DETERMINATION OF THE DIGITOXIN CONTENT OF *DIGITALIS PURPUREA*

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There is no simple method of determining the digitoxin content of leaves of *Digitalis purpurea* and often it is not easy to decide the suitability of a crop for the preparation of this glycoside other than by extracting a substantial quantity of leaf. It is well known that both the amount and composition of the glycosidal mixture are subject to wide variation depending on botanical, geographical and ecological factors. Neither biological nor chemical assays of the leaf or crude extracts prepared from it give a reliable indication of the amount of digitoxin present. However by using paper chromatography the separation of the glycosidal mixture is considerably simplified and the individual glycosides may be estimated colorimetrically. A method along these lines for the estimation of digitoxin in small samples of leaf is described here and results obtained in applying the procedure to several samples are reported.

Propylene dichloride was used to isolate the glycosides because it is more selective than chloroform[1] and the system carbon tetrachloride-ethanol-water[2] was found suitable for the chromatographic separation of digitoxin from the propylene dichloride-soluble glycosides. This system consists of readily available solvents which require no further purification and are easily removed from the completed chromatograms. One drawback is that the capacity of the system is small compared with those of systems based on formamide, but this is not serious as digitoxin is the main constituent of the glycosidal mixture and it was not intended that trace constituents should be studied. The best known and most sensitive of the colorimetric assays for digitoxin is that using sodium picrate but this needs 100 to 150 μg. of digitoxin to give a useful optical density. This would necessitate running a considerable number of spots and the extraction of a correspondingly large area of paper. A sensitive assay method with xanthydrol was developed from the qualitative test for digitoxin described by Arreguine and Pasqualis[3]; the digitoxose side chain is the basis of the reaction and quantities of the order of 20 μg. only are required.

Arreguine and Pasqualis heated the reaction mixture in a water bath until the red colour developed. Pesez[4] reported that the colour is stable if the mixture is heated in a boiling water bath for exactly three minutes followed by cooling in ice for five minutes. These conditions have been used by Tschesche, Grimmer and Seehofer[5] and by Tuzson and Vastagh[6] respectively in their quantitative chromatographic methods. The modification of the Arreguine and Pasqualis procedure described here, in which the reaction takes place at room temperature, avoids any critical conditions of heating and there is no risk of errors arising from uptake of

moisture. In addition, when using paper chromatograms, extraction of the paper and colour formation proceed simultaneously.

Preliminary autolytic fermentation is employed to hydrolyse the initial complexes to the secondary glycosides. Purpurea glycosides A and B are apparently not extracted by propylene dichloride, for on chromatograms they would be expected to remain close to the solvent front whereas no more than a trace of unidentified material has been found in this position on chromatograms from unfermented leaf. Moreover, after the preliminary hydrolysis both the content of propylene dichloride-soluble glycosides and of digitoxin show substantial increases. The breakdown of the secondary glycosides into aglycones does not take place and according to Neuwald[7] there are no enzymes in the leaf capable of such hydrolysis. In the present work no more than a trace of digitoxigenin has been detected and this is in agreement with Neuwald's findings.

