Qualitative and Quantitative Analysis of Flavor Additives on Tobacco Products Using SPME−GC−Mass Spectroscopy

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Headspace solid-phase microextraction−gas chromatography−mass spectroscopy (HS-SPME−GC−MS) has been used for both qualitative and quantitative analysis of flavor additives to tobacco. Sampling conditions for the 100 μm methyl silicone fiber, 65 μm polyacrylate fiber, 65 μm methyl silicone/divinylbenzene fiber, and 65 μm Carbowax/divinylbenzene fiber were investigated. Menthol, anethole, benzaldehyde, and tetramethylpyrazine were quantitated on spiked Kentucky Reference 1R1 tobacco. Major components in mandarin orange oil, nutmeg oil, and sweet fennel oil exhibited linear relationships with concentration of the essential oil. Limits of detection for 31 typical tobacco flavors have been determined.

Keywords: *Flavor additives; tobacco; solid-phase microextraction; gas chromatography; mass spectroscopy; headspace.*

INTRODUCTION

Over the past two decades, there has been an increased interest and usage of tobacco flavor additives (US DHHS, 1989). This has been due primarily to a decreased use of tobacco lamina in blends and the introduction of low-yield cigarette products. Natural tobacco flavor components vary depending upon crop year, genetic variants, and the process in which the tobacco was cured. An analytical procedure which can quantitate added and natural tobacco flavor materials would be beneficial to identify components which may be deficient in poor quality leaf. Accurate determination of such data would provide valuable information for the tobacco flavorist. In addition, a rapid procedure would provide an excellent tool to control the quality and to assure uniform addition of tobacco flavorants.

The evaluation of flavors and other tobacco constituents has been performed by several workers; however the analytical procedures are often laborious. Several methods such as the qualitative and quantitative identification of tobacco additives from steam distillates yield unreliable results (LaVoie et al., 1985, 1989). Steam distillation is a harsh isolation technique and frequently produces artifacts from the degradation or reaction of components. Incomplete removal or loss of volatile flavor materials also detracts from this methodology. Existing techniques have primarily reported the components natural to tobacco (Wu et al., 1992, 1989; Gordon et al., 1988; Mendell et al., 1984; Lloyd et al., 1976). Tobacco additives (Leffingwell, 1974) have been investigated to a lesser degree. Of these, sugars (Carmella et al., 1984) and humectants (Carugno and Lionetti, 1971) have been evaluated.

Solid-phase microextraction (SPME) is a relatively new sampling technique which involves exposure of a microfiber coated with a stationary phase directly to an aqueous solution or to the headspace above the analyte of interest. This technique has been utilized successfully for the evaluation of environmental samples (Zhang and Pawliszyn, 1993; Bucholz and Pawliszyn, 1994), flavor materials (Yang and Peppard, 1994; Pelusio et al., 1995), and cigarette smoke condensate (Clark and Bunch, 1996). We recently reported our results on the identification of tobacco varieties by headspace solid-phase microextraction−gas chromatography−mass spectrometry (HS-SPME−GC−MS) (Clark and Bunch, 1995; Mindrup, 1995).

In this study we have examined the application of solid-phase microextraction for the analysis of cigarette tobacco flavor additives. This technique provides a rapid and reliable approach to the quantitative analysis of flavor additives.

EXPERIMENTAL PROCEDURES

Instrumentation. A Hewlett-Packard (HP) 5890 gas chromatograph (Palo Alto, CA) directly interfaced with a HP 5970B mass selective Detector was used to separate and analyze the tobacco headspace components. The instrument was controlled by a HP G1030A DOS Chemstation using standard quantitation programming. Splitless injections were made at 250 °C using a manual solid phase microextraction (SPME) syringe equipped with commercially available SPME fibers (Supelco, Inc., Bellefonte, PA). Separation was accomplished using a HP 30 m DB-5MS column (0.23 mm i.d. × 0.20 μm film thickness). The initial oven temperature was 40 °C for 1 min and then increased to 250 °C at 5 °C/min. The GC−MS transfer line was maintained at 280 °C. Mass spectrometric detection was made in the scan mode.

