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Glucosinolate Analysis in Rapeseed and Canola— An Update

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Recent advances in the analysis of glucosinolates in rapeseed are reviewed. Individual glucosinolates have been determined by gas liquid chromatography of trimethylsilyl ethers after desulfation or by high performance liquid chromatography of either desulfated or intact ionic glucosinolates. Methods for estimating total glucosinolates as a group have been developed based on analysis of sulphate ion or glucose produced on hydrolysis or by spectrophotometric determination of a glucosinolate-palladium ion complex. Most methods require an ion exchange clean up procedure to isolate and concentrate glucosinolates prior to their estimation.

1 Introduction

Glucosinolates are thioesters of glucose or other sugars with the general formula shown in Fig.-1. About 90 different glucosinolates have been isolated from natural sources, principally from plants of the *Cruciferae*, *Capparidicae* and *Resedaceae* families. Glucosinolates occurring in *Brassica* species have been most studied, probably because these species contain so many food plants. Although considered to be nutritionally harmless when intact, glucosinolates have been shown to decompose in the presence of a thioglucosidase generally also present within the plant (Fig.-2). Many of the hydrolysis products have been found to be antinutrients. A general review of glucosinolates has been published recently¹⁾ and glucosinolates have often been considered in texts and reviews dealing with other naturally

occurring toxicants^{2), 3)}.

Rapeseed (*Brassica napus* L. and *Brassica campestris* L.) contains at least 16 different glucosinolates (Table-1). Other glucosinolates may be found in commercial rapeseed or rapeseed meal

Fig.-1 General structure of glucosinolates.

ISOTHIOCYANATES $R-N=C=S$
THIOCYANATES $R-S-C=N$
NITRILES $R-C\equiv N + \text{ELEMENTAL SULPHUR}$
AMINES $R-NH_2 + H_2S$

Fig.-2 Enzymatic hydrolysis of glucosinolates.

Table-1 Glucosinolates occurring in rapeseed and canola^{a)}.

Semi-systematic name of side chain	Trivial name
Major glucosinolates :	
<i>S</i> -2-Hydroxy-3-butenyl	Progoitrin
3-Butenyl	Gluconapin
4-Pentenyl	Glucobrasicanapin
1-Methoxy-3-indolyl=methyl	Neoglucobrassicin
Minor Glucosinolates :	
2-Propenyl (Allyl)	Sinigrin
<i>R</i> -2-Hydroxy-3-butenyl	epi-Progoitrin
2-Hydroxy-4-pentenyl	Gluconapoleiferin
4-Hydroxy-3-indolyl=methyl	4-Hydroxyglucobrassicin
3-Indolylmethyl	Glucobrassicin
3-Methylsulphinylpropyl	Glucoiberin
4-Methylsulphinylbutyl	Glucoraphanin
4-Methylsulphinyl-3-butenyl	Glucoraphenin
3-Methylthiopropyl	Glucoiberverin
4-Methylthiobutyl	Glucoerucin
5-Methylthiopentyl	Glucoberteroin
2-Phenethyl	Gluconasturtiin

a) D.I. McGregor, Agriculture Canada
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samples due to contamination with weed seeds. In Canadian samples, the most common weed seed contaminants have been wild mustard (*Sinapis arvensis* L.) which contains sinalbin (*p*-hydroxybenzyl glucosinolate) and stinkweed (*Thlaspi arvense* L.) which contains sinigrin (allyl glucosinolate).

The development of rapeseed cultivars containing low levels of glucosinolates has led to an increased interest in glucosinolate analysis. The general analytical chemistry of glucosinolates was reviewed in 1983⁴⁾ and brief reviews on the analysis of glucosinolates in European winter rapeseed were published as recently as the spring of 1985^{5), 6)}. This report considers the most recent developments in glucosinolate methodology, particularly developments which have occurred since the 1983 general review⁴⁾.

