Derivatization Solid-Phase Microextraction Gas Chromatographic–Mass Spectrometric Determination of Organic Acids in Tobacco

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Abstract

Isovaleric, valeric, hexanoic, benzoic, phenylacetic, 3-methylvaleric, heptanoic, octanoic, and nonanoic acids are converted to their methyl esters and quantitated for flue-cured tobacco grades of increasing stalk position using derivatization headspace solid-phase microextraction (SPME) with selected ion monitoring mode mass spectrometry. Qualitative analysis of the headspace of tobacco derivatized with methanolic hydrochloric acid using a 65-µm Carbowax–divinylbenzene SPME fiber indicates selectivity for relatively nonpolar volatile and semivolatile organic acid methyl esters. By contrast, direct exposure of an 85-µm polyacrylate SPME fiber to derivatives followed by GC–MS analysis provides the capability to evaluate significantly more polar organic acid methyl esters such as malic acid dimethylester and citric acid trimethylester.

Introduction

The organic acid content in tobacco is influenced by variety, curing practices, weather conditions, and quality (1–8). Smoke aroma and taste perception are highly dependent on the presence and concentrations of certain organic acids in tobacco (9). Accurate quantitative data for organic acids in tobacco is important for the assurance of consumer product uniformity.

Organic acids exist in several forms in tobacco including organic or inorganic salts, esters, and free acids (1). Quantitative data is therefore highly dependent on the mode of sample preparation and the analytical method utilized. Although many procedures have been reported for the analysis of acids in tobacco, most have evaluated "free" acids (1–4). There have been few reports that have identified and quantitated total organic acids in tobacco (5–8).

Due to the relative nonvolatile nature of carboxylic acids, they are most often converted to their more volatile methyl ester counterparts prior to analysis (5). Methyl ester derivatives are preferred due to their stability relative to the trimethylsilyloxy esters used in early studies (1). Methyl esterification can be performed in numerous ways. Direct treatment of tobacco with diazomethane provides methyl esters of the "free" acids (3). Current technology for the determination of total acids in tobacco requires reaction with methanolic acid solution followed by extraction of the derivatives with an organic solvent and sample cleanup using florisil (8). Although this methodology works well, sample preparation is lengthy, and the potential for sample loss exists. The samples are then analyzed using gas chromatography with mass spectrometry (GC–MS) or flame ionization detection.

Solid-phase microextraction (SPME) has been used for the analysis of low-molecular-weight fatty acids (10). This methodology relies upon the derivatization of carboxylic acids using 1-prenyldiazomethane on the SPME fiber. The technique has been applied to the analysis of a sewage sample (10).

The present study reports the qualitative and quantitative analysis of organic acids in tobacco using derivatization with methanolic hydrochloric acid followed by SPME GC–MS.

Experimental

Instrumentation

A Hewlett-Packard (HP) 5890 GC (Palo Alto, CA) with an HP 5970B mass selective detector was used for data collection.

Table I. Mass Spectral Detection Parameters

Carboxylic acid methyl ester	Retention time (min)	Ions monitored
Methyl isovalerate	5.72	74*, 59
Methyl valerate	6.66	74*, 59
Methyl 3-methylvalerate	7.88	74*, 59
Methyl hexanoate	8.66	74*, 87
Methyl heptanoate	10.61	74*, 87, 59
Methyl benzoate	12.09	105*
Methyl octanoate	12.42	74*, 87, 59
Methyl phenylacetate	13.46	91*
Methyl nonanoate	14.09	74*, 87, 59

*Ions used for quantitation.

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Splitless injections were made at 250°C using a manual SPME device and commercially available SPME fibers (Supelco, Bellefonte, PA). Separation was accomplished using a PTA-5 (Supelco) column (30 m × 0.23-mm i.d., 0.20-µm film thickness). The initial oven temperature was 50°C for 2 min, then increased to 250°C at a rate of 10°C/min. The GC–MS transfer line was maintained at 280°C.

