Phytochemistry, 1977, Vol. 16, pp. 1269–1272. Pergamon Press. Printed in England.

SEPARATION OF THEARUBIGINS ON SEPHADEX LH-20

DENIS J. CATTELL* and HARRY E. NURSTEN
Atkin-Thompson Laboratory, Procter Department of Food and Leather Science, The University, Leeds LS2 9JT, U.K.

(*Received* 7 *October* 1976)

Key Word Index—*Thea sinensis*; Theaceae; polyphenol separation; gel chromatography; thearubigins; flavonol glycosides; interaction with inorganic ions.

Abstract—The nature of the peaks eluted from Sephadex LH-20 using aqueous acetone as eluent, from whole tea brews and various ethyl acetate-soluble thearubigin fractions, were examined. Monitoring the columns at 380 nm revealed two extra peaks compared to monitoring at 460 nm, and these were attributed to the presence of two pairs of flavonol glycosides. Sharp peaks which occurred at 2.6 V_0 with 60% aqueous acetone as solvent were attributed to the action of inorganic ions contained in the samples under investigation. These, and other anomalous effects, were suppressed by electrolyte-containing eluents.

INTRODUCTION

The separation of polyphenol polymers by traditional column chromatographic methods has proved to be extremely difficult due to the strong adsorption of these compounds onto the usual support media, e.g. cellulose and silica gel. However, Sephadex G25, a crosslinked dextran designed for gel chromatography, when eluted with organic solvents of high polarity, proved suitable for partial separation of a number of such compounds [1–3]. Although the thearubigins, the polyphenol polymers found in tea, are very soluble in polar organic solvents, it has been shown that they were too highly adsorbed for useful examination by this method [4]. The alkylated crosslinked dextran, Sephadex LH-20, seemed to offer a material upon which the thearubigins should be separable and this was confirmed by their separation using 60% aqueous acetone [5]. Dimethylformamide, on the other hand, reduced adsorption and made molecular sieving the predominant effect, less separation resulting. Thus the balance between adsorption and molecular sieving seems to be critical [6, 7]. Furthermore, dimethylformamide appeared to cause degradation of some tea polyphenols.

In order to achieve the optimum balance between adsorption and molecular sieving for fractionation of the ethyl acetate-soluble thearubigins (see [8] and Fig. 1), a range of solvents was tested of which 60% aqueous acetone was found the best for most purposes and 70% aqueous acetone suitable in some cases.

RESULTS

Each thearubigin sample was chromatographed on columns of Sephadex LH-20 and successive eluted fractions examined by two-dimensional paper chromatography. Components were identified by their colour reactions and chromatographic position [9–11] (Fig. 1).

Whole tea brew

A depectinized whole tea brew was chromatographed on a column of Sephadex LH-20 with 60% aqueous acetone as eluent and the fractionation was monitored at 460 nm (Fig. 2). Two distinct peaks were observed, one at 2.6 V_0 and the other between 5.0 and 6.0 V_0. The former corresponded to the presence of ethyl acetate-insoluble or partially soluble thearubigins and the colourless chlorogenic acids, *p*-coumarylquinic acids, and theogallin, whilst the latter coincided with the presence of the theaflavins plus a few minor components (see Fig. 1). Coloured material was eluted from V_0–2.6 V_0 as well as between the two peaks and produced small shoulders but no other distinct peaks. The 380 nm trace exhibited two additional peaks, at 3.2 and 3.7 V_0, which, according to paper chromatographic evidence, corresponded to the position of two flavonol diglycosides and at least two flavonol monoglycosides, respectively, as well as to ethyl acetate-soluble thearubigins (see Fig. 1), the biflavanols, the flavan-3-ols and their gallates, and gallic acid. Since the flavonol glycosides, alone of these compounds, absorb significantly at 380 nm but negligibly at 460 nm, the additional two peaks in the 380 nm trace are clearly due to their presence.

Ethyl acetate extract

The fractionation of the ethyl acetate-soluble components of a decaffeinated tea infusion by Sephadex LH-20 chromatography with 60% aqueous acetone confirmed that the ethyl acetate-soluble thearubigins are almost entirely eluted between 2.5 and 6.0 V_0, the majority between 4.5 and 6.0 V_0. Two peaks were found at 3.2 and 3.7 V_0 in the 380 nm trace, corresponding to the previously mentioned flavonol glycosides.

