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PAPER

# AN INVESTIGATION OF THE ANTIOXIDANT ACTIVITIES AND SOME PHYSICOCHEMICAL CHARACTERISTICS OF STRAWBERRY ADDED YOGURT

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ABSTRACT

The effect of adding strawberry pulp to yogurt on its physicochemical properties, antioxidant activity and sensory characteristics was studied. For this purpose, experimental yogurts were produced by the addition of strawberry pulp (at 8, 12 and 16%) to plain yogurt and analyses were carried out through 14 days of cold storage. The titratable acidity and viscosity values of yogurts increased with the increasing of strawberry pulp concentration. Conversely, the total solid, fat, protein, ash, pH and syneresis values decreased. Antioxidant activity (β-caroten bleaching assay) and $IC_{50}$ (DPPH radical scavenging assay) in yogurts ranged from 77.71 to 86.12%, 205.70 to 2241.09 μg/mL during storage, respectively. For the sensory properties of yogurts, yogurt including 16% of strawberry pulp received the highest overall acceptability score on the first day of storage.

- Keywords: antioxidant activity, DPPH, Strawberry yogurt -

## INTRODUCTION

Yogurt, which is a type of fermented milk that is consumed all around the world, is a coagulated milk product obtained from lactic acid fermentation by the action of *Lactobacillus delbrueckii* spp. *bulgaricus* and *Streptococcus thermophilus* (LOURENS-HATTING and VILJOEN, 2001). Yogurt is one of the most frequently consumed dairy products in Turkey. In European countries, yogurt consumption per person each year is approximately 20kg, and this amount is reported as 36kg per person per year in Turkey (HUTKINS, 2006). Yogurt is also produced and consumed in flavoured and supplemented forms. In Turkey, the production and consumption of fruit-flavoured yogurt is lower than plain yogurt, although the mixture is widely made and consumed at home in most regions, especially during the winter months. The increasing annual consumption of yogurt in many countries has been attributed to the variety of fruit yogurts. Therefore, fruit-flavoured yogurts are produced by adding different fruit juices or pulps such as pear, apricot, peach, apple and plum. However, berry fruits such as bilberry, strawberry, raspberry, blackberry, blackcurrant and cherry are mainly used in fruit yogurt production. Many of these fruits are well known as being very good sources of anthocyanins (KONG *et al.*, 2003). These flavour ingredients lead to an increase in nutritional and therapeutic value, taste and product variability in the markets of milk products. However, the addition of flavour ingredients to the yogurt generally decreases product consistency.

Fruits and vegetables contain significant levels of biologically active components that possess health benefits beyond basic nutrients (OOMAH and MAZZA, 2000). Therefore, the consumption of foods containing these compounds as part of a diet may also be beneficial to human health. Strawberries are one of the richest sources of natural antioxidants among fruits (WANG and ZHENG, 2001). In addition to the usual nutrients such as vitamins and minerals, strawberries are also rich in β-carotene and phenolic compounds (OSZMİANSKI and WOJDYLO, 2009). Strawberries have a high scavenging capacity against peroxyl radicals (ROO*), superoxide radicals ($O_2$*-), hydrogen peroxide ($H_2O_2$), hydroxyl radical (OH*) and singlet oxygen ($^1O2$) (WANG and ZHENG 2001).

Strawberry [*Fragaria x ananassa* (Rosaceae Family)] production of Turkey in 2007 is 239.076 tons (TUİK 2008). Strawberries are widely consumed, both as fresh fruit and as an ingredient in processed products (jam, marmalade, juice, cake, milk, etc.) in Turkey. There have been a number of studies on the physiochemical, rheological, microbiological and sensory properties of fruit yogurts (OZTURK and ONER, 1999; KUCUKONER and TARAKCI, 2003; TARAKCI and KUCUKONER, 2003; CELIK *et al.*, 2006; TARAKCI, 2010). There is little information regarding the effect on the properties of yogurt produced by adding strawberry as an ingredient to yogurt. However, data on antioxidant activity of fruit added yogurt is relatively scarce in the scientific literature. A few studies have been documented on the total antioxidant activity of yogurts containing fruit (JIMÉNEA *et al.*, 2008; ZAINOLDIN and BABA, 2010).

The main purpose of the present study was to determine the antioxidant activity of yogurt extracts using the two commonly used spectrophotometric methods: DPPH (2,2-diphenyl-1- picrylhydrazyl) free radical-scavenging assay and BCB (β-carotene bleaching) assay. Furthermore, the other aim of this study was to evaluate the physico-chemical and sensory properties during storage for 14 days.

## MATERIALS AND METHODS

### Materials

Cows' milk was supplied from the dairy farm of Ataturk University located in the Erzurum province of Turkey. Direct-to-vat system yogurt starter culture (*S. thermophilus* and *Lb. bulgaricus*) was used in yogurt-manufacturing supplied from Chr. Hansen-Peyma, Istanbul, Turkey. Strawberries were hand-harvested at the mature stage from orchards in the Department of Horticulture, Faculty of Agriculture, Ataturk University, Turkey. Samples were immediately transported to the laboratory in cold chain and processed on the same day.

### Preparation of strawberry pulp

The fruit was washed under a tap by following the separation of the stalks, leaves and decayed berries. The washed fruit was placed in a boiler and 200 mL of water was added per kg of fruit. The fruit was preheated at 65°±2°C, and then strawberry pulp was obtained after filtration of the crushed fruit. The pulp was pasteurized at 90°±1°C for 5 minutes and hot filled into cleaned glass jars (KOKOSMANLI and KELES, 2000). The pulp (dry matter 11.25%, ash 0.57%, fat 0.12%, protein 0.17% and $IC_{50}$ 31.84 μg⁄mL) was stored at refrigerator temperature until use in yogurt production.

### Yogurt production

Raw cows' milk (milk fat 3.5%, protein 2.87%, dry matter 11.32%, pH 6.55 and titratable acidity 0.20%) was used for yogurt production. Skimmed milk powder (20g/L) and starch (1%) was added to increase the total solid. Milk was heated to 90°C for 10 minutes and then cooled to 42°C. The milk was then inoculated with DVS starter culture (20 g 100 $L^{-1}$) containing *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*. The inoc-

ulated milk was incubated at 44°±1°C until the pH reached 4.6 and then the yogurt was stored in a refrigerator (4°±1°C) for 24 hours. One batch of yogurt was then taken as the control and the remaining batches were prepared with one of the mixtures of $S_{8\%}$ [8% (w/w) strawberry pulp and 8% (w/w) sugar], $S_{12\%}$ [12% (w/w) strawberry pulp and 8% (w/w) sugar], $S_{16\%}$ [16% (w/w) strawberry pulp and 8% (w/w) sugar], respectively. The addition of strawberry pulp was made per 100 g yogurt. All experimental yogurts were divided into approximately 200g parts, stored at 4°±1°C for 14 days and analysed on the1st, 7th and 14th day of cold storage. The experimental yogurts were manufactured in duplicate.

**Chemical analyses**

Dry matter, fat, ash, titratable acidity (METIN, 2008) and protein contents (IDF 1993) of milk and yogurt samples were determined. Dry matter, fat, ash and protein contents (CEMEROGLU, 2010) of strawberry pulp were determined. The pH was measured with a pH meter (Crison, pH meter, Basic 20+) fitted with a combined glass electrode.

**Apparent viscosity**

The apparent viscosities of yogurt samples were measured during storage using Visco Star-L Fungilab viscometer equipped with a spindle (No: 6) and operated at a speed of 20rpm. The readings were taken by instrument directly at the point of the 30th second and were recorded in centipoises. All of the measurements were performed at a temperature of 4°±1°C in duplicate (ABRAHAMSEN and HOLMAN, 1980).

**Syneresis**

The yogurt samples were analysed for syneresis throughout storage according to the method described by ATAMER and SEZGIN (1986). Twenty-five grams of yogurt samples were weighed and filtered. After 120 minutes of drainage at 4°±1°C, the amount of collected whey (mL) in a flask was recorded and expressed as an index of syneresis.

**Preparation of the extracts for antioxidant activity**

10 mg of yogurt samples and strawberry pulp were extracted for 2 hours with 10 mL of water at room temperature on an orbital shaker set at 600rpm. The mixture was centrifuged at 6,000 rpm for 20 minutes, and the supernatant was collected. The suspension was filtered through Whatman No. l filter paper and stored at -20°C until analyses. The filtrate was further used as a stock solution for the analysis of antioxidant activity.

**Antioxidant activity**

*β-Carotene Bleaching (BCB) assay*

The antioxidant activity of yogurt and strawberry pulp extracts was determined according to the β-carotene bleaching method according to the procedure reported by KAUR and KAPOOR (2002) with certain modifications. Also the detailed explanation of method applied in fruit yogurt has been previously described by SENGUL *et al.* (2012)

*2,2-diphenyl- 1-picrylhydrazyl (DPPH*) radical scavenging assay*

For determining the free radical scavenging activity, the extracts were allowed to react with a stable free radical, 2.2-diphenyl-1-picryl hydrazyl radical (DPPH*) (GULCIN, 2010). In the assay, 0.1mM solution of DPPH was prepared in ethanol and 0.5 mL of this solution was added to 1.5 mL of extract solution in ethanol at different concentrations (20-100 μg/mL). These mixtures were incubated at 25°C for 30 minutes in the dark. The absorbance was measured at 517 nm against blank samples. The amount of concentration of sample required to scavenge 50% DPPH ($IC_{50}$) was calculated. The DPPH scavenging capacity was expressed as mM in the reaction medium and calculated from the calibration curve determined by linear regression.

DPPH scavenging effect (%) = [1-(As/Ac)]×100

Where $A_c$ is the absorbance at 517nm of the control reaction (containing DPPH solution without fruit extract), and $A_s$ is the absorbance of the test sample.

**Preliminary sensory acceptance**

The yogurt samples were assessed organoleptically at 1 and 14 days of storage by a panel of eight laboratory staff members (5 male and 3 female, 25-45 years old), well experienced and familiar with fruit added yogurt. Coded yogurt samples were left at room temperature for 10-15 minutes and were served with a glass of water a slice of bread for the panellists to cleanse their palates between samples. The yogurts were graded for six sensory attributes including colour and appearance, odour, texture, acidity, flavour and general acceptability of samples were scored on a point scale of 1 (poor) to 9 (excellent). The yogurt samples were evaluated according to the method of BODYFELT *et al.* (1988), and the sensory criteria were modified considering the characteristics of fruit yogurt.

**Statistical analysis**

The data obtained was analysed statistically using SPSS statistical software program version 13

Table 1 - Gross chemical composition of yogurt samples.

| Yogurt Samples | Dry Matter (%) | Ash (%) | Fat (%) | Protein (%) |
|---|---|---|---|---|
| $S_8$ | 19.12±0.01[c] | 0.69±0.01[a] | 2.90±0.14[b] | 2.76±0.02[b] |
| $S_{12}$ | 18.99±0.04[b] | 0.67±0.02[a] | 2.50±0.14[a] | 2.72±0.01[b] |
| $S_{16}$ | 18.69±0.01[a] | 0.64±0.02[a] | 2.45±0.07[a] | 2.62±0.01[a] |

Mean values followed by different letters in the same column are significantly different ($P$<0.05)
$S_8$: [8% (w/w) strawberry and 8% (w/w) sugar], $S_{12}$: [12% (w/w) strawberry and 8% (w/w) sugar], $S_{16}$: [16% (w/w) strawberry and 8% (w/w) sugar].

Table 2 - Changes in the physicochemical properties of yogurt samples during storage.

| Parameters | Storage time (day) | Yogurt samples | | |
|---|---|---|---|---|
| | | $S_8$ | $S_{12}$ | $S_{16}$ |
| Titratable acidity(%) | 1 | 0.83±0.01 [aA] | 0.87±0.01 [aAB] | 0.89±0.01 [aB] |
| | 7 | 0.86±0.01 [abA] | 0.88±0.01 [aA] | 0.90±0.01 [aA] |
| | 14 | 0.89±0.01 [bA] | 0.91±0.03 [aA] | 0.96±0.01 [bB] |
| pH | 1 | 4.39±0.01 [cC] | 4.31±0.03 [cB] | 4.16±0.04 [bA] |
| | 7 | 4.29±0.01 [bC] | 4.23±0.01 [bB] | 4.09±0.02 [bA] |
| | 14 | 4.21±0.01 [aC] | 4.16±0.01 [aB] | 4.00±0.01 [aA] |
| Syneresis (mL/25g) | 1 | 2.25±0.49 [aA] | 1.90±0.42 [aA] | 1.78±0.24 [aA] |
| | 7 | 3.63±0.04 [bB] | 3.43±0.04 [bB] | 2.65±0.21 [abA] |
| | 14 | 4.38±0.24 [bB] | 3.63±0.04 [bAB] | 3.15±0.35 [bA] |
| Viscosity (cP) | 1 | 2690±57 [bA] | 2620±57 [aA] | 3220±57 [aB] |
| | 7 | 2310±198 [abA] | 2375±120 [aA] | 2745±417 [aA] |
| | 14 | 2030±128 [aA] | 2335±233 [aA] | 2420±212[aA] |

[a–c]Means in the same column followed by different lowercase letters represent significant differences ($P$<0.05).
[A–C]Means in the same line followed by different uppercase letters represent significant differences ($P$<0.05)
C:$S_8$: [8% (w/w) strawberry and 8% (w/w) sugar], $S_{12}$: [12% (w/w) strawberry and 8% (w/w) sugar], $S_{16}$: [16% (w/w) strawberry and 8% (w/w) sugar].

(SPSS Inc., Chicago, IL, USA) Analysis of variance (ANOVA), and Duncan's Multiple Range Test was used to determine differences between results.

## RESULTS AND DISCUSSION

### Gross chemical composition

Some physical and chemical properties of strawberry added yogurt samples were analysed on the first day of storage and the results are presented in Table 1. The dry matter, ash, fat and protein content of yogurts ranged from 18.69 to 19.12%, 0.64 to 0.69%, 2.45 to 2.90% and 2.62 to 2.76%, respectively. There were significant differences in the dry matter, ash, fat and protein content of yogurts (P<0.05). These results were different than those reported by other researchers for fruit added yogurts (KUCUKONER and TARAKCI, 2003; AYAR *et al.*, 2006; BAKIRCI and KAVAZ, 2008; TARAKCI, 2010; SENGUL *et al.*, 2012). These differences may be derived from raw milk composition, fruit type, using of pulp or whole fruit, sugar content, composition of plain yogurt used in the fruit yogurt manufacture.

### Titratable acidity and pH

Titratable acidity values of yogurt samples during the storage period are shown in Table 2. There were significant differences ($P$<0.05) in titratable acidity among the fruit yogurts. The titratable acidity values of yogurts steadily increased during the storage period ($P$<0.05). This increase can be explained by microbial activity in fruit added yogurts. Similar results were also obtained by KUCUKONER and TARAKCI (2003), and BAKIRCI and KAVAZ (2008), TARAKCI (2010) and SENGUL *et al.* (2012). The lowest titratable acidity value (0.83%) was obtained in $S_{8\%}$ on the 1$^{th}$ day of storage, while the highest value (0.96%) was found on day 14 in $S_{16\%}$. VAHEDI *et al.* (2008) and SENGUL *et al.* (2012) also reported that acidity values increased during storage. The pH values of fruit yogurts during the storage period are presented in Table 2. The pH values of the fruit yogurts were affected by both the storage time and the added content of fruit. The lowest pH value was obtained in $S_{16\%}$ sample and this result suggests that pH values decreased with increasing concentration of fruit. Therefore, strawberries contributed to the yogurt acidity due to its na-

ture acidity. The pH of strawberry added yogurts decreased during the storage period. Also, some authors have reported a significant decrease in the pH values during refrigerated storage (VAHEDI *et al.*, 2008; SENGUL *et al.*, 2012).

**Viscosity**

Viscosity is an important quality parameter for fruit yogurts and the effect of various fruits on viscosity can be different. The viscosity of the strawberry added yogurts was not influenced significantly ($P$<0.05) by the rates of pulp concentration, with the exception of $S_{16\%}$ on the 1st day. The viscosity of the yogurt samples ranged from 2030 to 3220 cP. The highest viscosity value of 3220 cP was found in $S_{16}$ on the first day of storage and the lowest value (2030 cP) was determined in $S_{8\%}$ on the 14th day of storage. The viscosity of the strawberry added yogurts decreased during the storage period (Table 2). It was reported that the addition of concentrated fruit to yogurt decreases the water holding capacity of protein by diluting the protein content; therefore the viscosity decreases (RAMASWAMY and BASAK 1992). Similar viscosity patterns of yogurt during storage were reported by CELIK *et al.* (2006), and BAKIRCI and KAVAZ (2008) and TARAKCI (2010). Conversely, SENGUL *et al.* (2012) reported that the viscosity of the sour cherry added yogurts increased during the storage period. Viscosity increases with the increasing of yogurt acidity during storage. The natural acidity of sour cherry is higher than strawberry and ıt will increase the acidity of yogurt. Therefore, our results are different from the findings of SENGUL *et al.* (2012).

**Syneresis**

Syneresis is characterized as contraction of a gel and this occurs simultaneously with the expulsion of liquid. To prevent syneresis in yogurt, the total solids content of milk can be increased and stabilizers can be added to the stirred yogurt (LUCEY and SINGH 1998). The syneresis values of yogurts were affected significantly ($P$<0.05) by the rates of pulp concentration. The highest mean value (4.38 mL/25 g) of syneresis was found in sample $S_{8\%}$ on the 14th day of storage and the lowest mean value (1.78 mL/25 g) was found in sample $S_{16\%}$. The increased fruit addition decreased the syneresis values of all yogurt samples ($P$<0.05). Depending on the fruit type used in yogurt, the fibre from fruit can increase the water holding capacity. Therefore, the syneresis may be low by increasing the fruit concentration. However, the syneresis values of strawberry added yogurts increased significantly ($P$<0.05) during the storage period. Increase in syneresis and decrease in viscosity are common properties of fruit yogurts. The syneresis values during storage were similar to the results of KUCUKONER and TARAKCI (2003). Conversely, BAKIRCI and KAVAZ (2008), TARAKCI (2010) and SENGUL *et al.* (2012) reported different results to our results. Differences among studies may be related to the type of fruit used, the chemical and physical properties of fruit as well as its addition level to the yogurt and pH and acidity values obtained in yogurt.

**Antioxidant activity of yogurt**

For the determination of antioxidant activities of yogurts we chose 2 methods, which allowed us to analyse a lot of antioxidant components. In the β-carotene bleaching assay, hydroperoxides are produce by linoleic acid as free radicals during incubation at 50°C. The presence of antioxidants in the extract will minimise the oxidation of β-carotene by hydroperoxides. Hydroperoxides formed in this system will be neutralised by the antioxidants from the extracts. β-carotene is a physiologically important compound and shows strong biological activity (KUBOLA and SIRIAMORNPUN, 2008). The rate of β-carotene bleaching can be slowed down in the presence of antioxidants (KULISIC *et al.*, 2004). DPPH assay can determine the free radical-scavenging activity of various samples and pure compounds. A stable free radical with a characteristic absorption at 517 nm was used to study the radical-scavenging effects of extracts. As antioxidants donate protons to this radical, the absorption decreases (KUBOLA and SIRIAMORNPUN, 2008).

*The β-carotene bleaching (BCB) method*

Table 3 presents antioxidant activities of strawberry pulp extracts. Antioxidant activity of strawberry pulp determined by two different assays β-carotene bleaching assay (BCB) and DPPH (Table 3). In the BCB assay, linoleic acid produces hydroperoxides during incubation at 50°C. The presence of hydroperoxides cause rapid discoloration of β-carotene. However, hydroperoxides formed in this system can be neutralised by the antioxidants from the extracts (ISMAIL *et al.*, 2010). Table 3 shows the mean total antioxidant activity of strawberry pulp. The means of total antioxidant activity for strawberry pulp was 90.28%. BHA, used as the standard, had a higher antioxidant activity than strawberry pulp extracts.

Table 3 shows the antioxidant activity of the yogurt extracts in comparison with BHA and all yogurt extracts had lower antioxidant activities than BHA. The antioxidant activity of plain yogurt was 70.27%. The antioxidant activity of yogurt extracts followed the order: $S_{16}$ (82.42±1.41%) >$S_{12}$ (79.28±2.63%) >$S_8$ (78.42±1.41%) as shown in Table 3. There was a difference between the antioxidant activities of yogurt at the beginning of the storage period, although this difference was not statistically sig-

Table 3 - Changes in antioxidant capacity and total phenolic content of yogurt samples during storage.

| Parameters | Strawberry pulp | Plain yogurt | Storage time (day) | Yogurt samples | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | $S_8$ | $S_{12}$ | $S_{16}$ | BHA | Trolox |
| Antioxidant activity (β-caroten bleaching assay) (%) | 90.28 | 70.27 | 1 | 78.42±1.41 $^{aA}$ | 79.28±2.63 $^{bA}$ | 82.42±1.41 $^{aA}$ | 95.70 | - |
| | - | - | 7 | 77.71±1.61 $^{aA}$ | 80.71±0.61 $^{bA}$ | 82.14±1.41 $^{aA}$ | 95.70 | - |
| | - | - | 14 | 83.24±2.38 $^{aA}$ | 86.12±0.93 $^{aA}$ | 81.09±0.50 $^{aB}$ | 95.70 | - |
| DPPH ($IC_{50}$) (μg/mL) | 28.79 | 2450.85 | 1 | 2241.09±0.45 $^{cC}$ | 751.60±1.33 $^{cB}$ | 205.70±1.73 $^{aA}$ | - | 26.20 |
| | - | - | 7 | 1760.35±0.70 $^{bC}$ | 532.08±0.90 $^{bB}$ | 447.30±1.90$^{cA}$ | - | 26.20 |
| | - | - | 14 | 494.61±1.33 $^{aC}$ | 442.06±1.80 $^{aB}$ | 360.76±1.70 $^{bA}$ | - | 26.20 |

$^{a–c}$Means in the same column followed by different lowercase letters represent significant differences ($P < 0.01$).
$^{A–C}$Means in the same line followed by different uppercase letters represent significant differences ($P < 0.01$)
C:$S_8$: [8% (w/w) strawberry and 8% (w/w) sugar], $S_{12}$: [12% (w/w) strawberry and 8% (w/w) sugar], $S_{16}$: [16% (w/w) strawberry and 8% (w/w) sugar].
nd, not determined.

nificant. The degradation rate of β-carotene–linoleate depends on the antioxidant activity of the extracts. There was a correlation between the degradation rate and the bleaching of β-carotene; the extract with the lowest β-carotene degradation rate exhibited the highest antioxidant activity. Milk contains antioxidant compounds, beta-carotene and enzymatic systems, mainly represented by superoxide dismutase, catalase and glutathione peroxidase (CALLIGARIS *et al.*, 2004). Fermentation of milk by lactic acid bacteria releases a large number of peptides and amino acids with varying biological actions such as Angiotensin-Converting Enzyme (ACE) inhibitory and immune modulatory, opioid and antioxidant activities (FARVIN *et al.*, 2010). Dairy products can be beneficial for the oxidative defence of consumers by several mechanisms. Also, milk antioxidants play important roles in preventing lipid peroxidation and maintaining milk quality (CHEN *et al.*, 2003). In our study, the results indicated that the total antioxidant activity increased with the incremental strawberry concentration. For example, by increasing the strawberry concentration from 8 to 16%, the total antioxidant activity increased from 78.42 to 82.42%. However, the differences were not statistically significant. During the storage period, the total antioxidant activity decreased in $S_{16}$, whereas in $S_8$ and $S_{12}$ antioxidant activity increased, although it did not show a statistically significant variation (Table 3). Strawberries contain high levels of natural antioxidant compounds such as anthocyanins, flavonoids, and phenolic acids, vitamins, glutathione, among the fruits. These components provide protection against harmful free radical species (ZHENG *et al.*, 2007; JIN *et al.*, 2011). Antioxidant compounds have been associated with lower occurrences and mortality rates due to heart disease, as well as offering a number of other health benefits. There is strong evidence in the literature linking increased stress with high levels of free radicals, resulting in adverse effects on human health, including cancer (ZHENG *et al.*, 2007). Therefore, strawberries may decrease the risk of cancer and prevent various human diseases caused by oxidative stress (JIN *et al.*, 2011).

*DPPH (2.2-diphenyl-1-picrylhydrazyl) scavenging assay*

DPPH free radical-scavenging activities of strawberry pulp extract and trolox are shown in Table 3. The $IC_{50}$ values of the DPPH radical-scavenging activities were 28.79 μg/mL and 26.20 μg/mL for strawberry pulp and trolox, respectively.

DPPH free radical scavenging activities of yogurt extracts and trolox are presented in Table 3. In order to quantify the antioxidant activity of yogurt extracts in the course of storage, the $IC_{50}$ was calculated (Table 3). There is an inverse relationship between $IC_{50}$ and antioxidant activity. The lower the $IC_{50}$ value, the greater the free radical-scavenging activity (YANG and ZHAI, 2010). In DPPH assay, $IC_{50}$ values of plain yogurt were 1450.85 μg/mL. There was a significant difference ($P<0.05$) between the antioxidant activities of difference yogurt at the beginning of the storage period. The $IC_{50}$ values of the DPPH radical-scavenging activities were 2241.09±0.45, 751.60±1.33, 205.70±1.73 μg/mL and 26.20 for $S_8$, $S_{12}$, $S_{16}$ and Trolox, respectively. These results revealed that $S_8$, $S_{12}$ and $S_{16}$ had significantly ($p<0.01$) lower scavenge power than Trolox. In this method, the scavenging effect on DPPH radicals assay showed concentration-dependent activity and the antioxidant activity yogurt extracts increased with increasing strawberry concentration (Table 3). Strawberries have high oxygen radical absorbance activity against peroxyl radicals, superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen (ZHENG *et al.*, 2007). The DPPH assay is a simple method that provides infor-

mation about the radical scavenging activity of the antioxidant substances present in the examined sample. SKREDE *et al.* (2004) used the DPPH method to assess the antioxidant properties of berry/fermented milk blends. This method was also used to investigate the effects of tea supplementation on the antioxidant capacity of yogurt (NAJGEBAUER-LEJKO *et al.*, 2011). Earlier studies have shown that several dairy products and their fractions e.g. milk, skim milk, whey, casein and lactoferrin have antioxidant properties (CHEN *et al.*, 2003) presumably based on their ability to bind to transition metals and scavenge free radicals (FARVIN *et al.*, 2010). Antioxidant activity increased in yogurt during the storage period (Table 3) This increase may be explained by the fact that continued microbial growth, even during cold storage, may have changed some of the phenolic compounds and hence their antioxidant activities (BLUM, 1998; PAPADIMITRIOU *et al.*, 2007). Besides, the DPPH scavenging activity of yogurt extracts may also be dependent on the presence of small molecular compounds such as proteins, peptides and polysaccharides (O'SULLIVAN *et al.*, 2011). Organoleptic properties

The organoleptic properties of the yogurts are shown as a radar plot in Fig. 1 (a, b). The addition of a different proportion of strawberry fruit to yogurt significantly affected ($P<0.05$) the scores for colour, appearance and odour. Conversely, no significant effect ($P>0.05$) was observed for texture, flavour, acidity and general acceptability. Increased fruit addition also increased the colour, appearance, texture, flavour, odour and general acceptability scores. VAHEDI *et al.* (2008) determined that the texture score of strawberry added yogurts increased in the third day of storage. However, acidity scores decreased. It was also found that the storage duration did not significantly affect ($P>0.05$) the scores of all characteristics. The highest general acceptability score was obtained on day 1 of the storage of yogurt containing a 16% fruit ratio. Colour, appearance, odour, texture and flavour scores generally increased during the storage period. Conversely, the general acceptability and acidity scores generally decreased.

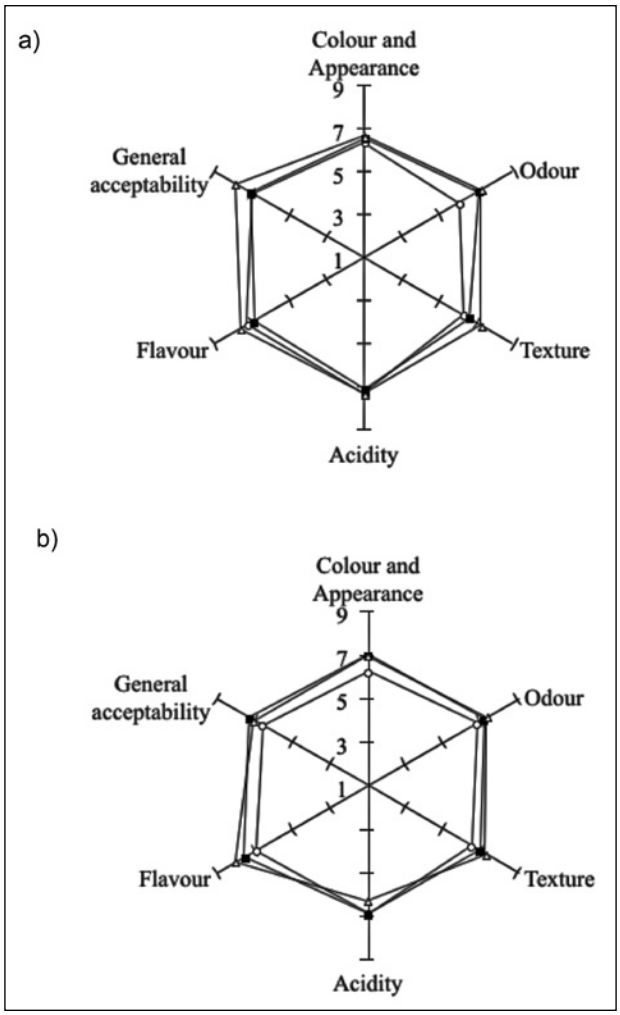



Fig. 1 - (a) Sensory profiles of yogurts containing strawberry at day 1 of storage (b) Sensory profiles of yogurts containing strawberry at day 14 of storage. strawberry concentrations: (○) 8% (w/w) strawberry, (■)12% (w/w) strawberry, (Δ)16% (w/w) strawberry.

## CONCLUSION

The increment of the strawberry pulp level in yogurt improved the structural properties of yogurts by resulting in a decrease in syneresis and an increase in viscosity. Conversely, due to spoilage of curd stability with prolonging of cold storage, syneresis values increased and viscosity values decreased in all yogurts. The results of this study indicated that the addition of various levels of strawberry to yogurt caused an increase in the antioxidant capacity of the yogurts. Yogurt including 16% strawberry pulp received the highest overall acceptability score on the first day of storage. Further studies on the antioxidant compounds of milk and dairy products are required, especially the identification and quantification of antioxidant compounds.

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PAPER

# RHEOLOGICAL AND CLUSTERING APPROACH TO CLASSIFY IRANIAN SOFT/LIQUID FOOD PRODUCTS TO MEET DYSPHAGIA DIET REQUIREMENTS

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ABSTRACT

The American Dietetic Association proposes a classification of foods for dysphagia diets based on viscosity data measured at shear rate of 50 ($s^{-1}$). This guideline has been used as a reference in many papers and therefore most of studies have only focused on steady state rheological parameters.

The purpose of present study was to cluster and to classify 39 Iranian viscous food products considering steady-state and viscoelastic parameters. Using clustering techniques, 39 samples were divided to six clusters. The results of this study indicate that more work is needed to determine the best rheological parameters to classify dysphagia oriented food products.

- Keywords: national dysphagia diet, dysphagia, viscoelastic properties, cluster classification, viscosity -

## INTRODUCTION

Dysphagia is a common disorder characterized by difficulty in swallowing or moving foods and liquids from the mouth to the stomach (FORSTER *et al.*, 2011). Two types of effect are created when a patient undergoes dysphagia: physical health issues and psycho-social and wellbeing problems (COUSINS *et al.*, 2013).

The key methods to treat dysphagia symptoms are as follows: (1) behavioral, (2) medical and (3) surgical. Among these behavioral methods, using thickened food products are widely used in healthcare systems (LOGEMANN 2007; HOUJAIJ *et al.*, 2009) and some hospitals have special menus for dysphagic patients (GERMAIN *et al.*, 2006). Medical food companies have also special food products to serve dysphagic patients (SOPADE *et al.*, 2007; SOPADE *et al.*, 2008; CASANOVAS *et al.*, 2011). Two types of food products are available: "ready-to-serve" and "powdered instant food thickeners". Regardless of DOP's type (beverage, main course and dessert), classification of the products for disphagic patients is a problematic issue among food science experts and nutritional staff. Having realized the need for standardization of dysphagia diet, miscellaneous practices have been performed and some manuals have been presented to the dietitians (PARDOE 1993; BAKHEIT 2001).

The first systematic guideline for DOP's was published by American Dietetic Association in 2002. In this taskforce called National Dysphagia Diet (NDD), the viscosity measured at the shear rate of 50 ($s^{-1}$) is considered the criterion to classify foodstuffs into four categories. The NDD guideline proposed a classification of foods based on viscosity values ranging from thin (1-50 cP), nectar-thick (51-350 cP), honey-thick (351-1750 cP) to spoon thick (>1750 cP) foodstuffs.

It is interesting to note that only one year after NDD's publication, McCallum described the American Dietetic Association's plan as less than useful in real world application. His aim was to assess efficacy of NDD level descriptors in a care facility and claimed that the descriptors were confounding. He proposed that two extra classes should be added to the NDD, namely the Dysphagia Pureed and Dysphagia Mechanically Altered ones (MCCALLUM 2003).

Previous studies in the area of rheology-dysphagia, have based their classification on the NDD guideline (DIETITIAN ASSOCIATION OF AUSTRALIA 2007; GABB 2007; LANGE *et al.*, 2007; WILSON and GREEN 2009; GARCIA *et al.*, 2010; HEISS *et al.*, 2010; MOMOSAKI *et al.*, 2011; O'LERAY *et al.*, 2011; PAYNE *et al.*, 2012; UMEMOTO *et al.*, 2012).

Nevertheless, there are still insufficient studies based on the role of viscoelstic parameters obtained from oscillatory rheological measurements in the classification of DOP's.

Therefore, this paper is aimed to propose a new clustering classification approach of some viscous Iranian food products including viscosity and viscoelastic parameters and to compare the results with the NDD classification.

## MATERIALS AND METHODS

### Materials

Thirty-nine commercial food products were selected from various sectors of market (beverages, desserts and main courses) and different brands (A to L in the tables). All the food products were purchased from a local supermarket (Tehran, Iran). This choice depends on the fact that there isn't any DOP in the Iranian market.

Samples were either for direct consumption or re-constituted in the Rheology Lab according to the manufacturers' instruction. All the samples were stored according to the package instruction.

### Methods

#### *Rheological measurements*

Rheological experiments were carried out by using rheometer (Anton Paar Physica MCR 301, Graz, Austria); temperature control was carried out using a Peltier system equipped with a fluid circulator.

In regard to temperature analysis, various conditions are used and reported in the literature i.e.: 8°C (GERMAIN *et al.*, 2006), 20°C (SOPADE *et al.*, 2007; SOPADE *et al.*, 2008; SOPADE *et al.*, 2008), 21°-22°C (DEWAR and JOYCE 2006) and 25°C (BUDKE *et al.*, 2008; CASANOVAS *et al.*, 2011; PAYNE *et al.*, 2012). Many parameters are involved in determining the serving temperature of provided foodstuffs for dysphagic patients such as initial temperature, needed serving time and so on.

In this study, rheological measurements were performed at temperatures different for each product based on the specific consumption habits. Therefore, a clinical pretest was performed for each sample and the exact serving temperature was measured. These test temperatures are presented in Table 1.

Vane geometry (ST14-4V-35-SN16727; d=0 mm) was used for main courses and dessert samples and concentric cylinder (CC27-SN16194; d=0 mm) was utilized for all the drink samples.

Before the steady and dynamic shear rheological measurements, all the samples were left standing for 5 min at the related temperature (Table 1) to allow for structure recovery and temperature equilibrium. Data analysis was conducted using Rheoplus software (version 3.21).

Table1 - Consistency index *m*, flow behavior *n*, apparent viscosity $\eta_0$, and yield stress $\tau_0$ of 39 samples. *NDD group*, indicates the group of samples, based on NDD classification as follows: 1: Non-thickened, 2: Nectar-like, 3: Honey-like and 4: Spoon-thick. 1 and 2 superscripts indicate the temperature at which rheological tests were performed, 10° and 45°C, respectively.

| | Name | Power law model model | | $\tau_0$ | ηa | NDD group |
|---|---|---|---|---|---|---|
| | | m(Pa.sn) | n | (Pa) | (cP) | |
| 1. | Guava Nectar (A)[1] | 0.08 | 0.56 | 0.04 | 14.71 | 1 |
| 2. | Apricot Nectar (A)[1] | 0.05 | 0.56 | 0.11 | 9.31 | 1 |
| 3. | Mango Nectar (A)[1] | 0.1 | 0.53 | 0.09 | 16.01 | 1 |
| 4. | Peach Nectar (A)[1] | 0.15 | 0.46 | 0.1 | 18.36 | 1 |
| 5. | Apricot Nectar (B)[1] | 0.15 | 0.45 | 0.11 | 17.71 | 1 |
| 6. | Mango Nectar (A)[1] | 0.18 | 0.45 | 0.14 | 21.69 | 1 |
| 7. | Peach-mango Nectar (C)[1] | 0.31 | 0.41 | 0.2 | 30.64 | 1 |
| 8. | Cappochino coconut drink(D)[1] | 10.53 | 0.25 | 3.15 | 589.04 | 3 |
| 9. | Strawberry drink (D)[1] | 1.6 | 0.43 | 9.73 | 163.14 | 2 |
| 10. | Melone drink (D)[1] | 2.95 | 0.38 | 0.69 | 251.81 | 2 |
| 11. | Chocolate coconut drink(D)[1] | 18.92 | 0.23 | 9.75 | 919.67 | 3 |
| 12. | Halim (E)[2] | 164.57 | 0.24 | 198.14 | 8455.03 | 4 |
| 13. | Halim (F)[2] | 5.64 | 0.37 | 1.36 | 451.15 | 3 |
| 14. | Sholezard (G)[1] | 347 | 0.28 | 89.27 | 20780.16 | 4 |
| 15. | Shirberenj (G) | 40 | 0.41 | 13.01 | 3911.92 | 4 |
| 16. | Vegetable soup (H)[2] | 3.33 | 0.39 | 0.64 | 281.41 | 2 |
| 17. | Chicken soup (I)[2] | 10.85 | 0.29 | 13.53 | 645.18 | 3 |
| 18. | Lentile soup (F)[2] | 19.1 | 0.19 | 8.46 | 736.37 | 3 |
| 19. | Eggplant dish (J)[2] | 95 | 0.12 | 123.2 | 2691.79 | 4 |
| 20. | Fig olive yoghurt (G)[1] | 72.32 | 0.25 | 58.12 | 3591.35 | 4 |
| 21. | Spinach yoghurt (G)[1] | 37.14 | 0.24 | 22.82 | 1825.83 | 4 |
| 22. | Celery yoghurt (G)[1] | 16 | 0.31 | 11.01 | 1069.39 | 3 |
| 23. | Cucumber yoghurt (G)[1] | 34 | 0.33 | 52.48 | 2466.97 | 4 |
| 24. | Seven yoghurt (G)[1] | 25.49 | 0.28 | 29.25 | 1504 | 3 |
| 25. | Ferni (K)[1] | 336 | 0.18 | 139.77 | 9818.05 | 4 |
| 26. | Saffron dessert (G)[1] | 293.15 | 0.15 | 206.48 | 10270.45 | 4 |
| 27. | Coffee dessert (G)[1] | 361.07 | 0.13 | 175.21 | 11635.63 | 4 |
| 28. | Chocolate dessert (D)[1] | 99.4 | 0.2 | 41.13 | 4188.36 | 4 |
| 29. | Banana dessert (D)[1] | 96.15 | 0.2 | 49.55 | 4017.8 | 4 |
| 30. | Vanilla dessert (D)[1] | 124.12 | 0.17 | 70.53 | 4538.02 | 4 |
| 31. | Biscuit dessert (D)[1] | 91.41 | 0.22 | 48.95 | 4204.23 | 4 |
| 32. | Strawberry dessert (D)[1] | 66.87 | 0.22 | 32.23 | 3057.89 | 4 |
| 33. | Caramel dessert (L)[1] | 73.72 | 0.17 | 31.35 | 2691.79 | 4 |
| 34. | Cocoa cream dessert (L)[1] | 349.14 | 0.17 | 130.64 | 13283.19 | 4 |
| 35. | Aloevera yoghurt (G)[1] | 19.54 | 0.4 | 35.18 | 1685.45 | 3 |
| 36. | Apple yoghurt (G)[1] | 21.82 | 0.32 | 23.73 | 1440.95 | 3 |
| 37. | Peach yoghurt (G)[1] | 56.88 | 0.26 | 58.57 | 3009.4 | 4 |
| 38. | Strawberry yoghurt (G)[1] | 31.89 | 0.32 | 42.9 | 2122.11 | 4 |
| 39. | Fig Cream (G)[1] | 97.47 | 0.33 | 74.01 | 7013.89 | 4 |

*Flow curves*

Flow curves were obtained at shear rates of 0.01-1,000 (1/s). The power law (Eq. (1)) (over mid-range shear rates) and the Cross model (Eq. (2)), were used to describe rheological properties of the samples. In the power law model (Eq. (1)), the flow behavior index (*n*) and consistency coefficient (*m*) values were obtained by fitting the shear rate versus apparent viscosity values.

$$\eta_a = m\dot{\gamma}^{n-1}$$

(Equation 1) Power law model

where $\eta a$ is apparent viscosity (Pa.sn), *m* is consistency coefficient (Pa.sn), is shear rate (1/s) and *n* is flow behavior index (dimensionless). Values for the yield stress, calculated by extrapolation of the steady-state shear stress to zero shear rate.

In the Cross model (Eq. (2)), and are asymptotic values of viscosity at zero and infinite shear rates, respectively, λ is the characteristic time and *k* rules the shear dependence in the power-law region.

$$\eta = \eta\infty + \frac{\eta_0 - \eta\infty}{1 + (\lambda\dot{\gamma})^{\kappa}}$$

(Equation 2) Cross model

*Oscillatory tests*

The extent of the linear visco-elastic region was determined by performing a strain (0.01-1,000%, 6.28 rad/s). To provide a direct view for whether the samples behaved as liquids or solids, tan δ was calculated at the $_{Lve}$ (ratio of loss modulus to elastic modulus). Shear stress at the limit of Lve range, $\tau_y$, and crossover point (G'=G") were also determined. After the determination of the Lve range, frequency sweep tests

were performed using a frequency ramp from 0.0628 to 94.2 rad/s.

The goal of the present study was first to characterize commercial food products and then cluster them; so, frequency dependence of G' and G" were described by three models:

$$G', G'' = a\omega^b$$
(Equation 3) power law model

$$G', G'' = a + bx + cx^2$$
(Equation 4) 2nd polynomial model

$$G', G'' = a + xb + x^{c.lnx}$$
(Equation 5) 2nd log polynomial model

In the power law model (Eq. (3)), $a$ is a constant and $b$ may be referred to as the frequency exponent. The values of $b$ which represent the extent of frequency dependence present expedient information regarding viscoelastic characteristics of food materials.

*Statistical analysis*

When comparable parameters were obtained, Matlab Fuzzy Logic Toolbox (R2012 a) was utilized for clustering the samples.

## RESULTS

The parameters of Cross model (Eq. (2)), were determined by fitting experimental data of steady-shear flow curves for all samples and just 17 samples showed high correlation ($R^2$>0.99, data are not shown). Therefore, power law model (Eq. (1)) parameters were calculated within 10-200 (1/s) for 39 samples (Table 1). The coefficients of determination ($R^2$) were 0.99 or higher for all the tested samples, indicating the appropriateness of the power law model to describe flow properties of all the different samples.

All 39 samples showed a non-Newtonian flow behaviour or pseudoplasticity. As the shear rate increased, apparent viscosity of all the samples decreased. This result is in agreement with those of previous studies on dysphagia-oriented products (GERMAIN *et al.*, 2006; SOPADE *et al.*, 2007; QUINCHIA *et al.*, 2011). Apparent viscosity at the shear rate of 50 ($s^{-1}$) (the NDD reference shear rate) and yield stress were also reported (Table 1). Table 1 also presents the classification of samples, based on NDD taskforce. As Table 1 shows, all seven nectars are classified in non-thickened group of NDD.

Limits of the linear viscoelastic domains for thirty-nine samples were determined and Fig. 1 depicts the tan δ of six representative samples.

The limiting value of linear viscoelastic range ($\gamma_l$), tan δ for the end of Lve region, shear stress on the ($\gamma_l$) and crossover point (G'=G") of 39 samples are listed in Table 2.

Strain sweep tests were performed to determine strain limits of linear viscoelastic behavior of the samples for the following frequency sweep measurements. Linear viscoelasticity is observed when the deformation encountered by the material is small enough and the material is negligibly disturbed from its equilibrium state. When viscoelastic food materials were tested beyond this region, irreversible structural changes occurred (GONG *et al.*, 2012).

In strain sweep test the tan $\delta_{Lve}$ parameter was also considered. It is the proportion of loss modulus to storage modulus at the end of Lve. Tan δ of the samples ranged from 0.19 for Ferni and saffron dessert to 0.88 for apricot nectar. Tan δ provided a direct view for whether the samples behaved as liquids or solids and higher tan δ indicates more liquid-like nature of food materials.

In Table 2 the flow point and the correspond-

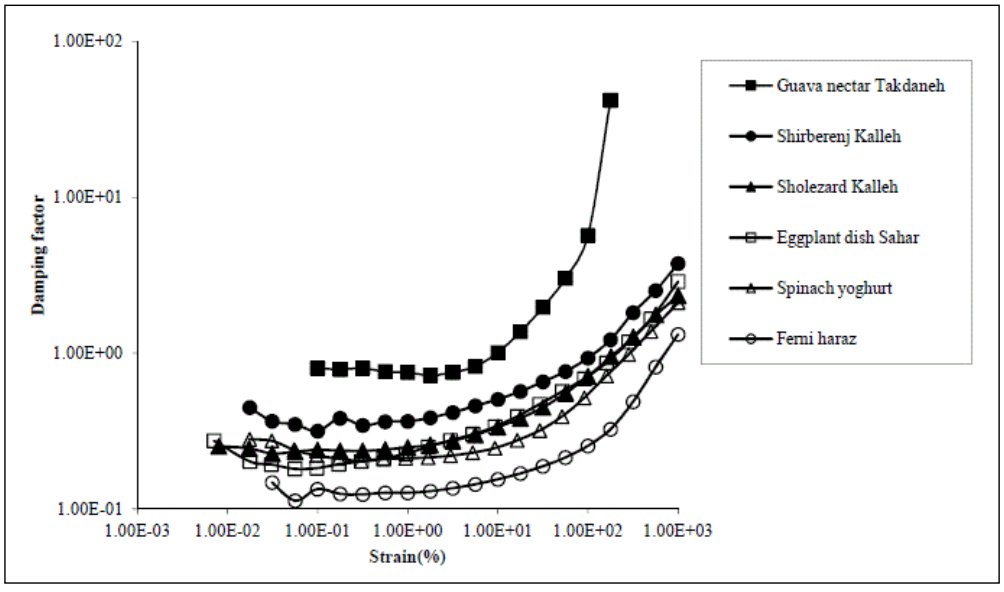



Fig. 1 - Strain sweep of five representative samples.

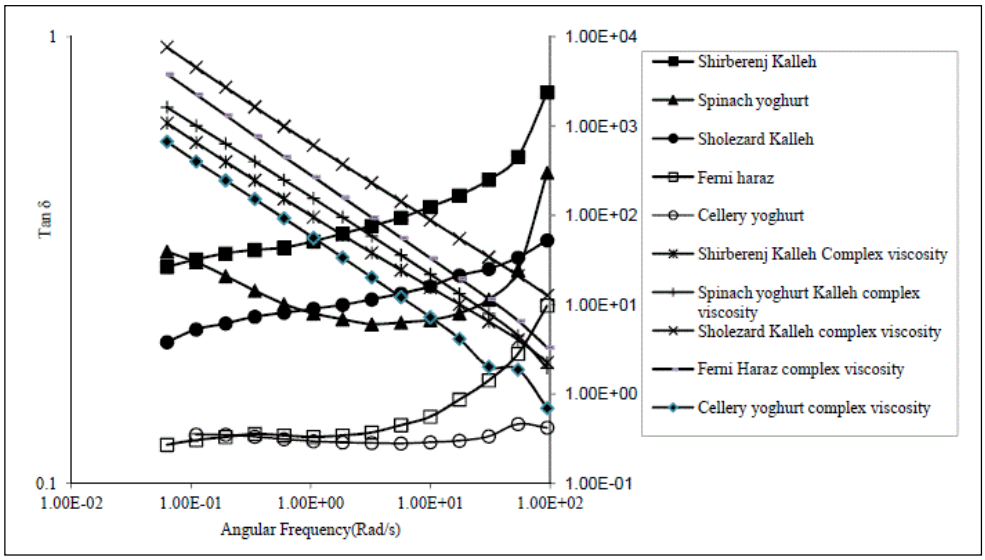



Fig. 2 - Frequency sweep rheogram of five representative samples.

ing coordinate at *G'*=*G''*, are reported. Ferni (with the lowest tan δ and the highest elastic feature) showed the most flow point stress and apricot nectar had the lowest. The flow of foodstuffs is started at the crossover point, in other words the implied stress ( ) leads to breaking the internal structure of food material.

Frequency sweep tests were carried out in the viscoelastic linear region at the constant strain previously determined, in the range from 0.628 to 94.2 (rad/s). Fig. 2 presents complex viscosity and tan δ of six representative samples.

Power-law model (Eq. (3)), is a largely used model to analyze frequency sweep data of food materials such as puddings (QUINCHIA *et al.*, 2011), gum solutions (NÚÑEZ-SANTIAGO and TECANTE 2007; WANG *et al.*, 2009; ISHIHARA *et al.*, 2011) and food emulsions (PARASKEVOPOULOU *et al.*, 1999).

The power-law model was fitted to both viscous and elastic modules (frequency sweep test) and 14 samples showed $R^2$ <0.98. Other models like 2nd polynomial and 2nd log polynomial were fitted to the mentioned samples and the results showed that the best fitted model was the 2nd log polynomial with correlation ratio of > 0.9901. These data are not shown in tables.

Furthermore, viscous and elastic moduli at three angular frequencies of 0.628, 1.84 and 17.4 (rad/s) were reported as representatives of low, moderate and high angular frequencies, respectively (Table 3).

