DEVELOPMENT OF A EUROPEAN COMMUNITY METHOD FOR THE ANALYSIS OF GLUCOSINOLATES IN OILSEED RAPE

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INTRODUCTION

In 1983 the Canadian Grain Commission introduced a revised method for the analysis of rapeseed glucosinolates based on the gas chromatography of silylated desulphoglucosinolates. The desulphation method was first described by Thies (Thies 1978) and later modified (Heaney and Fenwick 1980) by the introduction of temperature programming to take account of the indole glucosinolates. This procedure was further developed for use by the Canadian Grain Commission and in 1985 the European Commission adopted a modification of the Canadian protocol as an interim Official Method for the analysis of rapeseed for subsidy purposes. The most significant difference between the two methods was the inclusion of the indole glucosinolates in the calculation of total glucosinolate content, these compounds being omitted from the Canola specification. The method used sinigrin as the internal standard and relative response factors were assigned the value of 1.0 with no adjustment for carbon number.

Conditions for silylation were critical, particularly for indole glucosinolates (Heaney and Fenwick 1982). Although these compounds were of relatively minor importance in high glucosinolate rapeseed, they represent a high proportion of the total glucosinolates in low glucosinolate rapeseed and so methods of analysis should accurately quantitate this important group of compounds. Some of these problems have since been addressed (Slominski and Campbell 1987; Hase et al. 1988) and more quantitative versions of the method are now available. Nevertheless the method suffers from some inherent disadvantages, not least the time-consuming derivatisation step and so the European Commission set up an ad hoc group of analysts from six member countries (France, Germany, The Netherlands, Denmark, Belgium and the United Kingdom) charged with the task of producing a suitable replacement method.

In achieving this objective the group identified many problems, most of which could be resolved by focussed study by individual teams. These findings were incorporated into the final protocol only after detailed discussion within the group. This paper describes such problems and explains the scientific rationale behind the submission and subsequent adoption of the final method.

The use of high performance liquid chromatography (HPLC) for the analysis of glucosinolates had already been described (Møller et al. 1985; Spinks et al. 1984) and it was considered that with further refinement HPLC would provide a suitable reference method against which other methods (e.g. national alternative procedures) would be set, whilst also providing evidence of contamination by non-rapeseed material. In October 1986, a meeting was held at Gembloux (Wathelet 1987a) at which two fundamentally different HPLC approaches were proposed, each having advantages and disadvantages.

In the first method an extract containing intact

glucosinolates is absorbed onto an ion exchanger, washed free of contaminants and the glucosinolates desorbed with sodium hydrogen carbonate which is then neutralised with hydrochloric acid. Intact glucosinolates are separated using a reversed phase column with a mobile phase consisting of phosphate buffer containing an ion-pairing reagent. The inclusion of acetonitrile as a modifier rather than methanol as reported in earlier studies, resulted in an improvement in peak shape and definition (Bjerg and Sørensen 1987).

The basis of the second method is the conversion of the glucosinolates to desulphoglucosinolates, achieved by absorbing the compounds onto a column of anion exchanger and washing free of contaminants before addition of sulphatase enzyme. The resulting desulphoglucosinolates are eluted with water and separated on a reversed phase column with a gradient of water/acetonitrile.

The HPLC analysis of intact glucosinolates is widely used in Denmark and has the advantage that all glucosinolates are measured including bound-forms, such as cinnammoyl esters; it was however concluded at the Gembloux meeting that such compounds were currently not a problem in commercial rapeseed. The meeting accepted that such compounds might in the future be found in new rapeseed lines but argued that they should be monitored at the breeding stage and taken into account only when they become a problem in commercial rapeseed. Although the relatively lower cost of equipment needed for isocratic analysis was seen as a further advantage, the experience of other groups was with the earlier version using methanol as a modifier resulting in a loss of peak shape and separation and consequently several workers had already turned to the desulphoglucosinolate method. A proposal that after a common clean-up procedure, the tentative Official Method could accommodate the analysis of either intact or desulphated glucosinolates, was rejected by the Commission as unsatisfactory and the meeting elected to develop the desulphation method for Community purposes. Similar basic principles underlie all of the methods for the HPLC of desulphoglucosinolates but the variation in detail described in different papers was considerable. It was considered important to include the best features of each of these variants with (and this is equally important) the agreement of all participants. The HPLC method was accepted by the EC in July 1990 (EC 1990).

The features of the method which attracted the most study, in the order in which they occur, as follows:

1. Sample preparation
2. Seed moisture content
3. Choice of internal standard
4. Volume of ion-exchange column
5. Ion-exchange column eluant volume
6. Sulphatase activity
7. Choice of HPLC column
8. Glucosinolate response factors
9. Validation of the method
10. 4-Hydroxyindolylmethyl glucosinolate

1. <u>Sample Preparation</u>

The procedure for defatting rapeseed prior to extraction of the glucosinolates is time-consuming, potentially inaccurate and offers no advantages over the direct extraction

of milled whole seed. Quantitation errors are inherent in the defatting method due to the need to achieve a complete removal of the oil. Studies by Wathelet have shown that similar results are obtained whether glucosinolates are extracted from defatted seed meal or from ground whole seed (Wathelet 1987b; Heaney et al. 1988). Direct extraction also facilitates the expression of the results on a seed basis. Important features of the adopted procedure are the seed moisture content (see below) and the method of grinding. Because the seed is oily there is a tendency for the resulting meal to stick to the sides of the coffee mill. It is essential therefore to grind the sample twice with thorough mixing after each pass through the mill.