Classical methods for glucosinolate analysis have mostly relied on the estimation of one or more of the components formed on glucosinolate hydrolysis. These methods have been found to

Fig.-3 Comparison of results from glucosinolate procedure employing hydrolysis with results from the GLC-TMS ether method.

give erroneously low results when applied to samples with low levels of glucosinolates⁵⁾. An example of this problem is shown in **Fig.-3** where results from a collaborative study in which a hydrolysis based method⁷⁾ often gave low results in comparison with the non-hydrolytic method used by the other participants. In general, methods involving hydrolysis have been found to give problems because it is very difficult to control the direction of the reaction, particularly when certain enzyme cofactors may be present.

Two techniques have been applied in modern methods for glucosinolate analysis. One technique involves isolation of the glucosinolates followed by their chromatographic separation and individual estimation. Both gas liquid chromatography (GLC) and high performance liquid chromatography (HPLC) have been used. The second technique involves the isolation of the glucosinolates followed by a spectrophotometric estimation of the glucosinolate group. Chromophores have been produced either by complexation, or by an estimation of the glucose formed after hydrolysis of the isolated glucosinolates.

2 Reporting of Results

It has been difficult to compare results from glucosinolate analyses among much of the early literature, both because of the different methods used and because of the different ways in which the results were reported. Glucosinolates have been reported variously as a percentage (as intact glucosinolates), as mg/g or percentage as hydrolysis products, or as some function of a single hydrolysis product, often one such as butyl isothiocyanate or allyl isothiocyanate, which was not even present as a hydrolysis product in the sample being tested⁴⁾.

The molecular weight of glucosinolates present in rapeseed varies from 415 (sinigrin) to 532 (neoglucobrassicin) while the molecular weight of the biologically active hydrolysis products varies from 58 (thiocyanate ion) to 207 (1-methoxy-3-indolacetic acid). Since the relative composition of different glucosinolates and their hydrolysis products may vary from sample to sample, attempting to use the weight of a single component or even the total weight of all the components to represent the biological activity of the whole mixture could be misleading.

The problem of glucosinolate reporting was solved, to a great extent, when an international group of rapeseed analysts met in 1980 and agreed that the preferred method to express the glucosinolate content of rapeseed (and rapeseed related products) was as micromoles per gram of oil-free material⁵⁾. This removed the molecular weight discrepancy since one micromole of neoglucobrassicin is equal to one micromole of sinigrin or one micromole of thiocyanate ion or one micromole of indolacetic acid in terms of the number of molecules present. Glucosinolate reporting by this format has been generally accepted.

There has also been some confusion as to the exact reporting basis for glucosinolates. The canola definition⁶⁾ came close to supplying an exact reporting base by indicating which glucosinolates were included and the method which must be used to determine them. The problem with the canola definition was the definition of air-dried meals. For most samples in the author's laboratory, air-dried meal contained about 6% moisture. Depending on the

relative humidity in the laboratory, however, moistures ranged from 5% to 12% and the difference contributed significantly to the variation in results. It has been recommended that the glucosinolates be reported on a constant moisture basis or that the exact moisture basis be included in the analytical report¹⁰⁾.

Further confusion can arise if the glucosinolates included in a total figure are not defined. For example, the canola definition specifies the four aliphatic glucosinolates whose contents in rapeseed were reduced by plant breeders in developing low glucosinolate rapeseed. Recently, some glucosinolate analyses from Europe have included a measure of the indolyl glucosinolates as part of the total. The content of indolyl glucosinolates was not reduced in low glucosinolate rapeseed lines. They have been estimated to be present to approximately the same extent in all rapeseed and canola varieties. Since indolyl glucosinolates have been estimated to account for one third to one half the total of the major glucosinolates in canola, the inclusion of indolyl glucosinolates could have a large effect on the total figure and might cause confusion in canola trading if the results are not explained and qualified.

More confusion may occur if the EEC decides to base its glucosinolate standard on a seed basis rather than on an oil-free basis as has been the practice in the past. Most current low glucosinolate winter rapeseed varieties have more glucosinolates than the minimum defined by the canola definition but may appear to meet the canola standard if the result is expressed

Table-2 Glucosinolate content^{a)} of some *B. napus* rapeseed cultivars.

