HPLC Determination of Chlorophyll and Carotenoid Pigments in Processed Green Pea Cultivars (*Pisum sativum* L.)

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Chlorophyll and carotenoid pigments from six cultivars of processed green peas (Avola, Tristar, Rampart, Turon, Bella, and Greenshaft) were extracted with 100% acetone and analyzed by reversed-phase HPLC. A total of 17 pigments were identified in the pea cultivars including 8 xanthophylls ((*all-E*)-neoxanthin, (9′*Z*)-neoxanthin, (*all-E*)-violaxanthin, neochrome, (*all-E*)-lutein epoxide, (*all-E*)-lutein, neolutein B, neolutein A), 4 chlorophyll *b* related compounds (chlorophyll *b* derivative, chlorophyll *b*, chlorophyll *b*′, and pheophytin *b*), 4 chlorophyll *a* related compounds (chlorophyll *a* derivative, chlorophyll *a*, chlorophyll *a*′, and pheophytin *a*), and (*all-E*)-*β*-carotene. The efficiency of different extraction procedures using 100% acetone showed that initial extraction followed by three reextractions without holding time between gave a higher extraction yield than no reextraction and 30 or 60 min holding time. All six cultivars contained the same pigments, but the concentration of each pigment varied significantly. On average of the two years, the chlorophyll *a* concentration varied from 4800 to 7300 *μ*g/100 g fresh weight, the chlorophyll *b* concentration from 2100 to 2800 *μ*g/100 g fresh weight, the (*all-E*)-lutein concentration from 1200 to 1900 *μ*g/100 g fresh weight, and the (*all-E*)-*β*-carotene concentration from 300 to 490 *μ*g/100 g fresh weight in the processed pea cultivars. These differences in pigment concentration between the investigated cultivars are discussed in relation to maturity, product color and nutritional quality.

Keywords: *Pisum sativum; chlorophylls; carotenoids; HPLC; extraction method; cultivars*

INTRODUCTION

Color and appearance are important attributes when the consumers consider a food product for consumption. The color of foods is due to the presence of various pigments, either natural or artificial, produced during growth or after harvest or added during processing. The yellow, orange, and red colors of fruits and vegetables are due to carotenoids and/or anthocyanins and the green color to chlorophylls. The carotenoids and chlorophylls are found in all organisms capable of photosynthesis; however, the bright colors of many carotenoids are often masked by chlorophylls in photosynthetic tissues (*1*).

Green peas (*Pisum sativum* L. convar. *medullare* Alef.) for deep freezing is an important vegetable crop in Denmark. The market quality of this vegetable depends on several factors including flavor, texture, size, and color (*2*). When peas are sold from producer to retailer they are sold by alcohol insoluble solids (AIS), size, and color uniformity (personal communication). These parameters are influenced by genotype, growing conditions, maturity at harvest, processing conditions, and handling by consumers prior to consumption (*3*−*6*). The work reported here is part of an extensive research program focusing on pea quality aspects in the total chain from plant breeding to consumption (*2*, *7*−*12*).

Part of the program focusing on pea color and pigment composition of pea cultivars grown under natural and reduced light intensities included development of a method for the isolation and quantification of chlorophylls and carotenoids. Several methods for the extraction and isolation of carotenoids and chlorophylls have been described in the literature including methods for the quantification by high-performance liquid chromatography (HPLC) (*5*, *13*−*18*). A method for the extraction of chloroplast pigments in raw and processed peas was reported by Forni et al. (*5*), but they only quantified the chlorophylls and pheophytins *a* and *b*. Hart and Scott (*18*) developed an HPLC method for analysis of carotenoids in foods and analyzed the concentration of (*all-E*)-lutein, (*all-E*)-*β*-carotene, and (*all-Z*)-*β*-carotene in raw and processed peas in order to determine the nutritional value and potential beneficial effect of peas on human health. Quantification of the chlorophyll and carotenoid pigments of green peas, however, has never been reported. Perhaps the closest examples to the work reported here are those of Khachik et al. (*14*), Chen and Chen (*16*), and Yamauchi and Watada (*17*). They reported work on the separation, identification, and quantification of the major carotenoid and chlorophyll constituents in extracts of raw and processed green vegetables. The extraction procedures described by Khachik et al. (*14*) and Chen and Chen (*16*) were very complex and time consuming in contrast to the procedures of Yamauchi and Watada (*17*). Yamauchi and Watada (*17*) used 80% acetone as extraction solvent and reextraction until colorless (no green color) of the photosynthetic pigments in parsley leaves; however, the presence of water in their extraction solvent slowed the extraction process.

In the present work, we outline the development of an analytical method for the quantification of a wide

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10.1021/jf010569z CCC: $20.00
Published on Web 09/19/2001

Table 1. Plant Characteristics, Cultivation Data, and Physiochemical Attributes of Green Pea Cultivars. Average of Two Years

	cultivar					
characteristic	Avola	Tristar	Rampart	Turon	Bella	Greenshaft
leaf type	normal	normal	semileafless	normal	semileafless	normal
size grade class[a]	large	medium	small	small	large	large
days to harvest	67	74	76	76	73	73
tenderometer value	114	127	115	117	102	101
AIS (%)	14.0	16.8	14.8	15.5	12.7	13.6
dry matter (%)	26.3	28.6	26.5	26.6	24.7	25.7
lightness	44.5	42.5	41.1	41.9	41.6	41.6

[a] Principal size of processed peas, small 6.0−8.75 mm; medium 8.75−10.2 mm; large >10.2 mm.

range of chlorophyll and carotenoid pigments in processed green peas. The concentration of carotenoid and chlorophyll pigments was determined in six cultivars of processed green peas grown in Denmark, and the genetic differences between the cultivars are discussed in relation to maturity, product color, and nutritional quality.

