ISSN 1120-1770

ITALIAN JOURNAL OF FOOD SCIENCE

Rivista italiana di scienza degli alimenti

IJFS

Volume XXIII
Number 2
2011

CHIRIOTTI EDITORI

ITALIAN JOURNAL OF FOOD SCIENCE
(RIVISTA ITALIANA DI SCIENZA DEGLI ALIMENTI) 2nd series

Founded By Paolo Fantozzi under the aeges 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

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Publisher:
Alberto Chiriotti
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e-mail: info@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.
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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: 5-Year Impact Factor: 0.596 published in 2009 Journal of Citation Reports, Institute for Scientific Information; Index Copernicus Journal Master List 2009 (ICV): 13.19
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 page charge of € 25.00 each page.

Subscription Rate: IJFS is available on-line in PDF format only.
2011: Volume XXIII: Ordinary € 50.00 - Supporting € 1,000.00

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OPINION PAPER

APPLICATION OF THE HAZARD ANALYSIS AND CRITICAL CONTROL POINT (HACCP) SYSTEM ON THE MUSHROOM PROCESSING LINE FOR FRESH CONSUMPTION

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ABSTRACT

The Hazard Analysis and Critical Control Point (HACCP) is a preventive system that intends to guarantee the safety and harmlessness of food. It improves the quality of products as it eliminates possible defects during the process, and saves costs by practically eliminating final product inspection. This work describes the typical hazards encountered on the mushroom processing line for fresh consumption. Throughout the process, only the reception stage of mushrooms has been considered a critical control point (CCP). The main hazards at this stage were: the presence of unauthorised phytosanitary products; larger doses of such products than those permitted; the presence of pathogenic bacteria or thermo-stable enterotoxins. Putting into practice such knowledge would provide any industry that processes mushrooms for fresh consumption with a self-control HACCP-based system for its own productions.

- Key words: hygiene, safety, industry, self-control, quality -

INTRODUCTION

The Hazard Analysis and Critical Control Point (HACCP) is a preventive system that intends to guarantee food safety, identify the specific hazards associated with food or beverages, and establish the control systems which centre on prevention and not on the final product test (EEC, 1993). This system is based on principles which have been well defined internationally to offer firms a more detailed and systematic control over their various production stages and processes so they make better use of their often limited resources, and respond more swiftly and efficiently to possible contingencies. Besides, HACCP is an ongoing system that helps face new hazards originating from emerging pathogens and food toxic infections as a result of changes in and forms of consumption.

The HACCP system may be applied along the whole food chain, that is, from acquiring raw materials, to the production, distribution, sale and consumption of the product (WATSON, 1994). Apart from improving food safety, this system may offer other significant advantages, such as facilitating the inspections made by the regulatory organisations, cutting losses in the final product (eliminating the causes that alter them), and promoting international commerce by increasing the trust in food safety.

Basically owing to fierce competition and lower prices, a generalised drop in production in all the Countries of the European mushroom sector took place in 2003. Today, the sector is being restructured given the entrance of new Countries like Poland, currently one of the largest mushroom-producing Countries, and Hungary. European production is presently around 1,260,000 t of mushrooms (ASAJA, 2007). Traditional processing Countries like the Netherlands, France and Spain are being affected by imports from China. Both Poland and China have rapidly entered foreign markets in recent years, and have seriously affected Spanish exports. Therefore, producers are concerned about the possibility of the European Commission considering increased market quotas; that is, basically authorising imports from China. Therefore, implementing the HACCP system in this type of industries intends to both improve the quality of their products considerably and increase customer satisfaction and food safety by improving the firms' image and boosting their competitiveness by facilitating the possibility of opening new markets at the same time.

Spanish mushroom production increased until 2001, but has practically stabilised since then. Spain, presently with an approximate production of 165,000 t, is Europe's fourth mushroom-producing Country behind the Netherlands, Poland and France and followed by Italy (FAOSTAT, 2007). Spain's production is divided between two main areas: La Rioja and Castilla-la Mancha. There are other production centres scattered around different Spanish regions, but collectively they represent only 2% of Spanish production.

The main objective of this work is the implementation of the HACCP system in the mushroom processing line for fresh consumption. It will allow mushroom factories to design and implement a self-control system for their own productions. This objective would greatly facilitate official control tasks and offer a much more complete and objective vision of what actually takes place in the industry.

MATERIALS AND METHODS

This work has been the result of the implementation of the HACCP system in the mushroom processing line for fresh consumption in different factories in La Manchuela (Castilla-la Mancha, Spain). The first step involved the collection of information about the process and the physico-chemical and microbiological characteristics of the raw material used. Information about instructions on usage, and transport and storage conditions was also sought.

One important step in the methodology followed has been the design of a flow diagram showing the complete production process (Fig. 1). After designing, reviewing and checking this diagram, each stage of the process was reviewed to search for possible hazards (biological, physical or chemical) which may endanger food safety.

Having defined any hazards encountered, we then defined the possible preventive measures to mitigate or even eliminate the hazard. Afterwards, the critical control points (CCPs) were determined by means of a tree or sequence of decisions indicating the logical reasoning approach, as various International Organisations recommend (ICMSF, 1991; FAO, 2003).

When a stage is considered a CCP, the critical limits were established over or under which the process would be unacceptable. In order to detect these possible discrepancies, a monitoring or surveillance system was established to set up the CCP-programmed measurements or observations in relation to the critical limits.

Then the specific correcting measures required for all the CCPs were drawn up to face the possible deviations which could take place.

Finally, a documentation and recording system was set up to document all the HACCP system procedures, along with all the records that had to be registered to implement the HACCP system correctly.

RESULTS AND DISCUSSION

In this section the flow chart of the process (Fig. 1) is explained from the reception of auxiliary material to the dispatch of the finished product in accordance with the scope of the study and always on the basis of the real process observed in the various mushroom-processing factories for fresh consumption analysed.

Table 1 summarises the hazards analysis of the mushroom processing line for fresh consumption. The foreseeable main hazards (microbiological, chemical and physical) are described for each stage, along with the preventive measures to be taken with a view to minimising or eliminating such hazards. This table also indicates the stages that were considered CCPs after the decision tree was implemented. The answers to the different questions raised on the decision tree for each stage, as well as the comments explaining the reasons for not being included as a CCP, are shown in Table 2. The critical levels, monitoring system, including the frequency of surveillance, corrective actions and records taken for each stage, are also described in the summary table.

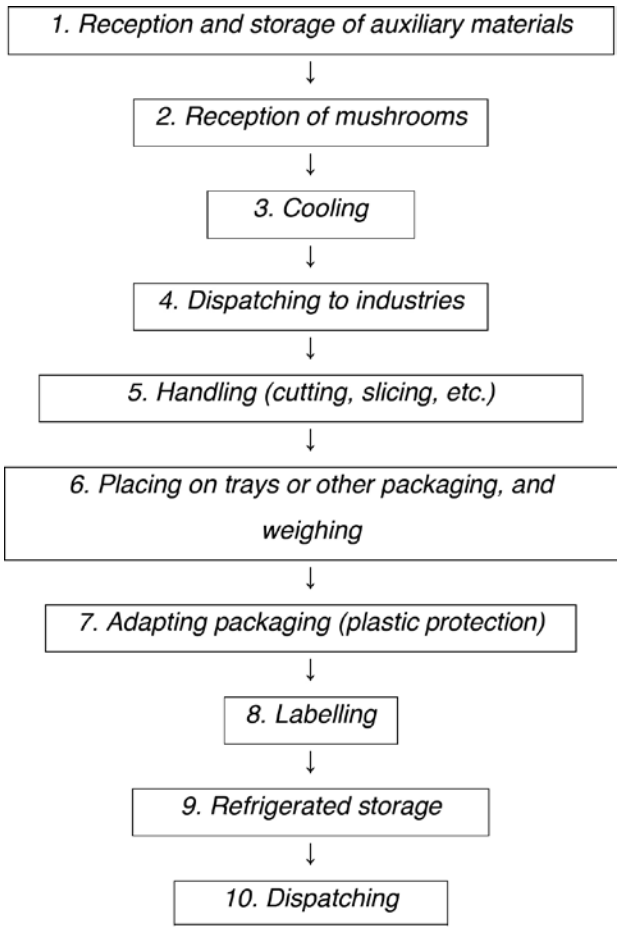


Fig. 1 - Flow diagram of the mushroom processing line for fresh consumption.

Stage 1. Reception and storage of auxiliary material

The auxiliary material used to carry the mushrooms from the production plant to the processing factory (boxes and pallets), and from this factory to the shops (trays, labels, plastic film, etc.) is received and subjected to a thorough visual inspection. Once this control has been passed, the auxiliary material can be stored in the storage area.

Hazards: One of the main hazards of this stage will be accepting material that does not meet the requirements for use with food or the basic sanitary standards.

Preventive measures: It will be necessary to officially approve the suppliers of the auxiliary materials, which will involve defining purchase specifications and demanding technical specifications for all the materials they supply. Prior to their official approval, visits will be organised to the suppliers to verify that they fulfil the necessary quality and hygiene regulations. Packaging should never be toxic, give off smells or flavours, or allow dyes to come into contact with the mushrooms, and should withstand being stacked.

Critical Control Point (CCP): This stage was not considered a CCP since the approval of suppliers ensures compliance with the specifications laid down in Royal Decree 397/1990 of 16 March, which approves the general conditions of the materials used for food, apart from polymers, as well as the hygienic quality of the material received.

Stage 2. Reception of mushrooms

The mushrooms that arrive at the factory are unloaded and weighed in the reception room. Mushrooms are received mainly in bulk (in boxes), although some may be received on trays. Mushrooms may be received without stalks, sliced or even placed on trays covered in plastic film. Mushrooms must be picked when mature, when the stalk has become flexible and the whole mushroom is softer when touched. Mushrooms should always be picked before the velum covering the hymenium breaks. Two boxes of each batch will be selected randomly for the purpose of determining the sensory quality. A Quality Committee will be in charge of the sample analysis, and will offer incentives or impose sanctions to the associates according to several analysed parameters. Delivering mushrooms early in the morning, when temperatures are as low as possible will be encouraged because the subsequent refrigeration of mushrooms is more effective. In this phase, all the batches received will be labelled in accordance with the firm's established tracking system. Labels will include the date, time, weight and a reference corresponding to the associate's

Table 1 - Summary table of the hazard analysis for the transformation line of mushrooms for fresh consumption.

STAGE	TYPE OF HAZARD			HAZARDS	PREVENTIVE MEASURES	CP	CRITICAL POINTS (CP)	SURVEILLANC/ FREQUENCY	CORRECTIVE MEASURES	RECORDS
	Physical	Chemical	Biological							
1. Reception and storage of auxiliary materials	X	X	X	*Packaging not suitable for food use. *Packaging without hygiene.	*Official approved for suppliers.	NO				
2. Reception of mushrooms		X	X	*Presence of phytosanitary products *Presence of bacterial pathogens, yeasts, mosses or thermostable enterotoxins.	*Forbidding non-authorised phytosanitary products. *Follow the harvesting and picking guidelines. *Careful, rapid and exclusive use of transport for mushroom in clean vehicles.	YES	*Respect the safety doses and periods. *Fulfil the harvesting and picking guidelines established by the firm.	*Visual inspection of each lot and sampling plan to ensure quality and lack of toxic residues. *Handing according to guidelines and their fulfilment. *Conditions of transport and boxes.	*Rejecting or sending away unsuitable lots. *Stress the harvesting and transport guidelines.	*Reception of lots. *Results of both the sampling plan and the quality of the rejected lots. *Incidences and corrective measures.
3. Cooling			X	*Altered microbials from high cooling temperatures or lack of hygiene in chambers.	*Preventive maintenance programme for equipment. *Good hygiene and handling practices.	NO				
4. Dispatching to industries			X	*Microbiological contamination caused by lack of hygiene among workers, in the dispatching area or during transport, or a problem with temperatures during transport.	*Suitable C+D plan in the dispatching area and in the transport vehicles. *Preventive measures completed in the refrigerated chambers of the transport vehicles.	NO				
5. Handling (cutting, slicing, etc.)	X		X	*Contamination caused by metal traces. *Microbiological contamination caused	*Utensils are maintained in good order (knives, cutters, etc.). *Suitable C+D plan.	NO				

Table 1 - Continued.

STAGE	TYPE OF HAZARD			HAZARDS	PREVENTIVE MEASURES	CP	CRITICAL POINTS (CP)	SURVEILLANC/ FREQUENCY	CORRECTIVE MEASURES	RECORDS
	Physical	Chemical	Biological							
				by lack of hygiene among workers or by unhygienic surfaces which come into contact with food.	*Application of good hygiene and handling practices. *Correct temperature in the premises.					
6. Placing onto trays or other packaging, and weighing	X		X	*Presence of foreign bodies. *Microbiological contamination owing to lack of hygiene of either workers or the working surfaces that food comes into contact with.	*Suitable C+D plan. *Application of good hygiene and handling practices. *Suitable temp. in the premises.	NO				
7. Adapting packaging (plastic protection)		X	X	*Inappropriate use of film.	*Approval of suppliers (demand characteristics and specifications). *Suitable temp. in the premises.	NO				
8. Labelling	X			*Labelling machine fails to work properly.	*Preventive maintenance programme for equipment.	NO				
9. Refrigerated storage	X		X	*Microbial growth by high cooling temperature. *Damaged trays.	*Preventive maintenance programme for equipment. *Good handling practices.	NO				
10. Dispatching	X		X	*Damage caused by badly stored trays. *Microbial growth due to high cooling temperature.	*Careful handling and transport. *Preventive maintenance programme for the refrigerated chambers on vehicles.	NO				

Table 2 - Determination of the processing stages as Critical Control Points (CCP), according to the answers given in the decision tree.

STAGE	HAZARD	Q1*	Q2*	Q3*	Q4*	CCP	COMMENTS
1. Reception and storage of auxiliary materials	- Packaging not suitable for food use. - Packaging without hygiene.	YES YES	NO NO	NO NO	- -	NO NO	Approval of suppliers to minimise the hazards in this phase.
2. Reception of mushrooms	- Presence of phytosanitary products. - Presence of bacterial pathogens, yeasts, mosses or thermostable enterotoxins.	YES YES	YES	-	-	YES	
3. Cooling	- Altered microbials from excessively high cooling temperatures or lack of hygiene in chambers.	YES	NO	NO	-	NO	Implementation of preventive measures (preventive maintenance of equipment and C+D plan) to minimise the hazards in this phase.
4. Dispatching to industries	- Microbiological contamination caused by lack of hygiene among workers, in the dispatching area or transport, or a problem with temperatures during transport.	YES	NO	NO	-	NO	Implementation of preventive measures (cleaning and disinfection programmes and preventive maintenance of equipment) to minimise the hazards in this phase.
5. Handling (cutting, slicing, etc.)	- Contamination caused by metal traces. - Microbiological contamination caused by lack of hygiene among the workers or on the working surfaces which come into contact with food.	YES YES	NO NO	NO NO	- -	NO NO	Implementation of preventive measures (preventive maintenance of equipment, C+D plan and good handling practices and hygiene) to minimize the hazards in this phase.
6. Placing onto trays or other packaging, and weighing	- Presence of foreign bodies. - Microbiological contamination owing to lack of hygiene among the workers or on the working surfaces which come into contact with food.	YES YES	NO NO	NO NO	- -	NO NO	Implementation of preventive measures (good handling practices and hygiene and C+D plan) to minimise the hazards in this phase.
7. Adapting packaging (plastic protection)	- Inappropriate use of film.	YES	NO	NO	-	NO	Approval of suppliers to minimise the hazards in this phase.
8. Labelling	- Labelling machine fails to work properly.	YES	NO	NO	-	NO	Implementation of preventive measures (preventive maintenance of equipment) to minimise the hazards in this phase.
9. Refrigerated storage	- Microbial growth caused by a high cooling temperature. - Damaged trays.	YES YES	NO NO	NO NO	- -	NO NO	Implementation of preventive measures (preventive maintenance of equipment and good handling practices) to minimise the hazards in this phase.
10. Dispatching	- Damage caused by badly stored trays. - Microbial growth caused by a high cooling temperature.	YES YES	NO NO	NO NO	- -	NO NO	Implementation of preventive measures (preventive maintenance of equipment and good handling practices) to minimise the hazards in this phase.

*Q1: Are there preventive measures for the identified hazard? *Q2: Has this stage been specifically designed to eliminate or lower the likelihood of this hazard appearing at an acceptable level?; *Q3: Could contamination appear or increase and reach unacceptable levels?; *Q4: Would a subsequent stage or action eliminate or mitigate the hazard at an acceptable level?

or farmer's number. Therefore, each batch will be identified with its farmer.

Hazards: The main hazard of this stage will be the presence of unauthorised phytosanitary products or in higher doses than those permitted for the mushrooms received. In addition, the microbiological contamination of mushrooms must be taken into account due to a possible lack of hygiene in cultivation chambers and in compost. The main pathogens associated with fresh mushrooms are *Clostridium botulinum*, *Escherichia coli*, *Campylobacter jejuni*, *Samonella* sp. and *Listeria monocytogenes* (GONZÁLEZ-FANDOS *et al.*, 2001; SAMADPOUR *et al.*, 2006; CHIKTHIMMAH *et al.*, 2007; KORSTEN, 2008). Deterioration caused by microbials after harvest are due to bacterial growth, mainly the genus *Pseudomonas*, which generates bacterial stains and also influences carpophore darkening (SIMÓN, 2004; PEÑARANDA, 2008), although in this case, the hygienic quality of the mushrooms is not affected.

Preventive measures: The use of phytosanitary products containing certain non-registered or unauthorised active matter will be forbidden. Similarly, all those recommendations or restrictions of use considered necessary to reduce residue content in the harvested product will be indicated (PARDO, 1994). To ensure suitable cultivation and harvesting so that the product arrives at the factory in safe conditions, the factory's recommendations will be followed, with special emphasis placed on the chambers and operators' hygiene. Transport will be organised as quickly as possible in clean, disinfected and well-ventilated vehicles to prevent microbial growth. In addition, exclusive transport for mushrooms will be used to avoid cross-contamination.

Critical levels: The farmer must respect the doses and safety periods of the phytosanitary products employed so that any residue exceeding the maximum residue limits set by legislation does not appear in the mushrooms. Farmers must comply with the growing and harvesting guidelines set by the firm as regards the hygiene of the cultivation chambers and operators. Good transport practices will be carried out and will be performed in the shortest possible time and under the appropriate hygiene conditions. Furthermore, vehicles should transport only mushrooms.

Monitoring: The firm shall have a sampling plan to control the quality of the mushrooms received, which includes monitoring the presence of toxic residues and pathogenic microorganisms. The firm must ensure that the cultivation and transportation guidelines have been given to each partner, and must check that these guidelines are being followed by organising surprise visits to cultivators. During mushroom reception, the person in charge will verify that exclusive transport for mushrooms was used and that the external appearance of the boxes is good.

Corrective actions: Whenever the firm detects that a batch does not arrive in acceptable conditions, this will be rejected or moved out of the way to seek the most appropriate solution; for example, sending such batches to the canning industry instead of using them for fresh consumption. If this problem persists with a given associate, its official approval status may be withdrawn. In addition, the company will be in charge of emphasising that the farming associates must learn and comply with the firm's farming, harvesting and transport guidelines.

Records: Records will be made on the delivery note of the associate/farmer's number and locality, weight, and the date and time the batch was admitted, along with the sanitary state it arrives in after the person in charge has visually inspected it. All the phytosanitary products used by the farmer will be recorded, as will the results of the residues analysis done of the phytosanitary products used. Any incidence occurring and any corrective measures carried out when the batch arrives will also be recorded.

Stage 3. Cooling

Once the mushrooms have passed the visual inspection to ensure their quality and weight, they will be placed inside the cooling chamber immediately to rapidly eliminate the heat they have absorbed in the cultivation chambers and to hold their oxidation (BURTON *et al.*, 1987). Containers with mushrooms will be stacked inside these chambers and maintained at a temperature of 2°-4°C. Storage in a cool atmosphere will stop the ripening process and will prevent deterioration (SIMÓN, 2004). Nonetheless, low temperatures will dry mushrooms due to the effect of evaporation in a cold, dry atmosphere, which could worsen the quality of the mushrooms and lower their weight. Therefore, refrigerated chambers require reliable modern humidity-controlled systems to maintain a relative humidity of 70-80%. Cooling, apart from prolonging the mushrooms' life and helping them better withstand subsequent handling, makes them more resistant to microbial contamination. The success of this stage depends on several factors: the time between picking mushrooms and cooling, the type of transport container, the product's initial and final temperatures, the velocity or amount of cold air, the cleaning of the air or water to be used while lowering the temperature in order to reduce the amount of decomposing microorganisms, and the maintenance of the recommended temperature after the cooling phase (PARDO *et al.*, 2004).

Chambers will consist in expanded polyurethane panels, powerful compressor fans, a sensor to determine the air temperature and sprinklers to maintain, and if necessary, to increase the relative humidity of the product. There will be a control panel on the outside of

each chamber to observe the temperature and the relative humidity inside the chamber at all times. These chambers will be used for the initial cooling phase and for subsequent conservation purposes since the conditions under which the product will be maintained must be equal in both stages.

Hazards: One of the main hazards to emerge in this stage will be the microbial alteration to mushrooms caused by excessively high cooling temperatures. The hazard involving microbiological alterations could also emerge owing to inadequately cleaned chambers.

Preventive measures: The first measure will consist in the preventive maintenance of the equipment used in the cooling chambers (fans, sprinklers, temperature sensors, etc.). A constant temperature of 2°-4°C must be maintained, and the relative humidity of the product must remain between 70-80%. Likewise, it is essential to ensure that the cleaning and disinfection of the chambers is appropriate to avoid contamination.

Critical Control Point (CCP): This was not considered a CCP as the preventive maintenance of equipment and the cleaning and disinfection programmes applied to the cooling chambers will ensure the proper working of the refrigeration equipment and the hygienic conditions inside the chambers, respectively.

Stage 4. Dispatching to industries

Generally, the firm itself handles the mushrooms after the cooling phase to obtain the final product (fresh mushrooms on trays). However in some cases, mushrooms could be sent to the canning industry before or after the handling stage (cutting, calibrating, slicing, etc.). If dispatched to industries, mushrooms will be removed from the refrigerated chambers or the processing room to be loaded onto lorries in the docks located in the dispatch room. The temperature in the transport vehicles could be somewhat higher, but should never exceed 10°C. However, it is preferable that the temperature inside these vehicles is the same as that inside the refrigerated chambers (2°-4°C) to avoid any deterioration of the mushrooms.

Hazards: The main hazard of this phase will be the microbiological contamination of the mushrooms caused by the poor hygiene of the delivery facilities or the transport vehicles, and excessively high temperatures during transport.

Preventive measures: The dispatching area, the boxes and the refrigerated chambers of the transport vehicles will be kept in perfect hygiene-sanitary conditions by suitably applying the established cleaning and disinfection plan in each case. A suitable temperature will be maintained in the dispatching area, and special care will be taken to avoid temperatures in excess of 12°C. Mushrooms will be left in this area for the shortest possible time to avoid their temperature dropping too low and any alterations to them. Another preventive measure in the refrigerated vehicle chambers will be to ensure the correct temperature (2°-4°C) and relative humidity (70-80%) during transport.

Critical Control Point (CCP): This was not considered a CCP since the cleaning and disinfection program implemented will guarantee the hygiene of the dispatching area and vehicles, while preventive maintenance will ensure the proper working of refrigeration equipments.

Stage 5. Handling (cutting, slicing, etc.)

In this stage, various operations will be carried out depending on the final product needed:

- Whole mushrooms with stalks: mushrooms are placed directly into boxes and are transferred to the next area without further handling.
- Whole mushrooms without stalks: stalks are removed and mushrooms are placed onto trays.
- Sliced mushrooms: mushrooms are sliced and placed onto trays.
- Combinations of spring onions, asparagus, sliced mushrooms: mushrooms are sliced and combined with the other products to be placed onto trays.

All these operations are done manually by the firm's manipulators. For these purposes, staff will use suitably sharpened stainless steel knives and cutters to ensure safe, perfect slices. Whole mushrooms must be calibrated before packing.

Hazards: The main hazard will be physical contamination caused by either the incorporation of metal traces from the knives and cutters used for removing stalks or for slicing, or the presence of foreign bodies or dirt on the working surfaces. Microbiological contamination caused by the manipulators' poor hygiene or the unhygienic conditions of the working surfaces will be another factor to bear in mind.

Preventive measures: The tools used for slicing or cutting mushrooms must be kept in perfect conditions and need to be suitably sharpened. The cleaning and disinfection plan for the handling area must be correctly carried out, particularly regarding working surfaces, and only authorised products for this purpose must be used. Knives and cutters will be disinfected at the start of each working day and each time a new batch of mushrooms is received for handling. Both the manipulators' hygiene and handling practices will be strictly fulfilled. The staff working in the handling area will wear a uniform comprising hygienic gloves, a white coat or working aprons, caps and, on specific occasions, masks. All the handling staff members will receive the appropriate training and will be made aware of the importance of appropriate hygiene and sanitary handling. The temperature in the handling area will always be maintained at below or equal to 12°C to avoid microbial proliferation.

Critical Control Point (CCP): This was not considered a CCP since the programme of preventive maintenance of equipment and utensils will avoid any traces of metals coming into contact with the mushrooms. In addition, the cleaning and disinfection programmes and the good handling and hygiene practices will prevent microbiological contamination.

Stage 6. Placing products onto trays or into other types of packaging, and weighing

Processed mushrooms are placed onto polystyrene trays and weighed on stainless steel balances. Damaged and very dirty mushrooms shall be removed and replaced to reach the required weight. Balances will be tared to exclude the weight of the tray from the product. Whole mushrooms that have not undergone handling will be received in this area in the boxes into which they were placed inside the refrigerated chambers. Therefore, workers will need trays to place and weigh them.

Hazards: One of the hazards at this stage will be the presence of foreign objects among mushrooms that cause mechanical damage, or even chemical or microbiological contamination. In this stage, the product could be microbiologically contaminated due to the unsuitable hygiene of either the workers or the working surfaces that mushrooms come into contact with.

Preventive measures: The main preventive measure will be the application of the hygiene and good handling practices, and carrying out the established cleaning and disinfection plans. Another important preventive measure will involve maintaining the temperature in this area at a maximum of 12°C to avoid or mitigate microbial proliferation.

Critical Control Point (CCP): This was not considered a CCP since the various programmes implemented (good handling, hygiene and cleaning practices and disinfection) will avoid hazards.

Stage 7. Adapting packagings (plastic protection)

In this stage, once the product has been placed on trays and weighed, it is transferred on a conveyor belt to the plasticising machine. Porous heat activating food film is used to facilitate the gas exchange and to avoid anaerobiosis conditions to prevent *Clostridium botulinum* from developing (SIMÓN, 2004).

Hazards: The main hazard at this stage will involve the use of unadequate or non-porous plastic film for food use, which can contaminate the mushrooms or enable pathogen formation of microorganisms like *C. botulinum*.

Preventive measures: The main preventive measure will consist in checking the validity of the transparent film used in accordance with the firm's suppliers. The approval of this supplier will ensure compliance with the terms of contract. For the purpose of reducing microbial proliferation, trays will be covered in plastic film immediately after being filled, and the temperature in the area will not exceed 12°C.

Critical Control Point (CCP): This was not considered a CCP since the approval of suppliers will ensure adequate film being used to meet the target objectives.

Stage 8. Labelling

Trays will leave the plasticising machine on a conveyor belt to be automatically transferred to the labelling machine. Labels will include details about the product, the commercial brand, the packing date, weight, conservation recommendations, plus a code consisting in one number and one letter. The number will correspond to the associate or farmer who grew the mushrooms, while the letter will indicate the worker or group of workers who handled them. This enables the product to be controlled until it reaches the destination market so it can be withdrawn should any problem be detected; it also helps identify its origin to help solve any problem earlier. Labelled trays will move on the conveyor belt to a rotating table where one or several workers will inspect them. If satisfactory, they will be placed suitably into boxes to be taken immediately to the refrigerated chambers.

Hazards: The only hazard at this stage will be incorrect tray labelling because the labelling machine does not work properly.

Preventive measures: The preventive maintenance programme for the labelling machine will be carried out.

Critical Control Point (CCP): This was not considered a CCP as the preventive maintenance of equipment will ensure the correct working of the labelling machine.

Stage 9. Refrigerated storage

The labelled trays are placed into boxes or containers, and are kept in refrigerated chambers to be dispatched as soon as possible.

Hazards: As in the cooling stage, one of the main hazards to emerge in this phase will be the microbial alteration of mushrooms due to excessively high refrigeration temperatures. Temperature fluctuations may cause water condensation on both the surface of the mushrooms and their packaging, which causes rotting. Another possible hazard will relate to breakages to packaging if handled wrongly, which will consequently contaminate the product.

Preventive measures: The first measure will consist in the preventive maintenance programme for the equipment inside the refrigerated chambers (fans, temperature sensors, etc.) to ensure a constant temperature of 2°-4°C. The workers' good handling practices on the will pre-

vent breakages to packaging, thus preventing subsequent microbial contamination.

Critical Control Point (CCP): This was not considered a CCP as the preventive maintenance programms for equipment and good handling practices will prevent inappropriate temperatures and breakages to packaging, respectively.

Stage 10. Dispatching

Once the product is ready, it will be dispatched for its commercialisation as soon as possible. It will be transported in refrigerated lorries at a temperature below 12°C at all times, even though they are normally offered for sale at room temperature when the cold chain benefits are lost (FROST *et al.*, 1989). The processing date and destination point will be checked before loading mushrooms onto lorries to foresee any replacements and control traceability.

Hazards: Packaging could become physically damaged as a result of bad handling practices or inadequate transport, which could lead to product contamination. Another factor to control will be microbial proliferation caused by excessively high temperatures during transport.

Preventive measures: Operators' good handling practices in the dispatching room and during transport will prevent breakages to trays and avoid consequent micobial contamination. Final products must be transported carefully to avoid mechanical damage to mushrooms, which could cause microbial contamination. The preventive maintenance of the refrigerated chambers on the vehicles will avoid inappropriate temperatures.

Critical Control Point (CCP): This was not considered a CCP as the preventive maintenance programmes for equipment and good handling practices will prevent inappropriate temperatures and breakages to packaging, respectively.

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Paper received February 3, 2010 Accepted September 22, 2010

PAPER

APPLICATION OF MODIFIED FATS BY ENZYMATIC INTERESTERIFICATION IN EMULSIONS

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ABSTRACT

Beef tallow was interesterified with sunflower oil using immobilized lipase from *Rhizomucor miehei*. The fats obtained were used as a fat base of the emulsions. Selected physicochemical properties of fats obtained (the content of free fatty acids, the polar fraction content and oxidative stability) were determined in the initial and modified fats. The emulsions with the interesterified fats (interesterification in the presence of Lipozyme IM with 10 and 15% water in the preparation) showed high stability. For the emulsions containing noninteresterified fats and interesterified products, where the content of water in the preparation was 4%, the opposite dependence was observed.

- Key words: beef tallow, emulsions, immobilized lipase, interesterification, sunflower oil -

INTRODUCTION

Beef tallow has a characteristic composition of triacylglycerols which are responsible for such properties as a high melting point and high resistance to temperature. Beef tallow is a source of fatty acids: stearic and palmitic acids. It is used in the cosmetic industry as a material resource for creams or preparations designed for children. In the food industry, it is used for edible purposes such as frying fats or shortenings. For technical purposes, beef tallow is used to obtain detergents and energy (fuel) (KOWALSKA *et al.*, 2007; KOWALSKI *et al.*, 2004). It is one of the most important byproducts of the meat industry.

In Europe, alone annual production of beef tallow is over one million tons (GUNSTONE, 2000). Because of its high melting point and low level of unsaturated fatty acids (C18:2, C18:3) tallow practically cannot be directly used for edible purposes. For edible use, it has to be fractionated and/or modified by interesterification with edible oils (BHATTACHARYYA *et al.*, 2000; RODRIGUEZ *et al.*, 2001).

Sunflower oil is a vegetable oil containing acid from the group of essential unsaturated fatty acids such as linoleic acid and also containing a significant quantity of oleic acid. This kind of fat is used in the food industry (as a component of "low trans" margarine and mayonnaise oils) and also in the cosmetic industry to produce preparations for the bath and as hair conditioners. It is obtained by extraction from *Helianthus annuus* seeds (KOWALSKI *et al.*, 2004; GRUCZYŃSKA *et al.*, 2002).

Interesterification is a process which is used in the oils and fats industry to modify the properties of triacylglycerol mixtures. There are two types of interesterification presently in use: chemical and enzymatic interesterification. Enzymatic interesterification relies on the use of random or regiospecific and fatty acid-specific lipases as catalysts. By exploitation of the specificity of the lipases, it is possible to manufacture a useful glyceride mixture which cannot be obtained in another way, for example by conventional chemical interesterification processes.

In this work the beef tallow and sunflower oil were used to obtain fat blends which were interesterified by a biocatalyst. New fats were used as a material resource for the emulsions.

The components of the blends are available, and there is a need to utilize beef tallow, so the interesterification of beef tallow and sunflower oil blends can be reduced to practice.

The objective of this study was to determine the usefulness of modified beef tallow for production of fat emulsions. Properties of emulsions containing interesterified fats were compared with those of the starting blends containing noninteresterified fats.

MATERIALS AND METHODS

This work was divided into two parts. In the first one, the fat mixtures were prepared, and in the second one, fat mixtures were used as the fat base in the emulsions. First, the properties of interesterified mixtures were compared to those of the initial blends. Next, selected properties of the emulsions prepared from the interesterified fat blends were determined.

MATERIALS

Sunflower oil (SFO)

It was purchased at a local market. Its main fatty acid composition was as follows: C16:0 (6.0%), C 18:0 (3.3%), C 18:1 *cis* 9 (27.8%), C 18:2 all *cis* (60.1%).

Beef tallow (L) acid value < 1.0 mg KOH/g

It was laboratory refined, bleached and deodorized under vacuum at 105°C. Fatty acid composition of beef tallow was: C 14:0 (3.7%), C 16:0 (29.2%), C 16:1 *cis* 9 (4.7%), C 18:0 (14.9%), C 18:1 *cis* 9 (34.3%), C 18:2 all *cis* (1.6%), C 18:2 all *cis* (0.5%), C 20:0 (0.5%), C 20:1 *cis* 9 (0.4%).

BHT (2,6 di-t-butyl-6-methylphenol)

The antioxidant produced by Rhein Chemie Rheinau GmbH Bayer Company was added to selected samples of fats in the amount of 0.02% to the mass of fat.

Catalyst

As a catalyst during enzymatic interesterification Lipozyme IM (Novozymes, Bagsvaerd, Denmark) was used. Lipozyme IM contains immobilized lipase from *Rhizomucor miehei* and has a water content of 4%.

Methylcellulose

It is produced by Mikro-Technik GmbH & Co. KG, Germany. It was used as a thickener.

METHODS

According to the chart presented in Fig. 1 emulsions were manufactured with eight different fats: 1, 2, 3, 4, 5, 6, 7, and 8.

Fat blends preparation

Beef tallow was mixed at 70°C under nitrogen with sunflower oil in the L:SFO proportions 3:2 and 3:3. Selected properties of beef tallow and sunflower oil and the starting mixtures are given in Table 1.

Enzymatic interesterification of fats

Flasks containing fat blends were positioned in a thermostated mineral oil shaker bath. After

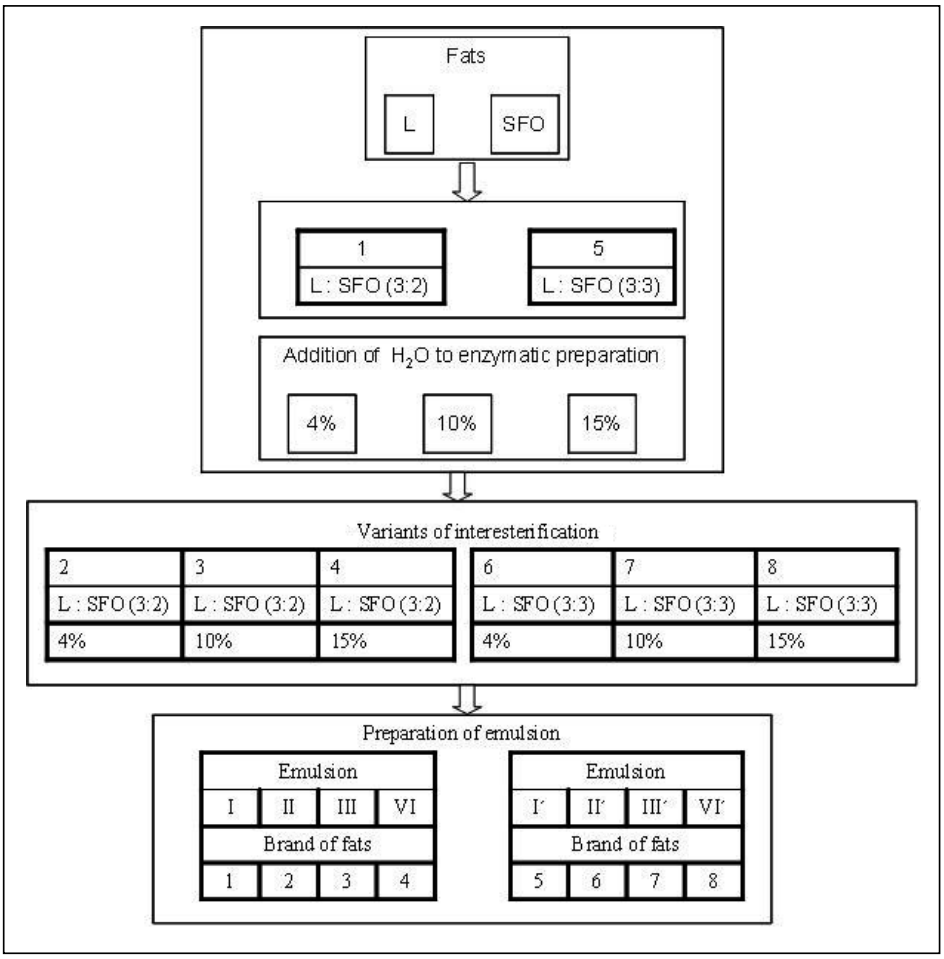


Fig. 1 - Variants of experimental emulsions depending on used fat.

Table 1 - Characteristics of raw materials and fat blends.

Symbol of fat		Acid value (mgKOH/g fat)	FFA (%)	FP (%)	TAG (%)	MAG+DAG (%)
	Characteristics of raw materials					
L	Beef tallow	2.3±0.04	1.1±0.02^{c}	2.0±0.02 ^{b}	98.0	0.9±0.02^{a}
SFO	Sunflower oil	0.1±0.02	0.1±0.01^{a}	0.5±0.01 ^{a}	99.5	0.4±0.01^{a}
	Characteristics of fat blends L:SFO (3:2)					
1	Initial blend (noninteresterified blend)	1.1±0.04	0.5±0.03^{b}	2.0±0.03^{b}	98.0	1.5±0.04^{b}
2	Fat blend interesterified by Lipozyme IM containing 4% water in the enzymatic preparation	5.2±0.12	2.3±0.05^{d}	8.0±0.96^{c}	92.0	5.7±0.08^{c}
3	Fat blend interesterified by Lipozyme IM containing 10% water in the enzymatic preparation	10.8±0.86	4.8±0.22^{f}	17.0±2.11^{f}	83.0	12.2±2.11^{f}
4	Fat blend interesterified by Lipozyme IM containing 15% water in the enzymatic preparation	23.1±2.02	10.2±2.10^{h}	32.0±3.12^{h}	68.0	21.8±2.56^{h}
	Characteristic of fat blends L:SFO (3:3)					
5	Initial blend (noninterestrified blend)	1.0±0.04	0.5±0.02^{b}	2.0±0.0^{b}	98.0	1.5±0.02^{a}
6	Fat blend interesterified by Lipozyme IM containing 4% water in the enzymatic preparation	5.4±0.22	2.6±0.24^{e}	11.0±2.12^{d}	89.0	8.4±0.04^{d}
7	Fat blend interesterified by Lipozyme IM containing 10% water in the enzymatic preparation	11.1±1.24	4.9±0.2	16.0±1.25^{e}	84.0	11.1±1.26^{e}
8	Fat blend interesterified by Lipozyme IM containing 15% water in the enzymatic preparation	22.2±2.75	10.0±2.00^{h}	28.0±2.24^{g}	72.0	18.0±2.02^{g}

Initial blend - noninteresterified blend
FFA - free fatty acids; FP - polar fraction; TAG - triacylglycerol fraction; MAG - monoacylglycerol fraction; DAG - diacylglycerol fraction.
^{a,b,c} Bearing the same letter do not differ significantly from each other.

thermal equilibration of the fat blend at the desired temperature of 60°C, 8% of Lipozyme IM was added to the blend. To obtain a greater quantity of polar fraction, especially mono- and diacylglycerols (MAG+DAG), in the reaction environment, the balance between interesterification and hydrolysis was changed, by addition of water (10, 15%) to the enzymatic preparation. It is believed that the present of polar fraction (MAG+DAG) can stabilize the emulsions. The water content in the biocatalyst was adjusted by the addition of water directly before the reaction. The interesterification was performed with continuous shaking. After a predetermined time (6 h) the samples were filtered to stop the interesterification reaction. As the filtering bed contained a drying agent, water was also removed from the fat.

Determination of analyses of fats

Acid value

It was determined according to the ISO standard (ISO 660, 1996).

Polar fraction content

It was determined by column chromatography according to the ISO standard (ISO 8420, 2004).

Gas-liquid chromatographic analyses

The fatty acid composition of beef tallow and sunflower oil was determined by gas-liquid chromatography (GLC) after conversion of the fats to fatty acid methyl esters. The apparatus and procedure are reported elsewhere (KOWALSKI *et al.*, 2000).

Resistance of fats to oxidation

The determination was carried out using Rancimat method according to the ISO standard (ISO 68861997). The tests were carried out at 120°C with 2.5 g ± 0.02 of fat. Air flow rates were set at 20 dm^3/h. The average induction time was given in hours. The induction times for oxidation were measured using Methrom Rancimat apparatus model 679 (Herisau, Switzerland).

The effectiveness was expressed as the stabilization factor, F:

$$F=IPx/IPo$$

where: IPx is the induction period in the presence of the antioxidant,
IPo is the induction period in the absence of the additive.

Emulsion preparation

The emulsions were prepared according to the following formula: fat 10% (w/w), thickener 2% (w/w), and distilled water up to 100% (w/w). Emulsions were prepared by adding together the water and the fat phase and stirring with the mixer RW 20 DZM (Janke & Kunkle, Germany) for 3 min with the velocity of 13,500 rpm. Each emulsion was prepared in the same way at room temperature (23°C).

Determination of values
for the emulsions obtained

Determination of mean particle size and particle size distribution of fat emulsions

Determination was carried out in the range 0.12-704 μm by laser scattering using a Microtrac Particle Size Analyzer (Leeds & Northrup, USA) after preparation. The emulsions were diluted 1:200 with distilled water.

Determination of dispersion index

It was carried out on the basis of the determination of particle size of the emulsion using laser diffraction (ELWELL *et al.*, 2004).

Determination of emulsion density

According to Polish Standard (PN-92/C-04504, 1992).

Determination of emulsion viscosity

Determination was carried out by using a viscometer (Rheotest-2 Typ RV 2). 15 g of each sample (accurate to 0.01 g) was weighed out. The sample was placed in the measurement apparatus. A measurement roller with the following parameters was used to take measurements: *Z* (device constant 2.4428 N/m^2), *Dr* (rate of shear 80.2/s), η (viscosity Pa·s).

The measurement was taken by reading the injunction of an angular factor [α]. Emulsion viscosity was calculated according to the following formula:

$$\eta = Z \cdot \alpha \times 100/Dr$$

Determination of emulsion pH

Determination was carried out by using pH-meter N517. Each sample was placed in a measurement dish in a way which did not allow air bubbles to appear. The combination pH electrode was used to take measurements. Determination was performed at room temperature. Determination was performed according to Polish Standard BN 77/6140-01/08

Determination of emulsion stability by using temperature test at 35°C and –3°C

All emulsions were stored in the dryer at 35°C and in the refrigerator at –3°C alternately for five days, with a change every 24 hours. If the emulsion remains homogeneous in such conditions, it has proper stability.

