COMMISSION REGULATION (EEC) No 1864/90

of 29 June 1990

amending Regulation (EEC) No 1470/68 on the drawing and reduction of samples and on methods of analysis in respect of 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 2902/89 [2], and in particular Article 24a thereof,

Whereas the 'double zero' designation for rapeseed depend on its glucosinolate content; whereas, in order to determine that content, provision should be made for the most suitable method;

Whereas Commission Regulation (EEC) No 1470/68 [3], as last amended by Regulation (EEC) No 2435/86 [4], defines the common Community method for determining the glucosinolate content of rapeseed; whereas since then a better method of analysis has been developed and tested; whereas the common Community method for determining the glucosinolate content should be amended;

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

Annex VIII to Regulation (EEC) No 1470/68 is hereby replaced by the Annex hereto.

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 from 1 July 1990.

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

Done at Brussels, 29 June 1990.

For the Commission

Ray MAC SHARRY

Member of the Commission

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(1) OJ No 172, 30. 9. 1966, p. 3025/66.
(2) OJ No L 280, 29. 9. 1989, p. 2.
(3) OJ No L 239, 28. 9. 1968, p. 2.
(4) OJ No L 210, 1. 8. 1986, p. 55.

ANNEX

'ANNEX VIII

Oil seeds — determination of glucosinolates

High Performance Liquid Chromatography

1. FIELD OF APPLICATION

This Annex specifies a method for the determination of different glucosinolates in oil seeds of the *Brassica* species, particularly colza, using high performance liquid chromatography. This method does not measure glucosinolates which are substituted on the glucose molecule but these compounds are of little importance in commercial rape seed.

2. REFERENCES

Preparation of samples for analysis must be carried out in accordance with the relevant Annexes to this Regulation. In particular, the following Annexes are important:

Annex II — Reduction of contract samples to analysis samples.

Annex III — Determination of moisture and volatile matter content.

3. PRINCIPLE

Extraction of glucosinolates in a methanol solution, then purification and enzymatic desulphation on ion exchange resins. Determination using reversed-phase high-performance liquid chromatography with elution gradient and UV detection.

4. REAGENTS AND MATERIALS

All the reagents shall be of reagent grade and the water used shall be distilled water or water of at least equivalent purity.

4.1. Methanol, solution at 70 % (v/V) in water

4.2. Sodium acetate, 0,02 mol/l solution at pH 4,0

4.3. Sodium acetate, 0,2 mol/l solution

4.4. Imidazole formate, 6 mol/l solution

Dissolve 204 g of imidazole in 113 ml of formic acid in a 500 ml volumetric flask. Dilute to 500 ml with water.

4.5. **Internal standard**

Use sinigrin (potassium allylglucosinolate monohydrate, MW = 415,49) of which the purity should be checked as indicated in 4.5.3.

4.5.1. *Sinigrin solution 5 mmol/l*

Dissolve 207,7 mg of potassium allylglucosinolate monohydrate in water in a 100 ml volumetric flask and make up to the mark.

4.5.2. *Sinigrin solution 20 mmol/l*

Dissolve 831,0 mg of potassium allylglucosinolate monohydrate in water in a 100 ml volumetric flask and make up to the mark.

Store these solutions in a refrigerator at approximately 4 °C if the storage is for a short period (e.g. a week or less) or in a freezer at − 18 °C for longer periods.

4.5.3. *Sinigrin purity check*

Use one or more of the three following tests:

— analysis by HPLC using the present method should only give one major peak

— analysis of the intact sinigrin by HPLC (ion-pair technique) should only give one major peak

— analysis of the de-sulphated and silylated sinigrin by gas chromotography should only give one major peak

In these tests the major peak should represent at least 98 % of the total peak area.

Confirm by determining the quantity of glucose released after hydrolysis with myrosinase (thioglucoside glucohydrolase EC 3.2.3.1). The glucose should be measured by enzymatic means using a commercial test kit. It is necessary to take into account any free glucose present which should be determined without addition of myrosinase. The molar concentration of glucose measured should be at least 98 % of the molar concentration of the sinigrin solution tested.

4.5.4. *Alternative internal standard: Glucotropaeolin*

In certain seeds of the *Brassica* species or in seeds of similar species (cultivated or self-propagated) sinigrin may be present naturally. Where naturally occurring sinigrin is present, an alternative internal standard, glucotropaeolin (benzylglucosinolate, potassium salt, MW = 447,52) should be used but it is sometimes difficult to separate from other natural minor glucosinolates.

