X-RAY FLUORESCENCE SPECTROSCOPY - A NEW METHOD FOR PRECISE, RAPID AND SIMPLE DETERMINATION OF TOTAL GLUCOSINOLATE CONTENT IN RAPESEED AND MEAL

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Introduction

The introduction of 00-oilseed rape into plant production necessitates analytical techniques which allow to separate yield batches of oilseed rape before storing respectively further process. Complementary to the time- and work-consuming reference methods of the EC (temperature controlled gaschromatography at the moment (GC) (ANONYM,1986), later on HPLC), simple rapid methods are required which produce precise results. Chemical methods either rely on an enzymatic release of glucose from the glucosinolate molecule (LEIN, 1970; SMITH and DACOMBE, 1987; SMITH et al., 1985; THIES, 1985) or formation of a coloured complex of palladium with glucosinolates (THIES, 1982). Insufficiencies with regard to correctitude, reproducibility and realization make this methods problematic for usage (MARQUARD and SCHLESINGER, 1985; FIEBIG and KALLWEIT, 1987). Common methods for the determination of glucosinolates in oilseed rape require intact molecules, so that they are not convenient for the investigation of meals.
The determination of the total glucosinolate content by X-ray fluorescence (SCHNUG and HANEKLAUS, 1987 a) is a new physical method which is applicable to the investigations of seed and meal of oilseed rape. The following contribution displays the theoretic and technical basis of the X-RF method as well as differences between this and common methods within glucosinolate analysis are discussed.

Theoretic principle of the X-RF method

The X-RF method is based on a tight relation between total S- and total glucosinolate content in rapeseed. Therefore possible causes and factors influencing the variability of this relation are of fundamental meaning for the X-RF method.

- Causes of a genetically fixed low glucosinolate content in rapeseed

JOSEFSSON and APPELQVIST (1968) observed in 1967 that BRONOWSKI, a polish spring oilseed rape variety, showed a genetically fixed low content of glucosinolates. All 00-varieties derive from this cultivar. The reason for the low glucosinolate content seems to be a metabolic block in the biochemical pathway of glucosinolates (JOSEFSSON, 1970). Neither a reduction in the uptake of sulfate, a raised enzymatic degradation of glucosinolates nor changes in the metabolism of sulfur containing amino acids (as precusors of glucosinolates) are responsible for a low glucosinolate content (JOSEFSSON, 1970; LARSEN, 1981; UNDERHILL,1980). For this reason 0- and 00- oilssed rape show equal sulfur contents in the vegetative plant material on the supposition that growth and nutritional conditions are the same (table 1). Therefore in 00-varieties a certain amount of sulfur must be bound with intermediary products of the glucosinolate metabolism.
LEIN (1972) stated that there is no de novo synthesis of glucosinolates in seeds of oilseed rape, but found out that they are produced in the siliques and to a smaller extent in other vegetative plant parts. However, there exists a mechanism which only allows a transport of intact glucosinolates into seeds. This mechanism also reasons out the lower S-concentrations in seeds of 00-oilseed rape in comparison with 0-varieties, whereas the S-content of the silique walls is correspondingly high in 00-varieties.

- Variability of sulfur fractions in seeds of oilseed rape

Sulfur is mainly bound in proteins and glucosinolates in seeds of oilseed rape; other sulfur containing compounds (e.g. APS, PAPS, sulfolipids) do not vary to such an extent and therefore their variability is unimportant (LARSEN, 1981; UNDERHILL, 1980; FRENTZEN and HEINZ, 1983).

Table 1: Sulfur content in leaves, silique walls and seeds of 0- and 00-oilseed rape grown side by side under field conditions

cultivar	plant part	glucosinolates (µmol/g)	total-S (%)	sulfate-S (%)
JET NEUF	leaves*	34.2	0.421	0.022
	silique walls**	27.4	0.147	0.051
	seeds**	100.3	0.915	0.007
RUBIN	leaves*	7.[illegible]	0.449	0.029
	silique walls**	8.5	0.411	0.296
	seeds**	17.1	0.446	0.006

(comment: * at shooting stage; ** at harvest)

In contrast with vegetative plant parts the sulfate concentration in seeds is very low (table 1). For that reason the relation between total S- and total glucosinolate content in seed is very close (r^2 96-99%), which is the presupposition for the X-RF method (SCHNUG and HANEKLAUS, 1987 a+b).
The sulfur concentration in the protein fraction amounts to 30% in 0-cultivars and 70% in 00-cultivars as regards the total S-content. Therefore the variability of amount and constitution of the protein fraction are of great importance concerning possible sources of faults made by an indirect determination of glucosinolates like the X-RF method:

