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Analysis of Heterocyclic Amines in Human Urine Using Multiple Solid-Phase Extraction by Liquid Chromatography/Mass Spectrometry

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A multiple solid-phase extraction (SPE) method was used with liquid chromatography, coupled with mass spectrometry (LC/MS), for the analysis of heterocyclic amines (HCAs) in human urine. Separation efficiencies based on the pH of the mobile phase and the types of columns were compared. An amide column showed better baseline separation and narrower HCA peak widths at pH 5.0 for the mobile phase than a C_8 column. Each SPE step, HLB, MCX, and HybridSPE, was optimized by controlling the pH conditions. The combined method with the three SPEs effectively removed interfering species that cause ion-suppression during HCA detection. Validation of the method, performed with SIM and SRM detection, showed correlation coefficients above 0.991 in the range 0.3 - 16.7 ng/mL. Recovery rates were 45.4 - 97.3% on the C_8 column and 71.8 - 101.4% on the amide column, and method detection limits were 0.11 - 0.65 ng/mL on the C_8 column and 0.12 - 0.48 ng/mL on the amide column. This method using multiple SPEs offers significant benefits for high-throughput determination of HCAs in urine.

Key Words: Heterocyclic amines, Urine, LC/MS, Solid-phase extraction

Introduction

Heterocyclic amines (HCAs) have been known to form during cooking foods such as fish and meat at high temperatures.[1-4] Toxicity studies of various organs of mice and rats suggest that HCAs are potent mutagenic and carcinogenic agents.[5-7] HCAs can be classified into amino-imidazoazarene and amino-carboline types based on the cooking temperature of protein-rich food. The former are known as thermic HCAs, which are formed by the Maillard reaction of free amino acids, creatine, and hexoses below 300 °C. The latter are pyrolytic HCAs, which are produced through the radical reaction of amino acids and proteins at temperatures above 300 °C. To date, more than 20 HCAs have been recorded in cooked foods,[8,9] and eight have been classified as either probable (class 2A) or possible (class 2B) carcinogens by the International Agency for Research on Cancer (IARC).[10]

The HCAs most frequently detected in cooked foods are 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), usually at concentrations of a few ng/g (ppb).[1,11-14] After consumption, the HCAs are rapidly absorbed and distributed across tissues and blood, and only 1 - 3% of ingested HCAs are excreted as intact compounds via urine.[15-17] In addition to intact compounds, HCAs can also be metabolized to conjugated compounds by hydroxylation, sulfation, glucuronidation and binding with DNA in human organs.[18-22] However, the amounts of intact and metabolized HCAs detected in urine are quite low (ng/mL).[23-25] Thus, for assessing human exposure to HCAs, an analytical method of urine analysis with high sensitivity, selectivity, and reproducibility would be of practical value.

Various extraction methods have been used for detecting HCAs in food, including liquid-liquid extraction (LLE),[26,27] supercritical fluid extraction (SFE),[28] and solid phase extraction (SPE),[29-32] but analysis of HCAs in urine has primarily been performed using SPE methods. SPE sorbents, such as Blue Rayon,[33,34] Blue Cotton,[25,35,36] and Blue Chitin[17,37] offer high selectivity for polycyclic aromatic compounds[38-40] and selectively isolate target HCAs from urine. However, the major obstacle in the processes of adsorption and desorption using SPE is the extraction efficiency. Recovery rates of HCAs using sorbents are around 50%, even using reliable methods.[15,23,41,42] Thus, the analytical problem of low recovery rates during the cleanup process indicates a need for a better routine analysis to quantify low amounts of HCAs.

HCAs are polar and non-volatile compounds that require chemical derivatization methods to improve detection selectivity and sensitivity when using gas chromatography.[26,43-45] However, yields from the derivatization reactions depend strongly on the class of HCAs and are limited to specific HCAs. For this reason, high-pressure liquid chromatography (HPLC) using reverse-phase columns has been preferred for the analysis of HCAs in urine.[46-49] UV detection has been a popular method, due to the high absorption coefficients of HCAs.[50-54] Additionally, mass spectrometry (MS) using electrospray (ESI)[55-59] or atmospheric pressure chemical ionization (APCI)[60-62] has been used in the detection of HCAs. These approaches provided a reduced matrix effect and sensitivity and selectivity were increased by using the selected ion monitoring (SIM)[13,30,34,63] and single reaction monitoring (SRM)[57,62,64,65] techniques. Both SIM and SRM detection in ESI mode are useful for obtaining low detection limits (DLs) to determine trace levels of HCAs in urine, but the SRM method may provide identification that is more distinct from interference than the SIM method.

To establish a reliable analytical method for quantification of HCAs in urine, chromatographic conditions need to be optimized according to pH, sample cleanup, and detection method(s). In this study, we compared separation efficiency using eight

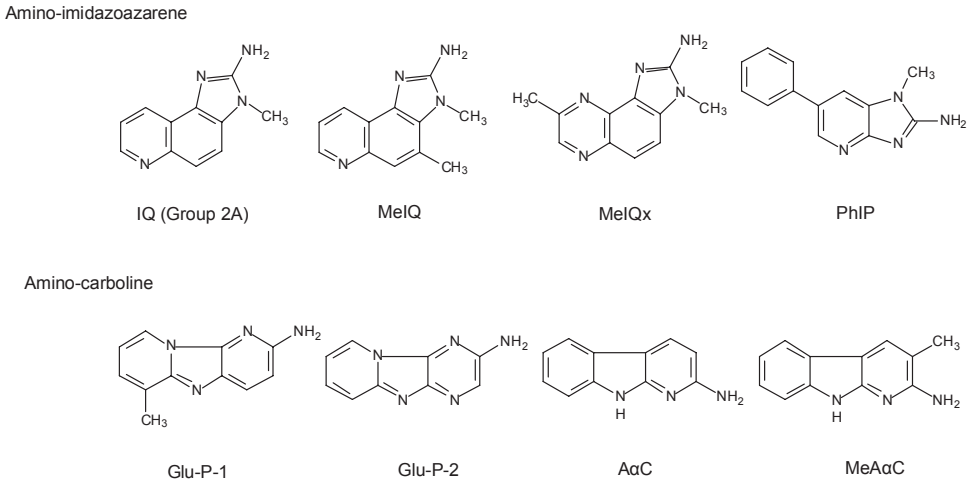

Figure 1. Chemical structures and abbreviated names of eight HCAs.

