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Separation by solid phase extraction and quantification by reverse phase HPLC of sulforaphane in broccoli

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The separation and quantification of sulforaphane [1-isothiocyanato-4-(methylsulfinyl)-butane] from the florets, stalks and leaves of broccoli is described. The procedure uses solvent extraction, followed by purification of extracts using solid phase extraction (SPE) and reverse phase HPLC analysis. The HPLC method is compared with a spectrophotometric assay. To obtain information about the usefulness of acid hydrolysis of glucosinolates, the florets were left to autolyze at room temperature or were treated with concentrated hydrochloric acid, then analysed. The method proves reliable and reproducible as regards both SPE purification and chromatographic determination. Quantities of sulforaphane were found in the florets, stalks and leaves. The highest content of sulforaphane ($110\,\mu g\,g^{-1}$) was found in the leaves. © 1998 Elsevier Science Ltd. All rights reserved.

INTRODUCTION

Interest in food with chemoprevention properties has been steadily increasing in recent years as a result of a growing body of epidemiological evidence pointing to the low incidence of some types of tumour in populations, or sections thereof, whose diet includes large quantities of certain vegetables. Cruciferous vegetables in particular, have attracted a great deal of attention, since they are rich in aromatic and aliphatic isothiocyanates (Huang *et al.*, 1994). Of these, sulforaphane [1-isothiocyanato-4-(methylsulfinyl)-butane], which has been identified in broccoli as a product of enzymatic or acid hydrolysis of the corresponding ω-(methylsulfinyl)-alkyl-glucosinolate (glucoraphanin), has recently aroused interest (Zhang *et al.*, 1992b; Kore *et al.*, 1993).

Sulforaphane has been shown to reduce the incidence of a number of forms of tumour in various experimental models, both *in vivo* in animals and *in vitro* in cell cultures (Zhang *et al.*, 1994).

The chemoprotective effect of sulforaphane was thought to be due solely to its ability to behave as a monofunctional inducer of phase II enzymes, which are known to represent the most important group of detoxication enzymes of the human organism (Prestera *et al.*, 1993; Prestera and Talalay, 1995). More recently, however, sulforaphane has also been shown to inhibit the CYP2E1 isoenzyme of the cytochrome P450, thus emerging as an inhibitor of phase I enzymes (Barcelo *et al.*, 1996).

The information in current literature regarding the sulforaphane content of cruciferous vegetables is sketchy, and the few existing studies on the extraction of sulforaphane from the seeds and florets of broccoli do not give precise data about the content (Zhang *et al.*, 1992b; Kore *et al.*, 1993). The only method available for the direct analysis of free sulforaphane as such in food is one which determines isothiocyanates in general, but is not specific for sulforaphane (Zhang *et al.*, 1992a).

Given the considerable interest in sulforaphane, we therefore set out to devise an analytical method for the quali-quantitative determination of sulforaphane that, consequently, should apply not only to food, but to other biological materials as well, and should be precise, reproducible and rapid.

The present study describes an HPLC method for the determination of sulforaphane in vegetables. This method is compared with the spectrophotometric assay of isothiocyanates, which relies on a reaction with 1-2 benzenedithiol (BDT) (Zhang *et al.*, 1992a). It also describes the isolation and purification of sulforaphane from crude extracts by solid phase extraction (SPE).

To test the efficiency of the method, it was applied to broccoli samples previously subjected to acid or

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enzymatic hydrolysis of glucosinolates; also, given the limited market availability of sulforaphane, it was synthesized as a reference compound to establish the best condition for analysis.

GC–MS was used to confirm the presence of sulforaphane in sample extracts and to validate the SPE method.

MATERIALS AND METHODS

Samples of broccoli (*Brassica oleracea* L. var. *italica*, cv. *Calabrese*) were bought in a retail outlet in Italy. After purchasing, the broccoli was processed as quickly as possible. All chemical reagents were of analytical grade and all solvents were of HPLC grade.

Sulforaphane was synthesized by treating 4-bromo butylphthalimide with 1 equivalent of sodium methanthiolate in hexamethylphosphoramide under nitrogen atmosphere at 80°C (Cogolli *et al.*, 1979).

