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Characterization and Quantification of Flavonoids and Hydroxycinnamic Acids in Curly Kale (*Brassica oleracea* L. Convar. *acephala* Var. *sabellica*) by HPLC-DAD-ESI-MS^n

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Kale is a leafy green vegetable belonging to the Brassicaceae family, a group of vegetables including cabbage, broccoli, cauliflower, and Brussels sprouts, with a high content of health-promoting phytochemicals. The flavonoids and hydroxycinammic acids of curly kale (*Brassica oleracea* L. ssp. *oleracea* convar. *acephala* (DC.) Alef. var. *sabellica* L.), a variety of kale, were characterized and identified primarily through HPLC-DAD-ESI-MS^n analysis. Thirty-two phenolic compounds including glycosides of quercetin and kaempferol and derivatives of *p*-coumaric, ferulic, sinapic, and caffeic acid were tentatively identified, providing a more complete identification of phenolic compounds in curly kale than previously reported. Moreover, three hydroxycinnamic acids and one flavonoid with an unusual high grade of glycosylation, quercetin-3-disinapoyl-triglucoside-7-diglucoside, have been tentatively identified for the first time. The influence of different extraction conditions (extraction method, solvent type, solvent/solid ratio, and duration of extraction) was investigated. The total flavonol and hydroxycinnamic acid contents in curly kale determined as rutin equivalents (RE) were 646 and 204 mg of RE/100 g of fresh weight (fw), respectively. The contents of individual flavonoids ranged from 2 to 159 mg of RE/100 g of fw, with main compounds kaempferol-3-sinapoyl-diglucoside-7-diglucoside (18.7%) and quercetin-3-sinapoyl-diglucoside-7-diglucoside (16.5%). After acidic hydrolysis, two flavonol aglycones were identified in curly kale, quercetin and kaempferol, with total contents of 44 and 58 mg/100 g of fw, respectively.

KEYWORDS: Curly kale; *Brassica oleracea* L. convar. *acephala* var. *sabellica*; polyphenols; flavonoids; glycosides; hydroxycinnamic acids; extraction; liquid chromatography; mass spectrometry; metabolite profiling

INTRODUCTION

Kale is a leafy green vegetable that belongs to the cabbage family (Brassicaceae), a group of vegetables including cabbage, broccoli, cauliflower and Brussels sprouts that recently have gained increased attention due to their high content of health-promoting phytochemicals. Kale has a high nutritive value due to its richness of phytochemicals, with high concentration of vitamins, minerals, dietary fiber, glucosinolates, and antioxidative compounds, including polyphenols and phenolic acids (*1, 2*). The curly kale varieties (*Brassica oleracea* L. convar. *acephala* var. *sabellica*) have ruffled leaves and fibrous stalks, and they are usually deep green in color. Curly kale is a robust plant tolerating a broad range of agricultural and climate conditions; freezing temperatures even make the kale taste sweeter (*3*).

One group of polyphenols, the flavonoids, is a large family of low molecular weight phenolic compounds. The flavonoids can be divided into subclasses according to the structure of the $C_6-C_3-C_6$ flavone skeleton, with variation in the number and distribution of phenolic hydroxyl groups across the molecules and differences in substitution (*4*). This variability gives rise to more than 8000 different flavonoids at present found in the plant kingdom, and the number is constantly growing with newly identified compounds. The most ubiquitous subclass of flavonoids in plant foods is the flavonols, with the main aglycones quercetin and kaempferol (*5*).

The flavonoid composition in different edible *Brassica* species, for example, pak choi (*Brassica campestris*) (*6, 7*), broccoli (*Brassica oleracea*) (*8*), cauliflower (*Brassica oleracea*) (*9*), turnip tops (*Brassica rapa*) (*10, 11*), and tronchuda cabbage (*Brassica oleracea*) (*12*), has been investigated. The flavonoids found in *Brassica* vegetables are mainly derivatives of the flavonols kaempferol and quercetin, whereas isorhamnetin and myricetin are less common. The flavonols occur in the plants

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as complex conjugates, with one to five sugar moieties bound to the aglycone, and they are often acylated with hydroxycinnamic acids. Variation in the polyphenolic content is related to the biosynthesis in the plant, which is influenced by many factors, such as cultivar, climate, postharvest treatments, and agricultural and environmental factors (*1*). Total polyphenol content in edible parts of kale has been reported to be 2-fold higher than in Brussels sprouts, cauliflower, and broccoli (*13*). In previous papers, only the content of flavonol aglycones in curly kale has been reported (*14*−*16*). The total concentration of quercetin and kaempferol varied between 8 and 91 mg/100 g of fresh weight (fw). Five hydroxybenzoic acids derivatives and four hydroxycinnamic acids have been identified in leaves of kale (*17*), and the total content was reported to be 1.24 mg/100 g of fw.

It is well accepted that fruits and vegetables are important components of a healthy diet and that their consumption helps to prevent a wide range of diseases. Epidemiological data have shown that persons with a high consumption of fruits and vegetables are at a lower risk of several types of cancers (*18*−*20*) and cardiovascular diseases (*21*) and have reduced mortality compared to persons with a low consumption of fruits and vegetables (*22*). These beneficial properties have been associated with the presence of bioactive phytochemicals, such as polyphenols, glucosinolates, carotenoids, tocopherols, and ascorbic acid in fruits and vegetables (*23*). *Brassica* vegetables are known to contain high concentrations of phytochemicals, such as polyphenols (*24*) and vitamins (*2*), and to have high antioxidant capacity (*25*, *26*). However, the exact mechanisms of the positive effects of flavonoids and other polyphenols remain to be unravelled. The bioavailability of a compound is a crucial factor for the assessment of any health-promoting functions in humans. In recent years, several papers on the bioavailability of flavonoids in humans have been published (*27*, *28*). Bioavailability is dependent on both the chemical structure of the polyphenols and the food matrix (*20*). The bioavailability of the flavonol aglycone is affected by the degree and nature of modification and substitution on the flavonol. To be able to understand the bioavailability and functions of flavonoids from plant foods in the human body, it is important to reveal their exact chemical structure and reactive groups. Recent studies on the characterization and identification of individual metabolites in complex plant extracts have shown that HPLC-DAD-ESI-MS^n is a powerful tool for structure elucidation. ESI is considered to provide soft ionization where only protonated or deprotonated molecules are produced. MS/MS, particularly multistage MS/MS with an ion trap, provides further information about the structures of the compounds under analysis. The aim of this work was to identify and quantify the naturally occurring polyphenols in the edible curly kale leaves, after extraction optimization, by using HPLC-DAD-ESI-MS^n.

MATERIALS AND METHODS

Chemicals. Gallic acid, quercetin, quercetin-3-rhamnosylglucoside (rutin), ferulic acid, and Folin−Ciocalteu's phenol reagent were purchased from Sigma Chemical Co. (St. Louis, MO). Kaempferol and sinapic acid were purchased from Fluka Chemie GmbH (Buchs, Switzerland). Sodium carbonate, methanol, acetonitrile, and formic acid were obtained from Merck KGAa (Darmstadt, Germany). All solvents were of HPLC grade, and water was of Milli-Q quality (Millipore Corp., Bedford, MA).