Method Development. Tobacco (1.0 g) was removed from Kentucky Reference 1R1 (Tobacco and Health Research Institute, University of Kentucky, Lexington, KY) cigarettes and placed into a headspace vial (20 mL). Two microliters of 2,6-dichlorotoluene (ISTD) (1 μg/μL) solution and 1 μL of the flavor spike mixture (1 μg/μL each of benzaldehyde, tetramethylpyrazine, menthol, and anethole in ethanol) was added via a Hamilton syringe. The samples were sealed and allowed to equilibrate for 2 h at room temperature before analysis. Sample conditions including SPME fiber exposure time, sample temperature, and influence of salt addition were evaluated as indicated in Table 1. A 100 μm poly(dimethylsiloxane) SPME fiber was used for these experiments, and each experiment was performed in duplicate. Extreme care was taken to ensure the fiber was placed in the same location for each exposure to the headspace. The manual SPME device was operated according to the manufacturer's instructions (Supelco, Inc., Bellefonte, PA).

Conditions producing the highest signal to noise ratio (S/N) for the 100 μm poly(dimethylsiloxane) fiber (1 g of Kentucky

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Table 1. Sample Conditions Used with 100 μm Poly(dimethylsiloxane) SPME Fiber

sample Kentucky Reference 1R1 (1 g)	SPME fiber exposure time (min)	sample temp (°C)	3 M KCl solution (mL)
1	5	25	
2	15	25	
3	30	25	
4	45	25	
5	5	50	
6	15	50	
7	30	50	
8	45	50	
9	5	95	
10	15	95	
11	30	95	
12	45	95	
13	15	145	
14	15	25	1
15	30	25	1
16	15	25	2
17	30	25	2
18	15	25	3
19	30	25	3
20	15	95	1
21	30	95	1
22	15	95	2
23	30	95	2
24	15	95	3
25	30	95	3

Reference 1R1 tobacco at 95 °C with 1 mL of 3 M KCl solution) were utilized to determine optimal fiber exposure times for the 65 μm polyacrylate fiber, 65 μm poly(dimethylsiloxane)/divinylbenzene fiber and 65 μm Carbowax/divinylbenzene fiber by performing analysis with each fiber after 5, 15, 30, or 45 min fiber exposure time. The temperature was maintained using a dry bath equipped with a heating block for 25 mm vials (Fisher Scientific, Raleigh, NC). The 65 μm Carbowax/divinylbenzene fiber using 15 min fiber exposure time provided the highest S/N ratio with minimal inteferences from the background matrix. This SPME fiber was selected for use in quantitative analysis studies.

Quantitative Analysis. Headspace SPME at 95 °C was performed on tobacco samples (1 g) treated with 2 μL of 2,6-dichlorotoluene ISTD (1 μg/μL), 1 mL of 3 M KCl solution, and 1, 3, or 5 μg of a spike flavor mixture (1 μg/μL each of benzaldehyde, tetramethylpyrazine, menthol, and anethole in ethanol) using the 65 μm Carbowax/divinylbenzene fiber. Calibration curves for menthol, benzaldehyde, tetramethylpyrazine, and anethole were constructed, and the correlation coefficients approached unity for each flavor material. Samples containing 2.5 μg of the spike flavor mixture were analyzed in the same manner and quantitated. Five replicate analyses were performed.

