Effect of Variety, Processing, and Storage on the Flavonoid Glycoside Content and Composition of Lettuce and Endive

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Eight varieties of lettuce (*Lactuca sativum*) and three varieties of endive (*Cichorium endivia*) were analyzed for flavonoid composition and content. Total flavonoid contents, expressed as units of aglycon for fresh material, were in the ranges of 0.3−229 μg/g for lettuce and 44−248 μg/g for endive. Five quercetin conjugates [quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-glucuronide, quercetin 3-*O*-(6-*O*-malonyl)glucoside, and quercetin 3-*O*-rhamnoside] and luteolin 7-*O*-glucuronide were measured in the green-leafed lettuce and an additional two cyanidin conjugates [cyanidin 3-*O*-glucoside and cyanidin 3-*O*-[(6-*O*-malonyl)glucoside]] in the red-leafed varieties. Three kaempferol conjugates [kaempferol 3-*O*-glucoside, kaempferol 3-*O*-glucuronide, and kaempferol 3-*O*-[6-*O*-malonyl)glucoside]] were measured in each of the endive varieties. The presence and identity of kaempferol 3-*O*-(6-*O*-malonyl)glucoside in endive was shown for the first time. Shredding of lettuce leaf followed by exposure to light produced significant losses of the flavonoid moiety in the green oak leaf (94%), red oak leaf (43%), iceberg (36%), green batavia (25%), lollo biondo (24%), and lollo rosso (6%) samples, whereas cos and green salad bowl samples did not show an overall loss. Shredding of endive also produced loss of the flavonoid moiety in escarole (32%), fine frisee (13%), and coarse frisee (8%). Significant demalonation was observed for both the quercetin and cyanidin glucosides in lettuce, whereas a similar degradation of the kaempferol analogue was found in endive tissue. Storage of whole heads of both lettuce and endive in the dark at 1 °C and 98% humidity for 7 days resulted in losses of total flavonol glycosides in the range of 7−46%. The identification of the amounts, position of substitution, and nature of the sugars is important for understanding the potential bioavailability and biological activities of flavonoids in salads.

Keywords: *Lettuce; endive; HPLC; flavonoid; flavonoid conjugates*

INTRODUCTION

A diet containing a high proportion of fruit and vegetables has been advocated as one of the best ways to reduce the incidence of chronic disease in the Western world (Block, 1992). The protective effect, which this type of diet confers, is believed to be due to the antioxidant and other activities of the flavonoids, a class of compounds found in significant levels in many fruits and vegetables (Hertog et al., 1993). The antioxidant activity of some of the subclasses of the flavonoids, such as the flavonols and anthocyanins, has been reported to be greater than that of either vitamin C or E (Rice-Evans et al., 1995).

Although tea, onion, broccoli, apple, and green bean supply the majority of the flavonols in the U.K. diet (Hertog et al., 1993), salad foods such as lettuce and endive are consumed in increasing amounts due to their perception as being “healthier” foods. It is important to further understand the mode of action of flavonols as biologically active components of the human diet. For this reason, both their composition and content in foods have been the subject of much research (Hertog et al., 1993; Price and Rhodes, 1997; Price et al., 1997, 1998a; Crozier et al., 1997). The flavonols in all of these foods are based on just five aglycons, namely, myricetin, quercetin, kaempferol, luteolin, and cyanidin, although they are present only in the edible portions of the food plants in the form of a multiplicity of conjugates. Recent work demonstrates the importance of the chemical nature of these conjugates to their bioactivity and bioavailability. These findings suggest the degree of hydroxylation is important in determining the antioxidant activity (Plumb et al., 1997) and ability to induce phase II enzymes such as quinone reductase (Uda et al., 1997), whereas the type and degree of glycosylation may be important in determining the ability of these compounds to cross the intestinal wall (Gee et al., 1998) and to be absorbed in humans (Hollman et al., 1999).

This work reports on both the composition and content of the flavonoid conjugates, which are based on the four aglycons (quercetin, kaempferol, luteolin, and cyanidin) found in eight commercial varieties of lettuce and three varieties of endive grown in the United Kingdom. Confirmation of the chemical structure of a kaempferol conjugate, previously suggested by Woldecke and Herrmann (1974), has been made. The effects of shredding and exposure to light and also to storage, which are similar to the commercial treatment of salads, are reported.

MATERIALS AND METHODS

Lettuce (*Lactuca sativum*) varieties lollo biondo cv. Ciereo, lollo rosso cv. Malibu, green salad bowl cv. Hrizet, iceberg cv. Saladin, cos cv. Remus, green batavia cv. Vanity, green oak leaf, and red oak leaf and endive (*Cichorium endivia*) varieties fine frisee cv. Glory, coarse frisee, and escarole were grown in Kent, U.K., and collected as a normal commercial harvest on September 20, 1998. Five whole heads of each variety were

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taken on the day of harvest, divided, and weighed. Half was immediately immersed in liquid nitrogen prior to freeze-drying, and the other half was processed by hand-shredding with a knife, sealing in a polythene bag, and exposing to fluorescent light (370 lx, on bench) at 22 °C for 48 h before immersion in liquid nitrogen and subsequent freeze-drying. Two heads of each of the varieties of lettuce and endive were stored in an icebank cooler (1 °C, relative humidity of 98%) for 7 days, and no visual deterioration of any of the samples was noted. Each head was cut into halves and frozen immediately or processed (shredding and exposure light) as in the case of the fresh samples. Each sample was ground to a fine powder in a domestic food processor and weighed, and the samples were kept at −40 °C until extraction and analysis.

All solvents were of AnalaR or HPLC grade where appropriate, and the water was purified via a Millex Q-plus system (Millipore Ltd., Watford, U.K.). MN polyamide SC6 was purchased from Macherey-Nagel GmbH & Co. β-Glucuronidase from *Escherichia coli* K12 was purchased from Boehringer Mannheim GmbH.