Following conversion of the 4-methylthiobutylphthalimide to the corresponding 4-methylsulfoxybutylphthalimide using hydrogen peroxide in acetic acid, the intermediate was converted to 4-methylsulfoxybutylamine with hydrazine in the usual manner. The latter intermediate was converted to the isothiocyanate by treatment with carbon disulfide and ethyl chloroformate (Moore and Crossley, 1946; Holland *et al.*, 1994, 1995).

Preparation and purification of extracts

The broccoli was sorted into florets, stems and leaves and the samples prepared by grinding and homogenizing 100 g of vegetable with water.

In order to obtain information about the usefulness of acid hydrolysis of glucosinolates, the florets were divided into two equal parts; the first was left to autolyze at room temperature for 24 h, while the second was treated with concentrated hydrochloric acid to reduce the pH to 3 and then left at room temperature for 24 h. Stalks and leaves were treated only at pH 3. The respective products were then freeze-dried for use in powder form.

The powders were extracted twice with methylene chloride at room temperature, the first time with 30 ml g^{-1} for 7 h, the second time with 10 ml g^{-1} for 4 h, and the organic extracts were pooled and evaporated in a rotary evaporator (35°C). The concentrate was repeatedly washed with water. The aqueous layers were filtered, pooled and saturated with sodium chloride and then re-extracted with methylene chloride. The methylene chloride layers were dried over anhydrous sodium sulfate and stored at −20°C until processed (Kore *et al.*, 1993).

The organic broccoli extracts were purified by SPE using Bakerbond SPE* silica gel (SiOH) 3 ml disposable columns; the extraction cartridge was inserted on an SPE vacuum manifold column processor (J and T Baker, Division of Mallin Ckroot Baker Inc., Deventer, Holland) and the flow rate adjusted to 1.5 ml min^{-1} by means of a slight vacuum. Prior to use, the silica gel cartridge was conditioned with 3 ml of methylene chloride. Sulforaphane was extracted by passing 3 ml of organic extract through the cartridge, washing the cartridge with 3 ml of ethylacetate, which was then discarded, and eluting the sulforaphane with 3 ml of methanol.

GC–MS analysis

A Varian model 3400 gas chromatograph, equipped with a Finnigan MAT model SSQ 710A mass spectrometer was used for detection. A J and W DB5, 30 × 0.25 mm i.d. capillary column delivered compounds to the mass spectrometer.

The operating conditions were as follows: injection volume, 1 μl; injector temperature, 250°C; helium velocity, 40 cm s^{-1}; oven temperature, 50°C for 5 min increasing to 250°C in increments of 10°C min^{-1} and remaining steady at 250°C for 10 min; ion source temperature, 180°C; electronic impact energy, 70 eV.

Quantitative assay

Sulforaphane content was determined by two different methods.

Determination by reaction with BDT

This method, as described in detail by Zhang *et al.* (1992a), relies on the fact that all isothiocyanates react quantitatively with an excess of vicinal dithiols in methanol at pH 8.5; absorbance at 365 nm (due to the reaction product) was evaluated.

Determination by reverse phase HPLC

This method envisages the use of a reverse phase technique using a C18 column. Samples were analysed with a Waters 3000 HPLC apparatus equipped with Waters 600 Multisolvent delivery, Waters 717 Autosampler, Waters Lambda Max 481 Lc spectrophotometer set at 201 nm and a Waters 745 Data Module (Millipore Corporation, Milford, MA, U.S.A.). The column used was a Lichrospher 100 RP-18 250 × 4 mm i.d. with RP 18

Table 1. Sulforafane content in samples of broccoli: comparison of methods

	Determination by reaction with BDT		Determination by HPLC	
	Mean μg g^{-1}	CV%	Mean μg g^{-1}	CV%
Broccoli florets A1	12.67	10.57	17.99	2.50
Broccoli florets A2	11.51	3.90	12.69	2.36
Broccoli florets H1	26.12	5.89	31.82	2.26
Broccoli florets H2	35.28	1.27	49.28	1.68
Broccoli stalks H	16.09	18.39	17.45	2.86
Broccoli leaves H	87.20	5.27	110.03	3.05

A = autolyzed samples, H = pH 3 treated samples, BDT = 1-2 benzenedithiol.

