Note

High-performance liquid chromatographic assay for theaflavins

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(Received October 7th, 1980)

Tea has a uniquely high polyphenols content comprising about 30% of the dry weight of tea leaf[1]. Theaflavins, representing about 4% of the total polyphenolic content, are the most studied group of compounds[1]. Many workers have claimed that theaflavins are important for the quality attributes of tea[2].

Theaflavins have been determined previously using solvent extraction and optical density measurement[3], chromatography on Sephadex LH-20[4] and extraction, derivatization and subsequent assay by gas chromatography (GC)[5]. All of these procedures have inherent disadvantages.

However, in recent years high-performance liquid chromatographic (HPLC) methods have been used for the separation of phenolic acids in wine[6], and of flavanols, procyanidins and prodelphinidins in beer[7]. A preliminary investigation by Hoefler and Coggon[8] indicated that some tea polyphenols, including theaflavins, could also be separated by HPLC.

The present work is a systematic examination of the factors involved in obtaining an automatic, reproducible and quantitative determination of theaflavins in tea extracts by reversed-phase HPLC and lays down guidelines which could be used for the development of any reversed-phase HPLC separation.

MATERIALS AND METHODS

HPLC system

The HPLC system used was a Waters Assoc. (Milford, MA, U.S.A.) ALC/GPC-204 with two 6000A pumps, 660 solvent programmer, a W.I.S.P. 710A automatic sample injector, and a 440 absorbance unit. Eluent was also monitored with a Perkin-Elmer LC 75 spectrophotometer which has a linear response to 3.0 O.D. units. Peak areas were determined with a Hewlett-Packard 3388A computing integrator. Columns used were: Partisil 5 C_{22} (35,000 plates/m), 10 × 0.46 cm I.D. (HPLC Technology, Wilmslow, Great Britain); Partisil 5 ODS-2 (44,000 plates/m) and Partisil 10 ODS-1 (15,000 plates/m), 25 × 0.4 cm I.D., (Whatman, Maidstone, Great Britain) and Spherisorb 5 ODS (45,000 plates/m) 25 × 0.4 cm I.D. (Hichrom, Woodley, Great Britain). Sep-paks (C_{18}) were obtained from Waters Assoc.

Solvent systems

All solvents were obtained from Rathburn (Walkerburn, Great Britain) and

were filtered and degassed under vacuum prior to use. The solvent system used was based on the acetone–water mixture recommended by Hoefler and Coggon[8], though other solvents were also tried as described below. The pH was adjusted using acetic acid[8] or phosphoric acid and phosphate buffers.

Sample preparation

Tea infusions were prepared by the addition of boiling deionised water (100 ml) to black tea leaf (2.0 g). The infusions were stirred for five minutes, in a covered 150-ml beaker, with a magnetic stirrer. An aliquot was immediately taken from the liquor and filtered through 0.45 μm type HA Millipore filters prior to injection. For automatic sample injection a stable sample which would not form tea cream[9] had to be prepared. Tea liquor (10.0 ml) was passed through a methanol–water conditioned Sep-pak C_{18}, followed by water (5.0 ml) to wash out the syringe. Polar material was eluted with 35% (v/v) methanol in water (5.0 ml), followed by the theaflavin fraction using 80% (v/v) methanol in water (2.0 ml). Recovery of theaflavins was 98–100%. The theaflavin fraction was diluted to a known volume with the same solvent prior to injection.

To aid peak identification, an enriched extract of theaflavins was prepared using the isolation procedure of Lea and Crispin[4]. Individual components were then purified by semi-preparative HPLC on Partisil 10 ODS-1 using the solvent system of Fig. 4.

RESULTS AND DISCUSSION

The factors which could be important in controlling the separation and resolution of theaflavins by HPLC were examined in turn, using the method of Hoefler and Coggon[8] as the starting point (Fig. 1).

Sample preparation

The sample preparation procedure which was developed had a number of advantages as follows:

(a) After Sep-pak treatment no loss of theaflavins from the sample was found after 48 h at room temperature.

(b) Automatic sampling was feasible as cream formation did not occur in 80% methanol.

(c) A five-fold increase in concentration was possible. (It was possible to increase the concentration of the sample still further by passing more than 10 ml of tea liquor through the Sep-pak). This concentration increase permitted a reduction of sample injection volume, resulting in greater resolution.

(d) The reduction in amount of polar material rendered baseline separation of the theaflavins easier.

(e) Retention of material such as green pigments on the Sep-pak prolonged column life.

