Glucosinolates in Members of the Family Brassicaceae: Separation and Identification by LC/ESI-MS-MS

Bertrand Matthäus*,† and Heinrich Luftmann‡

Institut für Chemie und Physik der Fette der Bundesanstalt für Getreide-, Kartoffel- und Fettforschung, Postfach 1705, 48006 Münster, Germany, and Organisch Chemisches Institut der Westfälischen Wilhelms-Universität Münster, Corrensstrasse 40, 48149 Münster, Germany

Seeds of 14 different members of the family Brassicaceae were investigated with regard to their content and composition of glucosinolates by HPLC-UV/ESI-MS-MS coupling. The seeds were extracted with hot methanol/water (70:30 v/v) and the desulfoglucosinolates isolated by anion-exchange chromatography with solid-phase extraction columns. The desulfoglucosinolates were detected by UV and identified by ESI-MS/MS with the neutral loss method. Nineteen different glucosinolates were detected in the seeds with a wide range of contents (10−200 μmol/g) and a great variation in the composition.

Keywords: *Brassicaceae; desulfoglucosinolates; glucosinolates; HPLC; LC/ESI-MS-MS*

INTRODUCTION

Glucosinolates are a group of β-D-thioglucosides that are mainly found in the family Brassicaceae. Many plants of this family are used in agriculture and nutrition, for example, rapeseed, wintercress, false flax, crambe, Brussels sprouts, radish, or cauliflower. More than 100 different glucosinolates are known (Kjaer and Skrydstrup, 1987). Glucosinolates are relatively nontoxic (Bell, 1984), but they gain importance from the fact that the products of myrosinase [thioglucoside glucohydrolase (EC 3.2.3.1)] induced degradation adversely affect animal growth, reproduction, and performance as well as intake and palatability of fodder. The degradation products also cause goiter and abnormalities in internal organs of animals (Mawson et al., 1993, 1994a−c).

Several methods have been developed to detect glucosinolates and their hydrolysis products. Methods that indicate the total amount of glucosinolates are often based on the measurement of released glucose (Daxenbichler and vanEtten, 1977; Thies, 1985; Sjödin and Sundqvist, 1988) or bisulfate ions (Fiebig and Sendfeld, 1989; Sendfeld et al., 1988) by enzymatic assays. The method of choice is the determination of individual glucosinolates by HPLC of the desulfoglucosinolates, which became established as the official reference method (high-pressure liquid chromatography of desulfoglucosinolates) of the European Community (EC, 1990), and the International Organization for Standardization (ISO) followed the same method (ISO 9167-1, 1992). For the determination of the glucosinolate content in rapeseeds, this method is excellently suitable, but for other samples the identification and classification of peaks are very difficult. The identification of desulfoglucosinolates has been carried out by gas−liquid chromatography/mass spectrometry (GC-MS) (Hogge et al., 1988), capillary gas chromatography (GC)/positive-ion chemical ionization mass spectrometry (PICIMS) (Shaw et al., 1989), fast-atom bombardment mass spectrometry (FAB/MS) (Fenwick et al., 1982), or gas chromatography/chemical ionization mass spectrometry (GC-CIMS) (Eagles et al., 1981). Besides a difficult pretreatment of the samples these methods have some limitations for the GC analysis, because desulfoglucosinolates with sulfinyl or indolic groups are not volatile enough (Heaney and Fenwick, 1982) and some glucosinolates are sensitive to higher temperatures (Christensen et al., 1982).

Rapid development in liquid chromatography−mass spectrometry (LC-MS) in the past years has eliminated the difficulties in the GC-MS analysis of glucosinolates and desulfoglucosinolates and combines the excellent separation of HPLC with the possibility of direct identification by MS. These methods use liquid chromatography/atmospheric pressure chemical ionization mass spectrometry (LC/APCI-MS) (Ishida et al., 1997), thermospray liquid chromatography−mass spectrometry (Lange and Petrzika, 1990; Hogge et al., 1988; Lange et al., 1995), LC coupled with frit to fast-atom bombardment mass spectrometry (Kokkonen et al., 1989), or liquid chromatography/electrospray mass spectrometry (Zrybko et al., 1997).