Mandarin orange oil (1, 5, or 10 ppm) (Mother Murphies Laboratories, Greensboro, NC) was spiked onto Kentucky Reference 1R1 tobacco (1 g) and analyzed by HS-SPME−GC−MS. The limonene peak (ion 71) was used to construct a calibration curve. Nutmeg oil (1, 5, or 10 ppm) (Mother Murphies Laboratories, Greensboro, NC) was then added to Kentucky Reference 1R1 tobacco (1 g) and analyzed as described. Two separate calibration curves were constructed, one for the peak corresponding to safrole (ion 162) and one for myristicin (ion 192). Finally, Kentucky Reference 1R1 tobacco (1 g) was treated with sweet fennel oil (1, 5, or 10 ppm) (Mother Murphies Laboratories, Greensboro, NC) and analyzed as described, and the peak for anethole (ion 148) was used to construct a calibration curve.

Detection Limits. Kentucky reference 1R1 tobacco (1 g) was treated with 1, 5, or 10 μg of ethyl nonanoate, ethyl decanoate, ethyl octanoate, ethyl hexanoate, isoamyl phenylacetate, diethyl malonate, benzyl benzoate, ethyl phenylacetate, linalool, geraniol, benzyl alcohol, hexanal, cinnamaldehyde, octanal, decanal, menthone, acetophenone, pinene, trimethylpyrazine, 2,6-dimethylpyrazine, vanillin, ethylvanillin, maltol, salicaldehyde, hexalactone, heptalactone, benzaldehyde, tetramethylpyrazine, menthol, and anethole. One milliliter of 3 M potassium chloride solution was added, the samples were heated at 95 °C and allowed to equilibrate for 15 min, and a 65 μm Carbowax/divinylbenzene SPME fiber was exposed to the headspace for 15 min. The samples were introduced into the GC−MS injector and analyzed as described. Extracted ion chromatograms for the major ion of each flavorant were used to estimate detection limits at a 10:1 S/N ratio.

RESULTS AND DISCUSSION

Method Optimization. Several factors have been shown to increase sensitivity of the SPME technique including the sample matrix, sample temperature, SPME fiber exposure time, ionic strength of the matrix, analyte structure, and fiber polarity. Each of these factors has been investigated to determine optimum conditions for the analysis of flavor additives on tobacco products.

Matrix Effects. Sample matrix changes provide significant differences in signal intensity of analytes of varying structure. The equilibrium effects between matrix, headspace, and SPME fibers has been elegantly described in detail by others (Louch et al., 1992; Chaintreau et al., 1995). Results from our laboratory have shown that the tobacco matrix has a strong affinity for many of the analytes of interest. For example, the headspace above 1 μg each of benzaldehyde, tetramethylpyrazine, menthol, anethole, and 2,6-dichlorotoluene in 1 mL of water at 25 °C was extracted using a 100 μm poly(dimethylsiloxane) fiber. Fiber exposure times of 5, 15, 30, and 45 min were evaluated. The analytes were easily detected by GC−mass spectroscopy. However, when 1 μg of each component was added to 1 g of tobacco, the analytes provided peaks of significantly lower intensity. A 65 μm polyacrylate fiber, 65 μm poly(dimethylsiloxane)/divinylbenzene fiber, and 65 μm Carbowax/divinylbenzene fiber were also evaluated and similar results were observed.

Temperature Effects. Several headspace techniques have been reported for the analysis of tobacco (Coleman, 1992; Heinzer et al., 1988; Dirinck et al., 1980). Increase in sample temperature has been used in these traditional techniques to release volatiles into the headspace. Sample temperatures ranging from ambient to 145 °C have been evaluated for the analysis of flavorants on tobacco. Headspace SPME of spiked tobacco samples at 25 °C provided an extremely low signal to noise ratio (S/N) independent of the SPME fiber coating. An increase in temperature to 50 °C provided a slight increase in signal to noise ratio for menthol and anethole, but the signals for benzaldehyde and tetramethylpyrazine decreased (Figure 1). At 95 °C sample temperature, benzaldehyde and tetramethylpyrazine intensities were unchanged from 50 °C. However, menthol and anethole signal intensities were greatly enhanced. A further increase in temperature to 145 °C did not increase the signal to noise ratios. As the temperature was increased above 95 °C, a broad signal for glycerol, a known additive to Kentucky Reference 1R1 tobacco, was intensified, thus masking the peak for menthol. This extreme temperature dependence indicates that appropriate conditions for each class of analytes should be developed for best performance of the method.

