Characterization of Cigar Tobaccos by Gas Chromatographic/Mass Spectrometric Analysis of Nonvolatile Organic Acids: Application to the Authentication of Cuban Cigars

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A reliable method based on gas chromatographic/mass spectrometric (GC/MS) profiling of nonvolatile organic acids is described for the characterization of cigars. The method involves an aqueous extraction of ground tobacco and selective isolation of the acids by simply stirring strong anion exchange (SAX) disks in the aqueous tobacco extract. The acids are then directly silylated on the disk with *N*-methyl-*N*-trimethylsilyl-trifluroacetamide (MSTFA) in acetonitrile in an autosampler vial. Elution of the derivatized acids in situ allows the sample to be directly analyzed by GC/MS without further sample handling. Compared to the conventional disk-extraction technique using a vacuum manifold, this method is much less labor intensive, and is desirable for multiple sample analysis. Nicotinic acid, succinic acid, glyceric acid, malic acid, pyroglutamic acid, threonic acid, citric acid, uracil, and an unidentified acid were reproducibly quantified in tobacco samples. Principal component analysis (PCA) of the acid profiles of the filler tobaccos of 18 Cuban cigars and 31 non-Cuban cigars shows separation of the two groups, indicating that the acid profiles are potentially useful in the authentication of Cuban cigars.

Keywords: *Cuban cigars; organic acids; anion-exchange disk extraction; silylation, multivariate analysis; gas chromatography/mass spectrometry*

INTRODUCTION

Cuban cigars are acknowledged as among the finest in the world. Illegal sales of smuggled or counterfeit Cuban cigars have sprung up in recent years to meet the high demand for the commodity. In addition to violating criminal codes, such activities result in the loss of millions of dollars in national revenues. The Laboratory and Scientific Services Directorate is responsible for providing analytical services to support protection of Canadian taxation revenues on various commodities including tobacco products. Effective chemical approaches for assessing the origin of agricultural products usually involve chemometric investigation of the chemical composition (*1*), and stable isotope analysis (*2*) of the commodity. To our knowledge, there has been no report that addresses the issue of assessing the origin of cigars.

Tobacco contains nicotine and solanone as characteristic compounds (*3*), and a wide range of basic biochemical components such as proteins, carbohydrates, and lipids. Cigar tobacco leaves are usually air-cured, fermented, and aged before being manufactured into cigars. During these processes, some of these basic chemicals undergo degradation and yield additional components. The relative amounts of the chemical constituents in cigar tobaccos are related to the agricultural and processing practices, in addition to the genetic factor of the tobacco plant, and the climate and soil environments. Cuban cigars are expected to yield reproducible and characteristic analytical profiles because the tobaccos used are grown in specific regions from seeds that are highly controlled to prevent any gene mutation. Moreover, the cigars are manufactured from tobacco plants that are grown and processed under a carefully designed set of treatments and conditions.

It has been reported that the combination of nine analytical variables, namely, alkaloids, reducing sugars, Kjeldahl N, soluble NH_4, ash, chloride, phosphate, nitrate, and sulfate, has shown correlation with the country of origin of tobaccos (*4*). To analyze all these components demands a great deal of time and resources. A more practical approach involves targeting a class of compounds. Organic acids have been reported to be very useful in distinguishing different tobacco types: flue-cured, Burley, and Turkish (*3*), although they are not known to be used for determining country of origin. This study investigated the correlation of the organic acid profiles to geographical origins.

Nonvolatile organic acids, whether in free acid or salt form, can be readily extracted from tobacco into water. Aqueous acids are usually analyzed by HPLC techniques. For laboratory data to be legally defensible, it is desirable to have additional information other than chromatographic results. This is achieved by using highly powerful separation/identification techniques such as GC/MS or LC/MS; the former was used for this study. To be amenable to GC/MS analysis, these compounds must be converted to more volatile derivatives. Silylation is well-known for its effectiveness to derivatize polyfunctional acids, in which hydroxy, amino, and carboxylic functions can be simultaneously silylated. However, this method requires that acids be isolated from the aqueous medium because silylating reagents

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are not compatible with water. A previous method (*5*) employed freeze-drying to remove water from the aqueous tobacco extract before silylation. The procedure, when repeated in our laboratory, did not yield reproducible results, mainly because some of the acids were lost by sublimation during freeze-drying. Solid-phase extraction (SPE) using strong anion-exchange (SAX) disk has been shown as an effective technique to isolate ionizable acid herbicides from groundwater and eliminate nonionic impurities (*6*). Conversion of a wide range of organic acids recovered from alcoholic beverages and soils to trimethylsilyl (TMS) derivatives has previously been carried out simply and efficiently on Empore anion-exchange disks using *N*-methyl-*N*-trimethylsilyl-trifluroacetamide (MSTFA, *7*) and *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA, *8*). The silylated derivatives were eluted in the same vial where derivatization took place before being analyzed by GC/MS.