HCAs on three columns, C_{18}, C_8, and amide, under different pH conditions of the mobile phase. The use of a multiple SPE method to eliminate interference from human urine was also evaluated, and an optimized clean-up procedure is presented. Quality control using an analytical method based on two different acquisition modes, SIM and SRM, was established to evaluate human exposure to carcinogenic HCAs.

Experimental

Chemicals. The eight HCAs studied, 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), 2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole (Glu-P-1), 2-amino-dipyrodo[1,2-a:3',2'-d]-imidazole (Glu-P-2), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ), 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), 2-amino-9*H*-pyrodo[2,3-b]indole (AαC), and 2-amino-3-methyl-9*H*-pyrodo[2,3-b]indole (MeAαC), were purchased from Toronto Research Chemicals Inc. (Toronto, Canada); their chemical structures are shown in Figure 1. The internal standard, 1-naphthyl amine, was purchased from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile and methanol were purchased from J.T. Baker (Phillipsburg, NJ, USA), and water was purified using a Milli-Q system (Millipore Co., Bedford, MA, USA). Solutions of 25% ammonia and 37% hydrochloric acid were purchased from Merck (Darmstadt, Germany). Ammonium acetate, acetic acid, and formic acid were used as modifiers and were purchased from Fluka (Buchs, Switzerland). For SPE, an Oasis HLB cartridge (60 mg) and an Oasis MCX cartridge (30 mg) were purchased from Waters (Milford, MA, USA), and HybridSPE (30 mg) was purchased from Supelco (Bellefonte, PA, USA).

LC/ESI-MS. Chromatographic separation of eight HCAs using HPLC (NANOSPACE SI-2, Shiseido, Japan) was performed with Luna C_{18} (150 × 1 mm, 5 μm, Phenomenex, CA, USA), Luna C_8 (150 × 1 mm, 5 μm, Phenomenex), and Ascentis Express RP-Amide (150 × 2.1 mm, 2.7 μm, Supelco) columns. Efficiency in separating HCAs was studied based on pH, using mobile phases of water (A) containing 0.1% formic acid, 0.1% acetic acid, or 10 mM ammonium acetate with pH values of 2.6, 3.2, and 5, respectively. A gradient program was used with the C_{18} and C_8 separations for 0 - 20 min (5 - 70% B acetonitrile in A), and then the columns were equilibrated with 5% B for 20 min at a flow rate of 50 μL/min. For the amide column, a gradient program was used with 0 - 20 min (20 - 70% B acetonitrile in A), and then the column was equilibrated with 20% B for 15 min at a flow rate of 100 μL/min. A 2 μL sample aliquot was injected into the HPLC. The HPLC system was interfaced to an LCQ DECA XP MS system (Thermo Finnigan, San Jose, CA, USA) operated with an ESI source in positive mode with a spray voltage of 4.5 kV under N_2 sheath gas flow at 50 arbitrary units. The capillary temperature was maintained at 275 °C. Total ion chromatograms (TICs) of HCAs were obtained in the *m*/*z* range 100 - 300. The MS/MS spectra were acquired using different collision energies, depending on the type of HCA; detailed parameters are described in Table 1. Information for the quantitation ions used in SIM and SRM detection are also listed in Table 1.

Analytical method. A 3 mL urine sample was loaded into the HLB cartridge (60 mg), preconditioned with 1 mL of methanol (MeOH) and 1 mL of water. After washing with 2 mL of 1% NH_3, the eluent was collected using 3.5 mL of MeOH. The HLB eluent was loaded into the HybridSPE (30 mg), followed by addition of 1 mL of MeOH. The filtrate was collected and combined with 0.5 mL of 0.1 M HCl. The solution was loaded into the MCX cartridge (30 mg), preconditioned with 1 mL of MeOH. After washing with 1 mL of 0.1 M HCl and 1 mL of MeOH, the eluent was collected using 3 mL of 10% NH_3 in MeOH. Finally, the extract was dried under a stream of nitrogen gas and reconstituted with 100 μL of 1-naphthylamine (5 μg/mL) as an internal standard. An aliquot of 2 μL was injected into the LC/MS system described above. The analytical procedure for detection of HCAs in urine is shown in Figure 2.

Method validation. For linearity validation, standard solutions of eight HCAs in the range 0.3 - 16.7 ng/mL were prepared and measured at seven concentration points. The peak areas of the HCAs relative to the internal standard were plotted against

Table 1. SIM ions and characteristic ions observed in MS/MS spectra of HCAs

HCAs	Molecular weight (u)	$[M+H]^+$ or SIM ions (*m*/*z*)	Collision energy (%)	Characterized ions[a] (*m*/*z*)
IQ	198	199	45	199 $[M+H]^+$, **184[M+H-CH_3]$^+$**, 157[M+H-CH_3-HCN]$^+$
Glu-P-1	198	199	49	199[M+H]$^+$, 184[M+H-CH_3]$^+$, **182[M+H-NH_3]$^+$**, 172[M+H-NCN]$^+$, 145[M+H-$(HCN)_2$]$^+$
Glu-P-2	184	185	47	185 [M+H]$^+$, **168 [M+H-NH_3]$^+$**, 158[M+H-HCN]$^+$, 131 [M+H-$(HCN)_2$]$^+$
MeIQx	213	214	47	214[M+H]$^+$, **199[M+H-CH_3]$^+$**, 173[M+H-CH_3-CN]$^+$, 172[M+H-CH_3-HCN-CN]$^+$
MeIQ	212	213	45	213 [M+H]$^+$, **198[M+H-CH_3]$^+$**, 197[M+H-CH_4]$^+$, 172[M+H-CH_3-CN]$^+$, 145[M+H-CH_3-HCN-CN]$^+$
PhIP	224	225	49	225[M+H]$^+$, **210[M+H-CH_3]$^+$**
AαC	183	184	45	184[M+H]$^+$, **167[M+H-NH_3]$^+$**, 140[M+H-NH_3-HCN]$^+$
MeAαC	197	198	43	198[M+H]$^+$, 183[M+H-CH_3]$^+$, **181[M+H-NH_3]$^+$**, 154[M+H-NH_3-HCN]$^+$, 129 [M+H-$C_3H_5N_2$]$^+$