Experimental Methods

Propylene Dichloride-Soluble Glycosides

20 g. of dried powdered leaf was mixed with 20 ml. of distilled water, placed in a 250 ml. flask and incubated in a water bath at 37° C. for 65 hours. At the end of this time 40 ml. of distilled water and 140 ml. of industrial absolute ethanol were added. The flask was then shaken for an hour and the 70 per cent. ethanol extract was obtained by filtering through a fluted 531 paper. To 50 ml. of the extract was added 100 ml. of distilled water and the solution distilled under vacuum until free from ethanol, using a silicone antifoam to prevent frothing. The glycosides were then extracted with four quantities each of 25 ml. of propylene dichloride taking care to avoid emulsification. These extracts were mixed together and washed with two quantities each of 25 ml. of 1 per cent. sodium carbonate solution (2·7 per cent. of $Na_2CO_3 \cdot 10H_2O$) and finally two volumes each of 25 ml. of water. The propylene dichloride was then removed under vacuum and the residue taken up in 100 ml. of *iso*propanol. 5 ml. portions of this solutions were mixed with 5 ml. of sodium picrate reagent (1 per cent. aqueous solution of trinitrophenol 95 ml., 10 per cent. sodium hydroxide solution 5 ml.) and the maximum optical density at 495 mμ measured (filter 623 in the EEL photoelectric colorimeter). The results were calculated from a linear calibration curve relating optical density with biological potency, previously prepared using samples of digitoxin of known biological potency. As no solid fraction is isolated in this method results were expressed as units per g. of leaf.

Chromatography

Equal volumes of carbon tetrachloride B.P., industrial 95 per cent. ethanol and distilled water were shaken together and allowed to separate. The lower layer then formed the mobile phase and the upper layer was placed in the bottom of the tank. The solution to be chromatographed was obtained by distilling to dryness, *in vacuo*, 50 ml. of the above prepared *iso*propanol solution and taking up the residue in 5 ml. of dry methanol.

0·01 ml. quantities of this were applied to the paper using an Agla micrometer syringe. A strip of Whatman No. 1 paper 46 × 9 cm. was used and on this were pencilled (*a*) a starting line 19 cm. from one end and (*b*) two longitudinal lines running one along each side, 1·5 cm. from the edge. Six spots were placed on the starting line, one in the centre of each of the side divisions and four, one centimetre apart, in the middle of the strips, the outside spots of the latter group thus being 1·5 cm. from the parallel markings. The solvent system in the tank was allowed to come to equilibrium as usual but no preliminary saturation of the paper was used. The strip together with a blank marked in the same way was dipped into the trough and downward development was continued until the solvent front was near the bottom of the test strip (about seven hours) by which time the solvent front on the blank had already reached the bottom. The papers were then removed and allowed to dry in the air. By cutting along the two pencilled lines the chromatograms corresponding to the two outer spots were obtained and the position of the glycosides on these was revealed by spraying with trichloroacetic acid in chloroform, "aged" with hydrogen peroxide. By placing these strips alongside the unsprayed part of the original strip the positions of the zones in the latter were determined and these were then cut out. The corresponding areas of the blank paper were also cut out to serve as controls in the colorimetry. In preliminary experiments the glycosides were also located by using antimony trichloride[8], phosphoric aid[9] and xanthydrol, respectively.

Colorimetric Assays using Xanthydrol Reagent

The rectangular pieces of paper were then further cut up and placed in small beakers, one to each zone. To each was added 10 ml. of a 0·125 per cent. solution of xanthydrol in glacial acetic acid (A.R.) and 0·1 ml. of hydrochloric acid. The contents of each beaker were then stirred and the beakers placed under inverted amber glass jars. At the same time a reagent blank consisting of 10 ml. of xanthydrol solution mixed with 0·1 ml. of hydrochloric acid was placed in a corked colorimeter tube. At the end of two hours, during which time the contents of the beakers were stirred occasionally, the solutions were poured into colorimeter tubes and corked. Readings of optical density were taken, using filter 624, on the EEL Colorimeter, the instrument being balanced to zero with the reagent blank in position. Further measurements were made at intervals until the maximum density was reached (2–4 hours from the time of mixing) the tubes being kept away from strong light between observations. The optical densities recorded from the areas of blank paper were then subtracted from those for the corresponding areas of the chromatogram and the glycosidal contents calculated from a standard graph based on digitoxose.