The aim of this paper was to show the application of the ESI-MS-MS on the identification of desulfoglucosinolates, in combination with the separation and calculation of the content of each glucosinolate using the EC standard method. The presented method was applied to the determination of glucosinolates in seeds of different members of the family Brassicaceae.

MATERIALS AND METHODS

Plant Material and Chemicals. Seeds of American wintercress (*Barbarea verna*), wintercress (*Barbarea vulgaris*), *Brassica carinata*, Brussels sprouts (*Brassica oleracea* L. var.

* Author to whom correspondence should be addressed (telephone ++49 251 43510; fax ++49 251 519275; e-mail matthaus@uni-muenster.de).

† Institut für Chemie und Physik der Fette der Bundesanstalt für Getreide-, Kartoffel- und Fettforschung.

‡ Organisch Chemisches Institut der Westfälischen Wilhelms-Universität Münster.

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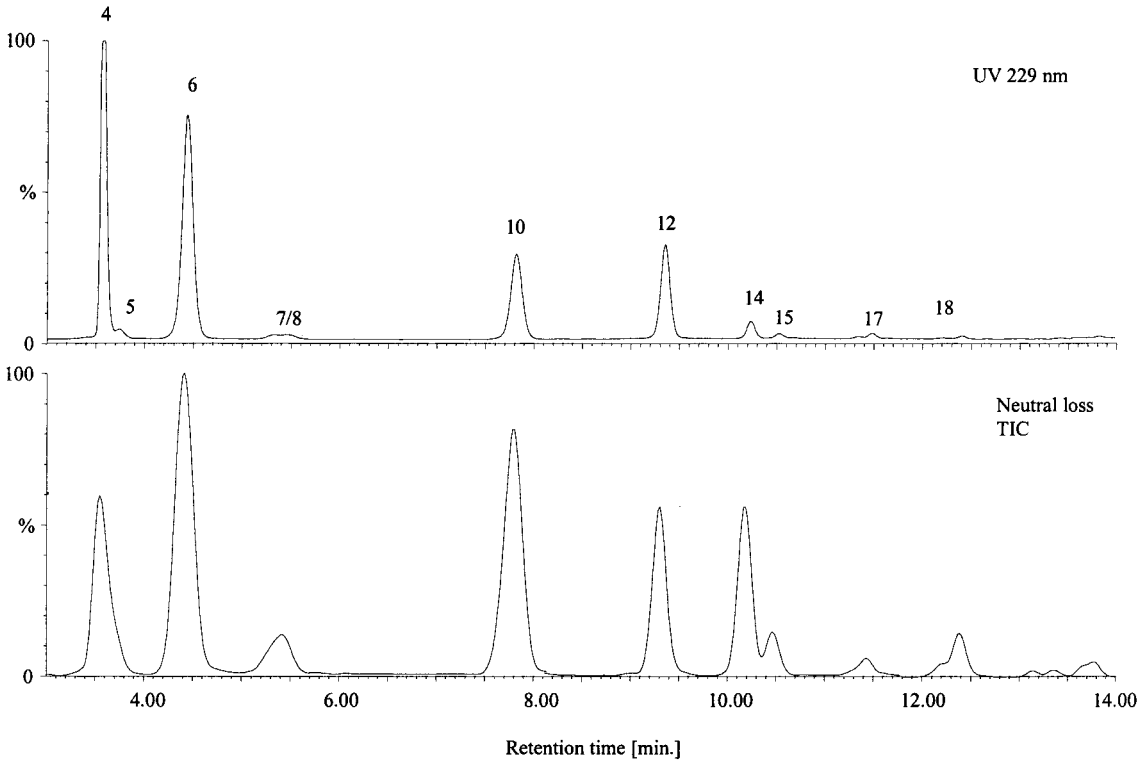

