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Class targeted metabolomics: ESI ion trap screening methods for glucosinolates based on MS^n fragmentation

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Abstract

Glucosinolates are naturally occurring anionic secondary plant metabolites incorporating a thioglucosidic link to the carbon of a sulphonated oxime. There are a large number of naturally occurring glucosinolates and they are found in relatively large quantities in many plant species within the family Crucifereae. These metabolites are of interest for both their anticancer and flavour properties and in the study of nitrogen and sulphur metabolism in model plants such as *Arabidopsis*. Parent ion mapping is an analytical mass spectrometry approach that allows rapid assessment of glucosinolate content. Ion mapping proved to be highly sensitive and the glucosinolate sinigrin could be detected at three parts per trillion. This method takes advantage of the glucosinolate anion fragmentation which consistently produces a sulphonate ring-opened glucose moiety in the ion trap mass spectrometer, *m/z* 259. An intramolecular transfer mechanism for this fragmentation is presented here for the first time. This fragmentation can be exploited as a general identifier of the glucosinolate class of metabolites in plant extracts and in LCMS^n can be employed provide positive identification and quantification of individual glucosinolates. Such approaches offer sensitive tools for focused metabolomics analysis and screening of plant breeding lines.

Keywords: Glucosinolates; LCMS; Ion trap mass spectrometry; *Brassica sp.*; Metabolomics; Crucifereae

1. Introduction

Glucosinolates are widely distributed in the genus *Brassica* and the family Crucifereae, and have generated significant interest with respect to their proposed role in cancer prevention. Interest has focused on glucoraphanin which is hydrolysed by the enzyme myrosinase to form isothiocyanates and sulforaphane when the enzyme is released from the plant vacuoles after mechanical stress, e.g. during cutting or chewing. Sulforaphane, derived from glucoraphanin, is the active component that has been shown to inhibit carcinogenesis by several means, including the inhibition of Phase 1 enzymes, the induction of Phase II enzymes, cell proliferation and angiogenesis inhibition and induction of apoptosis (Fahey et al., 1997; Juge et al., 2007; Nestle, 1998; Pereira et al., 2002). Another glucosinolate, sinigrin, breaks down to produce the characteristic taste in mustard seed. Others, for example, progoitrin, are toxic when ingested in high levels.

The breeding emphasis in canola has been on decreasing the total seed glucosinolate concentration to less than 20 μmol g^{-1} and to less than 30 μmol g^{-1} in the oil extracted meal as the presence of glucosinolates in the meal reduces the feed quality for livestock particularly poultry (Tripathi and Mishra, 2007). These deleterious effects on livestock include problems with the thyroid, liver and kidneys, however there is no evidence that moderate concentrations of glucosinolates are harmful in humans. In contrast, there is mounting evidence that isothiocyanates

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are beneficial to humans as they may protect against cancer through several mechanisms (Fahey et al., 1997; Juge et al., 2007; Nestle, 1998; Pereira et al., 2002).

Studies of glucosinolate regulation in model plants such as *Arabidopsis* have also been important in furthering understanding of the biosynthesis and metabolism of these metabolites and also the regulation of sulphur and nitrogen metabolism in plants in general (Bones and Rossiter, 2006; Halkier and Gershenzon, 2006; Hirai et al., 2005, 2007, 2004; Maruyama-Nakashita et al., 2006; Sasaki-Sekimoto et al., 2005). The biological significance of certain glucosinolates has resulted in renewed efforts to accurately assess glucosinolate level and composition in plant materials.

There have been several studies evaluating glucosinolate composition by mass spectrometry. These have included the utilisation of several different techniques, with those coupled to LC being the most common. Tolra et al. (2000) examined the glucosinolates by LC-APCI mass spectrometry (single quadrupole) with a detection limit of 2.85 ng for sinigrin. Tian et al. (2005) have employed LC-ESI tandem mass spectrometry to achieve even greater detection limits (down to 0.6 pmol for some glucosinolates). This is not to say the LC is the only tool used. Bringmann et al. (2005) demonstrated that CE-ESI-TOF-MS may also be an efficient method of glucosinolate detection, while Botting et al. (2002) have applied a separation free MALDI-TOF technique for the analysis of intact glucosinolates. An early study also demonstrated the utility of Fast Atom Bombardment (FAB) MS for the detection of glucosinolates and their charged fragments (Bojesen and Larsen, 1991). More recently ESI ion-trap mass spectrometry has proved to be an important technique for the classification and quantification of glucosinolates, with Cataldi et al. (2007) describing the characterization of some 20 glucosinolates.

MS fragmentations of the glucosinolate class are important. Several studies have utilised the fragmentation behaviour of glucosinolates for targeted analysis. Glucosinolates undergo consistent neutral losses during MS fragmentation. Matthaus and Luftmann (2000) utilised the loss of the glucose moiety (−162) in a LC-ESI-tandem mass spectrometry study on various plants of the family Brassicacea. Other authors have used the *m*/*z* 96 or 97 ion ($[SO_3H]^-$) as being indicative for glucosinolates (Hill, 2006; Mellon et al., 2002).

