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PAPER

DETERMINATION OF CONTAMINATION STAGES OF FROZEN CHICKEN DÖNER

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ABSTRACT

"Döner" is a traditional food product generally produced with beef, lamb, and/or chicken meat, and is a very popular product in Turkey. The aim of this study was to investigate the possible contamination sources during the processing stages of Chicken Döner. Total viable psychrotrophs, total lactic acid bacteria, *Enterobacteriaceae* spp., *Escherichia coli*, total yeast and mold, *Salmonella* spp., and *Staphylococcus aureus* counts were determined at 13 processing points. At the freezing stage, lactic acid bacteria and *Enterobacteriaceae* counts can decrease, but at this point the elimination of *E. coli* and *S. aureus* contaminations is also very important.

- Keywords: chicken döner, contamination, meat processing, microbiological quality, ready-to-eat -

INTRODUCTION

"Döner" (sometimes known by other names, such as gyro, donair, dona kebab, and shawarma) is generally made of beef, lamb, veal, and/or poultry meat and is a traditional meat product in the Middle East (TODD *et al.*, 1986; KILIÇ, 2003; KAYAARDI *et al.*, 2005). The traditional "Döner" is made from intact muscle, ground muscle, or slices of meat interleaved with layers of raw meat resembling minced meat (TODD *et al.*, 1986; KILIÇ, 2003; VAZGEÇER *et al.*, 2004). Meat pieces (thickness ranging from 1-6 mm) or ground meat is marinated (for 3-6 h) according to the producers' preferences with red or black pepper, salt, diced onion, onion juice, or onion powder; diced tomatoes or tomato sauce; olive oil; lemon juice or vinegar; white sugar; cumin; allspice; thyme; grape juice; milk or milk powder; yoghurt, and egg at 4°C for 12 h (KAYAHAN and WELZ, 1992; ANONYMOUS, 1995; VAZGEÇER *et al.*, 2004; KAYAARDI *et al.*, 2005). Then, döner dough is impaled on a vertical stainless steel döner stick. The döner is grilled in a vertical position in front of heating equipment (open gas or electric oven to cook the surface). When the meat surface is cooked, it is shaved off, and the cooked döner is thinly sliced. "Döner" slices are served either on a plate or on bread with sliced tomato, onions, and lettuce (ANONYMOUS, 1995; KAYIŞOĞLU *et al.*, 2003).

Recently, the use of chicken and turkey breast meat in "Döner" production has become very popular because of the low cost of production, the high nutritional value, and the ease of digesting the product (CHOULIARA *et al.*, 2007). When chicken breast meat is used to manufacture döner as described above, it is called "Chicken Döner" (KILIÇ, 2003). In recent years, Döner has become a ready-to-eat food product that is prepared in meat processing plants. After preparation, it is packaged in a plastic tray and held in refrigerated (+4°C, 24 h) or frozen conditions (-18°C, 6 months) until it is ready to be cooked. The aim of freezing is to control microbiological activity (KILIÇ, 2003; ERGÖNÜL and KUNDAKÇI, 2007).

The purpose of this study was to investigate the hygienic conditions and possible contamination sources in the processing stages of "Chicken Döner" that limit its shelf-life.

MATERIALS AND METHODS

Material

Sampling procedures

"Chicken Döners" were prepared four times in a local meat factory in Balıkesir, Turkey, at different times, and each sample from collected processing stages was analyzed twice (n = 4 x 2). Fresh, deboned chicken breast meat was obtained from another company, and all bone, skin, and subcutaneous fat were removed before use. Chicken breast fillets were marinated in sauce (tomato paste, yoghurt, vegetable oil, water, garlic, onion) for 12 h at 4°C. Immediately after, the fillets were massaged for 25 min at 10°C, after which they were given a cone shape and placed on a stainless steel döner spit. Plastic stretch film was used as the packaging material. The döner samples were frozen at -40°C, and subsequently stored at -18°C.

The microbiological analyses were conducted for the various stages of chicken döner production on the day of production. A sample of 100 g was collected from each stage of the production process, placed in a sterile Stomacher bag, and stored in a cooler for transportation to the laboratory, where the analyses were conducted within 2 h. The stages of sampling were as follows: 1) raw chicken breast meat (with bone), 2) the hands of the employees who were chopping the meat, 3) the meat cutting board and knife, 4) the meat collecting tray, 5) the fillets, 6) sauce, 7) marinated fillets, 8) fillets in sauce before massaging, 9) fillet in sauce after massaging, 10) the board used to skewer the fillets, 11) the hands of the employees who skewered the fillets, 12) the pre-frozen ready-to-eat chicken döner, and, 13) the post-frozen product.

Methods

Microbiological analysis

For the microbiological analyses, 25-g samples of chicken döner were weighed out aseptically, 225 mL of a sterile buffered peptone water (Oxoid CM0509) were added, and the mixture was homogenized in a Stomacher blender (Masticator, IUL Instruments, Spain) for 60 s at room temperature (20° ± 1°C). Decimal dilutions of the buffered peptone water solution were prepared and duplicated, and 1 or 0.1 mL of at least three appropriate dilutions were mixed or spread on the following agar media: Plate Count Agar (PCA; Oxoid CMO325) for total viable (TV) and psychrotroph counts, incubated at 35°C for two days and at 7°C for seven days, respectively (BERRUGA *et al.*, 2005); de Man-Rogosa-Sharp medium (MRS; Oxoid CM0361) for lactic acid bacteria, overlaid with the same medium and incubated at 37°C for 48 h and Rose Bengal Chloramphenicol Agar (RBC; Oxoid CM 549 supplemented with SR 78) for yeasts and molds, incubated at 25°C for five days (COULIARA *et al.*, 2007); Violet Red Bile Glucose Agar (VRBGA; Merck 1.10275) for *Enterobacteriacea* counts, incubated at 37°C for 24 h (GOVARIS *et al.*, 2007); Tryptone Bile X-glucuronide Agar (TBX - Oxoid, CM0945) medium for *E. coli* counts (ÇOLAK *et al.*, 2008), incubated at 37°C for 48 h; Streptomycin Thallous Acetate Actidione Agar (STAA, Oxoid CM0881 supplemented with SR0151) for *Brochothrix thermosphacta,* incubated at 22°-25°C for 48-72 h (LIN and LIN, 2002). Presumptive *Staphylococcus aureus* were determined on Baird-Parker Agar base

(Oxoid CM0275) enriched with Egg Yolk Tellurite (SR0054) and plates were incubated aerobically at 35°C for 2 d. For the detection of *Salmonella spp.*, 25 g of sample was inoculated for pre-enrichment in 225 mL of buffered peptone water, and homogenized in a stomacher blender and incubated at 35°-37°C. After 16-20 h, the pre-enrichment culture was transferred into Rappaport Vassiliadis Enrichment Broth (Oxoid CM0669), and Tetrathionate Broth (Oxoid CM0029) base media with a ratio of 0.1/10 and 1/10, incubated at 42°-43°C for 24 h, and 37°C for 24 h, respectively. A loopful of these two enrichments were streaked onto Xylose Lysine Desoxycholate Agar, (Oxoid CM0469) and MacConkey Agar (Oxoid CM0007) for selective growth, and were incubated at 35°-37°C for 18-24 h. The plates were examined for the presence of typical suspect colonies of *Salmonella*, i.e. pink colonies with or without black centers on XLD Agar, and colorless colonies on MacConkey Agar. Presumptive *Salmonella* colonies were then subjected to initial screening tests using Triple Sugar Iron Agar (Oxoid CM0277), Lysine Iron Agar (Merck, 1.11640.0500), Urea Broth (Merck, 1.08483.0500), and Lysine Decarboxylase Broth (Oxoid, CM308). All biochemical tests were performed at 37°C for 18-24 h. Positives were confirmed serologically utilizing *Salmonella* O grouping antisera (TEMELLI *et al.*, 2006; ÇOLAK *et al.*, 2008). Swab methods were used for the microbiological analyses of the meat collection tray, the cutting board and knife (EISEL *et al.*, 1998). Glove methods were used for the employees' hands (DE WIT and KAMPELMACHER, 1988). All samples were analyzed in duplicate, and the results were averaged for statistical analysis. Analyses were conducted separately on the materials from each package. All microbial counts were expressed as base-10 logarithms of colony forming (log CFU, g^{-1} or mL or swabbed sample), except *Salmonella* spp. In this study, the presence of *Salmonella* spp. was assessed using 25-g samples and the minimum detectable level from presence/absence test is typically one organism in a 25-g sample.

Statistical analysis

The SPSS 16.0 Professional Statistics package (SPSS Inc., Chicago, IL, USA) was used to determine mean ± standard deviation values of the microbiological counts.

RESULTS AND DISCUSSION

The results of the microbiological analysis showed: 4.45, 5.04, and 3.89 log CFU/g total viable count (TVC); 3.35, 3.61, and 3.88 log CFU/g psychrotrophs; 5.33, 4.12, and 3.50 log CFU/g total yeast and mold; 2.79, 3.67, and 2.68 log CFU/g *Enterobacteriaceae* for the raw chicken breast meat, the fillets, and the ready-to-eat frozen "Chicken Döner," respectively (Table 1).

Table 1 - Results of the microbiological analyses of the Chicken Döner Kebab samples and sauce (n = 4 x 2) collected from production stages.

Samples	Total viable count [a,b]	Psychrotrophs	Lactic acid bacteria	*B. thermosphacta*	*Enterobacteriaceae*	*E. coli*	Total yeast-mold	*S.aureus*
Raw chicken breast meat-bone	4.45±0.07	3.35±0.02	2.96±0.07	2.26±0.09	2.79±0.17	2.11±0.07	5.33±0.02	3.79±0.17
Fillet	5.04±0.02	3.61±0.06	2.66±0.06	2.13±0.15	3.67±0.12	2.34±0.06	4.12±0.41	3.75±0.24
Sauce	6.35±0.09	2.80±0.09	6.04±0.13	1.00±0.00	2.79±0.17	2.41±0.15	4.44±0.05	1.00±0.00
Marinated fillet	4.83±0.06	3.07±0.12	4.23±0.21	3.12±0.07	2.70±0.25	2.47±0.10	3.44±0.19	3.69±0.32
Fillet in sauce pre-massaging	4.89±0.06	3.24±0.05	4.23±0.18	3.24±0.32	3.83±0.21	2.73±0.02	3.26±0.32	3.29±0.05
Fillet in sauce post-massaging	4.97±0.04	3.49±0.09	4.26±0.04	3.37±0.16	3.92±0.05	2.90±0.09	3.29±0.45	3.32±0.09
Ready-to-eat döner pre-frozen	3.95±0.05	2.86±0.09	5.12±0.05	3.34±0.09	2.77±0.08	2.65±0.21	2.96±0.16	2.98±0.03
Ready-to-eat döner post-frozen	3.89±0.07	3.88±0.16	3.46±0.02	3.29±0.05	2.68±0.04	2.46±0.26	3.50±0.02	2.80±0.23

[a] Mean (± standard deviation) counts determined samples; [b] log CFU/g for chicken döner kebab samples and sauce.

Also, the TVC in fillets with sauce after marination (4.83 log CFU/g) were similar to raw chicken breast meat, although the sauce had a TVC that was considered high (6.35 log CFU/g). It was thought that because the marination was conducted in cold conditions (+4°C), this restricted the increase of TVC.

According to OKONKWO *et al.* (1994), unhygienic processing conditions and/or recontamination due to poor post-processing handling are usually responsible for the high viable counts of organisms in meat products. The influence of environmental sanitation on the microbial population is a highly significant factor that affects the quality of meat products. "Chicken Döner" have higher aerobic plate counts and psychrotrophic bacteria counts than beef döners, therefore, these could be a potential hazard for public health because of low hygienic quality (KAYIŞOGLU *et al.*, 2003). Bacterial counts (aerobes, *Salmonella* spp., *E. coli*) are higher on the breast area of broiler carcasses than on the thigh and drum areas. In addition, some microorganisms, particularly *Salmonella* spp., attach to the skin of the poultry and they are difficult to remove (KOTULA and DAVIS, 1999). A great risk may be incurred from these pathogens if chicken skin is added to the chicken döners (VAZGEÇER *et al.*, 2004). Also, hygienic quality is expected because of the normal prevailing conditions in the processing sites and the personal hygiene of the processors. This situation was explained by ELMALI *et al.* (2005), and ABDULLAHI *et al.* (2006), indicating that contamination of meat products can occur due to inadequate cleaning of processing equipment, such as the meat chopping board, knife, and the meat collecting tray, infected or unhygienic status of the handlers, and contamination from raw beef, among others.

Lactic acid bacteria (LAB) and *Brochothrix thermosphacta* are found to be significant components of the microflora of products stored at refrigeration temperatures, and both have been associated with the spoilage of meat and meat products (STOLLE *et al.*, 1993; SAMELIS *et al.*, 2000; CAYRE *et al.*, 2005). STOLLE *et al.* (1993) reported that LAB counts ranged from less than 2.30 log CFU/g to 7.32 log CFU/g in döner. In this study herein, the LAB count in raw meat (2.96±0.07 log CFU/g) was approximately two log units lower than marinated fillet (4.23±0.21 log CFU/g). It was thought that the use of sauce that contained yoghurt was responsible for increasing the count of LAB in the fillets with sauce after marination. Also, LAB counts were increased in the ready-to-eat, pre-frozen chicken döner due to cross-contamination caused by the staff, and the board that was used to skewer the fillets. However, there was no change in the counts of *B. thermosphacta* in the stages of the process. Nevertheless, it was identified that LAB counts were decreased after freezing. It is thought that LAB were subjected to adverse conditions, such as water crystallization and low temperatures, producing a degree of protein denaturation and bacterial membrane injury, with consequent decrease in viability and loss of reproductive capability, similar to that reported by DE GIULIO *et al.* (2005)

Species of *Enterobacteriacea*e, such as *Salmonella* spp. and *E. coli*, as well as gram-positives such as *Staphylococcus aureus*, may be found in considerable numbers in "Döners" and may create a high potential risk of foodborne diseases for consumers (KAYISOGLU *et al.*, 2003; VAZGECER *et al.*, 2004; ELMALI *et al.*, 2005; ARUN *et al.*, 2007). Among these, the presence of *E. coli* in both raw and cooked döners is a matter of particular concern (JOCKEL and STENGEL, 1984; ELMALI *et al.*, 2005). Ensuring the microbial safety of meat and meat products is based on application of proper process hygiene, managed through a HACCP-based system.

The results of the microbiological analyses showed: 2.11, 2.34, and 2.46 log CFU/g *E. coli* counts, and 3.79, 3.75, and 2.80 log CFU/g *S. aureus* for the raw chicken breast meat, fillet, and ready-to-eat frozen chicken döners, respectively (Table 1). Among the investigated bacteria, the maximum counts calculated from [log CFU/g], i.e., 3.70 for *E. coli*, and 3.70 for *S. aureus* for the raw meat used for various types of döners were in compliance with the Turkish Food Codex (ANONYMOUS, 2006) and, therefore, the meat samples tested in this study could be considered to have good hygienic quality. Nevertheless, it is widely known that humans are the primary sources of contamination in most of the poisonings caused by *S. aureus*, where its growth is inhibited at temperatures under 5°C. In the EU, verification of the hygienic functioning of the manufacturing process for meat/meat products is done through microbiological testing to determine whether process hygiene indicator organisms ("aerobic colony count" and/or *E. coli* count) are within given acceptable ranges (ANONYMOUS, 2005). The detection of these bacteria in raw chicken meat is a very important indicator of the cross-contamination caused by staff during the production process.

Results of the microbiological analysis of samples collected from equipment and the hands of personnel revealed that counts were notably high from the aspect of total viable count, *E. coli*, and *S. aureus* (Table 2). This may be an indication of insufficient sanitary applications in the plant.

Salmonella spp., which can cause infections even with low counts (1-10 cell/g), have been reported to form the natural microflora in poultry farms, where they are raised, and therefore, can be a risk factor for humans and animals. In our study, all samples taken from the various stages of production were assumed to have *Salmonella* colonies, but these were not detected in the samples by serological tests. Similar

Table 2 - Results of the microbiological analyses of possible contamination stages (n = 4 x 2) collected from Chicken Döner Kebab production stages.

Samples	Total viable count [a,b]	Psychrotrophs	Lactic acid bacteria	*B. thermosphacta*	*Enterobacteriaceae*	*E. coli*	Total yeast-mold	*S. aureus*
Hands of staff-meat chopping	4.44±0.03	2.96±0.08	1.00±0.00	3.28±0.03	1.00±0.00	1.00±0.00	4.95±0.03	3.39±0.05
Meat cutting board and knife	4.49±0.04	3.34±0.12	1.00±0.00	3.30±0.14	2.82±0.05	2.31±0.03	4.23±0.12	1.00±0.00
Meat carriage-collecting tray	4.04±0.05	4.65±0.04	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00	5.20±0.08	1.00±0.00
Board of skewering fillets	3.59±0.09	3.04±0.03	3.02±0.09	3.46±0.06	1.74±0.35	1.00±0.00	1.00±0.00	1.00±0.00
Hands of staff-skewering fillets	5.42±0.03	3.72±0.05	4.09±0.16	3.34±0.02	2.16±0.12	1.00±0.00	2.96±0.04	3.24±0.12

[a] Mean (± standard deviation) counts determined processing stages; [b] CFU/swabbed sample for collection tray, cutting board and knife; CFU/mL for staff hands.

results were reported for "Döners" by VAZGECER *et al.* (2004).

In this study, yeast and mold count, which were at high levels, had decreased from 5.33 to 3.44 log cfu/g after marination. After this processing stage, the population of yeast-mold occurred steadily throughout the frozen periods (Tables 1 and 2). The high levels of yeast and mold counts before marination, can be related to contamination from the raw material, and poor hygiene applied in the plant. It was also thought that yeast and mold contamination is a problem in meat production and to avoid problems with these, it is important to monitor the hygienic quality of the environmental air and equipment, and follow up with corrective actions to prevent further contamination.

CONCLUSIONS

Based on the critical appraisal of the chicken döner production process, some of the potential safety and quality hazards should be appropriately controlled. Potential hazards associated with the production line and considerations include: i) the raw material could contribute to the possible growth of pathogenic bacteria, so it should be purchased only from reliable suppliers; and ii) the production process is subjected to significant contamination from both human and environmental sources, such as the cutting board and knife, the meat collecting tray, and the hands of the staff; therefore strict compliance with hygienic regulations, such as those required by the HACCP (Hazard Analytical Critical Control Points) and GMP (Good Manufacturing Practice) programs is very important at all stages, from the production to the consumption of chicken döner.

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PAPER

VOLATILE ORGANIC COMPOUNDS OF PARMA DRY-CURED HAM AS MARKERS OF AGEING TIME AND AGED HAM AROMA

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ABSTRACT

Extension of Parma ham ageing time resulted in several changes in volatile organic compounds in the ham headspace, enhancing signals for branched aldehydes/alcohols and ethanol/ethyl esters. The NaCl content of dry-cured hams was found to be positively related to volatile analytes with low solubility in dry muscle and molecules coming from lipid oxidation, but negatively to certain branched aldehydes originating from amino acids. In PLS regression relating volatile compounds to matured dry-cured ham aroma, branched aldehydes and several oxidation compounds were found to be influential in the sensory perception of matured ham odour. In this respect, oxidative mechanisms (lipid oxidation and oxidative degradation of amino acids) would seem to prevail over other biochemical pathways in increasing the odour of aged dry-cured ham.

- Keywords: aged dry-cured ham odour, ageing time, volatile compounds, ethyl esters, oxidation compounds, salt -

INTRODUCTION

The dry-cured ham known as 'Prosciutto di Parma' is among the most popular PDO products and its manufacture is regulated by the Consortium for its protection (Regulation EEC 2081/92).

In the early stages of traditional processing, the raw hams remain at low temperatures (0°-3°C), including the double salting with a mixture of dry and wet salt, and a resting phase for inner salt equalization and muscle dehydration. Subsequently, the hams are moved to cellars kept at room temperature for the drying and maturing phases, in a process that can take anywhere from 12 months (minimum time for brand apposition) to 30 months and beyond. Salt intake and dehydration play an important role in microbial control by reducing water activity (a_w) in the outer and inner zones of dry-cured hams; in addition, temperature, relative humidity and airflow in the processing rooms are kept under control to achieve a safe and high quality product (ASEFA *et al.*, 2011). No addition of preservatives and ingredients other than sodium chloride is permitted.

The flavour of dry-cured ham is the result of biochemical and microbial processes which take place during the processing time, when volatile organic compounds (VOCs) are generated (TOLDRÁ *et al.*, 1997). Most of these VOCs belong to the chemical categories of alcohols, esters, aldehydes, ketones, sulphur compounds, aromatic and aliphatic hydrocarbons. Differing VOC profiles in dry-cured hams have been ascribed to geographic origin (SANCHEZ-PENA *et al.*, 2005; SABIO *et al.*, 1998), feeding (JURADO *et al.*, 2007), surface microbiota (MARTIN *et al.*, 2006), processing type and length (FLORES *et al.*, 1997; RUIZ *et al.*, 1999) and chemical composition (PEREZ-JUAN *et al.*, 2006).

The subject of VOC dynamics during the extended ageing time of Parma ham has been tackled in the past (HINRICHSEN and PEDERSEN, 1995), but the possible effect of the ham's composition was not taken into account. Notwithstanding, due to the characteristics of this product, which features large amounts of moisture and inner a_w values that continue to exceed 0.90 even after two years of processing (VIRGILI *et al.*, 2007), the identification of headspace VOCs associated with the effective sensory perception of ageing could prove useful in detecting analytical markers of sensory quality.

Consistent with these preliminary remarks, the aim of this work is to detect markers of ageing time of Parma ham among identified VOCs, and their relationships with the sensory descriptor "matured dry-cured ham odour".

MATERIALS AND METHODS

Ham sampling

A total of 34 dry-cured hams, average deboned weight = 7.40 ± 0.5 kg, were taken in numerically homogeneous batches for each ageing time from two manufacturing plant facilities. Each facility provided 22 (8 standard maturation, 7 extended maturation, and 7 extended ageing) and 12 (3 standard maturation, 5 extended maturation, and 4 extended ageing) dry-cured hams respectively. The two plants were operating in accordance with mandatory basic regulations for Protected Designation of Origin (PDO) products, even though potential differences occurring in applied processing conditions can be a variability source for final outcomes. Both plants processed green hams obtained from the domestic heavy pigs allowed for Parma ham production (at least 9 months old at slaughtering), but no further information about the raw material (e.g. crossbreeding, feeding, slaughtering weight) was available.

After ham de-boning and partial removal of the rind, two adjacent ham portions, 10 mm thick, including the whole slice with intramuscular, intermuscular and subcutaneous fat (Fig. 1a), were cut with an electric slicer from the centre of each ham, perpendicularly to the femur bone and below the boundary trimming, for proximate composition and volatile organic compound (VOC) analyses. Both slices included the sections of muscles shown in Fig. 1a, intramuscular, intermuscular and exterior fat; the external fat layer was standardized to 1 cm thickness. The remaining part of the ham, from the centre to the butt, was kept for sensory analysis, while the shank was not used. Each part was separately stored in a vacuum-sealed bag; the slice for proximate composition and the portion for sensory analysis were stored at 2°C and analysed within one week from sampling, while the slice for VOC analysis was kept frozen at -20°C and analysed within three months.

Proximate composition

The whole slice was thoroughly minced and its major chemical components were determined. Moisture was determined as the weight loss of ca. 2 g per minced slice after drying at 100°-102°C for 16-18 h according to the AOAC method (2002a). Salt content was estimated at 10 g per minced slice as chlorides, which were extracted with warm water (40°C) and quantified using the Carpenter-Volhard method, according to the AOAC (2002b). Proteins were determined as total nitrogen in ca. 2 g of minced muscle using the Kjeldal method and calculated as N × 6.25 following the AOAC (2002c). The estimated fat content was calculated as the difference

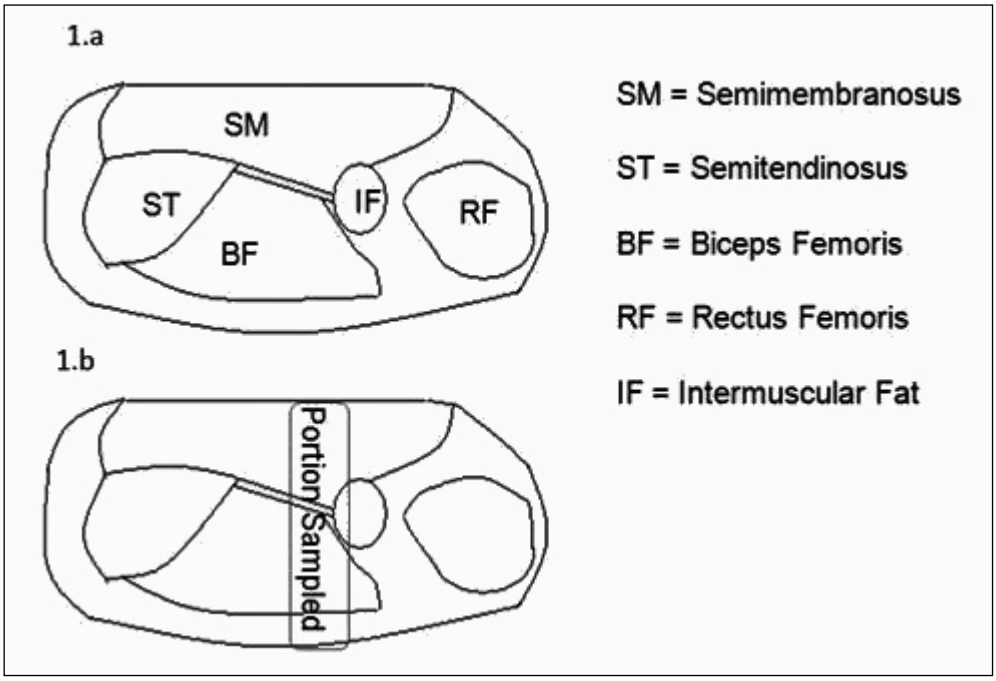


Fig. 1 - a) 10 mm thick slice of Parma ham used for proximate composition analyses; b) the centre of the slice (labelled as "portion sampled") is the portion removed from the frozen slice for volatile HS-SPME-GC-MS analysis.

from 100 of the summed proximate data. Moisture, salt and protein content were expressed as grams per 100 grams wet slice and as grams per 100 grams dry matter.

Sampling and headspace analysis

The central portion of the slice (Fig. 1b), including fat and sections of *biceps femoris* and *semimembranosus* muscles, was removed from the frozen slice: this sampling position was established to minimize the variability in fat and lean distribution occurring in the whole slice. The ham portion was fragmented with a knife into particles of approximately 1-2 mg weight. 3 g of the sample was weighed into a 20 mL glass vial tightly capped with a PTFE septum and left for 10 min at 40°C to allow temperature equilibration. Volatile components were extracted by Headspace Solid-Phase Micro-Extraction technique (HS-SPME), piercing the septum covering each vial with a needle equipped with a Carboxen/PDMS/DVB (Supelco, Bellefonte, PA) coated fibre. Prior to the collection of volatiles, this fibre was preconditioned at 250°C for 40 minutes in the GC injection port and exposed to the headspace for 180 min (SANCHEZ-PEÑA *et al.*, 2005) at 40°C. Then the fibre was inserted into the injector port of the GC for 1 min at 250°C using the split mode (split ratio 1:10). Temperature, time of incubation and injection were controlled by means of a TriPlus Autosampler (Thermo Electron Corporation) using Excalibur 1.4 software (Thermo Electron Corporation).

Gas chromatography-Mass spectrometry (GC-MS) analysis

The volatile compounds were separated using a SLB-5ms column (Supelco; 60 m × 0.25 mm id × 0.25 μm film thickness) installed on a Trace GC Ultra (Thermo Electron Corporation). The carrier gas was helium. The oven temperature was kept at 36°C for 15 min, programmed to rise by 10°C/min to 250°C and then held for 5 min. The GC-MS transfer line temperature was 280°C. The mass spectrometer was operating in the electron impact mode, with an electron energy of 70eV, and a scan rate of 1.4 s^{-1} over a range of m/z from 35 to 350 in full scan mode for data collection.

The identification of volatile compounds by GC-MS was carried out using a DSQII mass-selective detector (Thermo Electron Corporation). Volatile compounds were tentatively identified by comparison with reference spectra from the NIST 2005 version 2.0 spectral library database and with Kovats retention indices in accordance with the literature (NIST, Gaithersburg, MD, USA).

Peak integration and relative quantification were based on the signal area, computed on an arbitrary scale, according to the Single Ion Monitoring (SIM) mode and based on a single ion selected for each compound according to its fragmentation pattern, in order to improve selectivity and remove noise due to background and co-eluting peaks. Two replicates were run and averaged for each sample. An aqueous solution of ethyl propionate (0.25 mg/L) was used as exter-

nal standard twice a day to correct the chromatograms according to instrumental performance.

Sensory analysis

Samples were evaluated by quantitative descriptive analysis (MEILGAARD *et al.,* 1999). The panel, consisting of 8 members, were trained (ISO 8586-1: 1993; ISO 8586-2: 1994) in three preliminary sessions in the use of the attribute "odour of aged dry-cured ham" defined as the complex and characteristic dry-cured ham odour related to the presence of aged fat and aged muscle (GUÀRDIA *et al.,* 2010). This attribute was scored on a non-structured 0-9 intensity scale (0= not detected, 9= maximum perception of the attribute) and anchored to the given scale values by providing the panel with dry-cured hams corresponding to perceptions of the attributes covering most of the intensity scale. Dry-cured ham slices 1.0 mm thick with 1 cm of covering fat, obtained using an electric slicer a few minutes before testing, were presented at 15°C, according to a randomized order. Analysis of the 34 hams took no. 7 sessions (no. 5 samples per session). At the beginning of the session, each dry-cured ham was sliced and presented to the panellists in an open tray. At the end of the testing sessions, the sensory rating for each ham sample was averaged over the panel (to be kept, scores had to fall within the panel mean ± 3 standard deviations).

Statistical analysis

SPSS 14 for Windows was used for statistical analysis, running the procedures ANOVA (one-way analysis of variance) and General Linear Model (GLM); in GLM, the processing length was the sole main effect, while salt content was included as covariate. The Least Square Means (LSM) of volatile compounds of each group of processing length were estimated and the Bonferroni test was performed to statistically separate them ($P < 0.05$). Partial-Least-Square (PLS) analysis (Unscrambler ver. 9.7, CAMO Software AS, Norway) was applied to relate sensory scores of "aged ham odour" (dependent variable) with the set of HS volatiles (independent variables) and Martens' uncertainty test was run to detect significant independent variables; two PLS components were run to achieve variance reduction of the dependent variables (calibration model) by means of the Full Cross Validation Method.

RESULTS AND DISCUSSION

Identified VOCs and proximate composition

Identified VOCs belonged to chemical categories of aldehydes, alcohols, ketones, esters, acids, sulphur compounds, aliphatic and aromatic hydrocarbons are reported in Table 1. Most of the more than 60 identified volatile compounds have been previously reported in studies dealing with dry-cured ham odour (with the exception of 3-heptanone and 2,3-dimethylphenol), and their origin and olfactory notes have been thoroughly discussed (THERON *et al.,* 2010; GARCÍA-GONZÁLEZ *et al.,* 2008; MARCO *et al.,* 2006). Amino acid catabolism, auto- and β-oxidation of lipids, esterase activity and carbohydrate catabolism are the biochemical pathways leading to the generation of most of the VOCs reported in Table 1.

The amount of volatile molecules in the headspace is related to their concentration in the substrate as well as interaction with food components (DE ROOS, 2000). Applying the same experimental conditions to all tested samples, the portion of the analyte absorbed by the fibre is related to its original concentration in the sample and to the analyte distribution coefficient sample/headspace as reported by WANG *et al.* (2005).

In general, lipids and proteins reduce the volatility of aroma compounds, while salt, through the salting-out phenomenon (FLORES *et al.,* 2007), can influence the release of analytes into the headspace depending on the nature of the VOCs (PEREZ-JUAN *et al., 2*007). As a consequence, the composition of dry-cured hams is a potential source of variability in the release of volatile compounds into the headspace, because of the sample/headspace distribution coefficient and the analytes absorbed by the fibre; all to be taken into account when differences due to ageing times are investigated.

The proximate composition data of ham slices, expressed on a wet and dry matter basis and grouped according to assayed ageing times are reported in Table 2. Dry-cured hams with scheduled processing times showed no difference in estimated fat and protein content; the expected decrease in moisture due to progressive dehydration occurring during protracted ageing, was not significant ($P > 0.05$). These findings can be ascribed to variability in ham slices in terms of lean and fat distribution masking moisture changes occurring in single muscles during ageing (VIRGILI *et al.,* 2007). The most aged hams were the least salty (on a dry matter basis), as a result of the common practice of using less salt for hams manufactured for extended ageing, in order to prevent excessive salt concentration in the dried muscles. Even if dry-cured ham groups differed only in salt content, the sensory score given to the descriptor "aged ham aroma" was higher in longer-aged hams than in lesser aged ones (6.38 *vs* 5.76, $P < 0.05$). Consequently, the volatile release into the headspace may be affected by salt differences between dry-cured ham groups, masking the actual concentration in the substrate. Furthermore, during ham processing, salt, well-known as a proteolysis inhibitor, may be decreasing the production of VOCs generat-

Table 1 - Volatile organic compounds identified in Parma ham grouped into chemical categories. Kovats retention indices (KI) and ions used for peak integration according to the Single Ion Monitoring (SIM) mode are reported for each listed compound.

Volatile compound	compound origin[a]	KI[b]	ion (m/z)
Alcohols			
Ethanol	CC	<500	45
2-Methyl-1-butanol	AAC	748	57
3-Methyl-1-butanol	AAC	744	55
1-Pentanol	LO	778	70
2-Pentanol	β-O	707	55
1-Penten-3-ol	β-O	683	57
1-Hexanol	LO	876	69
1-Octen-3-ol	β-O	983	57
Aldehydes			
2-Methyl-propanal	AAC	553	72
3-Methyl-thiopropanal	AAC	910	104
2-Methyl-butanal	AAC	648	58
3-Methyl-butanal	AAC	659	57
Pentanal	LO	701	58
Hexanal	LO	803	72
Heptanal	LO	904	96
Octanal	LO	1005	84
Nonanal	LO	1108	82
2-Decenal	LO	1270	85
2-Undecenal	LO	1372	70
Benzaldehyde	AAC	968	106
Benzenacetaldehyde	AAC	1055	91
Ketones			
2-Propanone (acetone)	CC	<500	58
2-Butanone	CC	600	72
2,3-Butanedione (diacetyl)	CC	592	86
3-Hydroxy-2-butanone (acetoin)	CC	714	88
2-Pentanone	β-O	686	86
2-Hexanone	β-O	793	58
2-Heptanone	β-O	889	114
3-Heptanone	β-O	892	99
2,3-Octanedione	β-O	986	99
Acids			
Acetic acid	CC	578	60
Butanoic acid	CC	787	60
3-Methyl-butanoic acid	AAC	850	60
Pentanoic acid	LO	848	60
Octanoic acid	LO	1164	60
Esters			
Ethyl acetate	EA	611	61
Ethyl 2-Methyl-propanoate	EA	763	71
Ethyl 2-Methyl-butanoate	EA	854	102
Ethyl 3-Methyl-butanoate	EA	859	88
Ethyl pentanoate	EA	902	88
Ethyl hexanoate	EA	998	88
Ethyl heptanoate	EA	1096	88
Ethyl octanoate	EA	1193	88
Ethyl decanoate	EA	1392	88
Sulphur compounds			
Dimethyl-sulfide	AAC	517	62
Dimethyl-disulfide	AAC	746	94
Alifatic and aromatic hydrocarbons			
Pentane	LO	500	72
Hexane	LO	600	86
Heptane	LO	700	100
Octane	LO	800	85
Nonane	LO	900	85
Undecane	LO	1100	85
Tetradecane	LO	1300	57
2-Octene	LO	815	112
Toluene	AAC	769	91
Ethylbenzene	AAC	864	91
2,3-Dimethyl phenol	AAC	922	122
p-xylene	U	861	91
o-xylene	U	895	91
Furans and lactones			
2,5-Dimethyl furan	β-O	706	96
2-Pentyl furan	β-O	992	81
γ-Hexalactone	β-O	1064	85
γ-Octalactone	β-O	1288	75

[a]Compound origin. CC: carbohydrate catabolism; AAC: amino acid catabolism; LO: lipid oxidation; β-O: β-oxidation; EA: esterase activity; U: uncertain origin.
[b]Kovats retention index of the compounds injected in the GC-MS using a SLB-5ms column (Supelco, 60 m × 0.25 mm id × 0.25 µm film thickness).

ed by amino acid catabolism (PEREZ-JUAN *et al.*, 2006), and accelerating lipid peroxidation by acting as a pro-oxidant agent (KANNER *et al.*, 1991).

Comparison of VOCs detected in Parma hams with different ageing times

Differences in volatile compounds due to extended ageing times were evaluated by means of GLM analysis: the processing length was the sole main effect of the model, while salt was included as covariate to estimate salt coefficients for HS-VOCs (Table 3): a significant covariate estimate coefficient ($P < 0.05$), denotes a relationship between ham salt content and the corresponding volatile compound. Further sources of uncontrolled bias in dry-cured hams with different ageing times may be due to the processing conditions applied in the plants of origin, on top of raw material properties.

Most branched aldehydes originating from amino acid catabolism proved to be positively influenced by maturing time: 3-methyl-butanal and benzeneacetaldehyde rose sharply in the most aged samples, according to previous findings (JURADO *et al.*, 2007). Branched volatile compounds represent the final step of the proteolytic events that occur during dry-cured ham maturation and produce large amounts of free amino acids (FAA). Both chemical oxidative Strecker degradation and microbial amino acid catabolism (Erlich pathway) have been postulated as accounting for the formation pathway of branched volatile compounds in dry-cured

Table 2 - Proximate components of Parma dry-cured hams grouped according to processing times, expressed on wet slice and on dry matter as % component (± standard deviation)[a].

	14-15 months (n = 11)	17-18 months (n = 12)	21-22 months (n = 11)	P value
g/100 g wet slice				
NaCl	4.78±0.39	4.74±0.47	4.39±0.54	0.114
estimated fat	19.6±4.15	20.0±4.75	21.1±4.20	0.715
protein	24.9±2.12	25.9±1.83	26.2±3.12	0.460
moisture	49.7±2.11	48.9±2.71	47.3±2.37	0.079
g/100 g dry matter				
NaCl	9.53±0.89[a]	9.32±1.23[a,b]	8.33±0.89[b]	0.020
estimated fat	38.7±6.69	38.8±7.34	39.9±6.93	0.999
protein	49.8±5.92	49.9±6.18	49.9±6.37	0.907

[a]Estimated means within a row with different lower case letters are different (Bonferroni multiple comparison method, P < 0.05).

Table 3 - Effect of maturation time on volatile organic compounds of Parma ham, expressed in arbitrary area units × 10^{-4} according to the Single Ion Monitoring (SIM) mode, and correlation with NaCl content of the ham slice. Least Square Means (LSM) of VOCs with significant differences (P < 0.05) along the processing time were reported[a].

