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Solid phase microextraction of alkenylbenzenes and other flavor-related compounds from tobacco for analysis by selected ion monitoring gas chromatography–mass spectrometry

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Abstract

Some constituents found in natural flavorings are known to exhibit toxic properties. We developed a rapid method for quantifying 12 flavor-related compounds in cigarette tobacco using headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry. Using selected ion monitoring, we quantified and positively identified coumarin; pulegone; piperonal and nine alkenylbenzenes, including *trans*-anethole, safrole, methyleugenol and myristicin in one or more brands of cigarettes. In 62% of 68 brands analyzed, we detected one or more of the flavor-related compounds ranging from 0.0018 to 43 μg/g. Toxic properties of these flavor-related compounds may constitute an additional health risk related to cigarette smoking. Published by Elsevier Science B.V.

Keywords: Tobacco; Alkenylbenzenes; Pulegone; Piperonal; Coumarin

1. Introduction

Cigarette tobacco is a very complex physicochemical mixture, containing naturally-occurring constituents and chemical additives, including sugars, humectants and flavor components. Tobacco flavor additives include individually-added compounds (natural and synthetic) and botanical preparations that impart organoleptic characteristics to tobacco and tobacco smoke [1]. Botanical preparations, which include extracts, essential oils, spices, powders and oleoresins, are complex mixtures containing numerous chemical constituents. Alkenylbenzenes, a class of allyl- and propenylbenzenes with methoxy and methylenedioxy ring substitutions, are constituents of some of the botanicals preparations (e.g., anise, basil, nutmeg) used to flavor tobacco [2,3].

Genotoxicity or carcinogenicity has been reported for several alkenylbenzenes, including safrole, estragole, methyleugenol [3,4], eugenol [5] and *trans*-anethole [6]. Safrole has subsequently been banned as a flavorant in the United States [7]. Inhalation of the compound eugenol has been shown to cause pulmonary edema [8] and has been estimated to be approximately 250-times more toxic when inhaled than when ingested [9]. Additionally, it has been demonstrated that myristicin and elemicin, constituents of nutmeg, exhibit genotoxic [10] and

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hallucinogenic properties [11,12]. Piperonal, a compound structurally similar to safrole, has been shown to elicit depression in the central nervous systems of rodents [3]. In addition, the terpene ketone, pulegone, has been shown to cause irreversible destruction of cytochrome P_{450} [13], depletion of glutathione [14], and cell necrosis in liver and lung tissue in rodents [15]. Liver damage in rodents has also been found to result from exposure to coumarin, which has subsequently been banned as a flavor in the United States [7,16].

Research has established that flavoring compounds are distilled from the tobacco of a burning cigarette and transferred to mainstream smoke. In addition, some compounds may be pyrolyzed to form additional compounds present in the cigarette smoke [17,18]. A clearer knowledge of the level of certain flavor-related compounds found in commercially-available cigarette tobacco is crucial to assessing the potential health risks associated with the long-term inhalation of these compounds in mainstream cigarette smoke.

Solid phase microextraction (SPME) is a rapid, sensitive and solvent-free means of extracting chemicals directly from sample headspace. When a SPME fiber is exposed to sample headspace, volatile and semi-volatile compounds with a chemical affinity for the SPME fiber coating are absorbed and retained. Analysis of these compounds is accomplished by thermally desorbing the analytes from the fiber in the inlet port of a gas chromatograph [19]. This sampling technique has proven beneficial for extracting aroma chemicals from matrices including water [20], beverages [21], spices [22], and food [23]. In addition, SPME has been demonstrated as an excellent tool for detecting specific compounds that suggest the presence of certain botanical preparations present in flavored tobaccos [24].

In our research we quantified the concentrations of specific flavor-related compounds present in USA cigarette brands using the headspace SPME technique with a Carbowax–divinylbenzene fiber. Chemical analysis was performed using selected-ion monitoring gas chromatography–mass spectrometry (GC–MS-SIM) in the low nanogram-to-low microgram per gram range. Using a SPME protocol developed in our laboratory, we analyzed tobaccos from 68 cigarette brands for pulegone, piperonal, coumarin and nine alkenylbenzenes.