This work describes in more detail, the fragmentation of a wide range of aliphatic, alkenyl and indole glucosinolates and demonstrates the utility of a sulphated glucose moiety fragment (*m*/*z* 259) for both targeted and untargeted analysis of glucosinolates using ESI-ion trap mass spectrometry. Also described, is a novel parent ion mapping approach that allows rapid assessment of glucosinolate content. This method takes advantage of the fragment (*m*/*z* 259) which is consistently produced by the disassociation of glucosinolates in the ion trap mass spectrometer. This fragmentation can also be employed for quantification via LC-ion trap-MS.

2. Results and discussion

2.1. Mechanism of gas phase fragmentation

Glucosinolates are important plant compounds and several studies of the mass spectral properties of these metabolites have been published in the recent literature. Along with other researchers our studies identified that the MS/MS fragmentation of glucosinolates give a number of generic ions. The most discussed is the HSO_4^- ion at *m*/*z* 97. However, the more diagnostic ion is the *m*/*z* 259 ion, since the loss of sulphate may occur in other sulphated metabolites, such as sulphated sterols. The larger *m*/*z* 259 ion is due to a sulphated glucose moiety and has been observed by several authors (Hill, 2006; Mellon et al., 2002). Although the structure has been described as a sulphated glucose, there has been no attempt to describe the gas phase reaction leading to this ion. Fig. 1 offers a possible explanation for the formation of *m*/*z* 259. Further fragmentation of the *m*/*z* 259 ion resulted in the spectrum shown in Fig. 2. These fragmentations support the ring-opened structure proposed. Accurate mass measurements (Table 1) support the fragmentations depicted in Figs. 1 and 2. The *m*/*z* 259 ion was consistently produced from glucosinolates, though the relative abundance of this ion was not uniform. For example, although the MS^2 spectrum of sinigrin was dominated by the *m*/*z* 259 ion, glucoraphanin preferentially fragmented to *m*/*z* 372 (the loss of the methyl sulphoxide moiety) (Fig. 3a). While the *m*/*z* 259 was present in the MS^2 spectrum of glucoraphanin, it was more abundant in the MS^3 spectrum (Fig. 3b).

2.2. Ion mapping experiments

LCMS techniques have been employed to investigate the glucosinolates content of many plant extracts. For high

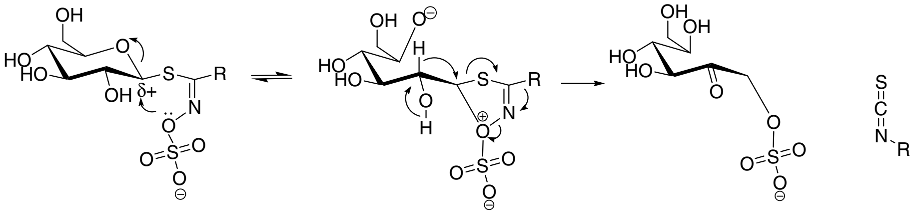

Fig. 1. Breakdown of glucosinolates to produce the ubiquitous *m*/*z* 259 ion.

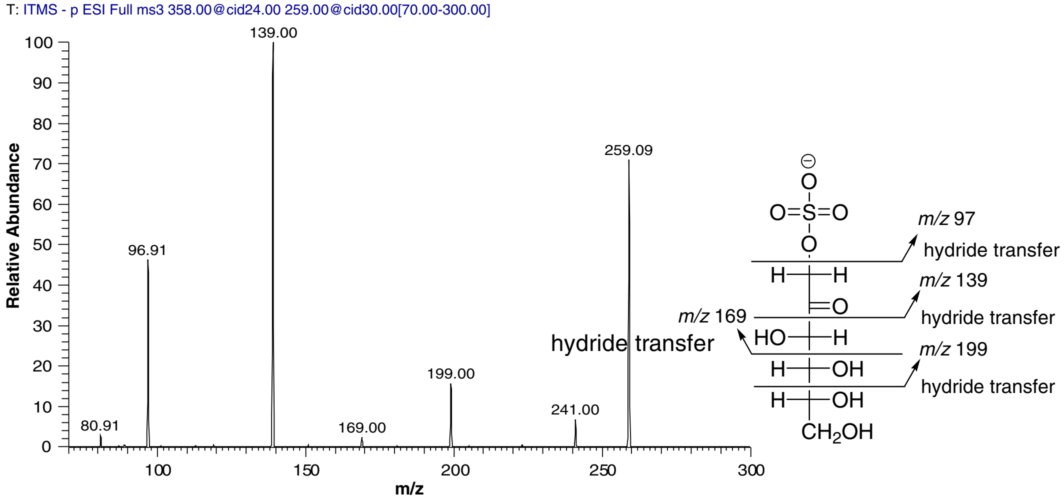

Fig. 2. Fragmentation of *m*/*z* 259.

