Quantitation of Flavor-Related Alkenylbenzenes in Tobacco Smoke Particulate by Selected Ion Monitoring Gas Chromatography−Mass Spectrometry

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Little is known about the possible health effects associated with inhaling alkenylbenzenes through cigarette smoking, even though these flavor-related compounds have known toxic effects in animals. We developed a rapid and sensitive solid-phase extraction (SPE) method to quantify seven alkenylbenzenes and piperonal in mainstream cigarette smoke particulate. The smoke particulate fraction of a single cigarette was collected on Cambridge filter pads, solvent extracted, concentrated, purified with SPE, and analyzed by selected ion monitoring gas chromatography−mass spectrometry. We positively identified and quantified five alkenylbenzenes compounds (eugenol, isoeugenol, methyleugenol myristicin, and elemicin) and piperonal in the smoke particulate from eight U.S. brands with mean levels (measured in triplicate) ranging from 6.6 to 4210 ng per cigarette. Additionally, complete blocking of nearly invisible ventilation holes in the cigarette filter increased 2- to 7-fold the percent transfer of alkenylbenzenes from tobacco to the particulate fraction of mainstream smoke.

Keywords: *Tobacco; mainstream smoke; particulate fraction; alkenylbenzenes; flavor additive; solid-phase extraction; gas chromatography; mass spectrometry; selected ion monitoring*

INTRODUCTION

Currently, several hundred additives with a "Generally Recognized As Safe" status for use in food (National Academy of Science, Food Protection Committee, 1972), including individual compounds (natural and synthetic) and botanical preparations, are used to flavor cigarette tobacco (Tobacco Reporter Staff, 1994). Some botanical preparations (e.g., extracts, essential oils, powders, oleoresins, etc.) contain alkenylbenzenes, a class of allyl- and propenylbenzene compounds with methoxy and methylenedioxy ring substitutions (Leung, 1980). Alkenylbenzenes including safrole [5-(2-propenyl)-1,3-benzodioxole], eugenol [(1-hydroxy-2-methoxy-allylbenzene], isoeugenol [1-hydroxy-2-methoxy- propenylbenzene], methyleugenol [1,2-dimethoxy-4-allylbenzene], methylisoeugenol [1,2-dimethoxy-4-(1-propenyl)-benzene], myristicin [4-methoxy-6-(2-propenyl)-1,3-benzodioxole], and elemicin [3,4,5-trimethoxyallylbenzene] are not added individually as flavorings (Tobacco Reporter Staff, 1994) but are constituents of tobacco flavor additives such as cinnamon and nutmeg (Leung, 1980). An additional compound, piperonal [1,3-benzodioxole-5-carboxaldehyde] has a chemical structure similar to that of safrole and is used individually to flavor tobacco (Tobacco Reporter Staff, 1994). Recently in our laboratory, we quantified several alkenylbenzenes (including safrole, eugenol, methyleugenol, isoeugenol, methylisoeugenol, myristicin, and elemicin) and piperonal in several commercial U.S. cigarette tobaccos at concentrations ranging from low nanogram to low microgram per gram of tobacco (Stanfill and Ashley, 1999). Further use of the term "alkenylbenzene" will also include piperonal.

Most flavor additives enter the human body through ingestion of food. After absorption from the gastrointestinal tract into the blood stream, compounds are carried directly by the hepatic portal vein to the liver where they may be converted to less toxic and more water-soluble metabolites and eliminated before entering the systemic circulation (Kulkarni and Byczkowski, 1994; Lu, 1996; Riviere, 1994).

In contrast to oral exposure, flavor-related compounds present in cigarette tobacco are pyrolyzed by the burning cigarette coal to form new compounds or distilled unchanged from the tobacco and transferred into the smoke (Green et al., 1989). Smoke inhaled through the mouthpiece of the cigarette is termed mainstream smoke and is composed of smoke particles (particulate fraction) and gases (vapor fraction) (Hoffmann and Hoffmann, 1997). As a smoker draws on a ventilated cigarette, air enters through nearly-invisible ventilation holes in the cigarette filter and dilutes the mainstream smoke (Hoffmann and Hoffmann, 1997). The concentration of certain smoke constituents (i.e., nicotine, CO, etc.) inhaled is increased if the ventilation holes are covered (unintentional or intentionally) by the smoker's lips or fingers; vent blocking has been reported to occur commonly among smokers. Occasionally, vent holes are intentionally blocked by covering the vent holes with tape (Kozlowski et al., 1982). Within the lungs, inhaled compounds are rapidly and efficiently transferred into the circulation due to the large surface area for gas exchange (85 m^2), thin cell boundary, and the abundant blood supply in close proximity to the inhaled air. The inspired compounds are absorbed through the alveolar cells and subsequently pass through the capillary endothelial cells and into the arterioles for systemic circulation; compounds circulate throughout the body prior to passing through the liver for potential detoxification and excretion (Sabourin, 1994; Stine and Brown, 1996).

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Published on Web 03/22/2000

There has been extensive research on the acute toxic properties of alkenylbenzenes. For example, carcinogenic activity has been reported for safrole and methyleugenol in rodents (Hirono, 1987; Miller et al., 1982) and mutagenicity for eugenol in the Ames *S. typhimurium* assay (Leleng et al., 1982). Myristicin and elemicin, constituents of nutmeg, are thought to elicit the genotoxic (Hasheminejad and Caldwell, 1994) and hallucinogenic effects of the spice observed in humans (Weil, 1965; Braun and Kalbhen, 1973). Piperonal has been shown to cause depression of the central nervous system in rodents (Hirono, 1987). Inhalation of eugenol causes pulmonary edema in rabbits (McDonald and Heffner, 1991) and pulmonary edema and acute emphysema in hamsters. Toxic properties of eugenol differ significantly for inhalation and oral routes of exposure; its inhalation toxicity in rodents is 250 times greater than its oral toxicity (LaVoie et al., 1986). It is not known whether other alkenylbenzenes, particularly those chemically similar to eugenol, exhibit inhalation toxicities greater than their oral toxicities in smokers (Hoffmann and Hoffmann, 1997). Presently, very little is known about the extent to which alkenylbenzenes are transferred into mainstream cigarette smoke and the chronic health effects associated with their repetitive, long-term inhalation.

