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Carotenoid composition of kale as influenced by maturity, season and minimal processing

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Abstract: The principal carotenoids of kale were identified by chemical reactions, high-performance liquid chromatography/mass spectrometry and high-performance liquid chromatography/photodiode array detection and were quantified by the last technique. In kale taken from conventional farms, the β-carotene and lutein contents were significantly higher in the mature leaves, violaxanthin was at an unusually high level in the young leaves, and neoxanthin had practically the same concentration at both stages of maturity. In samples taken from an organic farm, the carotenoid contents were essentially the same in the young and the mature leaves. Except for β-carotene, which did not differ with season, the carotenoid concentrations of marketed minimally processed kale were found to be significantly higher in the summer than in the winter, reflecting seasonal rather than processing effects. In minimally processed kale monitored during 5 days of storage at 7–9 °C, β-carotene, lutein, violaxanthin and neoxanthin were reduced by 14, 27, 20 and 31% respectively. Thus minimal processing, seasonal and maturity factors were found to have an influence on the carotenoid content of kale.

Keywords: carotenoids; kale; minimal processing; maturity; seasonal effects

INTRODUCTION

Aside from their importance as natural pigments and their well-known role in human health as vitamin A precursors, carotenoids are among the phytochemicals most cited as responsible for a lowered risk of developing degenerative diseases such as cancer, cardiovascular diseases and macular degeneration.[1–3] Thus fruits and vegetables are widely recommended as healthy foods.

Although the colour of the carotenoids is masked by chlorophyll, leafy vegetables are good sources of β-carotene, the most important provitamin A, and of lutein, which, together with zeaxanthin, is considered responsible for a reduced risk of cataract and macular degeneration.[4,5] Consumption of spinach and other green leafy vegetables has been associated with lower incidence of cataract[6–11] and macular degeneration,[12] with kale and collard being specifically cited in two studies.[9,12]

There is growing recognition that optimisation of the nutrient/phytochemical contents and profiles of foods, through conventional plant breeding and agronomic practices or genetic manipulation, is a viable strategy.[13–16] This has to be complemented by postharvest handling, processing and storage procedures that avoid loss of the desired food components.[17] In order to put this strategy into practice, the variation of the phytochemical content throughout the food chain has to be known.

Minimal processing is a current trend for marketing fruits and vegetables, stimulated by increasing consumer demand for high-quality, nutritive, fresh-like and convenient-to-use products. Usually the fruits and vegetables are washed, trimmed, peeled, sliced or shredded and packaged before being sold to restaurants, hotels and retail markets or groceries. Since thermal and other drastic processing conditions are not used, minimally processed products are expected to retain fresh or fresh-like properties and have good nutritive quality. However, tissue disruption by cutting or shredding allows substrate–enzyme interactions and makes these products more prone to rapid physiological and biochemical changes than intact raw commodities. In addition, exposure of plant components, including nutrients and phytochemicals, to oxygen also enhances oxidative degradation. Carotenoid contents, for example, have been shown to decline in mini-peeled carrots[18] and jalapeno pepper rings.[19] There is a paucity of information and more research is urgently needed to gain an insight on the chemical and biochemical alterations that occur in minimally processed foods.

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In a previous paper, cultivar differences, seasonal variation and the effect of farming technique on the carotenoid composition of kale have been studied.[20] The present work was carried out to verify changes in the carotenoid content of kale due to maturity at harvest, season and minimal processing.

MATERIALS AND METHODS

Sampling

Kale (*Brassica oleracea*) of the common cultivar 'Manteiga' was used throughout the present work. To study the effect of maturity, the young and the mature leaves from the same bunch of kale (about 290 g) were separated. Although smaller, the young leaves of kale (averages of 12 cm wide and 14 cm long) had the same green colour as the mature leaves (averages of 26 cm wide and 29 cm long). Young and mature leaves collected directly from an organic farm were also analysed for comparison with those taken from conventional farms in the same vicinity. The leaves from the organic farm were smaller (averages of 4 cm wide and 8 cm long for the young leaves and 14 cm wide and 16 cm long for the mature leaves) than those from the conventional farms.

