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Analysis of Intact Glucosinolates by MALDI-TOF Mass Spectrometry

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Glucosinolates are naturally occurring plant compounds that may be important in the dietary prevention of cancer. This study shows that they can be detected in their intact form by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) with a high degree of sensitivity. The methodology was used to characterize a number of individual glucosinolates either produced by synthetic chemistry or isolated from plants. The method was used for crude plant extracts to rapidly examine the glucosinolate profile of the plant. The results for a range of plant extracts showed good agreement with previous LC−MS analysis of the desulfoglucosinolates from the same samples.

KEYWORDS: Glucosinolates; *Brassica* sp.; MALDI-TOF; metabolomics

INTRODUCTION

Glucosinolates **1** are a class of plant secondary metabolites (*1*) with some structural variability. The generalized structure contains a β-D-glucose linked to a thiohydroximate which is sulfated. The side chain R is variable, and over 100 different glucosinolates have been identified and characterized. R is derived from modified amino acid side chains, which may be chain extended, hydroxylated, methylated, etc. (*2*). Glucosinolates are mainly found in species belonging to the order Capparales and are particularly abundant in the Cruciferae, including *Brassica* sp. vegetables such as Brussels sprouts, broccoli, and cabbage (*3*).

The glucosinolates are stored in plant vacuoles and are hydrolyzed by the enzyme myrosinase (thioglucosidase glucohydrolase, EC 3.2.3.1) following tissue damage, to produce a variety of volatile and nonvolatile bioactive compounds (*1*, *4*) (**Scheme 1**). The main product is the isothiocyanate **2**, although factors such as pH, the presence of various myrosinase-associated proteins, and the nature of the side chain R, have a major influence on the product distribution (*1*, *3*, *5*). The glucosinolates and their breakdown products are thought to play an important role in the plant's general defense mechanism against fungal infection and nonadapted herbivores (*4*). However, in some instances these compounds are also involved in host-plant recognition by specialized pests (*6*). For example, leaf-surface glucosinolates can act as oviposition (egg-laying) stimulants for *Brassica* sp. adapted insects, e.g. cabbage and turnip root flies (*7*, *8*). The volatile isothiocyanates produced on glucosinolate breakdown can also attract pests to their host plants (*9*).

In mammals, the consumption of high concentrations of glucosinolates has been shown to produce anti-nutritional and potentially toxic effects (*10*). However, recent work has demonstrated that *Brassica* sp. consumption in humans is associated with a reduced risk of certain cancers such as colorectal cancer (*11*). Attention has focused on the isothiocyanates that are formed from glucosinolate breakdown as the active compounds. The most active appears to be sulforaphane, produced from the breakdown of the glucosinolate glucoraphanin found in broccoli, which appears to inhibit phase I detoxification enzymes, induce mammalian phase II detoxification enzymes, and protect against the initiation of cancer (*12*, *13*).

Because the specific biological effects of the different glucosinolates vary considerably it is important to be able to accurately identify the specific glucosinolates in any plant tissue under investigation. Rapid examination of the glucosinolate profile of a particular plant is important in plant breeding studies in which the focus is on optimizing resistance to pests. It may also be important when screening the anti-cancer activities of vegetables.

Methods for the analysis of glucosinolates have been recently reviewed (*14*, *15*). GC−MS analysis of glucosinolate breakdown products has often been used, but it has limitations as some glucosinolate side chains (e.g., indolyl or hydroxylated side chains) produce poorly volatile or unstable breakdown products.