Determination of emulsion stability by using centrifugal test

Determination was performed in the centrifugal machine at 2,500 rpm. The test tube was filled with 15 mL of the emulsion and was cen-

trifuged for 30 min. If the emulsion stays homogeneous after this time it means that it has proper stability.

Sensory parameters of emulsions

Such parameters as adhesion, consistency, homogeneity, distribution, absorption, stickiness, and greasiness of each emulsion were determined. The emulsion was described by laboratory personnel (10 people) according to the criteria mentioned above.

Moisturizing parameters of emulsions

They were determined by using Corneometer type CM 825 PC. On the surface of the forearm six squares and one additional control square were drawn. After that, the probe with constant pressure, directed perpendicularly to the skin surface, was applied to each square (five measurements). The results were noted. Subsequently, the emulsion was applied to the tagged squares. After five minutes the measurement was taken and noted down. After 10, 15, 20, 30, 60, 90, and 120 min the measurements were repeated and noted down.

Statistical analysis of results

The results were subjected to one-way ANOVA. Duncan test was used to assess the differences between means. The level of $P<0.05$ was considered significant. Statgraphics plus 4.0 package (Statistical Graphics Corp., USA) was used. The above analyses were carried out in three replicates.

RESULTS AND DISCUSSION

During interesterification of fats apart from new triacylglycerols, free fatty acids, mono- and diacylglycerols are also formed (KOWALSKA *et al.*, 2005). These products were determined in the reaction products and results are shown in Table 1. The free fatty acids (FFA) and MAG+DAG contents in interesterified samples increased compared to the starting blends. For the fat mixture interesterified by Lipozyme IM with 15% water in the enzymatic preparation, the highest acid value was observed. Consequently, the content of polar fraction (MAG+DAG) was also the highest for the same sample. These increases are in agreement with the findings reported in the literature (KOWALSKI *et al.*, 2004; KOWALSKA *et al.*, 2005). Due to positional (sn-1,3) specificity of Lipozyme IM, the interesterification in these cases occurred mainly at the sn-1,3 positions of triacylglycerols (TAGs) (KOWALSKI *et al.*, 2004). It is believed that occurrence of free fatty acids such as stearic and palmitic acid during interesterification is possible. These acids are mostly located at sn-1,3 positions both in beef tallow and sunflower oil triacylglycerols. The presence of these acids could be very desirable, for example in cosmetic emulsions. They can be used for the production of creams, as emulsion preparations and also as substances reducing the action of detergents. Moreover, they show coating and emulsifying properties (IMKE PETERS, 2005).

Appearance of MAGs and DAGs during interesterification is very desirable. They can stabilize emulsions (LEDÓCHOWSKA and DATTA, 1998). They can be used in different kinds of industry. Beside applications in the food and dairy industry, MAGs are also used in medicine (e.g. for controlled release of medical substances) and in other areas such as the plastics industry (plasticizers) and cosmetics, e.g. toothpastes and deodorants (BOBA'LOWA, 2006).

The cosmetics industry takes advantage of monoacylglycerols because of their stability, chemical inertia in the presence of active substances, tolerance shown by skin and mucosa, penetration effectiveness, indifferent colors and aroma. Pure MAGs of lauric, palmitic and stearic acids quickly penetrate through the skin and thus it is naturally protected. MAGs of lauric acid are used for deodorants to suppress sweating and MAGs of stearic acid are used in hair products. Moreover, MAGs are widely applied in the production of face creams and lotions due to their capacity for water/oil/water emulsion stabilization. Antimicrobial effects of MAGs from lauric and myristic acids are utilized in the production of toothpastes and mouthwashes (ISAACS *et al.*, 1995). Sulfated derivatives of MAGs from coconut oil have been known for a long time in cosmetic products such as shower gels and foam baths or shampoos.

Oxidative stability is a very important parameter describing fats (RATUSZ *et al.*, 2002). In this work the induction period of interesterified fats as well as noninteresterified blends was determined (Table 2). Initial blends had the longest induction time [L:SFO (3:3) = 1.22 h; L:SFO (3:2) = 0.98 h]. This meant that they were much more resistant to temperature than their interesterified counterparts. Also, the presence of linoleic and oleic acids in fat blends derived from sunflower oil causes these blends to be much more unstable at high temperatures (temperature during the test was 120°C). The Rancimat test was carried out on the fat samples containing BHT at the level of 0.02%. As expected, the induction time of samples was lengthened (on average from 0.15 to 0.30 h) (Table 2).

Obviously, the dependencies for antioxidant showing a protective effect are not linear. Fat blends L:SFO (3:2) are easier to stabilize at 120°C: F from 1.15 to 1.52. The best results were obtained by using BHT in fat blend no. 3 (the blend interesterified by Lipozyme IM containing 10% water in the enzymatic preparation) (Fig. 2).

As mentioned above, fats obtained during mixing and interesterification are used as a fat base in cosmetic emulsions. There are many factors which play a significant role in stability of the emulsions.

Table 2 - Results of determinations of fat blends using Rancimat method.

Fat blend	Induction time for initial blend (h)	Induction time for interesterified blends by using Lipozyme IM during interesterification containing different amount of water in the enzymatic preparation (h)		
		4% water	10% water	15% water
L:SFO(3:2)	0.98±0.02	0.80±0.02	0.53±0.04	0.77±0.02
+BHT L:SFO(3:2)	1.13±0.01	1.1±0.04	0.81±0.02	0.92±0.01
L:SFO(3:3)	1.22±0.02	1.18±0.04	0.82±0.01	1.06±0.02
+BHT L:SFO(3:3)	1.25±0.04	1.22±0.20	1.04±0.04	1.12±0.08

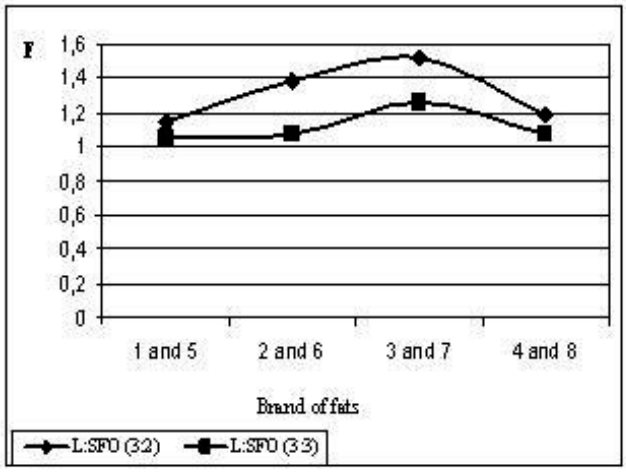


Fig. 2 - Dependence between stabilization factor and types of fats.

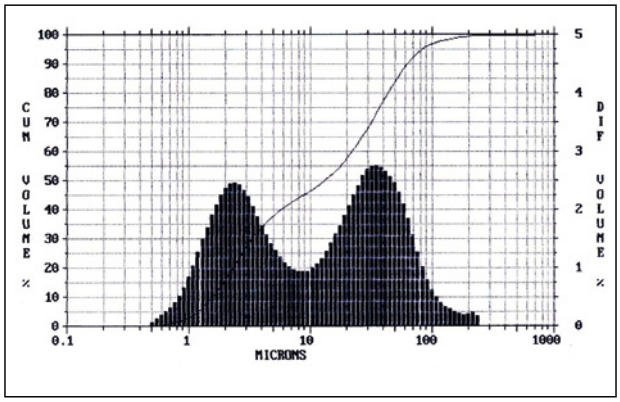


Fig. 3 - The distribution of average particle size of emulsion II.

Limitation of destabilization processes in the emulsions can be achieved among others by obtaining the proper dispersion degree (DURAND *et al.*, 2007; DŁUŻEWSKA *et al.*, 2005; COUPLAND and MCCLEMENTS, 2001; CYR and TAGNIT-HAMOU, 2000). In this study the particle size of the emulsions was determined for each emulsion (Table 3).

It was found that the emulsion with fat 2 had the biggest average particle size (Table 3). Its value was 25.19×10^{-6} m; however, the emulsion with fat 8 had the smallest average particle size of emulsion, 3.96×10^{-6} m. The emulsion with fat 4 was also characterized by a relatively small droplet size. Its dispersion index was comparable with the dispersion index of the emulsion with fat 8. The value of the dispersion index was 2.8 and 2.5 for emulsion with fat 4 and 8, respectively. These were the lowest values for the dispersion index.

The emulsions with both noninteresterified and interesterified fats (4% water in the enzymatic preparation) showed unstable character (Fig. 3-6). Their dispersion index was in the range 2.8 to 6.0 (Table 3).

In most cases, two fractions were obtained; only the emulsions containing interesterified fats (where the content of water in the enzymatic preparation during interesterification was 15%) had one fraction (Fig. 7). The results of the amount of fractions are shown in Table 3.

In this work to determine the stability of the emulsions centrifuge and temperature tests at 35° and –3°C were applied. The results are given in Table 4.

As seen in Table 4 the emulsions containing

Table 3 - Results of particle size determinations of the emulsions containing prepared fat blends.

Symbol of emulsion	Emulsion	Average particle size of emulsion ($\times10^{-6}$m)	Number of fractions	Dispersion index
I	Emulsion containing fat 1	15.09±1.54^{g}	2	6.0
II	Emulsion containing fat 2	25.32±2.38^{h}	2	4.6
III	Emulsion containing fat 3	6.21±0.52^{c}	2	3.2
IV	Emulsion containing fat 4	4.74±0.08^{b}	1	2.8
I'	Emulsion containing fat 5	6.95±0.82^{d}	2	2.8
II'	Emulsion containing fat 6	12.44±2.46^{e}	2	5.2
III'	Emulsion containing fat 7	13.78±1.68^{f}	2	3.2
IV'	Emulsion containing fat 8	3.96±0.04^{a}	1	2.5

^{a,b,c} Bearing the same letter do not differ significantly from each other.

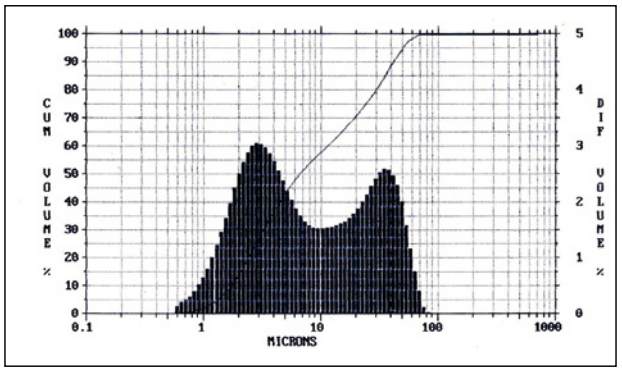


Fig. 4 - The distribution of average particle size of emulsion I.

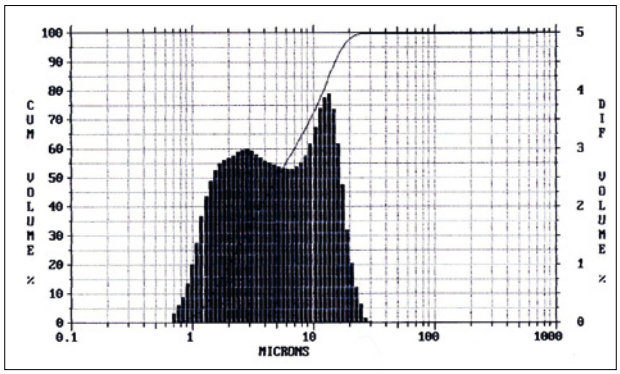


Fig. 6 - The distribution of average particle size of emulsion I´.

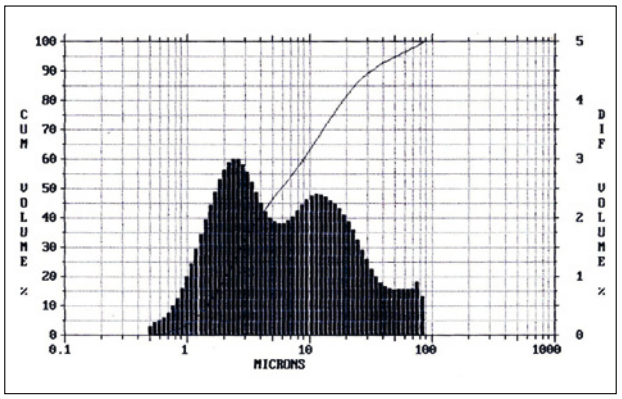


Fig. 5 - The distribution of average particle size of emulsion II´.

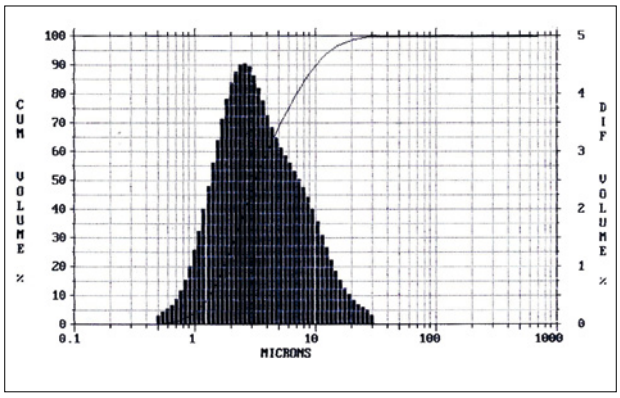


Fig. 7 - The distribution of average particle size of emulsion IV.

noninteresterified fat were completely unstable. Similar trends were observed for the emulsions containing fats 2 and 6. In emulsions with fats 3, 4, 7, and 8, the opposite situation was observed. These emulsions showed high stability during the centrifuge test. As expected, greater amounts of MAGs and DAGs (natural emulsifiers) were generated during interesterification.

The next parameter which can determine the quality of the emulsions is thickness. In this work the thickness of all emulsions was described (Table 5).

As seen from this table most of the emulsions reached a comparable level of thickness. Only two samples were significantly different ($P<0.05$) (Table 5).

Viscosity has a very significant influence on rheological properties of the emulsions (ZIELIŃSKI, 2000). From the commodity science view it is very important because it gives an opportunity to get information about the expiry date of the usefulness of the emulsion.

In the present research the viscosity was measured after 24 h and 30 days of emulsion formation. The changes of viscosity were in the range 724-784.9 mPa and 256.6-709 mPa for the emulsions formed after 24 h and 30 days, respectively (Fig. 8a,b). It was observed that in all cases the viscosity decreased after 30 days; however, the most significant changes were noticed for the emulsions containing noninteresterified fats and emulsions with fats 2 and 6. For

Table 4 - Results of the stability of the emulsions using centrifuge test and temperature test.

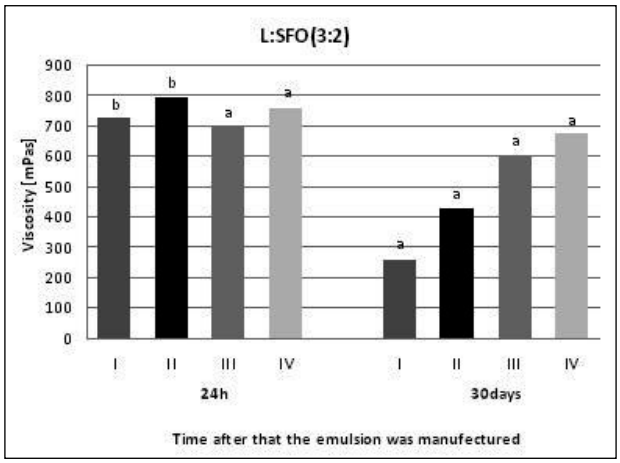
Emulsion	Results of centrifuge and temperature test
I	Complete stratification
II	Stratification, heterogenous
III	Stable
IV	Stable, homogenous
I'	Complete stratification
II'	Stratification, heterogenous
III'	Stable
IV'	Stable, homogenous

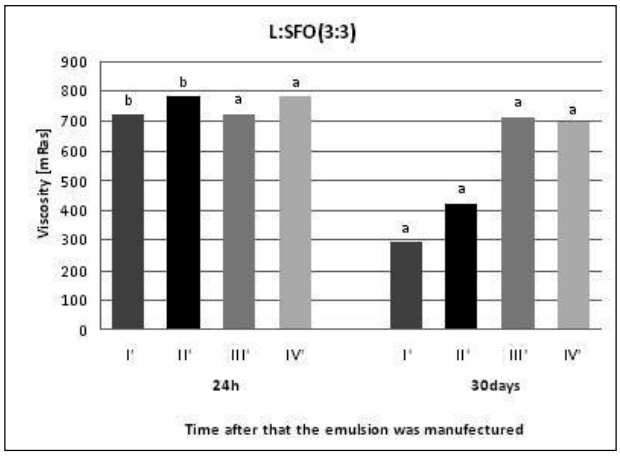
Determinations of emulsions are the same as in Table 3.

Table 5 - Results of the emulsion thickness.

Type of emulsion	Thickness (g/cm^3)
I	0.9949±0.0002^{b}
II	0.9944±0.0001^{ab}
III	0.9944±0.0002^{ab}
IV	0.9941±0.0001^{ab}
I'	0.9941±0.0001^{ab}
II'	0.9944±0.0000^{ab}
III'	0.9942±0.0001^{ab}
IV'	0.9931±0.0002^{a}

Determinations of emulsions are the same as in Table 3.





Figg. 8ab - Dependence of viscosity changes on: applied fat blend in emulsion and the time of preparation of the emulsion (24 hours and 30 days).

the other emulsions significant changes in the viscosity were not observed. It is only suggested that the presence of polar fraction, especially MAG + DAG, in these emulsions could stabilize them.

pH is a very important parameter of emulsions. In the present investigations, pH was measured. The determined value of pH for all samples was in the range 4.7- 4.9. The proper pH should be close to pH of skin (5.5), so the samples showed a rather weak acid reaction.

In this study, sensory and moisturizing parameters of emulsions were also determined. It was noted that all emulsions had very good distribution on the skin. The emulsions containing fats 3, 4, 7, and 8 gave good smoothness of skin; however, the skin adhesiveness parameter received a comparatively low value for all emulsions. It is presumed that the consistency of emulsions was too thin. On the other hand, such consistency of emulsions can be used to form cosmetic milks, creams or body balms.

Considering moisturizing parameters of the emulsions it was noted that in all cases the best moisturizing appeared 5 minutes after application on the skin. After the next 5 minutes a decrease of moisturizing was observed. In subsequent measurements a decreasing tendency of moisturizing for all applied emulsions was noted. After 120 min from applying the emulsions on the epidermis the level of moisturizing was close to the level of moisturizing measured before application of the emulsion.

CONCLUSIONS

The emulsions prepared on the basis of fats which were formed during interesterification in the presence of the catalyst Lipozyme IM (where the amount of water was 10 and 15%) were the most stabilized emulsions. They successfully passed the stability test. The addition of water to the enzymatic preparation (10, 15%) caused the balance of interesterification and hydrolysis to move in the direction of hydrolysis. As a result, more polar fraction appeared in the reaction environment. As regards remaining emulsions, they showed instability of structure. These results were also confirmed by the determination of particle size of emulsions, their distribution and the dispersion index.

The results obtained in this work showed that interesterification of beef tallow and sunflower oil blend resulted in new fats which are unprecedented in the environment. Such fats with very interesting characteristics of triacylglycerols can be used as a fat basis in various applications, for example in the cosmetics and food industries. Moreover, from an economic point of view beef tallow is a rather cheap, useless raw material, so its use for the production of fat blends and as a fat base for emulsions is justifiable in the opinion of the authors.

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Paper received April 1, 2010 Accepted September 3, 2010

PAPER

INFLUENCE OF NUTRITIONAL STATUS, ENVIRONMENTAL AND PEDOLOGICAL PARAMETERS ON *CITRUS MEDICA* CV DIAMANTE PEEL ESSENTIAL OIL CHEMICAL COMPOSITION

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ABSTRACT

This work aimed at evaluating the influence of pedological parameters and nutritional status on the fruit quality and chemical composition of *Citrus medica* cv Diamante peel oil. With this purpose 16 samples were obtained from fruits collected at sea level (zone A) and at 300 meters above sea level (zone B). The oils were found to be rich in monoterpene hydrocarbons. The main components were limonene and γ-terpinene. Other abundant compounds that characterized *Citrus medica* cv Diamante peel essential oils were α-pinene, β-pinene, β-myrcene, neral and geranial. The soil in the plain at sea level is characterized by a major sand content (67.6%) and consequently by a lower of P_2O_5 and K_2O. On the contrary, the soil in the area in the hills showed a lower content of total lime (0.89%) in comparison with the plain at sea level (26.78%). A similar trend was observed for the pH value. The differences highlighted in carpometric characteristics of fruits could be attributed to the soil composition. Nevertheless, none of the studied parameters influenced the amount of the main peel essential oil components.

- Key words: *Citrus medica* cv Diamante, essential oil, fruit quality, GC-MS, macro-micro nutrient, soil analysis -

INTRODUCTION

Plants of the genus *Citrus* are primarily valued for their edible fruit, but they also have traditional medicinal value. The peel of *Citrus* fruits has been used in traditional Asian medicine for centuries (HUANG and WANG, 1993; WICHTL and BISSET, 1994; BLUMENTHAL *et al.*, 1998). The *C. medica* was the first *Citrus* fruit to come to the notice of Europeans and was for many years the only one known. This plant has an ancient origin; the more accredited provenance is from India but it probably arrived in Italy through the Hebrews who introduced the cultivation of the citron on the Calabrian coasts. The cultivars are divided into two groups: sweet and acidic citron. Among the better-known *C. medica* cultivars are "Corsican", "Diamante", "Etrog" and "Fingered citron" (FERSINI *et al.*, 1973). *C. medica* cv Diamante is the most diffused cultivar in Italy and in particular, in Calabria where the cultivation of citron extends along the high Thyrrenium coast from Diamante to Tortora, called the Coast of citron. Citron was sought by industry for preparation of liquors, ice cream, jams and canned products. Besides such use of ripe fruits, fruits of various developing stage are specifically preferred in preparation such as beverage. The ripe fruits are big, with a thin, smooth and lemon-yellow peel and the pulp is poor of juice. The most important part of the Diamante citron is the peel which is a fairly important article in international trade. The main products obtained from citron are candy and liqueurs, while the peel essential oil is a minor product, used as flavouring in sweets and beverages (CUTULI *et al.*, 1985). In our previous work we have evaluated the chemical composition and the *in vitro* antioxidant, hypoglycaemic and anticholinesterase properties of *C. medica* L. cv Diamante *n*-hexane peel extract (CONFORTI *et al.* 2007). This extract was characterized by the presence of monoterpenes and sesquiterpenes. The most abundant constituents were two monoterpenes: limonene and γ-terpinene. The extract showed significant antioxidant activity that was carried out using different assays (DPPH test, β-carotene bleaching test and bovine brain peroxidation assay). This non polar extract weakly inhibited α-amlyase enzyme (IC_{50} 625 mg/mL).

In spite of previous studies that investigated the chemical composition of oils obtained from *C. medica* cv Diamante collected in Calabria and in Greece (POIANA *et al.*, 1998; VEKIARI *et al.*, 2004; GABRIELE *et al.*, 2009), to our knowledge, this is the first report that evaluated the influence of environmental and pedological parameters, nutritional status on chemical composition and fruits carpometric characteristics. Some studies have been also reported on the influence of soil characteristics on the yield and composition of essentials oils from various aromatic plants (FRANZ, 1983; DIATLOFF, 1990; SCHALLER and SCHNITZLER, 2000). For instance, DIALTOFF (1990) has studied the effect of nitrogen fertilizers on the yield of essential oil of *Leptospermum* species, while DETHIER *et al.* (1997) have reported the influence of cultural treatment and harvest time on Vetiver oil quality. More recently, RAZIC *et al.* (2006) have studied the relations between mineral content (eleven metals) of seven herbal drugs and corresponding soils.

INTRIGLIOLO *et al.* (1999) reported the effect of some pedologic parameters on the yields and chemical composition of another Calabrian *Citrus* fruits essential oil namely *C. bergamia*. The study demonstrated that soil parameters (total $CaCO_3$, pH and Mg) positively influenced the oil. For this purpose in this study 16 farms were selected in two districts different for soil composition, altitude and way of cultivation. In Italy, the cultivation of *C. medica* cv Diamante is restricted to Calabria characterized by mild winters and summer temperatures not very high. The trees are very sensitive to low temperatures, wind and subject to serious infections such as "mal secco" (*Phoma tracheiphila*), in consequence, must therefore be protected with suitable covers, made with different modes. The quality of the fruits is also influenced by harvesting, handling, transportation, and storage as reviewed by RAMANA *et al.* (1981).

The aim of this work was to highlight for the first time the influence of the environmental parameters, cultivation management, physicochemical parameters of soil and leaves nutrients with the fruit quality and chemical composition of *Citrus medica* cv Diamante peel oil. For this purpose two different areas of growth were analysed. These findings could help cultivators to improve fruit quality suitable for industrial products.

MATERIALS AND METHODS

Plant material

Fruits of *C. medica* L. cv Diamante (Rutaceae) used in this study were collected in October 2006 in Calabria (Southern Italy), loc. Santa Maria del Cedro (CS) from 16 farms selected in two districts namely zone A and B. The authentication of the *C. medica* L. cv Diamante samples was carried out at Natural History Museum of Calabria and Botanic Garden, University of Calabria, Italy.

Zones are characterized by different altitudes (zone A with farms in the plain at sea level and zone B with farms in the hills at 300 meters above sea level). Moreover, the areas are different based on the cultivation method and various cultivation techniques. In zone B the aver-

age size of farms is very small (0.16 hectares) with plant density up to 1,200 plants/ha, while in zone A the majority of the farms have an extension from 1 to 6 hectares. Besides in the zone A are used and has been made with modern criteria that allow the introduction of rational farming techniques (i.e.: working with agricultural machinery, irrigation, pesticide treatments, foliar fertilizers).

The *C. medica* cv Diamante needs a mild climate, as constant as possible during the growing season, being particularly sensitive to temperature changes, especially those caused by cold winds that dry up the twigs. In the plain, the summer temperatures reach maximum values of 40°C, while winter temperatures arrive at 4°C with differences of 2°-4°C above the hill. To create appropriate temperature conditions during the winter period, plants were covered with dark green plastic nets.

Harvesting and sample preparation

The experimental design included fruits randomly harvested on 50 healthy homogeneous trees per grove. Twenty fruits (weight >300 g and length 20-30 cm) were collected. Fruits were examined for integrity and absence of dust and insect contamination. The following physical characteristics of citron fruits namely fruits number-picked, weight, volume, equatorial diameter, longitudinal diameter, firmness (by means of a penetrometer TRpenetrometer-Italy), firmness without peel, peel thickness, width of central axis, weight and volume fruit without peel were determined. Samples were freeze-dried and stored at -20°C until they were analyzed. Fruits were peeled off mechanically. Citron essential oil was obtained using the traditional cold-pressing procedure. The essential oil is present in oil sacs or oil glands located at different depths in the peel and the cuticles of the fruit. Peel and cuticle oils are removed mechanically by cold pressing and since cold pressing yields a watery emulsion, this emulsion is then centrifuged in order to separate the essential oil out (BOUSBIA *et al.*, 2009). The essential oils were dried (anh. Na_2SO_4) to remove traces of moisture and stored at 4°-8°C in a brown bottle to prevent the negative effect of light.

Soil and leaves analysis

Soils were sampled in February before the annual application of fertilizers using a manual drill. In each grove, four soil specimens were collected at depths of 0-30 cm and 30-60 cm and mixed to form a single sample. Total $CaCO_3$ (%) was determined by a De Astis calcimeter (MIRAAF, 1994). Organic matter (%) was assessed using the Walkley-Black method (SISS, 1985), total N (%) with the Kjeldahl method and available P (mg/g) using the Olsen method (MIRAAF, 1994, SISS 1985). Exchangeable K, Ca, Mg, and Na cations (mg/g) were extracted with CH_3COONH_4 and determined by atomic absorption spectrophotometry (SISS 1985); pH was determined by PH211 pH-meter (HANNA Instruments).

The leaves were picked in November when the level of the elements was stable and in a period far from the fertilizers application. The leaves collected for analysis were 5-7 months old.

The foliar analyses was performed on 20 leaves of the index trees picked from non fruit bearing terminal shoots of the year's spring flush (INTRIGLIOLO *et al.*, 1999; EMBLETON *et al.*, 1973). Determinations of total nitrogen were made with the Kjeldahl method, the rest macro and micro elements have been detected by the inductively spectrometer plasma (ICP) (WHITE *et al.*, 1999).

Volatile compounds analysis

To determine the *C. medica* cv Diamante peel essential oil composition, GC-MS analyses were performed using a Hewlett-Packard 6890N GC, equipped with a fused capillary column (stationary phase methylsilicone SE30, film thickness of 0.25 μm, a length of 30 m and an internal diameter of 0.25 mm) and a mass spectrometer Hewlett Packard 5973N. The carrier gas was helium, at a flow rate of 1 ml/min. Electron impact ionization was carried out at energy of 70 eV; acquisition mass range 25-500 m/z (scan speed: 1,470 amu/s, 2.94 scan/s) multiplier energy 1,340 V, solvent delay 3 min. Injector and detector temperatures were set at 250° and 280°C, respectively. Column temperature was initially 50°C for 5 min, then gradually increased to 250° at 5°C/min, and finally held for 10 min at 250°C. The essential oils were diluted 1:100 (v/v) with dichloromethane and 1.0 mL of the diluted samples was directly injected using a splitless injection technique. Constituents of *C. medica* cv Diamante peel essential oils were identified by comparison of their GC retention indices with those of the literature or with those of standards available in our laboratory. The retention indices were determined in relation to a homologous series of *n*-alkanes (C_8-C_{24}) under the same operating conditions. Further identification was made by comparison of their mass spectra with those stored in Wiley 138 and NIST 98 libraries or with mass spectra from literature (ADAMS, 1995). The quantitative determination of the most abundant identified compounds was obtained by the external standard method. As standards were used α-pinene, sabinene, α-terpinene, limonene, neral, linalool, *trans*-caryophyllene, β-bisabolene and citropten. Experimental results are expressed as the mean values.

Statistics

To achieve the objectives set, the data obtained were processed using statistical procedures that tend to highlight any significant relationships of nutrients in the leaves and in the physico-chemical parameters of soil with the components of the essential oils extracted from peel. Moreover differences between zone A and B were underlined. For this purpose ANOVA test and correlation analysis with the statistical package SPSS (PASW Statistics 18) was applied.

RESULTS AND DISCUSSION

Sixteen essential oils were obtained from fruits collected in districts namely zone A and B. These zones are characterized by different soil composition and altitude (zone A with farms in the plain at sea level and zone B with farms in the hills at 300 meters above sea level). The input of macroelements with fertilization was higher than actual needs of the plants with differences in the two areas especially for nitrogen (150-200 g of N to plant in zone A and 250-300 in B) (CALABRETTA *et al.*, 2010).

Soil, leaves and fruits parameters

The results of soil analyses showed that almost all physical and chemical parameters analyzed were significantly different (Table 1). The soil in the plain at sea level is characterized by a major sand content (67.6%) and consequently by a lower of P_2O_5 and K_2O. On the contrary, the soil in the area in the hills showed a lower content of total lime (0.89%) in comparison with the plain at sea level (26.78%). A similar trend was observed for pH value.

Leaf analysis is used to determine the nutrient status of the tree and to understand the nutritional requirements of the plant. However, the data obtained should be used in combination with other known parameters. Table 2 evidenced that the leaf content of macro and micro-nutrients was in the optimal range. The nitrogen content is particularly high in zone B (3.39%), probably caused by the higher intake of

Table 1 - Physical and chemical parameters of soils in the zone A and B.

Parameters	Zone A	Zone B	Significance
Clay (%)	13.22±2.93	21.31±2.37	***
Silt (%)	19.13±7.16	21.38±1.91	-
Sand (%)	67.66±8.15	57.31±0.88	**
Total N (‰)	1.23±0.24	1.05±0.38	-
P_2O_5 (mg kg^{-1})	28.07±82.43	188.07±26.64	***
K_2O (mg kg^{-1})	41.38±111.51	205.38±41.11	***
Na (mg kg^{-1})	19.21±14.94	24.68±10.86	-
MgO (mg kg^{-1})	183.38±68.93	213.13±57.28	-
TOC (%)	0.98±0.30	1.05±0.34	-
S.O. (%)	1.69±0.49	1.79±0.58	-
Total lime ($CaCO_3$ %)	26.78±11.08	0.89±5.27	***
Active lime (%)	3.35±1.27	0.59±0.66	***
pH	8.31±0.29	7.34±0.29	***
Electrical conductivity (mS/cm 25°C)	1.07±0.31	1.03±0.23	-
C.S.C. (meq/100 g soil)	10.82±1.38	13.08± 0.61	***

Data are expressed as means ± S.D. (n= 8). **$p \leq 0.01$, ***$p \leq 0.001$; - not significant.

Table 2 - Leaf analysis of *C. medica* cv Diamante.

Parameters	Zone A	Zone B	Significant
N (%)	2.74±0.22	3.39±0.29	***
P (%)	0.20±0.03	0.25±0.06	*
K (%)	1.16±0.19	1.21±0.30	-
Ca (%)	7.84±1.58	5.73±0.74	-
Mg (%)	0.40±0.09	0.38±0.05	-
Fe (mg kg^{-1})	129.32±24.68	128.80±23.93	-
Zn (mg kg^{-1})	90.91±39.91	19.85±6.79	***
Mn (mg kg^{-1})	54.24±20.76	44.43±18.80	-

Data are expressed as means ± S.D. (n= 8). *$p \leq 0.05$, ***$p \leq 0.001$; - not significant.

Table 3 - Carpometric characteristics of *C. medica* cv Diamante fruits.

Parameters	Zone A	Zone B	Significant
Fruit weigh (g)	775.10±205.92	504.17±133.39	**
Equatorial diameter (cm)	10.14±0.51	8.40±0.88	***
Longitudinal diameter (cm)	17.72±2.09	13.99±1.15	**
Fruit firmness (kg/cm^2)	13.47±3.88	17.92±1.36	***
Peel thickness (cm)	24.66±3.56	19.29±3.07	***
Central axis (cm)	13.21±2.00	9.79±1.96	**
Weigh/volume ratio (whole fruit)	0.86±0.06	0.94±0.02	**
Weigh/volume ratio (without peel)	0.90±0.03	0.99±0.03	**
Pulp (%)	10.30±4.07	8.6±2.42	-
Peel (%)	89.70±4.07	91.44±2.42	-

Data are expressed as means ± S.D. (n= 8). **$p \leq 0.01$, ***$p \leq 0.001$; - not significant.

nitrogen fertilizer. Significant differences were noticed for Zn with higher levels in plain area.

Fruit carpometric characteristics showed some statistical significant differences between zone A and B (Table 3). In particular, fruits collected in plain at sea level are characterized by a higher weight, equatorial and longitudinal diameter and a lower fruit firmness (determined on a portion of the peel and "albedo") than fruits collected in zone B, probably due to more balanced nutritional status of plants. On the contrary, fruit weight/volume *ratio* was significantly higher in sample in the hills. In zone A, it is evident that the greater volume of the fruit, the greater the consistency of albedo. Previously, the effect of some pedologic parameters on the yields and chemical composition of the peel oil obtained from *C. bergamia* was analyzed (INTRIGLIOLO *et al.*, 1999). Obtained results that available phosphorus seemed to affect only some of the minor oil constituents (β-pinene, β-myrcene and β-caryophyllene). Total $CaCO_3$, pH and Mg positively influenced the oil yield and quality.

Volatile compounds analysis

Sixteen *C. medica* cv Diamante peel essential oils (8 from the zone A and 8 from the zone B) were analyzed in order to highlight the relationships of leaves nutrients and soil pedological parameters with chemical composition of the essential oil.

A total of 34 constituents were identified as major essential oil components. The components, their retention indices and their percentage composition are summarized in Table 4 and 5. The essential oils were found to be rich in monoterpene hydrocarbons. The main component was limonene with values in the 42.7-55.3% range for zone A and 40.2-55.8% range in zone B, followed by g-terpinene with values in the 18.3-24.2% range for zone A and 20.8-28.0% range for zone B. According to POIANA *et al.* (1998), the high content of limonene and g-terpinene is a significant characteristic of this citron cultivar. Generally, the content of other more abundant compounds, namely thujene, α-pinene, β-pinene, β-mircene and terpinolene, was higher in zone A. Thujene content ranged from 1.0 to 1.7% for zone A and from 0.8 to 1.5% in zone B. A same trend was observed for α-pinene with values in the 2.2-3.6% range for zone A and 1.9-3.0% range in zone B. The analysis of terpinolene content in all samples collected at sea level evidenced values in the 1.1-1.7% range for zone A except for sample 4 (0.2%). In the hills the same monoterpene presented values of 0.6-1.4%. According to VERZERA *et al.* (2005), the sesquiterpene fraction was less represented; the main components were β-bisabolene with values in the 0.5-1.3% range for zone A and 0.5-1.5% range in zone B, and *trans*-caryophyllene with values in the 0.2-0.5% range for zone A and 0.3-0.7% range in zone B. Other sesquiterpenes identified, namely α-bergamotene, β-elemene, β-cubebene, α-humulene, and *trans*-β-farnesene, were not representative of all *C. medica* cv Diamante peel essential oils. Among oxygenated compounds, carbonyl compounds showed the highest amount, with neral and geranial as the main components; their average content is 0.7-3.2% for neral and 0.8-4.5% for geranial, respectively. Alcohols and esters are less represented. Two coumarins namely citropten and oxypeucedanin were also identified; their average content is 0.2-0.9% for citropten (not identified in samples 4, 9, 11, 12 and 16) and <0.1-0.8% for oxypeucedanin (not identified in samples 1-4, 9-11 and 16).

Our results are in agreement with the results reported by VERZERA *et al.* (2005). Also in this study the essential oil obtained by manual pressure from *C. medica* L. cv Diamante peel showed limonene (51.95%) and γ-terpinene (27.71%) as the most abundant components. On the other hand, the hydrodistilled peel oil obtained by LOTA *et al.* (2005) showed a high content of limonene (70.4%) but a very low abundance of γ-terpinene (≤ 0.05%).

Table 4 - Major components of the essential oil from *C. medica* cv Diamante peel collected in zone A.

Compound	I[a]	%[b]								ID Method[c]
		1	2	3	4	5	6	7	8	
Thujene	926	1.2±0.4	1.3±0.5	1.3±0.6	1.0±0.4	1.0±0.1	1.4±0.2	1.7±0.4	1.5±0.5	*I*, MS
α-Pinene	936	2.5±0.9	2.6±0.9	2.8±0.9	2.3±0.7	2.2±0.5	3.3±0.9	3.6±0.7	3.1±0.5	*I*, MS, Co-GC
Camphene	953	0.6±0.02	tr	tr		0.1±0.02	tr	tr	0.2±0.02	*I*, MS
Sabinene	973	0.4±0.01	0.5±0.02	2.9±0.02	0.3±0.01	0.3±0.01	tr			*I*, MS, Co-GC
β-Pinene	978	2.1±0.02	2.2±0.06	0.6±0.06	1.9±0.05	1.8±0.1	3.0±0.4	3.4±0.5	3.1±0.02	*I*, MS, Co-GC
β-Myrcene	986	1.9±0.8	1.7±0.05	2.0±0.05	1.6±0.05	1.8±0.7	2.7±0.9	0.8±0.05	2.2±0.05	*I*, MS, Co-GC
α-Phellandrene	1005		tr	0.1±0.02	tr	tr		tr	0.1±0.02	*I*, MS
α-Terpinene	1016	0.6±0.3	0.7±0.01	0.9±0.01	0.4±0.01		0.1±0.01		0.1±0.02	*I*, MS, Co-GC
Limonene	1032	55.3±1.5	53.7±1.7	46.9±1.9	52.9±2.2	45.7±1.5	42.7±1.5	47.2±2.8	45.0±1.2	*I*, MS, Co-GC
(*E*)-β-Ocimene	1047		1.3±0.08	2.4±0.08	1.4±0.08		1.2±0.2	1.4±0.08	2.9±0.5	*I*, MS
γ-Terpinene	1059	24.1±1.4	24.2±1.2	22.7±1.2	23.6±1.2	21.3±1.9	18.3±1.8	23.7±1.0	24.2±1.9	*I*, MS, Co-GC
cis-Sabinene hydrate	1068		0.1±0.02	0.1±0.02		tr	0.1±0.02		0.1±0.02	*I*, MS
Terpinolene	1089	1.2±0.02	1.4±0.07	1.7±0.07	0.2±0.07	1.3±0.04	1.4±0.07	1.3±0.07	1.1±0.07	*I*, MS, Co-GC
Linalool	1098	0.2±0.01	0.1±0.02	0.2±0.01	0.2±0.02	0.2±0.01	tr		0.2±0.02	*I*, MS, Co-GC
n-Nonanal	1100	tr	tr	0.1±0.01		tr				*I*, MS
Citronellal	1156	0.2±0.02	0.1±0.02	0.1±0.02	tr	0.1±0.03	0.1±0.02	0.1±0.02	0.2±0.02	*I*, MS
Terpinen-4-ol	1178	0.4±0.08	0.3±0.02	0.1±0.03		0.1±0.07	0.1±0.01	0.1±0.02		*I*, MS, Co-GC
α-Terpineol	1189	0.4±0.06	0.4±0.01	0.5±0.01	0.2±0.01	0.5±0.07	0.6±0.01	0.6±0.03	0.4±0.02	*I*, MS
n-Decanal	1205	tr	0.1±0.02	0.1±0.03		tr		0.1±0.02	0.1±0.02	*I*, MS
Nerol	1232	0.2±0.04	0.1±0.03	0.1±0.01	tr	0.1±0.04	tr	tr	tr	*I*, MS
Neral	1242	1.3±0.02	1.6±0.02	1.7±0.08	1.5±0.02	2.7±0.02	3.1±0.5	3.2±0.6	1.6±0.04	*I*, MS, Co-GC
Geraniol	1255	0.3±0.02	0.5±0.02	0.1±0.04	0.4±0.02	0.1±0.02				*I*, MS
Geranial	1275	1.2±0.9	2.1±0.08	2.3±0.07	2.0±0.08	3.8±0.9	4.5±0.9	4.4±0.8	2.4±0.08	*I*, MS
Neril acetate	1370	0.3±0.02	0.2±0.01	0.2±0.01	0.1±0.01	0.4±0.02		0.3± 0.01	0.6±0.02	*I*, MS
Geranil acetate	1388	0.2±0.02	0.2±0.03	0.2±0.04	0.1±0.03	0.7±0.02	0.6±0.03	0.5±0.01	0.5±0.02	*I*, MS
β-Elemene	1390	0.1±0.02	0.2±0.02	0.2±0.02						*I*, MS
β-Cubebene	1392	0.4±0.02		tr		tr	0.2±0.04	0.2±0.02		*I*, MS
α-Bergamotene	1403	0.5±0.02	0.5±0.02	tr			tr	0.8±0.04	1.0±0.02	*I*, MS
trans- Caryophyllene	1418	0.3±0.02	0.2±0.03	0.3±0.01	0.2±0.03	0.5±0.02	0.5±0.01	0.4±0.03	0.4±0.03	*I*, MS, Co-GC
trans-β-Farnesene	1441	tr	0.1±0.02	0.1±0.01			0.1±0.02	0.1±0.02		*I*, MS
α-Humulene	1454	0.2±0.01	0.3±0.05	0.1±0.01		0.1±0.01	0.1±0.02	0.1±0.02	tr	*I*, MS, Co-GC
β-Bisabolene	1508	0.7±0.02	0.8±0.04	0.5±0.04	0.8±0.04	1.2±0.2	1.3±0.4	1.1±0.3	1.3±0.04	*I*, MS, Co-GC
Citropten	1993	0.9±0.03	0.4±0.04	0.5±0.04		0.7±0.02	0.5±0.04	0.3±0.01	0.3±0.02	MS, Co-GC
Oxypeucedanin	2420					0.4±0.03	0.2±0.01	0.3±0.01	0.1±0.01	MS

[a]Retention Index on SE-30 MS column. [b]Mean abundance value ± standard error, *n* = three independent determinations. Compositional values less than 0.1% are denoted as traces (tr). [c]I, Retention index; MS, mass spectrum; Co-GC: co-injection with authentic compound.

