PROGRESS OF THE X-RF METHOD FOR THE DETERMINATION OF THE TOTAL GLUCOSINOLATE CONTENT IN OILSEED RAPE AND PROCESSED MEAL

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INTRODUCTION

The change of rapeseed cropping to varieties with low glucosinolate content requires analytical methods which are fast, precise and reliable for the analysis of seeds as well as for the evaluation of the quality of processed rapeseed meal. The latest and most successful development in the field of glucosinolate (GSL) analysis is based on X-ray fluorescence spectroscopy ("X-RF method" according to Schnug and Haneklaus, 1987), which since its first international presentation 1987 on the occasion of the 7th International Rapeseed Congress in Poland, has become the most popular method for this analysis within the European Community; more than 90% of all analyses for intervention purposes have been carried out by the X-RF method during the last four years.

The X-RF method is based on two major principles: the close relationship between total sulphur (S) and total GSL content in rapeseed and the distinctive applicabilities of X-ray fluorescence spectroscopy for total S determination in organic matter.

The contribution will give a brief explanation of principles of the X-RF method as applied to seeds and processed meals and deal with the most recent protocols of analytical procedures.

APPLICATION OF THE X-RF METHOD TO SEEDS

Principles

The reason for the close relationship between total S and total GSL content is based on the fact that more than 99% of the S in rapeseed is bound in proteins and GSL, whereof the S in the protein fraction is a fairly constant factor. Therefore genetic and environmental factors exclusively cause variations in the total S content of Brassica seeds (Schnug, 1989).

According to literature and experimental data the (theoretical) maximum error by calculating the GSL content from the total S concentration caused by natural variations in the aminoacid composition is supposed to be lower than 0.5 µmol/g; as experience shows changes in total protein content of seeds within one species will result in maximum errors of less than 2 µmol/g for each percent deviation below 19 or above 25 % protein for oilseed rape (Schnug et al., 1990).

Thus the total GSL content determined by the X-RF method in seeds of a certain Brassica species is defined as the total S content minus those amounts that are bound in proteins or

single GSL that cannot be determined by direct reference methods, divided by the average stoichiometric number of S atoms occurring in the GSL fraction typical for the investigated Brassica species. This assumes that the non-protein S in Brassica seeds is predominately bound to true GSL. S-containing GSL-breakdown products may occur under some circumstances in seeds. Some examples are where samples are treated with high temperatures (e.g. oven drying with more than 120°C) or attacked by microorganisms (e.g. by mould in samples stored at more than 13% (room temperature) to 18% (refrigerated) moisture content, Aitzetmueller, 1988; Kallweit and Schnug, 1988). However, by the X-ray fluorescence method this S will still be correctly attributed to true GSL, which is an important advantage over chemical and chromatographic methods.

Analytical procedure for seeds

Materials

Reference materials. Three suitable reference materials for Brassica napus with certified total S concentrations and GSL are now available from the Commission of the European Communities (Commission of the European Communities - Community Bureau of Reference; BCR-XII/C/5; Rue de la Loi 200; B-1049 Brussels; Belgium; fax: +32 2 235 8072)

Synthetic samples for re-calibration covering the range of S content are of interest. These "setup samples" should be prepared of durable materials such as glass or plastics. It is recommended to dissolve sulphates in a lithium borate glass (Norrish and Chappel, 1967), or to impregnate cellulose powder (Linters, ashless quality, acid washed e.g. Macherey, Nagel & Co. MN 2200) with ammonium sulphate to compress after mixing with HOECHST wax "C" in aluminium cups under a pressure of 1t/cm^2. Due to a higher intensity yield in the synthetic samples (Schnug and Haneklaus, 1990a) final S concentrations between 1 and 7 mg S/g cover the whole range of interest for the purpose of total GSL analysis in Brassica seeds.

Apparatus. Spoon with a capacity corresponding to approximately 20g (about 30 ml) of seeds; Ventilated oven maintained at 85°C or microwave oven (600 W) for drying seeds if necessary; Blender (coffee mill type): 100 cm^2 volume, 8 cm diameter, 180 W power; Spatula; liquid cuvettes (e.g. CHEMPLEX 1540) covered with mylar film 6 μm gauge (e.g. CHEMPLEX 250); hand-press allowing (repeatable) application of a defined pressure (e.g. SH69-1540 ILLIT-GmbH, Hansastrasse 18, D-2300 Kiel, (FRG)); high dispersive X-ray spectrometer (dispersion better than 50 eV) with vacuum equipment (vacuum better than 1 torr is sufficient (Schnug and Haneklaus, 1990b) or helium purge (recommended e.g. OXFORD QX with the proposed settings: Rh-tube (40 kV, 4.5 mA), Ge-crystal, Ar-CH_4 flow counter, He purge, 100 seconds counting time).

