STUDIES IN THE GENUS *DIGITALIS*

PART I. THE COLORIMETRIC ESTIMATION OF DIGITOXIN AND OF PREPARATIONS OF *DIGITALIS PURPUREA*

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IN recent years a number of different methods have been proposed for the quantitative estimation by colorimetric methods of either digitoxin or the mixture of glycosides obtained by extraction from the leaves of *Digitalis purpurea*. These processes, which have been reviewed in two recent publications,[1,2] depend upon the interaction of either the aglycone portion of the glycoside molecule, essentially the butenolide group, or of the digitoxose sugar part of the molecule with suitable colour-producing reagents. Alkaline picrate or several different dinitro aromatic compounds have been employed for interaction with the butenolide group of the glycoside molecule; such processes are more or less specific for this linkage but do not distinguish between the various closely related glycosides or their aglycones which may be present in the leaf of *D. purpurea*. The Keller-Kiliani reagent has been used for the quantitative estimation of digitoxose. It is not specific, but several workers have claimed that in a partially hydrolysed glycoside mixture the unhydrolysed digitalis glycosides along with aglycones may be separated from free digitoxose by solvent extraction; when the solvent is removed the sugar moiety of the unhydrolysed glycoside only is estimated by the Keller-Kiliani reagent. The intensity of colour produced in each of these processes has been measured spectrophotometrically, but in a number of instances the time allowed for colour development, the selection of a blank for comparison in the spectrophotometer and the choice of wavelength of light for observations have not been critically examined.

For the quantitative evaluations of powdered digitalis leaf by means of these colorimetric processes different methods of leaf extraction by means of ethanol or water have been employed, also different methods for the preliminary decolorisation of these extracts have been recommended by the different workers. The results obtained by these processes have, in the majority of cases, been compared with those obtained by one or other of the biological methods of assay but no worker has attempted to prove the efficiency of each process, and no critical comparison of the different proposed processes of extraction of leaf, decolorisation of extracts and of methods of colorimetric estimation, have been made. Fuchs and his co-workers[2] have examined leaf samples from 8 different species of *Digitalis* by different colorimetric and biological methods of estimation but using the same process of extraction and decolorisation for all samples; and their method of extraction will be shown in this paper to be inaccurate.

The work to be reported in this paper includes a critical examination both of the methods for colorimetric estimation of digitoxin and of the efficiency of processes for the decolorisation of digitalis leaf tinctures or infusions, prior to applying the colorimetric methods of estimation.

1. The Colorimetric Estimation of Digitoxin

A commercial sample of digitoxin containing 1735 I.U./g. has been employed in this work. All spectrophotometric readings were made by means of the "Unicam" quartz spectrophotometer and in every estimation the 1 cm. cells were used.

(*a*) *The Aglycone* (*Butenolide*) *Estimations*

The alkaline picrate method has been very extensively examined by Bell and Krantz[3] and it is now official in the United States Pharmacopeia XIV for the quantitative estimation of digitoxin in tablets. The process depends upon the interaction of the glycosides in ethanolic solution with a freshly prepared mixture of aqueous or ethanolic solution of trinitrophenol and sodium hydroxide; an orange colour develops after standing, the intensity of which is measured at wavelength 525 mμ in a spectrophotometer using a dilution of the reagent as a blank. Bell and Krantz prepared standard graphs for the relation of colour intensity to digitoxin content up to 15 mg. per cent., and found that these were not straight lines and that the Beer-Lambert law was not obeyed.

For the following work a solution of digitoxin in ethanol (50 per cent.) and aqueous sodium picrate reagent were used. A volume of solution equivalent to from 0·1 to 0·4 mg. of digitoxin was measured into a stoppered 10 ml. cylinder, adjusted to 5 ml. with ethanol (50 per cent.), and 5 ml. of fresh picrate reagent added [this reagent is a mixture of 95 ml. of 1 per cent. aqueous trinitrophenol with 5 ml. of 10 per cent. sodium hydroxide solution]. At the same time a blank consisting of 5 ml. of ethanol (50 per cent.) and 5 ml. of picrate reagent was also prepared. Colour densities of these solutions reached a maximum some 10 minutes after mixing and remained constant for a further 20 minutes before fading commenced. Thus future readings were taken 20 minutes after mixing. Colour densities of 3 different digitoxin concentrations of 1, 2 and 3 mg. per cent. were determined at wavelengths between 400 and 600 mμ. These are shown in Figure 1, from which it is seen that maximum colour measurement was obtained at wavelengths of 490 to 500 mμ. Finally the colour densities of a range of digitoxin levels from 0·5 to 4 mg. per cent. were determined at wavelength 495 mμ. The graph obtained by plotting these values of colour densities against digitoxin concentration between 1 and 3·5 mg. per cent. was a straight line corresponding to k (1 mg. per cent. digitoxin) = 0·190. Thus between these concentrations the reaction obeys the Beer-Lambert law.

The suggestion by Bell and Krantz[4] that a more intense colour was developed if tetraethylammonium hydroxide in high concentration replaced sodium hydroxide in the reaction, was not confirmed. For the same concentration of digitoxin in final reaction mixture the use of this

