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Trace Analysis of Mutagenic Heterocyclic Aromatic Amines in Cigarette Smoke Condensate and its Base Fractions via Silylation-GC-MS *

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SUMMARY

Among the more than 5000 chemicals reported in cigarette smoke condensate (CSC), heterocyclic aromatic amines (HAAs) are considered to be a contributor to observed biological activity. HAAs are non-volatile and are reported at ppb levels in CSC. A new method for HAA analysis at the trace level is reported here. *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane was employed to derivatize amino groups by heating the reagent containing a sample of CSC at 80 °C for 30 min followed by analysis employing gas chromatography-mass spectroscopy (GC-MS) in the selected-ion-monitoring (SIM) mode. This derivatization method afforded symmetrical peak shapes on a ZB-50 stationary phase and achieved instrumental limits of quantification (LOQ) at 10:1 S/N from ~1 ng/mL for AαC to120 ng/mL for Glu-P-1. The chemical identity of each derivative was confirmed by comparison of retention time and mass spectra of standards. The latter were characterized by the following ions: $M^{\bullet+}$ or $[M-1]^+$, $[M-15]^+$, and m/z 73 (i.e., trimethylsilyl). CSC and its base sub-fractions were studied using the GC-MS method. Ten HAAs were screened and five were quantified in cigarette smoke condensate, while 2-5 HAAs were quantified in each of three base sub-fractions. Values obtained with the new procedure agree well with values reported in the literature and with results obtained from a commercial laboratory via a different analytical method. The potential contribution of each HAA to the overall mutagenic activity observed for CSC and its base fractions is discussed. When considered together, HAAs account for only a small portion (~7.8%) of the observed mutagenicity of the CSC. [Beitr. Tabakforsch. Int. 25 (2013) 550–562]

ZUSAMMENFASSUNG

Unter den über 5.000 bekannten Chemikalien in Zigarettenrauchkondensat (CSC) wird von heterozyklischen aromatischen Aminen (HAA) angenommen, dass sie zur beobachteten biologischen Aktivität beitragen. HAA sind nicht flüchtig und sind im CSC in ppb-Konzentrationen nachweisbar. Es wird über eine neue Methode für die HAA-Analyse in Spurenmengen berichtet. *N,O*-Bis(trimethylsilyl)trifluoroacetamid (BSTFA) mit einem Trimethylchlorosilangehalt von 1% wurde eingesetzt zur Derivatisierung der Aminogruppen bei 80 °C für 30 min, gefolgt von einer Analyse mittels Gaschromatographie-Massenspektrometrie (GC-MS) im Selected-Ion-Monitoring-Modus (SIM-Modus). Diese Derivatisierungsmethode führte zu symmetrischen Peakformen auf einer stationären ZB-50-Phase und erreichte instrumentelle Bestimmungsgrenzen (LOQ) bei S/N 10:1 von ~1 ng/ml für AαC bis 120 ng/ml für Glu-P-1. Die chemische Identität jedes Derivats wurde durch Vergleich der Retentionszeiten und Massenspektren mit Standardsubstanzen bestätigt. Letztere wurden durch die folgenden Ionen charakterisiert: $M^{\bullet+}$ oder $[M-1]^+$, $[M-15]^+$ und m/z 73 (d. h. Trimethylsilyl-Reste). Das CSC und seine basischen Subfraktionen wurden mittels GC-MS-Verfahren untersucht. Zehn HAA wurden gescreent, fünf wurden im CSC quantifiziert und

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2–5 HAA wurden jeweils in drei basischen Subfraktionen quantifiziert. Die mit dem neuen Verfahren erhaltenen Werte entsprechen den Werten, die in der Literatur berichtet wurden und den Ergebnissen eines kommerziellen Labors unter Anwendung eines anderen analytischen Verfahrens. Es wird der mögliche Beitrag jedes HAA zur gesamten mutagenen Aktivität, die für CSC beobachtet wurde, und seiner basischen Fraktionen diskutiert. Zusammengenommen sind HAA nur für einen geringen Anteil (~7,8 %) der beobachteten Mutagenität des CSC verantwortlich. [Beitr. Tabakforsch. Int. 25 (2013) 550–562]

RESUME

Parmi les plus de 5000 composants chimiques répertoriés dans le condensat de fumée de cigarette (CFC), les amines aromatiques hétérocycliques (AAH) sont considérées comme contribuant à l'activité biologique observée. Les AAH sont non volatiles et ont été rapportées à des niveaux de ppb dans le CFC. Une nouvelle méthode d'analyse des AAH présents à l'état de traces est présentée ici. Le *N,O*-bis(triméthylsilyl)trifluoroacétamide (BSTFA) contenant 1% de triméthylchlorosilane a été employé pour dériver des groupes aminos en chauffant le réactif contenant un échantillon de CFC à 80 °C durant 30 minutes et en effectuant ensuite une analyse employant la chromatographie en phase gazeuse-spectrométrie de masse (GC-MS) en mode de détection sélective d'un ion (SIM). Cette méthode de dérivation a nécessité des pics symétriques sur une phase stationnaire ZB-50 et a atteint les limites instrumentales de quantification (LOQ) à 10:1 S/N à partir de ~1 ng/mL pour AαC jusqu'à 120 ng/mL pour Glu-P-1. L'identité chimique de chaque dérivé a été confirmée par comparaison du temps de rétention et du spectre de masse des standards. Ces derniers ont été caractérisés par les ions suivants : $M^{\bullet+}$ ou $[M-1]^+$, $[M-15]^+$, et m/z 73 (c.-à-d. triméthylsilyl). Le CFC et ses sous-fractions basiques ont été étudiés en utilisant la méthode GC-MS. Dix AAH ont été décelés et cinq quantifiés dans le condensat de fumée de cigarette, tandis que 2–5 AAH ont été quantifiés dans chacune des trois sous-fractions basiques. Les valeurs obtenues au moyen de cette nouvelle procédure sont tout à fait conformes aux valeurs rapportées dans la littérature et aux résultats obtenus d'un laboratoire commercial au moyen d'une méthode d'analyse différente. La contribution potentielle de chaque AAH à l'activité mutagène globale observée pour les CFC et ces fractions basiques est discutée. Considérés ensemble, les AAH contribuent à une petite portion (~7,8 %) de la mutagénicité du CFC observé. [Beitr. Tabakforsch. Int. 25 (2013) 550–562]

INTRODUCTION

Approximately twenty heterocyclic aromatic amines (HAAs) have been identified as potent mutagens by the Ames/*Salmonella* mutagenicity test. These compounds contain 2–5 condensed aromatic rings with one or more nitrogen atoms in the ring system and one exocyclic primary amino group (1). HAAs are formed during the heating of organic products containing nitrogenous compounds. The HAAs formed at 100–300 °C are known as thermic HAAs (i.e., IQ-type). All other HAAs formed above 300 °C are known as pyrolytic HAAs. Based on available data, the International Agency for Research on Cancer (IARC) has designated one HAA (IQ) as a probable human carcinogen and nine HAAs (MeIQ, $MeIQ_x$, PhIP, AαC, MeAαC, Trp-P-1, Trp-P-2, Glu-P-1, and Glu-P-2) as possible human carcinogens. (See Figure 1 for molecular structures.) IARC has recommended reducing exposure to these compounds (2) which are present in many substances including some cooked foods (3), coffee, alcoholic beverages, cigarette smoke, and polluted water and air. In 2012 the U.S. Food and Drug Administration placed IQ, PhIP, AαC, MeAαC, Trp-P-1, Trp-P-2, Glu-P-1, and Glu-P-2 on a list of harmful and potentially harmful constituents in tobacco products and tobacco smoke (4).

