Characterization of flavonol conjugates in immature leaves of pak choi [Brassica rapa L. Ssp chinensis L. (Hanelt.)] by HPLC-DAD and LC-MS/MS

ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · JULY 2006

Impact Factor: 2.91 · DOI: 10.1021/jf060154j · Source: PubMed

5 AUTHORS, INCLUDING:

Simone Rochfort
Department of Environment and Primary Ind…
67 PUBLICATIONS **1,264** CITATIONS
SEE PROFILE

Bruce Tomkins
Department of Environment and Primary Ind…
26 PUBLICATIONS **290** CITATIONS
SEE PROFILE

Characterization of Flavonol Conjugates in Immature Leaves of Pak Choi [*Brassica rapa* L. Ssp. *chinensis* L. (Hanelt.)] by HPLC-DAD and LC-MS/MS

SIMONE J. ROCHFORT,*,† MICHAEL IMSIC,‡ ROD JONES,‡
V. CRAIGE TRENERRY,† AND BRUCE TOMKINS‡

Department of Primary Industries, Primary Industries Research Victoria, Werribee Centre, 621 Sneydes Road, Werribee 3030, Victoria, Australia, and Department of Primary Industries, Primary Industries Research Victoria, Knoxfield Centre, 621 Burwood Highway, Knoxfield 3152, Victoria, Australia

The flavonoid composition of immature leaves of pak choi [*Brassica rapa* L. ssp. *chinensis* L. (Hanelt.)] was investigated. Flavonol aglycone content was measured in 11 pak choi varieties, indicating significant differences ($P < 0.05$) in content between varieties and relatively high contents of kaempferol and isorhamnetin. Levels of quercetin ranged from 3.2 to 6.1 mg/100 g of dry weight (DW), whereas levels of isorhamnetin and kaempferol were significantly higher (8.1−35.1 and 36.0−102.6 mg/100 g of DW, respectively). A large number of glycoside and hydroxycinnamic acid derivatives of quercetin, kaempferol, and isorhamnetin were identified in cv. 'Shanghai' by LC/UV-DAD/ESI-MS/MS. The UV-DAD data allowed identification of hydroxycinnamic acid derivatives, but detailed MS/MS fragmentations were required for the structure elucidation. Pak choi could be a potentially important source of dietary flavonols, in particular, kaempferol and isorhamnetin.

KEYWORDS: Food analysis; pak choi; flavonol; glycosides; hydroxycinnamic acid esters; liquid chromatography, mass spectrometry

INTRODUCTION

There is increasing evidence that flavonols demonstrate beneficial properties for human health. The major flavonols found in commonly consumed vegetables are quercetin and kaempferol (*1*); isorhamnetin is less common. In particular, quercetin has been the focus of several biological studies in which the compound has demonstrated in vitro activity against multiple types of cancer (*2*, *3*). Quercetin is known to be a potent free radical scavenger and antioxidant and is also considered to be protective against cardiovascular disease (*4*, *5*). More recently, the closely related compound, kaempferol, has also been shown to possess strong antioxidant activity in its own right (*6*). Quercetin and kaempferol were recently found to act synergistically in the inhibition of cell proliferation in human gut cancer lines (*7*). As well as being contained in glycosylated form in some vegetables, such as fennel (*8*), isorhamnetin is the major metabolite of quercetin in plasma and is known to have potent vasodilator effects (*9*, *10*). Analysis for the presence of these compounds has therefore become more important.

Many recent in vitro studies have been conducted with a focus on the aglycone cores, hence the importance of the characterization and quantitation of aglycones for comparison, but in plant foods the flavonols do not necessarily occur as free compounds but rather as complex conjugates. In vegetables such as onions, there is a considerable body of work that demonstrates that flavonols occur as relatively simple mono- or diglucose conjugates (*11*). More recent work has demonstrated that in *Brassica* species such as cauliflower and broccoli, the flavonoids are more complex, with up to five sugar residues present, and that these may be further substituted with hydroxycinnamic residues (*12*, *13*). It may be expected that the different glycosides possess different biological properties for human consumers. For instance, the degree and nature of modification may affect the bioavailability of the flavonol core; therefore, it is important to be able to characterize the different glycosides.

Pak choi [*Brassica rapa* L. ssp. *chinensis* L. (Hanelt.)], also known as bok or buk choy, is gaining increasing popularity in Western diets. Often the mature plants are consumed steamed or sautéed. However, the immature leaves are also commonly consumed as part of salad mixes. Despite this, there is little in the scientific literature regarding the phenolic composition of this vegetable. The aim of this work was to analyze pak choi both for total flavonol (aglycone) content and to identify the major conjugated flavonols. The complexity of the pak choi flavonoid extract necessitated in-depth analysis by liquid chromatography−ultraviolet diode array detection−electrospray ionization tandem mass spectrometry (LC/UV-DAD/ESI-MS/MS).

* Author to whom correspondence should be addressed (telephone +61 3 97428704; e-mail simone.rochfort@dpi.vic.gov.au).
† Primary Industries Research Victoria, Werribee.
‡ Primary Industries Research Victoria, Knoxfield.