Training and advice on use and application of the X-RF method are available on request from the Institute for Agricultural Ecological Innovations and Technologies (ILLIT); Hansastrasse 18; D-2300 Kiel 1; FRG.

Method

Sample preparation. If the moisture of the seed sample exceeds 10%, it must be reduced to 7-9% (this moisture content corresponds to the term "air-dry") by drying about 40 g (60 ml) of the seeds spread out in a thin layer either in a ventilated oven for 75 minutes at 85°C or in a microwave oven for 2

minutes at 600 W (Schnug and Haneklaus, 1990b). Allow samples to cool to room temperature before proceeding.

Spoon about 20 g (30 ml) of air dry seed into the blender and grind for 30 seconds. Scrape off any meal adhering to the wall of the blender by means of the spatula and grind the sample again for not more than one second, to ensure homogenisation.

Fill a liquid cuvette with homogenised meal to the upper edge. Handle the cuvettes gently after filling to avoid separation of particles of different size. Compress the meal by means of a hand-press under a pressure that reduces the volume of the meal to 35% of the original volume. In either case, the pressure applied to all samples, including the reference samples, should be the same within ±10% deviation. The amount of ground material used in the aluminium cups or liquid cuvettes depends only on their size and does not influence the determination itself. These amounts however should be kept constant for one laboratory and apparatus.

A detailed description of errors caused by different sources of variations in any step of the preparation procedure for the X-RF method is given by Schnug and Haneklaus (1990b).

Calibration for seeds

Due to the high oil content it is impossible to grind whole seeds of Brassica to the particle size which is usually required for the X-RF analysis of light elements. As the intensity peak depends upon the particle size distribution in the ground seed sample (Schnug and Haneklaus, 1990b) it is neccessary to standardise all steps of the sample preparation within one laboratory. Thus for the basic calibration of a certain X-RF spectrometer for total S in Brassica seed reference materials prepared from whole Brassica seeds are vital. To overcome the natural inhomogeneity of rapeseed samples (Schnug and Haneklaus, 1989) measurements of the intensity of the S-Kα radiation for calibration purposes must be made on three separate sub-samples of each rapeseed reference material.

In each laboratory this calibration has to be transferred to physically durable synthetic samples. It should be mentioned that differences in the intensity yield influence the S concentrations calculated for the synthetic samples after calibrating with seed standards and do not therefore represent the true S concentration in the synthetic material. These calculated values for the synthetic samples are only valid for measurement of seed samples from which the original calibration has been derived.

During routine usage the calibration of the spectrometer has to be verified twice a day by use of the synthetic standards. A check by use of whole seed standard reference material is recommended on a monthly basis.

From the total S content in the seeds the total GSL concentrations can be derived either directly from stoichiometric calculations or indirectly according to calibrations against results from direct methods for total GSL analysis, like HPLC.

For stoichiometric calculations the total GSL content in seeds of Brassica species (Y in μmol/g) can be calculated directly from the total concentrations of S (S in mg/g) and nitrogen (N in mg/g) by means of the following formula:

$$Y = (S-(N/11.5)) * 14.2$$

The direct calculation procedure, however, requires an additional total nitrogen determination in the seeds, but might be useful hints when results obtained from calibrations against certain reference methods need to be verified.

Several calibration systems for the X-RF method have been released during the three years since the X-RF method has been established (Schnug et al., 1990) which were necessary due to tremendous changes in the field of methods for direct GSL analysis. Since 1991 a set of three reference materials consisting of seeds of Brassica napus for the calibration of the X-RF method are available from the Community Bureau of Reference. For this set of samples the relation between the total concentrations for S and GSL is demonstrated in Figure 1.

