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SPECIFIC SCREENING METHODS FOR GLUCOSINOLATES IN SPROUT EXTRACTS USING ON-LINE THERMOSPRAY LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY

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SUMMARY

Considering the growing importance of the determination and identification of (desulpho-)glucosinolates in extracts of plant materials, rapid screening methods for desulphoglucosinolates were developed, based on thermospray liquid chromatography–mass spectrometry (LC–MS) and LC–MS–MS. In thermospray LC–MS the screening of plant extracts containing various desulphoglucosinolates can be based on the group-specific fragment at m/z 214, but identification of the peaks is not always straightforward owing to the lack of compound-specific fragments in the mass spectra. Using a constant neutral loss of 162 a.m.u., corresponding to the loss of the sugar ring, in thermospray LC–MS–MS the screening and identification of the various desulphoglucosinolates gives better results, especially because the signal in the MS–MS mode is less dependent on the fluctuating thermospray conditions. In some applications direct thermospray MS–MS analysis of mixtures, injected in the flow-injection mode, can give satisfactory results.

INTRODUCTION

Glucosinolates are found naturally in cruciferous plants, such as brassica vegetables, which are important in the human diet. The qualitative and quantitative analysis of glucosinolates (general formula and examples are shown in Table I) has received much attention in recent years[1]. Glucosinolates are of particular interest in food research, because the enzymatically released aglucones are responsable for the flavour and odour of many vegetables, *e.g.*, sprouts[2,3]. A number of them may have adverse effects on health[3].

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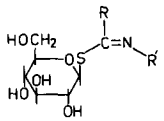
TABLE I

GENERAL STRUCTURES OF GLUCOSINOLATES AND DESULPHOGLUCOSINOLATES AND THE COMPOSITION OF THE R GROUP OF THE COMPOUNDS ANALYZED



R′ = SO_4, glucosinolates; R′ = OH, desulphoglucosinolates.

Desulphoglucosinolate	*Side-chain (R)*	*Mass*	*Molecular weight*
Glucobrassicin	CH_3–	15	253
Sinigrin	$CH_2=CHCH_2$–	41	279
Gluconapin	$CH_2=CH(CH_2)_2$–	55	293
Progoitrin	$CH_2=CHCHOHCH_2$–	71	309
Glucotropaeolin	$C_6H_5CH_2$–	91	329
Glucoiberin	$CH_3SO(CH_2)_3$–	105	343
Gluconasturtiin	$C_6H_5(CH_2)_2$–	105	343
Glucobrassicin	Indole-3-CH_2–	130	368
Neo-glucobrassicin	4-Methoxyindole-CH_2–	160	398

The separation and analysis of glucosinolates can be performed by either gas chromatography (GC) or liquid chromatography (LC), using flame ionization detection and UV detection at 230 nm, respectively. However for GC separation, application of trimethylsilyl derivatization is necessary[4]. During the silylation the glucosinolates also undergo desulphation, so pertrimethylsilyl desulphoglucosinolates are obtained. For direct analysis of glucosinolates, various high-performance liquid chromatography (HPLC) systems have been developed. Separation of glucosinolates can be performed by ion-exchange chromatography[5] or by reversed-phase ion-pair LC[6]. By these methods the glucosinolate fraction can be well separated from the other plant constituents.

A major breakthrough in glucosinolate analysis has been achieved with the introduction of enzymatic on-column desulphation using aryl sulphatase[7]. The enzymatic procedure, which converts the glucosinolates into their desulpho analogues, also acts as a specific clean-up method. Desulphoglucosinolates can be analysed either by GC, after silylation to the pertrimethylsilyl derivatives[8], or by reversed-phase HPLC with gradient elution using acetonitrile–water mixtures as the mobile phase and UV detection[9].

Several mass spectrometric techniques have been investigated for structure elucidation of the various (desulpho-)glucosinolates, *e.g.*, direct probe electron impact[10], chemical ionization[11] and fast atom bombardment[12]. Considerable structural information can be obtained with these techniques.

As the glucosinolates are present in complex mixtures, much attention has also been given to combined chromatography mass spectrometry. Gas chromatography–mass spectrometry (GC–MS) of the pertrimethylsilyl desulpho derivatives has been performed with either electron impact or ammonia chemical ionization. The latter technique appears to provide more useful structural information[13]. Recently, some preliminary experiments on the analysis of underivatized glucosinolates have been

performed in our laboratory, using continuous flow fast atom bombardment mass spectrometry. The results obtained appear to be very promising[14].