This report describes the development of a reproducible method based on anion-exchange disk extraction for sampling tobacco acids, and the evaluation of the usefulness of the GC/MS acid profiles by multivariate statistical analysis for distinguishing Cuban cigars from those manufactured in Nicaragua, the Dominican Republic, Honduras, Mexico, the United States, etc. These non-Cuban countries were chosen because cigars from these locations were deemed most likely to be used in counterfeiting Cuban cigars.

MATERIALS AND METHODS

Materials. Citric acid >99.5%, fumaric acid >99%, glycolic acid 99%, pyruvic acid 98%, threonic acid, calcium salt, and 3,3-dimethylglutaric acid 98% were purchased from Aldrich (Oakville, ON). Succinic acid >99% and glyceric acid, hemicalcium salt hydrate >98% were obtained from Sigma (Oakville, ON). Pyroglutamic acid >99%, malic acid >99%, nicotine > 99%, and uracil >99% were supplied by Fluka (Oakville, ON). Acetone, acetonitrile, and methanol (HPLC grade) were obtained from J. T. Baker (Phillipsburg, NJ). Acetonitrile used in silylation was dried over molecular sieve 4 A. MSTFA was supplied by Supelco (Oakville, ON). NaOH 1N solution was prepared from the solid obtained from Anachemia Canada Inc (Montreal, PQ).

Eighteen different Cuban cigars were supplied by Havana House (Toronto, ON), which is the authorized distributor of Cuban cigars in Canada. Thirty-one non-Cuban cigars manufactured in different countries were obtained from local cigar stores, of which 10 were from Honduras, 6 were from Dominican Republic, 5 each were from the U.S. and Nicaragua, 2 were from Mexico, and 1 each were from Brazil, Jamaica, and Aruba. They were kept in a humidor at 20 °C and 70% humidity until used.

Stock Standardizing Solution. Various organic acids were dissolved in water to give a solution containing 20 ng μL^{-1} of nicotinic acid and succinic acid, 35 ng μL^{-1} of maleic acid, uracil, fumaric acid, threonic acid, and glyceric acid, and 500 ng uL^{-1} of citric acid and malic acid. To mimic the aqueous tobacco extracts that have a pH of 7 and contain nicotine as one of the major components, the stock solution was brought to the same pH by addition of nicotine.

Disk Conditioning. Small 9-mm diameter disks were cut from commercially available 47-mm Empore Anion Exchange-SR disks (VWR, Mississauga, ON) using a cork-boring tool. The disks were conditioned by being immersed in acetone, methanol, and water successively for 0.5 h, then transferred to a 1 N NaOH solution and soaked for 1 h. They were repeatedly washed with deionized water until the pH of the wash was ~9. They were then kept at this pH in deionized water and stored in the refrigerator until used.

Sample Preparation. Each cigar was separated into different parts, i.e., wrapper, binder, and filler. The wrapper and the filler were ground separately in a Wiley mill (Arthur H. Thomas Co., Philadelphia, PA), passed through a 20-mesh sieve, and used without further treatment. Values for % moisture of fillers were determined by using an HR73 Moisture Analyzer from Mettler Toledo (VWR Canlab, Montreal, PQ). For tobacco with a moisture content of 12 ± 2%, 500 mg of the ground sample (without drying) was used for aqueous extraction. The sample size was adjusted accordingly if the moisture content fell outside the range. The weighed sample was mixed with 200 mL of deionized water and agitated at 200 rpm for 1 h on an orbital shaker. About 4 mL of the supernatant liquid was pipetted from the mixture through a 0.45 μm Nylon filter.

The filtered tobacco extract (2 mL) or the stock standardizing solution (1 mL) was mixed with 1 mL of the internal standard (ISTD) solution (100 mg L^{-1} 3,3-dimethylglutaric acid) and diluted to 100 mL with deionized water in a 250-mL Erlenmeyer flask. Two preconditioned disks were dropped into the tobacco solution. The extraction was carried out on an orbital shaker at 200 rpm for 1 h. The disks were removed by pouring the content of the flask through a stainless steel strainer within 10 min after the extraction was completed. After the wet disks were rinsed with deionized water, they were pressed between filter papers to remove as much water as possible before being automatically dried to constant weight in the moisture analyzer (3−4 min).