The presence of HAAs in tobacco smoke was first reported in 1962 (5). The compounds were harman and norharman (e.g., *β*-carbolines) which are not mutagenic, but may become co-mutagenic in the presence of other compounds including aromatic amines (6). Harman and norharman have also been observed to inhibit the mutagenicity of some compounds, including benzo[*a*]pyrene (7). Later, two amino-*α*-carbolines were identified in cigarette smoke condensate via HPLC with fluorescence detection (8). These compounds were 2-amino-9*H*-pyridol[2,3-*b*]indole and 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (i.e., AαC and MeAαC, respectively). It should be noted, however, that prior to this time other biologically active aromatic amines (as opposed to heterocyclic aromatic amines) had been identified in mainstream cigarette smoke (9). The late 1980s and early 1990s saw the identification of additional HAAs such as 2-amino-3-methyl-3*H*-imidazo[4,5-*f*]quinoline (IQ) (10) and then 2-amino-1-methyl-6-phenyl-1*H*-imidazo[4,5-*b*]pyridine (PhIP) (11) in cigarette smoke. MANABE (12) also reported measurable quantities of additional HAAs during this time. Unfortunately, published values of HAAs in cigarette smoke at this point ranged over orders of magnitude for any particular analyte. The consensus by workers in the field was that the uncertainty in HAA values was more a problem of sample preparation and analysis than variations in the composition of tobacco products.

During the past decade, considerable work has been devoted to the development of faster and more sensitive analytical methods for HAAs. Concurrently, heterocyclic aromatic amines with greater complexity have been identified for analysis. Some methods have involved a pre-analytical derivatization step and a tandem sample preparation procedure. For example, KATAOKA *et al.* (13) and KATAOKA and KIJIMA (14) developed a method for the determination of HAAs by gas chromatography with nitrogen-phosphorus selective detection in which the compounds were analyzed as their *N*-dimethylaminomethylene derivative. A blue cotton/rayon absorption method was employed to achieve efficient isolation and pre-concentration of the HAAs. Nevertheless, recoveries of HAAs spiked to cigarette smoke samples varied from 40 to 100%. Among the amines tested in the study, AαC, Trp-P-1, IQ, and MeIQ were detected in cigarette smoke. Data were presented that indicated the amount of HAAs in the smoke per gram of tobacco burned. After correction for recoveries, HAAs

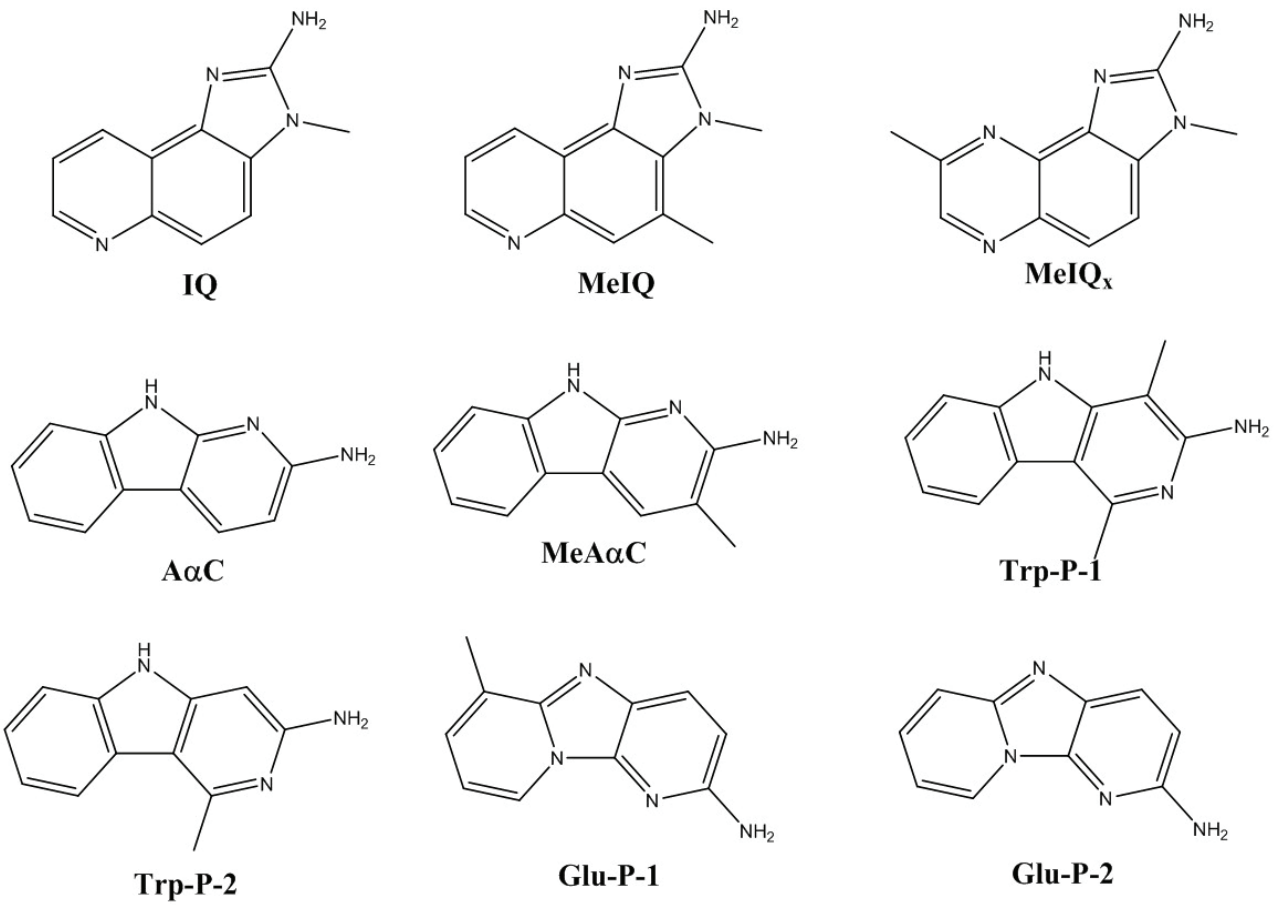

Figure 1. Molecular structure of HAAs discussed in this manuscript.

were reported in the range of ~0.2–5.0 ng/g for the two cigarettes tested.

In an effort to improve analytical sensitivity for these analytes in the CSC matrix, SASAKI *et al.* (15) developed a GC-MS method using negative chemical ionization and single ion monitoring detection. The procedure was based on a two-step derivatization by first acetylation with heptafluorobutyric anhydride and second, methylation with *N,N*-dimethylformamide dimethyl acetal. Using this procedure, PhIP and IQ could be analyzed at levels between 0.3 and 5.0 ng/cig. The technique was not considered sensitive enough for analysis of AαC, MeAαC, and Trp-P-1. Furthermore, levels of Trp-P-2, Glu-P-1, and Glu-P-2 were stated to be below the detection limit, which was estimated at about 0.1–0.5 ng/cig.

This method was later modified for study of simpler aromatic amines to include two solid-phase extraction steps of different chemistries rather than a single solid-phase step with basically one retention mechanism (16). The first step used a cation-exchange resin followed by a second step that used a solid-phase resin with hydrophobic retention character. The aromatic amines, after elution from the second SPE cartridge, were then derivatized with heptafluorobutyric anhydride, and analyzed via GC-MS as described above. Detection limits ranged from 0.02 ng/cig for toluidine to 1.41 ng/cig for aniline, and spiked recoveries were 79 to 109%, respectively. No HAAs were measured in the investigation.

