Simultaneous Determination of Isothiocyanates, Indoles, and Oxazolidinethiones in Myrosinase Digests of Rapeseeds and Rapeseed Meal by HPLC

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HPLC has been used for the analysis of isothiocyanates, indoles, and oxazolidinethiones in rapeseeds and rapeseed meal. The samples were treated with myrosinase and the released hydrolysis products extracted with dichloromethane. The separation was performed on an RP-18 column using a gradient system with acetonitrile and water. Use of a programmable UV detector permitted the detection of the compounds at their absorption maxima of 210 and 240 nm, respectively. Response factors of eight standard compounds were calculated for 240 nm. The contents of glucosinolates calculated with the results of this method showed a significant linear correlation ($r = 0.9995$; $P < 0.005$) with the contents of glucosinolates evaluated with the results of the HPLC method of desulfoglucosinolates.

Keywords: *HPLC; indoles; isothiocyanates; oxazolidinethiones; rapeseed*

INTRODUCTION

The importance of rapeseed as oilseed has increased in the last decades. Besides the high oil content of about 40%, the yield of protein and the favorable nutritional value of its amino acid composition play important roles (Eggum, 1981). However, the use of rapeseed and rapeseed meal as a protein source of nutrients for farm livestock is limited by the presence of antinutritive compounds such as glucosinolates.

Glucosinolates are relatively nontoxic (Bell, 1984), but their hydrolysis products, formed by myrosinase (thioglucoside glucohydrolase [EC 3.2.3.1]), adversely affect animal growth, reproduction, and performance and cause goiter and abnormalities in internal organs of animals (Mawson et al., 1994a−c). Myrosinase occurs in several microbial species inhabiting the gastrointestinal tract (Larsen, 1981) or in compartments of the rapeseeds (Höglund et al., 1991). The hydrolysis products are isothiocyanates, thiocyanates, nitriles, or oxazolidinethiones, dependent on the pH value of the reaction medium (Benn, 1977).

Several methods have been developed to follow the trend of glucosinolates and their hydrolysis products in rapeseeds. Methods that indicate the total amount of glucosinolates are often based on the measurement of released glucose (vanEtten et al., 1974; Thies, 1985; Fiebig and Kallweit, 1987) or bisulfate ions (Fiebig and Sendfeld, 1989; Sendfeld et al., 1988) by enzymatic assays. Other methods include the palladium test (Thies, 1982), the near-infrared method (Renard et al., 1987), and the X-ray fluorescence method (Schnug and Haneklaus, 1987, 1988; Haneklaus et al., 1994). Individual glucosinolates were determined directly by GLC analysis of the trimethylsilyl derivates of glucosinolates (Thies, 1976), HPLC analysis of the intact glucosinolates (Helboe et al., 1980), and HPLC analysis of desulfoglucosinolates (Minchinton et al., 1982; Spinks et al., 1984; Fiebig and Jörden, 1990) or indirectly by GLC analysis of the enzymatic hydrolysis products (Daxenbichler and vanEtten, 1977; vanEtten and Daxenbichler, 1977). A few methods used the determination of the enzymatic hydrolysis products by HPLC for the detection of oxazolidinethione in milk (Benns et al., 1979), the separation of a mixture of standard organic isothiocyanates (Mullin, 1978), the simultaneous quantitation of individual isothiocyanates and oxazolidinethione (Maheshwari et al., 1979), or the determination of oxazolidinethione in feeding stuffs (Harris et al., 1979). The sample preparation for these methods was very fast and simple but not applicable to current rapeseeds or rapeseed meals with glucosinolate contents of less than 20 μmol/g. Besides, the detection of the indoles was disregarded in these investigations.

The objective of this paper is to describe the sample preparation and HPLC method for the simultaneous detection of isothiocyanates, indoles, and oxazolidinethiones in rapeseeds and rapeseed meals after myrosinase digestion and to compare the results of the HPLC method of desulfoglucosinolates.