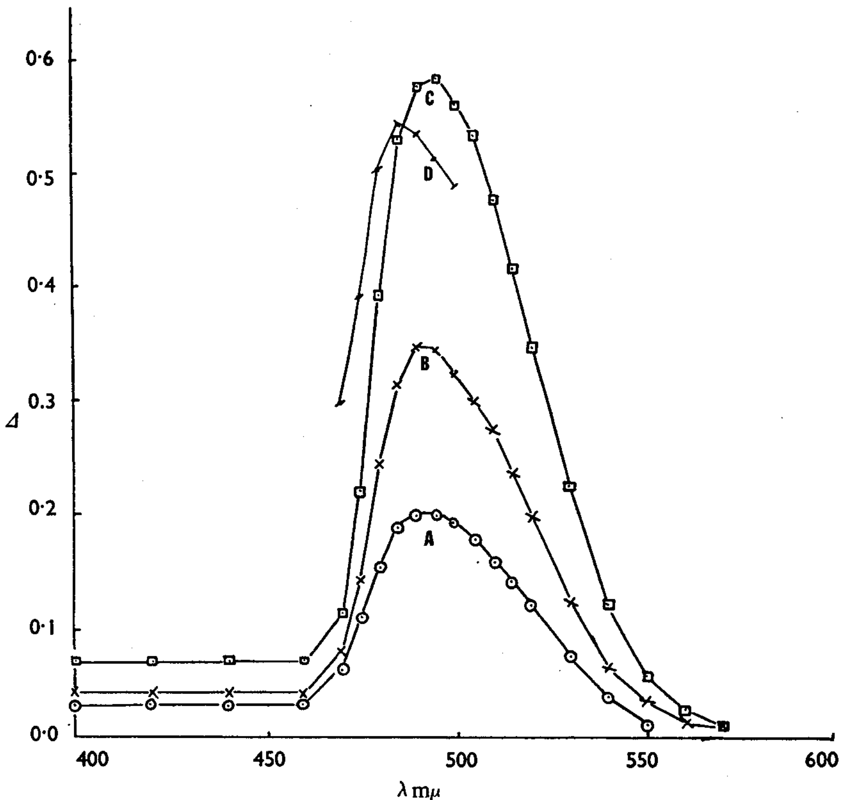

FIG. 1. Alkaline picrate reagent.
A. Digitoxin 1 mg. per cent.
B. Digitoxin 2 mg. per cent.
C. Digitoxin 3 mg. per cent.
D. Tincture of digitalis 3 units per cent.

quaternary hydroxide gave a weaker colour, but with its peak at the same wavelength of 495 mμ; k (1 mg. per cent. digitoxin) = 0·134.

The metadinitrobenzene process (the Raymond process) has been used by Canbäck for the estimation of digitoxin and other digitalis glycosides, but has not been applied to the estimation of digitalis tinctures. The process depends upon the production of a blue colour when a mixed ethanolic solution of digitoxin and *m*-dinitrobenzene is rendered strongly alkaline with sodium hydroxide. The blue colour fades rapidly but logarithmically in relation to time: thus colour densities of the reaction mixture are determined at exact half-minute intervals, from mixing, at a wavelength of 620 mμ: and by graphic extrapolation the colour density at zero time is calculated.

Repeatable results by this process have been obtained for a number of estimations of the same solution of digitoxin. Colour density readings at different wavelengths were plotted, giving a curve with a well defined

broad peak at 600 to 650 mμ, thus confirming Canbäck's choice of a wavelength of 620 mμ. At this wavelength digitoxin concentrations of 1 to 4 mg. per cent. were shown to obey the Beer-Lambert law, k (1 mg. per cent. digitoxin) = 0·192.

The dinitrobenzoic acid process (*Kedde*). This method is described by Canbäck[1] as a modification of the Raymond process which should be efficient. Kedde[6] added a 2 per cent. solution of 3:5 dinitrobenzoic acid in ethanol to a decolorised tincture of digitalis, more ethanol was added followed by normal sodium hydroxide and adjusted to volume with water. The brown colour was measured at wavelength 530 mμ after standing for 1 hour and using as blank a dilution of the decolorised tincture. This choice of blank is wrong, for the dinitrobenzoic acid solution is lemon yellow, gradually becoming brown after adding alkali. The method was investigated by using as test solution a mixture of 0·1 to 0·8 mg. of digitoxin in ethanol (50 per cent.) with 2 ml. of a 2 per cent. ethanolic solution of dinitrobenzoic acid and ethanol (50 per cent.) to 9 ml., followed by 1 ml. of N sodium hydroxide, and, as blank, this solution without digitoxin. Observations at 530 mμ showed that the colour density increased up to 6 minutes after mixing, remained constant up to 12 minutes and faded progressively up to 75 minutes, when the experiment was stopped. Hence Kedde's choice of measurement 1 hour after mixing is wrong. Colour densities of 3 different concentrations of digitoxin were determined at different wavelengths; the peak values were between 525 and 550 mμ with a shallow maximum at 535 mμ as shown in Figure 2 and this wavelength was used in subsequent work. Concentrations of 1 to 8 mg. per cent. of digitoxin were then examined and it was found that the reaction obeyed the Beer-Lambert law.

This process was easy to handle, the reagents are stable, no difficulties were experienced in making any observations, the concentration range of 2 to 8 mg. per cent. is convenient, also the process is rapid.

(*b*) *The Digitoxose Estimations*

The Keller-Kiliani process. This qualitative test has been modified by James and his co-workers,[7] also by Soos,[8] as a quantitative process. Water must be excluded from the reaction mixture. Soos dissolved dried digitoxin equivalent to 10 to 20 mg. per cent. in a freshly prepared reagent of strong acetic acid, ferric chloride and strong sulphuric acid: after standing for 5 hours colour densities were measured at wavelength 570 mμ every half-hour until maximum values were obtained.