Use glucotropaeolin in the same way (5 and 20 mmol/l solutions) as sinigrin, after checking its purity according to the procedure described in 4.5.3, and verifying its response factor, in comparison with sinigrin, corresponds to that indicated in 7.2.

4.6. **Mobile phases**

4.6.1. Eluant A : (HPLC quality)

4.6.2. Eluant B : acetonitrile (HPLC quality) 20 % (v/V) solution in water (HPLC quality). The concentration may need to be modified depending on the column used.

4.7. **Ion exchange resin**

4.7.1. DEAE Sepharose Cl-6B in suspension and sold ready for use.

4.7.2. DEAE Sephadex A25 suspension prepared as follows :

Mix 10 g of resin in excess acetic acid (2 mol/l). Leave to settle. Add 2 mol/l of acetic acid until the volume of the suspension is equal to twice the volume of the settled material.

4.8. Sulphatase, *Helix pomatia* type HI (EC 3.1.6.1), purified previously and checked as indicated below, then diluted.

4.8.1. *Preparation of ion exchange columns*

Cut five Pasteur pipettes (5.9) 7 cms above the neck and place a glass wool plug (5.8) in the neck. Locate the pipettes vertically on a support and place in each a suspension of ion exchange resin DEAE Sepharose Cl-6B (4.7.1) such that, once the water has drained off, a volume of 500 µl of resin is obtained.

Pour 1 ml of imidazole formate solution 6 mol/l (4.4) into each pipette and rinse twice with 1 ml of water.

4.8.2. *Purification of sulphatase*

Weigh, to the nearest 0,1 mg, 25 mg of *Helix pomatia* type H1, dissolve in 2,5 ml of water and transfer 500 µl of this solution into each of the regenerated columns (4.8.1). Wash each column with 1,5 ml of water removing the effluent. Then add 1,5 ml of a 0,2 mol/l sodium acetate solution (4.3), recover and collect the eluates from the five columns in a test tube.

Concentrate the eluates by filtration on a Millipore PTGC 11K25 filter until the residue of approximately 100 µl is obtained (sulphatase with a molar mass in excess of 5 000 is not removed). Add 2,5 ml of water and concentrate once more by filtration until a residue of approximately 100 µl is obtained. Dilute to 2,5 ml with water and store the purified sulphatase in a freezer at − 18 °C (in small amounts in order to allow defrosting of the amount necessary for immediate use).

4.8.3. *Test of the sulphatase activity*

4.8.3.1. Preparation of a sinigrin solution 0,15 mmol/l, buffered to pH 5,8

Prepare the following solutions in succession :

(a) Transfer 1 ml of acetic acid in a 500 ml flask and make up to the mark with water.

(b) Transfer 1 ml of ethylene diamine into a 500 ml flask and make up to the mark with water.

(c) Mix 73 ml of solution (a) and 40 ml of solution (b).

(d) Adjust to pH 5,8 with solutions (a) or (b).

Pour 3 ml of the 5 mmol/l sinigrin solution (4.5.1) into a 100 ml flask and make up to the mark with solution (c).

4.8.3.2. Test of activity

A unit of activity is expressed as the production of 1 micromole of desulphated sinigrin per minute at 30 °C and pH 5,8.

For the test, transfer 2 ml of the buffered sinigrin solution (4.8.3.1) into the reference and measuring cells of the spectrometer (5.3) adjusted to a wavelength of 228 nm with a cell temperature of 30 °C. At time t = 0 ; place 50 µl of purified sulphatase (4.8.2) in the measuring cell and switch on the recorder. Stop the recorder when absorbance no longer varies (Ae), plot the tangent to the point t = 0 and measure its gradient $\frac{\Delta A}{\Delta t}$.

The activity of the sulphatase, expressed in units of activity per millilitre of sulphatase solution, is equal to

$$\frac{\Delta A}{\Delta t} \times \frac{V}{\Delta E} \times \frac{1000}{50} \times 10^6$$

where :

$\frac{\Delta A}{\Delta t}$ is the gradient of the tangent to the point t = 0, in absorbance unit per minute

V is the volume, in litres of the reacting medium (i.e. $2{,}0510 \times 10^{-3}$ l)

ΔE is the difference between the molar extinction coefficients of sinigrin and of desulphosinigrin at 228 nm

$$\Delta E = \frac{\Delta Ae}{e \times c}$$

where :

ΔAe is the difference between absorbance at equilibrium of the desulphated sinigrin and absorbance at time t = 0

e is the cell length, in centimetres (i.e. 1 cm)

c is the concentration of desulphated sinigrin at equilibrium, in moles per litre (i.e.

$$c = \frac{0{,}15 \times 10^{-3} \times 0{,}95 \times 2}{2{,}05} = 1{,}39 \times 10^{-4} \text{ mol/l}$$

The yield at equilibrium of the desulphation of the sinigrin is 0,95.

