Determination of Carbohydrates in Tobacco Products by Liquid Chromatography–Mass Spectrometry/Mass Spectrometry: A Comparison with Ion Chromatography and Application to Product Discrimination

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Carbohydrates in commercial tobacco products were quantified utilizing a liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS) technique. The method utilizes negative ion electrospray with multiple reactions monitoring and an internal standard calibration. Snuffs, chewing tobaccos, cigars, and cigarettes were analyzed. Product type differentiation was possible using the carbohydrate levels coupled with pH and moisture contents. The LC-MS/MS method was compared to a method utilizing ion chromatography with pulsed amperometric detection. The LC-MS/MS method provided improved selectivity and specificity, demonstrated better precision, and had a larger dynamic range for glucose, fructose, and sucrose in tobacco extracts.

KEYWORDS: LC-MS/MS; carbohydrates, tobacco; ion chromatography

INTRODUCTION

As major components of tobacco, carbohydrates are valuable for product characterization and differentiation. Tobacco products in the United States may fall into several taxable categories including cigars, cigarettes, snuff, chewing tobacco, pipe tobacco, and roll-your-own. The Alcohol and Tobacco Tax and Trade Bureau (TTB) is responsible for determining proper tax classification. This classification will determine the amount of tax owed. As an example, the 2005 federal tax on cigarettes is $0.39 per pack as compared to $0.04 for a pack of little cigars.

Prior work published by Zook et al. (*1*) found differences in the carbohydrate profiles of cigars and cigarettes. Predominately flue-cured cigarettes have higher concentrations of carbohydrates than cigar tobaccos consisting of predominately air-cured and fermented tobaccos. Both air curing and fermentation lead to a reduction in carbohydrate levels. Furthermore, sugars, usually invert or partially invert, are often added to cigarettes by the manufacturers. Although most products contain a blend of tobaccos, significant differences in carbohydrate levels of cigars and cigarettes have been demonstrated (*1*).

The predominant sugars found in tobacco are glucose, sucrose, and fructose. Glycerin is often added as a humectant and can also be found in very high concentrations. Many other naturally occurring and added carbohydrates can be found in tobacco products. As an example, maltose and isomaltose have also been identified in pouch and plug chewing tobacco (*2*).

Carbohydrates are usually hydrophilic, neutral, and lack satisfactory chromophores for UV detection. Derivatization is often used to improve sensitivity and chromatographic resolution (*3*). Alternately, ion-exchange chromatography with pulsed amperometric detection (IC-PAD) has been applied to numerous matrices including tobacco without derivatization (*1, 4−7*). In IC-PAD, the use of alkaline eluents causes carbohydrates to be ionized. This allows separation by anion-exchange mechanisms. Detection occurs by measuring the current generated when the carbohydrates are oxidized on a gold electrode. Optimum detection potentials for tobacco carbohydrates were examined previously (*1*). Although the use of an appropriate detection potential makes this a very selective technique for carbohydrates, the possibility for coeluting carbohydrates or other compounds in tobacco matrices exists. Coelution of compounds can be discovered using mass spectrometry provided that the mass/charge ratios of the coeluting compounds are different. An additional advantage of mass spectrometry is the ability to screen the extracts for other components that might be useful for product discrimination.

The purpose of this work is to develop an liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS) method for common carbohydrates in tobacco and to examine the classification of different tobacco products using percent total carbohydrates. This method was applied to cigarettes, cigars, chewing tobaccos, and snuff. In addition, the pH and moisture of the products were examined as complementary

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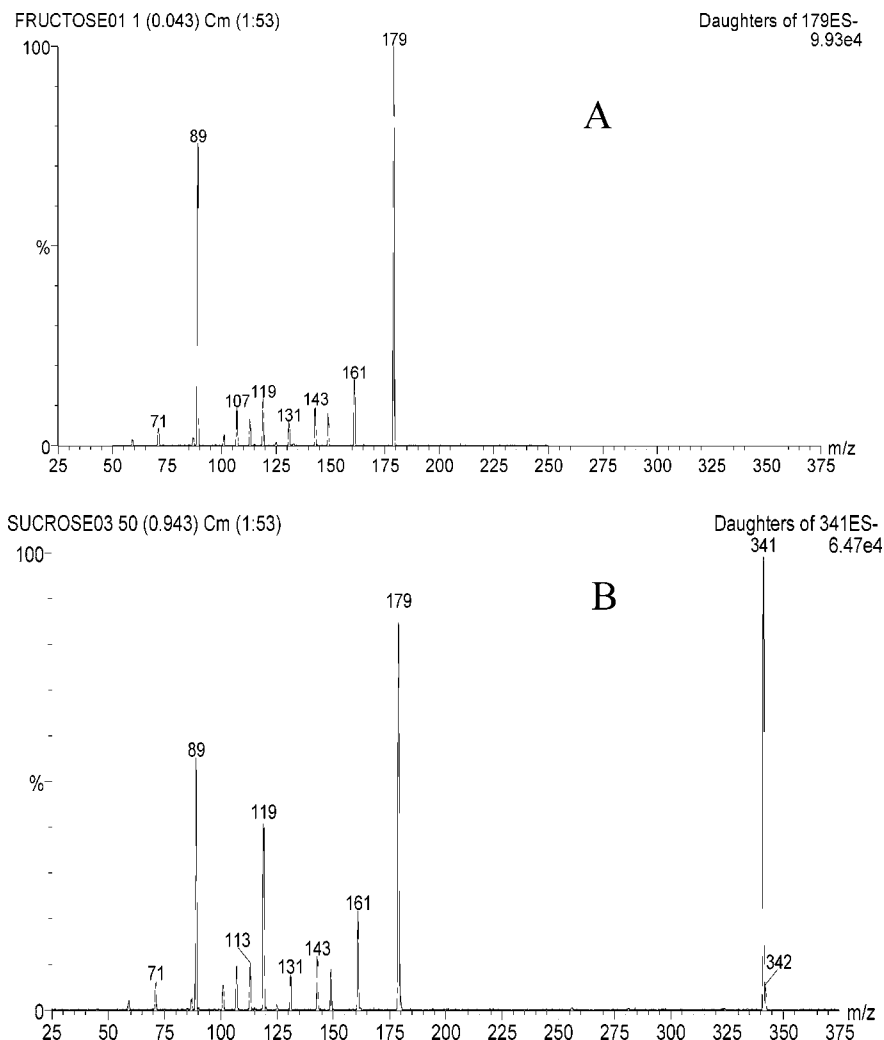§ The Johns Hopkins University.

