

# THE COLORIMETRIC ESTIMATION OF GITOXIGENIN IN PRESENCE OF DIGITOXIGENIN

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FOR the investigation of gitoxigenin in presence of digitoxigenin different reagents are known<sup>1,2,3,4</sup>. The first to indicate a reagent for qualitative purposes was Kiliani<sup>2</sup>; it consists of a mixture of 100 ml. of concentrated sulphuric acid and 1 ml. of a 5 per cent. aqueous ferric sulphate solution. This reagent was applied by Kraft<sup>5</sup>, Windaus and Schwarte<sup>6</sup> and Cloetta and Fischer<sup>7</sup>. The reagent of Kiliani is not satisfactory for the quantitative estimation of gitoxigenin in presence of digitoxigenin. The reaction develops too quickly and moreover the reagent gives too deep a colour with digitoxigenin. When concentrated sulphuric acid is used without ferric chloride<sup>3</sup> the time required for obtaining a colour with gitoxigenin is rather long, namely 24 hours, and digitoxigenin also gives a colour. The proportion of the extinctions of equal quantities of gitoxigenin and digitoxigenin is 5 to 7.

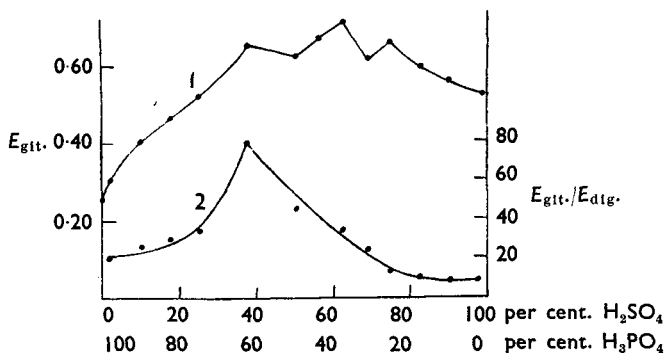


FIG. 1.

It has been proved by some workers<sup>4,8</sup> that concentrated phosphoric acid reacts with gitoxigenin under certain conditions. We therefore investigated the influence of phosphoric acid on the reagent of Kiliani. 1 g. of a 5 per cent. solution of ferric chloride in phosphoric acid was added to a mixture of concentrated sulphuric acid with syrupy phosphoric acid (85 per cent.). The composition of the reagent was varied and the results of the reaction with gitoxigenin are given in Figure 1.

The upper graph (1) shows the maximal extinctions of the colour which, after a shorter or longer lapse of time, develops by the reaction of 10 ml. of reagent with 0.1 mg. of gitoxigenin. The absorption was determined at a wavelength of 5800 Å.

The lower graph (2) represents the proportion of the extinction of equal quantities of gitoxigenin and digitoxigenin ( $= E_{glt.}/E_{dtg.}$ ), obtained

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by varying the composition of the reagent. The time of reaction with the digitoxigenin is the same as that in which gitoxigenin attained its maximal value using the reagent of the same composition. From the two curves it can be seen that the most favourable results are obtained when the concentration of phosphoric acid is 62.5 per cent. w/w.

In many cases the absorption spectrum of the colour obtained by the influence of the reagents on gitoxigenin was determined. In the experiments represented in Figure 2 we used for this purpose the reagent of the following composition:—concentrated sulphuric acid, 37.5 per cent. w/w; phosphoric acid, 62.5 per cent. w/w; hydrated ferric chloride, 0.05 per cent. w/w, added as a 5 per cent. w/w solution in the phosphoric acid. The measurements were carried out with a spectrophotometer (Bleeker, Holland).

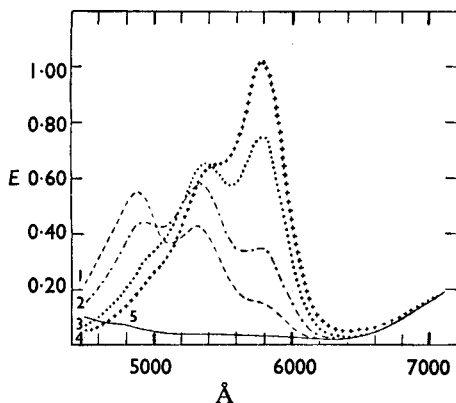


FIG. 2.

