An HPLC Method for Simultaneous Quantitation of Individual Isothiocyanates and Oxazolidinethione in Myrosinase Digests of Rapeseed Meal

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ABSTRACT

A simple, rapid and precise method for simultaneous quantitation of individual isothiocyanates and oxazolidinethione in myrosinase digests of rapeseed meal has been developed. The method consists of inactivation of native myrosinase activity present in the seedmeal, followed by digestion with mustard myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) to hydrolyze rapeseed glucosinolates quantitatively to isothiocyanates and oxazolidinethione. These hydrolytic products are extracted in methylene chloride as soon as they are formed and finally resolved by a reverse phase high pressure liquid chromatography (HPLC) technique on a μ Bondapak C_{18} column using aqueous acetonitrile as solvent and an ultraviolet (UV) absorbance detector set at 254 nm. The lower limits of quantitation by this method in a single aliquot applied to the column were 0.2 μg for the isothiocyanates and 0.01 μg for the oxazolidinethione. Recoveries of allyl isothiocyanate, oxazolidinethione and sinigrin added to *B. juncea*, prior to digestion, were quantitative and averaged at 94.5, 93.0 and 91.2 percent with standard deviations of 1.5, 3.3 and 2.8 percent, respectively. The butenyl and pentenyl isothiocyanates and oxazolidinethione in Tower (*B. napus*) and Candle (*B. campestris*) rapeseeds, and allyl isothiocyanate in *B. juncea* were the major hydrolytic products of glucosinolates. The identity of peaks corresponding to these compounds on a HPLC chromatogram was confirmed by mass spectroscopy.

INTRODUCTION

Rapeseed is the major oilseed crop of Canada (1), an important oilseed crop of India, and presently ranks fifth in world production of oilseeds (2). This crop is primarily used as a source of vegetable oil since the oil content reaches ca. 45% (dry, dehulled basis). The residual meal contains ca. 40% protein (d.b., N x 5.53) and is a good source of quality protein since rapeseed proteins possess a well balanced essential amino acid composition and are, for plant proteins, high in lysine and methionine (3,4). However, the utilization of rapesed meal as a protein source in nonruminant rations and human diets is severely limited, mainly due to the presence of glucosinolates. Glucosinolates in rapeseed are a source of goitrogens. As such, the glucosinolates are relatively nontoxic, but their hydrolytic products, following the action of thioglucoside glucohydrolase (EC 3.2.3.1), are isothiocyanates, thiocyanates, nitriles, and oxazolidinethiones which are goitrogenic (5). In addition, these hydrolytic products in nonruminants produce varying manifestations of toxicity ranging from depressed growth and weight gain, loss of reproductive potential, and enlarged thyroids and kidneys to death in rats and other species of experimental animals (6,7).

Previously, several technological attempts have been made to overcome the problems of toxicity associated with rapeseed. These include heat inactivation of the enzyme and the removal or destruction of glucosinolates and their hydrolytic products. An alternative approach considered genetic improvement and the removal of glucosinolates from rapeseed through plant breeding. In either case, several methods have been developed for monitoring the removal of glucosinolates including: (i) gas liquid chromatographic (GLC) analysis of trimethylsilyl derivatives of glucosinolates (8-12); (ii) enzymatic hydrolysis of the glucosinolates followed by quantitation of the released aglucons by spectrophotometry or gas chromatography (GC) (13-16); (iii) hydrolysis of glucosinolates by thioglucoside glucohydrolase (EC 3.2.3.1) and measurement of the released glucose by enzymatic (17-20) and GLC assays (21), and the bisulfate ion by gravimetric (22) and volumetric techniques (23,24). However, many of these methods lack precision and reproducibility and often require additional clean-up steps before final quantitation. In addition, the aglucon assay procedures are generally suspect since enzymatic hydrolysis may not go to completion or may proceed by other routes (25).

Recently, high pressure liquid chromatography (HPLC) has been used for the detection of oxazolidinethione in milk (26) and separation of a mixture of standard organic isothiocyanates (27). In this paper, we describe a simple, rapid and precise HPLC method for the detection and simultaneous quantitation of individual isothiocyanates and oxazolidinethione in myrosinase digests of rapeseed meal.