GABRIELE *et al.* (2009) evaluated the composition of *C. medica* cv Diamante essential oil from fruits of three types: green citron of little size, green citron of big size and yellow citron after 1 month from the harvest. The essential oils extracted using three different methods differ only in the quantitative composition, while the qualitative profile was the same. In agreement with our results, the volatile fraction of every sample is characterized by a high content of limonene, followed by γ-terpinene. Differences can be noted between our oils and the samples analyzed by GABRIELE *et al.* (2009) regarding the quantitative composition of limonene, α-pinene and β-pinene. In particular, our samples are characterized by a lower content of limonene (40.2-55.8% in comparison with 57-60%), but a higher content of g-terpinene (21.3-28.0% except for samples 6 and 13, in comparison with 20.9-24.4%), α-pinene (1.9-3.6% in comparison with 0.7-1.5%) and β-pinene (1.2-3.4% except for sample 3, in comparison with 0.9-1.4%). Oxypeucedanin was the main component of the oxygenated heterocyclic fraction in the extracts of green fruits, while citropten was the major oxygenated compound in the oil obtained from yellow citron.

The essential oil isolated from peel of Cretan origin *C. medica* cv Diamante revealed that limonene was the main constituent. The oil also contained a high content of neral and geranial, with β-pinene and myrcene being the most abundant monoterpene hydrocarbons following limonene. Moreover, the peel oil was characterized by appreciable proportions of citronellol, nerol and geraniol (VEKIARI *et al.*, 2004).

Correlations

The physical soil constituent and leaves micro and macro-nutrient content attained statistical significance with some of the components of the oils. Table 6 shows the correlation matrix between

Table 5 - Major components of the essential oil from *C. medica* cv Diamante peel collected in zone B.

Compound	I[a]	%[b]								ID Method[c]
		9	10	11	12	13	14	15	16	
Thujene	926	0.8±0.5	1.2±0.6	1.1±0.8	1.0±0.6	1.4±0.3	1.5±0.6	0.9±0.8	1.1±0.6	*I*, MS
α-Pinene	936	1.9±0.5	2.6±0.9	2.5±0.4	2.5±0.9	3.0±0.7	3.0±0.9	2.1±0.5	2.4±0.9	*I*, MS, Co-GC
Camphene	953					tr	tr	tr	0.1±0.02	*I*, MS
Sabinene	973	1.8±0.07	tr	0.3±0.02	0.4±0.02				0.3±0.01	*I*, MS, Co-GC
β-Pinene	978	1.2±0.02	2.1±0.06	2.2±0.09	2.2±0.06	2.9±0.3	3.2±0.06	2.9±0.11	2.1±0.06	*I*, MS, Co-GC
β-Myrcene	986	1.0±0.05	1.8±0.05	1.6±0.05	1.7±0.05	2.5±0.05	1.5±0.05	1.0±0.05	1.4±0.09	*I*, MS, Co-GC
α-Phellandrene	1005			0.1±0.01			tr	0.1±0.01	tr	*I*, MS
α-Terpinene	1016		0.6±0.01	0.3±0.02	0.3±0.02	tr	tr	0.2±0.01	0.2±0.02	*I*, MS, Co-GC
Limonene	1032	55.8±1.2	52.3±1.7	51.3±1.9	48.5±1.7	48.7±2.6	40.2±1.7	50.2±1.9	54.4±2.1	*I*, MS, Co-GC
(*E*)-β-Ocimene	1047	1.1±0.5	0.3±0.08	1.7±0.08	2.1±0.08	1.3±0.08	1.1±0.08	1.8±0.08	1.9±0.06	*I*, MS
γ-Terpinene	1059	26.5±1.9	24.6±1.2	27.5±1.2	22.2±1.2	20.8±1.5	23.0±1.2	26.1±1.2	28.0±1.1	*I*, MS, Co-GC
cis-Sabinene hydrate	1068		tr			tr	0.1±0.02	tr		*I*, MS
Terpinolene	1089	1.1±0.07	1.2±0.07	1.0±0.07	1.1±0.07	1.4±0.03	0.7±0.03	1.1±0.06	0.6±0.03	*I*, MS, Co-GC
Linalool	1098		0.1±0.02		0.1±0.02	0.2±0.02	0.2±0.01	0.1±0.01	tr	*I*, MS, Co-GC
n-Nonanal	1100					0.1±0.02				*I*, MS
Citronellal	1156		0.1±0.02		tr	tr	0.2±0.01	0.1±0.01	tr	*I*, MS
Terpinen-4-ol	1178		0.3±0.02	0.3±0.02	0.3±0.02	tr		0.2±0.02		*I*, MS, Co-GC
α-Terpineol	1189		0.5±0.01			0.4±0.01	0.6±0.01	0.4±0.01	0.3±0.02	*I*, MS
n-Decanal	1205				0.1±0.02			tr	tr	*I*, MS
Nerol	1232		0.5±0.03		0.1±0.03	tr	0.3±0.03	tr	tr	*I*, MS
Neral	1242	0.9±0.04	1.5±0.02	0.7±0.02	1.1±0.02	2.9±0.9	1.5±0.02	1.3±0.06	1.4±0.07	*I*, MS, Co-GC
Geraniol	1255				0.5±0.02		0.3±0.01	tr	tr	*I*, MS
Geranial	1275	1.2±0.08	2.1±0.08	0.8±0.08	2.9±0.08	4.2±0.08	2.0±0.08	1.8±0.08	2.0±0.08	*I*, MS
Neril acetate	1370		0.2±0.01	tr	0.2±0.01	0.4±0.01	0.7±0.02	0.2±0.01	0.2±0.01	*I*, MS
Geranil acetate	1388		0.3±0.03	0.7±0.08	0.3±0.03	0.7±0.03	0.7±0.03	0.2±0.08	0.2±0.03	*I*, MS
β-Elemene	1390			0.6±0.08		tr	tr	0.2±0.08	0.2±0.01	*I*, MS
β-Cubebene	1392					0.2±0.01			tr	*I*, MS
α-Bergamotene	1403		0.6±0.02		tr	tr	1.1±0.09	tr	tr	*I*, MS
trans-Caryophyllene	1418	0.7±0.03	0.4±0.03	0.3±0.03	0.3±0.03	0.5±0.01	0.4±0.03	0.3±0.05	0.3±0.03	*I*, MS, Co-GC
trans-β-Farnesene	1441		0.2±0.02			0.1±0.02	0.1±0.02	0.1±0.01	tr	*I*, MS
α-Humulene	1454					0.1±0.01				*I*, MS, Co-GC
β-Bisabolene	1508	1.0±0.04	0.8±0.04	0.5±0.04	1.3±0.04	1.1±0.7	1.5±0.08	1.0±0.04	0.8±0.05	*I*, MS, Co-GC
Citropten	1993		0.8±0.04			0.4±0.04	0.6±0.04	0.2±0.01		MS, Co-GC
Oxypeucedanin	2420				0.8±0.04	0.1±0.02	0.2±0.01	tr		MS

[a]Retention Index on SE-30 MS column. [b]Mean abundance value ± standard error, n = three independent determinations. Compositional values less than 0.1% are denoted as traces (tr). [c]I, Retention index; MS, mass spectrum; Co-GC: co-injection with authentic compound.

Table 6 - Correlation between soil, leaves and essential oil components.

Parameters	Essential oil components				
	Thujene	α-Pinene	α-Terpineol	*trans*-β-Farnesene	Terpinolene
Soil					
Silt (%)	-0.589**	-0.595**	-	-	-
Sand (%)	0.615**	0.595**	-	-	-
K (%)	-0.553*	-0.534*	-	-	-
N (‰)	-	-	0.500*	0.572*	-
Na (mg kg^{-1})	-0.532*	-0.532*	-	-	-
Mg (%)	-0.532*	-0.529*	-	-	-
C.S.C. (meq/100 g soil)	-0.491*	-	-	-	-
Leaves					
Ca (%)	0.685**	0.654**	-	-	-
Mg (%)	-	-	-	-	-0.6191**

*$p \leq 0.05$, **$p \leq 0.01$, - not significant.

the most significative parameters of the soil and leaf and the individual components of the essential oils. The sand positively influenced the content of monoterpene thujene (r= 0.615, P= 0.11) and the α-pinene (r= 0.595, P= 0.15). On the contrary, a negative correlation was observed with exchangeable potassium, sodium and magnesium ions. The total nitrogen positively influenced the α-terpineol and *trans*-β-farnesene content (P< 0.05). Among the parameters of leaves nutritional status calcium positively influenced the thujene and α-pinene content (r= 0.685, P= 0.03 and 0.654, P= 0.06, respectively), and magnesium conditioned the terpinolene content (r= 0.760, P= 0.01).

CONCLUSIONS

In this study the essential oil obtained from the peel of *C. medica* cv Diamante was analysed and investigated to evaluate the influence of the environmental parameters, cultivation management, physico-chemical parameters of soil and nutrients in the leaves on its chemical composition. Differences highlighted in essential oil composition and carpometric characteristics of fruits collected in the two different zones could be attributed to the soil composition and in particular to sand, and P_2O_5 and K_2O content. Results demonstrated as fruits collected in plain at sea level are characterized by a higher weight, and equatorial and longitudinal diameter and lower fruit firmness lower than fruits collected in zone B.

These findings could help cultivators to improve fruits quality suitable for industrial products such as candy.

ACKNOWLEDGEMENTS

This work was realized in the Project CITROMED "Caratterizzazione fitochimica, biologica e agropedoclimatica del cedro (*Citrus medica* L. var. Liscio Diamante) prodotto in Calabria: biodiversità prospettive farmaceutiche, cosmetologiche ed alimentari", funded by Italian Ministry of Agriculture, Food and Forestry Policies (MiPAAF) - Research agreements No. 2/07.
The Authors are grateful to farms for supplying the samples.

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Paper received April 16, 2010 Accepted January 10, 2011

PAPER

BIOCHEMICAL, MICROBIOLOGICAL AND SENSORY CHARACTERISTICS OF PROBIOTIC YOGURT CONTAINING VARIOUS PREBIOTIC COMPOUNDS

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ABSTRACT

Effects of addition of several well-known commercially available prebiotics or fibres (inulin, Hi-maize, lactitol, lactulose, β-glucan, and maltodextrin) in two levels (1.5 and 3.0%) on biochemical, microbiological and sensory characteristics of ABY-type probiotic yogurt (*Lactobacillus acidophilus* LA-5, *Bifidobacterium animalis* ssp. *lactis* BB-12 and yogurt bacteria) were investigated. Experimental parameters analyzed during fermentation were changes in pH, acidity and redox potential. At the end of fermentation (pH 4.5), the mean pH drop, mean titrable acidity increase and mean redox potential increase rates, concentrations of lactic and acetic acids, incubation time, viability of probiotic organisms, and sensory attributes of yogurts were determined. Viability of probiotics in selected yogurts was also assessed during 21 days of refrigerated storage (5°C). Addition of prebiotics significantly changed the biochemical characteristics of different yogurts and substantially enhanced the viability of probiotics/fibres immediately after fermentation and during the cold storage. Many prebiotic/fibre-containing yogurts had lower sensory acceptability compared to the control. In an overall approach, yogurts containing 1.5% β-glucan and 1.5% Hi-maize were considered as the best choices.

- Key words: prebiotic, probiotic, sensory, viability, yogurt -

INTRODUCTION

Nowadays, many types of probiotic dairy products are available worldwide. Probiotic yogurt is still among the most popular probiotic products (KORBEKANDI *et al.*, 2010; TAMIME *et al.*, 2005). To provide health benefits related to probiotic organisms, the minimum viable counts of each probiotic strain in gram or milliliter of probiotic products is a critical value. Generally, the amount of 10^6 cfu/mL or cfu/g has been accepted as minimum level and the amounts of 10^7 and 10^8 cfu/mL or cfu/g as satisfactory levels. In Japan, the "Fermented Milks and Lactic acid Bacteria Association" have developed a standard which requires a minimum of 10^7 cfu/mL viable probiotic cells to be present in dairy products (TAMIME *et al.*, 2005). In Iran, National standard requires a minimum of 10^6 cfu/mL viable probiotic cells in yogurt (ANON, 2009). Probiotic products should be consumed regularly with an approximate amount not less than 100 g/d in order to deliver about 10^9 viable cells into the intestine (KORBEKANDI *et al.*, 2010; TAMIME *et al.*, 2005). Many intrinsic and extrinsic factors in food products significantly affect viability of probiotic microorganisms in fermented milks such as pH, titrable acidity, molecular oxygen, redox potential, hydrogen peroxide, bacteriocins, biorelationships among starter bacteria and microbial competitions, short chain fatty acids, some flavouring agents, anti-microbial preservatives, packaging materials and conditions, rate and proportion of inoculation, step-wise/stage-wise fermentation, microencapsulation, supplementation of milk with nutrients, heat yogurt of product, incubation temperature, storage temperature, carbonation, addition of salt, sugar and sweeteners, cooling rate of product and scale of production (KORBEKANDI *et al.*, 2010; TAMIME *et al.*, 2005; CHAMPAGNE and RASTALL, 2009).

Prebiotics are non-viable and non-digestible (or very low digestible) food ingredients selectively metabolized by beneficial intestinal bacteria and enhance their growth and/or activity. They are mostly sugar-like compounds comprising between two and ten monomers that largely resist digestion by pancreatic and brush board enzymes. The term "symbiotic" is used to describe products that contain both probiotics and prebiotics (HOLZAPFEL and SCHILLINGER, 2001; KORBEKANDI *et al.*, 2010; ARYANA *et al.*, 2007). Fructooligosaccharides (FOSs) (linear or branched forms) are among the most important prebiotic compounds (ARYANAET *et al.*, 2007; AKALIN *et al.*, 2004).

Nowadays, prebiotics are included in many products in order to promote the growth of probiotics in the intestine. Therefore, incorporation of prebiotics into fermented milks such as yogurt, especially those containing probiotic bacteria, would potentially lead to a healthier product. It has been known that prebiotics may aid survival of probiotics in fermented milks during processing and storage (CAPELA *et al.*, 2006). Although much has been written about the beneficial impact of prebiotic on probiotic growth and/or activity under *in vivo* (colonic) conditions, there are a few years that the effects of prebiotic compounds on viability of mentioned organisms in fermented milks as well as on qualitative properties of final products has been under investigation and limited reports are present (ARYANA *et al.*, 2007; AKALIN *et al.*, 2004; CAPELA *et al.*, 2006; ARYANA and MCGREW, 2007; DONKOR *et al.*, 2007; MARTINEZ-VILLALUENGA *et al.*, 2006; *et al.*, 2009; ÖZER and ÖZER, 2005; TABATABAIE and MORTAZAVI, 2008; VASILJEVIC *et al.*, 2007). The vast majority of these studies dedicate to the effects of prebiotics on probiotic stability during the cold storage of product rather than during the fermentation period.

There are conflicting reports on the effect of prebiotics on viability of probiotics in fermented milks. Many reports indicate significant increase in survival of bifidobacteria (AKALIN *et al.*, 2004; CAPELA *et al.*, 2006; NOUBAKHTI *et al.*, 2009; ÖZER and ÖZER, 2005; TABATABAIE and MORTAZAVI, 2008; VASILJEVIC *et al.*, 2007; BRUNO *et al.*, 2002; CHEN *et al.*, 2004; SHIN, LEE *et al.*, 2000), *Lactobacillus acidophilus* (ARYANA *et al.*, 2007; CAPELA *et al.*, 2006; DONKOR *et al.*, 2007; TABATABAIE and MORTAZAVI, 2008; RASTALL and MAITIN, 2002; SADEK *et al.*, 2004), *L. casei* (CAPELA *et al.*, 2006; ARYANA and MCGREW, 2007; DONKOR *et al.*, 2007) and *L. rahmnosus* (CAPELA *et al.*, 2006; TABATABAIE and MORTAZAVI, 2008; SADEK *et al.*, 2004), and some detect no significant impact (*L. acidophilus*) (ÖZER and ÖZER, 2005) or even lower viability compared to the control (*L. acidophilus*) (NOUBAKHTI *et al.*, 2009). It seems that factors such as the type of prebiotic, prebiotic chemical properties and the level of incorporation determine its efficiency and impact. For example, shorter chain fructooligosaccharides (FOSs) are the first to be consumed by bifidobacteria (PERRIN *et al.*, 2002); However, long-chain fructans mostly display non-prebiotic-specific health benefits such as enhancing bioavailability of minerals, decreasing fat absorption, preventing constipation, lowering blood cholesterol, boosting body defenses, decreasing incidence of colon cancer, and helping the body to eliminate toxins (ARYANA *et al.*, 2007; JENKINS *et al.*, 1999). There are also conflicting results about the effects of prebiotics on sensory properties of fermented milks. Some researches indicate good or better flavor and texture profiles compared to control (inulin) (IBRAHIM *et al.*, 2004; SEYDIN *et al.*, 2005), whereas, some detect no significant differences compared to the control (inulin or wheat fiber or bamboo fiber) (DELLO STAFFOLO *et al.*, 2004; HASSAN *et al.*, 1999) and some represent weaker sensory properties compared to the control (inulin) (GUVEN *et al.*, 2005). The aim of this study

was to investigate the effects of addition of several well-known commercially available prebiotic/fibre compounds (inulin, Hi-maize, lactitol, lactulose, β-glucan, and maltodextrin; 1.5 or 3%) on biochemical, microbiological and sensory characteristics of yogurt containing *Lactobacillus acidophilus* La-5, *Bifidobacterium animalis* ssp. *lactis* BB-12, and yogurt bacteria.

MATERIALS AND METHODS

Starter culture and prebiotic compounds

Starters of commercial lyophilized ABY culture (containing *Lactobacillus acidophilus* LA-5, *Bifidobacterium animalis* ssp. *lactis* BB-12, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*) that are known as "FD-DVS ABY-1" were supplied by Chr-Hansen (Horsholm, Denmark). This culture is currently used by dairy industry to produce probiotic dairy fermented products. The cultures were maintained according to manufacturer's instructions at -18°C until used.

The fine powders of six commercially available prebiotics/fibres (inulin, Hi-maize, lactitol, lactulose, β-glucan, and maltodextrin) were supplied by North Pacific (IIDEA, Jalisco, Mexico). They were stored at room temperature and under dry condition until were used.

Study design and sample preparation

Twelve yogurts including those containing 1.5 or 3.0% of inulin, Hi-maize, lactitol, lactulose, β-glucan, or maltodextrin were formulated using reconstituted skim milk powder and sterilized potable water. A control yogurt (without prebiotic) was also prepared. The final milk non-fat dry matter content of yogurt samples was 11% (W/W). After heat yogurt (90°C - 15 min) and cooling of yogurt milk samples, and starter culture inoculation, fermentation was carried out at 42°C until pH reached 4.5. Biochemical parameters including changes in pH, acidity and redox potential were measured during fermentation period. These parameters were recorded per 30-minute time intervals. The mean pH drop, mean acidity increase and mean redox potential increase rates, incubation time, and final titrable acidity were determined at the end of fermentation. The final samples were cooled down and kept at 5°C until the probiotic organisms were enumerated and the concentrations of lactic and acetic acids were determined.

Microbiological analysis

MRS-bile agar medium (MRS agar by Merck, Darmstadt, Germany and bile by Sigma-Aldrich, Inc., Reyde, USA) was used for the selective enumeration of *L. acidophilus* and bifidobacteria in ABY culture composition according to MORTAZAVIAN *et al.* (2007). The plates were incubated aerobically and anaerobically at 37°C for at least 72 hours. Yogurt bacteria (*L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*) did not growth in presence of bile. *L. acidophilus* cells were selectively counted under aerobic conditions, while selective enumeration of bifidobacteria achieved using subtractive enumeration method (SEM) under anaerobic conditions (MORTAZAVIAN *et al.*, 2007). Anaerobic conditions were produced using the GasPac system (Merck, Darmstadt, Germany).

Growth proportion index (GPI) of probiotic microorganism at the end of period was calculated as following (MORTAZAVIAN *et al.*, 2010):

$$GPI = \frac{\text{Final cell population (cfu/mL)}}{\text{initial cell population (cfu/mL)}}$$

Chemical analysis

pH values and redox potential of the samples were measured at room temperature using a pH meter (MA235, Mettler, Toledo, Switzerland).

Titrable acidity was determined after mixing 10 mL of sample with 10 mL of distilled water and titrating with 0.1 N NaOH using 0.5% phenolphthalein according to MORTAZAVIAN *et al.* (2010).

Parameters of pH mean drop rate, mean acidity increase rate, and mean redox potential increased rate were calculated as following (MORTAZAVIAN *et al.*, 2010):

- pH drop rate = (final pH value – initial pH value) / incubation time [pH value/min]
- Acidity increase rate = (final acidity value – initial acidity value) / incubation time [Dornic degree/min]
- Redox potential increase rate = (final value – initial value) / incubation time [mV/min]

Quantification of lactic and acetic acids was carried out by High Performance Liquid Chromatography (CE 4200- Instrument, Cecil, Milton Technical Center, Cambridge CB46AZ, UK) according to SHAFIEE *et al.* (2010). Briefly, for extraction of acids, 4.0 g of sample was diluted to 25 mL with 0.1 N H_2SO_4, homogenized and centrifuged at 5,000 *g* for 10 min. The supernatant was filtered through Whatman #1 filter paper and through a 0.20 μm membrane filter, and was immediately analyzed. A Jasco UV-980 detector and a Nucleosil 100-5C_{18} column (Macherey Nagel, Duren, Germany) were used. The mobile phase was 0.009 N H_2SO_4 at a flow rate of 0.5 mL/min. The wavelength of detection was optimized at 210 nm. The standard solutions of lactic and acetic acids (Merck, Darmstadt, Germany) were prepared in distilled water. The retention times for lactic and acetic ac-

ids were 3.45 and 3.58 min and the standard curve regression coefficients were 0.989 and 0.991, respectively.

Sensory analysis

Consumer panel of nine panelists were used. First, yogurts containing the same prebiotic/fibre compounds with different levels (1.5 or 3.0%) were compared using "Paired comparison test (DUO-test)" (KHOSROKHAVAR and MORTAZAVIAN, 2010). The sensory parameters studied at this phase were gel firmness and scoopability of the gel, texture smoothness, sourness, flavour acceptability and total acceptability. Selected yogurts were analyzed and compared using "scoring methodology" according to the Iran national standard (ANON, 2008). The sensory parameters for the second phase were flavour, oral texture and mouthfeel, non-oral texture (pouring, stirring and scoopability), and appearance (colour, syneresis, and surface and texture homogeneity). Each of these parameters was scored in a five-point scale including 0 = inconsumable, 1 = unacceptable, 2 = acceptable, 3 = satisfactory, and 4 = excellent. The given numbers for each sensory parameter were multiplied with the relevant coefficients, namely, 6 for flavour, 3.5 for oral texture and mouthfeel, 2 for appearance, and 1 for non-oral texture.

Statistical analysis

Experiments were performed in triplicate and the significant differences among means/values were analyzed using the Duncan's test (on the basis of complete randomized design) from MSTATC software (Pussell D. Freed, Crop and Soil Science Department, Michigan State University, Version 2.10).

RESULTS AND DISCUSSION

Biochemical characteristics of yogurt during fermentation and at the end of fermentation

Fig. 1 shows changes in pH, acidity and redox potential during fermentation. Table 1 presents mean pH drop rate, mean acidity increase rate, mean redox potential increase rate, incubation time, final titrable acidity, and lactic and acetic acid contents (%) in different yogurts throughout the fermentation or at the end of this period. As shown in Fig. 1, three distinguished phases could be observed in charts related to changes in pH decline, acidity increase and redox potential increase of the yogurts, namely, lag and pre-log phases (initial part of the charts with relatively low slopes), log phase (with considering higher slope), and late log and stationary phases (with significantly decrease in chart slop compared to previous phase). According to Table 1, there is no difference in mean pH drop rate during fermentation among different yogurts. The greatest (p<0.05) mean acidity increase rate was observed in the yogurt containing 1.5% lactitol (Lactitol-1.5) and then, Control, Inulin-1.5, and Maltodextrin 1.5. In contrast, Lactitol-3.0 and β-glucan-3.0 had the lowest mean acidity increase rate during fermentation. It seems that the addition of prebiotic compounds instead of milk solid non-fat did not significantly affect buffering capacity of the product during fermentation since all yogurts showed a similar mean pH drop rate. It is not exactly clear whether the higher mean acidity increase rate in some yogurts was mainly due to stimulating growth and/or activity of yogurt bacteria or probiotic cells. For instance, Lactitol-1.5 which exhibited the highest mean acidity increase rate (Table 1) was from the yogurts with the lowest viability of both probiotic bacteria (Section 3.2 and Table 2); indicating unsurprisingly that the acidification was mainly due to yogurt bacteria and not by probiotics. Generally, yogurts with higher mean acidity increase rate had also higher mean redox potential increase rate because organic acids produced during fermentation increase redox potential (KORBEKANDI *et al.*, 2010). The highest levels of final titrable acidity were observed in Maltodextrin-1.5 and after it, Lactulose-1.5, Lactitol-1.5, inulin-1.5 and Control, whereas, the lowest were found in Hi-maize-3.0 and Lactulose-3.0. The longest incubation times were observed for β-glucan-1.5 & 3.0, Maltodextrin-1.5, Lactitol-3.0 and inulin-3.0, whereas Hi-maize-1.5 showed the shortest incubation time (Table 1). A correlation between the mean acidity increase rate and incubation time was observed: the yogurts with the lowest mean acidity increase rates displayed the longest incubation times (Table 1). In comparison with the Control, only Hi-maize-1.5 and Lactitol-1.5 showed significantly shorter fermentation time which was either the same or longer than for Control.

There are conflicting reports on the effects of prebiotic/fibre addition on acidification or post-acidification (during the refrigerated storage) rate of fermented milks containing probiotics and yogurt bacteria. HARDI and SLACANAE (2000) found that the rate of pH decease of fermented milk was increased by inulin supplementation. This is controversial to our results because in this study (Table 1), addition of inulin (1.5 or 3.0) did not significantly affect the mean acidity increase rate and did not change the mean pH decrease rate during fermentation. Also, control yogurt had incubation time less than Inulin-1.5 and Inulin-3.0. ZHU (2004) reported that the acidity and pH of yogurt were not affected by the addition of 4% fructooligosaccharide. According to GUVEN *et al.* (2005),

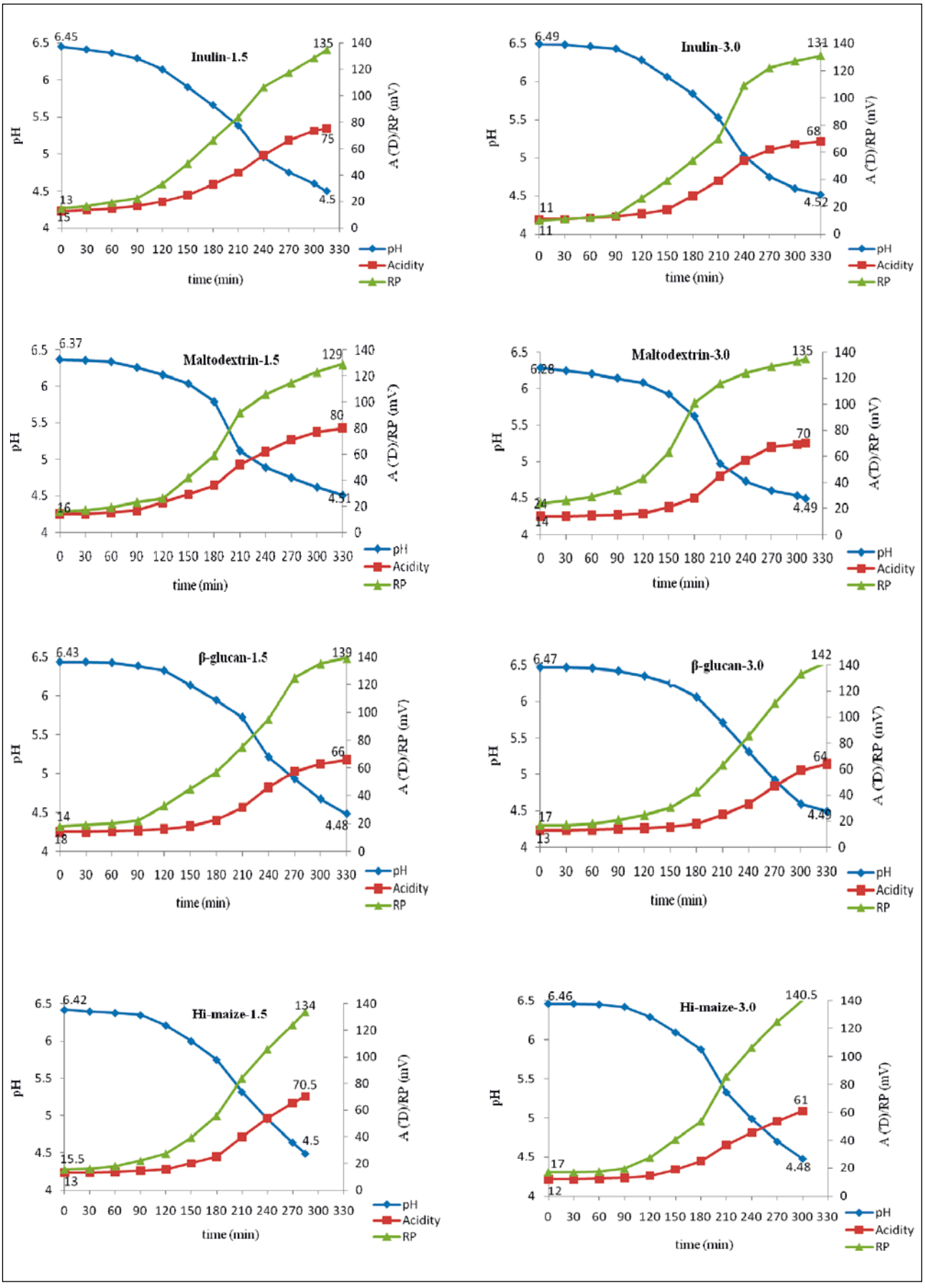


Fig. 1 - Changes in pH drop, acidity increase and redox potential increase during fermentation period in different yogurts (continues).

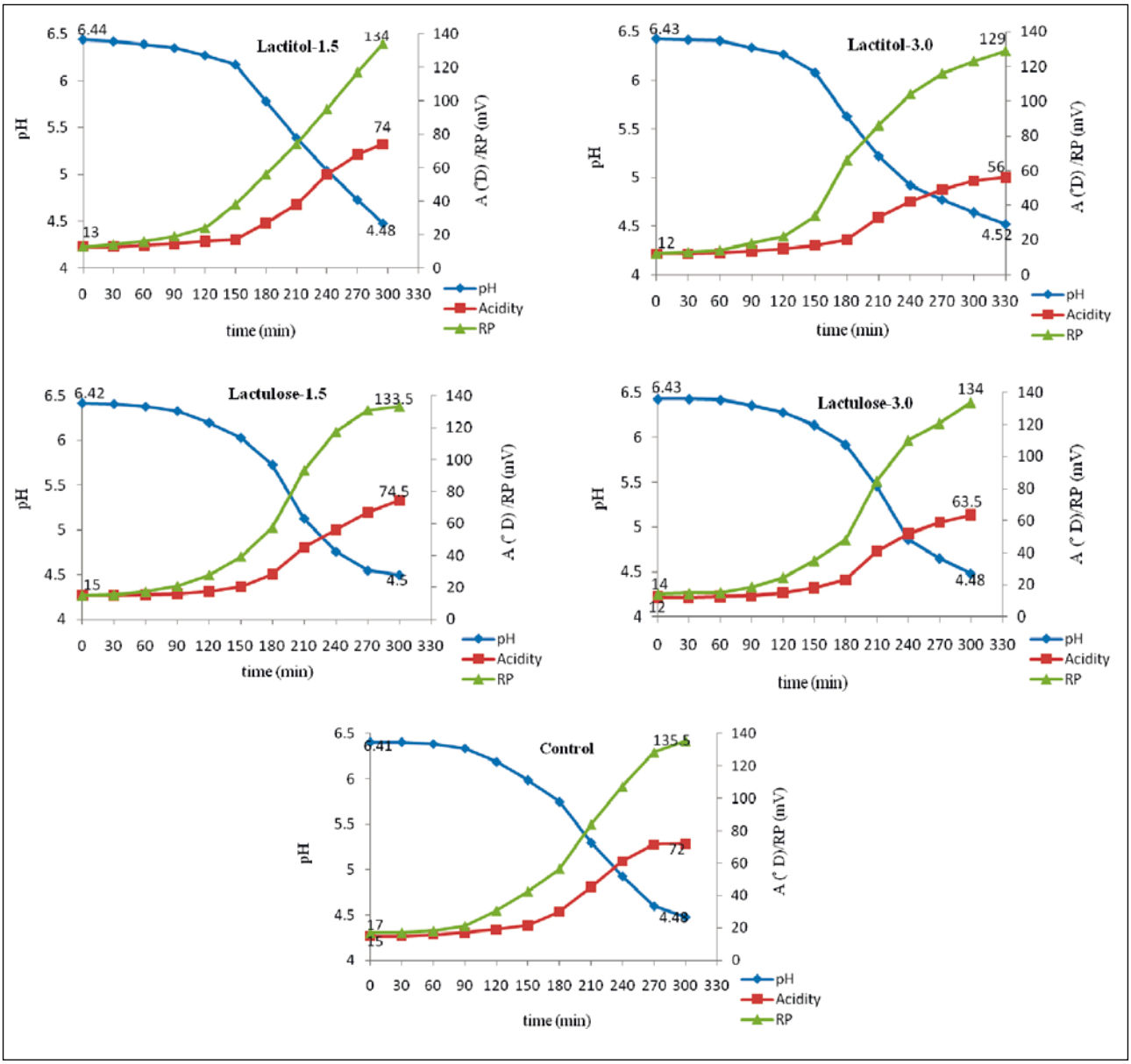


Fig. 1 - Continued.

inulin did not affect the pH and titrable acidity of yogurt. Yogurt containing inulin had a stable pH over 28 days of storage period (DELLO STAFFOLO *et al.*, 2004). TABATABAIE and MORTAZAVI (2008) found that the pH decrease rate of the control was higher compared to the yogurts containing lactulose (1.0 and 3.0%).

The concentration of lactic acid was directly proportional to the final titrable acidity (Table 1). For instance, yogurts containing Maltodextrin-1.5, Control, Inulin 1.5, Lactitol-1.5 and Lactulose-1.5 which had the highest amounts of titrable acidity, rendered also the highest values of lactic acid percentage. The same pattern was observed for the lowest amounts (Table 1). Acetic acid concentration in yogurts was in the range of 0.05-0.12%. Sum of other organic acids except lactic acid were less than 0.03%.

Viability of probiotic bacteria at the end of fermentation and during 21 days of storage

Table 2 shows viability of probiotic microorganisms as well as the relevant growth proportion index (GPI) in different yogurts immediately after fermentation. Table 3 indicates viability of these organisms during a 21-day refrigerated storage period (5°C) in selected yogurts. As shown in Table 2, for *L. acidophilus*, except Inulin-3.0 and Lactulose-3.0, addition of prebiotics significantly increased the viability of this bacterium compared to the control. The highest levels of viability of *L. acidophilus* were observed in yogurts containing Maltodextrin-3.0>Maltodextrin-1.5>β-glucan-1.5>β-glucan-3.0 & Hi-maize-1.5. The lowest viabilities of this bacterium were seen in Inulin-3.0 and Control. For bifidobacteria,

Table 1 - Mean pH drop rate, mean acidity increase rate, mean redox potential increase rate, incubation time, final acidity, and lactic and acetic acid contents in different yogurts throughout the fermentation or at the end of fermentation (final pH 4.5)*.

	Parameters						
Prebiotic concentration (%)	pH-DR** (pH/min)	A-IR (°D/min)	RP-IR (mV/min)	Incubation time (min)	Final acidity (°D)	Lactic acid percentage	Acetic acid percentage
Control	0.006^{a}	0.20^{ab}	0.40^{ab}	300^{c}	76.0^{b}	0.69^{a}	0.05^{cd}
Inulin-1.5	0.006^{a}	0.20^{ab}	0.38^{b}	315^{b}	76.1^{b}	0.61^{bc}	0.12^{a}
Inulin-3.0	0.006^{a}	0.17^{bc}	0.37^{b}	330^{a}	68.0^{cd}	0.58^{c}	0.08^{b}
Hi-maize-1.5	0.007^{a}	0.20^{ab}	0.42^{a}	285^{d}	70.5^{c}	0.60^{bc}	0.09^{b}
Hi-maize-3.0	0.006^{a}	0.16^{c}	0.41^{a}	300^{c}	61.09^{ef}	0.53^{cd}	0.06^{c}
Lactitol-1.5	0.006^{a}	0.21^{a}	0.41^{a}	295^{c}	74.0^{bc}	0.64^{b}	0.07^{bc}
Lactitol-3.0	0.006^{a}	0.13^{e}	0.35^{c}	330^{a}	56.0^{g}	0.47^{e}	0.07^{bc}
Lactulose-1.5	0.006^{a}	0.20^{ab}	0.40^{ab}	300^{c}	74.5^{bc}	0.66^{ab}	0.06^{c}
Lactulose-3.0	0.006^{a}	0.17^{bc}	0.40^{ab}	300^{c}	63.5^{e}	0.56^{c}	0.06^{c}
Maltodextrin-1.5	0.006^{a}	0.20^{ab}	0.34^{c}	330^{a}	80.0^{a}	0.68^{a}	0.11^{a}
Maltodextrin-3.0	0.006^{a}	0.18^{b}	0.36^{c}	310^{bc}	70.0^{c}	0.57^{c}	0.11^{a}
β-glucan-1.5	0.006^{a}	0.16^{c}	0.37^{b}	330^{a}	66.0^{d}	0.54^{cd}	0.10^{ab}
β-glucan-3.0	0.006^{a}	0.15^{d}	0.38^{b}	330^{a}	64.0^{de}	0.53^{cd}	0.08^{b}

*Means in the same column shown with different letters are significantly different (p<0.05).
**pH-DR = man pH drop rate, A-IR = mean acidity increase rate, RP-IR = mean redox potential increase rate.

except Lactulose-3.0, addition of prebiotics significantly increased their viability compared to the control. The greatest viabilities of bifidobacteria cells were related to the yogurts containing Inulin-1.5>Maltodextrin-1.5>Maltodextrin-3.0>β-glucan-1.5 & β-glucan-3.0 & Inulin-3.0. The lowest viabilities of bifidobacteria were in yogurts containing Lactitol-3.0 and Control. Generally, Increasing the concentration of prebiotic from 1.5 to 3.0% resulted in a neutral or decreasing effect on viability of the two probiotic bacteria The positive effect of prebiotics on viability of probiotics seems to be different during fermentation compared to refrigerated storage. For *L. acidophilus*, from d 7 onwards, Hi-maize-1.5 that resulted in the highest viability throughout the storage period and β-glucan-1.5 which caused the highest viability among the 4 yogurts immediately after fermentation, resulted in the lowest viability throughout the storage period. For bifidobacteria, from d 7 onwards, β-glucan-1.5 led to the highest viability throughout the storage period.

There are controversial reports regarding

Table 2 - Viability of probiotic microorganisms and the relevant growth proportion index in different yogurts at the end of fermentation (final pH 4.5)*.

Prebiotic concentration	Initial population (log cfu mL^{-1})			Final population (log cfu mL^{-1})			GPI**		
(%)	A***	B	A+B	A	B	A+B	A	B	A+B
Control	6.2	6.4	6.6	7.0^{fAB}	7.0^{fA}	7.3^{k}	6.1	3.9	4.8
Inulin-1.5	6.2	6.4	6.6	7.1^{deB}	7.6^{aA}	7.7^{c}	7.4	13.8	11.5
Inulin-3.0	6.2	6.4	6.6	7.1^{eB}	7.4^{cA}	7.6^{f}	7.1	9.3	8.5
Hi-maize-1.5	6.2	6.4	6.6	7.1^{dAB}	7.2^{eA}	7.5^{h}	8.5	5.5	6.6
Hi-maize-3.0	6.2	6.4	6.6	7.1^{deAB}	7.2^{eA}	7.4^{i}	7.6	5.1	6.0
Lactitol-1.5	6.2	6.4	6.6	7.1^{dB}	7.3^{dA}	7.5^{g}	8.5	6.6	7.2
Lactitol-3.0	6.2	6.4	6.6	7.0^{efA}	7.1^{fA}	7.3^{jk}	6.2	4.1	5.0
Lactulose-1.5	6.2	6.4	6.6	7.0^{efAB}	7.1^{efA}	7.4^{j}	6.6	4.5	5.2
Lactulose-3.0	6.2	6.4	6.6	7.0^{gAB}	7.1^{efA}	7.3^{jk}	6.3	4.3	5.0
Maltodextrin-1.5	6.2	6.4	6.6	7.4^{bB}	7.6^{abA}	7.8^{b}	15.8	12.9	14.1
Maltodextrin-3.0	6.2	6.4	6.6	7.5^{aA}	7.5^{bA}	7.8^{a}	20.4	12.3	15.5
β-glucan-1.5	6.2	6.4	6.6	7.3^{cB}	7.4^{cA}	7.6^{d}	11.2	9.3	10.0
β-glucan-3.0	6.2	6.4	6.6	7.2^{cdB}	7.4^{cA}	7.6^{de}	9.3	9.1	9.6

*Means shown with small and capital letters represent significant differences (p<0.05) in the same columns (between the yogurts) and rows (between the two probiotic bacteria in each yogurt sample), respectively.
**GPI = Growth Proportion Index.
***A = *L. acidophilus*, B = bifidobacteria, A + B = total probiotics.

stimulatory impact of various prebiotic compounds on viability of probiotic bacteria, some of these are in agreement and some are in disagreement to our findings. NOUBAKHTI *et al.* (2009) reported that addition of 3.0% Hi-maize into milk resulted in the highest viability of *B. Lactis* BB-12 and the lowest viability of *L. acidophilus* LA-5 at the end of fermentation (pH4.2) compared to the control and inulin (1 and 3%). AKALIN *et al.* (2004) announced that adding FOSs considerably increased the viability of *B. animalis* BB-12 and *B. longum* BB-46 (especially *B. longum* BB-46) during the 28 days refrigerated storage at 4°C. Adding of β-glucan from two different cereal sources into milk improved the *B. lactis* BB-12 stability during 28 days cold storage more efficiently than inulin (VASILJEVIC *et al.*, 2007). ARYANA and MCGREW (2007) reported that addition of inulin improved the viability of *L. casei* 01 during five weeks of cold storage compared to control. According to SADEK *et al.* (2004), the viability of *L. acidophilus* LA-5 and *L. rhamnosus* ASCC was enhanced in fermented milk containing 2% inulin or lactulose. However, ÖZER and OZER (2005) reported that the growth of *L. acidophilus* LA-5 as well as yogurt bacteria was not stimulated by inulin in Acidophilus-Bifidus yogurt. In contrast, the viability of *B. bifidum* BB-02 was enhanced to a great extent. Generally, lactulose was more effective growth promoter for both probiotic strains compared to inulin. TABATABAIE and MORTAZAVI (2008) found that in yogurt containing lactulose (1 and 3%) during 5 weeks of cold storage, the survival of *L. rhamnosus* LBA and *B. bifidum* CECT was slightly and considerably improved, respectively. RASTALL and MAITIN (2002) found that the largest increase in bifidobacteria population was seen by addition of xylo-ligosaccharide and lactulose, whilst, the highest growth for lactobacilli was obtained by addition of FOSs. DONKOR *et al.* (2006) reported that in the presence of inulin, *L. acidophilus* L10 and *L. casei* L26 had better viability compared to that of Hi-maize (Table 2). Lower concentrations of inulin were found to be sufficient to stimulated the growth and retain the viability of the probiotic organisms and yogurt bacteria. This is controversial to the results of present study, because inulin did not considerably increase the viability of *L. acidophilus*. ARYANA *et al.* (2007) found that inulins with different chain lengths (short, medium and long) resulted in the significant higher viability of *L. acidophilus* LAC4 than control during 22-day refrigerated storage. Chain length of inulin did not significantly affect the viability of mentioned bacterium. According to Table 2, addition of 1.5% Hi-maize enhanced the viability of probiotic bacteria (especially *L. acidophilus*). BRUNO *et al.* (2002) found that Hi-maize was more efficient in retention of viability of bifidobacterial strains than inulin or raftilose which was in contrast to the results of this study. It seems that the stimulatory impacts of prebiotics on probiotic viability depends on several factors such as probiotic strain type, type and purity of prebiotics as well as their chain length (especially for inulin), and the formulating and process factors such as intensity of milk heat treatment, incubation temperature, final pH of fermentation and refrigerated storage temperature.