Cyanidin 3,5-di-β-D-*O*-glucoside, quercetin 3-*O*-α-L-rhamnosyl-β-glucoside (rutin), quercetin 3-*O*-β-D-galactoside, quercetin 3-*O*-β-D-glucoside, quercetin 3-*O*-α-L-rhamnoside, kaempferol 3-*O*-α-L-rhamnoside, luteolin 7-*O*-β-D-glucoside, quercetin, luteolin, and kaempferol were from Apin Chemicals Ltd., Abingdon, Oxford, U.K. Cyanidin 3-*O*-β-D-glucoside, quercetin 3-*O*-β-D-glucoside, and kaempferol 3-*O*-β-D-glucoside from Extrasynthese, Genay, France, were used for identification and for quantitative calibration. Quercetin 3-*O*-β-D-[(6-*O*-malonyl)-glucoside] 7-glucoside, cyanidin 3-*O*-β-D-(6-O-malonyl)glucoside, and quercetin 3-*O*-β-D-(6-*O*-malonyl)glucoside from lollo rosso lettuce were gifts, kindly donated by Francisco A. Tomas-Barberan, Department of Food Science and Technology, CEBAS (CSIC), Murcia, Spain; see Ferreres et al. (1997) for confirmation of structures from NMR spectral data. Quercetin 3-*O*-β-D-glucuronide and kaempferol 3-*O*-β-D-glucuronide were isolated from green beans (Price et al., 1998a), and quercetin 3-*O*-β-D-xyloside was isolated from apples (Price et al., 1999).

Quantitative Extraction. Duplicate samples of the dry powder (2 g) were homogenized three times in methanol/water/acetic acid 70:30:5 (50 mL) at 1200 rpm for 1 min (Ultra Turrax, Janke & Kunkel) and kept cool on ice. The homogenate was filtered under reduced pressure through filter paper (Whatman No. 541). The combined fractions were evaporated to dryness in vacuo at 40 °C and made up to 20 mL with methanol/acetic acid 95:5. Aliquots (5 mL) were evaporated to dryness, taken up in water (10 mL), and added to a polyamide (1 g) column preconditioned with methanol (20 mL) followed by water (60 mL). The column was washed with water (20 mL) and eluted with methanol/20 M ammonium hydroxide 99.5:0.5 (50 mL) to elute the flavonol glycosides, which were immediately readjusted to pH 5 with acetic acid. Each extract was evaporated to dryness under reduced pressure at 40 °C, redissolved in methanol/acetic acid 95:5 (1 mL), and filtered prior to HPLC analysis (0.2 μm).

Isolation of Flavonol Conjugates. Lollo rosso and coarse frisee samples were freeze-dried and powdered. The dry powder (40 g) was homogenized three times in methanol/water/acetic acid 70:30:5 (800 mL) at 1200 rpm (cooled on ice) for 2 min (Pro400 homogenizer) and the homogenate filtered under reduced pressure through filter paper (Whatman No. 541). The combined fractions were reduced in volume (100 mL) in vacuo at 40 °C to remove the methanol. The aqueous sample was fractionated in two portions on a polyamide column (50 g) that had been preconditioned with methanol (500 mL) followed by water (1 L). The column was washed with water (1 L) and further eluted, under pressure (10 psi nitrogen) with methanol (1 L) to elute the neutral flavonols and with methanol/ammonium hydroxide 99.5:0.5 (1 L) to elute the acidic flavonols. For coarse frisee, the corresponding acidic extract (after pH adjustment to neutral) was evaporated in vacuo at 40 °C and rechromatographed on a polyamide (50 g) column as described above to give two fractions. The five extracts were evaporated under reduced pressure (40 °C) for subsequent preparative high-pressure liquid chromatography (HPLC).

HPLC. *Preparative HPLC.* A Prodigy 5 μm ODS3 reversed phase silica (250 mm × 21.2 mm i.d., Phenomenex Ltd., Macclesfield, U.K.) column was used at a flow rate of 5 mL/min with a gradient solvent of A [0.1% trifluoroacetic acid (TFA)] and B (acetonitrile) used in the proportion of a (for lollo rosso) 17% B for 2 min increasing to 20% B after 10 min to 26% B at 75 min and to 27% B at 85 min and b (for coarse frisee) 17% B for 2 min increasing to 20% B after 5 min to 25% B at 75 min and to 27% B at 95 min. A column cleanup stage was used by increasing B to 90% after a further 5 min and finally re-equilibration for 20 min at 17% B. The column effluent was monitored at 270 and 370 nm, and fractions were collected using a Gilson fraction collector.

Analytical HPLC. A Hewlett-Packard 1100 system comprising an autosampler and a quaternary pump coupled to a diode array detector and controlled by Chemstation software was used with a solvent gradient of A [water/tetrahydrofuran (THF)/TFA 98:2:0.1] and B (acetonitrile) used in the proportion of 17% B for 2 min increasing to 20% B after 10 min to 27% B at 35 min and to 90% B after another 5 min. A column cleanup stage was used at 90% for a further 5 min and finally re-equilibration for 15 min at 17% B. The column used was packed with Prodigy 5 μm ODS3 reversed phase silica (250 mm × 4.6 mm i.d., Phenomenex Ltd.), temperature 30 °C, and the effluent (1 mL/min) was monitored by a diode array detector.

In experiments that required identification of the aglycon the solvent gradient was A (water/THF/TFA 98:2:0.1) and B (acetonitrile) used in the proportion of 17% B for 2 min increasing to 25% B after 5 min to 35% B after a further 8 min and to 50% B after 5 min followed by a cleanup stage and equilibration (Price et al., 1998a).

External standards of quercetin 3-*O*-glucose for green-leafed lettuce and, in addition, cyanidin 3-*O*-glucose for red-leafed lettuce or kaempferol 3-*O*-glucose for endive (0.5, 1.5, 4.5, and 10 μg injected) were included between replicate samples (injected at two levels in duplicate), that is, every eight runs on the HPLC.

Peak Identification. *HPLC.* Standards were used to identify peaks by retention times and cochromatography. Diode array spectral characteristics were matched to standards and to library spectra. Standards prepared in this laboratory were characterized by NMR and MS as described previously (Price et al., 1998a, 1999).