Table 1. Quercetin, Kaempferol, and Isorhamnetin Aglycone Content in the Immature Leaves of 11 Cultivars of Pak Choi[a]

	mg/100 g of DW		
cultivar	quercetin	kaempferol	isorhamnetin
Choko	5.4	72.3	21.9
Diggers	4.2	72.1	16.3
Green	4.8	68.3	18.6
Green Fortune	4.2	102.6	16.3
Joi Choi 1	6.1	60.4	24.0
Joi Choi 2	5.9	50.5	15.0
Lunar Queen	5.4	76.5	20.7
Mei Qing	2.9	71.4	12.1
Shanghai	4.7	92.0	19.8
Tokyo Belle	4.8	73.8	8.1
Sumo	7.1	81.1	35.1
LSD ($P < 0.05$)	3.2	36.0	11.6

[a] Plants were grown under identical glasshouse conditions and harvested 8 weeks after germination. Contents are expressed as mean values ($n = 5$), and LSD at $P < 0.05$.

MATERIALS AND METHODS

Materials. Pak choi [*B. rapa* L. ssp. *chinensis* L. (Hanelt.)] seedlings were transplanted into 150 mm pots containing a soil-free potting mix ≈6 weeks after germination. Seedlings were grown in a glasshouse with a mean minimum temperature of 13.1 °C and mean maximum temperature of 25.4 °C and harvested 28 days after transplanting. Mean maximum light levels during the growing period were 53.8 klx. Pak choi seedlings were planted in a replicated block design with $n = 5$ and were harvested 28 days after transplanting. Seedlings were separated from root tissue, immediately snap frozen, and then freeze-dried in preparation for flavonol analysis. Chemicals and reagents were obtained from the following commercial sources: rutin (Sigma), quercetin (Sigma), kaempferol (Sigma), isorhamnetin (Carl Roth) formic acid (Ajax Finechemicals), NaOH (Ajax Finechemicals), *tert*-butylhydroquinone (Sigma), and hydrochloric acid (Merck). Methanol (HPLC grade) was purchased from J. T. Baker (Deventer, Netherlands). All solvents were of HPLC grade (Mallinchkrodt Chemicals). All samples, solutions, and buffers were prepared using Milli-Q water.

Quantitative Analysis of Pak Choi Cultivars. *Acid Hydrolysis.* The flavonoid aglycones were prepared by acidic hydrolysis as follows: 40 mL of 62.5% aqueous methanol containing 2 g/L *tert*-butylhydroquinone was added per 0.5 g of freeze-dried, powdered pak choi. Ten milliliters of 8 M HCl was added to the stirring solution, and the mixture was refluxed for 4 h. After this time, the mixture was cooled, diluted to 100 mL with MeOH, and sonicated for 10 min. The extract was filtered through a 0.45 μm Teflon filter before HPLC analysis. The method was optimized in terms of sample size, acid concentration, and hydrolysis period for pak choi and characterized by spiking recovery studies.

HPLC Analysis of Aglycone Concentration. Flavonoid aglycones were separated on a 250 × 4.6 mm id., 5 μm, Alltima HP C-18 column (Alltech Associates) and a GBC HPLC (Melbourne, Australia) equipped with a PDA detector using a binary gradient of 0.5% orthophosphoric acid (v/v) in 30% methanol (v/v) (mobile phase A) and 0.5% orthophosphoric acid (v/v) in methanol (mobile phase B) according to the following program: The proportion of B was increased from 20% B to 35% B over the first 30 min, then to 85% B over the next 5 min, maintained at 85% B for 5 min, and finally returned to the initial condition on 20% B. The flow rate was 1 mL/min, and the column temperature was 30 °C. Flavonoid aglycone content was quantified by comparison with authentic compounds. Calibration was carried out with six points from 10 ng to 3 μg (on column amounts). Response was linear, and all analyses were within this range.

Statistical Analysis. Flavonol content in pak choi varieties (**Table 1**) was analyzed by ANOVA using Genstat vers. 8.

Qualitative Analysis of Pak Choi Cv. 'Shanghai'. *Preparation of Extract.* Freeze-dried, ground pak choi (1.0 g) was extracted with 20% H_2O/MeOH (100 mL) by stirring for 30 min. The material was sonicated for 5 min and then filtered under vacuum. Five milliliters of solution was dried under a stream of nitrogen. The material was resuspended in 30% H_2O/MeOH (600 μL) and filtered through a 0.45 μm nylon filter. Five microliters was injected onto the HPLC for analysis.

Hydrolysis. (a) Acid Hydrolysis. The free flavonoid aglycones were prepared by acidic hydrolysis as follows: 40 mL of 62.5% aqueous methanol containing 2 g/L *tert*-butylhydroquinone was added per gram of freeze-dried, powdered pak choi. Ten milliliters of 8 M HCl was added to the stirring solution and the mixture refluxed for 3.5 h. After this time, the mixture was cooled, diluted to 100 mL with MeOH, and sonicated for 5 min. The extract was filtered through a 0.45 μm Teflon filter before LC-MS/MS analysis.

(b) Alkaline Hydrolysis. Flavonoid glycones were prepared by alkaline hydrolysis as follows: freeze-dried, ground pak choi (1.0 g) was stirred in a 2.6 M 53% aq methanol solution of NaOH (60 mL) for 20 h. After this time, the mixture was filtered under vacuum, and a 50 μL subsample was analyzed by LC-MS/MS.

