A STUDY OF ISOTHIOCYANATE GLUCOSIDES OCCURRING IN RAPESEED

by

Horng-mou Tsay

A Thesis

Submitted to

the Faculty Council of Graduate Studies and Research

in Partial Fulfilment of the Requirements

for the Degree of Master of Science

The University of Manitoba

September 1964

ACKNOWLEDGEMENTS

The author gratefully acknowledges indebtedness to Dr. F.W. Hougen for the helpful advice, assistance and encouragement given during the course of the research and preparation of the thesis.

Many thanks are extended to Dr. D.T. Canvin, Dr. J.K. Nayar, Mr. B.R. Stefansson, and Dr. A.M. Unrau for their helpful advice and assistance throughout the course of the research.

ABSTRACT

A purpose of this research was to isolate thioglucosides which occur in rapeseed. Although sinalbin, glucoiberin and sinigrin have been isolated earlier, this work was repeated to make these substances available for further study. Plant extracts containing gluconapin were purified with anion exchangers. Gluconapin was not obtained in crystalline form. A purified fraction of gluconapin was acetylated and subsequently purified with the use of acid alumina. Gluconapin tetraacetate was obtained in crystalline form. Its infrared spectrum and elemental analysis were in agreement with the structural formula of gluconapin tetraacetate.

The anthrone method for estimation of thioglucosides was investigated and simplified for possible use in routine analysis of rapeseed.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Rapeseed is produced mainly for production of oil for edible purposes. Rapeseed meal, which remains after removal of the oil from the seed, is used as a protein supplement for livestock and poultry. This use of the meal constitutes a problem in that an excess intake of rapeseed meal may cause goitre and growth depression in livestock and poultry. Goitre is understood to be caused partly by thio-oxazolidone, an enzymatic fission product of progoitrin. Progoitrin is one of six thioglucosides found in rapeseed (1), namely progoitrin, sinalbin, glucoiberin, gluconasturtin, gluconapin and glucobrassicanapin. The effects on animals of thioglucosides other than progoitrin are not well understood.

With increasing production of rapeseed in Canada the toxicity of the meal has been causing some concern and more attention has been given to the study of the goitre factors.

For a further study of the effects of individual thioglucosides on animals it would be desirable to have pure thioglucosides available for animal feeding experiments. If pure individual thioglucosides were made available, they would also become useful in the study of analytical methods for their determination in rapeseed.

Of the six known thioglucosides in rapeseed, sinalbin (2), glucoiberin (3), gluconasturtin (4) and progoitrin (5) have been earlier isolated. In addition, a probably impure preparation of gluconapin has been separated (6). However, these thioglucosides are not commercially available.

The isolation from plant materials of thioglucosides occurring in rapeseed constitutes one part of this thesis.

Sinalbin, glucoiberin and the acetyl derivative of gluconapin were isolated. The latter has not earlier been described in the literature. A thioglucoside, sinigrin, which does not occur in rapeseed was also isolated in order to provide material for further studies.

Another part of this thesis is concerned with the study of methods for determination of the total thioglucosides in rapeseed. Such a method is required for use in plant breeding experiments for the purpose of possible elimination of the thioglucosides from the seed. The method by Schultz and Gmelin (7) for estimation of thioglucosides with anthrone reagent was selected for study as it was considered the most promising method for this purpose.

REVIEW OF LITERATURE

I. Historical Development in the Study of Thioglucosides.

In 1831 Robiquet and Bourtons (8) isolated a crystalline sulphur containing constituent, sinalbin, from seeds of white mustard (Sinapis alba L.). This substance was later recognized as a thioglucoside. In 1840 Bussy (9) demonstrated the enzymatic formation of a mustard oil from black mustard seed (Brassica nigra, Koch) in the presence of water by isolation of the crystalline parent compound, subsequently named sinigrin, and a preparation of its hydrolysing enzyme, later named myrosinase. During the second half of the nineteenth and the beginning of the twentieth century, some studies on the chemical structure of thioglucosides appeared in the literature. Gadamer (10 - 12) proposed structural formulae for sinigrin and sinalbin in 1897. The common basis of these two structures was for many years used as the general structure of thioglucosides. Up to 1952 three thioglucosides and eight mustard oils with established structures had been recorded. In the following years a rapid progress was made in this field of study, largely because improved techniques such as paper chromatography became available. Ettlinger and Lundeen (13) revised Gadamer's structure in 1956, and their revised general structure

for thioglucosides has been accepted as the correct one. Up to 1960 (14) nine thioglucosides had been isolated in crystalline form and seven of these also as the acetyl derivatives. An additional six thioglucosides had been isolated only as the acetyl derivatives. Thirty isothiocyanates were known as the enzymatic fission products of naturally occurring thioglucosides. Although speculations about the biosynthesis of thioglucosides have appeared many times in the literature (15,16), it was not until 1962 that Wetter et al. (17) reported the first experimental study on the biosynthesis of a thioglucoside.

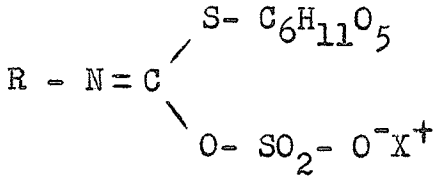
II. Elucidation of the General Structure of Thioglucosides.

Gadamer (lo - 12) in 1897 proposed structure (I) for sinigrin and sinalbin on basis of the following information:

(A) Will and Körner (18) had demonstrated in 1863 the formation of mainly allyl isothiocyanate, glucose and sulphate upon enzymatic decomposition of sinigrin.

(B) The general structure of isothiocyanates had been established as RNCS by Hofmann in 1868 (19).

(C) Gadamer found that reaction of one equivalent of silver nitrate with sinigrin or sinalbin resulted in the detachment of glucose and the appearance of a silver mercaptide which suggested that the glucose moiety was connected through a thioglucoside linkage in the original glucosides (10 - 12).



(I)

Sinigrin: R = CH_2= CH- CH_2 X= K

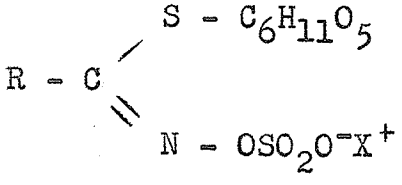
Sinalbin: R = (p)$HOC_6H_4CH_2$ X= Sinapine

Schneider and Wrede (20) later confirmed the presence of a thioglucoside linkage in sinigrin through reaction of the glucoside with potassium methoxide which yielded 1-thio-D-glucose. The configuration of the thioglucoside linkage was later determined by the same authors (21) as the β form in sinigrin and other thioglucosides.

It had long been known that enzymatic action on sinigrin and sinalbin produced, besides the main fission products, also smaller amounts of free sulphur, carbon disulphide and nitriles, which did not seem compatible with the formulae proposed by Gadamer. This led Ettlinger and Lundeen (13) to renewed investigations which on basis of the following evidence resulted in a revision of Gadamer's general structure for thioglucosides to (II):

(A) Hydrogenolysis of sinigrin with Raney nickel gave n-butylamine.

(B) Acid hydrolysis of sinigrin yielded vinylacetic acid and hydroxylamine.



(II)

All thioglucosides encountered thus far in nature are believed to have the same general structure as put forward by Ettlinger and Lundeen (13) for sinigrin. Thus the various thioglucosides differ only in the structure of the side chain and in the cation bound to the sulphate group.

The six thioglucosides known to occur in rapeseed have side chains and cations as shown in Table 1 (14).

TABLE 1

SIDE CHAINS (R) AND CATIONS (X^+) OF SIX THIOGLUCOSIDES FOUND IN RAPE SEED.

Thioglucosides	R-	X
Progoitrin	$CH_2=CHCH(OH)CH_2$-	Na
Gluconapin	$CH_2=CHCH_2CH_2$-	
Glucobrassicanapin	$CH_2=CHCH_2CH_2CH_2$-	
Glucoiberin	$CH_3SOCH_2CH_2CH_2$-	K
Sinalbin	$HOC_6H_4CH_2$-	Sinapine
Gluconasturtin	$C_6H_5CH_2CH_2$-	K

III. Preparation of Thioglucosides.

Thioglucosides have generally been difficult to isolate in crystalline form. Of more than thirty-five thioglucosides known to exist in plants, only nine have been brought to crystallisation. Among the nine crystalline thioglucosides only sinigrin, sinalbin and glucoiberin are considered to crystallize readily (14). Of the six thioglucosides known to occur in rape seed (1) gluconapin and glucobrassicanapin have not been obtained in pure crystalline form. Thioglucosides are readily extracted from plant materials with aqueous organic solvents such as methanol, ethanol and acetone. The solvent extracts usually contain impurities such as amino acids, tannins and pectins which interfere with crystallisation and should be removed. Some reported methods for purification of extracts of plant materials are briefly reviewed below:

For preparation of sinigrin from roots of horse radish (Cochlearia Armoracia L.) Stoll and Seebeck(22) removed sugars from the plant extract by fermentation followed by addition of calcium carbonate to precipitate the acid formed. The sugar-free solution was further purified by precipitation with lead acetate solution.

Schultz and Gmelin (23) isolated gluctropaeolin from

an extract of Lepidium sativum by employing adsorption chromatography on a column of cellulose powder with n-butanol: acetic acid: water as the developing solvent.

Electrophoresis with an acetic acid solution as buffer was employed by Schultz and Barthold (24) for purification of an extract of glucotropaeolin from Lepidium sativum. Although a pure thioglucoside fraction was not obtained by the electrophoretic procedure, a crystalline preparation of glucotropaeolin was obtained from the purified solution.

