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Short communication

Rapid analysis of methylated xanthines in teas by an improved high-performance liquid chromatographic method using a polyvinylpolypyrroridone pre-column

Hideo Nakakuki[a,*], Hideki Horie[b], Yuji Yamauchi[b], Katsunori Kohata[b]

[a] *Food Research Institute, Aichi Prefectural Government, 2-1-1 Shinpukuji-cho, Nishi-ku, Nagoya, Aichi 451-0083, Japan*

[b] *National Research Institute of Vegetables, Ornamental Plants and Tea (NIVOT), Ministry of Agriculture, Forestry and Fisheries, 2769 Kanaya, Shizuoka 428-8501, Japan*

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Abstract

We have developed a new high-performance liquid chromatography (HPLC) method for the analysis of methylated xanthines in tea by removing polyphenols using a polyvinylpolypyrroridone (PVPP) pre-column. The PVPP pre-column was connected with the upstream of analytical column to remove catechins in tea extract. Using this pre-column, caffeine and theobromine in tea, which belong to methylated xanthines, could be rapidly determined in less than 10 min with an isocratic solvent system. RSDs of standard solutions of caffeine and theobromine were about 0.3 and 0.3% for the retention time, and were about 1.6 and 2.5% for the peak area. The quantitation curves of caffeine and theobromine were linear from 5 to over 1000 ng.

Keywords: Tea; Food analysis; Polyvinylpolypyrroridone columns; Stationary phases, LC; Caffeine; Theobromine; Xanthines

1. Introduction

Caffeine and theobromine are important methylated xanthines found in tea, coffee, cocoa and other foods. Caffeine, one of the major components in tea, is contained in about 2–5% in dry leaves. The stimulating effect of tea beverage is due to this compound. Tea contains only a little amount of theobromine, while theobromine is one of the major components in cocoa, and chocolate. However, there are some cases that we can not disregard the existence of these methylated xanthines in tea, for example, in the case of serving patients or small children with tea, because of the stimulating effect. In these cases, it is important to precisely analyze methylated xanthines in tea leaves.

Simultaneous determination of caffeine and theobromine has been reported in cocoa [1], but not in tea. One of the problems for the determination of methylated xanthines in tea using HPLC, is the interference by polyphenols that are contained in more than 15% of dry leaves. There is some literature published on the simultaneous determination of caffeine and catechins in tea [2–4], but they do not mention theobromine. As some catechins and these xanthines show close retention times on the chro-

*Corresponding author.

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matogram of reversed-phase HPLC [2,3], we must select an adequate analytical column and mobile phase to analyze these components. An isocratic solvent system is simple and convenient, but some catechins, (−)-epicatechin gallate, show longer retention times [4], which would disturb the repeated analysis of these xanthines in a short interval, even if methylated xanthines could be separated from catechins. A gradient solvent system could shorten the analysis time to some extent [2,3], but it needs a more sophisticated system, and needs time to initialize the analytical column. Recently, pre-treatment using Sep-Pak C_{18} has been attempted [5], however, this method is not economical and still laborious.

Polyvinylpolypyrroridone (PVPP), a low-priced and an excellent absorbent of polyphenols [6], is popularly used to remove polyphenols in the case of analysis of caffeine [7], amino acids [8] and other components in tea. Generally, in this method (batch PVPP treatment), the tea extract must be mixed with PVPP powder for more than 30 min to remove polyphenols before the analysis. Most of the polyphenols are removed by this batch PVPP treatment, while a little amount of them still remain and interfere with the analysis of methylated xanthines by HPLC. Therefore, for the purpose of rapid analysis of caffeine, we have developed a method to remove the polyphenols completely without laborious treatments. We used a pre-column filled with PVPP powder for the on-line removal of the polyphenols, which not only enabled analysis of caffeine but also theobromine in tea.