Purified thearubigin extracts, T1

The fractionation of T1, an almost pure sample of

* Present address: Department of Humanities, North East London Polytechnic, Romford Road, London E15 4LZ.

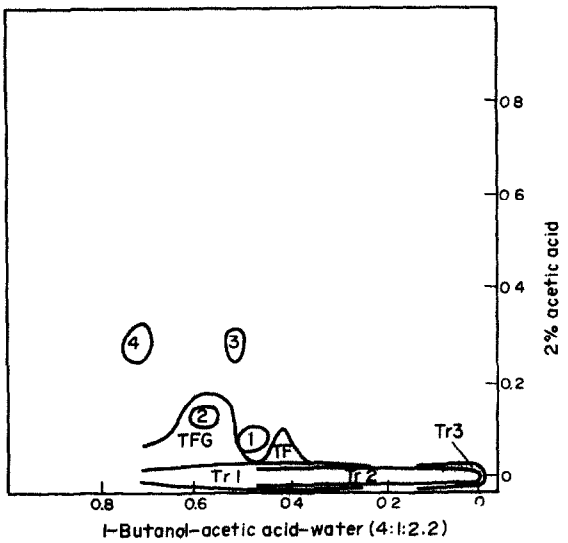

Fig. 1. Two-dimensional paper chromatography of selected tea components. pY = pale yellow; dO = dull orange; bO = bright orange; O-Br = orange-brown; dO-Br = dull orange-brown; Br = brown; a = absorption; dBr = dark brown; Y = yellow; GrY = green-yellow.

Component	Nature	Colour on chromatogram	Colour reactions		
			UV (350 nm)	UV + NH_3 (350 nm)	$AlCl_3$
1	Myricetin-3-glucoside	pY	a	Y	Y
2	Quercetin-3-glucoside	—	a	Y	Y
3	Quercetin-3-rhamnoglucoside	pY	a	Y	Y
4	Kaempferol-3-rhamnoglucoside	—	a	GrY	Y
TF	Theaflavin	dO	dBr	dBr	O
TFG	Theaflavin gallate	bO	dBr	dBr	O
Tr1	Ethyl acetate-soluble thearubigins	O-Br	dBr	dBr	—
Tr2	Partially ethyl acetate-soluble thearubigins	dO-Br	dBr	dBr	—
Tr3	Ethyl acetate-insoluble thearubigins	Br	dBr	dBr	—

ethyl acetate-soluble thearubigins, by Sephadex LH-20 chromatography with 60% aqueous acetone revealed two poorly resolved peaks at about 3.0 and 4.5 V_0, which is in keeping with the previous results. Unexpected, however, was the presence of coloured material eluted between V_0 and 2.5 V_0, which constituted about 20% of the total colour. Two-dimensional paper chromatography could not distinguish between any of the coloured material eluted, all of it producing the Tr1 streak (Fig. 1). Elution with 70% aqueous acetone produced a broadly similar result, though adsorption was reduced, so that all of the T1 thearubigins were eluted within 4.5 V_0.

The penultimate stage in the purification of T1 involved precipitation from cold water. The cold water-soluble fraction (T1-CWS) contained some coloured material, which when chromatographed on Sephadex LH-20 produced an unexpectedly sharp peak at 2.6 V_0 reminiscent of that recorded for the whole tea brew, as well as a broad flat peak between 4.0 and 5.0 V_0, consistent with the anticipated behaviour of ethyl acetate-soluble thearubigins. Again, two-dimensional paper chromatography revealed the same Tr1 streak for all of the coloured material eluted. This is in contrast to the result obtained for the corresponding peak at 2.6 V_0 in the whole brew fractionation, which was predominantly composed of ethyl acetate-insoluble thearubigins identified by the nature of their steaks (Tr2 and Tr3) on paper chromatograms (Fig. 1).

As anticipated for this fraction T1-CWS, the twin flavonol glycoside peaks at 3.2 and 3.7 V_0 were very pronounced.