EXPERIMENTAL PROCEDURES

Materials. Rapeseeds were obtained from the Deutsche Saatveredelung, Lippstadt, Germany, and the rapeseed meals from the Cereol Deutschland GmbH, Mannheim, Germany.

Some seeds of *Cardamine impatiens* L. were obtained from the Botanical Garden of Osnabrück, Germany.

Reagents. Allyl, propyl, and butyl isothiocyanates as well as indole-3-acetonitrile and indole-3-carbinol were purchased from Aldrich Chemie, Steinheim, Germany. Phenethyl isothiocyanate was obtained from Sigma Chemical, St. Louis, MO, and heptyl isothiocyanate from Eastman Kodak Co., Rochester, NY. Goitrin was purchased from Johnson Matthey Alfa Products, Karlsruhe, Germany.

Water, acetonitrile, dichloromethane, and *n*-hexane (Lichrosolv, chromatography grade) were obtained from Merck, Darmstadt, Germany, and myrosinase from InnoTech BioLog GmbH.

All sample preparations were carried out in phosphate−citrate buffer (pH = 7.0), prepared according to the method of Wetter and Youngs (1976).

HPLC. The HPLC analysis was conducted using a Merck-Hitachi low-pressure gradient system, fitted with a 655-12 pump and a L-5000 gradient controller, a Merck-Hitachi L-4250 UV/vis detector set at 240 nm, and a Knauer Euro-

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S0021-8561(96)00318-4 CCC: $12.00

chrom 2000 for Windows integration system. Ten microliters of the glucosinolate hydrolysis products-containing eluates was injected onto a 250 mm × 4 mm, 5 μm LiChrospher 60 RP-select B column (Merck, Darmstadt), used with a flow rate of 1 mL/min. The mobile phase used was water (A) vs acetonitrile (B), for a total running time of 60 min, and the gradient changed as follows: 80% A/ 20% B for 8 min, then in one step from 80% A/20% B to 60% A/ 40% B, and in 27 min to 40% A/60% B. Afterward, the column was equilibrated at 80% A/20% B for 25 min.

Sample Preparation. Ten grams of rapeseeds was crushed in a ball mill for 10 min, and then 2.00 g of the meal was defatted 3-fold with 20 mL of *n*-hexane in a 30 mL tube with a screw top (Fleischhacker, Germany) by shaking, centrifugation, and decantation of the oil-containing *n*-hexane phase. Afterward, the defatted sample was dried at 40 °C for a short time. Next, 10 mL of dichloromethane, 5 mL of phosphate−citrate buffer (pH = 7.0), and 30 mg of myrosinase were added to the dried meal; afterward, the sealed tube was shaken for 2 h at room temperature and then centrifuged at 5000*g* for 10 min. The aqueous layer could be discarded easily by decantation, and then 1 mL of a standard solution was added. The tube was shaken carefully for a short time to mix the standard and the sample solution, and then the dichloromethane phase was filtrated through a hydrophobic filter filled with 2 g of sodium sulfate into a 25 mL flask. Dichloromethane was removed by evaporating the solution to dryness at 0 °C and 190 mbar. The residue was dissolved in 1 mL of acetonitrile/water (60:40) and filtered through a 0.45 μm membrane filter (Millipore) into an HPLC vial.

Rapeseed meal and *Cardamine impatiens* L. were treated in the same way, but rapeseed meal was used without crushing and defatting the sample material.

Calibration and Evaluation. A calibration curve was established for propyl isothiocyanate with solutions containing 0.07−1.14 mg/mL acetonitrile. Each point of the calibration curve was measured five times, and mean values as well as standard deviations were calculated. The mean values were used for the calibration curve. To find the optimum evaporating conditions, 10 mL of dichloromethane containing 0.76 mg of propyl isothiocyanate was transfered into a 25 mL flask, and the solvent was removed by evaporating at 0 °C and different pressures in the range of 130−210 mbar. This procedure was done for each pressure five times, and the mean values were calculated. A statistical checkup was carried out by calculating the standard deviation.