Each dried disk was placed in a 2-mL autosampler vial with 1 mL of 1:1 MSTFA/acetonitrile (v/v). The vial was capped and heated at 80 °C for 45 min. It was cooled to room temperature before being placed on the autosampler carousel for GC/MS analysis. Each silylated sample was analyzed once.

Gas Chromatography/Mass Spectrometry. After in-vial derivatization and elution, the extracts were analyzed using a Hewlett-Packard gas chromatograph 6890 series II equipped with a 5973 mass selective detector (Hewlett-Packard, North Hollywood, CA). The column used was a 30-m DB5ms (5% diphenyl and 95% dimethyl polysiloxane) fused silica, 0.25 mm i.d., and 0.25-μm film thickness (J & W Scientific, Folsom, CA). A 1-μL aliquot was injected in the pulsed splitless mode with a head pressure of 20 psi for 45 s. The carrier gas was He and the flow rate was kept at 1.0 mL min^{-1} throughout the remainder of the run with an electronic pressure controller. The split/splitless injection port and the interface were at 260 °C and 290 °C, respectively. The oven temperature was programmed: 75 °C for 1.5 min, then ramped at 10 °C min^{-1} to 240 °C, followed by 30 °C min^{-1} to 300 °C, and held for 5 min. The solvent delay was set at 7.6 min. All mass spectra were acquired in the electron impact (EI) mode at 70 eV. The mass spectrometer was scanned in the range of 40 to 500 amu at a rate of 3.18 scans s^{-1}. Detection of the analytes and the ISTD was based on the retention time, the presence of the target ion, and the two qualifier ions chosen on the basis of their abundance and/or specificity to the compound (Table 1). Quantification was performed on the extracted ion chromatograms using auto-integration.

Treatment of Data. The integrated data were treated in two ways. In one treatment, nicotinic acid, succinic acid, uracil, malic acid, citric acid, and pyroglutamic acid were quantified, in mg g^{-1} of tobacco, based on one-point calibration using the standardizing solution, and response ratios of individual analytes to the ISTD. Glyceric acid and threonic acid were not identified until the end of this investigation, therefore, like unknown 1, they were expressed as response ratios to pyroglutamic acid. Pyroglutamic acid instead of ISTD was used because the response of pyroglutamic acid was more comparable to those of the two acids and the unknown. In the other treatment, all the targeted analytes were quantified as % normalized responses (analyte response/total response of all target analytes × 100).

Multivariate Analysis. Data sets resulting from the two treatments described above were separately subjected to multivariate analysis without further preprocessing. The commercial software package Pirouette (InfoMetrix, Seattle, WA) was employed for calculating principal components.

Table 1. List of Analytes

analyte	TMS derivative	M^+ (m/z)	target ion (m/z)	qualifiers (m/z)	retention time (min)
3,3-dimethylglutaric acid (ISTD)	$(TMS)_2$	304	83.1	186, 289	9.77
nicotinic acid	$(TMS)_1$	195	180.0	106, 136	7.90
succinic acid	$(TMS)_2$	262	147.1	247	8.19
glyceric acid	$(TMS)_3$	322	189.0	205, 292	8.54
uracil	$(TMS)_2$	256	241.0	99, 255	8.56
fumaric acid[a]	$(TMS)_2$	260	245.0	133, 143	8.61
malic acid	$(TMS)_3$	350	233.1	245, 335	10.51
pyroglutamic acid	$(TMS)_2$	273	156.1	230, 258	10.86
unknown 1	–	–	292.1	205, 220	11.26
threonic acid	$(TMS)_4$	424	292.1	205, 220	11.46
citric acid	$(TMS)_4$	480	273.2	347, 363, 465	14.30
unknown 2[a]	–	–	345.1	255, 372	14.78

[a] Fumaric acid and unknown 2 were removed from the list at the end of the method development process because they could not be measured reproducibly from day to day.

