were presented in the form of mean±standard deviation (SD). Data was statistically analyzed by one-way analysis of variance (ANOVA) with IBM SPSS software package (version 21.0). When *F* values were significant, mean differences were compared using Duncan's multiple range test at the 5% level of probability.

3. RESULTS AND DISCUSSION

3.1. Total phenolic and flavonoid contents

The results of the total phenolic and flavonoid contents of the extracts of coconut testa and bean seed coats were compared as shown in Table 1. The TPC ranged from 3.46 to 44.61 GAE (mg) per gram of dry sample with the highest phenolic content being displayed by TCO. All tested bean seed coat extracts were significantly ($p<0.05$) different in their TPC values except BB and RB. In the case of TFC, the values were found to range from 2.30 to 67.60 QE (mg) per gram of dry sample with the highest value (67.60 mg QE/g) being displayed by TCO. Among the bean seed coat extracts, RKB showed the highest TPC and TFC values. A number of previous studies discussed about the phenolic and flavonoid contents of beans. According to WU *et al.* (2004), RKB was ranked the first in the USDA's list of top 20 foods with high antioxidant capacity. However, the differences in phenolic contents of beans might also be due to genetic factors, variation between cultivars, and extraction methods (ZHAO et al., 2014) There was hardly any detailed study to compare the TPC and TFC of the TCO, which was higher than those of other extracts namely, RKB, RB, BEP, and BB. Although RAIHANA *et al*. (2015)previously discussed the food values of several fruit seed wastes, TCO was not mentioned despite it being a by-product of coconut processing. According to MARIKKAR and MADURAPPERUMA (2012) there was hardly any data on TPC of TCO but the fatty acid compositions of various oil samples were available. As SENEVIRATNE *et al*. (2009) compared the distribution of the phenolic contents in coconut oil extracted under two different methods, the phenolic content was increased with the addition of TCO into white coconut kernel. In another study, APPAIAH *et al*. (2014) confirmed that the oil extracted from TCO was composed of phenolics, phytosterols, tocopherols, tocotrienols etc. Generally, the outer layers such as seed coats and testa would have polyphenolic compounds due to their protective role in plants. Moreover, dark colours of seed coats were generally found to correlate with higher phenolic contents (KANATT *et al*., 2011).

Table 1. Total phenolic and flavonoid contents and ferric reducing antioxidant power of coconut testa and different bean seed coats.

Type of extract	Total Phenolic content (mgGAE/g)	Total flavonoid content (mgQE/g)	Reducing Power (FRAP) mmol FeSO4/100g
RKB	21.80±0.51b	24.38±1.22b	204.71±2.87b
RB	5.29±0.47c,d	6.29±1.21c	48.45±7.34d
TCO	44.61±0.56a	67.60±6.99a	546.10±36.90a
BEP	3.46±0.25d	2.30±0.38c	26.70±4.43d
BB	9.19±0.53c	7.35±1.30c	68.68±3.30c

Data represented as mean±sd of triplicate analysis. Mean values within the column with different superscript letters are significantly ($p < 0.05$) different. Abbreviations: RKB, red kidney bean seed coat; RB, red bean seed coat; TCO, testa of coconut; BEP, black-eyed pea seed coat; BB, black bean seed coat.

The HPLC- DAD analysis data shown in Table 2 compared the flavonoid and phenolic acid compositions of TCO and bean seed coat extracts. Phenolic compounds are essential constituents of plants due to their ability to scavenge free radicals; they are known for their alleviation of oxidative stress as well as beneficial effects on human health.

Table 2. Phenolics composition of coconut testa and selected beans seed coats by HPLC-DAD analysis[1].

Phenolic compounds (mg/g)	TCO	RKB	RB	BB	BEP
Gallic acid	0.42±0.03	0.01±0.01	0.16±0.02	0.11±0.02	0.04±0.00
Catechin	-	0.18±0.09	-	-	-
Epigallocatechin gallate	5.747±0.54	-	-	-	-
Chlorogenic acid	0.625±0.24	-	-	-	-
Caffeic acid	1.752±0.08	-	-	-	-
Ferulic acid	-	-	-	-	-
Ellagic acid	-	-	0.91±0.03	4.85±0.02	7.25±0.06
Rutin	-	-			1.210±0.01
Quercetin	-	-	0.17±0.00	0.69±0.27	0.05±0.00
Kaempferol	-	-	-	-	-

[1]Abbreviations: See Table 1.

According to IRONDI *et al*. (2014) the most abundant phenolic acid in plants is caffeic acid followed by chlorogenic acid while quercetin and rutin are two important flavonoids in a number of food items. According to Table 2, the extracts contain varied levels of phenolic compounds which include; gallic acid, ferulic acid, chlorogenic acid, ellagic acid, catechin, epicatechin, caffeic acid, quercetin, rutin and kaempferol. The concentrations of the different phenolic compounds ranged from 0.03-0.25 mg/mL for phenolic acids and 0.05-0.45 mg/mL for flavonoids. Gallic acid was the most common phenolic compound present among the extracts with the highest amount observed in TCO. Previously, SENEVIRATNE *et* al. (2009) noticed that coconut oil extracted by incorporating TCO had compounds such as gallic acid, epigallocatechin, catechin, p-hydroxybenzoic acid, epi-catechin and caffeic acid. The level of gallic acid in RB seed coat and RKB seed coat were quite comparable.

Some phenolic compounds such as ellagic acid, caffeic acid and rutin were noticeably higher in certain extracts while phenolic compounds such as ferulic acid and kaempferol were observed to be absent in all extracts.

3.2. Antioxidant activities

A comparison of the FRAP activities of the extracts of TCO and bean seed coats are presented in Table 1. The FRAP values ranged from 26.70 to 546.10 mmol $FeSO_4$/100g extracts with the highest and lowest values being displayed by TCO and BEP, respectively. According to our literature search, only few studies were available to compare the FRAP value of TCO. Previously, SENEVIRATNE *et al*. (2009) reported the phenolic-dependent antioxidant capacities of coconut oil without giving consideration for FRAP activity. The FRAP values of bean seed coats used in this study were found to range from 26.7 to 204.71 mmol $FeSO_4$/100g. The values displayed significant ($p<0.05$) differences among the extracts of RKB, RB, BB, and BEP. Interestingly, bean seed coat extracts of BEP, RKB and BB had displayed better antioxidant capacities than the seed extracts of the same reported previously by MARATHE *et al.* (2011). BOATENG *et al.* (2008) stated that dark coloured seed coats usually had higher FRAP values than the pale coloured ones, which might be due to higher amount of phenolic compounds in them. According to some other reports, high FRAP values of grains such as red bean were due to the high content of condensed tannins (ZOU and CHANG, 2014). The results of DPPH radical scavenging activities of the extracts of TCO and bean seed coats were compared as shown in Fig. 1.

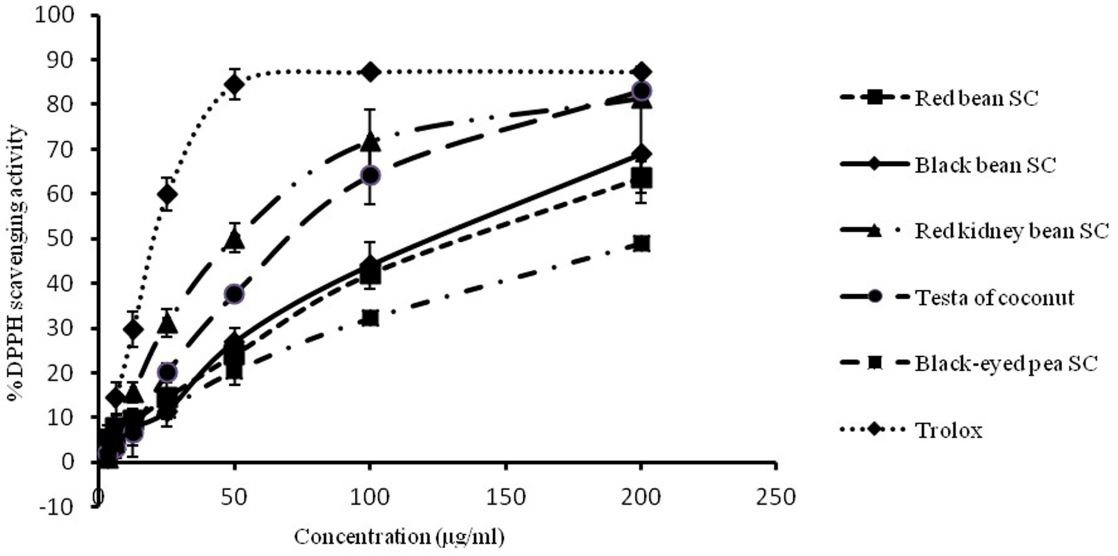


Figure 1. DPPH radical scavenging activities of coconut testa and different bean seed coats. Values represent mean±s.d. of triplicate analysis.

Previously, BOATENG *et al*. (2008) reported the DPPH radical scavenging activities of several common beans, but there was hardly any comparison with TCO. According to Fig. 1, the radical scavenging activities of all extracts were ranged from 48.97 to 83.21% as

observed at 200 μg/mL. The values displayed by TCO and RKB were 83.21% and 81.53%, respectively. The strong radical scavenging potentials of TCO and RKB might be due to their higher phenolic and flavonoid contents as seen before (Table 1).
According to Table 3, there was no significant ($p>0.05$) difference between the EC_{50} values of RB and BB while the EC_{50} of other tested extracts were significantly ($p<0.05$) different with respect to each other.

Table 3. DPPH and ABTS radical scavenging activities of coconut testa and selected bean seed coats.

Type of extract	DPPH EC50 (μg/mL)	ABTS EC50 (μg/mL)
RKB	63.6±3.5c	111.3±0.6c
RB	144.2±6.0b	266.5±36.2b
TCO	47.4±7.0d	125.7±6.7c
BEP	188.2±1.9a	375.7±11.6a
BB	135.4±19.0b	260.5±36.8b
TROLOX	22.4±1.7e	32.0±1.2d

Values represent mean±sd of triplicate analysis. Mean values within the column with different superscript letters are significantly ($p<0.05$) different; Abbreviations: See Table 1.

Previously, GARCIA-LAFUENTE *et al.*(2014) found that the extracts of RB displayed higher radical scavenging activity than those of white ones, which was in agreement with the results obtained in the current study. Separately, HUANG *et al*. (2012) stated that factors such as soil types, genes, temperature, light and agronomic conditions could also affect anthocyanin composition which was an important antioxidant constituent of plants. Seed coats are generally similar to hulls that are rich in polyphenolics and can act as natural antioxidants to counter the formation of free radicals that can lead to the onset of various diseases including diabetes, and cancer (BOATENG *et al*., 2008).
ABTS activities of the extracts of TCO and bean seed coats are shown in Fig. 2.
Both the RKB and TCO displayed maximum scavenging activities of 83.3% and 76.37%, respectively at the concentration of 200 μg/mL. According to Table 1, the phenolic and flavonoid contents of both RKB and TCO extracts were relatively higher than those of any other extract, which might have contributed to their strong antioxidant activity. It has been reported that phenolics with higher molecular weight (e.g. tannins) were greater in ability to scavenge free radicals (SIDDHURAJU and BECKER, 2007) The higher the phenolic content of an extract, the stronger the antioxidant activity that they tend to exhibit (GARCÍA-LAFUENTE *et al.*, 2014). ABTS values of the extracts of the other samples showed significant ($p<0.05$) differences. According to Table 3, there were no ($p>0.05$) significant differences in the EC_{50} values of the RKB and TCO, as well as the EC_{50} values of BB and RB extracts. However, significant ($p<0.05$) differences were observed between the extracts of BEP and other samples. Most of the extracts exhibited strong scavenging ability at all the tested concentrations (3.12 – 200 μg/mL). Extracts of RKB and TCO showed somewhat similar activities.