Hydrolysis Conditions for Production of Aglycons. Extracts were heated at 90 °C for 2 h in 1.2 M HCl in 50% aqueous methanol (Crozier et al., 1997), evaporated under reduced pressure, redissolved in methanol, and filtered prior to HPLC analysis.

Deglucuronidation. Glucuronide conjugates were dissolved in dilute phosphate buffer (pH 6), β-glucuronidase was added, and the mixture was incubated at 37 °C for 30 min. The reaction was stopped by the addition of an equal volume of methanol/1 mM ascorbate (Gee et al., 2000). The extracts were microfuged and the supernatant was filtered (0.2 μm) prior to HPLC.

Demalonation. Samples were dissolved in methanol/acetic acid (95:5), stored in the light at room temperature, and analyzed by HPLC at 0, 7, and 21 days.

Mass Spectrometry. Atmospheric pressure chemical ionization (APCI) spectra were obtained by continuous flow injection on a Platform benchtop mass spectrometer (Micromass, Manchester, U.K.) operated in the positive and negative ionization modes. The mobile phase was 60:40 water/acetonitrile at a flow rate of 200 μL/min. Typical tuning parameters were as follows: corona, 3.00 kV; high voltage lens, 0.10 kV; cone, 10 V; source temperature, 130 °C; APCI probe temperature, 550 °C.

NMR Spectroscopy. NMR spectra were obtained using a JEOL GX400 spectrometer operating at 400 MHz for ^{1}H and at 100 MHz for ^{13}C. Samples were dissolved in either methanol-d_4 or dimethyl-d_6 sulfoxide, and all spectra were run at 27 °C. Chemical shifts are given relative to TMS but were measured using the solvent methyl signal as secondary reference (δ values: ^{1}H, 3.30 ppm; ^{13}C, 49.0 ppm).

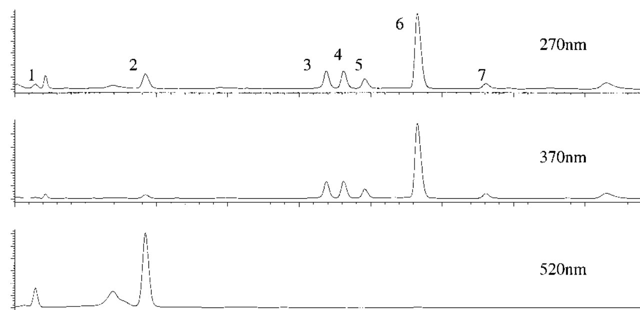

Figure 1. HPLC analysis of lettuce extract (lollo rosso): 1, cyanidin 3-*O*-glucoside; 2, cyanidin 3-*O*-(6-*O*-malonyl)glucoside; 3, quercetin 3-*O*-glucoside; 4, quercetin 3-*O*-glucuronide; 5, luteolin 7-glucuronide; 6, quercetin 3-*O*-(6-*O*-malonyl)-glucoside; 7, quercetin 3-*O*-rhamnoside.

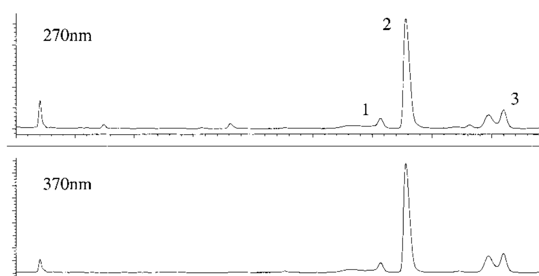

Figure 2. HPLC analysis of endive extract (coarse frisee): 1, kaempferol 3-*O*-glucoside; 2, kaempferol 3-*O*-glucuronide; 3, kaempferol 3-*O*-(6-*O*-malonyl)glucoside.

RESULTS AND DISCUSSION

Identification of Flavonoids. Each of the compounds was identified by combination of a comparison of the retention times of HPLC (see Figure 1 for lettuce extract and Figure 2 for endive extract), cochromatography with standard compounds, matching of UV–visible spectral characteristics, and both acid and enzymic hydrolysis for the following compounds: cyanidin 3,5-di-*O*-glucoside, cyanidin 3-*O*-glucoside, cyanidin 3-*O*-(6-*O*-malonyl)glucoside, quercetin-3-*O*-[(6-*O*-malonyl)-glucoside] 7-glucoside, quercetin 3-*O*-rhamnosylglucoside (rutin), quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-glucuronide, quercetin 3-*O*-(6-*O*-malonyl)glucoside, quercetin 3-*O*-rhamnoside, kaempferol 3-*O*-glucoside, kaempferol 3-*O*-glucuronide, and kaempferol 3-*O*-rhamnoside. A component with a retention time similar to that of quercetin 3-*O*-xyloside (Price et al., 1999) eluted from a polyamide column as a glucuronide and following hydrolysis yielded the aglycon, luteolin, which was identified by comparison with the standard compound. Luteolin 7-*O*-glucuronide has been identified as present in lettuce (Woldecke and Herrmann, 1974; Herrmann, 1976; Rees and Harborne, 1984), and it can therefore be proposed that the site of conjugation in this compound is also in the 7-position. NMR spectral data confirmed the identity of kaempferol 3-*O*-glucuronide, a major component isolated from endive, and quercetin 3-*O*-(6-*O*-malonyl)glucoside isolated from lettuce by comparison of data from other sources (Horowitz and Aspen, 1989; Wald et al., 1989; Berhow et al., 1991; Ferreres et al., 1997; Withopf et al., 1997; Price et al., 1998a,b).