Taking comparative goals and cluster classification of the samples into account and considering the fact that clustering should be done based on a fixed number of parameters, the authors decided to report only 2nd log polynomial model parameters.

By using clustering methodologies, a large number of samples are classified into clusters in which all members are similar to each other and are different from those of other clusters (KRAIC *et al.*, 2008).

At least two parameters are required for the cluster classification of large number of samples, but to produce a concise view for a cluster's behaviour, more parameters are required (Zimmerman 2005). Therefore, in the present study, rheological characterization of 39 samples was conducted by dynamic and steady-state rheological tests.

In the dynamic rheological tests, three parameters from strain sweep test and nine parameters from frequency sweep tests were obtained (Tables 2 and 3). Steady state tests also presented four parameters (Table 1). After analyzing rheogramms and gathering all 16 parameters, clustering was carried out by Fuzzy Logic Toolbox MATLAB.

The Fuzzy c-means (FCM) technique presented by Bezdec (1981) and K-means methods are common clustering methods. In the FCM method, data points are related to a cluster with a membership grade between 0 and 1. One of the advantages of this method over older methods like K-means method is the fact that a data point is not confined to one cluster and degree of membership is defined for other clusters (ANSARI *et al.*, 2010; MATHWORKS 2012).

FCM technique is practical when there is a clear idea on cluster numbers. Like the k-mean method, the result of clustering is related to initial partitioning; therefore, this initial guessing of cluster numbers is regarded as the "major bottleneck" of c-means and k-means methods (BUDAYAN *et al.*, 2009; TARI *et al.*, 2009). In the present study, as mentioned before, the products were selected from different types of food and drinks and there was not any previous rheological data in this field. Therefore, c-mean and k-mean methods were impractical and the subtractive clustering technique was employed as the clustering method (DUBOIS and PRADE 1980). One of our limitations, that could partially explain some of the results obtained, could be that the wide range of food products confined the use of a single rheolog-

Table 2 - Strain sweep parameters of 39 samples: limiting value of linear viscoelastic range $\gamma_l$ (%), tan δ at the end of Lve region tan $\delta_{Lv}$e, and crossover point $G_f$ (Pa) (G'=G"). 1 and 2 superscripts indicate the temperature at which rheological tests were performed, 10° and 45°C, respectively.

| Type | | Name | $\gamma_l$ (%) | tan $\delta_{Lve}$ | (pa) |
|---|---|---|---|---|---|
| Drinks | 1. | Guava Nectar (A)[1] | 5.55 | 0.82 | 0.25 |
| | 2. | Apricot Nectar (A)[1] | 1.85 | 0.88 | 0.13 |
| | 3. | Mango Nectar (A)[1] | 3.84 | 0.56 | 0.28 |
| | 4. | Peach Nectar (A)[1] | 2 | 0.44 | 0.32 |
| | 5. | Apricot Nectar (B)[1] | 1.85 | 0.38 | 0.40 |
| | 6. | Mango Nectar (A)[1] | 3.17 | 0.3 | 0.45 |
| | 7. | Peach-mango Nectar (C)[1] | 1.99 | 0.3 | 0.82 |
| | 8. | Cappochino coconut drink(D)[1] | 12.8 | 0.31 | 2.55 |
| | 9. | Strawberry drink (D)[1] | 35.8 | 0.76 | 0.62 |
| | 10. | Melone drink (D)[1] | 27.4 | 0.68 | 0.91 |
| | 11. | chocolate coconut (D)[1] | 23.6 | 0.24 | 4.70 |
| Main courses | 12. | Halim (E)[2] | 7.13 | 0.23 | 111.39 |
| | 13. | Halim (F)[2] | 0.47 | 0.24 | 22.89 |
| | 14. | Sholezard (G)[1] | 8.2 | 0.32 | 192.38 |
| | 15. | Shirberenj (G) | 3.5 | 0.42 | 31.86 |
| | 16. | Vegetable soup (H)[2] | 0.12 | 0.31 | 13.02 |
| | 17. | Chicken soup (I)[2] | 4.56 | 0.26 | 5.80 |
| | 18. | Lentile soup (F)[2] | 1.34 | 0.32 | 5.81 |
| | 19. | Eggplant dish (J)[2] | 1.69 | 0.25 | 44.61 |
| Desserts | 20. | Fig olive yoghurt (G)[1] | 10.6 | 0.2 | 42.54 |
| | 21. | Spinach yoghurt (G)[1] | 9.45 | 0.2 | 16.998 |
| | 22. | Celery yoghurt (G)[1] | 4.44 | 0.25 | 8.75 |
| | 23. | Cucumber yoghurt (G)[1] | 3.64 | 0.23 | 30.04 |
| | 24. | Seven yoghurt (G)[1] | 13.8 | 0.21 | 16.86 |
| | 25. | Ferni (K)[1] | 33.4 | 0.19 | 75.87 |
| | 26. | Saffron dessert (G)[1] | 12.8 | 0.19 | 67.69 |
| | 27. | Coffee dessert (G)[1] | 14.6 | 0.2 | 71.32 |
| | 28. | Chocolate dessert (D)[1] | 33.6 | 0.21 | 23.34 |
| | 29. | Banana dessert (D)[1] | 38.6 | 0.23 | 21.78 |
| | 30. | Vanilla dessert (D)[1] | 43.4 | 0.21 | 24.76 |
| | 31. | Biscuit dessert (D)[1] | 29.7 | 0.26 | 21.44 |
| | 32. | Strawberry dessert (D)[1] | 34.4 | 0.26 | 14.76 |
| | 33. | Caramel dessert (L)[1] | 71.1 | 0.28 | 21.32 |
| | 34. | Cocoa cream dessert (L)[1] | 9.46 | 0.23 | 96.49 |
| | 35. | Aloevera yoghurt (G)[1] | 8.6 | 0.21 | 21.08 |
| | 36. | Apple yoghurt (G)[1] | 10.1 | 0.2 | 15.43 |
| | 37. | Peach yoghurt (G)[1] | 17.2 | 0.21 | 26.70 |
| | 38. | Strawberry yoghurt (G)[1] | 7.61 | 0.23 | 20.93 |
| | 39. | Fig Cream (G)[1] | 0.22 | 0.23 | 93.01 |

ical model for all samples. Considering all 16 parameters, classification of the samples with subtractive method was performed. The results showed that, when 16 parameters were included in the classification, 39 samples were divided to 14 clusters. Since the objective of classification of data was to minimize the volume of large samples, this classification did not provide any useful hints. Consequently, clustering was performed using some selected parameters from each rheological test. Therefore, after analyzing different parameters and calculating the number of provided clusters (subtractive clustering), the authors finally decided to cluster the samples by 12 parameters.

These parameters included 3 strain sweep parameters (Lve (%), tan δ Lve and G'=G" (pa)), 6 frequency sweep parameters (G' (pa): ω= (0.0628, 1.84, 17.4 rad/s, G" (pa): ω= (0.0628, 1.84, 17.4 rad/s) and 3 steady state parameters (*m*, and $\tau_0$). Considering this procedure, the samples were classified into 6 clusters. Table 4 shows the membership degree of each sample to clusters.

## DISCUSSION

An important branch of dysphagia related literature is focused on setting a unified approach to characterize dysphagia diets and foodstuffs. The American Dietetic Association introduced a standard for dysphagia oriented food products. The key problem with this guideline is that it relies on a single steady-state rheological parameter, like apparent viscosity at the shear rate of 50 ($s^{-1}$).

Some hydrocolloid solutions show different swallowing profiles in a fixed shear viscosity.

Table 3 - Frequency sweep parameters of 39 samples. G'(Pa) and G" (Pa) of each sample in frequency sweep test was calculated in three angular frequencies, namely, low ω= 0.0628 (rad/s), medium ω= 1.84 (rad/s) and high ω= 17.4 (rad/s) angular frequency. 1 and 2 superscripts indicate the temperature at which rheological tests were performed, 10° and 45°C, respectively.

| Name | | G'(Pa) : | | | G"(Pa): | | |
|---|---|---|---|---|---|---|---|
| | | ω=(0.0628) | ω= (1.84) | ω= (17.4) | ω= (0.0628) | ω= (1.84) | ω= (17.4) |
| 1. | Guava Nectar (A)[1] | 0.22 | 0.24 | 0.24 | 0.04 | 0.12 | 0.52 |
| 2. | Apricot Nectar (A)[1] | 0.10 | 0.11 | 0.12 | 0.01 | 0.06 | 0.31 |
| 3. | Mango Nectar (A)[1] | 0.37 | 0.41 | 0.51 | 0.04 | 0.14 | 0.58 |
| 4. | Peach Nectar (A)[1] | 0.56 | 0.55 | 0.60 | 0.07 | 0.17 | 0.66 |
| 5. | Apricot Nectar (B)[1] | 0.74 | 0.74 | 0.83 | 0.08 | 0.21 | 0.76 |
| 6. | Mango Nectar (A)[1] | 0.90 | 0.99 | 1.12 | 0.10 | 0.23 | 0.82 |
| 7. | Peach-mango Nectar (C)[1] | 2.09 | 2.13 | 2.55 | 0.26 | 0.49 | 1.45 |
| 8. | Cappochino coconut drink(D)[1] | 5.16 | 8.75 | 7.63 | 1.21 | 2.37 | 4.79 |
| 9. | Strawberry drink (D)[1] | 0.63 | 1.45 | 1.62 | 0.20 | 0.54 | 3.61 |
| 10. | Melone drink (D)[1] | 1.2 | 2.65 | 2.87 | 0.39 | 0.81 | 4.42 |
| 11. | Chocolate coconut (D)[1] | 15.8 | 23.2 | 29.2 | 3.27 | 4.1 | 7.99 |
| 12. | Halim (E)[2] | 617 | 806 | 1010 | 116 | 151 | 240 |
| 13. | Halim (F)[2] | 41.4 | 45.5 | 62.6 | 14.7 | 19.5 | 29.9 |
| 14. | Sholezard (G)[1] | 470 | 665 | 919 | 97.2 | 167 | 269 |
| 15. | Shirberenj (G) | 64.8 | 104 | 160 | 19.8 | 37.6 | 70.7 |
| 16. | Vegetable soup (H)[2] | 17.5 | 19 | 28.7 | 11.6 | 17.6 | 28.6 |
| 17. | Chicken soup (I)[2] | 15.4 | 15.7 | 17.3 | 2.68 | 3.32 | 7.28 |
| 18. | Lentile soup (F)[2] | 14.7 | 15.8 | 18.3 | 2.96 | 5.03 | 10.3 |
| 19. | Eggplant dish (J)[2] | 531 | 645 | 859 | 91.1 | 164 | 224 |
| 20. | Fig olive yoghurt (G)[1] | 194 | 322 | 415 | 49.3 | 64.3 | 87.2 |
| 21. | Spinach yoghurt (G)[1] | 96.5 | 171 | 226 | 31.8 | 39.8 | 54.4 |
| 22. | Celery yoghurt (G)[1] | 39.7 | 59.6 | 68.5 | 13.9 | 16.8 | 23 |
| 23. | Cucumber yoghurt (G)[1] | 174 | 296 | 383 | 61.3 | 75.6 | 94.5 |
| 24. | Seven yoghurt (G)[1] | 76.1 | 132 | 170 | 22.4 | 27.6 | 36.5 |
| 25. | Ferni (K)[1] | 238 | 290 | 340 | 29 | 37 | 52.2 |
| 26. | Saffron dessert (G)[1] | 259 | 259 | 325 | 401 | 39.7 | 49.2 |
| 27. | Coffee dessert (G)[1] | 251 | 251 | 320 | 384 | 41.9 | 48.1 |
| 28. | Chocolate dessert (D)[1] | 86.6 | 122 | 145 | 15.4 | 17.4 | 28.5 |
| 29. | Banana dessert (D)[1] | 53.9 | 74.5 | 89.9 | 9.93 | 12.6 | 21 |
| 30. | Vanilla dessert (D)[1] | 78.9 | 110 | 133 | 12.4 | 15.9 | 26 |
| 31. | Biscuit dessert (D)[1] | 65.1 | 83.3 | 97.9 | 11.3 | 14.4 | 25.3 |
| 32. | Strawberry dessert (D)[1] | 38.3 | 56.3 | 67 | 8.37 | 10.7 | 18.5 |
| 33. | Caramel dessert (L)[1] | 41.3 | 117 | 151 | 7.95 | 17.9 | 28.8 |
| 34. | Cocoa cream dessert (L)[1] | 397 | 883 | 1220 | 110 | 197 | 267 |
| 35. | Aloevera yoghurt (G)[1] | 99.8 | 169 | 224 | 29.7 | 37.6 | 51.5 |
| 36. | Apple yoghurt (G)[1] | 70.1 | 113 | 145 | 25 | 25.7 | 31.1 |
| 37. | Peach yoghurt (G)[1] | 137 | 230 | 308 | 35.6 | 50 | 67.1 |
| 38. | Strawberry yoghurt (G)[1] | 113 | 189 | 253 | 34.2 | 45.6 | 61.8 |
| 39. | Fig Cream (G)[1] | 866 | 1090 | 1310 | 365 | 465 | 591 |

For example, in an equivalent shear viscosity (at 10 $s^{-1}$), xanthan gum and locust bean gum show different swallowing profiles; therefore, the only viscosity at a certain shear rate does not completely represent ease of swallowing (KUMAGAI *et al.*, 2009; NAKAUMA *et al.*, 2011). Moreover, this view was supported by KUMAGAI (2011) who introduced some viscoelastic parameters as suitable parameters for characterizing both liquid- and gel-like dysphagia-oriented products.

Considering the inescapable viscoelastic features of food products, it seems that the classification of dysphagia oriented products only by one single steady state rheological parameter may lead to misdirect investigations and taking inappropriate treatment policies.

It can be seen from the data in Table 4 that sixth group of cluster classification contains samples which have apparent viscosities five times more than the minimum limit of the spoon thick group of the NDD.

The findings of the current study are consistent with those of CASANOVAS (2011) who add a fifth class to conventional NDD classes which is called "pate class". In his major study on the "classification of DOPs", he proposed mixture of flow, thixotropy and oscillatory parameters as criterion for classification of DOPs (CASANOVAS *et al.*, 2011). Our findings further support the idea of adding more groups to conventional NDD groups.

The results of present study indicate that NDD categories became inaccurate to define all classes, as the rheological parameters were incorporated in the classification of DOPs, and the place of some samples probably changed. Such variations were observed in all classes of

Table 4 - Membership degree to the six classes considered for all the products studied.

| Name | | Cluster | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| 1. | Strawberry drink (D) | 0.99 | 0 | 0 | 0 | 0 | 0 |
| 2. | Melon drink (D) | 0.99 | 0 | 0 | 0 | 0 | 0 |
| 3. | Vegetable soup (H) | 0.99 | 0 | 0 | 0 | 0 | 0 |
| 4. | Guava Nectar (A) | 0.97 | 0 | 0.01 | 0 | 0 | 0 |
| 5. | Apricot Nectar (A) | 0.97 | 0 | 0.01 | 0 | 0 | 0 |
| 6. | Mango Nectar (C) | 0.97 | 0 | 0.01 | 0 | 0 | 0 |
| 7. | Peach Nectar (A) | 0.97 | 0 | 0.01 | 0 | 0 | 0 |
| 8. | Apricot Nectar (B) | 0.97 | 0 | 0.01 | 0 | 0 | 0 |
| 9. | Mango Nectar (A) | 0.97 | 0 | 0.01 | 0 | 0 | 0 |
| 10. | Peach-mango Nectar (C) | 0.97 | 0 | 0.01 | 0 | 0 | 0 |
| 11. | Halim (F) | 0.94 | 0 | 0.04 | 0 | 0 | 0 |
| 12. | Cappochino coconut drink (D) | 0.86 | 0 | 0.1 | 0 | 0.02 | 0 |
| 13. | Chicken soup (I) | 0.81 | 0 | 0.14 | 0.01 | 0.02 | 0 |
| 14. | Lentile soup (F) | 0.71 | 0 | 0.23 | 0.01 | 0.03 | 0 |
| 15. | Chocolate coconut (D) | 0.47 | 0 | 0.44 | 0.02 | 0.05 | 0 |
| 16. | Sholezard | 0 | 0.99 | 0 | 0 | 0 | 0 |
| 17. | Aloevera yoghurt (G) | 0 | 0 | 0.99 | 0 | 0 | 0 |
| 18. | seven yoghurt(G) | 0 | 0 | 0.98 | 0 | 0 | 0 |
| 19. | Apple yoghurt (G) | 0.02 | 0 | 0.96 | 0 | 0.01 | 0 |
| 20. | Spinach yoghurt (G) | 0.01 | 0 | 0.93 | 0 | 0.03 | 0 |
| 21. | Strawberry yoghurt (G) | 0.04 | 0 | 0.65 | 0.03 | 0.25 | 0 |
| 22. | Celery yoghurt (G) | 0.2 | 0 | 0.63 | 0.02 | 0.05 | 0 |
| 23. | Chocolate dessert (D) | 0 | 0 | 0 | 0.99 | 0 | 0 |
| 24. | Biscuit dessert (D) | 0 | 0 | 0 | 0.98 | 0 | 0 |
| 25. | Vanilla dessert (D) | 0 | 0 | 0 | 0.95 | 0.03 | 0 |
| 26. | Banana dessert (D) | 0 | 0 | 0.01 | 0.92 | 0.05 | 0 |
| 27. | Shirberenj (G) | 0 | 0 | 0.02 | 0.86 | 0.1 | 0 |
| 28. | Fig Cream (G) | 0.07 | 0.02 | 0.11 | 0.34 | 0.19 | 0.24 |
| 29. | Peach yoghurt (G) | 0 | 0 | 0 | 0 | 0.99 | 0 |
| 30. | Strawberry dessert (D) | 0.01 | 0 | 0.05 | 0.07 | 0.85 | 0 |
| 31. | Caramel dessert (L) | 0.01 | 0 | 0.08 | 0.04 | 0.85 | 0 |
| 32. | Cucumber yoghurt (G) | 0.03 | 0 | 0.2 | 0.05 | 0.70 | 0 |
| 33. | Eggplant dish (J) | 0.05 | 0 | 0.2 | 0.12 | 0.60 | 0 |
| 34. | Fig olive yoghurt (G) | 0.01 | 0 | 0.05 | 0.4 | 0.50 | 0 |
| 35. | Saffron dessert (G) | 0 | 0 | 0 | 0 | 0 | 0.94 |
| 36. | Coffee dessert (G) | 0 | 0.01 | 0 | 0.01 | 0.01 | 0.94 |
| 37. | 25.Ferni (K) | 0 | 0 | 0.01 | 0.02 | 0.01 | 0.92 |
| 38. | Cocoa cream dessert (L) | 0.03 | 0.09 | 0.03 | 0.06 | 0.04 | 0.72 |
| 39. | Halim (E) | 0.04 | 0.02 | 0.06 | 0.16 | 0.1 | 0.59 |

NDD, which indicated that effect of including viscoelastic parameters of foodstuffs, should be considered on NDD categories.

In the current study, based on the classification method (NDD or cluster classification), there were some differences between the numbers of members in the classes. For example, non-thickened group of NDD in this study contained all seven nectars. As shown in Table 4, class 1 of cluster classification consisted of these seven nectars in addition to eight other samples.

Taken together, the findings of this study suggested that further clinical trials are required to introduce the most effective rheological parameters for attaining a safe swallow and achieving a proper basis for classification of dysphagia-oriented products.

## CONCLUSIONS

This essay has argued National Dysphagia diet's classification criterion and explained the importance of including viscoelastic parameters in this classification. The evidence from this study suggests that more groups are necessary to accurately classify DOP's. There is, therefore, a definite need for encouraging the American Dietetic Association to update their taskforce and include more parameters and more categories in their classification.


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PAPER

# BIOACTIVE COMPOUNDS IN INDUSTRIAL TOMATO SAUCE AFTER PROCESSING AND STORAGE

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ABSTRACT

The effects of industrial processing, product parameters (lethality $F^{10}_{100}$ and °Brix) and storage (up to 24 months at $T_{room}$) on the content and antioxidant capacity (AC) of the bioactive compounds in industrial tomato sauce were studied. The total phenolic content (TPC) of both the hydrophilic and lipophilic fractions (using the Folin–Ciocalteu assay and a direct absorbance reading at 280 nm), in addition to the lycopene content and AC (using the $ABTS^+$ assay), were analysed. Statistical analysis showed that the content and activity of the studied bioactive constituents were positively influenced by the °Brix but not influenced by the thermal treatment severity (up to 40 min as $F^{10}_{100}$), that the TPC and its AC did not vary during storage, and that the lycopene AC decreased by 30% in the first year and then remained constant. The lycopene fraction and the hydrophilic fractions contributed 83% and 27%, respectively, of the sauce AC.

- Keywords: antioxidant activity, bioactive compounds, phenolics, shelf life, thermal treatment, tomato sauce -

## INTRODUCTION

Tomatoes, the second most consumed vegetable in the world after potatoes, are the main component of the Mediterranean diet. The world production of tomatoes for processing in 2012 was estimated at 33.44 million tons, with California, Italy and China being the largest producers (11.46, 4.50 and 3.23 million tons, respectively) (WPTC, 2013). Tomatoes are a very important dietary source of carotenoids, especially lycopene, which represents approximately 80-90% of the total carotenoid content in tomatoes and confers their characteristic red colour; β-carotene is the second most abundant carotenoid in tomatoes (3-5% total tomato carotenoid content) (SHI and LE MAGUER, 2000). Tomatoes, either fresh or as a processed product, contain different types of micronutrients with a high nutritional value, such as vitamins (C and E), folates, carotenoids and phenolic compounds (PERIAGO and GARCIA-ALONSO, 2009; SÁNCHEZ-RODRÍGUEZ *et al.*, 2012). In addition to being potent antioxidants, these compounds also have anti-inflammatory, antimutagenic and anticarcinogenic properties as well as the capacity to modulate key cellular enzyme functions (GIOVANNUCCI, 2005; VALLVERDÚ-QUERALT *et al.*, 2011a). It is known that thermal treatment and storage can cause a decrease in the nutritional value and a change in the colour of tomato products (CAPANOGLU *et al.*, 2010).

Numerous studies have investigated the micronutrient content of fresh and processed tomatoes, with the majority studying the loss of one or two types of micronutrients (SHARMA and LE MAGUER, 1996; RE *et al.*, 2002; GAHLER *et al.*, 2003; GRAZIANI *et al.*, 2003; LAVELLI and TORRESANI, 2011). However, to date, only a few studies have been published regarding the effect of technological processes or storage on antioxidants in tomatoes and tomato products (ABUSHITA *et al.*, 2000; GIOVANELLI and LAVELLI, 2002; LAVELLI and GIOVANELLI, 2003; CAPANOGLU *et al.*, 2008; MURCIA *et al.*, 2009; PÉREZ-CONESA *et al.*, 2009; CAPANOGLU *et al.*, 2010; COLLE *et al.*, 2010; VALLVERDÚ-QUERALT *et al.*, 2011a, 2011b, 2012). Furthermore, only a few of the cited authors have investigated changes in bioactive compounds due to processing at the industrial level, and none have investigated storage periods of longer than 12 months. The majority of the published studies involved laboratory-scale or pilot-scale experiments, and significant deviations have been found when comparisons were made with the end products of industrial-scale processes (CAPANOGLU *et al.*, 2010). Therefore, it is essential to examine actual commercial processes in order to obtain reliable data regarding the quality of the product that actually reaches the consumer.

In this context, the objectives of this work were to study the effects of various processes and product parameters, in particular the equivalent thermal effect or lethality (expressed as $F^{10}_{100}$, corresponding to equivalent minutes at 100°C, taking as reference a microorganism with a thermal resistance (z value) of 10°C), the °Brix, and prolonged storage periods (up to 24 months at room temperature, compared to an expected shelf-life of 3 years), on the content and activity of the bioactive compounds in industrial tomato sauce, applying simple and rapid analytical methods suitable for application in industrial quality control laboratories. The bioactive substances studied include lycopene and the total phenolics compounds.

## MATERIALS AND METHODS

### Reagents

Gallic acid, potassium persulfate, ascorbic acid and sodium carbonate were purchased from Carlo Erba (Milan, Italy), and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) was purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Folin–Ciocalteu reagent was purchased from Merck (Darmstadt, Germany), and butylated hydroxytoluene (BHT) was purchased from Sigma Aldrich (St. Louis, MO, USA).

Acetone, ethanol (95%), methanol and hexane, all solvents of analytical reagent grade, were obtained from Carlo Erba Reagents (Milan, Italy).

### Samples

Cans of tomato sauce (5 kg) were kindly provided by Steriltom Srl (Piacenza, Italy). This company uses both hydrostatic and continuous atmospheric retorts (AR). The products used in the present study were sterilised in two hydrostatic retorts (HRa and HRb). The temperature at the cold point of the product was measured using a Datatrace® Micropack III Temperature Data Logger for each sterilisation cycle. Industrial processing was carried out in order to achieve a minimum sterilising effect of 12 min as ($F^{10}_{100}$), which is required for the inactivation of spores of *Clostridium pasteurianum* and *Bacillus coagulans,* the most important spoilage-causing microorganisms in tomato products with a pH of 4.5 or less.

The total soluble solids were determined using a digital refractometer (RFM712, Bellingham + Stanley Ltd), and the results are reported as °Brix. The equivalent thermal effect obtained for each process was evaluated using Datatrace® equipment placed inside the can. For the specific aim of this study, the company provided samples of non-sterilised tomato (NS), for which the $F^{10}_{100}$ value was measured. Because preliminary studies had shown the influence of the position in the retort on the process lethality, for this investigation samples were collected from the hottest plant locations. Because the cans used to evaluate the F values could not be used for product analysis, the samples for experimentation were

collected from positions in the retort close to the can with the temperature data logger.

For the analyses, each can was opened, the content was manually homogenised with a glass rod, and the hydrophilic fraction (HF), lipophilic fraction (LF) and lycopene fraction (LYF) were obtained. The HF and LF were tested for total phenolic content (TPC) and antioxidant capacity (AC). The LYF was tested for lycopene content and AC. All data in this work were obtained from the analysis of three cans in triplicate.

**Experimental protocol**

Because the first aim of this study was to assess the influence of process lethality on the content and activity of bioactive compounds, products were analysed with the same °Brix and storage age. To achieve different equivalent thermal effect values, five series of samples with different $T_{hi}$ (temperature of the product cold point at the start of heating, measured by the thermocouple) were collected (Table 1). For each series, the samples from HRa and HRb were collected at the same day and time (to ensure the same °Brix). For the non-sterilised samples, the cans were not processed in the retorts but instead allowed to cool at room temperature, and a lethality value of 1.9 was assumed as the average of the $F^{10}_{100}$ values experimentally evaluated for the three different $T_{hi}$ values from the process $T_{hi}$ range; that is, $F^{10}_{100}$ values of 2.5, 1.8 and 1.4 min for $T_{hi}$ values of 84.9°, 83.6°C and 82°C, respectively.

The sampling of the cans was planned and carried out throughout the processing season in order to permit analysis of all the samples exactly 6 months after their collection and to sample the entire production from a single harvest. NS samples were unavailable for one of the series because the cans exploded before analysis; fortunately, this did not occur for the other series, demonstrating the high hygienic quality of the raw material. Furthermore, it was not possible to obtain exactly the same °Brix for all the samples (Table 1), and therefore the data were also elaborated to assess any possible influence of variations in the °Brix.

The second aim of the study was to assess the influence of long-term storage on the content and AC of bioactive compounds, analysing products with the same received lethality (21.3 and 1.8 min for sterilised and non-sterilised samples, respectively) and °Brix (6.15). All the samples were stored in a non-air-conditioned room and periodically analysed (after 0, 30, 64, 100, 133, 330, 407, 575 and 735 days). The non-sterilised samples were monitored for only 4 months.

**Separation of hydrophilic and lipophilic fractions**

The method was adapted from LARROSA *et al.* (2003) and DJURIC and POWELL (2001). The tomato sauce (35 g) was centrifuged in a Varifuge 20 RS (Heraeus Sepatech GmbH, Hanau, Germany) at 10,000 g and 4°C for 10 min. Two fractions were obtained: a nearly colourless supernatant and a red pellet. The pellet was washed three times with 20 mL of distilled water. The four aqueous supernatants were pooled, and the total volume was measured ($V_{HF}$) and filtered through filter paper (Whatman 595 ½) to give the hydrophilic fraction (HF). The red pellet was washed three times with 15 mL of acetone/methanol (70:30 v:v), shaken, and further centrifuged under the same conditions as specified for the HF. The reddish supernatants obtained were pooled, and the total volume was measured ($V_{LF}$) and filtered through filter paper (Whatman 595 ½) to give the hydrophobic or lipophilic fraction (LF). The resulting colourless pellet was discarded. The LF was concentrated using a rotavapour BÜCHI B – R114 (BÜCHI, Flawil, Switzerland) to eliminate the acetone that could have interfered in the

Table 1 - Sample series for the investigation of the influence of the process equivalent thermal effect (HR: hydrostatic retort; NS: non-sterilised sample; $T_{hi}$ temperature in the coldest point of the product at the beginning of heating; $T_{hmax}$ maximum temperature reached in the coldest point during heating).

| Series | Sample type | $T_{hi}$ (°C) | $T_{hmax}$ (°C) | pH | °Brix | $F^{10}_{100}$ (min) |
|---|---|---|---|---|---|---|
| 1 | HRa | 85.3 | 97.3 | 4.31 | 6.72 | 31.9 |
| | HRb | 86.0 | 98.5 | 4.32 | 6.70 | 38.7 |
| | NS | | | | 6.37 | 1.9 |
| 2 | HRa | 84.0 | 96.6 | 4.25 | 7.42 | 25.6 |
| | HRb | 84.6 | 97.4 | 4.26 | 7.37 | 27.8 |
| | NS | | | | 7.40 | 1.9 |
| 3 | HRa | 84.1 | 96.0 | 4.27 | 6.92 | 21.4 |
| | HRb | 84.9 | 97.4 | 4.26 | 7.10 | 31.2 |
| | NS | | | | 7.02 | 1.9 |
| 4 | HRa | 85.1 | 95.7 | 4.24 | 7.44 | 18.7 |
| | HRb | 83.9 | 95.7 | 4.26 | 7.53 | 16.5 |
| | NS | | | | 7.50 | 1.9 |
| 5 | HRa | 83.1 | 95.1 | 4.28 | 7.33 | 18.1 |
| | HRb | 85.6 | 95.7 | 4.29 | 7.41 | 37.2 |

spectrophotometric analysis. After removing the acetone, the extract was brought to 50 mL with methanol and homogenised through agitation.

**Recovery of Lycopene fraction**

The lycopene fraction (LYF) was recovered according to the method proposed by FISH *et al.* (2002). The tomato sauce (600 mg) was placed in an amber screw-top vial (40 mL) with 5 mL of 0.05% (w/v) BHT in acetone, 5 mL of ethanol, and 10 mL of hexane. The samples were extracted on an orbital shaker (HT INFORS AG CH-4103, Bottmingen, Switzerland) at 180 rpm for 15 min in an ice bath. After shaking, 3 mL of distilled water was added to each vial, and the samples were then shaken for 5 min on ice. The vials were left at room temperature for 5 min to allow the separation of the reddish upper hexane phase (LYF). For the estimation of AC, a BHT blank was obtained with the same extraction procedure but without tomato sauce, even though it is known from the literature that the ABTS response of BHT is negligible compared to that of lycopene (MÜLLER *et al.*, 2011).

The lycopene content was estimated by measuring the absorbance at 503 nm (Lambda Bio 40 UV-Vis spectrophotometer, Perkin-Elmer, Norwalk, CT) in a 1 cm path length quartz cuvette using hexane solvent as a reference. The total lycopene content in each sample was estimated from Eq. (1):

$$\text{Lycopene (mg kg}^{-1}) = \frac{A_{503}}{\varepsilon \times b} \times M_w \times \frac{\text{volume extract (L)}}{\text{tomato pulp (kg)}} = \frac{A_{503} \times 31.2}{g_{tissue}} \quad (1)$$

where: $A_{530}$ is the absorbance at 530 nm; ε is the molar extinction coefficient ($17.2 \times 10^4$ $M^{-1}cm^{-1}$) for lycopene in hexane, as reported by ZECHMEISTER *et al.* (1943); b is the path length of the quartz cuvette (1 cm); $M_w$ is the molecular weight of lycopene (536.9 g $mol^{-1}$ $cm^{-1}$); and volume extract is the LYF or hexane phase in $10^{-2}$ L.

Although it is not the lycopene maximum absorption wavelength, the absorbance at 503 nm was used to minimise interference from other carotenoids because it has been reported that their contribution to the absorbance at 503 nm is less than 4% in fresh red tomatoes (FISH *et al.*, 2002).

**Total phenolic content**

The total phenolics were determined using two different methods:

(1) Folin-Ciocalteu assay: this method was used because it is widely employed to study natural antioxidants and is considered the best method to determine the content of total phenolics (including tannins) (ENGELHARDT, 2001). Samples of HF and LF were analysed without dilution according to the method reported in AMENDOLA *et al.* (2010). Solid phase extraction to eliminate interference from, for example, ascorbic acid, amino acids and reductant sugars (VALLVERDÚ-QUERALT *et al.*, 2012), was not carried out in order to simplify the procedure for potential routine industrial product control. Regarding the possible interference from reducing sugars, considering the maximum °Brix of the samples, that in tomatoes °Brix is not given only by reducing sugars and sample dilution in the experimental procedure, it was estimated that the minimum 2% sugar concentration necessary for the correction of Folin's results (WATERHOUSE, 2002) was not reached. To address possible interference from ascorbic acid (which was not measured in the samples), blank trials were carried out. Based on the literature values for the ascorbic acid content in fresh tomatoes and processed products (SINGH *et al.*, 2008; CHANFORAN *et al.*, 2012; KOH *et al.*, 2012), a content of 125 and 250 mg/kg ascorbic acid in the product was assumed. Assuming that all the ascorbic acid was extracted in the HF, blanks of standard ascorbic acid were prepared at concentrations of 50 and 100 mg/L and analysed with the Folin assay. The results demonstrated absorbance values of 0.050±0.003 and 0.101±0.005 for the 50 and 100 mg/L concentrations, respectively, while all of our samples had absorbance values above 0.2.

(2) Direct reading of the absorbance of the sample at 280 nm: this is a more rapid procedure based on the absorbance of the aromatic ring (AMENDOLA *et al.*, 2010) and the wavelength used to detect phenolic compounds in tomato products (CHANFORAN *et al.*, 2012). Samples of HF and LF were analysed after 50 dilutions.

In both cases, the total phenolics were expressed as gallic acid equivalents (GAE-Folin and GAE-280) by means of calibration curves with standard gallic acid in the concentration range of 100-750 and 2.5-30 mg/L for methods 1 and 2, respectively. In particular, the results were reported as mg/kg content of tomato sauce considering the $V_{HF}$ or the $V_{LF}$ and the amount of tomato sauce used for extraction, according to Eq. (2).

$$\frac{mg_{GAE}}{kg_{sauce}} = \frac{mg_{GAE}}{L} \frac{L_{V_{HF}/V_{LF}}}{kg_{sauce}} \quad (2)$$

**Antioxidant capacity**

The antioxidant capacity was assessed using the ABTS assay (AMENDOLA *et al.*, 2010), which is based on the ability of antioxidants to interact with the ABTS radical, decreasing its absorbance at 734 nm. A radical solution (7 mM ABTS and 2.45 mM potassium persulfate) was prepared and kept in the dark at room temper-

ature for 12–16 h before use. This solution was then diluted with ethanol to obtain an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30 °C. For the analysis, cuvettes for the samples and blanks were prepared, kept in the dark for 6 minutes and then read on the spectrophotometer (absorbance at 734 nm against ethanol). For the samples, 2 mL of the diluted radical solution was mixed with 20 μL of sample (HF, LF or LYF), and for each sample, three replicates were analysed. Two different blanks were prepared; one for the solvent, consisting of 2 mL of diluted ABTS mixed with 20 μL of solvent extraction (water for HF, methanol for LF, and hexane for LYF), and one for the ABTS, consisting of 2 mL of diluted ABTS. For each trial, two replicate blank samples were included. The antioxidant power (AOP) was calculated as the percentage inhibition of the absorbance at 734 nm according to Eq. (3):

$$AOP = \%\ \text{inhibitionof}\ ABTS_{734\,nm} = \\ = \frac{Abs_{SolventBlank} \cdot Abs_{Sample}}{Abs_{ABTSBlank}}\ 100 \qquad (3)$$

For the analysis of LYF, the AOP values were corrected with the AOP of the blank of BHT, giving values in the 20-40% range.

For the analysis of the HF, blanks of ascorbic acid were prepared as described for the total phenolic content and analysed for the AOP, giving values of 12.01±.13% and 23.70±0.84% for the 50 and 100 mg/L concentrations, respectively. The AOP in the analysed HF samples was always in the range 35-60%, while in the LF it was < 10%.

For the samples with an AOP < 30-40% it was not possible to obtain a proper dose-response curve (in terms of the change in AOP as a function of the sample concentration). Therefore, for a better comparison of all the samples, the specific antioxidant power (AOP') was also determined either in reference to the concentration of bioactive compounds (total phenolics or lycopene) in the sample according to Eq. (4) or as the specific power (AOP'') referred to in tomato sauce according to Eq. (5) (AMENDOLA *et al.*, 2010; AMENDOLA *et al.*, 2012):

$$AOP' = \frac{AOP\,\%}{mg\ L^{-1}_{GAE_{Folin}\ or\ GAE_{280}\ or\ Lycopene}} \qquad (4)$$

$$AOP'' = \frac{AOP\,\%}{20\ \ 10^{-3}\ ml} \cdot \frac{ml_{HF\ or\ LYF}}{mg_{tomato\ sauce}} \qquad (5)$$

**Statistical analysis**

The results reported in this paper are the average of the replicates ± SD. The statistical software SPSS (version 19.0, SPSS Inc., Chicago, IL, USA) was used to assess the influence of the $F^{10}_{100}$, the °Brix and the storage time on the content and activity of the bioactive compounds using univariate analysis of variance (ANOVA) at a confidence level of over 99%. When significance was found, a post-hoc test was carried out for mean discrimination. When variance homogeneity (according to Levene's test) was found, Tukey's post-hoc test was applied, while in the presence of non-homogeneous variances, a Browne-Forsythe ANOVA and the Games Howell post-hoc test were applied.

## RESULTS AND DISCUSSION

**Analytical and methodological approach**

The spectral analysis at 280 nm of the total phenolic compounds can be subject to influences from aromatic compounds other than phenolics, and each class of phenolic substances has a different absorptivity. However, this method is not influenced by the oxidative status of the molecules (AMENDOLA *et al.*, 2010), and it is a very rapid method, suitable for monitoring the influence of process parameters on the product.

On the other hand, the Folin assay, although traditionally used to estimate the total phenolic content, can be considered an antioxidant capacity method based on a single electron transfer (SET) mechanism (PRIOR *et al.* 2005); the results are thus influenced by the oxidative status of the molecules. The absolute values of the total phenolic content, although expressed in both cases as GAE, cannot be compared due to the different analytical principles. Folin analysis of the LF consistently gave absorbance values below 0.1, so the results are not reported.

Although seemingly redundant with the Folin assay, the ABTS test is usually classified as an SET reaction, but the radical can be neutralised either by direct reduction *via* electron transfer or by radical quenching *via* H atom transfer (HAT) (PRIOR *et al.*, 2005). Therefore, even though currently a minimum of two assays are recommended in the evaluation of AC, we carried out only the Folin assay and the $ABTS_{F^{10}_{100}}$ test due to the screening nature of the present study.

Ascorbic acid is a recognised bioactive compound in tomatoes and related products (LAVELLI and GIOVANNELLI, 2003; SÁNCHEZ-MORENO *et al.*, 2006) and it can interfere with both the Folin and the ABTS assay. Analysis of blanks (prepared based on the ascorbic acid content of fresh tomatoes and of processed products, as reported in the literature) revealed that ascorbic acid could contribute up to 50% of the Folin reducing power or of the ABTS AOP. On one hand, this means that in the evaluation of the total AC of a tomato product, ascorbic acid should not be removed from the HF in the purification steps,

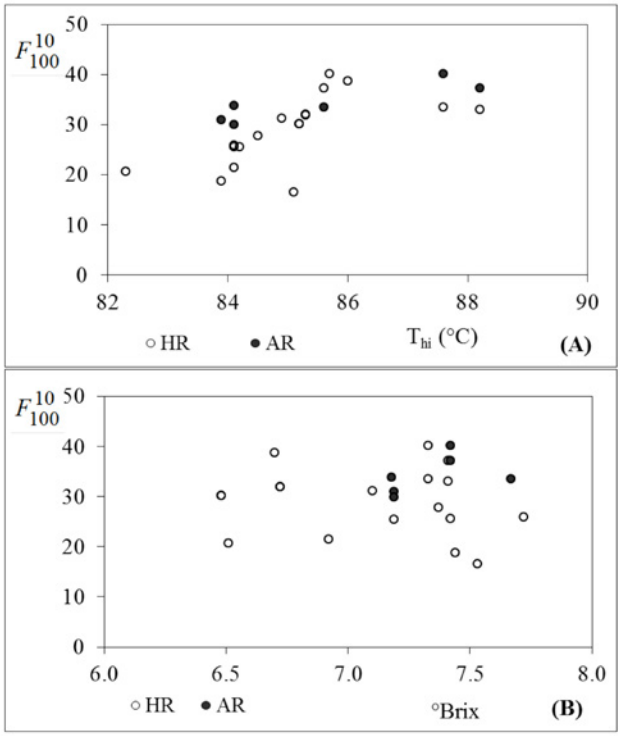



Fig. 1 - Influence of filling temperature (A) and °Brix (B) on process lethality ($F^{10}_{100}$) for products processed in hydrostatic (HR) or atmospheric (AR) retort.

as was done in the present work. On the other hand, this also means that evaluation of the ascorbic acid content in the HF is advised in order to properly reference the Folin and ABTS values only to the phenolic content.

### Effect of industrial process parameters

In the industrial process, the temperature of the product at filling is a pre-set value but inevitably undergoes some variations during operation. Similarly, the °Brix of the product varies slightly depending on the raw material and the harvest phase. Preliminary investigations had shown a great influence of $T_{hi}$ on the achieved lethality (Fig. 1a), independent of both the retort type and of °Brix, at least in the very narrow measured °Brix range (Figure 1b). Based on these assumptions, in the experimental protocol samples with different $T_{hi}$ were collected in order to obtain different lethality values. For statistical analysis, the samples were grouped according to different $F^{10}_{100}$ ranges (1.9 min; 17-21.5 min; 26.6-32 min; 37-39 min) and different °Brix (6.4-6.7; 6.9-7.3; 74-7.5). Considering the very limited °Brix range and that there should be no interaction between the °Brix and the process lethality for the solid contents (as also evidenced in Figure 1b), statistical analysis was carried out separately on the two factors. The ANOVA results are summarised in Table 2.

As was expected given its very limited variation, the °Brix was found to have a significant influence only on the total content of phenolic compounds and on the tomato sauce specific AOP" based on the HF, with a slight tendency toward higher values at higher °Brix degrees (Table 3).

The content and activity of the studied bioac-

Table 3 - Means discrimination of the total phenolics (as GAE, gallic acid equivalents) and of the specific antioxidant power of tomato sauce (AOP") in the hydrophilic fraction according to the °Brix (Tukey's test). Means of the same parameter with the same letter were not significantly different ($\alpha$ =0.01).

| °Brix | GAE-280 (mg kg$^{-1}$) | AOP" |
|---|---|---|
| 6.4-6.7 | 918.21$^{a}$ | 5.19$^{a}$ |
| 6.9-7.3 | 954.76$^{a,b}$ | 5.70$^{a,b}$ |
| 7.4-7.5 | 1034.94$^{b}$ | 6.31$^{b}$ |

Table 2 - Results of ANOVA analysis of the influence of the process parameters on the hydrophilic fraction (HF), lipophilic fraction (LF) and lycopene fraction (LYF) of sterilised (S) and non-sterilised samples (NS). GAE: gallic acid equivalents. AOP': specific Antioxidant Power of the different bioactive compounds. AOP": specific Antioxidant Power of tomato sauce.

| Final Product characteristic | | Sig. | | | |
|---|---|---|---|---|---|
| | | $F^{10}_{100}$ | °Brix | Storage time (S) | Storage time (NS) |
| HF | GAE-Folin (mg kg$^{-1}$) | 0.000 | 0.025 | 0.377 | 0.216 |
| | AOP' Folin | 0.221 | 0.218 | 0.029 | 0.209 |
| | GAE-280 (mg kg$^{-1}$) | 0.000 | 0.004 | 0.000 | 0.062 |
| | AOP' 280 | 0.030 | 0.373 | 0.000 | 0.074 |
| | AOP" | 0.837 | 0.001 | 0.000 | 0.169 |
| LF | GAE-280 (mg kg$^{-1}$) | 0.801 | 0.361 | n.d. | n.d. |
| | AOP' 280 | 0.329 | 0.270 | n.d. | n.d. |
| | AOP" | 0.299 | 0.046 | n.d. | n.d. |
| LYF | Lycopene (mg kg$^{-1}$) | 0.000* | 0.106 | 0.000 | 0.011 |
| | AOP' lycopene | 0.001* | 0.031 | 0.000 | 0.054 |
| | AOP" | 0.457 | 0.053 | 0.000 | 0.005 |

* non-homogeneous variances;
n.d. : not determined.

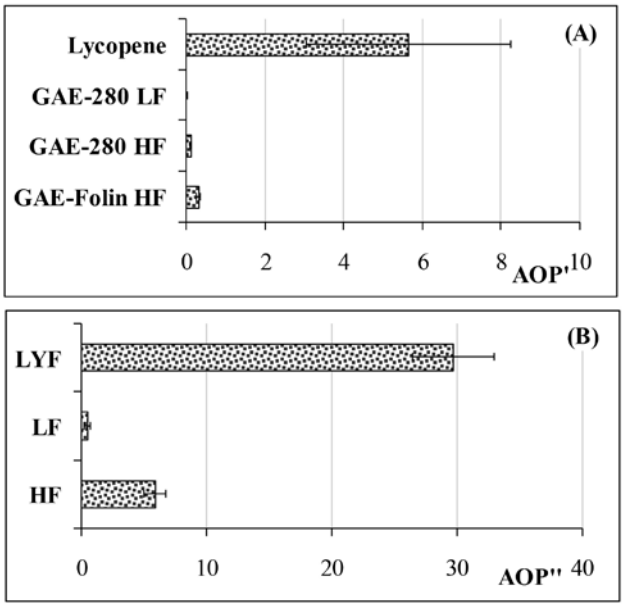



Fig. 2 - Specific antioxidant power of the different bioactive compounds [AOP' in (A)] and of tomato sauce [AOP'' in (B)] based on the different recovered fractions (values are the average of all the samples; error bars indicate ± SD; LF: lipophilic fraction; HF: hydrophilic fraction; LYF: lycopene fraction).

tive constituents of tomato sauce were separated according to the $F^{10}_{100}$ range only in some cases. Lethality was observed to influence the total phenolic content of HF according to both analytical methods, but the means discrimination (Table 4) did not allow for a clear distinction between the samples. Overall, it was observed that an increase in the severity of thermal treatment within the observed range (2-40) did not cause any variation in the phenolic content, as also reported by PEREZ-CONESA *et al.* (2009). Our GAE-Folin results are consistent with the phenolic content of tomato and tomato products (between 100 and 500 mg $kg^{-1}$) described in the literature (VALLVERDÚ-QUERALT *et al.*, 2011a). Considering the GAE-280 results and Eq. (2), the contribution of the HF to the total phenol content of the tomato sauce was calculated as the ratio between the $mg_{GAE\text{-}HF}/kg_{sauce}$ and the sum ($mg_{GAE\text{-}HF}/kg_{sauce}$ + $mg_{GAE\text{-}LF}/kg_{sauce}$), confirming that almost all of the polyphenols (83-91%) were contained in the hydrophilic fraction (TOOR and SAVAGE, 2005).

Confirming the Folin results, lethality did not influence either the AOP' or the AOP'', and a comparison among the hydrophilic, lipophilic and lycopene fractions showed that the greatest contribution to the AC of tomato sauce came from lycopene (Fig. 2). In fact, the LF should have given values close to those of LYF. This means that the procedure used to separate the LF was inadequate, while that used for lycopene extraction (in an ice bath and with the addition of an antioxidant) permitted the conservation of the bioactivity of the extracted compounds.

Likewise, for the lycopene content, despite the significant influence of lethality, the means could not be properly separated in the post-hoc test (Table 4), even though with increasing treatment severity an apparent trend of decreasing content and increasing activity was found. In the literature, it has been reported that heat treatment can exert a positive effect on carotenoid efficiency and absorption (ANESE *et al.*, 2002) and that carotenoids can undergo isomerisation and oxidation, depending on the conditions, but generally remain quite stable during processing (GIOVANELLI and LAVELLI, 2002). The values we obtained were consistent with data reported by other authors for tomato and tomato juices (GARCÍA-ALONSO *et al.*, 2009; PÉREZ-CONESA *et al.*, 2009; COLLE *et al.*, 2010).

### Effect of storage on bioactive compounds and antioxidant capacity

Based on the aforementioned conclusions about the inadequacy of the procedure used to separate the LF, the LF was not analysed for the storage samples.

The unsterilised samples did not significantly differ from their sterilised counterparts for any of the investigated parameters at any time, confirming the previous finding. Only a few exceptions were found for the total phenolic content (as GAE-280) of the HF (with lower values for the NS samples) and for the related AOP' (with higher values for the NS samples).

The significance of the influence of storage time on the analysed parameters and the results of the means discrimination are reported in Tables 2 and 5, respectively. For the unsterilised samples (monitored for 4 months), only the AOP'' based on the LYF was influenced by storage time, even though Tukey's post-hoc test could not discriminate the means. Regarding the processed samples, it can be concluded that the total phenolic content of the HF remained unvaried during the storage time, consistent with previous studies in which shorter periods were investigated (GIOVANELLI and PARADISO, 2002; GARCÍA-ALONSO *et al.*, 2009; VALLVERDÚ-QUERALT *et al.*, 2011a). However, a decrease in the specific antioxidant capacity of the tomato sauce (AOP'') occurred after one year of storage.

