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

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INTRODUCTION

As major components of tobacco, carbohydrates are valuable for product characterization. Tobacco products in the U.S. 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.

The purpose of this work is to compare the results for two methods used by the TTB Tobacco Laboratory for routine analysis of common carbohydrates in tobacco. Furthermore, the results will be used to examine the classification of different tobacco products using percent total carbohydrates. These methods were applied to cigarettes, cigars, chewing tobacco, snuff and cured tobacco leaf. The feasibility of using carbohydrates for product differentiation is discussed.

SAMPLE PREPARATION

Leaf tobacco samples were obtained from the USDA Cotton & Tobacco Program (Raleigh, NC). Samples of popular tobacco products were obtained from local stores. Only widely known products were used to establish a baseline for product comparison.

Cigar, cigarette and leaf tobaccos were prepared as follows. 3 to 5 grams of each sample was placed in a screen basket. The baskets were placed in a 90ºC convection oven for 1 hour. The dried samples were ground immediately after drying using a Wiley Mill Grinder with a 20 mesh screen. Chewing tobaccos, which are heavily laden with casing sauces, could not be ground with the Wiley Mill. Instead, these products were commuted to 1-2 mm with a razor. Snuff was used as received. Cigarettes and cigars were separated into wrapper and filler materials before grinding. Twenty cigarettes per pack were ground. Dried, ground samples were placed in air-tight containers and stored in a cold room.

Extraction for segmented flow analysis

100 mg of each dried and ground tobacco sample was accurately weighed into 125 ml Erlenmeyer flasks. The samples were extracted with 100 ml of 1% acetic acid solution, which was added to each flask with a Class A volumetric glass pipette. The flasks were stoppered and placed on an orbital shaker at 150 rpm for 30 minutes. Following agitation, extracts were filtered through Schleicher and Schuell #560 pleated filter paper. Filtered extracts were placed in 4 ml autosampler vials for analysis or placed in the refrigerator. Refrigerated samples are stable for 48 hours.

Extraction for analysis using ion chromatography with pulsed amperometric detection (IC-PAD)

Ground tobacco samples were accurately weighed into glass bottles. Approximately 200 mg of tobacco was extracted with 50 mL of 18.2 MΩ water which was added using a Class A volumetric glass pipette. The samples were shaken on a Burrel Wrist Action Shaker™ for 15 minutes and filtered directly into auto sampler vials using an Alltech 600mg C_{18} Maxi-Clean Cartridge and a Pall Acrodisc Nylon 0.2 µm filter in series. The first few milliliters were discarded. Lactose monohydrate was added at 50 mg/L to the extraction solution before samples and standards were prepared. This ensured that all standards contained the same concentration of internal standard as the tobacco extracts.

METHODS

IC-PAD

The IC-PAD method for carbohydrate analysis has been used by the TTB for many years (1). The chromatographic system used for IC-PAD analysis of carbohydrates consists of a Dionex ICS2500 Ion Chromatograph with Chromeleon Software, a GP50 Gradient Pump, an AS50 Auto sampler with a 20 µL loop, a Dionex CarboPac™ PA-1, 4X250mm column and a Dionex CarboPac™ PA-1, 4X50mm guard column. The mobile phase was deionized water, degassed with He and a 50%w/w NaOH solution was added to yield a final concentration of 150mM NaOH. The run was isocratic at 1 mL/min. The detector was a Dionex ED50 Electrochemical Detector with Ag/AgCl reference electrode and a gold working electrode. The potential waveform was: 0-0.4 sec, E_d = 0.10V, 0.4-0.42 sec, E = -2.00V, 0.43 sec, E = 0.60V, 0.44-0.50 sec, E = -0.10V. The injection volume was 10 µL and the run time was 15 min/sample.

