Modification of a Mini-column Method for Rapid Routine Determination of Total Glucosinolate Content of Rapeseed by Glucose Release

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The practicability of a glucose-release procedure for glucosinolate determination in rapeseed has been improved with consequent economy in operator time. Data are presented to support the modifications made. Currently available alternative procedures are noted.

KEY WORDS: Colza, cruciferous seed, glucosinolates, myrosinase, rapeseed, thioglucosidase.

The development of rapeseed (*Brassica napus* L. and *Brassica campestris* L.) as a crop in Europe has followed the same pattern as that in Canada. Low erucic acid varieties (single lows) developed to yield an oil utilizable in human foods are being superseded by varieties also low in toxic glucosinolates (double lows) to promote the use of rapeseed meal as a protein source in animal feedstuffs. In the U.S., the recent gaining of GRAS (Generally reviewed as safe) status for low erucic acid rapeseed (LEAR) oil has already led to considerable interest in rapeseed cultivation by oilseed producers.

Crucial to the development and monitoring of low glucosinolate varieties is the ability to determine glucosinolates (Fig. 1) in the seed and this may be done by any one of several methods with varying levels of practicability, accuracy and cost. As developmental activity has occurred since the most recently published review (1), it is appropriate to provide a summary.

Originally, attention was directed towards determination of the potentially toxic breakdown products of rapeseed glucosinolates generated by heat, moisture and endogenous myrosinase enzyme activity during the oil extraction process (1) and methods were developed to determine isothiocyanates (1,2), 5-vinylthiooxazolidone (goitrin) (2,3), nitriles, (4), thiocyanate (1) and bisulfate (5). Analysis for these compounds is most relevant in processed meals but use of added myrosinase also allows indirect estimation of intact glucosinolates.

Sulfatase enzyme (aryl-sulfatase sulfohydrolase, E.C. 3.1.6.1) may be used on intact glucosinolates to produce desulfoglucosinolates (6) which are determined by reverse-phase high performance liquid chromatography (HPLC) (7-10) or, after silylation, by gas liquid chromatography (GLC) using isothermal (11,12) or temperature-programmed (7,13) conditions. These methods allow the quantification of individual glucosinolates as does ion-pair HPLC of intact glucosinolates (14,15). HPLC is the method of choice for determination of individual glucosinolates (10), although problems such as poor 4-hydroxyglucobrassicin recovery may require further examination. Sulfate ion, generated stoichiometrically by enzymic desulfation, may be determined directly by ion chromatography (16), or $BaCl_2$ gravimetry (17), or indirectly by assay of excess dissolved Ba using X-ray fluoresence (XRF) (18).

Secondary instrumental methods have been sought for their ease and rapidity, but have high capital costs. Total glucosinolate content in rapeseed may be determined from the sulphur content of dried, ground and compressed seed by XRF (19) or by near-infrared reflectance spectrometry (NIR) on dried and ground seed (20,21).

Extraction and clean-up are the rate limiting factors for glucosinolate determination by any "wet" chemical procedure, the latter required because of the multiplicity of potential interfering compounds in the crude plant extract. However, wet methods generally have lower costs. Two such methods are complexiметric reaction with tetrachloropalladate II (22,23) and reaction with thymol-sulphuric acid (24,25). These methods are relatively quick, but use less-than-optimal reagents by reason of instability ($PdCl_42$-), are difficult to handle (thymol-H_2SO_4) or are toxic (Pd, thymol). Likewise, results obtained by the $PdCl_42$- procedure are relative only to the standards used, and the complex molar absorbance varies considerably for individual glucosinolates. The thymol-H_2SO_4 reaction is reportedly stoichiometric (22).

One wet chemical procedure which gives absolute results is that described as "glucose release". Myrosinase enzyme catalyzes the stoichiometric release of glucose from glucosinolate, and the glucose released may be determined using one of several specific enzymatic-colorimetric procedures. An elegant but time-consuming procedure described by Heaney *et al.* (7,26,27) involves clean-up of rapeseed extract on an ion exchange minicolumn, myrosinase being added to the purified glucosinolates on the column, and, after reaction, the glucose released is eluted and assayed. This method was studied with the aim of increasing practicability and reducing operator time for use in the commercial laboratory situation. Other attempts to produce a more rapid glucose release test involving elimination of a clean-up step and use of endogenous myrosinase (28; twice modified since publication) have proved unreliable in practice.