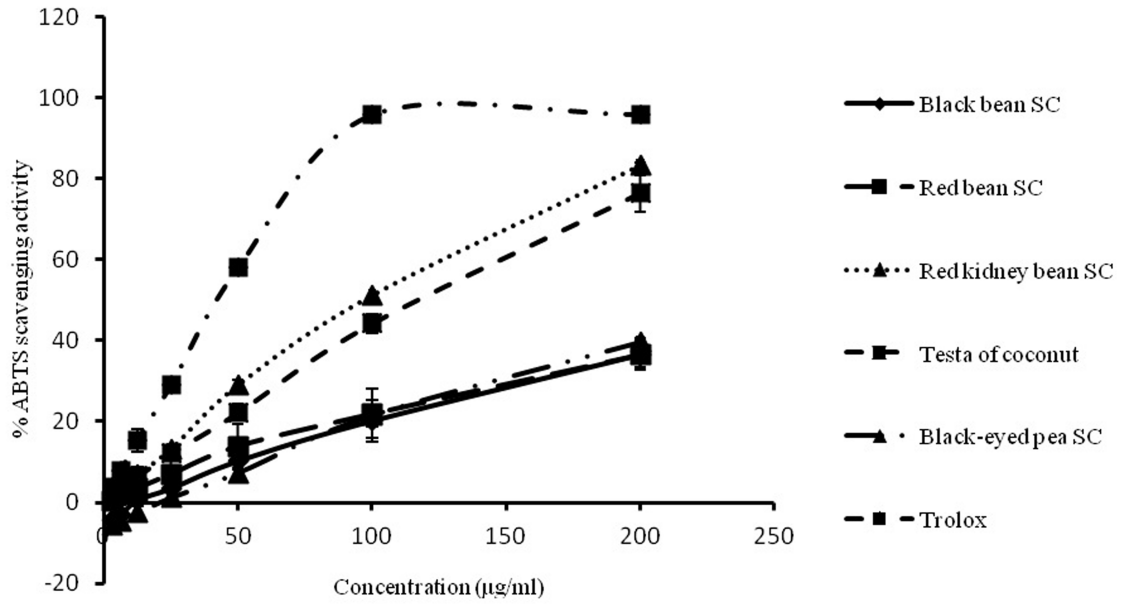


Figure 2. ABTS radical scavenging activities of coconut testa and different bean seed coats. Values represent mean±s.d. of triplicate analysis.

3.3. Enzyme inhibitory activities

3.3.1. α-Amylase inhibitory activity

The α-amylase inhibitory potentials of the extracts of TCO and bean seed coats were compared as shown in Table 4. Inhibition of carbohydrate degrading enzymes is one of the approaches to decrease the rate of glucose released into the bloodstream (ADEMILUYI and OBOH, 2013). MOJICA *et al.*(2015) stated that common beans contain bioactive substances such as phenolics, which help in inhibiting digestive enzymes by binding to these enzymes and modifying their activity. At the highest concentration of 200 μg/mL, the highest activity was displayed by RKB (77.02%) while the least activity was recorded for TCO (24.83%). When the efficacy of various extracts were compared using their IC_{50} values, the values of the test samples were ranged from 120.5 to 612.3 μg/mL while the IC_{50} of acarbose (positive control) was 115.9 µg/mL (Table 4). This indicated that the positive control exhibited stronger inhibition towards α-amylase when compared with the test extracts. Significant ($p<0.05$) differences were noticed among the extracts such as; RB, TCO, BB, and BEP except between RKB and the positive control, which showed no significant difference. In this study, the anti-amylase activity of RKB was significantly higher than that of TCO. Plant-derived phenolic compounds have the potential to inhibit digestive enzymes, thus serving as natural therapeutic agents for the management of diabetes and its associated complications (IRONDI *et al.*, 2014).

3.3.2. α-Glucosidase inhibitory activity

The α-glucosidase inhibitory potentials of extracts of TCO and bean seed coats were compared as shown in Table 4. Reducing the rate at which starch is being broken down and absorbed may be beneficial to manage insulin resistance in diabetic patients (UDDIN *et al.*, 2014). Various extracts showed a dose-dependent increase in their inhibition towards α-glucosidase.

Table 4. α-Amylase and α-Glycosidase inhibitory effects of coconut testa and bean seed coats[1].

Samples	α-Amylase IC50 (μg/mL)	α-Glucosidase IC50 (μg/mL)
RKB	120.5±15.4d	19.9±5. 7c
RB	612.3±113.0a	29.9±1.4b
TCO	532.8±68.0b	4.8±1.4d
BEP	573.6±68.1a,b	32.0±9.5b
BB	319.3±36.0c	14.9±4.2c
Acarbose	115.9±11.0d	43.4±3.7a

[1]Values represent mean±standard deviation of triplicate analysis. Mean values in the same column with different superscript letters are significantly different ($p<0.05$); RKB, red kidney bean seed coat, RB, red bean seed coat, TCO, testa of coconut, BEP, black-eyed pea seed coat, BB, black bean seed coat.

As shown in Table 4, the IC_{50} values of the test extracts in relation α-glucosidase inhibition were ranged from 4.84 to 32.03 μg/mL, with the IC_{50} value of acarbose being significantly ($p<0.05$) higher than those of RB, BB, RKB and BEP. However, IC_{50} values of BB and RKB were not significantly ($p>0.05$) different in the similar way between the IC_{50} values of RB and BEP. Since IC_{50} value of TCO was observed to be significantly lower than those of any other extract, it could be considered as potential alpha-glucosidase inhibitor for the management of diabetes. In fact, its inhibitory potential was even stronger than those of some other legumes reported previously by YAO *et al*. (2013).

4. CONCLUSIONS

In this study, polyphenolic contents, antioxidative properties, and α -amylase and α-glucosidase inhibitory potentials of TCO and selected bean seed coats were compared. Among the different extracts studied, TCO and RKB exhibited better antioxidant properties. This may be partly due to their high phenolic content; thus helping to scavenge free radicals and preventing oxidative damage in biological systems. TCO displayed a mild α-amylase inhibition but a strong α-glucosidase inhibition, which is a key characteristic feature of an effective anti-diabetic agent. Hence, TCO could possibly serve as a base material for preparation of food formulation suitable for diabetic patients.

ACKNOWLEDGEMENTS

The authors acknowledge partial funding from a research grant (FRGS/2/2013/SG01/UPM/02/5) awarded by Ministry of Higher Education Malaysia.

REFERENCES

Ain Najwa M.N., Shuhaimi M., Marikkar J.M.N., Yazid A.M.M. and Arbakariya A. 2016. Defatted coconut residue crude polysaccharides as potential prebiotics on proliferation and acidifying activity of probiotics in *in vitro*. J. Food Sci. Technol. In press.

Ademiluyi A.O. and Oboh G. 2013. Soybean phenolic-rich extracts inhibit key-enzymes linked to type 2 diabetes (α-amylase and α-glucosidase) and hypertension (angiotensin i converting enzyme) *in vitro*. Exp. Toxicol. Pathol. 65:305.

Appaiah P., Sunil L., Kumar P.K.P. and Krishna A.G.G. 2014. Composition of coconut testa, coconut kernel and its oil. J. Am. Oil Chem. Soc. 91:917.

Benzie I.F. and Szeto Y.T. 1999. Total antioxidant capacity of teas by the ferric reducing/antioxidant power assay. J. Agric. Food Chem. 47:633.

Bernfeld P. 1955. Amylases: alpha and beta. Methods Enzymol. **I:**149.

Blois M. 1958. Antioxidant determinations by the use of a stable free radical. Nature 181:1199.

Boateng J., Verghese M., Walker L.T. and Ogutu S. 2008. Effect of processing on antioxidant contents in selected dry beans (*Phaseolus spp.* L.). *LWT* - Food Sci. Technol., 41:1541.

Debmandal M. and Mandal S. 2011. Coconut (*cocos nucifera* l. :arecacea) :In health promotion and disease prevention. Asian Pac. J. Trop. Med. 1:241.

García-Lafuente A., Moro C., Manchón N., Gonzalo-Ruiz A., Villares A., Guillamón, Rostagno M. and Mateo-Vivaracho L. 2014. *In vitro* anti-inflammatory activity of phenolic rich extracts from white and red common beans. Food Chem. 161:216.

Huang W., Zhang H., Liu W. and Li C. 2012. Survey of antioxidant capacity and phenolic composition of blueberry, blackberry, and strawberry in Nanjing. J. Zhejiang Univ. Sci. B 13:94.

Irondi A.E., Oboh G., Akindahunsi A.A., Boligon A.A. and Athayde IM. 2014. Phenolic composition and inhibitory activity of *Magnifera indica* and *Mucuna urens* seeds extracts against key enzymes linked to the pathology and complications of type 2 diabetes. Asian Pac. J. Trop. Med. 4:903.

Kanatt S.R., Arjun K. and Sharma A. 2011. Antioxidant and antimicrobial activity of legume hulls. Food Res. Int. 44:3182.

Layer P., Carlson G.L. and DiMagno E.P. 1985. Partially purified white bean amylase inhibitor reduces starch digestion *in vitro* and inactivates intraduodenal amylase in humans. Gastroenterol., 88:1895.

Marathe S.A., Rajalakshmi V., Jamdar S.N. and Sharma A. 2011. Comparative study on antioxidant activity of different varieties of commonly consumed legumes in India. Food Chem. Toxicol. 49:2005.

Marikkar J.M.N. and Madhrapperuma W.S. 2012. Ch. 9. Coconut. In:"Tropical and Subtropical Fruits:Postharvest Physiology, Processing and Packaging", Muhammed Siddiq (Ed.). p. 159. John-Wiley Publishing Co., Ames, Iowa, USA.

Matsui T., Ueda T., Oki T., Sugita K., Terahara N. and Matsumoto K. 2001. α-glucosidase inhibitory action of natural acylated anthocyanins, 1. Survey of natural pigments with potent inhibitory activity. J. Agric. Food Chem. 49:1952.

Mojica L., Meyer A., Berhow M.A., González E. and Mejía D. 2015. Bean cultivars (*Phaseolus vulgaris L .*) have similar high antioxidant capacity, *in vitro* inhibition of α -amylase and α -glucosidase while diverse phenolic composition and concentration. *Food Res. Int.* 69:38.

Raihana A.R., Marikkar J.M.N., Ismail A., Musthafa S. 2015. A review on food values of selected fruits' seeds. Intel. J. Food Prop. 18:2380.

Re R., Pellegrini N., Pannala A., Yang M. and Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical. Free Radic. Biol. Med. 26:1231.

Salil G., Nevin K.G. and Rajamohan T. 2011. Arginin rich coconut kernel protein modulates diabetes in alloxan treated rats. Chem. Biol. Interact. 189:107.

Seneviratne K.N., Hapuarach C.D. and Ekanayake S. 2009. Comparison of the phenolic-dependent antioxidant properties of coconut oil extracted under cold and hot conditions. Food Chem. 114:1444.

Siddhuraju P. and Becker K. 2007. The antioxidant and free radical scavenging activities of processed cowpea (*Vigna unguiculata* (l.) walp.) seed extracts. Food Chem. 101:10.

Singleton V.L., Orthofer R. and Lamuela-Raventos R.M. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-ciocalteu reagent. Methods Enzymol. 299:152.

Tanko A.S., Marikkar N., Salleh A., Azlan A. and Jivan M. 2016. Evaluation of brans of different rice varieties for their antioxidative and antihyperglycemic potentials. J. Food Biochem. 41:e12295.

Trinidad T.P., Valdez D.H., Loyola A.S., Mallillin A.C., Askali F.C., Castillo J.C. and Masa D.B. 2003. Glycaemic index of different coconut (*Cocos nucifera*) – flour products in normal and diabetic subjects, Brit. J. of Nutri. 90**:**551.

Uddin R., Saha M.R., Subhan N., Hossain H., Jahan I.A., Akter R. and Alam A. 2014. HPLC-analysis of polyphenolic compounds in *Gardenia jasminoides* and determination of antioxidant activity by using free radical scavenging assays. Adv. Pharm. Bull. 4:273.

Wu X., Beecher G.R., Holden J.M., Haytowitz D.B., Gebhardt S.E., and Prior R.L. 2004. Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. J. Agric. Food Chem. 52:4026.

Xu B.J., Yuan S.H., and Chang S.K. 2007. Comparative analyses of phenolic composition, antioxidant capacity, and color of cool season legumes and other selected food legumes. J. Food Sci. **72**:167.

Yao Y., Yang X., Tian J., Liu C., Cheng X. and Ren G. 2013. Antioxidant and antidiabetic activities of black mung bean (*Vigna radiata* l.). J. Agric. Food Chem. 61:8104.

Zhao Y., Du S., Wang H. and Cai M. 2014. *In vitro* antioxidant activity of extracts from common legumes. Food Chem. 152:462.

Zhishen J., Mengcheng T. and Jianming W. 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem. 64:555.

Zou Y.P. and Chang S.K.C. 2014. Antioxidant and anti-proliferative properties of extract and fractions from small red bean (*Phaseolus vulgaris* L.). J. Food Nutri. 1:1.

Paper Received June 24, 2017 Accepted July 24, 2017

NUTRITIONAL VALUE OF RAW AND PROCESSED FILLETS OF BOLSENA LAKE WHITEFISH (*COREGONUS LAVARETUS* L.)

S. MATTIOLI[1]*, A. DAL BOSCO[1], E. ZINGONE[1], D. RANUCCI[2], C. CASTELLINI[1] and R. BRANCIARI[2]

[1]Department of Agricultural, Food and Environmental Science, University of Perugia, Borgo XX Giugno 74, 06121 Perugia, Italy

[2]Department of Veterinary Science, University of Perugia, Via S. Costanzo 1, 06124 Perugia, Italy

*E-mail address: simona.mattioli@hotmail.it

ABSTRACT

The aim of the study was to investigate and compare the nutritional value of raw and processed fillets (marinated and smoked) of whitefish from Bolsena Lake (Italy). The study was carried out in collaboration with the "Lago vivo" Fisherman Cooperative using 40 whitefish caught by net. The chemical composition showed increased nutrient (protein, lipid and carbohydrate) concentration with processing due to dehydration. Regarding the fatty acid profile of fillets, marinating was associated with higher levels of n-6 PUFAs, whereas the smoked and raw fillets showed higher concentrations of α-linolenic, eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids. Whitefish fillets were characterized by high nutritional value and good oxidative stability, mainly as smoked products.