As for the LYF, the lycopene content remained almost significantly unchanged. In fact, only

Table 4 - Means discrimination by either Tukey's test (total phenolics of hydrophilic fraction) or Games Howell's test (lycopene content and lycopene specific activity) according to the process equivalent thermal effect ($F^{10}_{100}$). GAE: Gallic Acid Equivalents. AOP': specific Antioxidant Power.

| $F^{10}_{100}$ (min) | GAE-Folin (mg $kg^{-1}$) | GAE-280 (mg $kg^{-1}$) | Lycopene (mg $kg^{-1}$) | Lycopene AOP' |
|---|---|---|---|---|
| 1.9 | $379.96^{b}$ | $902.43^{a}$ | $141.67^{a}$ | $4.32^{a}$ |
| 16.5-21.4 | $395.19^{b}$ | $1061.85^{b}$ | $131.67^{a}$ | $4.41^{a}$ |
| 25.6-31.9 | $333.75^{a}$ | $980.31^{a,b}$ | $89.17^{b}$ | $7.78^{a}$ |
| 37.2-38.7 | $359.50^{a,b}$ | $1019.96^{a,b}$ | $110.00^{a,b}$ | $6.16^{a}$ |

Table 5 - Means discrimination by Tukey's test for sterilised samples and non-sterilised samples (*NS*) of the hydrophilic fraction (HF) and of the lycopene fraction (LYF) according to storage time. Means of the same parameter with the same letter were not significantly different (α =0.01). GAE: Gallic Acid Equivalents. AOP': specific Antioxidant Power of the different bioactive compounds. AOP": specific Antioxidant Power of tomato sauce.

| Storage time (days) | GAE-Folin | | GAE-280 | | Lycopene | | | |
|---|---|---|---|---|---|---|---|---|
| | (mg kg$^{-1}$) (HF) | AOP' (HF) | (mg kg$^{-1}$) (HF) | AOP' (HF) | AOP" (HF) | (mg kg$^{-1}$) | AOP' | AOP" (LYF) |
| 0 | 337.58 | 0.39 | | | | | | |
| *NS* | *346.56*$^{a}$ | *0.33*$^{a}$ | 804.43$^{cd}$ | 0.16$^{a}$ | 6.52$^{a}$ | 103.02$^{a}$ | 6.17$^{a}$ | 31.47$^{a}$ |
| | $^{a}$ | $^{a}$ | *775.13*$^{d}$ | *0.15*$^{abc}$ | *5.80*$^{ab}$ | *102.01*$^{a}$ | *6.31*$^{a}$ | *32.15*$^{a}$ |
| 30 | 333.44$^{a}$ | 0.28$^{a}$ | 894.11$^{ab}$ | 0.11$^{bcde}$ | 4.72$^{abc}$ | 97.43$^{ab}$ | 6.70$^{a}$ | 32.61$^{a}$ |
| *NS* | *372.88*$^{a}$ | *0.31*$^{a}$ | *824.61*$^{bcd}$ | *014*$^{abcde}$ | *5.60*$^{ab}$ | *106.19*$^{a}$ | *6.14*$^{a}$ | *32.55*$^{a}$ |
| 64 | 333.16$^{a}$ | 0.28$^{a}$ | 839.33$^{bcd}$ | 0.11$^{bcde}$ | 4.72$^{abc}$ | 98.67$^{ab}$ | 5.84$^{ab}$ | 28.75$^{a}$ |
| *NS* | *357.23*$^{a}$ | *0.32*$^{a}$ | *683.12*$^{e}$ | *0.17*$^{a}$ | *5.73*$^{ab}$ | *88.22*$^{ab}$ | *6.78*$^{a}$ | *29.57*$^{a}$ |
| 100 | 355.19$^{a}$ | 0.32$^{a}$ | 810.34$^{cd}$ | 0.14$^{abcde}$ | 5.62$^{ab}$ | 94.13$^{ab}$ | 6.44$^{a}$ | 30.17$^{a}$ |
| *NS* | *308.93*$^{a}$ | *0.37*$^{a}$ | *777.13*$^{d}$ | *0.15*$^{abcd}$ | *5.76*$^{ab}$ | *101.74*$^{a}$ | *5.71*$^{abc}$ | *28.98*$^{a}$ |
| 133 | 313.32$^{a}$ | 0.31$^{a}$ | 929.26$^{ab}$ | 0.10$^{cde}$ | 4.84$^{abc}$ | 90.57$^{ab}$ | 6.38$^{a}$ | 28.80$^{a}$ |
| *NS* | *320.01*$^{a}$ | *0.31*$^{a}$ | *626.79*$^{e}$ | *0.16*$^{ab}$ | *4.91*$^{abc}$ | *103.36*$^{a}$ | *5.88*$^{ab}$ | *30.37*$^{a}$ |
| 330 | 342.07$^{a}$ | 0.25$^{a}$ | 851.06$^{cd}$ | 0.10$^{de}$ | 4.37$^{bc}$ | 92.63$^{ab}$ | 4.25$^{cd}$ | 19.55$^{b}$ |
| 407 | 347.69$^{a}$ | 0.23$^{a}$ | 828.18$^{bcd}$ | 0.09$^{e}$ | 3.91$^{bc}$ | 93.30$^{ab}$ | 3.91$^{c}$ | 18.06$^{b}$ |
| 575 | 355.99$^{a}$ | 0.26$^{a}$ | 872.03$^{abc}$ | 0.10$^{cde}$ | 4.57$^{abc}$ | 76.55$^{b}$ | 4.64$^{bcd}$ | 17.67$^{b}$ |
| 735 | 314.72$^{a}$ | 0.27$^{a}$ | 883.72$^{abc}$ | 0.10$^{cde}$ | 3.34$^{c}$ | 78.93$^{b}$ | 4.59$^{bcd}$ | 18.18$^{b}$ |

two homogenous groups could be discriminated by Tukey's test, one from 0 to 407 days, and a second from 30 to 735 days. This is consistent with the findings of GARCÍA-ALONSO *et al.* (2009), although other authors have reported final losses of approximately 60-70% (MIN *et al.*, 2003; LIN and CHEN, 2005; ODRIOZOLA-SERRANO *et al.*, 2008). However, comparisons between literature data are difficult due to differing tomato products and processing conditions. The specific antioxidant power of lycopene, and as a consequence the specific tomato sauce activity, decreased appreciably (30%) between 5 and 11 months of storage, and then remained constant thereafter up to 24 months.

## CONCLUSIONS

The present study showed that the sterilisation step of tomato sauce processing, in a severity of up to almost 40 min (as $F^{10}_{100}$), influenced neither the content nor the AC of the hydrophilic (related to phenolics content) or the lycopene fractions. However, the extraction method can greatly influence the results because the addition of an antioxidant compound (BHT) and low temperature conditions are both required to prevent lycopene degradation.

Industrial tomato sauce has been confirmed as a potential rich dietary source of bioactive compounds, although the lycopene-associated nutritional antioxidant profile showed a 30% reduction between 5 and 11 months of storage at room temperature and then remained stable until 24 months. Furthermore, the effects of further processing during home cooking on antioxidant activity must be taken into account, given that tomato sauce is generally further cooked (*i.e.*, for pasta or pizza dressing) before consumption.


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PAPER

# PREDICTION OF THE MAXIMAL GROWTH RATE OF *LISTERIA MONOCYTOGENES* IN SLICED MORTADELLA BY THE SQUARE ROOT TYPE MODEL

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ABSTRACT

The growth parameter (maximum specific growth rate, $\mu_{max}$) of two different strains of *Listeria monocytogenes* on sliced Mortadella were calculated. Three batches of sliced Mortadella were vacuum packed, stored at 8°C and samples collected at different time intervals were enumerated for *L. monocytogenes*. The pathogen counts were fitted using the DMFit version 2.1 Excel® add-in, based on Baranyi model, to determine the specific growth rates. At 8°C the sliced Mortadella supported rapid and prolific *L. monocytogenes* growth: the $\mu_{max}$ varied between 0.035/h and 0.044/h, and the pathogen counts increased up to $10^8$ CFU/g after 21-27 days. A square root type model, Ratkowsky model, was used to describe how $\mu_{max}$ changes as a function of storage temperature. Growth kinetics data could be useful for establishing a safe shelf life, once the product could be post process contaminated and stored under different handling scenarios.

- Keywords: challenge test, *Listeria monocytogenes*, predictive microbiology -

## INTRODUCTION

*Listeria monocytogenes* is a ubiquitous bacterium which is widely distributed in the environment. This pathogen may contaminate Ready To Eat (RTE) meat products through raw meat, ingredients or processing equipment, and/or through post-processing contamination (NISSEN and HOLCK, 1998; BARBUTI and PAROLARI, 2002; GOMBAS *et al.*, 2003; COLAK *et al.*, 2007), in particular, the slicing is an important stage to check for *L. monocytogenes* contamination, since it is the last processing step, without further thermal treatment, before consumption (LIN *et al.*, 2006). Temperature is identified as one of the crucial factors controlling growth of *L. monocytogenes* in RTE food products during the shelf-life (EFSA, 2008; FAO/ WHO, 2004). Processing and retail temperature are usually well controlled. The mean temperature of retail refrigerators in Europe ranged from 3.7 to 5.6°C (LIKAR and JEVSNIK, 2006; KOUTSOUMANIS and ANGELIDIS, 2007; EFSA, 2008). However, most of RTE-products spend a considerable part of their shelf-life in domestic refrigerators of which the temperature is less controlled. The mean temperature of eleven surveys cited in EFSA (2008) ranged from 5.2° to 7.2°C. Besides, 20 to 35% of refrigeration temperature in Europe was above 8°C. A more recent study in Spain showed that the temperature of the food product in the refrigerator was on average 7.9°C (n=33) (GARRIDO *et al.*, 2010).

Predictive microbiology combines knowledge of bacterial growth responses over a range of conditions with the power of mathematical modeling to enable predictions of growth.

Models are used to describe the behavior of microorganisms at different physical and chemical conditions, such as temperature, pH, and water activity. They can be used to predict microbial safety or shelf life of products, to find critical points in the process, and to optimize production and distribution chains. A major factor determining the specific growth rate of microorganisms in chilled foods is temperature. Predictive microbial models can be classified into primary and secondary models. While a primary model describes the growth of bacteria over a time period under constant environmental conditions such as temperature, the secondary model describes the effect of environmental conditions on the parameters of the primary model (WHITING, 1995).

Thus, in the present paper, a microbiological challenge test was carried out, and the primary model of BARANYI and ROBERTS (1994) was used to evaluate the maximum specific growth rate ($\mu_{max}$) of *L. monocytogenes* in artificially contaminated pork Mortadella stored in moderate thermic abuse. The effect of temperature on the $\mu_{max}$ of a microbial population can be described using the simple square root model of RATKOWSKY *et al.* (1982) in agreement with the European Union Community Reference Laboratory for *L. monocytogenes* (EU CRL, 2008). Therefore, the square root model was used to evaluate the specific growth rate at a chosen temperature, and to establish the time to reach the critical legal limit of 100 CFU/g in Mortadella product, in a domestic scenario (EUROPEAN COMMISSION, 2005).

## MATERIALS AND METHODS

### Mortadella samples

To evaluate the growth rate of *L. monocytogenes* on sliced Mortadella, three batches obtained from a commercial processing plant, were used for this trial. The basic Mortadella formulation consists of fresh pork (30% fat), trimmings, sodium chloride, dextrose, corn syrup solids, polyphosphate, sodium nitrite, spices and flavouring. The meat and non-meat ingredients are emulsified, processed, and cooked (core temperature at least 72°C). Mortadella is then cooled down and stored at refrigerator temperature. The fibrous cellulose casing is removed and the Mortadella is sliced. Thirty vacuum-packed trays, contained almost 100 g of sliced, were considered for each batch. Mortadella trays were transferred to the Microbiology laboratory for inoculation, storage and analysis.

### Bacterial cultures and inoculum preparation

*L. monocytogenes* serotype 4b was selected to carry out this challenge test on the basis of its well-recognized pathogenesis (CARRASCO *et al.*, 2008; NORRUNG 2000). Two strains of *L. monocytogenes* serotype 4b were used in these experiments: ATCC® 19115™ and Lm171767 as wild strain isolated from pork meat and classified by *Eco*RI ribotyping (DUP 1038).

Each stock cultures were kept frozen (-80°C) in Brain Heart Infusion (BHI; Oxoid, Milano, Italy) supplemented with 20% glycerol, separately regenerated by transferring into BHI and incubating at 37°C for 24 h. Each culture was centrifuged at 4,000x g for 60 min at 4°C, and the pellet was resuspended in sterile physiological solution and appropriately diluted. Counts were confirmed by serial decimal dilution and inoculation in Agar Listeria according to Ottaviani-Agosti (ALOA; Microbiol Diagnostici, Cagliari; Italy) plates.

### Samples inoculation

Each batch was inoculated separately with both strains of *L. monocytogenes*. The trays of Mortadella were aseptically opened and the slices were inoculated on the top surface with 1 % v/wt of *L. monocytogenes* suspension to a final concentration of about 1.5-2 log CFU $g^{-1}$ (con-

taminated samples) or with 1% v/wt of the sterile physiological solution (control samples). The inoculum was distributed over the entire surface with a sterile L-shaped plastic cell spreader (Incofar, Modena, Italy) and the slices were then vacuum packed using S100-Tecnovac equipment (Tecnovac, Bergamo, Italy).

### Microbiological and physico-chemical analysis

To verify the natural meat contamination level, three control samples were analysed at time zero for the presence of *L. monocytogenes* according to ISO 11290-1 (ISO, 2004a). For microbiological analysis, Mortadella sliced (100 g) were transferred into plastic one-chamber filter stomacher bags (NEOMED, Milano, Italy) and homogenized 1:3 (wt:v) in sterile peptone water (PW) (CONDA, Madrid, Spain) for 3 min in a Stomacher 400 blender (Seward Medical, London, UK). Decimal dilutions in sterile PW were prepared from each bag. On control samples, the lactic acid bacteria enumeration was carried out using the de Man Rogosa Sharpe agar (MRS; Microbiol Diagnostici) in micro-aerophilic condition at 37°C for 48-72 h. The quantitative analysis for *L. monocytogenes* enumeration was carried out on contaminated samples according to ISO 11290-2 (ISO, 2004b).

Measurement of pH (Hanna Instrument, USA) and water activity ($a_w$) (Decagon Devices, Inc., Pullman, USA) were carried out on control samples at the beginning and at the end of storage time. The packages of sliced Mortadella were stored at 8°C for 60 days.

### Mathematical models

Growth curves for each strain inoculated in each batch of sliced Mortadella were built separately by fitting data to the Baranyi model (BARANYI and ROBERTS, 1994) using DMFit version 2.1 Excel® add-in (www.combase.cc) in order to estimate the growth rate of *L. monocytogenes*.

The growth rate, derived from fitting the log values *versus* time (hours), was transformed to maximum specific growth rate, $\mu_{max}$ ($h^{-1}$) by multiplying it with ln(10).

Therefore, the highest $\mu_{max}$ ($\mu_{max\ ref}$) calculated at 8°C ($T_{ref}$), was used to estimate the $\mu_{max}$ at another temperature (T) using the square root secondary model (RATKOWSKY *et al.*, 1982; ZWIETERING *et al.*, 1996) (Eq. 1):

$$\mu_{max} = \mu_{max\,ref} \frac{(T - T_{min})^2}{(T_{ref} - T_{min})^2} \qquad \text{(Eq. 1)}$$

where $T_{min}$ is a minimal growth temperature for *L. monocytogenes* (-2°C) (EU CRL, 2008).

To establish the time to reach the critical legal limit of 100 CFU/g in Mortadella product, in a domestic scenario, was calculated the doubling time, *td* (h) of *L. monocytogenes* during the exponential growth phase (Eq. 2):

$$td = \frac{\ln(2)}{\mu_{max}} \text{ (Eq. 2)} \qquad \text{(Eq. 2)}$$

### Statistical analysis

Counting results were expressed as colony forming unit (CFU) per gram. Microbial counts were reported in terms of log CFU/g. Means and standard deviations were calculated and analysis of variance (ANOVA) followed by Tukey's test was used to check significant differences ($p$>0.05) regarding the microbiological and physico-chemical properties of three batches of sliced Mortadella. The data were statistically analysed using R statistical software version 2.7.0 (R Development Core Team, 2008).

## RESULTS AND DISCUSSION

The average values of lactic acid bacteria, pH and $a_w$ measured in three batches of sliced Mortadella at the beginning (day 0) and at the end (day 60) of the storage time were shown in Table 1.

In control samples (non-contaminated) at day 0, *L. monocytogenes* was absent (<1 CFU/25 g) and direct plating revealed even the absence of lactic acid bacteria (≤ 1.0 log CFU/g) (data not shown). At the end of the storage time, the average of lactic acid bacteria count was 7.57±0.64

Table 1 - Evolution of pH, $a_w$ and lactic acid bacteria (Lab) during the shelf life of sliced Mortadella. Values are means ± standard deviation of three replicates samples for each batch.

| Parameter | Shelf life (days) | |
|---|---|---|
| | 0 | 60 |
| **pH** | | |
| Batch 1 | 6.18±0.01aA[a] | 6.12±0.15aA |
| Batch 2 | 6.18±0.01aA | 6.15±0.24aA |
| Batch 3 | 6.22±0.05aA | 6.01±0.30aA |
| **$a_w$** | | |
| Batch 1 | 0.968±0.003aA | 0.972±0.003aA |
| Batch 2 | 0.970±0.001aA | 0.969±0.002aA |
| Batch 3 | 0.968±0.003aA | 0.969±0.002aA |
| **Lab[b]** | | |
| Batch 1 | ND[c] | 8.09±0.07a |
| Batch 2 | ND | 6.77±0.22b |
| Batch 3 | ND | 7.85±0.18c |

[a]Means with different lowercase letters within a column for each parameter are significantly different ($p$<0.05). Means with different uppercase letters within a row for each parameter are significantly different ($p$<0.05).
[b]Values are means log CFU/g ± standard deviation.
[c]ND; not detected by either direct plating.

log CFU/g (range 6.61-8.14 log CFU/g). This growth can be due to the fact that reducing the redox potential by vacuum-packaging and the storage at refrigerated temperatures are two of the factors able to enhance growth of lactic acid bacteria in this type of product (BARMPALIA *et al.*, 2005).

The average initial values of pH and $a_w$ were about 6.19 ± 0.03 (range 6.16-6.25) and 0.968 ± 0.003 (range 0.962-0.971) respectively. No statistical difference ($p > 0.05$) was observed at the end of the storage time (Table 1). The physico-chemical and microbiological properties of sliced Mortadella investigated in this study are in agreement with those obtained by other authors (GEORNARAS *et al.*, 2005; SHAND 2000; BARMPALIA *et al.*, 2005) in similar products. In contrast to these studies, the pH of Mortadella did not reduce during storage.

Three serotypes of *L. monocytogenes* (1/2a, 1/2b and 4b) are associated with the majority of sporadic cases of listeriosis, and the serotype 4b is linked to almost all recent outbreaks (ROCOURT and BILLE 1997). Therefore, this study was conducted using *L. monocytogenes* 4b in order to provide information on the growth of this serotype. The results of challenge testing of vacuum packed Mortadella slices are shown in Fig. 1. It is clear that vacuum packed Mortadella slices well support the growth of the pathogen.

Growth data for *L. monocytogenes* in sliced Mortadella were fitted by primary model at isothermal conditions (Fig. 1). At 8°C the sliced Mortadella supported rapid and prolific *L. monocytogenes* growth. In accordance with these results, in fact a wide range of cooked RTE food products supports multiplication of *L. monocytogenes*. In a study by BEUMER *et al.* (1996), growth of *L. monocytogenes* on vacuum packed luncheon meat, cooked ham, and cooked chicken breast meat was similar to that of modified atmosphere packaged stored meats, with counts increasing up to $10^8$ CFU/g after 35 days at 7°C. In a study by UYTTENDAELE *et al.* (2004) rapid

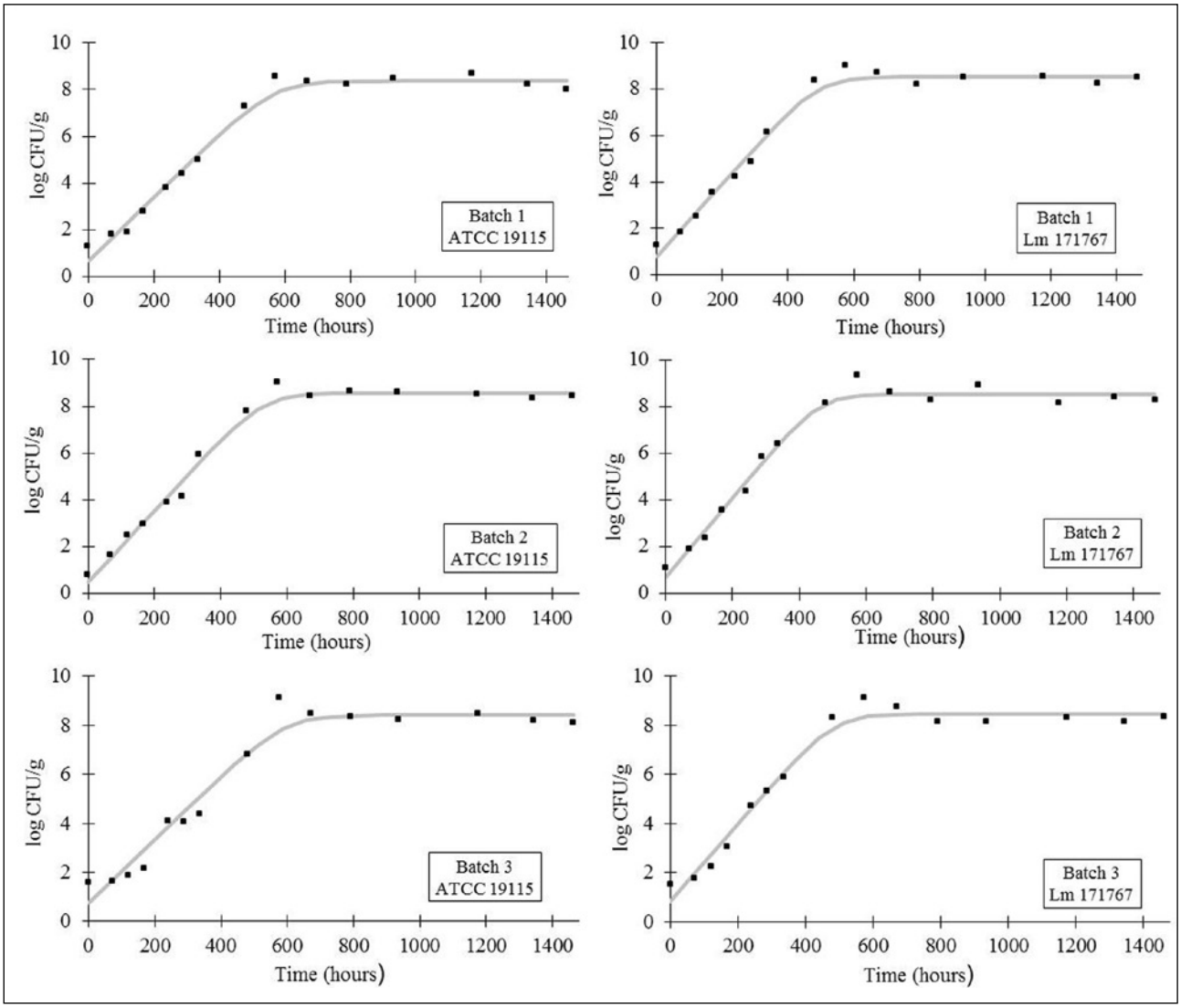



Fig. 1 - Fitting (—) of *L. monocytogenes* counts (■) in different batches to the Baranyi model.

Table 2 - Growth parameters, maximum specific growth rate ($\mu_{max}$) in hours$^{-1}$ of *L. monocytogenes* strains calculated by non-linear regression (BARANYI and ROBERTS, 1994) and doubling time in hours. The goodness of fit is indicated by the coefficient of determination (R-square value; $R^2$).

| Batch | Strain | Growth parameters | | |
|---|---|---|---|---|
| | | $\mu_{max}$ (hours$^{-1}$) | Doubling time (hours) | $R^2$ |
| Batch 1 | ATCC 19115 | 0.038 | 18.24 | 0.993 |
| | Lm 171767 | 0.043 | 16.12 | 0.99 |
| Batch 2 | ATCC 19115 | 0.035 | 19.8 | 0.986 |
| | Lm 171767 | 0.039 | 17.77 | 0.983 |
| Batch 3 | ATCC 19115 | 0.039 | 17.77 | 0.977 |
| | Lm 171767 | 0.044 | 15.75 | 0.986 |

growth of *L. monocytogenes* was noticed on vacuum packed sliced cooked ham. The concentration of 100 CFU/g, the safety limit considered as low risk for causing listeriosis, was exceeded after 5 days whereas ca. $10^5$ CFU/g were obtained after 14 days. Within the storage time of the investigated products low initial numbers of *L. monocytogenes* increased up to $10^8$ CFU/g after 21-27 days. It has often been reported that lactic acid bacteria might inhibit the growth of *Listeria* mainly due to the production of antimicrobial metabolites and nutrient competition. (MCKELLAR *et al.*, 1994; SCHMIDT 1995; DEVLIEGHERE *et al.*, 2001). As can be seen from Fig. 1, development of *L. monocytotogenes* is not inhibited by the presence of lactic acid bacteria. This can be explained by the fact that the initial level of lactic acid bacteria (< 10 CFU/g) were lower compared to contamination level of *Listeria* (about 100 CFU/g). Because of this, mutual interaction between the (micro) colonies formed was unlikely (BEUMER *et al.*, 1996; MELLEFONT *et al.*, 2008).

The fitting of the observed *L. monocytogenes* data counts (Table 2) indicated that, during storage at 8°C, the contaminated scenario that resulted in the highest maximum specific growth rate, $\mu_{max}$ ($h^{-1}$), was the combination of strain Lm 171769 in Mortadella batch 3, followed by the combination of strain Lm 171769 in batch 1. Origins of the strain affect the ability of adaptation of the microorganism and the growth rate in the food matrix.

This result is in agreement with those obtained by UYTTENDAELE *et al.*, 2004.

For each *L. monocytogenes* strain separately inoculated into three batches of sliced Mortadella, was calculated the doubling time during the exponential phase of growth (Table 2) (linear in log scale). Considering the worst-case scenario, the doubling time of *L. monocytogenes* was 15.7 h. Then, assuming a contamination of 1 CFU in 25 grams of product at the beginning of shelf life, the normed limit of 100 CFU/g is reached in about 7 days. For RTE products, the initial contamination levels are typically low (UYTTENDAELE *et al.*, 2004) so the ability to support growth of *L. monocytogenes* (qualitatively) and the growth kinetics (quantitatively) will determine if a food product can comply with the upcoming legislative requirements on European scale (EUROPEAN COMMISSION, 2005).

The secondary model, allowed the estimation of the specific growth rate of *L. monocytogenes* at different hypothetical storage temperatures (Table 3). The results show that, even if the product were to be stored at 2°C, the doubling time of *L. monocytogenes* is 99 h. Therefore, assuming an initial contamination of 1 CFU in 25 g of product (0.04 CFU/g), *L. monocytogenes* would reach the limit of 100 CFU/g in 46 days.

The prevalence and level of *L. monocytogenes* in RTE meat products, in which can survive for long period are well documented (LAHTI *et al.*, 2001; GOMBAS *et al.*, 2003; GIANFRANSCESCHI *et al.*, 2006; ANGELIDIS and KOUTSOUMANIS, 2006). In general, the primary source of food contamination by *L. monocytogenes* before release to consumers appears to be the processing environment (KATHARIOU 2002). Thus, the quality of the hygiene in the slicing and packaging room have a great importance since the higher the contamination at this internship is, the shorter the shelf life will be, regardless storage conditions (SAMELIS and GEORGIADOU, 2000). In order to extend the shelf life of the product, it would be indicated to change the type of packing in modified atmosphere, although many authors assert that no significant differences in the growth of *L. monocytogenes* has been observed in RTE pork products packaged under vacuum

Table 3 - Predicted specific growth rate ($\mu_{max}$) in hours$^{-1}$ of the *L. monocytogenes* at different storage temperature and doubling time in hours.

| Temperature (°C) | $\mu_{max}$ (hours$^{-1}$) | Doubling time (hours) |
|---|---|---|
| 2 | 0.007 | 99 |
| 4 | 0.016 | 43.3 |
| 6 | 0.028 | 24.8 |
| 12 | 0.085 | 8.2 |

or in modified atmosphere (BOEREMA *et al.*, 1993; METAXOPOULOS *et al.*, 2002).

In conclusion, sliced Mortadella vacuum packed and stored in conditions of mildly abusive temperature (8°C) is shown to be a product that supports the rapid development of *L. monocytogenes*. The use of predictive microbiology has allowed us to determine the growth rate of *L. monocytogenes* in the same product at different storage temperatures, indicating how the storage temperature of the product should be considered a crucial point for consumer safety. Through mathematical models, in fact, it was possible to predict the development of *L. monocytogenes* in different scenarios of conservation, including domestic, indicating that an abuse of temperature makes the product unsafe, if contaminated, within a few days.

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PAPER

# EFFECTS OF TEMPERATURE, SHEAR RATE AND PROCESSING ON THE RHEOLOGICAL PROPERTIES OF SALEP DRINK

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ABSTRACT

The effects of shear rate, temperature and processing on the rheological properties of commercial salep drinks (UHT processed in liquid form, powdered forms both instant and traditionally cooked type) are investigated. The empirical power law model is used for the determination of the flow behavior and consistency index. Apparent viscosity of salep drink decreased with increasing shear rate. The salep drink exhibited a pseudoplastic or shear thinning behavior. Processing affected the flow behavior and consistency indices significantly ($p \leq 0.05$). The flow behavior index of samples was in the range of 0.51-0.74 while the consistency index changed in the range of 0.14-2.68 Pa.sn. The lowest flow behavior index and the highest consistency index value are determined with the instant salep drink, while the highest K value was recorded for it. The flow behavior index increased and the consistency index decreased with processing temperature. In sensory analysis, salep drinks were evaluated for flavor, consistency and overall acceptability. A single rheological model is developed to express combined effects of shear rate (by Power law model) and temperature (by Arrhenius equation) on the apparent viscosity of an instant salep drink preferred by the sensory panelists in all samples. This study is expected to standardize the product formulation and facilitate equipment and process design.

- Keywords: salep, processing, rheology, sensory -

## INTRODUCTION

Traditional salep drink, generally consumed in winter, is regarded as nutritious, healthy and delicious beverage in Turkey. There are two types of salep drinks in the market. The first type is in liquid form which is UHT processed and the second type is in powder form, which is prepared by traditionally cooking or adding hot milk instantly. Although powdered salep drink is the most widely consumed product, the UHT processed liquid salep drink is also gaining consumers' preference (ARDUZLAR, 2003).

Salep powder is milled dried tubers of a wild terrestrial orchid (*Orchis anatolica*) (Kaya and Tekin, 2001). The roots or tubers of orchids are processed by boiling in milk or ayran to inhibit the enzymatic activity and reduce the loss of water-soluble ingredients (Kaya and TekIn, 2001). Salep is an essential ingredient for the production of hot salep drink and ice-cream (DOGAN and KAYACIER, 2004).

The most important constituent of salep is glucomannose ranging from 16 to 55% (TEKINSEN, 1996; KAYA and TEKIN, 2001). Salep also contains starch (2.7%), moisture (12%), and ash (2.4%) (TEKINSEN and KARACABEY, 1984; KAYA and TEKIN, 2001; ALPARSLAN and HAYTA, 2007). Its incorporation into a formulation contributes to the development of desired aroma and flavor of product and it also serves as a thickening and stabilizing agent (KAYACIER and DOGAN, 2006; GEORGIADIS *et al.*, 2012).

Knowledge on rheological properties is very important for designing process equipments like pumps, pipes and mixers, for determining the function of an ingredient in a formulation, and for controlling the viscosity of the products (TELCIOGLU and KAYACIER, 2007; STEFFE, 1996). An optimum consistency provides a good mouthfeel in a hot salep drink. As there are no standard procedures for the manufacture of salep, understanding the changes in the flow behavior is definitely necessary for producing an acceptable product with consistent quality (DOGAN and KAYACIER, 2004).

The rheological properties of salep drink were studied (ARDUZLAR, 2003; DOGAN and KAYACIER, 2004; ALPASLAN and HAYTA, 2007; TELCIOGLU and KAYACIER, 2007; YASAR *et al.*, 2009; KARAMAN and KAYACIER, 2010, YILMAZ *et al.*, 2010), but the information available in the literature is still limited. Furthermore, some researchers also recently investigated the rheological properties of ice cream or dessert containing salep and of gum-salep solutions (KAYA and TEKIN, 2001; GUVEN *et al.*, 2003; KUS *et al.*, 2005; KAYACIER and DOGAN, 2006, FARHOOSH and RIAZI, 2006; AYAR *et al.*, 2009; BAHRAMPARVAR *et al.*, 2010; VAHID *et al.*, 2011; KARAMAN and KAYACIER, 2012; KARAMAN *et al.*, 2013; YILMAZ *et al.*, 2013). It was reported that salep drinks generally exhibited a pseudoplastic or shear thinning behavior.

There is recently recorded data in literature expressing combined effects of shear rate (by Power law model) and temperature (by Arrhenius equation) on salep drink (KARAMAN and KAYACIER, 2010). The objectives of this study were to determine rheological properties of commercial salep drink types and investigate the effects of such technological parameters as shear rate, temperature and processing on the viscosity of the product. A single rheological model is developed to express combined effects of shear rate (by Power law model) and temperature (by Arrhenius equation) on the apparent viscosity. It is known that rheological properties of liquid affect the consumer preference so the results obtained in this research may be used in the development of new salep drink formulations and to standardize the product and facilitate equipment and process design.

## MATERIALS AND METHODS

Commercial salep samples covering UHT processed liquid salep (2 brands) and powdered form salep (6 instant brands and 3 traditionally cooked type brands) and UHT milk were obtained from local markets in Istanbul.

Instant salep drink was prepared by adding boiled milk (100 mL) to powder mixture (8-13 g). All solid particles in the sample were thoroughly dissolved by vigorously stirring for about 5 min. using a glass bar. Traditionally cooked type salep powder mixture (8-15 g) was added to milk (100 mL) and cooked about 10 min at 85°C by vigorously stirring on the oven. All salep drinks (11 commercial brands) were prepared according to instruction on the package label. The samples were heated (Electromantle, UK) to 20° (storage temperature) - 55°C (consumption temperature) prior to the rheological measurements.

### Rheological analysis

Shear measurements on the samples were conducted using a rheometer (Haake Rotovisco RT20, Karlsruhe, Germany) which is equipped with a circulating water bath and a Z20 concentric cylinder sensor system (cup diameter 10.85 mm, bob diameter 10 mm, gap width 0.85 mm, diameter ratio 0.92, sample volume 8.2 mL). The shear rate range was 0.13-300 $s^{-1}$. All rheological tests were done in duplicate. Samples were allowed to rest for 10 min. before measurement at constant temperature.

The steady shear behavior of salep drink was described by the power law model (Eq. 1).

$$\eta = K.\gamma^{(n-1)} \quad (1)$$

where $\eta$ is the apparent viscosity (Pa.s), $\gamma$ is the shear rate ($s^{-1}$), K is the consistency index ($Pa.s^n$) and n (dimensionless) is the flow behavior index.

The effect of temperature on the rheological properties of salep drink was investigated at 20, 30°, 40°, 50° and 55°C.

An Arrhenius type relationship (Eq. 2) was used to describe the temperature effect on the consistency index.

$$K = K_{\infty}.exp(E_a/RT) \quad (2)$$

where $K_{\infty}$ is the Arrhenius constant (Pa $s^n$), $E_a$ is the activation energy of flow (J $mol^{-1}$), R is the gas constant (8.314 J $mol^{-1}$ $K^{-1}$) and T is the absolute temperature (K). K and n values were calculated using the Rheometer RM Software (Version 3.30, Haake).

### Sensory analysis

Sensory analysis (ranking test) was performed by using 20 educated panelists (15 female, 5 male) from Food Engineering Department, who were familiar with the salep drink and sensory analysis. Panelist ranked the samples (11 commercial brands) according to their preference. Salep drinks were analyzed for flavor, consistency and overall acceptability. A section for the panelist's comments was present in the evaluation form. Samples for sensory evaluation were kept in a water bath (Nuve, TR) to maintain serving temperature (55°C). All the samples, randomly coded using a three-digit number, were serviced in white 50 mL plastic cups. Panelists received a tray containing the samples, a glass of water and unsalted crackers and evaluation form.

### Statistical analysis

All tests were replicated four times and the average values were used for each samples. Analysis of variance results were derived with SPSS 9.0 for Windows software package. The mean comparison was carried out with Duncan's multiple range test ($p \leq 0.05$).

## RESULTS AND DISCUSSION

The apparent viscosity values of selected samples are given in Figs. 1, 2 and 3. As the rheological results showed that, all commercial salep drink samples showed a non-Newtonian behaviour. As expected, viscosity values of samples decreased with increasing shear rate, and therefore salep samples showed a shear thinning behavior. The reduction in the viscosity with shear rate has been related to the increased alignment of the constituent molecules (KAYA and TEKIN, 2001). Shear-thinning behavior of sample was expected since weak physical bonds, electrostatic, and hydrophobic interactions affect the rheology of milk products (ABU-JDAYIL and MOHAMEED, 2002; DOGAN and KAYACIER, 2004). Similar results were reported in the literature for salep containing products in terms of flow behavior (KAYA and TEKIN, 2001; DOGAN and KAYACIER, 2004; KUS *et al.*, 2005; TELCIOGLU and KAYACIER, 2007; ALPASLAN and HAYTA, 2007; FARHOOSH and RIAZI, 2007; YASAR *et al.*, 2009; KARAMAN and KAYACIER, 2010; YILMAZ *et al.*, 2010; BahramParvar *et al.*, 2010; KARAMAN and KAYACIER, 2012).

The power law model is the most widely employed model for non-Newtonian fluids (BAHRAMPARVAR *et al.*, 2010). In this study, this model was used to describe the rheological properties of samples. The fitting of the experimental data was satisfactory with high correlation coefficients ($r^2$). As shown in Table 1, the consistency index (K) generally decreased with increasing temperature. Previous studies reveal that the smaller the *n* values the greater the departure from Newtonian behavior. Lower K values indicate a decrease in viscosity (ALPASLAN and HAY-

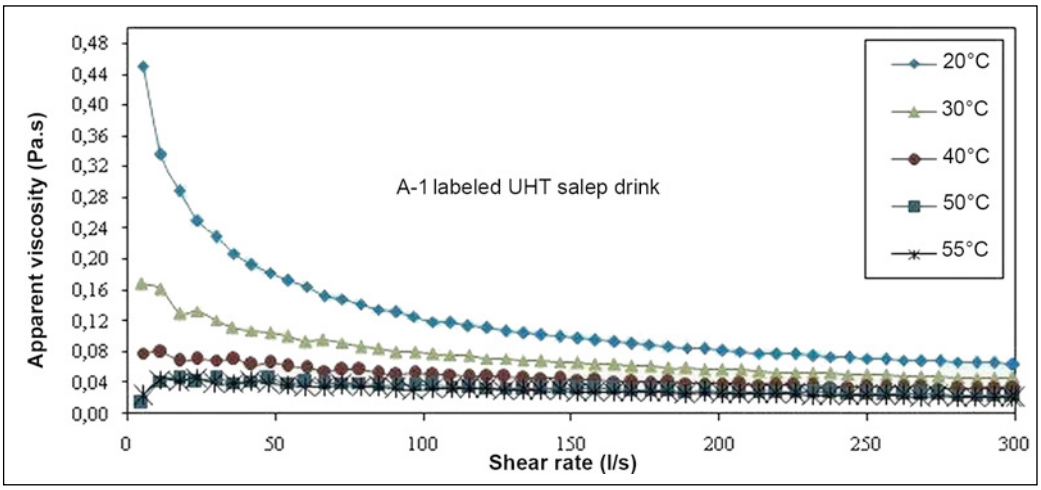



Fig. 1 - The viscosity values of A-1 labeled UHT salep drink at 20°, 30°, 40°, 50° and 55°C.

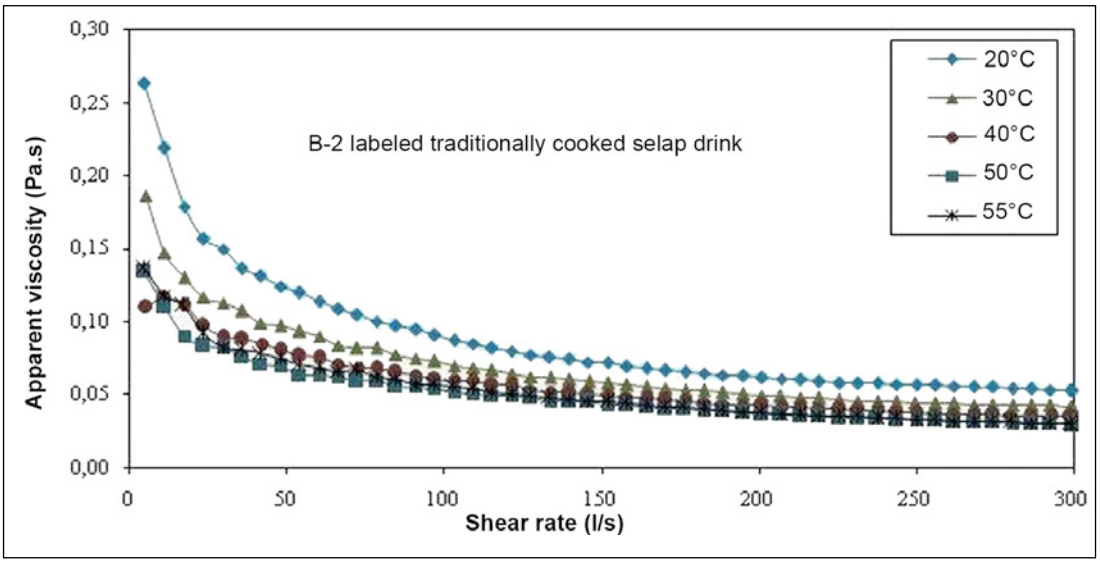



Fig. 2 - The viscosity values of B-2 labeled traditionally cooked salep drink at 20°, 30°, 40°, 50° and 55°C.

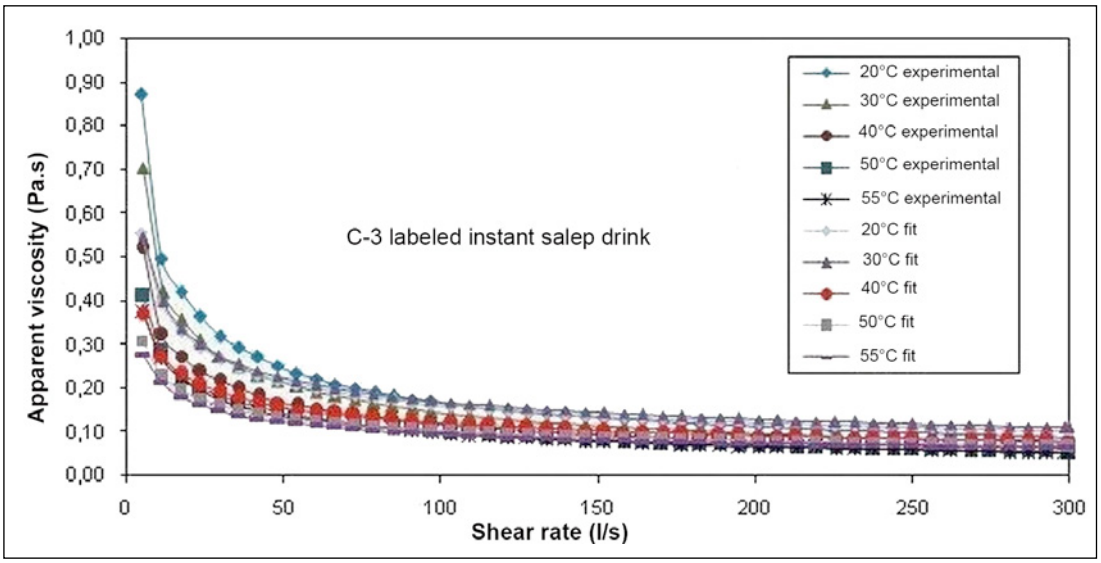



Fig. 3 - The viscosity (experimental and model fit) values of C-3 labeled instant salep drink at 20°, 30°, 40°, 50° and 55°C.

TA, 2007). Usually, flow behaviors of fluids tend to Newtonian behavior from non-Newtonian behavior at high the temperature. A decrease in the consistency index was observed with increasing the temperature indicating a decrease in apparent viscosity at higher temperatures. As temperature increases, thermal energy of the molecules increases and molecular distances develop due to reduction of intermolecular forces hence viscosity of the fluid declines (FARHOOSH and RIAZI, 2007). It is said that the viscosity changes resulting from heat treatments are related to heat-induced denaturation and subsequent aggregation of proteins (ALPASLAN and HAYTA, 2007).

Table 1 shows the power law parameters (K, n) for three commercial salep drinks at all temperatures studied. It is concluded from Table 1 that process (salep drink type) affected the n and K values which were found to be significantly different in samples ($p \leq 0.05$). K and n values for instant salep drink were different from those for UHT and traditionally cooked salep drink. The flow behavior index of samples was in the range of 0.51-0.74 while the consistency index changed in the range of 0.14-2.68 Pa.sn for all temperatures. The lowest n value was determined for instant salep drink, while the highest K value was recorded for it. As expected, the viscosity and consistency index decreased with increasing temperature. The decrease in consistency index with increasing temperature was reported by several other researchers in the literature for various samples (DOGAN and KAYACIER, 2004; KUS *et al.*, 2005; KAYACIER and DOGAN, 2006; ALPASLAN and HAYTA, 2007; KARAMAN and KAYACIER, 2010). The flow behavior index increased with increasing temperature. Whereas

Table 1 - K and n values for all commercial salep drink types.

| T (°C) | Salep Drink Sample | $K_{ave}$ (Pa.s$^n$) | $n_{ave}$ |
|---|---|---|---|
| 20 | UHT[a] | 0.62±0.03a | 0.66±0.02a |
| | Traditionally Cooked[b] | 0.31±0.08a | 0.69±0.03a |
| | Instant[c] | 2.68±1.67b | 0.51±0.10b |
| 30 | UHT[a] | 0.47±0.25a | 0.68±0.07a |
| | Traditionally Cooked[b] | 0.22±0.07a | 0.71±0.03a |
| | Instant[c] | 2.15±1.38b | 0.51±0.10b |
| 40 | UHT[a] | 0.44±0.47a | 0.68±0.09a |
| | Traditionally Cooked[b] | 0.17±0.07a | 0.72±0.04a |
| | Instant[c] | 1.52±0.99b | 0.55±0.08b |
| 50 | UHT[a] | 0.29±0.38a | 0.72±0.20a |
| | Traditionally Cooked[b] | 0.16±0.06a | 0.72±0.05a |
| | Instant[c] | 1.29±0.96b | 0.57±0.08b |
| 55 | UHT[a] | 0.14±0.10a | 0.74±0.11a |
| | Traditionally Cooked[b] | 0.18±0.08a | 0.69±0.07a |
| | Instant[c] | 1.31±1.07b | 0.57±0.09b |

[a]The average values of 2 commercial brands.
[b]The average values of 3 commercial brands.
[c]The average values of 6 commercial brands.

temperature effect on K and n values found to be insignificantly different in samples.

Some literature references can be found reporting power law parameters. For example, KAYA and TEKIN, (2001) studied the rheological characteristics of a ice cream prepared from whole cows' milk or water-salep-sugar mix. They reported that the power law index values (n) of milk-salep-sugar and water-salep-sugar mixes were in the range of 0.95-0.77 and 0.99-0.85 at 10, 20, 30, 40 and 50°C. In another research has been done by DOGAN and KAYACIER (2004), rheological properties of the reconstituted hot salep beverage prepared by adding water or milk to the commercial instant salep powder in the temperature range of 10°-50°C were evaluated. It was observed that the n values of milk-salep and water-salep mixes were 0.44-0.57 and 0.25-0.44, K values of milk-salep and water-salep mixes were 564-1453 mPas$^n$ and 107-407 mPas$^n$, respectively. In the study of Kus *et al.* (2005), the effect of salep concentration (0.5–1.5% salep content) on the rheological characteristics of ice-cream mixes was determined. The consistency coefficient decreased with increasing temperature while the flow behavior index increased. The flow behavior index of ice cream mixes at different temperatures ranged from 0.33 to 0.81 while consistency index changed between 0.15 and 31.05 Pas$^n$. In another research, the rheological properties of guar, xanthan and alginate solutions prepared (0.25, 0.50, 0.75 and 1.0%) with salep addition (0.05 and 0.1%) were evaluated. In this study, KAYACIER and DOGAN, (2006) reported that n values of samples was in the range of 0.196–0.971 while K values changed 35.576–3189.9 mPas$^n$. In the experiments performed by ALPASLAN and HAYTA, (2007), salep drink prepared by soymilk mixed cow's milk (0, 25, 50, and 75%) were studied. It was shown that K and n values were found between 0.147-1.276 and 0.4094-0.7303. In addition, effects of sweeteners and milk type on the rheological and sensorial properties of reduced calorie salep drink were studied by TELCIOGLU and KAYACIER (2007). It was reported that the average K values of salep sample with sugar was 232.64 mPas$^n$, that was 236.89, 261.54 and 249.64 mPas$^n$ for saccharine, cyclamate and aspartame, respectively. KARAMAN and KAYACIER, (2010) investigated the rheological characteristics of salep drink flavored with cocoa powder (1, 2, 3%) at 10°, 20° and 30°C. They concluded that the flow behavior index of salep drink flavored with cocoa powder samples changed between 0.231 and 0.326 while the consistency coefficient ranged from 7.365 to 21.277 Pas$^n$. These findings are in accordance with our K and values.

