原　著

HPLC Determination of Caffeine Using a Photodiode Array Detector and Applying a Derivative Processing to Chromatograms

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Abstract : We assessed caffeine levels in bathwater and diluted urine samples by derivative processing of chromatograms obtained through the use of HPLC with photodiode array detection. Chromatograms were differentiated on the basis of d (Abs) /d (min). The peak position of the target analyte, caffeine, was observed to shift depending on the derivative order applied, but was constant when applying the same derivative order, such that retention times were constant even after differentiation. When the method detection limit was examined using standard solutions, the varying absorption of acetonitrile during a linear gradient elution from 1 to 100 % acetonitrile was shown to have an effect, such that the linearity of the peak height deteriorated if caffeine was present at low concentrations. Recovery of caffeine from real samples was optimized in the case of both linear gradient elution and isocratic elution (10 % acetonitrile) when applying the second derivative and using a detection wavelength of 259 nm. Interestingly, this does not correspond to the maximum absorption of caffeine. Thus the optimum wavelength for use with this differential chromatographic method is one that is not affected by the absorbance of the eluent and at which the target analyte shows significant absorbance, even if it is not the maximum absorbance. Such conditions were found to be necessary for the high-sensitivity measurement of caffeine. It is believed that this method is applicable to the HPLC analysis of compounds other than caffeine and this technique is expected to be effective when applied to an automated analyzer and should be helpful to analysts unfamiliar with HPLC.

Key words : derivative chromatographic method, caffeine, urine

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Introduction

Caffeine is an alkaloid that naturally occurs in various plants; its name stems from its initial extraction from coffee beans. It is also present in tea, cola beverages, chocolate and some energy drinks. It has cardiotonic and stimulating effects and is used for medicinal purposes owing to its stimulant and diuretic properties. It is important, however, to limit caffeine intake, since it has several side effects, including headache, dizziness and fatigue,[1,2] and the LD_{50} of caffeine is about 200 mg/kg in a healthy adult.[3] In warm weather, the intake of caffeinated beverages can lead to dehydration owing to its stimulant and diuretic effects.[4] This effect is rarely seen in Japan, but a significant percentage of people of European descent possess a low tolerance for the

effects of caffeine. Therefore, in Europe and the United States, decaffeinated beverages that contain little to no caffeine are widely available. Recently, commercial beverages intended to act as simulants and to increase wakefulness (so-called "energy drinks") have become available in Japan. Since some of these drinks contain a large amount of caffeine, it is important for consumers to pay attention to their intake of such beverages, particularly in the case of persons suffering from heart disease. There have in fact, been reports of severe symptoms resulting from excessive intake of caffeinated beverages.[5] Based on these facts, it is vital to determine the caffeine contents of various foods and beverages and also to examine the degree to which caffeine is excreted in urine and perspiration, since such data helps us to understand the degree to which caffeine is absorbed into the body.

High performance liquid chromatography (HPLC) is a versatile technique which is used in the analysis of many compounds in a wide range of samples and in many different applications. To date, there have been numerous research reports detailing the development of HPLC methods and providing the data obtained from measurement of various samples. As a result, the theoretical basis of HPLC is well developed and the selection of suitable condition for the analysis of specific materials or samples is generally straightforward. Even now, the development of HPLC detectors and separation columns is still ongoing, since the sensitivity of common HPLC detectors is sometimes insufficient. In addition, even though HPLC has been a very common technique, some users will not be overly familiar with the process and thus will have difficulty in efficiently determining certain analytes. In contrast, others who have an advanced knowledge of HPLC may make use of their own experience and literature searches to determine the optimum conditions for a given analysis. However, even an advanced user may have to employ many analytical methods for various analytes, and may thus have little time to devote to optimizing the HPLC analytical conditions. As a result, we suspect that many users have difficulty in determining the optimal conditions for a given HPLC analysis.

A derivative spectrum chromatographic method[6-11] was recently reported by Yamamoto *et al.*, based on the use of the conventional photodiode array (PDA) detector. The PDA allows the simultaneous detection of multiple wavelengths during an HPLC run and is widely used. However, the Yamamoto method differs from the normal usage of this detector, in which multiple wavelengths are scanned and the absorption spectrum of the analyte is obtained, since this method takes the derivative of the analyte spectrum.[12-15] By using the derivative of the spectrum (d(Abs)/d(nm)), peaks corresponding to target compounds that elute in close proximity to one another may be separated and the isolation of analyte peaks from the background matrix noise is also improved.[11] Since continuous data (in the form of a 3D spectrum based on absorbance, wavelength and time) is collected by the PDA detector, by applying various mathematical operations after a spectrum is obtained, it is possible to optimize the derivative order and wavelength. This approach has the advantage of reducing the number of trials required for method optimization and therefore the use of mobile phase and other reagents.[9] The application of derivative-based processing therefore has the potential to assist in general HPLC method optimization based on computational analysis of the data. Unfortunately, this method requires that spectral data must be processed by using a computer language.[6-11]

We have recently devised a new differential chromatographic method, based on the use of absorbance and time data, d(Abs)/d(min), that employs standard HPLC software. The effectiveness and limitations of this method were examined in terms of increasing the sensitivity of an HPLC method for the detection of caffeine as a test compound. Several test samples were also used during the investigation of this new method, including urine specimens and bathwaters that were expected to contain excretions such as sweat.

