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PAPER

DETERMINATION OF FLAVONOIDS IN *ERUCA* AND *DIPLOTAXIS* ROCKET SALAD SPECIES: MEKC-DAD METHOD ESTABLISHMENT AND COMPARISON WITH HPLC-DAD-MS

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ABSTRACT

The phenolic compounds in ten rocket salad samples were determined in this study using micellar electrokinetic chromatography (MEKC). A detailed method optimisation plan was performed to separate the phenols in rocket salad with the following four separation parameters: buffer concentration, buffer pH, applied voltage and temperature. The best results in terms of peak resolution and reproducibility among separations were obtained with a MEKC running buffer (pH 9.0) composed of 40 mM sodium tetraborate and 15 mM SDS. Using this new MEKC method, it was possible to separate the flavonoids in less than 5 min.

The results obtained by MEKC analysis were compared with the data obtained by high-performance liquid chromatography (HPLC) coupled with DAD and MS detectors. The polyphenols study by HPLC-DAD-MS allowed the identification of two different classes of compounds. Quercetin and kaempferol derivatives were determined in the Diplotaxis and Eruca species, respectively. A positive correlation of phenolic compounds identified by MEKC and HPLC was found.

- Key words: rocket salads, *Eruca vesicaria* (L.) Cav. *subsp. Sativa*, *Diplotaxis tenuifolia* L., capillary electrophoresis, phenolic compounds, HPLC-DAD-MS -

INTRODUCTION

Brassicaceae vegetables are a good source of antioxidants because of their high phenolic and glucosinolate contents (JAHANGIR *et al.*, 2009).

Leafy green salads of the *Brassicaceae* family, such as rocket salad species, have been grown in the Mediterranean area since Roman times, and they are now cultivated and used in various places mainly as salad and soup ingredients. In recent years, these Brassica species have gained greater importance as salad vegetables and spices, especially among Middle Eastern populations and Europeans (LAMY *et al.*, 2008; ALQASOUMI *et al.*, 2009).

Rocket is the general name attributed to the species belonging to *Eruca* and *Diplotaxis* genera, such as *Eruca sativa* Mill. and *Diplotaxis tenuifolia* L., which are also known as "salad rocket" and "wild rocket", respectively.

The content of polyphenols can be influenced by various factors such as the following: variety, climatic conditions, biotic and abiotic stress caused by the preharvest and postharvest conditions (SOUSA *et al.*, 2008; SELMA *et al.*, 2010). JIN and co-workers (2009) established that there are substantial differences between the *Eruca* and *Diplotaxis* genera, and they suggested that *Eruca sativa* (cultivated rocket) may have a greater potential for a positive impact on human health than *Diplotaxis tenuifolia* (wild rocket).

Tissues in the rocket species studied to date contain significant levels of polyglycosylated flavonoids. Metabolite profiling of flavonoids has revealed that *Eruca vesicaria* leaves contain kaempferol derivatives as the principal compounds and that *Diplotaxis tenuifolia* accumulate quercetin derivatives (MARTINEZ-SANCHEZ *et al.*, 2007).

The characterisation of polyphenols and other polar compounds from rocket salad has been successfully carried out by RP-HPLC with spectrophotometric detection (SELMA *et al.*, 2010; MARTINEZ-SANCHEZ *et al.*, 2006; ARABBI *et al.*, 2004; HUBER *et al.*, 2009) coupled with MS (MARTINEZ-SANCHEZ *et al.*, 2007; MARTINEZ-SANCHEZ *et al.*, 2008; BENNETT *et al.*, 2006; DEGL'INNOOCENTI *et al.*, 2008; HEIMLER *et al.*, 2007; WECKERLE *et al.*, 2001). Nevertheless, to the best of our knowledge, MEKC has not been previously applied to rocket salad analysis.

In recent years, considerable interest has been generated in capillary electrophoresis (HPCE). This method is applicable in the analysis of a wide range of compounds, including natural products and plant metabolites. Moreover, among other electrophoretic methods, HPCE has demonstrated high efficiency and resolution to separate phenolic antioxidants. As compared with LC methods, capillary electrophoresis methods have some advantages, such as a high speed of analysis, use of small sample volumes and less production of waste (DELGADO-ZAMARREÑO *et al.*, 2007).

The main objective of the present study was to compare the polyphenol compounds in *Eruca sativa* L. (cultivated rocket) and *Diplotaxis tenuifolia* L. (wild rocket), which are used as salad ingredients. In this study, a capillary zone electrophoretic method with UV detection was developed and validated for quantitative analysis of *Eruca* and *Diplotaxis* flavonoids. A comparison between MEKC and HPLC was also carried out, which has not been previously reported for these vegetables.

MATERIALS AND METHODS

Plant material

Ten rocket salad accessions, including five *Eruca sativa* L. (cultivated rocket) and five *Diplotaxis tenuifolia* L. (wild rocket), were evaluated. The plants were grown in the open field in the spring of 2008 at the Martorano 5 experimental farm (near Cesena in Northern Italy) in four-row, unreplicated plots. Mature leaves were harvested at the beginning of the flowering time from the central two rows of the plot. Leaf samples were immediately frozen in encoded plastic bags at -20°C and then freeze-dried (Thermo Heto PowerDry Lyolab 3000; Waltham, USA). After drying, the samples were ground to a fine powder in a blender mixer (Ika-Werke M20; Staufen, Germany). A subsample was used for the determination of dry matter.

Reagents and chemicals

Unless otherwise stated, solvents were of analytical grade from Merck (Darmstad, Germany). The rutin standard, SDS and sodium tetraborate were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Phenolic compounds extraction

To collect the phenolic compounds, the optimised extraction protocol by MARTINEZ SANCHEZ *et al.* (2007) was used. Briefly, the freeze-dried sample (0.25 g) was homogenised with 2.5 mL of methanol/water (*v:v*) using an Ultra-Turrax homogeniser, and the mixture was then sonicated at 40°C for 20 minutes. The extract was then centrifuged (10,500 g) for 5 min, and the supernatant was filtered through a 0.45 μm membrane filter (Pall Life Sciences, Ann Arbor, MI, USA). Each extraction was replicated three times (n=3), and the extracts were stored at -18°C until analysis.

MEKC analysis

Uncoated fused silica tubing (50 μm ID x 375 μm OD; Beckman, Fullerton, CA, USA) was used.

The effective capillary length was 40 cm. UV-DAD detection was performed at 330 nm. The optimum separation condition of MEKC was achieved using a 40 mM sodium tetraborate buffer (pH 9.0) containing 15 mM SDS. A voltage of 25 kV with normal polarity was applied for 10 min, and the capillary temperature was maintained at 28°C. The capillary tube was conditioned prior to use with 1 M sodium hydroxide (5 min), 0.1 M sodium hydroxide (5 min), HPCE grade water (5 min) and running buffer (5 min). Before runs, the capillary was rinsed with 0.1 M NaOH for 2 min followed by HPCE grade water for 2 min and running buffer for 2 min. Standards and samples were injected hydrodynamically for 3.0 s at the anodic end in a low-pressure mode (0.5 psi). After each electrophoretic cycle, the capillary was rinsed with HPCE grade water (2 min). All washing steps were performed at the same temperature of the run. The running buffer was changed after three runs. Calibration curves of rutin ranged from 1 to 1,000 μg/mL. The capillary electrophoretic analysis was repeated three times for each extract (n=3) and for each calibration point (n=3). Data were collected with the System Gold Software data system (Beckman Coulter, Fullerton, CA).

HPLC-DAD-MS analysis

The RP-HPLC analysis was performed by a HP 1100 Series instrument with UV-diode array absorption and mass spectrometry detections (UV-DAD/MSD). Integration and data elaboration were performed using Chemstation software (Hewlett Packard).

A C18 Luna column (5 μm; 250 x 3.00 mm ID; Phenomenex, Torrance, CA, USA) with a C18 precolumn filter was used. All solvents were HPLC grade and filtered with a 0.45 μm filter disk. A gradient elution was performed using the following solvent system: mobile phase A composed of water/formic acid (99.9:0.1; v/v); and mobile phase B composed of methanol. The gradient program (flow of 0.5 mL/min) was as follows: from 0 to 30 min with 20% B; from 30 to 50 min with 50% B; from 50 to 52 min with 80% B; from 52 to 60 min with 95% B; and a post run for 5 minutes. The chromatograms were registered at 330 nm. The injection volume was 10 μL. All analyses were performed at room temperature. MS analyses were performed using an electrospray ionisation (ESI) interface using both positive and negative ionisation at the following conditions: drying gas flow (N_2) of 11.0 L/min; nebuliser pressure of 60 psi; gas drying temperature of 350°C; capillary voltage of 4000 V; fragmentor voltage; and scan range variables. The fragmentor voltage and m/z ranges used for HPLC-ESI/MSD analyses were 80 V and m/z 50-1350 in negative mode, respectively. The calibration curve of rutin ranged from 1 to 1,000 μg/mL at six concentration levels, and the curve was constructed by plotting peak area versus analyte concentration. The HPLC analysis was repeated three times for each extract (n=3) and for each calibration point (n=3).

Statistical analysis

The results represent the averages of three repetitions (n=3). Tukey's honest significant difference multiple comparison (one-way ANOVA) and Pearson correlations were performed using Statistica 6.0 software (2001, StatSoft, Tulsa, OK, USA).

RESULTS AND DISCUSSION

Optimisation of the micellar electrokinetic chromatography method

Advantages of capillary electrophoresis, such as high efficiency, low cost, short analysis time and easy implementation, result in its wide applications for analysis of phytochemical bioactive compounds. However, several aspects, including sample preparation, separation, and detection, have significant effects on the electrophoretic analysis. Therefore, optimisation of these procedures is necessary to establish the method. Separation factors, including buffer type, buffer concentration, buffer pH, additives, voltage and temperature, were considered to optimise the capillary electrophoresis analysis on phenolic compounds in rocket salad.

A wide variety of buffers can be employed in capillary electrophoresis methods. In this study, tetraborate and phosphate buffers were used.

Solutions of sodium tetraborate and phosphate (100 mM; pH 9) were used to separate the phenolic compounds in *Eruca* extracts. The separation was performed at 28°C and 28 kV, and registration of the electropherogram was performed at 330 nm. Phosphate buffer showed unsatisfactory results concerning separation and noise, which confirmed the different selectivity of a complexing tetraborate buffer as compared to a noncomplexing phosphate electrophoretic buffer as reported by MORIN and DREUX (1993).

The influence of buffer ionic strength was evaluated at a constant pH with increasing tetraborate concentrations from 10 to 100 mM. Fig. 1 shows the effect of buffer ionic strength on peak resolution. The migration time increased when the tetraborate concentration increased. This result may be due to the strong interaction between the tetraborate buffer and molecules containing polyhydroxyl groups, which impart a charge on neutral carbohydrates resulting in their migration in the electric field (LI *et al.*, 2006). The compromise between the time of analysis and peak separation was obtained with a tetraborate concentration of 40 mM.

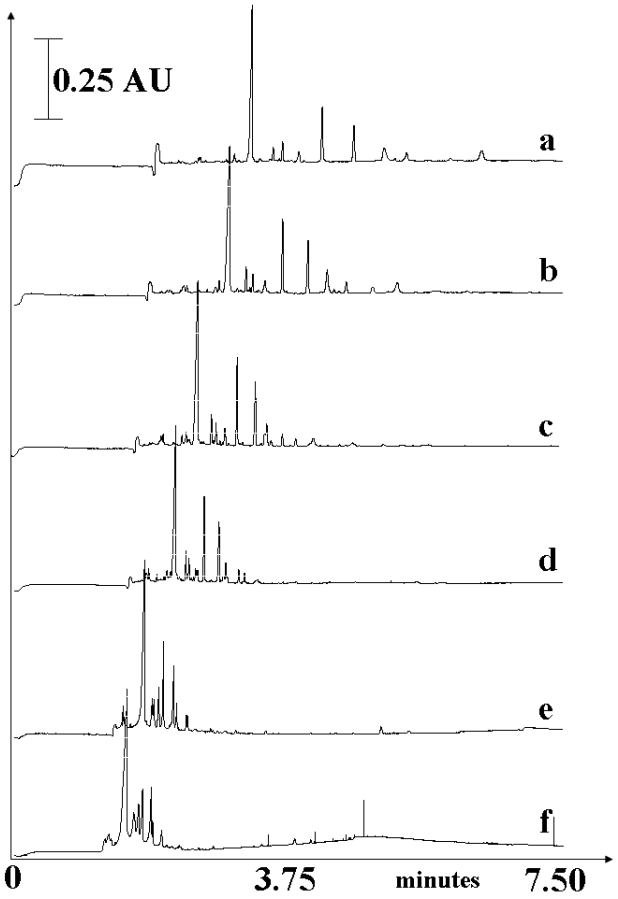

Fig. 1 - Effect of tetraborate concentrations on the capillary electrophoresis separation of *Eruca* phenolic compounds. Running buffer: (a) 100 mM sodium tetraborate, (b) 80 mM sodium tetraborate, (c) 60 mM sodium tetraborate, (d) 40 mM sodium tetraborate, (e) 20 mM sodium tetraborate, (f) 10 mM sodium tetraborate.

Recently, micellar electrokinetic chromatography (MEKC) has been successfully used to separate quercetin and quercetin glycoside (DADAKOVA and KALINOVA, 2010). For this, optimum separation has been achieved using a 40 mM sodium tetraborate solution containing SDS as a surfactant. Fig. 2 shows the effect of SDS on electrophoretic separation. When SDS was added to the tetraborate buffer, the peak resolution between 2.1 and 2.7 minutes increased. The best separation was achieved with a SDS concentration of 15 mM.

In addition, the effect of phosphate in the tetraborate/SDS buffer was evaluated to improve separation quality, but no peak resolution was found with the addition of phosphate.

The effect of pH was tested by adjusting the buffer (40 mM sodium tetraborate with 15 mM SDS) pH between 8.0 and 10.0. Sodium hydroxide and hydrochloric acid solutions were used to modify the pH level. As shown in Fig. 3, the peak migration times increased when the buffer pH was increased as a result of the higher ionisation state of the phenols and increased ionic strength, which caused a lower electroosmotic flow. The best peak resolution was reached using a buffer with a pH level of 9.0.

Voltage and temperature were changed from 20 to 30 kV and from 25° to 35°C, respectively. When the applied voltage and temperature were increased, peak migration times decreased as a result of the positive effect of these two parameters on electrophoretic mobility. A voltage of 25 kV and a temperature of 28°C were selected as optimal conditions.

Validation of the MECK method

Repeatability was assessed for the *Eruca* extract (both undiluted and 10-fold diluted) as reported by BONOLI *et al.* (2004). Both samples were injected 10 times on the same day (intraday precision; n = 10) and on three consecutive days (interday precision; n = 30). The relative standard deviations of the peak areas and migration times were determined for each electrophoretic peak detected. Intraday repeatability of the migration times was 0.25-0.59% for the undiluted extract and 0.37-0.68% for the 10-fold diluted extract. Interday repeatability was 0.58-0.70% for the undiluted extract and 0.67-0.86% for the 10-fold diluted extract. Intraday repeata-

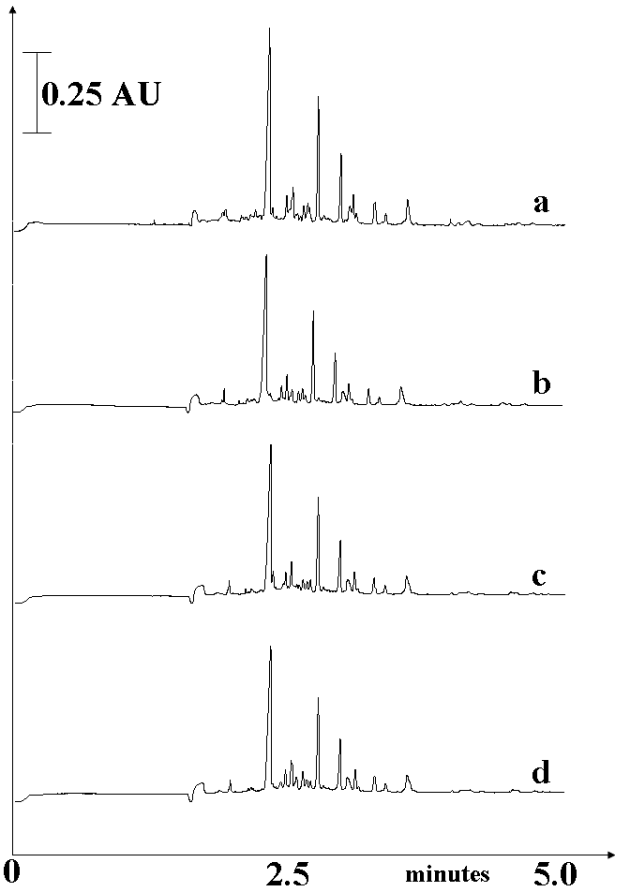

Fig. 2 - Effect of SDS concentrations on the capillary electrophoresis separation of *Eruca* phenolic compounds. Running buffer: 40 mM sodium tetraborate with (a) 0 mM SDS, (b) 5 mM SDS, (c) 10 mM SDS, (d) 15 mM SDS.

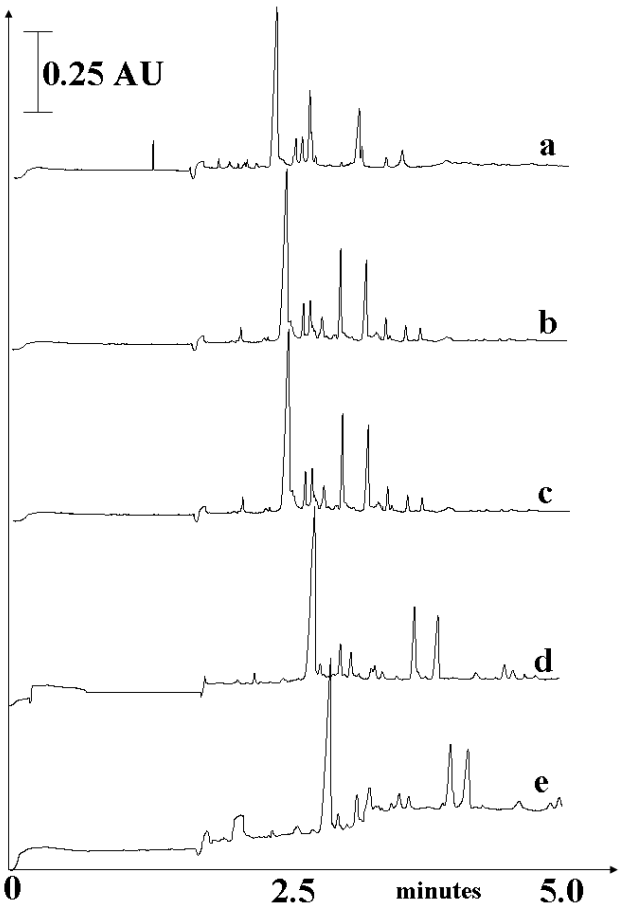

Fig. 3 - Electrophoretic separation of *Eruca* sample with 40 mM sodium tetraborate + 15 mM SDS buffer at different pH values: (a) 8.0, (b) 8.5, (c) 9.0, (d) 9.5, (e) 10.

bility of the total peak area was 4.31 and 7.08% for the undiluted extract and the 10-fold diluted extract, respectively. Interday repeatability of the total peak area was 6.65 and 8.92% for the undiluted extract and the 10-fold diluted extract, respectively. The limit of detection (LOD), which corresponds to a signal-to-noise ratio (S/N) of approximately 3, and the limit of quantification (LOQ), which corresponds to a S/N value of approximately 10, were evaluated using a rutin standard. At 330 nm, rutin had LOD and LOQ values of 0.2 and 0.6 μg/mL, respectively.

Determination of phenolic compounds in rocket salads

The determination of phenolic compounds in rocket salad samples was carried out under optimum conditions obtained for the MEKC method and with HPLC-DAD-MS according to MARTINEZ-SANCHEZ *et al.* (2007).

HPLC characterisation of the phenolic compounds was based on chromatographic behaviour, mass spectra obtained under electron spray ionisation (ESI) conditions and comparison with reference compounds and scientific publications. Moreover, the UV spectra obtained by MEKC separation were compared with UV and MS data obtained with HPLC-DAD-MS. The naturally occurring phenolic compounds were quantified as rutin equivalents because commercial standards were not available for these compounds. Fig. 4 shows the electropherogram (A) and chromatogram (B) of an *Eruca* sample. According to MARTINEZ SANCHEZ and co-workers (2007), the HPLC-MS data demonstrate that the *Eruca* samples were characterised by the presence of kaempferol derivatives (Table 1 and Fig. 4). Moreover, the UV spectra obtained by HPLC were compared with the UV spectra of compounds separated by MEKC. The peaks that were taken into account had an UV spectrum with two principal maxima in the range of 251-269 and 329-348 nm. According to OLSEN *et al.* (2009), these compounds are considered derivatives of kaempferol, quercetin and isorhamnetin.

The HPLC-DAD-MS results demonstrated that four of the eight compounds identified in the

Table 1 - Characterisation of phenolic compounds (expressed as mg of rutin/kg d.w.) in methanol extracts of *Eruca*. Different letters in the same line means significantly different values ($p < 0.05$).

Peak HPLC	Eruca 1	Eruca 2	Eruca 3	Eruca 4	Eruca 5
1	0.71 ^{b}	0.60 ^{c}	0.77 ^{b}	0.58 ^{c}	1.55 ^{a}
2	0.23 ^{d}	1.02 ^{c}	2.81 ^{a,b}	3.50 ^{a}	1.26 ^{c}
3	103.10 ^{d}	128.28 ^{c}	174.54 ^{b}	129.26 ^{c}	230.90 ^{a}
4	21.06 ^{c}	17.66 ^{d}	38.17 ^{b}	21.17 ^{c}	47.45 ^{a}
5	3.43 ^{b}	3.08 ^{c}	3.81 ^{b}	3.61 ^{b}	8.68 ^{a}
6	1.40 ^{c}	1.94 ^{b}	1.12 ^{c,d}	2.00 ^{b}	2.55 ^{a}
7	4.10 ^{c}	4.66 ^{c}	8.51 ^{a}	9.21 ^{a}	7.12 ^{b}
8	4.68 ^{c}	7.23 ^{b}	4.66 ^{c}	3.33 ^{d}	14.33 ^{a}
HPLC total phenols	138.70 ^{d}	164.47 ^{c}	234.41 ^{b}	172.67 ^{c}	313.86 ^{a}
MEKC total phenols	146.59 ^{d}	171.61 ^{c}	252.87 ^{b}	183.44 ^{c}	316.90 ^{a}

Compounds: **1**, kaempferol-3-diglucoside-7-glucoside, **2**, Q-3-(Fer/Fer-triGlc)-7-Rhamn, **3**, kaempferol-3,4'-diglucoside; **4**, isorhamnetin-3,4'-di-glucoside; **5**, quercetin-3-glucoside; **6**, kaempferol-3-glucoside; **7**, isorhamnetin-3-glucoside; **8**, kaempferol- 3-(2-sinapoyl-glucoside)-4'-glucoside.

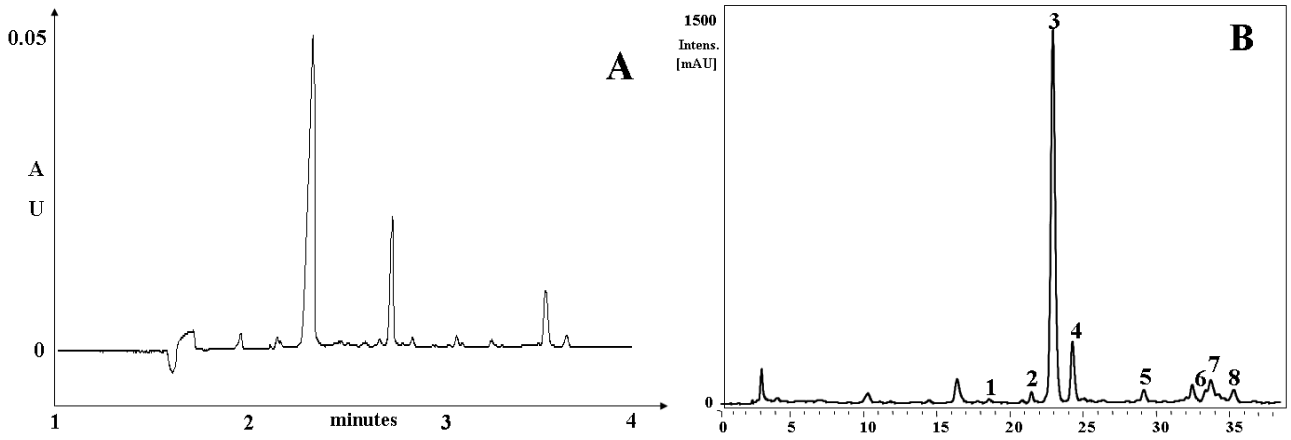

Fig. 4 - Electrophoretic (A) and chromatographic (B) profile of the hydroalcholic extracts of *Eruca*. Compounds: **1**, kaempferol-3-diglucoside-7-glucoside, **2**, Q-3-(Fer/Fer-triGlc)-7-Rhamn, **3**, kaempferol-3,4'-diglucoside; **4**, isorhamnetin-3,4'-diglucoside; **5**, quercetin-3-glucoside; **6**, kaempferol-3-glucoside; **7**, isorhamnetin-3-glucoside; **8**, kaempferol- 3-(2-sinapoyl-glucoside)-4'-glucoside.

Eruca samples were glycosylated acylated flavonoids derived from kaempferol and that these compounds represented 77.2 to 83.9% of the total flavonoid content.

Other compounds were identified as isorhamnetin derivatives (13.6-19.9%) and quercetin derivatives (2.5-4.1%). Table 1 shows that kaempferol-3,4'-diglucoside was the principal component in all *Eruca* accessions and that its content ranged from 103 to 231 mg of rutin/kg dry weight (dw). The second most abundant component was isorhamnetin-3,4'-diglucoside, and its content was in the range of 17.7–47.4 mg of rutin/kg dw. The total flavonoid content varied from 139 to 314 mg of rutin/kg dw with an average of 205 mg of rutin/kg dw. These results showed a high variability among the samples, and the flavonoid content data had a coefficient of variation equal to 34.3. The quantification of phenolic compounds by MEKC resulted in a flavonoid content in the range of 147-317 mg of rutin/kg dw with an average of 214 mg of rutin/kg dw and a coefficient of variation of 32.5.

An analysis of the *Diplotaxis* samples by HPLC-DAD-MS demonstrated that quercetin and quercetin derivatives were the principal components in these samples. The electropherogram and chromatogram of a methanolic extract from a *Diplotaxis* sample are shown in Fig.s 5A and 5B.

The total content of flavonoids in the *Diplotaxis* samples, as determined by HPLC, varied between 47 and 198 mg of rutin/kg dw with a mean value of 123 mg of rutin/kg dw (Table 2).

Table 2 - Characterisation of phenolic compounds (expressed as mg of rutin/kg d.w.) in methanol extracts of Diplotaxis. Different letters in the same line means significantly different values ($p < 0.05$).

Peak HPLC	Diplotaxis 1	Diplotaxis 2	Diplotaxis 3	Diplotaxis 4	Diplotaxis 5
9	12.29 ^{d}	46.48 ^{b}	58.09 ^{a}	11.78 ^{d}	31.03 ^{c}
10	1.24 ^{a}	1.11 ^{a,b}	0.09 ^{d}	0.40 ^{c}	1.32 ^{a}
11	16.61 ^{a}	8.89 ^{b}	5.09 ^{c}	5.36 ^{c}	7.72 ^{b}
4	10.83 ^{b}	13.44 ^{b}	22.08 ^{a}	4.21 ^{d}	6.49 ^{c}
12	21.13 ^{b}	54.23 ^{a}	11.54 ^{c}	14.59 ^{c}	46.06 ^{a}
13	7.56 ^{a}	4.79 ^{b}	0.09 ^{e}	1.20 ^{d}	3.07 ^{c}
14	3.13 ^{a}	1.51 ^{b}	1.11 ^{b}	0.90 ^{c}	3.20 ^{a}
15	15.14 ^{b}	25.46 ^{b}	80.33 ^{a}	6.39 ^{c}	13.94 ^{b}
16	5.38 ^{c}	9.45 ^{b}	18.40 ^{a}	1.88 ^{d}	2.00 ^{d}
17	1.47 ^{a}	1.42 ^{a}	1.26 ^{a}	0.09 ^{b}	0.09 ^{b}
Sum HPLC	90.66 ^{d}	176.69 ^{a,b}	198.10 ^{a}	46.82 ^{e}	114.92 ^{c}
Sum MEKC	103.45 ^{d}	199.34 ^{b}	211.66 ^{a}	58.99 ^{e}	126.45 ^{c}

Compounds: **9**, quercetin-3,3',4'-triglucoside; **10**, quercetin-3,4'-di-glucoside-3'-(6-methoxycaffeoyl-glucoside); **11**, quercetin-3,4'-di-glucoside-3'-(6-caffeoylglucoside); **4**, isorhamnetin-3,4'-di-glucoside; **12**, quercetin-3,4'-di-glucoside-3'-(6-sinapoyl-glucoside); **13**, quercetin-3,4'-di-glucoside-3'-(6-feruloyl-glucoside); **14**, quercetin-3,4'-di-glucoside-3'-(6-p-coumaroyl-glucoside); **15**, quercetin-3-(2-sinapoyl-glucoside)-3'-(6-sinapoyl-glucoside)-4'-glucoside; **16**, quercetin-3-(2-feruloyl-glucoside)-3'-(6-sinapoyl-glucoside)-4'-glucoside; **17**, quercetin-3-(2-feruloyl-glucoside)-3'-(6-feruloyl-glucoside)-4'-glucoside.

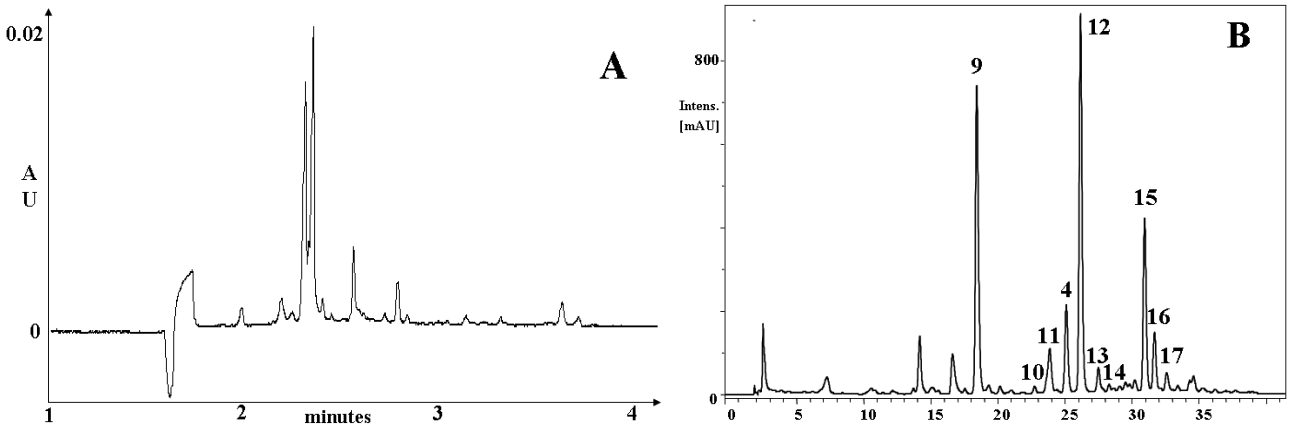

Fig. 5 - Electrophoretic (A) and chromatographic (B) profile of the hydroalcholic extracts of *Diplotaxis*. Compounds: **9**, quercetin-3,3',4'-triglucoside; **10**, quercetin-3,4'-di-glucoside-3'-(6-methoxycaffeoyl-glucoside); **11**, quercetin-3,4'-di-glucoside-3'-(6-caffeoylglucoside); **4**, isorhamnetin-3,4'-di-glucoside; **12**, quercetin-3,4'-di-glucoside-3'-(6-sinapoyl-glucoside); **13**, quercetin-3,4'-di-glucoside-3'-(6-feruloyl-glucoside); **14**, quercetin-3,4'-di-glucoside-3'-(6-p-coumaroyl-glucoside); **15**, quercetin-3-(2-sinapoyl-glucoside)-3'-(6-sinapoyl-glucoside)-4'-glucoside; **16**, quercetin-3-(2-feruloyl-glucoside)-3'-(6-sinapoyl-glucoside)-4'-glucoside; **17**, quercetin-3-(2-feruloyl-glucoside)-3'-(6-feruloyl-glucoside)-4'-glucoside.

A high variability of flavonoid contents among the *Eruca* samples was also found (coefficient of variation equal to 49.5). Quercetin derivatives represented 88.0-94.3% of the total flavonoid content. The remaining compound was identified to be isorhamnetin-3,4'-diglucoside, which was the only compound in common with the *Eruca* samples.

To quantify the flavonoids by MEKC, the same approach utilised for the *Eruca* samples was used. The MEKC analysis confirmed the data obtained by HPLC-DAD-MS. According to the MEKC analysis, the flavonoid content was in the range of 59-212 mg of rutin/kg dw (coefficient of variation 46.2).

The positive correlation found between the MEKC and HPLC methods ($r^2 = 0.9887$; $p < 0.001$) confirmed the good selection of phenolic compounds quantified by the two analytical techniques. Moreover, the positive correlations also confirmed the good agreement between these two techniques, which gave results with the same order of magnitude.

CONCLUSIONS

A MEKC method was established allowing the quantification of flavonoids in *Eruca* and *Diplotaxis* samples in less than 5 min. To the best of our knowledge, this is the first report showing capillary electrophoresis determination of flavonoids in rocket salads. This method provided good repeatability for the same day and between different days, and it positively correlated with the HPLC method. As reported by MARTINEZ SANCHEZ and co-workers (2007), the data obtained by HPLC-DAD-MS demonstrate that *Eruca* samples were characterised by the presence of kaempferol derivatives and that *Diplotaxis* samples contained mostly quercetin derivatives. Moreover, significant differences were found between the samples.

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PAPER

DETERMINATION OF ANTIOXIDANT CAPACITIES AND CHEMICAL CHARACTERISTICS OF POMEGRANATE (*PUNICA GRANATUM* L.) GROWN IN THE SIIRT DISTRICT

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ABSTRACT

In this study, we measured the concentrations of organic acids, phenolic compounds, sugars and vitamin C as well as the total antioxidant capacities of pomegranate genotype cultivars grown in the Siirt province of Turkey. Our analysis of organic acids found citric acid concentrations between 0.66-2.75 g/L and malic acid concentrations between 0.61-3.88 g/L. Our analysis of phenolic compounds found catechin concentrations between 0.88-2.96 g/L and chlorogenic acid concentrations between 0.04-0.43 g/L. Further analyses revealed fructose concentrations between 4.76-11.74 g/100 g and glucose concentrations between 2.98-8.43 g/100 g. In addition, the concentrations of vitamin C and the total antioxidant capacities contents ranged between 11.40-45.07 mg/L and 4.56-16.64 mmol/L, respectively.

- Key words: pomegranate, organic acids, phenolic compounds, vitamin C, antioxidant capacity, HPLC -

INTRODUCTION

The pomegranate (*Punica granatum* L.) is an ancient fruit that has been utilized for diverse purposes. Because climatic factors are a determinant of its cultivation, global pomegranate production is insufficient to meet market demand. The interest in and demand for this fruit have been continuously growing for centuries.

Pomegranate is a highly valuable fruit in human nutrition due to the strong antioxidant effects of its flavonoids. Pomegranate juice and oil were reported to extend longevity and to prevent cardiovascular diseases and cancer (LANSKY *et al.*, 1998). The recent reports of anti-cancer effects for some flavonoids have increased demand for fruits containing anthocyanidin and anthocyanin (TOSUN and ARTIK, 1998). Antioxidants and phenolic compounds have the ability to neutralize free radicals, which are molecules created in the body as part of normal cellular metabolic activities that are short-lived but have very harmful effects (ÖZGEN *et al.*, 2006). As a result of this ability, it is essential to determine the phenolic compound content and antioxidant capacities of fruits and vegetables. This knowledge will contribute to clinical studies of some specific types of cancer (AMES *et al.*, 1993; STEİNMETZ and POTTER, 1996; KAUR and KAPOOR, 2001).

As their salts are alkaline forming elements, fruits are very important in human nutrition (SCHOBİNGER, 1988; SAVRAN, 1999). Organic acids are important sources of respiratory energy in fruits. The most common organic acids in fruits are malic acid, citric acid and tartaric acid. The most abundant acid in many fruits is either citric acid or malic acid, which is followed by phenolic acids (CEMEROĞLU *et al.*, 2004).

Recent studies have determined the chemical compounds in pomegranate juice (POYRAZOĞLU *et al.*, 2002; ÖZGEN *et al.*, 2008; ÇAM *et al.*, 2009; EKŞİ and ÖZHAMAMCI, 2009). These studies were mainly conducted in Mediterranean, Aegean and Marmara regions. In the Southeast Anatolian region, preferable selection and pomological studies were performed (KAZANKAYA *et al.*, 2003; MURADOĞLU *et al.*, 2006; GÜNDOĞDU *et al.*, 2010). Because the study region of Southeast Anatolia is associated with very rich biological diversity and fruit genotypes and with abundant microclimates due to its topographic features, the region has a high potential for pomegranate cultivation. This study was conducted in Siirt, a province of this region to determine the antioxidant capacities and concentrations of organic acids, phenolic compounds, vitamin C and sugar in juice extracted from local pomegranate genotypes. This is the first such study conducted in Siirt province.

MATERIAL AND METHOD

Preparation of fruit samples

The pomegranate cultivation areas in Siirt province were determined during harvest time of pomegranate fruits. Ten fruit samples were collected from this province because they represent the local cultivars. These samples were placed in cloth bags and then transferred to the laboratory. Pomegranates were stripped from the skin and membranes and then granulated. The granules were pressed manually in cheesecloth to obtain their juice. The juice was stored at -20°C until analysis.

Analysis of vitamin C

Samples of 5 mL pomegranate juice were added to 5 mL 2.5% M phosphoric acid. The mixtures were centrifuged at 6,500 x g for 10 min at 4°C. One-half milliliter of clear supernatant was taken from each centrifuge tube and supplemented with 2.5% M phosphoric acid solution to prepare a 10 mL mixture. This mixture was filtered through a Teflon filter and injected into an HPLC instrument. HPLC analyses of vitamin C were performed in a C_{18} column (Phenomenex Luna C_{18}, 250 x 4.60 mm, 5 µ) with the column oven temperature set at 25°C. For the mobile phase, ultra-pure distilled water (adjusted to a pH of 2.2 with H_2SO_4) was used at a 1-mL/min flow rate. Quantitations were performed with a diode array detector (DAD) at a wavelength of 254 nm. For the identification of peaks and amounts of vitamin C, a series of L-ascorbic acid (Sigma A5960) of concentrations (50, 100, 500, 1,000, and 2,000 ppm) were used (CEMEROĞLU, 2007).

Analysis of organic acids

The standards used in organic acid analyses (tartaric acid, succinic acid, malic acid, oxalic acid, lactic acid, fumaric acid and acetic acid) were obtained from Sigma (St. Louis, MO, USA), and chromatographically pure H_2SO_4 was supplied by Merck (Darmstadt, Germany). Milli-Q (Bedford, MA, USA) deionized water was used for the preparation of standards and samples. The method of BEVILACQUA and CALIFANO (1989) was modified and used to extract organic acids. Mixtures containing 5 mL pomegranate juice and 20 mL 0.009 N H_2SO_4 homogenized (Heidolph Silent Crusher M, Almanya). The mixtures were blended by a shaker (Heidolph Unimax 1010, Germany) for 1 hour and then were centrifuged for 15 min at 15,000 rpm. The supernatants were filtered first through filter paper and then twice through a 0.45 µm membrane filter (Millipore Millex-HV Hydrophilic PVDF, Millipore, USA) before being passed through a SEP-PAK C_{18} cartridge.

An Aminex HPX - 87 H, 300 mm x 7.8 mm

column (Bio-Rad Laboratories, Richmond, CA, USA) was used in the HPLC system, and the instrument was controlled by a PC with Agilent software. The DAD detector in the system (Agilent. USA) was set at wavelengths of 214 and 280 nm. The mobile phase was 0.009 N H_2SO_4 that had been filtered through a 0.45 μm membrane filter.

Analysis of sugars

Sugars were analyzed using a method modified from MELGAREJO *et al.* (2000) With standards of fructose, glucose, sucrose and maltose. Pomegranates were stripped of their skin and membranes and then granulated. The granules were pressed manually in cheesecloth to obtain their juice. The juice was centrifuged for 2 min at 12,000 rpm and passed through a SEP-PAK C_{18} cartridge. The supernatant was stored at -20°C until analysis. The sugar levels in the filtered pomegranate juice were quantitated using a μbondapak-NH_2 column with 85% acetonitrile liquid as the mobile phase in an HPLC instrument with refractive index detector. Concentration calculations were based on externally provided standards.

Trolox equivalent antioxidant capacity (TEAC)

For a standard TEAC measurement, the ABTS reagent was dissolved in acetate buffer and prepared with potassium persulfate (OZGEN *et al.*, 2006). For preserving stability, ABTS was diluted in an acidic medium of 20 mM sodium acetate buffer (pH 4.5) to an absorbance of 0.700±0.01 at 734 nm. For spectrophotometric measurements, 3 mL $ABTS^+$ solution was mixed with 20 μL fruit extract, incubated for 10 min and absorbance values were measured at 734 nm.

Analysis of phenolic compounds

We analyzed the phenolic compounds of gallic acid, protocatechuic acid, catechin, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, 0-coumaric acid, phloridzin, vanillic acid, rutin, syringic acid and quercetin.

Phenolic compounds were separated by HPLC using the method described by RODRIGUEZ-DELGADO *et al.* (2001). The pomegranate juices were diluted 1:1 with distilled water and centrifuged for 15 min at 15,000 rpm. The supernatant was then filtered through 0.45 μm Millipore filters and injected into an HPLC. Chromatographic separation was performed in an Agilent 1100 (Agilent, USA) HPLC pump equipped with a DAD detector (Agilent. USA) and an ODS column (250x4.6 mm, 4 μm; HiChrom, USA). Solvent A [methanol:acetic acid:water (10:2:88)] and Solvent B [methanol:acetic acid:water (90:2:8)] were used as the mobile phase and a gradient elution program was applied as presented in Table 1. Separation was achieved at 254 nm and 280 nm; flow rate and injection volume were 1 mL/min and 20 μL, respectively.

Table 1 - Gradient elution program.

Time (min)	Solvent A (%)	Solvent B (%)
0	100	0
15	85	15
25	50	50
35	15	85
45	0	100

Statistical analysis

Statistical analysis was conducted using the program SPSS11.0 (www. Spss.com). One-way analysis of variance was used to compare the organic and phenolic acids concentrations in pomegranates of various fruit genotypes. Statistical differences between organic and phenolic acids concentrations of the pomegranate genotypes were estimated using Duncan's multiple range test.

RESULTS AND DISCUSSION

The organic acid concentrations of juices from the pomegranate genotypes grown in Siirt province were measured in this study. Concentration ranges for each of the organic acids we measured were as follows: 0.18-1.19 g/L oxalic acid, 0.66-2.75 g/L citric acid, 0.61-3.88 g/L malic acid, 0.03-0.16 g/L succinic acid and 0.01-0.14 g/L tartaric acid. Whereas some cultivars had no fumaric acid, others had concentrations between 0.01-0.02 mg/L. These findings are similar to those reported MELGAREJO *et al.* (2000), who found fumaric acid in some cultivars but not in others. In their study conducted in different pomegranate cultivation areas in Turkey, POYRAZOĞLU *et al.* (2002) pomegranate juice was reported to contain the following: 0.33-8.96 g/L citric acid, 0.56-6.86 g/L malic acid, 0.28-2.83 g/L tartaric acid, 0.02-6.72 g/L oxalic acid and 0.00-1.54 g/L succinic acid. The findings of our study are in agreement with the findings of other researchers. Similar findings were also reported by a number of researchers (MELGAREJO *et al.*, 2000; ÖZGEN *et al.*, 2008; TEZCAN *et al.*, 2009). Analysis of the organic acid profiles of juice from the various pomegranate genotypes identified citric acid as the predominant organic acid. The citric acid concentration was followed by malic acid, oxalic acid, succinic acid, tartaric acid and fumaric acid (Tables 2 and 3).