The electrophoretic behavior of glucotropaeolin led Schultz, Gmelin and Keller (25) to explore the applicability of anion exchangers for purification of thioglucosides contained in crude plant extracts. They found chromatography through various anion exchange columns to be very effective for this purpose. Lewatit M1, Amberlite IR-400 and Amberlite IR-4B in hydroxide, carbonate, acetate and chloride forms were examined, and the Amberlite IR-4B in chloride form was found the most suitable.

Schultz and Gmelin (26) later found that acid alumina functioned as an anion exchanger for thioglucosides and, further, that this adsorbent effected a good separation of thioglucosides from coloured material in the plant extracts. Schultz and Wagner (27) purified plant extracts

by lead acetate precipitation followed by chromatography on acid alumina, and other workers (28,29) have similarly with succes combined lead acetate precipitation and chromatography on anion exchange resins.

Greer (5) purified an extract of progoitrin from rutabaga seed (Brassica campestris L.) by chromatography on a column of alcohol-washed neutral alumina using 80 per cent alcohol as the eluting agent. Subsequent crystallisation of the progoitrin, however, occurred only after more than half a year. Greer (30) later isolated progoitrin more effectively with the use of Amberlite IR-4B in chloride form.

Counter-current distribution with a solvent system of n-butanol: water: pyridine was applied by Hietala (31) for fractionation of a mixture of thioglucosides. The crude extract had been previously chromatographed on ion exchange columns to remove amino acids and organic acids. While pure fractions of progoitrin and gluconapin were reported to have been obtained by this method, only progoitrin was obtained in crystalline form.

For isolation of sinigrin from an extract of horse radish leaves, Wetter et al. (17) employed a column of Amberlite IR-120 to remove amino acids and a column of Amberlite IR-4B to remove organic acids. The dimensions

of the latter column were such as to retain only organic acids and to allow sinigrin to pass through.

Nayar and Thorsteinson (6) found thin layer chromatography to effect a further purification of crude fractions of thioglucosides which had been previously chromatographed on neutral alumina.

Anion exchange resins have been used also for introducing cations such as sodium (30), tetramethylammonium (13) and rubidium (29) into thioglucosides.

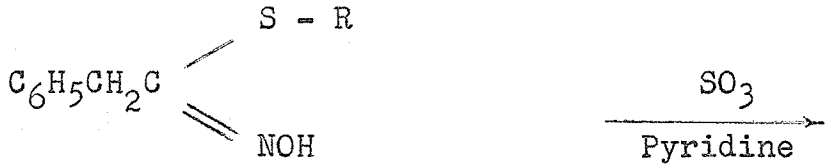
Schultz and Wagner (27) found that the poor crystallizing properties connected with some thioglucosides were greatly improved in the corresponding acetyl derivatives.

Besides isolation from natural sources thioglucosides may also be prepared synthetically. To date one thioglucoside has been synthesised. Ettlinger and Lundeen (16) synthesised glucotropaeolin by the following scheme:

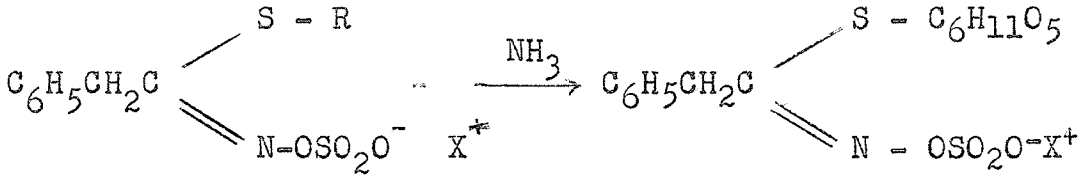
$$C_6H_5CH_2MgCl \xrightarrow{CS_2} C_6H_5CH_2CSS^- \xrightarrow{NH_3OH^+}$$

Benzyl magnesium chloride — Dithiophenyl acetate ion

Phenylaceto-thiohydroxamic acid



S-β-D-1-(Tetraacetyl glucopyranosyl)-phenylaceto-thiohydroxamic acid



X = $(CH_3)_4N$ Glucotropaeolin

R = Tetraacetyl-β-D-1-glucopyranosyl

Benn (32) used an alternative method to synthesize glucotropaeolin.

III Estimation of Thioglucosides.

Most of the available methods for determination of thioglucosides are based on the estimation of products formed by acid or enzymatic hydrolysis of the thioglucosides.

Acid hydrolysis of thioglucosides produces glucose, sulfate, carboxylic acids and free and substituted hydroxylamines. Quantitative yields are obtained of the former two products and these may be used for indirect determination of the thioglucosides. The latter two products are not produced in quantitative yields.

Enzymatic hydrolysis of thioglucosides gives rise to the three main products, glucose, sulfate and isothiocyanates, besides small amounts of byproducts such as carbon disulfide, elementary sulfur and nitriles (18). It is not certain at present whether any of the three main products may be obtained in quantitative yields.

Literature related to the estimation of thioglucosides through determinations of glucose, sulfate and isothiocyanates is briefly reviewed below:

(A) Determination of glucose.

Sandberg (33) determined the glucose content of an enzymatic hydrolysate of pure sinigrin by the method of Folin and Malmros (34), which is based on the reaction

of reducing sugar and ferricyanide to generate a blue color which is measured photometrically.

Nagashima and Uchiyama (35) determined the amounts of glucose produced during enzymatic hydrolysis of pure sinigrin by the use of 3,5-dinitrosalicylic acid according to Sumner (36). They pointed out an advantage with Sumner's method in that sinigrin exerted no reducing effect on the 3,5-dinitrosalicylic acid reagent, while it did have a reducing effect on the reagents used in other methods for determination of reducing sugars. In a similar study of enzymatic hydrolysis of sinigrin Reese et al. (37) obtained near quantitative yields of glucose as estimated by Sumner's method and they strongly recommended the determination of glucose rather than sulfate or isothiocyanates in enzymatic hydrolysates of thioglucosides.

Schultz and Gmelin (7) applied Dreywood's method (38) for determination of glucose and glucosides with anthrone reagent to the estimation of thioglucosides. Sulfuric acid contained in the reagent caused complete hydrolysis of the thioglucosides during heating and the glucose thus produced reacted with anthrone to form a blue color which was measured photometrically. For determination of the thioglucoside content in a plant material, they first purified the plant extract by chromatography on paper or on a column of acid alumina.

(B) Determination of sulfate.

Gadamer (39) determined the amount of sinigrin in solutions by hydrolysis with hydrochloric acid followed by precipitation of the sulfate as barium sulfate which was determined gravimetrically.

Sandberg and Holly (33) employed Hubbard's method (40) to precipitate sulfate with benzidine from a hydrolysate of sinigrin. The benzidine sulfate was subsequently determined colorimetrically. Alternatively, benzidine sulfate obtained as above has been determined titrimetrically by Nagashima and Uchiyama (35) by a modification of McKittrick and Smith's titrimetric method (41).

(C) Determination of isothiocyanates.

Schmid and Karrer (42) estimated isothiocyanates by reaction with an excess ammoniacal silver nitrate solution to form silver sulphide. The unreacted silver nitrate was determined by titration with potassium thiocyanate solution with ferric ammonium sulfate as indicator. Stoll and Jucker (43) in a similar study isolated the precipitated silver sulphide and determined it gravimetrically.

Astwood et al. (44) reported a method for spectrophotometric determination of the cyclic compound, 5-vinyl-2-thiooxazolidone, which is formed by spontaneous rearrangement of the isothiocyanate released from progoitrin by

enzymatic hydrolysis.

Wetter (45) reported a method, essentially as by Schmid and Karrer (42), for estimation of volatile isothiocyanates released from rape seed by enzymatic hydrolysis. He further used Astwood's method (44) for determination of the nonvolatile thiooxazolidone remaining in the sample after removal of isothiocyanates by steam distillation (46).

Kjaer (47) determined isothiocyanates by reaction with ammonium hydroxide to form thioureas which were subsequently determined spectrophotometrically.

MATERIALS AND METHODS

I. Relevant to Isolation of Thioglucosides.

Plant material: Seed of Brassica campestris L. var. Sarson (yellow seed) Prain and a commercial sample of Brassica napus L. were used in experiments for isolation of gluconapin. These samples are referred to as "variety sarson" and "a commercial sample of rapeseed" in the text.

Seeds of Brassica hirta (white mustard), Iberis amara (candytuft) and Brassica nigra Koch (black mustard) were used for isolation of sinalbin, glucoiberin and sinigrin, respectively.

Melting points: Melting points were measured with a Fisher-Johns melting point apparatus. The thermometer was not corrected.

Specific rotation: Optical rotations were measured in aqueous solution at 28° with a Carl-Zeiss polarimeter with scale reading to 0.01°. A 1 dm. tube with 2 mm. inner diameter was used.

Infrared absorption spectra: Infrared absorption spectra were recorded with a Perkin-Elmer Infracord Model 137. Substances were measured in a Nujol mull made into a thin film between two sodium chloride discs.

Elemental analyses: Elemental analyses of gluconapin tetraacetate were done by Geller Laboratories, Charleston, West Virginia. The samples used for determination of carbon, hydrogen and sulfur weighed 3.043, 4.271 and 1.124 mg., respectively.

Preparation of meal: Plant seeds were ground with a sample grinder. Petroleum ether (1 litre) ("Skellysolve F", b.p. 36-58°, Skelly Oil Co., Kansas City, Missouri), previously warmed to boiling, was added to the ground seed (250 g.) in a round bottom flask (2 litres), refluxed for 2 hrs., and filtered. The residual meal was used for extraction of thioglucosides.

Extraction of thioglucosides: A mixture (1.2 litres) of methanol: acetone: water (10:2:3, v/v/v), previously warmed to boiling, was added to the meal obtained above, refluxed for 3 hrs., and filtered. The filtrate was evaporated at 40° to approximately 100 ml. volume using a flash evaporator. This concentrate is referred to as a "plant extract".

