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# Total and individual carotenoids and phenolic acids content in fresh, refrigerated and processed spinach (*Spinacia oleracea* L.)

Andrea Bunea [a], Mirjana Andjelkovic [b,c,*], Carmen Socaciu [a], Otilia Bobis [a], Madalina Neacsu [a], Roland Verhé [c], John Van Camp [b]

[a] *Department of Chemistry and Biochemistry, University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania*
[b] *Department of Food Safety and Food Quality, Faculty of Bioscience Engineering, Ghent University, Belgium*
[c] *Department of Organic Chemistry, Faculty of Bioscience Engineering, Ghent University, Belgium*

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**Abstract**

The carotenoid and phenolic acid contents in fresh, stored and processed (blanched, frozen and boiled) spinach were comparatively determined by spectrophotometric and HPLC analyses. The major carotenoids identified after HPLC analysis in saponified samples were lutein (37–53 μg/kg), β-carotene (18–31 μg/kg), violaxanthin (9–23 μg/kg) and neoxanthin (10–22 μg/kg). These carotenoids were all affected by storage and/or heating. The content of carotenoids was best preserved after storage for one day at 4 °C.

The total phenolic content in the fresh spinach was 2088 mg GAE/kg FW. After LC–MS analysis three phenolic acids were identified and quantified. These being *ortho*-coumaric acid (28–60 mg/kg FW), ferulic acid (10–35 mg/kg) and *para*-coumaric acid (1–30 mg/kg) depending on the sample type. After storage of spinach at different temperatures (4 °C or −18 °C) the amount of total phenolic compounds decreased by around 20%, while the amount of individual phenolic acids increased by four times on average.


*Keywords:* Carotenoids; Phenolic compounds; Storage; Processing; Chromatography; Spectrophotometry; Mass-spectroscopy

## 1. Introduction

Increased intake of vegetables is generally associated with a reduced risk of cancer and cardiovascular disease (Kris-Etherton et al., 2002). This association is based on the presence of different phytochemicals in vegetables with either potential or proven beneficial effects on human health, like carotenoids and phenolic acids (Mattila & Kumpulainen, 2002). Processing and preparation, especially thermal treatment, which are applied prior to consumption, may affect these phytochemicals.

The reduction of vitamin levels in vegetables during processing and cooking can vary largely depending on the cooking method and the type of food (Leskova et al., 2006). In addition, Bergquist, Gertsson, and Olsson (2006) reported that post-harvest storage may influence the nutritional quality of vegetables. They demonstrated that the temperature during storage plays an essential role in the preservation of bioactive molecules such as carotenoids.

In the production of frozen vegetables, blanching is performed to reduce the microbial load and inactivate undesirable enzymes (Bahceci, Serpen, Gokmen, & Acar, 2005). At the same time, blanching may also remove tissue gases, shrink the product, clean and stabilize colours, but also can lead to leaching of nutrients (Price, Casuscelli, Colquhoun, & Rhodes, 1998). Blanching may also inactivate particular enzymes which degrade phytochemicals depending on the duration and temperature applied. However, overblanching may result in an undesirable loss of colour, flavour, texture and nutrient quality in addition to excessive energy requirements and need to use and dispose large quantities of waste (Puuponen-Pimia et al., 2003).

* Corresponding author. Address: Department of Food Safety and Food Quality, Faculty of Bioscience Engineering, Ghent University, Belgium. Tel.: +32 9 264 6161; fax: +32 9 264 6215.
*E-mail address:* mirjana.andjelkovic@ugent.be (M. Andjelkovic).

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One of the vegetables considered to have a high nutritional value is spinach (*Spinacia oleracea* L.). Spinach is often consumed fresh or stored frozen after cooking in boiling water. Frozen spinach is preferred by most consumers due to its prolonged shelf-life which enables it to be available throughout the year. Spinach has been suggested to be a rich source of carotenoids (yellow, orange or red), which are masked by green chlorophyll. In spite of being susceptible to light oxidation (acting as efficient antioxidants), carotenoids have been proven to be more stable during thermal processing compared to chlorophylls (Bergquist et al., 2006). Carotenoids from cooked or processed foods have also been reported to have a better bioavailability than those from raw commodities (Gartner, Stahl, & Sies, 1997; Hedren, Diaz, & Svanberg, 2002; Stahl & Sies, 1992). Moreover, when stored for short periods at different temperatures, carotenoids are better preserved than ascorbic acid (Bergquist et al., 2006). The stability of these nutrients is dependent on their location and distribution in plant tissues; therefore there is a need to investigate their behaviour in various vegetable matrices. Whereas data about the stability of carotenoids during processing in different vegetables has been published, their stability in spinach subjected to different storage conditions has been addressed only in a few reports (Bergquist et al., 2006; Kopaslane & Warthesen, 1995).

