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Separation and Identification of Desulfoglucosinolates in Japanese Rapessed by LC/APCI-MS

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Abstract

To identify seed glucosinolates in Japanese rapeseed (*Brassica napus* L.), a method for the separation and identification of various desulfoglucosinolates that were prepared by digestion with sulfatase from glucosinolates, was developed using liquid chromatography/atmospheric pressure chemical ionization mass spectrometry (LC/APCI-MS). Desulfoglucosinolates were separated by a high-performance liquid chromatography (HPLC) system and were analyzed by APCL-MS. Nine desulfoglucosinolates were easily identified from the protonated molecular ions $(M + N)^+$ and the group-specific fragment ions $(M + H - C_6H_{10}O_5)^+$. The seed of Japanese rapeseed contained the same glucosinolates as the exotic cultivars. These results revealed that LC/APCI-MS analysis is suitable for the identification of desulfoglucosinolates. HPLC analysis showed that there were remarkable varietal differences in the total and individual glucosinolate contents among the 50 main Japanese cultivars. Among these cultivars, Norin 18 showed the lowest glucosinolate content.

Discipline: Plant breeding
Additional key words: seed glucosinolate, HPLC, mass spectrometry, *Brassica napus*, oilseed rape

Introduction

Cruciferous plants including rapeseed (*Brassica napus* L.) usually contain various kinds of glucosinolates in tissues[21]. When the cells of the tissues are crushed, several hydrolysates such as oxazolidinethion and isothiocyanate, which are known to be toxic compounds, are released from glucosinolates by the action of myrosinase[12]. Table 1 shows the general structural formula of glucosinolates (R = a variety of side chains). The seed glucosinolates in rapeseed are divided into 3 major classes: the aliphatic glucosinolates derived from methionine, the indolyl glucosinolates derived from tryptophan, and alkyl glucosinolates derived from phenylalanine[13].

Seed meals of rapeseed have a well-balanced amino acid composition and a high content of proteins. However, there was a serious constraint on their utilization as animal feeds because of the high aliphatic glucosinolate content. Since Polish cv. Bronowski that contains a considerable amount of reduced aliphatic glucosinolates was discovered, low glucosinolate cultivars were bred in Canada and Germany, etc.[15]. However, low glucosinolate cultivars have not yet been bred in Japan. Furthermore, seed glucosinolate content and composition of the Japanese cultivars have not been determined.

In the glucosinolate analysis, glucosinolates are generally converted to desulfoglucosinolates by sulfatase action, because no derivatization of the desulfoglucosinolates is necessary and the eluate arising from on-column sulfatase digestion can be injected directly onto the high-performance liquid chromatography (HPLC), and the indolyl glucosinolates and non-indolyl glucosinolates are well resolved from one chromatogram[18]. These desulfoglucosinolates are analyzed by reversed-phase HPLC system with linear gradient elution with acetonitrile-water as the mobile phase and UV detection[7]. Since qualitative information on HPLC analysis is scanty, the identification of desulfoglucosinolates has been carried out

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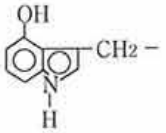
Table 1. Structure of separated glucosinolates in this study

Glucosinolate — Desulfoglucosinolate

No.*	Structure of R group	Common name
1.	$CH_2=CH-\underset{\underset{OH}{|}}{CH}-CH_2-$	Progoitrin
2.	" "	Epi-progoitrin
3.	$CH_3SO-CH=CH-CH_2-CH_2-$	Glucoraphanin
4.	$CH_2=CH-CH_2-\underset{\underset{OH}{|}}{CH}-CH_2-$	Napoleiferin
5.	$CH_2=CH-CH_2-CH_2-$	Gluconapin
6.		4-Hydroxy-glucobrassicin
7.	$CH_2=CH-CH_2-CH_2-CH_2-$	Glucobrassicanapin
8.	$CH_3-S-CH_2-CH_2-CH_2-CH_2-$	Glucoerucin
9.		Glucobrassicin
10.	$C_6H_5-CH_2-CH_2-$	Gluconasturtiin

*Elution order of desulfoglucosinolates from HPLC.

using gas liquid chromatography/mass spectrometry (GC/MS)[2], fast atom bombardment/MS[3], direct probe electron impact/MS[4], chemical ionization/MS[5] and field desorption/MS[10] after complicated pre-treatment.