A further component (peak 3, Figure 2), with UV spectrum indicative of a kaempferol conjugate and a retention time, by analogy with the quercetin components in the lettuce extract, was indicative of a malonated form of the kaempferol 3-*O*-glucoside (Woldecke and Herrmann, 1974). The chemical structure of this component was confirmed after preparative isolation and subsequent mass spectroscopy [FAB, positive mode, *m*/*z* 557 (4%, MNa^+), 535 (100%, MH^+), 491 (5.3%, $MH - CO_2^+$), and 287 (9%, MH − $gluM^+$) and, negative mode, *m*/*z* 533 (33%, M − H^-), 489 (100%, M − H − CO_2^-), 447 (1%, M − H − M^-), 285 (3%, M − H − $gluM^-$); NMR spectroscopy (^{13}C NMR, DMSO-d_6, 27 °C) δ 41.0 (malonate CH_2), 63.4 (C-6″), 69.5 (C-4″), 73.8 (C-5″), 74.0 (C-2″), 76.1 (C-3″), 93.7 (C-8), 98.7 (C-6), 101.2 (C-1″), 103.9 (C-10), 115.0 (C-3′,C-5′), 120.7 (C-1′), 130.8 (C-2′, C-6′), 133.1 (C-3), 156.4 (C-2), 156.6 (C-9), 160.0 (C-4), 161.1 (C-5), 164.1 (C-7), 166.4 (malonate CO_2R), 167.6 (malonate CO_2H), 177.3 (C-4)] as kaempferol 3-*O*-(6-*O*-malonyl)glucoside. This compound was indicated as a possibility in endive from chromatographic retention times and UV spectra by Woldecke and Herrmann (1974), but this is the first time the presence of kaempferol 3-*O*-(6-*O*-malonyl)glucoside has been demonstrated.

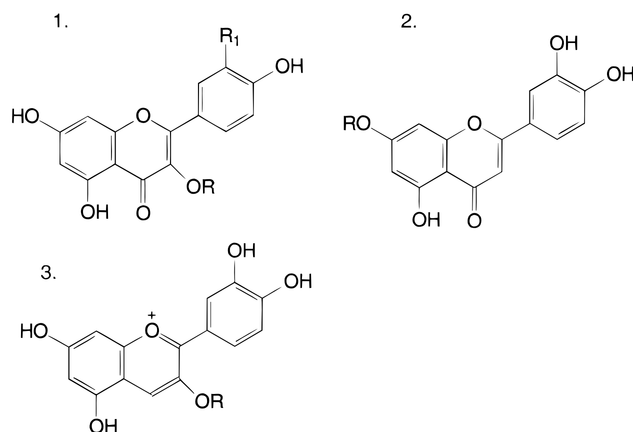

Figure 3. Chemical structures of flavonoids found in lettuce and endive: **1**, flavonol glycosides; **2**, flavone glycoside; **3**, anthocyanidin glycosides (R_1 = H = kaempferol; R_1 = OH = quercetin) (R = glycoside).

Table 1. Stability of Malonyl Conjugates, with Time, in Lettuce and Endive Extracts Exposed to Light at Room Temperature

	% loss (by wt)					
day	C-3-gluM	C-3-glu	Q-3-gluM	Q-3-glu	K-3-gluM	K-3-glu
0	92	8	66	34	69	31
7	88	12	53	47	51	49
21	40	60	13	87	0	100

In addition, HPLC resulted in the separation of the hydroxycinnamoyl derivatives: caffeoylmalate, caffeoylquinate (chlorogenic acid), dicaffeoylquinate, caffeoylmalate, and dicaffeoylmalate. The latter is the major phenolic compound (<50%) in the lettuce and endive samples (Winter and Herrmann, 1986; Goupy et al., 1990; Tomas-Barberan et al., 1997; Ferreres et al., 1997).

The presence of malonyl esters of both cyanidin and quercetin glucosides in lettuce and the corresponding kaempferol analogue in endive and their lack of stability in extracts were confirmed as noted previously (Woldecke and Herrmann, 1974; Herrmann, 1976; Rees and Harborne, 1984; Bridle et al., 1984; Yamaguchi et al., 1996; Ferreres et al., 1997). A quantitative loss of malonate and corresponding increase in glucoside after the extract was exposed to light for 21 days were observed, whereas the glucuronide content was unchanged (Table 1).

The flavonoids present in the lettuce leaves of the eight varieties studied here contained only conjugates of quercetin and luteolin (and cyanidin in the red-leafed

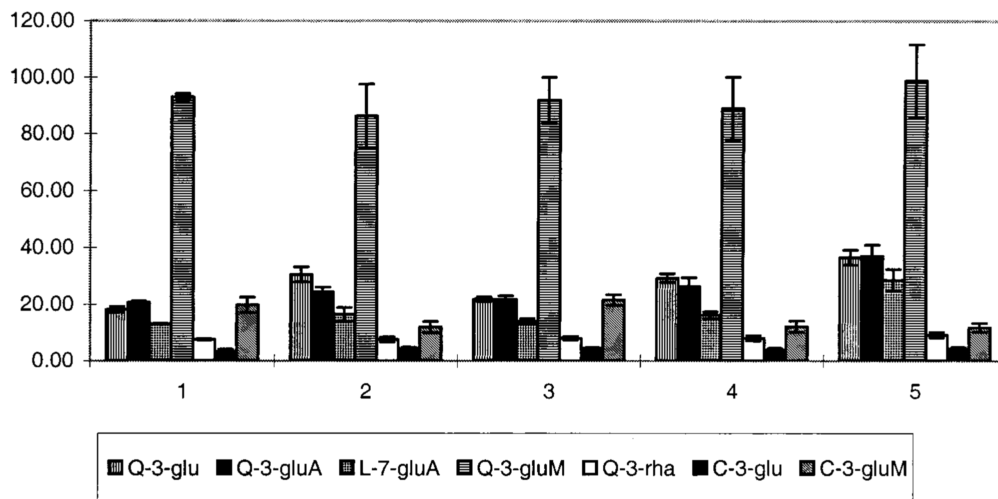

Figure 4. Comparison of flavonoid glycoside composition in five heads of lettuce var. lollo rosso.