- - protein quality: There exist no significant differences between oilseed rape varieties in their amino acid sequence (FORSTER, 1978; JOSEFSSON and APPELQVIST, 1968). The genetic constancy of this feature reasons for the unsuccessful trial to influence the protein quality by breeding.
The concentration of sulfur containing amino acids (especially cysteine and methionine) is little dependant on environmental factors. All trials in order to change the amino acid sequence by fertilization significantly (normal physiological conditions assumed) failed (AUGUSTINUSSEN et al., 1983; NORDESTGAARD et al., 1984; FORSTER, 1978; JOSEFSSON, 1970; WETTER et al., 1970; JOSEFSSON and APPELQVIST, 1968). The N:S ratio in the protein of oilseed rape amounts to 12:1 and expands only under extreme sulfur deficiency (JOSEFSSON, 1971). The high constancy of this ratio within one plant species (DIJKSHOORN and van WIJK, 1967) can be attributed to a regulative link of the nitrogen and sulfur reduction in plant metabolism (REUVENY and FILNER, 1977; REUVENY et al., 1977). In oilseed rape plants there is still another mechanism that guarantees the constancy of the N:S ratio even under a scanty sulfur supply, namely the dependence of the glucose splitting enzyme myrosinase on the sulfur level in the plant: UNDERHILL (1980) observed an increased myrosinase activity, when the sulfate concentration at the roots were low. Namely in a second step the enzyme myrosinase severs sulfate that becomes again available in the plant metabolism. A decreasing sulfur supply of oilseed rape will therefore first lead to a decrease in the glucosinolate content and only under extreme deficiency there will be qualitative and quantitative changes in the protein fraction (SCHNUG, 1987).

- - protein content: In comparison with protein quality the total protein content (normally 22%) shows a certain fluctuation. The average variability of ±1.5% in the protein content depends on genetic and environmental influences (BUNDESSORTENAMT, 1984; RAKOW, 1983; FINLAYSON, 1976; BLAIR and SCOUGALL, 1975).
Deviations in the protein concentration of samples from the reference material of ±1% would result in a fault of ±2 µmol/g by the X-RF method. A higher protein concentration is

a sign of a lower glucosinolate content and vice versa. The experiences with the X-RF method show that generally this correction is not necessary.
In the case of a protein correction it is possible to perform the calculation by means of the oil content, as there exists a close relation between oil and protein in oilseed rape. According to HOLMES (1980) the protein content in rapeseed can be calculated from the oil content using the following equation:

$$Y = -0.98 * X + 64.1 \quad (X = \text{oil content (\%)};\ Y = \text{protein content (\%)})$$

In meal of oilseed rape, used as feedstuff, it is also possible to determine the total glucosinolate content by the X-RF method, corresponding to the values of the seeds previously inserted in oil production. Losses of sulfur containing compounds during the whole crushing process are very low (DAUN and HOUGEN, 1976) and therefore without any meaning as concerned possible analytical mistakes.

- Relations between total S-content in seeds of oilseed rape and glucosinolate content according to different reference methods

Indirect methods like the X-RF method require a calibration with reference samples for the interpretation of the results. The X-RF method determines the total S-content of seeds , which absolute correctitude is guaranteed by use of standard reference materials respectively can be verified by independent analytical procedures (SCHNUG, 1984).
This precise as well as reproducible value is faced with chromatographically determined glucosinolate contents, which differ greatly in dependence of the method used. These differences result from the fact that all methods only seize a part of the glucosinolates occuring in seeds of oilseed rape. Gaschromatographically there are 3 (THIES, 1982) respectively 6 (ANONYM,1986) and by HPLC up to 13 (BUCHNER, 1987) of 27 (BJERG and SORENSEN,1986) glucosinolates are quantified. Correspondingly different are the relations between total S- and glucosinolate content referring to each reference method (table 2).

Table 2: Relations between the total sulfur content of rapeseed in % (X) and the total glucosinolate content according to different reference methods in µmol/g (Y)

referencemethod	regression	difference tothe stoichiometric relation	
		ppm S	µmol/g GSL
1: GC (THIES, 1982)	Y = 177.3*X - 62.0	566.9	8.5
2: GC (ANONYM, 1986)	Y = 177.7*X - 58.1	340.7	5.1
3: HPLC (BUCHNER, 1987*)	Y = 152.2*X - 48.9	280.9	4.2
4: HPLC (BUCHNER, 1987*)	Y = 160.8*X - 47.4	15.0	0.2
5: stoichiometric **	Y = 177.0*X - 51.9	---.-	-.-

