Colorimetric Determination of Eriodictyol and Its Glycoside, Eriocitrin

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Abstract □ A procedure has been devised to quantitate the flavanone, eriodictyol, and its glycoside, eriocitrin, by use of the color reagent, 2-aminoethyl diphenylborate. Also, some parameters in the use of this reagent in quantitative analysis have been studied. Application of the procedure to lemon bioflavonoid complex gave an average content of 2.5% eriocitrin.

Keyphrases □ Eriodictyol, eriocitrin—determination □ Lemon bioflavonoid complex—eriodictyol, eriocitrin determination □ 2-Aminoethyl diphenylborate—color reagent □ Colorimetric analysis—spectrophotometer

Several investigators (1–4) have shown interest in the biological and clinical effects of eriodictyol, a flavanone which occurs as the glycoside in *Citrus limon* (5–7).

In the course of an investigation of the biological effects of this flavonoid, it was found necessary to devise an analytical method for assaying the eriodictyol content of the materials being evaluated. Several methods for the quantitation of flavonoids are known. In general, they are either nonspecific (8–13) or involve time-consuming chromatographic separations (14–19). A control method avoiding these disadvantages was desired.

The following work describes assay methods for eriodictyol, both in the free aglycone form and in the form of eriodictyol glycoside, as it is present in the commercial product known as lemon bioflavonoid complex[1]. By TLC, the authors found this glycoside to coincide with the lemon glycoside described by Horowitz and Gentili (5) and characterized by them as eriodictyol 7-β-rutinoside (eriocitrin).

Neu (20) investigated the analytical use of diarylboric acids, including the photometric determination of flavones, and described the preparation of 2-aminoethyl diphenylborate. This reagent has come into use as a flavonoid spray on TLC and papergrams (21). Ogura *et al.* (8) presented UV spectra of the reaction products of aminoethyl diphenylborate with rutin and quercetin.

Except for eriodictyol and eriocitrin, available flavonoids which give colored aminoethyl diphenylborate reaction products have absorption peaks[2] ranging from 350 to 467 nm. Only eriodictyol and eriocitrin give red colors, with peaks at 505 and 520 nm., respectively (Table I) (Fig. 1). Of great interest was the observation that hesperidin and hesperetin, the most likely interferences in the samples, give negligible absorbance above 500 nm. Based on these observations, the following procedure was devised for eriodictyol in the absence of its glycoside. This procedure was then used as the basis of a procedure to assay for eriocitrin in lemon bioflavonoid complex.

Table I—Comparison of Absorbance Peaks of Some Flavonoids after Aminoethyl Diphenylborate Treatment under Standardized Conditions Using 0.1 mg. of the Compound

Flavonoids	$\lambda_{max.}$	A_{250}	A_{505}
Glycosides			
Flavanones			
Eriocitrin	520	0.33[a]	–
Hesperidin	420	0.00	–
Nonflavanones			
Quercitrin	453	0.03	–
Phlorizin	–	0.00	–
Aglycones			
Flavanones			
Eriodictyol	505	–	0.43
Hesperetin	417	–	0.00
Dihydroquercetin[b]	467	–	0.09
Nonflavanones			
Quercetin	467	–	0.22
Apigenin	350	–	0.00
Luteolin	445	–	0.08
Phloretin	–	–	0.00

[a] Calculated from lemon bioflavonoid complex reference standard results. [b] Dihydroquercetin may be classed as a 3-hydroxyflavanone or a dihydroflavonol; UV spectra of flavanones and dihydroflavonols are very similar.

When the methanol extractives from lemon bioflavonoid complex react with aminoethyl diphenylborate, the resultant solution is orange, as opposed to the red obtained from eriocitrin. The yellow component of this color is due to hesperidin and small amounts of other flavonoids, but it does not contribute significantly to the A_{520}, which can therefore be used as a measure of the eriocitrin content, relative to a standard lemon bioflavonoid complex run simultaneously. Because pure eriodictyol was available as a standard, a quantitative hydrolysis–extraction procedure was devised for assaying this (secondary) lemon bioflavonoid complex standard in terms of its eriodictyol (aglycone) equivalence, which may be recalculated to eriocitrin by multiplying by the ratio of the molecular weights. In this procedure, the use of hemicellulase as hydrolytic enzyme and ethyl acetate as extractant was adapted from work reported by Horowitz and Gentili (5). Acid hydrolysis was found to be too drastic.

