Chromatographic Determination of Changes in Pigments in Spinach (*Spinacia oleracea* L.) During Processing

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Abstract

The content of individual chlorophyll and carotenoid pigments is determined in three spinach varieties (Lorelei, Springfield, and Ballet) after processing. Raw spinach and spinach that is steam-blanched for 3, 9, or 15 min is stored frozen at –24°C for 6 months. In addition, spinach is air-dried at 75°C, packed in atmospheric air or nitrogen, and stored at ambient temperature for 6 months. Processing has a significant effect on the content of individual chlorophyll and carotenoid pigments; however, there are no differences between varieties in their content of total and individual pigments in raw, frozen spinach. Increasing blanching time resulted in decreased contents of chlorophyll *a* and *b* and increased contents of chlorophyll *a*' and *b*' and pheophytin *a* and *b* because of pheophytinization. Changes in the color because of pheophytinization are only detected after 15 min blanching. The carotenoid pigments are more stable than the chlorophyll pigments during blanching. (*all-E*)-Violaxanthin is significantly reduced, caused by degradation to other xanthophylls, such as neochrome, during blanching. There are no significant differences in the content of chlorophyll *a* and *b* of dried spinach and blanched, frozen spinach. Formation of chlorophyll *a*' and *b*', pheophytin *a* and *b*, and chlorophyll *a-1* and *b-1* is observed after drying. The content of pheophytin *a* and *b* is significantly lower in dried versus blanched frozen samples. In dried spinach that is stored in atmospheric air, the content of β-carotene [599 mg/kg dry matter (DM)] is significantly lower compared with nitrogen (766 mg/kg DM), and the content of (*all-E*)-lutein is lower than in blanched frozen spinach. Neochrome is not detected in raw spinach but in steam-blanched and dried spinach. No differences are observed in the content of (*all-E*)-neoxanthin, (9'*Z*)-neoxanthin, (*all-E*)-violaxanthin, (*all-E*)-lutein epoxide, or neolutein A and B between spinach that is stored frozen after 3 min blanching and dried spinach.

Introduction

Color is one of the main attributes that contribute to the sensory quality of vegetables. The green color of spinach is an indication of the "freshness" of the product. The color of vegetables, for example spinach, is attributable the presence of various pigments, which primarily are the green chlorophylls and the yellow, orange, and red carotenoids. In green leafy vegetables such as spinach, only the green chlorophylls are seen because they mask the bright colors of the carotenoids (1).

The green color of spinach may change during post-harvest storage and during thermal processing and freezing (1). Degradation or conversion (or both) of the green chlorophylls (chlorophyll *a* and *b*) into the olive-brown degradation products (pheophytins, pheophorbides, pyropheophytins, and pyropheophorbides) leads to color changes (1). The conversion depends on temperature, length of the heat treatment, pH, and water activity (a_w) (the proportion between the water vapor pressure above the food and the water vapor pressure above pure water, a_w is an indication of available water in the food), as for example in dried foods (2,3). The chlorophylls *a* and *b* are also converted to green-colored degradation products, including the chlorophyllides, pyrochlorophylls, and hydrochlorophylls (4).

In raw spinach, β-carotene, (*all-E*)-lutein, (*all-E*)-violaxanthin, and (*all-E*)-neoxanthin are the four most abundant carotenoids (5,6). Apart from acting as pigments with a photoprotective function, the carotenoids do also have an impact on the nutritional quality and the health-benefit properties of spinach. Some of the carotenoids possess provitamin A activity as they are converted to vitamin A in the human body. In leafy vegetables, including spinach, the vitamin A effect is mainly because of the content of β-carotene (7). In addition, there is considerable evidence that some of the carotenoids have protective properties against certain forms of cancers, cardiovascular diseases, and specific eye diseases such as age-related macular degeneration (8–10). During thermal treatment of spinach, degradation or *trans*- or *cis*-isomerization (or both) of the carotenoids may occur. Moreover, the changes are promoted by processing conditions such as high temperature, long time of heat treatment, the presence of oxygen and light, and low water activity (6,7,11,12).