Another group of phytochemicals that have been determined in high amounts in vegetables such as broccoli, kale, cabbage, and onions are polyphenolic compounds (Ismail, Marjan, & Foong, 2004; Lanzotti, 2006; Podsedek, 2007); especially phenolic acids and flavonoid derivatives with antioxidant capacity (Rice-Evans, Miller, & Paganga, 1996). Phenolic acids are hydroxylated derivatives of benzoic or cinnamic acids found in fruits and vegetables, and other plant derived foods (Naczk & Shahidi, 2004).

Although the effects of storage and processing on the content of total flavonoids and some high molecular weight polyphenols in fresh-cut spinach has been studied (Gil, Ferreres, & Tomas-Barberan, 1999; Turkmen, Sari, & Velioglu, 2005), there is a lack of studies concerning the phenolic acids in spinach and their stability after processing.

The aim of this study was to evaluate the carotenoid and phenolic acid profiles in spinach. The stability of these compounds was evaluated during storage at 4 and −18 °C and after different processing techniques. In this study the effect of blanching and freezing followed or not by subsequent cooking on the nutritional quality of spinach was assessed and compared.

## 2. Materials and methods

### 2.1. Chemicals

Methanol, potassium hydroxide, triethylamine, sodium chloride, anhydrous sodium sulphates were purchased from Sigma Chemical Co. (Bornem, Belgium). The purity of donated carotenoid standards, β-carotene and lutein, was estimated by registering their UV–Vis spectra and by an individual HPLC run. The β-carotene and lutein were found to be 95% and 98.5% pure, respectively. Standards of analytical grade phenolic acids (gallic acid, ferulic acid, *para*-coumaric acid, *ortho*-coumaric acid, and *trans*-cinnamic acid) (Sigma–Aldrich, Bornem, Belgium) were used for HPLC validations. Solvents used for carotenoid analysis (ethylacetate, acetonitrile, petroleum-ether, and water, purchased from Biosolve, BV, Valkenswaard, The Netherlands) were of HPLC grade whereas those used for phenolic analysis (methanol, acetonitrile and acetic acid from Acros Organics, Geel, Belgium) were of LC–MS grade.

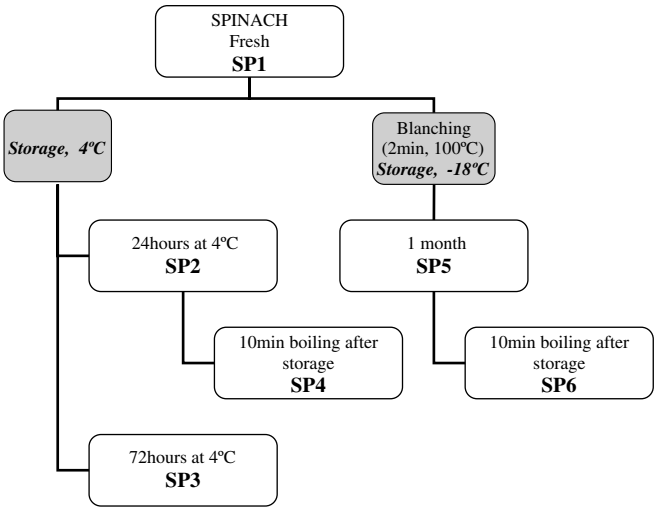


Fig. 1. Flow chart of spinach processing resulting in six different samples (SP1–SP6). Storage and processing conditions of each sample (SP) are indicated in a successive order in the boxes.

### 2.2. Sample preparation

Samples of spinach (*Spinacia oleraceea*), variety *Leopold leopard* grown in Belgium were used in the study. Leaves were freshly harvested at the end of May 2005, sampled and processed the following day in the laboratory.

The fresh spinach was cleaned and washed under running tap water and divided in two parts: one for storage at 4 °C (A) and one for thermal treatment (blanching) (B), as shown in the processing flow chart (Fig. 1). One raw spinach sample (SP1), was taken prior to processing or storage. Other spinach samples (SP2 and SP3), were stored at 4 °C for 24 and 72 h, respectively. After storage, half of SP2 sample was boiled for 10 min, resulting in sample SP4. In order to inhibit degrading enzymes, raw spinach sample SP1 was blanched for 2 min in boiling water at 100 °C, in a 1/1 (w/w) ratio, then frozen and stored at −18 °C for 1 month, resulting in sample SP5. The frozen batch (SP5) was subsequently boiled 10 min in water, resulting in sample SP6. The range of temperatures and times were chosen to reflect those of some domestic handling practices.