SMITH *et al*. (17) reported the analysis of harman, norharman, AαC, and MeAαC in the mainstream smoke of three reference cigarettes wherein solid-phase extraction using an exchange resin which possessed reverse-phase character was employed. In the study, GC-MS was employed without derivatization for quantification in the single ion mode. Greater amounts of carbolines were found in "full-flavor" than in "ultralight" cigarette smoke condensate. Norharman was found to be approximately three times more abundant than harman; AαC and MeAαC were 10–50 times less abundant than harman.

Most recently, ZHANG *et al*. (18) reported the analysis of harman, norharman, AαC, MeAαC, Trp-P-1, and Trp-P-2 in mainstream cigarette smoke using isotope dilution liquid chromatography-electrospray ionization tandem mass spectrometry. Without derivatization, HAA concentrations were determined in the mainstream smoke from U.S. commercial, Kentucky reference and custom-made unfiltered cigarettes. These samples were generated with two different smoking regimens. After smoking, sample preparation consisted of adding an internal standard to a Cambridge filter extract prior to instrumental analysis. Greater amounts of carbolines were found in mainstream smoke from cigarettes made from Burley tobacco than from cigarettes made with flue-cured tobacco. Mainstream smoke carboline yields were also greater with the Canadian in-

tense smoking regimen than with the standard ISO smoking regimen. On average, norharman was found to be approximately two times more abundant than harman; AαC, MeAαC, Trp-P-1, and Trp-P-2 were 1–3 orders of magnitude less abundant than harman.

While much research has been conducted to determine the chemical basis for the biological activity observed for cigarette smoke, the relative contribution of individual compounds to specific biological responses generally remains unknown. To address this issue in part, a new method for HAA analysis at the trace level is reported here. The method has been successfully applied to CSC and its base sub-fractions. Ten HAAs were screened and five were quantified in cigarette smoke condensate; while 2–5 HAAs were quantified in each of three base sub-fractions. The potential contribution of HAAs to the overall mutagenic activity observed for CSC and its base fractions is discussed.

EXPERIMENTAL

Materials

The following chemicals were purchased in order to establish protocols for standardization and quantification: 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC), 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC), 2-amino-3-methyl-3*H*-imidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethyl-3*H*-imidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethyl-imidazo[4,5-*f*]quinoxaline ($MeIQ_x$), 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole acetate (Trp-P-1 acetate), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole acetate (Trp-P-2 acetate), 2-amino-6-methyldipyrido[1,2-*a*:3',2'-*d*]imidazole hydrochloride hydrate (Glu-P-1 hydrochloride hydrate), and 2-aminodipyrido[1,2-*a*:3',2-*d*]imidazole hydrochloride (Glu-P-2 hydrochloride) (Toronto Research Chemicals Inc., Toronto, Canada). For reference purposes molecular structures for these compounds are shown in Figure 1. Also purchased from Toronto Research Chemicals was 2-amino-3-trideuteromethyl-3*H*-imidazo[4,5-*f*]quinoline which served as the internal standard (IS) in this study. *N,O*-Bis(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS), *N,N*-dimethylformamide (DMF), and norharman were obtained commercially from Sigma-Aldrich (St. Louis, MO). Solid-phase extraction cartridges were purchased from both EMD Chemicals (Gibbstown, NJ) and Waters Corp. (Milford, MA). The initial cartridges were Extrelut-1 (1 g) followed by MCX solid-phase extraction cartridges which contained a mixed mode cation exchange resin (6 mL/150 mg). Sodium sulfate, dichloromethane, ethyl acetate, methanol, hexane, and acetic acid sources were Sigma-Aldrich. Methyl *tert*-butyl ether (MTBE) came from Acros Organics (Brussels, Belgium).

Stock solutions

Each HAA, which was analytical reagent grade unless noted otherwise, was freshly dissolved in methanol to make a stock solution (20 µg/mL) as needed on the day of analysis. Glu-P-1 and Glu-P-2 (which were not analytical reagent grade) were found to have limited stability with both derivatized compounds, significantly degrading over time in solution under ambient conditions.

CSC collection

CSC was collected by smoking 2R4F Kentucky reference cigarettes (Tobacco and Health Research Institute, Lexington, KY) on an AMESA Type 1300C smoke generator (Amesa Aeromecanique, Geneva, Switzerland) under historical Federal Trade Commission (FTC) smoking conditions (35 mL puff of 2 s duration taken once/min to a butt-length of 3 mm beyond the filter overwrap) (19).

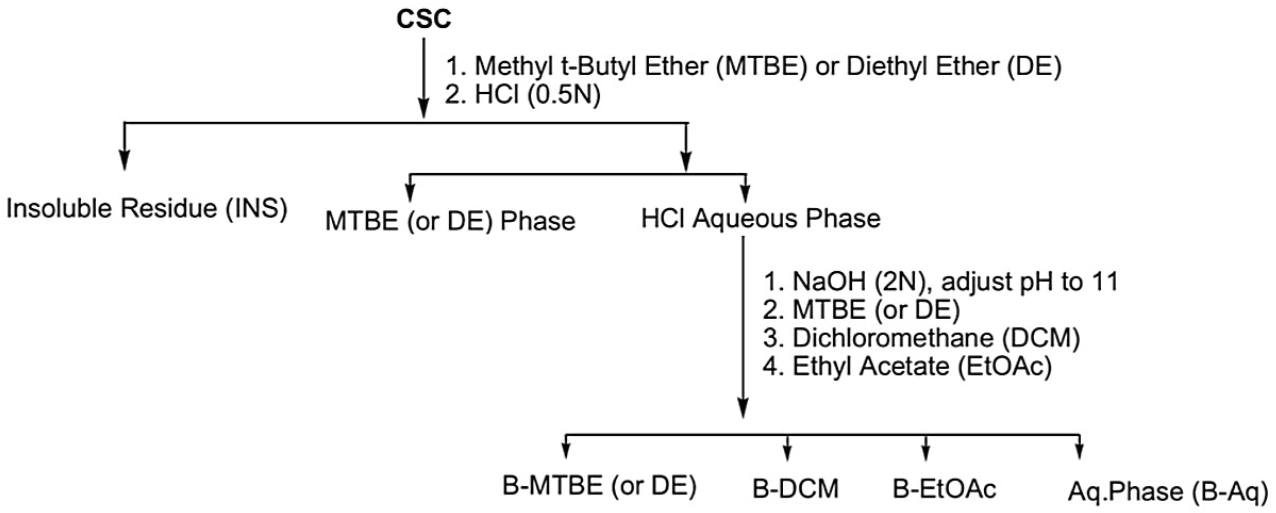

Figure 2. CSC Fractionation scheme.

Cigarette smoke was delivered to a CSC cold-trapping system with a temperature range of −10 °C to −70 °C (20). Condensate sub-sample preparations were kept frozen at −20 °C until they were extracted from the trap by rinsing with high-purity acetone. Several daily condensate sub-sample collections were then combined to create a pooled CSC sample. The acetone was partly removed using rotary evaporation at 40 °C until the concentration of CSC approached ~80–100 mg/mL.