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Figure 1. Typical MS/MS mass spectra for glucose and sucrose. (**A**) MS/MS mass spectrum of glucose obtained at a collision energy of 10 V. Fructose and glucose yielded identical mass spectra. The MS/MS mass spectrum for sucrose obtained at a collision energy of 15 V is shown in panel **B**.

Table 1. Summary of pH, Moisture, and Carbohydrate Data

product type	snuff ($n = 11$)[a]	cigar ($n = 10$)[a]	cigarette ($n = 10$)[a]	chewing tobacco ($n = 9$)[a]
pH range	7.13–8.93	5.31–7.36	5.50–6.26	5.12–6.23
avg. pH	7.64	6.61	5.92	5.65
σ, pH	0.51	0.72	0.27	0.39
moisture range	36.79–54.32	11.08–17.76	9.54–15.2	15.65–26.34
avg. moisture	50.38	14.48	13.37	23.04
σ, moisture	4.78	2.11	1.77	3.30
glucose range	not detected–0.16	0.002–0.348	0.94–2.52	0–10.43
avg. glucose	0.02	0.047	1.75	5.88
σ, glucose	0.05	0.107	0.50	3.25
fructose range	not detected–0.02	not detected–0.320	3.98–5.76	1.89–20.96
avg. fructose	0.01	0.054	4.92	8.96
σ, fructose	0.01	0.097	0.60	7.08
sucrose range	not detected	not detected–0.355	0.52–4.72	0.026–22.46
avg. sucrose	not detected	0.040	2.79	12.83
σ, sucrose	not detected	0.111	1.53	6.70
total sugars avg.	not detected–0.19	0.002–1.023	6.67–12.29	5.96[b]–40.71
avg. total sugars	0.03	0.141	9.45	27.67
σ total sugars	0.06	0.313	2.08	9.98

[a] Total number of samples analyzed indicated by "$n =$". [b] One sample labeled "plain and nonsweet" measured 5.96%; all others ranged between 23.7 and 40.7%.

indicators of product type. The feasibility of using carbohydrates as well as pH and moisture for product differentiation is discussed. Finally, the LC-MS/MS method is compared to an IC-PAD method utilized in this laboratory.