Volatile Organic Compounds	Processing time[b]			P-value	Pooled SEM[c]	Covariate (NaCl) estimate coefficient[d]
	1 (n =11)	2 (n = 12)	3 (n = 11)			
Aminoacid catabolism						
2-Methyl-propanal	7.74[b]	9.40[a,b]	11.2[a]	0.037	0.51	-2.34*
3-Methyl-thiopropanal	7.71[b]	11.0[a]	13.2[a]	0.001	0.51	
2-Methyl-butanal	60.8[b]	74.7[a,b]	98. 5[a]	0.018	5.10	
3-Methyl-butanal	77.3[b]	84.7[b]	122[a]	< 0.001	4.21	-26.4**
Benzenacetaldehyde	128[b]	187[b]	357[a]	< 0.001	15.9	
2-Methyl-1- butanol	6.41[b]	8.73[a,b]	19.3[a]	0.019	1.88	
2, 3-Dimethyl phenol						1.31**
Toluene	39.6[a,b]	35.0[b]	44.5[a]	0.023	1.35	9.72**
Ethylbenzene						37.2*
Lipid autoxidation						
1-Pentanol	168[a]	118[b]	76.4[c]	< 0.001	6.21	
1-Hexanol						48.5**
Hexanal	467[a]	378[a,b]	322[b]	0.048	23.7	
γ-Hexalactone						24.5**
Octanoic acid	11.7[b]	16.7[a]	15.4[a,b]	0.012	0.68	
Nonane	15.1[b,c]	26.1[a,b]	38.8[a]	0.004	2.64	
Tetradecane						14.5*
Lipid β-oxidation						
1-Penten-3-ol	54.1[a]	55.4[a]	36.4[b]	0.008	2.49	
2-Pentanone						70.6*
2-Heptanone						7.70**
Carbohydrate catabolism						
Ethanol	1850[b]	2550[a,b]	3540[a]	0.014	214	
Butanoic acid	33.8[b]	53.6[a]	33.1[b]	0.009	3.09	
Esterase activity						
Ethyl acetate	9.12[b]	14.3[a,b]	19.8[a]	0.012	1.34	
Ethyl 2-methyl-propanoate	3.21[b]	8.60[a]	6.85[a,b]	0.029		
Ethyl 2-methyl-butanoate	12. 8[b]	28.1[a]	28.9[a]	0.020	2.58	
Ethyl 3-methyl-butanoate	29.4[b]	69.9[a]	66.1[a]	0.021	6.47	
Ethyl pentanoate	13.7[b]	24.1[a,b]	36.7[a]	0.005	2.67	
Ethyl hexanoate	188[b]	322[a,b]	444[a]	0.009	31.3	
Ethyl heptanoate	4.77[b]	7.65[a,b]	9.26[a]	0.035	0.68	
Ethyl octanoate	25.8[b]	48.3[b]	98.9[a]	0.001	7.17	
Ethyl decanoate	11.2[b]	20.5[b]	46.3[a]	0.001	3.57	
Unknown origin						
p-xylene						44.7**
m-xylene						19.4**

[a]Estimated means within a row with different lower case letters are different (Bonferroni multiple test, P < 0.05); [b]1 = 14-15 months; 2 = 17-18 months; 3 = 21-22 months; [c]Values for SEM represent the pooled standard error of the mean; [d]NaCl content of dry-cured ham slice was included in the model as covariate. Significant effect: *, P < 0.05; **, P < 0.01; non-significant covariate estimate coefficients were not reported.

ham (MARTIN *et al.,* 2006; ANDRADE *et al.*, 2009). During the maturation of Parma ham up to 24 months, a progressive increase in free amino acids (FAA) has been detected (VIRGILI *et al.,* 2007), matching the increase in amino acid-related volatile compounds found in the present study. Among branched VOCs, salt estimate coefficient was significant for 2-methyl-propanal and 3-methyl-butanal: the negative sign may be accounted for by the inhibition exerted by sodium chloride on proteolytic activities (TOLDRÀ *et al.,* 1997; JURADO *et al.,* 2007), proteolysis, and free amino acid release in dry-cured ham (VIRGILI *et al.,* 1999). In this respect, the above-mentioned branched aldehydes, having low odour thresholds and odour notes relevant for dry-cured meat (THERON *et al.,* 2010; CARRAPISO *et al.,* 2010), which would be expected to increase in the headspace of dry-cured hams with low salt and high free amino acid content. 2-methyl-1-butanol and characterized by malty olfactory notes coming from the microbial reduction of 2-methyl-butanal, remarkably increased in the headspace of the longest aged dry-cured hams. Toluene, ascribed to the catabolism of phenylalanine (BERDAGUÉ *et al.,* 1991), or deriving from the cyclization of unsaturated carboxylic chains produced by lipid degradation (MIN *et al.,* 1977), increased in the headspace as a result of both ageing and salt (Table 3). The positive sign of the salt covariate can be explained by the salting-out phenomenon enhancing the release of analytes which are poorly soluble in dried muscle such as toluene; in the same way, ethyl benzene and 2,3-dimethyl-phenol, generated by amino acid catabolism, could be increased in the headspace of more salty dry-cured hams, masking the expected variations due to ageing time.

Most volatile compounds generated by fatty acid oxidation either decreased during the ageing of dry-cured ham (1-pentanol and hexanal) (BOLZONI *et al.,* 1996), or remained fairly constant, e.g. octanoic acid.

Among the VOCs originating from β-oxidation, 1-penten-3-ol decreased during ageing; the positive sign of the salt covariate for 2-pentanone may be increasing this molecule in the headspace of the saltiest samples, mainly belonging to the least aged group (Table 1). Oxidation compounds characterized the first ageing deadline (14-15 months), with the exception of nonane, which displayed a significant increase during the extended ageing of assayed hams. Several VOCs generated by oxidation pathways had positive significant relationships with salt content (1-hexanol, γ-hexalactone, tetradecane, 2-pentanone, 2-heptanone).

Butanoic acid, generated by carbohydrate fermentation, showed a rather slight decrease during the extended ageing of dry-cured hams, as a possible consequence of the decrease in carbohydrate availability, while ethanol was characterised by a sharp increase during ageing. Ethanol has been recognized as most likely originating from pyruvate (SPAZIANI *et al.,* 2009) produced by the microbial metabolism of free organic acids and amino acids (LIU, 2003). The accumulation of amino acid sources during ageing is consistent with the progressive increase in ethanol during ham ageing.

Ethyl ester formation was related to the presence of ethanol and was reported as being the result of microbial enzymatic mechanisms such as esterification and alcoholysis (TALON *et al.,* 1998; LIU, 2003). The enzymes needed for ethyl esters biosynthesis are present in *Micrococcaceae* (MONTEL *et al.,* 1996) and in many species of yeast and moulds (JELÉN and WASOWICZ, 1998), i.e., microbial populations detected at high levels in Parma hams (LORI *et al.,* 2005; SIMONCINI *et al.,* 2007). The present investigation found a rise in ethyl esters during the extended ageing of Parma dry-cured hams, unaffected by salt variations. SABIO *et al.* (1998), in comparing the volatile compounds of six types of European dry-cured hams, reported that Parma hams were more characterised by ethyl esters than other types of ham. According to the data shown in Table 3, ethyl esters can be regarded as a class of volatile compounds highly representative of fully-aged Parma dry-cured hams: the processing method (PAROLARI, 1996), the a_w values still around 0.90 after two years of processing (VIRGILI *et al.,* 2007) and the absence of preservatives other than salt, may account for the increased microbial esterase activities in comparison with other dry-cured ham types (HINRICHSEN and PEDERSEN, 1995).

Relationship between "matured ham smell" and volatile compounds

Several volatile markers of the investigated time range were evidenced, but it may be that molecules highly representative of increasing ageing time had a different influence on the sensory perception of aged ham aroma. Since the sensory quality of dry-cured ham is influenced by descriptors related to the intensity of dry-cured ham flavour and aroma (RESANO *et al.*, 2010), the relationship between volatile molecules corresponding to scheduled ageing times and the sensory perception of the matured dry-cured ham smell was investigated.

The algorithm used was the PLS regression (MARTENS and MARTENS, 1986); this technique constrains the components obtained by linear combination of the x-variables (volatile compounds listed in Table 1) to be orthogonal, thereby overcoming the problem of related variables; the use of the full cross validation method prevents non-significant relations even if the model is run on more variables than objects. The regression model achieved by means of two PCs, gave a reduction in % Y-variance corresponding to 42.7 (PC1) and 67.6 (PC1 + PC2). Table 4

Table 4 - Matured ham aroma vs. volatile compounds in Parma dry-cured ham. Coefficients of volatile compounds in the PLS regression are reported in standardized form[a]. The two PLS-factors account for 42.7 and 24.9% of the variance, respectively.

Variables	1st factor Coefficients	2nd factor Coefficients
Aged Odour Alcohols	**0.144**	**0.133**
Ethanol	*0.283*	*-0.088*
1-Pentanol	*-0.209*	*0.159*
2-Pentanol	0.105	-0.039
1-Penten-3-ol	-0.118	0.050
1-Hexanol	-0.098	0.208
1,2-Hexantriol	0.039	0.199
1-Octen-3-ol	-0.090	0.125
Aldehydes		
2-Methyl-propanal	0.205	-0.073
3-Methyl-thiopropanal	*0.238*	*0.090*
2-Methyl-butanal	*0.161*	*-0.053*
3-Methyl-butanal	0.228	-0.060
Benzaldehyde	*0.158*	*0.187*
Benzenacetaldehyde	*0.209*	*0.079*
Pentanal	-0.013	0.230
Hexanal	-0.035	0.274
Heptanal	-0.075	0.281
Octanal	-0.019	0.279
Nonanal	0.037	0.286
2-Decenal	0.051	0.259
2-Undecenal	-0.008	0.279
Ketones		
2-Propanone (acetone)	-0.117	0.102
2-Butanone	-0.155	0.162
2,3-Butanedione (diacetyl)	0.189	-0.121
3-Hydroxy-2-butanone (acetoin)	0.111	-0.143
2-Pentanone	*-0.104*	*0.118*
2-Heptanone	0.022	0.119
2,3-octanedione	0.012	0.277
Acids		
Butanoic acid	0.013	-0.114
3-Methyl-butanoic acid	0.050	-0.121
Octanoic acid	0.164	-0.109
Esters		
Ethyl acetate	0.236	-0.220
Ethyl 2-Methyl-propanoate	0.201	-0.237
Ethyl 2-Methyl-butanoate	0.247	-0.194
Ethyl 3-Methyl-butanoate	0.235	-0.213
Ethyl pentanoate	0.304	-0.129
Ethyl hexanoate	0.258	-0.218
Ethyl heptanoate	0.245	-0.098
Ethyl octanoate	0.302	-0.160
Ethyl decanoate	0.291	-0.104
Alifatic and aromatic hydrocarbons		
Heptane	-0.159	0.070
Octane	-0.161	0.084
Nonane	0.287	-0.141
Tetradecane	0.164	0.237
Toluene	-0.017	0.124
Ethylbenzene	-0.084	0.144
2,3-Dimethyl-phenol	-0.017	0.224
Furans and lactones		
Hexalactone	0.000	0.202
Octalactone	0.040	0.268
2-Pentyl furan	-0.152	0.111

Only volatiles with coefficients of PC1 or PC2 > 0.1 were listed.
Variables reported in italics were significant (P < 0.05) according to the Martens' uncertainty test.

presents the results of PLS analysis, expressed by the loadings of the dependent and independent variables: the coefficients of the first and second factor were reported (only coefficients of PC1 or PC2 > 0.1 are listed). In general, the size of the regression coefficients provides an approximate assessment of the importance: if the regression coefficient is greater than 0.2 in absolute value, the effect of the variable is most probably important, while if it is smaller than 0.1 then the effect is negligible. Significant independent variables (Martens' uncertainty test) are reported in italics in Table 4.

The loadings of "matured ham smell" are positive on PC1 and PC2: the first factor highlighted the opposition between volatiles with negative loadings such as linear alcohols (1-pentanol, 1-hexanol), methyl ketones (2-propanone, 2-butanone and 2-pentanone), hydrocarbons like heptane, octane, ethylbenzene, 2-pentyl furane, and volatiles with positive scores like branched aldehydes, ethanol/ethyl esters, diacetyl and acetoin and long-chain alkanes nonane and tetradecane. Ethyl esters, though having positive loadings along PC1 are in opposition along PC2 (accounting for nearly 25% of the total explained variance), indicating a less important role than branched aldehydes in enhancing "matured dry-cured ham smell". Furthermore, four branched aldehydes but no ester passed the test for a significant effect on the Y variable (Table 4). On PC2, oxidation compounds such as linear aldehydes, lactones and tetradecane, and some branched aldehydes, positively counteracted ethyl esters and organic acids.

Variable loadings and sample scores were normalized to the interval ± 1 and projected onto the PC1-PC2 plane (bi-plot), to display variables and sample interrelationships; each quarter of the bi-plot corresponding to different combinations of PC1 and PC2 signs was named from Q1 to Q4. Ham samples were labelled by processing the time group number (1 or 2 or 3) and the symbol surrounding the number identifies the sensory score as low (< 5.5), medium-low (5.5 - 6.0), medium-high (6.0 - 6.5) and high (> 6.5) (Fig. 2).

As a consequence of the overall decrease in VOCs, the samples projected onto Q3 (Fig. 2) and belonging to ageing groups 1 or 2, warranted low scores for "aged ham odour". On the contrary, most samples placed in Q4, though having low ageing times on average, achieved increased scores thanks to volatiles yielded by oxidation mechanisms. Samples located in Q1 are characterised by high scores for "aged ham odour": these mostly correspond to ageing groups 2 and 3, even if two samples from group 1 also earned a high rating. Carbonyl compounds, including branched and linear aldehydes, lactones, ketones, resulted the dominant chemical class involved with the sensory perception of "matured dry-cured ham smell" in Parma hams. GARCÍA-GONZÁLES *et al.* (2008), reported for the "acorn

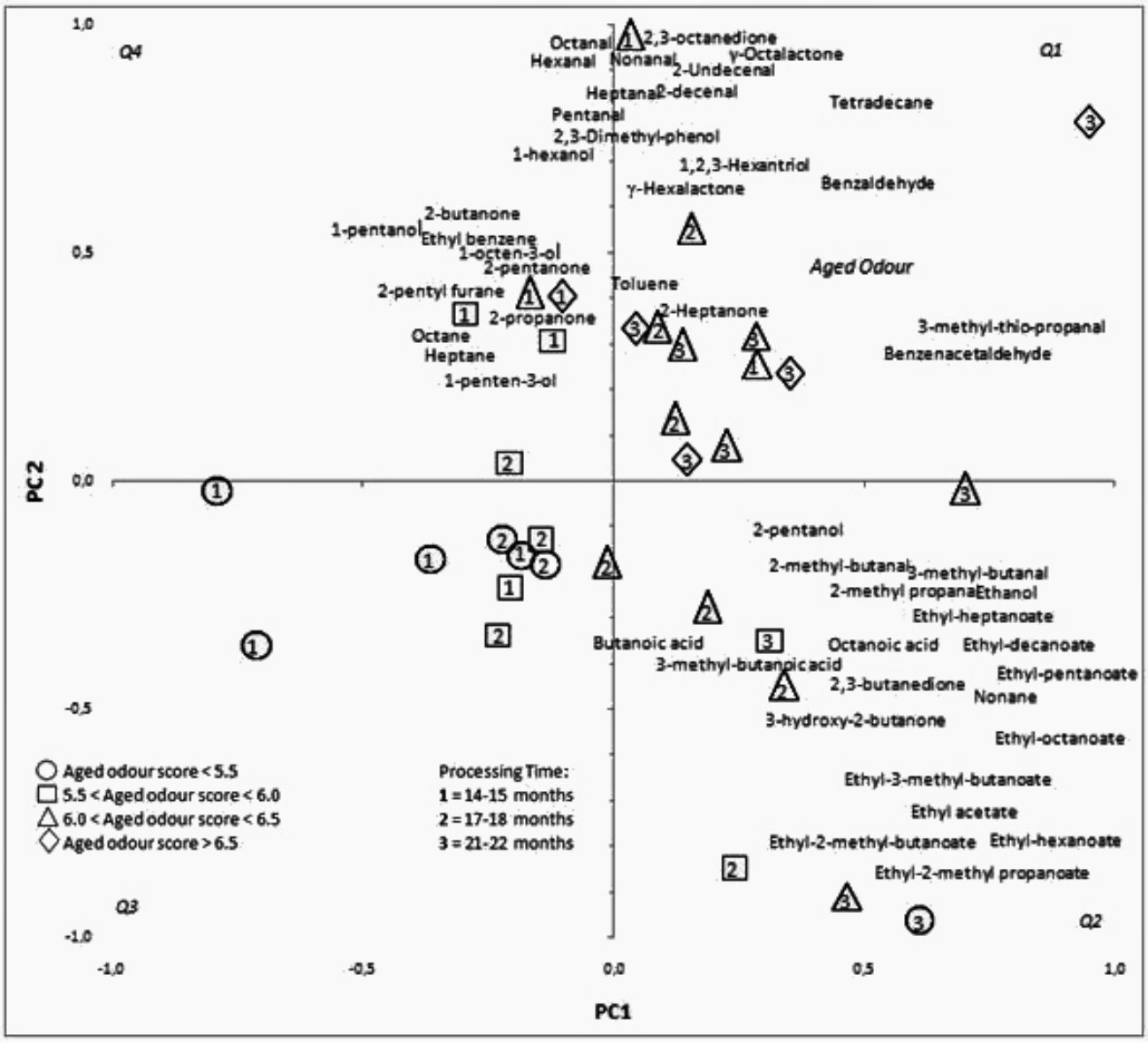


Fig. 2 - Projection of sample scores and variable loadings onto the PC1-PC2 plane (bi-plot). The two principal components account for 42.7 and 24.9% of variance respectively (67.6% in total). Parma ham samples are identified with a number corresponding to processing time and surrounded by a symbol changing according to the aged-odour score.

odour" descriptor, a relevant contribution of benzaldehyde, 2-heptanone and 3-methylbutanal, in accordance with our results for matured ham smell, as shown in Fig. 2. Even if the abovementioned attributes describe different sensory perceptions (acorn odour rates the acorn flavour perception mainly due to pig feeding), both of these are positively related to dry-cured ham quality and acceptability (RESANO *et al.*, 2010).

The volatiles loadings projected onto the Q2 area of the bi-plot include ethyl esters, organic acids, and molecules related to carbohydrate fermentation such as ethanol, acetoin, diacetyl and butanoic acid. Though all coming from ageing groups 2 and 3, samples that fell into Q2 decreased their ratings if compared to Q1 cases.

Even if the model reported in Table 4 does not explain the 32.4% in aged ham odour variability, it does seem that, although both branched aldehydes and ethyl esters are positive markers of ageing time extension, the former contributes to aged ham odour perception more than the latter. A recent GC-O study on odour-active compounds of Bayonne ham found that fruity notes due to ethyl esters were masked by the overall flavour of dry-cured ham (THERON *et al.*, 2010). Moreover, volatiles generated by lipid oxidation, although not related to extended ageing time, can improve aged ham odour perception. Salt, being positively associated with some oxidation compounds and molecules like toluene, ethylbenzene, tetradecane, and 2,3-dimethyl-phenol (salting-out effect), may enhance aged ham aroma when aged odour-impact molecules such as branched aldehydes, that need extended ageing time to increase, are still at low levels.

CONCLUSIONS

The results of the present investigation suggest that, among the volatile molecules identified, the markers of Parma dry-cured ham extended ageing time do not overlap the markers of "matured ham smell" sensory perception. The main information provided by the present study is that the volatile compounds increased by the ageing time of Parma ham are not equally aged-odour active, or involved in processes that positively contribute to matured ham smell perception. In the case of some dry-cured hams with shorter processing times, the achievement of a high "aged odour" score can more be ascribed to VOCs generated through oxidative processes than to volatiles molecules increasing with ageing time. As a consequence, even if the extension of ageing time does have a positive influence on aged ham odour perception, further ways should be investigated for their effectiveness in yielding aged odour-active volatile profile. Environmental factors in maturing rooms, their effects on ham composition and on microbial populations growing in the outer and inner layers of Parma hams, could be key parameters in throwing light on mechanisms suitable for selective VOC generation.

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PAPER

EFFECTS OF INCORPORATION OF CLEAR FLOUR ON THE QUALITY OF CHINESE NOODLES

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ABSTRACT

Clear flour, the lowest quality of all commercial grades of flour, was incorporated alone or in combination for making Chinese noodles. Some of the RVA (Rapid Visco Analyzer) parameters and Farinograph parameters of wheat flour-clear flour blends, as well as cooked noodle brightness and whiteness index decrease as the clear flour proportion in the blends increased. The water absorption and the mixing tolerance index for wheat flour-clear flour blends showed the reverse tendency. However, incorporation of clear flour improved the tensile force, the textural attributes of cooked noodle, and revealed the greater mouth-feel and overall acceptance.

- Keywords: clear flour, Chinese noodles, noodles quality -

INTRODUCTION

Clear flour is the by-product of straight flour that remains after patent flour has been removed. Clear flour is darker in color than straight flour and patent flour, as it is made from the part of the endosperm closest to the bran (HUI, 2006). Clear flour may have a protein content as high as 17% and an ash content of 0.7 to 0.8% (GISSLEN, 2005). It is less expensive than patent flour, but the gluten formed from clear flour is typically of less quality than that from patent flour. Clear flour is usually separated into more than one grade (FIGONI, 2007). First clear is dark flour that is often used in rye and whole grain breads, where its dark color is not noticed and its high protein content contributes much-needed gluten (GISSLEN, 2005).

Chinese noodles, which originated in northern China, are generally made from wheat flour of high protein content (10.5-13.0%), and gradually became a staple food in many Asian countries (HOU and KRUK, 1998; HUANG, 1996; MISKELLY, 1993). Today, they make up more than 40% of the total wheat flour consumed, and have become a popular food source in Asia (CROSBIE *et al.*, 1990). In the simplest form, noodles are prepared from a dough containing wheat flour, water and salt; the procedure involves mixing, resting, compounding, sheeting and cutting (OH *et al.*, 1983). In general, a good quality noodle has a bright and creamy appearance, and a smooth, soft and elastic texture (CROSBIE *et al.*, 1998). Because of the simple formula, the characteristics of protein and starch in wheat flour are known to have important effects on the eating quality of noodles. The chewiness of cooked noodles has a high correlation to protein content as well as to sodium dodecyl sulfate (SDS) sedimentation volume (BAIK *et al.*, 1994). In addition, many reports also indicate that protein quantity and quality of wheat flour are highly correlated with noodle quality, especially hardness (BAIK *et al.*, 1994; HATCHER *et al.*, 1999; KRUGER *et al.*, 1994; MISKELLY, 1984; MISKELLY and MOSS, 1985; MORRIS *et al.*, 2000; OH *et al.*, 1985b; ROSS *et al.*, 1997; TOYOKAWA *et al.*, 1989; YUN *et al.*, 1996).

The importance of the pasting properties of starch to the texture of cooked noodles has been well-documented (CROSBIE, 2005). Noodles made from flour with high swelling starches have softer texture than those with low swelling starch (FU, 2008). In addition, starch properties appear to play a role in instant noodle quality; some manufacturers prefer flour with low gelatinization temperatures for rapid hydration during cooking (FU, 2008). Many researches point out that wheat flour with low amylose content, high peak paste viscosity as well as high gelatinization temperature and enthalpy provide white salted noodles with the preferred soft and elastic eating texture (BAIK and LEE, 2003; KONIK *et al.*, 1992; MIURA and TANII, 1993; MORRIS, 1998; TOYOKAWA *et al.*, 1989; ZHAO *et al.*, 1998).

Previous studies have focused on the issue of understanding the role of lipids, protein and starch properties on the textural qualities of noodles. However, relatively little attention has been devoted to the influence of clear flour used alone or in combination on the quality attributes of noodles. In our pretest, clear flour was used to replace partially wheat flour to successfully make noodles. Hence, the objective of this study was to illustrate the potential of incorporating increasing amounts of clear flour with wheat flour for making noodles. In this study, different ratios of clear flour:wheat flour were mixed to form the dough and then made into noodles. The pasting properties, farinograph characteristics of clear flour-wheat flour blends were evaluated, as was the qualities of the noodles, including: color, tensile force, tensile distance, as well as textural and sensory properties. Our aim was to develop Chinese noodles with clear flour, which exhibit the consumer-satisfying texture and taste.

MATERIALS AND METHODS

Materials

Wheat flour with medium strength and clear flour were purchased from Lien Hwa Fukang plant (Taoyuan County, Taiwan). Clear flour was obtained by milling of hard red spring wheat. Clear flour has a concentrate particle size distribution between 50-125 μm, and wheat flour has a particle size between 25-200 μm.

Preparation of flour blends

Wheat flour was blender with clear flour in ratios of 0:100, 15:85, 25:75, 50:50, and 100:0 (wheat flour:clear flour, w:w).

Chemical analysis of flours

Protein, lipid, ash, dietary fiber (DF), soluble DF, and insoluble DF content of samples were determined according to Approved Methods 44-15A, 46-13, 30-20, 08-01, and 32-21, respectively (AACC methods, 2000). The peak (PV), holding strength (HS), breakdown (BV), final (FV), and setback (SB) viscosities were determined using the RVA SUPER 3(Newport Scientific, Australia) according to AACC Method 76-21 (AACC methods, 2000). The water absorption (WA), dough development time (DDT), dough stability (DS), mixing tolerance index (MTI), and dough breakdown time (DBT) were determined using a Brabender Farinograph Resistograph according to AACC Method 54-21 (AACC methods, 2000).

Noodles color measurement

Colors (L*, a*, and b* values) of flours and dough sheet were measured, in triplicate, with a chromameter (CR-410, Konica Minolta, Tokyo, Japan). A white tile (L* 92.30, a* 0.32, and b* 0.33) was used as standard. The L*, a* and b* data were transformed to a Whiteness Index score using the equation $100-[(100-L)^2+a^2+b^2]^{0.5}$ (BOLIN and HUXSOLL, 1991). Dough sheet color measurements were taken on each side of the two reserved dough sheets at 0 and 24 h after sheeting.

Cooking properties and textural analysis of noodles

Noodles preparation

The Chinese white salted noodles of this study were prepared by initially mixing the wheat flour and clear flour in different ratios, namely, 100:0, 85:15, 75:25, 50:50, and 0:100. The experiment was replicated three times. To this mixture was added 2% NaCl and 30% distilled water (on flour basia) (in the previous test, 30% water added can maintain the structure of dough sheet). The suspension was mixed at low speeds for 2 min and then for 4 min at higher speed in a Spar mixer. The dough was allowed to rest in a polyethylene bag for 30 min then rolled into a 3-mm thick sheet, folded, and rolled into a 5-mm thick sheet. These steps were repeated twice. After resting for an addition 30 min, the thickness of the dough sheet was reduced by pressing it through rollers with a gap of 1.2 mm. The dough sheet was cut into 0.33×30 (width×length) strands and stored in a plastic bag for 1 h before cooking and texture evaluation. Raw noodles were cooked in boiling distilled water (1:10, w/w), then rinsed with cool water, drained, wiped using paper towels, and kept covered in Petri dishes for 5 min at room temperature before the TPA analysis.

Cooking loss and cooking yield

Cooking yield and cooking loss of the noodles were determined as described in AACC method 66-50 (AACC methods, 2000). Thirty grams of the noodles were added to a beaker containing about 300 mL of boiling water. The beaker was covered with a watch glass and cooked for 5 min (the optimum cooking time, measured according to AACC Method 16-50, 2000) with slight agitation. The cooked noodles allowed to drain for 5 min and were then weighed and the cooking yield calculated. Cooking loss was determined by evaporating the cooking water in a hot air oven at 105°C to constant weight.

Tensile force and distance

Tensile force and distance of cooked noodles were measured on a TA-XT2i® Texture Analyser (Stable Micro Systems, Surrey, England), using Spaghetti Tensile grips (A/SPR) at a pre-test speed of 10 mm/sec, a test speed of 10 mm/sec and a post-test speed of 10 mm/sec. Noodles were tested individually within 5 min after cooking and night noodles strands from the different batches were measured for each sample.

Texture analysis

TPA (Texture profile analysis) of cooked noodles was performed with a 3.5 mm radius probe. Instrument settings were compression mode, trigger type, auto; pretest speed, 2.0 mm/s; post-test speed, 5.0 mm/s; test speed, 1.0 mm/s; compression, 50%; interval between two compressions, 1 s; load cell, 5 kg. From force-distance curves, five texture parameters can be obtained: hardness (g), springiness, cohesiveness and resilience (EPSTEIN *et al.*, 2002). Noodles were tested individually within 5 min after cooking and night noodles strands from the different batch were measured for each sample.

Sensory evaluation

The sensory evaluation of noodles was performed with an evaluation panel of 12 trained members. They were professionally trained for evaluating the following characteristics: appearance, color, mouth feel, and overall using a 1-7 hedonic scale. The scale is verbally anchored with seven categories, as follows: extremely like, very like, like, neither like or dislike, dislike, very dislike, and extremely dislike.

Statistical analysis

Analysis of variance and the significance of differences among samples were, respectively, analyzed with the ANOVA procedure and Duncan's multiple range test of the SAS for Windows R 6.12 (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Flour quality characteristics

The values in wheat flour and clear flour of total dietary fiber content (TDF) were 2.67 and 4.74%, respectively, of which soluble dietary fiber (SDF) contents were 0.48 and 0.37% and insoluble dietary fiber (IDF) contents were 2.19 and 4.37%, respectively (Table 1). Indeed, significant improvements in the amount of DF can be made by incorporating clear flour into noodles generally made from wheat flour, which are not generally considered to be a good source of fiber.

Flour protein, ash content and flour-pasting characteristics are major specifications for achieving the desired eating quality of each noodle type (HOU and KRUK, 1998). The content of protein, lipid, ash, total dietary fiber, and insoluble dietary fiber were higher in clear flour than in wheat flour (Table 1). Generally, protein content has a positive correlation with noodle hardness, and a negative correlation with noodle

Table 1 - Chemical composition (on a dry weight basis) and color properties of wheat flour and clear flour.

	WF[a]	CF
Crude protein (%)	13.73±0.29[a]	18.78±0.20[b]
Crude fat (%)	1.17±0.06[a]	2.57±0.05[b]
Ash (%)	0.40±0.03[a]	1.18±0.02[b]
IDF (%)	2.19±0.37[a]	4.37±0.01[b]
SDF (%)	0.48±0.12[a]	0.37±0.10[a]
L*	92.97±0.15[b]	89.37±0.06[a]
a*	0.33±0.12[a]	1.17±0.06[b]
b*	8.23±0.06[a]	10.40±0.10[b]
W.I.	89.17	85.08

Means of 3 replicates ± standard deviations; means followed by different letters in a row differ significantly (P < 0.05).
[a]WF, wheat flour; CF, clear flour; IDF, insoluble dietary fiber; SDF, soluble dietary fiber.

brightness. Thus, there is an optimum protein content of flour required for each noodle type (HOU and KRUK, 1998). Chinese noodles require hard wheat flour with high protein content (10.5-13.1%), giving a firmer bite and springy texture (BAIK *et al.*, 1994; HOU and KRUK, 1998). In our research, clear flour was obtained by milling hard red spring wheat cultivar. Its 18.78% protein content provided more protein to improve the texture of noodles and showed a greater quantity than that of soft wheat cultivar (12.7%) (FUSTIER *et al.*, 2007).

Flour ash content has been rated as one of the important specifications because it negatively affects noodle color. Flour ash content is largely determined by the wheat's ash content. Wheat with an ash content of 1.4% or less always enjoys an advantage (HOU and KRUK, 1998). In our search, wheat flour of 0.4% ash content is usually suitable for making premium quality noodles. With the addition of clear flour of 1.18% ash content an undesirable color of noodles may result. Sometimes, flour color may be more related to noodle color. Flour color L*>90 measured with Chroma Meter is often required (HOU and KRUK, 1998). Clear flour of L*89.37 (Table 1), close to the standard stated above, should not significantly influence the color of noodles.

Finally, the pasting characteristics of flour (as measured on the amylograph or Rapid Visco Analyzer) also play an important role. Previous results reported that peak paste viscosity was highly correlated to the organoleptic eating quality of Japanese- and Korean-style white salted noodles (PANOZZO and MCCORMICK, 1993). RVA peak viscosity, holding strength and breakdown were the main indicted quality parameters of noodles, which significantly correlated positively with stickiness, appearance, smoothness, and total score (LIU *et al.*, 2003; KONIK and MOSS, 1992). In our research, all RVA parameters of wheat flour were higher than those of clear flour (Table 2). This may be attributed to the higher dietary fiber in clear flour. So, the presence of clear flour in the flour will reduce all RVA parameters, thereby influence the eating quality of noodles.

Dough properties measured by farinograph are often also included in noodle flour specifications because they affect noodle processing behavior and noodle eating quality (HOU and KRUK, 1998). Incorporation clear flour at 0, 15, 25, 50 and 100% levels revealed differences to the dough mixing behavior as measured by the Farinograph. Table 3 shows the main parameters registered in the farinogram. The addition of clear flour at different levels mainly caused an increase in the farinograph water absorption from 19.7 to 23.42%. This phenomenon would be contributed to the higher fiber content of clear flour because the incorporation of high fiber ingredients results in a dramatic increase in the amount of water required to make a dough (IZYDORCZYK *et al.*, 2005).

Dough development time and breakdown time were decreased slightly by the addition of clear flour at 15 and 25%, and rapidly by 50 and 100%. The stability time of clear flour is one third that of wheat flour. But, it was increased with the incorporation of 15 and 25% clear flour in the flour blends, and with a rapid decrease at 50%. Stability, in general, gives some indication of the tolerance of mixing the flour (FAUBION and HOSENEY, 1989). The higher protein content of clear flour would increase the tolerance when the incorporation of 15 and 25% clear flour in the mixture. However, decrease of tolerance results in weakness of the flour blends, as the DF content increased with increasing clear flour from 50 to 100%. Mixing tolerance index, the degree of softening during mixing, increased slightly at the incorporation of 15 and 25% clear flour. Although clear flour generally contained a higher percentage of protein than did wheat flour, the quality of the protein was lower, significantly influencing the farinograph parameters. Overall, from the farinograph results, the incorporation of 15 and 25% clear flour was acceptable.

Table 2 - Pasting evaluation of wheat flour incorporated with clear flour by rapid visco analyzer.

WF:CF[a]	PV (RVU)	HS (RVU)	BD (RVU)	FV (RVU)	Setback (RVU)
100:0	290±6[e]	180±4[e]	110±3[d]	355±4[e]	65.78±3.97[e]
85:15	269±3[d]	165±1[d]	104±3[bc]	325±1[d]	56.50±3.18[d]
75:25	259±3[c]	153±3[c]	106±0[c]	307±6[c]	48.05±3.74[c]
50:50	236±3[b]	135±5[b]	101±2[bc]	272±4[b]	36.31±2.16[b]
0:100	206±6[a]	108±7[a]	98±1[a]	218±9[a]	11.58±2.60[a]

Means of 3 replicates ± standard deviations; means followed by different letters in a column differ significantly (P < 0.05).
[a]WF, wheat flour; CF, clear flour; PV, peak viscosity; HS, holding strength; BD, breakdown; FV, final viscosity.

Table 3 - Farinograph parameters of wheat flour incorporated with clear flour.

WF:CF[a]	WA (mL)	DDT (min)	DS (min)	MTI	DBT (min)
100:0	59.30±0.00[a]	25.9±0.00[e]	28.10±0.28[c]	0.00±1.41[a]	48.80±1.27[e]
85:15	59.45±0.07[b]	20.20±0.14[d]	36.90±0.00[d]	1.50±0.71[a]	39.50±0.70[c]
75:25	60.40±0.14[c]	17.60±0.14[c]	37.25±0.35[d]	6.50±0.71[b]	44.70±0.00[d]
50:50	63.95±0.07[d]	8.50±0.14[b]	14.10±0.71[b]	19.00±1.41[d]	16.95±0.07[a]
0:100	70.25±0.07[e]	7.60±0.00[a]	10.90±0.99[a]	13.00±1.41[c]	20.40±1.41[b]

Means of 3 replicates ± standard deviations; means followed by different letters in a column differ significantly (P < 0.05).
[a]WF, wheat flour; CF, clear flour; WA, water absorption; DDT, dough development time; DS, dough stability; MTI, mixing tolerance index; DBT, dough breakdown time.

Raw noodle color

The influence of time-dependent color changes in raw noodles prepared by incorporating clear flour can be seen in Table 4. Significant linear decreases (R^2=0.9908) in raw noodle brightness (L*) were observed with the progressive increase in clear flour content. Coincident with this was a general decrease in the raw noodle whiteness index (W.I.). Furthermore, 24-hour storage at room temperature of raw noodles also showed the same tendency, but the values were less than the counterparts of fresh noodles (0-hour storage). There was a linear increase in raw noodle redness, a*. Therefore, the observed changes can be attributed to the color of clear flour being darker than wheat flour which was proved by the value of W.I. of clear flour to be less than that of wheat flour (Table 1).

Quality of noodles

Noodle qualities were evaluated by solid loss during cooking, tensile strength and textural tests. The results indicated that incorporating clear flour insignificantly affected the solid loss of cooked noodles in 5 minutes of cooking time (Table 5). The previous observation was that the quantity of protein content in noodles will not affect its cooking loss (OH *et al.*, 1985a). Thus, incorporating clear flour with higher protein content in noodles showed an insignificant influence in the solid loss of cooked noodles. However, the cooking yield of noodles was decreased by increasing the addition of clear flour (Table 5). The water absorption of noodles prepared from clear flour, about 29.17%, is less than that of wheat flour (32.93%) with 5 minutes of cooking time (data not shown). Therefore, the addition of clear flour would decrease the cooking yield of noodles.

The tensile forces of cooked noodles were increased with the increased incorporation of clear flour. The cooked noodles containing 100% clear flour were found to have a similar tensile distance compared to 100% wheat flour. However, incorporating clear flour improves the tensile distance of cooked noodles, and incorporating 20% clear flour revealed the maximum tensile distance (Table 5).

Texture profile analysis (TPA) results of cooked noodles prepared with 100:0, 85:15, 75:25, 50:50, and 0:100 wheat flour:clear flour are summarized in Table 5. According to the previous observation, hardness of cooked noodles increased significantly as protein content was increased (PARK *et al.*, 2003). When the noodles were made from clear flour with higher protein content, they showed higher scores for hardness and chewiness. Thus, by increasing the incorporation of clear flour from 25 to 100%, these parameters increased signifi-

Table 4 - Color changes in raw noodles prepared by incorporating clear flour.

	AF:CF[a]	100:0	85:15	75:25	50:50	0:100
0 h	L*1	79.73±0.06[e]	75.83±0.06[d]	73.23±0.06[c]	68.73±0.12[b]	60.23±0.06[a]
	a*1	-4.07±0.06[a]	-2.13±0.06[b]	-1.40±0.00[c]	0.07±0.06[d]	2.37±0.06[e]
	b*1	20.60±0.00[a]	20.63±0.06[ab]	21.00±0.00[c]	20.73±0.12[b]	20.53±0.06[a]
	W.I.**	70.82±0.04[e]	68.15±0.01[d]	65.95±0.05[c]	62.48±0.14[b]	55.18±0.07[a]
24 h	L*2	72.73±0.06[e]	65.53±0.06[d]	62.43±0.06[c]	55.83±0.06[b]	49.9±0.00[a]
	a*2	-3.2±0.00[a]	-1.4±0.00[b]	-0.4±0.00[c]	1.7±0.00[d]	3.57±0.06[e]
	b*2	21.27±0.06[e]	19.6±0.00[d]	19.1±0.00[c]	18.7±0.00[b]	17.67±0.06[a]
	W.I.**	65.27±0.08[e]	60.33±0.05[d]	57.85±0.05[c]	52.01±0.05[b]	46.76±0.02[a]

Means of 3 replicates ± standard deviations; means followed by different letters in a row differ significantly (P < 0.05).
[a]WF, wheat flour; CF, clear flour.

Table 5 - Cooking loss, cooking yield, tensile force, tensile distance, textural properties, and sensory evaluation of noodles with different ratio of clear flour.

Sample (WF:CF)[a]	100:0	85:15	75:25	50:50	0:100
Cooking loss (%)	4.48±0.26^{a}	4.49±0.15^{a}	4.50±0.36^{a}	4.73±0.19^{a}	4.83±0.26^{a}
Cooking yield (%)	211±1^{d}	210±0^{d}	205±1^{c}	199±1^{b}	179±1^{a}
Tensile force (g)	36±1^{a}	40±1^{b}	43±1^{c}	48±0^{d}	61±1^{e}
Tensile distance (mm)	114±1^{a}	118±1^{b}	133±1^{d}	126±2^{c}	113±0^{a}
Textural properties					
Product height (mm)	1.74±0.07^{a}	1.77±0.03^{a}	1.76±0.05^{a}	1.76±0.20^{a}	1.74±0.08^{a}
Hardness (g)	3265±36^{a}	3261±48^{a}	3709±63^{b}	3718±55^{b}	3928±28^{c}
Adhesiveness (g)	-141±20^{ab}	-94±16^{b}	-124±24^{ab}	-171±55^{a}	-115±55^{ab}
Springiness	0.93±0.04^{ab}	0.96±0.02^{b}	0.95±0.02^{ab}	0.92±0.04^{a}	0.95±0.02^{ab}
Cohesineness	0.55±0.01^{ab}	0.57±0.02^{c}	0.54±0.01^{bc}	0.53±0.02^{a}	0.55±0.02^{ab}
Chewiness (g)	1681±94^{a}	1763±60^{ab}	1908±84^{c}	1804±72^{b}	2065±99^{d}
Resilience	0.42±0.01^{a}	0.44±0.01^{b}	0.44±0.01^{ab}	0.44±0.01^{ab}	0.45±0.01^{b}
Sensory evaluation					
Appearance	4.92±0.79^{c}	4.58±0.79^{bc}	4.83±0.58^{c}	4.08±0.79^{ab}	3.58±0.90^{a}
Color	5.33±0.78^{c}	4.83±0.83^{bc}	4.92±0.79^{c}	4.25±0.83^{ab}	3.50±1.00^{a}
Mouth feel	3.50±1.00^{a}	4.08±0.67^{ab}	4.00±0.60^{ab}	4.50±0.80^{b}	4.33±1.44^{ab}
Overall	3.92±0.90^{a}	4.08±0.67^{a}	4.42±0.67^{a}	4.33±1.07^{a}	4.08±1.16^{a}

Means followed by different letters in a row differ significantly ($P < 0.05$).
[a]WF, wheat flour; CF, clear flour.

cantly. Meanwhile, hardness and chewiness of noodles were insignificantly influenced by the incorporation of 15% clear flour. Otherwise, springiness and cohesiveness of cooked noodles were not influenced by the incorporation of clear flour. A similar phenomenon was observed in resilience, although noodles prepared with 100% clear flour had the higher resilience (Table 5). Several investigations reported that noodles made from wheat flour with high protein had higher scores for hardness, cohesiveness and chewiness (BAIK *et al.*, 1994; PARK *et al.*, 2003). Thus, the higher protein content in clear flour would be the major factor influencing the textural properties of noodles prepared with clear flour-wheat flour blends.