Figure 1. Comparison of the chromatograms obtained by UV detection at 229 nm and TIC obtained by the neutral loss method from the extract of *B. napus* seeds.

gemmifera DC), rapeseed (*Brassica napus*), turnip (*Brassica rapa* var. *rapa*), wallflower (*Cheiranthus cheiri*), field pepperwort (*Lepidium campestre*), garden cress (*Lepidium sativum*), *Lesquerella fendleri*, sweet allysum [*Lobularia maritima* (L.) *Desv*], winter radish [*Raphanus sativus* L. var. *niger* (Mill) S. Kerner], oil radish (*Raphanus sativus* L. var. *oleiformis* Pers), and small radish (*Raphanus sativus* L. var. *sativus*) were used for the investigations.

Water, methanol, and acetonitrile (Lichrosolv, chromatography grade) were obtained from Merck (Darmstadt, Germany). Sulfatase type H-1 from *Helix pomatia* was purchased from Sigma (St. Louis, MO).

Sample Preparation. One gram of the seeds was crushed in a Z 100 centrifugal mill (Retsch, Haan, Germany), and 200 mg of each was transferred into two 20 mL tubes with screw tops (Fleischhacker, Germany). Five milliliters of hot 70% methanol and in the case of the quantitative HPLC 5 and 20 μmol, respectively, of sinigrin or glucotropaeolin as internal standard were added. The loosely capped vials were placed in an 80 °C hot ultrasonic bath for 15 min. Afterward, the solution was centrifuged and was ready for the isolation of the desulfoglucosinolates by anion-exchange chromatography.

Columns used were strong anion exchanger (SAX, 500 mg) for solid-phase extraction (SPE) (Merck), which were conditioned with 2 mL of 70% methanol. Afterward, 2 mL of the crude extract was put onto the column, the column was washed twice with 1 mL of water, and 2 mL of acetate buffer (pH 4.0) was added. The release of the desulfoglucosinolates was achieved on column by the addition of 1 mL of a sulfatase solution (0.2 mg/mL). The column was closed and allowed to stand alone for ~16 h. The desulfoglucosinolates were eluted with 1.5 mL of water into an HPLC vessel.

HPLC. The HPLC analysis was conducted using a Merck-Hitachi low-pressure gradient system, fitted with an L-7140 pump, a Merck-Hitachi L-4250 UV−vis detector set at 229 nm, and a Knauer Eurochrom 2000 for Windows integration system. Thirty microliters of the desulfoglucosinolate-containing eluates was injected by an AS-4000 autosampler onto a 125 × 4 mm, 5 μm LiChrospher 60 RP-select B column (Merck) used with a flow rate of 1 mL/min. The mobile phase used was water (A) versus acetonitrile (B) for a total running time of 45 min, and the gradient changed as follows: 95% A/5% B for 2 min, then in 14 min to 59% A/41% B. After 2 min isocratic, in 4 min to 5% A/95% B, again 5 min isocratic, and then in 2 min back to 95% A/5% B. Afterward the column was equilibrated at 95% A/5% B for 15 min.

Calculation of Each Glucosinolate. The calculation of each glucosinolate identified in the samples was done by evaluation of the chromatograms obtained by UV detection at 229 nm as described in the EC standard method. The content of each glucosinolate was calculated and expressed in micromoles per gram of seed. Statistical parameters, such as precision, repeatability, and reproducibility for the calculation of each glucosinolate, were given in the EC standard method.

HLPC and Coupling to the MS. Injection was done manually by a 20 μL loop injector (Rheodyne). The separation was carried out on an HP1100 (Hewlett-Packard) LC with vacuum degasser, binary pump, and variable wavelength detector. All other separation conditions are identical to the above-mentioned.

The outlet of the UV detector was connected to a mixing T-piece where a flow of 50 μL/min of 10% formic acid was added by a separate pump (LKB 2150). To reduce the load of the ion source, the flow was divided by a T-piece with an appropriate restrictor to give 0.3 mL/min flow to the source.

