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Determination of Glucosinolates in Canola Meal and Protein Products by Desulfation and Capillary Gas-Liquid Chromatography

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A rapid procedure for the quantitation of intact glucosinolates, including the indole glucosinolates, in rapeseed and canola meal is described. Me_3Si derivatives of the desulfoglucosinolates were separated on a glass capillary column by GLC within 10 min. Low-glucosinolate selections of *Brassica campestris* and *Brassica napus* were shown to contain relatively high concentrations of (1-methoxy-3-indolyl)methyl glucosinolate. Procedures designed to extract the glucosinolates from canola flours were not efficient, and plant breeding to reduce the levels of glucosinolates will continue to be necessary for seed improvement.

Glucosinolates are common constituents in species of Cruciferae, which are utilized for food and feed purposes. These sulfur-containing organic compounds contribute the characteristic pungent odor and sharp taste to many of the Cruciferae crops. However, the consumption of these compounds has been associated with goitrogenicity, toxicity, and antinutritional effects in experimental and farm animals. Recent reviews have dealt comprehensively with the nature of glucosinolates in food plants (Fenwick et al., 1983), the biological role of glucosinolates in plants (Kjaer, 1981), the effects of toxic products from glucosinolates on animals (Tookey et al., 1980), and analyses of glucosinolates (Olsen and Sørensen, 1981).

Glucosinolates occur as salts throughout the various plant parts of Cruciferae including the seeds but are readily hydrolyzed, under moist conditions, by an accompanying native enzyme, thioglucoside glucohydrolase (myrosinase). Usually the products of enzymatic decomposition are β-D-glucose and an organic aglucon moiety. Depending on the kind of glucosinolate and environmental conditions, the aglucon may undergo an intramolecular rearrangement and/or fragmentation to yield one or more products: isothiocyanates (Ettlinger and Lundeen, 1957), thiocyanates (Gmelin and Virtanen, 1960), nitriles (VanEtten et al., 1966), cyanides (Saarivirta, 1973), or oxazolidinethiones (Astwood et al., 1949; Daxenbichler and VanEtten, 1977).

Because of the instability of intact glucosinolates, enzyme-catalyzed hydrolysis under controlled conditions has been used in the determination of glucosinolate content. While total aglucon contents can be measured spectrophotometrically (Wetter and Youngs, 1976), most investigators have quantitated the individual degradation products by GLC (Youngs and Wetter, 1967; Daxenbichler and VanEtten, 1977) and, recently, HPLC (Mullin, 1978; Maheshwari et al., 1979). In these procedures, some glucosinolates escape detection (Olsen and Sørensen, 1980, 1981), and especially in the low glucosinolate cultivars, the proportion of undetected glucosinolates may be large.

Therefore, efforts have been made to elaborate procedures for analyses of intact glucosinolates. The GLC method of Underhill and Kirkland (1971) has been modified and greatly improved (Thies, 1980; Heaney and Fenwick, 1980; Olsen and Sørensen, 1980; Daun and McGregor, 1981). In the Heaney and Fenwick (1980) procedure, glucosinolates were extracted from the sample, purified, and desulfated with sulfatase on a DEAE-Sephadex A-25 column. The resultant desulfoglucosinolates were derivatized, separated, and quantified by GLC. Using a packed column, pertrimethylsilylated (Me_3Si) derivatives of 12 desulfoglucosinolates, including indole glucosinolates of low volatility, were satisfactorily separated in just over 30 min on a gas chromatograph.

The separation of Me_3Si derivatives of glucosinolates on glass capillary columns has been shown to reduce the elution time of the major glucosinolates quite markedly as compared to that on the packed column (Hiltunen et al., 1980). However, uneven base lines, numerous interfering peaks of sugars and other contaminants, and the absence of the indole glucosinolates had discouraged the adoption of this rapid technique.

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The objectives of the present study were to investigate the separation of desulfoglucosinolates by capillary GLC with emphasis on the quantitative recovery of indole (thiocyanate-releasing) glucosinolates. The analyses were applied to samples of low glucosinolate rapeseed (canola) cultivars of both *Brassica campestris* and *Brassica napus* from which the lipids had been removed with hexane. A further objective of the present study was to determine the efficiency of glucosinolate removal by the various published procedures for aqueous and alcoholic extraction of seed, meats, and flour. The resulting protein flours (concentrates) and isolates had been processed to be comparatively free of butenyl and pentenyl isothiocyanates and, especially, oxazolidinethiones (Sosulski et al., 1972; El Nockrashy et al., 1977; Jones, 1977), but the compositions of indole glucosinolates were not considered in these earlier investigations.