Methods

Reagents

Special grade caffeine for use in the preparation of standards was obtained from Wako Pure Chemical Industries (Osaka, Japan) and special grade acetonitrile was purchased from Nacalai Tesque (Tokyo, Japan). Ultrapure water was employed in all trials, purified to a resistance value of 18 MΩ・cm or greater using a SimpliLab ultrapure water system (Merck Millipore, Billerica, MA, USA). All other reagents were the highest possible commercially available grade.

Test samples

An aqueous stock solution of caffeine was initially prepared at a concentration of 250 mg/L and standard solutions in the concentration range of 0.001 − 100 mg/L (for use in ascertaining the detection sensitivity or producing calibration curves) were then obtained by

diluting the stock solution with ultrapure water. Test samples consisted of two urine specimens and the water resulting from the bathing of one of the authors. These samples were prepared for recovery tests as follows. Urine samples were diluted 1：100 with ultrapure water while bath water samples were not diluted, and both types of specimens were passed through membrane filters (Millipore HA, 0.22 μm pore size, Merck Millipore). Spiked samples for analysis on the HPLC were prepared by mixing a caffeine standard solution and the urine/bath water filtrate at a ratio of 1：9.

Instrumentation

The HPLC instrument used was a Prominence system manufactured by Shimadzu (Kyoto, Japan). This system consisted of an LC-20AT pump unit operating at a total flow of 1 mL/min (pumping a mixing of water and acetonitrile), a low-pressure LC-20AD/T gradient unit, a DGU-20A3 degasser, a CTO-20AC column oven held at 40 ℃, an SPD-M20A PDA detector (190－400 nm, 1 nm detection interval, 640 ms time constant), and an SIL-10AF autosampler (10 μL sample injection volume). The instrument was controlled by a CBM-20A control unit and an LC Solution Multi-PDA workstation and was able to detect and display changes in detector response down to a level of 1 μAU. Each chromatographic run spanned 10 minutes. Unless otherwise noted, the HPLC was continuously operated under the above conditions.

HPLC conditions

A Lachrom C18 (100 × 4.6 mm i.d.) column was employed (Hitachi, Ibaraki, Japan). Two HPLC mobile phase conditions were applied during the study; isocratic elution with a 10 % acetonitrile mobile phase to detect caffeine as early as possible following the end of the solvent front (the time at which substances with no affinity for the column packing are eluted) and linear gradient elution, transitioning the mobile phase from 0.1 to 100 % acetonitrile. During the linear gradient elution, the water and acetonitrile eluents were pumped separately towards the analytical column and combined in the appropriate ratios by a mixing unit within the column oven at low pressure. The eluent conditions were not necessarily optimized with regard to minimizing baseline fluctuations during application of the gradient or the effects of matrix contaminants. Rather, we attempted to separate the caffeine peak from other peaks by differentiation of the resulting chromatograms. In addition, we analyzed the manner in which the differentiation process affected quantitative performance and detection sensitivity.

Differential chromatogram analysis

Differential processing on the basis of d (Abs) /d (min) was applied to chromatograms with time continuity to improve the separation of the caffeine peak by narrowing the peak width (Fig. 2). Because it is necessary to obtain the absorbance changes (d (Abs) /d (nm)) at multiple points near the wavelength used for detection of the analyte, the derivative spectra chromatographic method requires time-consuming computations and thus analysis in semi-real-time is difficult.[8] However, applying the present method, it was possible to perform simple data conversion using the standard HPLC control software, since this approximate differential processing is based on same principle as the Savitzky-Golay method. The software used in this study was able to calculate the maximum absorbance values from standard chromatograms (i.e., without applying differentiation) based on a simple procedure, and was also able to plot primary and secondary derivative spectra in the wavelength and time directions. However, calculation of the maximum wavelength and absorbance values based on derivative orders of three or higher was not possible. Additionally, the instrument software was only able to automatically calculate peak heights to a maximum value of one. Accordingly, the third to fifth order derivatives of chromatograms were obtained by repeated calculations of the first or second order derivatives of the same data. The height of the positive peak in each of the chromatograms following zero to fifth order differentiation was subsequently used for quantitative analysis. In the event of multiple peaks following differential processing, we selected the central peak.

After injecting standard caffeine solutions ranging in concentration from 10 μg/L to 10 mg/L, differential spectra were obtained by applying from zero to fifth order differential processing of the absorption data at the maximum peak heights in the chromatograms. Chromatograms were subsequently generated using the maximum wavelength obtained from each order of differentiation.

Results and Discussion

Choice of wavelength used for the differential chromatograms

Based on the absorption spectrum of caffeine obtained under the isocratic elution conditions, in which the

spectrum is not affected by variation in the eluent, wavelength maxima are observed at 273 and 205 nm (Fig. 1). The wavelength maxima obtained in the differentiated absorption spectra were: 198/259 nm (first derivative), 221/293 nm (second), 210/287 nm (third), 205/231 nm (fourth) and 200/226 nm (fifth). Therefore, these 11 wavelengths were used for the purposes of HPLC measurement in this study and various combinations of differential orders and wavelengths were employed to assess the optimum conditions for this analysis. Significant absorption of the acetonitrile in the eluent was observed at low wavelengths of 250 nm or less. This absorption compromised the quantitative calculations based on peak height at various wavelengths and differential orders and also resulted in baseline drift when a gradient elution was applied, as described below. Additionally, because of limitations in the mixing efficiency of the instrument, changes in absorbance were not always linear during the application of a gradient.