Spinach is a commonly consumed vegetable in developed and developing countries. In developed countries, spinach is consumed fresh as ready-to-use fresh-cut or processed as blanched and frozen or more rarely as canned spinach. Dehydration, however, is used in many developing countries to prolong the shelf life of spinach (12,13). Dehydration can be conducted by conven-

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tional air-drying, vacuum drying, drum drying, osmotic dehydration, or freeze-drying. Because expensive equipment is not available in many developing countries, simple sun drying is often used (12). Drying is known to result in changes in the color, texture, and nutritional value of spinach in various degrees. However, the changes depend on the processing conditions (12,14). Direct comparison of the changes in pigments in spinach subjected to commonly used industrial methods for processing (such as freezing, blanching, and drying) have not been very intensively studied.

Changes in individual pigments during processing can be evaluated by analytical high-performance liquid chromatography (HPLC). Several HPLC methods have been developed for analysis of chlorophyll and carotenoid pigments in green vegetables (15–17). In order to discriminate between individual carotenoids and chlorophylls, as well as between the different degradation products, Edelenbos et al. (17) developed an HPLC method for the separation of 8 chlorophyll and 12 carotenoid pigments in raw and processed green peas.

The aim of the present work was to evaluate the changes in the content of individual pigments in spinach varieties that were subjected to different processing and storage conditions (drying, blanching, freezing, and storage at ambient temperature in air and nitrogen). The qualitative and quantitative changes of the chlorophyll and carotenoid pigments in spinach were analyzed by analytical HPLC.

Experimental

Plant material

Three spinach varieties Lorelei, Springfield, and Ballet that differed in leaf shape, leaf color, and content of oxalate after processing were selected (Table I). The varieties were grown at a local organic farm near Research Centre Aarslev, (Aarslev, Denmark). Cultivation was carried out after normal organic practice. The material was sown in two replications in August and harvested manually in October 1999.

Plant registration and sensory evaluation of the raw material

The registration was carried out in the field, and the sensory evaluation was carried out immediately after harvest by a trained expert panel. The expert panel consisted of judges with great expertise in evaluating spinach. The shape of the leaves was described as round, oval, triangular, or pointed. The degree of uprightness of the leaves were evaluated as very upright, if the angle between the surface of the earth and the position of the leaves was 90°, and not upright, if the angle between the surface of the earth and the position of the leaves was 0°. The upright position of the leaf blades and the color and smoothness of the leaves were evaluated on a scale from 1 (not upright, light green, very savoy) to 9 (very upright, dark green, very smooth). "Very savoy" is a very rough, uneven surface similar to Savoy leaves. The taste was evaluated as very strong, medium strong, mild, and very mild. The surface color of 10 blades at three positions was measured using a Hunterlab Color Difference Meter (Hunter Lab Model DP-9000, Reston, VA) and expressed as a L-value (lightness).

Table I. Plant Characteristics and Physiochemical and Sensory Attributes of Spinach Varieties*

Variety	Lorelei	Springfield	Ballet
Leaf shape	Triangular/pointed	Oval/triangular	Oval/round
Upright position†	4	3	3
Leaf smoothness†	7	5	5
Sensory characteristics	Mild, nutty taste	Medium spinach taste with weak bitterness	Mild taste of pea pod
Color†	8	5	5
Lightness‡	30.4	35.2	33.6
Dry matter, raw (%)	9.4	8.8	8.5
Dry matter, dried (%)	95.7	93.8	95.9
Oxalate (mg/100 g DM)	7738 a††	5742 b	6447 b
β-Carotene (mg/kg DM)	745 a	607 a	695 a
Sum of carotenoids§ (mg/kg DM)	1809 a	1437 a	1736 a
Sum of chlorophylls**(mg/kg DM)	10995 a	8136 a	9709 a

* Average of two determinations.
† Upright position, leaf smoothness and color was evaluated on a scale from 1 = (not upright, very savoy, very light green) to 9 = (very upright, very smooth, very dark green).
‡ Lightness of the raw leaves was measured using a Hunterlab Color Difference Meter on a scale from black (0) to white (100).
§ The sum of (*all-E*)-neoxanthin, (9′*Z*)-neoxanthin, (*all-E*)-violaxanthin, (*all-E*)-lutein, neolutein A, neolutein B, lutein epoxide, and three unidentified carotenoids, determined in raw frozen samples.
** The sum of chlorophyll *a* and *b*, chlorophyll *a-1* and *b-1*, chlorophyll *a′* and *b′*, and pheophytin *a* and *b*, determined in raw frozen samples.
†† Means within a row followed by different letters are significantly different ($P \leq 0.05$).