2. Experimental

2.1. Conditions for HPLC

The HPLC system consisted of a Shimadzu LC-6A pump (Shimadzu, Kyoto, Japan), a Shimadzu SPD-6A UV–Visible spectrophotometer, a Shimadzu CTO-6A column oven, a Jasco 851-AS auto-sampler (JASCO, Tokyo, Japan). The analytical column was a Capcell Pak C_{18} UG-120A (250×4.6 mm, Shiseido, Tokyo, Japan) equipped with a guard column (10×4 mm, Shiseido).

Water–acetonitrile–methanol–phosphoric acid (82.5:11:6:0.5, v/v) [4] was used as the mobile phase. The flow-rate of the mobile phase was 1 ml/min. The temperature of the column oven was set at 40°C. A 10-μl volume was injected for each analysis. The detection wavelengths were set at 272 nm.

2.2. Chemicals and samples

Caffeine and theobromine were purchased from Wako (Osaka, Japan). Four major tea catechins, (−)-epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin and (−)-epigallocatechin gallate were purchased from Kurita Kogyo (Tokyo, Japan). PVPP was purchased from Sigma. Acetonitrile and methanol used for the mobile phase were of HPLC grade. All the other chemicals were of analytical grade and were used without further purification.

Green tea of first flush was produced in NIVOT and stored under −20°C until use. Black tea, broken orange pekoe (BOP) grade, was purchased from the market in Aichi Pref., and was stored in a 4°C refrigerator. Both of the tea samples were milled by a Cyclone Sample Mill Model 3010-018 (Udy, USA).

2.3. Preparation of PVPP pre-column

The PVPP pre-column was prepared by filling an empty guard column (10×4.6 mm, Nomura, Seto, Japan) with PVPP powder, and this column was washed with the mobile phase for about 10 min. After confirming that the pressure of the mobile phase did not rise appreciably, the PVPP pre-column was connected to the upstream of the analytical column.

2.4. Sample preparation for HPLC

The powdery tea sample (500 mg) was extracted with approximately 80 ml of 50% (v/v) aqueous acetonitrile [9] in an ultra-sonic bath for 30 min. It was filled up to 100 ml with the same solvent, filtered through No. 2 filter paper and diluted ten times with distilled water. After passing through the 0.45-μm membrane filter (DISMIC 13HP045AN,

Advantec Toyo, Tokyo, Japan), the filtrate was used as the tea extract for HPLC analysis.

Batch treatment with PVPP powder was performed as follows; 200 mg of PVPP was added to the 50 ml of diluted tea extract for removing polyphenols. The suspension was allowed to stand for about 30 min with frequent mixing and filtered through No. 2 filter paper, followed by the above mentioned membrane filter for HPLC analysis.

3. Results and discussion

3.1. Performance of PVPP pre-column

Fig. 1 shows the HPLC chromatograms of green tea with different PVPP treatments. We compared the polyphenol removing efficiency without PVPP treatment (control), batch PVPP treatment (batch treatment) and PVPP pre-column method (on-line treatment). As shown in Fig. 1A (control), the four major catechins and caffeine in the green tea extract could be well separated, while theobromine was not separated. Moreover, it needs more than 25 min for one analysis because of the long retention time of (−)-epicatechin gallate. In the case of batch treatment, the peak areas of catechins decreased considerably, however, there still remained small peaks of catechins (Fig. 1B). On the other hand, the on-line treatment removed catechins and other polyphenols completely (Fig. 1C), and the peaks of caffeine and theobromine could be clearly separated. It shows that the on-line treatment is much more effective to remove catechins than the batch treatment.

In the case of the black tea extract, catechins and caffeine seemed to be well separated within 25 min (Fig. 2A). However, some polyphenolic compounds, theaflavines, which are not shown in Fig. 2A because of much longer retention times, often cause the drift of baseline after the continuous analysis of black tea in these conditions. We observed that the PVPP pre-column could completely remove theaflavines (data not shown), and caffeine and theobromine could be separated well using this pre-column (Fig. 2C).

As shown in Figs. 1 and 2, the analysis of caffeine and theobromine could be performed in less than 10 min using this on-line treatment without observing the drift of the baseline caused by the compounds with longer retention times even after dozens of injections of 10 min intervals. Therefore, the analytical time could be effectively shortened and the solvent system could be simplified using the PVPP pre-column.