All samples (SP1–SP6) were lyophilized prior to spectrophotometric and HPLC analyses. These lyophilized samples were kept at −18 °C until analysis.

### 2.3. Quantification of carotenoids

Total carotenoids were extracted from 5 g lyophilized spinach using a mixture of methanol/ethyl acetate/petroleum ether (1:1:1, v/v/v). After filtering the extract, the residue was re-extracted two times with the same solvent mixture, following the procedure described by Breithaupt and Schwack (2000). The extracts were combined before being partitioned in a separation funnel, successively with water, diethyl ether and saturated saline solution. The ether phase was evaporated to dryness under vacuum, using a rotary evaporator at 35 °C. The evaporated residue (oleoresin) was dissolved in 15 ml of petroleum ether. Half of the oleoresin was dissolved in diethyl ether and saponified overnight, in the dark, at room temperature using 30% methanolic KOH. The saponification step removes chlorophylls, released carotenes and free xanthophylls.

The saponified extract was washed with saturated saline solution and distilled water, eliminating the soaps and alkaline excess. The organic layer containing carotenoids was dried over anhydrous sodium sulphate and evaporated to dryness. The samples were kept under nitrogen, at −20 °C until further utilization.

HPLC analyses for individual carotenoids were carried out on a Finnigan Surveyor Thermo system with PDA detector (Thermo Finnigan, San Jose, California) using a reversed phase Lichrosorb C18 column (250 × 4.6 mm), 5 μm. A dual gradient mobile phase was used, and made of acetonitrile: water (9:1, v/v), with 0.25% triethylamine (solvent A) and ethyl acetate with 0.25% triethylamine (solvent B). The gradient started with 0% B to 60% B from 0 to 16 min and continued isocratically up to 20 min. The flow rate was 1 ml/min. All chromatograms were monitored at 450 nm. The HPLC peaks were identified either by using parallel HPLC runs with carotenoid standards, or by co-chromatography of each sample with lutein and β-carotene standards, as well by recording the UV–Vis spectra specific to each carotenoid peak. Calibration curves were made using five different concentrations (0–0.2 mg/ml) of pure lutein and β-carotene, the linear regression factor of the calibration curves were higher than 0.977 in all cases (data not shown). The individual and total carotenoids were then determined according to the calibration curves. The total carotenoid content was also estimated spectrophotometrically for the saponified extracts, all of them dissolved in diethyl ether by measuring the maximum absorption at 450 nm ($\lambda_{max}$) using 2500 as the specific absorption coefficient (Britton, 1995). All results were expressed as mg carotenoid/kg fresh spinach.

### 2.4. Quantification of total phenolics and phenolic acids

For the extraction and quantification of phenolics, the method of Rodriguez-Delgado, Malovana, Perez, Borges, and Montelongo (2001), optimized by Neacsu, Varga, Socaciu, and Van Camp (2004) was used. Lyophilized samples of approximately 1 g weight were extracted in 50 ml mixture of methanol, water, and hydrochloric acid (50:40:10). After 1 h extraction and protected from light, the extract was cotton-filtered and centrifuged 10 min at 1000*g*. The supernatant was collected, and the pellet re-extracted further 3 times. The mixture was concentrated up to 90% in a rotary evaporator at 35 °C. Overnight hydrolysis (16 h) was performed at pH values near to 0. During the hydrolysis the extracts were protected from light and continuously stirred in a water bath at 35 °C. The hydrolyzed extract was further extracted 4–5 times with diethyl ether. The extracts were collected, evaporated to dryness and kept under nitrogen at −20 °C until further utilization.

The total phenolics in the samples was determined by a modified method of Singleton, Orthofer, & Lamuela-Raventos (1999), using Folin–Ciocalteu reagent. A calibration curve with different concentrations of gallic acid in methanol, in the range 0.5–1.3 mg/100 ml was obtained, with an $R^2$ value of 0.990. The results were expressed as milligrams of gallic acid equivalents (GAE) per kg of fresh weight (FW) spinach.

Briefly, the procedure was performed as follows. Dry extracts of spinach were diluted in 10 ml of LC–MS grade methanol. 250 μl of the resulting solution was thoroughly mixed with 10 ml of water and 1.25 ml of Folin–Ciocalteu reagent. After 5 min incubation, 3.75 ml of 20% sodium carbonate was added and the volume adjusted with water to 25 ml. After 2 h incubation the absorbance was read at 750 nm using a Cary UV 50 spectrophotometer.

