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Development of an analytical method for polycyclic aromatic hydrocarbons in coffee beverages and dark beer using novel high-sensitivity technique of supercritical fluid chromatography/mass spectrometry

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Polycyclic aromatic hydrocarbons (PAHs) are carcinogenic substances that are mainly generated during heating in food; therefore, the European Union (EU) has regulated the amount of benzo[*a*]pyrene and PAH4 in various types of food. In addition, the Scientific Committee on Food of the EU and the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives have recommended that 16 PAHs should be monitored. Since coffee beverages and dark beer are roasted during manufacture, monitoring these 16 PAHs is of great importance. On the other hand, supercritical fluid chromatography (SFC) is a separation method that has garnered attention in recent years as a complement for liquid and gas chromatography. Therefore, we developed a rapid high-sensitivity analytical method for the above-mentioned 16 PAHs in coffee beverages and dark beer involving supercritical fluid chromatography/atmospheric pressure chemical ionization-mass spectrometry (SFC/APCI-MS) and simple sample preparation. In this study, we developed a novel analytical technique that increased the sensitivity of MS detection by varying the back-pressure in SFC depending on the elution of PAHs. In addition, analysis of commercially available coffee and dark beer samples in Japan showed that the risk of containing the 16 PAHs may be low.

[**Key words:** Supercritical fluid chromatography; Mass spectrometry; Back pressure regulator; Polycyclic aromatic hydrocarbons; Coffee beverage; Dark beer]

Polycyclic aromatic hydrocarbons (PAHs) are carcinogenic substances; the most well-known PAH, benzo[*a*]pyrene (BaP), has been classified in group 1 (carcinogenic to humans) by the International Agency for Research on Cancer. Since PAHs are generated by the combustion of organic matter (1,2), they are known to be produced via heating processes like roasting and smoking in food manufacturing (3,4). Therefore, limits have been set for PAHs in various foods in several countries. The European Union (EU) has set maximum levels (MLs) for BaP and PAH4 (the sum of BaP, benzo[*a*]anthracene (BaA), chrysene (Chr), and benzo[*b*]fluoranthene (BbF)) (5). The strictest MLs of BaP and PAH4 are set to 1 μg/kg in foods for infants and young children. In addition, the Scientific Committee on Food (SCF) of the EU concluded that analyses of 15 PAHs should continue so that data can be collected on them in order to evaluate food contamination (6–8). Furthermore, the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA) also recommended that data should be collected on 14 PAHs (8,9), including benzo[*c*]fluorene (BcF), which was not mentioned by the SCF. Total 15+1 PAHs (PAH_{15+1}, Fig. S1) were target compounds. In addition, Collins reported that dibenzo[*a,h*]pyrene (DBahP), dibenzo[*a,i*]pyrene (DBaiP), and dibenzo[*a,l*]pyrene (DBalP) are ten times as carcinogenic as BaP (10). The data collection of these PAH_{15+1} recommended by the SCF and JECFA is of great importance. Since the manufacturing processes of coffee beverages and dark beer involve roasting of the raw materials (3,11–16), they may contain the PAH_{15+1}.

The most common analytical method for PAHs in food and beverages is gas chromatography-mass spectrometry (GC/MS), but elution of BaP requires 20–30 min, so the analysis time for the PAH_{15+1} is 30–40 min (17–21). There have been reports of combinations of liquid chromatography (LC) with a UV detector, fluorescence detector (FLD), or MS for PAHs analysis. With a UV detector, the selectivity and sensitivity are limited (22–25). LC-FLD shows good sensitivity except for cyclopenta[*c,d*]pyrene (CPP) (8,23–25), and LC-MS analyses show moderate sensitivity with two types of ionization: atmospheric pressure chemical ionization (APCI) (26–29) and atmospheric pressure photoionization (APPI) (30–34).

On the other hand, supercritical fluid chromatography (SFC) is a separation method that has garnered attention in recent years for using supercritical carbon dioxide as the mobile phase. Since supercritical carbon dioxide has low viscosity, it allows analysis with a high flow rate without increasing the pressure and a short analysis time (35). The polarity of supercritical carbon dioxide is similar to that of *n*-hexane (35); therefore, it is suitable for the analysis of low-polarity substances like PAHs. PAH analysis using SFC with a packed column has been reported only combined with atmospheric pressure laser ionization–time-of-flight MS, and the sensitivities for some PAHs were insufficient (36).

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In the present study, we developed a rapid high-sensitivity analytical method for the PAH_{15+1} in coffee beverages and dark beer using SFC/APCI-MS (limits of quantification (LOQs) below 1 μg/kg). In addition, using a novel technique in which the back-pressure in SFC is varied with the elution of compounds, we achieved higher sensitivity than that of the conventional SFC/MS method. Samples were prepared simply via extraction with cyclohexane and purification with a solid-phase extraction (SPE) cartridge. Using the developed method, commercially available coffee beverages and dark beer in Japan were analyzed.

