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SHORT COMMUNICATION

DETECTION AND DETERMINATION OF ELLAGITANNINS

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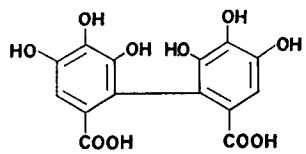
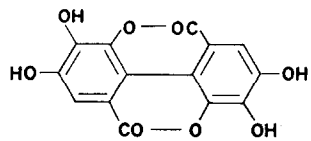
Abstract—Ellagic acid combined in the form of esters of hexahydroxydiphenic acid (HHDP) can be quantitatively determined by taking advantage of the formation of a coloured product with nitrous acid. Under specified conditions, monoesters with glucose have molecular absorption coefficients between 2150 and 2300 at 600 nm. Although the ϵ values per HHDP unit of the more complex esters such as pedunculagin and chebulagic acid are considerably higher, the method can be applied to estimate combined ellagic acid in the majority of plant extracts.

INTRODUCTION

ELLAGITANNINS are esters of glucose with hexahydroxydiphenic acid (I), which are of widespread occurrence in woody dicotyledons. When hydrolysed, they yield ellagic acid (II), the dilactone of (I). When so combined, but not otherwise, ellagic acid reacts with



(I)



(II)

nitrous acid giving a coloured product which can be employed for its detection in plant tissues,[1] and is the basis of a method for the determination of ellagitannins. This reaction is akin to the first step in the Hoepfner reaction[2] for chlorogenic acid which involves the production of a deep red colour on subsequent addition of sodium hydroxide. This reaction is given by other esters of caffeic acid,[3] but with esters of ferulic and sinapic acids the colour produced with strong base is fugitive; it is, however, stable when sodium carbonate is used.

In the case of ellagitannins, the colour reaction with nitrous acid occurs in weak acid. As recommended earlier, this step is best carried out in a methanolic extract of the plant tissue acidified with dilute acetic acid. On addition of sodium nitrite, esters of the hydroxycinnamic acids slowly give a deep yellow coloration, while ellagitannins give a red colour which rapidly becomes blue. In nitrogen atmosphere the colour is fairly stable (Fig. 1) but rapidly changes to an orange–yellow in air.

Quantitative Aspects

The course of the reaction in N_2 is qualitatively similar under a wide variety of conditions, but it is affected by temperature, pH and the concentration of methanol. Variations in all

[1] E. C. BATE-SMITH, *Sci. Proc. Roy. Dublin Soc.* **27**, 365 (1956).
[2] W. HOEPFNER, *Chem. Ztg.* **56**, 991 (1932).
[3] E. C. BATE-SMITH, *Phytochem.* **7**, 459 (1968).

these parameters have been studied and optimum conditions have been worked out. The effect of variation in methanol concentration is important because of its bearing on the method of extraction of the plant tissues. The extraction of tannins from tissues containing proteins and other constituents with which they form insoluble complexes is difficult. The best compromise seems to be exhaustive extraction with aqueous methanol.[4,5] In the present investigation concentrations of methanol between 0 and 86% have been studied using chebulagic acid as a test substance. Differences were found both in λ_{max} of the product and in the time taken to reach the maximum absorbance, but the $E^{1\%}_{1cm}$ at 600 nm was constant between 17 and 70% MeOH. At any given temperature, the lower the concentration of MeOH the more rapid was the formation of the blue product. Between 20 and 35°, the temperature of the reaction has a marked effect on rate, but little effect on the value of ε and measurements have usually been done at 25° since this involves no special apparatus.

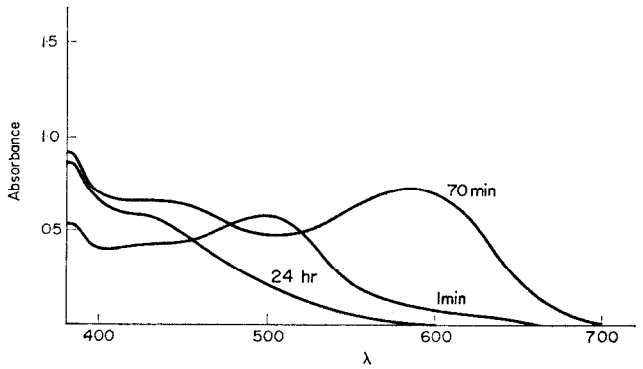
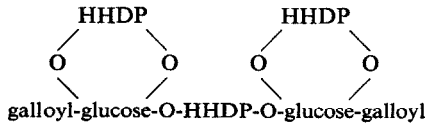
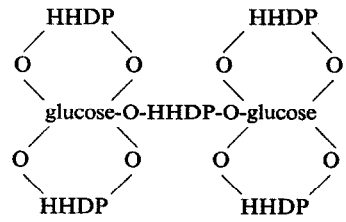


FIG. 1. 2,3-HEXAHYDROXYDIPHENOYLGLUCOSE (HHDPG), 43% aq. MeOH, 25°, ANAEROBIC, $E^{1\%}_{1cm}$ 600 nm = 51·5.

The observed pH of any solution in aqueous methanol depends upon the methanol concentration. The pH of the standard reaction mixture (AcOH 0·4%, $NaNO_2$ 0·4%) in water is 4·0; between 40 and 60% MeOH it is 4·5; and at 86%, 5·6. Adjustment of pH showed, however, that the reaction of HHDP esters with HNO_2 in 80% MeOH followed a similar course at all values of pH between 4·5 and 6·7 but was most rapid at 5·7.