Qualitative analysis

Headspace SPME

Tobacco samples were dried at 70°C in a convection oven and ground to a fine powder. Tobacco (10 mg) and 2-ethylhexanoic acid (surrogate, 500 ng) were placed in a 4-mL screw cap vial with methanolic HCl (400 µL) (Supelco) and heated at 55°C overnight. The samples were then cooled in an ice bath, and 1M sodium bicarbonate solution (1600 µL) (Fisher Scientific, Raleigh, NC) was added. A 65-µm Carbowax–divinylbenzene SPME fiber was exposed to the headspace above the sample (while being stirred at 400 rpm) for 15 min, then desorbed for 0.5 min at 250°C into the injection port of the GC. The injections were analyzed by MS. The MS was operated at 70 eV in the scan mode for ions with molecular weights in the range of 30–350 amu.

Direct SPME

Tobacco samples were dried at 70°C in a convection oven and ground to a fine powder. Tobacco (10 mg) and 2-ethylhexanoic acid (surrogate, 500 ng) were placed in a 4-mL screw cap vial with methanolic HCl (400 µL) (Supelco) and heated at 55°C overnight. The samples were then cooled in an ice bath, and 1M sodium bicarbonate solution (1600 µL) (Fisher Scientific) was added. Samples were filtered through a 45-µm nylon Whatman (Clifton, NJ) filter, and an 85-µm polyacrylate SPME fiber was immersed directly into the stirred solution (400 rpm) for 15 min. The samples were desorbed for 0.5 min at 250°C into the GC injection port and analyzed by MS.

Table II. Carboxylic Acid Methyl Esters Detected Using Derivatization SPME GC–MS

Carboxylic acid methyl ester	Retention time (min)	Carbowax–divinylbenzene	Polyacrylate
Acetic acid	2.35	+	+
2-Butenoic acid	5.43	–	+
3-Isovaleric acid	5.72	+	+
Valeric acid	6.66	+	+
2-Methyl-2-butenoic acid	7.50	–	+
3-Methylvaleric acid	7.88	+	+
Hexanoic acid	8.66	+	–
Malonic acid	8.64	+	+
2-Furancarboxylic acid	9.69	+	+
4-Oxopentanoic acid	9.89	+	+
Maleic acid	10.54	–	+
Heptanoic acid	10.61	+	–
Succinic acid	10.68	+	+
2-Methyl-3-furancarboxylic acid	11.86	+	+
Benzoic acid	12.09	+	+
Octanoic acid	12.42	+	+
Malic acid	12.43	–	+
Phenylacetic acid	13.46	+	+
3-Pyridinecarboxylic acid	12.78	–	+
2-Hydroxybenzoic acid	13.83	+	+
4-Methylbenzoic acid	13.92	–	+
Nonanoic acid	14.09	+	+
Decanoic acid	15.62	+	–
3-Methoxybenzoic acid	15.86	–	+
Pimelic acid	15.91	–	+
Cinnamic acid	16.74	–	+
Suberic acid	17.40	–	+
4-Hydroxybenzoic acid	17.60	–	+
3-Hydroxybenzoic acid	17.63	–	+
Citric acid	17.72	–	+
4-Hydroxyphenylacetic acid	18.06	–	+
Lauric acid	18.47	+	–
4-Hydroxy-3-methoxybenzoic acid	18.63	–	+
2,4-Dimethoxybenzoic acid	18.69	–	+
Azelaic acid	18.79	+	+
Sebacic acid	20.09	–	+
Tetradecanoic acid	21.02	+	–
Undecanedioic acid	21.32	–	+
Pentadecanoic acid	22.19	+	–
Palmitic acid	23.42	+	+