Plant Material. Curly kale (*B. oleracea* L. ssp. *oleracea* convar. *acephala* (DC.) Alef. var. *sabellica* L., 'Reflex') was grown at the Norwegian University of Life Sciences (59° 40′ N) in the same growing field. Five plants of the curly kale cultivar were harvested in October 2007, 140 days after transplantation into the field. The leaves were separated from the stem, and one-third of the leaves at each plant were sampled as a representative selection from the top to the bottom. The leaves were immersed in liquid nitrogen, crushed to a coarse powder in a mortar, and stored at −80 °C for 12 weeks, until extraction.

Extractions of Phenolic Compounds. Before the kale leaf samples were subjected to extraction, the extraction procedure earlier used for cabbage (*29*) was optimized. Ultrasound sonication versus mechanical homogenization was tested. During the first extraction step, 15 mL of methanol was added to 5 g of frozen curly kale. Samples were either ultrasound sonicated for 15 min in an ultrasound bath (10 °C) or homogenized with a Polytron, PT3100 homogenizer (Kinematica AG, Littau, Switzerland) with a speed of 27000 rpm for 45 s. After 15 min on ice (extraction duration time), the samples were centrifuged (31000*g*, 10 min, 4 °C), the supernatants were collected, and the pellets were re-extracted with 10 mL of 80% methanol in water.

Acetone and methanol were tested as the extraction solvents, using 5 g of plant material, a 15 min extraction duration time, and 15 mL of solvent during the first extraction and 10 mL of 80% solvent in water during the re-extraction of the pellet. No difference between methanol and acetone as the extraction solvent was found. Methanol was used in the further experiments.

Three different sample concentrations, 3, 4, and 5 g in 25 mL of aqueous methanol, were also tested. Methanol (15 mL) was added to the frozen curly kale powder, and the sample was homogenized with the Polytron. After 15 min on ice, the sample was centrifuged, the supernatant was collected, and the pellet was re-extracted with 10 mL of 80% methanol in water.

Finally, the influence of the extraction duration, that is, the time between the homogenization and centrifugation, was tested. Duration times of 5, 10, 15, 20, and 60 min were tested. The sample size was 5 g, and the extraction procedure, including Polytron homogenizer and volumes of solvents, was as described above. The extraction efficiency was evaluated by determining the total phenolic (TP) in the extracts. All extractions were made in triplicates and on the same batch of frozen, crushed plant leaves.

On the basis of the results from the testing described above, the most efficient extraction procedure was used. From each of the five plants, 5 g of frozen, ground leaf material was extracted in a two-step procedure using a Polytron homogenizer in a total volume of 25 mL, left for 15 min on ice before centrifugation. The supernatants were combined and stored at −80 °C until analysis. The extractions were made in duplicates.

Total Phenolics. TP content in curly kale extracts was determined according to the Folin−Ciocalteu procedure (*30*, *31*). The extract of curly kale was diluted 1:20 with water, mixed with 1.0 mL of Folin−Ciocalteu's reagent (1:10 v/v, diluted with water), and incubated for 2 min before 0.8 mL of sodium carbonate (7.5% w/v) was added. The mixture was incubated for 60 min at room temperature before absorption was measured at 765 nm in a UV−vis spectrophotometer (Agilent Technologies, 8453 UV−visible, Waldbronn, Germany). TP content was expressed as gallic acid equivalents (GAE) in milligrams per 100 g of fresh weight of curly kale (mg of GAE/100 g of fw). All extracts were analyzed in duplicate.

Acidic Hydrolysis. To determine the content of flavonol aglycones, the curly kale methanolic extracts were subjected to total acidic hydrolysis (*3*). The extract (2 mL) was mixed with 2 M hydrogen chloride (2 mL) in a headspace crimp-top vial (Agilent Technologies). The vials were flushed with nitrogen gas for 15 s, sealed with crimp caps with silicone septa, and kept in a water bath at 94 °C for 60 min. Different duration times of hydrolysis (15, 30, 60, and 90 min) were tested. Sixty minutes of hydrolysis gave the highest yield of the aglycones and was used during quantification of the aglycones. The extract was filtered through a 0.45 μm Millex HV filter (Millipore, Cork, Ireland) and immediately analyzed by HPLC-DAD-ESI-MS^n.

Alkaline Hydrolysis. Alkaline hydrolysis was performed as described by Ferreres et al. (*32*) to obtain information about the acylation of the flavonoids. The extract (2 mL) was mixed with 4 M sodium hydroxide (2 mL) in a headspace crimp-top vial (Agilent Technologies). The vials were flushed with nitrogen gas (15 s), sealed with crimp caps with silicone septa, and kept at room temperature for 16 h. The samples

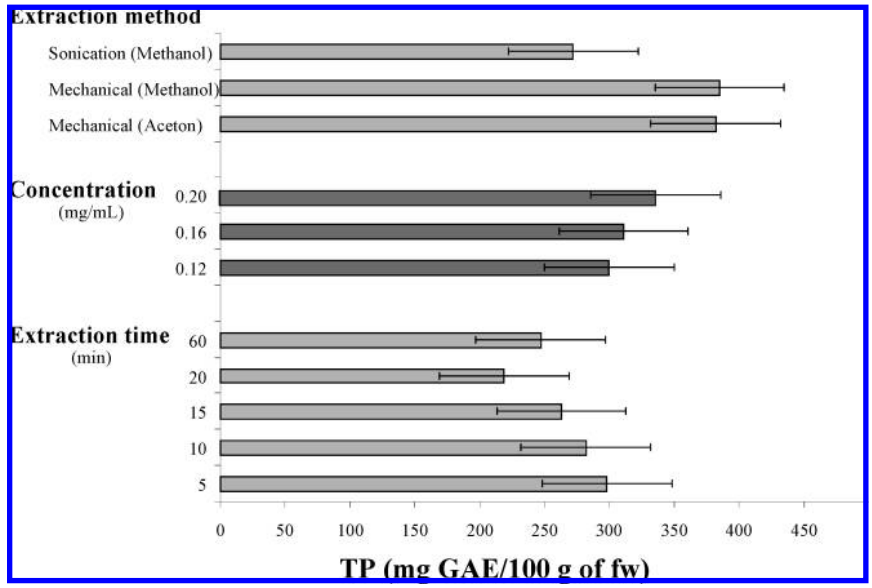

Figure 1. Extraction efficiency of different extraction conditions determined by TP (mg of GAE/100 g of fw) in curly kale extracts: (top) effects of solvent (methanol versus acetone) and mechanical (in methanol) versus sonication (in methanol); (middle) effects of curly kale concentrations; (bottom) effect of extraction durations. All extraction procedures were repeated three times ($n = 3$).

were then acidified with concentrated hydrogen chloride until pH 1−2, filtered through a 0.45 μm Millex HV filter (Millipore), and analyzed by HPLC-DAD-ESI-MS^n.

