Microdetermination of the Major Individual Isothiocyanates and Oxazolidinethione in Rapeseed[1]

C. G. YOUNGS and L. R. WETTER, National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan

Abstract

A method has been developed for the quantitative determination of the major individual thioglucosides (as their aglycones) in rapeseed on a micro scale. The thioglucosides in the meal were hydrolyzed with myrosinase (E.C. 3.2.3.1, thioglucoside glucohydrolase), and the released aglycones were extracted with methylene chloride. The 3-butenyl and 4-pentenyl isothiocyanates were determined by gas-liquid chromatography. The 5-vinyl-2-oxazolidinethione was determined on an aliquot of the same extract by ultraviolet absorption.

Five to 20 milligrams of seed or meal were used, and one operator could analyze 24 samples per day. The standard deviation was ±3% for the isothiocyanates and ±6% for the oxazolidinethione. The limit of detection was 0.1 mg per gram of meal for the isothiocyanates and 0.3 mg per gram of meal for the oxazolidinethione. Small amounts of phenylethyl isothiocyanate and two unidentified isothiocyanates were also found.

Introduction

THE THIOGLUCOSIDES IN RAPESEED are a deterrent to greater use of this crop as a source of oil and oil meal. Since there are differences in the amounts and types of these glucosides in different varieties of rape and in related species, it may be possible to reduce or eliminate these compounds through plant breeding. However, to study the genetic control of the thioglucosides, a method is required for the quantitative determination of the individual thioglucosides. Such a method should be applicable to small samples and suitable for the analysis of the large number of samples required for determination of inheritance patterns.

The major thioglucosides in rapeseed are progoitrin, gluconapin, and glucobrassicanapin (1,2,3,4), which on enzymatic cleavage give rise to 5-vinyl-2-ozazolidinethione, 3-butenyl isothiocyanate, and 4-pentenyl isothiocyanate respectively. Although the thioglucosides may be resolved by paper chromatography (5,6,7), quantitative determinations are usually based on the products of enzymatic hydrolysis. The total thioglucoside content may be determined by the enzymatic release of glucose (8,9) or sulfate (10). Determination of the amounts of the individual thioglucosides has been based on the analysis of the released mustard oils. 5-Vinyl-2-oxazolidinethione may be determined by UV absorption measurements (1,11). The high specific extinction coefficient at 240–250 mμ of this compound makes this a sensitive method, and the ease of measurement allows analysis of a large number of samples. Quantitative analyses of volatile isothiocyanates are generally based on argentiometric methods (12,13). However these methods require relatively large samples and give only the total volatile isothiocyanates. Conversion of the isothiocyanates to thioureas gives material with a high UV absorption similar to oxazolidinethione and provides a sensitive method of analysis (14,15). Separation of the individual thioureas by paper chromatography (14) is not suitable for routine analysis of a large number of samples.

Recently gas-liquid chromatography (GLC) has been used for identification of volatile isothiocyanates and for quantitative relations between isothiocyanates and other products from thioglucosides, such as thiocyanates and nitriles (16,17,18). The high sensitivity of flame ionization detectors for GLC analysis should make this an excellent method for the quantitative microdetermination of volatile isothiocyanates.

The present paper outlines the development of a micro method for the determination of the individual thioglucosides in rapeseed, in which the volatile isothiocyanates are determined by GLC and the oxazolidinethione by UV absorption.

Experimental Section and Results

GLC Analysis of Isothiocyanates

All analyses were done by using an F&M Model 810 GLC unit, operated isothermally with a single column and a flame ionization detector. A solution of 4 mg each of ethyl, allyl, and n-butyl isothiocyanates[2] in 100 ml of methylene chloride was used to establish operating conditions and response factors. The dilution was based on the approximate concentration that would be obtained if the isothiocyanates released from 10 mg of rapeseed meal were obtained in ½ ml of solvent. Initially a 6-ft × ¼-in. stainless steel column packed with 10% SE 30[3] on 60–80 mesh Anakrom ABS[4] was used. Satisfactory operation was obtained at an oven temperature of 110C and helium flow rate of 60 ml per min. Injector and detector temperatures were 160C, and the sample size was 4 μl.

