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INVESTIGATION ON CHEMICAL METHODS OF ANALYSIS FOR GLUCOSINOLATES IN RAPESEED

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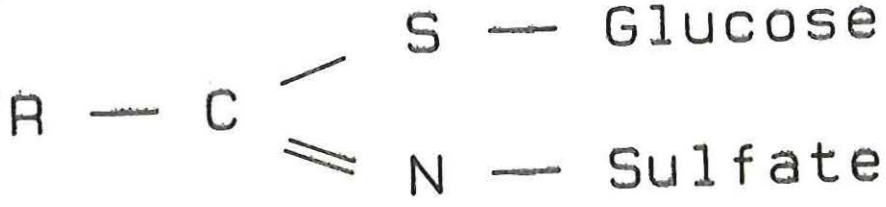
GLUCOSINOLATES



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Summary:

During the period 1985-1986 a study was done on request by the European Commission to select methods for the determination of glucosinolates (GSL) in rapeseed with low GSL content ("00"-rapeseed).

Several methods have been studied. The ISO 5504 did not determine the indolyl GSL which is one of the disadvantages of this procedure. Other methods for the determination of individual GSL in rapeseed have also been examined, methods which include the indolyls and have not the disadvantages of molecular shifts which occur after hydrolysis with the myrosinase enzyme. The different methods were compared with each other and with methods which determine the total GSL content like palladium test or glucose determinations.

Finally we concluded that the HPLC separation of desulfated GSL offers a reliable method for the control of rapeseed varieties on the content of the different GSL. However for analysing Brassica seed residues intended for animal feed this method is less suitable since it only determines the intact glucosinolates in the seed.

The calculation of the HPLC results needs calibration factors being the reciprocal value of the molar extinction coefficients in the ultraviolet range.

These molar extinction coefficients have been determined by comparing the results with gaschromatography. For the indolyl-GSL a calibration factor relative to the internal standard sinigrin is established of 0.21 at 226 nm and for progoitrin of 1.37.

To the EC Commission the HPLC method for the determination of the desulfo glucosinolates is proposed as reference method for the control on "00" rapeseed.

Contents

1. Introduction and description of the work

In January 1985 the European Commission decided to study a method for the determination of the glucosinolate content in "00" rapeseed. In the meantime the Commission accepted a Canadian method as official method for the control on the limit of 35 uMol/g seed. This gaschromatographic (GC) method determines the desulfoglucosinolates and needs silylation of the GSL. The method is laborious, vulnerable and gives artefacts.

Investigations had to be done by a group of experts to obtain a reliable HPLC method to replace the GC method. Among others the RIKILT was contracted by the EC. In this report our investigation on methods of analysis for the determination of glucosinolates in rapeseed of the "00" type is described.

April 1986 an interim report has been sent to the Commission with preliminary results of the work (RIKILT report 85.128).

1.1 Objective

The objectives of the study are:

1. Comparison of methods of interest.
2. Elaboration of the preferable method.
3. Proposal to the EC Commission.

2. Survey of methods which have been examined

2.1 Principles of the different methods

In literature a lot of methods have been described. These methods can be divided in a group using an enzymic hydrolysis of GSL with myrosinase and in a group of methods without this hydrolysis. In the schedule these methods are shown.

Method	Principle	Measured compound	Result	Unit	
With hydrolysis					
EC	Argentometric	ITC	Total ITC	-	%
ISO	GC	ITC	ITC's, separated	uMol	%
ISO	Fotometric	VTO	Progoitrin derivative	uMol	%
Litt	Enzymic	Glucose	Total GSL	uMol	-
Litt	Thymol test	Glucose	Total GSL	uMol	-

Method Principle	Measured compound	Result		Unit	
Without hydrolysis					
EC GC	Desulfo GSL	Major GSL	separated	uMol	%
Litt HPLC	Intact GSL	All GSL	separated	uMol	%
Litt HPLC	Desulfo GSL	All GSL	separated	uMol	%
Litt Palladium	Intact GSL	Total GSL		uMol	-
Litt Palladium	Desulfo GSL	Total GSL		uMol	-

2.2 Possibilities of the different methods.

2.2.1. Methods with hydrolysis of the glucosinolates.
The oldest method is the argentometric titration of the distilled volatile isothiocyanates as described in the official guideline of the European Community for control of animal feed (71/250 EEG, 1971). The hydroxy GSL compounds like progoitrin however have to be analysed separately. Also the method of ISO 5504 analyses the isothiocyanates separately from the hydroxy-GSL but separates the isothiocyanates with gaschromatography and therefore gives information on the individual alkyl isothiocyanates. The indolyl-GSL however will not be determined by this method.
For the determination of the total content of GSL also hydrolysis with myrosinase is used to liberate the glucose from the GSL followed by determination of the glucose.
The determination of glucose is possible by enzymatic methods using hexokinase or glucose oxidase but also by the very sensitive thymol method (Brzezinsky 1984, McGregor 1986, Rugraff 1986).

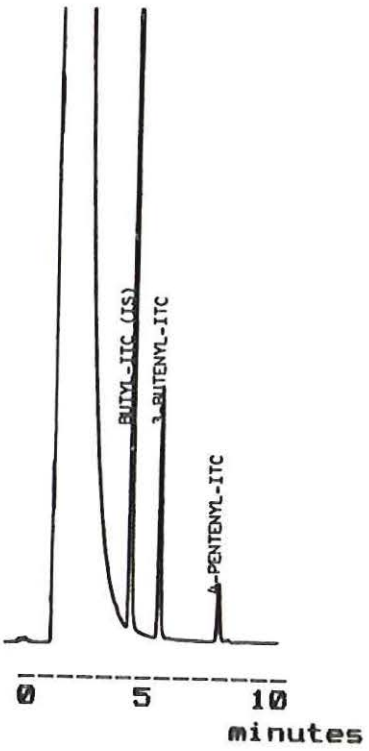
2.2.2 Intact and desulfated glucosinolate analysis.
The disadvantage of using hydrolysis for the chromatographic analysis of the individual GSL can be omitted by analysing the intact or desulfated GSL. In literature several methods are offered using these procedures. Procedures in which the intact GSL are analysed are first described by Thies (1976) applying GC and by Helboe (1980) applying HPLC.

