

COMMISSION REGULATION (EEC) No 2435/86

of 29 July 1986

amending Regulation (EEC) No 1470/68 on the drawing and reduction of samples and the determination of the oil content, impurities and moisture in oil seeds

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Economic Community,

Having regard to Council Regulation No 136/66/EEC of 22 September 1966 on the establishment of a common organization of the market in oils and fats⁽¹⁾, as last amended by Regulation (EEC) No 1454/86⁽²⁾, and in particular Article 24a thereof,

Whereas, as a result of the successive amendments to Commission Regulation (EEC) No 1470/68⁽³⁾, as last amended by Regulation (EEC) No 3519/84⁽⁴⁾, the title of the said Regulation only partially describes its content; whereas its designation should be adjusted accordingly;

Whereas the term 'double zero' as applied to the seed of rape and turnip rape depends on the level of glucosinolates in the seed; whereas a suitable method should be laid down for determining this level;

Whereas, under Commission Regulation No 282/67/EEC of 11 July 1967, on detailed rules for intervention for oil seeds⁽⁵⁾, as last amended by Regulation (EEC) No 2436/86⁽⁶⁾, and Commission Regulation (EEC) No 2681/83 of 21 September 1983 laying down detailed rules for the application of the subsidy system for oil seeds⁽⁷⁾, as last amended by Regulation (EEC) No 2434/86⁽⁸⁾, a single Community method should be laid down for determining glucosinolate levels;

Whereas the measures provided for in this Regulation are in accordance with the opinion of the Management Committee for Oils and Fats,

HAS ADOPTED THIS REGULATION:

Article 1

Regulation (EEC) No 1470/68 is hereby amended as follows:

1. The title is replaced by the following:

'on the drawing and reduction of samples and on methods of analysis in respect of oil seeds';

2. The following Article 2c is inserted:

Article 2c

The determination of glucosinolate levels as referred to in Article 4 of Regulation No 282/67/EEC and Article 32 of Regulation (EEC) No 2681/83 shall be carried out using the method laid down in Annex VIII hereto, without prejudice to the transitional arrangements referred to in the abovementioned Articles.'

3. The Annex to this Regulation is added as Annex VIII.

Article 2

This Regulation shall enter into force on the day of its publication in the *Official Journal of the European Communities*.

It shall apply with effect from 1 July 1986.

This Regulation shall be binding in its entirety and directly applicable in all Member States.

Done at Brussels, 29 July 1986.

For the Commission

Frans ANDRIESEN

Vice-President

⁽¹⁾ OJ No 172, 30. 9. 1966, p. 3025/66.

⁽²⁾ OJ No L 133, 21. 5. 1986, p. 8.

⁽³⁾ OJ No L 239, 28. 9. 1968, p. 2.

⁽⁴⁾ OJ No L 328, 15. 12. 1984, p. 12.

⁽⁵⁾ OJ No 151, 13. 7. 1967, p. 1.

⁽⁶⁾ See page 61 of this Official Journal.

⁽⁷⁾ OJ No L 266, 28. 9. 1983, p. 1.

⁽⁸⁾ See page 51 of this Official Journal.

ANNEX

ANNEX VIII

COLZA AND RAPE SEED

Determination of the content of glucosinolates

1. SUBJECT

The present method is designed for determining the main glucosinolate composition and content of colza and rape seed.

2. PRINCIPLE

- 2.1. Measurement of trimethylsilyl derivatives of enzymatically desulphated glucosinolates by gas chromatography with temperature programming, using sinigrine as internal standard.
- 2.2. The method provides the quantities, in micromoles per gram of air-dry seed, of six major glucosinolates contained in colza and rape seed, and two glucosinolates contained in mustard seed which may be contaminated.

3. MAJOR REAGENTS

- 3.1. DEAE Sephadex A-25
- 3.2. SP Sephadex C-25
- 3.3. Sulphatase type H-1
- 3.4. Allyl glucosinolate (Sinnigrin)
- 3.5. Barium acetate
- 3.6. Lead acetate
- 3.7. Pyridine (Silylation grade)
- 3.8. N-methyl-N-trimethylsilyl heptafluorobutyramide (MSHFBA)
- 3.9. Trimethylchlorosilane (TMCS)
- 3.10. 1-Methylimidazole

4. MAJOR EQUIPMENT

- 4.1. Swedish extraction tubes, 70 ml, stainless steel, 18 mm ID neck, 17 mm ball bearings, No 3 fluoro-silicone rubber stoppers and horizontal shaker (Troeng 1955), or equivalent steelball shaker.
- 4.2. High speed coffee grinder.
- 4.3. Forced-air oven.
- 4.4. Gas chromatograph with temperature programming capability and flame ionization detector.
- 4.5. Gas chromatographic column of glass, length about 2 metres, internal diameter 2 mm filed with 2 % OV-07 on diatomite CLQ 80-100 meshor.

