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Comparison of Segmented Flow Analysis and Ion Chromatography for the Quantitative Characterization of Carbohydrates in Tobacco Products

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ABSTRACT: Segmented flow analysis (SFA) and ion chromatography with pulsed amperometric detection (IC-PAD) are widely used analytical techniques for the analysis of glucose, fructose, and sucrose in tobacco. In the work presented here, 27 cured tobacco leaves and 21 tobacco products were analyzed for sugars using SFA and IC. The results of these analyses demonstrated that both techniques identified the same trends in sugar content across tobacco leaf and tobacco product types. However, comparison of results between techniques was limited by the selectivity of the SFA method, which relies on the specificity of the reaction of *p*-hydroxybenzoic acid hydrazide (PAHBAH) with glucose and fructose to generate a detectable derivative. Sugar amines and chlorogenic acid, which are found in tobacco, are also known to react with PAHBAH to form a reaction product that interferes with the analysis of fructose and glucose. To mitigate this problem, solid phase extraction (SPE) was used to remove interferences such as sugar amines and chlorogenic acid from sample matrices prior to SFA. A combination of C18 and cation exchange solid phase extraction cartridges was used, and the results from SFA and IC analyses showed significant convergence in the results of both analytical methods. For example, the average difference between the results from the SFA and IC analyses for flue-cured tobacco samples dropped by 73% when the two-step C18/cation exchange resin sample cleanup was used.

KEYWORDS: *tobacco, carbohydrates, SFA, IC-PAD, PAHBAH*

INTRODUCTION

Tobacco products such as cigars and cigarettes contain blends of tobacco from a variety of leaf grades and, in the case of cigarettes, several curing processes. Many of the significant chemical differences between cigar and cigarette tobaccos are related to the type of curing and fermentation processes used to manufacture them. One of the most significant chemical differences is the level of sugars, specifically glucose, fructose, and sucrose, which vary dramatically depending on processing.[1] Flue-cured tobaccos, which are high in these sugars, are prevalent in cigarettes sold on the U.S. market. Furthermore, sugars are often added directly to products such as cigarettes, chewing tobaccos, and pipe tobaccos. In contrast, cigars are manufactured from processed tobaccos, air-cured or fermented, that contain relatively low levels of glucose, fructose, and sucrose.

Established analytical methods for the analysis of carbohydrates in tobacco include ion chromatography with pulsed amperometric detection (IC-PAD) and segmented flow analysis (SFA). Both have been discussed in the literature[2−5] and used extensively to study sugar content in tobacco leaves and products. The most common SFA method is based on the reaction of *p*-hydroxybenzoic acid hydrazide (PAHBAH) with fructose and glucose, the most prominent reducing sugars found in cured tobacco leaves.[6] The test method for total reducing sugars (Astoria test method A250[7]) uses invertase to hydrolyze sucrose to form the reducing monosaccharides, fructose and glucose. Reducing sugars react with PAHBAH in an alkaline media to form a colored complex, described as a hydrazone that can be measured at 410 nm.[6,8] SFA is used to automate the reaction described above so it can be applied efficiently to a large group of samples.

Although chemical interferences in the PAHBAH reaction have been discussed in the literature, the method is considered by the instrument manufacturer, Astoria-Pacific International, to be specific for reducing sugars in tobacco.[7] It is important to note that most of the developmental work on this method, including investigations of chemical interference, was centered on applications in the clinical setting, specifically serum glucose levels.[5,9] Two investigations involving the application of the PAHBAH reaction to the analysis of reducing sugars determined that chlorogenic acid and glucosamine, which are found in tobacco, are reactive as well.[4,8] An investigation of the influence of chlorogenic acid on the determination of reducing sugars in tobacco suggested that its influence on the results of the analysis would be negligible.[4]

IC-PAD has been used for years to analyze carbohydrates in tobacco.[2,3] An anion exchange stationary phase is used to separate weakly acidic carbohydrates at high pH. Amperometry can be a very selective detection technique because only certain compounds will undergo a redox reaction under specific voltage conditions.[10] The carbohydrates' hydroxyl groups are oxidized on a working electrode surface, and the resulting current is measured. The oxidation of a carbohydrate is performed at a specific potential and results in the loss of a proton. The

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Table 1. Recovery Experiments for Sucrose, Fructose, and Glucose after Solid Phase Extraction Using C18 Syringe Cartridges and a Two-Step SPE Sample Cleanup Process Involving C18E and SCX Sorbents (n = 3)[a]

analyte	preparation	concn (ppm)	av (%)	SD (%)	preparation	av (%)	SD (%)
50:50 fructose and glucose	Maxi-Clean C18 syringe cartridge	400	98.9	0.5	SPE-C18E and SCX sorbents	99.2	0.6
		200	98.8	0.7		98.8	0.9
		20	98.3	0.9		99.0	0.3
sucrose	Maxi-Clean C18 syringe cartridge	20	99.3	1.8	SPE-C18E and SCX sorbents	99.1	2.1
		200	99.1	0.9		98.0	0.5
		400	99.5	1.5		99.2	1.8

[a]This study was performed on the segmented flow analyzer using the PAHBAH reaction and is intended to compare the recovery of sugar analytes using the sample preparation for the IC-PAD method as described by Clarke et al.[3] to the two-step C18E/SCX procedure described in this paper.

resulting current can then be measured at that potential, ensuring selective and sensitive detection.

Although IC-PAD is more selective than the SFA PAHBAH method, it does not share its high-throughput capabilities. As a wet chemical technique, the PAHBAH method is laborious and time intensive. Adaptation of this reaction to SFA automates complex procedures and dramatically increases sample throughput.[4,5] Using the SFA method, up to 72 samples can be analyzed in 1 h. Under the conditions described in this paper, throughput using the IC-PAD method is limited to 4 samples per hour. Given the dramatic difference in sample throughput, it is important to understand the relative merits of these analyses when one is deciding how and when to apply them in a routine testing environment.

The U.S. Alcohol and Tobacco Tax and Trade Bureau (TTB) is responsible for determining the proper tax classification of tobacco products. Tobacco products in the United States may fall into several taxable categories including cigars, cigarettes, snuff, chewing tobacco, pipe tobacco, and roll-your-own. As major components of tobacco, carbohydrates are valuable for characterization and differentiation. The purpose of this paper is to compare the results for two methods used by the TTB Tobacco Laboratory for routine analysis of common carbohydrates in tobacco. These methods were applied to cigarettes, cigars, pipe tobacco cigars, chewing tobacco, and cured tobacco leaf. Notable differences in the results obtained from these analytical methods were found. These differences will be discussed in the context of the sample preparation steps and relative selectivities of the methods. It will be shown that trends in each data set can provide similar conclusions when relative carbohydrate contents in leaf samples and tobacco products are compared.