2. Experimental

2.1. Materials

2.1.1. Storage of tobacco samples

For this study, we purchased commercially available cigarette brands on the open market, labeled the pack with an adhesive identification code, logged the brand information into a database, and sealed the cigarette packages in zip-lock bags. The samples were then stored in an ultra-low freezer (Revco Scientific, Asheville, NC, USA) set at −70°C. Before analysis, we thawed the cigarettes, separated the tobacco from the filter plug and paper wrapper, and weighed the tobacco.

2.1.2. Chemicals

All reagents, used for either standard or sample preparation were checked by GC–MS for chemical purity and used without additional purification. Potassium chloride, coumarin, estragole, eugenol, piperonal, pulegone, safrole, *trans*-anethole, *trans*-isoeugenol, methyleugenol, *trans*-methylisoeugenol and 3′,4′-methylenedioxyacetophenone (MDA) were purchased from Aldrich (Milwaukee, WI, USA). Myristicin was acquired from Sigma (St. Louis, MO, USA). Elemicin was the generous gift of Dr. Peter Cadby at Firmenich (Geneva, Switzerland). Chemical standards and internal standards were prepared by dilution in ethanol (dehydrated 200 proof) that we obtained from Quantum (Tuscola, IL, USA).

2.1.3. Standards

A solution of chemical standards (i.e., estragole, elemicin, eugenol, coumarin, methyleugenol, myristicin, piperonal, pulegone, safrole, *trans*-anethole, *trans*-isoeugenol and *trans*-methylisoeugenol) and internal standard (MDA), were prepared in ethanol by dilution from the neat material. The isomeric designation (*trans*-) for anethole, isoeugenol and methylisoeugenol are omitted below. In this study we weighed materials to the nearest 0.1 mg on a research-grade analytical balance (Sartorius, Waukegan, IL, USA) and performed further manipulation of the stock solution with a Gilson Microman positive-displacement pipette (Rainin, Woburn, MA, USA). We also prepared a 3 *M* potassium chloride solution in ultra-pure water [25].

2.1.4. Equipment

The tobacco samples and KCl were weighed on a PB302 Mettler Toledo Balance (Worthington, OH, USA). Custom-made PTFE/silicone septa, open-center crimp seals, 10-ml SPME vials, were purchased from Supelco (Bellefonte, PA, USA). Septa spacer O-rings were custom ordered from Varian (Sugarland, TX, USA). Before use, serum vials, crimp seals, septa and O-rings were heated overnight at 80°C in a vacuum oven evacuated with a mechanical pump. The 65-μm Carbowax–divinylbenzene SPME fibers, manual fiber holder, sampling stand, GC inlet guide, and SPME injection sleeve used in this study were purchased from Supelco. Before initial use, SPME fibers were pre-conditioned at 250°C for 30 min. Chemical standard and internal standard additions were made with a positive displacement pipettor (Dade International, Miami, FL, USA).

2.2. Procedure

2.2.1. Tobacco spiking for standard curve analysis

For the standard curve and recovery measurements, a blank matrix was prepared using tobacco from commercial cigarettes with levels of all of the analytes of interest, except piperonal (1.1 μg/g), below the limits of detection (LODs). Approximately 100 g of tobacco was combined and mixed in a 2000-ml beaker. We transferred the tobacco (0.7-g portions) into 10-ml SPME vials and then stored the vials in the ultra-low freezer until analyzed. We prepared nine spiking solutions, one containing ethanol alone, and eight ethanol solutions containing increasing concentrations of flavor-related compounds. To each vial we added an aliquot (125 μl) of internal standard solution and an aliquot (250 μl) of one of the spiking solutions and then immediately crimp-sealed each vial. The vials were placed on a hematology mixer (Fisher Scientific, Pittsburgh, PA, USA) for 18 to 20 h to allow the contents to mix. After mixing, we performed headspace SPME as described in Section 2.2.3.

2.2.2. Sample preparation for cigarette brand analysis

For the cigarette brand analysis we weighed the tobacco from a single cigarette and deposited it into a 10-ml SPME vial. Two cigarettes were combined in one case when only a small amount of tobacco was present in a single cigarette. To each sample, we added an aliquot of internal standard solution (125 μl) and an aliquot of ethanol (250 μl), immediately crimp-sealed the vial, and mixed the contents for 18 to 20 h. After mixing, we performed headspace SPME as described in Section 2.2.3.