Handling and processing after harvest

Pretreatment

After harvesting, the samples were stored in boxes at 1°C until processing within 20 h. The samples were sorted in usable and unusable material (broken and ill leaves), respectively. The unusable leaves were discarded. The usable leaves were almost the same size and, thus, the same age. Leaf stalks of the usable leaves were removed and the blades were subsequently washed twice in cold tap water. Surface water was removed by spin-drying at 1000 rpm [≈ relative centrifugal force (RCF) = 105 g] for 1 min (AEG, Type SV 2514, Nürnberg, Germany).

Blanching

Replicate samples of 250 g of each variety were blanched in a steam blancher (Hounø APS, Type TS 13, Spenstrup, Denmark) at 90°C for 3, 9, or 15 min. After blanching, the samples were chilled in a CO_2-chiller (AGA Frigoscandia A/S, AGA-freeze FK/CO2, Copenhagen, Denmark) with a conveyer belt speed of 1.2 m/min and then frozen in a cryogenic freezer (AGA Frigoscandia A/S, AGAfreeze Mini30-06) at –40°C with a conveyer belt speed of 0.38 m/min. Following freezing, the samples were packed in aluminium foil pouches (PETP12/ALU9/LLDPE75, 120- × 200-mm) and stored at –24°C for 6 months.

Frozen storage

Replicate reference samples of 300 g raw material of each variety were chilled and frozen as

described previously. Following freezing, the samples were packed in aluminium foil pouches (PETP12/ALU9/LLDPE75, 120- × 200-mm) and stored at –24°C for 6 months.

Storage of dried samples

Two replicates of each variety were dried at 75°C in a heating cabinet (Lytzen, CBM-spec, Herlev, Denmark) for 3 h. Following drying, the samples were packed in aluminium foil pouches (PETP12/ALU9/LLDPE75, 120- × 200-mm) and flushed with either atmospheric air [21 % oxygen (O_2)] or 100% nitrogen (N_2). After packaging, the samples were stored at ambient temperature for 6 months.

Analysis of the raw, blanched, and dried material

Determination of dry matter

Dry matter (DM) content was determined in the raw or dried samples. DM content was determined twice on an aliquot sample by drying at 80°C in a heating cabinet (Lytzen, CBM-spec, Herlev, Denmark) with forced air convection for 20 h.

Determination of oxalate

Water soluble oxalate (potassium, sodium, and magnesium oxalate) was extracted from 1.5 g dried spinach or 5 g fresh spinach samples with 50 mL ultrapure boiling water [ultrapure water was generated by an Elgastat Maxima Analytica Water Purification System (Elga Ltd., High Wycombs, U.K.)] for 3 h at room temperature, as described by Hels et al. (18). The extract was filtered through a 0.45-µm Minisart SRP 25 filter (Bie & Berntsen, Rødovre, Denmark) directly into a 4-mL brown vial (Merck Kebo Lab, Albertslund, Denmark). The samples were analyzed by ion chromatography that was performed on a Dionex HPLC system (Dionex Denmark, Rødovre, Denmark) equipped with an ED-40 conductivity detector, GP-40 gradient pump, and LC30 chromatography oven. The chromatography oven was equipped with an ASRS-ULTRA anion suppressor (4 mm), DS3-detection stabilizer, IonPac ATC-1 Trap column (24- × 9-mm i.d.) and an analytical IonPac AS11 column (250- × 4-mm i.d.) protected with an IonPac AG11 guard cartridge (50- × 4-mm i.d.). Separations were performed at 30°C by gradient elution with a sodium hydroxide (NaOH) eluent, consisting of solvent A (100mM NaOH) and solvent B (H_2O). The elution profile was as follows: 0 min 99% B, 8 min 99% B, 28 min 70% B, 35 min 70% B, 36 min 40% B, 37 min 0% B, 40 min 0% B, 48.1 min 99% B, and 55 min 99% B. All changes in the gradient were linear programmed. The flow rate was 1.5 mL/min, and the injection volume was 20 µL. Oxalate was identified by peak addition of an authentic standard and quantitated in extracts using oxalate as external standard (18). The concentration of oxalate was calculated mg/100 g DM, so that the data were comparable.