Sensory evaluation of noodles prepared with wheat flour and clear flour

The sensory evaluations of noodles prepared with 100:0, 85:15, 75:25, 50:50, and 0:100 wheat flour:clear flour were compared for appearance, color, mouth feel, and overall acceptance. The appearance of noodles is important to consumers. The results indicated a nearly statistical similarity for the appearance of noodles with 0, 15, and 25% clear flour; noodles with 50 and 100% clear flour revealed the worst appearance. Similarly, the color of noodles showed the same phenomenon, and noodles with 100% clear flour had the worst value of color. The poor appearance and color of noodles with clear flour are attributed to the darker color of clear flour. However, the mouth feel and overall result were not significantly affected by the incorporation of clear flour, even at the 100% substitution (Table 5).

CONCLUSIONS

It is possible to produce noodles of acceptable texture from wheat flour containing clear flour, even when the substitution of clear flour is up to 100%. Noodle color is really influenced by the original color of clear flour. In addition, the pasting and farinograph characteristics of wheat flour-clear flour blends are significantly affected by the substitution of clear flour above 25%. Therefore, the textural and sensory properties of cooked noodles reveal a positive improvement from adding clear flour. From the viewpoint of consumer acceptance, noodles made with clear flour will ultimately be welcomed in the marketplace. Meanwhile, the use of clear flour in noodle manufacturing will decrease the cost and improve the added-value of clear flour. Overall, Data from this study can be used for the development of clear flour-based products. It also provides a basis for clear flour-wheat flour blends and quality evaluation in the noodle manufacturing industry.

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PAPER

MOISTURE ADSORPTION CHARACTERISTICS OF PISTACHIO NUT CREAM: A NEW FOOD PRODUCT

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ABSTRACT

Pistachio is used in different ways in food industry. Pistachio nut cream is a new delicate food product within this field. Some properties of this product are required to be known to determine the best packaging materials and storage conditions. We determined the moisture sorption isotherms of this product at 4° and 20°C using mathematical equations under saturated salt solutions. It was concluded that Halsey and Oswin equations were the most suitable equations with higher regression coefficients, and lower SSE and RMSE% values. We recommend these equations for use to determine the moisture adsorption characteristics of similar products to increase the shelf-life.

- Keywords: cream, moisture isotherm, nut cream, pistachio nut, water activity -

INTRODUCTION

Pistachio, which belongs to genus *Pistacia vera L,* is a fruit rich in oil, growing mainly between 30°-45° latitudes both at North and South hemispheres. Pistachio is indigenous to the Southeastern region of Turkey. The fruit is mainly consumed as a snack, either fresh or roasted and salted, or as an ingredient in various products such as baklava, ice cream, pistachio paste, and all these significantly contribute to regional economy.

Pistachio paste is a kind of dessert consumed particularly in Southeastern Turkey and Middle East, West-central Asia and USA (GAMLI and HAYOGLU, 2007). Nut based-spreadable creams for general purpose are produced as follows: Nut is roasted; its skin is removed, milled, and then mixed with various ingredients such as milk powder, sugar and margarine. The mix is kneaded, refined, homogenized, during which lecithin and vanillin are added, and the resultant product is cooled and packaged (ALTAN, 2000; YILMAZ, 1997).

Nut cream takes a special place in breakfast in Turkey; especially, the children and the teenagers consume it preferably (GAMLI and HAYOGLU, 2007). Some nut creams contain sucrose (20-30%), nut (5-13%), milk powder (4-6%), lecithin (0.5-1.0%), vanillin (0.5%), fat (30-38%), whey powder (2%), non-fat cacao powder (6%), and soybean flour (4-6%) (GAMLI, 2009). The texture of pistachio cream depends on water content; it is rather dry and tough, when it is $a_w <$ 0.15, and soft, when $a_w >$ 0.85. However, the high water content may cause it to be browned due to Maillard reaction (MASKAN and GÖGÜŞ, 1997).

The main non-enzymatic reaction that occurs in sugar containing foods is the Maillard reaction, the rate of which is increased by increasing water activity, reaching a maximum rate in the range between 0.6 and 0.7 a_w values. Out of this range, the rate of this reaction will decrease (SALDAMLI, 2007).

Many studies regarding moisture isotherms (MI) for sweetened food were carried out, and they were evaluated using different mathematical equations. For example, the MI of lokum (Turkish delight), which contains 72% invert sugar, 13.4% starch, and 14.6% moisture, was determined at temperatures of 10°, 20° and 30°C. Due to its starch content, lokum exhibits rather complicate structure than sugar and invert sugar alone. At low a_w values, it has a hard and brittle structure, but at higher a_w values, it exhibits two different structures (GÖGÜŞ *et al.*, 1998).

Isotherm curves (IC) obtained at different temperatures may cross each other, partly due to the dissolution of sugar. Among the mathematical models, Chung-Pfost equation was generally reported to be the most suitable model for the starchy foods. As the sugar modifies the structure of the product, Henderson, Chung-Pfost and Halsey equations are more suitable for sugar containing products. Iglesias-Chirife model had higher regression value only within 0.078-0.92 a_w values (Us, 2007; GÖGÜŞ *et al.*, 1998).

According to BET classification, the food stuffs are divided into three groups: Type I is exhibited by the food stuffs containing crystal sugar; Type II is exhibited by dry food stuffs, Type III is exhibited by the food stuffs that contain agglomeration agents (LABUZA, 1984).

Isotherm curves identify mathematical relations between the moisture content of the solids and the relative humidity of their surroundings. There are three basic approaches for modeling the moisture isotherms (MI) mathematically mainly based on kinetics, potential and condensation in capillary regions (US, 2007). Among the equations that are used for modeling isotherms are BET, Oswin, Halsey, GAB, Kübik, Peleg, Henderson, Chung and Pfost, Iglesias and Chiriffe, and Kuhn equations (GÖGÜŞ *et al.*, 1997; GAMLI, 2009; MASKAN and GÖGÜŞ 1997; BOKI and OHNO, 1991). In general, the GAB is reported as the best equation to identify the sorption isotherms of the food stuffs. However, Oswin equation is more suitable for fruit, spice, tea and coffee and Halsey for meat and milk products, while both Halsey and Oswin equations are suitable for starchy foods, nuts, oil seeds and vegetables. The loss of quality and the deterioration of food during the storage and processing are mainly affected by a_w, and the rate of chemical reactions, which are controlled by moisture content of food stuffs. The most important three factors that control the a_w of foodstuff are colligative, capillarity, and surface interactions (US, 2007).

The moisture isotherms are used to predict the potential changes in stability of the food stuff, and to select the best packaging materials and additives. The monolayer moisture content, M_o (g water 100 g^{-1} dry matter), is calculated from the moisture isotherms using BET and GAB equations. Foods with monolayer moisture content are reported to have the longest shelf-life, whereas, those having higher or lower moisture contents have a shorter shelf-life (LABUZA, 1984).

Limited information is available in the literature about sorption properties of similar products, such as pistachio nut paste, especially the spreadable pistachio cream.

The objective of the present study was to determine the most suitable mathematical equation for pistachio cream, based on the adsorption curves as obtained at 4° and 20°C to help finding the best packaging and storage facilities, and to increase the shelf-life of this product.

MATERIALS AND METHODS

Preparation of samples

The unshelled pistachio kernels were roasted at 160°C and the skins were removed, and milled, and then well-mixed with milk powder, margarine and sugar. After adding lecithin and vanillin, the mixture was kneaded, until a spreadable consistency could be obtained.

Table 1 - Some mathematical equations used to express the moisture adsorption of pistachio curves.

Model	Equation	Reference
BET	$M_e = M_o Ca_w/[(1-a_w)(1-a_w+Ca_w)]$	Chirife and Iglesias (1978)
Halsey	$M_e = A(-Lna_w)^B$	Lomauro *et al*. (1985)
Oswin	$M_e = A[(a_w/(1-a_w))]^B$	Saldamlı (2007)
Kuhn	$M_e = A(-Lna_w)^B + C$	Hayoglu and Gamli (2007)
Filonenko-Chuprin	$M_e = A/(1-Ba_w) + C$	Hayoglu and Gamli (2007)
Peleg	$M_e = Aa_w^B + Ca_w^D$	Hayoglu and Gamli (2007)
GAB	$M_e/M_o = C\ k\ a_w/[(1-ka_w)(1-k\ a_w+Ck\ a_w)]$	Mok and Hettiarachchy (1990)
Henderson	$(1-a_w) = \exp(-kM_e)^C$	Boquet *et al*. (1978); Ertugay and Certel (1999)
Chung-Pfost	$M_e = -1/k[(Ln\ a_w)]^{RT/C}$	Chung-Pfost (1967)
Kubik	$M = A + Ba_w + Ca_w^2 + Da_w^3$	Mok (1990)

Three different pistachio cream samples were produced with the constant rates of milk powder (6%), sugar (30%), lecithin (0.5%), vanillin (0.5%), and three different levels of pistachio (5, 10, and 15%).

Obtaining the adsorption curves

The adsorption curves for the samples were obtained at 4° and 20°C using saturated solutions of KOH, $MgCl_2$, K_2CO_3, $Mg(NO_3)_2$, KI, $(NH_4)_2SO_4$ and K_2SO_4 that possess different water activities (a_w). The samples of 1.5 ± 0.001 g were placed on the suitable dishes, and then, they were transferred into a chamber containing the saturated solutions, and weighed periodically, until they reached a constant weight (35-45 d). The moisture gained by the samples was calculated by drying the samples at 70°C (LABUZA, 1983). All the analyses were repeated three times.

Determination of a_w values with PEC method

The a_w values of pistachio cream samples were determined using proximity equilibration cell (PEC) method. According to this method, the filter papers were dried, and they were kept in chambers containing saturated solutions, until a constant weight is reached. A standard curve was produced using the amount of equilibrium moisture gained by the filter papers. The water activity values of the cream samples studied were determined using the standard curves (McCUNE *et al.*, 1981). The mathematical equations used for moisture adsorption curves of pistachio cream samples at 4° and 20°C were given in the Table 1. The parameters and coefficients of mathematical models include M_e, equilibrium moisture (g water 100 g^{-1} dry matter), a_w, mathematical model coefficients of A, *B, C*, *D*, and k.

Matlab (R2009b) was used to establish the nonlinear coefficients. The degree of suitability of mathematical equations was determined using the regression coefficient (R^2), the root mean square error (RMSE%), and the sum of square error (SSE).

$$\%\ RMSE = [(\Sigma((m_{ob}-m_{es})/m_{ob})^2)/n]^{0.5} \cdot 100$$

(MOK and HETTIARACHCHY, 1990)

$$SSE = 1/N[\Sigma(m_{ob}-m_{es})^2]$$

(ARIFOGLU, 2005; DOYMAZ, 2007)

RESULTS AND DISCUSSION

The water activity values of the samples with a 5, 10 and 15% pistachio content were 0.6273, 0.6317 and 0.6333, respectively. The adsorption curves obtained at 4° and 20°C for samples with three different levels of pistachio were given in the Fig.1. The regression coefficients (R^2), SSE,

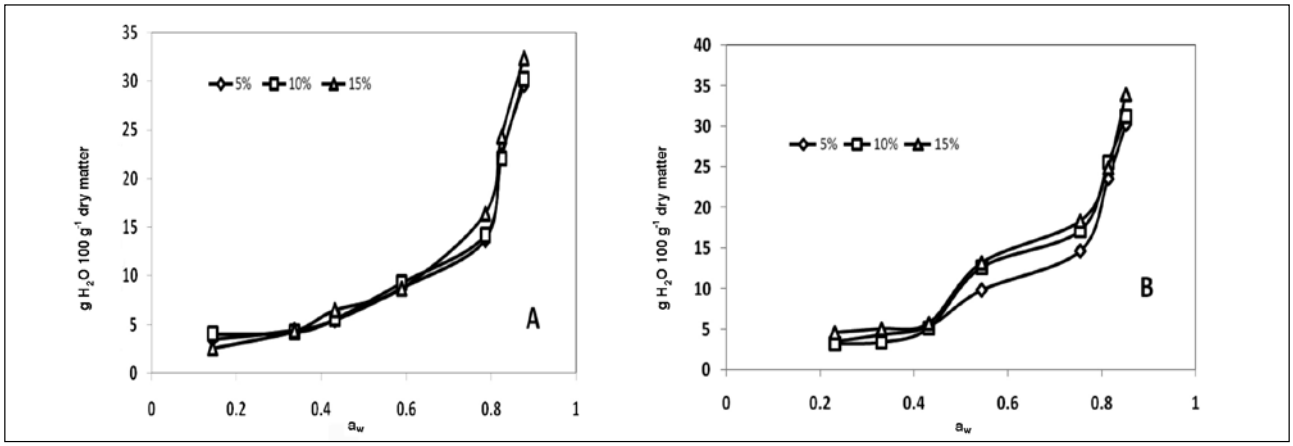


Fig. 1 - Adsorption curves for pistachio cream samples at 4°C (A) and 20°C (B).

Table 2 - Regression coefficients, RMSE% and SSE values for pistachio cream at 4° and 20°C, according to the mathematical models.

Model	Coefficients	4°C			20°C		
		% 5	% 10	% 15	% 5	% 10	% 15
BET	R^2	0.973	0.9806	0.9902	0.9652	0.9731	0.9758
	RMSE	1.647	1.536	1.215	2.114	2.004	1.918
	SSE	19	18.72	9.894	65.99	96.46	121.8
	M_o	3.653	3.684	4.103	4.219	4.923	5.026
	C	11.6	17.41	5.943	-9.466	3.803	6.794
Halsey	R^2	0.9763	0.9784	0.9901	0.9849	0.9718	0.9749
	RMSE	1.691	1.623	1.223	1.394	2.055	1.954
	SSE	14.56	11.45	9.69	10.57	9.96	17.45
	A	4.477	4.634	4.803	4.816	5.536	6.223
	B	-0.9279	-0.917	-0.943	-0.9965	-0.9524	-0.9102
Oswin	R^2	0.9606	0.9677	0.9861	0.9791	0.9745	0.9716
	RMSE	1.991	1.983	1.449	1.635	1.953	2.078
	SSE	19.81	19.66	10.5	13.37	19.07	21.58
	A	6.242	6.442	6.746	7.049	8.009	8.895
	B	0.7815	0.7715	0.7936	0.8149	0.7762	0.7381
Kuhn	R^2	0.9802	0.9841	0.9903	0.9125	0.946	0.9149
	RMSE	1.730	1.553	1.355	3.744	3.177	4.025
	SSE	32.45	47.56	23.36	88.79	124.6	216.4
	A	2.784	2.583	4.342	7.915	-7.686	-8.591
	B	-1.136	-1.174	-0.9863	0.00139	0.0016	0.00135
	C	2.102	2.55	0.5579	7.921	7.692	8.599
Filonenko-Chuprin	R^2	0.9700	0.9841	0.9901	0.357	0.6708	0.6241
	RMSE	1.737	1.556	1.370	10.14	7.845	8.457
	SSE	281.3	273.4	349	549.3	20.47	19.18
	A	3.424	3.288	4.511	$1.47e^4$	$1.54e^4$	$1.57e^4$
	B	1.013	1.018	0.9932	5941	1975	2353
	C	-0.6048	-0.1463	-2.276	18.5	31.14	29.72
Peleg	R^2	0.8894	0.8281	0.9275	0.9012	0.9537	0.751
	RMSE	4.716	5.907	4.274	4.595	3.398	7.947
	SSE	153.5	262.3	227.3	5.45	14.49	56.54
	A	0.7164	-2317	41.52	-2430	8.551	5567
	B	1.784	2.523	2.675	2.831	4.325	2.634
	C	33.97	2344	-1.08	2462	33.88	-5549
	D	2.455	2.51	2.832	2.814	2.059	2.652
Kubik	R^2	0.9349	0.9374	0.953	0.952	0.9593	0.9488
	RMSE	3.134	3.088	2.979	2.773	2.758	3.122
	SSE	22.3	24.04	14.24	16.87	26.99	32.03
	A	6.455	6.956	5.376	7.266	2.862	6.215
	B	-14.34	-15.33	-11.6	-15.27	-1.081	-8.468
	C	47.69	48.01	49.74	53.02	43.14	50.21
GAB	R^2	0.9554	0.9538	0.7223	0.9701	0.3198	0.9583
	RMSE	2.592	2.651	7.244	2.188	11.28	2.816
	SSE	12.14	40.43	7.273	76.41	19.23	277.5
	M_o	6.796	12.92	16.45	4.561	19.57	16.44
	C	0.462	0.3181	0.9754	0.6105	18.32	0.3945
	k	1.766	1.389	1.71	2.424	0.1805	1.451
Henderson	R^2	0.8928	0.889	0.8038	0.9062	0.8513	0.8300
	RMSE	3.597	3.67	10.26	3.468	4.716	5.086
	SSE	102.3	117.9	217.1	245.8	326.7	348.8
	k	5.453	3.523	5.214	28.66	3.769	22.46
	C	-9.047	-5.872	-7.129	-52.34	-7.007	-42.37
Chung-Pfost	R^2	0.911	0.813	0.871	0.918	0.827	0.839
	RMSE	3.663	20.68	17.11	23.26	41.23	26.58
	SSE	44.78	58.96	87.41	119.4	187.4	215.1
	k	$2.0e^{-5}$	0.0017	0.0018	0.0102	0.0145	0.00197
	C	0.7446	0.7411	0.849	0.748	0.668	0.837

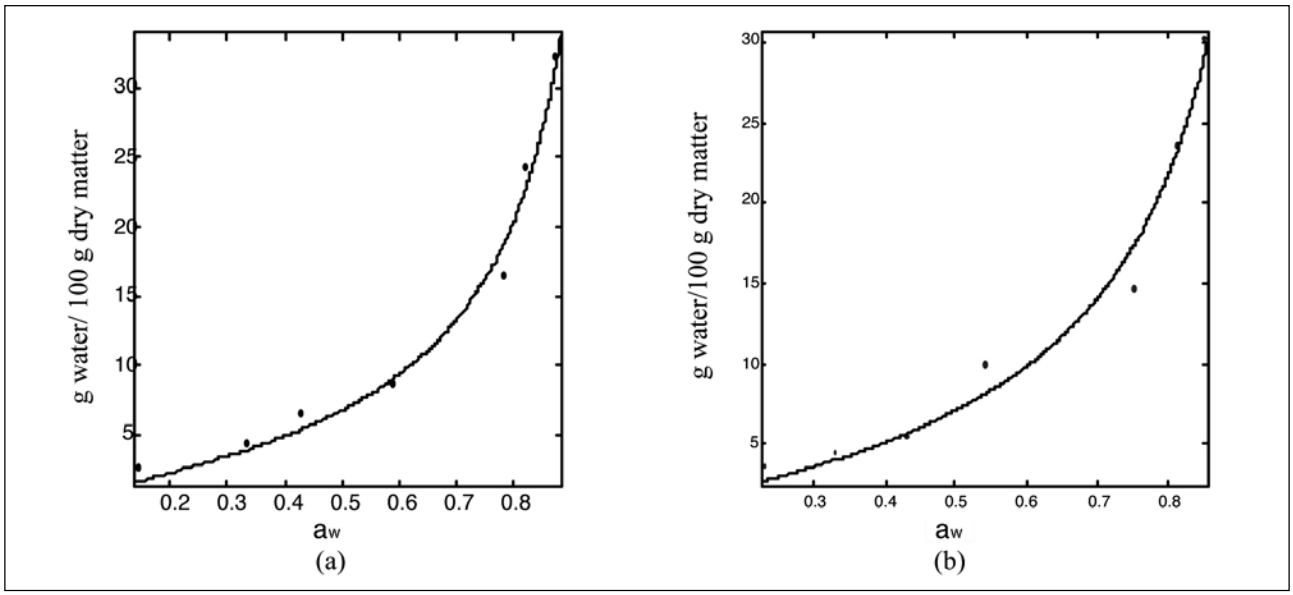


Fig. 2 - Regression curves of moisture equilibrium curves at 4°C (a) and 20°C (b) according to Halsey equation (4°C, % 5, r^2: 0.9901; 20°C, % 15, r^2: 0.9849).

RMSE% and the constants for different mathematical equations were presented in the Table 2.

Halsey and Oswin equations were found to have higher regression coefficients (R^2) and lower RMSE% and SSE values than the others (Fig. 2).

BET equation is reported to be more suitable at 0-0.45 a_w values (LABUZA, 1983; MASKAN and GÖGÜŞ, 1997). BET and GAB equations had higher R^2 coefficient; however, due to their higher SSE values, they were not suitable for pistachio cream.

The Henderson, Chung and Pfost, Kübik, Iglesias and Chiriffe, Filonenko and Chuprin, and Peleg equations were found to have low regression coefficient, but high RMSE % and SSE values, indicating that they were not suitable for pistachio cream, as well.

M_o values for pistachio cream obtained using BET equation ranged between 3.653 and 4.103 at 4°C, and between 4.219 and 5.026 at 20°C. M_o values for raisin, fig, dry prune and carrot at 30°C were reported as 12.5, 11.7, 13.3 and 15.1 g water 100 g^{-1} dry matter, respectively. As also reported by MAROULIS *et al.* (1988), these M_o values decreased as the temperature increased.

It was established that M_o value of the pistachio cream increased with the increased temperature, which was not consistent with the previous results reported by MAROULIS *et al.* (1988), and MOK and HETTIARACHCHY (1990). Peleg model was reported to be suitable to express the moisture isotherm of pistachio paste.

M_o (g water 100 g^{-1} dry matter) values of the pistachio paste were reported to be 1.237 and 1.982 g water 100 g^{-1} dry matter at 4° and 20°C, respectively (HAYOGLU and GAMLI, 2007); however, higher values were obtained for pistachio cream. M_o values for dehydrated meat, pea, potato and coffee are 4.0-6.19; 3.64; 5.1-7.8 and 8.3 (g water 100 g^{-1} dry matter), respectively (LABUZA, 1984).

CONCLUSIONS

The water activity of pistachio cream samples that contained 5, 10 and 15% pistachio were determined at 20°C as 0.6273, 0.6317 and 0.6333, respectively. Of the mathematical equations to determine the adsorption curves for pistachio cream, Halsey and Oswin equations resulted in higher regression coefficient (R^2), and lower values of SSE and RMSE %. M_o values of these samples ranged between 3.653 and 5.026 g water 100 g^{-1} dry matter. According to BET classification, the moisture adsorption curve of pistachio cream fell into the group Type I. We recommend these equations for use to determine the moisture adsorption characteristics of similar products so that the suitable packaging material and storage conditions and period can be determined.

Abbreviations	Description
A, B, C, D, k	Coefficients of models
R	Gas constant (8.314 j/mol.K)
T	Absolute temperature, K
Me	Equilibrium moisture content, g water 100 g^{-1} dry matter
R^2	Regression coefficient
M_o	Monolayer moisture value, g water 100 g^{-1} dry matter
rmse	root mean square error
sse	sum of square error
a_w	Water activity
m_{ob}	Expected equilibrium moisture content, g water 100 g^{-1} dry matter
m_{es}	Calculated equilibrium moisture content, g water 100 g^{-1} dry matter
y_i	i value of the measured equilibrium moisture content, g water 100 g^{-1} dry matter

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PAPER

DEGRADATION KINETICS OF TOTAL PHENOLS, ANTHOCYANINS AND ANTIRADICAL ACTIVITY OF BLACKCURRANT NECTARS STORED AT ROOM AND REFRIGERATOR TEMPERATURES

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ABSTRACT

Total phenol (TP) and total anthocyanin (TA) contents as well as 2,2-diphenyl-1-picrylhydrazyl (DPPH) antiradical activity (AA) were monitored during storage of three industrial blackcurrant nectars and one blackcurrant-aronia mix at two temperatures (4° and 22°C) for 10 days. Severe loss in the TP and TA contents and a substantial loss in the AA were observed in blackcurrant nectars stored at both temperatures. The thermal degradation of TP, TA and AA followed first-order reaction kinetics. The kinetic constants of anthocyanin degradation were higher at 22°C, while the constants for AA degradation were not significantly different at 22° and 4°C.

- Keywords: anthocyanins, blackcurrant, degradation kinetics, DPPH antiradical activity, temperature effects, total phenols -

INTRODUCTION

Fruit juices are an important source of phenolic antioxidants, which have been proven to exert a profound protective effect against the onset of cardiovascular diseases (ZAFRA-STONE *et al.*, 2007), cancers (SEERAM, 2008; DUTHIE, 2007) and even aging (AMES *et al.*, 1993). The effectiveness of phenolic compounds has been primarily attributed to their ability to act as antioxidants and quench the free radicals involved in the onset of mentioned pathological processes. The radical-scavenging capability of phenolics is due to their hydrogen/electron donating ability, therefore, the greater the number of hydroxyl groups from which they can release an electron or a hydrogen atom the greater their antiradical potency. The antiradical effectiveness is also directly related to the redox potential of individual polyphenols, the availability of their OH groups, and the ability of the aromatic structure to delocalize the extra charge around the π-electron system. In our previous study of wines and catechin's antioxidant properties, it was concluded that the OH groups in the *ortho* position on the B-ring of flavonoids are easily oxidized, which is followed by release of a hydrogen atom (PILJAC *et al.*, 2004a). Besides the chemical structure of phenolic compounds, other important factors that influence the reactivity of phenolics from plant extracts or food products primarily include: (1) temperature, (2) chemical characteristics of the solvent and (3) the duration of exposure to these conditions (PINELO *et al.*, 2004).

In the past few years, an increasing body of literature has been published on characterization of phenolic antioxidants from natural sources, especially fruit juices (VAN der SLUIS *et al.*, 2005; LICHTENTHÄLER and MARX, 2005; BERMÚDEZ-SOTO and TOMAS-BARBERAN, 2004) and berries (DVARANAUSKAITÉ *et al.*, 2006; ARAMWIT *et al.*, 2010; PILJAC-ŽEGARAC and ŠAMEC, 2011; ŠAMEC and PILJAC-ŽEGARAC, 2011). Blackcurrant antioxidants have already been the subject of several studies (HALVERSEN *et al.*, 2002; NIELSEN *et al.*, 2003; EHALA *et al.*, 2005; HEINONEN, 2007). On Croatian market blackcurrant beverages are usually distributed as blackcurrent nectars with a minimum 25% fruit juice content. Several studies reported changes in anthocyanins and antioxidant capacity during blackcurrant juice (MIKKELSEN and POLL, 2002; LANDBO and MAYER, 2004) and nectar (IVERSEN, 1999) processing but there are limited studies where specific correlations have been drawn between degradation kinetics of anthocyanins and phenols and antiradical activity during storage of blackcurrant nectars at the two most common temperatures, 4° and 22°C.

The aim of the present research is to study the effects of storage time and temperature on the degradation of phenolics and anthocyanins, as well as antiradical activity, of three industrial blackcurrant (BC) nectars and one blackcurrant-aronia (BC-A) (*Aronia melanocarpa*) mix. We will also mathematically quantify the thermal degradation kinetics of all three parameters in studied blackcurrant juices.

MATERIALS AND METHODS

Juice samples and storage treatments

Three blackcurrant (BC_1-BC_3) nectars from three different producers readily available on the Croatian market and one blend of blackcurrant (6.5% v/v) and aronia (3.5% v/v) nectars (BC-A) were obtained right after packaging in 1 L aluminum foil-enforced cardboard containers. The countries of origin for analyzed juices were as follows: BC-A (Slovenia), BC_1 (Croatia), BC_2 (Slovenia) and BC_3 (Croatia). On the first day of measurement, the nectars were mixed in their original containers to ensure homogeneous distribution of constituents, aliquoted into five sterile 15 mL falcon tubes and tightly capped. Each set of five samples for each nectar was stored in the dark at 4°C (refrigerator) and 22°C (room temperature). One falcon tube was opened and sampled for measurement every 48 h for a period of ten days until mould growth was observed.

Measurement of total phenols, total anthocyanins and antiradical activity

The total phenol (TP) content of nectars was determined using the Folin-Ciocalteau method (SINGLETON and ROSSI, 1965), modified for smaller volumes. The absorbance of each juice (diluted 10×), as well as the absorbance of the gallic acid standard in the concentration range 0 - 500 mg/L, were determined at 765 nm against the blank (the "0 mL" solution). A calibration curve absorbance *vs c* (gallic acid) was used to derive the gallic acid equivalent (GAE) concentrations for juices.

Total anthocyanins (TA) were determined according to the bisulfite bleaching protocol (OUGH and AMERINE, 1988). 1 mL of nectar was mixed with 1 mL of 0.1% hydrochloric acid solution in 95% ethanol, and 20 mL of 2% hydrochloric acid. From this mixture, 10 mL aliquots were pipetted into two different test tubes, 4 mL of 15% sodium bisulfite solution was added to the first tube and 4 mL of water to the second. After 20 min, absorbance of each mixture was measured at 520 nm and the anthocyanin content determined according to the following formula derived from the standard curve: c (mg/L) = 615 (A_1-A_2).

Antiradical activity was determined according to a modified method of BRAND-WILLIAMS *et al.* (1995). Five microliters of nectar was mixed with 995 μL of 0.094 mmol/L 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol. The free radical scavenging activity was evaluated by measuring the absorbance at 515 nm after 60 min of reaction at 20°C in a spectrophotometer. The

DPPH antiradical activity, expressed as mmol/L Trolox Equivalent Antioxidant Capacity (TEAC), was derived from a calibration curve designed for Trolox (vitamin E analogue).

Statistical analysis

The data were analyzed using One-way analysis of variance (ANOVA) accepting $p<0.05$ as the level of significance. The influence of storage time and temperature on the content of TP, TA and AA was evaluated separately. Error bars drawn in graphs represent standard deviation values.

Degradation of TP, TA and AA was described using first-order reaction kinetics with respect to time in storage. A first-order dependency may be expressed by the following set of equations:

$$Y_t = (Y_0)^{kt} \quad (1)$$

$$t_{1/2} = - \ln 0.5/k \quad (2)$$

where Y_0 is the initial content of polyphenols or anthocyanins, or the initial antiradical activity, and Y_t is the content of polyphenols or anthocyanins, or antiradical activity, at time t, while k represents the first order kinetic constant. $t_{1/2}$ is the half-life, or the time necessary for degradation of 50% of polyphenols and anthocyanins, or the time at which 50% loss in activity is observed.

RESULTS AND DISCUSSION

Blackcurrant nectars were chosen for this study because in an initial screening (data not shown) of more than 15 industrial fruit nectars (sour cherry, multivitamin preparations, blueberry and blackcurrant) they repeatedly exhibited the highest values of TP, TA and antiradical activity. Under ordinary household storage conditions, fruit nectars are stored at 4°C (refrigerator) or 22°C (room temperature) for daily consumption, thus, these two temperatures were selected for our experiment. The changes in TP and TA contents as well as antiradical activities were monitored for 10 days or until severe degradation of phenolic content (>30%) coupled with a significant decrease (>15%) in antiradical activity and deterioration of sensory properties were observed. Conclusions were drawn about the influence of storage time and temperature on all three observed parameters.

Phenolics, anthocyanins and antiradical activity

The initial TP and TA contents, as well as the antiradical activity detected upon the opening of nectars, are shown in Table 1. The TP content ranged from 1,640-2,173 mg GAE /L, while the TEAC ranged from 5.07-5.72 mmol/L. The TP content (2,173 mg GAE/L) was the greatest in the sample of BC-A nectar while TEAC (5.72 mmol Trolox/L) was the greatest in BC_3. BC-A had a 1.3 fold greater TP content in comparison to BC_1 and BC_3. BC_2 exhibited an intermediate TP content of 2,012 mg/L. The lowest antiradical activity value, significantly smaller than all others, coincided with the lowest TP value (5.07 mmol Trolox/L and 1,640 mg/L GAE, respectively) and was determined for BC_1 nectar. The observation that BC_3 exhibited the lowest TA content, second to lowest TP content, but at the same time very high antioxidant activity may be explained by the fact that other compounds, such as vitamin C, significantly contribute to the overall antioxidant capacity of blackcurrant preparations (VIBERG *et al.*, 1997).

The TA content in the four tested samples varied greatly, with BC_3 exhibiting the lowest value of 109 mg/L and BC_2 exhibiting the highest value of 265 mg/L, which corresponds to about a 2.5 fold difference ($p<0.05$). BC-A mix and BC_1 exhibited similar TA contents of 230 mg/L and 215 mg/L, respectively. The range of obtained values is not surprising; NIELSEN *et al.* (2003) tested 13 different commercially available blackcurrant beverages where the TA content ranged from 1.4 to 492 mg/L. The variation in anthocyanin content is most probably due to various environmental factors that influence the formation of anthocyanins, such as, stress, light, temperature, and agronomic practices (KALT *et al.*, 1999), in addition to the postharvest treatment of berries prior to processing and the technological processes involved in juice and nectar preparation.

There are no published studies available comparing the phenolic and anthocyanin contents and DPPH antiradical activity of BC nectars during storage at different temperatures. However, BC juice has been shown previously to exhibit the highest antioxidant activity in six different *in vitro* assays compared to tea, apple juice and tomato juice (SCHLESIER *et al.*, 2002). HALVORSEN *et al.* (2002) used the FRAP assay in comparing the total antioxidant concentrations of berries, and found that BC takes the high fourth place, above strawberries, blackberries, sour cherries, blueberry and raspberry, in a group of 19 analyzed berry samples. In their study of commercial red fruit juice concentrates, BERMÚDEZ-SOTO and TOMÁS-BARBERÁN (2004) confirmed that the BC concentrate is very rich in phenolic compounds, mainly anthocyanins, and that BC along with chokeberry and elderberry exhibit the highest antioxidant capacities in the group of nine common berry fruits. In the present study, three pure BC juices and one BC-A mix showed TP content greater, on the average, by about 500-700 mg GAE/L in comparison to a set of 15 blueberry, sour cherry and multivitamin industrial juice preparations screened initially (data not shown). The results of the present study of monitoring the TP and TA contents, as well as the DPPH antiradical activity over ten days, at two temperatures, are in accordance with the results of previous studies which indicate that all three parameters are dependent on the tem-

Table 1 - Total phenols (TP), total antocyanins (TA) and antioxidant capacity (AA) of nectars on the first day.

	TP (mg GAE/L)	TA (mg/L)	AA (mmol Trolox/L)
BC-A	2,173±99^{b}	230±5^{c}	5.44±0.12^{b}
BC_1	1,640±76^{a}	215±6^{b}	5.07±0.31^{a}
BC_2	2,012±79^{b}	265±9^{d}	5.37±0.18^{b}
BC_3	1,654±58^{a}	109±7^{a}	5.72±0.10^{c}

Abbreviations: BC (blackcurrant), BC-A (blackcurrant-aronia mix), TP (total phenols), TA (total anthocyanins), GAE (gallic acid equivalents), AA (antiradical activity wih respect to the DPPH free radical). The results are an average of three measurements ± SD. Different letters next to the values from three assays represent significant differences.

perature and length of storage (KATSABOXAKIS *et al.*, 1998; FIORE *et al.*, 2005).

The average TP value for our four samples (1,870 mg/L) is lower than that obtained for pomegranate juice tested by GIL *et al.* (2000), 2,566 mg/L, but still places BC juice at the high end of the TP content scale of natural fruit juices. Pomegranate juice had an antiradical activity even higher than that of red wine or green tea, while the values for our BC samples (between 5.07 and 5.72 mmol Trolox/L) fall in the range of those expected for red wines (PILJAC *et al.*, 2004b).

Storage time and temperature effects - degradation kinetics of TP, TA and AA

All three parameters, the antiradical activity, the TP and TA contents were significantly affected ($p<0.05$) by storage time after the 10-day storage period (Figs. 1-3). An initial increase in the antiradical activity was observed for BC-A and BC_1 at both temperatures and for BC_2 at 22°C after 48 h. This increase was followed by a rapid drop in the next 48 h and a gradual decline which resulted in all samples, except in BC-A, in a final loss of activity between 6-39% at 4°C and 16-39% at 22°C after the 10-day storage period. In comparing both storage temperatures (Fig. 1), it becomes evident that the observed total percent loss in activity after 10 days in storage is smaller for those samples stored at 4°C (on the average by about 2% and in the case of BC-A mix by 10%). This indicates that 4°C is a better storage temperature for BC and BC-A juices in question. However, the average loss in activity (22%) observed for samples stored at 4°C is not significantly ($p>0.05$) different from the average loss in activity observed for samples stored at 22°C. The greatest loss of activity over ten days was observed for BC_3 at 4° and 22°C, even though this sample had the highest initial antiradical activity (5.72 mmol Trolox/L).

All four samples showed a decrease in the TP content with time at both storage temperatures (Fig. 2). The most abrupt drop (between approximately 20 and 30%) in the TP value of all samples was observed between the 48 and 92 h in storage at both and it coincided with the sharp drop in the antioxidant activity. The continual trend in degradation of polyphenols was similar at both temperatures, with the smallest drop between 92 and 140 h where the samples almost reached plateau values, and a final decrease which resulted in a total percent loss between 38-58% at 4°C and between 26-46% at 22°C. BC_2 was the most influenced by time in storage, at 4°C, a 58% loss in the TP content was observed after 10 days in storage, at 22°C a 46% loss. On the other hand, storage temperature did not exert a significant effect on the TP content of these nectars.

The four analyzed nectars showed a varying influence of storage time and temperature on the TA content (Fig. 3). BC_1 and BC-A mix were the least prone to anthocyanin degradation at both temperatures; the percent loss in TA for these samples varied from 11-36% loss. Similar color loss (below 20%) was determined for pigmented orange anthocyanins stored for shorter storage periods, less than one month (KATSABOXAKIS *et al.* 1998). Except for BC-A mix, the percent loss in TA was less pronounced at 4°C, just as in the mentioned study of orange anthocyanins where a higher rate of anthocyanin degradation

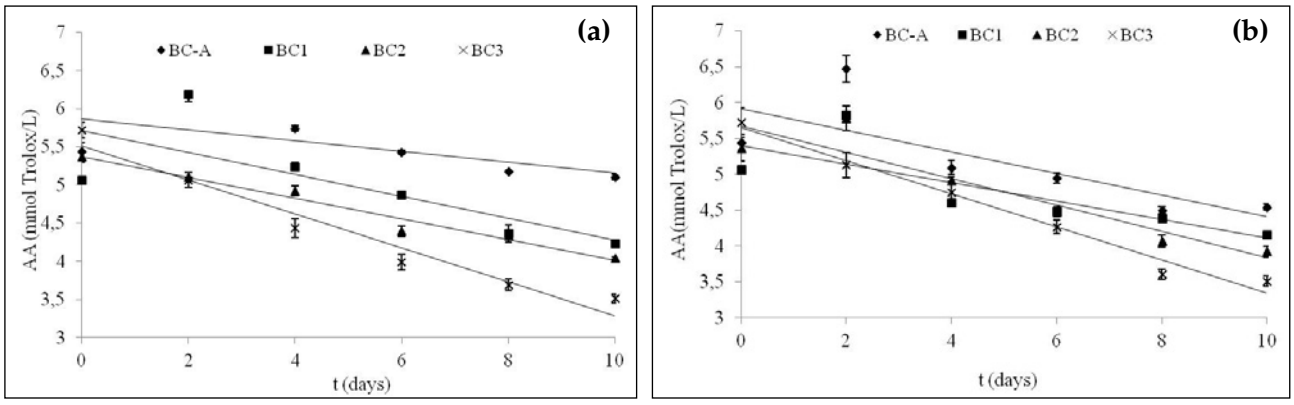


Fig. 1 - Antiradical activity of blackcurrant and blackcurrant aronia juices after 10-day storage at: a) 4°C, b) 22°C. Data points are shown as mean of three measurements ± SD.