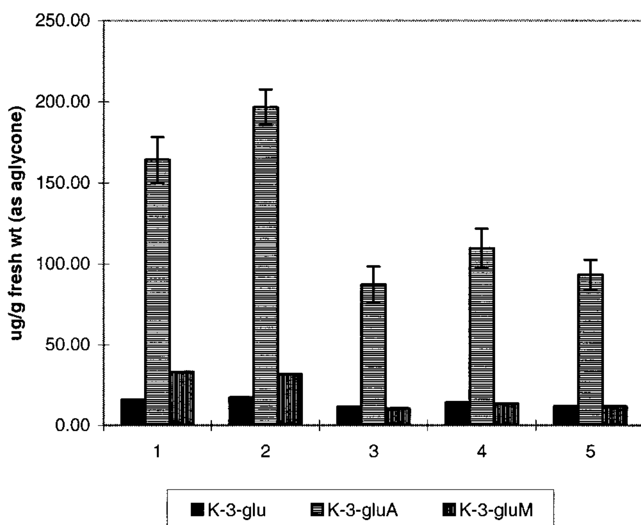

Figure 5. Comparison of flavonoid glycoside composition in five heads of endive var. coarse frisee.

varieties), whereas the leaves of the endive samples (three varieties) contained only conjugates of kaempferol. The chemical structures are shown in Figure 3.

Effect on Composition within Variety. The variations in both overall flavonoid content and composition in five heads of lettuce (var. lollo rosso) (Figure 4) and in five heads of endive (var. coarse frisee) all harvested at the same time (Figure 5) were determined. The total flavonoid content in each of the heads of lollo rosso lettuce were 198 ± 11, 199 ± 3, 201 ± 11, 210 ± 8, and 229 ± 13 μg/g (mean of 207 ± 28 μg/g of fresh weight expressed as aglycon). This compares with levels found in different parts of the plant tissue for lollo rosso of 43 μg/g in white tissues, 244 μg/g in green tissues, and 1384 μg/g in red tissues (Ferreres et al., 1997). There were major differences in the total levels found in coarse Frisee with values of 214 ± 14, 246 ± 11, 109 ± 11, 137 ± 14, and 117 ± 9 μg/g, with a mean content of 165 ± 80 μg/g of fresh weight (expressed as aglycon). These compare with the highly variable levels obtained by Hertog et al. (1992) of endive (variety unspecified) of 46 ± 42 μg/g of fresh weight (mean). Variations within variety, head to head, could be due to differing agronomic conditions, outdoor or glasshouse lighting, and tissue type from red to green or white and from outer or inner leaves (Herrmann, 1976; Bilyk et al., 1985; Goupy et al., 1990; Crozier et al., 1997).

The composition of the quercetin glycosides in each of the five heads of lollo rosso showed the malonated form of quercetin 3-*O*-glucoside as the dominant form, followed by the glucoside and glucuronide, whereas the rhamnoside, galactoside, and rutinoside were the minor quercetin conjugates in all cases. The flavone luteolin 7-glucuronide was also present in all varieties. The malonated form of cyanidin glucoside was more abundant than the nonmalonated form, and the latter was not measured in lollo rosso by Ferreres et al. (1997).

In coarse frisee, all of the conjugates were based on kaempferol and analogous to the quercetin conjugates present in lettuce; however, the predominant form was the glucuronide followed by the malonated glucoside, then glucoside, and then rhamnoside.

Varietal Effect on Composition. Five quercetin conjugates [quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-glucuronide, quercetin 3-*O*-(6-*O*-malonyl)glucoside, and quercetin 3-*O*-rhamnoside] plus luteolin 7-*O*-glucuronide were identified in all of the green-leafed lettuces. Rutin (quercetin 3-*O*-rhamnosylglucoside) was found only in the cos variety, and quercetin-3-*O*-[6-*O*-malonyl)glucoside] 7-glucoside was measured only in the two lollo varieties, in agreement with the findings of Ferreres et al. (1997). Two cyanidin conjugates [cyanidin 3-*O*-glucoside and cyanidin 3-*O*-(6-*O*-malonyl)glucoside] were identified in the red-leafed varieties, lollo rosso and red oak leaf, together with trace amounts of cyanidin 3,5-di-*O*-glucoside. The total flavonol content ranged from 0.5 μg/g (fresh weight, expressed as aglycon) for iceberg to 207 μg/g (fresh weight, expressed as aglycon) for lollo rosso (average value of five heads, Table 2). The cyanidin conjugates in the two red-leafed varieties lollo rosso and red oak leaf contributed 12.9 and 16.9%, respectively, to the total flavonoid content. In red oak leaf, iceberg, green batavia, and the two lollo varieties, the quercetin 3-*O*-(6-*O*-malonyl)glucoside predominated, whereas the quercetin 3-*O*-glucuronide was the main conjugate in the remaining three varieties. No kaempferol conjugates were found in any of the lettuce varieties and, similarly, no quercetin conjugates were detected in the endive varieties. The three varieties of endive, fine frisee, escarole, and coarse frisee, each possessed three flavonol conjugates and were analogous to the quercetin conju-

Table 2. Content of Individual Flavonoid Conjugates in Eight Varieties of Lettuce[a]

variety	Q-3-rut	Q-3-gal	Q-3-glu	Q-3-gluA	L-7-gluA	Q-3-gluM	Q-3-rha	g7Q-3-gluM	C-3-glu	C-3-gluM	total
iceberg	0.0	0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.0	0.0	0.0	0.0	0.3 ± 0.05
green batavia	0.0	0.0	0.1 ± 0.01	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.0	0.0	0.0	0.0	0.7 ± 0.05
cos remus	1.2 ± 0.1	0.9 ± 0.1	0.8 ± 0.2	4.2 ± 0.2	1.3 ± 0.1	3.3 ± 0.2	0.0	0.0	0.0	0.0	9.6 ± 0.9
green salad bowl	0.0	0.3 ± 0.0	1.3 ± 0.1	9.2 ± 1.0	1.3 ± 0.2	7.6 ± 0.6	0.5 ± 0.0	0.0	0.0	0.0	19.9 ± 2.0
green oak leaf	0.0	1.2 ± 0.2	2.2 ± 0.3	14.1 ± 0.9	2.5 ± 0.1	13.0 ± 0.6	1.1 ± 0.2	0.0	0.0	0.0	32.9 ± 2.3
red oak leaf	0.0	0.0	5.5 ± 0.1	8.6 ± 0.6	7.8 ± 0.5	38.5 ± 1.6	3.0 ± 0.2	0.0	2.2 ± 0.2	10.6 ± 0.1	76.2 ± 4.0
lollo biondo	0.0	1.7 ± 0.2	10.2 ± 1.1	23.4 ± 0.6	9.5 ± 0.7	47.1 ± 1.4	3.8 ± 0.1	1.6 ± 0.6	0.0	0.0	95.7 ± 4.2
lollo rosso	0.0	1.8 ± 0.1	23.1 ± 0.8	24.9 ± 0.4	23.3 ± 0.8	95.7 ± 3.1	8.5 ± 0.6	3.2 ± 0.4	5.8 ± 0.3	20.7 ± 0.5	207 ± 13.0