RESULTS

Study of the Isolation Procedure

A number of estimations were made using one of the leaf samples (A24) in order to study the period of fermentation required to complete

enzymatic hydrolysis, the proportion of water needed for this process and the completeness of extraction into propylene dichloride. It was found that the content of propylene dichloride-soluble glycosides indicated by assay with sodium picrate increased rapidly during the first eight hours of fermentation, then increased slowly to reach a maximum in about three days (Table I). Variations in the amount of water used, with corresponding alterations in the quantity added with ethanol for extraction, showed that the ratio one part of water to one part of leaf is satisfactory (Table II). By assaying each 25 ml. volume of propylene dichloride in the extraction process, after washing with sodium carbonate solution and with water, it was found that the total yield could be increased by about five per cent. if a fifth volume of the solvent was used (Table III).

TABLE I

EFFECT OF THE DURATION OF FERMENTATION ON THE APPARENT CONTENT OF PROPYLENE DICHLORIDE-SOLUBLE GLYCOSIDES (ASSAYED WITH SODIUM PICRATE)

Length of fermentation at 37° C. in hours	Units per g. of leaf propylene dichloride-soluble fraction
0	1·36
8	2·52
18	2·60
24	2·56
48	2·60
72	2·75
115	2·75

TABLE II

EFFECT OF THE PROPORTION OF WATER USED DURING FERMENTATION FOR 65 HOURS AT 37° C. ON THE APPARENT CONTENT OF PROPYLENE DICHLORIDE-SOLUBLE GLYCOSIDES (ASSAYED WITH SODIUM PICRATE)

Weight of water/Weight of leaf	Units per g. of leaf propylene dichloride-soluble fraction
0	1·28
0·25	2·12
0·5	2·40
1·0	See Table I
3·0	2·45

TABLE III

EXTRACTION OF PROPYLENE DICHLORIDE-SOLUBLE GLYCOSIDES INTO SUCCESSIVE PORTIONS OF THE SOLVENT (ASSAYED WITH SODIUM PICRATE)

	Units per g. of leaf propylene dichloride-soluble fraction	
	Per portion	Progressive total
1	1·13	1·13
2	0·59	1·72
3	0·36	2·08
4	0·22	2·30
5	0·13	2·43

Chromatography of the Propylene Dichloride Soluble Glycosides

Chromatograms were prepared of the propylene dichloride-soluble glycosides obtained without preliminary fermentation and those obtained after 72 hours of such treatment. The two strips were similar in appearance but the zones from the latter were denser. Digitoxin formed elongated spots extending from R_F 0·26 to R_F 0·42 and assays with xanthydrol reagent showed the apparent digitoxin content of the unfermented leaf to be 0·073 per cent. and that of the fermented leaf, 0·157 per cent. Further runs of the "72 hours" sample were made on wider sheets of paper with digitoxin, gitoxin and the aglycones as controls. Such a run, in which the solvent has been allowed to drip from the bottom of the paper is shown in Figure 1A. By continuing development for 24 to 48 hours, further resolution of the slower running spots into additional components takes place but no attempt has been made to study them further. A typical chromatogram of the propylene dichloride-soluble glycosides is shown in Figure 1B. There is sometimes a trace of digitoxigenin running

faster than digitoxin and normally some gitoxigenin slightly slower than digitoxin. Somewhat slower still is the spot due to gitoxin and between this and the starting line is an unidentified member of the B series. There is usually a slight "ghost" on the starting line itself.

Assays with Xanthydrol Reagent

When a solution of digitoxin is mixed with xanthydrol reagent and warmed in a water bath the initial yellow colour rapidly changes to an intense red, then becoming brown and finally green. At lower temperatures the reaction is much slower, the red colour reaching maximum intensity in two to four hours at room temperature. The maximum sensitivity is shown when using filter 624 (about 520 mμ), and Beer's Law is obeyed. Some fading takes place in sunlight and the presence of a trace of water greatly impairs colour formation, 1 per cent. producing a significant effect. Gitoxin on treatment gives a colour with an optical density similar to that of digitoxin while, as expected, the aglycones produce no colour at all. The reaction is about four and a half times as sensitive as that with sodium picrate, the value of E (1 per cent. 1 cm.) using digitoxin being about 900. Because of the difficulty in obtaining a pure sample of digitoxin, digitoxose was used as standard and a value of E (1 per cent. 1 cm,) = 1520 was obtained.