According to our sensory analysis results (Table 2), A-1 labeled UHT salep drink, B-2

Table 2 - Ranking sensory analysis results of commercial salep drink for flavour, consistency and overall acceptability by consumers.

| | Salep Drink Sample | Consistency | Flavour | Overall acceptability |
|---|---|---|---|---|
| UHT | A-1 | 1.20±0.41 | 1.20±0.41 | 1.20±0.41 |
| | A-2 | 1.80±0.41 | 1.80±0.41 | 1.80±0.41 |
| Traditionally cooked | B-1 | 2.70±0.57 | 2.5±0.69 | 2.45±0.69 |
| | B-2 | 1.65±0.67 | 1.40±0.60 | 1.50±0.69 |
| | B-3 | 1.65±0.75 | 2.10±0.79 | 2.05±0.83 |
| Instant | C-1 | 5.05±1.96 | 4.30±2.05 | 4.40±1.98 |
| | C-2 | 3.45±1.23 | 4.20±1.61 | 3.95±1.64 |
| | C-3 | 2.75±1.48 | 2.40±1.47 | 2.35±1.42 |
| | C-4 | 4.80±1.32 | 5.0±1.30 | 5.20±1.47 |
| | C-5 | 2.85±1.63 | 3.10±1.65 | 3.20±1.44 |
| | C-6 | 2.95±2.14 | 2.55±1.32 | 2.60±1.54 |

labeled traditionally cooked salep drink and C-3 labeled instant salep drink was the most preferred for flavour, consistency and overall acceptability by consumers in each group ($p \leq 0.05$). An overall evaluation of the results leads to the conclusion that salep drink should have a high enough viscosity for good mouthfeel. Most people prefer instant foods and beverages due to their easy preparation and long shelf life (DOGAN *et al.*, 2013). Instant type salep drink is more common than the other types in the market (ARDUZLAR, 2003). Considering to the sensory analysis results (Table 2), a single rheological model was developed to express combined effects of shear rate (by Power law model) and temperature (by Arrhenius equation) on the viscosity of C-3 labeled commercial instant salep drink. In the literature there is a rheological model expressing combined effects of shear rate (by Power law model) and temperature (by Arrhenius equation) on rheological flow of some food samples (IBANOGLU and IBANOGLU, 1998; SIMUANG *et al.*, 2004; KAYA and SOZER; 2005; ALTAY and AK, 2005; YOGURTCU and KAMISLI, 2006; DAK *et al.*, 2007; FARHOOSH and RIAZI, 2007; KARAMAN and KAYACIER, 2010). The Arrhenius model was used to express the influence of temperature on apparent viscosity of salep samples (Eqs. 1 and 2).
The model was successfully described by Eq. (3) with high correlation coefficients.

$$\eta = (1.03 \times 10^{-3}) \times e^{\frac{2035.70}{T(K)}} \times \gamma^{\left[-0.4569+0.0028T(^{\circ}C)-0.00002T^{2}(^{\circ}C)\right]} \quad (3)$$

## CONCLUSIONS

In this study effects of temperature, shear rate and processing on the rheological properties of the commercial salep drink were determined. Salep drink generally exhibited a pseudoplastic or shear thinning behavior. Processing (salep drink type) affected the n (flow behavior index) and K (consistency index) values which were found to be significantly different in samples ($p \leq 0.05$). K and n values for instant salep drink were different from those for UHT and traditionally cooked salep drink. The lowest n value was determined for instant salep drink, while the highest K value was recorded for it. The n value increased with increasing temperature whereas the K value generally decreased with increasing temperature. Whereas temperature effect on K and n values found to be insignificantly different in samples. Furthermore, a single rheological model was developed to express combined effects of shear rate (by Power law model) and temperature (by Arrhenius equation) on the viscosity of selected salep sample. As rheological properties of food sample affect the consumer preferences; the results obtained in this study could be used in standardization of the formulation, to facilitate better design of equipment and processes.


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PAPER

# THE EFFECT OF TECHNOLOGICAL PROCESSING ON THE CHEMICAL COMPOSITION OF CAULIFLOWER

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ABSTRACT

The aim of the study was to assess the effect of a technological processing method (blanching, cooking and freezing) on the content of dry matter, protein, fat, ash, mineral compounds as well as dietary fiber. The research material was cauliflower with white floret and cauliflower with conical, green-yellow floret. Our results showed that the processing method significantly influences the concentrations of all nutrients and dietary fiber. The major factor, determining the extent of nutrients' losses seems to be the period of water exposure. The results indicate that the cooking of raw cauliflower results in lower losses of nutrients than cooking frozen.

- Keywords: blanching, cauliflower, chemical composition, cooking, freezing -

## INTRODUCTION

There is strong evidence that the composition of our diet is crucial for human health and well-being (VOLDEN *et al.*, 2008). It is necessary to provide vegetables and fruits because they are rich sources of vitamins, minerals, fiber, and many other biologically active compounds. These components show antibacterial, antiviral, and antioxidative effects in the organism and influence on metabolic pathways (e.g. detoxification), the immune system, as well as decreased blood pressure (TOTUSEK *et al.*, 2011). The consumption of Brassicaceae family (Cruciferous) vegetables is also associated with a reduced incidence of cancer, particularly colorectal cancer (SARIKAMIS, 2009, TOTUSEK *et al.*, 2011). Dietary behaviour has shown to influence the acid-base balance. The Western-type diet, due to the excessive consumption of animal-derived protein and an inadequate intake of fruits and vegetables, shows an acidogenic effect. This is associated with a high dietary acid load and a low intake of base-forming dietary minerals (KÖNIG *et al.*, 2008). A mismatch between acid- and base-forming nutrients may result in subclinical low-grade acidosis. In the mineral composition of vegetables dominate potassium, magnesium, calcium. A proper intake of minerals is crucial for growth, development, metabolism pathways of an organism, as well as prevention of several chronic diseases, i.e. cardio-vascular disorders, osteoporosis (KÖNIG *et al.*, 2008). It is important to maintain an adequate consumption level of vegetables in all seasons in the fresh form and after appropriate technological processing (to maintain as high nutritional value as possible). The World Health Organization (WHO) recommends consumption of at least 400g of vegetables per day. Cauliflower is one of the most popular vegetables. In recent years, some new varieties of cauliflower (including these with green floret) have appeared on the market, and could enrich the offer of the frozen food industry (GĘBCZYŃSKI and KMIECIK, 2007). Due to seasonality, only small amounts of these vegetables are eaten as fresh ones. Most of them need to be preserved, usually by freezing. Cauliflower is a popular vegetable consumed as a dish or its' component. Due to its gustatory and nutritional value, as well as bioactive compounds content, cauliflower is recommended by nutritionists. Moreover, cauliflower, similarly as broccoli, is the present in the diet of small children. This vegetable is subjected to various forms of processing to make it suitable for consumption. Wet thermal processes influence the dietary constituents because of their thermal breakdown and the migration of components due to the change of integrity of cell structure (DAVEY *et al.*, 2000; VOLDEN *et al.*, 2008). Their leakage can be minimized by optimal conditions of thermal processing. This is why the knowledge of the effects of wet thermal treatments is crucial (VOLDEN *et al.*, 2008). The aim of the study was to investigate the effects of blanching, boiling (fresh as well as frozen material), and freezing on the levels of health-related parameters (selected minerals, protein, fat and dietary fiber) in two varieties of cauliflower. We also examined the mass changes after technological processing, in order to try to determine the types of loss during processing.

## MATERIAL AND METHODS

### Material and processing methods

The experimental material was cauliflower with white floret (Rober variety) and cauliflower with conical, green-yellow floret (*Romanesco* type, Amfora variety). The vegetables were cultivated in the Polan Plant and Horticultural Seed Production Centre in Krakow, Poland (Experimental Station in Igołomia). The experimental field was located in the eastern outskirts of the city. The cauliflower was grown in black soil on loess framework, with good horticulture and neutral pH. Mineral fertilization (taking into consideration the soil composition and nutritional requirements of the species) included: 400 kg Polifoski PK (MgS) 15-24-(6-7), 100 kg of Saletrzak $NH_4NO_3$ + $CaCO_3$ and twice foliar application of Folicare NPK 18:18:18; 5:17:40. Conditioning treatments (i.e. mechanical weed control, as well as diseases and pests) were carried out during the growing season, depending on soil and weather conditions. Research materials were: fresh vegetables (treated as raw material), edible parts of vegetables subjected to blanching, vegetables cooked to consumption softness, frozen cauliflowers, and also cauliflower cooked after earlier freezing. In all cases, freezing of cauliflower was preceded by blanching process. The first step of processing (before thermal treatment) included leaf removing, washing in running water, dividing the heads into florets (about 5 cm in diameter). The blanching was carried out in a stainless-steel pot, by putting vegetables into the water of 96-98°C temperature for approximately 3 min (immediately after placing raw material into the container, the temperature of water was decreased to 80°C). After blanching, material was immediately chilled by placing it into cold water and then drained. One half of the blanched vegetables were packaged in polyethylene pouches (max. 5 cm of vegetable lawyer) and frozen at –22°C. Vegetables (fresh and frozen) were cooked in a stainless-steel pot (by the use of electric stove), starting with boiling water (the highest stove heating power). After the second boiling the heating power was reduced to medium heating power. In the beginning phase of thermal processing, it was conducted without a lid. The cooking of fresh vegetables was carried out for 10 -15 min, and frozen cauliflowers

5-8 min. Frozen vegetables were taken for analysis after 24 hours of storage (–22°C). Material was partially freeze-dried using a Christ Alpha 1-4 freeze-dryer and stored at refrigerator temperature until the end of analysis. All technological processes were conducted in triplicate.

**Chemical analysis**

Determination of dry matter content was performed by PN-ISO 712:2002 method, by the use of Venticell 55Plus dryer. The nitrogen content was assessed by Dumas method (PN-EN ISO 16634-1:2008E) (TruSpec N, LECO, USA), and then calculated on protein content (using 5.9 coefficient). The method's correctness and recovery were determined using certified reference material NCS ZC73009(GSB-2) (China National Analysis Center for Iron and Steel, Beijing, China). The content of fat was analyzed by carbon dioxide (purity of 99.995%) in supercritical state (extraction method) in TFE2000 (LECO, USA) analyzer. 2 g of sample were mixed with about 1 g Leco Dry (LECO, USA), placed in a metal tube, and then taken for analysis. The analysis parameters were set as follows: sample temperature - 100°C, $CO_2$ pressure - 62 MPa, the flow rate - 2 L/min, time of static extraction – 15 min., time of dynamic extraction 35 min. The determination of ash content was performed, according to PN-EN ISO 2171: 2010 and by the use of a muffle stove (Nabertherm model LE6/11/B150, Poland). Minerals such as calcium, magnesium, potassium, and sodium were analyzed by the flame atomic absorption spectrometric method FAAS (AA240FS, Varian, USA), according to PN-EN 15505:2009, and zinc and manganese – according to PN-EN 14084:2004. The mineralization of samples was conducted in teflon containers by the use of high pressure microwave method (MarsX-Pres, CEM, USA), with 65% nitric acid (Suprapur, MERCK, Poland, kat. Nr 1.00441), in the amount of 10 mL/ 0.5 g sample. Max. temperature was set to 200°C, mineralization time – 40 min. Buffer solution of Schuhknecht and Schinkel (cesium chlorate and aluminium nitrate in the concentrations 50 g/l and 250 g/l respectively – MERCK, Poland, cat. no 102037, for potassium and sodium determination as well as buffer solution of Schinkel (cesium chloride and lanthanum chloride in the concentrations of 10g/l – MERCK, Poland, cat. no 1.16755), for the calcium and magnesium analysis were added. The wavelengths for the determination of minerals were as follows: K – 766.5 nm, Ca – 222.7 nm, Mg – 285.2 nm, Na – 589.0, Zn – 213.9 nm, Mn – 279.5 nm. The accuracy of methods used was verified on the basis of certified reference material NCS ZC73012 - GSB-5, China National Analysis Center for Iron and Steel, Beijing, China). All the methods used are fully validated, and checked by internal quality control procedure (according to PN-EN 13804) and interlaboratory/proficiency tests.

The content of dietary fiber content was determined by enzymatic-gravimetric method AOAC 991.43 (Megazyme kit, Ireland, cat. No. K-TDFR). The method's correctness was verified by the use of TDF Controls KIT (Megazyme kit, Ireland, cat. No. K-TDFC,). All the chemical analysis's were performed in triplicates.

**Statistical analysis**

The results were expressed as treatment means with standard deviations (SD). The data was subjected to one-factorial analysis of variance. The differences between treatment means were evaluated using *post hoc* Duncan's test. Treatment effects and differences between treatment means were considered significant at $P< 0.05$. The Statistica 9 software package (StatSoft, Tulsa, OK) was used for statistical analysis.

## RESULTS AND DISCUSSION

All the results in Tables 2 and 3 were presented after calculation on dry matter. It permits us to know whether the changes of hydrothermal treatment affect the retention of particular compounds in ready-to-eat material. Percentage losses of compounds (determined as the effect of hydrothermal processing) were calculated in compliance with mass balance changes. This way of presenting data eliminated the influence of thinning the content of the element (with water), and demonstrated only the influence of the processing technique applied.

The dry matter content in cauliflower of Rober variety was 7.8 g/100 g. Cauliflower of the Amfora variety was characterized by higher amount of dry matter – 9.6 g/100 g (Table 1). The level of dry matter in cauliflower with green floret was 9.1 g/100 g in the study conducted by GAJEWSKI (2001), and 9.3 g/100 g in the study by GĘBCZYŃSKI and KMIECIK (2007). Hydrothermal processes led to decreasing of dry matter content in cauliflower of both varieties (Table 1). As a result of blanching (an indispensable element of pre-treatments to the freezing of vegetables) – being a treatment connected with enzymes' inactivation, venting, and reducing microbial amount – a small decrease of dry matter was observed. Reducing of dry matter content in vegetables was also observed by other authors (GĘBCZYŃSKI, 2003; LENTAS and WITROWA-RAJCHERT, 2008). Cooking of fresh cauliflowers caused significant decrease of dry matter level. Cauliflower of the Rober variety was characterized by 9.5% less its' amount, in comparison to raw vegetable, and cauliflower of the Amfora variety – by 13.1% (Table 1). Similar losses of dry matter content (12.5%) in cooked vegetables were obtained by GEBHARDT and THOM-

Table 1 - The dry matter content (mean ± SD) and mass changes after technological processing of Amfora and Rober var. cauliflower.

| | Content [g/100 g] | | Mass changes [%] | | Losses* [%] | |
|---|---|---|---|---|---|---|
| | Amfora | Rober | Amfora | Rober | Amfora | Rober |
| Raw | 9.6$^{d}$±0.1 | 7.8$^{d}$±0.1 | 100 | 100 | - | - |
| Blanched | 9.1$^{c}$±0.5 | 7.1$^{c}$±0.2 | 105 | 105 | 0.5$^{a}$ | 4.4$^{a}$ |
| Cooked | 7.8$^{b}$±0.7 | 6.6$^{b}$±0.0 | 107 | 107 | 13.1$^{b}$ | 9.5$^{b}$ |
| Frozen | 9.0$^{c}$±0.9 | 7.1$^{c}$±0.6 | 105 | 105 | 1.6$^{a}$ | 4.4$^{a}$ |
| Frozen and cooked | 5.5$^{a}$±0.1 | 4.4$^{a}$±0.1 | 102 | 102 | 41.6$^{c}$ | 42.5$^{b}$ |

*Percentage losses of dry matter as the effect of hydrothermal processing were calculated in compliance with mass balance changes.

Table 2 - The content (mean ± SD) of protein, fat, ash and dietary fiber of Amfora and Rober var. cauliflower subjected to various technological processing (g/100 g d.m.).

| Cauliflower | Protein | | Fat | | Ash | | Dietary Fibre | |
|---|---|---|---|---|---|---|---|---|
| | Content | Losses* [%] | Content | Losses* [%] | Content | Losses* [%] | Content | Losses* [%] |
| **Amfora** | | | | | | | | |
| Raw | 35.4$^{b}$±0.37 | - | 1.5$^{b}$±0.03 | - | 8.6$^{c}$±0.32 | - | 21.2$^{b}$±0.13 | - |
| Blanched | 36.2$^{c}$±0.08 | 1.6$^{a}$ | 1.2$^{a}$±0.05 | 0.4$^{a}$ | 8.9$^{c}$±0.05 | 0.4$^{a}$ | 18.6$^{a}$±0.40 | 12.4$^{a}$ |
| Cooked | 34.7$^{a}$±0.02 | 14.7$^{b}$ | 1.5$^{b}$±0.02 | 11.5$^{c}$ | 6.9$^{b}$±0.11 | 28.6$^{b}$ | 21.2$^{b}$±0.55 | 17.3$^{b}$ |
| Frozen | 35.2$^{b}$±0.03 | 2.3$^{a}$ | 1.5$^{b}$±0.15 | 6.1$^{b}$ | 8.6$^{c}$±0.02 | 3.6$^{a}$ | 17.8$^{a}$±0.12 | 13.1$^{a}$ |
| Frozen and cooked | 34.5$^{a}$±0.39 | 43.1$^{c}$ | 2.4$^{c}$±0.01 | 21.1$^{d}$ | 4.9$^{a}$±0.12 | 65.5$^{c}$ | 30.0$^{c}$±0.27 | 17.2$^{b}$ |
| **Rober** | | | | | | | | |
| Raw | 24.2$^{b}$±0.12 | - | 2.0$^{b}$±0.13 | - | 11.8$^{c}$±0.04 | - | 25.6$^{a}$±0.30 | - |
| Blanched | 23.4$^{a}$±1.01 | 4.4$^{b}$ | 2.2$^{b}$±0.04 | 0.9$^{a}$ | 10.4$^{c}$±0.10 | 16.9$^{a}$ | 23.7$^{a}$±0.50 | 11.2$^{b}$ |
| Cooked | 23.7$^{a}$±0.15 | 6.3$^{c}$ | 1.5$^{a}$±0.06 | 39.2$^{b}$ | 9.8$^{b}$±0.03 | 27.5$^{b}$ | 26.4$^{b}$±0.15 | 3.2$^{a}$ |
| Frozen | 24.2$^{b}$±0.51 | 2.5$^{a}$ | 1.2$^{a}$±0.09 | 32.8$^{b}$ | 10.1$^{c}$±0.08 | 19.3$^{a}$ | 23.8$^{a}$±0.49 | 10.7$^{b}$ |
| Frozen and cooked | 28.2$^{c}$±0.35 | 18.3$^{d}$ | 2.5$^{c}$±0.17 | 50.3$^{c}$ | 5.7$^{a}$±0.09 | 80.0$^{c}$ | 37.0$^{c}$±0.50 | 16.4$^{c}$ |

The values in the same columns denoted with different letters a,b,c differ statistically significant at $p \leq 0.05$.
*Percentage losses of compounds determined by the effects of hydrothermal processing were calculated in compliance with mass balance changes.

AS (2002). On the other hand, in the study of GĘBCZYŃSKI and KMIECIK (2007) the increase of this parameter was observed during this process, possibly because of the loss of water from the tissue and the shrinkage of the raw material (RAMESH, 2000).

Among hydrothermal treatment used, the cooking of frozen vegetables led to the highest changes in the level of dry matter (Table 1) – its' losses in cauliflower of the Rober and Amfora varieties were 42.5 and 41.6%, respectively. Frozen and stored (by 24 hours, in -22°C) cauliflower was characterized by similar ranges of dry matter losses as compared with blanched vegetables. One can conclude that the changes of dry matter content were the result of blanching (water and temperature exposure).

The reason could be the extraction of soluble compounds to water and/or absorption of a slight amount of water by tissues; it was proven by mass balance analysis (Table 1). Previous studies have shown the loss of dry matter (ranging from 11 to 31%) during blanching in peas, Brussels sprouts, carrots and swedes, (WENNBERG *et al.*, 2006). In their study, about 50% of dry matter loss during blanching is connected with soluble sugars. The leakage of dry matter is possibly a function of the osmotic gradient of soluble compounds. VOLDEN *et al.* (2008) showed that DM loss was higher in blanching experiment (3 min) than in the boiling experiment (10 min), so the time of the process was less important than the kind of treatment. However, the results of our study are in opposition to this theory, because of higher dry matter losses during cooking process than after blanching. Protein content in dry matter of the variety Amfora cauliflower was 35.4 g/100 g, and cauliflower of the variety Rober was lower (24.2 g/100 g), as was presented in Table 2. Taking into consideration mass balance, it was reported that cooking, particularly of vegetables previously frozen, led to significant losses of this compound. In the case of Amfora variety cauliflower (cooked after freezing) losses mentioned above were over 40% (Table 2). The awareness of this fact is indispensable for the consumer to make a right choice of raw material (fresh or frozen) and

cooking method. The fat content in cauliflower was minimal. Similar results were reported by KUNACHOWICZ *et al.* (2005) and USDA National Nutrient database. There was no clear tendency for the fat content in dry matter in dependence of treatment (Table 2). However, the fat content was significantly reduced depending on processes used, if mass changes were considered in calculations. Higher value of dietary fiber content in raw material was reported for the Rober var. cauliflower (Table 2) than in the Amfora var. vegetable (Table 2). Blanching as well as freezing led to similar losses of this compound (10.7-13.1%), as was shown in Table 2. The physico-chemical properties of the dietary fiber (e.g., the proportion of soluble and insoluble fiber, the viscosity and the molecular weight) are changed after blanching. Our results showed that the cooking of Rober var. cauliflower led to lower dietary fiber loses than blanching. The reason could be higher levels of the resistant starch content during cooking in comparison with blanching. During wet heat treatment, insoluble fiber can be solubilized. Additionally, it may be degraded into smaller fragments and, in turn, lost to the water (WENNBERG *et al.*, 2006). These changes are the consequence of cleavage of glycosidic linkages within the polysaccharides. Breakage of weak bonds between dietary fiber polymers is also possible (WENNBERG *et al.*, 2006). Insoluble fiber can reduce the risk of constipation and colonic cancer. During heat treatment glycosidic linkages may be broken, and as a result the bulking capacity may decrease (WENNBERG *et al.*, 2006). According to PUUPPONEN-PIMIA *et al.* (2003), soluble fraction constitutes 22% of cauliflower's dietary fiber. There are evidences that soluble and viscous fibers added to a carbohydrate-rich meal, lowered postprandial glucose levels (ANDERSON *et al.*, 2009; WENNBERG *et al.*, 2006). Cauliflower of the Rober variety contained ash on the level of 11.8 g/100g, and in cauliflower of the variety Amfora – 8.6 g/100 g. Similar values were presented by KUNACHOWICZ *et al.* (2005). Only cooking process (raw and frozen material) caused significant decrease of this compound in dry matter (Table 2). Decrease of this compound in blanched material and also in material cooked after freezing was observed by LISIEWSKA *et al.* (2008) and LISIEWSKA *et al.* (2009). The share of calcium content in dry matter of cooked (after freezing) Rober variety cauliflower was significantly higher than in raw material. The other processes led to reduction of this element share in dry matter (Table 3). Simultaneously, losses of this mineral (in compliance with mass changes) were observed. A similar tendency was reported for cauliflower of the Amfora variety - the losses of calcium were from 6.3 to 29.7% (Table 3). Hydrothermal processes caused reduction in magnesium, potassium, sodium, manganese and zinc levels (in dry matter of cauliflower), as was presented in Table 3. Taking into consideration mass balance, lowering of all mineral compounds concentration was observed. As an example, cauliflower cooked after freezing contained 65.0 - 70.9 less magnesium, 67.9-70.4 potassium, 26.3-66.5 sodium, 58.4-67.2 manganese as well as 51.5-63.2 zinc (Table 3). The cooking of frozen cauliflower resulted in higher losses of minerals as well as other nutrients in comparison to cooking of raw cauliflower. The reason could be probably a longer time of water exposure. In the case of raw cauliflower, it was only the time of cooking process, and in the case of frozen vegetables – losses were raised from blanching and cooking processes. The reduction of mineral compounds could be

Table 3 - The content (mean ± SD) of selected minerals in Amfora and Rober var. cauliflower subjected to different technological processing (mg/kg d.m.).

| Cauliflower | Calcium | | Magnesium | | Potassium | | Sodium | | Manganese | | Zinc | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Content | Losses*[%] | Content | Losses*[%] | Content | Losses*[%] | Content | Losses*[%] | Content | Losses*[%] | Content | Losses*[%] |
| **Amfora** | | | | | | | | | | | | |
| Raw | 1676.3$^{c}$±39.9 | - | 2156.8$^{e}$±1.3 | - | 27238$^{d}$±929 | - | 126.8$^{b}$±0.6 | - | 36.5$^{d}$±1.3 | - | 67.3$^{e}$±1.8 | - |
| Blanched | 1573.0$^{b}$±2.6 | 6.3$^{a}$ | 1804.1$^{d}$±3.1 | 20.5$^{a}$ | 25512$^{c}$±920 | 8.0$^{a}$ | 114.7$^{a}$±8.4 | 9.3$^{a}$ | 28.8$^{c}$±0.1 | 25.1$^{a}$ | 53.7$^{d}$±0.8 | 24.1$^{a}$ |
| Cooked | 1856.1$^{d}$±40.1 | 10.8$^{b}$ | 1320.2$^{b}$±1.4 | 48.2$^{c}$ | 18717$^{b}$±324 | 41.9$^{b}$ | 121.7$^{b}$±2.6 | 18.8$^{c}$ | 26.9$^{b}$±0.4 | 37.8$^{b}$ | 39.3$^{b}$±1.9 | 50.6$^{c}$ |
| Frozen | 1563.2$^{a}$±4.0 | 8.5$^{a}$ | 1568.8$^{c}$±2.2 | 28.6$^{b}$ | 25520$^{c}$±221 | 11.0$^{a}$ | 117.1$^{a}$±3.9 | 14.0$^{b}$ | 27.3$^{c}$±0.1 | 26.6$^{a}$ | 48.0$^{c}$±0.7 | 29.9$^{b}$ |
| Frozen and cooked | 1830.7$^{d}$±5.2 | 29.7$^{c}$ | 1173.2$^{a}$±4.5 | 65.0$^{d}$ | 12529$^{a}$±25 | 70.4$^{c}$ | 145.2$^{c}$±0.6 | 26.3$^{d}$ | 23.6$^{a}$±0.3 | 58.4$^{c}$ | 38.4$^{a}$±1.3 | 63.2$^{d}$ |
| **Rober** | | | | | | | | | | | | |
| Raw | 1536.7$^{b}$±0.6 | - | 2663.7$^{c}$±1.0 | - | 40045$^{e}$±821 | - | 137.6$^{e}$±8.5 | - | 33.3$^{d}$±1.0 | - | 59.3$^{d}$±0.1 | - |
| Blanched | 1465.2$^{b}$±1.7 | 14.9$^{a}$ | 2064.8$^{b}$±2.6 | 30.8$^{a}$ | 27238$^{d}$±929 | 39.3$^{a}$ | 96.1$^{d}$±6.7 | 37.7$^{a}$ | 30.1$^{c}$±2.6 | 19.3$^{a}$ | 59.1$^{d}$±1.8 | 12.7$^{a}$ |
| Cooked | 1480.2$^{b}$±32.5 | 29.6$^{b}$ | 1398.9$^{a}$±0.4 | 53.8$^{c}$ | 18081$^{a}$±84 | 60.3$^{c}$ | 71.5$^{b}$±0.6 | 59.3$^{b}$ | 17.9$^{b}$±0.0 | 52.5$^{b}$ | 46.0$^{b}$±0.4 | 42.6$^{c}$ |
| Frozen | 1132.1$^{a}$±39.2 | 15.3$^{a}$ | 1446.2$^{a}$±0.4 | 48.1$^{b}$ | 19447$^{b}$±143 | 53.6$^{b}$ | 58.7$^{a}$±0.8 | 54.3$^{b}$ | 18.4$^{b}$±0.4 | 47.1$^{b}$ | 35.7$^{a}$±0.6 | 31.7$^{b}$ |
| Frozen and cooked | 1722.9$^{c}$±27.1 | 38.0$^{c}$ | 1399.4$^{a}$±2.6 | 70.9$^{d}$ | 23243$^{c}$±327 | 67.9$^{d}$ | 83.5$^{c}$±1.2 | 66.5$^{c}$ | 19.7$^{a}$±0.4 | 67.2$^{c}$ | 52.0$^{c}$±1.6 | 51.5$^{d}$ |

The values in the same columns denoted with different letters a,b,c differ statistically significant at p≤0.05.
*Percentage losses of minerals determined by the effect of hydrothermal processing were calculated in compliance with mass balance changes.

Table 4 - Percentage of recommended daily intake (RDA) for calcium, magnesium, manganese and zinc as well as adequate intake (AI) for potassium and sodium after consumption of 100g of cauliflower portion by a man or woman aged 31-50.

| Minerals | Calcium | | Magnesium | | Potassium | | Sodium | | Manganese | | Zinc | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cauliflower | Amfora | Rober | Amfora | Rober | Amfora | Rober | Amfora | Rober | Amfora | Rober | Amfora | Rober |
| Raw | 1.6* | 1.2* | 4.9*/5.2** | 4.9*/6.5** | 5.6* | 6.6* | 0.1* | 0.1* | 7.8* | 5.8* | 5.9*/6.5** | 4.2*/5.8** |
| Cooked | 1.4* | 1.0* | 2.4*/2.7** | 2.2*/2.9** | 3.1* | 2.5* | 0.1* | 0.0* | 4.7* | 2.6* | 2.8*/3.2** | 2.8*/3.8** |
| Frozen and cooked | 1.0* | 0.8* | 1.5*/1.6** | 1.5*/1.9** | 1.5* | 2.2* | 0.0* | 0.0* | 2.9* | 1.9* | 1.9*/2.1** | 2.1*/2.8** |

* value for man; ** value for woman.

affected by different factors. One of them could be the denaturation of metal-biocomplexes present in material, it is typical for protein complexes. The other one could be a reduction of total elements content connected with their elution into water solution. This effect could be enhanced by the expanded external surface of vegetables (KOPLIK *et al.*, 2004). SANTOS *et al.* (2003) as well as ONYEIKE *et al.* (2003) also observed changes of mineral content in different vegetables under the influence of thermal processes. According to WENNBERG *et al.* (2006), during wet treatment (such as blanching), losses of low-molecular-weight components, such as minerals, from the plant cells into the processing water are observed. It could lead to a relative increase in some components in the plant, e.g., the content of dietary fiber. Taking into consideration the general health in the population, a good knowledge of the impact of culinary processing on dietary compounds content, is of pivotal importance. This is why the results obtained for mineral compounds in raw and cooked cauliflower (for comparison) were used for the calculation of percentage RDA (*Recommended Daily Allowances*) or AI (*Adequate Intake*) for an adult man and woman (Table 4). It was shown that cooking, especially frozen cauliflower, provided less amounts of minerals (in comparison to recommended dose) after consumption of one vegetable portion (100g). However, consumption of the Amfora var. cauliflower provided more minerals, independent of material (raw or frozen) cooked.

## CONCLUSION

The findings in this study indicate that the method of cauliflower processing significantly influences the content of all nutrients as well as dietary fiber. This awareness is indispensable for the consumer to make a correct choice of raw material (fresh or frozen) and cooking method. Losses of health-related constituents are thus likely to be a function of type and physical form, and parameters of hydrothermal processes (such as time, temperature, amount of water). The major factor, in this study, determined the extent of losses of nutrients seems to be the period of water exposure. The estimation of hydrothermal processing's real impact on nutrients content in cauliflower should include mass changes. The results of the present studies indicate that the cooking of raw cauliflower is more advantageous method (lower losses of nutrients) in comparison with the cooking of frozen vegetable. In comparison with Rober var. cauliflower, consumption of Amfora var. cauliflower provides more minerals, independent of material (raw or frozen) cooked.

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PAPER

# ANGIOTENSIN I CONVERTING ENZYME INHIBITORY AND ANTIOXIDANT ACTIVITY OF ADLAY (*COIX LACRYMA-JOBI* L.VAR. *MA-YUEN STAPF*) GLUTELIN HYDROLYSATE

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ABSTRACT

*Coix* seed is an important herbal medicine in China and it has various medicinal and nutritional uses. In this study, *Coix* glutelin was hydrolyzed by five different enzymes and the angiotensin I converting enzyme (ACE) inhibitory activity was evaluated. The result showed that pepsin hydrolysate had the best ACE inhibitory activity. The optimized conditions for hydrolysis of glutelin with pepsin were then determined with hydrolysis degree as the indicative parameter. The pepsin-enzymatic glutelin markedly inhibited ACE activity with an $IC_{50}$ value of 52.34 ± 3.71 μg/mL. Our results strongly suggest that *Coix* glutelin peptides (CGP) are a beneficial ingredient in functional foods for pharmaceutical utilization.

- Keywords: angiotensin I converting enzyme, *Coix*, glutelin, hydrolysis degree, peptides -

## INTRODUCTION

Adlay (soft-shelled Job's tears, *Coix lacryma-jobi* L. var. *ma-yuen Stapf*) is a member of the grass family in the tribe Maydeae and has many medicinal and nutritional uses. Extensive research has revealed that *Coix* seed and its extracts possess a wide spectrum of bioactivities. The methanolic extracts from the hull of adlay seeds exhibited antiproliferative effects in human histolytic lymphoma U937 monocytic cells (KUO *et al.*, 2001). Free fatty acids of *Coix* seed extracted by acetone possessed antitumor activity (NUMATA *et al.*, 1994). *Coix* seed oil played a role in enhancing immunocompetence and preventing decreases in white blood cells during chemical therapy (HU *et al.*, 2007). SUNG OK KIM *et al.* (2004) discovered the hypolipidemic effects of the crude extract of adlay seed and considered its potential therapeutic usage for obesity control. *Coix* seeds could also effectively aid against viral infections by enhancing cytotoxic activity (HIDAKA *et al.*, 1992).

The endosperm of the *Coix* seed is rich in protein, accounting for 20% of fresh weight (OTTOBONI *et al.*, 1990). The protein may not show any bioactivity alone in an intact state, but it can be decomposed to release bioactive peptides during enzymatic digestion as well as through food processing (KORHONEN and PIHLANTO, 2003). Currently, the enzymatic catalyzation of proteins using selected proteolytic enzyme preparations to cleave specific peptide bonds is widely used in food industry.

The angiotensin-converting enzyme (ACE) is a dipeptidyl carboxypeptidase that catalyzes the conversion of angiotensin I to the potent vasoconstrictor angiotensin II (Ang II). It can also catalyze the degradation of bradykinin, a vasodilatory peptide (WU *et al.*, 2002). ACE inhibitory peptides may therefore have the ability to lower blood pressure *in vivo* by limiting the vasoconstrictory effects of Ang II and potentiating the vasodilatory effects of bradykinin (MURRAY and FITZGERALD, 2007). In recent years, ACE inhibitory peptides obtained from various food sources such as buckwheat (AOYAGI, 2006), soybean (RHO *et al.*, 2009), rapeseed (MARCZAK *et al.*, 2003) and many other crops have received great attention.

Reactive oxygen species (ROS) are physiological metabolites formed as a result of respiration in aerobic organisms. In humans, it is believed that ROS are involved in many health disorders such as diabetes mellitus, cancer, neurodegenerative disorders, gastric ulcers, ischemic reperfusion, arthritis and inflammatory diseases (JE *et al.*, 2009). Therefore, oxidation inhibitors such as superoxide dismutase (SOD), glutathione and vitamin C are essential for human health. There are various methods to estimate antioxidant activity *in vitro* and DPPH radical quenching activity is one of the most important analysis methods because it is simple, rapid and convenient.

The aims of this study are: (1) to find the appropriate enzyme whose catalytic hydrolysate shows the highest ACE inhibitory activity, (2) to optimize hydrolytic conditions by single factor and orthogonal experimental design with hydrolysis degree as indicative parameter and (3) to estimate ACE inhibitory activity and antioxidant activity of CGP *in vitro*.

## MATERIALS AND METHODS

### Extraction of *Coix* seed glutelin

The *Coix* seed powder was defatted with cooled petroleum ether and dried overnight. Albumin, globulin, prolamin and glutelin were then sequentially extracted from the dried powder with deionized water, 0.5 M NaCl, 70% ethanol (containing 5% β-mercaptoethanol) and 12.5 mM sodium borate buffer (containing 1% SDS and 2% β-mercaptoethanol), respectively (WALLACE *et al.* 1990). Proteins were extracted in a shaker three times successively, each with a solid-liquid ratio of 1:10 for 1 h. The extraction solution was centrifuged for 15 min at 10,000 rpm and the supernatant was collected. The target glutelin, dialyzed (3.5 KD molecular weight cut off) against distilled water for 24 h at 4°C, was vacuum freeze-dried for further analysis.

### SDS-PAGE analysis of molecular weight distribution

SDS-PAGE was conducted to determine the molecular weight distribution of each type of protein. All the proteins were run for approximately half an hour in 5% stacking gel with a voltage of 80 V and then were run for another 2.5 h in 15% separating gel with a voltage of 100 V. Afterwards the gel was dyed with Coomassie brilliant blue for approximately 5 h and then the gel was decolored until the strips were seen clearly.

### Preparation of bioactive peptides by hydrolyzing *Coix* glutelin

The enzymatic hydrolysis method for preparing bioactive peptides was applied according to GUÉRARD *et al.* (2001) with some modifications. The glutelin was exactly weighed in quintuplicate. Each batch was replicated 3 times and we then added an equal quality of enzyme. The five different kinds of enzyme applied in this research were pepsin (Sigma, P7000), papain (Sigma, P3250), α-chymotrypsin (Sigma, C3142), thermolysin (Sigma, P1512) and flavourzyme (Sigma, P6110). Their temperatures for hydrolysis are 37°, 65°, 37°, 65° and 65°C, respectively. The hydrolysis reactions were all

conducted under the same conditions: an enzyme-to-substrate (E/S) ratio of 1:10 and a substrate concentration of 2% for 48 h. Tris buffer (pH 8.0) was used for all enzymes except pepsin. For pepsin, 0.01 M HCl was used as the reaction buffer. The reaction was terminated by water bath heating at 85°C for 5 min and ice cooling for 10 min. The supernatant of glutelin hydrolysate was collected and passed through a 3,000 molecular weight (MW) cutoff ultrafiltration membrane. Peptides with molecular weight less than 3 KD were freeze dried for further analysis.

### Determination of ACE inhibitory activity *in vitro*

The determination of *in vitro* ACE inhibitory activity was performed by the reverse-phase high-performance liquid chromatography (RP-HPLC) method as described by WU and DING (2002) with slight modification. ACE extracted from rabbit lung acetone powder (A6778), hippuryl-L-histidyl-L-leucine (HHL, H1635) and hippuric acid (HA, 112003) were purchased from Sigma Chemical Co. ACE inhibitory activity was confirmed by monitoring formation of HA which was generated by HHL under enzymatic hydrolysis at 228 nm. The lyophilized peptide preparation was dissolved in the borate buffer solution (pH 8.3) (KIM *et al.*, 2001). The reaction system was made up of 10 μL of samples, 20 μL ACE (2 mU) and 20 μL 2 mM HHL. The mixture was incubated at 37°C for 10 min after adding ACE and samples. The mixture was then incubated for 60 min after adding HHL until the appropriate amount of acetonitrile was added to terminate the reaction.

Samples were analyzed on a $C_{18}$ column (250×4.6 mm, 5μm, Tianhe) and eluted by a mobile phase of acetonitrile/water (0.05% TFA) at a volume ratio of 25:75 (MENG *et al.*, 1995). Captopril and borate buffer solution were used as positive and blank control, respectively. The ACE inhibitory ratio of each sample was calculated as follows:

$$\text{Inhibitory activity (\%)} = [\,(A-B)/A] \times 100\%$$

where A is for the chromatographic peak area of HA from the blank and B is for the peak area of HA in the presence of the ACE inhibitor.

### Optimization of hydrolytic conditions

In this study the OPA method was applied to determine the degree of hydrolysis (DH) and the reagents were prepared according to WANASUNDARA *et al.* (2002) with serine as the standard and deionized water as blank control. The OPA reagents were mixed with the sample, standard and blank solution, respectively. After 2 minutes of reaction, the mixture was taken out to measure its absorbance at 340 nm. The DH of each sample was calculated as shown below:

$$\text{Serine}_{-NH_2} = \frac{OD_{sample} - OD_{blank}}{OD_{standard} - OD_{blank}} \times \frac{0.9516 mmol/L}{X \times P}$$

$$DH = (\text{serine}_{-NH_2} - b)/a\ /h_{tot} \times 100\%$$

(X = g sample; P = protein % in sample)

Hydrolytic conditions were optimized using a single factor experiment followed by a multifactor orthogonal design. The three factors of hydrolysis duration, E/S ratio and substrate concentration were examined in the single-factor experiment and the DH of each group was detected. Then, three levels of each parameter were selected and an orthogonal array was applied to determine the optimal combination of the experimental conditions.

### Radical scavenging assay of DPPH

DPPH radical scavenging activity was measured by using the methods described by NANJO *et al.* (1996). DPPH was a stable free radical in methanolic solution and in its oxidized form, the DPPH radical had a maximum absorbance centered at 517 nm. The DPPH solution (25 μg/mL) was prepared using methanol as solvent and the CGP was made into linear concentrations (0.25, 0.5, 0.75, 1, 1.25 and 1.5 mg/mL). The typical antioxidant - glutathione was used as positive control and methanol as blank control. After reacting in the dark at ambient temperature for 0.5 h and 1 h, the absorbance of the mixture was measured at 517 nm.

### Statistical analysis

All the data in this paper were derived from the average of three replications and were subjected to one-way analysis of variance using the SPSS 16.0 statistical software. Duncans' new multiple range test was performed to determine the significant differences at the 5% probability level.

## RESULTS

### Molecular weight distribution of *Coix* seed protein

Albumin, globulin, prolamin and glutelin were sequentially obtained from four different solvents and their molecular weight (MW) distributions were detected by SDS-PAGE. The results showed that each protein from *Coix* had a wide range of MW distribution (Fig. 1). The albumin contained protein bands with molecular weights ranging from 97 to 10 KD and there was no significant difference between each band. Wide MW distribution was also detected in globulin and the

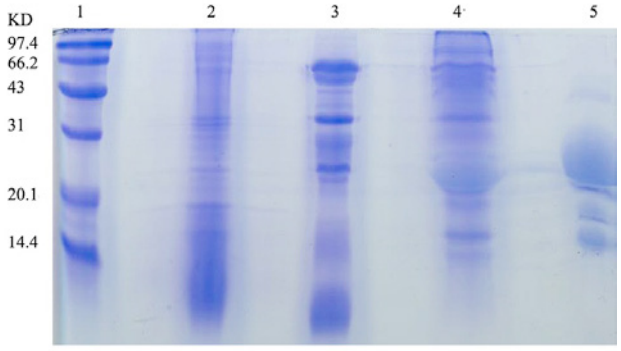



Fig. 1 - The MW (molecular weight) distribution of four proteins extracted from *Coix*. lane 1, marker; lane 2, albumin; lane 3, globulin; lane 4, glutelin; lane 5, prolamin.

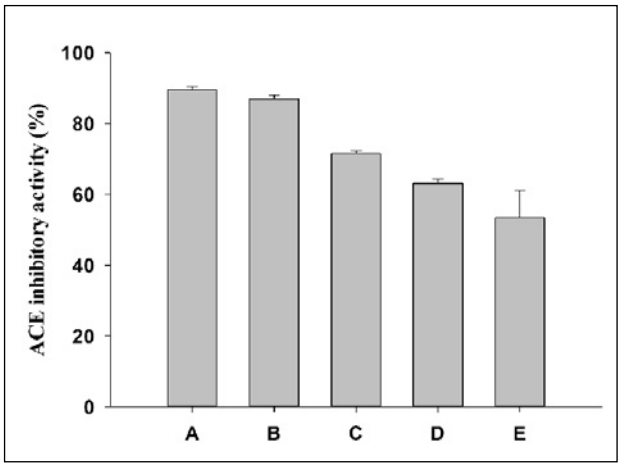



Fig. 2 - The ACE inhibitory activity of glutelin hydrolysates catalyzed by (A) pepsin, (B) thermolysin, (C) papain, (D) flavourzyme protease and (E) α-chymotrypsin.

proteins of 66, 35, 27 and 10 KD were highly expressed. The bands of glutelin were heterogeneous ranging from 97 to 15 KD and exhibited high expressions in approximately 66, 55, 35, 25 and 17 KD. There were four molecular weight classes of prolamin, namely 27, 22, 19 and 16 KD.

### Selection of protease for hydrolyzing *Coix* glutelin

Peptides were obtained by hydrolyzing glutelin with different enzymes since each enzyme had its specific sites of action. To select suitable proteases for glutelin hydrolysis, *Coix* glutelin was independently hydrolyzed with pepsin, papain, α-chymotrypsin, thermolysin and flavourzyme. Among them, the hydrolysate digested with pepsin and thermolysin revealed rather high ACE inhibitory activity at the concentration of 0.20 mg/mL, showing inhibitory ratios of 89.62±0.78% and 86.91±1.11%, respectively. However, there was no significant difference between them. The ACE inhibitory activity of the peptides from the other three enzymes decreased strikingly compared to the former two ($p = 0.05$, Fig. 2) with inhibitory ratios from papain, flavourzyme and α-chymotrypsin hydrolysates of 71.63±0.60%, 63.06±1.27% and 53.51±7.47%, respectively. Thus, pepsin was chosen for economic reasons as the optimal enzyme in further bioactive peptide production from *Coix* glutelin.

### Optimization of hydrolytic conditions

In the test of the single factor experiment, hydrolysis reaction time was first explored with a constant substrate concentration of 2% and an E/S ratio of 1:10. The result indicated that a longer time was more favorable to the DH of glutelin. There was a dramatic increase over the first 12 h, which reached a plateau state at 72 h (Fig. 3). Glutelin hydrolysis under different amounts of substrate and protease were also monitored over 72 h. Results reported in Fig. 3a showed that the DH was promoted by increasing the E/S ratio. A significant difference could be detected among given time points, except for the data at 12 h reaction time. On the other hand, lower substrate concentration was beneficial for the DH, with the minimum concentration of 1% as an exception (Fig. 3b). The orthogonal experiment was designed

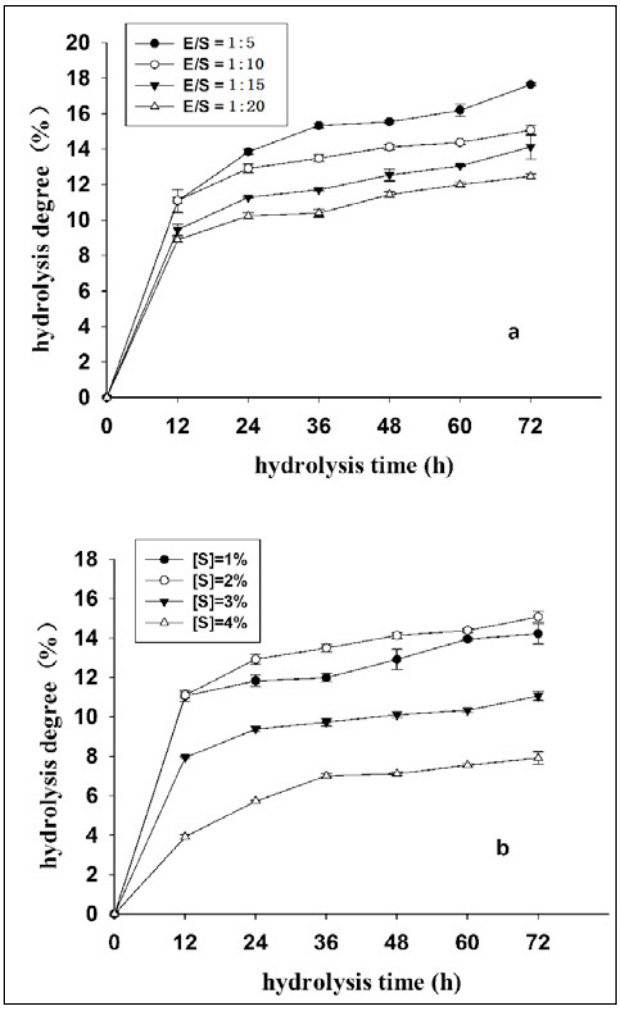



Fig. 3 - The result of hydrolyzing *Coix* seed glutelin with pepsin by the single factor experiment. (a) the DH of glutelin at different E/S ratios. (b) the DH of glutelin at different substrate concentrations.

Table 1 - The orthogonal results of hydrolyzing *Coix* seed glutelin with pepsin.

| Experiment | Hydrolysis duration (h) | Enzyme-to- substrate ratio | Substrate concentration (%) | Degree of hydrolysis (%) |
|---|---|---|---|---|
| 1 | 36 | 1:5 | 2 | 14.9±0.2 [a] |
| 6 | 48 | 1:15 | 2 | 13.4±0.5 [b] |
| 8 | 60 | 1:10 | 2 | 13.1±0.3 [b] |
| 4 | 48 | 1:5 | 3 | 12.9±0.2 [b] |
| 7 | 60 | 1:5 | 4 | 12.1±0.2 [c] |
| 2 | 36 | 1:10 | 3 | 11.3±0.4 [d] |
| 9 | 60 | 1:15 | 3 | 10.8±0.3 [d] |
| 5 | 48 | 1:10 | 4 | 10.0±0.1 [e] |
| 3 | 36 | 1:15 | 4 | 8.2±0.2 [f] |
| $K_1$ | 34.4 | 39.9 | 41.4 | |
| $K_2$ | 36.3 | 34.4 | 35.0 | |
| $K_3$ | 36.0 | 32.4 | 30.3 | |
| $k_1$ | 11.5 | 13.3 | 13.8 | |
| $k_2$ | 12.1 | 11.5 | 11.7 | |
| $k_3$ | 12.0 | 10.8 | 10.1 | |
| R | 0.6 | 2.5 | 3.7 | |

Each value of degree of hydrolysis was overall means ± SE from three independent experiments. The same letter means that the variance of two samples was not significantly different (P = 0.05). K represents the sum of degree of hydrolysis (DH) value at each level while lowercase k stands for the average DH value at each level. R represents the sum of deviation squares.

based on the results of the single factor test (Table 1). LSD multiple comparison of orthogonal results was also conducted (P=0.05). The optimized hydrolytic condition of pepsin-hydrolyzed glutelin was determined to be an E/S ratio of 1:5, a substrate concentration of 2% and hydrolysis duration of 36 h.

### ACE inhibitory activity of pepsin-catalytic glutelin

The RP-HPLC method was utilized to estimate ACE inhibitory activity. HA and HHL standard eluted out of the $C_{18}$ column at 5.53 and 9.19 min, respectively (Fig. 4a, b) and were well separated in the assay mixture (Fig. 4c, d, e, f). The control had no inhibitory activity and thus displayed a strong peak area of HA (Fig. 4c), while captopril ($2\times10^{-8}$ mol/L) manifested a strong ACE inhibition ratio of 87.36±0.32% (Fig. 4d). CGP acted as an inhibitor of ACE with an $IC_{50}$ of 52.34±3.71 μg/mL (Fig. 5). The results implied that the observed inhibitory effects of CGP, although not as powerful as captopril, contributed to antihypertensive activities.