After the addition of ammonium acetate, commonly used reversed-phase HPLC mobile phases are suitable for thermospray liquid chromatography–mass spectrometry (TSP LC–MS) in the TSP buffer ionization mode. The desulphoglucosinolates show considerable fragmentation in TSP LC–MS, yielding weakly or even non-detectable protonated molecules, several ions due to the glucosyl and thioglucosyl moieties and ions giving information on specific parts of the molecule[15,16].

There is a need for a rapid and specific screening method for glucosinolates in plant material, *e.g.*, sprout extracts. It should provide detection of desulphoglucosinolates and either confirmation of their identity or identification. This paper describes the investigation of the applicability of TSP LC–MS and TSP LC–MS–MS for this purpose. The results obtained with TSP LC–MS confirm most of the findings of others[15,16], but provides additional information with respect to the use of tandem mass spectrometry (MS–MS). The possibility of analysing sprout extracts directly by TSP MS–MS without chromatographic separation was also investigated.

EXPERIMENTAL

Samples and sample pretreatment

The plant material studied consisted of Brussels sprouts, variety "Roger". The total glucosinolate content, as determined by the glucose-release method[17], was 3800 μmol/kg on a fresh weight basis. The sprouts were extracted with boiling 100% methanol and re-extracted with boiling 70% methanol. After removal of the volatile solvent *in vacuo* the sample was stored at −20°C until required.

An aliquot of the sample was analysed by HPLC with UV detection at 230 nm[9]. Based on retention times, the following glucosinolates were identified (the concentrations found, in μmol/kg on a fresh weight basis, are given in parentheses): glucoiberin (68), progoitrin (414), sinigrin (1832), gluconapin (117), glucobrassicin (1080) and neoglucobrassicin (118). The sum of the six glucosinolates identified amounted to 96% of the total glucosinolate content as determined by the glucose-release method.

Liquid chromatography

The HPLC system, combined with the mass spectrometer, consisted of two Model 2150 high-pressure pumps, a Model 2152 gradient controller (LKB, Bromma, Sweden), a Model 7125 injection valve (Rheodyne, Berkeley, CA, U.S.A.) and a Spherisorb ODS-2 (5 μm) column (250 × 4.6 mm I.D.) (Tracer Analytica, Barcelona, Spain).

During a gradient run, the amount of eluent B [acetonitrile–water (20:80) containing 50 mmol/l ammonium acetate] in eluent A (50 mmol/l ammonium acetate in water) was increased from 1 to 50% within 11 min, from 50 to 99% in 5 min and kept at 99% for 14 min. The eluent flow-rate was 1.0–1.5 ml/min.

Mass spectrometry

LC–MS and LC–MS–MS experiments were performed using a Finnigan-MAT TSQ 70 triple quadrupole MS–MS system (Finnigan-MAT, San José, CA, U.S.A.), equipped with a Finnigan-MAT TSP interface. The vaporizer temperature and repeller potential were optimized. The block temperature was kept at 200°C.

MS–MS was performed in the daughter and neutral loss scan modes. The collision energy was optimized for the various compounds. Air was used as collision gas with a pressure of 0.05 Pa, measured in the collision quadrupole. Because the pressure in the analyser quadrupole is rather high owing to the TSP conditions in the ion source, the admittance of a collision gas in the collision cell is not always necessary.

RESULTS AND DISCUSSION

Reversed-phase LC using gradient elution with acetonitrile as the organic modifier can be used to separate desulphoglucosinolates in plant extracts[9]. This solvent system can be used for TSP LC–MS when ammonium acetate is added. The chromatographic separation of the desulphoglucosinolates is not adversely influenced. The ions observed in the TSP mass spectra obtained from some standards (*e.g.*, sinigrin) were similar to those observed by others[15,16]. The general appearance of the thermospray LC–MS traces of the desulphoglucosinolates is summarized in Table II and illustrated for glucobrassicin. The assignment of the various fragments must be considered tentative until supporting evidence is forthcoming. Usually a protonated molecule is observed (for some compounds only with low intensity), sometimes accompanied by small sodium cationized molecules. Ammoniated molecules, which were reported by Mellon *et al.*[15], were not found in these experiments. Characteristic peaks related to the (thio-)glucosidic part of the molecule (group-specific fragments), which occur at *m/z* 180, 198, 214 and 240 are explained in Table II. Some compound-specific fragments containing the characteristic R group were also found. A rearrangement peak of general formula $(RN{=}C{=}O + NH_4)^+$ was observed for most of the desulphoglucosinolates studied. Some of the other specific fragments, which have been reported by Hogge *et al.*[16], were only observed in some of the spectra and others were not found at all. Some of the compound-specific fragments appear at relatively low masses, *i.e.*, below *m/z* 150, where the background ions, *e.g.*, solvent cluster ions, are also present with relatively high intensity. This hinders the interpretation of the mass spectra of the desulphoglucosinolates with molecular weights below 330 a.m.u. In principle, identification of the various compounds can be