Analysis of the phenolic compounds in juice from the various pomegranate genotypes gave the following concentrations: 0.26-3.21 g/L gallic acid, 0.88-2.96 g/L catechin, 0.04-0.43 g/L

Table 2 - Organic acid concentrations of juice from Siirt province pomegranate genotypes.

Genotype	Oxalic acid (g/L)	Citric acid (g/L)	Malic acid (g/L)
56-01	0.75±0.05 cd*	2.75±0.00 a*	2.12±0.08 ab*
56-02	1.07±0.01 a	1.92±0.00 def	1.37±0.06 b
56-03	0.68±0.07 de	2.05±0.04 cd	1.48±0.71 b
56-04	0.80±0.08 bc	1.88±0.13 efg	1.38±0.09 b
56-05	0.29±0.07 fg	1.17±0.07 kl	1.07±0.01 b
56-06	0.80±0.06 bc	2.61±0.02 a	3.88±0.53 a
56-07	0.60±0.08 cde	2.18±0.07 c	0.97±0.08 b
56-08	0.31±0.01 fg	1.96±0.00 de	1.47±0.07 b
56-09	0.78±0.06 bc	2.73±0.06 a	0.70±0.08 b
56-10	0.29±0.07 fg	1.74±0.01 gh	1.12±0.09 b
56-11	0.37±0.07 f	1.79±0.04 fg	0.61±0.01 b
56-12	0.30±0.01 fg	1.54±0.02 ij	0.91±0.10 b
56-13	0.37±0.10 f	0.88±0.14 m	1.27±0.10 b
56-14	0.59±0.06 e	1.10±0.01 kl	1.06±0.04 b
56-15	0.18±0.11 g	0.66±0.04 n	0.96±0.02 b
56-16	0.29±0.09 fg	0.96±0.08 lm	1.07±0.06 b
56-17	0.38±0.02 f	1.64±0.11 hı	0.72±0.04 b
56-18	0.90±0.03 b	2.36±0.06 b	0.71±0.06 b
56-19	0.29±0.03 fg	1.49±0.09 j	1.14±0.05 b
56-20	1.19±0.05 a	2.11±0.03 c	0.73±0.07 b
56-21	0.34±0.06 f	0.93±0.93 m	0.73±0.00 b

* Different letters in columns indicate significantly different values at $p \leq 0.05$.

Table 3 - Organic acid concentrations of juice from Siirt province pomegranate genotypes.

Genotype	Succinic acid (g/L)	Tartaric acid (g/L)	Fumaric acid (mg/L)
56-01	0.11±0.01 cd*	0.02±0.00 m*	0.02±0.00 ab*
56-02	0.03±0.00 h	0.03±0.00 k	0.01±0.00 c
56-03	0.12±0.00 cd	0.05±0.00 f	0.01±0.00 bc
56-04	0.08±0.08 e	0.01±0.00 p	0.01±0.00 bc
56-05	0.10±0.00 d	0.04±0.00 h	
56-06	0.15±0.01 ab	0.06±0.00 d	
56-07	0.14±0.01 b	0.04±0.00 h	
56-08	0.05±0.00 fg	0.05±0.00 f	
56-09	0.12±0.01 cd	0.01±0.00 n	0.01±0.00 abc
56-10	0.11±0.00 d	0.14±0.00 a	
56-11	0.10±0.00 d	0.09±0.00 b	0.01±0.04 bc
56-12	0.10±0.00 d	0.07±0.00 c	0.01±0.00 bc
56-13	0.05±0.00 fgh	0.01±0.00 n	0.01±0.00 bc
56-14	0.06±0.00 ef	0.07±0.00 c	
56-15	0.04±0.00 gh	0.03±0.00 j	
56-16	0.06±0.00 ef	0.02±0.00 l	
56-17	0.11±0.00 cd	0.05±0.00 g	0.01±0.02 abc
56-18	0.13±0.00 cd	0.06±0.00 e	
56-19	0.16±0.01 a	0.04±0.00 i	
56-20	0.12±0.00 cd	0.05±0.00 g	0.02±0.00 a
56-21	0.03±0.00 gh	0.02±0.00 l	

* Different letters in columns indicate significantly different values at $p \leq 0.05$.

chlorogenic acid, 0.01-0.06 g/L caffeic acid, 0.01-0.53 g/L syringic acid, 0.03-0.17 g/L p-coumaric acid, 0.02-0.13 g/L ferulic acid, 0.03-0.16 g/L o-coumaric acid, 0.03-0.96 g/L phloridzin, 0.02-0.23 g/L protocatechuic acid, 0.00-0.04 g/L vanillic acid, 0.10-0.66 g/L rutin and 0.17-0.81 g/L quercetin. The differences between the phenolic compound contents of examined genotypes were statistically significant (Tables 4, 5 and 6). Analysis of the phenolic profiles of the various pomegranate genotypes showed that catechin the most abundant phenolic compound, followed by gallic acid, quercetin and rutin. Low amounts of caffeic acid, vanillic acid and ferulic acid were measured in the study. In their study conducted in different pomegranate cultivation areas in Turkey, POYRAZOĞLU *et al.* (2002) reported the following concentration ranges: 0.03-30.86 g/L gallic acid, 0.12-2.09 g/L protocatechuic acid, 0.13-8.44 g/L catechin, 0.00-4.72 g/L chlorogenic acid, 0.08-2.89 g/L caffeic acid, 0.02-0.21 g/L p-coumaric acid, 0.01-0.06 g/L ferulic acid, 0.03-0.30 g/L 0-coumaric acid, 0.03-4.93 g/L phloridzin and 0.23-5.30 g/L quercetin. The findings of our study are in agreement with the findings of other researchers. Phenolic compounds that have an important role in the fruit juice processing industry are also effective in blurring and sedimenting drinks such as fruit juices and wines (CEMEROĞLU *et al.*, 2004).

Fructose, glucose and sucrose concentrations of the various pomegranate genotypes were also determined in this study. Fructose and glucose concentrations ranged between 4.76-11.74 g/100 g, and 2.98-8.43 g/100 g, respectively. Sucrose, on the other hand, was only found in genotypes 56-02, 56-05, 56-13 and 56-14 and could not be found in the remaining genotypes (Table 7). MELGAREJO *et al.* (2000) also reported not finding sucrose in some genotypes. In another study of the sugar concentrations of pomegranate juice, fructose ranged between 3.50-5.96% and glucose ranged between 3.40-6.40% (FADAVİ *et al.*, 2005). Our findings are in agreement with those of other studies. Sugar analysis identified fructose as the most abundant sugars, and glucose and fructose comprise most of the total sugar in fruits. Monosaccharides have an important role in terms of their utilization in the food processing technology (CEMEROĞLU *et al.*, 2004).

The vitamin C contents of the various genotypes ranged between 11.40-45.07 mg/L and total antioxidant capacities were between 4.56-16.64 mmol TE/L (Table 7). The vitamin C concentrations of the juices 10 pomegranate genotypes were studied in Iran and ranged between 0.09-0.40 mg/100 g (FADAVİ *et al.*, 2005). The vitamin C concentrations identified in our study are higher. Vitamin C is highly sensitive to such factors as temperature and light and degrades quickly (CEMEROĞLU *et al.*, 2004). Hence, the differences in the vitamin C concentrations of

Table 4 - Phenolic acid concentrations of juice from Siirt province pomegranate genotypes.

Genotype	Gallic acid(g/L)	Catechin (g/L)	Chlorogenic acid (g/L)	Caffeic acid (g/L)
56-01	0.36±0.00 d*	0.96±0.00 o*	0.05±0.00 k*	0.04±0.00 g*
56-02	0.76±0.00 cd	2.42±0.00 c	0.34±0.01 b	0.03±0.00 j
56-03	0.26±0.00 d	1.61±0.00 i	0.04±0.00 k	0.02±0.00 k
56-04	0.39±0.00 d	1.77±0.00 g	0.05±0.00 k	0.04±0.00 f
56-05	0.46±0.00 d	1.14±0.00 n	0.13±0.00 gh	0.02±0.00 l
56-06	0.42±0.00 d	2.21±0.00 d	0.11±0.00 hi	0.05±0.00 d
56-07	0.56±0.00 d	1.47±0.00 k	0.21±0.00 e	0.03±0.00 h
56-08	0.44±0.00 d	1.61±0.00 i	0.14±0.00 g	0.06±0.00 a
56-09	1.33±0.00 bc	1.15±0.00 mn	0.07±0.00 j	0.02±0.00 k
56-10	0.70±0.00 cd	1.94±0.01 f	0.10±0.00 i	0.01±0.00 m
56-11	0.32±0.00 d	1.12±0.00 n	0.07±0.00 j	0.06±0.00 b
56-12	0.58±0.00 d	2.75±0.00 b	0.17±0.00 f	0.06±0.00 b
56-13	0.35±0.00 d	1.73±0.00 h	0.11±0.00 i	0.03±0.00 i
56-14	0.44±0.00 d	1.55±0.00 j	0.08±0.00 j	0.01±0.00 m
56-15	0.53±0.00 d	1.16±0.00 m	0.18±0.00 f	0.01±0.00 n
56-16	0.63±0.00 d	1.34±0.01 l	0.29±0.00 c	0.06±0.00 c
56-17	0.50±0.00 d	0.88±0.00 p	0.43±0.00 a	0.06±0.00 ab
56-18	1.51±0.00 b	2.96±0.00 a	0.26±0.00 d	0.02±0.00 k
56-19	0.62±0.00 d	1.47±0.00 jk	0.05±0.00 k	0.01±0.00 m
56-20	0.43±0.00 d	2.12±0.02 e	0.12±0.00 hi	0.04±0.00 e
56-21	3.21±0.00 a	1.53±0.00 j	0.14±0.00 g	0.01±0.00 o

* Different letters in columns indicate significantly different values at $p \leq 0.05$.

the various genotypes are likely to be the result of degradation. Analysis of the antioxidant capacities of pomegranate juice examined eight pomegranate genotypes. TEAC measurements showed the highest antioxidant capacity in genotype I8 (418.3 mg/100 mL) the lowest in genotype Zivzik (221.1 mg/100 mL) (ÇAM *et al.*, 2009). MOUSAVİNEJAD *et al.* (2009) studied the antioxidant capacities of juice from eight pomegranate genotypes grown in Iran. Their TEAC measurements revealed antioxidant capacities of juices between 18.6-42.8 mM. Our results are within this range.

Pomegranate is an important fruit for human

Table 5 - Phenolic acid content of juice from Siirt province pomegranate genotypes.

Genotype	Syringic acid (g/L)	P-coumaric acid (g/L)	Ferulic acid (g/L)	O-coumaric acid (g/L)
56-01	0,04±0.00 d*	0,03±0,00 k*	0,07±0,00 e*	0,07±0,00 l*
56-02	0,02±0,00 j	0,17±0,00 a	0,13±0,00 a	0,09±0,00 j
56-03	0,01±0,00 k	0,06±0,00 g	0,04±0,00 fg	0,08±0,00 k
56-04	0,04±0,00 g	0,03±0,00 k	0,04±0,00 f	0,03±0,00 p
56-05	0,02±0,00 j	0,10±0,00 e	0,08±0,00 de	0,11±0,00 g
56-06	0,04±0,00 g	0,11±0,00 d	0,09±0,00 cd	0,11±0,00 f
56-07	0,01±0,00 l	0,06±0,00 h	0,11±0,00 bc	0,05±0,00 n
56-08	0,07±0,00 d	0,10±0,00 e	0,11±0,00 ab	0,14±0,00 b
56-09	0,03±0,00 i	0,05±0,00 i	0,09±0,00 cd	0,12±0,00 e
56-10	0,02±0,00 j	0,09±0,00 f	0,02±0,00 h	0,06±0,00 m
56-11	0,06±0,00 e	0,03±0,00 k	0,09±0,00 cd	0,05±0,00 n
56-12	0,09±0,00c	0,13±0,00 c	0,10±0,00 bcd	0,13±0,00 d
56-13	0,03±0,00 i	0,10±0,00 e	0,08±0,00 de	0,13±0,00 cd
56-14	0,03±0,00 i	0,05±0,00 i	0,09±0,00 cd	0,10±0,00 h
56-15	0,02±0,00 j	0,05±0,00 i	0,09±0,00 cd	0,03±0,00 n
56-16	0,10±0,00 b	0,12±0,00 d	0,10±0,00 bcd	0,13±0,00 c
56-17	0,53±0,00 a	0,05±0,00 i	0,10±0,00 bcd	0,12±0,00 d
56-18	0,04±0,00 g	0,14±0,00 b	0,10±0,00 bcd	0,16±0,00 a
56-19	0,03±0,00 h	0,06±0,00 g	0,03±0,00 fgh	0,03±0,00 n
56-20	0,05±0,00 f	0,03±0,00 k	0,09±0,00 cd	0,12±0,00 e
56-21	0,05±0,00 f	0,04±0,00 j	0,02±0,00 gh	0,10±0,00 i

* Different letters in columns indicate significantly different values at $p \leq 0.05$.

Table 6 - Phenolic acid content of juice from Siirt province pomegranate genotypes.

Genotype	Phloridzin (g/L)	Protocatechuic acid (g/L)	Vanillic acid (g/L)	Rutin (g/L)	Quercetin (g/L)
56-01	0,07±0,00 def*	0,02±0,00 kl*	0,01±0,00 efg*	0,28±0,00 f*	0,30±0,00 h*
56-02	0,04±0,00 ef	0,04±0,00 fgh	0,01±0,00 ef	0,65±0,00 a	0,81±0,00 a
56-03	0,05±0,00 ef	0,07±0,00 d	0,01±0,00 efgh	0,17±0,00 j	0,23±0,00 k
56-04	0,13±0,00 cdef	0,03±0,00 ijk	0,02±0,00 cd	0,10±0,00 k	0,21±0,00 k
56-05	0,11±0,00 def	0,04±0,00 fg	0,00±0,00 hi	0,23±0,00 h	0,33±0,00 g
56-06	0,13±0,00 cdef	0,03±0,00 ij	0,02±0,00 c	0,23±0,00 h	0,38±0,00 f
56-07	0,16±0,00 cdef	0,06±0,00 e	0,01±0,00 efg	0,30±0,00 f	0,28±0,00 i
56-08	0,20±0,00 cd	0,16±0,00 b	0,02±0,00 cd	0,39±0,00 d	0,17±0,00 m
56-09	0,14±0,00 cdef	0,03±0,00 hi	0,02±0,00 cd	0,26±0,00 g	0,30±0,00 h
56-10	0,21±0,01 cd	0,05±0,00 e	0,01±0,00 ghi	0,47±0,00 c	0,25±0,00 j
56-11	0,03±0,00 f	0,05±0,00 f	0,02±0,00 d	0,29±0,00 f	0,39±0,00 f
56-12	0,17±0,00 cde	0,05±0,00 fg	0,04±0,00 a	0,53±0,00 b	0,73±0,00 b
56-13	0,16±0,00 cdef	0,02±0,00 l	0,01±0,00 efg	0,35±0,00 e	0,24±0,00 j
56-14	0,13±0,01 def	0,04±0,00 fgh	0,00±0,00 ij	0,23±0,00 h	0,55±0,00 c
56-15	0,03±0,00 f	0,04±0,00 gh	0,00±0,00 j	0,18±0,00 i	0,52±0,00 d
56-16	0,26±0,00 c	0,07±0,00 d	0,01±0,00 fgh	0,29±0,00 f	0,19±0,00 l
56-17	0,16±0,00 cdef	0,23±0,00 a	0,03±0,00 b	0,29±0,00 f	0,47±0,00 e
56-18	0,49±0,00 b	0,15±0,00 b	0,01±0,00 fgh	0,47±0,00 c	0,48±0,00 e
56-19	0,03±0,00 f	0,08±0,00 c	0,01±0,00 e	0,48±0,00 c	0,56±0,00 c
56-20	0,13±0,00 cdef	0,03±0,00 jkl	0,01±0,00 efgh	0,23±0,01 h	0,55±0,00 c
56-21	0,96±0,00 a	0,03±0,00 ij	0,02±0,00 cd	0,66±0,01 a	0,32±0,00 g

* Different letters in columns indicate significantly different values at $p \leq 0.05$.

Table 7 - The sugar, vitamin C and TEAC concentrations of juice from Siirt province Pomegranate genotypes.

Genotype	Fructose (g/100 g)	Glucose (g/100 g)	Sucrose (g/100 g)	Vitamin C (mg/L)	TEAC (mmol/L)
56-01	5,81±0,23 jk	3,51±0,07 i		32,15±0,72 cd	13,79±0,04 b
56-02	8,17±0,40 de	5,39±0,06 de	0,07±0,00 b	11,40±1,02 k	14,23±0,00 b
56-03	6,42±0,12 ij	4,30±0,03 h		12,90±0,21 jk	11,56±0,15 f
56-04	10,02±0,90 b	6,90±0,64 b		15,86±0,27 j	10,25±0,67 g
56-05	7,55±0,14 efg	4,88±0,28 efg	0,09±0,00 b	11,91±0,16 k	13,27±0,04 bcd
56-06	6,80±0,23 ghi	4,52±0,21 gh		13,25±0,37 jk	12,70±0,43 cde
56-07	7,60±0,04 efgh	4,83±0,14 fg		27,31±0,00 ef	4,56±0,78 k
56-08	7,07±0,16 ghi	5,17±0,19 def		29,60±1,23 de	12,19±0,18 ef
56-09	5,20±0,16 kl	3,25±0,27 ij		35,55±1,27 b	12,54±0,15 def
56-10	6,74±0,04 hi	5,19±0,18 def		36,46±0,60 b	16,64±0,48 a
56-11	8,78±0,18 cd	6,23±0,26 c		35,41±0,74 b	6,23±0,02 j
56-12	7,39±0,05 efgh	5,28±0,08 def		24,24±1,68 fgh	7,23±0,64 i
56-13	9,27±0,25 bc	6,12±0,16 c	0,20±0,01 a	23,00±1,22 gh	6,10±0,48 j
56-14	11,74±0,93 a	8,43±0,15 a	0,09±0,00 b	19,40±1,18 i	12,02±0,41 ef
56-15	5,09±0,33 kl	2,98±0,05 j		11,95±0,38 k	13,62±0,54 bc
56-16	7,64±0,04 efgh	5,62±0,27 d		24,70±0,11 fg	12,76±0,04 cde
56-17	4,76±0,50 l	2,90±0,30 j		35,03±2,08 bc	12,19±0,80 k
56-18	7,25±0,24 fghi	5,34±0,20 def		25,66±1,42 fg	9,71±0,09 gh
56-19	7,68±0,28 efg	6,21±0,12 c		21,10±2,33 hi	8,77±0,06 h
56-20	7,24±0,48 fghi	5,12±0,06 def		45,07±3,26 a	9,80±0,76 g
56-21	8,05±0,09 def	6,17±0,21 c		12,55±1,71 k	9,40±0,26 gh

* Different letters in columns indicate significantly different values at $p \leq 0.05$.

health and nutrition (BEŞİKCİ and ARIOĞLU, 2010). In this study, we analyzed the chemical characteristics of various pomegranate genotypes grown in Siirt province, which is one of Turkey's most important pomegranate cultivation areas. Comprehensive chemical analyses conducted on this fruit enhance the value of this study. Furthermore, this is the first study to report the chemical characteristics of these local pomegranate genotypes, including the concentrations of phenolic compounds, organic acids, and sugars and total antioxidant capacities.

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PAPER

DETECTION OF COW'S MILK, FAT OR WHEY IN EWE AND BUFFALO RICOTTA BY HPLC DETERMINATION OF β-CAROTENE

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ABSTRACT

The addition of cow's milk, fat or whey to ewe or buffalo ricotta is a common form of fraud in the dairy industry because of its economic convenience. β-carotene may serve as an excellent indicator of the presence of cow's milk in ewe or buffalo ricotta because it has been noted that β-carotene is naturally present in cow's milk alone. Therefore, a simple and sensitive isocratic reversed-phase HPLC method has been developed for the determination of β-carotene in ricotta. This method involves fat saponification followed by extraction with a mixture of organic solvents. A linear correlation between the concentration of β-carotene and the area response has been obtained over the concentration range of 0.045 to 0.900 μg/mL, which corresponds to about 1 to 22 μg/100 g in ricotta. The detection limit was 0.7 ng of injected sample, and the recovery evaluated in spiked ricotta samples was always higher than 90%.

- Key words: ricotta, whey, β-carotene, fraud, HPLC -

INTRODUCTION

"Ricotta" is a high-moisture soft dairy product that is traditionally prepared by heating (85°-90°C) and acidifying whey with a suitable acidulant (lactic, acetic or citric acid). Due to its nutritional and dietetic characteristics, it is considered a precious food and plays an important role in the modern diet. Ricottas produced from both ewe and buffalo cheese wheys are particularly appreciated by consumers. Both these products have been registered as Protected Designation of Origin (PDO) products, as "Ricotta Romana" and "Ricotta di Bufala Campana," respectively. Thus, products carrying the PDO label must conform to the specifications outlined by the European Commission (EU COMMISSION, 2006). To increase its yield and to improve its texture and flavour, ricotta is often produced by adding a small amount of milk and/or cream. In manufacturing PDO "Ricotta Romana," up to 15% of ewe's milk can be added to whey, whereas for "Ricotta di Bufala Campana," the addition of whey with up to 6% of milk and up to 5% of cream is permitted, provided that both these ingredients are from buffalo. However, the addition of bovine ingredients (milk, whey or cream) to ewe and buffalo ricotta are motivated by decreased manufacturing costs and greater market availability. However, several issues arise with this type of fraud, such as misleading and deceiving the consumer, unfair competition against manufacturers who operate legitimately, and damage to the consumer perception of food products. To alleviate these issues, the authenticity of ricotta should therefore be controlled. To this end, suitable analytical methods are necessary.

Several analytical methods based on different principles and techniques have been proposed to detect the addition of cow's milk to the milk and cheeses of other species (DE LA FUENTE and JUAREZ, 2005). Some of these are based on the discrimination of specific casein or whey protein fractions by gel electrophoresis (MOIO *et al.*, 1990; IANNIBELLI *et al.*, 1999; CERQUAGLIA *et al.* 2004), by RP-HPLC (PELLEGRINO *et al.*, 1991; PELLEGRINO *et al.*, 1992), by cation exchange HPLC (MAYER *et al.*, 1997) or by new approaches such as polymerase chain reaction (MAFRA *et al.*, 2006). However, the application of the above-mentioned methods can be ineffective when whey and/or cream from cow's milk are added to ewe and buffalo dairy products such as ricotta, as specific whey proteins may be fully denatured during whey heating and the casein content of cream is low. In contrast, the observation that both ewe and buffalo milk do not contain a detectable level of β-carotene may represent a useful principle to identify the presence of cow's milk.

Among milk fat-soluble micronutrients, carotenoids directly influence the antioxidant properties, as well as the colour, of the derived dairy products. For this reason, the influence of both dietary and nondietary factors on β-carotene concentration in cow's milk have been studied in-depth, and wide variability in this component has been observed (NOZIERE *et al.*, 2006a).

Several methods have been reported for the determination of β-carotene in food products, most of which are based on HPLC (MANZI *et al.*, 1996; OLIVER and PALOU, 2000; AKHTAR and BRYAN, 2008) because of the significant advantages that this technique offers in terms of simplicity, sensitivity, specificity and precision. However, as these methods are often intended for the simultaneous determination of more than one molecule, including lutein, lycopene, zeaxanthin, retinol, tocopherols and sterols, they are time consuming. Furthermore, these analyses are expensive because of the amount of solvent required to achieve chromatographic separation. Recently, CHAUVEAU-DURIOT *et al.* (2010) proposed the simultaneous separation of carotenoids, tocopherols and retinol by UPLC. Although their method was tested for forages, bovine plasma and milk, the authors concluded that for the last two products, the relatively few types of carotenoids present make it possible to use a shorter run-time analysis on HPLC.

This work aimed to develop a rapid, inexpensive, robust and accurate analytical HPLC method to detect and quantify β-carotene in ewe and buffalo ricotta cheese. This method is a suitable tool to probe and regulate fraudulent practices in the dairy sector.

MATERIALS AND METHODS

Materials and reagents

HPLC grade methanol and isopropanol; analytical grade *n*-hexane, ethyl acetate, ethyl alcohol 96% and L-Ascorbic acid were purchased from Carlo Erba (Rodano, MI, Italy). Tert-butylated hydroxytoluene (BHT) was obtained from Fluka Co. (Buchs SG, Switzerland). Trans- β-carotene was obtained from Sigma-Aldrich Co. (St. Louis, MO, Usa). EDTA was purchased from USB Co. (Cleveland, OH, Usa).

Commercial samples of fresh ricotta, all from different manufacturers and including both industrial and artisanal products, were as follows: five samples were from ewe, two samples were from buffalo, four samples were from cow, and four samples were from ewe/cow mixtures. One β-carotene-free ricotta was produced under controlled conditions from genuine ewe's milk and used as a blank sample.

Preparation of stock and working standard solutions

A stock solution (0.45 mg/mL) of β-carotene was prepared in hexane containing 0.1% (w/v)

butylhidroxytoluene (BHT) and stored at -20°C in the dark until it was ready for use. The stock solution was diluted with a methanol/isopropanol (50/50, v/v) solution containing 0.1% (w/v) BHT to prepare an intermediate standard solution with an approximate concentration of 4.5 μg/mL. The exact concentration of the intermediate standard solution was determined by measuring the absorbance at 450 nm using a dual-beam Lambda 25 spectrophotometer (Perkin-Elmer Wellesley, MA, Usa) and calculated using the molar extinction coefficient ($\varepsilon_{1\%}$) of 2,592 for β-carotene in hexane (SHARPLESS *et al.*, 1999). Five working standard solutions (0.045, 0.225, 0.450, 0.675 e 0.900 μg/mL) were prepared from the intermediate standard solution with appropriate dilutions in the HPLC mobile phase, and these were injected on three different days to construct the calibration curve of peak area counts versus β-carotene concentrations (μg/mL) by the least squares method.

Sample preparation

Ricotta samples were manipulated in a room that was protected from direct sunlight. Aliquots of 20 g samples (in order to have a sufficient amount of fat), 100 mg BHT, and 50 mg EDTA were directly weighed into a 250-mL round-bottom flask. One hundred millilitres ethanol and 5 mL KOH solution (50% p/v) were added to the round-bottom flask and left to saponify for 30 min under reflux, under a stream of nitrogen. The flask was gently rotated a few times to avoid losing material. After saponification, about 20 mL distilled water was added into the flask through the condenser, and the flask was removed and cooled to room temperature. The contents of the flask were transferred quantitatively into a separatory funnel (500 mL), and the saponified solution was extracted with a 100 mL hexane:ethyl acetate mixture (9:1, v/v) by carefully shaking and rotating the funnel several times. When the layers separated, the lower aqueous-alcoholic layer was collected, and two more extractions were performed with 50 mL of the same mixture. The collected fractions were combined and washed with distilled water until they were at a neutral pH. The neutral extract was filtered through phase separators and evaporated in a rotary evaporator. The residue was carefully dissolved in 5 mL mobile phase and analysed by HPLC.

RP-HPLC analysis

Waters (Rochester, MN, USA) chromatographic equipment was used, including the 600E multisolvent delivery system, a column temperature control unit set at 30°C and a Rheodyne 7010 injector fitted with a 20 μL loop. Separation was carried out on a Supelcosil LC-18 column (250 x 4.6 mm i.d., 5 μm particle size) (Sigma Aldrich, Saint Louis, MO, Usa). The column eluate was monitored at 450 nm using a Waters Model 994 diode array detector. TotalChrom version 6.3.1 software (Perkin-Elmer Wellesley, MA, Usa) was used for instrument control and data acquisition and processing. Isocratic elution was performed with methanol/isopropanol (50/50, v/v) at a flow rate of 1 mL/min, and a run-to-run time of 15 min was adopted.

Method validation

To assess the performance of this method, linearity, precision, limit of detection (LOD), limit of quantification (LOQ), and recovery were determined.

The un-weighted linear regression analysis was applied to the plot of the peak area counts versus β-carotene concentration (0.045, 0.225, 0.450, 0.675, and 0.900 μg/mL). Linearity was checked between 0.045 and 0.900 μg/mL by calculating the correlation coefficient (r) and testing its significance by a t-test. The LOD is considered the lowest concentration of β-carotene that can be detected but not necessarily determined quantitatively, and it is fixed at 3 times the standard deviation of the signal of a solution with a concentration near the blank ($3\sigma_B$) divided by the slope value. The LOQ corresponds to the lowest concentration of β-carotene that can be determined quantitatively with reasonable accuracy, and it is fixed at 10 times the standard deviation of the signal of a solution with a concentration near the blank ($10\sigma_B$) divided by the slope value. Nevertheless, a fundamental assumption of the un-weighted least squares method is that each point on the curve, including the point representing the "blank" signal, has a normally distributed variation (in the y-direction alone) with a standard deviation estimated by σ_{res} (MILLER and MILLER, 2005). It is therefore appropriate to evaluate LOD and LOQ by using the standard deviation of the response estimated through the standard deviation of the y-residuals (σ_{res}) rather than by calculating σ_B, which also saves a considerable amount of time.

To evaluate the recovery, aliquots of β-carotene-free ewe ricotta (four aliquots for each analyte level) were weighed in round-bottom flasks, and the working standard solutions (0.18, 0.30 and 0.45 μg/mL) were added to the sample portions to provide for three levels of analyte (4.50, 7.50 and 11.25 μg/100 g). Then, the entire analytical procedure was applied as described above. The precision of the method was determined by calculating the coefficient of variation (CV) of the four independent analyses used for the recovery study.

RESULTS AND CONCLUSION

The method proposed in the present work is suitable for detecting and quantifying β-carotene content. However, this method cannot accurately quantify the amount of cow's milk added to a sample because the fat content in ricotta samples varies widely. In fact, a variety of raw materials (i.e., whey, cream, milk) are added in ricotta manufacturing; therefore, the fat content of the finished ricotta may vary. According to product specifications, the fat content of "Ricotta Romana DOP" (ewe's milk) should be in the range of 17% to 29% based on dry matter. For "Ricotta di Bufala Campana DOP," the fat content should be higher than 12% based on the product, with a maximum of 75% humidity provided. CONTARINI *et al.* (2002) report average values of 29.8% (SD: 3.02), 35.8% (SD: 10.64) and 32.4% (SD: 5.23) for fat content based on dry matter in ricotta of ewe, buffalo and bovine origin, respectively. Furthermore, the β-carotene content itself may vary widely in cow's milk depending on several factors, including the season, type of feeding, cow breed, stage of lactation, and fat content (NOZIERE *et al.* 2006b; AGABRIEL *et al.*, 2007). Taking these aspects into consideration, our first aim was to find the appropriate sample size for analysis in order to achieve the highest sensitivity permitted by the analytical conditions, regardless of the fat and water content of ricotta. To prevent the oxidation of the characteristic conjugated double bond of the β-carotene molecule, both BHT and EDTA were added to the sample during the saponification and extraction steps, a stream of nitrogen was used, and the whole process was conducted away from direct sunlight.

To optimize the chromatographic conditions, different mobile phases were tested. The methanol/isopropanol system was chosen as the mobile phase because it allowed the β-carotene peak to elute under isocratic conditions in a clean area of the chromatogram. To improve the sharpness of the β-carotene peak, the optimal column temperature was determined by running samples at 20°, 25° and 30°C. The column operating temperature was chosen to be 30°C, and it should be noted that the temperature did not significantly alter the retention time. This finding is in agreement with recent literature (AKHTAR and BRYAN, 2008) reporting that carotenoids do not degrade during HPLC analysis when the column is maintained at 30°C because of the relatively short residence time in the column (retention time 10-11 min).

Representative chromatograms of ewe, buffalo, and cow ricotta are presented in Fig. 1. The β-carotene peak in these samples was identified based on its retention time with respect to the retention time of the pure standard solution of β-carotene. As expected, β-carotene was present only in the samples originating from cows. Thus, β-carotene is an excellent marker to indicate the fraudulent addition of whey, milk or cream of bovine origin. Under the adopted chromatographic conditions, a small peak elutes just after the main peak of β-carotene, both in the standard solution and in ricotta samples. This peak is probably due to light- or heat-induced geometrical isomerization (SCHUEP and SCHIERLE, 1997). Nevertheless, this additional peak is irrelevant for the purposes of this work.

In this study, the linearity of peak area versus β-carotene concentration was examined (Fig. 2).

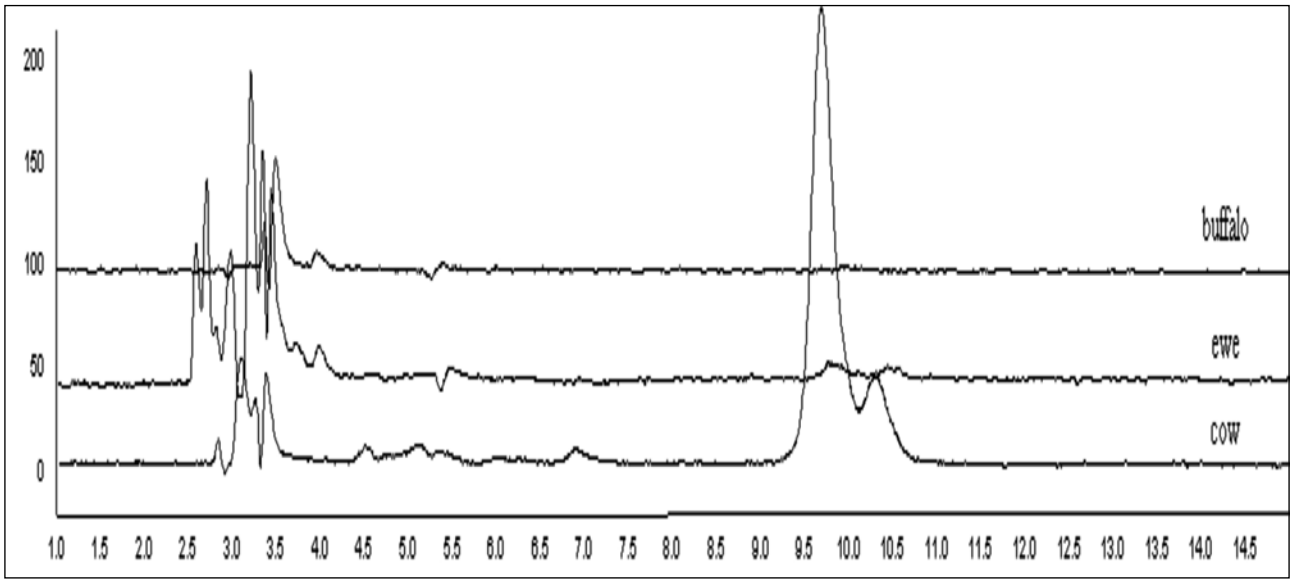

Fig. 1 - HPLC profile of buffalo, ewe and cow ricotta samples analysed with the proposed method.

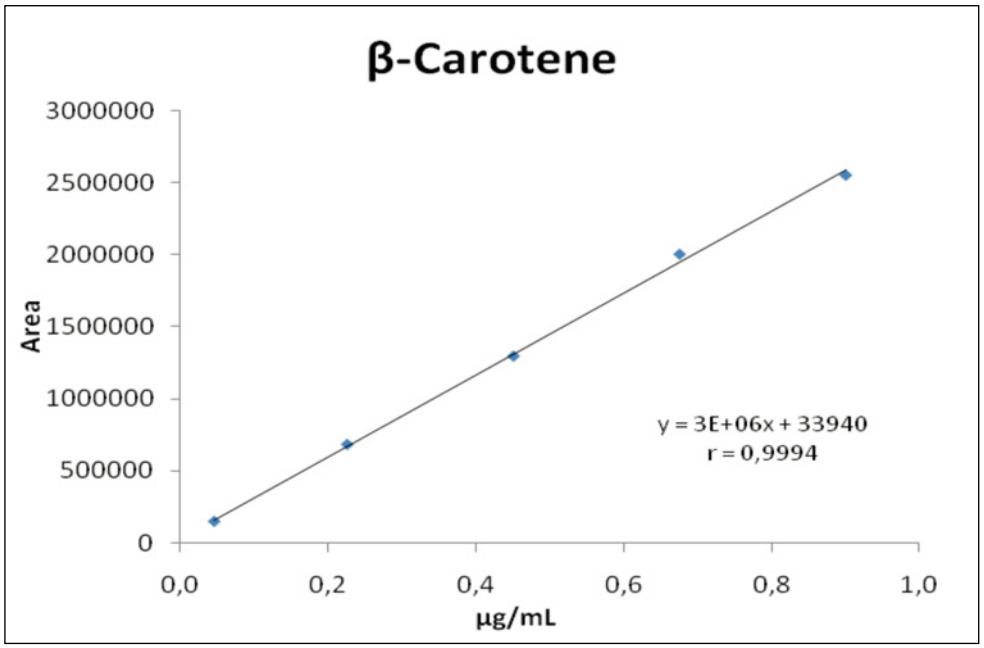

Fig. 2 - Regression line between the concentration and peak area of β-carotene standard solutions.

The correlation coefficient was r = 0.9994 and the calculated value of t (48.9) was greater than the tabulated one (3.18) at a significance level of 0.05 and three degrees of freedom.

The β-carotene concentration in the sample solution (C_s), expressed as μg/100 mL, was calculated by the calibration curve. The analyte concentration in fresh ricotta samples (C_a), as μg/100 g, was calculated using the following equation:

$$C_a = \frac{C_s \times 5 \times 100}{m}$$

where m is the sample mass (g).

The LOD, determined from a working standard solution of β-carotene, was 0.036 μg/mL, corresponding to approximately 0.9 μg/100 g ricotta. The LOQ was 0.12 μg/mL, corresponding to 3.0 μg/100 g ricotta.

These results provide strong evidence that recovery was good at all levels of analyte and ranged from 91 to 93%, proving that the extraction procedure was highly efficient (Table 1). The analytical precision was evaluated by submitting ricotta samples spiked with three different levels of β-carotene to the whole procedure four times on different days. The coefficients of variation were always lower than 2%.

This newly developed method was utilized for the analysis of commercial ewe, buffalo, cow and blended ewe/cow ricotta samples. The content of β-carotene found in cow ricotta ranged from 7 to 40 μg/100 g, whereas the β-carotene content of blended ewe/cow ricotta samples varied from 9 to 15 μg/100 g. No β-carotene was found in the samples labelled as ewe or buffalo ricotta, either PDO or generic samples.

In conclusion, this work presents a new method based on β-carotene determination that allows for the detection of cow's milk, fat or whey in ewe or buffalo ricotta.

The results of the validation procedure show that this method is accurate, sensitive and suitable for the routine determination of β-carotene. Thus, this method is capable of evaluating the quality of both traditional and PDO ricotta types. Furthermore, this method could also be a useful tool for correctly classifying other dairy products such as mozzarella or ripened cheeses made with buffalo or ewe milk. Compared to other methods, this method has the advantages of isocratic elution and a short retention time, which allows for the analysis of a large number of samples in a short period of time.

Because of the high variability of β-carotene content in cow's milk, it is not possible to quantify the amount of bovine ingredients added into ewe or buffalo ricotta manufacturing. Nevertheless, the proposed method has great poten-

Table 1 - Inter-day repeatability of the method and analytical recovery of β-carotene in samples of ewe ricotta spiked with three different levels of the analyte.

Level	Theoretical ug/mL	Found ug/mL	Recovery (%)
1	**0.180**		
Mean		0.166	92.5
CV %		1.9	9.0
2	**0.300**		
Mean		0.277	92.5
CV %		1.9	9.0
3	**0.450**		
Mean		0.408	90.7
CV %		1.4	5.0

tial for adoption by official control laboratories with the aim of eliminating fraud and protecting both consumers and manufacturers from illegal behaviour.

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PAPER

WATER-SOLUBLE VITAMINS FROM CHINESE AND TUNISIAN POMEGRANATE (*PUNICA GRANATUM* L.) FRUITS

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ABSTRACT

Vitamins from twelve pomegranate cultivars (6 from Tunisia and 6 from China) were studied using high-performance liquid chromatography analysis. Six common vitamins found in fruit juices were identified and quantified. Ascorbate contents for all cultivars ranged from 6 to 18 mg per 100 g. Thiamin ranged from 0.08 to 0.24 mg per 100 g, riboflavin from traces to 0.009 mg per 100 g, niacin from 0.21 to 1.32 mg per 100 g, pyridoxin from traces to 0.14 mg per 100 g and cobalamin from traces to 0.039 μg per 100 g. Present findings may be used to guide the content of vitamins included in fruits as supplements for commonly diets.

- Key words*: Punica granatum* L., pomegranate fruit, juices, HPLC, water-soluble vitamins -

INTRODUCTION

Pomegranate (*Punica granatum* L.) as one of the oldest edible fruit is spread around the Mediterranean Basin and eastwards to India and China. Pomegranates are grown in the hanging gardens of Babylon. Ancient Egypt considered this fruit as a symbol of prosperity and ambition (BRAGA *et al.*, 2005). Pomegranate is now cultivated throughout the tropics and subtropics. The Babylonians regarded pomegranate as an agent of resurrection. The Persians considered this species as conferring invincibility on the battlefield and for ancient Chinese the red juice was regarded as a "soul concentrate" homologous to human blood and capable of coffering on a person longevity or even immortality (MADIHASSAN, 1984; OPARA *et al.*, 2009).

About 50% of the total pomegranate fruit weight corresponds to the peel, which is an important source of bioactive compounds such as phenolics, flavonoids, ellagitannins and proanthocyanidin compounds (LI *et al.*, 2006), minerals (MIRDEHGHAN and RAHEMI, 2007), and complex polysaccharides (JAHFAR *et al.*, 2003). The chemical composition of the pomegranate fruits differs depending on the cultivar, growing region, climate, maturity, cultivation practice, and storage conditions (FADAVI *et al.*, 2005). Significant variations in organic acids, phenolic compounds, sugars, water-soluble vitamins, and minerals of pomegranate cultivars have been reported (TEZCAN *et al.*, 2009). The edible pulp may be eaten fresh when scooped or sucked out and the juice can be used for drinks, wine and syrup.

Fruits (juices) and vegetables are known to protect against most aging-related and chronic diseases, such as cardiovascular disorders and cancer. Antioxidants chemicals (polyphenols, flavonoids, vitamins) are supposed to protect against these chronic conditions (LIU *et al.*, 2000).

Mostly, thirteen vitamins are recognized in human nutrition and these may be conveniently classified as either water-soluble (C or B complex) or fat-soluble (A, D, E and K) (Ball, 2006). The water-soluble vitamins comprise vitamin C and members of the vitamin B group, namely thiamin (vitamin B1), riboflavin (vitamin B2), niacin (vitamin B3), pyridoxin (vitamin B6), pantothenic acid (vitamin B5), folate (vitamin B9), and cobalamin (vitamin B12).

Vitamin B1 aids in promoting normal appetite and digestion and maintains a healthy nervous system and prevents irritability. Vitamin B2 aids in absorption of nutrients, maintains healthy skin, tongue and lips. Vitamin C maintains healthy bones, teeth and blood vessels, aids in the development of tissue between cells and promotes healing of wounds and broken bones (ASARD *et al.*, 2004).

It is also known that several B-group vitamins serve as coenzymes for enzymes (BALL, 2006). A typical coenzyme consists of a protein to which the vitamin is attached. The vitamin portion of the coenzyme is usually responsible for the attachment of the enzyme to the substrate. For some B-group vitamins, clinical deficiency results in a biochemical defect, which is manifested as a disease with characteristic symptoms (BALL, 2004). Other vitamins have less dramatic deficiency symptoms in humans, but their deficiency in certain animal species may give rise to distinctive signs. Some human individuals can benefit from vitamin supplements, indicating that they may have been subclinically vitamin deficient (BALL, 2004).

There has been a virtual explosion of interest in the pomegranate as a medicinal and nutritional product because of its multi-functionality and its great benefit in the human diet as it contains several groups of substances that are useful in disease risk reduction. Worldwide, there are many pomegranate cultivars, broadly divided into sweet sour-sweet and sour taste. The aim of this study is to report water-soluble vitamin contents of twelve pomegranate cultivars; six were collected from Tunisian regions and six from Chinese provinces. The studied cultivars are subdivided into sweet, sour-sweet and sour pomegranate groups based on their organoleptic characteristics and chemical compositions. This study gives more value to non-valorised sour cultivars, which may be of both industrial and nutritional interest. The data may be used to guide the content of vitamins included in fruits as supplements for commonly diets.