As is evident in Table 1, the concentration of acetic acid is greatest in Inulin-1.5 and β-glucan-1.5. The lowest concentrations were observed in Control and Lactulose-1.5 & 3.0. Therefore, the viability of bifidobacteria was directly proportional to the concentration of acetic acids in yogurts; best viabilities were observed in yogurts containing the highest amounts of this acid. This phenomenon was in agreement to the findings of MORTAZAVIAN *et al.* (2010) and SHAFIEE *et al.* (2010).

Sensory attributes of yogurts at the end of fermentation

Table 4 shows paired comparison tests (DUO tests) between the yogurts containing the same prebiotics with different concentrations (1.5 or 3.0%). Table 5 represents comparative sensory analysis among the selected yogurts (Section 2.5) using scoring methodology. According to Table 3, the sourness was more appreciable in the yogurts with 1.5% prebiotics, representing masking effect of these compounds on the parameter. Except inulin and Hi-maize, increasing concentration of prebiotics led to a weaker sensorial gel firmness and scoopability probably due to depletion flocculation of milk proteins during fermentation. Except inulin, increasing the concentration of prebiotics resulted in the less smooth oral texture. Also, except inulin, higher concentration of prebiotics possessed less flavor acceptability and total acceptability. Yogurt with 1.5% inulin exhibited less sweetness and more unpleasant flavor. Yogurts with 3.0% lactitol and lactulose displayed an appreciable and a mild sweet taste, respectively. Maltodextrin-containing yogurts (especially 3.0%) showed a typical dusty and drug-like off flavor with was unacceptable to most of the panelists. According to the total acceptability of the yogurts, inulin-3.0, Hi-maize-1.5, Lactitol-1.5, Lactulose-1.5, β-glucan-1.5, Maltodextrin-1.5 and Control were selected for further sensory comparison (Table 4) using scoring method (Section 2.5). Considering Table 4, Control, β-glucan-1.5, and Hi-maize-1.5 had the best flavor profile and Maltodextrin-1.5 rendered the weakest. The former yogurt along with Lactitol-1.5 showed the best oral texture and mouthfeel, whilst Maltodextrin-1.5 and Inulin-1.5 had the lowest scores. All yogurt samples did not show significant difference from

Table 3 - Viability of probiotic microorganisms (log cfu mL^{-1}) in selected yogurts during a 21-day refrigerated storage period (5°C)*.

Prebiotic concentration (%)	Storage time (day)											
	0			7			14			21		
	A**	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
Control	7.0^{d}	7.0^{d}	7.3^{d}	6.9^{a}	6.9^{bc}	7.2^{c}	6.8^{a}	6.9^{b}	7.2^{a}	6.1^{ab}	6.5^{b}	6.7^{b}
Inulin-1.5	7.1^{bc}	7.6^{a}	7.7^{a}	6.9^{a}	7.1^{b}	7.3^{b}	6.7^{a}	6.9^{b}	7.1^{b}	6.0^{ab}	6.5^{b}	6.6^{b}
Hi-maize-1.5	7.1^{b}	7.2^{c}	7.5^{c}	6.9^{a}	7.1^{b}	7.3^{b}	6.7^{a}	6.9^{b}	7.1^{a}	6.5^{a}	6.6^{b}	6.8^{ab}
β-glucan-1.5	7.3^{a}	7.4^{b}	7.6^{b}	6.8^{ab}	7.4^{a}	7.5^{a}	6.0^{b}	7.1^{a}	7.2^{a}	5.5^{c}	7.0^{a}	7.02^{a}

*Means shown with small letters represent significant differences ($p<0.05$) in the same columns.
**A = *L. acidophilus*, B = bifidobacteria, A+B = total probiotics.

Table 4 - The "paired comparison tests (DUO tests)" between yogurts containing the same prebiotic compounds with different concentrations (1.5 or 3.0%)*.

Treatments	Parameters					
	Gel firmness and scoopability	Smoothness	Sourness	Flavor acceptability	Total acceptability	Notes
Inulin	3.0>1.5	1.5=3.0	1.5>3.0	3>1.5	3.0>1.5	1.5% Possessed less sweetness and more unpleasant flavor
Hi-maize	3.0>1.5	1.5>3.0	1.5>3.0	1.5>3.0	1.5>3.0	3.0% Had higher unpleasant flavor
Lactitol	1.5>3.0	1.5>3.0	1.5>3.0	1.5>3.0	1.5>3.0	3.0% Possessed appreciable sweet taste
Lactulose	1.5>3.0	1.5>3.0	1.5>3.0	1.5>3.0	1.5>3.0	3.0% Exhibited a mild sweetness
β-glucan	1.5>3.0	1.5>3.0	1.5>3.0	1.5>3.0	1.5>3.0	3.0% Was sweeter than 1.5%
Maltodextrin	1.5>3.0	1.5>3.0	1.5>3.0	1.5>3.0	1.5>3.0	3.0% Displayed typical unpleasant dusty and drug-like off flavor

*The signs ">" and "=" represent significant and insignificant differences, respectively.

Table 5 - Comparative sensory analysis among the selected yogurts using "scoring methodology"*.

Treatments	Parameters**				
	Flavor	Oral texture and mouthfeel	Appearance	Non-oral texture	Total score
Control	18^{a}	10.5^{a}	8^{a}	3^{a}	39.5^{a}
Inulin-3.0	11.2^{cd}	7^{c}	8^{a}	1.9^{h}	28.1^{d}
Hi-maize-1.5	17.2^{ab}	10^{ab}	8^{a}	3^{a}	38.2^{ab}
Lactitol-1.5	12^{c}	10.5^{a}	8^{a}	3^{a}	33.5^{c}
Lactulose-1.5	16.2^{b}	9.5^{b}	8^{a}	3^{a}	36.7^{b}
β-glucan-1.5	18^{a}	10.5^{a}	8^{a}	3^{a}	39.5^{a}
Maltodextrin-1.5	6^{e}	7^{c}	7.4^{a}	1.7^{b}	22.1^{e}***

*Means shown with small letters represent significant differences ($p<0.05$) in the same columns.
**Every data is mean of 9 replications (9 panelists).
***The flavor profile of the yogurt with maltodextrin was recognized "unacceptable" by all members of panel group.

appearance point of view. Inulin-3.0 and Maltodextrin-1.5 showed the lowest scores of non-oral texture. Overall, Control, β-glucan-1.5 and Hi-maize-1.5 exhibited the highest total acceptability scores, whilst Maltodextrin-1.5 was unacceptable due to the off flavor. Literatures about the effects of prebiotics on sensory attributes of fermented milk products are rather conflicting. SEYDIN *et al.* (2005) found that yogurts containing inulin had a good flavor and smooth texture. Also, IBRAHIM *et al.* (2004) reported that organoleptic scores for set fermented milk containing inulin and *B. bifidum* were higher than their controls. This result was not in agreement to the results obtained from this study. DELLO STAFFOLO *et al.* (2004) analyzed

consumer acceptability, color, texture, flavor and aroma and reported that the yogurt containing inulin was not significantly different from the control yogurt and the yogurts containing wheat or bamboo fibers. Also, HASSAN *et al.* (1999) stated that organoleptic properties of yogurt containing inulin were comparable with the organoleptic properties of yogurt prepared with commercial stabilizers. However, GUVEN *et al.* (2005) found that organoleptic quality of yogurt decreased with increasing inulin concentration. The latter report is in agreement to the results of our study because the yogurt containing inulin had lower sensory score compared to the control.

Selection of the best yogurts from probiotic viability and sensory profile points of view

From overall aspects, Hi-maize-1.5 and β-glucan-1.5 can be selected as the best prebiotics/fibres for yogurt. Maltodextrin-containing yogurts although good from viability point of view, are discounted due to unacceptable off flavor (Section 3.3). With respect to the good impact of Inulin-1.5 on viable counts of bifidobacteria and total probiotics, this prebiotic composition can be also considered as a good alternative.

CONCLUSIONS

Effects of addition of several commercially available prebiotics in concentrations of 1.5 and 3.0% on biochemical, microbiological and sensory characteristics of ABY-type probiotic yogurt were investigated in this study. Addition of these compounds significantly changed the biochemical characteristics of yogurts and substantially enhanced the viability of probiotics immediately after fermentation and during the refrigerated storage. Many prebiotic-containing yogurts had a lower sensory acceptability compared to the control. In overall (from viability and sensory point of view), Hi-maize-1.5 and β-glucan-1.5 were found the best prebiotics for yogurt.

ACKNOWLEDGMENTS

This study was made possible with the financial support of Pak Dairy Co. (Tehran, Iran). Authors specially appreciate Mr. Taleb, Mr. Rohani and Mr. Dolatkhah-nejad for their sincere helps.

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Paper received May 3, 2010 Accepted June 29, 2010

PAPER

DSC EVALUATION OF OLIVE OIL DURING ACCELERATED OXIDATION

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ABSTRACT

Changes in differential scanning calorimetry cooling thermal properties of an extra virgin olive oil in the presence and absence of its phenolic fraction were evaluated at different times of accelerated storage treatment (up to 4 weeks at 60°C under air) and related to lipid oxidation molecules (measured with k_{232} and k_{270} indices) and total phenol content. Phenols did not appear to directly influence crystallization of extra virgin olive oil as neither cooling profiles nor thermal properties differed significantly between the two oils at the beginning of storage. However, oil samples deprived of phenols showed more significant changes at the longest storage time in comparison with untreated oil. Cooling transitions were all deconvoluted into three peaks. Changes in thermal properties were more evident for the two transition peaks at the highest temperature in both oil samples. Thus, a marked influence of lipid oxidation products on the crystallization pattern of these two peaks may be hypothesized.

- Key words: differential scanning calorimetry, extra virgin olive oil, lipid oxidation, phenols, thermal properties, storage -

INTRODUCTION

Extra virgin olive oil (EVOO) plays an important role in the Mediterranean diet as its consumption has been associated with beneficial effects on health and prevention against several diseases (BENDINI *et al.*, 2007). EVOO has a high resistance to oxidative deterioration that is related not only to its fatty acid composition (high monounsaturated to polyunsaturated fatty acid ratio), but also to the presence of minor compounds with powerful antioxidant activity. Lipid oxidation of EVOO occurs mainly during processing and storage when the oil is in contact with oxygen, and is recognized as the main cause of deterioration of the oil during its shelf-life (FRANKEL, 1985). The influence of several factors on the rate of deterioration as well as the importance of its prevention and its impact on sensory and olfactory attributes of the oil have been recently reviewed (BENDINI *et al.*, 2009). Among the minor components, phenolic compounds, which are responsible for sensorial properties of EVOO such as bitterness, pungency and astringency (CERRETANI *et al.*, 2008), also provide resistance to auto-oxidation (BALDIOLI *et al.*, 1996). At the same time, phenolic compounds appear to be only partially affected by heat treatment by microwave (particularly lignans) (BRENES *et al.*, 2002; CERRETANI *et al.*, 2009).

The evaluation of the oxidation process of EVOO under realistic storage conditions is quite slow, and complete oxidation occurs over a period of 12 to >18 months. On the other hand, high-stress oxidation conditions evaluated by oxidative stability tests performed at high temperature (e.g. Rancimat) hardly mimic realistic storage conditions due to the widely divergent kinetics of lipid oxidation at the high temperatures employed. Thus, accelerated storage conditions at a maximum of 60°C have also been evaluated as they do not alter the oxidation mechanism, and correlate well with experiments performed under normal storage conditions (FRANKEL, 1993).

Differential scanning calorimetry (DSC) is a calorimetric technique widely employed for the characterization of the thermal behaviour of oils and fats as it does not require sample preparation or the use of solvents, resulting in a reduced time analysis and low environmental impact. The use of DSC for assessment of oxidative deterioration of vegetable oils is well known (TAN and CHE MAN, 2002). In addition, thermal parameters obtained by cooling and heating thermograms have been found to be related to the chemical composition of vegetable oils (TAN and CHE MAN, 2000), and relationships have also been documented for both major and minor components of EVOO (JIMÉNEZ MÁRQUEZ and BELTRÁN MAZA, 2003 and 2007; CHIAVARO *et al.*, 2007, 2008a, and 2010).

Several papers have been recently published on the application of DSC for assessment of quality factors of EVOO, such as the discrimination of commercial categories of olive oil (CHIAVARO *et al.*, 2008b), detection of adulterations of EVOO with less expensive vegetable oils (CHIAVARO *et al.*, 2008c and 2009a) and discrimination of oil samples according to cultivar-environment effects (KOTTI *et al.*, 2009). However, few reports have examined the effects of storage and/or heating treatment on the DSC thermal properties of EVOO in relation to chemical oxidative changes. Auto- and thermo-oxidation of EVOO have been evaluated by DSC cooling thermograms (VITTADINI *et al.*, 2003), and the amount of oxidized volatile compounds was found to be correlated with such thermal parameters as crystallization enthalpy and on-set transition temperature. More recently, changes in DSC thermal parameters and transition profiles upon cooling and heating were evaluated on different commercial categories of olive oil after microwave heating in relation to chemical composition and stability indices (CHIAVARO *et al.*, 2009b).

The aim of the present investigation was to evaluate DSC thermal parameters upon cooling of an EVOO, in the presence and absence of phenolic fraction during an accelerated storage treatment of up to 4 weeks at 60°C, and to relate these changes to the state of lipid oxidation (UV absorbance at 232 and 270 nm) and phenolic content. The potential role of phenols in the crystallization of EVOO was also evaluated on a preliminary basis.

MATERIALS AND METHODS

Samples and storage

An Italian sample of extra virgin olive oil from Tuscany (blend of Leccino, Moraiolo and Frantoio cultivars) was employed in this study. The sample was divided into two aliquots: extra virgin olive oil with phenols ($EVOO_p$) and extra virgin olive oil without phenols ($EVOO_{p0}$). Phenolic compounds were removed from $EVOO_p$ according to the procedure described by BONOLI-CARBOGNIN *et al.* (2008). Briefly, 35 g of $EVOO_p$ were washed with several aliquots of 0.5M NaOH (4x15 mL). To eliminate the aqueous phase, the mixture was centrifuged (1,000 x g, 5 min) after each washing. Combined olive oil fractions were then washed with 0.5M HCl (2x10 mL) and saturated NaCl solution (5x10 mL), centrifuged at 1,000 x g for 5 min, dried with anhydrous sodium sulphate, and finally filtered under vacuum. Dried $EVOO_{p0}$ was then obtained.

Both samples ($EVOO_p$ and $EVOO_{p0}$) were divided in 8 aliquots each (250 mL, 228.8 g) and kept in the dark at 60°C for 4 weeks. Each aliquot was stored in an individual open glass bottle

of 300 mL (i.d. = 6 cm; surface area exposed to air 28.3 cm^2). Bottles of $EVOO_p$ and $EVOO_{p0}$ were removed each week from the oven and analyzed.

Chemical analysis

Free acidity (free fatty acid content of the oil expressed as the percentage of oleic acid), peroxide value (amount of hydroperoxides expressed as mequiv of O_2 kg^{-1}oil) and UV absorbance at 232 and 270 nm (k_{232} and k_{270} provide a measurement of the state of oxidation) were performed according to the official methods of the European Commission (EUROPEAN COMMUNITY, 2003).

Phenolic compounds were extracted from oil samples by a liquid-liquid extraction procedure using a modified version of the method by PIRISI *et al.* (2000). Briefly, 4 g of oil (± 0.001 g) were dissolved in 4 mL of *n*-hexane, and the solution was extracted twice with four 2 ml portions of methanol:water (60:40, v/v). The combined extracts of the hydrophilic layer were concentrated and dried by evaporative centrifuge (Mivac Duo of Genevac Inc., Valley Cottage, NY, USA) at a temperature of 40°C. Finally, the residue was redissolved in 0.5 ml methanol:water (50:50, v/v) and filtered through a 0.20 µm nylon filter (Whatman, Clifton, NJ, USA). HPLC analysis was carried out using a HP 1100 system (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump delivery system, degasser, autosampler, diode array UV-vis detector (DAD) and mass spectrometer detector (MSD) using a reverse phase column C_{18} Luna 5 µm, 25 cm x 3.00 mm i.d. (Phenomenex, Torrance, CA, USA) according to ROTONDI *et al.* (2004). Each phenolic compound was expressed as mg 3,4-dihydroxyphenylacetic acid (3,4-DHPAA) kg^{-1} oil (calibration curve with r^2=0.9739).

Three replicates were analyzed per sample.

DSC analysis

Samples of oil (8-10 mg) were weighed in aluminium pans, covers were sealed into place and analyzed with a DSC Q100 (TA Instruments, New Castle, DE, USA). Indium (melting temperature 156.6°C, H_f = 28.45 J/g) and *n*-dodecane (melting temperature -9.65°C, H_f = 216.73 J/g) were used to calibrate the instrument and an empty pan was used as reference. Oil samples were equilibrated at 30°C for 3 min and then cooled at -80°C at a rate of 2°C/min. Dry nitrogen was purged in the DSC cell at a flow rate of 50 cm^3/min. Cooling thermograms were analyzed with Universal Analysis Software (Version 3.9A, TA Instruments) to obtain enthalpy (ΔH, J/g), onset temperature (T_{on}, °C) and offset temperature (T_{off}, °C) of the transitions. The range of the transitions was calculated as the temperature difference between T_{on} and T_{off}. Overlapping transitions of cooling thermograms were deconvoluted into individual constituent peaks using PeakFitTM software (Jandel Scientific, San Rafael, CA, USA). The following parameters were considered for each deconvoluted peak: T_{on}, T_{off} and peak temperatures (T_p), % peak area (percentage area of the total peak area) and range of the transitions. Three replicates were analyzed per sample.

Statistical analysis

Data were analysed using SPSS (Version 17.0, SPSS Inc., Chicago, IL, USA) statistical software. SPSS was used to perform one-way-analysis of variance (ANOVA) and least significant difference (LSD) test at a 95% confidence level ($p \leq 0.05$) to identify differences between storage times. A student *t* test ($p < 0.05$) was also used to identify differences between samples for the same parameter at each storage time.

RESULTS AND DISCUSSION

Chemical analysis

Free acidity and peroxide values were determined on both oil samples before storage to verify their conformity with legal limits established by the European Community (EUROPEAN COMMUNITY, 2003). The free acidity percentages of $EVOO_p$ and $EVOO_{p0}$ were largely under the limit established by the EC Regulation for EVOO (EUROPEAN COMMUNITY, 2003), and were 0.15 and 0.11%, respectively. Similarly, the peroxide values were below the legal limit (EUROPEAN COMMUNITY, 2003), ranging from 15.9 meq O_2 kg^{-1} oil to 13.6 meq O_2 kg^{-1} oil for $EVOO_p$ and $EVOO_{p0}$, respectively.

Phenol stripping was very efficient: after the removal process (BONOLI *et al.*, 2009) the total amount of phenolic compounds measured at storage time zero (t_0) decreased from 154.95 mg 3,4-DHPAA /kg^{-1} oil ($EVOO_p$) to a value lower than the limit of detection ($EVOO_{p0}$).

Oxidative status of $EVOO_p$ and $EVOO_{p0}$ was measured with conjugated diene (k_{232}) and triene (k_{270}) determination, as previously reported during an accelerated storage test carried out under the same experimental conditions (LERMA-GARCÍA *et al.*, 2009). In particular, k_{232} was previously reported to show a higher predictive value in the evaluation of the oxidative status of EVOO under accelerated storage test conditions (HRNCIRIK and FRITSCHE, 2005; MANCEBO-CAMPOS *et al.*, 2008). Before storage, both oil samples showed k_{232} and k_{270} values that were below the limits established by the EC Regulation for EVOO (EUROPEAN COMMUNITY, 2003), which corresponded to 2.50 and 0.22, respectively. $EVOO_p$ and $EVOO_{p0}$ also showed significant differences for k_{232} and k_{270} values at time 0, as previously observed (LERMA-GARCIA *et al.*,

2009), most likely because of the effects of phenol stripping on oxidation, as molecules with different polarity can be also extracted together with phenols.

Changes in oxidative indexes (k_{232} and k_{270}) and total phenol content were taken into account to evaluate the effects of storage on lipid oxidation and related to the DSC thermal properties. In general, k_{232} (conjugate dienes) and k_{270} (conjugate trienes) both increased with storage. After the first week, $EVOO_p$ and $EVOO_{p0}$ reached slightly higher k_{232} values than the legal limit (2.50), as shown in Table 1. After 2 weeks of storage, significantly higher values of k_{232} were also observed for $EVOO_{p0}$ in comparison with $EVOO_p$. This is probably due to very low content of phenolics in $EVOO_{p0}$ that was not able to inhibit oxidation, since the radical generation rate was too high for scavenging by antioxidants, as previously hypothesized under similar storage conditions (BENDINI *et al.*, 2006). However, lipid oxidation also occurred in $EVOO_p$. This is probably due to a partial oxidation of phenols, which was promoted by storage conditions applied (60°C under air), as previously reported (HRNCIRIK and FRITSCHE, 2005; LERMA-GARCIA *et al.*, 2009). In addition, k_{232} values appeared to reach a plateau after 3 weeks of storage for both samples. This is in accordance with a previous study where a stationary phase was observed for this index after a few weeks of storage at 60°C in EVOO samples with different phenol contents (MANCEBO-CAMPOS *et al.*, 2008).

The storage conditions used did not appear to prevent the increase of k_{270}, even in samples with phenol (Table 1). In addition, the $EVOO_p$ sample exceeded the legal value for k_{270} (0.22) after 3 weeks of storage, while $EVOO_{p0}$ samples exceeded this limit after 4 weeks (LERMA-GARCIA *et al.*, 2009), reaching a final value greater than $EVOO_p$. It can be hypothesized that hydroperoxide decomposition to hydroxy, keto and epoxy of fatty acids (FRANKEL, 1985) may be more marked in $EVOO_{p0}$ at the longest storage time due to the very low content of phenols that are well known to act as natural antioxidants (BALDIOLI *et al.*, 1996; CARRASCO-PANCORBO *et al.*, 2005).

Changes in phenolic content during storage for $EVOO_p$ are shown in Figure 1. At time 0, phenolic compounds were abundant in the $EVOO_p$ sample (154.95 mg 3,4-DHPAA/kg of oil). The total phenol content gradually and significantly decreased with increasing storage time (beginning from the first week of storage), reaching a value of 81.84 mg/kg^{-1} oil (-47.2%) after 4 weeks. Phenolic compounds have been previously found to decrease under the same storage conditions used in the present study (HRNCIRIK and FRITSCHE, 2005), and are transformed into oxidized molecules that have been tentatively identified (LERMA-GARCÍA *et al.*, 2009).

DSC analysis of cooling transition

Representative DSC cooling thermograms obtained for $EVOO_p$ and $EVOO_{p0}$ at 0 week and during accelerated storage are shown in Figg. 2A and 2B, respectively. Both samples showed curves similar to those previously reported by CHIAVARO *et al.*, (2007, 2008b and 2008c, 2009a and 2009b, 2010) with two well-defined exothermic events, namely a minor peak at the highest

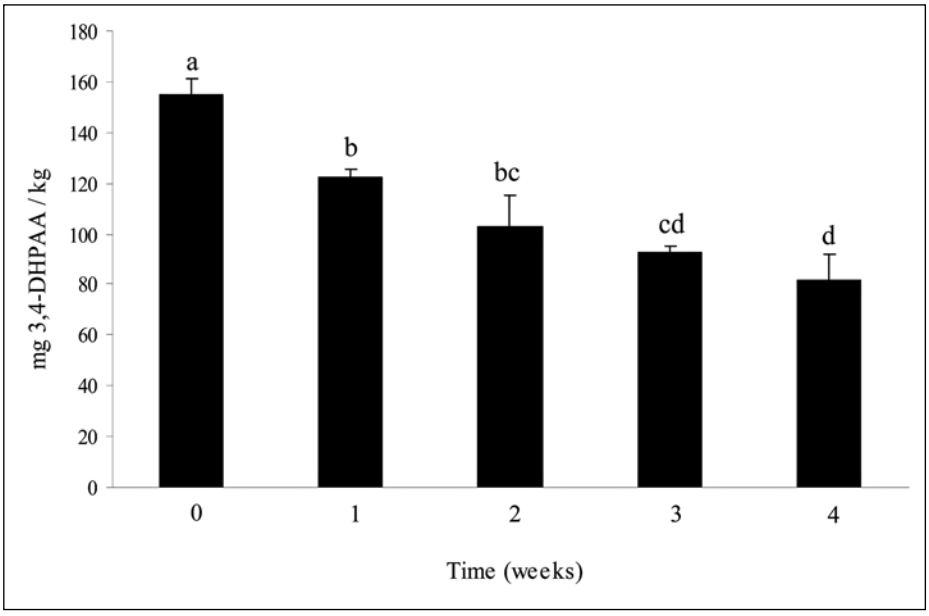


Fig. 1 - Changes in phenolic compounds in $EVOO_p$ at different storage times. Error bars represent +/- 1 standard deviation, (n = 3). Bars with the same letters are not significantly different ($p \leq 0.05$).

Table 1 - k_{232} and k_{270} values of $EVOO_p$ and $EVOO_{p0}$ samples at different storage times.

Storage time (weeks)	k_{232}		k_{270}	
	$EVOO_p$	$EVOO_{p0}$	$EVOO_p$	$EVOO_{p0}$
0	1.9 c	2.4 *c	0.125 *e	0.108 d
1	3.3 ab	3.2 bc	0.149 *d	0.108 d
2	3.9 b	4.5 *ab	0.191 *c	0.146 c
3	4.9 a	5.6 *a	0.250 *b	0.204 b
4	4.9 a	5.6 a	0.269 a	0.372 *a

a, b, c, d: The same letters within each column are not significantly different (n = 3, $p < 0.05$).
Means with an asterisk at the same storage time are significantly different (n = 3, $p < 0.05$).
RSD ≤ 3%.

and a major peak at lowest temperature. The major peak has been previously associated with crystallization of highly unsaturated triacylglycerols (TAG), in particular triolein (OOO), while the minor peak has been attributed to crystallization of more saturated TAG fractions, probably influenced by minor components (CHIAVARO *et al.*, 2007, 2010).

Oil samples with phenols ($EVOO_p$) did not exhibit changes upon storage except for a slight enlargement of the transition range, probably related to a shift in the onset temperature of crystallization towards a higher temperature at longer storage times. In contrast, the profile of $EVOO_{p0}$ showed more marked changes: in fact, the major peak height began to decrease after 3 weeks at 60°C, whereas the onset and offset temperatures of transition shifted towards higher and lower temperatures, respectively, which led to an increase of the crystallization range especially at the longest storage times. All these changes were most likely related to an increase in lipid oxidation products with storage, albeit less consistent for $EVOO_p$ (Table 1) where phenols have partially prevented oxidation. Molecules formed by hydrolysis and/or oxidation of lipids have been previously reported to interfere with TAG crystallization, hindering both contact and alignment of molecules under DSC experimental conditions in EVOO (VITTADINI *et al.*, 2003; CHIAVARO *et al.*, 2009b) and other vegetable oils (GLORIA and AGUILERA, 1998). A decrease of the height and a shift towards a lower temperature of the major exothermic transition was also observed by VITTADINI *et al.* (2003) for EVOO after 28 days of storage at 50°C when a 1.5% decrease of headspace oxygen content of the sample was measured. This different behaviour can be reasonably attributed to different experimental conditions of storage and/or initial lipid oxidation status of the oil.

Cooling thermal properties are reported in Table 2 for $EVOO_p$ and $EVOO_{p0}$ at different times of storage. At time 0, both oil samples did not show any significant differences in any thermal properties. Accordingly, it would appear that a different content of total phenol compounds did not have a direct influence on the DSC cooling thermal properties of EVOO. JIMÉNEZ MÁRQUEZ *et al.* (2007) found that the cooling thermal properties of an oil sample from the Arbequina variety with similar total phenol content than $EVOO_p$ did not significantly differ from those of the same oil sample deprived of phenolic compounds. However, phenolic compounds are partially dispersed in the water contained in olive oils (LERCKER *et al.*, 1994). Thus, their direct influence on crystallization transition cannot be excluded and requires further investigation.

Enthalpy did not exhibit significant changes for either sample (Table 2) during storage, except for $EVOO_{p0}$ at the longest storage time, which exhibited a significant decrease of the energy required for crystallization when the formation of conjugated dienes and trienes (increase of k_{232} and k_{270}, Table 1) from hydroperoxides became more pronounced. VITTADINI *et al.* (2003) also measured enthalpy among thermal properties and found a small decrease in this parameter for EVOO starting from about 14 days of storage at 50°C, when oxidation of the sample measured by depletion of headspace oxygen content in the dark slightly decreased.

Table 2 - DSC data from cooling thermograms of $EVOO_p$ and $EVOO_{p0}$ samples at different storage times.

Storage time (weeks)	ΔH (J/g)		T_{on} (°C)		T_{off} (°C)		Range[a] (°C)	
	$EVOO_p$	$EVOO_{p0}$	$EVOO_p$	$EVOO_{p0}$	$EVOO_p$	$EVOO_{p0}$	$EVOO_p$	$EVOO_{p0}$
0	56.2 a	56.4 a	-12.9 b	-13.0 b	-46.0 a	-44.8 a	33.3 b	31.9 c
1	55.5 a	55.6 a	-12.4 ab	-13.0 b	-45.9 a	-45.2 ab	33.5 b	32.2 bc
2	56.2 a	55.1 a	-11.8 a	-12.4 a	-45.9 a	-45.6 ab	34.1 ab	32.9 bc
3	55.4 a	54.8 a	-11.9 a	-12.5 a	-46.2 a	-46.2 b	34.5 a	33.4 b
4	56.4* a	53.5 b	-11.9 a	-12.3 a	-46.6 a	-51.1 *c	34.7 a	38.9 *a

a, b, c: The same letters within each column are not significantly different (n = 3, $p < 0.05$).
Means with an asterisk at the same storage time are significantly different (n = 3, $p < 0.05$). RSD ≤ 3%.
[a]Temperature difference between T_{on} and T_{off}.

Table 3 - Deconvolution parameters of cooling thermograms of $EVOO_p$ and $EVOO_{p0}$ samples at different storage times.

Storage time (weeks)	Area (%)		T_p (°C)		T_{on} (°C)		T_{off} (°C)		Range[a] (°C)	
	$EVOO_p$	$EVOO_{p0}$	$EVOO_p$	$EVOO_{p0}$	$EVOO_p$	$EVOO_{p0}$	$EVOO_p$	$EVOO_{p0}$	$EVOO_p$	$EVOO_{p0}$
Peak 1										
0	78.7 a	78.6 a	-39.2 a	-39.0 a	-33.0 a	-32.9 a	-45.1 a	-44.4 a	12.0 a	11.8 b
1	79.8 a	80.6 a	-39.3 a	-39.2 a	-33.5 a	-32.9 a	-45.4 a	-45.5 b	11.9 a	12.4 b
2	80.3 a	78.0 a	-39.1 a	-39.3 ab	-33.1 a	-33.0 ab	-44.6 a	-44.3 ab	11.7 a	11.8 b
3	80.4 a	79.8 a	-39.5 ab	-39.7 b	-33.3 a	-33.3 ab	-44.8 a	-45.2 ab	11.9 a	12.3 b
4	80.5 *a	73.4 b	-39.9 b	-42.3 *c	-33.1 a	-34.7 *b	-44.9 a	-50.1 *c	11.9 a	15.4 *a
Peak 2										
0	8.4 a	8.7 a	-33.0 a	-32.7 a	-27.0 a	-26.8 a	-36.5 a	-36.8 a	9.6 b	10.0 b
1	8.6 a	8.1 a	-33.4 a	-32.8 a	-27.6 a	-26.6 a	-37.0 a	-36.9 a	9.4 b	10.1 b
2	8.2 a	8.3 a	-33.1 a	-33.2 a	-27.4 a	-27.1 a	-36.5 a	-37.1 a	9.5 b	10.1 b
3	9.0 a	8.4 a	-33.4 a	-33.5 a	-27.0 a	-27.4 a	-37.4 a	-36.8 a	10.4 a	10.3 b
4	8.6 a	8.9 a	-33.3 a	-35.1 *b	-26.4 a	-27.9 *a	-36.9 a	-39.4 *b	10.5 a	11.5 *a
Peak 3										
0	11.8 a	11.7 a	-15.5 c	-15.3 b	-12.7 a	-11.3 b	-22.4 a	-22.7 a	10.2 b	11.3 b
1	11.6 a	11.3 a	-14.3 b	-15.3 b	-11.6 ab	-11.4 b	-21.9 a	-22.6 a	10.3 b	11.2 b
2	10.6 a	11.8 a	-13.2 a	-15.5 *b	-11.3 b	-11.7 b	-21.8 a	-22.9 a	10.5 b	11.2 b
3	10.6 a	11.8 a	-13.3 a	-14.4 *a	-11.2 b	-11.7 b	-22.5 a	-22.9 a	11.3 a	11.2 b
4	10.9 a	11.8 a	-13.4 a	-14.5 *a	-11.3 b	-10.3* a	-22.5 a	-24.2 *b	11.2 a	14.0 *a

a, b, c, d: The same letters within each column are not significantly different (n = 3, $p < 0.05$).
Means with an asterisk at the same storage time are significantly different (n = 3, $p < 0.05$). RSD ≤ 3%.
[a] Temperature difference between T_{on} and T_{off}.

The cooling thermal properties of $EVOO_p$ exhibited slight changes during storage. In particular, T_{on} shifted significantly towards a higher temperature after 2 weeks at 60°C, which also increased the range of transition. In contrast, $EVOO_{p0}$ exhibited more marked changes upon cooling, especially at the longest storage time, when oxidative changes were more evident (Table 1). In particular, T_{on} and T_{off} shifted towards higher and lower temperatures, respectively, and the range of transition significantly increased, becoming greater than in $EVOO_p$. These changes in thermal properties were related to the inhibition of TAG crystallization by molecules formed from lipid oxidation, and have already been observed in EVOO after thermal oxidation by microwave treatment (CHIAVARO *et al.*, 2009b).

Deconvolution analysis of cooling transition

Deconvolution of overlapping transitions has been previously applied to DSC cooling thermograms obtained for EVOO to better describe the complex nature of the crystallization process (CHIAVARO *et al.*, 2007) and to evaluate the ability of DSC to discriminate commercial categories of olive oil and/or identify oleic sunflower oil as an adulterant (CHIAVARO *et al.*, 2008b and 2009a). More recently, statistical correlations among the thermal properties of the deconvoluted peaks obtained by cooling thermograms and major and minor components such as diacylglycerols have been established (CHIAVARO *et al.*, 2010). In all these previous studies, cooling transitions of EVOO were deconvoluted into three peaks that were numbered starting from the lowest to the highest temperature, identified as peaks 1, 2, and 3. The predominant peak (peak 1) was an asymmetric double Gaussian function, with a rather symmetrical curve and a narrow profile, whilst peaks 2 and 3 were asymmetric double sigmoid functions and exhibited a more complex, asymmetrical shape.

In this study, deconvolution was applied for the first time to relate changes of thermal properties and cooling profiles to lipid oxidation. All cooling thermograms fit best with three peaks ($R^2 \geq 0.98$), as previously reported for EVOO (CHIAVARO *et al.*, 2007, 2008b, 2009a, and 2010), and the thermal properties are reported for $EVOO_p$ and $EVOO_{p0}$ at different storage times (Table 3). The thermal properties of peak 1 did not significantly change after up to 3 weeks of storage for either sample. In addition, no significant differences were found between $EVOO_p$ and $EVOO_{p0}$ up to the same storage period. However, at the end of storage, $EVOO_{p0}$ showed a significantly lower area% in comparison with $EVOO_p$, as well as a marked shift of peak 1 towards lower temperature, which also exhibited a larger range of transition. The thermal properties of peak 1, which accounted for the large

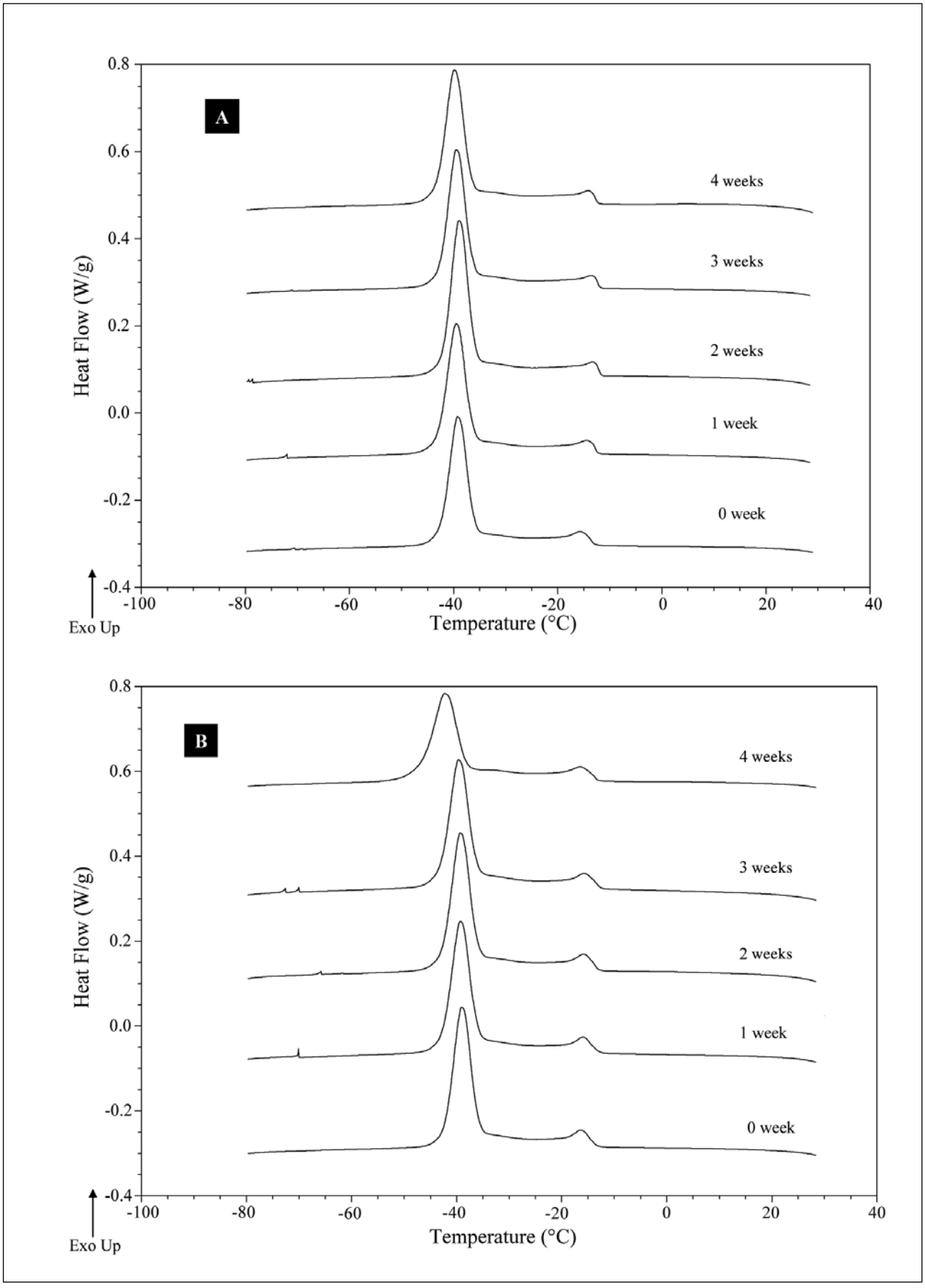


Fig. 2 - Representative DSC cooling thermograms of $EVOO_p$ (A) and $EVOO_{p0}$ (B) at different storage times.

fraction of crystallizing lipid, have been previously found to positively correlate with such major components of EVOO as OOO, oleic acid and monounsaturated fatty acid (MUFA), and negatively with linoleic acid and polyunsaturated fatty acid (PUFA) (CHIAVARO *et al.*, 2010). In addition, the thermal properties of peak 1 correlated well with the oxidative stability index (OSI) (CHIAVARO *et al.*, 2010). Thus, changes in thermal properties for peak 1 appeared to be strictly related to a larger extent of oxidation reached by the more unsaturated lipid fraction for $EVOO_{p0}$ at the end of storage (k_{232} and k_{270} values, Table 1), likely due to the absence of a protective effect by phenols. However, the thermal properties of peak 1 for $EVOO_{p}$ did not appear to be directly influenced by the decrease of phenolic compounds observed (Figure 1), and remained unchanged during storage.

The thermal properties of peak 2 did not show any significant changes during storage for $EVOO_{p}$. However, this deconvoluted peak clearly shifted towards a lower temperature for $EVOO_{p0}$ at the longest storage time compared with $EVOO_{p}$ (Table 3). A shift towards higher temperature was observed for peak 3 during storage for both samples, although more markedly for $EVOO_{p0}$ (Table 3). It has been previously hypothesized that the more complex crystallization pattern exhibited by these peaks in comparison with peak 1 may be related to the presence of minor chemical components like diacylglycerols and lipid oxidation products (CHIAVARO *et al.*, 2007). This hypothesis was recently confirmed by the high statistical correlation values found among these minor components and the thermal properties of the two peaks for EVOO (CHIAVARO *et al.*, 2010). The degree of lipid unsaturation (total fatty acid composition grouped as saturated, monounsaturated and polyunsaturated percentages) was found to clearly influence the thermal properties of these two peaks (CHIAVARO *et al.*, 2010).

Thus, it can be hypothesized that lipid oxidation products, derived from the reaction between hydroperoxides and unsaturated fatty acids (k_{232} and k_{270} values, Table 1), may have interfered with crystallization of the deconvoluted peaks 2 and 3 more markedly than for peak 1; in fact, some statistical differences were also found for $EVOO_{p}$ (where lipid oxidation was less pronounced) during longer storage times, especially for peak 3. On the other hand, the presence of phenols did not appear to directly influence the thermal properties of peaks 2 and 3, which were not significantly different between $EVOO_{p}$ and $EVOO_{p0}$ at time 0 of storage, as previously found for peak 1. Therefore, the thermal properties of peaks 2 and 3 appeared to be only indirectly influenced by phenols for $EVOO_{p}$, as their decrease probably led to a simultaneous increase in lipid oxidation products.

CONCLUSIONS

The results of this study confirm that cooling thermal properties and transition profiles of EVOO are influenced by lipid oxidation products formed by a relatively slow oxidation process that occurs during storage. Deconvolution analysis provided additional information about the relationship between lipid oxidation and cooling thermal properties. In particular, oxidized molecules appeared to influence the thermal properties of the two transitions, peaking at the highest temperatures independently of the extent of lipid oxidation related to the presence of antioxidant molecules such as phenols.

However, preliminary findings showed that the cooling thermal properties of EVOO did not seem to be influenced by phenols, although these results must be confirmed by the analysis of several oil samples with different phenolic content. Additionally, more information should be obtained to clarify the influence of both phenols and lipid oxidation products on EVOO crystallization by kinetic evaluation of this transition at different degrees of lipid oxidation and/or phenol depletion.

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Paper received May 14, 2010 Accepted October 5, 2010

PAPER

CHANGES IN THE FATTY ACIDS OF NEUTRAL AND POLAR LIPIDS OF *SILURUS GLANIS* AND *BARBUS CAPITO CAPITO* DURING AN ANNUAL CYCLE

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ABSTRACT

Seasonal changes in the fatty acid composition of neutral (NL) and polar lipid (PL) fractions of immature Wels (*Silurus glanis*) and Bulatmai barbel (*Barbus capito capito*) were investigated. The most abundant saturated, monounsaturated, and *n*-3 and *n*-6 polyunsaturated fatty acids (PUFA) for both fractions were palmitic, oleic, docosahexaenoic (DHA), and linoleic acid, respectively. The maximum *n*-3/*n*-6 PUFA ratio and eicosapentaenoic acid (EPA)+DHA content of the NL and PL fractions were found in the hot and cold seasons, respectively. The high *n*-3/*n*-6 ratio and the high EPA+DHA content of the NL fraction might be influenced by the composition of ingested food and that high amount in the PL fraction can be explained by the fact that fish acclimatize to the cold. Finally, these species are recommended for human consumption throughout the year, especially in winter due to the high *n*-3/*n*-6 ratio and the EPA+DHA content of the PL fraction during this season.

