ELSEVIER

Journal of Chromatography A, 767 (1997) 43–52

JOURNAL OF CHROMATOGRAPHY A

Determination of glucosinolates in domestic and wild mustard by high-performance liquid chromatography with confirmation by electrospray mass spectrometry and photodiode-array detection

Carol L. Zrybko[a,*], Elaine K. Fukuda[b], Robert T. Rosen[b]

[a]*Nabisco, Inc., 200 DeForest Avenue, East Hanover, NJ 07936, USA*

[b]*Center for Advanced Food Technology, Cook College, Rutgers University, New Brunswick, NJ 08903, USA*

Received 11 October 1996; revised 27 November 1996; accepted 29 November 1996

Abstract

A method has been developed to easily separate and quantify glucosinolates in mustard and winter cress plants using reversed-phase HPLC with volatile ion-pairing reagents to modify the mobile phase and enhance retention and selectivity. Volatile buffers are required for HPLC–electrospray mass spectrometry. Confirmation of identity was accomplished using both negative ion electrospray mass spectrometry and photodiode-array detection. This method provides a way to determine glucosinolates in *Brassica* and cruciferous vegetables, most of which are commercially unavailable. Glucosinolates were determined in various parts and sections of yellow and brown domestic mustards and winter cress (wild mustard). Results varied from 0.2 mg/g glucosinolate content in the roots of the plants to 26 mg/g in the seeds.

Keywords: Mustard; Ion-pairing reagents; Glucosinolates

1. Introduction

Domestic mustard and winter cress, commonly known as wild mustard, are both of the *Brassicacae* family of cruciferous vegetables. Mustard is harvested mainly in the northwestern United States and southwestern Canada for processing as a condiment. Winter cress grows ubiquitously in the eastern United States and is often mistaken for a true mustard due to similarities between the flowers and leaves of both plants. The most important components of these plants are the glucosinolates.

Glucosinolates are the nonvolatile flavor precursors to isothiocyanates, nitriles and thiocyanates which are responsible for the hot, pungent taste of the mustards. The structure of glucosinolates, as seen in Fig. 1, contains a β-D-glucopyranosyl unit, a cyano group and a sulfate group. While glucosinolates differ from one another with respect to their side chain, all glucosinolates identified to date are formed from glucose and amino acids through a common biosynthetic pathway. Each species may

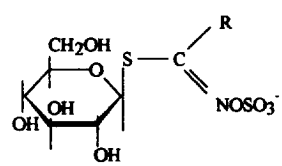

Fig. 1. Structure of glucosinolates.

*Corresponding author.

PII S0021-9673(96)01068-0

contain only two or three glucosinolates thereby providing a "finger print" with which to identify individual species.

Glucosinolates are important in that their myrosinase degradation products have been found to decrease the growth of yeast and microorganisms [1]. Some isothiocyanates formed from glucosinolates are known to be phase II anticarcinogens [2].

Glucosinolates of mustard, winter cress and other cruciferous vegetables have previously been analyzed using both wet chemistry and instrumental methods including paper, thin layer, gas and high-performance liquid chromatography. To date, most research into glucosinolate identification has been done by isolating glucosinolate breakdown products and then determining the original precursor [3]. This method of back tracking was favored because it was easy, inexpensive and fast. Ultraviolet spectroscopy and total glucose concentration by enzymatic methods have been utilized to estimate glucosinolate concentration [4]. Although the glucose method allows for determination of the total glucosinolate content, the separation and quantification of individual intact glucosinolates is often more important.

Betz and Fox [5] have recently determined the glucosinolate content of raw and cooked broccoli by applying reversed-phase HPLC with tetrabutylammonium sulfate as the ion-pairing agent. This method allowed for quantification of intact glucosinolates based on their retention times using known standards. The vast majority of glucosinolates are not available for purchase, therefore a method for identifying glucosinolates that uses HPLC–MS is advantageous.

Most glucosinolate analysis in the 1970s concentrated on running gas chromatography on volatile degradation products to determine the original structure of the glucosinolate prior to degradation [6].

Reversed-phase ion-pair liquid chromatography along with GC analysis of the trimethylsilylated (TMS) derivatives of glucosinolates were techniques utilized by Helboe et al. [7]. Desulfating and derivatizing glucosinolates was very popular in the early 1980s as seen by the number of laboratories utilizing this method [8]. This method, although effective, requires a more complicated sample preparation.