CSC fractionation

Anhydrous sodium sulfate (~25 g/3 g of CSC) was initially added to the CSC acetone solution to remove extraneous water. The resulting CSC solution was then evaporated to dryness under a nitrogen atmosphere to a constant weight. The dried material was next partially dissolved with stirring in a binary solution composed of 200 mL 0.5 M hydrochloric acid and either 200 mL of MTBE or diethyl-ether (DE). An insoluble precipitate (INS) was observed and collected in addition to the MTBE (or DE)-acidic water solution. The resulting two-phase solution was transferred to a separatory funnel where ether-water partitioning was accomplished. This procedure was repeated three times, after which the individual phases were subsequently combined. The aqueous phase that was assumed to contain basic salts was adjusted to pH 11 using 2 M NaOH. Liquid-liquid extraction was conducted again, but in a sequential manner using first MTBE (or DE), then dichloromethane (DCM), and finally EtOAc. After removing water with sodium sulfate as described before, each organic solvent phase was dried under a nitrogen atmosphere to constant weight to form three base sub-fractions (i.e., B-MTBE (or DE), B-DCM, and B-EtOAc). A TurboVap II concentration workstation (Caliper Life Sciences, Hopkinton, MA) was employed to evaporate the various organic phases using a nitrogen stream. After extensive organic solvent partitioning, the aqueous raffinate was adjusted to pH 7 with 2 M HCl. Aliquots of the resulting aqueous phase were added to 50 mL polypropylene conical tubes (Becton-Dickinson Labware, Franklin Lake, NJ) and frozen. The frozen sample was placed in a Virtis Genesis Model 25ES freeze dryer (Gardiner, NJ) and evacuated for 72 hours at room temperature. The dried aqueous residue (B-Aq) was weighed immediately and stored over indicating Drierite in a desiccator until use. Figure 2 summarizes the CSC fractionation scheme applied.

CSC and base sub-fraction sample preparation

After addition of 60 ng of the 2-amino-3-trideuteromethyl-3*H*-imidazo[4,5-*f*]quinoline internal standard (added as 150 µL of a 400 ng/mL solution) to a known amount of CSC (~81 mg) or base sub-fraction (Base-MTBE, 70 mg; Base- DCM, 70 mg; Base-EtOAc, 29 mg), the sample was dried at room temperature to constant weight via dynamic nitrogen purge at 40 psi. After drying, the sample was extracted with 20 mL of 0.1 M HCl/20 mM acetic acid with the aid of a Burrell wrist-action shaker for 30 min. The gravity-filtered extract solution (20 mL) was passed through a fresh Extrelut-1 SPE cartridge after which the eluent was loaded onto a fresh MCX mixed mode cation-exchange cartridge that had been pre-conditioned with 5 mL of acetone followed by 5 mL of water. The MCX cartridge was next washed with 5 mL of 0.1 N HCl and 5 mL acetone. The cartridge and its contents were vacuum dried at 10 mm Hg for 5 min then analytes were eluted with 5 mL of a mixture of acetone and NH_4OH (98:2, v/v). Eluents were dried and reserved for derivatization. The seven steps employed in this process are summarized in Figure 3.

Chemical derivatization

Trimethylsilyl derivatives of CSC and its base sub-fractions plus HAA standards were prepared following published procedures (21, 22). A known aliquot of the SPE eluent solution in a 2-mL GC vial was dried under nitrogen at 70 °C; then 50 µL DMF and 100 µL BSTFA with 1% TMCS were added to the vial. The mixture was then capped and heated at 80 °C for 30 min. The derivatized products were allowed to cool after which 2 µL of the vial contents was manually injected into the gas chromatograph for separation and mass spectral analysis, *vide infra*.

Instrumental analysis

The derivatized samples were analyzed with an Agilent Model 6890 gas chromatograph interfaced to an Agilent MSD 5973 mass spectrometer and Chemstation data system. Separation was performed on a ZB-50 (50% phenyl-50% dimethyl-polysiloxane, 30 m × 0.25 mm i.d. × 0.25 µm film thickness) column (Phenomenex, Torrence, CA). The gas chromatographic oven was held at 120 °C for 1 min then first increased to 200 °C at 20 °C/min, then increased at 5 °C/min to 280 °C and increased again to 315 °C at 20 °C/min where it was held for 10 min. The injection volume was 2 µL and the injection mode using a Uniliner® Liner (Restek, Bellefonte, PA) was pulsed splitless at 30 psi for 1 min. The mass spectrometer transfer line was heated at 260 °C, and the injector temperature was 280 °C. The helium flow rate was fixed at 1 mL/min. Ionization voltage was 70 eV and the mass spectrometer was operated in single-ion-monitoring (SIM) mode. SIM parameters for quantification of each of the HAAs are shown in Table 1. All sample analysis was conducted in triplicate.

Instrument calibration

To prepare calibration standards, each HAA was freshly dissolved in methanol to make a stock solution (20 µg/mL) as needed on the day of analysis. As previously stated, this was necessary because derivatized Glu-P-1 and Glu-P-2 severely degraded over time in solution under light and ambient environmental conditions. Five concentrations of each analyte were prepared by appropriate dilution of the stock standard to achieve calibration ranges (ng/mL) as follows: Norharman, 30–480; AαC, 2–32; MeAαC, 2–32; IQ, 10–160; MeIQ and $MeIQ_x$, 10–160; Trp-P-1, 30–480; Trp-P-2, 10–160; Glu-P-1, 120–1920; Glu-P-2, 120–1920. Calibration coefficients of linear regression obtained for each HAA were 0.99, or better.