Liquid chromatography/mass spectrometry (LC/MS) analysis which has made great progress in recent years, combines the outstanding separation ability of HPLC with the wide qualitative ability of mass spectrometry. LC/MS can directly separate well and identify the compounds which can not be readily analyzed by GC/MS due to their sensitivity to high temperature, nonvolatility, and derivatization requirement[17] like the indolyl glucosinolates.

This paper describes the separation and identification of desulfoglucosinolates from Japanese rapeseed using HPLC with atmospheric pressure chemical ionization mass spectrometry (APCI/MS). Moreover, total and individual glucosinolate contents of main cultivars were determined by HPLC analysis for a breeding program aimed at reducing the glucosinolate levels.

Materials and methods

Glucosinolates analyzed in this study were isolated from seed materials of 50 cultivars of Japanese rapeseed and 8 exotic low glucosinolate cultivars, which were grown in the field of Tohoku National Agricultural Experiment Station, from September 1992 to July 1993. Extraction of glucosinolates from seeds and preparation of desulfoglucosinolates with sulfatase digestion were carried out according to the method of Bjerg and Sorensen[1].

The HPLC system combined with a mass spectrometer, consisted of a Model L-6200 pump unit with low-pressure gradient system, a Model L-4200 UV detector (Hitachi, Tokyo, Japan), and a TSK gel ODS-80TM column (particle size 5 μm, 150 × 4.6 mm I.D.) (TOSOH, Tokyo, Japan). Desulfoglucosinolates were separated by gradient elution: 99% solvent A (water) + 1% solvent B (20% acetonitrile in water) for 5 min and then linear gradient during 25 min to 75% solvent A + 25%

solvent B. The eluent flow-rate was 1.0 ml/min. The temperature of the column oven was 40°C, the sample volume was 20 μl, and the UV detection wavelength was 230 nm.

Liquid chromatography-mass spectrometry (LC/MS) was performed using a Model M-1000 (Hitachi, Tokyo, Japan) with an atmospheric pressure ionization (API) interface. The ion accelerating voltage was 4 kV, and the drift voltage was 150 V. The vaporizer temperature and the desolvation region temperature were 250°C and 400°C, respectively.

The identification procedures of desulfoglucosinolates were carried out by comparing the mass spectrum with the reported mass spectrum for desulfoglucosinolates[8] and by comparison with published chromatograms[20]. Glucosinolate contents were calculated as described by the International Organization for Standardization[20].

Results and discussion

Desulfoglucosinolates in the seed extract of cv. Tokiwanatane were completely separated under the HPLC conditions applied to give 10 main peaks (Fig. 1). Fig. 2 shows the total ion current (TIC) chromatogram of the protonated molecules of desulfoglucosinolates of cv. Tokiwanatane. In the 10 main peaks, the delay time between the UV detector and the mass spectrometer was ca. 0.1 min. The mass spectra corresponding to the individual peaks in the TIC chromatogram are shown in Fig. 3. The protonated molecular ions $(M + H)^+$ which corresponded to each molecular species were observed. The characteristic clusters produced by water ion attachment to each protonated molecular ion $(M + H)^+$ were observed, too. Based on the results of the observation of the protonated molecular ions $(M + H)^+$ and the group-specific fragment ions $(M + H - C_6H_{10}O_5)^+$ which dissociate the glucose molecule, in the full scan mass spectra and the retention time compared with that described in a previous report, each peak was identified as 1: desulfoprogoitrin, 3: desulfoglucoraphanin, 4: desulfonapoleiferin, 5: desulfogluconapin, 6: desulfo-4-hydroxy-glucobrassin, 7: desulfoglucobrassicanapin, 8: desulfoglucoerucin, 9: desulfoglucobrassin, 10: desulfogluconasturtiin. Peak 2 corresponded to the optical isomer of desulfoprogoitrin (Table 1). The composition of the glucosinolates resembled that of the exotic cultivars reported by Sang and Salisbury[19].