Quantitative Analysis. Quantification of the aglycones was carried out after acid hydrolysis of the curly kale extracts from five plants. Analysis of the hydrolyzed extract was carried out on an Agilent 1100 series HPLC system (Agilent Technologies) equipped with an autosampler cooled to 4 °C and a diode array detector (DAD, 180−600 nm). Chromatographic separation was performed on an analytical Betasil RP-C_{18} column (250 × 2.1 mm i.d., 5 μm particle size) equipped with a C_{18} guard column (4.0 mm × 2.1 mm i.d., 5 μm particle size), both from Thermo Hypersil-Keystone (Bellefonte, PA). The column temperature was set to 30 °C, and the injection volume was 5 μL. Solvent A consisted of acetic acid/water (2:98, v/v), and solvent B consisted of acetonitrile/acetic acid/water (50:2:48, v/v/v). The elution gradient profile used was 10−45% B in 60 min, 45−100% B in 10 min, followed by 7 min 100% B, with a flow rate of 0.25 mL/min. The column was allowed to equilibrate for 10 min with solvent A between the injections. Aglycones were quantified on the basis of external standards of quercetin and kaempferol, in the concentration range of 40−105 μg/mL, with UV detection at 360 nm. Naturally occurring phenolic compounds were quantified using quercetin-3-rhamnosylglucoside (rutin), in the concentration range of 3−150 μg/mL, with UV detection executed at 330 nm.

Characterization of Phenolic Compounds by HPLC-DAD-ESI-MS^n. Phenolic compounds in the methanolic curly kale extracts and in the hydrolysates were characterized using an Agilent 1100 series HPLC system as described above, with an ESI interface and MSD XCT ion trap mass spectrometer (MS) (Agilent Technologies). The HPLC conditions used were as described above. The LC eluate was introduced directly into the ESI interface without splitting, at a flow rate of 0.25 mL/min, and the phenolic compounds were analyzed in both negative and positive ionization modes. Fragmentation (MS^{2-5}) was carried out in the automatic mode; that is, the two most abundant ions in MS^{1-4} were fragmented. The nebulizer pressure was 40 psi; dry gas flow, 10 mL/min; dry temperature, 350 °C; and capillary voltage, 3.5 kV. Analysis was carried out using scan from *m/z* 100 to 1800, with a scan speed of 27000 amu/s. Helium gas was used as the collision gas in the fragmentation experiments.

Statistical Analysis. The data were analyzed with one-way ANOVA, using the statistical program Minitab release 14.2 (Minitab Inc., State College, PA). Differences were considered to be statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

Extraction of Phenolic Compounds in Curly Kale. Prior to achieving the phenolic profile and quantification of the polyphenols in curly kale, the influence of different extraction conditions (extraction method, solvent type, solvent/solid ratio, and duration of extraction) was investigated. The results from the extraction experiments are shown in **Figure 1**. A significant difference ($p < 0.05$) was found between the extraction methods (mechanical homogenization and ultrasound sonication), with higher extraction yield using mechanical homogenization. The yields of phenolic compounds were equal when using aqueous methanol or aqueous acetone as the extraction solvent ($p > 0.05$). Methanol was chosen in this study because it is more convenient in chromatographic analysis. No significant differences ($p > 0.05$) were observed between the different concentrations of curly kale. Increasing the extraction duration time had neither positive nor negative effect on the extraction yield ($p > 0.05$).

Characterization of Phenolic Compounds by HPLC-DAD-ESI-MS^n. The analysis of the aqueous methanol extracts by LC-MS analysis revealed that flavonols were the major group of phenolic compounds in curly kale leaves. Thirty-two phenolic compounds, 23 flavonoids and 9 hydroxycinnamic acids, were characterized. Characterization of the phenolic compounds was based on chromatographic behavior, mass spectra obtained under electron spray ionization (ESI) conditions, UV−vis spectra, and comparison with reference compounds and scientific publications (*6*−*11*, *33*, *34*). Ionization of the phenolic compounds in negative mode gave the highest sensitivity and selectivity; therefore, only mass spectra and fragmentation patterns obtained in negative mode are discussed. However, ionization in positive mode provided extra certainty in the determination of the molecular masses. The results are summarized in **Table 1**. The chromatographic profiles, recorded at 330 nm, of the naturally occurring phenolic compounds in the curly kale extract and the deacylated phenolic compounds, obtained after alkaline hydrolysis, are shown in **Figure 2**, panels **A** and **B**, respectively.

Alkaline hydrolysis was performed to reduce the complexity of the naturally occurring compounds present in curly kale, due to the release of the hydroxycinnamic acids by cleavage of the

Table 1. Characterization of Phenolic Compounds in Methanol Extracts of Curly Kale and Their Alkaline Hydrolysates Using HPLC with Diode Array and Electrospray Ionization MS^n Detection[i]

