HPLC ANALYSIS OF GLUCOSINOLATES IN 00-RAPESEED

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With the introduction of the new 00-varieties containing low amounts of glucosinolates GLC analysis of glucosinolates was to be replaced by HPLC analysis. Different from GLC HPLC is able to measure all types of glucosinolates. Procedures using HPLC have been developed to analyse glucosinolates in their intact form (Møller et al. 1985) as well as after desulfatation (Thies 1979 and Spinks et al. 1984). The advantage of the latter "desulfo" method is a very good purification of the glucosinolates conditioned by their enzymatic desulfatation (use of sulfohydrolase H-1). But cinnamoyl derivatives of glucosinolates, the presence of which in rapeseed was reported by H.Sørensen (1986), are only detected by analysis of the intact glucosinolates. The same would meet glucosinolates carrying an additional negative charge.

This paper reports on the detailed optimization of the HPLC method of desulfo glucosinolate analysis with special attention to the indolyl glucosinolates, which play an important role in 00-rapeseed. The experiments included the following glucosinolates (GSLs): progoitrin, sinigrin, gluconapoleiferin, gluconapin, 4-hydroxy-glucobrassicin (4OHGBC), glucobrassicanapin, glucotropaeolin, glucobrassicin (GBC), gluconasturtiin and neoglucobrassicin (NeoGBC).

RESULTS AND DISCUSSION

Grinding:

No activity of myrosinase could be detected in grinded rapeseed containing less than 9 % humidity. Therefore grinding can take place before the beginning of the extraction procedure. Fineness of grinding is of lower importance for the way of extraction suggested below.

Extraction:

After some preliminary experiments work was concentrated on two ways of extraction: (1) Extraction using 2 ml of 70% methanol/water per 0.2 g seed meal at 75°C two times for 10 minutes; (2) preextraction with 1 ml 70% methanol/water for 2 minutes, after addition of 1 ml water continued extraction for another 8 minutes followed by a second extraction using 2ml of water for 10 minutes.

The results shown in Figure 1 indicate, that glucosinolates are generally better dissolved in the more aequeous solutions. Glucobrassicin is well dissolved by both procedures (not in the Figure). Both methods can be applied, but in all cases the second extraction step is necessary. Two times 5 minutes using 70 % methanol/water does not satisfy completely. Only sometimes degradation

of 4OHGBC could be observed during 20 minutes' extraction. Obvious loss occurred at extraction times of 40 minutes. Hence a special protection of 4OHGBC is not needed at an adequate extraction time of 10 minutes.

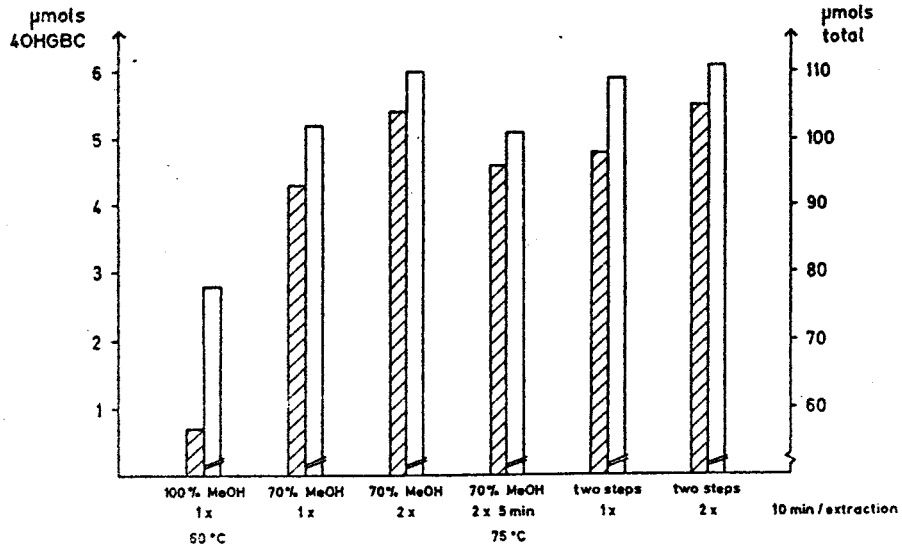

Fig. 1 Extraction of a standard sample using different solvents, numbers of extraction and times for each extraction step. Open columns represent the total sum of glucosinolates, sloped line columns 4OHGBC.

All solvents have to be added hot. The internal standard (200 µl) has to be put to the sample immediately after the first volume of solvent. After extraction volume was adjusted to 5 ml. The proposed procedures also meet the needs of an extraction of dried green material.