Preliminary experiments have been carried out using as reagent a mixture of 60 ml. of glacial acetic acid, 2 ml. of a 5 per cent. solution of ferric chloride and 5 ml. of strong sulphuric acid. A solution of digitoxin in ethanol was evaporated to dryness and dissolved in freshly prepared reagent to give a final volume of 10 ml., a blank of reagent only was employed. At 570 mμ it was found that colour densities increased for 15 minutes after mixing, remained constant for a further 15 or 20 minutes, then faded slowly up to 90 minutes. Taking readings in this constant period between 15 and 30 minutes, the colour densities of 2 different

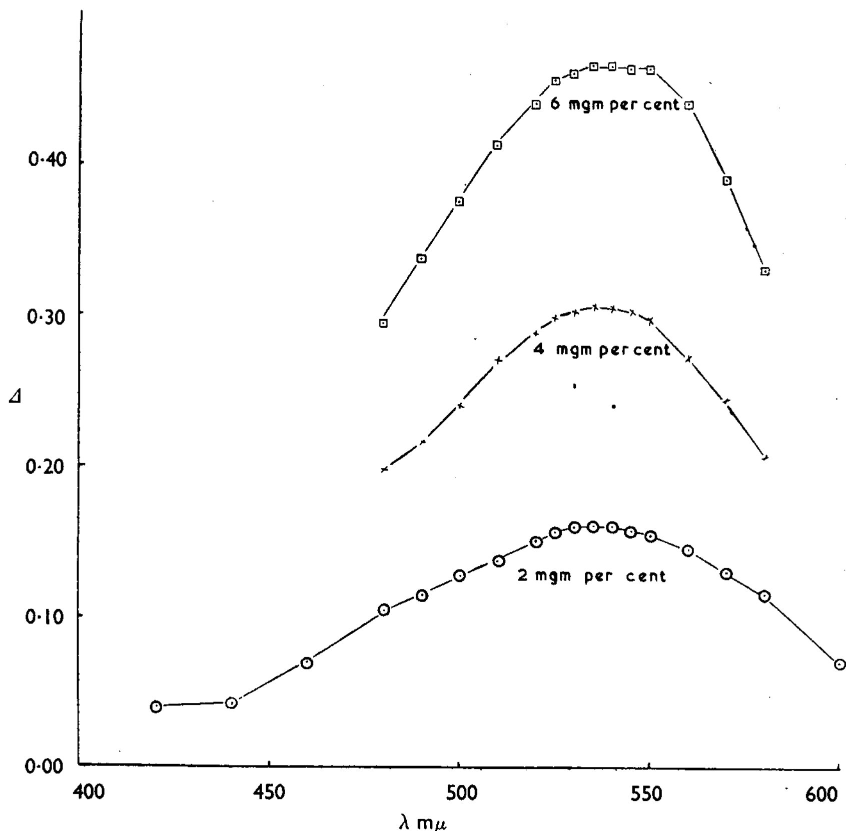

FIG. 2. 3:5-Dinitrobenzoic acid reagent and digitoxin.

digitoxin contents were determined at different wavelengths. These are plotted in Figure 3 where a sharp peak was observed at 590 mμ, and not at 570 mμ as used by Soos. A second peak was found at 470 mμ; but repeatable results were obtained at 590 mμ with greater ease than at 470 mμ and the higher wavelength was chosen for future use. Quantities of 1 to 6 mg. per cent. of digitoxin were found to obey the Beer-Lambert law under these conditions, and in general the process was considered satisfactory.

The influence of water upon the Keller-Kiliani reaction was investigated by preparing a series of batches of reagent containing from 0·5 to 5·0 per cent. of added water; a quantity of 4 mg. per cent. of digitoxin was then estimated by means of each reagent. The results are shown in Table I, from which it is seen that the colour density for the same amount of digitoxin decreases with increase in water content of the reagent, whilst the period of time required to develop the maximum colour is correspondingly increased.

From this group of investigations it was concluded that the picrate,

TABLE I

COLOUR DENSITY OF DIGITOXIN WITH KELLER-KILIANI REAGENT IN THE PRESENCE OF ADDED WATER $\lambda = 590$ mμ

k (1 mg. per cent. of digitoxin)

	Water added to reagent per cent.							
	0·0	0·5	1·0	1·5	2·0	2·5	3·5	5·0
k 1 mg. per cent. of digitoxin	0·118	0·112	0·112	0·112	0·109	0·106	0·101	No constant colour
Time for maximum colour development (minutes) ..	15	15	20	25	25	30	40	60

dinitrobenzoate and Keller-Kiliani processes for the estimation of digitalis glycosides should be further employed in the second stage of this work.

2. THE DECOLORISATION AND ESTIMATION OF TINCTURES OR LEAF EXTRACTS

Previous workers have extracted the leaves of *Digitalis purpurea* with water, either cold or hot, or with ethanol; from these extracts the pigments have been precipitated by the addition of either lead acetate or lead sub-

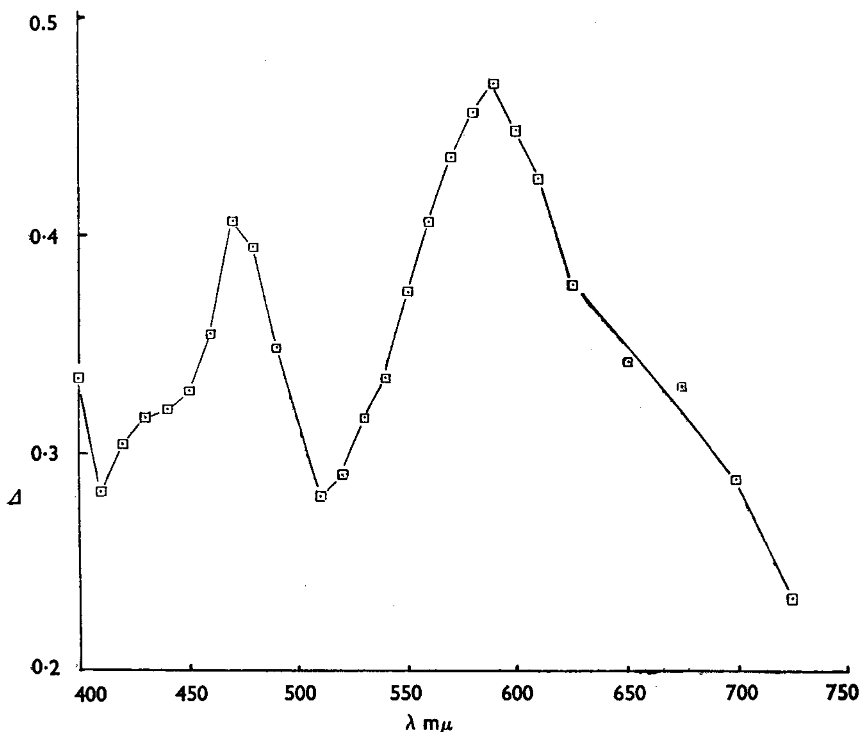

FIG. 3. Keller-Kiliani reagent and digitoxin 4 mg. per cent.

acetate solution, excess of lead being removed by means of disodium hydrogen phosphate; to the final clear filtrate representing an aqueous dilution of the original extract one or other of the chemical estimation processes have been applied. In order to compare the efficiencies of these different processes of decolorisation and to compare the results obtained by different methods of colorimetric estimation for each decolorisation process, a commercial sample of tincture of digitalis containing 1 I.U./ml. was employed for the following work.