Quantitative analysis

Calibration standards

Carboxylic acid standard solution in amounts of 1, 2, 5, 10, 20, or 40 µL (40 ng/µL) each of isovaleric, valeric, hexanoic, benzoic, phenylacetic, 3-methyl valeric, heptanoic, octanoic, and nonanoic acids (ChemService, Westchester, PA), 2-ethylhexanoic acid (surrogate, 500 ng), and methanolic HCl (400 µL) (Supelco) were heated at 55°C overnight. The samples were cooled in an ice bath, and 1M sodium bicarbonate solution (1600 µL) (Fisher Scientific) and 2,6-dichlorotoluene (internal standard, 500 ng in methanol) were added. A 65-µm Carbowax–divinylbenzene SPME fiber was exposed to the headspace above the sample (stirred at 400 rpm) for 15 min, then desorbed for 0.5 min at 250°C into the injection port of the GC and analyzed by selected ion monitoring (SIM) mode MS. The ions monitored are listed in Table I. Calibration curves were prepared, and all of them exhibited linearity (correlation coefficients were 0.997 or greater) over the range evaluated.

Tobacco samples

Tobacco (10 mg) and 2-ethylhexanoic acid (internal standard) (500 ng) were treated with methanolic hydrochloric acid as described. A 65-µm Carbowax–divinylbenzene SPME fiber was exposed to the headspace above the

sample (stirred at 400 rpm) for 15 min, then desorbed for 0.5 min at 250°C and analyzed by SIMMS. Quantitation was performed by linear regression analysis using standard HP ChemStation programming.

Results and Discussion

Derivatization

Total organic acids in flue-cured tobacco grades were simultaneously extracted and derivatized using methanolic hydrochloric acid. This derivatization reagent was utilized because carboxylic acids are efficiently converted to methyl esters regardless of their form (i.e., salts, esters, free acids, etc.). Boron trifloride in methanol is commonly used for methyl esterification (10). We found that this reagent reacted with carboxylic acids in tobacco; however, numerous undesired byproducts were detected in samples derivatized with this reagent.

Sampling of the organic acid methyl esters was accomplished through the use of SPME using either a 65-µm Carbowax–divinylbenzene SPME fiber or an 85-µm polyacrylate SPME fiber. Headspace sampling using a 65-µm Carbowax–divinylbenzene SPME fiber provided a means to sample nonpolar semivolatile organic acid methyl esters from the matrix. More polar organic acid methyl esters were preferentially trapped using direct exposure of an 85-µm polyacrylate SPME fiber to the derivatives. Two additional SPME fibers, a 100-µm polydimethylsiloxane SPME fiber and a 65-µm polydimethylsiloxane–divinylbenzene fiber, were also evaluated for performance. These fibers exhibited poor reproducibility for tobacco samples, presumably due to matrix effects.

SPME conditions

SPME fiber performance has been shown to rely on fiber exposure time, ionic strength of the matrix, and sample temperature (11–12). Exposure time profiles for volatile esters were constructed using a Carbowax–divinylbenzene SPME fiber with stirred samples at room temperature. Equilibration was completed in 20 min for all methyl esters quantitated in this study. The derivatization reaction produced a matrix containing approximately 0.6M sodium chloride solution. Attempts to improve sensitivity by "salting out" the carboxylic acid methyl esters with the addition of saturated potassium chloride to the matrix did not significantly increase the concentrations absorbed onto the fiber.

Qualitative analysis

Exposure of a 65-µm Carbowax–divinylbenzene fiber into the headspace above derivatized samples followed by GC–MS provided a rapid technique for the qualitative identification of numerous organic acid methyl esters. This method is extremely selective; most components detected in the headspace were semivolatile organic acid methyl esters (Table II).

More polar acids such as citric and malic acids were not detected using this methodology. However, polar carboxylic acids are amenable to analysis by SPME. Direct exposure of the more polar 85-µm polyacrylate SPME fiber to a stirred reaction mixture provided extraction and identification of the more polar organic acids, including citric and malic acids. Direct SPME also offers the advantage of detection of organic acids with higher boiling points. For example, sebacic acid dimethyl ester is readily detected by direct SPME, but this compound was not detected in the headspace. Carboxylic acid methyl esters detected by direct SPME are listed in Table II.