Type	Growing region	Cultivar	Glucosinolate content ($\mu\text{M/g}$)	
			Meal	Seed ^{b)}
Summer	Canada	Westar	10~15	5~8
		Regent	10~15	5~8
	Sweden	Global	10~15	5~8
		Hannah	10~15	5~8
Winter	France	Jet Neuf	120~150	67~83
		Tandem	40~60	22~33

a) Total of aliphatic glucosinolates as specified in the canola definition.

b) Assuming 45% oil (dry basis).

on a whole seed basis (Table-2).

3 Chromatographic Methods

3.1 Gas Liquid Chromatography

The first GLC separation of intact glucosinolates was carried out in 1971 by Underhill and Kirkland¹¹⁾ who directly derivatized aqueous extracts from rapeseed to their pertrimethylsilyldesulphoderivatives which rendered them sufficiently volatile to be separated by gas liquid chromatography. The methodology was refined by Thies who introduced ion exchange purification¹²⁾ and enzymatic desulphation¹³⁾.

Silylation reagents have included hexamethyldisilazane and trimethylchlorosilane¹¹⁾, *N,O*-bis-(trimethylsilyl) trifluoroacetamide¹⁴⁾, and, most preferably, *N*-methyl-*N*-trimethylsilyl-heptafluorobutyramide in combination with trimethylchlorosilane¹⁵⁾. This last mixture was found to give complete derivatization and to minimize detector contamination. A silylation temperature of 120°C was found to be necessary to give complete derivatization¹⁶⁾.

Originally, chromatographic separations were carried out isothermally, using packed columns

with SE-52 liquid phase. OV-7 liquid phase has been used more recently⁸⁾. Glass columns have been used as metal columns promoted decomposition of the TMS-derivatives. Isothermal conditions allowed only the alkenyl glucosinolates to be determined but the introduction of temperature programming by Heaney and Fenwick¹⁵⁾ allowed the separation of aliphatic and aromatic glucosinolates including indolyl glucosinolates. A typical chromatogram using the last technique is shown in Fig.-4.

Fig.-4 Separation of trimethylsilyldesulfoglucosinolates from rapeseed on a 2 m×4 mm column of Supelcoport with 2%OV-7 and temperature programming as described in Reference ¹⁰⁾. Glucosinolates identified: A=allyl-, B=butenyl, C=4-pentenyl, D=2-hydroxy-3-butenyl-, E=2-hydroxy-4-pentenyl, F=1-hydroxy-3-indolylmethyl.

Fig.-5 Separation of trimethylsilyldesulfoglucosinolates from rapeseed on a 10 m×0.32 mm fused silica column coated with SPB 20 (0.25 μm). Program 210°C (1 min), 10°C/min to 280°C (0.1 min). Flow 30 cm/s. Glucosinolates identified: A=3-butenyl, B=4-pentenyl, C=2-hydroxy-3-butenyl, D=2-hydroxy-4-pentenyl, E=indolylmethyl, F=1-hydroxy-3-indolylmethyl-. Chromatogram reproduced courtesy of F.W. Hougen, Dept. of Plant Science, University of Manitoba.

Table-3 Reproducibility of glucosinolate analyses : Results on a single sample analyzed by laboratories using GLC method of the Canadian Grain Commission : Canola check sample series¹⁸⁾.

Laboratory	Glucosinolate content ($\mu\text{M/g}$ oil-free dry basis ^{a)})							
	1980	1981	1982	1983	1984	Mean	Std. dev.	
1	14	9	—	15	14	13		2.7
2	—	15	17	17	14	16		1.5
3	—	12	—	15	15	14		1.7
4	13	12	12	14	14	13		1.0
5	—	—	13	14	15	14		1.0
Mean	14	12	14	15	14		14	
Std. dev.	0.7	2.5	2.6	1.2	0.6			1.7

a) Sum of 4 alkenyl glucosinolates as in the canola definition.

Open tubular GLC¹⁷⁾ has been used recently to separate rapeseed glucosinolates (**Fig.-5**).