SAMPLE PREPARATION

Leaf tobacco samples were obtained from the USDA Cotton and Tobacco Program (Raleigh, NC, USA). Samples of popular tobacco products were obtained from local retailers. Cigar, cigarette, and leaf tobaccos were prepared for analysis as follows. Approximately 3 g of each sample (product fill material or leaf) was placed in a screen basket. The baskets were placed in a 90 °C convection oven for 1 h. The samples were ground immediately after drying using a Wiley Mill Grinder with a 20 mesh screen, placed in airtight containers, and stored in a cold room at 4 °C. Chewing tobaccos (which are heavily laden with casing sauces) could not be ground with the Wiley Mill. These products were not dried and ground but instead were broken into smaller pieces with a razor.

Tobacco Samples: Extraction and Filtration. Tobacco samples were prepared for analysis at weight to volume ratios of 100 mg/100 mL and 200 mg/50 mL. The choice of weight to volume ratio was based on the curing process; the higher ratio was chosen for air-cured samples and cigars, and the lower ratio was chosen for cigarettes and chewing tobaccos. Samples were extracted with either deionized water or 1% acetic acid as described below:

Acetic Acid Extractions. Samples were accurately weighed into a 125 mL Erlenmeyer flask and extracted with 1% acetic acid, prepared from glacial acetic acid (Fisher) and 18.2 MΩ (Millipore Gradient A10) deionized water. The 1% acetic acid solution was added to the samples using Class A volumetric glass pipets. Samples were then placed on an orbital shaker at 150 rpm for 30 min and passed through Whatman 114 V pleated filter paper. Samples extracted with 1% acetic acid were analyzed by using the SFA PAHBAH method.

Water Extractions. Samples were accurately weighed into a 125 mL Erlenmeyer flask and extracted with 18.2 MΩ (Millipore Gradient A10) deionized water. Samples were then placed on an orbital shaker at 150 rpm for 30 min. Following agitation, tobacco extracts were prepared for analysis using the following cleanup procedures: (1) Samples that were filtered through Whatman 114 V pleated filter paper were analyzed by SFA PAHBAH. (2) Samples treated with C18E/SCX SPE were analyzed by SFA PAHBAH or IC-PAD. The C18E/SCX SPE process occurred in two steps that included Strata C18-E sorbent (Phenomenex, part 8B-S001-HBJ, 500 mg packing bed) followed by Strata SCX sorbent (Phenomenex, part 8B-S010-HBJ, 500 mg packing bed). A 12-port vacuum manifold was used to process the extracts. Strata SPE cartridges were conditioned using 6 mL of methanol (reagent grade) followed by 6 mL of 18.2 MΩ (Millipore Gradient A10) deionized water. Approximately 6 mL of the tobacco extract was passed through the C18-E SPE cartridge first. The first 2 mL was discarded, and the remaining 4 mL from the C18-E treated sample was collected and applied onto the SCX SPE cartridge. The first 2 mL was discarded, and the remaining 2 mL from the SCX SPE treated sample was collected for analysis.

Recovery Study Samples: Sugar Solutions. Aqueous solutions of fructose, glucose, and sucrose used in recovery studies were also processed using the paper filtration and two-step C18E/SCX SPE cleanup procedures described above. Additionally, these sugar solutions were treated with Maxi-Clean C18 SPE syringe filters.[3] All recovery study samples were analyzed using the SFA PAHBAH method. The results of these experiments are shown in Table 1. Maxi-Clean C18 SPE syringe filters (Grace Davison, part 20936, 600 mg packing bed) were conditioned using 5 mL of methanol (reagent grade) followed by 5 mL of 18.2 MΩ (Millipore Gradient A10) deionized water. Approximately 6 mL of each sample was passed through a Maxi-Clean C18 SPE syringe filter. The first 2 mL was discarded, and the remaining 4 mL was collected from the SPE-treated sample for analysis by using the SFA PAHBAH method.

METHODS

IC-PAD. The chromatographic system used for IC-PAD analysis of carbohydrates consists of a Dionex ICS5000 ion chromatograph with Chromeleon software, a DP-5 gradient pump, an AS-1 autosampler, a Dionex CarboPac PA-1, 4 × 250 mm, column, and a Dionex CarboPac PA-1, 4 × 50 mm, guard column. The mobile phase was prepared by adding 50% w/w NaOH solution (Fisher) to 18.2 MΩ water, degassed with helium, to yield a final concentration of 150 mM NaOH. The run was isocratic at 1 mL/min. The detector was a Dionex DC-5

electrochemical detector with a gold working electrode and a pH-Ag/AgCl reference electrode. The waveform was E_1 = 0.10 V, 0−0.4 s; E_2= −2.00 V, 0.4−0.42 s; E_3 = 0.60 V, 0.43 s; and E_4 = −0.10 V, 0.44−0.50 s; where E_1 is the detection potential and the remaining potentials clean and restore the electrode for subsequent detection. The injection volume was 10 μL, and the run time was 15 min/sample.

SFA. The SFA system used for analysis of carbohydrates was the Astoria-Pacific 2+2 Analyzer running Astoria Test Method A250.[7] All reagents used in Astoria-Pacific SFA method A250 were of ACS grade or better. Samples of tobacco extract were aspirated sequentially and transported to the total reducing sugars analytical cartridge with a peristaltic pump, where they were segmented with air to minimize sample-to-sample interaction. Following segmentation, reagents were introduced to the sample flow sequentially. Flow for all reagents and the analyte was maintained by the peristaltic pump turning at a constant rate. The flow rate for individual reagents was controlled by the inner diameter of the pump tubing chosen for each reagent. After the reagents and analytes were mixed, the reagent/analyte stream was passed through a heating block at 90 °C, initiating the reaction with PAHBAH. After exiting the heating block, the air bubbles were removed, allowing the reaction products to pass through the optics for detection.

QUANTITATIVE ANALYSIS

Each leaf or product sample was extracted twice, and each extract was analyzed twice with SFA and three times with IC-PAD. The low number of extracts per sample (n = 2) was determined by the need for an adequate tobacco sample population to observe trends in the results. Calibration standards for sucrose, glucose, and fructose were of ACS grade and purchased from Sigma-Aldrich. Fructose and glucose standards for SFA were prepared with chloroform as a preservative and were stable for 30 days. Standards for IC-PAD were prepared and used on the same day.