Marketed minimally processed samples of shredded kale (average of 3 mm strips) were purchased from two supermarkets in Campinas, São Paulo, Brazil. The vegetable was packed in polyethylene bags in 200 g units and sold within 5 days after preparation. Five sample lots for each season (summer and winter) were collected at different times during the season and analysed individually. These samples, as well as those of the maturity study, were analysed immediately after collection.

According to the food firms, minimal processing consisted of washing, trimming, cutting, washing with chlorinated water (100 ppm chlorine) and water, draining (centrifugation) and packaging.

To directly evaluate the degradation of carotenoids during the shelf-life of the minimally processed vegetable, kale was processed by a small industry in Campinas and brought to our laboratory immediately. Three packages were sampled and analysed individually on arrival in the laboratory and after 1, 2, 3 and 5 days of storage in the refrigerator (7–9 °C), simulating storage conditions in the groceries.

Preparation of carotenoid standards

Standards of β-carotene, lutein, violaxanthin and neoxanthin were isolated by open column chromatography according to Kimura and Rodriguez-Amaya.[21] Briefly, this involved extraction of the carotenoids of a leafy vegetable with cold acetone, partition to 10% ethyl ether in petroleum ether (PE), concentration in a rotary evaporator and separation of the carotenoids on an MgO:Hyflosupercel column (1:1, activated). Average purity of the isolated carotenoids as verified by high-performance liquid chromatography (HPLC) was 92, 96, 98 and 94% for β-carotene, lutein, violaxanthin and neoxanthin respectively. The concentrations of the standard solutions were corrected accordingly.

Carotenoid analysis

All the necessary precautions were taken to avoid alterations or losses of the carotenoids and other errors during analysis, so that analytical errors could not be mistaken for variations brought about by the factors being investigated.[22]

The contents of each package for minimally processed kale or the leaves of each bunch of fresh kale were finely cut or ground in a multipurpose household Walita food processor, mixed and 3–5 g samples were taken in duplicate. Carotenoid analysis was carried out according to Kimura and Rodriguez-Amaya.[21] Briefly, it involved extraction of the carotenoids with cold acetone using a mortar and pestle, which was found to be more efficient in the disintegration of small amounts of leaf samples than a Waring blender, partition to 10% ethyl ether (peroxide-free) in PE, concentration in a rotary evaporator, evaporation of the solvent to dryness with nitrogen and, immediately before injection, dissolution in acetone and filtration through a syringe filter (0.22 μm). The samples were not saponified to avoid losses, especially of the more polar carotenoids (lutein, violaxanthin and neoxanthin).

The carotenoids were identified by the combined use of the retention times, co-chromatography with authentic samples, the visible absorption spectra obtained spectrophotometrically and by the photodiode detector and chemical tests for the xanthophylls.[22] The % III/II (ratio of the peak height of the longest-wavelength absorption band and that of the middle absorption band, the minimum between the two peaks being taken as the baseline) was calculated to express the spectral fine structure.[23] Chemical tests such as acetylation with acetic anhydride of secondary hydroxyl groups, methylation with acidic methanol of allylic secondary hydroxyl groups and epoxide–furanoid rearrangement of 5,6-epoxides were undertaken with carotenoids isolated through an MgO:Hyflosupercel column as with the standards. Thin layer chromatography (TLC) was carried out on silica gel plates developed with 5% methanol in toluene. Confirmation of the identity of the carotenoids was also done by means of the mass spectra. Quantification was carried out by external standardisation.