Table 1
Accurate mass data for sinigrin MS^2 and MS^3 fragmentation

Fragment ion *m*/*z* (MS^n)	Measured mass/mu	Formula calculated	Δmass/mmu (measured − calculated)
358 (parent)	358.02682	$C_{10}H_{16}NS_2O_9$	0.72
259 (MS^2)	259.01199	$C_6H_{11}SO_9$	0.16
199 (MS^3)	198.99134	$C_4H_7SO_7$	0.64
139 (MS^3)	138.97003	$C_2H_3SO_5$	0.46
97 (MS^3)	96.95993	HSO_4	0.92

glucosinolate breeding programs the ability to rapidly screen prospective parental lines for glucosinolate content is of great value. On the other hand, with oil/forage crops such as canola, an aim of breeding is to reduce the total content of glucosinolates, and rapid screening of breeding lines for glucosinolates is also necessary. Ion mapping offers a sensitive, qualitative technique to rapidly and simultaneously assess a wide range of glucosinolate contents. The aqueous plant/seed extract is infused directly into the mass spectrometer and each analysis took less than three minutes for a mass range of 300–900 and less than 2 min for a mass range of 300–600. The ion mapping experiments identified any parent ion that gives rise to the daughter ion at *m*/*z* 259, an ion consistently generated in the ion trap fragmentation of glucosinolates. Infusion of the broccoli seed extract generated an intensity map of the parent ions and a spectrum view of the parent ions detected (Fig. 4). The glucosinolates were identified from the $[M–H]^-$ ion (confirmed by $LCMS^n$ analysis as described later) as follows, *m*/*z* 358 sinigrin, *m*/*z* 372 gluconapin, *m*/*z* 388 progoitrin, *m*/*z* 406 glucoiberverin, *m*/*z* 420 glucoerucin, *m*/*z* 422 glucoiberin, *m*/*z* 436 glucoraphanin, *m*/*z* 463 4-hydroxyglucobrassicin, *m*/*z* 477 neoglucobrassicin and/or 4-methoxyglucobrassicin. This analysis resulted in a very clean spectrum, devoid of confounding ions caused by other metabolites. For example, the spectrum generated by the infusion of mustard seed, essentially the only parent ion detected is *m*/*z* 358, due to sinigrin, results confirmed by LC-ESI-ion trap MS analysis. The method is also extremely sensitive. Serial dilution analysis of a sinigrin standard allowed detection of the parent ion at three parts per trillion.

2.3. $LCMS^n$ analysis

The apparently ubiquitous nature of the 259 fragmentation ion allows the rapid identification of glucosinolates in a complex mixture by $LCMS^n$ analysis. To test this hypothesis a mixture of glucosinolates containing glucoiberin (*m*/*z* 422), glucoraphanin (*m*/*z* 436), glucosinalbin (*m*/*z* 424), glucotropaeolin (*m*/*z* 408), glucoerucin (*m*/*z* 420) and neoglucobrassicin (*m*/*z* 477) was prepared. This mixture was analysed by LC-ESI-ion trap mass spectrometry using an unbiased analysis method (described in the method section). The LC-ion trap ms analysis allowed identification of all these compounds. An extraction of the dependant scans at *m*/*z* 259 correlated to each metabolite in the standard mix. To confirm that this ion was due to the sulphated sugar moiety, the mixed standards were subjected to enzymatic desulphation. This desulphated mixture was analysed by both the unbiased and targeted methods, with no detection of intact glucosinolates or the ion at *m*/*z* 259. The method was also applied to a mustard seed extract, broccoli seed extract and an extract of commercially available mixed brassica sprouts. In each case the *m*/*z* 259 ion was observed. The results are summarised in Table 2 with reference to the published literature for compound identification (Cataldi et al., 2007; Kensler et al., 2005). Fig. 5 details the result for brassica sprouts. The method produced a sufficient number of points across a parent ion peak to allow quantification. However, for the less abundant glucosinolates there were insufficient points using the *m*/*z* 259 ion for accurate quantification. To utilise the *m*/*z* 259 ion for quantification a more focused, targeted analysis was developed. Results of such an analysis for the brassica sprouts are shown in Fig. 6.