### DPPH scavenging activity of pepsin-catalytic glutelin

DPPH radical scavenging activity of CGP at 0.5 h and 1 h was measured by the spectrophotometric method. Scavenging activity was enhanced with increasing concentrations of CGP reaching 63.80±0.10% at 2.5 mg/mL after 1 h (Fig. 6). The calculated $IC_{50}$ values of DPPH radical scavenging activity for CGP were 1.92±0.09 mg/mL and 1.27±0.05 mg/mL at 0.5 h and 1 h, respectively. While the $IC_{50}$ of glutathione was significantly lower than CGP with 42.07±2.52 μg/mL and 23.82±1.06 μg/mL at the same corresponding times. The data showed that CGP manifested antioxidant ability to a certain extent although not as strongly as glutathione.

## DISCUSSION

There are different methods by which bioactive peptides can be produced from precursor proteins, e.g. enzymatic hydrolysis with protease, through the action of enzymes derived from proteolytic micro-organisms (NAKAMURA *et al.*, 1995) and microbial fermentation (MEISEL, 1998). Once the structure of bioactive peptides is obtained, chemical synthesis of them can be accomplished. Among these methods, enzymatic hydrolysis is the most commonly used method. The angiotensin I-converting enzyme is closely associated with the renin-angiotensin system, which regulates peripheral blood pressure. Inhibition of this enzyme can produce effective antihypertensive properties. ACE-inhibitory peptides have been isolated from the enzymatic digestion of various food proteins. At present, these peptides are the most studied bioactive peptide group, with diverse enzymes being used. In this study, pepsin enzymatic CGP showed satisfactory ACE inhibitory activity with an $IC_{50}$ of 52.34±3.71 μg/mL. ACE-inhibitory peptides were also released from soy protein alkaline hydrolysate after ultrafiltration and cation exchange resin fractionation by WU and DING (2002). Their $IC_{50}$ value was estimated to be 0.065 mg of protein/mL. Via proteolytic di-

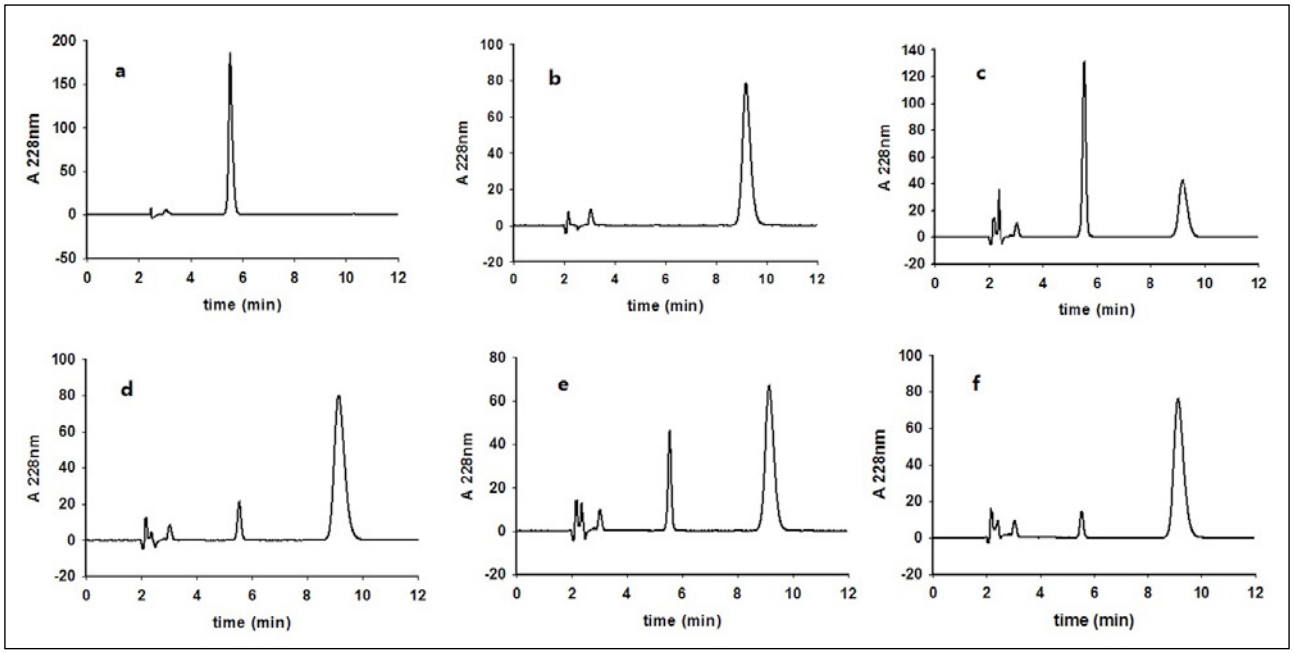



Fig. 4 - RP-HPLC chromatograms of (a) HA standard, (b) HHL standard, (c) blank control, (d) captopril of $2\times10^{-8}$ mol/L, (e) glutelin hydrolysate of 0.1 mg/mL and (f) glutelin hydrolysate of 0.4 mg/mL detected at 228 nm.

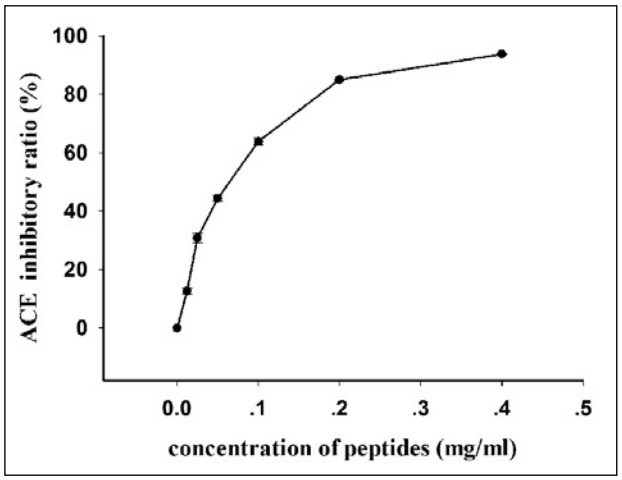



Fig. 5 - ACE inhibitory activity of CGP under different concentrations.

gestion of wheat germ protein with alkaline protease, MATSUI *et al.* (2000) also obtained a desired ACE inhibitor ($IC_{50}$ = 0.37 mg protein/mL). Thermolysin digestion of chicken muscle and the peptic digestion of ovalbumin were revealed to possess potent ACE-inhibitory activities with $IC_{50}$ of 45.0 and 45.3 μg/mL, respectively (FUJITA *et al.*, 2000). In addition, MIYOSHI *et al.* (1991) isolated ACE inhibitory peptides from alpha-zein thermolysin catalytic hydrolysate. There were also many ACE inhibitors derived from combinations of other sources and proteases. Every protein has an optimal enzyme according to its structure and properties. However, the use of pepsin was considered to be more economical and would reduce costs for mass production in future research.

It is widely recognized that most antihypertensive peptides have relatively low molecular weight (TOVAR-PÉREZ *et al.*, 2009; QUIRÓS *et al.*, 2007; SUETSUNA *et al.*, 2001; SAITO and CHEN, 2007; YAMAMOTO, 1997). In our study, ultrafiltration was used to obtain hydrolysate of less than 3 KD. In future research, bioactive peptides can be fractionated and enriched through various chromatographic methods such as cation exchange, gel filtration chromatography, preparative liquid chromatography and so on.

DH is a useful parameter for monitoring the extent of protein degradation and several meth-

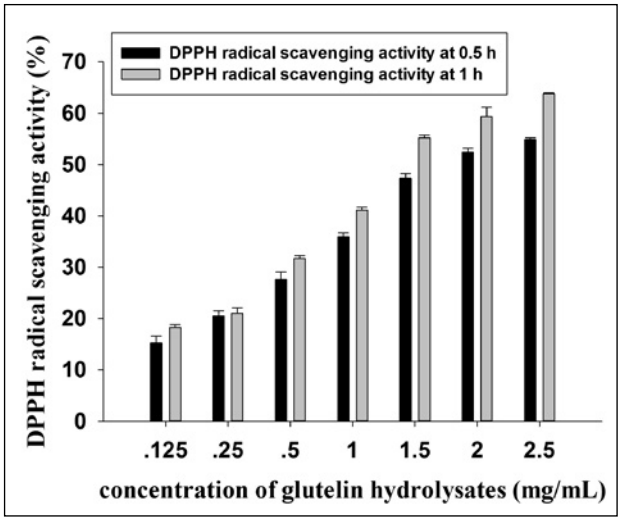



Fig. 6 - DPPH radical scavenging activity of CGP. Black column stands for DPPH scavenging activity of peptides at 0.5 h while the grey column represents activity at 1 h.

ods have been used for its estimation. The most commonly employed methods include OPA (ophthaldialdehyde), TNBS (trinitrobenzene-sulphonic acid) and pH stat (NIELSEN *et al.*, 2001). OPA is based on the specific reaction between OPA and primary amino groups in the presence of a thiol to form an isoindole which can be quantified spectrophotometrically at 340 nm (HERNANDEZ *et al.*, 1990). Similar to OPA, TNBS reacts specifically to primary amino groups to form a chromophore with a maximum absorbance at 340 nm. These two methods can detect most of the amino acids except the proline, which contains no primary amino group. In addition, OPA exhibits a weak reaction with cysteine and will lead to underestimation of DH of protein with high cysteine content (SPELLMAN *et al.*, 2003). Nevertheless, the OPA method is superior to TNBS due to the advantage of speed and low toxicity. pH stat is unaffected by the type of amino acids but is limited to pH conditions higher than 7. In this study, the OPA method was applied after full comparison of each method despite its possible underestimation of the DH. Our results showed that the DH increased with increasing incubation time and was nearly steady after 72 h at 37°C. GUERARD *et al.* (2002) speculated that this phenomenon may be due to a limitation of the enzyme activity by formation of reaction products at high degrees of hydrolysis. They also suggested that it might be due to a decrease in the concentration of peptide bonds available for hydrolysis, enzyme inhibition and enzyme deactivation.

Reactive oxygen species are related to many disorders and inflammatory diseases that are harmful to human health. DPPH radical quenching is one of the most important factors in estimating antioxidant activity *in vitro*. Our study revealed that CGP quenched DPPH radicals at a concentration of 1.92 mg/mL (0.5 h) and 1.27 mg/mL (1 h), suggesting that CGP has some potential ability for electron or hydrogen donation. This provides evidence for CGP's potential usefulness in preventing and treating health disorders and inflammation related diseases. Antioxidative peptides have also been isolated from other crops such as wheat, chickpea and peanut.

Our study mainly focused on the bioactivity of crude hydrolysate for *Coix* glutelin. After further separation, purification and structural identification of CGP, bioactive peptides with better antihypertensive and antioxidant activity might be obtained. Thus, functional foods beneficial for hypertension and related cardiovascular treatments could also be developed in the near future.

## CONCLUSION

Glutelin hydrolysate from *Coix* seed manifested high ACE inhibitory activity and some degree of antioxidant activity *in vitro*. This might contribute to further research into food derived antihypertensive compounds. This study also provides some reference for the clinical drug use of *Coix* in traditional Chinese medicine.


### ACKNOWLEDGEMENTS

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PAPER

# FACTORS LIMITING THE SHELF-LIFE OF SALAMI PIECES KEPT IN RETAILING CONDITIONS

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ABSTRACT

Different sensory, microbial and physical-chemical traits were evaluated as potential limiting factors of the shelf-life of small-size salami pieces packed in perforated bag and kept for up to 150 days at 10°C, 65% R.H. and 1,000 lx. Both pathogenic and fermentative bacteria were adequately inhibited during storage. Flavour deterioration associated with rancidity, bitterness and mouldy off-flavour, together with hardening and loss of juiciness, were identified as the main causes of loss of sensory quality. The storage time could be accurately monitored by using most of the sensory and physical-chemical traits. The shelf life of salami was established in 100 days.

- Keywords: dry-cured, fermented, pork, sausage, spoilage, storage -

## 2. INTRODUCTION

Product shelf life is very important for the meat industry. In the case of dry-cured fermented sausages, such as salami, the shelf-life is often limited by its loss of sensory quality, since health risks are controlled by the use of preservatives (salt, potassium nitrate and sodium nitrite), colourings (carminic acid or others), antioxidants (sodium ascorbate or others), starter cultures (Lactic Acid bacteria and Gram-positive, catalase-positive cocci) and covering moulds (*Penicillium* spp. and others) (LÜCKE, 1994). The shelf life of salami ends when its appearance becomes unacceptable, or when the subsequent eating quality is critically worsened by previous display (GYESLEY, 1991); however, sometimes it is not clear which unfavourable traits are really limiting product acceptance. The shelf life of salami will depend on its intrinsic characteristics (composition, mincing, casing type and size, ripening, etc.) and further processing (slicing, packing and retailing). Small-size salami (40 mm calibre and 30 cm length) is widely consumed in European Mediterranean countries, such as Spain, France or Italy. This salami is primarily made with pork, which is fermented, stuffed into natural or artificial casings covered by moulds and quickly dried. Because of its small size, this salami is often retailed in whole pieces packed (or not) in perforated bags to regulate dehydration, and consumed as slices without casing.

The time period that salami remains acceptable to consumers is largely determined by retailing conditions. There are some studies on the effects of packaging methods and storage time on the shelf life of salami slices (ZANARDI *et al.*, 2002; RUBIO *et al.*, 2007; ESTURK and AYHAN, 2009) and whole pieces (SUMMO *et al.*, 2006; MARCO *et al.*, 2008; LEE *et al.*, 2009). The sensory quality of salami would be mainly limited by deterioration of the odour and flavour, and, to a lesser extent, colour and texture. Flavour deterioration is associated with the degradation of volatile compounds resulting from spices and microbial activities, and the formation of volatiles through lipid oxidation, which would be responsible for rancidity and other off-flavours (SUMMO *et al.*, 2010; LORENZO *et al.*, 2013), while bitterness can also appear though the release of some hydrophobic amino acids (HENRIKSEN and STAHNKE, 1997). Nitrosylmyoglobin, the purplish-red pigment of dry-cured meat, may degrade during storage through the action of oxidizing agents, such as fluorescent lighting or an increase in temperature, resulting in discolouration and the appearance of brownish tonalities (ZANARDI *et al.*, 2002; RUBIO *et al.*, 2008). Packed-sliced salami at retail can also present changes in hardness, cohesiveness and elasticity through the alteration of meat proteins (RUBIO *et al.*, 2007). Spoilage can be controlled by packing under vacuum or in protective atmospheres, although lactic-acid bacteria may still proliferate in the salami. As regards whole pieces of salami unpacked or packed in perforated bags, spoilage might also involve to their appearance (mould growth, colour changes and casing wrinkling), odour and flavour (mouldy off-flavours and others) or texture (hardening, loss of juiciness and crusting), although little information is available in this respect. Bearing in mind that such differences may be regarded as a function of retailing conditions, it is necessary to monitor the spoilage phenomena that could limit the shelf life of salami.

The objective was to determine the shelf-life time of small-sized pork salami, stuffed into pig casing and retailed as whole pieces packed in perforated bags. Several sensory, microbial and physical-chemical traits that may contribute to sausage spoilage were evaluated.

## 3. MATERIAL AND METHODS

### 3.1. Sausage manufacture and sampling

The salamis were manufactured by a local company (Elaborados Cárnicos de Lorca, Murcia, Spain) using the following recipe (g $kg^{-1}$): boned pork shoulder (880), water (44), sodium chloride (22), black and white pepper (10), dextrose, lactose and sucrose (20), dextrin (20), potassium nitrate (0.15) and sodium nitrite (0.15), sodium isoascorbate (0.5), sodium citrate (0.3) sodium glutamate (2.5) and Ponceau 4R red (0.2). The commercial mixture of additives and spices was provided by Cargill Texturizing Solutions Barcelona, Spain. The meat was minced in an atmospheric mincer using a 6 mm plate (Laska GMBH, WW1302, Nu-Meat Technology, Girona, Spain). A commercial starter culture composed of (g per 100g culture) *Pediococcus pentosaceus* (50), *Staphylococcus carnosus* (25) and *Staphylococcus xylosus* (25) was provided by Degussa Ferment´s Aromatization SAS, La Ferté sous Jouarre, France. The lyophilised cultures were rehydrated (15g in 200 mL chlorine-free water) for 4 h and then sown in the mass at a rate of $6 \times 10^7$ cfu $g^{-1}$.

The meat was then mixed with the starter cultures, additives and spices in an AMU102 vacuum mixer (Maquinaria Vall, Miralcamp, Lleida, Spain). The paste was stuffed into the casing on a WF-612 automatic line (Albert Handtmann Mahcinefabrik, Biberach an der Riss, Germany). Swine casing, slightly curved, 40-43 mm calibre and 300-320 mm in length, was used. The casing was previously desalted and washed with de-chlorinated water. The recently stuffed pieces were bathed in mould (*Penicilium chrysogenum* PS5.1, Cargill Texturizing Solutions Cultures SAS, La Ferté Sous Jouarre, France) solution (0.8 g $l^{-1}$ water) and hung from steel racks 1.2 m wide, 1.2 m deep and 2.2 m

high. The loading density on the trolleys was 19 kg $m^3$. The trolleys were placed in an air-drying room (Sabroe S.A., Barcelona, Spain) set at 15°±1°C. The R.H. was gradually reduced, after the first day at ambient humidity to eliminate water through dripping; 6 days at 80±5% R.H. and 5 days at 70±5% RH. The temperature and R.H. were verified using a P 650 thermohygrometer (Dostmann Electronic GmbH, Wertheim-Reicholzheim, Germany) with a precision of 0.2°C and 0.5% RH. After drying, excess external microflora were eliminated by brushing in a chamber to avoid recontamination. For the study of shelf life, the salamis were packaged in perforated (6 mm diameter) polypropylene BA-85 bags (Plásticos Sierra del Oro, Abarán, Murcia, Spain) and then they were stored at 10°±1°C and 65±0.2% R.H. for 0, 30, 60, 90, 120 or 150 days in an open display cabinet (Booster Group, Santiago, Chile) continuously illuminated with white fluorescent light (1,000 lx), simulating retail display conditions. The cabinet had a chilling system capable of reaching average values of 10°±1°C and 65±0.2% R.H. during the retailing period, as was verified using a P 650 thermohygrometer.

### 3.2. Physical and chemical analysis

Moisture (g 100 $g^{-1}$) was determined by gravimetry after dehydration in an oven (Heraeus, Madrid, Spain) (ISO 1442, 1997). The water activity "$a_w$" was measured at 25°C using a water activity meter (Novasina TH200 Axair AG, Pfäffikon, Switzerland) (ISO 21807, 2004). The pH was measured with a Crison micropH 2001 pHmeter (Crison, Barcelona, Spain) using a combined Cat n. 52-22 electrode (Ingold, Inc. Wilmington, USA) (ISO 2917, 1999). Objective colour was measured using a CR-200/08 Chroma Meter II (Minolta Ltd., Milton Keynes, United Kingdom). The salami pieces were sliced (8 mm thick) with a MAS9101 slicer (Rober Bosch Hawgeräte GmbH, Ljubljana, Slovenia). Three measurements in different zones of each slice and a total of three slices per sausage unit were measured. A D65 illuminant and a CR-A3f light projection tube (aperture size of 10 mm diameter) were used. Results were expressed as Lightness ($L^*$), redness ($a^*$), yellowness ($b^*$), Chroma ($C^*$) and Hue angle ($H^*$) (sexagesimal degrees). $C^* = (a^{*2} + b^{*2})^{1/2}$ and $H^* = \tan^{-1}(b^*/a^*)$ (CIE $L^*a^*b^*$ units).

Texture profile analysis (TPA) and Warner-Bratzler shear force were measured using a texture analyser QTS-25 (Brookfield CNS Farnell, Borehamwood, Hertfordshire, England) equipped with a load cell of 25 kg and Texture Pro V. 2.1 software. Salami cubes of 20 x 25 x 25 mm were used for both texture measurements. For TPA, the samples were compressed with a 20 mm diameter cylindrical probe. The testing conditions were: 20°C room temperature; two consecutive cycles of 50% compression, with the cross-head moved at a constant speed of 50 mm/min and a trigger point of 0.05 N. Texture variables, hardness (expressed as N), cohesiveness (no units), springiness (expressed as mm), gumminess (expressed as N) and chewiness (expressed as N*mm). Five measurements per sausage unit sample were made. To measure shear force, salami samples were cut perpendicular to the muscle fibres with a Warner-Bratzler shear (plain vee type). The testing conditions were: 20 °C room temperature; 30 mm/min crosshead speed; 0.2 N trigger point; 40 mm travel distance and 1 mm calibration distance. Peak force (expressed as N) was determined, making five measurements per sausage unit.

### 3.3. Microbiological analysis

For microbiological assays, the salamis were aseptically manipulated in a 131 Bio-II-A microbiology cabinet (Telstar, Tarrasa, Spain) using sterile tweezers. The casing was removed and then the samples (ranging from 10 to 25 g, depending on the microbial analysis) were blended with peptone water, 0.1% w: w (Oxoid 133 CM0087 Tryptone water, Basingstoke, Hampshire, United Kingdom) into plastic bags using a stomacher (IUL Instruments, GMBH, Königswinter, Germany). Total viable counts "TVC" (log cfu $g^{-1}$) were determined on Plate Count Agar "PCA" (Oxoid CM0325) after incubation at 30*°C for* 72 h in a ST 6120 culture incubator (Heraeus S.A., Boadilla, Madrid, Spain) (ISO 4833, 2003). Lactic acid bacteria were counted on MRS Agar plates (de Man, Rogosa, Sharpe) (Oxoid Ltd. CM0361) and incubated at 30°C for 72 h in a ST 6120 culture incubator (Heraeus) (ISO 15214, 1998). *Staphylococcus* spp. were counted on Mannitol Salt Agar (Oxoid Ltd. CM0085) and incubated at 37°C for 72 h. *Enterobacteriaceae* (log cfu $g^{-1}$) was determined on Violet Red Bile Glucose Agar "VRBG" (CM04825, Oxoid) and incubated at 37*°C for 24 h (ISO* 21528-2, 2004). *Escherichia coli* and Total Coliform Counts "TCC" (log cfu $g^{-1}$) were determined using a chromogenic *E. coli* / coliforms medium (Rapid *E. coli* 2 Agar, Bio-Rad, Marnes La Coquette, France) after incubation at 37*°C for 24 h (ISO 483*2, 2006). *Salmonella* spp. (absence in 25 g) was determined according ISO 6579 (2002). Samples were pre-enriched by incubating in peptone water at 37°C for 18 h. A modified semi-solid Rappaport-Vassiliadis (MSRV) Agar (Oxoid CM1112) was used for the selective enrichment (at 41.5*°C for 24 h). Muller-Kauffmann* Tetrathionate "MKT" Broth Base (Oxoid CM0343) was used as culture medium for the isolation of *salmonellae* and the suppression of Proteus species (at 37 °C for 24 h). The culture medium for recovery of *Salmonella* spp. was Xylose-Lysine-Desoxycholate AGAR XLD (Oxoid PO1132) (at 37*°C for 24 h). The selective and diagnostic culture medium was Brilliant Green* Agar BGA (Oxoid: PO0171) (at

37°C *for 24 h). Clostridium* spp. (log cfu $g^{-1}$) was determined according ISO 7937 (2004). Samples were incubated at 37°C for 20 h under anaerobic conditions on Sulphite Polimixin Sulphadiazine "SPS" agar (Cat 1082.00; Laboratorios Conda, Torrejón de Ardoz, Madrid, Spain). Coagulase positive Staphylococci *(S. aureus* and other species) (log cfu $g^{-1}$) was determined according to ISO 6888-2 (1999). Samples were incubated at 37°C *for* 24-48 h on Baird Parker agar base (Oxoid: CM0961) supplemented with Rabbit Plasma Fibrinogen (Oxoid: SR0122). *Listeria monocytogenes* (absence in 25 g) was determined according ISO 11290-1 (1996) modified procedure, which has validated according ISO 16140 (2003) protocol. Samples were enriched at 30°C during 24 h using Fraser Agar before being incubated at 37°C *for 24 h* on Listeria agar containing phosphatidylinositol (Listeria Agar according Ottaviani and Agosti). Moulds and yeasts were counted on RB (Rose-Bengal) plates with chloramphenicol (Oxoid Ltd. CM0549) after incubating at 25°C for 120 h (ISO 21527-2, 2008).

### 3.4. Sensory analysis

A quantitative descriptive sensory analysis (QDA) was carried out according to ISO 4121 (2003). The salami pieces were skinned and then sliced (8 mm thick) using a MAS9101 slicer. Ten panellists were selected and trained according to ISO 8586 (2012). Most of the panellists had previous experience on QDA of dry-cured fermented sausage. There were six training sessions. In the first two sessions, the main sensory descriptors of freshly-made salami were identified and quantified in different samples; the next two sessions were concerned with identifying and selecting the sensory attributes related with the spoilage of salami (moulds growth, appearance changes, off-odours, off-flavours, hardening and casing wrinkling, among others). The final two sessions were concerned with quantifying colour, odour flavour, texture and overall quality using five-point intensity scales from 1 (minimum) to 5 (maximum), which was graduated in one-point intervals. Both extremes of each scale corresponded to the respective reference samples and their descriptions. Table 1 shows the scales and references for the different selected attributes used to monitor the shelf life of salami. Six different references of this type of salami were used: (i): Salami retailed for 6 months for strongly spoiled samples (wrinkling, fat yellowness, mouldy, rancidity, bitter, hardness and fattiness); (ii): Semi-ripened salami (dried for 6 days) for pre-ripened samples (deficient colour, odour and flavour, excessive acidity and softness); (iii): Iberian pork salami (ripened for 12 days) for high coloured and high flavoured samples; (iv): Turkey salami (ripened for 12 days) for lean and dry samples; and (v): Fresh-cured (without ripening) salami for samples lacking acidity. The outer halo, a quality defect consisting in a darkening of slice surface due excessive dehydration, was directly measured using a millimetre ruler.

Finally, the panel scored the overall quality using the same five-numeric scale. The maximum score of overall quality corresponded to freshly-made salami, while the minimum score of overall quality corresponded to the above men-

Table 1 - Sensory descriptors and reference scales used in the qualitative descriptive analysis of salami.

| | Scales | | Salami references | |
|---|---|---|---|---|
| Scoring | Minimum 1 | Maximum 5 | Minimum 1 | Maximum 5 |
| Wrinkling | Smooth | Wrinkled | Ripened for 6 days | Kept for 6 months |
| Mouldy off-colour | White | Greenish | Ripened for 6 days | Kept for 6 months |
| Lean colour | Pinkish-red | Purplish-red | Ripened for 6 days | Iberian |
| Fat colour | White | Yellowish | Ripened for 6 days | Kept for 6 months |
| Outer halo | 0 mm | 5 mm | | |
| Odour | Raw pork seasoned with pepper | Dry-cured pork seasoned with pepper | Ripened for 6 days | Iberian |
| Rancid odour | Undetectable | Rancid | Ripened for 6 days | Kept for 6 months |
| Mouldy off-odour | *P. chrysogenum* | Wet cardboard and pungent | Ripened for 6 days | Kept for 6 months |
| Flavour | Raw pork seasoned with pepper | Dry-cured pork seasoned with pepper | Ripened for 6 days | Iberian |
| Rancid flavour | Undetectable | Rancid | Ripened for 6 days | Kept for 6 months |
| Mouldy off-flavour | *P. chrysogenum* | Wet cardboard and bitter | Ripened for 6 days | Kept for 6 months |
| Acid flavour | Undetectable | Acid | Non-ripened | Ripened for 6 days |
| Bitter aftertaste | Undetectable | Bitter | Ripened for 6 days | Kept for 6 months |
| Hardness | Soft | Hard | Ripened for 6 days | Kept for 6 months |
| Juiciness | Dry | Juicy | Turkey | Iberian |
| Fattiness | Lean | Fatty | Turkey | Iberian |

Table 2 - Microbial counts of salami kept in retailing conditions for up to 150 days.

| | Storage days | | | | | |
|---|---|---|---|---|---|---|
| | 0<br>M±SD | 30<br>M±SD | 60<br>M±SD | 90<br>M±SD | 120<br>M±SD | 150<br>M±SD |
| Total Viable Counts | 8.64±0.28a | 8.58±0.09a | 8.70±0.36a | 8.60±0.26a | 8.31±0.39a | 7.79±0.27b |
| Lactic Acid Bacteria | 8.45±0.15a | 8.40±0.14a | 8.54±0.12a | 7.97±0.19b | 7.82±0.20b | 7.74±0.17b |
| Staphylococcus spp. | 5.52±0.13 | 5.93±0.38 | 5.51±0.79 | 5.12±0.98 | 4.98±0.78 | 4.77±0.33 |
| Moulds and yeasts | 5.46±0.24 | 5.51±0.27 | 5.55±0.13 | 5.53±0.28 | 5.70±0.15 | 5.62±0.16 |
| Enterobacteriaceae | 3.08±0.71 | <1 | <1 | <1 | <1 | <1 |
| *E. coli* and coliforms | <1 | <1 | <1 | <1 | <1 | <1 |
| Salmonella spp. | ND | ND | ND | ND | ND | ND |
| *L. monocytogenes* | ND | ND | ND | ND | ND | ND |
| Coagulase + Staphylococci | <1 | <1 | <1 | <1 | <1 | <1 |
| Clostridium spp. | ND | ND | ND | ND | ND | ND |
| aw | 0.85±0.01a | 0.82±0.02ab | 0.81±0.05ab | 0.77±0.07b | 0.70±0.04c | 0.69±0.06c |
| pH | 5.10±0.36b | 4.95±0.25b | 5.00±0.20b | 5.31±0.44ab | 5.25±0.19ab | 5.61±0.59a |

M±SD: means and standard deviations.
Means with different superscripts are different at P<0.05.
ND: not detected.

tioned spoiled salami. The relative loss of sensory quality (expressed as percentage) was calculated as the ratio between current overall quality (days 0, 30, 60, 90, 120 and 150) and the overall quality corresponding to the salami with all its sensory properties intact (day 0).

### 3.5. Statistics

The statistical model design was completely random, and the time of storage was considered as the main treatment. Nine sausage units per replicate (three) and storage time (five) were analysed in duplicate. The effect of storage time on the physical, chemical, microbial and sensory variables of salami was determined by one-way ANOVA. The least squares means "LSM" and significance of treatments were calculated using type IV sum of squares. The Scheffe Means Test was used to compare the LSM, which were considered to be statistically different when P<0.05. The statistical analysis was made with the Statistix 8.0 program for Windows (Analytical Software, Tallahassee, FL, USA). Different regression (linear and non-linear) equations between the storage time and the dependent variables were determined to monitor the spoilage and shelf life of salami (Excell 2010, Microsoft Corporation, Redmond, WA, USA). Polynomial regression equations presented the highest $R^2$ coefficients and were finally used to monitor changes in the different quality traits of salami throughout the retailing period. These equations permitted to predict the average values of each quality trait of salami at any control time. The loss of half of the initial overall quality was used to establish the maximum shelf life time of salami.

## 4. RESULTS

Table 2 shows the effect of storage time on the microbial counts and related parameters ($a_w$ and pH) of pork salami. TVC were higher than 8 log cfu $g^{-1}$ from day 0 to 120 but had significantly ($p<0.05$) decreased by day 150 with respect to initial TVC. The growth of fermentative bacteria was very limited in the salami kept in retailing conditions. LAB, the main fermentative group, stabilized ($p>0.05$) at around 8 log cfu $g^{-1}$ from day 0 to 60, decreased ($p<0.05$) from day 60 to 90 and then stabilized again from day 90 to 150. *Staphylococcus* spp., moulds and yeasts remained stable at around 5 log cfu $g^{-1}$ throughout the retailing period. As regards hygiene and safety, *Enterobacteriaceae* totalled 3 log cfu $g^{-1}$ in the freshly-made salami, probably resulting from to the use of pork casing, although they were below the detection limit on subsequent control days. None of studied pathogenic bacteria, *E. coli* and total coliforms, *Salmonella* spp., *Clostridium* spp., coagulase positive staphylococci, and *L. monocytogenes*, were found in the salami. The evolution of $a_w$ and pH values was coherent with the microbial data, since $a_w$ diminished from 0.85 (day 0) to 0.69 (day 150), the decrease being significant ($p<0.05$) from day 90 onwards. Mean pH values stabilized around 5 throughout storage, although the pH was higher ($P<0.05$) at day 150 with respect to initial pH. This evolution of pH values suggests the lack of additional lactic fermentation, an observation that could be extended to other bacterial groups even less capable of growing in the salami. It is therefore unlikely that microbial phenomena could have limited the shelf-life of the salami.

Table 3 - Appearance and colour changes of salami kept in retailing conditions for up to 150 days.

| | Storage days | | | | | |
|---|---|---|---|---|---|---|
| | 0<br>M±SD | 30<br>M±SD | 60<br>M±SD | 90<br>M±SD | 120<br>M±SD | 150<br>M±SD |
| *Sensory* | | | | | | |
| Wrinkling | 1.96±0.60b | 2.02±0.75b | 2.07±0.66b | 2.37±0.72ab | 2.53±0.80ab | 2.68±0.78a |
| Mould colour | 1.30±0.58e | 1.84±0.63d | 2.12±0.77cd | 2.38±0.88c | 3.08±0.87b | 3.53±0.87a |
| Lean colour | 3.47±0.62d | 3.65±0.63cd | 3.83±0.69bc | 4.07±0.69b | 4.34±0.55a | 4.46±0.57a |
| Fat colour | 2.36±0.75c | 2.29±0.67c | 2.50±0.67bc | 2.46±0.60bc | 2.72±0.71ab | 2.89±0.70a |
| Outer layer | 1.98±0.66a | 1.53±0.49b | 1.60±0.48b | 1.45±0.48bc | 1.44±0.53bc | 1.26±0.41c |
| *CIE colour* | | | | | | |
| L* | 44.9±3.80a | 39.5±1.96b | 38.2±2.69bc | 36.9±1.18cd | 36.3±2.48cd | 37.4±2.37bcd |
| a* | 13.4±1.83a | 11.1±0.78b | 11.2±1.51b | 10.6±1.71bc | 9.25±1.55c | 9.33±1.19c |
| b* | 1.88±0.97a | 1.51±0.45a | 0.85±0.88ab | 0.91±1.27ab | -0.08±1.05bc | -0.75±1.19c |
| C* | 13.5±1.90a | 11.2±0.82b | 11.2±1.52b | 10.7±1.83bc | 9.30±1.53c | 9.42±1.13c |
| H* | 7.82±3.40a | 7.83±1.43a | 4.08±4.65ab | 4.04±5.78ab | -1.32±6.81bc | -4.97±7.48c |

M±SD: means and standard deviations.
Means with different superscripts are different at P<0.05.

Table 3 shows the effect of the storage time on the appearance and colour of pork salami. The slight wrinkling of the freshly-made salami moderately increased during storage, and was particularly high at day 150, by which time dehydration was very intense. Mould colour tended to change from white (day 0) to greenish (day 150). Slight but significant mould greening was detected at day 30 and later. Changes in the colour of moulds are often due to the proliferation of contaminating microflora, since the *P. chrysogenum* culture used had white conidia. Mould greening may affect appearance and, therefore, reduce the shelf life of the pieces of salami. After slicing, the lean colour score increased from 3.5 (day 0) to 4.5 (day 150) and red-purplish colour was evaluated as more intense at day 60 (P <0.05) and later. The outer halo of salami slices diminished from 2 mm (day 0) to undetectable (day 150), this defect beginning to decrease at day 30 (p<0.05). As regards CIELab colour, L* decreased from 45 (day 0) to 37 (day 150) CIE units, this decrease being significant from day 90 onwards. The a* and b* coordinates decreased from 13.4 and 1.9 (day 0) to 9.3 and -0.7 (day 150) CIE units, respectively. This decrease in both coordinates was slight but significant at day 120 and later. C* and H* values proportionally decreased during storage. Both sensory and reflectance data indicated that the intensification of lean colour in salami slices was related with gradual dehydration and curing, while the fat colour was quite stable. The internal colour of the salami slices does not seem to be a factor limiting its shelf-life, since no lean browning and no relevant fat yellowing were detected. However, the slight greening observed in the covering moulds of casing might negatively

Table 4 - Odour and flavour changes of salami kept in retailing conditions for up to 150 days.

| | Storage days | | | | | |
|---|---|---|---|---|---|---|
| | 0<br>M±SD | 30<br>M±SD | 60<br>M±SD | 90<br>M±SD | 120<br>M±SD | 150<br>M±SD |
| Odour | 2.93±0.78a | 2.93±0.69a | 2.91±0.71a | 2.85±0.86a | 2.55±0.79b | 1.95±0.68c |
| Flavour | 3.16±0.73a | 3.16±0.59a | 2.88±0.77a | 2.89±0.99a | 2.38±0.76b | 1.85±0.71c |
| Rancid odour | 1.05±0.28d | 1.25±0.46d | 1.79±0.75c | 2.01±0.94c | 2.55±0.91b | 3.11±0.92a |
| Rancid flavour | 1.07±0.28d | 1.39±0.55d | 2.21±0.93c | 2.33±1.07c | 2.96±0.94b | 3.43±0.92a |
| Mould off-odour | 1.08±0.38d | 1.22±0.46d | 1.35±0.51cd | 1.52±0.80c | 1.80±0.95b | 2.26±0.91a |
| Mould off-flavour | 1.04±0.26d | 1.14±0.34d | 1.33±0.46cd | 1.55±0.88bc | 1.81±0.95ab | 2.08±1.05a |
| Acid flavour | 1.70±0.64ab | 1.41±0.48c | 1.48±0.52bc | 1.43±0.55c | 1.64±0.70abc | 1.75±0.66a |
| Bitter aftertaste | 1.21±0.47c | 1.37±0.53c | 2.12±0.90b | 2.17±1.12b | 2.95±0.98a | 3.11±1.17a |

M±SD: means and standard deviations.
Means with different superscripts are different at P<0.05.

affect the sensory quality of the whole pieces of salami at retail.

Table 4 shows the effect of the storage time on the odour and flavour of pork salami. The odour and flavour intensities were scored as moderate in the freshly made salami. They remained stable from day 0 to day 90, but significant losses in both attributes were noted at day 120 and later. Coinciding with this loss of odour and flavour, rancid odour and flavour were scored as imperceptible (day 0), detectable ($p<0.05$) (day 60) and moderate (day 150), which may indicate gradual lipid oxidation. Mouldy off-odour and off-flavour increased from imperceptible (day 0) to detectable (day 90) and then fell to weak (day 150). The mouldy smell was described as being like wet cardboard and pungent, while the mouldy off-taste was also described as bitter. Bitter aftertaste was marked as imperceptible (day 0), detectable (day 60) and finally medium (day 150). Mouldy off-flavour was less intense than rancidity and bitterness. Acid flavour scored weak throughout storage and showed only minor variations, reflecting the pH values obtained. The shelf life of salami was limited by the odour and flavour deterioration associated with incipient rancidity and bitter aftertaste.

Table 5 shows the effect of storage time on the texture and moisture of pork salami. The salami showed strong dehydration throughout the retailing period, passing from 33% moisture (day 0) to 18% moisture (day 150), despite being packed in perforated plastic bags. This resulted in marked changes in texture: early hardening was detected by the panel at day 30 ($p<0.05$), while loss of juiciness was detected at day 60 and later and the sense of fattiness had intensified by day 90 and remained intense thereafter. Instrumental texture data corroborated the above findings. The shear force increased from 51 (day 0) to 111 N (day 150). According TPA, hardness increased from 87 (day 0) to 202 N (day 120), springiness decreased from 6.4 (day 0) to 4.7 mm (day 120) and cohesiveness decreased from 0.5 (day 0) to 0.3 (day 120). The effects of storage time on hardness, springiness and cohesiveness were significant at day 60 and later. However, the TPA test could not be performed on day 150 because the hardness value was over 250 N, the maximum force measurable by the load cell used. Other texture parameters, such as adhesiveness, gumminess and chewiness, were not clearly affected by the storage time. Early hardening associated with losses of juiciness, springiness and cohesiveness, limited the shelf life of salami pieces under retail display conditions.

Finally, the contribution of the different limiting factors to the shelf-life of salami was assessed. Table 6 shows the polynomial regression coefficients between storage time and all the dependent variables. Polynomial regression provided higher $R^2$ coefficients than other types of non-linear regression. The storage time was accurately predicted by using most of the dependent variables. The highest $R^2$ were obtained for the variables associated with off-flavours and off-odours (moulds deterioration and rancidity), hardening and loss of juiciness (measured by TPA and sensory analysis) and lean purpling. These variables therefore might be used to calculate the shelf-life of the whole pieces of salami at retail. To confirm this, the

Table 5 - Texture changes of pork salami pieces kept in retailing conditions for up to 150 days.

| | Storage days | | | | | |
|---|---|---|---|---|---|---|
| | 0<br>M±SD | 30<br>M±SD | 60<br>M±SD | 90<br>M±SD | 120<br>M±SD | 150<br>M±SD |
| Moisture | 33.3±2.61$^{a}$ | 27.4±2.18$^{b}$ | 27.1±3.94$^{bc}$ | 23.5±4.64$^{c}$ | 18.5±1.67$^{d}$ | 18.0±3.93$^{d}$ |
| *Sensory* | | | | | | |
| Hardness | 2.76±0.62$^{d}$ | 3.29±0.58$^{c}$ | 3.49±0.59$^{c}$ | 3.80±0.60$^{b}$ | 4.18±0.70$^{a}$ | 4.27±0.70$^{a}$ |
| Juiciness | 3.28±0.64$^{a}$ | 3.10±0.57$^{ab}$ | 2.91±0.54$^{bc}$ | 2.79±0.65$^{c}$ | 2.50±0.79$^{d}$ | 2.15±0.71$^{e}$ |
| Fattiness | 2.87±0.67$^{b}$ | 3.18±0.57$^{ab}$ | 3.18±0.73$^{ab}$ | 3.34±0.65$^{a}$ | 3.37±0.92$^{a}$ | 3.37±1.15$^{a}$ |
| *Instrumental* | | | | | | |
| Shear Force | 51.4±12.8$^{b}$ | 56.2±18.8$^{b}$ | 70.8±14.5$^{b}$ | 69.0±15.5$^{b}$ | 110.9±37.0$^{a}$ | 128.1±44.6$^{a}$ |
| Hardness | 87.3±28.3$^{c}$ | 116.9±38.6$^{c}$ | 156.7±34.1$^{b}$ | 182.9±30.6$^{ab}$ | 201.9±50.9$^{a}$ | ND |
| Adhesiveness | -9.60±7.73 | -9.31±71.01 | -4.87±5.24 | -7.09±9.77 | -5.75±6.54 | ND |
| Cohesiveness | 0.50±0.04$^{a}$ | 0.47±0.05$^{a}$ | 0.37±0.06$^{b}$ | 0.31±0.04$^{bc}$ | 0.28±0.09$^{c}$ | ND |
| Springiness | 6.37±0.57$^{a}$ | 6.09±0.49$^{a}$ | 5.28±0.39$^{b}$ | 4.75±0.34$^{b}$ | 4.75±0.69$^{b}$ | ND |
| Gumminess | 46.1±8.98$^{b}$ | 53.4±11.55$^{ab}$ | 56.1±7.23$^{ab}$ | 57.0±10.75$^{a}$ | 53.6±7.71$^{ab}$ | ND |
| Chewiness | 290.2±63.9$^{ab}$ | 320.9±48.3$^{a}$ | 292.6±37.9$^{ab}$ | 271.5±57.3$^{ab}$ | 253.8±45.7$^{b}$ | ND |

M±SD: means and standard deviations.
Means with different superscripts are different at $P<0.05$.
ND: not determined.

Table 6 - Polynomial regression coefficients ($R^2$) between storage time and the dependent variables ordered from highest to lowest.

| Dependent variable | $R^2$ | Dependent variable | $R^2$ |
|---|---|---|---|
| Mould flavour | 0.9990 | TPA Cohesiveness | 0.9677 |
| TPA Hardness | 0.9951 | CIE b* Yellowness | 0.9643 |
| TPA Gumminess | 0.9944 | Water activity | 0.9573 |
| Lean colour | 0.9921 | Bitter aftertaste | 0.9550 |
| Mould odour | 0.9920 | Total Viable Counts | 0.9545 |
| Rancid odour | 0.9917 | Moisture | 0.9534 |
| Juiciness | 0.9917 | Shear Force | 0.9491 |
| Hardness | 0.9829 | TPA Springiness | 0.9484 |
| Mould colour | 0.9823 | Fattiness | 0.9368 |
| Rancid flavour | 0.9793 | Fat colour | 0.9336 |
| Flavour | 0.9726 | Outer halo | 0.9291 |
| Odour | 0.9700 | Chroma | 0.9047 |
| Wrinkling | 0.9693 | CIE a* Redness | 0.9021 |
| Lightness | 0.9688 | Lactic acid bacteria | 0.8323 |
| Hue angle | 0.9678 | pH | 0.8322 |

All the $R^2$ coefficients were significant for $p<0.05$.

trained panel quantified the loss of sensory quality of salami throughout the retailing period. Fig. 1 shows the effect of the storage time on the relative overall quality (real and estimated) of pork salami. The relative overall quality fell by half after 100 days of storage, which may also be estimated using a polynomial regression equation ($R^2$ = 0.9684). This $R^2$ even increased to 0.9986 when the relative overall quality at day 150 was not used for the calculations. Spoiled salami (day 100) had an intense purplish-red and was hard, while its odour and flavour were already masked by rancidity, bitter aftertaste, and, to a lesser extent, mouldy off-flavour. Most of the physical-chemical parameters studied also changed from day 0 to day 100. Thus, a reduction by 50% in the initial score for overall quality seemed to correspond to the end of the shelf-life of whole salami pieces kept in retailing conditions.

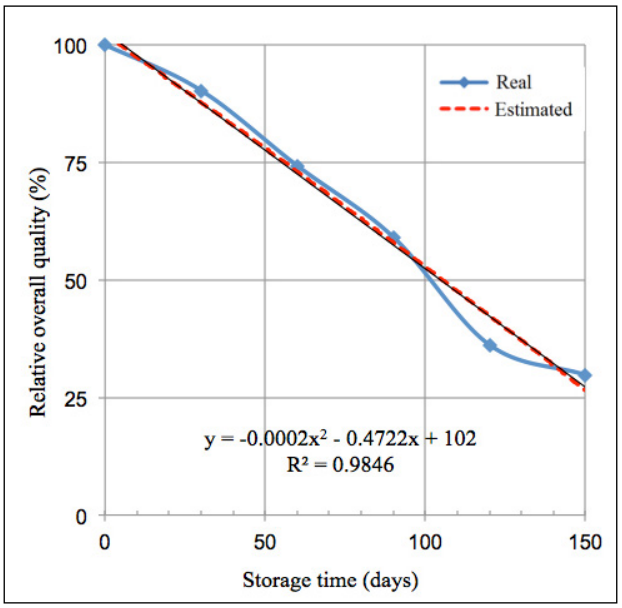



Fig. 1 - Estimation of the shelf-life under retail display conditions of salami pieces according to their loss of sensory quality.

## 5. DISCUSSION

Excessive acidification, microbial quality and other defects due to oxidation and dehydration were evaluated as potential limiting factors of the shelf-life of salami. No additional acidification by lactic fermentation was found during storage and the inhibition of pathogens and enteric bacteria was amply ensured throughout the retailing period. The microbial quality of salami strongly depends on its levels of fermentative bacteria, especially when LAB and Coagulase Negative Cocci are used as started cultures. Lactic-acid bacteria and Staphylococci are the predominant microflora present in this type of salami, where they are responsible for most of the total viable counts (MARTÍNEZ *et al.*, 2009). As can been seen, no additional growth of total viable and fermentative bacteria occurred during storage, although their final counts were low because $a_w$ decreased as a result of additional dehydration. Other authors have also reported the inhibition of lactic fermentation during storage in unpacked pieces (LEE *et al.*, 2009) or packed sliced salami (RUBIO *et al.*, 2007). The low $a_w$ might have limited the microbial and endogenous enzymatic activities responsible for pH changes in the salami. An increase in pH may occur as a result of the gradual release of peptides, amino acids and ammonia following protein breakdown (SPAZIANI *et al.*, 2009) or due to lactic acid degradation by moulds and internal yeasts (MENDONÇA *et al.*, 2013). Pathogenic and spoilage bacteria would have been strongly inhibited in the displayed salami as a result of decreases in $a_w$, pH and temperature, and preservation by salt, nitrite and culture bacteriocins. The *Enterobacteriaceae* that occasionally appeared in the first few days were probably due to the use of pork casing, although they were not detected on subsequent control days. No bacterial risk regarding the safety and quality was reported in salami stored for up to 7 months (IZQUIERDO *et al.*, 2006; RUBIO *et al.*, 2007; LEE *et al.*, 2009).

As regards the appearance of the salami pieces, wrinkling did not seem to be a major cause of loss of sensory quality, since a weak wrinkling was only assessed at day 150, when the salami was already strongly spoiled by other causes. Wrinkling is caused by excessive dehydration, although many dry sausages stuffed in pig gut can present some degree of natural wrinkling (ORDÓÑEZ *et al.*, 1999). However, slight greening of the mould covering was detected early, although it is difficult to assess this im-

pact on the sensory quality in the salami slices eaten without casing. Changes in the colour of moulds might be due to the growth of contaminating microflora present in the meat factories or retailing systems, whose proliferation would be favoured by the high relative humidity existing inside the perforated bag due to water evaporation and chilling. Mould cultures are used to prevent this defect. The *P. chrysogenum* culture used had white conidia and a particularly recognisable flavour; however, most *Penicillium* strains and other moulds can produce grey and green conidia (POSE *et al.*, 2004). The presence of contaminating moulds, such as *Penicillium*, *Aspergillus* and others, should be avoided for sensory reasons and because moulds may produce toxic metabolites, such as ochratoxin, and beta-lactams, which can spread to the meat (SUNESEN and STAHNKE, 2003; IACUMIN *et al.*, 2011). Mouldy odour and flavour are good discriminators of the eating quality of salami (RUÍZ *et al.*, 2005).