The extinctions were measured every 50 Å or, if necessary, every 25 Å. The spectral band width was 40 Å. For the estimation, 0.1600 mg. of gitoxigenin was mixed with 10 ml. of reagent and the absorption measurements were made in a 0.50 cm. cuvette against a blank of reagent only.

Figure 2 shows that at first (curve 1) a colour is formed with a maximal absorption at about 4900 Å. In curves 2 and 3 the maximum is altered, and the final result in curve 4 is that a peak is formed at 5800 Å. After a longer or shorter time, the colour begins to fade. In Figure 2 the absorption spectrum of the reaction product of 0.20 mg. digitoxigenin with the reagent is also reproduced (curve 5). This is quite different from curve 4.

McChesney, Nachod, Auerbach and Laquer<sup>3</sup>, who used pure concentrated sulphuric acid as reagent, recommend a wavelength of 5250 Å.

The influence of the quantities of ferric chloride and of water also of the temperature was determined, and it was also ascertained for how long the colour keeps its maximal extinction value. Table I shows the influence of ferric chloride. Of the reagents examined the sulphuric acid and phosphoric acid concentrations have been kept constant and only the percentage of ferric chloride varied. To shorten the time required for

attaining the maximal extinction, the temperature at which the reaction occurs must be raised by heating in a water bath at 65° C.

Table I shows there is an optimal concentration of ferric chloride. As the proportion  $E_{git}/E_{dig}$  always is approximately 80, the concentration of 0.05 per cent. of hydrated ferric chloride can be recommended as the most favourable one.

TABLE I  
INFLUENCE OF FERRIC CHLORIDE ON THE EXTINCTION

| H <sub>2</sub> SO <sub>4</sub><br>per cent. | H <sub>3</sub> PO <sub>4</sub><br>per cent. | FeCl <sub>3</sub> .6H <sub>2</sub> O<br>per cent. | Time<br>minutes | Tempera-<br>ture ° C. | λ max.<br>Å | E <sub>0.1 git.</sub> | E <sub>0.4 dig.</sub> | E <sub>git./E<sub>dig.</sub></sub> |
|---|---|---|-----------------|-----------------------|-------------|-----------------------|-----------------------|------------------------------------|
| 37.5  | 62.5  | —   | 30              | 65                    | 5800        | 0.44                  | 0.02                  | 85                                 |
| 37.5  | 62.5  | 0.005   | 11              | 65                    | 5800        | 0.61                  | 0.03                  | 80                                 |
| 37.5  | 62.5  | 0.050   | 9               | 65                    | 5800        | 0.65                  | 0.03                  | 85                                 |
| 37.5  | 62.5  | 0.250   | 3               | 65                    | 5800        | 0.58                  | 0.03                  | 80                                 |

In Table II the influence of water and of phosphoric acid on the reagent is shown. The quantities of ferric chloride and sulphuric acid were kept constant and the phosphoric acid was partly substituted by water.

TABLE II  
INFLUENCE OF THE QUANTITY OF WATER ON THE EXTINCTION

| H <sub>2</sub> SO <sub>4</sub><br>per cent. | H <sub>3</sub> PO <sub>4</sub><br>per cent. | FeCl <sub>3</sub> .6H <sub>2</sub> O<br>per cent. | H <sub>2</sub> O<br>per cent. | Time<br>minutes | Tempera-<br>ture °C. | λ max.<br>Å | E <sub>0.1 git.</sub> | E <sub>0.4 dig.</sub> | E <sub>git./E<sub>dig.</sub></sub> |
|---|---|---|-------------------------------|-----------------|----------------------|-------------|-----------------------|-----------------------|------------------------------------|
| 37.5  | 62.5  | 0.050   | —                             | 9               | 65                   | 5800        | 0.65                  | 0.03                  | 85                                 |
| 37.5  | 57.5  | 0.050   | 5                             | 6               | 65                   | 5800        | 0.625                 | 0.035                 | 70                                 |
| 37.5  | 52.5  | 0.050   | 10                            | 7               | 65                   | 5800        | 0.51                  | 0.03                  | 35                                 |
| 37.5  | 47.5  | 0.050   | 15                            | 7               | 65                   | 5800        | 0.37                  | 0.07                  | 20                                 |
| 37.5  | 42.5  | 0.050   | 20                            | 8               | 65                   | 5800        | 0.14                  | 0.025                 | 12                                 |

The extinction of the reaction product of 0.1 mg. of gitoxigenin with the reagent is continually reduced as the quantity of water is increased. The presence of a relatively small percentage of water only slightly affects the extinction. Small fluctuations in the percentage of water in the sulphuric and phosphoric acids used therefore have no significant influence on the application of the reagent.

