# Determination of the Total Glucosinolate Content in Canola by Reaction with Thymol and Sulfuric Acid[1]

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**Intact glucosinolates in seeds and meals of rapeseed and canola were isolated and purified on small DEAE ion-exchange columns. After being eluted with potassium sulfate the glucosinolates were hydrolyzed with sulfuric acid to produce thioglucose which was determined as a complex with thymol. The method was compared to a gas liquid chromatography (GLC) procedure which determines aliphatic glucosinolates. The extra amount of glucosinolates found (ca. 14 μmol/g oil-free) was equal to the sum of those not determined by GLC. The thymol method had a standard error of ±3 μmol/g compared with a standard error of ± 1 μmol/g for the GLC procedure for the same set of 18 samples ranging from 10 μmol/g to 100 μmol/g (oil-free, 8.5% moisture basis) of aliphatic glucosinolates.**

Many methods have been devised to determine the level of glucosinolates in seeds and other parts of cruciferous plants, and this methodology has been reviewed recently (1, 2). Gas liquid chromatography (GLC) was chosen as the official method for determination of glucosinolate content of rapeseed/canola in Canada (3, 4) and the EEC (5). Although GLC [or high performance liquid chromatography (HPLC)] permits measurement of the individual glucosinolates in a sample, these methods are time consuming, expensive, and require sophisticated equipment and extensive calibration. Plant breeders and the crushing industry continue to seek simpler procedures to screen large numbers of samples or to evaluate quality of delivered seed. Regulatory agencies also require accurate methods that are quicker than the existing chromatographic methods.

The thymol procedure described is based on the method reported by Brzezinski and Mendelewski (6). A modification of this method for use in Brassica breeding programs has been described (7), and it has also been used to establish response factors for the determination of glucosinolates by HPLC (8). The low glucosinolate varieties developed to date have had reduced levels of aliphatic glucosinolates but constant levels of indolyl glucosinolates. As plant breeders further reduce the level of aliphatic glucosinolates, the relative importance of the indolyl glucosinolates increases. The Canadian industry may soon require a method which conforms to the international practice of reporting total (aliphatic plus indolyl) glucosinolates on a whole-seed (full fat) basis. The present study was undertaken to compare the thymol method which determines all glucosinolates present (6) with the gas chromatography method (4) used in Canada which determines, with accuracy, only aliphatic glucosinolates. The study also evaluates the accuracy and precision of the thymol procedure for estimating the glucosinolate content of samples with relatively low amounts of glucosinolates. Modifications are described which allow for improved sample handling and precision of analysis.

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## EXPERIMENTAL

*Materials.* Canola seed (*Brassica nupus* L. and *Brassica campestris* L.) from the Grain Research Laboratory's new crop surveys and rapeseed from international collaborative studies were used. Sinigrin (allyl glucosinolate) was purchased from Aldrich Chemical Co., Milwaukee, Wisconsin; thymol (5-methyl-2-[1-methylethyl] phenol) from Sigma Chemical Co., St. Louis, Missouri; DEAE-Sephadex A-25 from Pharmacia Fine Chemicals, Uppsala, Sweden; and Econo-Column polypropylene columns with Luer-3-way nylon stopcocks from Bio-Rad Laboratories, Richmond, California.

Chemicals and solvents were of Reagent Grade or better, and all aqueous solutions were prepared with deionized water. The 0.5 M pyridine acetate solution was prepared by mixing 930 ml water, 30 ml glacial acetic acid and 40 ml pyridine.

*Extraction of glucosinolates.* The extraction procedure of Slominski and Campbell (9) was used to maximize extraction of indolyl glucosinolates. Defatted seed (100 mg) or ground whole-fat seed (200 mg) was weighed into a 16 × 75 mm Pyrex tube and placed in a heating block at 95°C for 15 min. Four ml boiling water was added, and the tube was fitted with a PTFE lined screw cap and mixed quickly on a vortex mixer to reduce cooling. The tube was returned to the heating block and heated at 95°C for three min. After cooling and centrifugation at 600 × g, the supernatant solution was transferred to a 10-ml graduated centrifuge tube containing 150 μl of a 0.5 M barium/lead acetate solution. The resulting pellet was extracted with an additional 4 ml of boiling water for three minutes at 95°C. After cooling and centrifuging, the supernatant from the second extraction was added to the first, and the total volume was adjusted to 10 ml. The total extract was centrifuged; if necessary, it could be refrigerated several days before testing.

