OIL CROPS: BRASSICA SUBNETWORK

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ABBAS OMRAN

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Edited by
Abbas Omran
Technical Advisor, Oilcrops Network

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DETERMINATION OF TOTAL GLUCOSINOLATE CONTENT OF RAPESEED/CANOLA USING IMMOBILIZED MYROSINASE AND GLUCOSE OXIDASE

X. Wang, Z.Y. Yuan and D.I. McGregor
Agriculture Canada Research Station, 107 Science Crescent, Saskatoon, Saskatchewan,S7N OX2, Canada

1. APPLICATION

The method is intended for determining the glucosinolate content of seed or meal samples of canola or near canola quality (1 to 70 μmoles per gram oil-extracted meal) rapeseed (*Brassica campestris* L. and *B. napus* L.), although it may be applied to the entire range (1 to 200 μmoles per gram oil-extracted meal) of rapeseed glucosinolate content. It may also be applied to the analysis of seed or meal samples of Brown or Oriental (yellow seeded) mustard, *B. juncea* L. Coss., and white mustard, *Sinapsis alba* L.

2. PRINCIPLE

Endogenous enzymes including myrosinase are inactivated glucosinolates extracted from oil-extracted meal using hot (boiling) water. The extracted glucosinolates are hydrolyzed in a bioreactor containing immobilized myrosinase. The glucose released is converted to gluconic acid and hydrogen peroxide and the amount of hydrogen peroxide measured in a biosensor consisting of a membrane containing immobilized glucose oxidase (GOD) attached to the end of a hydrogen peroxide sensitive electrode.

3. BACKGROUND

Accuracy, precision, speed, simplicity and low cost are desirable attributes of any analytical method particularly when there is a need for large numbers of analyses such as in breeding programs or in quality control associated with production, transportation and marketing. Over the last four decades many methods have been proposed or used to determine glucosinolate content. In part this proliferation has been due to deficiencies in one or other of the above attributes. Breeders, nutritionists and industry have required a simple, low cost method which is sufficiently rapid to allow identification of low glucosinolate seed and meal in only a few minutes time. Such a method would not only be useful to identify and segregate canola seed and meal in commerce, but would be particularly useful to pay premiums or penalties based on glucosinolate content in countries where low glucosinolate winter type cultivars are currently being introduced into commercial production. Also required is a method which, in addition to being rapid, is sufficiently sensitive to be suitable for analyzing seed and meal with very low glucosinolate content (<10 micromoles glucosinolate expressed per gram oil-extracted air-dried meal). Other methods with sufficient sensitivity are either too slow or too expensive to facilitate screening of very large numbers of samples. The availability of a rapid, sensitive low cost method for determining glucosinolate content could facilitate conversion of commercial production to glucosinolate free cultivars expected in the near future. Identification and segregation of glucosinolate free seed and meal throughout the production, transpor-tation and marketing system could give Canada the competitive edge enjoyed for the last decade with canola.

To date the most popular analytical approach to screen for glucosinolate content has involved myrosinase hydrolysis to release glucose and colorimetric measurement of the glucose utilizing glucose oxidase, peroxidase and a chromogen (Lein and Schon 1969, Lein 1970, McGregor et al. 1973, Daun and McGregor in press). In plant breeding programs the so-called "hammer test", in which a few seeds are crushed with a hammer, water added to allow endogenous myrosinase to hydrolyze the glucosinolates and the glucose measured with a test paper containing the glucose oxidase, peroxidase and a chromogen (Comer 1956), was extensively

