THE USE OF THE SULFURIC ACID REACTION FOR THE
ESTIMATION OF α- AND β-ESTRADIOLS AND OF
ESTRONE AND EQUILIN IN BINARY MIX-
TURES IN PURE SOLUTIONS*

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The problem of estimating quantitatively the components of a mixture
of naturally occurring estrogens has been attacked through the prepara-
tion of their p-phenylazobenzoyl (azoyl) esters and separating these esters
by the chromatographic adsorption technique (1). By this method, the
monoazaoates were quantitatively separated from the diazoates. Hydroly-
sis of these esters and suitable extraction procedures resulted in the quan-
titative recovery of the free estrogens. The present report describes a
study of a single phase sulfuric acid reaction for the further analysis of
the mixtures of free estrogens so obtained and for the positive identifica-
tion of a single free estrogen.

It was early observed that, when the natural estrogens are treated with
sulfuric acid, orange-colored solutions possessing a green fluorescence are
obtained (2-4). Kober (4) showed that the initial orange color obtained
with estrone was changed to a clear green-fluorescing red on warming with
water, and, in addition, that the intensity of the red color could be en-
hanced and the intensity of the yellow color and the fluorescence dimin-
ished by using a mixture of phenol and sulfuric acid in the initial stage of
the reaction instead of sulfuric acid alone. Since the orange or yellow
solutions resulting from the action of sulfuric acid on cholesterol, bile acids,
pregnanediol, and many other steroids are decolorized by dilution with
water, the test was claimed to be specific for the natural estrogens. Cohen
and Marrian (5), however, found that the presence of either cholesterol or
pregnanediol considerably decreased the intensity of the red color yielded
by given quantities of estrone or estriol in the Kober reaction. They
resorted, therefore, to an alkali extraction procedure which separated the

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phenolic estrogens from these interferers, and which, in effect, supplied the specificity claimed by the Kober reaction.

Various modifications of the Kober reaction designed to eliminate the yellow color and the fluorescence and to increase the specificity and sensitivity of the reaction have been published (5-11). Carol and Molitor (12) have described a modification of the Kober reaction which permits the determination of the components of a binary mixture of \( \alpha \)- and \( \beta \)-estradiols.

Mather (13) pointed out that the spectral absorption curves of the colors formed with the estrogens by phenol reagents are basically the same as those with sulfuric acid alone, and Cohen and Bates (14) have published the details of a method utilizing the two-stage Kober test without the use of phenol. Bates and Cohen (15) and Jailer (16) have described methods utilizing the fluorescence that develops with sulfuric acid for the quantitative determination of the estrogens, and Dr. Willard M. Allen, in unpublished work made known to the authors, has made use of the yellow component of the color produced by sulfuric acid and alcohol for the quantitative determination of the estrogens in human pregnancy urine.

**EXPERIMENTAL**

*Behavior of Natural Estrogens with Various Concentrations of Sulfuric Acid*

Solutions containing 30, 45, 60, 75, 90, and 100 per cent concentrated sulfuric acid were prepared by diluting a measured amount of concentrated sulfuric acid (Baker's Analyzed, c.p., assay 95.5 to 97.5 per cent) to volume in a volumetric flask with small amounts of distilled water, and cooling after each addition. For example, 90 per cent sulfuric acid was prepared by placing 90 ml. of concentrated sulfuric acid in a 100 ml. volumetric flask and diluting to the mark with distilled water. The reaction tubes were prepared by sealing off the end of the outer part of a 24/40 standard taper joint and closing with a 24/40 standard taper penny head stopper. To the tube was added an alcoholic solution of 25 \( \gamma \) of the estrogen and the alcohol was evaporated on the steam bath under a stream of air. The tube and stopper were dried over phosphorus pentoxide under a vacuum for 1 hour. The tube was then placed in an ice bath, 5.0 ml. of the diluted sulfuric acid were added, and the tube was stoppered, allowed to remain in the ice bath 5 minutes, and heated in a vigorously boiling water bath. During the heating period, the tube, without removal from the bath, was agitated at 15 second intervals during the 1st minute and at 1 minute intervals thereafter. On removal from the boiling water bath the tube was again placed in the ice bath for 1 minute. The absorption spectrum of the color produced was measured in the range from 400 to 560 m\( \mu \) on the Beckman spectrophotometer.
Absorption curves were determined for α-estradiol (Fig. 1), β-estradiol (Fig. 2), estrone (Fig. 3), and equilin (Fig. 4), after a 12 minute heating period. This heating period was chosen since α-estradiol and β-estradiol give equal intensities of absorption at 515 mμ after 12 minutes heating. It will be observed that with 60 per cent sulfuric acid the curves for all four estrogens are similar. The solutions are orange in color and fluoresce strongly. Two peaks of maximum absorption are shown, one in the neighborhood of 460 mμ and one in the neighborhood of 515 mμ. As the sulfuric acid concentration is increased, the solutions become yellow, with an increase in absorption at the lower wave-length relative to the absorption at the higher wave-length. The converse is true on decreasing the sulfuric acid concentration, and the solutions become red. Also as the concentration of sulfuric acid is increased, the absorption maxima are shifted towards the lower wave-lengths in the cases of α-estradiol, β-estradiol, and estrone. With equilin, however, these maxima become less distinct, there being general absorption throughout the region of the spec-