A Quattro LCZ (Micromass) two-analyzer quadrupole mass spectrometer with a collision cell between the quadrupoles equipped with an electrospray inlet was used.

The source conditions are as follows: capillary, 2.7 kV; cone, 17 V; extractor, 1 V; source block temperature, 110 °C; and desolvation temperaturee, 200 °C. The spray was stabilized by a nebulizer flow (N_2) of 76 L/h, and the desolvation gas flow was adjusted to 430 L/h.

RESULTS AND DISCUSSION

Identification of Desulfoglucosinolates by ESI-MS-MS. The separation, identification, and calculation of different desulfoglucosinolates from seeds of various Brassicaceae were performed by HPLC/UV-ESI-MS-MS coupling within 15 min (Figure 1). In Table 1 a list of

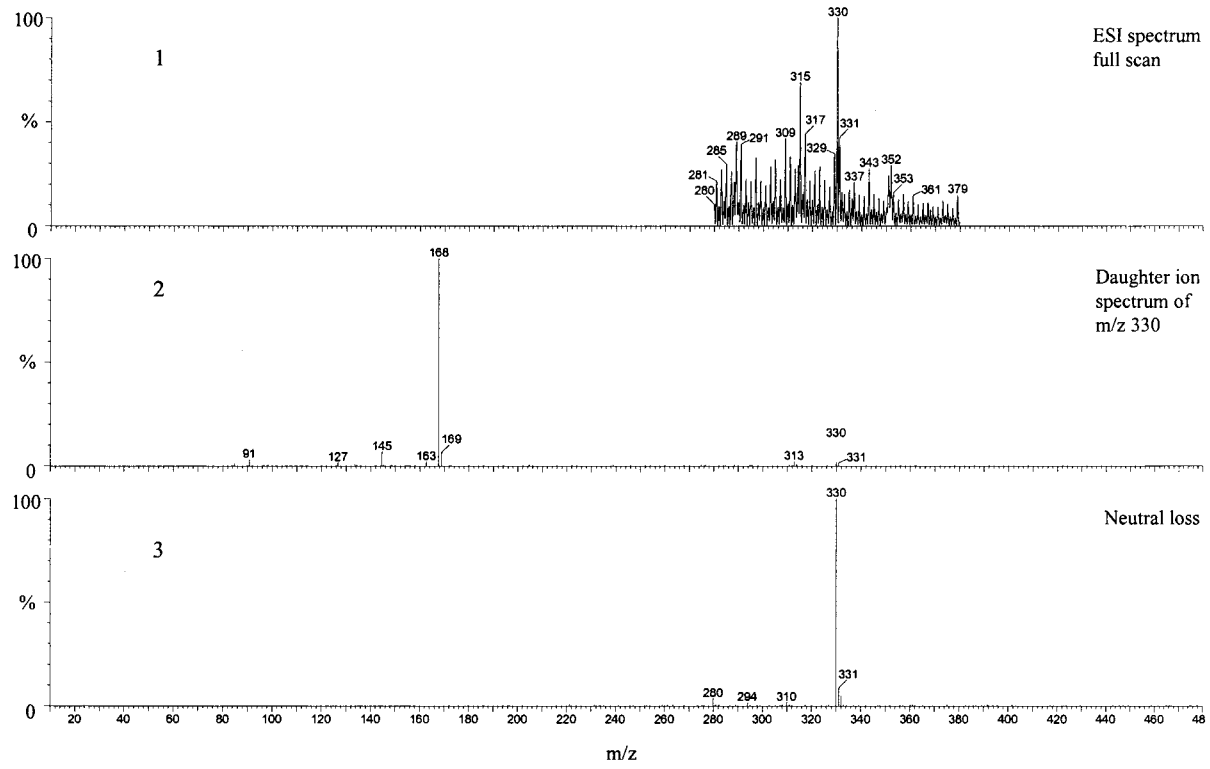

Figure 2. ESI spectrum of glucotropaeolin taken in full scan mode (1), as daughter ion spectrum (2), and as neutral loss scan (3).