Luminance is another good discriminator of the sensory quality of salami (RUÍZ *et al.*, 2005). The colour changes observed could be explained by the formation of pigments, together with gradual dehydration. Nitrosylmyoglobin exponentially increases at the beginning of drying as a result of the addition of nitrite and the nitrate-reductase activity of staphylococci, and may continue to increase throughout ripening, since strong oxidizing conditions are required to transform the nitrosylmyoglobin into metmyoglobin (GØTTERUP *et al.*, 2008). This would explain why no browning was detected in the salami stored for up to 5 months. A greater concentration of nitrosylmyoglobin in salami is associated with chroma increase and hue angle decrease (CHASCO *et al.*, 1996); however, a decrease in lightness due to dehydration may change the red tonality from pinkish to purplish. SUMMO *et al.* (2006) found no decrease in CIELab values in vacuum-packed salami stored for 40 days, while other authors reported a certain discoloration of salami as storage time increased (ZANARDI *et al.*, 2002; RUBIO *et al.*, 2008; LEE *et al.*, 2009). A dark outer halo appears in the slices through excessive evaporation of water on the surface due to rapid drying, and this can affect appearance (GUERRERO, 1992); however, the initial halo gradually disappeared because it was rehydrated by internal water. Fat yellowing was almost imperceptible, indicating modest fat oxidation during storage. The addition of sodium ascorbate and red colorant, together with the low exposure of whole pieces to fluorescent lighting might could also have contributed to colour stabilization.

Flavour deterioration (associated with rancidity and bitterness) resulted in a clear loss of sensory quality. Flavour deterioration during storage has also been reported by others (RUBIO *et al.*, 2007; SUMMO *et al.*, 2010), although the rate of deterioration is slower in vacuum-packed than in unpacked salami (SUMMO *et al.*, 2006 and 2010). Flavour deterioration in this type of salami is associated with the degradation of volatiles resulting from spices and microbial activities, and the formation of volatiles from lipid oxidation. Increases in heptanoic acid methyl ester, pentanoic acid methyl ester and hexanoic acid methyl ester and 2-heptenal are good indicators of the storage time (LORENZO *et al.*, 2013). Other studies (MARCO *et al.*, 2008; RUBIO *et al.*, 2007; SUMMO *et al.*, 2006) evaluated lipid oxidation through increases in the malondyaldehyde level of retailed salami, which was determined by distillation and spectrophotometry, although this method not to be advised due to the interferences between nitrites and sugars presents with the thiobarbituric acid reagent substances (DE LAS HERAS *et al.*, 2003). Lipid oxidation largely takes place by reaction with the oxygen occluded in the mass (FERNÁNDEZ *et al.*, 1997), depending on fat composition and the balance between pro-oxidant and antioxidant agents, such as sodium ascorbate (MORRISSEY *et al.*, 1998). Lipid autooxidation generates volatile and non-volatile compounds, such as aldehydes, ketones, alcohols, esters and alkanes, which contribute to the flavour of dry-cured sausages, including possible off-flavours (OLESEN *et al.*, 2004). Packing and retailing conditions being equal, salami whole pieces should have a lower degree of oxidation than sliced salami, since whole pieces may be more protected against the pro-oxidant factors involved in retailing systems, such as light and oxygen.

Bitterness has been associated with the free amino acids, such as methionine, valine, leucine, arginine, histidine, isoleucine, phenylalanine and tryptophan (HENRIKSEN and STAHNKE, 1997; ORDÓÑEZ *et al.*, 1999), formed, along with ammonia, by reactions catalysed by endogenous and microbial exopeptidases (DEMASI *et al.*, 1990; MOLLY *et al.*, 1997). These amino acids can adversely affect acceptance and can also be transformed into biogenic amines by the decarboxylase activity of some microorganisms, constituting a health risk (MASSON *et al.*, 1996; COLORETTI *et al.*, 2008). Mouldy off-flavours might be caused by the impregnation of slices by contaminating moulds from casing, since the *P. chrysogenum* culture used had a particularly recognisable flavour. The protease and lipase activites of moulds, together with their ability to degrade both free amino acids and fatty acids, leads to the formation of branched aldehydes and alcohols, methylketones and secondary alcohols, which are related with flavour in ripened sausage (HIERRO *et al.*, 2005). The worsening of texture clearly limited the shelf life of the salami pieces packed in perforated bags. The hardening, loss of juiciness and other textural changes noted would primarily have been related with the dehydration and denaturation of myofibrillar proteins (GONZÁLEZ-FERNÁNDEZ

*et al.*, 2006; LIAROS *et al.*, 2009; SPAZIANI *et al.*, 2009). This differs from other studies on vacuum-packed salami, where hardness, cohesiveness and elasticity slightly increased during storage (RUBIO *et al.*, 2007).

GYESLEY (1991) defined the shelf life as the length of time that food can be kept before it is considered unsuitable for sale, use, or consumption, or, as the recommended time that food can be stored, during which the defined quality of a specified proportion of the goods remains acceptable under specified conditions of distribution, storage and display. According to this definition, acceptance may be measured as purchase criterion based on consumer hedonism or preferences, or as the degree of preservation of properties of the freshly-made salami. We proposed the second method, since QDA is a highly sensitive tool for predicting the storage time, as regression analysis indicated. For example, hardening was detected earlier by QDA that by TPA or the Warner-Bratzler test and, in general, sensory scoring predicted the storage time more accurately than instrumental measurements. A reduction of 50% of the initial sensory quality scored by a trained panel can be used as a limit for establishing the maximum time that the eating quality of salami is critically affected by previous display, or to quantify its shelf life. Most of our findings (hardening, losses of juiciness and flavour, rancidity, bitterness and mould spoilage) found in the spoiled salami support this proposal. However, further studies should be directed at verifying whether the determination of shelf life of salami based on a quantification of its gradual sensory spoilage corresponds, or not, with purchase criteria based on consumer hedonism or preferences.

## 6. CONCLUSIONS

The relative loss of sensory quality, scored by a trained panel, is proposed as a criterion for establishing the shelf life of salami. The shelf-life of salami, retailed as small pieces covered by moulds and packed in perforated bags, was established at 100 days under the tested retail conditions. Microbiological criteria could not be applied to establishing the shelf-life, since the microbial quality was amply ensured and lactic fermentation stopped, favoured by the additional dehydration that occurred at retail. Flavour deterioration and a worsening in texture were identified as the main causes related with the loss of sensory quality of salami. Flavour deterioration was associated with incipient rancidity and bitterness, and, to a lesser extent, with mouldy off-flavour, which was described as "wet cardboard". Worsening of the texture involved hardening and a loss of juiciness. Most of the sensory and instrumental data determined in salami accurately predicted its storage time.

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PAPER

# THE EFFECT OF POLYSACCHARIDE CONCENTRATION AND PH ON PHYSICOCHEMICAL PROPERTIES OF FLAXSEED POLYSACCHARIDE-POTATO STARCH MIXTURES

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ABSTRACT

Physiochemical properties of starch and quality of the starch-based food can be modified by hydrocolloids such as flaxseed polysaccharide (FG), which is an anionic polysaccharide. In this paper the effect of flaxseed polysaccharide concentration and pH on the flaxseed polysaccharide-potato starch mixtures (FG-PS) were investigated. The results showed that both flaxseed polysaccharide concentration and pH had significant effect on the physiochemical properties of FG-PS. At pH 7, when compared with pure potato starch, pasting properties, swelling power and freeze-thaw stability of potato starch were improved with addition of flaxseed polysaccharide, while the gel hardness was decreased. Freeze-thaw stability of the mixtures under alkaline condition was higher than that under acidic condition due to the addition of FG, but the best one was exhibited at pH 7.

- Keywords: flaxseed polysaccharide, freeze-thaw stability, hardness, pH, potato starch, swelling power -

## 1. INTRODUCTION

The taste and texture of starch gel can be changed by addition of hydrocolloids. Therefore, hydrocolloids are widely used to improve the texture, stability and moisture migration of starch-based foods (AROCAS *et al.*, 2009; BRENNAN *et al.*, 2008; GULARTEA and ROSELL, 2011; KULICKE *et al.*, 1996; SIKORA *et al.*, 2007; SONG *et al.*, 2006). Till now, there are many investigations about the effects of hydrocolloids on the pasting properties, gelatinization, swelling power, freeze-thaw stability of starches. SHI and BEMILLER (2002) reported that pasting temperature of corn starch was decreased by addition of CMC, gellan gum, xanthan gum, guar gum and sodium alginate. Some other researches also reported that peak viscosity of potato starch was decreased significantly by addition of anionic polysaccharide, such as xanthan, for the electrostatic repulsive interaction between the anionic polysaccharide and the phosphate group of potato starch (CAI *et al.*, 2011).

The hardness of starch gel could also be influenced by the presence of hydrocolloids (PARADA *et al*, 2011; WANG *et al*, 2007). Possible reasons that could explain why the gel properties of starch could be modified by addition of hydrocolloids are as follows: (1) starch granules are embedded in the gel network of hydrocolloid; (2) the electrostatic repulsive force exists between starch granules and ionic hydrocolloids (GARCIA-OCHOA *et al.*, 2000; KATIA *et al.*, 2011).

Swelling power and freeze-thaw stability of starch could be also affected by hydrocolloids. Rosell *et al.* reported that swelling power of rice starch was reduced by guar gum, while changed oppositely by xanthan (ROSELL *et al.*, 2011). LEE *et al.* (2002) showed that freeze-thaw stability of sweet potato starch was improved significantly by anionic polysaccharides such as xanthan, guar gum, sodium alginate.

Potato starch (PS) is one of the most widely used tuber starch owing to its brilliant thickening capacity and paste transparency (SUDHAKAR *et al*, 1995; HASSAN *et al*, 2012). However in our previous studies, PS showed a weak thermo-stability and weak resistance to acid or alkali, which could be likely improved by addition of polysaccharides.

Flaxseed polysaccharide is a water soluble anionic polysaccharide which is consisting of acidic flaxseed polysaccharide (AFG) and neutral flaxseed polysaccharide (NFG). It presents suitable properties with thickening, weak gelling, emulsifying and stabilizing properties (CHEN *et al.*, 2006; MURALIKRISHAN and SALIMATH, 1987). Flaxseed polysaccharide solution with low concentration exhibited shear-thinning properties and weak thixotropic properties (CHEN, 2005; MAZZA and BILIADERIS, 1989). The weak gelling properties of flaxseed polysaccharide depend on the ratio of AFG to NFG and the flaxseed polysaccharide concentration. Additional researches, however, reported that gelling properties of flaxseed polysaccharide were weaker in the case of higher NFG content (CUI and MAZZA, 1996a; CUI *et al.*, 1994). Besides, flaxseed polysaccharide solution showed good water-and-oil holding capacities for its emulsifying and emulsion stability (DICKINSON, 2008; FEDENIUK and BILIADERIS, 1994).

More studies were carried out on the effects of hydrocolloids on pasting and gelling properties of starch. However, few reports were available about the effect of flaxseed polysaccharide on pasting, gelling, swelling properties, freeze-thaw stability of starch. WANG *et al* (2008) investigated the apparent viscosity of maize starch as affected by the addition of flaxseed polysaccharide. They concluded that apparent viscosity of maize starch was increased with adding flaxseed polysaccharide.

During food processing or storage, pH of food materials could be changed by adding some ingredients which would also affect the physicochemical properties of starch or hydrocolloid-starch mixtures, and that could influence the quality of final products (CUI and MAZZA, 1996b; MALI *et al.*, 2003; MAZZA and BILIADERIS, 1989; PARADA *et al.*, 2011; SEDDIK *et al.*, 2008). However, little information was available about the effect of pH on pasting, gelling, swelling properties and freeze-thaw stability of flaxseed polysaccharide-potato starch mixtures.

The objective of this study was to investigate: (1) the effect of flaxseed polysaccharide (FG) concentration on pasting, gelling, swelling properties and freeze-thaw stability of potato starch; (2) the effect of different pH on pasting, gelling, swelling properties and freeze-thaw stability of flaxseed polysaccharide-potato starch (FG-PS) mixtures.

## 2. MATERIALS AND METHODS

### 2.1 Materials

Flaxseed polysaccharide (moisture content: 9.86%) was gifted by Linseed Biological Technologies Co., Ltd (Sinkiang, China). Potato starch (moisture content: 16.56%) was purchased from Tingfung Starch Development Co., Ltd (Tianjin, China). All other reagents were of analytical grade.

### 2.2 Preparation of samples

Preparation of solutions with different pH: solutions at different pH were prepared by citric acid or $Na_2CO_3$ of 0.1M with a DELTA320 acidity meter (Mettler-Toredo Co., Shanghai, China).

Preparation of potato starch suspension (PS) at different pH: accurately weighed pota-

to starch was dispersed in distilled water or in solutions at various pH and in different concentration.

Preparation of flaxseed polysaccharide-potato starch mixtures (FG-PS) at different pH: Preliminarily, flaxseed polysaccharide was dispersed in either distilled water or in solutions at different pH under magnetic stirring for 30 min, then heated to 80°C for 8 min, and cooled to room temperature. Then, PS was added to the FG solutions. The mixtures were stirred sufficiently to avoid the formation of lumps.

### 2.3 Determination of pasting properties

An aliquot of 2.1 g (7%, w/w) of PS was dispersed in distilled water or solutions with different pH, and added with the following different FG amounts: 0, 0.03, 0.09, 0.15 g (corresponding to a concentration of 0, 0.1, 0.3, 0.5% based on the total dispersion system, w/w, and to a FG:PS ratio of 0, 1:70, 1:23, 1:14), respectively. Pasting properties of the FG-PS mixtures were examined with a Rapid Visco Analyzer (RVA Starchmaster, Newport Ltd., Australia) and pasting profiles were recorded. Aliquotes of 30 g of FG-PS mixtures were used in this measurement. The FG-PS mixtures were stirred at 960 rpm for 10 s before the shear input decreased and held constant at 160 rpm during the following heating and cooling cycles. The FG-PS mixtures were heated from 50° to 95°C at the rate of 12°C/min and held at 95°C for 2.5 min, then cooled to 50°C at the same rate. Pasting temperature, peak viscosity, final viscosity and breakdown value were recorded. All determinations were done in triplicate.

### 2.4 Gel hardness analysis

An aliquot of 2.8 g (7% w/w) of PS was dispersed in either distilled water or solutions with different pH, with the following different FG amounts: 0, 0.04, 0.12, 0.20 g (corresponding to a concentration of 0, 0.1, 0.3, 0.5% based on the total dispersion system, w/w, and to a FG:PS ratio of 0, 1:70, 1:23, 1:14), respectively.

An aliquot of FG-PS mixtures of 40 g was poured in a cylinder vessel. The mixtures were pasted in a boiling water bath for 10 min, then cooled in an ice water bath for 5 min, and stored at 4°C for 24 h to allow gel forming before analysis. Before measurement, the samples were restored to room temperature. Hardness analysis was performed with a Texture analyzer (TA-XT. Plus, Stable Micro Systems Ltd., England) using a P/0.5 (diameter of 0.5 inch) probe. The original height of the sample was 2.4 cm, diameter of 4 cm. Gel was penetrated to 90% of original height by the test speed of 0.5mm/s. The maximum of force at fracture of the gel was recorded as gel hardness. The result was reported as the average of quintuplicate measurements.

### 2.5 Determination of swelling power of the mixtures

The determination of swelling power was carried out according to the method of HYUN-SEOK and BEMILLER (2012) with slight modification. An aliquot of 0.4 g of PS (1% w/w) was dispersed in either distilled water or solutions with different pH, added with the following different FG amounts: 0, 0.01, 0.02, 0.03g (corresponding to a concentration of 0, 0.025, 0.05, 0.075% based on the total dispersion system, w/w, and to a FG:PS ratio of 0, 1:40, 1:20, 1:13), respectively. Aliquots of FG-PS mixtures of 40 g were put into centrifuge tubes with closed screw caps and heated at 95°C for 30 min . Then, the centrifuge tubes were immersed in an ice bath to be cooled to room temperature immediately. The samples were centrifuged at the speed of 2000×g for 20 min and then the supernatants were separated. The supernatants were dried to constant weight at 105°C. Dried supernatant (*A*) and wet precipitated paste (*P*) were weighed, respectively. All determinations were done in quintuplicate. The swelling power (*SP)* was calculated by the following formula:

$$SP = P/[W\times(1- A/W)]$$

where *W* is the initial weight of the starch in the centrifuge tube.

### 2.6 Determination of freeze-thawing stability

Freeze-thawing stability was determined according to the method of ARUNYANART and CHAROENREIN (2008). An aliquot of PS of 0.9 g (3%, w/w) was dispersed in either distilled water or solutions with different pH, added with the following different FG amounts: 0, 0.03, 0.06, 0.09g (corresponding to a concentration of 0, 0.1, 0.2, 0.3% based on the total dispersion system, w/w, and to a FG:PS ratio of 0, 1:30, 1:15, 1:10), respectively. The mixtures were pasted in a boiling water bath for 20 min with continuously stirring, and then cooled in an ice water bath for 5 min. Aliquots of the mixtures of 30 g was poured into n. 25 centrifuge tubes with closed screw caps (individually marked with No.1 to No. 25) and each of them was initially exactly weighted (*M*). All the samples were frozen at -18°C for 24 h, and then thawed at 37°C for 12 h. The above freeze-thaw procedure was repeated for five cycles. After each freeze-thaw cycle, 5 samples were taken and centrifuged at 4500×g for 10 min to remove supernatant, while the rest of the samples were left to the next cycle. The quantity of precipitate was weighed, *m*. All determinations were done in quintuplicate. The syneresis (*WS*) was calculated by the following formula:

$$WS(\%) = [(M-m)/M] \times 100$$

### 2.7 Statistical analysis

The statistical analysis of the results was conducted by analysis of variance (ANOVA, SPSS 17.0, SPSS Inc., Chicago, IL) and Tukey's test. All significant differences were reported at a significance level of 0.05. Values followed by different letter for the same parameter in the same column are significantly different.

## 3. RESULTS AND DISCUSSION

### 3.1 Effect of polysaccharide concentration and pH on pasting properties of potato starch

Pasting properties of PS in the presence or absence of FG determined by the RVA are summarized in Table 1. Addition of FG led to pronounced increases in pasting temperature of PS, result that was observed also by other researchers (ACHAYUTHAKAN and SUPHANTHARIKA, 2008; CAI *et al.*, 2011; CHAISAWANG and SUPHANTHARIKA, 2006; KIM and WANG, 1999). Compared with PS alone, pasting temperature was increased of 12.3°C by addition of 0.5% FG. This might be the strong electrostatic repulsions between the carboxyl group of FG (CHEN, 2005) and the phosphate group of PS. In addition, available water for pasting was reduced due to the increase in the hydration of FG. These made the swelling of PS granules more difficult and resulted in higher pasting temperatures (BRENNA *et al.*, 2006; HIRASHIMA *et al.*, 2005; SHI and BEMILLER, 2002). Peak viscosity of PS was increased significantly by the addition of FG in agreement with the results reported by CHAISAWANG and SUPHANTHARIKA (2006), GULARTE and ROSELL (2011), HYUN-SEOK and BEMILLER (2012), VITURAWONG *et al* (2008). However CAI *et al* (2011) found an inverse and decreasing trend of the peak viscosity of PS by addition of xanthan gum. These might be ascribed to the difference in molecular structure between xanthan gum and FG, which led to a different electrostatic repulsive force. FG is composed by both neutral polysaccharide and acidic polysaccharide, while xanthan gum is composed only by acidic polysaccharide (CHEN, 2005; ABDULMOLA, *et al.*, 1996). Peak viscosity of PS was increased by 101 RVU in presence of 0.5% FG by comparison with the PS alone. This might be attributed to the synergistic effect of the starch-hydrocolloid systems, in which FG is interspersed within the starch continuous phase as the starch granules absorbed water and swelled (CHAISAWANG and SUPHANTHARIKA, 2006; CHEN *et al.*, 2006; MURALIKRISHNA and SALIMATH, 1987; PTASZEK and GRZESIK, 2007; PTASZEK and PTASZEK, 2011; XU *et al.*, 2011).

The addition of FG showed slight effect on the final viscosity of PS, as also reported by PARADA *et al* (2011) that observed that the final viscosity of starch-based food changed slightly when low concentrations of guar gum were added.

Breakdown value of PS was promoted by the addition of FG. This is in agreement with other studies (ACHAYUTHAKAN and SUPHANTHARIKA, 2008; ARUNYANART and CHAROENREIN, 2008; CHAISAWANG and SUPHANTHARIKA, 2006), while CAI *et al* (2011) found a decrease for PS with addition of xanthan gum. This might be explained as the rigid and ordered chain conformation of xanthan in solution, which made it steadier than FG during heating (ABDULMOLA, *et al.*, 1996). The breakdown value of PS was increased by the addition of 0.5% FG, comparing with PS alone. This result indicates that the thermostability of PS was weakened by the addition of FG. The viscosity of FG was reduced at high temperature with constantly stirring and this determined an increase of the breakdown value of PS (CHAISAWANG and SUPHANTHARIKA, 2006; CHEN, 2005; CUI *et al.*, 1996; FEDENIUK and BILIADERIS, 1994).

In the case of PS alone, pasting properties were slightly affected by pH (Table 2). By comparing results with those at pH 7, peak viscosity, final viscosity and breakdown value were reduced significantly under acidic or alkaline conditions. MALI *et al* (2003) observed that peak viscosity and final viscosity of cassava starch decreased at pH 3. This might be the consequence of starch hydrolysis promoted by heat treatment under acidic or alkaline conditions (ROGOLS, 1986).

Table 1 - Pasting temperature, peak viscosity, final viscosity, breakdown value of PS added with different concentrations of FG[a,b].

| FG concentration/% | Pasting temperature/°C | Peak viscosity/RVU | Final viscosity/RVU | Breakdown value/RVU |
|---|---|---|---|---|
| 0 | 71.5±0.3$^{D}$ | 523±5$^{D}$ | 282±5$^{B}$ | 293±5$^{C}$ |
| 0.1 | 74.3±0.5$^{C}$ | 560±9$^{C}$ | 285±4$^{B}$ | 296±8$^{C}$ |
| 0.3 | 76.5±0.0$^{B}$ | 581±1$^{B}$ | 285±4$^{B}$ | 336±4$^{B}$ |
| 0.5 | 83.8±0.0$^{A}$ | 624±4$^{A}$ | 301±4$^{A}$ | 375±2$^{A}$ |

Note: [a]Values are the mean ± SD of triplicate. Values in the same column with different letters (A-D) are significantly different ($p<0.05$);
[b]pH value of the samples is about 7.

Table 2 - Pasting temperature, peak viscosity, final viscosity, breakdown value of FG-PS mixtures and of PS alone at different pH values[a,b].

| pH | Pasting temperature/°C | | Peak viscosity/RVU | | Final viscosity/RVU | | Breakdown/RVU | |
|---|---|---|---|---|---|---|---|---|
| | PS-FG | PS | PS-FG | PS | PS-FG | PS | PS-FG | PS |
| 4 | 77.8±0.2A | 70.9±0.1B | 505±4C | 468±3BC | 271±3B | 162±7D | 286±9E | 196±10D |
| 6 | 78.1±0.6A | 71.2±0.1AB | 537±1B | 492±3B | 282±2A | 241±2BC | 309±7D | 247±5B |
| 7 | 76.5±0.0B | 71.5±0.3A | 581±1A | 523±5A | 285±4A | 282±5A | 336±4B | 293±5A |
| 8 | 77.5±0.5A | 71.5±0.4A | 502±3C | 448±10C | 282±7A | 257±5B | 325±4C | 255±4B |
| 10 | 75.6±0.2B | 71.9±0.1A | 453±9D | 314±6D | 269±0B | 229±1C | 345±5A | 219±8C |

Note: [a]Values are the mean ± SD of triplicate. Values in the same column with different letters (A-D) are significantly different ($p<0.05$);
[b]FG concentration: 0.3%.

Pasting properties of FG-PS mixtures (PS of 7%, FG of 0.3% based on the total dispersion system, w/w) was influenced significantly by pH (Table 2). Pasting temperature and final viscosity of FG-PS mixtures changed slightly between pH 4 and pH 10. Pasting temperature of the mixtures at pH 10 was lower of 2.2°C than that at pH 4, which corresponds to the fact that the mixtures were pasted easier under alkaline condition than under acidic condition. By increasing the pH from 4 to 10 the peak viscosity of the mixtures was initially increased and then decreased. Compared with the mixtures at pH 7, peak viscosity of the mixtures was decreased by 76 RVU at pH 4 and 128 RVU at pH 10, respectively. The maximum peak viscosity was observed at pH 7. This might be ascribed to the degradation of flaxseed polysaccharide molecule and potato starch molecule under acidic or alkaline heat treatment (CHEN, 2005). Breakdown value of the mixtures was gradually decreased under acidic condition, while varied slightly under alkaline condition. Scarce information available on the effect of pH on starch-hydrocolloids mixtures and this will need further investigations.

### 3.2 Effect of polysaccharide concentration and pH on swelling power of potato starch

As shown in Fig.1a, swelling power of PS was increased gradually with increasing the FG level. In particular, an increase of 30% by the addition of 0.075 % FG, compared with PS alone occurred. XU *et al* (2011) reported that swelling power of corn starch was increased by addition of konjac, CMC or chitosan. WANG *et al* (2011) reported that swelling power of tapioca starch was increased with addition of sodium alginate. Chaisawang *et al.* reported an inverse trend that swelling power of cationic tapioca starch was lowered greatly by addition of xanthan (CHAISAWANG and SUPHANTHARIKA, 2005). It was generally accepted that starch with high swelling power also yielded high peak viscosity during pasting. This fact was reflected by higher peak viscosity obtained during pasting

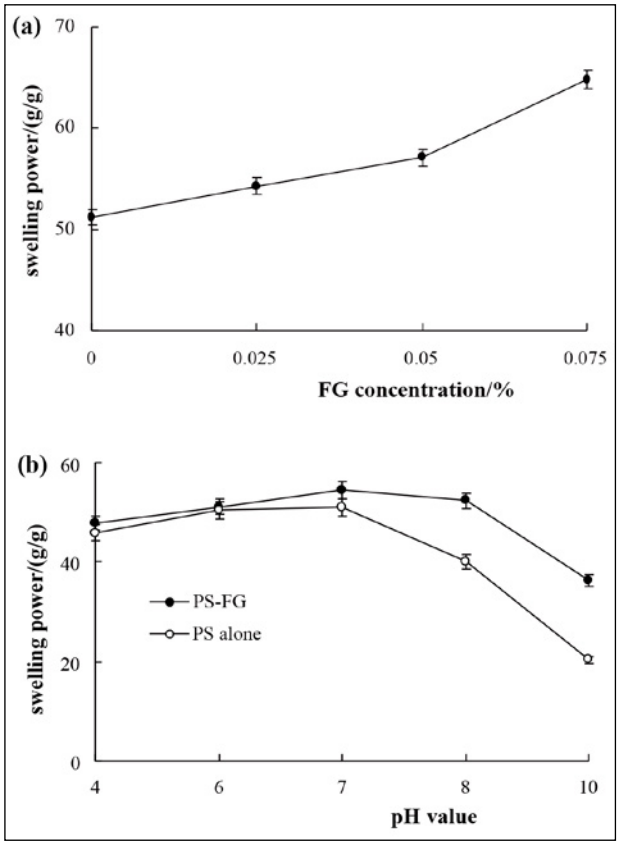



Fig. 1 - Swelling power of PS alone and of PS-FG mixtures at different pH.
(a) effect of FG concentration on swelling power of PS at pH7; (b) effect of pH on swelling power of PS alone and PS-FG mixtures at FG concentration of 0.025%.

of PS with addition of FG (Table 1). These results also implied that addition of FG could enhance swelling of starch granules at high temperature and this could be due to the fact that the FG molecules either interacted or associated with PS granules.

As shown in Fig. 1b, for PS alone, a decrease of the swelling power was observed under acidic or alkaline condition. This result might be associated to both the electrostatic interactions between phosphate groups of PS granules and the depo-

lymerization of starch molecules under acidic or alkaline condition (BRENNAN *et al.*, 2008). On the other hand, SIMONIN *et al* (2011) reported that gel swelling power of waxy rice starch showed a minimum at neutral pH and no significant effect of pH was observed on the gel swelling power of waxy corn starch.

Swelling power of FG-PS mixtures was affected significantly by pH as shown in Fig. 1b. Compared with pH 7, swelling power of the mixtures was decreased by both acidic and alkaline condition. The results exhibited a similar trend as that of the PS alone. Moreover, swelling power of the mixtures in alkaline condition was lower than that in acidic condition. Under acidic condition, electronic interactions and/or depolymerization of the mixtures could have increased the water binding capacity of swollen starch granules more than that under alkaline condition. The results of swelling power also are also in accordance with the data of the peak viscosity of the mixtures at different pH (Table 2).

### 3.3 Effect of polysaccharide concentrate and pH on gel hardness of potato starch

Gel hardness of PS was decreased with increasing FG level (Fig. 2a). Gel hardness of PS was decreased by 57% with addition of 0.5% FG,

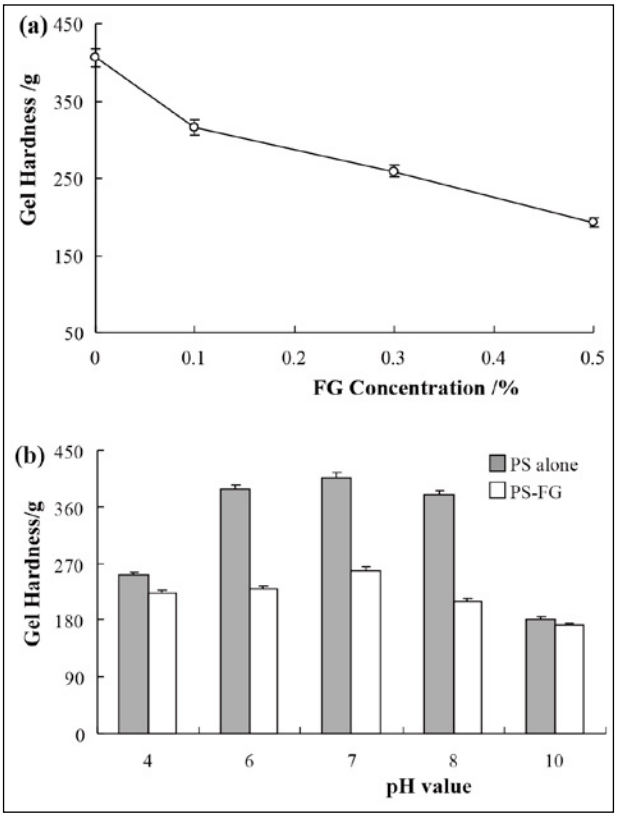



Fig. 2 - Gel hardness of PS alone and of PS-FG mixtures at different pH.
(a) effect of FG concentration on gel hardness of PS at pH7;
(b) effect of pH on gel hardness of PS alone and PS-FG mixture at FG concentration of 0.3%.

when data are compared with those of the PS alone. The result was in accordance with some other researches. SASAKI and KOHYAMA (2011) reported that addition of konjac gum or xanthan gum resulted in lower hardness of rice starch gels. With higher FG concentration, gel hardness of maize starch was significantly weakened (WANG *et al.*, 2011). PARADA *et al* (2011) showed that gel hardness of four kinds of starch-based products (maize, potato, rice or wheat starch) was decreased by guar gum. This might be ascribed to the weak-gel network formed by FG, which might disrupt the network of PS gel (CAI *et al.*, 2011; CHEN *et al.*, 2006; SASAKI and KOHYAMA, 2012).

By considering the effect of pH on the PS alone, gel hardness was strongly reduced under both acidic and alkaline conditions (as shown in Fig. 2b). At low pH this might be attributed to the decrease of net electrostatic repulsion for phosphate group of PS while at higher pH values, the alkaline depolymerization reaction, resulting in less junction zones could be considered as main cause (CHEN *et al.*, 2006).

For PS-FG mixtures, gel hardness was increased gradually and then decreased by the progressive increase of the pH (Fig. 2b) with a maximum gel hardness at pH 7. Gel hardness of the mixtures was decreased by 16% and 38% under acidic and alkaline conditions, respectively. This might be associated with the decrease of net electrostatic repulsion under acidic condition and degradation under alkaline condition for both FG and PS. The results showed a similar trend as that of PS alone and this may indicate that starch-acid/alkali interaction might be the predominant effect on the gel strength of FG-PS mixtures.

### 3.4 Effect of polysaccharide content and pH on freeze-thaw properties of potato starch

Freeze-thaw stability is an important property in food processing. During cold storage, the reorganization of starch molecules might lead to syneresis and affect the functional properties of starch-based food. As shown in Fig. 3a, freeze-thaw stability of PS was affected by the addition of FG. A positive correlation between syneresis and number of freeze-thaw cycles was observed for PS either with or without FG. This result may be due to the fact that the retrograded starch network could be easily disrupted by ice crystal formation and this led to a higher syneresis during the subsequent freezing and thawing process.

A negative correlation between syneresis and FG level was also observed likely resulting from the higher water binding capacity of the mixtures associated to both the higher water holding capacity of FG and the interaction between FG and PS (FERRERO *et al.*, 1994; LEE *et al.*, 2002; LIU and ESKIN, 1998). After the first freeze-thaw cycle, syneresis of PS was decreased by 32% with addition of 0.3% FG. The results suggested that

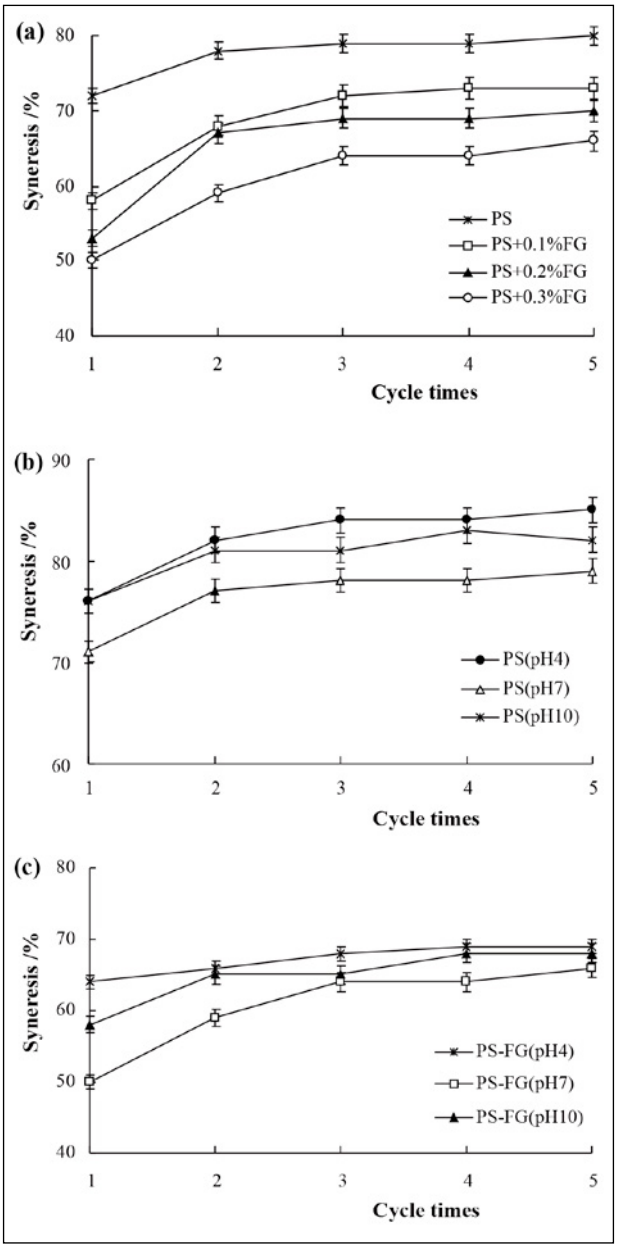



Fig. 3 - Syneresis of PS alone and of PS-FG mixtures at different pH.
(a) effect of FG concentration on syneresis of PS at pH7; (b) syneresis of PS alone at different pH; (c) effect of pH on synersis of PS-FG mixtures at FG concentration of 0.3%.

FG could minimize freeze-thaw damage and reduce the re-association of starch molecules and the damage to the network, with a decreased syneresis. WANG *et al.* (2011) reported a similar result as the freeze-thaw stability of tapioca starch was improved by sodium alginate or xanthan. PONGSAWATMANIT and SRIJUNTHONGSIRIA (2008) reported that freeze-thaw stability of tapioca starch could be also enhanced by the addition of xanthan, with results similar to our study. FG showed thus, to be able to effectively stabilize the starch gel against the repeated treatments of freeze-thaw cycles. The ionic groups of the starch and of FG molecules could immobilize adjacent water molecules and make more difficult their transformation into ice crystals (LEE *et al.*, 2002; SANDERSON, 1981).

As shown in Fig. 3b, syneresis of PS alone varied between pH 4 and pH 10 with the lowest water release at pH 7 and at pH 4 it was higher than that at pH 10. The results are in agreement with those of SAE-KANG and SUPHANTHARIKA (2006) that reported that the freeze-thaw stability of tapioca starch was also impaired under acidic condition.

As shown in Fig. 3c, syneresis of the FG-PS mixtures varied between pH 4 and pH 10, with a similar trend as shown by the PS alone and a minimum at pH 7. This result is similar to those of SAE-KANG and SUPHANTHARIKA(2006) that showed that the freeze-thaw stability of tapioca starch was improved by xanthan at pH 7 or pH 9. Syneresis of the mixtures under acidic condition was higher than that under alkaline condition at any given cycle number.

## 4. CONCLUSION

According to the results of the pasting properties, hardness, swelling power, and syneresis, the thickening capacity and the water binding capacity during freeze-thaw of PS was enhanced by the presence of FG, while the thermo-stability and gelling capacity were reduced.

The results of physicochemical properties of FG-PS mixtures under different pH indicated that pH showed significant effect on the physicochemical properties of the mixtures. Thickening capacity, thermo-stability, gelling capacity, and water binding capacity of FG-PS decreased in the presence of acidic or alkaline, meanwhile freeze-thaw stability of the mixtures exhibited the best was observed at pH7.

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PAPER

# PHENOTYPIC DIVERSITY AND TECHNOLOGICAL PROPERTIES OF YOGURT CULTURES ISOLATED FROM TRADITIONALLY PRODUCED TURKISH YOGURTS WITH COMPARISON TO COMMERCIAL STARTER CULTURES

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ABSTRACT

Sixty *Streptococcus thermophilus* and 111 *Lactobacillus delbrueckii* subsp. *bulgaricus* isolates from traditionally produced Turkish yogurts were biochemically identified and examined for their technologically important features. Acidification profiles of the isolates were examined. Isolates with acidification rates comparable to those of commercial isolates were also tested for final pHs and acetaldehyde production after 24-h incubation. Selected isolates were also examined for their bacteriophage resistance and proteolytic activity. Twenty-five *L. bulgaricus* and 22 *S. thermophilus* isolates were selected based on their technological properties other than bacteriophage resistance. Bacteriophage resistance experiments revealed that all *L. bulgaricus* isolates except two were sensitive to the bacteriophages, while *S. thermophilus* isolates were resistant to most of the bacteriophages. The results show that yogurt bacteria isolated from traditional Turkish yogurts have significant phenotypic diversity and the technological properties of these isolates are comparable to tested commercial cultures.

- Keywords: lactic acid bacteria, *Lactobacillus bulgaricus*, starter culture, *Streptococcus thermophilus*, technological property, yogurt -

## 1. INTRODUCTION

Yogurt is a fermented dairy product made by *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* as starter culture. The origin of yogurt was thought to be the Middle East (TAMIME and ROBINSON, 2007). The first yogurt is believed to have been produced by the Turks (TAMIME and DEETH, 1980; HAYALOGLU *et al.*, 2007).

Yogurt starter culture turns the lactose in milk into lactic acid and hence causes milk coagulation by decreasing pH (DE BRABANDERE and DE BAERDEMAEKER, 1999; TAMIME and ROBINSON, 2007). Additionally, these cultures are important to the development of characteristic yogurt aroma and flavour (DE BRABANDERE and DE BAERDEMAEKER, 1999). Since starter cultures affect the features and quality of the final product, the selection of an appropriate starter culture is crucial (MAYRA-MAKINEN and BIGRET, 1998). Although industrial starter cultures provide a safer process in terms of quality of the product and optimization of the manufacturing processes, they also narrow the diversity of fermented dairy products (WOUTERS *et al.*, 2002). Many scientists have studied native lactic acid bacteria isolated from fermented dairy products, particularly cheese, by examining their distinct and technologically important properties such as the production of volatile compounds (MORALES *et al.*, 2003), acid production activity (COGAN *et al.*, 1997; GAYA *et al.*, 1999; DURULU-OZKAYA *et al.*, 2001), proteolytic activity (COGAN *et al.*, 1997; GAYA *et al.*, 1999; DURULU-OZKAYA *et al.*, 2001), production of bacteriocin/bacteriocin-like compounds (COGAN *et al.*, 1997; GAYA *et al.*, 1999), exopolysaccharide production (COGAN *et al.*, 1997), phage sensitivity and prophage induction (SÁNCHEZ *et al.*, 2000). Nevertheless, other dairy products have been also used to isolate native lactic cultures such as laban (CHAMMAS *et al.*, 2006), yogurt (ERKUŞ, 2007; JAYASHREE *et al.*, 2010; TERZIC-VIDOJEVIC *et al.*, 2009), and yayik butter (SAĞDIÇ *et al.*, 2002). However, studies on native cultures from dairy products other than cheese are limited. In Turkey, native *S. thermophilus* and *L. bulgaricus* were previously isolated from different sources such as raw milk (AYHAN et. al., 2005), pasteurized milk (YILMAZ, 2002) and artisanal yogurt samples (ERKUŞ, 2007).

Traditional Turkish yogurt is produced by back-slopping. Therefore, it is likely to contain selected yogurt bacteria that have desirable technological properties and also provides features that could satisfy consumers who prefer traditional taste. In the present work, yogurt bacteria were isolated from traditional Turkish yogurts and identified biochemically. Additionally, the technological properties of these isolates were examined in comparison to commercial starter cultures.

## 2. MATERIAL AND METHODS

### 2.1. Yogurt samples and reference bacteria

Traditional Turkish yogurts were collected from the cities of Mersin (7), Antalya (4) (Mediterranean Region of Turkey) and Erzincan (1) (Eastern region of Turkey). Commercial starter cultures were additionally used for the isolation of yogurt starter cultures. The *L. bulgaricus* (DSM 20081$^T$) reference strain was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). The *S. thermophilus* (LMG 18311) reference strain was provided by the French National Institute for Agricultural Research (INRA).

### 2.2. Bacterial isolation, growth media and temperature

Yogurt samples were diluted using sterile peptone water (0.1% w/v). A loopful of the dilutions was streak-plated onto MRS agar (Merck; pH 5.7±0.2 at 25°C) for the isolation of *L. bulgaricus* and M17 agar (Merck; acidified to pH 6.8±0.1 at 25°C; TERZAGHI and SANDINE, 1975) for the isolation of *S. thermophilus*. Plates were incubated at 42°C for 48 hours under controlled atmospheric conditions using a gas pack (Anaerocult C, Merck). Colonies with similar morphological characteristics to that of reference strains were chosen from plates. Each isolated culture was streak-plated to obtain pure cultures. These cultures were examined for their cell morphology under a light microscope. Rod-shaped bacteria from MRS and cocci-shaped bacteria from M17 media were selected. Stock cultures were prepared in 20% glycerol and stored at –80°C.

### 2.3. Biochemical identification

Isolates were examined by Gram staining and the catalase test (Fig. 1). Gram-positive and catalase-negative isolates were further examined for gas production from glucose. Growth at 10°C was tested by incubating for 7 days in MRS or M17 broth with 0.04 g/*L* bromocresol purple. Carbohydrate fermentation tests were performed using the following carbohydrates: arabinose, cellobiose, esculin, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, melibiose, ribose, saccharose, salicin, sorbitol, trehalose, and xylose. The test for esculin hydrolysis was conducted in MRS broth containing 5 g/*L* esculin with no meat extract or glucose (TJANDRAATMADJA *et al.*, 1990). Positive results were identified via occurrence of turbidity and loss of fluorescence under UV light. *Lactobacillus casei* subsp. *casei* NRRLB 441 was included in the esculin hydrolysis test as a positive control. Fermentation tests for the rest of the carbohydrates were conducted using microtiter plates according to BULUT *et al.* (2005) and

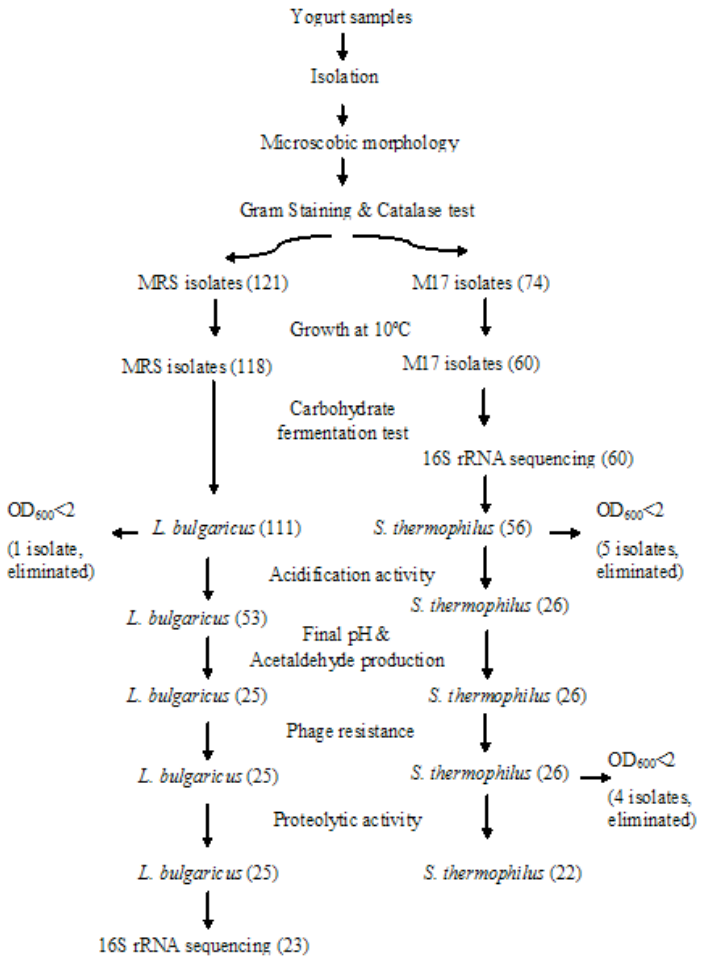



Fig. 1 - Experiments with isolate quantity. Isolate quantities were given in parenthesis.

ERKUŞ (2007) with some modifications. Modified MRS broth without glucose and meat extract (GÜRAKAN *et al.*, 1995) containing bromocresol purple (0.04 g/L) was used as the basal media and supplemented with each filter sterilized carbohydrate (0.22 μm) to a final concentration of 1%. Cultures incubated at 42°C for about 24 h in 10 mL MRS or M17 broth were centrifuged (1220xg for 15 min) and then washed with modified MRS. After placing the culture and sugar solution into wells, two drops of sterile mineral oil were placed in the wells.

### 2.4. Standardization of initial microbial load for technological characterization experiments

Microorganisms of interest were taken from stock culture and transferred twice into broth before the preparation of pre-cultures. Pre-cultures of *S. thermophilus* isolates were incubated for 13 h at 42°C, and their optical density ($OD_{600}$) was adjusted to 2. Pre-cultures of the putative *L. bulgaricus* isolates were incubated for 18 h at 42°C, and their $OD_{600}$ was adjusted to 2. After these adjustments, both pre-cultures contained approximately $10^8$ cfu/mL each.

### 2.5. Technological characterization of isolates

The technological characterization tests of isolates, except for bacteriophage resistance, were performed using reconstituted skim milk (RSM) (10% (w/v), Fluka) sterilized by autoclaving at 121°C for 5 min and inoculated with 2% standardized pre-culture. The isolates with $OD_{600}$ lower than 2 were not subjected to these technological property experiments since obtaining high-yield cultures is also considered another important property for starter cultures (AYAD *et al.*, 2004).

#### 2.5.1. Acidification activity

Acidification activity was determined measuring pH change with a pH meter (WTW pH 330) in 2-h intervals during a 10-h incubation at 42°C. The acidification rate was calculated as $\Delta pH = pH_{\text{at zero time}} - pH_{\text{at any time}}$ (AYAD *et al.*, 2004).

#### 2.5.2. Acetaldehyde production and final pH

The samples were incubated at 42°C for 24 h. The pH measurements were made to determine final pH after 24 h incubation at 42°C using a WTW pH 330 pH-meter. Acetaldehyde production by the isolates was examined using an acetaldehyde determination kit (R-biopharm Roche). Acetaldehyde production of *L. bulgaricus* isolates having comparable acetaldehyde production to that of commercial isolates was measured in three parallels, since *L. bulgaricus* is claimed as the major acetaldehyde producer (OBERMAN and LIBUDZISZ, 1998).

#### 2.5.3. Bacteriophage resistance

The bacteriophages supplied by Prof. Dr. Nezihe Tunail of the Department of Food Engineering at Ankara University were used to specify the bacteriophage resistance profiles of the isolates. The bacteriophages were isolated (KALELİ *et al.*, 2004; ACAR, 2002 and ACAR-SOYKUT, 2007) from various sources such as raw milk, yogurt, whey and bulk culture collected from dairy plants in Turkey.

Bacteriophage resistance was examined using a spot test as performed by ÖZYURT (2005) with some modifications. An aliquot (200 μL) of active *S. thermophilus* isolates, cultured in modified M17 (mM17) broth (KRUSCH *et al.*, 1987; ACAR, 2002) for 4-5 hours at 42°C, was added to 3 *mL* of mM17 soft-agar (KRUSCH *et al.*, 1987) (45°-50°C) and poured onto mM17 agar plates (KRUSCH *et al.*, 1987). Some of the isolates did not form bacterial lawns during incubation. These isolates were concentrated 6 times and then mixed with mM17 soft-agar. After the solidification of the second layer, 10 μL of bacteriophage lysates (≥$10^7$ pfu/*mL*) were dropped

on plates. The plates were incubated at ambient temperature for 10 min and then incubated at 42°C for 18 h and checked for any plaque formation. Four bacteriophages were examined on a single plate.

Active *L. bulgaricus* isolates were transferred to MRS broth and incubated for 5-6 hours at 42°C. An aliquot (200 μL) from this culture was mixed with 3 *mL* of MRS soft-agar (0.45% agar) at 45°-50°C, and 100 μL of sterile $CaCl_2$ (1M) were also added to obtain visible plaque formation (QUIBERONI *et al.*, 2004). This mixture was poured on MRS agar as double layer. The following steps were the same as that of *S. thermophilus* with the exception of anaerobic incubation applied for *L. bulgaricus*.