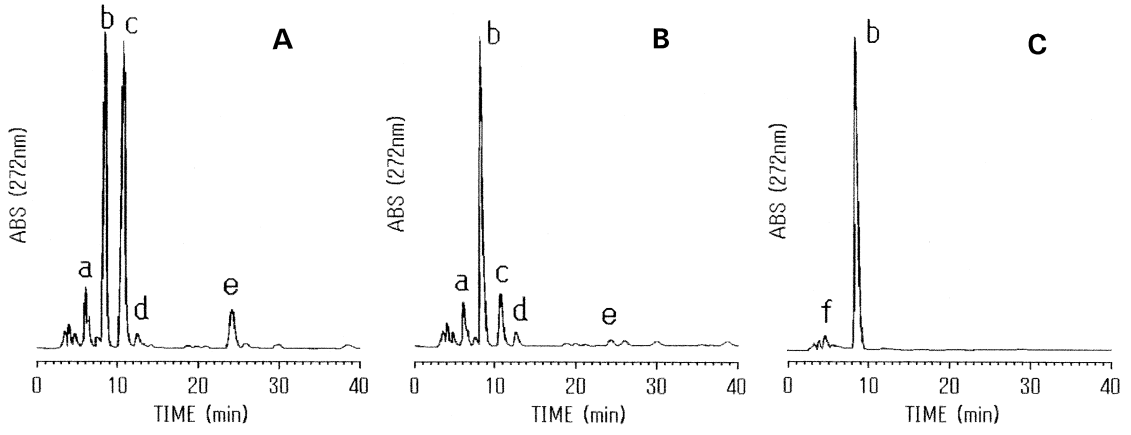

Fig. 1. HPLC chromatograms of green tea with different PVPP treatments (A) no PVPP treatment (control); (B) PVPP batch treatment; (C) PVPP on-line treatment. Peak: (a) (−)-epigallocatechin; (b) caffeine; (c) (−)-epigallocatechin gallate; (d) (−)-epicatechin; (e) (−)-epicatechin gallate; (f) theobromine. Of each sample, 10 μl was injected. The mobile phase was water–acetonitrile–methanol–phosphoric acid (82.5:11:6:0.5, v/v).

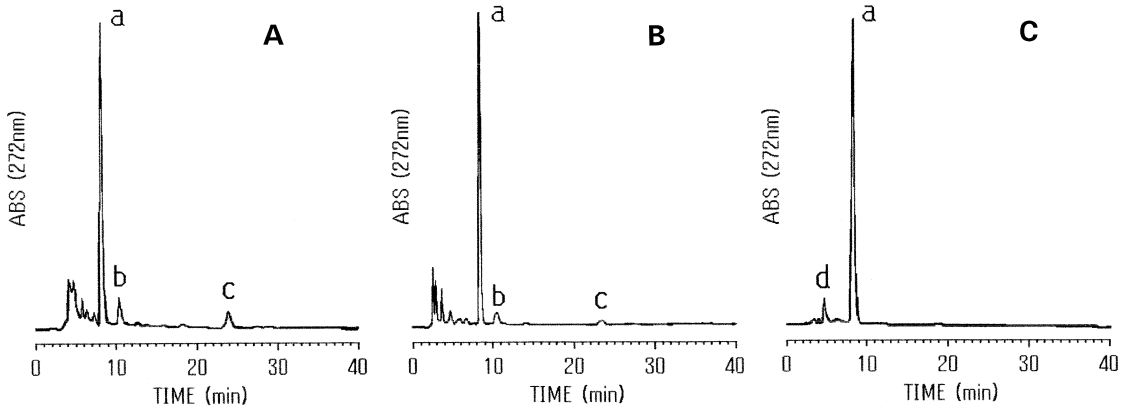

Fig. 2. HPLC chromatograms of black tea with different PVPP treatments (A) no PVPP treatment (control); (B) PVPP batch treatment; (C) PVPP on-line treatment; (a) caffeine; (b) (−)-epigallocatechin gallate; (c) (−)-epicatechin gallate; (d) theobromine. Of each sample, 10 μl was injected. The mobile phase was water–acetonitrile–methanol–phosphoric acid (82.5:11:6:0.5, v/v).