IC-PAD. All standards, samples, and blanks were subjected to the same cleanup procedures as described under Sample Preparation. Linear ranges of 3.5−140 mg/L were obtained for glucose, fructose, and sucrose.

SFA. Determination of the analyte concentrations was based on the comparison of sample peak height (signal intensity) to known calibration standards. This method has a linear range of 20−400 mg/L.

RESULTS

Recovery Study: Paper Filtration versus Solid Phase Extraction. Recovery studies were performed to determine the effect of SPE C18E/SCX SPE during sample preparation. Studies were undertaken using neat solutions of the sugars in water across the linear range of the SFA PAHBAH method, with simple paper filtration used as a baseline for comparison. The results show minimal effect of C18E/SCX SPE on the concentration of sugars (Table 1). Recovery studies were also performed on tobacco samples using extraction solutions spiked with sucrose at two levels. These results are shown in Table 2 and show comparable recoveries between the paper-filtered and C18E/SCX SPE-treated tobacco extracts. The recovery results for spiked tobacco extracts treated with C18E/SCX SPE in Table 2 are also comparable to the results for C18E/SCX SPE-treated standards shown in Table 1.

SFA and IC-PAD: Total Reducing Sugars (as Glucose, Fructose, and Sucrose). Figure 1 shows the results from SFA PAHBAH and IC-PAD experiments for cured leaf tobaccos obtained from the USDA. The SFA results were obtained from 1% acetic acid extracts using the Astoria-Pacific method A250[7] for total reducing sugars as described above. The IC-PAD results were obtained from aqueous extracts treated with C18E/SCX SPE and reported as the sum of the glucose, fructose, and sucrose concentrations. Overall, the levels

Table 2. Spiked Recovery Experiments Performed on Three Tobacco Leaf Samples Using the SFA PAHBAH Method[a]

tobacco type	extract treatment	sucrose spike (mg)	% recovery av	% recovery SD
Burley (C2L)	Whatman 114 V paper filter	8.74	99.8	1.2
		18.42	101.1	0.8
	C18E/SCX SPE	8.74	101.0	1.2
		18.42	101.8	0.4
Eastern Carolina (M4F)	Whatman 114 V paper filter	8.74	97.3	2.3
		18.42	99.7	1.9
	C18E/SCX SPE	8.74	100.1	4.4
		18.42	98.7	2.0
Eastern Carolina (M5KM)	Whatman 114 V paper filter	8.74	96.1	1.6
		18.42	98.5	0.9
	C18E/SCX SPE	8.74	97.7	1.3
		18.42	98.1	0.9

[a]Each leaf sample was extracted in duplicate using purified water solutions spiked with sucrose at two levels. Sample cleanup procedures for each spiked extract involved either filter paper or the two-step C18E/SCX SPE process. Each extract was analyzed twice.

determined from both analytical methods are consistent with levels reported in the literature for flue-cured and air-cured tobaccos.[1] Additionally, the differences observed between the IC-PAD and SFA PAHBAH results are consistent between samples. This same trend is observed for tobacco products and can be seen in Figure 2. With the exception of the chewing tobaccos shown in Figure 2, the results reported using SFA PAHBAH for total reducing sugars are 18−78% higher than results reported using IC-PAD. Because the SFA PAHBAH and IC-PAD methods use different sample preparation techniques, it was clear that the effect of sample preparation on the results needed to be explored before any conclusions could be drawn about the differences in analytical techniques.

The simplicity of the SFA PAHBAH method, including sample preparation, makes this approach appealing to investigators who are concerned about sample throughput for routine analysis. The SFA instrument is capable of analyzing up to 72 samples per hour. In addition, the instrument manufacturer describes their SFA application of the PAHBAH method as largely free of interferences.[7]

On the other hand, the IC-PAD method is significantly more labor intensive. The original IC-PAD method used in this laboratory employed C18 SPE syringe cartridges for treatment of aqueous extracts and 15 min chromatographic separations to complete the analysis.[3] Subsequent efforts to apply this preparation technique to samples analyzed with the Dionex ICS5000 met with poor peak area reproducibility and significant statistical variations in experimental results. This problem appeared to be related to the performance of the detector and was observed only when tobacco extracts were analyzed. On the basis of this observation, it was surmised that contaminants present in the tobacco extracts were affecting the detector and modifications to the sample preparation technique would be needed. To address this problem, C18 and strong cation exchange resins were used in series for sample preparation. This combination of resins was advantageous because neither stationary phase binds sugars. The overall impact on the sample preparation procedure was that, rather

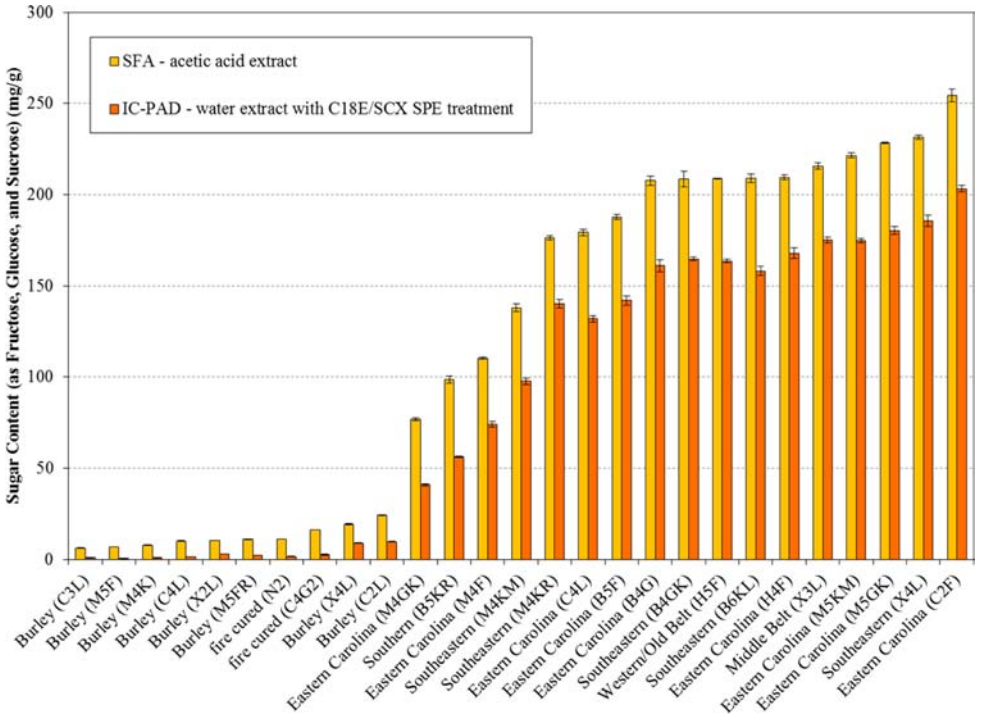

Figure 1. Comparison of sugar content from 27 cured leaf tobacco samples (n = 2). SFA experiments for total reducing sugars were run on 1% acetic acid extracts processed with paper filtration (yellow). IC-PAD experiments were run on water extracts treated with C18E/SCX SPE (orange). The error bars at the top of each column indicate the standard deviation of the measurement.