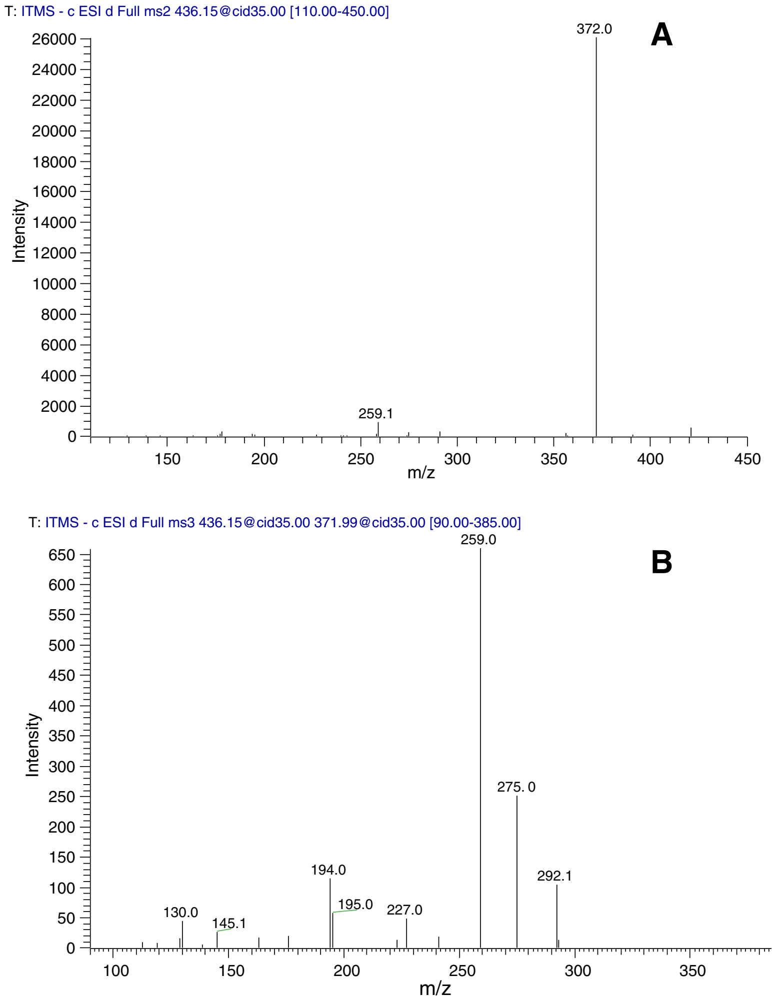

Fig. 3. Fragmentation of glucoraphanin: (A) MS^2 fragmentation results in a dominant ion at *m*/*z* 372 and (B) fragmentation of *m*/*z* 372 results in a MS^3 spectrum with *m*/*z* 259 dominating.

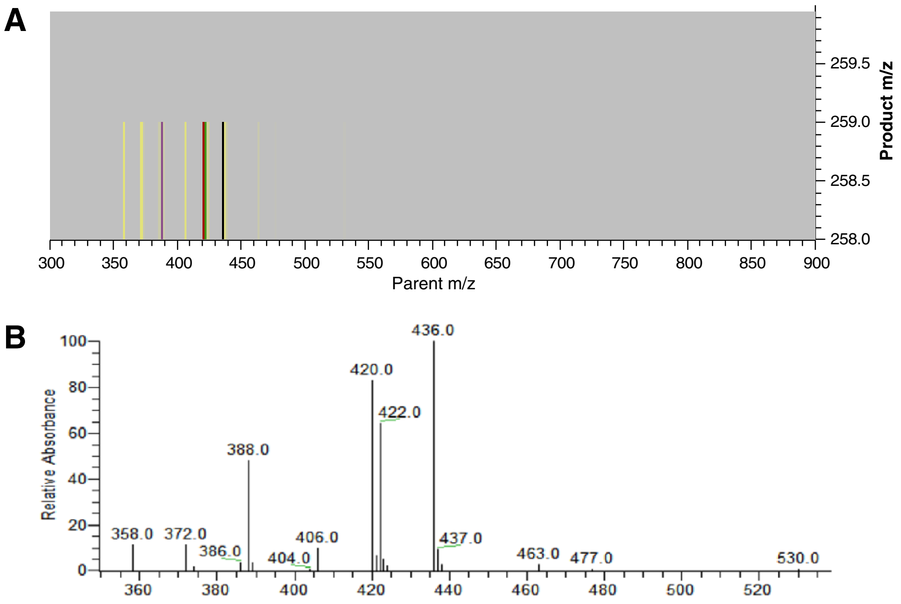

Fig. 4. Ion mapping result from a broccoli seed extract: (A) The map view generated from the experiment and (B) the spectrum view of the same data.