MATERIALS AND METHODS

Plant Material and Growing Conditions. Six genotypes of wrinkled-seeded peas were grown at the Department of Horticulture, Aarslev, Denmark (10° 27′ E, 55° 18′ N), during two seasons. The field experimental design was a complete block design with four replicates. In order to be able to harvest the peas as near to a tenderometer value (TV) of 110 as possible, which is the optimum harvest time in Denmark (*19*), the peas were harvested every second or third day from approximately a TV of 80. The TV was determined on a FMC 384 tenderometer (FMC Food Machinery, Parma, Italy). At harvest, botanical registrations were made on 10 plants per plot (Table 1) and all replicates within a cultivar were harvested. Plants in a plot was cut using a modified grass plot machine (Haldrup, Løgstør, Denmark) and threshed in a stationary "mini-viner" (Schepers Techniek BV, Hoogeveen, The Netherlands). The TV of the peas in each plot was determined on samples of threshed, washed, and cleaned raw peas. The remaining peas were steam blanched and individual frozen and packed in polyethylene pouches and stored at −24 °C until analysis (*11*). All pigment analysis were carried out on processed peas (blanched, frozen, and thawed) unless stated. Alcohol insoluble solids (AIS) and dry matter content were measured as previously described (*11*). Surface color was determined using a Hunterlab Tristimulus colorimeter system (D25M-9000, Hunter Associates Laboratory, Inc., Reston, VA) measuring reflective colors of surfaces. The system was calibrated against a standard white reference tile supplied by the manufacturer. Surface color was measured on 10 subsamples of 20 g with three readings of each subsample and expressed as lightness value. Lightness in peas relate to sensory perception of green color; e.g., peas with a high lightness value are brighter green than peas with a low value.

Chemicals. Acetone, chloroform ($CHCl_3$), dichloromethane (CH_2Cl_2), ethanol (EtOH), ethyl acetate (EtOAc), methanol (MeOH), and tetrahydrofuran (THF) were of Ratborn HPLC grade obtained from Aldrich (Steinheim, Germany). The water used for extraction and HPLC determination was ultrapure generated by an Elgastat Maxima Analytica Water Purification System (Elga Ltd., United Kingdom). All eluents for HPLC were filtered through a 0.45 μm Minisart SRP 25 filter (Bie & Berntsen, Rødovre, Denmark) and degassed with ultrasound for 20 min before use. Reference samples of (*all-E*)-lutein, (*all-E*)-β-carotene, and chlorophyll *a* and *b* were purchased from Sigma (Diesenhofen, Germany) and used without purification.

Extraction of Pigments. Pigments were extracted from peas at room temperature under dim laboratory light. Samples (10 g) were homogenized 60 s with 5 g of ultrapure water. A 10 g subsample was suspended in 10 mL of cold 100% acetone, homogenized for 30 s by ultrasonic agitation (Branson Sonifier 250, Merck Kebo Lab, Albertslund, Denmark), and centrifuged for 4 min using a Sorvall SA-600 head (G_{max} 20.845; Buch & Holm, Herlev, Denmark), and the resulting supernatant was saved. The sample was reextracted three times as described above, which ensured approximately >98% extraction of carotenoids and chlorophylls. The supernatants were pooled, diluted to 50 mL with 100% acetone, and 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) and analyzed directly by analytical HPLC. The reproducibility of this extraction method was determined on five true extractions of processed peas of cv. Tristar. This cultivar was also used for analytical control throughout the experiments. The above method was developed after a series of initial experiments where, e.g., the efficiency of extraction with 100% acetone with or without reextractions was studied.

Pigment Stability during Processing. For studies of pigment stability raw peas of cv. Bella were divided into three subsamples and each subsample was then divided into a sample for direct extraction (raw sample) and a sample for cooking and then extraction (cooked sample). Peas were cooked for 3 min in the double amount of boiling tap water, drained, and cooled to room temperature and then extracted.