*Preparation of Sephadex pyridine-acetate columns.* Dry DEAE-Sephadex A-25 (100 mg) was weighed into clean 0.8 × 4 cm Econo-Columns (Bio-Rad Laboratories) and was allowed to swell with deionized water while being stirred to remove air bubbles. Settled columns were refrigerated if stored for long periods. The procedure produced columns with bed volumes of 15 × 8 mm leaving the 10-ml reservoir at the top of the column for rinsing and sample application.

Prior to sample application, five ml of 0.5 N sodium hydroxide was passed through the columns, followed by 10 ml water to remove the excess sodium hydroxide. The eluate was monitored to ensure that

the pH was neutral. Columns were changed to acetate form by addition of five ml of 0.5 M pyridine acetate solution and then 10 ml water leaving a meniscus of water on the top of the column for sample application.

*Isolation of glucosinolates.* An aliquot (3.0 ml) of the total extract was added to the prepared columns. The columns then were washed with 2 × 2 ml water, 2 × 2 ml 30% formic acid, and 2 × 2 ml water, discarding the eluate each time. Glucosinolates were eluted with 2 × 4.75 ml of 0.3 M potassium sulfate and adjusted to 10.0 ml.

*Measurement of glucosinolates.* Aliquots of 1.0 ml were placed in clean borosilicate tubes (16 × 100 mm), and 7.0 ml of 80% sulfuric acid followed by 1.0 ml of 1% thymol in ethanol were added. Tubes were capped with PTFE-lined screw caps, thoroughly mixed, placed in a heating block at 100°C for 60 min, cooled under tap water, and mixed again. Absorbance was measured against 0.3 M potassium sulfate at 505 nM on a Bausch and Lomb Spectronic 1001 fitted with a test tube holder. The test tube holder permitted absorbance measurements to be made directly in the reaction tubes, eliminating the handling of the corrosive mixture.

*Calculation of total glucosinolate content.* The concentration of glucosinolates in the samples was calculated from the absorbance readings using the absorption factor of a sinigrin standard solution. The thymol-thioglucose complex follows Beer's law over a wide range of concentrations. Slight changes in reagent concentration, however, were found to affect the slope of the calibration curve and it was found necessary to carry out a single point calibration with each set of samples to ensure maximum accuracy. For this calibration, four blanks and four standards were prepared with each batch of samples. Blanks consisted of 1.0 ml of 0.3 M potassium sulfate, 7.0 ml 80% sulfuric acid, and 1.0 ml of 1% thymol solution. Standards were prepared with 1.0 ml sinigrin solution (0.3 μmol/ml), 7.0 ml 80% sulfuric acid, and 1.0 ml of 1% thymol solution. The absorbance of blanks and standards was determined before and after each sample batch. Absorbance readings of standards and blanks permitted calculation of the absorption factor (K):

$$K = (A_s - A_B)/C$$

where

- $K$ = absorption factor (usually about 2.8–3.2)
- $A_s$ = mean absorbance of standards
- $A_B$ = mean absorbance of blanks
- $C_s$ = concentration of the standards (μmol/ml)

The micromolar concentration of glucosinolates in the sample ($C_x$) was calculated as:

$$C_x = A_x/K \times DF/W$$

where

- $C_x$ = concentration of glucosinolates in sample (μmol/g)
- $A_x$ = absorbance of sample
- $K$ = absorption factor as calculated above
- $DF$ = dilution factor (depending on extraction volume and aliquot sizes)
- $W$ = weight of sample in grams