Table 2
Unbiased analysis of plant extracts and standards

Compound semi systematic name (common name)	Retention time (min)	Mass parent ion	MS^2 spectrum ions (base ion in bold)
Standard mixture			
3-Methylsulphinylpropyl (glucoiberin)	3.0	422	407, **358**, 275, 259
4-Methylsuphinylbutyl (glucoraphanin)	4.1	436	**372**, 259
p-OH-benzyl (glucosinalbin)	6.3	424	344, 291, 275, 261, **259**, 246, 231, 228, 182
Benzyl (glucotropaeolin)	16.8	408	328, 275, **259**, 241, 230, 212, 195, 166
4-Methylthiobutyl (glucoerucin)	18.4	420	340, 291, 275, **259**, 242, 227, 195, 178, 163
N-Methoxy-3-indolylmethyl (neoglucobrassicin)	34.2	477	447, **466**, 284, 259
Mustard seed			
2-Propenyl (sinigrin)	4.0	358	278, 275, **259**, 227, 195, 180, 162
4-OH-3-indolylmethyl (4-hydroxyglucobrassicin)	9.7	463	383, **285**, 275, 267, 259, 240
Broccoli seed			
3-Methylsulphinylpropyl (glucoiberin)	3.0	422	**358**, 259
(2R)-2-hydroxy-3-butenyl (progoitrin)	3.6	388	332, 308, 301, 275, **259**, 210, 195, 136
2-Propenyl (sinigrin)	3.8	358	275, **259**, 227, 162
4-Methylsuphinylbutyl (glucoraphanin)	4.0	436	**372**, 291, 259
3-Butenyl (gluconapin)	7.5	372	292, 275, **259**, 227, 195, 194, 176
4-Hydroxy-3-indolylmethyl (4-hydroxyglucobrassicin)	9.7	463	383, **285**, 275, 267, 259, 240
3-Methylthiopropyl (glucoiberverin)	13.0	406	326, 275, **259**, 228, 145
4-Methylthiobutyl (glucoerucin)	19.5	420	340, 291, 275, **259**, 242, 227, 224, 195, 178
4-Methoxy-3-indolylmethyl (4-methoxyglucobrassicin)	30.9	477	291, 275, **259**, 235, 227, 195
Brassica sprouts			
3-Methylsulphinylpropyl (glucoiberin)	2.9	422	**358**, 259
(2R)-2-hydroxy-3-butenyl (progoitrin)	3.7	388	332, 308, 301, 298, 275, **259**, 241, 227, 210, 195, 136
2-Propenyl (sinigrin)	3.8	358	278, 275, **259**, 227, 196, 180, 162
4-Methylsuphinylbutyl (glucoraphanin)	4.0	436	**372**, 259
3-Butenyl (gluconapin)	7.5	372	292, 275, **259**, 227, 195, 194, 176
4-Hydroxy-3-indolylmethyl (4-hydroxyglucobrassicin)	9.7	463	383, **285**, 275, 267, 259, 240, 176
3-Methylthiopropyl (glucoiberverin)	12.9	406	326, 275, **259**, 228, 195
4-Methylthiobutyl (glucoerucin)	19.4	420	340, 291, 275, **259**, 242, 227, 195, 178, 163
3-Indolylmethyl (glucobrassicin)	21.4	447	275, **259**, 251, 205
4-Methoxy-3-indolylmethyl (4-methoxyglucobrassicin)	31.2	477	463, 397, 299, 291, 275, **259**, 235, 227, 195
1-Methoxy-3-indolylmethyl (neoglucobrassicin)	34.6	477	447, **466**, 284, 259

2.4. Quantification

LC-ESI-ion trap mass spectrometry was also suitable for quantification. The method described in the experimental section was both robust and reproducible, with good area and retention time CVs and linearity over five concentration ranges. For glucoraphanin triplicate analysis of various concentrations results in an average CV of 5% or less for the molecular ion and the fragment ions *m*/*z* 372 and *m*/*z* 259 (2.5–3.4%, 1.5–2.6% and 3.3–9.1%, respectively). Fig. 7 depicts the linearity analysis for a glucoraphanin standard. The linearity for sinigrin was similar although the CVs for sinigrin were slightly higher for the molecular ion and the *m*/*z* 259 ion (average 6%), reflecting greater variation due to its early retention time. This method was successfully trialed using the mixed standards and extracts of broccoli seed, brassica sprouts, broccoli sprouts, broccoli florets, canola seeds and mustard seeds and with the appropriate standards could allow quantification of each of these glucosinolates. The method allowed sufficient data points for detection and quantification using both the parent ions in Table 3 and the *m*/*z* 259 ion. Seven replicate injections of both the standards and the plant extracts indicated that good CVs were typical across the chromatogram. The levels of sinigrin and glucoraphanin for mustard seeds and broccoli florets respectively, compared favourably with the levels determined concurrently using the in line PDA detector monitoring the $\lambda_{\max}$ of the glucosinolates. E.g. sinigrin: *m*/*z* 358, 6.9 g/100 g; *m*/*z* 259, 7.1 g/100 g; $\lambda_{\max 224\,\mathrm{nm}}$ 7.1 g/100 g: glucoraphanin *m*/*z* 436, 170 mg/100 g; *m*/*z* 372, 180 mg/100 g; *m*/*z* 259 120 mg/100 g; $\lambda_{\max 230\,\mathrm{nm}}$ 170 mg/100 g. These values are comparable to those obtained in previous studies (Font et al., 2004; Kushad et al., 1999; Smigielska and Schoenau, 2000; Trenerry et al., 2006; Tsao et al., 2002).