Alkenylbenzenes have been identified and quantified in spices (Archer, 1988), food (Carman et al., 1985), tobacco (LaVoie et al., 1985; Clark and Bunch, 1997), and tobacco smoke (Schmeltz, 1967). Several sample preparation techniques, including steam distillation (Lavoie et al., 1985), solvent extraction (Schmeltz, 1967), supercritical fluid extraction (Heikes, 1994), solid-phase extraction (Yates and England, 1982), and solid-phase microextraction (SPME) (Clark and Bunch, 1997), have been used to purify these compounds from various matrices for analysis. Analysis has been carried out using high-performance liquid chromatography (Wulf et al., 1978) and gas chromatography−mass spectrometry (GC−MS) (Heikes, 1994). Recently, we developed a method that detected nine alkenylbenzenes (including the analytes studied here), piperonal, coumarin, and pulegone in one or more U.S. cigarette tobaccos using headspace SPME coupled with GC−MS (Stanfill and Ashley, 1999).

In the past, measurement of alkenylbenzenes in cigarette smoke has proven to be a challenge. In the late 1960s, as much as 1 kg of smoke condensate (the equivalent of smoke from 50 000 cigarettes) was required to measure myristicin at submicrogram levels (Schmeltz, 1966). In addition, eugenol, isoeugenol, and methyleugenol (Rodgman and Cook, 1964) have been detected as smoke constituents; more recently, means of detecting eugenol in clove cigarette smoke have been reported (LaVoie et al., 1986). Accordingly, a rapid analytical method was required that could measure alkenylbenzenes in the mainstream smoke particulate of a single cigarette in the low nanogram range. In this study, we evaluate the use of SPE coupled with SIM-GC-MS to accomplish this analysis. The analytical method was developed for three reasons: (1) to facilitate the measurement of alkenylbenzenes in mainstream tobacco smoke particulate, (2) to allow comparison of alkenylbenzene levels among U.S. cigarette brands, and (3) to evaluate the effect of vent blocking on alkenylbenzene concentrations present in mainstream smoke particulate.

EXPERIMENTAL PROCEDURES

Materials. Commercially available cigarettes were purchased, labeled with identification codes that were then logged into a database, and placed in ziplock bags. The samples were stored in an ultralow freezer (Revco Scientific, Inc., Asheville, NC) set at −70 °C. Prior to analysis, cigarettes were removed from the freezer and thawed, and their weight and level of ventilation were determined.

Reagents and Chemicals. Methanol, tetrahydrofuran, toluene, and hexane were purchased from Burdick & Jackson (Muskegon, MI). Dehydrated 200 proof ethyl alcohol was obtained from Pharmcoproducts, Inc (Brookfield, CT). 3′,4′-Methylenedioxyacetophenone (MDA), eugenol, isoeugenol (trans isomer), methyleugenol, methylisoeugenol (trans isomer) [1,2-dimethoxy-4-(1-propenyl)-benzene], piperonal, and safrole were purchased from Aldrich (Milwaukee, WI). Myristicin was acquired from Sigma Chemical Company (St. Louis, MO). Elemicin was the generous gift of Dr. Peter Cadby of Firmenich (Geneva, Switzerland). Sample or standard preparation reagents were used as obtained. Helium and nitrogen gases were obtained from Holox (Norcross, GA) and Air Products (Allentown, PA). Glassware (beakers and conical tubes) was obtained from Corning-Costar Corporation (Action, MA) and Kimble Glass, Inc. (Vineland, NJ). GC autosampler vials and kim spring inserts were also obtained from Kimble Glass.

Standard Preparation. An alkenylbenzene standard solution (containing safrole, piperonal, methyleugenol, eugenol, isoeugenol, methylisoeugenol, myristicin, and elemicin) and an internal standard solution of MDA were prepared by weighing these components to the nearest 0.01 mg on a research-grade analytical balance (Sartorius, Waukegan, IL) and diluting them in hexane. Liquid standard materials were measured with an SMI positive displacement pipettor (Dade International Inc., Miami, FL). The purity of the standards was confirmed by comparing the mass spectra with the NIST ′98 mass spectral library (National Institute of Standards and Technology, Gaithersburg, MD).

Safety Considerations. A respirator and safety glasses were worn while working in the chamber housing the smoking machine to minimize environmental tobacco smoke exposure. During standards preparation, solvent evaporation, and SPE extraction, a lab coat, nitrile gloves (8 mil thickness), and safety glasses were worn. Work was done under a fume-hood with an appropriate sash level and an average flow velocity within safety specifications. A flexible air handling tube was mounted over the GC autosampler to vent away vapors released during sampling.

Smoking Parameters. Thawed cigarettes were weighed on a PB302 Mettler Toledo balance (Mettler Toledo Process, Worthington, OH), and cigarette tip ventilation was measured with a QTM5 Ventilation Test Module (Filtrona Instrument and Automation, Ltd., Richmond, VA). Vent blocking was carried out by covering half or all of the ventilation holes with cellophane tape. A clean Cambridge filter pad was placed in each of the filter holders on the 8-port Filtrona Harmonizer smoking machine housed in an environmental control chamber (Parameter Generation & Control, Black Mountain, NC) maintained at 22.2 °C and 60% relative humidity. Puff volumes were measured with the Filtrona volume indicator and calibrated to meet the specified 35 ± 0.3 mL volume. Air flow across the cigarette tip was measured with an air velocity anemometer (Schiltknedt, Gossau, Switzerland) interfaced with a Filtrona VMD 100 velocity measurement digitizer (Richmond, VA). The air flow was maintained at a velocity of 200 ± 30 mm at each port, conforming to a standard deviation of less than 20%. Cigarettes were "smoked" according to the Federal Trade Commission standard regimen (puff duration, 2 s; interpuff interval, 1 min; puff volume, 35 mL) (Ogg, 1964). Immediately after the cigarette was smoked, each filter pad (which captures the smoke particulate fraction) was removed, used to wipe the interior of the filter holder, and placed in a clean, labeled ziplock bag. A clean pair of nitrile gloves was used to handle each filter pad to prevent cross-contamination.

