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Lipid peroxidation and chlorophyll levels in spinach during refrigerated storage and after industrial processing

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Chlorophylls *a, b* and pheophytins *a, b* were quantitatively determined in raw, frozen and canned spinach. About 15.9% was lost during the freezing process and 99.9% after canning as a consequence of the heating used in industrial processing. Pheophytins *a* and *b* (3.28 and 3.00 mg kg^{-1}) were the predominant chlorophyll degradation derivatives for samples stored in a refrigerator at 8°C for three weeks. The degradation limit of stored samples was around 13–15 days with 2.54 and 1.30 mg kg^{-1} for pheophytins *a* and *b*, respectively. Lipid peroxidation during spinach senescence, as a consequence of being cold stored or processed, increased particularly during the canning process with 3.40 μmol equivalent MDA.

INTRODUCTION

Consumption of frozen food has increased in recent years and interest has thus grown concerning its quality and shelf-life. Appearance is taken as an index of freshness, palatability and nutritional value (Haisman and Clarke, 1975).

Chlorophylls *a* and *b* have been shown to be the main compounds responsible for the green colour of vegetables (Schwartz and von Elbe, 1983). Degradation of chlorophylls has been studied since their bright green colour is usually more pleasing to the consumer than the olive–brown colour of pheophytins (Schwartz and Lorenzo, 1991). Chemically, the colour change is due to the conversion of chlorophyll *a* and *b* to pheophytin *a* and *b* (Jones *et al.*, 1977; Canjura *et al.*, 1991).

Furthermore, the properties of chlorophyll in green tissues may depend on the nature of its association with lipoproteins of the chloroplast. Disruption of the chlorophyll structure cannot take place until the membrane array of chlorophyll has been disorganised (Haisman and Clarke, 1975). It is known that hydrogen ions can transform chlorophylls into their corresponding pheophytins by replacing the magnesium (Mg) atom in the porphyrin ring. (Lajollo *et al.*, 1971). In fact, the most common mechanism of chlorophyll degradation seems to be acid-catalysed transformation into pheophytin. Oxidation through the action of lipoxygenase and subsequent bleaching has also been observed in some foods (Lajollo and Lanfer-Marquez, 1982).

On the other hand, chlorophyll degradation in dehydrated foods is likely to occur either at high water activity (a_w, water available for chemical reaction) or low a_w (mechanism linked to nonenzymic browning or to lipid oxidation) (Lajollo and Lanfer-Marquez, 1982). Water activity has shown a definite influence on the rate of chlorophyll degradation in dried spinach (Lajollo *et al.*, 1971). Furthermore, several factors, such as temperature and duration of the heat treatment, which are required to achieve commercial sterility, will influence the quantity of chlorophyll retained during processing (Schwartz and Lorenzo, 1991).

Changes in ripening parameters and in lipid peroxidation products, such as malondialdehyde (MDA) and aldehydes, have been observed (Suda *et al.*, 1994). Similarly, changes in colour may be related to the development and beginning of ripening in vegetables (Suda *et al.*, 1994), to the chilling-injury index after harvest (Molla *et al.*, 1990), to senescence symptoms in the harvested leaf (Yamauchi and Watada, 1991), and to storage (Lajolo *et al.*, 1971; Fuke *et al.*, 1985).

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Senescence in plant tissues is known to be accompanied by changes in membrane permeability, and these changes have been correlated with a simultaneous decline in membrane lipids (Matsuo *et al.*, 1984). Free fatty acids accumulate in plant membranes after exposure of plants to environmental stress or during senescence. This is a consequence of the selective degradation of phospholipids by phospholipases (Barclay and McKersie, 1994) or by lipolytic acyl hydrolase, since these enzyme activities increase with senescence. The resulting free fatty acids may be oxidised by lipoxygenase to form hydroperoxides (Whitaker, 1990). All these products formed directly from the oxidation of polyunsaturated fatty acids (PUFA) can interact as the radical intermediates with constituents such as vitamins, and stimulate chlorophyll degradation (Yamauchi and Watada, 1991). Thiobarbituric acid (TBA) assays of MDA are increasingly used in plant senescence research (Du and Bramlage, 1992), since MDA, which is a major degradation product of lipid hydroperoxides, serves as a marker for the extent of lipid peroxidation (Botsoglou *et al.*, 1994).