used in the development of canola cultivars. In the marketplace the so-called "mortar test", in which a small mortar was used to analyze a larger sample of seed and powdered carbon added to adsorb inhibitors thus enhancing the color development (McGregor and Downey 1975), was extensively used to identify and segregate canola seed during the conversion in Canada to commercial production of canola cultivars. More recently, efforts have been made to improve the glucose oxidase approach to glucosinolate determination. Noteable attempts include the use of automation to increase throughput (Smith et al. 1985), the use of a reflectometer to estimate the color development and thereby improve precision (Thies 1985, Robbelen 1987), and the varying reagent to improve both accuracy and precision (Saini and Wratten 1987, Smith and Dacombe 1987). Nevertheless, an assay which is sufficiently simple, rapid and inexpensive which can be used by relatively unskilled analysts to obtain answers in a matter of minutes with little cost, which is sufficiently sensitive to be used to identify low glucosinolate, canola and very low glucosinolate seed and meal, and which is sufficiently accurate and precise to be used to pay premiums and penalties based on glucosinolate content, remains elusive.

A solution is the use of enzyme immobilization. Studies have been reported recently on the immobilization of myrosinase on solid supports (Iori and Palmieri 1988, Wang et al. 1989a, Wang et al. 1989b) and on the coupling of immobilized myrosinase with immobilized glucose oxidase to measure glucose released from glucosinolate extracts (Koshy et al. 1989, Wang et al. 1989). The later study showed that myrosinase can be immobilized using readily available commercial supports and bonding agents. Sufficient activity can be bound to as little as 0.2 g of support to allow hydrolysis the glucosinolates from seed and meal extracts in as little as 5 minutes. When incorporated into a bioreactor at least 500 analyses can be performed without the need for replenishment of the myrosinase. When the bioreactor is coupled to a biosensor, in which glucose oxidase is immobilized by entrapment on the end of a hydrogen peroxide sensitive electrode, the complete analysis, including the initial extraction of the glucosinolates from seed or oil-extracted meal, can be completed in less than an hour.

4. ADVANTAGES/DISADVANTAGES

Immobilization of myrosinase in a bioreactor in combination with immobilization of glucose oxidase to form an enzyme electrode offer some particular advantages over other methods for determining of glucosinolate content. Since both the myrosinase and the glucose oxidase are reusable the analysis is essentially "reagentless". This substantially reduces the cost of analysis. Using a hydrogen peroxide sensitive electrode eliminates the need for peroxidase.

Rapeseed and rapeseed meal contain inhibitors of peroxidase which must be removed, either with powdered carbon or ion-exchange chromatography, in methods that rely on glucose oxidase and peroxidase to colorimetrically determine myrosinase released glucose. Measuring the hydrogen peroxide formed by the action of glucose oxidase on myrosinase released glucose eliminates the need to isolate the glucosinolates or glucose from these inhibitors and thus speeds up the analysis substantially.

Recently it has been shown that rapeseed contains enzymes other than myrosinase which release glucose when endogenous myrosinase is used to hydrolyze the glucosinolates (Smith and Donald 1988). Studies (McGregor, unpublished) have shown that this non-glucosinolate glucose can amount to as much as 6 to 8 micromoles expressed per gram of oil-extracted air-dried meal and thus provide a substantial background interference in very low glucosinolate material. By in activating endogenous enzymes during extraction and relying on exogenous myrosinase in the bioreactor, the enzyme immobilization approach avoids the problem of non-glucosinolate glucose.

Sound rapeseed contains about 6 to 10 micromoles of free glucose while poor quality rapeseed may contain even greater amounts of free glucose. By bypassing the bioreactor and analyzing a sample of the glucosinolate extract directly with the enzyme electrode background glucose can be determined and a correction applied. This does not add substantially to the time required for analysis as analysis of glucose with the enzyme electrode requires only 90 seconds. Correction for non-glucosinolate background glucose in combination with high sensitivity allows for accurate and precise analyses of samples with less than 10 micromoles of glucosinolate per gram

oil-extracted air-dried meal.

Analysis of myrosinase released glucose estimates true glucosinolate content. Indole glucosinolates are estimated in addition to aliphatic glucosinolates. This rapid and simple method which estimates the true glucosinolate content of seed or meal with high accuracy, precision and throughput is ideally suited as a standard method of analysis. The improved ease of analysis and standardization can facilitate trade of canola and of glucosinolate-free seed and meal.