MATERIALS AND METHODS

Chemicals and reagents

HPLC grade solvents were used. The analytical reagent grade acetonitrile and methanol were obtained from Lab-Scan (Labscan Ltd, Dublin, Ireland). The water used in high-performance liquid chromatography and sampling was prepared with a Millipore Simplicity (Millipore S.A.S., Molsheim, France). All vitamin standards were of chromatography grade quality and were purchased from Sigma Chemical Co. (Poole, Dorset).

Plant material

Twelve (12) pomegranate cultivars were used in this work. Six of them were located in Tunisia (Zerkin, Arram, Tozeur, El-Alia and Testour) and others were from China (Sichuan, Shaanxi and Yunnan provinces). Table 1 shows more information about geographical origin of cultivars. The cultivars used in this study were subdivided into sweet, sour-sweet and sour pomegranate groups based on their organoleptic characteristics and

Table1 - Geographic location of the 12 studied pomegranate cultivars.

	Name	City, Country	Latitude	Longitude
Sweet	Gabsi 3	Zerkine, Tunisia	33°40'N	10°15'E
	Gabsi 2	Arram, Tunisia	33°45'N	10°11'E
	Sichuan 2	Sichuan, China	30°36'N	104°04'E
	Mengzi	Yunnan, China	23°21'N	103°23'E
Sour	Mezzi	Tozeur, Tunisia	33°54'N	8°08'E
	Garsi	Tozeur, Tunisia	33°37'N	10°17'E
	Shaanxi	Shaanxi, China	36°46 'N	112°37 'E
	Dali 1	Yunnan, China	25°35'N	100°16'E
Sour-sweet	Chetoui	El-Alia, Tunisia	37°10'N	10°01'E
	Zehri	Testour, Tunisia	36°33'N	9°25'E
	Sichuan 1	Sichuan, China	30°36'N	104°04'E
	Dali 2	Yunnan, China	25°35'N	100°16'E

chemical compositions. In the same way earlier pomegranate accessions were classified by several authors as sweet, sour–sweet and sour accessions (MARTÍNEZ *et al.*, 2006; MELGAREJO *et al.*, 2000). Pomegranates were randomly picked from three trees of each cultivar at harvest time (fully mature fruits). Fresh pomegranates were stored at room temperature for two weeks until used. To avoid any variation in analysed parameters: hydrosoluble vitamins, pH and acidity were analysed daily from fresh open pomegranate fruit.

Pomegranate juice acidity and pH

The seeds of the fruit containing the intact juice sacs were manual1y separated from the pericarps. The sacs were ruptured by very light agitation in an electric blender for 5-10 s. The resulting juice was then centrifuged at 2,500 rpm for 10 min. The supernatants from the centrifugation step of the pomegranate juice extract were recovered, filtered prior to experimentation. Pomegranate juice acidity was calculated as the percentage of citric acid, by titrating 10 mL pomegranate juice with NaOH 0.1 N solution until pH 8.1 using a Sartorius PB-10 pH meter (Sartorius, Germany).

Pomegranate ascorbic acid analysis

Vitamin C was extracted according to the modified method of ABDULNABI *et al.* (1997). The fresh pomegranate pulps (10 g) were blended and homogenized with an extracting solution containing metaphosphoric acid (0.3 M) and acetic acid (1.4 M). The mixture was placed in a conical flask (wrapped with aluminium foil) and agitated at 10,000 rpm for 15 min, at room temperature. The mixture was then filtered through a Whatman No. 4 filter. Samples were extracted in triplicates.

Ascorbic acid analysis was performed on an Agilent HPLC system. The chromatographic separation was achieved on a RP-HPLC column (Agilent ZORBAX Eclipse Plus Column C18: 250 mm × 4.6 mm i.d., 5 μm), through isocratic delivery of a mobile phase (A/B 33/67): A: 0.1 M potassium acetate (pH= 4.9), B: acetonitrile-water (50:50) at a flow rate of 1 mL/min. UV absorbance was recorded at 254 nm at room temperature.

Ascorbic acid identification was achieved by comparing the retention time of L-ascorbic acid purchased from Sigma (Sigma, Co. Chemical, St. Louis, USA). Ascorbic acid standard was prepared by dissolving 100 mg of L-ascorbic acid in a metaphosphoric acid (0.3 M) – acetic acid (1.4 M) solution at the final concentration of 0.1 mg/mL. The calibration line was divided into linear range based on four concentration levels. Each point was the average of three peak area measurements (r^2=0.99, data not shown).

Pomegranate Vitamin B analysis

Vitamin B group was extracted according to the method described in AOAC International (1990). More details about the vitamin B group extraction procedure were reported by WOOLLARD and INDYK (2002) and later by VIŇAS *et al.* (2003). Briefly blended Pomegranate pulps (10 g) were kept during 30 min at 121°C in 25 mL of H_2SO_4 (0.1 N) solution. Then, contents were cooled and adjusted into pH 4.5 with 2.5 M sodium acetate. Next, 50 mg takadiastase enzyme (Sigma) was added. The preparation was kept one night at 35°C. The mixture was then filtered through a Whatman No. 4 filter. Filtrate was diluted with 50 mL of pure water and filtered again through a micropore filter (0.45 μm). 20 μL of the filtrate was injected onto the HPLC system. The quantification was made by comparison to standards vitamins B. Standard stock solution for thiamin, riboflavin, niacin and pyridoxine were prepared as report-

ed in ASLAM *et al.* (2008). The limit of detection (LOD) and limit of quantification (LOQ) for the analysis were estimated as the signal-to-noise ratio of 3:1 and as the signal-to-noise ratio of 10:1, respectively. In the current study, respective LOD/LOQ values were 0.008/0.026 μg/mL for thiamine, 0.003/0.010 μg/mL for riboflavin, 0.028/0.090 μg/mL for niacin, 0.0012/0.040 μg/mL pyridoxine and 0.0042/0.014 μg/mL for cobalamin.

Vitamins separation condition was reported by BENMOUSSA *et al.* (2003). Chromatographic separation was achieved on a RP-HPLC column (Agilent ZORBAX Eclipse Plus C18: 250 mm × 4.6 mm i.d., 5 μm), through isocratic delivery mobile phase (A/B 33/67): A: MeOH, B: 0.023 M H_3PO_4 pH= 3.54 at a flow rate of 0.5 mL/min. UV absorbance was recorded at 270 nm at room temperature. Typical chromatogram of standards vitamin B and Chromatographic profile of B vitamins from juice of "Shaanxi" pomegranate cultivar were reported in Fig. 1.

Statistical and chemometric methods

All tests were carried out in triplicate and the results were presented as means±SD. Differences at $p < 0.05$ were considered statistically significant. Cultivar values for each compound were compared to the mean of all cultivars by calculating a confidence interval. One-way ANOVA analysis was used for mean differences.

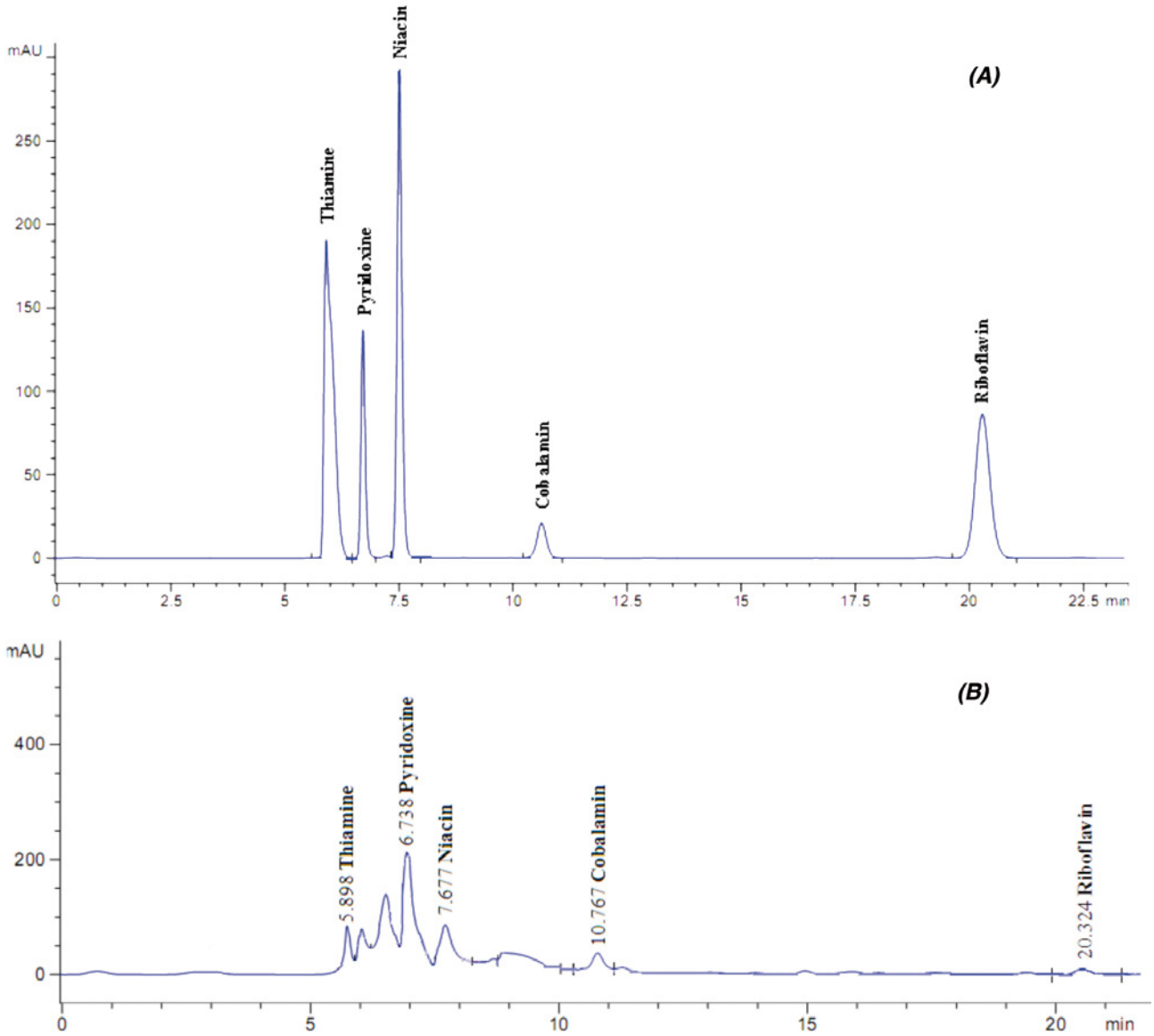

Fig. 1 - Chromatographic profile using RP-HPLC column (Agilent ZORBAX Eclipse Plus C18: 250 mm × 4.6 mm i.d., 5 μm), through isocratic delivery mobile phase: A/B (33/67); A: MeOH, B: 0.023 M H3PO4 pH= 3.54 at a flow rate of 0.5 mL/min. UV absorbance was recorded at 270 nm at room temperature. (**A**) Typical HPLC chromatogram of prepared vitamins B standards. **1**: Thiamin, **2**: Pyridoxin, 3: Niacin, **4**: Cobalamin, 5: Riboflavin. (**B**) Chromatographic profile of B vitamins from juice of "Shaanxi" pomegranate cultivar.

RESULTS AND DISCUSSION

Pomegranate juice acidity and pH

pH values of all studied pomegranate juice are acid (pH < 4.3) and titratable acidity ranged from 0.48 (Zehri cultivar) to 2.13 (Mezzi cultivar). Acidities are respectively 0.66, 0.70 and 1.42 for sweet, sour-sweet and sour cultivars. pH values are 4.17, 4.13 and 3.02 for sweet, sour-sweet and sour groups of pomegranate cultivars respectively (Table 2).

Results indicate distinction of sour group which characterized by low pH value and high acidity (Mezzi, Garsi, Shaanxi and Dali1 cultivars). However there is no difference between sour-sweet and sweet cultivars. In previous study we reported comparable pH and acidity value in Tunisian pomegranate juice (ELFALLEH *et al.*, 2009). Based on pH and titratable acidity CAM *et al.* (2009) reported statistically significant differences between pomegranates from Turkey; these values varied closely with the pomegranate taste.

Pomegranate vitamin contents

Table 2 shows ascorbic acid contents in the studied pomegranate cultivars. Quantitatively ascorbate contents ranged from 9.52 mg/100 g fresh weight basis (FW) (pomegranate sour-sweet group) to 10.85 mg/100 g FW (pomegranate sour group). No significant differences are found based on the *p*-values determined by Duncan test (*p*-value = 0.76) between sour, sweet and sour-sweet groups.

Based on Duncan's multiple range tests significant differences of ascorbic acid contents between cultivars were found. The contents ranged from 6.03 (Zehri, sour-sweet group) to 18.30 mg/100 g (Sichan1, sour-sweet group). Comparable results were reported by LEGUA *et al.* (2000) (4 to 12 mg/100 g). Whereas, results reported by LI *et al.* (2006) and OPARA *et al.* (2009) were significantly higher than our findings. LI *et al.* (2006) reported that in Chinese pomegranate fruit the content of ascorbic acid was 85 mg/100 g fresh weight basis (FW). OPARA *et al.* (2009) reported a significant variation in vitamin C content of pomegranate cultivars from India, Egypt and Oman, ranging from 52.8 to 72.0 mg/100 g FW for arils and 76.8 to 118.4 mg/100 g FW for peels. These differences according the pomegranate in some countries would be explained by i) the genotype and the organoleptic group effects, ii) the geographical sites, and iii) by the interaction genotype and environment, which is in agreement with previous studies (KIM *et al.*, 2007; TLILI *et al.*, 2011). Therefore, it is usually noted that the vitamin C content in the peel was higher.

Ascorbic acid contents of some vegetables and fruits are listed in Table 3. Reported values are typical of the observed concentrations found in these samples, but they can vary greatly and should not be taken as absolute. Indeed, genetic variation, maturity, climate, sunlight, method of harvesting, and storage conditions can affect the levels of vitamin C.

Vitamin C in pomegranate is greatly higher than date palm (0.40 mg/100 g) and is comparable to bananas (8.70 mg/100 g) or tomatoes

Table 2 - pH, acidity and ascorbic acid contents from Tunisian and Chinese pomegranate cultivars.

	Name	pH	Acidity (g/L H_2SO_4)	Ascorbic acid (mg/100 g)
Sweet	Gabsi 3	4.19±0.19 $^{a.b.c}$	0.64±0.18 $^{c.d.e}$	10.13±1.04 ^{d}
	Gabsi 2	4.10±0.24 $^{a.b.c}$	0.66±0.17 $^{c.d.e}$	6.67±0.04 $^{f.g}$
	Sichuan 2	4.30±0.20 $^{a.b}$	0.60±0.16 $^{d.e}$	16.42±0.24 ^{b}
	Mengzi	4.08±0.22 $^{b.c}$	0.75±0.18 $^{c.d.e}$	10.06±0.07 ^{d}
	Mean (Sweet)	**4.17±0.10**	**0.66±0.06**	**10.82±4.07**
Sour	Mezzi	2.58±0.17 ^{f}	2.13±0.28 ^{a}	10.02±0.05 ^{d}
	Garsi	3.48±0.26 ^{d}	0.92±0.21 ^{c}	10.04±0.33 ^{d}
	Shaanxi	2.99±0.53 ^{e}	1.31±0.27 ^{b}	15.25±0.13 ^{c}
	Dali 1	3.05±0.21 ^{e}	1.32±0.21 ^{b}	8.09±0.10 ^{e}
	Mean (Sour)	**3.02±0.37**	**1.42±0.50**	**10.85±3.07**
Sour-sweet	Chetoui	3.88±0.09 ^{c}	0.83±0.35 $^{c.d}$	6.34±0.04 ^{g}
	Zehri	4.54±0.20 ^{a}	0.48±0.09 ^{e}	6.03±0.16 ^{g}
	Sichuan 1	3.98±0.14 $^{b.c}$	0.75±0.14 $^{c.d.e}$	18.30±0.28 ^{a}
	Dali 2	4.11±0.20 $^{a.b.c}$	0.75±0.17 $^{c.d.e}$	7.39±0.08 $^{e.f}$
	Mean (Sour-sweet)	**4.13±0.28**	**0.70±0.15**	**9.52±5.88**

(1) Each value in the table is represented as mean±SD (n=3).

(2) Superscript letters with different letters in the same column of cultivar respectively indicate significant difference (P <0.05) analyzed by Duncan's multiple range test.

Table 3 - Vitamin C and B content of some vegetables and fruits (mg/100 g FW).

	Ascorbic acid	VB_1 (Thiamin)	VB_2 (Riboflavin)	VB_3 (Niacin)	VB_6 (Pyridoxin)
Pomegranate pulp (this study)	10.40	0.163	0.002	0.440	0.093
Bananas*	8.70	0.031	0.073	0.665	0.367
Orange juice*	50.00	0.090	0.030	0.400	0.040
Tomatoes*	9.00	0.041	0.047	1.179	0.056
Grapefruit*	33.30	0.037	0.030	0.269	0.043
Potatoes*	19.70	0.080	0.032	1.054	0.295
dates, "Deglet Noor"*	0.40	0.052	0.066	1.274	0.165

(*) Data shown in this table were taken from the United States Department of Agriculture (USDA, 2010).

(9.00 mg/100 g). Therefore, pomegranate is rich in vitamin C and would replace some fruits consumption.

In addition to vitamin C, vitamin B_1 known as thiamine is also analyzed. Vitamin B_1 plays an essential role in the metabolism of carbohydrates. In pomegranate fruit, thiamin contents ranged from 0.08 (Mengzi, sweet group) to 0.27 mg/100 g (Zehri, sour-sweet group). Duncan test do not show significant differences between groups. The average of thiamin content is 0.163 mg/100 g (Table 4).

Thiamin contents in pomegranate are much higher compared to bananas (0.031 mg/100 g), orange juice (0.090 mg/100 g), tomatoes (0.041mg/100 g), grapefruit (0.037 mg/100 g), potatoes (0.08 mg/100 g) or dates (0.052 mg/100 g) (Table 3).

Pomegranate pulp riboflavin contents ranged from traces to 0.009 mg/100 g (Shaanxi, sour group) averaging 0.002 mg/100 g for both groups sour and sour-sweet and 0.001 mg/100 g for sweet group (Table 4). From the twelve studied cultivars riboflavin content was almost zero in nine cultivars, detected only in three Chinese cultivars (Mengzi, Shaanxi and Dali 2). This content was relatively lower compared to data reported by the United States Department of Agriculture (0.053 mg/100 g of pomegranate pulp) (USDA, 2010). Table 3 shows that highest riboflavin content was found in bananas and date, 0.073 and 0.066 mg/100 g, respectively. Living cells require FMN and FAD as the prosthetic groups of enzymes variety, and hence the flavins were found, at least in small amounts, in all natural unprocessed foods (BALL *et al.*, 2006).

In pomegranate pulp niacin contents were significantly different between cultivars (Table

Table 4 - The vitamin B content of Tunisian and Chinese pomegranate cultivars.

	Name	VB_1 (Thiamin) mg/100 g	VB_2 (Riboflavin) mg/100 g	VB_3 (Niacin) mg/100 g	VB_6 (Pyridoxin) mg/100 g	VB_{12} (Cobalamin) µg/100 g
Sweet	Gabsi 3	0.14±0.11 ^{a}	ND	0.39±0.09 $^{b.c.d}$	0.13±0.00 $^{b.c}$	0.024±0.02 $^{a.b.c}$
	Gabsi 2	0.09±0.00 ^{a}	ND	0.60±0.07 $^{b.c}$	0.14±0.00 ^{b}	0.017±0.12 ^{c}
	Sichuan 2	0.18±0.06 ^{a}	ND	0.28±0.16 $^{c.d}$	0.07±0.03 ^{e}	ND
	Mengzi	0.08±0.01 ^{a}	0.005±0.007 $^{a.b}$	0.25±0.00 $^{c.d}$	0.09±0.00 $^{d.e}$	ND
		0.12±0.04	**0.001±0.003**	**0.38±0.15**	**0.11±0.03**	**0.010±0.012**
Sour	Mezzi	0.21±0.03 ^{a}	ND	0.47±0.23 $^{b.c.d}$	0.09±0.00 $^{d.e}$	0.024±0.08 $^{b.c}$
	Garsi	0.10±0.00 ^{a}	ND	0.65±0.40 ^{b}	ND	0.033±0.10 $^{a.b}$
	Shaanxi	0.19±0.16 ^{a}	0.009±0.00 ^{a}	0.21±0.00 ^{d}	0.11±0.00 $^{c.d}$	0.014±0.00 $^{c.d}$
	Dali 1	0.18±0.09 ^{a}	ND	0.38±0.00 $^{b.c.d}$	ND	ND
		0.17±0.04	**0.002±0.005**	**0.43±0.18**	**0.05±0.06**	**0.018±0.014**
Sour-sweet	Chetoui	0.18±0.00 ^{a}	ND	0.21±0.01 ^{d}	ND	0.014±0.00 ^{c}
	Zehri	0.27±0.00 ^{a}	ND	1.32±0.02 ^{a}	0.24±0.00 ^{a}	0.039±0.00 ^{a}
	Sichuan 1	0.24±0.16 ^{a}	ND	0.21±0.01 ^{d}	0.15±0.00 ^{b}	ND
	Dali 2	0.09±0.00 ^{a}	0.007±0.01 $^{a.b}$	0.28±0.00 $^{c.d}$	0.11±0.00 $^{c.d}$	ND
		0.20±0.08	**0.002±0.004**	**0.51±0.54**	**0.12±0.10**	**0.013±0.018**

(1) Each value in the table is represented as mean±SD (n=3).
(2) Superscript letters with different letters in the same column of cultivar respectively indicate significant difference (P <0.05) analyzed by Duncan's multiple range test.
*Thiamin, riboflavin, niacin and pyridoxin are expressed in mg per 100 g FW, Cobalamin was expressed in µg per 100 g FW.

4). Niacin is the generic descriptor for two vitamins, nicotinic acid and nicotinamide. Niacin contents ranged from 0.21 mg/100 g (Sichuan1, sour-sweet group) to 1.32 mg/100 g (Zehri, sour-sweet group). Theses contents (mean = 0.44 mg/100 g) are comparable to orange juice (0.4 mg/100 g) and greater than grapefruit (0.26 mg/100 g). High niacin values were recorded in date, tomatoes and potatoes with 1.27, 1.17 and 1.05 mg/100 g, respectively.

Vitamin B_6 in pomegranate pulp ranged from traces to 0.24mg/100 g (Zehri, sour-sweet group) Table 4. Sweet (0.11 mg/100 g) and sour-sweet (0.12 mg/100 g) groups have same contents and higher then sour cultivars (0.05 mg/100 g). The vitamin B_6 family includes pyridoxine, pyridoxal, pyridoxamine, and their phosphorylated counterparts; pyridoxine phosphate, pyridoxal phosphate and pyridoxamine phosphate.

Pyridoxin contents from pomegranate pulp are calculated and compared to some fruit and vegetable (Table 3). This value (0.093 mg/100 g) is greater than orange juice (0.04 mg/100 g), tomatoes (0.05 mg/100 g) or grapefruit (0.04 mg/100 g). However highest values were reported in bananas (0.36 mg/100 g) and potatoes (0.29 mg/100 g).

Generally, vitamin B6 is present in all natural unprocessed foods. Yeast extract, wheat bran, and liver containing particularly high concentrations. Other important sources include wholegrain cereals, nuts, pulses, lean meat, fish, kidney, potatoes, and other vegetables (SPINNEKER *et al.*, 2007). Vitamin B6 plays an important role in the development and maintenance of competent immune system. Because of this versatility, vitamin B6 is crucial for normal growth, development, and homeostasis (SPINNEKER *et al.*, 2007; GRIMBLE, 1998).

Cobalamin contents in pomegranate pulp ranged from 0 to 0.039 μg/100 g (ZEHRI, sour-sweet group). Cobalamin contents are 0.010, 0.013 and 0.018 μg/100 g in sweet, sour-sweet and sour cultivars, respectively (Table 4). The present study shows a very low cobalamin contents in pomegranate fruits. Generally the intake of vitamin B12 in the fruit and vegetable is almost zero. Plants do not naturally contain vitamin B12; they may carry some through microbial contamination.

CONCLUSION

The present study reveals that pomegranate fruit is rich with hydro-soluble vitamins particularly, ascorbic acid, thiamin, niacin and pyridoxin. The vitamin content in pomegranate makes it an appropriate source for use in the food industry and reinforces its nutritional value. The cultivar effect is the major factor on vitamin content variation independently of the organoleptic groups (sour, sour-sweet and sweet). Significant differences were observed between cultivars according to Duncan test; nevertheless based on taste (sour, sour-sweet and sweet) no differences were shown between the three groups. This comparable characteristic based on hydro-soluble vitamin content may allow us to think about a new approach for vitamin extraction from non commercial sour and sour-sweet fruit, and to encourage the valorisation of unexploited pomegranate product. The content of vitamins included in fruits may be used as supplements for commonly diets.

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PAPER

PHENOLIC COMPOSITION OF AGLIANICO AND NERO DI TROIA GRAPES AND WINES AS AFFECTED BY COVER CROPPING AND IRRIGATION

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ABSTRACT

The aim of this study was to evaluate over two seasons (2007 and 2008) the effect of cover cropping and irrigation on the phenolic composition of grapes from two red cultivars (Aglianico and Nero di Troia) grown in Southern Italy and of their corresponding wines. Cover cropping led to impoverishment in phenols of grapes and wines of Aglianico and to enrichment of Nero di Troia. For both varieties irrigation deficit led to higher contents of anthocyanins and total polyphenols, indicating that their concentration is closely related to water stress intensity. Some differences were found according to season: Aglianico grapes and wines produced in 2008 were richer in phenols than those produced in 2007, whereas an opposite trend was observed for Nero di Troia. The study demonstrated that the application of modulated water stress to the investigated cultivars allows one to obtain wines very rich in phenolic substances, thus improving the wine quality.

- Key words: Aglianico, anthocyanins, cover cropping, irrigation, Nero di Troia, phenol compounds -

INTRODUCTION

Red wine quality is strongly affected by the phenolic fraction, which is responsible for both the sensory characteristics such as colour, texture and taste (KOSIR *et al.*, 2004), and the multiple biological properties such as antioxidant, anti-inflammatory, anti-atherosclerosis, cardio protective and cancer protective effects (FRESCO *et al.*, 2006; SOLEAS *et al.*, 2006). Phenolic composition depends on the grape used, which, in turn, is greatly affected by ground, agronomic techniques and climate, and on winemaking technology.

Among the agronomic techniques, irrigation management is fundamental in order to obtain the grape quality necessary for wine production. Excess of water could lead to a luxuriant vegetative development and dense canopies that reduce fruit exposure and air movement, resulting in poor grape maturation and high risk of fungal attacks (SMART *et al.*, 1985; ZAHAVI *et al.*, 2001). Further, irrigation causes an increase of wine pH that, associated to a slight decrease in anthocyanin concentration, reduces colour intensity (INTRIGLIOLO and CASTEL, 2008). Conversely, strong water stress could lead to a block of maturation, which reduces yield and diminishes grape quality (MATTHEWS and ANDERSON, 1989). When water stress occurs during the postveraison period, fruit maturity could be delayed, resulting in both restricted sugar accumulation and poor flavour development (SMART and COOMBE, 1983). Regulated deficit irrigation can be applied as a strategy to reduce the possible negative impact of irrigation on grape and wine quality. In fact, moderate water stress increases pH and grape compounds such as soluble solid and phenols due to reduced vegetative growth and decreased berry weight (GINESTAR *et al.*, 1998; MATTHEWS and ANDERSON, 1988; REYNOLDS and NAYLOR, 1994).

Vineyard cover cropping is another agronomic strategy that can spatially and temporally modify the water regime of grapevine due to strong competition for water (CELETTE *et al.*, 2008). Other benefits related to cover cropping are prevention of erosion, easier mechanisation, ground cover, reduction of ground pressure and improvement of soil structure (FOROLUNSO *et al.*, 1992; GEOFFRION, 1999; GEOFFRION, 2000). Aglianico and Nero di Troia are two red grape cultivars, the former native to Basilicata region, Southern Italy, widely cultivated all-over Southern Italy area, and recently also in Australia, the latter native to Apulia region, Southern Italy. Both varieties produce red wines, some of which of controlled appellation of origin, whose organoleptic characteristics improve with aging thus showing the importance of their phenolic fraction content and composition.

The purpose of the present work was the evaluation of the effects exerted by cover cropping and water stress on the phenolic fraction of Aglianico and Nero di Troia grapes and wines, produced in 2007 and 2008 in Apulia region, Italy.

MATERIALS AND METHODS

Plant material

The research was conducted from 2007 to 2008 in two commercial vineyards located in the same farm on a hilly area (200 m above sea level), in the countryside of Minervino Murge (41° 08' 55" N - 16° 02' 32" E) (Apulia region, Southern Italy). The "SO4" rootstock (*Vitis berlandieri* x *Vitis Riparia*) was planted in 2002 and, one year later, *Vitis vinifera* L. Aglianico (a late-maturation grapevine) and Nero di Troia (a medium-late-maturation grapevine) were grafted on it. The plant density was 6,250 vines ha^{-1} (2.0 m x 0.8 m spacing). The rows were oriented NE-SW. The site was characterized by sandy-clay soil (sand 69.7%, clay 24.3%, silt 6%) deep and very homogeneous, with a 0-1% slope. The organic matter content was 0.5%. The vines were trained to a vertical trellis system (one armed cordon) and they were about 4-5 "two-bud" spurs/plant pruned. In this area a Mediterranean climate prevails, with an annual rainfall of about 650 mm, so water irrigation is necessary mainly from May to August. Irrigation is required due to the fact that rain generally falls during the dormant phase of the growing season and water storage in the soil profile is insufficient to meet vineyard evapotranspiration (ETc). According to average estimate for the last 20 years, a value of 2,710±720 $m^3 ha^{-1}$ is the necessary seasonal irrigation volume to counterbalance the evapotraspiration ratio for vineyard, during the whole productive cycle in Apulia. For each *cv*, two different soil managements were considered: soil tillage and permanent sown green using a mixture of essences. Herbicide applications were never used in the field having an organic management. From 2006, the soil was tilled at a depth of 10 cm, twice in a year, using universal vineyard plough: the first tillage was made after the bud breaking and the second after veraison. The cover crop mix was planted in November of 2006 at a rate of 80 kg ha^{-1} and it was mowed only once in May of 2007 due to the low grass vigor caused by the dry climate of the area and sandy soil of the side. The percentages of each species in the mix were *Festuca rubra* L. 20%, *Festuca ovina* L. 20%, and *Trifolium subterraneum* L. 60 %. From 2006 to 2008 in February, in both soil tillage and grass mix field trials, 6-0-0 organic fertilizer was applied. The application rate was 400 kg ha^{-1} yr^{-1} corresponding to 24 kg N ha^{-1} yr^{-1}. Vineyards were irrigated according to two controlled water deficits (CWD), which counterbalanced 24 and 48% of crop evapotranspira-

tion corresponding to severe (24 ETc) and medium water stress (48 ETc), respectively. A non-irrigated treatment was also included (0 ETc). The seasonal irrigation volumes imposed corresponding to two CWD were about 630 $m^3 ha^{-1}$ (63 mm ha^{-1}) and 1,260 $m^3 ha^{-1}$ (126 mm ha^{-1}), and were managed by two independent drip lines. A localized irrigation system consisting of 1.6 L h^{-1} pressure compensated drip emitters placed between the vines and at a distance of 0.80 m from each other was used to supply the water amount of 24% ETc. Another drip line was added in order to perform the trial at 48% ETc. Starting 10 days after the beginning of veraison (at 10% of berry softening) and until harvest, vineyards were irrigated 6 times and the interval between irrigation cycles was approximately 10 days. A volume of 105 and 219 m^3 ha^{-1} of water in 24 and 48% ETc trials, respectively, were given in each of the irrigation interventions scheduled. In 2007 the harvest time was 1st October for Nero di Troia and 4th October for Aglianico. In 2008 the harvest time was 13th October for Nero di Troia and 14th October for Aglianico.

Experimental design

The two cultivars were considered separately. Soil management (SM) and irrigation were arranged as a split plot design with three replications. SM represented the main plot (cover cropping and soil tillage; two randomized levels) while irrigation (two irrigation levels and non-irrigated vines; three randomized levels) represented the subplot. Each of the three replications was constituted by 12 rows of 220 vines. The main plot (SM) included 12 rows of 110 vines each, whereas the subplot (irrigation level, IL) was made of 4 rows of 110 vines each. A specific volumetric valve was used for separately managing each IL. For each subplot, only the central two rows and, for each of them, the central 100 vines were used for data collection.

Plant water status

From the beginning of June to the harvest time (first decade of October) the midday stem water potential (ψs) was measured 12 times. The irrigations dates during the course of the experiment were 18th and 29th July, 9th, 18th and 30th August and 10th September in 2007, and 12th, 22nd July and 1st, 12th, and 23rd August and 3rd September in 2008. Measures were made one day before irrigation and five days after. The measurements were performed on two mature leaves per vine selected from the canopy. The midday stem water potential was measured on non-transpiring leaves that had been bagged with plastic sheets inserted into aluminum foil at least 1 h before measurement. Bagging prevented leaf transpiration, so leaf water potential equaled stem water potential (BEGG and TURNER, 1970). Leaves were then detached and their ψs was measured immediately in the field by a model 600-pressure chamber instrument (PMS Instrument Company, Albany, USA). The measurements of stem ψ were collected during the steady period of the water potential diurnal curve (from 11.00 am to 14.00 pm).

Yield components

The mean cluster weight was calculated on 30 clusters (10 clusters per replicate, sampled from 10 different vines). Yield (kg $vine^{-1}$) was determined averaging five vines yield per subplot at harvesting. The mean berry weight was determined on 150 berries (50 berries per replicate).

Sampling and winemaking

From each field treatment three wines replicates were performed for a total of 18 winemaking per year. In particular, about 100 kg grapes from each replicate were de-stemmed and submitted to traditional red winemaking as follows: addition of yeast (*Saccharomyces cerevisiae*, Zymasil, 0.15 g kg^{-1}, AEB, Brescia, Italy), yeast activator (preparation based on ammonium phosphate bibasic, thiamine chlorohydrate, yeast cell walls, cellulose, Bioact Plus, 0.25 g kg^{-1}, Oliver Ogar, Montebello Vicentino, Italy) and potassium metabisulphite (0.15 g kg^{-1}), and 5 days of maceration at 25°C with 2 punching-down per day. At the end of alcoholic fermentation and after static decantation, wines were racked into dark green Bordeaux bottles.

Chemical analysis

For each replicate, a 300-berry sample was picked at vintage, cutting and leaving intact part of the peduncle, from different parts of bunches. For each sample, 3 lots consisting of 10 berries were taken for a total of 9 replicates per treatment (90 berries). To obtain skin extracts, berry skins were manually removed from the pulp, dried with filter paper, and then macerated in 25 mL of ethanol/water/HCl solution (70/30/1, v/v; ethanol-hydrochlorid acid solution) for 20 h in the dark at room temperature (DI STEFANO and CRAVERO, 1991). The pulp was pressed and the juice was analyzed for total soluble solids (TSS) (°Brix), pH and titratable acidity (TA) according to EEC 2676 standard procedure EEC methods (1990). Chemical characteristics of wines were assessed by analyzing ethanol (E), pH, TA, volatile acidity (VA), malic acid (MA), lactic acid (LA), dry reduced extract (DRE) and ashes by means of a FOSS WineScan FT120 FT-MIR spectrometer (FOSS, Padova, Italy).

Phenol analysis

Phenol composition of skin extracts and wines were determined according to the methods reported by DI STEFANO and CRAVERO (1991) using an UV-visible spectrophotometer (Shimadzu

UV 1601, Columbia, Maryland, USA). For flavonoids (F) and anthocyans (A) determination, skin extract and wine samples were diluted 100 and 25 times, respectively, with ethanol-hydrochloric acid solution and an absorbance spectrum in the range 230-700 nm was recorded. The F content was calculated according to the following formula:

$$F = E_{280} \cdot df \cdot 82.4$$

E_{280} = specific extinction coefficient at 280 nm assessed with the graphic method (absorbance corresponding to the segment, parallel to the y-axis, starting from the peak maximum at 280 nm and finishing on the tangent joining the points of minimum at the left and at the right of the peak).

df = dilution factor.

82.4 = value determined considering the ratio between the concentration (expressed as mg L^{-1}) and the corresponding E_{280} of pure (+)-catechin.

The A content was determined according to the following formula:

$$F = E_{maxvis} \cdot df \cdot 26.6$$

E_{maxvis} = specific extinction coefficient at the maximum of visible region (~ 520 nm) assessed with the graphic method as described before.

df = dilution factor

26.6 = value determined considering the molar extinction coefficient of an anthocyanins mixture deriving from grapes (ε = 18.800, MW medium = 500) (GLORIES, 1978).

The A content was determined using a calibration curve for malvidin-3-glucoside and expressed as mg L^{-1} of malvidin-3-glucoside.

The total polyphenols (TP) content was measured at 700 nm using the Folin-Ciocalteu reactive and quantified by the following formula:

$$TP = \frac{E_{700}}{V} \cdot 186.5$$

E_{700} = absorbance at 700 nm

V = skin extract volume (0.1 mL) or wine volume (1 mL)

186.5 = value determined considering the ratio between the concentration (expressed as mg L^{-1}) and the corresponding E_{700} of pure (+)-catechin.

In the case of flavans reacting with vanillin (FRV), skin extracts were diluted 10 times with methanol and absorbance at 500 nm was measured. The FRV content was calculated according to the following formula:

$$FRV = \Delta E \cdot df \cdot 290.8$$

ΔE = E_1-E_0 (difference between the absorbance assessed at 500 nm of the sample with and without vanillin).

df = dilution factor.

290.8 = value determined considering the ratio between the concentration (expressed as mg L^{-1}) and the corresponding E_{500} of pure (+)-catechin.

The proanthocyanidins (P) content was measured at 532 nm and calculated according to the following formula:

$$P = \frac{\Delta E}{V} \cdot 1162.5$$

ΔE = E_1-E_0 (difference between the specific extinction coefficient assessed at 532 nm with the graphic method of the sample after and before acid hydrolysis by heat).

V = skin extract volume (0.1 mL) or wine volume (1 mL).

1162.5 = value determined considering the ratio between the concentration (expressed as mg L^{-1}) and the corresponding ΔE of pure cyanidin chloride.

All data of skin extracts were converted from mg L^{-1} to mg kg^{-1} of berries multiplying the data by a factor 25/P, where 25 is the volume (mL) of extracts and P is the weight (g) of 10 berries.

HPLC-DAD anthocyanin analysis

Detection and quantification of anthocyanin compounds were carried out by high performance liquid chromatography (HPLC) using an Agilent 1200 apparatus (Agilent, Palo Alto, CA, USA) with a photodiode array detector (DAD) and an injection valve (20 mL loop). The samples, previously filtered on 0.45 mm membrane, were injected into a Zorbax SP C18 (100 x 4.6 mm, 1.8 mm, Agilent, Palo Alto, CA, USA) column and eluted at flow rate of 0.5 mL min^{-1} with water-acetonitrile (95:5, v/v) (solvent A), and water-acetonitrile (50:50, v/v) (solvent B), both adjusted to pH 1.8 with percloric acid. The gradient program of solvent A was as follow: 0 to 4.8 min from 95 to 90%, 4.8 to 16.8 min 80%, 16.8 to 21.6 min 70%, 21.6 to 31.2 min 60%, 31.2 to 40.8 min 55%, 40.8 to 48.0 min 0%, 48.0 to 58.0 min 0%, 58 to 60 min 95%, and 60 to 80 min 95%. Detection was performed at 520 nm and quantitative analysis was made according to external standard method on the basis of a calibration curve obtained by injection of solutions at different concentration of malvidin-3-glucoside (R^2 = 0.9991). Results were expressed as mg kg^{-1} berries for skin extracts and as mg L^{-1} for wines.

Statistical analysis

Chemical analyses were repeated 3 times for each sample. The analysis of variance (ANOVA) and the F test were performed by means of the Statistica 6.0 software (StatSoft, Inc. Tulsa, OK, USA) in order to separate the effects of soil management, irrigation and season. The Least Significant Difference (LSD) post hoc multiple range

test was used to compare means for main effects. Many interactions between SM and IL were observed, but only those with major technological impact such as A, TP and total anthocyanins for grapes, and A, TP, CI and total anthocyanins for wines, are discussed.

RESULTS AND DISCUSSION

Yield parameters

For both cultivars the average values of yield per vine and cluster weight were influenced by the season and the IL (Table 1). SM did not affect grape yield, with the exception of cluster weight of Aglianico that was higher for soil tillage treatment. For non-irrigated vines very low levels of ψs were reached: -2.20 and -2.34 MPa in 2007, and -2.00 and -2.13 MPa in 2008, for Aglianico and Nero di Troia, respectively (Table 2). SM did not affect ψs, whereas, as expected, IL induced higher values of ψs as a function of water volumes. The differences occurred 5 days after irrigation and less at the pre-irrigation stage. These differences induced increase of yield per vine ($P \leq 0.05$), from 0.44 to 0.73 to 1.36 kg/vine for Aglianico, and from 0.76 to 0.95 to 1.22 kg/vine for Nero di Troia, respectively for 0, 24 and 48% ETc (Table 1). Non-irrigated vines and vines subjected to severe water stress produced smaller berries with respect to medium water stress vines (18 and 12% for Aglianico and 33 and 17% for Nero di Troia, respectively), causing, consequently, a higher skin/flesh ratio in grapes (data not showed).

Qualitative characteristics of grapes

Table 3 shows the effect of season, SM, and IL on the main characteristics of grapes. Season exerted the major impact on all qualitative parameters of Aglianico. In particular, higher ripening indices were found in 2007 grapes associated to higher TSS, higher pH values and lower TA, whereas 2008 grapes were richer in all phenol compounds, except TP. The differences of phenols amounts should be attributed to the different intensity of water stress that occurred in the two years under study; in 2007 the level of water stress was probably excessive, resulting in reduced capability of vine to produce phenols. A very little impact was exerted on acidity. Cover cropping led to impoverishment in F, A, TP and PA, in contrast with previous results reported in literature on Cabernet Sauvignon grown in different areas (MONTEIRO and LOPES, 2007). Mild water stress caused by competition between the radical apparatus of vine and that of sward, commonly leads to lower canopy density and, consequently, to an increase of cluster exposure resulting in an improvement in phenols concentration (DOKOOZLIAN and KLIEWER, 1996; KELLER and HRAZDINA, 1998; SPAYD *et al.*, 2002). Our results evidenced that *cv* Aglianico is weakly affected by cover cropping. Concerning IL, medium water stress (48 ETc) led to a decrease in TA and A, which might be attributed to a dilution effect (INTRIGLIOLO and CASTEL, 2008),

Table 1 - Effect of season, soil management and irrigation level on yield components of Aglianico and Nero di Troia.

Source of variation	Yield (kg vine^{-1})	Cluster weight (g)	Berry weight (g)	Yield (kg vine^{-1})	Cluster weight (g)	Berry weight (g)
		Aglianico			Nero di Troia	
Season						
2007	0.68 b†	76 b	1.50 a	0.72 b	97 b	2.31 a
2008	1.01 a	108 a	1.46 a	1.27 a	123 a	2.41 a
Significance	*	*	ns	***	*	ns
Soil management (SM)						
Soil tillage	0.99 a	107 a	1.55 a	0.94 a	106 a	2.35 a
Cover crop	0.70 a	77 b	1.41 a	1.05 a	114 a	2.38 a
Significance	ns	*	ns	ns	ns	ns
Irrigation level (IL)						
0 ETc	0.44 c	59 c	1.35 b	0.76 b	78 c	1,90 c
24 ETc	0.73 b	80 b	1.45 ab	0.95 ab	114 b	2.35 b
48 ETc	1.36 a	136 a	1.65 a	1.27 a	148 a	2.85 a
Significance	**	**	**	**	***	***
Interaction						
SM*IL	ns	ns	ns	ns	ns	ns

†In columns data followed by different letters for each source of variation are significantly different by LSD post hoc multiple range test at $P = 0.05$.
ns, *, **, and ***, not significant or significant at $P \leq 0.05$, $P \leq 0.01$, or $P \leq 0.001$, respectively.

Table 2 - Effect of season, soil management and irrigation level on midday stem water potential (Ψs) of Aglianico and Nero di Troia grapevines.