Paper chromatography of thioglucosides: Paper chromatography of thioglucosides was essentially as by Schultz and Gmelin (48). Unknown samples together with reference samples of glucose, sinigrin, sinalbin, glucoiberin and an extract of rutabaga seed which contained predominantly progoitrin were applied to a sheet of Whatman No. 1 filter

paper (23 x 56.5 cm.). The paper was developed for 16 hrs. in a solvent system of n-butanol: acetic acid: water (4:1:3, v/v/v) by descending chromatography. The chromatogram was dried, sprayed with ammoniacal silver nitrate solution, and dried at 90° for 15 min. Thioglucosides appeared as black or black-brown spots on a slightly brown background. The intensities of the spots indicated the relative amounts of the compounds.

Methods for purification of plant extracts: Chromatographic and other methods were used for purification of plant extracts obtained from 100 or 250 g. batches of plant material. Effluents from column chromatograms were examined by paper chromatography for the presence of individual thioglucosides. Fractions containing thioglucosides were evaporated at 40° in vacuum using a flash evaporator.

a. Lead acetate precipitation:

A plant extract was shaken for 20 min. with lead hydroxide freshly prepared from lead acetate (6 g.) and filtered. Lead acetate solution was added to the filtrate until there was no further precipitation. The precipitate was filtered off and excess lead was removed from the solution by addition of sulfuric acid and filtering (22).

b. Decolorisation with activated carbon:

A plant extract was agitated with activated carbon (4 g.) and celite (4 g.), left to stand for 2 hrs., and filtered. The filter cake was washed with 96% ethanol. The filtrate and ethanol solution were combined and evaporated to a smaller volume.

c. Adsorption chromatography:

Columns of cellulose powder (3.6 x 18 cm., Solka-Flock, Brown Co.) and neutral alumina (2 x 20 cm., 100-200 mesh, Bio-Rad Laboratories, California) were packed dry and used with ethanol as developing solvent.

d. Anion exchange chromatography:

The general procedure for chromatography of a plant extract on anion exchangers was as follows: A plant extract was loaded on a column at a rate of 1 ml./min. and the column was washed with distilled water until the effluent was colorless. The column was subsequently eluted with 0.02N KOH solution until the pH of the effluent reached a value of 7. Alternatively, columns of Amberlite IR-4B were eluted in the same way with 0.1N calcium chloride, 0.1N barium chloride, 0.1N ammonium sulfate or 0.1N tetramethylammonium chloride solutions and columns of Dowex AG1x2 with 0.1N potassium sulfate or 0.02N potassium acetate solutions.

Columns of Amberlite IR-4B (2 x 20 cm., Fisher

Scientific Co.), Amberlite CG-50 (2.3 x 11 cm.), Dowex AG1x2 (1.5 x 10 cm., 200-400 mesh, Bio-Rad Laboratories, California) and acid alumina (1.5 x 20 cm. and 1.2 x 14 cm., 100-200 mesh, Bio-Rad Laboratories, California) were used. Columns of the former three resins were packed in a water slurry and the acid alumina was packed dry. Effluents were usually collected in 50 ml. fractions.

e. Cation exchange chromatography:

A column of the cation exchanger Amberlite CG-50 (2.3 x 11 cm., Fisher Scientific Co.) packed in a water slurry, was joined on top of an anion exchange column of Amberlite IR-4B. The plant extract was loaded on the upper column and passed through the two columns at a rate of 1 ml./min. with further elution with water (200 ml.).

Acetylation of thioglucosides: Thioglucosides were acetylated essentially as by Schultz and Gmelin (27). Acetic anhydride (10 ml.) and pyridine (10 ml.) were added to a plant extract previously purified by chromatography on Amberlite IR-4B. The mixture was left for 16 hrs. at room temperature and concentrated to 2 ml. volume. To the concentrate three portions of distilled water (3 x 5 ml.) were successively added and evaporated. The residue was precipitated repeatedly from 90% methanol (30 ml.) to give a white amorphous substance.

II. Relevant to Estimation of Thioglucosides with Anthrone Reagent.

Anthrone reagent. Anthrone (0.2 g.) (reagent grade, Fisher Scientific Co.) was dissolved in 95.5% sulfuric acid (100 ml.) (reagent grade, Canadian Industries, Ltd.). The reagent was prepared immediately before use.

Glucose solution. A stock solution was prepared with glucose (1.00 g.) (analytical grade, British Drug Houses Ltd.) dissolved in an 0.25% solution of benzoic acid in water and made up to 100 ml. with the same solvent. Two ml. of the stock solution were made up to 100 ml. with the 0.25% benzoic acid solution to give the final glucose solution which contained 200 µg. glucose/ml.

Thioglucoside solutions. Separate solutions were made of glucoiberin (102 mg.), sinigrin (88.2 mg.) and sinalbin (80 mg.) (prepared from Iberis amara, Brassica nigra Koch and Brassica hirta, respectively) in water containing 0.25% benzoic acid and made up to 100 ml. with this solvent. The corresponding glucose contents of these solutions were calculated as 400, 400 and 92.6 µg./ml., respectively.

Rapeseed. Seeds of variety sarson (Brassica campestris L. var. sarson (yellow seed) Prain), a commercial sample (Brassica napus L.) and variety Tanka (Brassica napus L.) were used for thioglucoside analyses.

Spectrophotometry. A Perkin-Elmer spectrophotometer Model 4000A and Pyrex cuvettes of 1 cm. light path were used.

Preparation of thioglucoside extracts from rapeseed. Ground rapeseed (0.5 g.) was added to a test tube (1.8 x 10 cm.) containing petroleum ether (15 ml.) ("Skellysolve B", b.p. 63-69°, Skelly Oil Co., Kansas City, Missouri). The tube joined with an air condenser was heated on a water bath for 30 min. for reflux of the solvent. The suspension was centrifuged and the supernatant discarded. Refluxing and centrifugation were repeated twice more with petroleum ether (2 x 15 ml.). The residue was refluxed 30 min. with a mixture (15 ml.) of methanol: acetone: water (5:2:3, v/v/v) and centrifuged. This extraction was repeated twice more. The combined supernatants were made up to 50 ml. volume with water and designated the "plant extract".

An aliquot (5 ml.) of a plant extract was passed through a column of acid alumina (1.0 x 7.0 cm., 100-200 mesh, Bio-Rad Laboratories, California) which had

been packed in a water slurry after trituration with water to remove fine particles. The column was eluted with distilled water (25 ml.) followed by KOH solution until a yellow band had moved to a distance of 1 cm. from the bottom of the column so that no elution of colored material occurred. The colorless eluate was made up to 50 ml. volume with water and designated the "thioglucoside extract". Aliquots (5 ml.) of this extract were used for analyses with anthrone reagent,

Used acid alumina was regenerated in larger portions in a column by washing with 1 N HCl followed by distilled water until a pH of approximately 4.5 was reached in the eluate.

In certain experiments the organic solvents were removed from the plant extract by evaporation in vacuo at 40°. The residue was made up to 50 ml. with distilled water before chromatography on acid alumina.

Determination of thioglucosides with anthrone reagent. Anthrone reagent (10 ml.) from a 100 ml. buret was delivered into a test tube (1.8 x 15 cm.). The solution to be analysed (5 ml. or less) was layered over the reagent. If less than 5 ml. of sample solution were used, the additional amount of distilled water required to make a total of 5 ml. was first added to the anthrone reagent. The test tube was placed in cold tap water

until the contents measured 15°. The two layers in the tube were mixed by blowing oxygen through a pipette dipped into the solution. The contents of the test tube were again cooled to 15° and subsequently heated in a boiling water bath for 15 min. unless otherwise stated. Duplicate blank solutions were determined with each set of samples analysed by employing 5 ml. of distilled water instead of a sample. The absorbance of the colored sample solution was measured with reference to that of a blank solution at 625 mμ. unless otherwise stated.

Color development versus heating time. For a study of color development in relation to heating time standard solutions of glucose (7.2 ml.) and glucoiberin (3.6 ml.) were diluted with distilled water (52.8 and 56.4 ml., respectively). Portions (5 ml.) of the diluted solutions and of rapeseed thioglucoside extracts were treated with anthrone reagent (10 ml.) for periods from 5 to 19 min.

Calculation of regression coefficients. Regression coefficients (b) were calculated by the equation

$$b = \frac{\Sigma(X - \bar{X})\,\Sigma(Y - \bar{Y})}{\Sigma(X - \bar{X})^2}$$

X = actual glucose content (in units of 10 µg.) in sample analysed

$\bar{X}$ = mean of actual glucose contents

Y = measured absorbance for sample analysed

$\bar{Y}$ = mean of measured absorbances

The regression coefficients are expressed in units of absorbance per 10 µg. glucose.

Calculation of analytical results. The thioglucoside content in a sample of rapeseed was expressed in terms of the corresponding glucose content as calculated from the measured absorbance of the analysed extract and the regression factor for glucoiberin (0.02953).

$$\text{Glucose content (mg. glucose/g. seed)} = \frac{\text{measured absorbance} \times 200}{0.02953}$$

RESULTS AND DISCUSSION

I. Isolation of Thioglucosides.

Gluconapin.

(A). Preliminary examination of gluconapin contents in two varieties of rapeseed.