Elevated temperatures (>100 °C) were expected to produce artifacts reminiscent of reactions between analytes. Indeed this was the case as evidence of Maillard

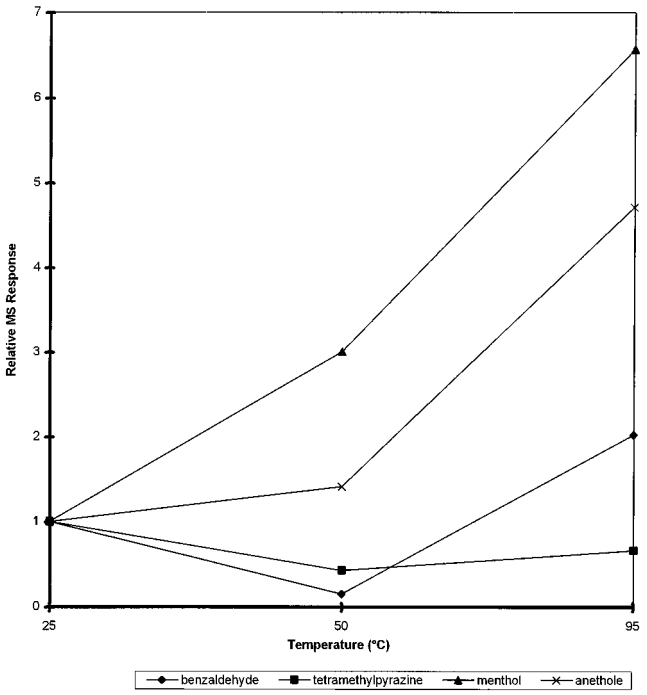

Figure 1. Sample temperature versus intensity, 15 min SPME. MS response relative to 25 °C signal intensity.

reaction and sugar degradation products was seen at 145 °C whereas it is not present at 95 °C (Bunch and Clark, 1995). Heating of an amino acid with fructose at 145 °C for 5 min provided a similar profile, whereas at 95 °C there was no indication of reaction products. This provides evidence suggestive of Maillard reactions between the amino acids in the tobacco and sugars at elevated temperatures. Many of the products from these reactions are common tobacco additives; therefore analysis at these elevated temperatures would not indicate accurate baseline concentrations.

SPME Fiber Exposure Time. Fiber exposure time has a dramatic effect on the signal intensity for flavor components. Exposure time profiles versus signal intensity for tobacco samples at 95 °C indicate that some components of a spike flavor mixture equilibrate to a maximum in 15 min (Figure 2). Signal intensity versus fiber exposure time is analyte dependent. For example benzaldehyde, menthol, and anethole indicate maximum signal intensity in 15 min. Tetramethylpyrazine signal intensity indicates a maximum at 5 min exposure time and then slowly decreases. Longer sampling times (>1 h) indicate no increase in signal intensity for analytes in the flavor mixture.

Salt Effects. The addition of salt solution to the sample matrix has varied effects on the equilibrium process dependent upon the analyte structure and properties. Sampling for 15 min at 95 °C exhibited increases in intensity for menthol and benzaldehyde, but not anethole and tetramethylpyrazine upon the addition of 1 mL of 3 M potassium chloride solution (Figure 3). Additional KCl solution provided an overall decrease in signal intensity. The decrease is most likely due to the dilution. Although signal intensities were higher with the addition of KCl solution at 30 min fiber exposure, glycerol absorption onto the fiber began to mask the menthol and internal standard peaks. A maximum signal to noise ratio with minimal inteferences was determined using 1 mL of 3 M potassium chloride solution and fiber exposure at 95 °C for 15 min. The chromatogram shown in Figure 4 was determined