MATERIALS AND METHODS

Materials. Rapeseed of the canola type, having low contents of erucic acid in the triglycerides and glucosinolates in the seeds, was used in the present investigation. Initial experiments were conducted on two low glucosinolate selections of spring-sown *B. campestris* and *B. napus* from the breeding program at the Agriculture Canada Research Station at Saskatoon, Canada. The seeds of the *B. campestris* strain were partially yellow-seeded as was the Candle cultivar of *B. campestris*, which was used for the glucosinolate-extraction experiment. The seeds of the experimental strains were flaked and the lipids removed with hexane. Seeds of Candle were dehulled and defatted before or during processing into protein flours.

Glucosinolates were extracted from Candle by four procedures, all but one of which were two-stage processes of 30 min each at seed, meats, or flour to solvent ratios of 1:5 (w/v). Diffusion extraction of seed was conducted with dilute alkali (0.01 N NaOH) at 80 °C, followed by dehulling, oven-drying, and oil extraction with hexane (Sosulski et al., 1972; Sosulski, 1978). After dehulling and a 3-min boiling water treatment to inactivate myrosinase, aqueous diffusion of intact meats was done at 30 °C and then at 80 °C, followed by oven-drying and oil extraction (Jones, 1977). Direct extraction of the defatted flour was done with 70% ethanol (1:5 w/v) in two stages at room temperature. Alternately, the defatted flour was extracted once with boiling water (1:5 w/v) for 15 min, followed by a second extraction with the same volume of hot water (80 °C) for 30 min.

To avoid darkening of the products, it was necessary to freeze-dry these extracted flours. The four extracted protein materials are referred to as "protein flours" in the present paper in order to distinguish them from the original Candle flour and true protein concentrates that should contain over 65% of protein.

The protein isolate was prepared by the two-step precipitation procedure of El Nockrashy et al. (1977), at pH 6.0 and then at pH 3.8 to improve the recovery of protein. Candle flour was extracted with 0.02 N NaOH, ratio 1:25 (w/v), for 90 min, followed by centrifugation of the slurry. The supernatant was decanted and adjusted to pH 6.0 for isoelectric precipitation of one protein fraction. After centrifugation and decantation, the remaining supernatant was adjusted to pH 3.8 to obtain a second protein precipitate. After the second precipitation, the whey was discarded and the two protein fractions were combined before freeze-drying.

The protein flours and protein isolate were analyzed for proximate constituents, and the colors were determined on the Hunterlab D25D2M digital color difference meter equipped with an M optical head. Total color difference $[\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2}]$ was calculated as a composite score for color development during processing.

Chemicals. The silylating reagents, *N*-methyl-*N*-(trimethylsilyl)heptafluorobutyramide (MSHFBA) and trimethylchlorosilane (TMCS), were obtained from Macherey-Nagel GmbH and Co., Duren, West Germany, and Pierce, Rockford, IL, respectively. Pyridine (silylation grade) was obtained from Pierce. Sulfatase (Type 1), from Sigma Chemical Co., St. Louis, MO, was purified according to Heaney and Fenwick (1980). 2-Propenyl glucosinolate (sinigrin) from Aldrich Chemical Co., Montreal, Canada, was used as the internal standard.

Sample Preparation. The meals, flour, protein flours, and isolate were prepared for capillary GLC analysis by the method of Heaney and Fenwick (1980) as modified by Daun and McGregor (1981). Exactly 100 mg of defatted sample was extracted with 1 mL of distilled water in a water bath at 95 °C for 15 min, which also inactivated the endogenous myrosinase. The mixture was cooled and 1.0 mL of internal standard (1.0 μmol/mL 2-propenyl glucosinolate) was added. After centrifugation for 10 min at 2000*g*, 1.0 mL of supernatant was mixed with 100 μL of a 1:1 (v/v) mixture of 0.5 M barium acetate and 0.5 M lead acetate. This mixture was centrifuged for 10 min at 2000*g*, and 1.0 mL of supernatant was applied to a narrow-bore DEAE-Sephadex A-25 column (prepared from an Eppendorf pipet tip, 101–1000-μL size, packed with 20 mg of Sephadex). The column was washed with 1.0 mL of 0.02 M pyridine acetate to remove carbohydrates. About 70 mg of sulfatase was washed with ethanol and taken up in 2 mL of water. The purified sulfatase solution (50 μL) was loaded on the column and allowed to stand overnight at room temperature. The desulfoglucosinolates were eluted with 1.5 mL of distilled water and evaporated to dryness under vacuum at 50 °C in a 4-mL vial with an aluminum foil lined cap. The pyridine (100 μL) and MSHFBA (100 μL) and TMCS (10 μL) were added to the vial, which was capped immediately, swirled, and heated at 120 °C for 20 min to complete the derivatization reaction.