2.2.3. Headspace solid-phase microextraction of tobacco

Through the vial septum, we introduced a 2-ml aliquot of 3 *M* KCl into the sample using a 5-ml airtight syringe (Hamilton, Reno, NV, USA) fitted with a 27-gauge hypodermic needle (Sherwood, St. Louis, MO, USA). To prevent leakage through the needle puncture, we fit a PTFE-faced silicone septa, with the PTFE side facing down, snugly against the SPME vial septa. The vials remained at room temperature for at least 1 h to equilibrate, and then we heated an individual vial in a heating block at 95°C for 5 min. The septa covering the SPME vial septa was removed, the SPME needle inserted into the sample headspace, and the fiber exposed for 2 min at 95°C. For each analysis, we were careful to insure that the SPME fiber was positioned in the vial headspace off-centered and at a constant depth directly above the tobacco. After this exposure, the SPME fiber was retracted into the needle assembly and removed from the sample. The holder was readjusted to a deeper needle depth, and the septum piercing needle was wiped with a lint-free laboratory tissue. For desorption, we introduced the SPME needle assembly, maintained at a constant needle depth, through a SPME inlet guide into the GC inlet (230°C). The fiber was immediately exposed and the analytical run initiated in rapid succession to prevent the formation of split chromatograph peaks. The fiber remained in the inlet for at least 10 min to ensure complete sample desorption.

2.3. Instrumental analysis

2.3.1. GC–MS analysis

Analytical measurements were performed using a Hewlett-Packard (HP) 6890 GC system coupled to a HP5973 mass-selective detector (Avondale, PA, USA). The GC oven was fitted a 30-m DB-5MS column (J&W Scientific, Folsom, CA, USA). Identity of the compounds was confirmed by retention time and full scan spectra of standards analyzed with the

same instrument conditions. Mass spectra were searched against the NIST '98 (National Institute of Standards and Technology, Gaithersburg, MD, USA) mass spectral library and exhibited excellent match purity.

2.3.2. Chromatography parameters

The GC inlet port was fitted with a narrow-bore (75 mm I.D.) SPME injection sleeve and maintained at 230°C in splitless mode. High purity grade (99.997%) helium, with a flow-rate of 1.2 ml/min, was the column carrier gas during all analyses. The GC oven was programmed as follows: hold at 55°C for 1 min, ramp at 30°C/min to 110°C, ramp at 3°C/min to 155°C, ramp at 30°C/min to 270°C. The transfer line was maintained at 280°C, and the quadrupole and source heaters were held at 110°C and 230°C, respectively. The mass spectrometer was routinely calibrated using Autotune provided in the HP ChemStation software. Peak areas were integrated using the ChemStation Integrator program in the HP Enhanced ChemStation software (version A.03.00).

2.3.3. SIM parameters

Mass spectra were acquired in the SIM mode with a quantitation and a confirmation ion for each analyte. Table 1 lists the quantitation ion, confirmation ion and number of SIM scans per ion. The dwell time for each SIM scan was 15 ms. The quantitation ion for each compound was the mass spectral peak of highest abundance and the least amount of signal interference from overlapping components. The confirmation ion was the next most abundant ion fragment with minimal background interferences. Analyte identity was established based on comparison of retention time and quantitation ion-to-confirmation ion ratios between the standards and the corresponding analyte in the sample. Relative response factors were calculated as the ratio of the area of the quantitation ion peak area divided by the peak area of the internal standard ion.

2.3.4. Data collection and statistical calculations

Chromatogram peak areas were determined automatically, checked for proper integration, and reintegrated manually if needed. Areas were transferred to a database designed specifically for this application in R:Base version 4.5 (Microrim, Bellevue, WA, USA). We carried out statistical determinations with Statistical Analysis System software (SAS Institute, Cary, NC, USA).