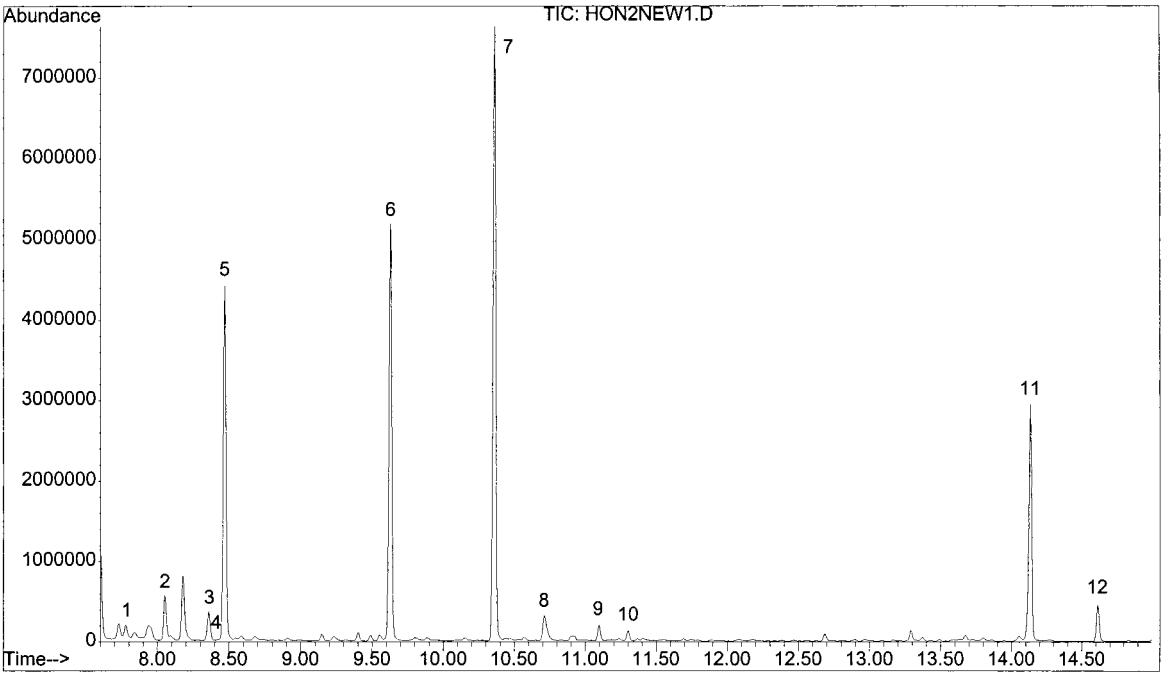

Figure 1. SAX/GC/MS profile of a typical cigar filler. Peaks: 1, nicotinic acid (TMS); 2, succinic acid $(TMS)_2$; 3, glyceric acid $(TMS)_3$; 4, uracil $(TMS)_2$; 5, fumaric acid $(TMS)_2$; 6, 3,3-dimethylglutaric acid $(TMS)_2$ (ISTD); 7, malic acid $(TMS)_3$; 8, pyroglutamic acid $(TMS)_2$; 9, unknown 1 $(TMS)_n$; 10, threonic acid $(TMS)_4$; 11, citric acid $(TMS)_4$; 12, unknown 2 $(TMS)_n$.

RESULTS AND DISCUSSION

Selection of Acids. Those acids that were detected in cigar tobaccos at easily quantifiable amounts were selected for the work and are listed in Table 1. The identities of these compounds, except unknowns 1 and 2, were confirmed by computerized library matching and by comparison with authentic chemicals. Unknown 1 is probably an isomer of threonic acid, as the mass spectra of silylated derivatives of both compounds are very similar. Uracil is usually not considered an acid, but was included in the list because it was always detected in the samples. Glycolic acid, pyruvic acid, and maleic acid were also detected; they were not included in the list because they could not be properly integrated due to the presence of interfering peaks often found in the tobacco extracts. Benzoic acid, which was always observed in the blank, was a manufacturing contaminant on the Empore disk (*8*). Although it might be present in the sample, the peak was ignored for practical reasons.

Chromatographic and Mass Spectrometric Characteristics. All analytes were detected as silyl derivatives with every labile proton on hydroxy, carboxyl, and amido functions replaced by a trimethylsilyl group. Under the GC/MS conditions described above, all the analytes and the ISTD were well separated as their silyl derivatives (Figure 1). The total ion chromatogram baseline was very stable. The extract was clean and there was no need to change the GC injector liner even after 75 injections.