(III)



(IV)

RESULTS

The results for a series of ellagitannins of known constitution are shown in Table 1. These were obtained under closely comparable conditions, but should not be regarded as

[4] W. E. HILLIS and T. SWAIN, *J. Sci. Food Agric.* **2**, 135 (1959).
[5] P. RIBÉREAU-GAYON, *Les Composés phénoliques des Végétaux*, Dunod, Paris (1968).

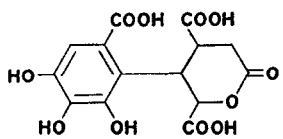
stoichiometrically accurate. The molecular absorption coefficients, ε, of the two monoesters, D1 and D4 are 2148 and 2165, respectively. The value for corilagin, 2300, is close to those of the monoesters, as also is that for D13, 2580 (accepting Seikel and Hillis's suggestion[6] that this might be a monoester containing two glucose residues).

Seikel and Hillis thought that D3 might have the constitution (III) which would give ε = 2800/HHDP group. The result for pedunculagin is interesting. Seikel and Hillis[6] suggest the diester structure shown in Table 1 but this would have a molecular absorption coefficient of 5950, two and a half times that of the monoesters. A possible answer would be a molecule having *five* HHDP residues attached to *two* glucose residues; in other words a structure identical with that proposed for D3, but with HHDP units in place of galloyl groups (IV).

TABLE 1

Fraction[6]	Constitution	Mol. wt	$E^{1\%}_{1\,cm}$ 600 nm	ε
D1	2,3-HHDP-glucose	482	51·5	2148
D4	4,6-HHDP-glucose	482	45	2165
D3			49	
D6			49·5	
D13			37	
Corilagin	3,6-HHDP-1-galloylglucose	634	36·5	2300
Pedunculagin	1,6,2,3-diHHDP-glucose?	784?	76	5950
Chebulagic acid	3,6-HHDP-1-galloyl-2,4-chebuloylglucose*	954	45	4300

* Chebulic acid is:



Chebulagic acid, which, together with chebulinic acid is a major constituent of myrobalans, the fruit of *Terminalia chebula* Retz., gives a much higher value of ε, (4300) than its HHDP content warrants. This is not due to the reaction of the chebuloyl residue with nitrite, because chebulinic acid does not produce any absorption between 500 nm or 600 nm. It seems likely that the high value of ε at 600 nm of chebulagic acid is due to an increase in the rate of formation of the blue product, since the peak at 500 nm is much less marked than in the case of the other esters studied (Fig. 2).

Ellagitannin Content of Natural Extracts

Not enough is known at present about the forms of combination of ellagic acid in plant tissues to be able to formulate any method of determination which would be universally applicable. However, the constancy of the molecular absorption coefficient with the method

[6] M. E. SEIKEL and W. E. HILLIS, *Phytochem.* **9**, 1115 (1970).

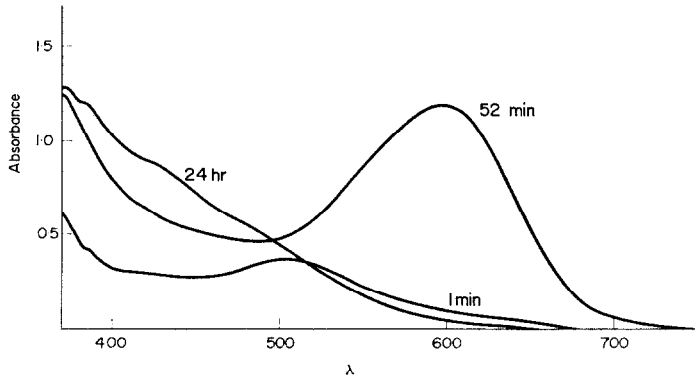


FIG. 2. CHEBULAGIC ACID, 43% aq. MeOH, 25°, ANAEROBIC, A = 45.

described here for the monoesters, provides a starting point. If more complex ellagitannins with higher molecular absorption coefficients, such as pedunculagin and chebulagic acid, are present, suitable adjustments can be made to the analytical values.

It is proposed, therefore, that the ellagitannin content of plant tissues should be expressed in the form of the percentage of hexahydroxydiphenoyl glucose (HHDPG) as determined by the absorbance at 600 nm of the reaction product with nitrous acid in approx. 50% aq. MeOH and 0·4% AcOH in absence of oxygen at 25°, each HHDPG residue having a molecular absorption coefficient in these circumstances of 2250.

EXPERIMENTAL

Solutions containing approximately 0·02% of the esters in 50% aq. MeOH (2·0 ml) and 0·16 ml of 6% HOAc were placed in a 1-cm dia. cuvette, oxygen free N_2 was bubbled for about 15 min. 0·16 ml of 6% aq. $NaNO_2$ was added, N_2 passed for 15 sec, and the cell then sealed. The absorption was measured as shown in Figs. 1 and 2, using suitable blanks.

The reaction tubes were kept at room temp. for a further 24 hr, during which time the blue reaction product changed completely into the orange–yellow product. A final tracing was then made, the absorption at 430 nm being noted.

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Key Word Index—Ellagitannins; analysis; Hoepfner-type reaction; hexahydroxydiphenic acid.