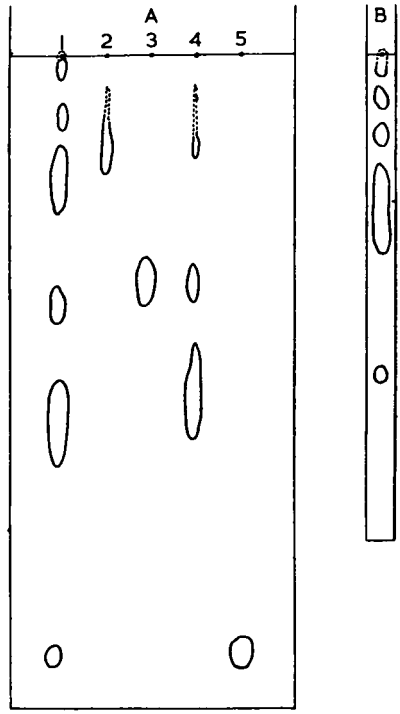

FIG. 1. Chromatograms of the propylene dichloride–soluble glycosides of digitalis. *A*. 15-hour run. *B*. 7-hour run.

1. 10 μg. Propylene dichloride–soluble glycosides. 2. 1 μg. Gitoxin. 3. 0·4 μg. Gitoxigenin. 4. 10 μg. Digitoxin. 5. 10 μg. Digitoxigenin.

Estimation of Digitoxin on Paper Strips

Eight samples of digitoxin all containing gitoxin were examined chromatographically, running duplicate chromatograms from 1 in 1000 solutions in methanol. The digitoxin and gitoxin zones were then assayed using xanthydrol reagent, and direct assays of the methanol solution were also made by placing 0·04 ml. quantities in colorimeter tubes, evaporating the solvent and adding the reagent to the residue. Recoveries from the strips were calculated by adding together the optical densities produced by the digitoxin and gitoxin zones and expressing the sum as a percentage

of that obtained by direct assay. The average recovery was found to be 95·1 per cent., the lowest being 90·5 per cent. and the highest 99 per cent. (Table IV).

On these chromatograms R_F values for digitoxin and gitoxin were somewhat lower than the R_F values for these substances when constituents of the propylene dichloride-soluble fraction, and the fringes of the two

TABLE IV

RECOVERY FROM CHROMATOGRAMS, 0·04 ml. PORTIONS OF APPROXIMATELY 1 IN 1000 SOLUTIONS OF DIGITOXIN ASSAYED WITH XANTHYDROL, DIRECTLY AND AFTER CHROMATOGRAPHY

Sample	Optical density × 100 direct assay (A)		Optical densities × 100 of chromatographic zones		$\frac{(d+g)}{A} \times 100$
			Digitoxin (d)	Gitoxin (g)	per cent.
1	28·0 27·0	27·5	21·5 24·5	4·3 2·0	94·0 96·2
2	31·0 31·0	31·0	20·6 22·0	8·0 6·3	92·3 91·2
3	33·5 33·5	33·5	30·0 29·6	2·6 2·0	97·4 94·5
4	32·5 32·5	32·5	26·6 24·9	5·0 4·6	97·2 91·0
5	27·5 27·5	27·5	23·8 22·5	3·4 3·7	99·0 95·2
6	32·0 31·0	31·5	27·0 29·0	3·8 1·8	97·7 97·7
7	34·5 35·0 35·5	35·0	24·6 26·2	7·0 6·5	90·5 93·5
8	36·0 35·5 36·5	36·0	27·1 28·5	8·3 6·0	98·5 96·0

zones tended to merge. Consequently with some of these samples although agreement between parallel runs was good in respect of total glycosides it was rather poorer for the two components due to the difficulty in deciding where to make the cut between them. Using this procedure to study the amount of gitoxin present in samples of digitoxin it was found better to develop the chromatograms for up to 15 hours, allowing the solvent to drip from the bottom of the paper, under which conditions good separation was obtained.