A recent review of thioglucosides by Kjaer (14) provided information on the distribution in plant materials of thioglucosides. On basis of this information and additional data by Kjaer (1), rapeseed was chosen as a promising source for isolation of gluconapin. Two samples of rapeseed were compared by analysis. The contents of volatile isothiocyanates as analysed by Wetter's method (45) were as follows:

Rapeseed	Allyl isothiocyanate (mg./g. of meal)
Variety *sarson* (*B. campestris* L.)	10.0
Commercial sample (*B. napus* L.)	3.5

An approximate estimate of the relative amounts of individual thioglucosides in the two samples of rapeseed was obtained by paper chromatography of crude extracts from the seed, as follows:

	Gluco-napin	Sinal-bin	Progoi-trin	Gluco-iberin	An unknown compound with Rf 0.274
Variety sarson	++++	++	+	+	
Commer-cial sample	++	++	+++	+	+

Of the four identified thioglucosides in the two samples, gluconapin alone produces a volatile isothiocyanate upon hydrolysis. The determined amounts of the total volatile isothiocyanates reflects, therefore, the amounts of gluconapin in these two samples, and it was concluded that variety sarson probably contained approximately three times more gluconapin than the commercial sample. Variety sarson was therefore chosen as the starting material for isolation of gluconapin.

(B). Preparation of meal

Oil-free meal of variety sarson seed was prepared by extraction of the ground seed with petroleum ether. Removal of fatty material has the advantage of facilitating filtration in the subsequent extraction of thioglucosides.

(C). Extraction of thioglucosides from meal.

Thioglucosides were extracted by refluxing the

meal with a mixture of methanol, acetone and water. In order to inactivate the enzyme myrosinase, the solvent mixture was applied at boiling temperature to the meal. If this precaution is not taken, the enzyme present in the meal might effect some destruction of the thioglucosides by hydrolysis.

Although 70 % methanol alone will effect extraction of the thioglucosides, it was found preferable to have some acetone in the extraction solvent or to use aqueous acetone alone as this facilitated the subsequent seperation of suspended fine solids from the solution.

Since thioglucosides tend to decompose at prolonged heating, evaporation of solvent from the extract was carried out in vacuo at 40°.

(D). Purification of the plant extract.

In order to facilitate possible crystallization of gluconapin from plant extracts of variety sarson, various methods of purification were applied to the extracts as described in the following. Effluents in chromatographic experiments were monitored for thioglucosides by paper chromatography (48). In these experiments crystallization of gluconapin was attempted from various solvents such as methanol, ethanol, acetone at -20°, and alcohol containing lactic or silicic acid. In no case were crystals

of gluconapin obtained.

a. A plant extract was treated with activated carbon and subsequently chromatographed on a column of cellulose powder with alcohol as developing solvent. Activated carbon had little effect in the decolorization of the gluconapin extract. The cellulose column also failed to separate coloured material from the thioglucosides.

b. A plant extract was chromatographed on a column of neutral alumina with alcohol as eluting agent. The eluted substance was a dark colored syrup. Although this method had been used succesfully by Greer (5) for isolation of progoitrin, a more satisfactory result was later obtained by the same author (30) by chromatography on Amberlite IR-4B in chloride form. Amberlite IR-4B was earlier used by Schultz et al (25) for isolation of glucoiberin.

c. A plant extract was chromatographed on a column of Amberlite IR-4B in chloride form. This anion exchanger was effective in retaining gluconapin and removing most of the colored material and other impurities. The eluted thioglucoside fractions contained gluconapin as the main thioglucoside constituent and smaller amounts of progoitrin and glucoiberin. Amberlite IR-4B columns had the advantage of not being blocked by the plant extracts, while blocking usually was encountered with columns of acid

alumina and Dowex AG1x2 resin.

d. A plant extract was first passed through a cation exchanger, Amberlite CG-50, to remove amino acids and subsequently through an anion exchanger, Amberlite IR-4B in chloride form, to retain thioglucosides and remove impurities. The failure in obtaining crystalline gluconapin indicated that impurities other than amino acids might have been present to interfere with the formation of crystals.

e. The anion exchanger Amberlite CG-45 in hydroxide form was tried for purification of a plant extract. The extent of exchange of thioglucosides with this exchanger was very low.

f. The anion exchanger Dowex AG1x2 was used in a number of experiments. A plant extract was passed through a column of Dowex AG1x2 in acetate form with potassium hydroxide solution as eluting agent. To avoid blocking of the column the plant extract was applied to the column in a relatively dilute solution which had been previously filtered. This column effectively adsorbed gluconapin, glucoiberin and progoitrin but not sinalbin. Elution of the thioglucosides gave fractions of gluconapin contaminated with small amounts of glucoiberin but free from progoitrin and sinalbin.

An experiment using Dowex AG1x2 in formate form gave essentially the same results as reported above for this exchanger in acetate form.

In a separate experiment potassium acetate was used instead of potassium hydroxide as eluting agent with no essential difference in results.

g. Since extracts of variety sarson had failed to furnish crystalline gluconapin, two experiments similar to the above with Dowex AG1x2 were performed with the commercial rapeseed sample as starting material. Preliminary examination had shown this material to contain essentially the same thioglucosides as the variety sarson but in different relative amounts. An extract of the commercial sample was chromatographed on a column of Dowex AG1x2 in acetate form with potassium hydroxide as eluting agent. The later fractions eluted from this column contained progoitrin contaminated with small amounts of glucoiberin but free from other thioglucosides contained in the seed. Crystalline gluconapin was not obtained.

In a similar experiment an extract of the commercial sample was eluted from the Dowex column with a potassium sulfate solution instead of the usual potassium hydroxide solution. The later fractions eluted from this

column contained progoitrin completely separated from other thioglucosides contained in the seed. Crystalline progoitrin was not obtained however.

h. An extract of variety sarson was chromatographed on a column of acid alumina with potassium hydroxide as eluting agent as used succesfully by Wagner (28) for fractionation of the thioglucosides. To avoid blocking of the column the plant extract was applied to the column in a relatively dilute solution which had been previously filtered. The results with acid alumina were similar to those with Dowex AG1x2 in acetate or formate form and Amberlite IR-4B in chloride form. Thus effective fractionation of thioglucosides was not obtained with acid alumina as has also been the experience of Kjaer (14).

i. The mixture of thioglucosides contained in the plant extract was converted in separate experiments to salts containing the cations ammonium, tetramethyl ammonium, barium and calcium by exchange on a column of Amberlite IR-4B in chloride form. Although the barium and calcium salts of other glucosides are recognised to have favorable crystallising properties, the present salts of the thioglucosides were not obtained crystalline. The barium salt, however, appeared the best of the four salts

prepared and gave a white amorphous powder.

Although crystalline gluconapin was not obtained in the present investigation, it is reported to have been obtained from leaves of Brassica napus L. by Nayar and Thorsteinson (6). There may be some doubt as to the identity of their preparation. The authors report that paper chromatography indicated the presence of small amounts of other thioglucosides in their preparation (6,51). The only other characterisation of their preparation was by melting point which was given as "above 100^{o}, decomposition". Further, a sample of their preparation as obtained by courtesy of these authors was examined by infrared absorption spectrophotometry in this laboratory. The sample gave a broad band in the region 1700 to 900 cm^{-1} and a characteristic band for the carbonyl group at 1760 cm^{-1}. These characteristics are not compatible with the structure of gluconapin and they are absent in the infrared spectra of glucoiberin (26).

Hietala (31) reported the isolation of an apparently pure fraction of gluconapin by counter-current distribution of an extract of rapeseed. Crystalline gluconapin was not obtained from this preparation however. Hietala's results corroborate the negative results of the present author in attempts at crystallising gluconapin from variously purified plant extracts.

(E). Preparation and isolation of gluconapin tetra-acetate.

It has been reported (27) that acetylated thio-glucosides possess better crystallising properties than the corresponding thioglucosides. Since all attempts at initiating crystallisation of gluconapin had failed, the tetraacetate was finally prepared. The mixture of thioglucosides contained in a plant extract from variety sarson was purified by chromatography on Amberlite IR-4B and acetylated with acetic anhydride in pyridine. The acetylation was complete as shown by the absence of thio-glucoside spots in a subsequent paper chromatogram. The acetylated mixture was precipitated from aqueous metha-nol to give a white amorphous substance. As repeated precipitations of the amorphous substance from the same solvent failed to give crystals, the substance was chro-matographed on a column of acid alumina. Potassium hy-droxide was used as eluting agent and the effluent was collected in ten successive fractions. As a specific reagent for detection of thioglucoside acetylates was not available the eluted fractions were evaporated to dryness and dissolved in 90 % ethanol for crystalli-sation. The seventh, eighth and ninth fractions produced clusters of acicular crystals. The crystals after being isolated and dried appeared as a thin film and

under the microscope as fine crystalline needles with some amorphous particles. Recrystallisation from methanol gave colorless needles without contamination of amorphous material as observed under the microscope.

The gluconapin tetraacetate was characterised by melting point, specific rotation, infrared absorption spectrum and elemental analysis. This substance has not earlier been described in the literature.

	m.p.	$[\alpha]_D^{28}$
Found	170-171.5°	-17.5°(c 2.46% in H_2O)

	C(%)	H(%)	N(%)	S(%)
Found	39.22	4.35	2.67	11.03
Calculated for $C_{19}H_{26}O_{13}S_2NK$	39.37	4.52	2.42	11.06

The above empirical formula represents gluconapin tetraacetate free from water of crystallisation.

The infrared spectrum (Fig.1) of gluconapin tetraacetate shows absorption peaks at:

2950 cm^{-1}: indicates $-CH_2-$ in sample and Nujol

1750 cm^{-1}: indicates C=O in the acetylated glucose moiety.

1630 cm^{-1}: indicates terminal $=CH_2$

1580 cm^{-1}: indicates C=N

1430 cm^{-1}: indicates $-CH_2-$ in sample and Nujol

1375 cm^{-1}: indicates $-CH_2-$ in sample and Nujol

1250 cm^{-1}: indicates C=O in the acetylated glucose moiety

1060 cm^{-1}: indicates $-OSO_3^-$

720 cm^{-1}: indicates C-S

These interpretations of the spectrum (30,52) are in agreement with the structure for gluconapin tetraacetate.