The GLC method developed by Thies¹³⁾ and modified by Heaney and Fenwick¹⁵⁾ was adopted as the official method for determining glucosinolates in canola. The method was published in detail by the Canadian Grain Commission⁸⁾ and has been used by the Canadian canola industry since 1982. Many Canadian laboratories have participated in an ongoing collaborative study¹⁸⁾ and results since 1982 indicate that an overall accuracy of $\pm 2 \mu\text{M/g}$ has been achieved by laboratories using the CGC procedure (**Table-3**). The ISO and the EEC have also considered a GLC method modelled on the Canadian method. A first collaborative study using the ISO method showed a within laboratory precision of $\pm 5\%$ and a between laboratory precision of $\pm 20\%$ ¹⁹⁾.

The proposed ISO method differed from the Canadian procedure in two ways. First, the ISO procedure allowed the analyst to carry out the analysis using full-fat ground seed. The Canadian procedure contains very specific instructions for oil removal and for obtaining a fine grind to ensure complete extraction of glucosinolates. Recent experiments, however, have suggested that fine grinding and oil removal may not be as important as previously supposed. Secondly, the ISO procedure assigned a response factor of one to all glucosinolates analysed while theoretical carbon number response factors were used in the Canadian procedure. The use of theoretical carbon number response factors gave almost the same values

Table-4 Effect of response factors on GLC analysis of glucosinolates.

Sample	Glucosinolate content ($\mu\text{M/g}$ oil-free dry basis ^{a)})	
	With response factors	Without response factors
Westar	15	14
Tobin	28	28
Torch	69	71

a) Sum of 4 alkenyl glucosinolates as in the canola definition.

for total glucosinolates in canola (**Table-4**) although values for individual glucosinolates differed. A further ISO sponsored round robin study has been planned for 1985/86.

3.2 High Performance Liquid Chromatography (HPLC)

It seems probable that by the time organizations such as the ISO or AOAC adopt a gas liquid chromatographic procedure, most laboratories will be using HPLC for glucosinolate separations. Glucosinolates are well suited for HPLC being water soluble and non-volatile. Analytical methods published to date have included determination of both intact glucosinolate (using a counter-ion technique), and determination of desulphoglucosinolates using conventional reversed phase HPLC.

HPLC of intact glucosinolates using a counter ion was first described in 1980²⁰⁾. An updated version of this procedure²¹⁾ has been adopted by many laboratories in Europe. Glucosinolates were extracted from ground seed with water. The extract was purified by ion exchange on Ecteola-cellulose acetate. The gluco-

Fig.-6 HPLC separation of intact glucosinolates isolated from rapeseed on a reversed phase C18 column. Mobile phase was 0.01 molar (pH 7) phosphate with 5 mM tetraheptylammonium bromide counter ion. Modified with 62.5% methanol²¹⁾. Glucosinolates identified: A=2-hydroxy-3-butenyl-, B=2-hydroxy-4-pentenyl-, C=3-butenyl-, D=4-hydroxy-3-indolylmethyl-, E=3-indolylmethyl.

sinolate ions were eluted with bicarbonate ion and the eluate neutralized with hydrochloric acid. The glucosinolates were separated on a C18 reversed phase column using a mobile phase of 62.5% methanol in 0.01 M phosphate buffer which was 5 mM in tetraheptylammonium bromide. Detection was at 235 nm (**Fig.-6**). Being isocratic, this method can be carried out using relatively inexpensive equipment. Unfortunately, the use of buffers and counter ions is hard on HPLC equipment and there is some question about the ability of the procedure to resolve some of the important glucosinolates in rapeseed. The preferred reference standard (2-hydroxy-2-phenethyl glucosinolate) is difficult to prepare outside of Europe. For quantitative analysis, it is necessary to use response factors, ranging from 1.16 to 0.27 depending on the molar extinction coefficient for different glucosinolates at 235 nm.