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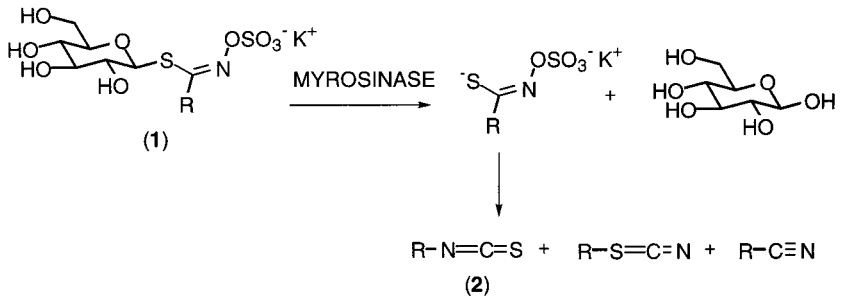
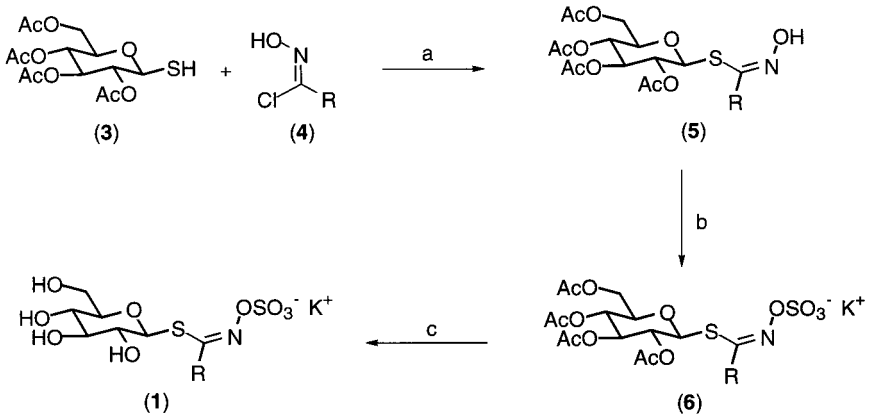
Scheme 1. Metabolism of Glucosinolates



Scheme 2 . Synthetic Route Used To Prepare Glucosinolates



a) Et_3N, THF; b) $ClSO_3H$, pyridine, CH_2Cl_2 then aq. K_2CO_3 ; c) KOMe, MeOH

The intact glucosinolates can be analyzed by HPLC, although this requires elaborate mobile phases and often gives poor resolution. The most satisfactory technique to date appears to be the HPLC separation of enzymatically desulfated glucosinolates (*16*, *17*). Desulfation results in better separation and cleaner samples and also makes the compounds easier to analyze by traditional mass spectrometric techniques. Both LC−thermospray-MS (*18*) and LC−APCI-MS (*17*) have been employed for desulfoglucosinolates, and the latter has been found to give the best sensitivity, with at least an order of magnitude improvement over UV detection and the added advantage of structural confirmation from the mass spectrum. The sensitivity can be further improved by the use of synthetic per-deuterated desulfoglucosinolates as internal standards (*17*, *19*). Analysis of intact glucosinolates by FAB-MS has been reported, but it has not become established as an analytical technique (*20*, *21*).

MALDI-TOF MS was introduced in 1987 and developed for use with nonvolatile and large biomolecules (*22*). It has some advantages over other methods including speed of analysis, high sensitivity, good tolerance toward contaminants, and the ability to analyze complex mixtures (*23*). MALDI-TOF MS thus has potential for the analysis of plant metabolites, both in the plant itself and in foodstuffs, as a profile of the sample can be obtained in only a few minutes. For example, a recent report described methodology for the analysis of soy isoflavones using this technique (*24*). The objective of this work was to develop a robust and reliable MALDI-TOF MS method for glucosinolate analysis, initially for confirmation of the structures of a series of synthetic glucosinolates and glucosinolates isolated and purified from plants. The method was then employed for analysis of crude plant samples, to allow rapid determination of the glucosinolate profile. Such a procedure could have great utility for screening *Brassica* sp., and other members of the Cruciferae, in breeding programs to increase pest resistance and in studies on anti-cancer activity.