Lead Barium Acetate Precipitation:
After the two above mentioned extraction procedures a lead barium acetate precipitation was carried out (200 µl 0.5 M). This precipitation cleans up for glucosinolates, which is of special interest for analyses of green material. Sometimes and more often in green material, slight losses of glucosinolates up to 10 % maximum (neoglucobrassicin: 20 %) were obtained. Only the yield of 4OHGBC increased somewhat. A difference between the two discussed extraction procedures could not be secured. Hence it is difficult, to recommend the use of the precipitation. Finally however comparisons of results obtained with and without this step may cause problems.

Desulfatation:
Sang and Truscott (1984) introduced the use of 2-mercaptoethanol at pH 8 and found a twofold increase of 4OHGBC. But the use of this toxic and smelling antioxidant is not acceptable for routine analyses. Therefore the influence of pH and temperature on the enzymatic desulfatation of glucosinolates, especially 4OHGBC, fixed on an ion exchange column was investigated.

Concerning the ion exchange materials those derived from aromatic compounds or amides are not suitable. Among different products based on derivatives of sugars (Ecteola, DEAE-Sepharose, DEAE-Sephadex) DEAE-Sephadex showed the best properties. An amount of 20 mg was found to be enough to fix 2.5 µmoles of glucosinolates even in 70 % methanol/water.

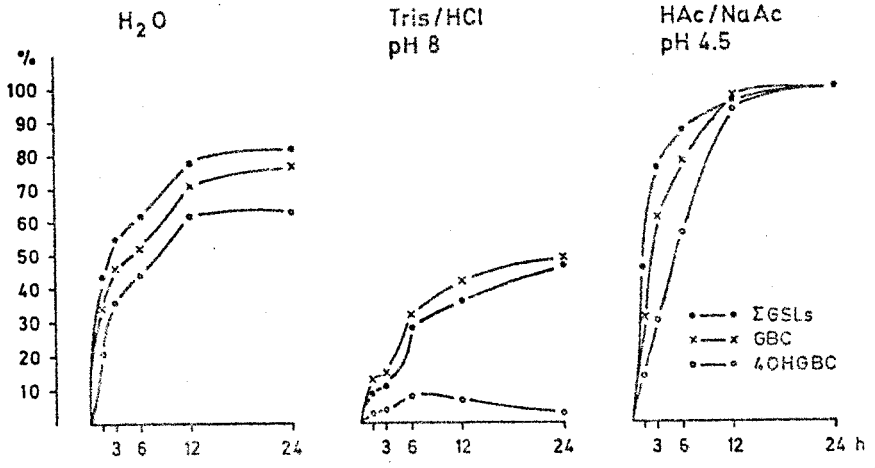

Fig.2 Desulfatation kinetics and yield of different glucosinolates (GBC, 4OHGBC) and the total amount glucosinolates (GSLs) at different pH at 39°C. The pH of the water was 4.5-5. The yield after 24 hours desulfatation at pH 4.5 is set to 100%. 75 µl of 1:5 diluted H-1 sulfohydrolase were added.

Figure 2 presents five important results:
(1) Reaching a stable glucosinolate level does not mean automatically the complete desulfatation of all glucosinolates of a sample.
(2) Because of the low yield the use of pH 8 is not suitable for the preparation of desulfo glucosinolates. Without the presence of an antioxidant degradation of 4OHGBC does take place.
(3) Low pH raises the yield of all glucosinolates, especially of 4OHGBC.
(4) It depends on the pH, which glucosinolate is preferred by the sulfatase (e.g. glucobrassicin!). This may become a source of problems, if different glucosinolates reach their maximum yield at different pH (not found yet).
(5) The use of water and buffer of identical pH gives different answers in the desulfatation process.

The maximum yield of all glucosinolates is influenced much more by pH than by temperature. The velocity of the desulfatation of glucosinolates is influenced much more by temperature than by pH. As an example for the effects of temperature Figure 3 shows its influence on 4OHGBC, which is the most affected glucosinolate.