TABLE II

DIAGNOSTIC FRAGMENTS IN THE TSP MASS SPECTRA OF DESULPHOGLUCOSINOLATES, ILLUSTRATED FOR GLUCOBRASSICIN (FIG. 2)

Group-specific fragments	*m/z*	*Compound-specific fragments*	*m/z*	*Glucobrassicin*
$[C_6H_{10}O_5 + NH_4]^+$ [a]	180	$[M + H]^+$		369
$[C_6H_{10}O_5 + H_2O + NH_4]^+$	198	$[M + Na]^+$		391
$[C_6H_{11}O_5SH + NH_4]^+$ [a]	214	$[M + K]^+$		407
$[C_6H_{11}O_5SCH{=}NOH + H]^+$	240	R^+ [b]		130
		$[RCN + H]^+$	R + 27	157
		$[RCN + NH_4]^+$	R + 44	174
		$[RN{=}C{=}O + NH_4]^+$ [a]	R + 60	190
		$[RC(=NOH)SH + H]^+$ [b]	R + 77	207

[a] Structure proposed by Mellon *et al.*[15].
[b] Structure proposed by Hogge *et al.*[16].

performed by interpretation of the compound-specific fragments. However, the identification is not always straightforward, as the relative intensities of the various fragment ions in the spectrum are strongly influenced by the TSP experimental conditions, which explains the differences observed compared with other studies. One of the drawbacks of TSP LC–MS is the difficulty of reproducing the ionization conditions. The appearance of the TSP mass spectrum is influenced by several interdependent parameters, such as the repeller potential, the vaporizer temperature, the block temperature, the composition of the mobile phase, the flow-rate, the contamination of the repeller electrode and the performance of the spray. Some of these experimental conditions cannot be reproduced from day to day, *e.g.*, the spray performance and the repeller electrode contamination.

As an illustration of the difficult reproducibility of TSP mass spectra, Fig. 1 shows two mass spectra of progoitrin, obtained under comparable conditions on different days. In Fig. 1B the compound-specific fragments $[M + H]^+$ (*m/z* 310),