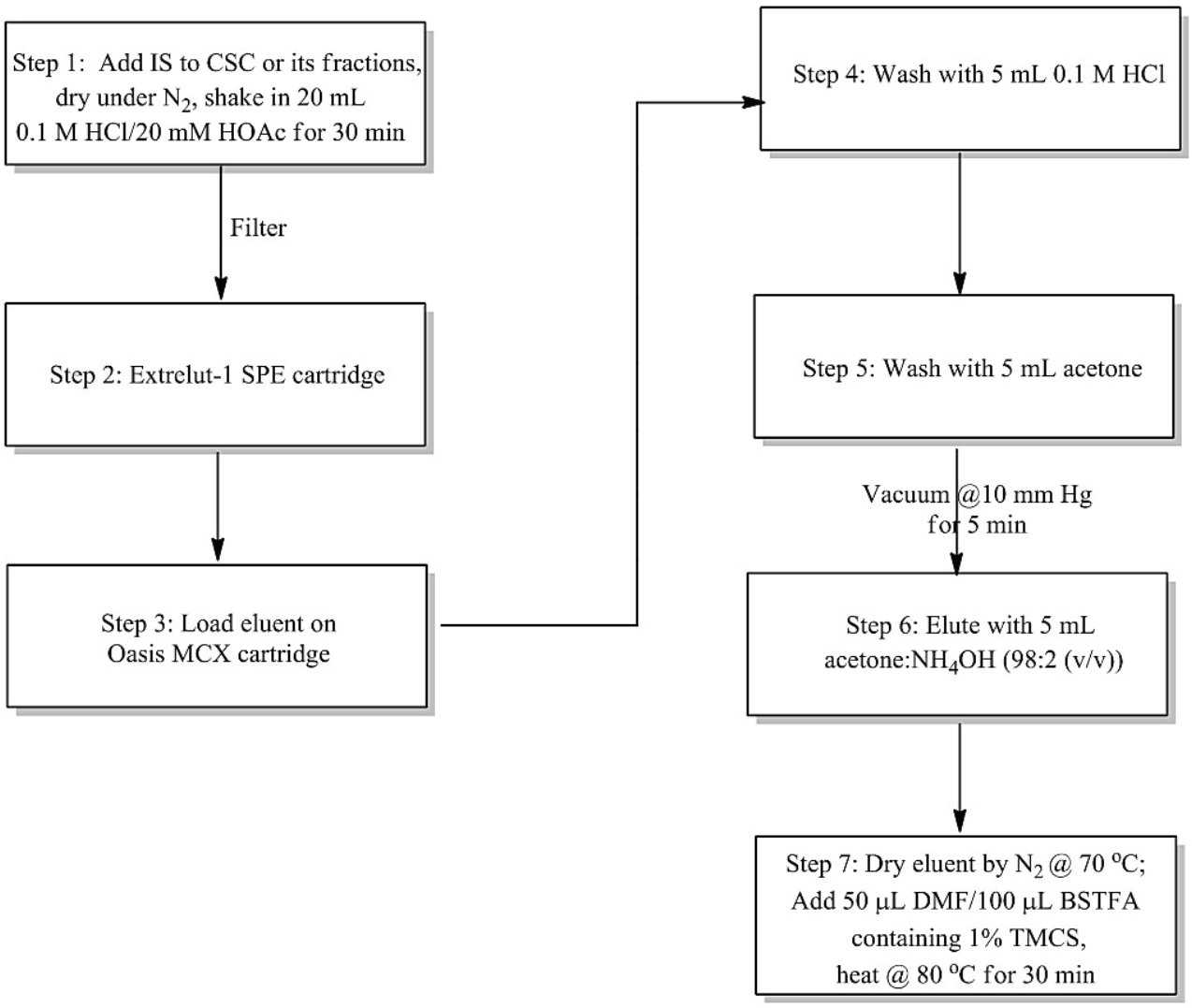

Figure 3. CSC sample preparation and subsequent derivatization procedure.

Table 1. SIM experimental parameters.

SIM	Start time (min)	Mass @ Dwell (ms)
Group 1	6.00	Norharman (240@100, 225@10) Glu-P-1 ((327@100, 342@10) Glu-P-2 (313@100, 328@10)
Group 2	15.00	AαC (327@100, 312@10) IQ (341@100, 327@10) MeAαC (341@100, 326@10) Trp-P-1 (340@100, 355@10) Trp-P-2 (326@100, 341@10) MeIQ (355@100, 341@10)
Group 3	19.00	$MeIQ_x$ (342@100, 357@10)

Contracted analyses of HAAs

LABSTAT INTERNATIONAL ULC (Kitchener, ON, Canada) conducted the determination of selected HAAs from the same CSC matrix using Labstat Method TMS-133 in order to corroborate the results obtained with the new method (23). Per Method TMS-133, 1 mL of cigarette smoke condensate extract (acetone) was transferred into a 15-mL culture tube and mixed well with 30 µL of D3-IQ internal standard before being evaporated to complete dryness under nitrogen at 55 °C. The sample was reconstituted with 15 mL of 0.1 M Hcl/20 mM acetic acid solution and purified by solid-phase extraction (SPE) using a 3 mL/200 mg cation-exchange resin cartridge. The HAAs were eluted with an acetonitrile/concentrated ammonium hydroxide solution. The eluent from the SPE cartridge was again evaporated to dryness and the residue reconstituted with 300 µL of acetonitrile for GC-MS analysis. The HAAs were separated on a 30 m × 0.25 mm i.d. × 0.25 µm film thickness ZB-50 column and quantified by using a Varian 1200 triple quadrupole mass spectrometer using multiple-reaction monitoring (MRM) mode.

Contracted Ames assay

A pre-incubation modification of the Ames *Salmonella*/microsome plate assay (24–25) was used to evaluate mutagenic activity. Assays were conducted by Labstat

Laboratories, ULC. *Salmonella* tester strain TA98 was used in the presence of the liver homogenate (S-9, 10% v/v). The S-9 (Mol-tox, Boone, NC) was prepared from Aroclor 1254-induced Sprague Dawley rat liver. A brief summary of the assay is presented here. 0.5 mL of S-9 mix was added to sterile tubes. 100 µL of tester strain and 50 µL of DMSO-diluted sample or positive control were then added. The solution was vortexed and incubated at 37 ± 1 °C for 20 ± 5 min prior to the addition of 2 mL of selective top agar. The contents were poured onto minimal glucose agar plates and incubated at 37 ± 1 °C for 48 to 72 hours before scoring. Test samples were dissolved in DMSO and at least five non-zero concentrations were assayed for each sample. Final concentration ranges evaluated (µg/plate) were as follows: IQ, 0.00003–0.03; MeIQ, 0.00001–0.3; $MeIQ_x$, 0.0003–0.3; Glu-P-1, 0.0003–0.3; Trp-P-2, 0.0005–0.1; Trp-P-1, 0.003–3; AαC, 0.05–33; MeAαC, 0.5–333; Glu-P-2, 0.05–333; Norharman, 10–500; CSC, 100–1000. Triplicate plates were used for each concentration. At least three non-toxic doses were required for determination of the dose-response curve.

RESULTS AND DISCUSSION

Overview

Both detection and quantification of HAAs present in CSC at parts per billion concentrations are analytically challenging. While BSTFA is often used to derivatize alcohols, phenols, and carboxylic acids to the corresponding silyl ether and ester moiety, its use with heterocyclic aromatic amines has not previously been reported. However, BATY *et al*. (26) employed BSTFA to replace the primary alkyl amine hydrogen of primaquine with a trimethylsilyl group. Chromatographic separation must be performed in order to (a) remove interferences, (b) pre-concentrate HAAs, and (c) isolate the analytes for quantitative analysis. CSC is sufficiently complex to warrant tandem sample preparation procedures, whereby different sorbents and eluents are combined to provide sample clean-up. For HAA analysis, however, a matrix with less interference does not necessarily guarantee a successful result. Most HAAs are polar and non-volatile, and when analyzed by GC they tend to elute as broad tailing peaks due most likely to their strong adsorption to the stationary phase and injector. For this reason, high performance liquid chromatographic methods with ultraviolet, electrochemical, or fluorescence detection have been used for quantification of HAAs (27). Gas chromatography following derivatization, on the other hand, affords many advantages for mass spectral detection of HAAs at their usual low concentrations versus HPLC. The following discussion describes an excellent new option for HAA analysis of CSC and its base sub-fractions via GC-MS. As will be disclosed, the method should find application in the analysis of mainstream cigarette smoke as well.

Spiked HAA recovery from CSC

HAAs are present at parts per billion in CSC; therefore, it is important to remove as many matrix components as possible in order to enhance the reliability, precision, and accuracy of HAA analysis. In this study, impurities in CSC were removed with Extrelut-1 followed by MCX cation-exchange SPE. The negatively charged sulfonate groups on the MCX resin are thought to have a strong electrostatic attraction toward protonated HAAs which allows efficient removal of other neutral and acidic CSC matrix components. In order to assess our analytical results concerning HAAs in CSC, *vide infra*, spiked recovery data were of interest. About 10 mg of CSC was spiked with 10 HAAs (1–4 mg/HAA) and an internal standard (150 µL of a 40 ng/mL solution containing 2-amino-3-trideuteromethyl-3*H*-imidazo[4,5-*f*]quinoline internal standard). The mixture was subjected to the tandem SPE procedure described in the Experimental section under "Sample preparation" followed by trimethylsilylation of the extracted HAAs. The recovery of each HAA was calculated against the concentration of original HAAs without CSC. Average recovery for eight HAAs ranged between 86.6 and 106.8% (Table 2). As observed in Table 2, recovery results were generally similar with and without the addition of internal standard. Regardless, an internal standard was employed in all subsequent analysis to compensate for possible HAA loss during the SPE cleanup step in sample preparation.