[a]Bold: ions selected for single reaction monitoring detection

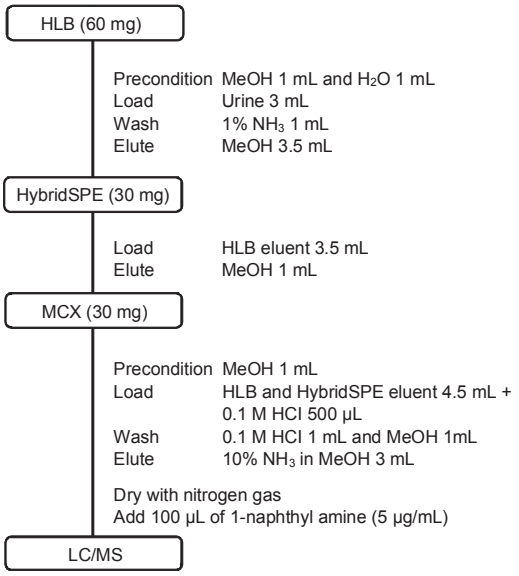

Figure 2. Analytical procedure for detection of HCAs in human urine.

the corresponding concentrations of the HCAs, and the linearity was calculated by a mean of the least-squares method. For accuracy and precision validation, HCA mixtures at two different concentrations (1.67 and 3.33 ng/mL) were spiked into HCA-free urine and nine replicate samples were analyzed according to an established analytical method. The limit of detection (LOD) and the limit of quantification (LOQ) were determined using urine spiked with 5 ng of the HCA mixture (n = 9), and the values were defined as 2.90 times the standard deviation (SD) and 10 times the SD, respectively.

Results and Discussion

Separation of HCAs using different columns and pH values. Mass detection using ESI requires ionized analytes to increase sensitivity after separation with a reverse-phase column. Thus, amine compounds such as HCAs require the use of an acid modifier in the mobile phase to maintain a lower pH than their pK_a during separation. However, at low pH ionized HCAs are quickly eluted, resulting in poorly resolved peaks on the reverse-phase columns, even though protonated HCAs provide enhanced sensitivity when using the ESI process. A pH effect is seen and maximum HCA sorption on the columns is attained between pH 3 and 7 and decreases steeply at pH < 3.[66]

When a conventional C_{18} column was used, the separation of polar IQ, MeIQ, MeIQx, Glu-P-1, and Glu-P-2 was better than that with less-polar PhIP, AαC, and MeAαC, based on LC parameters, such as flow rates and the pH of the mobile phase. The use of a 0.1% formic acid modifier of pH 2.6 in the mobile phase resulted in poor discrimination for IQ, MeIQ, and Glu-P-2 in the region of the void volume (Fig. 3a). The use of 10 mM ammonium acetate buffer of pH 5.0 resulted in sufficient interaction of polar HCAs with the C_{18} column and longer retention times, while MeIQx and Glu-P-1 were not separated as individual peaks (Fig. 3b).

C_8 columns are commonly used to retain HCAs longer, but the separation performance of a column is dependent on the type of stationary phase produced by the manufacturer. Similarly, at pH 2.6 on the C_8 column the polar HCAs were insufficiently separated in the region of shorter retention times (Fig. 3c). Additionally, the mobile phase of pH 3.2 containing a 0.1% acetic acid modifier did not provide sufficient separation using either the C_{18} or C_8 columns. In the pH-5.0 buffer, better peak separations of eight HCAs in the total ion chromatogram (TIC) with positive ESI were achieved using the C_8 column (Fig. 3d). For comparison with the C_8 column, an amide column with a polar stationary phase and orthogonal selectivity was used to achieve the appropriate separation performance. The amide column resulted in satisfactory peak resolution with narrower peak widths and shorter total runtimes than the C_{18} column (Fig. 4). In this study, the separation parameters of two C_8 and amide columns optimized at pH 5.0 were used to validate the established method described in Section 2.3.

HCAs are predominantly produced as protonated molecular

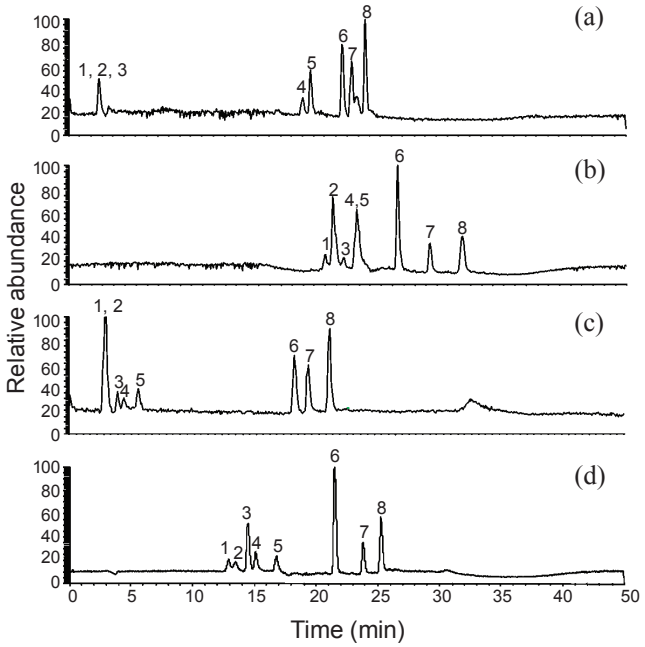

Figure 3. Total ion chromatograms of eight HCAs obtained at (a) pH 2.6 and (b) pH 5.0 using C_{18} columns, and (c) pH 2.6 and (d) pH 5.0 using C_8 columns by LC/ESI-MS. Peak identities are as follows: (1) IQ, (2) Glu-P-2, (3) MeIQx, (4) Glu-P-1, (5) MeIQ, (6) PhIP, (7) Aα C, and (8) MeAαC.