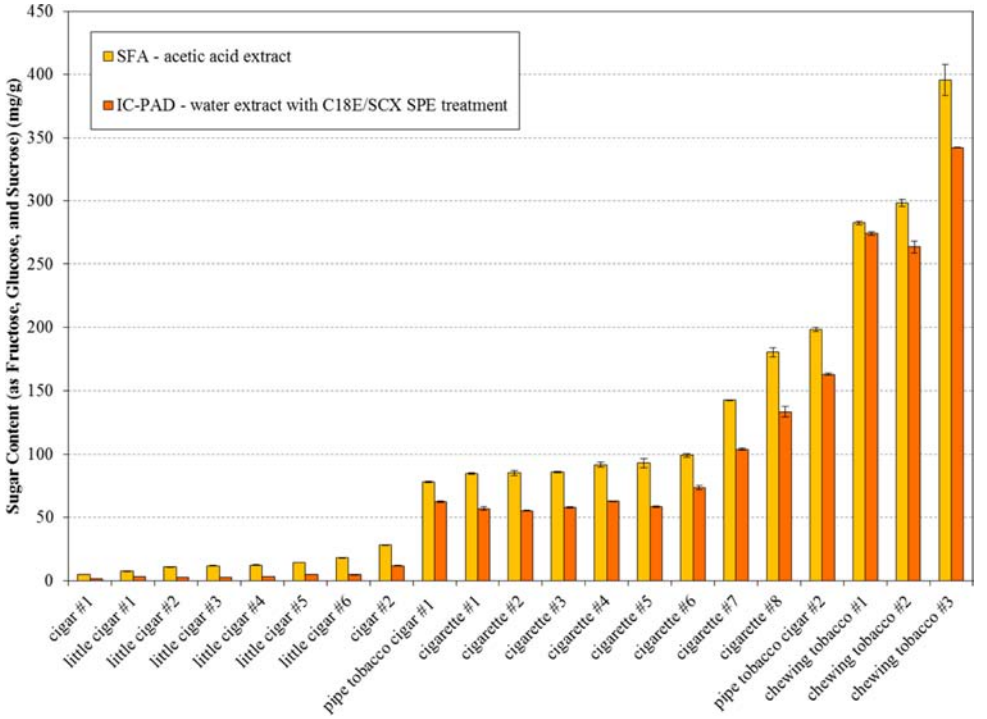

Figure 2. Comparison of sugar content from 21 tobacco products (n = 2). SFA experiments for total reducing sugars were run on 1% acetic acid extracts processed with paper filtration (yellow). IC-PAD experiments were run on water extracts treated with C18E/SCX SPE (orange). The error bars at the top of each column indicate the standard deviation of the measurement.

than making significant alterations, sample cleanup was simply augmented with a second SPE step. As was demonstrated in Tables 1 and 2, the two-step C18E/SCX SPE did not significantly alter the recovery of the reducing sugars relative to samples treated with paper filtration.

SFA: Interfering Chemical Species. Chlorogenic acid was described previously as a source of chemical interference in the SFA PAHBAH method.[4] Preliminary results from this laboratory for the HPLC analysis of 49 air-cured and flue-cured tobacco samples found chlorogenic acid at average

concentrations of 0.04 and 1.4% by weight, respectively. These concentrations are approximately an order of magnitude lower than corresponding levels of total reducing sugars in the same samples (as determined by SFA). SFA PAHBAH experiments showed that chlorogenic acid elicited a response that was approximately 38% of the response of fructose and 47% of the response of glucose (Table 3). This is approximately 4 times higher than previously reported.[4] For a flue-cured tobacco sample containing 14% total reducing sugars as determined by SFA PAHBAH, the interference from chlorogenic acid could be as high as 4% of the total response for reducing sugar. Furthermore, because chlorogenic acid is not electrochemically active under the conditions of the IC-PAD method, it cannot interfere with the chromatographic analysis. Given that the least significant differences between the SFA PAHBAH paper filtration results and IC-PAD results are in the 20% range (for flue-cured tobacco leaf), it is likely that chlorogenic acid is only one of several interfering species affecting the results of the SFA PAHBAH method.

Table 3. Comparison of SFA PAHBAH Results for Fructose, Glucose, Glucosamine, Mannosamine, and Chlorogenic Acid Showing Relative Responses to Fructose at 200 ppm

		SFA result	
analyte	prepared concn (ppm)	peak height	ratio to fructose
fructose	200	0.2750	1.00
glucose	200	0.2255	0.82
glucosamine	200	0.1933	0.70
mannosamine	200	0.1858	0.68
chlorogenic acid	200	0.1049	0.38

Amino sugars such as glucosamine have been reported to comprise as much as 1.5−2.0% of the weight of dried flue-cured tobacco.[11] Additionally, the presence of amino sugars in leaf tobacco samples has been verified by IC-PAD, as shown in Figure 3, for aqueous extracts of air- and flue-cured tobaccos that were processed through C18 syringe SPE cartridges. Glucosamine has been shown to react with PAHBAH,[6] and Table 3 shows that glucosamine has a response that is approximately 70% of glucose and 85% of fructose. Given the abundance of amino sugars in flue-cured tobacco as reported in the literature,[11] the impact of the glucosamine interference on the total response from the PAHBAH reaction could be significant. However, we want to avoid the implication that chlorogenic acid and the sugar amines are the only potential interferences of any significance. Our interest in them was a result of readily available data in the literature on chemical reactivity with PAHBAH and abundance in tobacco leaf. Although more work is required to identify all interfering species in tobacco extracts relevant to the PAHBAH reaction, it is clear that the aggregate effect of these interferences is an overestimation of the carbohydrate concentration relative to IC-PAD.