Segmented flow analysis

Segmented flow analysis is routinely used for carbohydrate analysis in the TTB Tobacco Laboratory. The Astoria 2+2 Analyzer is capable of running four distinct tobacco tests simultaneously. The test method for total reducing sugars (Astoria test method A250-A00) uses invertase to hydrolyse sucrose to form the reducing monosaccharides, fructose and glucose. Reducing sugars react with p-hydroxybenzoic acid hydrazide (PAHBAH) in an alkaline media to form a colored complex that can be measured at 410 nm. SFA is used to automate the reaction described above so it can be applied efficiently to a large group of samples. Samples of tobacco extract are aspirated sequentially and transported to the total reducing sugars analytical cartridge with a peristaltic pump. As soon as sample enters the analytical cartridge, it is segmented with air to minimize sample-to-sample interaction. Following segmentation, reagents are introduced to the sample flow sequentially. Flow for all reagents and the analyte is maintained by the peristaltic pump, which turns at a constant rate. Flow rate for individual reagents is controlled by the inner diameter of the pump tubing chosen for each reagent. Once all of the reagents and analyte are mixed, the reagent/analyte stream is passed through a heating block at 90ºC, where the sugars react with PAHBAH. Exiting the heating block, the reagent/analyte stream passes through a flow cell, where the air bubble is removed and the analyte passed through the optics for detection.

All reagents used in Astoria-Pacific SFA method A250-A00 for Total Sugars were ACS grade or better.

QUANTITATIVE ANALYSIS

Each leaf and product sample was extracted in duplicate and analyzed in triplicate using SFA and IC-PAD. Calibration standards for sucrose, glucose and fructose were 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

Calibration with an internal standard (lactose monohydrate) 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 clean-up procedures. Linear dynamic ranges of 3.5 to 140 mg/L were obtained for glucose, fructose and sucrose. The limits of quantification (LOQ's) for glucose, fructose, and sucrose were 3.5 mg/L as defined by the lower limit of the calibration curves. The precision of this method has been determined to be 5.3% (RSD).

Segmented flow analysis

Determination of the analyte concentrations is based on the comparison of sample peak height (signal intensity) to known calibration standards. The precision of this method has been determined to be 2.5% (RSD).

RESULTS

Total carbohydates (as glucose, fructose, and sucrose)

Figure 1 shows the results from SFA and IC-PAD for cured leaf tobaccos. The stark contrast in carbohydrate levels for the cured leaves is apparent. The carbohydrate levels observed here are consistent with levels reported in the literature for flue-cured and air-cured tobaccos (2). Comparing these results to the tobacco products in Figure 2, it is clear that, with the exception of one product, there are significant differences between the cigarettes and cigars collected for this study. Considering the tobacco blends used in American cigarettes, which contain a significant amount of flue-cured, and cigars, which are generally made from air-cured, the results shown in Figure 2 would be expected. The anomalous cigar result (red arrow in Figure 2 inset) shows a carbohydrate level that is approximately equal to observed levels for cigarettes. The carbohydrate results the chewing tobacco samples (Figure 2), which contain air-cured tobacco treated with sugar-based casing sauce, are expected for products of this type.

The difference between the results from the SFA and IC-PAD are significant. This difference is due in large part to the relative specificities of the two methods.

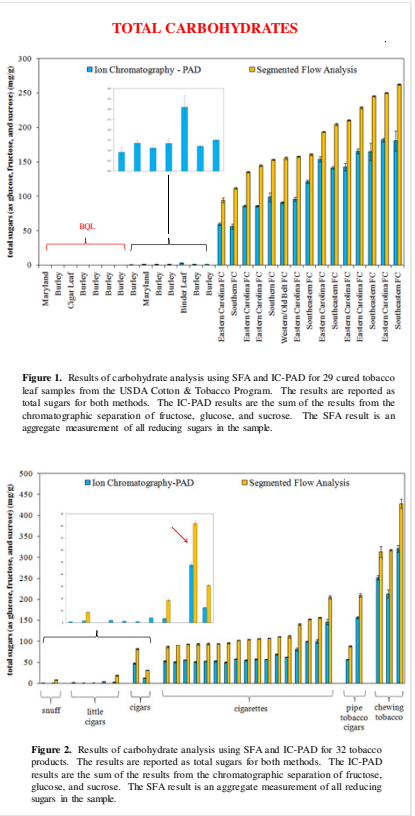

Figure 1. Results of carbohydrate analysis using SFA and IC-PAD for 29 cured tobacco leaf samples from the USDA Cotton & Tobacco Program. The results are reported as total sugars for both methods. The IC-PAD results are the sum of the results from the chromatographic separation of fructose, glucose, and sucrose. The SFA result is an aggregate measurement of all reducing sugars in the sample.

Figure 2. Results of carbohydrate analysis using SFA and IC-PAD for 32 tobacco products. The results are reported as total sugars for both methods. The IC-PAD results are the sum of the results from the chromatographic separation of fructose, glucose, and sucrose. The SFA result is an aggregate measurement of all reducing sugars in the sample.