Sinalbin

An extract of white mustard seed (Brassica hirta) was used for the isolation of sinalbin. In order to preserve the unique cation, sinapine, of this thioglucoside, the use of ion exchangers was avoided in the purification of the extract. The plant extract was chromatographed on a column of cellulose powder with 90 % ethanol as developing agent. The cellulose column did not effect a high degree of purification of the extract as judged by the deep color of the eluate. Repeated crystallisations of the evaporated eluate from methanol gave pure sinalbin as crystalline needles. The isolated sinalbin was cha-

racterised by its melting point which was in good agreement with a published value (14).

	m.p.
Found	83°
Reported (14)	83-84°

Glucoiberin

An extract of candytuft seed (Iberis amara) was used for the isolation of glucoiberin. The plant extract was chromatographed on a column of Amberlite IR-4B in chloride form with potassium hydroxide as eluting agent. The Amberlite column was considered satisfactory for separation of glucoiberin from impurities as shown by paper chromatography of the eluted fractions. The combined neutral eluates were evaporated to dryness and crystallised from 90 % methanol until colorless needles of glucoiberin of constant melting point were obtained.

The isolated glucoiberin apparently was not entirely pure, as indicated by its melting point, specific rotation and infrared absorption spectrum.

	m.p.	$[\alpha]_D^{28}$
Found	132-134° (decomposed)	-52.4° (c 2.1% in H_2O)
Recorded (14)	142-144°	$[\alpha]_D^{20}$ -55.3° (c 4.9% in H_2O)

The infrared spectrum (Fig.2) was identical to that reported in the literature (26) except for a small shift of the absorption peak at 740 cm^{-1}.

Sinigrin

An extract of black mustard seed (Brassica nigra Koch) was used for the isolation of sinigrin. In preliminary experiments the method of Stoll and Seebeck (22) was applied to the isolation of sinigrin by precipitation of impurities with lead acetate. Purification by chromatography on neutral alumina and cellulose powder was also attempted. Crystalline sinigrin was not obtained by these methods.

Ion exchange chromatography as used for glucoiberin subsequently proved a more satisfactory method. A plant extract was chromatographed on a column of Amberlite IR-4B in chloride form with potassium hydroxide as eluting agent. As with the isolation of glucoiberin the

Amberlite column was considered effective for separation of sinigrin from impurities. The combined neutral eluates were evaporated to dryness and crystallised from methanol without difficulty. The isolated sinigrin was characterised by melting point and specific rotation which were in good agreement with published values.

	m.p.	$[\alpha]_D^{28}$
Found	127-129°	-17.7°(c 3.72% in H_2O)
Reported (14)	127°	$[\alpha]_D^{27}$ -17.6°

II. Estimation of Thioglucosides with Anthrone Reagent.

Of the many methods known for determination of reducing sugars and their derivatives the colorimetric determination with anthrone reagent is particulary simple in operation. The substance to be analysed is added to a solution of anthrone in concentrated sulfuric acid and a blue-green color which developes with reducing sugars is measured colorimetrically or spectrophotometrically. The sulfuric acid simultaneously hydrolyses any polysaccharides or other glycosides with the effect that reducing sugar constituents of such molecules are included in the analysis. The anthrone method has been used for the determination of sucrose (53), glucose, lactose and glycogen (54), starch and cellulose (55), methyl cellulose (56) and dextran (50,57). Scott and Melvin (50) improved the precision and accuracy of the method by introducing a modification whereby the sample and anthrone reagent upon mixing were heated under controlled conditions for a measured length of time. Schultz and Gmelin (7) reported on the use of this method for estimation of thioglucosides.

The present investigation includes a study of the anthrone method as applied to three pure thioglucosides and to plant extracts of rapeseed. The absorption spectra

of the developed colors and the effect of heating time upon color development have been studied. The precision and accuracy of the method have been discussed.

(A). Preparation of thioglucoside extract from rapeseed.

A purified thioglucoside extract was prepared from half a gram of rapeseed by grinding of the seed, removal of fatty material, extraction of thioglucosides from the meal and chromatography of the crude extract on an acid alumina column.

For convenience, a relatively high boiling petroleum ether was used for extraction of fatty material since the cooling effect of an air condenser was sufficient to prevent losses of this solvent during refluxing.

As earlier mentioned, a solvent used for extraction of thioglucosides from the meal should contain a suitable amount of acetone as this was found to facilitate the separation of suspended fine particles from the solution. While Schultz and Gmelin (7) evaporated organic solvents from the crude extract and applied it in aqueous solution to the acid alumina column, it was found unnecessary in the present work to remove the organic solvents before chromatography. This was shown by an experiment in which crude extracts of rapeseed were analysed by the anthrone method

with and without removal of organic solvents prior to chromatography on acid alumina. The analytical results (Table 2.) were nearly identical with and without removal of the organic solvents.

TABLE 2

ANALYSIS OF RAPESEED EXTRACTS WITH AND WITHOUT REMOVAL OF ORGANIC SOLVENTS PRIOR TO ACID ALUMINA CHROMATOGRAPHY.

Rapeseed analysed	Commercial sample		Variety Tanka	
Organic solvents	removed	not removed	removed	not removed
Number of acid alumina column	1 2	3 4	5 6	7 8
Absorbance at 625 mμ. for duplicate color solutions	0.25 0.25 0.25 0.25	0.26 0.25 0.27 0.26	0.29 0.27 0.29 0.27	0.26 0.27 0.27 0.27

Chromatography of the crude extract on acid alumina was carried out essentially as by Schultz and Gmelin (7). It was found necessary to triturate the acid alumina with distilled water before use to remove fine particles which otherwise tended to sift into the eluate and cause inter-

ference with the color measurement. Elution of thioglucosides from the column was effected with potassium hydroxide.

A yellow band which moved down the column with the front of the potassium hydroxide solution served to indicate when to stop elution of the column. According to Schultz and Gmelin (7) the total thioglucosides are eluted in front of this band so that elution may be stopped when the yellow band reaches close to the end of the column. If elution is continued until colored material appears in the eluate the colorimetric analysis of the eluate will give incorrect results.

It was of interest to ascertain if an acid alumina column could be used again after regeneration with hydrochloric acid. For this purpose extracts of commercial rapeseed were purified in separate experiments with new and regenerated acid alumina and subsequently analysed with anthrone reagent. The analytical results (Table 3) which were nearly identical for new and regenerated alumina demonstrate that the alumina may be used again after regeneration and in this respect functions as an anion exchanger.

(B). Selection of wave length for absorption measurement.

McCready et al.(58) published a time curve for color

TABLE 3

ANALYSIS OF RAPESEED EXTRACTS AFTER CHROMATOGRAPHY ON NEW AND REGENERATED ACID ALUMINA.

Triplicate extracts of commercial rapeseed	1		2		3	
Acid alumina	new	regenerated	new	regenerated	new	regenerated
Absorbance at 625 mμ. for duplicate color solutions	0.20	0.20	0.21	0.20	0.21	0.20
	0.21	0.22	0.21	0.21	0.21	0.20

development at 100° showing that maximum absorption occurred at 625 mμ. and after 7.5 minutes. Scott and Melvin (50) in a similar experiment heated an 0.003 per cent glucose solution at 100° for 7.5 and 30 minutes. Absorption spectra of the developed colors showed peaks at 625, 502 and 427 mμ. They chose to use the absorption at 625 mμ for measurement in the analytical procedure because this absorption peak developed more rapidly than the other two. Schultz and Gmelin (7) in their study of thioglucosides

claimed that contamination of glucose with other fission products of thioglucosides did not affect the color development of the glucose as measured photometrically with the use of a filter. There was no information in their work, however, as to whether the absorption maxima obtained with thioglucosides occurred at the same wavelengths as with glucose.

In the present work, therefore, the absorption spectra were recorded of color solutions generated with solutions of glucose, glucoiberin, sinalbin and a thioglucoside extract from commercial rapeseed, using a 15 minutes development time. The absorption spectra (Fig. 3) show well defined peaks at 625 and 427 mμ. and a low absorption at 502 mμ. A comparison of the spectrum for glucose with those of the pure thioglucosides and the thioglucoside extract revealed that the absorbance at 427 mμ. relative to that at 625 mμ. was lower for glucose than for the other substances tested. This may indicate that these latter substances contain material other than glucose and that this material generates color with a higher absorption at 427 than at 625 mμ. In view of this observation the wavelength 625 mμ. was chosen for measurement of absorption in the anthrone method.

(C). Selection of heating time for color development.

The relationship between heating time and color development at 625 mμ. was determined for solutions of glucose, glucoiberin and a thioglucoside extract from seed of variety sarson (Fig.4). Of the three pure thioglucosides available, glucoiberin was chosen for this experiment since it occurs in rapeseed, while sinigrin does not, and while the third thioglucoside, sinalbin, although occurring in rapeseed, is reported to generate a stronger color with anthrone than can be explained by its glucose content (7). The concentrations of glucoiberin and glucose used in the experiment were chosen so that the theoretical amount of glucose released from glucoiberin was identical to the amount of pure glucose. The glucose and glucoiberin solutions gave maximum absorbances after 8 minutes and the thioglucoside extract after 9 to 10 minutes of heating. Considering the shapes of the three time/absorbance curves it appears that a development time chosen between 12 and 15 minutes would give the best conditions for reproducible color determinations as the curves slope the least over this interval. Although glucose used at a development time of 14 minutes might have been a satisfactory reference standard for the present work, glucoiberin was preferred for this purpose since its

curve coincided the most with that for rapeseed extract. A 15 minutes development time was chosen as standard for the method.