A plot of log retention time against carbon number was linear for the three isothiocyanates even though two of them were saturated and one was unsaturated, i.e., the silicone column did not distinguish between saturated and unsaturated isothiocyanates of the same carbon number. Extraction of myrosinase hydrolyzed rapeseed meal with methylene chloride gave two peaks on GLC analysis. The first peak had the same retention time as butyl isothiocyanate, and the second corresponded to a pentyl isothiocyanate on the plot of log retention time against carbon number. The two peaks were collected and treated in ethanol with aqueous ammonia. The resulting solution gave UV absorption spectra similar to that for allylthiourea, and on this basis it was concluded that the two peaks represented the expected butenyl and pentenyl isothiocyanates. Extraction of rapeseed meal which had not been treated with myrosinase gave no peaks under the GLC conditions, indicating that no interfering substances were extracted.

Subsequently it was found that a 6-ft × ¼-in. stainless steel column of 20% FFAP[5] on Anakrom ABS

[1] Issued as N.R.C. No. 9779.
[2] Eastman Organic Chemicals, Rochester, N.Y.
[3] Silicone gum rubber.
[4] Analabs, Hamden, Conn.
[5] Varian Aerograph, Walnut Creek, Calif.

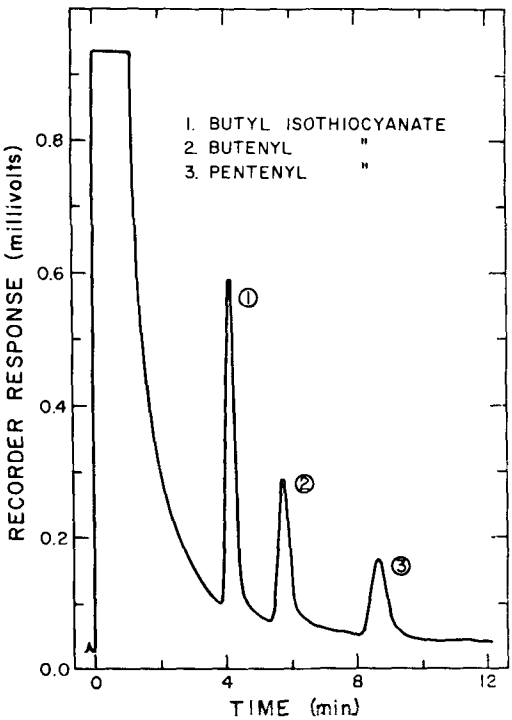

FIG. 1. GLC chart for isothiocyanates isolated from thioglucosides of rapeseed.

gave a better separation of the isothiocyanates from the solvent peak than was obtained on the silicone column. Operating conditions for the FFAP column were the same as for the silicone column except that the oven temperature was increased to 130C. The FFAP column separated the saturated and unsaturated isothiocyanates. Butyl isothiocyanate emerged immediately after allyl isothiocyanate, and butenyl isothiocyanate from rapeseed meal emerged some time after butyl isothiocyanate. A typical chromatographic trace is shown in Figure. 1.

For quantitative analysis, n-butyl isothiocyanate served as an internal standard since rapeseed does not contain a glucoside which gives rise to this isothiocyanate. The relative response factors for isothiocyanates on the flame ionization detector were determined by using known mixtures of the ethyl, allyl, and butyl isothiocyanates. The molar response for the GLC detector was found to correspond to the carbon number, excluding the isothiocyanate carbon, i.e., the peak areas divided by 2 for ethyl, 3 for allyl, and 4 for butyl isothiocyanate corresponded to the molar proportions of these materials in known mixtures. A similar response pattern was assumed for the butenyl and pentenyl isothiocyanates from rapeseed, i.e., a carbon number of 4 for butenyl and 5 for pentenyl. These factors were used in all subsequent calculations.