HPLC Determination of Pigments. A Shimadzu HPLC system equipped with a SPD 10AV UV−vis detector operating at 440 nm was used for routine analysis. Visible detection at 660 nm and fluorimetric detection (excitation 430 nm; emission 670 nm) using a RF-551 spectrofluorimeter equipped with a xenon 150 W lamp (Shimadzu, Kyoto, Japan) was used periodically to confirm spectral identity of pigments. The data were stored and processed by means of a Shimadzu C-R7A Chromatopac computing system. A Shimadzu SPD-M10A diode array detector was used to assess peak homogeneity and to confirm spectral identity of pigments. The data were stored and processed by means of a CLASS M10A computing system. The diode array detector was employed at 440 nm, and absorption spectra of carotenoids and chlorophylls were recorded between 300 and 600 nm. Separations were performed on a LiChrospher 100 RP-18 column (5 μm; 244 × 4 mm i.d., Merck Kebo Lab, Albertslund, Denmark) protected with a LiChrosorb RP-18 guard cartridge (5 μm; 15 × 4 mm i.d., Merck Kebo Lab, Albertslund, Denmark). The column temperature was maintained at 30 °C and the mobile phases consisted of solvent A (80% MeOH−20% H_2O) and solvent B (100% EtOAc). Separations were performed by the following solvent gradient: 0 min 20% B, 2.5 min 22.5% B, 20−22.5 min 50% B, 24−26 min 80% B, 31−34 min 100% B, 42−47 min 20% B. All increases of solvent B were linear programmed. The flow rate was 1 mL/min and the injection volume 25 μL.

Isolation of Pigments by Semipreparative HPLC and Flash Chromatography. Processed peas (600 g) of cv. Tristar were ground and extracted with CH_2Cl_2 at room temperature. The combined extracts were filtered and dried with anhydrous sodium sulfate and evaporated in a rotary evaporator at 25 °C to 10 mL. The extract was subjected to flash chromatography (silica gel 60, 230−400 mesh; Merck Kebo Lab, Albertslund, Denmark) eluting with a $CHCl_3$−MeOH gradient (98:2, 95:5, 90:10, 80:20, 60:40, 50:50, 20:80, and 0:100) which separated chlorophylls and hydrocarbon carotenoids from

xanthophylls. Extraction and separation by flash chromatography were carried out in dim laboratory light. Fractions of 50 mL were analyzed by analytical HPLC. Fractions containing the same pigments were combined and concentrated. Further purification of individual compounds was performed by semipreparative HPLC on a Shimadzu HPLC system equipped with a SPD-M10A diode array detector. A LiChrospher 100 RP-18 column (5 μm; 244 × 10 mm i.d.; Merck Kebo Lab, Albertslund, Denmark) protected with a LiChrosorb RP-18 guard cartridge (5 μm; 15 × 10 mm; Merck Kebo Lab, Albertslund, Denmark) was used. Separations were carried out at 25 °C with solvent A (90% [80% MeOH:20% H_2O]−10% EtOAc) and solvent B (100% EtOAc). Lutein and the mono-*Z*-isomers of lutein, neolutein B ((9*Z*)- or (9′*Z*)-lutein) and neolutein A ((13*Z*)- or (13′*Z*)-lutein) were separated by the same gradient as used for analytical HPLC. The other xanthophylls were separated by the following gradient: 0−10 min 0% B, 20 min 10% B, 30 min 15% B, 35 min 20% B, 40 min 30% B, 45 min 50% B, 50 min 75% B, 54 min 100% B, 55 min 90% B, 60 min 50% B, 70 min 0% B. All increases of solvent B were linear programmed. The flow was 3 mL/min and injection volume 50 μL. Absorption spectra of isolated pigments in EtOH were recorded on a Shimadzu MPS-2000 spectrophotometer (Shimadzu, Kyoto, Japan).

Identification and Quantification of Pigments. Identification was based on chromatographic behavior on reversed-phase HPLC (RP-HPLC), visible absorption spectra and their reaction with ethanolic 0.1 M HCl. Acid catalyzes the specific isomerization of 5,6-epoxides to 5,8-furanoid oxides (= 5,8-epoxides), resulting in a hypsochromic shift of approximately 20 nm for monoepoxides and 40 nm for diepoxides (*14*, *20*, *21*). The various carotenoids and chlorophylls in the peas were quantified using an external calibration method for (*all-E*)-lutein, (*all-E*)-β-carotene, and chlorophyll *a* and *b*. (*all-E*)-β-Carotene was dissolved in $CHCl_3$ (1 mg in 3 mL). The other pigments were dissolved in $CHCl_3$ (1 mg in 100 μL) to a final volume of 2 mL with 80% acetone. Several standard dilutions in 80% acetone were made from these stock solutions and the concentrations of (*all-E*)-lutein, (*all-E*)-β-carotene, and chlorophyll *a* and *b* determined spectrophotometrically. (*all-E*)-Lutein was determined with an absorption maximum at 453 nm in dioxane (ϵ 152.000) and (*all-E*)-β-carotene with absorption maximum at 450 nm in $CHCl_3$ (ϵ 139.057) (*22*). The chlorophyll *a* and *b* concentrations were calculated by the method of Lichtenthaler (*23*). The concentration calculated from the absorbance reading was corrected for pigment purity determined by analytical HPLC. All xanthophylls were calculated relative to (*all-E*)-lutein; chlorophyll *a* derivative, chlorophyll *a*′, and pheophytin *a* were calculated relative to chlorophyll *a*; and chlorophyll *b* derivative, chlorophyll *b*′, and pheophytin *b* were calculated relative to chlorophyll *b*.

Statistics. For statistical analyses of variances the general linear models (GLM) procedure of Statistical Analysis System (SAS Institute, Cary, NC) was used. The results were analyzed by one- or two-way analysis of variance (ANOVA) including test for normal distribution and variance homogeneity. The sources of variances for the one-way analysis were extraction technique, processing condition, or cultivar. The sources of variances for the two-way analysis were cultivar, year, and cultivar*year. Duncan's multiple range test was used to assess the significant differences.