After isolation and degradation of the nonvolatiles, identification of the volatile components is necessary. Truscott et al. [9], have used mass spectrometry and nuclear magnetic resonance spectroscopy on swede (*Brassica napobrassica*), also called rutabaga, to determine the isomeric structure of the indole glucosinolates. Use of NMR on the desulfated glucosinolates allowed these researchers to distinguish between 1-methoxyglucobrassicin (neoglucobrassin) and 4-methoxyglucobrassicin.

In our study, a novel method of reversed-phase ion pair HPLC followed by negative-ion electrospray mass spectrometry was utilized to isolate, identify, and quantify the nonvolatile flavor precursors in mustard and winter cress. Formic acid (F) and triethylamine (TEA) were used to produce triethylammonium formate which interacts with the glucosinolates in the aqueous phase of the system. Once bound, the glucosinolate-TEA.F complex has an increased affinity for the non-polar, organic stationary phase resulting in an increase in retention and elution of glucosinolates after the void volume. This ion-pairing reagent volatilizes during HPLC–MS, and therefore does not clog the LC–MS interface probe [10].

2. Experimental

2.1. Preparation of standards

Two standards, sinigrin (allylglucosinolate) (Aldrich, St. Louis, MO, USA), and gluconasturtiin (phenethylglucosinolate) (LKT Laboratories, St. Paul, MI, USA) were separately prepared at a concentration of 1.0 mg/ml in water and used as external standards for aliphatic and aromatic glucosinolates, respectively. No other glucosinolates were found to be commercially available.

2.2. Preparation of samples

Mustard plants (*Brassica hirta* and *Brassica juncea*) of approximately 4 months post germination were harvested in Canada and shipped by Continental Grain (Milk River, Canada) to New Jersey using overnight express delivery. Samples were packed with ice packs inside a Styrofoam cooler to prevent possible degradation of the glucosinolates.

Winter cress plants (*Barbarea vulgaris*) were collected from a field of wild flowers in Harmony, New Jersey in late April, once the flowers were completely open. To analyze the winter cress seeds, samples were collected in July from plants growing in the same field as the plants picked earlier. Samples were frozen at −20°F prior to extraction.

Mustard and winter cress samples were individually prepared by grinding 5.0 g of flowers, seed pods, leaves, stalks, roots and seeds in a Waring blender with 100 ml methanol. The methanol extracts were filtered through Whatman No. 4 filter paper into a 250 ml round bottom flask. The filtrate was evaporated to 2 ml volume under vacuum using a Rotovap set at 55°C with a rpm of 55. A final volume of 25 ml was obtained by diluting with HPLC-grade water. All samples were filtered using a 0.45-μm nylon Acrodisc (Gelman Sciences, Ann Arbor, MI, USA), as was used during standard preparation.

2.3. HPLC–photodiode-array analysis

Individual intact glucosinolates were separated on a Waters HPLC system (Milford, MA, USA) consisting of a Waters 600E System Controller, a Waters 490 UV detector set at a wavelength of 235 nm, and a 712 WISP auto-injector. Photodiode-array detection was established through the use of a Waters 996 PDA. The entire system was controlled using the Waters Millennium data acquisition software version 2.1. Separation of the glucosinolates was performed by injecting a 20 μl aliquot of sample into a Phenomenex (Torrence, CA, USA) 5 μm ODS (20) column measuring 250×4.6 mm with a Waters C_{18} guard column attached. The column was heated to 40°C.

The mobile phase solvents, HPLC-grade water (A) and methanol (B), were modified with an ion-pairing reagent consisting of 0.15% triethylamine and 0.18% formic acid. HPLC-grade water was generated in the laboratory through the use of a Milli-Q UV Plus Ultra-pure water system by Millipore (Milford, MA, USA). Methanol was purchased from Fisher Scientific (Pittsburgh, PA, USA). All solutions were filtered through 0.45-μm filters, sonicated under vacuum and sparged with Ultra-Pure helium throughout the chromatographic process at a rate of 50 ml/min. The mobile phase was run isocratically at 100% A for 10 min and then switched to a gradient of 100% A to 100% B over 60 min. The flow-rate was held constant at 1.0 ml/minute. After each run the initial mobile phase conditions were set and the system allowed to equilibrate.

2.4. HPLC–MS analysis

Identification of peaks was generated through the use of a Fisons (Danvers, MA, USA) VG Platform II mass spectrometer connected to a VG Mass Lynx data system. The system was operated in the negative ion electrospray mode. The ion source temperature was set at 150°C while the cone voltage was −60 V. The HPLC conditions were identical to those obtained off-line except that a Varian 9012 HPLC (Fernando, CA, USA) was used along with a Varian 9050 UV–Vis detector. This HPLC is interfaced to the mass spectrometer. Higher cone voltage experiments were evaluated so as to try to induce collisionally activated dissociation (CID) resulting in fragmentation patterns.