Keywords: fillet, nutritional quality, processed, whitefish, fatty acids, food value

1. INTRODUCTION

Bolsena Lake is the largest volcanic lake in Europe and the fifth largest in Italy. It is located in the province of Viterbo in Northern Lazio on the border with Umbria and Tuscany. It has a drainage basin of 243 km^2, nearly half of which is occupied by the lake itself. Unlike other Italian lakes, Bolsena Lake is populated by a large number of fish species, including native herbivorous or insectivorous species (i.e. tench, chub, carp, rudd and sand smelt) and some predatory species such as pike, perch and eel. These are supported by other fish species added for specific human interest, such as whitefish and gambusia, or placed unconsciously as in the cases of bluegill, a voracious predator of young fish and eggs.
The European whitefish (*Coregonus lavaretus*), widely distributed in the freshwaters of Northern Europe (GOEBEL *et al.*, 2016) and introduced into the major lakes of Northern Italy, is present in the lakes of Central Italy mainly as result of stock enhancement practices. However, it is an uncommon species in our country and rarely present on consumer tables. In addition, the reduced surface area of the Bolsena Lake basins and the strong national tradition linked mainly to marine species of our country are limiting marketing and thus the spread of freshwater fish species in the domestic markets. As result, there is very little knowledge of whitefish nutritive characteristics among consumers.
The high value of meat and the economic interest in the Bolsena Lake whitefish has placed a spotlight on the return of the product under the collective brand "*Tuscia-Viterbese*", which is also very popular. A license for this kind of product can be issued by fishing companies and/or companies that process and/or market the species covered by this brand; for this reason, it is considered a local product. Furthermore, considering the extreme perishability of foodstuffs, conservation techniques for whitefish products are needed (YEANNES and CASALES, 2007), some of which have already come up from fishermen 2000 years ago.
Nowadays, the various storage systems are also largely used to distinguish products and to attract consumers (SOGN-GRUNDVAG *et al.*, 2014). Accordingly, a potential contribution to the economy of the fishermen's cooperative is certainly represented by the possibility of placing on the market differentiated products, such as smoked or marinated fillets, and to define their nutritional characteristics. Indeed, in accordance with EC Regulation 1169/2011, nutrition labelling on food is mandatory to achieve a high level of health protection for consumers and to guarantee access to accurate information.
In a previous work, ORBAN *et al.* (2006) studied the nutritional quality and safety of whitefish caught in three different Italian lakes, underlining their good protein and mineral contents and low lipid levels in the fillet of such species; beginning with this study, we wanted to deepen the existing knowledge on nutritional aspects of whitefish living in Bolsena Lake, both raw and processed (marinated and smoked), in order to add value to local products on the market.

2. MATERIALS AND METHODS

The study was carried out in collaboration with the "*Lago vivo*" Fisherman's Cooperative of Bolsena (Viterbo, Italy). Forty whitefish were caught by net in April 2016. All fish were immediately dipped in a mixture of water and ice and successively transferred to polystyrene boxes containing ice and transported under refrigerated conditions to the laboratory of the cooperative for processing as described below.

2.1. Fillets and processed preparation

Whitefish with an average live weight of 250 g were eviscerated after washing with running water, the heads and tails were removed, dorsal and ventral fillets were dissected and 20 of them were taken for analysis. The preparation procedure of the threads and the process (smoking and marinating) adopted provided for manual filleting with subsequent removal of the skin and scraps. At this point, the process followed two different paths: firstly, for the smoked product, the obtained fillets (approximately 100 g per fish) were salted and then smoked at 60 ± 5°C over beech wood for 4 hours; the next step was vacuum packaging and storage in a special cold storage (0-4°C) awaiting shipment to the various sales outlets.
Concerning the production of processed fillets, these were marinated for almost 12 hours in vinegar, lemon juice and spices with addition of olive oil, onion, celery, pepper and salt (approximately 30 g/kg of fillet), then the whole fillet was packed in packages of various weights (from 100 to 1000 g) in a modified atmosphere and stored in special refrigerated cells (0-4°C) awaiting shipment to different outlets. All fillet samples, raw and processed (n=20 per type), were stored at -30°C at the laboratories of the Department of Agricultural, Food and Environmental Science and Department of Veterinary Medicine of the University of Perugia until analysis (2 weeks later).

2.2. Proximate analyses and fatty acid composition

All samples were analysed in duplicate to determine the proximate composition. In detail, moisture, ash and total nitrogen were assessed using the AOAC methods (2000 N. 950.46, 923.03 and 991.15, respectively). Total protein nitrogen was calculated by the Kjeldahl method using 6.25 as the conversion factor. Total lipids were extracted in duplicate from 10 g of each homogenized sample and calculated gravimetrically (FRANCESCHINI *et al.*, 2015). The energy value and caloric value of the raw and processed fillets expressed in kJ/g and kcal/g, respectively, was calculated following the criteria of EU Regulation 1169/2011.
Fatty acids (FA) were determined by gas chromatography after lipid extraction according to the method proposed by FRANCESCHINI *et al.* (2015). Approximately 10 g of fish were homogenized with 5 mL of 0.5 M sodium acetate-water solution using an Ultraturrax (T25 basic, IKA, Labortechnik, Germany). Then, another 8 mL of sodium acetate solution, 8 mL of methanol and 4 mL of chloroform were added to 4 g of this mixture and mechanically shaken for 3 minutes. After addition of 4 mL of chloroform, the mixture was shaken again for 2 minutes, and 8 mL of water was added in order to separate the chloroform and methanol phases. After centrifugation, the lower phase was collected in a flask.
Fatty acid methyl esters (FAME) were obtained as described by BRANCIARI *et al*. (2017). Fifty milligrams of the lipid fraction were dissolved in 2 mL of hexane, then 1 mL of hexane containing internal standard (methyl nonadecanoate 0.4 mg/mL; Sigma-Aldrich, Bellefonte, PA, USA) and 2 N KOH in methanol (0.5 mL) were added. The mixture was then shaken vigorously for 3 min, water (3 mL) was added and the upper organic phase was dried over anhydrous sodium sulphate, cooled in an ice bath and immediately injected into a high-resolution gas chromatograph. Separation of FAME was carried out on a capillary column CP-Select CB (100 m x 0.25 mm i.d., 0.39 μm, J&W, Agilent Technologies, Palo Alto, CA, US). We used helium as a carrier gas at a flow rate of 1.6 mL/min. The injector temperature was set at 270°C and the detector at 300°C. The oven temperature program was the following: starting from 60°C (maintained for 1 minute), the temperature was increased to 30°C/min up to 150°C; after 3 min, with an increase of 0.5°C/min, then, after 1 minute, it was increased to 220°C in increments of 1.5°C/min and

then maintained for 15 min. One μL of sample was injected in a split/splitless system (split ratio 1:5). Individual FAME were identified by comparison with a standard mixture (PUFA No. 1, Marine Source, 37 FAMEs by Sigma, methyl *cis*-7,10,13,16,19-docosapentaenoate, *trans*-11-vaccenic methyl ester, *cis*-11-vaccenic methyl ester, Supelco, Bellefonte PA, USA). The percentage of each FA was calculated by using the peak area of the samples corrected with the respective correction factors (AOAC, 2012). To assess the actual nutritional quality of fish fillets, fatty acids were quantified and expressed in mg/100 g tissue using the internal standard method outlined by JOSEPH and ACKMAN (1992). The following equation was applied:

$$\text{Fatty acids (mg/100 g food)} = [(A_x \times W_{is} \times \text{CRF}_x \times \text{CNF}x)/(\text{A}_{is} \times \text{W}s)] \times 1000 \times \text{W}_L,$$

where A_x is the eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) area, A_{is} is the internal standard area, CRF_x is the theoretical correction factor for EPA and DHA, CNF*x* is the conversion factor from FAME to the corresponding fatty acid, W_{is} is the weight of the internal standard added to the lipids, W*s* is the weight of the derivatized lipids and W_L is the percentage of sample lipid.
Based on current knowledge regarding the effect of specific fatty acids on cholesterol metabolism, the ratio between hypocholesterolaemic and hypercholesterolemic fatty acids (HH) was calculated using the following mathematical equation (SANTOS-SILVA *et al.*, 2002):

$$\text{HH} = (\text{C18:1n-9} + \text{C18:2n-6} + \text{C20:4n-6} + \text{C18:3n-3} + \text{C20:5n-3} + \text{C22:5n-3} + \text{+C22:6n-3})/(\text{C14:0} + \text{C16:0})$$

The mean value of each fatty acid was used to calculate the sum of the saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids and to calculate the peroxidability index (PI) according to the equation proposed by ARAKAWA and SAGAI (1986):

$$\text{PI} = (\%\ \text{monoenoic x } 0.025) + (\%\ \text{dienoic x } 1) + (\%\ \text{trienoic x } 2) + (\%\ \text{tetraenoic x } 4) + \text{+}(\%\ \text{pentaenoic x } 6) + (\%\ \text{hexaenoic x } 8).$$

The amount of each fatty acid was also used to calculate the atherogenicity (AI) and thrombogenicity (TI) indexes as proposed by ULBRICHT and SOUTHGATE (1991):

$$\text{AI} = (\text{C12:0} + 4\text{xC14:0} + \text{C16:0})/[(\sum\text{MUFA} + \sum (\text{n-6}) + \sum (\text{n-3})]$$
$$\text{TI} = (\text{C14:0} + \text{C16:0} + \text{C18:0})/[\ (0.5 \text{ x } \sum\text{MUFA} + 0.5 \text{ x } (\text{n-6}) + 3\text{x } (\text{n-3}) + (\text{n-3})/(\text{n-6})]$$

The index of nutritional quality (INQ) was calculated on the basis of the EPA + DHA acid level using the formula suggested by GODBE (1994).

2.3. Assessment of oxidative stability

The extent of lipid oxidation was quantified by spectrophotometry (Shimadzu 2025, Kyoto, Japan) as thiobarbituric acid reactive substances (TBARs) according to the method reported by KE *et al*. (1977) and using a molar extinction coefficient of 156 x 10^3 M/cm at λ = 532 nm. Results are expressed as malondialdehyde (MDA) equivalents per kg of tissue (mg MDA/kg).

2.4. Reagents

Unless otherwise noted, all chemicals were analytical grade and were purchased from Sigma Chemical Co (St Louis, MO, USA).

2.5. Statistical evaluation

The data were analysed with a one-way linear model (Statacorp®, 2015) evaluating the effect of technological treatment. Differences among whitefish products were evaluated by multiple samples *t*-test, reporting the mean and standard error of the mean (SEM). Results were considered significant at $p < 0.05$.

3. RESULTS AND DISCUSSIONS

3.1. Food labels of raw and processed whitefish fillets

The chemical characteristics of analysed whitefish fillets are reported in Table 1. The processing of whitefish, i.e. smoking and marinating, significantly affected the chemical parameters of fillets.
In particular, smoking reduced the water content of muscle fibres with respect to raw filets (64.82% vs. 76.53%) due to surface drying of the fillet and consequently, the fillets showed nutrient concentration. Even in marinated fillets, an increase in nutrient content due to dehydration was found (65.26%).

Table 1. Raw and processed whitefish fillets food label.

	Fillets			
	Raw	**Smoked**	**Marinated**	**SEM**
Moisture	76.53^{b}	64.82^{a}	65.26^{a}	4.52
Lipids	1.97^{a}	2.86^{a}	9.77^{b}	0.59
of which				
- saturates	30.82^{b}	30.21^{b}	14.50^{a}	1.18
- monounsaturates	33.60	33.54	36.35	2.11
- polyunsaturates	35.57^{a}	36.23^{a}	49.14^{b}	2.56
Carbohydrates	0.97^{a}	1.05^{a}	2.63^{b}	0.29
of which				
- sugar	n.d	n.d.	0.41	0.18
Protein	19.23^{a}	28.53^{b}	20.44^{a}	2.01
Ash	1.30^{a}	2.74^{b}	1.90^{ab}	0.35
Energy*	98^{a} 416^{a}	144^{b} 602^{b}	180^{c} 754^{c}	20.15 41.18

N=20 per group.
$^{a..c}$ Values within a row with different superscripts indicate a significant difference at $p < 0.05$.
Proximate composition was expressed as %; Energy was expressed as kcal/100 g on the first line values and kJ/100 g on the second line values.
* EU regulation 1169/2011.