[a] Micrograms per gram of fresh weight expressed as aglycon, three to five replicates.

Table 3. Content of Individual Flavonol Conjugates in Three Varieties of Endive[a]

variety	K-3-glu	K-3-gluA	K-3-gluM	K-3-rha	total
fine frisee	4.3 ± 0.2	32.1 ± 1.1	7.9 ± 0.9		44.3 ± 1.1
escarole	6.3 ± 0.2	92.4 ± 2.7	12.5 ± 0.2		111 ± 2.7
coarse frisee	17.4 ± 1.2	197 ± 7.9	31.8 ± 1.2	2.1 ± 0.3	246 ± 11

[a] Micrograms per gram of fresh weight expressed as aglycon, three to five replicates.

gates present in the lettuce. However, the predominant form was kaempferol 3-*O*-glucuronide in all of the varieties followed by kaempferol 3-*O*-(6-*O*-malonyl)-glucoside and kaempferol 3-*O*-glucoside. In addition, kaempferol 3-*O*-rhamnoside was measured in coarse frisee (average value of five heads, Table 3).

A survey carried out by Rees and Harborne (1984) on the different types of flavonoids present in Compositae suggests that the different species could be assigned according to their flavonoid profile. From our work it is clear that the *Lactuca* and *Cichorium* species, which are both from the Lactuceae (or Cichorieae) tribe, each have distinct profiles. *Lactuca* contains both luteolin and quercetin conjugates, whereas *Cichorium* contains only kaempferol conjugates, although *C. intybus*, which was not the subject of this study, has been recorded as also containing conjugates of isorhamnetin. These findings are in agreement with Herrmann (1976) but not with Goupy et al. (1990), who reported the presence of quercetin glycosides in endive (*C. endivia*), or Bilyk et al. (1985), who reported traces of kaempferol in the hydrolyzed extracts of lettuce. Bilyk et al. (1985), Crozier et al. (1997), and Hertog et al. (1992) failed to detect the presence of luteolin in the hydrolyzed extracts of lettuce.

The identities of the five dominant flavonoids in lettuce and endive in this study are in agreement with the findings of Woldecke and Herrmann (1974), Herrmann (1976), Rees and Harborne (1984), and Ferreres et al. (1997).

Our work has identified the presence of additional flavonoid conjugates in some varieties of lettuce, namely, quercetin 3-*O*-galactoside, quercetin 3-*O*-rhamnoside, and rutin (quercetin 3-*O*-rhamnosyl-glucoside), plus cyanidin 3,5-di-*O*-glucoside and cyanidin 3-*O*-glucoside in the red-leafed varieties. For the endives we report the presence of kaempferol 3-*O*-rhamnoside and demonstrate the presence of the malonated form of kaempferol 3-*O*-glucoside, which had previously only been postulated to be present by Woldecke and Herrmann (1974).

The large variation of flavonoid content, reported here, among varieties and heads of lettuce and endive each harvested at the same time is probably due to large variation in the levels in the different portions of the leaves. For instance, Herrmann (1976) reported levels of 60 μg/g for the outer parts and only 3.4 μg/g for the inner tissue for lettuce cv. Blanco and 462 and 7.6 μg/g, respectively, for lettuce cv. Valentine. This variation is likely to be due to changes in environmental conditions such as differing levels of exposure to sunlight. Because the aim of this work was to estimate the levels in lettuce as eaten and hence to enable dietary intake levels to be determined, measurement of the flavonoid content of the whole plant was appropriate.

Effect of Processing. The effects of shredding and subsequent exposure to light on both the level and composition of the flavonoids in the eight varieties of lettuce are shown in Table 4. Significant losses in the total flavonol content were found for the green and red oak leaf (94 and 43%, respectively), iceberg (36%), green batavia (25%), and lollo biondo and rosso varieties (24 and 6%, respectively). The large loss of flavonoid (94%) observed for green oak leaf corresponded to a visible amount of tissue degradation, which was not seen in the other varieties. Significant losses of the malonated forms of both quercetin glucoside and cyanidin glucoside were not matched on a quantitative basis by a corresponding increase in the glucoside, and these losses did not result in the formation of the respective aglycons.

To further understand the mechanism of these losses in red oak leaf tissue, whole leaf tissue was macerated in water with added rutin, either in water at room temperature or in boiling water. Complete loss of all endogenous flavonoids and the added rutin was observed at room temperature, whereas no losses were found in boiling water. Peroxidase, polyphenol oxidase, and phenolase activity reported in leaf tissues (Ke and Saltveit, 1988; Fujita et al., 1991; Yamasaki et al., 1997; Tomas-Barberan et al., 1997a,b) may be responsible for the rapid degradation of the flavonoid aglycon moiety in lettuce, increased accumulation of caffeic acid derivatives, and production of brown pigments on oxidation. Takahama (1986) and Takahama et al. (1991) showed the oxidation of rutin by peroxidase giving an ascorbate-reducible oxidized product after 1 min, which is an *o*-quinone derivative. Miller and Schreier (1985) and Schreier and Miller (1985) studied flavonol degradation by peroxidase resulting in characteristic spectral changes with a shift of maxima from 370 to 300 nm within 3 min. A number of oxidation products were fractionated and characterized mainly as benzoic acids following cleavage of the C-ring and following attack of the olefinic C-2/C-3 bond leading to the incorporation of oxygen into the flavonoid structure.