RESULTS AND DISCUSSION

The pigment constituents of green peas consisted of three classes of compounds. In order of chromatographic elution on a C_{18} reversed-phase column these were (a) xanthophylls (oxygenated carotenoids), (b) chlorophylls and their derivatives, and (c) hydrocarbon carotenoids ((*all-E*)-β-carotene). A total of 17 pigments were identified and quantified in raw and cooked green peas (Figure 1). Of these, 8 were xanthophylls ((*all-E*)-neoxanthin, (9′*Z*)-neoxanthin, (*all-E*)-violaxanthin, neochrome, (*all-E*)-lutein epoxide, (*all-E*)-lutein, neolutein B ((9*Z*)- or (9′*Z*)-lutein), and neolutein A ((13*Z*)- or (13′*Z*)-lutein)), 4 were chlorophyll *b* related compounds (chlorophyll *b* derivative, chlorophyll *b*, chlorophyll *b*′ (the C-10 epimeric isomer of chlorophyll *b*) and pheophytin *b*), 4 were chlorophyll *a* related compounds (chlorophyll *a* derivative, chlorophyll *a*, chlorophyll *a*′ (the C-10 epimeric isomer of chlorophyll *a*), and pheophytin *a*), and 1 was a carotene ((*all-E*)-β-carotene). In addition, three unidentified xanthophylls were quantified in green peas (Figure 1). Among the isolated pigments, only (*all-E*)-lutein, chlorophyll *a* and *b*, pheophytin *a* and *b*, and (*all-E*)-β-carotene have previously been reported in green peas (*5*, *24*).

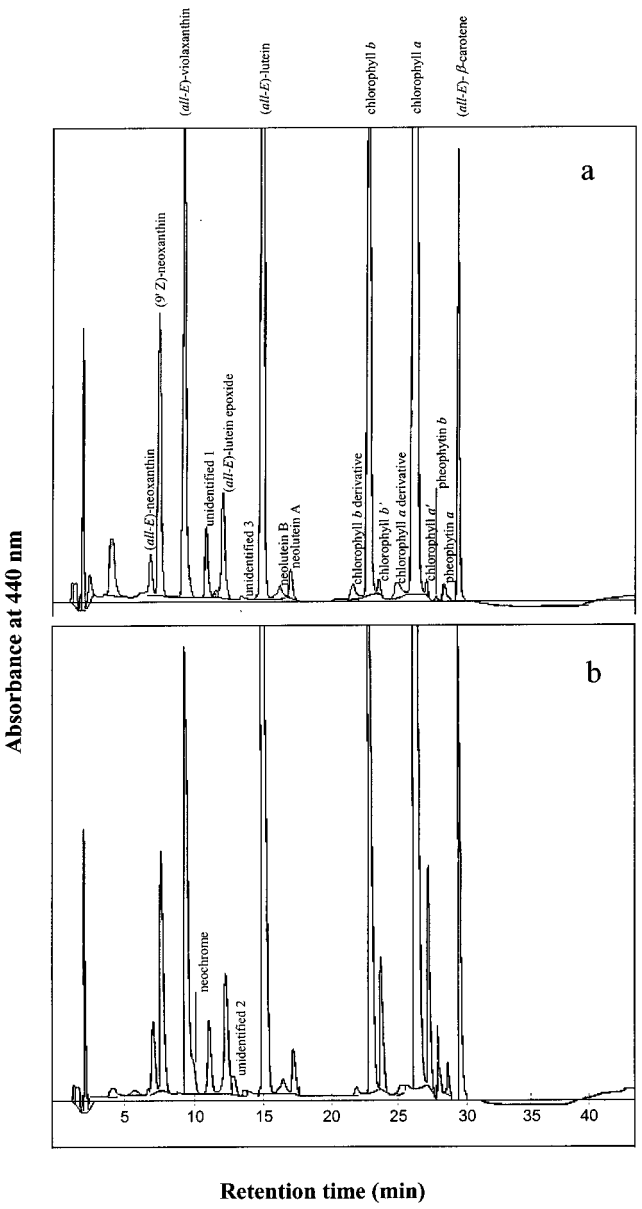

Figure 1. HPLC separation of carotenoids and chlorophylls from raw (a) and cooked (b) peas (cv. Bella).

Identification of Pigments. The pigments were identified by their chromatographic behavior on analytical RP-HPLC, absorption spectra (UV) and reaction with ethanolic 0.1 M HCl. All pigments are listed in Table 2 in order of chromatographic elution on analytical RP-HPLC.