Calculate ΔE, which shall be in the order of 1 500 $l.mol^{-1}.cm^{-1}$.

The activity of the sulphatase can also be calculated using the following simplified formula :

$$\text{Activity} = \frac{\Delta A \times 5{,}7}{\Delta t \times \Delta Ae}$$

The activity found should be in excess of 0,5 $\mu mol.min.^{-1}.ml^{-1}$ of purified sulphatase solution (4.8.2).

4.8.4. *Dilution*

Using a pipette, transfer 1 ml of purified sulphatase (4.8.2) to a 10 ml volumetric flask, make up to the mark with water and mix.

Divide the solution into small quantities and store at − 18 °C.

5. APPARATUS

Normal laboratory equipment, and in particular :

5.1. High performance liquid chromatograph, capable of providing an elution gradient and control of the temperature of the column at 30 °C and connected to a UV detector permitting measurements at 229 nm.

5.2. Chromatography column for HPLC, type C18, or C8 of particle size ≤ 5 µm, or for example :

Lichrosorb RP 18 Column	≤ 5 µm (150 mm × 4,6 mm)
Spherisorb ODS2 Column	≤ 5 µm (250 mm × 4-5 mm)
Novapak C18 Column	4 µm (150 mm × 4 mm)
Lichrospher RP8 Column	≤ 5 µm (125 mm × 4 mm)
Nucleosil C18 Column	≤ 5 µm (200 mm × 4 mm)

The performance of the chosen column should be regularly checked preferably using a reference sample of rape seed desulpho-glucosinolate preferably obtained from samples from the Community Reference Bureau. In particular, the column shall not degrade 4-hydroxyglucobrassicin, an important but relatively unstable glucosinolate.

New columns shall be conditioned by the use of this method until reproducible results are obtained.

5.3. Double beam spectrometer, capable of operating in ultra violet, and, if possible, at a controlled temperature of 30 °C, fitted with quartz cells and if possible a recording system.

5.4. Microgrinder, for example, a coffee mill.

5.5. Centrifuge, for tubes of 6 ml, capable of obtaining a centrifugal acceleration of 5 000 g.

5.6. Hot water bath or heating unit, adjustable to 75 °C.

5.7. Polypropylene tubes, of 6 ml capacity.

5.8. Glass wool.

5.9. Pasteur pipettes, 150 mm long.

6. PROCEDURE

6.1. **Preparation of the test sample**

Reduce the laboratory sample in accordance with Annex II and grind in the microgrinder (5.4) for 20 seconds. Mix the meal and grind for a further five seconds.

If the seeds have a moisture content in excess of 10 % (m/m), firstly dry them using an air current at a maximum of 45 °C.

Note : when treated seeds are analysed, wash them with dichloromethane before grinding.

6.2. **Determination of moisture and volatile matter content**

Determine the moisture and volatile matter content of the test sample in accordance with Annex III.

6.3. **Test portion**

Prepare two tubes (5.7) and transfer 200 mg of test sample of oil seeds, weighed to the nearest 0,1 mg, to each.

6.4. **Extraction of glucosinolates**

Place the tubes in the hot water bath or heating unit (5.6) adjusted to 75 °C and leave there for one min. Add 2 ml of a boiling solution of 70 % methanol (4.1) and then immediately add

— in the first tube A, 200 µl of 5 mmol/l sinigrin solution (4.5.1)

— in the second tube B, 200 µl of 20 mmol/l sinigrin solution (4.5.2)

Continue heating in the bath at 75 °C for min, agitating regularly. Mix the contents of each tube and centrifuge with an acceleration of 5 000 g for three min. Transfer each supernatant to another tube (5.7).

Add 2 ml of a 70 % boiling methanol solution (4.1) to each sediment and re-heat in the bath at 75 °C for 10 min, agitating regularly. Centrifuge for three min and add the supernatant to the original. Adjust the volume to approximately 5 ml with water and mix.

This extract can be stored for two weeks, in the dark, in a freezer at − 18 °C.

6.5. **Preparation of ion exchange columns**

Cut the required number of Pasteur pipettes (5.9) i.e. one pipette per sample, so as to leave a volume of 1,2 ml above the neck and place a glass wool plug (5.8) in the neck. Place the pipettes vertically on a support.

Deposit 0,5 ml of the well-mixed suspension of DEAE Sephadex A 25 (4.7.2) in each column and leave to settle.