5. PREPARATION

5.1. Preparation of DEAE Sephadex A-25 acetate form and Pyridine-acetate form

Weigh 10 g DEAE Sephadex A-25 into a 250 ml beaker, add 150 ml water and allow the Sephadex to swell overnight. Slurry the Sephadex onto a 20 × 400 mm column.

Pass 500 ml 0,5 N sodium hydroxide (10 g dissolved in water and made up to 500 ml) through the column. Wash the column with 250 ml water to remove excess sodium hydroxide checking to ensure the pH has dropped to neutrality,

Remove one-tenth of the Sephadex to be converted to the acetate form. Slurry in water and pour into a 15 × 200 mm column. Pass 100 ml 0,5 M acetic acid (2,9 ml glacial acetic acid made up to 100 ml) through the column. Wash with 250 ml water. Slurry into a 250 ml flask with water for storage.

Settle the remaining nine-tenths of the Sephadex in column with water. Pass 400 ml 0,5 M pyridine acetate (19,8 ml pyridine plus 15 ml glacial acetic acid made up to 500 ml with water) through the column. Wash with 250 ml water. Slurry into a 250 ml flask with water for storage.

5.2. Preparation of SP Sephadex C-25 Sodium form

Weigh 1 g of SP Sephadex C-25 into a 100 ml beaker, add 75 ml water and allow the Sephadex to swell overnight. Slurry the Sephadex onto a 15 × 200 mm column and wash with 250 ml water. Slurry into a 250 ml flash with water for storage.

5.3. Purification of sulphatase

Weigh 70 mg sulphatase type H-1 into a 16 × 150 mm test tube. Add 3 ml water to dissolve the sulphatase and dilute with an equal volume of ethanol. Centrifuge for 10 minutes at 2 000 x g. Decant off the supernatant into a second tube and discard the precipitate. Add 9 ml of ethanol to the supernatant and centrifuge again for 10 minutes at 2 000 x g. Discard the supernatant and dissolve the precipitate in 2 ml water.

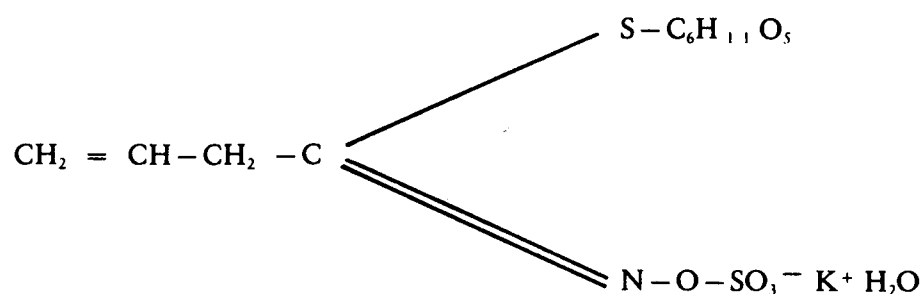
Place a small plug of glass wool in the tip of each of two pipettes.

To one add 100 microlitres of the aqueous layer above the DEAE Sephadex A-25 acetate form. Then to this add by volume the DEAE Sephadex A-25 acetate form to form a column 15 mm high, equivalent to 20 mg of dry Sephadex.

To the other add 100 microlitres of the aqueous layer above the SP Sephadex C-25 sodium form. Then to this add by volume the prepared SP Sephadex C-25 sodium form to form a similar column. Pass the aqueous enzyme solution first through the DEAE Sephadex A-25 acetate form column then through the SP Sephadex C-25 sodium form column. The sulphatase solution is used undiluted on pipette tip columns. Store the eluate at — 20 °C and thaw immediately before use.