TABLE I

Effect of pH on the Release of Aglycones from a *Brassica napus* L. meal

pH	3-Butenyl isothiocyanate [1]	4-Pentenyl isothiocyanate [1]	5-Vinyl-2-oxazolidinethione [1]
4.5	3.1	0.6	8.4
4.9	3.0	0.7	10.0
5.5	3.5	0.8	10.0
6.6	3.4	0.8	9.8
6.8	3.4	0.8	9.7
8.0	3.4	0.8	9.7

[1] The mg/g of oil-free moisture-free meal.

Enzymatic Hydrolysis and Extraction of Isothiocyanates and Oxazolidinethione

Ettlinger and Thompson (19) found that, if the enzymatic hydrolysis of glucosides was performed in the presence of a large excess of organic solvent, the isothiocyanates and oxazolidinethiones could be extracted as formed. This procedure was adapted for the present method. Ten milligrams of rapeseed meal were weighed into a 2-ml screw-cap vial with a polyethylene cap liner. Fifty microliters of a pH 7 citrate-phosphate buffer containing 6 mg of myrosinase [6] per ml were added, followed by 0.5 ml of methylene chloride containing 4 mg of internal standard (butyl isothiocyanate) per 100 ml. The vial containing a 3-mm steel ball was capped and shaken on an oscillating shaker at room temperature.

After incubation, four microliters of methylene chloride were injected into the GLC unit for the isothiocyanate determination. Another aliquot was used for the oxazolidinethione determination. By using a commercial sample of rapeseed meal, GLC analyses were run every 15 min to determine the time required for complete hydrolysis of the glucosides and extraction of the isothiocyanates. No change in the amounts of isothiocyanates were found after 1½ hr so a shaking time of 2 hr was used in all subsequent analyses.

The effect of pH was determined over the range of 4.5 to 8.0, and the results are given in Table I. These values were the mean of duplicates, and the maximum deviation was ±6% and the standard deviation ±3%. Although pH did not have any pronounced effect, the values for isothiocyanates at pH 4.5 and 4.9 were slightly lower than those from 5.5 to 8.0. Therefore, in agreement with Appelqvist and Josefsson (15), pH 7 was employed for all subsequent runs.

As found by the authors and reported by Appleqvist and Josefsson (15) and Van Etten et al. (21), rapeseed meal prepared by hexane extraction and air-dried without any heat treatment gives low, erratic values for the mustard oils released on enzymatic hydrolysis. Appelqvist and Josefsson (15) attributed this to an interaction of the mustard oils and protein. Recently Van Etten et al. (21) and Daxenbichler et al. (22) have shown that this is at least partly caused by formation of products other than isothiocyanates or oxazolidinethione, notably nitriles and epithionitriles. All workers have found that heating the meal prior to hydrolysis overcomes this difficulty.

The authors found that dry heating overnight at 110C was adequate whereas Appelqvist and Josefsson (15) recommend heating for a short period in the buffer solution. Four samples of rapeseed meal,

[6] Prepared from white mustard seed, according to a method described by Schwimmer (20).

TABLE II

Effect of Heat Treatments on the Release of Aglycones from Rapeseed Meal

	Sample No.			
	1	2	3	4
Heated overnight at 110C				
3-Butenyl isothiocyanate [1]	1.4	1.2	2.0	1.0
4-Pentenyl isothiocyanate [1]	1.9	1.9	0.3	0.2
5-Vinyl-2-oxazolidinethione [1]	1.8	1.9	11.0	6.6
Additional heating in buffer for 10 min at 96C				
3-Butenyl-isothiocyanate [1]	1.6	1.1	2.3	0.6
4-Pentenyl isothiocyanate [1]	1.8	1.5	0.4	<0.1
5-Vinyl-2-oxazolidinethione [1]	1.8	1.8	9.7	5.4