(*a*) *The influence of ethanol on alkaline picrate reagent.* A preliminary examination was made of the method proposed by Bell and Krantz[3] in which lead acetate, followed by disodium hydrogen phosphate, was used to decolorise the tincture. Quantities of 2 ml. of final clear filtrate, being a 1 in 10 dilution of the tincture, were diluted to 5 ml. with distilled water and were mixed with 5 ml. of freshly prepared sodium picrate reagent; as blank a mixture of sodium picrate reagent with an equal volume of water was used. Colour densities were measured spectrophotometrically at 495 mμ every 5 minutes until a constant value was obtained. A constancy of colour development, corresponding to k (1 ml. tincture) = 2·607, was obtained 35 minutes after mixing and was maintained for a further 30 minutes. This prolonged period of colour development was in contrast to the 10 minutes required when digitoxin was estimated by means of the same reagent. A further estimation of the decolorised tincture carried out with the addition of 2 ml. of ethanol (95 per cent.) to both test solution and blank required 1 hour to attain a maximum colour development corresponding to k (1 ml. tincture) = 1·782. The presence of ethanol in the blank thus exerted a marked influence upon the colour of that solution, and the general process of estimation by the alkaline picrate reagent was also influenced by the amount of ethanol in the reaction mixture.

To determine the extent of this influence of ethanol upon aqueous sodium picrate reagent, a blank containing the reagent diluted with an equal volume of water was compared with similar dilutions of reagent containing from 1·4 to 20 per cent. of ethanol in the final reaction mixtures. Colour densities measured at a wavelength of 495 mμ are shown in Figure 4,A, from which it is seen that a linear relation exists between ethanol content up to 10 per cent., and colour density produced in the picrate reagent. In other words this reagent gives a direct measure of the ethanol content of an aqueous-ethanolic mixture. It thus follows that if alkaline picrate reagent is to be used for the colorimetric estimation of digitalis glycosides the ethanol content of both test and blank solutions must be the same for each estimation.

To show the extent of the influence of ethanol within the reaction mixture a series of solutions were prepared containing 2 ml. of decolorised tincture (1 in 10 dilution as described above, ethanol content 7 per cent.) mixed with 5 ml. of fresh picrate reagent and sufficient diluted ethanol to produce 10 ml. of reaction mixture containing 1·4 to 25 per cent. of ethanol. Maximum colour densities were measured against blanks containing picrate reagent with the same amounts of ethanol. The

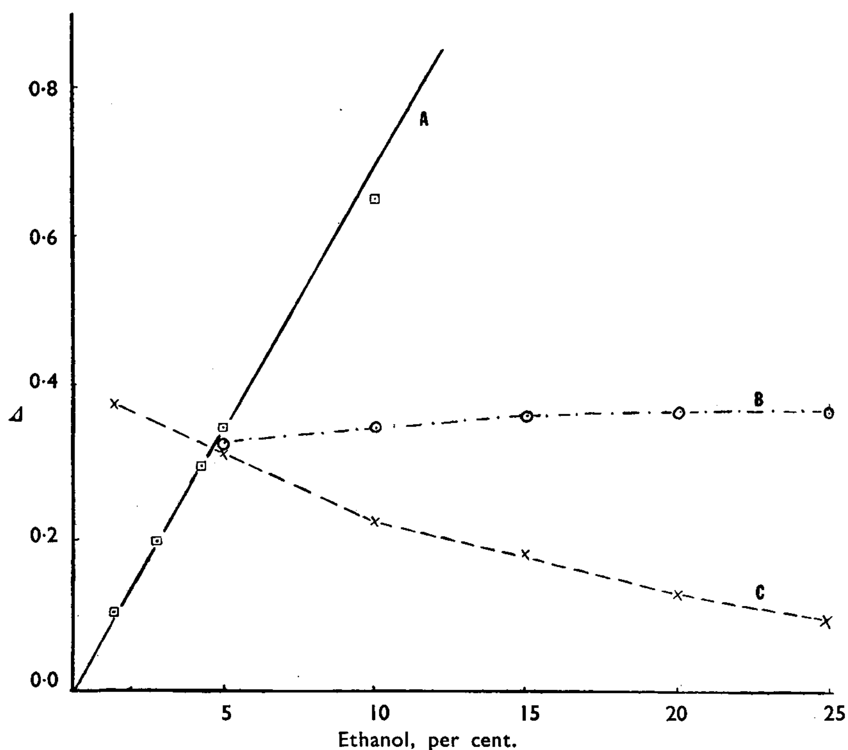

FIG. 4. Alkaline picrate reagent and ethanol.