Source of variation	Aglianico			Nero di Troia		
	Non-I†	Pre-I†	Post-I†	Non-I	Pre-I	Post-I
Season						
2007	-2.20±0.37	-1.83±0.23	-1.53±0.24	-2.34±0.34	-1.59±0.23	-1.35±0.24
2008	-2.00±0.34	-1.66±0.21	-1.39±0.21	-2.13±0.31	-1.45±0.21	-1.22±0.22
Significance	**	*	*	**	*	*
Soil management (SM)						
Soil tillage	-2.12±0.32	-1.74±0.24	-1.47±0.27	-2.21±0.36	-1.54±0.26	-1.29±0.17
Cover crop	-2.06±0.47	-1.75±0.24	-1.45±0.20	-2.25±0.34	-1.50±0.18	-1.28±0.29
Significance	ns	ns	ns	ns	ns	ns
Irrigation level (IL)						
0 ETc	-2.10±0.36			-2.23±0.34		
24 ETc		-1.81±0.27	-1.60±0.21		-1.70±0.18	-1.48±0.14
48 ETc		-1.68±0.19	-1.29±0.10		-1.37±0.13	-1.09±0.10
Significance		**	***		***	***
Interaction						
SM*IL		ns	ns		ns	ns

†Mean values (MPa ± SD) for non-irrigated (Non-I), 1 day before irrigation (Pre-I) and 5 days after irrigation (Post-I), respectively.
ns, *, **, and ***, not significant or significant at P ≤ 0.05, P ≤ 0.01, or P ≤ 0.001, respectively.

Table 3 - Influence of season, soil management and irrigation level on the main qualitative characteristics of grapes.

Source of variation	TSS (°Brix)	pH	TA (g L^{-1})	F (mg kg^{-1})	A (mg kg^{-1})	TP (mg kg^{-1})	FRV (mg kg^{-1})	P (mg kg^{-1})
	Aglianico							
Season								
2007	25.1a†	3.60a	4.91b	3748b	2246b	4416a	511b	2412b
2008	24.8b	3.36b	5.95a	4569a	3414a	3713b	1614a	4226a
Significance	**	***	***	***	***	***	***	***
Soil management (SM)								
Soil tillage	24.8b	3.49	5.49	4522a	3088a	4298a	1106	3500a
Cover crop	25.1a	3.47	5.37	3795b	2571b	3831b	1019	3138b
Significance	*	ns	ns	***	***	***	n.s.	**
Irrigation level (IL)								
0 ETc	24.7b	3.51	5.95a	3711b	2828b	3734c	1034b	3087c
24 ETc	25.3a	3.47	5.29b	4376a	2953a	4285a	989c	3501a
48 ETc	24.9b	3.46	5.06c	4389a	2709c	4175b	1164a	3370b
Significance	***	ns	***	***	*	***	*	**
Interaction								
SM*IL	**	**	***	**	ns	**	*	***
	Nero di Troia							
Season								
2007	23.0a	3.87	3.43	3424a	1933a	3623a	958b	3402b
2008	22.7b	3.89	3.56	2736b	1308b	2524b	1269a	3746a
Significance	***	ns	ns	***	***	***	***	***
Soil management (SM)								
Soil tillage	23.1a	3.86	3.45	3224a	1585b	2883b	1082b	3910a
Cover crop	22.6b	3.89	3.53	2937b	1655a	3265a	1144a	3238b
Significance	***	ns	ns	***	***	***	ns	***
Irrigation level (IL)								
0 ETc	22.7b	3.88ab	3.36b	3310a	1797a	3628a	1119b	4181a
24 ETc	22.8b	3.93a	3.36b	3103b	1648b	2945b	934c	3351b
48 ETc	23.0a	3.82b	3.76a	2827c	1414c	2649c	1287a	3190c
Significance	*	ns	***	***	***	***	***	***
Interaction								
SM*IL	***	ns	ns	***	***	***	ns	***

TSS, total soluble solids; TA, titratable acidity; F, flavonoids; A, anthocyans; TP, total polyphenols; FRV, flavans reagent with vanillin; P, proanthocyanidins.
† In columns, and for each cultivar, data followed by different letters for each source of variation are significantly different by LSD test at *P* = 0.05.
ns, *, **, and ***, not significant or significant at *P* ≤ 0.05, *P* ≤ 0.01, or *P* ≤ 0.001, respectively.

whereas a severe water stress (24 ETc) caused enrichment in TSS and in all phenol compounds, with the exception of FRV. In general, differences observed in phenols contents were significant from a statistical point of view, but they had little technological impact. This could be explained by the low ψs levels which would determine an early leaf senescence and a major exposure of bunches to sunlight in each treatment. Surprisingly, grapes from non-irrigated vines, although characterized by the smallest berry size, did not show the highest phenols values. Interactions between SM and IL were ascertained for all quality parameters, with the exception of anthocyanins. In particular, ST grapes from severe and medium water stress resulted richer in TP than CC grapes with the same water stress, whereas no differences in TP were observed between CC and ST non-irrigated grapes (Fig. 1a).

Concerning Nero di Troia, no significant differences due to season were found for pH and AT, whereas higher contents of F, A and TP and lower contents of FRV and PA were found in 2007 grapes with respect to those of 2008. Similarly to Aglianico, cover cropping did not influence pH, TA and FRV and led to a slight decrease of F and PA. In contrast with Aglianico and in agreement with results reported in literature (MONTEIRO and LOPES, 2007; XI *et al.*, 2010), this practice increased A and TP contents improving grapes colour and polyphenol concentrations. A higher TA content was found in grapes subjected to medium water stress as compared to those that suffered severe water stress. However, the quite low TA value (3.76 g L^{-1}) suggested that in any case acidity must be corrected by adding tartaric acid during winemaking or, at least, to the wine. Differently from Aglianico, the irrigation deficit led to a general accumulation of phenol compounds due to smaller berries size indicating that their content in *cv* Nero di Troia was greatly influenced by water stress intensity. Furthermore, bunches were differently exposed to solar radiation in function of vines IL causing marked differences in phenols values. In particular, bunches from non-irrigated vines were exposed for 10-15 days before the beginning of veraison until harvest; those with severe water stress were exposed from the end of veraison until harvest; those with medium water stress were exposed from defoliation (about 1 month after veraison) until harvest.

Differently from Aglianico, interaction between SM and IL was found only for TSS, F, A, TP and P quality parameters. CC grapes were richer in A than ST grapes, whereas no differences were observed between the two SM treatments subjected to severe water stress (Fig. 1b). At each IL, higher values of TP were found in CC grapes with respect to ST. However, a higher value was observed in ST non-irrigated grapes with respect to severe and medium water stress CC grapes (Fig. 1c).

Anthocyanin profile of grapes

Anthocyanins are the most abundant phenolics in red grape skins, and are directly responsible for colour. Anthocyanin composition and the total anthocyanins content of grapes determined by HPLC are reported in Table 4. The Aglianico grapes harvested in 2008 showed a slightly higher anthocyanins contents and quite different anthocyanin composition with respect to grapes harvested in 2007 indicating that season exerted a strong influence on these compounds. In particular, 2008 grapes presented a higher concentration of almost all anthocyanins, with the exception of peonidin-3-glucoside (Pn) and peonidin-3-acetylglucoside (Pn-Ac) which represented about 23% and 2% of total anthocyanins in 2007, and about 5% and less than 0.1% in 2008, respectively. The relative contents of acetylated and coumarylated forms were 4.5 and 22.2% in 2007 and 6.3 and 31.0% in 2008, respectively. These values are similar to those reported in literature for the same variety grown in nearby areas but in different years (LOVINO *et al.*, 2006; TAMBORRA and ESTI, 2010), confirming that analysis of anthocyanins could be a powerful tool in order to assess the authenticity of a variety. In 2008 higher contents of Delphinidin-3-glucoside (Df) and Cyanidin-3-glucoside (Cy) were found with respect to 2007. This result could be ascribed to a minimum of enzymatic activity in the last stage of ripening that reduced the conversion of these compounds to more stable forms such as petunidin-3-glucoside (Pt) and malvidin-3-glucoside (Mv) (ROGGERO *et al.*, 1986; ESTEBAN *et al.*, 2001). SM did not influence the content of anthocyanins, whereas it had a certain impact on anthocyanin composition. In fact, grapes deriving from cover cropping showed a higher content of Mv and malvidin-3-coumarylglucoside (Mv-Cm) and a lower content of Df, Cy and Pt. Grapes deriving from non-irrigated vines were found richer in almost all anthocyanins, followed in decreasing order by grapes subjected to severe water stress and medium water stress. Among anthocyanins, malvidin forms were found to be higher in grapes from non-irrigated vines whereas almost all forms were lower in grapes from medium water stress vines. These results demonstrated that severe water stress and, especially, absence of irrigation might induce a major accumulation of anthocyanins in skins, resulting in grapes with higher phenolic maturity to be devoted to the production of wines for aging. Interaction between SM and IL was found for all anthocyanins with the exception of Cy, Mv-Ac and Pt-Cm. Cover cropping of non-irrigated and severe water stress treatments led to the highest values of total anthocyanins, whereas a lowest value, similar to those of ST practice, was observed in medium water stress treatment (Fig. 1d).

As observed for Aglianico, season influenced

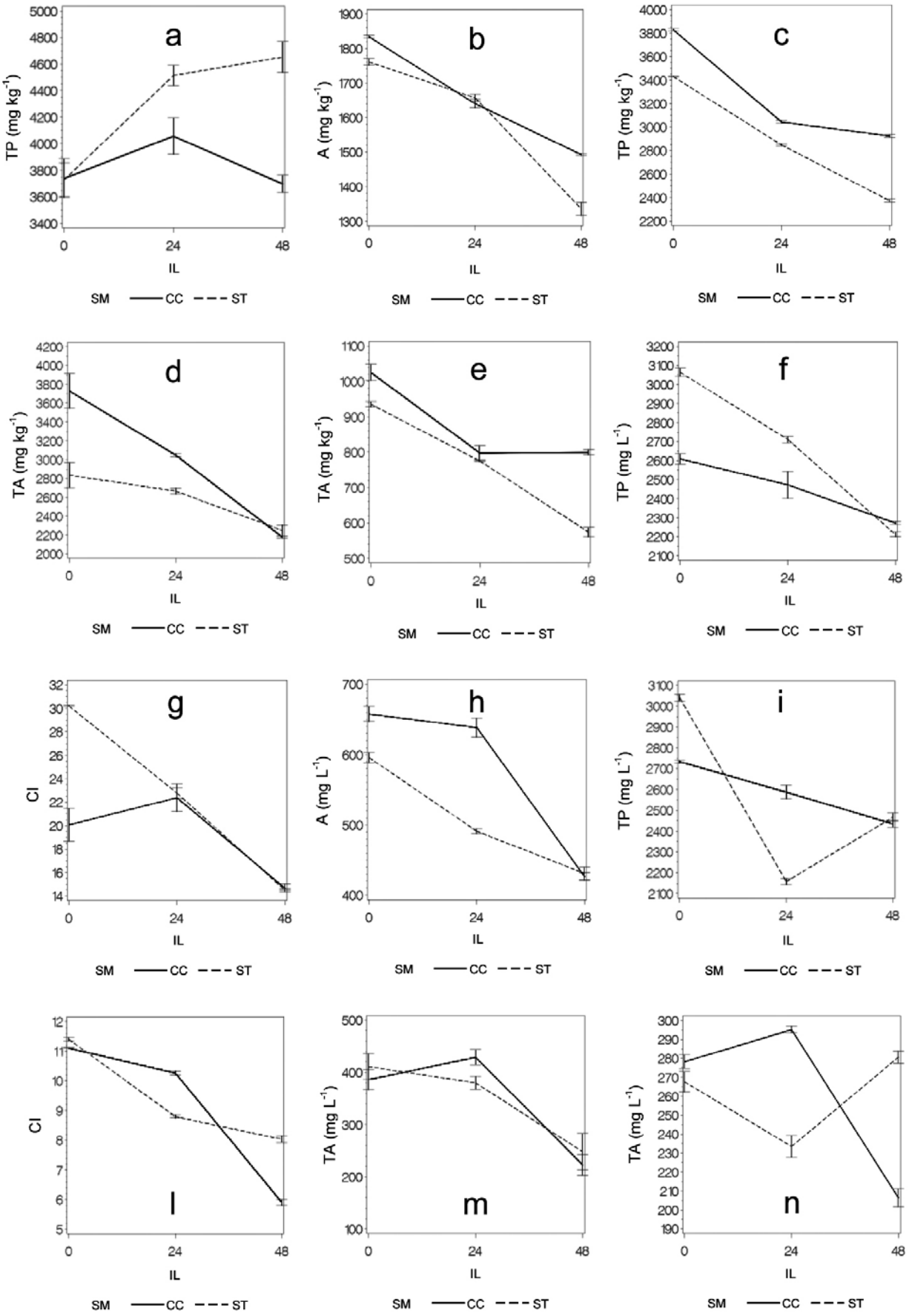

Fig. 1 - Interactions between soil management (SM) and irrigation level (IL) on main phenol compounds of Aglianico grapes (1a, 1d), Aglianico wines (1f, 1g, 1m), Nero di Troia grapes (1b, 1c, 1e) and Nero di Troia wines (1h, 1i, 1l, 1n). TP, total phenols; A, anthocyans; TA, total anthocyanins by HPLC; CI, colour intensity.

Table 4 - Influence of season, soil management and irrigation level on anthocyanin composition of grapes (mg kg^{-1} of berries).

Source of variation	Df	Cy	Pt	Pn	Mv	Df-Ac	Cy-Ac	Pt-Ac	Pn-Ac	Mv-Ac	Df-Cm	Cy-Cm	Pt-Cm	Pn-Cm	Mv-Cm	Total anthocyanins
Aglianico																
Season																
2007	57.0b†	39.1b	85.4b	439.1a	793.2a	tr	1.3a	tr	44.6a	40.8b	tr	tr	18.3b	tr	410.3b	1929.1b
2008	123.3a	93.7a	195.0a	106.2b	755.2b	tr	0.6b	tr	0.7b	126.3a	tr	tr	56.8a	tr	575.3a	2033.1a
Significance	***	***	***	***	*		***		***	***			***		***	*
Soil management (SM)																
Soil tillage	110.8a	78.2a	163.2a	288.7	754.2b	tr	0.8a	tr	21.8	82.6	tr	tr	34.3	tr	452.4b	1987.0
Cover crop	69.5b	54.6b	117.2b	256.5	794.1a	tr	0.5b	tr	22.8	84.4	tr	tr	40.8	tr	533.3a	1973.7
Significance	***	***	***	ns	*		***		ns	ns			ns		**	ns
Irrigation level (IL)																
0 ETc	102.7a	76.8a	159.5a	267.7b	883.3a	tr	0.4c	tr	19.9b	101.0a	tr	tr	42.4a	tr	581.5a	2235.2a
24 ETc	97.7a	66.5b	142.6b	313.7a	755.8b	tr	0.7b	tr	26.0a	80.0b	tr	tr	39.2a	tr	510.0b	1960.2b
48 ETc	70.1b	55.9c	118.6c	236.5c	683.5c	tr	0.9a	tr	21.0b	69.6b	tr	tr	31.0b	tr	387.0c	1674.1c
Significance	***	***	***	***	*		***		***	***			*		***	***
Interaction																
SM*IL	***	ns	***	***	*		***		***	ns			ns		***	**
Nero di Troia																
Season																
2007	22.8b	19.4b	40.5b	63.6b	173.0a	2.6b	8.1b	32.0a	22.8b	48.8b	11.2b	20.8a	16.9a	96.1a	182.7b	757.0b
2008	59.7a	32.8a	66.6a	72.3a	97.8b	13.6a	15.3a	12.2b	42.0a	92.9a	30.8a	13.5b	5.0b	64.1b	260.1a	878.7a
Significance	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
Soil management (SM)																
Soil tillage	36.4b	18.6b	50.1b	61.3b	127.6b	7.7	7.8b	16.6b	32.8	67.5b	21.8	17.2	7.1b	86.0a	202.7b	761.2b
Cover crop	46.1a	29.1a	57.0a	74.6a	143.5a	8.5	15.5a	27.5a	31.9	74.2a	20.2	17.1	14.8a	74.2b	240.1a	874.3a
Significance	***	***	***	***	***	ns	***	***	ns	***	ns	ns	***	***	***	***
Irrigation level (IL)																
0 ETc	48.4a	35.6a	67.8a	86.3a	164.3a	10.8a	13.1a	34.2a	38.0a	85.6a	29.2a	23.9a	19.4a	94.9a	228.3b	979.8a
24 ETc	37.7b	12.0c	45.8b	44.7c	136.4b	5.4c	11.8a	18.4b	29.4b	66.3b	17.1b	16.4b	8.4b	75.4b	261.1a	786.3b
48 ETc	37.6b	24.0b	47.1b	72.9b	105.9c	8.1b	10.2b	13.7c	29.7b	60.6c	16.8b	11.1c	5.0c	69.9c	174.8c	687.4c
Significance	***	***	***	***	***	***	**	***	***	***	***	***	***	***	***	***
Interaction																
SM*IL	***	***	***	***	***	***	***	***	***	***	***	ns	***	***	***	***

Df, delfinidin-3-glucoside; Cy, cyanidin-3-glucoside; Pt, petunidin-3-glucoside; Pn, peonidin-3-glucoside; Mv, malvidin-3-glucoside; Df-Ac, delfinidin-3-acetylglucoside; Cy-Ac, cyanidin-3-acetylglucoside; Pt-Ac, petunidin-3-acetylglucoside; Pn-Ac, peonidin-3-acetylglucoside; Mv-Ac, malvidin-3-acetylglucoside; Df-Cm, delfinidin-3-coumaroylglucoside; Cy-Cm, cyanidin-3-coumaroylglucoside; Pn-Cm, peonidin-3-coumaroylglucoside; Pt-Cm, petunidin-3-coumaroylglucoside; Mv-Cm, malvidin-3-coumaroylglucoside. †In columns, and for each cultivar, data followed by different letters for each source of variation are significantly different by LSD test at $P = 0.05$.
tr, traces (< 0.1 mg kg^{-1} of berries); ns, *, **, and ***, not significant or significant at $P \leq 0.05$, $P \leq 0.01$, or $P \leq 0.001$, respectively.

Table 5 - Influence of season, soil management and irrigation level on the chemical characteristics of wines.

Source of variation	E (% v/v)	pH	TA (g L^{-1})	VA (g L^{-1})	MA (g L^{-1})	LA (g L^{-1})	DRE (g L^{-1})	Ashes (g L^{-1})
	Aglianico							
Season								
2007	14.2a†	3.45a	7.48a	0.30	1.45a	0.39a	27.9a	2.97a
2008	14.0b	3.35b	7.06b	0.33	0.89b	0.12b	26.8b	2.73b
Significance	**	**	***	ns	***	***	***	***
Soil management (SM)								
Soil tillage	14.1	3.41	7.31	0.32	1.12b	0.30a	27.9a	2.87
Cover crop	14.0	3.39	7.23	0.31	1.22a	0.21b	26.7b	2.82
Significance	ns	ns	ns	ns	***	***	***	ns
Irrigation level (IL)								
0 ETc	14.0b	3.37b	7.57a	0.33	1.00c	0.38a	29.6a	2.83
24 ETc	14.0b	3.37b	7.34b	0.30	1.15b	0.22b	26.9b	2.85
48 ETc	14.3a	3.46a	6.90c	0.32	1.36a	0.16c	25.5c	2.87
Significance	***	*	***	ns	***	***	***	ns
Interaction (SM*IL)	***	ns	***	ns	***	***	***	ns
	Nero di Troia							
Season								
2007	13.3a	3.94	4.34a	0.36a	1.09a	0.83b	26.6a	3.80
2008	12.2b	3.92	3.37b	0.27b	0.24b	1.23a	24.5b	3.77
Significance	***	ns	***	***	***	***	***	ns
Soil management (SM)								
Soil tillage	12.9a	3.91	3.84	0.31	0.66	0.99b	25.5	3.84
Cover crop	12.7b	3.95	3.87	0.32	0.66	1.06a	25.6	3.73
Significance	***	ns	ns	ns	ns	***	ns	ns
Irrigation level (IL)								
0 ETc	12.8b	3.90b	3.72c	0.32	0.71a	0.81c	25.9a	3.79
24 ETc	12.6c	3.89b	4.00a	0.31	0.63b	1.05b	25.1b	3.87
48 ETc	12.9a	4.00a	3.85b	0.32	0.64b	1.22a	25.8a	3.70
Significance	***	*	***	ns	***	***	***	ns
Interaction SM*IL	***	ns	**	ns	***	***	***	***

E, ethanol; TA, titratable acidity; VA, volatile acidity; MA, malic acid; LA, lactic acid; DRE, dry reduced extract.
† In columns, and for each cultivar, data followed by different letters for each source of variation are significantly different by LSD test at $P = 0.05$.
ns, *, **, and ***, not significant or significant at $P \leq 0.05$, $P \leq 0.01$, or $P \leq 0.001$, respectively.

the content of anthocyanins also in Nero di Troia grapes. In fact, a higher content was found in 2008 grapes. The anthocyanin composition of grapes was different as well: higher contents of Mv, Pt-Ac, Cy-Cm, Pt-Cm and Pn-Cm were found in 2007 as compared to 2008. Similarly to Aglianico, percentages of acetylated and coumarylated forms were quite similar to those reported by other authors (LOVINO *et al.*, 2006; TAMBORRA and ESTI, 2010) whereas, differently from Aglianico, cover cropping led to an increase of total anthocyanins and of almost all anthocyanin compounds, with the sole exception of Pn-Cm. These results suggested a minor capacity of the radical apparatus of *cv* Nero di Troia to compete with sward for water, resulting in a higher accumulation of anthocyanins in skins. Concerning the water factor, the impact was very similar to that observed for Aglianico. Anthocianins concentration was inversely related to water availability confirming that water stress induces a greater accumulation of anthocyanins also in *cv* Nero di Troia and, consequently, it make it possible to obtain more coloured grapes which are more suitable to the production of red wines for aging. Interaction between SM and IL was found for all anthocyanins, with the sole exception of Cy-Cm. Total anthocyanins determined by HPLC (Fig. 1e) showed a trend of A similar to that assessed by spectrophotometry (Fig. 1b).

Chemical characteristics of wines

The chemical characteristics of wines are shown in Table 5. Data show that, in all wines, the malolactic fermentation was not completed since MA was not totally converted into LA. This effect could be ascribable to the winemaking protocol adopted, which did not include inoculation of malolactic bacteria. In addition, wine analyses

were performed after about three months from production, a period of time that could be not sufficient to ensure the completion of malolactic fermentation.

Concerning Aglianico, higher TA, DRE and ashes values were found in 2007 wines thus resulting more structured than those of 2008. The influence of SM was less evident. In fact, cover cropping led to a slight decrease of DRE only. Wines from medium water stress vines showed higher pH and lower TA values in agreement with results obtained for *cv* Tempranillo (INTRIGLIOLO and CASTEL, 2008), whereas severe water stress enhanced TA and DRE contents leading to more structured wines. Interaction between SM and IL was found for all quality parameters, with the exceptions of pH, VA and ashes.

Regarding Nero di Troia, season had a strong impact on wines structure. In fact, higher values of E, TA and DRE were found in 2007. However, TA values found in this *cv* are quite low, and, consequently, acidity must be increased in order to improve quality and safety of wines. Any influence was exerted by SM on chemical characteristics of wines. Regarding IL, a severe water stress led to a slight increase of TA and to a slight decrease of DRE. Finally, interaction between SM and IL was found for all quality parameters with the exceptions of pH and VA.

Phenolic composition of wines

The phenolic composition and colour indices of wines are reported in Table 6. In accordance with the characteristics of raw material, Aglianico wines produced in 2008 showed higher phenol contents, except for TP, and a greater colour intensity (CI) than those obtained in 2007, confirming that season affects the phenolic fraction. SM exerted a lesser effect. In fact, cover cropping led to a slight decrease in TP, FRV and CI. Water stress caused a general enrichment in phenols and CI. In particular, non-irrigation led to major enrichment in TP, P and CI with respect to severe and medium water stress. Medium water stress had some negative effects on wine colour, which were reduced due to pH increase and TA decrease (Table 5) and to a decrease in A concentration, in accordance with results reported by INTRIGLIOLO and CASTEL (2008). Interaction between SM and IL was found for all phenol parameters, with the exception of A and FRV. In contrast with grapes and likely due to the impact of the winemaking process, CC wines showed a highest value of TP in non-irrigated vines and a lowest value in medium water stress vines (Fig. 1f). The combination CC and 0 ETc led to the highest values of CI, whereas no differences were observed among other treatments (Fig. 1g).

Phenolic composition of Nero di Troia was also influenced by season but with some differences with respect to Aglianico. In fact, higher contents of F, A, TP and CI were found in 2007 resulting in more structured wines in phenols and colour, in accordance with the characteristics of the raw material. Regarding SM, cover cropping led to a slight enrichment in F, A and FRV showing a moderate influence on phenols accumulation. Conversely, the IL exerted a major influence. Phenolic structure and CI increased when the vines were not irrigated, as already observed for Aglianico, and in agreement with other authors' findings (SALÓN *et al.*, 2005; INTRIGLIOLO and CASTEL, 2008), suggesting that strong water stress increases phenol content and colour intensity of Nero di Troia wines. Interaction between SM and IL was found for all phenol parameters indicating that this cultivar is particularly susceptible to these practices. In particular, vines with a higher A content were obtained by CC practice applied to non-irrigated and to severe water stress vines, whereas no differences between CC and ST were observed when vines suffered medium water stress (Fig. 1h). CC practice showed a linear negative trend of TP as a function to IL, whereas ST induced a highest value in non-irrigated vines and a lowest value in severe water stress vines (Fig. 1i). Finally, CI showed a negative trend, more marked for CC, for both SM treatments as a function to IL (Fig. 1l).

Anthocyanin profiles of wines

Aglianico wines produced in 2008 contained about 25% more anthocyanins than those obtained in 2007 whereas the anthocyanin profiles were slight different (Table 7). Mv was the predominant compound representing about 65% and 62% of the total fraction in 2007 and 2008 wines, respectively. SM did not influence either the content or the composition of anthocyanins. Conversely, wines from vines suffering severe and very strong water stress (24 and 0 ETc, respectively) were richer in almost all anthocyanins than wines from medium water stress vines. This result is in agreement with the anthocyanins content and composition of grapes used for winemaking in this experimentation and with results reported in literature (SALÓN *et al.*, 2005; INTRIGLIOLO and CASTEL, 2008). Similarly to grapes, interaction between SM and IL was found in wines for all anthocyanins with the exceptions of Cy, Mv-Ac, Df-Cm, and Mv-Cm. The highest value of total anthocyanins was observed in vines submitted to CC and severe water stress, whereas a medium water stress induced lowest values in both SM treatments (Fig. 1m).

As for Nero di Troia, in spite of the low concentrations observed in the corresponding grapes, the 2007 wines exhibited a higher anthocyanins content than those of 2008. This could be ascribed to the different anthocyanin compositions of grapes of the two seasons. In fact, 2007 grapes were characterized by lower amounts of hydroxylated anthocyanins (unstable) and higher content of Mv (more stable) than 2008, result-

Table 6 - Influence of season, soil management and irrigation level on the phenolic composition of wines (mg L^{-1}).

Source of variation	F (mg L^{-1})	A (mg L^{-1})	TP (mg L^{-1})	FRV (mg L^{-1})	P (mg L^{-1})	CI	T
	Aglianico						
Season							
2007	1778b†	582b	2622a	637b	2612b	19.3b	0.51b
2008	2000a	707a	2491b	748a	3303a	22.2a	0.52a
Significance	***	***	***	***	***	***	**
Soil management (SM)							
Soil tillage	1872	645	2662a	727a	2963	22.5a	0.52
Cover crop	1907	644	2451b	658b	2952	19.0b	0.52
Significance	ns	ns	***	*	ns	***	ns
Irrigation level (IL)							
0 ETc	1991a	693a	2836a	721	3271a	25.1a	0.51b
24 ETc	1982a	714a	2592b	677	3063b	22.6b	0.50c
48 ETc	1695b	626b	2242c	678	2539c	14.6c	0.54a
Significance	***	***	***	ns	***	***	***
Interaction							
SM*IL	***	ns	***	ns	*	**	ns
	Nero di Troia						
Season							
2007	2291a	635a	2985a	789b	2410b	10.9a	0.72b
2008	1703b	445b	2155b	837a	3271a	7.6b	0.80a
Significance	***	***	***	***	***	***	***
Soil management (SM)							
Soil tillage	1899b	506b	2555	771b	2786	9.4a	0.78a
Cover crop	2095a	574a	2585	854a	2895	9.1b	0.74b
Significance	***	***	ns	***	ns	***	***
Irrigation level (IL)							
0 ETc	2193a	627a	2887a	884a	2816b	11.2a	0.73b
24 ETc	2022b	565b	2373c	824b	3051a	9.5b	0.78a
48 ETc	1776c	429c	2451b	730c	2655c	7.0c	0.78a
Significance	***	***	***	***	***	***	***
Interaction							
SM*IL	***	***	***	***	***	***	***

F, flavonoids; A, anthocyans; TP, total polyphenols; FRV, flavans reagent with vanillin; P, proanthocyanidins; CI, colour intensity; T, tonality.
†In columns, and for each cultivar, data followed by different letters for each source of variation are significantly different by LSD test at $P = 0.05$.
ns, *, **, and ***, not significant or significant at $P \leq 0.05$, $P \leq 0.01$, or $P \leq 0.001$, respectively.

ing in limited loss of anthocyanins during winemaking. Similarly to Aglianico, SM exerted little influence. The impact of IL was significant, but less than Aglianico. Wines from non-irrigated and severe water stress vines were characterized by higher anthocyanin contents. Finally, interaction between SM and IL found for all anthocyanins confirmed the higher susceptibility of this cultivar to viticultural practices with respect to Aglianico *cv* In particular, the association of CC and severe water stress and of ST and medium water stress produced wines rich in total anthocyanins (Fig. 1n).

CONCLUSIONS

Results obtained in this study indicated that season had a main influence on chemical and phenolic composition of grapes and wines of Aglianico and Nero di Troia cultivars grown in Apulia. Aglianico grapes and wines produced in 2008 were richer in phenol substances than those of 2007 probably due to the excessive water stress that occurred in the latter season. The opposite was observed for Nero di Troia. For both varieties, the anthocyan contents of grapes and wines were greatly influenced by season, whereas a minor influence was exerted on the anthocyanin profiles. Soil management had a low impact on phenols of grapes and wines. Cover cropping increased some phenol classes only in Nero di Troia. Irrigation highly affected all phenol substances in both varieties: non-irrigation and severe water stress caused a higher accumulation of phenolic compounds in grapes and wines with respect to medium water stress. Our results show that the phenolic content of grape and, consequently, of wine can be increased by modulating the water deficit applied to investigated varieties differently, thus improving the oenological potential of vines.

Table 7 - Influence of season, soil management and irrigation level on anthocyanin composition of wines (mg L^{-1}).

Source of variation	Df[A]	Cy	Pt	Pn	Mv	Df-Ac	Cy-Ac	Pt-Ac	Pn-Ac	Mv-Ac	Df-Cm	Cy-Cm	Pt-Cm	Pn-Cm	Mv-Cm	Total anthocyanins
	Aglianico															
Season																
2007	13.2b[†]	0.8b	19.3b	13.8b	202.0b	1.7b	8.0b	6.7a	2.4b	14.2b	1.6a	1.4	3.5a	1.8a	18.4	308.8b
2008	20.8a	1.6a	27.6a	27.6a	237.3a	9.7a	10.0a	4.9b	4.3a	16.8a	1.1b	1.5	2.7b	0.0b	18.2	383.1a
Significance	***	***	***	***	*	***	***	***	***	***	***	ns	***	***	ns	***
Soil management (SM)																
Soil tillage	16.6	1.2	22.3	21.6a	220.6	5.1b	8.6	5.7	3.1b	15.4	1.3	1.4	3.4a	0.7b	18.6	345.6
Cover crop	17.5	1.1	23.6	19.8b	218.7	6.2a	9.3	5.9	3.6a	15.6	1.3	1.5	2.9b	1.1a	18.0	346.1
Significance	ns	ns	ns	*	ns	*	ns	ns	**	ns	ns	ns	***	***	ns	ns
Irrigation level (IL)																
0 ETc	21.7a	1.2a	28.3a	24.3a	242.9a	10.6a	13.1a	7.5a	3.3b	17.3a	0.9c	1.7a	4.1a	0.6c	20.7a	398.2a
24 ETc	19.0b	1.3a	25.5b	21.5b	266.0a	3.6b	9.3b	5.4b	4.0a	18.0a	1.5b	1.5a	3.7b	1.3a	22.8a	404.4a
48 ETc	10.4c	1.0b	15.1c	16.4c	150.1b	2.8b	4.5c	4.4c	2.8c	11.2b	1.7a	1.2b	1.5c	0.9b	11.5b	235.5b
Significance	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
Interaction																
SM*IL	***	ns	***	***	*	***	***	**	***	ns	ns	ns	**	**	ns	***
	Nero di Troia															
Season																
2007	6.1a	0.6a	17.3a	15.3a	146.4a	7.6a	3.7a	8.1a	7.9a	58.3b	1.8a	2.5a	4.4b	2.9a	21.1a	304.0a
2008	1.71b	0.0b	9.4b	10.0b	103.9b	2.2b	1.2b	2.7b	0.0b	61.0a	0.8b	1.0b	5.2a	0.0b	17.2b	216.3b
Significance	***	***	***	***	***	***	***	***	***	*	***	***	***	***	***	***
Soil management (SM)																
Soil tillage	4.2a	0.3	13.6	12.4	130.0a	4.6b	2.8a	4.6b	4.4a	56.7b	1.2b	1.6b	4.0b	1.8a	18.1b	259.8
Cover crop	3.6b	0.3	13.1	12.8	120.2b	5.2a	2.2b	6.2a	3.5b	62.6a	1.4a	2.0a	5.5a	1.0b	20.2a	260.3
Significance	***	ns	ns	ns	***	**	***	***	***	***	***	***	***	***	***	ns
Irrigation level (IL)																
0 ETc	4.2a	0.3	14.2a	13.9a	131.7a	4.9	3.0a	5.4	3.4c	63.7a	1.6a	1.8	5.0b	0.8c	19.0	273.0a
24 ETc	4.3a	0.3	14.0a	13.2b	131.6a	5.2	2.0c	5.3	4.0b	57.3b	0.9c	1.7	3.7c	1.1b	19.7	264.3a
48 ETc	3.3b	0.3	11.9b	10.9c	112.0b	4.7	2.5b	5.5	4.5a	57.9b	1.4b	1.9	5.7a	2.5a	18.7	243.7b
Significance	***	ns	***	***	***	ns	***	ns	***	***	***	ns	***	***	ns	***
Interaction																
SM*IL	***	***	***	***	***	***	***	***	***	***	***	***	**	***	***	***

[A]The name of anthocyanins are explained in footnote of table 4.
[†]In columns, and for each cultivar, data followed by different letters for each source of variation are significantly different by LSD test at $P = 0.05$.
ns, *, **, and ***, not significant or significant at $P \leq 0.05$, $P \leq 0.01$, or $P \leq 0.001$, respectively.

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PAPER

EFFECT OF COOKING TREATMENTS ON NUTRIENT PROFILE OF DISHES BASED ON VEAL MEAT

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ABSTRACT

This study provides a picture of the compositional figure and nutritive value of veal based dishes typical of Italian culinary tradition: fillet (raw, in pan, roasted with bacon), top-side (raw, salt-imbocca, escalope, stewed, vitel tonnè), sirloin (raw, in pan, barbecued). The inclusion of vegetable or animal derived ingredients and cooking methods markedly influenced the nutritional profile of the dishes. The knowledge of changes in the concentration in several nutrients (eg. fatty acids, heme iron, vitamins, cholesterol, sodium) occurring upon cooking has a relevant nutritional significance and represents the most appropriate basis to formulate accurate diets.

- Key words: B-vitamins, vitamin E, fatty acids, cholesterol, heme iron, sodium -

INTRODUCTION

The increased awareness of the close relationship between food and health has led the interest of consumers towards the acquisition of more detailed information on quality characteristics of foods and also on their safety, with a clear demand to know the entire production chain of foods from farm to fork. The first step to meet these demands is to make available and easily accessible to consumers, as well as to the various stakeholders, data on the composition of the foods that are included in the daily diet. Getting data on the nutrient composition of foods as daily consumed, namely on the quality of processed food will allow consumers to make more careful choices of foods that constitute the overall daily diet. Both the preparation steps of meat and the utilization of different cooking methods can lead to changes in the chemical composition of meat, greatly varying the nutrient and micronutrient intake (LOMBARDI-BOCCIA *et al.*, 2005, D'EVOLI *et al.*, 2009). Losses in some vitamins, especially those more unstable to heat or to light exposure, can occur (YANG *et al.*, 1994; LOMBARDI-BOCCIA *et al.*, 2005, GERBER *et al.*, 2009). Furthermore, among trace elements, the knowledge of the level of heme iron in meat is of relevant interest because of its important nutritional value. The severity of the thermal processes in fact could induce the degradation of heme pigment with a consequent release of a less bioavailable iron form (IGENE *et al.*, 1979; LOMBARDI-BOCCIA *et al.*, 2002a). Therefore a careful estimation of the heme iron content in cooked meats and in meat-based dishes contributes to get a reliable index of its potential availability (D'EVOLI *et al.*, 2009), a necessary tool to plan suitable diets for specific segments of population.

The present study was addressed to evaluate the chemical composition and the nutritional value of culinary preparations based on veal meat. The recipes utilized in the study were selected among the most widespread ones in Italy and specific for the following veal meat cuts: fillet (raw, in pan, roasted with bacon), top-side (raw, saltimbocca, escalope, stewed, vitel tonnè), sirloin (raw, in pan, barbecued). Data on macro- and micro-nutrients, heme iron, cholesterol and energy value were reported. The influence of cooking methods and recipe formulation (ingredients) on the actual nutrient content was evaluated.

MATERIALS AND METHODS

Meat

Three veal cuts were utilised in this study: fillet, top-side and sirloin. Veals (Charolais and Limousine) were raised on commercial pellets (UNIFEED), a mixture of maize, wheat flour, hay, ensilage, and slaughtered conventionally at 7 months old. Cuts were trimmed away of external fat, vacuum packed and delivered to laboratory. Fillet, top-side and sirloin were subdivided in a number of equal aliquots: some aliquots were immediately taken for the analysis of raw meat, others were prepared to be cooked following recipes selected from the traditional Italian cuisine.

Dishes based on veal meat

The recipe formulation of the selected dishes (meat cuts, ingredients, cooking time) is reported in Table 1. Each selected dish was prepared three times in a test kitchen; stainless-steel tools were used. After cooking, each dish was homogenised and stored at -30°C for subsequent analyses.

Analyses

Moisture, ash, protein, lipid: analyses were performed following the AOAC method (1997).

Cholesterol: cholesterol was extracted by boiling meat sample under reflux in a 50 mL volumetrix flask with methanolic potassium hydroxide in the presence of isopropanol. Cholesterol content was determined by enzymatic oxidation (Boehringer Mannheim/R-Biopharm kit).

Energy: energy values were expressed in kilocalories (kcal) (GREENFIELD and SOUTHGATE, 1992).

Fatty acids: Intramuscular fat was extracted by the method of FOLCH *et al.* (1957) using chloroform/methanol (2/1, v/v). Fatty acids were esterified using boron trifluoride in methanol as esterification reagent (METCALFE *et al.*, 1966). The esterified fatty acids were quantified by gas-chromatography (HP 5890 II series,equipped with FID). Separations were accomplished for saturated fatty acid on a Supelcowax 10 TM (60 mx 0.25 mm i.d., 0.25 m film thickness column). Standard Reference Material: Beef/Pig Fat Blend (BCR 163, Community Bureau of Reference, Brussels) and F.A.M.E. Mix C4-C24 (Supelco, Bellofonte PA, USA) were analysed as a control of the accuracy of the analysis.

Minerals: Samples were analysed for macro elements (Ca, Mg, Na, K, P) and trace elements (Fe, Zn, Cu, Mn) content. Aliquots of the samples were liquid ashed (4mL HNO_3+1 mL H_2O_2) in a microwave digestion system. Analyses were performed by ICP-Plasma on a Perkin-Elmer (Norwalk, CT 06859, USA) Optima 3200XL. Standard Reference Material: Bovine muscle (BCR 184, Community Bureau of Reference, Brussels) and Bovine liver (NBS 1577°; National Bureau of Standards, Gaithersburg, MD, USA) were analysed as a control of the accuracy of the analysis.

Heme iron: heme iron was determined following the method described by LOMBARDI-BOCCIA *et al* (2002b).

Table - 1 Dishes composition and cooking treatments.

Veal Meat
In pan Ingredients: 850 g meat. Cooking method: in pan, 3-5 min.
Roasted with bacon 510 g meat, 25 g bacon, 30 mL extra-virgin olive oil, 3 g rosemary, 2 g salt, 0.4 g black pepper. Cooking method: in pan, 10 min.
Saltimbocca Roman style 450 g meat, 100 g ham, 25 g butter, 25 g wheat flour, 2.3 g salt, 0.3 g black pepper, 65 mL white wine, 4.5 g sauge (8 leaves). Cooking method: in pan, 10 min.
Escalope with Marsala wine Ingredients: 490 g meat, 27 g wheat flour, 40 mL extra-virgin olive oil, 150 mL white wine, 2 g salt., 55 mL Marsala wine. Cooking method: in casserole, 15 min.
Stewed 480 g meat, 8 g wheat flour, 1/2 onion (50 g), 40 mL extra-virgin olive oil, 60 mL red wine, 2.6 g salt, 0.2 g black pepper. Cooking method: in casserole, 45 min.
Vitel tonnè 600 g meat (top-side), 4 g salt. Cooking method: in casserole, 40 min. Maionnaise sauce: 1 egg (60 g), 180 mL extra-virgin olive oil, 160 g canned tuna, 33 g capperi, 6 anchovies (7.5 g), 1.2 g salt, 20 g lemon juice.
Barbecued Ingredients: 500 g meat. Cooking method: on the barbecue, 7 min.

B Vitamins: thiamine and riboflavin were separated and quantified by HPLC after acidic and enzymatic (Takadiastase) hydrolysis of the samples, following the procedure described by ARELLA *et al.* (1996). Niacin was quantified following the method described by LAHÉLY *et al.* (1999).

Vitamin E, t-retinol: were separated and quantified by HPLC following the method by ALBALÀ-HURTADO *et al.* (1997).

Validation and Quality control procedure: Analytical quality control was performed by the analysis of both accuracy and precision of the methods. For minerals and fatty acids the accuracy of the analysis was performed utilizing Reference Standard Materials. For vitamins the accuracy of the analysis was performed by the calculation of the recoveries. Recovery experiments were performed by spiking the samples with known standards concentration. Recoveries were over 85%. Precision of the methods used in this study was good: the reproducibility was performed extracting in triplicate the same lot. System suitability tests were performed during the analysis to verify that the resolution and the reproducibility of chromatographic system were adequate for the analysis.

Statistics: Data are presented as M±SD of three independent experiments. Compositional data were statistically processed utilizing the *student t-test* to compare raw vs. cooked meat cuts, differences were considered significant at $p<0.05$, $p<0.01$, $p<0.001$.

RESULTS AND DISCUSSION

Proximate composition

Proximate composition, cholesterol content and energy value of the selected meat-based dishes are reported in Table 2. Raw cuts were characterised by a similar protein content, by contrast total fat content was similar in fillet and sirloin (2.3% both) but slightly lower in top-side

Table 2 - Moisture, ash, macronutrients, cholesterol content and calorific value of raw veal cuts and respective dishes (f.w.).