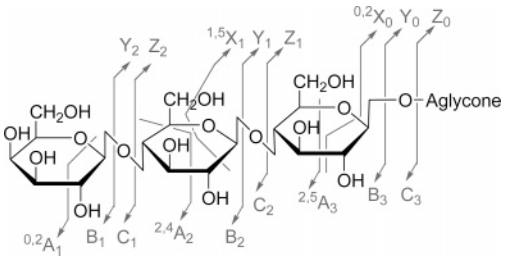
LC-MS/MS Analysis. For LC-MS/MS experiments, an Agilent 1100 series HPLC (Waldbronn, Germany) equipped with a quaternary gradient pump, autosampler with sample cooler (maintained at 4 °C), and diode array detector was coupled with a Thermo Electron LTQ ion trap mass spectrometer (Waltham, MA). Five microliters of extract was injected onto a 150 × 2.1 mm i.d., 3 μm, Thermo BDS Hypersil C18 column (Waltham, MA). The mobile phase consisted of three components, A (distilled water); B (acetonitrile); and C (10% aqueous formic acid), and followed the gradients at a flow rate of 0.2 mL/min with C maintained at 1% throughout: gradient 1, 0−5 min, 95% A; 5−40 min, 30% A; 45−46 min 95% A; 46−50 min (0.3 mL/min) 95% A; gradient 2, 0−15 min, 91% A; 15−27 min, 87% A; 27−35 min, 54% A; 35−53 min 9% A; 53−54 min, 91% A; 54−58 min (0.3 mL/min) 91% A. MS/MS data from both gradients were used to assemble **Tables 2**−**4**. The identities of kaempferol, quercetin, and isorhamnetin were confirmed by injection of pure compounds.

Mass Spectrometric Acquisition Parameters. For LC-MS/MS experiments a data-dependent protocol was used in ESI negative mode with a mass range of 200−2000 amu. Dynamic exclusion was engaged with a 20 s exclusion time. Data were acquired using automated MS/MS settings with a target of 30000, a normalized collision energy of 35, and an ion maximum time of 200 ms. The heated capillary was maintained at 200 °C, and the sheath, auxiliary, and sweep gases were at 25, 2, and 14 units, respectively. Source voltage was set to 3.4 kV with a capillary voltage of −43 V. Prior to data acquisition the system was tuned using a 250 μg/mL standard of rutin. The rutin was infused via syringe pump through a T-piece at a rate of 5 μL/min with an HPLC flow rate of 0.2 mL/min and a solvent composition of 49% A, 50% B, and 1% C.

RESULTS AND DISCUSSION

Analysis of the flavonol aglycone content in 11 pak choi cultivars was carried out by acid hydrolysis followed by HPLC analysis (gradient 1). Identification of the aglycones was confirmed by comparison of retention time, DAD information, and co-injection with standards. The results, summarized in **Table 1**, indicated that immature pak choi leaves may be a good additional source of both kaempferol and isorhamnetin in the Western diet. Kaempferol content ranged from 36 to 103 mg/100 g of dry weight (DW), which is equivalent to ≈4−10 mg/100 g of fresh weight (FW) [pak choi leaf water content equals ≈90% (*14*)]. By comparison with values in the USDA Database for the Flavonoid Content of Selected Foods (*8*), only kale and tarragon exceeded immature pak choi leaves in kaempferol content with means of 26.7 and 11.0 mg/100 g of FW, respectively. Comparable contents were found in broccoli and fennel, with 6.2 and 6.5 mg/100 g of FW, respectively. Isorhamnetin content in immature pak choi leaves ranged from 8.0 to 24.0 mg/100 g of DW, equivalent to 0.8−2.4 mg/100 g of FW (**Table 1**). These high levels of kaempferol and isorhamnetin prompted the study of the naturally occurring

Table 2. Analysis of the Alkaline Hydrolysate of Pak Choi, Showing Nomenclature for Carbohydrate Fragmentation



label	compound identity	UV (nm)	$[M-H]^-$ (*m/z*)	MS^2 $[M-H]^-$ (*m/z*) (%)					MS^3 $[MS^2\ (100\%)]^-$ (*m/z*) (%)			
				Y^{7-}_0 (–162)	$Y^{3-}_{1/0}$ (–162)	$^{0,2}X^-_{1/0}$ (–120)	Z^-_1 (–180)	Y^{3-}_0 (–324)	Y^{3-}_1 (–162)	$^{0,2}X^-_0$ (–120)	Z^-_0 (–180)	Y^{3-}_0 (–324)
A	Q-3-soph-7-gluc	196, 254, 352	787	625 (100)					463 (35)	505 (30)	445 (86)	300/301 (100)
B	K-3-soph-7-gluc	198, 266, 346	771	609 (100)					447 (35)	489 (30)	429 (100)	285 (95)
C	Q-3,7-digluc	194, 252, 348	625	463 (100)		505 (10)		301 (20)		343 (20)		300/301 (100)
D	K-3,7-digluc	196, 266, 344	609	447 (100)		489 (16)		285 (16)		327 (32)		284/285 (100)
E	I-3,7-digluc	202, 254, 352	639	477 (100)		519 (12)		315 (10)		357 (26)		314/15 (100)
F	Q-3-soph	198, 230, 340[a]	625		463 (70)	505 (20)	445 (70)	301 (100)				
G	K-3-soph	198, 265, 340[a]	609		447 (20)	489 (12)	429 (80)	285 (100)				
H	K-3-gluc	overlapping[b]	447		285 (100)	327 (18)						
I	I-3-gluc	overlapping[b]	477		315 (100)	357 (28)						

[a] Overlaps with a non-flavonoid peak; background subtraction used to extract spectrum. [b] Overlapping peaks with combined UV: 204, 254, 352.

