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Unexpected hyperchromic interactions during the chromatography of theafulvins and simple flavonoids

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Abstract

Theafulvins, isolated from black tea, chromatograph as a hump on reversed phase column packings. When whole black-tea brews are similarly analysed other phenolic compounds appear as more or less resolved peaks 'floating' on this hump. Model solutions of isolated theafulvins containing individual pure compounds (flavanols, flavonols and caffeine) have been prepared and their chromatographic behaviour compared with that of the pure compound chromatographed alone. In the case of quercetin, epicatechin and epicatechin gallate, a statistically significant hyperchromic effect has been observed in which the peak area 'floating' on top of the theafulvin hump is larger than expected from the area obtained for the pure compound alone. There was little or no effect with catechin and caffeine, and rutin showed a weak and inconsistent response. The data are discussed by reference to the established literature on copigmentation but it is not possible to define the mechanism responsible for the hyperchromism at this stage.

Keywords: Black tea; Theafulvins; Thearubigins; Flavanols; Quercetin; Copigmentation; Hyperchromic

1. Introduction

The dark pigments of black tea, known for thousands of years, have been studied since at least 1935 when Shaw (Shaw & Jones, 1935) described these non-nitrogenous acidic phenols as 'oxytheotannin'. In the late 1950s Roberts named the orange-red and brownish pigments theaflavins (TF) and thearubigins (TR), respectively (Roberts, Cartwright & Oldschool, 1957; Roberts, 1958a; Roberts, 1958b). The structure and origin of the TF is now known (Bryce et al., 1970; Davis, Cai & Davies, 1995; Takino & Imagawa, 1963) but that of TR is uncertain. TR chromatographed on paper (Roberts et al., 1957) either fail to move (2% aqueous acetic acid) or streak (n-butanol: acetic acid: water 4: 1: 2.2) and elute as a Gaussian hump from reverse phase packings upon which more or less well resolved peaks, corresponding to gallic acid, theogallin, theacitrins, flavanols, flavonol glycosides and theaflavins, can be seen 'floating' in most samples, if appropriate detection wavelengths are selected (Opie, 1992; Powell, 1995; Shao, Clifford & Powell, 1995a; Shao, Clifford & Powell, 1995b). It had been our custom to quantify these peaks by calculating the peak area above the hump rather than the area to the horizontal baseline which would incorporate the area of the underlying hump, but the validity of such an approach had never been tested in our laboratory, or elsewhere to our knowledge.

With the development (Bailey, Nursten & McDowell, 1992) and refinement of methods for the isolation of theafulvins (TFu) which chromatograph on reverse-phase packings as a hump free from associated peaks (and hence free from the chemically defined substances listed above) the opportunity became available to test the validity of this approach to quantification. Accordingly two series of solutions of quercetin were prepared—the first dissolved in aqueous methanol, the second in a solution of TFu dissolved in the same concentration of aqueous methanol. These were chromatographed using our normal conditions and the peak areas determined to the horizontal baseline and, in the case of the mixture, also to the top of the theafulvin hump. To our surprise, the results for the mixture gave unexpectedly large areas compared with the simple solutions **even when measured only to the top of the hump**. In this paper we report our systematic investigation of this phenomenon.

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2. Materials and methods

2.1. Materials

Theafulvin was prepared from Lattakari Assam black tea (Importers Ltd, Guildford, UK) as previously described (Powell et al., 1992; Powell, Clifford, Opie & Gibson, 1995a). (−)-Epicatechin gallate and (−)-epigallocatechin gallate (EGCG) were kindly supplied by Unilever, Sharnbrook, UK. All other reagents were standard commercial items from reputable sources.

2.2. Methods

The isolated TFu was freeze dried and stored at −20°C in the dark until reconstituted at 1 mg/ml in water as required and used immediately. The other phenols were dissolved in water (95% methanol for quercetin) at 1 mg/ml and diluted as required (3–25 μg/ml), using water as diluent in all cases. Equal volumes of test phenol and TFu were mixed by vigorous vortexing, transferred to small vials and placed in the autosampler. Test phenols, similarly diluted 1:1, solvent blanks and TFu (diluted 1:1) were interspersed with the mixtures and analysed under identical conditions. Replicate preparations and replicate injections were made as indicated in the results section. Peak areas were integrated using commercial software programmed to read either to the horizontal baseline or the 'top of the hump' as required (see Fig. 1).