3. Results and discussion

Nonvolatile compounds such as glucosinolates and derivitized isothiocyanates can be separated by high-performance liquid chromatography. In working with glucosinolates, the mobile phase may be modified with a buffer to increase retention on the column. Although nonvolatile buffers cannot be used in HPLC–MS, they may be used for quantitative analysis by HPLC.

Electrospray mass spectrometry is a powerful tool for identifying highly polar, heat labile compounds such as glucosinolates, present as ions in solution. In negative ion electrospray mass spectrometry (NIES), a high positive electrical potential is applied to a capillary tube carrying the solvent and sample at a rate of approximately 5–500 μl/min to give finely dispersed droplets. As the solvent containing the sample ions passes through the first pumping stage, a stream of N_2 gas evaporates the solvent leaving only the ions. The electrostatic lens is also given an electric potential, but of the opposite charge so that the ions are focused as they enter the skimmer. The ions are then swept into the quadrupole mass spec-

trometer where they are separated [11]. In NIES only the glucosinolate anion is observed. The use of CID (high energy collisions) was not successful for the aromatic glucosinolates. Cone voltages greater than 100, gave no useful fragmentation because of the stability of the aromatic anions.

The glucosinolates of yellow and brown mustard plants, along with winter cress plants, were successfully separated and semi-quantified by HPLC using external standard calibration. Samples were run in triplicate, with the mean and standard deviation reported. Response factors were assumed to be 1. UV chromatograms of the methanol extract of the flowers from all three plants are seen in Fig. 2. Identification of the major glucosinolates (Fig. 3) was established through the use of negative ion electrospray mass spectrometry with secondary confirmation by photodiode-array detection. The identifi-