Column support material

Increasing the number of theoretical plates should give a better separation, but the separation of the theaflavins was not as good on Partisil 5 ODS or Spherisorb
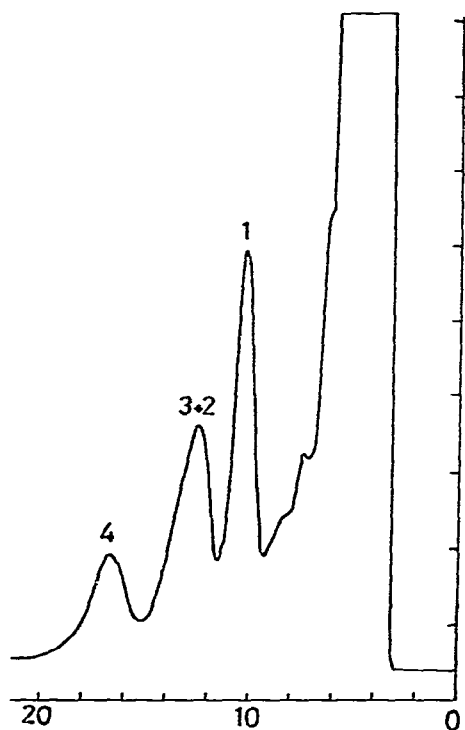
Fig. 1. Initial separation of theaflavins by method of Hoefler and Coggon[8]. Column: Partisil 10 ODS-1 (25 × 0.4 cm I.D.); temperature: 26°C; flow-rate: 2 ml/min; sample: 200 μl of 2% tea infusion; detection: 365 nm, 0.2 a u.f.s. Solvent: water–acetone–acetic acid (69:30:1). Peaks: 1 = theaflavin (tf); 2, 3 = unknown benzotropolone based compound and theaflavin monogallate (tmg); 4 = theaflavin bisgallate (tbg).

5 ODS as on Partisil 10 ODS-1, although peak shape was improved. Apparently, the presence of a proportion of free siloxane groups, due to the low carbon loading of the Partisil 10 ODS-1, had affected the separation of the theaflavins. The free siloxane sites on Partisil 5 after ODS coupling have been "capped" while the high carbon loading and regular particle shape of Spherisorb 5 result in few free siloxane sites.

An uncapped Partisil 5 C_{22} column was found to give an improved separation compared with Partisil 10 ODS-1. This could be due to both the reduction in particle size and the increase in chain length, as shown by the work of Little et al.[10]. Further details are given in a later section.

Solvent system

The resolving power of reversed-phase columns can be altered by changing the nature of both the organic and aqueous components of the solvent. They were examined in turn.

Organic modifiers. By the use of solvent gradient programming some improvement to the separation was achieved. The polar material was better separated

from the theaflavins and the peak shapes of the theaflavins were slightly improved. Methanol, tetrahydrofuran and dimethylformamide all lacked the selectivity of acetone. Although theaflavin gallate was not separated with a methanol based solvent system, peak shape was improved. The introduction of a ternary solvent system (acetone–water[8] containing up to 2% methanol) resulted in greatly improved peak shapes but had little deleterious effect on the separation of theaflavin gallates.

Inorganic modifiers. The separation of polyphenols is pH sensitive[7]. The pH range 2 to 6 was examined and the best separation was obtained at pH 2 (Fig. 2). As the figure shows, at pH 2 better resolution of the theaflavin monogallate from the unknown and of the theaflavin monogallate from theaflavin bisgallate was achieved compared with that at pH 4 (the approximate pH of the solvent system using acetic acid). The separation was not improved by increasing the salt concentration at constant pH.
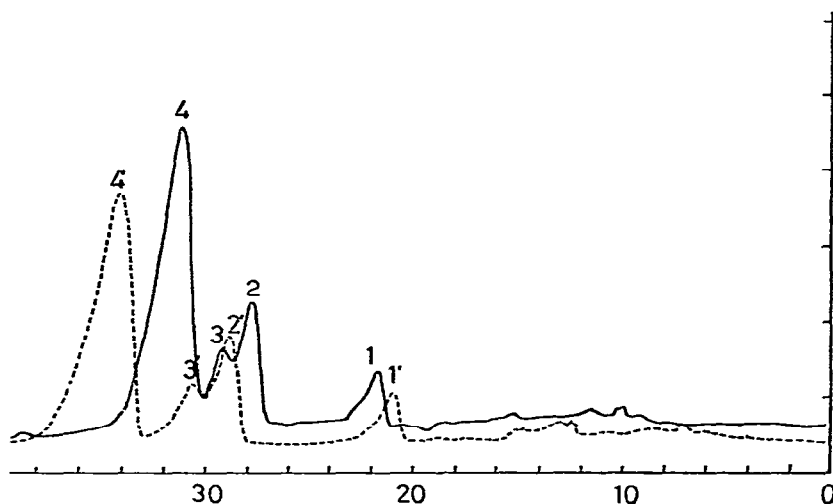