To determine individual phenolic acids, aliquots of spinach extracts were taken for LC–MS analysis under conditions described by Andjelkovic, Van Camp, Van Hoed, and Verhé (2005). Reversed-phase HPLC was performed in an Agilent 1100 Series LC–MSD system (Agilent Technologies, Waldbron, Germany). A Phenomenex C18 (ODS, Octadecyl) security guard and a Phenomenex Luna C18 (2) 100 Å column (4.6 mm i.d. × 250 mm; particle size = 5 μm), maintained at 35 °C, were used. Elution was performed at a flow rate of 1.0 ml/min, using as mobile phase a mixture of 0.2% acetic acid in water (solvent A), methanol (solvent B) and acetonitrile (solvent D). Chromatograms were registered at 280 and 320 nm, whereas mass spectrometric measurements were determined by an Agilent G1946D (SL) mass detector with an ion-trap mass spectrometer equipped with an electro spray ionisation (ESI) system. Nitrogen was used as the nebulizing gas at a pressure of 60 psi and the flow was adjusted to 1 l/min. The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. The full scan mass spectra of the phenolic compounds were measured from *m*/*z* 50 up to *m*/*z* 1000. Mass spectrometry data were acquired in the negative ionisation mode.

Using mass spectroscopy and UV–Vis spectrophotometry three phenolic acids, *para*-coumaric acid, ferulic acid and *ortho*-coumaric acid were identified. The data used for identification are summarized in Table 1. For the quantifica-

Table 1
Phenolic compounds and their pseudo molecular ions detected in spinach using HP LC–MS in negative ionisation mode ($MH^-$)

| Peak number | RT | Phenolic compound | $MH^-$ | Other ions | Max Abs |
|---|---|---|---|---|---|
| 1 | 16.4 | *para*-Coumaric acid | 163.2 | 119.5, 349.2 | 225, 310 |
| 2 | 17.2 | Ferulic acid | 193 | | 238, 295 |
| 3 | 22.6 | *ortho*-Coumaric acid | 163.2 | 119.5, 241.3 | 215, 277, 325 |

Their chromatographic characteristics (retention time, maximum absorbance bands) are presented.

tion of these individual phenolic acids calibration curves in the range 0.01–1.0 mg/ml with regression coefficients of $R^2 = 0.982$ (*para*-coumaric acid), $R^2 = 0.996$ (ferulic acid) and $R^2 = 0.992$ (*ortho*-coumaric acid) were obtained. The results were expressed in mg/kg FW of spinach.

### 2.5. Statistical analysis

Results were presented as a mean value from triplicate measurements with standard deviation. Statistical analysis was carried out using analysis of variance (ANOVA) with significance level set at $p < 0.05$ and a post hoc Tukey test for comparing differences between mean values of samples (SPSS, version 12.0 for Windows).

## 3. Results and discussion

The bioavailability of phytochemicals is influenced by the matrix and microstructure of the food they occur in, the storage conditions (light, oxygen, and temperature regime) and thermal processing they are subjected to (Parada and Aguilera (2007)). As a consequence, knowledge of the content and stability of phytochemicals in foods after processing is essential to evaluate the nutritional value of vegetables rich in these phytochemicals, like spinach.

### 3.1. Evaluation of total carotenoids by UV–Vis spectrophotometry and HPLC

Table 2 presents the individual and total carotenoid content in raw, refrigerated or processed spinach samples (SP1–SP6), as shown in the flow chart (Fig. 1). The total carotenoid content was measured both by HPLC (Total A) and by spectrophotometry (Total B). To assure the correct determination of carotenoids, a preliminary saponification was performed to remove lipids and chlorophylls which absorb light at wavelengths similar to those of carotenoids and have been proven to interfere with spectrophotometric measurements (Rodriguez-Bernaldo de Quiros & Costa, 2006).

The measurements made by spectrophotometry (absorption at 450 nm) although were less accurate than those by HPLC, generally gave similar or lower carotenoid concentrations (87–119 mg/kg) compared to those determined by HPLC (76–126.8 mg/kg). This range of concentrations is in good agreement with the data reported in previous studies (Kidmose, Knuthsen, Edelenbos, Justesen, & Hegelund, 2001; Kim, Lee, & Choe, 2003; Lisiewska, Kmiecik, & Slupski, 2004).

After storage at 4 °C for 24 h (SP2), the total carotenoid content did not change significantly compared to that of raw spinach. However, a slight decrease of total carotenoid content (around 15%) was observed when the samples were blanched and then frozen for one month (SP5). Even higher decreases of up to 64% were detected in samples that were boiled after a short storage (24 h or 72 h) at 4 °C (SP3 and SP4). This implies that boiling of spinach prior to consumption causes the greatest losses and may also negatively affect the amount of total carotenoids in spinach.