Examination of Leaf Samples

Samples of experimental crops of digitalis grown at Ware by Dr. Rowson were by his kind permission collected in September, 1954, dried at 55° C., powdered and stored in well closed tins. The propylene dichloride-soluble glycosides were estimated by the procedure outlined above, using two 20 g. samples of each batch. Duplicate chromatograms were run from each of the resulting extracts and the digitoxin contents were determined using xanthydrol reagent. The results are shown in Table V. The remaining glycosides in the chromatograms were estimated

as a single zone and direct assays of the methanol solution were also carried out. The percentage of the glycosides recovered from the chromatograms was somewhat lower than with samples of digitoxin, but averaged 91·5 per cent. For one strip it was only 83·5 per cent. but for the others it was above 86·5 per cent., while the highest was 99 per cent. (Table VI).

To find whether free digitoxose interferes in the determination of the digitoxin content of the leaf 25 mg. of digitoxose was dissolved in 100 ml. of distilled water and submitted to the isolation procedure. The propylene dichloride solution, after washing with sodium carbonate solution and with water, was distilled to dryness. No residue could be seen in the flask. However, glacial acetic acid was added and the solution assayed with xanthydrol reagent. No digitoxose was detected. Moreover when digitoxose was chromatographed it remained on the starting line and could not therefore be mistaken for digitoxin.

TABLE V

EXAMINATION OF SAMPLES OF DIGITALIS LEAF (i) PROPYLENE DICHLORIDE-SOLUBLE GLYCOSIDES (ii) DIGITOXIN CONTENT

Leaf	Propylene dichloride-soluble glycosides Units per g. of leaf	Percentage of digitoxin
A22	3·14 3·06	0·179 0·185
A23	2·64 2·54	0·115 0·113
A24	2·75 3·02	0·157 0·177
A25	2·84 2·76	0·164 0·166
A26	2·30 2·24	0·101 0·096
A27	2·50 2·72	0·139 0·150

It was thought desirable to test if digitoxin of the order indicated by these assays could be isolated from the leaf. The propylene dichloride-soluble glycosides were isolated from 1 kg. of leaf A22, yielding 5·17 g. of green solid assaying chemically at only 455 units per g. This solid was only partly soluble in 50 per cent. ethanol and the solution, after filtration, was treated with strong solution of lead subacetate, refiltered and the excess of lead removed as sulphate. The lead-free filtrate was distilled to remove the ethanol and the glycosides extracted with chloroform and isolated by precipitation of the concentrated dried chloroform solution in petroleum spirit. 2·08 g. of pale green solid was obtained assaying chemically (sodium picrate) at 905 units per g. and biologically at 1063 units per g. In the chromatographic assay the sample was found to contain 67 per cent. of digitoxin and 5·6 per cent. of gitoxin.

DISCUSSION

The results of the assays after various periods of fermentation, besides showing that most of the hydrolysis takes place in the first eight hours also serve to confirm the reproducibility of the method of extraction. The results in Table III indicate that it might be desirable to use a fifth volume of propylene dichloride in the extraction thereby increasing the total yield of propylene dichloride-soluble glycosides by about five per cent. Whether this is associated with a corresponding increase in the amount

of digitoxin detected on the paper chromatograms has not been studied and it is probable that further quantities of the solvent would continue to extract, in addition, small amounts of water-soluble glycosides for which the partition ratio into propylene dichloride is unfavourable. The sum of the glycosides extracted when using four volumes of propylene dichloride is somewhat lower than that indicated in Table I, but this is