## RESULTS AND DISCUSSION

The capacity of the ion exchange resin was evaluated using various amounts of sinigrin prepared from a stock solution. Glucosinolate levels were chosen to correspond to levels expected from the spring sown canola varieties grown in Canada. Aliquots containing 1 to 5 μmol were used to cover total glucosinolate concentrations in the 5–50 μmol/g range for 200 mg whole-fat samples. The aliquot of three ml applied to the ion exchange column from the total of 10 ml extract could be increased for samples with even lower glucosinolate content. Alternatively, the dilution factor and aliquot volume, prior to reaction with $H_2SO_4$ and thymol, could be adjusted to allow measurement of lower or higher glucosinolate levels. Ten ml of 0.3 M potassium sulfate was required to remove all the sinigrin from the ion-

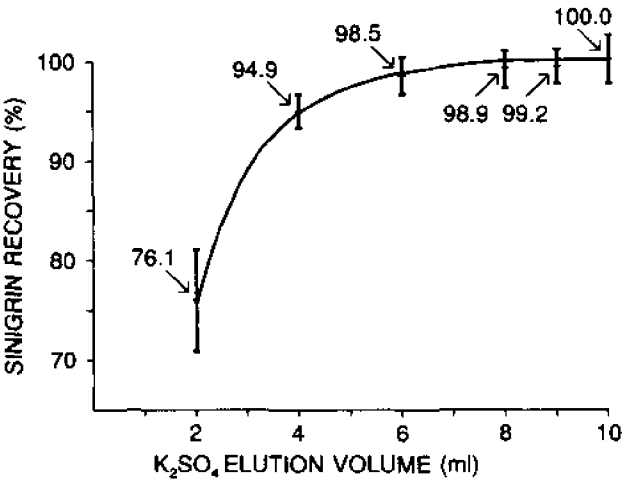


FIG. 1. Sinigrin recovery from 100 mg Sephadex A-25 ion-exchange columns. Each data point is the mean recovery of 1-μmol, 2-μmol, 3-μmol, 4-μ mol and 5-μmol loadings.

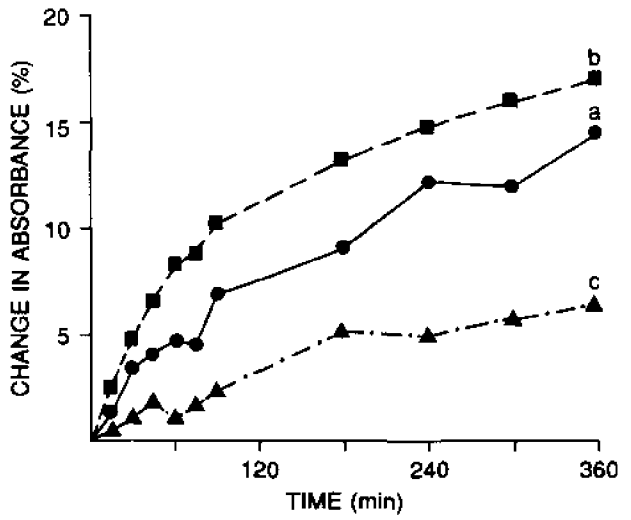


FIG. 2. Effect of tube choice on the stability of sulfuric acid/thymol digests. Each data point is the mean of five replicates for each of a) disposable borosilicate; b) corked disposable borosilicate, and c) cleaned borosilicate tubes fitted with Teflon lines screw caps.

exchange columns described in this study (Fig. 1). It is recommended that elution volumes required for complete recovery of glucosinolates be verified for columns of different size or shape.

The type and condition (cleanliness) of the tube used for the sulfuric acid/thymol digestion affected results (Fig. 2). Uncapped disposable tubes resulted in evaporation during heating and contamination by air-borne dust, while the use of corks was also unacceptable due to contamination from the corks. Clean borosilicate tubes fitted with PTFE-lined screw caps gave an absorbance drift of less than 2% during the first hour after digestion and less than 5% over six hr.