The possibility of coelution with minor sugars has been discussed with regard to the IC-PAD method (3). However, it is likely that, by virtue of the chromatographic separation, the IC-PAD method is less prone to interferences than the SFA method, which relies on the specificity of the PAHBAH reaction. Several potential interferences have been discussed in the literature (4,5) and our investigations have demonstrated that the SFA method is prone to interference from amino sugars and chlorogenic acid, both of which can be found in cured tobacco samples. Work done in the TTB Tobacco Laboratory has shown that chlorogenic acid is present in flue-cured and air-cured tobaccos at average levels of 1.4% and 0.04% respectively. Additionally, amino sugars such as glucosamine have been reported to comprise as much as 1.5 to 2.0% of the weight of dried flue-cured tobacco (6). The reactions of glucosamine, mannosamine, and chlorogenic acid with PAHBAH were confirmed with spiking studies on the SFA. Additionally, the presence of amino sugars in the leaf tobacco samples has been verified by IC-PAD. Examples of this are shown in Figure 5. While it is still unclear how much these interfering species influence the results of the SFA method, it is likely that the impact is an overestimation of the carbohydrate concentration relative to IC-PAD.

Analysis of individual sugars by IC-PAD

It is important to note that the tobacco leaf samples from the USDA contain very little sucrose (Figure 3). This is in contrast to the tobacco products shown in Figure 4, where sucrose is generally a significant component. Although sucrose has been identified as a constituent of flue-cured tobacco leaf (6), our experiments suggest that is, at best, a minor component in the carbohydrate profile of unprocessed leaf samples.

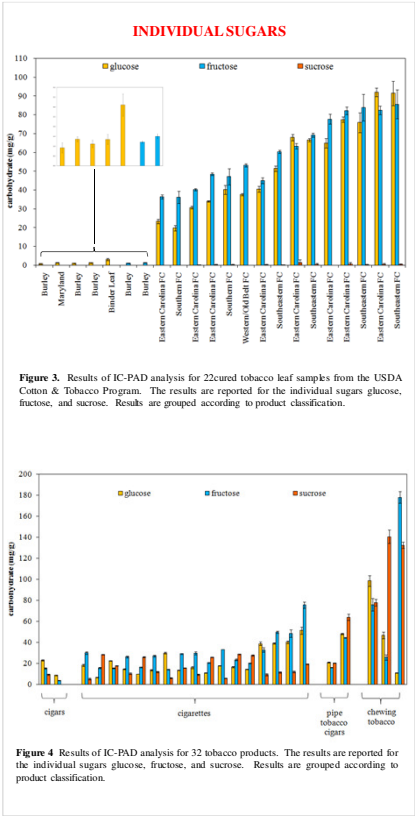

Figure 3. Results of IC-PAD analysis for 22cured tobacco leaf samples from the USDA Cotton & Tobacco Program. The results are reported for the individual sugars glucose, fructose, and sucrose. Results are grouped according to product classification.

Figure 4 Results of IC-PAD analysis for 32 tobacco products. The results are reported for the individual sugars glucose, fructose, and sucrose. Results are grouped according to product classification.

One interesting feature of the results in Figure 3 is the relationship between the absolute concentration of each sugar and the ratio between glucose and fructose. It appears that fructose is the predominant sugar at lower aggregate levels of carbohydrates. As the aggregate level increases, the ratio of fructose to glucose approaches 1.

This is contrasted by the results from the tobacco products in Figure 4, where the individual sugar levels will depend on two factors. The first is the blend used in the tobacco fill and the second involves the use of casing

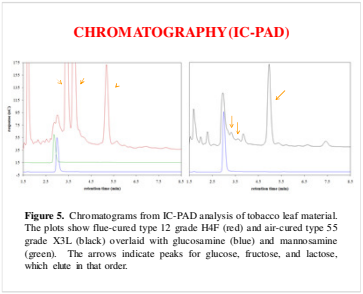

Figure 5. Chromatograms from IC-PAD analysis of tobacco leaf material. The plots show flue-cured type 12 grade H4F (red) and air-cured type 55 grade X3L (black) overlaid with glucosamine (blue) and mannosamine (green). The arrows indicate peaks for glucose, fructose, and lactose, which elute in that order.