Other calibration curves were established with goitrin, indole-3-carbinol, and heptyl isothiocyanate in the same manner. The solutions contained 0.012−0.114, 0.06−0.50, and 0.12−2.36 mg/mL, respectively. The recovery of the compounds was investigated in the way described above.

Standard solutions of allyl, propyl, butyl, phenethyl, and heptyl isothiocyanates as well as indole-3-acetonitrile, indole-3-carbinol, and goitrin were injected 10 times into the HPLC system in order to calculate the response factors for the different compounds.

A solution of heptyl isothiocyanate (13.73 μmol/mL) in dichloromethane was used as internal standard.

Preparative HPLC. Preparative fractionation for the isolation of compounds was performed with the same chromatograph and detection system employed for analytical HPLC. Detection was performed at 240 nm, and the peaks were collected automatically by a Merck-Hitachi L-5200 fraction collector according to time windows. A rapeseed sample with a high content of glucosinolates was prepared according the description above, and 25 μL of the extract was injected automatically with a intelligent autosampler AS 4000 (Merck-Hitachi), 20 times each. The fraction of each compound was evaporated to dryness at 40 °C and 50 mbar.

GC−MS Analysis. Identification of compounds by GC−MS analysis was performed using the electron ionization mode (70 eV) on a Hewlett Packard instrument, Model 5890, Series II/5989A. Separation was carried out with a 0.23 μm permabond OV-1 fused silica capillary column (Macherey-Nagel), 25 m × 0.32 mm i.d.

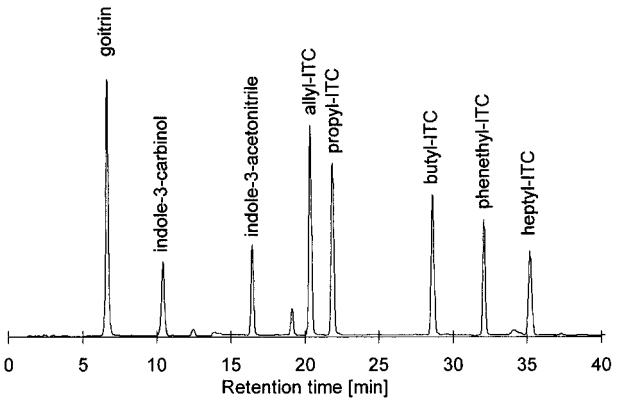

Figure 1. HPLC chromatogram of a standard solution measured with a detector wavelength of 240 nm.

Helium was used as carrier gas at a flow rate of 1.5 mL/min. The temperature program was set as follows: 10 min hold on 50 °C, then with 8 °C/min to 250 °C, and hold 20 min. Other operating conditions were splitless injector temperature of 230 °C, interface temperature of 280 °C, and ion source temperature of 200 °C.

Determination of Desulfoglucosinolates. The desulfoglucosinolates were determined as described by Fiebig and Jörden (1990).

Statistical Analysis. Data were analyzed by analysis of standard deviation and regression, and the statistical significance of the regression was tested by Kendall's τ test (Conover, 1971). Student's *t*-test to evaluate the statistical significance for independent and variables interactions was performed with two-tailed *t*-tests at $P = 0.005$. The data were evaluated using a computer program (Statgraphics).

RESULTS AND DISCUSSION

An alternative method for determination of the main hydrolysis products of glucosinolates of rapeseeds was developed. Instead of an isocratic HPLC system (Maheshwari et al., 1979), a gradient was used to separate the three compound classes, oxazolidinethiones, indoles, and isothiocyanates. Figure 1 shows the separation of a standard solution. With this gradient system, the separation was complete within 38 min, and the compounds were eluted in the order oxazolidinethiones, indoles, and isothiocyanates, as expected under these conditions. This is similar to the results of Maheshwari et al. (1979), who separated 5-vinyloxazolidine-2-thione (goitrin) and isothiocyanates with an isocratic HPLC system employing acetonitrile and water in 60:40 (v/v) proportion as mobile phase.