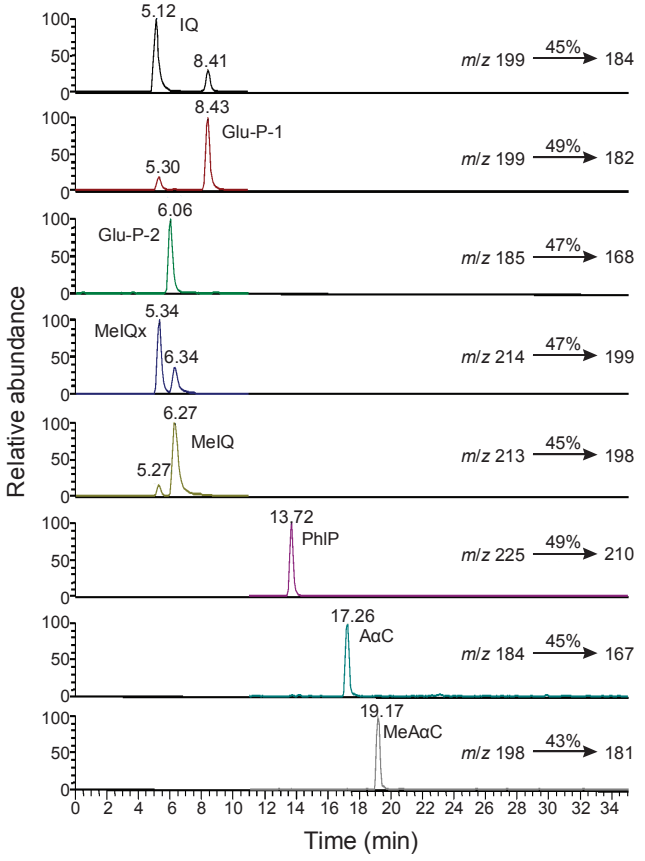

Figure 4. Single reaction monitoring chromatograms of eight HCAs at 20 ng/mL, separated using an amide column under the CID conditions described in Table 1.

ions, $[M+H]^+$, in the positive ion mode of ESI. To obtain structural information, the $[M+H]^+$ ions were selected as precursors for collision-induced dissociation (CID) to obtain MS/MS spectra. The maximum intensity of the fragment ions was provided to adjust the activation energies of the ion trap analyzer. The MS/MS spectra were classified into two groups according to the characteristic fragment ions produced. One was an aminoimidazoazarene group, which predominately produced the characteristic ions, $[M+H-CH_3]^+$, as base peaks in the MS/MS spectra. The product ion spectra of MeIQx showed another intense ion, $[M+H-C_2NH_3]^+$, representing the loss of 41 Da through the breaking of the aminoimidazole ring from the precursor ion. In the case of IQ, MeIQ, and PhIP, the neutral loss of C_2NH_3 resulted in minor fragment ions. The other group was an amino-carboline group, which commonly produced various fragment ions, $[M+H-NH_3+H_2O]^+$, $[M+H-NH_3]^+$, and $[M+H-HCN]^+$. MeAαC and Glu-P-1, which both contain a methyl group, produced additional fragment ions of $[M+H-CH_3]^+$.

Based on the mass spectra of HCAs, $[M+H]^+$ ions were selected for detection using the SIM mode for method development. Each characteristic ion, $[M+H-CH_3]^+$ for imidazoazarenes and $[M+H-NH_3]^+$ for carbolines, was also selected for SRM detection to validate the established method. The SIM and SRM parameters used in this study are summarized in Table 1.

Solid phase extraction (SPE) using various adsorbents. As discussed previously, pH is an important parameter in the SPE procedure and is related to reliable urine cleanup results. To evaluate the SPE procedure, a control water sample of 3 mL was prepared by spiking it with 20 μL of a mixture of eight HCAs (1 μg/mL). The HLB copolymer required a washing step (1 mL of 1% NH_3) to enhance adsorption of HCAs and remove interference from urine after loading the sample. No loss of HCAs occurred in this washing step. Unionized HCAs captured on the adsorbent in base condition using NH_3 were eluted with MeOH. The elution patterns of HCAs on the HLB sorbent showed that less polar HCAs eluted more slowly than the polar HCAs, due to strong interactions with the adsorbent. However, the eluent of 3.5 mL of MeOH was able to concentrate all HCAs (Fig. 5a). The recovery rates for this procedure ranged from 84.0 ± 15.2% to 105.6 ± 6.8%, indicating that the HLB cleanup procedure was appropriate.

In addition to the HLB procedure, an additional cleanup step was introduced to provide selective adsorption of basic HCAs. MCX is used as a cation exchange adsorbent and it requires low pH to adsorb positively ionized HCAs. Therefore, a washing step (0.1 M HCl) followed loading 20 ng of HCAs spiked into 3.5 mL of MeOH to capture HCAs on the MCX adsorbent. The eluent obtained by washing with HCl and MeOH contained no HCAs. A preliminary test of the elution behavior of HCAs using 60 mg of MCX adsorbent showed that less polar HCAs (i.e., PhIP, AαC, MeAαC) remained on the sorbent for a long time when eluting with a solution of 2, 5, or 10% NH_3 in MeOH. Therefore, MCX was unable to concentrate all HCAs within a small fraction, although interference from urine may have retained to this. This result is consistent with results reported by Sentellas *et al.*[24] MCX (30 mg) resulted in elution of all HCAs within 2 mL for 10% NH_3 in MeOH, as shown in the elution pattern in Figure 5b. The recovery rates of 80.2 ± 2.6% to 100.9 ±