The HPLC analysis was performed on a Waters (Milford, USA) separation module (model 2690), equipped with an autosampler and a UV-vis photodiode array detector (Waters model 996), controlled by a Millenium workstation (version 2010). Detection for quantification was at the wavelengths of maximum absorption (max plot), ie 441 nm for violaxanthin, 448 nm for lutein, 454 nm for β-carotene and 439 nm for neoxanthin. The column was a monomeric C_{18} Spherisorb ODS2, 3 μm, 4.6 mm × 150 mm. The mobile phase consisted of acetonitrile (containing

0.05% triethylamine), methanol and ethyl acetate, used at a flow rate of 0.5 ml min^{-1}. A concave gradient (curve 10) was applied from 95:5:0 to 60:20:20 in 20 min, maintaining this proportion until the end of the run. Re-equilibration took 15 min.

The electron impact mass spectra were taken with a Waters Integrity System equipped with a Thermabeam HPLC/MS interface, the expansion region and nebuliser temperatures being 80 and 90 °C respectively. The ionising voltage was 70 eV and the temperature of the ion source was 210 °C. The *m/z* range was 150–650.

Statistical analysis

The data obtained in relation to minimally processed kale stored for 5 days and the effect of stage of maturity were examined statistically by analysis of variance ($p \leq 0.05$), the means being compared by Tukey's test.

RESULTS AND DISCUSSION

Confirmation of identity of carotenoids

The colour and biological functions of carotenoids are intimately related to their structures. Thus the importance of conclusive identification of the carotenoids present in a food cannot be overemphasised. Because inconclusive or even incorrect identification can be noted in the literature, the following criteria were recommended as minimum requirements for identification:[24,25] (1) the visible (or ultraviolet for shorter chromophores) absorption spectrum (λ_{max} and fine structure) in at least two different solvents must be in agreement with the chromophore suggested; (2) chromatographic properties must be identical in two systems, preferably TLC (R_F) and HPLC (t_R), and co-chromatography with an authentic sample should be demonstrated; and (3) a mass spectrum should be obtained, which allows for at least the confirmation of the molecular mass. The present paper fulfilled these criteria. In addition, chemical reactions showing the type and position of functional groups were also carried out.

Unlike fruits in which the carotenoid composition varies qualitatively and quantitatively, leafy vegetables are known to have a constant qualitative pattern, with lutein, β-carotene, violaxanthin and neoxanthin being the principal carotenoids. This was confirmed in the present work on kale as shown in Fig 1 and Table 1. Identification of the carotenoids by their