- Key words: seasonal changes, fatty acid, neutral lipid, polar lipid, *Silurus glanis*, *Barbus capito capito*, *n*-3/*n*-6 PUFA, EPA+DHA -

INTRODUCTION

Fish oil contains a high percentage of *n*-3 polyunsaturated fatty acids (*n*-3 PUFA), including eicosapentaenoic acid (EPA; 20:5 *n*-3) and docosahexaenoic acid (DHA, 22:6 *n*-3) (HENDERSON and TOCHER, 1987; SHIRAI *et al.*, 2002), that are useful for preventing and treating cardiovascular disease, inflammation, aggression, depression, hypertension, auto-immune disorders and cancer (PIKE *et al.*, 1999). Because EPA and DHA are effectively synthesised by aquatic organisms, humans can obtain these essential components by consuming marine and freshwater products (SUSHCHIK *et al.*, 2007). Fish production in Turkey in 2007 was 772,000 metric tonnes, with a per capita consumption of 8.6 kg per year (ANONYMOUS, 2009), meaning that Turkish people consume about 165 g of fish per week. The Fish Intercommittee Sub-group of the UK Scientific Advisory Committee on Nutrition (UK SACN) suggested that at least two portions of fish (one portion = about 145 g), of which one should be oily, should be eaten weekly to decrease cardiovascular disease risk (UK SACN, 2004). Therefore, the amount of fish consumed by Turkish people does not fulfil the daily requirements of *n*-3 PUFA.

Wels (*Silurus glanis* Linnaeus) is the largest freshwater fish species of Europe and is naturally distributed in large rivers and lakes from central and eastern Europe to the southern part of central Asia. This fish is also present in Turkey's lakes and rivers (GELDİAY and BALIK, 1996). Furthermore, *Silurus glanis* is one of the most popular freshwater fish for human consumption throughout the word and is a good source of PUFA (HWANG *et al.*, 2004). Although the primary distribution areas of Bulatmai barbel (*Barbus capito capito* Güldenstädt) are the Caspian Sea and the Aral Lake basins, this species is also found in the Çoruh and Aras basins in the East Anatolian Region of Turkey (GELDİAY and BALIK, 1996). *Barbus capito capito* is considered to be a potential species for fish farm owing to its tasty flesh and rapid growth rate (ARAS *et al.*, 2009a; YANIK *et al.*, 2009). In Turkey, both *S. glanis* and *B. capito capito* are endangered because of illegal catching methods, human pressure, degradation of spawning habitats and pollution (BAYIR *et al.*, 2011).

The fatty acid composition of fish lipids is extremely variable, even within a species, and depends upon different abiotic and biotic factors such as the season, the type and amount of feed available, the water temperature, the pH, the salinity and the reproduction cycle (SHIRAI *et al.*, 2002; HALİLOĞLU *et al.*, 2005; ARAS *et al.*, 2009a-b). Although seasonal changes of the fatty acid contents of freshwater fish species have been documented in the literature (RASOARAHONA *et al.*, 2005; BAYIR *et al.*, 2010), changes in the fatty acid compositions of *S. glanis* and *B. capito capito* throughout their yearly cycles have never been measured. Therefore, the goal of this study was to determine the fatty acid compositions of neutral and polar lipid fractions of the edible flesh of *Silurus glanis* and *Barbus capito capito* from the East Anatolian Region of Turkey during different seasons.

MATERIAL AND METHODS

Collection of samples

Immature Wels and Bulatmai barbel were caught quarterly between autumn 2006 and summer 2007 from the Aras River, Karakurt, Kars, Turkey (altitude: 1,479 m; latitude: 040°09'36"N; longitude: 042°35'32.4"E). The mean weights of Wels and Bulatmai barbel were 807.0±41.2 g and 363.8±29.2 g in the autumn, 738.8±38.3 g and 373.6±50.8 g in the winter, 778.6±54.5 g and 309.0±40.3 g in the spring and 755.2±47.4 g and 334.4±39.4 g in the summer, respectively. The fish were caught using monofilament gill nets. Five fish of each species in each season were killed with a sharp blow to the head. Muscles excised from the point between the Linea lateral and the dorsal fin, were frozen immediately in liquid nitrogen. Samples were transferred to the laboratory and stored at -84°C until analysis.

Lipid extraction and quantification

The method described by FOLCH *et al.* (1957) was used for lipid extraction. According to this method, samples (*c.* 1 g) were homogenised in chloroform/methanol (2:1 v/v) containing 0.01% (w/v) butylated hydroxytoluene [Sigma, ≥ 99.0% (GC), product number: B1378] as an antioxidant (20 vol. (w/v)) for 1 min. The organic solvent was evaporated under a stream of nitrogen, and the amount of lipid was determined gravimetrically.

Lipid fraction separation

Crude muscle lipids were separated into the polar (phospholipids) and neutral lipid fractions using Sep Pak silica cartridges (Waters, Milford, MA, USA). The residual lipid (*c.* 0.15 g) was applied to a silica filter, and the neutral lipids were eluted with 30 mL chloroform [Sigma, ≥ 99-99.4% (GC), product number: 24216]. After the elution of the neutral lipids, the polar fractions were eluted with 30 mL methanol (Sigma, ≥ 99.9%, product number: 34885). Solvent from both fractions was then evaporated under nitrogen, and the amounts of neutral and polar lipids were determined gravimetrically (CZESNY and DABROWSKI, 1998).

Fatty acid analysis

Fatty acid methyl esters (FAMEs) were prepared from both fractions of lipids according to the method of METCALFE and SCHMITZ (1961). The crude lipid extract was saponified with sodium hydroxide in methanol, and FAMEs were prepared by transmethylation with boron trifluoride in methanol. FAMEs were obtained using an HP (Hewlet Packard) Agilent 6890 N model gas chromatography (GC) equipped with a flame ionisation detector and fitted with a DB 23 capillary column (60 m, 0.25 mm i.d. and 0.25 μm). The ejector and detector temperature program was 190°C for 35 min followed by an increase of 30°C per min up to 220°C; this temperature was maintained for 5 min. The carrier gas was hydrogen (2 mL min^{-1}) and split ratio was 30:1. The individual fatty acids (FAs) were identified by comparing their retention times to those of a standard mix of FAs (Supelco 37 component FAME mix, Cat No: 47885-U), and the FAs were quantified by comparing their peaks to those of the standards (DAVID *et al.*, 2003).

Data analysis

The statistical analyses were performed with SPSS version 10.0 for Windows (SPSS, 1996). Data are presented as the mean ± the standard deviation (SD) of the mean. Data were analysed by one-way analysis of variance (ANOVA). The significant means were compared by Duncan's multiple range tests at $\alpha = 0.05$ level (n=5).

RESULTS AND DISCUSSION

Total lipid, neutral lipid and polar lipid levels

The amounts of total, neutral and polar lipids of wet muscle of immature Wels and Bulatmai barbel for different seasons are presented in Table 1.

We found that the lipid contents (% wet weight of muscle tissues) of the two species were greatest in the winter (Table 1). Total lipids (% w/w) from the muscles of *S. glanis* and *B. capito capito* during the winter were 2.34 and 4.57%, respectively. Additionally, the highest polar lipid amounts were found in the winter and were 1.77 and 1.26%, respectively. However, the highest amounts of neutral lipid were obtained in the summer for *S. glanis* (0.84%) and in the winter for *B. capito capito* (3.31%). It has been reported that the amount of lipids in *Silurus asotus* during the winter was slightly higher than that during the summer, although the difference was not statistically significant (HWANG *et al.*, 2004). Lipids play a major role as regulators of body density when organisms adapt to specific habitat conditions (KOZLOVA and KHOTIMCHENKO, 2000). Phospholipids are a major component of biomembranes. As for other poikilothermic organisms, freshwater fish undergo alterations of the composition and amount of their biomembrane lipids in response to changes in the environmental temperature (HENDERSON and TOCHER, 1987; BAYIR *et al.*, 2010). Thus, the reason behind the high total lipid and polar lipid amounts of *S. glanis* and *B. capito capito* during the cold seasons is likely the response to the low water temperature (winter 4.1°C and autumn 6.2°C).

Fatty acid composition of neutral and polar lipid fractions

Tables 2-5 show the overall average fatty acid compositions of the neutral and polar lipid fractions of wet muscle tissues of *S. glanis* and *B. capito capito* together with the range of individual fatty acids observed.

Twenty-three fatty acids were identified in the neutral and polar lipid fractions of both species. The most abundant saturated (SFA), monounsaturated (MUFA) and *n*-3 and *n*-6 polyunsaturated (*n*-3 and *n*-6 PUFA) fatty acids were palmitic acid (16:0), oleic acid (18:1 *n*-9), DHA (22:6 *n*-3) and linoleic acid (18:2 *n*-6), respectively. These four fatty acids represented 56-63% of neutral lipids and 59-69% of polar lipids for *S. glanis* and 65-71% and 62-66% for *B. capito capito*, respectively. The most abundant polar SFA was palmitic acid; 23-26% for *S. glanis*

Table 1 - Total lipid, neutral lipid and polar lipid contents of *Silurus glanis* and *Barbus capito capito* in different seasons[1].

Species	Component	Autumn	Winter	Spring	Summer
Silurus glanis	Total lipid	2.33±0.25^{a}	2.34±0.06^{a}	1.11±0.19^{c}	1.31±0.41^{b}
	Neutral lipid	0.76±0.09^{a}	0.57±0.04^{b}	0.67±0.11^{ab}	0.84±0.22^{a}
	Phospholipid	1.57±0.19^{a}	1.77±0.04^{a}	0.43±0.15^{b}	0.46±0.27^{b}
Barbus capito capito	Total lipid	2.06±0.28^{b}	4.57±1.09^{a}	1.77±0.16^{c}	1.34±0.74^{d}
	Neutral lipid	0.95±0.12^{c}	3.31±1.19^{a}	1.29±0.20^{b}	0.74±0.05^{c}
	Phospholipid	1.11±0.16^{a}	1.26±0.14^{a}	0.48±0.09^{b}	0.60±0.04^{b}

[1]Values are means of five replicates (n=5) ± standard deviation (SD). Values in the rows with different superscript (a-d) are significantly different ($P < 0.05$).

Table 2 - Fatty acid content of neutral lipids of *Silurus glanis* along the year seasons[1,2].

Fatty acids	Neutral lipids			
	Autumn	Winter	Spring	Summer
14:0	0.35±0.0^{c}	0.44±0.0^{b}	0.57±0.0^{a}	0.35±0.0^{c}
15:0	0.16±0.0^{c}	0.38±0.0^{a}	0.34±0.0^{b}	0.19±0.0^{c}
16:0	17.3±0.2^{c}	20.3±0.2^{a}	19.6±0.3^{b}	17.4±0.0^{c}
17:0	0.65±0.1^{bc}	0.73±0.1^{b}	0.59±0.0^{c}	1.26±0.0^{a}
18:0	3.88±0.1^{d}	4.17±0.0^{c}	6.47±0.3^{a}	4.78±0.0^{b}
20:0	0.36±0.0^{b}	0.39±0.0^{b}	0.35±0.0^{b}	0.57±0.0^{a}
22:0	1.43±0.0^{b}	1.24±0.0^{c}	2.97±0.2^{a}	1.37±0.0^{bc}
24:0	0.23±0.0^{c}	0.50±0.0^{b}	0.77±0.0^{a}	0.21±0.0^{c}
Σ SFA	**22.9±0.2^{d}**	**27.0±0.2^{b}**	**28.7±0.3^{a}**	**24.8±0.1^{c}**
15:1	0.24±0.0^{d}	1.15±0.0^{a}	0.37±0.1^{c}	0.54±0.0^{b}
16:1 n-7	14.1±0.6^{d}	19.5±0.6^{a}	18.5±0.1^{b}	16.1±0.2^{c}
17:1	0.25±0.0^{c}	0.47±0.0^{b}	0.25±0.0^{c}	0.54±0.0^{a}
18:1 n-9	29.5±0.7^{a}	20.7±0.4^{c}	19.5±0.3^{d}	24.0±0.3^{b}
20:1 n-9	6.01±0.3^{a}	3.60±0.2^{d}	4.12±0.4^{c}	5.46±0.2^{b}
22:1 n-9	0.56±0.0^{c}	0.72±0.0^{b}	0.79±0.0^{a}	0.58±0.0^{c}
Σ MUFA	**50.1±0.7^{a}**	**45.5±0.9^{c}**	**42.7±0.4^{d}**	**46.7±0.3^{b}**
18:3 n-3	0.22±0.0^{b}	0.37±0.0^{a}	0.34±0.0^{a}	0.35±0.0^{a}
20:5 n-3	0.54±0.0^{c}	0.74±0.0^{a}	0.60±0.0^{b}	0.56±0.0^{c}
22:5 n-3	0.08±0.0^{c}	0.15±0.0^{c}	1.00±0.1^{a}	0.39±0.0^{b}
22:6 n-3	6.23±0.3^{b}	6.08±0.2^{b}	5.55±0.3^{c}	7.28±0.1^{a}
Σ n-3 PUFA	**7.08±0.4^{b}**	**7.34±0.2^{b}**	**7.50±0.4^{b}**	**8.58±0.1^{a}**
18:2 n-6	16.0±0.5^{a}	13.5±0.3^{c}	14.5±0.2^{b}	13.4±0.3^{c}
20:2 n-6	0.27±0.0^{c}	0.64±0.0^{a}	0.25±0.0^{c}	0.47±0.0^{b}
20:3 n-6	2.05±0.0^{b}	1.69±0.0^{c}	2.45±0.1^{a}	2.60±0.2^{a}
20:4 n-6	0.11±0.0^{d}	0.58±0.0^{c}	0.82±0.0^{a}	0.68±0.0^{b}
22:2 n-6	0.78±0.0^{b}	0.77±0.02	1.06±0.0^{a}	0.81±0.0^{b}
Σ n-6 PUFA	**19.2±0.5^{a}**	**17.2±0.4^{c}**	**19.1±0.2^{a}**	**17.9±0.3^{b}**
Unknown	**-**	**~2.07**	**~4.04**	**~2.25**
Σ PUFA	**26.3±0.62^{a}**	**26.2±0.5^{ab}**	**24.1±0.2^{c}**	**25.5±0.7^{b}**
Σ n-3/n-6 PUFA	**0.37±0.0^{d}**	**0.43±0.0^{b}**	**0.39±0.0^{c}**	**0.48±0.0^{a}**
EPA+DHA	**6.77±0.3^{b}**	**6.82±0.2^{b}**	**6.16±0.3^{c}**	**7.84±0.1^{a}**

[1]Values are expressed as percentages of total fatty acids.
[2]Values are means of five replicates (n=5) ± standard deviation (SD). Values in the rows with different superscript (a-d) are significantly different ($P < 0.05$).

Table 3 - Fatty acid content of polar lipids in the edible portions of immature *Silurus glanis* along the year seasons[1,2].

Fatty acids	Polar lipids			
	Autumn	Winter	Spring	Summer
14:0	0.24±0.0^{ab}	0.22±0.02^{b}	0.25±0.0^{a}	0.25±0.0^{a}
15:0	1.96±0.1^{c}	2.44±0.20^{a}	2.23±0.1^{b}	1.57±0.0^{d}
16:0	23.2±0.2^{b}	26.1±0.70^{a}	23.9±0.8^{b}	25.7±0.6^{a}
17:0	0.21±0.0^{b}	0.18±0.01^{b}	0.30±0.0^{a}	0.29±0.0^{a}
18:0	9.79±0.6^{a}	9.62±0.33^{a}	10.1±0.3^{a}	9.86±0.2^{a}
20:0	0.24±0.0^{b}	0.18±0.01^{c}	0.16±0.0^{c}	0.28±0.0^{a}
22:0	7.33±0.3^{c}	8.16±0.36^{b}	11.1±0.3^{a}	7.20±0.2^{c}
24:0	0.73±0.1^{b}	0.57±0.05^{b}	1.55±0.1^{a}	0.58±0.0^{b}
Σ SFA	**43.7±0.7^{d}**	**47.5±0.28^{b}**	**49.7±0.9^{a}**	**45.7±0.4^{c}**
15:1	0.05±0.0^{c}	0.07±0.01^{b}	0.25±0.0^{a}	0.06±0.0^{bc}
16:1 n-7	5.59±0.3^{b}	5.51±0.22^{b}	7.06±0.1^{a}	5.05±0.1^{c}
17:1	2.48±0.2^{a}	2.38±0.13^{a}	1.50±0.1^{c}	1.88±0.1^{b}
18:1 n-9	13.8±0.4^{a}	10.1±0.25^{b}	13.4±0.9^{a}	13.6±0.6^{a}
20:1 n-9	2.38±0.1^{b}	1.91±0.06^{a}	1.44±0.2^{d}	2.41±0.1^{c}
22:1 n-9	0.96±0.0^{b}	0.83±0.06^{c}	0.80±0.0^{c}	1.11±0.0^{a}
Σ MUFA	**25.2±1.0^{a}**	**20.8±0.28^{c}**	**24.4±0.8^{ab}**	**24.1±0.5^{b}**
18:3 n-3	0.30±0.0^{a}	0.17±0.03^{c}	0.18±0.0^{c}	0.23±0.0^{b}
20:5 n-3	1.33±0.0^{b}	1.50±0.08^{a}	1.03±0.0^{c}	1.12±0.0^{c}
22:5 n-3	0.04±0.0^{b}	0.04±0.00^{b}	0.11±0.0^{a}	0.02±0.0^{c}
22:6 n-3	11.9±0.5^{b}	15.2±0.34^{a}	7.94±0.1^{d}	10.7±0.2^{c}
Σ n-3 PUFA	**13.6±0.4^{b}**	**16.9±0.40^{a}**	**9.26±0.1^{d}**	**12.0±0.2^{c}**
18:2 n-6	13.4±1.0^{a}	11.4±0.59^{b}	11.2±0.2^{b}	13.8±0.4^{a}
20:2 n-6	0.21±0.1^{a}	0.30±0.03^{a}	0.23±0.0^{a}	0.24±0.0^{a}
20:3 n-6	1.45±0.1^{b}	1.09±0.05^{c}	1.89±0.0^{a}	1.81±0.1^{a}
20:4 n-6	1.43±0.2^{ab}	1.01±0.09^{c}	1.32±0.0^{b}	1.57±0.0^{a}
22:2 n-6	0.58±0.0^{a}	0.55±0.03^{a}	0.60±0.0^{a}	0.56±0.0^{a}
Σ n-6 PUFA	**17.0±1.1^{a}**	**14.2±0.72^{c}**	**15.3±0.3^{b}**	**18.0±0.3^{a}**
Σ PUFA	**30.0±0.2^{b}**	**30.6±1.11^{ab}**	**31.5±0.4^{a}**	**24.5±0.4^{c}**
Σ n-3/n-6 PUFA	**0.80±0.0^{b}**	**1.19±0.08^{a}**	**0.60±0.0^{c}**	**0.67±0.0^{c}**
EPA+DHA	**13.2±0.5^{b}**	**16.7±0.38^{a}**	**8.97±0.1^{d}**	**11.8±0.2^{c}**

[1]Values are expressed as percentages of total fatty acids.
[2]Values are means of five replicates (n=5) ± standard deviation (SD). Values in the rows with different superscript (a-d) are significantly different ($P < 0.05$).

(Table 3) and 22-28% for *B. capito capito* (Table 5). Numerous researchers have reported similar results for tilapia species, Bulatmai barbel (total lipids) and brown trout subspecies (RASOARAHONA *et al.*, 2005; ARAS *et al.*, 2009a; BAYIR *et al.*, 2010). It has been suggested that decreasing the water temperature results in a decrease in the 16:0 content (HENDERSON and TOCHER, 1987). However, an inverse result was found in this study except for the neutral lipids of *B. capito capito*; this discrepancy could be explained as a metabolic response of fish to extreme environmental conditions.

The highest and lowest total SFA contents of both fractions in *S. glanis* and *B. capito capito* were found during the hot (spring and summer) and cold seasons (autumn and winter), respectively (Tables 2-5) ($P < 0.05$). The SFA content of the polar lipid fraction was higher than that of the neutral lipid fraction (Tables 2-5). It has been suggested that in most freshwater fish species, the polar lipid fraction contains more PUFA than and similar amounts of SFA as the neutral lipid fraction (HENDERSON and TOCHER, 1987). However, it is also known that water temperature can have a substantial impact on the fatty acid profiles of fish (JANKOWSKA *et al.*, 2008). In this study, because a substantial temperature difference was observed between winter (4.1°C) and summer (20°C), it was assumed that the adaptation of fish to this extreme environmental condition was a possible reason for the higher SFA amounts in the polar lipid fraction than in the neutral lipid fraction. Similarly, in a previous study, we reported that the polar lipid fraction of *Salmo trutta* contained higher SFA levels than the neutral lipid fraction (BAYIR *et al.*, 2010).

As seen in Tables 3 and 5, the highest MUFA levels in the polar lipid fraction were recorded in the autumn (25% for Wels and 23% for Bulatmai barbel). Similarly, the maximum MUFA level in the neutral lipid fraction was also ob-

tained during the cold seasons (in the autumn for Wels (50%) and in the winter for Bulatmai barbel (49%); Tables 2 and 4). Oleic acid was the most abundant fatty acid among MUFAs of both fractions, which is a characteristic property of freshwater fish oils (GÜLER *et al.*, 2008), and the highest level of oleic acid was found during the cold seasons (Tables 2-5) ($P < 0.05$). However, CSENGERI *et al.* (1978) suggested that the low level oleic acid in carp was related to the low level oleic acid in their natural diets, which is a possible explanation for our results. Although the MUFA content in the neutral lipid fraction was higher than that of in the polar lipid fraction, PUFA contents of both fractions were similar for the two species. GUNSTONE *et al.* (1978) reported that the polar lipid fractions of fish contain much lower contents of MUFA than the neutral lipid fraction does. Thus, the findings of our study are in accordance with those published in literature.

There was no harmony in the seasonal fluctuations of the highest total PUFA value of polar lipids. While the maximum total PUFA value was measured in the spring for Wels (31%), the maximum value was observed in the winter for Bulatmai barbel (32%). In contrast, the total PUFA contents of neutral lipids in both species were higher in the autumn than during the other seasons (Tables 2-5) ($P < 0.05$). Additionally, we found that fish have more total PUFA during cold seasons than during hot seasons except for polar lipids in Bulatmai barbel. Similar to our results, FARKAS *et al.* (1980) reported that exposure to cold temperatures for only a few hours was sufficient to increase the PUFA content of the polar lipid fraction of the liver of *Cyprinus carpio*. It is also known that as the temperature decreases, the level of unsaturation tends to increase to assist in maintaining the freezing point below that of the surrounding water to ensure

Table 4 - Fatty acid content of neutral lipids in the edible portions of immature *Barbus capito capito* along the year seasons[1,2].

Fatty acid	Neutral lipids			
	Autumn	Winter	Spring	Summer
14:0	0.30±0.0^{c}	0.45±0.0^{b}	0.90±0.0^{a}	0.30±0.0^{c}
15:0	0.14±0.0^{b}	0.47±0.0^{a}	0.48±0.0^{a}	0.13±0.0^{b}
16:0	11.5±0.6^{b}	12.6±1.4^{b}	17.8±0.8^{a}	11.9±0.6^{b}
17:0	0.24±0.0^{b}	1.58±0.1^{c}	0.77±0.0^{c}	0.24±0.0^{a}
18:0	2.16±0.1^{b}	2.62±0.2^{a}	2.77±0.0^{a}	2.32±0.2^{b}
20:0	0.18±0.0^{d}	0.62±0.0^{a}	0.50±0.0^{b}	0.36±0.0^{c}
22:0	0.95±0.0^{b}	0.83±0.1^{b}	1.32±0.1^{a}	0.85±0.1^{b}
24:0	0.22±0.0^{c}	0.31±0.0^{b}	0.45±0.0^{a}	0.28±0.0^{bc}
Σ SFA	**14.7±0.6^{c}**	**18.4±1.3^{b}**	**23.6±0.9^{a}**	**15.4±0.8^{c}**
15:1	0.24±0.0^{c}	0.43±0.0^{b}	1.06±0.0^{a}	0.16±0.0^{d}
16:1 n-7	7.05±0.4^{c}	12.6±0.9^{b}	15.7±1.0^{a}	5.92±0.4^{d}
17:1	0.16±0.0^{c}	1.13±0.1^{a}	0.29±0.0^{b}	0.16±0.0^{c}
18:1 n-9	25.9±0.5^{ab}	26.4±1.0^{a}	24.9±1.1^{b}	26.5±0.4^{a}
20:1 n-9	12.3±0.4^{b}	8.83±0.9^{c}	4.92±0.3^{d}	14.8±0.4^{a}
22:1 n-9	0.27±0.0^{a}	0.26±0.0^{a}	0.28±0.0^{a}	0.27±0.0^{a}
Σ MUFA	**45.7±0.7^{c}**	**49.4±0.6^{a}**	**7.00±1.6^{bc}**	**47.7±0.7^{b}**
18:3 n-3	1.21±0.0^{a}	0.73±0.0^{c}	0.40±0.0^{d}	0.87±0.0^{b}
20:5 n-3	0.70±0.0^{a}	0.63±0.1^{a}	0.59±0.0^{a}	0.59±0.0^{a}
22:5 n-3	0.05±0.0^{c}	0.26±0.0^{a}	0.27±0.0^{a}	0.09±0.0^{b}
22:6 n-3	2.24±0.1^{b}	5.96±0.3^{a}	6.07±0.4^{a}	1.51±0.0^{c}
Σ n-3 PUFA	**4.19±0.1^{b}**	**7.57±0.2^{a}**	**7.32±0.4^{a}**	**3.05±0.1^{c}**
18:2 n-6	32.1±0.6^{a}	21.2±1.1^{c}	16.2±0.3^{d}	30.4±1.0^{b}
20:2 n-6	0.25±0.0^{b}	0.31±0.0^{a}	0.24±0.0^{b}	0.26±0.0^{b}
20:3 n-6	1.67±0.1^{b}	1.64±0.1^{b}	2.81±0.1^{a}	1.70±0.1^{b}
20:4 n-6	0.52±0.0^{c}	0.65±0.1^{b}	0.78±0.0^{a}	0.51±0.0^{c}
22:2 n-6	0.34±0.0^{a}	0.35±0.0^{a}	0.39±0.0^{a}	0.35±0.0^{a}
Σ n-6 PUFA	**34.8±0.6^{a}**	**24.1±1.2^{c}**	**20.4±0.4^{d}**	**33.2±1.1^{b}**
Σ PUFA	**39.0±0.4^{a}**	**31.7±1.3^{c}**	**27.8±0.8^{d}**	**36.2±1.0^{b}**
Σ n-3/n-6 PUFA	**0.12±0.0^{c}**	**0.31±0.0^{b}**	**0.36±0.0^{a}**	**0.09±0.0^{d}**
EPA+DHA	**2.94±0.1^{b}**	**6.59±0.2^{a}**	**6.66±0.4^{a}**	**2.09±0.1^{c}**

[1]Values are expressed as percentages of total fatty acids.
[2]Values are means of five replicates (n=5) ± standard deviation (SD). Values in the rows with different superscript (a-d) are significantly different ($P < 0.05$).

Table 5 - Fatty acid content of polar lipids in the edible portions of immature *Barbus capito capito* along the year seasons[1,2].

Fatty acid	Polar lipids			
	Autumn	Winter	Spring	Summer
14:0	0.18±0.0^{bc}	0.14±0.0^{c}	0.24±0.0^{b}	0.30±0.0^{a}
15:0	1.73±0.0^{b}	0.88±0.1^{c}	2.55±0.0^{a}	2.50±0.0^{a}
16:0	28.2±0.2^{a}	27.7±1.4^{a}	24.2±0.4^{b}	22.8±0.1^{c}
17:0	0.29±0.0^{b}	0.43±0.0^{a}	0.26±0.0^{b}	0.41±0.0^{a}
18:0	8.46±0.2^{c}	9.42±0.3^{b}	9.54±0.5^{b}	12.3±0.4^{a}
20:0	0.06±0.0^{c}	0.13±0.0^{b}	0.24±0.0^{a}	0.11±0.0^{b}
22:0	9.38±0.0^{c}	7.51±0.2^{d}	10.1±0.2^{b}	11.5±0.1^{a}
24:0	1.66±0.1^{a}	0.21±0.0^{c}	0.16±0.0^{c}	0.54±0.0^{b}
Σ SFA	**50.0±0.1^{a}**	**46.4±1.6^{b}**	**47.4±0.7^{b}**	**50.6±0.2^{a}**
15:1	0.50±0.0^{b}	0.54±0.0^{a}	0.55±0.0^{a}	0.38±0.0^{c}
16:1 n-7	2.77±0.1^{b}	3.66±0.1^{a}	3.68±0.2^{a}	2.21±0.0^{c}
17:1	0.12±0.0^{b}	0.15±0.0^{b}	0.24±0.0^{a}	0.25±0.0^{a}
18:1 n-9	14.1±0.4^{ab}	10.2±0.6^{c}	14.9±1.2^{a}	13.6±0.1^{b}
20:1 n-9	5.46±0.1^{b}	6.00±0.5^{a}	1.56±0.3^{d}	2.58±0.0^{c}
22:1 n-9	0.42±0.0^{c}	0.69±0.0^{a}	0.48±0.0^{b}	0.23±0.0^{d}
Σ MUFA	**23.4±0.6^{a}**	**21.3±0.9^{b}**	**21.4±0.9^{b}**	**19.3±0.1^{c}**
18:3 n-3	0.49±0.0^{a}	0.13±0.0^{c}	0.31±0.0^{b}	0.32±0.0^{b}
20:5 n-3	0.92±0.0^{a}	0.53±0.0^{c}	0.89±0.1^{a}	0.70±0.0^{b}
22:5 n-3	0.02±0.0^{a}	0.007±0.0^{c}	0.03±0.0^{b}	0.006±0.0^{bc}
22:6 n-3	6.55±0.4^{d}	16.9±0.5^{a}	16.0±0.9^{b}	11.2±0.1^{c}
Σ n-3 PUFA	**7.98±0.4^{c}**	**17.6±0.5^{a}**	**17.3±1.0^{a}**	**12.2±0.2^{b}**
18:2 n-6	14.7±0.2^{a}	10.3±0.5^{b}	11.1±0.9^{b}	14.7±0.2^{a}
20:2 n-6	0.75±0.0^{d}	2.30±0.1^{c}	0.30±0.0^{b}	1.01±0.1^{a}
20:3 n-6	1.97±0.0^{b}	0.29±0.0^{a}	0.24±0.0^{b}	0.24±0.0^{c}
20:4 n-6	0.12±0.0^{c}	0.26±0.0^{b}	1.61±0.1^{a}	1.55±0.0^{a}
22:2 n-6	0.56±0.0^{d}	1.39±0.0^{b}	0.35±0.0^{a}	0.16±0.0^{c}
Σ n-6 PUFA	**17.9±0.4^{a}**	**14.6±0.5^{b}**	**13.6±0.9^{c}**	**17.7±0.2^{a}**
Σ PUFA	**25.9±0.6^{c}**	**32.2±0.8^{a}**	**30.9±1.6^{ab}**	**29.9±0.2^{b}**
Σ n-3/n-6 PUFA	**0.44±0.0^{c}**	**1.21±0.0^{a}**	**1.27±0.0^{a}**	**0.69±0.0^{b}**
EPA+DHA	**7.47±0.4^{d}**	**17.4±0.5^{a}**	**16.9±1.0^{b}**	**11.9±0.2^{c}**

[1]Values are expressed as percentages of total fatty acids.
[2]Values are means of five replicates (n=5) ± standard deviation (SD). Values in the rows with different superscript (a-d) are significantly different ($P < 0.05$).

membrane fluidity and general body flexibility (MARTINO and CRUZ, 2004).

The *n*-3/*n*-6 ratio of the polar lipid fraction was higher than that of the neutral lipid fraction (Tables 2-5) ($P < 0.05$). Similarly, BAYIR *et al.* (2010) found that phospholipids have a higher *n*-3/*n*-6 ratio than neutral lipids in female *Salmo trutta.* Whereas the highest *n*-3/*n*-6 ratio of the neutral lipid fraction was observed during the summer for Wels (0.48) and during the spring for Bulatmai barbel (0.36), it was obtained in the winter for the polar lipid fraction of Wels (1.19) and in the spring and winter for Bulatmai barbel (1.27 and 1.21, respectively). Previously, RASOARAHONA *et al.* (2005) and GÜLER *et al.* (2008) suggested that the highest *n*-3/*n*-6 ratio of muscle lipids of tilapia and carp were present in the winter. On the other hand, SHIRAI *et al.* (2001) reported that there was no seasonal variation in the *n*-3/*n*-6 ratio of liver phosphatidylcholine in *Silurus asotus*. Because many factors may be responsible for the variations in the levels of lipids and fatty acids of fish, e.g., internal factors such as genetic variation, age, sex, condition, starvation and reproductive cycle and external factors such as diet, season and temperature (HALİLOĞLU *et al.*, 2004-2005; BAYIR *et al.*, 2006; KWETEGYEKA *et al.*, 2008), the fatty acid composition of fish lipids is extremely variable (SHIRAI *et al.*, 2002; ARAS *et al.*, 2009a-b). This variability may have influenced the results of this study.

The EPA+DHA content of the polar lipid fraction was higher than that of neutral lipid fraction for both species. This result was in accordance with that of PETURSDOTTIR *et al.* (2008), who found that dietary EPA and DHA were present in much higher proportions in the polar lipid fraction than in the neutral lipid fraction of the deep-sea redfish *Sebastes mentella.* In our present study, the highest amounts of EPA+DHA of both species were recorded during the summer for neutral lipids and during the winter for polar lipids ($P < 0.05$). The general assumption is that the fatty acid composition of the neutral storage lipids reflects trophic influences much better than the physiologically more important polar lipids (KWETEGYEKA *et al.*, 2008). The lipids of predatory fish such as *S. glanis* that prey on small fish are expected to be influenced by the fatty acid composition of their prey, which are rich sources of PUFA (HENDERSON and TOCHER, 1987). *B. capito capito* is an omnivorous fish, and its natural food items are invertebrates, algae, detritus, plant material, and small fishes (KOTTELAT and FREYHOF, 2007). The fatty acid composition of *B. capito capito* lipids is also influenced by its nutritional habits. These results indicate that the high EPA+DHA content of neutral lipids in hot seasons is considerably influenced by the composition of the ingested food and that low water temperature is the main reason for the high EPA+DHA content of the polar lipid fraction during the winter.

CONCLUSIONS

We found that the season significantly influences the fatty acid contents of the studied species. However, the *n*-3/*n*-6 ratio and the EPA+DHA content of the polar lipid fraction of edible flesh, which contained a higher amount of PUFA than did the neutral lipid fraction (ACKMAN, 1989), were higher during the winter than during other seasons. In recent years, *n*-3 PUFA has been determined to have greater potency in the amelioration of heart disorders than *n*-6 PUFA. Therefore, the *n*-3/*n*-6 PUFA ratio is a marker of the biomedical relevance of fish oils (HAZRA *et al.*, 1998). The International Society for the Study of Fatty Acids and Lipids advocates 500 mg per day EPA+DHA for cardiovascular health (ISSFAL, 2004). Therefore, we recommend eating about 2.5 portions of *S. glanis* or *B. capito capito* per week throughout the year. Moreover, to investigate in detail the fatty acid metabolism of these economically important and endangered species, controlled aquarium experiments should be conducted.

ACKNOWLEDGEMENTS

Research support was provided by The Scientific and Technological Research Council of Turkey (TUBITAK, Project Number: CAYDAG, 105Y094). Therefore, the Authors are thankful to TUBITAK to support this project.

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Paper received May 19, 2010 Accepted December 5, 2010

PAPER

CHEMICAL COMPOSITION AND ENERGY VALUE OF DWARF OATS GRAIN

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ABSTRACT

Grain samples were derived from the plot trials. The treatments were: nitrogen rates (40, 100 kg·ha^{-1}) and oat cultivars (dwarf lines: STH 6102 – naked, STH 735 – hulled and tall: Polar – naked, Krezus – hulled). The naked forms were characterised by a higher content of crude protein, ether extract, nitrogen free extract, and starch and a lower content of crude fibre and sugars in comparison with the hulled forms. Significant variation was found between the dwarf and the tall forms. The higher nitrogen treatment compared with the lower treatment resulted in an increase of crude protein and a decrease of: ether extract, nitrogen free extract, and starch.

- Key words: dwarf gene, energy value, hulled oat, naked oat, nitrogen fertiliser, nutrient content -

INTRODUCTION

Hulled and naked are the two representative forms of common oat which are grown at present. In the naked oat, a thin non-lignified husk on the outside of the grain falls off during threshing. Such a huskless grain has a better chemical composition, a higher nutrient content and an increased concentration of digestible energy (CONCIATORI *et al.*, 2000; GIVENS *et al.*, 2004). These properties make the oat cultivar a highly attractive feed plant for pigs, poultry, horses, and dairy cows in particular (BRAND *et al.*, 2003; FEARON *et al.*, 1996; HUSSEIN *et al.*, 2004; MACLEOD *et al.*, 2008; WHITE *et al.*, 2008). Due to the fact that naked oats contain considerable amounts of high quality proteins, fat rich in unsaturated fatty acids, antioxidant compounds and many other substances positively affecting the functioning of the body (HOLGUÍN-ACUÑA *et al.*, 2008; PETERSON, 2001). They are also included in the human diet.

Oat breeders are still working to increase the grain yield. It is particularly noticeable in Poland that the grain yield of the naked cultivars is 10% lower than the grain yield of the hulled forms. Spikelets with many flowers producing tiny grains are considered the main reason for such a low yield, as well as worse germination in field conditions, probably due to some microdamage occurring in the grain after mechanical harvesting (PELTONEN-SAINIO, 1994; VALENTINE and HALE, 1990). One of the opportunities to improve the yield is to introduce short naked oat cultivars with dwarf genes, which are an effective source of lodging control. This would enable applying intensive agro-chemical input, mainly high nitrogen treatment, as a factor maximising the grain yield. Shortening the plant's straw is considered a main factor contributing to the success in breeding high yield wheat and rice varieties in the second half of the 20th century (SLAFER, 2003). However, it results in many physiological and morphological changes in plants which are visible at the level of the components affecting the grain yield. The most important changes reported, include: (a) increased productive tillering due to lower intraspecific competition that led to higher panicle density, (b) higher number of developed and more filled up grains harvested from a surface unit which resulted in a rise in the harvest index, (c) photoperiodic changes that shortened the vegetation season, (d) decrease in development of the root system and disturbances in the proportion of the above ground plant parts and, as a consequence, increased the need of water and nutrients (BROWN *et al.*, 1980; MÄKELA *et al.*, 2004; PELTONEN-SAINIO *et al.*, 2003; RAJALA and PELTONEN-SAINIO, 2001). The present dwarf or semi-dwarf oat cultivars have been bred with the use of a dwarf gene – *Dw6* (MILACH *et al.*, 2002). Using the *Dw6* dwarf gene can cause some limitations in the growing process, namely a decrease in yield, size of grains and their quality (KIBITE and CLAYTON, 2000; MILACH and FEDERIZZI, 2001), thus finding consistent and high yielding cultivars is difficult.

So far, there is no information on the influence of dwarf genes on the quality properties of oats. The aim of this study was to compare the chemical composition and digestible energy of hulled and naked oat cultivars with an implemented dwarf gene, along with various nitrogen regimes.

MATERIALS AND METHODS

Oat samples were derived from the plot trials carried out in 2005-2006 on sandy clay soil type at the Lipnik Experimental Station (near Szczecin – 53°12'N; 14°27'E), Poland. The treatments were: 2 nitrogen fertilization rates: 40 kg N per ha (pre-sowing) and 100 kg N per ha (60 – pre-sowing and 40 kg N per ha – during shooting stage) and 4 oat cultivars (the best yielded cultivars in the Polish official trials): two short lines with the *Dw6* dwarf gene (STH 6102 – naked cultivar and STH 735 – hulled cultivar) and two tall cultivars as control (Krezus – hulled cultivar and Polar – naked cultivar). The nitrogen doses were randomized as a first level factor and cultivars as a second level factor on the sub-plots. Nitrogen fertilization was introduced in an ammonium nitrate form by means of the plot spreader. The grains were cleaned and rendered free of dust, then stored in tightly closed glass jars at room temperature until used.

The chemical compositions of all samples were determined by the following AOAC (1990) procedures: Dry matter was obtained by drying samples in an oven at 105°C until a constant weight was obtained; ether extract, by Soxhlet extraction with diethyl ether; crude ash, by incineration in a muffle furnace at 580°C for 8 h; crude protein (CP) (N×6.25), by the Kjeldahl method. Crude fibre was determined using the Hennenberg-Stohmann method. Nitrogen-free extract (NFE) was calculated as follows: % NFE = 100 - %(moisture + crude protein +lipid + ash + crude fibre). Total sugars were determined after the inversion, using the Luff-Schoorl method. The fibre components were determined using the detergent method according to VAN SOEST *et al.* (1991) performed with a fibre analyzer ANCOM 220. The measurement of neutral detergent fibre (NDF) was carried out on an ash-free basis, including alpha-amylase and dodecylsulfat natriumsaltz (Merc 8.22050.). The determination of acid detergent fibre (ADF) included hexadecyl-trimethyl-ammonium bromide (Merc 102342), while acid detergent lignin (ADL) was determined by hydrolysis of ADF sample in 72% sulphuric acid. Hemicellulose was calculated as the difference between NDF and ADF, while cel-

lulose – as a difference between ADF and ADL. Acid ether extract (AEE) was determined using a method described by ALDERMAN (1985). Starch was determined by a polarimetric method (ISO 10520:1997). A flame photometer (Flapho-4) was used to determine calcium, potassium and sodium. After oven-drying of the samples, phosphorus content was determined by a colorimetric method. Results were expressed as gram(g) per kilogram(kg) of dry matter (DM).

Amino acids were determined using an AAA 400 automatic amino acid analyser (INGOS, Czech Republic). Samples were subjected to an acid hydrolysis in the presence of 6 M HCl at 105°C for 24 hours. Sulphur-containing amino acids were determined separately in 6 M HCl after an oxidative hydrolysis (formic acid + hydrogen peroxide, 9:1 v/v, 20 h at 4°C). Tryptophan was determined according to a method described in AOAC (1990). The Amino acid composition was displayed as g per 16 g of nitrogen.

All results are given as means of double analysis.

Digestible energy (DE) concentration for pigs and apparent metabolisable energy (AME) values for poultry were predicted using equations of MAFF (1993) derived for use on compound feeds. These equations are:

$$\text{DE (pigs) (MJ·kg}^{-1}\text{ DM)} = 17.47 + (0.0079 \times \text{CP}) + (0.0158 \times \text{AEE}) - (0.0331 \times \text{ash}) - (0.014 \times \text{NDF}).$$

$$\text{AME (poultry) (MJ·kg}^{-1}\text{ DM)} = (0.01551 \times \text{CP}) + (0.03431 \times \text{AEE}) + (0.01669 \times \text{starch}) - (0.01301 \times \text{sugar}).$$

The differences between the means were tested by the standard ANOVA method. The year, cultivar and nitrogen treatment were the factors of analysis. To distinguish the differences between the forms of the cultivars (naked *vs* hulled and dwarf *vs* tall) a planned comparison technique using linear contrast method was used. Specific posthoc comparisons of means were made using the Tukey procedure at p=0.05. All calculations were made using the Statistica Data Analysis Software System (STATSOFT, INC. 2007).

RESULTS AND CONCLUSION

Significant differences between years were obtained for all of the parameters measured indicating that oat differed in its response to changes in environmental factors, especially water conditions during growth (Table 1). Reduced rainfalls in 2006, particularly in June-July period, resulted in higher ash, crude protein, cellulose, hemicellulose, and starch content but lower ether extract, crude fibre, nitrogen free extract, and sugars, as compared with the wet season of 2006. These results support the conclusion of a great impact of water deficiency on the grain quality

Table 1 - Probability level of F statistic from analysis of variance to test differences among years (Y), cultivars (C) and nitrogen treatments (N) for evaluated traits.