The enzymatic hydrolysis of the glucosinolates was performed with myrosinase in the presence of a large amount of dichloromethane, so the hydrolysis products could be extracted as formed (Ettlinger and Thompson, 1962; Youngs and Wetter, 1967). Adjustment to pH = 7 allowed selective enzymatic formation of isothiocyanates, indoles, and oxazolidinethiones from the glucosinolates in the samples (Benn, 1977; McGregor et al., 1983). After centrifugation, the extracted rapeseed meal was placed between the two different phases, so a neat separation of the aqueous buffer and the organic dichloromethane was achieved.

The transfer of the isolated compounds from dichloromethane into a water-miscible solvent to make it usable for HPLC was problematical. One way was to remove dichloromethane by evaporation and then dissolve the residue in an appropriate solvent. But difficulties occurred because of the volatility of isothiocy-

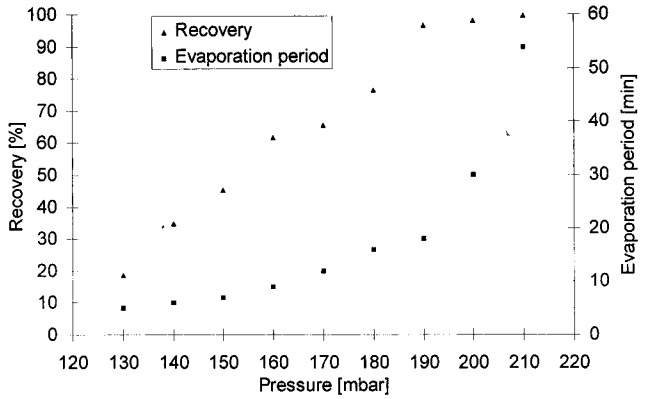

Figure 2. Recovery and the evaporating period of a solution of 0.76 mg of propyl isothiocyanate in 10 mL of dichloromethane, depending on the pressure.

anates (Hoffmann, 1978), which would be removed, too, if no appropriate evaporating conditions were kept.

Accuracy of the evaporation method was assessed by evaporating a solution of a definite amount of propyl isothiocyanate in dichloromethane to dryness at 0 °C and different pressures. Propyl isothiocyanate was selected because it belongs to the short-chain and very volatile isothiocyanates and so represents the worst thinkable case.

In Figure 2, the dependence of recovery and evaporation period on pressure is presented. With increasing pressure, the recovery of propyl isothiocyanate increases nearly linearly until a plateau is obtained at 190 mbar, with a recovery of about 97%. Further increase of the pressure results in only a small improvement of recovery. Instead of this, the evaporation period increases dramatically from about 20 min at 190 mbar to nearly 60 min at 210 mbar. Standard deviations of the different recoveries were calculated as 0.9−7.6. Experiments carried out with heptyl isothiocyanate in the same manner show similar results, but the recovery was better at 190 mbar. The pressure had no influence on the recovery of goitrin and indole-3-carbinol because they were very slightly volatile. To get a good recovery of isothiocyanates with an evaporation period as small as possible, the pressure was held at 190 mbar at 0 °C in the subsequent experiments.

The absorption behaviors of the different compound classes differ strongly; consequently, simultaneous determination of isothiocyanates, indoles, and oxazolidinethiones is difficult. In Figure 3, the dependence of the detector responses of eight standard compounds on the measurement at different wavelengths is presented. For better comparison the results of the standard compounds were recalculated on equimolar masses. As shown, the optimal measuring wavelength of goitrin is 240 nm just as for the isothiocyanates, whereas the optimal measuring wavelengths of indoles and phenethyl isothiocyanate are 220 and 210 nm, respectively. The detector responses at the optimal measuring wavelength of each compound differ very much. The sensitivity of the UV detector relating to isothiocyanates such as allyl or heptyl isothiocyanate is very low because of the compound's simple structure without absorbing groups (Silverstein and Bassler, 1964). However, to oxazolidinethiones, indoles, and phenethyl isothiocyanate with strongly absorbing OH-groups or ring systems, the UV detector reacts very sensitively, so the response of these compounds at the optimal wavelength is about 10−60-fold higher than the response of the simple isothiocyanates at the optimal wavelength of 240 nm.