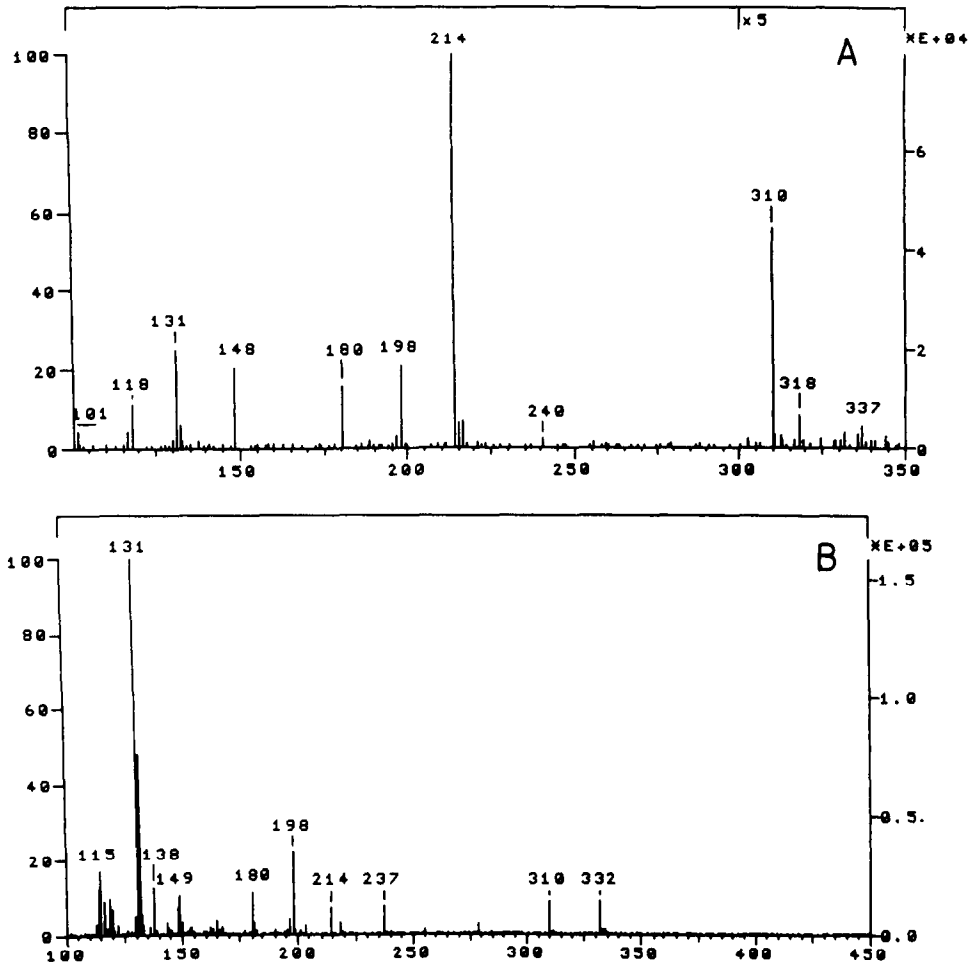


Fig. 1. TSP mass spectra of desulphoprogoitrin, obtained under comparable conditions on different days.

$[M + Na]^+$ (*m/z* 332) and $[RNCO + NH_4]^+$ (*m/z* 131) appear to be more intense than the group-specific fragments at *m/z* 180, 198, 214 and 240, which will make identification of the various compounds easier. In Fig. 1A the group-specific fragments are more intense. However, these effects have not been investigated in detail and more elaborate considerations of these effects will be given elsewhere[18].

The influence of some of the parameters was studied systematically. The results of these investigations are discussed here, demonstrating some of the striking features observed.

Changing the repeller potential from 0 to 100 V does not change the fragmentation pattern significantly; only the absolute intensities of the peaks are somewhat influenced. Optimization can give an increase in intensity of a factor of 2–3

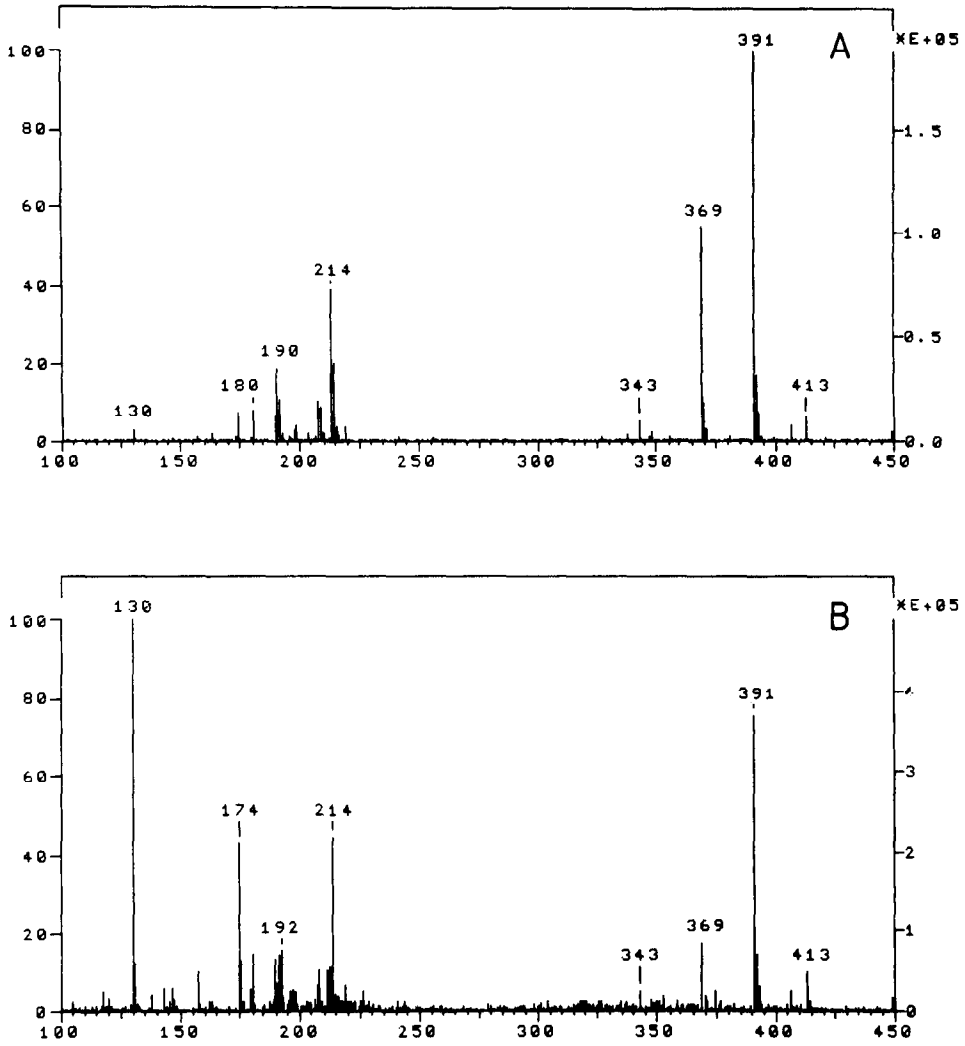


Fig. 2. TSP mass spectra of desulphoglucobrassicin, obtained at different vaporizer temperatures. (A) 100°C and (B) 130°C. Repeller potential, 50 V; modifier, methanol.