Trait	Source of variation						
	Y	C	N	Y×C	Y×N	C×N	Y×C×N
Dry matter	0,00	0,00	0,18	0,00	0,37	0,00	0,00
Ash	0,00	0,07	0,01	0,00	0,52	0,02	0,08
Crude protein (N×6.25)	0,00	0,00	0,00	0,00	0,09	0,00	0,02
Ether extract	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Crude fibre	0,01	0,00	0,68	0,00	0,00	0,03	0,00
Nitrogen free extract	0,00	0,00	0,00	0,00	0,05	0,24	0,01
Sugars	0,01	0,00	0,00	0,55	0,85	0,47	0,97
Starch	0,00	0,00	0,00	0,99	0,99	0,00	0,99
Neutral detergent fibre	0,00	0,00	0,02	0,00	0,14	0,00	0,00
Acid detergent fibre	0,00	0,00	0,43	0,00	0,12	0,22	0,00
Acid detergent lignin	0,01	0,00	0,05	0,16	0,60	0,17	0,21
Cellulose	0,00	0,00	0,95	0,00	0,40	0,16	0,00
Hemicellulose	0,00	0,00	0,11	0,00	0,01	0,00	0,00
Total amino acids	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Essential amino acids	0,00	0,00	0,01	0,00	0,00	0,00	0,00
Calcium	0,00	0,00	0,38	0,01	0,35	0,01	0,01
Phosphorus	0,00	0,00	0,00	0,02	0,09	0,34	0,44
Potassium	0,04	0,00	0,10	0,00	0,01	0,01	0,00
Sodium	0,00	0,00	0,89	0,00	0,04	0,04	0,12
Digestible energy	0,00	0,00	0,00	0,00	0,84	0,00	0,00
Apparent metabolisable energy	0,00	0,00	0,13	0,00	0,01	0,00	0,20

Cultivars (C) and nitrogen treatments (N) for evaluated traits.

of oat. The ANOVA data showed significant interaction between the cultivar and the year for most of the traits, except for sugars, starch, and acid detergent lignin content. An interaction between the nitrogen treatment and the year was also significant, but only in a few cases. Although the interaction between the main factors of the experiment and years was significant, rankings of differences among cultivars and between two nitrogen doses were consistent, regardless of the year. Therefore, the results were interpreted as average crops harvested in 2005 and 2006 without showing any interactions between the tested factors and years.

Differences due to cultivar x nitrogen interaction were significant for ash, crude protein, ether extract, crude fibre, and starch content and were not significant for nitrogen free extract and starch content (Table 1). However, the pattern of differences among cultivars was almost the same, with both nitrogen doses (Table 2).

The total protein content in oat grain was different in the tested cultivars, oat forms and it was subjected to changes due to applied nitrogen treatment (Table 2). In this study, both naked oat cultivars, Polar and STH 6102, contained more protein, fat, and starch than conventional oats, regardless of the nitrogen treatment (Table 2). Similar findings were also reported by other authors (BRAND and MERWE, 1996; MAURICE *et al.*, 1985). Polar, a model cultivar, contained the highest protein content (168.4 g, over nitrogen treatment) compared with the other cultivars. Although significant differences between the tall and dwarf cultivars were observed in terms of the total protein, fat, crude fibre, carbohydrates, sugars, and starch content, only the differences in sugars and starch content were consistent in character (increasing or decreasing trend). As for the others, the differences resulted from the dominance of an individual cultivar.

Increase the nitrogen rate to 100 kg·ha^{-1} significantly increased the grain protein level by approximately 27 g·kg^{-1} DM. A positive relationship between nitrogen treatment and the rise in grain protein level was also reported by other authors (GIVENS *et al.*, 2004; IZSÁKI, 2004; LÁSZTITY, 1998). Nitrogen treatment usually results in an increased protein content in oats, there are only discrepancies as to the extent of the increase and modifying impact of the environmental factors.

Due to increased nitrogen treatment, the fat content was reduced significantly while crude fibre content had no significant change (Table 2).

Due to the fact, that the monogastric animals are not able to digest fibre, the energetic value of fodder is lowered. Large amounts of fibre lower the utilization of nutrients, such as amino acids, therefore, whole oats are mostly fed to pigs and poultry with an addition of a suitable enzyme (SVIHUS and GULLORD, 2002; WELCH *et al.*, 1983).

This study confirmed the high fibre content of hulled oats. It is worth mentioning, however, that the STH 735 dwarf hulled oat contained significantly less fibre compared with the model hulled cultivar Krezus, in both nitrogen regimes

Table 2 - Effect of cultivar, nitrogen treatment and oat form on the chemical composition of oat grain (g·kg^{-1} DM).

Trait	N. fertilisation kg·ha^{-1}													
	Cultivar										Oat form			
	40					100								
	Polar^{NT}	STH 6102ND	Krezus^{HT}	STH 735^{HD}	Mean	Polar^{NT}	STH 6102ND	Krezus^{HT}	STH 735^{HD}	Mean	N	H	D	T
Dry matter (g·kg^{-1} fresh)	903*a*[1]	903*a*	902*a*	899*b*	901*A*[2]	906*a*	901*b*	899*c*	898*c*	901*A*	903*a*	899*b*	900*a*	902*a*
Ash	24,4*a*	22,6*b*	23,4*ab*	23,1*ab*	23,4*B*	24,5*ab*	25,1*a*	23,2*b*	24,2*ab*	24,2*A*	24,1*a*	23,4*a*	23,7*a*	23,9*a*
Crude protein (N×6.25)	153*a*	144*b*	108*d*	118*c*	131*B*	184*a*	172*b*	136*c*	139*c*	158*A*	163*a*	125*b*	143*b*	145*a*
Ether extract	89,5*a*	89,1*a*	47,7*c*	51,5*b*	69,4*A*	82,6*a*	82,5*a*	44,1*c*	51,5*b*	65,2*B*	85,9a	48,7b	68,6*a*	66,0*b*
Crude fibre	29,9*a*	28,5*a*	146,0*a*	128,2*b*	83,1*A*	29,3*a*	31,5*a*	139,4*a*	130,5*b*	82,7*A*	29,8*b*	136,0*a*	79,7*b*	86,1*a*
Nitrogen free extract	701*a*	716*a*	675*b*	679*b*	693*A*	680*a*	689*a*	657*b*	655*b*	670*B*	696*a*	667*b*	685*a*	678*b*
Sugars	13,8*d*	14,3*c*	16,2*b*	16,8*a*	15,3*B*	14,1*d*	14,5*c*	16,6*b*	17,4*a*	15,7*A*	14,2*b*	16,8*a*	15,8*a*	15,2*b*
Starch	597*a*	588*b*	550*c*	542*d*	569*A*	573*a*	566*b*	531*c*	532*c*	551*B*	581*a*	539*b*	557*b*	563*a*

Abbreviations: N – naked, H – hulled, D – dwarf, T – tall cultivar.

[1]Values for a common nitrogen levels and the oat forms within the row that bear the same letter do not differ significantly according to Tukey test (post hoc comparisons) and F test (planned comparisons) at p=0.05;

[2]means for the nitrogen effects in the same row followed by the same capital letter are not significantly different using Tukey test (post hoc comparisons) at p=0.05.

Table 3 - Effect of cultivar, nitrogen treatment and oat form on the fibre fraction (g·kg-1 DM).

Trait	N. fertilisation kg·ha^{-1}													
	Cultivar										Oat form			
	40					100								
	Polar^{NT}	STH 6102ND	Krezus^{HT}	STH 735^{HD}	Mean	Polar^{NT}	STH 6102ND	Krezus^{HT}	STH 735^{HD}	Mean	N	H	D	T
Neutral detergent fibre	184*b*	149*c*	403*a*	418*a*	288*A*	190*c*	152d	411*a*	376*b*	282*B*	169*b*	402*a*	274*b*	297*a*
Acid detergent fibre	53*b*	41*b*	185*a*	179*a*	115*A*	47*c*	41*c*	190*a*	173*b*	113*A*	45*b*	182*a*	109*b*	119*a*
Acid detergent lignin	4,6*b*	4,1*b*	23,4*a*	25,2*a*	14,3*A*	3,5*b*	4,7*b*	21,8*a*	20,9*a*	12,7*B*	4,2*b*	22,8*a*	13,7*a*	13,3*a*
Cellulose	49*b*	37*b*	161*a*	154*a*	100*A*	44*b*	36*b*	168*a*	152*a*	100*A*	41*b*	159*a*	95*b*	105*a*
Hemicellulose	131*b*	108*c*	219*b*	238*a*	174*A*	143*c*	111d	221*a*	203*b*	170*A*	123*b*	220*a*	165b	178*a*

See Table 2 for abbreviations and indicators.

(128,2 and 146,0 g·kg^{-1} DM at 40 N kg·ha^{-1} and 130,5 and 139,4 g·kg^{-1} DM at 100 N kg·ha^{-1}, respectively).

Table 3 shows the effect of cultivar's nitrogen treatment and the interaction between both factors on the selected samples of fibre which have a significant influence on the quality of fibre. By analising the amount of neutral detergent fibre and hemicellulose (Table 1), it became clear that the cultivars responded diversely to the change in nitrogen fertilisation. This was mainly due to a decrease in the STH 735 cultivar's properties at the higher nitrogen treatment in comparison with the basic nitrogen dose. The STH 6102 dwarf naked oat had a significantly lower concentration of NDF and ADF fractions (150 g and 41 g·kg^{-1} DM, respectively) after using average nitrogen treatments. Lignin content (ADL), which lowers feed digestibility and nutritional value the most, was ca. 80% lower in naked oat, compared with hulled oat. A similar trend may be observed in the amount of neutral detergent fibre as well as in acid detergent fibre, which was also reported by BRAND *et al.* (1996). A trend was noticed, for dwarf oats to have higher fibre content, compared with conventional oats. An increase in nitrogen treatment significantly lowered the levels of lignin (by 1.6 g·kg^{-1} DM) and NDF fraction (by 6.0 g·kg^{-1} DM). Furthermore, lower values of other fibre components were found when nitrogen rates were increased, however, the differences were not significant.

Oat protein had high levels of essential amino acids (Table 4). Cultivars, nitrogen fertiliser treatment and the interaction between them, all resulted in significant changes in amino acid composition (Table 4). The highest EAA content was detected in the STH 6102 dwarf naked oat (33.4 g·16 g^{-1} N, over nitrogen treatment). It is worth emphasizing that Krezus cultivar (tall hulled oat) had a significantly higher content of lysine (3.50 g·16 g N, over nitrogen treatment), i.e. the amino acid that most frequently limits the efficiency of cereal protein utilization (BOISEN *et al.*, 2000). On average, the naked cultivars were characterized by a significantly higher content of total and essential amino acids in comparison with the traditional hulled oat cultivars. Naked oats had higher levels of phe, thr, trp, asp, and glu and lower levels of pro compared to the hulled cultivars. The results indicate that the proteins from the naked grain have a higher nutritional value. PETERSON (1976) also reported that the higher cereal quality of oat proteins is due to a lower content of prolamine protein. On the other hand, the significant differences between tall and dwarf forms were obtained only for ser and pro content. The results of the studies conducted so far on the effect of nitrogen treatment, have not confirmed unequivocally the direction of changes in amino acid composition of oat protein in conditions of diverse supply of this nutrient (LÁSZTITY, 1998). In the studies conducted by GIVENS *et al.* (2004) increased nitrogen treatment (from 0 to 80-140 kg·ha^{-1}) led to increase in the concentration of all amino acids, proportional to the increased content of total protein. However, the authors also found that differences in the effect of nitrogen treatment, were not the same for hulled and naked oat. Regarding the non essential amino acids, a nitrogen rate of 100 kg·ha^{-1} resulted in an increased level of aspartic acid, glutamic acid and alanine (Table 4). Furthermore in this study, increased nitrogen treatment led to a significant rise in the levels of some essential amino acids (leu, phe, tyr), while only the content of lysine and cystine decreased significantly. Among the tested cultivars, STH 6102 (dwarf naked oat cultivar) showed the strongest positive response to increasing nitrogen fertilisation, especially for leu, phe, tyr, thr, gly, and ala.

Table 4 - Effect of cultivar, nitrogen treatment and oat form on the amino acid composition (g·16 g·N^{-1}).

Amino acid	N. fertilisation kg·ha^{-1}										Oat form			
	Cultivar													
	40					100								
	Polar^{NT}	STH 6102ND	Krezus^{HT}	STH 735^{HD}	Mean	Polar^{NT}	STH 6102ND	Krezus^{HT}	STH 735^{HD}	Mean	N	H	D	T
Essential amino acids														
Ile	2,77*b*	3,13*a*	2,93*ab*	2,89*ab*	2,93*A*	2,85*bc*	3,33*a*	2,98*b*	2,68*c*	2,96*A*	3,02*a*	2,87*a*	3,01*a*	2,88*a*
Leu	5,87*a*	6,22*a*	5,93*a*	5,99*a*	6,00*A*	6,07b	6,84*a*	6,24*b*	5,98*b*	6,28*A*	6,25*a*	6,03*a*	6,26*a*	6,03*a*
Lys	3,28a	3,49a	3,57a	3,39a	3,43*A*	3,28*a*	3,29*a*	3,43*a*	3,18*a*	3,29*B*	3,33*a*	3,39*a*	3,34*a*	3,39*a*
Met	1,30*b*	1,42*ab*	1,58*a*	1,27*b*	1,39*A*	1,52*a*	1,41*ab*	1,45*ab*	1,34*b*	1,43*A*	1,41*a*	1,41*a*	1,36*a*	1,46*a*
Cys	2,34*b*	2,23*b*	2,70*a*	2,30*b*	2,39*A*	2,36*a*	2,25*a*	2,22*a*	2,15*a*	2,24*B*	2,29*a*	2,34*a*	2,23*a*	2,40*a*
Phe	4,10*a*	4,41*a*	4,18*a*	4,12*a*	4,20*B*	4,43*b*	5,27*a*	4,46*b*	4,23*b*	4,60*A*	4,55*a*	4,25*b*	4,51*a*	4,29*a*
Tyr	2,90*a*	2,81*a*	2,86*a*	2,77*a*	2,83*B*	2,98*b*	3,44*a*	2,90*b*	2,95*b*	3,07*A*	3,03*a*	2,87*a*	2,99*a*	2,91*a*
Thr	2,91*ab*	2,99*a*	2,90*ab*	2,74*b*	2,88*A*	2,79*b*	3,39*a*	2,71*b*	2,61*b*	2,87*A*	3,02*a*	2,74*b*	2,93*a*	2,83*a*
Trp	1,27*a*	1,19*a*	1,06*a*	0,95*a*	1,12*A*	1,16*a*	1,17*a*	1,03*a*	0,93*a*	1,07*A*	1,20*a*	0,99*b*	1,06*a*	1,13*a*
Val	4,02*a*	4,18*a*	4,18*a*	4,06*a*	4,11*A*	4,11*a*	4,31*a*	4,11*a*	3,85*a*	4,09*A*	4,16*a*	4,05*a*	4,10*a*	4,10*a*
Nonessential amino acids														
His	1,82*a*	2,13*a*	2,06*a*	1,94*a*	1,99*A*	1,93*a*	2,06*a*	2,01*a*	1,83*a*	1,96*A*	1,99*a*	1,96*a*	1,99*a*	1,95*a*
Arg	6,95*a*	6,95*a*	7,04*a*	6,73*a*	6,92*A*	7,08*a*	7,08*a*	7,20*a*	6,44*a*	6,95*A*	7,01*a*	6,85*a*	6,80*a*	7,07*a*
Asp	8,03*a*	7,83*a*	7,85*a*	7,19*b*	7,72*B*	8,21*b*	8,87*a*	8,26*b*	7,57*c*	8,23*A*	8,24*a*	7,72*b*	7,86*a*	8,09*a*
Glu	21,1*a*	20,4*a*	19,7*a*	18,7*a*	19,9*B*	21,8*a*	21,6*a*	20,2*a*	19,1*a*	20,7*A*	21,2*a*	19,4*b*	19,9*a*	20,7*a*
Ser	3,78*a*	4,18*a*	4,04*a*	3,94*a*	3,98*A*	3,78*a*	4,32*a*	3,64*a*	3,87*a*	3,90*A*	4,02*a*	3,87*a*	4,08*a*	3,81*b*
Pro	4,45*c*	5,32*a*	4,72*bc*	4,95*b*	4,86*A*	4,35*c*	4,61*bc*	4,96*b*	5,46*a*	4,84*A*	4,68*b*	5,02*a*	5,08*a*	4,62*b*
Gly	4,42*a*	4,15*a*	4,46*a*	4,35*a*	4,35*A*	4,35*b*	4,85*a*	4,41*b*	4,17*b*	4,44*A*	4,44*a*	4,35*a*	4,38*a*	4,41*a*
Ala	4,05*ab*	3,72*b*	4,26*a*	4,26*a*	4,07*B*	4,00*b*	4,42*a*	4,26*ab*	4,30*ab*	4,24*A*	4,05a	4,27*a*	4,18*a*	4,14*a*
Total	85,3*a*	86,7*a*	86,0*a*	82,5b	85,1*B*	87,0*b*	92,5*a*	86,4*b*	82,6*c*	87,1*A*	87,9*a*	84,4*b*	86,1*a*	86,2*a*
EAA	30,7*bc*	32,1*a*	31,9*ab*	30,5*c*	31,3*B*	31,5*b*	34,7*a*	31,5*b*	29,9*c*	31,9*A*	32,3*a*	30,9*b*	31,8*a*	31,4*a*

See Table 2 for abbreviations and indicators.

Oat is a reservoir of mineral components, especially ash, that are of big importance from both nutritional and technological perspective. Ash content in the entire oat grain averaged 23.8 g·kg^{-1} DM. Table 5 presents the average concentration of selected mineral components in the tested oat grain. There was a significant difference in the content of mineral components between cultivars. The Krezus hulled cultivar had by far the highest content of calcium. Compared with the hulled oat, phosphorus levels where significantly higher while potassium and calcium levels, significantly lower in both naked oat cultivars. The highest level of sodium was detected in the STH 735 dwarf hulled oat. Compared with the tall forms, the

Table 5 - Effect of cultivar, nitrogen treatment and oat form on the mineral composition of oat grain (g·kg $^{-1}$ DM).

Trait	N. fertilisation kg·ha^{-1}										Oat form			
	Cultivar													
	40					100								
	Polar^{NT}	STH 6102ND	Krezus^{HT}	STH 735^{HD}	Mean	Polar^{NT}	STH 6102ND	Krezus^{HT}	STH 735^{HD}	Mean	N	H	D	T
Calcium	0,98*c*	1,05*bc*	1,38*a*	1,26*b*	1,17*A*	0,88*c*	1,36*ab*	1,43*a*	1,16*b*	1,21*A*	1,07*b*	1,31*a*	1,21*a*	1,17*a*
Phosphorus	4,65*a*	4,15*a*	3,27*b*	3,65*b*	3,93*B*	4,79*a*	4,78*a*	3,59*b*	3,89*b*	4,27*A*	4,59*a*	3,60*b*	4,12*a*	4,08*a*
Potassium	2,49*b*	2,12*b*	3,66*a*	3,59*a*	2,96*A*	2,35*c*	2,67*c*	4,12*a*	3,31*b*	3,11*A*	2,41*b*	3,67*a*	2,92*a*	3,16*a*
Sodium	0,16*b*	0,16*b*	0,15*b*	0,20*a*	0,17*A*	0,15*b*	0,19*a*	0,15*b*	0,19*a*	0,17*A*	0,16*a*	0,17*a*	0,19*a*	0,15*b*

See Table 2 for abbreviations and indicators.

Table 6 - Effect of cultivar, nitrogen treatment and oat form on the predicted energy value (MJ·kg^{-1} DM).

Trait	N. fertilisation kg·ha^{-1}													
	Cultivar										Oat form			
	40					100								
	Polar^{NT}	STH 6102ND	Krezus^{HT}	STH 735^{HD}	Mean	Polar^{NT}	STH 6102ND	Krezus^{HT}	STH 735^{HD}	Mean	N	H	D	T
Digestible energy	16,7*b*	17,2*a*	12,7*c*	12,6*c*	14,8*B*	16,8*b*	17,2*a*	12,7*d*	13,3*c*	15,0*A*	17,0*a*	12,8*b*	15,1*a*	14,7*b*
Apparent metabolisable energy	15,6*a*	15,3*b*	12,7*d*	12,9*c*	14,1*A*	15,4*a*	15,1*b*	12,7*d*	13,0*c*	14,1*A*	15,4*a*	12,8*b*	14,1*a*	14,1*a*

See Table 2 for abbreviations and indicators.

dwarf cultivars had a higher content of sodium and a similar content of calcium, phosphorus, and potassium. The increase in nitrogen treatment did not have a significant affect on the content of calcium, potassium, or sodium. Due to the increasing nitrogen treatment there was a significant increase in the phosphorus content of the oat grain, thereby confirming the findings reported by GIVENS *et al.* (2004). A significant interaction between cultivars and nitrogen treatment was detected in calcium, potassium and sodium levels. Increasing nitrogen fertilisation up to 100 kg·ha^{-1} resulted in a higher calcium content in the STH 6102 cultivar and higher potassium content in Krezus cultivar in comparison with other cultivars.

The naked cultivars had significantly higher energy value for pigs (Table 6). The dwarf cultivar had the highest DE level (17 MJ·kg^{-1} DM). A similar relationship was found in the studies conducted by BRAND *et al.* (2003) for pigs with the use of mobile nylon bags. They reported higher digestible energy for naked oat compared with hulled oat (18.0 and 12.6 MJ·kg^{-1} DM, respectively). Due to increased concentration of energy and high quality protein, naked oat may be a valuable component of pig feed (MORRIS and BURROWS, 1986). GIVENS *et al.* (2004) reported higher DE for hulled oat (on average 14 MJ·kg^{-1} DM), which probably resulted from a higher fat content than that found in this study (64.5 and 48.7 g·kg^{-1} DM, Table 2). The dwarf forms of oat had slightly higher values of digestible energy. In this study, increased nitrogen treatment significantly affected the digestible energy value as a result of higher crude protein concentration.

Hulled oat is reluctantly used as poultry feed due to it's high fibre content. The lack of hull in naked oat cultivars enables them to be used as feed. In this study, AME value for the hulled cultivars averaged 12.8 MJ·kg^{-1} DM and for the naked cultivars it was significantly higher – 15.4 MJ·kg^{-1} DM. This trend was also reported by GIVENS *et al.* (2004). Studies conducted by CAVE and BURROWS (1993) indicated that naked oat may be applied in feeding poultry.

The results obtained in this study indicate that oat cultivar and the amount of nitrogen treatment have a significant effect on chemical composition and energy value of grain. On the basis of this study it is difficult to determine clearly whether straw shortening by introducing the *Dw6* dwarf gene affects the quality properties of grain.

In this study, an improvement in the fibre (NDF, ADF, cellulose and hemicellulose) composition of dwarf forms was observed but for the other tested properties, it seems that the genotype of an individual cultivar was the decisive factor. Similarly ambiguous results were reported by KIBITE and CLAYTON (2000) who tested 7 pairs of oat near-isogenic lines, dwarf vs. tall. They observed adverse effects of the *Dw6* gene on fat content, however, protein content was dependent on the genetic background, not the dwarf gene. In conclusion, it is worth emphasizing that in this study the STH 6102 short naked oat, with an introduced dwarf gene had the highest quality properties of grain, i.e. high quality protein and significantly higher content of essential amino acids. The tested naked oat also had a beneficial fibre content in terms of NDF and ADF levels. Considerably lower fibre content and its beneficial composition, as well as an increased fat level affected the energy content of this cultivar. The STH 6102 oat had a high energy content for both pigs (DE) and poultry (AME).

ACKNOWLEDGEMENTS

The Authors wish to thank L. Kowalczyk for help with chemical analysis. We are also grateful to dr. Z. Nita of the Strzelce Plant Breeding Station for his co-operation and the providing of the seed samples.

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Paper received July 28, 2010 Accepted December 27, 2010

PAPER

FATTY ACID COMPOSITION OF SEED OILS FROM IMPORTANT SPANISH POMEGRANATE CULTIVARS

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ABSTRACT

The seed fatty acids composition from five Spanish pomegranate cultivars was determined. Pomegranate seeds yielded oil contents ranging from 69-81 g kg^{-1} of dry matter for sweet *cv.* to 131 g kg^{-1} and 105 g kg^{-1} for the sour-sweet and sour *cv.*, respectively. The highest levels of fatty acids were those of polyunsaturated ones (from 80.41 to 91.03%). The predominant unsaturated fatty acid was punicic (66.76-79.29%). Data on punicic acids were reported here for the first time for sour and sour-sweet Spanish pomegranate *cv.* This relatively high quantity of unsaturated fatty acids could be of interest for human nutrition and may definitively drive breeding selection programs.

- Key words: fatty acids, lipids, pomegranate, punicic acids -

INTRODUCTION

Although pomegranate (*Punica granatum* L.) seeds are mainly fresh consumed, they have been lately used for processing jams, jellies, refreshing juices and alcoholic drinks, as well as for colouring and flavouring drinks. Several researchers have already investigated the potential of pomegranate fruit for minimally processed ready to use fresh seeds and possible seed oil processing (MELGAREJO *et al.*, 1995, GIL *et al.*, 1996 and ARTÉS *et al.*, 1998). Moreover, there is an incipient interest worldwide in the functional and health benefits of pomegranate fruit (SUMNER *et al.*, 2005).

Seeds in pomegranate fruit are often abundant depending on the cultivar. They account for 50 to 70% of fruit fresh weight, including membranes and skin. The hard portion of pomegranate seeds (*tegmen*, cotyledons and embryo) represents 5-15% of the seed and is relatively rich in fibre and fat. The large fraction of seeds is the juicy *testa* (arils), being rich in soluble solids, sugars, pigments and organic acids.

From now on, and for study purposes, we will only refer as seed to the hard portion of the conventionally named pomegranate seed.

The seeds of diverse pomegranate cultivars are a rich source of lipids (FADAVI *et al.*, 2006 and KIRALAN *et al.*, 2009). Depending on the cultivar, lipids contents may range from 140 to 270 g kg^{-1} of dry matter (EL-SHAARAWY and NAHAPETIAN, 1983; EL-NEMR *et al.*, 1990; MELGAREJO, 1993; KIRALAN *et al.*, 2009). Pomegranate seed oil is also a natural source of punicic acid, representing about 80% of its total fatty acid composition (YAMASAKI *et al.*, 2006).

The determination of total lipid content along with the constituent fatty acids is an important mean of assessing pomegranate suitability for specific food and industrial utilisation (EKPA *et al.*, 1994). Consumers are particularly concerned about the saturated to unsaturated fatty acid ratio, and about contents of essential fatty acids as linoleic, linolenic and arachidonic, with especial emphasis on polyunsaturated ones. These fatty acids play a natural preventive role in cardiovascular disease and alleviating other health disorders (DE HOYA and MATA, 1989); they promote the reduction of both total and HDL cholesterol (MELGAREJO and ARTÉS, 2000). Furthermore, pomegranate seed oil and extracts might be used as an alternative or supplement (phytoestrogen medicaments) to conventional hormone replacement therapy in menopausal women (LANSKY, 1999).

Pomegranate seed oil contains considerable amounts of polyunsaturated fatty acids (PUFA), including conjugated linolenic acid (CLA) isomers as well as low saturated fatty acid content (YÜCEL, 2005). CLA isomers may prevent cancer incidence (YASUI *et al.*, 2005 and IGARASHI and MIYAZAWA, 2000). Furthermore, consumption of oils rich in highly unsaturated fatty acids (HUFA) protects against diseases such as colorectal, breast, colon and prostate cancers (GERBER *et al.*, 2005).

Punicic acid may also have beneficial anti-inflammatory effects. Oral administration of pure punicic acid, or pomegranate seed oil rich in punicic acid to rats prevented induced colitis and lowered induced-tissue damage (BOUSSETTA *et al.*, 2009).

The total lipid content plays an important role in several biochemical and nutritional studies. Not only the amount, but the type of fat has also an intense effect on human health. Determining the fatty acid profile of a substance helps in deciding its end use for both food and non-food applications.

Remarkable differences were found among diverse studies on the constituent fatty acids of pomegranate seeds (EL-SHAARAWY and NAHAPETIAN, 1983: EL-NEMR *et al.*, 1990; MELGAREJO *et al.*, 1995; MELGAREJO and ARTÉS, 2000; KIRALAN *et al.*, 2009; PANDE and AKOH, 2009). These compositional differences could be linked to agronomic and environmental effects as well as to different genotypes.

Thus, the goal of this study was to determine the total lipid content and the constituent fatty acids of five Spanish pomegranate *cv.*, cultivated under homogeneous conditions to prevent potential agronomic and environmental effects on plant genotypes. As far as we know, this probable aspect has not been carefully considered in any experiment previously reported. Moreover, the attained results could help breeders to select pomegranate cultivars according to not only external fruit quality and organoleptic traits (size, colour and appearance, soluble solids content, acidity and firmness), but also to total lipid content and constituent fatty acids, satisfying then consumers demand on beneficial fruit effects on human health.

MATERIALS AND METHODS

Pomegranate cultivars

The selected plant material belongs to the main pomegranate gene bank of the EU, located at the experimental field station of Miguel Hernandez University, in the province of Alicante (Spain) (02º03´50´´E, 38º03´50´´N, and 25 masl). The pomegranate collection held 64 different cultivars, with four repetitions of each and cultivated under homogeneous conditions. Established in 1992, the orchard is settled in a typical agricultural area close to the Mediterranean coast, obviously under the influence of moderate climatic conditions (MELGAREJO *et al.*, 1998). Conventional and commercial summer pruning along with drip irrigation and standard cultural practices were performed.

The studied cultivars were selected according to either their fresh consumption traits (sweet and sour-sweet *cv.*) or ornamental and processing aptitudes (sour *cv.*). While cultivars VA1 (Valenciana 1), ME16 (Mollar de Elche 16) and MA2 (Mollar de Albatera 2) were sweet, PTO8 (Piñón Tierno de Ojós 8) and BA1 (Borde de Albatera 1) were sour-sweet and sour cultivars, respectively. The complete pomological description of these pomegranate cultivars was previously reported by MELGAREJO (1993).

From mid-September to the end of October, fully ripe pomegranate fruits were harvested by hand during two consecutive years (2008-2009). Approximately 15 kg of fruit per cultivar were sampled as follows: 10 fruits per tree (5 located externally and five within tree canopy), and 4 trees per cultivar (n=40). Harvested fruits were taken to the laboratory afterwards, where those defective or even damaged were rejected (sunburns, cracking and bruises).

The following physical-chemical parameters were analysed at harvest to characterise the diverse *cv.*: size, weight, seeds, soluble solids content and titratable acidity of juice, as well as maturity index (data not shown).

Fatty acid extraction and analysis

Analytical methods were applied for preparing samples and methyl esters of the fatty acids. Preparation, extraction, separation and identification were performed as follows: triplicate samples of fresh seeds per cultivar (100 g each) were dried out in an oven for two days at 60°C; after drying, seeds were crushed and refluxed with 300 ml of petroleum ether in weighed flasks using a Soxhlet apparatus according to the AACC (1987). Lipids were recovered by distilling petroleum ether in a rotary evaporator at 60°C, and then dried out to constant weight in a vacuum oven at 90°C for 1 hour and weighted. Extracted lipids were dissolved with ethyl ether and kept at room temperature following the AACC protocol (1987).

Fatty acid methyl esters (FAMEs) were prepared according to the AOAC method (1990). Triglyceride purification was accomplished by evaporating until completely dried. Samples were resolved in hexane:ether (90:10 v/v) under a nitrogen flow, and kept in a silica column of 20 cm length and 0.9 cm diameter after elution with 50 ml hexane:ether (90:10 v/v). Every lipid sample (0.1 g) was methylated by transesterification with alcoholic KOH 2N (Merck Co, Darmstard, Germany). Methyl esters samples (1 µL) were analysed by a Hewlett-Packard gas-liquid chromatograph (5890 Series II, USA), with a silica funded column (DB-WAX, J&W scientific) of 25 m length, 0.25 mm diameter and 0.25 µm film thickness. The initial temperature was 140°C for 2 min, and then a 4°C ramp until 200°C for 20 min; nitrogen was the carrier gas (1 ml/min) with a 1:70 split relation. Methyl esters were derivatized to fix the unsaturating positions; it was accomplished by obtaining the oxazoline derivatives reaction (DMOX) with 2-amino-2 methyl 1 propanol. Samples of methyl esters and DMOX were analyzed by a Hewlett-Packard 5890 Series II gas-liquid chromatograph coupled to a high resolution mass spectrometer (Finnigan MAT 95, USA), equipped with a DB-WAX column similar to that previously described. Temperatures of the analyses were: injector 250°C, initial 180°C, rate 2°C/min until 220°C (240°C for DMOX derivates) for 20 min. Mass spectrums were accomplished by ionization: electronic impact at 70 eV with a resolution of 2,300 and a sweep range of 50-600 amu at 2 seg/dec.

Peaks were identified according to their retention times. All samples were run by triplicate using standard fatty acids methyl esters. All solvent/chemicals used were from Sigma (MO, USA) and Merck (Germany).

Statistical analysis

Analysis of variance (ANOVA) and Duncan's multiple range test were performed. The software package SPSS v.18.0 for Windows was used to evaluate the significance of differences among samples regarding oil contents and individual fatty acids composition at a confidence level of $p \leq 0.05$. Samples were analysed by triplicate and data expressed as mean values ± standard deviation.

RESULTS AND DISCUSSION

Total lipids and fatty acid composition are given in Table 1. Pomegranate seeds yielded total lipids contents ranging from 69-81 g kg^{-1} of dry matter for sweet *cv.* (VA1, ME16 and MA2) to 131 g kg^{-1} and 105 g kg^{-1} for the sour-sweet PTO8 and sour BA1, respectively.

Whereas the results on lipid contents agreed with those obtained by FADAVY *et al.*, (2006) dealing with Iranian cultivars, they differed from those found by EL-NEMR *et al.*, (1990) for an unidentified Egyptian cultivar (272 g kg^{-1} total lipids), and from these attained by EL-SHAARAWY and NAHAPETIAN (1983) for the Iranian sweet cultivar called *Shiring* (161 g kg^{-1} total lipids).

As previously said by FADAVI *et al.*, (2006), the Spanish pomegranate cultivars showed the following lipid content relation: sweet < sour < sour-sweet.

Other studies revealed remarkable differences in total lipid contents among pomegranate cultivars grown in Turkey, Iran and Georgia (13.95-24.13, 6.63-19.3 and 18.1-21.5%, respectively) (YÜCEL, 2005; KIRALAN *et al.*, 2009; PANDE and AKOH, 2009). This notable compositional divergence could be linked to agronomic and environ-

Table 1 - Lipid content (g kg^{-1} dry matter) and fatty acid composition of pomegranate seed oils (fatty acids esters as % w/w total fatty acid esters).

	No.	Cultivars	Lipids	Palmitic (C16:0)	Stearic (C18:0)	Oleic (C18:1)	Linoleic (C18:2)	Punicic (C18:3)	Σ Sat.	Σ Unsat.	Sat./Unsat.
Sweet	**1**	**ME16**	80.9±1.98^{a}	3.83±0.25^{a}	2.38±0.39^{a}	5.91±0.44^{ab}	7.74±0.32^{ab}	66.76±2.40^{a}	6.21±0.73^{ab}	80.41±1.53^{a}	0.077±0.01^{a}
	2	**MA2**	79.9±1.68^{a}	4.08±0.45^{a}	1.93±0.19^{ab}	6.85±0.58^{a}	8.54±0.42^{a}	75.28±1.96^{b}	6.02±0.54^{ab}	90.67±2.09^{b}	0.066±0.01^{ab}
	3	**VA1**	68.9±1.93^{b}	3.63±0.33^{ab}	1.60±0.15^{b}	5.23±0.41^{b}	7.30±0.45^{b}	78.50±1.18^{b}	5.23±0.24^{a}	91.03±2.18^{b}	0.057±0.01^{b}
Sour sweet	**4**	**PTO8**	130.9±2.49^{c}	4.30±0.52^{a}	2.62±0.29^{b}	6.49±0.58^{a}	7.48±0.23^{b}	75.39±1.81^{b}	6.92±0.41^{b}	89.36±1.70^{b}	0.077±0.01^{a}
Sour	**5**	**BA1**	104.9±2.20^{d}	2.99±0.47^{b}	1.64±0.23^{b}	4.70±0.33^{b}	4.98±0.40^{c}	79.29±1.90^{b}	4.63±0.40^{a}	88.97±2.76^{b}	0.052±0.01^{b}

Data shown as mean values±S.D. of three replicates. Different letters within the same column indicate significant differences among cultivars at the p≤ 0.05 level.

mental effects on pomegranate trees, as well as to genotypic differences. However, all cultivars in this study were cultivated under homogeneous growing conditions, and differences in total lipid contents and compositional fatty acid profiles should be entirely attributed to genotypic backgrounds.

Only five fatty acids were identified in all evaluated cultivars. Fatty acid compositional profiles are shown in Table 1 (fatty acids esters as % w/w total fatty acid esters).

The major saturated fatty acid in all cultivars was palmitic, which ranged from 2.9 to 4.3% for the sour BA1 and sour-sweet PTO8, respectively. These palmitic percentages were similar to those obtained by KIRALAN *et al.*, (2009) and YÜCEL (2005), 2.10-2.77 and 2%, respectively. On the contrary, the amounts of palmitic acid were lower than those obtained by MELGAREJO *et al.* (1995), MELGAREJO and ARTÉS (2000), and FADAVI *et al.* (2006), with palmitic percentages of 18.16-22.63, 2.58-14.91 and 2.8-16.7%, respectively.

The following saturated fatty acid was stearic (1.6% for the sour BA1 and 2.62% for the sour-sweet PTO8). These percentages were similar to those reported by YÜCEL (2005), FADAVI *et al.* (2006), and KIRALAN *et al.* (2009).

Arachidic and behenic acids were undetected in any evaluated cultivar. However, YÜCEL (2005), MELGAREJO and ARTÉS (2000) and KIRALAN *et al.* (2009) identified arachidic acid in pomegranate seed oil (3.0, 0-2.76 and 0.33-0.48%, respectively). Likewise, FADAVI *et al.* (2006) and KIRALAN *et al.* (2009) detected the presence of behenic acid (0-3.9 and 0.16-0.22%, respectively). The divergence in saturated fatty acid profiles could be certainly attributed to genotypic backgrounds.

Unsaturated fatty acids were predominant in all cultivars. The highest levels of fatty acids were those of polyunsaturated ones (80.41-91.03%), and between 66.76-79.29% were C18:3 fatty acids with double bonds in the 9, 11 and 13 positions, named all as punicic acids. These punicic acids were identified at different retention times, indicating the existence of different geometric isomers because of the invariable double bond positions. TAKAGI (1966) and KIRALAN *et al.* (2009) confirmed the presence of these and other isomers of the C18:3 fatty acid: specifically positions 9-*cis*, 11-*trans* and 13-*trans* (α-eleostearic acid), 9-*trans*, 11-trans and 13-*trans* (β-eleostearic acid), and 9-*trans*, 11-*trans* and 13-*cis* (catalpic acid). However, all identified isomers in the present study were considered as punicic acids.

The sour BA1 showed the highest content in punicic acid while sweet ME16 the lowest one. Results for sweet *cv.* confirmed previous findings obtained by EL-SHAARAWY and NAPETIAN (1983) (67.6% of punicic acids content). Furthermore, our results for sweet, sour-sweet and sour *cv.*

were similar to those obtained by FADAVI *et al.* (2006), KIRALAN *et al.* (2009), and PANDE and AKOH (2009). Data on punicic acids were reported here for the first time for sour and sour-sweet Spanish pomegranate *cv.*

The second most abundant fatty acid in all cultivars was linoleic, ranging from 4.98 (BA1) to 8.54% (MA2), followed by oleic acid (4.7-6.8%). Oleic and linoleic contents were similar to those of previous studies (MELGAREJO and ARTÉS, 2000; FADAVI *et al.*, 2006; KIRALAN *et al.*, 2009).

Unlike those detected by EL-NEMR *et al.* (1990), MELGAREJO and ARTÉS (2000), YÜCEL (2005) and KIRALAN *et al.* (2009), the presence of other fatty acids in pomegranate seed oil could not be confirmed [hexanoic (C6:0), octanoic (C8:0), decanoic (C10:0), lauric (C12:0), mirystic (C14:0), mirystoleic (C14:1), palmitoleic (C16:1), arachidic (C20:0), behenic (C22:0), lignoceric (C24:0) and gadoleic (C20:1)]. These compositional differences on the constituent fatty acids of pomegranate seeds could be positively linked to agronomic and environmental effects as well as to different genotypes (THOMPSON *et al.*, 1973; HOLADY and PEARSON, 1974; HARRIS *et al.*, 1978; ROBERTSON *et al.*, 1978; LAJARA *et al.*, 1990).

The evaluated Spanish cultivars were cultivated under homogeneous growing conditions, showing different fatty acid profiles of pomegranate seed oils than those Spanish *cv.* studied by MELGAREJO *et al.* (1995) and MELGAREJO and ARTÉS (2000), which were grown under heterogeneous and different climatic and environmental conditions.

The saturated/unsaturated fatty acid ratio was usually very low (Table1). These ratios were similar to those previously reported by MELGAREJO and ARTÉS (2000), FADAVI *et al.* (2006), and KIRALAN *et al.* (2009).

Moreover, some intervarietal differences regarding fatty acids compositions were established. The sour BA1 *cv.* showed the lowest content in oleic (C18:1), linoleic (C18:2) and palmitic (C16:0) acids and the highest one in punicic acid. While the sweet ME16 *cv* showed the lowest content in punicic acids, the sour-sweet PTO8 *cv.* showed high contents in oleic, linoleic and punicic acids.

CONCLUSIONS

Results clearly indicate that evaluated pomegranate seeds contained considerable amounts of oil depending on cultivars: sweet < sour < sour-sweet. Besides, it can be stated that unsaturated fatty acids were predominant in all evaluated Spanish pomegranate cultivars. The saturated/unsaturated fatty acid ratio was very low, ranging from 0.055 to 0.077. This relatively high quantity of unsaturated fatty acids could be of interest for human nutrition and may definitively drive breeding selection programs, in terms of pomegranate *cv.* lipid profiles.

Furthermore, it can be acknowledged that Mediterranean pomegranates differed from other Spanish and foreign cultivars with regards to fatty acid compositional profiles.

Finally, and since all evaluated pomegranates were grown under homogeneous conditions, differences in total lipid contents and fatty acid compositions among cultivars must be entirely due to genotypic backgrounds.

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Paper received September 2, 2010 Accepted November 26, 2010

SHORT COMMUNICATION

PROTEIN DENATURATION DURING THE PROCESSING OF SAUSAGE PREPARED WITH NILE TILAPIA FILLET AND MINCED FISH

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ABSTRACT

The objetive of this paper was to study changes in the state of the protein during the production of sausage with up to 100% of minced fish (MF) tilapia (*Oreochromis niloticus* L.). Differential scanning calorimetry (DSC) was used to study protein denaturation. The fillets and MF showed no difference in the myosin denaturation temperature, although the actin denaturation temperature was higher in the fillet than in the MF. After comminution, the denaturation temperature of actin decreased from 73,4° to 70,6°C when MF increased from 0 to 100%. The emulsification process caused an increase in the myosin and actin denaturation temperatures. The proteins of the sausages were denatured after approximately 1 h 20 min of cooking with a final temperature of 72°C.

- Key words: myosin, actin, fish sausage, DSC -

INTRODUCTION

Nile tilapia (*Oreochromis niloticus*) is one of the fish species most produced in Brazil, and production reached nearly 70 thousand tones in 2005 (FAO, 2006). However the fillet yield is considered low (30 to 35%) (GARDUÑO-LUGO *et al.*, 2003), consequently generating a large amount of waste. Filleting waste may be used for the extraction of minced fish (MF) (KRISTBERGSSON and ARASON, 2007), which is used as a raw material in the development of diverse products, amongst which cooked sausages (RAJU *et al.*, 2003; HU *et al.*, 2008; SINI *et al.*, 2008).

The production of these sausages implies in the formation of a good-quality gel, and for this the myofibrillar proteins must be in the non-denatured state before thermal processing, which will be responsible for the gel formation (SAEED and HOWELL, 2004). Thus a study of the denaturation of the proteins before sausage processing is of interest because the state of muscle proteins can be affected during frozen storage of the raw material (JENSEN *et al.*, 2003), or even during surimi processing (CHAN *et al.*, 1992). Some products such as sugars, phosphates and sorbitol, when mixed with meat, help protect the protein against denaturation during frozen storage, but they also affect protein denaturation during processing (PARK and LANIER, 1987; SYCH *et al.*, 1990, HERRERA *et al.*, 2001).