A. Ethanol and picrate reagent; aqueous picrate blank.
B. Digitoxin 2 mg. per cent.; ethanol in test and in blank.
C. Tincture of digitalis 2 units per cent.; ethanol in test and in blank.

values are shown in Figure 4,C, from which it is seen that there is a progressive decrease in colour density for the same volume of tincture, as the ethanol level of both reaction mixture and blank rise from 1·4 to 25 per cent. The calculated values for the tincture are k (1 ml.) = 1·895 in the presence of 1·4 per cent. of ethanol, and k (1 ml.) = 0·495 in the presence of 25 per cent. of ethanol. Digitoxin in quantities of 0·2 mg. was also estimated under similar variations of ethanol content from 5 to 25 per cent. Colour density values, shown in Figure 4,B, increase significantly with ethanol levels between 5 and 15 per cent. but only to a smaller extent at ethanol levels up to 25 per cent. [k (1 mg. per cent. digitoxin) = 0·165 in the presence of 5 per cent. of ethanol; = 0·184 in the presence of 15 per cent. of ethanol; = 0·187 in the presence of 25 per cent. of ethanol].

The further usefulness of the alkaline picrate process for the estimation of digitalis extracts depended upon the possibility of obtaining reproducible results with it under carefully controlled conditions of ethanol content in both reaction mixtures and blanks. Since curve C, Figure 4, shows continued change in colour density with variation in ethanol level, replicate estimations of the same tinctures were carried out as described

above at ethanol levels of both 3·5 per cent. and 20 per cent. Reproducible values, in agreement with those of Figure 4,C, were obtained at each ethanol level. A 3·5 per cent. ethanol content of reaction mixture was chosen for future work, since this is obtained when equal volumes of alkaline picrate reagent and decolorised filtrate, prepared as described above, from tincture of digitalis are mixed. If smaller proportions of filtrate were used, ethanol (7 per cent.) was added as a diluent. Under these conditions the wavelength of maximum absorption was redetermined and was found to be 485 mμ, as shown in Figure 1. At this wavelength of observation different volumes of filtrates were estimated and the colour densities were found to obey the Beer-Lambert law when volumes equivalent to from 0·05 to 0·3 ml. of tincture of digitalis were employed, giving an average value k (1 ml. tincture) = 1·93. When volumes of filtrate equivalent to 0·4 ml. of tincture were estimated the values obtained showed a 9 per cent. deviation from the Beer-Lambert law.

(*b*) *A comparison of decolorisation processes.* It has been shown above that the process for decolorisation of tincture of digitalis by means of lead acetate, followed by alkaline picrate estimations under carefully controlled conditions, may be used to obtain repeatable results. Kedde[6] has employed a 4-stage decolorisation of digitalis tincture, tannin being removed by ferric chloride, excess of ferric chloride by sodium hydroxide, pigments being precipitated with lead subacetate solution and excess of lead removed with disodium hydrogen phosphate; the final filtrate was a 5-fold dilution of the tincture and was estimated by the dinitrobenzoate process described above. This complete process was carried out upon the stock tincture and found to give reproducible values for colour density corresponding to k (1 ml. tincture) = 0·240. The first 2 stages of this decolorisation are superfluous since tannin would be removed by lead subacetate employed in the later stage; they were thus omitted and values for colour density corresponding to k (1 ml. tincture) = 0·285 were obtained. At the same time as the dinitrobenzoate estimations were made on these 4-stage and 2-stage decolorisations employing lead subacetate, the picrate estimations at ethanol level of 3·5 per cent. were also carried out upon each filtrate. Colour density values equivalent to k (1 ml. tincture) = 1·19 and 1·47 were obtained respectively and the alkaline picrate process appeared to work as smoothly upon lead subacetate decolorisations as upon the neutral lead acetate decolorisations. The percentage loss of colour density in the first 2 stages of the Kedde process were found to be of the same order when either the dinitrobenzoate or the picrate processes of estimation were employed; but the colour density equivalent to 1 ml. of tincture using the picrate method and the last 2 stages of the Kedde (lead subacetate) process (k = 1·47) is lower than when the picrate method follows the lead acetate decolorisation (k = 1·93), as shown in Table II.

When attempts were made to carry out the dinitrobenzoate estimations upon digitalis tincture decolorisations prepared by means of lead acetate, no results were obtained. Instead of a maximum colour developing some 6 minutes after mixing the filtrate and reagent, a continuously fading

colour was produced. This was overcome by reducing the amount of disodium hydrogen phosphate in the decolorisation process, until only just sufficient was added to precipitate the excess of lead, and at the same time, in the estimation process, the mixture of filtrate and ethanolic solution of 3:5 dinitrobenzoic acid was allowed to stand for up to 30 minutes before rendering alkaline. Constant colours over the period of 2 to 6 minutes from the time of rendering the mixture alkaline were obtained giving a colour density equivalent to k (1 ml. tincture) = 0·465, but the method was somewhat inflexible. A more simple procedure was to employ just sufficient sodium sulphate to remove excess of lead acetate in the decolorisation process in place of disodium hydrogen phosphate; the unmodified dinitrobenzoate process of estimation worked smoothly on this filtrate giving colour densities equivalent to k (1 ml. tincture) = 0·473. The picrate process of estimation at 3·5 per cent. ethanol level also yielded reproducible results upon this decolorisation filtrate, as shown in Table II.

TABLE II

COLOUR DENSITY EQUIVALENT TO 1 ML. OF DIGITALIS TINCTURE (IN 10 ML. OF REACTION MIXTURE)

Process of decolorisation	Picrate estimation (3·5 per cent. of ethanol)	Dinitrobenzoate estimation
Lead acetate and sodium phosphate (Bell and Krantz) ..	1·93	0·465 (prolonged nitration)
Lead acetate and sodium sulphate	1·73	0·473
Lead subacetate and sodium phosphate (Kedde: stages 3 and 4)	1·47	0·285