Gas Chromatographic Analysis. The derivatized desulfoglucosinolates were separated by using a 5880A Series Hewlett-Packard gas chromatograph equipped with a flame ionization detector and 3990A Hewlett-Packard peak area integrator. A WCOT fused silica OV-1 column (25 m × 0.20 mm i.d.) was used with helium as the carrier gas at a linear velocity of 25.7 cm s^{-1}. The oven temperature was kept at 210 °C for 3 min and then increased at 10 °C min^{-1} to 285 °C. Injection and detector temperatures were 250 and 300 °C, respectively. An attenuation of 0, corresponding to mV of maximum signal voltage at full-scale deflection, was used. The split injection mode was selected with a splitting ratio of 30 and an injection volume of 1 μL. Quantitative data were calculated on the basis of response factors and peak areas of their corresponding trimethylsilyl desulfoglucosinolate derivatives. The results are expressed in μmol/g of sample, dry weight basis, as means of duplicate determinations. Results obtained from 10 consecutive injections of the derivatized desulfoglucosinolates from Candle flour were used to calculate the instrument error for capillary GLC separation of trimethylsilyl desulfoglucosinolates.

RESULTS AND DISCUSSION

Separation and Quantitation. Excellent separation of the internal standard, 2-propenyl glucosinolate, and the six sample glucosinolates was obtained in each chromatogram (Figure 1). 3-Butenyl, 4-pentenyl, 2-hydroxy-3-butenyl, and 2-hydroxy-4-pentenyl glucosinolates eluted

Table I. Parameters for the Determination of Me_3Si Derivatives of Desulfoglucosinolates in Canola Meals (μmol/g of Oil-Free, Moisture-Free Meal)

peak no.	glucosinolate side chain	glucosinolate common name	response factor	retention time, min	*B. campestris* mean, μmol/g	*B. campestris* SD,[a] μmol/g	*B. napus* mean, μmol/g	*B. napus* SD,[a] μmol/g	instrument error, SD,[b] μmol/g
1	2-propenyl	sinigrin		3.71					
2	3-butenyl	gluconapin	0.96154	4.08	1.2	0.04	2.6	0.02	0.05
3	4-pentenyl	glucobrassicanapin	0.92593	4.51	1.2	0.11	0.3	0.01	0.06
4	2-hydroxy-3-butenyl	progoitrin	0.86207	4.97	3.0	0.08	4.0	0.13	0.10
5	2-hydroxy-4-pentenyl	gluconapoleiferin	0.83333	5.31	0.4	0.01	0.1	0.01	0.04
6	unidentified			6.24					
7	unidentified			6.53					
8	unidentified			6.90					
9	3-indolylmethyl	glucobrassicin	0.73529	9.55	2.1	0.14	1.0	0.08	0.01
10	(1-methoxy-3-indolyl)methyl	neoglucobrassicin	1.45161	9.89	10.2	1.31	9.1	2.00	0.45

[a] Based on analysis of duplicate samples. [b] Based on 10 injections of a single derivatized mixture from Candle flour.