MATERIALS AND METHODS

Glucosinolate Synthesis. The synthetic glucosinolates were prepared using the route shown in **Scheme 2**. This is the most commonly used procedure for glucosinolate synthesis (*25*−*27*). Tetraacetyl thioglucose **3** is coupled to an oximyl chloride derivative **4** to give the protected thiohydroximate **5** which is then sulfated with chlorosulfonic acid in pyridine and deprotected using potassium methoxide in methanol. The oximyl chloride can be prepared from either an aldehyde (*25*) or a nitroalkane (*28*). For the propyl, butyl, pentyl, heptyl, and nonyl glucosinolates and gluconasturtiin the commercially available aldehydes were used as starting materials (*29*, *16*). For the naphthylmethyl and 7-carboxymethyl glucosinolates extra synthetic steps were required to prepare the requisite aldehydes, as described previously (*29*). All the new synthetic glucosinolates have been fully characterized (^1H and ^{13}C NMR spectroscopy, mass spectrometry, microanalysis, etc.) to confirm their structure and purity (*16*, 29).

Glucosinolate Isolation. Glucotropaeolin, glucoraphanin, and glucoiberin were isolated from seeds of *Tropaeolum majus* cv. Empress of India (nasturtium), *Brassica oleracea* var. *botrytis* subvar. *cymosa* cv. Shogun F1 (calabrese), and *Iberis amara* L. (candytuft), respectively. Seeds were obtained from E. W. Kings, UK (*T. majus* and calabrese) and B & T World Seeds, Olonzac, France (candytuft). Briefly, the seeds were oven-dried at 100 °C for 24 h then extracted with 70% v/v MeOH at 70 °C for 30 min. The extracts were clarified by centrifugation and concentrated by rotary evaporation at 40 °C, protein was removed by addition of 0.1 M 1:1 Pb/Ba acetate followed by centrifugation, and the final supernatants were 0.2-μm filtered. Sub-samples (400 μL) of these extracts were loaded onto C_{18} SPE columns (column 1.2 cm i.d., 6.5 cm high, 3 mL bed volume) gravity-packed with Phenomenex (Macclesfield, Cheshire, UK) 50-μm C_{18} bulk packing silica. Sequential elution was carried out with 5-mL additions of aqueous MeOH, with increasing MeOH up to 100% MeOH (flow rate ca.1 mL/min). The

identities of the glucosinolates were confirmed by desulfation and analysis by HPLC (*16*).

Preparation of Crude Extracts. The vegetable samples (cauliflower, rutabaga (swede), and turnip) were diced and immediately dipped into liquid nitrogen, freeze-dried, and then milled using a laboratory mill fitted with a 1-mm screen. A 1-g sample of the freeze-dried powder was then placed in a 100-mL flask, and 20 mL of boiling 70% aqueous methanol was added. After swirling, the samples were refluxed for 15 min using a preheated water bath. After the sample was filtered the residue was re-extracted with a further 20 mL of boiling 70% methanol, and the combined filtrates were reduced by rotary evaporation to a final volume of 10 mL.

The *Lunaria annua* seeds were deactivated by heating at 100 °C for 10 min and then finely ground in a pestle and mortar. A 0.5-g sample was placed in a 100-mL round-bottomed flask and extracted using boiling 70% aqueous methanol, in the same manner as for the vegetable samples except that the combined filtrates were reduced to a final volume of 25 mL rather than 10 mL.

A portion of each sample (500 μL) was clarified by centrifugation, and the supernatant was lyophilized. The residue was redissolved in water (50 μL). Of this, 0.5 μL was applied to the target and analyzed as described below. This volume represented the extract from 0.2 to 0.5 mg of the plant material. Some samples appeared to contain too much salt to give good spectra (dried to give a glassy appearance). These samples were dialyzed using Spectra/Por Biotech Cellulose Ester membrane (Medicell International Ltd, London) with a 100 or 500 molecular weight cut off. A square of membrane approximately 1.5 × 1.5 cm was floated on about 50 mL of water in a Petri dish. Extract (5 μL) was applied to the center of the square and allowed to dialyze for 10 min. The dialyzed extract was then pipetted back up and used in the analysis as described below.