The purpose of this study was to quantify individual chlorophylls and their derivatives by comparing raw spinach with processed (frozen and canned) spinach and raw samples after three weeks of refrigerated storage (+8°C). These studies were designed to give some indication of what the user actually consumes. Another objective of this work was to determine lipid peroxidation levels in relation to these chlorophyll changes, by comparing fresh raw, processed and raw spinach samples during refrigerated storage, using high performance liquid chromatography (HPLC). This technique quantifies the removal of coloured contaminants and gives considerably better results for analysing MDA than those obtained through the spectrophotometric TBA assay.

MATERIALS AND METHODS

Samples

Samples of F1 Correta variety (slow bolring hybrid) *Spinacia oleracea*, L., a high yielding crop for summer and autumn, with stout, smooth, dark green leaves recommended for processing, were obtained from Industrias Prieto S.A. (Murcia, Spain) in April 1994. These samples were grown with 100 kg ha^{-1} NPK using cycloate (*N*-ethyl-cyclohexyl thiolcarbamate 5-ethyl) as herbicide in the first irrigation. All the leaves were harvested at one time and immediately sent by truck to the processing plant, where they were passed through shakers to remove any debris.

Industrial process

Spinach leaves were thrice washed rapidly with water. No important loss of nutrients would be expected during this process. Leaves were then blanched for 2 min at 95°C. Blanched spinach was washed in cold water (250 litres for min), and cooled to 10°C. Approximately 1 kg of sample was packed in heat-sealed unevacuated freezer pouches, which were then frozen (model Agacigoscandia) at −18°C and stored in the freezer (model Slos Freeze 26 B). Alternatively, following the water-blanching treatment, 350 g drained spinach were placed in 500 g enamel cans. The cans were filled with a hot filling (90–92°C) medium (2% NaCl in water) and sealed before being processed in a retort at 121°C (250°F) for 30 min.

Sample preparation for storage

A batch of raw spinach was transported, on the same day of picking (day 0), to the laboratory and divided into 15 batches. Twelve of these were stored at 8°C, and every two days one sample was lyophilised. The other three aliquots were frozen at −18°C immediately, and removed for lyophilisation on days 3, 15, and 22, as control.

Sample preparation after industrial processing

Samples (raw, frozen and canned) were lyophilised in a Virtis Quickseal Valves freeze-drier (The Virtis Company, New York, USA).

Moisture content

Freeze-dried spinach was ground to a fine powder with a Moulinex mill and the moisture content was then determined (Murcia and Rincón, 1991). Determination of the corrected moisture was performed by the Karl–Fischer method modified for food as described in Moibroek and Shahwecker (1984).

Chlorophyll content

The spectrophotometric procedure of Vernon (1960) and Robertson and Swinburne (1981) was used to determine the concentration of total chlorophyll and pheophytin, and also the percentage of chlorophyll *a* and chlorophyll *b* conversion into the corresponding pheophytins.

Lipid peroxidation by HPLC

Sample preparation

0.5 g of dried spinach samples was accurately weighed and subjected to the extraction procedure of Bligh and Dyer (1959). The extracts were evaporated under a stream of nitrogen and the residues dissolved in 2 ml ethanol and sonicated for 10 min. Following this, butylated hydroxytoluene (BHT) was added (at a final concentration of 10% w/v) and the extract filtered through a Millipore 0.22 μm GSWPO 1300 filter. The filtrate was then either frozen at −18°C for future analysis or

immediately analyzed for lipid peroxides using the HPLC-based TBA test as described by Chirico and Smith (1994).

Reagents and standards
All chemicals were obtained from E Merck, Darmstadt, Germany. Other compounds, including tetraethoxypropane (TEP), were obtained from Sigma Chemical Corp. Both methanol and water were of HPLC grade. TEP standards (dissolved in ethanol solution) at equivalent malondialdehyde (MDA) concentrations of 2.5, 5.0, and 10.0 μmol litre^{-1} were included in parallel with all samples to allow conversion of absorbances and peak heights into μmol MDA equivalents.