Table 1. Identified Desulfoglucosinolates in the Chromatogram

no.	MW (nominal)	compound	abbrev	retention time (min)	no.	MW (nominal)	compound	abbrev	retention time (min)
1	343	desulfoglucoiberin	GIB	3.4	11	385	desulfohesperin	GHP	8.3
2	357	desulfoglucoraphanin	GRA	3.6	12	384	desulfo-4-hydroxygluco-brassicin	4HGB	9.4
3	359	desulfoglucocheirolin	GCH	3.6					
4	309	desulfoprogoitrin	PRO	3.6	13	359	desulfobarbarin	BAR	9.6
5	309	desulfoepiprogoitrin	EPI	3.8	14	307	desulfoglucobrass	GBN	10.2
6	279	desulfosinigrin	SIN	4.4	15	329	desulfoglucotropaeolin	GTR	10.6
7	371	desulfoglucoalyssin	GAL	5.3	16	339	desulfoerucin	GER	11.0
8	323	desulfogluconapoleiferin	GNF	5.4	17	368	desulfoglucobrassicin	GBS	11.5
9	345	desulfosinalbin	SAN	7.5	18	343	desulfogluconasturtiin	GNA	12.4
10	293	desulfogluconapin	GNP	7.8	19	369	desulfolesquerellin	GLS	13.8

identified desulfoglucosinolates with the corresponding retention times and masses is presented. The desulfoglucosinolates were detected by the UV detector at 229 nm as described for the standard method, and afterward, formic acid was added to form protonated species in solution, which were freed from the surrounding solvent molecules by the electrospray process. As a result of the postcolumn addition of formic acid, the standard separation conditions were saved, so it is possible to transfer the results of the identification by ESI-MS-MS directly to the standard method, which makes it easier to classify the desulfoglucosinolates in the chromatogram. A drawback is that an additional HPLC pump was necessary. To improve the transfer of ions, the mass resolution was adjusted to unit resolution and MS-MS methods were used to enhance the signal to noise ratio (s/n). The desulfoglucosinolates share a common glucosyl structure, which produces the loss of a 162.1 Da neutral fragment under collision activation conditions. Because this fragmentation is typical for all desulfoglucosinolates, the neutral loss scan was chosen. With this the two mass analyzers were synchronously scanned with a difference of 162.1 Da. In Figure 2, spectrum 1, the ESI spectrum of glucotropaeolin is shown taken in full scan mode of the first analyzer. At *m/z* 330 is a signal that corresponds to the $[M + H]^+$ of glucotropaeolin. The s/n is low because of the coeluting chemical noise. The gain of specifity and the increase of the s/n ratio is obvious in spectrum 2, which is a daughter ion spectrum of *m/z* 330. The observed loss of 162.1 Da to *m/z* 168 is typical for glucosylated compounds. Because this is a common feature of the desulfoglucosinolates, the neutral loss scan gives the chance to detect the *m/z* values of their $[M + H]^+$ peaks. Spectrum 3 shows a neutral loss scan (−162.1 Da) of glucotropaeolin.

As a result of such scans, maps were produced to give a survey of the desulfoglucosinolates (better to say from all compounds losing 162.1 Da in the collision chamber). In these maps the masses of the desulfoglucosinolates are presented against the retention time, and the intensity is depicted as color or gray scale (Figure 3).

The full mass scan gives the maximum information, but long times are spent to scan across “empty” mass ranges. When a collection of interesting masses is found by the scanning method, it is favorable to use multiple reaction monitoring (MRM), which gives longer integration times by restricting the measurement on preselected masses of interest. Surely this 10−500-fold gain of senstivity is paid for by loss of information. A further