	Moisture g/100 g	Ash g/100 g	Protein g/100 g	Lipid g/100 g	Carb.(A) g/100 g	Cholesterol mg/100 g	Energy kcal
Fillet							
raw	76±0.2a	1.17±0.02a	19.0±0.6a	2.31±0.4a	—	75.4±2.1a	97
in pan	73±0.2c	1.12±0.04a	22.0±0.7b	3.12±0.7a		80.8±2.4a	116
roasted with bacon	65±0.1c	1.80±0.04c	19.1±0.9a	14.1±3.4b		99.5±3.8c	203
Top-side							
raw	77±0.4a	1.16±0.02a	20.6±0.4a	1.6±0.2a		63.0±3.7a	96
saltimbocca	64±0.2c	2.22±0.07c	23.6±0.2c	5.6±0.6c	4.6	80.4±5.5a	162
escalope	67±0.3c	1.27±0.02b	19.8±0.3a	6.6±0.4c	5.4	76.0±3.6a	160
stewed	60±0.3c	2.07±0.05c	26.3±0.8c	9.5±0.9c	2.1	85.0±7.1b	200
vitel tonné	58±0.2c	1.3±0.01c	25.2±0.7c	15.2±2.7c		129±16b	238
Sirloin							
raw	76±0.5a	1.15±0.06a	19.2±0.6a	2.3±0.2a		70.2±3.5a	98
in pan	74±0.3b	1.29±0.05a	23.4±0.4c	1.8±0.1a		74.0±3.1a	105
barbecued	67±0.2c	1.42±0.06b	27.3±1.2c	3.5±0.8a		99.7±12b	146

Values are the M±SD of three determinations. (A) = calculated by difference. Values (grouped for meat cut) in the same column, followed by different letters, are significantly different from the respective raw cut (a *vs* b= $p<0.01$; a *vs* c=$p<0.001$).

(1.6%) (Table 2). The highest cholesterol content in raw meat was found in fillet (75.4 mg/100 g) followed by sirloin (70.2 mg/100 g) and top-side (63 mg/100 g). RHEE *et al.* (1982) reported that low concentrations of cholesterol in meat generally coincided with tissue lower in fat. Total fat content determined in the raw veal cuts in this study was lower compared to both data reported in Italian Food Composition Tables (CARNOVALE and MARLETTA, 2000) and in a previous study dealing with top-side from bovine steers (D'EVOLI *et al.*, 2009). Proximate composition of the Italian traditional meat-based dishes analysed in this study (Table 2) was strongly influenced by the ingredients utilised in individual dish, and by the different cooking methods utilised. Among the dishes (see Table 1) only fillet cooked "in pan" and sirloin cooked both "in pan" and "barbecued" did not contain added ingredients (Table 1), so in these dishes the observed increases in protein, lipid and cholesterol content, with respect to raw cuts, were due to losses in moisture content (Table 2). The only exception was observed for sirloin cooked "in pan" where a slight decrease in lipid content was found. BADIANI *et al.* (2002) observed that heat processing parameters significantly affected the nutrient retention in meat. Conversely, the increases detected in both protein and lipid concentration in all the other meat-based dishes studied were mainly dependent on the added ingredients (extra-virgin olive oil, eggs, bacon, butter, ham, canned tuna, anchovies) (Table 1). This was the case especially of the two dishes which were characterized by the highest lipid increase: fillet "roasted with bacon" (14.1 mg/100 g; $p<0.01$) and "vitel tonné" (15.2 mg/100 g; $p<0.001$) (Table 2). As a consequence, the last two meat-based dishes were also characterised by the highest energy value: 238 and 203 kcal, respectively (Table 2). Significant differences in cholesterol content compared to the respective raw cut were found in fillet "roasted with bacon" ($p<0.001$), in both top-side cooked as "vitel tonné" ($p<0.01$) and "stewed" ($p<0.01$), in sirloin "barbecued" ($p<0.01$) (Table 2).

Fatty acids

The fatty acid profile of total extractable lipids and P/S ratio of the dishes based on veal meat is reported in Table 3. Among SFA palmitic (C16:0) and stearic acids (C18:0) were the most represented in the raw cuts. Palmitic acid content showed a significant increase only in "saltimbocca" dish ($p<0.001$) compared to the raw cut, this dish was the only utilizing butter instead of extra-virgin olive oil (Table 1). By contrast, significant reductions in palmitic acid content, from 56% detected in filled cooked "in pan" up to 69% detected in "barbecued" sirloin, was found in all the other dishes (Table 3). Stearic acid significantly decreased in fillet "roasted with bacon" ($p<0.001$) and in three of the of top-side cut dishes ("escalope", "stewed" and "vitel tonnè", $p<0.001$ for all) compared to the respective raw cuts. Among MUFA, oleic acid (C18:1) was the most represented fatty acid. Among the meat-based dishes cooked without added ingredients only fillet cooked "in pan" showed a significant decrease ($p<0.001$) in oleic acid content compared to the raw cut (Table 3). Cooking induces structural changes in muscle, some fatty acids are more or less easily released from tissues with droplets of fat, GERBER *et al.* (2009) observed a significant decrease in fatty acids content during cooking depending on the melting of fat and the meat cuts. On the other hand significant increases in oleic acid content were detected in all the top-side based dishes ($p<0.001$) except for "saltimbocca", the only dish that did not utilize vegetable fat (Table 3). This generalized increase in oleic acid content in the meat-based dishes compared with raw meat was explained by the utilization of extra-virgin olive oil as ingredient of the traditional Italian meat-based dishes, consequently the oleic acid content in some dishes (escalope, vitel tonné, stewed) was more than double the raw meat content. The most abundant PUFA in raw cuts were linoleic acid (C18:2) followed by arachidonic acid (C20:4). After cooking, the former was found in significantly lower concentration in top-side based dishes, with a decrease ranging from 62% detected in "saltimbocca" ($p<0.01$) to 72% in both "escalope" and "vitel tonnè" (both $p<0.001$). Arachidonic acid (C20:4) was found in significantly higher amounts in cuts cooked without added ingredients like fillet ($p<0.01$) and sirloin ($p<0.001$) cooked "in pan" and in "barbecued" sirloin ($p<0.05$). On the other hand in some top-side based dishes its content decreased significantly like in "saltimbocca"($p<0.01$), "escalope" and "vitel tonnè" (both $p<0.001$) (Table 3). The main changes detected in fatty acids content in the meat-based dishes analysed were due to the presence of added ingredients, first of all the extra-virgin olive oil responsible for the increase in oleic acid content, as well as eggs, bacon and butter, which further contribute to animal fat increase. The addition of culinary fat, necessary to the food preparation, thus affected both the total fat content and the fatty acid composition of the meat-based dishes. A recent study (HAAK *et al.*, 2007) found that pan-frying changed the fatty acid composition of pork meat depending on the type of culinary fat utilised. The recommended P/S ratio for a healthy diet is 0.45-0.65 (DEPT. of HEALTH, 1994). From a nutritional point of view total P/S ratio is thought to be of importance in relation to human health. The values of the P/S ratio found in this study ranged from 0.28 found in "saltimbocca" to a maximum value of 0.80 found in fillet cooked "in pan" (Table 3). Among the dishes analysed the most favourable P/S ratio was found in fil-

Table 3 - Content of the major fatty acids (% total fatty acids) of raw veal cuts and respective dishes (f.w.).

	FILLET						TOP-SIDE								SIRLOIN					
	Raw		In pan		Roasted+bacon		Raw		Saltimbocca		Escalope		Stewed		Vitel tonnè		Raw		In pan		Barbecued	
	mean	sd	mean	sd	media	sd	mean	sd	mean	sd	mean	sd	media	sd	media	sd	media	sd	media	sd
14:0	0.67	0.1	2.02	0.7	2.00	0.7	2.08	0.2	7.20	0.03	0.50	0.2	0.80	0.2	0.87	0.2	2.26	0.1	2.01	0.1	3.02	0.1
15:0	1.36	0.05	5.14	1.2	0.42	0.01	2.46	0.1	1.20	0.2	0.12	0.01	1.40	0.5			2.61	0.2	3.57	0.1	2.40	0.1
16:0	19.66a	0.6	15.6b	2.1	15.2c	0.9	17.9a	0.8	26.8d	0.3	13.6d	0.3	15.4c	0.07	14.5c	0.2	16.0a	0.6	17.3a	2.2	11.1b	0.8
17:0	2.39	0.2	3.49	0.8	1.20	0.7	1.61	0.2	0.95	0.2	0.33	0.1	1.12	0.6	1.11	0.2	3.48	0.3	1.84	0.9	4.30	0.3
18:0	13.1a	0.5	11.69a	1.2	9.80d	0.3	12.3a	0.6	11.6a	0.3	3.96d	0.2	5.40d	0.2	4.86d	0.13	11.8a	0.6	12.63a	1.1	12.5a	0.5
20:0					2.10	0.2			0.39	0.03	0.35	0.01	0.32	0.06	0.30	0.03						
21:0	1.61	0.01	1.72	0.2	0.70	0.01	1.79	0.04	0.38		0.26	0.02	0.6	0.3	0.22	0.05	1.84	0.02	2.33	0.2	3.50	0.2
14:1	1.20	0.1	1.27	0.1	0.96	0.05	0.91	0.1	0.62	0.02	0.46	0.3	0.20	0.02	0.12	0.02	1.18	0.1	0.94	0.1	1.20	0.1
16:1 n7	1.26	0.2	1.22	0.03	1.70	0.2	0.99	0.1	2.50	0.07	0.84	0.03	1.11	0.36	1.35	0.08	2.31	0.2	1.81	0.2	5.60	0.1
16:1 n9	2.95	0.2	1.70	0.7	0.30	0.02	2.23	0.2	0.72	0.04	0.55	0.02	0.17	0.02	0.31	0.16	1.12	0.1	0.74	0.2	1.00	0.01
17:1n8	0.92	0.03	2.17	0.9			1.65	0.1							0.31	0.2	1.47	0.2	1.55	1.1	2.00	0.2
18:1 n9	30.12a	2.04	16.34d	2.1	39.8a	12	24.9a	2.6	28.7a	1.0	66d	0.6	56.7d	6	61.5d	2.6	24.0a	2.1	24.26a	2.4	23.2a	3.1
18:1 n7	2.96	1.0	3	0.5	2.75	0.2	3.37	0.5	2.50	0.03					3.04		0.7	3.18	0.6	4.13	0.5	
18:2 n6	18.58a	1.1	24b	1.2	17.6a	4	19.72a	1.2	10.3d	0.3	10.2d	0.2	12.0b	3	11.4c	1.7	20.6a	1.3	20.66a	1.2	19.2a	1.6
18:3 n3	1.11	0.02	1.53	0.2	0.85	0.01	1.10	0.01	0.88	0.05	0.68	0.01	0.65	0.12	0.77	0.02	0.97	0.01	0.68	0.02	4.90	0.3
20:4 n6	4.0a	0.2	8.05d	0.4	4.24a	0.02	6.88a	0.2	2.6c	2.0	1.6d	0.5	6.14a	0.6	1.6d	0.5	7.12a	0.2	6.4a	0.3	2.50b	0.2
P/S	0.60		0.80		0.70		0.70		0.28		0.65		0.75		0.60		0.79		0.70		0.72	

Values are the M±SD of three determinations. Major fatty acids content in raw vs. cooked meat cuts are compared: values in the same line, followed by different letters are significantly different (a *vs* b= p<0.05; a vs. c= p<0.01; a *vs* d= p<0.001).

let and sirloin raw cuts and in top-side cooked as "escalope" (Table 3) which showed the highest reduction in SFA content upon cooking. The lowest P/S ratio was found in "saltimbocca", the meat-based dish with the highest SFA level. Our findings agree with previous studies showing that in cooked meat this ratio generally increased (ONO *et al.*, 1985; GERBER *et al.*, 2009). Figure 1 shows the percentage of SFA, MUFA and PUFA of the samples analysed. The main differences in the balance among the three fatty acid classes in the recipes compared to raw meat reflected the observed modifications in individual fatty acid content. In this study no major changes were observed among the three FA classes in the meat cuts cooked without added ingredients compared to the raw cut. In the dishes added with extra-virgin olive oil the ratio among SFA, MUFA and PUFA compared to raw meat was shifted in favour of MUFA. By contrast, the "saltimbocca" dish, which includes animal foods as main ingredients, showed the highest SFA content compared to the raw cut (Fig. 1).

Vitamins

Thiamin content in raw veal cuts was slightly higher than that reported previously (LOMBARDI-BOCCIA *et al.*, 2005), only trace amount of it was detected in raw sirloin but, after barbecuing, the marked water losses occurred made it detectable (Table 4). The amount of riboflavin was markedly higher than that reported by LOMBARDI-BOCCIA *et al.* (2005) and by GERBER *et al.* (2009). Vitamins content in meat-based dishes showed to be influenced by both dish composition and the severity of the adopted cooking methods (Table 4). Among the three cuts analysed a significant increase in thiamin concentration was detected in "saltimbocca" ($p<0.001$), probably due to the presence of ham in the dishes, on the other hand a significant decrease in this vitamin was detected in "escalope" ($p<0.05$). As far as riboflavin was concerned, significant increases in its concentration compared to raw meat were observed in both fillet based dishes ("in pan" $p<0.001$; roasted with bacon $p<0.05$) and in sirloin cooked "in pan" ($p<0.05$). On the other hand significant decreases were found in "saltimbocca" and "vitel tonnè" ($p<0.05$ both) and in "barbecued"

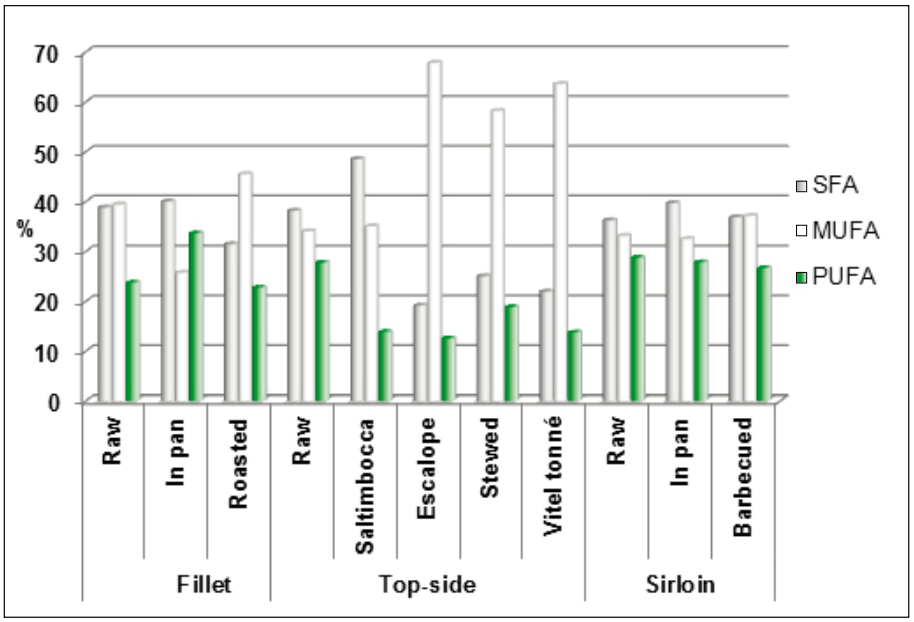

Fig. 1 - Percentage of SFA, MUFA and PUFA of raw veal cuts and respective dishes.

sirloin (p<0.001) (Table 4). Niacin content resulted significantly higher in the dishes without added ingredients like fillet "in pan" and "barbecued" sirloin (p<0.05 both), compared to the raw cuts. Among the meat-based dishes significant increases were detected in fillet "roasted with bacon" (p<0.05) and, among the top-side based dishes, in "saltimbocca" (p<0.001) probably because of the contribution of ham, otherwise a significant reduction was found in "escalope" (p<0.05) and in "stewed" (p<0.001) dishes. Among liposoluble vitamins only vitamin E was detectable; generally, a significant increase in vitamin E concentration was observed in the dishes including extra-virgin olive oil as main ingredient compared to the raw cut. This behaviour was detected in fillet "roasted with bacon" and "vitel tonnè" (both p<0.001), as well as in "stewed" and "escalope" (p<0.05 both) (Table 4), because most of the meat-based dishes studied included new sources of vitamin E from the ingredients utilised (especially extra-virgin olive oil) which masked losses due to heat. A similar behaviour in vitamins content in dishes made up with beef was reported (D'EVOLI *et al.*, 2009). The meat cuts cooked without added ingredients showed a significant loss in vitamin E like in fillet cooked "in pan" (p<0.05) or not relevant differences compared to the raw cut. *T*-retinol was detected only in trace amounts (Table 4).

Minerals

Mineral content in the dishes based on veal meat is reported in Table 5. Data on mineral content in the raw veal cuts found in this study were in the range previously reported, although among trace elements Fe and Zn were found in lower amount (LOMBARDI-BOCCIA *et al.*, 2005). The main differences between the raw cuts and the respective dishes were observed mainly in

Table 4 - Hydrosoluble and liposoluble vitamins in raw veal cuts and in the respective dishes (f.w.).

	Thiamin mg/100 g	Riboflavin mg/100 g	Niacin mg/100 g	Vit. E mg/100 g	*t-retinol* mg/100 g
Fillet					
raw	0.08±0.05a	0.33±0.01a	7.96±0.5a	0.21±0.03a	tr.
in pan	0.14±0.06a	0.95±0.1c	8.92±0.3b	0.14±0.03b	tr.
roasted with bacon	0.11±0.03a	0.45±0.03b	10.76±0.9b	0.47±0.01c	tr.
Top-side					
raw	0.08±0.03a	0.48±0.09a	8.29±0.3a	0.15±0.04a	tr.
saltimbocca	0.29±0.01c	0.33±0.01b	10.3±0.4c	0.12±0.09a	tr
escalope	0.02±0.01b	0.43±0.03a	6.71±0.3b	0.28±0.02b	tr.
stewed	0.14±0.06a	0.56±0.04a	5.54±0.5c	0.40±0.04b	tr.
vitel tonné	0.04±0.01a	0.29±0.04b	7.14±1.9a	1.95±0.35c	tr.
Sirloin					
raw	tr	0.62±0.04a	10.86±1.2a	0.16±0.01a	tr.
in pan	tr	1.06±0.11b	8.29±2.8a	0.14±0.03a	tr.
barbecued	0.07±0.01	0.24±0.01c	15.54±1.7b	0.21±0.02a	tr.

Values are the M±SD of three determinations. Values, in the same column (grouped for meat cut), followed by different letters are significanly different from the respective raw cut (a *vs* b= p<0.05; a *vs* c=p<0.001).

Table 5 - Minerals and trace elements in raw veal cuts and in the respective dishes (f.w.).

Top-side	Ca	Mg	P	K	Na	Fe	Zn	Cu	Mn
	mg/100 g								
Fillet									
raw	6.4±2.1a	20±1.7a	185±14a	408±20a	51±4a	0.54±0.01a	1.7±0.8a	0.05±0.05a	0.01±0.002a
in pan	6.3±0.6a	24.7±3a	226±20b	493±45b	66±7b	0.68±0.07b	1.9±0.15a	0.07±0.04a	0.17±0.021d
roasted with bacon	12.5±1.2b	22.2±2a	208±5a	496±10c	219±10d	0.69±0.02d	2.1±0.06a	0.06±0.06a	0.23±0.001d
Top-side									
raw	4.4±0.2a	20.7±0.3a	197±2a	395±6a	47±2.2a	0.50±0.02a	2.1±0.01a	0.04±0.007a	0.01±0.001a
saltimbocca	7.9±1.0c	22.4±0.5c	200±4a	525±5d	467±21d	0.69±0.10b	2.0±0.04a	0.04±0.002a	0.05±0.003d
escalope	5.9±0.8b	21.4±0.3b	197±3a	424±13b	108±2.2d	0.44±0.05a	1.2±0.02d	0.05±0.005a	0.04±0.004d
stewed	7.6±0.2d	24.8±0.2d	229±2d	554±6d	274±4d	0.68±0.03d	3.1±0.01d	0.06±0.001c	0.04±0.001d
vitel tonnè	6.9±0.7c	18±0.6c	169±6c	318±4d	274±18d	0.72±0.02d	2.9±0.13d	0.06±0.003c	0.01±0.001a
Sirloin									
raw	4.7±0.2a	19.4±1.1a	184±11a	397±7a	41±1a	0.56±0.03a	1.9±0.09a	0.03±0.002a	0.01±0.002a
in pan	4.6±0.2a	20.5±1.2a	195±11a	396±5a	46±3a	0.54±0.03a	1.8±0.01a	0.04±0.001d	0.01±0.001a
barbecued	7.9±0.4d	24.4±1.8b	234±14c	481±15d	50±5b	0.67±0.02c	2.5±0.02d	0.08±0.004d	0.02±0.002c

Values are the M±SD of three determinations. Values (grouped for meat cut), followed by different letters, are significantly different from the respective raw cut (a *vs* b= p<0.05; a *vs* c= p<0.01; a *vs* d= p<0.001).

Na content. With the exception of the "in pan" and "barbecued" dishes which were the only ones without added ingredients, all the other dishes showed significant increases (p<0.001 for all) in Na content compared to the respective raw cuts. These differences in Na concentration were strictly related to a discretionary salt amount added in each dish (Table 1). As far as trace elements are concerned, Fe content was significantly higher in both the fillet based dishes ("in pan" p<0.05, "roasted with bacon" p<0.001) compared to the raw cuts; among the top-side based dishes Fe content was significantly higher in "saltimbocca", "stewed" and "vitel tonnè" dishes (p<0.001), and among the sirloin dishes in the "barbecued" one (p<0.01) (Table 5) In meat-based dishes with no added ingredients, this raise is explained by moisture loss; in the case of composite dishes such as vitel tonnè (Table 1) the presence of canned tuna and anchovies yields to an additional amount of iron (canned tuna 1.6 mg/100 g; anchovies 4.1mg/100 g; FOOD STANDARDS AGENCY, 2002) and heme iron as well. Zinc content resulted significantly higher only in some top-side based dishes like "escalope", "stewed" and "vitel tonnè" and in sirloin "barbecued" (p<0.001 for all) compared to raw cuts (Table 5). Cu and Mn content also showed increased concentration in most of the veal based dishes analysed compared to the respective raw cuts. The differences observed in trace element content were always inherent to the ingredients (ham, bacon, tuna fish, anchovies), as sources of additional trace elements. In raw cuts heme-iron was from 73 to 82% of the total iron content (Fig. 2). Generally, an increase in heme iron content up to 8% was found in those dishes made up with ingredients which added new sources of heme iron. The highest increase was found in fillet "roasted with bacon" and in top-side cut cooked as "saltimbocca" or "vitel tonnè" (Fig. 2). By contrast, a previous study dealing with beef meat

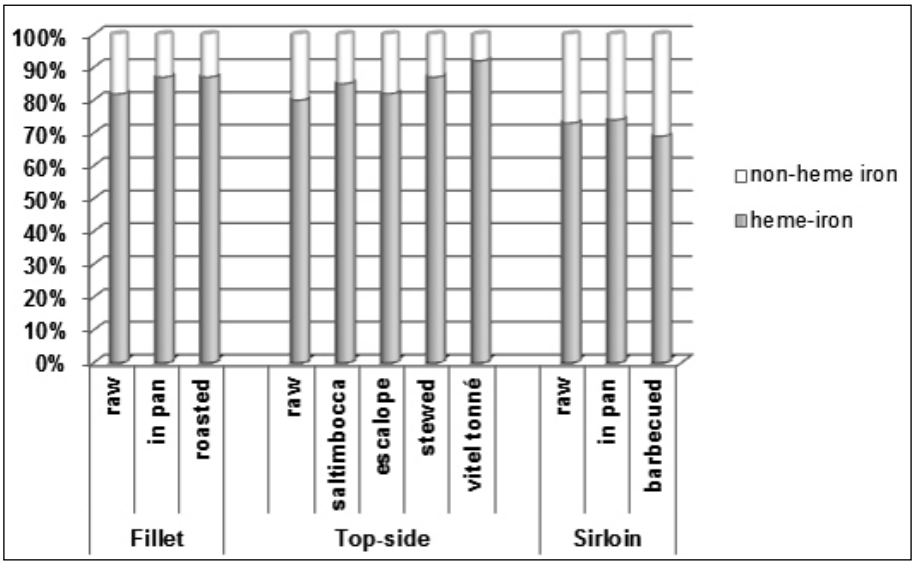

Fig. 2 - Percentage of heme-iron to toal iron of raw veal cuts and respective dishes.

cooked without the addition of heme iron rich ingredient, showed that heat treatments modified the heme/non-heme iron ratio, inducing marked reductions in heme iron concentration depending on the severity and the duration of the heat treatment (D'EVOLI *et al.*, 2009).

CONCLUSIONS

Meat consumption greatly contributes to nutrient supply but also provides less healthier molecules like saturated fatty acids and cholesterol. However, cooking methods can lead to important modifications in nutrient dietary intakes when foods are consumed as composite dishes. Our findings pointed out that when meat is cooked without any additional ingredient (in pan or barbecue), the nutritional profile was minimally altered. The variety of both the ingredients which make up the dishes and the cooking methods contribute to modify the nutritional profile of the meat. Factors like type of cooking (moist and dry heating), temperature, cooking time and ingredients, deeply contribute to modify the forecast data about the nutrient intake level. For example the consumption of meat in composite dishes containing vegetable fat instead of animal fat as added ingredient can change favourably the profile of fatty acids. In this study the proportion of fatty acids in the meat-based dishes changed with respect to both raw meat and meat cooked without ingredients, being influenced by the culinary fat added: the presence of extra-virgin olive oil gave rise to a marked increase in monounsaturated fatty acids. Similarly, the inclusion of extra-virgin olive oil in the dishes increased also the levels of vitamin E beneficial to human health. At the same time, other ingredients of the meat-based dishes can lead to a raise in some unhealthy molecules, such as an increased level of sodium (due to discretionary salt) or cholesterol. Anyway, the knowledge of the changes in nutritionally interesting molecules (eg. fatty acids, heme-iron, vitamins, cholesterol) occurring upon cooking is of importance because it allows to get a correct calculation of their actual intake. From a nutritional point of view, to collect data on the nutrient profile of meat-based dishes representative of the diverse worldwide eating habits has a relevant nutritional significance. This represents the most appropriate basis for reliable information on the actual nutrient intake and can be a proper and useful tool to formulate more accurate diets.

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PAPER

POLYPHENOL PROFILES OF TUNISIAN APPLES AND EVALUATION OF THEIR TOTAL ANTIOXIDANT CAPACITY

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ABSTRACT

Six varieties of Tunisian apples were studied on the basis of their detailed polyphenol profiles. Reversed Phase High performance Liquid Chromatography (RP-HPLC) was used to separate and identify the compounds. Thiolysis coupled to RP-HPLC was used to determine the nature and the proportion of the constitutive units as well as the concentration of procyanidins. The structural analysis of some phenolic compounds was carried out using an ion trap mass spectrometer equipped with an electrospray source (LC-ESI-MS) used in negative mode. The total antioxidant capacity was determined using the FRAP method.

- Key words: FRAP, *Malus domestica*, procyanidins, RP-HPLC, thiolysis -

1. INTRODUCTION

In recent years, the interest in polyphenols in fruits and vegetables has increased considerably, mainly in relation to their beneficial effects on human health often attributed to their antioxidant activity (SCALBERT and WILLIAMSON, 2000). Phenolic compounds contribute significantly to the quality and the diversity of apples and apple products, including juices and ciders. Polyphenols are largely involved in astringency, bitterness and color that are essential criteria in defining the character and diversity of apples (LEA, 1990). Apple is a source of polyphenols and its nutritional role is partly allotted to their antioxidant status (LU and FOO, 2000) which is correlated to its content of phenolic compounds (LEE *et al.*, 2003). Apples also contain ascorbic acid. However, its contribution to the total antioxidant capacity is rather poor (< 0.4%) (EBERHARDT *et al.*, 2000).

Apple phenolics have a wide variety of structures and can be divided into five main classes:

The flavan-3-ols (F3ols) which are subdivided in two classes: monomers (catechins) and oligomers and polymers, namely procyanidins (PCA) also called condensed tannins. The catechins are mainly (-)-epicatechin (EC) and (+)-catechin (CAT). The structure of procyanidins differs according to the nature and the number of constitutive units and also according to the nature, position and stereochemistry of the bonds linking the catechin units (THOMPSON *et al.*, 1972; PORTER, 1988). These compounds are the main phenolic class in apples (GUYOT *et al.*, 2002) and their concentration is generally high in cider varieties (SANONER *et al.*, 1999).

The second class, hydroxycinnamic acids, includes mainly 5-*O*-caffeoylquinic acid (CA) and *p*-coumaroylquinic acid (PCQ). Some other caffeoylquinic acid isomers have been detected in some varieties but always in minor concentrations.

Dihydrochalcones are not found in high concentrations and are very specific to apples (HERRMANN, 1990), although they have also been detected in very low concentrations in strawberries (HILT *et al.*, 2003). Phloridzin (PLZ) and phloretin xyloglucoside (XPL) are the principal compounds in this class.

Flavonols, which constitute the fourth class, are essentially located in the epidermis zone (peel) and contribute to the yellow color.

Finally, anthocyanins can be considered as an additional class since they are present in some varieties, contributing to the red color of the peel and the parenchyma in a few pink flesh varieties. However, they generally only constitute a very small proportion of all the polyphenols.

Many articles dealing with the separation and characterization of polyphenols in apple varieties using HPLC have been published. Most of them were carried out using reversed phase HPLC coupled to a diode array UV-visible detector. Some of them used thiolysis or phloroglucinolysis prior to HPLC analysis in order to improve the characterization and quantification of procyanidins. These studies were carried out with apples from several countries including France (SANONER *et al.*, 1999), England (MARKS *et al.*, 2007), Poland (WOJDYLO *et al.*, 2008), Germany (SCHIEBER *et al.*, 2001) and Italy (TAROZZI *et al.*, 2004). However, as far as we know, no data concerning the detailed polyphenol profiles of apple varieties that grow in south Mediterranean countries such as Tunisia are available. We formulated the hypothesis that the climate in this country may have some consequences on the biosynthesis of polyphenols in these fruits growing in particular conditions.

The objective of this study was therefore to determine the polyphenolic profiles in the tissue zones (flesh, peel and seeds) of six Tunisian apple varieties; two of them were local varieties. The total antioxidant capacity of each tissue was also evaluated using the FRAP method.

Thiolysis coupled to reversed phase High Performance Liquid Chromatography (RP-HPLC) was chosen to analyze the procyanidins (BETTS *et al.*, 1967) because unlike a single assay for total procyanidins, it also gives access to the nature and proportions of the constitutive units and to the average degree of polymerization (DPn). RP-HPLC without thiolysis was also performed in order to obtain the detailed profiles of phenolics belonging to other classes, namely catechins, hydroxycinnamic acids, flavonols and dihydrochalcones.

In addition, some individual molecules of the procyanidin class, for which standards were not available, were characterized using liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) in negative mode (LAZARUS *et al.*, 1999).

2. MATERIALS AND METHODS

2.1 Solvents, reagents and phenolic standards

Sodium fluoride (Prolabo, Fontenay-sous-bois, France); acetic acid (Biosolve, Fisher chemicals HPLC grade); Acetonitrile (Prolabo, HPLC gradient grade); Anhydrous methanol (Prolabo, HPLC grade); Toluene-α-thiol and hydrochloric acid were purchased from Merck (Darmstadt, Germany); Formic acid was purchased from Fischer scientific (Analytical grade, Loughborough, UK).

TPTZ, 2,4,6-tripyridyl-s-triazine (Flucka Chemicals Switzerland); Glacial acetic acid was purchased from Biosolve Ltd (Valkenswaard, Holland); Sodium acetate (Merck, Darmstadt, Germany); Pure $FeCl_3$ $6H_2O$ 99-102% (Flucka Chemicals Switzerland); pure $FeSO_4$ $7H_2O$ 99.5%

(Merck, Darmstadt, Germany); Ultrapure water was obtained using a Milli-Q water system (Millipore, Bedford, MA).

Phenolic standards: (-)-epicatechin (EC), (+)-catechin (CAT), 5-*O*-caffeoylquinic acid, *p*-coumaroylquinic acid, Quercetin and Phloridzin were purchased from Sigma (Sigma–Aldrich, Germany). Procyanidin B1 and B2 were purchased from Extrasynthèse (Lyon, France). (-)-Epicatechin benzylthioether used for the calibration was purified in the Laboratory. Its unambiguous identification was checked by comparison with our own work published previously (GUYOT *et al.*, 1997).

2.2 Plant materials

Six varieties of Tunisian apples were used in this study (*Zina*, *Chahla*, *Golden*, *Anna, Lorca*, Local *Golden*). The apples were harvested during the 2008 season in the regions of Jedeida (North-East of Tunis) and Sbiba (center of Tunisia). The fruits were kept at +2°C for one week. Before treatment, the fresh apples were washed with pure water. In order to take into account the variability of the fruit, three batches of nine fruits were constituted for each variety according to the method described by GUYOT *et al.* (1998). Tissues were manually separated (flesh, peel and seeds) and a sodium fluoride solution ($1 g.L^{-1}$ water) was sprayed on the tissues to inhibit oxidation. The tissues were then freeze-dried and transformed into a fine homogeneous powder by crushing with an electrical crusher (Retsch, model YGG, Bioblock scientific). The powders were kept in a desiccator until analysis.

2.3 Methanol extraction of freeze dried powders

Methanol extraction was used to extract small phenolic molecules including monomeric catechins, small procyanidin oligomers, hydroxycinnamic acids, flavonols and dihydrochalcones. Accurately weighed aliquots of powder (30 mg) were extracted with 1 mL of pure methanol containing 1% v/v acetic acid for 15 min in an ultrasonic bath (Brasson 2200, USA). The mixture was then filtered on PTFE filters (0.45 µm, Uptidisc interchim, France). The filtrate was ready for HPLC analysis.

2.4 Thiolysis reaction applied to freeze-dried powders

Thiolysis was used to complete the characterization and quantification of the procyanidins. When it is directly applied to apple powders without prior solvent extraction, this technique is very pertinent and informative since it allows the global characterization and the quantification of procyanidin molecules (particularly polymerized forms) that are not extracted by classical methanol extraction (GUYOT *et al.*, 2001). The first work concerning the use of thiolysis for the characterization of condensed tannins was published by Betts (BETTS *et al.*, 1967). However, the proposed structures for condensed tannins contained some errors. Then, in 1972, Thompson *et al.* published a more complete investigation on proanthocyanidin structures using thiolysis (THOMPSON *et al.*, 1972).

The method makes it possible to distinguish between the terminal units and the extension units of proanthocyanidins, the latter being converted into benzylthioether derivatives whereas the former are released as monomeric catechins. Consequently, when the extract also contains free monomeric catechins, these are not distinguished from the final units released by the thiolysis reaction.

The thiolysis reaction leads to the depolymerization of procyanidin structures by converting the flavanol extender units into their carbocations and the lower units into monomeric flavanols. The carbocations immediately combine with toluene-α-thiol, leading to the formation of thioether adducts. By making the distinction between terminal and extension units, HPLC analysis of thiolysis media allows the nature and the proportion of the constitutive units of procyanidins to be determined. Moreover, their average degree of polymerization (DP*n*) can thus be calculated (GUYOT *et al.*, 2003).

Freeze-dried samples (30 mg) were added to 800 µL of toluene-α-thiol 5% in methanol (v/v) and 400 µL of methanol acidified with HCl (0.4 N). After homogenization, the samples were heated to 40°C in a bain-marie for 30 min. The thiolysis reaction was stopped by a thermal shock in crushed ice for 5 min. The mixture was filtered on PTFE filters (0.45 µm, Uptidisc interchim, France). The filtrate was ready for HPLC analysis.

2.5 RP-HPLC of methanol extracts and thiolysis reaction medium

The samples were injected onto a RP-HPLC system composed of an automatic injector model WISP 717 (Waters, Milford, USA) thermostated at 4°C, a gradient pump Model 600 (Waters) and a diode array detector model 996 (Waters). The column was a RP18 Purospher endcapped 5 µm, 80 Å, 4 x 250 mm (Merck, Darmstadt, Germany) equipped with a 4 x 4 mm guard column of the same RP material and thermostated at 30°C. A gradient solvent system with solvent A (aqueous acetic acid, 2.5% v/v) and solvent B (gradient quality acetonitrile) was used and the following gradient was applied at a constant flow rate of 1 mL/min: initial, 3% B; 0-5 min, 9% B linear; 5-15 min, 16% B linear; 15-45 min, 50% B, linear followed by washing and reconditioning of the column. The solvents were degassed with helium (repeatedly bubbling for

20 seconds, stopping for 40 seconds and then bubbling again). The injection volume was 10 μL.

The acquisition, integration and processing of the signal were controlled using Millennium software 2010 version 2.1. Simultaneous monitoring was performed at 280 nm (Flavan-3-ols and dihydrochalcones), 320 nm (hydroxycinnamic acids) and 350 nm (flavonols). Spectra were recorded between 200 and 600 nm (GUYOT *et al.* 1998).

**Processing of the methanol extract chromatograms for the identification and assay of simple phenolics and procyanidin oligomers*

HPLC peaks were identified on chromatograms according to their retention times and their UV-visible spectra by comparison with standards. Caffeoylquinic acid, (+)-catechin, (-)-epicatechin, hyperoside and phloridzin were commercially available (Sigma-Aldrich). Phloretin xyloglucoside identification has already been performed in a previous study (SANONER *et al.*, 1999) and its quantification was expressed in phloridzin equivalents. For each compound, the quantification was performed by reporting the measured integration area in the calibration equation of the corresponding standard. Integrations were performed at 280 nm for flavanols and dihydrochalcones, at 320 nm for hydoxycinnamic compounds, or at 350 nm for flavonols.

**Processing of the thiolysis chromatograms for the characterization and assay of the whole procyanidin fraction*

Procyanidin concentrations were obtained by summing all the catechin equivalents assayed on the thiolysis chromatogram after subtraction of the amounts corresponding to native monomeric catechins assayed on the chromatogram without thiolysis.

The terminal units are directly released in their catechic form. The calculation of the average degree of polymerization (DPn) of the flavan-3-ols is given by the relationship (1).

$$\text{DPn flavan-3-ols} = \text{Total flavan-3-ols} / / [\text{T\_CAT} + \text{T\_EC}] \quad (1)$$

Total flavan-3-ols = [T_CAT + T_EC +T_EBTE]

[*T_CAT*] = total (+)-catechin concentration obtained after thioacidolysis.

[*T_EC*] = total (-)-epicatechin concentration obtained after thioacidolysis.

[*T*_EBTE] = (-)-epicatechin benzylthioether (extension units) concentration obtained after thioacidolysis.

For procyanidins *sensu stricto*, the selective quantification of these two types of unit allows the procyanidin DPn to be determined according to equation (2).

$$\text{DPn procyanidins} = (\text{Total procyanidins}) / / ([\text{T\_CAT}] + [\text{T\_EC}] - [\text{CAT}] - [\text{EC}]) \quad (2)$$

Total procyanidins = Total flavan-3-ols – ([CAT] + + [EC])

[*CAT*] = total (+) - catechin concentration.

[*EC*] = total (-)-epicatechin concentration.

2.6 Structural characterization of procyanidin oligomers and other phenolics using liquid chromatography coupled to mass spectrometry (LC-MS)

LC-MS was used to complete the structural characterization of the compounds that were detected on UV chromatograms and for which standards were not available. This particularly concerned procyanidin oligomers.

Weighed aliquots of powder (30 mg) were suspended in 1 mL of pure methanol containing 1% acetic acid (v/v) and extracted for 15 min in an ultrasonic bath (Brasson 2200, USA). The mixture was then filtered on PTFE filters (0.45 μm, Uptidisc Interchim, France). The filtrate was ready for LC-MS analysis.

Procyanidin oligomers were easily detected according to their $[M-H]^-$ pseudo-molecular ions by ESI MS in negative mode, as previously observed for condensed apple tannins (GUYOT *et al.*, 1997). The compounds were separated on an HPLC apparatus composed of an SCM1000 degasification system (ThermoQuest, San Jose, CA, Usa), an automatic injection system (ThermoFinnigan, San Jose, CA, USA), a binary pump 1100 Series (Agilent Technologies, Palo Alto, CA, USA) and a photodiode array detector Spectra system UV6000LP (ThermoFinnigan, San Jose, CA, USA). The mass spectrometer was an ion trap LCQ Deca (ThermoFinnigan San Jose, CA, Usa) equipped with an Electrospray ionization source (ESI). Samples injected (5 μL) were separated on a Zorbax Eclipse XDB-C18 column (2.1 x 150 mm, 3.5 μm, Agilent Technologies) of which the temperature was maintained at 30°C. The MS spectra were acquired in "*Full Scan*" negative ionization mode in the 50-2,000 m/z range. The source parameters were as follows: spray voltage (5 kV), capillary voltage (12.0 V), sheath gas (67 arbitrary units), auxiliary gas (5 arbitrary units), capillary temperature (240°C). Nitrogen was used as the nebulizing gas. The HPLC gradient conditions were as follows: solvent A (aqueous formic acid, 0.1% v/v) and solvent B (acetonitrile containing 0.1% formic acid v/v) with a flow rate of 0.2 mL/min; initial, 3% B; 0-5 min, 9% B, linear; 5-15 min, 16% B, linear; 15-45 min, 50% B, linear followed by washing and reconditioning of the column to restore the initial conditions. The injection volume was 3 μL.

Peaks giving *m/z* values corresponding to procyanidin on Total Ion Current chromatograms (TIC) were further investigated by LC-ESI/MS/MS. Helium gas was used as a collision gas and the potential change defining the collision energy was optimized in the range of 25-35 V to optimize the production of both parent and daughter ions.

2.7 Ferric reducing antioxidant power assay (FRAP)

The procedure was adapted from Benzie (BENZIE *et al.*, 1999). The method is based on the reduction of the colorless ferric-tripyridyltriazine complex (Fe^{III}-TPTZ) to its blue ferrous-tripyridyltriazine form (Fe^{II}-TPTZ) which can be measured by monitoring the change in absorbance at 593 nm. This change is directly linked to the oxidation of the antioxidants present in the medium. The FRAP values given were as described by Jiang (JIANG *et al.*, 2006).

Briefly, the FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), a solution of 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3$ $6H_2O$ at 10:1:1 (v/v/v) (BENZIE *et al.*, 1999). To 10 µL of control (ultrapure water), standard ($FeSO_4$ $7H_2O$ (1,000 µM)) or sample, were added 90 µL of ultrapure water and 200 µL of FRAP reagent (the plate was incubated at 37°C for the duration of the reaction). The absorbance was taken at 593 nm immediately after and at 1-min intervals for 4 min using a UV-vis microplate kinetics reader (Multichannel spectrophotometer SPECTRAmax PLUS, Model 384). The change in absorbance ΔA_{593nm} of a sample was translated into FRAP values by proportionality with the absorbance values of a standard solution ($FeSO_4$) of known concentration (Fe^{2+}, 1,000 µM). The data were collected and treated using SOFT max Pro 3.0 software.

3. RESULTS AND DISCUSSION

The qualitative and quantitative polyphenol profiles of Tunisian apple varieties were characterized by combining several approaches including LC-MS/MS of methanol extracts and LC-UV-visible quantification with or without thiolysis.

Initially, methanol extracts of apple samples were qualitatively analyzed using LC-DAD-MS. An example of an LC-MS extracted ion chromatogram is presented in Fig. 1. Then, the main phenolic compounds in the apple samples were identified or characterized by comparison with available standards and interpretation of the MS and UV-visible data corresponding to the main chromatographic peaks. These qualitative data including retention times (TR), λmax and pseudo molecular ions are presented in Table 1.

Three hydroxycinnamates were detected: 5-O-caffeoylquinic acid, caffeic acid and p-coumaroylquinic acid. Ten flavan-3-ols were detected: (+)-catechin, (-)-epicatechin, procyanidin B1, B2 and C1 and five additional procyanidin oligomers noted PCA_X1 to PCA_X5. Three dihydrochalcones, namely phloridzin, phloretin and phloretin xyloglucoside were also detected. These results are in agreement with those of Wojdylo (WOJDYLO *et al.*, 2008).