Table 3. Analysis of the Intact Pak Choi 7-Glucoside Derivatives

label	compound identity[a]	UV (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*) (%)	MS^3 $[MS^2\ (100\%)]^-$ (*m/z*) (%)				MS^4 $[MS^3\ (100\%)]^-$ (*m/z*) (%)			
				Y^{7-}_0 (–162)	$^{0,2}X^3_{1/0-}$ (–120)	$Y^{3-}_{1/0}$ (–162)	$Z^3_1{}^-$ (–180)	$Y^{3-}_{0/1}$ (–324/-R)	Y^{3-}_1 (–162)	$^{0,2}X^-_1$ (–120)	Z^-_1 (–180)	Y^{3-}_0 (–324)
1	A	196, 254, 352	787	625 (100)	505 (30)	463 (35)	445 (90)	300/301 (100)				
2	B	198, 266, 346	771	609 (100)	489 (30)	447 (35)	429 (100)	285 (95)				
3	A-Caf	194, 252, 336	949	787 (100)		625 (18)		625 (100)	463 (35)	505 (30)	445 (100)	301/303 (98)
4	B-OMeCaf	196, 266, 330	963	801 (100)				609 (100)	447 (35)	489 (8)	429 (100)	284/285 (95)
5	B-Caf	202, 268, 332	933	771 (100)				609 (100)	447 (12)	489 (10)	429 (100)	284/285 (85)
6	C	194, 252, 342	625	463 (100)	343 (20)	300/301 (100)						
7	B-Sin	198, 268, 334	977	815 (100)				609 (100)	447 (12)	489 (10)	429 (100)	284/285 (85)
8	B-Fer	overlapping	947	785 (100)				609 (100)	447 (12)	489 (2)	429 (100)	284/285 (79)
9	B-Cou	overlapping	917	755 (100)				609 (100)	447 (18)	489 (15)	429 (100)	284/285 (90)
10	E	198, 266, 346	639	477 (100)	357 (26)	314/315 (100)						

[a] Caf, caffeoyl; Sin, sinapoyl; Cou, coumaroyl; Fer, feruloyl; Cin, cinnamoyl.

Table 4. Analysis of the Intact Pak Choi 3-Glucoside Derivatives

label	compound identity	UV (nm)	$[M-H]^-$ (*m/z*)	MS^2 $[M-H]^-$ (*m/z*) (%)				MS^3 $[MS^2\ (100\%)]^-$ (*m/z*) (%)			
				$^{0,2}X^3_{1/0-}$ (–120)	$Y^{3-}_{2/3}$ (–162/R)	$Z^3_1{}^-$ (–180)	$Y^{3-}_{0/1}$ (–324/–R)	Y^{3-}_1 (–162)	$^{0,2}X^-_1$ (–120)	Z^-_1 (–180)	Y^{3-}_0 (–324)
11	F	198, 230, 340	625	505 (20)	463 (70)	445 (70)	301 (100)				
12	G- OMeCaf	194, 252, 336	801		609 (100)			447 (12)	489 (8)	429 (95)	284/285 (100)
13	G		609	489 (12)	447 (20)	429 (80)	285 (100)				
14	G-PerOMeCin	198, 246, 332	889		609 (100)			447 (13)	489 (10)	429 (100)	284/285 (95)
15	G-Caf	202, 246, 330	771		609 (100)			447 (14)	489 (8)	429 (100)	284/285 (85)
16	G-Sin*	*a*	815		609 (100)			447 (12)	489 (10)	429 (100)	284/285 (78)
17	G-Fer	196, 268, 318	785		609 (100)			447 (10)	489 (8)	429 (100)	284/285 (75)
18	G-Cou	196, 268, 318	755		609 (100)			447 (10)	489 (7)	429 (100)	284/285 (76)
19	H	overlapping	447	327 (28)	284/285 (100)						
20	I	overlapping	477	357 (25)	314/315 (100)						

[a] A number of overlapping peaks including this compound. See text for details.

flavonol conjugates in pak choi. One variety, Shanghai, was chosen for the more detailed LC-MS/MS analysis.

Analysis of the Shanghai variety of pak choi extract revealed a complex mixture of metabolites. An expansion of the chromatograms acquired for the extract under HPLC gradients 1 and 2 is shown in **Figure 1**. This level of complexity was not unexpected. Recent studies on flavonols in broccoli (*12*, *15*) and cauliflower (*13*) have led to the characterization of complex mixtures of acylated flavone glycosides. In these studies, assignments of the structures of individual metabolites benefited from several earlier, detailed studies of the fragmentation of carbohydrates by mass spectrometry. A common nomenclature for carbohydrate fragmentation was proposed in 1988 by Domon et al. (*16*) and has been adopted to describe the results in this