peak	tentative identification	t_R (min)	λ_{max} (nm)	MW	MS (*m/z*); ID	*m/z* of the main fragments (% intensity of base peak) MS^2 ions	MS^3 ions	MS^4 ions	concentration (mg of RE/100 g of fw)[a,b]	standard error[b] (±)	% distribution
quercetin glycosides											
33[c]	quercetin-3-triglucoside-7-glucoside	12.2	256, 267sh[d], 354	950	949 $[M-H]^-$	787	625 (83), **445**, 301 (31)	301	—[e]		
34[c]	quercetin-3-triglucoside-7-diglucoside	14.2	254, 267sh, 354	1112	1111 $[M-H]^-$	787	667 (45), **625**, 445 (69)	301	—		
2	quercetin-3-diglucoside-7-glucoside	13.2	254, 267sh, 352	788	787 $[M-H]^-$ 789 $[M+H]^+$	625 465	**445**, 301 (78) 303	301	16	2	1.9
35[c]	quercetin-3-diglucoside-7-diglucoside	15.0	256, 267sh, 350	950	949 $[M-H]^-$	**625**, 463 (8)	463	301	—		
3	quercetin-3-diglucoside-7-glucoside	14.5	ip[f]	788	787 $[M-H]^-$ 789 $[M+H]^+$	**625**, 463 (16) 627	**463**, 301 (15) 465	301303	7	1	0.8
11	quercetin-3-glucoside-7-glucoside	21.0	250, 267sh, 344	626	625 $[M-H]^-$	**463**, 301 (23)	301		nd[g]		
kaempferol glycosides											
36[c]	kaempferol-3-glucoside-7-glucoside	15.7	266, 340	610	609 $[M-H]^-$	**447**, 285 (3)	285		—		
37[c]	kaempferol-3-triglucoside-7-diglucoside	16.6	266, 348	1096	547 $[M-2H]^{2-}$	771	**609**, 285 (19)	285	—		
5	kaempferol-3-diglucoside-7-glucoside	16.1	266, 345	772	771 $[M-H]^-$ 773 $[M+H]^+$	609 611	**429**, 285 (67) 449	285 287	21	2	2.5
7	kaempferol-3-diglucoside-7-diglucoside	17.7	266, 344	934	933 $[M-H]^-$ 935 $[M+H]^+$	609 611	**429**, 285 (73) 287	285	9	0	1.1
19	kaempferol-3-diglucoside-7-glucoside	27.0	266, 346	772	771 $[M-H]^-$ 773 $[M+H]^+$	**609**, 447 (7), 611	447 (8), **285** 449	287	9	0	1.1
20	kaempferol-3-diglucoside-7-diglucoside	27.8	266, 346	934	933 $[M-H]^-$ 957 $[M+Na]^+$	609 611	285, 449		3	1	0.4
40[c]	kaempferol-3-diglucoside[h]	37.9	ip	610	609 $[M-H]^-$	**429**, 285 (60)	285		—		
acylated derivatives of quercetin glycosides											
4	quercetin-3-hydroxyferuloyl-diglucoside-7-glucoside	15.7	266, 340	980	979 $[M-H]^-$	**817**, 787 (89), 625 (78)	625	**445**, 301 (89)	27	2	3.2
6	quercetin-3-caffeoyl-diglucoside-7-glucoside	16.7	250, 340	950	949 $[M-H]^-$	**787**, 625 (60)	625	445 (80), **301**	7	1	0.8
12	quercetin-3-sinapoyl-diglucoside-7-diglucoside	21.7	248, 342	1156	1155 $[M-H]^-$ 1179 $[M+Na]^+$	**949**, 625 (21)	**625**, 445 (4)	625 (70), 445 (70), **301**	144	22	16.5
13	quercetin-3-feruloyl-diglucoside-7-glucoside	22.4	254, 338	964	963 $[M-H]^-$ 987 $[M+Na]^+$	**801**, 625 (68)	625	**445**, 301 (65)	14	1	1.7
14	quercetin-3-feruloyl-diglucoside-7-diglucoside	23.0	254, 338	1126	1125 $[M-H]^-$ 1149 $[M+Na]^+$	**949**, 801 (49), 625 (46)	625	301	nd		
21	quercetin-3-sinapoyl-diglucoside	37.2	ip	832	831 $[M-H]^-$	625	**445**, 301 (69)		4	0	0.5
24	quercetin-3-disinapoyl-triglucoside-7-diglucoside	46.4	242, 334	1524	761 $[M-2H]^{2-}$	599	993	**787**, 445 (13), 301 (5)	4	0	0.5
26	quercetin-3-disinapoyl-triglucoside-7-glucoside	48.0	240,332	1362	680 $[M-2H]^{2-}$	599	993	787 (28), **625**, 445 (50), 301 (49)	15	1	1.8
acylated derivatives of kaempferol glycosides											
8	kaempferol-3-hydroxyferuloyl-diglucoside-7-glucoside	18.6	240, 268, 300	964	963 $[M-H]^-$ 987 $[M+Na]^+$	**801**, 609 (6)	**609**, 429 (4)	**429**, 285 (74)	60	3	7.2
9	kaempferol-3-hydroxyferuloyl-diglucoside-7-diglucoside	19.5	238, 268, 332	1126	1125 $[M-H]^-$ 1151 $[M+Na]^+$	**801**, 609 (30)	609	**429**, 285 (60)	13	2	1.5
10	kaempferol-3-caffeoyl-diglucoside-7-glucoside	20.2	244, 268, 332	934	933 $[M-H]^-$ 957 $[M+Na]^+$	**771**, 609 (50) 509	609 347	**429**, 285 (65) 329	10	1	1.2
16	kaempferol-3-sinapoyl-triglucoside-7-diglucoside	24.0	238, 334	1302	1301 $[M-H]^-$	**977**, 771 (11), 488 (71)	771	**609**, 429, 285 (70)	6	1	0.8
17	kaempferol-3-sinapoyl-diglucoside-7-diglucoside	24.7	238, 268, 334	1140	1139 $[M-H]^-$ 1163 $[M+Na]^+$	815	609	**429**, 285 (70)	159	10	18.9
18	kaempferol-3-feruloyl-diglucoside-7-diglucoside	26.1	244, 268, 330	1110	1109 $[M-H]^-$ 1133 $[M+Na]^+$	**785**, 609 (30)	609	**429**, 285 (64)	32	1	3.8
27	kaempferol-3-disinapoyl-triglucoside-7-diglucoside	48.6	238, 332	1508	753 $[M-2H]^{2-}$	591	977	771 (6), **429**	4	0	0.5
28	kaempferol-3-disinapoyl-triglucoside-7-glucoside	50.4	236, 330	1346	672 $[M-2H]^{2-}$	591	977	771	5	0	0.6
hydroxycinnamic acids											
1	caffeoylquinic acid	9.4	300sh, 320	354	353 $[M-H]^-$	**191**, 179 (30)	127		73	5	8.7
38[c]	caffeic acid	20.5	236, 322	nd					—		
15	hydroxycinnamic derivative	23.3	238, 332	794	793 $[M-H]^-$	673	511	206	68	10	7.9
39[c]	ferulic acid	36.7	238, 322	nd					—		
22	sinapoyl-feruloyl-triglucoside	37.8	240, 324	886	885 $[M-H]^-$ 909 $[M+Na]^+$	**723**, 499 (29) 685	499 523	193 347, 329	6	0	0.8
41[c]	sinapic acid[g]	37.9	238, 324	nd					—		
23	diferuloyl-triglucoside	39.5	240, 300sh, 326	856	855 $[M-H]^-$	**693**, 499 (21)	499	193	2	0	0.2
25	disinapoyl-feruloyl-triglucoside	47.5	240,322	1092	1091 $[M-H]^-$	**929**, 705 (38)	705	499, 193	6	0	0.7
29	sinapoyl-feruloyl-diglucoside	57.5	240, 330	724	723 $[M-H]^-$ 747 $[M+Na]^+$	499	**193**, 179 (90)	134	56	6	6.6
30	disinapoyl-diglucoside	60.4	240, 300sh, 330	754	753 $[M-H]^-$ 777 $[M+Na]^+$	**529**, 223 (7)	223		31	5	3.6
31	trisinapoyl-diglucoside	66.2	240, 330	960	959 $[M-H]^-$	**735**, 511 (16)	529	223	13	2	1.6
32	disinapoyl-feruloyl-diglucoside	68.3	240, 330	930	929 $[M-H]^-$	**705**, 511 (7)	**499**, 223 (9)	193	18	2	2.1

[a] Concentrations are expressed as quercetin-3-rhamnosylglucoside (rutin) equivalents. [b] *n*, 5 plants. [c] Products obtained after alkaline hydrolysis. [d] sh, shoulder. [e] —, not measured. [f] ip, impure peak. [g] nd, not detectable. [h] Coelution of compounds **40** and **41**. The tentative identification of compound **40** is based on MS information and that of compound **41** on UV spectra information and coelution with authentic standard. [i] The native phenolic compounds are quantified as rutin equivalents (RE). Peak numbers refer to peaks in **Figure 2**.