The denaturation of proteins is a kind of order-disorder transition, which occurs when proteins are folding-unfolding due to some external agent (WRIGHT *et al.*, 1977; WALSTRA, 2003; MEDINA-VIVANCO *et al.*, 2007). Knowledge of the protein denaturation temperature and the enthalpy is useful to establish the final cooking temperature, as well as knowledge concerning the heat transfer rate and the cooking process of some products (PAREDI *et al.*, 1994).

The degree of protein denaturation can be measured from several indicators such as the myosin ATP-ase activity, solubility, availability of sulphide groups, viscosity, fluorescence and differential scanning calorimetry (DSC) (BEAS *et al.*, 1991; PAREDI *et al.*, 1994; FURUKAWA *et al.*, 2004), and the latter is widely used. The DSC technique is used in the determination of thermodynamic data, energy and the denaturation temperature of proteins under some industrial processing conditions (NAGAJ *et al.*, 1999).

The purpose of this paper was to study the changes in protein state during the processing of fish sausage produced with Nile tilapia fillet and minced fish obtained from the filleting waste. Thus the effects of the processes (comminution, emulsification and cooking) and the formulations (0, 20, 40, 60, 80 and 100% of minced fish in substitution of the fillet) on protein denaturation were studied using differential scanning calorimetry (DSC).

MATERIAL AND METHODS

Raw materials

The frozen tilapia fillets and filleting wastes from Nile tilapia (*Oreochromis niloticus*) were purchased on the local market and transported in thermal boxes to the laboratory. The wastes were composed of vertebral spinal columns without the head, skin and viscera. Nearly 24 h before processing, the wastes were thawed (7°±0.3°C), washed with water, cut in the abdominal region to remove excess fat and treated in an industrial fish deboning machine (HT 250, High Tech, Chapecó, Brazil). In this machine, a screw forced the filleting waste against separator blades, producing minced fish (MF) with a yield of 53%. The MF was packed into plastic bags, each containing 500 g of mass, and frozen (-40°C) in an ultra-fast plate freezer (UCE - 20, Eco, São Paulo, Brazil), for approximately 1 h 15 min.

Formulations

In the sausage formulations, the tilapia fillet was replaced by MF in different proportions: 0, 20, 40, 60, 80, and 100%. Other ingredients used were 4% of soy protein isolate (Nutrisoy, Colombo, Brazil), 2% of cassava starch, 1% of salt and other commercial additives, such as 0.25% curing salt (Cura, Ibrac, Rio Claro, Brazil), 0.5% antioxidant (Ibracor – serie 500, Ibrac, Rio Claro, Brazil), 0.25% stabilizer (Acordini – serie 700, Ibrac, Rio Claro, Brazil), 1% sausage seasoning (Kerry, Rio Claro, Brazil), and 2% natural onion.

Sausage processing

The raw materials (MF and fillet) were thawed in a refrigerator (7°±0.3°C) for approximately 24 h, weighed, comminuted and emulsified in a cutter (V80, Tecmafrig, São Paulo, Brazil) for 5 min together with the other ingredients. The temperature of the emulsion at the cutter outlet was 1°C. The emulsions were then filled into cellulose casing with a diameter of 25 mm (Viscofan, São Paulo, Brazil) with the aid of a pneumatic sausage filler (V25, Sirman, Curtarolo, Italy), handtied and cooked in an oven (SL 218, Arprotec, Valinhos, Brazil) with direct steam injection to an internal temperature of 72°C, attained in approximately 1 h 20 min (P410 digital thermometer, Incoterm, Porto Alegre, Brazil) (UYHARA *et al.*, 2008). After cooking, the sausages were cooled by spraying with cold water to an internal temperature of 40°C (P410 digital thermometer, Incoterm, Porto Alegre, Brazil), the casings manually removed, and the sausages then vacuum packaged (MI 60, Selovac, São Paulo, Brazil) in plastic bags (polyamide/polyethylene with a medium barrier to oxygen and water vapor) measuring 15 x 35 x 0.018 mm, (Selovac, São Paulo, Brazil) and stored in

a controlled temperature refrigerator (0°±0.3°C) until analyzed.

Protein and moisture contents

The protein and moisture contents of the raw materials (fillet and MF) and sausages were determined in triplicate according to the AOAC method (1999). The crude protein was determined using the micro-Kjeldahl method (N x 6.25) and the moisture gravimetrically determined in an oven at 105°C for 16 h.

Differential scanning calorimetry (DSC)

To evaluate the state of the proteins in the various processing steps, the samples were analysed by differential scanning calorimetry (DSC TA2010, controller TA5000, TA Instruments, New Castle, USA) operating at a flow rate of 45 mL/min of N_2, heating rate of 10°C/min, between 0° and 100°C, with a sealed empty pan as reference (FURUKAWA *et al.*, 2004). The instrument was calibrated with indium (T = 156.6°C and ΔH = 28.71 J/g). The samples, about 10 mg, were placed in hermetically sealed aluminum pans (TA Instruments) and weighed (±0.01 mg) on a precision scale (Ohaus, Analytical Plus, New Jersey, USA). The denaturation temperature (Td) and enthalpy (ΔHd) were identified as the peak temperature and area, respectively, on the endotherms, calculated using the Universal Analysis software (V.2.5H, TA Instruments, New Castle, USA). These tests were performed in duplicate on the raw materials, on the product obtained after comminution (mixtures) in the cutter, on the emulsion before filling and on the cooked sausages.

Experimental design
and statistical analysis

The experimental design was completely randomized for the different samples: raw materials (fillet and MF), mixtures, after comminution in the cutter, emulsions and sausages (0, 20, 40, 60, 80, and 100% of MF), with two replicates. The data were analyzed by ANOVA and whenever significant differences ($p<0.05$) were found, the Tukey test was applied. The statistical analyses were performed using the SAS statistical program version 9.1.3 (SAS/STAT®, Cary, USA).

RESULTS AND DISCUSSION

Protein and moisture contents

In order to explain the results of the differential scanning calorimetry (DSC) analyses, one must know the protein and moisture contents of the samples. It is worth mentioned that the sausages from all the treatments showed protein levels between 15.4 and 20.8%, above the minimum value required by the Brazilian legislation for regular sausages (made with other meats), which is 12% (UYHARA *et al.*, 2008), which makes this production interesting (Table 1). The protein levels of the samples were used to calculate the protein denaturation enthalpy (ΔHd) in joules/gram of protein.

Results of the DSC analysis

The results of all the DSC analyses are shown in Fig. 1, and the values relative to all the phenomena observed in these thermograms are presented in Table 2.

The DSC thermogram of the tilapia fillet (Fig. 1.1a), which may be considered as the control in order to compare those obtained with the processed samples, shows its first inflection (analyzing from low to high temperature) at 47.2°C, which may be associated with some fragments of myosin. Two clear endotherms were then observed one at 54.0°C, caused by denaturation of the myosin and the other at 73.9°C, associated with denaturation of the actin. At higher temperatures, an inflection in the baseline was observed at 84.3°C, which may have been a result of the denaturation of some actin fragments, followed by an exothermic peak at 94.0°C, which is probably related to the phenomenon of aggregation of the myofibrillar proteins (PARK and LANIER, 1987; PAREDI *et al.*, 1994; HERRERA *et al.*, 2001).

Table 1 - Moisture and protein content (%) of the samples[1] prepared for differential scanning calorimetry (DSC).

		Moisture (%)[1]	Protein (%)[1]
Raw material	Tilapia fillet	78.9±2.7	18.7±1.1
	Minced fish (MF)	75.5±0.2	12.8±0.6
Mixtures (%MF)	0	80.8±0.1	18.0±0.8
	20	81.1±0.1	14.9±0.2
	40	80.4±0.2	14.0±0.2
	60	79.0±0.3	13.3±0.3
	80	77.0±2.1	13.1±1.0
	100	75.6±0.3	12.3±0.1
Emulsion (%MF)	0	73.6±3.2	18.8±2.3
	20	73.3±2.3	17.9±1.8
	40	73.1±1.5	16.9±1.5
	60	72.6±0.8	16.1±1.4
	80	72.4±0.6	15.0±1.0
	100	71.7±0.9	13.7±0.7
Sausages (%MF)	0	71.5±3.7	20.8±2.5
	20	71.3±2.7	19.7±2.1
	40	70.8±1.6	18.7±1.7
	60	70.6±0.1	17.7±1.1
	80	70.5±0.6	16.1±0.4
	100	69.8±0.6	15.4±0.5

[1]Average ± standard deviation of three samples.

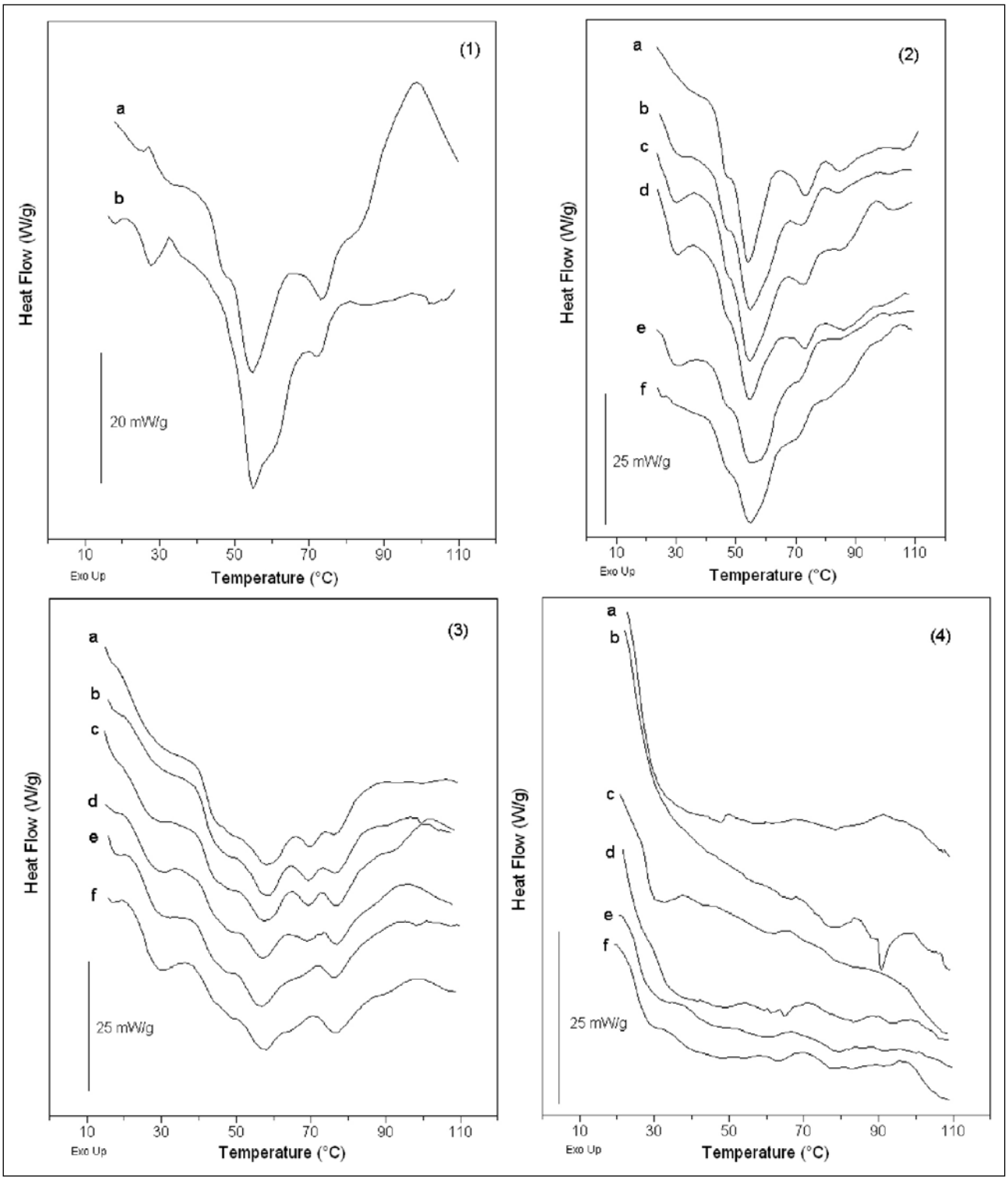


Fig. 1 - Examples of DSC curves of raw materials (1) (fillet-a, and minced fish (MF)-b), mixtures (2) (a-0%, b-20%, c-40%, d-60%, e-80% and f-100% of MF), emulsions (3) (a-0%, b-20%, c-40%, d-60%, e-80% and f-100% of MF), sausages (4) (a-0%, b-20%, c-40%, d-60%, e-80% and f-100% of MF) after cooking.

According to KO *et al.* (2007), the heating of myofibrilar proteins (such as actomyosin) may form a cluster of aggregates with noncovalent and disulfide bonds. The curve obtained was typical of the thermal behaviour of fish muscle proteins, and many authors have reported that the first denaturation peak corresponds to myosin, the second to the sarcoplasmic or stromatic (collagen) proteins, and the third to actin, with transition temperatures between 30° and 80°C (PARK and LANIER, 1987; NAGAJ *et al.* 1999; HERRERA *et al.*, 2001; JENSEN *et al.*, 2003). The values

reported by these researchers are in agreement with those found in the present study (Table 2).

MONTERREY-QUINTERO and SOBRAL (2000) found values very close to those of myosin denaturation (54.4°C) and slightly higher than those of actin denaturation (75.9°C) in a study with Nile tilapia muscle for the development of edible films. Similar values can be observed in the work of MEDINA-VIVANCO *et al.* (2007). Nevertheless, PARK and LANIER (1989), in a study with tilapia (*Oreochromis aureus*), observed higher values for myosin (58.3°C) and actin (78.6°C) denaturation than those found in this experiment. The different results found by different authors might suggest that the species of fish can also affect the behavior of protein denaturation. Moreover these small variations also depend on various factors such as pH, ionic strength and the presence of other compounds, amongst others (PARK and LANIER, 1989). The denaturation temperatures found in the present study for the myosin of the fillet (54.0°C) and MF (54.6°C) were close to those found for the denaturation of herring myosin (*Clupea harengus*) (53.0°C), but higher than those found for cod (*Gadus morhua*) (46°C) and silver hake (*Merluccius bilinearis*) (47.0°C) (CHAN *et al.*, 1992), and this variation was possibly due to the type of material used in the analysis (intact muscle or purified protein), as well as to the habitat of the fish (AKAHANE *et al.*, 1985). According to HOWELL *et al.* (1991) and POULTER *et al.* (1985), the thermal stability of fish myosin increases in species adapted to higher environmental temperatures.

On the other hand, in the thermogram of MF (Fig. 1.1b), two clear denaturation (endothermal) peaks can be seen with a small inflection

Table 2 - Denaturation temperature[1] (°C) and enthalpy[2] (ΔHd) (J/g) of the protein from raw materials, mixtures after comminution in cutter, ingredients, emulsions and Nile tilapia cooked sausages.

	T1 (°C)[1]	Myosin (°C)[1]	T3 (°C)[1]	Actin (°C) [1]	T5 (°C)[1]	ΔHd(J/g)[2]
Raw materials						
Fillet	47.2 ±0.6	54.0±1.0[a]	-	73.9±0.8[a]	84.3±1.2	24.8±5.0[a]
Minced fish (MF)	-	54.6±0.0[a]	59.1±0.7	71.8±0.1[b]	85.1±0.0	21.2±1.3[a]
Mixtures (%MF)						
0	47.0±0.5	54.5±0.7[a]	-	73.4±0.1[a]	86.1±1.9	23.5±5.4[a]
20	46.6±0.3	54.6±0.3[a]	-	72.4±0.8[a]	83.7±0.5	25.6±2.4[a]
40	46.8±0.5	54.6±0.1[a]	-	72.8±0.6[a]	84.9±0.8	26.9±7.0[a]
60	46.8±0.3	54.1±0.2[a]	-	72.6±0.6[a]	85.3±1.0	21.8±4.0[a]
80	46.4±0.3	55.3±1.7[a]	-	70.3±0.4[b]	-	16.7±0.4[a]
100	46.8±0.0	54.6±0.8[a]	-	70.6±0.8[b]	-	18.9±1.5[a]
Average	46.7±0.2	54.6±0.4	-	72.0±1.3	-	22.2±3.9
Ingredients						
Starch	-	-	67.9±0.4	-	-	-
SPI[3]	-	-	-	-	-	-
Emulsions (%MF)						
0	45.9±1.2	59.3±1.2[a]	70.1±0.2	76.5±0.3[a]	-	18.3±0.4[a]
20	47.2±0.9	58.1±0.9[a]	69.6±0.6	76.4±0.2[a]	-	13.9±5.0[a]
40	47.6±0.9	58.1±1.2[a]	69.6±0.6	76.7±0.1[a]	-	15.2±2.1[a]
60	44.5±1.2	57.8±0.1[a]	68.9±0.9	76.3±0.2[a]	-	14.7±0.1[a]
80	45.1±0.4	57.5±0.9[a]	68.6±0.1	76.4±0.5[a]	-	16.7±1.1[a]
100	43.7±0.3	57.5±0.1[a]	65.4±0.0	76.2±0.6[a]	-	15.3±0.9[a]
Average	45.7±1.5	58.1±0.7	68.7±1.7	76.4±0.2		15.7±1.6
Sausages (%MF)						
0	-	-	-	-	-	2.7±0.9[a]
20	-	-	-	-	-	1.7±0.0[a]
40	-	-	-	-	-	1.9±1.6[a]
60	-	-	-	-	-	1.7±1.2[a]
80	-	-	-	-	-	2.9±0.6[a]
100	-	-	-	-	-	2.2±0.2[a]
Average						2.2±0.5

[1]Peak temperatures of thermograms (average ± standard deviation);
[2]Different letters in same column indicate significant difference ($p<0.05$);
[3]SPI - soy protein isolate.

between them. The peak occurring at 54.6°C may be associated with the denaturation of myosin, while the peak at 71.8°C is associated with the denaturation of actin. The inflection, appearing as a shoulder at 59.1°C, may be associated with the denaturation of sarcoplasmic proteins and collagen (MONTERREY-QUINTERO and SOBRAL, 2000). The MF was obtained from filleting waste, which is composed of the vertebral column and the remains of muscles and fins, so it should contain a considerable amount of collagen. In addition, the MF was not submitted to a washing process, which could leach out the sarcoplasmic proteins, so the amount of sarcoplasmic protein present may be considerable (LEE, 1984). Moreover, in this thermogram (Fig. 1.1b), unlike that of the fillet (Fig. 1.1a), there was no rise in the baseline after actin denaturation, which could be a consequence of the processing, which may also affect the thermal stability of the proteins (PARK and LANIER, 1989; MONTERREY-QUINTERO and SOBRAL, 2000).

Comparing the results from the two thermograms shown in Fig. 1.1, a lower denaturation temperature of the MF actin (71.8°C) was observed in relation to that of the fillet (73.9°C) ($p<0.05$) (Table 2), similar to that observed by PARK and LANIER (1989) and MONTERREY-QUINTERO and SOBRAL (2000). According to these authors, this loss of thermal stability of the actin is difficult to explain, but is probably an outcome of the destruction of certain interactions between the myofibrils. On the other hand, it can be seen (Fig. 1.1b) that the temperature of actin peak for MF was lower than that of the muscle (Fig. 1.1a), which may indicate that the actin present in comminuted MF had already been denatured by some means. This denaturation was probably due to the mechanical strength of the industrial fish deboning machine used to obtain the MF. According to WALSTRA (2003), proteins may be denaturated by mechanical forces of friction. PARK and LANIER (1989) also observed a reduction in the actin denaturation temperature (85.0°C) after the grinding of tilapia (*Oreochromis aureus*) muscle (78.0°C) and also after the processing of surimi (75.0°C). However, these authors did not observe any difference in the myosin denaturation temperatures under these conditions, similar to that observed in the present work ($p> 0.05$).

In spite of the differences observed in the thermal stability of the actin, overall, the denaturation enthalpy (ΔHd) of the fillet proteins (24.8 J/g protein) showed no significant difference ($p>0.05$) from that of the MF (21.2 J/g protein) (Table 2). This result is probably due to the high thermal stability of the myosin in both materials. However, this is only a suggestion, because it is very difficult to quantify the enthalpy associated with each event in the DSC thermograms presented in Fig. 1 (SOBRAL and HABITANTE, 2001).

In general, it can be seen that the comminution process had practically no effect on the trends of the DSC thermograms for the fillet (Fig. 1.2a) and the MF (Fig. 1.2f) as compared with those in Fig. 1.1. Overall, the trends of the DSC thermograms varied as a function of the increment in MF, mainly concerning the event associated with the actin (Fig. 1.2). Neither the denaturation temperatures of the myosin fragment, which remained around 46.7°±0.2°C, nor that of the myosin, which remained close to 54.6°±0.4°C, were affected by the MF concentration ($p>0.05$) (Table 2). However, the actin denaturation temperature showed a difference ($p<0.05$) with the inclusion of MF in the mixtures, and decreased ($p<0.05$) from 73.4°C (0% of MF) to 70.6°C (100% of MF). The ΔHd showed no difference ($p>0.05$) between the treatments, with an average value of 22.2±3.9 J/g protein, although the treatment with 0% of MF was approximately 25% higher than the treatment with 100% of MF (Table 2).

Contrary to the effect of comminution, the emulsification process together with the ingredients added (soy protein isolate, cassava starch, salt, curing salt, antioxidant, stabilizer, sausage seasoning, and natural onion) caused a drastic change in the profiles of the thermograms (Fig. 1.3) when compared to the previous results (Fig. 1.1 and 1.2). A broadening of the peaks can be observed in the thermograms of the emulsions, probably due to the inclusion of additives in the mass. However, these results could also be caused by vigorous comminution as can be observed from the reduced values of the denaturation enthalpies (PARK and LANIER, 1989). These results are in agreement with those found for rabbit meat (FURUKAWA *et al.*, 2004) and hake (*Merluccius hubbsi*) surimi (BEAS *et al.*, 1991), where a broadening of the endotherms was also observed after the inclusion of additives to the muscles.

It is worth noting that the denaturation temperatures of the myosin and actin increased by about 4°C between the comminution and emulsification processes (Table 2). This result could be due to the polyphosphate (cryoprotectant agent) added to the emulsion, which may stabilize these two myofibrillar proteins and consequently increase the respective denaturation temperatures, or, in another words, may protect the proteins against heat denaturation (PARK and LANIER, 1987). Moreover, the soy protein isolate may have protected the myofibrillar proteins. In a study with protein additives in Alaska pollock (*Theragra chalcogramma*) surimi, the soy protein isolate stabilized the fish myofibrillar proteins, as indicated by an increase in the myosin denaturation temperature from 36.7° to 48.1°C (PARK, 1994).

On the other hand, cassava starch contrib-

uted to the stabilization of fish proteins, more specifically, of the sarcoplasmic or stromatic proteins. Thus the denaturation temperature of these proteins increased from 59.1°±0.7°C in the MF, to an average value of 68.7°±1.7°C (Table 2). It is worth saying that this value is even higher than the gelatinization temperature of starch, 67.9°±0.4°C (Table 2).

In emulsions, the salt is the ingredient responsible for the extraction and solubilization of the myofibrillar proteins (RUUSUNEN *et al.*, 2003), immobilizing the fat and water during heating and forming a stable emulsion (gel formation) (PAPPA *et al.*, 2000). However it is known that the salt causes destabilization of the proteins and, consequently, decreases the denaturation temperatures (HASTINGS *et al.*, 1985; FURUKAWA *et al.*, 2004). BEAS *et al.* (1991) investigated the destabilization of the myofibrillar proteins of hake surimi (*Merluccius hubbsi*) with added 3% salt, as indicated by the reduction in the myosin (46° to 42°C) and actin (75° to 65°C) denaturation temperatures, using DSC. A decrease in the actin denaturation temperature of 6°C with the inclusion of 3% salt was also verified by DSC in rabbit meat, showing the destabilization of this protein by salt (FURUKAWA *et al.*, 2004). However, in the present study, the salt added to the emulsion did not apparently cause this phenomenon, the myosin and actin denaturation temperatures increasing after the addition of this ingredient together with the others (Fig. 1.3). The denaturation temperatures of the myosin (58.1°±0.7°C) and actin (76.4°±0.2°C) and the ΔHd of the emulsions with different levels of MF inclusion (0 to 100%), showed no difference ($p>0.05$) from each other (Table 2).

No endothermic peaks were observed in the DSC thermograms of the cooked sausages (Fig. 2.4). The ΔHd were very low (2.2±0.5 J/g protein) (Table 2), indicating that the cooking time (1 h 20 min) and final cooking temperature (72°C) were sufficient to cook the product and promote denaturation of all the proteins present in the sausages.

CONCLUSIONS

The evaluation of the denaturation of the Nile tilapia proteins during sausage processing as shown by DSC, showed that the most important effect on denaturation was observed after obtaining the emulsion, in association with the formulation. The ingredients used during the emulsification process must stabilize the fish proteins, increasing their denaturation temperatures, although causing a broadening in the endothermal denaturation peaks. Finally, it was confirmed that the Nile tilapia proteins were completely denatured after cooking the sausages for approximately 1 h 20 min to a final temperature of 72°C.

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Paper received January 26, 2010 Accepted October 22, 2010

SHORT COMMUNICATION

EFFECT OF PROCESS TEMPERATURE ON GLUTEN FILM PROPERTIES

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ABSTRACT

Gluten has been proposed as raw material to develop edible film formulation. Gluten based edible films are characterised by good mechanical and gas barrier properties but poor water vapour barrier properties. Process conditions influence final film structure and performances. In particular, heating treatment is supposed to improve protein reticulation. In this study, different temperatures from 30° to 85°C were used to prepare film forming dispersions with the aim of studying the influence of temperature on mechanical and water vapour barrier properties of edible films. Differences in mechanical and barrier properties of films were observed only for samples prepared at 30° and 85°C. Size Exclusion-HPLC was performed to study the effect of temperature dispersion on molecular properties of gluten based films. Protein size distribution seemed not to change when dispersion temperature increased, suggesting the absence of modifications in protein chains. Therefore, the different behaviours observed for film prepared at different temperature are not due to changes in gluten fraction on the basis of molecular weight. Probably, heating leads to a more homogeneous gluten network caused by the protein unfolding, to the increase in hydrophobic interactions and a better plasticizer distribution.

- Key words: dispersion temperature, edible films, gluten, mechanical properties, WVP -

INTRODUCTION

In recent years, the potential of edible films to control water transfer, and to improve food quality and shelf-life, has received increasing attention from researchers and industry. Materials that can be used to make edible films include polysaccharides, proteins and lipids, or a combination of these. Different proteins, such as wheat gluten, corn zein and whey proteins have been proposed as raw materials to develop edible films formulation. Wheat gluten (WG) is a major functional food ingredient, inexpensive, biodegradable and with good film forming properties. Film forming and casting conditions influence final structure, mechanical and barrier properties of wheat gluten edible film. High pH conditions allow a better protein dispersion in film forming dispersion and a good final appearance, because reduction of the disulfide bonds to sulfhydryl groups is favoured. But, films processed in alkaline conditions are characterized by yellow colour and unpleasant taste (GENNADIOS *et al.*, 1993a). Wheat gluten films in acid conditions are very interesting even if they showed problems in protein dispersion. The solvent process is based on dispersing and solubilising proteins in various solvents and then casting, spraying, or dipping followed by drying.

Film formation from WG solutions or dispersions has been extensively studied (GONTARD *et al.*, 1992; GENNADIOS *et al.*, 1993a and b; KAYSERILIOGLU *et al.*, 2003; HERNÁNDEZ-MUÑOZ *et al.*, 2004; BRAVIN *et al.*, 2006). This process requires protein matrix formation and drying step, with the aim of obtaining a thin layer. WG proteins are not soluble in water and require a complex solvent system with basic or acid conditions in the presence of alcohols. Generally, pH of the medium strongly affects final film properties. Intermolecular (for glutenins) and intramolecular (for gliadins and glutenins) covalent S-S bonds are cleaved and reduced to SH groups when dispersing WG in alkaline environments. During drying, solvent and these volatile disruptive agents are progressively eliminated, allowing gluten active sites for bond formation getting free and close enough to each other to create new interactions. New hydrogen bonds, hydrophobic interactions, and S-S bonds contribute to formation of a three-dimensional network. The addition of plasticizers is essential to avoid film brittleness. Final films properties are highly dependent on film formation conditions, including the nature, concentration, and order of the addition of the solvents, drying conditions, nature and amount of plasticizers (GUILBERT *et al.*, 2002). Disulphide bonds, formed between the sulphydryl groups of cysteine residues, are important in stabilising the folded conformations of gluten proteins. Strong interactions were observed between pH and ethanol concentration affecting film opacity, solubility and water vapour permeability. However, the visual disadvantage of alkaline films is offset by their significantly higher tensile strength. The effect of heating treatment on the final film properties had been studied for both dispersion and film after drying (HERNANDEZ-MUNOZ *et al.*, 2004; KAYSERILIOGLU *et al.*, 2003; MICARD *et al.*, 2000; ROY *et al.*, 1999). Different heating treatments were applied on aqueous liquid dispersion in order to investigate changes in proteins (SINGH and MACRITCHIE, 2004). At temperatures below 100°C, glutenins appeared to polymerize but polymerization of gliadins occurs only at higher temperatures. Size exclusion-High Performance Liquid Chromatography (SE-HPLC) has shown that the gliadin peak is reduced and the glutenin peaks increased for gluten samples that had been heated above 130°C. The polymerization of glutenin may involve oxidation of sulphydryl groups whereas sulphydryl-disulfide interchange may be the mechanism for cross-linking of gliadin. Heat treatment resulted in aggregation of proteins by cross-linking (hydrophobic and disulfide bonding). ROY *et al.* (1999) investigated the effect of heating film-forming solutions on physical and molecular properties of cast wheat gluten films. Film-forming solutions prepared in alkaline conditions were heated at 55°, 75° or 95°C for 10 min. Protein solubility of films in water decreased with increasing temperature, suggesting a protein denaturation due to the heating treatment, which reduced existing S-S bonds, and revealed previously unexposed SH groups. Heating treatments had been applied on gluten based films, after the drying step, in order to investigate the effect on final film properties (HERNANDEZ-MUNOZ *et al.*, 2004; MICARD *et al.*, 2000). The effect of thermal treatments on properties of gliadin- and glutenin- rich films was investigated by HERNANDEZ-MUNOZ *et al.* (2004). The heating treatment after drying was performed at 40°, 55°, 70°, 85°, 95° and 115°C for 24 h. Thermal treatments at temperatures higher than 70° and 85°C, respectively, for glutenin and gliadin-rich films improved their water barrier properties, making them less extensible due to the promotion of cross-linking of polymer chains through disulphide bonds. Other Authors investigated the effect of post drying heating on final film properties: the application of short time-high temperature heat curing treatment caused a tensile strength increase at the expense of the film extensibility (MICARD *et al.*, 2000). The post treatment caused also drastic protein insolubilisation, probably due to an increase in covalent binding between glutenins. Thermal treatment induced changes in film colour. Many works were carried out about the formulation set up and process condition effect on final film properties, but only few Authors investigated the possibility of large scale productions and industrialization. The purpose

of this work was to study the effect of film forming conditions on functional properties of gluten films. The influence of dispersion temperature was investigated from 30° to 85°C.

MATERIALS

Wheat gluten (80% purity), glycerol, ethanol, potassium acetate, magnesium nitrate were supplied by Sigma-Aldrich (USA).

METHODS

Film preparation

The first film-forming dispersion was prepared using 10 g of wheat gluten, 3.2 g of glycerol and water-ethanol mixture (40:60, v/v). The pH of film forming dispersion was adjusted to 3 by adding hydrochloric acid and stirred for 15 min at a temperature of 30°, 55°, 70° or 85°C. Film-forming dispersions were spread on PVC-coated glass plates using a TLC spreader and dried overnight at 25°C and 40% RH. The thickness of dried films was 75±5 μm and 85±5 μm (Elcometer 345 digital micrometer, Elcometer Instruments, UK). Before measurements, films were maintained at a constant relative humidity (RH) of 53% and a temperature of 25°C for 10 days, using a saturated magnesium nitrate solution.

Water vapour permeability (WVP)

Water vapour transfer rate (WVTR) was measured gravimetrically at a constant difference of RH and at 25°C, using a modification of ASTM standard method E 96-80. The test film was sealed to a glass permeation cell containing distilled water, and the cell was placed in a climatic room maintained at 22% RH, with a saturated salt solution of potassium acetate. The climatic room was equipped with a fan to eliminate stagnant air above the test cell. Cell weight was determined to evaluate stationary-state water vapour transfer. WVTR (g m^{-2} s^{-1}) and WVP (g m^{-1} s^{-1} Pa^{-1}) were calculated using the equations

$$WVTR = \frac{\Delta m}{A \Delta t} \quad (1)$$

$$WVP = \frac{WVTR \cdot x}{p_1 - p_2} \quad (2)$$

where Δm is weight loss of the permeation cell, with 4.9×10^{-4} m^2 exposed area (A), over time (Δt), x is film thickness, and $p_1 - p_2$ is real vapour partial pressure difference (Pa) across the film. The real vapour partial pressure at the film inner surface (p_1) was corrected for the stagnant air gap inside the test cell, according to the method of GENNADIOS *et al.* (1994).

Mechanical properties

Tensile strength (TS, MPa) and percentage elongation (E, %) at breakpoint were measured uniaxially by stretching the specimen (10 x 2.5 cm) in one direction at 50 mm/min using an Instron Universal Testing Instrument (model 4301, England). The films were analyzed in a climatic room at 53% RH and 25°C. Initial grip separation was set at 4 cm. TS was calculated by dividing the maximum load by the cross-sectional area of the film, E being expressed as a percentage of change in the original specimen length between grips (4 cm), according to ASTM standard method D 882-88.

Protein size distribution

Protein was extracted and was analysed by SE-HPLC according to NIGHTINGALE *et al.* (*1999*) with minor modifications. SDS-soluble gluten was analysed by SE-HPLC (1% SDS in 0.1 M sodium phosphate buffer at pH 7.0) using a Phenomenex BIOSEP-SEC-S 4000 precolumn (35 x 7.8 mm) and a column (300 x 7.8 mm). Analytes were eluted with an isocratic separation at a flow rate of 0.5 mL/min with 50% aqueous acetonitrile solution, containing 0.05% trifluoroacetic acid. Proteins were detected at a wavelength of 210 nm. Areas for the glutenin peak and the gliadin peak were measured by Star Chromatography Workstation (v 5.31, Varian Associates, Inc., Copyright©). Total gluten content and SDS-soluble gluten fraction were determined according to Kjeldahl method (AOAC, 1980).

RESULTS AND DISCUSSION

In order to investigate the effect of temperature dispersion on structure of gluten-based film prepared in acidic conditions, different properties were evaluated: tensile behaviour, water vapour permeability, solubility, and changes in protein fractions. Temperatures ranging from 30° to 85°C were selected, since gluten is known to be a complex mixture of proteins with different susceptibility to heat. The glutenin fraction is affected predominantly between 55°-70°C, while changes in gliadin proteins occur at temperatures greater than 70°C (SCHOFIELD *et al.*, 1985). A reference sample without heating was prepared at a dispersion temperature of 30°C. This study was motivated by our results (unpublished data) on gluten-based film prepared in alkaline conditions (pH = 11), which showed large changes in its properties due to dispersion heating at 70°C for 15 min. SDS-solubility of this film, for example, decreased from 89.4 to 11.8% when dispersion temperature was increased

from 30° to 70°C indicating that heat and alkaline treatment had resulted in cross-linking (KAYSERILIOGLU *et al.*, 2001; ROY *et al.*, 1999).

Changes in mechanical properties of edible film as a function of dispersion temperature are reported in Fig. 1. Similar TS and E values were reported by GENNADIOS *et al.* (1993). Tensile properties are related to cross-linking density in the gluten network and polymer plasticization. Tensile strength decreased increasing dispersion temperature from 30° to 70°C, while a trend to enhance was observed above 70°C (Fig. 1). Elongation was gradually increased by heating up to 85°C. The inverse relationship observed between TS and E in the temperature range 30°-70°C could be attributed to differences in the polymer plasticization. Gluten is characterised by high contents of glutamine and proline residues inducing hydrogen and hydrophobic bonding among the protein molecules. Above 30°C, these non-covalent bonds are probably broken altering the proteins to a more open structure (WEEGELS and HAMER, 1998). This might favour glycerol-protein hydrogen bonds, which replace polymer-polymer interaction in the film. This phenomenon in turn led to lower strength and higher values of elongation at break or film extensibility.

For a dispersion temperature higher than 70°C, TS increased (Fig. 1). We interpret this behaviour as a result of cross-linking formation. Generally, the surface hydrophobicity increases upon heating due to the increase in exposure of hydrophobic groups at the outer surface of the molecule. Increased hydrophobicity leads to protein aggregation (WEEGELS and HAMER, 1998; WEEGELS *et al.*, 1994). Due to low sensitivity to heat, changes in the gliadin fraction are visible above 70°C. Cross-linking promotes generally the increase in TS and decrease in E of film. This is not our case: elongation increased above 70°C (Fig. 1). An explanation could be gliadin-glycerol interaction, favoured by protein unfold at high temperature. An additional hypothesis is simply the formation of a more homogeneous protein network promoted by non-covalent interactions, which render high extensibility and high strength.

BRAVIN *et al.* (2006b) observed TS values about four times higher, and E values about four times lower for gluten films in alkaline medium than our samples, probably due to strong gluten network. Due to problem in protein dispersion, films prepared in acid conditions showed large aggregates of gluten that affected film aspect. In order to minimize this disadvantage, glycerol content was increased compared to the formulation prepared in alkaline conditions by BRAVIN *et al.* (2006b). The difference between our results and those obtained for film prepared in alkaline conditions are partly related to the quantity of plasticizer that strongly affects mechanical properties.

No significant differences in WVP were observed for films prepared at different temperatures of dispersion (Table 1). Wheat gluten films have high permeability to polar substances, such as water vapour. The WVP is strongly affected by the presence of glycerol, because of its hydrophilicity. Probably, in this study, the high concentration of plasticizer, due to the

Table 1 - WVP for wheat gluten films at different thickness.

	WVP (10^{-10} gm^{-1}s^{-1}Pa^{-1})	
Temperature (°C)	75 µm	85 µm
30	8.21±0.47	8.88±0.93
55	8.16±0.50	10.23±0.68
70	9.06±0.80	10.15±0.91
85	8.49±0.87	9.38±1.12

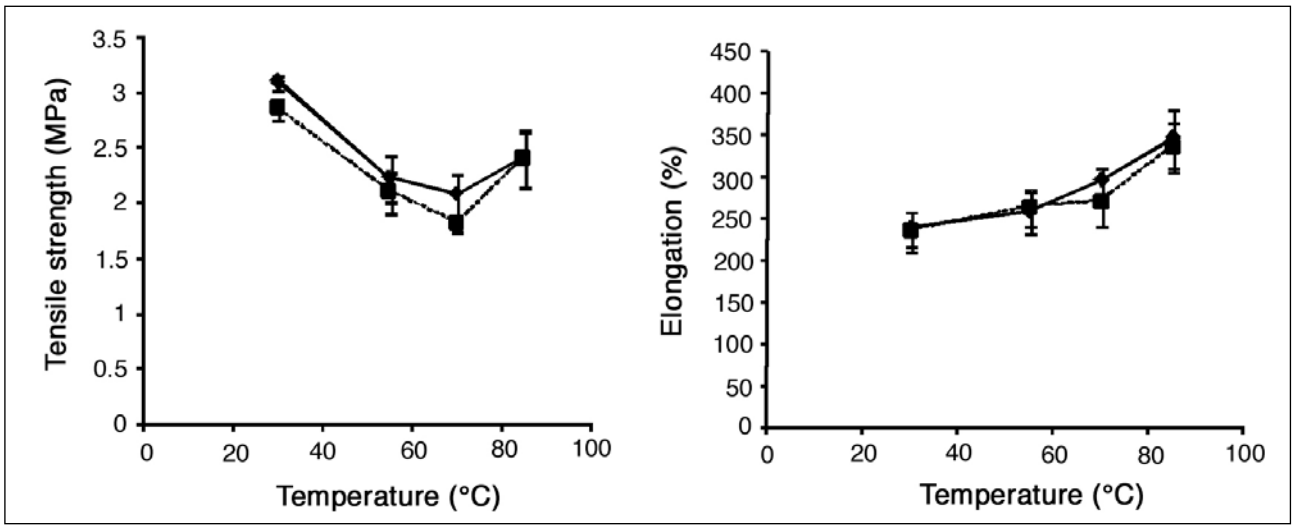


Fig. 1 - TS and E of wheat gluten edible films as a function of dispersion temperature (◆ 75 µm and ■ 85 µm).

Table 2 - SDS soluble and insoluble fractions of native gluten and gluten films (wg film).

Sample	SDS soluble fraction (%)	SDS soluble gliadins (%)	SDS soluble glutenins (%)	SDS insoluble fraction (%)
wheat gluten	89.91±0.27	54.59±0.21	35.32±0.07	10.09±0.28
30°C wg film	79.69±4.55	53.55±0.95	29.86±2.10	16.60±1.15
85°C wg film	83.40±1.15	52.91±3.47	26.79±1.08	20.31±4.55

problem in protein dispersion, strongly influenced WVP and did not allow to observe the effect of the heating treatment on the film barrier properties. According to other Authors (BRAVIN *et al.*, 2006a), WVP increased when film thickness increased.

In order to better understand the effect of temperature dispersion on gluten at molecular level, protein was extracted from native gluten and gluten films prepared at 30° and 85°C and analysed by SE-HPLC (Table 2). SDS-soluble protein fraction decreased from 90% (native gluten) to 79-83% due to film preparation. Dispersion temperature did not induce large changes in protein solubility of gliadin and glutenin fractions. SDS is able to break hydrophobic and hydrophilic bonds and to highlight the contribution of disulfide bonds. No significant differences between temperature dispersion of 30° and 85°C were observed suggesting that heating influenced mainly non-covalent interactions between protein chains.

Decrease in SDS-gluten solubility of films can not be explained only by protein denaturation, because a sample was prepared at low temperature. ROY *et al.* (1999) observed thiol-disulphide interchanges at temperature above 55°C for alkaline films. In acid media (pH=5.5), SINGH and MACRITCHIE (2004) observed a shift towards lower elution times for gliadin peak, when the temperature was above 70°C. HERALD *et al.* (1995) reported a glutenin solubility decrease in acid media according to our results.

CONCLUSIONS

Different dispersion temperatures were applied to gluten film forming dispersion, prepared in acid conditions, with the aim of improving protein reticulation. Differences in mechanical and barrier properties of films were observed only for samples prepared at 30° and 85°C. Protein size distribution seemed not to change when dispersion temperature increased, suggesting the absence of modifications in protein chains. When heating treatment was applied, protein changed its conformation because of non-covalent bonding ruptures: the exposure of hydrophilic groups allowed more interactions between protein chains and solvent (HERALD *et al.*, 1995). In this way, glycerol distribution was favoured inducing the decrease of intermolecular forces, as observed for film mechanical properties.

Therefore, the different behaviours observed for film prepared at different temperature are not due to gluten components polymerization, but probably to a more homogeneous gluten network caused by the protein unfolding with the increasing temperature, the enhancement of hydrophobic interaction and a better plasticizer distribution.

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Paper received February 25, 2010 Accepted September 20, 2010

SHORT COMMUNICATION

SAUSAGE CASINGS - MICROSTRUCTURE OF REGULAR AND COATED CELLULOSE, FABRIC AND PLASTIC CASINGS

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ABSTRACT

The microstructures of cellulose, fabric and plastic casings were evaluated by polarized light and scanning electron microscopy. The small diameter casings (18 mm), typically used for frankfurters, showed a fine structure of cellulose particles (pulp) adhering to each other. Birefringence of the cellulose was clearly visible by polarized light. The large diameter cellulose casing (90 mm) was three times thicker and also contained large distinct cellulose fibers embedded in its matrix. The fibers are used to enhance the strength required to hold heavy products as well as prevent bulging during the process of smoking and cooking that is commonly done at high relative humidity.

Cellulose casings with either inside or outside plastic (PVDC polymer) coating showed the interactions of the two components which are used to increase strength, and reduce moisture/gas permeability. While the inside coated casing requires a high humidity processing environment, the outside coated can be processed dry but it does not exert the same pressure on the product during cooking. Regular and coated fabric casings showed the unique woven structure of threaded fibers, within their matrix, which is used to support heavy products. Scanning electron microscopy revealed some hollow fibers. The all plastic casing appeared to be composed of two distinct layers. The inner layer appeared as a very dense matrix and the outer layer as a sheet/layer structure which also contributed to the shiny appearance. Overall, this casing was very thin yet very strong and could handle a 100 mm diameter product.