(comment: *: No 3 based on 9, No 4 based on 13 individual glucosinolates analyzed by HPLC; **: concerning reference samples with 22 % crude protein; N:S = 12:1; average S-content of glucosinolates = 16,2% (SCHNUG and HANEKLAUS, 1987 a))

Seeds of oilseed rape that contain a normal protein content of 22% would show a sulfur content bound in proteins of 0,293% under the consumption of a N:S ratio of 12:1 (cp JOSEFSSON and APPELQVIST, 1968). Although the missing sulfur content can be attributed to the glucosinolate fraction nearly exclusively there is a lack of glucosinolates from the stoichiometric calculation for all reference methods (table 2).
Knowing about the problems of a determination of the real and total glucosinolate content by chromatographic procedures the idea is close to derive the glucosinolate content from the exact determinative total S-content, possibly under consideration of the protein or total N-content in seed samples, in order to achieve a precise, uniform and reproducible value for the judgement of quality. The total glucosinolate content can be calculated by means of the following equation that takes the protein content into account or by table 2/No 5:

$$\mu\text{mol/g GSL} = (A-(B/75)) * 177 \quad (A = \text{total sulfur (\%)};\ B = \text{crude protein (\%)})$$

Technical basis of the X-RF method

The indirect determination of the total S-content necessitates a precise and reproducible sulfur analysis. The X-RF analysis is especially suited for this; other methods of sulfur analysis are either time- and work-consuming (e.g. gravimetry and turbidimetry) or have not the required accuracy (e.g. combustion followed by chromatographic or conductometric detection of SO_2 (CARLO-ERBA, LECO, STRÖHLEIN).

- Preparation of samples for the X-RF method

For all physical methods of elemental analysis like the X-RF method the preparation of the samples is of special importance, in particular the reproducibility of the results requires a defined and constant structure of the samples. For the X-RF method the preparation of the seeds consists of only two steps: the grinding in a common coffee mill (e.g. BOSCH TYPE K6) and the pelleting in a hydraulic press. The water content in the seeds may not exceed 10%, because this is the limit for an unproblematic grinding, otherwise water and oil in the sample lead to an emulsion during grinding.
The X-RF method does not require intact glucosinolates, so that, though the molecular structure is partly destroyed (MAHESWARI et al., 1980) a quick drying by microwaves or high temperatures is possible (SCHNUG and HANEKLAUS, 1987 b). An adequate degree of grinding for a constant and reproducible result requires a defined amount of oilseed rape (usually 20g, whereby a volumetric measure is sufficiently accurate) and a minimum time of grinding of 30 seconds; shorter times result in slightly lower values (max. 10%), however, longer times have no influence on the results (SCHNUG and HANEKLAUS, 1987 a).
The pelleting of the samples guarantees a defined volume of the sample as well as reproducible values and moreover the sample can easily be stored in case of repetition of the analysis to a later time. The amount of grinded oilseed rape (e.g. 5g) which is put into aluminium caps can vary more than $\pm$ 20% with regard to the target without any influence on the result.
The height of pressure during pelleting has only little influence on the result, but it should be nearly constant with regard to a high reproducibility. A sufficient hardness of the tablets can be achieved with pressures of more than 0.8 t/cm^2. Another possibility to prepare the grinded samples is to put them into liquid cuvettes with windows of polycarbonat films and press them only slightly, if a storage of the samples is not necessary. The high oil content of oilseed rape prevents troubles of the measurements by matrix effects (SCHNUG and HANEKLAUS, 1987 a). Therefore meal samples should be diluted 1:1 by HOECHST-Wax-"C".

- Determination of the total S-content by X-ray-fluorescence

X-RF analysis is based on the excitation of electrons by X-rays, whereby a fluorescence radiation is emitted that is characteristic of each element. Contradictionary with common emission spectrometry (e.g. flame fotometry) the electrons of the inner shells are concerned (K- and L-shells). Tubes with Cr-anodes are preferably suited for the excitation of the S K_α-radiation. The characteristic elemental fluorescence radiation is isolated by means of an organic crystal (e.g. pentaerythrit) and then lead on a detector. The measure has to take place in vacuum or under normal atmospheric pressure in a heliumatmosphere, because the absorption of the sulfur fluorescence radiation is high. As rapeseed contains other light elements (P, K, Ca, Cl) in sometimes slightly changing concentrations, only wavelength dispersive (crystal) or high solving energy dispersive spectrometers (semiconductors) are convenient for a precise determination of sulfur in rapeseed. Energy dispersive spectrometers of simple construction (excitation with radioactive isotopes; proportional counter tube as enrgydispersive detector) are unfit, because a sufficient selectivity of measuring sulfur is not guaranteed. An equipment of those instruments with so called "ROSS-FILTERS" may raise the selectivity of the measure, but impairs sensitivity and precision at the same time. so that these instruments are not suitable for the indirect determination of glucosinolates in rapeseed and meal.
The time for measuring depends on the possible impulse rate and is therefore proportional to the capacity of the X-ray tube used. Provided a tube capacity of 1800 W, usually 1.7

counts/ppm S * second are gained. Under this consumption the detection limit amounts to 1.2 µmol/g glucosinolates for the total procedure (preparation of samples included) (SCHNUG and HANEKLAUS, 1987 a).