at most. Above about 100 V the peak intensity decreases dramatically. The optimum repeller potential is dependent on the contamination of the repeller, as this changes the effective potential. With a clean repeller electrode the optimum intensities are obtained at lower potentials than with a contaminated repeller electrode. The influence of the flow-rate and the vaporizer temperature was also investigated. When the vaporizer temperature is increased at a constant flow-rate, more fragmentation will be induced. This is especially important when the compounds studied are thermolabile, such as desulphoglucosinolates. For instance, at a flow-rate of 2.0 ml/min and a vaporizer temperature of 100°C the sodium cationized molecule $[M + Na]^+$ of glucobrassicin (*m/z* 391) is the base peak and hardly any fragmentation is observed. On increasing the temperature, fragmentation also increases, *e.g.*, at a vaporizer temperature of 130°C the fragment at *m/z* 130, $[R]^+$, has become the base peak (see Fig. 2), probably resulting from thermal degradation.

After optimization of the various parameters, TSP LC–MS was applied to a sprout extract. Because all of the desulphoglucosinolates studied showed a relatively intense peak at *m/z* 214, irrespective of the ionization conditions, screening for glucosinolates was performed with a mass chromatogram of this mass-to-charge ratio. In Fig. 3 a mass chromatogram (*m/z* 214) of sprout extract is shown, in which at least six desulphoglucosinolates can be observed. The mass spectra obtained confirm the structures of progoitrin, sinigrin, gluconapin and glucobrassicin, which are expected to be present by virtue of the chromatographic retention data. The other two peaks might be glucoiberin and neo-glucobrassicin, but the evidence is not very convincing, because the characteristic peaks, such as $[M + H]^+$ or $[RNCO + NH_4]^+$ are very small or even absent. No other desulphoglucosinolates than those expected from the retention data of standard compounds are observed. Screening for desulphoglucosinolates using this approach is possible, but the identification of observed desulphoglucosinolates in a mixture is not always straightforward.

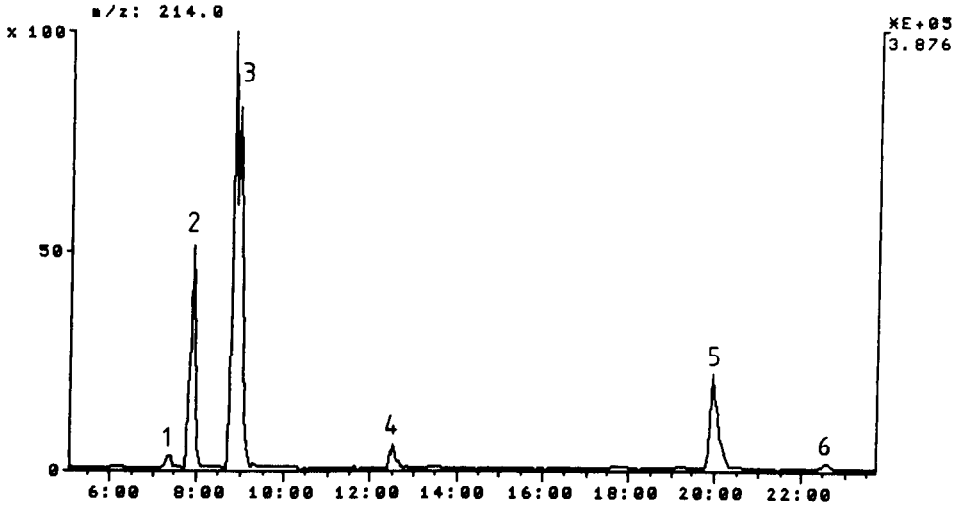


Fig. 3. Mass chromatogram (*m/z* 214) of a sprout extract. Modifier, acetonitrile; repeller potential, 50 V; vaporizer temperature, 100°C. Peaks: 1 = glucoiberin (?); 2 = progoitrin; 3 = sinigrin; 4 = gluconapin; 5 = glucobrassicin; 6 = neo-glucobrassicin (?).