2. Seed Moisture Content

In order to eliminate any possibility of glucosinolate loss due to hydrolysis by myrosinase, it was agreed to recommend the drying of seed samples to a moisture content of 10% or less. Seed grinding is also facilitated by a lower moisture content. Moist seed samples are dried in a current of air at 45°C.

3. Choice of Internal Standard

The original GC method in use by the Commission specified the use of sinigrin as an internal standard, the justification for which was firstly, the absence of this glucosinolate from commercial lines of B. napus and B. campestris and secondly its availability in relatively pure form at low cost. However, the possibility that sinigrin might occur as a contaminant due to inclusion of weed seeds necessitated the inclusion of a blank run without standard, thus increasing analysis time. Various alternative standards were suggested, including o-nitrophenyl-β-D-galactoside but the consensus was that the compound should ideally be a glucosinolate. Glucobarbarin, a glucosinolate readily isolated from natural sources, was proposed as a suitable compound since it had never been identified in rapeseed and was reported to elute from the HPLC column in a position free of other possible interfering glucosinolates. Sørensen multiplied seed for this purpose but at the time that the method was drafted, the compound had not been produced in quantity. Commercial availability of the internal standard at reasonable cost was also considered to be a prerequisite. Although already marketed by the Canadian Grain Commission and available from the Institute of Food Research, the glucosinolate glucotropaeolin was not commercially obtainable in Europe until 1988, followed by a synthesised product in 1989. Notwithstanding the small possibility that the elution position of this compound may be complicated by the presence of other compounds, glucotropaeolin has been used successfully by several laboratories for both routine purposes and for ring tests usually with a control run without standard.

In order to obviate the need for a control run the use of both sinigrin and glucotropaeolin together was considered. The presence of a contaminating peak at either internal standard position would thus be evident. This was rejected as unacceptable for reasons which included the need to stock two different standards. The final protocol incorporates a proposal from Herrmann, in which the internal standard sinigrin is included at two different levels. Calculation of results at each inclusion level would reveal any significant

contamination of the sinigrin peak and a third analysis without the internal standard would then become obligatory. Provision is made for the alternative use of glucotropaeolin when sinigrin is present naturally.

4/5. Volume of Ion-exchange Column and Eluant Volume

The volume of the ion-exchange column is determined by two factors. It should be of a sufficient capacity to absorb all the glucosinolates and other anions from the applied sample and after hydrolysis with sulphatase, the resulting desulphoglucosinolates should be eluted in a minimum amount of eluant. These conditions were determined by Thies (Thies 1978) but in the belief that a larger column equates with increased accuracy, some workers have found that a larger eluant volume is then required. The column volume specified equates approximately to the dry weight suggested by Thies but the eluant volume has been increased from 1.5 to 2ml to ensure complete elution of indole glucosinolates. This conclusion was reached on the basis of the studies of Buchner (Buchner 1987a).

6. Sulphatase Activity

The activity and purity of the sulphatase solution are of great importance and the recommended procedure to control these follows closely the findings of Quinsac and Ribaillier (1987) who studied this topic in considerable detail. After purification by passage of the sulphatase solution through columns of DEAE Sepharose Cl 6B, the eluate is concentrated with further removal of low molecular weight impurities, by filtration through a membrane filter system.

7. Choice of HPLC Column

The original method submitted to the Commission in 1987 specified two types of HPLC column. Due to perceived problems with the stability and recovery of 4-hydroxyindolylmethyl glucosinolate (see 10 below) and the improved peak separation experienced by users of different columns, it was decided that a free choice of column type should be allowed with the provision that the performance of the column should be regularly checked with a reference sample (see 9 below); in particular the column should not degrade the 4-hydroxyindolyl compound. Similarly, although two examples of solvent gradient profiles are given in the method, the analyst is free to select solvent conditions which give optimum peak separation.

8. Response Factors

It seems unlikely that samples of all glucosinolates of interest will become available in sufficient purity to permit the direct determination of response factors. The values included in the Official Method are the same as in the 1987 protocol and are based on values calculated for a detector wavelength of 229nm (Buchner 1987b) Agreement between these figures and response factors recalculated from 226nm wavelength published by other workers is close and it is considered unlikely they will be seriously in error. However, should this be shown to be the case, the method provides for a revision at a later date.

9. Validation of the Method

The Community Bureau of Reference (BCR) has recently made

available three rapeseed samples covering low to high glucosinolate content. It is recommended that the method is validated by performing replicate analyses of these samples and comparing the results with the certified values according to the directions supplied with the samples.

10. <u>4-Hydroxyindolylmethyl Glucosinolate</u>
This compound merits special mention because of its relative importance in current low glucosinolate rapeseed. 4-Hydroxyindolylmethyl glucosinolate is however, relatively unstable and much effort has been directed towards minimising losses of this compound during analysis. Extraction, desulphation and HPLC separation are just three areas where the compound has been shown to be particularly vulnerable. The possible benefit of longer extraction and desulphation times is thus negated due to increasing losses of this glucosinolate. Similarly, high HPLC column temperatures should be avoided. The final protocol is necessarily a compromise which aims to take all these facts into consideration.

CONCLUSION

The development of this EC method has been the result of dedicated study and much discussion over a long period by many scientists from Germany, France, Denmark, Belgium, The Netherlands and the UK. During this time many more groups from other European countries have taken part in ring tests. Scientists are all individuals who often hold very strong views about particular matters which may not always find support from colleagues. At such times the consensus view holds sway and a degree of compromise becomes necessary. The difficulties in achieving the goal of producing a highly complex protocol, agreed in every detail should not be underestimated and it is perhaps unsurprising that some aspects of the final procedure are not endorsed unanimously by all participants. However, we believe that the method, carefully applied by skilled analysts provides a suitable reference method, against which other more rapid techniques may be judged.

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