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Note

Reversed-phase high-performance liquid chromatography of tea constituents

ANDREW C. HOEFLER and PHILIP COGGON
Thomas J. Lipton, Inc., Englewood Cliffs, N.J. 07632 (U.S.A.)
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Historically, a major proportion of the analysis of non-volatile tea constituents has been achieved using paper chromatography[1] and low-pressure column chromatography[2]. More recently, gas chromatographic procedures were introduced[3] to improve the quantitative nature of the analysis. The advent of high-pressure liquid chromatography (HPLC), especially when coupled with microparticulate reversed-phase packings, has given us reason to review the methods used in our laboratory for the analysis of tea constituents. This change from gas phase to liquid phase chromatography has given us the opportunity to avoid the previously required sample work up procedures of solvent extraction and derivative formation, while retaining the quantitative aspects inherent in the gas chromatographic analysis.

All of the analyses described below utilized reversed-phase column packing material and resulted in the elimination of sample work up procedures, in that direct injection of an aqueous tea solution gave the desired separation and quantification.

EXPERIMENTAL

Procedure

A commercially available high-pressure liquid chromatograph, Model ALC/GPC-244/6000A/U6K, was used as supplied by Waters Assoc. (Milford, Mass., U.S.A.). A flow-rate of 0–9.9 ml/min is delivered at pressures up to 6000 p.s.i. by a pair of positive displacement reciprocating pistons. Their UV detector, Model 440, was used with 254, 280, and 365 nm filters. A 30 cm × 4 mm I.D. stainless-steel column packed with μBondapak C_{18} reversed-phase packing (10 μm) was used, also as supplied by Waters Assoc. All solvent systems were filtered prior to use. Aqueous solvents and aqueous tea samples were filtered through Millipore Type HA 0.45-μm cellulose filters, and organic solvent systems were filtered using Millipore Type FH 0.50-μm PTFE filters.

The mobile phase compositions used were: (A) acetic acid–acetone–water (1:60:139); (B) 0.02 *M*, pH 4.5, citrate–phosphate buffer; (C) methanol–0.1 *M*, pH 7.0, citrate–phosphate buffer (20:80); (D) acetic acid–methanol–dimethylformamide–water (1:2:40:157).

Materials

All solvents are commercially available spectrograde reagents. Analytical-

grade samples were obtained as reference materials for caffeine, theobromine, theophyline, and gallic acid. The flavanols were obtained from green tea[4] and are abbreviated as follows: EGCG = (−)epigallocatechin gallate, EGC = (−)epigallocatechin, ECG = (−)epicatechin gallate, EC = (−)epicatechin, and +C = (+)-catechin. The theaflavins[5] were provided by Dr. P. D. Collier, Unilever Research Lab. (Colworth House, Sharnbrook, Great Britain) and are abbreviated as follows: TF1 = theaflavin, TF2A = theaflavin-3-gallate, TF2B = theaflavin-3′-gallate, TF3 = theaflavin-3,3′-digallate, and TF4 = (−)epitheaflavic acid. ENZECO tannase was supplied by Enzyme Development Corp. (New York, N.Y., U.S.A.).

RESULTS AND DISCUSSION

HPLC coupled with the recently developed reversed-phase packing materials has opened up an important new approach to the analysis of beverages, and in this discussion, specifically tea. The direct injection of a small sample of the tea beverage, essentially a dilute aqueous solution, has virtually eliminated the need for sample work up procedures. Direct injection, after filtering using a 0.45-μm cellulose filter, of a black tea infusion, for example, allows the estimation of theaflavins (Fig. 1) using mobile phase A, gallic acid (Fig. 2) using mobile phase B, and caffeine (Fig. 3)

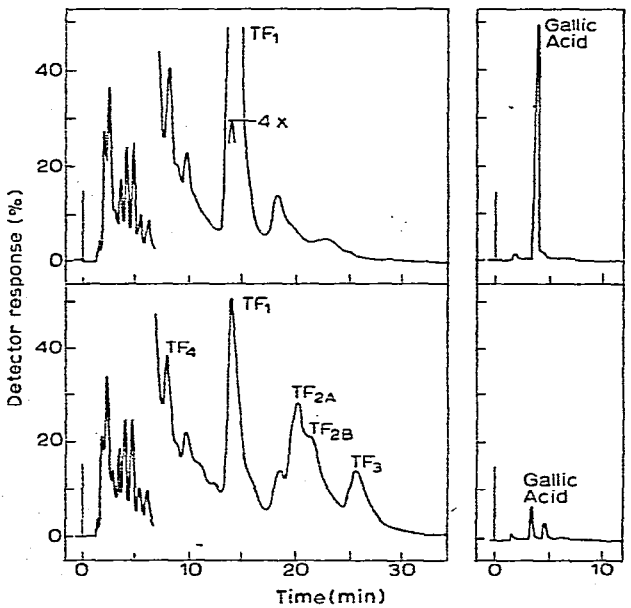

Fig. 1. HPLC analysis for theaflavins in a black tea infusion, before (lower trace) and after (upper trace) tannase treatment. Mobile phase A; 200-μl injection; 365-nm detector; 1.0 a.u.f.s. for first 7 min and 0.05 a.u.f.s. for remainder; flow-rate, 2.0 ml/min; temperature, 23°; pressure, 2,500–3,500 p.s.i.