To study the differences between the results from the IC-PAD and SFA PAHBAH experiments, several manipulations of the sample preparation were used. Tobacco samples extracted with 1% acetic acid could not be analyzed using the IC-PAD method due to the interference of the acetate ion with the retention of the sugars on the IC column. To make a complete comparison between the IC-PAD and SFA PAHBAH results, sample extracts had to be prepared in both water and 1% acetic acid. Water extracts were prepared for analysis using both paper filtration and C18E/SCX SPE procedures, whereas 1% acetic acid extracts were prepared using only paper filtration. The results of these studies are shown in Figure 4 for tobacco leaf samples and in Figure 5 for tobacco products.

The results for tobacco leaf shown in Figure 4 reveal several important trends. Differences were observed between the SFA PAHBAH results from paper-filtered samples extracted with water and paper-filtered samples extracted with 1% acetic acid. Although these differences appear to be relatively small, they were found to be statistically significant using the paired *t* test at a 95% confidence interval. Results for samples treated with C18E/SCX SPE from both the SFA PAHBAH and IC-PAD analyses are significantly lower than the results from the paper-filtered SFA PAHBAH experiments using the same paired *t* test criteria. Additionally, the relatively small differences between the SFA PAHBAH C18E/SCX SPE and IC-PAD results, as shown in Figure 4, were found to be statistically significant.

Although the same general trends for the tobacco leaf samples were observed for the tobacco products shown in

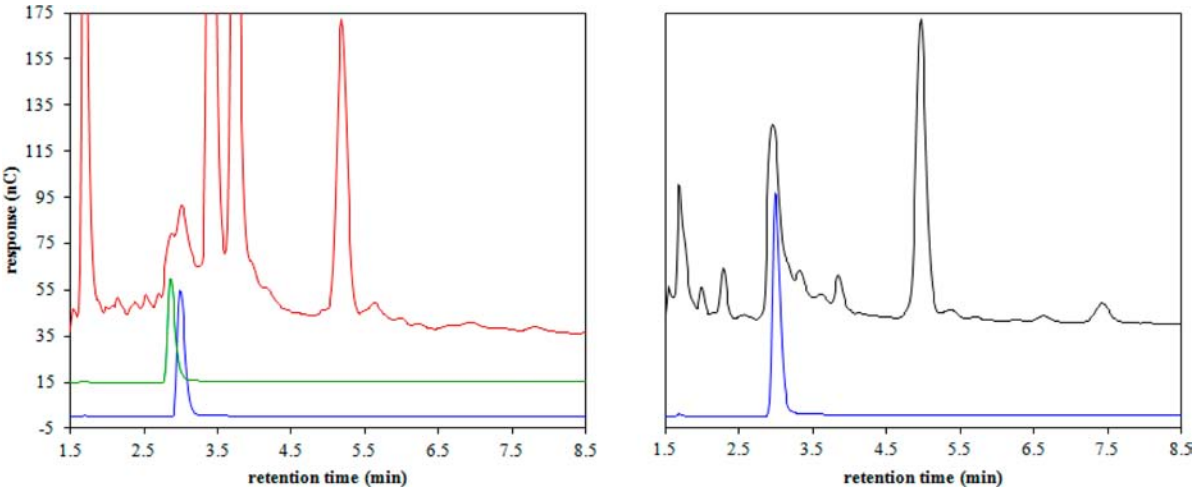

Figure 3. Chromatograms from IC-PAD analysis of tobacco leaf material. The plots show flue-cured (red) and air-cured (black) overlaid with glucosamine (blue) and mannosamine (green). Glucose and fructose elute at 3.5 and 3.8 min, respectively. These chromatograms were generated from analytes that were processed through Maxi-Clean C18 cartridges only.

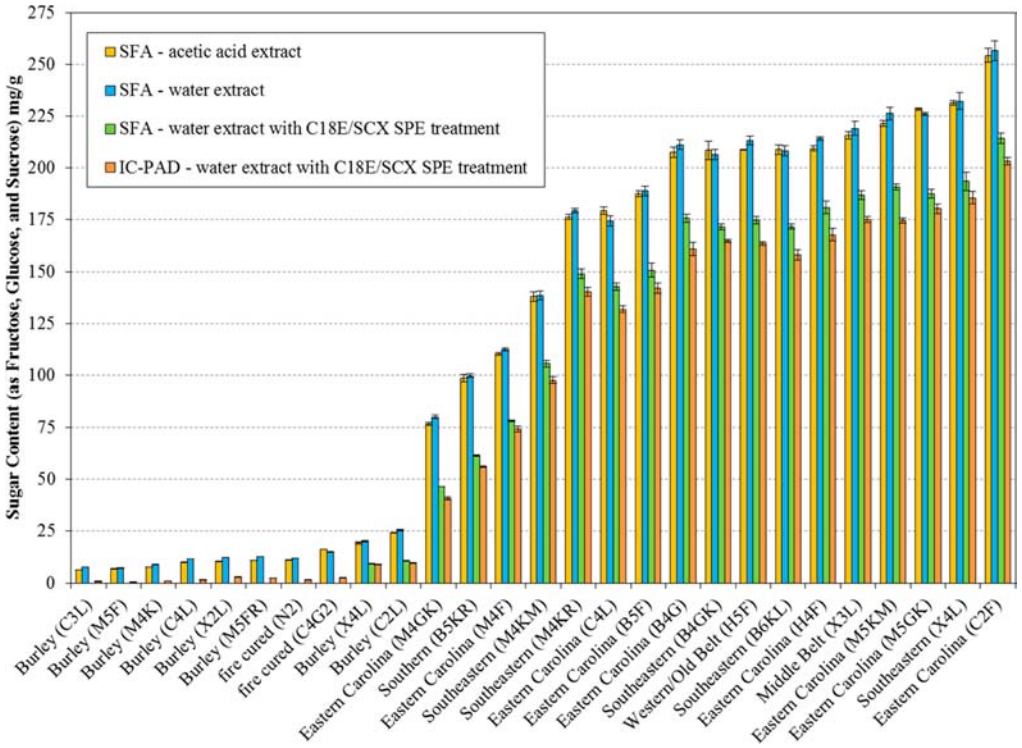

Figure 4. Comparison of results from segmented flow analysis and ion chromatography with pulsed amperometric detection for 27 tobacco leaf samples (n = 2). Sample preparation techniques included extraction in 1% acetic acid followed by filtration with Whatman 114 V filter paper, extraction with water followed by filtration with Whatman 114 V filter paper, and water extraction followed by C18E/SCX SPE cleanup. Samples processed with paper filtration were analyzed using the SFA PAHBAH method (yellow and blue). C18E/SCX SPE-treated samples were analyzed using both SFA PAHBAH and IC-PAD (green and orange). It is important to note that some of the SFA PAHBAH results for air-cured tobaccos were below the linear range of the method and could not be reported for the SPE-treated samples. The error bars at the top of each column indicate the standard deviation of the measurement.