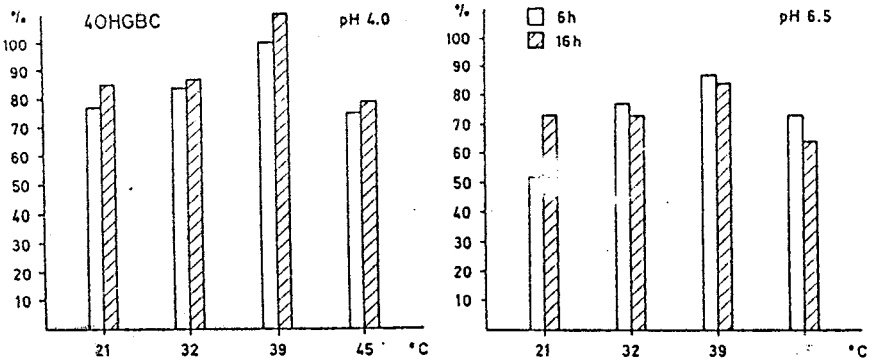

Fig. 3 Amount of 4OHGBC after 6 and 16 hours desulfatation at 4 different temperatures and 2 different pH. Yield after 6 hours desulfatation at 39°C and pH 4.0 is set to 100%. 75 µl of 1:1.5 diluted H-1 sulfohydrolase stem solution were added.

As shown in Figure 4 maximum yield of glucosinolates was reached at pH 4.0 and 39°C. Results were better than those obtained with 2-mercaptoethanol in an unbuffered environment. A glucose oxidase activity could not be detected during desulfatation under the above mentioned conditions. Good stability of the desulfo glucosinolates on the ion exchange columns was observed for a period of 16 hours. However stability especially of glucotropaeolin (internal standard!) was not satisfying at a pH above 4.5.

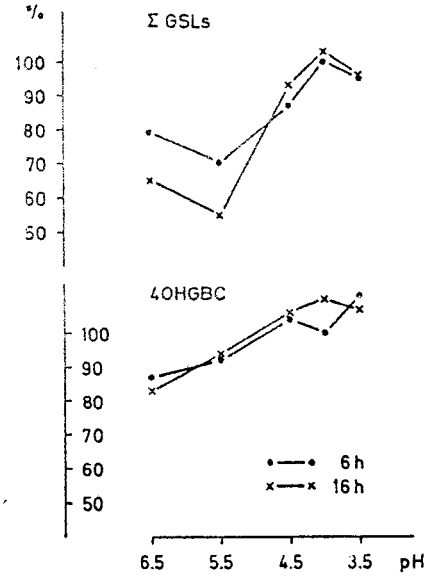

Fig. 4 Yield and stability of the total sum of glucosinolates and 4OHGBC at different pH at 39°C. Yield after 6 hours desulfatation at pH 4.0 is set to 100%. 75 µl of 1:1.5 diluted H-1 sulfohydrolase stem solution were added

Washing of the on column fixed glucosinolates with two times 1 ml of 20 mM sodium acetate buffer was most suitable to set the proposed pH conditions. 1.5 ml of water were necessary for the complete elution of all glucosinolates.

HPLC Analysis:
(Reversed phase; detection wave length: 229 nm)
The influence of HPLC-equipment and conditions of analysis on the relative UV response of single glucosinolates can exclude the use of commonly accepted relative response factors for glucosinolates.

The effect of different flow speeds on the absolute UV response of glucosinolates could be eliminated by simple calculation:
area(2) = area(1) x speed (2) / speed(1). Hence the use of an internal standard eliminates the influence of the flow speed. For an analysis time of 10 to 30 minutes the influence of different acetonitrile contents and gradient slopes was also limited to the absolute response of the glucosinolates. Relative response factors were not affected.

Temperature effected the relative response of 4OHGBC dramatically. The absolute response of all alkenyl glucosinolates, glucotropaeolin, glucobrassicin and neoglucobrassicin remained stable (Figure 5).

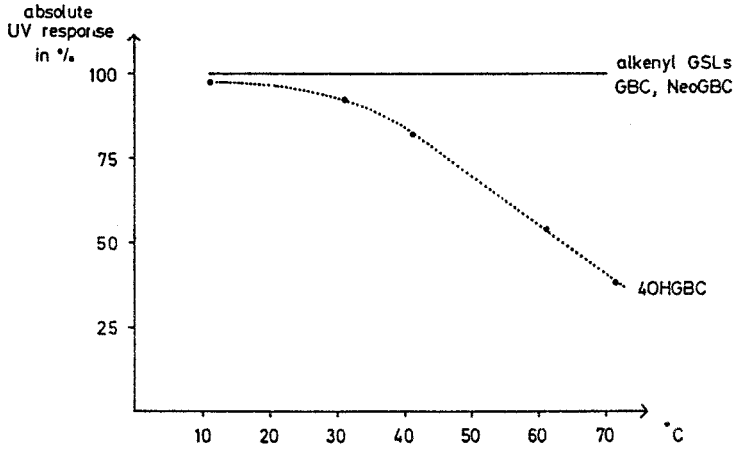

Fig. 5 One desulfo glucosinolate sample run at different temperatures of the HPLC column. The starting point of the 4OHGBC curve at the left hand side is set at random.