[1] The mg/g of oil-free, moisture-free meal. Sample No. 1 and 2, *Brassica campestris* L. Sample No. 3 and 4, *Brassica napus* L.

which had been heated overnight at 110C, were heated for an additional 10 min in buffer at 96C. Results of analysis of these meals are given in Table II. Since heating with the buffer gave no increase in the values obtained, overnight heating was deemed adequate. Commercial meals which had been heat-treated in processing showed no increase on further heating.

Ettlinger and Thompson (19) reported that hydrolysis of the thioglucosides and recovery of products could be accomplished without prior extraction of the oil from crushed seeds. This was also found to be the case in the present method. A sample of rapeseed was ground, and a portion of the ground seed was extracted with hexane. Both the full-fat meal and the defatted meal were heated overnight and analyzed as described. Results on the full-fat meal, corrected for fat content, were within ±10% of those obtained on the defatted meal.

As a final check on the quantitative aspect of the method, under the conditions arrived at above, crystalline sinigrin [7] was assayed. Results obtained for allyl isothiocyanate were 95 to 105% of the theoretical values.

Determination of Oxazolidinethione

The 5-vinyl-2-oxazolidinethione was determined by UV absorption by using a Spectracord [8] recording spectrophotometer. No attempt was made to determine 5-allyl-2-oxazolidinethione, which has recently been demonstrated in *Brassica napus* L. by Tapper and MacGibbon (23). On the basis of the work of Wetter (11) a dilution of 100 μl of the methylene chloride extract (as described in the previous section) to 3 ml would give a suitable concentration for assay. On dilution with ethyl ether virtually no absorption was found. However, on dilution with ethanol, absorption was obtained and this increased with time. It was concluded that the 2-hydroxy-3-butenylisothiocyanate released from the progoitrin did not cyclize in methylene chloride or ethyl ether but did in the more polar solvent, ethanol.

The absorption in ethanol reached a maximum after 4 hr at room temperature and did not change over the next 20 hr. Therefore 100 μl of the methylene chloride extract were diluted to 3 ml with ethanol and allowed to stand for at least 4 hr at room temperature prior to absorption measurements. One hundred μl of methylene chloride diluted to 3 ml with ethanol was used as a blank. A typical absorption curve is shown in Figure 2. A straight baseline was drawn, and the optical density was measured as indicated in Figure 2. The isothiocyanates present in the methylene chloride extract have a small absorption in the same range as the oxazolidinethione. The optical density owing to isothiocyanate content, as

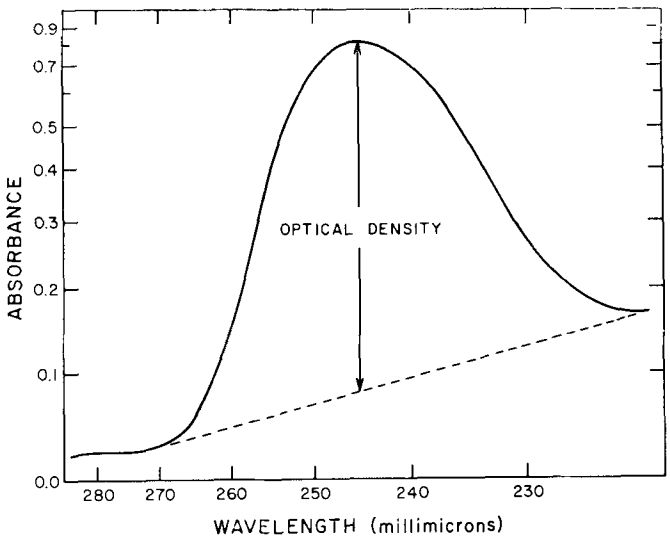

FIG. 2. UV absorption curve for oxazolinethione obtained from rapeseed meal.

determined by GLC, was calculated on the basis of a specific extinction coefficient of 1,000 (25) and subtracted from the observed value. The oxazolidinethione content was obtained from the corrected optical density by using the standard curve of Wetter (11).