Fig. 2. Effect of pH on the resolution of theaflavins. Column: Partisil 10 ODS-1 (25 × 0.4 cm I.D.); temperature: 26°C; flow-rate: 1 ml/min; sample: 50 μl of enriched theaflavins extract; detection: 365 nm, 0.5 a.u.f.s. Solvent: a linear gradient (25 min) from 20% acetone in water to 35% acetone in water containing orthophosphoric acid. – – –, pH 2, ———, pH 4. Peaks: 1, 1′ = tf; 2, 2′ = unknown; 3, 3′ = tmg; 4, 4′ = tbg.

Temperature

The optimum temperature for maximum resolution and peak area using the acetone–water system with acetic acid was found to be 26°C. When phosphoric acid was substituted for acetic acid it was possible to increase the column temperature without causing degradation of the sample. No reduction of peak area was then recorded up to a column temperature of 80°C, which was the maximum tried.

Increasing the temperature increased the mass transfer rate, with an improved resolution due to a reduction of peak width and peak trailing. Fig. 3 illustrates this effect using identical solvent conditions. The increase in temperature also increased
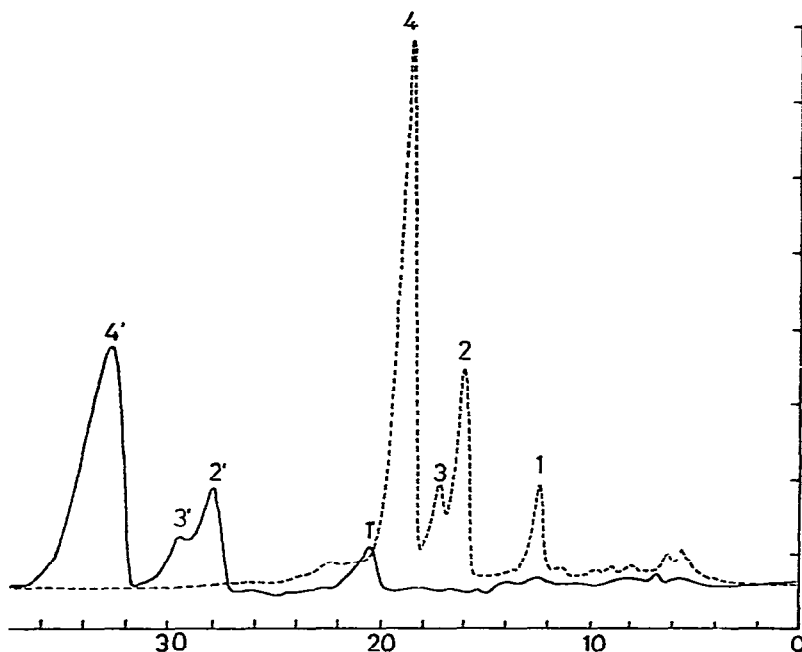
Fig. 3. Effect of temperature at constant solvent strength on the resolution of theaflavins. Column: Partisil 10 ODS-1 (25 × 0.4 cm I.D.); temperature: ——, 26°C; – – –, 80°C; flow-rate: 1 ml/min; sample: 50 µl of enriched theaflavins extract; detection: 365 nm, 0.5 a.u.f.s. Solvent: a linear gradient (25 min) from 20% acetone in water to 35% acetone in water at pH 2. Peaks: 1, 1′ = tf; 2, 2′ = unknown; 3, 3′ = tmg; 4, 4′ = tbg.

the rate of elution of components. The solvent strength was therefore reduced to improve separation (Fig. 4). The result of this was that the component which previously eluted with theaflavin bisgallate[8] was now totally resolved and good resolution was achieved between the monogallate and the unknown (peak 2).

Thus temperature is not only essential for reproducible reversed-phase chromatography but is also one of the critical factors in determining the best conditions for high resolution.

FINAL CHROMATOGRAPHIC SYSTEM

The results of the above studies on sample preparation, solvent systems and temperature using a Partisil 10 ODS-1 column, were applied to a Partisil 5 C_{22} column. After adjustment of the proportion of acetone in the solvents, the improved separation shown in Fig. 5 was achieved.