5.4. Preparation of internal standard

Allyl glucosinolate (monohydrate potassium salt)



Molecular weight :

C	10	×	12,011	=	120,110
H	18	×	1,008	+	18,144
N	1	×	14,008	=	14,008
S	2	×	32,006	=	64,132
O	10	×	16,000	=	160,000
K	1	×	39,100	=	39,100
					<u>415,494</u>

" To prepare a 1 micromole per ml solution weight out 41,5 mg and make up to 100 ml.

5.5. Preparation of OV-7 gas chromatography column

First wash the inside surface of a 4' × 0,25" OD, 2 mm ID glass column by attaching one end of the column with a rubber hose to a water aspirator and applying a low suction. Draw through the column 100 ml chloroform, 100 ml acetone, then 100 ml of petroleum ether (bp 30-60°C). Draw air through to dry, then dry in forced-air oven.

Plug one end with glass wool. Apply suction to this end of the column with a vacuum line. Through a small funnel, attached to the other end of the column by means of a short length of tygon tubing, add column packing, 2 % OV-7 on Diatomite CLQ 80-100 mesh. Tap the column gently to encourage flow of the packing material around the coils until full and evenly packed. Remove the funnel and tygon tubing. Blow out enough packing material with a Pasteur pipette and rubber bulb to allow the end to be plugged with glass wool.

Mount the column to the injection part of the gas chromatograph using a stainless steel nut, a back ferrule mounted backwards and a graphite ferrule. Pass helium carrier gas through the column and temperature programme from 100 °C at 1 °C per minute to 290 °C and hold there overnight. Cool the oven and attach the column to the detector using stainless steel nut and back ferrule and graphite front ferrule.

Set the injector temperature at 250 °C and detector temperature at 300 °C, column temperature at 200 °C, the flow rate of helium carrier gas at 40 ml per minute, the flow rates of the hydrogen and air at 50 and 500 ml per minute respectively to give optimum detector response, the range of 1 and the attenuation at 64.

6. PROCEDURE

6.1. Preparation of samples

The drawing of the seed samples and the reduction of the laboratory samples to samples for analysis shall be carried out in accordance with the procedures respectively described in Annexes I and II to Regulation (EEC) No 1470/68.

A standard seed sample should be analysed at least once per batch of samples. Data from standard sample analyses are useful for the control of the precision and accuracy of the method.

If the seed sample is moist, dry 20 g in a forced-air oven overnight at 45 °C in order to obtain 7 % moisture.

Grind 20 g of dried seed in a coffee mill.

Extract oil from 3 g of ground seed with 40 ml petroleum ether (bp 30-60 °C), or n-hexane, in a Swedish tube containing three steel balls, closed with a fluorsilicone stopper. Shake horizontally in a mechanical shaker for 45 minutes. Filter under suction through Whatman No 1 paper in a conical funnel and wash twice with fresh solvent. Air dry the meal sample in a fumehood overnight. Break up any lumps when dry by screening over 280 micrometer. More than 90 % of the sample must pass the screen.

6.2. Inactivation of myrosinase and extraction of the glucosinolates

Weigh meal (100 mg) into a test tube. Heat the tube and sample in a boiling (95 °C) water bath. After two minutes add boiling water (1 ml). After a further two minutes add internal standard (1 ml) (allyl glucosinolate). The concentration of the internal standard depends upon the estimated glucosinolate content of the sample as indicated in the table below. Continue heating at 95 °C for 15 minutes.

In a second assay the internal standard is not added, to determine the allyl glucosinolate content of the original sample.

Cool the solution and add 100 microlitre of a solution containing 0,5 ml each of barium acetate and lead acetate per litre of water and mix. Centrifuge at 2 000 x g. Save the supernatant.

Content of glucosinolates (micromole/g seed)	Concentration of internal standard (micromole/ml)	Amount of extract to be applied to Sephadex (ml)
< 15	1	1,0
15 — 40	2	0,5
> 40	3	0,2

6.3. Preparation of DEAE — Sephadex A-25 minicolumns

Suitable columns may be prepared using 1 ml plastic pipette tips or alternatively using shortened pasteur pipettes. Place a small plug of glass wool in the bottom of the 'minicolumn' and wash with water (1 ml). Add a suspension of DEAE Sephadex A-25 (pyridine-acetate form) equivalent to 20 mg dry weight of Sephadex. This is most simply achieved by adding a predetermined volume of a suspension (well mixed). Allow the gel to settle and wash with water.

