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Purification of sulforaphane from *Brassica oleracea* seed meal using low-pressure column chromatography

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Abstract

Sulforaphane is an isothiocyanate that is present naturally in widely consumed *Brassica oleracea* vegetables and has been shown to block the formation of tumors. The contents of sulforaphane in five groups of *B. oleracea* seeds (broccoli, Brussels sprouts, cabbage, cauliflower and kale) were determined by RP-HPLC using linear gradient of acetonitrile in water. A new low-cost method to isolate and purify natural sulforaphane from *B. oleracea* seed meal was described in this work. Crude sulforaphane was first separated from *B. oleracea* seed meal by using immiscible solvent extraction with ethyl acetate, 10% ethanol and hexane, and the crude sulforaphane was used as raw materials to prepare high purity sulforaphane by low-pressure column chromatography of silica gel (200–300 mesh) with different eluents and elution modes. Compared with these different elution methods, the gradient elution was preferable to the isocratic elution for reducing the elution time and the eluent consumption and increasing the purity of sulforaphane product. The purity and recovery of sulforaphane were more than 90% in gradient elution.

Keywords: Sulforaphane; Purification; Low-pressure column chromatography (LPCC); *Brassica oleracea* seed

1. Introduction

Epidemiological data show that a diet rich in *Brassica oleracea* vegetables, such as broccoli, cabbage, Brussels sprouts, cauliflower and kale, can reduce the risk from a number of cancers. The underlying mechanism for the reduction of cancer by *B. oleracea* vegetables is not clear. However, these vegetables are rich in glucosinolates. When vegetables are ground or chopped, myrosinase enzyme (thioglucoside glucohydrolase, EC 3.2.3.1) and glucosinolates come into contact. Myrosinase breaks the β-thioglucoside bond of glucosinolate molecules, producing glucose, sulfate, and a diverse group of aglycone products. The resultant aglycones then undergo non-enzymetic, intramolecular rearrangement to yield isothiocyanates, thiocyanates or nitriles. Sulforaphane (4-methylsulfinybutyl isothiocyanate), derived from glucoraphanin (4-methylsulfinybutyl glucosinolate), is the most potent naturally occurring inducer of phase II enzymes, including quinone reductase and glutathione *S*-transferase [1]. Moreover, sulforaphane can reduce the incident of a number of forms of tumors and induce cell cycle arrest and apoptosis in various experimental models [2–6]. Significantly, even at dietary doses, sulforaphane can modulate the xenobiotic-metabolising enzyme systems, shifting the balance of carcinogen metabolism toward deactivation [7].

With the increase in application of sulforaphane in nourishment, the demands for high purity sulforaphane are rapidly increasing. However, the high purity sulforaphane do not meet the needs of various fields because of the limit to its high price at present. Consequently, a new low-cost technology for the purification of sulforaphane is worth of great importance for extending application.

Purified sulforaphane can be prepared by chemical synthesis [8,9] and purification from plant [10,11]. Chemical synthesis requires several highly toxic substances, and final products from these reactions still contain toxic residues and require further purification. This disadvantage limits synthesized sulforaphane to be used as food additives. Thus, natural sulforaphane is more favorable for common consumer. Purified sulforaphane from plant are usually prepared by using preparative reverse phase high performance liquid chromatography

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(RP-HPLC). Separation efficiency of preparative RP-HPLC is the best since small diameter and suitable expensive packing can be used. However, the sample purified by preparative RP-HPLC has to be treated through elaborate pre-separation in order to remove large quantity of contaminants. Moreover, the equipment of preparative RP-HPLC is expensive and the cost of operation and maintenance are also high. Therefore, preparative RP-HPLC was not appropriate for the purification of sulforaphane as industry process with low cost. Compared with preparative RP-HPLC, low-pressure column chromatography (LPCC) exhibits a great potentiality for industrial production owing to its operation simplicity, low cost and high yield. The aim of the present study is to develop an economic LPCC process for purification of sulforaphane from *B. oleracea* seed meal. Therefore, irregular, large diameter, and cheap silica gel packing material and simple solvents that are easy to be recovered and reutilized were chosen in our procedure. The samples do not need elaborate treatment before purified by LPCC. All of these are worth decreasing separation cost of LPCC.