(D). Precision and Accuracy in Determination of Glucose and Pure Thioglucosides.

Five series of analyses with glucose, glucoiberin and sinigrin in different concentrations and one series with sinalbin were carried out (Tables 4-7). The amounts of each substance used in the analyses, with exception of sinalbin, were calculated so that corresponding samples for the different series theoretically contained the same amounts of glucose after hydrolysis of the thioglucosides.

In each of the series analysed for all four substances, the obtained color intensities (absorbances) were proportional to the amount of sample analysed. When plotted as graphs, the absorbance as a function of amount of substance analysed in all cases gave apparent straight lines interceptimg at origin thus demonstrating that the measurements fit Lambert Beer's law. The slopes of these lines are slightly different for the different series analysed but not sufficiently different to be clearly demonstrated in graphic form. In order to express a

measurement of the slopes of the lines, the regression coefficients (b) were calculated for each series of analysis (Tables 4-7). The regression coefficients are expressed in units of absorbance per 10 micrograms of glucose. The mean values of the regression coefficients for the five series relating to each substance were computed, except for sinalbin where only one series had been analysed.

The standard deviation from the mean and the relative standard deviation (Tables 4-6) give an expression of the precision of analysis in these experiments. On basis of the standard deviations, the analyses of sinigrin appear the most precise amongst the three substances used. As discussed in an earlier chapter, glucoiberin was nevertheless chosen as the standard for further analytical work with rapeseed since this substance occurs in rapeseed. The regression coefficient for glucoiberin was thus used in calculations of glucose contents in analyses of rapeseed.

The regression coefficients were very similar for all substances analysed with exception of sinalbin which had a regression coefficient 1.57 times larger than that of glucoiberin. This demonstrates that the development of color in the analysis of glucose, glucoiberin and

TABLE 4.

ABSORBANCES AND REGRESSION COEFFICIENTS FOR FIVE SERIES OF GLUCOSE ANALYSES

Glucose solution (ml.)	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	Regression
Glucose content (µg x 10⁻¹)	20	18	16	14	12	10	8	6	4	coefficient (b)
Absorbance (625 mµ.)										
Series No. 1	0.55	0.49	0.45	0.39	0.33	0.27	0.22	0.16	0.12	0.02742
2	0.55	0.49	0.43	0.40	0.34	0.28	0.24	0.18	0.11	0.02658
3	0.52	0.45	0.42	0.35	0.30	0.24	0.19	0.13	0.07	0.02983
4	0.55	0.50	0.44	0.38	0.34	0.28	0.22	0.16	0.10	0.02653
5.	0.50	0.46	0.41	0.35	0.30	0.26	0.20	0.15	0.10	0.02533
Mean value										0.02714
Standard deviation										0.00169
Relative standard deviation										6.19

TABLE 5

ABSORBANCES AND REGRESSION COEFFICIENTS FOR FIVE SERIES OF CLUCOIBERIN ANALYSES

Glucoiberin solution (ml.)	0.50	0.45	0.40	0.35	0.30	0.25	0.20	0.15	
Glucoiberin content (µg x 10⁻¹)	51.0	45.8	40.8	35.7	30.6	25.5	20.4	15.3	Regression coefficient (b)
Theoretical glucose content (µg x 10⁻¹)	20	18	16	14	12	10	8	6	
Absorbance (625 mµ.)									
Series No. 1.	0.63	0.56	0.47	0.41	0.35	0.29	0.22	0.18	0.03247
2.	0.57	0.51	0.45	0.38	0.33	0.27	0.21	0.16	0.02956
3.	0.55	0.48	0.42	0.38	0.32	0.25	0.20	0.15	0.02839
4.	0.57	0.50	0.46	0.39	0.33	0.27	0.21	0.16	0.02988
5.	0.55	0.52	0.44	0.37	0.31	0.26	0.20	0.16	0.02935
Mean value									0.02953
Standard deviation									0.00158
Relative standard deviation									5.35

TABLE 6

ABSORBANCES AND REGRESSION COEFFICIENTS FOR FIVE SERIES OF SINIGRIN ANALYSES

Sinigrin solution (ml.)		0.50	0.45	0.40	0.35	0.30	0.25	0.20	0.15	
Sinigrin content (µg x 10^{-1})		44.10	39.69	35.28	30.87	26.46	22.05	17.64	13.23	Regression coefficient (b)
Theoretical glucose content (µg x 10^{-1})		20	18	16	14	12	10	8	6	
Absorbance (625 mµ.) Series No.	1.	0.52	0.47	0.42	0.37	0.32	0.26	0.21	0.16	0.02589
	2.	0.52	0.46	0.42	0.37	0.34	0.28	0.21	0.16	0.02464
	3.	0.49	0.41	0.37	0.33	0.28	0.24	0.17	0.12	0.02518
	4.	0.52	0.46	0.42	0.36	0.31	0.25	0.20	0.15	0.02643
	5.	0.55	0.46	0.41	0.35	0.30	0.26	0.20	0.15	0.02530
Mean value										0.02549
Standard deviation										0.00069
Relative standard deviation										2.7

TABLE 7

ABSORBANCES AND REGRESSION COEFFICIENT OF ONE SERIES OF SINALBIN ANALYSES

Sinalbin solution (ml.)	1.8	1.6	1.4	1.0	0.8	0.6	0.4	
Sinalbin content (µg x 10)	144	128	112	80	64	48	32	Regression coefficient (b)
Theoretical glucose content (µg x 10)	16.67	14.82	12.96	9.26	7.41	5.56	3.70	
Absorbance (625 mµ.)	0.85	0.74	0.67	0.49	0.41	0.31	0.22	0.04658

sinigrin can be quantitatively accounted for by the glucose contents of these substances while the color development in sinalbin is considerably higher than can be accounted for by its glucose content. This latter observation regarding sinalbin is in agreement with the results of Schultz and Gmelin (7).

As implied in the above discussion relevant to sinalbin, the differences between the mean regression coefficients for each of the four substances express the degree of accuracy of the method. The regression coefficients for glucoiberin, and sinigrin differ only little from the coefficient of glucose. This indicates a high accuracy of the anthrone method in determining these two pure thioglucosides. The accuracy in the determination of sinalbin is poor, as expressed by the large difference between its regression coefficient and that of glucose. Since sinalbin constitutes a relatively small proportion of the total thioglucosides in rapeseed, this inaccuracy in the determination of sinalbin should not greatly influence the accuracy in determination of the total thioglucosides in rapeseed.

(E). Precision in Determination of Thioglucosides in Rapeseed.

A number of determinations of thioglucoside contents

in rapeseed was made to determine the precision of the anthrone method in such analyses (Table 8). Six extracts were made of seed of variety sarson. The extracts were each divided in two parts to give a total of twelve samples which were purified by chromatography on acid alumina and analysed by the anthrone method. The twelve analyses were done simultaneously and all under the same conditions.

The differences in absorbance between two thioglucoside fractions from the same plant extract were at the most 0.01 which is the average error of the spectrophotometer used. There were slightly larger differences in absorbance among the six pairs of thioglucoside fractions.

The absorbances were converted into the corresponding glucose contents by division with the regression coefficient for glucoiberin. The glucose contents are expressed in units of 10 micrograms in the Table. The mean value of the glucose contents for the twelve analyses and the standard deviation from the mean were calculated (Table 8). The mean value multiplied by a dilution factor of 200 provided the corresponding glucose content in one gram of rapeseed. Thus the thioglucoside content in the seed of variety sarson can be expressed as 21.34 ± 0.86 milligrams of glucose per gram of seed.

TABLE 8

ANALYSIS OF GLUCOSE CONTENTS IN THIOGLUCOSIDE EXTRACTS FROM VARIETY SARSON

No. of plant extract	1		2		3		4		5		6	
No. of acid alumina column	1	2	3	4	5	6	7	8	9	10	11	12
Absorbance (625 mμ.)	0.32	0.31	0.32	0.32	0.32	0.33	0.33	0.33	0.30	0.31	0.29	0.30
Glucose content (μg x 10^{-1})	10.84	10.50	10.84	10.84	10.84	11.18	11.18	11.18	10.16	10.50	9.82	11.16
Mean value (μg x 10^{-1})	10.67											
Standard deviation	0.43											