MALDI-TOF Mass Spectrometry. The sample was dissolved in methanol or water, and 0.5 μL was applied to the stainless steel target along with 0.5 μL of 3-aminoquinoline (10 mg/mL in a 1:1 mix of methanol and 0.1% TFA) (Fluka, Gillingham, Dorset, UK). The spot was allowed to dry and then analyzed, in the negative ion mode, by MALDI-TOF mass spectrometry using a TofSpec 2E (Micromass, Manchester, UK) in the reflectron mode, using, in the first instance, a laser coarse setting of 50% and a laser fine setting of 30%. This was reduced downward, if possible, while still allowing clear signals to be obtained. One hundred shots were combined. Signals were obtained which correspond to M−H ion. External calibration was performed using glucose sulfate (M−H (monoisotopic) 259.01) and ATP (M−H (monoisotopic) 505.99) as calibrants. The TofSpec 2E gives an accuracy of 100 ppm or better for a 2465 Da peptide by external calibration. Dilutions of propyl glucosinolate were made in order to determine the limit of detection of this technique.

RESULTS AND DISCUSSION

The initial aim of this study was to develop a suitable MALDI-TOF MS procedure for the analysis of samples of intact synthetic glucosinolates. This arose from the need to characterize a number of synthetic glucosinolates prepared in our laboratory, which were to be studied to determine their activity as oviposition stimuli for cabbage and turnip root flies. It was often found difficult to establish by any other spectroscopic techniques that the sulfation of the glucosinolate precursor had taken place. The effect of sulfation on most of the spectroscopic properties of the molecule is minimal, and although both FAB-MS and electrospray-MS had been employed to observe the increase in mass following sulfation, they did not give reliable results. MALDI-TOF MS was chosen as it is a technique suitable for the analysis of nonvolatile species such as sulfate salts. By using the spectrometer in the negative ion mode and using high concentrations of sample relative to matrix one can see strong signals in the 350−500 Da mass range for the glucosinolates despite the matrix background (**Figure 1**). As the glucosinolates are already salts (normally potassium) they require only volatilization from the target plate. MALDI-TOF MS is also rapid and easy to perform and analyze as only signals for the intact molecule are obtained.

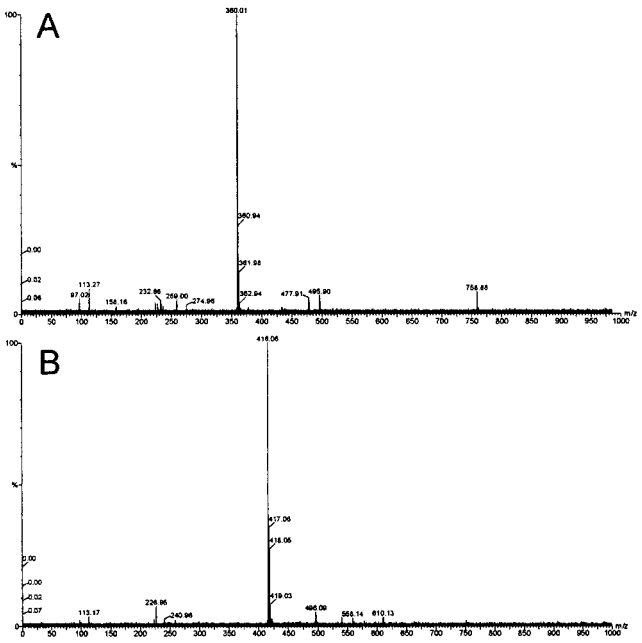


Figure 1. MALDI-TOF mass spectra of (A) propyl glucosinolate and (B) pentyl glucosinolate with 3-aminoquinoline (10 mg/mL in 1:1 methanol and 0.1% trifluoroacetic acid) as matrix, acquired in negative ion reflectron mode, using glucose sulfate and ATP as external calibrants.