![Graph](http://www.jbc.org/)
trum observed. With very high concentrations of sulfuric acid, \( \alpha \)-estradiol exhibits a new maximum at 425 to 430 \( \mu\mu \). This new maximum appears to be specific for \( \alpha \)-estradiol, although it is faintly evident in the cases of \( \beta \)-estradiol and equilin with 100 per cent sulfuric acid.

The changes in absorption intensity at 460 and 515 \( \mu\mu \) with concentration of sulfuric acid for the estradiols are shown in Fig. 5. There is clearly an optimal concentration of sulfuric acid for both wave-lengths which, however, differs for the two isomers. \( \beta \)-Estradiol reacts more strongly at the lower concentrations, while \( \alpha \)-estradiol reacts more strongly at the higher concentrations. Equal intensities of absorption were given with 60 per cent sulfuric acid at 515 \( \mu\mu \) and with 73.5 per cent sulfuric acid at 460 \( \mu\mu \). \( \beta \)-Estradiol is the only estrogen so far tested which shows appreciable reaction with sulfuric acid concentrations of 30 per cent or lower. No conditions were found at which the \( \alpha \) isomer reacts exclusively.

Based on the above observations, methods were developed for the quantitative estimation of \( \alpha \)-estradiol and \( \beta \)-estradiol in a binary mixture, and of estrone and equilin in a binary mixture. Methods could probably be developed for determining any two of the estrogens in a binary mixture.
Determination of $\beta$-Estradiol

In an attempt to determine $\beta$-estradiol quantitatively in the presence of $\alpha$-estradiol with 30 per cent sulfuric acid as the reagent, cloudy solutions developed owing to the lower solubility of the $\alpha$ isomer. This condition was remedied by the addition of $n$-butyl alcohol to 20 per cent by volume. With this 30 per cent sulfuric acid-20 per cent $n$-butyl alcohol reagent, optimal conditions were obtained when the time of heating was 6 minutes and the readings were made at 524 m$\mu$. The color was stable for at least 1 hour. Fig. 6 shows the results of a series of determinations of $\beta$-estradiol under these conditions. Beer's law is followed up to 50 $\gamma$. Fig. 6 indicates that on addition of 100 $\gamma$ of $\alpha$-estradiol to the $\beta$-estradiol there is no interference by this compound. Estrone, equilin, $\alpha$-dihydroequilin, equilenin, and estriol fail to give a color under these conditions.
in amounts of 50 to 100 γ. Other dihydro compounds have not yet been tested.

**Determination of Total Estradiols**

Since no conditions were found in which α-estradiol reacted exclusively, a method was developed for determining total estradiols. α-Estradiol can then be determined indirectly as the difference between the value for total estradiols and the value for β-estradiol.

![Graph](http://www.jbc.org/)

**Fig. 4.** Effect of concentration of sulfuric acid on spectral absorption given by 25 γ of equilin when heated with 5.0 ml. of various concentrations of sulfuric acid for 12 minutes.

While the α- and β-estradiols gave equal intensities of absorption at 515 mμ when heated with 60 per cent sulfuric acid for 12 minutes, there was an enhancement of the absorption when the two were mixed, resulting in an overestimation of total estradiols of from 5 to 20 per cent.