MATERIALS AND METHODS

Materials

Canadian grown rapeseed seeds of *B. campestris* variety Candle, and *B. juncea* (oriental mustard) were obtained from the Saskatoon Research Station of Agriculture Canada. These seeds were dehulled using the Palyi pneumatic small-seed dehulling unit (28). Dehulled rapeseed, *B. napus* variety Tower, was obtained locally. Laboratory seed meals were prepared by grinding dehulled rapeseeds in a Wiley mill and extracting them in *n*-hexane.

Crystalline sinigrin monohydrate, No. S330-5, was purchased from Aldrich Chemical Co. Inc., Milwaukee, WI. Allyl isothiocyanate was purchased from Eastman Kodak Co., Rochester, New York. An authentic sample of goitrin [(S)-5-vinyloxazolidine-2-thione] was obtained from another laboratory. Certified HPLC grade acetonitrile, certified ACS grade methylene chloride, *n*-hexane, citric acid, and sodium phosphate (dibasic) were purchased from Fisher Scientific Co. Glass-distilled deionized water was used throughout the course of this investigation.

Phosphate-citrate buffer (pH 7.0) was prepared according to the method of Wetter and Youngs (14) by mixing 17.5 ml of 0.1 M citric acid solution with 82.5 ml of 0.2 M dibasic sodium phosphate solution and adjusting the pH to 7.0. The myrosinase preparation used in these experiments was obtained from *B. juncea* (oriental mustard), according to the procedure of Schwimmer (29).

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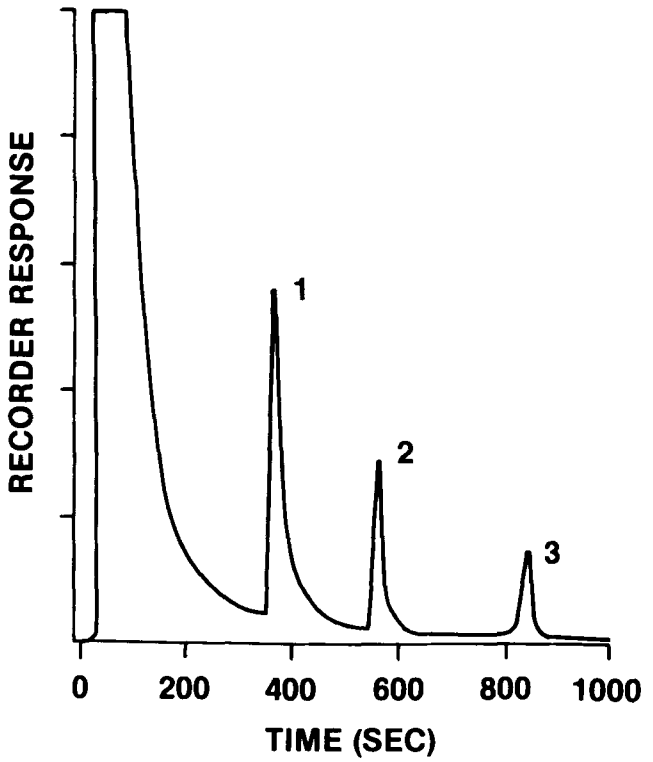


FIG. 1. Typical GLC chromatogram (FFAP column) for the three major isothiocyanates found in myrosinase digests of rapeseed meal. 1, allyl isothiocyanate (rt = 366 sec); 2, butenyl isothiocyanate (rt = 565 sec); 3, pentenyl isothiocyanate (rt = 826 sec); (rt = retention time).