Crude sulforaphane extracted from *B. oleracea* seed meal by using immiscible solvent was used as raw materials to prepare high purity sulforaphane by LPCC. The separation conditions of LPCC were optimized by using various eluents and elution modes.

2. Experimental

2.1. Chemicals

B. oleracea seeds (broccoli, Brussels sprouts, cabbage, cauliflower and Kale) were kindly provided by Vegetables and Flowers Institute of China Academy of Agriculture Science. Sulforaphane standard was purchased from Sigma Chemical Co. (St. Louis, MO.). Acetonitrile was HPLC grade. Methylene chloride, methanol, ethanol, ethyl acetate, hexane and anhydrous sodium sulfate were of analytical grade. Silica gel (200–300 mesh, irregular) was obtained from Haiyang Chemical Group (Qingdao, China).

2.2. Seed extraction

Fifty grams of seeds was homogenized in an analytical grinder. Ground seed meal was added to 250 ml of pure water, and the mixture was allowed to autolyze for 2 h at 25 °C. This paste was extracted three times with equal volumes of ethyl acetate, which were combined and dried at 35 °C under vacuum in a rotary evaporator. The residue was dissolved in 250 ml 10% ethanol in water (v/v) and washed three times with equal volumes of hexane to remove nonpolar contaminants. Following this, the aqueous phase was extracted three times with equal volumes of ethyl acetate. The ethyl acetate layers were pooled, dried over anhydrous sodium sulfate, and filtered through the 0.45 μm membrane. The filtrate was dried at 35 °C under vacuum in a rotary evaporator. The residue was dissolved in a measured volume of methanol solvent and then quantitatively analyzed by HPLC.

2.3. Analytical methods

2.3.1. HPLC

Sulforaphane was analyzed with a Hitachi HPLC apparatus equipped with Hitachi model L-7100 pumps, L-7420 tunable absorbance detector, reversed-phase C_{18} column (250 mm × 4.6 mm, 5 μm, Diamodsil^{TM}). The solvent system consists of 20% acetonitrile in water, then changes linearly over 10 min to 60% acetonitrile, and maintained 100% acetonitrile for 2 min to purge the column. Column oven temperature was set at 30 °C. The flow rate was 1 ml/min, and 10 μl portions were injected into the column. Sulforaphane was detected by UV 254 nm.

2.3.2. MS

To characterize purified sulforaphane, electron-impact (EI) mass spectra were obtained by the Mass Spectrometry Laboratory, Center of Analysis, Beijing University of Chemical Technology. A Micromass 70-VSE mass spectrometer was used with an ion source temperature of 200 °C and a probe temperature of 25 °C. The spectrum was scanned at 70 eV from *m*/*e* 30–300.

2.4. Preparative low-pressure column chromatography

The methanol phase containing compounds of interest was dried at 35 °C under vacuum in a rotary evaporator, and the residue was purified by preparative low-pressure column chromatography. A 500 mm × 18 mm glass tube was used as the preparative chromatography column. Silica gel about 60 g (200–300mesh) was activated 60 min at 120 °C, and then slurry packed into the column. Elution was performed using Waters model PreLC pumps to deliver a constant flow rate. Single solvent and binary mixed solvents were used as eluents, and different elution modes were tried. Compound separation was monitored with a Waters 2487 dual λ absorbance detector set at 254 nm.