The relationship between the GLC procedure and the thymol procedure for 60 defatted samples is shown in Figure 3. Values ranged from 9.5 to 122.5 μmol/g (oil-free, 8.5% moisture basis) for the sum of the aliphatic glucosinolates by the GLC method. Values for the thymol method ranged from 20.3 to 134.8 μmol/g. Total values obtained by the thymol procedure were higher than the GLC values because the GLC procedure determined only those glucosinolates named in the canola definition, (3-butenyl, 4-pentenyl, 2-hydroxy-3-butenyl, and 2-hydroxy-4-pentenyl glucosinolates). Other minor glucosinolates, the indolyl glucosinolates, and glucosinolates from cruciferous weed seed admixtures also contribute to the total glucosinolate value determined by the thymol procedure. In pedigreed and top grade canola seed most of the difference was due to the presence of the indolyl and minor glucosinolates. As the samples in this study were chosen from No. 1 Canada Canola and certified seed, the intercept value of about 14 μmol/g can be considered to represent the amount of indolyl and minor glucosinolates measured by the thymol procedure but not included in the canola definition. This is in good agreement with the value of 10–15 μmol/g estimated as the amount of indolyl glucosinolates in current Canadian canola cultivars (10). Although indolyl glucosinolates may be measured by GLC, results are variable (11).

To assess the accuracy and precision of the thymol procedure, a set of 18 unknown samples was defatted and analyzed in duplicate by the GLC and thymol techniques. Analysis of the samples in duplicate by GLC yielded a standard error of ± 1.1 μmol/g for the sum of the aliphatics. Analysis of the same samples in duplicate by the thymol method yielded a standard error of ± 3.0 μmol/g. Results ranged from 13.4 to 103.3 μmol/g for the sum of the aliphatic glucosinolates by GLC and 23.1 to 128.4 by the thymol procedure. The relationship determined in Figure 3 was used to "correct" the thymol results to give "predicted" GLC results. Regression of the predicted GLC means with the actual means determined by GLC yielded a slope of 1.18 and an intercept of −3.2 (Fig. 4). This relationship could be used to correct for the additional glucosinolates determined by the thymol method and give results comparable to those from the GLC method, the method of reference for canola quality in Canada.

It is estimated that one technician in an eight-hour period can perform 20 to 30 thymol analyses starting with oil-extracted samples (meals). Defatting of samples, although considered essential in traditional methods of glucosinolate analysis, may involve as much as an extra day's work, depending on the equipment used. To maximize sample throughout, the analysis of full-fat ground seed is desirable. Four samples were analyzed by the thymol procedure on a defatted (meal) and a seed basis. When expressed on a uniform oil and moisture basis, analysis of seed and meal sample gave similar results over a wide range of glucosinolate values (Table 1). Two samples of canola, starting from the whole seed, were analyzed on 10 different days. The total glucosinolate content was 13.4 ± 1.0 and 21.1 ± 1.5 μmol/g, whole-seed, dry basis. When these samples were analyzed 10 times on the same day, the results were 12.8 ± 0.5 and 20.7 ± 1.1 μmol/g whole-seed, dry basis.

A comparison of the thymol procedure with other commonly used glucosinolate methods (Fig. 5) sug-

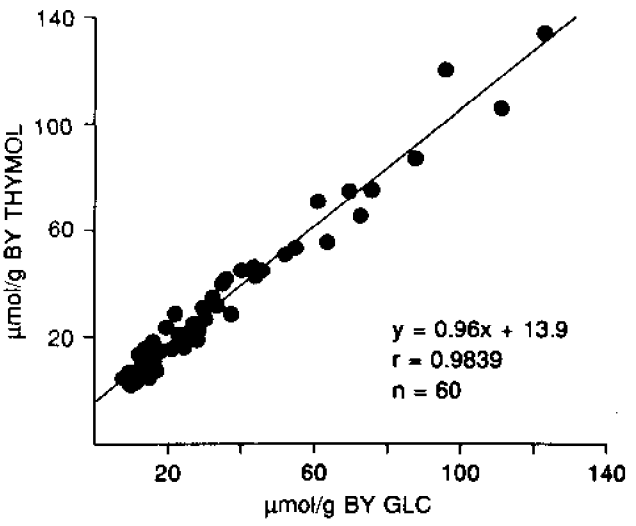


FIG. 3. Relationship between total glucosinolates determined by the thymol procedure and the four aliphatic glucosinolates determined by GLC.