Methods

Gas Liquid Chormatography. GLC analyses were carried out using a Hewlett-Packard Model 7620A research gas chromatograph equipped with dual flame ionization detectors, a Coleman recorder and Autolab System IV (Spectra Physics) computing integrator for automatic integration of the peak areas. Stainless steel columns (1.8 m x 3.2 mm ID) packed with 20% FFAP on 80/100 mesh Chromosorb W AW DMCS (Chromatographic Specialities, Brockville, Ontario) were used for analysis of isothiocyanates. The GLC unit was operated isothermally at an oven temperature of 130 C. The injection port and detector temperatures were 160 C (15). Glass columns (1.8 m x 4 mm ID) packed with 1% EGSS-X on 100/120 mesh Gas-Chrom Q (Applied Science Laboratories Inc., State College, Pa.) were used for the detection of 5-vinyloxazolidine-2-thione (30). Initially the column was operated isothermally at 120 C for a period of 5 min. Thereafter, the column oven temperature was programmed at 10 C/min to 210 C which was maintained for the remainder of the chromatogram (31). The injection port and the detector temperatures were 220 and 240 C, respectively.

Carrier gas (nitrogen, 65 psig) was introduced with a rotameter setting of 2 cm. The FFAP and EGSS-X columns were conditioned overnight with a reduced flow of nitrogen at 140 and 215 C, respectively.

High Pressure Liquid Chromatography. The HPLC system adopted consisted of a Waters' Liquid Chromatograph equipped with a Model 6000A solvent delivery system, a septumless injector U6K with 2 ml sample loading loop, a 30 cm x 3.9 mm ID μBondapak C_{18} column with monomolecular layer of organosilane permanently bonded to μPorasil (polar, fully porous silica particles of 10 μm size), a Model 440 UV absorbance detector set at 254 nm which combines high sensitivity (0.005 absorbance units full scale) with a wide dynamic range (2.0 to 0.005 absorbance units full scale) and a Beckman 25.4 cm strip chart recorder with an adjustable chart speed of 0.254 cm to 25.4 cm per min. The column was eluted with aqueous acetonitrile (60:40, 50:50 or 40:60 v/v) at a flow rate of 2 ml/min.

Gas Chromatography-Mass Spectroscopy (GC-MS). The GC-MS analyses were performed on a double focussing VG Micromass 7070 F mass spectrometer (VG Micromass Ltd., Winsford, England) equipped with a magnetic mass analyzer. The glass columns used for GC were 1.8 m x 4 mm ID with FFAP and EGSS-X packings. The FFAP and EGSS-X columns were operated at 140 and 210 C, respectively. The separator and transfer lines were maintained at 200 C. Ions were produced with a 70 eV electron beam, and a mass range of 0-200 was scanned.

Calibration curves. Calibration curves were established for allyl isothiocyanate on both GLC and HPLC and for 5-vinyloxazolidine-2-thione on HPLC only. The standard solutions of both these compounds were prepared in methylene chloride. The series of allyl isothiocyanate solutions contained 0.04-1.0 mg/ml for use on GLC and up to 5 mg/ml for use on HPLC. The sample quantities of pure 5-vinyloxazolidine-2-thione were weighed in a Cahn Gram Electrobalance to prepare solutions containing 0.01-0.25 mg of oxazolidinethione per milliliter.

Calibration curves and response factors for butenyl and pentenyl isothiocyanates were not established because of the unavailability of these compounds in reference preparations of high purity. In this situation an indirect method was adopted to calculate the amounts of each of these aglucons in rapeseed samples. This indirect method has been based on the assumption that the peak areas on the GLC chromatogram for each of the isothiocyanates of a homologous series are proportional to their amounts (by weight) present in the sample analyzed and that their recoveries on the analytical column and run conditions employed are quantitative. Thus, the amounts of butenyl or pentenyl isothiocyanates in any given sample was taken as being equal to the slope of the calibration curve for allyl isothiocyanate multiplied by the peak areas for butenyl or pentenyl isothiocyanates obtained for the sample. At the same time, if an equal aliquot of the same sample is analyzed by HPLC, the peak area on the HPLC chromatogram for an isothiocyanate corresponds to the amount present and calculated earlier from the GLC chromatogram for the sample. Considering a linear relationship between the peak area and the amount of isothiocyanate present in the aliquot analyzed, a factor similar to the slope of the calibration curve is calculated for HPLC analysis to be used subsequently for successive quantitative analytical determinations.