Desulfoglucosinolates, prepared as described for GLC also have been analyzed by HPLC. Since desulfoglucosinolates are nonionic, they have been separated by conventional reversed phase HPLC^{22), 23)}. To date, C18 reversed phase columns, and gradient elution from 1 to 20% acetonitrile in water have been used to obtain separations (**Fig.-7**). In a method which will be proposed to AOAC, McGregor has simplified the calculation of response factors by collection of peaks from the chromatograph

Fig.-7 HPLC separation of desulfoglucosinolates isolated from rapeseed on a reversed phase C18 column. Gradient elution from 2% acetonitrile in water to 22% acetonitrile. Glucosinolates identified: A=2-hydroxy-3-butenyl-, B=2-hydroxy-4-pentenyl-, C=3-butenyl-, D=4-hydroxy-3-indolylmethyl-, E=4-pentenyl-, F=3-indolylmethyl-.

followed by determination of the sugar moiety in each peak using thymol-sulphuric acid. Comparison with the internal standard (sinigrin or *o*-nitrophenyl-B-D-galactopyranoside) allowed relatively easy calculation of response factors. At 226 nm, response factors ranging from 0.22 to 1.11 have been calculated. (D.I. McGregor, Agriculture Canada, Saskatoon, Personal communication.)

It will be interesting to see which of the two HPLC systems becomes most popular. The ion pair system has the advantage of speed and simplicity which might be gained at the expense of greater equipment wear. The desulphation system uses simpler reagents but requires more time to complete the analysis and more expensive chromatography equipment.

4 Universal Procedures

Procedures for estimating glucosinolates as a group have depended on the determination of either sulphate ion or glucose released on hydrolysis or on spectrophotometric determination of a palladium complex with glucosinolates or desulphoglucosinolates. The advantage of sulphate or glucose estimation is that relatively uncontrolled hydrolysis conditions can be used as opposed to methods which rely on estimation of isothiocyanates or other "R" group hydrolysis products. Glucosinolates yield one mole of sul-

phate and one mole of glucose on hydrolysis under almost any conditions but the proportion of various "R" group products has been shown to vary according to pH and the presence of enzyme cofactors.

Sulphate has been determined both gravimetrically and titrimetrically. Gravimetric procedures have been found to be tedious and have not been suitable for determinations in rapeseed or canola. In the most recent method to be published²⁴⁾ aqueous extracts of glucosinolates from rapeseed were clarified, adjusted to pH 6 with alkali, and bisulphate ion released after myrosinase hydrolysis was titrated to pH 6.

Glucose has been measured after either enzymatic or chemical hydrolysis of the glucosinolate ion. Enzymatic glucose determinations have relied on the use of either the hexokinase/glucose-6-phosphate dehydrogenase system²⁵⁾ or the glucose oxidase/peroxidase system²⁶⁾. The latter system has been incorporated into a test paper system which, although useful for screening seed samples for plant breeding was not sufficiently accurate for regulatory purposes²⁷⁾. A recent publication²⁸⁾ suggested that the use of a pocket reflectometer to read the test paper color may improve precision. The glucose oxidase system was found to be partially inhibited by the presence of seed phenolics and it was necessary to isolate the glucosinolates prior to analysis. Similarly, impurities have contributed to high background readings in the hexokinase assay. A recently published procedure²⁹⁾ used the isolation techniques developed for determination of glucosinolates as TMS-ethers. The glucosinolates were hydrolyzed on column by myrosinase and the glucose released was determined enzymatically.

Possibly the most promising method for determining total glucosinolates involves chemical hydrolysis of the glucosinolates followed by glucose determination. This method was first used on glucosinolates in 1954³⁰⁾ when the glucosinolates were hydrolyzed by sulphuric acid and the liberated glucose determined by anthrone reagent. A recent publication³¹⁾ described a method for isolating glucosinolates using ion exchange, similar to the method used in preparing intact glucosinolates for ion-paired HPLC. The isolated glucosinolates were reacted

with thymol/sulphuric acid to give a colored complex. This method has been used successfully in some Canadian plant breeding programs within the last year. Attempts to use thymol or anthrone reagents to determine glucose eluted from columns after myrosinase treatment have encountered high blank values due to interfering components from the enzymes.