Unlike gas chromatography, or high performance liquid chromatography, this method does not provide detailed information on glucosinolate profiles. Unlike the glucose oxidase/ferric nitrate method, it does not provide information on the amount of the various (aliphatic and indole) glucosinolate classes.

Compared to the hammer method, mortar method, or microtiter plate method of glucosinolate analysis accuracy and precision is considerably higher, but throughput is lower.

5. EQUIPMENT

Standard laboratory apparatus and in particular:

5.1 Water bath, 70 °C.

5.2 Scintered glass funnel, 60 mL:

Fisher Scientific Ltd.
Cat. No. 1- 358H

5.3 Forced-air oven.

5.4 Vibrax shaker, Model VXR 7:

Terochem Scientific Ltd.

5.5 Eppendorf repeater pipete tips, 5 mL:

Fisher Scientific Ltd.
Cat. No. 21-381-101

5.6 Disposable plastic syringes, 5 mL Disposable syringe needles, 20guage, 38 mm long:

Fisher Scientific Ltd.
Cat. No. 14-823-85
Cat. No. 14-826-5C

or, preferably: **Eppendorf repeater pipette tips, 5 mL**

Fisher Scientific Ltd.
Cat. No. 21-381-101

5.7 Porous polyethylene sheeting, (Bel-Art) 1.6 mm 35 um pore size:

Mandel Scientific Co. Ltd.
Cat. No. F13638-5116

5.8 Cork bore, #6.

5.9 Disposable micropets, 50:

Fisher Scientific Ltd.
Cat. No. 21-164-2G

5.10 Vials, 10 mL with polyethylene cap:

Fisher Scientific Ltd.
Cat. No. 03-339-10C

5.11 Cuvette, 0.1 cm pathlength:

Sargent-Welch Scientific Ltd.
Cat. No. S75735-11-A

5.12 Culture tubes, 125 x 15 mm with PTFE-lined screw-cap:

Fisher Scientific Ltd.
Cat. No. 14-930-10E

5.13 Vial, 8 mL:

Fisher Scientific Ltd.
Cat. No. 06-408C

5.14 Water bath, boiling.

5.15 Centrifuge, bench top.

5.16 Water bath, 70 °C.

5.17 Glucose analyzer, Model 27 with 25µL syringepet:

Yellow Springs Instrument Co.

6. REAGENTS

Standard laboratory reagents and in particular:

6.1 -Aminopropyltriethoxysilane (APTS):

Pierce Chemical Co.
Cat. No. 80370

6.2 Citric acid, monohydrate.

6.3 Controlled Pore Glass, CPG/Uncoated:

500Å pore diameter
80/120 mesh
125-177um particle size

Pierce Chemical Co.
Cat. No. 23808

6.4 Controlled pore glass, CPG/ Aminopropyl 80/120 mesh 125-177 um particle size:

Pierce Chemical Co.
Cat. No. 23909

6.5 ß-D-Glucose:

Sigma Chemical Co.
Cat. No. G-5250

6.6 Glutaraldehyde, 25% in water:

Eastman Kodak Ltd.
Cat. No. P 8648

Available from North American Scientific Chemical Ltd.

6.7 Myrosinase:

Prepared from yellow mustard (*Sinapsis alba* L.) seed.
or
Biocatalysts Ltd.
or
Boehringer Mannheim Ltd.
Cat. No. 1088 769

6.8 Parafilm:

Fisher Scientific Ltd.
Cat. No. 13-374-5

6.9 Sodium borate.

6.10 Sodium chloride.

6.11 Sinigrin, potassium salt, monohydrate:

Sigma Chemical Co.
Cat. No. S7508

6.12 Sodium phosphate, dibasic anhydrous.

6.13 2,4,6-Trinitrobenzenesulfonic acid, hydrate (TNBSA):