The percent protein content of smoked fillets was significantly higher than that of raw and marinated ones (28.53% vs. 19.23% and 20.44%, respectively). However, the smoking process can cause a reduction of protein digestibility attributable to the formation of oxidized forms of sulphur amino acids and carbohydrate-protein complexes called Maillard compounds (MENSA-WILMOT *et al.*, 2001), as demonstrated by the low percentage of carbohydrates relative to that in the marinated fillets (1.05% vs. 2.63%). Instead, the higher proportion of carbohydrates in marinated samples was the result of the addition of onion and celery as ingredients, which contain approximately 5.7% and 2.4% carbohydrates, respectively (MARLETTA and CARNOVALE, 2000).
A prominent difference was found in the lipid content, which was higher in the marinated fillet (9.77%), probably because of the addition of olive oil; indeed, the main fatty acids were represented by PUFA and MUFA. The total mineral content was significantly different only between raw and smoked fillets, with lower values in the former (1.30% vs. 2.74%), possibly attributed to the concentration process previously cited.
FUENTES *et al.* (2010) reported a strong correlation of the smoking process with several physico-chemical parameters (moisture, water activity, pH and colour) in commercial smoked products (anchovy and salmon). They suggested that the ability of the smoking process to preserve fish is due to the synergistic action of salt incorporation, smoke compounds and dehydration during the smoking process.
Similarly, marinated fish are products consisting of raw, frozen or salted fish or portions of fish processed by treatment with edible organic acids, usually acetic acid, and salt and added to sauces, creams or oil (MEYER, 1965). They represent semi-preserved fish products, ready-to-eat with no heat treatment (GRAM and HUSS, 1996), and they are considered as a high-value delicacy, as are cold-smoked fish. In the present study, marinated fish were prepared with lemon juice and vinegar, then with natural citric and acetic acids. HAMM (1960) outlined that, in the presence of acid alone, the pH of the muscle was on the acidic side of the isoelectric point, and the electrostatic repulsion allowed for an increase in water holding capacity and a decrease in firmness. However, when salt was added (as in our case), the repulsion decreased and the structure became firmer.
The differing proportions of nutrients in the raw and processed fillets also affected the energy content of the studied products, with higher values found in marinated fillets, followed by smoked and non-processed whitefish (180, 144 and 95 kcal/100 g and 754, 602 and 416 kJ/100 g, respectively).

3.2. Fatty acid profiles of raw and processed whitefish fillets

The fatty acid composition (mg/100 g) of the samples is shown in Table 2. A total of 33 fatty acids were identified in the present study. The fatty acid composition of raw and smoked fillets was nearly identical. The slight differences in concentration were due to the lower moisture content of the smoked fillets, as demonstrated by chemical composition analysis. In raw and smoked fillets, the most represented fatty acid was oleic acid (C18:1n-9cis); this MUFA was present in an amount equal to 327.26 mg/100 g in raw fillets, whereas in smoked ones, a concentration of 483.85 mg/100 g was recorded, followed by palmitic acid (C16:0). Conversely, ORBAN *et al.* (2006) found a higher proportion of the latter fatty acid, followed by oleic (C18:1n-9) and palmitoleic (C16:1) acids in raw whitefish fillets.
In the marinated fillet, the most represented fatty acid was linoleic acid (C18:2n-6, LA; 4005.84 mg/100 g). This higher value, compared with that recorded in raw and smoked fillets, was justified by the marinating preparation method, which included the addition of olive oil with an average linoleic acid value of 12%. The same was also true for oleic acid

(2999.25 mg/100 g), which is present at almost 83% in olive oil, and for palmitic and stearic acids (786.81, 296 and 17 mg/100 g, respectively), which are present from 5% to 20% (BOSKOU, 2015).

Table 2. Fatty acid composition (mg/100 g tissue) of raw and processed whitefish fillets.

	Fillets			
	Raw	**Smoked**	**Marinated**	**SEM**
C12:0	1.18[a]	1.68[a]	8.73[b]	0.98
C13:0	0.57[b]	0.86[b]	0.00[a]	0.12
C14:0	119.08[b]	174.69[c]	63.93[a]	15.21
C14:1	22.62[b]	33.77[c]	12.74[a]	1.25
C15:0	15.19[b]	22.21[c]	9.70[a]	1.05
C15:1	0.80[b]	1.07[b]	0.00[a]	0.08
C16:0	319.22[a]	444.88[a]	786.81[b]	50.16
C16:1	214.52[b]	303.12[c]	147.19[a]	17.51
C17:0	9.08[a]	13.24[b]	8.11[a]	0.89
C17:1	0.54[b]	0.73[b]	0.00[a]	0.10
C18:0	50.42[a]	77.03[a]	296.17[b]	20.31
C18:1n-9t	4.52[b]	7.25[b]	0.00[a]	0.32
C18:1n-9c	327.26[a]	483.85[a]	2999.25[b]	124.21
C18:1n-7c	67.31[a]	93.46[b]	124.66[c]	24.15
C18:2n-6t	0.62[b]	0.97[b]	0.00[a]	0.12
C18:2n-6c	93.52[a]	136.69[a]	4005.84[b]	66.58
C18:3n-6	8.93[a]	13.54[b]	6.00[a]	1.24
C18:3n-3	138.30[b]	198.28[c]	82.65[a]	26.21
C20:0	3.22[a]	4.45[a]	24.20[b]	2.14
C18:4n-3	86.75[b]	128.08[c]	38.69[a]	15.48
C20:1n-9	8.98[a]	12.80[a]	20.39[b]	2.36
C21:0	5.30[a]	7.35[b]	7.32[b]	0.87
C20:3n-6	3.46[a]	5.20[b]	3.13[a]	0.69
C20:4n-6	41.20[b]	59.20[c]	28.71[a]	3.25
C20:3n-3	8.96[b]	12.12[c]	4.58[a]	1.05
C22:0	5.49[a]	8.15[a]	48.87[b]	2.36
C22:1n-9	1.37[b]	1.93[b]	0.00[a]	0.14
C20:5n-3	128.64[b]	186.87[c]	70.58[a]	3.36
C22:2	0.94[a]	1.32[b]	2.73[c]	0.25
C24:0	0.98[a]	1.40[a]	17.46[b]	1.28
C24:1	1.47[a]	2.09[a]	7.44[b]	0.86
C22:5n-3	38.10[b]	59.11[c]	27.32[a]	3.72
C22:6n-3	61.96[b]	105.25[c]	37.68[a]	3.14

N=20 per group.

[a-c] Values within a row with different superscripts indicate a significant difference at $p < 0.05$.

Oleic acid plays an important role in physical well-being and is responsible for the reduction of plasma cholesterol levels and the improvement of the low density/high

density lipoprotein ratio (LDL/HDL, SECCHIARI, 2008), both of which are well-known, important risk factors for cardiovascular disease (ALTHAUS *et al.*, 1988). Furthermore, considering the cultural value of olive oil in our country (DE LEONARDIS, 2014), its use in fishery products could represent an important value added.
Concerning PUFA, the fatty acid with the highest concentration was α-linolenic acid (ALA, C18:3n-3), followed by EPA (C20:5n-3) in all studied samples. Even the ALA content, both in raw and smoked fillets was higher than that in marinated ones (138.30 and 198.28 vs 82.65 mg/100 g, respectively). EPA and DHA are long-chain n-3 fatty acids, synthesized by the human body only in small amount (DE FILIPPIS and SPERLING, 2006); therefore, their dietary consumption is required. In particular, EPA and DHA intake has demonstrated physiological benefits in terms of blood pressure, heart rate, triglycerides and inflammation; moreover, a reduced risk of foetal coronary heart disease (CHD) and sudden cardiac death has been associated with the consumption of ~250 mg/day of EPA plus DHA (GISSI-HF, 2008; MOZAFFARIAN and RIMM, 2006). Accordingly, whitefish fillet is an excellent source of long-chain n-3 PUFA, as 100 g of raw fillet provides approximately 190 mg of EPA + DHA, and smoked filet exceeds the recommended requirement (292.18 mg/100 g).

3.3. Nutritional indexes and oxidative stability of raw and processed whitefish fillets

The total amounts of different fatty acid series, nutritional indexes and oxidative stability of whitefish fillets are reported in Table 3. When the three processing methods were compared, raw and smoked fillets had different amounts of SFA (529.73 vs 755.94 mg/100 g, respectively), due, as previously mentioned, to the concentration of nutrients during the smoking process. The same trend was observed in the MUFA and PUFA levels.

Table 3. Total saturated, monounsaturated and polyunsaturated fatty acids (mg/100 g), nutritional indexes and oxidative status of raw and processed whitefish fillets.

	Fillets			
	Raw	**Smoked**	**Marinated**	**SEM**
SFA	529.73^{a}	755.94^{b}	1271.28^{c}	50.26
MUFA	577.57^{c}	839.37^{b}	3187.01^{c}	62.52
PUFA	611.39^{a}	906.63^{b}	4307.91^{c}	59.85
Σ n-3	462.71^{b}	689.70^{c}	261.50^{a}	21.36
Σ n-6	147.74^{a}	215.61^{b}	4043.68^{c}	78.15
n-6/n-3	0.32^{a}	0.31^{a}	15.46^{b}	2.26
INQ	5.95^{b}	6.24^{b}	1.85^{a}	1.85
HH	1.16^{a}	1.21^{a}	5.00^{b}	5.00
Peroxidability index	112.60^{b}	117.83^{b}	91.14^{a}	21.14
Atherogenic index	0.67^{b}	0.66^{b}	0.14^{a}	0.14
Trombogenic index	0.28	0.27	0.26	0.26
TBARs	0.22^{a}	0.11^{a}	3.60^{b}	0.60

N=20 per group.
$^{a-c}$ Values within a row with different superscripts indicate a significant difference at $p < 0.05$.
SFA, MUFA, PUFA, Σ n-3 and Σ n-6 are expressed as mg/100 g tissue; TBARs are expressed as mg MDA/kg tissue.

Several studies have shown a direct relationship between the consumption of SFA in the diet and the risk of cardiovascular disease (DAYTON *et al.*, 1968). The negative effect of dietary SFA is mainly due to the increase in blood LDL cholesterol (MENSINK *et al.*, 1992). However, the heterogeneity of the saturated fatty acids and their effect as risk factors are worthy of note. For example, stearic acid, little represented in the raw fillet, is not a hypercholesterolemic agent (HUNTER *et al.*, 2010), whereas the myristic acid, which was present in a higher amount than stearic acid, is dangerous to human health because it increases serum cholesterol by four times more than palmitic acid (SECCHIARI, 2008).
Regarding the marinated fillet, the levels of SFA, MUFA and PUFA were significantly higher respect to control or smoked one. The SFA and MUFA amounts were 1271.28 mg/100 g and 3187.01 mg/100 g, respectively, mainly due to the addition of olive oil to the product (Table 3). This ingredient affected also the PUFA levels (4307.91 mg/100 g), which were greatly elevated (BOSKOU, 2015). The prevalence of PUFA over MUFA and SFA may reflect a high inherent capability of freshwater fish to desaturate and elongate enzymatically dietary precursors into long-chain highly unsaturated fatty acids (HENDERSON and TOCHER, 1987).
The smoked fillet had 689.70 mg/100 g of n-3 fatty acids, whereas during the marinating process, the n-3 fatty acid concentration decreased to 261.50 mg/100 g, of which 70.58 mg was EPA, and 37.68 mg was DHA. These results were not due to the oxidation of the product resulting from manipulation (FRANKEL *et al.*, 2014) but rather to the high quantity of PUFA found. In fact, the lipid oxidative status, evaluated with the TBARs test, was worse in marinated than in raw and smoked fillets (3.60 in marinated vs. 0.22 and 0.11 mg MDA/kg in raw and smoked samples, respectively), whereas the IP index, which measures the susceptibility to oxidation on the basis of the fatty acid composition, was lower in marinated than in raw and smoked fillets (91.14 in marinated vs. 112.60 and 117.83, in raw and smoked samples, respectively).
We observed a correlation between the amount of long chain n-3 fatty acids and oxidative status. As already mentioned, the raw and smoked fillets had a higher content of n-3 fatty acids than the marinated fillet. For fish products subjected to processing after slaughter, it is necessary to take precautions to avoid over-oxygenation of the product, which would make it more susceptible to oxidation. The higher TBARs value was related to the worse oxidative status found in the marinated samples, and to the content of unsaturated fatty acids, which are the primary targets of the free radicals (DAL BOSCO *et al.*, 2010).
The trend for n-6 fatty acids was different to that of n-3 fatty acids. Raw and smoked fillets showed a lower content of total n-6 fatty acids (147.74 and 215.61 mg/100 g, respectively), while in the marinated fillet, the concentration is almost 20-times higher, as also shown by the n-6/n-3 ratio (Table 3).
Nowadays, fish products are the main source of n-3 PUFA in the human diet and, in consideration of the high intake of n-6 PUFA in industrialized countries, an increment of their consumption is recommended by dietary guidelines in order to re-establish a healthy balance between n-3 and n-6 PUFA (SIMOPOULOS, 2003). Several studies have demonstrated the role of n-3 fatty acids in the prevention of human diseases. In particular, these compounds also have a beneficial effect on the control and prevention of cardiovascular diseases and play crucial roles in brain development and visual activity (RIZOS *et al.*, 2012).
All the studied indexes reflect the composition of single fatty acids of fillets (Table 3).
The HH index measures the ratio of hypocholesterolemic and hypercolosterolemic fatty acids. The marinated fillet contained a higher concentration of hypocholesterolemic fatty acids (MUFA + PUFA), with a HH value of 5.00, than raw and smoked fillets. This result is noteworthy considering that the main factor in the onset of cardiovascular diseases is the oxidation of low-density lipoproteins (LDL cholesterol; HU and WILLETT, 2002);