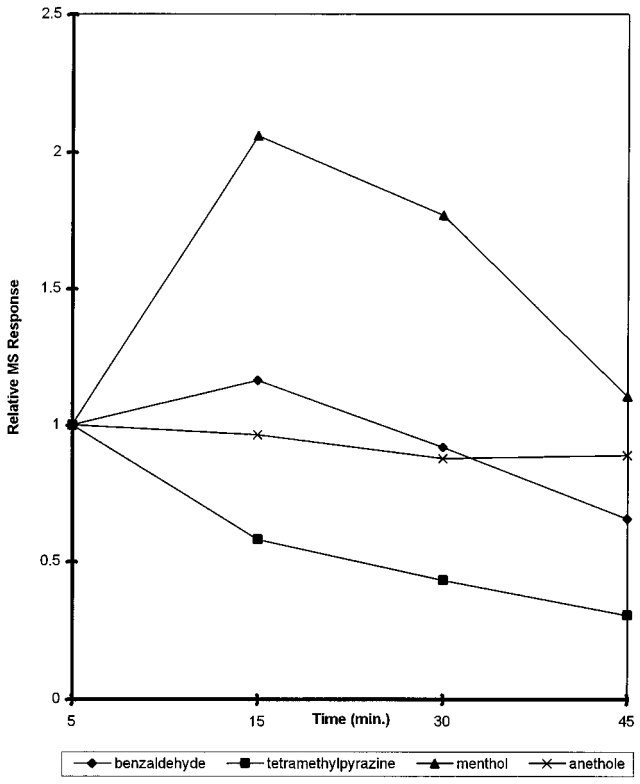

Figure 2. Sampling time versus intensity, 95 °C. MS response relative to 5 min fiber exposure time.

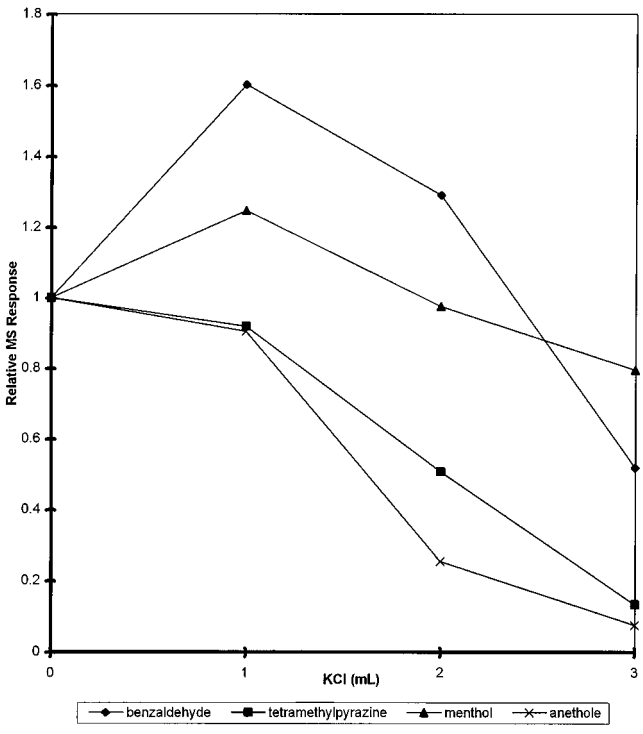

Figure 3. Amount of KCl versus intensity, 95 °C, 30 min. MS response relative to no KCl solution added.

using these conditions. Figure 4 indicates the complexity of the Kentucky Reference 1R1 tobacco matrix and the relative intensity of the components added in the spike flavor mixture.