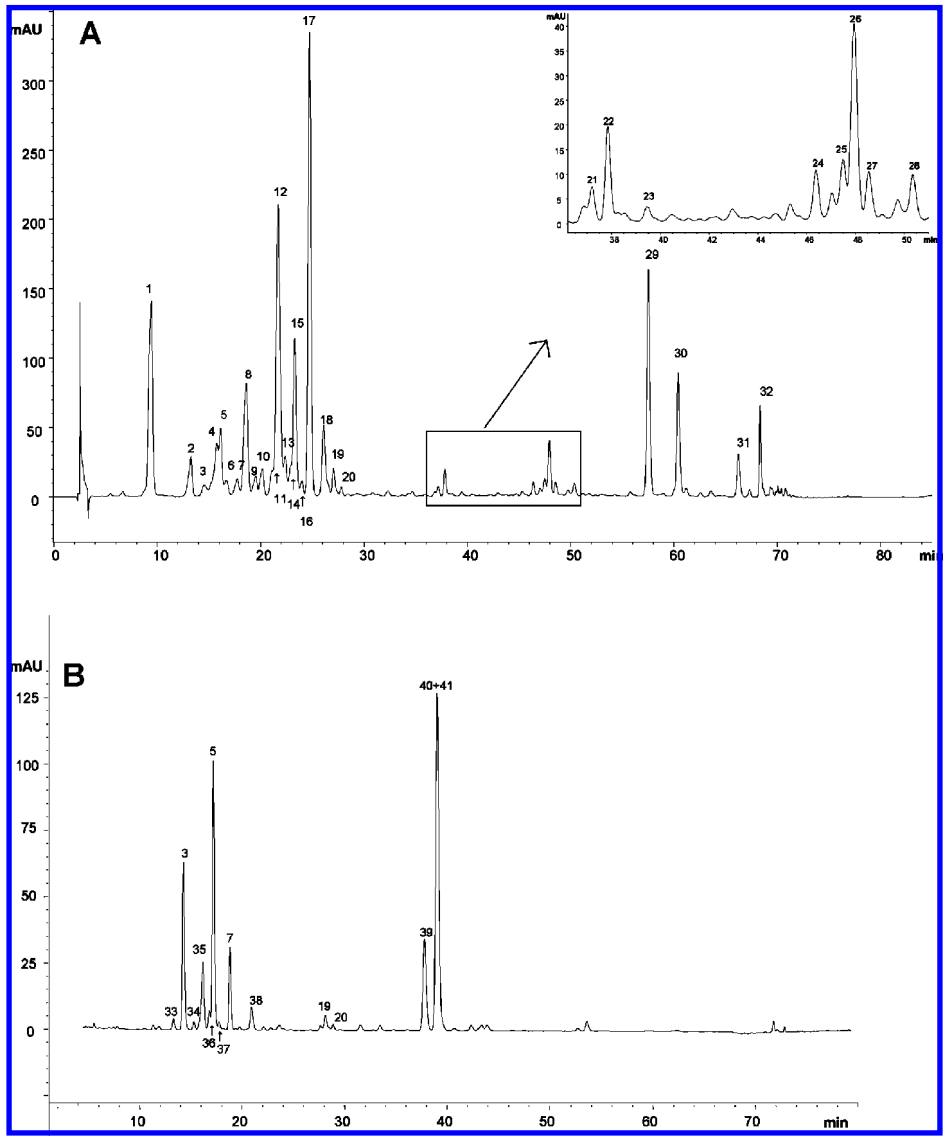

Figure 2. Chromatographic profiles acquired by HPLC-DAD at 330 nm of a methanol extract of curly kale (**A**) and its alkaline hydrolysate (**B**). Peak numbers refer to **Table 1**.

ester linkage between the acids and the glycosides (*35*). The study of the alkaline hydrolysate showed the presence of caffeic acid, ferulic acid, and sinapic acid. The presence of these hydroxycinnamic acids is in accordance with earlier findings in kale (*17*). In the present study, MS information was not very helpful due to low sensitivity of the phenolic acids; thus, the identification was based on UV spectra and coelution with reference standards.

Complete acid hydrolysis of flavonols releases the sugar moiety from the flavonol aglycone by deglycosylation (*32*). In the acid hydrolysate, kaempferol and quercetin aglycones were identified on the basis of retention times, UV spectra, and MS information using commercial reference compounds. Quercetin and kaempferol occurred as mono-, di-, tri-, tetra-, and penta-glycosides, some of them acylated with different hydroxycinnamic acids, which is in accordance with previous findings in other *Brassica* species (*6*−*11, 33*). Previous characterization studies on *B. oleracea* have revealed glucose as the sugar moiety of the glycosylated flavonoids (*6*−*11, 36, 37*). **Figure 3** illustrates an acylated flavonol glycoside, including the structures of the aglycones and the hydroxycinnamic acids found in curly kale.

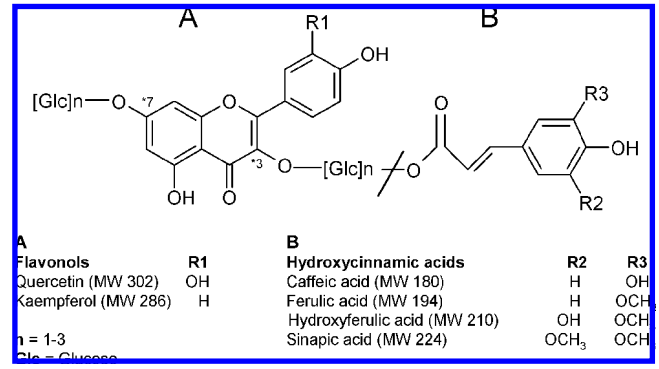

Figure 3. Structure of flavonol glycosides present in curly kale including the aglycones (**A**) and the hydroxycinnamoyl moieties (**B**). Glycosylation of the flavonol occurs at the 3- and 7-positions, marked with an asterisk.

In MS analysis, the first fragmentation of the deprotonated molecular ion $[M - H]^-$ is expected to always be due to the breakdown of the O-glycosidic bond at the 7-position, leading to the fragmentation $[(M - H) - 162]^-$ for monohexosides and $[(M - H) - 324]^-$ for dihexosides (*8, 9*). The remaining glucose moieties of the flavonoid molecule are expected to be linked to

the hydroxyl at the 3-position on the flavonol aglycone. Previous publications (*6*, *32*, *36*, *38*) suggest that the disaccharide moieties of the flavonoids in *Brassica* mainly are sophorosides, that is, two glucose molecules with 1→2 interglucoside linkage. MS fragmentation of sophorosides can produce three characteristic losses of 180, 162, or 120 amu (*6*, *32*). Fragmentation that gives only a loss of 324 amu and in some cases low abundance of 162 amu corresponds to a diglucoside with a 1→6 linkage, that is, gentiobioside (*32*). During the assessment of identifying the flavonoids in curly kale, this information has been used for allocating the position of the glucoside moieties and the interglucosidic linkage.

A typical flavonol UV spectrum consists of two maxima in the ranges of 250−295 nm (band II) and 310−370 nm (band I) (*39*). Quercetin has UV maxima at 256 and 370 nm, and kaempferol has UV maxima at 264 and 366 nm. However, different substitutions of the hydroxyl groups lead to alteration in wavelength and relative intensities of these maxima. The introduction of a glucose moiety at the 7-position has been reported to have minimal effect on the wavelength maximum and the spectrum shape. In contrast, blocking of the hydroxyl at the 3-position causes a hypsochromic shift of band I (*40*). Acylation with aromatic acids will also influence the UV spectrum of the flavonoids. However, using UV spectra for identification required baseline-separated peaks.