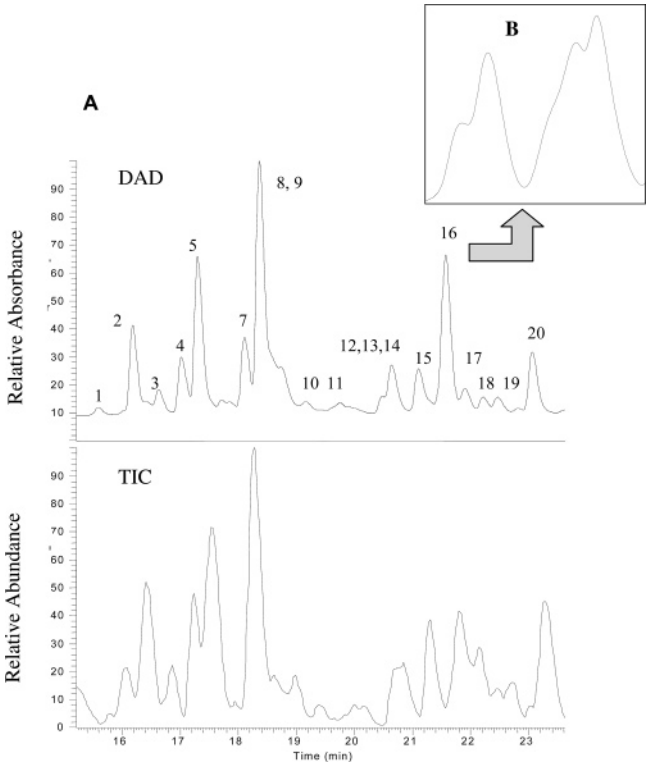


Figure 1. HPLC analysis of the methanolic extract of pak choi with gradient 1: (**A**) DAD trace at 360–380 nm and TIC showing an expansion of the chromatogram between 15 and 23 min; (**B**) expansion of one peak (16) run under gradient 2 conditions—peak 16 is now resolved into at least four peaks (35–37 min), demonstrating the complexity of pak choi extract.

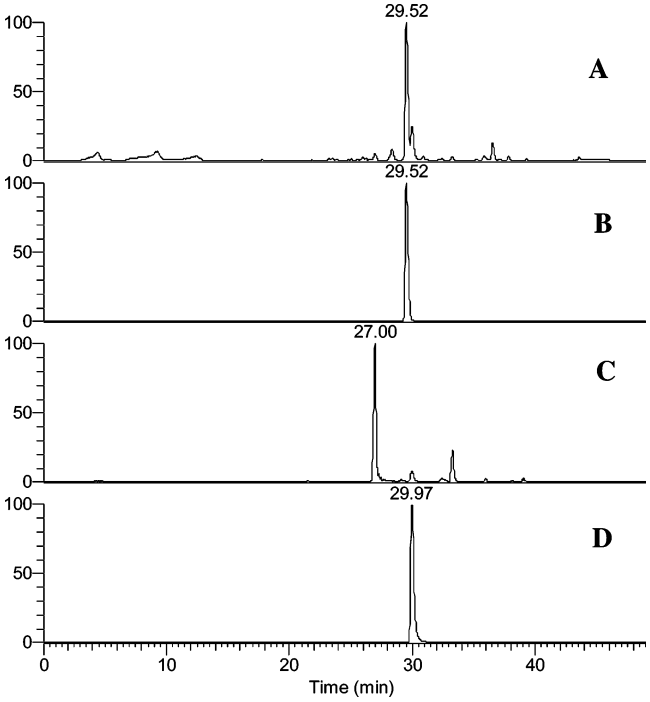


Figure 2. LC-MS analysis of the acid hydrolysate of pak choi using gradient 1: (**A**) total ion chromatogram (TIC) for *m*/*z* 200–2000, showing a simple chromatogram with a peak at 29.52 min dominating; (**B**) selective ion extraction for kaempferol (base peak, *m*/*z* 285–286) showing that kaempferol is the dominant aglycone after hydrolysis; (**C**) quercetin (27.00 min) detected by selective ion extraction (base peak, *m*/*z* 301–302); (**D**) isorhamnetin (29.97 min) detected by selective ion extraction (base peak, *m*/*z* 315–316).

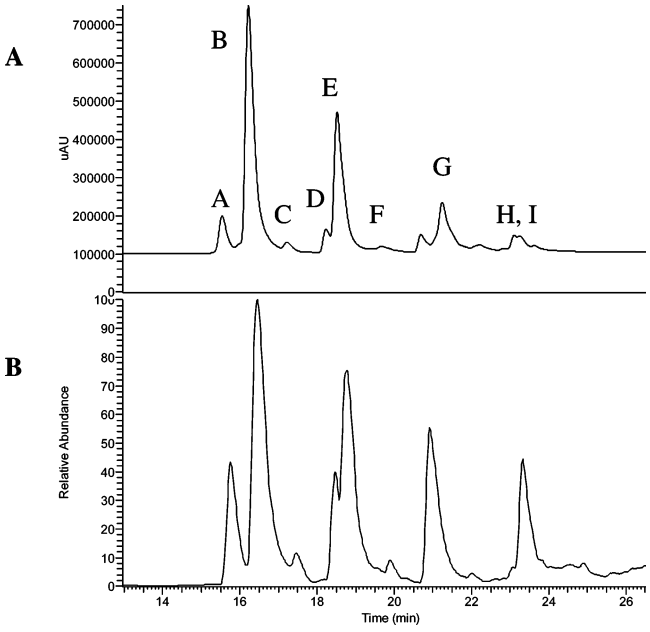


Figure 3. Analysis of the alkaline hydrolysate of pak choi: (**A**) PDA chromatogram (extracted wavelength, 360–380 nm) showing the presence of two major glycones and nine minor glycones; (**B**) TIC (*m*/*z* 200–2000) showing a similar pattern.

study (**Table 2**). A number of earlier studies also examined the specific MS fragmentation behavior of flavonol glycosides, characterizing the fragmentation patterns from the flavonol core for 1→2 and 1→6 glycosides (*17*), which has been important for the structure assignments made in this study (*12*, *18*, *19*). Investigation of the UV spectra from the chromatograms shown in **Figure 1** suggested that many of these metabolites were acylated with hydroxycinnamic acid derivatives, through the presence of a broad maximum present around 330 nm (**Table 2**) (*12*).