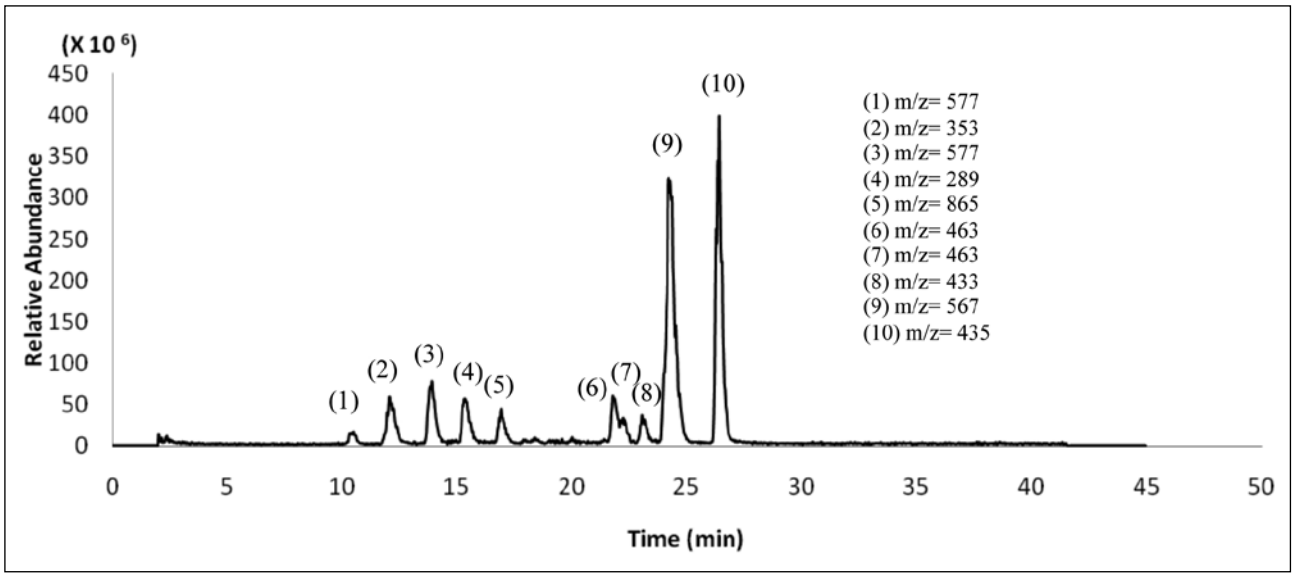

Fig. 1 - Full MS extracted ion chromatogram of apple peel methanol extract (Zina variety). *Specific pseudo-molecular ions corresponding to hydroxycinnamic acids, flavanols; flavonols and dihydrochalcones are selectively extracted from the Full MS data: (1), procyanidin dimers B1; (2),5-O-caffeoylquinic acid; (3), procyanidin dimers B2; (4), (-)-epicatechin; (5), procyanidin trimers C1; (6), isoquercitrin; (7), hyperoside; (8), reynoutrin; (9), phloretin xyloglucoside; (10), phloridzin.*

As already observed in previous works on apple phenolics (OLESZEK *et al.*, 1988; TEUBER *et al.*, 1978; MAYR *et al.*, 1995), the main flavonols detected were avicularin (Avi), quercitrin (QCl), isoquercitrin (isoQ), hyperoside (HYP), reynoutrin (REY) and rutin (RUT), and were primarily located in the peel.

Characterization of procyanidin oligomers by LC-ESI-MS in negative mode

The UV-visible spectra of apple catechins and procyanidins are very similar. For this reason, only compounds available as certified standards can be identified by DAD UV-visible detection on HPLC chromatograms. LC-ESI/MS is very useful to complete the characterization of procyanidins for which standards are not easily available, at least through the determination of their degree of polymerization. It also allows B-type and A-type procyanidins to be distinguished. The procyanidin composition of apple was characterized by comparing retention times with the procyanidin standard and by extracting ions from the TIC corresponding to each oligomeric group and generating reconstructed ion chromatograms. HPLC chromatograms and mass spectra indicated that the apples contained homogeneous amounts of B-type oligomers and contained trimers, tetramers and pentamers (Table 1).

3.1 DPn of flavan-3-ols and DPn of procyanidins

The average degree of polymerization was estimated by reversed phase HPLC analysis following thiolysis. The seeds were characterized by a higher average degree of polymerization (DPn) of flavanols in comparison with those observed in the flesh and the peel, except for the *Golden* variety, where DPn in the seeds (5.7) was lower than in the peel (7.5). For all the varieties, the DPn of F3ols in the peel was higher than in the flesh. These results (Table 2) are in accordance with those previously found by Guyot (GUYOT *et al.*, 2003).

A mixture of procyanidins (PCA) containing a range of molecules with different DP, was characterized by its DPn. Depending on the apple extract studied, the DPn of procyanidins varied from 3 to 77.7. The values found for the seeds oscillated between 6.8 for the *Golden* variety and 77.7 for *Chahla* (Table 2). On the other hand, the values were between 3.1 (*Zina*) and 20.3 (*Golden*) in the flesh and between 7.3 (*Zina*) and 16.3 (Golden) in the peel (Table 2). The results highlighted the existence, in some varieties, of particularly high DPn ($\approx$78) in the seeds (Table 2).

Table 1 - Negative mode LC-UV-visible-MS identification of some phenolic compounds in the peel methanol extracts of the Zina variety.

Identification	RT [a] (min)	λ_{max} (nm)	$[M-H]^-$ m/z	Major fragments	References
Flavan-3-ols					
(+)-catechin	11.88	278.3	289	245; 205; 203; 179	Wojdylo *at al*., 2008
(-)-epicatechin	15.42	278.3	289	245; 205; 203; 179	Wojdylo *at al*., 2008
procyanidin dimer B1	10.38	279.6	577	451; 425; 407; 289	Wojdylo *at al*., 2008
procyanidin dimer B2	13.86	279.6	577	451; 425; 407; 289	Wojdylo *at al*., 2008
procyanidin trimer C1	16.91	280.5	865	577	Wojdylo *at al*., 2008
Procyanidin isomers					
PCA_X1 (trimer)	16.98	280.6	865	577	-
PCA_X2 (tetramer)	17.98	280.6	1,153	865; 577; 289	-
PCA_X3 (pentamer)	19.28	280.6	1,441	1153; 289	-
PCA_X4 (trimer)	20.03	280.6	865	865; 577	-
PCA_X5 (tetramer)	21.30	280.6	1,153	865; 577; 289	-
hydroxycinnamic acids					
5-O-caffeoylquinic acid	12.11	325.5	353	191; 179	Wojdylo *at al*., 2008
caffeic acid	14.15	323.1	179	179; 135	-
p-coumaroylquinic acid	15.68	311.3	337	337; 289; 173	Wojdylo *at al*., 2008
Flavonols					
isoquercitrin	21.85	265; 355	463	300	Marks *at al.,* 2007
Hyperoside	22.27	261.9; 364.3	463	300; 225; 179	Marks *at al.,* 2007
Reynoutrin	23.18	267.1; 355	433	301;	-
Avicularin	24.05	259.9; 353.9	433	300	-
Quercitrin	24.56	256.9; 349.3	447	301	Marks *at al.,* 2007
Rutin	21.43	256.9; 354.1	609	301;179	-
Dihydrochalcones					
phloridzin	26.45	285.5	435	273; 167	Hilt *at al*., 2003
phloretin	26.35	285.5	273	167; 125	Wojdylo *at al*., 2008
phloretin xyloglucoside	24.31	285.8	567	433; 273; 167	Wojdylo *at al*., 2008

[a] RT: Retention Time.

Table 2 - DPn of flavan-3-ols and Procyanidins in the powder samples of the apple varieties.

Varieties[a]	DPn F3ols [b]						DPn PCA [c]					
	Flesh		Peel		Seeds		Flesh		Peel		Seeds	
Z	3.1	0.12	4	0.41	11.4	0.33	3.1	0.12	7.3	1.52	73.7	4.13
C	3.1	0.02	3.9	0.06	22.9	0.59	5.2	0.47	8.7	0.38	77.7	9.28
G	4.7	0.1	6.2	0.4	-	-	7	0.21	7.4	0.52	4.9	0.41
A	4.3	0.12	4.4	0.21	13.5	0.78	7	0.48	7.4	0.28	17.5	1.88
L	3.1	0.16	4.3	0.24	10	0.18	7.4	0.32	7.6	0.59	15.7	0.54
LG	5.8	0.11	7.5	0.15	5.7	0.08	20.3	0.36	16.3	1.14	6.8	0.11

[a] varieties: Z = Zina; C = Chahla; G= Golden; A= Anna; L= Lorca; LG= Local Golden.
[b] DPn F3ols: average degree of polymerization of the flavan-3ols; [c] DPn PCA: average degree of polymerization of the procyanidins; values in italics correspond to standard deviations (n=3).

In the case of the flesh and peel, the values are in accordance with those observed by SANONER *et al.* (1999) and HAMAUZU *et al.* (2008).

3.2 Concentrations of phenolic compounds in the different tissues

In the Peel

Concerning the catechin class, we noted that the ratio between (-)-epicatechin and (+)-catechin was highly variable according to the variety, with values varying from 0 to 37% of total flavanols in Golden and Lorca varieties, respectively. Several procyanidins were identified and characterized in the peels studied, including procyanidin B1 and B2 dimers, C1 trimers and five other procyanidin isomers named PCA_X1 to PCA_X5 with DP ranging from 3 to 5 (Table 3). Procyanidins ranged from 965 to 12187 mg/kg of DW depending on the variety.

As previously described in apples (GUYOT *et al.*, 2002), phloridzin, phloretin xyloglucoside and phloretin were the three identified representatives of the dihydrochalcones class. Phloridzin was present in the peel of all the varieties studied, accounting for 56 and 94% of total dihydrochalcones in the *Zina* and Lorca varieties, respectively. Phloretin was only detected in the *Zina* variety at very low levels (less than 0.37% of total dihydrochalcones).

As already observed (OLESZEK *et al.*, 1988; BURDA *et al.*, 1990), the flavonols: (Table 3) avicularin (Avi), quercitrin (QCI), isoquercitrin (isoQ), hyperoside (HYP), reynoutrin (REY) and rutin (RUT), were essentially localized in the epidermis zone (peel). Flavonols accounted for 18% (Chahla) to 52% (Golden) of total polyphenols in the peel, depending on the variety.

Hydroxycinnamic acids corresponded to an important class of polyphenols in apples (Table 3), except for the *Golden* variety that showed the lowest concentrations. In all varieties, caffeoylquinic acid (CA) was the main compound in this class. Caffeic acid was only detected as a minor compound in the *Chahla* variety, representing less than 3% of total hydroxycinnamic acids.

In the flesh

In contrast with the observations in the peel, the class of hydroxycinnamic acids was present in high levels in the flesh (Table 4). For all varieties, caffeoylquinic acid was the predominating compound in this class with concentrations varying from 53 to 95% of total hydroxycinnamic acids for the *Lorca* and *Zina* varieties, respectively. Interestingly, *p*-coumaroylquinic acid was present in higher proportions in the *Lorca* variety (46% of total hydroxycinnamic acids).

Concerning catechin and procyanidin classes, the results (Table 4) indicate the presence of (+)-catechin (CAT) in rather high quantities in the *Lorca* and *Local Golden* varieties although (-)-epicatechin remained largely prevalent in all the varieties studied. Several procyanidins were identified and characterized in the flesh. Procyanidin B2 was also present in significant amounts in the flesh, accounting for 16 to 58% of total procyanidins in the *Zina* and *Golden* varieties, respectively.

The class of dihydrochalcones is mainly constituted of phloridzin and phloretin xyloglucoside. In all the varieties studied (Table 4), the flavonols corresponded to the class with the lowest concentrations.

In the seeds

Seeds contained high concentrations of dihydrochalcones, with levels varying from 58% to 77% of total polyphenols for *Local Golden* and *Anna* varieties, respectively (Table 5).

Phloridzin was always present in very high concentrations compared to phloretin xyloglucoside, the latter accounting for only 0.8 and 4% in the *Lorca* and *Zina* varieties, respectively.

In the seeds, the other main polyphenol classes were hydroxycinnamic acids and flavanols. Caffeoylquinic acid (CA) was largely dominating (Table 5) whereas p-coumaroylquinic acids (PCQ) were only present in very low concentrations, except in the *Local Golden* variety in which it accounted for 15% of total HCA. Interestingly, hydroxycinnamic acids were absent in the *Golden* variety. The class of flavan-3-ols was characterized by

Table 3 - Concentration of phenolic compounds (mg/kg of DW) in the peel of the varieties studied.

Phenolic compounds[a]	Varieties											
	Zina		*Chahla*		*Golden*		*Anna*		*Lorca*		*Local Golden*	
Hydroxycinnamic Acids												
CA	3010	*126* [b]	1649	*64*	291	*27*	77	*6*	2053	*22*	6342	*16*
CAF	nd [c]	-	75	*10*	nd	-	nd	-	nd	-	nd	-
PCQ	108	*2*	516	*31*	44	*6*	51	*4*	1309	*78*	2753	*21*
PCQ_iso1	54	*1*	nd	-	nd	-	nd	-	33	*1*	102	*8*
PCQ_iso2	262	*13*	110	*14*	67	*9*	102	*7*	156	*11*	214	*5*
Total acids	3435	*144*	2351	*119*	404	*43*	231	*18*	3552	*113*	9412	*59*
Flavan-3-ols												
CAT	349	*36*	730	*18*	nd	-	446	*11*	1379	*18*	1466	*53*
EC	3636	*149*	5834	*81*	741	*51*	1630	*77*	3657	*92*	5607	*41*
B1	428	*18*	932	*49*	346	*16*	318	*11*	773,8	*15*	420	*19*
B2	1463	*34*	2718	*104*	711	*76*	783	*21*	1798	*39*	2229	*50*
C1	1241	*67*	3075	*43*	442	*30*	526	*13*	nd	-	nd	-
PCA_X1	640	*57*	1287	*59*	220	*19*	288	*13*	4154	*27*	8589	*48*
PCA_X2	449	*22*	886	*10*	121	*15*	230	*14*	nd	-	1028	*31*
PCA_X3	136	*13*	394	*18*	nd	-	nd	-	719	*38*	892	*16*
PCA_X4	186	*12*	298	*46*	nd	-	118	*6*	499	*25*	1489	*43*
PCA_X5	260	*24*	741	*17*	nd	-	nd	-	nd	-	nd	-
Total flavan-3-ols	8791	*436*	16899	*447*	2583	*210*	4341	*167*	12980	*255*	21977	*302*
Dihydrochalcones												
PLT	23	*0,6*	nd	-	nd	-	nd	-	nd	-	nd	-
PLZ	3497	*82*	4604	*69*	1445	*18*	2533	*57*	4723	*106*	5585	*71*
XPL	2723	*46*	5169	*86*	750	*46*	1909	*79*	296	*12*	623	*28*
Total dihydrochalcones	6243	*129*	9773	*155*	2195	*64*	4442	*136*	5019	*118*	6208	*99*
Flavonols												
AVI	1942	*99*	2814	*54*	886	*25*	2574	*71*	73	*6*	2725	*17*
QCI	743	*14*	1167	*44*	404	*12*	952	*16*	1465	*19*	1247	*18*
IsoQ	908	*18*	577	*13*	774	*32*	539	*13*	1162	*29*	nd	-
HYP	437	*16*	1001	*33*	2915	*123*	1705	*27*	2265	*11*	3341	*95*
REY	744	*18*	927	*21*	543	*15*	869	*7*	692	*25*	825	*31*
RUT	176	*5*	86	*9*	133	*4*	59	*5*	nd	-	nd	-
Total flavonols	4952	*172*	6572	*174*	5658	*213*	6699	*140*	5657	*91*	7883	*161*
Total polyphenols	23423	*883*	35596	*896*	10842	*532*	15715	*463*	27210	*579*	45528	*623*

[a] CA: caffeoylquinic acid; CAF: caffeic acid; PCQ: para coumaroylquinic Acid; PCQ_Iso1 and PCQ_Iso2: isomers of para-coumaroylquinic acid; CAT: (+)-catechin; EC: (-)-epicatechin; B1: procyanidin dimer B1; B2: procyanidin dimer B2; C1: procyanidin trimer C1; PCA_ X1 to X5: isomers of procyanidins; PLT: phloretin; PLZ: phloridzin; XPL: phloretin xyloglucoside; Avi: avicularin; QCI: quercitrin; IsoQ: isoquercitrin; HYP: hyperoside; REY: reynoutrin; RUT: rutin.
[b] Standard deviation (n=3); [c] nd: not detected.

the absence of (+)-catechin (CAT) and the presence of (-)-epicatechin in all the varieties studied.

A decreasing gradient of hydroxycinnamic acid concentrations from the flesh to the peel was generally observed and was consistent with previous observations in apples (MACHEIX *et al.*, 1977; RISH and HERMANN, 1988). Quantitatively, quantities of (+)-catechin (CAT) are always minor in comparison with (-)-epicatechin (EC), whatever tissue considered (Table 5). This result is also in agreement with the literature (LU and FOO, 2000; GUYOT *et al.*, 2003; MOSEL and HERMANN, 1974). Although in low concentrations, dihydrochalcones were nevertheless present in all parts of the fruit. They were more particularly concentrated in the seeds. This result is in accordance with previous work on apple polyphenols (SANONER *et al.*, 1999; GUYOT *et al.*, 1997; DURKEE and POAPST, 1965).

3.3 Antioxidant capacity of apple tissue determined by the FRAP method

For several years, the study of the antioxidant activity of polyphenols in plants has been of increasing interest. Many *in vitro* experi-

ments employing various methods to evaluate the antioxidant capacity of plant extracts have been described in the literature. One can quote the work of Pearson (PEARSON *et al.*, 1999) who evaluated the antioxidant capacity of apple juices by estimating the potential of inhibition of lipoprotein oxidation. LU and FOO (2000) studied the aptitude of polyphenols from apple pomace to trap free radicals. These authors showed that the main apple polyphenols have greater antioxidant potential than vitamins C and E, which are however regarded as the leading antioxidants.

The results obtained (Table 6) show that, in the peel, the antioxidant capacity determined by the FRAP method, expressed in μM, varied between 896±42 μM in the *Golden* variety and 2,713±125 μM in the *Local Golden*. This capacity was higher than that found in the flesh, which oscillates between 687±15 μM in the *Golden* variety and 2,694±66 μM in the *Local Golden* variety. For the *Zina* and *Anna* varieties, the seeds presented a greater antioxidant capacity (respectively 2,786±71 and 2,159± 53 μM) than the peel. These results were in accordance with those previously found by Tsao (TSAO *et al.*, 2005).

3.4 Correlation between FRAP values and quantities of polyphenols determined by HPLC

A better correlation was observed between the FRAP values (Table 6) and the quantities of total polyphenols (Tables 3, 4, 5) in the peel (R^2 = 0.74) than in the flesh (R^2 = 0.49). These results can most probably be explained by the high levels of polyphenols in the peel. The values found were very similar to those obtained by Khanizadeh (KHANIZADEH *et al.*, 2008). Moreover, a good correlation was also found between the total polyphenol levels in the seeds and the FRAP values (R^2 = 0.66), which is probably due to the high polyphenol concentrations observed in the seeds.

Table 4 - Concentrations of phenolic compounds (mg/kg of DW) in the flesh of the varieties studied.

Phenolic compounds	**Varieties**											
	Zina		***Chahla***		***Golden***		***Anna***		***Lorca***		***Local Golden***	
Hydroxycinnamic Acids												
CA	5549	*38* [a]	3358	*92*	527	*7*	709	*31*	3389	*49*	8128	*116*
CAF	nd [b]	-	68	*5*	nd	-	34	*6*	nd	-	nd	-
PCQ	285	*9*	1088	*16*	73		331	*26*	2567	*68*	4273	*92*
PCQ_iso1	nd	-	nd	-	178	*7*	nd	-	118	*11*	nd	-
PCQ_iso2	nd	-	320	*4*	nd [c]	-	96	*20*	278	*10*	203	*14*
Total acids	5834	*47*	4834	*117*	809	*26*	1171	*83*	6353	*138*	12604	*222*
Flavan-3-ols												
CAT	nd	-	1082	*33*	nd	-	258	*9*	2502	*21*	1716	*38*
EC	2335	*24*	6087	*52*	954	*24*	1189	*19*	4415	*43*	7613	*43*
B1	299	*38*	1258	*24*	nd	-	230	*11*	1342	*39*	423	*19*
B2	740	*36*	3614	*18*	564	*17*	739	*8*	2398	*44*	2473	*28*
C1	2136	*27*	4955	*116*	nd	-	1104	*12*	nd	-	nd	-
PCA_X1	961	*16*	1167	*36*	136	*8*	198	*31*	4199	*109*	nd	-
PCA_X2	253	*5*	648	*8*	80	*2*	142	*13*	647	*12*	1252	*42*
PCA_X3	197	*14*	176	*11*	nd	-	nd	-	537	*21*	1251	*18*
PCA_X4	nd	-	nd	-	nd	-	nd	-	309	*22*	1850	*25*
PCA_X5	nd	-	348	*27*	183	*14*	nd	-	nd	-	nd	-
Total flavan-3-ols	6923	*161*	19355	*327*	1919	*67*	3863	*104*	16349	*312*	16578	*213*
Dihydrochalcones												
PLZ	831	*21*	1426	*25*	491	*33*	489	*28*	1525	*54*	4857	*49*
XPL	nd	-	2214	*20*	131	*10*	749	*21*	nd	-	982	*12*
Total dihydrochalcones	831	*21*	3640	*45*	623	*43*	1239	*49*	1525	*54*	5839	*61*
Flavonols												
AVI	nd	-	151	*11*	15	*1,6*	69	*9*	nd	-	191	*9*
QCI	101	*6*	94	*5*	10	*0,4*	37	*4,3*	96	*8*	76	*4*
IsoQ	nd	-	nd	-	nd	-	12	*0,7*	nd	-	nd	-
HYP	nd	-	nd	-	nd	-	nd	-	nd	-	13	*0,1*
REY	nd	-	15	*2*	nd	-	nd	-	nd	-	62	*7,9*
Total flavonols	101	*6*	261	*18*	25	*2*	118	*14*	96	*8*	343	*21*
Total polyphenols	13691	*237*	28091	*508*	3377	*140*	6392	*252*	24324	*513*	35367	*519*

[a] Standard deviation (n=3); [b] nd: not detected.

Table 5 - Concentrations of phenolic compounds (mg/kg of DW) in the seeds of the varieties studied.

Phenolic compounds	Varieties											
	Zina		*Chahla*		*Golden*		*Anna*		*Lorca*		*Local Golden*	
Hydroxycinnamic Acids												
CA	4861	*105* [a]	3420	*104*	nd	-	3288	*16*	1614	*60*	5008	*116*
PCQ	65	*7*	nd [b]	-	nd	-	nd	-	273	*17*	411	*25*
PCQ_iso2	783	*20*	705	*13*	nd	-	205	*15*	nd	-	1418	*33*
Total acides	5709	*132*	4125	*117*	nd	-	3493	*31*	1887	*77*	6837	*174*
Flavan-3-ols												
EC	3014	*60*	2494	*56*	184	*1*	1538	*77*	1343	*25*	4143	*65*
B2	911	*37*	926	*16*	149	*14*	nd	-	836	*37*	1199	*45*
C1	572	*37*	nd	-	nd	-	849	*12*	nd	-	nd	-
PCA_X1	307	*1*	750	*27*	nd	-	354	*19*	505	*16*	1020	*15*
PCA_X2	252	*12*	466	*11*	nd	-	299	*21*	nd	-	836	*24*
PCA_X3	nd [c]	-	336	*11*	nd	-	nd	-	379	*12*	812	*7*
PCA_X4	100	*1*	nd	-	nd	-	224	*12*	206	*11*	1391	*11*
PCA_X5	142	*1*	nd	-	nd	-	nd	-	752	*10*	nd	-
Total flavan-3-ols	5301	*151*	4973	*122*	333	*15*	3265	*141*	4024	*113*	9401	*167*
Dihydrochalcones												
PLT	76	*5*	282	*14*	60	*3*	125	*14*	58	*2*	256	*4*
PLZ	16712	*144*	14806	*119*	3832	*158*	22439	*141*	17830	*161*	22494	*125*
XPL	676	*20*	237	*16*	237	*3*	833	*24*	145	*5*	1129	*11*
Total dihydrochalcones	17464	*169*	15326	*149*	4129	*164*	23398	*179*	18033	*168*	23879	*140*
Flavonols												
AVI	65	*7*	159	*9*	170	*12*	146	*6*	59	*2*	210	*12*
QCI	24	*3*	115	*4*	85	*6*	29	*1,5*	90	*5*	127	*11*
IsoQ	nd	-	81	*4*	117	*4*	24	*0,5*	nd	-	nd	-
HYP	nd	-	nd	-	418	*13*	nd	-	nd	-	nd	-
REY	34	*1*	200	*6*	87	*3*	63	*3*	42	*2*	86	*4*
Total flavonols	123	*11*	556	*23*	879	*38*	264	*11*	191	*9*	423	*27*
Total polyphenols	28598	*465*	24982	*414*	5342	*219*	30422	*364*	24138	*369*	40543	*511*

[a] Standard deviation (n=3); [b] nd: not detected.

Table 6 - Total antioxidant capacity according to the FRAP method.

Varieties[a]	FRAP (µM)		
	Flesh	Peel	Seeds
Z	1291±*52*[b]	2243±*64*	2786 ±*71*
C	1915±*32*	2117±*57*	1734±*22*
G	687±*15*	896±*42*	712±*35*
A	849±*26*	1425±*85*	2159±*53*
L	2107±*19*	2462±*14*	1733±*48*
LG	2694±*66*	2713±*125*	2169±*65*

[a] Variety: Z = *Zina*; C = *Chahla*; G= *Golden*; A= *Anna*; L= *Lorca*; LG= *Local Golden*.
[b] Average±*standard deviation* (n= 9).

As a whole, most of these correlations suggest that, in apple, the antioxidant capacity is mainly dependent on the quantities of polyphenols present in the tissue considered. Nevertheless, total antioxidant capacity can be also partly attributed to non-polyphenolic compounds with antioxidant character. For example, vitamin C, which is generally present in low concentrations in apples, can contribute to this capacity (SINGLETON and ROSSI, 1965).

CONCLUSION

For the six apple varieties, data concerning the polyphenol composition of apple tissues were wholly consistent with results previously published showing the great variability of polyphenol profiles depending on the variety. Globally, the Tunisian varieties studied showed higher polyphenol concentrations than the very widespread dessert apple *Golden*, which also exhibited the lowest antioxidant capacity. In all the varieties, procyanidins were the main class of polyphenols, their constitutive units being essentially (-)-epicatechin. Seeds contained very high concentrations of dihydrochalcones and were characterized by DPn of F3ols superior to those measured in the flesh and the peel.

ABBREVIATIONS

Z, *Zina*; C, *Chahla*; G, *Golden*; A, *Anna*; L, *Lorca*; LG, *Local Golden*; F3ols, flavan-3-ols (i.e., catechins + procyanidins); CA, caffeoylquinic acid; PCQ, *p*- coumaroylquinic acid; CAF, caffeic acid; CAT, (+)-catechin; EC, (-) epicatechin; B1, procyanidin B1; B2, procyanidin B2; C1, procyanidin C1; PCA, procyanidins; PLT, phloretin; PLZ, phloridzin; XPL, phloretin xyloglucoside; AVI, Avicularin; QCI, Quercitrin; iQCI, Isoquercitrin; HYP, Hyperoside; REY, Reynoutrin; RUT, rutin; DP*n*, average degree of polymerization; RT: Retention time.

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PAPER

CHEMICAL AND PHYSICAL CHANGES OCCURRING IN EXTRA VIRGIN OLIVE OIL USED AS A COVERING MEDIUM FOR VEGETABLE PRESERVES

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ABSTRACT

This paper evaluates the effect of the production process and storage on the quality of extra virgin olive oil used as a covering medium for vegetable preserves. Acidity, peroxide value, spectrophotometric indices (K_{232}, K_{270} and ΔK) and total polyphenols were evaluated for 6 to 12 months in the oil drained from vegetable preserves hand-canned in extra virgin olive oil. Our results showed that many oil-vegetable interactions occurred in the preserves, and the extra virgin olive oil's chemical characteristics experienced significant variation after processing and during conservation. Polyphenolic compounds showed a dramatic drop after pasteurisation and remained almost stable during the period of conservation, whereas significant changes during the entire period were revealed in the oxidative status of the oil, as measured by peroxide levels and spectrophotometric indices. The most important changes were found in K_{270}, which, after a few months, reached values that were not compatible with those required for classification as an extra virgin olive oil. These results make the parameters normally used for assessing the quality and genuineness of extra virgin olive oil inadequate when they are used for vegetable preservation.

- Key words: in-oil vegetable preserves, extra virgin olive oil, quality parameters -

INTRODUCTION

The qualitative characteristics of an in-oil vegetable preserve depend on the interactions between traits of the canned vegetables and those of the oil used as a covering medium. During preservation, many bioactive molecules migrate from the vegetables to the oil and from the oil to the vegetables, reaching a dynamic equilibrium which not only depends on the characteristics of oil and vegetables but also hinges on the technology of the preparation, the heat treatment, the amount of oxidative and hydrolytic degradation and, finally, the duration and conditions of the conservation (DE GIORGI *et al.*, 2000). This means that any vegetable oil preserve is not just a vegetable mixed together with oil, but is itself a new food whose characteristics are not the simple sum of the properties of original oil and vegetable (BALLARINI *et al.*, 2004).

During processing, vegetables undergo important modifications from mechanical and thermal shock: production of ethylene, degradation of the cell membranes (and the consequent dispersal of enzymes and substrates), creation of cell walls, development of oxidative and enzymatic darkening reactions, increases in cellular respiration and production of secondary metabolites (ketones, flavonoids, terpenes, alkaloids, tannins and alcohols) (BALLARINI *et al.*, 2004).

In addition, over time, the oil itself undergoes significant modification, primarily due to hydrolytic and oxidative processes in triglycerides, which are the main cause of quality deterioration of oil. The oxidative processes initially produce hydroperoxides, which, in turn, generate alcohols, aldehydes, ketones and carboxylic acids, provoking the deterioration of the preserve (DE GIORGI *et al.*, 2000). Hydrolysis of triglycerides determines the increase in free acids, which contributes to the oxidation. The amount of oxidative degradation occurring during conservation is the result of the interaction between prooxidant (oxygen, unsaturated fatty acids, free acids, catalytic metals, exposure to light, heat) and anti-oxidant (carotenoids, tocopherols, phenolic compounds) agents (DE GIORGI *et al.*, 2000; BENDINI *et al.*, 2009).

These chemical and physical processes start with pasteurisation and continue during conservation (BOCCA *et al.*, 1990), greatly altering the chemical and physical characteristics of both vegetables and oil. In turn, the organoleptic properties of the vegetables and oil, as well as those of the preserve as a whole, are strongly modified. In particular, oxidation has a strong impact on both the aroma and the taste of oil; it affects 1) the presence of volatile compounds, including a drastic reduction in C6 aldehydes, alcohols and esters and an increase in several C5-C11 saturated aldehydes that are responsible for rancidity and 2) the presence of phenolic compounds that are responsible for the bitter and pungent attributes of oil (BENDINI *et al.*, 2009).

These chemical transformations have very strong implications for the interpretation of standards regulating canned vegetables with oil when the label declares that extra virgin olive oil is used. If one of the parameters of the oil falls outside established limits, the oil itself, and thus all the preserved food, becomes legally "inedible", even if correctly processed. This makes the standards established for extra virgin oil inadequate when applied to oils used as a covering medium in vegetable preserves.

A second relevant problem is related to the selection of tools to fight fraud. It is essential to define whether the parameters normally used to classify olive oil as extra virgin can also be used for the oil in vegetable preserves.

Data on this matter in the scientific literature are scant and out-of-date. This paper illustrates the modification of oil quality indicators in different vegetable in-oil preserves produced through a mild, hand-canning process. The main aim of our work is to validate the indicators used to assert the quality and genuineness of oil used as a covering medium for preserves.

MATERIALS AND METHODS

Extra virgin olive oil

Three different extra virgin oils were used: monocultivar leccino, monocultivar moraiolo and a blended extra virgin olive oil produced from a mixture of leccino, frantoio, moraiolo, itrana, pendolino and canino olives. Each extra virgin olive oil was produced from handpicked olives using a new mechanical two-phase continuous oil mill (running below 27°C) from the Pieralisi Company, which is a major Italian oil-mill factory in production since 1888.

Vegetables

Zucchini, sweet peppers, asparagus, artichokes and eggplants were harvested and processed within a week, as reported in Table 1. For each vegetable, two different levels of acidification with vinegar were used. For asparagus and artichokes, three different cooking times were applied. When processed, the vegetables were hand-canned separately with the three different oils in 500-grams glass jars and stored at room temperature in the dark. The evolution of the main quality parameters was evaluated for each of the different vegetables over 6 to 12 months.

Olive paste

Olives were milled after washing and destoning and were canned in 180-grams glass jars covered with oil in a ratio of one part oil to nine

Table 1 - Description of the preparation of the vegetables before in-oil canning, and the oil:vegetable ratio.

	Water/vinegar		NaCl	Cooking time	Pasteurisation	Oil/vegetable ratio
Artichokes	2.5:10	3:10	40 g/L	5; 10; 15 min	35 min	2.2:10
Asparagus	2.5:10	3.75:10	40 g/L	5; 10; 15 min	28 min	5.2:10
Eggplants	3:10	3.2:10	40 g/L	3 min	35 min	2.5:10
Sweet peppers	2.9:10	3.2:10	40 g/L	6 min	34 min	2.5:10
Zucchini	2.9:10	3.3:10	40 g/L	5 min	38 min	2.5:10
Olive paste	-		-	-	40	

parts olive paste. Jars were then put through a 40-minutes pasteurisation process and stored at room temperature in the dark.

Analyses

The indicators used to assess the quality of extra virgin olive oil are those indicated by the European Council Regulation 1513/2001 of 23 July 2001: 1) acidity, indicating the presence of free fatty acids and, thus, degree of hydrolysis of the triglycerides; 2) peroxide levels, to define the level of rancidity of the oil; 3) spectrophotometric indices (K232, K270 and ΔK) to detect the presence of secondary products of the oxidation and/or refining (conjugated dienes and trienes).

Total polyphenols were analysed as an indicator of the presence of antioxidants. Acidity, peroxides and spectrophotometric parameters were analysed according the EC methods (EEC 1991) after percolation through anhydrous sodium sulphate. Polyphenols were extracted in methanol and quantified using the Folin-Ciocalteau method (SINGLETON and ROSSI, 1965) at 765 nm using gallic acid as a reference standard.

Expression of the results

The different vegetable preserves were prepared at different times of year (according to the seasonality of the different vegetables), whereas the oils were produced once, in early November. For this reason, to account for the different characteristics of the oil when it was added as covering medium (due to the different "ages" of the oil), the results in the tables and figures for each parameter are expressed as the difference between the values analysed in the oil drained from the vegetable preserves (covering oil) and those of the same oil, preserved in small dark bottles, under the same environmental conditions, for the same period (crude oil).

For all the vegetables, the differences in the preserves prepared with different oils, different levels of acidification and different cooking times were negligible and not statistically significant for all the parameters. The results are therefore presented here according to vegetable type, considering only the time elapsed from processing and canning.

Statistical analysis

Data are presented as the means ± standard deviations (SD). Statistical analysis was performed using repeated-measures analysis of variance (ANOVA), followed by Tukey's test for multiple comparisons. Analyses were performed with Statistica software (StatSoft Inc.). P values < 0.05 were considered statistically significant.

RESULTS

The oils

Table 2 shows the quality parameters of the three crude oils, and the evolution of these parameters during 12 months of conservation in 100 ml glass bottles under the same conditions as the vegetable preserves.

During the 12 months of preservation, the acidity of all three oils remained almost constant, fully below 0.8%, which is the limit for being classified as an extra virgin olive oil. On the contrary, peroxides and spectrophotometric parameters showed a progressive worsening, with indices above legal limits after 6 months for peroxides and 12 months for K232.

K270 and ΔK were still within the limits, even after 12 months. Polyphenols tended to decrease after six months, reaching about 75-80% of their initial value after one year of conservation.

The vegetable preserves

Figure 1 shows the free acidity of the covering oils. Two days after processing (time 0 in the table), acidity increased slightly in all preserves with respect to the oil used as covering medium. During the experiment, acidity remained almost constant in all the vegetable preserves, with a negligible and non-significant increase of 0.1-0.2% after 1 year. However, the covering oil of the olive paste showed a significant increase of acidity soon after pasteurisation that exceeded the maximum limit for extra virgin olive oil.

Peroxide value is indicative of the level of rancidity that normally occurs in oils due to progressive unsaturated fatty acid oxidation. In all vegetable preserves, peroxide values decreased just af-

Table 2 - Evolution of acidity, peroxides, polyphenols and spectrophotometric parameters of extra virgin olive oil preserved under the same condition as the vegetable preserves.

	Time 0	1 month	2 months	3 months	6 months	9 months	12 months
Acidity (% oleic acid)							
Leccino	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Moraiolo	0.3	0.3	0.3	0.4	0.4	0.4	0.4
Blend	0.3	0.3	0.3	0.3	0.2	0.3	0.4
Peroxides (meq 0_2/kg)							
Leccino	13	13.4	16.8	17.6	22.3	24.2	23
Moraiolo	16.1	15.7	18.5	22.7	24.8	27.3	26.1
Blend	10.2	11.5	13.7	15.6	22.1	20.9	20.2
K_{232}							
Leccino	0.7164	1.922	2.093	2.040	2.412	2.290	2.502
Moraiolo	0.7502	1.954	2.017	2.278	2.492	2.550	2.662
Blend	0.6240	1.649	1.658	1.832	2.280	2.432	2.525
K_{270}							
Leccino	0.031	0.094	0.101	0.098	0.112	0.100	0.149
Moraiolo	0.042	0.106	0.094	0.112	0.113	0.118	0.171
Blend	0.048	0.108	0.094	0.096	0.135	0.113	0.123
Delta K							
Leccino	-0.0022	-0.0070	-0.0091	-0.0089	-0.0136	-0.0135	0.0020
Moraiolo	-0.0019	-0.0060	-0.0077	-0.0077	-0.0123	-0.0123	0.0015
Blend	-0.0013	-0.0040	-0.0046	-0.0067	-0.0125	-0.0113	0.0017
Polyphenols (mg/kg)							
Leccino	115	116	113	112	86	96	85
Moraiolo	130	121	115	121	96	90	85
Blend	125	131	116	119	117	113	105

ter processing (Fig. 2). The peroxides remained almost constant for the first month of conservation, likely because of the presence of oil and vegetable antioxidants. Then, except for eggplants, the index tended to strongly decrease in the following months, as peroxide compounds were progressively decomposed. However, unlike eggplants covering oil, which exceeded the limit for extra virgin olive oil (<20 meq O_2/kg) after six months, the oil used as covering medium for artichokes, asparagus, sweet pepper, zucchini and olive paste still remained within the limits even after one year.

Spectrophotometric indices are used to evaluate the oxidation status of the oil by examining

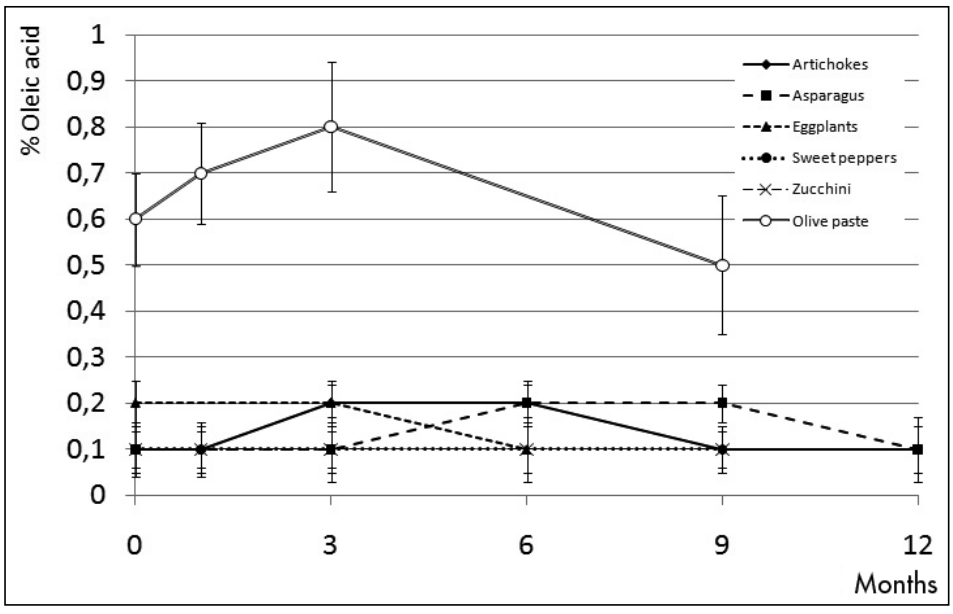

Fig. 1 - Acidity (% oleic acid) of oil used as covering medium in the vegetal preserves as compared to those of the oil conserved in the same environmental conditions (mean ± SD). Results are expressed as the difference between those obtained in the oil drained from the preserves and those obtained in the oil used as a control. All differences as respect to time 0 are not significant.

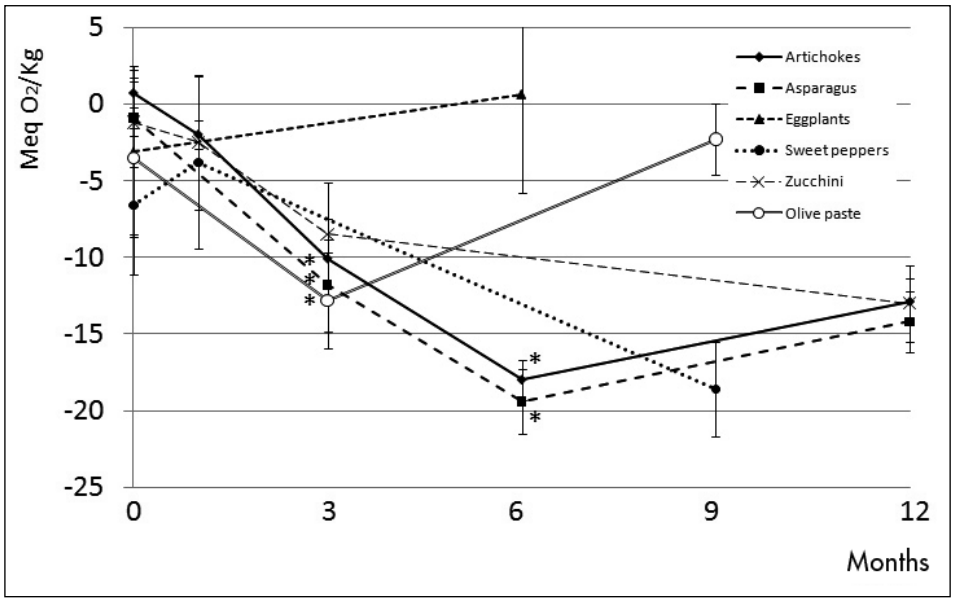

Fig. 2 - Differences of peroxide values (meq O_2/kg) of oil used as covering medium in the vegetal preserves as compared to those of the oil conserved in the same environmental conditions (mean ± SD). Results are expressed as the difference between those obtained in the oil drained from the preserves and those obtained in the oil used as a control. (*) $P < 0.05$ as respect to time 0 by One way ANOVA, Turkey HSD for unequal N (Spjotvoll/ Stoline Test).

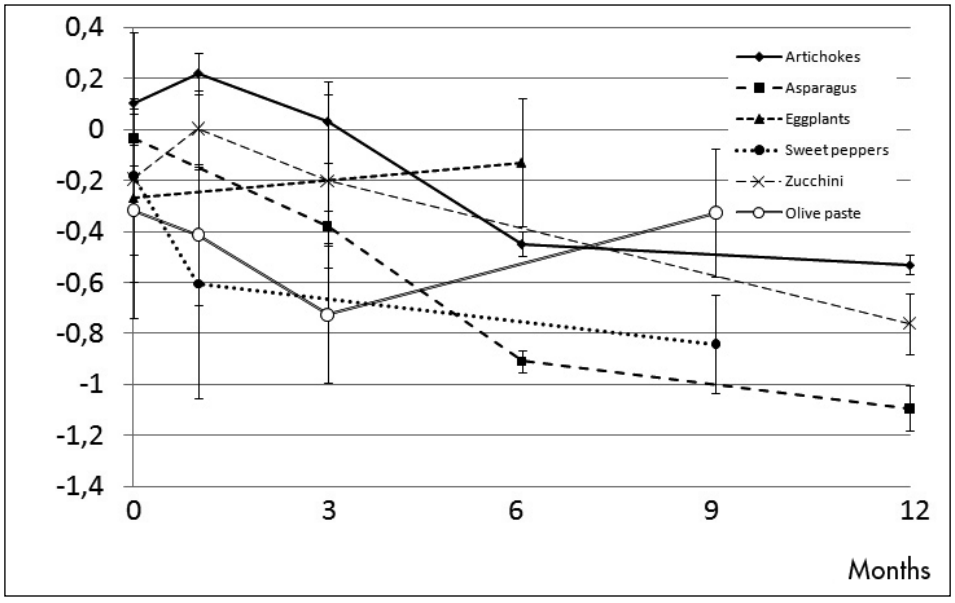

Fig. 3 - Differences of K_{232} of oil used as covering medium in the vegetal preserves as compared to those of the oil conserved in the same environmental conditions of oil used as covering medium in the vegetal preserves (mean ± SD). Results are expressed as the difference between those obtained in the oil drained from the preserves and those obtained in the oil used as a control. (*) $P < 0.05$ as respect to time 0 by One way ANOVA, Tukey HSD for unequal N (Spjotvoll/ Stoline Test).

the absorbance at wavelengths of double conjugated bonds (232 nm) and aldehydes, ketones and other carbonyl compounds (270 nm). High values of K232 thus indicate the beginning of the oxidative processes, whereas high K270 value reveals the presence of secondary products of the oxidation. The K232 levels of the oils used as a covering medium of the vegetable preserves tended to decrease during the entire period, compared with the control oils (Fig. 3), reaching absolute values that still remained within the limits for extra virgin olive oil for almost the entire year.