Quantitative analysis

Tobacco grades of similar color and quality from each stalk position were treated under the described conditions, and the headspace was collected using a 65-µm Carbowax–divinylbenzene SPME fiber. These samples were desorbed into a GC injector at 250°C and quantitated using SIMMS. Tobacco samples derivatized in the same manner were extracted with

Table III. Carboxylic Acids Present in Flue-Cured Tobacco Grades

Carboxylic acid	P4F* SPME (µg/g [%RSD])	P4F* Extract** (µg/g [%RSD])	X4F† SPME (µg/g [%RSD])	X4F† Extract** (µg/g [%RSD])	C4F‡ SPME (µg/g [%RSD])	C4F‡ Extract** (µg/g [%RSD])	B3F§ SPME (µg/g [%RSD])	B3F§ Extract** (µg/g [%RSD])
Isovaleric	75.4 (16)	–	90.4 (26)	147.7 (26)	64.4 (27)	99.5 (6)	63.8 (16)	136.3 (10)
Valeric	19.1 (24)	10.3 (20)	23.1 (41)	18.6 (7)	12.2 (31)	12.1 (23)	6.4 (18)	13.1 (37)
Hexanoic	32.3 (9)	95.5 (20)	40.6 (16)	71.1 (32)	24.5 (34)	85.5 (21)	23.6 (24)	81.9 (17)
Benzoic	20.5 (17)	30.8 (21)	29.0 (24)	39.0 (28)	12.2 (12)	21.0 (28)	10.5 (15)	25.7 (51)
Phenylacetic	98.5 (31)	115.5 (7)	90.0 (15)	44.0 (3.2)	30.5 (12)	37.6 (4)	59.1 (19)	50.4 (12)
3-Methylvaleric	10.0 (24)	7.0 (8)	9.3 (20)	3.5 (34)	7.3 (18)	3.6 (33)	5.7 (22)	3.5 (11)
Heptanoic	3.5 (26)	2.5 (23)	4.7 (20)	2.3 (27)	2.1 (16)	1.8 (55)	2.8 (12)	3.7 (58)
Octanoic	4.5 (28)	8.8 (28)	11.1 (46)	12.4 (13)	4.7 (14)	8.5 (19)	8.8 (14)	12.3 (24)
Nonanoic	0.7 (41)	2.1 (33)	0.96 (42)	3.3 (22)	0.45 (11)	2.8 (46)	0.8 (20)	3.4 (45)

* P4F designation: priming, orange color with 70% uniformity, 30% waste.
† X4F designation: lug, orange color with 70% uniformity, 30% waste.
‡ C4F designation: cutter, orange color with 70% uniformity, 20% injury tolerance, and 5% waste.
§ B3F designation: leaf, orange color with 80% uniformity, 15% injury tolerance.
** 10 mg of tobacco derivatized using 400 µL of methanolic HCl at 55°C overnight was shaken with hexane (100 µL) for 15 min, and the hexane layer was analyzed by GC–SIMMS. All tobaccos were from the 1995 crop year.

hexane and analyzed for comparison (Table III). Overall, both methods provided similar results, thus confirming that the derivatization–headspace SPME–GC–SIMMS technique was comparable to the widely acceptable method for the determination of organic acids in tobacco. SPME offered the advantage of minimal sample preparation.

Conclusion

Headspace SPME analysis is an extremely sensitive technique for the analysis of carboxylic acid methyl esters in tobacco. The detection limits ranged from 20 µg/g of tobacco for isovaleric acid to 22 ng/g of tobacco for nonanoic acid. Sensitivity can be increased significantly by using larger quantities of tobacco because up to 100 mg of tobacco could be derivatized using 400 µL of methanolic hydrochloric acid. Consequently, a tenfold increase in sensitivity was gained.

In summary, derivatization–headspace SPME–GC–MS provided a rapid and efficient method for the evaluation of total organic acids in tobacco. This SPME technology exhibited selectivity dependent on the SPME fiber used and the use of direct headspace sampling.

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