The recoveries of Glu-P-1 and Glu-P-2 were of special interest as their derivatives were prone to degradation. Acceptable stability for these analytes in calibration standards was achieved by freezing freshly prepared, derivatized standard solutions at −20 °C, thus quenching compound degradation. Using standard solutions prepared in this manner, Glu-P-1 and Glu-P-2 were found to be present at extremely low concentration in CSC and its three base subfractions. Also, investigation of Glu-P-1 and Glu-P-2 stability in CSC via the method of standard addition demonstrated that the two analytes did not degrade in CSC.

Table 2. Average percent recovery (n = 3) of spiked HAAs from CSC after tandem SPE and trimethylsilylation with and without Internal Standard (IS).

Analyte	With IS [a]		Without IS	
	Recovery	RSD (%)	Recovery	RSD (%)
Norharman	94.3	6.3	86.6	11.1
AαC	106.8	5.7	106.5	4.7
MeAαC	100.9	2.9	101.6	3.1
IQ	98.8	0.7	99.6	4.2
MeIQ	100.2	1.5	101.0	2.5
$MeIQ_x$	93.5	2.7	93.0	2.9
Trp-P-1	100.8	5.4	101.6	3.0
Trp-P-2	93.7	7.0	94.1	8.0
Glu-P-1	106.2	6.3	106.2	6.3
Glu-P-2	92.9	9.7	90.9	13.4

[a] 2-amino-3-trideuteromethyl-3H-imidazo[4,5-f]quinoline was added as the internal standard (IS) before sample preparation

Limits of quantification

In 2001, SASAKI *et al.* (15) utilized a two-step chemical derivatization in which HAAs were first acetylated by heptafluorobutyric acid anhydride to give the amide, and then the second hydrogen was methylated with *N,N*-

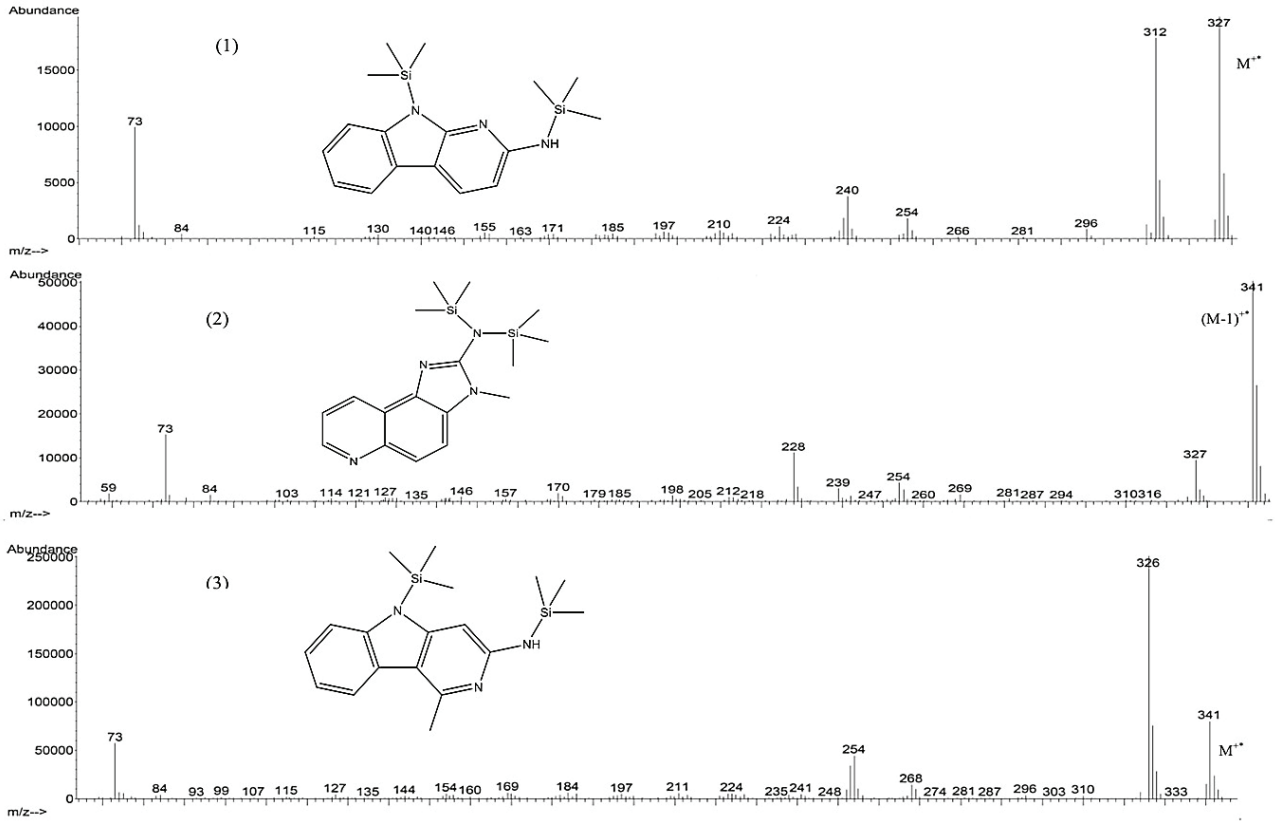

Figure 4. Mass spectra of silylated HAAs. (1) A C, (2) IQ, and (3) Trp-P-2. Mass spectrometer ionizing voltage was 70 eV. GC conditions were as stated in Figure 5.

dimethylformamide dimethyl acetal. Low ppb detection limits were achieved for some HAAs using this derivatization technique with GC-MS analysis. A novel approach involving trimethylsilylation of HAAs and GC-MS analysis was applied in this study with the expectation that silylated HAAs would be very volatile and exhibit symmetrical chromatographic peaks, thus potentially improving limits of quantification.

The molecular composition of silylated HAAs has been previously elucidated by GC-MS analysis (28). Trimethylsilyl derivatives of HAAs were characterized by (a) molecular ion $M^{\bullet+}$ or $[M-1]^+$, (b) $[M-15]^+$ ($M-CH_3$), and (c) trimethylsilyl m/z 73. The mass spectra for selected derivatized HAA standards prepared in this work are shown in Figure 4. These fragments coupled with measured retention times have led to unambiguous identification of HAAs in the cigarette-derived matrix.

In addition to forming chromatographically well-behaved derivatives, a Uniliner® Liner (Restek, part #21054) was employed to potentially improve sensitivity of the new method. A Uniliner® Liner improves sensitivity by essentially introducing all vaporized analyte into the GC-MS. GC-MS analysis of a mixture of eight derivatized HAAs under these conditions showed excellent sensitivity and baseline resolution with the ZB-50 stationary phase (Figure 5).

Table 3 summarizes limits of quantification found in this work. Based upon a chromatographic peak signal-to-noise of 10:1, instrumental limits of quantification (LOQ) from ~1 ng/mL for AαC to 120 ng/mL for Glu-P-1 were achieved with the new method. Overall method LOQs ranging from ~0.004–0.222 µg/g CSC for the new method and ~0.06–1.6 µg/g CSC for Labstat method TMS-133. In the case of the new method, LOQs were calculated based upon the lowest instrument calibration concentration, the amount of sample derivatized, and the final sample volume (Table 3). While our investigation yielded results greater than or equal to the Labstat LOQs for six HAAs, other reports which involved either mass spectrometric detection or nitrogen-phosphorus detection (14, 15, 18) were not as good. Thus, use of a tandem SPE matrix cleanup approach, trimethylsilylation, a special injection port liner, and GC-MS in the SIM mode provided limits of quantification either comparable, or somewhat improved, compared to other methods (14, 16, 17).