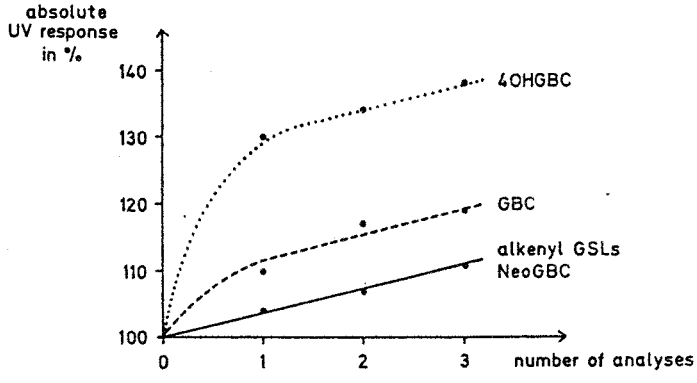

Fig. 6 Effect of replacement of water in the HPLC by 1 mM sodium phosphate solution pH 4.0 on the absolute UV response of glucosinolates.

A decreäse of the absolute response of 4OHGBC with increasing temperature could not be observed, if water was replaced by a sodium phosphate solution in the HPLC. The use of 1 mM sodium phosphate solution (pH 4.0) to protect 4OHGBC from a possible degradation on the HPLC column produced a constant increase of the absolute response of all glucosinolates at room temperature (Figure 6).

In particular the response of 4OHGBC but also that of glucobrassicin increased extraordinarily in the first run. The effect of a 5mM phosphate solution was nearly the same as shown in Figure 6. However in consequence of the instability of the response of glucosinolates the use of a phosphate solution for HPLC analyses can not be recommended.

A repetition of the experiment presented in Figure 6 one month later using the same HPLC column produced almost the same picture, but the initial strong rise of the response of 4OHGBC reached only 124%. In addition it was observed, that the detected area of 4OHGBC depends on the age and the product of the HPLC column material. As a result it is guessed, that 4OHGBC and perhaps also glucobrassicin are degraded or retained on HPLC reversed phase columns.

UV response factors: To determine HPLC response factors for glucosinolates an approach of McGregor (1985) involving the "thymol-test" has been used. The determination was performed by collection of single peaks behind the UV detector and freeze drying of the samples followed by resolving in water and execution of the "thymol-test". Results are presented in Table 1. Note, that the influence of the different typical moieties of the different glucosinolates on the colour development in the test is not known!

Table 1 HPLC response factors for some glucosinolates at 229 nm determined indirectly by use of the "thymol-test". The absolute response factors refer to conversion in µmoles glucosinolates for the HPLC equipment in Göttingen for a defined gradient system.

Glucosinolate	Number of determinations	s%	Absolute R_F-value	Relative R_F
Iberin	3	7.6	1.25518×10^{-9}	1.07
Progoitrin	13	4.1	1.27933×10^{-9}	1.09
Sinigrin	16	4.7	1.17242×10^{-9}	1.00
Gluconapin	19	5.9	1.30683×10^{-9}	1.11
4OH-Glucobrassicin	10	10.2	0.33120×10^{-9}	0.28
Glucobrassicanapin	7	5.5	1.34634×10^{-9}	1.15
Glucotropaeolin	13	5.1	1.11111×10^{-9}	0.95
Glucobrassicin	11	6.8	0.33988×10^{-9}	0.29
Neoglucobrassicin	5	5.5	0.23869×10^{-9}	0.20

A comparison of the relative response factors determined in other labs (Muuse 1987, Nyman 1986, McGregor 1985, Sang et al. 1984, Spinks et al. 1984) gives the impression, that for all alkenyl and sulfur glucosinolates the approximate relative response is close to 1, for indolyl glucosinolates 0.25 and for benzyl glucosinolates 0.9 at 229 nm.

European Collaborative Studies

The first collaborative study was finished in January 1987. The second one is on its way. R.K.Heaney, United Kingdom, B.G.Muuse, Netherlands, D.Ribaillier, France, H.Sørensen, Denmark, J-P.Wathelet, Belgium and the author participate in the studies.