therefore, fish products have an important role in the prevention of such pathologies (VALFRÈ *et al.*, 2003).
The INQ index describes the nutritional quality of a food, based on the satisfaction of the daily requirements of EPA + DHA. In the present study, the raw and smoked fillet had higher values than the marinated ones (5.95 and 6.24 vs. 1.85, respectively), in agreement with the EPA + DHA contents previously described.
Finally, the atherogenic (AI) and thrombogenicity indexes (TI), which measure the quality of the lipids in the fillets, are inversely correlated with the ability of fatty acids to reduce the lipid content in the blood and to reduce platelet activity, respectively (ULBRICHT and SOUTHGATE, 1991). The results obtained differed significantly between all three sample treatments, and they were better in the marinated fillet. In raw and smoked fillets, AI values of 0.67 and 0.66, respectively, were recorded, whereas for the marinated fillet, this value was 0.14. However, the TI value remained constant in all the samples (0.28, 0.27 and 0.26 in raw, smoked and marinated, respectively). These similar results could be explained by n-3 and n-6 PUFA values considered in the equations suggested by ULBRICHT and SOUTHGATE (1991). Indeed, they were equally weighted for the AI index, but not for the TI, where n-3 PUFA obtained a higher weight and then, equalizing the ratio between n-6 and n-3, was not significantly different between groups.

4. CONCLUSIONS

The data from the present study suggest that whitefish from Bolsena Lake is characterized by high nutritional quality (mainly due to EPA and DHA content) and a good oxidative stability of raw fillets. However, nutritional characteristics of fillets differed between processing methods: the smoked fillet showed a higher INQ and a lower n-6/n-3 ratio, HH index and TBARs value compared with the marinated one. In contrast, the marinated fillet offered an added value given by olive oil presence (lowest PI and highest PUFA content).
To conclude, the nutritional characterization of local products represents a good strategy to increase their economic value. Further studies focusing on the environment in which the fish lives and the one from which it originates, or on its history (as it is caught and processed on board the grounded boats and transformed yourself), are needed to evaluate such Umbrian fishery products (raw or processed).

ACKNOWLEDGEMENTS

Authors are grateful to "*Lago vivo*" Fisherman Cooperative of Bolsena (Viterbo, Italy).

REFERENCES

Althaus B.U., Staub J.J., Leche A.R.D.E., Oberhänsli A, Stähelin H.B. 1988. LDL/HDL changes in subclinical hypothyroidism:possible risk factors for coronary heart disease. Clinical endocrinology 28(2):157-163.

Arakawa K. and Sagai M. 1986. Species differences in lipid peroxide levels in lung tissue and investigation of their determining factors. Lipids 21:769.

Association of Official Analytical Chemists. 2000. Official Methods of Analysis 17th Ed. Washington, DC.

Association of Official Analytical Chemists. 2012. Official Methods of Analysis" 19th Ed. Washington, DC.

Boskou D. 2015. Olive oil: chemistry and technology. Elsevier, Ed. Champaign, Illinois.

Branciari R., Ranucci D., Urbani E., Valiani A., Trabalza-Marinucci M., Dal Bosco A. and Franceschini R. 2017. Freshwater fish burgers made from four different fish species as a valuable strategy appreciated by consumers for introducing EPA and DHA into a human diet. Journal of Aquatic Food Product Technology. In press.

Dal Bosco A., Mourvaki E, Mugnai C., Ruggeri S. and Castellini C. 2010. Nutritional evaluation of fillets, pulp and croquettes of wild caught lake Trasimeno goldfish (*Carassius auratus* L.). Italian Journal of Food Science 22(2):192-199.

Dayton S., Pearce M.L., Goldman H., Harnish A., Plotkin D., Shickman M., Winfield M., Zager A. and Dixon W. 1968. Controlled trial of a diet high in unsaturated fat for prevention of atherosclerotic complications. Lancet 2:1060-1062.

De Filippis A. and Sperling L. 2006. Understanding omega-3's. American Heart Journal 151:564-570.

De Leonardis A. 2014. Virgin olive oil: Production, composition, uses and benefits for man. Pages 1-392. Nova Science Publishers, Inc.

European Commission 2011. Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers. Official Journal of the European Union 22.11.2011, L304/18-L304/63. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri5OJ:L:2011:304:0018:0063:EN:PDF (accessed November 2012).

Franceschini R., Orrù M., Miraglia D., Ranucci D., Asdrubali F., Altissimi S., Valiani A. and Branciari R. 2015. Profilo acidico e caratteristiche sensoriali di un preparato a base di polpa di pesce del lago trasimeno. Rivista Italiana delle sostanze grasse 1(2):3.

Frankel E.N. 1998. Lipid oxidation. Page 300 in: The Oil Press. Dundee, Scotland.

Fuentes A., Fernández-Segovia I., Barat J.M. and Serra J.A. 2010. Physicochemical characterization of some smoked and marinated fish products. Journal of Food Processing and Preservation 34(1):83-103.

Gissi-Hf I. 2008. Effect of n-3 polyunsaturated fatty acids in patients with chronic heart failure (the GISSI-HF trial):a randomised, double-blind, placebo-controlled trial. Lancet 372:1223-1230.

Godbe J.S. 1994. Nutritional value of muscle foods. Page 430 in: Muscle Foods: Meat, Poultry and Seafood Technology. D.M. Kinsman, A.W. Kotula and B.C. Breidenstein, Eds. Chapman & Hall, New York, NY.

Goebel S.E., Gaye-Siessegger J., Baer J. and Geist, J. 2017. Comparison of body composition and sensory quality of wild and farmed whitefish (*Coregonus macrophthalmus* [Nüsslin, 1882]). Journal of Applied Ichthyology 00:1-8.

Gram L. and Huss H.H. 1996. Microbial spoilage of fish and fish products. Italian Journal of Food Microbiology 33:121–137.

Hamm R. 1960. Biochemistry of meat hydration. Advances in Food Research 10:144.

Henderson R.J. and Tocher D.R. 1987. The lipid composition and biochemistry of freshwater fish. Progress in Lipid Research 26:281–347.

Hu F.B. and Willett W.C. 2002. Optimal diets for prevention of coronary heart disease. Jama 288(20):2569-2578.

Hunter J.E., Zhang J. and Kris-Etherton P.M. 2010. Cardiovascular disease risk of dietary stearic acid compared with trans, other saturated, and unsaturated fatty acids: a systematic review. American Journal of Clinical Nutrition 91:46-63.

Joseph J.D. and Ackman R.G. 1992. Capillary column gas chromatographic method for analysis of encapsulated fish oils and fish oil ethyl esters: collaborative study. Journal of AOAC International 75(3):488-506.

Ke P.J., Ackman R.G., Linke B.H. and Nash D.M. 1977. Differential lipid oxidation products in various parts of frozen mackerel. Journal of Food Technology 12:37.

Marletta L. and Carnovale E. 2000. Tabelle di composizione degli Alimenti. Milano: Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione.

Mensa-Wilmot Y., Phillips R.D. and Hargrove J.L. 2001. Protein Quality Evaluation of Cowpea-Based Extrusion Cooked Cereal/Legume Weaning Mixtures. Nutrition Research 21:849-857.

Mensink R.P. and Katan M.B. 1992. Effect of dietary fatty acids on serum lipid and lipoproteins. A meta analysis of 27 trials. Arteriosclerosis, Thrombosis, and Vascular Biology 12(8):911-919.

Meyer V. 1965. Marinades. Page 221in:Fish as Food, Vol. 3, Part 1, Borgstrom G., Ed. Academic Press, New York.

Mozaffarian D. and Rimm E.B. 2006. Fish intake, contaminants, and human health: evaluating the risks and the benefits. JAMA 296(15):1885-1899.

Orban E. 2007. Le specie emergenti del mercato ittico ed i prodotti dell'acquacoltura nazionale: qualità alimentare a confronto. Acquacoltura 11.

Orban, E., Masci, M., Nevigato, T., Di Lena, G., Casini, I., Caproni, R. and Rampacci, M. 2006. Nutritional quality and safety of whitefish (*Coregonus lavaretus*) from Italian lakes. Journal of Food Composition and Analysis 19(6):737-746.

Rizos E.C., Ntzani E.E., Bika E., Kostapanos M.S. and Elisaf M.S. 2012. Association between omega-3 fatty acid supplementation and risk of major cardiovascular disease events: a systematic review and meta-analysis. JAMA 308(10):1024-1033.

Santos-Silva J., Bessa R.J.B. and Santos-Silva F. 2002. Effect of genotype, feeding system and slaughter weight on the quality of light lambs. II. Fatty acid composition of meat. Livestock Production Science 77:187.

Secchiari P.L. 2008. Aspetti del valore nutrizionale e nutraceutico degli alimenti di origine animale. Italian Journal of Agronomy 1:73-101

Simopoulos A.P. 2003. Importance of the ratio omega-6/omega-3 essential fatty acids: evolutionary aspects. Pages 1-22 in:Omega-6/Omega-3 Essential Fatty Acid Ratio: The Scientific Evidence. Simopoulos, A.P. and Cleland, L.G., Eds. World Review of Nutrition and Dietetics vol. 92. Karger, Basel.

Sogn-Grundvåg G., Larsen T.A. and Young J.A. 2014. Product differentiation with credence attributes and private labels:the case of whitefish in UK supermarkets. Journal of Agricultural Economics 65(2), 368-382.

StataCorp 2015. Stata Statistical Software: Release 14. Stata Corp L.P., College Station, TX.
Ulbricht T.L. and Southgate D.A.T. 1991. Coronary heart disease: seven dietary factors. The Lancet 338:985.

Valfré F., Caprino F. and Turchini, G.M. 2003. The health benefit of seafood. Veterinary Research Communications 27:507-512.

Yeannes M.I. and Casales M.R. 2008. Modifications in the chemical compounds and sensorial attributes of Engraulis anchoita fillet during marinating process. Food Science and Technology 28(4):798-803.

Paper Received January 1, 2017 Accepted July 20, 2017

SHORT COMMUNICATION

PHENOLIC ACIDS, FLAVONOIDS, ASCORBIC ACID, β-GLUCANS AND ANTIOXIDANT ACTIVITY IN MEXICAN WILD EDIBLE MUSHROOMS

E. LÓPEZ-VÁZQUEZ[a], F. PRIETO-GARCÍA*[a], M. GAYOSSO-CANALES[b], E.M. OTAZO SÁNCHEZ[a] and J.R. VILLAGÓMEZ IBARRA[a]

[a]Instituto de Ciencias Básicas e Ingeniería, Universidad Autónoma del Estado de Hidalgo Carretera Pachuca-Tulancingo km. 4.5, C.P. 42076, Pachuca, Hidalgo, México

[b]Instituto de Ciencias Agropecuarias, Universidad Autónoma del Estado de Hidalgo, Rancho Universitario Av. Universidad Km. 1, Ex–Hda. de Aquetzalpa C.P. 43600, Tulancingo, Hidalgo, México

*Corresponding author. Tel.: +52 7712211289;
E-mail address: prietog@uaeh.edu.mx

ABSTRACT

Five wild edible mushrooms (*Amanita caesarea*, *Boletus edulis*, *Cantharellus cibarius*, *Lactarius indigo* and *Ramaria* sp.) from Mexico were studied for their total phenolic content, antioxidant activity, flavonoids, ascorbic acid, gallic acid, and cinnamic and chlorogenic acids. *B. edulis* showed the highest contents, expressed in dry weight, of flavonoids (0.92 mg of quercetin equivalents/g), total phenolic acids (8.66 mg of gallic acid equivalents/g), chlorogenic acid (1001.67 μg/g) and cinnamic acid (10.68 μg/g), and it also presented the greatest antioxidant activity (48.6% DPPH scavenging activity and 59.48% reductive power) among the species analyzed. Overall, the Mexican wild edible mushrooms had high concentrations of chlorogenic and ascorbic acids and flavonoids. This study constitutes the first report in Mexico about nutraceuticals from wild edible mushrooms.