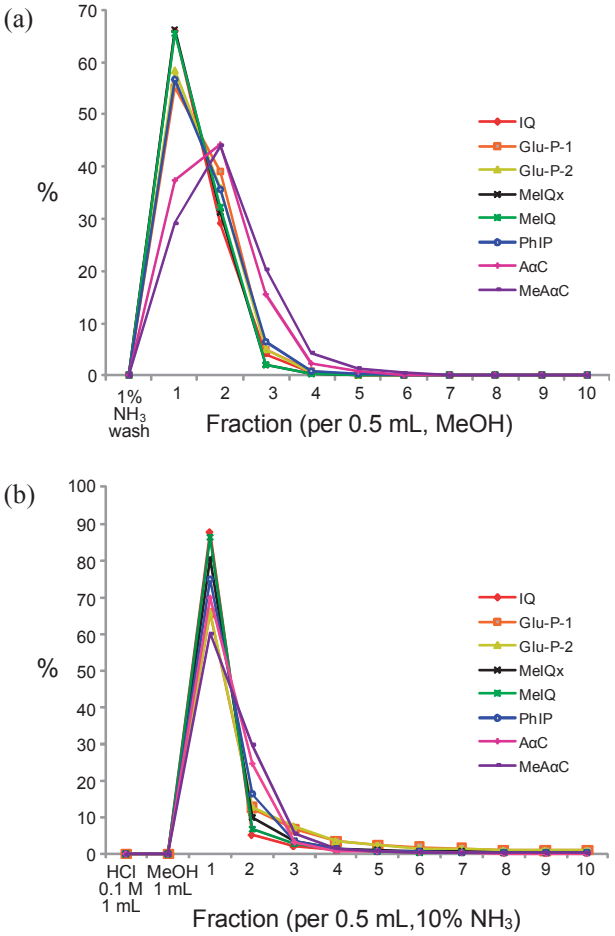

Figure 5. Elution patterns of eight HCAs using (a) HLB and (b) MCX sorbents.

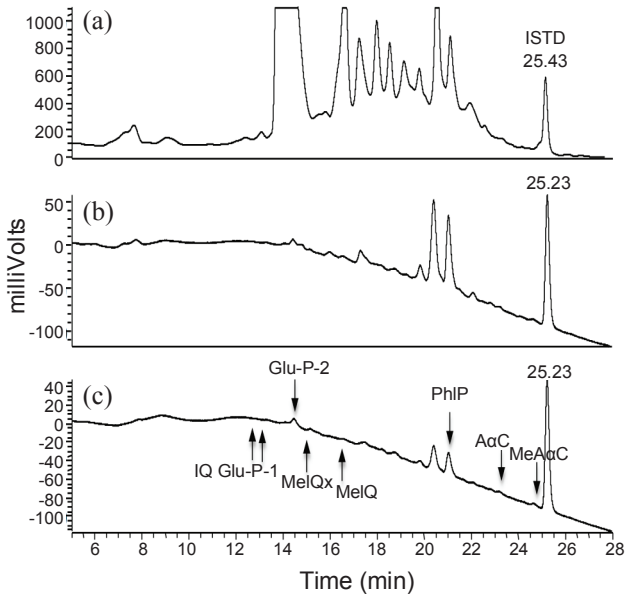

Figure 6. UV chromatograms of human urine spiked with 20 ng of HCAs after (a) HLB, (b) HLB and MCX, (c) HLB, HybridSPE, and MCX SPE.

Table 2. Equations of calibration curves for seven concentrations ranging from 0.3 to 16.7 ng/mL

HCAs	C_8 column		Amide column	
	Calibration equations	R^2	Calibration equations	R^2
IQ	0.3073x + 0.1223	0.991	1.7949x + 0.7649	0.995
Glu-P-1	1.0903x − 0.0984	0.999	2.6469x + 0.1937	0.998
Glu-P-2	0.4930x − 0.0171	0.997	2.2430x + 0.5660	0.993
MeIQx	0.7473x + 0.1222	0.998	1.8622x + 1.1720	0.992
MeIQ	0.7731x + 0.1083	0.997	2.4954x + 0.9165	0.991
PhIP	1.4692x + 1.3087	0.997	2.1195x + 1.9675	0.991
AαC	0.2934x + 0.0955	0.993	0.4195x − 0.2802	0.995
MeAαC	0.6106x + 0.2840	0.993	1.1792x − 0.7512	0.992

13.0% indicate that the adsorbent capacity was appropriate for the clean-up of all HCAs.

In a water matrix spiked with HCAs, recovery rates using the multiple SPE method were 51.4 ± 11.9% to 102.4 ± 4.5%, whereas the extraction efficiency for HCAs spiked into human urine using a combination of HLB and MCX cartridges was low, between 35.6 ± 10.0% and 85.2 ± 8.3%. It is likely that interference from urine that was co-eluted during chromatographic separation suppressed ion formation of HCAs during the ESI measurements. The lowest recovery rates were for PhIP, which has frequently been detected in human urine.[67,68] The ion suppression problem was revealed by UV chromatograms at 213 nm for each step of the SPE method. The chromatogram in Figure 6b shows that applying the MCX process after the HLB SPE (Fig. 6a) removed interference eluting in the range of shorter retention times on the C_8 column. However, interference eluting in similar regions with the retention time of PhIP was insufficiently washed out after these combined SPE steps, resulting in low recovery of PhIP. Thus, an additional cleanup step was needed to decrease the matrix effect. HybridSPE, which has been used primarily for precipitating proteins and removing phospholipids in plasma, was used as an alternative. The adsorbent, consisting of zirconia-coated silica particles, provided acid-base interaction and removed interfering species from urine that remained in the eluent after HLB-MCX SPE. Briefly, the basic eluent from the HLB adsorbent passed through the HybridSPE cartridge, and an additional 1 mL of MeOH was eluted. Then, the combined eluent was transferred onto the MCX cartridge. The tandem SPE method of HLB-HybridSPE-MCX showed successful wash-out of interference (Fig. 6c). The recovery rates also improved, to 54.7 ± 10.9% to 90.4 ± 7.8%, which are comparable with literature results. The recovery rate of HCAs has commonly been measured at 50%,[41,42] and the recovery rate of PhIP is generally lower than that of other HCAs.[38-40,67,68] The analytical procedure, optimized using tandem SPEs, is summarized in Figure 2.