Xanthophylls. (*all-E*)-Violaxanthin was identified from its UV−visible data and its reaction with acid. The UV

Table 2. Peak Identification of the Various Pigments in Processed Green Peas

chemical class	compounds	R_t, min	λ_{max}, nm (EtOH)[b]	hypsochromic shifts of absorption max[c]
xanthophylls	(*all-E*)-neoxanthin[a]	7.1	420 (80), 443 (100), 469 (84)	21 nm
	(9′*Z*)-neoxanthin[a]	7.8	415 (68), 438 (100), 466 (95)	16 nm
	(*all-E*)-violaxanthin[a]	9.62	418 (68), 443 (100), 471 (92)	40 nm
	neochrome	9.64	400 (70), 422 (100), 451 (94)	*d*
	unidentified 1	11.3	416 (78), 438 (100), 465 (86)	*d*
	(*all-E*)-lutein epoxide[a]	12.6	418 (70), 443 (100), 471 (98)	21 nm
	unidentified 2	13.1	406 (68), 428 (100), 453 (94)	*d*
	unidentified 3	13.9	*e*	*d*
	(*all-E*)-lutein	15.4	422 (73), 447 (100), 475 (90)	*f*
	neolutein B[g]	16.8	420 (73), 443 (100), 469 (87)	*d*
	neolutein A[h]	17.6	332 (54), 419 (74), 440 (100), 467 (85)	*d*
chlorophylls	chlorophyll *b* derivative	22.2	438sh (33), 463 (100)	*d*
	chlorophyll *b*	23.3	438sh (33), 463 (100)	28 nm
	chlorophyll *b*′[a,i]	24.1	438sh (32), 463 (100)	*d*
	chlorophyll *a* derivative	25.6	338 (43), 386 (64), 414 (86), 432 (100)	*d*
	chlorophyll *a*	26.7	338 (41), 386 (63), 414 (88), 432 (100)	22 nm
	chlorophyll *a*′[a,j]	27.6	338 (44), 386 (65), 414 (90), 432 (100)	*d*
	pheophytin *b*	28.3	414 (55), 436 (100)	*d*
	pheophytin *a*	29.0	328 (26), 410 (100), 505 (11), 535 (10)	*d*
carotenes	(*all-E*)-β-carotene	29.9	430sh (75), 455 (100), 479 (90)	*d*

[a] Tentatively identified. [b] Relative absorbance (in %) is given in parentheses. [c] A few drops of ethanolic 0.1 M HCl was added to a solution containing the purified xanthophyll/chlorophyll. [d] Hypsochromic shifts not investigated. [e] No pure UV spectrum could be obtained. [f] No hypsochromic shifts was observed. (*all-E*)-Lutein was partly converted into neolutein A and neolutein B by refluxing the compound in hexane under an atmosphere of N_2 for 5 h. [g] Neolutein B is a mono-*Z*-isomer of (*all-E*)-lutein and constitutes of (9*Z*)- or (9′*Z*)-lutein or a mixture of these. [h] Neolutein A is a mono-*Z*-isomer of (*all-E*)-lutein and constitutes of (13*Z*)- or (13′*Z*)-lutein or a mixture of these. [i] The C-10 epimeric isomer of chlorophyll *b*. [j] The C-10 epimeric isomer of chlorophyll *a*.

Table 3. Pigment Concentration (μg/100 g fresh weight) in Processed Peas (cv. Tristar) Using Different Extraction Techniques with 100% Acetone[a]

	no. of reextractions				CV[b] (%)	
	0	0	1	3	extraction	HPLC
holding time, min	30	60	10	0		
(*all-E*)-neoxanthin	73a	79a	72a	71a	15.3	1.4
(9′*Z*)-neoxanthin	210c	200c	240b	260a	7.0	1.4
(*all-E*)-violaxanthin[c]	210c	210c	230b	240a	6.2	1.5
unidentified 1	47b	47b	51a	53a	3.0	1.2
(*all-E*)-lutein epoxide	110b	110b	120a	130a	5.8	1.5
unidentified 2	29b	27b	32a	33a	11.9	2.5
unidentified 3	10a	10a	11a	9a	5.4	9.1
(*all-E*)-lutein	1480b	1490b	1650a	1670a	4.5	1.3
neolutein B	18ab	27a	13b	11b	60.3	10.5
neolutein A	64a	76a	67a	63a	32.1	11.6
total xanthophylls	2250b	2280b	2480a	2530a	5.2	1.3
(*all-E*)-β-carotene	490a	500a	440b	510a	5.5	5.0
total carotenoids	2740b	2780b	2920a	3040a	5.7	1.6
chlorophyll *b* derivative	4a	15a	12a	5a	8.7	15.2
chlorophyll *b*	2140b	2240b	2350a	2360a	5.0	1.7
chlorophyll *b*′	690b	720b	750b	760a	7.1	1.6
pheophytin *b*	130a	110b	140a	130a	16.7	6.6
total chlorophyll *b*	2960c	3090b	3250a	3250a	5.4	1.7
chlorophyll *a* derivative	78b	65c	91a	73bc	10.6	17.2
chlorophyll *a*	7030c	7320b	7570ab	7690a	5.9	1.7
chlorophyll *a*′	840c	910b	880b	950a	7.2	1.7
pheophytin *a*	90a	90a	100a	110a	13.1	3.9
total chlorophyll *a*	8030c	8380b	8640b	8820a	6.1	1.8
total chlorophylls	11000c	11500b	11900a	12000a	5.9	1.8
total pigments	13700c	14300b	14800a	15100a	5.7	1.7