Keywords: wild mushrooms, antioxidants, flavonoids, phenolics, β-glucans

1. INTRODUCTION

Some species of mushrooms are appreciated as food not only for their color, texture, flavor and odor but also as valuable sources of nutrients and nutraceuticals (TAOFIQ *et al*., 2015; WANG *et al*., 2014). Several studies of mushrooms from Portugal (HELENO *et al*., 2015a), Italy (MANZI *et al*., 2004), Poland (NOWACKA *et al*., 2014), China (SIU *et al*., 2014), Ethiopia (WOLDEGIORGIS *et al*., 2014) and Turkey (SARIKURCKU *et al*., 2015) have described mushrooms as naturally rich sources of phenolics and flavonoids. These compounds exhibit antioxidant, antibacterial, antiviral, anticarcinogenic and anti-inflammatory activities (HELENO *et al*., 2015b; REIS *et al*., 2011; WANG *et al*., 2014).
In addition, mushrooms are recognized for having other bioactive compounds that are beneficial for human health such as β-glucans and ascorbic acid (KAGIMURA *et al*., 2015; RUTHES *et al*., 2015). β-glucans are polysaccharidic compounds that display anticoagulant, antithrombotic, antioxidant, and anti-inflammatory activities. Therefore, these compounds play an effective role in the prevention of cardiovascular problems, the reduction of blood cholesterol levels and treatment of illnesses such as various cancers and diabetes (ZHU *et al*., 2015). Ascorbic acid is an antioxidant vitamin common in mushrooms (VAZ *et al*., 2011).
In spite of the fact that since precolonial times in Mexico, the fruiting body of mushrooms has been known as nanacatl (mushroom) from Nahuatl, and that a great edible mushroom diversity of approximately 300 species is known in the country (MORENO, 2014), there are no reports with respect to the content of phenolic compounds and phenolics acids in mushrooms. Therefore, thorough studies of the chemical composition of the wild edible mushrooms are needed to identify and quantify the bioactive compounds. The selection of the mushroom species was based on their appreciation as food in Mexico: *A. caesarea*, *B. edulis*, *C. cibarius*, *L. indigo* and *Ramaria* sp. (MORENO, 2014). The aim of this study was to determine the chemical composition of five Mexican wild edible mushrooms in terms of flavonoids, phenolic acids, ascorbic acid, β-glucans and antioxidant activity.

2. MATERIALS AND METHODS

2.1. Samples, standards and reagents

The mushroom samples were collected in different regions of Hidalgo State in Mexico. The regions were Los Reyes, Acaxochitlán (latitude 20.152° and longitude -98.185°, 2199 m a.s.l.), El Susto (latitude 20.074° and longitude -98.509°, 2620 m a.s.l.) and Paxtepec (latitude 20.033° and longitude -98.426°, 2381 m a.s.l.). The sampling was conducted during the rainy season (August-October) of 2014. The taxonomic identification of the sporocarps was made according to mycology keys (LÆSSØE, 2013).
The standards of chlorogenic, cinnamic, gallic, and (L)-ascorbic acid and schizophyllan, the reagents Folin-Ciocalteu (FC) reagent, 2,2-diphenyl-1-picrilhydrazyl radical (DPPH) and 2,6-dichloroindophenol, and the LC solvents methanol 99.8% and acetonitrile 99.9% were all purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). The methanol, hydrochloric acid, phosphoric acid and red Congo dye were purchased from J. T. Baker (Philipsburg, USA). All other chemicals of analytical grade, including sodium hydroxide, metaphosphoric acid, potassium hydroxide, sodium carbonate, citric acid, sodium nitrite, aluminum chloride, ferric chloride, trichloroacetic acid, potassium ferricyanide, sodium phosphate mono and dibasic, were purchased from Meyer Co (Mexico).

The fruiting bodies were cleaned with a brush, cut into slices and dried in a drying oven in a range between 40-60°C until a constant weight was achieved. The dried samples were reduced to fine powder (20 mesh) using an electric mill (IKA A11 basic) and stored in amber plastic bottles until analysis.

2.2. Analysis of antioxidant activity

Preparation of the extracts. The preparation of the extracts was made using the methodologies proposed by PAN *et al*. (2003) and ÖZYUREK *et al*. (2014) with some modifications. Each sample (~200 mg) was extracted with methanol:water (80:20, v/v; 25 mL), using a conventional microwave oven (900 W, 10% power). The mixtures were irradiated as follows: 7 s power on (heating without superboiling of the solution) and 30 s power off (cooling in an ice bath and mixed on a vortex) then 7 s power on and 30 s power off and so on to reach 5 min of heating time. The obtained extracts were filtered through a filter paper (Whatman No. 4), then the filtered volume was adjusted with methanol:water (80:20 v/v) to 25 mL and kept at 4°C in amber bottles until analysis.

DPPH radical scavenging activity assay. Aliquots of 60 μL of extract were mixed with 540 μL of a methanolic DPPH radical solution (0.06 mM). The mixture was left to stand for 60 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm (Genesys 10S VIS, Thermo Scientific, USA). L-ascorbic acid (8 mg/mL) was used as a standard. The radical scavenging activity (RSA) was calculated as a percentage of the DPPH pink discoloration using the following equation:

$$\% \text{ RSA} = [(A_{DPPH} - A_s)/A_{DPPH}] \times 100$$

where A_s is the absorbance of the solution containing the sample, and A_{DPPH} is the absorbance of the DPPH solution (VAZ *et al*., 2011).

Reducing power. The reducing power was determined according to BARROS *et al*. (2011). The methanolic extract (500 μL) was mixed with a sodium phosphate buffer (500 μL, 200 mM, pH 6.6) and potassium ferricyanide (500 μL, 1% w/v). The mixture was incubated at 50-60°C for 20 min, then trichloroacetic acid (500 μL, 10% w/v) was added. An aliquot of the reaction mixture (800 μL) was transferred to vials containing distilled water (800 μL) and ferric chloride (160 μL, 1% w/v). After 90 min, the absorbance was measured at 690 nm (Genesys 10S VIS spectrophotometer, Thermo Scientific). The reducing power was obtained as a percentage of the conversion of an orange Fe^{3+} ferricyanide complex to the Prussian blue Fe^{2+} ferrocyanide form in the presence of reductants (antioxidants from the mushroom extracts). BHT (1 mg/mL) was used as a control by measuring its absorbance in the assay and assigning it 100% of the reductive power.

Total phenolics. The total phenolic content was determined spectrophotometrically with the Folin-Ciocalteu (FC) test, which is an assay based on the capability of the phenolic compounds in an alkaline solution to reduce a colorimetric reagent composed of a mixture of phosphotungstic and phosphomolibdic acids from Mo(VI) to Mo(V) to produce a blue color (HUANG *et al*., 2002). According to HELENO *et al*. (2015a) 100 μL of methanolic extract were mixed with the FC reagent (750 μL, 10% v/v) for 1 min. After 5 min, a solution of Na_2CO_3 (750 μL, 10% w/v) was added to the mixture. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm (Genesys 10S VIS spectrophotometer, Thermo Scientific, USA). Gallic acid was used to obtain the standard curve (20 - 600 μg/mL). The total phenolic content is expressed as mg of gallic acid equivalents (GAE) per g of dry mushroom.

2.3. Determination of Phenolic Compounds by HPLC

The methanolic extracts were filtered through a 0.45 μm disposable LC filter disk for the HPLC analysis. The phenolic acids determination was performed using an Agilent 1260 CA, USA series liquid chromatograph equipped with a diode array detector. The separation was carried out using 5 μL of extract on a Poroshell 120 EC-C_{18} Agilent CA, USA, column (4.6 x 50 mm, 2.7 μm) thermostated at 25°C. The mobile phase was (A) 0.1% phosphoric acid in water, (B) HPLC-grade acetonitrile and (C) HPLC-grade methanol. The gradient used was 95% A, 5% B, and a flow rate of 1.0 mL/min for 2 min, 50% A, 25% B, 25% C, and a flow rate to 0.5 mL/min for 8 min. The phenolic acids were quantified by comparison of the area of their peaks recorded at 280 nm with the calibration curves obtained from the commercial standards of each acid. The calibration curves were obtained from a concentration range between 20-1000 μg/mL and 2-100 μg/mL for chlorogenic and cynamic acid respectively. The results are expressed in μg per g of dry mushroom.

2.4. Extraction and quantification of β-glucans

Mushroom β-glucans were isolated and quantified according to NITSCHKE *et al*. (2011): 750 mg of dry mushroom powder was heated with 60 mL 1 M KOH during 20 min at 60°C under constant stirring. Then, the suspension was filtered, the filter cake was washed with distilled water and the filtrate was collected and neutralized with 6 M HCl. The neutralized filtrate volume was adjusted to 100 mL with distilled water in a volumetric flask. This fraction was called KOH-fraction. The filter cake was resuspended in 65 mL of 0.58 M HCl and heated in an oil bath at 100°C for 1 h. The suspension was again filtered and the filter cake was washed with distilled water. The collected filtrate was neutralized with 6 M NaOH, transferred to a 100 mL volumetric flask and the volume adjusted with distilled water. This fraction was named HCl-fraction. The filter cake was again resuspended with 60 mL of 1 M NaOH and heated at 60°C for 20 min. The suspension was filtered and the filter cake was washed with distilled water, then the filtrate was neutralized with 6 M HCl. The filtrate was transferred to a 100 mL volumetric flask and the volume was augmented with distilled water. This fraction was called NaOH-fraction. The three fractions were used for the β-glucans determination.
For quantification of the β-glucans, 350 μL of each fraction were mixed with 300 μL of 0.2 M citric acid/sodium hydroxide buffer pH 7 and 50 μL of dye solution (8 mg of Congo red diluted in 10 mL of buffer) was added. The mixture absorbances were read at 523 nm against 350 μL of distilled water, 300 μL of buffer and 50 μL of dye solution as a blank. Because of the light brownish color of some of the fractions, a measurement of the background absorption at 523 nm was necessary. Therefore, 350 μL of the sample was mixed with 350 μL of the buffer, and the absorption was measured at 523 nm. The calibration curve was obtained with stock schizophyllan solutions in the range of 225-600 μg/mL. All analyses were performed in triplicate. The total content of the β-glucan is expressed as mg of β-glucan per g of dry mushroom.

2.5. Ascorbic acid

For the ascorbic acid determination, the mushroom powder (150 mg) was extracted with 10 of 1% (w/v) metaphosphoric acid for 45 min at room temperature under constant stirring and filtered through a Whatman N° 4 filter paper. The filtrate (0.1 mL) was mixed with 0.9 mL of 2,6-dichloroindophenol (0.00125% w/v) and was left to stand 30 min, then the absorbance was measured at 515 nm against a blank (VAZ *et al*., 2011). The ascorbic

acid content was calculated based on the calibration curve of the L-ascorbic acid (0.5-2.01 μg/mL), and the results are expressed as mg of ascorbic acid per g of dry mushroom.

2.6. Flavonoids

The flavonoid quantification was carried out according to PEREIRA *et al.* (2012). A total of 500 μL of methanolic extract was mixed with 150 μL of a 5% sodium nitrite solution and 2 mL of distilled water.
After 5 min, 150 μL of a 10% aluminum chloride solution was added, the reaction was kept for 6 min, then 2 mL of 4% sodium hydroxide solution and 200 μL of distilled water were added to the mixture. The reaction was mixed, and after 15 min the absorbance was measured at 510 nm. Quercetin was used to calculate the calibration curve (1 - 20 μg/mL) and the results are expressed as mg of quercetin equivalents (QE) per g of dry mushroom.

2.7. Statistical analysis

All assays were carried out in triplicate. The results are expressed as the mean values and standard deviation (SD). The results were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. These analyses were carried out using the SPSS v. 22.0 program (IBM Corp., USA).

3. RESULTS AND CONCLUSIONS

3.1. Antioxidant activity and total phenolics

The antioxidant activity of extracts was evaluated through the scavenging activity on the DPPH radicals and the reducing power. The antioxidant activity showed highly significant differences ($p < 0.001$) between the different mushrooms in both assays. The B. edulis extracts had the highest DPPH radical scavenging activity (48.06%) and reducing power (59.48%). These results are consistent with its higher content in phenolic compounds (8.66 GAE mg/g of dry mushroom) compared to the other mushrooms (Table 1).

Table 1. Total phenolics and antioxidant activity of methanolic extracts of wild edible mushrooms from Mexico.

Mushroom	Phenolics (mg of GAE/g)	% DPPH inhibition	% Reducing power
A. caesarea	2.90±0.16^{b}	6.15±1.76^{d}	36.69±2.37^{b}
B. edulis	8.66±0.69^{a}	48.06±6.60^{a}	59.48±0.99^{a}
C. cibarius	1.47±0.10^{c}	30.48±3.94^{b}	23.30±1.97^{c}
L. indigo	1.91±0.23^{c}	31.28±2.42^{b}	18.63±0.55^{c}
Ramaria sp	2.07±0.03^{c}	20.88±3.91^{c}	14.73±5.11^{c}

In each column, the letters imply significant differences ($p < 0.001$). Mean±SD; n = 3.