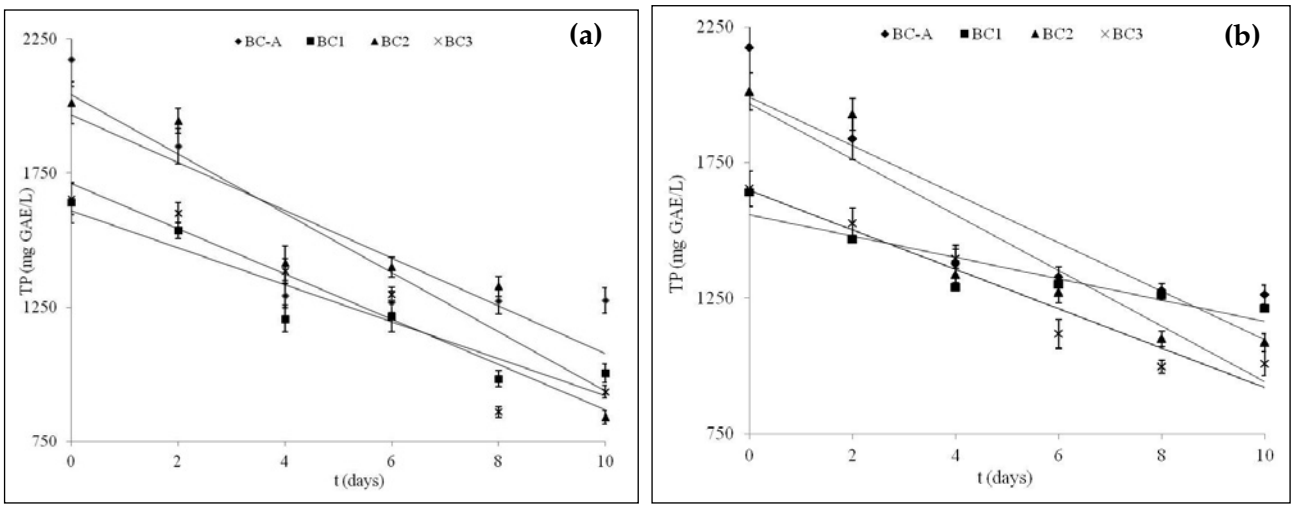


Fig. 2 - Total phenol content of blackcurrant and blackcurrant aronia juices after 10-day storage at: a) 4°C, b) 22°C. Data points are shown as mean of three measurements ± SD.

was observed at 30°C in comparison to 4°C. The most abrupt degradation of anthocyanins, ranging from ~10-20%, was observed for BC_2 and BC_3 between 92 and 140 h in storage at both temperatures. Also, BC_3 exhibited the greatest overall percent loss in TA content at both temperatures. The other juice samples exhibited a more or less continual decrease of TA content with time. In all samples, the loss of pigmented anthocyanins was marked by a subtle change in juice color, from a bright red-purple, to a darker red-brownish color. Our findings of temperature-facilitated anthocyanin degradation are in accordance with the findings of KIRCA and CEMEROGLU (2003) who noted that blood orange anthocyanins decreased very rapidly for samples stored at 37° and 20°C, whereas samples stored at 5°C showed a remarkably slower degradation.

Considering the observation that the drop in TP content in analyzed samples was by about 1.5-fold greater than the drop in TA content (in the case of BC_2 even 2-fold), it is plausible to conclude that anthocyanins were more stable in analyzed BC samples than some other groups of polyphenolic compounds. According to EIRO and HEINONEN (2002), anthocyanins owe their stability to copigmentation reactions they undergo with the other polyphenolic compounds present in the beverage.

Just as in the case of our BC juices, increases in antioxidant activity during storage have been observed after several hours of incubation of apple juice at elevated temperatures (VAN der SLUIS *et al.*, 2005) and in our earlier study of fruit juices in refrigerated storage (PILJAC-ŽEGARAC *et al.*, 2009). The observed increase after the first 48 h in storage was explained by the tendency of flavonoid polyphenols to undergo polymerization reactions (PINELO *et al.*, 2005). The resulting oligomer molecules have larger areas available for charge delocalization, thus yielding higher antiradical activity values. When the degree of polymerization becomes too great, the steric hindrance in the molecule reduces the availability of hydroxyl groups in the oligomer, causing the resultant decrease in antioxidant capacity over time. Considering that flavonoids (anthocyanins, favan-3-ols and flavonols) account for the majority of phenolic

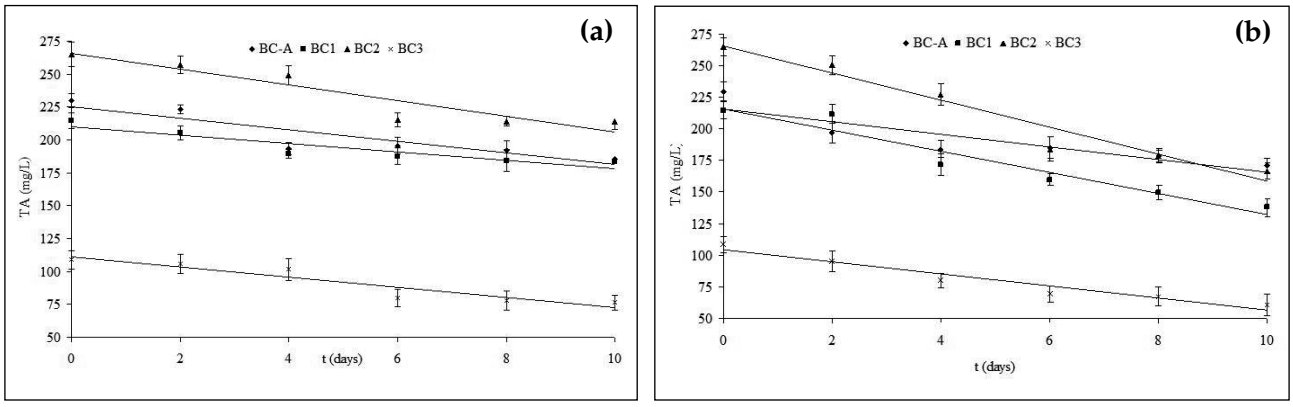


Fig. 3 - Total anthocyanin content of blackcurrant and blackcurrant aronia juices after 10-day storage at: a) 4°C, b) 22°C. Data points are shown as mean of three measurements ± SD.

Table 2 - Kinetic constants for nectars.

		$k \cdot 10^3$ (day^{-1})		$t_{1/2}$ (days)		r^2	
		4°C	22°C	4°C	22°C	4°C	22°C
TP	BC-A	54.4^{b}	54.8^{bc}	10.7^{b}	11.0^{b}	0.7285	0.8223
	BC_1	54.1^{b}	28.1^{a}	12.7^{c}	22.8^{d}	0.9150	0.8499
	BC_2	78.9^{e}	68.8^{d}	9.5^{a}	9.9^{a}	0.8713	0.9003
	BC_3	68.3^{d}	56.8^{c}	11.1^{b}	12.4^{c}	0.8681	0.9404
TA	BC-A	21.4^{b}	25.3^{c}	31.5^{e}	24.9^{d}	0.8210	0.8274
	BC_1	16.0^{a}	47.6^{e}	42.0^{f}	14.9^{b}	0.8593	0.9525
	BC_2	25.2^{c}	50.7^{f}	27.7^{de}	13.9^{ab}	0.8706	0.9533
	BC_3	41.8^{d}	58.3^{g}	17.2^{c}	11.3^{a}	0.8875	0.9635
AA	BC-A	12.7^{a}	28.9^{c}	60.5^{f}	26.9^{d}	0.4673	0.6418
	BC_1	28.8^{c}	26.8^{b}	28.3^{e}	28.2^{e}	0.6189	0.6753
	BC_2	28.9$_{c}$	38.8^{d}	24.2^{c}	19.5^{b}	0.9698	0.8900
	BC_3	49.8^{e}	51.6^{e}	13.4^{a}	13.5^{a}	0.9753	0.9803

Abbreviations: AA (antiradical activity wih respect to the DPPH free radical), BC (blackcurrant), BC-A (blackcurrant-aronia mix), TP (total phenols),TA (total anthocyanins).
Different letters next to k and $t_{1/2}$ values calculated for three assays represent significant differences.

compounds in blackcurrant (BERMÚDEZ-SOTO and TOMÁS-BARBERÁN, 2004), a similar pattern of behavior was expected for the antiradical activity of BC juices as for catechin and quercetin solutions studied earlier by PINELO *et al.* (2004; 2005). When comparing the two storage temperatures (Figs. 1-3), storage at 4°C for 10 days resulted in a smaller loss in antioxidant activity in individual juices (by about 2%) and in the case of BC-A mix by more than 10%. Our findings for BC samples are similar to the findings of other authors, which indicate that antiradical activity of phenolic antioxidants is best preserved at lower temperatures for an extended period of time (VAN der SLUIS *et al.*, 2005).

The continual trend in degradation of polyphenols and anthocyanins in BC juices at both temperatures, which resulted in a final average percent loss in polyphenols of 38% at 4°C and 45% at 22°C, and a final average percent loss in TA of 21% at 4°C and 36% at 22°C, indicates that the samples under study were highly susceptible to degradation of phenolic antioxidants during storage. Results from Figs. 1-3 indicate that the degradation of anthocyanins and total phenols, as well as loss in antiradical activity, followed first-order reaction kinetics (Table 2) with respect to time in storage.

The correlation coefficients for first-order loss in antiradical activity (Table 2) were somewhat lower than those observed for anthocyanin and TP degradation due to the increase in antiradical activity observed in most juices after 48 h in storage (Fig. 1). IVERSEN (1999) also observed first order reaction kinetics for the degradation of anthocyanins and ascorbic acid in blackcurrant juices and nectars stored at 20 °C in daylight and in darkness. First-order degradation of anthocyanins was also observed in blackberry (WANG and XU, 2007) and blood orange juices (KIRCA and CEMEROĞLU, 2003). Our observed kinetic constants for TA content (range: 16.0-41.8 day^{-1} at 4°C and 25.3-58.3 day^{-1} at 22°C) were comparable to the kinetic constants for degradation of TA in blackberry juice (21.6 day^{1}) stored at 25°C (WANG and XU, 2007) and blood orange juice (12.4 day^{-1}) stored at 5°C (KIRCA and CEMEROĞLU, 2003). Significantly larger kinetic constants and smaller half-life values for TA and AA were observed in 3/4 analyzed juices at 22°C, in comparison to 4°C, indicating that the higher storage temperature facilitates the loss in anthocyanins and antiradical activity. The situation was reversed in the case of TP, where slightly higher kinetic constants and smaller half-lives were observed at 4°C in comparison to 22°C. In addition, in 3/4 studied juices, half-life values for TP degradation were not significantly different at 22° and 4°C indicating that storage at refrigerator temperature does not significantly affect the degradation of total phenols in these juices.

CONCLUSIONS

This study has shown a severe loss of the TP and TA contents and, consequently, a substantial loss in the DPPH antiradical activity of analyzed blackcurrant juices, stored at 4° and 22°C. First-order reaction kinetics were observed for degradation of polyphenols, anthocyanins and antiradical activity, at both temperatures, with respect to time in storage. Although the initial high content in polyphenolic antioxidants places the blackcurrant juice preparations in the 'healthy foods' category, the antioxidant properties of juices strongly depend on the time in storage. Additional studies focused on storage condi-

tions-dependent stability of antioxidants in fruit juices, employing a large sample set, are needed before making consumer recommendations.

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ABBREVIATIONS

AA, antiradical activity; BC, blackcurrant; BC-A, blackcurrant-aronia mix; 2,2-diphenyl-1-picrylhydrazyl, DPPH; GAE, gallic acid equivalents; TA, total anthocyanins; TEAC, Trolox equivalent antioxidant capacity; TP, total phenols.

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PAPER

EVALUATION OF RIBOFLAVIN CONTENT IN DAIRY PRODUCTS AND NON-DAIRY SUBSTITUTES

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ABSTRACT

The riboflavin content was determined in different types of milk and non-dairy substitutes. A protein precipitation with $HClO_4$ and quantification of riboflavin by isocratic HPLC-FLD, that allows enough sensitivity, precision and accuracy are presented. The advantages of the method are the use of small volumes of less toxic solvents and reagents, simple sample pre-treatment and short time of analysis. The mean value of the riboflavin content was 1.81 μg/mL in UHT milk, 1.51 μg/mL in pasteurized milk and 1.34 μg/mL in yogurt. The average riboflavin content of 2.3 μg/mL was found in raw goat milk, 1.66 μg/mL in raw cow milk, 0.14 μg/mL in soymilk, and 4.79 μg/mL in rice milk. These data show the wide variability in the levels of riboflavin in different types of milk and dairy products. When UHT milks packages were opened and stored in refrigerator for 5 days, the significant degradation of riboflavin was not observed ($p < 0.01$). The loss of riboflavin was in the range from 0.66 to 7.14% for these storage conditions.

- Keywords: HPLC, milk, riboflavin -

INTRODUCTION

Riboflavin (vitamin B_2) is a very important constituent of all living cells, and is one of the most widely distributed water-soluble vitamins. Vitamin B_2 can be found in nature as riboflavin (RF), but in most biological materials, it occurs predominantly in the form of two coenzymes, flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD) (POWERS, 2003). As animals and humans cannot synthesize riboflavin, and there is a short-term storage capacity for this vitamin in the liver, they must consume it in their diet (BÖHLES, 1997; VIÑAS *et al.*, 2004; BALL, 2006). The Recommended Daily Allowances (RDA) for riboflavin for adult men and women are 1.3 and 1.1 mg per day, respectively. For infants, the RDA is 0.3-0.4 mg per day and for children it is 0.6-0.9 mg per day (POWERS, 2003). Milk and milk products are the most important and common dietary sources of riboflavin. In cow milk, at least 90% of riboflavin is in the free form, but in other sources, it occurs mostly bound to proteins.

Riboflavin is relatively heat-stable, although it can be altered or destroyed during prolonged heating (JEDLICKA and KLIMES, 2005). On the other hand, riboflavin is readily degraded under the influence of light or oxygen. The riboflavin loss is dependent on light intensity, wavelength, exposure time and packaging materials. It is known that milk kept in glass bottles might be particularly susceptible to the loss of riboflavin. This is also associated with flavor changes, because the products of photolysis can damage milk lipids (HALL *et al.*, 2010).

Analytical techniques for the determination of vitamin B_2 in milk and food include fluorometric, liquid chromatographic, and capillary electrophoretic methods (ALBALÁ-HURTADO *et al.*, 1997; GLISZCZYŃSKA-ŚWIGLO and KOZIOLOWA, 2000; VIÑAS *et al.*, 2004; GATTI and GIOIA, 2005; JEDLICKA and KLIMES, 2005; BUENO-SOLANO *et al.*, 2009). Both fluorometric and microbiological assays are official methods of the AOAC International (WOOLLARD and INDYK, 2002). Riboflavin has a strong natural fluorescence and can be detected very specifically with high sensitivity at its maximum fluorescence intensity at pH 4-7. Although HPLC method with reversed-phase columns and UV/VIS detection can also be applied, the combination of HPLC and fluorescence detection is more rapid, selective and sensitive for the determination of riboflavin in complex matrices such as milk or dairy products (WOOLLARD and INDYK, 2002; BUENO-SOLANO *et al.*, 2009).

The aim of this work was estimation of riboflavin content in the raw cow and goat milk, commercial dairy products (UHT, pasteurized milk and yogurt) and non-dairy substitutes such as rice and soy milk from the market. HPLC with fluorescence detection was chosen as the most appropriate method for detection and quantification of this vitamin.

MATERIALS AND METHODS

Reagents

The standard stock solution of riboflavin in concentration of 5 μg/mL was prepared by dissolving 0.25 mg of solid riboflavin (98% purity, Acros Organic, New Yersey, USA) in 50 mL of hydrochloric acid (5%, w/w). Deionized water was obtained using a Smart2Pure Ultrapure water system from Thermo Electron LED GmbH (Niederelbert, Germany). The working standard solutions, in concentration ranging from 0.04 to 4 μg/mL, were prepared by dilution of starting standard stock solution with deionized water. These working solutions were stored in the darkness under refrigeration at 4°C, for the maximum period of 3 weeks. The following solvent and solution were also used: methanol (HPLC grade, Merck, Darmstadt, Germany) and 0.005M ammonium acetate (NH_4Ac) (Sigma-Aldrich, St. Louis, USA). All the other chemicals used were at least analytical reagent grade.

Apparatus and chromatographic conditions

Using a Series 1200 Agilent HPLC system (Santa Clara, CA, USA), consisting of the binary pump, solvent degasser system and fluorescence detector (FLD) set at λ_{ex}=440 nm and λ_{em}=520 nm, the chromatographic determination of riboflavin was achieved at 30°C. The separation was performed using RESTEK Ultra IBD column (4.6 mm x 150 mm, 5 μm) by isocratic elution with the mobile phase consisted of 36.0 (%, v/v) methanol and 64.0 (%, v/v) 0.005M NH_4Ac solution (pH 5.0). The flow rate was kept at 1.2 mL/min and 10 μL of the sample was injected. The total analysis time was below 6 min. In order to confirm riboflavin presence in the analyzed samples, as well as to investigate the possibility of interference by the matrix, diode array detector (DAD) was used simultaneously with FLD. DAD was set at 223, 267, 373, and 440 nm.

Sample preparation

Considering that riboflavin is sensitive to the light, oxygen, pH or heat, the preparation of all types of samples was carefully performed by using test tubes covered with aluminum foil. Raw cow and goat's milk were obtained from the local farms in the same mountainous area in the winter period. The animals were fed only with natural food (forages, grass, hay, straw) without any vitamin supplements. The milk was kept refrigerated at 4°C until it was processed. Pasteurized, UHT milk and yogurt with a different fat content (0.5-3.2%) were collected from local stores. Powder rice milk and soy

milk were also purchased from the local markets. All products were analyzed immediately after opening.

The procedure for sample preparation was: 2 mL aliquot of the sample, in a test tube, was treated with 2 mL of perchloric acid (12%, w/w), then mixed vigorously and allowed to stand for 15 min at the room temperature protected from the light. After proteins precipitation followed by centrifugation for 15 min at 4,000 rpm, 80 μL of mixture 2M K_2CO_3/6M KOH was added to the 2 mL of supernatant to adjust the pH value of the sample to 5.0. Then, the formed insoluble perchlorate was also removed by centrifugation (15 min at 4,000 rpm). An aliquot of the obtained supernatant was filtered through Econofilter 25 mm /0.45 μm membrane syringe filter (Agilent Techologies) before the HPLC analysis. All samples were analyzed in triplicate.

For rice milk, amount of 20 g of powder was weighted in a test tube and dissolved in 200 mL of deionized water and kept in a warm water bath for 10 min. After complete dissolving, the test tube was cooled and 2 mL of the sample was treated according to the procedure for the other samples, described above.

Statistical analysis

All samples were analyzed at least in triplicate. The results are presented as mean value ± SD. The statistical analysis was performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, California, USA). An independent Student's t-test was used to compare the concentrations of riboflavin in all types of analyzed samples. The effect of refrigerated storage on riboflavin content in opened milk packages was examined by paired t-test. Statistical significance was assigned to $p < 0.05$.

RESULTS

Optimisation of the chromatographic conditions

During the development of the method, different mobile phases were tested to optimize the separation of riboflavin and to minimize the run time. The influence of the mobile phase composition on the riboflavin retention parameters was studied using mixtures of 5 mM NH_4Ac solution (pH 5.0) and MeOH in different proportions. The organic solvent was varied between 30 and 36% v/v in order to achieve better separation of the peaks and retention parameters. The final chromatographic system comprising a reverse-phase C18 column (4.6 mm x 150 mm, 5 μm) with a mobile phase consisting of 36.0% methanol and 64.0% 0.005M NH_4Ac solution (pH 5.0) in an isocratic elution at the flow rate of 1.2 mL/min was found to be optimal. Fluorescence detection was performed at λ_{ex}=440 nm and λ_{em}=520 nm. The run time was below 6 min and the retention time for riboflavin was 3.2 min.

Table 1 - Characteristic parameters for the linear regression equations.

Parameters	Riboflavin[a]	Riboflavin[b]
Number of points	9	7
Linear range (μg/mL)	0.2-5.0	0.04-0.2
Slope ± S.E.	90.284±1.191	438.20±6.313
Intercept ± S.E.	1.958±2.976	-0.402±0.782
Correlation coefficient	0.9994	0.9995
LOD (μg/mL)	0.099	0.0059
LOQ (μg/mL)	0.330	0.0178

S.E.: standard error.
[a]Calibration graph used for raw, UHT, pasteurized, rice milk and yogurt samples analysis;
[b]Calibration graph used for soymilk samples analysis.

Linearity

Working standard solutions of riboflavin were prepared as described in Experimental Section. Two different standard calibration graphs, covering the entire concentration range of riboflavin in analyzed diet samples, were constructed. Because of the different intervals of the expected riboflavin concentrations in analyzed samples (in soy milk these concentrations were significantly lower than in other samples), it was necessary to construct two calibration curves. The calibration equations were determined by the linear regression analysis. Characteristic parameters for the linear regression equations are shown in Table 1.

Precision and repeatability

To study the precision of the method, both intra- and inter-day precision were calculated. Intra-day precision was studied by taking three replicates of three concentrations of standard solutions. The relative standard deviations (RSD) were 0.40 and 0.82% for intra- and inter-day precision, respectively.

Limit of detection (LOD) and limit of quantification (LOQ)

The limits of detection (LOD) and quantification (LOQ) were calculated according to International Conference on Harmonisation (ICH) guidelines using the equations: $LOD = 3.3 \cdot SE_a/b$ and $LOQ = 10 \cdot SE_a/b$, where SE_a is the standard error of the intercept, and b is the slope of the calibration curve. The obtained values for LOD and LOQ are given in Table 1.

Optimization of the extraction procedure

Determination of riboflavin requires an appropriate extraction method because it is sensitive to light, extremely acid and alkaline conditions, as well as to phosphatase enzymes. In order to protect riboflavin from light-induced degradation, the extraction procedure was performed in covering test tubes with aluminum foil. According to other authors, extraction procedures were carried out with deionized water, buffer solutions, aqueous solutions of different inorganic acids, organic solvents such as methanol, ethanol, acetonitrile, glacial acetic acid, formic acid, or their mixtures (VIÑAS *et al.*, 2004; GATTI and GIOIA, 2005; JEDLICKA and KLIMES, 2005; PARK *et al.*, 2007; BUENO-SOLANO *et al.*, 2009). Perchloric acid causes a direct acid hydrolysis of the esters bonds of the riboflavin coenzyme forms and transformation of FMN and FAD into the free riboflavin. This step enabled determination of riboflavin as the total riboflavin content in all analyzed samples. Perchloric acid solutions in three different concentrations (8, 10 and 12%, w/w) were chosen for the riboflavin extraction from the milk samples. Comparing retention times and peak areas, the best results were obtained by extraction with 12% solution.

After proteins precipitation, K_2CO_3/KOH mixture was added to the supernatant to adjust the pH value of the sample to 5.0. The riboflavin's maximum fluorescence intensity is at the range of pH 4-7, but it is important to maintain pH at 5.0 to prevent riboflavin photodegradation to lumichrome and lumiflavin.

The yield of the extraction method was confirmed by the recovery study. The milk samples were spiked with known amounts of riboflavin at two concentration levels (0.38 and 0.75 μg per 1 mL of milk). Recovery data for the extraction method were 99.53 and 99.24%, respectively. Also, the values obtained for RSD were lower than 2% and show good reproducibility of the method.

Analysis of milk samples

The chromatograms of blank milk and milk spiked with standard solution of riboflavin are shown in Fig. 1. A well separated peak of riboflavin with good symmetry and peak width was obtained at approximately 3.2 min. Also, UV spectra of riboflavin in unspiked and spiked milk (recorded by DAD detector) were well matched with that of the riboflavin standard. This indicated that interferences by the matrix were not observed.

Our results of the analysis of raw milks obtained from the same geographical area showed that riboflavin content of raw goat milk was in the range from 1.94 to 2.78 μg/mL, while raw cow milk had 1.30 to 2.12 μg/mL of riboflavin (Table 2). The raw cow milk had a significantly lower amount of riboflavin than raw goat milk ($p < 0.01$). Comparing UHT and pasteurized milk from the same producer with the same fat content, statistically significant difference of riboflavin content in these samples was found ($p < 0.05$). Also, the general observation (Table 2) is that analyzed UHT milks have significantly higher amounts of riboflavin (1.81 μg/mL) than pasteurized milks (1.51 μg/mL) ($p < 0.01$). During prolonged heating in pasteurization process, sensitive components of milk such as water-soluble vitamins may be altered or destroyed. Riboflavin is relatively heat-stable but observed differences may arise from other technological parameters which are used in UHT and pasteurization procedure.

For yogurt, our results showed that average riboflavin content was 1.34 μg/mL, which is sig-

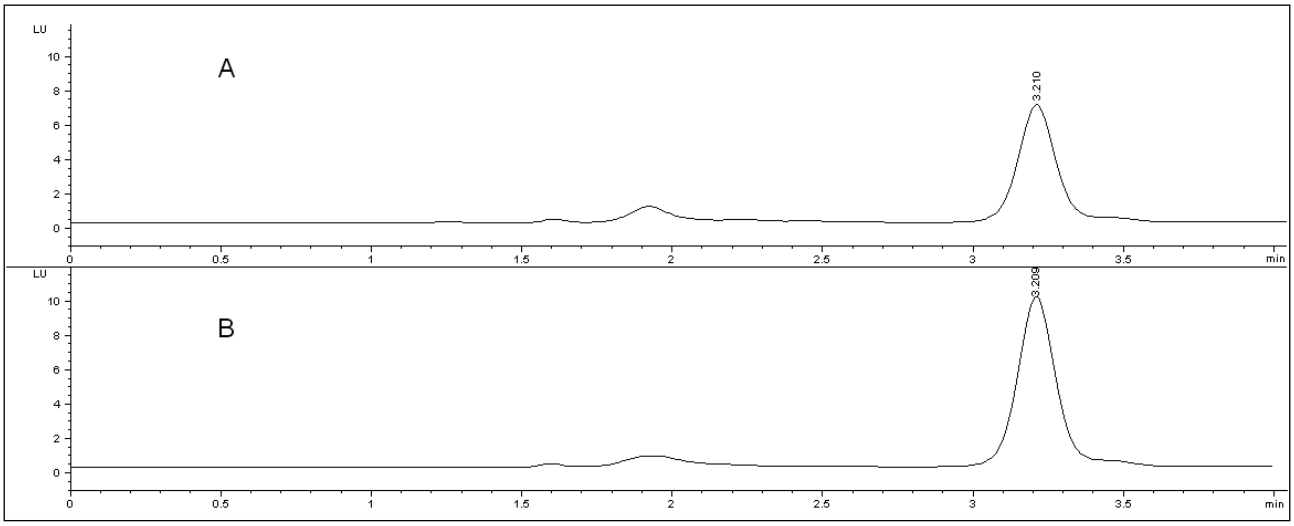


Fig. 1 - The chromatograms of blank milk (A) and milk spiked with standard solution of riboflavin (B).

Table 2 - Results of HPLC analysis of riboflavin in dairy products and non-dairy substitutes.

Sample			Fat content (%)	Concentration of riboflavin (μg/mL of milk)[a] ± SD	RSD (%)
Cow milk	Raw	S_1		2.12±0.04	1.89
		S_2		1.56±0.01	0.64
		S_3		1.30±0.03	2.31
	UHT	P_1	0.5	1.68±0.02	1.25
		P_1	1.6	1.92±0.02	0.85
		P_1	2.8	1.87±0.03	1.66
		P_2	1.6	1.82±0.03	1.54
		P_2	2.8	1.74±0.04	2.18
	Pasteurized	P_1	1.6	1.71±0.01	0.64
		P_2	1.6	1.30±0.01	0.77
		P_2	2.8	1.65±0.04	2.42
		P_3	2.8	1.35±0.03	2.22
Goat milk	Raw	S_1		1.94±0.02	1.03
		S_2		2.78±0.01	0.36
		S_3		2.19±0.02	0.91
	UHT	P_4	3.0	1.52±0.04	2.63
Yogurt	Tetra Pak	P_1	3.2	1.43±0.04	2.80
		P_2	2.8	1.26±0.01	0.79
Rice milk	Reconstituted	P_5		4.78±0.35	0.73
Soy milk	Tetra Pak	P_6		0.14 ±0.01	7.14

S: sample; P: producer; [a]Mean value of triplicate.

nificantly lower than UHT ($p < 0.01$) and pasteurized milk ($p < 0.05$).

In this work we also studied the effect of refrigerated storage (+4°C) on the riboflavin content in opened UHT cow and goat milk packages. The stability of riboflavin depends on external factors such as presence of oxygen, light and acids (SMET *et al.*, 2008). Also, the packaging materials can affect the shelf-life of pasteurized and UHT milk. MUÑOZ *et al.* (1994) have reported the riboflavin loss between 12.5 and 23.4% in milk and non-diary substitutes of milk during refrigerated storage. Our results showed that freshly opened samples had amounts of riboflavin in the range of 1.52-1.92 μg/mL. Similar riboflavin contents (1.51-1.87 μg/mL) were found in opened samples which were stored in refrigerator (+4°C) in the darkness for 5 days (Table 3). The loss of riboflavin was in the range of 0.66 -7.14%, but no statistically significant loss was found ($p > 0.01$). The chromatograms of freshly opened milk and milk after refrigeration are shown in Fig. 2. Although the opened packages were exposed to oxygen, the significant degradation of riboflavin was not observed. Furthermore, the packaging materials, plastic containers and UHT aseptic packaging in the absence of light had no noteworthy influence on the riboflavin content during refrigerated storage.

As regard non-dairy milk substitutes such as soy and rice milk, we found that rice milk had an average content of riboflavin 4.78 μg/mL which is significantly higher than soy milk (0.14 μg/mL) ($p < 0.001$) (Table 2). Comparing the riboflavin content in all analyzed products, rice milk had the significantly highest levels of

Table 3 - Concentration of riboflavin in milk samples after opening (C_0) and after 5 days at 4°C in refrigerator (C_5).

Sample	Fat content (%)	C_0 (μg/mL of milk)[a] ± SD	C_5 (μg/mL of milk)[a] ± SD	Loss of riboflavin (%)
UHT milk	0.5	1.68±0.02	1.56±0.01	7.14
UHT milk	1.6	1.92±0.02	1.87±0.05	2.60
UHT milk	2.8	1.87±0.03	1.77±0.01	5.35
Sterilized goat milk	3.0	1.52±0.04	1.51±0.02	0.66

[a]Mean value of triplicate.

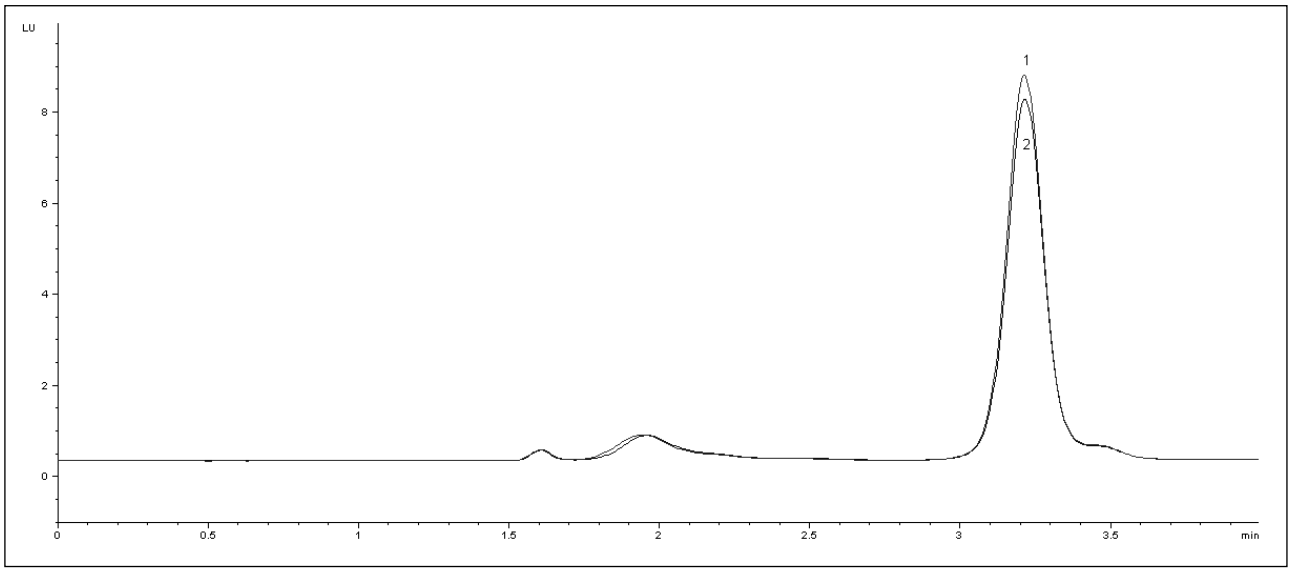


Fig. 2 - The chromatograms of freshly opened milk (1) and milk after refrigeration (2).

this vitamin ($p < 0.01$). Also, the significantly lowest ($p < 0.001$) amount of vitamin B_2 was found in soy milk.

These data show the wide variability in the levels of riboflavin in different types of milk and dairy products. Since the rice milk has high content of riboflavin, its consummation could easily supply the daily requirement for this vitamin. This fact could be important for increasing the use of this type of foodstuff in the daily diet of people.

CONCLUSIONS

The reversed-phase HPLC method with fluorescence detection was applied for determination of the riboflavin content in different types of milk, dairy products and non-dairy milk substitutes. Raw goat milk had high content of riboflavin. This type of milk could be recommended as a good source of vitamin B_2 and it would be important to increase the use of goat milk in the daily diet of people. Furthermore, high levels of riboflavin were found in rice milk, so it could be used as an important non-dairy foodstuff for people with a greater riboflavin deficiency. The other analyzed commercially available dairy products (UHT, pasteurized milk and yogurt) also had satisfactory amounts of riboflavin. Our results showed that analyzed commercial soy milk had a very low amount of vitamin B_2.

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PAPER

ACCELERATED KASHAR CHEESE RIPENING WITH ENCAPSULATED LIPASE AND PROTEASE ENZYMES

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ABSTRACT

In this study, lipase and protease enzymes were encapsulated in κ-carrageenan, gellan and sodium alginate by using emulsion and extrusion technique and were then added in cheese milk together with rennet. The effects of the encapsulating material and ripening period on the chemical characteristics of Kashar cheese were investigated. The study demonstrated that κ-carrageenan, gellan and sodium alginate could successfully be used as lipase and protease carrier systems to accelerate Kashar cheese ripening. Those samples treated with κ-carrageenan capsules showed the highest rate of lipolysis and proteolysis compared to those treated with the other capsules.

- Keywords: kashar cheese, enzyme encapsulation, κ-carrageenan, gellan, sodium alginate -

INTRODUCTION

After White cheese, Kashar cheese is the most commonly produced and consumed cheese in Turkey, the Balkan, Peninsula and the Mediterranean region. The main problem in manufacturing Kashar cheese is the long maturation period which increases the cost of handling significantly. Maturation is very important in developing the unique flavor, aroma and texture of the cheese before marketing. The time required to develop this characteristic flavor and texture varies from a few weeks for soft cheese up to 3 years for very hard cheese varieties (GRIPON *et al.*, 1991). However, the long maturation period increases the price of the cheese (FOX, 1993).

Several attempts have been made to reduce the ripening period by the addition of individual and mixed lipase, protease and β-galactosidase enzymes, some of which have been reported to halve the normal maturation period of cheese (LAW, 2001). Lipolysis and proteolysis play an important role in cheese ripening, and a large number of studies dealing with the acceleration of lipolysis and proteolysis through the addition of free lipolytic and proteolytic enzymes to either cheese milk or curd have been published (KOCAK *et al.*, 1996; CAGLAR and CAKMAKCI, 1998). The addition of free lipases has resulted in premature attack leading to excessive lipolysis and texture and flavor defects (KOCAK *et al.*, 1996). Direct addition of protease enzyme to the cheese milk was not successful due to loss of enzymes in the whey, poor enzyme distribution, reduced yield and poor-quality cheese. Incorporation of encapsulated enzyme eliminated the problems associated with direct enzyme addition. The use of microencapsulated enzymes has been proposed to circumvent these drawbacks. Enzyme microcapsules physically separate the enzyme from the substrate in the curd and the enzyme is only released into the curd upon capsule breakdown during ripening (LAW, 2001). Milk-fat-coated capsules were first developed by MAGEE and OLSON (1981) and used to encapsulate a wide variety of substances.

Vegetable gels such as Konjac, liposomes, milk fat, some food gums and hydrophilic hydrocolloids are used for enzyme encapsulation. The use of liposomes as enzyme-encapsulating substances has some drawbacks. They may be expensive and are generally not regarded as safe and edible. A group of substances that exhibit excellent encapsulating abilities includes food gums or hydrophilic hydrocolloids. Gum capsules are easy to prepare, and gums are relatively widely available, cheap, and biologically compatible (KAILASAPAHTY and LAM, 2005). Because of the aforementioned drawbacks, the addition of enzymes directly or encapsulated in milk fat to cheese milk has not been successful.

Limited information is available on the accelerated ripening of Cheddar cheese using encapsulated enzymes (CAGLAR and CAKMAKCI, 1998). We investigated food gums as an alternative to liposomes for enzyme encapsulation to accelerate cheese ripening. Three gums (κ-carrageenan, gellan and sodium alginate) were used to encapsulate enzymes for application to cheese milk. The objective of this work was to study the effect of encapsulated lipase and protease enzymes cocktails added to Kashar cheese milk on the lipolysis and proteolysis of the cheese during storage.

MATERIALS AND METHODS

Gums, enzymes and chemicals

κ-carrageenan, gellan and sodium alginate gums were supplied by Sigma Chemicals (İstanbul, Turkey). The enzyme Palatase 20000 L (LUN 00217) and Flavourzyme 1,000 L were obtained from Novozymes (İstanbul, Turkey). Direct-set frozen lactic acid starter cultures (Ezal MA014) containing *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* were obtained from Ezal (France). Rennet (ECOREN 200) was obtained from Maysa Gıda (İstanbul). All other reagents used were of analytical grade.

Preparation of gum capsules

κ-carrageenan enzyme capsules were prepared by a modified method of AUDET and LACROIX (1989). Gum powder (1.5 g) was suspended in three lots of 50 mL deionized water, heated to 80°C, stirred and kept at that temperature for 20 min to completely dissolve the polymer. The solutions were cooled to 40°C and mixed with 0.133 mL of 7.5% (w/v) solution of Palatase 20000 L and 13.3 mL of 7.5% (w/v) solution of Flavourzyme 1000 L to produce of capsules. The mixture was rapidly poured into 150 mL soybean oil containing 0.2% emulsifier in a beaker maintained at 40°C while stirring with a magnetic stirrer. The water-in-oil emulsions were cooled to 25°C to allow the gum droplets to gel. The oil phase was decanted, and the resulting capsules were harvested by centrifugation (100 *g*, 2 min). The gel beads were washed twice with distilled water, and the capsules were separated from the supernatant by sieving. The beads formed were hardened by soaking in 0.07% calcium chloride solution for 2 h.

Gellan capsules were prepared by dispersing 0.3 g of gellan powder in 50 mL of deionized water. The dispersion was heated to 90°C under magnetic stirring for 10 min. The solutions were cooled to 45°C and mixed with 0.133 mL of a 7.5% (w/v) solution of Palatase 20000 L and 13.3 mL of 7.5% (w/v) solution of Flavourzyme 1000 L to produce capsules. The rest of the preparation procedure was performed as described for κ-carrageenan.