Comparison of the X-RF method with common rapid tests for the determination of the total glucosinolate content in oilseed rape and meal

Suitable rapid tests for the determination of the total glucosinolate content in rapeseed must satisfy the following demands:
- high agreement of the results with the reference methods of the EC
- low time-limit for the analysis
- easy performance and high security respectively tolerance of errors and
- acceptable costs

The X-RF method is in tense agreement with the official reference methods. Results which correspond with those of GC respectively HPLC methods can be obtained by a suited calibration (table 2).
Series of analysis revealed correlation coefficients of 0.96 - 0.999 by a deviation of the average values from less than 4% (SCHNUG and HANEKLAUS, 1987 b). Enzymatic (FIEBIG and KALLWEIT, 1987) or colorimetric (MARQUARD and SCHLESINGER, 1985) methods do not approximately achieve this grade of agreement. The time of 2-4 minutes for one glucosinolate analysis according to the X-RF method is very short (cleaning of the equipment included); further on drying of the samples is possible in 1-2 minutes by microwaves or high temperatures (SCHNUG and HANEKLAUS, 1987b). Common chemical rapid tests at least need 45 minutes for constant results (SMITH et al., 1985). A drying by warm air will further last 40 minutes (RÖBBELEN, 1986). Physical, non-destructive procedures confine possible faults because of their simple handling in comparison with chemical methods. The security towards errors is very high by the X-RF method, because there are only a few steps of preparation and the tolerance of errors during grinding and pelleting is also great. There are only slight deviations from the correct result even if weight, time of grinding and pressure vary to a great extent; the measure in the spectrometer itself depends not on external factors. In contrast with the X-RF method there are more than 30 steps necessary for GC and HPLC analyses and enzymatic methods which determine glucose as well as the palladium test require about 15-20 steps. Further the tolerance of errors of each step is small (precise weighing, precise addition of chemicals, exact timing of reaction times, dependence of storing conditions upon enzyme activity). Correspondingly all enzymatic tests only allow a semiquantitative determination of the glucosinolate content in comparison with the X-RF method though the procedure is complicated and time-consuming.
The costs of investment for the X-RF method are relatively high with 25000£, especially in comparison with simple rapid tests (see above). Considering factors like realization (of special interest for commerce with unskilled staff repectively great amount of samples) and agreement with the reference methods (GC and HPLC) relevant to justice, this disadvantage of the X-RF method becomes less important. The variable costs are much lower for the X-RF method than for chemical tests, because the only expendable material are aluminium caps and He-gas. Further on the costs for wages per analysis are low for a high rate of samples as well as the possibility to insert unskilled persons. The calculation of costs and time by the VDO-Mannheim (KROLL, 1986) reveals a price of about 13£ for enzymatic tests, so that under consideration of fixed and variable costs, an equilization of costs of the X-RF method with chemical tests is achieved at 2400 analyses. However, exceeding numbers of analyses lower the costs per analysis by use of the X-RF method. Therefore the costs of a single analysis depends on the amortization time of the X-RF spectrometer. A space of 8 years supposed (this corresponds to the time necessary in order to produce pure 00-qualities of less than 20 µmol/g (SAUERMANN, 1987)), the productiveness of the X-RF method will be given at an annual need of more than 300 analyses. With regard to double analyses and check up's this would correspond to 200 samples/year. Next to economic aspects of costs the particular advantages like practicability, security and precision of the X-RF method should be considered.

Summary

The introduction of 00-oilseed rape, poor in glucosinolates, necessitates rapid, precise and simple methods of analysis in order to separate different qualities of seeds. Concerned are commerce, crushers as well as official laboratories. The X-RF method is a new technique which is based on the tight relation between total sulfur - and total glucosinolate content and that is suited for rapeseed and meal in an equal manner.
The preparation of samples only consists of two easy steps: grinding and pelleting. The total procedure (preparation, measuring, cleaning of tools) needs only about 2-4 minutes per sample. Moist samples can be dried quickly by microwaves or high temperatures, because the X-RF method does not require intact glucosinolates. This new method distinguishes from common rapid tests by a high security and tolerance to errors, and furthermore a high agreement with the results from official reference methods.

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