Procedures which analysis the GSL after desulfatization are first described by Thies (1979) and Heany (1980 and 1982). They used gaschromatography for the separation of the desulfated and silylated compounds. HPLC separations of the desulfo-GSL are first described by Minchinton 1982 and Spinks 1984.
A method for the fotometric determination of the total GSL content is described by Thies (1982) and uses Palladium chloride as a chromophoor. This method is shown to be applicable for the intact GSL (Möller 1986) and also for the desulfo GSL.

2.3 Comparison of results from the different methods.

2.3.1. Methods with hydrolysis of the glucosinolates.
The argentometric European Community method for animal feed control has been compared with the ISO 5504 method. The results were identical within a difference of 10% relative. The ISO 5504 method however gives also some information on the composition of the GSL.
Progoitrin is not included in these procedures and had to be determined by the seperate fotometric method of ISO 5504. The indolyl-GSL is also not found in the gaschromatogram of ISO 5504. From the argentometric method it is not known whether the indolyl-GSL are included in the measurement or not.
A chromatogram of the ISO 5504 is given in fig.1. The GC has been applied with capillary column CP wax 57 CB; 25m x 0.22 mm.
In table 1 the results are given of a comparison of the ISO and also the Thymol test with other methods and shows that the Thymol test gives the same results as the official Community method.
The data of all individual results are tabulated in annex III-A and plotted against the HPLC desulfo-GSL results in annex IV-A.

Figure 1. Gaschromatogram of ITC's from the ISO 5504 method applied on "00"rapeseed.



2.3.2 Intact and desulfated GSL analysis.

The intact GSL can be separated by HPLC as is shown by Sörensen (1986 in Press) but the results in our lab were not reproducable since the separation was not as good as was obtained by Bjerg & Sörensen (1986). The followed procedure is described in annex I.

On the contrary the desulfated GSL analysed with HPLC were separated easily, without special precautions, giving a flat baseline and the results showed a good repeatability. In fig.2 a chromatogram is given obtained with the method for "00"-rapeseed. The procedure is described on a flowchart in annex II.

These desulfated GSL have also been separated with gaschromatography which is the official EC procedure for the control on "00" rapeseed during the period 1986-1988. Fig.3 shows a chromatogram of rapeseed analysed with this GC procedure. This method is also given on the flowchart in annex II.

Another possibility to analyse the total content of intact or desulfated GSL is given by the fotometric determination of a palladium complex with the GSL. This procedure needs the same clean up preparation as the chromatographic methods followed by the addition of palladium chloride and measuring of the absorption at 425 nm.

In table 1 the overall results are given of the GCC, HPLC and Palladium method beside the results of the methods with hydrolysis. The data of all individual results are given in annex III-B.

Table 1. General results for level comparison of different methods, expressed as recovery percentages related to the desulfo GCC method.

	ISO	GCC desulfo	HPLC desulfo	Pd	Thymol
Gluconapin	119	100	90	-	-
Glucobrassicanapin	108	100	83	-	-
Progoitrin	-	100	76	-	-
Gluconapoleiferin	-	100	68	-	-
4-OH glucobrassicin	-	100	485	-	-
Total GSL	-	100	-	115	100

Figure 2. HPLC pattern of desulfo glucosinolates in "00" rapeseed. Gradient 0-30% acetonitril in water.

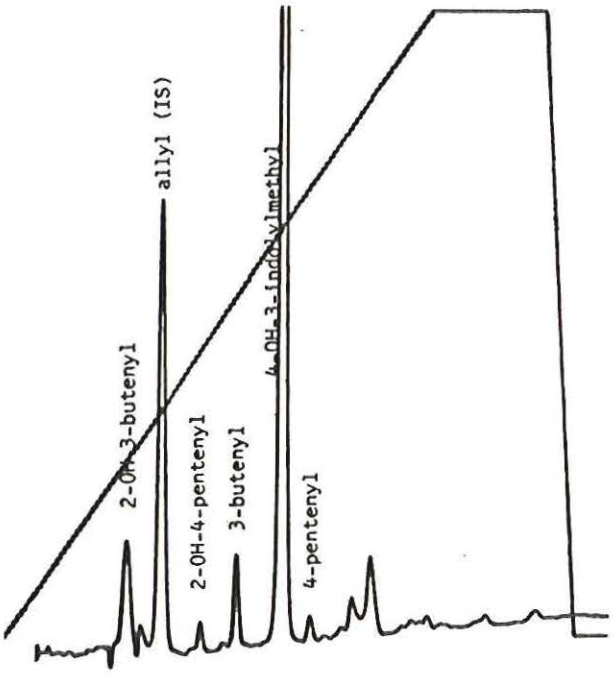
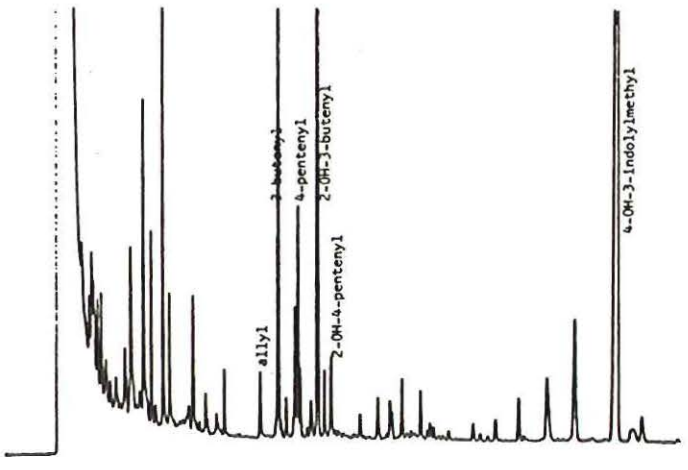


Figure 3. GCC chromatogramme of desulfo glucosinolates in "00" rapeseed.