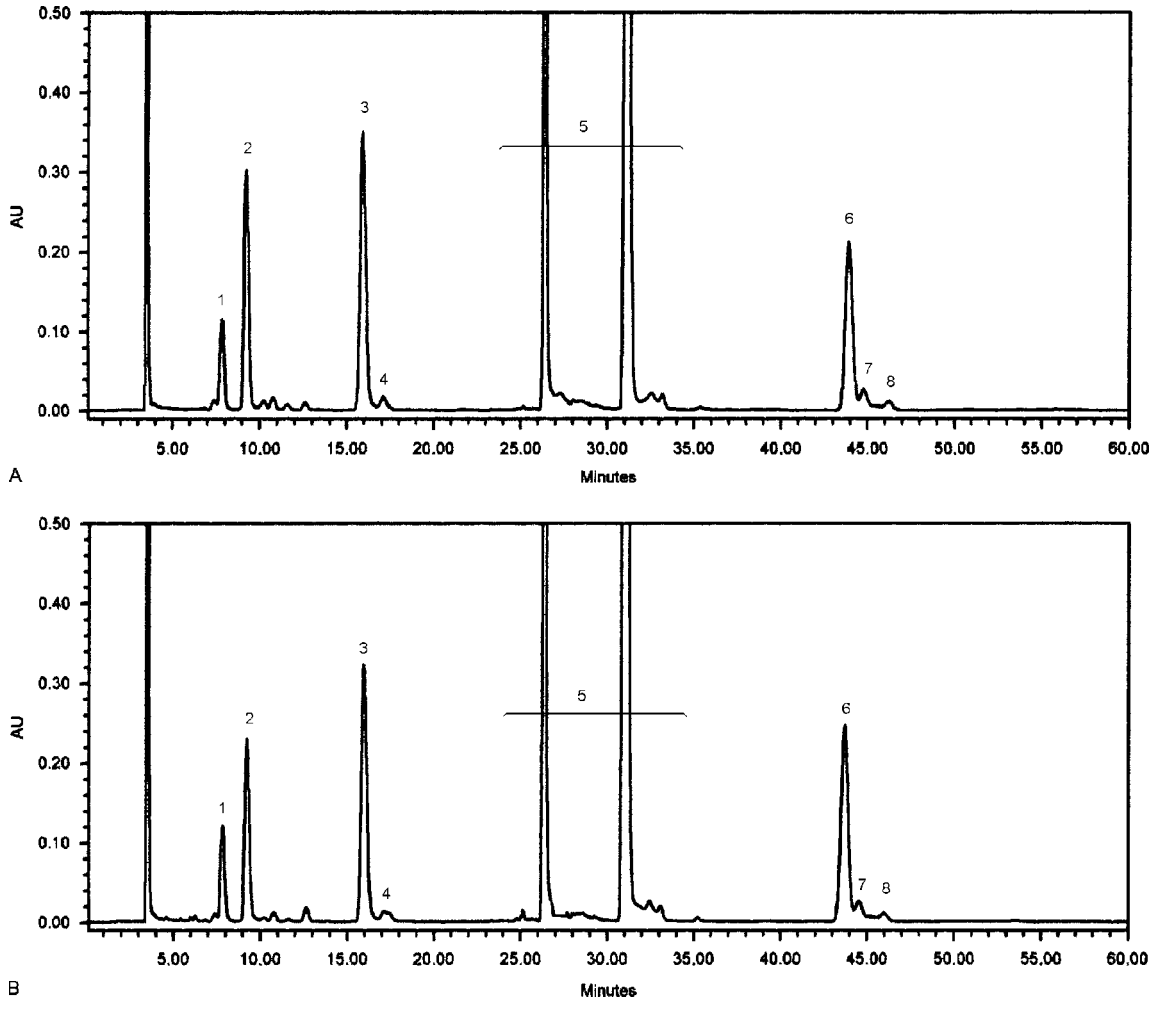

Figure 1. HPLC chromatograms of the carotenoids of (A) young and (B) mature kale leaves taken from a conventional farm. Peak identification: 1, neoxanthin; 2, violaxanthin; 3, lutein; 4, zeaxanthin; 5, chlorophylls; 6, *trans*-β-carotene; 7, 9-*cis*-β-carotene; 8, 13-*cis*-β-carotene. HPLC conditions are described in the text.