Apply to the column the supernatant from the barium/lead treatment. Allow to drain and wash the column with 1 ml water followed by 1 ml pyridine acetate (0,02M). Allow to drain and then add to the column 50 microlitres purified sulphatase solution and allow to stand at room temperature overnight. Elute the desulphoglucosinolates with water (3 x 0,5 ml).

6.4. Derivatization of the desulphoglucosinolates

Prepare the silylating reagent by mixing MSHFBA (100 microlitres), TMCS (10 microlitres) and diluted 1-methylimidazole (50 microlitres). The diluted 1-methylimidazole is prepared with 1-methylimidazole (50 microlitres) and acetone (950 microlitres).

Dry a sample of desulphoglucosinolate eluate in a small vial capable of efficient sealing. Small quantities (5 to 10 microlitres) may be dried by heating at 120 °C for 10 minutes.

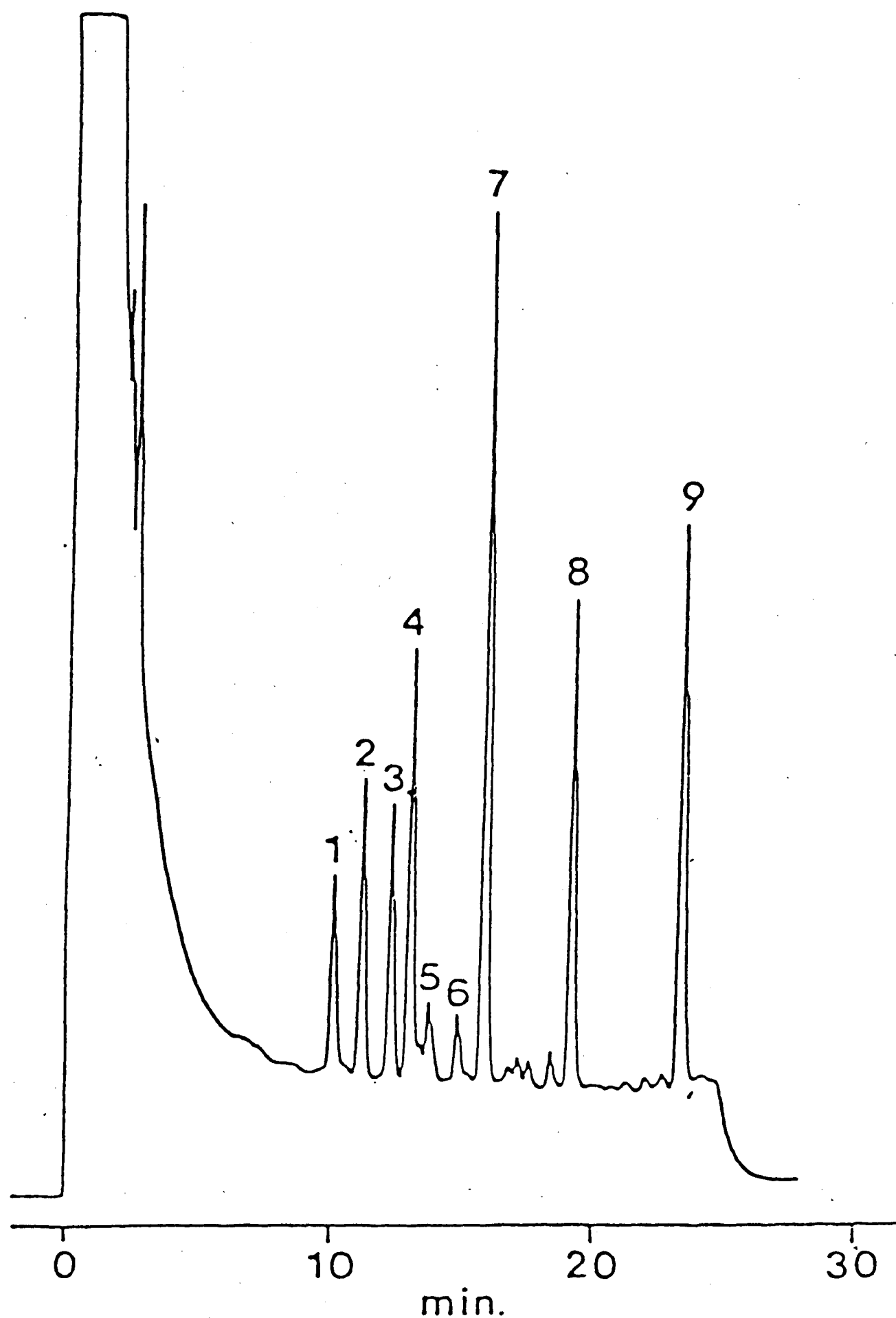
Alternatively, dry in a vacuum dessicator over P₂O₅. To the dried sample add an equal volume of silylating reagent and seal the vial. Heat the vial at 120 °C (5 minutes) to complete derivatization.