As a consequence, the measurements have been carried out at 240 nm in order to determine the optimal sensitivity of the UV detector for the weakly absorbing isothiocyanates (Figure 4A). With a programmable UV detector, the sensitivity for phenethyl isothiocyanate or other isothiocyanates with a phenyl ring system can be increased by measuring the different compounds at the optimal wavelength of 210 nm (Figure 4B). In this case, the sensitivity for phenethyl isothiocyanate was about twice as high as that measured at 240 nm.

The HPLC method was assessed for precision with pure solutions of goitrin, indole-3-carbinol hydrate, and propyl and heptyl isothiocyanates. Peak areas vs concentrations were linearly correlated over the concentration ranges employed for the four compounds (r = 0.9998, 0.9999, 0.9933, and 0.9960, respectively). The correlations were statistically signifigant ($P < 0.005$).

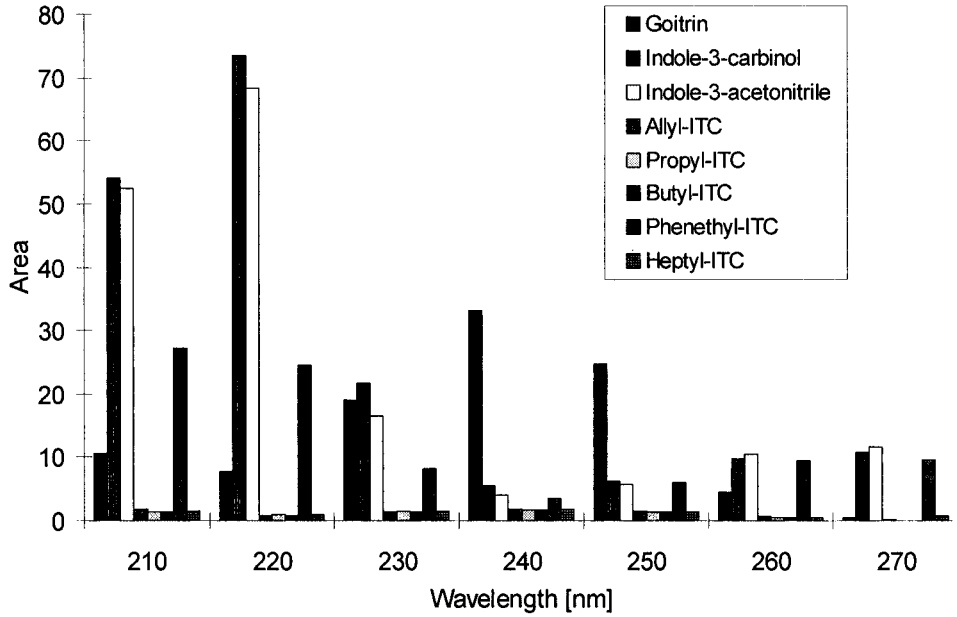

Figure 3. Dependence of different compounds on the measuring wavelength of the UV detector. The values presented were recalculated on equimolar masses.