Procedure
0.25 ml of sample (filtrate) or standard solution were added to 1.5 ml of 0.44 mmol phosphoric acid (H_3PO_4). After 20 min at 3000 rpm, 0.5 ml of the supernatant was microfiltered. After 10 min at room temperature, 0.5 ml of freshly prepared TBA reagent (0.6% w/v TBA in water, heated gently to 60°C to dissolve the TBA) were added to each tube, followed by mixing for 20 s and heating at 90°C for 10 min. Finally BHT was added (10% final w/v).

HPLC analysis
A sample (20 μl) was injected into a Spherisorb 5 ODS_2 (C_{18}) (25 cm × 4.9 mm) column (HPLC Technology, Macclesfield, Cheshire, UK) fitted with a guard (Hiber C_8, HPLC Technology). HPLC was carried out using a Waters Millipore (Watford, Hertfordshire, UK) Model A6000 HPLC system. The sample was eluted with 65% (v/v) 50 mmol KH_2PO_4–KOH buffer pH 6.0–6.8 and 35% methanol at a flow rate of 1 ml min^{-1}. A sharp peak corresponding to the $(TBA)_2$–MDA complex elutes at a retention time of approximately 4.8 min, as detected by a uv/visible detector set at 532 nm.

RESULTS AND DISCUSSION

Industrial processing

Figure 1 shows the chlorophyll and pheophytin derivatives formed after industrial processing. Chlorophylls and pheophytins were expressed as mg kg^{-1} of raw, frozen and canned samples on a dry weight basis.

Raw samples

Chlorophylls *a* and *b* were the most abundant (almost the only) pigments detected in raw spinach (Fig. 1), with chlorophyll *a* levels being higher than those of chlorophyll *b* (18.1 and 5.84 mg kg^{-1} respectively). Only trace amounts of chlorophylls were present in canned samples. The level of pheophytin *b* was higher than that of pheophytin *a* in raw samples, suggesting that the chlorophyll *b* is a better substrate for the chlorophyllase

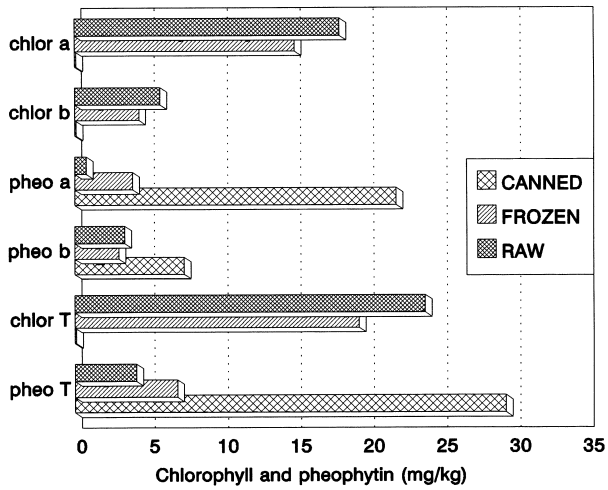

Fig. 1. The contents of chlorophyll *a* (chlor *a*), chlorophyll *b* (chlor *b*), pheophytin *a* (pheo *a*), pheophytin *b* (pheo *b*), total chlorophylls (chlor T) and total pheophytins (pheo T) expressed as mg kg^{-1} in spinach (*Spinacia oleracea*, L.) raw, frozen and canned.

enzyme action. Canjura and Schwartz (1991) reported high chlorophyllase activity in spinach leaves, and Mínguez-Mosquera *et al.* (1994) reported a much higher affinity of chlorophyllase for the substrate, chlorophyll *b*.

Frozen samples

In frozen spinach, chlorophylls *a* and *b* were slightly degraded (Fig. 1), but only small quantities of pheophytins *a* and *b* were detected (3.97 and 3.03 mg kg^{-1} respectively) because the spinach had been blanched. Haisman and Clarke (1975) showed that thermal treatment changed chlorophyll into pheophytin, because cellular acids are released during heating, as demonstrated by LaBorde and von Elbe (1994). Pheophytin *a* levels were slightly higher than those of pheophytin *b*.

Canned samples

In canned spinach, only trace amounts of chlorophylls were present (Fig. 1), almost all chlorophyll having been converted to pheophytin *a* and *b*, which is in agreement with Schwartz *et al.* (1981). If the heat treatment was prolonged, the concentration of pheophytin increased, and there was a significant decrease in the concentration of chlorophyll as reported also by Canjura and Schwartz (1991).