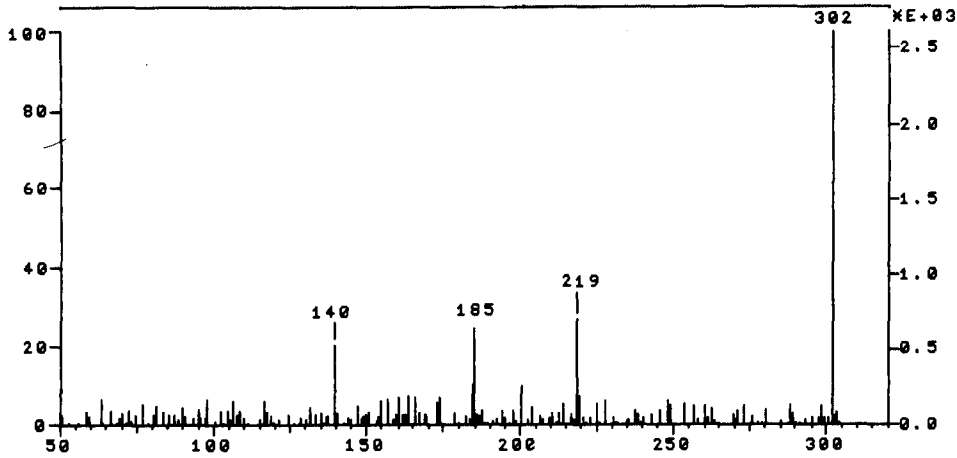


Fig. 4. Daughter spectrum of the sodium cationized molecule of desulphosinigrin (*m/z* 302). Collision gas, on; collision energy, 30 V.

Because of the increased selectivity, MS–MS in combination with HPLC was also investigated for the analysis of desulphoglucosinolates. Daughter spectra were obtained from the protonated and sodium cationized molecules of various desulphoglucosinolate standards. Fig. 4 shows the daughter spectrum of the sodium cationized molecule of sinigrin (*m/z* 302). Three fragments are formed at *m/z* 140, 185 and 219, which correspond to $[M + Na - C_6H_{10}O_5]^+$, $[C_6H_{10}O_5 + Na]^+$ and $[M + Na - RNCO]^+$, respectively. In the daughter spectrum of the protonated molecule of sinigrin (*m/z* 280), one intense fragment at *m/z* 118 is observed (see Fig. 5), which

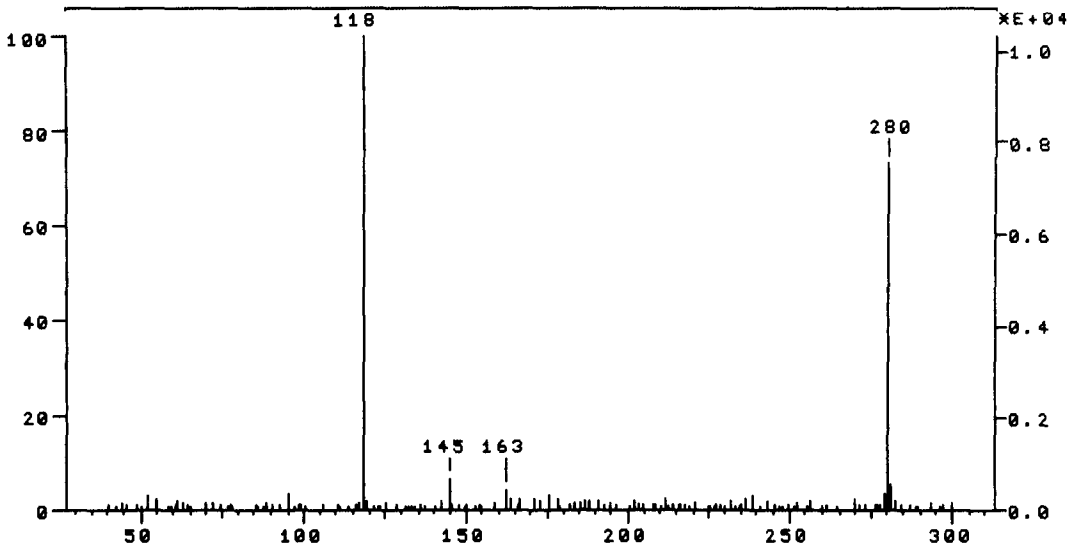


Fig. 5. Daughter spectrum of the protonated molecule of desulphosinigrin (*m/z* 280). Collision gas, off; collision energy, 20 V.