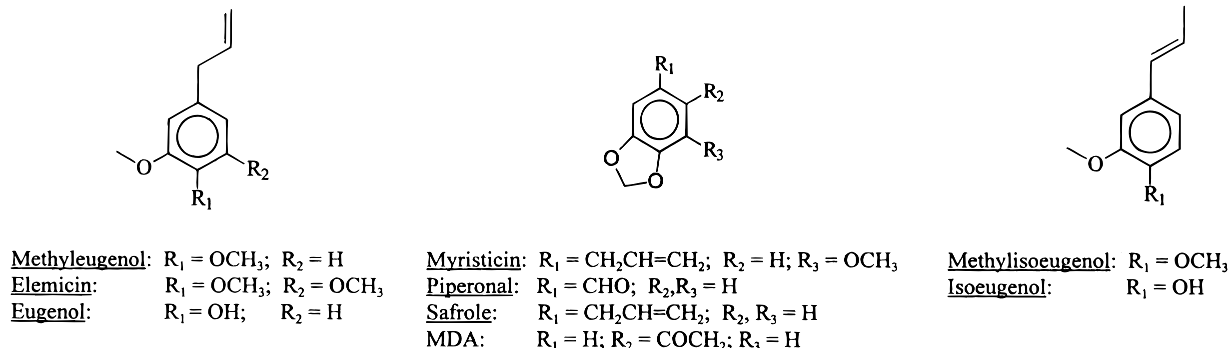

Figure 1. Common names and chemical structures of the eight alkenylbenzenes and the internal standard, 3′,4′-(methylenedioxy)-acetophenone (MDA), utilized in this study.

Filter holders were removed and washed with methanol and hexane prior to subsequent use.

Solvent Extraction of Smoke Particulate Fraction. Each filter pad (with the stained side facing up) was placed in a clean, labeled 250-mL beaker. A 25-mL portion of hexane and 25 μL of the 5 ng/μL MDA solution were added to each beaker. The beakers was covered with foil to minimize solvent volatilization and agitated for 1 h at ambient temperature on an orbital shaker (Labline Instruments, Inc., Melrose Park, IL) set at 90 rpm. After the extraction, the solution in each beaker was decanted into a 50-mL graduated conical centrifuge tube. The solution was then evaporated to a volume of 5 mL under a continuous nitrogen stream in an N-EVAP analytical evaporator (Organomation Associates Inc., South Berlin, MA).

Preliminary experiments revealed that two fractions of the hexane extract were necessary to quantitate the analytes. One fraction was an aliquot of the concentrated hexane extract, designated as the pre-SPE fraction. The other fraction was the eluent resulting from purifying the concentrated hexane extract by SPE, designated as the post-SPE fraction. The cyanopropyl SPE cartridge was chosen following investigation of analyte cleanup using various SPE sorbents and solvent regimes.

Preparation of Pre-SPE Fraction. A 1-mL aliquot of each concentrated hexane extract was transferred directly to a 15-mL graduated conical tube. A 200-μL aliquot of toluene was added to the extract as a keeper solvent, and the solution was evaporated to a volume between 100 and 300 μL with an AS290 SpeedVac evaporator (Savant Instruments Inc., Farmingdale, NY). The resulting sample, designated as the pre-SPE fraction, was transferred to a 1.8-mL autosampler vial fitted with a 250-μL kim spring insert for GC−MS analysis.

Preparation of Post-SPE Fraction. Another 1-mL aliquot of each concentrated hexane extract was purified by SPE using 3-mL, 500-mg cyanopropyl cartridges (Varian, Harbor City, CA) placed in a 12-port Visiprep vacuum manifold (Supelco, Bellefont, PA). The sorbent bed of the cartridge was conditioned with a 3-mL aliquot of hexane prior to adding a 1-mL aliquot of the extract. The SPE cartridge was then washed with 2 mL of hexane and aspirated to dryness using a house vacuum. The conditioning and wash solutions were captured in a disposable test tube, which was removed and replaced with a 15-mL graduated conical tube prior to elution. Analytes were eluted from the cartridge with 1.5 mL of a 85% hexane, 5% toluene, 10% tetrahydrofuran mixture. The eluent was evaporated to a volume between 100 and 300 μL in the SpeedVac evaporator. No toluene was added as a keeper solvent, because it was already present in the eluent. The resulting sample, designated as the post-SPE fraction, was transferred into a 1.8-mL autosampler vial fitted with a 250-μL kim spring insert for GC−MS analysis.

GC−MS Instrumentation. Analytical measurements were carried out on a Hewlett-Packard (HP) 6890 GC coupled to a HP5973 mass selective detector (Avondale, PA). The GC was fitted with a 30-m DB-5MS column with 0.25 mm i.d. and 0.25 μm film thickness (J&W Scientific, Folsom, CA). A 1- to 3-m length of deactivated, fused silica precolumn (J&W Scientific, Folsom, CA) was installed in front of the analytical column; this precolumn was trimmed and replaced periodically, as needed. A HP 6890 series autosampler (Avondale, PA) was used to make splitless injections into the injector port fitted with a narrow-bore (0.75 mm i.d.) injection sleeve (Supelco, Bellefonte, PA) and maintained at 250 °C. Helium, which was used as the carrier gas, was held at a constant flow of 1.2 mL/min. The oven was heated and held at 40 °C for 1 min, increased to 110 °C at 25 °C/min, increased to 155 °C at 3 °C/min, and increased to 270 °C at 25 °C/min. The GC−MS transfer line was held at a constant temperature of 280 °C, and the MS quadrupole and source heaters were maintained at 110 and 230 °C, respectively.