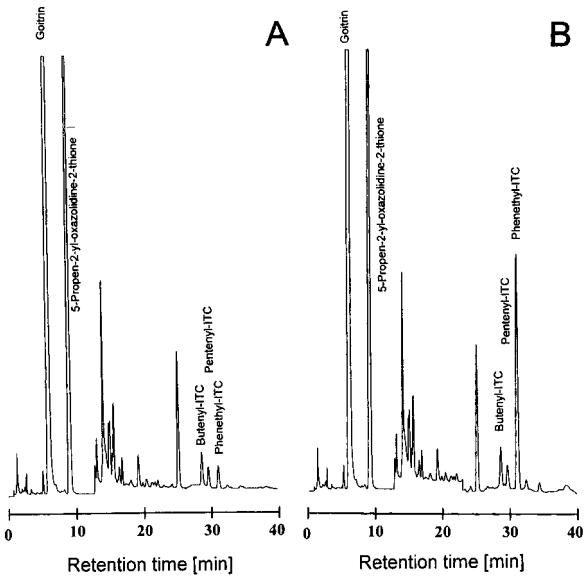

Figure 4. HPLC chromatograms of hydrolysis products of a rapeseed sample measured (A) at 240 nm and (B) with a programable wavelength detector (0−24 min, 240 nm; 24.1−40 min, 210 nm).

Table 1. Response Factors of Eight Available Standard Compounds Calculated Relative to Heptyl Isothiocyanate at 240 nm

compound	response factor[a]
goitrin	0.04 (0.0036)
indole-3-carbinol	0.31 (0.0027)
indole-3-acetonitrile	0.43 (0.015)
allyl ITC	0.94 (0.037)
propyl ITC	1.04 (0.039)
butyl ITC	1.04 (0.043)
phenethyl ITC	0.77 (0.028)
heptyl ITC	1.00

[a] Mean values of 10 determinations. Standard deviation of 10 injections is given in parentheses.

Heptyl isothiocyanate was selected as internal standard for further investigations because of its better recovery at 190 mbar and the retention time. Heptyl isothiocyanate eluted after all other compounds occurring in the chromatogram of rapeseed samples (Figure 5).

For the quantitative determination of the main hydrolysis products of glucosinolates in rapeseed or rapeseed meal, response factors for different available standard compounds were calculated relative to the internal standard heptyl isothiocyanate from 10 injections (Table 1). The wavelength of the UV detector was set at 240 nm. There was no significant difference recognizable between the 10 calculated response factors of each compound ($P < 0.005$).

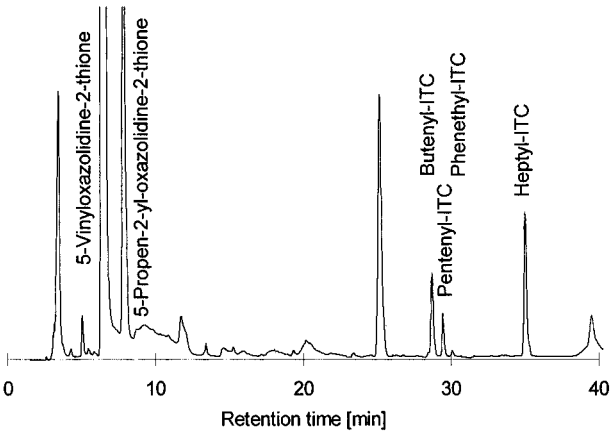

Figure 5. HPLC chromatogram of the hydrolysis products of a rapeseed sample with heptyl isothiocyanate as internal standard, measured at 240 nm.

The response factors of the homologue compounds propyl, butyl, and heptyl isothiocyanates were identical, whereas the response factor of allyl isothiocyanate was a little lower because of its double bond in the structure. In the subsequent work, the amount of butenyl and pentenyl isothiocyanates in rapeseed samples was calculated assuming that their response factors were the same as for the homologue allyl isothiocyanate, because it was impossible to obtain these compounds by synthesis or isolation in sufficient purity. The same assumption was made for goitrin and 5-propen-2-yl-oxazolidine-2-thione.