Table II summarises the colour density values obtained by 2 different processes of estimation when applied to the same tincture of digitalis decolorised by 3 different methods; from which it is seen that the results by the picrate process are not paralleled by those of the dinitrobenzoate method. This may be due to some loss of glycosides in, as well as to interference with the estimation methods by, the different precipitation processes. The processes reported in Table II are based on those employed by previous workers and differ in volume of tincture, ratio of lead to tincture and to total volume as well as to the nature of the lead salts used. A series of decolorisations of 10 ml. quantities of the same tincture were prepared using either lead acetate or lead subacetate in the ratios of 20, 30, 40, and 50 mg. Pb. to 1 ml. of tincture, excess of lead was removed by adding sodium sulphate equivalent to total lead added and the final filtrates represented a 4-fold dilution of tincture. Each filtrate was estimated both by the dinitrobenzoate and by the controlled picrate processes. Results are shown in Figure 5, from which it was concluded that results for the same volume of tincture varied with the lead level of decolorisation when neutral lead acetate was employed (Figure 5, A and C). When lead subacetate was employed as the pigment precipitant, estimations of the same amount of tincture were identical at different lead levels, as shown by the dinitrobenzoate estimation process (Figure 5, D);

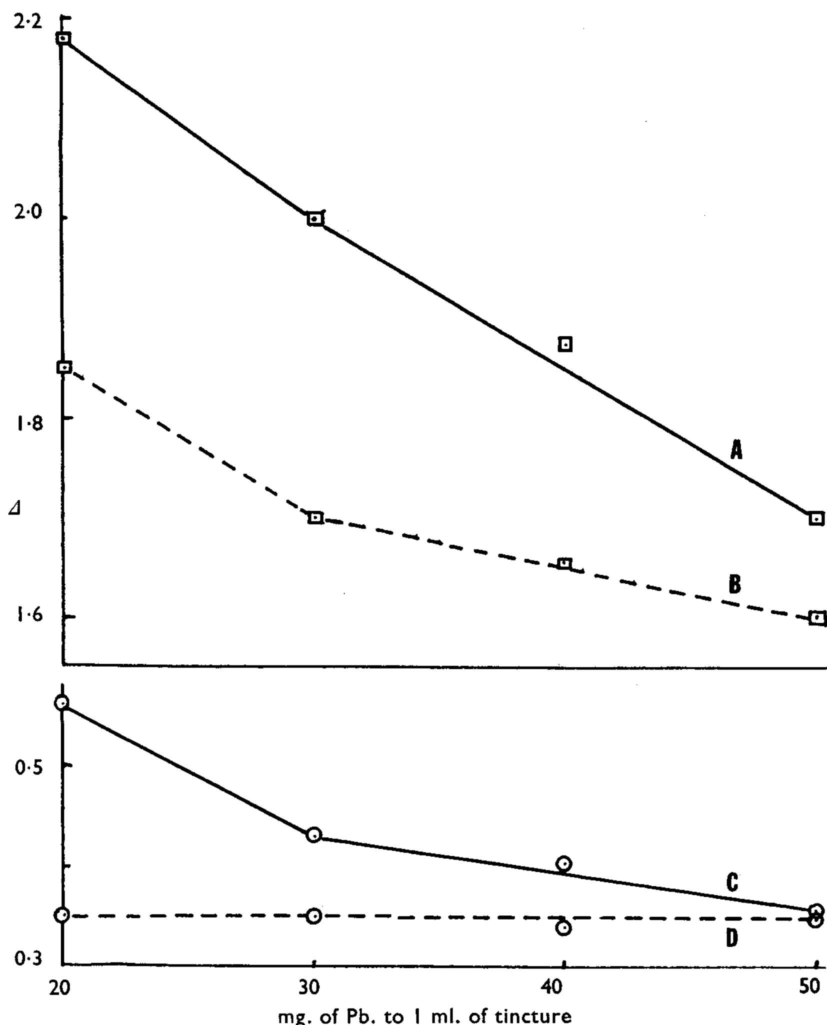

FIG. 5. Tincture of digitalis estimations; decolorisation at varying lead levels. k (1 ml. tincture).

A. Lead acetate decolorisation, picrate estimation.
B. Lead subacetate decolorisation, picrate estimation.
C. Lead acetate decolorisation, dinitrobenzoate estimation.
D. Lead subacetate decolorisation, dinitrobenzoate estimation.

estimations by the picrate process upon these same filtrates showed some variation in values (Fig. 5,B).

Figure 5 may indicate that a progressive precipitation of glycosides is occurring with increased amounts of lead added to decolorise the tincture as suggested also by Table II. However, if this were the only cause, there should be a parallelism between graphs A and C and between B and D

which does not occur; hence side reactions must also be interfering with the processes. To investigate these points, parallel experiments to the above were set up for the estimation of (*a*) digitoxin; (*b*) digitoxin and a 1 in 10 tincture of grass meal in ethanol (70 per cent.); (*c*) a second sample of tincture of digitalis containing added digitoxin. It was found that the decolorisation by either lead acetate or lead subacetate of digitoxin solution, alone or in the presence of tincture of grass, resulted in a constant loss of about 15 per cent. of glycoside as shown by both estimation processes and concordant results between the different processes were obtained; thus differing from Figure 5. Similar concordance of results was obtained for the estimations of mixed tincture of digitalis and digitoxin. These values are expressed in Table III and also the colour density values determined for the same quantities of digitoxin and of the tincture of digitalis when estimated separately under similar conditions.

From these results it is apparent that fictitiously high values were obtained for tincture of digitalis when decolorised by means of lead acetate followed by dinitrobenzoate estimation; consistency of deviations in the last line of Table III for the other 3 methods is a measure of the losses involved in the decolorisation processes.