Preparation of Myrosinase Digests of Rapeseed Meals. A 0.25 g sample of ground, defatted, air-dried rapeseed meal was weighed into a 5 ml vial containing a glass bead and held in an oven at 100 ± 2 C for 10 min. Then 1 ml of boiling citrate-phosphate buffer was added, and the sample was heated for an additional 5 min. This treatment inactivated the native enzyme while keeping the glucosinolates intact in the sample. After cooling the sample to room temperature, 1 ml of buffer containing 15 mg of thioglucoside glucohydrolase (EC 3.2.3.1) and 1 ml of methylene chloride for Tower and Candle or 2 ml of methylene chloride for *B. juncea*, meal samples were added to the vial which was sealed with a teflon-lined cap. The methylene chloride did not contain an isothiocyanate marker. The capped vial was shaken on a Burrell's wrist-arm shaker for 4 hr at 22 C. This procedure, based on results of previous studies (13, 30, 32), ensured quantitative and selective hydrolysis of rapeseed glucosinolates to isothiocyanates and 5-vinyl-oxazolidinine-2-thione. At the end of the incubation period, the vial was centrifuged at 20 C in a Sorval centrifuge at 1000 x g for 20 min, thus separating the aqueous

phase to the top, sample meal to the center and methylene chloride layer to the bottom. The aqueous phase was discarded, and the methylene chloride layer was analyzed for its aglucon content.

Preparation of Samples for GC-MS Analysis. Methylene chloride extracts were used to identify the aglucons in the thioglucoside glucohydrolase digests of rapeseed meal. An HPLC fraction corresponding to the oxazolidinethione peak on the chromatogram was evaporated to dryness on a rotary evaporator connected to a water aspirator at 45 C, and the residue was dissolved in fresh methylene chloride. The HPLC fractions corresponding to various isothiocyanates were extracted three times with petroleum ether. The petroleum ether extracts were then concentrated to a solution volume of ca. 1.5-2.0 ml by evaporating the excess solvent under a gentle stream of nitrogen. All extracts were filtered through anhydrous sodium sulfate using Whatman No. 42 filter paper.

RESULTS AND DISCUSSION

A typical GLC trace of the three major isothiocyanates found in thioglucoside glucohydrolase digests of rapeseed (Fig. 1) contains allyl isothiocyanate from sinigrin, the major glucosinolate of *B. juncea,* and butenyl and pentenyl isothiocyanates from gluconapin and glucobrassicanapin, two major glucosinolates in *B. napus* and *B. campestris* species of rapeseed. A plot of the logarithm of retention time against carbon number was reasonably linear for these three unsaturated and homologous isothiocyanates. The calibration curve obtained for allyl isothiocyanate was linear over the concentration range (0-5 μg) analyzed; linear regression of peak area vs. concentration of allyl isothiocyanate gave a correlation coefficient of 0.9994. The limit of detection was 0.02 mg/ml. These results are similar to the findings of Youngs and Wetter (15) and support this method for obtaining a reasonably accurate estimation of butenyl and pentenyl isothiocyanates indirectly.

Although the GLC method of Youngs and Wetter (15) is precise for quantitation of individual isothiocyanates, the aglucon, 2-hydroxy-3-butenyl isothiocyanate, a product of a major glucosinolate, progoitrin, in rapeseed, is unstable and cyclizes spontaneously to form 5-vinyl-oxazolidine-2-thione or goitrin (33). This compound cannot be quantitated by this procedure and, hence, is generally determined by a spectroscopic procedure based on its specific UV absorbance. More accurate and sensitive GLC methods for analysis of 5-vinyloxazolidine-2-thione and all other aglucons have been described (16,30), but the analysis is relatively slow. As an alternative, a HPLC technique was investigated for simultaneous separation of individual isothiocyanates from oxazolidinethione and quantitation of each of these aglucons in thioglucoside glucohydrolase digests of rapeseed meal.