Pierce Chemical Co.
Cat. No. 28999

7. SUPPLIERS

7.1 Biocatalysts Ltd.

Main Avenue, Treforest Industrial Estate Pontypridd, Wales CF37 5YT
Telephone: 044385 3712
Telex: 497126 BIOCAT G

7.2 Boehringer Mannheim Ltd.

11450 Cote de Liesse, Dorval, PQ CANADA H9P 1A9
Telephone: (514) 636-6760
Telex: 05-8222677

7.3 Chromatographic Specialties Ltd.

P.O. Bag 1150, 300 Laurier Blvd.
Brockville, ON, CANADA K6V 5W1
Telephone: (613) 342-4678

7.4 Fisher Scientific Ltd.

P.O. Box 3840 Station D, Edmonton, AB, CANADA T5L 4K2

7.5 Mandel Scientific Co. Ltd.

9840-47th Ave., Unit #2, Edmonton, AB, CANADA T6E 5P3
Telephone: (403) 436-0665

7.6 North American Scientific Chemicals Ltd.

7058 "F" Farrell Road S.E.
P.O. Box 5961 Station "A"
Calgary, AB, CANADA T2H 1Y4
Telephone: (403) 253-0456
Telex: 03-825749

7.7 Pierce Chemical Co.

P.O. Box 117, Rockford, IL USA 61105
Telephone: (910) 631-3419
Telefax: (815) 968-7316

7.8 Sargent-Welch Scientific Ltd.

285 Garyray Drive, Weston, ON CANADA M9L 1P3
Telephone: (416) 741-5210

7.9 Sigma Chemical Co.

P.O. Box 14508, St. Lois, MO USA 63178
Telephone: (314) 771-5750
Telefax: (314) 771-5757

7.10 Terochem Scientific Ltd.

5729 92 Street, Edmonton, AB CANADA T6E 3A4
Telephone: (403) 438-2222
Telex: 0373022
Telefax: (403) 434-3362

7.11 Yellow Springs Instrument Co.

Yellow Springs OH, USA 45387
Telephone: (513) 767-7241
Telex: 205437
Telefax: (513) 767-9353

8. PREPARATION

8.1 Silanization of CPG Glass

. Weigh 1 g of CPG\uncoated glass

into a 100 mL round bottom flask.
- Add 20 mL of 10% APTS (18 mL water and 2 mL APTS).
- Reflux in a water bath with the flask fitted with a cold water-jacketed condenser at 70 °C for 4 hours.
- Transfer the glass to a medium porosity scintered-glass filter funnel and wash with water under suction to remove the APTS.
- Dry in a forced-air oven at 120 °C for 5 hours.
- To a small amount of silanized CPG glass (approximately 10 mg) add approximately 2 mL of saturated sodium borate.
- Add 5 drops of a 3% aqueous solution of TNBSA and mix. If the glass has been sufficiently silanized it should turn orange within a couple of minutes.

8.2 Construction of the Bioreactor:

(**NOTE:** The bioreactor may be constructed with a 5 mL plastic syringe. However, if available, a 5 mL Eppendorf Repeater Pipette tip is preferred. Its one piece construction and flat base, which allows for snug fitting of the porous polyethylene frit, minimizes retention of eluate. Also, the plastic plunger has less tendency to stick with repeated use.)

- Insert a disk of porous polyethylene cut from a sheet with a #6 cork bore into the shorter bottom piece of the Eppendorf Repeater Pipette tip.

8.3 Activation of the Silanized Glass:

- Weigh 0.2 g of alkylamine glass (as prepared above, or preferably Controlled Pore Glass, CPG/Aminopropyl), into the 5 mL bioreactor.
- Add 2 mL 6.6% glutaraldehyde in citrate-phosphate buffer (2.5 mL 25% glutaraldehyde and 7.5 mL 0.2 M citrate-phosphate buffer pH 7) 14.1 g sodium phosphate, dibasic anhydrous, dissolved in 500 mL water and 2.1 g citric acid, monohydrate, dissolved in 100 mL water, citric acid solution added to 450 mL sodium phosphate to obtain pH 7).
- Seal the tube with its plunger and a small piece of Parafilm and shake on the Vibrax shaker for 2 hours.
- Wash with water until all of the glutaraldehyde has disappeared.