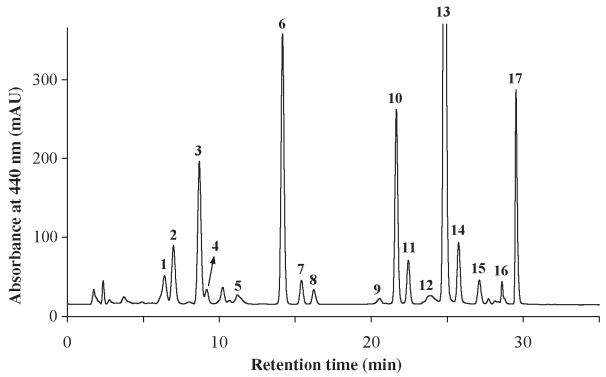

Figure 1. HPLC chromatogram of major carotenoids and chlorophylls in blanched, frozen spinach. (*all-E*)-Neoxanthin (1), (9'*Z*)-neoxanthin (2), (*all-E*)-violaxanthin (3), neochrome (4), (*all-E*)-lutein epoxide (5), (*all-E*)-lutein (6), neolutein B (7), neolutein A (8), chlorophyll *b*-1 (= chlorophyll *b* derivate, 9), chlorophyll *b* (10), chlorophyll *b*' (11), chlorophyll *a*-1 (= chlorophyll *a* derivate, 12), chlorophyll *a* (13), chlorophyll *a*' (14), pheophytin *b* (15), pheophytin *a* (16), (*all-E*)-β-carotene (17).

Determination of pigments

Chemicals. Acetone, ethanol, etyl acetate (EtOAc), methanol (MeOH), and tetrahydrofuran were Rathborn HPLC-grade (Sigma-Aldrich, Steinheim, Germany). All eluents for HPLC were degassed with an ultrasound for 20 min before use. Standards [(*all-E*)-lutein, (*all-E*)-β-carotene, and chlorophyll a and *b*] were purchased from Sigma-Aldrich (Steinheim, Germany).

Extraction of pigments. The carotenoids and the chlorophylls were extracted under red light according to the procedure described by Edelenbos et al. (17). After homogenisation for 60 s with ultrapure water (generated by the Elgastat Maxima analytica water purification system), a subsample was suspended in 10 mL 100% cold acetone and homogenized for 30 s by ultrasonic agitation [Branson Sonifier 250 (Merck Kebo), power setting of 7 corresponding to an output of 38 Watts] and centrifuged (15,000 rpm; RCF = 34,000 g) for 4 min (Sorvall RC-5B Plus, Axeb lab & test, Albertslund, Denmark). The residue was reextracted until it was colorless, in total three times. The supernatants were pooled, diluted to 50 mL with 100% acetone, and filtered through a 0.45-µm Minisart SRP 25 filter (Bie & Berntsen) directly into a 4 mL brown vial (Merck Kebo).

HPLC determination of pigments. The extracts were analyzed by reversed-phase (RP)-HPLC using a SPD-10 AV-UV–vis detector with a path length of 10 mm, operated at 440 nm with a LiChrospher 100 RP-18 column, as described by Edelenbos et al. (17). The mobile phase consisted of 80% MeOH–20% H_2O (solvent A) and 100% EtOAc (solvent B). Solvent B was increased from 20% to 22.5% in 2.5 min, to 50% in 17.5 min, and from 50% to 80% in 1.5 min (2-min holding time) from 80 to 100 min in 5 min and 3-min holding time. The flow rate was 1 mL/min and the injection volume was 25 µL. Identifications were based on chromatographic behavior, visible absorption spectra, and specific chemical reactions as previously described by Edelenbos et al. (17). Eight individual chlorophylls (chlorophyll *b*, chlorophyll *a*, chlorophyll *b*', chlorophyll *a*', chlorophyll *b*-1, chlorophyll *a*-1, pheophytin *b*, and pheophytin *a*), 8 individual xantophylls [(*all-E*)-neoxanthin, (9'*Z*)-neoxanthin, (*all-E*)-violaxanthin, neochrome, (*all-E*)-lutein epoxide, (*all-E*)-lutein, neolutein B, and neolutein A), and β-carotene were separated and identified in the spinach samples (Figure 1). Quantitation was carried out as described by Edelenbos et al. (17) using an external calibration method for (*all-E*)-lutein, (*all-E*)-β-carotene, and chlorophyll *a* and *b*. All xanthophylls were calculated relative to (*all-E*)-lutein, whereas chlorophyll *a*-1, *a*', and pheophytin *a* were calculated relative to chlorophyll *a*, and chlorophyll *b*-1, *b*', and pheophytin *b*

were calculated to relative to chlorophyll *b*. The concentrations of all the pigments were calculated as mg/kg DM, so that the data were directly comparable.