TABLE III

COLOUR DENSITIES PRODUCED BY A MIXTURE OF DIGITOXIN AND TINCTURE OF DIGITALIS UNDER DIFFERENT CONDITIONS OF DECOLORISATION AND ESTIMATION

	Dinitrobenzoate estimation of 0·5 ml. of tincture + 0·7 mg. of digitoxin		Picrate estimation of 0·15 ml. of tincture + 0·21 mg. of digitoxin	
Decolorisation (20 mg. of Pb per ml. of tincture) by	Lead subacetate	Lead acetate	Lead subacetate	Lead acetate
Colour density of mixture	0·693	0·654	0·587	0·623
Colour due to digitoxin present	0·572	0·554	0·356	0·365
Calculated colour density due to tincture of digitalis	0·121	0·100	0·231	0·258
Results found for tincture of digitalis alone ..	0·150	0·271	0·300	0·317
Difference between calculated and observed values for tincture per cent.	19	.63	23	19

The general conclusion to be drawn from this work is that decolorisation of digitalis tincture by means of lead subacetate followed by sodium sulphate and estimation by means of the dinitrobenzoate process, gives concordant results not subject to variations in lead levels or to changes in the ethanol content, within the range of 20 to 50 per cent., of the reaction mixture. The recommended process for estimation is as follows. Mix 10 ml. of tincture of digitalis with about 7 ml. of water, add 1 ml. of strong solution of lead subacetate B.P.C., adjust the volume to 20 ml., shake and filter. To 10 ml. of clear filtrate add 2 ml. of a 6·3 per cent. solution of sodium sulphate (Na_2SO_4 $10H_2O$), adjust the volume to 20 ml., shake and filter through a fine filter paper (Whatman No. 42). 1 ml. of filtrate is equivalent to 0·25 ml. of tincture and has ethanol content

17·5 per cent. [This decolorisation employs 25 mg. of Pb. to 1 ml. of tincture; if it is desired to employ a ratio of 50 mg. of Pb. to 1 ml. of tincture, the quantities of strong solution of lead subacetate and of sodium sulphate solution should be doubled.] Measure a suitable volume of this filtrate (usually 2 to 4 ml.) into a 10-ml. stoppered measuring cylinder, adjust to 4 ml. by the addition of ethanol (17·5 per cent.), add 5 ml. of a freshly prepared 0·8 per cent. solution of 3:5-dinitrobenzoic acid in ethanol (95 per cent.), mix and add 1 ml. of N sodium hydroxide, at the same time prepare a blank solution as above but omitting the decolorised filtrate. Transfer both solutions to a spectrophotometer and measure the colour density of the test solution relative to the blank at wavelength 535 mμ every minute until a maximum value is obtained.

The process proposed by Soos[8] for the extraction of digitalis leaf, followed by decolorisation and estimation, employing the Keller-Kiliani method, was investigated in 2 stages. Soos extracted powdered digitalis leaf by shaking with water for 1 hour; this solution was decolorised by adding lead subacetate and filtering; the filtrate was shaken out with 3 quantities of chloroform which leaves any free digitoxose in the aqueous layer but extracts the total glycoside and any aglycone; in this mixture the Keller-Kiliani process estimates glycoside only. To test the possibility of totally extracting all glycoside and aglycone with chloroform from an aqueous solution, a bulk of tincture was decolorised by lead subacetate and, as a control, an aliquot part was estimated by the dinitrobenzoate and the Keller-Kiliani processes. The bulk of filtrate was then diluted with water and extracted with successive 25-ml. quantities of chloroform, from the separated chloroform layers the solvent was removed and the extracted glycosides determined. It was found that 3 quantities of chloroform extracted about 73 per cent. of the glycosides present, 6 quantities extracted 78 to **82** to 86 per cent. and 12 quantities of solvent extracted less than 90 per cent. of glycosides. Thus the process did not seem very promising and the Soos claim of extraction with 3 quantities of chloroform is wrong. The aqueous extraction of a powdered leaf sample was then attempted by the Soos method; the aqueous filtrate being extracted with 6 quantities of chloroform. Since the efficiency of the aqueous extraction alone was under test, the dinitrobenzoate estimation process was used. 3 repeats of the process gave values of 45 to **47** to 55 per cent., of those obtained when the same sample of digitalis leaf was estimated independently by the recommended process for estimation described above. If we assume the chloroform extractions to have an 82 per cent. efficiency this means that the aqueous extraction yields 60 per cent. of the activity present in the sample. Thus both stages of this process are considered inefficient as at present proposed.

Discussion of Results

Alkaline picrate reagent has been extensively used for the quantitative estimation of either digitoxin or decolorised preparations from digitalis tinctures by many different workers and has been hotly criticised as non-specific and untrustworthy by other workers. The present investigations

have shown that the ethanol level of the reaction mixture is the most important factor to be controlled when this reagent is employed. Figure 4,A, shows that sodium picrate reagent may be employed for the colorimetric estimation of ethanol present in aqueous dilutions and that the reaction follows the Beer-Lambert law with a steeply rising graph of colour density plotted against ethanol content. It thus follows that when this reagent is used for quantitative estimations by spectrophotometric methods employing a control solution as blank, that control solution must contain exactly the same amount of ethanol as is present in the reaction mixture in order to balance, in both solutions, the colour produced when solvent and reagent interact. By a careful observance of these conditions the picrate reagent has been shown to yield concordant results for the estimation of either digitoxin or preparations from powdered digitalis leaves. These reactions have been shown in each instance to follow the Beer-Lambert law over a reasonably wide range of concentrations which may be employed in such quantitative estimations. An examination of the published work dealing with the use of this reagent in the investigation of digitalis glycosides suggests that this significance of ethanol levels has not been appreciated. Some workers, perhaps fortuitously, have employed the same concentrations of ethanol in both blank and reaction mixtures, whilst others have not done so and in these instances such lack of balance may explain why the reaction did not follow the Beer-Lambert law.

Under a number of conditions of estimation the behaviour of tincture preparations have not paralleled those of solutions of digitoxin. This is seen in the slower development of maximum colour density of the former with sodium picrate reagent, and this colour density reaches a maximum at wavelength 485 mμ for tincture preparations, but the maximum is at wavelength 495 mμ for the samples of digitoxin examined, as shown in Figure 1. Moreover, the behaviour of digitoxin and tincture preparations differ when estimated by sodium picrate reagent in different concentrations of ethanol, as shown in Figure 4,B and C, the colour density of the former increasing somewhat with increase in ethanol concentration of both test and blank for the same concentration of digitoxin; the latter showing considerable decrease in colour density under the same conditions. It thus follows that colour density values for tincture preparations cannot be equated to those of digitoxin under these conditions of estimation with sodium picrate with the object of expressing the results for tinctures as an equivalent digitoxin content.