Figure 5, the results shown for chewing tobacco are inconsistent with the rest of the samples. Whereas a significant difference was observed when the SFA PAHBAH results from paper-filtered water extracts were compared to SFA PAHBAH results from C18E/SCX SPE samples, the chewing tobaccos shown in Figure 5 appear to show little difference. A possible explanation for these results might be the presence of maltose, which is often added to chewing tobacco casing sauce.[12] Maltose, which is not retained by any of the SPE cartridges used in this study, is reported to be slightly more reactive with PAHBAH than is fructose.[6] It is also well resolved from the other sugars in the IC-PAD experiments so it would not have affected the determination of fructose, glucose, and sucrose.

Cigars and air-cured tobacco products, which typically contain low amounts of fructose, glucose, and sucrose, showed the most substantial overall differences in sugar content found by paper filtration versus C18E/SCX SPE, as seen in Figures 4 and 5. The differences in the results for air-cured leaf samples are so large that virtually none of the sugar content reported in the SFA-analyzed paper-filtered samples appears to be related to fructose, glucose, or sucrose. The pattern is largely repeated in the cigars shown in Figure 5, although several of these products might have added sugar or a tobacco blend that includes flue-cured leaf. It is important to note that some of the SFA PAHBAH results for cigars and air-cured tobaccos were below the linear range of the method and could not be reported for the C18E/SCX extracts shown in Figures 4 and 5.

IC-PAD Analysis of Individual Sugars. The results for individual sugars from IC-PAD experiments are shown in Figures 6 and 7 for tobacco leaf and tobacco products, respectively. A review of these results illustrates that, in all but a few samples in which significant amounts of sugars are present, the dominant reducing sugar is fructose. Prominent exceptions to this trend are found in the Eastern Carolina (C2F) leaf sample in Figure 6 and in chewing tobaccos 1 and 2 shown in Figure 7, in which glucose is the predominant reducing sugar. It should also be noted that many of the products shown in Figure 7 contain substantial amounts of sucrose. These trends in the distribution of individual sugars in tobacco products are heavily influenced by sugars that are added during manufacturing. Sugars such as fructose, glucose, sucrose, and maltose are added to tobacco products at various levels depending on product type.

The predominance of fructose in Figures 6 and 7 is important to the discussion of the results shown in Figures 4 and 5. Focusing on the C18E/SCX SPE results in Figures 4 and 5, the SFA PAHBAH results are generally higher than the IC-PAD results. This trend should be considered in the context of the difference in response of glucose and fructose on the SFA, where glucose has a concentration response that is approximately 80% of fructose (see Table 3). It is because of the difference in concentration response that the SFA instrument manufacturer requires a 50:50 mixture of glucose and fructose in the calibrant solutions. Furthermore, the mixed standard approach acknowledges that tobacco samples generally contain a mixture of the two reducing sugars.

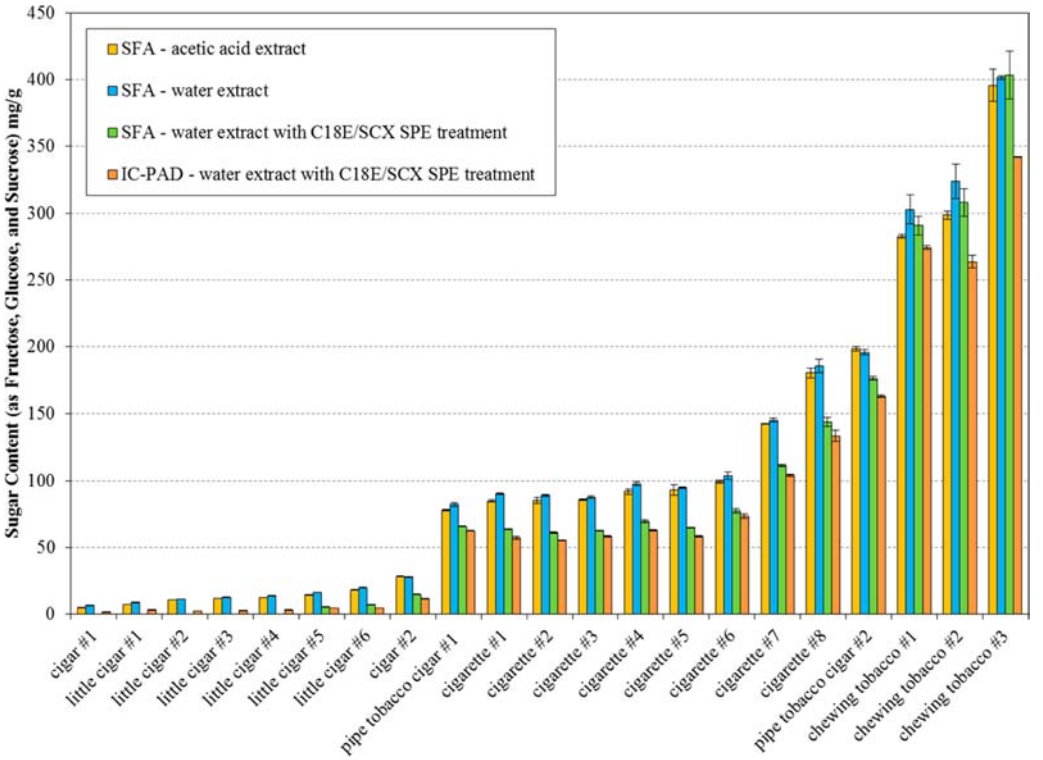

Figure 5. Comparison of results from segmented flow analysis and ion chromatography with pulsed amperometric detection for 21 tobacco products (n = 2). Sample preparation techniques included extraction in 1% acetic acid followed by filtration with Whatman 114 V filter paper, extraction with water followed by filtration with Whatman 114 V filter paper, and water extraction followed by C18E/SCX SPE cleanup. Samples processed with paper filtration were analyzed using the SFA PAHBAH method (yellow and blue). C18E/SCX SPE-treated samples were analyzed using both SFA PAHBAH and IC-PAD (green and orange). It is important to note that some of the SFA PAHBAH results for cigars were below the linear range of the method and could not be reported for the SPE-treated samples. The error bars at the top of each column indicate the standard deviation of the measurement.