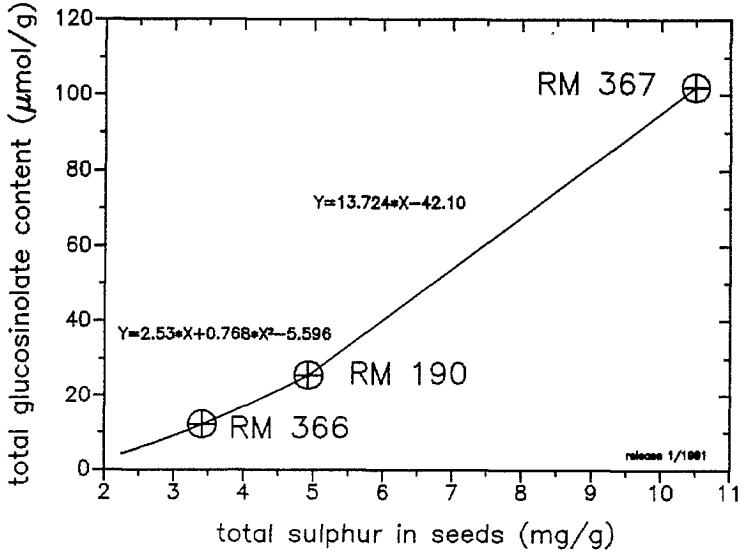

Figure 1. Relation between the total concentrations for S and GSL in three BCR reference materials and calibration functions for the X-RF method.

The calibration function for seeds of Brassica napus given in figure 1 is split into two sections with different algorithms recommended for the calculation of the total GSL content from the total S content. In the lower range the calibration function shows a non-linear shape. This is due to complex physiological interactions in the source sink relationship for S in Brassica species (Schnug, 1990 and 1988b). Typical statistical data derived from wavelength dispersive spectrometers in ringtests performed with a central calibration are 1.9 µmol/g for repeatability and 3.8 µmol/g for reproducibility (Schnug and Kallweit, 1987).

APPLICATION OF THE X-RF METHOD TO PROCESSED MEAL

Principles

The striking problem of the analysis of GSL in meals is the fact that approximately 50% of the GSL, which are originally in the seeds, are decomposed during processing and

crushing (Daun, 1986), yielding a wide spectrum of breakdown products (Gardrat and Prevot, 1987). In a model experiment Buchner (1988) detected beside intact GSL, 18 different decomposition products in hydrolysed rapeseed meal from which only 12 could be identified. In addition to this, the toxicological importance of individual intact GSL and their respective breakdown products is not fully clear.

The proposed new way for the evaluation of the GSL quality of the meal is based on the idea of a close relationship between the total S content in seeds and corresponding meals after crushing. One example supporting this relationship is the fact that only very low amounts of S are lost during crushing to extract the oil (Abraham and Man, 1987) and that the loss of volatile compounds can be evaluated from the share of steam volatile GSL in the seeds. Thus it should be possible to calculate the total S content and in that way also the total GSL content of the seeds originally used for crushing from the total S content of the meal.

This principle of a "re-identification" of the quality of the original seed from which the meal has been produced has two main advantages: first, that the total S content in organic materials can be analysed easily, rapidly and precisely by use of X-ray fluorescence spectroscopy; second the possibility to determine the meal quality (which currently has no legal definition) by seed quality, which is exactly defined by law. So a "double low rapeseed meal" could legally be defined as a meal, which is definitively produced by the crushing of a double low rapeseed.

Analytical procedure for processed meals

Materials

Reference materials. National Bureau of Standards (NBS) Washington "Citrus Leaves" NBS 1572.

Synthetic standards. A set of synthetic standards has to be prepared by spiking samples of double low meal with S by wetting the samples with ammoniumsulphate solution resulting in final S concentrations of 3-12 mg/g S. Homogenisation and grinding after drying to less than 2 % moisture are carried out as described below.

Synthetic samples for re-calibration covering the range of S (3-12 mg/g S) as described above.

Apparatus. Additional to the equipment necessary for the application of the X-RF method to seeds (listed above) only a glass plate and a glass powder-funnel of 20 cm and 15 cm diameter respectively are needed.

Method

Sample preparation. Spoon about 20 g (30 ml) of the meal onto the glass plate, spread it out in a thin layer, cover it with the glass powder-funnel (which acts as condenser for the moisture in the sample) and heat it for 60 seconds in a microwave oven.