Fig. 2. HPLC analysis for gallic acid using the same samples as in Fig. 1. Mobile phase B; 20-μl injection; 254-nm detector; 1.0 a.u.f.s.; flow-rate, 2.0 ml/min; temperature, 23°; pressure, 2,500–3,500 p.s.i.

using mobile phase C. These three analyses eliminate the time required for an ethyl acetate extraction and trimethylsilyl derivative formation needed for gas chromatographic analysis of the theaflavins[3] of black tea, a diethyl ether extraction and derivatization required for gallic acid[6], and a chloroform extraction for caffeine[7]. The retention time for each component analyzed was checked using authentic pure compounds.

In the analysis of the theaflavins, the identity of the gallated compounds was checked further by tannase treatment of the black tea infusion, when as expected[8], the peaks associated with the three gallated theaflavins, TF2A, TF2B, and TF3, were lost (Fig. 1) with a concomitant increase in the height of the non-gallated theaflavin peak, TF1. An unknown compound can be seen to be co-chromatographing with TF2B but this can be subtracted after a tannase treatment whenever a quantitative analysis is required. The tannase treatment of a tea extract also results in an increase in the gallic acid content which can be readily measured using mobile phase B (Fig. 2). Not all of the gallic acid liberated by the tannase action is attributable to the gallated theaflavins, however, as there are other gallated polyphenolic compounds (thearubigins) present in a black tea infusion.

A decaffeinated tea sample was prepared by solvent extraction and was used to show that there were no compounds interfering in the analysis for caffeine. Two components of tea which cause problems with the spectrophotometric estimation of caffeine, are theophyline and theobromine. In the new HPLC analysis (Fig. 3) they are well resolved from caffeine and may be quantified separately. A similar separation of these three components has been reported by Wildanger[9], who uses silica gel with dichloromethane–ethanol–water as the mobile phase.

Another analysis which has been simplified is the estimation of flavanols in fresh green tea. The gas chromatographic analysis of their trimethylsilyl derivatives[3]

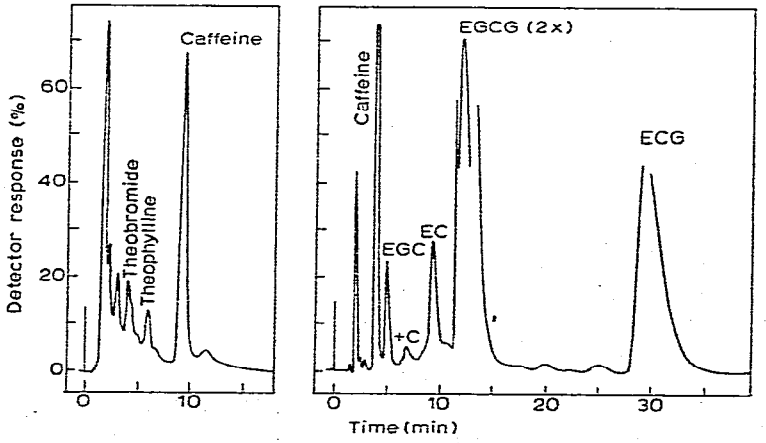

Fig. 3. HPLC analysis for caffeine in a black tea infusion. Mobile phase C; 25-μl injection; 254-nm detector; 0.2 a.u.f.s.; flow-rate, 2.0 ml/min; temperature, 25°; pressure, 2,500–3,500 p.s.i.

Fig. 4. HPLC analysis for tea flavanols in 400 μg crude green tea extract. Mobile phase D; 20-μl injection; 280-nm detector; 0.5 a.u.f.s.; flow-rate, 2.0 ml/min; temperature, 23°; pressure, 2,500–3,500 p.s.i.

must be preceeded by an aqueous acetone extraction and an ethyl acetate extraction of the aqueous solution after removal of acetone. This analysis may now be done on the aqueous acetone extract after the removal of acetone by direct injection onto the reversed-phase column. The analysis (Fig. 4) uses mobile phase D, which was devised using Snyder's approach[10] after it had been found that a 5% aqueous acetic acid mobile phase, suggested by Waters Assoc.[11], did not fully resolve the minor constituents EC and +C from EGCG and EGC, respectively. Mobile phase D can also be used to analyze an aqueous extract or infusion of fired green tea leaf by direct injection of the beverage.

Of the four analyses described above, three may be done using aqueous acetic acid at various concentrations to give suitable retention times. Thus, pure gallic acid, caffeine, and tea flavanols may be used in the preparation of standard curves. Indeed, the analysis of caffeine, together with saccharin and sodium benzoate, in soft drinks has been described[12] in which reversed-phase chromatography is used with 5% acetic acid as the mobile phase. However, when fresh black tea infusions are analyzed, the low pH of this mobile phase causes precipitation. To avoid this problem, different mobile phases were tried. A buffered pH approach was used successfully for two of the analyses (mobile phases B and C) and the incorporation of an organic solvent aided in the other (mobile phase A). Cream formation in these fresh tea infusions[13], as opposed to instant tea solutions, is overcome by keeping the samples warm (40–50°) and making the injection within 15 min.

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