EXPERIMENTAL

Eriodictyol Assay—The standard preparation is 0.1 mg. reference standard eriodictyol per milliliter in methanol. The sample preparation is 0.1 mg./ml. in methanol. The reagent is 2-aminoethyl diphenylborate[3]. Prepare a 2.5% solution in methanol. For the buffer, mix 1 volume 2 *M* aqueous acetic acid and 1 volume 2 *M* sodium acetate; adjust pH to 4.6.

[1] The lemon bioflavonoid complex used in this work was manufactured by Sunkist Growers, Inc., Corona, Calif., and by Test Laboratories Inc., Reseda, Calif.

[2] A Cary 15 spectrophotometer was used throughout this work.

[3] Diphenylboric acid–ethanolamine complex, Aldrich Chemical Co., Milwaukee, Wis.

Table II—Effect of Amount of Aminoethyl Diphenylborate Reagent on Color[a]

Aminoethyl Diphenylborate, mg.	$A_{max.}$ at 4 hr.	$A_{max.}$ at 95 hr.
25	0.284	0.629
50	0.430	0.879
75	0.522	0.979
100	0.601	1.090
125	0.645	1.128
150	0.676	1.188
175	0.729	1.218
200	0.733	1.198

[a] Buffer pH 4.6; 0.1 mg. eriodictyol.

Pipet 1 ml. sample and standard preparations into 10-ml. volumetric flasks. Add 1.00 ml. buffer, then add 4.00 ml. aminoethyl diphenylborate reagent, and bring to volume with methanol. Let stand exactly 4 hr. Measure A_{505} in 1-cm. cells against a reagent blank. Calculate purity from the absorbance ratio.

Assay of Lemon Bioflavonoid Complex for Its Eriocitrin Content–Method A—Mix 100 mg. lemon bioflavonoid complex, 200 mg. hemicellulase[4], and 10 ml. distilled water in a stoppered flask and store at 32° for about 20 hr. Using ethyl acetate, rinse quantitatively into a separator and extract with three 10-ml. portions of ethyl acetate. Transfer the combined organic extracts, with the aid of additional ethyl acetate, to a rotary evaporator flask, and evaporate to dryness *in vacuo*, below 50°. Rinse the residue with small portions of methanol into a 25-ml. volumetric flask, and add methanol to the mark. Pipet a 2-ml. aliquot, as sample preparation, into a 10-ml. volumetric flask, and proceed as directed under *Eriodictyol Assay*.

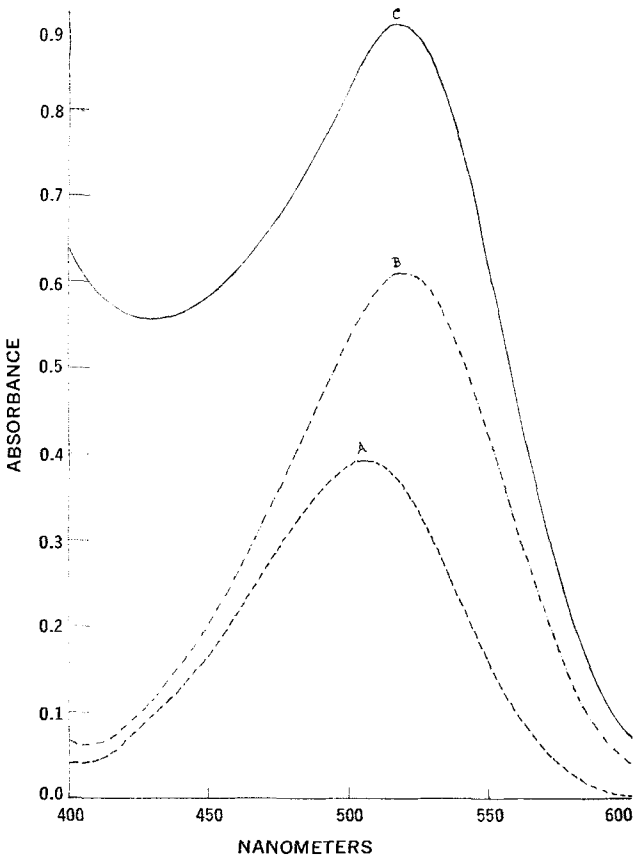

Figure 1—*Spectra of colored complex of eriodictyol, eriocitrin, and lemon bioflavonoid complex. Key: A, --- eriodictyol; B, -·-, eriocitrin; and C, ——, lemon bioflavonoid complex.*

[4] Nutritional Biochemicals Corp., Cleveland, Ohio.