In derivative spectrum chromatography, the absorption spectrum of the compound of interest is first obtained. In past studies, the maximum wavelength obtained by differentiating the chromatogram based on the approximations associated with the Savitzky-Golay method[16] has been used for detection purposes.[6] This is because the use of the derivative of the wavelength to find the peak position increases the absorbance value and the quantitative sensitivity is improved as a result. However, even at the maximum absorbance

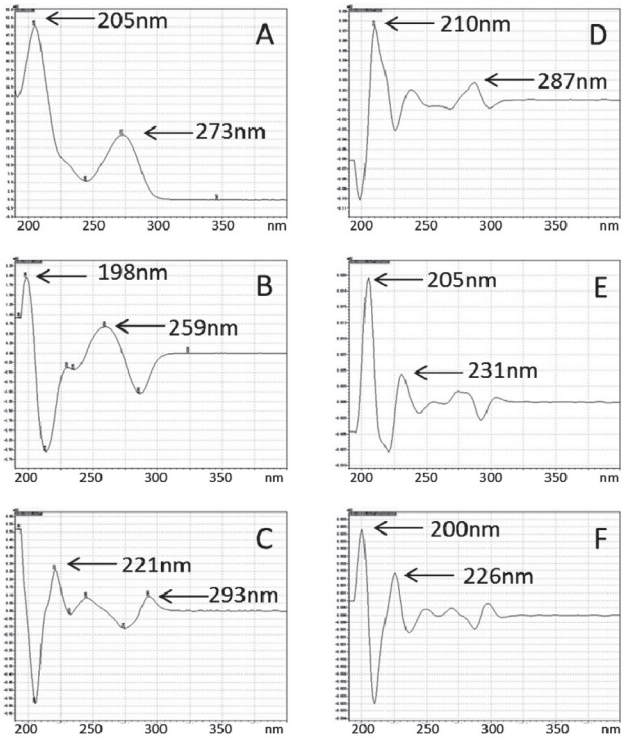

Fig. 1. Caffeine spectra obtained by SPD-M20A PDA. (A) Original spectrum and (B–F) applying first to fifth order differentials. The two highest absorption wavelengths are indicated in each spectrum.

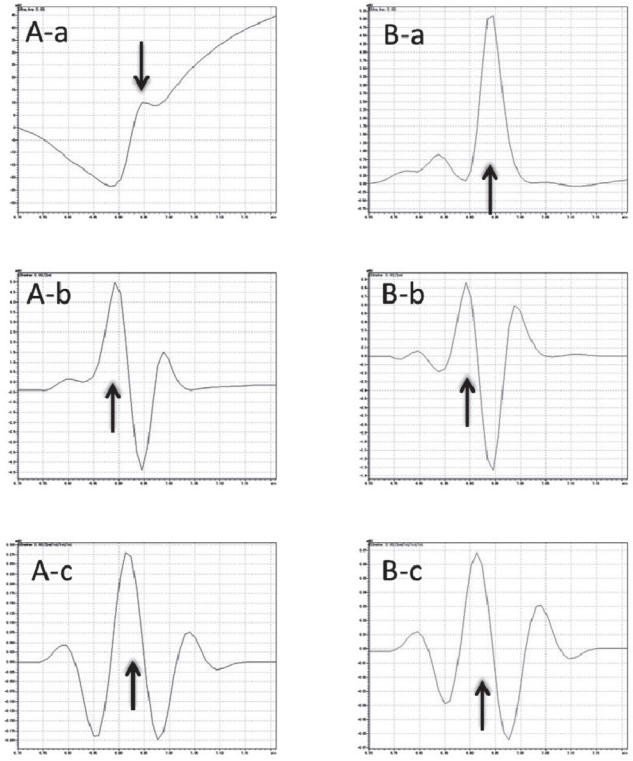

Fig. 2. Typical caffeine derivative chromatograms obtained for diluted urine (1 : 100) + 1 mg/L caffeine, applying detection wavelengths of (A) 205 and (B) 259 nm and differential orders of (a) 0 (original), (b) second and (c) fifth. Eluent conditions: linear gradient from 0.1 to 100 % acetonitrile over 10 min. Arrows indicate the peak used for quantitation. The horizontal axis in each chromatogram spans 6.7 − 7.2 min. Vertical axes (mAU) : A-a, −35 ～ 45; A-b, −4.5 ～ 5; A-c, −0.2 ～ 0.2; B-a, −1 ～ 5; B-b, −1.5 ～ 1; B-c, −0.07 ～ 0.07.

wavelength of an analyte, there may be interference by co-eluting substances as well as deleterious effects due to variations in the absorbance of the eluent. In a previous paper,[7] we reported on the analysis of caffeine by HPLC using a differential chromatographic method. When the chromatographic analysis was performed at 205 nm, which is not the maximum wavelength for this analyte, caffeine could still be detected. From this result, we concluded that the maximum analyte wavelength is not always the optimum analytical wavelength, and may in fact produce inferior results. Therefore, we also examined the use of wavelengths which did not represent the maximum absorbance values at each order of differentiation.