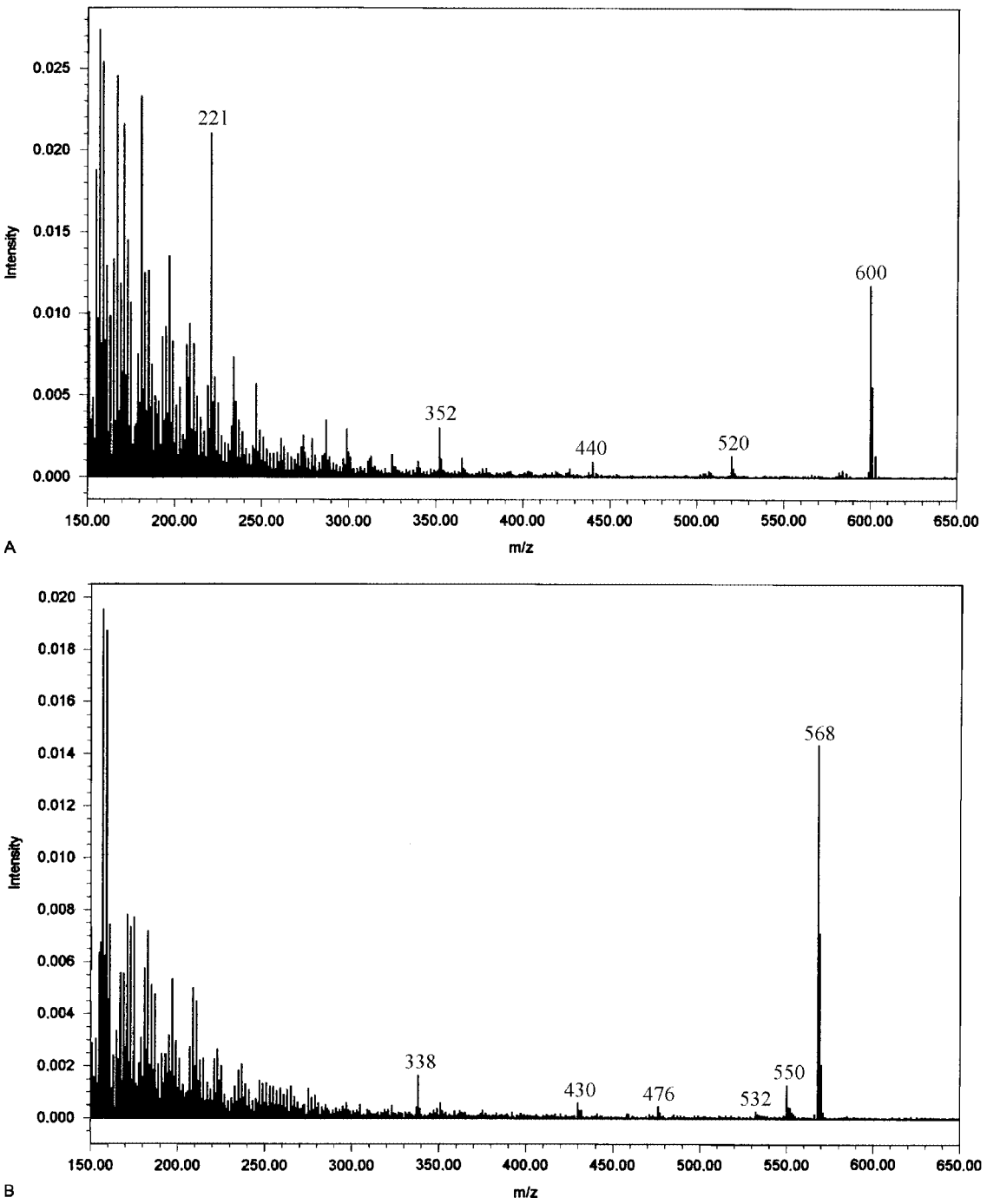

Figure 2. Mass spectra of (A) violaxanthin and (B) lutein obtained on-line. MS conditions are described in the text.

chromatographic behaviour, visible absorption spectra and chemical reactions agreed with the electron impact mass spectra. As examples, the mass spectra taken on-line of lutein and violaxanthin are presented in Fig 2, showing the prominent peaks of the molecular ions and some of the characteristic mass fragments.[26] The identifying characteristics of each carotenoid are described in detail as follows.

Neoxanthin (5′,6′-epoxy-6,7-didehydro-5,6,5′,6′-tetrahydro-β,β-carotene-3,5,3′-triol) displayed a visible absorption spectrum (λ_{max} in PE = 414, 438, 466 nm; λ_{max} in the mobile phase = 415, 439, 467 nm) with defined spectral fine structure (% III/II = 88), reflecting a chromophore of eight conjugated double bonds and an allenic group in the polyene chain. The presence of three hydroxyl groups, indicated initially by the chromatographic behaviour (t_R = 7.8 min, R_F = 0.07), was confirmed by the positive response to acetylation. A hypsochromic shift of 20 nm on addition of dilute HCl manifested the rearrangement of a 5,6-epoxide to a 5,8-epoxide. The mass spectrum showed the molecular ion at *m/z* 600, corresponding to $C_{40}H_{56}O_4$, and mass fragments at *m/z* 221, 181 and 172, due to the elimination of a β-ring with epoxy and hydroxy groups.