EXPERIMENTAL PROCEDURES

Materials and Methods. *Reagents and Samples.* D-Glucose, D-fructose (99+% purity), sucrose (99.5% purity), and D-glucose (98 atom % D2) were from Sigma-Aldrich. Standards were used within 24 h of
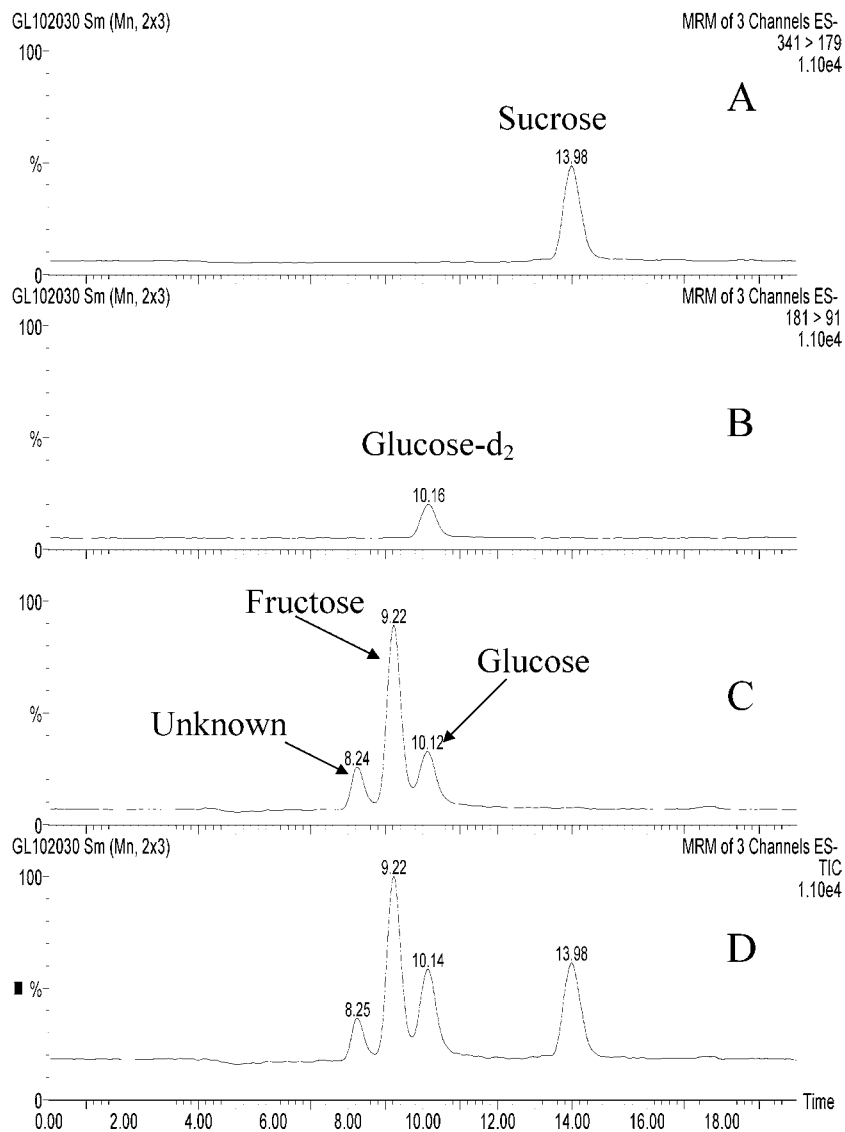
Figure 2. Negative ion electrospray LC-MS/MS of cigar filler tobacco. (**A**) MRM of sucrose; (**B**) MRM of D-glucose-d_2 (internal standard); (**C**) glucose (at 10.23 min), fructose (at 9.22 min), and unidentified isomer (at 8.24 min); and (**D**) the total ion chromatograph.
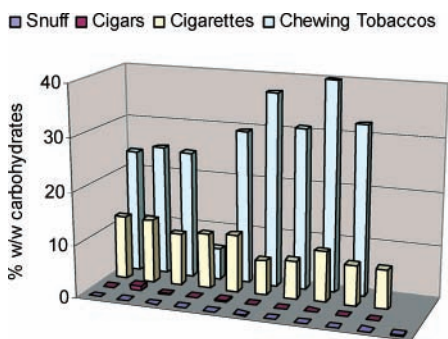

Figure 3. Total carbohydrate levels in snuffs, cigars, cigarettes, and chewing tobaccos. Data are shown as percent by weight (*z*-axis) without moisture correction.

preparation and were refrigerated between use if necessary. Samples of popular tobacco products were obtained from local stores, TTB field personnel, and tobacco manufacturers. Only widely known products were used to establish a baseline for product comparison.

Sample Preparation and Handling. Cigar and cigarette tobaccos were ground in a Wiley Mill (Thomas Scientific, Swedesboro, NJ) equipped with a 20 mm mesh screen. Chewing tobaccos were cut into 1−2 mm with a razor. Snuff was used as received. Cigars were separated into

wrapper and filler materials. Ten cigarettes per pack were ground. Samples were stored in a cold room.

pH and Moisture Determination. The pH was determined by weighing 500 mg of ground tobacco in duplicate into 125 mL Erlenmeyer flasks. Twenty-five milliliters of Milli-Q 18 MΩ cm water was added, and the flask was swirled to wet the tobacco. An additional 25 mL of Milli-Q 18 MΩ cm water was transferred by pipet to wash down the sides of the flask. The flask was sealed with a stopper, and the mixture was gently shaken for 24 h at room temperature. The pH was determined with a Metrohm 744 pH meter equipped with a Metrohm Solitrode pH electrode.

Moisture contents were determined by weighing 1.0 g samples in triplicate using 70 mL aluminum dishes. The samples were evenly distributed over the bottom of the dish and placed in a convection oven at 110 °C for 4 h. They were cooled to room temperature in a desiccator prior to reweighing.