Until now, several mass spectrometric investigations based on the thermospray (TSP) HPLC/MS

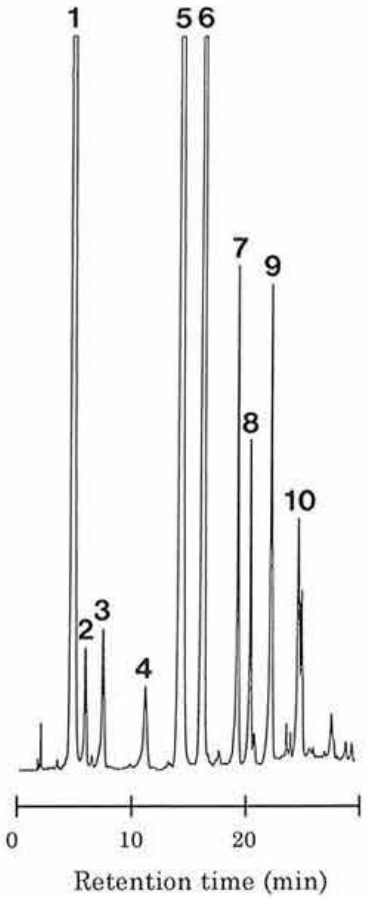


Fig. 1. HPLC chromatogram of desulfoglucosinolates of Japanese rapeseed cv. Tokiwanatane
Peak numbers correspond to compounds.
1: desulfoprogoitrin, 2: optical isomer of desulfoprogoitrin, 3: desulfoglucoraphanin, 4: desulfonapoleiferin, 5: desulfogluconapin, 6: desulfo-4-hydroxy-glucobrassicin, 7: desulfoglucobrassicanapin, 8: desulfoglucoerucin, 9: desulfoglucobrassicin, 10: desulfogluconasturtiin.

system had been reported for the determination and identification of various desulfoglucosinolates[8,14]. However, the drawback of TSP LC/MS is that it is difficult to reproduce the ionization conditions. Heeremans et al.[6] reported that the appearance of the TSP mass spectrum was strongly influenced by experimental conditions, such as the repeller potential, the vaporizer temperature, the composition of the mobile phase, the flow rate, etc. In contrast, APCI interface is not influenced by these conditions. Moreover, since the addition of ammonium acetate is not necessary, the kind of group-specific fragment ions is simple, and the interpretation of the APCI mass spectra is easy in comparison with TSP mass spectra. On the other hand, the drawback of the APCI method is that it is difficult to obtain infor-

mation except for the protonated molecular ions, owing to the limited information from APCI mass spectra. In this study, LC/APCI-MS enabled to easily separate and identify desulfoglucosinolates based on the difference of m/z and the group-specific fragment ions $(M + H - C_6H_{10}O_5)^+$. These results indicate that the identification of desulfoglucosinolates by LC/APCI-MS is highly reliable.

Fifty cultivars of Japanese rapeseed showed a considerable variation in the content of several