DISCUSSION

Given the reported specificity of the SFA PAHBAH method for total reducing sugars,[4] it might be expected that the result for any given sample would be substantially free of interferences. However, the potential for contribution of interferences to the results of the SFA PAHBAH method could be more significant than previously reported. In fact, the results presented in Figures 4 and 5 demonstrate that, in some cases, the contribution of nonreducing sugar reactants is larger than the contribution from the sugars themselves.

An important conclusion to be drawn from the results in Figures 4 and 5 is that, in general, the most significant differences in the data from SFA and IC-PAD are a result of the sample cleanup procedures. Given the affinities of the interfering species discussed previously for the sorbents used in the SPE cleanup, some convergence of the SFA PAHBAH and IC-PAD results after C18E/SCX SPE would be expected. Chlorogenic acid is retained on the C18 stationary phase and, under the conditions of the sample preparation, would almost certainly be removed from the sample matrix. Likewise, sugar amines have a high degree of affinity for cation exchange resin and appear to be removed from the sample matrix by the SCX cartridge. Both mannosamine and glucosamine were observed in IC-PAD chromatography when only the C18 syringe cartridge was used for sample cleanup (Figure 3) and absent from chromatograms when the C18E/SCX two-step cleanup was used. Although more work is required to identify the interfering species present in tobacco, the results shown here demonstrate that the C18E/SCX SPE process is effective at removing interferences in the PAHBAH reaction from the sample matrix.

With regard to the apparent differences observed in the C18E/SCX SPE results from SFA PAHBAH and IC-PAD shown in Figures 4 and 5, it is possible that at least part of this trend is due to the aforementioned difference in the responses of glucose and fructose on the SFA. Because the SFA PAHBAH method uses a 50:50 mixture of glucose and fructose in the calibrant solutions, it is reasonable to assume that results for samples containing fructose and glucose at ratios favoring one or the other sugar will be artificially high or low depending on the predominant sugar. The results shown in Figures 6 and 7 demonstrate that in all but a few samples the predominant sugar is fructose. This could contribute to the somewhat higher sugar content observed in the SFA PAHBAH results from C18E/SCX SPE relative to the IC-PAD results for many of the samples. However, some results are inconsistent, especially for the chewing tobaccos in Figure 5. There are several samples that show nearly equivalent levels of fructose and glucose in Figures 6 and 7, whereas the trend showing higher sugar content from SFA PAHBAH runs of C18E/SCX SPE samples persists in Figures 4 and 5.

The data presented here demonstrate that, overall, the two methods are consistent with respect to trends in carbohydrate content. It is clear that relative differences in SFA results between samples are generally paralleled by the results from the IC-PAD experiments. Ultimately, each data set can provide similar conclusions regarding relative carbohydrate contents of tobacco leaves and products. However, comparison of absolute

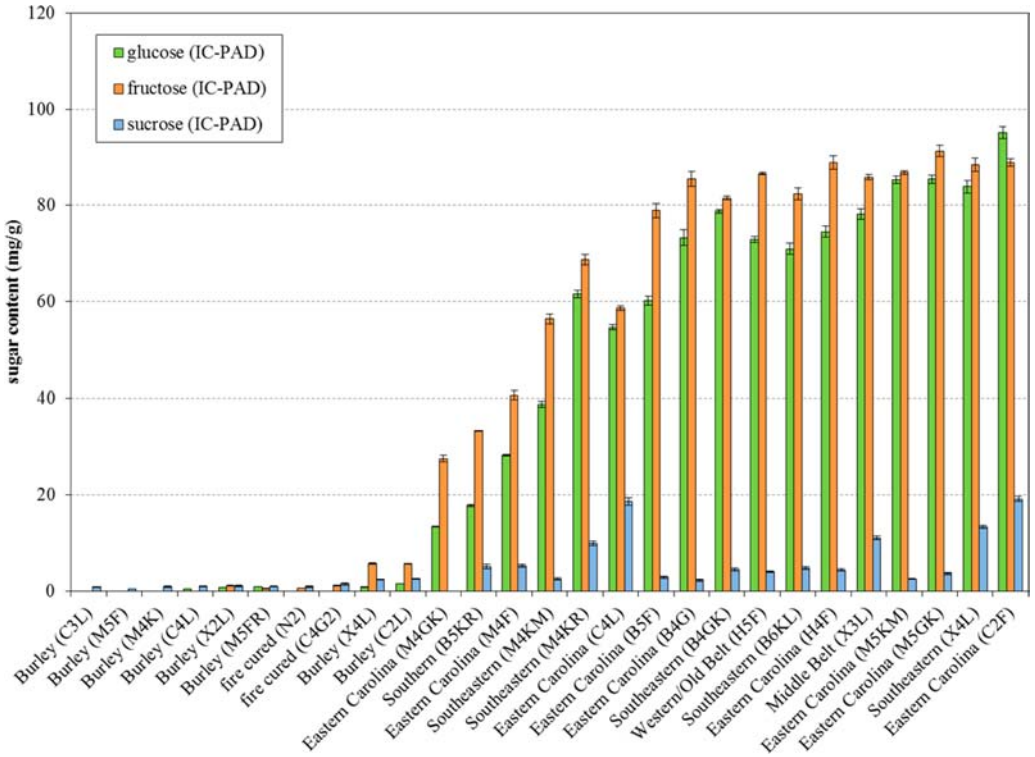

Figure 6. Results of IC-PAD analysis for 27 cured tobacco leaf samples (n = 2). Water extracts of leaf tobacco samples were prepared for analysis using the two-step C18E/SCX SPE cleanup. The results are reported for the individual sugars glucose (green), fructose (orange), and sucrose (blue). The error bars at the top of each column indicate the standard deviation of the measurement.