Extraction for LC-MS/MS Analyses. Approximately 500 mg of the ground or cut-up tobacco samples was weighed into glass bottles. The samples were extracted using Milli-Q 18 MΩ cm water with D-flucose-d_2, 98 atom % D, as an internal standard. The ratio of tobacco/extracting solution was adjusted to stay within the working range of the method but did not exceed 500 mg/25 mL. As an example, for cigarettes, 500 mg of sample was extracted with 100 mL of water. The samples were shaken on a Burrel Wrist Action Shaker for 10 min and filtered directly into autosampler vials using an Alltech 600 mg C18 Maxi-Clean
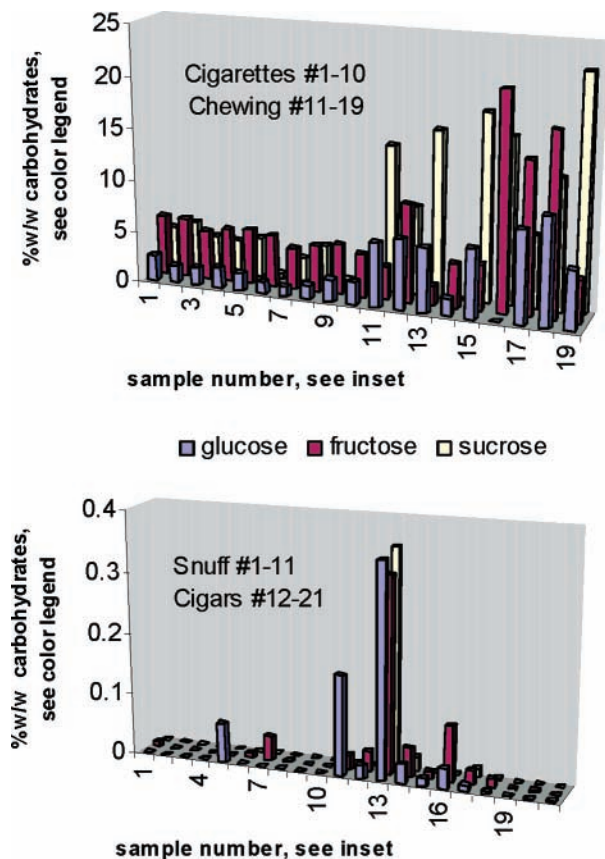
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Figure 4. Percent by weight (*z*-axis) of glucose (blue), fructose (red), and sucrose (yellow) without moisture correction as a function of product type. Insets on graphs identify sample types by sample numbers listed on the *x*-axis. The top graph shows cigarettes and cigars. The bottom graph shows cigars and snuffs.
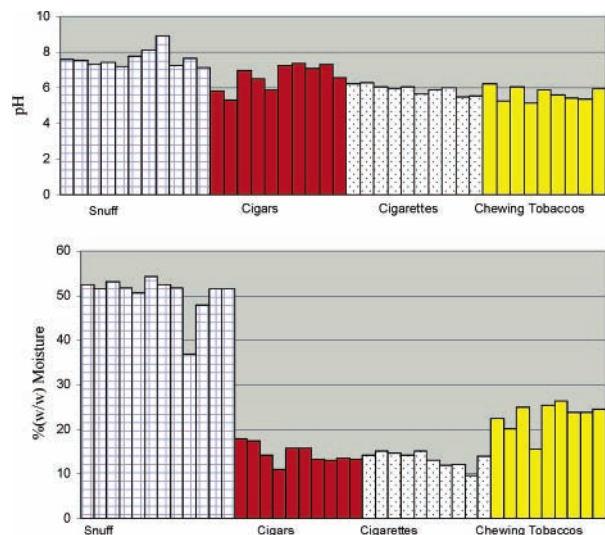

Figure 5. Top, pH as a function of product type; bottom, moisture content as a function of product type.

Cartridge and a Fisherbrand Nylon 0.2 μm filter in series. The first few milliliters were discarded. When necessary, the filtrates were diluted with the extraction solution. All standards contained the same concentration of internal standard as the extraction solution.

Carbohydrate concentrations as a function of time were measured for the extraction of a cigar and a cigarette in water. In both cases, the concentrations reached a plateau in less than 10 min. The concentration was also measured after 24 h of extraction and remained constant within analytical error.
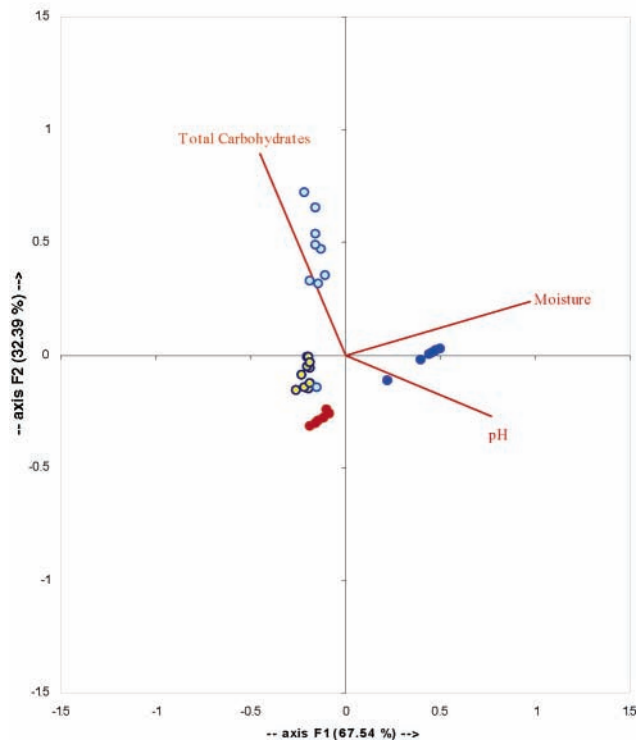