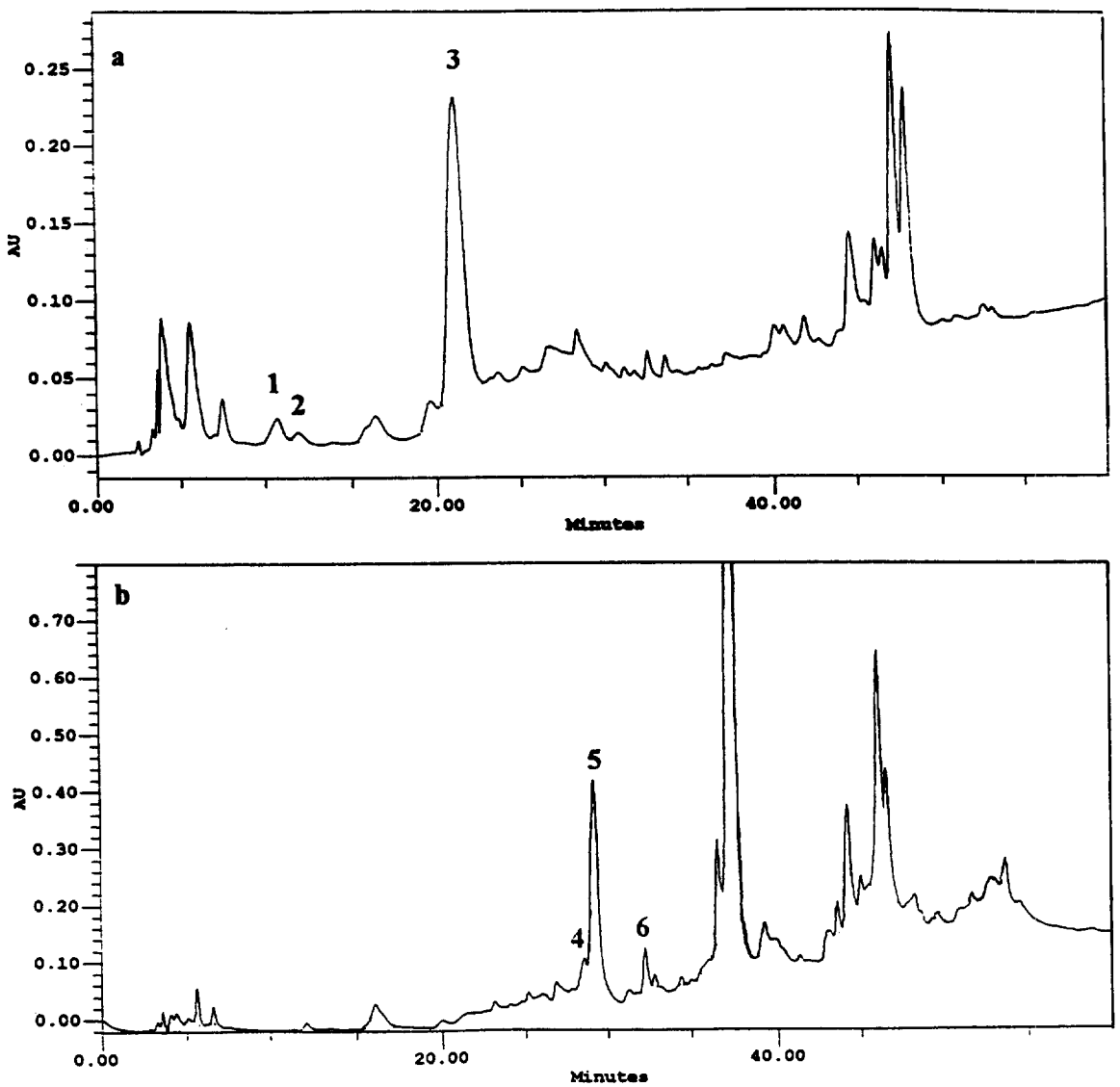

Fig. 2. HPLC separation of glucosinolates from methanol extracts of (a) yellow mustard (*Brassica hirta*) and (b) winter cress (*Barbarea vulgaris*) flowers.

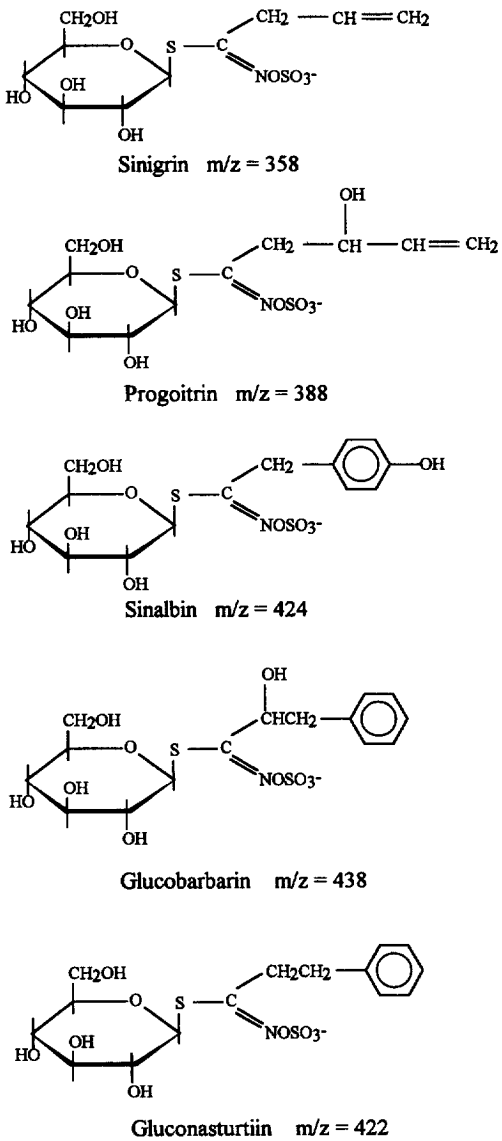

Fig. 3. Structures of identified glucosinolate anions.

cation of glucosinolates which were found in mustard and winter cress using this method, along with the molecular ion and UV maxima, are noted in Table 1.

Results on the quantification of glucosinolates in mustard (Table 2) show that the three glucosinolates, sinigrin, progoitrin and sinalbin, were found at the highest level in the flowers of the plant. As demonstrated by other researchers [12,13], the concentration decreased going down the plant from the flowers to the roots, the roots having the lowest concentration of glucosinolates.

Yellow and brown mustard are often combined with vinegar and seasonings to produce the hot, pungent mustard we use as a condiment. The major volatile flavor compound in brown mustard is allylisothiocyanate (AIT) which is produced through enzymatic degradation of sinigrin. The peak at m/z in the NIES mass spectrum represents the negatively charged portion (anion) of the compound. Although sinigrin is found in nature as a potassium salt, the MS sees only the anion in the negative ion mode. Secondary confirmation of sinigrin was determined by comparing the retention time of the sinigrin standard. Also, the diode-array spectrum matches that of the standard with a maxima of 224 nm (Fig. 4a).

Progoitrin was identified in yellow mustard directly from the mass spectrum. Progoitrin is the precursor to goitrin (5-vinyl-2-thiooxazolidine) which has been found to cause hypothyroidism in farm animals fed large quantities of cruciferous vegetables [14]. The peak at m/z 388 confirms that the compound with a retention time of 11 min on the chromatogram of yellow mustard (Fig. 2a) is indeed progoitrin.