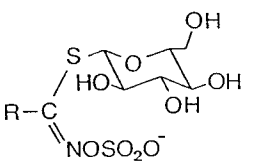

FIG. 1. General structure of glucosinolates (R = functional group: alkenyl, aromatic, indolyl).

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EXPERIMENTAL PROCEDURES

The following paragraphs describe the procedure as developed.

Sample preparation. Reduction of a laboratory sample, typically 100-1500 g, to an analysis sample of 20-100 g was by the ISO method (29). When done, removal of impurities also was according to the ISO method (30). The analysis sample was ground in a mill, e.g., a domestic coffee grinder, to a meal of which 80% passed a 1 mm sieve. If the moisture content in the seed much exceeded 10%, fine grinding was not possible and pre-drying was necessary. Moisture content was determined by the ISO method (31) on milled seed (32). The full-fat meal was taken directly for testing. Glucosinolate levels in ground seed of low moisture content were stable for several days if the meal was properly stored.

Extraction of glucosinolates. Extraction was done using heat provided by a sand bath (not exceeding 150°C at the core) or heating block. Full-fat meal was weighed into a 25 mL volumetric flask (0.500 g) or a 10 mL graduated tube (0.200 g). The container and meal were heated for 1-2 min, taking care the meal did not char, until moisture heated from the meal could be seen to condense on the upper parts of the container. Almost boiling 50% vol/vol aqueous methanol (2-5 mL) was added. As the liquid began to boil again, further volumes of hot extractant were added, with washing down of the inside of the container. Gentle boiling (approximately 80°C) was continued for 20 min. The container and contents were cooled and made to volume with extractant. The container was stoppered, shaken and the contents transferred to a stoppered, polypropylene centrifuge tube and centrifuged for 10-15 min at 3000 × g. The supernatant was decanted off and could be stored without change at -18°C for several weeks if analysis was not continued immediately.

Minicolumn-enzyme procedure. Disposable Bond Elut™ 1 mL DEA columns were equilibrated by passing the following solutions successively through them: 1 mL 0.5 M pyridine-acetate (PA) buffer, 1 mL distilled water, 1 mL 0.02 M PA buffer, 1 mL water. (PA buffer, 0.5 M, was prepared by mixing 930 mL distilled water with 30 mL glacial acetic acid and 40 mL pyridine.) Positive or negative pressure was necessary to generate flow with the Bond Elut system. Vacuum, applied to a Vac Elut™ manifold, was used where the eluate was not collected, but positive pressure (50 mL disposable Luer-tip syringe and Bond Elut adaptor) was easiest where the eluate was collected. The seed extract (1000 μL) was applied to the column and drawn through. The column was washed with 2 × 0.5 mL water, followed by 2 × 0.5 mL 0.02 M PA buffer. Effluents were discarded. Myrosinase (thioglucosidase; E.C. 3:2:3:1) solution (250 μL; 10 mg mL^{-1} in 0.02 M PA buffer; biocatalysts lyophilized enzyme preparation 200S; 0.5 unit) was applied to the column. Sufficient pressure was applied to force the enzyme into, but not through, the column. The eluate was collected. The column, with collecting tube, was kept at 35°C for 15 min in an incubator and was then drained by positive pressure. After two washes with distilled water (2 × 0.5 mL), the total eluate (1.25 mL) was mixed carefully on a vortex mixer and used for glucose analysis. Sorbent columns were discarded after a single use.

Glucose assay. A glucose assay kit (Sigma No. 115-A) was used. Eluate, typically 500 μL, was made to a total sample volume of 1000 μL with water. This was reacted with the test reagent, 1 mL, and the reaction stopped with 5 or 10 mL 0.1M HCl. A calibration series was run concurrently on every occasion using dilutions of a standard glucose solution (1 mg mL^{-1}). Colorimetric readings were made at 530 nm. All other conditions for glucose assay were as defined in the manufacturer's instructions.