Quality control. The validity of the HPLC method used for determination of the pigments was checked with regard to accuracy, linearity, and precision. The coefficient of variance (CV) was 5.5% between five extractions and 5.0% between five injections of one extract. The analysis of the pigments was carried out as two extractions, and the CV was below 10% of the major peaks between two extractions for all samples analyzed.

Statistical analysis

A general linear model procedure in the statistical analyses system (SAS) was used for analyses of different color parameters and individual pigments with variety, blanching time, or the three treatments (blanched, frozen spinach, and dried spinach that was packed in atmospheric air or nitrogen) as the main factors (SAS, Cary, N.C.). Unless stated, all significant differences are at $P \leq 0.05$. Duncan's multiple range test was used to assess the significant differences. Data on (*all-E*)-neoxanthin, (*all-E*)-violaxanthin, (*all-E*)-lutein epoxide, chlorophyll *a,* and chlorophyll *a*' were ln-transformed to fit normal distribution.

Results and Discussion

Characteristics of the spinach varieties

The three varieties varied in leaf shape, smoothness, taste, and color (Table I). The sensory evaluation and the objective color measurements showed that Lorelei was obviously the darkest green and had the smoothest leaves, whereas Springfield and Ballet were lighter green and had less smooth leaves (Table I). The oxalate content in Lorelei was significantly higher than in the two other varieties (Table I). The level of oxalate in the investigated varieties was in accordance with earlier investigations in which a range from 605 mg/100 g fresh weight (FW) to 806 mg/100 g FW was observed between 12 varieties (19). The mean values of oxalate for the three varieties are presented in Table I because no treatment and interaction effects were observed (Table I).

The subjective and objective color measurements were in accordance with the total chlorophyll content in raw, frozen spinach, as Lorelei was clearly the darkest green variety and contained the highest content of total chlorophyll, although the differences were not significant (Table I). In raw, frozen spinach, chlorophyll *a* and *b* dominated, whereas the contents of chlorophyll *a*' and *b*' and pheophytin *a* and *b* were rather low. The content of chlorophyll *a*' and *b*' varied between 0.1–0.3% and 1.5–1.7%, respectively, and the content of pheophytin *a* and *b* varied between 1.5–3.4% and 1.2–5.4%, respectively, in the three varieties. The highest content of pheophytins was found in Lorelei, which also had the highest chlorophyll content.

The individual carotenoids detected in raw, frozen spinach were the same as found in raw spinach, although the concentration levels are not directly comparable (15). The four most abundant carotenoids in raw, frozen spinach were β-carotene, (*all-E*)-lutein, (*all-E*)-violaxanthin, and (*all-E*)- and (9'*Z*)-neoxanthin, which is in accordance with previous investigations (5,6). The most abundant carotenoid was β-carotene, which confirms that spinach is an excellent provitamin A source. The total carotenoid content on a fresh weight basis in the three varieties ranged between 127 and 170 mg/kg FW (Table I), which are similar levels reported previously in fresh spinach (60–150 mg/kg FW) by Gross (7) and in frozen spinach varieties (177–226 mg/kg FW) by Kidmose et al. (6). None of the other individual carotenoids (data not shown) or the total content of carotenoids varied significantly in raw frozen samples of the three varieties (Table I).

Changes in the pigment content during blanching

Because no interaction between variety and blanching time was seen on the individual chlorophylls and carotenoids in the statistical analysis, except for pheophytin *b* and chlorophyll *b*', data are presented as mean values for the three varieties.