Detection of peak retention times

In Table 1, the average retention times and associated coefficients of variations of the caffeine peak after various differential treatments are shown. It was known that the shape of the chromatogram would be changed by differentiation and that the ability to identify the subject compound based on its original retention time would therefore be impaired. However, we also found that, when the derivative order was kept constant, the caffeine peak retention time was very repeatable and could be determined with high accuracy. Thus it is believed that by comparing the peak positions in a differential processed chromatogram of a sample with that of a standard solution it is possible to identify whether or not caffeine is present. The elution time of caffeine in underivatized chromatograms was in good agreement with the time at which a value of zero was obtained in the third order differential or the time at which a maximum value was observed in the second derivative chromatogram. Therefore, it was concluded that differential processing by the HPLC software was a reasonable approach and quantitative analysis was performed, as described below.

Lower limit of quantitation

We next investigated combinations of either the original chromatogram or first to fifth order derivatives and the 11 wavelengths determined in the previous section. We also constructed linear calibration curves to assess the relationships between peak heights and the concentration of caffeine. The caffeine concentrations used in these tests ranged from 0.001 to 100 mg/L. The results of the assessments of lower determination (recovery shows 90－110 %) and detection limits (S/N=3) are shown in Tables 2 and 3. The upper quantification limit was observed to be 100 mg/L or higher when applying any combination of wavelength, derivative order, and isocratic or gradient elution. In the short wavelength region of 198－221 nm, the determination limit tended to increase up to a maximum value of 10 mg/L, especially in the case of gradient elution. This is presumably due to fluctuations in the baseline resulting from the absorption of acetonitrile in the eluent. In almost all cases, the limit of detection was found to be greatly improved by taking the derivative.

Table 1. Caffeine peak retention times

		0.1 mg/L caffeine		0.3 mg/L		1 mg/L		3 mg/L	
		Average (min)	CV (%)	Average	CV	Average	CV	Average	CV
Differential order	0	2.226 6.981	0.87 0.72	2.219 6.953	0.66 0.16	2.222 6.949	0.86 0.43	2.216 6.951	0.33 0.42
	1	2.196 6.967	0.74 0.76	2.192 6.942	1.16 0.55	2.189 6.932	0.74 0.52	2.188 6.935	0.46 0.48
	2	2.171 6.917	0.39 0.24	2.167 6.905	0.46 0.17	2.164 6.894	0.54 0.15	2.162 6.898	0.61 0.20
	3	2.249 6.995	0.36 0.21	2.248 6.985	0.84 0.15	2.250 6.979	1.14 0.36	2.253 6.980	0.36 0.16
	4	2.225 6.969	1.38 0.20	2.224 6.958	0.38 0.17	2.219 6.954	1.04 0.35	2.222 6.953	0.39 0.15
	5	2.201 6.941	0.74 0.20	2.194 6.929	0.58 0.21	2.188 6.918	0.79 0.17	2.188 6.920	0.43 0.22

Upper: isocratic elution (10 % acetonitrile) ; Lower: gradient elution (0.1－100 % acetonitrile).
Data are based on retention times determined in triplicate at 259 nm.

Table 2. Lower determination limits of caffeine (mg/L)

		Wavelength (nm)										
		198	200	205	210	221	226	231	259	273	287	293
Differential order	0	0.3 10	0.3 10	0.3 10	0.1 10	0.3 10	0.3 3	0.1 10	0.3 1	0.1 0.3	0.1 0.3	1 1
	1	0.03 3	0.03 3	0.03 3	0.03 3	0.03 10	0.03 3	0.03 3	0.03 0.3	0.01 0.1	0.03 0.3	0.1 0.3
	2	0.03 3	0.03 3	0.03 3	0.03 3	0.03 3	0.03 3	0.03 3	0.1 0.3	0.03 0.1	0.1 0.1	0.3 0.1
	3	0.03 10	0.03 3	0.01 3	0.01 3	0.03 3	0.1 1	0.1 0.3	0.1 0.1	0.03 0.03	0.1 0.1	0.3 0.1
	4	0.03 1	0.03 3	0.03 3	0.03 3	0.1 3	0.1 0.3	0.1 0.3	0.1 0.1	0.1 0.1	0.1 0.1	0.3 0.3
	5	0.1 3	0.1 3	0.1 1	0.1 1	0.3 3	0.3 1	0.3 1	0.3 0.3	0.3 0.1	0.3 0.3	1 1

Upper: isocratic elution (10 % acetonitrile) ; Lower: gradient elution (0.1 − 100 % acetonitrile).
These values represent lower levels of peak height and concentration is proportional, based on trials performed in triplicate.

Table 3. Lower detection limits of caffeine (mg/L)

		Wavelength (nm)										
		198	200	205	210	221	226	231	259	273	287	293
Differential order	0	0.1 1	0.03 1	0.1 1	0.01 1	0.1 1	0.1 1	0.03 0.1	0.03 0.03	0.01 0.01	0.03 0.03	0.1 0.1
	1	0.003 0.03	0.003 0.03	0.003 0.03	0.003 0.03	0.003 0.03	0.01 0.03	0.01 0.03	0.01 0.01	0.003 0.01	0.01 0.03	0.03 0.1
	2	0.003 0.03	0.003 0.03	0.003 0.03	0.003 0.03	0.01 0.03	0.01 0.03	0.01 0.03	0.03 0.03	0.01 0.01	0.03 0.03	0.1 0.1
	3	0.01 0.03	0.01 0.03	0.003 0.03	0.003 0.03	0.01 0.03	0.03 0.03	0.03 0.03	0.03 0.03	0.01 0.01	0.03 0.03	0.1 0.03
	4	0.01 0.03	0.01 0.03	0.01 0.03	0.01 0.03	0.03 0.03	0.03 0.03	0.03 0.03	0.03 0.03	0.03 0.03	0.03 0.03	0.1 0.1
	5	0.03 0.03	0.03 0.03	0.03 0.03	0.03 0.03	0.1 0.03	0.1 0.03	0.1 0.03	0.1 0.03	0.1 0.03	0.1 0.03	0.1 0.1