On the other hand, total estradiols were satisfactorily determined by heating with 5.0 ml. of 73.5 per cent sulfuric acid for 12 minutes. Under these conditions, the maximum sensitivity was obtained by reading at 455 mμ. There appeared to be no tendency towards either enhancement or inhibition of absorption in mixtures. Spectral absorption curves for the two isomers were practically identical in the region from 400 to 470 mμ.
but α-estradiol gave greater absorption in the region from 470 to 560 μm, as would be predicted by the curves in Fig. 5.

**Simultaneous Determination of Estrone and Equilin in Binary Mixture**

Since there was considerable difference in the spectral absorption curves given by estrone and equilin when heated with 90 per cent sulfuric acid, they could be determined simultaneously in mixtures by reading at two wave-lengths and solving simultaneous equations to obtain the concentration of each component. General formulas for such a calculation are given by Knudson, Meloche, and Juday for the simultaneous determination of iron and aluminum in water by the hematoxylin method (17). This method can be used only when there is no appreciable interference between the development of colors by the two components. To test this, estrone and equilin were heated separately and in an equal mixture

![Graph showing absorption at different wave-lengths](image-url)
with 5.0 ml. of 90 per cent sulfuric acid for 5 minutes and the spectral absorption curves determined. A 5 minute heating period was used instead of a 12 minute period to reduce the amount of color due to charring which might be expected in an impure solution. The curves so obtained are shown in Fig. 7. Curve A is the absorption curve of 20 \( \gamma \) of equilin alone and Curve B that of 20 \( \gamma \) of estrone. Curve C was obtained with a mixture of 20 \( \gamma \) each of equilin and estrone. Curve D is the arithmetical sum of Curves A and B and should have coincided with Curve C had there been no interference. There appears to be an inhibition of color throughout the entire spectrum studied. However, the color interference is negligible, as demonstrated by the results on the determination of a series of mixtures shown in Table I.

Identification of Natural Estrogens by Means of Sulfuric Acid Reaction

The spectral absorption curves obtained when the natural estrogens are heated with 90 per cent sulfuric acid for 12 minutes are sufficiently charac-
Fig. 7. Absorption spectra of colors produced by heating estrone and equilin separately and in mixtures with 5.0 ml. of 90 per cent sulfuric acid for 5 minutes. Curve A, 20 \(\gamma\) of equilin; Curve B, 20 \(\gamma\) of estrone; Curve C, 20 \(\gamma\) each of estrone and equilin; Curve D, arithmetical sum of values for Curves A and B.

Table I

Results Obtained in Determination of Estrone and Equilin in Mixtures in Pure Solution by Heating with 90 Per Cent Sulfuric Acid for 5 Minutes

Density readings at 450 and 480 m\(\mu\).

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Estrone added</th>
<th>Estrone found</th>
<th>Equilin added</th>
<th>Equilin found</th>
</tr>
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<tr>
<td>1</td>
<td>0</td>
<td>1</td>
<td>25</td>
<td>26</td>
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<td>2</td>
<td>5</td>
<td>5</td>
<td>40</td>
<td>42</td>
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<tr>
<td>10</td>
<td>25</td>
<td>26</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>
Fig. 8. Spectral absorption curves for six natural estrogens obtained by heating 25 γ of each with 5.0 ml. of 90 per cent sulfuric acid for 12 minutes. Curve A, estrone; Curve B, equilenin; Curve C, α-estradiol; Curve D, equilin; Curve E, β-estradiol; Curve F, estriol.

### Table II

Identification of Natural Estrogens by Heating with 90 Per Cent Sulfuric Acid for 12 Minutes and Determining Density Values at 430, 450, 480, and 520 m̅µ

<table>
<thead>
<tr>
<th>Estrogen</th>
<th>Identification No.</th>
<th>( X = \frac{(D_{450})^2}{D_{480} \times D_{430}} )</th>
<th>( Y = \frac{(D_{480})^2}{D_{520} \times D_{430}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrone</td>
<td></td>
<td>14.92</td>
<td>0.46</td>
</tr>
<tr>
<td>Equilin</td>
<td></td>
<td>1.11</td>
<td>3.03</td>
</tr>
<tr>
<td>Equilenin</td>
<td></td>
<td>1.09</td>
<td>6.94</td>
</tr>
<tr>
<td>α-Estradiol</td>
<td></td>
<td>1.92</td>
<td>1.24</td>
</tr>
<tr>
<td>β-Estradiol</td>
<td></td>
<td>5.87</td>
<td>0.86</td>
</tr>
<tr>
<td>Estriol</td>
<td></td>
<td>3.80</td>
<td>1.36</td>
</tr>
</tbody>
</table>
It is not necessary to measure the entire spectral absorption curve in order to obtain an identification. Density readings can be made at 430, 450, 480, and 520 m\(\mu\), and from a relationship between these values identification numbers can be calculated. For our purposes, we have calculated an identification number \(X\) and an identification number \(Y\). The formulas for their calculation are as follows:

\[
X = \frac{(D_{450})^2}{D_{430} \times D_{490}}
\]

\[
Y = \frac{(D_{490})^2}{D_{480} \times D_{520}}
\]

Identification numbers calculated in this manner for the estrogens so far studied are shown in Table II.

**DISCUSSION**

The methods described here involve a single-stage reaction with sulfuric acid. The Kober test, on the other hand, is a two-stage reaction in which the estrogen is first heated with a relatively high concentration of sulfuric acid and then with a low concentration of sulfuric acid. Cohen and Bates (14) have shown that essentially the same results can be obtained in the Kober test without the use of phenol. Apparently, the phenol serves the purpose of a simple diluent in the first stage of the reaction and of quenching the fluorescence in the second stage. As shown here, \(\beta\)-estradiol is the only estrogen so far studied which gives a red color with low concentrations of sulfuric acid. The preliminary heating period with concentrated sulfuric acid used in the Kober test, therefore, must activate the other estrogens in such a way as to cause them to give a red color when heated with the diluted sulfuric acid. This was clearly demonstrated by Cohen and Bates (14) in the case of estrone, when, after a preliminary treatment with concentrated sulfuric acid, a yellow color was obtained if the dilution in the second stage was made with 1:1 sulfuric acid or higher concentrations, and a red color if the dilutions were made with water or dilute concentrations of sulfuric acid. Since \(\beta\)-estradiol is the only compound so far studied which gives a red color with 30 per cent sulfuric acid, it is apparent that it is more reactive and does not need to be activated before it will react. This would explain the results of Carol and Molitor (12) in which \(\beta\)-estradiol reacted with the Kober reagent without a preliminary heating period. It is significant that \(\beta\)-estradiol gives an almost immediate yellow color with 60 per cent sulfuric acid, even at room tem-
temperature, while $\alpha$-estradiol gives little or no color under these conditions. With 73.5 per cent sulfuric acid, however, these observations are reversed.

Just as with the Kober reaction, rigid adherence to manipulative details and concentrations of reagents must be maintained. As illustrated in Fig. 5, the intensity of the absorption varies greatly with the concentration of sulfuric acid. This is particularly of importance in the determination of total estradiols with the 73.5 per cent sulfuric acid. However, in the determination of $\beta$-estradiol and the simultaneous determination of estrone and equilin, many of these variables are eliminated by including standards along with the determinations. Since $\beta$-estradiol is sensitive to even traces of sulfuric acid, the reaction tubes must be carefully cleaned before each determination. After the tubes are washed exhaustively with tap water, once with a saturated solution of sodium carbonate, and again exhaustively with tap water, they are rinsed out with distilled water, and finally with absolute alcohol.

In addition to being simpler to carry out, the sulfuric acid reaction possesses an advantage over the Kober reaction in that it provides a means of identifying the estrogen in minute amounts. The colors produced in the Kober reaction are in general qualitatively the same. Also, the ultraviolet absorption curves for the estradiols, estrone, and equilin are identical. They can, however, be distinguished by their infra-red absorption (18).

**SUMMARY**

The behavior of several natural estrogens with various concentrations of sulfuric acid has been studied in detail. The colors produced vary with the estrogen, the time of heating, and the concentration of sulfuric acid. The relationship of the sulfuric acid reaction to the Kober reaction has been discussed.

Spectral absorption curves of the colors produced by sulfuric acid with the natural estrogens are sufficiently characteristic to serve as a means of identification of the individual estrogens in minute amounts.

Methods for the quantitative estimation of $\alpha$-estradiol and $\beta$-estradiol and of estrone and equilin in binary mixtures in pure solution have been described.

**BIBLIOGRAPHY**

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