Quality assurance. Regular checks were made on procedures and operators by testing samples with known glucosinolate content. Extracted rapeseed meals, with inactivated endogenous myrosinase, were used to check the extraction procedure and full-fat rapeseed was used to evaluate the inactivation of endogenous enzyme by the brief pre-extraction heating stage.

Development of this procedure was done by an examination of each stage of the methodology by suitable experimentation, the results of which are described below. Other methods used in the study were to determine oil (32-34), free fatty acids (FFA) in oil (35-37) and fatty acid composition (38,39). Defatted meals were prepared by continuous extraction of ground seed with light petroleum, b.p. 40-60°C—the defatted powder passed a 250 μm sieve. Glucotropaeolin was used as internal standard for the HPLC determination of glucosinolates as desulfoglucosinolates (7). Rapeseed and mustard seed were commercial samples from various origin countries, but were mainly from the U.K. Standard glucosinolates used were sinigrin (Sigma S7508) and glucotropaeolin (AFRC Norwich Food Research Institute, U.K.).

RESULTS AND DISCUSSION

Sample preparation. The condition of a rapeseed sample dictates the preparation necessary. Impurities may be present which influence the overall glucosinolate content. Rape plant parts other than seed, e.g., pod, stalk, and most contaminating seeds, tend to have a lower glucosinolate content than rapeseed, as does the fine dust found in commercial samples. For example, in a double low sample with 19.4 μmoles glucosinolates g^{-1} seed, the nonseed rape plant material contained 3.6 μmoles g^{-1}, and the fine dust (0.5 mm sieve pass) 10.4 μmoles g^{-1} glucosinolates. Equally, some seeds present in admixture (e.g., mustard) may increase overall glucosinolate content.

Pre-drying may be required for wet seed since endogenous myrosinase may decompose glucosinolates in the interval between grinding and testing, although this was not found to occur when ground seed was stored at -18°C for short periods. Self-heating due to microbial action or moulding of damp seed also leads to glucosinolate breakdown. However, most commercial seed samples are dried on farm or in store to 5-10% moisture, at which level the glucosinolate content of milled seed was found to be stable for up to 10 days if properly stored (Table 1).

Extraction of glucosinolates. Many methods require the defatting of ground rapeseed prior to glucosinolate extraction. Tests done using ground full-fat or defatted material gave equivalent results (Fig. 2), confirming that a defatted step is unnecessary. Glucosinolates are water-

TABLE 1

Decline with Time of Total Glucosinolate Content in Ground Full-Fat Rapeseed[a]

Days after grinding:	Total glucosinolates, μmol g^{-1} seed[b]				
	0	3	10	17	24
	7.5 ± 0.0	8.6 ± 0.4	8.2 ± 0.0	6.6 ± 0.4	6.8 ± 0.0
	21.7 ± 2.8	22.8 ± 1.8	23.7 ± 1.4	19.3 ± 0.9	19.6 ± 1.2
	64.5 ± 1.9	64.0 ± 0.0	65.3 ± 2.1	59.8 ± 1.3	52.6 ± 2.4
	105.2 ± 2.5	108.7 ± 0.0	110.3 ± 3.4	107.0 ± 2.3	98.5 ± 2.4

[a]Moisture contents in range 6.4-8.2%. Meals kept in sealed plastic jars at ambient temperature out of direct sunlight. During 24 days of test, FFA in oil increased from 0.3-0.5% to 0.8-2.9%.

[b]Results are mean ± standard deviation for duplicate analyses.

TABLE 2

Comparative Extent of Extraction of Glucosinolates with Different Strengths of Aqueous Methanol

Methanol/water (%vol/vol):	Total glucosinolates, μmol g^{-1} seed[a]			
	0	25	50	75
	7.5 ± 0.4	8.8 ± 0.7	9.4 ± 0.7	10.2 ± 0.4
	20.4 ± 0.6	22.8 ± 1.1	24.2 ± 2.1	24.0 ± 1.7
	59.3 ± 0.7	64.3 ± 2.1	65.7 ± 2.2	62.7 ± 0.8
	101.7 ± 4.2	102.8 ± 2.5	97.2 ± 3.3	94.5 ± 1.1

[a]Results are mean ± standard deviation for quadruplicate analyses.