Addition of potassium chloride to the purified thearubigin extract, T1

Potassium chloride added to T1 (1:2.5 w/w), prior to Sephadex LH-20 chromatography with 60% aqueous acetone resulted in a very sharp peak at 2.6 V_0. A

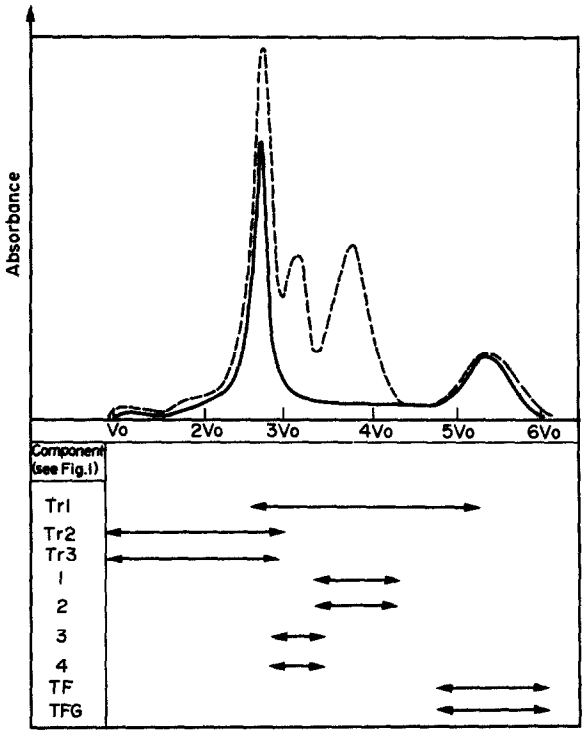

Fig. 2. Sephadex LH-20 chromatography of a depectinized whole tea brew. Eluent: 60% aqueous acetone. Absorbance at 380 nm,-----; absorbance at 460 nm, ———.

similar sharp peak at 3.0 V_0 occurred when 70% aqueous acetone was employed as eluent. On further investigation, it was found that similar peaks were formed when a small amount of pH 3.5 citric acid/phosphate buffer was added to the applied sample or when T1 was pre-heated with 0.1 M HCl and dried before application to the column. Even prolonged standing of solutions of T1 in contact with borosilicate glass created a small, broad peak or shoulder around 2.6 V_0 with 60% aqueous acetone as solvent, as well as a general tendency for more coloured material to be eluted between V_0 and 2.0 V_0.

The fraction containing the 3.0 V_0 peak (70% aqueous acetone as eluent) created by one of the above treatments (addition of buffer) was collected, dried, and rechromatographed on Sephadex LH-20 equilibrated with 60% aqueous acetone as solvent. A very sharp peak occurred at 2.6 V_0, confirming the correlation between the two sharp peaks observed with the different eluents, as well as the anticipated broad peak between 4.0 and 5.0 V_0. The fraction containing the very sharp 2.6 V_0 peak was collected, dried, and rechromatographed again with 70% aqueous acetone, whereupon all the coloured material was eluted as a very sharp peak between 2.0 and 3.0 V_0. Paper chromatography of this material gave the Tr1 streak (Fig. 1) and the absorption spectrum of it was very similar to that of the whole T1 fraction, which in turn resembled that published by Roberts for his S_{ty} thearubigins [12].

Chromatography of thearubigin samples with buffered eluents

Samples of T1 and the cold water-soluble thearubigin fraction (T1-CWS) were individually chromatographed on Sephadex LH-20 with 60% aqueous acetone containing 0.5% NaCl as eluent. The resulting chromatograms showed that the thearubigins in both fractions were eluted between 2.0 and 6.0 V_0 as a single broad peak with a maximum around 4.0 V_0, even though their behaviour with 60% aqueous acetone alone was markedly different. A similar result was obtained using 60% aqueous acetone containing 1 ml/l of concentrated HCl as eluent.