8.4 Covalent Attachment of Myrosinase:

- Add 2 mL of freshly prepared myrosinase (7 mg/mL) to the 5 mL bioreactor.
- Seal the tube with its plunger and a small piece of Parafilm and shake on the Vibrax shaker overnight at 4 °C.
- Wash with 0.5 N NaCl (2.9 g dissolved in 100 mL of water), water, then 0.05 M citrate-phosphate buffer pH 7 (0.2 M citrate-phosphate buffer pH 7 diluted 4 fold.)
- Store with sufficient 0.05 M citrate-phosphate buffer to cover the glass at 4 °C.

8.5 Analysis of Immobilized Myrosinase Activity:

- Transfer a known weight (approximately 5 mg) of CPG glass containing the immobilized myrosinase to a second 5 mL bioreactor.

(**NOTE:** Transfer of a known weight of CPG glass may be facilitated by constructing a transfer device consisting of a 5 mL plastic syringe or Eppendorf Repeater Pipette tip to which is attached a short piece of tygon tubing containing a porous polyethylene frit disk and a short length of glass tubing (30 mm of a 50 disposable Micropet) into which can be drawn and expelled a known weight of glass. The porous frit permits removal of the buffer.)

- Rinse the bioreactor twice with 0.5 mL 2 mM sinigrin (41.5 mg/100 mL) and remove With aspiration.
- Without delay add 1 mL 2 mM sinigrin after last washing.
- After 3,6,9,12 or 15 minutes transfer the filtrate to a 4 mL vial, cap and heat at 70 °C for 5 minutes.
- Transfer the filtrate from the vial to a 1 mm pathlength cuvette and read the absorbance at 227 nm.
- Rinse the CPG glass by addition of water and with removal by aspiration, and repeat the above steps for additional times.
- Upon completion replace the CPG glass with immobilized myrosinase in the original bioreactor.

To express activity in μmoles per minute per 10 mg CPG glass: (ΔT) after 3 minutes, i.e. the initial change in absorbance per minute excluding the initial 3 minutes.

- **$(\Delta A/\Delta T)/\epsilon$ * 1/(pathlength) * 10/(mg of glass assayed) =μmoles min^{-1} per 10 mg CPG glass**

where:
$-\Delta A/\Delta T$ is the change in absorbance per minute.
$-\epsilon(6.784)$ is the extinction coefficient for sinigrin.
-1/(pathlength) is the correction for the sort (less that 1 cm) pathlength of the cuvette.
-10/(mg of glass assayed) is the correction to express the result per 10 mg CPG glass

Thus if:
-The change in absorbance per minute is 0.0475,
-The pathlength of the cuvette is 0.1 cm,
-The weight of glass assayed in 4.16 mg,
then:

0.0475/6.784 * 1/0.1 * 10/4.16 = 0.144 μmoles min^{-1} per 10 mg CPG glass.

An activity of 0.1 μmoles min^{-1} per 10 mg CPG glass is sufficien or a 0.2 g bioreactor to hydrolyse 0.5 mL of an extract of 30 μmole glucosinolate per g oil-extracted meal in 5 minutes, or 0.5 mL of an extract of 180 μmole glucosinolate /g oil-extracted meal in 15 min.

The performance of the bioreactor should be checked periodically with either the above procedure or by applying a meal extract and hydrolyzing for varying periods of time.

8.6 Glucose standard:

- Weigh 180.2 mg of b-D-glucose in to an 100 mL volumetric flask, dissolve and make to volume with water.
- Let stand overnight for mutarotation to equilibrium between α- and ß-D-glucose forms, 37% and 63%, respectively.