Method Development. The method procedure includes (1) aqueous extraction of acids from the tobacco sample; (2) concentration of acids from the aqueous extract by disk extraction; (3) silylation of acids on the disk and in situ elution of the derivatives; and, (4) GC/MS analysis. A standard solution was processed through steps 2 to 4 to provide calibration of the known analytes. In multi-analyte analysis, it is impossible to optimize the conditions for each compound. However, attempts were made to obtain satisfactory chromatographic detection and measurement precision for most of the target analytes, while minimizing sample handling.

Aqueous Extraction of Acids from Tobacco. A 500-mg portion of the ground tobacco (20 mesh) was

used for the extraction. This sample size has been reported to be representative of tobacco of this particle size for the extraction of sugars (*9*), and has been proven to yield reproducible results as shown later in this report. Deionized water was used to extract the acids from the filler. The use of water has the advantage of avoiding defatting, which is the major problem in plant analysis. Responses of individual analytes in three test tobacco samples were monitored after 0.5, 1, 2, 4, 7, and 24 h of extraction in 200 mL of water at room temperature. They were found to level off after 0.5 h, indicating completion of the extraction process. All samples were extracted for 1 h throughout this study to ensure negligible effect of time on analyte responses.

Recovery of Acids from Aqueous Tobacco Extract using SAX Disk. For silylation of the acids and elution of the derivatives to occur together in the autosampler vial, the disk must be small enough to be placed directly in the vial and fully immersed in the 1 mL of MSTFA/acetonitrile solution. Disks of 9-mm diameter were found to be desirable. Traditionally, disk extraction is carried out by applying vacuum to pull the conditioning solvents, and subsequently the samples, through the disk, and each disk is treated separately and manually. In the current method, the disks are conditioned in batches by being soaked successively in acetone, methanol, water, and a NaOH solution. Moreover, disk extractions of multiple samples can be carried out simultaneously and automatically by simply stirring the disks in the aqueous tobacco extracts on an orbital shaker. For multiple sample analysis, this procedure is definitely much less labor-intensive as compared to the conventional operation.

The pH of the aqueous tobacco extracts was ~7, which is at least 2 units higher than the pK_a of the analytes (except uracil that has a pK of 9.45). At this pH, most of the analytes exist in the anion form, amenable to ion exchange with the disk. Therefore, the aqueous extract was used for disk extraction without any pH adjustment. The aqueous tobacco extract was diluted 50 times with deionized water before disk extraction. In principle, multiple disks can be used for recovering acids from the same aqueous tobacco solution. This is desirable since an increase in the number of analyses will lead to an improvement in the statistical reliability of the analysis results. However, GC/MS analysis will be impracticably lengthened if too many replicates are analyzed (each GC/MS analysis requires 30 min). Subsequently, all disk extractions were performed in duplicate. The adsorption time profile of a standard solution containing equal concentration (0.001 mM) of several analytes and the ISTD is shown in Figure 2. After 8 h, the responses of most of the analytes started to level off, and those of uracil and pyroglutamic acid decreased. This is the point when the exchange sites on the disk were approaching depletion. Uracil is not ionized at the sample pH and is only weakly adsorbed on the disk, possibly through hydrogen bonding or another form of interaction with the adsorbent. As the number of available free exchange sites on the disk dwindles on approaching the saturation point, uracil can no longer compete and is replaced by the acid analytes with higher affinities for the disk. It is not clear why pyroglutamic acid, a heterocyclic monoacid, also displayed a similar but more gradual downward trend. A similar adsorption profile was obtained with the standardizing solution that had a composition mimicking that of a typical tobacco extract.

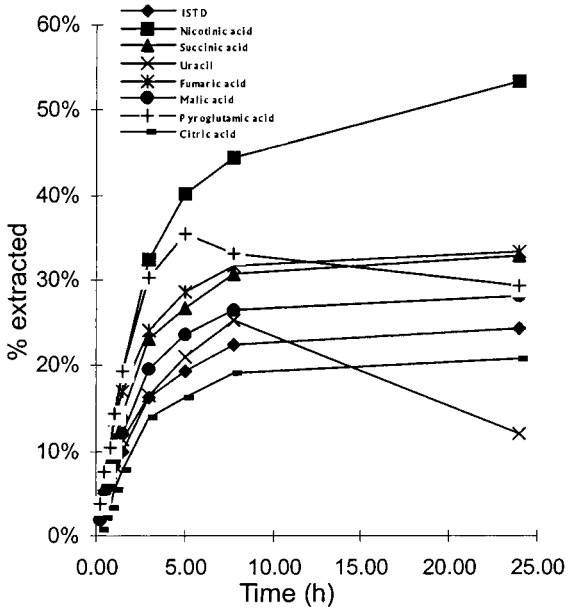

Figure 2. Disk extraction time profile of individual analytes and the ISTD. The amount of each analyte extracted is expressed as a percentage of the amount of the corresponding analyte present in the aqueous solution before disk extraction.