Violaxanthin (5,6,5′,6′-diepoxy-5,6,5′,6′-tetrahydro-β,β-carotene-3,3′-diol) had a spectrum (λ_{max} in PE = 416, 440, 468 nm; λ_{max} in the mobile

Table 1. Identifying characteristics of the principal carotenoids of kale

Carotenoid	λ_{max} (nm) in petroleum ether[a]	λ_{max} (nm) in the mobile phase[a]	Response to chemical tests	Molecular ion (*m/z*)
Neoxanthin	414, 438, 466 % III/II = 88	415, 439, 467	Positive to acetylation (3 OH groups); positive to 5,6-epoxide test (1 group);	600
Violaxanthin	416, 440, 468 % III/II = 98	417, 441, 470	Positive to acetylation (2 OH groups); positive to 5,6-epoxide test (2 groups).	600
Lutein	421, 443, 472 % III/II = 60	423, 447, 475	Positive to acetylation (2 OH groups); positive to methylation (1 allylic OH)	568
β-Carotene	(424), 448, 476 % III/II = 25	(428), 454, 480	No substituent	536
Zeaxanthin	(424), 448, 476 % III/II = 25	(428), 454, 480	Positive to acetylation (2 OH groups); negative to methylation	
9-*cis*-β-Carotene		346, 449, 477	No substituent	
13-*cis*-β-Carotene		341, 448, 476	No substituent	

[a] Parentheses indicate a shoulder.

phase = 417, 441, 470 nm) with well-defined fine structure (% III/II = 98), in agreement with a chromophore consisting of nine conjugated double bonds in the polyene chain. The chromatographic behaviour (t_R = 9.2 min, R_F = 0.12) and positive acetylation demonstrated the presence of two hydroxyl substituents. Epoxide–furanoxide rearrangement resulted in a hypsochromic shift of 40 nm, showing the presence of two epoxides at the 5,6- and 5′,6′-positions. The mass spectrum presented the molecular ion at *m/z* 600, consistent with $C_{40}H_{56}O_4$, and fragments at *m/z* 520 $[M - 80]^{\bullet+}$ and 440 $[M - 80 - 80]^{\bullet+}$ due to the elimination of one and two epoxide groups respectively. Other fragments at *m/z* 352 and 221 indicated that the epoxy substituents were in rings with a hydroxy group.

Lutein (β,ε-carotene-3,3′-diol) exhibited a visible spectrum (λ_{max} in PE = 421, 443, 472 nm; λ_{max} in the mobile phase = 423, 447, 475 nm) with less-defined fine structure (% III/II = 60), consistent with a carotenoid of 10 conjugated double bonds, nine in the polyene chain and one in a β-ring. The presence of two hydroxyl substituents was shown by the chromatographic behaviour (t_R = 15.9 min, R_F = 0.21) and the positive reaction to acetylation, the allylic position of one of them being shown by the positive response to methylation, producing a monohydroxylated carotenoid. The molecular ion as shown in the mass spectrum was at *m/z* 568, corresponding to $C_{40}H_{56}O_2$, and mass fragments appeared at *m/z* 550 $[M - 18]^{\bullet+}$ and 532 $[M - 18 - 18]^{\bullet+}$, due to the loss of one and two molecules of water respectively. Also encountered were fragments at *m/z* 476, representing the loss of toluene from the polyene chain, *m/z* 430 $[M - 138]^{\bullet+}$ and *m/z* 328 $[M - \text{toluene} - 138]^{\bullet+}$, in which 138 corresponded to the elimination of either the ε- or β-end group of lutein.

β-Carotene (β,β-carotene) had the typical spectrum (λ_{max} in PE = 448, 476 nm and a shoulder at 424 nm; λ_{max} in the mobile phase = 454, 480 nm and a shoulder at 428 nm) of a carotenoid with 11 conjugated double bonds, two of which were located in β-rings, thus lacking spectral fine structure (% III/II = 25). The absence of functional groups was manifested by the chromatographic behaviour (t_R = 43.8 min, R_F = 0.99) and confirmed by the mass spectrum with the molecular ion at *m/z* 536, corresponding to $C_{40}H_{56}$. Mass fragments at *m/z* 444 $[M - 92]^{\bullet+}$, 430 $[M - 106]^{\bullet+}$ and 399 $[M - 137]^{\bullet+}$ indicated the elimination of toluene and xylene from the polyene chain and the loss of a β-ring respectively.