Flavonol Glycosides. Numbers refer to peaks in **Figure 2** and the corresponding compounds in **Table 1**. Three peaks, 2, 3, and 11, had MS fragmentation ions at *m*/*z* 301 and 303 in negative and positive mode, respectively, and were recognized as derivatives of quercetin. Compounds **2** and **3** both had deprotonated molecular ions at *m*/*z* 787, corresponding to quercetin triglucoside, and lost one hexose moiety (162 amu) at the 7-position during MS^2 fragmentation. However, further fragmentation of compounds **2** and **3** differed. MS^3 fragmentation of compound **2** gave a loss of 180 amu, which corresponds to a sophoroside in the 3-position (*6*, *32*). During further fragmentation (MS^3 and MS^4) of compound **3**, a loss of 162 amu, but not 180 amu, was observed, indicating a different interglycosidic linkage than in compound **2**. Compound **2** had absorption maxima at ∼254 and ∼352 nm, indicating glycosylation at the 7- and 3-positions, whereas the UV spectrum of peak 3 was impure and could not be interpreted. Compound **2** was tentatively identified as quercetin-3-sophoroside-7-glucoside, and compound **3** was tentatively identified as quercetin-3-diglucoside-7-glucoside. Compound **11**, detected in small quantities in curly kale, was identified as quercetin-3-glucoside-7-glucoside. The fragmentation pattern (*m*/*z* 625→463→301) of compound **11** revealed the presence of a glucoside residue (loss of 162 amu) in both positions 3 and 7.

Four compounds (**5**, **7**, **19**, and **20**) in curly kale were identified as kaempferol glucosides on the basis of their mass spectra and UV spectra. From the UV spectra, MS fragmentation patterns, and previous publication (*8*), compounds **5** and **19** were both tentatively assigned as kaempferol-3-diglucoside-7-glucoside. However, the retention times of the two compounds differed by 10.9 min, indicating two distinct interglycosidic linkages, that is, 1→2 (compound **5**) and 1→6 (compound **19**). In accordance with the literature (*33*), kaempferols containing diglucosides with a 1→6 linkage have longer retention times compared to kaempferols containing 1→2-linked diglucosides, further supporting the determination of the interglucoside linkage. Fragmentation of compound **7** (*m*/*z* 933→609→429→285) revealed the presence of a diglucoside moiety at the 7-position and a sophoroside (1→2 linkage) in the 3-position. Compound **20**, with a similar molecular mass, eluted 10 min later, and fragmentation of this compound (*m*/*z* 933→609→285) revealed diglucoside residues in both positions 3 and 7 and a 1→6 interglycosidic linkage at the 3-position. Both compounds **7** and **20** were thus identified as kaempferol-3-diglucoside-7-diglucoside, a 3-*O*-sophoroside and a 3-*O*-gentiobioside, respectively.

After alkaline hydrolysis, all of the native flavonol glucosides (compounds **2**, **3**, **5**, **7**, **11**, **19**, and **20**) remained present, but five new flavonoids (compounds **33**−**37**) were observed in the chromatogram (**Figure 2B**). These compounds with the exception of **35** have relatively low abundance, due to their low abundance of corresponding native acylated derivatives. The presence of kaempferol-3-diglucoside (compound **40**) in the alkaline hydrolysate was unexpected because no kaempferols with only glucosides in the 3-position were present in the methanolic extracts. The occurrence of kaempferol-3-diglucoside indicates that also 7-O-glycosidic bonds, in addition to ester bonds, were cleaved during alkaline hydrolysis. This finding substantiates that the O-glycosidic bond at the 7-position is the weakest glycosidic linkage in the flavonol molecule, which supports the assumptions made by Llorach et al. (*9*). In the HPLC chromatogram (**Figure 2B**), sinapic acid (**41**) coeluted with **40** and dominated the UV spectrum of this peak.

Acylated Derivatives of Quercetin- and Kaempferol-Glycosides. Several of the flavonols in the curly kale extract had UV spectra with a broad maximum around 330−340 nm (**Table 1**), suggesting they were acylated with hydroxycinnamic acids (*39*). Acylation with hydroxycinnamic acids makes band I of the UV spectrum shift to lower wavelengths with an increase in intensity depending on the number of acyl residues present in the molecule (*39*). A total of 16 acylated flavonol glycosides (compounds **4**, **6**, **8**, **9**, **10**, **12**−**14**, **16**−**18**, **21**, **24**, and **26**−**28**) were detected in the curly kale extracts. All acylated derivatives of quercetin and kaempferol disappeared after alkaline hydrolysis due to cleavage of the ester linkage (**Figure 2**).

During the MS fragmentation of compounds **4**, **8**, and **9**, a loss of 192 amu, corresponding to hydroxyferulic acid, in the terminal 3-position, previously reported in *Brassica* (*6*−*8*, *10*, *33*), was observed. Compound **4** first lost 192 amu, followed by loss of 162 amu, that is, a glucoside residue at the 7-position and a diglucoside moiety at the 3-position (loss of 324 amu); thus, compound **4** was assigned as quercetin-3-hydroxyferuloyl-diglucoside-7-glucoside. A similar fragmentation pattern was observed for compound **8**, with a loss of 162 amu corresponding to a glucoside residue at the 7-position. Further fragmentation revealed a loss of 192 amu (hydroxyferulic acid) and a diglucoside moiety at the 3-position (loss of 325 amu) and kaempferol as the aglycone (*m*/*z* 285). Compound **8** was tentatively identified as kaempferol-3-hydroxyferuloyl-diglucoside-7-glucoside. The MS analysis of compound **9** showed that fragmentation of the molecular ion (*m*/*z* 1125) produced a fragment at *m*/*z* 801 due to the loss of two glucosyl residues from position 7. Further fragmentation of the *m*/*z* 801 ion showed the sequential loss of hydroxyferulic acid (192 amu), followed by a loss of two glucosyl residues from position 3, resulting in the kaempferol aglycone ion (*m*/*z* 285); thus, compound **9** was assigned as kaempferol-3-hydroxyferuloyl-diglucoside-7-diglucoside. During alkaline hydrolysis, compounds **4**, **8**, and **9** disappeared, while the corresponding flavonol glycosides, compounds **3**, **5**, and **7**, increased (**Figure 2**), substantiating the presence of these acylated flavonol glycosides.

The MS analyses of compounds **6** and **10** showed that the deprotonated molecular ions at *m*/*z* 949 and 933 lost a monohexose residue from position 7, giving fragments at *m*/*z*