[a] Mean of triplicate analysis per method. Numbers within a row followed by different letters are significantly different at $P = 0.05$ by Duncan's multiple range test. [b] Coefficient of variance (CV) between five extractions of peas using the standard method and between five HPLC-injections of one extract. Holding time and temperature between each injection was 1 h at 5 °C. [c] Approximately 20% of the content was neochrome.

spectrum of (*all-E*)-violaxanthin with λ_{max} at 443 nm (Table 2) was in accordance with literature values (*14*, *21*). Further evidence for the identity of (*all-E*)-violaxanthin was confirmed by treating this pigment with a few drops of 0.1 M ethanolic HCl resulting in the expected hypsochromic shift for diepoxides of 40 nm (Table 2). The UV−visible data of a xanthophyll with λ_{max} at 443 nm indicated the presence of the monoepoxide (*all-E*)-neoxanthin (*14*). This was confirmed by addition of a few drops of 0.1 M ethanolic HCl, which produced the characteristic epoxide−furanoxide rearrangement (*14*, *21*) to afford neochrome with the expected hypsochromic shift of 21 nm (λ_{max} at 422 nm) (Table 2). A further xanthophyll with λ_{max} at 438 nm with a retention time on analytical RP-HPLC close to that of (*all-E*)-neoxanthin (Figure 1 and Table 2) indicated the presence of natural neoxanthin ((9′*Z*)-neoxanthin) (*14*). This was confirmed upon reaction with

acid, which produced the expected hypsochromic shift of 16 nm to afford neochrome. Neochrome was only present in cooked peas, clearly indicating that this compound was an artifact (*14*, *21*). The major xanthophyll found in green peas was identified as (*all-E*)-lutein from its UV spectrum and retention time on analytical RP-HPLC (Table 2), which were identical with that of an authentic sample. In both raw and cooked pea extracts (*all-E*)-lutein was accompanied by two minor *Z*-isomers of lutein, which were identified as neolutein B ((9*Z*)- or (9′*Z*)-lutein) and neolutein A ((13*Z*)- or (13′*Z*)-lutein), respectively, from their UV spectra (*14*, *21*). After refluxing (*all-E*)-lutein in hexane under an atmosphere of nitrogen for 5 h, the mixture was found to consist of (*all-E*)-lutein and neolutein B and A, respectively (*14*). The HPLC retention times and visible absorption spectra of the produced *Z*-isomers of lutein were identical with those found in green pea extracts. A further xanthophyll that was closely related to (*all-E*)-lutein was detected in green pea extracts. The UV spectrum with λ_{max} at 443 nm indicated the presence of (*all-E*)-lutein epoxide, the precursor to lutein. By treating the compound with a few drops of ethanolic 0.1 M HCl it resulted in a hypsochromic shift of 21 nm in accordance with the compound being (*all-E*)-lutein epoxide (*14*, *21*). Finally, three further compounds were detected by analytical RP-HPLC in raw and cooked pea extracts (Figure 1) of which, however, only two were present in concentrations high enough to obtain pure visible absorptions spectra. The UV spectra of these compounds were similar to the characteristic UV spectra of xanthophylls, clearly indicating that these were xanthophylls (Table 2). β-Cryptoxanthin, a xanthophyll reported in small amounts in green peas (*24*), was not detected using our method, probably because minute amounts of this compound was masked by chlorophyll *a*. When we added β-cryptoxanthin to a processed pea extract, the retention times of these compounds were very close.

Chlorophylls and Their Derivatives. All chlorophylls and their derivatives were quantified at 440 nm but specific methods such as visible detection at 660 nm and fluorimetric detection were also used to confirm the identity of this group of compounds. The major chlorophylls found in green peas were identified as chlorophylls *a* and *b* from their UV spectra and HPLC retention times, which were identical with those of authentic samples. Chlorophylls *a* and *b* were both accompanied by minor quantities of their C-10 epimeric isomers, known as chlorophylls *a*′ and *b*′, respectively (*14*, *21*). The UV spectra of chlorophylls *a*′ and *b*′ were identical with those of chlorophylls *a* and *b*, respectively (Table 2). Pheophytins *a* and *b*, the most common derivatives of chlorophylls *a* and *b* (*21*, *25*), were also identified. The conversion of chlorophylls to pheophytins is readily effected as a result of heat or acid treatment (*14*, *21*). A few drops of ethanolic 0.1 M HCl to a solution of chlorophyll *a* and *b* gave pheophytin *a* and *b*, respectively. Furthermore, two chlorophyll derivatives with the same characteristics as chlorophyll *a* and *b*, respectively, were isolated (Table 2). The chlorophyll *a* derivative has previously been detected in kiwi cultivars (*21*) and recently these derivatives were also reported in extracts from rehydrated spinach (*26*). In spinach these pigments were identified as hydroxychlorophyll *a* and *b*, respectively. Gauthier-Jaques et al. (*26*) showed that hydroxychlorophyll *a* is formed by allomerization