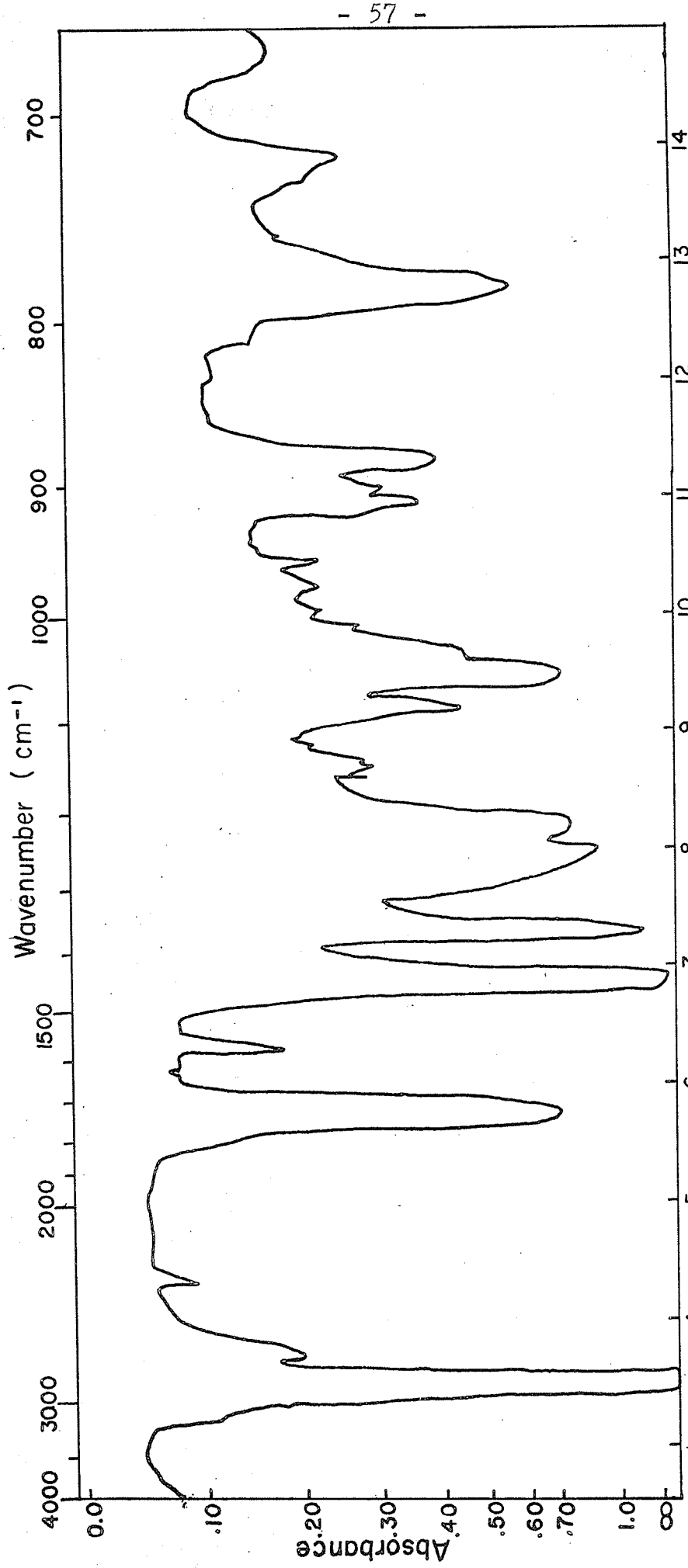


Fig. I Infrared spectrum of gluconapin tetraacetate in Nujol mull.

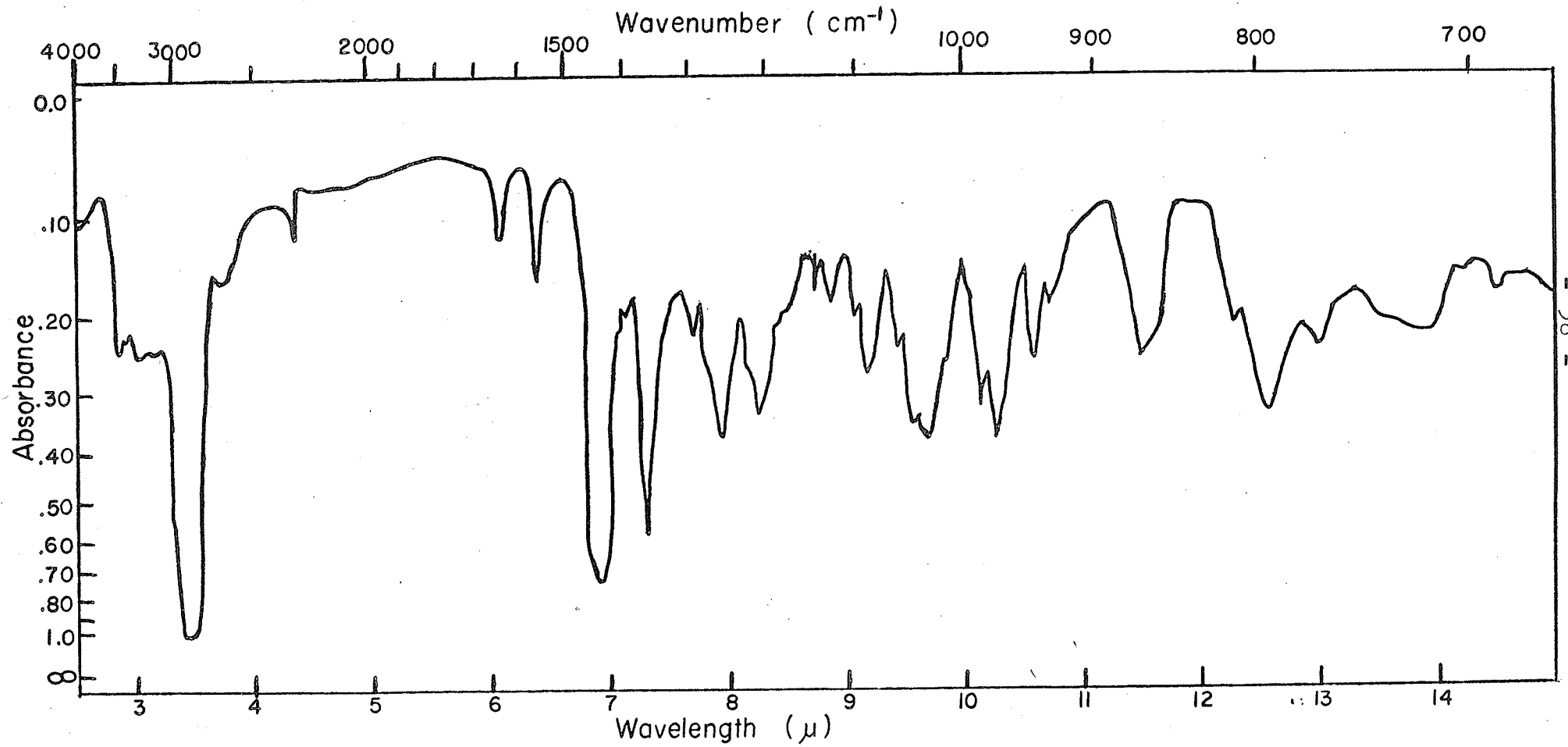


Fig. 2 Infrared spectrum of glucoiberin in Nujol mull

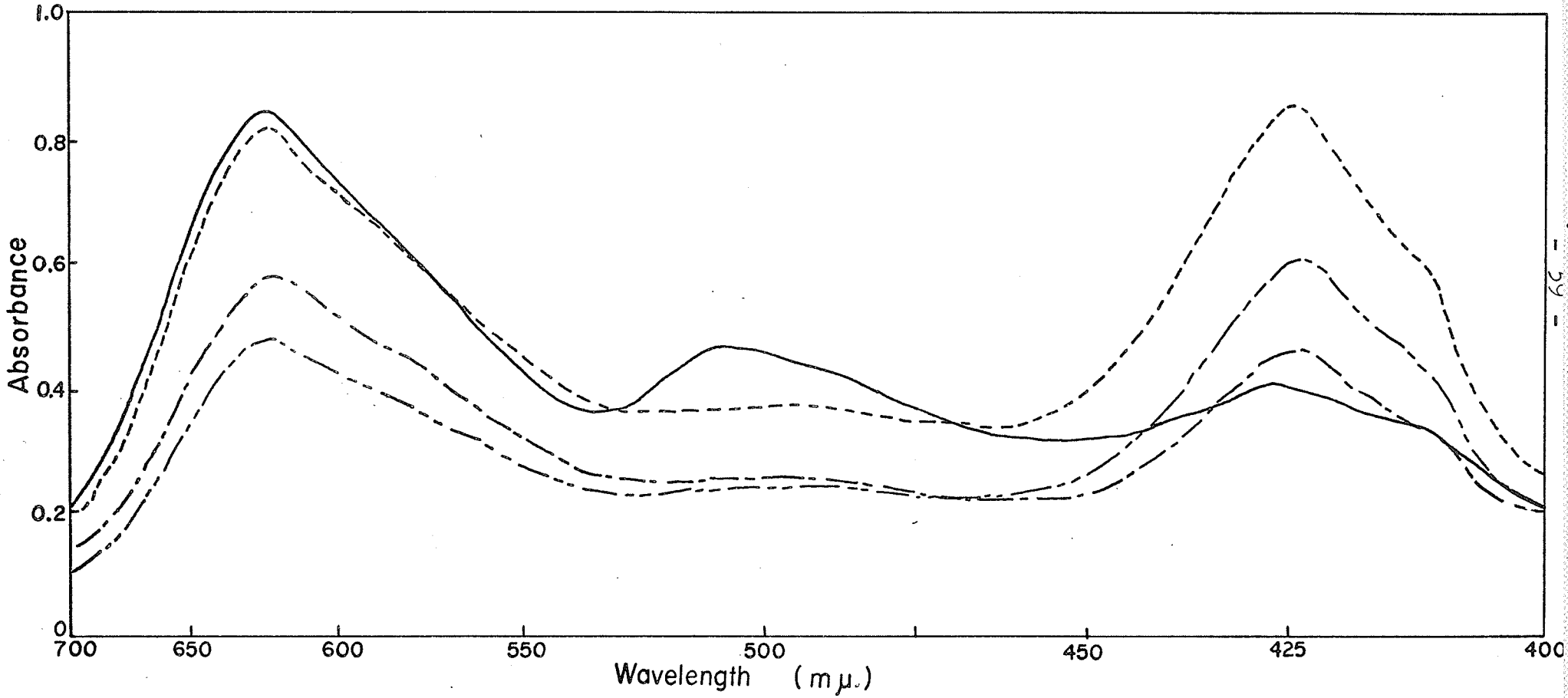


Fig. 3 Absorption spectra of colored solutions generated by anthrone reagent with glucose (——), sinalbin (- - -), glucoibrin (—— - ——), and a thioglucoside fraction from rapeseed (B. napus) (—— - - ——).