Table 1. Analysis of Individual Synthetic and Isolated Glucosinolates by MALDI-TOF Mass Spectrometry

name	side chain (R)	M–H expected	M–H observed
n-propyl glucosinolate[a]	$CH_2CH_2CH_3$	360.04	360.01
n-butyl glucosinolate[a]	$(CH_2)_3CH_3$	374.06	373.99
n-pentyl glucosinolate[a]	$(CH_2)_4CH_3$	388.06	388.04
n-heptyl glucosinolate[a]	$(CH_2)_6CH_3$	416.10	416.06
n-nonyl glucosinolate[a]	$(CH_2)_8CH_3$	444.14	444.10
naphthylmethyl glucosinolate[a]	$CH_2C_{10}H_7$	458.06	458.05
7-carboxymethyl glucosinolate[a]	$(CH_2)_6COOCH_3$	460.09	460.11
tropaeolin[b]	CH_2Ph	408.04	408.10
gluconasturtiin[a]	CH_2CH_2Ph	422.06	421.85
brassicin[a]	CH_2indole	447.05	446.98
glucoiberin[b]	$(CH_2)_3SOCH_3$	422.02	422.05
glucoraphanin[b]	$(CH_2)_4SOCH_3$	436.04	436.04

[a] Synthetic. [b] Isolated from plant material.

MALDI-TOF MS of Synthetic and Isolated Glucosinolates. A wide range of matrixes is available for use in MALDI-TOF mass spectrometry. The matrix 3-aminoquinoline (*30*) which is known to give good signals for sugars, among other molecules, was found to be the matrix of choice for this analysis. All the glucosinolates analyzed gave good signals, in negative ion mode, corresponding to the M−H ion of the intact glucosinolate (**Table 1**; **Figure 1**). The external calibrants chosen, glucose sulfate and ATP, span the mass range of all the possible glucosinolates and, being functionalized sugar molecules themselves, are very appropriate calibrants.

Dilutions of propyl glucosinolate showed that the minimum application to the plate that could be detected was 0.5 ng. It should be noted that only a very small proportion of this sample is actually consumed in the ionization process. The remainder could therefore theoretically be redissolved and removed from the target after analysis, although the sample would contain a large excess of the matrix 3-aminoquinoline. However, this