### 3.2. Identification and quantification of individual carotenoids by HPLC

The losses of individual carotenoids during storage and processing were determined by HPLC (Table 2). The four carotenoids separated by HPLC and identified in all spinach samples were lutein, β-carotene, violaxanthin and neoxanthin (Fig. 2). Lutein and β-carotene, were determined as major carotenoids, occurring in the range from 33.5 to 53.0 mg/kg and 18.9 to 31.5 mg/kg, respectively. The behaviour of these two carotenoids was rather similar during storage and processing. When compared to their content in raw spinach, lutein and β-carotene were reduced by up to 50% when storage was done at 4 °C (SP3 and SP4). However, initial blanching kept them stable during one month storage at −18 °C (SP5 and SP6). Violaxanthin

Table 2
Quantitative analysis of total and individual carotenoids in saponified extracts of raw and processed spinach

| Sample | Neoxanthin | Violaxanthin | Lutein | β-Carotene | Total A[1] | Total B[2] |
|---|---|---|---|---|---|---|
| SP1 | $15.3 \pm 2.7^{a,b,*}$ | $26.6 \pm 1.4^{a}$ | $52.2 \pm 0.9^{a}$ | $31.5 \pm 2.0^{a}$ | $125.6 \pm 6.5^{a}$ | $119.0 \pm 4.9^{a}$ |
| SP2 | $21.8 \pm 3.6^{b}$ | $23.0 \pm 2.0^{a}$ | $51.5 \pm 1.0^{a}$ | $30.5 \pm 2.0^{a}$ | $126.8 \pm 8.5^{a}$ | $88.9 \pm 3.2^{b}$ |
| SP3 | $9.7 \pm 5.5^{a,c}$ | $14.0 \pm 2.0^{b}$ | $33.5 \pm 2.2^{b}$ | $18.9 \pm 5.7^{b}$ | $76.0 \pm 15.2^{b}$ | $91.4 \pm 6.4^{b}$ |
| SP4 | $10.8 \pm 1.0^{a,c}$ | $9.2 \pm 5.0^{b}$ | $37.5 \pm 2.0^{b}$ | $24.9 \pm 2.5^{a,b}$ | $82.4 \pm 10.0^{b,c}$ | $87.0 \pm 4.1^{b}$ |
| SP5 | $16.0 \pm 3.3^{a}$ | $12.4 \pm 2.3^{b}$ | $50.9 \pm 5.7^{a}$ | $30.7 \pm 4.3^{a}$ | $110.0 \pm 15.5^{a,c}$ | $100.3 \pm 7.5^{b,c}$ |
| SP6 | $14.5 \pm 4.4^{a}$ | $13.9 \pm 2.2^{b}$ | $53.0 \pm 0.9^{a}$ | $31.4 \pm 2.7^{a}$ | $112.8 \pm 10.2^{a,c}$ | $110.2 \pm 6.4^{a,c}$ |

* Different letters within columns represent significant difference at $p < 0.05$. Data are expressed as average of triplicate measurements with standard deviation (mg/kg FW).

[1] Total A, sum of four carotenoids quantified by HPLC analysis.

[2] Total B, amount of total carotenoids quantified spectrophotometrically.