Validation of analytical method. Calibration curves were generated using the peak ratios for eight HCAs against the internal standard of 1-naphthylamine. Standard mixtures of the eight HCAs were analyzed at seven concentrations, in the range 0.3 - 16.7 ng/mL. The concentration range of calibration is sufficient to measure amounts of HCAs in urine. The calibration curves

Table 3. Recovery rates, MDL and PQL measured by SIM and SRM detection using C_8 and amide columns

HCAs	C_8 column								Amide column							
	SIM				MRM				SIM				MRM			
	Recovery (%)		MDL	PQL	Recovery (%)		MDL	PQL	Recovery (%)		MDL	PQL	Recovery (%)		MDL	PQL
	1.67 ng/mL	3.33 ng/mL	(ng/mL)		1.67 ng/mL	3.33 ng/mL	(ng/mL)		1.67 ng/mL	3.33 ng/mL	(ng/mL)		1.67 ng/mL	3.33 ng/mL	(ng/mL)	
IQ	94.7 ± 5.7	71.4 ± 8.3	0.26	0.90	73.6 ± 7.7	83.9 ± 7.1	0.27	0.94	101.4 ± 4.5	78.3 ± 5.4	0.22	0.75	86.6 ± 10.2	81.5 ± 6.4	0.42	1.47
Glu-P-1	82.6 ± 5.5	57.3 ± 6.7	0.22	0.75	67.8 ± 8.6	61.1 ± 9.8	0.28	0.97	99.2 ± 5.3	80.3 ± 6.4	0.25	0.87	96.8 ± 5.4	81.9 ± 9.5	0.25	0.86
Glu-P-2	95.4 ± 5.4	81.4 ± 6	0.25	0.85	81.2 ± 5.9	78.6 ± 7.4	0.23	0.80	93.7 ± 6.2	77.3 ± 7.0	0.28	0.96	71.8 ± 8.3	75.0 ± 9.0	0.29	0.99
MeIQx	97.2 ± 6.7	67.7 ± 9.3	0.31	1.08	82.8 ± 16.4	77.8 ± 9.9	0.65	2.25	90.5 ± 6.6	83.9 ± 4.8	0.29	0.99	82.3 ± 12.1	81.4 ± 6.6	0.48	1.65
MeIQ	97.3 ± 4.7	75.3 ± 12.9	0.22	0.75	84.7 ± 2.8	86.7 ± 6.9	0.11	0.39	99.7 ± 6.1	92.9 ± 3.6	0.29	1.01	86.5 ± 7.2	92.3 ± 6.7	0.30	1.03
PhIP	58.2 ± 7.6	54.2 ± 10.8	0.21	0.74	45.4 ± 6.6	60.8 ± 6.9	0.14	0.50	88.6 ± 6.1	73.0 ± 6.3	0.26	0.89	83.9 ± 7.1	86.0 ± 8.5	0.29	0.99
AαC	75.2 ± 4.3	51.5 ±7 .5	0.16	0.54	78.2 ± 10.8	77.9 ± 7.1	0.41	1.41	98.0 ± 2.5	69.1 ± 6.1	0.12	0.41	83.3 ± 9.7	94.0 ± 7.4	0.39	1.35
MeAαC	88.6 ± 6.2	51.6 ± 9.8	0.26	0.91	75.4 ± 12.6	64.7 ± 7.1	0.46	1.58	83.9 ± 5.8	68.7 ± 5.8	0.23	0.80	80.1 ± 10.5	86.8 ± 8.6	0.41	1.40

of the HCAs on C_8 and amide separations showed good linearity within the given concentration ranges. The correlation coefficients ranged from 0.991 - 0.999 for the C_8 column and 0.991 - 0.998 for the amide column (Table 2).

To validate the accuracy and precision of the analytical method, two analytical concentrations, 1.67 and 3.33 ng/mL, were spiked into human urine. The samples (n = 9) were analyzed according to the established analytical procedure using SIM and SRM on two columns. The results are summarized in Table 3. The recovery rate did not depend on the detection method, SIM or SRM, nor on the amount of HCAs spiked into the urine. The amide column provided consistently higher recovery rates than the C_8 column, particularly for detection of PhIP. This was due to interference from matrix components that co-eluted with HCAs during separation on the C_8 column, but these components were resolved using amide separation due to the dissimilar interaction mechanisms of the columns.

The LODs were determined by performing nine analyses with 3 mL of urine spiked with 5 ng of the HCA mixture. The LOD values were 0.16 - 0.31 ng/mL for SIM and 0.11 - 0.65 ng/mL for SRM using the C_8 column, and 0.12 - 0.29 ng/mL for SIM and 0.25 - 0.48 ng/mL for SRM using the amide column. The SIM detection tool was more sensitive for detection of HCAs in urine than the SRM detection tool, although SRM is a more selective technique. The LOQs were defined as ten times the standard deviation, calculated when determining LODs. The LOQs represent the lowest concentration that could reliably be quantified. The LOD and LOQ results are shown in Table 3.