High-Resolution Mass Spectrometry. Exact mass measurements (±0.0001 amu) were made using a HP 6890 Plus GC, fitted with a J&W DB-5MS column, coupled to a 70SE high-resolution magnetic sector mass spectrometer (Micromass, Beverly, MA) operated in selected ion ratio mode at a resolution of 10 000.

SIM Parameters. Mass spectra were acquired in SIM mode with a quantitation and confirmation ion for each analyte. The molecular structure of each compound is shown in Figure 1. The quantitation ion for each compound was the molecular ion $(M)^+$, with the exception of piperonal ($(M - H)^+$). The confirmation ion was the ion of greatest abundance that exhibited the least background interference. In the case of eugenol, isoeugenol, methylisoeugenol, and elemicin, the $(M - CH_3)^+$ fragment was used as the confirmation ion. For methyleuegnol and myristicin, the $(M - OCH_3)^+$ and $(M - CH{=}CH_2)^+$ fragments, respectively, were used as the confirmation ions. For safrole and piperonal, the $(M - H)^+$ fragment and the $(M)^+$ ion, respectively, were utilized as the confirmation ions. The peak area for the internal standard, MDA, was determined from the $(M - CH_3)^+$ fragment. The ratio of the areas of the quantitation ion and the confirmation ion was used as an interference quality control check; ion ratios were calculated by dividing the peak area of the quantitation ion by the peak area of the confirmation ion. SIM parameters are listed in Table 1. The primary criteria for choosing a fraction for the analysis of a particular analyte were based on the chromatographic clarity of the quantitation and confirmation ion peak shape and the absence of coeluting, interfering substances. A secondary criterion involved choosing the fraction that yielded the best reproducibility and calibration curve correlation coefficients. The identity of the alkenylbenzenes was confirmed on the basis of retention times, and ion ratios were compared to standards spiked onto Cambridge filters with trapped smoke particulate.

Data Analysis. Chromatogram peak areas were determined automatically using the ChemStation Integrator program in the HP Enhanced ChemStation software version A.03.00 (Avondale, PA). Each peak was checked for proper integration and reintegrated manually, if needed. Areas were transferred into R:Base version 4.5++ (Microrim Inc., Bellevue, WA). Statistical determinations were performed using Statistical Analysis System software version 6.12 (SAS Institute, Cary, NC).

Table 1. Summary of SIM Parameters Used for the Analysis of Pre- or Post-SPE Fractions of Tobacco Smoke Particulate

analyte	RT[a] (min)	RRT[b]	quantitation mass (amu[c]) [SIM scans[d]]	confirmation mass (amu[c]) [SIM scans[d]]	pre-SPE[e]	post-SPE[f]
MDA[g]	14.50	1.00	149 [7]	*h*	X[i]	X
safrole	10.36	0.71	162 [14]	161 [13]	X	
piperonal	11.65	0.80	149 [7]	150 [6]	X	
eugenol	11.96	0.83	164 [13]	149 [14]		X
methyleugenol	13.31	0.92	178 [10]	147 [10]		X
isoeugenol	14.87	1.03	164 [14]	149 [13]	X	
methylisoeugenol	16.37	1.13	178 [14]	163 [13]	X	
myristicin	17.25	1.19	192 [14]	165 [13]	X	
elemicin	18.11	1.25	208 [14]	193 [13]	X	

[a] Retention time. [b] Relative retention time referenced to MDA[g] (no units). [c] Atomic mass units. [d] Number of selected ion monitoring scans; dwell time per SIM scan = 20 ms). [e] Pre-solid-phase extraction fraction. [f] Post- solid-phase extraction fraction. [g] 3′,4′-methylenedioxyacetophenone (used as internal standard). [h] Confirmation mass was not monitored for IS. [i] Fraction chosen for analysis; MDA used as IS in both fractions.

RESULTS AND DISCUSSION

Pre- and Post-SPE Fractions. The SPE fractions (pre or post) used for each analyte are listed in Table 1. Piperonal, isoeugenol, myristicin, and elemicin were present in both fractions, but the pre-SPE fraction was chosen for these analytes in accordance with the criteria mentioned above. Removal of the interfering compounds by SPE yielded resolved peaks and allowed eugenol and methyleugenol to be quantified in the post-SPE fraction. Nicotine, which interferes with eugenol, was present in the pre-SPE fraction but not present in the post-SPE fraction. Safrole (in standards only) was observed only in the pre-SPE fraction because it was not eluted from the SPE cartridge. In standards, methylisoeugenol exhibited excellent peak shape for quantitation and confirmation ions and a linear curve; however, the peak shape and ion ratios were unacceptable at the levels encountered in the smoke particulate from the commercial cigarette tobacco analyzed.

Gas Chromatographic Profile. Combined selected ion chromatograms of a pre-SPE fraction and post-SPE fraction of smoke particulate from brand B, a brand that contained six alkenylbenzenes (piperonal, eugenol, methyleugenol, isoeugenol, myristicin, elemicin), is shown in Figures 2A and B, respectively. In general, the pre-SPE fraction exhibited higher chemical background than the post-SPE fraction. The ion profiles of the quantitation ion for each analyte (shown in the insets) exhibited good peak resolution. MDA eluted at 14.50 min in both fractions. Isoeugenol, which eluted at 14.87 min, was the largest peak in the chromatograms of both fractions. A DB-5MS column was used in this study so that the analysis of alkenylbenzenes in both unburned tobacco (Stanfill and Ashley, 1999) and smoke particulate could be performed with the same analytical column.