DISCUSSION

Evidence obtained from the paper chromatography of fractions eluted with 60% aqueous acetone in Sephadex LH-20 chromatography of whole tea brews indicated that the two peaks at 3.2 and 3.7 V_0 in the 380 nm trace were produced by the presence of two pairs of flavonol glycosides, tentatively identified as quercetin- and kaempferol-3-rhamnoglucosides, and myricetin- and quercetin-3-glucosides, respectively. Similar evidence from the chromatography of other fractions, e.g., the ethyl acetate extract of tea brews, support this conclusion. These peaks certainly cannot be due to thearubigins, as has been suggested [13], since these compounds would have produced mirror peaks in the 460 nm trace, being orange-brown in colour and so absorbing significantly at this wavelength. Thus monitoring thearubigin separations at 380 nm is clearly unsuitable when flavonol glycosides are also present.

For a long time the overall quality of teas has been correlated with the value of the ratio of thearubigin absorbance at 380 nm to that at 460 nm, Roberts having used this ratio to characterize his different thearubigin fractions [14]. These ratios were measured directly on crude thearubigin fractions after removal of theaflavins. However, reference to Fig. 2 shows that about 35% of the total absorbance at 380 nm is due primarily to flavonol glycosides, which absorb negligibly at 460 nm. Thus, their presence in tea fractions significantly increases the value of the 380/460 nm absorption ratio and many teas said to contain thearubigins of high ratios may well be better described as teas with a high flavonol glycoside content.

The Sephadex LH-20 fractionation of the ethyl acetate-soluble extract of tea brews indicates that the thearubigins it contains are eluted between 2.5 and 6.0 V_0. However, fractions purified further contained coloured material which was eluted earlier, e.g., 20% of all the coloured material of T1 was eluted before 2.5 V_0. Moreover, these more highly purified fractions sometimes showed a tendency to form a peak or shoulder at 2.6 V_0. This effect was particularly pronounced in the case of T1-CWS.

Initially it was felt that these effects were caused by polymerization occurring during the purification processes, since higher MW material would be expected to be eluted earlier as the Sephadex LH-20 was clearly operating, at least in part, as a molecular sieve. However, later it was found (a) that electrolyte-containing eluents suppress both effects and (b) that for columns equilibrated with 70% acetone the equivalent sharp peak occurred

at a later rather than an earlier point of the elution, which suggests that molecular size has not increased. Moreover, the behaviour on paper chromatograms of the coloured material eluted before 2.5 V_0 was similar to that of the thearubigins eluted later. Finally, the very sharpness of the peak at 2.6 V_0 does not suggest a more polymerized form of compounds, which themselves give rise to broad peaks.

It was noticed that Sephadex LH-20 chromatograms run for thearubigin fractions, solutions of which had previously been subjected to prolonged standing in contact with glassware, frequently exhibited the peak at 2.6 V_0 (in 60% aqueous acetone), as well as elution of thearubigins before 2.6 V_0. These fractions were also found to contain higher than usual quantities of non-volatile matter, which suggested that the polyphenolic compounds had picked up inorganic ions, a tendency associated with such compounds. To test whether the presence of inorganic ions could affect Sephadex separations, small additions of potassium chloride were made to samples of T1 prior to elution. The resulting chromatograms indicated that for elution with both 60 and 70% aqueous acetone the sharp peaks (at 2.6 V_0 and 3.0 V_0, respectively) were considerably enhanced, though no tendency towards earlier elution was observed. Indeed, prior treatment with a number of other inorganic compounds and even 0.01 M HCl produced similar effects. The peak occurring at 3.0 V_0 (70% aqueous acetone) after one such treatment was cleanly isolated from a column, but the thearubigins contained in it had paper chromatographic and electronic absorption characteristics similar to those of the whole T1 fraction and so were not thought to represent an individual species of thearubigin.

In order to minimize these apparently anomalous effects created by the action of ionic material, eluents containing electrolytes were investigated, whereupon it was found that not only the sharp peaks (as in T1-CWS), but also the tendency to elute before 2.5 V_0 (as in T1), was suppressed when 60% aqueous acetone containing 0.5% NaCl or 1 ml/l. concentrated HCl was employed. Exploration of the use of such solvents for all routine Sephadex LH-20 separations of tea fractions therefore seems important.