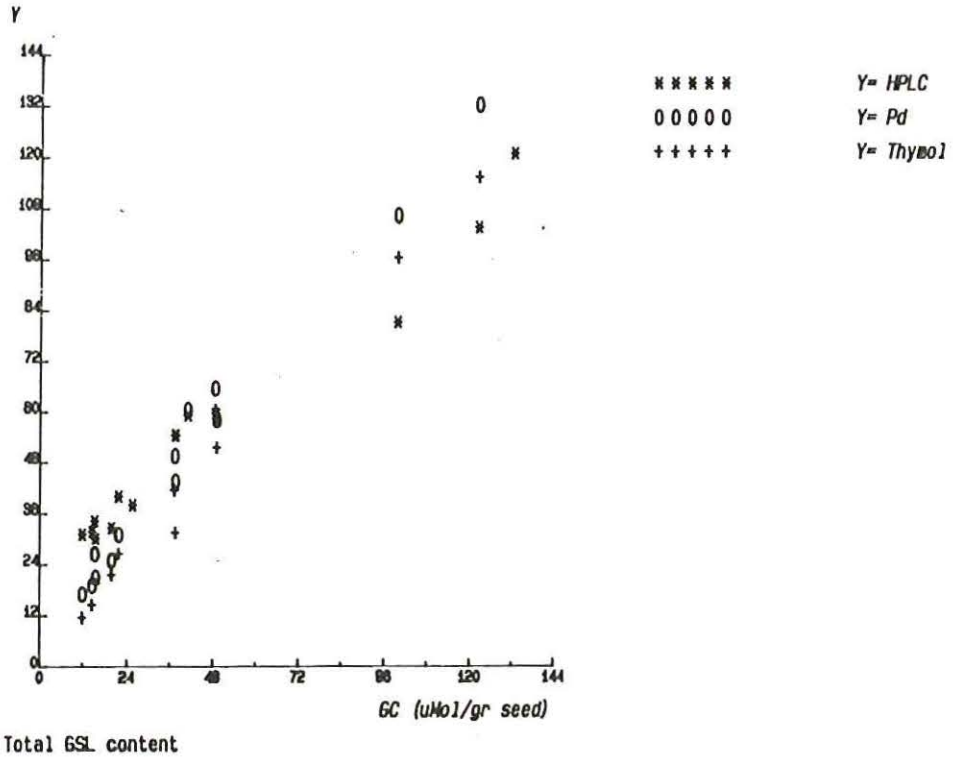


2.3.3. Comparison of GCC and HPLC results of desulfo-GSL and total GSL content of the different methods. In fig.2 and 3 chromatogrammes of the GCC and the HPLC analyses are shown. The GCC separation of the GSL is good for the alkyls, the hydroxy-alkyls as well as the hydroxy-indolyl-GSL. The other peaks are unknown but possibly a lot of artifacts are found caused by the silylation procedure. The HPLC pattern on the contrary is very clean with good separations of peaks which are all from glucosinolates.

From the results in table 1 it was concluded that the differences between the HPLC and the GCC method are caused by the lack of respons factors for the HPLC analysis. The data of the area percentages of the GCC against the HPLC method have been plotted in annex IV-B for the different glucosinolates. From the regression lines through these data the respons factors of the HPLC method were estimated. These respons factors have been applied on the HPLC data.

The results of the different glucosinolates have been summarised and the total GSL content obtained with the different methods have been plotted in figure 4 showing a good similarity of the methods.

Figure 4. Comparison of the total GSL content obtained with HPLC, GCC, Palladium and Thymol test. (DATA ANNEX IIIC)



3. Conclusions from the preliminary study.

The aim of the European Commission is to obtain a reference method for the determination of the total GSL in rapeseed with low GSL content (20-35 umol/G seed). Methods which meet this criterium and which are shown to be practicable are: the total GSL determining methods with Thymol (colorimetric determination of glucose) or with Palladium (complexometric determination of GSL) and the individual desulfo- GSL determining GCC or HPLC methods.

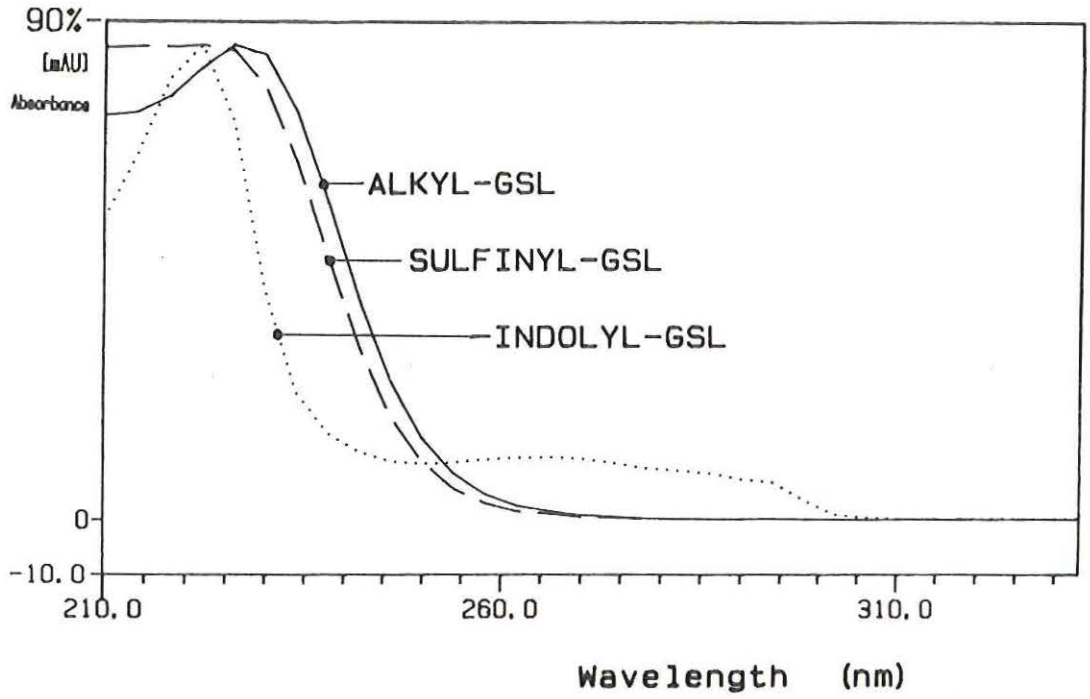
The best choice for a reference method is, in our opinion, the HPLC analysis of the desulfo GSL since this method determines all individual glucosinolates which gives more information about the rapeseed than the empirical methods of Thymol and Palladium.
The GCC method of desulfo GSL is also applicable but needs carefull and skillfull work with the problem of artifacts caused by a laborious silylation procedure.