The complexation of glucosinolate with metal ions formed the basis for a spectrophotometric determination of total glucosinolates using Pd^{+4} ions³²⁾. In the initial method, seed were homogenized with boiling water and an aliquot of the extract was reacted with palladium tetrachloropalladate solution. The increase in absorption at 450 nm was determined to be proportional to the glucosinolate content. In later studies, it was determined that several compounds could interfere with the procedure and a recent method³³⁾ included a purification step similar to the one used to prepare glucosinolates for HPLC. This method has been carefully studied at the authors laboratory and at the Agriculture Research Station in Saskatoon. Careful control of the acid concentration or the use of surfactants was found to be necessary to avoid precipitation of palladium complex polymers. Results from both laboratories have shown that although useful correlations could be found for a wide range of glucosinolate concentrations (**Fig.-8**), the method was not useful for glucosinolate concentrations less than $30 \mu\text{M/g}$. The palladium complex method has been adopted by some laboratories in Europe but is no longer used in Canada.

Universal methods give an estimate of the total amount of glucosinolates available in a sample with no information on the amount of any individual glucosinolate. They are of some use in the identification of canola, however since the glucosinolates not included in the canola definition seem to be relatively constant in quantity. For example, the y-intercept in **Fig.-8** is about $12 \mu\text{M/g}$, approximately equal to the amount of indolyl glucosinolate by gas chromatography. Similarly, **Table-5** shows that the difference between the GLC results for aliphatic glucosinolates and the total results by glucose estimation ranged from $9 \mu\text{M/g}$ to $15 \mu\text{M/g}$, for samples with a wide range in gluco-

Fig.-8 Comparison of GLC and palladium complex methods for glucosinolate determinations.

Table-5 Comparison of glucosinolate content of rapeseed determined by different procedures.

Sample	Glucosinolate content ($\mu\text{M/g}$)		
	GLC ^{a)}	Universal ^{b)}	Difference
Westar	15	25	10
Tobin	28	40	12
Candle	13	22	9
Regent	23	38	15
Altex	21	35	14
Torch	78	108	30 ^{c)}

a) Method of the Canadian Grain Commission, aliphatic glucosinolates only.

b) Method as described in reference 10.

c) Some minor glucosinolates (methylthio-group and phenethyl-group) may be present in larger quantities than in canola types.

sinolate content. Subtraction of a constant amount from the total estimate may allow the universal methods to be used as quality control procedures in the canola industry.

5 Conclusions

Two different approaches to glucosinolate analysis have been identified. One approach involves separation and analysis of each glucosinolate in the mixture, either by gas liquid chromatography or by high performance liquid chromatography. These techniques are especially useful when the content of each individual glucosinolate in a mixture must be known, such as in nutritional studies, or where the sum of certain glucosinolates must be expressed as a total as in certification of canola.

The other approach involves estimation of the total amount of glucosinolates present in a sample without providing information on the

amount of any individual glucosinolate. Usually a universal hydrolysis product such as glucose is used as the basis for measurement. These methods may be useful as screening procedures, especially in plant breeding programs, and possibly as quality control procedures at crushing plants.

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最近のカナダにおけるなたね（カノ ーラ）中のグルコシノレートの分析 法について

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なたね中のグルコシノレートの分析に関する最近の進歩について総説する。個々のグルコシノレート含量の測定には、硫酸塩除去後トリメチルシリルエーテルを用いるガスクロマトグラフィーにより、あるいは硫酸塩除去後またはイオン性グルコシノレートのまま高速液体クロマトグラフィーにより求められてきた。グルコシノレート総量の測定には、加水分解によって生成する硫酸塩イオンまたはグルコースを分析する方法、もしくはグルコシノレート——パラジウムイオン錯化合物を分光分析法により求める方法が開発されてきた。ほとんどの測定法はあらかじめグルコシノレートを分離・濃縮するのにイオン交換樹脂による試料中の妨害物を除去する操作を必要とする。