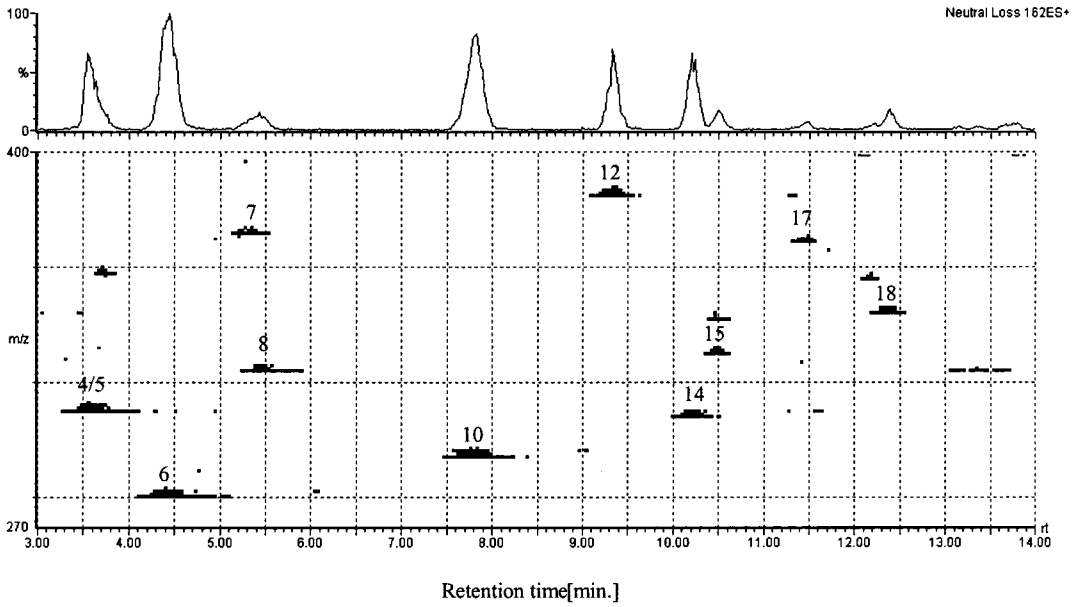

Figure 3. Resulting map with retention time as *x*-axis, *m*/*z* of the generator ion as *y*-axis, and intensity as gray scale.