4. Study on the HPLC analysis of desulfo-GSL

4.1 Identification of the HPLC peaks

The HPLC peaks in the chromatograms have been classified by a diode array scan of the peaks. The indolyl and benzyl GSL show a second absorption area as is illustrated in fig.5. Also with the help of the GCC results the HPLC chromatogram was identified and compared to the results in literature (Spinks 1984, Minchinton 1982, Sang 1984).

Figure 5. UV-spectrum of some desulfo-GSL Measured with diode array detection.



4.2 Determination of HPLC respons factors for UV detection.

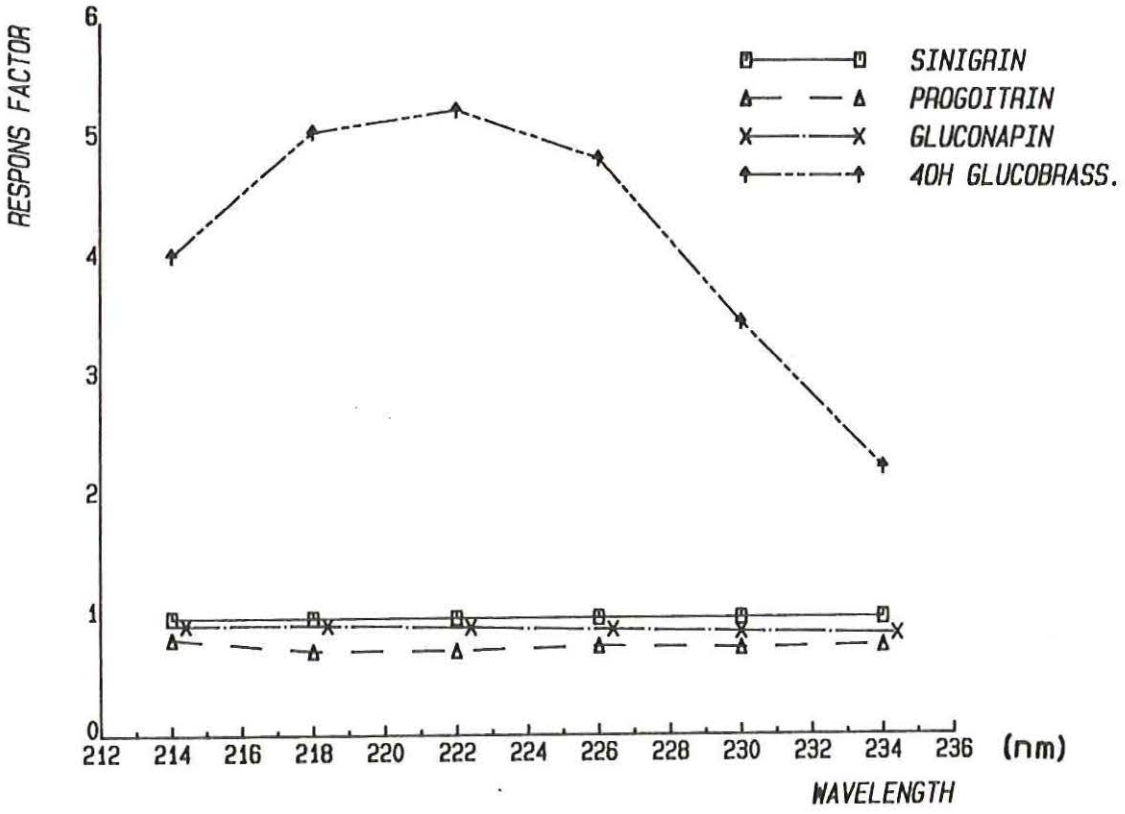
For the quantification of the results the GCC method is chosen as reference method because the flame ionisation detector (FID) is proposed to give uniform responses for the silylated compounds whilst for the HPLC measurement the molar extinction coefficients of the different GSL at 220 to 230 nm are unknown and not predictable.

The HPLC results calibrated with the GCC results show high wavelength dependent respons factors for the hydroxy-indolyl-desulfo-GSL and to a lesser extent for the alkyl-GSL. In fig.6 these respons factors relative to the internal standard sinigrin are shown at different wavelength. To convert the peak areas to the weight by mass, the areas must be divided by the corresponding respons factor.

From the graphics in annex IV-B it was concluded that at the wavelength of 226 nm calibration factors are needed for the HPLC analyses which are equal to the reciprocal respons factors. Also in literature these respons factors are studied (Sang 1984) and seem to be slightly dependent of the lab. For 4-hydroxyglucobrassicin for instance Sang (1984) published 0.27 and for progoitrin 1.01 while we found 0.21 and 1.37 respectively.

Our results have been controlled by applying the palladium test on the collected HPLC fractions of the separated desulfo-GSL and were found to be correct. Also the results of table 3 confirm the reliability of these factors compared to the thymol test for the total GSL content. The comparison of this thymol test to the GCC analysis of the desulfo-GSL has been made by McGregor (1986) showing the equality of these methods. Nevertheless the respons factors seem to be slightly system dependent and can not be applied universally by different labs. To prevent the need for each lab to investigate these respons factors, reference materials should be made.

Figure 6. Molar extinction coefficient of some GSL types relative to sinigrin and related to the wavelength.



4.4 Precision of the method.

The precision of the method is measured for our laboratorium by calculation of the internal reproducibility from threefold HPLC analyses of 15 samples over a period of 1 year. For the different glucosinolates level independent coefficients of variation (CV) for the internal reproducibility are found as tabulated in table 2.

Table 2. Coefficient of variation (CV) for the internal reproducibility for the major glucosinolates analysed with HPLC, obtained from 15 samples in 3 fold.

	concentration level uMol/g	CV
Gluconapin	1-25	6.9
Glucobrassicanapin	0-5	13.1
Progoitrin	4-90	4.8
Gluconapoleiferin	0-7	12.6
4-OH glucobrassicin	2-6	19.1
total GSL	5-100	6.2

5. Application of the HPLC Desulfo-GSL method on a cultivar line of rapeseed.

5.1 Materials and methods.

From a cultivar line of Lingot eight "00" rapeseed varieties are obtained from the Research Centre of Cultivare Testing in Slupia Wielka (Poland). These samples have been analysed by Brzezinsky with the Thymol method (Brzezinsky 1984) and by us with the HPLC desulfo-GSL method (Annex II).