Analysis of the endive samples (Table 5) showed losses of flavonol in all three varieties (8.4−31.8 wt %). Although there was a net gain of kaempferol 3-glucoside for both frisee varieties, there was a greater equivalent loss of the corresponding malonated glucoside. There

Table 4. Content of Individual Flavonol Conjugates in Eight Varieties of Lettuce both before and after Processing and Showing the Percentage Weight Changes of the Individual Conjugates[a]

variety	Q-3-rut	SD	Q-3-gal	SD	Q-3-glu	SD	Q-3-gluA	SD	L-7-gluA	SD	Q-3-gluM	SD	Q-3-rha	SD	g7Q-3-gluM	SD	C-3-glu	SD	C-3-gluM	SD	total FG	SD
lollo biondo																						
control	0.0	0.0	29.6	3.3	177	18.9	416	11.3	154	12.1	963	27.9	63.9	2.3	1.6	0.6	0.0	0.0	0.0	0.0	1775	72.5
processed	0.0	0.0	23.8	2.2	136	11.5	350	19.9	114	8.6	670	108	51.7	16.9	1.4	0.4	0.0	0.0	0.0	0.0	1346	197
wt % loss			19.8		22.8		15.9		26.2		30.4		19.1		12.5						24.1	
lollo rosso																						
control	0.0	0.0	33.8	3.1	518	18.7	532	28.5	312	21.1	2592	230.3	184	15.0	3.2	0.4	111	3.5	621	53.7	4874	371
processed	0.0	0.0	32.2	3.3	1020	13.5	517	3.7	344	5.0	1853	7.1	198	3.1	3.6	0.4	184	9.0	434	30.6	4585	325
wt % loss			4.8		−96.8		2.9		−10.2		28.5		−7.8		−11.3		−65.4		30.2		6.0	
green batavia																						
control	0.0	0.0	0.0	0.0	1.1	0.2	3.6	0.2	4.1	0.3	9.4	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	18.2	1.3
processed	0.0	0.0	0.0	0.0	2.6	1.2	3.4	1.0	0.0	0.0	7.7	1.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	13.7	3.6
wt % loss					−129		6.4		100		17.3										24.7	
cos remus																						
control	21.5	1.2	16.3	1.1	14.8	3.0	79.9	11.2	22.5	2.4	72.2	8.2	15.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	189	24.8
processed	19.8	0.8	14.4	1.2	24.0	5.1	74.1	6.6	17.6	1.9	66.7	4.0	21.5	1.6	0.0	0.0	0.0	0.0	0.0	0.0	238	21.7
wt % loss	7.9		11.6		−62.1		7.2		21.7		7.6		7.6								−25.7	
iceberg																						
control	0.0	0.0	0.0	0.0	0.6	0.1	2.7	0.3	1.0	0.1	4.5	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	8.8	1.1
processed	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.6	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.6	0.7
wt % loss					100		100		100		−24.9										36.5	
green salad bowl																						
control	0.0	0.0	7.2	0.5	27.7	3.2	200	22.5	26.0	3.6	192	16.1	9.8	0.9	0.0	0.0	0.0	0.0	0.0	0.0	456	46.3
processed	0.0	0.0	7.8	0.7	100	8.5	204	7.2	29.3	1.0	125	4.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	466	46.5
wt % loss			−8.1		−263		−1.8		−12.5		35.1		100								−2.2	
green oak leaf																						
control	0.0	0.0	20.4	2.7	36.3	4.8	238	15.0	37.8	2.1	253	12.3	16.7	3.4	0.0	0.0	0.0	0.0	0.0	0.0	582	37.7
processed	0.0	0.0	2.2	0.3	6.2	1.0	12.0	2.2	4.2	0.8	7.2	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	31.8	5.4
wt % loss			89.1		82.8		94.9		89.0		97.2		100								94.5	
red oak leaf																						
control	0.0	0.0	0.0	0.0	83.5	10.8	135	8.7	110	7.6	693	28.1	44.6	2.5	0.0	0.0	34.3	3.6	195.4	1.7	1295	63.1
processed	0.0	0.0	0.0	0.0	10.5	2.3	55.5	10.4	84.2	13.3	96.5	7.8	59.7	0.2	0.0	0.0	401	61.6	29.6	6.2	737	97.7
wt % loss					87.4		58.8		23.6		86.1		−34.0				−1071		84.8		43.1	

[a] Micrograms per gram of dry weight expressed as aglycon, three replicates.

Table 5. Content of Individual Flavonol Conjugates in Three Varieties of Endive both before and after Processing and Showing the Percentage Weight Changes of the Individual Conjugates[a]

	K-3-glu	SD	K-3-gluA	SD	K-3-gluM	SD	K-3-rha	SD	total FG
coarse frissee									
control	216	15.0	2516	140	471	62.4	25.6	2.0	3228
processed	311	47.8	2362	342	282	10.4	0.0	0.0	2955
wt % loss	−44.4		6.1		40.1		100		8.4
fine frissee									
control	65.8	3.2	505	49.5	144	52.7	0.0	0.0	767
processed	77.1	15.9	498	84.6	89.8	8.3	0.0	0.0	665
wt % loss	−17.2		1.4		37.4				13.3
escarole									
control	89.5	3.8	1342	39.5	210	3.6	0.0	0.0	1686
processed	77.9	2.9	864	5.0	209	5.1	0.0	0.0	1150
wt % loss	13.0		35.6		0.9				31.8

[a] Micrograms per gram of dry weight expressed as aglycon, three replicates.

was no significant loss of the glucuronide in either frisee variety, whereas for escarole most of the overall loss was due to a loss of the glucuronide.