Rinse the columns with 2 ml of 6 mol/l imidazole formate (4.4) and then twice with 1 ml of water.

6.6. **Purification and desulphatation**

Deposit 1 ml of extract (6.4) in the prepared column (6.5) without disturbing the resin surface and leave to drain. Add twice 1 ml of sodium acetate buffer at 0,02 mol/l of pH 4,0 (4.2) leaving it to drain each time.

Deposit 75 µl of diluted purified sulphatase solution (4.8.4). Leave to act overnight at ambient temperature. Elute the desulphoglucosinolate obtained with two times 1 ml of water (HPLC quality) which is left to drain between each addition. Collect the eluate in a tube. Mix the eluate well and store, in the dark, in a freezer at — 18 °C if it is not be subjected immediately to chromatography.

6.7. **Reference test**

If required (see 7.3) carry out a reference test under the same conditions on an identical test portion, but omitting the sinigrin internal standard solution in order to detect and quantify any possible sinigrin present in the test portion.

6.8. **Chromatography**

6.8.1. *Adjustment of the apparatus*

Flow rate of the mobile phase : depends on the nature of the column (see 6.8.2)

Temperature of the column : 30 °C

Detection wavelength : 229 nm

6.8.2. *Test and elution gradient*

Using the operating instructions for the apparatus inject not more than 50 µl of the desulphoglucosinolate solution obtained in 6.6.

According to the column used a number of gradients may be appropriate :

— Spherisorb RP18 5-µm column (150 mm × 4,6 mm)
  — 100 % of eluant A (4.6.1) + 0 % of eluant B (4.6.2) for 1 minute
  — a linear elution gradient in 20 minutes until 0 % of eluant A and 100 % of eluant B are obtained
  — a linear elution gradient in 5 minutes until 100 % of eluant A and 0 % of eluant B are obtained
  — 100 % of eluant A and 0 % of eluant B for 5 minutes for equilibrating
— Lichrospher RP8 5-µm column (125 mm × 4,0 mm)
  — 100 % of eluant A for 2 minutes 30 seconds
  — a linear elution gradient in 18 minutes until 0 % of eluant A + 100 % of eluant B
  — 100 % of eluant B for 5 minutes
  — a linear elution gradient in 2 minutes of 0 % of eluant A + 100 % of eluant B until 100 % of eluant A and 0 % of eluant B are obtained
  — equilibrate for 5 minutes

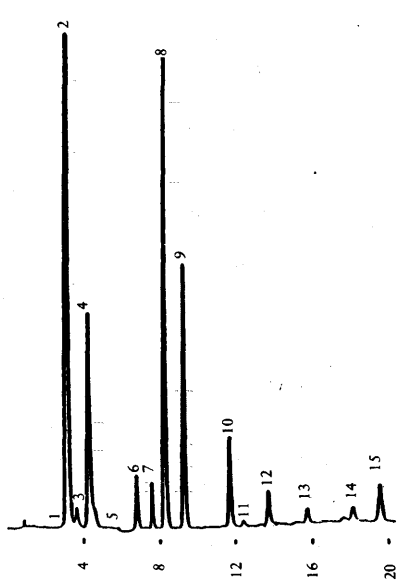
Note : the gradient profiles can be modified to give optimum separations according to the columns used.

6.8.3. *Examination of chromatograms*

Take into account only the glucosinolate peaks of area greater than 1 % of the sum total of the peak areas.

The order of elution of the peaks with a type C18 column and elution gradient of 6.8.2 is generally as follows :

1. Desulphoglucolberin
2. Desulphoprogoitrin
3. Desulphoepi-progoitrin
4. Desulphosinigrin
5. Desulphoglucoraphanin
6. Desulphogluconapoleiferin
7. Desulphoglucoalyssin
8. Desulphogluconapin
9. Desulpho-4-hydroxyglucobrassicin
10. Desulphoglucobrassicanapin
11. Desulphoglucotropaeolin
12. Desulphoglucobrassicin
13. Desulphogluconasturtlin
14. Desulpho-4-methoxyglucobrassicin
15. Desulphoneoglucobrassicin



7. EXPRESSION OF RESULTS

7.1. **Calculation of the content of each glucosinolate**

The content of each glucosinolate, expressed in micromoles per gram of whole dried seeds, is equal to :

$$\frac{\text{desulphoglucosinolate peak area}}{\text{desulphosinigrin peak area}} \times \frac{\text{internal standard quantity added to the tube in 6.4 (in µmoles)}}{\text{weight of extracted seed sample}}$$

$$\times \text{ response factor of desulphoglucosinolate (7.2)} \times \frac{100}{100 - H}$$

where :