Table III—Effect of Buffer pH on Color Development[a]

Buffer pH	Absorbance at $\lambda_{max.}$: 4 hr. A	4 hr. nm.	22 hr. A	At Max. A	At Max. nm.	At Max. Days	29 Days A	29 Days nm.
2.15	0.190	507	0.642	1.545	514	22	1.520	513
3.00	0.230	506	0.754	1.376	513	19	1.273	512
4.05	0.387	505	0.920	1.260	512	19	1.114	512
4.60	0.540	505	0.879	1.385	513	19	1.315	511
5.90	0.313	493	0.461					
6.72	0.251	488	0.358	Not determined				
8.50	0.191	485	0.276					

[a] 0.1 mg. eriodictyol, 100 mg. aminoethyl diphenylborate reagent, 1 ml. aqueous acetate buffer, and 9 ml. methanol.

Calculate the eriodictyol content from the absorbance ratio. Then, 2.07 × % eriodictyol × dilution factor = % eriocitrin in sample.

Alternative Assay of Lemon Bioflavonoid Complex–Method B—By using, as the reference standard, a lemon bioflavonoid complex that has been assayed by Method A, other lemon bioflavonoid complex samples can be assayed as follows.

Standard Preparation—Shake well 100 mg. reference standard lemon bioflavonoid complex with 100 ml. anhydrous methanol in a 100-ml. volumetric flask; then let stand overnight.

Sample Preparations—Similarly treat 100 mg. lemon bioflavonoid complex sample.

Aminoethyl Diphenylborate Reagent and Buffer—Proceed as described under *Eriodictyol Assay*.

Pipet 4 ml. clear supernatant solutions from standard and sample into 10-ml. volumetric flasks, and proceed as described under *Eriodictyol Assay*. Measure the absorbances at 520 nm. after exactly 4 hr. Calculate the eriocitrin content from the absorbance ratio and the assay value of the reference standard.

RESULTS AND DISCUSSION

Eriodictyol Assay—*Study of Conditions*—Absorbance values were found to vary somewhat between experiments. However, duplicates run at the same time always checked closely. Table II shows that even when aminoethyl diphenylborate concentration was increased to high values, an absorbance plateau could not be attained either at 4 or at 95 hr. Therefore, 100 mg. was chosen for the arbitrary standard conditions. The 4-hr. reaction time is also arbitrary. If more time is available, more intense color develops. When the pH 4.6 buffer is used, the reaction mixture pH is quite stable at 6.65 and is not influenced by addition of eriodictyol, lemon bioflavonoid complex, or aminoethyl diphenylborate. The pH values were measured by glass and calomel electrodes with a Beckman Zeromatic pH meter.

Table III shows that pH influences the rate of color development. The highest absorbance value for the 4-hr. measurement is attained when pH 4.6 buffer is used. There is a shift in wavelength of the absorbance peak when the pH or color development time is changed. No quantitative study was made of temperature dependence, but strong heating resulted in side reactions, while slight temperature variations had little effect. Artificial light had no noticeable effect. Absorbance is directly proportional to sample size up to at least 0.25 mg. eriodictyol.

Lemon Bioflavonoid Complex Assay–Method A—*Validity—Estimate of Interferences*—To estimate the contribution from other lemon bioflavonoid complex ingredients to the A_{505}, the methanolic solution obtained after hydrolysis of Sunkist lemon bioflavonoid complex was chromatographed on Whatman No. 1 paper by descending technique, using the solvent system of benzene–acetic acid–water (125:72:3) (22). The dried paper was edge-sprayed with 1% methanolic aminoethyl diphenylborate reagent; then the unsprayed part was cut into bands and separate color-forming ingredients were eluted with methanol. Several bands gave varying shades of yellow and there was the red eriodictyol band. By running the aminoethyl diphenylborate assay on these solutions in parallel, it was found that the total A_{505} due to noneriodictyol bands was about 5% of that from eriodictyol.