A 1-h extraction time was chosen for recovering the tobacco acids because it yielded good sensitivity for the minor analytes without causing saturation of the detector for the major analytes.

The effect of the stirring rate on the analyte responses was studied. Increasing the agitation rate from 200 to 400 rpm resulted in enhancing the responses by 30−80% depending on the analytes, and the response ratios (analyte/ISTD) were also affected significantly. Prolonged contact between the disk and the tobacco solution for over 10 min after agitation is stopped was found to affect the adsorption of analytes. In view of these findings, it is critical to keep the stirring rate constant during the extraction, and to remove the disks from the solution within 10 min once the extraction is completed.

Derivatization and Elution. After drying, the disks are ready for silylation. In conventional SPE, the analytes are eluted before derivatization is carried out. Elution preceding silylation in the present method is not possible because anions cannot be displaced by acetonitrile. In fact, none of the analytes were detected in the acetonitrile solution in which a SAX disk containing analytes had been heated for 30 min at 80 °C. When the SAX disk is heated with MSTFA in CH_3CN, it is most likely that derivatization occurs on the disk, followed by elution of the analytes from the disk as silylated derivatives. CH_3CN possibly serves to swell the disks, thus facilitating the penetration of MSTFA to the exchange sites to react with the acids. This is supported by the observation that individual analyte responses were reduced by 3 times when MSTFA was used alone without CH_3CN; whereas in the absence of a disk, the responses were not affected by the presence of CH_3CN when the acids were silylated by MSTFA. Lou et al. (*8*) reported that the presence of a residual amount of water on SAX disks is necessary to ensure good recovery of several herbicide acids when BSTFA was used in the absence of any solvent to silylate the analytes. They suggested that in the presence of water, the disk remained swollen and thus allowed free passage of BSTFA. We have found that incomplete drying of the

Table 2. Lot-to-Lot Reproducibility[a]

	ISTD counts	nicotinic mg g^{-1}	succinic mg g^{-1}	glyceric resp. ratio[b]	uracil mg g^{-1}	malic acid mg g^{-1}	pyroglutamic mg g^{-1}	unknown 1 resp. ratio[b]	threonic resp. ratio[b]	citric mg g^{-1}
a non-Cuban cigar										
lot# 720141	4380568	0.27	1.63	19.25	0.13	49.84	1.81	10.95	5.56	28.03
		(1.18)	(14.59)	(2.67)	(0.4)	(41.98)	(13.88)	(1.52)	(0.77)	(23.01)
lot# 720175	4353595	0.28	1.60	19.50	0.13	51.55	2.01	11.09	5.17	29.25
		(1.15)	(13.71)	(2.89)	(0.40)	(41.65)	(14.80)	(1.64)	(0.77)	(22.99)
a Cuban cigar										
lot# 720141	4375282	0.79	2.13	10.49	0.23	18.81	1.47	3.24	0.00	23.5
		(4.86)	(26.75)	(1.66)	(1.00)	(22.28)	(15.86)	(0.51)	(0.00)	(27.08)
lot# 720175	4307979	0.68	2.22	8.96	0.20	20.28	1.24	2.73	0.00	22.11
		(4.71)	(27.27)	(1.41)	(1.04)	(24.94)	(15.76)	(0.43)	(0.00)	(26.38)

[a] All numbers are average results from duplicate runs of each sample. Numbers in parentheses are percent normalized responses (100× analyte response/total response of target analytes). [b] All response ratios are 100× analyte response/response of pyroglutamic acid.

disks led to a dramatic reduction of the analyte responses. This may be due to the fact that in the current method, MSTFA is diluted by CH_3CN and is mostly consumed by the relatively large amount of water present in the disk, such that little MSTFA is left for reacting with the analytes.

The time required to complete the combined process of derivatization and elution was determined by monitoring the responses of the analytes extracted from a tobacco sample after 15, 30, 45, and 60 min of heating at 80 °C. The responses of individual analytes except fumaric acid leveled off after 45 min. Derivatization of all analytes, including fumaric acid, was completed in the first 15 min when a solution of standards reacted with the silylating reagent. These results clearly show that heterogeneous silylation involving the disk is different from homogeneous derivatization. It is likely that silylation on the disk is an equilibrium reaction. For all the analytes except fumaric acid, the equilibrium is established after 45 min; at this point there are still some analytes left on the disk to maintain the equilibrium. This is supported by the finding that heating of the disk in a fresh MSTFA/CH_3CN solution for repeated silylation resulted in the recovery of significant amounts of analytes (ca 20% of the amounts recovered in the first treatment). In all subsequent experiments, silylation was carried out for 45 min.