Due to the complexity of the extract, the freeze-dried pak choi leaves were subjected to acid hydrolysis to simplify the analysis of the flavonol cores. The acid hydrolysis of pak choi was analyzed by LC-MS under gradient 1 conditions to generate the chromatogram shown in **Figure 2**. As anticipated from the pak choi variety hydrolysis study (**Table 1**), the kaempferol aglycone was the major flavonol found in the hydrolysis mixture, but also present were isorhamnetin and quercetin, in trace amounts. The identities of these aglycones were confirmed by the comparison of the LC-MS-DAD data of the hydrosylate to those of commercial standards, confirming the results summarized in **Table 1**. This result was similar to previously reported observations for broccoli, with kaempferol demonstrated to be the major aglycone. However, for broccoli, the studies found isorhamnetin rather than quercetin to be the minor constituent (*12*).

Having confirmed the identity of the three flavonol aglycones, a second aliquot of the Shanghai pak choi was hydrolyzed under milder alkaline conditions to enable analysis of the flavonol glycones (**Figure 3**). Nine individual compounds could be identified by analysis of the UV spectra and MS fragmentation patterns (**Table 2**). Sugar substitution on flavonols usually occurs as the *O*-glycosides, mainly at the 3-, 7-, and 4′-positions (*19*). Inspection of the UV spectra for the nine compounds (**Table 2**) suggests that the 3-hydroxyl is blocked (maxima, 347−355 nm), indicating that there is glycosylation at this

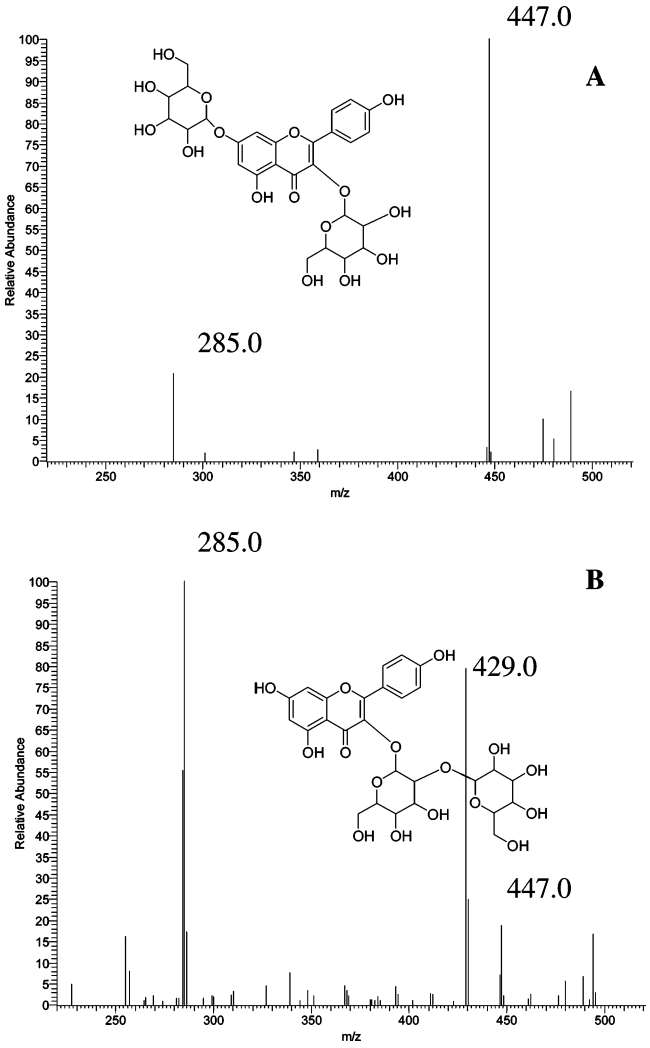


Figure 4. MS^2 analysis of peaks D (**A**) and G (**B**). The Z_1^- (–180) ion (*m*/*z* 429) is clearly visible for G, distinguishing the 3-sophoroside from D, the 3,7-diglycoside.