Preparation of sodium alginate capsules by using emulsion and extrusion techniques

The modified method of SHEU and MARSHALL (1993) was used. A 2% alginate mixture containing 2% Hi-maize resistant starch and 0.133 mL of 7.5% (w/v) solution of Palatase 20000 L and 13.3 mL of 7.5% (w/v) solution of Flavourzyme 1000 L were prepared. The mixture was dropped into oil containing Tween 80 (0.02%). After the dropping was completed, the mixture was stirred vigorously until it was emulsified and appeared creamy. A solution of 0.1 M calcium chloride was then added quickly along the side of the beaker; the phase separation of the oil/water emulsion then occurred. The mixture was left to stand for 30 min to allow the calcium-alginate beads to separate and settle at the bottom of the calcium chloride layer. The oil layer was drained, and beads were collected by low-speed centrifugation (350 *g*, 15 min), washed once with 0.9% saline containing 5% glycerol, and stored at 4°C. Size separation of the beads was performed using 500 and 150 µm steel sieves.

The extrusion technique of KRASAEKOOPT *et al.* (2003) was used. In this study, the lipase in the 0.133 mL of Palatase 20000 L solution and the protease in the 13.3 mL Flavourzyme 1000 L solution was mixed with 20 mL of 2% (w/v) sodium alginate solution (Sigma Aldrich Steinheim, Germany). The suspension was injected through a 0.11 mm needle into 0.05 M $CaCl_2$. The beads were allowed to stand for 30 min to gelate, then rinsed with 0.9% saline containing 5% glycerol and subsequently kept in at 4°C.

Rates of enzyme entrapment

The efficiency of lipase and protease enzyme encapsulation for the three types of capsules were measured separately for lipase and protease according to the methods of TENG and XU (2007) and SARATH *et al.* (1989) respectively, and was used to express the entrapment efficiencies (EE). The rate of EE was the percentage of enzyme encapsulated (expressed as units enzyme activity) in capsules divided by the total units of enzyme in bulk solution multiplied by 100.

Capsules prepared by the addition of 0.133 mL of a 7.5% (w/v) solution of Palatase 20000 L to the encapsulant solutions were used for this purpose. The total enzyme activity was determined from a bulk solution of capsules (before separation of capsules from the un-encapsulated material). The bulk solutions (10 mL) containing κ-carrageenan, gellan and sodium alginate capsules were separately dispersed in 50 mL of 0.4% trisodium citrate solutions and stirred for 30 min at room temperature (23°-24°C) until completely dissolved. Separated gum capsules were treated similarly in trisodium citrate solution.

The dissolved capsule solution was mixed with 0.5 mL stock solution (10 mM pNPP [Sigma] in n-heptane). Approximately 30 µL ethanol (1 M) was added to the reaction mixture and incubated at 40°C at a shaking speed of 200 rpm for 5 to 30 min. After allowing the lipase in the reaction mixture to settle for 30 s, 25 µL of the clear supernatant was taken and then immediately mixed with 1 mL of 0.1 M NaOH in a 1 mL cuvette. The pNP liberated was extracted through the aqueous alkaline phase; the extraction was then analyzed at 410 nm against a blank without enzyme using a UV-visible spectrophotometer.

Proteolytic activity of the enzyme was determined as trichloroacetic acid (TCA) soluble peptides and amino acids following the precipitation of intact casein with TCA. Capsules prepared by addition of 5.0 mL of a 7.5% solution of Flavourzyme 1000 L to the encapsulant solutions were used for this purpose. The total enzyme activity was determined from a bulk solution of capsules (before separation of capsules from the un-encapsulated material). The bulk solutions (10 mL) containing k-carrageenan and gellan capsules were separately dispersed in 50 mL of 0.4% trisodium citrate solutions and stirred for 30 min at room temperature (23°-24°C) until completely dissolved. Separated gum capsules were treated similarly in trisodium citrate solution.

One unit of specific enzyme activity was defined as the increase in absorbance at 280nm across a 1 cm path length caused by a unit amount (1 mg) of enzyme (expressed as total nitrogen) under the conditions of the assay.

The diameters of 100 randomly selected beads of each treatment were measured with an eyepiece micrometer on an optical microscope at a magnification of 100x.

Cheesemaking

A 7.5% (w/v) solution of Palatase M (20,000 LU/g) and a 7.5% (w/v) solution of Flavourzyme 1000 L (1,000 LAPU/g) were encapsulated in sodium alginate, κ-carrageenan and gellan gums as described above.

Cheese production was done in the Dairy Pilot Plant of the Food Engineering Department of Harran University. One hundred twenty kilograms of standardized milk was used for each batch (1 control (coded A) and 4 treatments). The fat content of the milk was standardized to 2.5%. All batches were pasteurized at 72°C for 1 min and then cooled to 34°C. Starter culture (1%) and $CaCl_2$ (0.02%) were then added. For the experimental cheeses, enzyme capsules made of sodium alginate by emulsion techniques, sodium alginate by extrusion techniques, κ-carrageenan and gellan gums, were introduced into the cheese milk at 34°C, just before the addition of rennet, and the samples were coded B, C, D and E respectively. Stir-

ring was continued after enzyme capsule addition up to the point of rennet addition. These quantities corresponded 0.133 mL Palatase M (20,000 LU/g) per kilogram milk fat and 13.4 mL Flavourzyme 1000 L (1,000 LAPU/g) per kilogram of cheese. When the pH of the milk reached 6.2-6.3, rennet diluted with pure water was added. Cutting was performed 30 min later. The curd was cut with a curd knife into cubes of 1 cm^3. The cut curd was allowed to settle for 15 min. Cooking was performed by increasing the temperature from 34° to 40°C over 30 min. The heating rate was an increase of 1°C for every 4-5 min. At the end of cooking, a third of the whey content was drained from each batch. At the same time, the cheese curd was agitated. The cheese curd was fermented until it reached a pH level of 5.0. The remaining whey was then drained. Cheese whey was collected during the manufacturing and strained using a 120-µm stainless steel sieve. The capsules were collected on the sieve and re-added to the curd. The curd was hand-stretched in a 6% brine at 74°C for 2 min for all the cheeses studied. Brine was strained using a 120 µm stainless steel sieve and the capsules were collected on the sieve and re-added to curd while the curd was kneading. The curds were placed into cylindrical stainless steel molds and turned 30 min later to provide a flat surface. All cheeses were cooled at room temperature, and the molds were removed. Then, the cheeses were allowed to gain their yellow color for 24 h at 15°±2°C.

The mass of one block of fresh Kashar cheese was approximately 600 g. The blocks of cheeses were surface-salted for 1 week and stored at 4°-6°C for 180 days. Cheese samples were taken for chemical analyses on the 1st, 15th, 30th, 60th, 90th, 120th and 150th days of storage. Cheese was manufactured in triplicate for each group.

Cheese composition

The pH of the milk (TSE, 1994) and cheeses (TSE, 1995) was measured using a digital pH-meter (model of Orion 250 A, Orion Research Inc., Boston, USA). The protein content of the milk and cheeses were determined by the Kjeldahl method (GRİPON *et al.*, 1975). The total fat and dry matter contents of the cheese samples were determined using the method proposed by Gerber (TSE, 1994) and gravimetric methods, respectively. The salt content of the cheeses was determined by the Mohr titration method (AOAC, 1990).

Determination of free fatty acid analysis

The total free fatty acids (TFFAs) were the sum of the contents of butyric ($C_{4:0}$), caproic ($C_{6:0}$), caprylic ($C_{8:0}$), capric ($C_{10:0}$), lauric ($C_{12:0}$), myristic ($C_{14:0}$), palmitic ($C_{16:0}$), stearic ($C_{18:0}$), oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) acids.

Fat was extracted from cheese samples as described by GARCIA-LOPEZ *et al.* (1994) and methylated according to the procedure of SUKHIJA and PALMQUIST (1988). Fatty acid methyl esters were analyzed using Gas Chromatography (Thermo Quest) equipped with a flame ionization detector (FID) and fitted with a fused silica capillary column (SP-2380, 30 m, 0.25 mm; Supelco Inc., Bellefonte, PA). The temperature of the injector and detector temperature was 250°C.

The initial oven temperature was set to 40°C for 1.0 min, and then increased to 240°C at a rate of 5°C/min. The final temperature was maintained for 10 min. Nonanoic acid was used as the internal standard. A standard fatty acid mixture containing 37 fatty acids (Sigma-Aldrich Chemicals 189-19) was used to provide standard retention times. Fatty acids were identified by comparing their retention times with those of fatty acids in standard samples. An auto system Thermoquest GC-MS equipped with a flame ionization detector (FID) was used to analyze the FFAs of the cheese samples. The carrier gas was helium, flowing at 2 mL min^{-1}. A 1 µL sample was applied with a split ratio of 1:30 into the injector.

Determination of proteolysis

Cheese protein (casein) degradation during ripening was evaluated after 1st, 15th, 30th, 60th, 90th, 120th and 150th days using by mini urea polyacrylamide gel electrophoresis (Urea-PAGE). Electrophoresis was carried out on a vertical slab unit (Bio-Rad Laboratories, Inc. 1000 Alfred Nobel Drive, Herculus, California, USA) and the stacking gel system described by CREAMER (1991). Samples were prepared by gratting 0.5 g of each cheese into 25 mL of sample buffer (0.092 g EDTA, 1.08 G Tris, 0.55 g boric acid and 36 g urea made up to 100 mL and adjusted to pH 8.4). Each sample was centrifuged at 10,000 *g* for 10 min and 2 mL from the middle portion was taken. All samples were mixed with 3% each of 0.1% (w/v) bromphenol blue solution and mercaptoethanol. 5 µL of the 2% cheese solutions were used for electrophoresis. For dying the gels were stained with Coosmassie Blue R-250 dye solution was used.

Statistical analyses

Each cheese experiment was repeated three times, and each analysis was done in duplicate. The experiment was designed according to a 5x8x3 (capsule material x storage time x repeation) factorial design. All statistical analyses were performed using the SPSS statistical software program (version 5.0). Statistically different groups were determined by the LSD (Least Significant Difference) test (BEK and EFE, 1995).

RESULTS AND DISCUSSION

Enzyme encapsulation

The encapsulation efficiencies of Palatase M (20,000 LU/g) in κ-carrageenan, gellan gums, sodium alginate produced by emulsion technique or sodium alginate by extrusion technique were, respectively, 58.7±0.51, 51.0±0.43, 42.50±0.36 and 53.1±0.35% of the initial activity (mean of 3 separate trials). Encapsulation efficiencies of Flavourzyme 1000 (1,000 LAPU/g) in κ-carragennan, gellan gums, sodium alginate by emulsion technique or sodium alginate by extrusion technique were, respectively, 54.1±0.64, 47.3±0.56, 39.2±0.71 and 47.9±0.63% of the initial activity (mean of 3 separate trials). The encapsulation efficiencies of the four capsulants were statistically significantly different from each other ($p<0.01$). The ionic strength of the capsule-hardening solution (calcium chloride) may have had an effect on the activity of the enzymes (KAILASAPATHY and LAM, 2005).

The beads were globular in shape. The capsule materials influenced on the size of the beads. The sodium alginate capsules by extrusion technique were bigger than the other capsules. The diameter of beads of κ-carragennan, gellan gums, sodium alginate by emulsion technique was 1.68 mm, which was significantly lower than that of sodium alginate by extrusion technique beads (1.90 mm).

Chemical composition

The gross chemical composition of the control and experimental cheeses is given in Table 1. Significant differences were observed between the control and experimental cheeses. The cheese curds had a significantly higher moisture content and titratable acidity but lower pH contents as compared to the control ($p<0.01$). The high moisture content of the gum-capsule-treated cheese curds was due to the hydrophilic nature of sodium alginate, carrageenan, and gellan gums, which retained moisture in the cheeses. The protein, fat and salt contents of the experimental cheeses were close to those of the control. Similar results were reported for capsule-treated cheeses by KHEADR *et al.* (2002 and 2003), KAILASAPATHY and LAM (2005).

Free fatty acid production

The free fatty acid (FFA) contents of the control and experimental cheeses were quantified throughout a 180-day of ripening period (Table 2). The production of FFAs was markedly stimulated by the introduction of encapsulated lipases and proteases. This effect was noticeable even on the day of manufacture. At that time, the experimental cheeses exhibited substantial increases in FFAs. This suggests that a portion of the enzyme is immobilized on the external capsule membrane and/or a loss of capsule integrity during cheese manufacture leads to the release of the encapsulated enzymes.

Upon aging, the amount of FFA produced increased with the ripening period; moreover, the experimental cheeses had accumulated higher quantities of individual FFAs, depending on the type of capsule, as compared to control ($p<0.01$) (Table 2). The higher incidence of FFAs in the κ-carrageenan capsules-treated cheeses. The conditions to which cheeses are subjected, such as the presence of ions and lactic acid, appeared to influence the stability of gum capsules in the cheeses. The interaction between milk proteins and κ-carrageenan gum capsules most likely enhanced the interaction between the encapsulated enzyme and milk proteins (KAILASAPATHY and LAM, 2005). On the other hand, no report is available in the literature about the effect of the lipase and protease enzyme encapsulated in sodium alginate on cheese properties, to which a part of this work has been addressed. The lower FFA content of sodium-alginate-treated cheeses suggests that these capsules probably release their enzyme contents very slowly. As the cheeses aged, the progressive increase in the

Table 1 - Chemical composition of control and encapsulated lipase and protease-treated cheeses on day of manufacture.

	Cheeses*				
Chemical parameter	**A**	**B**	**C**	**D**	**E**
pH	5.37±0.02^{a}	5.29±0.04^{b}	5.32±0.04^{b}	5.17±0.02^{c}	5.08±0.04^{c}
Titratable acidity(% l.a.)	1.15±0.021^{d}	1.278±0.021^{c}	1.170±0.021^{d}	1.332±0.03^{b}	1.404±0.021^{a}
Moisture (%)	45.48±0.32^{a}	47.84±0.38^{b}	46.40±0.35^{c}	47.13±0.37^{b}	47.88±0.37^{b}
Protein (%)	23.52±0.34^{a}	22.38±0.22^{b}	23.88±0.27^{a}	23.52±0.24^{a}	22.77±0.19^{b}
F/D	51.81±0.23^{b}	51.77±0.3^{b}	52.54±0.11^{a}	51.86±0.42^{b}	51.48±0.08^{b}
Salt (%)	1.76±0.05^{a}	1.66±0.07^{c}	1.76±0.05^{a}	1.70±0.05^{b}	1.40±0.06^{d}

*A: control cheese; B, C, D, and E cheeses contain Palatase M and Flavourzyme 1000 in sodium alginate capsules produced by emulsion techniques, in sodium alginate capsules produced by extrusion techniques, in κ-carrageenan capsules, in gellan capsules, respectively. l.a. : lactic acid.
Different letters following numbers in the same row denote significant differences ($p<0.05$).

Table 2 - Production of free fatty acids in control and enzyme- (lipase and protease) treated cheeses during ripening period (n=3).

Cheeses*	Ripening period (day)	Free fatty acids mg 100 g^{-1} cheese										
		$C_{4:0}$	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	Total
A	1	0.48^{a2}	0.54^{a1}	0.51^{a3}	0.65^{a3}	1.81^{c4}	4.08^{b3}	13.11^{a4}	4.37^{b5}	7.82^{b4}	1.50^{a2}	34.87^{b5}
	15	0.55^{a2}	0.65^{a1}	0.70^{b3}	1.00^{b3}	2.22^{c4}	5.15^{b3}	18.22^{b4}	5.85^{c4}	9.27^{c4}	2.22^{a2}	45.83^{d4}
	90	1.27^{b1}	1.51^{b1}	1.60^{e2}	2.29^{d2}	4.14^{e3}	9.27^{e2}	42.97^{b3}	10.80^{e3}	16.83^{e3}	4.72^{d1}	95.41^{e3}
	120	1.52^{c1}	1.78^{b1}	1.91^{e2}	2.96^{e2}	5.27^{e2}	10.65^{e2}	52.54^{c2}	12.41^{e2}	20.74^{e2}	5.62^{e1}	115.40^{e2}
	180	2.48^{c1}	2.82^{c1}	3.10^{e1}	4.19^{e1}	7.81^{e1}	14.14^{e1}	74.56^{c1}	16.24^{e1}	30.08^{e1}	6.45^{e1}	161.87^{e1}
B	1	0.74^{a3}	0.73^{a3}	0.83^{a5}	1.12^{a4}	2.20^{b5}	7.57^{a4}	15.16^{a4}	8.01^{a5}	12.21^{a5}	1.68^{a4}	50.24^{a5}
	15	1.35^{a3}	1.47^{a3}	1.51^{a4}	2.04^{a4}	3.61^{b4}	8.93^{a4}	22.14^{a4}	9.50^{a4}	15.07^{b4}	3.01^{a4}	68.64^{c4}
	90	4.79^{a2}	5.10^{a2}	5.42^{d3}	8.37^{c3}	11.83^{d3}	23.71^{d3}	49.55^{b3}	25.50^{d3}	29.11^{d3}	8.24^{c3}	171.63^{d3}
	120	6.07^{a2}	6.51^{a2}	7.06^{d2}	11.09^{d2}	14.40^{d2}	29.04^{d2}	63.22^{b2}	34.59^{d2}	38.08^{d2}	12.55^{d2}	222.61^{d2}
	180	10.13^{b1}	11.21^{b1}	11.01^{d1}	20.74^{d1}	26.15^{d1}	39.20^{d1}	93.01^{b1}	46.07^{d1}	52.35^{d1}	18.08^{d1}	328.04^{d1}
C	1	0.70^{a3}	0.77^{a3}	0.82^{a5}	1.19^{a5}	2.32^{a5}	7.79^{a4}	15.14^{a4}	8.20^{a5}	12.10^{a5}	1.76^{a4}	50.81^{a5}
	15	1.36^{a3}	1.55^{a3}	1.63^{a4}	2.32^{a4}	3.81^{b4}	9.44^{a4}	23.23^{a4}	10.00^{a4}	15.64^{b4}	3.42^{a4}	72.41^{c4}
	90	4.93^{a2}	5.53^{a2}	6.22^{c3}	9.21^{b3}	13.27^{c3}	25.90^{c3}	54.07^{a3}	27.84^{c3}	33.62^{c3}	10.46^{b3}	191.06^{c3}
	120	6.55^{a2}	7.04^{a2}	7.95^{c2}	12.41^{c2}	17.42^{c2}	32.37^{c2}	69.99^{b2}	37.51^{c2}	43.91^{c2}	15.08^{c2}	250.23^{c2}
	180	10.79^{a1}	12.45^{a1}	12.71^{c1}	23.11^{c1}	30.23^{c1}	43.53^{c1}	99.41^{b1}	51.92^{c1}	58.74^{c1}	22.14^{c1}	365.03^{c1}
D	1	0.82^{a3}	0.83^{a3}	0.81^{a5}	1.35^{a5}	2.64^{a5}	8.17^{a4}	16.14^{a5}	8.57^{a5}	12.45^{a5}	2.08^{a4}	53.86^{a5}
	15	1.51^{a3}	1.73^{a3}	1.83^{a4}	2.71^{a4}	4.41^{a4}	10.74^{a4}	25.47^{a4}	11.42^{a4}	17.14^{a4}	4.17^{a4}	81.13^{a4}
	90	5.74^{a2}	6.48^{a2}	7.34^{a3}	10.95^{a3}	15.79^{a3}	30.45^{a3}	63.28^{a3}	32.53^{a3}	43.01^{a3}	14.14^{a3}	229.71^{a3}
	120	7.59^{a2}	8.39^{a2}	9.44^{a2}	15.59^{a2}	21.05^{a2}	39.52^{a2}	79.72^{a2}	43.72^{a2}	54.12^{a2}	20.66^{a2}	299.81^{a2}
	180	12.01^{a1}	14.16^{a1}	16.18^{a1}	27.11^{a1}	37.95^{a1}	53.08^{a1}	110.94^{a1}	61.88^{a1}	72.84^{a1}	30.15^{a1}	436.28^{a1}
E	1	0.76^{a3}	0.82^{a3}	0.84^{a5}	1.27^{a5}	2.49^{a5}	7.92^{a4}	15.74^{a4}	8.40^{a5}	12.04^{a5}	1.88^{a4}	52.16^{a5}
	15	1.48^{a3}	1.69^{a3}	1.74^{a4}	2.51^{a4}	4.20^{a4}	10.09^{a4}	24.40^{a4}	10.71^{a4}	16.34^{a4}	3.81^{a4}	76.96^{b4}
	90	5.35^{a2}	6.04^{a2}	6.83^{b3}	9.94^{b3}	14.51^{b3}	28.10^{b3}	59.03^{a3}	30.17^{b3}	38.82^{b3}	12.85^{a3}	211.64^{b3}
	120	7.18^{a2}	7.77^{a2}	8.65^{b2}	14.03^{b2}	19.22^{b2}	36.15^{b2}	74.70^{a2}	40.35^{b2}	49.07^{b2}	18.08^{b2}	275.21^{b2}
	180	11.41^{a1}	13.35^{a1}	14.52^{b1}	25.08^{b1}	34.17^{b1}	48.09^{b1}	100.21^{a1}	56.11^{b1}	65.43^{b1}	26.09^{b1}	394.46^{b1}

*A: control cheese; B, C, D, and E cheeses contain Palatase M and Flavourzyme 1000 in sodium alginate capsules produced by emulsion techniques, in sodium alginate capsules produced by extrusion techniques, in κ-carrageenan capsules, in gellan capsules, respectively.
Different letters in the same column denote significant differences for capsule materials ($p<0.01$).
Different numbers in the same column denote significant differences for storage period ($p<0.01$).

concentration of such FFAs suggested a gradual release of the encapsulated lipases.

Despite a rise in the rate of lipolysis, a limited amount of volatile fatty acids was produced. Capric acid, which was the main volatile free fatty acid, accounted for 1.53 and 2.75 to 3.08% of the TFFA, as observed in the 180-day-old control and experimental cheeses, respectively. The importance of volatile fatty acids in the development of cheese flavor has been discussed by other researchers (WOO *et al.*, 1984), who have reported that a certain concentration of such volatile fatty acids was needed to develop a typical cheese flavor. However, a high production of these fatty acids could lead to off-flavor. Among the nonvolatile fatty acids, the amount of palmitic acid increased at the fastest rate, followed by that of oleic, stearic, myristic and lauric acids.

Proteolysis

There were significantly ($p<0.01$) higher levels of proteolysis in enzyme treated cheeses as compared to the control cheese (Figs. 1-5). Cheeses treated with κ-carrageenan capsules showed a high rate of increase in β-, α_{s1}-casein degradation. The higher rate of β-, α_{s1}-casein hydrolysis in the κ-carrageenan capsules-treated cheeses was probably due to the low stability of κ-carrageenan gels in solutions with acidic pH (KAILASAPATHY and LAM, 2005), similar to that observed in ripening cheese. The observed slower rate of β- and α_{s1}-casein degradation in cheeses treated with sodium alginate capsules suggests that these capsules probably release their enzyme contents very slowly.

An increasing trend for β- and α_{s1}-casein degradation was observed during the 180-day of ripening period for all cheeses ($p<0.01$). This was expected because amino groups are produced in cheese as a consequence of protein breakdown during ripening (FOX *et al.*, 1993). Thus an increase in proteolysis in treated cheese over that of control at any given time during ripening indicates an acceleration of ripening.

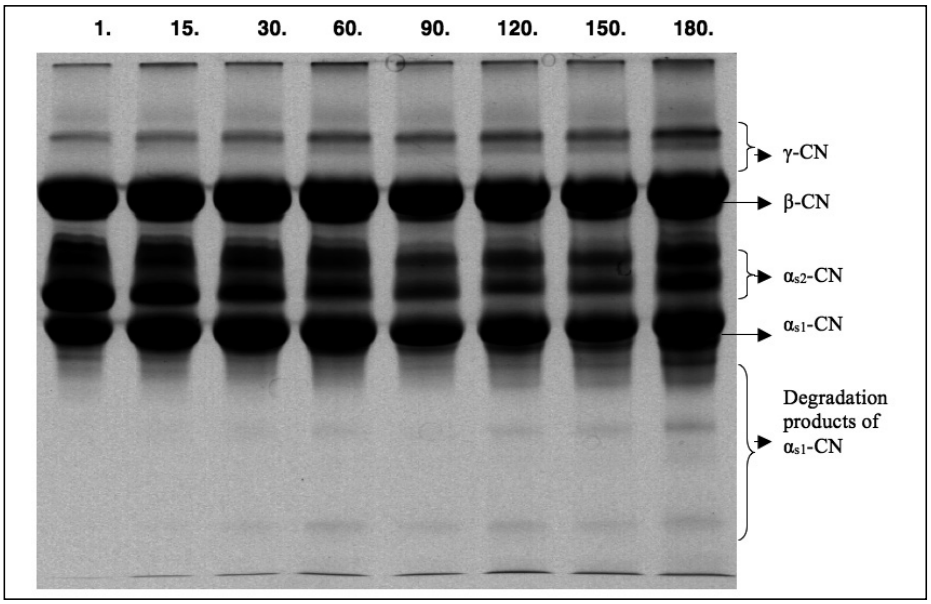


Fig. 1 - Electrophoretograms for casein of control cheese during 180 days of ripening.

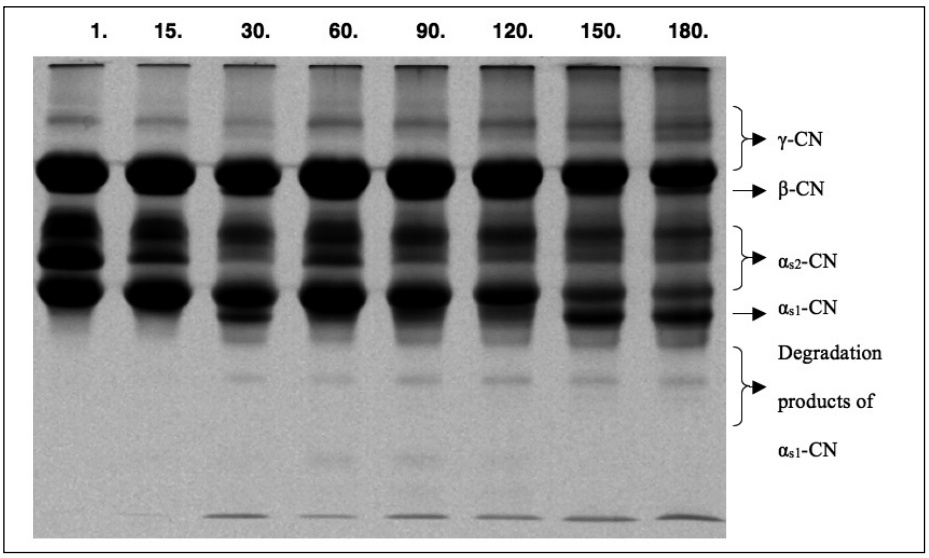


Fig. 2 - Electrophoretograms for casein of contain Palatase M and Flavourzyme 1000 in sodium alginate capsules produced by emulsion technique cheeses during 180 days of ripening.

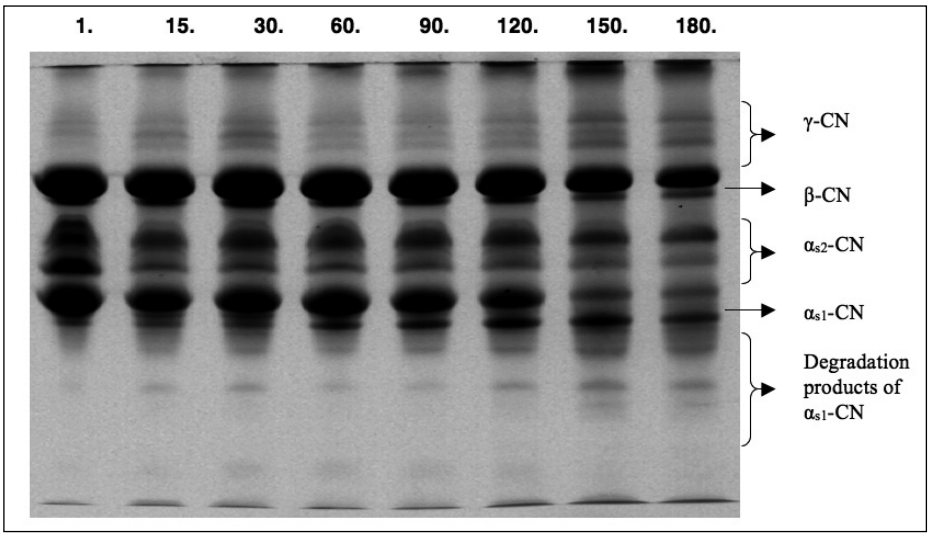


Fig. 3 - Electrophoretograms for casein of contain Palatase M and Flavourzyme 1000 in sodium alginate capsules produced by extrusion technique cheeses during 180 days of ripening.

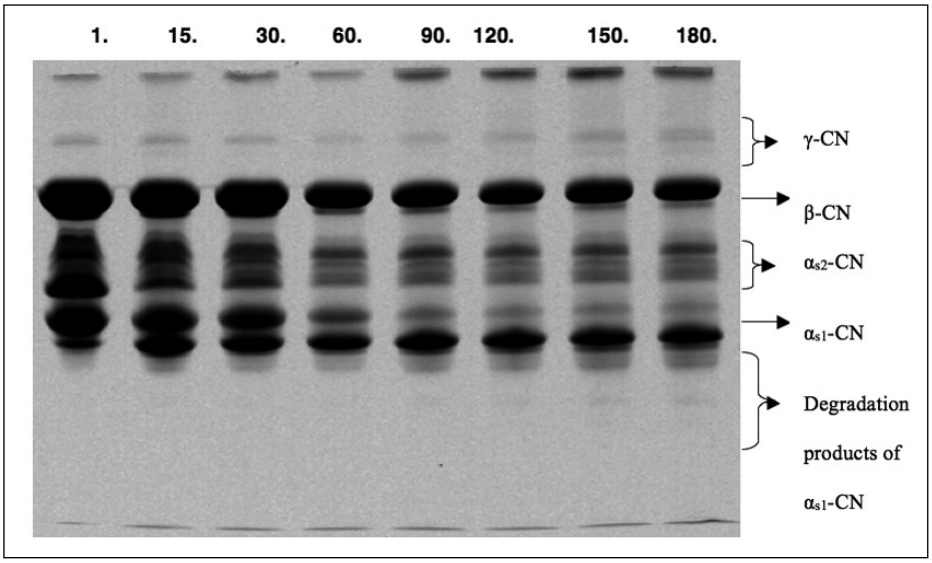


Fig. 4 - Electrophoretograms for casein of contain Palatase M and Flavourzyme 1000 in κ-carrageenan capsules cheeses during 180 days of ripening.

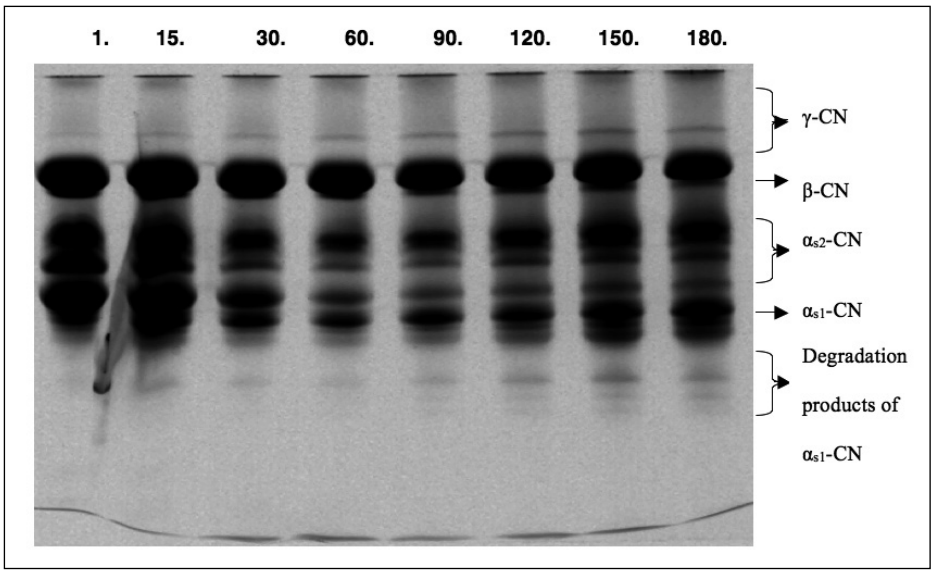


Fig. 5 - Electrophoretograms for casein of contain Palatase M and Flavourzyme 1000 in capsules cheeses during 180 days of ripening.

CONCLUSIONS

According to the results, it was concluded that κ-carrageenan, gellan and sodium alginate could successfully be used as lipase and protease carrier systems in order to accelerate the fat and protein breakdown process during Kashar cheese ripening. The use of this system could be considered as a way to avoid the flavor defects that usually result from the addition of free lipases or proteases to either cheese milk or curd. This system was relatively shortened Kashar cheese ripening.

This study confirms that the impact of encapsulated enzymes in cheese ripening is influenced greatly by the nature of the encapsulating gums themselves. Cheeses treated with κ-carrageenan capsules showed the highest rate of lipolysis and proteolysis compared to those treated with gellan or sodium alginate capsules. Sodium alginate capsules disrupted under cheese manufacturing conditions appeared to be more suitable than gum capsules as enzyme encapsulants for use in accelerating cheese ripening. However, the easily ruptured gum capsules under cheese manufacturing conditions may lead to the rapid release of enzymes and excessive lipolysis during early ripening.

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PAPER

EFFECT OF ENZYMATIC HYDROLYSIS ON THE RESISTANT STARCH YIELD

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ABSTRACT

Cereal starch has a great benefit on human health through its resistant starch content. Enzyme hydrolysis of native starch is very helpful in understanding the structure of starch granules and utilizing them. In this study, the resistant starch formation by pullulanase hydrolysis of corn starch was investigated. Resistant starch was prepared by debranching of normal corn starch and the autoclaving. The objective of this study was to optimize starch debranching with pullulanase for the production of resistant starch. The formation of resistant starch was studied with pullulanase at different incubation temperatures, pH and time. Debranched starch samples with a maximum yield of resistant starch was 17.13%. X-ray diffraction showed qualitative differences among the crystallities formed at different yields of resistant starch. Corn starch (A pattern) was different from resistant starch formed by pullulanase in conjunction with termostable α-amylase (B pattern). Determination of reducing sugars showed that amylose fractions with different number average chain lengths (DP 22-260) were obtained. Under the experimental conditions, the yield of RS increased within a region of DP of 22-75.

- Keywords: resistant starch, pullulanase, termostable α-amylase, degree of hydrolysis -

INTRODUCTION

Starch consists of two D-glucan polymers: amylose, which is a linear polymer made up of D-glucose units, and amylopectin, which is a highly branched polymer of glucose found in plants. A part of the dietary starch, called resistant starch (RS), escapes enzymatic digestion in the small intestine. Although it is not digested in the small intestine, it may be fermented by microorganisms in the large intestine. RS activities are similar to dietary fiber, altering lipid metabolism and improving cholesterol metabolism (CHENG and LAI, 2000; NUGENT, 2005). Besides physiological benefits in humans, RS has been showed to have potential as a unique ingredient with improved oral tactile perception and taste (BROWN, 2004). Formation of RS depends on many factors, such as pH, temperature, amylose/amylopectin ratio and incubation time (KIM *et al.*, 2006).

The corn starch contains a lower percentage of resistant starch, it may tend to increase with amylose content (WALTER *et al.*, 2005). Some studies showed that debranching using pullulanase had been used to produce starch with linear and recrystallization polymer chains, which were enhanced resistant starch content (YIN *et al.*, 2007; PONGJANTA *et al.*, 2008). As normal maize starch contains both linear amylose and branched amylopectin with short and long chains of different sizes, our approach was to degrade it through physical and enzymatic treatments to attenuate starch digestion properties.

The aim of the present work was to optimize starch debranching with pullulanase for RS production and to investigate how RS formation is influenced by the degree of enzymatic hydrolysis and the amylose chain length. Therefore, it seemed to be of great interest to investigate the degree to which RS formation is influenced by the chain length of amylose.

MATERIALS AND METHODS

Materials

Corn (moisture, 12.1%; starch content, 86.7%; amylose content, 25.4%) starch was purchased from a local producer. Termostable α-amylase (120,000 NU/g), glucoamylase (100,000 u/mL) and pullulanase (1,350 NPUN/g starch) were purchased from Novozymes (Bagsvaerd, Denmark).

Optimization of pullulanase debranching

The experimental design was chosen to investigate the optimal conditions for RS formation from the debranched and autoclaved starch. At first, the effect of pullulanase on RS formation was investigated. Starch sample (30 g) were suspended in 100 mL distilled water. The starch samples were debranched (using pullulanase enzymes) for 5 h in water bath. The debranched samples are then autoclaved for 30 min at 121°C. The samples were taken out of the autoclave and then the samples were cooled to room temperature, they were incubated at 4°C for 12 h. A three-factor, five-level quadratic regression rotation combination design was used for optimization of the pullulanase debranched conditions. The selected process variables were shown in Table 1. The range of each factor and level was based on the results of preliminary experiments.

Determine the incubation time of pullulanase

The effect of time on RS yield was examined with different time gradient (2, 4, 6, 8, 10, 12, and 24 h).

Collaborative preparation of RS with pullulanase and α-amylase

After the starch samples were debranched, pH was adjusted to 6.0~7.0, α-amylase was added in order to determine the synergy of two enzymes.

Determination of RS content

The resistant starch content was determined according to SAURA *et al.* (1993). A freshly prepared 2M KOH solution (6 mL) was added to the sample, mixed thoroughly and shaken continuously for 30 min at room temperature. Acetate buffer (0.4M, pH 4.75, 3 mL) was added as well as 4 mL 2N HCl. pH was adjusted to 4.75 using 2N HCl. Then 60 μL amyloglucosidase suspension (E.C. 3.2.1.3) was added, and the solution mixed and incubated 30 min at 60°C, with continuous agitation. Samples were cen-

Table 1 - Levels for the main factors.

Parameters	Levels				
	-1.682	-1	0	1	1.682
Temperature/°C	38	45	55	65	72
pH	4.2	4.5	5.0	5.5	5.8
Enzyme/(NPUN/g starch)	1.0	2.0	3.5	5.0	6.0

trifuged 15 min at 3,000×g and the supernatants collected. The pellet was resuspended in 10 mL distilled water and the centrifugation repeated. The supernatants were combined with the water washes.

Total glucose was analyzed using a GOD-POD reagent (Glucose Oxidase/Peroxidase). A glucose standard solution (10-50 mg/mL) was used. In a glass tube 0.5 mL of supernatant was pipetted and 1 mL of the reagent solution added from the combined kit for glucose determination. The solution was mixed thoroughly and the tubes were placed in a water bath at 37°C for 30 min. Absorbances at 500 nm were measured against blank reagent. The resistant starch was calculated as glucose (mg) ×0.9.

Determination of the degree of polymerization (DP)

The degree of polymerization of the samples was analyzed as described in YANG (2000). The DP was calculated as the ratio of glucosyl monomer concentration determined by the phenol-sulfuric acid method divided by the reducing-end concentration determined by the method according to SOMOGYI (1952).

X-ray diffraction

X-ray powder diffraction was performed with dried starch samples. Freeze-dried samples was done in a freeze dryer at 4°C. Wide-angle X-ray diffraction analysis was performed using a diffractometer (JEOL, JDX-3530, Janpan) operating at 40 kV and 30 mA. Dried starch samples were packed in an aluminum frame. Diffractograms of samples were acquired at an angular range of 2θ from 5° to 40° with step size 6°/min. Counting time was 1 s on each step. The data was analyzed with program MDI Jade 6.5.

Statistical analysis

The test data were statistically analyzed using one-way analysis of variance (ANOVA). P values < 0.05 were considered to be significant. Triplicate determinations were performed for each test and the results reported are mean values.

RESULTS

Optimization of pullulanase debranching conditions for RS formation

The optimization condition for RS formation was obtained from the test design (Table 2). The experiments contained 23 runs. The levels of each variable were coded as −1, 0, and +1 for statistical analysis. The dependent variable was the yield of RS. The results for yield of RS are shown in Table 2. The impact of pullulanase on resistant starch formation is significant. Based on the results of experiment, different condition has yielded different content of resistant starch.

Table 2 - Levels for the main factors in the quadratic regression rotation design.

Run number	X_1 Temperature (°C)	X_2 pH	X_3 Enzyme (NPUN/g starch)	y RS (%)
1	1	1	1	16.09
2	1	1	-1	15.12
3	1	-1	1	15.86
4	1	-1	-1	14.52
5	-1	1	1	15.18
6	-1	1	-1	14.06
7	-1	-1	1	15.38
8	-1	-1	-1	14.40
9	1.682	0	0	14.82
10	-1.682	0	0	15.06
11	0	1.682	0	13.29
12	0	-1.682	0	14.75
13	0	0	1.682	14.94
14	0	0	-1.682	14.47
15	0	0	0	17.25
16	0	0	0	16.67
17	0	0	0	17.08
18	0	0	0	15.64
19	0	0	0	16.33
20	0	0	0	15.87
21	0	0	0	16.71
22	0	0	0	14.84
23	0	0	0	16.26

Table 3 - The variance analysis.