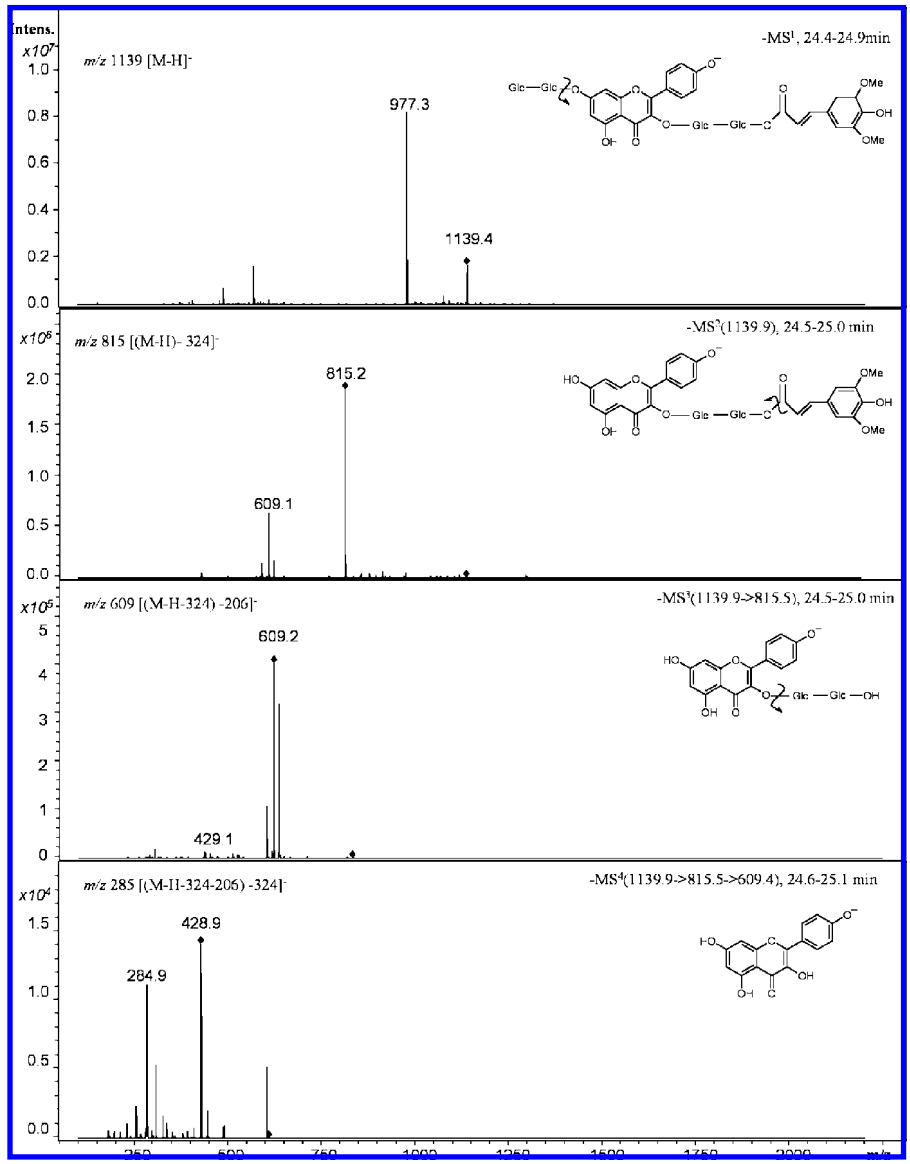

Figure 4. MS^{1-4} analysis (negative mode) and fragmentation pathway of compound **17**, tentatively identified as kaempferol-3-sinapoyl-diglucoside-7-diglucoside; sequential fragmentation of the ions *m*/*z* 1139 and the derived major fragmented ions. The arrow drawn in the structure indicates the position of the next fragmentation.

787 and 771, respectively. Further fragmentation revealed a loss of 162 amu, corresponding to caffeic acid, which was further confirmed by the UV spectra (maximum ~330 nm) and disappearance after alkaline hydrolysis. The MS^3 fragmentation gave a loss of a dihexose residue at the 3-position (324 amu), leading to the aglycones. Thus, compounds **6** and **10** were tentatively identified as quercetin-3-caffeoyl-diglucoside-7-glucoside and kaempferol-3-caffeoyl-diglucoside-7-glucoside, respectively.

Compounds **12** and **17** displayed similar glycosylations and acylations patterns, but they had different aglycones, that is, MS^4 ions at *m*/*z* 301 and 285, respectively. Compound **12** was identified as quercetin-3-sinapoyl-diglucoside-7-diglucoside. Compound **17** was the most abundant flavonoid in the curly kale and was identified as kaempferol-3-sinapoyl-diglucoside-7-diglucoside with a molecular weight of 1140. **Figure 4** illustrates the MS fragmentation steps of compound **17**. The arrows indicate the sequential fragmentation occurring in the mass spectrometer, starting with the breakdown of the O-glucosidic bond at the 7-position leading to the $[(M - H) - 324]^-$ ion. Further fragmentation of the acylated ion at *m*/*z* 815 resulted in the loss of the hydroxycinnamic acid residue (sinapoyl), cleavage of the 3-O-glycosidic bond, and finally the loss of a diglucosyl residue (sophoroside), producing the aglycone ion (*m*/*z* 285). The last fragmentation gave ions at both *m*/*z* 429 (loss of 180 amu) and *m*/*z* 285 (loss of 324 amu), with relative abundances of 100 and 70%, respectively.

Compound **24** had $[M - 2H]^{2-}$ at *m*/*z* 761, which corresponded to a molecular weight of 1524. The major MS^2 fragments were double-charged ions at *m*/*z* 599 (loss of 324 amu). Fragmentation of *m*/*z* 599 caused single-charged ions at *m*/*z* 993, by loss of a sinapoyl residue (206 amu). Additional fragmentation revealed the presence of another sinapoyl residue (loss of 206 amu) followed by the loss of three glucosyl residues from position 3 to obtain the quercetin aglycone. Compound **24** was thus tentatively identified, for the first time in *Brassica*, as quercetin-3-disinapoyl-triglucoside-7-diglucoside. A similar fragmentation pattern was observed for the acylated flavonol-glycosides **26**, **27**, and **28**. Compound **26** was assigned as quercetin-3-disinapoyl-triglucoside-7-glucoside, previously detected in broccoli (*8*). Compounds **27** and **28**, tentatively identified as kaempferol-3-disinapoyl-triglucoside-7-diglucoside

Table 2. Contents of Total Phenolics (TP), Total Aglycones (Kaempferol and Quercetin after Acid Hydrolysis), Total Flavonols, and Total Hydroxycinnamic Acid Derivatives in Methanol Extracts of Five Plants of Curly Kale (A−E)

sample	TP (mg of GAE[a]/100 g of fw)	kaempferol (mg/100 g of fw)	quercetin (mg/100 g of fw)	total native flavonols (mg of RE[b]/100 g of fw)	total native hydroxycinnamic acids (mg of RE[b]/100 g of fw)
A	389	52.2	44.0	601	250
B	305	57.7	34.2	491	158
C	384	58.0	44.7	644	230
D	363	60.7	41.7	661	148
E	478	61.4	56.2	831	234
average	384 ± 62	58 ± 4	44 ± 8	646 ± 123	204 ± 47

[a] TP was determined as gallic acid equivalents (GAE) according to Folin−Ciocalteu's method. [b] Native glycosylated flavonols and hydroxycinnamic acids were determined as rutin equivalents (RE).

and kaempferol-3-disinapoyl-triglucoside-7-glucoside, respectively, have recently been found in small amounts in cauliflower (*9*).

The loss of 176 amu during fragmentations of compounds **13**, **14**, and **18** revealed the presence of feruloyl derivatives. The fragmentation pattern of compounds **13** and **14** were *m*/*z* 963→801→625→301 and *m*/*z* 1125→949→625→301, respectively, indicating a glucoside residue (loss of 162) in the 7-position in compound **13** and a diglucoside residue (loss of 324 amu) in the 7-position in compound **14**. In both compounds a dihexose residue (loss of 324 amu) and a terminal feruloyl derivative (loss of 176 amu) in the 3-position was observed. Compounds **13** and **14** had the same UV maxima and the same aglycone (quercetin) and were assigned as quercetin-3-feruloyl-diglucoside-7-glucoside and quercetin-3-feruloyl-diglucoside-7-diglucoside, respectively. MS analysis of compound **18** showed a deprotonated molecular ion at *m*/*z* 1109. Fragmentation produced ions at *m*/*z* 785, corresponding to loss of a diglucose moiety. MS^3 of the produced ion (*m*/*z* 785) led to loss of 176, corresponding to ferulic acid. The last cleavage led to loss of the glucosidic fraction (324 amu), and the aglycone ion (*m*/*z* 285) was observed. In accordance with a study on flavonoids in cauliflower (*9*), compound **18** was tentatively identified as kaempferol-3-feruloyl-diglucoside-7-diglucoside.