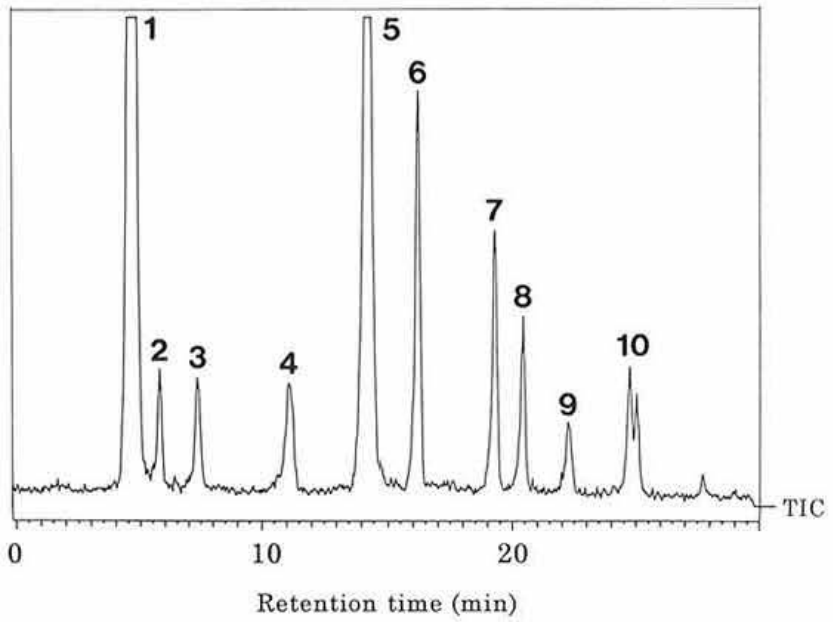


Fig. 2. Total ion current (TIC) chromatogram of protonated molecules of desulfoglucosinolates
Peak numbers correspnd to compounds.
1: desulfoprogoitrin, 2: optical isomer of desulfoprogoitrin, 3: desulfoglucoraphanin, 4: desulfonapoleiferin, 5: desulfogluconapin, 6: desulfo-4-hydroxy-glucobrassicin, 7: desulfoglucobrassicanapin, 8: desulfoglucoerucin, 9: desulfoglucobrassicin, 10: desulfogluconasturtiin.

Table 2. Range and mean contents of the major and total glucosinolates in 50 Japanese rapeseed cultivars and 8 exotic low glucosinolate cultivars

	Glucosinolates (μmol/g)				
	Progoitrin	Gluconapin	4-Hydroxy-glucobrassicin	Glucobrassi-canapin	Total
Japanese cv.					
Max	96.9 (Norin 12)*	44.9 (Norin 1)	10.8 (Norin 7)	9.8 (Koganenatane)	136.7 (Norin 7)
Min	35.8 (Norin 18)	14.5 (Norin 18)	2.3 (Kizakinonatane)	1.5 (Aburamasari)	63.3 (Norin 18)
Mean	56.6	27.4	6.4	4.6	100.0
Range	61.1	30.5	8.5	8.3	73.4
D.S.	11.3	5.5	1.8	1.9	14.3
Exotic cv.					
Mean	5.5	2.4	7.4	0.3	16.8
Range	9.0	4.3	4.7	0.4	18.0
D.S.	3.4	1.3	1.8	0.1	6.6

* Name of cultivar.

glucosinolates (Table 2). In these cultivars, the mean value for the total glucosinolate content was 102.9 μmol/g with a range from 63.3 μmol/g (Norin 18) to 136.7 μmol/g (Norin 7). These results revealed that the total glucosinolate content of Japanese cultivars was higher than that of exotic low glucosinolate cultivars with a content of less than 30 μmol/g. The 4 glucosinolates, progoitrin, gluconapin, 4-hydroxy-glucobrassicin and glucobrassicanapin, which were considered to be the major glucosinolates, accounted for more than 94.7% of the mean total content in 50 Japanese cultivars. The contents of the major glucosinolates ranged from 35.8 to 96.9 μmol/g for progoitrin, 14.5 to 44.9 μmol/g for gluconapin, 2.3 to 10.8 μmol/g for 4-hydroxy-glucobrassicin and 1.5 to 9.8 μmol/g for glucobrassicanapin. In a previous report[9], we revealed the varietal diversity of the content of seed glucosinolates in Japanese rapeseed by using the palladium-glucosinolate complex method, and Norin 18 showed the lowest glucosinolate content of 60.5 μmol/g. The results of HPLC analysis in this study were consistent with those obtained by the palladium-glucosinolate complex method.

Total glucosinolate content is known to be influenced by aliphatic glucosinolates, especially progoitrin and gluconapin contents[11]. Actually, low glucosinolate cultivars showed a considerable reduction of the aliphatic glucosinolate content. Although cultivars with a low glucosinolate content were not detected in Japanese cultivars, they exhibited a considerable variation in the total content and content of several glucosinolates (Table 2). Norin 18 showed the lowest glucosinolate content of 35.8 μmol/g for progoitrin, 14.5 μmol/g for gluconapin and 63.3 μmol/g for total glucosinolates. Since the glucosinolates of rapeseed are considered to be genetically controlled by a polygenic system[16], Norin 18 is expected to be a suitable genetic resource for the breeding of low glucosinolate cultivars.

The genotypic variations detected in this study indicate that novel genetic resources in terms of glucosinolate content could be identified in Japanese rapeseed germplasm. We are currently studying and evaluating the glucosinolate content and composition of Japanese rapeseed germplasm by HPLC analysis.