The *L. indigo*, *C. cibarius* and *Ramaria* sp. presented similar levels of the DPPH scavenging activity, reducing power and total phenolics. Despite the *A. caesarea* extracts showing the lowest (6.15%) DPPH scavenging activity, these extracts registered a high reducing power

(36.69%) and a low phenolic compound content (2.90 GAE mg/g of dry mushroom). The scavenging effect of the L-ascorbic acid was higher with 94.4% inhibition at 8 mg/mL. PALACIOS et al. (2011) reported the phenolics content for the *B. edulis* and *C. cibarius* of 5.5 and 2.5 mg of GAE/g of dry mushroom respectively, while we found 8.66 and 1.47. The differences observed may be due to the nutrimental and environmental conditions that affect the production of these metabolites (HELENO et al., 2015a).

The concentrations of chlorogenic acid range from 108.22 to 1001.67 μg/g of dry mushroom (Table 2), thus this acid constitutes 7.36 to 24.64% of the total phenolics present in the wild edible mushrooms studied. *B. edulis* showed the highest concentration of chlorogenic acid, while *C. cibarius* registered the lowest concentration. Other studies report chlorogenic acid concentrations of 4.55 to 63.73 μg/g (KIM *et al.*, 2008, PALACIOS *et al.*, 2011; WOLDEGIORGIS *et al.*, 2014) in mushrooms. Compared to those reports, the mushrooms evaluated in this study had very high amounts of chlorogenic acid. Our results suggest that chlorogenic acid could make a significant contribution to the DPPH radical scavenging activity of all mushrooms evaluated, except *A. caesarea*.

Cinnamic acid was only detected in *B. edulis* (10.68 μg/g of dry mushroom) and in *A. caesarea* (4.93 μg/g of dry mushroom). While HELENO *et al.* (2015a) report 3.1 μg/g of cinnamic acid in *B. edulis*, TAOFIQ *et al.*, (2015) found 14.2 μg/g. The content of this acid in *A. caesarea* reported by FERNANDES *et al.* (2015) was 24.8 μg/g, and REIS *et al.* (2011) found 0.3 μg/g. Thus, the results obtained in this study are somewhat comparable with those studies.

Table 2. Content of chlorogenic and cinnamic acids (μg/g of dry weight) in wild edible mushrooms from Mexico.

Mushroom species	Chlorogenic acid	Cinnamic acid
A. caesarea	564.45±41.99^{b}	4.93±0.61
B. edulis	1001.67±2.74^{a}	10.68±2.15
C. cibarius	108.22±2.61^{d}	Nd
L. indigo	222.42±1.55^{c}	Nd
Ramaria sp	510.11±8.13^{b}	Nd

In each row, different letters imply significant differences ($p< 0.05$). Mean±SD; n = 3.

3.2. Ascorbic acid

The ascorbic acid concentration ranging from 2.08 to 3.65 mg/g of dry mushroom reported elsewhere for the *L. indigo* reveals highly significant differences ($p< 0.01$) as compared to the mushrooms investigated herein (Table 3). The *L. indigo* registered the highest concentration and the *A. caesarea registered* the lowest. Although BARROS *et al.* (2007) report amounts of ascorbic acid of 0.13 to 0.35 mg/g in mushrooms, REIS *et al.*, (2011) found 1.75 to 8.99 mg/g. Hence, our results are in agreement with the latter study. The presence of ascorbic acid in the mushroom is not surprising as this metabolite is required as part of the defense mechanisms against radicals to avoid oxidative stress; therefore, the concentrations of ascorbic acid may be subject to the effects of geographic locations, maturating stage of the fruiting body, genotype and weather conditions (FERREIRA *et al.*, 2009).

3.3. Flavonoids

The flavonoid content of the mushrooms studied is shown in the Table 3. The concentration varies depending the mushroom species, and the differences are highly significant ($p < 0.001$). The results of the present study are similar to those reported by WOLDEGIORGIS *et al.* (2014) who found concentrations of 0.17 to 1.97 mg catechin equivalent/g. Although KORZASKI *et al.* (2015) report that *C. cibarius* is a rich source of flavonoids (42.9 mg of catechin equivalents/g of dry mushroom), this does not agree with our finding. The results suggest that flavonoids may play an important role in the total antioxidant activity, specifically in reducing power.

Table 3. Concentration of ascorbic acid, flavonoids and β-glucans in wild edible mushrooms from Mexico.

Mushroom	Ascorbic acid (mg/g)	Flavonoids (mg QE/g)	β-glucans (mg/g)
A. caesarea	2.08±0.16^{d}	0.39±0.04^{b}	214.92±2.09^{a}
B. edulis	2.61±0.05^{c}	0.92±0.02^{a}	39.97±0.91^{c}
C. cibarius	2.63±0.09^{c}	0.34±0.02^{b}	nd
L. indigo	3.65±0.00^{a}	0.25±0.00^{c}	88.34±3.62^{b}
Ramaria sp	3.14±0.04^{b}	Nd	nd

nd = not detected. In each row, different letters imply significant differences ($p< 0.001$). Mean±SD; n = 3.

3.4. β-glucans

β-glucans are the most abundant polysaccharide on the fungal cell wall (FESEL and ZUCARO, 2015) and the colorimetric method with Congo red detects β-1,3-1,6 glucans from mushrooms with high precision and without extensive clean-up (ZHU *et al.*, 2015). Thus, the β-glucans content determined by this study showed highly significant differences ($p< 0.01$) between the mushroom species (Table 3). The highest β-glucans content was detected in *A. caesarea* with 214.92 mg/g of dry mushroom, while *Ramaria* sp. and *C. cibarius* did not show detectable β-glucans contents. Despite the fact that there are several reports on β-glucans in mushrooms in other countries (SYNYTSYA and NOVÁK, 2013; RUTHES *et al.*, 2015), the species described here have been not explored yet, except for *B. edulis*. MANZI *et al.* (2004) reported that rehydrated dry samples of *B. edulis* had a β-glucans content of 43.3 mg/g, which is similar to our finding for this mushroom (39.97 mg/g). It seems that *A. caesarea* is a rich source of β-glucans, which will produce health benefits when consumed.

The Mexican wild edible mushrooms studied in this research shown high contents of chlorogenic and ascorbic acids and flavonoids. *B. edulis* has the highest antioxidant activity, which correlates with its contents of chlorogenic and cinnamic acids and flavonoids. *L. indigo* has the highest content of ascorbic acid, and *A. caesarea* registered the highest concentration of β-glucans. The presence of these biologically active molecules in the mushrooms studied reveals the nutraceutical potential of the mushrooms.

REFERENCES

Barros L., Ferreira J.M., Queiroz B. and Ferreira I.C.F. R. 2007. Total phenols, ascorbic acid, b-carotene and lycopene in Portuguese wild edible mushrooms and their antioxidant activities. Food Chem. 103:413.

Barros L., Barreira J. C. M., Grangeia C., Batista C., Cadavez V. A. P., and Ferreira I. C. F. R. 2011. Beef Burger patties incorporated with *Boletus edulis*extracs: Lipid peroxidation inhibition effects. Eur. J. Lipid Sci. Tech. 113: 737.

Fernandes A., Barreira Antonio A.L., Rafalski A., Beatriz M.B.P.P., Martins A. and Ferreira I.C.F.R. 2015. How does electron beam irradiation dose affect the chemical and antioxidant profiles of wild dried *Amanita* mushrooms? Food Chem. 182, 309.

Ferreira I.C.F.R., Barros L. and Abreu R.M. 2009. Antioxidants in wild mushrooms. Curr. Med. Chem. 16(12):1543.

Fesel P.H. and Zuccaro A. 2015. β-glucan: crucial component of the fungal cell wall and elusive MAMP in plants, Fungal Genetics and Biol., doi: http://dx.doi.org/10.1016/j.fgb.2015.12.004

Heleno S.A., Ferreira R.C., Antonio A.L., Queiroz M.J.R.P., Barros L. and Ferreira I.C.F.R. 2015a. Nutritional value, bioactive compounds and antioxidant properties of three edible mushrooms from Poland. Food Biosc. 11:48.

Heleno S.A., Martins A., Queiroz M.J.R.P. and Ferreira. 2015b. Bioactivity of phenolic acids: Metabolites *versus* parent compounds: A review. Food Chem. 173:501.

Huang D., Ou B. and Prior R.L. 2002. The chemistry behind antioxidant capacity assays. J. Agric. Food Chem., 50:3122.

Jung J.Y., Lee I.K., Seok S.J., Lee H.J., Kim Y.H. and Yun B.S. 2008. Antioxidant polyphenols from the mycelial culture of the medicinal fungi *Inonotusxeranticus* and *Phellinuslinteus*. J. App. Microb. 104:1824.

Kagimura F.Y., Cunha M.A.A., Barbosa A.M., Dekker R.F and Malfatti C.R.M. 2015. Biological activities of derivatized D-glucans: A review. Int. J. Biol. Macromol. 72:588.

Kim Y.M., Seguin P., Ahn J.K., Kim J.J., Chun S.C., Kim E.H., Seo S.H., Eun Y.K., Sun L.K., Yool J.P., Hee M.R. and Ill. M.C.2008. Phenolic compound concentration and antioxidant activities of edible and medicinal mushrooms from Korea. J. Agric. Food Chem. 56: 7265.

Korzaski M., Klaus A., Vunduk J., Zizak Z., Niksic M., Jakovljevic D., Miroslav M.V. and Leo J.L.D.V.G. 2015. Nutraceutical properties of the methanolic extract of edible mushroom *Cantharelluscibarius* (Fries): primary mechanisms. Food Funct. 6:1875.

Læssøe. 2013. Mushrooms. How to identify and gather wild mushrooms and other fungi, DK.

Manzi P., Marconi S., Aguzzi A. and Pizzoferrato L. 2004. Commercial mushrooms: nutritional quality and effect cooking. Food Chem. 84:201.

Moreno F. A. 2014. Un recurso alimentario de los grupos originarios y mestizos de México: Los Hongos Silvestres. An. Antrop. 48-I, 241, ISSSN: 0815-1225.

Nitschke J., Modick H., Busch E., Wantoch V. R. Altenbach and Mölleken H. 2011. A new colorimetric method to quantify β-1,3-1,6-glucans in comparison with total β-1,3-glucans in edible mushrooms. Food Chem. 127:791.

Nowacka N., Nowak R., Drozd M., Olech M. and Los R. 2014. Analysis of phenolic constituents, antiradical and antimicrobial activity of edible mushrooms growing wild in Poland. LWT Food Sci. Technol. 59:689.

Özen T. and Türkeful I. 2010. Antioxidant activities of *Sarcodonimbricatus* wildly grown in the Black Sea Region of Turkey. Phcog. Mag. 6(22):89.

Özyurek M., Bener M., Güçlü K., and Apal R. 2014. Antioxidant/antiradical properties of microwave-assisted extracts of three wild edible mushrooms. Food Chem. 157:323.

Palacios I., Lozano M., Moro C., D'arrigo M., Rostagno M.A., Martínez J.A., García L.A., Guillamón E. and Villares A. 2011. Antioxidant properties of phenolic compounds occurring in edible mushrooms. Food Chem. 128:674.

Pan X., Niu G. and Liu H. 2003. Microwave-assisted extraction of tea polyphenols and tea caffeine from green tea leaves. Chem. Eng. Process 42:129.

Pereira E., Barros L., Martins A. and Ferreira I.C.F. R. 2012. Towards chemical and nutrition inventory of Portuguese wild edible mushrooms in different habitats. Food Chem. 130:394.

Reis F.S., Heleno S.A., Barros L., Sousa M.J., Martins A., Buelga C.S. and Ferreira I.C.F.R. 2011. Toward the antioxidant and chemical characterization of mycorrhizal mushrooms from Northeast Portugal. J. Food Sci. 76(6):C824-C830.

Ruthes A. C., SmiderleRibeiro F. and Iacomini M. 2015. D-Glucans from edible mushrooms: A review on the extraction, purification and chemical characterization approaches. Carbohyd. Polym. 117:753.

Sarikurcku C., Tepe B., Kocak M.S. and Uren M.C. 2015. Metal concentration and antioxidant activity of edible mushrooms from Turkey. Food Chem. 175:549.

Siu K.C., Chen X. and Wu J.Y. 2014. Constituents actually responsible for the antioxidant activities of crude polysaccharides isolated from mushrooms. J. Funct. Foods 11:548.

Synytsya A. and Novák M. 2013. Structural diversity of fungal glucans. Carbohyd. Polym. 92:792.

Taofiq, O., Calhelha, R. C., Heleno, S., Barros, L., Martins, A., Santos, C., Queiroz, M. J. R. P., and Ferreira, I. C. F. R. 2015. The contribution of phenolics acids to the anti-inflammatory activity of mushrooms: Screening in phenolic extracts, individual parent molecules and synthesized glucuronated and methylated derivatives. Food Res. Int. 76:821.