The objective of the studies is to receive knowledge about
- the repeatability and reproducibility of HPLC-analysis of glucosinolates, if sample preparation is the same;
- the correct identification of peaks;
- the influence of the use of fixed relative UV response factors on the reproducibility.

Although preparation and analysis of the samples was not identical in the different labs, the results of the first collaborative study showed considerable agreement. Figure 7 presents the mean of 4OHGBC, gluconapin, progoitrin and total glucosinolate content over triple analyses of 10 different rapeseed varieties performed in the six European labs.

As expected the biggest variation occurred in the measurements of 4OHGBC. This is also expressed in the reproducibility (Figure 8), which is only high for 4OHGBC. Repeatability is about 40 %, as is reproducibility. Both were calculated in accordance with ISO/DIN5725. Agreement of the results obtained with the desulfo method to those received from the intact glucosinolates method (only performed by lab 1) is unexpectedly high (Figure 7).

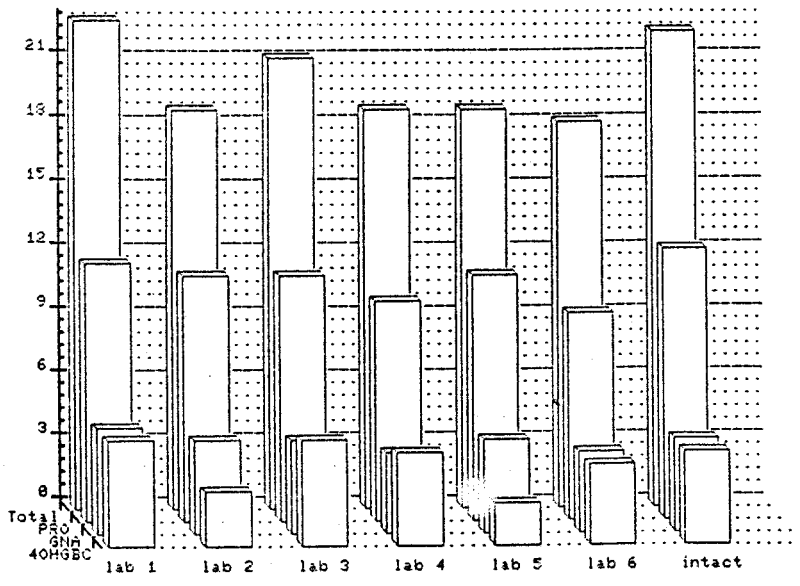

Fig. 7 First European Collaborative Study: Mean of 10 varieties: triple desulfo glucosinolate analyses in 6 labs (µmoles/g seed). Last columns represent results obtained with intact glucosinolates by lab 1.

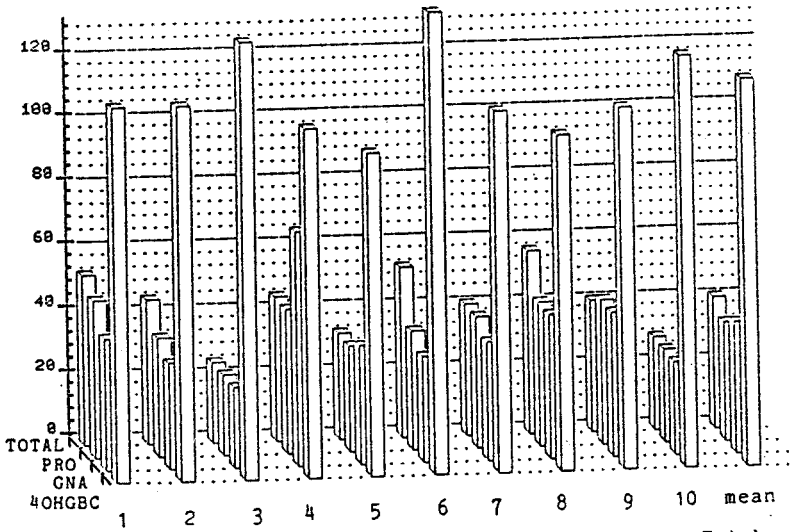

Fig 8 Reproducibility of 10 varieties in % of the mean: Triple analyses of desulfo glucosinolates in 6 labs as in Figure 7.

Gluconapoleiferin was identified by different labs and sometimes by the same lab in different samples as different peaks. Altogether there exist at least four peaks in front of gluconapin in rapeseed chromato gramms. The second peak might be glucoalyssin, the third one gluconapoleiferin and the forth one is an unknown phenyl- or benzyl-type glucosinolate.

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