Sinalbin is not found in nature as a potassium salt. The cation, sinapine, is found quite often in the *Brassica* family. The identification of sinalbin was established through the mass spectrum with a peak at m/z 424 (Fig. 5) and UV maximum of 226 nm. Sinapine, a choline ester of 3,5-dimethoxy-4-hydroxy cinnamic acid, was also found by NIES in the seeds of both mustard and wintercress, with a peak on the mass spectrum at m/z 354 (Fig. 6). Peaks seen at 46 mass units higher than the peak at 354 indicate that the compound is forming an adduct with formic acid. The peak at m/z at 354 represents 310 mass units from sinapine and 45 from the formate ion (minus a proton). It is of interest to note that the positively charged sinapine was not observed in ESI+ but the adduct gave a strong signal in ESI−.

Results for the quantification of glucosinolates in the flowers, immature seed pods, mature seeds picked three months later, stalks and roots of the winter cress plant (*Barbarea vulgaris)* can be seen in Table 3.

Table 1
Glucosinolates identified in mustard and winter cress

t_R	Peak No.	Molecular anion	UV maxima	Identification
Mustard				
10.5 min	1	358	224	Allylglucosinolate (sinigrin)
11	2	388	–[a]	2-Hydroxy-3-butenylglucosinolate (progoitrin)
21	3	424	226	*p*-Hydroxybenzylglucosinolate (sinalbin)
Winter Cress				
26.8 min	4	438	236[b]	(2*R*)-Hydroxy-phenylethylglucosinolate (glucobarbarin)
27.5	5	438	236[b]	(2*S*)-Hydroxy-phenylethylglucosinolate (glucobarbarin)
33.5	6	422	238	Phenethylglucosinolate (gluconasturtiin)

[a] Too small to determine.
[b] In general agreement with Ref. [15].

The glucobarbarin isomers were easily separated by the HPLC method utilized in this study. Although Haung et al. [15], were also able to separate the isomers using a C-18 column with acetonitrile and water as the mobile phase, their method required the samples to be desulfated before liquid chromatography. The direct method is much quicker and easier, with no desulfating or derivatizing necessary.

The identification of (2*R*)-glucobarbarin, with a retention time of 26.8 min, was determined directly from the negative ion electrospray mass spectrum. The molecular anion of glucobarbarin is indicated by a peak at *m/z* 438. The (2*S*)-glucobarbarin isomer with a retention time of 27.5 min, was also identified by MS, and as seen in the mass chromatogram (data not shown), the two species are distinctly separate. The photodiode-array spectrum also confirms the identity of these two compounds as glucobarbarin. Fenwick et al. [14], determined the identity of the two isomers based on the optical rotation (2*R* is more dextrorotatory), ^{1}H and ^{13}C NMR, and concluded that the 2*R* elutes earlier in reversed-phase HPLC. We relied on this data for deciding which optical isomer eluted first on our system.

Table 2
Quantification of glucosinolates in mustard

	Sinigrin	Progoitrin (mg/g)	Sinalbin
Yellow Mustard Plants (Brassica hirta)			
Flowers	0.25±0.06	0.06±0.02	4.8±0.8
Seed Pods	0.11±0.00	0.06±0.01	3.8±0.0
Seeds	–	0.56±0.10	25±2
Leaves	0.06±0.00	–	2.2±0.4
Stems	0.08±0.00	–	1.8±0.5
Stalks	–	–	–
Roots	–	–	–
Brown Mustard Plants (Brassica juncea)			
Flowers	3.5±0.6	0.04±0.00	–
Seed Pods	0.82±0.23	–	–
Seeds	8.8±2.0	–	–
Leaves	0.80±0.25	–	–
Stems	1.0±0.0	–	–
Stalks	–	–	–
Roots	–	–	–

C.V.=<10% except when the concentration is less than 1 mg/g.

Gluconasturtiin, also present in nature as a potassium salt, was identified by comparison of retention time for the external standard, the photodiode-array spectrum (Fig. 4b), and the mass spectrum. This glucosinolate anion is represented by a peak at *m/z* 422.

Results show that (2*S*)-glucobarbarin and gluconasturtiin are found at the highest concentration in the mature seeds while (2*R*)-glucobarbarin is found at very low concentrations throughout the plant. Overall, the concentration of gluconasturtiin is much higher than that of both isomers of glucobarbarin in all plant components.