Table 1
Retention time, relative retention time, quantitation and confirmation ions

	Retention time (min)	Relative retention time	Quantitation mass (u[a], SIM[b] scans)	Confirmation mass (u[a], SIM[b] scans)
MDA[c] (I.S.[d])	12.51	1.00	149 (4)[e]	164 (4)
Estragole	6.71	0.54	148 (4)	147 (4)
Anethole	8.53	0.68	148 (2)	147 (2)
Safrole	8.65	0.69	162 (3)	161 (3)
Eugenol	10.14	0.81	137 (7)	149 (5)
Methyleugenol	11.40	0.91	178 (5)	147 (5)
Isoeugenol	12.86	1.03	164 (4)	149 (4)
Methylisoeugenol	14.32	1.14	178 (5)	163 (6)
Myristicin	15.14	1.21	192 (4)	165 (5)
Elemicin	15.98	1.28	208 (6)	177 (6)
Pulegone	7.52	0.60	152 (6)	81 (4)
Piperonal	9.80	0.78	149 (1)	150 (1)
Coumarin	12.56	1.00	146 (3)	90 (4)

[a] Atomic mass units.
[b] Selected ion monitoring.
[c] 3′,4′-Methylenedioxyacetophenone.
[d] Internal standard.
[e] Dwell time per SIM scan=15 ms.

3. Results and discussion

3.1. SPME chromatogram

SIM chromatograms of tobacco from a non-menthol cigarette with high alkenylbenzene levels (Brand B) and tobacco from a menthol cigarette with moderate levels of alkenylbenzenes (Brand G) are displayed in Fig. 1A and B, respectively. The identity of compounds that are indicated in the chromatograms was confirmed in full scan mode using the NIST library. The prominent peak at 12.50 min corresponded to the internal standard, MDA. We observed the naturally-occurring tobacco constituents, solanone and myosmine [26], in both chromatograms at 10.30 and 12.22 min, respectively. In addition, the flavor-related compound, ethyl cinnamate [2] was observed in both chromatograms at 13.48 min. For Brand B (Fig. 1A), the most prominent analyte peaks in this chromatogram were myristicin, methyleugenol, safrole, elemicin, and a smaller piperonal peak (9.80 min). Less abundant peaks included eugenol, isoeugenol and methylisoeugenol.

For Brand G (Fig. 1B) the most prominent analyte peaks in the chromatogram were piperonal, pulegone and myristicin (15.16 min). We tentatively identified the prominent peak at 7.49 min as menthomenthene, which was found in all 24 menthol brands analyzed. Additionally, we found two terpene compounds, tentatively identified as bisabolene and sequiphellan-