A close similarity exists between the peak found at 2.6 V_0 for the various purified ethyl acetate-soluble thearubigin extracts, e.g., T1-CWS, and that exhibited by the whole tea brew (Fig. 2). Since such tea brews are known to contain sizeable quantities of inorganic ions [15], it may well be that the exceptionally sharp peak at 2.6 V_0 owes something to their presence. Crispin *et al.* [13] have attributed this totally to high MW material, which from the evidence of paper chromatograms is undoubtedly eluted at this point, but the existence of the peak and particularly its excessive sharpness may well be due to the presence of ions.

The positions of the 2.6 V_0 (60% acetone) and the 3.0 V_0 (70% acetone) peaks coincide with those at which compounds which are neither excluded from the gel nor strongly adsorbed onto it are eluted, e.g., theogallin, but a thorough investigation into how these anomalous effects are created by the action of ionic material still needs to be instituted, possibly with model compounds.

EXPERIMENTAL

Sephadex LH-20 chromatography. Preparation of the gel and the packing of the column beds (39–44 cm × 1.64 cm) was carried out according to the manufacturer's instructions [16]. Fresh columns were always first treated with EDTA, followed by a surplus sample of thearubigins, to ensure the removal of all metallic ions. The samples were applied in about 2 ml of the appropriate eluent and flow rates were adjusted to about 25 ml/hr. Elution was monitored at 380 and 460 nm. Individual or pooled fractions were examined by 2-D PC after concn below 30°.

Paper chromatography. 2-D ascending PC (Whatman No. 2) was carried out with BuOH–HOAc–H_2O (4:1:2.2) and 2% HOAc. All papers were examined in UV, with and without NH_3, and either sprayed with 1% EtOH–$AlCl_3$ or dipped in 0.3% $FeCl_3$–0.3% $K_3Fe(CN_6)$ (1:1), followed by washing in 0.1 M HCl and H_2O.

Preparation of the whole tea brew. Tea leaf (40 g) was added to previously boiled-out H_2O (250 ml) at 95°. After 10 min, the infusion was rapidly filtered through a fine polyester cloth into Me_2CO at 0° and brought to 60% Me_2CO. The pptd pectin was centrifuged and the clear supernatant applied directly to the Sephadex LH-20 column.

Acknowledgements—We are gratful for advice to Dr. L. G. Plaskett and his colleagues and for financial support to Brooke Bond Liebig Limited.

REFERENCES

1. Forrest, G. J. and Bendall, D. S. (1969) *Biochem. J.* **113**, 757.
2. Somers, T. C. (1966) *Nature* **209**, 368.
3. Nilsson, A. (1967) *Acta. Chem. Scand.* **16**, 31.
4. Millin, D. J. and Swaine, D. personal communication.
5. Millin, D. J., Swaine, D. and Dix, P. L. (1969) *J. Sci. Fd Agric.* **20**, 296.
6. Johnston, K. M., Stern, D. J. and Waiss, A. C., Jr, (1968) *J. Chromatog.* **33**, 539.
7. Lewark, S. (1968) *Phytochemistry* **7**, 665.
8. Cattell, D. J. and Nursten, H. E. (1976) *Phytochemistry* **15**, 1967.
9. Roberts, E. A. H., Cartwright, R. A. and Oldschool, M. (1957) *J. Sci. Fd Agric.* **8**, 72.
10. Vuataz, L. and Brandenberger, H. (1961) *J. Chromatog.* **5**, 17.
11. Harborne, J. B. (1967) *Comparative Biochemistry of the Flavonoids.* Academic Press, London.
12. Roberts, E. A. H. (1962) in *The Chemistry of Flavonoid Compounds*, (Geissman, T. A., ed), p. 468. Pergamon Press, Oxford.
13. Crispin, D. J., Payne, R. H. and Swaine, D. (1968) *J. Chromatog.* **37**, 118.
14. Roberts, E. A. H. (1960) *Ann. Rep. Tocklai Expt. Sta.* 341.
15. Tsutsumi, C. (1967) *Nippon Shokuhin Kogyo, Gakkeishi* **14**, 308.
16. *Sephadex-Gel Filtration in Theory and Practice*, (Dec. 1966) Pharmacia Fine Chemicals, Uppsala, Sweden.