HAAs found in CSC and base fractions

CSC and the three base sub-fractions were analyzed for each of the ten HAAs using the optimized methods discussed above (Table 4). Relative standard deviations were generally less than 10% for each measureable HAA. Norharman was found in CSC and all three base sub-fractions. AαC was observed in all samples except Base-DCM; whereas IQ was not observed in Base-EtOAc. On the other hand, MeIQ, $MeIQ_x$,Trp-P-1, and Glu-P-1 were not found in any of the four samples. The determination of selected HAAs in mainstream cigarette smoke from the 1R4F and

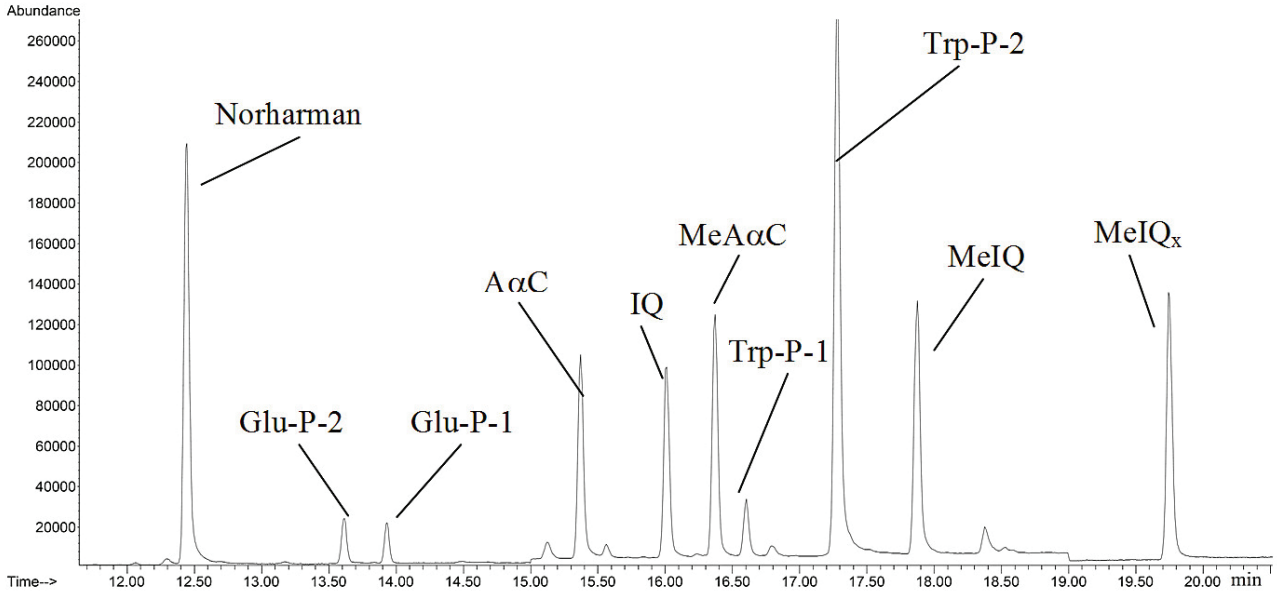

Figure 5. Chromatogram of a solution of silylated HAAs. (A C and MeA C: 32 ng/mL; IQ, MeIQ, MeIQ$_x$, and Trp-P-2: 160 ng/mL; norharman, Trp-P-1: 480 ng/mL; Glu-P-1, Glu-P-2: 1920 ng/mL) obtained using a ZB-50 column. The gas chromatographic oven was held at 120 °C for 1 min then increased to 200 °C at 20 °C/min and then increased at 5 °C/min to 280 °C then increased to 315 °C at 20 °C/min where it was held for 10 min.

2R4F Kentucky reference cigarettes has been previously reported in the literature, and these results are summarized in Table 5 for comparison with data reported here (14, 16, 18). While the analytical methods applied in these earlier studies were not identical to our work and mainstream smoke (rather than CSC) was studied, it is interesting to note that norharman and MeAαC results from this work are in good general agreement with previously reported values (16, 18). In order to compare our analytical results with those independently prepared by another laboratory, CSC samples were also sent to an external analytical laboratory (Labstat International ULC) for HAA analyses. Per Labstat method TMS-133, HAAs were not derivatized prior to analysis. Three HAAs were quantified and the results obtained matched our CSC data very well. Additionally, AαC results (both from the new method reported here and from the contract laboratory analysis) were approximately three times lower than results for the same cigarette reported by SMITH *et al*. (16). Trp-P-2 results with the new method are in general agreement with the results of ZHANG *et al*. (18). Glu-P-2 was not reported in any of the cited references; however, it was detected in this work, possibly due to improved method sensitivity.

Table 3. Limits of Quantification (LOQ).

Analyte	New method			Labstat method LOQ (mg/g CSC) [c]
	Instrument LOQ (ng/mL) [a]	Lowest calibration standard (ng/mL)	Method LOQ (mg/g CSC) [b]	
AαC	0.9	2	0.004	0.229
MeAαC	1.4	2	0.004	—
IQ	5.3	10	0.019	0.064
MeIQ	4.3	10	0.019	—
MeIQ$_x$	4.8	10	0.019	—
Trp-P-1	30	30	0.056	—
Trp-P-2	1.4	10	0.019	—
Norharman	1.7	30	0.056	1.600
Glu-P-1	120	120	0.222	—
Glu-P-2	57	120	0.222	—

[a] Based on peak height S/N = 10.
[b] Based on the lowest calibration standard, an initial sample concentration of 81 mg CSC/mL, a 1-mL sample size and a final sample volume of 0.15 mL.
[c] Labstat Method TMS-133. Based on an initial sample concentration of 81 mg CSC/mL, a 1-mL sample size and a final sample volume of 0.30 mL

Understanding the chemical basis for the biological activity observed with cigarette mainstream smoke and CSC is a key hurdle to the development of new reduced exposure and reduced risk tobacco products. Establishing the relative contribution of individual compounds and compound classes to specific biological responses provides guidance for the development of new tobacco products. As such, the relative contributions of HAAs to the observed mutagenicity of both CSC and the base sub-fractions have been evaluated. The potential contribution from HAAs to observed CSC and base sub-fraction mutagenic activity was calculated as follows:

$$\mathbf{Rev} = \sum_{i=1}^{n} \mathbf{Rev}_i \mathbf{C}_i$$

Where Rev$_i$ is the specific activity (revertants/µg) for the individual HAA, C$_i$ is the measured concentration (µg/g) of the HAA in the CSC or base sub-fraction and Rev is the total calculated contribution of the specific HAA to the

Table 4. Quantified HAAs in 2R4F CSC and three base sub-fractions (µg/g, n = 3).