Hydroxycinnamic Acids. Nine hydroxycinnamic acid derivatives in the curly kale were characterized and eight of them identified (**Table 1**). In accordance with the literature (*41*), the hydroxycinnamic acid derivatives had UV spectra with absorptions maxima at ∼240 and ∼320−330 nm. During alkaline hydrolysis the ester linkage between the hydroxycinnamic acid and the glycoside was cleaved.

The presence of 3-*O*-caffeoylquinic acid (chlorogenic acid) as compound **1** was confirmed by MS spectra, fragmentation pattern *m*/*z* 353→191 (base peak), 179 (∼50% of the base peak) (*42*), and coelution with reference standard and was in accordance with previous findings in *Brassica* (*7*, *43*).

During MS fragmentation of hydroxycinnamic acids containing sinapic acids, that is, compounds **22**, **25**, and **29**−**31**, loss of sinapic acid (224 amu) was observed. MS analysis of compounds containing ferulic acids, that is, compounds **22**, **23**, **25**, **29**, and **32**, gave ions at *m*/*z* 193 upon fragmentation. The loss of 306 amu represents the loss of a diglucoside moiety, in accordance with earlier findings in *Brassica* (*7*). The MS analysis of compound **25** gave the following fragmentation ions: *m*/*z* 1091→929→705→499→193, corresponding to the sequential loss of a glucoside residue (162 amu), a sinapic acid (224 amu), a sinapic acid residue (206 amu), and finally a loss of 306 amu corresponding to a diglucoside moiety. The presence of MS^4 fragments at *m*/*z* 193 substantiated the presence of a ferulic acid. Compound **25** was thus assigned as disinapoyl-feruloyl-triglucoside. Similar fragmentation patterns were observed for compounds **22**, **29**, and **32**, which were identified as sinapoyl-feruloyl-triglucoside, sinapoyl-feruloyl-diglucoside, and disinapoyl-feruloyl-diglucoside, respectively. Compounds **30** and **31** contained a glucoside moiety (loss of 306 amu) and two or three sinapic acid residues, respectively, and were tentatively identified as disinapoyl-diglucoside and trisinapoyl-diglucoside. Fragmentations of compound **23** revealed the presence of two ferulic acid residues, and three glucose molecules; thus, the compound was identified as diferuloyl-triglucoside. Compounds **1**, **29**, **30**, **31**, and **32** have previously been described in different *Brassica* species, such as cauliflower (*9*), turnip tops (*11*), and pak choi (*7*). To the best of our knowledge, the characterization of compounds **22**, **23**, and **25** has not previously been reported in any *Brassica* vegetables.

According to UV spectra and mass spectral data, peak 15 had structural features similar to the compounds identified above. Furthermore, peak 15 disappeared after alkaline hydrolysis. The MS analysis revealed $[M - H]^-$ at *m*/*z* 793, and MS^{2-4} fragmentation led to ions at *m*/*z* 673, 511, and 206, with losses of 120, 162, and 305 amu, respectively. However, a more exact identification could not be accomplished.

Quantitative Determination of Polyphenolic Compounds in Curly Kale. Quantitative studies on kale and other *Brassica* species have previously been performed with the focus on the flavonol aglycones (*14*−*16*). The concentrations of quercetin and kaempferol, which were the two identified aglycones in leaves of curly kale, are shown in **Table 2**. The quercetin content in the five plants varied between 34.2 and 56.2 mg/100 g of fw, with a mean value of 44.1 mg/100 g of fw. The variation of the kaempferol content in the five plants was from 52.2 to 61.4 mg/100 g of fw, with a mean value of 58.0 mg/100 g of fw. Previous studies on flavonol aglycones in kale have reported a total content of quercetin between 7.7 and 31.8 mg/100 g of fw and kaempferol levels between 23.5 and 90.3 mg/100 g of fw (*14*−*16*).

The naturally occurring flavonols were quantified as rutin equivalents (RE), as commercial standards are not available for these compounds. The concentrations of the individual compounds ranged from 2 to 159 mg of RE/100 g of fw (**Table 1**). The compounds present at the highest levels in the kale extract were kaempferol-3-sinapoyl-diglucoside-7-diglucoside (compound **17**), quercetin-3-sinapoyl-diglucoside-7-diglucoside (compound **12**), and caffeoylquinic acid (compound **1**), representing 18.7, 16.5, and 8.7% of the total amount, respectively. The total content of naturally occurring flavonol glycosides in the five plants varied between 491 and 831 mg of RE/100 g of fw, with a mean value 646 mg of RE/100 g of fw (**Table 2**). The total content of naturally occurring hydroxycinnamic acids in the five plants varied between 148 and 250 mg of RE/100 g of fw, with a mean value of 204 mg of RE/100 g of fw (**Table 2**).

TP content, as determined with the Folin−Ciocalteu method, in the curly kale methanol extract was 377 ± 64 mg of GAE/

100 g of fw. The content in the five analyzed plants were significantly different ($p < 0.05$) and varied from 305 to 478 mg of GAE/100 g of fw. Total phenolics in kale (*B. oleracea* var. *acephala* cv. Winterbor) have been reported to be 773 mg of chlorogenic equiv/100 g of fw (*13*). Another study on *B. oleracea* var. *acephala*, of unknown cultivar, reported TP content to be 136 mg of GAE/100 g of fw (*17*).

In the present study, considerable variations (16−23%) were found in the phenolic content, total flavonols, and total hydroxycinnamic acids in curly kale plants from the same cultivar, grown and harvested from the same field at the same time (**Table 2**). These variations may be related to the differences in the biosynthesis of the phenolic compounds in the plants and the selection of leaf materials, making comparison of data from different studies and varieties difficult. However, it should be pointed out that the total phenolics in curly kale are higher (377 mg of GAE/100 g of fw, or, based on dry weight, 1700 mg of GAE/100 g) compared to other *Brassica* species (531−1285 mg of GAE/100 g of dry weight) (*44*).

In summary, this is the first study characterizing and quantifying the flavonoids present in curly kale. The main naturally occurring phenolic compounds identified by HPLC-DAD-ESI-MS^n were flavonols and hydroxycinnamic acids, both highly glycosylated and acylated. It is expected that different glycosylation and acylation of the flavonol give rise to variation in biological activities in the human body, due to differences in absorption, distribution, and metabolism profiles (*45*). Furthermore, this study shows that curly kale is a considerable source of polyphenols, especially flavonoids, with a total content of 0.6 g of RE/100 g of fw. It is hoped that information on the identity of the polyphenols in one of the nutritionally richest vegetables can be used in future databases. Both the high content of polyphenols and the number and high complexity of the flavonols and hydroxycinnamic acids present indicate that curly kale is interesting for bioactivity studies in humans.

ABBREVIATIONS USED

amu, atomic mass unit; ANOVA, analysis of variance; DAD, diode array absorbance detector; ESI, electrospray ionization; fw, fresh weight; GAE, gallic acid equivalents; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometer; MW, molecular weight; RE, rutin equivalents; TP, total phenolics; UV−vis, ultraviolet−visible light.

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