Upper: isocratic elution (10 % acetonitrile) ; Lower: gradient elution (0.1 − 100 % acetonitrile).
Values represent minimum detectable concentrations based on triplicate analyses of peak heights.

Recovery of analyte from real-world specimens

Spikes of 0.1 − 3 mg/L caffeine were added to the bath water and 1 : 100 diluted urine samples, and the recovery of the analyte was determined from peak heights. The percent recovery was calculated as(C − B) /A × 100, where A is the expected peak height based on the quantity of caffeine standard added as a spike, B is the peak height of the unspiked sample and C is the peak height of the spiked sample. The results are shown in Tables 4 − 7. Good recovery was observed when relatively high concentrations of caffeine were added and isocratic elution was employed. In contrast, the use of gradient elution slightly lowered recovery rates in the low wavelength region where the absorption of acetonitrile is significant. The samples with lower levels of caffeine added also showed that absorbance changes in the eluent affected the calculation of the peak height of caffeine, since good recovery was not always obtained. Additionally, because the higher-order derivatives of the chromatogram sometimes produced sharp peaks due to variations in the eluent absorbance, there were several cases where the recovery was artificially high. In both the urine and bathwater samples, the best recoveries were observed when applying the second derivative at 259 nm, which is not the maximum absorbance wavelength of caffeine. The use of a longer

wavelength is believed to be preferable in these trials for the same reasons as noted in the previous section. Sharper peaks were observed on increasing the order of the differential, but their heights were also lowered. Since this method used the instrument's processing software to calculate derivatives, minimum detectable peak height of the analysis was fixed. Therefore, under conditions where a higher-order derivative is applied at a wavelength at which the analyte has relatively low absorbance, it may not be possible to obtain sufficient peak height. Caffeine concentrations in the urine and bathwater calculated based on the optimal conditions were in the ranges of 0.0049 − 0.0078 mg/L and 0.053 − 0.076 mg/L, respectively. Those values which are below the lower limit of determination are provided solely for reference purposes.

Using the differential chromatographic method, it is possible to reduce the peak widths and thus minimize the influence of background noise. As a result, this technique allows simultaneous analysis of multicomponent mixtures in a relatively short time frame. However, the differentiation process cannot be expected to analyze chromatograms where two or more peak maxima are completely coincident, and will not produce accurate values under such conditions. Based on the results of these trials, it is helpful to

Table 4. Recovery of 3mg/L Caffeine

Differential order	Wavelength (nm) 198	200	205	210	221	226	231	259	273	287	293
0	101.26 ± 1.57	101.38 ± 1.31	101.56 ± 1.05	101.57 ± 1.01	101.42 ± 1.37	101.29 ± 1.75	100.89 ± 2.51	102.66 ± 0.87	102.16 ± 0.92	102.13 ± 0.98	101.44 ± 2.08
	95.15 ± 7.97	96.29 ± 7.69	97.04 ± 7.41	97.18 ± 7.76	97.99 ± 9.50	117.28 ± 7.06	195.43 ± 5.85	104.44 ± 2.41	102.90 ± 2.06	104.23 ± 2.22	103.33 ± 2.75
1	101.98 ± 1.09	101.88 ± 1.07	101.82 ± 1.06	101.82 ± 1.06	101.79 ± 1.03	101.76 ± 1.02	101.59 ± 1.00	101.96 ± 1.06	101.87 ± 1.07	101.82 ± 1.07	101.90 ± 1.05
	130.30 ± 12.04	131.03 ± 10.94	127.44 ± 9.78	129.94 ± 11.24	158.31 ± 14.76	140.99 ± 10.64	128.24 ± 6.86	102.37 ± 2.12	101.85 ± 1.80	103.09 ± 1.83	105.88 ± 1.98
2	104.71 ± 2.19	104.10 ± 1.93	103.65 ± 1.60	103.75 ± 1.39	104.59 ± 1.64	104.32 ± 1.73	103.45 ± 1.72	102.31 ± 1.41	102.26 ± 1.29	102.56 ± 1.37	103.15 ± 1.42
	104.56 ± 0.97	104.24 ± 0.91	103.91 ± 0.91	104.14 ± 0.99	105.08 ± 1.67	103.91 ± 1.89	102.26 ± 1.59	100.48 ± 0.89	100.45 ± 0.83	100.86 ± 0.84	101.43 ± 1.14
3	102.10 ± 0.95	102.16 ± 0.99	102.20 ± 1.03	102.19 ± 1.02	102.29 ± 1.10	102.23 ± 1.18	102.13 ± 1.32	102.36 ± 1.08	102.34 ± 1.05	102.26 ± 1.08	102.11 ± 1.13
	103.16 ± 5.55	102.96 ± 5.21	102.80 ± 4.85	102.89 ± 4.91	103.93 ± 5.26	103.08 ± 4.08	102.33 ± 2.93	101.05 ± 2.07	100.97 ± 2.07	101.23 ± 2.14	101.12 ± 2.13
4	103.37 ± 0.97	103.33 ± 1.04	103.17 ± 1.03	103.16 ± 1.04	103.25 ± 1.05	103.54 ± 0.97	103.08 ± 0.95	103.14 ± 1.02	103.04 ± 1.04	103.03 ± 1.08	103.21 ± 1.18
	103.24 ± 4.26	103.11 ± 4.00	102.92 ± 3.72	103.03 ± 3.76	103.96 ± 3.97	103.46 ± 3.05	102.28 ± 2.44	101.29 ± 1.69	101.07 ± 1.69	101.44 ± 1.77	101.49 ± 1.76
5	103.53 ± 1.08	103.34 ± 1.08	103.00 ± 1.05	102.97 ± 1.02	102.98 ± 1.02	102.95 ± 1.03	102.64 ± 1.05	103.34 ± 1.02	103.10 ± 0.93	103.19 ± 1.04	102.17 ± 1.75
	105.01 ± 3.49	104.61 ± 3.20	104.22 ± 2.92	104.30 ± 2.94	104.46 ± 3.43	102.39 ± 2.97	101.24 ± 2.88	100.59 ± 1.23	100.79 ± 1.19	101.00 ± 1.29	102.34 ± 1.51