2.4.1. Isocratic elution

The residue was dissolved with 5 ml eluent, injected into the silica gel column, and eluted at a flow rate of 10 ml/min. The eluents were collected according to 100 fractions, each of which was 10 ml. The fractions containing sulforaphane were combined together and removed solvent under vacuum at 35 °C. Purity and recovery of the sulforaphane product were determined by using HPLC. The purity of a given product was defined for this study as the sulforaphane percentage of the product. Recovery of a given product was evaluated by dividing the quantity of sulforaphane in the product obtained by that in the loading sample. Hexane–ethanol (6:4, v/v), hexane–ethanol (5:5, v/v), hexane–ethanol (4:6, v/v) were used as eluents, respectively. The column was packed again and the above procedure was repeated when the eluent changed.

2.4.2. Gradient elution

The residue was dissolved with 5 ml eluent of the hexane-ethanol (6:4, v/v), injected into the silica gel column, and eluted

with three different gradient modes at a constant flow rate. The gradient elution was performed by A and B solvent systems, from 0 to 100% B in 100 min. The eluent A, hexane–ethanol (6:4, v/v), was the same in three modes. The eluent B was different in various elution modes, which is hexane–ethanol (5:5, v/v) in Mode 1, hexane–ethanol (4:6, v/v) in Mode 2 and ethanol in Mode 3, respectively. The column was packed again with the changes in gradient modes. The fractions were treated as those in isocratic elution.

3. Results and discussions

3.1. High performance liquid chromatography (HPLC)

Bertelli et al. [12] used the mixture of water and tetrahydrofuran as mobile phase for analyzing the contents of sulforaphane in the edible tissues of broccoli. In our study, a rapid HPLC method with the linear gradient of acetonitrile–water as mobile phase was established.

Linear regression analysis of the peak area responses (y) versus the theoretical concentration (x) gave the following equation: $y = 11949 + 160377.80x$, $r^2 = 0.9998$. The correlation coefficient demonstrated linearity of the method over the concentration range. The system precision was determined by chromatographing six injections of the standard solution and calculating the relative standard deviation (R.S.D.) of the peak area responses. The method precision was established by assaying six replicates of authentic sample with the proposed chromatographic method. The R.S.D.% for standard and samples were 0.96 and 1.05, respectively. Furthermore, triplicate analyses of a broccoli seed sample following the addition of known amounts of sulforaphane standard provided recoveries (mean ± R.S.D.) for sulforaphane of 96.5 ± 0.8%. This HPLC method was used to quantitatively determine sulforaphane in several kinds of *B. oleracea* seeds. Sulforaphane in the seeds of broccoli, Brussels sprouts, cabbage, cauliflower, and kale was evaluated in order to determine variation in amounts across groups and using the same analytical procedure. The HPLC chromatogram of crude sulforaphane extracts from broccoli seed meal is show in Fig. 1. Table 1 shows the contents of sulforaphane in the seeds of *B. oleracea*. It is worth noting that there were significant differences among *B. oleracea* seeds tested. The lowest content of sulforaphane was found in cauliflower seed, while the highest content was found in broccoli seed. However, differences in the content of sulforaphane among the broccoli accessions were relatively slight. The high desired compound contents indicate that broccoli seeds are good raw materials for preparing sulforaphane.

3.2. Extraction of crude sulforaphane

The primary contaminants in the seed meal extracts were fatty acids that can be removed after hexane defatting. In an attempt to raise the initial sulforaphane purity, defatted seed meal was used for crude extraction by many researchers [10,11]. However, it is found that hexane defatting maybe make some myrosinase inactive and decrease the formation of sulforaphane.