TABLE VI

RECOVERY FROM CHROMATOGRAMS. SOLUTIONS OF PROPYLENE DICHLORIDE-SOLUBLE GLYCOSIDES ASSAYED WITH XANTHYDROL, DIRECTLY AND AFTER CHROMATOGRAPHY

Sample	Optical density × 100 direct assay (A)		Optical densities × 100 of chromatographic zones		$\frac{(d+g)}{A} \times 100$ per cent.
			Digitoxin (d)	Other glycosides (g)	
A22 1	43·5 44·0 44·5	44·0	30·7	6·0	83·5
			32·5	7·0	89·5
A22 2	40·5 41·5 40·0 41·0	40·7	32·4	4·0	89·5
			33·1	2·9	89·0
A23 1	30·5 30·5	30·5	20·7	7·7	93·0
			20·0	7·3	89·5
A23 2	29·5 28·5	29·0	20·2	6·8	93·0
			20·1	6·0	90·0
A24 1	39·0 39·0	39·0	28·5	7·0	91·0
			26·7	7·0	86·5
A24 2	40·5 40·5	40·5	30·4	6·3	90·6
			32·5	7·3	98·5
A25 1	39·0 39·0	39·0	27·9	6·8	89·0
			30·0	6·2	93·0
A25 2	36·0 35·5 35·5 36·5	35·9	29·8	5·7	99·0
			28·8	6·4	98·0
A26 1	28·5 29·5 28·0	28·7	17·6	9·2	93·5
			18·0	8·6	92·6
A26 2	26·5 26·5 25·5 26·5	26·2	16·9	8·2	96·0
			17·1	7·0	92·0
A27 1	39·0 39·0	39·0	24·6	9·5	87·5
			24·5	9·8	88·0
A27 2	37·5 38·0	37·7	26·3	8·2	91·5
			26·8	8·6	94·0

probably due to losses during the washing of each volume twice with alkali and twice with water.

Assays with sodium picrate were carried out to follow the progress of extraction for, although chemical determinations give, for crude materials, results higher than biological assays[10] the optical densities in these assays appear to run parallel with the content of digitoxin. Thus both the digitoxin content (chromatographically) and the content of propylene dichloride-soluble glycosides (assayed with sodium picrate) are greater

when fermented leaf is examined, while in Table VI the leaf samples assaying highest by the sodium picrate method also have the greatest digitoxin content. However in a routine estimation of the digitoxin content of a sample of leaf, the picrate method of assay is unnecessary and may be omitted, the residue from the distillation of the propylene dichloride being therefore dissolved in methanol for application to the paper strips.

From Tables IV and V it is seen that elution from the chromatograms is practically complete, but to ensure full recovery it is necessary to treat a larger area of paper than that representing the limits of the fluorescent zones. Thus in estimating digitoxin a further 1–2 cm. below the lower limit of the spot should be included and at the other end of the zone a cut midway between the apparent edges of the digitoxin and gitoxin zones is suitable. The gitoxigenin present in this region is not determined in the assay.

The yield and potency of the solid isolated from leaf A22 are in agreement with the digitoxin content indicated by the assay. No attempt was made to purify the product further, for in one or more stages of recrystallisation considerable weight losses could be expected, but it is evident that the isolated solid is a potential source of digitoxin of high potency. Leaf samples A22–A27 are believed to represent specially selected strains and the digitoxin contents are considered distinctly higher than those of commercial samples of digitalis although no assays on such samples have so far been carried out.

SUMMARY

1. A new method of estimating the digitoxin content of digitalis leaf has been described.

2. This consists in preliminary autolytic fermentation followed by extraction of the propylene dichloride-soluble glycosides from which digitoxin is separated chromatographically and estimated colorimetrically.

3. During the preliminary fermentation the residual plant enzymes release digitoxin from the initial complexes, but there is no evidence of any further breakdown into digitoxigenin.