Sample Fraction Characterization. Pre- and post-SPE fractions of cigarette smoke particulate was analyzed to characterize the diversity of compounds extracted from the particulate-imbedded Cambridge filters. The analysis was made using the mass spectrometer in scan mode (m/z 40−250) under the same chromatographic conditions used in the SIM analysis. Identification of unknown compounds in smoke extracts was made by searching the full scan mass spectra against the NIST ′98 mass spectral library. The pre-SPE fraction contained compounds tentatively identified as nicotine, as well as less intense peaks for methylphenol, mysomine, nicotyrine, skatole, solanone, and vanillin. In contrast, the post-SPE fraction lacked nicotine but contained compounds tentatively identified as benzyl benzoate, cresol, guaiacol, indole, limonene, megastimatrienone, and xylenol.

Calibration Curve. A seven-point calibration curve was produced for the eight compounds with concentrations spanning 3 orders of magnitude in the nanogram range. Clean Cambridge filter pads were spiked with the alkenylbenzene standards and carried through the extraction and concentration procedures. Characteristics of the least-squares linear regression fit for each analyte are shown in Table 2. Myristicin, methylisoeugenol, and piperonal had the best correlation coefficients, and isoeugenol, safrole, and elemicin had somewhat lower values; however, all of the calibration curves exhibited linearity over the concentration ranges investigated.

Limits of Detection. Limits of detection (LOD) (Table 3) were determined by spiking alkenylbenzene standards onto Cambridge filters, on which smoke particulate from a cigarette had been trapped. The blank (an unspiked Cambridge filter with trapped smoke particulate) did not contain detectable levels of any analytes, except for isoeugenol, which was present at levels at or slightly above the LOD but much less than the level of the lowest spiking solution used below. The spiking solutions contained differing concentrations of the eight analytes and were designed to represent the low, medium, and high concentration ranges. For each of three concentration levels, five particulate-deposited filters were spiked with a 250-μL aliquot of an alkenylbenzene mixture. LOD was calculated as 3 times the standard deviation at zero concentration (Taylor, 1987). The lowest LOD values observed were for elemicin and methylisoeugenol; the highest LOD values were for isoeugenol and eugenol, probably because of variability in the measurable baseline levels.

Analyte Recovery. Generally, the mean recoveries were consistent across the spike concentrations (Table 3). The recoveries were closest to 100% for piperonal, myristicin, and elemicin with average mean recoveries of approximately 95%, 98%, and 111%, respectively. Safrole, eugenol, and methyleugenol had consistently low recoveries at all three spike levels, with mean recoveries of about 80% for each analyte. Isoeugenol exhibited recoveries above 150% for the low and medium spikes and a recovery of 122% at the highest level; however, the cause of the elevated recoveries for isoeugenol are not fully understood. Methylisoeugenol exhibited recoveries closer to 100% except at the low spike level (recovery = 184%). The elevated recovery for methylisoeugenol at the lower concentrations may be