Chlorophylls

The content of chlorophyll *a* and *b* decreased significantly with increasing blanching time (Table II). The content decreased 5%, 25%, and 32% during 3, 9, and 15 min blanching, respectively, as compared with storage of raw samples at –24°C for 6 months. A decrease of 5% in the chlorophyll *a* and *b* content after 3 min blanching of spinach was in accordance with the decrease of approximately 7% observed in green peas (17). The actual loss because of blanching may be even greater, as the total content of chlorophyll *a* and *b* were not measured in raw spinach immediately after freezing, and it is well known that enzymatic degrada-

Table II. The Content (mg/kg DM) of Carotenoid and Chlorophyll Pigments after 0, 3, 9, and 15 min Blanching at 90°C and 6 Month Frozen Storage at –24°C

	Blanching time			
	0 min	3 min	9 min	15 min
Visual color	Green	Green	Green	Green with brown tinge
Chlorophylls				
Chlorophyll *a*	6480 a*	6362 a	4947 b	4253 b
Chlorophyll *b*	2646 a	2321 b	1909 c	1984 c
Chlorophyll *a*'	16.3 b	483 a	634 a	589 a
Chlorophyll *b*'	41.5 b	520 a	522 a	567 a
Chlorophyll *a*-1 + *b*-1	197 a	118 a	158 a	156 a
Pheophytin *a*	179 c	293 bc	377 b	566 a
Pheophytin *b*	67.8 d	320 c	601 b	1025 a
Carotenoids				
(*all-E*)-Neoxanthin	43.9 a	62.9 a	56.0 a	67.4 a
(9'*Z*)-Neoxanthin	146 a	178 a	131 a	125 a
(*all-E*)-Violaxanthin	206 a	182 ab	103 bc	53.7 c
Neochrome	–	40.6 a	43.7 a	48.0 a
(*all-E*)-Lutein epoxide	14.0 a	7.0 b	7.0 b	7.6 b
(*all-E*)-Lutein	475 b	583 a	479 b	460 b
Neolutein A	28.7 a	39.3 a	31.2 a	44.4 a
Neolutein B	59.3 a	36.1 a	74.1 a	81.1 a
(*all-E*)-β-carotene	684 a	757 a	694 a	773 a

* Means within a row followed by different letters are significantly different ($P \leq 0.05$), not detected. Neochrome is formed during blanching (17).

tion of chlorophyll *a* and *b* caused by chlorophyllase may occur during frozen storage (1). The content of chlorophyll *a* and *b* was significantly reduced after 9 min blanching compared with 3 min blanching, whereas additionally 6 min blanching did not lead to further reduction in the content of chlorophyll *a* and *b*.

Chlorophyll *a*' and *b*' were formed during blanching, however, no significant differences were observed between blanching times (Table II). Although frozen storage may cause formation of these epimers (4), the present study clearly indicates that these chlorophyll epimers also are formed during heating. The length of the heat treatment, however, seemed to be of minor importance. Olive-brown pheophytins were also formed during heating. Pheophytins are progressively formed from chlorophyll *a* and *b* with increasing temperatures above 60°C (20). The content of pheophytins increased with blanching time (Table II). Increasing blanching time resulted in a more pronounced formation of pheophytin b compared to the formation of pheophytin *a*. Fifteen minutes of blanching resulted in a 316% increase of pheophytin *a* and 1510% increase of pheophytin *b* compared with the content in the raw, frozen samples (Table II). The total content of chlorophyll *a*-1 and *b*-1 did not change significantly during blanching from 0 to 15 min (Table II).

The perception of the colors of chlorophyll *a*, *b*, *a*', and *b*', and the hydroxychlorophylls are very similar, as they are all blue-green in color. The presence of olive brown pheophytins may influence the color of spinach, however, a change in visual color because of pronounced pheophytin formation was only detected after 15 min blanching (Table II).

Table III. The Content (mg/kg DM) of Carotenoid and Chlorophyll Pigments after Frozen Storage of 3 min Blanched Spinach or Storage of Dried Spinach at Ambient Temperature Packed in Atmospheric Air (Air) or Nitrogen (N_2)*