5.2 Results and discussion.

In table 3 the results of of the glucosinolate composition from a cultivar line from Lingot is shown (Brzezinsky et al 1986). Lingot is the original rapeseed whereas BOH-484 is the ultimate "00" rapeseed. The hydroxy-indolylmethyl GSL is increased relatively and goes up to 50% of the total GSL content. The progoitrin and gluconapin go equally down to nearly zero.

Table 3. Content of glucosinolates in rapeseed varieties of a cultivar line from Lingot expressed in uMol/g defatted meal.
The total glucosinolate content is determined by HPLC of the desulfo-GSL and by thymol colorreaction on glucose.

Variety	Glucosinolate content					
	Thymol	HPLC				
	Total	Total	PRO	GNA	HGB	rest
Lingot	94.6	100.9	71.3	22.7	0.6	6.3
Tandem	59.3	59.2	40.4	13.0	3.3	2.5
Darmor	49.9	54.5	36.8	12.2	3.9	1.6
Linglandor	40.3	40.3	21.0	8.0	5.3	6.0
BOH 183*	25.1	24.5	12.6	4.9	5.1	1.9
Jantar	19.8	19.3	10.2	4.0	4.0	1.1
BOH 384	17.5	17.6	9.2	3.5	3.8	1.1
BOH 183*	13.3	15.5	6.9	2.7	4.7	1.2
BOH 484	9.7	11.4	4.0	1.5	5.1	0.8

* FROM DIFFERENT SOIL EN CLIMATE REGIONS
PRO=Progoitrin
GNA=Gluconapin
HGB=4-Hydroxy Glucobrassicin
(In Press: Brzezinsky et al, Cruciferae Newsletter 1986)

The thymol results compared to the HPLC total contents are very similar. From this table it is clear that the use of the respons factor for 4-hydroxy glucobrassicin of 4.85 at 226 nm is of great importance for the true value of the GSL content especially for the "00" varieties and also that the use of the respons factor for progoitrin of 0.76 is of essential influence on the results found for rapeseeds with high amounts of GSL.

6. Conclusions

The results of the different methods applied in our lab show no great differences per individual glucosinolate, either with the argentometric method or with the ISO method or the gaschromatographic determination of silylated desulfo-GSL or HPLC determination of desulfo-GSL. Also a good agreement has been found between the palladium complexation method applied on the individual desulfo glucosinolates which were obtained with HPLC and between the total GSL content measured with the Thymol test and the HPLC or GCC method.
For the HPLC analyses calibration factors have been found at 226 nm for hydroxy-indolyl desulfo GSL: 0.21 and for desulfo-progoitrin: 1.37 (equal to respons factors 4.85 resp. 0.76).
The HPLC determination of desulfoglucosinolates is found to be generally applicable without laborious precautions and resulting in clear chromatogrammes without artefacts as is the case by the gaschromatographic analysis.
When only the total GSL content is needed, fotometric methods can be used such as palladium test, thymol test or enzymic methods which determine the glucose from glucosinolates.

As reference method for the EC regulation on "00"-rapeseed we propose the HPLC method of the desulfo-GSL.

Acknowledgement

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HPLC ANALYSIS OF INTACT GLUCOSINOLATES

500 mg sample

3 X hot extraction with methanol/water 7/3

centrifugation

adsorption on Ecteola 23

elution with NaHCO3 0.1M; pH 9.0

ion pair HPLC separation

Circumstances:

Varian LC 5000
Varichrom UV detector
Lichrosorb RP18 5um, 4.6mm ID x 15 cm
Gradient: Methanol/water 55/45 ---> 65/35 (20 min)
with Fosfatebuffer 0.01M and THAB 0.006M
Flow 0.9 ml/min
UV detection 235nm
Integration peak areas with Spectra
Physics data system.

HPLC/GCC ANALYSIS OF DESULFO- GLUCOSINOLATES

100 mg sample

extraction with 2 ml water of 95°C

internal standard (sinigrine)

removal of sulfate by Ba- and Pb-acetate

centrifugation

adsorption on Sephadex DEAE A-25

washing with water

enzymatic desulfatisation overnight

elution with 3 x 0.5 ml water

HPLC	GCC
Injection of 25 ul	Evaporation of 300 ul till dry. Silylation of the GSL with MSHFBA, TMCS, Pyridin at 120 °C during 15 min.

HPLC separation

Circumstances:

Varian LC 5000
Varichrom UV detector
Lichrosorb RP18 5um, 4.6mm ID x 15 cm
Gradient: 100% water ---> 30/70 acetonitril/water (20 min.)
Flow 1 ml/min
UV detection 226nm
Integration peak areas with Spectra Physics data system.

GCC separaton

Circumstances:

Varian GC 3700
CP sil 5 CB, 25m x 0.22mm ID
Temp. 200 -----> 290°C, 3°C/min
Carriergas Helium 1.0 bar
Detector FID
Injection split mode 1: 100
Integration peak areas with Spectra Physics data system.

Comparison of ISO 5504 and HPLC method.

(Results expressed as uMol/g seed)

Sample nr	But1	But3	Pent1	Pent3
1	28.6	33.8	5.9	5.0
2	28.3	35.5	16.0	18.9
3	44.8	54.3	30.5	37.4
5	7.7	6.8	2.6	1.5
6	8.0	7.1	2.0	1.4
7	27.5	35.0	4.7	6.0
8	23.7	35.2	3.8	5.3
9	19.4	24.1	18.2	19.3
10	63.2	77.4	17.1	19.8
11	29.8	37.6	4.9	5.7
12	5.3	6.2	1.3	0.9
13	5.1	5.7	1.4	1.0
14	29.2	35.6	5.9	5.0
15	29.7	37.6	6.5	5.7
16	22.6	31.3	4.8	4.6

Explanation:

But = Gluconapin
Pent = Glucobrassicanapin

1 = HPLC determination of desulfo-GSL calculated from peak areas without respons factors

3 = ISO 5504 determination of isothiocyanates from But and Pent calculated with the respons factors as stated in the method.