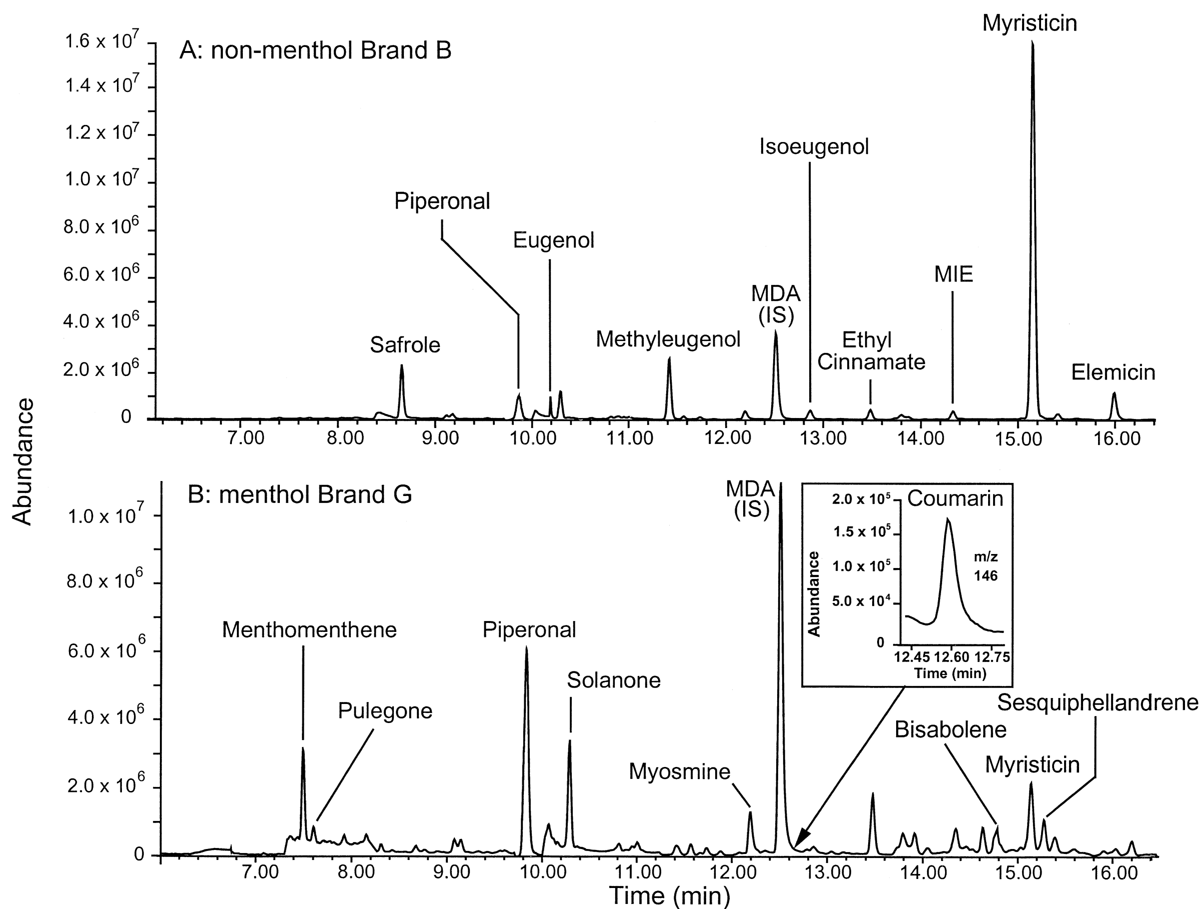

Fig. 1. Selected ion monitoring GC chromatogram of (A) non-menthol Brand B and (B) menthol Brand G. Quantitation ion of coumarin (m/z 146) is shown in (B insert). I.S. denotes internal standard, in this case, MDA (3′,4′-methylenedioxyacetophenone). Methylisoeugenol is abbreviated as MIE.

drene, in Brand G at 14.79 and 15.28 min, respectively. The SIM chromatograms were characterized by symmetrical peak shape, excellent resolution and low levels of signal interference. All compounds identified with the NIST library exhibited excellent match purities. Table 1 presents the retention time, relative retention time versus MDA, the quantitation ion and the confirmation ion for each analyte.

3.2. Calibration curve

For the purpose of quantitation, we generated a calibration curve for nine alkenylbenzenes and three other flavor-related compounds with concentrations ranging over five-orders of magnitude in the low nanogram to low microgram range. The standards were spiked directly onto a ''blank'' tobacco that had low levels of the analytes investigated in this study. The concentration ranges and correlation coefficients of the least-squares linear regression fit, as well as, the slopes, *y*-intercepts and standard errors are presented in Table 2. The average R^2 value for all analytes is 0.96, which indicates good linearity in the concentration ranges investigated for these analytes.

3.3. Limits of detection

The LODs were determined by spiking standards onto blank tobacco from a cigarette with low levels of the compounds being investigated. We spiked three different spike concentrations onto five blank samples each. In addition, we spiked five blank tobacco with ethanol alone to provide a baseline level. The LOD values were calculated as three-times the standard deviation at zero concentration [27]. The lowest LOD for the analytes varied somewhat but were generally in the low ng/g range. The highest LOD observed was for piperonal (1.1 μg) due to elevated baseline levels of the piperonal present in the blank tobacco. The LODs for compounds spiked onto the blank tobacco are presented in Table 3.

3.4. Recovery study of analytes

Mean recovery for alkenylbenzenes and other flavors spiked onto blank tobacco are presented in Table 3. The most consistent recoveries across all three spike concentrations were observed for myristicin, anethole, eugenol and estragole. The medium and high spike levels for all analytes showed excellent recoveries with values ranging from 97% to 116%. The recoveries at the low spike were between 94% (eugenol) and 120% (methyleugenol) with a few exceptions. The recovery of the low spike level was significantly different from 100% for elemicin (51%), coumarin (159%), methylisoeugenol (166%) and safrole (194%). The low recovery value for elemicin and the elevated values for coumarin, methylisoeugenol and safrole at the low spike level

Table 2
Concentration range, slope, *y*-intercept and correlation coefficient of multiple-point standard curve for 12 common flavor additives in cigarette tobacco