corresponds to a loss of the sugar ring ($C_6H_{10}O_5$) (162 a.m.u.). The daughter spectra of the sodium cationized molecule and the protonated molecule both show a loss of the sugar ring (162 a.m.u.) resulting in fragment ions at *m/z* 140 and 118. All the desulphoglucosinolates studied show a similar fragmentation of the protonated molecule under these conditions, *i.e.*, a loss of 162 a.m.u. A common fragmentation in the collision-induced dissociation spectra of this class of compounds indicates the possibility of using the neutral loss scan mode in tandem mass spectrometry. Therefore, LC–MS–MS in the neutral loss mode, *i.e.*, Q1 and Q3 both scanning with a mass difference of 162 a.m.u., was performed on various desulphoglucosinolate standards and the sprout extract. Fig. 6 shows the mass chromatograms based on the neutral loss experiments on the protonated molecules of various desulphogluco-

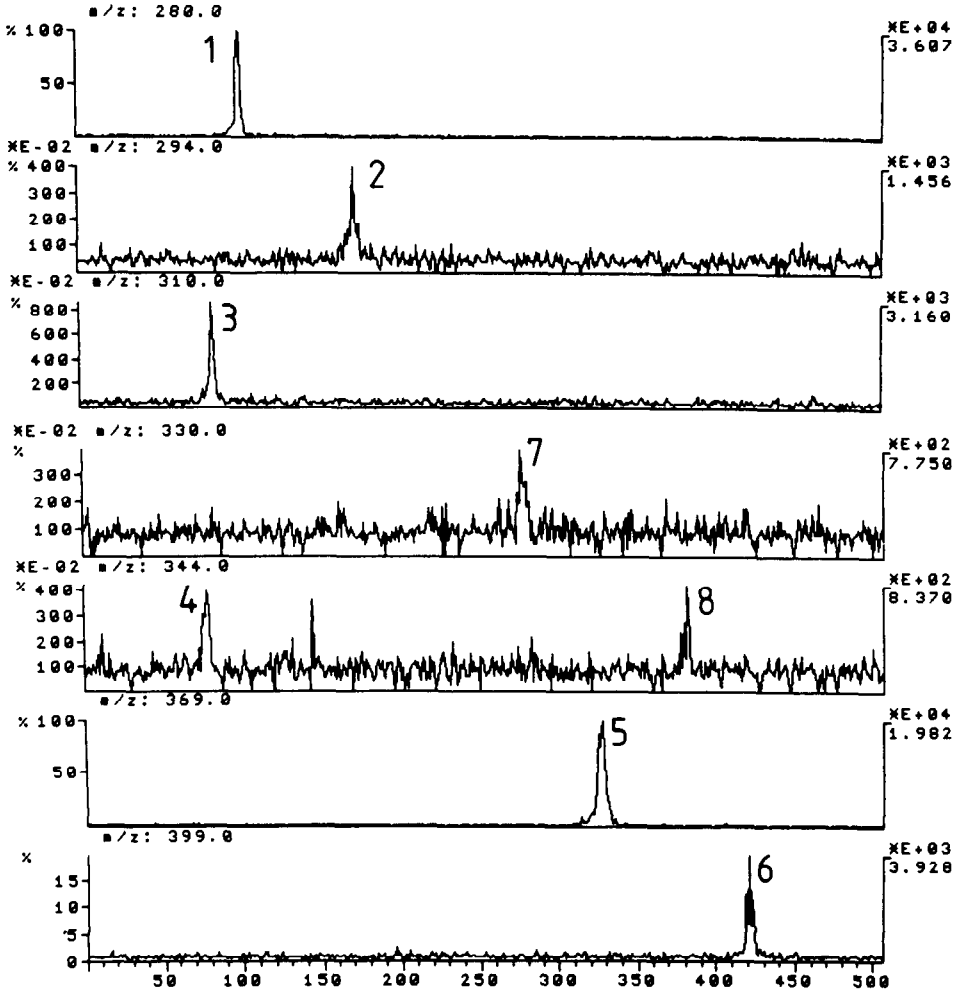


Fig. 6. Mass chromatograms of the protonated molecules of desulphoglucosinolates in a sprout extract obtained in the neutral loss (162 a.m.u.) scanning mode. Peaks: 1 = sinigrin; 2 = gluconapin; 3 = progoitrin; 4 = glucoiberin; 5 = glucobrassicin; 6 = neo-glucobrassicin; 7 = glucotropaeolin; 8 = gluconasturtiin. Collision gas, on; collision energy, 30 V.