ANNEX III-B

Comparison of HPLC and GCC method.

(Results expressed as uMol/g seed,
calculated without the use of respons factors)

NR.	But1	But2	Pent1	Pent2	Prog1	Prog2	Nap1	Nap2	HGB1	HGB2
21	6.3	6.9	3.2	4.0	16.6	21.8	3.9	2.0	27.7	5.2
22	1.9	2.5	0.4	0.9	3.2	5.0	0.8	0.2	26.4	5.7
23	21.0	24.5	3.1	4.8	67.2	92.8	3.2	4.4	25.1	5.1
24	2.5	3.0	0.5	1.0	9.9	14.0	0.9	1.2	22.7	5.4
31	21.3	23.8	4.5	5.6	60.5	85.3	3.2	4.1	12.7	2.8
41	1.4	1.6	0.4	0.6	2.8	3.0	0.5	0.2	24.5	5.5
42	2.3	2.5	0.6	0.8	4.9	5.5	0.6	0.3	21.8	4.4
43	3.1	3.3	0.5	0.7	6.4	6.5	0.6	0.3	18.0	3.7
44	3.5	4.5	0.6	0.9	7.4	8.7	0.5	0.3	19.1	4.4
45	4.3	4.6	1.0	1.2	8.7	9.2	0.9	0.3	23.6	5.4
46	5.5	6.3	1.9	2.8	16.0	21.3	2.1	0.9	27.4	5.3
47	7.1	8.2	3.0	4.1	15.2	18.2	3.1	1.0	24.5	4.8
48	10.6	11.9	1.0	0.6	26.0	31.1	0.7	0.6	18.3	3.8
49	11.1	11.2	1.4	1.9	29.0	30.5	1.1	0.9	15.5	3.1
50	19.6	20.1	3.3	4.6	50.8	72.7	2.9	0.4	3.1	0.9

.=missing

Explanation:

But = Gluconapin
Pent = Glucobrassicanapin
Prog = Progoitrin
Nap = Napoleiferin
HGB = 4-Hydroxy glucobrassicin

1 = HPLC determination of desulfo-GSL calculated on the internal standard Sinigrin

2 = GCC determination of silylated desulfo-GSL (EC method) calculated on the internal standard Sinigrin

Comparison of total GSL content from different methods
(Results expressed as uMol/g seed)

Sample nr	HPLC	GC (uMol/gr	Pd	Thymol
21	57.7	39.9	59.0	.
22	32.7	14.3	25.0	.
23	119.6	131.6	.	.
24	36.5	24.6	.	.
31	102.2	121.6	131.0	114
41	29.6	10.9	15.5	10
42	30.2	13.5	17.5	13
43	28.6	14.5	19.5	18
44	31.1	18.8	23.5	20
45	38.5	20.7	29.5	25
46	52.9	36.6	42.0	30
47	52.9	36.3	48.0	40
48	56.6	48.0	56.5	50
49	58.1	47.6	64.0	59
50	79.7	98.7	105.0	95

.=missing

Explanation:

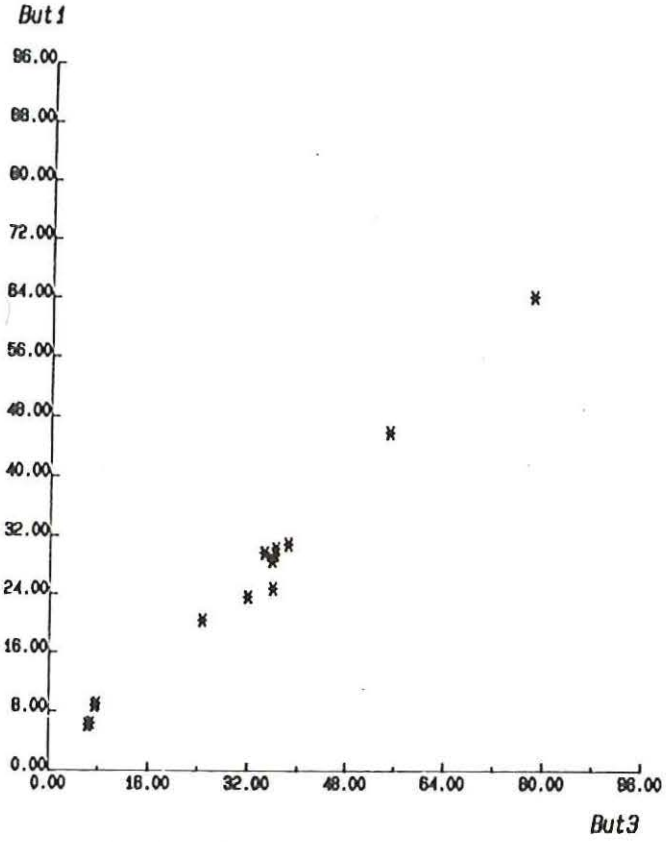
Tot HPLC = Summarized HPLC results of the different GSL calculated with the presented respons factors

Tot GC = Summarized GCC results of the different GSL calculated without respons factors

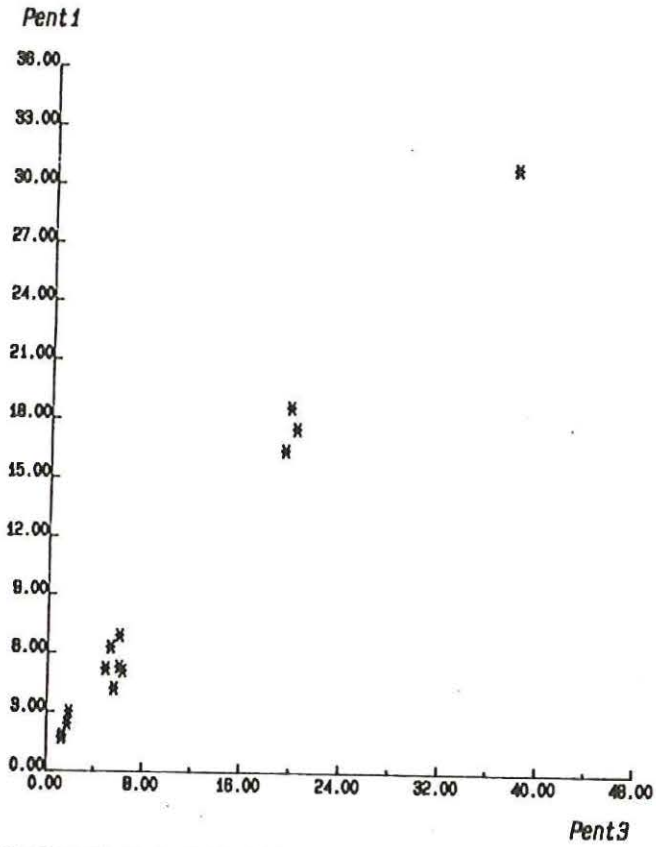
Pd = Results of the Palladium fotometric method