3. Experimental

Broccoli seeds (*Brassica oleracea* spp *italica cv*. 'Marathon') were obtained from New Gippsland Seeds and Bulbs, Silvan, Victoria, Australia. The seeds were sprouted

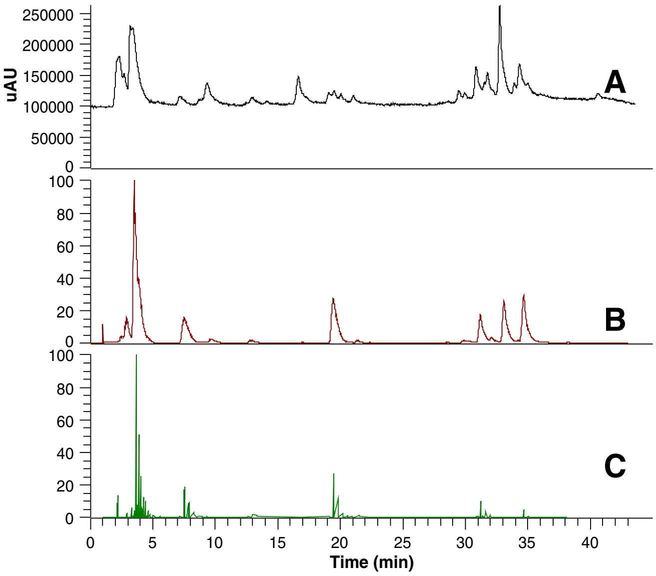

Fig. 5. Untargeted analysis of brassica sprouts depicting MS^n identification of glucosinolates: (A) HPLC PDA chromatograph (total scan 200–600 nm); (B) LCMS base peak chromatograph (C) LCMS chromatograph displaying *m*/*z* 259 extraction in data dependant scans. Refer to Table 5 for compound details.

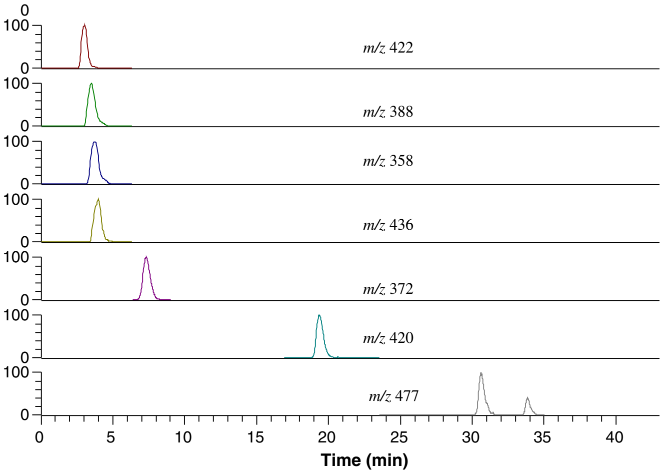

Fig. 6. Targeted analysis of brassica sprouts depicting parent ion and MS^n identification of glucosinolates via extraction of the *m*/*z* 259 ion. *m*/*z* 422 glucoiberin, *m*/*z* 388 progoitrin, *m*/*z* 358 sinigrin, *m*/*z* 436 glucoraphanin, *m*/*z* 372 gluconapin, *m*/*z* 420 glucoerucin, *m*/*z* 477 4-methoxyglucobrassicin and neoglucobrassicin.

using an Easygreen Mikrofarm sprouter (Seed and Grain Technologies, LLC, Pahrump, Nevada, USA) and grown for seven days before harvest.

Two commercial Brassica oilseed samples were obtained from Department of Primary Industries, Victoria trials and consisted of a canola-quality *Brassica juncea;(condiment mustard) cv* AC Vulcan and canola *Brassica napus; cv* AV Sapphire.

Commercial ‘broccoli’ sprouts were obtained from a local supermarket in Knoxfield, Victoria, Australia. These are referred to here after as brassica sprouts since it is likely these were not a pure sample of one cultivar.

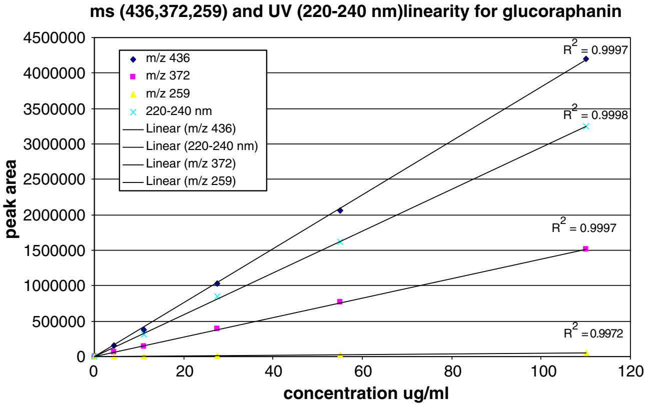

Fig. 7. Linearity results for glucoraphanin. Good R^2 values (0.9972–0.9998) were obtained for measurement of the peak area by UV and MS. The figure demonstrates the linearity for the parent ion and two fragment ions.