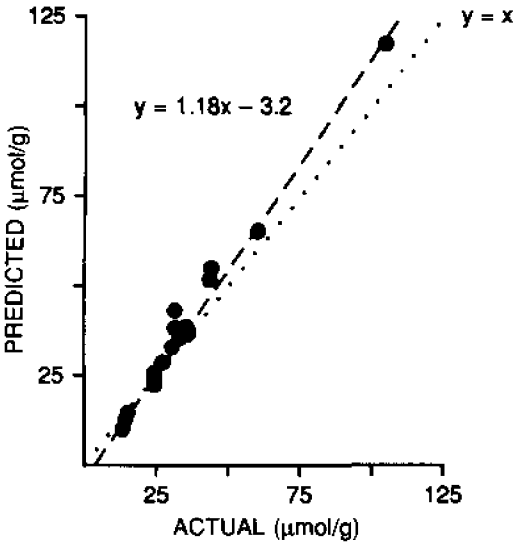


FIG. 4. Comparison of total aliphatic glucosinolate values predicted by the thymol procedure with GLC analysis.

TABLE 1

**Effect of Defatting on Glucosinolate Determination by Thymol Procedure**

| Sample | Day | Meal[a] (μmol/g) | | Seed[b] (μmol/g) | |
|---|---|---|---|---|---|
| | | Mean[c] | C.V. (%) | Mean[c] | C.V. (%) |
| A | 1 | 25.2 | 1.6 | 24.1 | 3.7 |
| | 2 | 24.5 | 4.1 | 25.5 | 10.6 |
| B | 1 | 37.8 | 2.6 | 37.9 | 2.6 |
| | 2 | 39.3 | 4.3 | 40.1 | 2.7 |
| C | 1 | 75.8 | 1.3 | 78.8 | 1.0 |
| | 2 | 76.2 | 2.5 | 76.3 | 4.2 |
| D | 1 | 174.6 | 3.2 | 168.7 | 2.0 |
| | 2 | 163.3 | 1.9 | 170.5 | 4.7 |

[a]Corrected for moisture on a dry basis.
[b]Corrected for oil content and moisture on a dry basis.
[c]Each value represents a mean of four replicates.

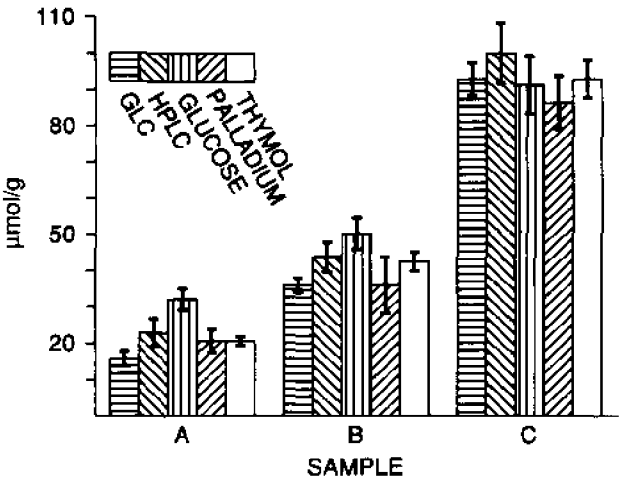


**FIG. 5. Comparison of several methods for determining total glucosinolates. Data for other methods from international round-robin study sponsored by ISO (12).**

gests it should be given consideration for determining total glucosinolate content. The thymol procedure is an inexpensive technique that requires no enzymatic preparations, eliminating concern over enzymatic activity or purity.

The elimination of an on-column waiting period (e.g., for hydrolysis or desulfation of glucosinolates) also makes the analysis quicker. The use of whole-seed samples eliminates the laborious defatting step. If an oil content value is available, whole-seed results can be expressed on an oil-free basis which is required for canola. The high sensitivity of the technique should permit the use of small sample sizes as is required in breeding programs.

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