Source Threshold	Sum of squares	Degrees of freedom	Mean Squares	F value
Re regression $F_{0.1}$ (9, 13) = 2.20	D = 14.889	f = 9	1.654	F_2 = 2.803
Remainder	D = 7.664	f = 13	0.590	
Fitting $F_{0.05}$ (5, 8) = 3.69	D = 3.094	f = 5	0.619	F_1 = 1.083
error	D = 4.571	f = 8	0.571	
sum	D = 22.553	f = 22		

The quadratic model from regression analysis was expressed as in the following equation:

$$y=16.28+0.16x_1-0.16x_2+0.38x_3-$$
$$+0.35x_1^2+0.17x_1x_2+0.03x_1x_3-0.67x_2^2-0.03x_2x_3-$$
$$+0.43x_3^2$$

Where y is the dependent variable, x_1 and x_2 are reaction temperature and pH, respectively, and x_3 is amount of enzyme.

F (minimum) = (-1.68 1.68 -1.68) = 10.66
F (maximum) = (0.22 -0.1 0.45) = 16.39

The results from the model analysis of variance indicated that the relationship between the RS and the variables was significant, with a *p* value less than 0.1 and a satisfied correlation coefficient of 0.9 (Table 3). Results from the regression analysis showed that the RS was primarily affected by the amount of pullulanase and pH, followed by the reaction temperature. The negative coefficients for the interaction terms suggested that the RS was decreased at certain levels of pH and pullulanase contents and at certain temperature. These results indicated that there was an optimum value for the RS.

Effect of experimental factors on the yield of RS

(1) Optimization of pH

Reaction temperature and enzyme level were fixed at -1, 0, 1 level. Several pH conditions were applied to study the effect of pH on the content of RS (Fig. 1). Increasing the pH in the reaction resulted in an increase of the RS. However, at high levels of pH, the side reaction of pH became more significant. A decrease in the RS was observed. This trend was observed for all incubation pH. When pH was 5.0, the maximum of RS was 16.28%.

(2) Optimization of incubation temperature

Several temperatures were applied to study the effect of temperature on the content of RS (Fig. 2). The RS content of the debranched samples increased with increasing incubation temperature from 38° to 55°C. The increase in incubation temperature from 38° to 55°C had a considerable effect on the RS contents. Further increase in incubation temperature from 55° to 72°C reduced the formation of RS.

(3) Optimization of concentration of pullulanase

Different enzyme concentrations were applied to study the effect of enzyme concentration on the content of RS (Fig. 3). The addition of the higher amount of enzyme did not result in increase in RS yield, it was likely due to the abundance of short chains that were unable to form RS structure, thus the concentration (3.5-5.0 NPUN/g) was suitable for starch debranching in this work.

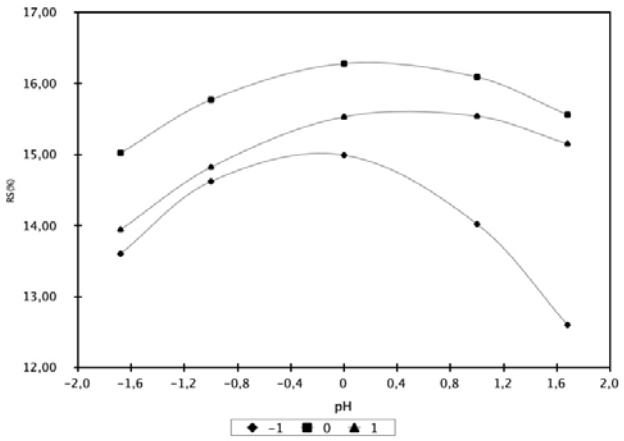


Fig. 1 - Response contour plot of RS *vs* pH.

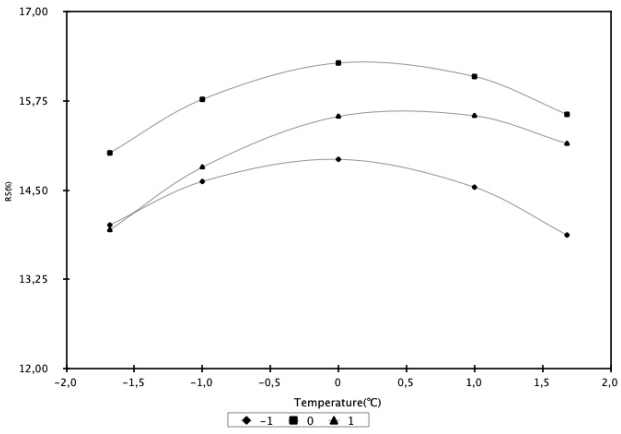


Fig. 2 - Response contour plot of RS *vs* temperature.

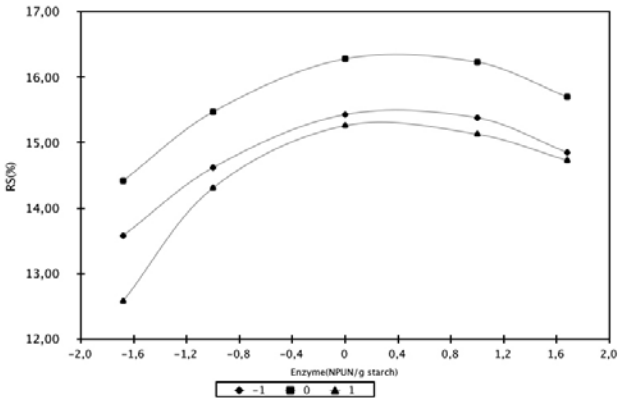


Fig. 3 - Response contour plot of RS *vs* pullulanase.

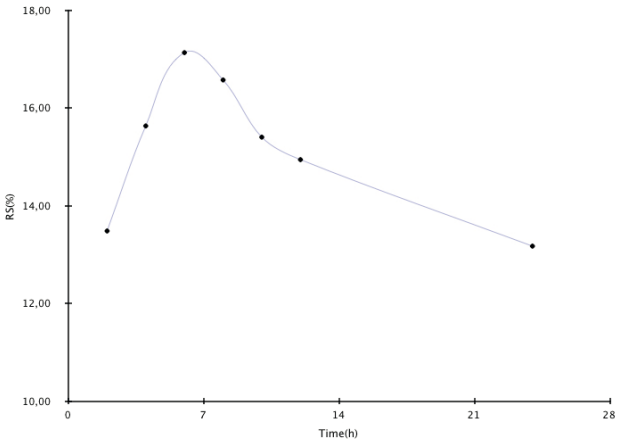


Fig. 4 - Influence of the reaction time of the pullulanase on the yield of RS.

Verification test

The optimum pullulanase debranching conditions were found as follows: Temperature 56°C; pH 5.0; the amount of pullulanase 3.9PUN/g dry starch. Under the optimum conditions, the experimental values of RS (16.73%) were found to be very close to the predicted values (16.39%).

Effect of the reaction time of the pullulanase on the RS yield

A gradual increase in the RS content of the corn starch samples was observed with increasing debranching time (Fig. 4). The corn starch had a very low RS content (0.95%) compared to the debranched samples. The RS content increased significantly from 0.95 to 17.13% after 6 h of debranching. The increment of RS content from 0 to 6 h indicated that the debranching process has released medium to small chains which were able to form RS. The lower RS obtained from 6 to 24 h debranched samples was likely due to the abundance of short chains that were unable to form RS structure. As reported by EERLINGEN *et al.* (1993), the yield of RS varied with average chain length of the amylose and the yield of RS formed in amylose with short chains can be low. After 6 h no significant increase in RS content was observed. Based on the result, a high level of debranching had occurred during the first 6 h of debranching. As can be seen in Fig. 4, the RS content after 6 h of debranching was 17.13% compared to 18.2% reported by ESCARPA *et al.* (1996). The results of GONZÁLEZ-SOTO *et al.* (2004) demonstrated that debranching of banana starch with pullulanase for 5 h was sufficient to obtain a high RS level (18%). These authors observed increased RS yields when the amylose content in the studied starches was higher. The differences in the results found in this study and those determined by other researchers might be due to the source of the starch and the conditions used for the RS preparation.

Synergistic action of two enzymes on the RS formation

Fig. 5 shows the yield of RS as a function of the DP of the amylose from which it was formed. The yield of RS varied with average chain length of the amylose. The DP of the commercial corn starch was 256. Under the experimental conditions, a maximum yield of 22.37% RS was obtained (DP 75). Amylose with shorter chains obviously gave lower yields, whereas the amount of RS obtained from amylose with higher DP was still lower.

Amylose with DP smaller than 75 contains a relatively high number of chains that do not have dimensions critical for incorporation in the crystal structure. It is likely that chains shorter than those of the crystalline zones will not take part very effectively in the crystallization process. It is reasonable to expect that the yields of RS formed in amylose with short chains can be low. On the other hand, amylose samples with high DP contain relatively high proportions of long chains. Such chains can easily interact simultaneously over different regions of the chain with other amylose molecules. The results sug-

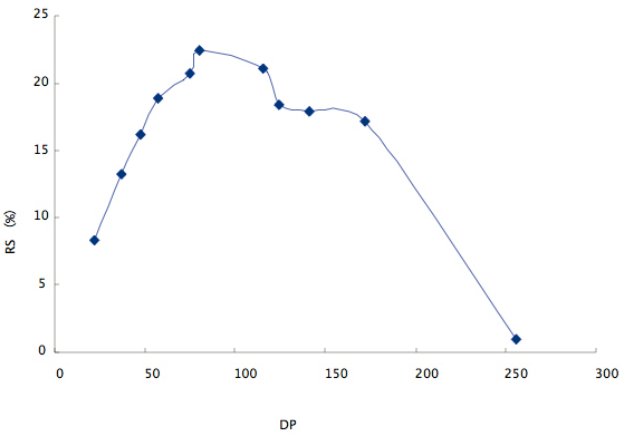


Fig. 5 - Relationship between DP of samples and RS.

gest that even the dimensions of the largest amylose molecules (DP 260) used in the study are sufficient to impair diffusion of the amylose molecules to the crystallization nuclei.

X-ray diffraction

The diffraction pattern obtained from native corn starch is shown in Fig. 6. This starch is classified as an A-type starch that was indicated by typical peaks at 15.1, 17.1 and 23.16° of diffraction angle 2θ. These values are in agreement with those reported for native cereal starch. When the starch was subjected to debranching and retrogradation treatments in this study, the RS samples showed completely different pattern from the native corn starch. As can be seen from Fig. 7, diffraction patterns of the treated samples differ from that of the corn starch, as the peak at 2θ = 22.5 appeared. Furthermore, it can be clearly seen that different incubation condition has led to the formation of different diffraction patterns. An additional conclusion that may be drawn from the Fig. 7 is that the enzymatic procedure affected the polymorph structure. One more observation that can be made is that the peak at 2θ = 22.5 looks sharper and more emphasized in the corn starch pattern compared to the treated sample pattern.

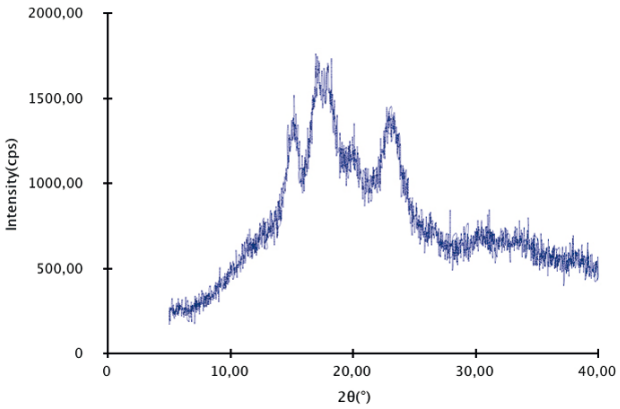


Fig. 6 - X-ray diffraction pattern of the raw corn starch (0.95% RS).

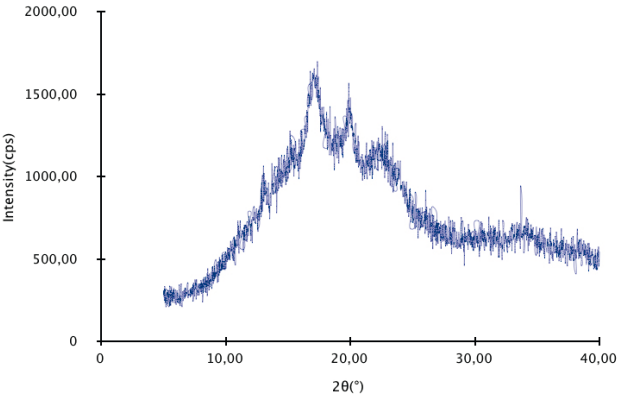


Fig. 7 - X-ray diffraction pattern of the corn starch treated with pullulanase and termostable α-amylase (22.37% RS).

Fig. 7 showed a strong diffraction peak at 2θ = 22.5 and a few small peaks at around 2θ values of 17° and 20°. This indicated that RS derived from corn starch that was highly degree of hydrolysis influenced the crystalline structure. This may be attributed to debranching and retrogradation which reorganized the structure of starch into a helical complex to that of B-type diffraction pattern. On the basis, it could be possible that the mechanism of cross-linking amylase was via junction zones of aggregated single helices.

DISCUSSION

In the present study, debranching using pullulanase was applied to produce a sample with linear, low molecular weight and recrystallizable polymer chains. This finding could be useful for developing an efficient process of RS production using this enzyme. Pullulanase rapidly hydrolyzed only the α-1, 6-glucosidic bonds, releasing a mixture of long and shorter unit chains from the parent amylopectin molecule. These fragments could rapidly produce retrograded starch by chain elongation and folding. This recrystallization could reduce digestibility of the starch (TOVAR *et al.*, 2002). These newly formed crystals were more perfect and firmer than the crystals of native starch granules and resist digestion in the small intestine. It has been suggested that crystalline polymorphs, accessibility of substrate to enzyme, fraction of crystalline structures and granule size (NODA *et al.*, 2005) influence the enzyme susceptibility for granular starches. When the equal amount of starch bulk is compared, total surface area of larger starch granules is smaller than that of smaller ones and this might reduce the opportunity for amylase to absorb. SRICHUWONG *et al.* (2005) showed that large granule size of potato and edible canna starches might partly account for their low digestibility. Among these factors, granular structure is considered to be the most important in defining the extent of enzymatic hydrolysis (ZHANG and OATES, 1999). SRICHUWONG *et al.* (2005) showed that pinholes were observed on the surfaces of some granules prepared from corn and rice at high magnification using scanning electron microscope, whereas the granules of the other plant species were smooth. It has been postulated that pinholes are the result of enzyme degradation during seed germination (JANE *et al.*, 2003). External chains of amylopectin chain length distribution to the enzyme digestibility of starches should be further discussed.

ESCARPA *et al.* (1996) reported the RS content ranged from 7.6% for waxy starch (0% amylose) to 36.4% for purified amylose. These Authors found that the RS yield increased with increasing amylose content in the starches under study.

MILASINOVIC *et al.* (2010) showed that the starch concentration (10 or 20%) did not significantly affect the RS yields. After three autoclaving-cooling cycles, the RS yields increased by 2.1% for both starch concentrations. The differences in the results found in this study and those determined by other researchers might be due to the source of the starch and the conditions applied for the RS preparation.

RS can result from a highly retrograded amylose fraction, the quantity formed being directly proportional to the amylose content of the starch (ANNISON and TOPPING, 1994). It has been shown that, after debranching of starch, the linear chains can contribute to a high RS content. In addition, partial acid hydrolysis and debranching of amylopectin are very effective in generating RS from various starches (BRUMOVSKY and THOMPSON, 2001). RS was initially supposed as a crystalline state of amylose double helices. Later, the non-crystalline part of retrograded starch was also found to be enzyme resistant (SIEVERT *et al.*, 1991). It is shown that, the amylose/amylopectin ratio in the native starch has some close relationship with RS content. Similarly, the crystals with different shape and property will be influenced by retrograded from the starch paste with the treatment of pullulanase. Thus, different pullulanase hydrolysis conditions exert different effect on amylose/amylopectin ratio, which will lead to the difference in RS content. But the RS contents did not always increase with the amylose content. More amylose chains are expected to facilitate recrystallization (LEONG *et al.*, 2007). GAO *et al.* (2011) reported that an increase in amylose content was observed from RE4.5 to RS14%, but a decrease was found from RS14 to RS19%. The RS contents did not always increase with the amylose content. More amylose chains are expected to facilitate recrystallization (LEONG *et al.*, 2007).

Cold storage seems to support an increase in the content of resistant starch during low temperature storage. MILASINOVIC *et al.* (2010) reported that the storage conditions (4° or -20°C) did not significantly affect the formation of RS. HASJIM and JANE (2005) also found that freezing at -20°C had no effect on the RS content of starch samples. MILASINOVIC *et al.* (2010) reported that native maize starch had a low RS content (0.6%) compared to the debranched samples. The RS content increased significantly from 0.6 to 25% after 11 h of debranching. After 24 h of debranching, the RS content slightly increased to 25.5%. The relationship between temperature of hydrolysis, pH, enzyme and amounts of RS formed could be interpreted in terms of crystallization in a crystalline polymer system. It is logical that the amount of RS formed depended on the overall crystallization, which is a function of nucleation and propagation. Both nucleation and crystal growth were affected by the degree of hydrolysis and DP. It has been showed that polymer chain length has a profound effect on such aggregation processes. The precipitate, the gel strength all depend on the amylose chain length.

PONGJANTA *et al.* (2009) found that the RS content in 48 h hydrolysis of HARS (High Amylose Rice Starch) preheated at 121°C was the highest (19.19%) among RS formation. As can be seen in Fig. 4, the RS content after 6 h of debranching was 17.13% compared to 18.2% reported by ESCARPA *et al.* (1996). GONZÁLEZ-SOTO *et al.* (2004) demonstrated that debranching of banana starch with pullulanase for 5 h was sufficient to obtain a high RS content of 18%. Different pullulanase hydrolysis conditions exert different effect on the amylose content, which will lead to the difference in RS content. As only a maximum of 22.37% of the amylose molecules became enzyme resistant, the question arose whether amylose molecules with similar chain length or a certain interval of chain lengths are responsible for the enzyme resistance. Under normal crystallization conditions, the complete alignment of the polymer chains is not possible for reasons of entropy. So the molecules are aligned only over relatively small regions of the chain. Micelles can be formed by aggregation of a number of different molecules over a particular region of the chain in an ordered structure interspersed with amorphous regions (LEVINE and SLADE, 1990). Formation of lamellar structures by chain folding is also a possibility. As far as we can see, micelle formation and chain folding both account for our results. In addition, resistant starch content in the samples was significantly different from the commercial high-maize resistant starch (48%) and the native corn starch. Overall comparison indicated that the pullulanase hydrolysis produced the higher RS content than the non debranched corn starch. These may be attributed to the formation of several linear glucan fractions from pullulanase hydrolysis of the corn starch that was recrystallization. The recrystallization of these linear starch fractions may permit a rearrangement of granules thereby preventing access to amylase digestion (ENGLYST *et al.*, 1992). It is supposed that the amorphous matrix of starch is readily exposed to the digestive enzymes whereas most of the crystallites reformed during retrogradation are embedded in the matrix (GRANFELDT *et al.*, 2000). SRICHUWONG *et al.* (2005) found that the hydrolysis degree of B-type starches did not significantly change with increased incubation time and only 2.5-7.2% of starch was hydrolyzed after 72 h. It is likely that the penetration of α-amylase at surface of starch granules is influenced by the chain length of amylopectin. GAO *et al.* (2011) Reported that higher RS content can be achieved when α-amylase was added to starch sample. The RS of α-amylase hydrolysis was increased by 1.2 to 6.5%. The action of α-amylase was to cleave the α-1, 4 glycosidic bonds of starch chains, thus lowing the viscosi-

ty of starch paste rapidly. If too much α-amylase was added, the viscosity of starch paste was too dilute. The probability for the linear chains to be close to each other became low. It is difficult to form RS. Not only the amylose content, but also the molecular weight of amylose is a key factor affecting crystallization. The amylose with lower molecular weight move more quickly than those with greater molecular weight, so the probability of collision is small and it is not easy to obtain RS.

Chain lengths of about DP 50 (RUSSELL *et al.*, 1989) and 65 (SILJESTROM *et al.*, 1989) have been found for RS formed in autoclaved wheat starch gels. RS formation, generally accepted to be amylose retrogradation, strongly depends on the experimental conditions used. Thus, the chain length of the retrograded amylose in the crystalline zones might be influenced by factors such as concentration, temperature. The existence of shorter chains, forming short or weak double helices, would produce inferior crystalline structures. Longer chains would strengthen hydrogen bonds between chains (JANE *et al.*, 1999). In our experiments, RS was formed under experimental conditions different from those applied by the cited authors. Thus, differences in the DP values can be rationalized by invoking differences in experimental conditions used for the RS formation and in hydrolytic enzymes employed for the isolation of the RS. Our results have shown that α-amylase treatment of corn starch resulted in a broadened chain length distribution from DP 22 to DP 141.

Crystalline nature of starches plays a dominant role in determining swelling and enzymatic behaviour of starches. These properties vary with different treatments. X-ray diffraction studies are important in understanding the digestion aspects of these starches. It is well-known that starches in their native state are semi-crystalline. Fig. 7 showed a B-type X-ray diffraction pattern. The patterns suggested that chain fragments, packed in a B-type crystalline structure with a slightly enlarged crystal lattice, contribute to formation of RS from amylose starch. Structures resistant to enzymatic hydrolysis differed in crystallinity depending on the treatment of amylose starch. Extent of digestibility is known to be related to crystalline polymorphic forms, and it is accepted that starch with A type X-ray diffraction is more susceptible to amylolysis than that with a B type pattern (JANE *et al.*, 1997). A-type starch is thought to be more densely packed in helical structures (WANG *et al.*, 1998). In addition, the amylopectin of A-type starch is thought to contain a higher number of chains per cluster than B-type starch (TAKEDA and HANASHIRO, 2003). RS formed in starch gel also shows a B-type diffraction pattern when it is formed at low temperatures (SIEVERT *et al.*, 1991; EERLINGEN *et al.*, 1993). GONZALEZ-SOTO *et al.* (2007) suggested that the RS developed peaks, due to the process of starch chain re-ordering or retrogradation. PONGJANTA *et al.* (2009) and LEONG *et al.* (2007) reported that, not only amylose chains were recrystallized, but also linear debranched amylopectin chains, which would help form RS crystalline structure in the period of heating and cooling. Differences in association between the chain fragments, presumably originating from amylose and varying proportions of interspersed, less-ordered segments, were assumed to account for these findings. Conditions applied for preparation of RS, such as type of enzyme used in the procedure, also affected thermal and crystallographic properties of RS (SIEVERT *et al.*, 1991). Recently, labeling techniques with a fluorophore have been introduced in the structural analysis of amylose and amylopectin (TAKEDA *et al.*, 2003). Chain-length distribution of labeled chains can be performed by the size-exclusion chromatographic method. The structural analysis of RS using fluorophore-assisted capillary electrophoresis will be reported in our future study.

CONCLUSIONS

RS was prepared from corn starch by the treatment of enzyme hydrolysis, autoclaving and retrogradation. It is very important to control the hydrolysis conditions during the course of preparation. The optimum conditions to obtain RS were as follows: pH, 5.0; temperature, 56°C; amount of pullulanase 3.9 NPUN/g, with reaction time of 6 h. After debranching of starch with pullulanase at 56°C, pH 5.0 and subsequent retrogradation at 4°C, RS yield was 17.13%. The RS formation from enzymatically-debranching of corn starch had higher resistant content than those of the native corn starch. The extent of resistant starch content was positively corrected with the hydrolysis by pullulanase and α-amylase. The highest RS yield in the starch samples was 22.37% after treatment with pullulanase and α-amylase. Under the experimental conditions, the yield of RS increased with DP to plateau values of 20-22.37% RS. The RS samples had B-type patterns and consisted of short chains (DP 75). The results of the presented investigation suggest that corn starches with high amylose content can be used for the development of an efficient process for RS production with pullulanase.

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PAPER

THE EFFECT OF CHIPS PROCESSING ON THE CONTENT OF TOXIC COMPOUNDS

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ABSTRACT

The aim of the experiment was focused on studying the content of glycoalkaloids, nitrates and acrylamide in raw materials, semi and final products coming directly from the processing line of fabricated chips in snacks' factory. On the ground of the results it was stated that there was found less glycoalkaloids and nitrates by ca. 50% in obtained dough after mixing of dry components and water. The quantity of nitrates in fried chips was lower by 84% comparing to the content of those substances in used raw materials, and glycoalkaloids by 89%. The process of fabricated chips affected on farther quantities of acrylamide formation. In raw materials the content of this toxic component was determined in ranges from 32 μg kg^{-1} (flakes) up to 65 μg kg^{-1} (granules) but chips contained ten times more acrylamide content, i.e. about 397 μg kg^{-1}.

- Keywords: fabricated chips, glycoalkaloids, nitrates, acrylamide -

INTRODUCTION

Chips made from potato dough owed their popularity to the large variety of flavors, crisp texture and attractive potato smell. They are especially popular among European and Asian consumers. In the USA the production of chips made from dehydrated potato products started in the 1970s (WEISS *et al.*, 1977); in 1997 "Pringles" chips made from potato dough accounted for 12% of the total sales of crisps in the Country. In Europe, the production of such chips began after their commercial success in the United States and it was based on the technology developed in the USA. Dehydrated cooked potatoes and water are mixed in suitable proportions to achieve about 40% moisture, then rolled, cut into various shapes and fried. This method of production allows a wide range of different forms and flavors of the final product.

The main component of crisps are dehydrated potatoes, influencing on the organoleptic characteristics of the finished crisps and the content of toxic and anti-nutritional components, related to their initial amount in the tubers. Some of the naturally occurring potato toxins are glycoalkaloids: α-chaconine and α-solanine, potent poisons (FRIEDMAN *et al.*, 1991; FRIEDMAN, 2006; DONALD, 2008; HAASE, 2010; LERI *et al.*, 2011) with toxicity similar to strychnine or arsenic (JECFA, 1992; HAASE, 2010); at 3-5 mg per kilogram of body weight they are lethal to humans. Potatoes used for direct consumption or processing in food industry should contain less than 10 mg glycoalkaloids per 100 g of potatoes (FRIEDMAN and MCDONALD, 1997; SPEIJERS, 1998; PĘKSA *et al.*, 2002; KNUTHSEN *et al.*, 2009). Importantly, processes used in the manufacture of dehydrated potato products, French fries or chips, do not remove all of the toxic compounds. According to the literature (RYTEL *et al.*, 2005; FRIEDMAN 2006; PĘKSA *et al.*, 2006; TAJNER-CZOPEK *et al.*, 2008), fried potato products still have about 10-20% of the initial content of glycoalkaloids.

Apart from glycoalkaloids, potatoes and potato products may contain nitrates, which are classified as anti-nutritional compounds. They are not toxic to humans, but intestinal microflora may reduce them to class 3 nitrites which can participate in the production of carcinogenic nitrosamines. The permissible amount of class 3 nitrates in potatoes should not exceed 200 mgNO_3^-/kg (HILL, 1999) and toxic nitrites should be below 5 mg $NaNO^3$ kg^{-1} (CIEŚLIK, 1995). Potato products usually contain about 20-30% of the initial amounts of these compounds in potatoes (CIEŚLIK, 1995; RYTEL *et al.*, 2005; PĘKSA *et al.*, 2006).

Dehydrated potato products also contain acrylamide, which may additionally be produced during frying Chipsletten. Acrylamide is mainly found in fried and baked high starch foods exposed to temperatures above 120°C (FRIEDMAN, 2003). In French fries and chips acrylamide content may vary from 30 to 2,300 μg kg^{-1} (KITA *et al.*, 2009). The production of acrylamide in the final product depends on the amount of reducing sugars and amino acid (asparagine) in the raw material, the temperature and duration of drying or frying (FRIEDMAN, 2003). A potential mechanism for the formation of acrylamide in foods is associated with the reaction between asparagine and reducing sugars during exposure of high starch products to high temperature (FRIEDMAN, 2003). This mechanism is accompanied by a Maillard reaction occurring between free amino groups and carbonyl groups of reducing sugars (glucose and fructose) during baking, frying or drying (FRIEDMAN, 2003).

The production process of Chipselleten may induce changes in the content of anti-nutritional and toxic compounds in the raw material or dehydrated potatoes, and also may increase the content of acrylamide in the finished product. For this reason it is important to determine technological factors that have an effect on the content of toxic compounds in the semi and final potato products.

The aim of the experiment was focused on determined the content of glycoalkaloids, nitrates and acrylamide in raw materials, semi and final products coming directly from the processing line of Chipselleten in the snacks' factory.

MATERIAL AND METHODS

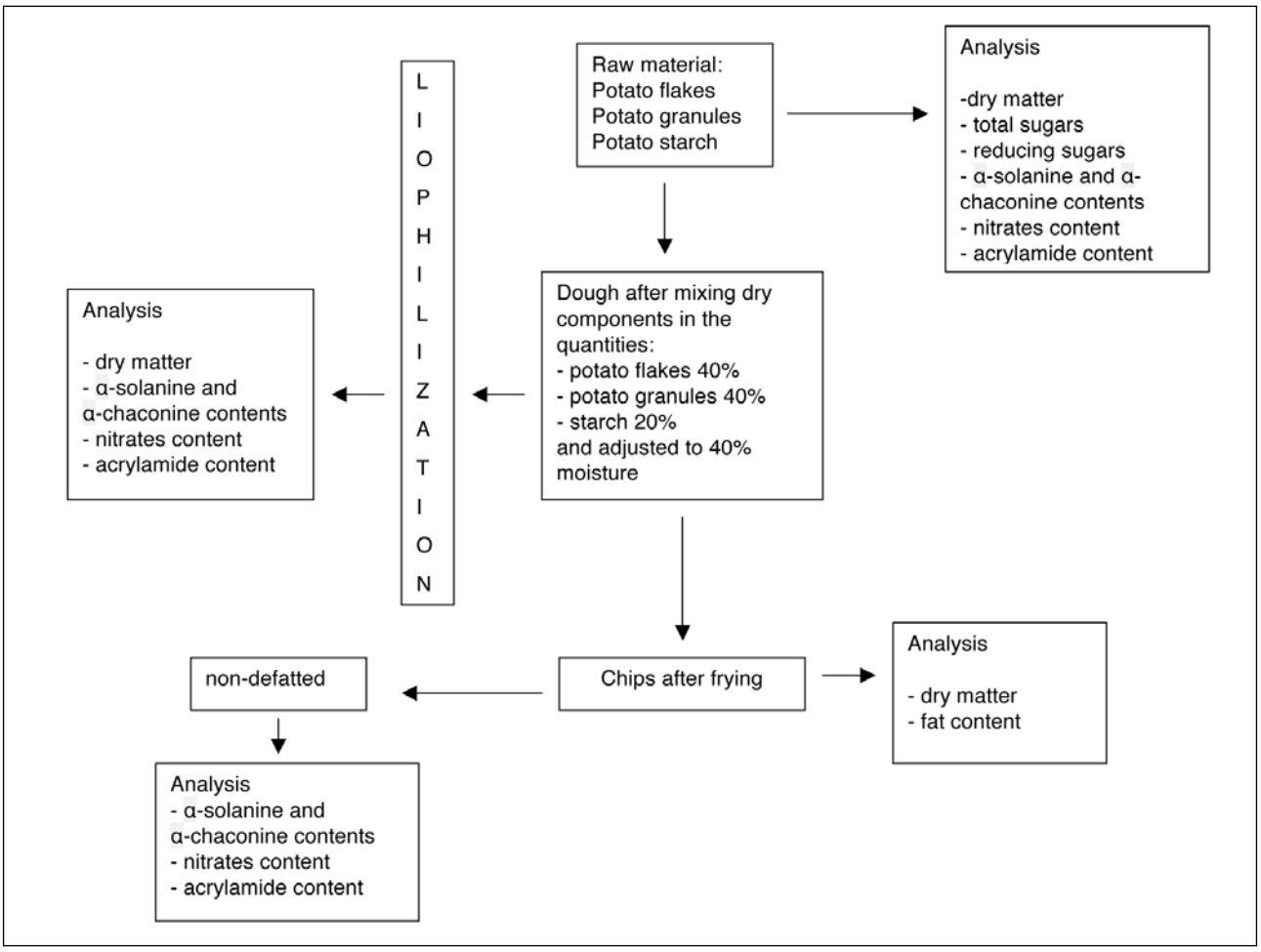
Material

Potato flakes, granules and starch were used in the experiment as the raw materials. Samples were taken directly from the processing line of formed potato chips. Samples were obtained from snacks factory three times yearly in the period of 2009-2010, from the following stages of the process: raw material (granules, flakes and modified potato starch) before dough preparation, formed dough pieces and ready-to-eat fried chips (before flavouring).

Potato samples preparation for the analysis

The content of dry matter, total sugars, reducing sugars, nitrates, glycoalkaloids (α-solanine i α-chaconine) and acrylamide were determined in raw material. The content of nitrates, glycoalkaloids (α-solanine i α-chaconine) and acrylamide were determined in freeze dried dough and ready fried non-defatted chips.

The scheme of analytical studies is presented below (Scheme 1).



Scheme 1 - The scheme of analytical studies.

Glycoalkaloids analysis

Apparatus

A high-pressure liquid chromatograph HPLC (pro Star) was used, made by Varian company (Walnut Creek, CA, USA), equipped with UV detector – 310 type, analytical column Mikrosorb NH2 (25x46 cm LD) (firm- Rainin Instrument, Woburn, Ma, USA) and a computer system monitoring the chromatograph (Varian Chromatography System).

Conditions of glycoalkaloids separation

As an eluent, a mixture of the tetrahydrofuran (firm Merck, Germany), acetonitrile, water 50:20:30 + KH_2PO_4 (1.02 g) per liter was used. The process was carried out at the temperature of 35°C, with the speed of flow of 2 $cm^3 min^{-1}$ and pressure of 11,3 MPa, applying the light wavelength of 208.

Sample preparation
for chromatographic analysis

1 g of potato flakes, granules, starch, freeze-dried dough and non-defatted chips were homogenized with 4 cm^3 of water and 30 cm^3 of methanol (by Labscan, Ireland) for 2 minutes, followed by filtration. The filtrate was brought to a final volume of 50 cm^3 with methanol. A 5 cm^3 aliquot of extract was cleaned up on the SPE column (Bond Elut C18; 500 mg; 6.0 mL firm Varian, USA). Glycoalkaloids were rinsed with methanol and evaporated to dryness in vacuo at the temperature of 50°C. The residue formed was dissolved in 1 cm^3 of THF:ACN:H_2O – 50:20:30. Before application into the column, the sample was cleand up by using filters of 0.45 μm. The volume of the injection was 10 μm. Standard solutions (1 mg/cm^3) were prepared by dissolving 10 mg of α-solanine and α-chaconine (Sigma) in 10 cm^3 of methanol; 10 μL, containing from 1 to 50 μg/cm^3 for both α-solanine and α-chaconine, were injected.

Acrylamide analysis

Reagents

All reagents were of analytical grade unless otherwise stated. Acrylamide (2-propene amide) and d_3-acrylamide (standard solution) were purchased from Fluka, Switzerland. Methanol HPLC-grade (methanol) was purchased from

Merck (Darmstadt, Germany), water Milli-Q (deionized water) from a Millipore purification system (Millipore, Bedford, MA, USA) and HPLC-grade acetonitrile from Lab-Scan (Ireland). Two Supelco SPE columns (Bellfonte PA, USA) were used for purification of the samples. The upper one (MCAX) of 300 mg and 3 mL in volume was placed directly on the lower one (DSC C-18 of 1 g and 6 mL in volume). Two columns were connected with each other by an adapter.

Standard solutions
and calibration standard solutions

Preparation and calibration of a standard solution were performed by modifying the method described by (ROŚEN and HELLENÄS, 2002; AURAND and TRINH, 2005). The stock solutions of acrylamide and d_3-acrylamide-standard solution as well as calibration standard were prepared in acetonitrile concentration of 500 mg×L^{-1} (500 ppm) (Fluka Co.). The standard solution was protected from light and stored in a refrigerator. The stock solutions of standards 500 mg×L^{-1} (500 ppm), were prepared in acetonitrile and refrigerated. A working standard solution, for spiking samples as well as for the standard curve, was obtained by dilution in acetonitrile. Concentrations for the standard curve were 0, 1, 5, 10, 30, 50, 100, 500, 1,000, 1,500 and 2,000 ng×mL^{-1}, all with acrylamide-d_3 at 50 ng×mL^{-1} for potato chips.

Apparatus

The acrylamide content of potato flakes, granules, starch, freeze-dried dough and non-defatted chips was obtained using LC-MS-MS 1200 L (Varian, Walnut Creek, CA, USA) performed using a triple quadruple with interfacial electrospray (ESI) a HPLC system Model a ProStar of a Varian, Walnut Creek, USA. HPLC-MS-MS 1200L system was equipped with one ProStar 210 Pump and ProStar 430 Autosampler. (Nitrogen generator, domnick hunter, model G 4510 E, UK). The samples were separated by the Pursuit XRs 3 u C-18 column (150 x 2.0 mm) – with a Metaguard Pursuit XRs 3 u C18 column (2.0 mm, Varian, Walnut Creek, CA, USA), using the mobile phase of 0.5% methanol in aqueous 0.1% acetic acid, at a flow rate of 0.2 mL·min^{-1}. The volume of each injected sample was 20 μL.

MS-MS condition
for the acrylamide determination

The LC-MS-MS was operated in the positive electrospray mode, needle voltage 5000V, nebulizing gas (compressed air or N_2) 54 psi, capillary voltage 25V, drying gas (N_2, 99.5%, 400°C, 22 psi). The collision cell gas (Ar 99.999%) pressure was 1.86 mTorr) and the detector voltage was set to 1,300 V. Acrylamide was determined by multiple reaction monitoring (MRM). The MRM mode was performed by monitoring the 72–54.9 *m/z* transition for acrylamide (collision energy-10V) and 75–58 *m/z* transition for acrylamide-d_3 (collision energy -10 V). In all the MRM transitions, the dwell and inter scan delay times were 1 sec, respectively, SIM width 0.7 atm. The methods detection of limit values were calculated for S/N = 3 for the summed signals of both fragment ions.

Sample preparation
for the LC-MS-MS analysis

The samples were prepared by modifying the methods described by (ROŚEN and HELLENÄS, 2002; AURAND and TRINH, 2005; PEDRESCHI *et al.*, 2007). Samples of potato flakes, granules, starch, freeze-dried dough and non-defatted chips weighed 2 g (0.0001 g). The samples (dough and chips) were ground using a GM 200 cutting mill (Retsch, GmbH, Germany) at a speed of 2,000 rev·min^{-1} for 16 seconds (2 x 16 seconds). The samples were next placed in 50 mL Falcon centrifuge tubes and added 40 mL of deionised water. Parallel, the samples containing 200 μg/L of internal standard (akrylamid-d_3), at a concentration of 10 μg·mL^{-1} were prepared. The samples were shaken using a Multi Reax shaker (Heidolph Instruments GmbH & Co.KG. Germany) for 10 minutes. Next, the samples were centrifuged in 3 KI 5 centrifuge (Sigma) at a speed of 9,500 rev·min^{-1} and 4°C for 15 minutes.

Solid phase extraction (SPE). Solid phase extraction (SPE) was performed by modifying the method described by AURAND and TRINH (2005) and KIM *et al.* (2007). 1 mL of transparent (clarified) solution (IA) was placed in SPA columns, which had been previously conditioned with 1 mL of methanol and 1 mL of deionized water, and vacuum-dried (Vac Elut 20 Manifold, Varian, Walut Creek, CA, USA). The samples in the columns were washed with 1 mL of deionized water. The upper SPE columns (MCAX) and the filtrate were removed. Acrylamide from the lower SPE columns (C-18) were eluted with 2 mL of methanol and the filtrate obtained was nitrogen-dried (at 30°C). The dry residue was dissolved in 500 μL (0.5 mL) of deionized water and placed in an autosampler using liquid chromatography-tandem mass spectrometry (LC-MS-MS). The final acrylamide content of the finished product was expressed in [μg·kg^1] = [ppb].