To determine the accuracy and precision, brown mustard samples were spiked with sinigrin only and the percent recovery calculated. Recovery ranged from 94–110%. The relative standard deviation was calculated on multiple replicates to determine the

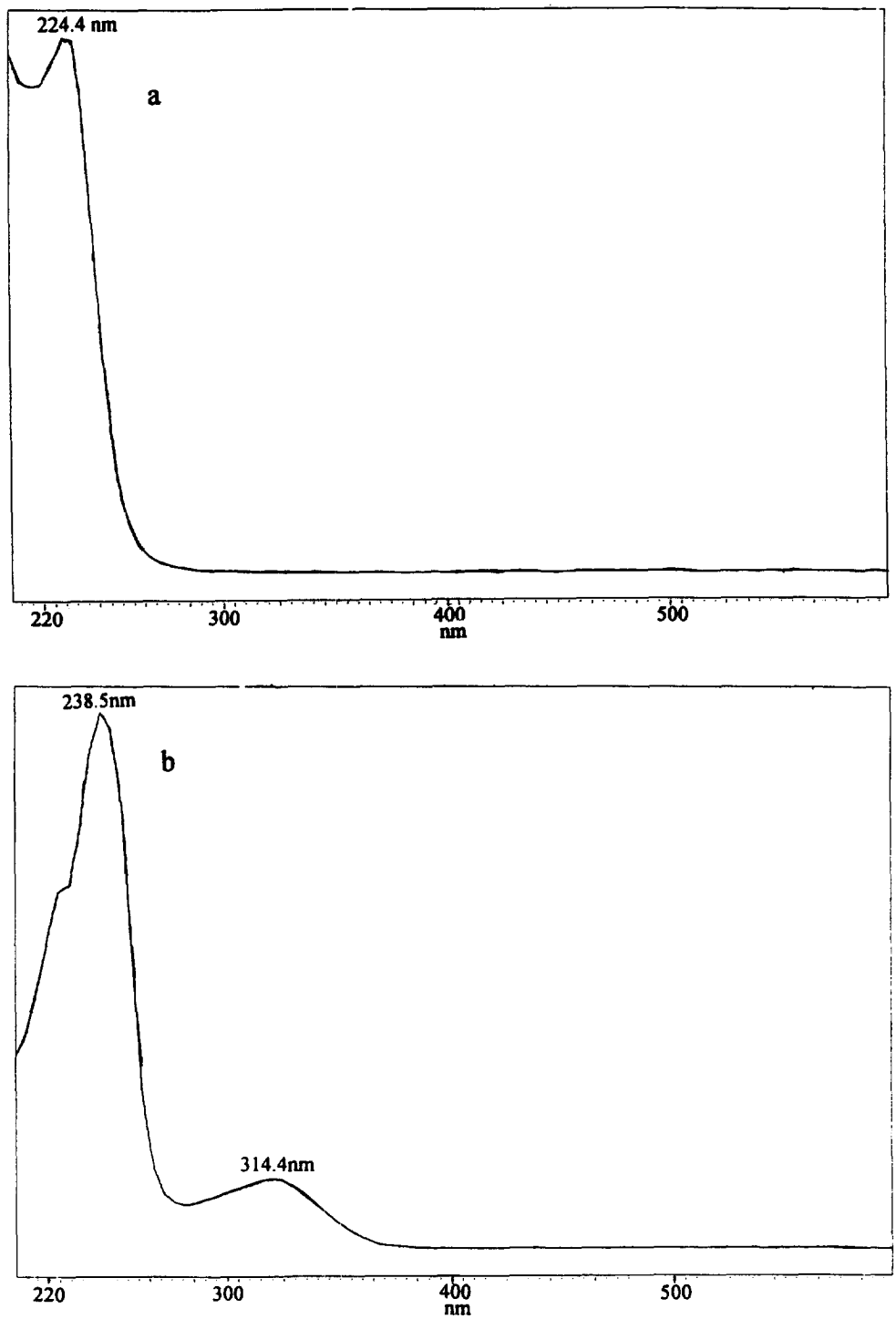

Fig. 4. Photodiode array spectrum of (a) sinigrin and (b) gluconasturtiin.