### 2.5.4. Proteolytic activity

Proteolytic activity of the selected isolates was examined using the *o*-phthaldialdehyde (OPA) method of CHURCH *et al.* (1983). The assay was carried out at least in duplicate for each isolate. Aliquots of 5 mL RSM were inoculated with 2% cell solution (obtained by washing 5 mL pre-culture with 0.32 mM sodium phosphate (pH 7.2) and resuspended to its original volume in the same solution) and incubated for 6 h at 42°C. A non-inoculated RSM was also incubated under the same conditions as the control. After incubation, 1 mL $H_2O$ and 10 mL of 0.75 N trichloroacetic acid prepared daily was added into 5 mL of sample. The sample was incubated at ambient temperature for 10 min and then filtered using filter paper. The filtrates were stored at –80°C until the assay. A small amount of filtrate (150 μL) was mixed with 3 mL of daily prepared OPA reagent (CHURCH *et al.* 1983; DONKOR *et al.*, 2006), and this solution was incubated for 2 min at ambient temperature. The absorbance was then measured at 340 nm using a spectrophotometer (Shimadzu UV1700). Triplicate samples were analysed from each permeate. The absorbance values of non-inoculated RSM were subtracted from the absorbance readings of the samples, and hence ΔAbsorbance (ΔAbs) values were obtained as indicators of proteolytic activity.

## 3. RESULTS AND CONCLUSIONS

Gram-positive and catalase-negative rod-shaped (121 total) and cocci-shaped (74 total) isolates were obtained from MRS media and M17 media, respectively. The isolates that grew at 10°C were eliminated (Fig. 1). The rest of the isolates and reference strains were subjected to carbohydrate fermentation tests. The carbohydrate fermentation patterns of the isolates from M17 were observed to be similar to that of *S. thermophilus* LMG 18311. The lactose and glucose fermentations tests resulted in acid production for all cocci-shaped isolates. Almost all cocci-shaped isolates showed acidification upon fermentation on saccharose except one isolate. Some diversity was detected for fermentation of select carbohydrates such as fructose, galactose, maltose and mannose. The cocci-shaped isolates did not show any acidification on the following test carbohydrates: esculin, arabinose, cellobiose, mannitol, melibiose, ribose, salicin, sorbitol, trehalose and xylose. VAN DEN BOGAARD *et al.* (2004) observed utilization of glucose, lactose and sucrose among almost all of their *S. thermophilus* strains. Galactose and fructose utilizing *S. thermophilus* isolates were also reported by the same authors. In our study, acid production from galactose was commonly observed among the cocci-shaped isolates. Identification of 56 cocci-shaped isolates was confirmed as *S. thermophilus* by 16S rRNA gene sequencing (ALTAY DEDE, 2010).

The majority of isolates from MRS (111) had similar carbohydrate fermentation patterns to *L. bulgaricus* DSM 20081$^T$ and previously reported patterns of *L. bulgaricus* (BUCHANAN and GIBBONS, 1974; BADIS *et al.*, 2004; GOMEZ-ZAVAGLIA *et al.*, 1999). Fermentation of fructose, glucose, lactose and mannose was observed among most of these isolates. Eighty-one isolates showed acid production only from these four carbohydrates. Some of the isolates showed acid production from one or two of the following carbohydrates: esculin, galactose, maltose, mannitol, ribose, saccharose and trehalose, in addition to fructose, glucose, lactose and mannose. Because of the probability of atypical strains, these isolates were retained in culture collection. The remainder of the rod-shaped isolates (7) were eliminated from this study because their carbohydrate fermentation patterns differed from *L. bulgaricus*.

Acidification activities of 51 *S. thermophilus* isolates and 110 putative *L. bulgaricus* isolates were measured (Table 1). The isolates were classified as good, medium and fair acidifiers based on their ability to acidify RSM. The classification of the isolates was performed by comparing ΔpH after 4 h and 6 h incubation at 42°C for *S. thermophilus* and putative *L. bulgaricus* isolates, respectively. The classification parameters were defined based on ΔpH of commercial iso-

Table 1 - Classification of *Streptococcus thermophilus* and putative *Lactobacillus bulgaricus* isolates based on their acidification properties.

| | Good | Medium | Fair |
|---|---|---|---|
| *S. thermophilus* | 4 | 22 | 25 |
| *L. bulgaricus* | 44 | 11 | 55 |

Classification parameters for *S. thermophilus*: ΔpH≥1.4 (Good), 1.4>ΔpH≥1.3 (Medium), 1.3>ΔpH (Fair)
Classification parameters for *L. bulgaricus*: ΔpH≥1.5 (Good), 1.5>ΔpH≥1.4 (Medium), 1.4>ΔpH (Fair)

lates. The isolates having fair acidification were eliminated from culture collection. Nearly 50% of the isolates from each species showed medium or good acidification. Isolation of these cultures from yogurts produced via back-slopping could be the reason for this high ratio of desirable acidification.

Acetaldehyde is known as the major compound that provides the typical yogurt aroma (TAMIME and ROBINSON, 2007). Therefore, acetaldehyde production of the isolates was studied in order to exhibit the effect of the isolates on yogurt aroma. Twenty-five putative *L. bulgaricus* isolates out of 53 were selected because the amount of acetaldehyde they produced was comparable with that of commercial isolates. The amount of acetaldehyde production by putative *L. bulgaricus* isolates ranged from 6.95 to 18.78 μg/g (Fig. 2) and by *S. thermophilus* isolates (26) ranged from 3.96 to 7.02 μg/g (Fig. 3). Acetaldehyde produced by *S. thermophilus* isolates was comparable with commercial isolates. Greater acetaldehyde producers, compared with commercial isolates, were detected among putative *L. bulgaricus* isolates.

Acetaldehyde levels produced by *S. thermophilus* and *L. bulgaricus* isolates were found to be in agreement with previous observations summarized by TAMIME and ROBINSON (2007). ÇELİK (2007) reported that the acetaldehyde level ranged from 1.2 to 31.5 mg/L among majority of *L. bulgaricus* strains and acetaldehyde levels produced by *S. thermophilus* and some *L. bulgaricus* strains were not in considerable level in their study. AYHAN *et al.* (2005) reported that acetaldehyde production was 25-55 ppm and 27.28-47.74 ppm for their native *S. thermophilus* and *L. bugaricus* isolates, respectively.

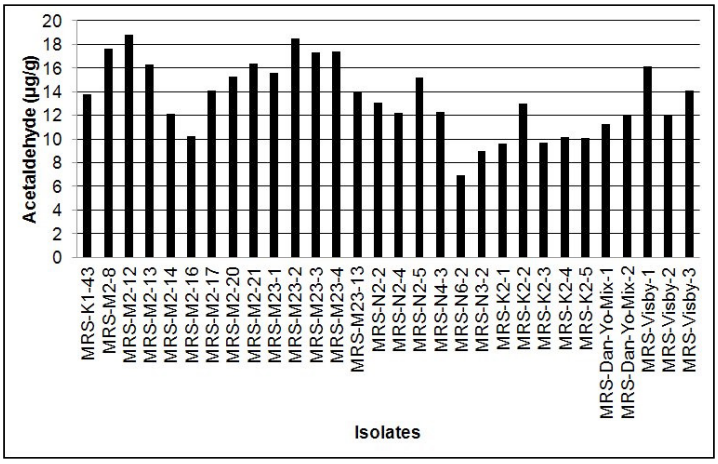



Fig. 2 - Acetaldehyde production of putative *Lactobacillus bulgaricus* isolates.

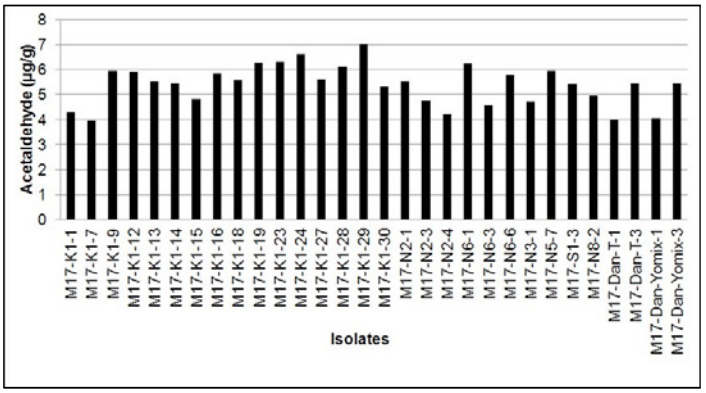



Fig. 3 - Acetaldehyde production of *Streptococcus thermophilus* isolates.

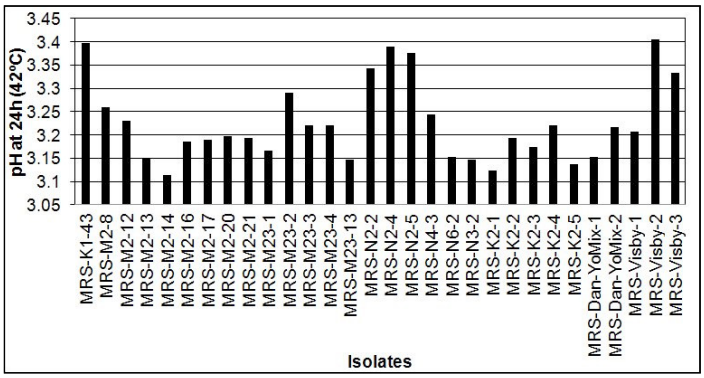



Fig. 4 - Final pH of putative *Lactobacillus bulgaricus* isolates reached after 24-h incubation at 42°C.

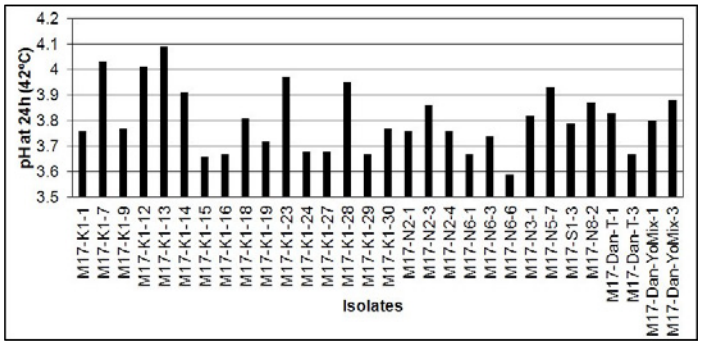



Fig. 5 - Final pH of *Streptococcus thermophilus* isolates reached after 24-h incubation at 42°C.

The final pH that putative *L. bulgaricus* isolates reached ranged from 3.11 to 3.39 (Fig. 4), while the final pH of *S. thermophilus* isolates ranged from 3.59 to 4.09 (Fig. 5). *L. bulgaricus* isolates reduced the pH to lower levels when compared to *S. thermophilus* isolates. This is consistent with the general properties of yogurt bacteria because the inhibition of *S. thermophilus* occurs in higher pH than *L. bulgaricus* (JAY *et al.*, 2005). *L. bulgaricus* isolates with comparably higher final pHs were detected, which could be important in eliminating post-acidification since *L. bulgaricus* is considered to be the main source of undesirable post-acidification during the storage of yogurt (MOLLET, 1999; RENAULT, 2002; LEROY and DE VUYST, 2004).

The 16S rRNA gene sequencing was performed on 23 *L. bulgaricus* isolates (out of 25) (CEBE-

Cİ AYDIN, 2008), and the biochemical identification of these 23 isolates was confirmed by CEBECİ AYDIN (2008).

The majority of *L. bulgaricus* isolates including commercial cultures were sensitive to *L. bulgaricus* bacteriophages, while two traditional isolates (MRS-M2-16 and MRS-M23-2) were resistant to most of the bacteriophages (Table 2). Additionally, there were *L. bulgaricus* isolates that were resistant to different bacteriophages in the collection (MRS-M2-21 and MRS-M2-16), which could be used together as members of the same starter culture. Phage sensitivity among native *L. bulgaricus* strains isolated in Turkey has been previously reported in the literature (TUNAİL *et al.*, 2002; AYHAN *et al.*, 2005). Conversely, *S. thermophilus* isolates were generally resistant to the bacteriophages. However, some of the *S. thermophilus* isolates from K1 yogurt, showed a clear ring-like area (a zone with the shape of ring with a less dense of bacterial growth within it) (Table 3). The presence of bacteriophage-resistant mutants in culture could cause this uncommon response to the bacteriophages. The remainder of *S. thermophilus* isolates (18) and commercial isolates (4) were resistant to all *S. thermophilus* bacteriophages tested in this study. KALELİ *et al.* (2004) reported that all native *S. thermophilus* strains in their study were resistant to tested phages.

In lactococci, the loss of bacteriophage resistance encoding plasmids can cause phage sensitivity since many of the phage resistance systems are related to plasmids instead of chromosome (JOSEPHSEN and NEVE, 1998). However, plasmids were seen infrequently in *S. thermophilus* and *L. bulgaricus* (MERCENIER and LEMOINE, 1989; PRIDMORE *et al.*, 2000; LEE *et al.*, 2007), even though a restriction modification system, which is coded on a plasmid, has been shown to exist in *S. thermophilus* by SOLOW and SOMKUTI (2001). Therefore, the common bacteriophage sensitivity among *L. bulgaricus* could not be explained with plasmid loss. The reason behind this bacteriophage sensitivity should further be investigated. However, preparation of yogurt starter cultures containing greater *S. thermophilus* to *L. bulgaricus* ratios (i.e. 1:10-1000) is the new approach (TUNAİL, 2009), and this could decrease the need for *L. bulgaricus* in yogurt pro-

Table 2 - Bacteriophage resistances of putative *Lactobacillus bulgaricus* isolates challenged with *L. bulgaricus* bacteriophages.

| | ***L. bulgaricus* bacteriophages** | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Isolates** | **ΦY4-X1** | **ΦY4-X2** | **ΦY4-X3** | **ΦY4-X4** | **ΦY4-X5** | **ΦY4-X6** | **ΦY4-X7** | **ΦY4-X8** | **ΦY4-X9** | **ΦY4-X10** | **ΦY4-X11** | **ΦY4L-A** | **ΦV1-X20** | **ΦLbA-A** | **ΦLbA-Z** |
| MRS-K1-43 | + | + | + | + | + | + | (+) | + | + | + | + | + | + | – | + |
| MRS-M2-8 | – | – | (+) | (+) | (+) | (+) | – | – | + | – | (+) | – | (+) | – | (+) |
| MRS-M2-12 | (+) | (+) | – | – | – | – | (+) | (+) | (+) | – | (+) | – | – | + | + |
| MRS-M2-13 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| MRS-M2-14 | (+) | + | (+) | + | + | + | + | + | + | + | + | + | + | + | + |
| MRS-M2-16 | – | – | – | – | (+) | – | – | – | – | – | – | – | – | (+) | – |
| MRS-M2-17 | (+) | (+) | – | – | (+) | (+) | (+) | (+) | (+) | (+) | (+) | (+) | (+) | (+) | (+) |
| MRS-M2-20 | (+) | – | – | (+) | (+) | – | (+) | – | (+) | (+) | – | – | – | (+) | (+) |
| MRS-M2-21 | + | (+) | (+) | (+) | – | – | – | – | – | – | – | – | – | – | – |
| MRS-M23-1 | (+) | + | – | – | (+) | – | (+) | – | + | + | + | (+) | – | – | – |
| MRS-M23-2 | – | – | – | – | – | – | – | – | + | – | – | + | – | – | – |
| MRS-M23-3 | – | + | + | + | + | + | + | (+) | + | + | (+) | + | – | – | – |
| MRS-M23-4 | + | + | + | + | – | – | – | (+) | + | – | – | + | – | – | – |
| MRS-M23-13 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| MRS-N2-2 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| MRS-N2-4 | (+) | – | – | (+) | (+) | – | (+) | (+) | (+) | – | – | – | – | – | – |
| MRS-N2-5 | (+) | + | (+) | + | + | + | (+) | (+) | + | + | (+) | (+) | + | + | + |
| MRS-N4-3 | (+) | (+) | – | (+) | (+) | (+) | (+) | (+) | (+) | (+) | – | (+) | – | (+) | – |
| MRS-N6-2 | (+) | (+) | (+) | (+) | (+) | (+) | (+) | (+) | (+) | (+) | + | + | + | – | – |
| MRS-N3-2 | (+) | (+) | – | – | (+) | (+) | – | – | (+) | (+) | (+) | – | (+) | – | – |
| MRS-K2-1 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| MRS-K2-2 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| MRS-K2-3 | + | + | + | + | + | (+) | + | (+) | + | + | + | + | + | + | + |
| MRS-K2-4 | + | + | (+) | + | + | + | + | + | + | + | + | (+) | + | + | + |
| MRS-K2-5 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| MRS-Dan-Yo-Mix-1 | (+) | (+) | (+) | (+) | (+) | (+) | (+) | (+) | (+) | (+) | (+) | (+) | (+) | (+) | (+) |
| MRS-Visby-1 | + | + | + | + | (+) | (+) | (+) | (+) | + | + | + | (+) | (+) | (+) | – |
| MRS-Visby-2 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| MRS-Visby-3 | + | + | + | + | + | + | + | + | + | + | + | + | + | (+) | (+) |

+: sensitive, -: resistant, (+): 1 or 2 plaque were detected.

Table 3 - Bacteriophage resistances of *Streptococcus thermophilus* isolates, which gave a clear ring-like area.

| | ***S. thermophilus* bacteriophages** | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Isolates** | **Φ1B3-A** | **Φ2B3-A** | **Φ709S-B1** | **Φ709-X1** | **Φ709-X2** | **Φ709-X3** | **Φ709-X4** | **Φ709-X5** | **Φ231SAlmö** | **Φ231SB1** | **Φ231-X6** | **Φ231-X7** | **Φ231-X8** | **Φ231-X9** | **Φ231-X10** | **Φ231-X22** | **Φ231-X23** | **ΦB3S-B1** | **ΦB3-X11** | **ΦB3-X12** | **ΦB3-X13** | **ΦB3-X14** | **ΦB3-X15** | **ΦB3-X16** | **ΦB3-X17** | **ΦB3-X18** | **ΦB3-X19** | **ΦB3-X20** |
| M17-K1-1 | – | (+) | – | (+) | (+) | – | (+) | (+) | (+) | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | (+) | (+) | – |
| M17-K1-7 | – | – | – | (+) | (+) | – | (+) | – | – | – | – | – | – | – | – | – | – | – | (+) | – | – | – | – | – | – | – | (+) | (+) |
| M17-K1-13 | – | – | – | (+) | – | (+) | (+) | (+) | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| M17-K1-16 | – | – | – | – | – | – | (+) | (+) | – | – | – | – | – | – | – | – | – | – | (+) | – | – | – | – | – | – | – | – | – |
| M17-K1-19 | – | – | – | – | – | – | (+) | (+) | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| M17-K1-27 | – | – | – | – | (+) | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| M17-K1-28 | – | – | – | – | – | – | – | (+) | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| M17-K1-30 | – | – | (+) | – | (+) | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |

-: resistant, (+): a zone having a shape of ring with a less dense of bacterial growth within it was detected.

duction. The bacteriophage-resistant property of traditional *S. thermophilus* isolates revealed that these isolates could be used as starter cultures alone or in a rotation with commercial *S. thermophilus* cultures to overcome the bacteriophage problem.

In this study, putative *L. bulgaricus* isolates (25 native and 4 commercial) were also challenged with nine of the *S. thermophilus* bacteriophages, and *S. thermophilus* isolates (26 native and 4 commercial) were challenged with the 15 L*. bulgaricus* bacteriophages since some of these bacteriophages used were previously reported as broad-host-range phages (ÖZYURT, 2005; ACARSOYKUT, 2007). However, the heterologous host characteristic was not observed within these isolates (data not shown).

*L. bulgaricus* isolates, including commercial isolates, were more diverse in terms of their proteolytic activities, ranging from 0.052 to 0.416 (ΔAbs 340 nm) (Fig. 6). Proteolytic activity of *S. thermophilus* isolates, including commercial isolates, ranged from 0.042 to 0.079 (ΔAbs 340 nm) (Fig. 7). *L. bulgaricus* isolates revealed higher proteolytic activity than *S. thermophilus* isolates. Higher proteolytic activity by *L. bulgaricus* than *S. thermophilus* has been previously observed by others (SLOCUM *et al.*, 1988a; RAJAGOPAL and SANDINE, 1990; COURTIN and RUL, 2004), even though the opposite case has also been reported previously (SHIHATA and SHAH, 2000). Diversity within *L. bulgaricus* isolates was observed in terms of their proteolytic activity. Four *L. bulgaricus* cultures (MRS-M23-4, MRS-M23-13, MRS-N4-3 and MRS-K2-5) revealed higher proteolytic activity than commercial cultures, while the proteolytic activity of the remaining of *L. bulgaricus* isolates was consistent with that of the commercial isolates. It is known that excessive proteolysis may be the cause of the bitter taste in yogurt (SLOCUM *et al.*, 1988b). On the other hand, since proteolytic activity of these species is essential in their associative growth (TAMIME and ROBINSON, 2007), *L. bulgaricus* isolates that have different proteolytic activities in a yogurt culture collection could be important to match consistent *L. bulgaricus* and *S. thermophilus* isolates for yogurt production (GÜRAKAN and ALTAY, 2010).

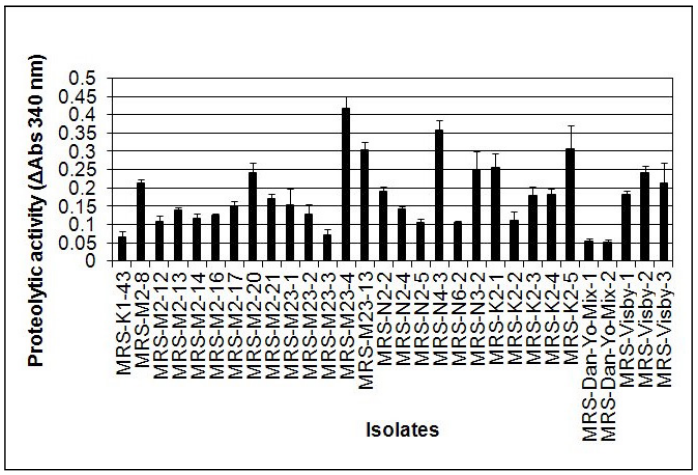



Fig. 6 - Proteolytic activity of putative *Lactobacillus bulgaricus* isolates.

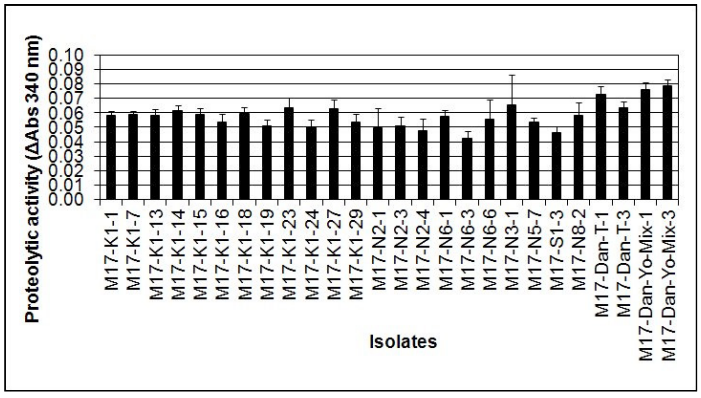



Fig. 7 - Proteolytic activity of *Streptococcus thermophilus* isolates.

Technological property studies revealed considerable phenotypic diversity within traditional yogurt cultures and demonstrated the high potential of traditionally produced yogurts as a source of starter cultures in Turkey. Our future work deals with the investigation of genotypic diversity among the isolates using the tools of molecular biology.

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PAPER

# POLYPHENOLIC PROFILE OF INTERSPECIFIC CROSSES OF ROWAN (*SORBUS AUCUPARIA* L.)

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ABSTRACT

The aim of the work was to make reference to antioxidant properties of selected cultivars of interspecific crosses of rowan. Altogether 6 cultivars were compared. The total phenolic content, antioxidant capacity, total flavonoid content and scavenging activity of the methanolic extract of fruit on nitric oxide were determined. Moreover, the polyphenolic profile of cultivars was screened.

The highest values of phenolic and flavonoid content were found to be in the fruit of the Likernaja cultivar (5.82 g of gallic acid · $kg^{-1}$ FW; 3.85±0.18 g of rutin · $kg^{-1}$ FW). Likernaja cultivar displayed the highest content of chlorogenic acid and quercetin (100.8 mg · 100 $g^{-1}$ FW, 51.4 mg · 100 $g^{-1}$ FW). The correlation coefficient between total phenolic content (TPC) and nitric oxide scavenging activity (NO) was $r^2$ = 0.885 and between total flavonoid content (TFC) and NO was $r^2$ = 0.810.

- Keywords: antioxidants, flavonoids, nitric oxide, polyphenolic compounds, *Sorbus* -

## INTRODUCTION

Nowadays, lesser-known fruit species are a potential source of antioxidants. This also applies to interspecific crosses of rowan. The ornamental plants with edible fruits can play several important functions, liver and gall bladder diseases (WILSKA-JESZKA, 1992).

Rowanberry has been described as an important source of flavonoids and their antioxidant activity affects reactive oxygen species and lipid peroxidation (BALOGHOVA, 2009; GIL-IZQUIERDO and MELLENTHIN, 2001). In an attempt to grow new fruit species but also cultivars which would be resistant to low temperature and adapted to a shorter vegetation period, from the start of the 20th century interspecific and intergeneric crossing of fruit woody plants have been conducted. The fruits of interspecific crosses seem to be a suitable source of antioxidants and a promising fruit species in relation to modern civilization diseases (Ehlenfeldt, 2009). The hybrid cultivars of *Sorbus* sp. were developed by cross-breeding rowan with Apple - *Malus*, pear - *Pyrus*, chokeberry - *Aronia*, hawthorn - *Crateagus* and medlar - *Mespilus* (HUKKANEN *et al.*, 2006; POYRAZOGLU, 2004).

In the cross - breeding considerable frost resistance, fertility needed and required characteristics of fruit were accomplished so that the crosses could be suitable for consumption and further processing such as making fruit compotes, for the production of liquors, wines or spirits (WIDODO *et al.*, 1995). In general, cultivars of interspecific crosses have a less astringent taste and are bigger in comparison with the fruit of Moravian rowan (*Sorbus aucuparia* subsp. moravica) (HUKKANEN *et al.*, 2006). The fruits of interspecific crosses of rowan have a high content of tannins, which could be noticeable in an unfavourable way in taste in products (although it is the case of Moravian rowan). The consumption of fresh rowanberries can lead to light poisoning which might be caused by the presence of parasorbic acid and hydrogen cyanide (VOLDRICH and KYZLINK, 1992). Nevertheless, by drying or cooking the toxicity of rowanberries disappears completely. High antioxidant activity of fruits is generally connected with anticarcinogenic effects (MAFFEI, 2007). The great contribution of these crosses is high antioxidant capacity (HUKKANEN *et al.*, 2006) caused, in particular, by the content of phenolics (ROP *et al.*, 2011a). The rowanberries cultivars and hybrids studied by Hukkanen *et al.* (2006) (Burka, Dessertnaja, Eliit, Granatnaja, Kubovaja, Rosina, Rubinovaja, Titan, and Zholtaja) displayed high antioxidant correlated to phenolic contents. Cultivation of rowanberries resulted in increased anthocyanin content, but this did not diminish their bioactivity in comparison to wild rowanberries rich in caffeoylquinic acids (HUKKANEN *et al.*, 2006; KYLLI *et al.,* 2010).

The ripe wild rowanberries have traditionally been used for jellies and jams, but their use as a food ingredient has been less popular because of their bitter taste. Sweeter and less astringent than wild rowanberries are different cultivars of sweet rowanberries and hybrids with other species, especially Granatnaja and Moravica cultivars (BERNA, 2012). Both wild and cultivated rowanberry phenolics exhibited a bacteriostatic effect toward *Staphylococcus aureus*. In addition, the phenolic extract from Zoltaja was weakly inhibitory toward *Salmonella typhimurium*, whereas both Zoltaja and Granatnaja-derived phenolics retarded *Escherichia coli* growth (KYLLI *et al.*, 2010). Polyphenol-rich extracts from certain berries inhibited a glucosidase activity in vitro (GRUSSU *et al.*, 2011). These extracts exhibited the potential to replace acarbose (or reduce the dose required) in its current clinical use in improving post-prandial glycaemic control in type 2 diabetics (BOATH, 2012).

The main objective of this study was to determine antioxidant properties - the total phenolic content (TPC), total antioxidant capacity (TAC), total flavonoid content and scavenging activities of 25% methanolic extracts of fruit on nitric oxide (NO) of fruit of six interspecific crosses of rowan cultivars used. Moreover, cultivars were analysed in term of selected polyphenolic compounds analysed by HPLC - ED method (chlorogenic acid, gallic acid, catalposide, rutin, quercetin, quercitrin).

## MATERIAL AND METHODS

Fruit were harvested in an experimental genefund orchard of Mendel University in Brno. This orchard is situated in the area of the village Zabcice, approximately 20 km southwards from Brno, the Czech Republic. The average annual temperature and a fifty-year average sum of precipitation are 9°C (during the growing season 15.6°C) and 553 mm (during the growing season 356 mm), respectively. Soils are classified as gleyed alluvial soils developed on the Holocene calciferous sediments with a marked accumulation of organic compounds. Concerning the texture, the topsoil is loamy and the subsoil clayey-loamy.

Fruit was harvested in consume ripeness from three trees of each cultivar under study in the course of September 2012 and 2013. 30 randomly chosen fruits from each tree were used for analyses (i.e. altogether 90 per each cultivar). The fruit of particular cultivars was processed immediately after the harvest (not later than within two days). Harvested fruits were puréed in a mixer and the average sample was obtained by dividing into quarters. Each parameter was measured in five replications.

The following cultivars were used:

´Burka´ - the cross between *Sorbus aucuparia* x (*Sorbus aria* x *Aronia arbutifolia*)

´Dessertnaja´ - the cross between *Sorbus aucuparia* x *Aronia melanocarpa* x *Mespilus germanica*

´Granatina´ - the cross between *Sorbus aucuparia* x *Crataegus sanquinea*

´Granatnaja´ - the cross between *Sorbus aucuparia* x *Crataegus sanquinea* x *Crataegus laevigata*

´Likernaja´ - the cross between *Sorbus aucuparia* x *Aronia melanocarpa*

´Titovnaja´ - the cross between *Sorbus aucuparia* x *Malus* spp.

**Extraction procedure**

The extraction was performed according to the method described by Kim *et al.* (2003), using the following procedure: 10 g of a fresh sample were homogenized for 10 s in 100 mL of methanol in a laboratory grinder SJ500 (MEZOS, Hradec Kralove, the Czech Republic). The resulting paste was placed into Erlenmeyer flasks (120 mL) and let to stand in a water bath with the temperature of 25°C for a period of 24 h. After the extraction the content of the flask was filtrated through paper Filtrapak No. 390 (Petr Lukes, Uhersky Brod, the Czech Republic) and stored at 4°C for further use. For analyses the final volume of extract was 100 mL.

*Total phenolic content assay*

To measure total contents of phenolic substances (TPC), 0.5 mL of extract was taken and diluted with water in a 50 mL volumetric flask. Thereafter, 2.5 mL of Folin-Ciocalteau reagent and 7.5 mL of a 20% solution of sodium carbonate were added. The resulting absorbance was measured in the spectrophotometer LIBRA S6 (Biochrom Ltd., Cambridge, the UK) at the wavelength of 765 nm against a blind sample (water extract without adding Folin reagent), which was used as reference. A calibration curve using gallic acid (GAE) · $kg^{-1}$ of fresh mass (FW) was measured.

*Antioxidant activity by the DPPH test assay*

The DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay was conducted according to the method of PAREJO *et al.* (2000). This test is based on reduction of DPPH*. In its radical form, DPPH* absorbs light at 515 nm, but upon reduction by an antioxidant or a radical species, the absorption disappears.

The stock solution was prepared by dissolving 24 mg of DPPH with 100 mL of methanol and then stored at -20°C until needed. The working solution was obtained by mixing 10 mL of the stock solution with 45 mL of methanol to obtain the absorbance of 1.1±0.02 units at 515 nm using the spectrophotometer LIBRA S6 (Biochrom Ltd., Cambridge, the UK). Fruit extracts (150 μL) were allowed to react with 2.850 μL of the diluted DPPH solution for 24 h in the dark. Then the absorbance was taken at 515 nm. The results of absorbance were converted using a calibration curve of the standard and expressed in ascorbic acid equivalents (AAE).

*Total flavonoid content assay*

The total flavonoid content (TFC) was determined following PARK *et al.* (2008). In a 10 mL Eppendorf tube, 0.3 mL of the fruit extract, 3.4 mL of 30% ethanol, 0.15 mL of $NaNO_2$ (c = 0.5 mol·$L^{-1}$) and 0.15 nmol·$L^{-1}$ of $AlCl_3 \cdot 6H_2O$ (c = 0.3 mol·$L^{-1}$) were added and mixed. After 5 min, 1 mL of NaOH (c = 1 mol·$L^{-1}$) was added, and the mixture was measured at the wavelength of 506 nm using the spectrophotometer LIBRA S6 (Biochrom Ltd., Cambridge, the UK). The total flavonoid concentration was calculated from a calibration curve using rutin as the standard. The results were expressed in mg · $kg^{-1}$ FW.

*Nitric oxide scavenging activity assay*

For the measurement of nitric oxide activity a 25% fruit extract (NO) (25 g fruits filled up to 100 mL with extracting buffer) was prepared in the phosphate buffer (c = 50 mmol · $L^{-1}$, pH 7.0). The assay of nitric oxide scavenging activity was performed according to the method described by GREEN *et al.* (1981). 1 mL of the extract was mixed with 1 mL of the reaction solution containing sodium nitroprusside (10 mmol · $L^{-1}$) in the phosphate buffer (20 mmol · $L^{-1}$, pH 7.4). The incubation at 37 °C for 1 h followed and 0.5 mL of the aliquot was then mixed with 0.5 mL of Griess reagent. The absorbance was measured at 540 nm using the spectrophotometer LIBRA S6 (Biochrom Ltd., Cambridge, the UK) and expressed in per cent of inhibition.

*HPLC profile of selected antioxidants*

For the determination of the HPLC profiles of the individual cultivars, high performance liquid chromatography (HPLC) with electrochemical and UV-VIS detection was used. The system consisted of two Model 582 ESA chromatographic pumps (ESA Inc., Chelmsford, MA, USA) with a working range from 0.001 to 9.999 mL · $min^{-1}$ and a Zorbax SB C18 (150 × 4.6; size of particles 5 μm, Agilent Technologies, USA) reverse phase chromatographic column. For UV detection, a Model 528 ESA UV detector was used. A twelve-channel Coul Array detector (ESA) was used for electrochemical detection. Samples were injected automatically by an autosampler (Model 542, ESA), which has incorporated a thermostatic space for a column.

Flow rate was 1 mL· $min^{-1}$. Chromatographic column was thermostated to 35°C. Compounds

were eluted by following linear increasing gradient: 0→1 min (5% ), 1→4 min (6%),

4→20 min (25% ), 20→30 min (100% ), 30→36 min (100%), 36→38 min (5%), 38→45 min (5%). Detection on ED detector was carried out at 600 mV. Time of one analysis was 45 min.

## RESULTS AND DISCUSSION

Phenolic content of *Sorbus* sp. cultivars was analysed as total phenolic content, compound classes - phenolic acids and flavonoids (chlorogenic acid, gallic acid, catalposide, rutin, quercetin and quercitrin).

The results of chemical analyses of the samples of interspecific crosses of rowan cultivars are shown in Figs. 1, 2 and 3. The results were expressed as an average of a two-year experiment since there was not a statistically significant difference among the years in any parameter investigated.

According to studies PAULOVICSOVA *et al.* (2009) less common fruit species according to their free radical scavenging ability can be included to the following order: cornelian cherries of the genotype 13 (88.85±5.58%) > cornelian cherries of the genotype 44 (84.56±0.27%) > mulberries of the genotype M 410 (81.85±0.99%) > cornelian cherries of the genotype 55 (77.59±0.25%) > mulberries of the genotype M 104 (77.25±1.47%) > rowanberries of the variant ´Sladkoplodá Moravská (76.84±1.22%) > blackberries of the variant Thornfree´ (62.80±1.73%) > honeyberries and their clones (30.03±0.24 - 33.00±0.22%).

Moreover, recent studies also proved that total phenolics, total flavonoids, total proanthocyanidins and chlorogenic acid isomers represented the major group of phenolic compounds determined antioxidant activity of wild rowanberry fruits (OLSZEWSKA, 2009). The phenolic content varied between 550 and 1,014 mg · 100 $g^{-1}$ FW. Berries contain a large variety of different phenolic compounds such as anthocyanins, flavonols, tannins, and phenolic acids. Only the few qualitative studies in rowanberry fruits examined polyphenolic compounds as quercetin, isoquercetin, hyperin, rutin, meratin, kaempferol-O-glycoside, catechin, epicatechin and compounds from the anthocyanin group: cyanidin-3-galactoside, and cyanidin 3,5 diglucoside (HÄKKINEN, 1998). According to studies HAKKINEN *et al.* (1999) in rowans ferrulic acid and quercetin were the most abundant phenolic. On the other hand, the HPLC and LC-MS analyses of phenolic composition provided by KYLLI *et al.* (2010) revealed that the main phenolic constituents were caffeoylquinic acids, varying from 56 to 80% total phenolics. Hydroxycinnamic acids (neochlorogenic and chlorogenic acid) and procyanidins were the main phenolics in rowanberry juice (WOJDYLO and OSZMIANSKI, 2009). Rowanberries contain also carotenoids, vitamin E, and vitamin C, which also contribute to their antioxidant capacity. Piir - Niiberg (2003) examined that carotenoid levels in sweet rowanberries are as high as those in carrots. The cultivated rowanberry fruits contained from 7.25 mg · 100 $g^{-1}$ FW to 10.48 mg · 100 $g^{-1}$ FW of carotenoids (Kampuss, 2009). Levels of vitamin C are close to those of strawberries, varying from 12 - 21 mg · 100 $g^{-1}$ (Granatnaja) to 86 mg · 100 $g^{-1}$ (Zholtaja) (HAKKINEN *et al.*, 1999). According to studies KAMPUSS *et al.* (2009) the vitamin C content in rowanberry fruits was equal to 131.3 mg · 100 $g^{-1}$ FW.The antioxidant activity is defined as an ability of the compound (or mixture of compounds) to inhibit oxidative reaction of various biomolecules (e.g. prevent the peroxidation of lipids) (POHANKA *et al.*, 2012; ROP *et al.*, 2011a; SOCHOR *et al.*, 2010a; SOCHOR *et al.*, 2011; SOCHOR *et al.*, 2010c). Methods of the antioxidant activity determination are usually based on the direct reaction of the studied molecule with radicals (scavenging) or on the reaction with transition metals (JURIKOVA *et al.*, 2012a; JURIKOVA *et al.*, 2012b; ROP *et al.*, 2012; ROP *et al.*, 2011b; SOCHOR *et al.*, 2010b). Berries with a strong purple color, such as crowberry, aronia, bilberry, and whortleberry, had clearly higher phenolic contents (28.7 - 50.8 mg GAE · $g^{-1}$ FW) than the yellowish rowanberries and cloudberries (18.7 and 16.2 mg GAE · $g^{-1}$ FW), but there were no significant differences between the antioxidant activities (KAHKONEN *et al.*, 1999). The total polyphenolic content of wild rowanberry (*Sorbus aucuparia*) was 522 mg · 100 $g^{-1}$ FW (KAHKONEN *et al.*, 2001). The total phenolic content can vary greatly among the sweet rowanberry cultivars ranging from 550 - 1014 mg GAE · 100 $g^{-1}$ of fresh weight of berries. In the study presented, the total polyphenolic and flavonoid content in the fruits of rowanberry interspecific crosses reached up 3.65±0.10 g of GAE · $kg^{-1}$ FW; 2.55±0.1 g of rutin · $kg^{-1}$ FW (Granatnja) up to 5.82±0.14 g of GAE · $kg^{-1}$ FW; 3.85±0.18 g of rutin · $kg^{-1}$ FW (Likernaja). In the same way, the highest phenol content was detected in the rowanberry cultivar Likernaja (in average 484.9 mg GAE · 100 $g^{-1}$ FW) in the studies of KAMPUSE *et al.* (2010). Black chokeberries grafted onto European rowan tree exhibited the highest content of total phenols (14.25±0.33 mg GAE · $g^{-1}$ FW), and flavonoids (7.87±0.25 mg CE · $g^{-1}$ FW) also in studies of ŠAMEC *et al.* (2009) but in comparison with presented study they measured higher values of TPC and TFC (Fig. 1). The assayed cultivars displayed higher content of TPC in comparison with studies of KAMPUSS *et al.* (2009) noticed the total phenol content of 8 rowanberry cultivar fruits and the fruits of their hybrids with *Rosaceae* L. between 162 and 485 mg GAE · 100$g^{-1}$ FW .

The Likernaja cultivar displayed the highest

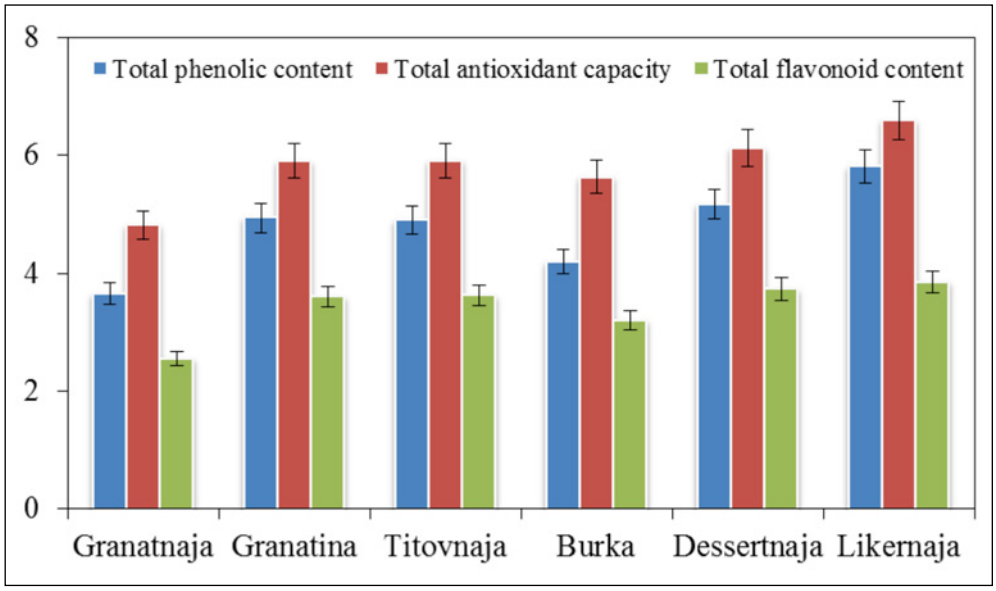



Fig. 1 - Total phenolic content (expressed as grams of gallic acid · kg$^{-1}$ FW), total antioxidant capacity (expressed as grams of AAE · kg$^{-1}$ FW) and total flavonoid content (expressed as grams of rutin · kg$^{-1}$ FW) n = 15.

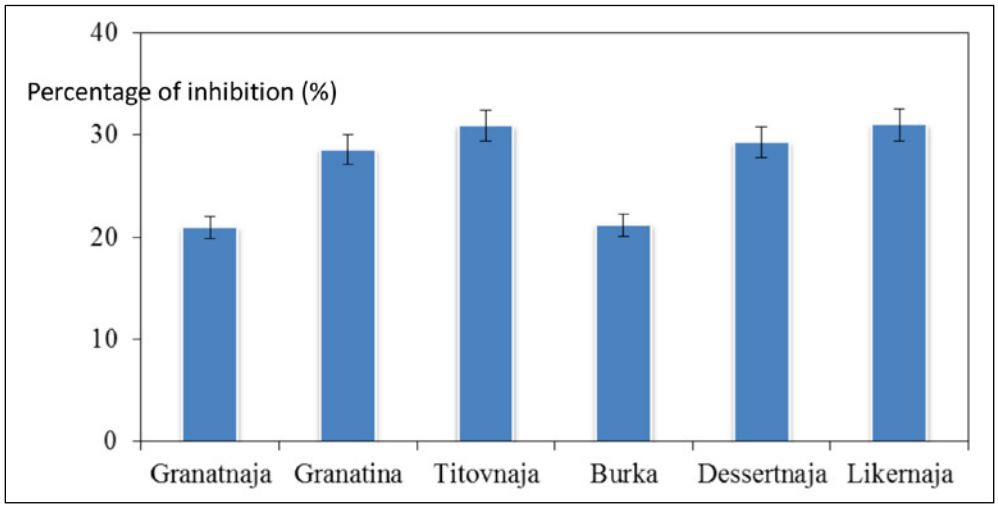



Fig. 2 - Scavenging effect of 25% methanolic extract of fruit on nitric oxide (percentage of inhibition), n = 15.

value of TAC 6.59 g of AAE · kg$^{-1}$ FW followed by the Dezertnaja cultivar 6.12 g of AAE · kg$^{-1}$ FW. Likernaja had by 50% higher antiradical activity (11.2 g of berries per 1 g of DPPH radical) KAMPUSE *et al.* (2010) than in assayed hybrids.

In previous studies high antioxidant capacity strongly correlating with phenolic levels has been found for numerous species representing the genus *Sorbus* s.s., including *S. aucuparia* (OLSZEWSKA *et al.*, 2010; OLSZEWSKA, 2009). As regards TPC and TFC, they were significantly in correlation with TAC ($r^2$ = 0.9479; y = 0.7572x + 2.2085) and ($r^2$ = 0.890; y = 1.115x + 2.041). In accord with our results a high correlation between the antioxidant capacity and total phenolic contents of sweet rowanberries cultivars and hybrids was established by HUKKANEN *et al.*, (2006) and KAMPUSS *et al.*, (2009).

Both antioxidant effects measured by ABTS and DPPH test of the *Sorbus* sp. extracts were significantly (R > 0.8097, p < 0.05) influenced by the total phenolic content (TPC) as it was determined by the OLSEWSKA (2012).

Significant linear correlations (R in the range of 0.75 - 0.98) between DPPH, FRAP method and the contents of total phenolics, total flavonoids, total proanthocyanidins and chlorogenic acid isomers showed that the listed phenolic compounds are determinants of the antioxidant capacity tested (OLSZEWSKA, 2009).

When comparing other species of pomaceous fruit, the quality of fruits of the rowan cultivars investigated is evident in relation to antioxidant properties (THAIPONG *et al.*, 2006). For example, in apples the average value is 2.50 g of AAE · kg$^{-1}$ FW, in pears it is on average 1.90 g of AAE · kg$^{-1}$

FW. Similarly, cherries reach 1 g of AAE · $kg^{-1}$ FW and plums have around 5 g of AAE · $kg^{-1}$ FW (ROP *et al.*, 2010).

Moreover, for verifying the results the measurement of scavenging activity of 25% methanolic extracts on NO was performed. The highest values were found to be in the Likernaja, Dessertnaja, Granatina and Titovnaja cultivars - in concrete terms, 30.97%, 29.26%, 28.56% and 30.89%, respectively. The correlation coefficient between TPC and scavenging activity of NO was $r^2 = 0.885$; $y = 8.742x - 2.719$ and between TFC and scavenging activity of NO was $r^2 = 0.810$; $y = 5.526x + 0.544$

KAKHONEN *et al.* (2001) analyzed the phenolic profiles of a total of 26 berry samples by HPLC-ED technique. The results of experiment showed that phenolic contents among different berry genera varied considerably, while phenolic acids were dominant in rowanberries (genus *Sorbus*). Sweet rowanberry is especially rich in chlorogenic and neochlorogenic acid content (CLIFFORD, 2000) as well as caffeic acid (HALLMANN, 2012). In our experiment the highest content of chlorogenic acid was examined in Likernaja 100.88 mg · 100 $g^{-1}$ FW together with Granatnaja 90.62 mg · 100 $g^{-1}$ FW that are by 60% lower values in comparison with HALLMANN (2012). The content of gallic acid varied from 4.33 mg · 100 $g^{-1}$ FW (Titovnaja) up to 26.35 mg · 100 $g^{-1}$ FW (Burka) (Fig. 3).

Quercetin is the main flavonol presented in *Sorbus* sp. (HALLMANN, 2012; GIL-IZQUIERDO and MELLENTHIN, 2001). In assayed interspecific crosses of *Sorbus* sp. the content quercetin represented values from 44.03 mg · 100 $g^{-1}$ FW up to 51.41 mg · 100 $g^{-1}$ FW (Likernaja). In generally, quercetin is the predominant flavonol in fruit reach up lesser amount than 15 - 30 mg · $kg^{-1}$ FW (MANACH *et al.*, 2004) but in all assayed interspecific crosses reached up higher values. Quercetin was determined in all berries lesser known fruit species analysed by HAKKINEN *et al.* (1999), the contents being higher in bog whortleberry (158 mg · $kg^{-1}$, FW), lingonberry (74 and 146 mg · $kg^{-1}$), cranberry (83 and 121 mg · $kg^{-1}$), chokeberry (89 mg · $kg^{-1}$), sweet rowan (85 mg · $kg^{-1}$), rowanberry (63 mg · $kg^{-1}$), sea buckthorn berry (62 mg · $kg^{-1}$). In crowberry (53 and 56 mg · $kg^{-1}$ FW) the content of quercetin is comparable to Likernaja. Except for quercetin assayed hybrids contained significant amount of rutin 14.43 mg · 100 $g^{-1}$ FW (Burka) up to 75.95 mg · 100 $g^{-1}$ FW (Granatina).

The content of quercitrin in assayed samples reached up 8.54 mg · 100 $g^{-1}$ FW (Titovnaja) to 38.79 mg · 100 $g^{-1}$ FW (Burka). Quercitrin was found in lower amount in all the berries such as whortleberry (15.8 mg · 100 $g^{-1}$ FW), ligonberry (7.4 - 14.6 mg · 100 $g^{-1}$), cranberry (8.3 - 12.1 mg · 100 $g^{-1}$), chokeberry (8.9 mg · 100 $g^{-1}$), sweet rowan (8.5 mg · 100 $g^{-1}$), rowanberry (6.3 mg · 100 $g^{-1}$), sea buckthorn (6.2 mg · 100 $g^{-1}$) and crowbery (5.3 - 5.6 mg · 100 $g^{-1}$) (HÄKKINEN, 1998). It is also evident, that interspecific crosses of *Sorbus* sp. presented reacher sources of quercitrin than wild species.

The results presented are unique and in interspecific crosses of rowan they have not been presented in such a way so far. In particular, it concerns nitric oxide which belongs to reactive oxygen species. These develop as a response of the human immune system to infections but are also produced in stressful situations - then they have a negative impact on human tissues, damage biological membranes and blood vessels (BAE and SUH, 2007). No data on phenolics content of hybrids presented in paper have been published previously.