Method Validation. ***Precision.*** Precision is the key factor that determines the suitability of the method for profiling purposes, especially when the profiles are used to build a reference database. The two measurements for each analyte obtained from duplicate disk extractions, whether expressed as mg g^{-1} tobacco, response ratios, or percent normalized responses, typically agree within 8%. The day-to-day precisions were determined by using the data obtained from one Cuban and one non-Cuban cigar filler samples analyzed on five nonconsecutive days over a one month period. Most of the analytes were quantified reproducibly from day-to-day with 2−14% RSD. The exceptions were fumaric acid and unknown 2 that were measured with >20% RSD, although the peaks were strong and easily integrated. Because the reasons for this observation remain to be investigated, these two analytes were not used to characterize cigar tobacco.

It should be noted that the disks used in the study of day-to-day precision were conditioned on the same day and kept in the refrigerator when not in use. The results indicate that the performance of the disks was not significantly affected by the storage over a one-month period. Thus, SAX disks can be conditioned in batches and stored in water for later use. Unlike the traditional operation, which requires the disks to be used immediately after conditioning, the present procedure provides a great deal of convenience to analysts. Lot-to-lot reproducibility of the SAX disks was also investigated by using disks of two different lots to extract acids from one Cuban and one non-Cuban cigar filler tobacco samples. The results presented in Table 2 show that the performance of the two lots was not significantly different.

Stability of the Silylated Derivatives. In multiple-sample analysis, samples are not analyzed by GC/MS immediately after derivatization. During the delay, the silylated derivatives might degrade, adversely affecting the reliability of the measurements. The stability of the TMS derivatives of individual analytes in the silylating solution containing the disk at room temperature was determined by monitoring the responses after 0, 5, 10, 18, and 24 h. For three test tobacco samples, the responses or response ratios to ISTD remained constant throughout the first 24 h period, suggesting that at least 48 samples can be run in a long sequence.

Application to Tobacco Samples. Two groups of samples were used to examine the usefulness of the acid profiles generated by this method for differentiating Cuban from non-Cuban cigars. They included 18 authentic Cuban cigars and 31 cigars manufactured in the United States, Honduras, the Dominican Republic, Nicaragua, Mexico, Brazil, Jamaica, and Aruba.

The wrappers, binders, and fillers of Cuban cigars are always made with tobaccos of Cuban origin, whereas the three tobacco parts of any of the non-Cuban cigars can be derived from different geographical origins (*10*, *11*). Therefore, the wrappers and fillers of the non-Cuban cigars must not be assumed to be originated from the manufacturing country. These cigars are collectively grouped as non-Cuban, because it is highly unlikely that they contain any Cuban component.

The acid data were subjected to principal component analysis (PCA). PCA provides a simple graphical picture of the trend in a set of data. It is a statistical multivariate technique of reducing the dimensionality of the data by constructing a series of new data axes, called principal components, which are linear combinations of the original variables in the data set. In effect, a projection of the data onto the new axes allows one to visualize graphically the relationship among the samples on a two-dimensional plot, if the first two principal components can account for most of the variance of the data. In general, similar samples tend to stay close together in the score plot. Figure 3 presents the PCA results of fillers, which captures 98% of the variance of the data based on the concentrations (mg g^{-1} tobacco) of nicotinic acid, succinic acid, uracil, malic acid, citric acid, and pyroglutamic acid, as well as the response