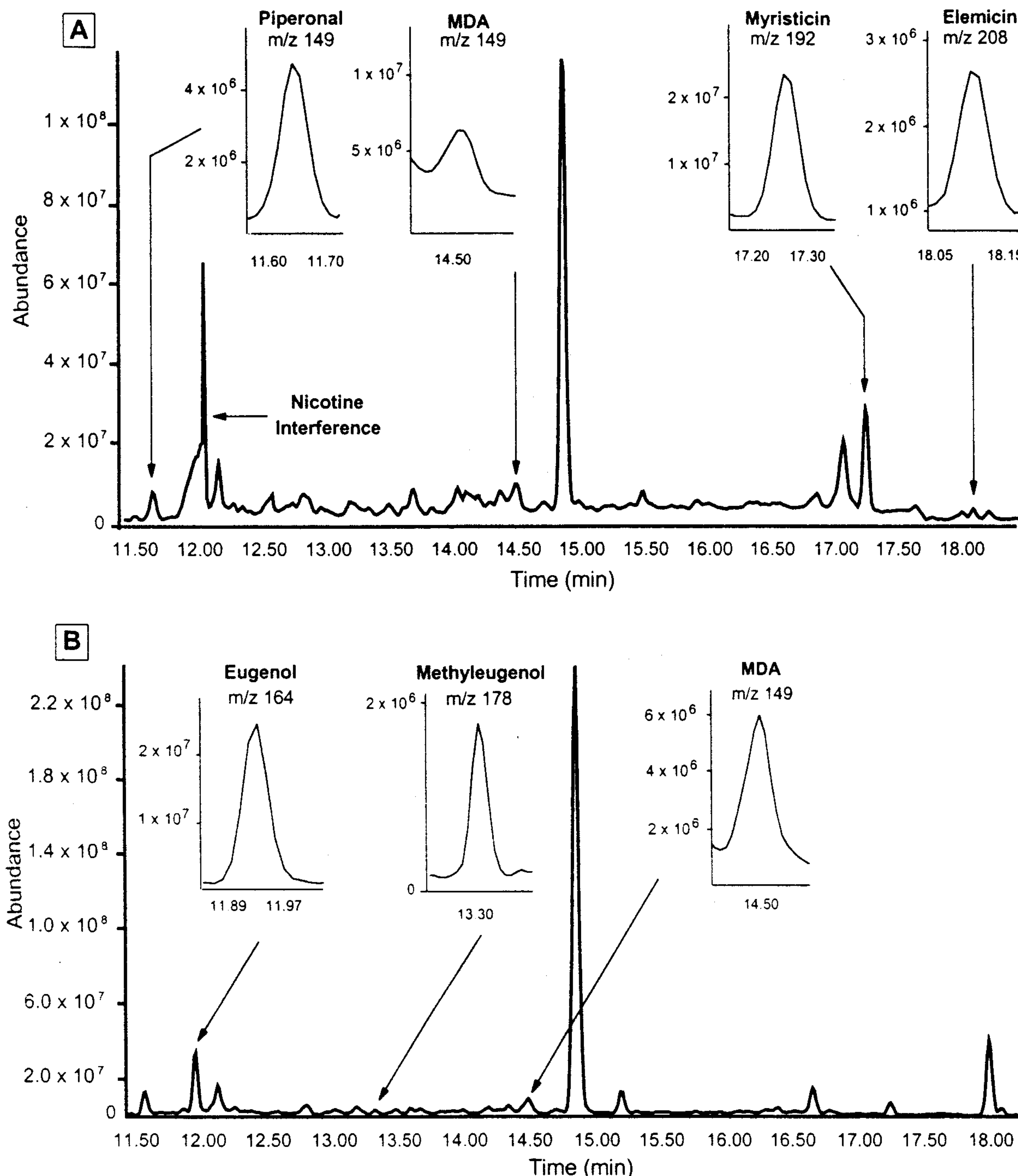

Figure 2. SIM-GC-MS chromatogram and quantitation ion profiles of alkenylbenzenes in the (A) pre- and (B) post-SPE fractions of brand B. The chromatogram was acquired by monitoring both the quantitation and confirmation ions for each analyte and a single ion for the internal standard. The compound, ions (quantitation and confirmation) monitored, and acquisition start time (in parentheses) for each sequential group of ions are as follows: safrole, 162, 161 (9.0 min); piperonal, 149, 150 (10.8 min); eugenol, 164, 149 (11.6 min); methyleugenol, 178, 147 (12.8 min); isoeugenol, 164, 149 (14.0 min); methylisoeugenol, 178, 163 (15.7 min); myristicin, 192, 165 (16.5 min); elemicin, 208, 193 (17.3 min). The base peak in both chromatograms was isoeugenol observed at 14.87 min. For the internal standard MDA, mass 149, was acquired starting at 12.8 min with the masses for methyleugenol. The large spike-like peak at 12.05 min in Figure 2A is the trailing edge of the nicotine peak, which coeluted with eugenol and interfered with its quantitation in the chromatogram of the pre-SPE fraction.

due to signal interferences associated with measurements made close to the analyte detection limits; methylisoeugenol was not detected at levels above the LOD in any of the commercial tobaccos. Excellent reproducibility was observed for the coefficient of variation (CV), which was below 11% for all analyte concentrations and had an average value for all analyte concentrations of approximately 7%. Very minimal cleanup of samples was done in this study; however, data suggest that only eugenol and methyleugenol where sufficiently affected by interferences in the pre-SPE fraction to warrant SPE cleanup of those compounds.

Cigarette Smoke Analysis. Initially, 25 top-selling brands (Davenport & Company LLC, 1998) were "smoked", and the particulate was analyzed to determine the brands that had the highest alkenylbenzene concentrations. Eight cigarette brands containing vari-

Table 2. Characteristics of the Least-Squares Linear Regression for Alkenylbenzene Analytes

analyte	concn range (ng)	slope (±SE[a])	*y*-intercept (±SE[a])	correlation coefficient
myristicin	3.1−1230	0.00628 ± 0.00006	−0.01184 ± 0.00459	0.995
methylisoeugenol	1.0−407	0.00804 ± 0.00008	−0.00413 ± 0.00226	0.994
piperonal	2.1−821	0.00177 ± 0.00002	−0.00170 ± 0.00111	0.992
eugenol	3.2−1280	0.00503 ± 0.00008	−0.01454 ± 0.00770	0.986
methyleugenol	1.0−411	0.00410 ± 0.00007	−0.00342 ± 0.00199	0.984
isoeugenol	20.1−8040	0.00403 ± 0.00008	−0.01491 ± 0.044	0.974
safrole	1.1−436	0.00598 ± 0.00014	0.01219 ± 0.04362	0.963
elemicin	1.1−420	0.00746 ± 0.00023	−0.00336 ± 0.00628	0.944

[a] SE (standard error of predicted value).

Table 3. Determination of Detection Limits, Recovery, and Reproducibility of Alkenylbenzenes Spiked on Cambridge Filters Containing Captured Smoke Particulates from a Single Cigarette

analyte	limit of detection (ng)	spiking concn (ng)	mean recovery[a] (%)	CV[b] (%)
safrole	5.4	27.2	72.1	10.3
		109	83.2	6.2
		436	90.6	10.4
piperonal	3.8	51.3	100.0	2.4
		205	95.8	3.1
		821	94.9	8.8
eugenol	27.8	79.7	75.0	9.6
		319	85.6	8.7
		1280	84.2	10.6
methyleugenol	5.1	25.7	83.1	8.6
		103	80.3	9.6
		411	78.8	9.7
isoeugenol	20.1	502	156	9.2
		2010	153	4.9
		8040	122	7.8
methylisoeugenol	1.1	25.4	184	7.5
		102	121	2.9
		407	105	8.1
myristicin	15.4	77.2	97.4	2.2
		309	98.4	2.0
		1230	97.2	7.9
elemicin	5.2	26.2	116	2.9
		105	109	1.6
		420	107	7.4

[a] Average of five measurements. [b] Coefficient of variation.

ous concentrations of alkenylbenzenes were chosen for subsequent analysis. Table 4 shows the amounts of alkenylbenzenes detected in the smoke particulate fraction of eight commercial brands. We used the brand designations from our earlier study (Stanfill and Ashley, 1999), in which we measured alkenylbenzene concentrations in the cigarette tobacco of top-selling brands (Davenport & Company LLC, 1998). Six of the eight analytes studied were detected in smoke particulate above the LOD in one or more of brands analyzed; safrole and methylisoeugenol were not detected at levels above the LOD in any of the smoke particulate samples. Among the brands tested, brand B contained the greatest number and highest concentrations of alkenylbenzenes, except for piperonal and isoeugenol, which were highest in brand E. Isoeugenol and eugenol were both present in the smoke particulate fraction of seven of the eight brands analyzed, with mean values of 2780 and 260 ng, respectively (Table 4). Interestingly, these compounds were found at elevated levels (approximately 3500 ng) in the smoke particulate fraction of brands E, K, and L, which contained no detectable levels of isoeugenol (LOD = 33 ng) and eugenol (LOD = 10.5 ng) in the unburned tobacco (Stanfill and Ashley, 1999). An additional highly ventilated brand (J), which lacked detectable eugenol and isoeugenol in the tobacco, contained a moderate level of eugenol (444 ng) in the smoke particulate fraction. Both eugenol and isoeugenol have been demonstrated as pyrolysis products of lignins, structural components of plants (Rodgman and Cook, 1964; Scholtzhauer and Chortyk, 1987).