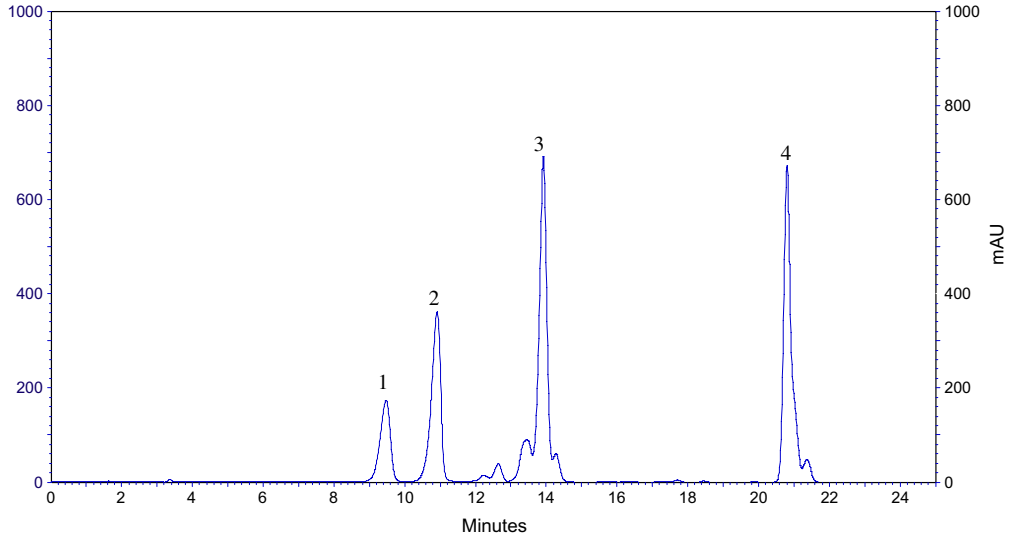


Fig. 2. HPLC chromatogram of carotenoids after saponification and extraction from raw spinach. The HPLC conditions are described in the text. Four carotenoids are identified: (1) neoxanthin, (2) violaxanthin, (3) lutein, and (4) β-carotene.

was the least stable under these conditions. Other authors found similar contents like 45 mg/kg of β-carotene and 63 mg/kg of lutein in cooked spinach (Granado, Olmedilla, Blanco, & Rojas-Hidalgo, 1992). Increase in the amount of all-trans-β-carotene by cooking of raw spinach, 30.53 mg/kg against 27.30 mg/kg in raw samples, has also been reported (Hulshof, Xu, van de Bovenkamp, & West, 1997). It is suggested that physical alteration of the food matrix by blending, chopping, freezing, blanching, and cooking generally may release carotenoids from the food matrix (Hedren et al., 2002). In our study, as mentioned above, blanching was used as an intermediate step before storage; however its influence was not assessed separately.

As depicted in Table 2, in the raw spinach (SP1) the major carotenoid was lutein (52.2 mg/g) representing about 41.5% of the total carotenoid content, followed by β-carotene (25.1%), violaxanthin (21.2%), and neoxanthin (12.2%). In agreement with our data, Calvo (2005) found values for lutein in spinach to be in the range 41–59 mg/kg (wet basis) suggesting its predominant presence compared to the other carotenoids. Moreover, after the different treatments applied to the spinach samples, lutein was the most stable carotenoid (37–46% of total carotenoid content). Food processing has both positive and negative effects on the levels of carotenoids in food, but overall it is more evident that processing may be beneficial through disruption of matrix (cell walls) which facilitates their release and solubilization as free or esterified/glucosylated forms in appropriate solvents (oils or water, respectively), or after long boiling times, leading to chemical changes (isomerization and degradation) (Granado-Lorencio & Olmedilla-Alonso, 2003).

Likewise, evaluation of carotenoids in the spinach samples that were stored for one-day at 4 °C (SP3) revealed some significant losses. Up to 48% of violaxanthin and 40% of β-carotene were lost, whereas after boiling (SP4) the content of β-carotene was only around 20% lower than that of raw spinach. Similar trend was found for neoxanthin and lutein indicating that boiling may have a less negative effect on these compounds. Nevertheless, boiling did not change the general profile of carotenoids, in accordance with the results reported by Granado et al. (1992). These authors have also shown that the carotenoid content may increase after boiling which can be attributed to the improved solubility of carotenoids and their better extractability due to the heat treatment (Mayer-Miebach & Spiess, 2003). In our experiments, lutein and β-carotene were more stable compared to violaxanthin, which was found to be the most labile. This may be due to either its weaker interaction with the sample matrix, higher solubility in aqueous environments or instability, as observed by Gross (1991) and Aman, Schieber, and Carle (2005). Moreover, the behaviour of carotenoids after different processing procedures depends on the matrix they are contained in: while carrot carotenoids are stable (Kopaslane and Warthesen, 1995), spinach carotenoids have been observed to be less stable (Leskova et al., 2006). This should be taken into account when comparing carotenoid stability in different types of vegetables, where their physical state and location are important factors for their thermal stability (Schieber & Carle, 2005). A similar effect of heat treatment was observed in broccoli (Zhang & Hamauzu, 2004), in which, as a function of boiling period, lutein levels increased by almost 27% after 5 min, while β-carotene and violaxanthin content decreased by 80% and 54%, respectively.

In the same manner, spinach that was blanched and frozen for 1 month (SP5) revealed a similar stability trend with regard to its carotenoids. Violaxanthin was the least stable. Furthermore, boiling, after blanching and freezing, did not change the carotenoid profile nor their content (SP5 vs SP6).

### 3.3. Evaluation of total phenolics by VIS spectrophotometry

Using all samples processed according to the flow chart in Fig. 1, free phenolic acids were identified and quantified by HPLC in raw (SP1), stored (SP2, SP3) and processed (SP4, SP5, and SP6) spinach samples and in parallel the total phenolics content was measured by spectrophotometry (Table 3).