Thymol = Results of the Thymol test (glucose determination)

Plot of data from ISO 5504 (3) versus HPLC (without respons factors) (1) for gluconapin (But) and glucobrassicanapin (Pent). Results expressed as uMol/ g seed.

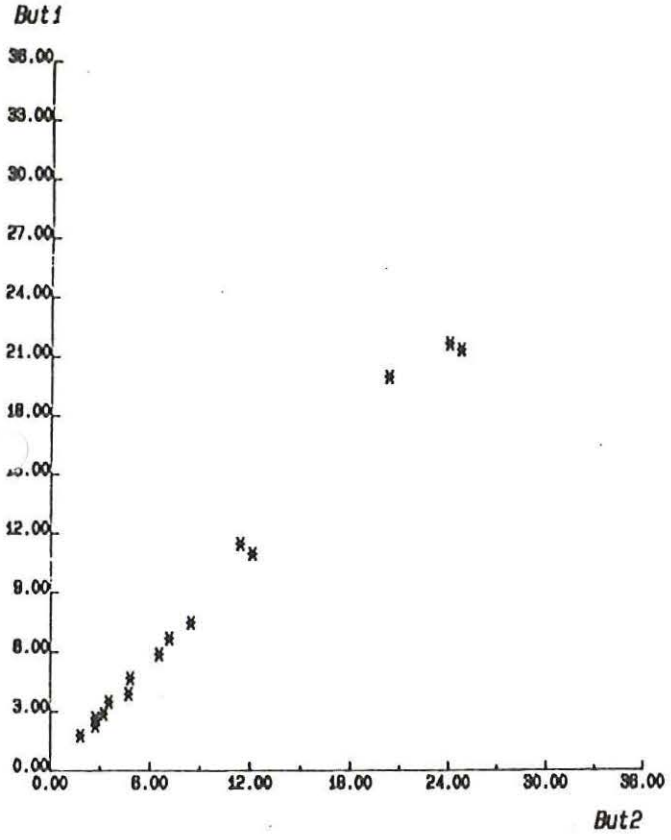


GLUCOSINOLATES IN RAPESEED



GLUCOSINOLATES IN RAPESEED

Plot of data from GCC (2) versus HPLC (without respons factors) (1) for respectively gluconapin (But), glucobrassicanapin (Pent), progoitrin (Pro), gluconapoleiferin (Nap) and 4-hydroxy glucobrassicin (HGB).
Results expressed as uMol/g seed.



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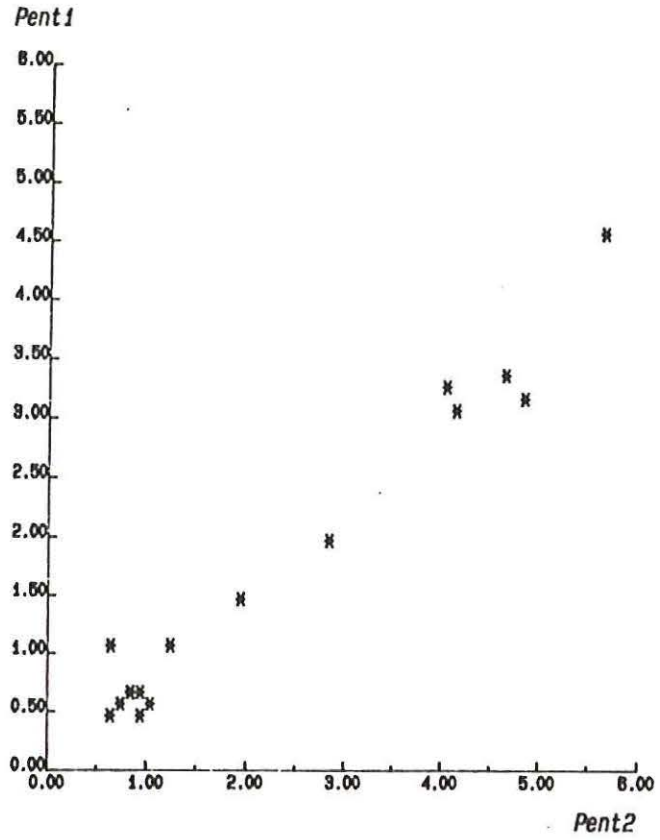

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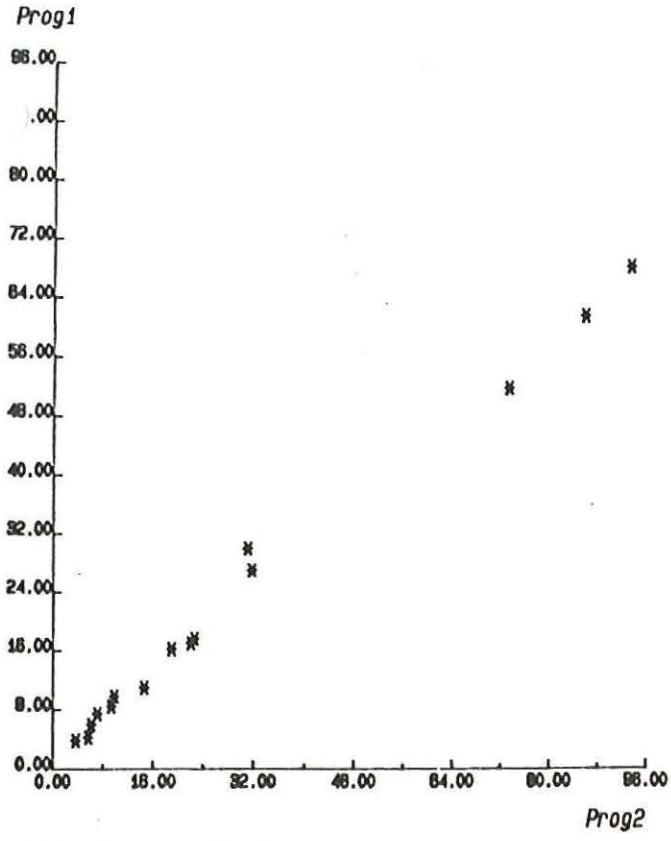