Analyte Confirmation by HRMS Analysis. Compounds detected by SIM-GC-MS were also detected by HRMS and had quantitation/confirmation ion ratios that were within the expected limits as compared with the ratios of the standards. The exact quantitation mass for methyleugenol (m/z 178.0994), which corresponds to the $(M)^+$ ion, was present at the expected retention time in both the standards and unknown tobacco samples. The exact confirmation mass for methyleugenol (m/z 163.0759) was detected in the standards but was not detected in the samples, which had analyte concentrations that were much lower than the standard levels. The absence of a detectable confirmation ion fragment in the samples may be due to factors such as the low analyte concentrations in samples and the softer ionization process (30 eV) used in the HRMS analysis, as compared with the SIM-GC-MS analysis at 70 eV, which generally produces more intense fragment ions. In addition, the recoveries for methyleugenol were less than 100% (about 80%), which also suggests that interferences did not contribute to the reported values. The low recoveries for methyleugenol may cause a slight negative bias to the data where the reported values are less than the actual amount present in the sample. In general, the HRMS data suggest that interfering compounds did not contribute to any analyte levels reported here.

Effect of Ventilation on Smoke Composition. Table 5 shows the effect of vent hole blocking on the transfer of alkenylbenzenes from tobacco to the smoke particulate fraction of brand C. Tobacco from brand C contained detectable levels of safrole, piperonal, eugenol, methyleugenol, isoeugenol, myristicin, and elemicin (Stanfill and Ashley, 1999). In this study, vent blocking reduced the ventilation, concentrated the smoke particulate (as evidenced by the Cambridge pad stain), and increased the levels of alkenylbenzenes transferred (percent transfer) from tobacco to smoke particulate fraction.

Partial vent blocking yielded levels of piperonal, myristicin, elemicin, and isoeugenol about 2.5 times greater than those observed in the smoke particulate of an unblocked cigarette. Complete vent blocking caused the percent transfer of alkenylbenzenes to increase by 3- to 7-fold. Accordingly, the percent transfer of piperonal increased by 786% when the vent holes were completely blocked as compared to the unblocked. Methyleugenol and eugenol, which were not detected above the LOD in the unblocked cigarette smoke, were detected when the ventilation holes were partially or fully blocked. Research utilizing ^{13}C-labeled flavor

Table 4. Amount of Alkenylbenzenes Detected in the Smoke Particulate Fraction of a Single Cigarette from Eight Commercial U.S. Brands

		mean[b], ng (CV[c], %)					
brand	ventilation[a] (%)	piperonal	eugenol	methyleugenol	isoeugenol	myristicin	elemicin
B[d]	1	490 (10.6)	608 (23.0)	46.5 (19.6)	4050 (11.9)	615 (14.8)	38.9 (12.8)
C[e]	83	66.6 (44.0)	ND[f]	ND	265 (25.3)	33.6 (27.4)	6.6 (21.5)
D[e]	22	ND	192 (18.8)	ND	2020 (19.5)	45.1 (3.1)	ND
E[d]	22	1010 (13.1)	501 (9.0)	ND	4210 (11.4)	ND	ND
F[e]	68	ND	77.8 (24.2)	ND	2810 (2.9)	ND	ND
J[e]	21	ND	444 (36.0)	ND	ND	ND	ND
K[e]	2	ND	308 (16.1)	ND	3120 (5.2)	ND	ND
L[g]	2	ND	271 (1.5)	ND	3020 (6.5)	ND	ND

[a] Average of three ventilation measurements. [b] Average of three measurements. [c] Coefficient of variation. [d] Unfiltered brand, nonmenthol brand. [e] Filtered, nonmenthol brand. [f] Below limit of detection. [g] Filtered, menthol brand.

Table 5. Effect of Filter Vent Blocking on the Recovery of Several Alkenylbenzenes in the Smoke Particulate Fraction of Brand C

		smoke particulate concn mean[c], ng[d] (CV[e], %)		
analyte	cigarette tobacco concn[a] mean, ng/cig[b]	unblocked (79% ventilation)[f]	partially blocked (59% ventilation)	completely blocked (10% ventilation)
isoeugenol	188	226 (25.6)	525 (30.0)	1030 (4.3)
piperonal	21300	48.6 (31.1)	167 (35.9)	382 (17.9)
myristicin	3570	28.3 (29.2)	63.9 (13.4)	130 (5.5)
eugenol	57.6	ND[g]	41.1 (45.6)	85.2 (8.0)
elemicin	76.8	5.8 (27.8)	10.2 (10.9)	18.3 (12.2)
methyleugenol	81.0	ND	6.4 (42.6)	10.8 (9.9)

[a] Calculated from amount measured in brand C cigarette tobacco, see: Stanfill and Ashley, *J. Chromatogr., A* **1999**, *858*, 79−89. [b] Tobacco concn (ng/cig) = mean ng/g conc (cigarette tobacco) × mean cigarette wt; three measurements were made for each result, except for eugenol ($n = 2$). [c] Average of three measurements. [d] Amount (ng) of analyte trapped on a Cambridge pad on which the mainstream smoke particulate of a single cigarette is deposited. Cigarettes were smoked according to the FTC method with the vent blocking regimens as designated. [e] Coefficient of variation. [f] Average of three ventilation measurements. [g] Below limit of detection.

compounds has shown that during the smoking process these compounds are distributed between mainstream smoke, sidestream smoke (smoke produced by the cigarette but not inhaled directly through the filter tip), filter butt, and tobacco residue; under certain conditions (i.e., elevated coal temperature) some compounds may be pyrolyzed (Green et al., 1989). Vent blocking is known to increase both cigarette coal temperature (Lendvay and Lazlo, 1974) and the concentration of components in the mainstream smoke (Kozlowski et al., 1982). These effects are both likely to enhance the transfer of compounds from the tobacco to smoke (Hoffmann and Hoffmann, 1997) and increase the concentration and number of compounds formed through pyrolysis (Lendvay and Lazlo, 1974; Kozlowski et al., 1982).