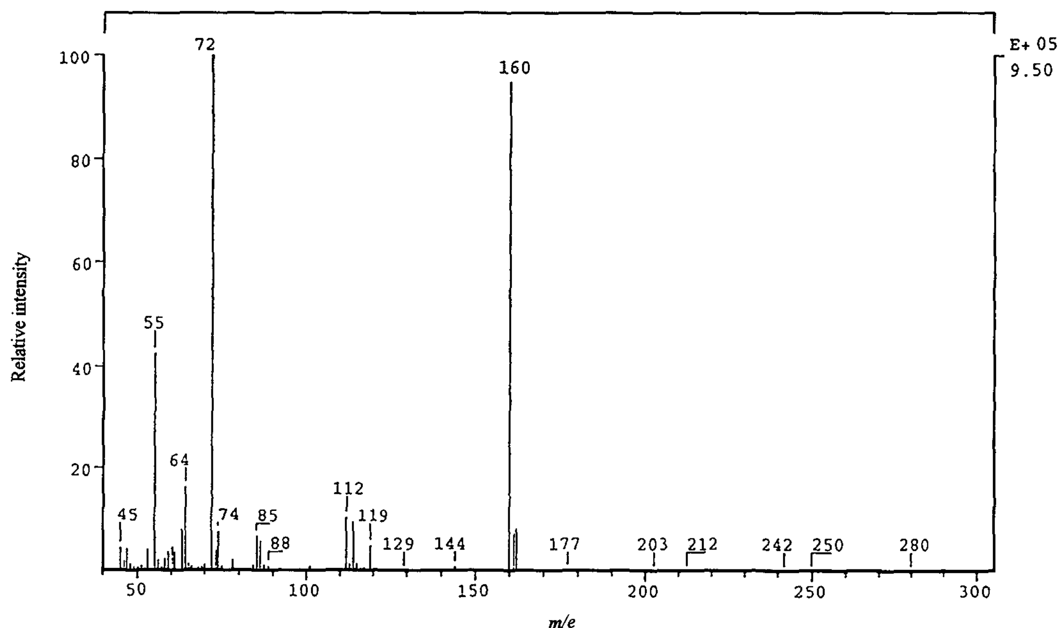

Fig. 1. Mass spectrum of synthetic sulforaphane.

guard column (Merck, Darmstadt, Germany). The mobile phase consisted of water–tetrahydrofuran (95:5 v/v) and the flow rate was 1 ml min^{-1}.

The methanol solutions obtained by SPE were evaporated, the residue was suspended in water–tetrahydrofuran (95:5 v/v) and filtered with Millex Hv and then 200 μl of this solution was injected on to the column.

RESULTS AND DISCUSSION

Preparation and purification of extracts

The analytical results for acid or enzymatic hydrolyzed broccoli florets (Table 1) show that acid hydrolysis performed before extraction consistently enhances the yield of sulforaphane; therefore, stalks and leaves were treated only at pH 3.

The purification and isolation of sulforaphane from the crude organic broccoli extracts by the proposed SPE method proved to be simple and selective, as well as being qualitatively and quantitatively reliable. A comparison between the extract and elute gas chromatographs shows in fact that sulforaphane is purified by most of the substances extracted (Fig. 1).

GC–MS

The GC–MS technique was used to confirm the presence of sulforaphane in broccoli extracts and to evaluate the SPE procedure.

The synthetic product was used to establish the analytical conditions and provide a reference spectrum; the retention time proved to be 14 min.