Transfer the hot sample immediately (do not allow to cool!) to the blender and grind it for 30 seconds. Scrape off any meal adhering to the wall of the blender by means of the spatula and grind the sample again for not more than one second, to ensure homogenisation.

Fill a liquid cuvette with homogenised meal to the upper edge. Handle the cuvettes gently after filling to avoid separation of particles of different size. Compress the meal by

means of a hand-press under a pressure that reduces the volume of the meal to 35% of the original volume. In either case, the pressure applied to all samples, including the reference samples, should be the same within ±10% deviation. The amount of ground material used in the aluminium cups or liquid cuvettes depends only on their size and does not influence the determination itself. These amounts however should be kept constant for one laboratory and apparatus.

A comprehensive description of the evaluation of details of the method is given by Schnug and Haneklaus (1990c).

Calibration for processed meal

As shown by Schnug and Haneklaus (1990c) a linear relation exists between the S concentrations and the S-Kα intensity in meal samples prepared as described above. The total sulphur in the sample is derived by comparing the intensity of the S-Kα radiation with those derived from synthetic standards and the reference material.

To recalculate the total GSL content of the original seed from the total S-content of the extracted meal three principle conversions of the total S-content derived from the X-ray analysis are necessary: conversions for changes in oil and moisture content, a consideration of S-losses during processing and finally the calculation of the total GSL content in the seeds by the corrected S-content of the meal.

During processing of rapeseeds changes in oil and water content take place which cause a concentration of S in the meal. The first step in calculating the S content of the original seed (S_{seed}) from the S content in the meal (S_{meal}) is therefore the correction for changes in water and oil concentrations by use of a concentration factor (F; default value = 1,12) in the algorithm:

$$S_{seed} = S_{meal}/F$$

in which F derives from the formula:

$$F = 100/(100-((O_{seed}-O_{meal})+(M_{seed}-M_{meal}))$$

O_{seed}= oil content in seeds (default value = 42%); O_{meal}= oil content in the meal already prepared for analysis (default value = 3%); M_{seed}= moisture content in seeds (default value = 8%); M_{meal}= moisture content in the meal already prepared for analysis (default value = 2%)

Sulphur losses with the extracted oil are extremely low (Abraham and Man, 1987) and need therefore not to be considered in the calculations. The alkenyl GSL gluconapin and glucobrassicanapin,however, are steam volatile compounds and therefore may be completely lost during processing. The seed content of steam volatile GSL is in close relation to the total GSL content of the seeds the loss of S bound to those compounds during processing is at least a function of the total glucosinolate and thus the S content of the original seed (Schnug and Haneklaus, 1990c).

By the following algorithm the S content of the original seed (S_{seed}, *including S in the steam volatile GSL*) *from the* total S content in the meal (S_{seed}):

$$S_{seed} = ((S_{meal}-1.845)/(0.708/(2/F)))/F$$

Below S concentrations of 6.3 mg/g in the meal a correction for steam volatile GSL seems to be unnecessary (Schnug and Haneklaus, 1990c).

For the final calculation of the GSL content by the S concentrations in the original seed the algorithms given in figure 1 are used again.

CONCLUSIONS

The X-RF method for GSL in rapeseeds has become successful and a widely used method of which reliability improved by the availability of a central calibration system by the BCR program of the Commission of the European Communities and at least the decision of the ISO to standardises the analytical procedure.

The extension of the X-RF method to processed meals is the logical consequence of the idea that glucosinolates are important antinutritive compounds in animal diets. The proposed principle of "re-identification" assumes a linear dependency between the total GSL content of a certain seed batch and the utilization of the meal for foodstuff production, but gives no direct information about the toxicity of the meal itself. One possible way to partly compensate for this lack of information seems to be the evaluation of the amount of GSL destroyed during processing. This parameter can be calculated from the amount of free sulphate in the meal, because this compound is absent in rapeseeds and only released during decomposition by biochemical or physicochemical processes. In order to avoid enzymatic destruction of GSL during the analysis an extraction with 1m ascorbic acid is recommended. For further analytical details one should refer to Schnug (1987).

The proposed method enables a quick and precise determination of the GSL content of the seeds, which have been used for the production of a certain rapeseed meal. This method also opens the discussion about a new and simple way for the definition of the quality of rapeseed meals based on the principle: " a double low meal is one which has been processed by ensuring a double low rapeseed".

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