Except for the oil used as covering medium for artichokes, K270 remained almost constant after the processing and canning (time 0 in Fig 4) and then, beginning in the first month of conservation, showed a progressive increase (Fig. 4). In artichokes, the K270 showed a substantial increase during the first month of conservation, compared with the crude oil (Fig. 4), and reached a value that exceeded the maximum value allowed for extra virgin oil soon after pasteurisation and canning. This happened in the oils covering the other vegetables only after 6-12 months.

The same trend was observed in ΔK (Fig. 5). Except for the oil covering artichokes, which showed a sizable and significant increase in the ΔK value both after processing and in the first month of conservation, all the analysed oils scored very well for the entire period with a negligible increase in ΔK, compared with the crude oils.

Total polyphenols in crude oils ranged from 115-130 mg/kg and were almost stable for the first month but started to decrease, reaching approximately 80-90% of their initial value, after one year (Tab. 2). In covering oils, polyphenols decreased sharply during processing (time 0 in Fig. 6). This was particularly remarkable

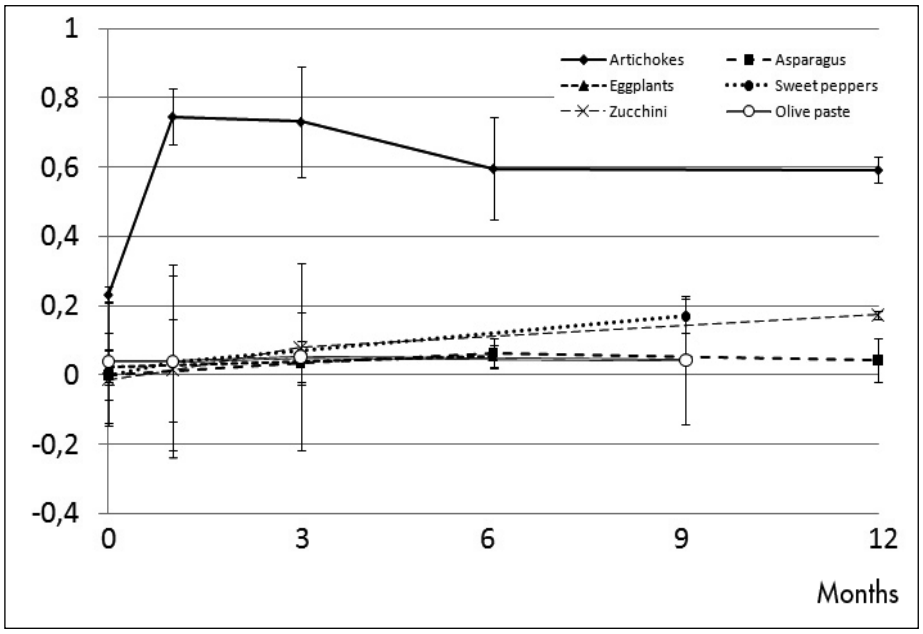

Fig. 4 - Differences of K_{270} of oil used as covering medium in the vegetal preserves as compared to those of the oil conserved in the same environmental conditions of oil used as covering medium in the vegetal preserves (mean ± SD). Results are expressed as the difference between those obtained in the oil drained from the preserves and those obtained in the oil used as a control. (*) $P < 0.05$ as respect to time 0 by One way ANOVA, Tukey HSD for unequal N (Spjotvoll/ Stoline Test).

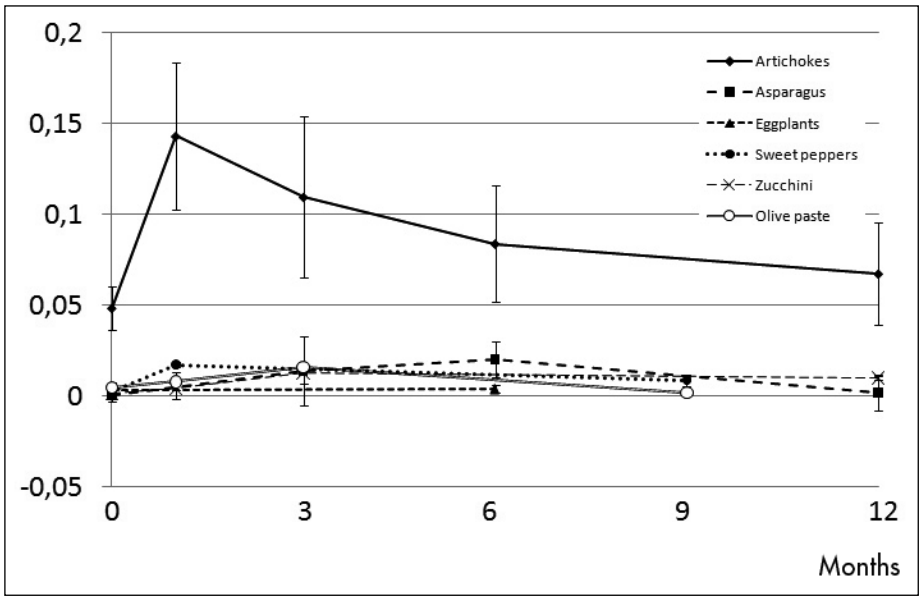

Fig. 5 - Differences of ΔK of oil used as covering medium in the vegetal preserves as compared to those of the oil conserved in the same environmental conditions of oil used as covering medium in the vegetal preserves (mean ± SD). Results are expressed as the difference between those obtained in the oil drained from the preserves and those obtained in the oil used as a control (*) $P < 0.05$ as respect to time 0 by One way ANOVA, Tukey HSD for unequal N (Spjotvoll/ Stoline Test).

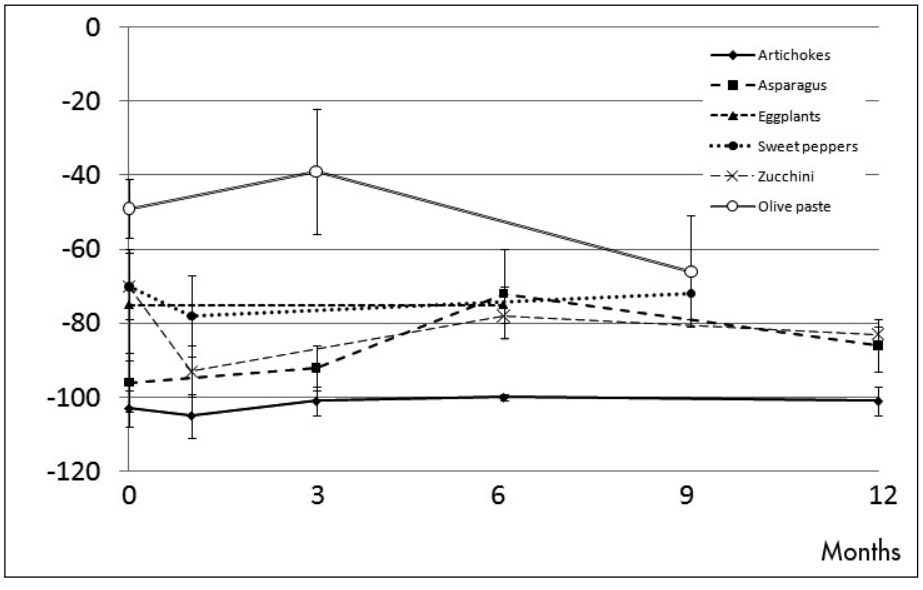

Fig. 6 - Differences of Total polyphenols (mg/kg) of oil used as covering medium in the vegetal preserves as compared to those of the oil conserved in the same environmental conditions of oil used as covering medium in the vegetal preserves (mean ± SD). Results are expressed as the difference between those obtained in the oil drained from the preserves and those obtained in the oil used as a control. (*) $P < 0.05$ as respect to time 0 by One way ANOVA, Tukey HSD for unequal N (Spjotvoll/ Stoline Test).

in the oil preserving artichokes and asparagus (which lost almost 80-85% of polyphenols during processing) but also was seen in the oil covering the other vegetables (approximately -60% of polyphenols) and in the olive paste (-40%). After processing and during conservation, polyphenols in vegetables declined in a similar manner to that observed in the crude oils.

DISCUSSION

Our results showed that, in in-oil handcanned vegetables, an oil-vegetable interaction occurred and that the chemical characteristics of extra virgin olive oil, when used as a covering medium for canned vegetables, experience significant variation after pasteurisation and during preservation.

The acidity of the oil in vegetable preserves is not significantly affected during preservation. The slight increase in acidity that was found after pasteurisation and canning is probably due to the release of free fatty acids that occurs when fats are heated in presence of moisture (NAWAR 1969; WARDELL *et al.*, 2008). On the contrary, the more sizable increase in acidity found in the oil of the olive paste just after pasteurisation may likely be attributed to lipolytic enzymes in the milled olives.

The oxidative status of the oil of the vegetable preserves was very dynamic, with a significant decrease in peroxide values during the first 6 months of conservation, followed by a slight and non-significant increase. K232, tended to decrease throughout the year, whereas a significant and constant increase was found in K270. The reduction in peroxide value and K232 cannot be explained only by the decomposition of hydroperoxides and conjugated trienes, yielding carbonyl compounds (as indicated by the increase of K270). Therefore, it could be hypothesized a dynamic migration of metabolites from vegetables to covering medium and vice versa.

All of the oils used for canning the different vegetables behaved similarly, except those used for artichoke preserves. The oil used for covering artichokes showed higher values of K232 and a sizeable and significant increase in K270 soon after pasteurisation and after one month of conservation, which was accompanied by a high loss of polyphenolic compounds. This could be due to the elemental composition of these vegetables, which are significantly richer in some transition metals (such as iron and copper), compared with the other studied vegetables (INRAN, 2000). Transition metals, in fact, can catalyse the decomposition of hydroperoxides into lipid peroxyl and alcoxyl radicals that initiate free radical chain oxidation (BENDINI *et al.*, 2009).

The dramatic loss of polyphenols during thermal treatment of oils has been already described (PELLEGRINI *et al.*, 2001; BRENES *et al.*, 2002; SACCHI *et al.*, 2002), and it has also been demonstrated that their decrease can be higher in the presence of vegetables (SILVA *et al.*, 2010). This finding was confirmed in our study, because total polyphenols in the covering oils strongly decreased immediately after the pasteurisation process and canning. This reduction could also be boosted by the low pH caused by the vinegar used in the preliminary cooking of vegetables. In fact, it has been demonstrated that at low pH, the loss of virgin olive oil polyphenols during thermal treatments is increased (BRENES *et al.*, 2002). This loss of polyphenols in the oil of the vegetable preserves could also be due to their partial migration from the lipophilic environment into the hydrophilic phase of the vegetables.

The findings of this study demonstrate the existence of significant changes in the characteristics of extra virgin olive oil when used as covering medium for vegetable preserves. Further studies will be needed to better understand the changes occurring in the oil used as covering medium in vegetable preserves, particularly the influence of fatty acids, different antioxidants, and volatile compounds, to validate our hypothesised explanations for the changes we found in the peroxide values and the spectrophotometric parameters.

One final consideration is the implication of these findings on the rules regulating in-oil canned vegetables, particularly when the label states that extra virgin olive oil was used. The regulations do not specify whether the quality parameters of extra virgin olive oil must maintained up to the time of the consumption of the preserve or if it is sufficient to use extra virgin olive at the canning process.

This is particularly true for K270, which was within the limits for extra virgin olive oils during processing but started to increase just after the contact with vegetables and continued to increase during conservation, reaching values that are not compatible with extra virgin olive oil. Although K232 and ΔK also changed after processing and storage, they remained close to values accepted for extra virgin olive oil even after one year of conservation.

This is not just a theoretical exercise. In fact, if only one of the parameters used to classify an extra virgin olive oil exceeds the limit allowed by the standards, the oil (and thus the preserve) becomes unmarketable. This means that, with this approach, almost all the in-extra virgin olive oil preserves turn out to be unmarketable soon after being processed. If this is the case, it is then obvious that the processes used for canning vegetables are not suitable when extra virgin olive oil is used, and they should then be modified using, for instance, inert gases during the whole process (PAGANUZZI *et al.*, 1995).

However, even though the statement "in extra virgin olive oil" just refers to the character-

istics of the oil during the preparation and canning, a relevant problem still exists. The classical chemical parameters used for classifying an olive oil as extra virgin are not discriminatory enough to guarantee the recognition of the genuineness of the olive oil used as covering medium in vegetable preserves nor to verify the absence of refined olive oil. For this reason, analysis of stigmastadienes is indicated for detecting the presence of refined vegetable oils (olive, olive-pomace, sunflower, soybean, palm) in virgin olive oil (IOC 2001), as these steroid hydrocarbons increase with deodorisation and physical refining (LEON-CAMACHO *et al.*, 2004; GARCIA-GONZALEZ *et al.*, 2008) and are not significantly influenced by a correct processing for the preparation of vegetable preserves.

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SHORT COMMUNICATION

PHYSICAL PROPERTIES, NUTRIENTS AND ESTIMATED VOLATILES OF *AGARICUS BISPORUS* (WHITE) AT TWO HARVESTS

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ABSTRACT

Agaricus bisporus was investigated in two harvest terms with three flushes in each. Cultivated mushrooms like *Agaricus bisporus* were produced in multiple flushes in the same compost substrate. Texture, diameter, protein, ash, moisture, vitamin and mineral contents were determined, and results from the harvests and flushes were compared. Mean values of texture, diameter, and protein content increased in the second harvest term were compared to the first and they were higher in the second and third flushes for both harvests. Volatiles of mushrooms were identified using gas chromatography and mass spectrophotometer (GC-MS) employing a library catalog. Estimated aroma volatiles and ratios differed among harvest time and flushes.

- Key words: *Agaricus bisporus*, fruit body, flush stages, harvest, nutrients, volatiles, aroma, texture, diameter -

INTRODUCTION

The most frequently cultivated mushroom species in Turkey are *Agaricus bisporus* (button) mushroom, *Portabella* (*Agaricus bisporus*, brown) mushroom, oyster mushroom (*Pleurotus spp.),* shiitake (Japanase mushroom, *Lentinula edodes*), and also some more exotic and medicinal mushroom species such as *Agrocybe cylindracea, Ganoderma lucidum, Grifola frondosa, Hericium erinaceus, Pleurotus citrino-pileatus, P. djamor* too (İLBAY and ATMACA, 2004). Production of cultivated mushroom in Turkey was 45.000 tones in 2009 (TUK, 2009).

Nutrients of mushrooms in relation to human nutrition have been thoroughly investigated (ÇAĞLARIRMAK, 2007; 2009). The components: proteins, minerals and vitamins, and functional properties were carried out by MIZUNO (1995), MANZI *et al.* (1999), MATILLA *et al.* (2000), and YANG *et al.* (2001). The changes in nutrients of mushrooms as a function of processing technologies, such as blanching and canning were also studied (ÇAĞLARIRMAK *et al.*, 1999; 2001).

Physical parameters of mushrooms were studied against some factors like temperature and pH (ZIVANOVIC *et al.*, 2003). Texture and diameter were evaluated as physical quality criteria for mushrooms (ALTUĞ *et al.*, 2000). Firmness is resistance to determination under applied force for puncturing. Firmness of mushrooms was determined when subjected to food. TSAI *et al.* (2007) established properties of fruit body at different stage of maturity from the first stage to the fifth stage. The cap diameter varied from 20.09 to 61.05 mm.

Proximate constituents have been determined under different conditions like different stages of maturity, on a dry weight basis high ash content is 12.5 and 11.9% for immature and also mature samples (DIKEMAN *et al.*, 2005). TSAI *et al.* (2007) studied proximate conditions and some of physical properties of *A. bisporus* in different stages and they reported that the moisture contents were in the range of 89.3-92.3% in wet weight and crude protein in the range of 21.3-27.0%, crude ash in the range of 157-260 mg/g, crude fat in range of 2.53-3.92%.

Vitamins of *A. bisporus* were reported in fresh and processed conditions like canning, blanching or compost prepared by different manures (MATILLA *et al.*, 2001). It was established that vitamin contents of mushrooms have been affected by compost types prepared according to different formulas (ÜNAL *et al.*, 1996).

Mushrooms contain sufficient quantities of B-complex vitamins and vitamin C (ÇAĞLARIRMAK, 2009). Contents of edible fungi have been examined by comparing with other vegetables and meats. German Academy for Nutritional Medicine has compiled an edible fungi databank giving contents of both nutrients and heavy metals. Average protein digestibility is about 70%. Protein levels were comparable to those of cauliflower and whole milk. Vitamin B contents could be increased by growth on suitable substrates (TIMMEL and KLUTHE, similar results showing that mushrooms have rich biochemical composition and have functional properties and also medicinal importance were obtained (MATTILA *et al.*, 2000; WASSER and WEIS, 1999; PARDO *et al.*, 2001). TORIJA-ISASAE and ORZAEZ VILANUEVA (1987) studied vitamin C contents of *A. bisporus* and found mean value 42 mg/100 g, ranged (0.46-5.65 mg ascorbic acid/100 g).

Minerals of *A. bisporus* were carried out by many authors, i.e. ÜNAL and *et al.* (1996) and MATILLA *et al.* (2001). Minerals of mushrooms can be affected by various factors like compost preparation, species, and partially mushrooms (FALANDYSZ *et al.*, 1993; LATIFF *et al.*, 1996). It was found that the growing conditions (composition of compost) have a determinative role. Minerals of mushrooms can contribute to human nutrition (LANOS *et al.*, 1993; VETTER, 1994; FLEGG, 1998; KALAC and SWOBODA, 2000).

The aroma compounds affect the taste and acceptance of *A. bisporus* (MAU *et al.*, 1997). Mushrooms have been used as food flavoring. Typical flavor substances of mushrooms contain volatile and non-volatile compounds (MAGA, 1981). The most important aroma components form a series of eight-carbon compounds; 1-octen-3-ol, which is common for most of mushroom species (MAU *et al.*, 1997; CRUZ *et al.*, 1997).

Flavor compounds of *A. bisporus* are carried out in the three developmental stages; 1-octen-3-ol was the major flavor compound in developmental stages (CRONIN and WARD, 1971; CRUZ *et al.*, 1997). They isolated and identified some volatiles by steam distillation. Major volatiles components were 3-methylbutanol, 3-octanone, oct-1-en-3-one, 3-octanol, oct-1-en-3-ol, furfural, benzaldehyde, phenyl acetaldehyde, and benzylacohol. Mushroom species process creates odor that can be described as "mushroom-like," which is special and native to mushrooms. This flavor is formed because of the presence of aliphatic eight-carbon compounds. The other compounds contribute to the flavor (JANG and BIRMINGHAM, 1993). The typical flavor of mushrooms causes preference of consumption and attributed the most important quality criteria in cultivated mushrooms (MAU, 2005).

The purpose of this study is to establish variations of nutrients, texture, diameter, and volatiles in three different flushes and in two different harvest terms in order to provide new data.

In this research, the changes of texture, diameter, moisture, ash and protein, vitamin C, B group vitamins and minerals and volatiles of *Agaricus bisporus* (A15) are investigated in flushes in two different harvest terms. Variety of A. *bis-*

porus (A15, Sylvan) is explained as; Mid-Range Hybrid. It is middle size hybrid variety. They are hybrid species in Off-White species class.

1. MATERIAL AND METHODS

1.1. Materials

Button mushroom (*Agaricus bisporus*) A15, Sylvan; Mid-Range-Hybrid, a variety of middle size hybrid, variety was used. It belongs to a hybrid species in Off-White species class. Samples were obtained according to flush terms in two different harvest terms from PEMA mushroom producing company. The mushroom company is situated in Menemen a town of İzmir province in west of Turkey. This company from which samples are obtained have produced their own compost; hen and horse manure with straw, and grow mushroom species *A. bisporus* cv. Portabella (Brown Mushroom), *Pleurotus ostreatus*. *A. bisporus* planted spawn A15 was used in the experiment and the three flushes of two harvest terms were investigated. Flush l, FII, FIII were in (l, 2.) first harvest terms, all of the analyses were carried out in triplicate.

1.2. Physical properties

Texture and diameter of ten caps of three batches of mushrooms were measured. The measurements were performed with fresh samples.

Texture: mushroom caps were measured by fruit hardness tester FHR-5, max pressure kg/mm^2, Cat. No5. (FHR-5) and 510-1 (FHR-1).

Diameter: diameter was measured by a pair of compasses. These measurements were evaluated according to ALTUĞ *et al.* (2000).

1.3. Chemical analysis

Ash; (AOAC 29.013); moisture were determined Sartarius automatic moisture measurement by using device; protein (AOAC 1990, PN-75/A04018). Total protein contents were determined by Kjeldhal method. Calculated nitrogen was multiplied by 4.38 (CRISAN and SANDS, 1978; MANZI *et al.*, 1999).

1.4. Mineral analysis

Was carried out according to the AOAC standards (1990). Ash was dissolved in 5 mL 20% HCI, diluted and filtered through 0.45 μm pore size filter. Lanthanum was added to overcome interferences for Ca and Mg determination. Minerals were established by AAS (atomic absorption spectrophotometer) except for Na, K which were detected by FES (flame emission spectrophotometer).

1.5. Water soluble vitamins

L-ascorbic acid (vitamin-C), B1 (thiamin), B2 (riboflavin), folic acid, niacin were determined. Vitamin C was analyzed by the 2,6 dichlorophenolindophenol titration method (ÇAĞLARIRMAK, 2007; 2009). Analysis of B complex vitamins B1 (thiamin), B2 (riboflavin), folic acid, niacin was determined according to FINGLAS and FOULKS (1984), KAMMAN *et al.*, 1980, Method Dionex Vydac Application Note, 1994.

1.6. B complex vitamins sample preparation

Ten samples (fruit bodies) were (fruit bodies) weighed and put into a 250 mL flask. Thirty mL 0.1 M HCl was added and the flask was plugged with cotton, then covered with aluminum and autoclaved at 121°C, 30 min. After this step, the pH of samples was adjusted to 6.5 and 4.5 with sodium acetate and HCI the volume was completed with distilled water, filtered with a normal filter paper. If turbidity occurred, the sample would be centrifuged for 10 min at 6,000 rpm. If turbidity contained, sample would be filtered by using a filter of a 0.45 μm pore size. The sample was ready to for measuring.

1.7 HPLC (high performance liquid chromatography) conditions

A colon oven was used. Colon: C18 Omni Sphere 5, 250-4.6 (mm), λ: 254 nm, flow rate: 1.9 mL/min., injection volume is 20 μL, mobile phase: 1,000 mL phosphate solvent + 360 mL methanol mixture, Press: 150-160 bar, running time is 22 min.

1.8. Analysis of aroma compounds by GC-MS (gas chromatography and mass spectrometer)

Extraction of aroma volatiles: 150 g sample was cut into small cubes then blended with 300 distilled water. Homogenized sample was rested to sample was matured to form forming aroma compounds enzymatically (VENKATESHWARLU *et al.*, 1999). The sample was placed in a liquid-liquid extraction apparatus (HEATH and REINECCIUS, 1986). GC parameters: Instrument name: Inst 1, instrument type: PE (Perkin-Elmer) autosystem XL GC Perkin-Elmer Torbomas, column: OV 17 (% 50 dimethy) length, 30 m; inside diameter: 0.25 mm, film tickness (HP-50): 0.25 μm. Carrier gas: helium, flow rate of 146. carrier gas: 5 mL/min; temperature program: 50°C (2 min), 10°C/min/240°C (19 min), injection temperature: 230°C, injection quantity: 0.5 μL, injection mode: splitless, electron energy: 70 eV, MS mass weight range: 40-400, MS library: WILLEY and NIST libraries. Determination of aroma compounds was performed by comparing mass spectra with those in the MS library.

Table 1 - Physical and chemical values of *A. bisporus* in three flushes and two harvest terms.

Flush terms	Texture sd*	Diameter (cm) sd	**Moisture (%) sd	**ash (%) sd	Protein***(%) sd
FI	1.86±0.036	2.18±0.020	91.06±0.108	1.07±0.01	2.30±0.09
FII	1.95±0.132	3.83±0.29	91.87±0.64	1.03±0.01	2.46±0.08
FIII	1.81±0.763	3.33±0.51	90.13±0.84	1.81±0.01	2.75±0.07
Mean	**1.88**	**3.11**	**91.02**	**1.31**	**2.51**
FI	1.92±0.02	4.71±0.25	89.12±0.586b	1.10±0.00	3.17±0.02
FII	2.04±0.01	4.06±0.15	91.36±1.635a	1.16±0.00	3.78±0.03
FIII	1.75±0.01	2.85±0.02	91.26±0.234	1.37±0.01	3.53±0.43
Mean	**1.90**	**3.87**	**90.58**	**1.21**	**3.49**

*sd (standard deviation); **wb (wet basis); ***N x 4,38.

Table 2 - Mean of physical and chemical values of *A. bisporus* in two harvest terms.

Flush terms	Texture sd*	Diameter (cm) sd	**Moisture (%) sd	**ash (%) sd	Protein***(%) sd
FI	1.89±0.04a	3.45±1.40a	90.09±0.586a	1.09±0.01a	2.74±0.05a
FII	1.99±0.10b	3.95±0.24a	91.61±1.635b	1.10±0.00a	3.12±0.07b
FIII	1.78±0.06c	3.09±0.42a	90.69±0.234ab	1.60±0.02b	3.14±0.51b
Mean	**1.88**	**3.88**	**90.58**	**1.26**	**3.00**

*sd (standard deviation); **wb (wet basis); ***N x 4,38.

Table 3 - Vitamin contents of *A. bisporus* in three flushes and two harvest terms (mg/100 g wb).

Flush terms	Vitamin C sd*	Folic acid sd	Thiamin sd	Riboflavin sd	Niacin sd
FI	5.06±0.41	0.06±0046	0.09±0.00	0.22±0.01	4.26±0.03
FII	11.02±0.80	0.05±0.00	0.07±0.00	0.29±0.01	3.08±0.00
FIII	8.47±0.87	0.04±0.00	0.1±0.04	0.46±0.05	3.99±0.14
Mean	**8.18**	**0.05**	**0.09**	**0.33**	**3.78**
FI	3.49±0.17	0.08±0.00	0.09±0.00	0.28±0.00	2.58±0.01
FII	9.84±0.19	0.09±0.00	0.07±0.00	0.25±0.01	2.00±0.01
FIII	4.28±0.13	0.05±0.00	0.10±0.00	0.12±0.05	3.37±0.02
Mean	**5.87**	**0.075**	**0.87**	**0.22**	**2.65**

*sd (standard deviation).

1.9. *Statistical analysis*

Statistical analysis was calculated according to randomized block design (Duncan's $P<0.05$).

2. RESULTS AND DISCUSSION

Physical properties and diameter of mushrooms were measured in the flushes in two different harvest terms and also moisture, ash, protein contents were determined (Tables 1 and 2).

When values of three flush terms in the two different harvest terms were examined, texture values were the highest in the second flush terms, diameters were the greatest in the flush II of the first harvest term, whereas in case of the second harvesting time the diameter of the first flush had the highest value.

The moisture contents of the second flushes in the both harvest terms were the highest; 91.87 and 91.36% respectively. The third ash contents of both harvest terms were the highest; 1.81 and 1.37% on wet basis, respectively. In the first harvest term protein contents were ranged between 2.30 and 2.75%. In the flush I, the protein content was the lowest Table 1). In the second harvest term, the protein contents were ranged between (3.17 and 3.78%), in the flush I, the protein content was the lowest (3.17%) (Table 1). In both harvesting periods, variations of protein contents in the both harvest terms have shown similar trend, the first flushes have the highest protein contents. Average protein values of the second harvest term were higher than first one (3.49 and 2.51% wb respectively).

Vitamin C and riboflavin increased after first flush terms (5.06 and 8.47 mg/100 g wb; 0.22-0.46 mg % 100 g wb respectively) (Table 3). Thi-

amin changed between (0.07 and 0.1 mg/100 g wb), Folic acid, and niacin decreased from first flush terms to third flush terms. They ranged from (0.09-0.04 mg/100 g wb to 4.26-3.99 mg/100 wb respectively) (Table 3).

Minerals were given in Tables 5 and 6 in flush and harvest terms.

Minerals of mushrooms can be affected by various factors easily, like preparation compost, environment conditions, and using some insecticide, or some agricultural artificial chemical plant pesticides, fungicides or other agricultural chemical materials like fumigations (FALANDYSZ *et al.*, 1993; PROCIDA *et al.*, 1995; RACZ *et al.*, 1995). In the flush terms, in the first harvesting time, contents of Zn, Fe, P, Mg had been increased, Na had been reduced, Ca and K had been increased in the second flush term and reduced in the third flush term (Table 5).

The following mean of mineral contents increased in the second harvest term (mg/kg wb). Zn; (7.70-7.99), Fe; (5.62-6.24), K; (1801.4-2038.4), Na; (547.37-576.7). The other mean of mineral contents decreased in the second harvest term (mg/kg wb); P; (943.6-831.79), Ca; (109.3-88.70), Mg; (225.3-213.5).

2.1. Statistical evaluation of Tables 1, 2 and 3

Differences of vitamin C between the harvest terms were significant; P=0.00, and differences between the flushes were significant P=0.00. Differences of folic acid contents between the crops were significant, P=0.28, and never significant (P=0.082) for the flushes. Similar trends were found for thiamin and riboflavin analysis at these P levels, 0.024, 0.07, 0.032, 0.764 respectively and for niacin both differences were significant, P= 0.00 (Table 3).

Differences of Zn between first and second harvests were never significant; P= 0.0605, between the flushes were significant; P=0.00, differences of Fe for both crops and flushes were significant; P=0.00. P differences between the crops were never significant, P= 0.255, and differences between the flushes were significant; P= 0.038. Differences of Ca were significant P=0.018 and P=0.00 for both between the crop terms and flushes and never significant for Mg; P= 0.686, P= 0.353 respectively. Similar trends with Ca and Mg were determined for K and Na, P=0.03, P= 0.00 were significant and never significant for Na because P= 0.664 and P= 0.194 for harvest periods and flushes (Table 4).

Volatiles and aroma compounds were given in Tables 7 and 8.

It more flavor compounds could be detected when compared to the first flush term 3, 4-dimethyl pentanol. (54.95%) 3-oct en-1-ol (9.65%) and were the highest ratios respectively (Table 7). These aroma compounds should be basic or detectable compounds by GC-MS.

Fatty acids are the precursors of mushroom volatile. Linoleic is the precursor of 1-octen-3-ol, which is the mushroom alcohol. It gives typical mushroom aroma (CRUZ *et al.*, 1997). In the research, in third flush term, mostly 9-octadecanoic acid and similar aroma com-

Table 4 - Mean of vitamin contents of *A. bisporus* in two harvest terms (mg/100 g wb).

Flush terms	Vitamin C sd*	Folic acid sd	Thiamin sd	Riboflavin sd	Niacin sd
FI	4.28±0.86a	0.08±0.03	0.09±0.00b	0.25±0.031a	3.42±0.91b
FII	10.43±0.66c	0.09±0.024	0.05±0.01a	0.27±0.026a	2.54±0.59a
FIII	6.37±2.29b	0.05±0.00	0.04±0.04ab	0.29±0.19a	3.68±0.35b
Mean	**7.03**	**0.07**	**0.062**	**0.27**	**3.21**

*sd (standard deviation).

Table 5 - Mineral contents of *A. bisporus* in three flushes and two harvest terms (mg/kg wb).

Flush terms	Zn sd*	Fe sd	P sd	Ca sd	Mg sd	K sd	Na sd
FI	7.60±0.13	4.41±0.03	795.1±1.59	113.4±0.06	189.0±1.39	2012.67±0.57	610.0±1.15
FII	6.90±0.01	6.31±0.03	929.7±12.08	114.6±0.21	213.7±0.62	1499.33±289.54	571.2±1.71
FIII	8.59±0.03	6.15±0.06	1106±7.81	99.80±0.12	273.1±0.60	1892.33.±3.21	460.0±0.057
Mean	**7.70**	**5.62**	**943.6**	**109.3**	**225.30**	**1801.4**	**547.37**
FI	5.59±0.01	5.10±0.023	730.0±1.97	110.2±0.20	256.7±0.56	1807.7±4.93	460.4±0.72
FII	6.74±0.03	6.90±0.05	1224.7±15.52	117.7±0.35	273.7±0.15	1925.3±3.51	430.0±0.10
FIII	11.64±0.02	6.71±0.51	547.81±3.60	38.23±0.60	110.03±0.86	2382.3±6.8	839.9±7.30
Mean	**7.99**	**6.24**	**831.79**	**88.70**	**213.5**	**2038.4**	**57 6.7**

*sd (standard deviation).

Table 6 - Mean of mineral contents of *A. bisporus* in two harvest terms (mg/kg wb).

Flush terms	Zn	sd*	Fe	sd	P	sd	Ca	sd	Mg	sd	K	sd	Na	sd
FI	6.59±1.10a		4.41±0.03a		762.0±35.86a		112.06±2.04a		222.85±37.09a		1910.17±112.3 ab		535.13±81.90a	
FII	6.82±0.09a		6.30±0.03b		1077.18±161.74b		116.15±1.68a		243.71±32.82a		1712.33±296.16a		501.10±77.89a	
FIII	10.11±1.67b		6.15±0.06b		823.69.1±236.46a		68.99±33.70b		191.570.2±89.31a		2137.33±268.42b		649.95±208.09a	
Mean	**7.84**		**5.62**		**887.69**		**99.68**		**219.4**		**1919.94**		**562.06**	

*sd (standard deviation).

Table 7 - Volatiles of second and third flush term in I. harvest term*.

Volatile compounds	Mean % ratios
3,4-dimethy pentanol-1	54.95
N-octanol	3.84
2-heptamine	2.51
N-nonalaldehyde	4.24
Heptaminol	0.67
2,6 prazynediamin	1.68
2,5 hexandiol, 1-hexanol	tr
1-hexanol	tr
N-nonal aldehyde	tr
3,4 dimethyl penyanol-1	3.48
Decalaldhyde	1.15
Hexadecanoic acid (palmitinic acid)	0.74
Hexadecenoic acid	0.36
Akuamilan-17-ol	0.87
1,1 dodecanediol, diacetate	0.59
9-octadecenoic acid	6.34
3-oct en-1-ol	19.65
8-nitro-oxododecanoic acid	1.87
1-pentanol	0.60
9-octadecanoic acid (capric acid)	7.81
9,12 octadecadienal acid	76.12
9,12 0ctadecdienoil acid, methyl ester	5.24
2,6 pyrazinediamine	3.78
9-octadecanomide	3.20
Akuammilan-17-ol, 10-methoxy	0.40

*All of the analysis was established on wet basis.

Table 8 - Volatiles of second and third flushes in II. harvest term*.

Volatile compounds	Mean % ratios
Benzyl alcohol	3.93
Hexadecanoic acid (palmitic acid)	32.49
Octadecanoic acid propenyl ester	5.60
Linoleic acid methyl ester	25.09
DL-Limonene	4.49
Thiozele	3.66
Hexadecenoic acid, methyl ester	12.32
Hexadecenaic acid, ethyl ester	2.43
Eicosanoic acid tetra decananoic acid	38.45
Octadecanoic acid, 2-propenyl ester	4.08
Cyclodecane-8-ethyl heptadencanol	2.47
Hexadecenoic acid	7.61
Heptadecane, 4-propyl-4-propyl decane	2.86
9-octadecenoic acid, octadcyl ester	2.62
12-0ctadecenoic methyl ester	4.54
9-octadecenoic acid, 4-tridecanol	12.46
9,12 decadienoic acid, methyl ester	2.39
Heptadecane, 4-propyl	2.95
1-hydroxytridecan-5-one	1.98
1,2 benzendicarboxylic acid, diactyl ester	9.37

*All of the analysis was established on wet basis.

pounds were determined (Table 7). These compounds were derivatives of some fatty acids of mushrooms. The mushrooms reached typical and rich aroma in the second and third flush terms (Table 7).

When Table 8 was examined for second flush and second harvest term volatile compounds, there were fatty acid derivatives of the flavor components like hexadecenioc acid (32.49%) and 9-octadecenoic acid and its derivatives ranged from 2.62 to 25.09% wb). Determinations of volatile compounds were made by comparing library catalog of device. GC separation of volatile compounds and identification by spectrometry depends on various factors; technically like, extraction conditions resolution number (SKOOG *et al.*, 1991) and nature of samples like flush terms, harvest term, compost preparation (CRUZ *et al.*, 1997). In this research, it was observed that a larger variation of volatiles of *A. bisporus* was examined in the literature. The recognized flavor substances were not repeated for every flush stages and crops since quantities of aroma substances were so law and changeable in various conditions. It was established by the best separated on GC (gas chromatography) and then identified by MS (mass spectrometer) by comparison device library. Dominant and the best resolvable and separated volatiles were obtained and identified in different flushes and harvest terms. The others were obtained depending on the factors mentioned above, thus estimated that aroma and flavor of mushroom (*A. bisporus*) could be changed or not stationary in every staged and harvest term.

Table 8 gives typical mushroom compounds like DL-limonene and 1, 2 benzendicarboxylic acids, diactyl ester and 9-octedecenoic acid and their derivatives, which were identified by GC-MS mostly. *A. bisporus* has a rich flavor compounds and there were some differences between the harvest terms. In each of harvest terms it was found that typical aroma components derived from both fatty acids and other chemical compounds.

3. CONCLUSION

The comparison of chemical composition, physical properties and volatile compounds of button mushroom (*A. bisporus*,) revealed in the

different flushes and harvest terms. Chemical contents or nutritients and physical properties exhibited different trends from the first flushes to the third flushes and between the two harvest terms. Different quantities of nutrients of *A. bisporus* in the three flushes and two different harvest terms, cannot affect quality of human nutrition. Mean contents of nutritients can contribute to nutrition. Physical properties have not varied in large range, so significant physical quality criteria of *A. bisporus* were almost same in the flushes and harvests. Results of research should be original and useful about nutritional value and flavor of the button mushroom (*A. bisporus*). Volatiles were evaluated as an estimated approach therefore they were investigated and by well development device; GC-MS (gas chromatography and mass spectrometer). So *Agaricus bisporus* (white) has typical and rich aroma volatiles.

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SHORT COMMUNICATION

MONITORING OF MARINE BIOTOXINS IN *MYTILUS GALLOPROVINCIALIS* OF CENTRAL ADRIATIC SEA (2006-2009)

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ABSTRACT

A monitoring program aimed at detecting marine biotoxins in coastal bivalves (*Mytilus galloprovincialis*) of central Adriatic Sea was carried out in the years 2006-2009. The presence of Diarrhoetic Shellfish Poisoning and Paralytic Shellfish Poisoning toxins was assessed by the Mouse Bioassay, as established by the Italian Decree of the Ministry of Health of May, 16, 2002, whereas the Amnesic Shellfish Poisoning toxins were detected by HPLC. No biotoxins were determined in all the samples. Since the number of the examined samples was directly proportional to the number of shellfish aquaculture plans located in Abruzzo and Molise regions, these results provide an estimate of the possible shellfish contamination in the considered area.

- Key words: diarrhoetic shellfish poisoning, amnesic shellfish poisoning, paralytic shellfish poisoning, central Adriatic sea, *Mytilus galloprovincialis*, Mouse Bioassay -

INTRODUCTION

The Adriatic Sea is an elongated basin, with its major axis in the Northwest-Southeast direction, located between the Italian peninsula and the Balkans. It is subjected to strong forcing functions, producing a clear seasonal variability in both the circulation and the ecosystem (ZAVATARELLI *et al.*, 1998). In recent years eutrophic phenomena have frequently been reported in the Italian coastal waters (PENNA *et al.*, 2004). Eutrophication has been shown to cause major changes in species composition, structure and function of marine communities over large areas. It also promotes proliferation of macroalgae and filamentous algae. A general shift from diatoms to dinoflagellates as well as predominance of small size nanoplankton (e.g. microflagellates and coccoids) are generally observed (ISLAM and TANAKA, 2004). These massive growths of phytoplankton may contain highly toxic chemicals that cause illness and even death to aquatic organisms and humans. Elevated growth and subsequent decay of phytoplankton have caused widespread areas of seasonally oxygen depleted water. The global distribution of frequently occurring oxygen depleted zones (MALAKOFF, 1998) dominates the highly developed industrial areas that include much of Europe, central and North America and some parts of the Asia-Pacific (ISLAM and TANAKA, 2004). The Italian coast of the Adriatic Sea is periodically subjected to the high-intensity algal blooms. It is hypothesized that eutrophic phenomena are due above all to the discharge of nutrients from the Po River, but also to particular climatic conditions or when wind and currents push the river waters (PENNA *et al.*, 2004). Marine biotoxins consist of more or less complex molecules with various origins that can accumulate in marine and fresh water systems. In terms of their solubility, they can be distinguished in water-soluble and fat-soluble. In particular, the toxins soluble in water are those of Paralytic Shellfish Poisoning (PSP), Amnesic Shellfish Poisoning (ASP), while the toxins soluble in fat are those of Diarrhoetic Shellfish Poisoning (DSP) and Neurotoxic Shellfish Poisoning (NSP). These toxins are produced by marine phytoplankton species with high variability due to both intrinsic and environmental factors – temperature, salinity, macronutrients and trace elements (DOUCETTE *et al.*, 2009). The frequency of occurrence and intensity of the so-called harmful algal blooms (HABs) appear to be increasing on a global scale, becoming the subject of new research over the past decade (LEE *et al.*, 2005). The production of PSP toxin is caused almost exclusively by micro-algae belonging to the Order Dinoflagellata. The name saxitoxin (STX) derives from the species of the bivalve mollusc (*Saxidomus giganteus*) from which it was extracted. The symptoms of poisoning are similar to paralytic phenomena such as cramp, signs of paralysis and blocking of respiration. PSP toxins are potential neurotoxins which specifically block the excitation current in nerve and muscle cells resulting in signs of paralysis (BERND and BERND, 2008). The domoic acid is the toxin responsible for ASP. It acts as a glutamic acid agonist which explains the amnesia since glutamic acid plays an important role for the storage information (TODD, 1993). The symptoms include vomiting and diarrhoea followed in some case by confusion, loss of memory, disorientation and coma. Among liposoluble toxins, the DSP syndrome is the best-known due to the serious and prolonged gastrointestinal disorders (diarrhoea, nausea, vomiting and abdominal cramps) it causes to people. These toxins are divided into four structural classes: okadaic acid (OA) and its derivates (dinophysistoxins or DTXs), pectenotoxins (PTXs), yessotoxin and its derivates (YTXs) and azaspiracid (AZP). Initially the DSP was exclusively due to the presence of OA, but afterwards yessotoxin and other analogues were isolated and characterized, some of which seemed to be unique to the North-Western Adriatic Sea (GUERRINI *et al.*, 2007). The NSP is caused by the consumption of shellfish contaminated by brevetoxins or brevetoxin analogues. It involves a cluster of gastrointestinal and neurological symptoms: nausea and vomiting, paraesthesias of the mouth, lips and tongue as well as distal paraesthesias, ataxia, slurred speech and dizziness. Neurological symptoms can progress to partial paralysis; respiratory distress has been recorded (WATKINS *et al.*, 2008). The European Union established specific rules for official controls concerning live bivalve molluscs from classified production areas. These areas have to be periodically monitored in order to check the presence of biotoxins. The sampling frequency is, as a general rule, to be weekly in the periods when harvesting is allowed. If any changes in toxin populations that may lead to toxin accumulation are detected, the sampling frequency of molluscs is to be increased or precautionary closures of the areas are to be established until the results of toxin analysis are obtained (Regulation (EC) No 854/2004). According to Regulation (EC) No 853/2004 the overall maximum level of PSP and ASP allowable for the consumption of live bivalve molluscs is 800 μg kg^{-1} and 20 mg kg^{-1} respectively, while for OA, DTXs, PTXs together the limit is 160 μg of OA equivalents per kg. The maximum level for YTXs and AZP is 1 mg of yessotoxin equivalents per kg and 160 μg of azaspiracid equivalents per kg respectively (Regulation (EC) No 853/2004). The aim of this study was the control and monitoring of marine biotoxins in bivalves (*Mytilus galloprovincialis*) of

central Adriatic Sea according to Regulation (EC) No 854/2004.