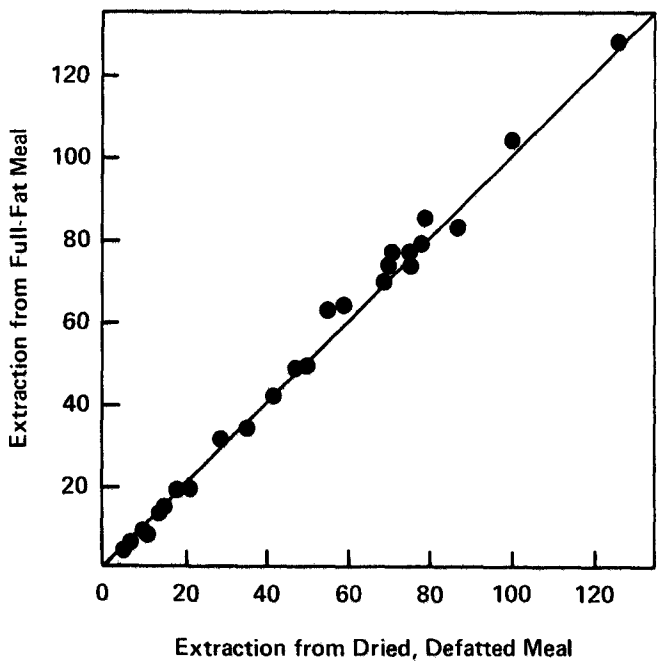

FIG. 2. Comparative results for glucosinolate analysis done on ground full-fat or defatted rapeseed. Line represents equivalence only.

soluble and the use of aqueous methanol extraction solutions, 40-70% vol/vol, is well established (40). A range of aqueous methanol concentrations was tested and these were found to be more effective than water alone (Table 2)—aqueous methanol 50% vol/vol was adopted for routine use. The greater effectiveness of methanol solutions may be attributed in part to lower boiling temperatures allowing gentler reflux and lessening the likelihood of indole glucosinolate breakdown.

Heaney *et al.* (7,26) described the use of two consecutive water extractions or three consecutive 70% vol/vol aqueous methanol extractions (27). Using 50% vol/vol aqueous methanol, the proportion of total glucosinolates extracted in the first extraction was high (89.4-96.7%) and relatively constant (Table 3). If a single extraction was done and transfers avoided using a volumetric container as the extraction vessel, a factor of 1.075 was included in the calculation with negligible loss of accuracy.

Mini-column enzyme procedure. Preparation of DEAE-Sephadex™ mini-columns is described in several methods (7,26) but the use of commercially available, disposable bonded silica mini-columns, (e.g., Bond Elut™) is much more convenient, although more costly. Equilibration to the acetate form was done with PA buffer (41), which has the advantage of being wholly

TABLE 3

Yield of Glucosinolates by Single and Double Extraction with 50% vol/vol Aqueous Methanol

Total glucosinolates, μmol g^{-1} seed			
First extraction	Second extraction	Total	Factor for multiplication of first extraction result to give total result
11.8	0.4	12.2	1.034
14.3	0.8	15.1	1.056
14.8	0.8	15.6	1.054
15.6	1.3	16.9	1.083
16.5	1.7	18.2	1.103
18.2	2.1	20.3	1.115
22.8	2.1	24.9	1.092
23.7	2.1	25.8	1.089
25.4	3.0	28.4	1.118
37.6	1.3	38.9	1.035
36.6	2.5	39.1	1.068
44.4	3.0	47.4	1.068
			Mean 1.076 ± 0.03

TABLE 4

Comparative Results with Different Bond Elut™ Sorbent Columns[a]

Total glucosinolates, μmol g^{-1} seed[b]			
NH_2	DEA	SAX	Mean
9.0 ± 0.4	9.6 ± 0.4	9.3 ± 0.0	9.3 ± 0.4
36.3 ± 0.4	36.8 ± 0.4	37.4 ± 0.4	36.8 ± 0.6
47.6 ± 1.6	48.7 ± 3.3	49.9 ± 1.6	48.7 ± 2.1
83.5 ± 3.3	89.3 ± 4.9	90.5 ± 0.0	87.8 ± 4.2

[a]Bond Elut 1 mL, 100 mg sorbent columns with functional groups: NH_2, aminopropyl; DEA, diethylaminopropyl; and SAX, trimethylaminopropyl.