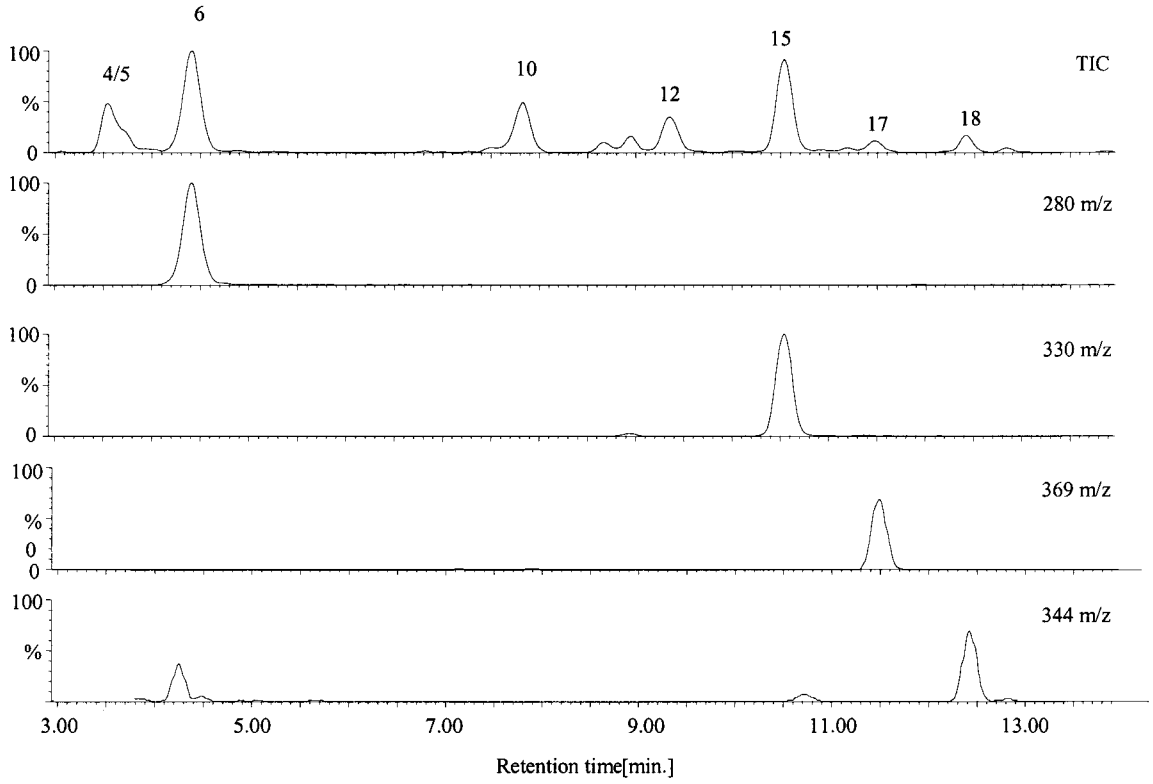

Figure 4. TIC of the extract of Brussels sprouts seeds and the specific chromatograms of some selected masses obtained by MRM.

gain of sensitivity by a factor of 2−8 could be achieved by declaring time windows where (a few) expected masses should appear instead of registering all interesting masses all of the time. This approach was not used.

Figure 4 shows the TIC of an extract of *Brassica oleracea* L. var. *gemmifera* DC and the specific chromatograms of some selected masses obtained by MRM. From *m*/*z* 344 and 369 it is obvious that it is possible to improve the sensitivity of the method based on this scan by restricting the scan to the relevant masses.

Calculation of the Content of Each Glucosinolate by the Chromatogram of the UV Detector. The peaks in the chromatogram of the UV detector and the neutral loss show only a short delay, resulting from different points of detection. Otherwise, both chromatograms have a roughly identical form. This indicates that the compounds detected by neutral loss are almost exclusively desulfoglucosinolates (Figure 1). There is some loss of separation and s/n ratio, but for the specific mass traces the s/n is much better even for minor compounds.

The calculation of the content of each glucosinolate in the different samples was carried out according to the EC standard method. An interlaboratory test, carried out at the international level in 1988, in which 11 laboratories participated, proved the statistical results

Table 2. Content[a] and Composition of Glucosinolates of the Investigated Seeds

species	GIB	GCH	PRO	EPI	SIN	GNF	BAR	GRA	SAN	GNP	GHP	4HGB	GBN	GAL	GBS	GTR	GER	GNA	GLS	sum
B. verna																		71.0		71.0
B. vulgaris							80.4					1.5			0.4			9.1		91.3
B. carinata			1.6		71.7					0.7		0.8			0.3	2.0				77.1
B. napus			3.9							1.8		3.2	0.7		0.1			0.5		10.0
B. oleracea L. var. *gemmifera* DC			28.0	1.1	21.7				0.4	3.4		1.4			0.3	5.6		0.4		62.4
B. rapa var. rapa			8.0	3.0						27.4		0.2	10.5	1.1	0.2			0.9		51.3
Ch. cheiri		66.0																		66.0
L. campestre			1.5						179.9					18.6			0.1	0.7		200.9
L. sativum L.																191.3				191.3
L. fendleri	27.5																			27.5
L. maritima			0.2							34.6	5.9		4.2			0.6		0.8	36.2	82.6
R. sativus L. var. *nigher* (Mill) S. Kerner			1.9	73.0				8.5	0.5			1.4		3.8			2.8	1.6		93.4
R. sativus L. var. *oleiformis* Pers	29.1		1.1	54.9				7.2				1.9					0.9			95.1
R. sativus L. var. *sativus*			0.7	54.2					0.7			1.3		2.7			1.2	0.6		61.3

[a] Contents in micromoles per gram.

of precision, repeatability, and reproducibility of the HPLC method for the determination of glucosinolate content (EC, 1990). Thus, in the presented work the description of the statistical results was not repeated.