Analytical methods

The dry matter of potato flakes, granules, starch, dough, chips and freeze-dried dough was determined by the reduced weight after drying at 102°C until constant weight was achieved (AOAC, 1995). The content of total and reducing sugar were determined chemically. The nitrate content in potato granules, flakes, starch,

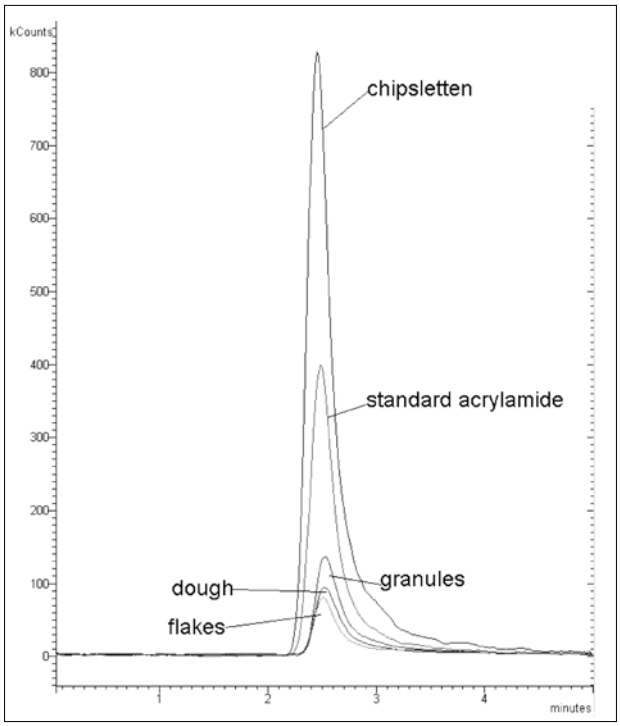


Fig. 1 - Chosen chromatograph of acrylamide standard, potato flakes, granules, dough and Chipsletten.

dough and chips was determined by the used the colorimetric method (RYTEL *et al.*, 2005). The quantities of α-solanine and α-chaconine were determined by the method of PĘKSA *et al.* (2002) and SAITO *et al.* (1990). All analyses were carried out in duplicate. Chosen chromatograph of acrylamide standard, potato flakes, granules, dough and Chipsletten is presented on Fig. 1.

Statistical analysis

The data obtained in the study were analyzed statistically using the Statistica 9.0 software package. For comparison, the results obtained were analyzed using multi-way analysis of variance with the application of Duncan's test ($P \leq 0.05$). The results presented in the tables and figures are the mean values of two-years experiments.

RESULTS AND DISCUSSION

Changes in the content of glycoalkaloids and nitrates

The process of manufacturing chips made from potato dough had an impact on the content of glycoalkaloids and nitrates in semi and final products (Table 1). Semi and final potato products contain the same as potatoes pro- and anti-nutritional ingredients but in different amounts, depending on the product and enrichment with other components, such as fat (French fries, chips). The high content of glycoalkaloids in potatoes results in a high amount of these compounds in potato products, as the production process often does not remove them completely. Therefore dehydrated potato products should be made from varieties with low susceptibility to glycoalkaloid synthesis.

In this study, the amount of glycoalkaloids in dehydrated potato products averaged 8.61 mg 100 g^{-1} (Table 1). A high content of these compounds was found in potato granules- 5.37 mg 100 g^{-1}, flakes contained 3.57 mg 100 g^{-1} while the potato starch did not contain any glycoalkaloids. According to the study of SMITH *et al.* (1996), the content of potato glycoalkaloids varies depending on the type of products, with more TGA found in potato flour derived from cooked potatoes (from 6.5 to 7.5 mg 100 g^{-1}) than in potato flakes from 1.5-2.3 mg 100 g^{-1}. Also MÄDER *et al.* (2009) observed a low amount of glycoalkaloids in potato flakes at 0.6 mg 100 g^{-1}, while FRIEDMAN and DAO (1992) noted a considerably higher level of these compounds in potato pancake powder at 4.5 mg 100 g^{-1} level. All the aforementioned differences in concentration of glycoalkaloids in dehydrated potato products depend not only on the type of product but also on treatments during manufacture. Many Authors (FRIEDMAN, 2006; RYTEL *et al.*, 2005; PĘKSA *et al.*, 2006) argue that most thermal treatments used in the industry and households have little effect on decreasing the amount of glycoalkaloids due to the compounds' high thermal stability and low solubility at the temperatures below 170°C (FRIEDMAN, 2006).

Table 1 - Characteristic of the compounds used for chipsletten production.

Compounds	Total sugers [%]	Reducing sugers [%]	Glycoalkaloids [mg 100 g^{-1}]	Nitrates [mg $NaNO_3 kg^{-1}$]	Acrylamide [μg kg^{-1}]
Granules	$0,37^{a}$±0,07	$0,14^{a}$±0,04	$5,37^{b}$±0.07	175^{a}±20,0	65^{a}±10,0
Flakes	$0,39^{a}$±0,04	$0,17^{a}$±0,05	$3,24^{a}$±0,10	163^{a}±10,0	32^{a}±10,0
Modified starch	nd	nd	nd	nd	nd

nd: non determined.
Values are given as mean ±SD, n=6.
^{a,b}: differences superscripts within columns indicate significant differences (Duncan test, p<0.05).

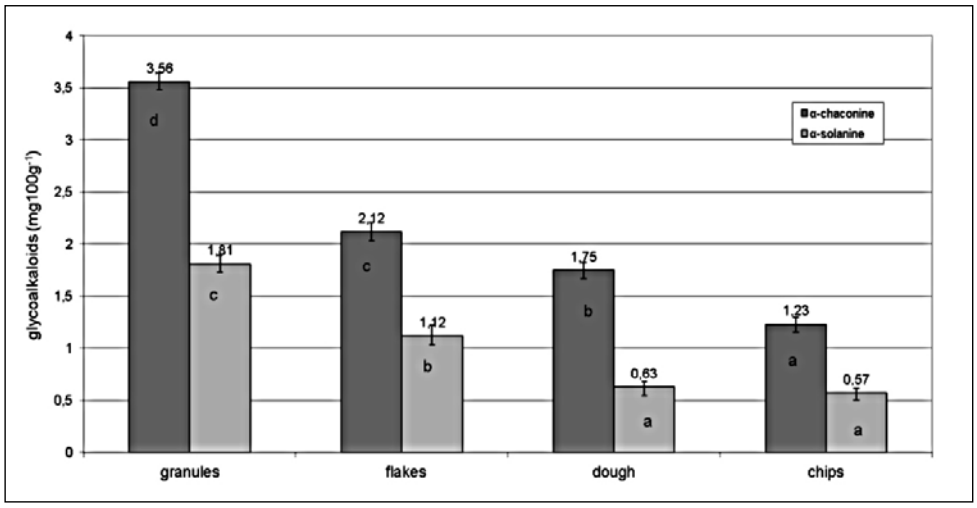


Fig. 2 - The content of total glycoalkaloids in row material, dough and Chipsletten.

Values are given as mean ±SD, n=6.
[a,b,c,d]: differences superscripts within columns indicate significant differences (Duncan test, p<0.05).

In this study, in the analyzed raw materials used in Chipsletten production, the highest nitrate concentration was observed in granules (175 mg $NaNO_3kg^{-1}$), then flakes (163 $NaNO_3kg^{-1}$), whereas in the starch nitrates it was not detected (Table 1). Technological procedures used during the production of dehydrated potato products do not remove the entire amount of nitrate contained in the potatoes, although many authors (CIEŚLIK, 1995; RYTEL *et al.*, 2005; PĘKSA *et al.*, 2006) report nitrites' good water solubility and low resistance to high temperatures used during thermal processes. The content in potatoes products (flakes, granules) during drying can only be decreased by very high temperatures, while the lack of leaching agents such as water may be the reason of a large amount of these compounds remain in the product (Table 1).

The analyzed potato dough taken from the Chipsletten technological line, a mixture of 40% flakes, 40% potato granules and 20% potato starch, contained about half as much glycoalkaloids and nitrates in comparison with raw materials (Figs. 2 and 3). The content of glycoalkaloids decreased from 5.37 (granules) and 3.24 (flakes) to 2.38 (dough) (Fig. 2), and nitrate content decreased from 175 (granules) and 163 (flakes) to 104 (dough) (Fig. 3). The decrease in the content of these compounds in the finished dough was due to the mixing of granules and flakes with potato starch which did not contain glycoalkaloids or nitrates. Furthermore, in order to obtain dough with an appropriate 40% moisture, the dry mixture was supplemented with large quantities of water which further reduced the concentration of these compounds. In the process of preparing dough from dried potato products, there is no factor that would result in leaching

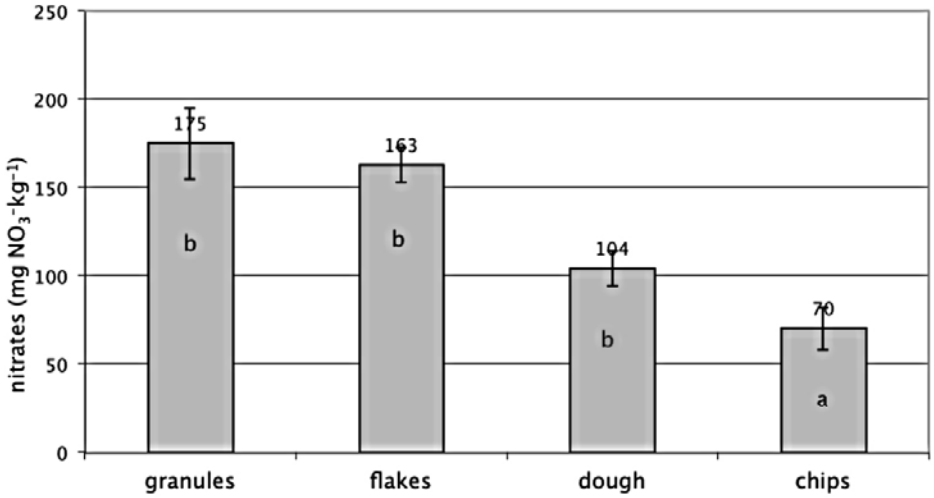


Fig. 3 - The content of nitrates in row material, dough and Chipsletten.

Values are given as mean ±SD, n=6.
[a,b]: differences superscripts within columns indicate significant differences (Duncan test, p<0.05).

or degradation of these compounds. The content of toxic compounds in the dough depends mainly on the recipe used by the plant.

The last stage was frying chips made of potato dough. Obtained chipsletten contained 35-37% fat. Ready-to-eat chips contained on average ca. 30% less chakonine (1,23 mg 100g^{-1} α-chaconine) and ca. 9% less solanine (0,57 mg 100 g^{-1} α-solanine) than potato dough (1,75 mg 100 g^{-1} α-chaconine and 0,63 mg 100 g^{-1} α-solanine) (Fig. 2). Process of frying chips didn't influence significanltly on the loss of solanine, despite the high temperature. From the studies presented by PĘKSA *et al.* (2006) it results that loss of α-solanine after frying potato chips are smaller than α-chakonine. Frying had a significant impact on lowering the amount of toxic compounds, including glycoalkaloids. The obtained chipsletten contained 1.8 mg 100 g^{-1} TGA, which represents 19% of the original content in raw material (Figs. 2 and 3). According to RYTEL *et al.* (2005) after the 2nd stage of frying, French fries contained below 10% of glycoalkaloids and chips contained less than 18% compared to raw unpeeled potatoes (PĘKSA *et al.*, 2006). In the process of frying potato chips or French fries, the high loss of toxic substances was due to the facilitated penetration of oil and high temperature (above 170°C). According to FRIEDMANN and MCDONALD (1997) during this process glycoalkaloids are removed from the material partly by leaching and partly through their degradation. Also the amount of nitrates reduced significantly after frying Chipsletten, to 16% of the initial content in dehydrated potatoes (Fig. 3). This is mainly due to their high solubility, especially at higher temperatures. According to PĘKSA *et al.* (2006) potato chips contain 18% of the original nitrate content in raw material; CIEŚLIK (1995) presents a higher level, at 20-30%.

Acrylamide

The amount of acrylamide increased during the production process of Chipsletten. The most significant factor that influenced on the amount of acrylamide in raw material, intermediates and crisps, was the content of reducing sugars. In this study the amount of reducing sugars in dehydrated potato samples was low, less than 0.2 g 100 g^{-1} (Table 1). The content of reducing sugars in dried potato depends on the initial amount in the potato tubers. Due to the well demonstrated high toxicity of acrylamide (FREDMAN, 2003; PEDRESCHI *et al.*, 2004), food manufacturers are trying to lower the reducing sugar content in potatoes by selection of varieties and appropriate storage conditions. The level of reducing sugars required by the producers of dehydrated potato and French fries is 0.3%, and during the production of chips even 0.2% (TAJNER-CZOPEK *et al.*, 2008; KITA *et al.*, 2009), which is beneficial not only for the colour of finished products but also due to the lower amount of acrylamide produced during frying.

Potato chips made of dough are prepared from intermediate products (granules, flakes), the quality of which should be subjected to thorough examination. In our experiment, potato granules contained 65 μg kg^{-1} acrylamide and flakes 32 μg kg^{-1} (Table 1). In the dough the amount of acrylamide decreased by about 40% as a result of adding water during this stage. After frying, the level of acrylamide in chips increased significantly and reached 397 μg kg^{-1} (Fig. 4). It was the result of frying in oil at the temperature of 185°C and the reaction of reducing sugars with asparagine.

The content of acrylamide in fried and dehydrated potato products may vary greatly; e.g. chips may contain from 50 to 1,900 μg kg^{-1} acr-

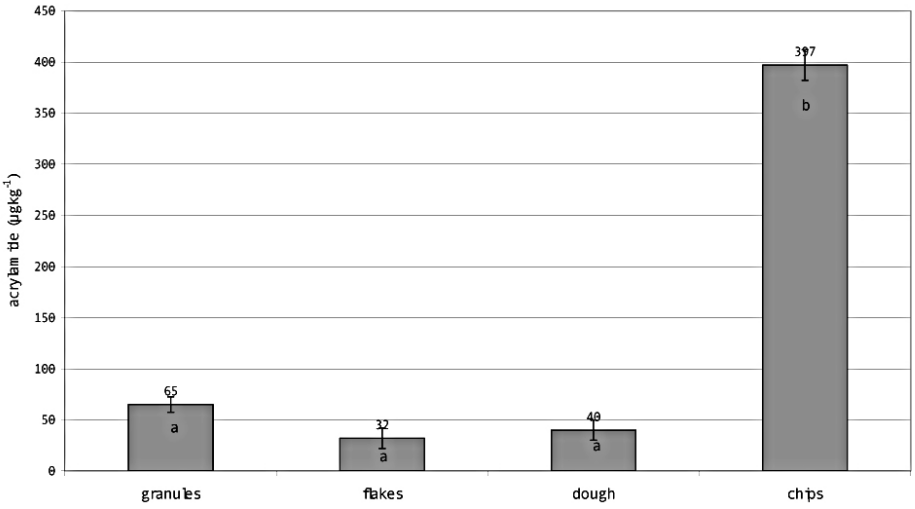


Fig. 4 - The content of akrylamide in row material, dough and Chipsletten.

Values are given as mean ±SD, n=6.
[a,b]: differences superscripts within columns indicate significant differences (Duncan test, p<0.05).

ylamide (PEDRESCHI *et al.*, 2004; TAJNER-CZOPEK *et al.,* 2008) and crisps may contain 170 to 3,700 μg kg^{-1} acrylamide (FRIEDMAN, 2003). Hence the necessity of efforts of scientists and food manufacturers to induce substantial reductions in the quantities of acrylamide formed in these products.

It should be noted that dehydrated potato products are often a semi product that is added to food which undergoes further thermal treatment (baking, frying), making it very important to analyze potato products with regard to the content of toxic and anti-nutritional compounds.

CONCLUSIONS

On the ground of the results obtained it was stated that there was found less glycoalkaloids and nitrates by ca. 50% in obtained dough after mixing of dry components and water. The quantity of nitrates in fried chips was lower by 84% comparing to the content of those substances in used raw materials, and glycoalkaloids by 89%. The process of fabricated chips affected on farther quantities of acrylamide formation. In raw materials the content of this toxic component was determined in ranges from 32 μg kg^{-1} (flakes) up to 65 μg kg^{-1} (granules) but chipsletten contained ten times more acrylamide content, i.e. about 397 μg kg^{-1}.

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SHORT COMMUNICATION

COMPARISON BETWEEN NERO D'AVOLA WINES PRODUCED WITH GRAPES GROWN IN SICILY AND TUSCANY

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ABSTRACT

Nero d'Avola is a red autochthonous cultivar from Sicily, a region of southern Italy. The aim of this study is to compare the quality of Nero d'Avola wines produced from vines grown in Sicily and Tuscany (central Italy) between 2003 and 2005. Chemical-physical parameters and polyphenol content were evaluated in the grapes and wines and sensory profiles for the wines were obtained. These results highlighted the strong performance of the Nero d'Avola cultivar outside its main production area, characterised by different climate conditions.

- Keywords: autochthonous vine, Nero d'Avola, phenolics, wine sensory profile -

INTRODUCTION

Nero d'Avola is a red autochthonous cultivar from Sicily, a region of southern Italy that is well known for producing the world-famous IGT (Typical Geographical Indication) Sicily wine. Nero d'Avola is used, together with other Sicilian or international cultivars, to produce high-quality red wines. The Nero d'Avola grape is an early ripening cultivar with considerable vegetative vigour. In recent years, studies into the influence of yeast on the composition of Nero d'Avola wines (CORONA, 2007) and the phenolic content of grapes and wines (CORONA *et al.*, 2004; CORONA and FINOLI, 2004; LOVINO *et al.,* 2006) have been undertaken. A study into the anthocyanin patterns of Sicilian wines (CASAVECCHIA *et al.,* 2007) showed that Nero d'Avola was distinct from other allochthonous varieties (Syrah, Merlot and Cabernet Sauvignon). Nero d'Avola grape quality depends on the irrigation strategy and canopy management practices, such as leaf removal (BARBAGALLO *et al.*, 2007).

Nero d'Avola has been studied in experimental vineyards to evaluate its adaptability to the different regions of Italy (CALÒ *et al.,* 2002; BUCELLI and STORCHI, 2007) and an earlier study, undertaken in Piedmont (northern Italy), reported that the Nero d'Avola cultivar was late ripening and produced a wine with a high degree of acidity (BONIFACINO *et al.,* 2004).

The aim of this study was to compare the quality of Nero d'Avola wines produced from grapes grown in Tuscany (Central Italy) and from their native area, Sicily.

MATERIALS AND METHODS

The grapes were grown in experimental vineyards in Tuscany (Montescudaio, Pisa) and Sicily (Biesina, Trapani). These vineyards belonged to a group of 24 vineyards located in 17 Italian regions and planted with local varieties as well as 20 varieties that were common to all 24 vineyards as described in CALÒ *et al.* (2002). The aim of that project was to assess the potential of introducing some allochthonous and autochthonous Italian varieties into regions other than their native areas and containing different "*terroir*".

The two vineyards chosen for this study were similar in age (1993), had the same training system (vertical trellis) and were at a similar altitude (80 m a.s.l). The planting material had the same origin and rootstock (SO4) and the planting system was similar. The wines were produced in duplicate under the same conditions in 2003, 2004 and 2005 in both areas. Grape samples (120 kg) were crushed and destemmed. SO_2 (60 mg kg^{-1}) was added and the mixture inoculated with a "pied de cuve" (starter Fermicru VR5, 0.25 g L^{-1}). Maceration lasted 8-10 days and, during this period, the cap was punched down twice a day. The free-run wine was inoculated with *Oenococcus oeni (Uvaferm alpha)* to induce malolactic fermentation. The wines were then decanted three or four times and another 30 mg L^{-1} of SO_2 was added. Following this, the wine was kept at -5°C for 10 days to allow tartaric stabilization. The level of SO_2 was then checked and the wine was filtered, using a cartridge line with decreasing porosity, and finally bottled in the spring of the year following the vintage (April - May). The chemical–physical and sensory analyses of the wines were undertaken during the summer following the vintage year. The chemical-physical analyses, consisting of alcohol, dry extract, total acidity (g L^{-1} tartaric acid), volatile acidity (g L^{-1} acetic acid) and pH analysis, were performed according to standard methods (G.U. C.E. n. 272 3/10/1990). Polyphenol indices (DI STEFANO *et al.*, 1989), colour intensity and hue were also measured. Tasting sessions took place in duplicate in an ISO tasting room (8589-2007) with ISO approved glasses (3591-1977). The wine sensory profiles were constructed by a trained panel from the CRA-ENO (14 tasters), following a procedure derived from the ISO standards (11035-1994). The wines were described using a predefined odour list (GUINARD and NOBLE, 1986) and included visual and gustative descriptions. The attributes were chosen if their identification frequency was greater than:

$$(n° \text{ assessors} \times n° \text{ wines}) / 2.$$

The third level olfactory descriptors (violet, raspberry, cherry, prunes, jam, just-cut grass, hay) were chosen if their identification frequency was higher than:

$$(n° \text{ assessors} \times n° \text{ wines}) / 4.$$

A tasting sheet was created in order to measure the intensity of each chosen descriptor using an unstructured intensity scale presented on a wheel.

A fixed model ANOVA (three-way) was performed on the raw data from each of the sensory descriptors evaluated by the panel in order to analyze: assessor, effects of vinification, replications of the tasting session, assessor × effects of vinification, assessor × replication and effects of vinification × replication. This was conducted in order to obtain information on panel performance, in terms of individual differences among assessors. Three-way ANOVA was performed on the entire data set according the following model: region, effects of vinification, vintage and the interactions among the main factors. Chemical data were also submitted to three-way ANOVA (effects of vinification, region, vintage and their interactions).

Table 1 - Chemical-physical properties of Nero d'Avola wines obtained from grapes grown in the two regions. (Means in the same row followed by a different letter are significantly different at $p \leq 0.05$ using Duncan's test).

	Tuscany		Sicily	
	average	s.d.	average	s.d.
Alcohol (% vol.)	13.60	0.88	13.63	0.58
pH	3.46	0.07	3.40	0.23
Total acidity (g L^{-1})	5.68	0.70	6.58	1.26
Colour intensity ($E_{420} + E_{520}$)	**0.654 b**	0.154	**0.576 a**	0.084
Colour hue (E_{420}/E_{520})	0.677	0.012	0.674	0.108
Total anthocyanins (mg L^{-1})	171	16	205	35
Total flavonoids (mg L^{-1})	1505	44	2240	70
Total phenols (mg L^{-1})	**1642 a**	29	**2173 b**	46
Proanthocyanidins (mg L^{-1})	**2392 a**	49	**3433 b**	104
Flavans reactive to vanilline (mg L^{-1})	829	15	1089	20

Table 2 - Chemical - physical properties of Nero d'Avola wines recorded over the three years for both regions. (Means in the same row followed by a different letter are significantly different at $p \leq 0.05$ using Duncan's test).

	2003		2004		2005	
	average	s.d.	average	s.d.	average	s.d.
Alcohol (% vol.)	13.79	1.16	13.49	0.55	13.56	0.68
pH	3.34	0.28	3.48	0.49	3.48	0.10
Total acidity (g L^{-1})	6.63	0.94	5.73	0.60	6.04	0.64
Colour intensity ($E_{420} + E_{520}$)	**0.517 a**	0.030	**0.591 b**	0.010	**0.738 c**	0.092
Colour hue (E_{420}/E_{520})	**0.617 a**	0.017	**0.736 b**	0.077	**0.673 ab**	0.081
Total anthocyanins (mg L^{-1})	178	23	160	3	227	21
Total flavonoids (mg L^{-1})	**2444 b**	59	**1322 a**	109	**1851 ab**	117
Total phenols (mg L^{-1})	**2304 c**	39	**1561 a**	77	**1858 b**	31
Proanthocyanidins (mg L^{-1})	**3468 b**	65	**2033 a**	1121	**3237 b**	115
Flavans reactive to vanilline (mg L^{-1})	1165	20	847	39	866	2

RESULTS AND DISCUSSION

No effects of the vinification and replications of the tasting session were found. Many physical-chemical parameters (alcohol, pH, total acidity, colour hue) were similar in Sicilian and Tuscan wines (Table 1). Colour intensity was significantly higher in the Tuscan products, whereas total phenols and proanthocyanidins were significantly higher in the Sicilian wines. Generally all the phenolic parameters were higher in the Sicilian wines, although for most of them (total anthocyanins, total flavonoids and flavans reactive to vanilline) the difference was not statistically significant.

There was a significant effect of vintage (Table 2) on colour intensity and hue; the lowest values were recorded in 2003, a year characterized by extreme summer temperatures. The only statistically significant interactions were "region × vintage" for the colour intensity and for the total anthocyanins content, which were significantly lower in the Sicilian wines in 2003. This could be explained by changes in the biosynthetic pathway leading to the synthesis of anthocyanins (DI STEFANO *et al.*, 2008; CHORTI *et al.*, 2010): excessive temperature levels or hydric stress in southern Italy may have caused the low anthocyanins content in the grapes.

The recorded phenolic content (Table 2) was lowest in 2004. However, specifically, only total flavonoids, total phenols and proanthocyanidins were significantly lower than in 2003 and 2005. This could be due to the unfavorable climatic conditions. In both vineyards, the highest rainfall and the lowest average temperatures, in the period after veraison, were recorded in 2004 (data not shown).

The sensory analysis confirmed the results of the chemical analysis. The wine sensory profiles (Fig. 1) were also similar. The Sicilian wines were statistically different only at the highest intensity of "raspberry" odour. A statistical difference in colour intensity (Table 1) was not perceived at a sensory level, possibly because the wines both had an intense ruby red colour (Fig. 1).

Vintage (Fig. 2) significantly influenced the sensory profiles, with the exception of "hay" odour and astringency. Generally, the 2003 wines showed significantly lower intensities for all the descriptors. The 2003 vintage was characterized by anomalous thermal events, which could explain the low intensity of the wine odour characteristics (TOMASI *et al.*, 2007).

In the 2004 wines, the sensory perception of acidity and astringency were lower (Fig. 2) according to the chemical data for total acidity and tannin parameters (proanthocyanidins and flavans reactive to vanilline) – reported in Tab. 2. However, only the acidity perception was statistically significantly different (Fig. 2).

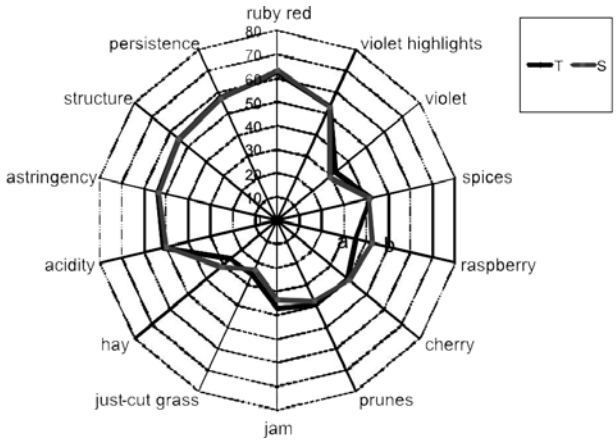


Fig. 1 - Nero d'Avola sensory profiles - Region Effect.
Three year average of the sensory profiles for the Tuscan and Sicilian wines.
(Descriptors followed by a different letter are significantly different at $p \leq 0.05$ using Duncan's test).

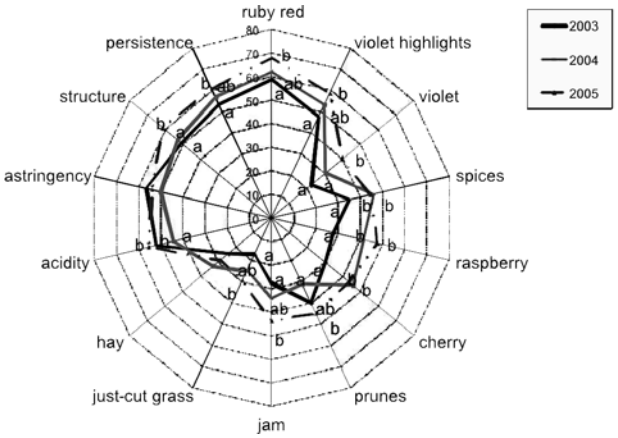


Fig. 2 - Nero d'Avola sensory profiles - Vintage Effect.
Average of the sensory profiles for the Tuscan and Sicilian wines in the three vintages.
(Descriptors followed by a different letter are significantly different at $p \leq 0.05$ using Duncan's test).

CONCLUSIONS

This 3-year survey showed the effects of various environmental conditions on the composition of Nero d'Avola wines. The analyses showed that the alcohol content and the polyphenolic compounds in the wines produced in central and southern Italy were similar. The Tuscan wine results confirmed previous results by BUCELLI and STORCHI (2007) and BORSA *et al.* (2007). However, although the environmental conditions were different in the two regions, the visual and olfactory descriptors were still the same for both the Sicilian and the Tuscan wines. These results suggest that Nero d'Avola could be a promising variety to grow outside Sicily.

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DURUM AND COMMON WHEAT IMPORTS INTO PUGLIA DURING 2010: MYCOTOXINS AND GRAIN-QUALITY MONITORING

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ABSTRACT

The Istituto Zooprofilattico of Puglia and Basilicata is an accredited analytical laboratory for major mycotoxins (Article 12, EC Regulation 882/2004). During 2010, analytical controls for mycotoxins were started on durum and common wheat imported through the Italian Sea, Air and Frontier Health Offices of Bari and Manfredonia. In the mycotoxins analysis, >80% of the tested samples were contaminated by detectable levels of the mycotoxins, with 5% with an aflatoxin B1 content over the EC Regulations limit, and 1.7% with a deoxynilvalenol content over the EC Regulations limit. These samples were also analysed for quality traits, which showed: 25% of all of the samples had test weights <80 kg; 4.2% had protein content <13%; 40% had gluten index <66; 30% had yellow index b* <24; about 50% had flour strength W between 100 and 200; and almost 40% had a tenacity to extensibility (P/L) ratio >1.5.

- Keywords: mycotoxins, aflatoxin B1, ochratoxin A, zearalenone, deoxynivalenol, durum wheat, common wheat, quality -

INTRODUCTION

In recent years, increasing attention has been paid to food safety, including that of cereals, which are one of the food matrices at highest risk of contamination by mycotoxins. According to data obtained from monitoring studies conducted worldwide and to results of toxicological studies, maximum limits for mycotoxin concentrations in cereals and cereal-based foods were established in the European Commission (EC) Regulations 1881/2006 and 1126/2007. For instance, for the mycotoxin deoxynivalenol, the tolerable daily intake is 1 μg/kg body weight (EC Scientific Committee for Food, 856/2005).

In human, mycotoxins have immunosuppressive, organotoxic, teratogenic and carcinogenic effects (PESTKA and SMOLINSKI, 2005; GALLO *et al.*, 2010). From the data provided by the Food and Agricultural Organisation of the United Nations, about 25% of crops around the world are contaminated (CALONI and NEBBIA, 2009).

Recent studies have shown a considerable increase in the levels of deoxynivalenol and its conjugate deoxynivalenol-3-glucoside in malt and beer that has occurred through unprocessed barley (KOSTELANSKA *et al.*, 2009). Baking processes generally reduce the levels of deoxynivalenol in bread (BRETZ *et al.*, 2006). However, recently studies have shown that when improver enzyme mixtures are used in bakeries as a dough ingredient, there can be increases of up to 145% of this deoxynivalenol-3-glucoside conjugated form in the fermented dough, and up to about a 120% increase in the bread (KOSTELANSKA *et al.*, 2011).

The Italian pasta-making industry produces pasta with durum wheat semolina that is obtained by blending the best quality national and foreign durum wheat semolina. The quality of pasta, as is currently understood, is determined mainly by the technological characteristics of the semolina, which must have adequate amounts of gluten and carotenoids to guarantee a product with excellent nutritional and cooking qualities. The quantity and quality of gluten are the basis in the making of pasta according to high organoleptic standards. The presence of an adequate amount of carotenoids is crucial for the colour of the pasta.

The aim of the present study was to report the results of a survey on safety and quality characteristics of common and durum wheat imported into Italy during 2010 and in the first two months of 2011.

MATERIALS AND METHODS

Sample collection and milling

The grain in the holds of ships or trucks was sampled by the technical staff of the Italian Sea, Air and Frontier Health Offices (*Uffici di Sanità Marittima, Aerea e di Frontiera*; USMAF) of Bari and Manfredonia, in accordance with the procedures established by EC Regulation 401/2006. The grain lots of around 1,500 tons were divided into sub-lots of 500 tons, which then form a number of incremental samples of about 100 tons, from which three laboratory samples of 10 kg are taken. Generally dynamic sampling was used, with the laboratory samples collected at different times from the moving grain on conveyor belts, or using automatic samplers. This procedure ensures more uniform distribution of mycotoxins in the samples, as these are generally shown a patchy distribution (CALONI and NEBBIA, 2009).

Extraction and quantification of mycotoxins

The procedures specified by manufacturers of the relevant immunoassay kits were used for the extraction of the mycotoxins from these wheat samples. According to the validity of tests defined by EC Decision 2002/657/EC and EC Reg. 401/2006, kits were used for the following mycotoxins: aflatoxin B1 (Tecna; code Ma314), deoxynivalenol (R-Biopharm; code 5906), ochratoxin A (R-Biopharm; code R1311), and zearalenone (R-Biopharm; code R1401).

The ELISA competitive enzyme immunoassays for quantitative analysis of these mycotoxins were developed according to the manufacturer instructions. The results of the analysis were obtained photometrically at a wavelength of 450 nm, using a microplate reader (Anthos HT-2). The concentrations given by the assays were then corrected by a factor that takes into account the recovery of the method for the sample preparation from the original cereal, as calculated during the validation. These enzyme immunoassay kits have been validated according to EC Decision 2002/657/EC, relating to the performance of analytical methods and the interpretation of the results. These kits are also periodically checked to ensure their correct operation by the Food Analysis Performance Assessment Scheme (FAPAS) and the Associazione Italiana Allevatori (AIA).

Grain quality

The protein content and test weights were determined directly on the clean grain samples using an Infratec grain analyser (Foss Electric; 1241).

Prior to milling, the clean grain of the durum wheat samples (800 g) were tempered to 16.5% humidity for 18 h; they were then milled with a laboratory mill that separates the semolina from the bran and shorts (Bona, Monza, Italy). The common wheat flour samples were obtained after grain samples had been moistened at 14% humidity for 18 h, and then also milled with a

laboratory mill that separates the flour from the bran and shorts (Brabender TYPE 27900 for common wheat; Belotti Strumenti S.r.l. Milan, Italy). The extraction rates for the grain samples ranged from 55 to 60% for both the semolina and the flour.

The dry gluten content (GC) and the gluten index (GI) were determined in triplicate on the semolina, according to the approved methods of the *Ente Italiano di Unificazione* (UNI) 10689 (UNI, 1997a) and 10690 (UNI, 1997b), respectively, using the Glutomatic 2200 system (Perten, Sweden). For determination of GC, the wet gluten was dried with a Glutorx at 150°C for 5 min, and left to cool for 3 min. The scores applied to the GI were: <30, weak gluten quality; 30-60, sufficient gluten quality; 60-80 very good gluten quality; >80 excellent gluten quality (approved method UNI 10940; UNI, 1997c).

The rheological characteristics of the semolina samples were determined as the deformation energy, or the flour strength, defined as the W index, and the ratio of resistance to extension or tenacity (P), to extensibility (L). These were evaluated using a Chopin alveograph (Chopin, Cedex France), according to the approved method UNI 10453 (UNI, 1995).

The yellow index (YI) was used to define the semolina colour, using a chromameter (ChromaMeter CR-200 Minolta, Tokyo, Japan) equipped with a xenon arc lamp. The chromameter was calibrated on a standard white tile of L* = 97.34, a* = -0.53 and b* = 2.39 (CIE 1976), with the International Commission on Illumination (CIE) illuminant D65. Each measure was performed in triplicate.

Statistical analysis

The parameters considered for mycotoxin validation according to EC Decision 2002/657/EC, were: specificity, ß-error checking, precision and robustness, using statistical tests for the calculation of variance (ANOVA test), Fisher's tests and Students' t-tests. All of the positive samples in the ELISA screening were then confirmed through a high performance liquid chromatography method, which showed superimposable analytical data (DE PACE *et al.*, 2009; DE PACE *et al.*, 2010). All of the qualitative data were replicated three times, and the mean values and standard deviation (±SD) are reported here.

RESULTS AND DISCUSSION

Mycotoxins contamination

Fig. 1 shows an overview of the continents of origin of the imported cereals. According to a survey conducted during 1998 by FARES *et al.* (1999), for the non-EU countries, Canada is the leading exporter of cereals to Italy, followed by eastern European countries, and finally Australia. Table 1 gives the levels of aflatoxin B1, deoxynivalenol, zearalenone and ochratoxin A found in the full 60 samples that were analysed,

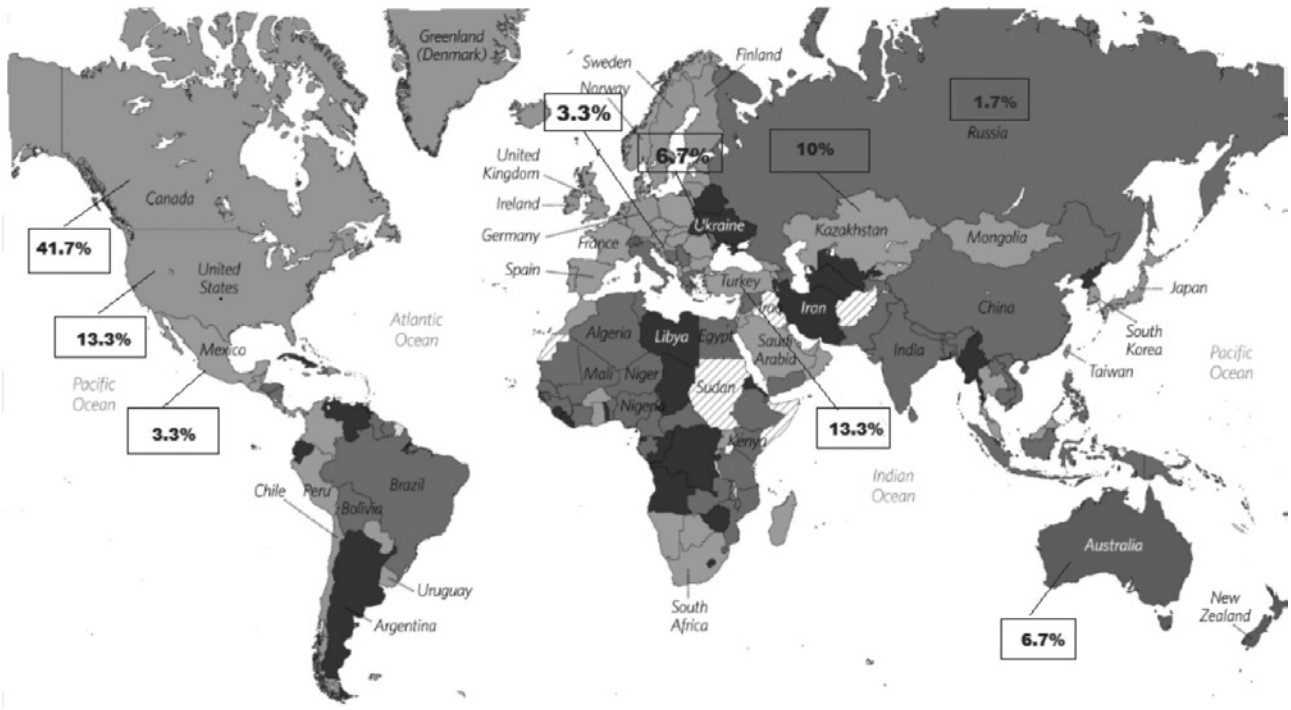


Fig. 1 - Countries of origin of the durum and common wheat samples (60 in total) collected for the present study by the USMAFF of Bari and Manfredonia from January 2010 to February 2011.

Table 1 - Countries of origin of the durum wheat samples, and their mycotoxin concentrations.