SPME Fiber Coating. The stationary phase of the SPME fiber has a key role in determining the signal intensity. Nonpolar stationary phases such as methyl silicone are expected to prefer nonpolar analytes. Conversely, a polar stationary phase such as polyacrylate

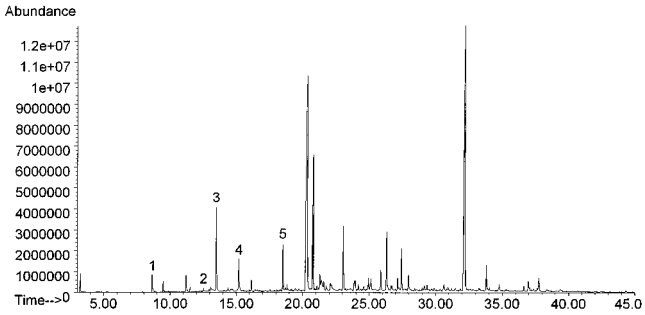

Figure 4. HS-SPME–GC–MS chromatogram of Kentucky Reference 1R1 tobacco with 1 μg of the spike flavor mixture. (1) benzaldehyde, (2) tetramethylpyrazine, (3) 2,6-dichlorotoluene, (4) menthol, (5) anethole. Conditions: 1 g of Kentucky Reference 1R1 tobacco with 1 μg of spike flavor mixture, 1 μg of ISTD, and 1 mL of 3 M KCl solution. The sample was heated at 95 °C for 30 min. SPME was performed by exposure of a 65 μm Carbowax/divinylbenzene SPME fiber to the headspace.

Table 2. Optimum Conditions for SPME Fiber Types

SPME fiber	fiber exposure time (min)	sample temp (°C)	3 M KCl (mL)	fiber polarity[a]
100 μm methyl silicone	15	95	1	nonpolar
65 μm methyl silicone/divinylbenzene	30	95	1	sl polar
65 μm Carbowax/ divinylbenzene	30	95	1	mod polar
65 μm polyacrylate	30	95	1	polar

[a] sl = slight, mod = moderate.

will exhibit some selectivity for polar analytes. Analysis performed using a polyacrylate fiber indicated the amount of potassium chloride solution and temperature required for maximum signal to noise ratio were similar to those determined for the methyl silicone fiber. Fiber exposure times providing the highest S/N ratio were determined for the 65 μm polyacrylate fiber, 65 μm methyl silicone/divinylbenzene fiber, and 65 μm Carbowax/divinylbenzene fiber using the same temperature and potassium chloride concentration as determined (highest S/N ratio) for the 100 μm methyl silicone fiber (Table 2). The methyl silicone/divinylbenzene and Carbowax/divinylbenzene fibers are intermediate in polarity and were assumed to behave similarly to the 100 μm methyl silicone fiber.

In order to determine which fiber is most suited for the analysis of flavor additives on tobacco, several factors were addressed. First, the flavor materials must be considered as they vary in structure, polarity, and volatility. The fiber polarity must also be addressed if analysis of flavorants with varying polarity is desired. Flavor solvents such as propylene glycol, glycerol, and ethanol should be retained on the fiber at a minimal level. Lastly, due to the extremely low concentrations of many flavor materials which may be added to tobacco, the most sensitive fiber should be utilized.

The more polar fibers tend to lend more diversity for the analysis of tobacco headspace volatiles. There are less interferences by highly nonpolar volatiles; thus flavor additives are more easily identified. As most flavor additives are slightly polar molecules, these materials are easily retained by the more polar fibers and provide high intensity signals. Fibers containing methyl silicone have a strong affinity for nonpolar analytes. Tobacco has numerous natural components which are nonpolar and may have similar retention behavior as flavor materials, thus interfering with quantitation. Although the polyacrylate fiber has the highest retention for the flavor materials evaluated, it also retains glycerol quite well. The poly(dimethylsiloxane)/divinylbenzene fiber has the next highest retention for flavor materials; however this fiber has a high affinity for hydrocarbons. Therefore, the background matrix contributes significantly for this fiber, and flavor additives are difficult to detect. The 65 μm Carbowax/divinylbenzene fiber is preferred as flavor additives are easily identified and there are fewer interferences from tobacco matrix components. This selection has been made as the preferred fiber for overall performance with multicomponent analysis of flavorants with various structural differences. Figure 5 indicates the relative mass spectrometer response relative to fiber coating for each of the four analytes. Each fiber type offers advantages for particular classes of compounds and should be chosen accordingly.