Analyte	CSC [a]		Base-MTBE [a]		Base-DCM [a]		Base-EtOAc [a]	
	mean	RSD	mean	RSD	mean	RSD	mean	RSD
Norharman	247	9	1736	10	30	5	27	2
AαC	2	5	2.5	3	ND [b]	ND	0.7	7
MeAαC	0.6	4	2.5	2	ND	ND	ND	ND
IQ	0.2	21	0.5	1	0.7	1	ND	ND
MeIQ	ND [b]	ND	ND	ND	ND	ND	ND	ND
$MeIQ_x$	ND	ND	ND	ND	ND	ND	ND	ND
Trp-P-1	ND	ND	ND	ND	ND	ND	ND	ND
Trp-P-2	0.01	12	0.1	4	ND	ND	ND	ND
Glu-P-1	ND	ND	ND	ND	ND	ND	ND	ND
Glu-P-2	0.2	NA [c]	ND	ND	ND	ND	ND	ND

a Sample derivatized as described in Figure 2
b ND: not detected
c NA: not available

Table 5. Comparison of HAAs determined in CSC (µg/g) with literature values reported for mainstream smoke.

Analyte	Cigarette smoke condensate (CSC)		Mainstream smoke [a]		
	New method [b] (2R4F)	Contract laboratory [c] (2R4F)	SMITH *et al.* (16) (2R4F)	SASAKI *et al.* (14) (1R4F)	ZHANG *et al.* (18) (2R4F)
Norharman	247 ± 21 [d]	204 ± 7	163 ± 7	NA	189
AαC	1.8 ± 0.09	2.0 ± 0.01	5.7 ± 0.2	NA	7.1
MeAαC	0.6 ± 0.02	< 0.4 but ≥ 0.1	0.9 ± 0.03	NA	0.5
IQ	0.2 ± 0.04	ND	NA	0.2 ± 0.01	NA
MeIQ	ND [e]	ND	NA	NA	NA
$MeIQ_x$	ND	NA [f]	NA	NA	NA
Trp-P-1	ND	NA	NA	NA	0.1
Trp-P-2	0.1 ± 0.02	NA	NA	ND	0.3
Glu-P-1	ND	NA	NA	ND	NA
Glu-P-2	0.2 ± 0.01	NA	NA	ND	NA

a 1R4F and 2R4F mainstream smoke total particulate matter, yield is assumed to be 10.6 mg/cig for the purpose of unit conversion
b 2R4F CSC sample derivatized as described in Figure 2
c 2R4F CSC sample analyzed according to Labstat method TMS-133 (no derivatization)
d Mean ± SD
e ND: not detected
f NA: not available

observed mutagenic activity (revertants/g). Such calculations assume that the specific activity of the individual compounds is preserved in the complex chemical mixtures that comprise CSC and the base sub-fractions. As such, potential synergistic and/or antagonistic effects that may occur in the complex mixtures are not accounted for by this calculation.

The accurate determination of HAA specific activities (i.e., mutagenicity per HAA mass) is challenging because HAAs are highly mutagenic. For example, mutagenic activity of HAAs in the Ames assay have been reported by SUGIMURA (29), SMITH *et al*. (30), and LEE *et al*. (31), with some TA98 + S-9 values differing by a factor of approximately 2–3, or more, from one study to the next. To address this uncertainty, mutagenic activity of CSC, base sub-fractions and neat HAAs were determined in a single laboratory, providing a common basis from which to calculate the potential contribution of HAAs to the observed mutagenicity of both CSC and its base sub-fractions.

Table 6 summarizes HAA specific activities determined in this work and the calculated potential contributions of each HAA to the Ames activity of CSC and base sub-fractions. HAAs were calculated to contribute 7.8% of the mutagenicity observed for CSC (CSC: 634 Rev/mg), 7.5% of the observed base-MTBE fraction mutagenicity (MTBE fraction: 1681 Rev/mg), 15% of the base-DCM fraction mutagenicity (DCM fraction: 1213 Rev/mg), and 0.003% of the observed base-EtOAc fraction mutagenicity (EtOAc fraction: 649 Rev/mg). The principal contribution to the overall 7.8% activity comes from IQ. If HAA contributions to observed CSC Ames activity are calculated based upon specific activities previously reported in the literature (29–31), HAAs may contribute from 9–15% of the CSC activity. Further, if typical Ames activity values reported for mainstream smoke TPM are considered (e.g., 2507 Rev/mg TPM (32)) HAAs may contribute as little as ~2% of the observed Ames activity.

The HAA specific mutagenic activities and the concentrations of individual HAAs determined in CSC are generally consistent with other reports in the scientific literature.

Table 6. Potential HAA contribution to observed mutagenicity of 2R4F CSC and base sub-fractions [a].

Analyte		Calculated contribution to observed mutagenicity (Rev/g)			
	HAA specific activity [b]	CSC	B-MTBE fraction	B-DCM fraction	B-EtOAc fraction
Norharman	zero	NA [c]	NA	NA	NA
AαC	238	425	584	NA	17
MeAαC	145	80	363	NA	NA
IQ	248036	47623	124514	181810	NA
MeIQ	466251	NA	NA	NA	NA
$MeIQ_x$	61635	NA	NA	NA	NA
Trp-P-1	3956	NA	NA	NA	NA
Trp-P-2	10485	1384	1258	NA	NA
Glu-P-1	29538	NA	NA	NA	NA
Glu-P-2	162	39	NA	NA	NA

[a] HAA and Ames assay mutagenicity results were derived from two identical but independently prepared chemical fractionations of 2R4F CSC conducted by two different chemists

[b] Values determined in this work (Rev/µg)

[c] NA = not applicable

Collectively, the results from this study and previous reports suggest that the chemical basis for the mutagenic activity observed with CSC (and mainstream smoke, in general) as determined by the Ames assay derives primarily from compounds and compound classes other than HAAs. Considering the amounts of individual HAAs found in CSC and mainstream smoke, the various specific mutagenic activities reported for the HAAs, and the mutagenic activities of CSC and mainstream smoke, it is apparent that HAAs contribute as little as ~2% and as much as ~15% of the observed mutagenic activity. Therefore, the development of new cigarette designs intended to reduce exposure to toxicants and reduce the risks of smoking will need to address a range of toxicants present in cigarette smoke in order to reduce the Ames activity of cigarette smoke.

Study limitations

It should be noted that the calculations of potential HAA contribution to observed biological activity are based on data derived from two identical but independent chemical fractionations of 2R4F reference cigarette CSC conducted by two different chemists. Samples from one fractionation were used to obtain HAA results and samples from the other fractionation were used to obtain Ames assay results. While this does not affect the calculated contributions of each HAA to the Ames activity of CSC, it is possible that the calculated contributions of each HAA to the Ames activity of CSC base sub-fractions are affected by the two independent CSC fractionations. Future research efforts will address the generation of HAA and Ames assay data from common CSC fractions. The full complement of CSC fractions (base, acid and neutral) will also be examined in order to more fully explore a HAA materials balance.

CONCLUSIONS

An optimized method for the determination of HAAs in CSC and its base fractions has been established. The method provides excellent selectivity and sensitivity for detection of HAAs at sub part-per-billion concentrations. HAA results obtained with the new method compare favorably with values reported in the literature and results obtained from a commercial laboratory that employed a different analytical method. HAAs were calculated to potentially contribute 7.8% of the mutagenicity observed for CSC, 7.5% of the observed base-MTBE fraction mutagenicity, 15% of the base-DCM fraction mutagenicity, and 0.003% of the observed base-EtOAc fraction mutagenicity. Considering the amounts of individual HAAs found in CSC and mainstream smoke, the various specific mutagenic activities reported for the HAAs, and the mutagenic activities of CSC and mainstream smoke, it is apparent that HAAs potentially contribute as little as ~2% and as much as ~15% of the observed mutagenic activity.

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