Country of origin	Mycotoxin levels			
	Aflatoxin B1 (µg/kg) (2)[a]	Deoxynivalenol (µg/kg) (1,750)[a]	Zearalenone (µg/kg) (100)[a]	Ochratoxin A (µg/kg) (5)[a]
Canada	<0.05[c]	<92.5[c]	<35[c]	<1.25[c]
Canada	<0.05[c]	<92.5[c]	<35[c]	1.73[d]
Canada	1.30[d]	<92.5[c]	<35[c]	<1.25[c]
Canada	0.80[d]	<92.5[c]	<35[c]	<1.25[c]
Canada	0.85[d]	<92.5[c]	<35[c]	<1.25[c]
Canada	0.88[d]	<92.5[c]	<35[c]	<1.25[c]
Canada	<0.05[c]	<92.5[c]	<35[c]	<1.25[c]
Canada	<0.05[c]	<92.5[c]	<35[c]	<1.25[c]
Canada	0.96[d]	<92.5[c]	<35[c]	<1.25[c]
Canada	1.14[d]	<92.5[c]	<35[c]	<1.25[c]
Canada	<0.05[c]	<92.5[c]	<35[c]	<1.25[c]
Canada	<0.05[c]	<92.5[c]	<35[c]	3.31[d]
Canada	<0.05[c]	<92.5[c]	<35[c]	<1.25[c]
Canada	<0.05[c]	200[d]	<35[c]	2.68[d]
Canada	1.10[d]	<92.5[c]	<35[c]	<1.25[c]
Canada	<0.05[c]	<92.5[c]	<35[c]	2.29[d]
Canada	<0.05[c]	<92.5[c]	<35[c]	<1.25[c]
Canada	<0.05[c]	<92.5[c]	<35[c]	<1.25[c]
Canada	<0.05[c]	970[d]	<35[c]	3.6[d]
Canada	<0.05[c]	1300[d]	<35[c]	<1.25[c]
Canada	1.40[d]	860[d]	<35[c]	<1.25[c]
Canada	0.66[d]	1240[d]	<35[c]	<1.25[c]
Canada	1.03[d]	160[d]	<35[c]	2.57[d]
Canada	0.76[d]	350[d]	<35[c]	2.14[d]
Canada	<0.05[c]	130[d]	<35[c]	<1.25[c]
Turkey	1.91[d]	<92.5[c]	<35[c]	<1.25[c]
Turkey	2.07	<92.5[c]	<35[c]	<1.25[c]
Turkey	1.62[d]	<92.5[c]	<35[c]	<1.25[c]
Turkey	1.83[d]	<92.5[c]	<35[c]	<1.25[c]
Turkey	1.62[d]	<92.5[c]	<35[c]	<1.25[c]
Turkey	1.74[d]	<92.5[c]	45[d]	<1.25[c]
Turkey	1.67[d]	<92.5[c]	<35[c]	<1.25[c]
Turkey	1.84[d]	<92.5[c]	<35[c]	<1.25[c]
USA	1.02[d]	<92.5[c]	<35[c]	<1.25[c]
USA	1.46[d]	875.3[d]	<35[c]	<1.25[c]
USA	1.89[d]	786.38[d]	<35[c]	<1.25[c]
USA	1.28[d]	731.13[d]	<35[c]	<1.25[c]
USA	0.66[d]	<92.5	<35[c]	<1.25[c]
USA	1.19[d]	693.24[d]	<35[c]	<1.25[c]
USA	0.98[d]	968.97[d]	<35[c]	<1.25[c]
USA	<0.05[c]	719.5[d]	<35[c]	<1.25[c]
Kazakhistan	1.46[d]	<92.5[c]	<35[c]	<1.25[c]
Kazakhistan	1.13[d]	<92.5[c]	<35[c]	<1.25[c]
Kazakhistan	1.39[d]	<92.5[c]	<35[c]	<1.25[c]
Kazakhistan	1.39[d]	<92.5[c]	<35[c]	<1.25[c]
Kazakhistan	<0.05[c]	<92.5[c]	<35[c]	<1.25[c]
Kazakhistan	<0.05[c]	<92.5[c]	<35[c]	1.71[d]
Australia	<0.05[c]	390[d]	<35[c]	<1.25[c]
Australia	<0.05[c]	<92.5[c]	<35[c]	<1.25[c]
Australia	<0.05[c]	150[d]	<35[c]	<1.25[c]
Australia	<0.05[c]	<92.5[c]	<35[c]	<1.25[c]
Moldova	1.93[d]	<92.5[c]	<35[c]	<1.25[c]
Moldova	1.93[d]	<92.5[c]	<35[c]	<1.25[c]
Moldova	2.29	<92.5[c]	<35[c]	<1.25[c]
Moldova	2.48	<92.5[c]	<35[c]	<1.25[c]
Croatia[b]	<0.05[c]	2,020	52.66[d]	<1.25[c]
Croatia[b]	<0.05[c]	1,370[d]	58.81[d]	<1.25[c]
Mexico	<0.05[c]	<92.5[c]	<35[c]	2.23[d]
Mexico	<0.05[c]	<92.5[c]	<35[c]	1.64[d]
Russian Fed.	1.20[d]	<92.5[c]	<35[c]	<1.25[c]
Minimum	0.05 µg/kg	<92.5 µg/kg	<35 µg/kg	<1.25 µg/kg
Maximum	2.48 µg/kg	2,020 µg/kg	52.66 µg/kg	3.60 µg/kg

[a]Limits according to EC Regulation 1126/2007.
[b]Common wheat samples.
[c]Below the given limit of detection of the method applied.
[d]Values of mycotoxins not considered as a risk for human health.

along with the limits according to EC Regulations 1881/2006 and 1127/2007 and the limits of the detection methods applied (<0.05, <92.5, <35, and <1.25 μg/kg, respectively). In all, 5.0% of these samples was over the EC Regulation limit for aflatoxin B1 (2 μg/kg); these were the durum wheat samples from Turkey (1 sample) and Moldova (2 samples) (Table 1, Fig. 2). Also, 1.7% of the samples was over the EC Regulation limit for deoxynivalenol (1,750 μg/kg), which was one of the common wheat samples from Croatia (Table 1, Fig. 2). Thus the remaining 93.3% of the samples was below the legal limits set by the EC Regulations 1881/2006 and 1127/2007 for all of these mycotoxins. Thus, although overall the assessments carried out showed that the 6.7% of the analysed samples was over the EC Regulations, it can also be noted that the remaining samples examined were not completely free from mycotoxins, according to the detection limits of the analyses. These lower levels should, however, not pose significant risk for human health in terms of studies on the tolerable daily intakes. As also indicated in Table 1, the maximum acceptable levels for these mycotoxins are: aflatoxin B1, 2.0 μg/kg (for all cereals); deoxynivalenol, 1,750 μg/kg (for unprocessed wheat); zearalenone, 100 μg/kg (for unprocessed wheat); and ochratoxin A, 5.0 μg/kg (for unprocessed cereals).

Weather conditions affect the fungal growth on plants. In North America, the weather was quite favourable to crop growth, while North-Eastern Europe was characterised by a climate with frequent rainfall and fluctuating temperatures that enhanced the Fusarium attack and resulted in yield reductions. Our data thus reflect the climatic conditions that occurred in the origin areas of the contaminated samples (Turkey, Moldova, Croatia). Indeed, AURELI *et al.* (2009) showed the importance of some of the factors that can influence deoxynivalenol contamination of crops on samples of Italian origin. The factors that were of particular significance were the year, the geographical distribution of the fields, and the varietal response. Also among these main factors, the climate is the only really imponderable factor, as its effects on the crops cannot be predicted, at least not through mathematical forecasting models.

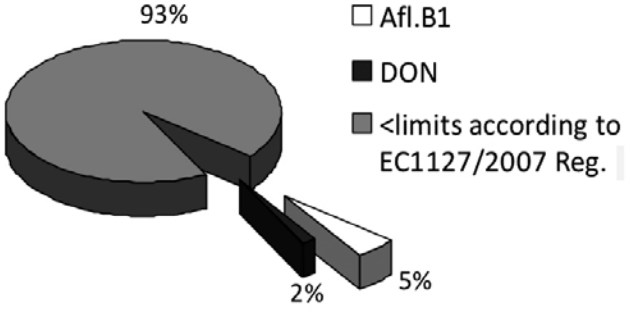


Fig. 2 - Relative levels (%) of infected samples across the full set of samples analysed in the present study.

The mycotoxin levels reported in the present study show lower concentrations with respect to those reported during 2010 in the Italian Network of Varietal Tests on Cereals (AURELI *et al.*, 2010). In this other investigation that was carried out in Italy, the authors found that for durum wheat samples, the average values of deoxynivalenol contamination were around 3,767, 1,874 and 115 ppb for Northern, Central and Southern Italy, respectively. The climatic conditions in Italy during 2010 were characterized by frequent rainfall and fluctuating temperatures, especially in the North.

The European Union (EU) legislation, as defined by the various regulations, provide a very comprehensive regulatory package. In the USA, the regulations on mycotoxins allow five-fold greater total aflatoxin concentrations in all foods, while for deoxynivalenol, the USA limits for grain and its derivatives is lower than in the EU. Canadian legislation allows the highest maximum for deoxynivalenol on common wheat (2,000 μg/kg).

In all, >80% of the samples analysed showed contamination by at least one of these mycotoxins, although these levels were not necessarily over those stated in the EC regulations. However, this does indicate that these mycotoxins must be checked with attention, particularly if these grains are intended for the whole-foods market. Indeed, as this market is also showing a significant increase, these cereals must be continually monitored, for the safety of the consumer.

Quality evaluation

Together with the colour, the protein and gluten contents represent the basic parameters of quality assessment of durum wheat (AUTRAN *et al.*, 1986). Table 2 shows the results of our analysis of the durum wheat samples under study, which also includes four common wheat samples from Croatia (3 samples) and Turkey (1 sample). The number of durum wheat samples (30) was lower than that of the samples analysed for mycotoxins (60), as several samples from the same vessel were combined. Fig. 3 shows an overview of the durum wheat grain quality classes for gluten index, protein content, test weight, alveographic indices (W and P/L), and yellow index.

The measure of test weight provides a good estimation of the milling yield and also indicates the 'history' of a crop; namely, it indicates whether the climate course was favourable for crop growing or not. This quality characteristic of the samples was good, and if we consider a test weight based on ≥80 kg, <25% of the samples (5 of 21 measured) was under 80 kg (Fig. 3).

The amount of protein in wheat is considered as a good indicator of the quality of the durum wheat pasta, and a content of ≥13% is generally accepted as a good threshold value for the pasta industry. The protein content in these durum wheat samples ranged from 10.4 to >15% (maximum, 16.7%), with only 1 sample (from Turkey; at 10.4% protein content) of the 24 measured (4.2%) below the accepted level of 13%. Therefore, according to the protein content and the test weighs of the durum wheat samples analysed, these durum wheat fall into a good class of quality, as indicated by the UNI 10709 rule.

Fares *et al.* (1999) reported that most durum wheat samples imported into Italy (about 70%) have a high protein content (>13%, with peaks of 16%). Indeed, these authors suggested that durum wheat imports with high protein content were necessary at that time, to compensate for the Italian national durum wheat production of 1998, which was characterized by an overall low protein content, of 12.8%.

The other tests that were performed on the durum wheat semolina confirmed the good characteristics found for these grains (Fig. 3). The gluten index is a measure of gluten strength and therefore provides a good estimation of protein

Table 2 - Quality traits of the durum wheat samples.

	Quality trait							
Country of origin	Moisture (%)	Protein content (% dm)	Gluten content (% dm)	Test weight (kg)	Gluten index	Yellow index (b*)	W (10^{-4} joule)	P/L
Canada	*n.e.*	*n.e.*	*n.e.*	*n.e.*	95±4	29.6±0.0	189	2.06
Canada	*n.e.*	*n.e.*	*n.e.*	*n.e.*	87±3	29.3±0.1	152	1.24
Canada	11.3	14.5±0.4	10.4±0.2	*n.e.*	75±4	29.8±0.1	144	0.97
Canada	11.9	14.2±0.5	10.1±0.1	*n.e.*	87±1	29.3±0.2	164	1.10
Canada	10.3	14.6±0.7	9.6±0.3	*n.e.*	71±5	25.2±0.0	168	1.62
Canada	11.7	15.0±0.4	10.8±0.1	86.5	76±2	28.6±0.1	156	1.03
Canada	11.9	14.8±0.1	10.8±0.1	86.3	58±2	29.3±0.0	184	1.04
Canada	11.6	14.9±0.2	10.8±0.2	85.6	69±1	29.9±0.2	152	1.03
Canada	10.9	14.5±0.2	9.8±0.3	85.6	68±2	24.5±0.1	165	1.15
Canada	9.9	14.7±0.7	9.5±0.4	86.1	51±3	29.3±0.1	208	2.06
Canada	11.9	14.9±0.3	11.1±0.1	84.7	60±2	29.2±0.0	195	0.96
Canada	12.5	14.8±0.6	11.0±0.1	80.7	49±3	27.7±0.0	188	0.71
Turkey	*n.e.*	*n.e.*	*n.e.*	*n.e.*	65±2	18.4±0.0	20	1.00
Turkey	*n.e.*	*n.e.*	*n.e.*	*n.e.*	20±1	18.0±0.1	29	0.78
Turkey[a]	*n.e.*	*n.e.*	*n.e.*	*n.e.*	98±2	11.8±0.0	264	1.43
Turkey	8.9	10.4±0.5	5.0±0.1	78.8	42±4	17.3±0.0	nd	nd
USA	9.1	14.3±09	9.4±0.2	84.8	98±3	28.6±0.2	224	3.07
USA	11.3	15.2±0.1	10.1±0.3	79.7	54±4	27.7±0.0	169	0.51
Kazakhistan	11.9	15.7±0.3	11.1±0.1	80.7	44±2	23.3±0.0	56	0.95
Kazakhistan	11.7	15.5±0.6	11.0±0.2	81.2	41±4	23.1±0.2	57	1.00
Kazakhistan	11.2	15.8±0.2	11.4±0.4	83.7	69±1	23.4±0.1	98	0.81
Kazakhistan	11.5	15.4±0.6	11.2±0.5	83.2	65±1	23.6±0.1	88	0.63
Kazakhistan	11.6	15.1±0.8	11.0±0.2	84.3	50±4	23.6±0.1	95	1.63
Kazakhistan	11.2	15.7±0.2	11.4±0.3	84.9	41±2	23.6±0.0	108	0.84
Australia	*n.e.*	*n.e.*	*n.e.*	*n.e.*	92±5	28.9±0.0	363	3.38
Australia	*n.e.*	*n.e.*	*n.e.*	*n.e.*	95±1	29.4±0.1	283	2.29
Australia	9.1	16.2±0.5	11.7±0.1	84.9	94±3	29.3±0.0	338	2.16
Australia	8.8	16.7±0.4	12.2±0.2	84.0	91±3	29.3±0.1	341	2.28
Moldova	13.0	14.4±0.6	9.1±0.2	76.6	99±5	25.7±0.0	150	1.87
Moldova	12.7	13.9±0.1	8.8±0.1	76.6	100±1	25.3±0.1	139	1.88
Moldova	12.8	14.0±0.7	8.8±0.2	76.6	100±4	25.5±0.0	292	1.45
Croatia[a]	13.1	13.1±0.3	7.54±0.1	*n.e.*	97±3	15.1±0.1	230	2.39
Croatia[a]	13.0	13.0±0.1	7.6±0.4	*n.e.*	97±1	14.8±0.2	209	2.81
Croatia[a]	*n.e.*	*n.e.*	*n.e.*	*n.e.*	96±1	13.3±0.0	236	3.69

W, deformation energy or flour strength; P, resistance to extension or tenacity; L, extensibility.
nd, not detectable; *n.e.*, not enough sample for analysis.
[a]Common wheat samples.

quality. Just <60% of the durum wheat samples had gluten index values >66 (maximum 100 here; Table 2), which includes both the good and excellent quality classes (Fig. 3). The colour (yellow index), as measured by b*, shows that >50% of these durum wheat produced semolina with an index >27, 16.7% between 24 and 27, with the remaining 30.0% at <24. The high b* values found in the semolina samples are significant for enhancing the yellow amber colour of the pasta products.

The characteristics assessed according to the alveograph indices show that just >50% of the durum wheat samples had flour strengths (W) between 100 and 200, while just <40% had a P/L ratio over 1.5; these results are in agreement with the data for the gluten index, and they confirm that the quality of the durum wheat semolina samples was suitable for high quality pasta production. The common wheat samples from Croatia (Table 2) were much too tenacious (P/L, >2.3) even if the values of W were >200. Comparing the present data to the survey conducted in 1988 (FARES *et al.*, 1999), the overall quality of the imported durum wheat has improved considerably, especially if we consider the yellow index, and also the quality of the gluten (Table 2, Fig. 3, b* and GI). Therefore the durum wheat imports have reached very high quality standards that are significantly better than those in the 1988 survey (FARES *et al.*, 1999). As seen for 1998 (FARES *et al.*, 1999), these imports can be considered as technical tools to improve the performance of the national durum wheat production.

CONCLUSIONS

The examinations that are carried out on imported products are closely related to economic aspects. In recent years, there has been a decrease in the quantity of grain entering the ports of Bari and Manfredonia, and consequently there has been a decrease in the number of samples analysed. In particular, the recent global economic crisis has had serious repercussions on these activities. The overall technological quality of imported cereals in the year 2010 and early 2011 was fair, although some samples were of very poor quality. This proportion of poor quality samples included the greatest number of positive reports for mycotoxins (6.7%). The remaining samples (>80%) were below the maximum mycotoxin levels allowed by EC Regulations 1881/2006 and 1127/2007, and although these

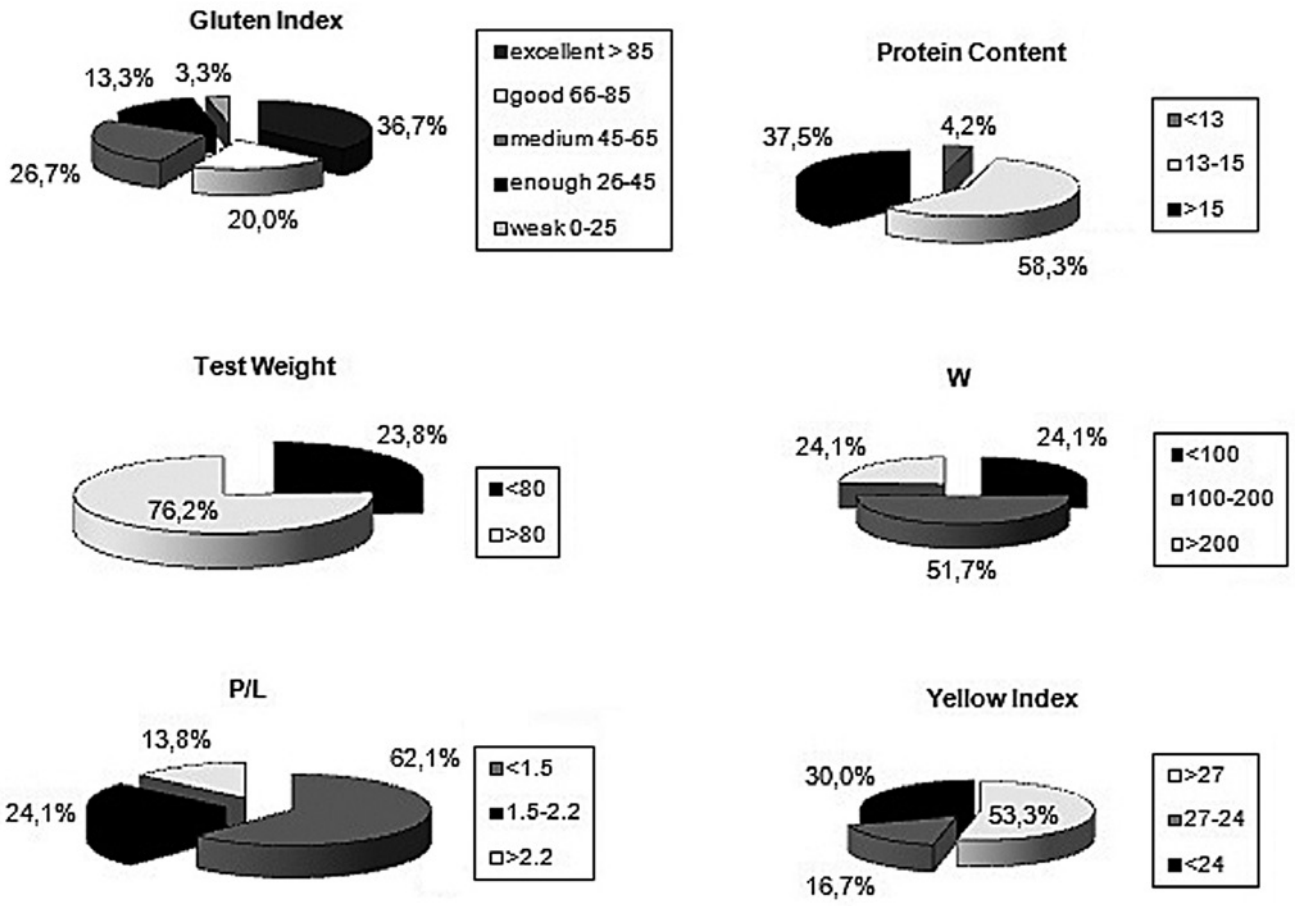


Fig. 3 - Relative quality evaluations (%) of the imported durum wheat analysed in the present study.

were not completely free from mycotoxins, they should not pose a risk for human health in reference to the studies on tolerable daily intakes. However we must consider that these grains are the raw materials, and so they undergo further physical treatments, such as pearling and milling, which can also promote reductions in the mycotoxin residues.

It is important to continue the surveillance activities always, to ensure the protection of consumer health against the risks that can derive from mycotoxin contamination. The data reported in the present study also show that durum wheat imports have reached relatively high quality standards, with the samples analysed in the present study significantly better than those analysed in a 1988 survey (FARES *et al.*, 1999).

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A SURVEY OF ERGOSTEROL CONTENT OF TOMATO PRODUCTS IN TURKEY DURING 2006-2010

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ABSTRACT

Ergosterol is an important constituent of fungal cell wall and measurement of it is a useful method to detect fungal activity, which correspond to mycotoxine production. Monitoring of ergosterol content in tomato products in Turkey has been carried out since 2006. The results of monitoring over the period 2006-2010 are presented. During this time 344 samples of six different tomato products (home-made tomato paste, commercial tomato paste, diced or crushed tomato, tomato juice, ketchup and tomato puree) were analysed for ergosterol using high-performance liquid chromatography. The detection limit of ergosterol was 0.1 mg/kg. Recovery experiments were carried out with spiked samples in the range 2-10 mg/kg of ergosterol. Average ergosterol contents were highest in home-made tomato paste (1,074 mg/kg), and lowest in tomato juice (4.5 mg/kg). Ergosterol exceeded the Turkish legal limit of 15 mg/kg in 65 of 88 commercial tomato paste samples; however all home-made tomato paste were >15 mg/kg. This paper reports data of the first survey for the presence of ergosterol in tomato products in Turkey.

- Keywords: ergosterol, home-made, HPLC, tomato products, Turkey -

INTRODUCTION

Tomato paste is an important tomato product consumed worldwide. Other important tomato products are peeled tomatoes, diced or crushed tomatoes, tomato juice, strained tomatoes, tomato sauces and ketchup (KADAKAL and ARTIK, 2004). Mould amount and colour are important quality parameters for tomato products. The presence of mold in processed foods represents unsanitary conditions of processing and/or unacceptable raw ingredient quality (COUSIN *et al.*, 1984). Ergosterol is an important constituent of fungal cell wall and used as an indicator of fungal growth, which correspond to mycotoxine production (SAXENA *et al.*, 2001). Mycotoxins can show carcinogenic, mutagenic, toxic, teratogenic or immunotoxic effects (MAYER *et al.*, 2008). Ergosterol is present in two forms, as free ergosterol and esterified ergosterol, and the relative abundances of free to esterified ergosterol are different among the various species (DE SIO *et al.*, 2000). However, because of the similarity between tomato tissue and cells with mould mycelium, it is quite difficult to determine the type and the amount of moulds in tomato products (BATTILANI *et al.*, 1996; KADAKAL and ARTIK, 2004). Fungal contamination of food, generally derived from poor manufacturing practices utilizing mouldy plant material, is usually determined by microbial counting techniques, such as the Howard mould count (HMC) method utilized for tomato products (DE SIO *et al.*, 2000). These methods are subjective and require special training and experienced microscopists. The poor precision of the "percentage of discarded fruits" and "HMC" methods have increased the importance of ergosterol for the microbiological quality evaluation of tomato and tomato products. Consequently, many papers have been published about the quantitative determination of ergosterol in tomato and tomato products with High-performance liquid chromatography (HPLC) (GRASELLI *et al.*, 1993; BERTONI *et al.*, 1994; BOCCHI *et al.*, 1995; GHIRETTI *et al.*, 1995).

When a fungal biomass cannot be separated from a solid substrate, growth of the fungus can be monitored by measuring the concentration of a chemical component. Ergosterol has not been widely used to monitor fungal growth, even though it is the predominant sterol component of most fungi and is either absent or a minor constituent in most higher plants. Ergosterol, a major fungal sterol, was reported as a relatively specific product of fungi with a straightforward method, suggesting ergosterol as a measure of fungal growth in plant materials (SEITZ *et al.*, 1979; KADAKAL *et al.*, 2005). In bacteria, ergosterol is present only in traces. The amount of sterols found in some bacteria are never higher than 0.01% of cell dry weight, and only a very small part of this percentage consists of ergosterol. Ergosterol is only a minor component of the sterol mixture of several plants and animals; therefore its occurrence, if any, in tomato products can be ascribed almost exclusively to the presence of moulds (GHIRETTI *et al.*, 1995). For that reason, ergosterol has been recently recognized as a potential objective parameter useful for the characterization of the quality of processed tomatoes (GRASELLI *et al.*, 1993). On the basis of the different specific biosyntheses and growth kinetics, ergosterol total production/day of mycelium growth was significantly higher in some of the most important tomato-contaminating moulds (*Rhizopus oryzae*, *Penicillium chrysogenum, Aspergillus oryzae, Mucor spinescens, Botrytis cinerea* and *Alternaria alternatae)* (GHIRETTI *et al.*, 1995) and reliably and rapidly determined by HPLC method (SEITZ *et al.*, 1979).

Several researchers have tried to ascertain whether there is any correlation between the HMC and ergosterol level or, at least, whether it is possible to find a correlation between ergosterol level and incidence of mould contamination of the raw material (GRASELLI *et al.*, 1993; GHIRETTI *et al.*, 1995; BERTONI *et al.*, 1996). In particular, GRASELLI *et al.*, (1993), when testing the possible application to tomato products of ergosterol quantitative analysis, observed that ergosterol determination was more effective than the Howard Mold Count (HMC) for classifying tomato products obtained from raw materials with different degrees of spoilage. However, some researchers have reported minimal or poor correlation between the total ergosterol level and HMC (GRASELLI *et al.*, 1993; BATTILANI *et al.*, 1996). In addition, a much higher correlation between the content of rotten tomatoes with total ergosterol was reported than with HMC (BERTONI *et al.*, 1994). With the current given knowledge, ergosterol has been recently recognized as a potential objective parameter useful for the characterization of the quality of processing tomatoes. Thus, a limiting value of 15 mg of total ergosterol/kg total solids has been approved as an index of acceptable quality for tomato products (BERTONI *et al.*, 1994; BOCCHI *et al.*, 1995; GHIRETTI *et al.*, 1995; DE SIO *et al.*, 2000; ANON, 2008). However, the importance of ergosterol for quality control of tomato and tomato products has not been fully exploited.

The purpose of the article is to present the results of the monitoring of ergosterol content in tomato products in Turkey over the period 2006-2010 and to compare them with the limiting value of 15 mg of total ergosterol/kg total solids, approved as an index of acceptable quality for tomato products.

MATERIAL AND METHODS

Material

In this study, a total of 344 samples were collected from September 2006 to December 2010 in Turkey. Commercial tomato paste (n = 88), diced or crushed tomato (n = 35), tomato juice (n = 30), ketchup (n = 77) and tomato puree samples (n = 34) were purchased from different markets and supermarkets all over Turkey. The samples were kept at 4°C until analysis. In addition, a total of 180 home-made tomato paste samples were purchased (minimum 2,000 g per sample) from villagers all over Turkey during the period 2006-2010. Home-made tomato paste samples were taken to the laboratory using cold-storage bags and homogenized by blending before analysis. Sampling program and provenance were given in Table 1.

METHODS

Determination of moisture

The moisture content of all samples was determined by vacuum oven method (AOAC, 1995). Samples (10 g) were weighed into aluminium weighing dishes (VWR, West Chester, PA, USA) and placed in a vacuum oven (EV 018 Model, NUVE Company, Ankara, Turkey) at 60°C until the weight of the sample reached a constant value.

Ergosterol determination

The HPLC method of SCHWADORF and MULLER (1989), modified by GHIRETTI *et al.* (1995), was used for the determination of ergosterol in the samples. Ergosterol in the crystalline form (analytical reagent grade) was obtained from Sigma (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany). Ten grams of sample were saponified with 75 mL of methanol, 50 mL of ethanol and 10 g of potassium hydroxide. The mixture was boiled for 1 h and the resultant reflux was filtered, followed by separation in a separatory funnel, with water and n-hexane (water/hexane; 1:2) and shaking for 1 min. After that, the lower layer was collected in an Erlenmeyer flask and the upper layer filtered over anhydrous sodium sulphate (Na_2SO_4). The lower layer was re-transferred to a separatory funnel and shaken for 2 min after adding 50 mL of n-hexane, followed by filtration over Na_2SO_4. The combined organic extracts were evaporated using a rotary evaporator to approximately 1 mL and transferred into a 10 mL test tube, and the contents of the tubes evaporated to dryness at 40°C (heating block) under a gentle stream of nitrogen. The residue was dissolved in 5 mL of n-hexane and a 20 μL portion of the solution was injected into the HPLC for analysis. The mobile phase was n-hexane and isoamyl alcohol (95/5; v/v) with a flow rate of 2 mL/min.

Apparatus for HPLC

For the analysis, a Nucleosil 100-7 C18 (250 mm X 4.6 ID mm) column, a photodiode array detector (Shimadzu, model SPD-M10 Avp) set at 282 nm, a LC-10AT-VP Shimadzu HPLC pump, a column oven (Shimadzu, CTO-10AS) set at 25°C and a software programme (Shimadzu) were used. The sample (20 μL) was injected with a syringe (Hamilton Co., Reno, NV, USA) into the HPLC system. Coefficient of determination (r^2) was 99.7% for ergosterol.

The detection limits (Signal to Noise Ratio, S/N= 3) (LI and CHEN, 2000) ranged from 0.1 to 0.5 mg/L.

Table 1 - The program of sampling of tomato products in the period 2006-2010.

	Year of sampling						
Sample	**2006**	**2007**	**2008**	**2009**	**2010**	**Total**	**Sample provenance in Turkey**
Tomato paste (home-made)	32	36	30	39	43	180	Denizli, Izmir, Manisa, Balıkesir, Gaziantep, Hatay, Bursa, Kahramanmaraş, Tokat
Tomato paste (commercial)	17	15	18	19	19	88	Denizli, Istanbul, Izmir, Ankara, Gaziantep, Hatay, Gümüşhane, Balıkesir, Bursa Manisa
Diced or crushed tomato	8	6	6	6	9	35	Denizli, Istanbul, Izmir, Ankara, Gaziantep, Hatay, Balıkesir, Bursa Manisa
Tomato juice	6	6	6	6	6	30	Denizli, Istanbul, Izmir, Ankara, Gaziantep, Hatay, Balıkesir, Bursa, Manisa
Ketchup	13	16	16	17	15	77	Denizli, Istanbul, Izmir, Ankara, Gaziantep, Hatay, Gümüşhane, Balıkesir, Bursa Manisa
Tomato puree	6	6	7	7	8	34	Denizli, Istanbul, Izmir, Ankara, Gaziantep, Hatay, Balıkesir, Bursa Manisa
Total	84	85	83	84	98	344	

Recovery of ergosterol

In the recovery experiment, samples of six different tomato products (home-made tomato paste, commercial tomato paste, diced or crushed tomato, tomato juice, ketchup and tomato puree), for which ergosterol concentrations were predetermined, were spiked with the different concentrations of ergosterol, using aliquots at 1, 10, 20, 50 and 100 mg/L, to determine the recovery of the extraction procedure in the initial step. Three determinations were carried out for each addition level.

Calculation method

The content of ergosterol was expressed in mg/kg of dry matter. The results of analyses were processed using Minitab 16 Statistical Software.

RESULTS AND DISCUSSION

Figure 1 illustrates the separation of ergosterol of home-made tomato paste sample by HPLC. The analytical method proved reliable, with a detection limit of 0.1 mg/kg for ergosterol. Ergosterol was baseline separated from other matrix components with good resolution. Identification of the compound was achieved by comparing its retention time and UV spectrum with the data obtained from the pure reference substance. Also, HPLC chromatogram of a tomato juice sample containing no ergosterol is given in Fig. 2. Moreover, expected retention time for ergosterol is shown in Fig. 2. As seen in Figure 2, ergosterol was not detected in tomato juice sample. The average percentage recovery of ergosterol was 97.16% (range 96.41-98.81%), for the five different added concentrations. The concentrations of ergosterol in tomato products were corrected for the average percentage recoveries.

Ergosterol contents of tomato products have been measured as total ergosterol, which includes free and esterified ergosterol. The ergosterol concentrations in home-made tomato paste, commercial tomato paste, diced or crushed tomato, tomato juice, ketchup and tomato puree were summarized in Table 2. During the period 2006-2010 there was a relatively high variability in ergosterol contents detected between and within the individual types of products (Table 2). The standard deviation for the home-made tomato paste has been determined larger (91.5) than that for other types of tomato products. Moreover, the least standard deviation value (1.7) has been obtained for the tomato juice. The ergosterol concentrations in commercial and home-made tomato paste samples ranged from 6.2 to 28.4, and 52.1 to 854 mg/kg, respectively. The highest average ergosterol contents were in home-made tomato paste samples (174 mg/kg), while in commercial tomato paste the ergosterol content was 13.4 mg/kg. A limiting value of 15 mg of total ergosterol/kg total solids is an official legal limit of acceptable quality for tomato products in Turkey (ANON, 2008). However, all 180 home-made tomato paste samples had ergosterol >15 mg/kg. From a total of 88 commercial tomato paste samples analysed, in 28 samples (or 32%) the ergosterol contents were >15 mg/kg. Five of those

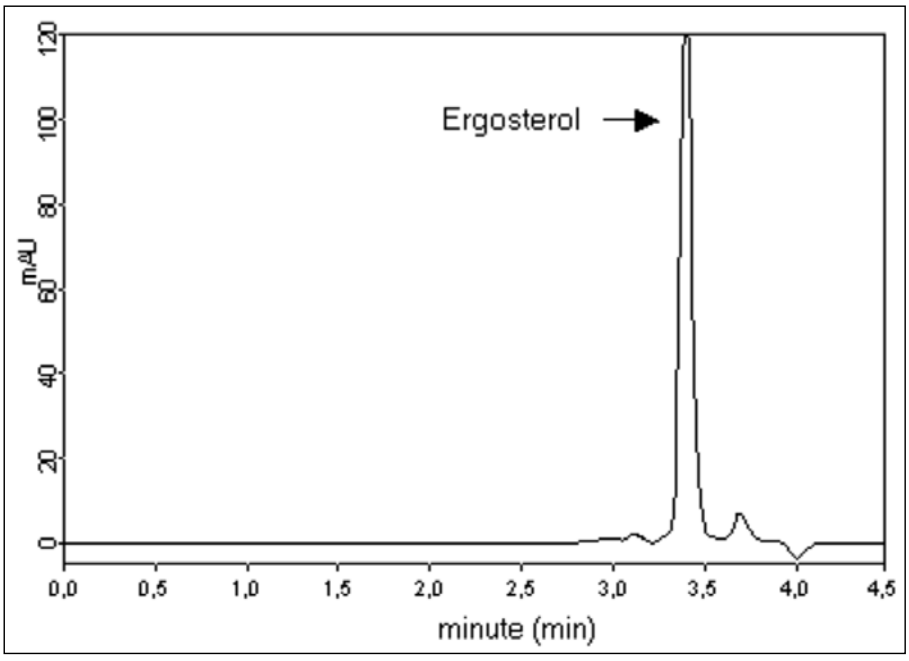


Fig. 1 - HPLC chromatogram of ergosterol for a sample of home-made tomato paste.

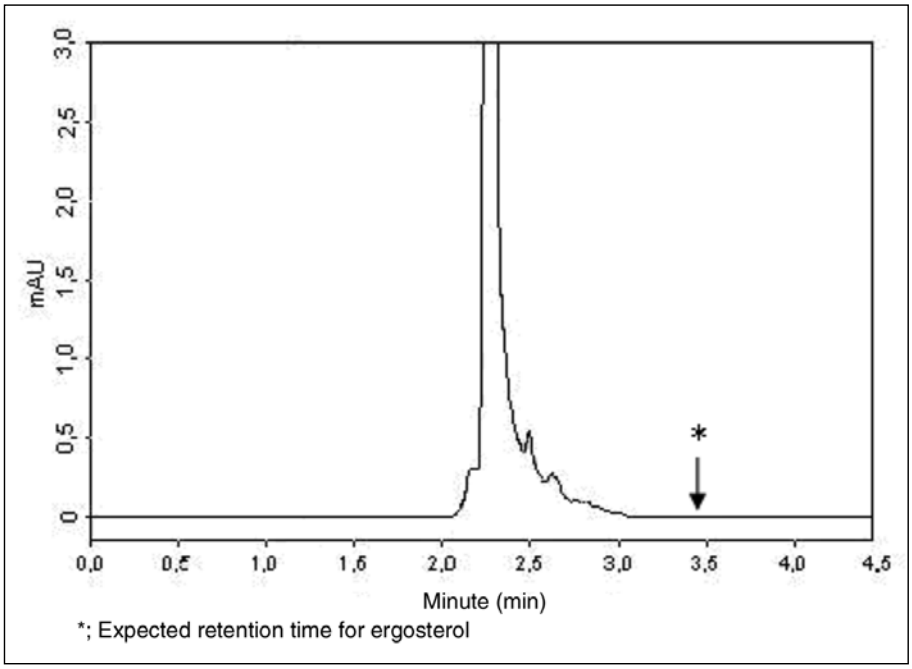


Fig. 2 - HPLC chromatogram of a tomato juice sample containing no ergosterol.

samples were analysed in 2006, three in 2007, seven in 2008, eight in 2009 and five in 2010. Home-made tomato paste is a sun dried (drying in shadow about 2 days) product. Therefore, the high ergosterol values in home-made tomato paste compared to commercial one may be attributable to the growing of mould in home-made tomato paste during the sun drying in shadow. It is well known that mould can propagate under sun drying conditions in shadow.

Of 35 diced or crushed tomato samples, seven samples were over the legal limit of 15 mg/kg. One of those samples was analysed in 2006, one in 2008 and five in 2010. However, of 30 tomato juice, 77 ketchup and 34 tomato puree samples, none were >15 mg/kg. The lowest average ergosterol contents were in tomato juice samples (4.5 mg /kg) while in ketchup the ergosterol content was 8.1 mg/kg. The results indicate that the Turkish tomato juice, ketchup and tomato puree investigated were likely safe for human consumption.

CONCLUSION

This is the only report known to the authors concerning the presence of ergosterol in tomato products. It is also the first time that tomato products were examined in Turkey for the presence of ergosterol. In addition, there are no published data available from other countries for comparison of results of ergosterol in Turkish tomato products. Higher levels of ergosterol were found in home-made tomato paste, and much lower amounts in tomato juice. There was

Table 2 - Ergosterol contents in tomato products.

Sample	Number of samples	Ergosterol concentrations (mg/kg dry weight)				Classes of ergosterol concentrations (%)			
		Min	Max	Mean	SD[a]	<5 I	5-15 II	15-30 III	>30 IV
Tomato paste (home-made)	180	52.1	854	174	91.5	-	-	-	100
Tomato paste (commercial)	88	6.2	28.4	13.4	8.6	-	68	32	-
Diced or crushed tomato	35	4.5	18.4	6.3	4.0	5	88	7	-
Tomato juice	30	1.8	6.7	4.5	1.7	81	19	-	-
Ketchup	77	4.1	12.8	8.1	3.2	6	94	-	-
Tomato puree	34	3.8	12.4	7.6	4.1	15	85	-	-

[a]SD = Standard Deviation.

a relatively high variability in ergosterol contents detected between and within particular tomato products. The results indicate that Turkish tomato products investigated are safe for human consumption, except for home-made tomato paste and some commercial tomato paste. In this study all home-made tomato paste samples and 28 of 88 commercial tomato paste samples exceeded the legal limit of 15 mg/kg.

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GUIDE FOR AUTHORS
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Acknowledgments. Acknowledgments of assistance are appropriate provided they are not related to analyses or other services performed for a fee. Financial support, thanks for assistance, artic1e number or thesis fulfilment may be included.

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