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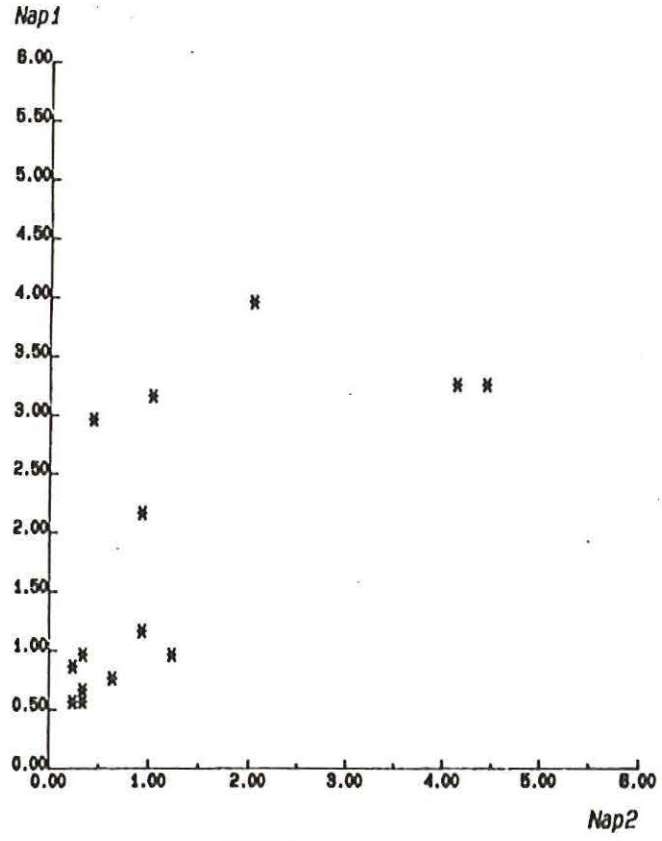

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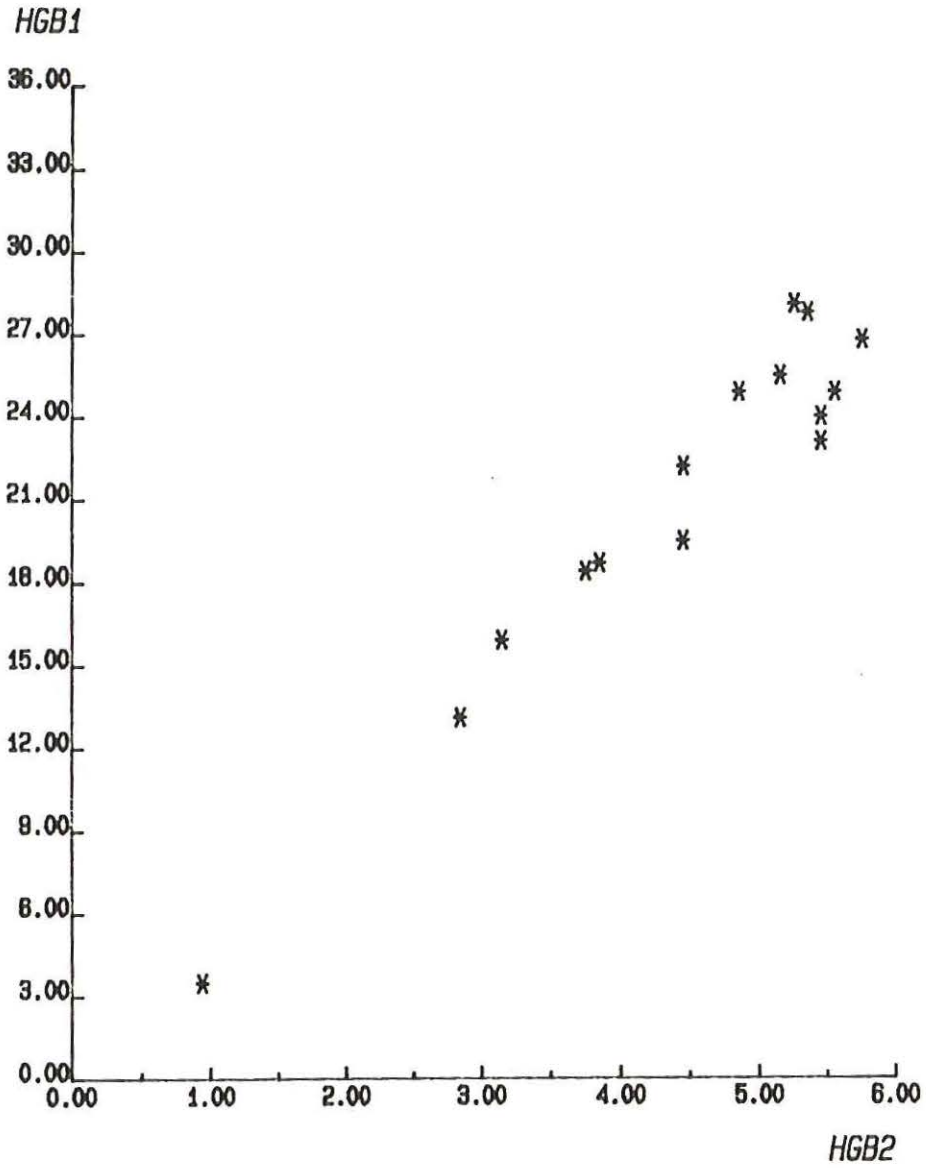

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