Vaz, J. A., Barros, L., Martins, A., Buelga, C., Vasconcelos M. H., and Ferreira, I. C. F. R. 2011. Chemical composition of wild edible mushrooms and antioxidant properties of their water soluble polysaccharidic and ethanolic fractions. Food Chem. 126:610.

Wang, X. M., Zhang, J., Wu, L. H., Li, J. Q., Wang, Y.Z., Liu, H. G. 2014. A mini-review of chemical composition and nutritional value of edible wild-grown mushroom from China. Food Chem. 151:279.

Woldegiorgis, A. Z., Abate, D., Haki, G., and Ziegler, G. R. (2014). Antioxidant property of edible mushrooms collected from Ethiopia. Food Chem. 157:30.

Zhu, F., Du, B., Bian, Z. and Xu, B. 2015. Beta-glucans from edible and medicinal mushrooms: Characteristics, physicochemical and biological activities. J. Food Comp. Anal. 41:165.

Paper Received April 24, 2017 Accepted September 8, 2017

GUIDE FOR AUTHORS

Editor-in-Chief: **Paolo Fantozzi**
Dipartimento di Scienze Agrarie, Alimentari ed Ambientali, Università di Perugia, Via S. Costanzo, I-06126 Perugia, Italy - Tel. +39 075 5857910 - Telefax +39 075 5857939-5857943 - e-mail: paolo.fantozzi@ijfs.eu

IJFS has a publication charge of € 350.00 each article

Publication Ethics and Publication Malpractice

Italian Journal of Food Science is committed to upholding the highest standards of publication ethics and takes all possible measures against any publication malpractices. All Authors submitting their works to Italian Journal of Food Science for publication as original articles attest that the submitted works represent their Authors' contributions and have not been copied or plagiarized in whole or in part from other works. The Authors acknowledge that they have disclosed all and any actual or potential conflicts of interest with their work or partial benefits associated with it. In the same manner, Italian Journal of Food Science is committed to objective and fair Editor(s) review of the submitted for publication works and to prevent any actual or potential conflict of interests between the editorial personnel and the reviewed material. Any departures from the above-defined rules should be reported directly to the Editor-in-Chief, who is unequivocally committed to providing swift resolutions to any of such a type of problems.

1. Manuscript Submission

Manuscripts must submitted as an electronic version to
http://www.chiriottieditori.it/ojs/index.php/IJFS/index
All the Authors must be registered with initals, surname and e-mail. The word processor used to generate the file should be indicated and the files should be saved in format "Text only"; **graphs, pictures and diagrams must be saved in JPG format**. Manuscripts must be typed single-space, using Times New Roman 12 pt as the advised font; employs italics, rather than underlining (except with URL addresses); **lines must be numbered**. A single file must contain the manuscript and all the tables and figures inserted at the appropriate points.

English is the official language. The Editor-in-Chief and/or Co-Editors reserve the right to make literary corrections and to make suggestions to improve brevity, but **the paper must be previously revised for English by the authors**. If English is not the mother tongue of authors, they **must seek** help from one of the following agencies (or other similar official agencies):
WWW.JOURNALEXPERTS.COM
WWW.SCIENCEDOCS.COM
WWW.INTERNATIONALSCIENCEEDITING.COM
WWW.WRITESCIENCERIGHT.COM

When the paper will be accepted, copy of the English Translator Certificate of the revised version must be uploaded.

2. Manuscript Preparation

(1) The paper should be divided under the following headings in this order:
Title. Informative of the content of the article (<50 characters + spaces).
Author(s). Initials and Surname, omit professional and official titles. The institute and address where the research was carried out and the current address of each author should be given on the title page.
Abstract. Clearly state the objective of the study, give a concise description of experiment(s), observations, results and conclusions. No references should be cited. **Do not exceed 100 words**.
Key words. Up to six words, in alphabetical order, which describe the document must be given to aid data retrieval and indexing.
Introduction. Review pertinent previous work and cite appropriate references. State the purpose of the investigation.
Materials and Methods. Indicate apparatus, instruments, reagents, etc., giving sufficient detail to allow the work to be repeated.
Results and Conclusions. Results and Conclusions may be presented together or separately. Concisely present results using tables and figures to help justify conclusions (do not present the same information in both forms). Use statistical analysis when appropriate. Unsupported hypotheses should be avoided. Conclusions should point out the significance of the findings and, if possible, relate the new findings to some problem in Food Science and Technology.
Acknowledgments. Acknowledgments of assistance are appropriate provided they are not related to analyses or other services performed for a fee. Financial support, thanks for assistance, article number or thesis fulfilment may be included.
Units. A list of units particular to the paper may be included.
References. References in the Reference list should be arranged alphabetically (initials of first name, only), and, for the same author, should be arranged consecutively by year, typed double-spaced. Each individual reference should begin flush left (no indentation). Refer to attached examples taken from "Style Guide for Research Papers" by the Institute of Food Technologists (Chicago - I1linois - USA). Literature citations in the text should be referred to by Surname and year in parentheses. If there are more than two authors, give the surname of the first author and add et al. and the year in parentheses. Examples: (SMITH, 2007), (SMITH and JONES, 2008) (SMITH et al., 2008).

(2) Tables should be as few and as simple as possible and include only essential data. Each table must be saved within the text at the appropriate points, and have an Arabic number, e.g. Table 4 NOT Tab. 4. Legends must be self-explanatory. Use lower-case letters for footnotes in tables and explain below the table in the order in which they appear in the table.

(3) Figures must be prepared and saved in **JPEG format,** and inserted in the manuscript at the appropriate points. They should be prepared so that on 50% reduction, lines, figures and symbols will be clearly legible and not overcrowded. All figures must be given Arabic numbers, e.g. Fig. 3. Legends for figures must be self-explanatory.

(4) Standard Usage, Abbreviations and Units. The Concise Oxford and Webster's English Dictionaries are the references for spelling and hyphenation. Statistics and measurements should always be given in figures, e.g. 10 min, except when the number begins a sentence. When the number does not refer to a unit of measurement it should be spelled out unless it is 100 or greater. Abbreviations should be used sparingly, only when long or unwieldy names occur frequently, and never in the title; they should be given at the first mention of the name. International Standard abbreviations should generally be used except where they conflict with current practice or are confusing. For example, 3 mm rather than $3x10^{-3}$ m. Abbreviations should be defined the first time they are used in the text and they should be used consistently thereafter. Temperatures should be expressed in the Celsius (centigrade) scale. Chemical formulae and solutions must specify the form used, e.g. anhydrous or hydrated, and the concentration must be in c1early defined units.
Common species names should be followed by the Latin binomial (italics) at the first mention. For subsequent use, the generic name should be contracted to a single letter if it is unambiguous.

3. Editorial and Review Policy

Scientific contributions in one of the following forms may be submitted:
Reviews –They can be submitted directly to the Editor-in-Chief or articles can be requested directly by the Editor-in-Chief.
Short Communications, Surveys and Opinions – They do not need to have the formal organization of a research paper; they will receive priority in publication; maximum of five pages allowed.
Papers – The paper must follow the guidelines as specified under the section Manuscript Preparation.

Reviews, Papers, Short Communications and Surveys will be **subjected to critical review by referees.**
(1) Manuscripts will be processed in the order received. The Editor-in-Chief will select papers to enter into the reviewing system based on originality and innovation. A letter will be sent to the authors acknowledging receipt of the manuscript along with a Declaration form stating that it has NOT been previously published, accepted or submitted for publication elsewhere and agreeing to the page charges upon acceptance of the paper. On receipt of the signed Declaration form, the Editor-in-Chief will send the manuscript to a Co-Editor and/or referees for evaluation.
(2) Referees may not be from the same institution as the author. Referees should make their comments and questions in detail and return the paper to the Editor-in-Chief and/or Co-Editor as soon as possible, usually within three weeks. The identity and report of the referees are made know to the Editor-in-Chief, but only the anonymous referee report is sent to the author(s). If all referees recommend acceptance or rejection, the decision stands. If the opinions of the referees tie, the Editor-in-Chief and/or Co-Editors have the freedom to decide upon acceptance or rejection of the paper.
(3) The results of the refereeing process, accompanied by a letter from the Editor-in-Chief or the Co-Editor, will be sent to the author(s). **Papers needing revision must be returned to the Co-Editor within four weeks, otherwise the paper will be considered as withdrawn**. A letter announcing acceptance of the manuscript will be sent to the author(s) upon acceptance by the referees.
(4) The authors **will receive galley proofs** of the manuscript along **with the invoice** for the article charge which **must be paid in order to allow for publication**. The

proofs will be sent to the corresponding author as a PDF file by e-mail, only.
Once the page charge is paid, the DOI will be sent to the Author.

REFERENCE EXAMPLES

EXAMPLES of use in a Reference list are given below. The bold-faced parenthetical type of citation above the example is indicated ONLY for information and is NOT to be included in the reference list.

(Anonymous)
Anonymous. 1982. Tomato product invention merits CTRI Award. Food Technol. 36(9): 23.

(Book)
AOAC. 1980. "Official Methods of Analysis" Association of Official Analytical Chemists, Washington, DC.
Weast, R.C. (Ed.). 1981 "Handbook of Chemistry" 62nd ed. The Chemical Rubber Co. Cleveland, OH.

(Bulletin, circular)
Willets C.O. and Hill, C.H. 1976. Maple syrup producers manual Agric. Handbook No. 134, U.S. Dept. of Agriculture, Washington, DC.

(Chapter of book)
Hood L.F. 1982. Current concepts of starch structure. Ch. 13. In "Food Carbohydrates". D.R. Lineback and G.E. Inglett (Ed.), p. 217. AVI Publishing Co., Westport, CT.

(Journal)
Cardello A.V. and Maller O. 1982. Acceptability of water, selected beverages and foods as a function of serving temperature. J. Food Sci. 47: 1549.
IFT Sensory Evaluation Div. 1981a. Sensory evaluation guide for testing food and beverage products. Food Technol. 35 (11): 50.
IFT Sensory Evaluation Div. 1981b. Guidelines for the prepaation and review of papers reporting sensory evaluation data. Food Technol. 35(4): 16.

(Non-English reference)
Minguez-Mosquera M.I., Franquelo Camacho A, and Fernandez Diez M.J. 1981. Pastas de pimiento. Normalizacion de la medida del color. Grasas y Aceites 33 (1): 1.

(Paper accepted)
Bhowmik S.R. and Hayakawa, K. 1983. Influence of selected thermal processing conditions on steam consumption and on mass average sterilizing values. J. Food Sci. In press.

(Paper presented)
Takeguchi C.A. 1982. Regulatory aspects of food irradiation. Paper No. 8, presented at 42nd Annual Meeting of Inst. of Food Technologists, Las Vegas, NV, June 22-25.

(Patent)
Nezbed R.I. 1974. Amorphous beta lactose for tableting U.S. patent 3,802,911, April 9.

(Secondary source)
Sakata R., Ohso M. and Nagata Y. 1981. Effect of porcine muscle conditions on the color of cooked cured meat. Agric. & Biol. Chem. 45 (9): 2077. (In Food Sci. Technol. Abstr. (1982) 14 (5): 5S877).

(Thesis)
Gejl-Hansen F. 1977. Microstructure and stability of Freeze dried solute containing oil-in-water emulsions Sc. D. Thesis, Massachusetts Inst. of Technology, Cambridge.

(Unpublished data/letter)
Peleg M. 1982. Unpublished data. Dept. of Food Engineering., Univ. of Massachusetts, Amherst.
Bills D.D. 1982. Private communication. USDA-ARS. Eastern Regional Research Center, Philadelphia, PA.

CONTRIBUTORS

Gratitude is expressed to the following entities for contributing to the realization of the Journal by being supporting subscribers for 2017.

ASSOCIATIONS and COMPANIES

Associazione Italiana di Tecnologia Alimentare (A.I.T.A.)
Parma
Fax +39-0521-230507
www.aita-nazionale.it

Società Italiana di Scienze e Tecnologie Alimentari (S.I.S.T.Al)
Perugia
Fax +39-075-5857939
www.sistal.org

RESEARCH INSTITUTES

Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali (DI.VA.P.R.A.), Sezione Microbiologia ed Industrie Agrarie, Università di Torino
Grugliasco
Fax +39-011-6708549

ITALIAN JOURNAL OF FOOD SCIENCE
Rivista Italiana di Scienza degli Alimenti
DIRETTORE RESPONSABILE: Alberto Chiriotti
AUTORIZZAZIONE: n. 3/89 in data 31/1/1989
del Tribunale di Perugia

ISSN 1120-1770

CHIRIOTTI EDITORI srl - 10064 Pinerolo - Italy

publishes the technical magazines:

VOLUME XXIX IJFS No. 4, 2017

CONTENTS