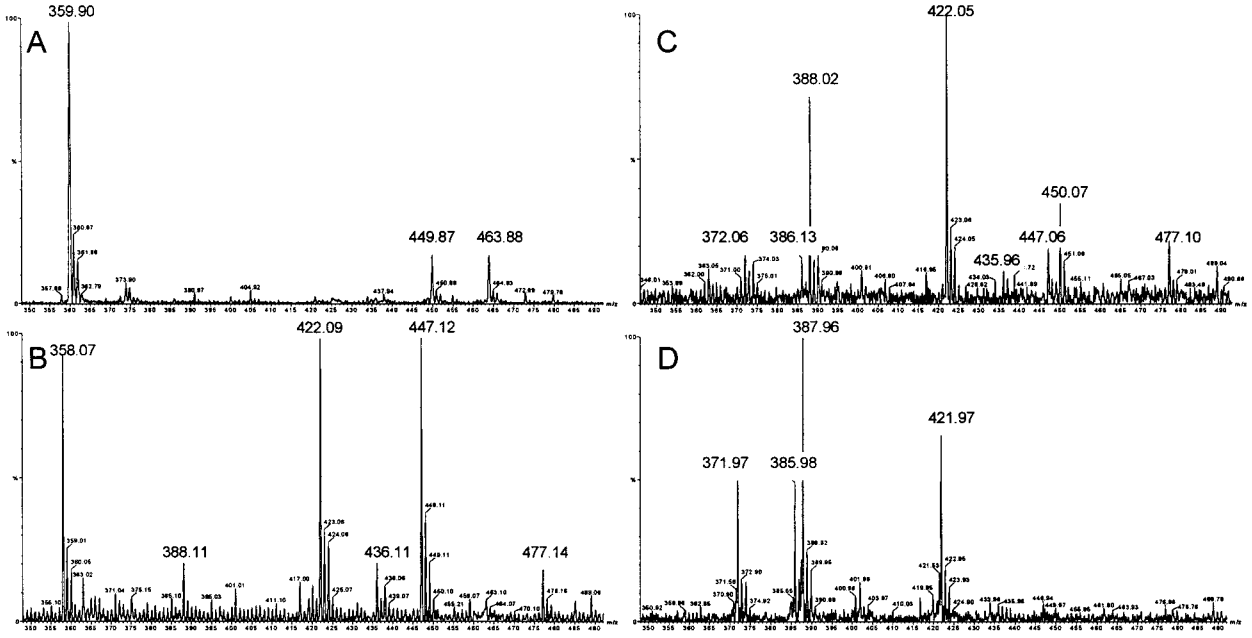


Figure 2. MALDI-TOF mass spectra of extracts from (A) *L. annua* seeds; (B) cauliflower florets; (C) rutabaga (swede) peel; and (D) turnip. Spectra were acquired in negative ion reflectron mode, on a Micromass TofSpec 2E, with 3-aminoquinoline (10 mg/mL in 1:1 methanol and 0.1% trifluoroacetic acid) as matrix, using glucose sulfate and ATP as external calibrants.

Table 2. Results from Analysis of Crude Plant Extracts by MALDI-TOF MS

M−H observed	corresponding glucosinolate	side chain (R)	M−H expected
	Lunaria annua		
359.90	*i*-propyl glucosinolate	$(CH_3)_2CH$	360.04
449.87	5-methylsulfinylpentyl glucosinolate	$(CH_2)_5SOCH_3$	450.06
463.88	6-methylsulfinylhexyl glucosinolate	$(CH_2)_6SOCH_3$	464.07
	Cauliflower Florets		
358.07	prop-2-enyl glucosinolate	CH_2CHCH_2	358.03
388.11	2-hydroxybut-3-enyl glucosinolate	$CH_2CH(OH)CHCH_2$	388.04
422.09	phenylethyl (gluconasturtiin) or	CH_2CH_2Ph	422.06
	3-methylsulfinylpropyl glucosinolate (glucoiberin)	$(CH_2)_3SOCH_3$	422.02
436.11	4-methylsulfinylbutyl glucosinolate (glucoraphanin)	$(CH_2)_4SOCH_3$	436.04
447.12	3-indolylmethyl glucosinolate (brassicin)	CH_2indole	447.05
477.14	1-methoxy-3-indolylmethyl glucosinolate	CH_2indoleOCH_3	477.06
	Rutabaga Peel		
372.06	but-3-enyl glucosinolate	$(CH_2)_2CHCH_2$	372.04
386.13	pent-4-enyl glucosinolate	$(CH_2)_3CHCH_2$	386.06
388.02	2-hydroxybut-3-enyl glucosinolate	$CH_2CH(OH)CHCH_2$	388.04
422.05	phenylethyl (gluconasturtiin) or	CH_2CH_2Ph	422.06
	3-methylsulfinylpropyl glucosinolate (glucoiberin)	$(CH_2)_3SOCH_3$	422.02
435.96	4-methylsulfinylbutyl glucosinolate (glucoraphanin)	$(CH_2)_4SOCH_3$	436.04
447.06	3-indolylmethyl glucosinolate (brassicin)	CH_2indole	447.05
450.07	5-methylsulfinylpentyl glucosinolate	$(CH_2)_5SOCH_3$	450.06
477.10	1-methoxy-3-indolylmethyl glucosinolate	CH_2indoleOCH_3	477.06
	Turnip		
371.97	but-3-enyl glucosinolate	$(CH_2)_2CHCH_2$	372.04
385.98	pent-4-enyl glucosinolate	$(CH_2)_3CHCH_2$	386.06
387.96	2-hydroxybut-3-enyl glucosinolate	$CH_2CH(OH)CHCH_2$	388.04
421.97	phenylethyl (gluconasturtiin) or	CH_2CH_2Ph	422.06
	3-methylsulfinylpropyl glucosinolate (glucoiberin)	$(CH_2)_3SOCH_3$	422.02

could be removed by dialysis. The sensitivity levels achieved here compare favorably with those of LC−APCI-MS methods where the detection limit was found to be 0.1−0.2 mg/mL, corresponding to 0.5−1 ng of sample on column, using the single ion monitoring mode (*17*).