Analyte	Concentration range (μg)	Slope (±S.E.[a])	*y*-Intercept (±S.E.[a])	Correlation coefficient
Isoeugenol	0.012–1.6	0.425±0.006	0.0073±0.0013	0.988
Coumarin	0.0091–1.7	0.100±0.002	0.00024±0.00013	0.985
Methylisoeugenol	0.010–2.0	1.07±0.02	0.0091±0.0014	0.985
Piperonal	0.033–26	0.0370±0.006	0.089±0.003	0.985
Myristicin	0.057–14	0.619±0.012	0.0059±0.0006	0.983
Eugenol	0.010–1.6	0.479±0.009	0.0046±0.0009	0.981
Methyleugenol	0.0022–1.5	1.91±0.04	0.0035±0.0004	0.972
Anethole	0.0026–2.0	0.550±0.016	0.00065±0.00010	0.953
Elemicin	0.010–0.94	1.96±0.06	–0.015±0.003	0.946
Pulegone	0.0030–1.8	0.947±0.032	0.0010±0.0009	0.938
Estragole	0.0029–1.9	1.68±0.06	0.0041±0.0013	0.928
Safrole	0.0016–2.2	1.03±0.05	0.0026±0.0003	0.895

[a] S.E.=Standard error of predicted value.

Table 3
Detection limits, mean recovery and reproducibility for common flavor additives spiked onto blank tobacco (n=5)

Analyte	Detection limit (μg)	Spiking concentration (μg)	Mean recovery (%)	RSD[a] (%)
Estragole	0.0029	0.029	109	17
		0.24[b]	100	13
		1.9	116	12
Pulegone	0.015	0.030	102	16
		0.23[b]	97	12
		1.8	115	13
Anethole	0.0026	0.026	110	15
		0.25[b]	101	10
		2.0	114	11
Safrole	0.0016	0.016	194	16
		0.28	97	10
		2.2[b]	112	11
Eugenol	0.010	0.020	94	21
		0.20[b]	99	8.3
		1.6	108	6.7
Methyleugenol	0.0022	0.022	120	12
		0.19[b]	104	7.9
		1.5	109	8.4
Coumarin	0.013	0.018	159	25
		0.22[b]	100	6.1
		1.7	106	3.2
Methylisoeugenol	0.010	0.02	166	11
		0.25[b]	104	8.6
		2.0	104	9.6
Myristicin	0.016	0.16	97	14
		1.7[b]	102	9.4
		14	97	7.2
Elemicin	0.010	0.02	51	13
		0.12[b]	116	5.8
		0.94	107	7.9
Isoeugenol	0.033	0.21[b]	114	6.7
		1.6	112	7.4
Piperonal	1.1	3.3[b]	109	6.9
		26	107	3.2

[a] Relative standard deviation.
[b] n=4.

are likely due to variations associated with making measurements near the LOD. The low spike level for elemicin, coumarin and methylisoeugenol was within twice the LOD, and for safrole, was the lowest spiking level reported for all the analytes. No data are given for the low spike levels for isoeugenol and piperonal since these levels were below the LOD.

Many flavor additives are added to tobacco in a process called top flavoring, which involves spraying a rum or ethanol mixture, containing the flavoring agents onto tobacco in a rotating cylinder or on a moving belt [1]. These flavor compounds may be absorbed by the tobacco matrix as the tobacco ages. In order to generate our standard curve and determine recovery, we added flavors to commercial cigarette tobacco in an ethanol solution and rotated the tobacco in a sealed vial for 18 h in order to mimic the actual flavoring process. The quantification and validation steps of our method were designed to measure these added compounds and not necessarily other compounds that were more tightly bound within the matrix. The results of the recovery study indicated that the amount of analyte added to the tobacco was almost completely recovered from the matrix. The high recoveries are probably due to the elevated temperature and concentrated salt solution use to liberate the analytes from the matrix during extraction.

3.5. Sample analysis precision

The relative standard deviation (RSD) values at the high and medium spike concentrations were 8% and 9%, respectively (Table 3). The RSDs at the low spike levels were between 11% and 17%, with the exception of eugenol (21%) and coumarin (25%). The elevated RSD values for eugenol and coumarin at the lowest spike level are likely due to increased analytical error associated with measurements that approach the LOD. The average RSD value for all concentrations was 11%, showing good reproducibility.

3.6. Cigarette brand survey

In this study we investigated the level of 12 flavors in top-selling USA brands of cigarettes. These brands were chosen from 1998 first quarter sales estimates for the cigarette industry. Private-label brands were not analyzed in this study [28]. We examined 68 USA brands, including 45 non-menthol and 23 menthol brands.