Initially, the qualitative solutions of pure 5-vinyloxazolidine-2-thione, pure allyl isothiocyanate, and a mixture of the two were prepared in methylene chloride and applied to a μBondapak C_{18} column to select a suitable mobile phase for the separation of these compounds. Aqueous acetonitrile proved effective, 5-vinyloxazolidine-2-thione and allyl isothiocyanate having retention times of 2.0 and 6.5 min, respectively, when acetonitrile and water in 40:60 (v/v) proportion constituted the mobile phase. However, speed and resolution were lost when Tower and Candle meal digests were applied to the column with this mobile phase. Increasing the proportion of acetonitrile to 50% (v/v) in the mobile phase restored the speed and resolution of the HPLC technique. Even more rapid analysis and better resolution was achieved by employing acetonitrile and water in 60:40 (v/v) proportion as the mobile phase (Fig. 2).

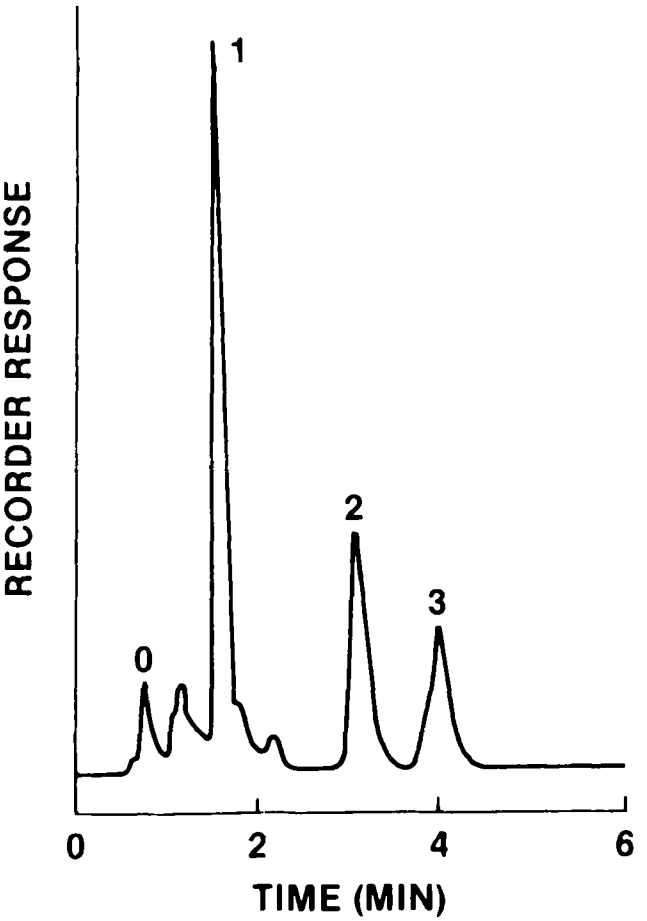


FIG. 2. HPLC chromatogram of thioglucoside glucohydrolase digest of dehulled defatted Candle meal. Mobile phase – acetonitrile/water (60:40 (v/v)). Recorder response for first 2.5 min was 0.2 absorbance units full scale, then 0.02 absorbance units full scale. 1, 5-vinyloxazolidine-2-thione (rt = 1.6 min); 2, butenyl isothiocyanate (rt = 3.1 min), 3, pentenyl isothiocyanate (rt = 4.0 min).

The HPLC method was assessed for accuracy with pure allyl isothiocyanate and 5-vinyloxazolidine-2-thione. Peak areas were measured manually with an OTT compensating polar planimeter in vernier units, and the peak absorbances were obtained from the peak height on the calibrated recorder chart. Plots of peak area vs. concentration and peak absorbance vs. concentration were linear over the concentration ranges employed for both allyl isothiocyanate (Fig. 3) and 5-vinyloxazolidine-2-thione (Fig. 4). However, statistical analysis of the data suggested that the peak area was a more accurate measure of concentration than peak absorbance for these compounds. The lower limits of detection in a single aliquot applied to the column were estimated at 0.01 μg for allyl isothiocyanate and 0.0005 μg (0.5 ng) for 5-vinyloxazolidine-2-thione, but the practically determined lower limits of quantitation by this method were 0.2 μg for the isothiocyanates and 0.01 μg for the oxazolidinethione.