Classification of the Different Glucosinolates by ESI-MS-MS and UV Detection. On the basis of the results of the neutral loss and the resulting maps of (*m*/*z*) values, 19 different desulfoglucosinolates were identified in the various seeds. With regard to retention time desulfoglucoraphanin, desulfoglucocheirolin, and desulfoprogoitrin are critical compounds because they all elute after 3.6 min from the HPLC column. Thus, an identification and differentiation only by means of UV detection is impossible, but the different masses of these desulfoglucosinolates allow a clear identification of the three compounds. Another critical pair is desulfoprogoitrin and its optical isomer desulfoepiprogoitrin, which have the same mass. In this case the identification of the compounds is possible by means of the different retention times. Desulfoepiprogoitrin elutes shortly after desulfoprogoitrin. All other desulfoglucosinolates were identified and classified by their retention times and/or their masses.

Comparison of ESI with Other LC-MS Systems. A comparison with commonly used LC-MS methods for the determination of glucosinolates, such as TSP (Hogge et al., 1988) or APCI (Ishida et al., 1997), shows that ESI is comparable with APCI. Both ESI and APCI are liquid-based inlet systems. With APCI the spraying and desolvation are accomplished by a heated gas flow with subsequent ionization by a plasma. ESI uses ions that are preformed in solution; their spraying and desolvation are caused by coulomb repulsion. This technique is effective for the ionization of medium-polarity to high-polarity compounds, whereas APCI extends the range to low polarity (Careri et al., 1998). ESI appears to be superior for the detection of nonfragmented ions of labile compounds, which in our case is a prerequisite for the neutral loss scan.

In contrast to the techniques above, TSP is a quite old interface/ionization system and therefore much more sensitive to the influence of experimental conditions such as vaporizer temperature, block temperature, composition of the mobile phase, flow rate, and performance of the spray, so that it is in some cases impossible to reproduce results from day to day (Heeremans et al., 1989).

Glucosinolate Contents of Different Seeds. Seeds from 14 different members of the family Brassicaceae were investigated with regard to their composition and content of glucosinolates with the method described above. The seeds show a great diversity in glucosinolate composition as well as in content of glucosinolates. Table 2 summarizes the results of these investigations. The total content of glucosinolates ranges from 10 μmol/g (rapeseed) to 200.9 μmol/g (*L. campestre*).

Almost all seeds show a great variation in the glucosinolate composition. Only *B. verna*, *Ch. cheiri*, and *L. fendleri* are dominated by one glucosinolate. In *B. verna* this is gluconasturtiin, and the only glucosinolates of *L. fendleri* and *Ch. cheiris* are glucoiberin and glucocheirolin, respectively. Other glucosinolates are detectable only in traces in these seeds.

Most of the other seeds consist of one main glucosinolate and different minor glucosinolates. The main glucosinolate varies in the different seeds. In seeds of turnip, Brussels sprouts, oil radish, and rapeseed, the number of glucosinolates that make a higher contribution to the total amount of glucosinolates is higher than in the other seeds. Most of the investigated seeds contain progoitrin, which causes thyroid enlargement. In Brussels sprouts and rapeseed this glucosinolate represents one of the main glucosinolates [3.9 μmol/g of rapeseed (39% of the total glucosinolates) and 28 μmol/g of Brussels sprouts (44.8%)]. Turnip contains 8.0 μmol/g progoitrin, but this corresponds only to 16% of the total glucosinolates in the seeds.

Conclusion. The unequivocal identification and quantification of glucosinolates in samples of different seeds by an HPLC standard method with ESI-MS-MS and UV detection are possible. The loss of 162.1 Da under MS-MS conditions was used as a characteristic feature of the desulfoglucosinolates. By the neutral loss scan it is possible to detect only molecules having this property, which increases the sensitivity. Although it is possible to quantify with the MS-MS signal, the calculation of each glucosinolate was done according to the standard method by the chromatogram of the UV detector. By combination of ESI-MS-MS and UV detection it is feasible to identify unknown compounds in a sample without changing the conditions of the standard method for the determination of glucosinolates.

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