Using this chromatographic system excellent reproducibility of peak areas was obtained for all the theaflavins (maximum range ±1.5% for 10 successive injections), and retention times have been within ±1% for many hundreds of injections.
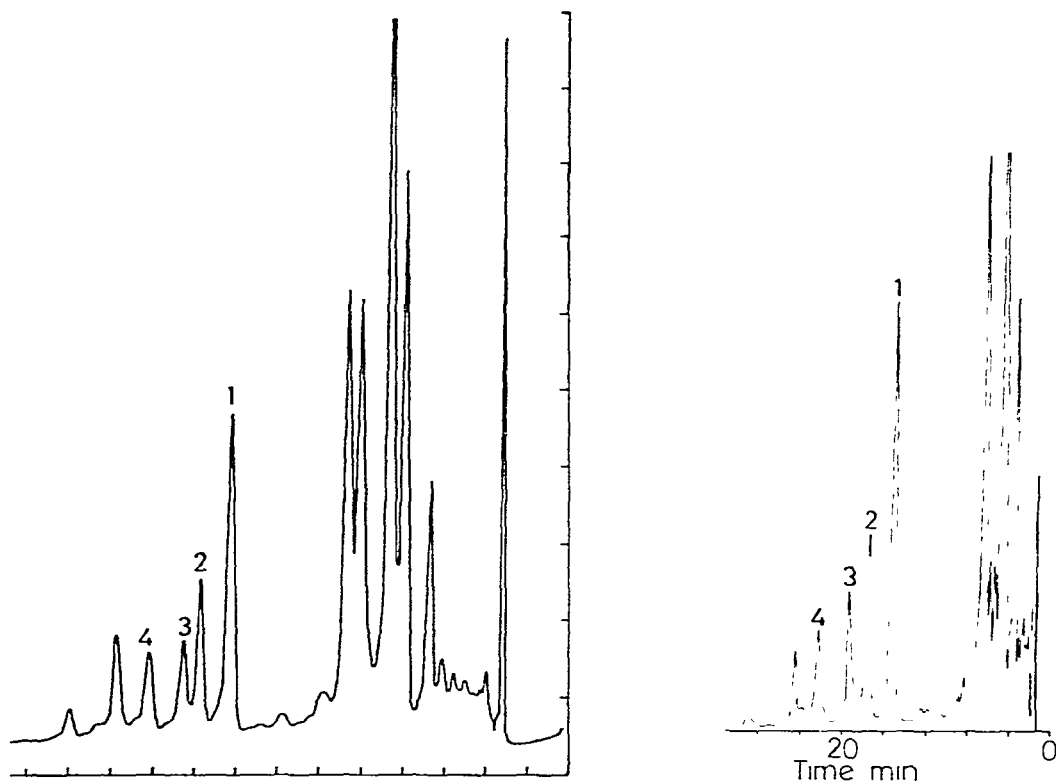
Fig. 4. Separation of theaflavins in a tea infusion using the chromatographic conditions developed for Partisil 10 ODS 1 columns. Column: Partisil 10 ODS-1 (25 × 0 4 cm I.D.); temperature· 80°C; flow-rate: 1 ml/min; sample: 10 μl of a 2% tea infusion after Sep-pak treatment (5-fold concentration); detection: 365 nm, 0.2 a.u.f s. Solvent· a linear gradient (20 min) from acetone–water–methanol (15.83 2), pH 2, to acetone–water–methanol (25:73·2), pH 2. Peaks. 1 = tf; 2 = unknown, 3 = tmg; 4 = tbg.

Fig. 5. Separation of theaflavins in a tea infusion using the chromatographic conditions developed for Partisil 5 C₂₂ columns. Column· Partisil 5 C₂₂ (10 ∕ 0 46 cm I D.), temperature: 80°C; flow-rate: 1 ml/min; sample: 10 μl of a 2% tea infusion after Sep-pak treatment (5 fold concentration), detection: 372 nm, 0.32 a.u.f.s. Solvent: a linear gradient (20 min) from acetone–water–methanol (69:323:8), pH 2, to acetone–water–methanol (109.283:8), pH 2. Peaks· 1 = tf; 2 = unknown; 3 = tmg; 4 = tbg.

CONCLUSIONS

An improved method of separation of theaflavins has resulted from the study of the following parameters: sample preparation; column temperature; the use of a ternary solvent system and solvent gradient programming. The system developed is amenable to automation for the routine determination of the theaflavin contents of tea.

ACKNOWLEDGEMENTS

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