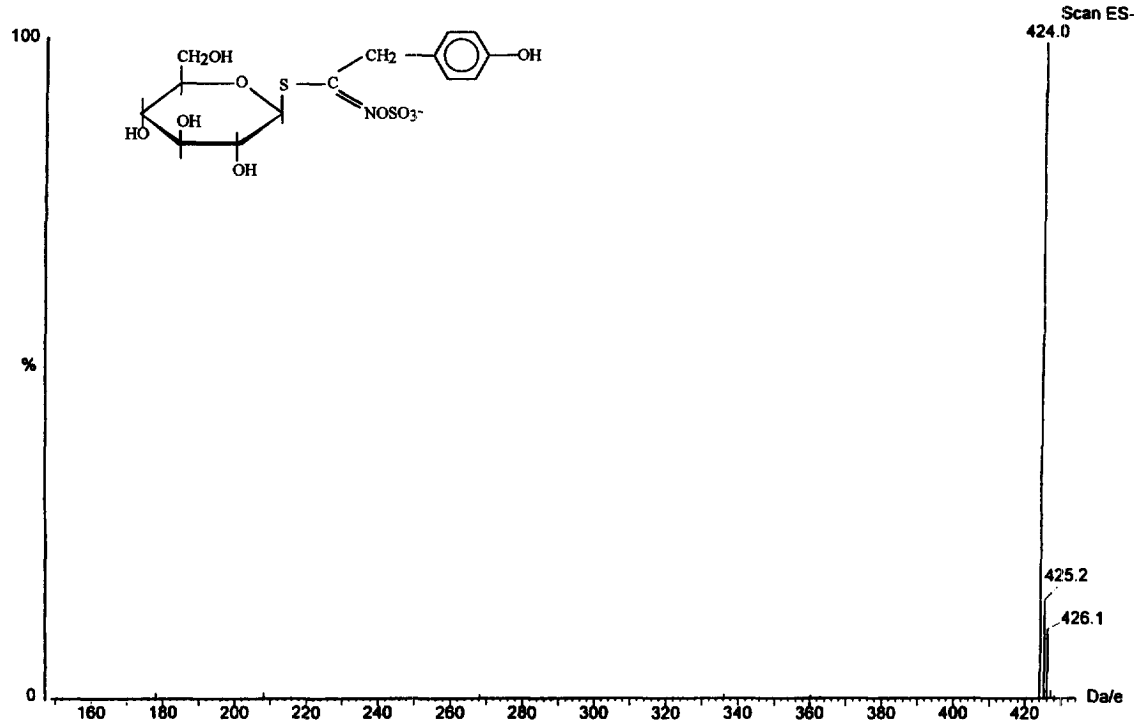

Fig. 5. Negative ion electrospray mass spectrum of sinalbin.

precision of the method. In most cases the R.S.D. for mustard and winter cress flowers, leaves, and seeds was often less than 10%. In some cases where the concentration of the glucosinolate was less than 1 mg/g, such as in the roots, the R.S.D. was higher indicating that the glucosinolate level was close to the limit of detection.

4. Conclusion

To date, the major nonvolatiles in mustard and winter cress have been separated, identified and quantified by reversed-phase HPLC using ion-pairing reagents and negative ion electrospray mass spectrometry as well as diode-array detection. This method works well for identifying intact glucosinolates and therefore eliminates the need for desulfating and derivatizing glucosinolates as seen in previous studies. Negative ion electrospray mass spectrometry is ideal for identifying glucosinolates as anions in solution. Although other peaks were evident in the UV chromatogram, they are not visible by this mass spectrometry method indicating that these compounds are uncharged and are neutral organics. For these systems, only the glucosinolates and sinapine gave signals by NIES mass spectrometry. By using this method we were able to distinguish between glucosinolates and other nonvolatile compounds present in cruciferous vegetables. Use of mass spectrometry, with secondary confirmation by photodiode-array detection, allowed us to identify six glucosinolates.

Acknowledgments

We acknowledge the Center for Advanced Food Technology (CAFT) mass spectrometry facility for providing instrumentation support. CAFT is an initiative of the New Jersey Commission on Science and Technology. This is New Jersey Agricultural