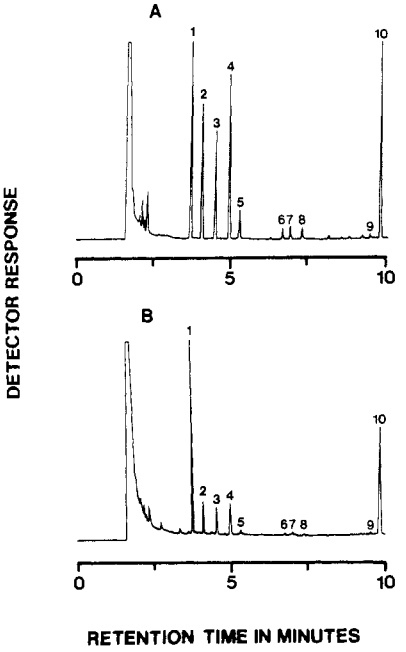

Figure 1. WCOT capillary chromatograms of Me_3Si derivatives of desulfoglucosinolates in Candle flour (A) and in the protein flour (B) after the two-stage extraction with 70% ethanol. Peak numbers are identified in Table I.

within 5.5 min, and the 3-indolylmethyl and (1-methoxy-3-indolyl)methyl glucosinolates appeared prior to the 10-min mark (Table I). The three unidentified compounds eluting at 6.24, 6.53, and 6.90 min may be methylsulfinyl glucosinolates (Olson and Sørensen, 1980). These elution times were substantially les than the chromatographic run times of 31 min reported by Heaney and Fenwick (1980) and 23 min obtained by Daun and McGregor (1981) for packed columns. In addition to the excellent resolution of each glucosinolate, there was no evidence of interfering peaks. The stable base line permitted quantitation of the very low concentrations of 3-indolylmethyl glucosinolate.

The *B. campestris* selection contained 12.3 μmol of indole glucosinolates/g of meal while the hydroxy glucosinolates constituted 3.4 μmol/g of sample (Table I). The *B. napus* selection showed a similar distribution of glucosinolates, but the 3-butenyl and 2-hydroxy-3-butenyl glucosinolate contents were characteristically higher and 4-pentenyl and 2-hydroxy-4-pentenyl glucosinolate levels were lower than those obtained for *B. campestris*. When compared on a fat-free basis, the values for 3-butenyl, 4-pentenyl, 2-hydroxy-3-butenyl, and 2-hydroxy-4-pentenyl glucosinolates in *B. campestris* and *B. napus* were comparable to those of Olsen and Sørensen (1980) except for the low value for 4-pentenyl glucosinolate in *B. napus* in this study. The 2-phenylethyl glucosinolate (gluconasturtiin) quantitated by Olsen and Sørensen (1980) was not found in the present sample; the sinalbin (*p*-hydroxybenzyl glucosinolate) reported by these authors is a constituent of wild *Brassica* species such as *Brassica kaber*. The recoveries of 3-indolylmethyl and (1-methoxy-3-indolyl)methyl glucosinolates were higher than the levels reported by Heaney and Fenwick (1980) for meals from cultivars grown in West Germany.

Table II. Effects of Extraction or Isolation Procedure on Yields of Protein Products, Proximate Composition, and Hunterlab Color Difference Values (Dry Basis)

extraction process	yield, % of seed	proximate composition, % protein[a]	lipid	ash	fiber	total color difference, ΔE
control flour	36.5	48.2	1.1	7.5	8.8	
seed diffusion	29.2	59.0	1.8	6.6	10.1	5.2
meats diffusion	27.4	60.7	1.5	7.8	10.8	2.2
flour—alcohol extracted	27.4	58.1	0.8	8.3	11.0	2.4
flour—water extracted	25.2	54.2	2.8	8.0	11.2	13.5
protein isolation	13.6	80.9	2.8	3.6	0.5	29.0

[a] $N \times 6.25$.

The standard deviations (SD) for duplicate determinations of the two *Brassica* species varied between 0.01 and 0.14 μmol/g of meal for all desulfoglucosinolates except (methoxyindolyl)methyl glucosinolate, which gave highly variable results (Table I). The variability was evaluated further by comparing the SD of 10 injections of the same mixture of derivatives from Candle flour where the glucosinolate concentration was almost double that of the two selections. The instrument error for most Me_3Si derivatives was found to be quite low, but the Me_3Si derivative of the desulfated (methoxyindolyl)methyl glucosinolate appeared to be relatively unstable.

Detoxification Procedures. Because of an excellent balance of essential amino acids, rapeseed and canola flours have been investigated as potential protein supplements in foods (Olson et al., 1978). Since even low levels of glucosinolates, especially the hydroxy glucosinolates or oxazolidinethiones, would be considered unsafe for human consumption, procedures have been developed for glucosinolate extraction during protein concentration or isolation. However, their effectiveness in the extraction of intact glucosinolates including, in particular, the indole glucosinolates has not been determined.