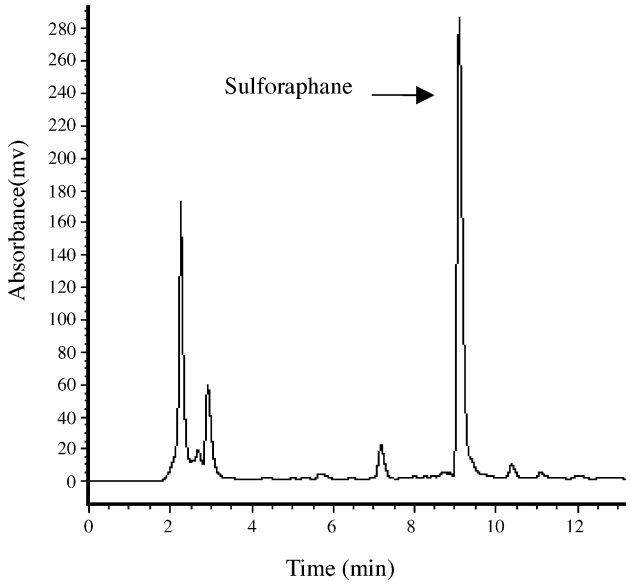

Fig. 1. HPLC chromatogram of crude sulforaphane extract from broccoli seed meal detected by absorbance at 254 nm. The column was reversed-phase C_{18} (250 mm × 4.6 mm, 5 μm, Diamodsil™). The solvent system consisted of 20% acetonitrile in water, then changed linearly over 10 min to 60% acetonitrile, and maintained 100% acetonitrile for 2 min to purge the column. Column oven temperature was set at 30 °C. The flow rate was 1 ml/min.

Thus, fresh seed meal was used as extracting material in our work. Four different solvents were tested for extraction of sulforaphane from equal amount of seed meal. Solvents tested included methylene chloride, ethyl acetate, chloroform, and hexane. A 25 ml portion of each solvent was added to 25 ml of autolyzed seed meal. After three times extraction, the solvent was combined and dried at 35 °C under vacuum in a rotary evaporator. The residue was dissolved in a measured volume of methanol and tested by HPLC to check the sulforaphane solubility in equal volumes of each solvent. The largest amount of sulforaphane was extracted by methylene chloride, followed by

Table 1
Sulforaphane content in the seeds of *B. oleracea*

Groups	Accessions	Sulforaphane (μg/g)	
Broccoli	Zhongqing II	4748[a]	2841[b]
	Greenshirt	4548	
	Greenyu	2036	
	Broccoli 70	1619	
	Greenball	1349	
	Greenwave	2746	
Brussels sprouts	Brussels 08	201	162
	Brussels 09	123	
Cabbage	CA 03	161	261
	CA 05	350	
	CA 08	272	
	CA 09	261	
Cauliflower	Snowflower 01	75.6	62.5
	Snowflower 02	49.4	
Kale	K11	499	340
	K15	181	

[a] Values represent the mean of three replicates per accession.
[b] Values represent the mean. Means were calculated from the following accessions: broccoli 6, Brussels sprouts 2, cabbage 4, cauliflower 2, kale 2.

ethyl acetate. Hexane solvent did not extract detectable amount of sulforaphane. However, the least amount of contaminants was extracted by ethyl acetate than the other three solvents. This led to further method development using ethyl acetate extraction because this solvent can be used for food-grade extraction of sulforaphane.

To remove these primary oil contaminants, the concentrated crude extracts, after being resuspended in 10% ethanol in water and washed with hexane. The immiscible solvent wash of concentrated crude extract increased sulforaphane purity and removed most organic solvent soluble impurities before the final LPCC purification. Yield was calculated to be 27% (w/w) of the sulforaphane in the crude extract after the immiscible solvent wash and extraction steps.

3.3. Preparative low-pressure column chromatography

3.3.1. Selection of eluents in isocratic

For the purpose of reducing the cost of production, a simple solvent system seems favorable for using as eluent. Since the sulforaphane is hexane-insoluble, pure hexane could not be used as eluents. Otherwise, sulforaphane would block the column during elution. Therefore, only mixed solvents were used as eluents when the crude sulforaphane extracts were used for separation as raw materials.

The results of separation with isocratic elution for the crude sulforaphane extracts as raw material are given in Table 2.