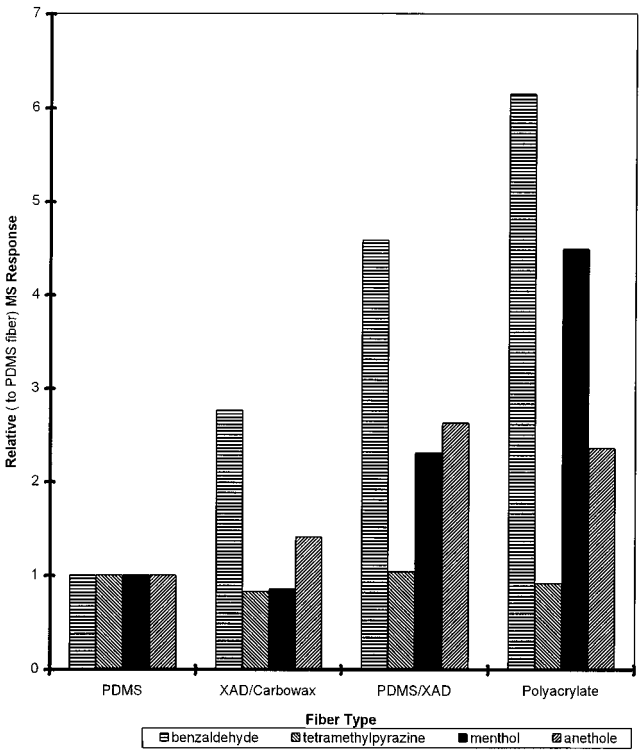

Figure 5. Fiber type versus intensity, 95 °C, 1 mL of KCl, 30 min.

Quantitation of Flavor Additives. Using the method described (65 μm Carbowax/divinylbenzene fiber, 1 g of tobacco at 95 °C with 1 mL 3 M KCl solution, fiber exposure time 15 min), Kentucky Reference 1R1 tobacco treated with a spike flavor mixture was analyzed by HS-SPME–GC–MS. Calibration curves for each flavor material exhibited linear behavior over the specified range. Samples prepared with 2.5 μg of each standard were analyzed and quantitated using the calibration curves. Tetramethylpyrazine was recovered quantitatively. Benzaldehyde recovery concentrations were determined to be 128%, whereas menthol and anethole were recovered in 72 and 79%, respectively. The samples were evaluated for five replicate analyses, and the relative standard deviations are below 20% for each flavor component (Table 3).

This methodology can also be applied to the quantitative analysis of flavor additives with multiple components to provide good estimates of the concentrations present. In order to demonstrate this methodology, individual Kentucky Reference 1R1 tobacco samples were treated with either mandarin orange oil, nutmeg

Table 3. Quantitative Analysis of Flavors on Kentucky Reference 1R1 Tobacco[a]

component	t_R (min)	av recov (μg)	% RSD
benzaldehyde	8.646	3.19	11.7
tetramethylpyrazine	12.502	2.48	5.3
menthol	15.188	1.98	14.9
anethole	18.561	1.79	17.6

[a] 2.50 μg of each analyte and 1 mL of 3 M potassium chloride solution were added to 1 g of tobacco; 2 μg of 2,6-dichlorotoluene was added as ISTD. The sample was heated at 95 °C, and a Carbowax/divinylbenzene SPME fiber was exposed for 15 min. Analysis was performed as described under Experimental Procedures.