Butenyl and pentenyl isothiocyanates were identified in the chromatogram by comparison with the retention times of the main compounds of *Cardamine impatiens* L. This result was confirmed by GC−MS analysis of the dichloromethane extract of *Cardamine impatiens* L. Other unknown peaks in the HPLC chromatogram were fractionated by preparative HPLC. 5-Propen-2-yloxazolidine-2-thione was identified and unequivocally assigned by GC−MS analysis. Goitrin and phenethyl isothiocyanate were identified in the chromatogram by comparison with the retention times of standard compounds. Indoles of the samples were not clearly identified. Neither UV spectrum of the different peaks occurring in the range of 10−17 min in the chromatograms nor GC−MS analysis of fractionated peaks within this range results in an unequivocal evidence for indoles.

The HPLC method for the enzymatically released products of rapeseed samples was compared with the HPLC method for desulfoglucosinolates, which was adopted by the European Committee for Standardization (CEN) in 1995 (International Organization for Standardization, 1995). In Table 2, the mean values of the results of both methods are reported.

Table 2. Contents of Glucosinolates Calculated from the Results of the HPLC Method for (A) Desulfoglucosinolates and (B) Hydrolysis Products[a]

sample no.	progoitrin (μmol/g)	gluconapoleiferin (μmol/g)	gluconapin (μmol/g)	glucobrassicanapin (μmol/g)	gluconasturtiin (μmol/g)	sum (μmol/g)	part of total content (%)
A							
1	42.65 (1.23)	1.56 (0.03)	16.03 (0.12)	3.42 (0.15)	1.85 (0.13)	65.51	95.96
2	5.85 (0.21)	0.30 (0.07)	2.35 (0.06)	0.29 (0.09)	0.18 (0.04)	8.97	73.28
3	15.03 (0.34)	0.57 (0.02)	5.81 (0.11)	1.00 (0.07)	0.41 (0.03)	22.82	86.21
4	36.65 (0.67)	1.40 (0.06)	12.60 (0.15)	3.67 (0.21)	1.04 (0.06)	55.36	86.34
B							
1	41.97 (2.84)	1.15 (0.15)	15.12 (0.42)	2.97 (0.47)	1.44 (0.21)	61.50	90.08
2	5.80 (0.84)	0.24 (0.23)	2.01 (0.21)	0.26 (0.17)	0.11 (0.11)	8.18	66.83
3	13.57 (0.56)	0.44 (0.07)	5.11 (0.33)	0.81 (0.12)	0.25 (0.09)	19.74	74.57
4	35.71 (1.19)	1.12 (0.18)	11.04 (0.32)	3.21 (0.32)	0.95 (0.30)	50.91	79.40

[a] Mean values of five determinations. Standard deviation of five determinations is given in parentheses.

Only the results for the main glucosinolates were listed. 4-Hydroxyglucobrassicin as another main glucosinolate was not included in the table, because the unequivocal identification of the indoles was not successful. Besides the results, the standard deviations are given. The contents of the different glucosinolates calculated from the results of the determination of the hydrolysis products correlated extremely well with those calculated from the results of the determination of desulfoglucosinolates. The correlation between both methods (r = 0.9995) was statistically significant. There was no significant difference recognizable between the mean values of the resulting glucosinolate contents of both methods ($P < 0.005$). The standard deviations of the results of the HPLC method for hydrolysis products were higher than the standard deviations of the results of the other method. In addition, all results of the HPLC method for hydrolysis products were smaller than the corresponding results for the desulfoglucosinolate method. Probably, losses occurred during the release and extraction of the hydrolysis products from the samples. Nevertheless, the HPLC method for hydrolysis products is very suitable for the determination of glucosinolates, being a screening method for the main glucosinolates.

It is also possible to determine the main hydrolysis products of rapeseed meal, although the content of isothiocyanates is lower than that in rapeseeds, because of losses during the expeller pressing and extraction combined with toasting (Velisek et al., 1991).

ACKNOWLEDGMENT

We thank Dr. K. Vosmann of the institute for providing the results of the identification of hydrolysis products by GC−MS.

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Received for review May 1, 1996. Accepted September 19, 1996.[⊗]

JF9603184

[⊗] Abstract published in *Advance ACS Abstracts,* November 15, 1996.