The study demonstrates the advantages of combining regular and polarized light as well as electron microscopy to study casings microstructure. In the case of inside versus outside PVDC coated fibrous casings, the microstructure could be used to explain differences in soaking requirements and problems that may be associated with post cooking splitting.

- Key words: casings, cellulose, fabric, fibrous, meat, microscopy, microstructure, plastic, sausage -

INTRODUCTION

There are many types of casings on the market today. They influence the manufacturing and characteristics of sausages and therefore should be carefully selected for each application. Casing materials range from natural biopolymers, such as collagen (natural or manufactured) and cellulose, to synthetic polymers such as polyethylene and polyvinylidene dichloride. Natural collagen casings are the oldest form of casings, which have been used for thousands of years. The international trade in natural casings has been estimated at about $2.5 billion a year (KOOLMEES *et al.*, 2004). Over the past few decades, manufactured cellulose casings (i.e. prepared from derivatized fibers), fabric casings (woven fibers), and more recently plastic casings (extruded polymers) have been introduced on the market (RUST and OLSSON, 1988; BARBUT, 2002). Exact trade figures for the different categories of manufactured casings are difficult to obtain, but they are used in a substantial number of sausages manufactured today. The increased popularity of cellulose casings has been in part due to the relatively high cost of using natural casings (e.g. preparation cost, labour at the meat processing plant), their strength, ease of processing and shelf life considerations (BAKKER *et al.*, 1999; ZAVIC and ZAVIC, 2002). It should also be mentioned that because of the uniformity of the cellulose family of casings and plastic families of casings more automation and mechanization can be employed; both are essential in high speed/high volume production lines. For generations, casings have been used to stuff different meats (whole muscle chunks, coarse ground, emulsified) as well as other food products (dairy) for storage, preservation (by fermentation) and processing. An example could be a semi-liquid raw comminuted meat batter, which after heating gels and forms a self-supporting structure which can then be handled without the casings. The casings play both direct and indirect roles in the volumetric, structural and chemical changes that occur in the sausage throughout the processing steps (SAVIC and SAVIC, 2002; BARBUT, 2010). Therefore the unique requirements of each sausage should be matched with the characteristics of the casings to be used. To accomplish this goal, the casing industry offers a variety of products with different functional properties. In spite of the large volume of this industry, there is very little or no information in the scientific literature on the microstructure of cellulose, fabric and plastic casings used for meat products. The objective of this study was to examine and compare the structure of some representative cellulose and fabric casings with and without the use of plastic polymer coating.

MATERIAL AND METHODS

Sausage preparation

Seven different casings (Table 1) were stuffed with a common sausage meat batter at the University of Guelph Meat Science Laboratory. The common meat batter was made with 20% chilled beef trims (80/20), 25% pork trims (80/20), 20% pork jowls, 15% pork back fat, and 20% ice. The non meat ingredients were: 1.7% salt, 1.0% starch, 0.6% seasonings, 0.02% phosphate, 0.003% sodium erythorbate, and 0.0016% sodium nitrite. The meat was ground through a 12 mm plate, chopped in a bowl cutter (Schneidmeister SMK 40, Berlin, Germany) while adding all the non meat ingredients for 4 min; temperature maintained below 12°C. The meat was then stuffed (Mainca model EM20R, Berkshire, UK) into the different casings. Sausages were cooked in a smokehouse (Enviro-Pak, model CVU 490E, Clackhamas, OR) up to an internal temperature of 72°C. Casings were evaluated by cutting thin sausage samples (5 mm), and embedding the whole section or a portion of the section (depending on product's diameter) in paraffin. In the case of the "easy release" type casing, which would not stay attached to the meat product after slicing the sausage, a casing sample was attached onto 2x2 cm rigid cardboard squares (using stainless steel pins) prior to embedding.

Light microscopy

Samples were fixed in 10% neutral buffered formalin for 1.5 h, followed by dehydrating in 70, 95, and 100% isopropanol; each for 2 h. The samples were soaked in xylene for 2 h and embedded in paraffin for 3 h, using an automated vacuum infiltration unit (Sakura Tissue-Tek VIP, Sakura Finetek, Torrance, CA). The embedded samples were sectioned (Microtome HM 200, Ergostar, Walldoft, Germany) into 5-7 micron sections, allowed to float on water, and transferred onto a glass slide. Slides were dried, and stained with Hematoxyline/Eosin. A computerized image analysis system attached to microscope (Model BX60F5, Olympus Optical Co, Ltd., Japan) was used to view (x 200 magnification) the samples, and capture images (Image-Pro Plus, Version 4.5-1.29, Media Cybernetics Inc., Silver Spring, MD).

Scanning electron microscopy

Five to six cubes (approx. 1 cm^3) from the edge of each raw sausage were fixed overnight in 2.5% glutaraldehyde washed with a Sorensen's buffer, post fixed in 1% OsO_4, dehydrated in ethanol, critical point dried, fractured to expose inside surfaces, placed on stubs and sputter-coated with 200-300Å of gold/palladium (Emitech

K550; Ashford, Kent, England). The scanning electron microscope (S-570, Hitachi, Japan) was used at an accelerating voltage of 5 kV.

RESULTS AND DISCUSSION

The thin cellulose casings, regularly used for a small diameter frankfurter product (18 mm diameter; Table 1), show a very uniform profile (Fig. 1a) with a thickness of 50 μm. It consists of cellulose material that is clearly visible under polarized light (Fig. 1b) as cellulose fibers/pulp show birefringence characteristics. This results from the anisotrophic nature of the fibers (KRISHNA-IYER *et al.,* 1983; PETERSSON *et al.,* 2009) which have two or more principle refractive indices. This particular casing is commonly marketed under the name "easy release". In this experiment, the casing also did not adhere to the thin slice of the product and therefore was prepared as a casing sample by itself (i.e. not showing the meat mass underneath as is shown in all the other samples) (Fig. 1). Such casings are preferred in high capacity production of cooked smoked frankfurters where the casing is later removed by automatic peeling equipment, prior to vacuum packing at the plant. SAVIC and SAVIC (2002) indicated that this type of casings are usually available in diameters ranging from 15 to 38, or less often at 50 mm, and are generally produced in flat or shirred (i.e., applied to a dummy transfer horn) forms with a wall thickness of about 30 μm. They do not require presoaking in water, and are directly inserted on the stuffer's nozzle, ready for packing. This casing is permeable to small molecules such as water and short hydrocarbon molecules carried within wood smoke; i.e., the spaces between the fine cellulose pulp particles allow migration of the small molecules, but usually not the passage of large molecules such as of fat and protein. They are designed to resist breakage and withstand conditions used in a smokehouse (e.g., high relative humidity).

The large diameter fibrous casing (Fig. 1c) was substantially thicker (150 μm) as it is designed to hold a 90 mm diameter product (Table 1). Another difference from the thin casing is the presence of larger and distinct cellulose fibers which are embedded within the fine cel-

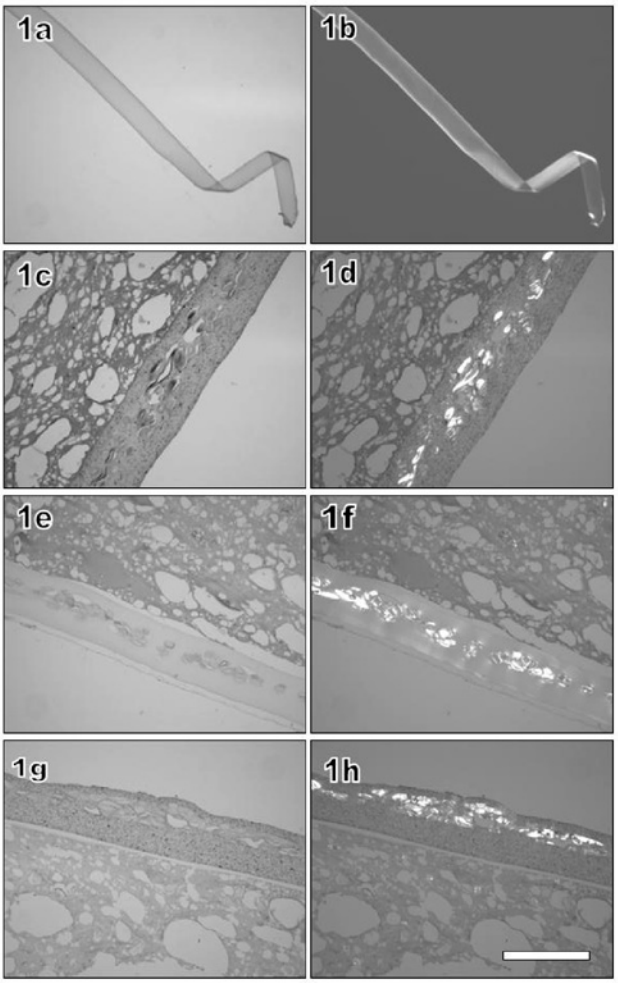


Fig. 1 - Light micrographs of cellulose casings: (a) and (b) thin, "easy release" frankfurter type; (c) and (d) large diameter fibrous casings covering the actual meat batter; (e) and (f) outside polyvinylidene dichloride (PVDC) coated fibrous casings; (g) and (h) inside PVDC coated fibrous. Polarized light was used in (b), (d), (f), and (h) to reveal cellulose fibers. Bar = 200 μm.

Table 1 - Cellulose, fibrous, and plastic casings evaluated and some of their characteristics.

Casings type	Stuffed diameter (mm)	Appearance	Treatment prior to stuffing	Supplier[1]
1. Cellulose - "easy release"	18	Very thin, clear with 2 dark strips	None	Naturin
2. Fibrous - "regular"	90	Thick, brown color	Soak in water	Kalle
3. Fibrous-outside coated[2]	98	Thick, shiny, red color	Soak / no Soak	Kalle
4. Fibrous -inside coated[2]	66	Thick, orange color	Soak in water	Kalle
5. Fabric - regular	Ave. 65	Bag type, printed pattern	None	Texda
6. Fabric - outside coated	Ave. 52	Bag type, shiny, printed pattern	None	Texda
7. Plastic - extruded	100	Thin, shiny, black color	None	Supralon

[1]Naturin/Viscofan, Montgomery, AL, USA. Kalle, Wiesbaden, Germany. Texda, Osnabruck, Germany. Supralon, Massy, France.
[2]Coated with-polyvinylidene dichloride (PVDC).

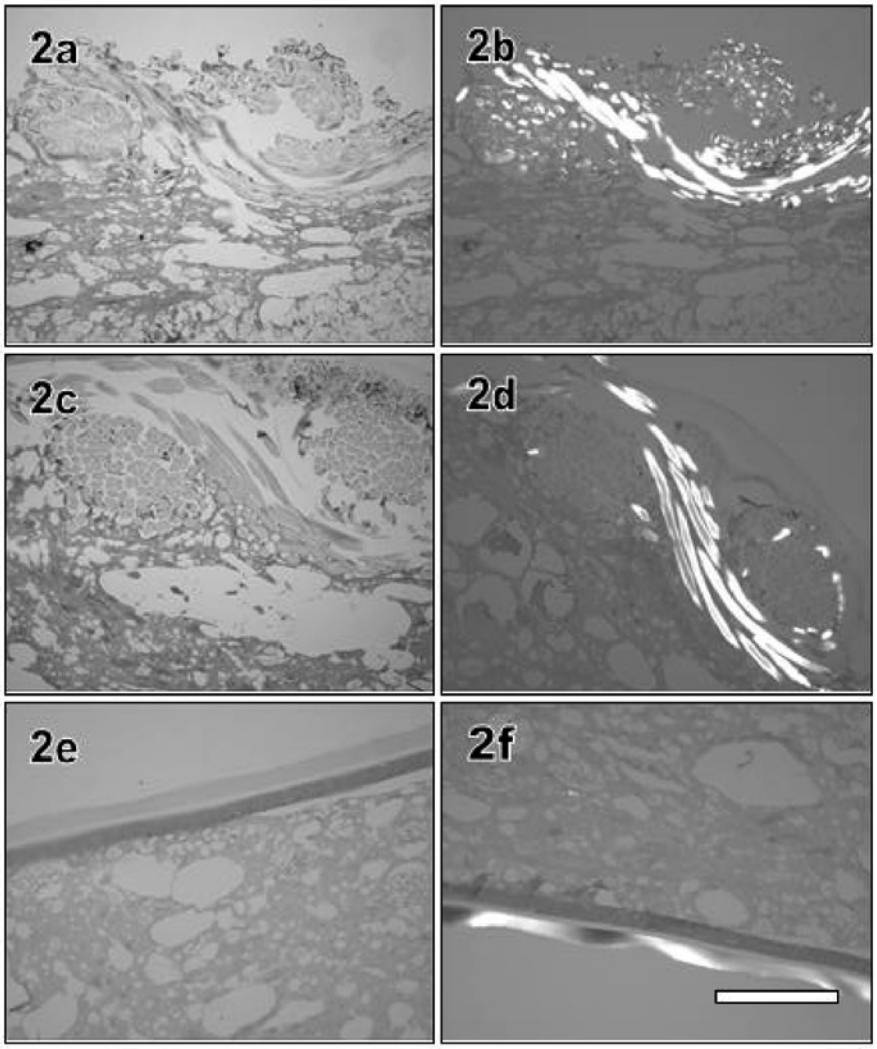


Fig. 2 - Light micrographs of fabric and plastic casings: (a) and (b) fabric casing covering a meat batter; (c) and (d) outside coated fabric; (e) and (f) extruded plastic casing. Polarized light was used in (b), (d) and (f) to reveal cellulose fibers/special plastic. Bar = 200 μm.

lulose (e.g., usually regenerated from alpha cellulose) matrix (Fig. 1d; large fibers visible by polarized light), which are about 10 to 20 μm in diameter. These fibers provide the casing with the strength needed to hold the meat mass (SAVIC and SAVIC, 2002); in this case, in excess of 1 kg while the product was going through the smoking and cooking cycle. This is a very important point because heavy sausages hanging on a smoke house tree are subjected to fairly substantial physical stresses (e.g., vibration, temperature and relative humidity changes) as well as strict requirements (e.g., no uneven expansion of diameter especially at the lower part of the sausage as the casing is hanging in a humid environment for a few hours). A closer look at the cellulose fibers, using a scanning electron microscope (Fig. 3b), shows the larger cellulose fibers embedded in a fine cellulose matrix. In this cross section the four round fibers appearing at the top are running along the sausage's length and the ones underneath are positioned around the product to provide more radial support. It is also interesting to note that some of the fibers have a hollow core. In contrast, the thin frankfurters casing (Fig. 3a) does not have any such large fiber embedded within its matrix and is composed of what appears to be fine cellulose particulates (pulp) "glued" together. This is consistent with the light micrographs (Figs. 1a and 1b) not showing any distinct large fibers. Large cellulose fibrous casings require soaking in water prior to filling (usually 0.1-0.5 hr) to allow stretching of the fibers. It is then recommended to fill them tightly. Later during cooking and drying (within the smoke house) the large fibers start to shrink and by doing so they put pressure on the product's surface and prevent fat/jelly pocket formation. The thicker the casings' wall the higher the shrink/pressure can be. This type of casing is commonly used for various large diameter products (e.g., luncheon meats) as it allows water and smoke migration.

The outside polyvinylidene chloride (PVDC)

coated fibrous casings (Table 1) show cellulose fibers under the plastic layer (Figs. 1e and 1f). The cellulose appears to be divided into middle dense material and bottom (inside, close to the meat) fine structured matrix of short cellulose fibers/pulp. The PVDC is a crystalline thermoplastic polymer $(CH_2CCl_2-)_n$ of vinylidene dichloride. A relatively small pendent group, such as chlorine, yields a distinct level of crystallinity. The polymer can be formed into waterproof and chemically resistant filaments, films, and fabrics (SAVIC and SAVIC, 2002). The outside PVDC coated casing is usually impermeable to smoke and water (note: but not always – depending on the manufacturing process; processor needs to check specific transmission data), has a shiny (gloss) surface and because the cellulose material is inside, it is recommended to soak the casings prior to stuffing (Table 1). If the processor does not soak the casing, in order to get the cellulose fibers hydrated, the water will have to come from the meat (note: some shirred casings today are pre-humidified). As for filling pressure, they are usually stuffed very firm because the plastic layer provides substantial strength. Cooking and later cooling can be done in a dry or wet environment since the outer plastic layer is not sensitive to humidity. Under higher magnification (Fig. 3c), a bunch of fibers is seen in a cross section of the casing, and the smooth plastic matrix is seen on the top. The individual fiber diameter is about 10-20 μm which agrees with the light microscopy results.

The inside PVDC coated casings (Figs. 1g and 1h) show the cellulose fibers all positioned on the outside surface (away from the meat mass). The thickness of the casings is about 125 μm. Under high magnification (Fig. 3d) a cross section of fibers is visible on top of the smooth plastic matrix. The inner layer of plastic is very dense compared to the cellulose layer. With this casing, the processor needs to soak the casing prior to use (usually recommended 0.5 hr in warm water) so the cellulose fibers will get hydrated, be more flexible, and be able to stretch. The filling pressure should be fairly firm (not to exceed 5% of nominal caliber) (SAVIC and SAVIC, 2002) but not as much as with the outside coated casing, since the cellulose fibers on the outside would also exert pressure on the product during the cooking operation. In terms of heat processing, this casing should be cooked in a steam environment or water. A dry environment will cause drying and shrinking of the fibers, which will eventually result in splitting of the casings. The cooling stage is also critical and should be done under water or a humid environment otherwise the casing would split. The advantage of this casing is the continuous pressure it exerts on the product; however, if this casing is allowed to dry during the process (heating and cooling), there is a high risk of bursting. EFFENBERGER (1996) has also recommended wetting the casings, with a wet fine cloth or gauze, prior to slicing if a relatively dry environment is used for storage.

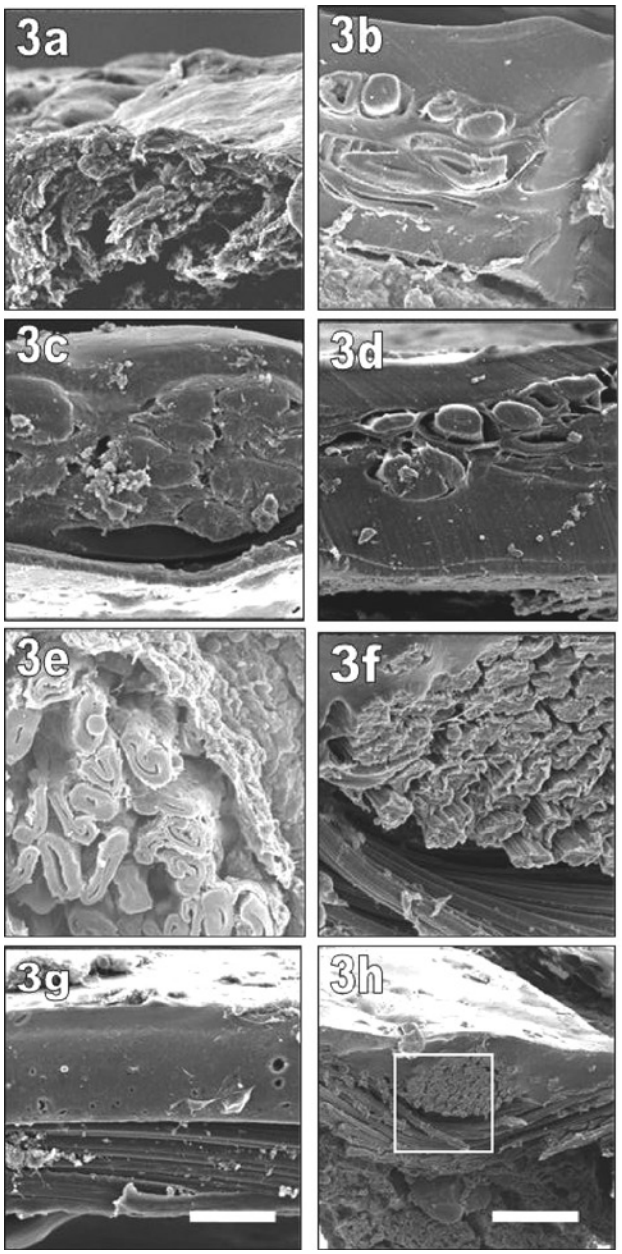


Fig. 3 - Scanning electron micrographs of casings: (a) thin cellulose "easy release" frankfurter type; (b) large diameter fibrous sausage; (c) outside polyvinylidene dichloride (PVDC) coated fibrous; (d) inside PVDC coated fibrous; (e) regular fabric; (f) outside coated fabric; (g) extruded plastic; and (h) lower magnification of micrograph "f" - see box. Bars = 25 μm for all, except (h) which is 120 μm.

The fabric casings (also known as cloth) which are commonly produced from woven soft wood pulp or less often today from cotton fibers (Figs. 2a and 2b) show a unique pattern of fibers arranged around and parallel to the product's length. The woven fiber arrangement is more easily viewed under polarized light (Fig. 2b) where the small dots (diameter of about 5 to 20 μm) are the ones running parallel to the product's length; i.e. part of a cross section of a cotton thread. The elongated fibers are positioned around the

product and seen here in lengthwise section. A higher magnification of the fibers' cross section obtained by SEM (Fig. 3e) shows the inner microstructure of several fibers which form a thread. This casing is permeable to both moisture and smoke as the spaces among the fibers allow small molecule to migrate. The casing can be made very strong by including more threads and/or interconnecting the threads. The diameter of the product stuffed in this casing was not so uniform and as reported in Table 1, the average was 65 (actual 65±3 mm) for this bag-type casing. This was probably done to give the casings a more "natural appearance". Fabric casings with a consistent diameter are also available on the market.

The outside coated fabric casings (Figs. 2c and 2d) reveal a very ordered pattern of the soft wood pulp fibers positioned around the circumference of the product. Some of the fibers run along the product's length (appear as small dots arranged in a concentric pattern on the right hand side; Fig. 2d). They appear to be woven with some other fibers (possible plastic polymer fibers) that do not show birefringence properties under polarized light (Fig. 2d). A closer look at the fiber arrangement (Fig. 3h) shows a group of fibers running along the product's length (the group is surrounded by a square box and further magnified in Fig. 3f) and fibers running along the product's circumference. This arrangement provides support in both radial and lengthwise directions, which is important in keeping the shape of the product (e.g., constant diameter), and allowing high pressure filling. Because of the plastic coating, this casing is usually impermeable to moisture and smoke and therefore can also be cooked in water.

The extruded plastic casing is composed of two distinct polymer layers (Figs. 2e and 2f). The inner one appears as a dense material, with a thickness of 30 μm. The outer one (about 25 μm) possesses some birefringency and appears bright under polarized light. This outer layer provided a very shiny appearance to the product (Table 1). Under higher magnification (Fig. 3g) the inner layer appears as a very dense matrix (thickness 30 μm), and the outer one composed of several sheets/layers. This sheet/layer structure appears to be the reason for the unique light reflectance pattern seen under polarized light (Fig. 2f). It should also be mentioned that the appearance of the two-layer structure can also be resulting from different densities of the same polymer used for manufacturing. Overall, this plastic casing is relatively thin but yet very strong, as well as impermeable to moisture and smoke. The reasons for that can be explained by the very dense inner layer's structure, and the plastic polymer composition. The casing used in this experiment was for a 100 mm in diameter sausage, and was strong enough to hold a > 1.5 kg product tightly stuffed and provided a very firm raw product. No deviation in diameter was observed before or after cooking.

ACKNOWLEDGMENTS

The financial support from the Ontario Ministry of Agriculture and Food, technical assistance of A. Smith, and casing contribution by the Hermann Laue Spice Company are appreciated.

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Paper received March 5, 2010 Accepted October 10, 2010

SHORT COMMUNICATION

A CHARACTERIZATION OF CONTENT, COMPOSITION AND ANTIOXIDANT CAPACITY OF PHENOLIC COMPOUNDS IN CELERY ROOTS

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ABSTRACT

In this paper, we examine the contents and composition of phenolic compounds and antioxidant capacity of celery roots. The phenolic compounds content ranged from 265.44 to 368.51 μmol of chlorogenic acid per g of dry extract. The antioxidant capacity was determined by DPPH method and EC_{50} value ranged from 2.41 to 3.14 mg/mL. By HPLC analysis 24 compounds were identified. The main components are genkwanin glucosides (30.03 to 35.80%), kaempferol glucosides (17.19 to 24.25%), and quercetin glucosides (2.23 to 17.85%).

- Key words: antioxidant capacity, celery roots, phenolic compounds -

INTRODUCTION

Phenolic compounds have free radical scavenging abilities, take part in antimutagenic and anticancerogenic activities and have ability to modify the gene expression (NAKAMURA *et al.* 2003). For this reason there is increased interest for these compounds in food, today. Numerous studies have confirmed relationship between the dietary intake of phenolic compounds, especially flavonoids, and the reduction in cardiovascular and carcinogenic risk (COOK and SAMMAN, 1996).

Phenolic compounds are one of the most numerous and widely distributed group of substances in the plant kingdom with more than 8,000 structures (HARBORNE, 1993). They are a product of secondary metabolism of plants which arise biogenetically from the main synthetic pathways: shikimate and the acetate pathway (BRAVO, 1998). The phenolic compounds content varies during the process of development (MARINOVA *et al.*, 2005) and the antioxidant capacity depends on the exposure to stress such as light, temperature, water (KAYS, 1997; DIOXIN and PAIAVA, 1995), nutritional deficiencies (SALTIVET, 1997), type of vegetable tissue (SONIA and ALICIA, 2007), mechanical damage such as wounding (FERNANDO *et al.*, 2007), maturation stages (ÖZGEN *et al.*, 2009), etc. Their function as natural antioxidants has already been discussed (ASTELY, 2003; TREWAVAS and STEWART, 2003) and especially that of quercetin (HERTOG and HOLLMAN, 1996). Their antioxidant capacity is based on the free radical scavenging capacity and several methods for its determination have been developed, typically based on spectrometric measurements of the disappearance of free radicals such as 2,2 diphenil-1-picrylhydrazyl radical (DPPH radical) (SÁNCHES-MORENO *et al.*, 1998; CHOI *et al.*, 2002).

In celery *(Apium graveolens* L.) leaves phenolic compounds content was 113.0 mg gallic acid equivalents/100 g of fresh weight, while in celery fruit the content was 436.1 mg/100 mg gallic acid equivalents/100 g of dry weight. Following a cutting process, the total phenolic compounds content changed and the flavonoids identified by HPLC were apigenin and luteolin (SONIA and ALICIA, 2007).

The purpose of this study was to examine whether the content and composition of phenolic compounds and their antioxidative capacity depend on whether the celery roots had the same duration of development period and different growth levels. The present work was undertaken with the following objectives: (1) to determine the content and composition of phenolic compounds in different size celery roots; (2) to determine their antioxidant capacity and (3) to find the correlations between antioxidant capacity and total phenolic compounds and the main phenolic compounds content.

MATERIALS AND METHODS

Plant material

Celery *cv* Golden Boy roots, grown in Serbia were grouped into a C1 (root size 55±5 g), C2 (130±10 g), and C3 (450±20 g) group. The roots had the same period of development and were obtained from an experimental lot of the Agricultural school, Leskovac, Serbia. Five samples of roots were collected for each group. The roots were cut into cube shape (1.5x1.5x1.5 cm), dried at 45°C during 6 h and left at room temperature for 1 h. Then the five samples were mixed into each group and milled to average particle size of 0.5 mm.

Moisture content

The plant material moisture content after drying was determined by using the analyzer (Scaltec SMO 01, Scaltec instruments, Germany). The plant material (3 g) was dried at 110°C until a constant weight and moisture content was read out on the analyzer display. Triplicate determinations were performed.

Preparation of extracts

Plant extracts preparations for measurements of the phenolic compounds content were done according to DOKHANI *et al.* (2005) procedure. Plant material (3.0 g) was measured and 80 mL of 80% (v/v) ethanol was added. The mixture was stirred by IKA MR1 magnetic stirrer, for 10 minutes at 200 min^{-1} and vacuum filtered through No. 54 Wathman filter paper. The solids were re-extracted with 60 mL of 80% (v/v) ethanol, the filtrates combined and made to a final volume of 250 mL. Three replicate extractions from each celery group were made. For the measurement of total phenolic compounds content, 10 mL of each extract was filtered through a 0.45 μm membrane filter. For antioxidant capacity measurements and HPLC analysis, 200 milliliters of each extract were evaporated in vacuum at 45°C until dry and dissolved in 96% v/v ethanol.

Total phenolic compounds content

For the total phenolic compounds content measurement, a standard curve for five chlorogenic acid (Sigma Chemical Co., St. Louis, USA) concentrations, covering the range from 50 to 1,500 μmol, was made. In a test tube, 0.25 mL of 0.1% HCl in 95% ethanol, 4.50 mL of 2% HCl and 0.25 mL of chlorogenic acid standard solutions were added, mixed by vortex and allowed to stand for approximately 15 min. Then the absorbance was read at 280 nm as described by GLORIES (1998). For the measuring total phenolic compounds content in celery extracts, 0.25 mL of 0.1% HCl in 95% ethanol, 4.50 mL of 2%

HCl and 0.25 mL of filtered extracts were added into test tube and further treated as standard solutions of chlorogenic acid. The total phenolic compound content was determined as follows:

$$TPC = \frac{A - 0{,}1083}{4{,}890 \cdot 10^{-4}} \text{ (µmol of chlorogenic acid/L)}$$

and calculated as µmol per gram of dry extract.

Antioxidant capacity

The antioxidant capacity of extract diluted by ethanol to concentrations ranging from 0.2 to 6 mg/mL, was determined by the DPPH test (CHOI *et al.*, 2002). Thus, an ethanol solution of DPPH radicals, 1 mL of a 0.3 mM, was added to 2.5 mL of the investigated extract and allowed to react at room temperature during 30 min. Then the Ab value was measured at 518 nm on a VARIAN UV–Vis Cary-100 Spectrophotometer, and converted into the percentage of scavenging capacity by using the equation, as described by LUCIANA *et al.*, 2001:

Scavenging capacity (%) = 100 - [(Ab of sample – Ab of blank) x 100/Ab of control],

where "Ab of sample" is the absorbance at 518 nm of the ethanol solution of the extract treated with the DPPH radical solution; "Ab of blank" is the absorbance at 518 nm of the ethanol solution of the extract (1 mL of ethanol added to 2.5 mL of extract), and "Ab of control" is the absorbance at 518 nm of the ethanol solution of DPPH radical (1 mL of a 0.3 mM added to 2.5 mL of ethanol). The final results are presented as EC_{50} value, i.e. concentration of investigated extracts sufficient to decrease the initial DPPH concentration by 50%.

Qualitative HPLC analysis

The HPLC analyses were performed on an Agilent 1100 Series HPLC system consisting of a micro vacuum degasser, binary pump, thermostatted column compartment, and variable wavelength detector. An original VIET *et al.* (1995) method was used. Column: Agilent Eclipse XDB-C18 4.6 mm ID x 150 mm (5 µm) 80 Å. Elution profile: A = 0,15% phosphoric acid in H_2O:MeOH, 77:23 (v/v, pH=2); B = MeOH. Isocratic: 0-3.6 min 100% A; gradient: 3.6 min 100% A; linear-24.0 min; 80.5% A-isocratic-30 min linear-60 min; 51.8% A-linear-67.2 min; 100% B; Flow rate: 1 mL/min. The dosing volume of an ethanol extract diluted to a concentration of 5 mg/mL, was 20 µL. The spectrophotometric detection in the UV region at 350 nm was used; therefore, all the used solvents had to have sufficient light permeability at this wavelength. Due to the poor reproducibility of retention times in HPLC chromatograms and the relative retention times in HPLC chromatograms, the relative retention indices were calculated as the ratio between retention time of the component in question and naringenin as the internal standard. The peak identity was checked by comparison of the relative retention indices with the previous ones (VIET *et al.*, 1995) and with reference compounds quercetin 3-O-rhamnoside, genkwanin 5-O-glucoside, genkwanin 5-O-(6"-O-malonylglucoside, kaempferol 7-O-rhamnoside, apigenin 5-O-(6"-O-malonylglycosid and naringenin, obtained from Sigma Chemical Co. (St. Luis, USA); methanol and ortho-phosphoic acid from Merck (Darmstad, Germany). The mass of analyzed compounds present in the samples was calculated by introducing the areas of peaks into the corresponding calibration curves. Those curves were made with reference compounds. Standard solutions of at least 5 concentration levels were injected three times each into the HPLC and the corresponding peak areas were plotted against concentration. Statistical analysis showed that all standard solutions had good linearity within the concentration range examined in this work, as shown by the high correlation coefficients. The validity of the calibration curve was checked every day of measurement by means of one of the 5 calibration solutions mentioned above and kept cool in a refrigerator. The percentage composition of the extracts was computed based on HPLC peak area.

Statistical analysis

Statistica version 5.0 software was used to perform the statistical analysis. The mean and standard deviations were obtained by Descriptive Statistics, marking the Median & Quartiles and Confirm Limits for Means.

RESULTS AND CONCLUSIONS

The investigation results of moisture content, extract yield and total phenolic compounds content, expressed as µmol of chlorogenic acid per g of dry extract are presented in Table 1.

FERNANDO *et al.* (2007) found the total phenolic compounds content in fresh celery root after being wounded and stored at 15°C for 2 days, was about 250 mg of chlorogenic acid per kg of fresh weight. MARINOVA *et al.* (2005) published that celery leaves cut into small pieces, frozen with liquid nitrogen, freeze-dried and vacuumized in hermetically sealed packages, contained 113 mg of gallic acid equivalents per 100 g of fresh mass, while according to Souri *et al.* (2008) results, the phenolic content in celery fruit was 436.05 mg of gallic acid equivalent per 100 g of dry weight.

Table 1 - Moisture content, extract yield and total phenolic compounds content in celery roots.

Group	C1	C2	C3
Plant material moisture content (%)[a]	8.28±0.2	9.62±0.3	8.41±0.2
Extract yield (g per g of plant material dry weight)[a]	0.28±0.03	0.32±0.03	0.33±0.02
Total phenolic compounds content (μmol chlorogenic acid per g of dry extract)[a]	368.51±15.3	297.13±14.7	265.44±16.1
EC_{50} (mg/mL)[a]	3.14±0.11	2.97±0.08	2.41±0.06

[a]Mean value of three determinations ±SD.

Our results showed that the phenolic compounds varied ranging from 265.44 to 368.51 μmol of chlorogenic acid per g of dry extract and the highest phenolic compounds content was in the C1 group where the samples with the smallest root size were grouped. In order to be able to compare our results to those of FERNANDO *et al.* (2007), where the content of 250 mg of chlorogenic acid per kg of fresh weight was found, and considering that dry matter content in celery samples was 4%, the phenolic compounds content is calculated to be 193.81 μmol chlorogenic acid per g of dry extract. So, the phenolic compounds content of 265.44 μmol of chlorogenic acid per g of dry extract in the C3 celery sample had a similar value while the content found in samples C1 was almost twice as high as the one found in celery root samples in FERNANDO *et al.* (2007). Hence, root size influenced on phenolic compounds content.

The results of antioxidant capacity of phenolic extracts from celery roots as EC_{50} values are shown in Table 1. The scavenging capacity ranged from 64.6 to 69.9%. Generally, the lower EC_{50} value indicates higher antioxidant capacity, the roots of C1 group had a slightly better antioxidant capacity than the others. For Trolox, the concentration necessary for reaching EC_{50} value was 3.67 μg/mL, so the investigated samples showed much lower antioxidant capacity than this compound.

Phenolic compounds composition

The HPLC chromatogram of 80% (v/v) ethanol extract of phenolic compounds from C2 sample is presented in Fig. 1.

In the investigated samples of dried celery roots, 24 compounds were identified, of which 20 are presented in Table 2, and the others are phenolic "p3" and "p5" found in C1 sample with a content of 2.82 and 1.16%, and phenolic "p7" and quercetin 3-O-rutinoside found in sample C3 in content of 1.66 and 2.22%, respectively. The content of the quercetin 3-O-rutinoside compound was added to the total content of quercetin glucoside for C3 sample in Table 2.

The highest content of identified compound in samples C1 and C2 was kaempferol 3-O-rhamnoside (18.63 and 19.03%, respectively) and

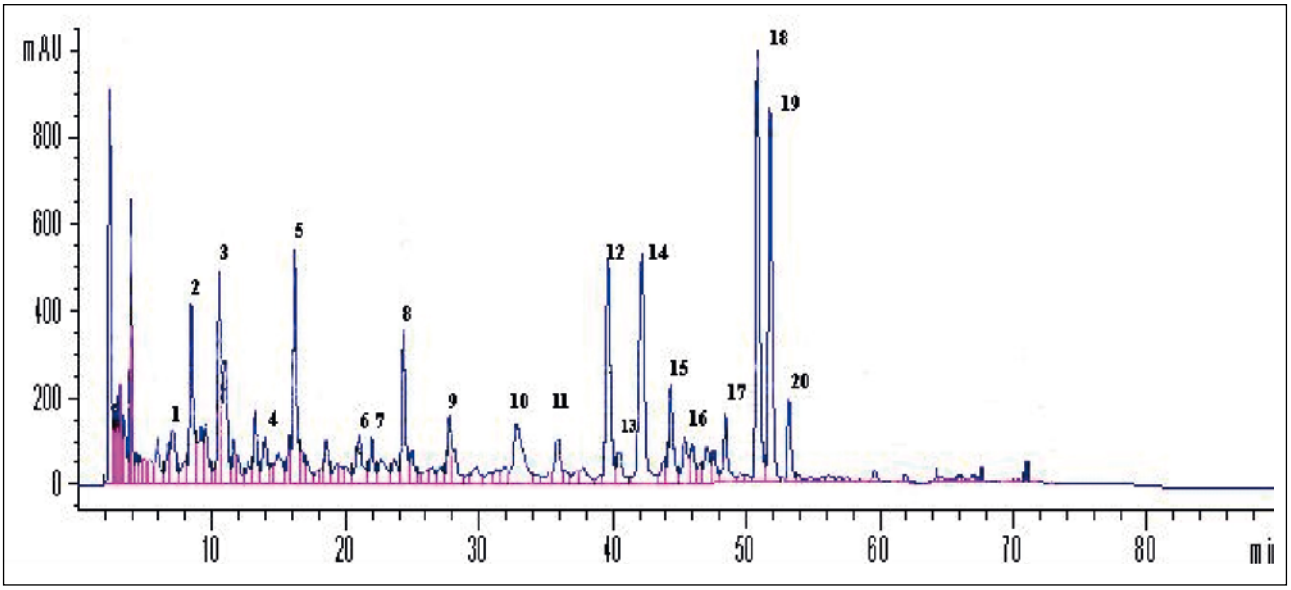


Fig. 1 - The HPLC chromatogram of 80% (v/v) ethanol extract of phenolic compounds from C2 group of celery roots.

Table 2 - Phenolic compounds identified in celery roots by HPLC analysis.

Component	Relative peak area, %		
	C1	C2	C3
1. Kaempferol glucoside "p1"	0.63[1]	0.58	1.05
2. Hydroxycinnamic acid "C1"	3.73	3.99	1.45
3. Monocaffeol-*meso*-tartaric acid	2.09	2.73	0.65
4. Protoapigenin-4-glucoside	0.57	0.67	1.91
5. 5-O-Caffeoylshikimic acid	2.83	2.52	1.31
6. Kaempferol 3-O-(6"-O-malonylglucoside)-7-O-glucoside	1.16	4.03	2.82
7. Phenolic "P5"	1.12	2.42	2.04
8. Caffeic acid methylester	1.52	1.93	3.52
9. Luteolin 5-O-glucoside	0.24	0.59	0.82
10. Quercetin 3-O-glucoside	1.67	2.61	1.56
11. Quercetin 3-O-(6"-O-malonylglucoside)	0.32	1.38	0.74
12. Quercetin 3-O-rhamnoside	10.24	11.16	13.33
13. Kaempferol 3-O-glucoside	2.05	1.95	1.50
14. Genkwanin 5-O-glucoside	11.3	11.97	15.16
15. Apigenin 5-O-(6"-O-malonylglucoside)	2.09	2.61	2.37
16. Naringenin	2.21	2.54	1.52
17. Kaempferol 3-O-rhamnoside	1.78	1.44	1.53
18. Genkwanin 5-O-(6"-O-malonylglucoside)	17.90	18.41	18.86
10. Kaempferol 7-O-rhamnoside	18.63	19.03	10.29
20. Genkwanin 4'-O-glucoside	0.83	1.83	1.78
Total Kaempferol glucoside (%)	**24.25**	**27.03**	**17.19**
Total Quercetin glucoside (%)	**12.23**	**15.15**	**17.85**
Total Genkwanin glucoside (%)	**30.03**	**32.21**	**35.80**
Total Apigenin glucoside (%)	**2.66**	**3.28**	**4.28**
Total Luteolin glucoside (%)	**0.24**	**0.59**	**0.69**
Total Naringenin (%)	**2.21**	**2.54**	**1.52**

genkwanin 5-O-(6"-malonylglucoside in sample C3 (18.86%).

As many derivates of kaempferol, quercetin, genkwanin, apigenin and luteolin compound are detected, in order to facilitate the comparison of our results to literature data; in Table 2, the total contents of these compounds are presented. Thus, the total content of kampferol glucoside is the sum of kaempferol glucoside "p1", kaempferol 3-O-(6"-O-malonylglucoside)-7-O-glucoside, kaempferol 3-O-glucoside, kaempferol 3-O-rhamnoside and kaempferol 7-O-rhamnoside, the total content of quercetin glucoside is the sum of quercetin 3-O-glucoside, quercetin 3-O-(6"-O-malonylglucoside) and quercetin 3-O-rhamnoside, the total content of genkwanin glucoside is the sum of genkwanin 5-O-glucoside and genkwanin 5-O-(6"-O-malonylglucoside), the total content of apigenin glucoside is the sum of protoapigenin-4-glucoside and apigenin 5-O-(6"-O-malonylglucoside) and the total content of luteolin is only that of luteolin 5-O-glucoside.

SONIA *et al.* (2007) found that the initial luteolin content in the celery petioles was 0.88 while apigenin initial content was 4.61 μg per g of fresh tissue. These contents represent 1.5 and 8% of total phenols content, respectively. ALAN *et al.* (1997) have also identified luteolin and apigenin in several celery varieties in whole celery plants, leaves and petioles. For hydrolyzed celery extract, considerable amounts of both compounds, 38 μg of luteolin and 97 μg apigenin were found, but the Authors pointed that quercetin and kampferol were not detected. The differences between the results of SONIA *et al.* (2007) and ALAN *et al.* (1997) indicate that the flavonoid content in petioles is markedly lower than in leaves. After storage at different temperatures of minimally processed celery, luteolin content increased up to approximately 8 and apigenin content up to approximately 35 nmol per g of fresh tissue (SONIA *et al.* 2006).

Contrary to ALAN *et al.* (1997) results that quercetin and kaempferol were absent, in all of our investigated size celery roots samples, besides luteolin and apigenin, kampferol, quercetin, genkwanin and naringenin were also detected. Different parts (petioles, leaves or root) and state (fresh or dried) of celery plant and different extraction conditions could be the reason for this. So, in the total sum, the main components are genkwanin glucosides (30.03 to 35.80%), kaempferol glucosides (17.19 to 24.25%), and quercetin glucosides (2.23 to 17.85%). The content of other components, apigenin glucosides, luteolin glucosides and naringenin were below 5%.

ACKNOWLEDGEMENTS

This work was supported under the project No.19048 by the Ministry of Science and Technology of the Republic of Serbia.

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Paper received March 15, 2010 Accepted July 30, 2010

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Gratitude is expressed to the following entities for contributing to the realization of the Journal by being supporting subscribers for 2010.

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ITALIAN JOURNAL OF FOOD SCIENCE
Rivista Italiana di Scienza degli Alimenti
DIRETTORE RESPONSABILE: Giovanni Chiriotti
AUTORIZZAZIONE: n. 3/89 in data 31/1/1989
del Tribunale di Perugia
Proprietà dell'Università di Perugia
TIPOGRAFIA Giuseppini - Pinerolo
Una copia € 20.00

ISSN 1120-1770 © 2011

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