It is demonstrated in Table III that the temperature plays an important part in the velocity of colour formation.

TABLE III  
INFLUENCE OF TEMPERATURE ON THE VELOCITY OF THE REACTION

| Time         | Temperature<br>° C. | λ max. Å | E <sub>0.1 git.</sub> |
|--------------|---------------------|----------|-----------------------|
| 9 hours      | 20                  | 5800     | 0.65                  |
| 42.5 minutes | 50                  | 5800     | 0.64                  |
| 25 "         | 55                  | 5800     | 0.64                  |
| 14 "         | 60                  | 5800     | 0.63                  |
| 9 "          | 65                  | 5800     | 0.65                  |

The time of reaction is much shortened by rise of temperature and small differences of temperature have a relatively great influence. Moreover, when the heating time is too short, the extinction reading is too

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low, but a little extra heating time is without effect. It is therefore necessary to proceed very accurately regarding both time and temperature.

Finally, the length of time for which the extinction of the colour remains constant was investigated. Table IV shows that this is about 2 hours.

TABLE IV  
INFLUENCE OF THE TIME ON THE EXTINCTION

|                           |      |      |      |      |      |      |      |      |      |
|---------------------------|------|------|------|------|------|------|------|------|------|
| Extinctions .. ..         | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.56 |
| Measured after minutes .. | 5    | 15   | 30   | 45   | 60   | 75   | 90   | 105  | 120  |

### CONCLUSION

For the estimation of gitoxigenin in presence of digitoxigenin concentrated sulphuric acid or a mixture of this acid and a small quantity of ferric chloride is less suitable than a reagent containing syrupy phosphoric acid. A concentration of 62.5 per cent. of phosphoric acid gives the best results. The ratio of the extinctions of equal quantities of gitoxigenin and digitoxigenin is in that case approximately 80. An optimal value of 0.050 per cent. was found for the concentration of the ferric chloride. Therefore a reagent of the composition given on page 477 is recommended. Addition of water to the reagent is not advantageous, though small quantities have but little influence. The reaction must be carried out at a raised temperature. As the duration of heating is dependent on the temperature, the heating must be always carried out in the same way.

The estimation is carried out as follows:—10 ml. of reagent is mixed with a quantity of gitoxigenin or of a mixture of gitoxigenin and digitoxigenin. When solution is complete the mixture is warmed for 9 minutes in a water bath at 65° C. The vessel is then cooled for 1 minute in running water and kept for another 5 minutes in water at room temperature. The absorption is measured in a 0.50-cm. cuvette against a blank of reagent only at a wavelength of 5800 Å. The spectral band width is 50 Å. As the peak at about 5800 Å is a very narrow one (Fig. 2), it is advisable to measure at 5750 Å with a broader spectral band width. The colour obtained remains constant for 2 hours.

The graph obtained by plotting the colour densities against gitoxigenin concentration between 0 and (at least) 1.6 mg. per cent. was a straight line;  $k$  (1 mg. per cent. of gitoxigenin) = 1.30. The reaction therefore obeys the Lambert-Beer law.

### SUMMARY

1. A new reagent, consisting of a mixture of concentrated sulphuric acid, syrupy phosphoric acid and ferric chloride is recommended for the colorimetric estimation of gitoxigenin in presence of digitoxigenin. Various experiments showed that the most favourable results are achieved if the composition is the following:—concentrated sulphuric acid, 37.5 per cent. w/w; syrupy phosphoric acid (85 per cent.), 62.5 per cent. w/w; and hydrated ferric chloride, 0.050 per cent. w/w.

2. The red colour originating from the interaction of this reagent with gitoxigenin must be measured at 5750 Å.

3. The extinction of the colour obtained with gitoxigenin is approximately 80 times as great as that of an equal quantity of digitoxigenin and keeps a constant value for at least 2 hours.

#### REFERENCES

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