Figure 6. Biplot from principal component analyses. The product types are indicated as follows: chewing tobaccos, light blue; cigarettes, yellow; cigars, red; and snuffs, dark blue. All 40 products tested were used in the model; however, some data points overlap due to tight clustering.
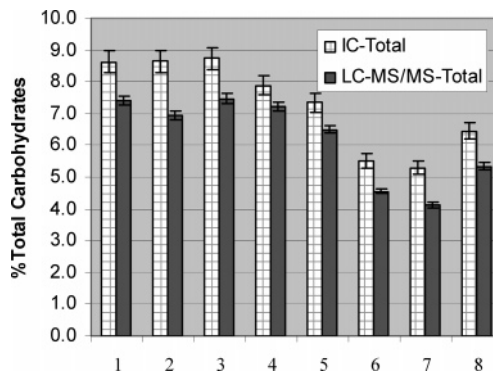

Figure 7. Total carbohydrate levels in eight cigarette products as determined by IC-PAD and LC-MS/MS. Values shown are without moisture correction and are the average of three replicate injections. Error bars represent 4% for IC-PAD and 2% for LC-MS/MS.

*Extraction for Comparative Study of IC-*PAD and LC-MS/MS. For method comparison, a variety of tobacco samples were analyzed by both methods. The tobaccos were extracted in water and filtered utilizing the procedures given above. Lactose, the internal standard for the IC method and d-glucose-d_2, 98 atom % D, the internal standard for the LC-MS/MS method, were quantitatively added to the autosampler vials. This minimized any error between the methods during extraction procedures. The possibility of coeluting peaks with lactose cannot be eliminated due to the complex nature of tobacco products. Therefore, lactose was utilized only to evaluate elution time. It was not used in quantification.

Methods. LC-MS/MS analyses were performed on a Waters Alliance 2690 LC separation module coupled to a Micromass Quattro II triple quadrupole mass spectrometer system equipped with an electrospray source. The 2690 LC system was equipped with a quaternary pump, an in-line vacuum degasser, and a photodiode array detector. Chro-
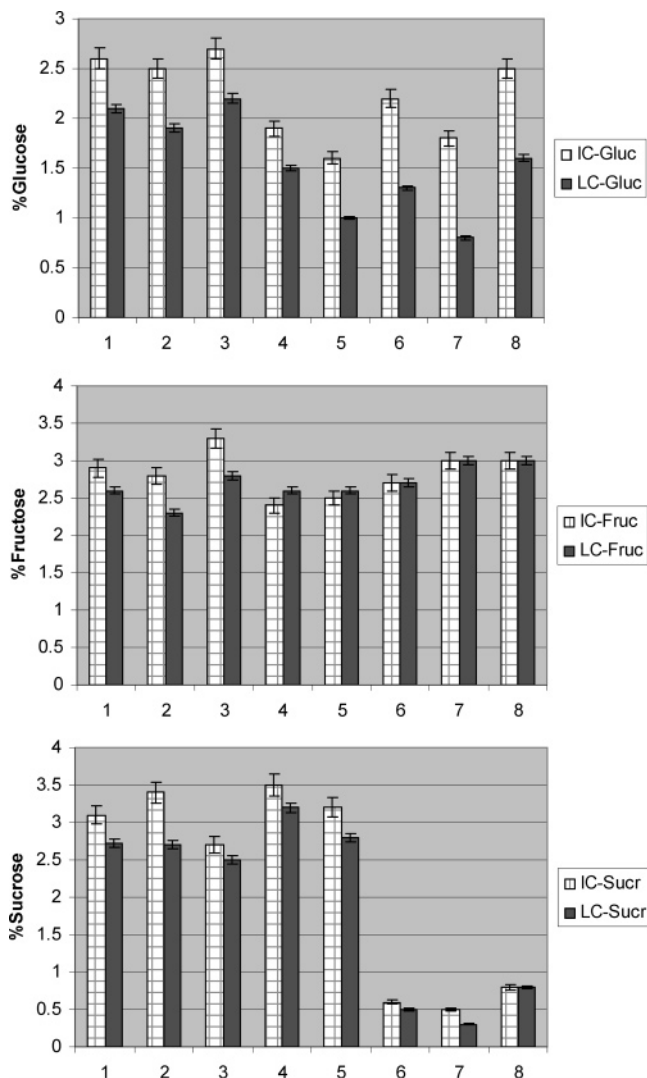
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Figure 8. Percent glucose (top), fructose (middle), and sucrose (bottom) in eight cigarette products as determined by IC-PAD and LC-MS/MS. Values shown are without moisture correction and are the average of three replicate injections. Error bars represent 4% for IC-PAD and 2% for LC-MS/MS.

matographic separation was achieved using a Supelcosil LC-NH₂, aminopropyl bonded phase, column (3.0 mm × 250 mm, 5 μm particle size) with a Supelguard LC-NH₂ guard column. The LC elution conditions were used as follows: a linear gradient of 20% water with 10 mM formic acid and 80% acetonitrile at a flow rate of 300 μL/min and injection volume of 5 μL. The column temperature was maintained at 45 °C.