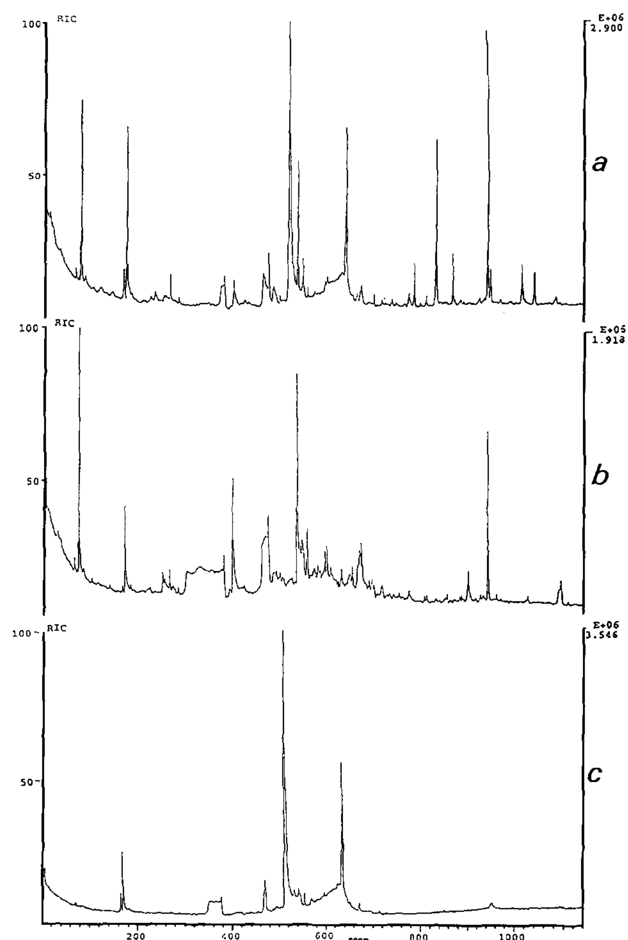

Fig. 2. GC–MS reconstructed ionization chromatographs for solid phase extraction fractions: (a) crude broccoli extract; (b) washing fraction; (c) elution fraction. Sulforaphane (scan 640).

Table 2. Data obtained for calibration curve of spectrophotometric and HPLC methods from standard solutions of sulforaphane

Reaction with BDT			HPLC		
Molarity	Means (abs. at 365 nm)	CV%	Molarity	Means (peak area)	CV%
0.04	0.029	12.25			
			0.05	438444	1.52
0.08	0.047	10.32			
			0.1	855342	2.2
0.12	0.079	10.35			
0.16	0.106	8.55			
0.20	0.137	5.21	0.2	1913167	1.6
0.24	0.161	3.00			
0.28	0.189	2.69			
0.32	0.210	3.12			
0.36	0.239	4.20			
0.40	0.263	3.30	0.4	3969139	2.0
0.44	0.305	2.10			
0.48	0.333	1.89			
0.52	0.352	5.56			
0.56	0.379	6.59			
0.60	0.392	7.23	0.6	5695603	2.86
0.64	0.415	6.53			
0.68	0.437	9.58			
0.72	0.446	13.22			
			0.8	7665562	1.92
			1	9697900	2.3
r^2	0.9949		r^2	0.9995	

The mass spectrum of synthetic sulforaphane is reported in Fig. 2. The *m/e* values obtained were: 45(6); 55(45); 60(7); 64(16); 72(100); 85(10); 86(9); 98(1); 101(1); 112(13); 114(11); 119(6); 144(1); 160(96); 162(11); M = 177(1).

The *m/e* values obtained for sulforaphane extracted from broccoli were: 45(5); 55(45); 60(15); 64(15); 72(100); 85(7); 86(6); 98(1); 101(2); 112(8); 114(9); 119(5); 144(1); 160(94); 162(6); M = 177(1).

Unfortunately, the poor quality of the peak of sulforaphane, due to degradation on the column, makes this method unsuitable for the quantitative analysis of extracts.

Quantitative assay

The sulforaphane content in all the samples analysed by the two methods is reported in Table 1. The results are the means of three samples each analysed three times on different days.

Reaction with BDT

For this reaction, the methanol solutions obtained by SPE are used after dilution. The sulforaphane content of samples was determined by reference to a calibration curve prepared with different isothiocyanates or with sulforaphane. The curve is linear up to concentrations of 600 μmol. However, the marked variability of the results, above all at the highest and lowest concentrations, points to the need to eventually increase or decrease the amount of sample used in order to remain within a range of optimal concentration (Table 2).