By using the Folin–Ciocalteu method, both free and conjugated forms of polyphenols were evaluated (Singleton et al., 1999) and considered as total phenolics. Their concentration in fresh spinach samples (SP1), expressed as gallic acid equivalents (GAE) was around 2000 mg GAE/kg FW, which was higher than previously reported by Turkmen et al. (2005) probably due to the different extraction methods. This concentration is comparable to that determined in cabbage, but lower than that of cauliflower and broccoli (Puuponen-Pimia et al., 2003). On the other hand, this level is similar to the total phenols determined in some fruits like bananas and cherries (Vinson, Su, Zubik, & Bose, 2001).

The storage of spinach, for different periods and different temperature conditions, induced a decrease in the total phenolics content; up to 50% for SP6 spinach samples. While the total phenolics in spinach decreased slightly (7.6%) by refrigeration at 4 °C for 24 h (SP2), a more intense decrease (11.2%) was observed after 72 h of storage (SP3). According to some reports this loss of phenolics in green leafy vegetables stored at 4 °C is limited in comparison to the effect of storage at 25 °C (Chu, Chang, & Hsu, 2000).

Stronger losses of total phenolics (49%) were noticed in spinach samples that were boiled after being subjected to blanching, freezing, and storage at −18 °C (SP6). As these treatments largely resemble domestic preparation practices, significant losses of phenolics may occur when vegetables are prepared for consumption. Similar decreases were reported for kale, cauliflower and broccoli (Podsedek, 2007). Zhang and Hamauzu (2004) suggested that leaching of phenolic compounds into the cooking water would cause the decrease in total phenolics. In contradiction to these results, Turkmen et al. (2005) observed a slight increase in total phenolics in spinach following different types of cooking methods. This could be explained by the fact that phenolic compounds (which are usually stored in vegetables in pectin or cellulose networks) can be released during thermal processes making them more extractable. As mentioned above, blanching applied as a pre-freezing treatment, may either induce a decrease in polyphenol content (Gebczynski & Lisiewska, 2006) due to an increase in their water solubility and changes of matrix constituents during thermal and enzymatic degradation or, in contrast, blanching may cleave the phenolic-sugar glycosidic bonds, leading to the formation of phenolic aglycons, which react better with the Folin–Ciocalteu reagent leading to increased values of total phenolics (Singleton et al., 1999).

Table 3
Amount of free phenolic acids (mg/kg FW) and total phenolic compounds (mg gallic acid equivalent per kg of fresh spinach weight, GAE/kg FW) in raw and processed spinach

| Sample | Phenolic acids | | | Total phenolic compounds |
|---|---|---|---|---|
| | *ortho*-Coumaric acid | Ferulic acid | *para*-Coumaric acid | |
| SP1 | $27.8 \pm 2.7^{a,b,*}$ | $9.9 \pm 0.4^{a}$ | $1.3 \pm 0.1^{a}$ | $2088.9 \pm 30.4^{a}$ |
| SP2 | $35.3 \pm 0.2^{b,c}$ | $13.3 \pm 0.9^{a}$ | $7.1 \pm 2.8^{b}$ | $1929.7 \pm 53.1^{b}$ |
| SP3 | $55.8 \pm 3.1^{d}$ | $15.2 \pm 7.9^{a}$ | $10.6 \pm 1.7^{b}$ | $1854.7 \pm 3.7^{b}$ |
| SP4 | $23.7 \pm 0.6^{a}$ | $9.9 \pm 2.0^{a}$ | $9.7 \pm 2.0^{b}$ | $1911.6 \pm 29.6^{b}$ |
| SP5 | $37.5 \pm 2.4^{c}$ | $20.0 \pm 4.7^{a}$ | $7.1 \pm 3.3^{b}$ | $2108.8 \pm 14.9^{a}$ |
| SP6 | $53.2 \pm 5.0^{d}$ | $37.3 \pm 3.5^{b}$ | $28.7 \pm 0.1^{c}$ | $1067.4 \pm 7.3^{c}$ |

* Different letters within columns represent significant difference at $p < 0.05$. Data are expressed as a mean of triplicate measurements with indicated standard deviation.