6.5. Separation of the derivatized desulphoglucosinolates by gas chromatography

Inject 2 microlitres onto the OV-7 column and hold at 200 °C for five minutes, then temperature programme at 5 °C per minute to 280 °C. Hold at this final temperature for 15 minutes.

Collect the peak areas for: 3-butenyl glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3-butenyl glucosinolate, 2-hydroxy-4-pentenyl glucosinolate, indolyl-3-methyl glucosinolate, 4-OH-indolyl-3-methyl glucosinolate and when present: allyl glucosinolate 4-hydroxy benzyl glucosinolate.

When analysing samples with a wide range of glucosinolate content, or when starting analysis after a period of some hours of inactivity, repeated injections of a sample is necessary until constant results are obtained.



Separation of derivatized desulphoglucosinolates by temperature programmed gas chromatography.

- (1) allyl-,
- (2) 3-butenyl-,
- (3) 4-pentenyl-,
- (4) 2-hydroxy-3-butenyl-,
- (5) 2-hydroxy-4-pentenyl-,
- (6) sucrose,
- (7) benzyl-,
- (8) 4-hydroxy-benzyl,
- (9) 4-hydroxyindolyl-3-methyl-.

6.6. Quantitation of results

The first task is to determine the area of allyl glucosinolate which is attributable to contamination, if any.

The area for allyl glucosinolate obtained in the analysis without added internal standard is normalized using the areas for 2-hydroxy-3-butenyl glucosinolate from both analyses, with and without added internal standard:

$$\frac{\text{area allyl glucosinolate TMS (without internal standard)}}{\text{area 2-OH-3-butenyl glucosinolate TMS (without internal standard)}} = \frac{\text{area 2-OH-3-butenyl glucosinolate TMS (with internal standard)}}{\text{area 2-OH-3-butenyl glucosinolate TMS (without internal standard)}} = \frac{\text{allyl glucosinolate TMS from contamination}}{\text{allyl glucosinolate TMS from added internal}}$$

The second task is to obtain the area of allyl glucosinolate attributable to internal standard. The previous result is subtracted from the total area for allyl glucosinolate obtained in the analysis with added internal standard:

$$\text{allyl glucosinolate TMS (with internal standard)} - \text{allyl glucosinolate TMS from contamination} = \text{allyl glucosinolate TMS from added internal}$$

The areas for the individual glucosinolate TMS derivatives obtained in the analysis with added internal standard are each in turn divided by the area attributable to allyl glucosinolate added as internal standard, then multiplied by the micromoles of allyl glucosinolate added per g of oil-extracted air-dry meal to obtain the micromoles glucosinolate per g oil-extracted air-dry meal:

$$\frac{\text{area glucosinolate TMS}}{\text{area allyl glucosinolate TMS from added internal standard}} \times \frac{\text{micromoles allyl glucosinolate}}{\text{g oil-extracted air-dry meal}} = \frac{\text{micromoles glucosinolate}}{\text{g oil-extracted air-dry meal}}$$

To express results on air-dry seed basis:

$$\frac{\text{micromoles glucosinolate}}{\text{oil-extracted air-dry meal}} \times \frac{100 - \% \text{ oil}}{100} = \frac{\text{micromoles glucosinolate}}{\text{g air-dry seed}}$$

7. REPORTING OF RESULTS

The amounts of 3-butenyl glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3-butenyl glucosinolate, 2-hydroxy-4-pentenyl glucosinolate, Indolyl-3-methyl glucosinolate, 4-OH-indolyl-3-methyl glucosinolate are summed and reported together. Allyl glucosinolate and 4-hydroxybenzyl glucosinolate, if present, are reported separately as an indication of contamination with mustard or other cruciferous weed seeds.

Results are reported in micromoles per g of air-dry seed, as mean of double determinations and indication of the range R. (R = higher value minus lower value of the double determination).