The analytical HPLC was performed essentially as described by Powell et al. (1992) except that the conditions were as follows:

Injection volume: 100 μl
Mobile phase:
Solvent A: 5 ml/l aqueous acetic acid
Solvent B: 5 ml acetic acid and 350 ml acetonitrile made up to 1 l with distilled water

Gradient:	100% solvent A to 100% solvent B over a non-linear gradient of 35 min followed by 100% solvent B for 5 min. The equation for the gradient is: $B\% = 0.36 + 17.34$ t where t = time (min)
Flow rate:	1 ml min^{-1}
Detection:	280 or 365 nm as appropriate
Column:	250×4.6 mm Hypersil ODS 3 μm (Hichrom, Theale, UK)

3. Results and discussion

The investigation was performed in two stages. Initially, the test phenols were examined at a limited number of concentrations, in the range 3–25 μg/ml, but varying slightly depending on the phenol, and without replication to conserve the supply of TFu. This first phase of the study allowed the optimum conditions to be identified and in the second phase, replicates were also run (never less than $N = 3$ and for quercetin $N = 8$) but it was necessary to use a different preparation of TFu. Although this second sample had been prepared from the same batch of black tea leaf using our standardised methodology, the results obtained in phase 2 of the study were similar but not identical to those obtained in phase 1, suggesting that there were some chemical differences between the two TFu preparations.

The peak area data collected were converted to calibration curves and a least squares regression equation calculated to pass through the origin, as illustrated for quercetin in Fig. 2. These regressions were all highly linear with small and random values for the residuals, which implied that the Beer–Lambert relationship was obeyed. Accordingly, for each test phenol, the slope of each individual data point was also calculated in order

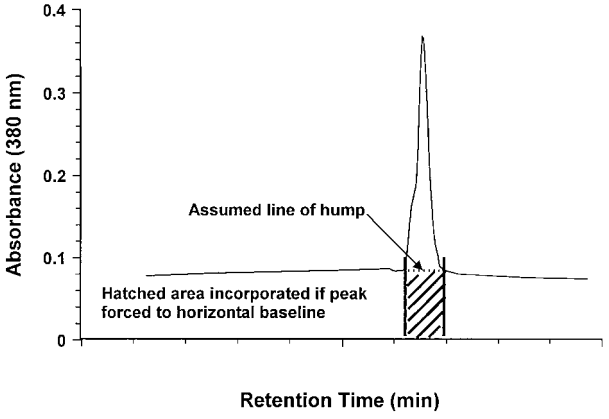

Fig. 1. Diagrammatic representation of the two ways in which the 'floating' peak areas could have been calculated, i.e. by forcing the peak to the baseline, or by integrating to the assumed line of the hump.

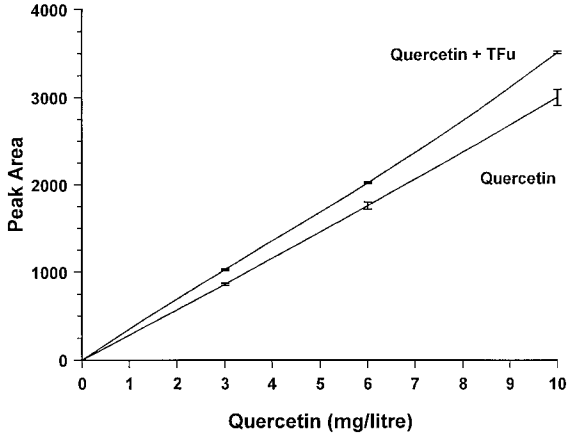

Fig. 2. Calibration curves for quercetin alone and quercetin in theafulvin (TFu). Error bars are SEM (quercetin alone, $N = 8$; quercetin + TFu, $N = 6$).

Table 1
The effect of theafulvins on the relative absorptivity of caffeine and flavanols

Test phenol	Phase of study	Concentration range (μg ml^{-1})	Replicates × concentrations ($N \times C$)	Gradient 1 (with TFu)			Gradient 2 (without TFu)			$\frac{\text{Gradient 1}}{\text{Gradient 2}} \times 100$	p
				Mean	Standard Error	R^2	Mean	Standard error	R^2		
(−)-Epicatechin	1	3–25	1×6	85298	5021	0.9955	69903	2722	0.9928	122	0.02
	2	3–12	3×3	94152	4824	0.9985	85411	1101	0.9995	110	< 0.001
(−)-Epicatechin gallate	1	5–25	1×6	138709	2205	0.9986	102123	1466	0.9917	136	< 0.001
	2	5–25	3×3	120129	3917	0.9978	80643	3116	0.9898	149	< 0.001
(+)-Catechin	1	10	3×1	90051			89789	228		103	NS
Caffeine	1	10	3×1	314362			310956	466		101	NS
Rutin	1	5–25	1×5	141306	12844	0.9960	134352	7252	0.9904	105	0.15
	2	5–25	3×3	137050	2113	0.9997	141973	1906	0.9983	96	0.2
Quercetin	2	3–10	8×3				294219	6782	0.9940	117	< 0.001
	2	3–10	6×3	343017	7195	0.9942					

to derive a standard error and facilitate a statistical comparison (t test) of the values obtained with and without TFu.