Effect of Storage. There was a significant loss of flavonol glycosides in icebank-stored whole heads of lettuce except for iceberg, although the relatively low level of flavonol glycosides present in this variety made interpretation of the data difficult. The losses observed (Table 6) ranged from 7 to 46%, and although there was evidence of demalonation of the quercetin conjugate in all varieties, red oak leaf and green batavia were the only varieties to show a net increase in the corresponding quercetin 3-*O*-glucoside on storage. On modified atmosphere packaging/storage and minimal processing of lollo rosso, Gil et al. (1998) showed some losses in both malonated cyanidin and quercetin conjugates with small but not complete corresponding increases in glucosides and suggested that demalonation led first to glucosides with further degradation to acetyl derivatives. Horowitz and Asen (1989) stated that flavonoid malonylglycosides can lose carbon dioxide to give corresponding flavonoid acetylglycosides; these were not identified in the lettuce samples. The apparent gain of quercetin galactoside in green salad bowl, lollo biondo, and lollo rosso may be due to release of this component from an unidentified precursor. Shredding of the stored heads of lettuce showed further losses due to demalonation, although, with the exception of green batavia, these corresponded to some increases in levels of quercetin glucoside.

Following storage, all of the endive varieties produced a significant amount of demalonation of the kaempferol conjugate, although only fine frisee showed a gain in the flavonol glucoside (Table 7). This variety was the most resistant to flavonoid loss with an apparent increase in glucuronide content. Losses of all conjugates, 54.7% on storage and 65.5% on further processing, were shown for coarse frisee.

During minimal processing and wounding, Tomas-Barberan et al. (1997a,b) suggested dependency on

Table 6. Lettuce Varieties: Effect of Storage in Icebank for 7 Days and Effect of Storage in Icebank for 7 Days followed by Shredding and Exposure to Light for 2 Days[a]

		wt % loss									
variety	process	Q-3-rut	Q-3-gal	Q-3-glu	Q-3-gluA	L-7-gluA	Q-3-gluM	Q-3-rha	C-3-glu	C-3-gluM	total FG
iceberg	storage			100	100	100	−84.6				1.2
	+ processing			−363	−1.1	100	20.0				−9.7
green batavia	storage			−29.7	41.1	100	37.9				39.4
	+ processing			100	76.7	100	76.7				82.1
cos remus	storage	100	100	30.3	−18.5	2.1	−2.7	−100			7.5
	+ processing	100	−59.0	14.5	−21.8	−1.4	−3.9	−195			1.0
green salad bowl	storage		−48.7	71.3	47.4	47.7	43.1	60.1			46.3
	+ processing		−403	−263	20.2	28.4	52.1	32.1			16.1
green oak leaf	storage		70.6	24.4	13.8	29.4	6.5	100			16.3
	+ processing		nm	nm	nm	nm	nm	nm			nm
red oak leaf	storage			−46.1	4.2	−7.0	21.5	10.5	67.8	75.4	34.1
	+ processing			nm	nm	nm	nm	nm	nm	nm	nm
lollo biondo	storage		−52.0	54.2	11.8	3.3	19.9	43.3			19.3
	+ processing		−99.0	22.0	6.0	12.4	24.7	26.1			17.3
lollo rosso	storage		−71.5	47.8	6.5	−14.0	11.0	19.9	55.3	23.9	17.6
	+ processing		−85.5	17.2	−25.1	6.1	15.5	14.3	45.7	34.6	18.4

[a] Two replicates.

Table 7. Endive Varieties: Effect of Storage in Icebank for 7 Days and Effect of Storage in Icebank for 7 Days followed by Shredding and Exposure to Light for 2 Days[a]

		wt % loss				
variety	process	K-3-glu	K-3-gluA	K-3-gluM	K-3-rha	total FG
coarse frisee	storage	39.8	54.4	60.2	100	54.7
	+ processing	45.7	66.9	65.5	100	65.5
fine frisee	storage	−33.5	−29.3	49.1		−12.4
	+ processing	−42.0	−36.3	43.3		−19.2
escarole	storage	2.0	45.5	56.1		44.8
	+ processing	nm	nm	nm		nm

[a] Two replicates.

temperature and variety for increased action of phenylalanine lyase, polyphenol oxidase, and peroxidase as a response to stress and wounding and showed spectral shifts of degradation of flavonols and phenols giving browning with phenolic metabolism. Yamasaki et al. (1997) linked stress protection and detoxification to the flavonoid−peroxidase reaction with accumulation of flavonoids in response to wounding.

This study has identified the individual conjugates of the flavonoids and their levels present in a range of commonly eaten lettuce and endive varieties. Processing and storage of these foods have also been shown to alter both their levels and compositions. These data are important because the flavonoids, acting as natural antioxidants, may be part of the reason for the protective effect against degenerative diseases when this type of food is a significant part of the diet. However, to further understand the protective effects of dietary phytochemicals in general, the chemical form of the putative factors, as eaten, is the first essential step to enable the study of their subsequent absorption and metabolism. Only from these studies will the biological activity and hence significance of flavonoids in terms of improved human health through change in diet be determined.

ABBREVIATIONS USED

C-3-glu, cyanidin 3-*O*-glucoside; C-3-gluM, cyanidin 3-*O*-(6-*O*-malonyl)glucoside; Q-3-rut, quercetin 3-*O*-rhamnosylglucoside (rutin); Q-3-gal, quercetin 3-*O*-galactoside; Q-3-glu, quercetin 3-*O*-glucoside; Q-3-gluA, quercetin 3-*O*-glucuronide; Q-3-gluM, quercetin 3-*O*-(6-*O*-malonyl)glucoside; Q-3-rha, quercetin 3-*O*-rhamnoside; g7Q-3-gluM, quercetin 3-*O*-[(6-*O*-malonyl)glucoside] 7-glucoside; L-7-gluA, luteolin 7-*O*-glucuronide; K-3-glu, kaempferol 3-*O*-glucoside; K-3-gluA, kaempferol 3-*O*-glucuronide; K-3-gluM, kaempferol 3-*O*-(6-*O*-malonyl)glucoside; K-3-rha, kaempferol 3-*O*-rhamnoside; FG, flavonol glycosides; nm, not measured.

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