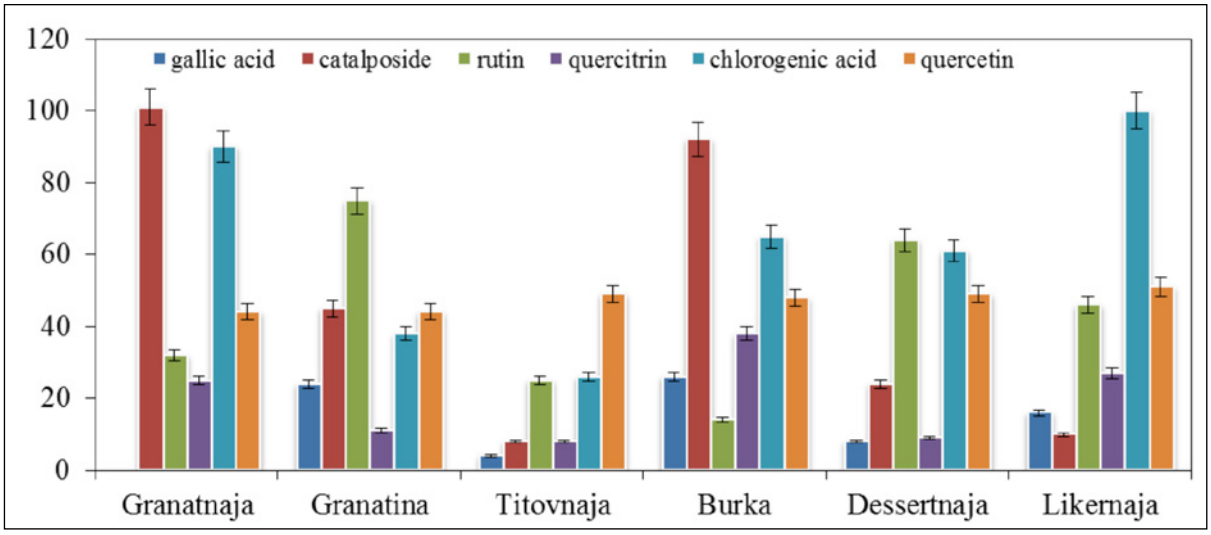



Fig. 3 - The content of selected polyphenolic compouns in cultivars of *Sorbus* sp. (mg · 100$g^{-1}$ FW).

## CONCLUSION

The results obtained point to possibilities of using interspecific crosses of rowan in human nutrition as a good substitute for other edible rowan species. The evaluated cultivars displayed high antioxidant activity significantly related to total polyphenolic and flavonoid content. The Likernaja cultivar displayed the highest content of TPC, TFC, antioxidant activity, chlorogenic acid and quercetin. This information about fruit quality is interesting for nutritionists as well as berry growers and breeders who can promote the cultivation of species and new cultivars with higher phenolic content an d antioxidant activity. Moreover, assayed interspecific crosses excel in low requirements for growing conditions and high resistance to frost. What is also interesting is that their yield is mostly higher in comparison with Moravian rowan. Therefore, these crosses are found in Northern European countries as well as the Siberian regions of Russia.


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SHORT COMMUNICATION

# OCCURRENCE OF AFLATOXIN M1 IN MILK IN QOM, IRAN

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ABSTRACT

Aflatoxins are potent fungal toxins, that may be transmitted and present in foods; this is possible if aflatoxin have infected the primary product source, animal or agricultural product. There can be major negative health effects if exposed to very high aflatoxin levels, these levels are known to produce cirrhosis, necrosis and possible hepatic failure as well as changes in the digestive/absorption and metabolism. As a result, aflatoxin presents a major concern for the food industries and their products produced for human consumption. The objective of this study was to investigate the probable fungal infection of AfM1 in milk product produced in the province of Qom, Iran (2013). For testing purposes, forty random milk samples were collected from eight milk-collecting centers, for evaluation and analysis of AfM1 content, HPLC method was used for evaluating all samples. All samples tested positive for Aflatoxin $M_1$, the average concentration of 0.170 μg $L^{-1}$, in one-hundred percent of the samples. AfM1 levels were greater than EU limit (0.05 μg $L^{-1}$) and 87.5% of samples exceeded legal limit of national standard of Iran.

- Keywords: aflatoxin M1, raw milk, food safety, HPLC, immunoaffinity column -

## INTRODUCTION

Aflatoxins subset of mycotoxins, consist of a large group of extremely toxic components, which are produced by certain species of fungi, specifically by *Aspergillus flavus* and *Aspergillus parasiticus* (FALLAH, 2010). These fungi contaminate a wide range of agricultural products mainly cereal grains, during the pre- and post-harvest stages (EL KHOURY *et al.*, 2011, GUAN *et al.*, 2011). Factors such as season, humidity, temperature and drought in field as well as storage conditions (i.e. relatively high humidity and high temperatures of extended periods) play a critical role in production of aflatoxins (KANGETHE and LANGA, 2010; DASHTI *et al.*, 2009; EFSA, 2004). These metabolites are highly carcinogenic, mutagenic and teratogenic components, which pose a serious health threat to humans and economic concern to the food industry hence; consumables should be monitored closely for these potentially hazardous bacteria (SADIA *et al.*, 2012; ELZUPIR and ELHUSSEIN, 2010). Among the four common aflatoxins available (aflatoxin B1, B2, G1, and G2), aflatoxin B1 (AfB1) is the most prevalent and poisonous molecule and categorized as group 1 human carcinogen by IARC (FALLAH, 2010; GUAN *et al.*, 2011; EL KHOURY *et al.*, 2011). When lactating mammals ingest feeds containing aflatoxin B1, it is converted to AfM1 by hydroxylation (RUANGWISES and RUANGWISES, 20120). AfM1 is secreted into the milk and subsequently digested by humans through the consumption of milk and other dairy products (PRANDINI *et al.*, 2009). AfM1 have teratogenic and mutagenic implication and recently, likewise AfB1, classified as first group human carcinogen (SASSAHARA *et al.*, 2005; ALMEIDA PICININ *et al.*, 2012). Similarly, other aflatoxins, AfM1 are thermo stable thus, during thermal treatment, storage and/or other processing methods do not affect the AfM1 bacteria (ALMEIDA PICININ *et al.*, 2012; ZHENG *et al.*, 2013).There are several regulations for AfM1 limitation in milk and dairy products in many countries. According to Codex Alimentarius and European Community legislations, permitted level of AfM1 concentration in milk and dairy product are fixed at 0.05 μg $L^{-1}$ (COMMISSION, 2001; EC, 2006). The Institute of Standards and Industrial Research of Iran (ISIRI) has set national legal limit to 0.1 μg $L^{-1}$ for milk (ISIRI, 2005). There were no previous studies, records or reportsof AfM1 bacterial presence in the raw milk in the province of Qom. We determined further monitoring and testing was necessary for ruling out any possibility of the AfM1 bacteria; random samples of raw milk product were collected by sterile procedure, from the different milk-collecting centers of Qom province, Iran.

## MATERIALS AND METHODS

### Sample collection

Forty samples were randomly collected from the eight milk-collecting centers (C1-C8) in the Qom province, during the month of January 2013. Samples were collected and prepared in sterile plastic container and refrigerated at 4°C pending analysis, that were carried out later the same day.

### Chemical and reagents

AfM1 standard were purchased from Sigma-Aldrich. AfM1 Immunoaffinity columns were obtained from Vicam (USA). HPLC grade solvents were purchased from Merck, Riedel (Darmstadt, Germany).

Water used was purified by a Waters-Millipore (Milford, MA, USA).

### Analysis of AfM1

The method used for analysis of AfM1 was the AOAC Official Method 2000.08 as reported by (DRAGACCI *et al.*, 2001) with a minor modification. 40 ml of homogenate milk were defatted by centrifuge at 3,500 rpms for 30 min, the supernatant was removed and the resulting skimmed milk was poured through filter paper (Whatman no. 4) to remove any remnants of debris. Filtrate was then attached into an Immunoaffinity column. To remove matrix interferes, the column were washed with 30 mL of water. AfM1 were eluted with 4 ml of acetonitrile: the liquid(1:3, v/v) and elutes were evaporated at 40°C under a stream of nitrogen gas, and redissolved in 500 μL of water: acetonitrile (1:3, v/v). A volume of 100 μL was injected into the HPLC system. All samples were analyzed in duplicate. The HPLC system consisted of an A Hewlett Packard HPLC system (Hewlett Packard, Agilent 1100, Palo Alto, Ca, USA) equipped with an auto sampler Agilent 1100 Series and a HP Agilent 1100 fluorescence detector. Detection was set at 360 and 340 nm for excitation and emission, respectively. Temperature in column oven was 40°C. The mobile phase was water: acetonitrile (1:3, v/v) at a flow rate of 1 ml/min. The retention time for AfM1 detection was 4.1-4.3 min. To determine the calibration curve, 6 fortified solution with AfM at the following concentrations 0.025, 0.05, 0.075, 0.1, 0.2, 0.5 μg $L^{-1}$ were used and calculated by least squares linear regression analysis of the peak areas versus milk concentrations of AfM. The coefficients of variation (CV) and the mean recoveries were calculated for each solution. The average recoveries were 77.1%. Limit of quantitation were determined using the Q2B method of US FDA (FDA, 1996). The precision of the method expressed as percentage CV ranged from 3.5 to 5.1% (not shown).

## RESULTS AND DISCUSSION

Levels of AfM1 are summarized in Table 1. AfM1 were found in all of 40 analyzed samples and the total average of AfM1 concentration were 0.170 μg $L^{-1}$. Statistical analysis showed no significant difference in the mean amount of AfM1 between points analyzed. According to the national standard of Iran, 87.5% of samples exceeded the legal limit of AfM1 content in the raw milk (0.1 μg $L^{-1}$) and regarding Codex Alimentarius and European Community regulation, one-hundred percent of samples were above the permitted level (0.05 μg $L^{-1}$). The results were in near agreement with (OVEISI *et al.*, 2007), that reported AfM1 contamination in 78% of milk samples exceeded EU action level (0.05 μg $L^{-1}$). KAMKARA (2005) also pointed out high contamination in 111 raw milk samples produced in Sarab, Iran, in which AfM1 detections rates were 76% of the samples collected ranged between 0.015 to 0.28 μg $L^{-1}$ whereas 40% of the positive samples were above EU limit. In similar studies in Iran, AfM1 contamination in raw milk samples exceeded EU limit with percentage of 36% (RAHIMI and AMERI, 2012), 33% (NEMATI *et al.*, 2010), 25% (RAHIMI and AMERI, 2012), 12.5% (TAJIK *et al.*, 2007), 11.76% (GHIYASIAN *et al.*, 2007), 4.4% (KHEZRI *et al.*, 2010).

A probable explanation of high values observed could be attributed to winter sampling that are commonly associated with improper feed storage and favorable condition for fungal growth and AfB1 production. Previous study analyses demonstrated a high AflaM1 content in milk that was analyzed in winter, with that compared to the summer season (KAMKAR, 2005; TAJKARIMI *et al.*, 2007; NEMATI *et al.*, 2010; GHIYASIAN *et al.*, 2007; HERZALLAH, 2009; HUSSAIN and ANWAR 2008). The combination of high humidity and warm temperature accelerate fungi growth in the stored feeds, which results in a contaminated food supply for the dairy cow, which inherently infects the raw milk withAfM1 in (DASHTI *et al.*, 2009; GRYBAUSKA *et al.*, 2000). However, we have no information regarding the feed given to cows by the local farmers in the sampling regions. Whereas the traditional dairy farming industry, it is more susceptible to this bacterial contamination due to the improperly stored feed, the present storage methods create a perfect environment for growth of the mould (heat and moisture) and AfBl accumulation. Crop residues and dried wheat bread are given as a food supplement to the animals; these products are often visually contaminated with mould and are predispose to AfB1 production. Silage making constitutes a chief component of feed given to cows during winter, these are a major contamination source of AfB1 (PEREYRA *et al.*, 2011). Our study seems to indicate that the dairy farmers require a comprehensive training program, for proper handling of the agricultural products for feed storage and silage production; this may well play a major role in the reduction in the contamination of AfM1 content in raw milk. A probable explanation of high bacterial infections values observed could be attributed to the winter sampling that are commonly associated with improper feed storage and the favorable conditions (heat and moisture) for fungal growth and AfB1 production. There are studies that implicate high AflaM1 content in the milk analyzed in the winter as compared to the summer season analyses, (KAMKAR 2005; TAJKARIMI *et al.*, 2007; NEMATI *et al.*, 2010; GHIYASIAN *et al.*, 2007; HERZALLAH, 2009; HUSSAIN and ANWAR, 2008). The combinations of high humidity and warm temperature during feed storage accelerate fungi growth, which resulted in AfM1 contamination of the raw milk (DASHTI *et al.*, 2009; GRYBAUSKAS *et al.*, 2000).

## CONCLUSION

Bearing in mind the fact that the amount of AfM1 in all milk examined were above EU limit, it is highly recommended that further controlling measures be implemented immediately to reduce the danger and further contamination of food and agricultural products. More stringent regulations and controls are required by the food administrations' and of the National Standard of Iran. (i.e. GAP and HACCP), proper feed storage and training programs for the dairy industry/

Table 1 - Aflatoxin M1 concentration (μg/L) in raw milk in different milk collecting center in Qom, Iran.

| Milk collecting centers | Number of samples | Mean | Range |
|---|---|---|---|
| Center 1 | 5 | 0.163 | 0.160-0.167 |
| Center 2 | 5 | 0.171 | 0.170-0.174 |
| Center 3 | 5 | 0.198 | 0.195-0.203 |
| Center 4 | 5 | 0.199 | 0.197-0.201 |
| Center 5 | 5 | 0.083 | 0.079-0.087 |
| Center 6 | 5 | 0.201 | 0.197-0.205 |
| Center 7 | 5 | 0.173 | 0.170-0.175 |
| Center 8 | 5 | 0.173 | 0.170-0.176 |
| **Total** | **40** | **0.170** | **0.079-0.205** |

farmers are a necessity, to aid in reducing and controlling this bacterial infection. Additionally, it is suggested further in-depth studies be conducted on AfB1 contamination in the different stages and in the feed preparation, and further studies on which factors might possibly be involved in the contamination AfB1 process.

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# SHORT FOOD SUPPLY CHAIN AND LOCALLY PRODUCED WINES: FACTORS AFFECTING CONSUMER BEHAVIOR

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ABSTRACT

This study explores a short food supply chain by analyzing direct sales of locally produced wine in Italy. The paper discusses the main factors influencing the purchase of locally produced wine through econometric analysis of a sample of Italian consumers using a binary logit model. The results reveal the profile of wine consumers who buy directly from wine producers including their attitudes and preferences. The findings provide important insight to managers and policy makers in terms of marketing and policy development.

- Keyword: short food supply chain, locally produced wines, local wine consumption, direct sale -

## INTRODUCTION

The short food supply chain (SFSC) has become an important issue in international as well regional agro-food systems (GUPTILL and WILKINS, 2002; STEPHENSON and LEV, 2004; SELFA and QAZI, 2005; LOMBARDI *et al.*, 2013; D'AMICO *et al.*, 2014) and has been widely explored in rural development studies (GALE, 1997; RENTING *et al.*, 2003; RIZZO and GIUDICE, 2013; D'AMICO *et al.*, 2013). The SFSC is based on the direct relationship between the producer and consumer, being characterized by benefits to both parties including a reduced number of trade intermediaries (LA TROBE, 2001; AGUGLIA *et al.*, 2009).

Among the alternative short food chains identified by RENTING *et al.* (2003), direct sales of a locally produced food - such as by farm shops, farmers' markets, pick-your-own, roadside markets, mail order, e-commerce, box schemes and home deliveries - allows consumers to verify the quality of the food and appreciate their favorable attributes such as the freshness, genuineness and seasonality. At the same time, consumers can buy higher quality products at lower or more competitive prices than those purchased in supermarkets (BROWN, 2003; WOLF *et al.*, 2005; AGUGLIA *et al.*, 2012). Several studies have observed that the high reputation of locally produced food is attracting the attention of the modern consumer. The increasing success of such SFSC is due to the growing environmental awareness of consumers; in fact, ecological sustainability is often associated with local food production given its low environmental impact (WOLF *et al.*, 2005; CRANFIELD *et al.*, 2012).

Many authors report that farmers' markets and direct selling at farm shops play a prominent role in enabling farmers to earn a higher income (NAYGA *et al.*, 1995; GOVINDASAMY *et al.*, 1999; DI VITA *et al.*, 2013c) as a consequence of better profits margins and reduced transport and packaging costs (JAROSZ, 2008; AGUGLIA *et al.*, 2009). Simultaneously, direct selling allows producers to regain their active role in the agro-food market, gaining new market opportunities and receiving a fair price for their products. In particular, direct marketing and direct sales represent an interesting economic opportunity in the light of the ongoing economic crisis that has weakened the power of household spending (Di Vita *et al.*, 2014) and reduced the profitability of small wine grape growers in southern European countries (LANFRANCHI and GIANNETTO, 2013; DI VITA, D'AMICO and BRACCO, 2013; PAPPALARDO *et al.*, 2013; SPAMPINATO *et al.*, 2013).

Studies on direct farm marketing have mainly addressed local food produce (WOLF, 1997; RENTING *et al.*, 2003) from the aspect of the social and economic features of direct market consumption (ONIANWA *et al.*, 2005; NAYGA *et al.*, 1995). Many authors have examined in depth the behavior of consumers toward locally produced food (GRACIA *et al.*, 2012; CRANFIELD *et al.*, 2012) and their willingness to purchase it (JEKANOWSKI *et al.*, 2000; LOUREIRO and HINE, 2002; CAMPBELL, 2013).

Consumers' studies on farmers' markets (EASTWOOD, 1996; BROOKER *et al.*, 1993; HINRICHS, 2000; WOLF *et al.*, 2005) have highlighted the relevance of the preferences, habits and socio-demographic characteristics of local consumers in urban as well in rural areas (BROWN, 2003; WEATHERELL *et al.*, 2003). Although the profile of local consumers has been analyzed in depth, studies have mainly been directed towards food produce in general. Few studies have focused on a specific product, such as locally produced wine. Given local wine production represents an important issue in the international scientific debate and there is a lack of empirical evidence regarding locally produced wines, the purpose of our study is to analyze in depth the main features and aspects of the direct selling of wine.

The main objective of the present paper is to identify the profile of Italian consumers with respect to locally produced wine by analyzing the main socio-economic components that directly influence their choice and purchasing behavior. This study additionally attempts to identify which factors influence the purchase of wine directly from wineries. The paper is divided as follows: Section 2 briefly describes the survey design for the study and the empirical model. Section 3 reports the main findings of Italian consumers who buy wine directly from wineries. Section 4 discusses the most important implications of the findings.

## MATERIALS AND METHODS

Data were collected using a questionnaire submitted to a random sample of 953 wine consumers who were interviewed between July and September 2011. The questionnaire was structured in 4 parts: The first part covered the features of the wine purchases (wine typology, quantity consumption, shopping frequency, etc.). The second part included questions on sensory attributes, such as packaging and origin. The third part focused on the distribution channel and selling location. The fourth and final part collected the social and economic variables of the interviewees. The questionnaires, utilizing 26 closed-ended questions, were administered through face-to-face interviews in the three main areas of Italy to obtain a representative sample of the Italian consumer: Southern Italy (Palermo and Catania), Central Italy (Rome) and Northern Italy (Milan). Respondents were recruited both at a large retail store and at specialized wine shops in each

Table 1 - Demographic characteristics of the sample.

| Category | Variable | Wine consumers | | Buyers at wineries | |
|---|---|---|---|---|---|
| | | n | % | n | % |
| Gender | Female | 261 | 27.4 | 45 | 15.3 |
| | Male | 692 | 72.6 | 250 | 84.7 |
| Age | 18-30 | 80 | 8.4 | 20 | 6.8 |
| | 31-45 | 349 | 36.6 | 89 | 30.2 |
| | 46-60 | 358 | 37.6 | 104 | 35.2 |
| | > 60 | 166 | 17.4 | 82 | 27.8 |
| Education | Primary | 90 | 9.4 | 49 | 16.6 |
| | Secondary | 380 | 39.9 | 112 | 38.0 |
| | Graduate/Postgraduate | 483 | 50.7 | 134 | 45.4 |
| Annual Income | - < 15,000 Euros | 114 | 12.0 | 43 | 14.6 |
| | - 15-30,000 Euros | 476 | 50.0 | 151 | 51.2 |
| | - 30-50,000 Euros | 273 | 28.6 | 75 | 25.4 |
| | - > 50,000 Euros | 90 | 9.4 | 26 | 8.8 |
| **Respondents** | | **953** | **100.0** | **295** | **100.0** |

of the study areas, and were selected according to their willingness to cooperate with the survey.

The study aimed at capturing the habits and socio-economic characteristics of consumers to identify the profile of consumers who buy wine directly from wine producers. The survey was carried out on 295 consumers who declared they buy wine directly from wine producers, which represent around 31% of the total sample. Table 1 provides a brief description of the respondents' demographic statistics that were collected from the survey.

Analysis was carried out using a logit model, where the dependent variable, defined as "consumers who buy wine directly from a wine producer", is dichotomous and assumes the value of 1 for consumers who buy wine at wineries and 0 for respondents who do not buy wine at wineries.

Binary models such as that used in our survey are particularly suitable for the analysis of an individual's choice, especially in surveys where individuals must make a choice between two distinct alternatives. This approach tends to give greater weight to the individual sphere, compared with the dimension of time in which the choices are made.

The econometric model we chose was the binary logit model estimated with maximum likelihood (ML). This is because such a functional form is relative to its assimilation to the normal distribution that generally leads to dissimilar results compared with those of the probit model (AMEMIYA, 1981). The specification of the logit model is as follows:

$$\Pr(y_i = 1 \mid x_i, \beta) = 1 - (e^{-x_i'\beta}/(1 + e^{-x_i'\beta})) = e^{x_i'\beta}/(1 + e^{x_i'\beta})$$

The independent variables in our survey were linked to a) *socio-economic features of consumer*, such as age, gender, number of household members, level of education, and four different levels of income; b) *characteristics of wines*, such as typologies (red, white, sparkling, bubbling, rosè, sweet and novella wines), sensory attributes (colour, taste, appearance and flavour) and Product Designation of Origin (PDO)/Protected Geographical Indication (PGI) designation; and c) *habits of buying*, such as shopping frequency (weekly, monthly and occasionally). Table 2 reports the variables used in the ordered analysis and their descriptive statistics.

## RESULTS

Table 3 presents the results of the binary logit model. The explanatory variables describe a specific short supply chain channel configuration: direct sales from wine producer to final wine consumer, revealing the profile and characteristics of wine consumers buying at wineries. In the econometric model we employed social and economic variables (age, gender, income, number of

household members, education and occupation), the typology of purchased wine (red, white, sparkling, bubbly, rosè, sweet and novello, wines) and the features of the purchasing process including shopping frequency, according to the post-modern consumer approach (CICIA *et al.*, 2011; CEMBALO *et al.*, 2013). Among the variables employed in the estimated model, we report only those with a statistical significance of at least 10% (Table 3). The estimated logit model shows that all the signs of the coefficients of each variable are significant and the results are in line with expectations, thus inferring how to better target the marketing of wines sold directly from wineries. The findings of our study provide for first time insight into the consumer profile of purchasers of locally produced wine and their attitudes towards the main attributes of wines

According to the descriptive statistics of our study, the results confirm a high percentage of

Table 2 - Variables implemented in the econometric logit.

| Variable | Type* | Description | Mean | Std. Dev. |
|---|---|---|---|---|
| **Age** | c | age in years | 48.01 | 12.669 |
| **Gender** | d | 1 if female | 0.27 | 0.446 |
| **Total household members** | c | number total | 2.99 | 1.239 |
| **Adults** | c | number adults | 2.35 | 0.990 |
| **Education** | | | | |
| - Primary | d | 1 if present or 0 | 0.09 | 0.282 |
| - Secondary | d | 1 if present or 0 | 0.40 | 0.490 |
| - Degree | d | 1 if present or 0 | 0.51 | 0.500 |
| **Occupation** | | | | |
| - Executive | d | 1 if present or 0 | 0.07 | 0.250 |
| - Unemployed | d | 1 if present or 0 | 0.01 | 0.091 |
| - Clerk | d | 1 if present or 0 | 0.49 | 0.500 |
| - Worker | d | 1 if present or 0 | 0.04 | 0.186 |
| - Pensioner | d | 1 if present or 0 | 0.11 | 0.313 |
| - Self-employed | d | 1 if present or 0 | 0.02 | 0.136 |
| **Annual Income** | | | | |
| - Income 1 (< 15,000 euros) | d | 1 if present or 0 | 0.12 | 0.325 |
| - Income 2 (15,000 - 30,000 euros) | d | 1 if present or 0 | 0.50 | 0.500 |
| - Income 3 (30,000 - 50,000 euros) | d | 1 if present or 0 | 0.29 | 0.452 |
| - Income 4 (> 50,000 euros) | d | 1 if present or 0 | 0.09 | 0.293 |
| **Typology** | | | | |
| - Red wine | cat | from 1 to 7 | 6.54 | 0.948 |
| - White wine | cat | from 1 to 7 | 5.28 | 1.762 |
| - Sparkling wine | cat | from 1 to 7 | 3.17 | 1.557 |
| - Bubbly wine | cat | from 1 to 7 | 2.98 | 1.581 |
| - Rosé wine | cat | from 1 to 7 | 3.22 | 1.940 |
| - Sweet wine | cat | from 1 to 7 | 3.04 | 1.740 |
| - NovelloWine | cat | from 1 to 7 | 2.67 | 1.786 |
| **Shopping frequency** | | | | |
| -Weekly | d | 1 if present or 0 | 0.19 | 0.389 |
| - Monthly | d | 1 if present or 0 | 0.25 | 0.434 |
| - Occasionally | d | 1 if present or 0 | 0.31 | 0.461 |
| **Sensory attributes (color, flavour, etc.)** | | | | |
| - Color | cat | from 1 to 4 | 2.53 | 1.072 |
| - Taste | cat | from 1 to 5 | 3.23 | 1.006 |
| - Clearness | cat | from 1 to 4 | 1.83 | 0.937 |
| - Flavour | cat | from 1 to 4 | 2.43 | 0.993 |
| **Origin Designation (PDO and PGI)** | d | 1 if Dop or IGP | 0.63 | 0.482 |

** Variable typology. C: continuous; D: dummy; Cat: categorial.*

Table 3 - Regression results of the binary logit model.

| Variable | Coefficient | Std. Error |
|---|---|---|
| CONSTANT | -4.231807*** | 1.031073 |
| GENDER | -0.779159*** | 0.198223 |
| AGE | 0.014543** | 0.006456 |
| RED | 0.484353*** | 0.130146 |
| ROSÈ | -0.223688*** | 0.044612 |
| SPARKLING | -0.122695** | 0.051737 |
| INCOME 1 | 0.537901** | 0.272357 |
| INCOME 2 | 0.380466** | 0.173488 |
| SECONDARY SCHOOL | -0.287534* | 0.167308 |
| SELF-EMPLOYED | 0.925121* | 0.534248 |
| TOTAL HOUSEHOLD MEMBERS | -0.255076*** | 0.093079 |
| ONCE A MONTH | -0.709668*** | 0.197114 |
| ADULTS | 0.616366*** | 0.112080 |
| *McFadden R-squared* | 0.145591 | |
| *Log likelihood* | -502.8064 | |
| *Probability(LR stat)* | 0.000000 | |
| ** 10%; ** 5%; *** 1%* | | |

consumers (31%) who buy wine directly from wine producers. These findings are consistent and in line with the findings of a recent study that estimated that over 30% of Italian consumers prefer to buy directly from wine producers (MEDIOBANCA, 2013).

On the basis of our regression results, the profile of consumers who buy wine directly from wineries is characterized by self-employed adult males with low to medium income and a larger than average household. With respect to consumer attitudes, it was found that the purchase of wines was not frequent, given most consumers only purchased wine once a month. An interesting insight can be derived by taking into account the variables related to the characteristics of the typology of the wine. Consumers tended to prefer red wines followed by white wines, being less interested in other typologies such as sparkling or rosé wines. Moreover, we observed that consumers are uninterested in product differentiation such as the PDO and PGI designations since these variables estimated in our models did not significantly differ from zero.

## CONCLUSION

Although the relevance of SFSC and local food product consumption has been widely reported in the economic literature (RENTING *et al.*, 2003; Stephenson and Lev, 2004), the majority of studies have focused on identifying factors affecting consumers' behavior towards locally produced food (WEATHERELL *et al.*, 2003; CRANFIELD *et al.*, 2012; DI VITA *et al.*, 2013b).

Our results show for first time the behavior of wine consumers buying directly from wineries and their profile, attitudes and preferences. The high number of wine consumers that bought wine directly from wineries indicates that SFSC represents a very relevant phenomena in the Italian agro-food system. Thus, our findings provide important insights to sales and marketing wine managers, wine experts and policy makers in terms of marketing and policy development.

Our findings highlight the importance of socio-demographic characteristics such as income and occupation in influencing the behavior of consumers of locally produced products. The results presented in this study also confirm the role of direct marketing as a strategic tool for consumers of winemaking areas (CANIGLIA *et al.*, 2008; BORSELLINO *et al.*, 2012; DI VITA *et al.*, 2013c), and suggests, as previously REPORTED (BROWN, 2003), that price does not play a prominent role in the choice of local products consumers at SFSC.

This study also has policy implications for wine-producing countries with respect to the enhancement of economic sustainability for rural areas. The findings suggest using public fund-

ing to support these forms of short channel markets to generate benefits for both wine consumers and wine producers. Within this context, it could be useful to stimulate the expansion of free and unconstrained direct sales of wine, releasing the wine market from tariff barriers and bureaucratic rules that negatively affect wine business.

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# ANALYSIS OF PRODUCERS' KNOWLEDGE ABOUT FARMERS' MARKETS

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ABSTRACT

The objective of this work is to determine the feasibility of establishing a farmer's market in Messina (Italy). For this purpose, a "motivational" snapshot was obtained through the traditional methodology of a descriptive research survey by questionnaire. The research methodology adopted was quantitative and random. Prior to conducting the survey, their search team developed the hypothesis of the study, constructed the survey questions and delimited the area of research. The questionnaire comprises 16 multiple choice questions that area series of general questions put to agricultural producers on the situation of local business, including the characteristics of conducting local business, the size of production and the local economy, agricultural products, distribution channels and marketing. The questions relate exclusively to knowledge of short chain markets in general and farmers' markets in particular. The sample size was fixed taking into account the objectives of the research and the resources available.

- Keywords: buying groups, farmer's market, market analysis, short chain, statistical analysis -

## INTRODUCTION: ECONOMIC EFFECTS OF SHORT CHAIN MARKETS

When considering rural development and multi-functionality in agriculture, we must take into account strategies and objectives that aim to keep people on the land, resources, traditions, and socio-economic and cultural aspects indispensable for revitalizing rural areas. In this respect, the short chain can be considered a component of rural development falling within the concept of multifunctional agriculture (DE BLASI *et al.*, 2009), helping to boost economic growth and consequently contributing to the permanence of the population and businesses in rural areas. The term "short chain," now in common use, includes reference to short circuits of production and consumption, agro-food networks, and alternative or sustainable food chains (LANFRANCHI, 2010). From a commercial point of view, the implementation of short chains is an alternative to the logic and organizational modalities of the agro-food system dominant up to now. Short chains shorten the distances not only spatially but also socially, culturally and economically between the agricultural world and the final consumer (SCHIFANI and MIGLIORE, 2011). Although, the concept of the short chain is not new, today it has become important in value. The short chain has its roots in sales made in local and traditional markets. In fact, buying or selling with zero km distances was born some time ago, when the farmer would take his locally produced vegetables and livestock products to the local village or town square to sell. Thereafter, large-scale distribution came into force, affecting consumer habits and breaking down one of the main pillars of agricultural enterprise, namely, the seasonality of products, by offering the consumer food out of season. Today, in fact, you can buy any agricultural food or products at any time of the year. The superiority of the food chain, conquered thanks to intensive agriculture, globalization of cultures, and technological advancement of packaging and transport, allows the consumer to tear down time issues (buying products out of season) and spatial issues (purchase of products made in other countries). The process of globalization has consequently deepened the crisis for small producers who have been ousted from the markets now occupied mainly by large producers (SPAMPINATO *et al.*, 2013). These large producers have farming practices not entirely environmentally sustainable but meet the needs of consumers who are increasingly accustomed to highly differentiated products. This economic-cultural change has intensified the process of pollution, mainly due to the advent of organized retailing and distribution that has seen the long-distance transportation of all agricultural goods, such as by road, ship, aircraft, railway. Such transportation methods are inefficient in terms of logistics and are major contributors to greenhouse gas emissions on a global scale (CAPUTO *et al.*, 2013). Thus, availability of food choice is a significant contributor to environmental pollution. Therefore, to respect the environment, and rediscover the seasonality, freshness and flavours of local products, it is important to buy and consume foods of ones' own local area (ANTONELLI and VIGANÒ, 2009). To achieve such a "zero cost km" model of purchase requires direct contact between producer and consumer at local markets (for example, farmers' markets), or selling directly from the producer's farm, where the cost of packing and shipping would be minimal. Analyzing this context, we can summarize the efficiency of the short chain into four areas of efficiency: logistic-organizational efficiency, informational efficiency, distribution efficiency and environmental efficiency (CHINNICI *et al.*, 2013). The development of short chain food production is not only a commercial alternative to the current globalized distribution process, but is an important tool to support the policies of local rural development (LANFRANCHI and GIANNETTO, 2013).

## TYPES OF SHORT CHAIN ACTIVITY

In recent years, the world agro-food industry has been characterized by the presence of a long chain comprising many actors placing a greater and greater distance between producers and consumers. Producer prices of agricultural products have generally decreased, while consumer prices have remained stable if not increased. In this context, shortening the supply chain seems the best solution to reduce costs for both producer and consumer (BELLETTI and MARESCOTTI, 2013). The short chain is a particular type of business that allows consumers to buy food directly from producers, resulting in a shortening of the number of commercial intermediaries and lowering the final price. One way of achieving a short chain are farmers' markets, which markets are usually held outdoors where producers make direct sales of their products to consumers. Farmers' markets established themselves United States and Europe in the early '70s and spread to Northern Europe in the 1990s. In Italy, their success has been very recent, and can in fact be traced back to the Decree of the Ministry of Agriculture and Agro-Forestry of 20 November 2007, in which specific reference is made to *"the creation of markets reserved for direct sale by farmers in order to meet the needs of consumers so as to purchase agricultural products which have a direct link with the territory of production"* (Official Gazette number 301 of 29-12-2007). This recognition at the legislative level has resulted

in considerable expansion of farmers' markets in Italy. In fact, since 2008,the strongest growth in employment has been in the area of direct sales of food products. In 2008 alone, about 52% of the Italian population bought food products from at least once directly from the primary producer. Unlike in other countries, Italy does not have a trade association establishing common requirements for farmers' markets. For this reason, farmers' markets in Italy often have very different characteristics. Farmers' markets are generally are organized through collaboration, such as by agricultural organizations, producer associations, organic organizations (AIAB, Associazione Italiana Agricoltura Biologica; Italian Association of Agricultural Biology) and other similar organizations. The farmer's market is not characterized simply by sales from producer to consumer, but infers a much deeper relationship established between the two parties, creating knowledge about products and confidence in the producer that are essential elements for the consumer.

Farmers' markets, in fact, according to the Decree of the Ministry of Agriculture and Agro-Forestry, must be (Official Gazette number 301 of 29-12-2007):

- organized by professional organizations or farmers' market associations constituted locally by farm producers;
- always carried out in the same area at least once a week because longer periods would create logistical problems for producers, forcing them to carry almost all their produce to the usual markets, thus reducing their presence in the farmer's market;
- organized in itinerant form so that they can be regularly found in other areas but always in the same province;
- an expression of the territory, offering only typical and traditional products;
- an expression of those farmers who synergistically promote sales of their own locally grown products either through specific associations of the farmer's market or via similar associations that have provided support from the their beginning;
- created by producers, preferably located as closely as possible to the market and should ordinarily have the nature of seasonality. If the producer runs out of their own products for that year, they also lose their presence in the market. A limited number of outlets provide better knowledge and collaboration between producers, increasing sales per producer and thus protecting their presence in the market. In large centres, several farmers' markets with the same characteristics can be established.

An important aspect of farmers' markets is the leading role of the director of a farmer's market, who acts as a key guarantee for both consumers and producers. The director's functions range from the organization of the logistics of the market itself (such as sales, turnover of producers, development of a register of market areas, planned production, production periods and so on) activities of promotion and communication, careful control of the products in the field, assessment of the cultivation techniques used in the production and compliance with rules. Following these principles, the farmers' markets are supported by consumers, who not only see the economic benefits and advantages but also the social benefits. Another alternative form in the marketing of food products are the solidarity-based purchasing groups (Gruppi di Acquisto Solidale; Solidarity Purchase Groups, GAS) (BRUNORI *et al.*, 2010) - groups of people who buy together. Based on the principle of solidarity, these groups prefer to buy from small producers who respect the local environment and from people with which they can remain in direct contact. The first GAS in Italy emerged in Fidenza in 1994, arising from criticism of an unjust and unsustainable development model. The group was established as an alternative to the dominant distribution system and the desire to be able to consume healthy and tasty foods. Such groups developed rapidly especially in the north of Italy. There are currently over 600 GAS. A substantial difference compared to farmers' markets, is that GAS are not promoted by manufacturers but by consumers. In particular, the aim of GAS members is to buy environmentally- and people-friendly products with the aim of constructing an economy that is closer to the needs of man and the environment that will lead to sharing instead of competition. Each GAS develops its own principles, but there is often a common base theme of criticism towards the consumption pattern and dominant global economy that has emerged in recent years.

Another classification of direct sales, is the "box scheme," whereby seasonal agricultural products are distributed by the producer at agreed intervals, generally to individual's homes or groups of consumers. Usually this type of direct distribution is carried out by individual producers. However, producers may also work in collaboration, forming associations or cooperatives, to expand the basket of products offered. Consumers, in this regard, may decide not only the quantity of product to buy, but also, the content, which varies according to the products available by a specific producer. This method also allows manufacturers to better manage the seasonal nature of agricultural production and to plan production activities. Box scheme distribution is widespread, especially in the United States, Canada and in Northern Europe. In some cases, it can cover a considerable share of the market of a specific product due to the amount of orders that can be really huge.

## METHODOLOGY

The research methodology adopted was quantitative and random. The random search refers to identification of factors that depend on the behaviour of producers in a specific location -Messina (Italy) in the case of our study - and evaluation of cause-effect relationships in a given community. Prior to conducting the survey, research members developed the research hypothesis, constructed the survey questions and delimited the area of research. The questionnaire consisted of 16 multiple-choice questions, which were a series of questions put to agricultural producers regarding the situation of local enterprises and with reference to the characteristics of the producer, the size of production and the local economy, agricultural products, sales distribution channels and the area of marketing (ASIOLI *et al.*, 2012). Some questions included knowledge about farmers' markets and the short chain market. The number of questions was limited to avoid discouraging participants. The survey was conducted between April 1, 2013, and June 28, 2013, on a sample of 300 producers in Messina. The survey was conducted mainly on farms, interviewing a sample of producers directly face-to-face, using a questionnaire prepared for the specific purpose. The sample size was fixed taking into account the objectives of the research and the resources available. The data collected from the responses was favourable and supportive of the creation of a single farmer's market in the area covered by this survey.

## DEGREE OF FEASIBILITY OF ESTABLISHING A FARMER'S MARKET IN THE MUNICIPALITY OF MESSINA (ITALY)

The purpose of the survey was to determine the feasibility of establishing a farmer's market in Messina (LANFRANCHI *et al.*, 2012). For this reason, the study was of the "motivational" type, conducted through a traditional survey methodology of descriptive research by questionnaire. Producers who responded to the questionnaire were far from the usual entrepreneur of the area of Messina: they were active entrepreneurs, already sensitized to the concept of short chain and informed about the operational and economic problems arising from the numerous supply chain steps of products of the earth. Respondents were mostly men with an average age of 58, reflecting a significant lack of young entrepreneurs, with the majority of respondents (69%) having a university degree or diploma in agriculture and most of the remaining (21%) having primary or middle school education. The analysis of the data makes apparent the need to establish a farmer's market, not only according to agricultural resources available in the territory of Messina, but also as an initiative to encourage young people to enter the agricultural sector by stimulating their knowledge and interest in an occupation that connects land, food and health, which are closely linked to the life of all of us. The survey findings and summary of type of sales by producers in Messina are shown in Table 1.

The data reveal there is no one homogeneous producer behavior. Although there is no common distribution channel, producers do have direct contact with consumers. All the respondents lean towards a strong respect for the seasonality of the products, only30 producers (10%) claiming to produce the same food products all year round.

From the analysis of the data, it is noted that-113farmers (37.5% of the sample interviewed) sell their products directly from the farm and have close contact with consumers, but 74 producers (25%) have never done this type of sale.

Regarding knowledge about farmers' markets (Table 2), 15% of respondents know fully about the function of the market, 25% know enough about it and only 12.6% lack knowledge. Nevertheless, the data highlight the high percentage of entrepreneurs who have not yet done direct sales but would like to do so: 127 out of 300 (42.5%) would be willing to sell their products in a farmer's market.

On the other hand, 15% of the producers

Table 1 - Type of sale including seasonality of products.

| Frequency of sales/production | Sale of own-produced agricultural products exclusively to supermarkets | Sale of own-produced agricultural products to both supermarkets and local markets | Sale of own-produced agricultural products directly to the consumer | Production of agricultural products all the year round |
|---|---|---|---|---|
| Never | 76 | 60 | 74 | 90 |
| Rarely | 36 | 0 | 15 | 39 |
| Occasionally | 90 | 67 | 38 | 60 |
| Often | 38 | 143 | 60 | 81 |
| Always | 60 | 30 | 113 | 30 |
| Total | 300 | 300 | 300 | 300 |

Source: data collected by questionnaire.

Table 2 - Questions related to knowledge of farmers' markets.

| | You know about farmers'markets | You would be willing to sell your products in the marketplace | You believe a better understanding of local products by consumers could counter the purchase of products from abroad in favour of local products | You believe the existence of a farmer's market in the area would bring benefits to both producers and consumers |
|---|---|---|---|---|
| Not At All | 38 | 45 | 8 | 23 |
| A Little | 52 | 15 | 22 | 15 |
| Average | 90 | 38 | 30 | 112 |
| Enough | 75 | 75 | 45 | 75 |
| A Lot | 45 | 127 | 195 | 75 |
| Total | 300 | 300 | 300 | 300 |

Source: data collected by questionnaire.

interviewed are not willing to reduce the time spent working to dedicate their time to making sales in markets or directly from the farm. Some of them, in fact, believe that the existence of a farmer's market in the territory would not bring benefits to producers and consumers. However, 65% of respondents think that a better understanding of local products by consumers would reduce the purchase of products from abroad in favour of local products. The producers surveyed have a varied product portfolio; most produce not only oil but also wines, vegetables, cheese and fruit. Only a few farms specialize in one product.

## RESULTS

The results from the survey reveal wide spread perception that direct sales in markets is little or undeveloped in the reference area, Messina. Despite this, however, many producers state they would sell their products in a farmer's market. Although most producers know about operation of farmers' markets, many still need to be better informed as well as encouraged to participate in this type of short chain. Most producers believe that the existence of a farmer's market in the area would bring benefits to both producers and consumers. The existence of asymmetry information on consumer demand is an element that must be removed. It is believed that more accurate information about the benefits related to the farmer's market could help reduce the import of agricultural products from abroad by encouraging the purchase and consumption of local products.

## CONCLUSIONS

This study provides useful data for the assessment of the potential role that short chain markets have in the process of revitalizing the economy of rural areas. In this sense, the spread of farmers' markets has created an alternative local economy. This, however, needs sufficient time to stabilize. The experiences of areas similar to that of the study area at the time of the survey appear to be well established (TUDISCA *et al.*, 2013). On one hand, the increasing propensity of consumers to seek out and establish a direct relationship with agricultural producers is contributing to the creation of an alternative local economy as opposed to the dominant global one. On the other hand, the growth of organizational skills and interpersonal relations by producers has influenced the emergence of this, both as a sales channel and also as an alternative approach to the production-consumption process. However, this process of shortening the supply chain is not yet sufficiently consolidated and only partly affects the dominant socio-technological system as represented by large supermarkets (SCHIMMENTI *et al.*, 2013). Nevertheless, it has begun to impact various levels of society and the economy at the local level.

Gradual integration between markets for producers and GAS will bring about a transition from intensive agriculture - industrialized and controlled by transnational corporations - to a more sustainable agricultural system based on the rational use of natural and social resources available (LANFRANCHI, 2012). The analysis in this paper identified a lack of a network of relationships between producers and consumers, which represents a major obstacle to the shortening of the agricultural supply chain. Another critical issue that emerged from the survey is the level of producers' knowledge of the short chain, agriculture and production processes, and consequently, the level of awareness in choosing this sales method. In fact, the majority of producers do not interpret this alternative form of trade as a concrete and sustainable economic opportunity for both consumers and producers.

In essence, consumers have yet to acquire sufficient knowledge of the problems that plague agriculture and have yet to abandon the traditional consumption associated with large retail organizations.

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**2. Manuscript Preparation**

(1) The paper should be divided under the following headings in this order:

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**Author(s).** Initials and Surname, omit professional and official titles. The institute and address where the research was carried out and the current address of each author should be given on the title page.

**Abstract.** Clearly state the objective of the study, give a concise description of experiment(s), observations, results and conclusions. No references should be cited. **Do not exceed 100 words**.

**Key words.** Up to six words, in alphabetical order, which describe the document must be given to aid data retrieval and indexing.

**Introduction.** Review pertinent previous work and cite appropriate references. State the purpose of the investigation.

**Materials and Methods.** Indicate apparatus, instruments, reagents, etc., giving sufficient detail to allow the work to be repeated.

**Results and Conclusions**. Results and Conclusions may be presented together or separately. Concisely present results using tables and figures to help justify conclusions (do not present the same information in both forms). Use statistical analysis when appropriate. Unsupported hypotheses should be avoided. Conclusions should point out the significance of the findings and, if possible, relate the new findings to some problem in Food Science and Technology.

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

**Units.** A list of units particular to the paper may be included.

**References**. References in the Reference list should be arranged alphabetically (initials of first name, only), and, for the same author, should be arranged consecutively by year, typed double-spaced. Each individual reference should begin flush left (no indentation). Refer to attached examples taken from "Style Guide for Research Papers" by the Institute of Food Technologists (Chicago - I1linois - USA). Literature citations in the text should be referred to by Surname and year in parentheses. If there are more than two authors, give the surname of the first author and add et al. and the year in parentheses. Examples: (SMITH, 2007), (SMITH and JONES, 2008) (SMITH et al., 2008).

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**Short Communications, Surveys and Opinions** – They do not need to have the formal organization of a research paper; they will receive priority in publication; maximum of five pages allowed.

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# CONTENTS

PAPERS

An Investigation of the Antioxidant Activities and Some Physicochemical Characteristics of Strawberry Added Yogurt
***M.S. Engül, T. Erkaya, M.S. Engül and H. Yildiz*** ..... *235*

Rheological and Clustering Approach to Classify Iranian Soft/Liquid Food Products to Meet Dysphagia Diet Requirements
***A. Zargaraa, Y. Omaraee, R. Rastmanesh, N. Taheri, Gh. Fadavi, F. Zaeri and M.A Mohammadifar*** ..... *243*

Bioactive Compounds in Industrial Tomato Sauce After Processing and Storage
***G. Spigno, L. Maggi, D. Amendola, J. Ramoscelli, S. Marcello and D.M. De Faveri*** ..... *252*

Prediction of the Maximal Growth Rate of *Listeria monocytogenes* in Sliced Mortadella by the Square Root Type Model
***P. Daminelli, E. Dalzini, E. Cosciani-Cunico, G. Finazzi, S. D'Amico and M.N. Losio*** ..... *261*

Effect of Temperature Shear Rate and Processing on the Rheological Properties of Salep Drink
***D. Arduzlar Kagan, M. Hikmet Boyacioglu and D. Boyacioglu*** ..... *268*

The Effect of Technological Processing on the Chemical Composition of Cauliflower
***A. Florkiewicz, A. Filipiak-Florkiewicz, K. Topolska, E. Cieslik and R.B. Kostogrys*** ..... *275*

Angiotensin I Converting Enzyme Inhibitory and Antioxidant Activity of Adlay (*Coix lacrymajobi* L. var. *ma-yuen Stapf*) Glutelin Hydrolysate
***J. Yuan, Yingxi Liang, S. Cui, X. Zhang, L. Wang and Y. Qiao*** ..... *282*

Factors Limiting the Shelf-Life of Salami Pieces Kept in Retailing Conditions
***S. Bañón, R. Serrano and M. Bedia*** ..... *289*

The Effect of Polysaccharide Concentration and pH on Physicochemical Properties of Flaxseed Polysaccharide-Potato Starch Mixtures
***Hai-Hua Chen, Yu-Sheng Wang, Yun Leng, Yang Zhao and Xia Zhao*** ..... *300*

Phenotypic Diversity and Technological Properties of Yogurt Cultures Isolated from Traditionally Produced Turkish Yogurts with Comparison to Commercial Starter Cultures
***N. Altay Dede, E. Acar-Soykut and G. C. Gürakan*** ..... *308*

Polyphenolic Profile of Interspecific Crosses of Rowan (*Sorbus aucuparia* L.)
***T. Jurikova, J. Sochor, J. MLcek, S. Balla, B. Klejdus, M. Baron, S. Ercisli and S. Ozturk Yilmaz*** ..... *317*

SHORT COMMUNICATION

Occurrence of Aflatoxin M1 in Milk in Qom, Iran
***M. Rezaei, M. Parviz, M. Eshaghi Gorji, N. shariatifar, M.A. Hosseini and S. Habibi*** ..... *325*

SURVEYS

Short Supply Chain and Locally Produced Wines: Factors Affecting Consumer Behaviour
***M. D'Amico, G. Di Vita, G. Chinnici, G. Pappalardo and B. Pecorinoiu*** ..... *329*

Analysis of Producers' Knowledge about Farmers' Markets
***M. Lanfranchi and C. Giannetto*** ..... *335*

PREVIEW ON VOLUME XXIV No. 4, 2014 ..... *341*
GUIDE FOR AUTHORS ..... *342*
CONTRIBUTORS ..... *345*