[b]Results are mean ± standard deviation for duplicate analyses; mean is mean of the six individual analyses.

volatile if an eluate is subsequently required to be dried, as for derivatization. In addition to the DEA variant, other Bond Elut anion exchange columns gave satisfactory results (Table 4). Indeed, the quaternary SAX variant would have very high bonding affinity, possibly irreversible, for the sulfonate moiety of the glucosinolate molecule.

Recovery of sinigrin 98.0 ± 4.1%, eight tests) and glucotropaeolin (105.2 ± 3.2%, eight tests) standards from DEA Bond Elut columns was acceptable, and the capacity of a 1 mL 100 mg DEA column was at least 2.4 μmoles glucosinolate equivalent to 1 mL extract of seed 120 μmoles g^{-1} total glucosinolate content. Other checks were made on the mini-column procedure described here: loss through application to a DEA column was negligible (<1 μmoles g^{-1} seed equivalent); myrosinase-generated glucose was fully eluted in 1.25 mL total eluate; re-treatment of an eluted column with myrosinase and re-elution gave no additional glucose. Re-use of columns was not possible as no satisfactory regenerative procedure was found.

The incubation period required for on-column myrosinase treatment at ambient temperature was noted as 2 hr by Heaney *et al.* (7,26,27), but elevation of the incubation temperature to 35°C showed that the time period could be correspondingly reduced to 15 min (Fig. 3). Commercially available myrosinase is inexpensive but should be checked for glucose contamination before use.

Evaluation of overall method and results. Repeatability of the method presented was good (Table 5) and results obtained were comparable with those by the method of Heaney *et al.* (Table 6). For commercial usage, the typical coefficient of variation at 5-10% implies a tolerable accuracy of ± 1-2 unit at 20 μmoles total

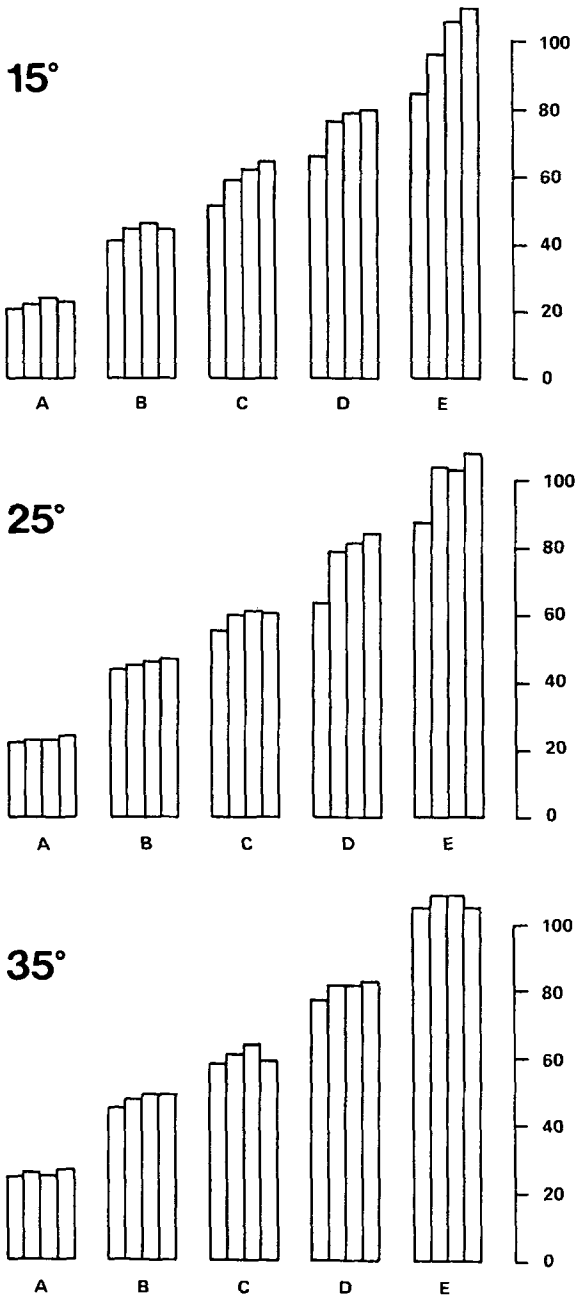

FIG. 3. Time course of glucose release reaction on sorbent column at three incubation temperatures (15, 25, and 35°C). Four bars (left to right) for each of five samples (A-E) represent results, μmoles g^{-1} seed, found at 15, 30, 60 and 120 min. Myrosinase, ≃ 0.5 unit, was added to 1 mL extract on column.