Upper: isocratic elution (10% acetonitrile); Lower: gradient elution (0.1→100% acetonitrile).
The recoveries (%) were shown as the average and SD of four samples.
Shaded cells indicate cases where the recovery was 95-105% and the SD was less than 5%.

Table 5. Recovery of 1mg/L Caffeine

Differential order	Wavelength (nm) 198	200	205	210	221	226	231	259	273	287	293
0	98.64 ± 6.56	98.52 ± 4.56	99.08 ± 1.54	99.42 ± 0.97	99.02 ± 3.69	98.56 ± 5.87	99.08 ± 7.19	101.08 ± 0.74	99.46 ± 0.73	99.34 ± 0.65	100.17 ± 1.25
	121.11 ± 32.58	117.35 ± 29.66	117.89 ± 29.21	124.04 ± 37.96	38.56 ± 54.80	-1121.29 ± 156.34	-3.92 ± 5.35	99.74 ± 1.58	98.56 ± 1.47	98.31 ± 1.58	98.01 ± 2.08
1	100.63 ± 1.59	101.56 ± 0.97	100.51 ± 0.80	99.72 ± 0.81	99.84 ± 1.12	99.19 ± 1.97	99.12 ± 2.38	100.38 ± 0.86	99.32 ± 0.92	99.12 ± 0.74	99.20 ± 0.92
	58.57 ± 27.30	62.21 ± 23.90	64.63 ± 20.49	60.80 ± 20.95	42.81 ± 22.56	49.71 ± 16.55	74.30 ± 9.41	98.33 ± 2.85	98.69 ± 1.76	97.47 ± 1.21	99.68 ± 2.02
2	103.15 ± 3.79	102.25 ± 2.57	102.26 ± 1.19	102.78 ± 0.80	104.16 ± 2.12	101.71 ± 3.49	99.63 ± 4.19	97.86 ± 0.73	97.97 ± 0.91	98.94 ± 1.04	99.17 ± 1.48
	102.71 ± 3.65	102.65 ± 3.35	102.35 ± 3.07	101.72 ± 3.40	99.22 ± 8.41	97.93 ± 12.45	98.24 ± 12.77	100.36 ± 3.23	99.99 ± 1.55	99.78 ± 0.94	99.20 ± 1.44
3	95.19 ± 5.02	96.90 ± 3.48	98.39 ± 1.90	98.54 ± 1.51	97.28 ± 3.14	95.42 ± 4.94	95.18 ± 5.60	99.01 ± 1.32	99.40 ± 1.01	99.79 ± 0.59	97.89 ± 0.77
	118.31 ± 14.16	115.78 ± 12.32	113.66 ± 10.49	114.83 ± 10.82	124.70 ± 11.97	110.77 ± 6.54	107.03 ± 3.81	98.88 ± 1.73	98.50 ± 1.65	98.88 ± 1.67	100.77 ± 1.88
4	95.15 ± 4.05	95.88 ± 3.10	97.20 ± 1.89	97.18 ± 1.54	96.35 ± 2.84	96.15 ± 4.02	94.89 ± 4.51	97.85 ± 1.71	98.04 ± 1.12	97.41 ± 1.03	97.75 ± 1.73
	102.54 ± 13.34	102.70 ± 11.30	102.74 ± 9.34	102.97 ± 9.72	105.51 ± 10.79	106.16 ± 5.96	103.59 ± 3.99	99.90 ± 1.87	99.63 ± 1.46	100.00 ± 1.44	102.76 ± 1.69
5	95.47 ± 3.73	96.93 ± 2.85	97.62 ± 1.72	97.32 ± 1.43	96.25 ± 3.05	97.33 ± 3.57	95.60 ± 4.26	98.48 ± 0.85	97.81 ± 1.29	98.42 ± 2.00	100.00 ± 0.00
	107.05 ± 12.16	106.00 ± 10.80	104.67 ± 9.39	104.74 ± 9.78	105.54 ± 11.81	103.24 ± 10.44	99.34 ± 9.32	98.86 ± 2.52	99.26 ± 1.57	100.00 ± 1.10	99.12 ± 1.75

Upper: isocratic elution (10% acetonitrile); Lower: gradient elution (0.1→100% acetonitrile).
The recoveries (%) were shown as the average and SD of four samples.
When recovery is 95-105% and within 5% SD, a fill color made.