Treatment	Frozen storage of blanched samples	Dried samples stored in air	Dried samples stored in N_2
Visual color	Green	Light green	Light green
Chlorophylls			
Chlorophyll *a*	6362 a†	5890 a	6284 a
Chlorophyll *b*	2321 a	1989 a	2117 a
Chlorophyll *a*'	483 b	718 a	783 a
Chlorophyll *b*'	520 a	321 b	351 b
Chlorophyll *a*-1 + *b*-1	118 a	319 a	327 a
Pheophytin *a*	293 a	134 b	131 b
Pheophytin *b*	320 a	46.1 b	43.3 b
Carotenoids			
(*all-E*)-neoxanthin	62.9 a	58.7 a	67.3 a
(9'*Z*)-neoxanthin	178 a	166.0 a	179 a
(*all-E*)-violaxanthin	182 a	212.6 a	247 a
Neochrome	40.6 a	23.7 b	38.9 a
(*all-E*)-lutein epoxide	7.0 a	13.9 a	8.0 a
(*all-E*)-lutein	583 a	497 b	550 ab
Neolutein A	39.3 a	39.9 a	46.5 a
Neolutein B	36.1 a	32.0 a	35.2 a
(*all-E*)-β-carotene	757 a	599 b	766 a

* Data are average of 3 varieties and 2 determinations on each variety.
† Means within a row followed by different letters are significantly different ($P \geq 0.05$).

In general, the carotenoid pigments were more stable during blanching than the chlorophyll pigments. (*all-E*)-Violaxanthin was the only carotenoid that decreased significantly by blanching, and the content of (*all-E*)-violaxanthin was reduced by approximately 74% after 15 min blanching compared with no blanching (Table II). This is in accordance with previous studies, reporting that (*all-E*)-violaxanthin is one of the most thermolabile carotenoids present in vegetables that is easily destroyed by steam blanching (21). (*all-E*)-Lutein epoxide was slightly lower in blanched samples compared with raw, frozen spinach, however the blanching time did not affect the content. A slight nonsignificant increase in the content of β-carotene and a significant increase in the content of (*all-E*)-lutein were observed in the blanched, frozen spinach as compared with raw, frozen spinach (Table II). This is in accordance with earlier investigations, which showed a better resistance of β-carotene in green beans towards degradation, when samples were blanched prior to freezing for 12 months (22). A slight increase in the (*all-E*)-lutein content has previously been reported during water and steam-blanching of peas and spinach (15,17). The slightly higher content of these carotenoids in blanched spinach may be explained by a higher enzymatic degradation of the carotenoids in raw spinach during frozen storage as compared with the degradation occurring during blanching. Enhanced chemical extractability of these carotenoids from plant cells, which change structure during blanching (11) may be another feasible explanation of the observed differences. Thus, whether blanching and freezing resulted in an increase or a degradation of carotenoids, the changes were similar between samples that were frozen raw or after blanching. Neochrome was not present in raw spinach, but was formed when spinach was subjected to blanching (Table II). This is in accordance with Edelenbos et al. (17), who observed neochrome in cooked peas. No significant differences were observed during blanching for the other carotenoids, including (*all-E*)- and (9'*Z*)-neoxanthin and the neoluteins.

In summary, the pigments in all three varieties act very similar when subjected to blanching and freezing. However, the chlorophyll pigments were more susceptible to heating than the carotenoid pigments, and the individual pigments were differently affected by blanching.

Changes in the pigment content during frozen storage and storage after drying

Because no interaction between variety and different storage treatments were seen on the individual chlorophylls and carotenoids except for (*all-E*)-violaxanthin, the data are represented as mean values for the three varieties.

Chlorophylls

No significant effect on the chlorophyll *a* content was observed between storage of dried spinach and frozen storage of blanched spinach (Table III). The degradation rates of chlorophyll *a*, which was reported twice as fast as chlorophyll *b* in broccoli juice (23),

and chlorophyll *b* were apparently similar in spinach regardless of processing and storage treatment (Table III). However, drying of spinach resulted in a higher degradation of chlorophyll *b* than frozen storage of raw samples (Tables II and III). Drying promoted the formation of chlorophyll *a*', whereas blanching seemed to promote the formation of chlorophyll *b*' (Tables II and III). Drying at 75°C for 3 h followed by storage at ambient temperature resulted in less formation of olive-brown colored pheophytins than after steam blanching at 90°C followed by frozen storage (Table III), indicating that the temperature is crucial for the conversion of chlorophyll to pheophytin (20). The contents of pheophytin *a* and *b* were similar or slightly lower after drying than in raw, frozen samples (Tables II and III), which indicate that the pheophytin formation at 75°C is equal to the formation during frozen storage. The presence of oxygen during storage of dried spinach had no effect on pheophytinization compared with storage in nitrogen (Table III). Storage in atmospheric air compared with storage in nitrogen of dried spinach samples did not affect the formation of chlorophyll epimers (Table III). It is rather surprising that the total content of chlorophyll *a* and *b* was higher and the pheophytin content lower after drying than after frozen storage of blanched samples because pheophytinization was reported during storage of material with low water activity such as dried samples (2).