Accuracy of the method was further assessed by measuring recovery upon addition of known amounts of pure allyl isothiocyanate and 5-vinyloxazolidine-2-thione and by analysis of enzymatically released allyl isothiocyanate upon addition of known amounts of sinigrin. The experimental results (Table I) showed that the recoveries were acceptable for the added compounds. The recovery of the oxazolidinethione at the two levels studied was reasonably constant indicating that the isothiocyanate content of the meal digest had apparently no effect on the recovery of oxazolidinethione. However, the recoveries of allyl isothio-

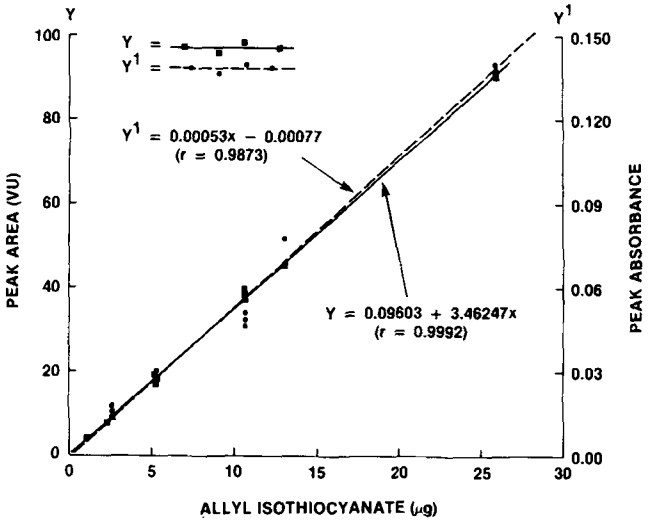


FIG. 3. HPLC calibration curves for pure allyl isothiocyanate (μBondapak C_{18} column). Mobile phase – acetonitrile/water (40:60 (v/v)). Recorder chart speed, 12.7 mm/min.

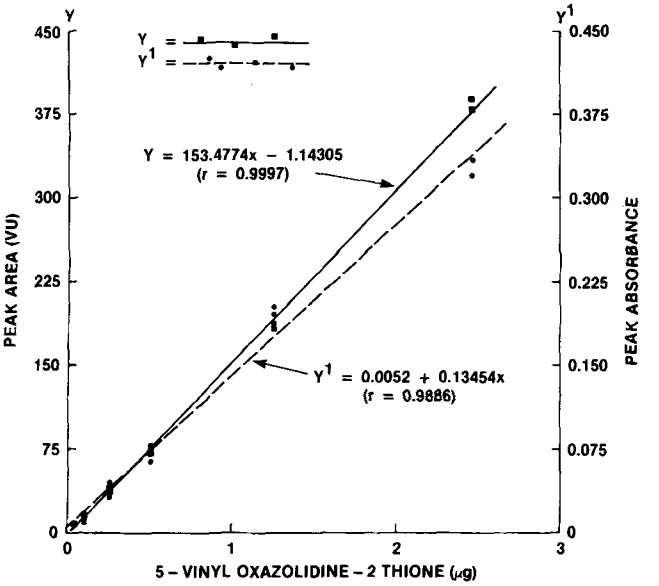
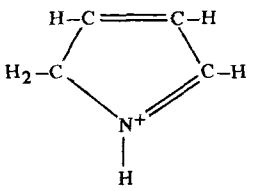


FIG. 4. HPLC calibration curves for pure 5-vinyloxazolidine-2-thione (μBondapak C_{18} column). Mobile phase – acetonitrile/water (40:60 (v/v)). Recorder chart speed, 50.8 mm/min.

cyanate and sinigrin for low levels of addition were lower, perhaps due to some interference of the natural concentrations of sinigrin in the meal and allyl isothiocyanate in the enzyme digest.

The HPLC method was utilized to determine the glucosinolate contents of rapeseed samples by assaying the isothiocyanates and 5-vinyloxazolidine-2-thione in thioglucoside glucohydrolase digests of sample meals (Table II). These data indicated that the major isothiocyanates were allyl- in *B. juncea*, butenyl- in Tower, and butenyl- and pentenyl- in Candle digests. 5-Vinyloxazolidine-2-thione was present in Tower and Candle digests. The HPLC chromatogram of *B. juncea* digest showed a small peak with a retention time close to that of 5-vinyloxazolidine-2-thione. However, the GC-MS analysis of this digest did not provide any evidence for the presence of 5-vinyloxazolidine-2-thione in enzyme digests of *B. juncea*. The identity of this unknown compound was not established.