Table 3
MS acquisition settings for targeted analysis

Segment number	Segment duration (min)	Number of scan events	Ions for targeted MS^2 fragmentation
1	6.34	9	388, 358, 436, 422
2	2.7	5	424, 372
3	7.89	5	406, 463
4	6.61	7	420, 408, 447
5	11.53	3	477
6	7.95	3	Untargeted

Chemicals and reagents were obtained from the following commercial sources: sinigrin and Sulfatase from *Helix pomatia* type H-1 (Sigma–Aldrich, Sydney Australia), ammonium acetate (BDH Chemicals, Australia), methanol (HPLC grade) was purchased from J.T. Baker (Deventer, Netherlands). Formic acid (85%) was supplied by (Sigma–Aldrich, Sydney, Australia), MS grade solvent, water with 0.1% ammonium acetate and methanol with 0.1% ammonium acetate (Sigma–Aldrich, Sydney, Australia) were used for targeted studies. All samples, solutions, and buffers were prepared from distilled water.

3.1. Extraction of plant material

3.1.1. Mustard, canola and broccoli seeds

The extraction followed the protocol outlined by Nuchanart et al. (2002). In brief: Hot (90 °C) water (15 mL) was added to 1 g of seeds and the solution boiled for 5 min. The mixture was transferred to a mortar and the seeds/water ground to a paste. The paste was transferred to a 100 mL volumetric flask with water (90 mL) and the mixture sonicated for 5 min. For mustard and broccoli seeds, the solution was made to volume (100 mL), mixed thoroughly and allowed to separate (20 min). A portion of the upper layer was filtered through a 0.45 µm cellulose acetate syringe filter disc for analysis. For canola seeds, the final volume of the solution was 50 mL.

3.1.2. Brassica/broccoli sprouts and florets

Hot (90 °C) water (70 mL) was added to 10 g of sprouts/florets and the solution boiled for 5 min. The mixture was then blended with a Bamix blender for 5 min, and transferred to a 100 mL volumetric flask with water (final volume 90 mL) and the mixture sonicated for 5 min. The solution was made to volume (100 mL), mixed thoroughly and allowed to separate (20 min). A portion of the upper layer was filtered through a 0.45 μm cellulose acetate syringe filter disc for analysis.

3.2. Preparation of standard mix

Glucosinolates were purified in our laboratory by ion-exchange chromatography and combined to form a standard mix. Glucosinolates were isolated from the following sources: glucoerucin from Rocket seeds (*Eruca sativa*); glucosinalbin from white mustard seeds (*Sinapsis alba*); glucotropaeolin from garden cress (*Lepidium sativum*); glucoiberin, glucoraphanin and neoglucobrassicin from broccoli seeds (*Brassica oleracea* cv *italica*) following the general protocol of Hanley et al. (1983).

3.3. Enzymatic desulphation of standard mix

Glucosinolates were desulfated by combining 500 μL of the glucosinolate standard mix with 50 μL of sulfatase (0.5 units/mL) in 50 mM acetate buffer (pH 5.0) and incubating at room temperature overnight.

3.4. ESI-ion trap mass spectrometry

3.4.1. Mass spectrometer parameters

3.4.1.1. Ion map parameters. Prior to data acquisition the system was tuned using a 250 μg/mL standard of sinigrin infused into the mass spectrometer at a flow rate of 10 μL/min. The heated capillary was maintained at 300 °C and the sheath, auxiliary and sweep gases were at 13, 6 and 8 units, respectively. Source voltage was set to 3.4 kV with a capillary voltage of −29 V. The ion map data was acquired with a parent mass range of 300–900, with a 1 mass unit step and an isolation width of 1 mass unit. Normalised collision energy was set at 35 with an activation time of 30 ms. The product mass scanned for was *m/z* 259 with a mass width of 1.5. Sample for analysis was introduced into the spectrometer with an infusion flow rate 10 μL/min.

3.4.2. LCMS parameters

3.4.2.1. HPLC analysis. The mixtures were analysed using a 150 mm × 2.1 mm BDS Hypersil 3 μm C18 HPLC column (Waltham, MA) fitted to an Agilent series 1100 high performance liquid chromatograph (quaternary gradient pump, cooled autosampler maintained at 4 °C, column heater maintained at 30 °C and photodiode array detector) (Agilent, Walbronn). For the unbiased analysis the compounds were eluted from the column using a gradient mobile phase consisting of a mixture of three solvents, A (50 mM ammonium acetate in water), B (water) and C (methanol) (see Table 4 for gradient details). For the targeted analysis the compounds were eluted from the column using a gradient mobile phase consisting of a mixture of two solvents, A (0.1% ammonium acetate in water) and B (0.1% ammonium acetate in methanol) (see Table 5 for gradient details). For quantifying sinigrin in mustard seed and glucoraphanin in broccoli florets, the mobile phase consisted of 0.1% formic acid in water (isocratic). Flow rates were maintained at 0.2 mL/min. The compounds were detected with a Thermo Fisher LTQ ESI-ion trap mass spectrometry (Waltham, MA), operating in the negative ion mode.