The results are summarised in Table 1. In phase 1 of the study it was not possible to detect any hyperchromic effect for (+)-catechin or caffeine. Weak hyperchromic effects were detected with (−)-epicatechin, (−)-epicatechin gallate and quercetin, but these preliminary data for quercetin were poor, probably due to precipitation of insoluble complexes before injection or on the column, and are not shown. A very weak hyperchromic effect was indicated for rutin (quercetin-3-rutinoside). The effect in every case was associated with the peaks rather than the underlying hump. Unexpectedly, EGCG proved unstable under the conditions used and useful data could not be obtained.

In phase 2 of the study, the hyperchromic effect was clearly demonstrated for quercetin, (−)-epicatechin and (−)-epicatechin gallate, albeit at magnitudes somewhat different from those seen in phase 1. However, it was not possible to demonstrate any hyperchromic effect with rutin suggesting that there were some chemical differences between the two TFu preparations despite both samples having been prepared from the same batch of black tea leaf using our standardised methodology.

It is known that TFu is heterogeneous, with apparent masses in the range 1000–2500 daltons, but free from flavanols, flavonol glycosides and theaflavins (Clifford et al., 1996; Clifford & Powell, 1996; Powell 1995; Powell et al., 1992; Powell, Clifford, Opie & Gibson, 1995b). Apart from the theacitrins (Davis et al., 1997), no other components have so far been characterised and it is quite possible that the hyperchromic effect observed in this study involves an interaction with only certain components of the TFu, and that these may vary in concentration from one preparation to another.

It should be noted that, while this interaction does not affect the retention time of the test phenols, these compounds do differ in their retention times and thus are superimposed on different points on the hump. Thus variations in the behaviour of the phenols tested might be a reflection of variations in the nature of the hump underlying particular peaks as well as of variations in the intrinsic properties of the phenols themselves.

The mechanism leading to this hyperchromic effect is not known, but we have wondered if it might be a manifestation of copigmentation, of which the general effects are to change the pigment absorption maximum both in position (bathochromism) and intensity (hyperchromism). According to Davies and Mazza (1993) the magnitude of the effect is a function of the structure of both pigment and copigment.

Possibly anthocyanins provide the best known examples of copigmentation and there have been reports of the presence in black tea (Bailey, Nursten & McDowell, 1991; Coggon, Moss, Graham & Sanderson, 1973; Roberts & Williams, 1958) of anthocyanin-like compounds and/or desoxyanthocyanidins. Copigmentation involves interaction of the flavylium cation or quinoidal base of the anthocyanin with the planar electronically unsaturated part of the copigment. This displaces several water molecules from the pigment's solvation shells and protects the flavylium chromophore from the nucleophilic attack of water, so-called covalent hydration, which leads to the colourless anthocyanin forms (hemiacetal and chalcone) (Brouillard & Dangles, 1994). Copigmentation is maximal in fully aqueous solution, generally in the range pH 3–5. The association is spontaneous and exothermic, and the copigmentation decreases markedly with temperature in the range 10–70°C. For strong copigments, e.g. rutin, a significant effect is seen at close to a 1:1 molar ratio. For copigments such as flavanols and caffeine a 10–100-fold greater concentration of copigment is required (Baranac, Petranovic & Dimitric-Markovic, 1996). It has been shown that a third species can also interact, either enhancing or diminishing the copigmentation (Dangles & Brouillard, 1992).

It is clear, from the foregoing, that copigmentation is a complex phenomenon and, as described above, does not exactly match the observations made in this study. In particular, it was not possible to detect a bathochromic effect, even when using a rapid scanning detector suggesting that this effect, is small (less than 5 nm) or absent (although the conditions used—pH 2.8 in the presence of acetonitrile—were not ideal). Also, in contrast to model systems containing anthocyanins, the hyperchromic effect observed in this study was restricted to the colourless copigment. Effects of copigmentation on the UV spectrum have received little attention, but inspection of published spectra shows that hypechromic effects may be observed below 400 nm for mixtures of malvin with rutin (Baranac et al., 1996) or malvin with chlorogenic acid (Brouillard, Figuieredo, Elhabri & Dangles, 1997). The lack of response in this study with rutin and the relatively strong response from epicatechin further suggests that the phenomenon observed differs from classic copigmentation and might not involve anthocyanin-like structures at all.

While this phenomenon is of considerable academic interest, its practical significance is less clear. It is unlikely that the colour of black tea beverage is much influenced since the effect is restricted to the UV region of the spectrum. It follows that a chromatographic procedure that allows the chemically characterised tea constituents to be resolved from the polyphenol hump is likely to produce more accurate and possibly less variable quantitative data for their contents. The same approach may be advantageous also for the analysis of other polyphenol-rich commodities in which similar chromatographic humps are found.

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