MATERIALS AND METHODS

Specimens of *Mytilus galloprovincialis* were collected from shellfish aquaculture plans located in the central Adriatic Sea along the coasts of Abruzzo (a total of 324) and Molise regions (a total of 91) during monitoring programs over the years 2006-2009 (Fig. 1). Then, the shellfish aquaculture plans were 7 and 2, respectively in Abruzzo and Molise regions. Precisely, in Abruzzo region 45 samples were collected in 2006 and 2007, 72 and 162 samples in 2008 and 2009, respectively. In Molise region the number of samples was 37 in 2006, 18 in 2007, 22 in 2008 and 24 in 2009.

The sampling was made according to Regulation (EC) No 854/2004 and to the regional plan of monitoring for marine biotoxins (P.P.R.I.C. 2008-2010; P.R. 2008).

According to Regulation (EC) No 854/2004 the sampling frequency for toxin analysis in the molluscs is to be weekly in the periods when harvesting is allowed. However, sampling plans must take into particular account the possible variations linked to the specific areas, the number of the shellfish aquaculture plans, the weather conditions and the periods when harvesting is forbidden because of the age of molluscs. Due to these reasons, the sampling frequency becomes three or six-monthly (P.P.R.I.C. 2008-2010). Therefore, the number of the examined samples was directly proportional to the number of shellfish aquaculture plans located in Abruzzo and Molise regions.

The detection of OA, DTXs, PTXs, AZP and YTXs in shellfish was carried out by the Mouse Bioassay based on YASUMOTO *et al.* (FERNÁNDEZ *et al.*, 2002; YASUMOTO *et al.*, 1984) on extract from the whole body or the edible part of molluscs. We used the first Protocol included in the Italian Decree issued by the Ministry of Health on May, 16, 2002 (Decree, May, 16, 2002). This method was based on the extraction of lipophilic compounds with acetone and methanol followed by a partition between dietylether and water. The organic solvent was evaporated and the resultant residue suspended in an aqueous solution of 1% Tween 60. One mL

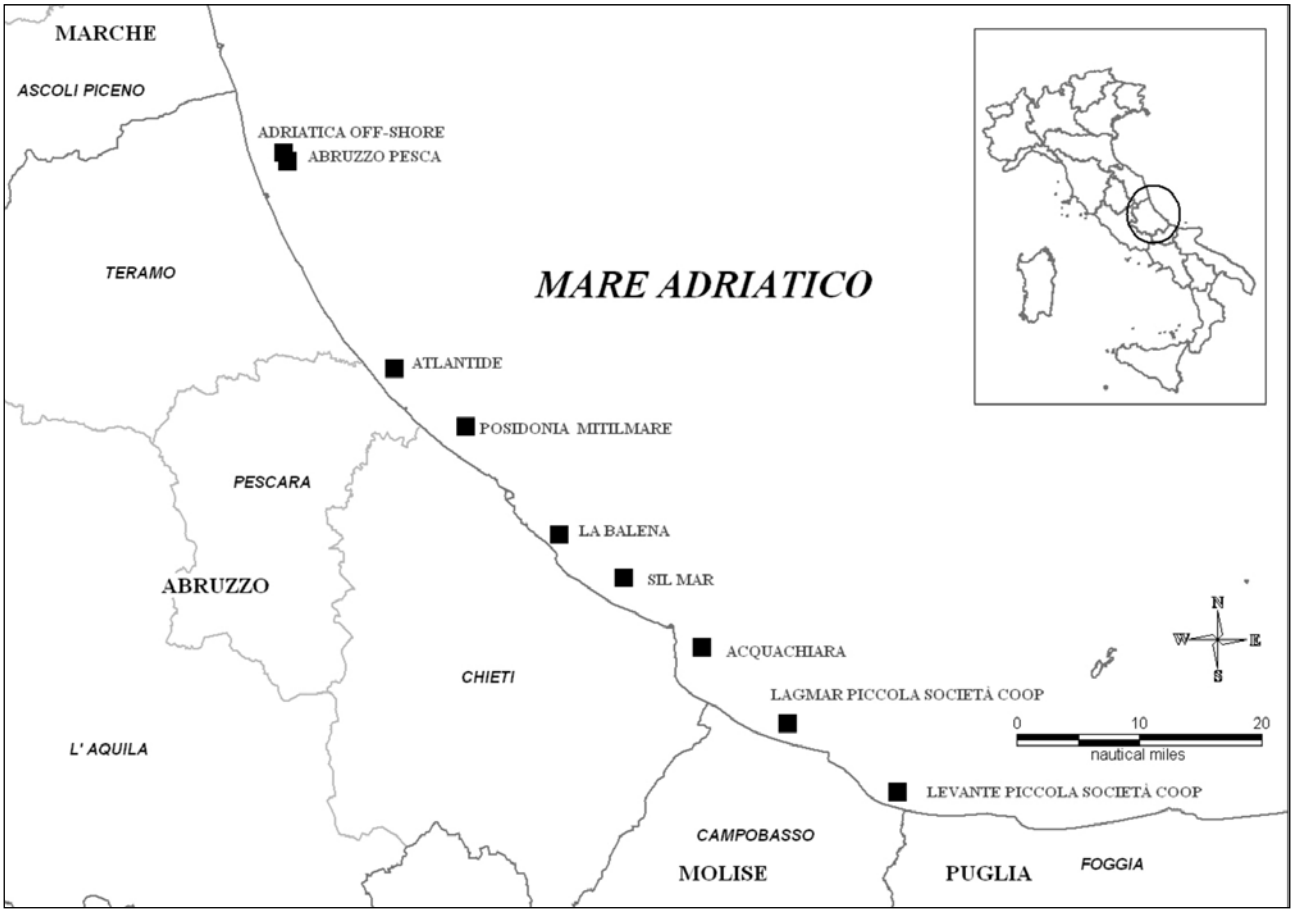

Fig. 1 - A map of study area (central Adriatic Sea) showing sampling locations.

of this extract was injected intraperitoneally (i.p.) into three albino mice weighing 18-20 g. The evaluation of the toxicity was based upon the death of the injected mice. The death of two out of three mice within 24 hours could be interpreted as the presence of one or more toxins of the following groups: OAs, DTXs, PTXs, YTXs and AZP at levels above those established in the Regulation (EC) No 853/2004. The presence of YTXs or the co-occurrence of YTXs with the other toxins of the group could be suspected if mice died showing PSP-like symptoms – as regards these cases we used the second Protocol included in the Italian Decree issued by the Ministry of Health on May, 16, 2002. It was based on the extraction of the toxins with acetone and methanol followed by partitioning into dichloromethane (60%)/methanol:water. The presence of OA, DTXs, PTXs and AZP was investigated in the dichloromethane extract whereas the presence of YTXs was investigated in the methanol:water extract. The selectivity of the assay was based on the choice of the solvents used. The detection of PSP was carried out by the Mouse Bioassay (AOAC International, 2005). The biological analysis was based on the dose of PSP (expressed as the equivalent amount of saxitoxin) that caused a fixed death time (from 1 to 60 min) in albino mice Swiss strain injected i.p. with 1 mL of an acid extract of bivalve molluscs. The determination of Domoic Acid (DA) in bivalves was carried out by HPLC-UV (QUILLIAM *et al.*, 1995). The method used a single step extraction with 50% methanol and after filtration the quantification was performed using a Shimadzu HPLC equipped with a UV/VIS detector (wavelength 242 nm), an autosampler and a column RP-18 (250 x 4 mm, 5 μm). This procedure included several steps as described below:

1) Extraction: 16 mL extraction solvent (methanol/water 1:1) was added to the sample omogenated and extracted for 3 min by the Ultra-Turrax;

2) Standards preparation: standards were prepared to set up the calibration curve, respectively: 0.4, 2.0, 4.0 and 8.0 μg/mL;

3) Samples free from Domoic Acid with added standard preparation: 2 additions were made to standards equal 2 μg/g and 20 μg/g.

The chromatographic conditions were the following: Eluent acetonitrile/water 10/90 + 0.2 mL of trifluoroacetic acid/L; Flow Rate 1 mL/min; Injection Volume 20 μL; Column Temperature 40°C. The detection limit (LOD) was 0.2 μg of Domoic Acid. The qualitative determination of domoic acid in the sample solution was based on the comparison among the retention times (RT), with an acceptability ± 2.5%. The quantitative determination was obtained by the interpolation of DA peak area in the sample extract on the calibration curve. The quality control data were the following: Correlation coefficient > 0.990; Recovery 95.8%; Detection limit < 0.2 μg/g. Value calculated with a 5:1 signal to noise.

RESULTS AND CONCLUSIONS

Monitoring of toxins in seafood and risk assessment for human exposure are important tasks for food control. Consumption of seafood contaminated with marine biotoxins may cause serious diseases. This paper described the 2006-2009 monitoring program of marine biotoxins profile of central Adriatic Sea. The results showed no presence of the detected biotoxins in all the analyzed samples. They demonstrated a good condition of the monitored marine zone in spite of the high intensity of algal bloom frequently reported in the Adriatic Sea (PAVELA-VRANČIČ *et al.*, 2002; GUERRINI *et al.*, 2007) as well as the safety of bivalves farmed along the coasts of Abruzzo and Molise regions. However, the production of toxins within and among toxigenic phytoplankton species can be highly variable, thus reflecting not only genetic traits, but also the influence of environmental factors, such as temperature, salinity, macronutrients and trace of oligoelements (DOUCETTE *et al.*, 2009).

CIMINIELLO *et al.* (2010) reported the toxin profile of *Mytilus galloprovincialis* from the Adriatic sea along the Emilia Romagna coasts and revealed the presence of the three major spirolides produced by the alga *Alexandrium ostenfeldii* as well as of the yessotoxin and its analogues.

Afterwards, other authors (VIRGILIO *et al.*, 2010) evaluated the presence of PSP toxins in mussels of Sardinia region at levels above 800 μg kg^{-1} using the mouse test according to the Italian Decree issued by the Ministry of Health on May, 16, 2002. They required specific protocols written by local health agencies in order to control if a high level of toxin detection occurs, thus managing the non-conformity cases. However, there is currently a high degree of dependence on mouse-based bioassays but there is an increasing acceptance of the need to develop and implement non-animal based methods. Recent advances in analytical equipment have enabled the development of alternative methods aimed at determining the possible presence of marine biotoxins at very low concentrations (BERND and BERND, 2008).

In recent years the increased complexity of multiple toxin classes found in bivalves has considered it necessary the development of additional tools to be implemented in the monitoring of contaminated seafood by individual toxin. Hence new efforts are particularly required to further refine the analytical assays for the detection of all toxins and to better organize periodical controls in order to obtain more reliable, accurate estimates of bloom toxicity and their potential impacts.

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SHORT COMMUNICATION

DIFFERENCES IN NUTRITIONAL COMPOSITION BETWEEN CULTURED AND WILD GREEN TIGER SHRIMP (*PENAEUS SEMISULCATUS*)

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ABSTRACT

This study was carried out to determine if there were differences in nutritional compositions between cultured and wild green tiger shrimp (*Penaeus semisulcatus*). Lipid content of cultured shrimp was significantly higher than that of wild shrimp ($p<0.05$). The percentages of total polyunsaturated fatty acids as well as the n-3/n-6 ratio were higher in the wild shrimp than the cultured one. On the other hand, no significant difference was found in terms of both the quality and quantity of amino acids between these two shrimps ($p>0.05$). Cultured shrimp contained higher concentrations of P, K and Zn than the wild one, while wild shrimp included higher amounts of Ca, Mg and Na than the cultured one. Total carotenoid contents in both the muscles and carapace of wild shrimp were higher than those in the cultured one ($p<0.05$).

- Key words: wild and cultured shrimp, fatty acid, amino acid, mineral content, carotenoid content -

INTRODUCTION

Green tiger shrimp, *Penaeus semisulcatus*, is one of the most important commercial shrimp in Eastern Mediterranean. Increased demand for shrimp in world markets has encouraged many developing countries to engage in shrimp farming. Consumers are increasingly getting interested in food safety and traceability of the production from egg to plate, and becoming more concerned about how or where animal are produced. Consumers are becoming more concerned about nutritional differences between cultured and wild animals. Chemical composition and sensory parameters in some cultured fish and shellfish, excluding shrimp, are known to differ from those of their wild counterparts. Specifically higher lipid levels in cultured sea food is a general phenomenon observed in a variety of species studied so far (GRIGORAKIS *et al.*, 2002; SAGLIK *et al.*, 2003). Fatty acid composition in sea foods varies depending on commercial diet fed cultured animals or season and geographic region of wild animals (GRIGORAKIS *et al.*, 2002; CALABRETTI *et al.*, 2003; YANAR and CELIK, 2005). Although numerous studies have been conducted on differences in nutritional compositions between cultured and wild fish or shellfish, this matter has not yet been studied on green tiger shrimp or other shrimp species.

The present study was designed to verify differences in proximate composition, fatty acid and amino acid profiles, mineral and total carotenoid contents in cultured and wild green tiger shrimp.

MATERIALS AND METHODS

Experimental shrimp

The cultured shrimp (average weight of 19.7 ± 0.21 g and length of 133.3 ± 3.90 mm) used in this study were reared for a period of 8 months in 1x5x10 m earthen pond at Yumurtalik Marine Research Station of the University of Cukurova, while the wild ones (average weight of 19.0 ± 0.07 g and length of 128.6 ± 1.30 mm) were caught off Yumurtalik on the coast of eastern Mediterranean of Turkey. Harvest time for both shrimps was in November 2005, and water temperature was about 6°-17°C. The cultured shrimp was fed a commercial feed, containing 47% protein, 10% fat, 2% fibre, 12% moisture and 15% ash (%, dry matter) obtained from Çamlı Yem A.Ş., İzmir, Turkey.

The shrimp samples were kept in ice until they were taken to the laboratory, and then deheaded, peeled, and divided. Only the edible portion was used for all analyses, whereas both edible portion and carapace of the shrimps were used for total carotenoid analysis. Four lots of each treatment were analysed in duplicate. All lots were comprised of at least twenty individuals.

Proximate composition analysis

Ash and moisture contents were determined as described by AOAC (1984) and crude protein content was calculated by converting the nitrogen content determined by Kjeldahl's method (6.25 x N) (AOAC, 1984).

Fatty acid analysis

Lipids were extracted by the method of BLIGH and DYER (1959) and were stored under nitrogen at -20°C for further analysis. The fatty acids in the total lipid were esterified into methyl esters by saponification with 0.5 N methanolic NaOH and transesterified with 14% BF_3 (w/v) in methanol (IUPAC, 1979). The esterified sample was analysed using a Thermoquest Trace gas chromatograph equipped with a Supelco-SP-2330 fused-silica capillary column (30x0.25 mm i.d., 0.20 μm film thickness of polyethylene glycol) (Supelco, Inc., Bellefonte, PA, USA) and flame-ionisation detector (FID). The chromatographic conditions were: initial temperature 120°C; heating rate 5°C/min; final temperature 220°C; injector temperature 240°C; carrier gas, helium; split ratio 1/150; column flow 75 mL/min (He); sample injection 0.5 μL. The fatty acids methyl esters were identified by comparison of their retention times with those of chromatographic standards (Sigma Chemical Company; the fatty acid methyl mixture No. 189-19).

Amino acid analysis

The shrimp samples were hydrolysed in 6 N HCl at 110°C for 24 h (AOAC, 1984) in an evacuated sealed ampoule. Excess acid from the hydrolysate was removed by flash evaporation under reduced pressure. The analysis was carried out using an Eppendorf Biotronic LC 3000 Amino Acid Analyzer (Eppendorf-Biotronic, Hamburg, Germany), according to the standard program.

Mineral analysis

The shrimp samples were wet digested with HNO_3/HCO_4 (AOAC, 1996). The elements: calcium, magnesium, sodium, potassium and iron were determined using a Perkin-Elmer AA 700 atomic absorption spectrophotometer (Perkin-Elmer Corporation, Norwalk, CT).

Total carotenoid analysis

The total carotenoids of shrimp were extracted according to the method of TORISSEN and NAEVDAL (1984). Carotenoids analysis was conducted on muscle and carapace of shrimps.

Four shrimp were randomly sampled from each treatment and used for carotenoid analyses, which were carried out in triplicate. Samples of about 0.5 g muscle or carapace were collected from shrimp and then transferred to 10 mL pre-weighed glass tubes. After the samples were ground in acetone containing anhydrous sodium sulphate with a homogenizer (Ultra-turrax Roche T 25 Basic), the extractions were made up to 10 mL with acetone. The samples were stored for 3 days at 4°C in a refrigerator, and then extracted three or four times until no more colours could be obtained. The solutions were centrifuged at 5,000 rpm for 5 min, and then absorptions were measured in a spectrophotometer (Shimadzu, UV mini 1240). Total carotenoid concentration in the samples was determined spectrophotometrically in acetone using extinction coefficients ($E_{1\%, 1cm}$) of 1900 for astaxanthin at 475 nm (FOSS *et al.*, 1984).

Statistical analysis

All data were expressed as mean ± standard error. The statistical significance of any intergroup differences was assessed using Student's t test wherever appropriate, and "P" value less than 0.05 was considered statistically significant. All statistical procedures were performed by using the SPSS 12.0 software for Windows. Experiments were conducted according to the European Council Directive 86/609/EEC regarding the protection of animals used for experimental and other scientific purposes.

RESULTS AND DISCUSSION

The results of proximate composition of cultured and wild green tiger shrimp are shown in Table 1. Protein, ash and moisture contents did not differ significantly ($p>0.05$) between the shrimp**.** Protein is considered to be a stable component of seafood in respect to diet and feeding level depending mainly on fish weight (SHEARER, 1994). The lipid content in cultured shrimp (1.44%) was significantly higher ($p<0.05$) when compared to wild shrimp (0.76%). Higher lipid content in cultured shrimp may be attributed to high lipids content of its diet (12%). In fact, in many studies (GRIGORAKIS *et al.*, 2002; GONZÁLEZ *et al.*, 2006), it has been reported that lipid level in cultured seafood was higher than the wild one.

The results of fatty acids analysis demonstrated that although cultured and wild shrimp contained the same fatty acid type, their concentrations displayed significant differences (Table 2). The predominant fatty acids in both shrimp were found as: C16:0 (palmitic acid) and C18:0 (stearic acid) in saturated fatty acids (SFA); C18:1 cis9 (oleic acid) in monounsaturated fatty acid (MUFA); C20:5 n-3 (eicosapentaenoic acid, EPA) and C22:6 n-3 (docosahexaenoic acid, DHA) in polyunsaturated fatty acid (PUFA). These five fatty acids represented 60 and 68% of the total fatty acid content in cultured and wild shrimp respectively. Similar results were reported on some other shrimp species (BRAGAGNOLO and RODRIGUEZ-AMAYA, 2001; ROSA and NUNES, 2003; YANAR and ÇELIK, 2006).

Total SFA content of cultured shrimp was similar to that of the wild shrimp ($p>0.05$). C16:0

Table 1 - Proximate composition (%) in the muscle of wild and cultured green tiger shrimp (*P. semisulcatus*).

Component	Cultured	Wild
Protein	22.76±0.07[a]*	23.53±0.09[a]
Lipid	1.44±0.03[a]	0.76±0.01[b]
Moisture	75.10±0.02[a]	75.18±0.02[a]
Ash	1.36±0.01[a]	1.62±0.01[a]

* Means±SE in the same row with different letter indices differ at a significance level of $p<0.05$.

Table 2 - Fatty acids (% Total Fatty Acids) in the muscle of wild and cultured green tiger shrimp (*P. semisulcatus*).

Fatty acids	Cultured	Wild
C10:0	0.09±0.00[a]*	0.02±0.00[b]
C11:0	0.15±0.00[a]	0.02±0.00[b]
C14:0	0.90±0.01[b]	1.24±0.03[a]
C15:0	0.09±0.00[b]	0.90±0.00[a]
C16:0	16.01±0.21[b]	18.44±0.11[a]
C17:0	1.79±0.02[a]	1.92±0.00[a]
C18:0	11.66±0.26[a]	6.60±0.16[b]
C20:0	0.34±0.02[a]	0.24±0.01[a]
C21:0	0.12±0.00[a]	0.11±0.00[a]
C22:0	0.28±0.01[a]	0.23±0.00[a]
C23:0	0.12±0.01[a]	0.10±0.00[a]
C24:0	0.28±0.00[a]	0.15±0.00[a]
Total saturated	31.83±0.3[a]	29.97±0.4[a]
C15:1	0.13±0.00[a]	0.05±0.00[b]
C16:1	2.34±0.10[b]	4.24±0.01[a]
C17:1	3.13±0.06[a]	1.74±0.04[b]
C18:1n-9t	0.15±0.01[b]	0.43±0.02[a]
C18:1n-9c	12.42±0.09[a]	8.96±0.03[b]
C20:1n-9	0.55±0.01[a]	0.62±0.01[a]
C22:1n-9	1.05±0.02[a]	1.05±0.01[a]
C24:1n-9	2.36±0.01[a]	0.34±0.00[b]
Total monoenoic	22.13±0.2[a]	17.43±0.3[b]
C18:2n-6t	0.24±0.02[a]	0.32±0.01[a]
C18:2n-6c	3.78±0.08[a]	2.30±0.01[b]
C20:2n-6	0.92±0.01[a]	0.89±0.00[a]
C18:3n-6	0.16±0.01[a]	0.13±0.01[a]
C18:3n-3	0.32±0.03[a]	0.18±0.02[b]
C20:3n-3	0.07±0.00[a]	0.03±0.00[a]
C20:4n-6	3.60±0.07[a]	3.56±0.02[a]
C20:5n-3	8.47±0.13[b]	13.76±0.05[a]
C22:6n-3	11.39±0.17[b]	17.48±0.03[a]
Total polyenoic	28.95±0.3[b]	38.65±0.2[a]
Σn-3: Σn-6	2.33±0.02[b]	4.37±0.03[a]
EPA/DHA	0.74±0.01[a]	0.79±0.01[a]

* Means±SE in the same row with different letter indices differ at a significance level of $p<0.05$.

was the primary saturated fatty acid followed by C18:0 for both shrimp. The content of C16:0 was lower while that of C18:0 was higher in cultured shrimp ($p<0.05$). C16:0 was prominent in all shellfish, contributing to high percentage of SFAs in shellfish.

Cultured shrimp was characterized by higher amounts of monounsaturated fatty acids (MUFA) such as, C17:1 (heptadecanoic acid), 18:1 n-9 (oleic acid) and 24:1 n-9 (nervonic acid), which was the quantitative dominant in these groups. The higher amount of C18:1 n-9 in cultured shrimp has been reported to arise from its dominance in the feed although it can also be synthesized from C18:0 (KRAJNOVIC-OZRETIC *et al.*, 1994; GRIGORAKIS *et al.*, 2002). The percentage of PUFA was higher in wild shrimp compared with the cultured one ($p<0.05$). EPA and DHA were identified as the main n-3 PUFA. DHA was the principal n-3 PUFA in both shrimp but its content was higher in the wild shrimp than in the cultured one and the difference was statistically significant. The percentage of EPA occurred in larger quantities in wild shrimp than in the cultured one ($p<0.05$). The wild marine species have higher n-3 PUFA because they feed on marine phytoplankton and other marine organisms rich in these fatty acids (ACKMAN and TAKEUCHI, 1986). For the group of n-6 PUFA, the primary fatty acid was C18:2 n-6 (linoleic acid) and C20:4 n-6 (arachidonic acid) for both shrimp. C18:2 n-6 was observed in higher concentrations in cultured than in wild shrimp. This fatty acid has a higher concentration in cultured shrimp because the plant oil in the feed provided it (YAMADA *et al.*, 1980). C20:4 n-6 content of cultured shrimp was similar to those of the wild shrimp ($p>0.05$). As a consequence of this dependence, the quantity of PUFA in cultured shrimp was lower ($p<0.05$). Similarities with the current results are found in data regarding various marine fish species (SAGLIK *et al.*, 2003) and fresh water species (JANKOWSKA *et al.*, 2008).

Table 3 - Amino acid profile (mg/100 g) in the muscle of wild and cultured green tiger shrimps (*P. semisulcatus*).

Amino acids (AA)	Cultured	Wild
Essential (EAA)		
Threonine	0.80±0.00[b*]	0.99±0.01[a]
Methionine	0.25±0.00[a]	0.19±0.00[b]
Isoleucine	1.41±0.00[b]	1.71±0.01[a]
Leucine	1.88±0.00[a]	1.86±0.00[a]
Phenylalanine	1.24±0.01[b]	1.41±0.01[a]
Valine	1.32±0.01[b]	1.85±0.00[a]
Lysine	1.66±0.01[b]	1.74±0.01[a]
Histidine	0.50±0.01[a]	0.53±0.00[a]
Arginine	0.99±0.01[a]	0.79±0.00[b]
ΣEAA	10.04±0.95[a]	11.07±1.26[a]
Non-essential (NEAA)		
Aspartic acid	1.99±0.01[b]	2.32±0.01[a]
Serine	0.83±0.01[a]	0.84±0.01[a]
Glutamic acid	3.25±0.01[b]	3.53±0.02[a]
Glycine	1.79±0.00[a]	1.51±0.00[b]
Alanine	1.88±0.00[b]	2.22±0.01[a]
Tyrosine	0.93±0.00[a]	0.92±0.00[a]
Proline	1.02±0.01[a]	0.99±0.00[b]
ΣNEAA	11.68±1.34[a]	12.32±1.24[a]
EAA/NEAA	0.86±0.01[a]	0.90±0.00[a]
ΣAA	21.72±2.58[a]	23.39±2.60[a]

*Means±SE in the same row with different letter indices differ at a significance level of $p<0.05$.

The ratio of n-3 to n-6 fatty acids was higher in wild shrimp (4.37) than in the cultured one (2.33), in agreement with the data from other marine species (SEROT *et al.*, 1998; GRIGORAKIS *et al.*, 2002). PIGGOTT and TUCKER (1990) suggested that the n-3/n-6 ratio was the better index of relative nutritional value from different species. The lower proportion of n-3 PUFA in cultured shrimp may reduce the nutritional quality of their lipid component, but the amplitude of these differences between wild and cultured shrimp depended on the dietary fatty acid composition.

Amino acid profiles in the shrimp are presented in Table 3. In both shrimp, the quantitatively most important essential amino acids (EAA), by order of decreasing magnitude, were valine, leucine and lysine; in relation to non-EAAs (NEAA), the major were glutamic acid, aspartic acid, glycine and alanine. These results resemble those presented by ROSA and NUNES (2003) where the amino acid profile of different shrimp species; red shrimp (*Aristeus antennatus*) and pink shrimp (*Parapenaeus longirostris*) were compared. YANAR and CELIK (2006) reported that glutamic acid, aspartic acid, arginine, lysine and leucine were the major amino acids in green tiger shrimp (*Penaeus semisulcatus*) and speckled shrimp (*Metapenaeus monoceros*) raised at different seasons, similar to the present study. Methionine and histidine were found to be the first and second limiting amino acids in both shrimp. In fact, the sulphur-containing amino acids are generally found to be first limiting in shellfish and fish meals (IWASAKI and HARADA, 1985; ACTON and RUDD, 1987). Total amino acid (TAA), EAA and NEAA contents did not differ significantly ($p>0.05$) between wild and cultured shrimp. The ratio of EAA and NEAA for wild and cultured shrimp were 0.86 and 0.90 respectively (Table 3), which reveals that both shrimp are well balanced with respect to EAA and may be considered as a food source of high-quality protein.

Mineral content in the muscle of wild and cultured shrimp are shown in Table 4. Cultured shrimp contained higher concentrations of phosphorous (P), potassium (K) and zinc (Zn) than the wild one, while wild shrimp included higher amount of calcium (Ca), magnesium (Mg) and sodium (Na) than the cultured one ($p<0.05$). But,

Table 4 - Mineral content (mg/kg) in the muscle of wild and cultured green tiger shrimp (*P. semisulcatus*).

Mineral content	Cultured	Wild
Fe	19.84±0.17[a*]	20.19±0.02[a]
Ca	89.77±0.17[b]	107.36±0.24[a]
Mg	579.54±0.34[b]	691.31±0.42[a]
Mn	1.14±0.01[a]	1.33±0.01[a]
Zn	25.26±0.02[a]	23.65±0.3[b]
Na	2947.30±4.63[b]	3246±6.65[a]
P	2901.60±6.77[a]	2444.6±4.17[b]
K	4725±6.00[a]	3656±12.00[b]

* Means±SE in the same row with different letter indices differ at a significance level of p<0.05.

iron (Fe) and manganese (Mn) concentration did not differ significantly between them (p>0.05).

It is generally accepted that mineral contents of sea food are influenced by their diets and water quality. Although both shrimps were subjected to the same sea water and climate conditions, there were naturally some differences between them, in terms of their diets and water quality. These differences were most probably derived from mineral passed from soil in earthen ponds to water or feeding artificial diet or live feed. For example, higher phosphorous level in cultured shrimp may result from the protein source, such as fish meal which is known to contain high level of phosphorus, present in feed (SUGİURA and HARDY, 2000).

As shown in Table 5, total carotenoid contents in wild shrimp were higher ($p < 0.05$) than those of the cultured shrimp (35% higher for muscle and 41% higher for carapace). The aquatic animals such as crustaceans are unable to produce carotenoids *de novo* (GOODWIN, 1984), and these components are obtained from only plant or protista occurring in their natural environment. Therefore, as expected, wild shrimp exhibited higher intensive colouring compared to shrimp reared in intensive conditions in which they were deprived of carotenoids. Total carotenoid contents in carapaces of two shrimps were higher than their muscles (highest being 89.47 mg/kg in the carapace, 16.16 mg/kg in the muscle of wild shrimp). YANAR *et al.* (2004) reported that the carotenoid contents in the muscle of wild *P. semisulcatus* and *M. monoceros* were 14.1 and 16.9 mg/kg, respectively. The reported values (SACHINDRA *et al.*, 2005) for carotenoid content in shrimp from shallow waters of India were 10.4-17.4 for muscle, 59.8-104.7 for carapace. Our results display similarities with these results.

Table 5 - Total carotenoid contents of wild and cultured shrimp muscle and carapace (mg/kg).

	Cultured	Wild
Muscle	10.43±0.17[b]	16.16±0.15[a]
Carapace	63.38±0.78[b]	89.47±0.78[a]

* Means±SE in the same row with different letter indices differ at a significance level of p<0.05.

CONCLUSION

The results of the current study indicate that the culture of green tiger shrimp on commercial feed produces shrimp whose muscle contain more total lipid and are characterized by a different fatty acid profile than the wild shrimp inhabiting the natural environment. Wild shrimp is a better source of PUFA than their cultured counterparts. The most predominant PUFA were EPA and DHA in both wild and cultured shrimp. Total amino acid, essential amino acid and non essential amino acid contents did not differ significantly between wild and cultured shrimp. Some minerals (Ca, Mg, Mn, Zn, Na, P, and K) were found to be significantly different between the muscle of cultured and wild shrimp (except for Fe and Mn contents). The carotenoid content in the muscle and carapace from wild shrimp was higher than that of the cultured shrimp.

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SHORT COMMUNICATION

EFFECTS OF CANNING ON EXTRACTION OF HEAVY METALS FROM TUNA

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ABSTRACT

In this study, mercury, lead, cadmium and copper were determined in oils and meat of canned tuna. Also two tuna species of Persian Gulf and Oman Sea and vegetable oil were analyzed for these metals. The results of this study indicate that these heavy metals could be extracted to oil or water in canned tuna. In the case of lead, because of impurity of galvanized cans to it, concentration in oil and water is higher than meats in some samples. Overall, the majority of canned tuna from the Persian Gulf (area of Iran) have concentrations below the maximum permissible levels for these toxic metals but concentration of heavy metals is considerable in oil and water of canned tuna. It should be due to the extraction of them to organic phase. Since most toxic organometallic compounds extract faster than inorganic compounds it could be very important in toxicology.

- Key words: heavy metals, canned tuna, oil, meat, extraction -

1. INTRODUCTION

Over the last few decades, there has been growing interest in determining heavy metal levels in the environment and attention was drawn to the measurement of contamination levels in public food supplies. Toxicological and environmental studies have prompted interest in the determination of toxic elements in food (ASHRAF, 2006).

Fish and seafood can be an important part of a balanced diet for humans; however, some fish accumulate contaminants present in the aquatic environment including mercury (Hg), cadmium (Cd) and lead (Pb), which are tolerated only at very low levels (LOURENÇO *et al.*, 2004). Tuna was recognized as a predator able to concentrate large amounts of heavy metals. For example, mercury concentrations ranging from 50 to 120 $\mu g\ g^{-1}$ in internal organs of Japanese tuna has been reported (ASHRAF, 2006).

Canned tuna fish are frequently and largely eaten all around the world, so their toxic metals content should be of concern to human health. The present study was therefore carried out in view of the scarcity of information about the effect of processing steps on the concentration of heavy metals in canned tuna.

Levels of heavy metals in the oil and meats of canned tuna and fresh fish were analyzed. The results of this study show how heavy metals could be extracted to oil or water in canned tuna. It will help in generating data needed for the assessment of heavy metals intake from this source especially from oil of canned tuna.

2. MATERIALS AND METHODS

2.1. Instrumentation

All glassware was soaked over night in 10% (v/v) nitric acid, followed by washing with 10% (v/v) hydrochloric acid, and rinsed with double distilled water and dried before using. A Varian Model AA-220 atomic absorption spectrometer equipped with a deuterium background corrector was used for the determination of heavy metals and the mercury /hydride generator was a Varian model VGA 77 in a continuous system.

2.2. Reagents

All reagents and solvents were of analytical reagent grade (E. Merck, Darmstadt, Germany).

Solutions were prepared using deionized water from a Nanopure Water system, Millipore Corporation (Bedford, MA, USA) with specific resistivity of 18.3 $M\Omega cm^{-1}$.

Aqueous standard solutions for Hg (II), Pb (II), Cu (II) and Cd (II) were prepared by appropriate dilution of 1,000 mg L^{-1} stock solutions.

A certified reference material Dorm 3 (fish protein certified reference material for trace metal) was obtained from the national research council of Canada (NRCC, Ottawa, Canada).

Also a certified reference oily matrix $Certi_{PUR}$, containing 100 mg kg^{-1} of Cd (II), Cu (II) and Pb (II) was purchased from Merck (Darmstadt, Germany).

2.3. Sampling

Tuna fish caught by commercial vessels from the coasts of the Iran are canned as chunks at a commercial factory on land.

Nineteen brands of canned tuna were purchased from local stores in 2009. Canned tuna included 2 types: tuna in water and tuna in oil.

After opening each can oil/broth was drained off and separated.

Oil and meat of 1 lot of each brand was analyzed. From each lot, the total contents of 14 cans were combined, and then meat was homogenized thoroughly in a food blender with stainless steel cutters. Also samples of two species of tuna (n=7) were collected from canned tuna factories in Tehran-Iran, which provide their fish from the Persian Gulf and Oman Sea.

The fish edible muscle was homogenized by stainless steel cutters.

2.4. Procedures for sample preparations

2.4.1. Sample preparation for meats of canned tuna and fresh fish.

The homogenized sample (3,000 g) was weighed into a 0.5 L glass digestion tube, and for mercury, 10 mL of conc. HNO_3 and 5 mL of conc. H_2SO_4 were slowly added. The tube was then placed on top of a steam bath unit to complete dissolution.

It was then removed from the steam bath, cooled and the solution transferred carefully into a 50 mL volumetric flask; for the reduction of mercury 5 mL $SnCl_2$ were used. For the determination of lead, cadmium and copper, about 2 ± 0.001 g of homogenized sample were weighed into a 200 mL beaker and 10 mL of conc. HNO_3 were added. The beaker was covered with a watch glass and, after most of the sample had dissolved by standing overnight, heated on a hot plate with boiling until any vigorous reaction had subsided. The solution was allowed to cool, transferred into a 50 mL volumetric flask and diluted to the mark with distilled water Ministry of health and family welfare government of India, (2005) and HASHEMI-MOGHADDAM and SABER-TEHRANI (2008).

Table 1 - Recovery of some heavy metals from oil and meat of canned tuna.

Metals	Heavy metal content (μg g^{-1})		Concentration of added (μg g^{-1})		Concentration of recovered (μg g^{-1})		%Recovery	
	Meat	Oil	Meat	Oil	Meat	Oil	Meat	Oil
Lead			0.40	0.40	1.57	1.79	95	94
	1.25	1.50	0.80	0.80	2.01	2.23	98	97
			1.60	1.60	2.74	2.97	96	96
Cadmium			0.050	0.050	0.095	0.099	92	93
	0.052	0.057	0.100	0.100	0.144	0.146	95	93
			0.150	0.150	0.194	0.197	96	96
Copper			0.40	0.40	0.94	1.14	94	95
	0.60	0.80	0.80	0.80	1.32	1.50	94	94
			1.60	1.60	2.12	2.34	97	97
Mercury			0.400	0.400	0.707	0.578	94	96
	0.350	0.202	0.800	0.800	1.109	0.975	96	97
			1.200	1.200	1.511	1.337	97	95

Table 2 - Analysis of certified reference materials expressed in μg g^{-1}.

Elements	Dorm3			Certified reference oily matrix Certi$_{PUR}$ (1 ppm)
	Certified values	Measured values	Recovery	Recovery
Pb	0.395±0.050	0.372±0.092	94	94
Cd	0.290±0.020	0.277±0.052	95	96
Cu	15.5±0.63	15.1±0.81	97	96
Hg	0.382±0.060	0.374±0.083	98	-

2.4.2. Sample preparation for oil of canned tuna.

A 3,000 g aliquot of oil, 2.0 mL of 35% H_2O_2 and 10.0 mL of 36% hydrochloric acid were placed in a teflon beaker. The extraction was carried out for about 30 min under magnetic stirring at 90°C. The mixture was cooled and then centrifuged at 4,800 rpm for 5 min: the acid phase was taken apart in a 50 mL volumetric flask, while the organic layer was extracted again for 10 min with 8.0 mL of boiling water under the same conditions described earlier; after the centrifugation, the second extract was added to the volumetric flask and brought to the mark with ultra pure water (DUGO *et al.*, 2004)

2.5. Accuracy test

The accuracy of the method was investigated by spiking different amount of these metals to meats and oils samples. Results tabulated in Table 1. Good recoveries of spiked samples demonstrate the accuracy of the methods.

Also the accuracy of the applied analytical method was estimated on certified reference materials (CRMs), Dorm3 (fish protein certified reference material for trace metal) and a certified reference oily matrix Certi$_{PUR}$. The certified oil, which contained 100 mg kg^{-1} of each metal, was diluted with n-hexane to obtain 1 mg kg^{-1} standard oily matrices. The dilution was performed in order to obtained concentration similar to those nearly found in oils.

The standard oily solution was subjected to extraction procedure describe above, each extract analyzed three times. The results obtained on the CRMs are presented in Table 2, showing good agreement with the certified values for all metals.

3. RESULTS AND DISCUSSIONS

Nineteen brands (266 samples) of canned tuna (in oil and water), two species of fresh tuna (14 samples) and 4 samples of commercial vegetable oil were analyzed for lead, cadmium, copper and mercury. The metal contents, expressed in μg g^{-1} wet weight, varied from 0.082 to 1.162 and 0.008 to 0.788 with an average values of 0.464 and 0.202 for mercury in meat and oil respectively, from 0.22 to 3.52 and 0.15 to15.51 with an average values of 1.23 and 2.33 for lead in meat and oil respectively, from non detectable to 0.114 and non detectable to 0.075 with an average values of 0.042 and 0.017 for cadmium in meat and oil respectively and from 0.025 to 32.114 and non detectable to 22.521 with an average values of 2.734 and 1.765 for copper in meat and oil respectively. The results showed in Tables 3 and 4.

Table 3 - Concentration of some heavy metals in meat and oils of canned tuna, expressed in μg g^{-1} wet weight.

Type of canned tuna		Hg		Cu		Cd		Pb	
		Meat	Oil	Meat	Oil	Meat	Oil	Meat	Oil
Canned tuna in oil	Range	0.22-3.52	0.15-15.51	0-0.114	0-0.075	0.025-32.114	0-22.521	0.082-1.162	0.008-0.788
	Mean	1.23	2.33	0.042	0.017	2.734	1.765	0.464	0.202
Canned tuna in water	Range	0-6.25	0.75-5.25	0-0.125	0-0.114	0.512-7.451	0-4.612	0.031-1.625	0.135-0.244
	Mean	2.19	2.25	0.038	0.047	2.559	1.153	0.681	0.213

Table 4 - Concentration of some heavy metals in two tuna species (edible parts) of the Persian Gulf and Oman Sea and vegetable oil, expressed in μg g^{-1} wet weight.

Matrices	n	Pb	Cd	Cu	Hg
Yellofine	7	0.47±0.12	0.09±0.01	0.7±0.1	0.59±0.12
Skipjack	7	0.39±0.09	0.07±0.01	0.5±0.1	0.41±0.12
Vegetable oil	4	ND	ND	0.09±5	ND

These results could give the following consideration:

1. Heavy metals can be extracted from meat in canned tuna.

Comparison results of this work and other independent work for determination of heavy metals in canned tuna and fresh fish reveal decrease concentration of heavy metals in canned tuna (GANJAVI *et al.*, 2010; SUPPIN *et al.*, 2005; STORELLI *et al.*, 2010; KNOWLESY *et al.*, 2003; FORSYTH *et al.*, 2004; DABEKA *et al.*, 2004; KUMAR *et al.*, 2003) (Table 5). Moisture content of tuna decreases after conservation, so heavy metals concentration should increase in meat. Nevertheless it decreases because of extraction to oil or water. Comparison of heavy metal concentration in fresh and canned tuna in case of Pb is out of the ordinary. A galvanized sheet which surface is tin plated and covered with special varnish is used for preparation of canned fish in Iran. Pb is an impurity in galvanized sheet and high concentration of this metal in canned tuna could be because of unsuitable packing in some samples.

2. Relative concentration of cadmium is higher than mercury in the water phase. It could be because of a large amount of organomercury in fish, which are more soluble in oil phase.

3. Because of lead impurity of some packages, mean concentration of lead in canned tuna oil and tissue is higher than fresh samples.

4. Assessment of extraction of copper is not possible because of presence of copper in vegetable oil,

5. Maximum permissible limits for lead, cadmium, copper and mercury are 2, 0.2, 20 and 0.5 μg/g (wet weight), respectively. Concentrations heavy metals of almost all canned tuna are below the permissible levels.

Table 5 - Comparison of heavy metal concentration in fresh and canned tuna fish.

Region	Type of Fish	Metals					
		Mercury		Lead		Cadmium	
		Fresh	Canned	Fresh	Canned	Fresh	Canned
Persian Gulf,	Tuna[a]	0.50	0.46	0.43	1.21	0.08	0.04
Oman Sea	Yellowfin[b]			0.441	0.154	0.084	0.029
	Skipjack[b]			0.218	0.072	0.062	0.016
Austria	Tuna	0.19	0.011	0.013	0.014	0.014	0.016
Italy	Tuna	0.61	0.41	0.07	0.06	0.01	0.04
UK	Tuna	0.401	0.190				
	Salmon	0.050	0.033				
		0.950	0.047				
Canada	Tuna	0.673[c]	0.024[c]				
		0.929	0.153				
Fiji	Tuna	0.34	0.20				
US FDA Data	Tuna	0.32	0.19				

[a]: Present work; [b]: Home canning; [c]: Methyl mercury.

4. CONCLUSION

It is often assumed (even in fish advisories) that fresh tuna or tuna steaks have higher heavy metals levels than canned tuna, but the literature does not provide clear evidence for this. Reasons for decrease of heavy metals concentration in canned tuna could be revealed by this study. Heavy metals can bind sulfur groups (i.e., cysteine) and those extracted into oil during the processing is possible. Also organic mercury and lead in presence of chloride can extract in oil easily based of westoo extraction (SABER-TEHRANI *et al.*, 2006), as more toxic organometallic compounds extract faster than inorganic compounds, this could be very important in toxicology.

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AUTHOR INDEX

VOLUME XXIII (2011)

CONTENTS OF VOLUME XXIII - 2011

OPINION PAPER

PAPERS

SHORT COMMUNICATIONS

PAPERS

SHORT COMMUNICATIONS

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CONTENTS

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SHORT COMMUNICATIONS