A CsI solution was used for mass calibration of the Quattro II mass spectrometer. Mass spectrometric parameters for carbohydrates were optimized by infusing carbohydrate standards at a flow rate of 5 μL/min into a stream of mobile phase flowing at a rate of 295 μL/min using a flow splitter. Split flow provided optimization of mass spectrometry parameters using the actual analyte and mobile phase composition. MassLynx software (3.4) was used for data acquisition and reduction. Nitrogen was used both as the nebulizing gas and as the drying gas. The following mass spectrometric conditions were used for the analysis of glucose, fructose, and sucrose in negative ion electrospray mode: capillary voltage, 2.50 kV; extractor voltage, 7 V; source block temperature, 110 °C; desolvation temperature, 400 °C; drying gas flow, 250 L/h; and ESI nebulizer gas flow, 20 L/h. The collision gas was filled with argon at a pressure of 2−3 mbar, and the collision energy was optimized for each transition ion. Quantitative analysis of carbohydrates was performed using multiple reaction monitoring (MRM) mode. The MRM mass transitions used for

quantitative determination of carbohydrates in tobacco products were obtained under the following conditions: For glucose and fructose, a transition of 179 → 89 was achieved at a cone voltage of 15 V and a collision energy of 10 V; for sucrose, a transition of 341 → 179 was achieved at a cone voltage of 25 V and a collision energy of 15 V; for the internal standard (glucose-d_2), a transition of 181 → 91 was achieved at a cone voltage of 20 V and a collision energy of 15 V. MRM detection was performed by switching between the different collision energies with a dwell time of 0.2 s.

The IC-PAD method is routinely used in TTB's laboratory for carbohydrate analyses. A Dionex ICS2500 Ion Chromatograph with Chromeleon Software, a GP50 Gradient Pump, an AS50 Autosampler with a 10 μL loop, a Dionex CarboPac PA-1, 4 mm × 250 mm column, and a Dionex CarboPac PA-1, 4 mm × 50 mm guard column, were used. The mobile phase was Milli-Q 18 MΩ cm, degassed with He, and a 50%w/w NaOH solution was added to yield a final concentration of 150 mM NaOH. The run was isocratic at 1 mL/min. The detector was a Dionex ED50A Electrochemical Detector with Ag/AgCl reference electrode and a gold working electrode. The potential waveform was as follows: 0−0.4 s, E_d = 0.10 V; 0.4−0.42 s, E = −2.00 V; 0.43 s, E = 0.60 V; and 0.44−0.50 s, E = −0.10 V. The injection volume was 10 μL. The run time was 15 min per sample. The approximate elution times were as follows: glucose, 3.5 min; fructose, 3.8 min; lactose, 5.6 min; and sucrose, 7.1 min.

RESULTS AND DISCUSSION

LC-MS/MS Method Performance. The detection of glucose, fructose, and sucrose in different tobacco products was accomplished using the LC-MS/MS method with an electrospray ion source operating in negative ion mode. Calibration with an internal standard was employed for quantification. All standards, analytes, and blanks were spiked with a known amount of internal standard and were subjected to the same sample extraction and cleanup procedures. Linear dynamic ranges of 0.5−100 μg/mL were obtained for glucose, fructose, and sucrose with coefficients of determination (r^2) greater than 0.999. The limits of detection were 0.05 μg/mL for glucose and sucrose and 0.025 μg/mL for fructose. The limits of quantification for glucose, fructose, and sucrose were 0.5 μg/mL.

LC-MS/MS analysis of a neat sucrose standard showed a minor peak at m/z 179, which corresponds to the hydrogen abstraction from the molecular ion, $[M − H]^-$, of fructose or glucose. However, the minor decomposition of sucrose observed in the analysis of both standards and analytes appears to be linear with concentration and did not affect the dynamic range and quantification of sucrose in tobacco products.

Daughter ion spectra obtained from the analysis of glucose, fructose, and sucrose standards are displayed in **Figure 1**. Glucose and fructose yield identical mass spectra (**Figure 1A**) with a strong deprotonated molecular ion at m/z 179 and a major fragment ion at m/z 89. The mass spectrum for sucrose (**Figure 1B**) exhibits a strong deprotonated molecular ion at m/z 341 and major fragment ions at m/z 179 and at m/z 89. As expected, the mass spectrum of sucrose (**Figure 1B**) yielded identical fragment ions as glucose and fructose.

A typical negative ion electrospray LC-MS/MS spectrum of a tobacco product is shown in **Figure 2**. A baseline separation of glucose and fructose is achieved with the addition of 10 mM formic acid into the aqueous mobile phase. In addition to the detection of the target analytes (glucose, fructose, and sucrose), another MRM transition ion (179 → 89) corresponding to the isomers of glucose and fructose is typically observed in the analysis of most tobacco products.