The identities of various aglucons in thioglucoside glucohydrolase digests of rapeseed samples and in HPLC fractions corresponding to various peaks on the HPLC chromatogram were established by GC-MS analysis. The mass spectra of isothiocyanates gave peaks corresponding to their molecular ions (m/e 99 for allyl-, m/e 113 for butenyl-, and m/e 127 for pentenyl- isothiocyanates) and a m/e 72 corresponding to $^{+}CH_2$-N=C=S ion. The mass spectra of allyl- and butenyl- isothiocyanate) also had a prominent peak for R^+ ion (m/e 41, corresponding to CH_2=CH-CH_2^+ for allyl-, and m/e 55, corresponding to CH_2=CH-CH_2-CH_2^+ for butenyl- ions for the two isothiocyanates, respectively). However, this peak for R^+ ion was not present in the mass spectrum of pentenyl isothiocyanate. Instead, there was a peak at m/e 70 corresponding to $(R+1)^+$ ion. The spectrum of pentenyl isothiocyanate was also unusual in that it included a prominent peak at m/e 126 corresponding to $(M-1)^+$ ion. This peculiarity was previously noted and discussed by Daxenbichler et al. (34). The mass spectrum of 5-vinyloxazolidine-2-thione showed an intense molecular peak at m/e 129 and fragment ion peaks at m/e 68, corresponding to



$(C_4H_6N^+)$ ion

and at m/e 57, corresponding to $(CH_2$=CH-$CH_2O)^+$ ion.

The HPLC method described simultaneously quantitates individual isothiocyanates and 5-vinyloxazolidine-2-thione in selectively prepared thioglucoside glucohydrolase digests of rapeseed meals and provides an estimate for each glucosinolate present. This method does not require the use of alcohol in preparation of samples and, therefore, eliminates

TABLE I

Recoveries of Added Allyl Isothiocyanate, 5-Vinyloxazolidine-2-thione and Sinigrin from *B. juncea* Meal Using HPLC Method

Additives	Quantities added[a]	Percent mean recoveries		
		Allyl isothiocyanate	Oxazolidinethione	Sinigrin
Allyl isothiocyanate	2.1164 mg	94.5 [1.5][b]	—	—
5-Vinyloxazolidine-2-thione	0.1016 mg	—	93.0 [3.3][b]	—
Allyl isothiocyanate	1.0584 mg	91.2	—	—
+	+			
5-Vinyloxazolidine-2-thione	0.0254 mg	—	93.2	—
Sinigrin	7.7 mg	—	—	87.7
	15.0 mg	—	—	93.7 91.2[2.8][b]

[a]To 0.25 g of dehulled, defatted, air-dried meal in 2 ml of methylene chloride. The natural allyl isothiocyanate content of the meal was 28.37 mg/g.

[b]Figures in brackets represent standard deviations.

TABLE II

Glucosinolate Contents of Some Rapeseed Samples Assayed as Isothiocyanates and 5-Vinyloxazolidine-2-Thione by HPLC Method

Sample	mg per g dehulled, defatted, dry meal			
	Isothiocyanates			
	Allyl	Butenyl	Pentenyl	Oxazolidinethione
B. juncea (oriental mustard)	28.37[a]	—[b]	—[b]	—[b]
B. napus var. Tower	—[b]	1.020	trace	1.925
B. campestris var. Candle	—[b]	0.520	0.472	0.687

[a]Average of duplicates.
[b]Not found.

the possibility of erroneous results due to the formation of isothiocyanate-alcohol addition compounds (27). The improved precision and low detection limits for this method make it particularly suitable for analysis of low glucosinolate rapeseeds and processed rapeseed protein products. The speed of analysis and the higher capacity of HPLC method should make this method attractive for routine analysis in processing plants and useful in screening rapeseed strains largely free from glucosinolates in plant breeding work. Also, it may be of use in evaluating the toxicity of processed rapeseed meal and its protein products in nutritional studies.

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