No differences in the chlorophyll derivates (chlorophyll *a*-1 and *b*-1) were seen between the three treatments, whereas other chlorophyll a and b derivatives, which were not identified, were also formed during drying. The content of these chlorophyll derivates were approximately twice as high in dried samples as compared to raw, frozen samples (data not shown). Apart from the chlorophylls, epimers, and pheophytins, Gauthier-Jaques et al. (4) also detected hydrochlorophylls *a* and *b* as well as an unknown chlorophyll *b* derivate in dried, rehydrated spinach powder. The unidentified chlorophyll *a* and *b* derivates in the present study might have been hydrochlorophylls *a* and *b*.

Before consumption, it is necessary to rehydrate or heat (or both) dried and frozen spinach. These losses/changes in the pigments that occur during these kind of preparations before consumption were not included in the present study.

Carotenoids

The individual carotenoids, (*all-E*)-β-carotene, (*all-E*)-lutein, neochrome, and two of the unknown carotenoids varied significantly between the three treatments (Table III). Oxygen is known to be a critical factor in β-carotene degradation, which was observed in the present study, as the content of β-carotene was significantly lower in dried spinach stored in atmospheric air compared with spinach storage in nitrogen (Table III). (*all-E*)-Lutein was also slightly higher after frozen storage of blanched spinach than after drying and storage in atmospheric air (Table III). However, a similar effect of oxygen on degradation of other carotenoids in spinach was not observed (Table III).

As stated previously, (*all-E*)-violaxanthin is easily destroyed by blanching above 90°C (21). In contrast, drying at 75°C for 3 h did not promote degradation of (*all-E*)-violaxanthin, because the content after drying was similar to the content after frozen storage of raw and 3 min blanched spinach (Tables II and III). Neochrome was also formed when spinach was subjected to drying (Table III). Two of the unknown carotenoids were formed during drying (Table III). The content of (*all-E*)-neoxanthin, (9'*Z*)-neoxanthin, (*all-E*)-lutein epoxide, neolutein A and B, and one of the unidentified carotenoids was rather unaffected by freezing and drying.

In summary, the individual carotenoids were affected differently by the investigated processing treatments, and the lowest content of (*all-E*)-β-carotene was obtained when dried samples were stored in atmospheric air. However, storage of dried samples in nitrogen resulted in similar content of β-carotene compared with frozen samples. because the lowest β-carotene content was found in the dried samples that were stored in the presence of oxygen, these samples also had the lowest Vitamin A activity.

The visual color of Lorelei was dark green in the frozen samples and light green in the dried samples. The raw and the blanched samples of Springfield and Ballet were both green, whereas the dried samples were light green. The visual color differences between the treatments could not directly be correlated to the changes in the individual chlorophylls. As the yellow and orange colors of the carotenoids were masked by the green color in spinach, the differences in the carotenoids between the three treatments did not directly affect the visual color of the spinach.

Conclusion

The three spinach varieties varied in leaf shape, smoothness and color. Springfield and Ballet had low levels of soluble oxalate as compared to Lorelei, which may be important when they are consumed without precipitation with calcium, but similar levels of total and individual carotenoids, including β-carotene. The three varieties reacted similarly to freezing and drying and storage for 6 months. All treatments had very little effect on visual green color but affected individual pigments. Blanching prior to frozen storage resulted in a loss of chlorophylls and formation of pheophytins. Storage of dried spinach in presence of oxygen lowered the β-carotene content and thus the vitamin A value compared to the other treatments. Blanching, frozen storage, and drying did not affect individual pigments in the same way. Differences in stability were observed between the carotenoids and the chlorophylls and the individual pigments reacted differently. This fact makes it very difficult to predict the content of individual pigments from spectrophotometric measurements of carotenoids and chlorophylls and consequently the nutritional value and the health-benefit properties of spinach exposed to different food processing treatments.

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