3.2. Analysis of caffeine and theobromine in tea leaves

The chromatograms of a standard mixture of caffeine and theobromine are shown in Fig. 3. The retention times of caffeine and theobromine were a little delayed when the PVPP pre-column was used. While the peak heights become lower, the peak areas were almost the same as those obtained without the PVPP pre-column. The performance of this on-line treatment was compared with control and batch treatment in Table 1. It means that either caffeine or theobromine was not absorbed on PVPP. RSDs of standard solutions of caffeine and theobromine were about 0.3 and 0.3% for the retention time, and were about 1.6 and 2.5% for the peak area. Quantitation curves of caffeine and theobromine using the PVPP pre-column were linear from 5 to over 1000 ng. The PVPP pre-column method (on-line treatment) was an ideal one for the simultaneous determination of caffeine and theobromine in tea extract, and it enabled the continuous analysis of these two compounds in 10 min interval. The data measured by this method were shown in Table 2.

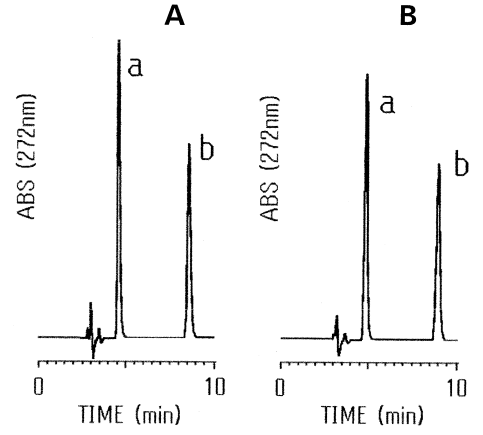

Fig. 3. HPLC chromatograms of standard caffeine and theobromine (A) without PVPP pre-column; (B) with PVPP pre-column; (a) theobromine; (b) caffeine. Each sample was injected 10 μl of 1 mg per 100 ml solution. Mobile phase was water–acetonitrile–methanol–phosphoric acid (82.5:11:6:0.5, v/v).

After the analyses of more than 200 samples of tea extracts, appreciable changes in the chromatograms were not observed. It means that this PVPP pre-column has the high enough absorption capacity of polyphenols for the continuous analysis of caffeine and theobromine in tea extract.

The merits of the PVPP pre-column method are (1) detection of theobromine with caffeine, (2) reduction of the analysis time (within 10 min) and (3) simplification of the laborious pre-treatment of tea extract. Moreover, this column may protect the analytical column from an irreversible adsorption of polyphenols.

Table 1
Reproducibility of the retention time and peak area with different PVPP treatments[a]

	Control	Batch treatment	On-line treatment
Theobromine			
Retention time (min) (n=16)	4.51	4.51	4.73
RSD (%)	0.3	0.3	0.3
Peak Area (%) (n=8)	100	99.6	101.6
RSD (%)	0.5	1.0	2.5
Caffeine			
Retention time (min) (n=16)	8.53	8.53	8.84
RSD (%)	0.2	0.2	0.3
Peak area (%) (n=8)	100	99.9	101.2
RSD (%)	0.5	0.7	1.6

[a] All values given are the average of n injections.

Table 2
Analysis of caffeine and theobromine in tea leaves with different PVPP treatments

Samples	Control caffeine(%)[a]	Batch treatment caffeine(%)	On-line treatment	
			Caffeine(%)	Theobromine(%)
Green Tea	2.33 (0.09)[b]	2.19 (0.02)	2.21 (0.03)	0.06 (0.01)
Black Tea	2.89 (0.02)	2.80 (0.03)	2.74 (0.02)	0.15 (0.02)

[a] All values given are the average of four extracted samples.
[b] Values in parentheses are the standard deviations.

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