sinolates observed in the sprout extract. Although the neutral loss scan mode is less dependent on the TSP ionization conditions, the presence of the protonated molecule is obligatory. The instrument used in these experiments displays the mass of the parent ion in the recorded mass spectra or mass chromatograms and not the mass of the detected daughter ion. Six desulphoglucosinolates can be identified at once, *viz.*, glucoiberin, progoitrin, sinigrin, gluconapin, glucobrassicin and neo-glucobrassicin, the presence of which was also suggested using HPLC with UV detection. Progoitrin, sinigrin, gluconapin and glucobrassicin were also identified in the MS mode, but for the presence of glucoiberin and neo-glucobrassicin there was only some slight evidence. A more detailed search of the MS–MS data also gives some evidence for the presence of glucotropaeolin and gluconasturtiin ($[M + H]^+$, m/z 330 and 344, respectively). For the presence of glucocapparin (m/z 254) (not shown in Fig. 6) there is only a slight indication. Our HPLC analysis with UV detection failed to detect these glucosinolates, probably owing to their low concentrations. The presence of gluconasturtiin in Brussels sprouts has been reported[3].

The enzymatic desulphation procedure appears to be a selective sample pretreatment, resulting in clean samples, containing few components that will rapidly contaminate the ion source. Therefore, the direct analysis of sprout extracts by means of TSP MS–MS, operating in the bypass or flow-injection mode, can be considered. The mass spectrum obtained by injecting the sprout extract directly without chromatographic separation and analysing in the neutral loss scan mode as earlier is shown in Fig. 7. Various desulphoglucosinolates can be identified rapidly in this way, *e.g.*, sinigrin from the peak at m/z 280, gluconapin from m/z 294, progoitrin from m/z 310 and glucobrassicin from m/z 369. However, because of the large differences in the concentrations of the various desulphoglucosinolates in the sprout extract, and because the sodium cationized molecules show a similar neutral loss of 162 a.m.u., the identification of minor components is not easy. Further, isomeric compounds cannot be differentiated by the flow-injection procedure. The selected reaction monitoring

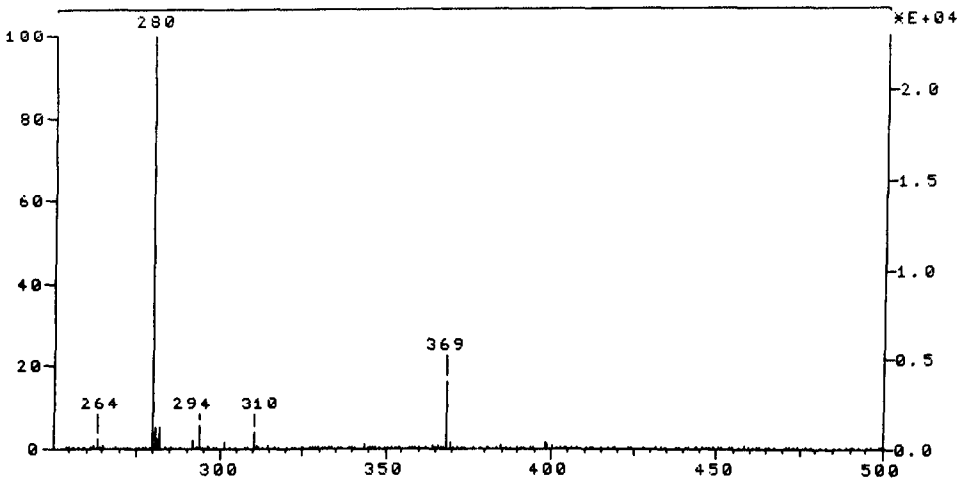


Fig. 7. MS–MS trace of a sprout extract obtained by flow injection in the neutral loss (162 a.m.u.) scanning mode.

mode (SRM) can be used to increase the selectivity further for certain applications. The choice between very rapid and simple direct analysis in with the flow-injection mode, taking only a few minutes, and the chromatographic analysis, taking about 1 h, will depend on the specific problem involved.

CONCLUSIONS

The combination of reversed-phase HPLC and either MS or MS–MS has been demonstrated to be a very useful method for the detection and identification of desulphoglucosinolates in sprout extracts.

In the MS mode sprout extracts can be easily screened for the presence of desulphoglucosinolates by the use of *m/z* 214 mass chromatograms. Identification of unknown compounds is not always straightforward, especially as some of the experimental parameters can exert a large influence on the TSP mass spectra. In the MS–MS mode desulphoglucosinolates can be identified owing to the monitoring of a highly selective reaction in the neutral loss scan mode, *i.e.*, the loss of the sugar ring (162 a.m.u.). Depending on the purpose, the analysis of desulphoglucosinolates can be performed by either chromatographic separation or direct analysis in the flow-injection mode, with the tandem mass spectrometer used in the neutral loss (162 a.m.u.) mode, either scanning or selective reaction monitoring.

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