Comparisons are made on the basis of per gram concentration (μg/g) in order to remove bias due to differences in mass for various brands. The amount (μg) of analyte detected in a cigarette was divided by the cigarette tobacco mass analyzed (g) and expressed in μg/g. The average mass of cigarette tobacco in this study was 0.73 g.

In 62% of the 68 brands we found one or more of the 12 analytes at levels above the analyte LOD values. Twenty-three of non-menthol brands (51%) and 18 of menthol brands (78%) contained detectable levels of at least one of the compounds. Table 4 summarizes the minimum and maximum, mean and median values of flavor-related compounds found in the 68 USA brands. Anethole, myristicin and safrole were the compounds detected most frequently in the brands, with each found above the LOD in at least 20% of the 68 brands surveyed. Pulegone, piperonal and methyleugenol were each present in at least 10% of the 68 brands. We detected estragole, methylisoeugenol, isoeugenol and elemicin only in non-menthol cigarettes. Anethole, piperonal, eugenol, methyleugenol and myristicin had approximately the same frequency and concentrations in both menthol and non-menthol cigarettes. Approximately half of the menthol cigarette brands lacked detectable levels of pulegone and the other half contained pulegone concentrations ranging from 0.037 to 0.29 μg/g. We did not detect pulegone in any of the non-menthol brands. Coumarin was found above the LOD in a single menthol brand at 0.39 μg/g.

Table 5 contains the values found in nine USA brands for the eight most frequently occurring analytes. The alkenylbenzenes, myristicin, safrole and elemicin, in order of concentration, were found together in four brands (A, B, C and D). The average stoichiometric ratio of myristicin, safrole and elemicin was approximately 60:2:1, for these four brands. Brand A, which contained the highest concentration of myristicin, contained the three alkenylbenzenes mentioned above, as well as methyleugenol, eugenol, isoeugenol and methylisoeugenol. Generally, as the level of myris-

Table 4
Statistical summary of 12 flavor-related compounds found in 68 USA brands of cigarette at levels above the method LOD

Compounds	Number detected[a]	% Detected	LOD[b] (μg/g)[c]	Minimum (μg/g)	Maximum (μg/g)	Mean (μg/g)	Median (μg/g)
Anethole	21/68	31	0.0026	0.0046	0.23	0.043	0.012
Myristicin	19/68	28	0.016	0.025	12	1.5	0.086
Safrole	16/68	24	0.0016	0.0018	0.59	0.066	0.0052
Piperonal	13/68	19	1.1	1.6	43	11	3.0
Pulegone	12/68	18	0.015	0.024	0.29	0.16	0.15
Methyleugenol	9/68	13	0.0022	0.0031	0.54	0.082	0.0059
Eugenol	6/68	8.8	0.010	0.010	0.15	0.073	0.070
Elemicin	4/68	5.9	0.01	0.015	0.30	0.16	0.16
Estragole	4/68	5.9	0.0029	0.0053	0.018	0.011	0.0099
Isoeugenol	4/68	5.9	0.033	0.068	0.38	0.24	0.25
Methylisoeugenol	1/68	1.5	0.01	0.019	0.019	0.019	0.019
Coumarin	1/68	1.5	0.013	0.39	0.39	0.39	0.39
Pulegone (Menthol only)	12/23	52	0.015	0.024	0.29	0.16	0.15

[a] Number detected is expressed as (brands with detected analyte)/(total brands analyzed).
[b] Limit of detection.
[c] Calculated limits of detection in μg/g assume a tobacco mass of 1 g.

ticin decreased from brand to brand, the levels of these other alkenylbenzenes also decreased until they were below the analyte LOD. This was evident when we compared the levels of these flavor-related analytes in brands B, C and D. Brands with myristicin concentrations less than those present in Brand D were found to contain no detectable levels of elemicin, as was the case with Brand E. Safrole, which had a lower LOD (0.0016 μg/g) than elemicin, was found to be present in 80% of the brands that contained myristicin. We found myristicin alone in five brands with concentrations ranging from 0.031 to 0.044 μg/g, concentrations just above the LOD for myristicin (0.016 μg/g). These results

Table 5
Amount of the eight most frequently detected flavors in nine USA cigarette brands (brands were analyzed in triplicate unless otherwise noted)