3.4.2.2. Unbiased analysis. Flow was diverted to waste initially and MS data acquisition triggered one minute after the run began. A data dependant protocol was used in ESI negative mode with a mass range of 260–1000 amu. Three scan events were employed. The first scan event was a full scan (260–1000), the second scan event was a dependant scan triggering MS^2 acquisition on the most abundant ion in the first scan event and the third scan event was a dependant scan triggering MS^3 acquisition on the most abundant ion in the second scan event. Dynamic exclusion was engaged with a repeat count of 3, a repeat duration of 10 s and a 20 s exclusion time. Data were acquired with a target of 30,000, normalized collision energy of 35 and an ion max time of 200 ms. The heated capillary was maintained at 250 °C and the sheath, auxiliary and sweep gases were at 13, 6 and 8 units, respectively.

Table 4
Gradient information for HPLC analysis

Program time (min)	Flow rate (mL/min)	Mobile phase %A (50 mM NH_4OAc)	Mobile phase %B water	Mobile phase %C MeOH
0	0.2	80	20	0
4	0.2	80	20	0
14	0.2	80	10	10
20	0.2	80	10	10
25	0.2	75	0	25
35	0.2	75	0	25
36	0.2	80	20	0
43	0.2	80	20	0

Table 5
Gradient information for targeted analysis

Program time (min)	Flow rate (mL/min)	Mobile phase %A (0.1% NH_4OAc)	Mobile phase %B (0.1% NH_4OAc)
0	0.2	100	0
4	0.2	100	0
14	0.2	90	10
20	0.2	90	10
25	0.2	75	25
35	0.2	75	25
36	0.2	100	0
43	0.2	100	0

Source voltage was set to 3.4 kV with a capillary voltage of −50 V. Prior to data acquisition the system was tuned using a 250 μg/mL standard of sinigrin in 50% methanol/water. The sinigrin was infused via syringe pump through a T-piece at a rate of 20 μL/min and mixed with a HPLC solvent (water, 50 mM ammonium acetate) at a flow rate of 0.2 mL/min.

3.4.2.3. Targeted analysis. Flow was diverted to waste initially and MS data acquisition triggered one minute after the run began. The chromatogram was divided into six segments for this analysis (Table 3). The first scan of each segment was a full scan event (220–1000). The next scans targeted specific masses (Table 3) and carried out a MS^2 fragmentation on each specified mass found, followed by a MS^3 event on the major ion in each MS^2 spectrum. MS^n data were acquired with a target of 30,000, normalized collision energy of 35 and an ion max time of 200 ms.

3.4.2.4. Quantification. The levels of sinigrin in mustard seeds and glucoraphanin in broccoli florets were determined using the specific target ions of *m*/*z* 358 and 259 for sinigrin and at *m*/*z* 436, 372 and 259 for glucoraphanin.

3.4.2.5. Accurate mass measurements. Accurate masses were measured on a ThermoFisher LTQ-FT-ICR mass spectrometer (Waltham, MA), using sinigrin standard material (Table 1, Fig. 2). Sinigrin solution was introduced by infusion and isolated in SIM mode with a range of *m*/*z* 353–363 amu. Sinigrin was fragmented with a collision energy of 30 and accurate mass measurements obtained on the fragments. The *m*/*z* 259 ion was further fragmented (activation energy 24) and accurate mass measurements obtained on the following ions in SIM mode: *m*/*z* 246 (range 236–246 amu); *m*/*z* 199 (range 194–204 amu); *m*/*z* 139 (range 134–144 amu) and *m*/*z* 97 (range 92–102 amu).

4. Conclusions

The glucosinolates are an important class of secondary plant metabolites due to their prospective role in cancer prevention and undesired presence in seed oils. The techniques described here allow for the rapid assessment of these metabolites in the study of foods but also for targeted metabolomics in non-food plant materials. The reproducible fragmentation of glucosinolates to a sulphated glucose anion, *m*/*z* 259, can be exploited to assess glucosinolate content of plant extracts. The highly sensitive parent ion mapping experiment allows rapid qualitative description of glucosinolates. Further analysis using unbiased LCMS techniques allows retention time information to be gathered and used for structure identification. The targeted analysis presented here may be used for quantification on either the parent ion or the *m*/*z* 259 ion. The methods are robust for identification and quantification in complex mixtures and have used both the common LCMS solvent modifiers for this class of molecules (ammonium acetate and formic acid). It is anticipated that the ion mapping experiments, which allows rapid determination of the number of glucosinolates present and indication which specific metabolites are present, will be an invaluable, rapid screening tool for plant breeders, particularly for the economically important crop canola.

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