The yield of flour after removal of hulls and oil from Candle seed was not high, being 36.5% of the seed weight (Table II). Diffusion extraction of the seed before dehulling and defatting resulted in a further 20% loss of flour

Table III. Glucosinolates in Candle Flour (*B. campestris*) and Residual Glucosinolates in Protein Flours and Isolates Prepared by Extraction with Water, Alkali, or Alcohol (μmol/g of Product, Moisture-Free Basis)

peak no.	glucosinolate side chain	candle flour	seed diffusion, alkali	meat diffusion, water	flour extraction, alcohol	flour extraction, water	protein isolate, alkali
2	3-butenyl	6.9	0.9	1.4	0.7	ND	ND
3	4-pentenyl	5.2	1.0	1.2	0.7	ND	ND
4	2-hydroxy-3-butenyl	7.6	0.8	1.2	0.3	0.1	0.1
5	2-hydroxy-4-pentenyl	1.3	0.8	0.2	0.1	0.5	1.0
9	3-indolylmethyl	0.2	ND[a]	ND	ND	ND	ND
10	(1-methoxy-3-indolyl)methyl	16.7	4.4	6.8	7.5	0.1	0.3
	total	37.9	7.9	10.8	9.3	0.7	1.4
	% of flour glucosinolates	100	21	28	25	2	4

[a] ND = not detected.

dry matter with a corresponding increase in protein content. Aqueous extraction of the dehulled meats before oil extraction resulted in a 25% loss of flour dry matter and a corresponding enrichment of protein in the final product. Extractions of the Candle flour with alcohol and water resulted in 25 and 30%, respectively, losses of flour solids, including a significant proportion of the protein, especially during aqueous extraction. The yield of protein isolate was only 37% of the flour weight and nearly 20% of the isolate was non-protein material. The low yield of protein flours or isolates has been one of the principal factors limiting the commercial development of food-grade rapeseed protein products.

Except for the ethanol-extracted product, each protein flour showed higher levels of lipid than the control flour (Table II). Also, ash contents remained high in all protein flours, and was not reduced to the levels expected in the protein isolate. The Candle flour contained 8.8% of crude fiber and the protein flours were enriched to 10–11% fiber, which would almost certainly reduce the digestibility fo the products, as was found by Olson et al. (1978) for the diffusion-extracted product. Thus, the degree of protein concentration was substantially less than can be effected in soybean by similar techniques, and the contents of indigestible components remained high after processing.

Protein flours prepared by aqueous diffusion of seed or meats and alcohol extraction of the flour were similar in appearance to the control flour (Table II). However, the direct aqueous extraction of the flour gave a grey product after freeze-drying. The protein isolate was dark brown.

About 70–80% of the total glucosinolates in canola flour was extracted by seed or meats diffusion or alcohol extraction of the flour (Table III). The contents of hydroxy glucosinolates were most effectively reduced by the alcohol extraction, but the residual indole glucosinolate remained high in the resulting protein flour (Figure 1). Water extraction of the flour was most effective in the removal of glucosinolates; only 2% of the original glucosinolates was present in the protein flour. Unfortunately, this process resulted in heat denaturation of the protein, high losses of dry matter and protein, and darkening of the final product during drying.

The protein isolation process was also effective in the removal of indole glucosinolates, but the residual hydroxypentenyl glucosinolate content was high (Table III). However, the major problems with protein isolation of rapeseed or canola flours or meals would be the high cost of the process, associated with low yield and dark appearance of the product.

On the basis of the analysis of total glucosinolates (Table III), the extraction procedures proved to be inefficient in glucosinolate removal and/or protein concentration. It appears that plant breeding will continue to be the primary method for overcoming the glucosinolate problem in canola cultivars. The present capillary GLC procedure for analysis of desulfoglucosinolates offers a rapid and precise method for the screening of large plant populations. In the future, emphasis will need to be given to breeding for low contents of indole glucosinolates.

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Registry No. Neoglucobrassicin, 5187-84-8; gluconapin, 19041-09-9; glucobrassicanapin, 19041-10-2; progoitrin, 585-95-5; gluconapoleiferin, 19764-03-5; glucobrassicin, 4356-52-9.

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