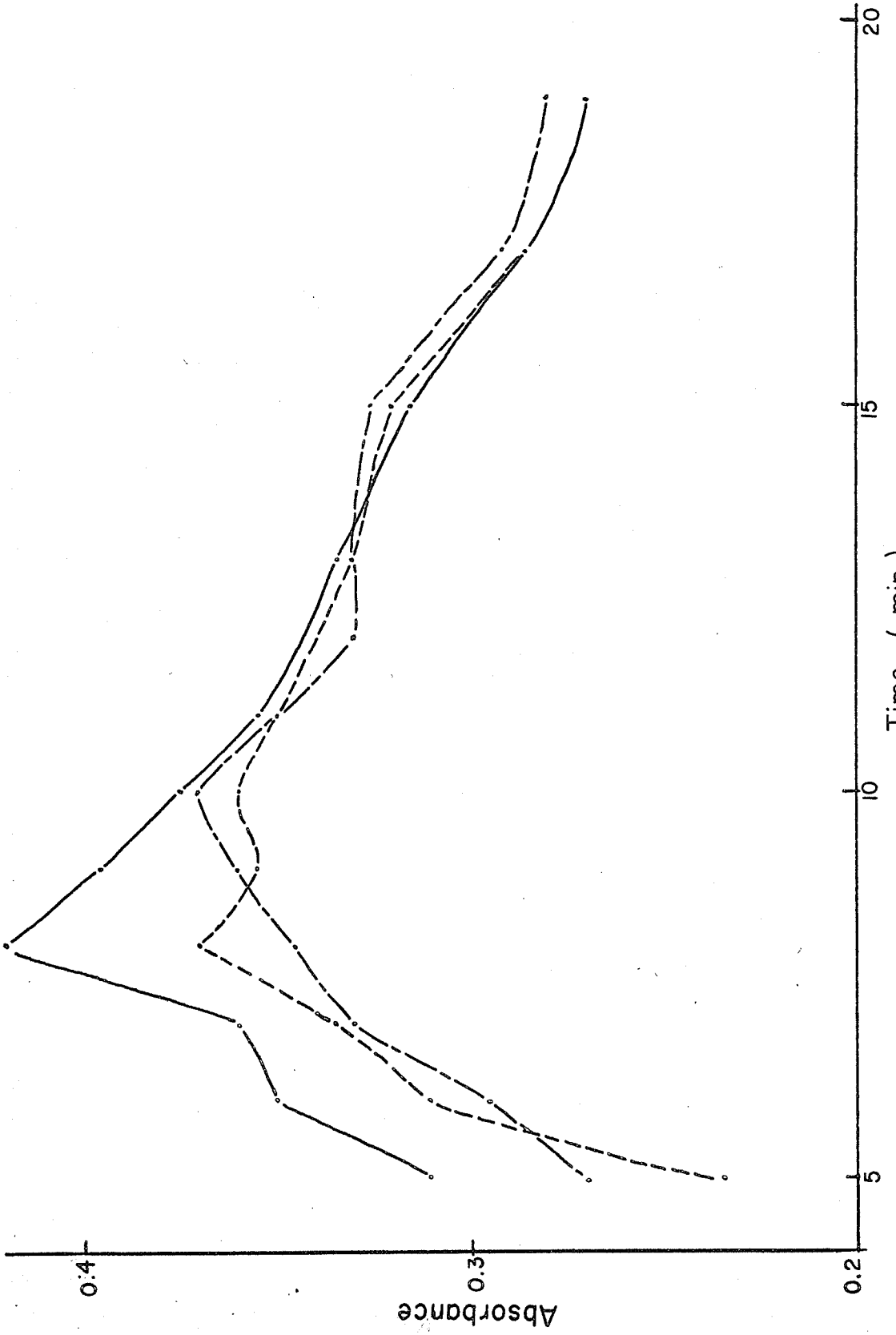


Fig. 4 Relation of heating time and intensity of color generated by anthrone reagent with glucose (——), glucoiberin (- - -), and a thioglucoside fraction from rapeseed (B. campestris) (—— - ——). (Absorbance at 625 mμ.)

REFERENCES

1. A.Kjaer. Acta. Chem. Scand. 10, 1365 (1956).

2. W. Schneider. In:Handbuch der Pflanzenanalyse. Bd. III/2. Wien: J. Springer. Berlin. 1932. S.1063.

3. O.E. Schultz and R. Gmelin. Arch. Pharm. 287/59, 404 (1954).

4. B.R. Das, P.A. Kurup and P.L.N. Rao. Indian J. Med. Research 45, 191 (1957).

5. M.A. Greer, J.Am. Chem. Soc. 78, 1260 (1956).

6. J.K. Nayar and A.J. Thorsteinson. Can. J. Zool. 41, 923 (1963).

7. O.E. Schultz and R. Gmelin. Z. Naturforsch. 9b, 27 (1954).

8. Robiquet et. Bourtons. J. pharm.chim. 17, 270 (1831).

9. A. Bussy. J. pharmac. chim. 26, 39 (1840).

10. J. Gadamer. Arch. Pharm. 235, 44 (1897).

11. J. Gadamer. Ber. 30, 2322 (1897).

12. J. Gadamer. Ber. 30, 2327 (1897).

13. M.G. Ettlinger and A.J. Lundeen. J. Am. Chem. Soc. 78, 4172 (1956).

14. A. Kjaer. In: L. Zechmeister. Progress in the Chemistry of Organic Natural Products. Vol. XVIII. Springer-Verlag, Wien. 1960. p.122.

15. A. Kjaer. Acta. Chem. Scand. 8, 1110 (1954).

16. M.G. Ettlinger and A.J. Lundeen. J. Am. Chem. Soc. 79, 1764 (1957).

17. E.W. Underhill, M.D. Chisholm and L.R. Wetter. Can. J. Biochem. and Physiol. 40, 1505 (1962).

18. H. Will and W. Körner. Liebigs Ann. Chem. 125, 257 (1863).

19. A.W. Hofmann. Ber. 1, 169 (1868).

20. W. Schneider and F. Wrede. Ber. 47, 2225 (1914).

21. W.Schneider, H. Fischer and W. Specht. Ber. 63, 2787 (1930).

22. A. Stoll and E. Seebeck. Helv. Chim. Acta. 31, 1432 (1948).

23. O.E. Schultz and R. Gmelin. Arzneimittel-Forsch. 2, 568 (1952).

24. O.E. Schultz and E. Barthold. Arzneimittel-Forsch. 2, 532 (1952).

25. O.E. Schultz and R. Gmelin and A. Keller. Z. Naturforsch. 8b, 14 (1953).

26. O.E. Schultz and R. Gmelin. Arch. Pharm. 287/59, 405 (1954).

27. O.E. Schultz and W. Wagner. Arch. Pharm. 288/60, 525 (1955).

28. W. Wagner. Dissertation, Universität Tübingen. 1956

29. A. Kjaer. Acta. Chem. Scand. 13, 851 (1959).

30. M.A. Greer. Arch. Biochem. Biophys. 99, 369 (1962).

31. P. Hietala. Ann. Acad. Sci. Fennicae. Ser. AII, No. 100, 69 (1960).

32. M.H. Benn. Can. J. Chem. 41, 2836 (1963).

33. M. Sandberg and O.M. Holly. J. Biol. Chem. 96, 443 (1932).

34. O. Folin and H. Malmros. J. Biol. Chem. 83, 115 (1929).

35. Z. Nagashima and M. Uchiyama. Bull. Agr. Chem. Soc. Japan. 33, 478 (1959).

36. J.B. Sumner. J. Biol. Chem. 65, 393 (1925).

37. E.T. Reese, R.C. Clapp and M. Mandels. Arch. Biochem. Biophys. 75, 228 (1958).

38. R. Dreywood. Anal. Chem. 18, 499 (1946).

39. J. Gadamer. Arch. Pharm. 237, 120 (1899).

40. R.S. Hubbard. J. Biol. Chem. 88, 663 (1930).

41. D.S. McKittrick and C.L.A. Smith. Arch. Biochem. Biophys. 6, 411 (1945).

42. H. Schmid and P. Karrer. Helv. Chim. Acta. 31, 1017 (1948).

43. A. Stoll and E. Jucker. In: K. Paech and M.V. Tracey. Moderne Methoden Der Pflanzenanalyse. Bd. IV. Springer-Verlag. Berlin. 1955. S. 689.

44. E.B. Astwood, M.A. Greer and M.G. Ettlinger. J. Biol. Chem. 181, 121 (1949).

45. L.R. Wetter. Can. J. Biochem. and Physiol. 38, 980 (1955).

46. L.R. Wetter. Can. J. Biochem. and Physiol. 35, 293 (1957).

47. A. Kjaer. Acta. Chem. Scand. 7, 518 (1953).

48. O.E. Schultz and W. Wagner. Z. Naturforsch. 11b, 73 (1956).

50. T.A. Scott. Jr. and E.H. Melvin. Anal. Chem. 25, (1953).

51. J.K. Nayar. Private communication.

52. L.J. Bellamy. The infrared spectra of complex molecules. John Wiley and Sons, Inc., New York. 1958.

53. E.E. Morse. Anal. Chem. 19, 1012 (1947).

54. D.L. Morris. Science 107, 254 (1948).

55. F.J. Viles. Jr. and L. Silverman. Anal. Chem. 21, 950 (1949).

56. E.P. Smasel and R.A. Delap. Ibid 23, 1795 (1951).

57. W.L. Bloom and M.L. Willcox. Proc. Soc. Exptl. Biol. Med. 76, 3 (1951).

58. R.M. McCready, J. Guggolz, V. Silviera and H.S. Owens. Ibid 22, 1156 (1950).