Zeaxanthin (β,β-carotene-3,3′-diol), having the same chromophore as β-carotene, had a spectrum with the same characteristics as that of β-carotene. The chromatographic behaviour (t_R = 17.2 min, R_F = 0.19) and positive acetylation confirmed the presence of two hydroxyl groups, in non-allylic positions as demonstrated by the negative reaction to methylation.

9-*cis*-β-Carotene and 13-*cis*-β-carotene were detected in the HPLC chromatogram and identified by λ_{max}s slightly lower than those of *trans*-β-carotene (449, 477 nm and 448, 476 nm respectively in the mobile phase) and a weak *cis* peak at 346 nm for the former and a more intense *cis* peak at 341 nm for the latter.

Zeaxanthin and the *cis* isomers of β-carotene were present at very low concentrations, so they were not quantified and their on-line spectra were not be obtained.

Effect of maturity

Maturation in vegetables or ripening in fruits is usually accompanied by enhanced carotenogenesis.[27–29] In this study, β-carotene and lutein of the kale samples from conventional farms had significantly higher levels in the mature leaves compared with the young leaves. Violaxanthin had an unusually high concentration in the young leaves. Neoxanthin had practically the same concentration in the young and mature leaves. The carotenoid concentrations in the young and mature leaves of kale taken from the organic farm were generally similar.

The high levels of violaxanthin (Table 2) can be related to the participation of violaxanthin in the xanthophyll cycle, which is believed to have a role in

Table 2. Concentrations of the principal carotenoids of kale as affected by stage of maturity

	Concentration ($\mu g\,g^{-1}$)			
Vegetable/maturity	β-Carotene	Lutein	Violaxanthin	Neoxanthin
Lot 1				
Young leaves	30.7b	44.0b	39.8a	12.0b
Mature leaves	39.4a	49.6a	30.5b	15.0a
Lot 2				
Young leaves	37.3b	44.4b	42.2a	12.8a
Mature leaves	42.2a	48.2a	32.5b	12.6a
Lot 3				
Young leaves	37.2b	48.7b	39.4a	15.1a
Mature leaves	41.9a	51.9a	27.4b	17.1a
Lot 4				
Young leaves	36.3b	57.4a	29.2a	17.1a
Mature leaves	42.4a	56.7a	29.4a	18.3a
Lot 5				
Young leaves	41.9a	54.0a	30.5a	23.5a
Mature leaves	40.1a	55.2a	30.7a	25.9a

Values are means of duplicate analyses. Different letters within a column for each lot indicate significant difference ($p \leq 0.05$). Lots 1–3 came from conventional farms and lots 4 and 5 from an organic farm.

photoprotection in plants.[30,31] This cycle involves the de-epoxidation of violaxanthin to zeaxanthin under excess light and the epoxidation of zeaxanthin to violaxanthin under limiting light. The considerable amount of violaxanthin was determined in kale cultivated in winter, when the light period is substantially shorter.

Carotenoid content of marketed minimally processed leafy vegetables

Table 3 shows the concentrations of the principal carotenoids of marketed minimally processed kale in the summer and in the winter. There was no significant difference in the carotenoid concentrations of samples taken from two supermarkets, thus the results are presented jointly.