Table 6. Recovery of 0.3mg/L Caffeine

Differential order	Wavelength (nm) 198	200	205	210	221	226	231	259	273	287	293
0	120.08 ± 44.09 N.C.	112.23 ± 28.61 N.C.	106.35 ± 12.22 N.C.	103.60 ± 7.30 N.C.	107.28 ± 14.19 N.C.	112.00 ± 24.31 90.37 ± 3.10	113.08 ± 26.01 101.34 ± 6.61	104.06 ± 6.56 121.70 ± 4.06	99.09 ± 4.36 110.23 ± 1.79	100.67 ± 2.07 112.75 ± 1.91	101.80 ± 4.55 130.99 ± 1.67
1	58.73 ± 61.25 14.42 ± 11.05	68.49 ± 44.43 14.41 ± 11.64	83.56 ± 21.54 14.34 ± 12.20	87.99 ± 16.04 14.33 ± 11.63	68.47 ± 42.95 13.86 ± 8.73	53.12 ± 71.29 14.17 ± 10.23	42.80 ± 84.37 -3.53 ± 10.04	94.61 ± 9.10 102.45 ± 10.18	90.62 ± 2.27 105.41 ± 4.51	87.70 ± 1.20 105.02 ± 5.31	76.70 ± 2.02 99.28 ± 11.27
2	113.07 ± 49.58 622.26 ± 146.18	106.53 ± 45.59 398.01 ± 80.61	103.19 ± 34.14 294.31 ± 68.16	103.42 ± 27.58 313.00 ± 60.40	101.50 ± 48.06 489.78 ± 114.48	99.19 ± 44.02 195.00 ± 32.83	89.66 ± 38.77 127.40 ± 12.96	**96.01 ± 1.63** **104.29 ± 3.79**	97.81 ± 3.01 106.31 ± 2.92	98.56 ± 2.05 107.42 ± 3.77	90.15 ± 4.56 99.49 ± 4.10
3	92.70 ± 11.59 45.84 ± 24.84	93.86 ± 9.05 42.41 ± 25.66	96.71 ± 5.42 36.59 ± 26.26	95.76 ± 4.70 35.63 ± 26.73	90.69 ± 12.11 34.41 ± 30.71	85.73 ± 17.74 -113.21 ± 125.02	82.74 ± 20.27 137.67 ± 16.20	98.24 ± 4.66 117.39 ± 2.77	100.27 ± 1.62 110.11 ± 2.16	101.09 ± 2.00 105.34 ± 2.36	99.31 ± 2.41 101.62 ± 5.40
4	94.25 ± 20.17 93.57 ± 41.99	95.91 ± 16.19 16.41 ± 42.79	97.32 ± 10.20 3.76 ± 50.95	101.18 ± 16.62 4.65 ± 48.94	95.92 ± 16.13 2.78 ± 45.85	90.38 ± 22.65 -111.41 ± 63.93	91.07 ± 25.03 130.00 ± 99.04	97.20 ± 7.98 111.61 ± 2.88	99.66 ± 1.16 108.99 ± 1.06	100.29 ± 1.96 105.31 ± 1.91	103.79 ± 1.52 108.75 ± 3.61
5	89.01 ± 21.70 -20.69 ± 46.70	91.67 ± 18.45 -35.05 ± 53.40	94.64 ± 12.18 -59.57 ± 63.92	95.68 ± 9.89 -56.98 ± 62.84	90.53 ± 18.32 -50.63 ± 57.83	87.78 ± 23.13 N.C.	82.97 ± 25.33 130.56 ± 35.19	97.78 ± 7.70 99.57 ± 4.95	**99.64 ± 1.82** **103.98 ± 2.71**	103.03 ± 4.29 105.23 ± 1.16	110.42 ± 7.98 106.25 ± 3.84

Upper: isocratic elution (10% acetonitrile); Lower: gradient elution (0.1→100% acetonitrile).
The recoveries (%) were shown as the average and SD of four samples.
N.C.: not calculated, since the denominator of the formula was equal to zero.
Shaded cells indicate cases where the recovery was 95-105% and the SD was less than 5%.