position (*12*). Glycosylation has also been assigned at C7 on the basis of similar studies in cauliflower and broccoli (*12*). In these earlier studies, Vallejo et al. (*12*) demonstrated that the first fragmentation from $[M - H]^-$ is always due to the breakdown of the 7-*O*-glycosidic bond, leading to a characteristic base peak Y^{7-}_0. For monohexosides, this leads to Y^{7-}_0 $[M - H - 162]^-$ or, for dihexosides, Y^{7-}_0 $[M - H - 324]^-$ (*19*). Analysis of the flavonols detected in the hydrolysis mixture (**Figure 3**) suggested that peaks A and B were glycosylated at the 7-position, with a clear Y^{7-}_0 $[M - H - 162]^-$ base peak in the MS^2 (**Table 3**). By contrast, peaks C−I showed multiple peaks in the MS^2, suggesting they were either 3,7-diglycosides or unsubstituted at C7. The 3,7-diglycosides were characterized by a base peak corresponding to Y^{7-}_0 $[M - H - 162]^-$ in the MS^2, whereas the 3-diglycosides were characterized by a base peak of Y^{3-}_0 $[M - H - 324]^-$. These 3-diglycosides could be identified as sophorosides [Glc(1→2)Glc] by characteristic fragments corresponding to $^{0,2}X_0^-$ (−120) and Z_1^- (−180), fragments that are typically not detected for 1→6 glycosides (*19*). The MS^2 spectra of D and G (**Figure 4**) clearly demonstrate this differential fragmentation, with the major difference being the presence of the Z_1^- (−180) ion (*m*/*z* 429) visible only for G, the 3-sophoroside.

The analysis of the natural metabolites (**Figure 1**) was carried out using the same rationale. The base peak, Y^{7-}_0 $[M - H - 162]^-$, in the MS^2 characterized the 7-glycosides, whereas the 3-sophoroside derivatives all yielded strong Z_1^- (−180) fragments (**Table 4**). This analysis revealed that the majority of the deacetylated compounds produced through alkaline hydrolysis also occurred naturally, although the acylated metabolites dominated the mixture. As might have been expected from the hydrolysis analysis, the kaempferol conjugates were the most numerous compounds in the extract.

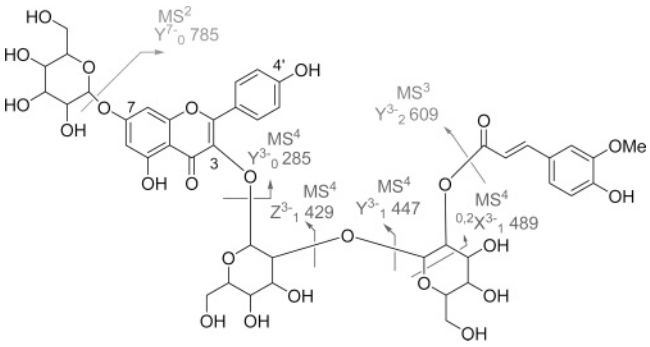


Figure 5. Fragmentation for compound 8-kaempferol-3-sophoroside-(feruloyl)-7-glucoside.

The structure of the acyl portion of the compounds was deduced by MS fragmentation analysis and by comparison to the flavonoid acylated derivatives identified from broccoli. The acyl fragments are tentatively identified on this basis; however, the MS study cannot eliminate the possibility of regioisomers in the subunits. The majority of the acyl fragments were identified as the commonly occurring units, including coumaroyl (−146), caffeoyl (−162), feruloyl (−176), and sinapoyl (−206). Less common derivatives were indicated by the loss of 192 or 280 amu and were tentatively assigned as a dihydroxy methoxycinnamic acid and permethoxy (pentamethoxy) cinnamic acid derivatives, respectively. **Figure 5** exemplifies the fragmentation analysis for the metabolite 8-kaempferol-3-sophoroside-(feruloyl)-7-glucoside.

In addition to the flavonol glycosides described in **Tables 3** and **4** there are a number of co-eluting metabolites in peak 16 that do not appear to be flavonols (**Figure 1**). Expansion of the peak under gradient 2 conditions reveals that this peak is now resolved into at least four peaks (35−37 min). The identity of the peaks on either side of this region with gradient 2 have the same UV and MS characteristics as peaks 15 and 17 under gradient 1 conditions (**Table 4**). This confirms that it is peak 16 that is now showing the additional resolution. The UV spectra of these metabolites suggest that they contain conjugated aromatics, with UV maxima at 196, 240, and 328 nm, and that they may be monoglycosides acylated with hydoxycinnamic acid derivatives, similar to previously reported feruloyl gentiobioses (*20*). There are two main compounds with *m*/*z* 309 and 339, respectively. The identities of these compounds could not be determined with certainty from the available data.

The major point of difference between the natural metabolites identified in this study and the metabolites identified in the earlier study of broccoli is the number of sugar residues present on the flavonol core. Vallejo et al. (*12*) reported the presence of metabolites with up to five sugars substituted on a flavonol, including 3-sophorotriosides-7-sophorosides. By comparison, the pak choi is less complex with no 7-diglucosides or sophorotriosides detected. The complexity of the *Brassica* flavonol constituents in general compared to vegetables such as onions represents an interesting biological question and may possibly relate to the aerial nature of broccoli florets and pak choi leaves compared to onion bulbs. It may also be expected that the

different glycosides possess different biological activities for human consumers. The absorption, distribution, metabolism, and excretion (ADME) profiles of the compounds could be expected to vary, and this alone may effect, for example, their anticancer activity. The majority of these metabolites have never been isolated or studied independently, and this represents an interesting avenue for future research.

LITERATURE CITED

(1) Hertog, M. G. L.; Hollman, P. C. H.; Katan, M. B. Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in The Netherlands. *J. Agric. Food Chem.* **1992**, *40*, 2379−2383.