Two hours of shaking time were adequate for hydrolysis and extraction of the oxazolidinethione; pH 7 and overnight heating of the meal prior to analysis was also satisfactory, as shown by Tables I and II. No significant difference was found between full-fat meals and defatted meals. Unfortunately no pure sample of progoitrin was available for checking the values obtained for oxazolidinethione.

Values obtained on a number of meals were checked against those obtained by the method of Wetter (11), in which 2 g of meals were hydrolyzed overnight with myrosinase in 100 ml of pH 7 buffer. The volatile isothiocyanates were removed by steam distillation, and the 5-vinyl-2-oxazolidinethione was extracted from an aliquot of the residue by ethyl ether. Values obtained by the two methods are given in Table III and show good agreement.

Comparison of Released Sulfate and Aglycones in Rapeseed Meal

As a further check on the precision and accuracy of the over-all method, two types of rapeseed were analyzed in quadruplicate and the total aglycone was compared with the enzymatically released sulfate. Released sulfate was determined gravimetrically as barium sulfate, as described by McGee et al. (26). Results are given in Table IV. The highest standard deviation for the isothiocyanates was ±3.4% and for the oxazolidinethione ±6.0%. The total mmoles of aglycone agreed within experimental error with the mmoles of sulfate released for Tanka rapeseed. There is however appreciably more sulfate than

[7] Prepared from horseradish root, as described by Wetter (24).
[8] Warren Electronics Inc., Bound Brook, N.J.

TABLE III

A Comparison of Two Methods for the Determination of 5-Vinyl-2-Oxazolidinethione in Rapeseed

Sample No.	5-Vinyl-2-Oxazolidinethione [1]	
	Present method	Wetter's method (11) at pH 7
1	1.8	1.8
2	1.9	1.9
3	1.9	2.0
4	6.6	6.4

[1] The mg/g of oil-free, moisture-free meal. Sample No. 1, 2, and 3, *Brassica campestris* L. Sample No. 4, *Brassica napus* L.

TABLE IV

Comparison of Released Aglycone and Released Sulfate

	Tanka meal [1]	Arlo meal [2]
3-Butenyl-isothiocyanate [3]	1.82 ± .03	2.36 ± .08
4-Pentenyl isothiocyanate [3]	0.72 ± .02	2.06 ± .03
5-Vinyl-2-oxazolidinethione [3]	8.14 ± .25	2.16 ± .13
Total aglycone [4]	.082 ± .002	.053 ± .002
Released sulfate [4]	0.80 ± .002[5]	.065 ± .002[5]

[1] *Brassica napus* L.
[2] *Brassica campestris* L.
[3] The mg/g of oil-free, moisture-free meal.
[4] The mmole/g of oil-free, moisture-free meal.
[5] Average of duplicates.

aglycone in the Arlo rapeseed. On raising the temperature of the FFAP column to 200C and injecting the methylene chloride extract for these two meals, one additional peak was found for the Tanka sample and three additional peaks for the Arlo sample.

These peaks were not present if the meals were extracted without treatment with myrosinase. The one peak in the Tanka sample and one of the peaks in the Arlo sample had the same retention time as phenylethyl isothiocyanate. Collection of this peak from a preparative run of GLC gave an oil with an infrared spectrum identical to that of phenylethyl isothiocyanate. The infrared spectra of the additional two peaks in the Arlo sample showed that these were also isothiocyanates. The spectra were quite similar and indicated that the compounds were not aromatic. Further work is being done to characterize these two compounds.