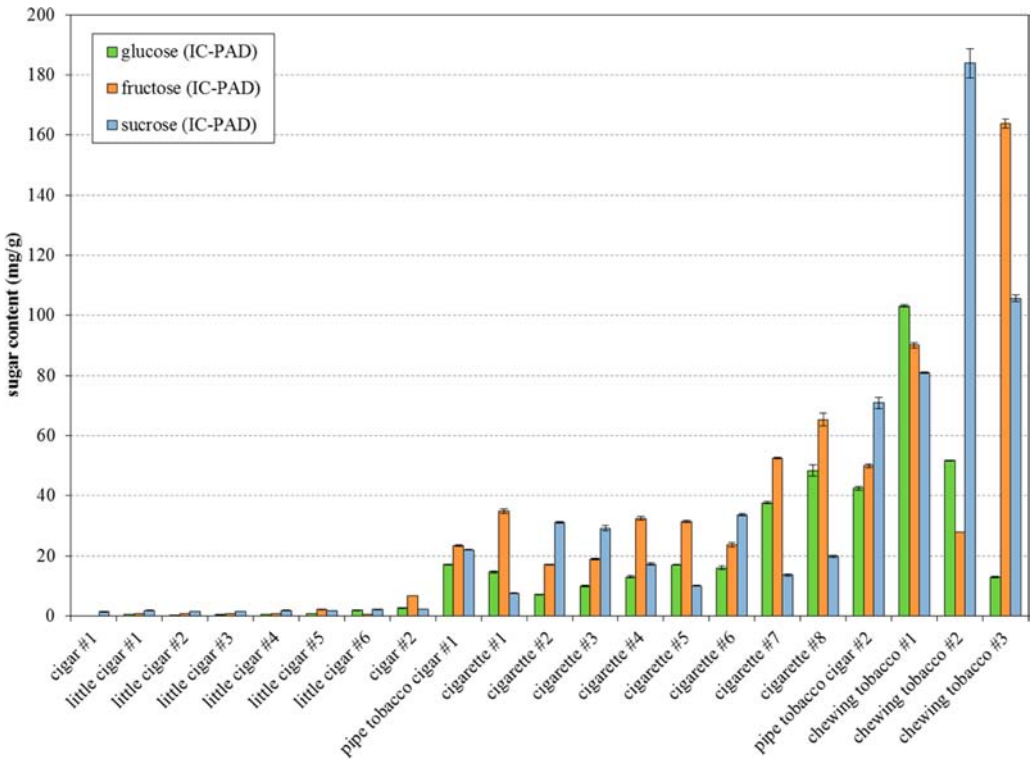

Figure 7. Results of IC-PAD analysis for 21 tobacco products (n = 2). Water extracts of leaf tobacco products were prepared for analysis using the two-step C18E/SCX SPE cleanup. The results are reported for the individual sugars glucose (green), fructose (orange), and sucrose (blue). Results are grouped according to product classification. The error bars at the top of each column indicate the standard deviation of the measurement.

total reducing sugar results across the two methods is complicated by the relative selectivities of the analytical techniques. Whereas the IC-PAD method relies on both the resolving power of the ion-exchange resin and the specificity of

the detector to differentiate sugars from potential interfering species, the SFA PAHBAH method relies solely on the selectivity of the chemical reaction that generates the detectable derivative. Using C18E/SCX SPE to remove many of the inferencesinterferences, the data demonstrate convergence in the results of both analytical methods. The existence of unresolved chemical interferences in the SFA method may explain differences that persist. Furthermore, the contribution of ratio mismatches between fructose and glucose in the calibrants and samples cannot be disregarded as a contributor to the differences in results from the IC-PAD and SFA PAHBAH methods. Developing a complete understanding of chemical interferences in the PAHBAH reaction and fructose/glucose ratio mismatches will be the subject of a future publication.

Although the C18E/SCX SPE steps appear to eliminate most of the chemical interferences in the acid hydrazide reaction with reducing sugars in tobacco, using SPE to replace paper filtration in the SFA PAHBAH method will significantly increase the cost of the analysis. Furthermore, the need to resolve chemical interferences must be considered in light of the fact that the SFA PAHBAH and IC-PAD results are consistent with respect to trends in carbohydrate content. Unless there is a need to compare the SFA PAHBAH results to results from another analytical technique, modification of the method to accommodate SPE sample cleanup may be unnecessary.

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Notes

Any mention of certain commercial equipment, materials, or methodologies in this paper is intended to adequately specify the experimental conditions and procedures. Such identification does not imply a recommendation or endorsement.

The authors declare no competing financial interest.

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ABBREVIATIONS USED

SFA, segmented flow analysis; PAHBAH, *p*-hydroxybenzoic acid hydrazide; IC-PAD, ion chromatography with pulsed amperometric detection; SPE, solid phase extraction; SCX, strong cation exchange.

REFERENCES

(1) Hoffman, D.; Hoffman, I. Chemistry and toxicology. In *Smoking and Tobacco Control Monograph No. 9: Cigars: Health Effects and Trends*; Amacher, R. H., Burns, D. M., Hoffman, D., Cummings, K. M., Eds.; National Cancer Institute, U.S. National Institutes of Health: Rockville, MD, 1998; pp 55−104.

(2) Zook, C. M.; Patel, P. M.; LaCourse, W. R.; Ralapati, S. Characterization of tobacco products by high-performance anion exchange chromatography-pulsed amperometric detection. *J. Agric. Food Chem.* **1996**, *44*, 1773−1779.

(3) Clarke, M. B.; Bezabeh, D. Z.; Howard, C. T. Determination of carbohydrates in tobacco products by liquid chromatography-mass spectrometry/mass spectrometry: a comparison with ion chromatography and application to product discrimination. *J. Agric. Food Chem.* **2006**, *54*, 1975−1981.

(4) Davis, R. E. A combined automated procedure for the determination of reducing sugars and nicotine alkaloids in tobacco products using a new reducing sugar method. *Tobacco Sci.* **1976**, *XX*, 139−144.

(5) Fingerhut, B. Automated method for serum glucose with *p*-hydroxybenzoic acid hydrazide. *Clin. Chem.* **1973**, *19*, 1022−1026.

(6) Lever, M. A new reaction for colorimetric determination of carbohydrates. *Anal. Biochem.* **1972**, *47*, 273−279.

(7) *Total Reducing Sugars Method A250*, revision D; Astoria-Pacific International: Clackamas, OR, 2003.

(8) Lever, M. Colorimetric and fluorometric carbohydrate determination with *p*-hydroxybenzoic acid hydrazide. *Biochem. Med.* **1973**, *7*, 274−281.

(9) Lever, M.; Powell, J. C.; Killip, M.; Small, C. W. A comparison of 4-hydroxybenzoic acid hydrazide (PAHBAH) with other reagents for the determination of glucose. *J. Lab. Clin. Med.* **1973**, *82*, 649−655.

(10) *Analysis of Carbohydrates by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD)*; Technical Note 20; Thermo Scientific: Sunnyvale, CA, 2004; pp 1−13.

(11) Leffingwell, J. C. Basic chemical constituents of tobacco leaf and differences among tobacco types. In *Tobacco Production, Chemistry and Technology*; Davis, D. L., Nielsen, M. T., Eds.; Blackwell: Ames, IA, 1999; pp 265−284.

(12) Hsu, S. C.; Pollack, R. L.; Hsu, A. F.; Going, R. E. Sugars present in tobacco extracts. *J. Am. Dental Assoc.* **1980**, *101*, 915−918.