Data reported in Table 1 show that the method is sensitive, but not very reproducible, as is evident from the high CV%; the analyst had to carry out a large number of analyses for each sample in order to obtain statistically significant data. Also, it is rather long and laborious.

Chromatographic method

This method is quantitatively and qualitatively reproducible, as well as being sensitive. The sulforaphane is eluted at 25.8 min and is well separated from any other components in the samples (Fig. 3). The calibration curve obtained with the synthetic reference compound is linear up to concentrations of 1 mmol (Table 2). The detection limit is 0.025 mmol. To estimate the recovery rate of sulforaphane, some fractions of broccoli

Table 3. Recovery of sulforaphane from spiked broccoli samples with solid phase extraction and HPLC techniques

Samples	Present $\mu g\,g^{-1}$	Spiked $\mu g\,g^{-1}$	Found $\mu g\,g^{-1}$	CV%	Recovery %
Broccoli florets H1	31.82	30	60.50	2.80	97.80
Broccoli florets A2	12.69	15	25.26	1.96	91.22

A = autolyzed samples, H = pH 3 treated samples.

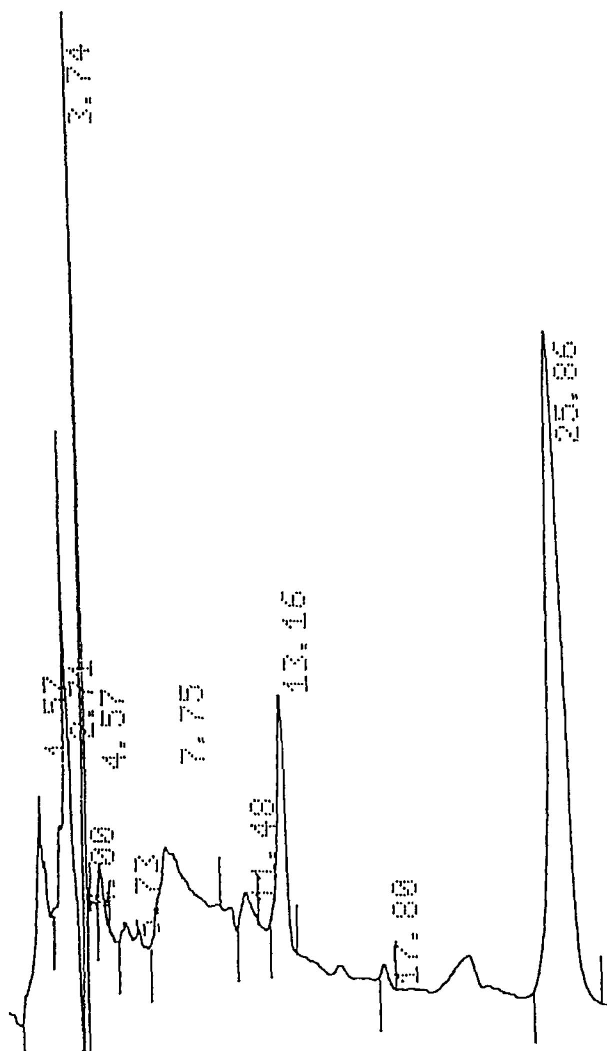

Fig. 3. HPLC elution profile of purified broccoli extract; sulforaphane (rt = 25.86).

homogenates were spiked with standard sulforaphane solutions. The recovery rate is summarized in Table 3. It is clear that the chromatographic method afforded better results with greater reproducibility, as borne out by the lower CV%.

CONCLUSIONS

The method proposed proved reliable and reproducible as regards both SPE purification and chromatographic determination. The SPE method led to a rapid and efficient extraction of sulforaphane. Both analytical methods offer reliable quantitative analysis, but the BDT method is less satisfactory on account of the marked variability and poor reproducibility of the results. Quantities of sulforaphane were found in all the samples analysed. Whereas the above-cited literature refers to the presence of sulforaphane in broccoli florets and seeds, the present findings show that sulforaphane is also contained in the stalks and leaves, the highest content overall being found in the latter.

Accordingly, we propose to continue our research in this direction, extending our studies to other samples of broccoli and brassicas in order to confirm our findings.

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