Canned samples contained less pheophytin *b* than *a* (Fig. 1). Canjura and Schwartz (1991) studied the relative degradation of chlorophyll *a* with respect to *b* and showed, too, that chlorophyll *a* degrades faster than chlorophyll *b*, depending on the temperature. Furthermore, it has been described how, following the conversion of chlorophyll *b* to pheophytin *b* during the thermal processing of canned peas, the latter compound continued to degrade (Jones *et al.*, 1977).

Chlorophyll and pheophytin changes during storage in refrigeration

Spinach samples were kept in darkness in a refrigerator to simulate domestic conditions. The pheophytin contents during this refrigerated storage of raw spinach are shown in Fig. 2. Chlorophylls of raw spinach are degraded to pheophytins (Vernon, 1960). Figure 2 shows that pheophytin *a* and *b* levels increased only slightly during storage until 3.28 and 3.00 mg kg^{-1}, with very small variations being observed because of the small variations in the chlorophyll levels. It is well established that variations occur in chlorophyll levels due to the different degrees of maturity of harvested leaves which were exposed to sunlight (Aparicio *et al.*, 1989). Furthermore, these variations may be attributed to the capacity of some green tissues to biosynthesise chlorophyll and this is probably a form of chlorophyll restoration (Barth *et al.*, 1993). Very small amounts of pheophytins were detected during storage in these raw samples, with pheophytin *b* levels being higher than those of pheophytin *a*. There was a small change after 24 days of storage, as also reported by Schwartz *et al.* (1981).

Three of the raw samples (used as control), which were frozen immediately on arrival at the laboratory and defrosted on days 5, 13 and 18, showed levels of 0.1 to 0.6 mg kg^{-1} for pheophytin *a* and 1.87 to 1.95 mg kg^{-1} for pheophytin *b*. In fact, these levels were similar to those of recently harvested raw samples.

The storage life of green vegetables is partly determined by the level of chlorophyll loss. Barth *et al.* (1993) assumed that the product would be acceptable until 20% of chlorophyll had been converted to pheophytin. The percentage of chlorophyll loss for raw samples during refrigerated storage in this study ranged from 12 to 27%. The 20% loss limit, which defines the limit of acceptability, was reached after 13–15 days. The fact that the total amount of pigment remained relatively constant suggests that there was no other type of important reaction that affected the pigments. In fact, the lipid peroxidation levels shown in the next paragraph were not very high.

Lipid peroxidation with processing and storage

Malondialdehyde is a major cytotoxic product of lipid peroxidation and acts as an indicator of free radical production (Pardha *et al.*, 1995).

The level of lipid peroxidation was measured by TBA-reactive substances. These levels in canned, frozen (industrial processing) or raw (day 1) were found to be 3.40, 1.86 and 2.54 μmol equivalent MDA, respectively. Processing, particularly canning, increased lipid peroxide levels in the spinach samples analysed. The effect of storing the raw samples at 8°C (Fig. 3) showed a linear relationship ($r = 0.90$, $y = 0.0279x + 2.4952$) with lipid peroxidation. There was a steady increase in lipid peroxidation during storage from 2.58 to 3.35 μmol.

Three of the raw samples (used as control), which were frozen immediately on arrival at the laboratory and defrosted on days 5, 13 and 18, showed changes in lipid peroxidation with levels of 1.48, 1.74 and 1.74 μmol, respectively. Our levels of lipid peroxidation are very similar to those obtained by Matsuo *et al.* (1984) by HPLC for senescent spinach leaves.

Freezing the spinach shortly after harvesting, significantly ($p \leq 0.05$) retarded the oxidation of lipids similar to a level to that in spinach frozen at the factory. Lipid peroxide levels for the control frozen samples were slightly lower than those in the industrially frozen samples, perhaps because transport from the field to the laboratory was faster and in isothermic containers. In the factory, on the other hand, the spinach was blanched prior to freezing.