9. PROCEDURE

9.1 Glucosinolate Extraction:

- Weigh 250 mg of oil-extracted meal into a 125 x 15 mm culture tube.
- Place the tube in a boiling water bath for 1 minute.
- Add 3 mL of hot (<90 °C) water to the tube and, without allowing the contents to cool, mix to ensure that the meal is thoroughly wetted and continue heating for 5 min.
- Centrifuge at 2000 g for 10 min. and transfer the supernatant to a 5 mL volumetric flask.
- Wash the pellet twice with 1.5 mL of water, pool the super-natants, and make to volume with water.

9.2 Myrosinase Hydrolysis:

- Ensure that the bioreactor has been rinsed well with water and excess water removed by aspiration.

(**NOTE:** It is important to rinse the bioreactor with water prior to addition of the sample. Rinsing the bioreactor with sample results in apparent binding of gluco-sinolate to the active sights of the immobilized myrosinase. This leads to over estimation of the glucosinolate content of the sample.)

- Transfer 0.5 mL of sample into a test tube and draw up into the bioreactor.
- Shake the bioreactor and sample on the Vibrax shaker for 10 minutes.
- Transfer the sample to a 8 mL screw-cap vial.
- Cap and heat in water bath (70 °C) for 1 minute.

(**NOTE:** It is important to ensure that the glucose released from the glucosinolates is completely mutarotated to equilibration between the α-glucose and ß-glucose forms. Glucose released from the gluco-sinolates is the ß-glucose form. As glucose oxidase reacts only with ß-glucose and the glucose oxidase reacts only with ß-glucose and the glucose analyzer is calibrated with an equilibrated α-glucose and ß-glucose solution, it is important to heat the sample to ensure that the ß-glucose released from the gluco-sinolate is completely mutarotated to equilibration between the α-glucose and ß-glucose forms.

Failure to equilibrate the sample can result in an artificially high measurement.

9.3 Glucose Measurement

- Stabalize glucose analyzer and perform analyses using 0.05 M citrate-phosphate buffer pH 7 (0.2 M citrate-phosphate buffer pH 7 diluted 4 fold).

- Inject 25 μL of a 30 mg/dL or 180

mg/dL glucose standard and calibrate the glucose analyzer if necessary.

(**NOTE**: For best results calibrate before analyzing each sample. Use a standard concentration of glucose close to that of the glucosinolate content of the sample.)

- Inject 25 μL of the sample and record the glucose value.

To correct for dilution of the sample in the bioreactor:

- Ensure that the bioreactor has been rinsed well with water and excess water removed by aspiration.
- Transfer 0.5 mL of a 30 mg/dL or 180 mg/dL glucose solution into a test tube.
- Draw up into the bioreactor.
- Shake the bioreactor and glucose standard on the Vibrax shaker for 1 minute.
- Transfer the filtrate to an 8 mL vial.
- Inject 25 μL of the sample and record the glucose value.

10. CALCULATION AND REPORTING OF RESULTS

10.1 μMoles per Gram Meal:

- Divide the concentration of the glucose standard (30 mg/dL or 180 mg/dL) by the glucose analyzer value obtained with addition of the glucose standard to the bioreactor to determine the dilution factor, i.e. 30 (mg/dL)/value (mg/dL) obtained for 30 mg/dL glucose standard in bioractor = dilution factor.

- Correct the glucose analyzer reading for the hydrolyzed sample for dilution by multiplying by the dilution factor to obtain a dilution corrected glucose analyzer reading.
- Subtract the corrected glucose analyzer reading for the hydrolyzed sample from the non-hydrolyzed (non-corrected) glucose analyzer reading.

Glucosinolate content is determined as:

▲glucose analyzer reads (mg/dL)/20*1000/250 * 1/0.1802 = μmoles/g oil-extracted meal

where:
-The factor **20** converts from 1 dL to the extract volume (5 mL).
-The factor **1000/250** converts to a gram basis the weight of oil-extracted meal (250 mg) from which the glucosinolates were extracted.
-The factor **1/0.1802** converts to μmoles from miligrams.

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