Product Discrimination Based on Carbohydrate, pH, and Moisture Data. Table 1 summarizes the carbohydrate, pH, and moisture data. The carbohydrates are listed as percent by weight
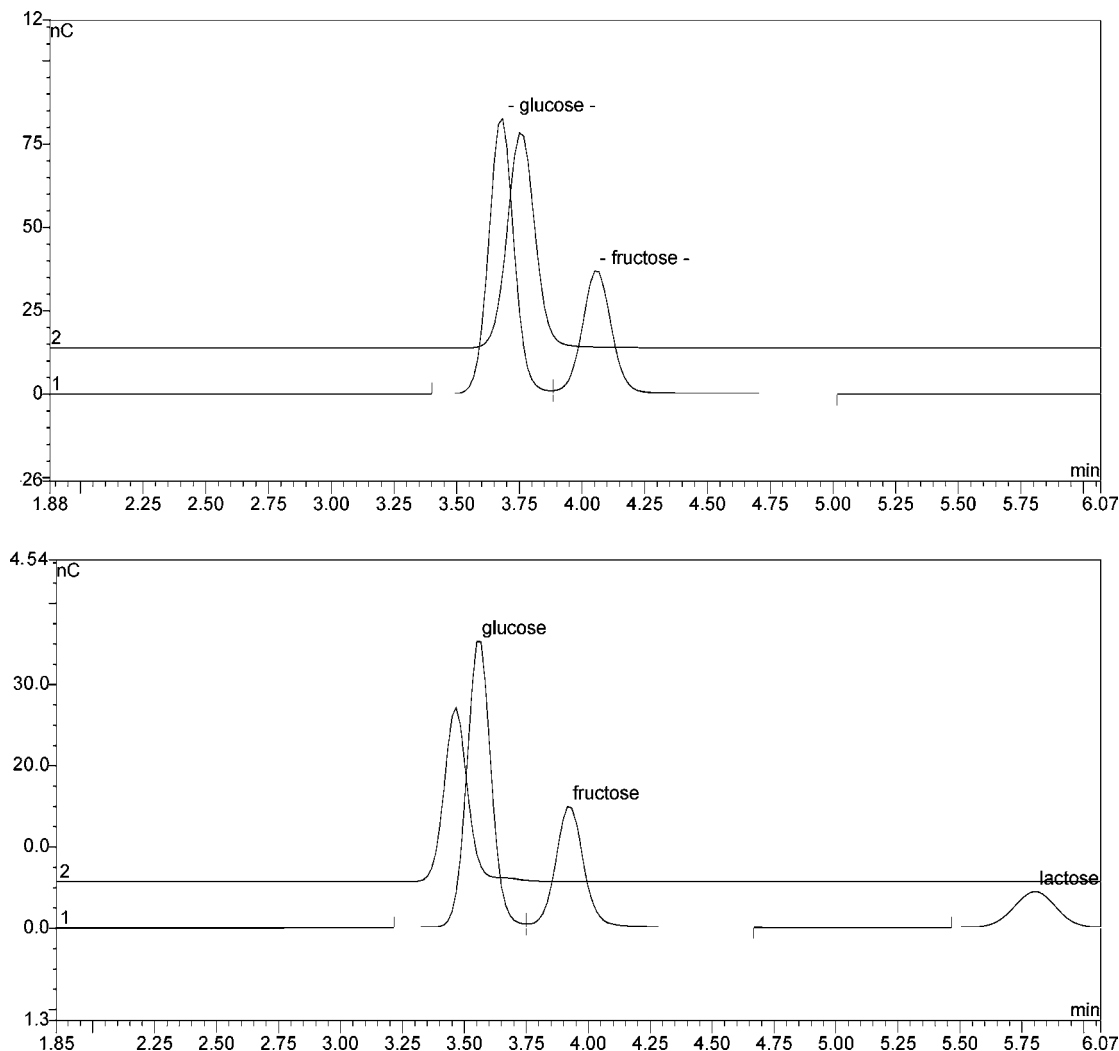
Figure 9. IC-PAD chromatograms. Top: overlay of 10 ppm galactose, shown offset and labeled as trace 2, on 10 ppm carbohydrate mixture labeled as trace 1. Bottom: overlay of 10 ppm mannose, shown offset and labeled as trace 2, on 10 ppm carbohydrate mixture labeled as trace 1. The top and bottom chromatograms were obtained approximately 1 month apart. Lactose was not added as an internal standard in top chromatograms. Sucrose (not shown) elutes at approximately 7.1 min.

as received, without moisture correction, and are the averages of three replicate injections. Total carbohydrates are calculated as the sum of glucose, fructose, and sucrose. The data are represented graphically in **Figures 3–5**. The carbohydrate levels of all of the products were calculated on a dry weight basis and compared. The same trends were observed with and without moisture correction. Unless specified otherwise, the values listed are without moisture correction. Snuffs contained very little or, in many cases, no quantifiable reducing sugars. Cigars also had very low concentrations of reducing sugars with total values ranging between 0.002 and 1.023%. Cigarettes and chewing tobaccos had much higher levels ranging between 6.667 and 12.285% and 5.963 and 40.710%, respectively. It should be noted that the one chewing tobacco with a total carbohydrate level of 5.963% was inconsistent with the other chewing tobaccos tested, which had levels exceeding 22%.

The carbohydrate data are influenced by the types of tobaccos used as well as flavors and humectants added by manufacturers. Snuffs produced in the United States are generally made with fire-cured and air-cured dark tobaccos that have characteristically low sugar concentrations (*8*). Chewing tobaccos generally use air-cured tobacco leaves that are treated with a sweet casing solution. The high levels of sucrose in the products tested indicate the addition of sugar. The packaging of the chewing

tobacco with the uncharacteristically low total carbohydrate levels indicated that the product was "plain" and "nonsweet". This product had only 0.03% sucrose consistent with a lack of added sweetener. Additionally, the moisture content of this product was 15.6%, much lower than the other chewing tobaccos, which ranged from 20.2 to 26.3%. These data also demonstrate the importance of carbohydrate levels in distinguishing products, particularly cigars and cigarettes. Although the moisture contents and pH values overlapped, there is a clear difference in the levels of reducing sugars, particularly in the sucrose levels, which were very low in the cigars measured.