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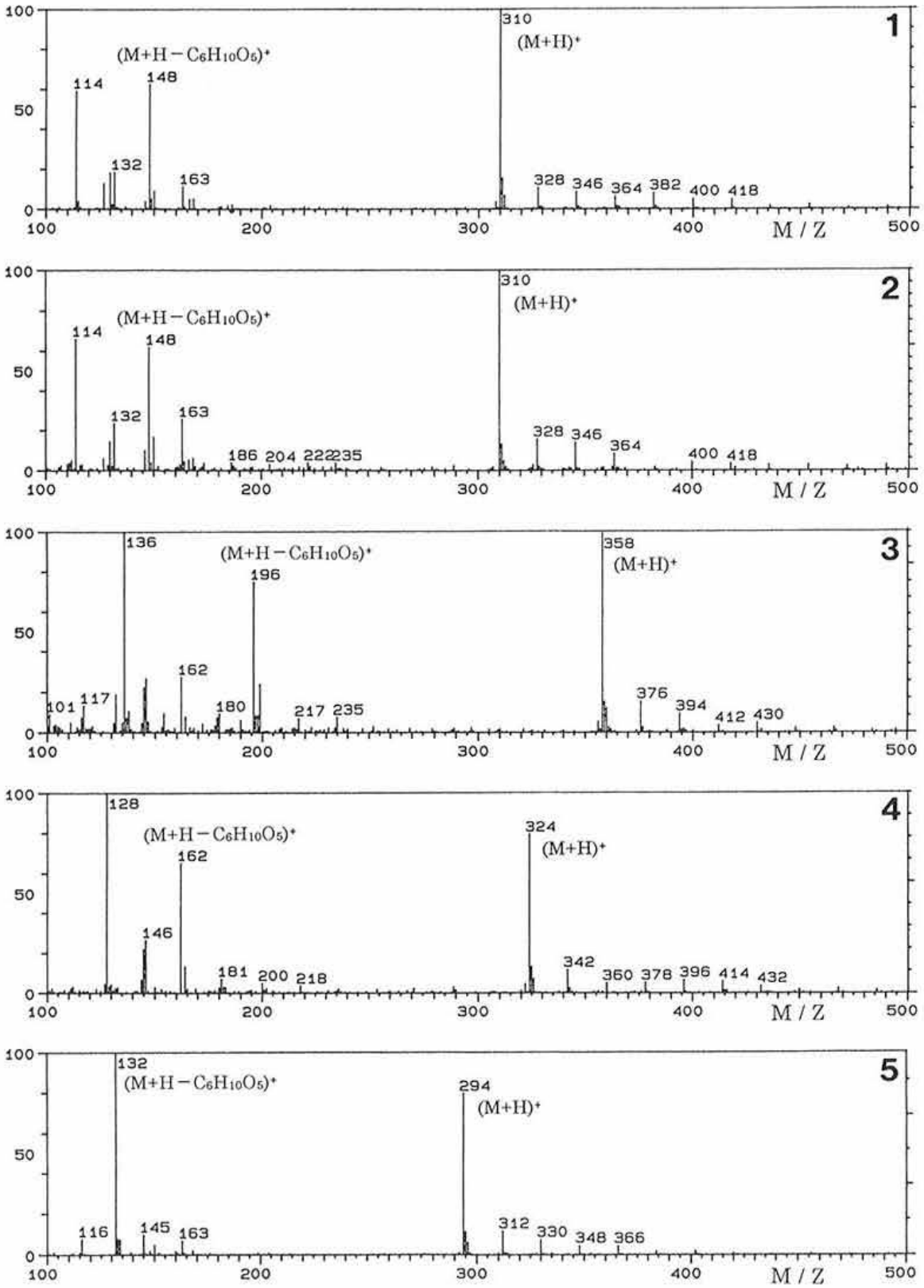
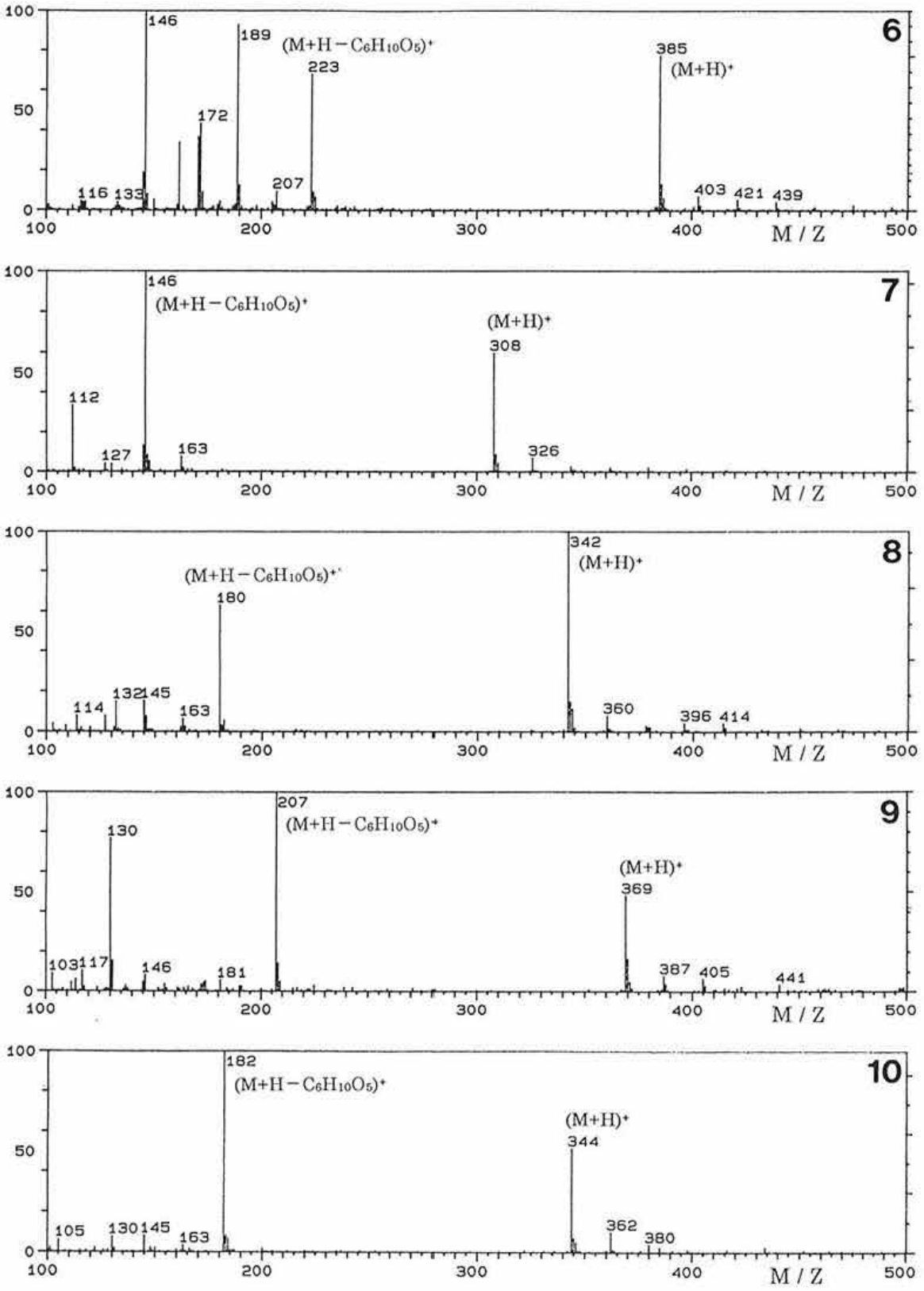


Fig. 3. Mass spectra of desulfoglucosinolates
1: desulfoprogoitrin, 2: optical isomer of desulfoprogoitrin, 3: desulfoglucoraphanin, 4: desulfonapoleiferin, 5: desulfogluconapin,



(Fig. 3. continued.)
6: desulfo-4-hydroxy-glucobrassicin, 7: desulfoglucobrassicanapin,
8: desulfoglucoerucin, 9: desulfoglucobrassicin, 10: desulfogluconasturtiin.

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