It is suggested that care should be exercised with the canning process to minimise increases in the levels of lipid peroxides, which arise from the high levels of polyunsaturated fatty acids in the spinach. Careful combination and use of antioxidants might improve the food matrix during the canning process. The main source of substrate for free radical-mediated peroxidation is the esterified PUFA contained in the phospholipids. Initial fatty acid composition is very important in determining membrane response because it influences

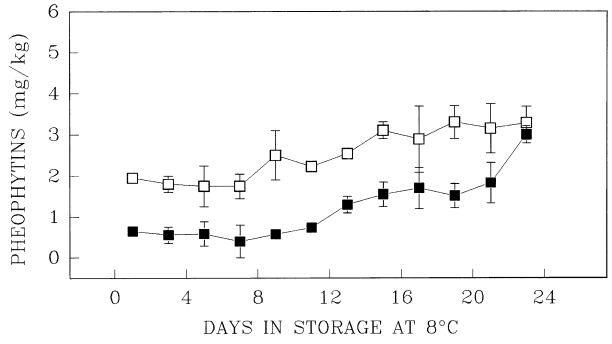

Fig. 2. Influence of refrigerated storage on pheophytin *a*(■) and pheophytin *b*(□) contents in raw spinach (*Spinacia oleracea*, L.) expressed as mg kg^{-1}.

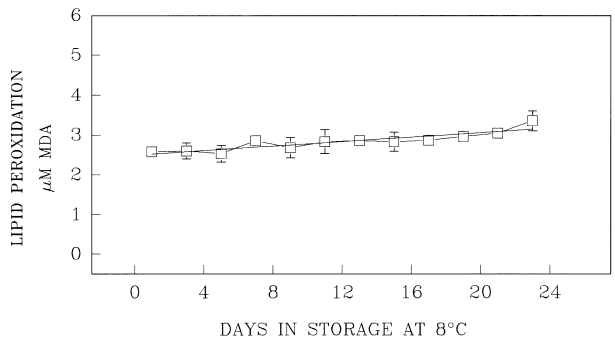

Fig. 3. Influence of refrigerated storage on lipid peroxidation in raw spinach (*Spinacia oleracea*, L.) expressed as μmol MDA.

their susceptibility to free radical attack. The tertiary carbon of the glycerol backbone of phospholipids has been proposed as the predominant site of free radical attack (Barclay and McKersie, 1994).

The high levels of MDA equivalent reported in this present paper agree with the high levels of polyunsaturated fatty acids in spinach samples reported by us in a former paper (Murcia *et al*., 1992*a*).

Free fatty acids accumulate in plant membranes after exposure of plants to environmental stress. Free radical reactions catalyse the selective degradation of phospholipids, after periods of environmental stress or during plant senescence. This has been cited as evidence for the involvement of phospholipases in these degenerative processes (Barclay and McKersie, 1994).

Plants chilled in strong light produced superoxide and singlet oxygen, which initiate lipid peroxidation reactions in the thylakoids. In developmental processes such as senescence, oxygen free radicals have been shown to mediate degradative reactions leading to selective phospholipid catabolism (Barclay and McKersie, 1994).It is known that α-tocopherol quenches singlet oxygen (Jung *et al*., 1991). The levels of α-tocopherols were studied by us in raw, frozen and canned spinach (Murcia *et al*., 1992*b*). An increase in the levels of lipid peroxidation is concurrent with a decrease in the tocopherol content resulting from industrial processing (Murcia *et al*., 1992*b*).

There are also data indicating that pheophytins have stronger pro-oxidant activity than chlorophylls, and the pheophytin content has been use to predict the shelf life of products such as oil (Ward *et al*., 1994), although minor changes were detected in oil samples that had been frozen (Ward *et al*., 1994). This pheophytin pro-oxidant activity would explain the increase in lipid peroxidation in canned spinach samples observed in this study.

In conclusion, during the freezing process about 15.9% of chlorophylls were degraded but, during the canning process, chlorophylls *a* and *b* were completely destroyed (up to 99.9%), which is in agreement with Robertson and Swinburne (1981).

This study shows quite clearly that the lipid peroxide levels increase with storage even in raw spinach samples stored at 8°C. Freezing the vegetable shortly after harvesting retards the oxidation of lipids significantly. Canning increases the level of lipid peroxides principally by increasing pheophytins and free PUFA and by decreasing the level of natural antioxidants such as tocopherols present in the spinach samples (Murcia *et al*., 1992*b*).

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