The results in Table 2 shows that the purities of the sulforaphane products were slightly changed with different mixed solvents as eluents. With the increase of ethanol content in the mixed solvent, the purities of the sulforaphane product increased (the range of changing from75.2 to 76.9%), and the elution time and the consumption of eluent decreased in a great degree (from76 to 57 min and from 370 to 270 ml, respectively) in isocratic elution. However, the purity of sulforaphane products decreased greatly when ethanol was used as single solvent to eluent. Therefore, it was very difficult to select single or mixed solvent as eluent in isocratic elution to obtain high purity sulforaphane.

3.3.2. Selection of eluents in gradient elution

In isocratic elution, the elution time was long and the eluent was consumed much when binary mixed solvents was used as eluent. This problem could be overcome by using gradient elution. The results of separation with gradient elution for the crude sulforaphane extracts as raw materials are listed in Table 3.

Table 2
The results of separation with isocratic elution for purifying sulforaphane

Eluent	Sulforaphane		
	Collection time (min)	Purity (%)	Recovery (%)
Hexane–ethanol (6:4, v/v)	39–76	75.2	90.1
Hexane–ethanol (5:5, v/v)	36–67	75.8	91.3
Hexane–ethanol (4:6, v/v)	30–57	76.9	90.8
Ethanol	21–32	52.5	92.6

Condition: column, 500 mm × 18 mm glass column packed with 60 g silica gel (200–300 mesh); flow rate, 10 ml/min.

Table 3
The results of separation with gradient elution for purifying sulforaphane

Gradient	Sulforaphane		
	Collection time (min)	Purity (%)	Recovery (%)
Mode 1	36–69	82.6	91.2
Mode 2	36–63	86.8	90.8
Mode 3	35–55	91.5	90.1

Condition: column, 500 mm × 18 mm glass column packed with 60 g silica gel (200–300 mesh); flow rate, 10 ml/min. The gradient elution was performed by A and B solvent systems, from 0 to 100% B in 100 min. The eluent A, hexane–ethanol (6:4, v/v), was the same in three modes. The eluent B were different in various elution modes, which is hexane–ethanol (5:5, v/v) in Mode 1, hexane–ethanol (4:6, v/v) in Mode 2 and ethanol in Mode 3, respectively.

The purities of the sulforaphane products were greatly different in three gradient elution modes. With the increase of ethanol content in B solvent system, the purities of the sulforaphane product increased (the range of changing from 82.6 to 91.5%), and the elution time and the consumption of eluent decreased (from 69 to 55 min and from 330 to 200 ml, respectively). When single ethanol was used as B solvent, the elution time and the consumption of eluent decreased to least. Thus, the purity and the elution time of the sulforaphane products were greatly affected by different strength of the solvents to be used as B solvent systems in gradient elution. With the increase of the strength of the B solvents, the total separation time and solvent consumption decreased. Consequently, the Mode 3, in which single ethanol was used as B solvent, should be selected in gradient elution for reducing the cost and preparing high purified sulforaphane (see Fig. 2). Fig. 3 shows the HPLC chromatogram of purified sulforaphane prepared by gradient elution with Mode 3.

Compared with the results between isocratic elution and gradient elution, the purities of the sulforaphane product prepared