MATERIALS AND METHODS

Reagents and chemicals Standard solutions from Kanto Chemical (Tokyo, Japan) were used in the experiments: PAH-mix 170 (Mixture of BaA, BbF, benzo[*j*]fluoranthene (BjF), benzo[*k*]fluoranthene (BkF), benzo[*g,h,i*]perylene (BghiP), BaP, Chr, CPP, dibenzo[*a,h*]anthracene (DBahA), dibenzo[*a,e*]pyrene (DBaeP), dibenzo[*a,h*]pyrene (DBahP), dibenzo[*a,i*]pyrene (DBaiP), dibenzo[*a,l*]pyrene (DBalP), indeno[1,2,3-*c,d*]pyrene (IP), and 5-methylchrysene (MCh), 10,000 ng/mL of each compound) and BcF (10,000 ng/mL). As a surrogate for the internal standard (IS), a mixed solution of PAH-mix 9 deuterated (containing BaA-d12, BbF-d12, BkF-d12, BghiP-d12, BaP-d12, Chr-d12, DBahA-d14, and IP-d12, 10,000 ng/mL of each compound) from Kanto Chemical and a solution of DBaiP-d14 (200,000 ng/mL) from Wako Pure Chemical Industries (Osaka, Japan) were used. Other solvents and reagents used included methanol (LC/MS grade), *n*-hexane, acetone (pesticide residue analysis grade), and cyclohexane (special grade) from Kanto Chemical. Formic acid (LC/MS grade) and acetonitrile (LC/MS grade) were obtained from Wako Pure Chemical Industries. Anisole (99.7%) and Supel QuE Citrate (EN) Tubes ($MgSO_4$ 4 g, NaCl 1 g, trisodium citrate 1 g, and disodium citrate 0.5 g) from Sigma–Aldrich (St. Louis, MO, USA) were also used. Bond Elut Alumina-N (AL-N) SPE cartridges (500 mg/10 mL) from Agilent Technologies (Santa Clara, CA, USA) were used. As an analytical sample filter, a 0.2-μm polytetrafluoroethylene (PTFE) syringe filter from Merck Millipore (Billerica, MA, USA) was used.

For the standard solution, PAH-mix 170 and BcF were mixed and diluted with hexane, while for the surrogate, PAH-mix 9 deuterated and DBaiP-d14 were mixed and diluted with hexane: a 100 ng/mL mixed standard solution and a 500 ng/mL mixed surrogate were prepared.

SFC/MS analytical conditions For SFC, Acquity UPC^2 from Waters (Milford, MA, USA) was employed. For MS, Quattro Premier/XE equipped with an APPI/APCI dual ion source from Waters was used. As the analytical column, an Inertsil ODS-P column (150 mm × 3.0 mm, 3 μm) from GL Science (Tokyo, Japan) was used. Elution of compounds was performed in the linear gradient mode. Solvents A (CO_2) and B (0.5% formic acid in acetonitrile) were used as mobile phases as follows: 0–0.2 min, 0.1% B; 6.7 min, 16.3% B; 8.4 min, 26.2% B; 9.4–12.5 min, 60% B; and 13.2–14 min, 0.1% B for a total of 14 min. During analysis, different flow rates were employed with the following linear gradient: 0–10.05 min, 2.5 mL/min; 10.15–12.4 min, 2.25 mL/min; and 12.5–14 min, 2.5 mL/min. The column oven temperature was set at 40°C. The injection volume was 15 μL. A back-pressure regulator was used to adjust the pressure in a linear as follows: 0–1.8 min, 13.8 MPa; 2.05–2.2 min, 20.7 MPa; 2.3–2.75 min, 13.8 MPa; 2.95–3.63 min, 20.7 MPa; 3.73–4.25 min, 13.8 MPa; 4.45–5.1 min, 20.7 MPa; 5.2–5.5 min, 13.8 MPa; 5.7–6.12 min, 20.7 MPa; 6.22–6.38 min, 13.8 MPa; 6.55–7.29 min, 20.7 MPa; 7.32–7.43 min, 13.8 MPa; 7.56–8.3 min, 20.7 MPa; 8.4–9.02 min, 13.8 MPa; 9.06–9.28 min, 15.5 MPa; 9.31–10.1 min, 13.8 MPa; 10.2–10.84 min, 17.2 MPa; 10.9–11.13 min, 13.8 MPa; 11.23–11.85 min, 17.2 MPa; and 11.9–14 min, 13.8 MPa.