Conclusions

In this study, a tandem SPE method was designed to achieve higher recovery rates and better reproducibility for detection of HCAs in urine. Although the clean-up steps, as well as the column separation of HCAs, were sensitive to pH, verification of each step led to the development of an analytical method with high throughput. Separation of HCAs on the amide column allowed faster run times and narrower peak widths than with the C_8 column, although the difference was not always significant. The poor recovery of PhIP using a combination of HLB and MCX SPE was overcome by the addition of another ion exchange SPE, HybridSPE. Two SIM and SRM detection techniques in positive ESI were also evaluated for detection of HCAs. The responses were linear from the LOQ up to 16.7 ng/mL in urine, and the recovery rates for two concentrations of HCAs indicated acceptable accuracy and precision for analysis of HCAs in urine. In comparison with a C_8 column, an amide column, which operates on a different separation mechanism, provides better recovery. The analytical method established using multiple SPEs may be useful for detecting HCAs in urine to assess human exposure.

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References

1. Sugimura, T. *Carcinogenesis* **2000**, *21*, 387.
2. Sugimura, T. *Mutat. Res.* **1997**, *376*, 211.
3. Skog, K. I.; Johansson, M. A. E.; Jägerstad, M. I. *Food Chem. Toxicol*. **1998**, *36*, 879.
4. Alaejos, M. S.; González, V.; Afonso, A. M. *Food Additives & Contaminants* **2008**, *25*, 2.
5. Adamson, R. H.; Takayama, S.; Sugimura, T.; Thorgeirsson, U. P. *Environmental Health Perspectives* **1994**, *102*, 190.
6. Ohgaki, H.; Takayama, S.; Sugimura, T. *Mutat. Res*. **1991**, *259*, 399.
7. Wakabayashi, K.; Nagao, M.; Esumi, H.; Sugimura, T. *Cancer Res.* **1992**, *52*, 2092s.
8. Sanz Alaejos, M.; Ayala, J. H.; González, V.; Afonso, A. M. *J. Chromatogr. B* **2008**, *862*, 15.
9. Felton, J. S., Knize, M. G., Cooper, C. S., Grover, P. L., Eds.; *Handbook of Experimental Pharmacology*; Springer-Verlag: Berlin 1990; p 471.
10. IARC, Monographs on the evaluation of carcinogenic risk to humans, *Some Naturally Occurring Substances: Food items and constituents, Heterocyclic Aromatic Amines and Mycotoxins*; Lyon 1993; Vol. 56.
11. Gross, G. A.; Gruter, A. *J. Chromatogr. A* **1992**, *592*, 271.
12. Toribio, F.; Busquets, R.; Puignou, L.; Galceran, M. T. *Food Chem. Toxicol*. **2007**, *45*, 667.
13. Iwasaki, M.; Kataoka, H.; Ishihara, J.; Takachi, R.; Hamada, G. S.; Sharma, S.; Marchand, L. L.; Tsugane, S. *Journal of Food Com-*