TABLE 5

Repeatability of the Modified Glucose Release Method

Total glucosinolates, μmol g^{-1} seed[a]	
Range	Mean ± standard deviation
8.2 - 10.6	9.5 ± 0.8
21.2 - 27.2	23.7 ± 1.7
62.7 - 69.2	64.3 ± 2.0
94.1 - 105.9	100.6 ± 3.9

[a]Results are for 12 replicate analyses of each sample.

TABLE 6

Comparison of Determinations Done by the Method of Heaney *et al.* (1986) and this Modification

Total glucosinolates, μmol g^{-1} seed[a]	
Modification	Heaney *et al.* (1986)[b]
7.8 ± 0.4	9.3 ± 0.3
12.2 ± 1.1	11.4 ± 0.8
18.3 ± 0.7	16.7 ± 0.4
22.0 ± 0.6	24.4 ± 1.3
38.7 ± 0.4	36.3 ± 1.1
43.1 ± 2.1	43.9 ± 1.8
49.2 ± 1.5	48.6 ± 3.0

[a]Results are mean ± standard deviation for duplicate analyses.
[b]References 7 and 26.

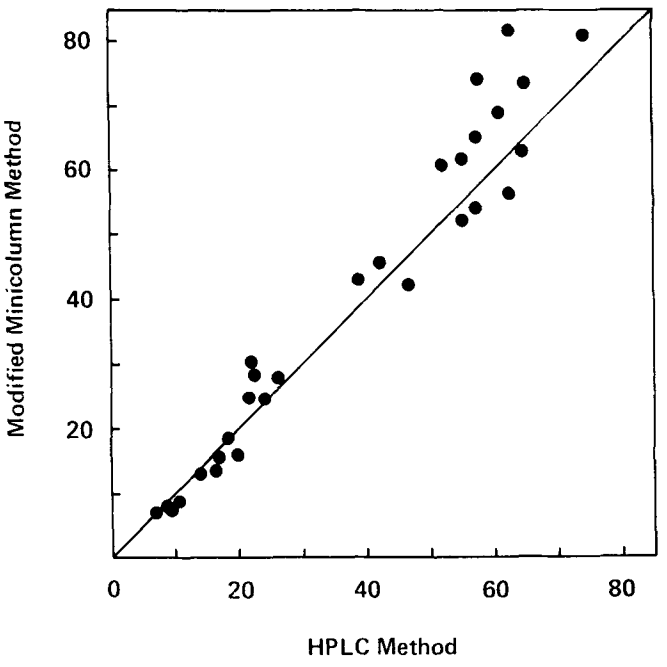

FIG. 4. Comparison of glucosinolate content found by this glucose release procedure and an HPLC method. Line represents equivalence only.

glucosinolate g^{-1} seed, certainly within the sampling error for bulk rapeseed parcels.

The modifications made here to the Heaney *et al.* procedures (7,26,27) have reduced the lead-in time to first result from 3.5 hr to 1.25 hr, the time requirement per test to about 10 min, and one experienced operator can comfortably cope with 30-40 samples per working day. The method is also suitable for use on an occasion basis, provided proper controls are done. The equipment required is not specialized and the method provides a cheap, useful alternative to the expensive XRF.

Comparison with an HPLC procedure (7) showed closer agreement at lower glucosinolate levels (Fig. 4) — better inter-method agreement is usually found at lower levels. For example, a recent BCR (European Community Bureau of Reference) exercise set a standard reference rapeseed material at 24.5 ± 0.9 μmoles g^{-1} seed (26.6 ± 2.3, 5 replicates, by the procedure described here) (42), whereas results were much more widely spread at higher glucosinolate levels. This suggests that instrument calibrations should possibly by weighted towards reliable calibrants at lower, and currently more relevant, glucosinolate levels.

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