The positive mode of APCI was used for detection with MS. A make-up solvent, 5% anisole in methanol, was introduced from SFC to MS as a dopant at a flow rate of 0.04 mL/min. The instrument settings for ionization were as follows: source temperature of 120°C, desolvation gas temperature of 500°C, desolvation gas flow rate of 500 L/h, and corona pin current value of 5.0 μA. Measurements were performed in selected-ion monitoring (SIM) mode. Precursor ions of the standard substances and the IS are $[M]^+$ ions. Table 1 shows the SIM conditions.

Sample preparation Commercially available coffee beverages and dark beer were analyzed in our study. The sample (10 g) was placed in a 50-mL centrifuge tube and spiked with 50 μL of a 500 ng/mL mixed surrogate solution. After adding 10 mL of methanol and 5 mL of cyclohexane, the contents of a Supel QuE Citrate (EN) Tube were added. Subsequently, samples were shaken for 5 min with a sample shaker and centrifuged for 8 min at 3500 rpm. While ensuring that the lower layer (water) does not infiltrate the upper layer, the upper layer (cyclohexane) was transferred to a 15-mL centrifuge tube. To the remaining lower layer, 3 mL of cyclohexane was added, and the sample was shaken for 5 min with a sample shaker and centrifuged for 8 min at 3500 rpm. The upper layer was transferred to the above-mentioned 15-mL centrifuge tube. Approximately 8 mL of the cyclohexane that was obtained was concentrated and dried under a stream of nitrogen at 40°C. A sample solution re-dissolved with 1 mL of hexane was loaded onto an AL-N SPE cartridge that had already been conditioned with 8 mL of hexane, and the eluate was transferred to a 15-mL test tube. Furthermore, the centrifuge tube was washed with 1 mL of hexane, and the sample was eluted with 9 mL of hexane. In total, 11 mL of eluate was collected. The eluate was concentrated and dried under a stream of nitrogen at 40°C, and after adding 0.5 mL of hexane/acetone (v/v, 95/5) as a dissolving solvent, the sample was dissolved using an ultrasound bath. Using a 0.2-μm PTFE syringe filter, the solution was filtered into a vial.

The absolute calibration method using an IS, in which the analytical sample was spiked with a surrogate substance, was employed for quantitation. In the case of substances for which surrogate substances are not commercially available, a substitute surrogate substance that elutes similarly was used.

RESULTS AND DISCUSSION

Investigation of ionization condition for MS We examined APPI and APCI, which are suitable for low-polarity substances without easily ionizable functional groups, such as PAHs. For APPI, anisole was used as a dopant, as described in the literature (31,33). For APCI, the most favorable signal-to-noise ratio (S/N) was obtained by using 5% anisole in methanol as the dopant (29). With APPI and APCI, 5 and 50 ng/mL of BaA and BaP were analyzed. For APPI, results showed that the background was high and detection at 5 ng/mL was not possible. On the other hand, for APCI, the background was low and detection was possible even at 5 ng/mL (Fig. S2). Based on these results, APCI was used for ionization.

LC column selection For the separation of the PAH_{15+1}, an XSelect HSS C18 SB column (150 mm × 3.0 mm, 3.5 μm, Waters) and Inertsil ODS-P column were studied. The HSS C18 SB column was not favorable for the separation of similar compounds such as benzofluoranthenes and dibenzopyrenes. On the other hand, with the ODS-P column, in which octadecyl groups are densely bonded and which is suited for the plane recognition ability of compounds, the PAHs were moderately separated and elution could be complete in 14 min (Fig. S3).

Investigation of the novel highly sensitive technique Using the UPC^2 with an MS, the SFC line is branched prior to the MS. One

TABLE 1. SIM parameters of standards and IS of PAH_{15+1}.

No.	STD[a]	PI[b] (*m/z*)	CV[c] (V)	No.	STD[a]	PI[b] (*m/z*)	CV[c] (V)	IS	PI[b] (*m/z*)	CV[c] (V)	Corresponding STD[a] No.
1	BcF	215.1	82	9	BaP	252.0	75	BaA-d12	240.0	70	1, 2
2	BaA	228.0	70	10	DBahA	278.1	70	Chr-d12	240.0	70	3–5
3	MCh	241.2	70	11	DBalP	302.1	85	BbF-d12	264.0	75	6, 7
4	CPP	226.0	90	12	IP	276.1	87	BkF-d12	264.0	75	8
5	Chr	228.0	70	13	BghiP	276.1	87	BaP-d12	264.0	75	9
6	BjF	252.0	70	14	DBaeP	302.1	95	DBahA-d14	292.2	65	10, 11
7	BbF	252.0	70	15	DBaiP	302.1	95	IP-d12	288.1	79	12
8	BkF	252.0	70	16	DBahP	302.1	95	BghiP-d12	288.1	79	13
								DBaiP-d14	315.6	95	14–16

[a] Standard.
[b] Precursor ion.
[c] Cone voltage.

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