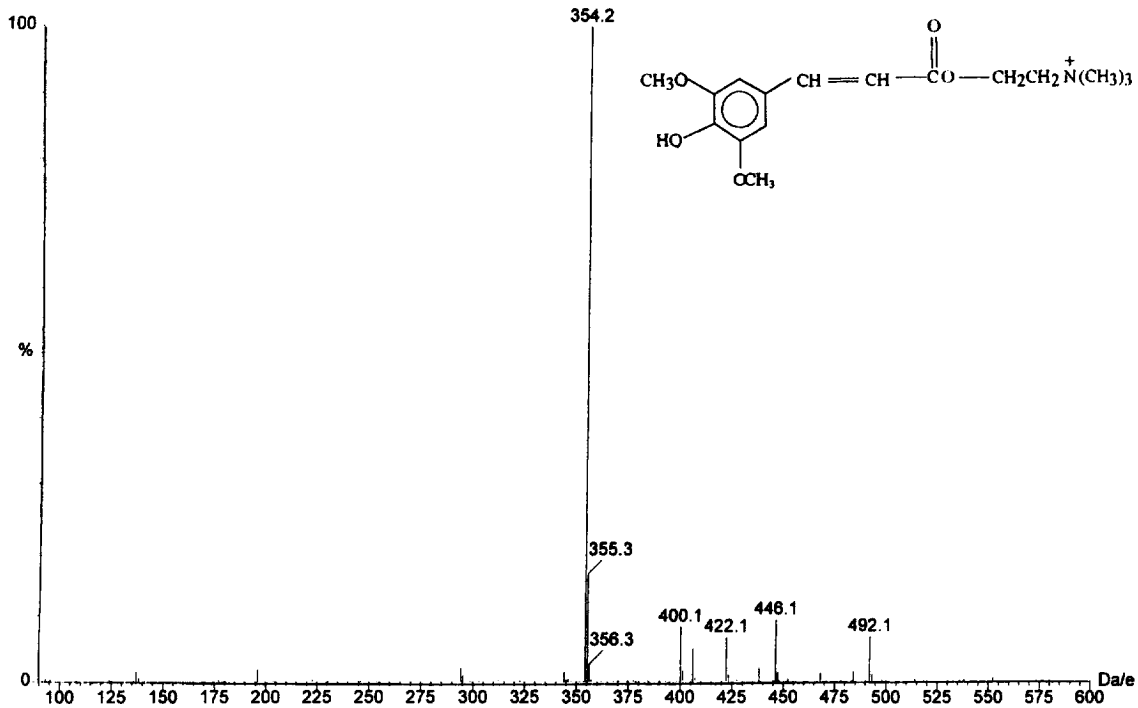

Fig. 6. Negative ion electrospray mass spectrum of sinapine.

Table 3
Quantification of glucosinolates in winter cress plants

	(2*R*)-Glucobarbarin (mg/g)	(2*S*)-Glucobarbarin (mg/g)	Gluconasturtiin (mg/g)
Winter Cress (*Barbarea vulgaris*)			
Flowers	0.29±0.05	3.3±0.9	2.7±0.2
Seed Pods	–	–	11±1
Seeds (Mature)	1.1±0.2	16±2	26±2
Leaves	0.03±0.05	0.02±0.12	2.3±0.2
Stalks	–	0.06±0.09	3.8±0.4
Roots	0.02±0.12	0.29±0.03	3.8±0.2

C.V.=<10% except when the concentration is less than 1 mg/g.

Experiment Station (NJAES) publication No. D10569-1-97.

References

[1] P.J. Delaquis and G. Mazza, Food Technol., 11 (1995) 73–84.
[2] C.A. Bradfield and L.F. Bjeldanes, J. Agric. Food Chem. 35 (1987) 46–49.
[3] O. Oleson and H. Sorensen. J. Am. Oil Chem. Soc., (1981) 857–865.
[4] C.H. VanEtten, C.E. McGrew and M. Daxenbichler, J. Agric. Food Chem., 22 (1974) 483–487.
[5] J.M. Betz and W.D. Fox, in M. Huang, T. Osawa, C. Ho and R.T. Rosen (Editors), Food Phytochemicals for Cancer Prevention I, ACS Symposium Series, 1994, Ch. 14, p. 181–196.
[6] M.E. Daxenbichler and C.H. VanEtten, J. AOAC, 60 (1977a) 950–953.
[7] P. Helboe, O. Olsen and H. Sorensen, J. Chromatogr., 197 (1980) 199–205.

[8] R.J.W. Truscott, I. Minchinton and J. Sang, J. Sci. Food Agric., 34 (1983) 247–254.
[9] R.J.W. Truscott, P.K. Johnstone, I.R. Minchinton and J.P. Sang, J. Agric. Food Chem., 31 (1983) 863–869.
[10] R. Hiserodt, T.G. Hartman, C. Ho and R.T. Rosen, J. Chromatogr. A, 740 (1996) 51–63.
[11] W.M.A. Niessen and J. van der Greef, Liquid Chromatography–Mass Spectrometry, Principles and Applications, Marcel Dekker, New York, 1992, p. 3–56.
[12] P.S. Sukhija, A. Loomba, K.L. Ahuja and S.K. Munshi, Plant Sci., 40 (1985) 1–6.
[13] S. Kaur, S.K. Gupta, P.S. Sukhija and S.K. Munshi, Plant Sci., 66 (1990) 181–184.
[14] G.R. Fenwick, R.K. Heaney and W.J. Mullin, CRC Crit. Rev. Food Nut., 18 (1983) 123–201.
[15] X. Haung, J.A.A. Renwick and K. Sachdev-Gupta, J. Chem. Ecology, 20 (1994) 423–438.