(2) Verma, A. K.; Johnson, J. A.; Gould, M. N.; Tanner, M. A. Inhibition of 7,12-dimethylbenz[*a*]anthacene and *N*-nitrosomethylurea induced rat mammary cancer by dietary flavanol quercetin. *Cancer Res.* **1988**, *48*, 5754−5788.

(3) Elongavan, V.; Sekar, N.; Govindasamy, S. Chemopreventative potential of dietary bioflavonoids against 20-methylcholanthrene-induced tumourigenesis. *Cancer Lett.* **1994**, *87*, 107−113.

(4) Gil, M. I.; Ferreres, F.; Tomás-Barberán, F. A. Effect of postharvest storage and processing on the antioxidant constituents (flavonoids and vitamin C) of fresh-cut spinach. *J. Agric. Food Chem.* **1999**, *47*, 2213−2217.

(5) Yan-Hwa, C.; Chao-Lin, C.; Hsia-Fen, H. Flavonoid content of several vegetables and their antioxidant activity. *J. Sci. Food Agric.* **2000**, *80*, 561−566.

(6) Kim, J. D.; Liu, L.; Guo, W.; Meydani, M. Chemical structure of flavonols in relation to modulation of angiogenesis and immune-endothelial cell adhesion. *J. Nutr. Biochem. Sep.* **2005**, *Sep 15*, Epub.

(7) Ackland, L.; van de Waarsenburg, S.; Jones, R. B. Synergistic antiproliferative effect of the flavonols quercetin and kaempferol in cultured human cancer cell lines. *Int. J. Exp. Clin. Pathophysiol. Drug Res.* **2005**, *19*, 69−76.

(8) USDA database. http://www.nal.usda.gov/fnic/foodcomp/Data/Flav/flav.html, accessed Dec 11,2005.

(9) Ibarra, M.; Perez-Vizciano, F.; Cogolludo, A.; Duarte, J.; Zaragoza-Arnae, F.; Lopez-Lopez, J. G. T. J. Cardiovascular effects of isorhamnetin and quercetin in isolated rat and porcine vascular smooth muscle and isolated rat atria. *Planta Med.* **2002**, *68*, 307−310.

(10) Ibarra, M.; Moreno, L.; Vera, R.; Cogolludo, A.; Duarte, J.; Tomargo, J.; Perez-Vizciano, F. Effects of the flavonoid quercetin and its methylated metabolite isorhamnetin in isolated arteries from spontaneously hypertensive rats. *Planta Med.* **2003**, *69*, 995−1000.

(11) Price, K. R.; Rhodes, M. J. C. Analysis of the major flavonol glycosides present in four varieties of onion (*Allium cepa*) and changes in composition resulting from autolysis. *J. Sci. Food Agric.* **1997**, *74*, 331−339.

(12) Vallejo, F.; Tomás-Barberán, F. A.; Ferreres, F. Characterisation of flavonols in broccoli (*Brassica oleracea* L. var. *italica*) by liquid chromatography-UV diode-array detection-electrospray ionisation mass spectrometry. *J. Chromatogr., A* **2004**, *1054*, 181−193.

(13) Llorach, R.; Gil-Izquierdo, A.; Ferreres, F.; Tomás-Barberán, F. A. HPLC-DAD-MS/MS ESI characterizaton of unusual highly glycosylated acylated flavonoids from cauliflower (*Brassica oleracea* L. var. *botrytis*) agroindustrial byproducts. *J. Agric. Food Chem.* **2003**, *51*, 3895−3899.

(14) Lako, J.; Trenerry, V. C.; Wahlqvist, M.; Wattanapenpaiboon, N.; Sotheesaran, S.; Premier, R. Phytochemical flavonols, carotenoids and the antioxidant properties of a wide selection of Fijian fruit, vegetables and other readily available foods. *Food Chem.* **2006**, in press.

(15) Sasakia, K.; Takahashib, T. A flavonoid from *Brassica rapa* flower as the UV-absorbing nectar guide. *Phytochemistry* **2002**, *61*, 339−343.

(16) Domon, B.; Costello, C. A systematic nomenclature for carbohydrate fragmentation in FAB-MS/MS spectra of glycoconjugates. *Glycoconjugate J.* **2005**, *5*, 397−409.

(17) Cuyckens, F.; Rozenberg, R.; de Hoffmann, E.; Claeys, M. Structure characterization of flavonoid *O*-diglycosides by positive and negative nano-electrospray ionization ion trap mass spectrometry. *J. Mass Spectrom.* **2001**, *36*, 1203−1210.

(18) Cuyckens, F.; Claeys, M. Mass spectrometry in the structural analysis of flavonoids. *J. Mass Spectrom.* **2004**, *39*, 1−15.

(19) Ferreres, F.; Llorach, R.; Gil-Izquierdo, A. Characterization of the interglycosidic linkage in di-, tri-, tetra- and pentaglycosylated flavonoids and differentiation of positional isomers by liquid chromatography/electrospray ionization tandem mass spectrometry. *J. Mass Spectrom.* **2004**, *39*, 312−321.

(20) Price, K. R.; Casuscelli, F.; Colquhoun, I. J.; Rhodes, M. J. C. Hydroxycinnamic acid esters from broccoli florets. *Phytochemistry* **1997**, *45*, 1683−1687.

Received for review January 19, 2006. Revised manuscript received April 4, 2006. Accepted April 6, 2006.

JF060154J