oil, or sweet fennel oil and were analyzed by the same method as described. The major component in mandarin orange oil is limonene (Bauer, 1985); therefore the peak corresponding to limonene was assumed to be representative to the concentration of orange oil. Similarly, safrole and myristicin are indicative of nutmeg oil, and anethole is indicative of fennel oil (Bauer, 1985). The peaks for each of these essential oils exhibits linearity over the concentration range evaluated. The concentration of the essential oils could be readily determined by the method described for the individual chemical flavorants. Essential oils and compounded flavorants do, however, pose some complexities which could lead to mistaken qualitative assignments. For example, sweet fennel oil contains limonene (Bauer, 1985); thus it would be difficult to determine if a limonene peak is due to the presence of orange oil, sweet fennel oil, or another essential oil. Also, it is extremely difficult to distinguish between orange, lemon, or other citrus oils, especially when applied to a tobacco matrix. Anethole is an excellent marker component of fennel oil; however it is also found in anise, basil, and licorice flavors. Synthetic anethole is also commonly used as a flavorant (Bauer, 1985). This indicates the complexity for analysis of commercial products as there are numerous essential oils which contain like components. Also, the relative amounts of particular essential oil components are subject to their geographic origin, means of isolation, and purification procedures. Due to these complexities, both positive identification and true quantitation is extremely difficult even with the exact essential oil for comparison.

Limits of Detection. In order to demonstrate the flexibility of this technique for the analysis of tobacco flavor additives, the limits of detection for 31 classical tobacco flavor additives have been determined and are listed in Table 4. Typically as little as 10 ng/g to 6 μg/g of an additive can be detected, identified with confidence and quantitated from a tobacco matrix. Sensitivity could be increased by operation of the mass spectrometer in the selected ion mode; however this is normally not necessary as these concentrations are typically within the range normally added as flavorants.

Method Limitations. Data from the evaluation of consumer products should be interpreted with some skepticism. For example, flavor additives migrate throughout commercial cigarettes. Flavorants initially added to tobacco can be found in the cellulose acetate filter, plugwrap, cigarette wrapper, and packaging material within days after their production (Bunch and Clark, 1995). The degree of migration is dependent upon the structure of the flavor additive and partition coefficients with the various surfaces in which absorption may occur. Many common flavor additives are natural to particular varieties of tobacco; therefore an increased level of a component may reflect the blend rather than a flavor additive. Any quantitative data obtained from commercial products is subjective as values determined could be lower than initially introduced into the product and components could be present due to the blend rather than as an additive.

Table 4. Limits of Detection for Flavor Additives[a]

flavor additive	detection limit (ng/g)	flavor additive	detection limit (ng/g)
acetophenone	41	hexalactone	3020
anethole	16	hexanal	1336
benzaldehyde	66	heptalactone	855
benzyl alcohol	1500	isoamyl phenylacetate	125
benzyl benzoate	324	linalool	154
cinnamaldehyde	495	maltol	5985
decanal	192	menthol	120
diethyl malonate	166	menthone	116
2,6-dimethylpyrazine	168	nonanal	144
ethyl decanoate	65	octanal	484
ethyl hexanoate	221	pinene	714
ethyl nonanoate	78	salicaldehyde	15
ethyl octanoate	93	tetramethylpyrazine	163
ethyl phenylacetate	18	trimethylpyrazine	140
ethylvanillin	2108	vanillin	2396
geraniol	325		

[a] 1, 5, or 10 μg of each analyte and 1 mL of 3 M potassium chloride solution were added to 1 g of tobacco; 2 μg of 2,6-dichlorotoluene was added as ISTD. The sample was heated at 95 °C, and a Carbowax/divinylbenzene SPME fiber was exposed for 15 min. Analysis was performed as described in the Experimental Section. Detection limits were estimated by extrapolation of the lowest standard on the calibration curve. The LOD was estimated on tobacco matrix for analytes producing a signal to noise ratio of 5:1.

Summary. In summary, headspace solid-phase microextraction−gas chromatography−mass spectroscopy provides an excellent methodology for the qualitative and quantitative determination of flavor additives on tobacco. Both single-component flavor additives as well as essential oils can be positively identified and quantitated with a high degree of accuracy. Our experience with tobacco flavor systems leads us to believe that this methodology could be adapted for the analysis of most any consumer agricultural product.

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