Brand	Amount (μg/g, RSD[a], %)							
	Anethole	Piperonal	Pulegone	Safrole	Eugenol	Methyleugenol	Myristicin	Elemicin
Non-menthol brands								
A	ND[b]	ND	ND	0.26[e] (24)	0.15[e] (13)	0.083 (18)	12[d] (18)	0.16[e] (6.4)
B	0.0046[e] (15)	6.8[f] (20)	ND	0.59[f] (18)	0.13[f] (10)	0.54[f] (39)	7.9 (8.3)	0.30[f] (35)
C	0.021 (23)	43 (3.8)	ND	0.11 (25)	0.12[c] (3.6)	0.16 (13)	7.1 (16)	0.15 (10)
D	ND	ND	ND	0.055 (22)	0.016 (8.2)	0.011 (13)	1.2 (14)	0.015[c] (15)
E	0.11 (1.6)	15.3 (4.2)	ND	0.013 (3.5)	ND	0.0041 (0.71)	0.16 (5.5)	ND
F	0.12 (13)	ND	ND	ND	ND	ND	0.032 (16)	ND
Menthol brands								
G	ND	20 (28)	0.037 (37)	0.015 (35)	0.021 (40)	0.0059 (30)	0.44 (31)	ND
H	0.043 (25)	ND	ND	0.022 (25)	ND	0.0034[c] (9.6)	0.16 (6.5)	ND
I	ND	2.0 (5.8)	ND	ND	ND	ND	ND	ND

[a] Relative standard deviation.
[b] Result less than limit of detection.
[c] $n=2$.
[d] $n=4$.
[e] $n=5$.
[f] $n=6$.

suggest a common botanical source for these alkenylbenzenes, which are found together in tobacco flavorings, such as those derived from nutmeg (*Myristica fragrans* Houtt.) [29].

In comparing the menthol and non-menthol varieties of the same brand, we did not observe any no clear combinations of flavor-related compounds among the 16 pairs investigated. Five of the pairs had none of the 12 analytes at levels above the LOD, and three other pairs contained only pulegone in the menthol variety. In a few cases, the non-menthol variety contained no detectable flavor-related compounds, whereas the menthol variety contained a single analyte, such as anethole, piperonal, or eugenol with or without the presence of pulegone. In another case, the non-menthol variety (Brand A) contained elevated levels of several flavor-related compounds, but the menthol variety had no detectable levels of any analyte. By contrast, in a different pair, the non-menthol (Brand C) and menthol (Brand G) varieties both had similar compounds but with lower levels in the menthol variety. These data suggest that the menthol variety of a brand does not always contain the same flavor-related compounds as the non-menthol variety.

The single internal standard used in this study, MDA, was not optimum for quantifying all of the analytes because R^2 values were lower for such compounds as safrole, estragole and pulegone. The use of isotopically labeled reference compounds would significantly improve the method reproducibility. In addition, the variability in the analytical results for the brands may have been due to within-pack sample variation due to such factors as non-uniformity of flavor addition or analytical variation from run-to-run. Lot-to-lot variation undoubtedly exists but was not addressed in this study because all samples were taken from a single pack.

Matrix differences may exist between different types of tobaccos (e.g., burly, bright and oriental) due to their different curing processes and sugar and moisture contents. USA blended cigarettes, though differing somewhat in makeup, have a similar distribution of these tobacco types, in general. Since all of the cigarette tobaccos measured and reported here are USA blended cigarettes, the matrix effects between the test samples and calibration materials are expected to be minor compared to the significant differences seen in various brands.

Research is currently under way in our laboratory to address the levels of alkenylbenzenes transferred to mainstream smoke and the effects of ventilation on that process. Subsequent research will address the presence of other flavor-related compounds present in tobacco and in tobacco smoke.

4. Conclusion

In this study we used SPME to quantify the levels of alkenylbenzenes and other flavor-related compounds in cigarette tobacco from commercial cigarettes. The biological activity of these compounds and the variation in their concentration in cigarette tobacco suggest that the health risks from these additives should be evaluated. Because of the nature of smoking, exposure to the same flavor-related compounds occurs on a repetitive basis if a single brand is smoked regularly over an extended period. Currently, the long-term health effects are associated with inhaling these compounds in tobacco smoke are not known.

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