4. Xanthydrol reagent is suitable for the colorimetric estimation of the digitalis glycosides eluted from chromatographic zones. The reaction is four and a half times as sensitive as that with sodium picrate.

5. The recoveries from chromatograms average 95·1 per cent. using samples of digitoxin and 91·5 per cent. for the propylene dichloride-soluble glycosides.

6. The method has been applied to the estimation of the digitoxin content of six samples of leaf; crude digitoxin of the order indicated by assay has been isolated from one of these.

The Author thanks Dr. J. M. Rowson for the samples of experimental crops of digitalis, Dr. H. O. J. Collier for the biological assay and Mr. G. L. Matchett for technical assistance in running the chromatograms.

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DISCUSSION

The paper was presented by MR. E. H. B. SELLWOOD.

DR. J. M. ROWSON said that the xanthydrol reaction was a useful tool, but the time of 2 to 4 hours required before peak colour development was a disadvantage. The best known colorimetric agent might be sodium picrate, but it was not the most sensitive. Had the author proved the efficiency of his extraction of the leaf with propylene dichloride and had he any idea of the absolute efficiency of this solvent to extract digitoxin? What else did it extract, e.g., how much gitoxin? Describing some unpublished work, he said he had tested six samples from the same clones as those examined by Mr. Sellwood and found the potency by chemical assay to be between 13 and 17 units/g. That was equivalent to 0·4 to 0·7 per cent. of total glycosides. He had found in those six samples that the content of the B series (gitoxin) comprised from 44 to 75 per cent. of the total. If it were assumed that the A series made up the remainder, then he estimated this to be from 25 to 56 per cent. of the total glycosidal complex. Therefore, the amount of digitoxin present in the leaves would be 0·14–0·25 per cent., which agreed with the author's results in Table V. On behalf of Mr. Rigby he asked what tests the author had applied to assess the purity of the digitoxose used as a standard.

DR. G. E. FOSTER (Dartford) said he was not sure whether the author was trying to estimate the amount of digitoxin in *Digitalis purpurea*, or whether he was suggesting that the method should be used for standardising *Digitalis purpurea*. In Table I he referred to units/g. of leaf and the highest figure was 2·75. Powdered digitalis was standardised to contain 10 units of activity per g.; was there any connection between the digitoxin content and the biological activity of the leaf examined?

DR. S. E. WRIGHT (Sydney) pointed out that in Table IV the author showed that 40 μg. of digitoxin were put on the paper, but on the chromatograms it was observed that the amounts were never more than 10 μg. It would seem that 40 μg. was an excessive loading; what was the separation between gitoxin and digitoxin when the paper was loaded to that extent? Perhaps the author would also comment on the temperature sensitivity of the xanthydrol reaction.

MR. E. H. B. SELLWOOD, in reply, agreed that the time of development of the colour of 2 to 4 hours was quite long, but in practice it was balanced by the fact that there was no critical condition of heating. He agreed that he should have said the sodium picrate method was the more sensitive of the older and better known methods. It was very difficult to decide the efficiency of the extraction with propylene dichloride without using some other extraction procedure. It was gratifying to learn that the percentages of digitoxin found were in fair agreement with those determined by Dr. Rowson. Whereas chemical methods in general tended to overestimate digitoxin content, he had obtained a somewhat low result. The digitoxose used was prepared by the standard method of hydrolysis and had a satisfactory melting point. The object of the work was to develop an assay procedure which would indicate whether a sample of leaf was suitable for extraction of digitoxin on a manufacturing scale. The figures for units/g. quoted were for the propylene dichloride soluble fraction and not for the total activity of the leaf. It was indicated in the paper that the spots were in all cases for 10 μg. digitoxin and the 40 μg. were made up of four separate spots of 10 μg., so there was no overloading. He could give no indication at all of the temperature sensitivity of the xanthydrol reaction: his work had been done as closely as possible to 20° C.