No explanations are offered in the present paper for the differences in behaviour of digitoxin and of preparations of tincture of digitalis discussed above and also reported in Figure 5 and Tables II and III. It was however concluded that sodium picrate reagent under very carefully controlled conditions might be used for the estimation of digitalis preparations. The control of ethanol levels in the extraction and estimation of powdered leaves is practicable but the examination of galenical preparations would necessitate the determination of their ethanol contents. The choice of the correct wavelength for observation is somewhat critical

involving the use of a suitable spectrophotometer as shown by the shape of the curve peaks in Figure 1. Figure 1 also supports the work of Abrams[9] in criticising the choice of wavelength 525 mμ by Bell and Krantz and employed also in the United States Pharmacopoeia XIV for the reaction. Wavelength 495 mμ should be used for digitoxin estimations and 485 mμ for digitalis tincture estimations.

The use of 3:5-dinitrobenzoic acid in alkaline solution for these estimations was smooth and rapid. It was not susceptible to variations between 20 to 50 per cent. of ethanol present in the final reaction mixture, hence the control of conditions in which this reagent may be employed is not so stringent as for the picrate reagent. The dinitrobenzoate reagent develops a maximum colour density with either digitoxin or tincture preparations in about 6 minutes after mixing, when measured at wavelength 535 mμ and compared with a blank of diluted reagent. These conditions result in the reaction following the Beer-Lambert law. They differ from those proposed by Kedde[6] who measured colour densities 1 hour after mixing and used as a blank a dilution of decolorised tincture only. The shape of the curves in Figure 2 suggests that the wavelength of observation need not be so critically observed as for the picrate reagent; Kedde has employed wavelength 530 mμ for making his observations. Canbäck[1] has stated that the modifications of the Raymond process using *m*-dinitrobenzene or dinitrobenzoic acid are to be preferred to the use of picrate for the estimation of digitoxin; throughout the work reported in this paper the dinitrobenzoic acid reagent has also been found satisfactory and flexible when employed as the recommended process for estimation.

Canbäck's process for the estimation of digitoxin and other isolated glycosides by means of *m*-dinitrobenzene in ethanol (95 per cent.) was found to be satisfactory in operation but did not offer any obvious advantage over the dinitrobenzoic acid method. It has not been applied to tincture preparations and the need to maintain an ethanol level of at least 40 per cent. in the final reaction mixture would present some difficulties in the handling of such preparations. Also the method of obtaining colour density values at zero time by graphic extrapolation demands a critical accuracy in timing the observations to be extrapolated. In the first 2 minutes after mixing the colour fade is very rapid at room temperatures and readings must be made at exact half-minute intervals; this is difficult to secure with a spectrophotometer circuit that must be balanced against a colour-changing blank for each observation. With these considerations in mind the dinitrobenzene process has not been explored further.

The results obtained in the series of experiments based upon the use of either lead acetate or lead subacetate for the decolorisation of tinctures of digitalis have been discussed above. The deductions which were made have led to the recommended process for decolorisation and estimation. It was found that digitoxin either alone or in the presence of a chlorophyll solution in ethanol (70 per cent.), extracted from grass meal, could be submitted to the decolorisation process using different amounts of either

lead acetate or lead subacetate. Under these variable conditions a constant estimation figure for the same amount of glycoside was obtained, showing a loss of about 15 per cent. of glycoside in the process by either the dinitrobenzoate or controlled picrate estimations. Similar results, shown in Table III, were obtained for a mixture of digitoxin and tincture of digitalis, showing a loss in the process of about 20 per cent. except for the decolorisation using lead acetate, followed by dinitrobenzoate estimations where an abnormally high assay figure was obtained. This is in agreement with the values shown in Figure 5 and in consequence lead subacetate was preferred for decolorisation of tinctures. The lack of parallelism between the picrate and dinitrobenzoate estimations of the same series of solutions shown in Figure 5,B and D, is of interest and it suggests that the picrate process is more susceptible to interference.

The process employed by Soos[8] for the extraction of powdered digitalis leaf by shaking with cold water for 1 hour has been shown to extract only 60 per cent. of the activity yielded to ethanol (70 per cent.) after a 48 hours' maceration. Also it has been found that the glycosides present in such an aqueous extract cannot be conveniently extracted by means of chloroform. In consequence this method for the estimation of the unhydrolysed glycosides of digitalis leaf by means of the Keller-Kiliani reaction has not, as yet, been investigated further.

Summary and Conclusions

1. Colorimetric processes have been examined for the estimation of digitoxin by means of alkaline picrate, alkaline *m*-dinitrobenzene, alkaline 3:5-dinitrobenzoic acid or the Keller-Kiliani reagents. The conditions under which these reactions obey the Beer-Lambert law, when measured spectrophotometrically, have been investigated.

2. A process is recommended for the decolorisation of digitalis tinctures and their estimation by means of alkaline solution of 3:5-dinitrobenzoic acid.

3. Lead subacetate is recommended for the decolorisation of tinctures of digitalis in preference to lead acetate.

4. Alkaline picrate reagent produces a colour with ethanol which interferes with the use of this reagent for the estimation of digitalis glycosides, unless the ethanol contents of test solution and blank are identical.

5. The colour density produced by decolorised tincture of digitalis and alkaline picrate reagent decreases with increase in ethanol content of reaction mixture; digitoxin under similar conditions gives increases in colour densities.

6. Alkaline picrate reagent may be used for the quantitative estimation of digitalis glycosides when the ethanol levels are controlled, but the reaction is sensitive to changes in concentration levels of reagents employed for the decolorisation of tinctures.

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