position and Analysis **2010**, *23*, 61.
14. Persson, E.; Oroszvári, B. K.; Tornberg, E.; Sjöholm, I.; Skog, K. *International Journal of Food Science & Technology* **2007**, *43*, 62.
15. Malfatti, M. A.; Kulp, K. S.; Knize, M. G.; Davis, C.; Massengill, J. P.; Williams, S.; Nowell, S.; MacLeod, S.; Dingley, K. H.; Turteltaub, K. W.; Lang, N. P.; Felton, J. S. *Carcinogenesis* **1999**, *20*, 705.
16. Turesky, R. J.; Garner, R. C. *Chem. Res. Toxicol.* **1998**, *11*, 217.
17. Bang, J.; Frandsen, H.; Skog, K. *Chromatographia* **2004**, *60*, 651.
18. Zhou, H.; Josephy, P. D.; Kim, D.; Guengerich, F. P. *Biochemistry* **2004**, *43*, 981.
19. Rich, K. J.; Murray, B. P.; Lewis, I.; Rendell, N. B.; Davies, D. S.; Gooderham, N. J.; Boobis, A. R. *Carcinogenesis* **1992**, *13*, 2221.
20. Frandsen, H.; Nielsen, P. A.; Grivas, S.; Larsen, J. C. *Mutagenesis* **1994**, *9*, 59.
21. Buonarati, M. H.; Turteltaub, K. W.; Shen, N. H.; Felton, J. S. *Mutat. Res.***1990**, *245*, 185.
22. Lin, D.; Meyer, D. J.; Ketterer, B.; Lang, N. P.; Kadlubar, F. F. *Cancer Res.***1994**, *54*, 4920.
23. Reistad, R.; Rossland, O. J.; Latva-Kala, K. J.; Rasmussen, T.; Vikse, R.; Becher, G.; Alexander, J. *Food Chem. Toxicol.* **1997**, *35*, 945
24. Sentellas, S.; Moyano, E.; Puignou, L.; Galceran, M. T. *J. Chromatogr. A* **2004**, *1032*, 193.
25. Ushiyama, H.; Wakabayashi, K.; Hirose, M.; Itoh, H.; Sugimura, T.; Nagao, M. *Carcinogenesis* **1991**, *12*, 1417.
26. Murray, S.; Lynch, A. M.; Knize, M. G.; Gooderham, N. J. *J. Chromatogr.* **1993**, *616*, 211.
27. Tikkanen, L. M.; Latva-Kala, K. J.; Heiniö, R. L. *Food Chem. Toxicol.* **1996**, *34*, 725.
28. Thiebaud, H. P.; Knize, M. G.; Kuzmicky, P. A.; Felton, J. S.; Hsieh, D. P. *J. Agric. Food Chem.* **1994**, *42*, 1502.
29. Kondjoyan, A.; Chevolleau, S.; Greve, E.; Gatellier, P.; Sante-Lhoutellier, V.; Bruel, S.; Touzet, C.; Portanguen, S.; Debrauwer, L. *Food Chem.* **2010**, *119*, 19.
30. Persson, E.; Oroszvari, B. K.; Tornberg, E.; Sjoholm, I.; Skog, K. *International Journal of Food Science and Technology* **2007**, *43*, 62.
31. Turesky, R. J.; Goodenough, A. K.; Ni, W.; McNaughton, L.; LeMaster, D. M.; Holland, R. D.; Wu, R. W.; Felton, J. S. *Chem. Res. Toxicol.* **2007**, *20*, 520.
32. Oz, F.; Kaban, G.; Kaya, M. *Food Chem.* **2007**, *104*, 67.
33. Wu, J.; Wong, M. K.; Lee, H. K.; Ong, C. N. *J. Chromatogr. Sci.* **1995**, *33*, 712.
34. Kataoka, H.; Pawliszyn, J. C*hromatographia* **1999**, *50*, 532.
35. Murkovic, M.; Friedrich, M.; Pfannhauser, W. *Z. Lebensm.Unters. Forsch. A* **1997**, *205*, 347.
36. Janoszka, B.; Blaszczyk, U.; Warzecha, L.; Strozyk, M.; Damasiewicz-Bodzek, A.; Bodzek, D. *J. Chromatogr. A* **2001**, *938*, 155.
37. Bang, J.; Nukaya, H.; Skog, K. *J. Chromatogr. A* **2002**, *977*, 97.
38. Skog, K. *J. Chromatogr. B* **2004**, *802*, 39.
39. Yasuyoshi S, K. N.; Hitoshi, U.; Rika, G. *Mutat. Res.* **1993**, *300*, 207.
40. Masahiko, T.; Kenji, Y.; Taketoshi, N. *J. Chromatogr. A* **2001**, *928*, 53.
41. Viberg, P.; Wahlund, K. G.; Skog, K. *J. Chromatogr. A* **2006**, *1133*, 347.
42. Wakabayashi, K.; Ushiyama, H.; Takahashi, M.; Nukaya, H.; Kim, S. B.; Hirose, M.; Ochiai, M.; Sugimura, T.; Nagao, M. *Environ. Health Persp.* **1993**, *99*, 129.
43. Kataoka, H.; Kijima, K.; Maruo, G. *Bull. Environ. Contam. Toxicol.* **1998**, *60*, 60.
44. Skog, K.; Solyakov, A.; Arvidsson, P.; Jägerstad, M. *J. Chromatogr. A* **1998**, *803*, 227.
45. Murray, S.; Gooderham, N. J.; Boobis, A. R.; Davies, D. S. *Carcinogenesis* **1988**, *9*, 321.
46. Galceran, M. T.; Moyano, E.; Puignou, L.; Pais, P. *J. Chromatogr. A* **1996**, *730*, 185.
47. Toribio, F.; Moyano, E.; Puignou, L.; Galceran, M. T. *J. Chromatogr. A* **2000**, *869*, 307.
48. Ardvidsson, P.; van Boekel, M. A. J. S.; Skog, K.; Jägerstad, M. *J. Food Sci.* **1997**, *62*, 911.
49. Pais, P.; Salmon, C. P.; Knize, M. G.; Felton, J. S. *J. Argric. Food Chem.* **1999**, *47*, 1098
50. Shah, F. U.; Barri, T.; Jönsson, J. Å.; Skog, K.*J. Chromatogr. B* **2008**, *870*, 203.
51. Johansson, M. A. E.; Jagerstad, M. *Carcinogenesis* **1994**, *15*, 1511.
52. Skog, K.; Steineck, G.; Augustsson, K.; Jagerstad, M. *Carcinogenesis* **1995**, *16*, 861.
53. Knize, M. G.; Dolbeare, F. A.; Carroll, K. L.; Moore, D. H.; Felton, J. S. *Food Chem. Toxicol.* **1994**, *32*, 595.
54. Karamanos, N. K.; Tsegenidis, T. *J. Liq. Chromatogr. Rel. Technol.* **1996**, *19*, 2247.
55. Ni, W.; Mcnaughton, L.; Lemaster, D. M.; Sinha, R.; Turesky, R. J. *J. Agric. Food Chem.* **2008**, *56*, 68.
56. Turesky, R. J.; Taylor, J.; Schnackenberg, L.; Freeman, J. P.; Holland, R. D. *J. Agric. Food Chem.* **2005**, *53*, 3248.
57. Barceló-Barrachina, E.; Moyano , E.; Puignou, L.; Galceran, M. T. *J. Chromatogr. B* **2004**, *802*, 45.
58. Prabhu, S.; Lee, M. J.; Hu, W. Y.; Winnik, B.; Yang, I.; Buckley, B.; Hong, J. Y. *Anal. Biochem.* **2001**, *298*, 306.
59. Holland, R. D.; Taylor, J.; Schoenbachler, L.; Jones, R. C.; Freeman, J. P.; Miller, D. W.; Lake, B. G.; Gooderham, N. J.; Turesky, R. J. *Chem. Res. Toxicol.* **2004**, *17*, 1121.
60. Toribio, F.; Busquets, R.; Puignou, L.; Galceran, M. T. *Food Chem. Toxicol.* **2007**, *45*, 667.
61. Paisa, P.; Knize, M. G. *J. Chromatogr. B* **2000**, *747*, 139.
62. Guy, P. A.; Gremaud, E.; Richoz, J.; Turesky, R. J. *J. Chromatogr. A* **2000**, *883*, 89.
63. Gross, G. A.; Turesky, R. J.; Fay, L. B.; Stillwell, W. G.; Skipper, P. L.; Tannenbaum, S. R. *Carcinogenesis* **1993**, *14*, 2313.
64. Toribio, F.; Moyano, E.; Puignou, L.; Galceran, M. T. *J. Mass Spectrom.* **2002**, *37*, 812.
65. Busquets, R.; Bordas, M.; Toribio, F.; Puignou, L.; Galceran, M. T. *J. Chromatogr. B* **2004**, *802*, 79.
66. Inbaraj, B. S.; Chiu, C. P.; Chiu, Y. T.; Ho, G. H.; Yang, J.; Chen, B. H. *J. Agric. Food Chem.* **2006**, *54*, 6452.
67. Zimmerli, B.; Rhyn, P.; Zoller, O.; Schlatter, J. *Food Addit. Contam.* **2001**, *18*, 533.
68. Ristic, A.; Cichna, M.; Sontag, G. *J. Chromatogr. B* **2004**, *802*, 87.