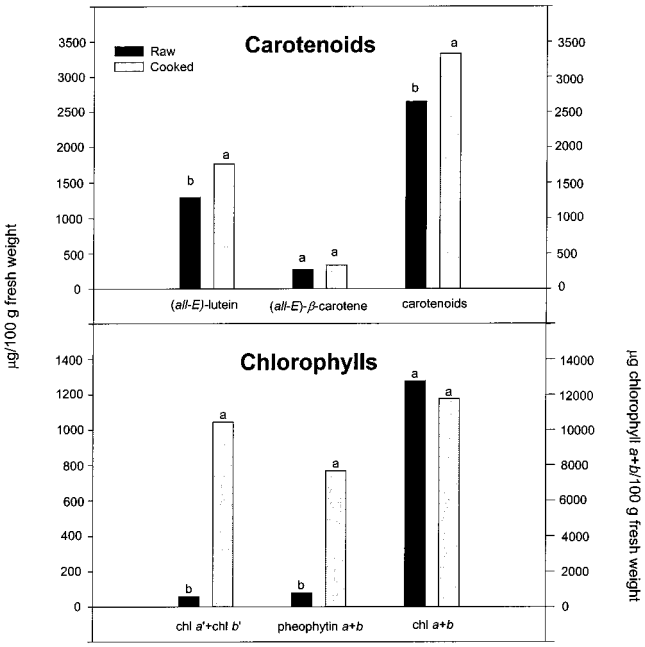

Figure 2. The concentration of carotenoids and chlorophylls in raw and cooked peas of cv. Bella. Mean of three extractions. Bars of pigments topped by different letters are significantly different at $P = 0.05$ by Duncan's multiple range test.

of chlorophyll *a* in a water-free oxygenated methanolic solution; however, these derivatives were also observed during boiling of leaves (*27*).

Hydrocarbon Carotenoids. (*all-E*)-β-Carotene was the only carotene detected in green peas. It was identified by comparing its UV spectrum and RP-HPLC retention time with that of an authentic sample. No α-carotene was detected in the pea extracts. The lack of detection of this compound in the pea extracts could be due to almost complete conversion to (*all-E*)-lutein (*14*, *21*).

Extraction of Chlorophyll and Carotenoid Pigments. The efficiency of different extraction procedures using 100% acetone showed that initial extraction followed by three reextractions without holding time between resulted in a significant higher chlorophyll and carotenoid concentration than no reextraction and 30 or 60 min holding time (Table 3). The reproducibility of the extraction procedure and the HPLC runs was in general good as indicated by a CV < 10% for most compounds (Table 3). Exceptions were the unstable neolutein A and B, which had CV > 30% between extractions. These high CV could be due to artifact formation and/or isomerization between the neoluteins and (*all-E*)-lutein during extraction and/or chromatography (*14*, *21*).

Pigment Stability during Heat Treatment. The (*all-E*)-lutein concentration increased significantly with cooking from 1300 µg/100 fresh weight to 1800 µg/100 fresh weight (Figure 2). The increase in the concentration of (*all-E*)-lutein in cooked peas as compared to raw was, however, not caused by loss of water during cooking. The water content was 78.0% in raw and 77.7% in cooked peas. Neochrome and an unidentified xanthophyll (number 2) were only present in cooked peas, where neochrome either formed a shoulder or separated from (*all-E*)-violaxanthin (Figure 1). The concentration of neochrome varied from 21 to 36 µg/100 g fresh weight in the processed pea samples. The concentration of (*all-E*)-β-carotene also increased with cooking; however, this difference was not significant (Figure 2). Others re-

Table 4. Concentration of Carotenoids and Chlorophylls in Processed Green Pea Cultivars[a]

compounds	cultivars, μg/100 g fresh weight					
	Avola	Tristar	Rampart	Turon	Bella	Greenshaft
(*all-E*)-neoxanthin	84a	87a	114a	99a	109a	98a
(9′*Z*)-neoxanthin	140c	180b	250a	200b	200b	190b
(*all-E*)-violaxanthin[b]	170a	220a	260a	240a	220a	220a
(*all-E*)-lutein epoxide	110a	130a	150a	150a	110a	120a
(*all-E*)-lutein	1200a	1500a	1900a	1600a	1400a	1400a
total xanthophylls[c]	1900c	2400b	2900a	2600ab	2300bc	2200bc
(*all-E*)-β-carotene	300c	460ab	490a	400b	420ab	430ab
total carotenoids	2200c	2800b	3400a	3000ab	2700bc	2700bc
chlorophyll *b*	2100c	2300b	2800a	2400b	2500b	2400b
chlorophyll *b*′	680a	780a	870a	820a	840a	800a
pheophytin *b*	620a	600a	740a	720a	710a	780a
chlorophyll *a*	4800c	6200b	7300a	6200b	6000b	5600bc
chlorophyll *a*′	780c	1040ab	1280a	1100ab	1060ab	1030b
pheophytin *a*	250bc	210c	300bc	300bc	410ab	470a
total chlorophylls	9300c	11000b	13400a	11700b	11600b	11100b
total pigments	11500c	14100b	16800a	14700b	14300b	13800b
vitamin A value (RAE[d]/100 g)	25c	38ab	41a	33b	35ab	36ab