The variability within each group of products is also important to note. These inconsistencies are not unexpected and demonstrate the importance of examining additional chemical and physical parameters as well as product packaging and labeling when determining tax classification. TTB's determination of tax classification involves consideration of both laboratory analyses and packaging and labeling.

To further examine the data, principal component analysis was performed using XLSTAT-Pro, Addinsoft, 2004. Over 99% of the variance was captured by two principal components. The resulting biplot is shown in **Figure 6**. As expected, the different

product types were easily resolved based on moisture, pH, and total carbohydrates except for the one anomalous chewing tobacco.

Comparison of LC-MS/MS and IC-PAD. The determination of carbohydrate levels in cigars and cigarettes by IC-PAD is discussed by Zook et al. (*1*). They measured glucose, fructose, and sucrose and determined total sugar concentrations as a percent by weight in the dried sample. Using the measured moisture concentrations, the data generated here were calculated on a percent by dry weight basis for comparison. The levels found in cigars using the LC-MS/MS data, 0.002−1.24%, are consistent with the levels measured by Zook et al. between none detected and 1.04%. However, the sugar concentrations in cigarettes are much higher in this work, 8.17−14.30%, as compared to the values reported by Zook et al., 1.58−4.7%. This difference may be due to a number of factors including the nature of the products sampled or a change in blending/manufacturing since the Zook et al. publication in 1996. Of these factors, the type of cigarettes sampled is likely the dominant factor. The products sampled for this work were all American-blended cigarettes (ABC). A recent publication by Abdallah (*9*) indicates that the typical ABC cigarette contains between 10 and 12% total sugars, which is consistent with the majority of the values found in this study.

To further compare carbohydrate levels found by the LC-MS/MS method and IC-PAD, eight cigarette products were analyzed. The products included light, full flavor, and menthol products. Each product was extracted with water and filtered, and aliquots were spiked with the appropriate internal standard and run by the two methods on the same day. The average of three replicate injections was calculated. The %RSDs were ≤4% for IC-PAD and ≤2% for LC-MS/MS. The results are presented graphically in **Figures 7** and **8**. The IC-PAD method gave consistently higher results. This experiment was repeated with different products, and the trend was consistent. The total carbohydrate levels in the cigarettes were on average 17% higher when measured by IC-PAD with approximately two-thirds of this difference attributable to glucose. The sucrose was 4% higher on average. The fructose was generally within the analytical error of the methods although it was elevated in three of the eight samples. One possible reason for this discrepancy would be the coelution of other carbohydrates. Tobacco contains a wide variety of carbohydrates, and additional carbohydrates may be added as sweeteners and humectants (*8*, *10*). Several materials including melibiose, maltose, mannose, and galactose were examined. Both mannose and galactose coeluted with glucose. The chromatograms are shown in **Figure 9**. Although an exhaustive examination of interferents was not conducted, it is immediately evident from these data that the potential for coelution of carbohydrates in tobacco matrices exists with the IC-PAD method utilized here.

Conclusion. This work confirmed that the assessment of carbohydrate levels is significant for the determination of tobacco product type, particularly when utilized in conjunction with other physical and chemical measurements. Furthermore, it has demonstrated that LC-MS/MS is a sensitive and selective tool for the analyses of carbohydrates in tobacco products. LC-MS/MS provides an advantage over the IC-PAD method utilized here for the analysis of specific carbohydrates. The ability to analyze the individual carbohydrates in tobacco products is especially significant for products such as little cigars and cigarettes, where the sucrose level in cigarettes is clearly higher than in little cigars.

Another advantage of the LC-MS/MS method is the increase in the linear range and precision. For the IC-PAD method, the linear range is approximately 1−100 ppm for glucose and fructose and 1−30 ppm for sucrose and the %RSDs were less than 4% for the individual components and total carbohydrate levels. In comparison, the LC-MS/MS linear range for glucose, fructose, and sucrose is 0.5−100 ppm and the %RSDs were ≤2% for the individual components and total carbohydrate levels.

It is important to recognize that the coelution of carbohydrates in tobacco matrices does not eliminate the use of the IC-PAD method or favor the LC-MS/MS method for tax classification. Currently, TTB evaluates total carbohydrate levels for discrimination of product type, not individual carbohydrates. The IC-PAD method utilized in this study is simple, fast, and reproducible. Furthermore, carbohydrate levels in hundreds of products have been analyzed by TTB's laboratory using this method and these data are utilized for comparison. To adopt the LC-MS/MS method for regulatory purposes, a similar database of products would need to be established with the method. TTB will continue to evaluate LC-MS/MS for product differentiation based on specific carbohydrates. This work will be done in conjunction with the evaluation of other physical and chemical properties such as microscopic characteristics, pH, moisture, and flavorings.

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