Comparison of peak sizes with that of known amounts of phenylethyl isothiocyanate gave a total additional amount of isothiocyanate of 0.3 mg/g for the Tanka meal and 1.1 mg/g for the Arlo meal. The total aglycone released for the two samples then becomes 0.084 mmole/g and 0.060 mmole/g compared with 0.080 and 0.065 mmole/g of sulfate. These results confirm that the micromethod is a quantitative assay of isothiocyanates and oxazolidinethione in rapeseed meal.

REFERENCES

1. Astwood, E. B., M. A. Greer and M. G. Ettlinger, J. Biol. Chem. *181*, 121–130 (1949).
2. Raciszewski, Z. M., E. Y. Spencer and L. W. Trevoy, Can. J. Technol. *33*, 129–133 (1955).
3. Kjaer, A., J. Conti and K. A. Jensen, Acta Chem. Scand. *7*, 1271–1275 (1953).
4. Kjaer, A., and R. B. Jensen, Ibid. *10*, 1365–1371 (1956).
5. Schultz, O. E., and R. Gmelin, Z. Naturforsch *7b*, 500–506 (1952).
6. Schultz, O. E., and R. Gmelin, Ibid. *8b*, 151–156 (1953).
7. Schultz, O. E., and W. Wagner, Ibid. *11b*, 73–78 (1956).
8. Schultz, O. E., and R. Gmelin, Ibid. *9b*, 27–29 (1954).
9. Hougen, F. W., and C. C. Tai, "Determination of Thioglucosides in Rapeseed," presented at the 49th Meeting of the Chemical Institute of Canada, Saskatoon, Sask., June, 1966.
10. Van Etten, C. H., M. E. Daxenbichler, J. E. Peters, I. A. Wolf and A. N. Booth, J. Agr. Food Chem. *13*, 24–27 (1965).
11. Wetter, L. R., Can. J. Biochem. Physiol. *35*, 293–297 (1957).
12. Andre, M. E., and M. Maille, Ann. Inst. Natl. Recherche Agron., Ser. A, Ann. agron. *2*, 442–455 (1951).
13. Wetter, L. R., Can. J. Biochem. Physiol. *33*, 980–984 (1955).
14. Kjaer, A., J. Conti and I. Larsen, Acta Chem. Scand. *7*, 1276–1282 (1953).
15. Appelqvist, L. A., and E. Josefsson, Ibid. *19*, 1242–1244 (1965).
16. Jart, A., Ibid. *15*, 1223–1230 (1961).
17. Kirk, L. D., L. T. Black and G. C. Mustakas, JAOCS *41*, 599–602 (1964).
18. Miller, H. E., "The Aglucone of Sinigrin," Master's Thesis, Rice University, Houston, Tex., 1965.
19. Ettlinger, M. G., and C. P. Thompson, "Studies of Mustard Oil Glucosides (II)," Final Report Contract DA 19–129–QM–1689, Office of Technical Services, U.S. Department of Commerce, 1962.
20. Schwimmer, S., Acta Chem. Scand. *15*, 535–544 (1961).
21. Van Etten, C. H., M. E. Daxenbichler, J. E. Peters and H. L. Tookey, J. Agric. Food Chem. *14*, 426–430 (1966).
22. Daxenbichler, M. E., C. H. Van Etten and I. A. Wolff, Chem. Commun. *No. 15*, 526–527 (1966).
23. Tapper, B. A., and D. B. MacGibbon, Phytochem. *6*, 749–753 (1967).
24. Wetter, L. R., Ibid. *3*, 57–64 (1964).
25. Ettlinger, M. G., and J. E. Hodgkins, J. Org. Chem. *21*, 204–205 (1956).
26. McGee, J. E., L. D. Kirk and G. C. Mustakas, JAOCS *42*, 889–891 (1965).

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Addendum

The two unknown isothiocyanates have subsequently been identified by the authors as 4-methylthio-butyl isothiocyanate and 5-methylthio-pentyl isothiocyanate.