[a] Data are mean of two years and four samples of each cultivar per year. Numbers within a row followed by different letters are significantly different at $P = 0.05$ by Duncan's multiple range test using cultivar*year as error in the statistical analysis. [b] Approximately 20% of the content was neochrome. [c] Sum of (*all-E*)-neoxanthin, (9′*Z*)-neoxanthin, (*all-E*)-violaxanthin, (*all-E*)-lutein epoxide, (*all-E*)-lutein, unidentified 1, 2 and 3, and neolutein A and B. [d] RAE, micrograms of retinol activity equivalent (0.083 μg (*all-E*)-β-carotene).

ported that the carotene concentration was higher in processed than in raw green peas (*28*). Although cooking procedures may result in loss of carotenoids in some vegetables, heat treatment increases the chemical extractability of carotenoids (*15*). Cooking had no significant effect on the total chlorophyll concentration but the chlorophyll *a* and *b* concentration decreased and the chlorophyll *a*′ and *b*′ and pheophytin *a* and *b* concentration increased, respectively (Figure 2). As chlorophyll *a*′ and *b*′ have the same absorption spectrum as natural chlorophylls, formation of these compounds will not affect visual pea color. In contrast, removal of magnesium from the green chlorophylls leading to pheophytin *a* and *b* (*29*) may affect pea color because pheophytins are brown (*26*) and have another absorption spectrum (Table 2). Measurements of the lightness value of several pea cultivars before and after cooking showed that raw peas had a higher lightness value (47) than cooked peas (40) indicating that the color became darker during cooking (unpublished data).

Quantitative Distribution of Xanthophylls, Chlorophylls, and Carotene. The quantitative distribution of xanthophylls, chlorophylls, and carotenes in the green pea cultivars is given in Table 4. The interaction between cultivar and year was significant for many of the pea pigments and therefore it was taken into account in the statistical analysis. The cultivars contained the same constituents, but the average concentration of each class varied significantly. The highest concentration of xanthophylls, chlorophylls, carotene, and total pigments in the cultivars that were harvested at the same average TV was found in the dark green cv. Rampart (lightness 41), intermediate in cv. Turon, and lowest in the bright green cv. Avola (lightness 45) (Tables 1 and 4). Cultivar Avola, which was characterized by normal pea leaves and a large seed size, was harvested approximately 10 days before cvs. Rampart and Turon, which were characterized by a small seed size and semileafless or normal leaves, respectively (Table 1). When the total pigment content was set to 100 in cv. Rampart it was 88 in cv. Turon and 68 in cv. Avola. In general, all cultivars were harvested at a higher TV level the second year with the exception of the cvs. Bella and Greenshaft (data not shown) and these differences in maturity had a significant effect on the pigment concentration. Table 5 shows the average concentration of the major pigments in the cultivars harvested at comparable maturities within a year (cv. Avola, cv. Rampart, and cv. Turon). A higher maturity level as indicated by a higher TV and AIS content resulted in a significant lower concentration of (*all-E*)-β-carotene, chlorophylls and total pigments (Table 5).

Table 5. Physicochemical Attributes and Pigment Concentration of Processed Peas Grown during Two Years. Average over cv. Avola, cv. Rampart, and cv. Turon[a]

parameters	year 1	year 2
tenderometer value	104b	126a
AIS (%)	13.3b	16.4a
(*all-E*)-lutein	1520b	1640a
(*all-E*)-β-carotene	410a	370b
total chlorophylls	11900a	11000b
total pigments	14700a	14000b

[a] Data are mean over three cultivars and four samples per cultivar. Numbers within a row followed by different letters are significantly different at $P = 0.05$ by Duncan's multiple range test.

There were similarities in the distribution percentage of the various pigments. Chlorophyll *a* accounted for 41−44% of the total pigment content, chlorophyll *b* for 17−18%, (*all-E*)-lutein for 10−11%, and (*all-E*)-β-carotene for 2.6−3.2%. The quantitative data on chlorophylls *a* and *b*, pheophytins *a* and *b*, and (*all-E*)-lutein and (*all-E*)-β-carotene were similar to those of Forni et al. (*5*), Hart and Scott (*18*), and Heinonen et al. (*24*). On average of the two years, the chlorophyll *a* concentration varied from 4800 to 7300 μg/100 g fresh weight, the chlorophyll *b* concentration from 2100 to 2800 μg/100 g fresh weight, the (*all-E*)-lutein concentration from 1200 to 1900 μg/100 g fresh weight, and the (*all-E*)-β-carotene concentration from 300 to 490 μg/100 g fresh weight in the processed pea cultivars. The vitamin A value, which was calculated from (*all-E*)-β-carotene, varied from 25 retinol activity equivalents (RAE)/100 g fresh weight in cv. Avola to 41 RAE/100 g in cv. Rampart (Table 4). Our results indicated that there was a consistent difference between the cultivars in the concentration of major pea pigments when the maturity level was taken into account. This result may be used in future breeding of new pea cultivars for deep freezing with a darker green color and a higher content of

constituents with nutritional and protective properties for human health.

ACKNOWLEDGMENT

We gratefully acknowledge the excellent technical assistance of Lisbeth Stage and Birgitte Foged.

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Received for review May 2, 2001. Revised manuscript received July 19, 2001. Accepted July 30, 2001. This research was part of a FØTEK-project Strategic Green Peas (no. 1992-503/09904-161) supported by the Danish Directorate for Agriculture and Development, Danisco Foods, and Danisco Seed.

JF010569Z