UV Spectrum—The isolation procedure specified in Method A gives a methanol solution whose UV curve is characteristic for

flavanones, such as hesperetin and eriodictyol ($\lambda_{max.}$ = 287 nm.), with hardly any distortion, which would be due to appreciable amounts of other flavonoids. That is, flavonoids which might interfere in the color test, *e.g.*, luteolin and quercetin (Table I), do not appear in the flavanone solution. Actually, at least 90% of the UV absorbance, in this case, was accountable to eriodictyol, with most of the balance probably due to hesperetin formed by partial hydrolysis and recovery from the hesperidin present in the lemon bioflavonoid complex. Dihydroquercetin was shown by TLC to be absent from the methanolic extract, and diosmin seems not to be present in lemon bioflavonoid complex in interfering quantity.

The UV spectrum of the aqueous layer remaining after ethyl acetate extraction of the enzymatic hydrolyzate showed the presence of different absorbers, probably other flavonoids. Examination by TLC and by the borohydride test (9) confirmed the absence of flavanones.

Reaction Course—Varying the enzymatic hydrolysis conditions by stirring magnetically for various times or by letting the reaction continue for 3 days did not affect the result.

Monitoring the reaction by TLC showed that, when insufficient time was allowed for complete hydrolysis, a product with a different R_f value, also giving a red color with aminoethyl diphenylborate spray, was formed as an intermediate. This may be eriodictyol glucoside. When this spot and the eriocitrin spot were no longer present, and only a product having the R_f value of eriodictyol gave a red product with aminoethyl diphenylborate spray, the hydrolysis was complete.

To determine if there was any loss of eriodictyol between hydrolysis and color measurement, two 100-mg. portions of reference standard lemon bioflavonoid complex were taken. To one of these, exactly 1 mg. of standard eriodictyol was added. Both were carried through the Method A procedure simultaneously. The difference between the resultant absorbances showed that very close to 100% of the added eriodictyol was recovered. Evidently the enzyme converts eriocitrin completely to eriodictyol and has no effect on the eriodictyol formed.

Lemon Bioflavonoid Complex Assay–Method B—*Study of Conditions—Extraction*—To justify the simple extraction procedure, varying proportions of methanol and lemon bioflavonoid complex were mixed. From each flask, an aliquot equivalent to the soluble portion from 4 mg. lemon bioflavonoid complex was carried through the aminoethyl diphenylborate reaction. Since no significant difference was observed, it may be concluded that solubility in methanol is not a color-limiting factor. In another experiment, a 2-week-old mixture of lemon bioflavonoid complex–methanol gave the same result.

Color Reaction—The reaction conditions for eriodictyol were adapted to the assay of eriocitrin in lemon bioflavonoid complex, and the procedure was standardized as described earlier. As in the case of eriodictyol, duplicate samples run simultaneously checked closely, even when reaction time was extended to 94 hr. Also, as with eriodictyol, the peak slowly shifts to higher wavelengths. Also, varying the amount of aminoethyl diphenylborate reagent had qualitatively the same effect on the $A_{max.}$ in the case of lemon bioflavonoid complex as with eriodictyol. Beer's law is obeyed up to at least 10 mg. lemon bioflavonoid complex.

Validity—To obtain an estimate of the contribution of interfering lemon bioflavonoid complex ingredients to the A_{520}, a concentrated methanolic extract of Sunkist lemon bioflavonoid complex was chromatographed on Whatman No. 1 paper by descending technique, using the solvent system of *n*-butanol–acetic acid–water (40:10:50). The separated ingredients were treated as in the case of the chromatographed hydrolyzed lemon bioflavonoid complex previously described. In addition to the eriocitrin band, several other bands giving colors with aminoethyl diphenylborate spray were observed. The total A_{520} due to noneriocitrin bands was not more than 5% of that from eriocitrin.

Roughly similar results were obtained when this experiment was repeated using Testlab lemon bioflavonoid complex.

APPLICATIONS

Assay of five different lots of lemon bioflavonoid complex from the two manufacturers gave eriocitrin contents of 2.1–3.0% when tested by Method A. When Method B was used, the absorbances gave the expected proportionality, with a maximum deviation of 10%.

A sample of orange bioflavonoid complex (Sunkist) was examined both by TLC and by the tests described previously. It failed to show the presence of any eriocitrin.

A lemon bioflavonoid complex sample tested after heating to 45° for 1 week gave the same assay result as the fresh material.

Preliminary experiments in the determination of eriodictyol in blood plasma by extraction and reaction with aminoethyl diphenylborate have been performed and have given promising results.

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