Table 7. Recovery of 0.1mg/L Caffeine

Differential order	Wavelength (nm) 198	200	205	210	221	226	231	259	273	287	293
0	402.66 ± 344.53 N.C.	312.42 ± 238.04 N.C.	143.57 ± 83.71 N.C.	128.38 ± 58.96 N.C.	172.99 ± 122.06 N.C.	295.22 ± 194.99 N.C.	241.26 ± 208.52 100.35 ± 4.87	134.04 ± 19.22 222.84 ± 28.52	106.73 ± 2.73 127.86 ± 3.53	102.87 ± 2.50 112.76 ± 3.02	103.53 ± 4.62 90.44 ± 32.35
1	119.95 ± 295.50 5.02 ± 6.04	110.28 ± 203.19 5.09 ± 6.02	75.89 ± 47.38 4.88 ± 5.81	61.44 ± 34.63 4.61 ± 5.30	40.37 ± 109.08 4.10 ± 4.40	3.20 ± 197.33 6.48 ± 5.41	-10.02 ± 229.78 0.74 ± 5.85	65.78 ± 5.22 163.24 ± 20.34	79.60 ± 1.64 126.02 ± 13.98	70.57 ± 1.43 204.27 ± 15.53	20.00 ± 1.84 N.C.
2	259.50 ± 113.59 -56.10 ± 9.19	214.07 ± 84.40 -65.32 ± 10.26	175.87 ± 9.60 -78.85 ± 11.23	191.67 ± 7.30 -76.16 ± 9.73	207.69 ± 91.34 -69.25 ± 9.28	166.41 ± 70.67 -138.93 ± 36.99	82.05 ± 109.26 4415.00 ± 842.12	**101.37 ± 5.94** **96.15 ± 2.87**	101.01 ± 3.32 114.31 ± 2.85	106.37 ± 2.83 131.34 ± 5.42	113.19 ± 6.16 81.01 ± 8.52
3	116.80 ± 31.50 N.C.	106.98 ± 10.71 N.C.	96.45 ± 12.91 N.C.	93.40 ± 11.62 N.C.	107.20 ± 13.67 N.C.	111.69 ± 17.33 N.C.	119.92 ± 31.72 N.C.	96.38 ± 21.31 291.25 ± 4.33	105.72 ± 2.53 167.82 ± 5.42	106.58 ± 3.62 142.06 ± 2.75	92.71 ± 3.99 105.36 ± 16.10
4	123.03 ± 38.74 10.13 ± 11.21	110.94 ± 13.27 9.63 ± 11.27	100.17 ± 10.11 8.50 ± 12.27	103.76 ± 3.83 7.86 ± 12.06	116.96 ± 37.70 -5.19 ± 14.93	142.76 ± 89.25 -29.81 ± 10.12	132.26 ± 64.89 1387.50 ± 193.11	97.97 ± 7.76 109.24 ± 4.82	103.23 ± 2.04 126.98 ± 2.90	108.33 ± 3.55 118.94 ± 2.90	93.75 ± 7.98 114.68 ± 17.18
5	88.07 ± 29.13 -6.21 ± 9.25	86.00 ± 25.87 -7.34 ± 10.40	83.04 ± 34.52 -9.49 ± 10.63	90.09 ± 11.57 -10.43 ± 9.96	97.62 ± 25.94 -16.67 ± 5.87	108.33 ± 63.33 -48.91 ± 9.64	120.83 ± 85.12 -1100.00 ± 294.39	88.33 ± 13.74 118.33 ± 13.74	104.55 ± 6.43 105.56 ± 6.42	89.58 ± 12.50 127.08 ± 4.17	131.25 ± 23.94 87.50 ± 15.96

Upper: isocratic elution (10% acetonitrile); Lower: gradient elution (0.1→100% acetonitrile).
The recoveries (%) were shown as the average and SD of four samples.
N.C.: not calculated, since the denominator of the formula was equal to zero.
Shaded cells indicate cases where the recovery was 95-105% and the SD was less than 5%.

choose a detection wavelength that avoids as much as possible changes in absorbance due to variations in mobile phase composition. It is also important to add the appropriate spike concentration to samples when testing for recovery, and to apply the optimal degree of differentiation.

Conclusions

The use of a differential chromatogram method has been shown to reduce the effects of the baseline and thus lead to an increase in sensitivity. It is believed that the method outlined in this study should be applicable to the measurement of compounds other than caffeine. This technique may also be helpful to analysts who lack computational resources and in cases where automated analysis is required,[18] and during the development of new HPLC methods.

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フォトダイオードアレイ検出 HPLC を用いた微分クロマトグラフ法によるカフェインの定量

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要　　約

フォトダイオードアレイ検出 HPLC を用いた微分クロマトグラフ法による希釈尿あるいは風呂水からのカフェインの回収について検討した。

通常のクロマトグラムを微分（d（Abs）/d（min））し，微分クロマトグラムを得る。目的の化合物のピーク位置は，微分次数によるずれるが，同じ微分次数で比較すると一定しており，保持時間による定性能は微分後も維持されていた。標準溶液を用いた定量下限値は，リニアグラジエント溶出（1 → 100 %アセトニトリル）では溶離液として用いたアセトニトリルの吸収が影響を与え，カフェインが低濃度の場合にはピーク高さとの直線性が悪化した。実試料からのカフェインの回収は，波長 259 nm，2 次微分実行時にリニアグラジエント溶出，アイソクラティック溶出（10 %アセトニトリル）共最適であった。この条件は必ずしもカフェインが極大吸収を持つ波長，微分次数ではない。本結果は微分クロマトグラフ法での検出条件として，1）溶離液吸収の影響を受け難い波長を選択すること，2）目的物質であるカフェインの吸光度が極大でなくてもある程度大きいことが高感度測定に必要であることを示す。

今回の結果はカフェインのみならず，他の化合物を HPLC 分析する場合，特に HPLC に不慣れな使用者が条件検討する場合や自動分析装置に応用する場合にも利用可能な知見であると考えられる。

キーワード：微分クロマトグラフ法，カフェイン，尿

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