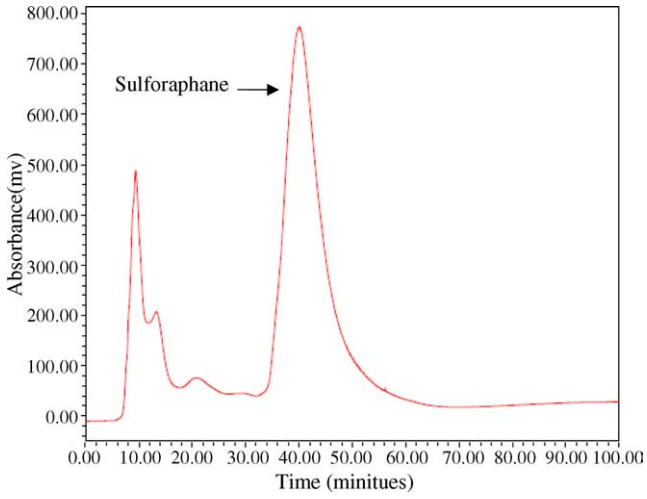

Fig. 2. LPCC chromatogram for preparing sulforaphane by gradient elution with Mode 3. The gradient elution was performed by A and B solvent systems, from 0 to 100% B in 100 min. The eluent A was hexane–ethanol (6:4, v/v), and the eluent B was ethanol. The flow rate was 10 ml/min. The detected absorbance set at 254 nm.

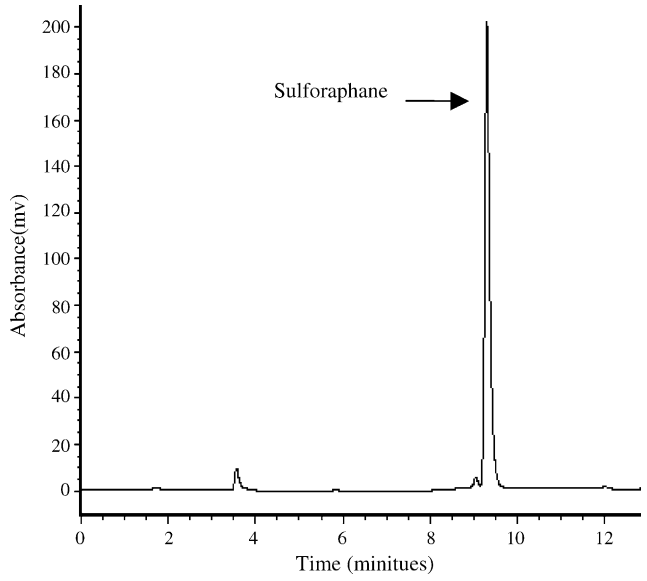

Fig. 3. HPLC chromatogram of purified sulforaphane by gradient elution with Mode 3. The detected absorbance set at 254 nm. The column was reversed-phase C_{18} (250 mm × 4.6 mm, 5 μm, Diamodsil™). The solvent system consisted of 20% acetonitrile in water, then changed linearly over 10 min to 60% acetonitrile, and maintained 100% acetonitrile for 2 min to purge the column. Column oven temperature was set at 30 °C. The flow rate was 1 ml/min.

by gradient elution were higher than those by isocratic elution. Moreover, the elution time and the consumption of eluent were less in gradient elution than those in isocratic elution for separation and purification of sulforaphane from *B. oleracea* seed meal. Higher purity and recovery of the sulforaphane products could be obtained if the smaller diameter and higher efficient silica gel or more complex solvent system was used. However, it was not our aim when taking the increase of the production cost into consideration.

3.3.3. Optimizing preparative purification strategy in gradient elution

Effects of loading amount and the flow-rate on separation of column chromatography were shown in Table 4. Different amounts (from 0.25 to 1.0 g) crude sulforaphane were separated by gradient Mode 3 at a constant flow rate of 10 ml/min, and 0.25 g crude sulforaphane was separated by gradient Mode 3 with different flow-rate to be controlled (from 8 to 14 ml/min). The other conditions and procedures were the same. The fractions containing sulforaphane should be combined for guaranteeing the recoveries of sulforaphane to be more than 90%. The purities of the products prepared from the crude sulforaphane were all high (>90%) when sample amount was less than 0.5 g and the flow-rate was under 10 ml/min. Once the loading amount was more than 0.5 g or the flow-rate was more than 10 ml/min, the purities of the sulforaphane decreased sharply. Consequently, the loading capacity should be limited to less than 0.5 g/60 g silica gel, and the appropriate flow-rate was controlled under 10 ml/min for preparation of the purified sulforaphane product from the crude extracts.