All four principal carotenoids had higher concentrations in the summer than in the winter. Two processes occur in photosynthetic tissues with opposing effects on carotenoid levels: biosynthesis and photodegradation. Exposure to sunlight and high temperature enhances biosynthesis, increasing the carotenoid concentrations, but also promotes photodegradation, which lowers the carotenoid contents. In leafy vegetables grown in open fields, the carotenoid levels have been found to be lower in the summer,[32,33] indicating that the effect of sunlight and high temperature on photodegradation prevailed. In the present work the vegetables intended for minimal processing were cultivated in plots protected with polyethylene roofs. It is possible that the plants were protected from excessive sunlight during the summer, thus favouring carotenogenesis instead of photodegradation. On the other hand, the polyethylene protection might have restricted exposure to sunlight in winter, thus carotenoid production was not enhanced. In a previous paper, hydroponic curly lettuce, which also had a polyethylene covering, had lower lutein, β-carotene, violaxanthin and neoxanthin contents than the same variety of lettuce, at the same maturity, taken from a neighbouring open field, both samples being collected at the same time in winter.[34] Tomatoes produced in winter in greenhouses had only one-third of the total carotenoid content of outdoor produce.[35]

In spite of the difference observed between the two seasons, the values obtained for β-carotene in the minimally processed vegetables fall within the ranges encountered in kale ($35 \pm 13\,\mu g\,g^{-1}$) analysed at different times during the year.[32] Thus the effect of minimal processing did not appear appreciable or might have been masked by other factors. Consumers of both fresh and minimally processed leafy vegetables are therefore likely to get the same nutritional or health benefits in terms of β-carotene. Lutein was underestimated in the previous paper, and violaxanthin and neoxanthin were not quantified, thus comparison could not be made with these other carotenoids.

Table 3. Concentrations of the principal carotenoids of marketed minimally processed kale

	Concentration ($\mu g\,g^{-1}$)			
Season	β-Carotene	Lutein	Violaxanthin	Neoxanthin
Summer	34.2 ± 5.7a	52.4 ± 3.6a	26.7 ± 2.0a	20.1 ± 2.8a
Winter	33.3 ± 1.8a	44.4 ± 2.1b	16.7 ± 2.9b	8.8 ± 2.3b

Values are means and standard deviations of five different sample lots collected at different times during the season. Different letters within a column indicate significant difference ($p \leq 0.05$).

Carotenoid concentration changes during storage of minimally processed kale

To evaluate the effect of minimal processing directly, the carotenoid levels of minimally processed kale stored for 5 days at 7–9 °C were determined (Table 4). All four carotenoids decreased significantly; lutein (27%), violaxanthin (20%) and neoxanthin (31%) had greater losses than β-carotene (14%). Reduction of carotenoids was greater in the first or second day of storage, most probably owing to the expected enzymatic reactions.

Table 4. Concentrations of the principal carotenoids of kale as affected by minimal processing and storage

Time after	Concentration ($\mu g\,g^{-1}$)			
processing (days)	β-Carotene	Lutein	Violaxanthin	Neoxanthin
0	28.7a	44.8a	20.5a	13.2a
1	24.8b	37.1b	16.7b	9.5b
2	22.8b	35.1b,c	16.1b	8.9b
3	24.0b	33.4c	16.1b	9.2b
5	24.7b	32.9c	16.1b	8.8b

Values are means of three packages analysed individually. Different letters within a column indicate significant difference ($p \leq 0.05$).

The losses observed in this study occurred under unoptimised packaging and storage conditions. Retention of the carotenoids could be improved, for example, by using modified atmosphere packaging and a lower storage temperature.

In unpacked fresh broccoli, pillow-packaged stalk-bisected broccoli, unpacked fresh green pepper and pillow-, partial vacuum- and total vacuum-packaged sliced green pepper, no significant loss of β-carotene has been observed during 10 days of storage at 4 °C.[36] However, the standard deviations ($n = 12$) were high, ranging from 3600 to 4730 $\mu g\,g^{-1}$ for means of 8420–9500 $\mu g\,g^{-1}$ in broccoli. For green pepper the standard deviations varied from 500 to 680 $\mu g\,g^{-1}$, the mean range being 1850–2120 $\mu g\,g^{-1}$. Thus the effects of processing and storage could have been masked by the variability of the samples.

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