In this publication, percent transfer is defined as the proportion of compounds present in the unburned tobacco that is transferred into the mainstream smoke particulate. In Table 6, we compare the effect of vent blocking on the percent transfer of alkenylbenzenes from tobacco to tobacco smoke. When the vents were unblocked, 0.79% of the myristicin in brand C was transferred to the smoke particulate; however, when the holes were completely blocked, 3.6% of the myristicin was found in the particulate. When the vents were completely blocked, the percent transfer for eugenol (150%) and isoeugenol (550%) exceeded 100%. The fact that more of these substances were found in the tobacco smoke than in the tobacco itself suggests that at least a portion of the eugenol and isoeugenol found in smoke was formed during burning of the tobacco. Studies are planned to address the effect of ventilation on the transfer of flavor-related compounds from tobacco to tobacco smoke using isotopically labeled compounds.

The smoke particulate fraction does not contain all of the flavor-related compounds transferred from the tobacco into the mainstream smoke. The vapor fraction will contain a significant amount of these compounds, depending on the individual compound volatility, chemical stability, its ionization state, its tendency to distill off as the burning coal moves down the cigarette rod, and other properties. It is important to determine the flavor-related compounds transferred into this fraction also, and we plan to expand the use of the techniques described in this work to include the vapor fraction. Thus, the results presented here represent only a portion of the flavor-related compounds transferred from tobacco into mainstream smoke.

Table 6. Effect of Filter Vent Blocking on the Percent Transfer of Several Alkenylbenzenes from the Cigarette Tobacco to Mainstream Smoke Particulate in Brand C

	percent transfer[a]		
analyte	unblocked (79% ventilation)[b]	partially blocked (59% ventilation)	completely blocked (10% ventilation)
isoeugenol	120	280	550
piperonal	0.23	0.78	1.8
myristicin	0.79	1.8	3.6
eugenol	ND	71	150
elemicin	7.6	13	24
methyleugenol	ND[c]	7.9	13

[a] Percent transfer = (smoke particulate mean concentration/tobacco mean concentration) × 100. [b] Ventilation is an average of three measurements. [c] Below limit of detection.

CONCLUSION

Smokers inhale from tobacco smoke tobacco-related compounds, and in some cases, alkenylbenzenes, which are flavor-related compounds that have known toxic properties. Finding piperonal, methyleugenol, eugenol,

isoeugenol, myristicin, and elemicin in smoke from several commercial cigarette brands shows that these compounds are present not only in the tobacco but are transferred from tobacco to mainstream smoke particulate. Because of the repetitiveness of smoking, over a 30-year span, a two-pack-a-day smoker, who is exposed to a seemingly small amount of alkenylbenzenes on a per cigarette basis, could inhale up to milligram amounts from the particulate alone. Additionally, vent blocking causes the delivered amounts of alkenylbenzenes to increase by 3- to 7-fold. More research is required to more clearly characterize the inhalation toxicology and the chronic health effects of repetitive inhalation of these compounds.

ABBREVIATIONS USED

amu, atomic mass units; CV, coefficient of variation; GC, gas chromatograph and gas chromatography; HRMS, high-resolution mass spectrometer or high-resolution mass spectrometry; LOD, limit of detection; MDA, 3′,4′-(methylenedioxy)acetophenone; MS, mass spectrometer or mass spectrometry; ND, below limit of detection; RT, retention time; RRT, relative retention time, referenced to MDA; SE, standard error of predicted value; SIM, selected ion monitoring; SIM-GC-MS, selected ion monitoring gas chromatography−mass spectrometry; SPE, solid-phase extraction; SPME, solid-phase microextraction

REGISTRY NUMBERS (SUPPLIED BY AUTHOR)

IUPAC name [common name, if available], CAS number: hexane, CAS 110-54-3; methanol, CAS 67-56-1; tetrahydrofuran, CAS 109-99-9; toluene, CAS 108-88-3; 1-hydroxy-2-methoxy-allylbenzene [eugenol], CAS 97-53-0; 1-hydroxy-2-methoxy- propenylbenzene [*trans*-isoeugenol], CAS 97-54-1; 1,2-dimethoxy-4-allylbenzene [methyleugenol], CAS 93-15-2; 1,2-dimethoxy-4-(1-propenyl)-benzene [*trans*-methylisoeugenol], CAS 6379-72-2; 1,3-benzodioxole-5-carboxaldehyde [piperonal], CAS 120-57-0; 3′,4′-(methylenedioxy)acetophenone [MDA], CAS 3162-29-6; 3,4,5-trimethoxyallylbenzene [elemicin], CAS 487-11-6; 4-methoxy-6-(2-propenyl)-1,3-benzodioxole [myristicin], CAS 607-91-0; 5-(2-propenyl)-1,3-benzodioxole [safrole], CAS 94-59-7.

ACKNOWLEDGMENT

We acknowledge the financial support received from the Office on Smoking and Health, National Center for Chronic Disease Prevention and Health Promotion, Centers for Disease Control and Prevention for this research. We thank Dr. Dana Barr for training and technical input in the development of the SPE method. We thank Mr. Fred Cardinali (GC/MS optimization) and Dr. Jim Myrick (smoking machine) for their technical assistance in this project. We also would like to thank Mr. Chet Lapeza for performing confirmational analysis of the samples by GC/HRMS.

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Received for review July 14, 1999. Revised manuscript received January 19, 2000. Accepted January 25, 2000.

JF990772I