### 3.4. LC–MS identification and quantification of individual phenolic acids

Using an appropriate LC–MS protocol, the major spinach phenolic acids (*ortho*-coumaric, ferulic and *para*-coumaric acids) were identified by comparing their absorbance and MS spectra, to those of the reference compounds, as illustrated in Table 1. Furthermore, using the calibration curves generated for all three phenolic acids in the range 10–500 mg per kg of sample their content was quantified and is presented in Table 3. *ortho*-Coumaric acid was determined to be the major phenolic compound with concentrations ranging from 23.7 to 55.8 mg/kg. Ferulic acid occurred at concentrations between 9.9 and 37.3 mg/kg, while *para*-coumaric acid occurred at concentrations ranging from 1.3 to 28.7 mg/kg. Recently, in frozen spinach, Mattila and Hellstrom (2007) found *para*-coumaric acid and ferulic acid; they also additionally found vanillic acid at lower concentrations. In other vegetables, like sweet pepper, *para*-coumaric acid was found at similar level as in frozen spinach (24 mg/kg) whereas ferulic acid at 74 mg/kg was slightly higher than that found in red cabbage (Mattila and Hellstrom, 2007). Bergman, Varshavsky, Gottlieb, and Grossman (2001) reported the presence of isomers of *para*-coumaric acid in spinach by assessing the NMR spectra of phenolic fractions obtained from spinach phenolic extract.

The data presented for individual phenolic acids in Table 3 illustrate their contradictory behaviour compared to the variations in total phenolics in spinach. Increase in concentrations of phenolic acids after storage and processing may be explained either by their better stability compared to other phenolics or due to their better release from the matrix, as mentioned above. For example, although the total phenolic content decreased during storage at 4 °C, the level of individual phenolic acids slightly increased.

Boiling of spinach after storage brought unexpected changes in phenolics content. Boiling after 24 h storage at 4 °C (SP4) induced significant decreases in *ortho*-coumaric

acid compared to storage without boiling (SP2), whereas *para*-coumaric acid content did not change significantly. However, boiling applied after prolonged storage (SP6) resulted in an increase in concentration for all phenolic acids, with the highest significant increase being for *para*-coumaric acid. The possible explanation for these results could be the degradation of complex phenolic structures, like tannins or flavonoids into simple phenolics, like phenolic acids. Ewald, Fjelkner-Modig, Johansson, Sjoholm, and Akesson (1999) found that flavonoids like quercetin and kaempherol, decreased during blanching, boiling or microwave cooking. Other authors have also reported the loss of flavonoids, e.g. during boiling of onions (Lombard, Peffley, Geoffriau, Thompson, & Herring, 2005). In contrast to this, vanillic acid, present in olive oil was reduced only after 12 months storage (Morello, Motilva, Tovar, & Romero, 2004). Thus, processing may negatively affect the total amount of phenolics while some of the simple phenolics can increase as a result of breakdown of supramolecular structures containing phenolic groups.

Phenolic acids may exert their antioxidant properties and can contribute to the total antioxidant capacity of a certain food product, but processing of vegetables reduces the total antioxidant capacity, concomitantly with the decrease in total phenolic content (Gebczynski and Lisiewska, 2006; Nicoli, Anese, & Parpinel, 1999). Therefore, the nutrional quality of the food, e.g. spinach, will depend on the type of phenolics present and the way the vegetable is processed.

## 4. Conclusion

Food processing may both have beneficial and detrimental effects on phytochemicals in vegetables. In this study, the behaviour of these compounds in spinach as a function of storage and heat processing was evaluated. It was shown that spinach is rich in at least two categories of phytochemicals: carotenoids and phenolics. Further, this study demonstrated that the carotenoid content of spinach may be affected during storage and also by different storage conditions (refrigeration at 4 °C or freezing). On the other hand, thermal treatment, in particular blanching in combination with subsequent freezing and boiling did not have a large influence on the level of carotenoids. However, in comparison to other processing techniques, the results indicate that boiling may affect to a higher extent the content of these compounds. Among individual carotenoids, lutein was most stable, followed by β-carotene, while violaxanthin being more polar and soluble became susceptible to degradation.

The total amount of phenolic compounds in spinach was comparable to that of other vegetables, like cabbage. It was shown in this study that the total amount of phenolic compounds in spinach decreased during processing while individual phenolic acids behaved differently. The three major phenolic acids found in spinach, namely *para*-coumaric, *ortho*-coumaric and ferulic acid, gradually increased during refrigeration, blanching and boiling processes, which demonstrates that not only the stability of these compounds but also the extent of their release from the vegetable matrix due to processing is essential for the evaluation.

Our data confirm that storage and minimal preparation techniques like blanching and boiling can significantly affect the level and stability of carotenoids and phenolic acids in spinach. These modifications have to be taken into account when evaluating the nutritional value of vegetables and their preparations. Additionally, the behaviour of some phytochemicals in vegetables may be influenced by the food matrix itself which should be further evaluated.

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