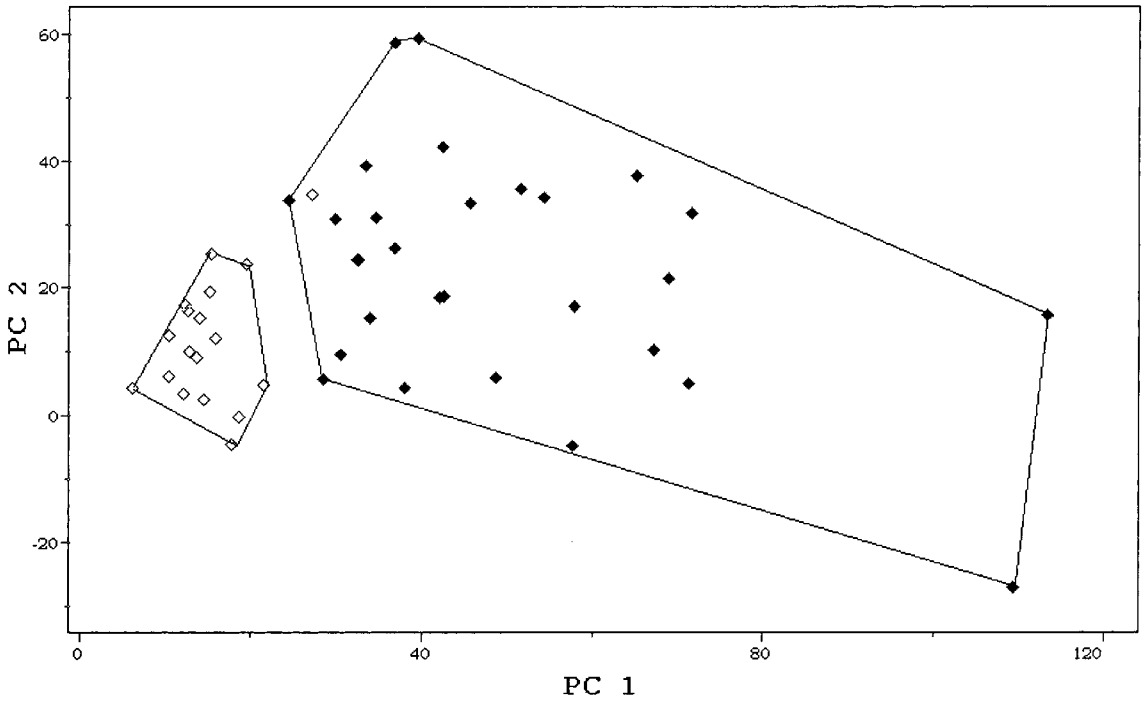

Figure 3. Plot of the first two principal components of cigar fillers based on acid data. Total variance, 98.3%. Bounded area on the left contains the Cuban fillers (◇), the bigger area on the right contains the non-Cuban fillers (◆) and a Cuban filler. The boundaries were drawn for ease of visualization, they were not determined statistically.

ratios of glyceric acid, threonic acid, and unknown 1 to pyroglutamic acid. It shows that the Cuban cigars, except for one sample, are grouped toward the left while the non-Cuban cigars are scattered over the right side of the score plot. As expected, the Cuban cluster appears tighter than the non-Cuban counterpart, because the latter is made up of fillers of various geographical origins. The PCA results clearly demonstrate the discriminatory power of the acid profiles of cigar fillers. However, the presence of an outlier suggests that the method by itself cannot unequivocally classify a sample. To accurately authenticate a sample, other powerful origin-probing techniques such as stable isotope or trace element analysis would be necessary to complement the current method. These techniques are presently under investigation.

The level of malic acid, and the response ratios of glyceric acid and unknown 1 to pyroglutamic acid were generally higher in the non-Cuban cigars than in the Cubans. These variables were identified by PCA as the most useful measurements to differentiate the Cuban cigars from the others. Similar separation of the two groups of cigar fillers was also observed when the data set based on percent normalized responses for individual analytes was used.

Results obtained from analysis of wrappers were less encouraging. The PCA plot shows no sign of natural grouping based on the acid profiles. This finding is not surprising in view of the fact that the quality and characteristics of a cigar are contributed mainly by the filler.

On the basis of this study, it is concluded that the SAX disk extraction /silylation/GC/MS analysis technique described herein is technically feasible for selective extraction of nonvolatile acids and reliable characterization of tobacco. The current disk conditioning and extraction procedure is much less labor intensive than the conventional protocol. The method yields reproducible day-to-day profiles based on uracil, nicotinic acid, succinic acid, malic acid, pyroglutamic acid, glyceric acid, threonic acid, citric acid. and an unknown analyte that is probably an isomer of threonic acid. Thus, the method is suitable for use in the establishment of a data bank on a continuing basis. Principal component analysis on 49 cigar filler samples demonstrates the usefulness of the quantitative acid profile in recognizing Cuban cigars among those from non-Cuban sources. Although only cigar tobaccos were investigated in this study, this method is potentially applicable to other tobacco products such as cigarettes or raw leaves.

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