Table 4
Effects of loading amount and the flow-rate on purity of sulforaphane products

Flow-rate (10 ml/min)	Loading amount (g)			
	0.25	0.50	0.75	1.0
Purity (%)	91.5	90.2	82.6	76.6
Loading amount (0.25 g)	Flow-rate (ml/min)			
	8	10	12	14
Purity (%)	90.8	91.5	83.2	75.3

Condition: column, 500 mm × 18 mm glass column packed with 60 g silica gel (200–300mesh). The gradient elution was performed by A and B solvent systems, from 0 to 100% B in 100 min. The eluent A was hexane–ethanol (6:4, v/v). The eluent B was ethanol.

In order to raise producing rate and decrease solvent consumption, additional modification and refinements to Mode 3 in gradient elution were carried out. Once the target compound was eluted and collected (about 60 min), the concentration of ethanol increased to 100% achieving a complete elution of undesired compounds in only 5 min. Thus, cross-contamination between sequential injections is avoided. To minimize equilibration time, the system is hold under initial condition (hexane–ethanol, 6:4, v/v) for 15 min before next injection. The cycle time for this approach including column equilibration time is 80 min. Compared with Mode 3 in gradient elution, producing rate of this approach raised 25% (from 0.5 g crude extracts/100 min to 0.5 g crude extracts/80 min), and solvent consumption decreased 20% (from more than 1000 to 800 ml). Consequently, the purification strategy has been found to be efficient and robust and is well established for separation and purification of sulforaphane from *B. oleracea* seed meal in our research laboratories.

3.4. MS

Purified samples were analyzed by MS (see Fig. 4). Mass spectrum (EI) of sulforaphane, *m/z*: 39, 55, 64, 72, 86, 114, 160, and 177. Electron impact mass spectrometry gave a small molecular ion (M^+) at 177, and prominent fragment ions with masses of 160 and 72. Precise masses of molecular and fragment ions obtained by electron impact mass spectrometry were 177.0286 (calculated for $C_6H_{11}NOS_2$, 177.0283), 160.0257 (calculated for $C_6H_{10}NS_2$, 160.0255), and 71.9909 (calculated for C_2H_2NS, 71.9908). MS (EI) analysis of the purified sulforaphane sample was consistent with those previous reported [10–12].

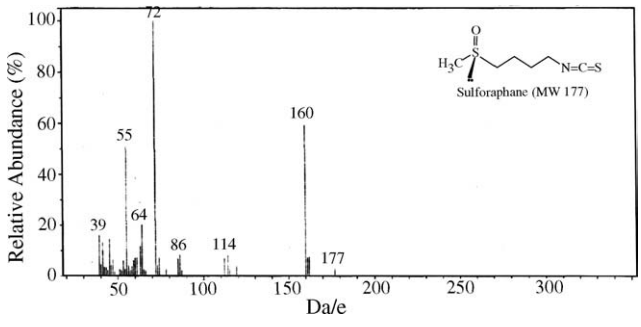

Fig. 4. Mass spectra of sulforaphane purified by LPCC.

4. Conclusion

A new low-cost method for separation and purification of sulforaphane from the seeds of *B. oleracea* was described in our work. In order to remove large quantity of oil contaminant, the immiscible solvent extraction was applied. As a result, ethyl acetate, 10% ethanol and hexane system was the best method to remove oil contaminant and decrease the loss of the derived compound.

Crude sulforaphane extracts were purified by isocratic or gradient elution with hexane and ethanol as eluent. Compared with the results between isocratic elution and gradient elution, it was better that purified sulforaphane product was prepared by gradient elution with hexane and ethanol as eluent. Moreover, the elution time and the consumption of eluent were less in gradient elution than those in isocratic elution. In order to decrease cost and raise producing rate, the gradient eluent was optimized by significant improvement in loading amount, flow-rate and cycle time. These results indicated that the proposed LPCC process could provide effective purification, high recovery, sustained usability and economy.

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