MALDI-TOF MS of Crude Plant Extracts. The strength and clarity of the MALDI-TOF mass spectra obtained for the single glucosinolates were such that it was decided to explore the technique's applicability to the screening of crude plant samples, as a rapid method for determination of the glucosinolate profile. Glucosinolate-containing extracts were prepared from the seeds of *L. annua* and from cauliflower florets, rutabaga peel, and turnip. The material was extracted with refluxing 70% aqueous methanol, the organic solvent was removed by rotary evaporation, and then the aqueous extract was examined directly by MALDI-TOF MS. Very clear spectra were obtained from the extracts with strong peaks which corresponded to the expected masses of specific glucosinolates (see **Figure 2** and **Table 2**). These results showed excellent agreement with previously published results of studies in which LC−MS was

used to separate and identify the desulfoglucosinolates in *L. annua* seeds and similar work performed on the vegetable species (*17*). The spectrum obtained from the *L. annua* extract clearly showed three strong peaks at *m*/*z* 360, 450, and 464 as the only signals in the mass range 350 to 500 Da (**Figure 2A**). These masses correspond to glucosinolates with isopropyl, 5-methylsulfinyl, and 6-methylsulfinyl side chains, which were also the three glucosinolates identified by LC−MS, and in previous work based upon the analysis of myrosinase-induced breakdown products (*31, 32*). The sensitivity of the MALDI-TOF MS technique is such that these species could be detected from a sample of just 0.2 mg of seeds.

The MALDI-TOF mass spectra of the samples from cauliflower, rutabaga, and turnip were also very clean, and the glucosinolates could be clearly observed. The data from MALDI-TOF MS again gave very good agreement with LC−MS analysis (*17*), showing the same spread of glucosinolates, and there was some correlation in terms of relative amounts.

MALDI-TOF mass spectrometry thus represents a quick and simple method for the analysis of glucosinolates. Very little purification is required, and the method also has the added advantage that it does not require a desulfonation step. However, its obvious disadvantage is that it will not distinguish glucosinolates of near identical mass but different structure. For example, glucoiberin (3-methylsulfinylpropyl glucosinolate) and gluconasturtiin (phenylethyl glucosinolate) both give M−H ions of molecular weight 422, differing by only 0.04 Da, and because under the conditions employed only the intact molecular ion is obtained, there is no fragmentation pattern to help distinguish between these glucosinolates. Furthermore, without much more extensive work comparing the relative ionization abilities, and therefore signal intensities, of the glucosinolates found, no detailed quantitative data can be obtained from this study. However, there does seem to be a general correlation between signal intensity and the amount of glucosinolate present, with those described as the most abundant species in previous studies giving strong signals here and those described as being at trace levels giving weak signals. To obtain truly quantitative data the use of labeled internal standards would be required. Each individual glucosinolate would be required in a labeled form, displaying a mass increase of at least 2 Da. In our laboratory we have previously used deuterated desulfoglucosinolates as internal standards for LC−MS analysis (*17*, *19*), and the analogous use of deuterated glucosinolates as internal standards for MALDI-TOF MS is currently under investigation.

In conclusion, it has been demonstrated that MALDI-TOF mass spectrometry is a sensitive technique for the analysis of intact glucosinolates. The technique has been used to analyze synthetically produced glucosinolates, glucosinolates which have been isolated and purified, and those extracted in a crude form directly from plant matter. It is therefore a useful addition to the methods currently available for the analysis of glucosinolates.

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