H is the moisture and volatile matter content; expressed as a percentage by mass, of the test sample (6.2)

In certain cases, the expression of results is required at a specified moisture level (presently 9 %). In such cases multiply the result obtained for dry matter by

$$\frac{(100 - \text{moisture})}{100}$$

7.2. **Response factors**

The response factor values have been determined experimentally; they have been fixed by consensus between the different laboratories who took part and may need official revision in due course :

Desulphoglucolberin	1,07
Desulphoglucobrassicanapin	1,15
Desulphoglucoraphanin	1,07
Desulpho-4-hydroxyglucobrassicin	0,28
Desulphoglucoalyssin	1,07
Desulphoglucobrassicin	0,29
Desulphoprogoitrin	1,09
Desulphoneoglucobrassicin	0,20
Desulphoepi-progoitrin	1,09
Desulphoglucotropaeolin	0,95
Desulphosinigrin	1,00
Desulphogluconasturtlin	0,95
Desulphogluconapin	1,11
Desulphogluconapolelferin	1,00
Desulpho-4-methoxyglucobrassicin	0,25
Other desulphoglucosinolates	1,00

7.3. **Total glucosinolate content**

The total glucosinolate content, expressed in micromoles per gram of dry matter of the sample, is equal to the sum of each glucosinolate, the correspondent peak area of which is greater than 1 % of the total sum of the peak areas.

The use of the internal reference standards at two concentrations (4.5.1 and 4.5.2) makes it possible to allow for sinigrin contained in the sample or the presence of an unknown compound co-eluting with sinigrin.

— If the difference between the total glucosinolate content results of the two repeats comply with the repeatability conditions (8.2) there is no contamination of the internal reference. The result is the arithmetic average of the two determinations.

— If the difference between the results is outside the repeatability conditions (8.2) repeat the determination on two other test samples and perform a reference test (6.7) omitting the internal standard solution. The contaminant area is deducted from the internal standard to give the true area of the internal reference used in the formula in 7.1. The result is the arithmetic average of the results of the two new determinations.

8. PRECISION AND ACCURACY

8.1. **PRECISION (repeatability and reproducibility)**

An international collaborative study organized in 1988 which involved 12 laboratories each of whom made duplicate determinations on 4 colza samples gave the repeatability and reproducibility values as presented in Table 1.

The results were evaluated in accordance with ISO 5725 (Precision of test methods-determination of repeatability and reproducibility for a standard test method by inter-laboratory tests).

Table 1 : Repeatability and reproducibility values derived from a collaborative study (1988)

Samples	Rapeseed A	Rapeseed B	Rapeseed C	Rapeseed D
Number of laboratories after elimination of outliers	11	1	11	11
Average	20,6	14,1	4,9	25,6
Standard deviation of repeatability	1,7	0,6	0,3	0,8
Repeatability co-efficient of variation	8,5 %	4,4 %	6,7 %	3,3 %
Repeatability	4,9	1,7	0,9	2,4
Standard deviation of reproducibility	3,4	2,5	1,5	2,4
Reproducibility co-efficient of variation	17 %	18 %	31 %	9,4 %
Reproducibility	9,6	7,1	1,4	6,8

8.1.1. *Repeatability*

On the basis of the above (8.1) results, the difference between the value of the two determinations, carried out rapidly one after the other, by the same analyst using the same apparatus on the same trial sample, should not exceed 2 µmoles/g for contents less than 20 µmoles/g and 4 µmoles/g for the contents lying between 20 and 35 µmoles/g.

8.1.2. *Reproducibility*

On the basis of the above (8.1) results, the difference between the values of the final result obtained by two laboratories using the present method to analyse the same laboratory sample, should not exceed 4 µmoles/g for contents less than 20 µmoles/g and 8 µmoles/g for the contents lying between 20 and 35 µmoles/g.

8.2. **Accuracy**

The correct application of the method shall be verified by making replicate measurements on reference colza materials having certified total glucosinolate contents and covering the range of concern.

Suitable certified reference materials may be obtained from the Community Bureau of Reference (BCR) programme of the Commission of the European Communities.

The procedure for comparing the laboratory results obtained on the reference material with the certified value is described in the section "instruction for use" of the report which is provided with the reference materials.

9. TEST REPORT

The test report shall state the method used and the result obtained. It shall also mention any operating details not specified in the International Standard or regarded as optional, together with any events likely to have had an effect on the result.

The test report shall give all information necessary for the complete identification of the sample.'