common name	type/ grade	Total Sugars as sucrose, fructose, and glucose IC-PAD mg/g ave	s.d.	Total Sugars as sucrose, fructose, and glucose SFA mg/g ave	s.d.	product	Total Sugars as sucrose, fructose, and glucose IC-PAD mg/g ave	s.d.	Total Sugars as sucrose, fructose, and glucose SFA mg/g ave	s.d.
Maryland	32/C2V	BQL		BQL		Little Cigar 1	1.9	0.2	BQL	
Maryland	32/C2F	1.4	0.1	BQL		Little Cigar 2	1.33	0.05	BQL	
Burley	31/X3L	1.1	0.2	BQL		Little Cigar 3	0.8	0.1	BQL	
Burley	31/M4FR	BQL		BQL		Little Cigar 4	3.97	0.08	BQL	
Burley	31/M4F	1.3	0.3	BQL		Little Cigar 5	3.5	0.4	18.5	0.9
Cigar Leaf	55/X3L	BQL		BQL		Little Cigar 6	BQL		BQL	
Binder Leaf	51/N2	3.1	0.6	BQL		Cigar 1	48	2	82	2
Burley	31/X4L	1.21	0.05	BQL		Cigar 2	12.4	0.4	30.9	0.5
Burley	31/M4K	BQL		BQL		Cigar 3	BQL		BQL	
Burley	31/X2L	BQL		BQL		Cigarette 1	53	2	87	2
Burley	31/C3L	BQL		BQL		Cigarette 2	51	1	91.1	0.3
Burley	31/M5F	1.5	0.1	BQL		Cigarette 3	55.7	0.7	94	1
Burley	31/C4L	BQL		BQL		Cigarette 4	51	1	94	2
Burley	31/M5FR	0.9	0.3	BQL		Cigarette 5	52.3	0.5	94	2
Eastern Carolina	12/H4F	60	2	94	4	Cigarette 6	52	2	95	1
Southern	14/B5KR	56	4	112	2	Cigarette 7	50	1	96	1
Eastern Carolina	12/C4L	86	1	135	1	Cigarette 8	58.4	0.5	102.6	0.8
Eastern Carolina	12/B5F	86	1	144.7	1.2	Cigarette 9	55	2	104.4	0.5
Southern	14/B5KV	99	7	153.2	0.9	Cigarette 10	57.6	0.7	106	1
Western/Old Belt	11a/H5F	91	1	155	2	Cigarette 11	57.1	0.6	107.6	0.7
Eastern Carolina	12/M4F	96	3	157.7	0.7	Cigarette 12	69	2	110.8	0.7
Southeastern	13/M4KR	121	2	161	1	Cigarette 13	62.1	0.5	112	3
Eastern Carolina	12/M5F	154	4	193.6	0.8	Cigarette 14	81	4	141	2
Southeastern	13/M4KM	141	2	205	2	Cigarette 15	100	1	152.8	0.9
Eastern Carolina	12/B4G	143	5	210	1	Cigarette 16	101	5	157	2
Eastern Carolina	12/M5KM	166	3	229	1	Cigarette 17	146	6	205	3
Southeastern	13/B6KL	165	13	245.2	0.9	Pipe Tob Cigar 1	57.2	0.8	89	2
Eastern Carolina	12/M5GK	182	3	250.3	0.6	Pipe Tob Cigar 2	156	3	210	4
Southeastern	13/B4GK	181	14	263	1	Snuff 1	0.63	0.02	BQL	
						Snuff 2	1.5	0.1	8.6	0.1
						Snuff 3	BQL		BQL	
						Chewing Tob 1	252	5	314	13
						Chewing Tob 2	213	10	318	2
						Chewing Tob 3	321	8	428	11

Table 1. Tabulation of total sugars from the analysis of tobacco leaf and tobacco products using SFA and IC-PAD. In this table, "not detected" and "below quantitation limit" are represented by ND and BQL.

sauces in the processing of the tobacco fill material. Casing sauces may contain cane sugar or other sweeteners that are high in fructose or glucose. The results for the pipe tobacco cigars are particularly interesting since cigars are generally made from air-cured tobaccos. The high sugar content is likely due, in part, to the use of sweeteners in the casing sauce. The high sucrose content most strongly suggests the addition of sweeteners. However, some blending involving flue-cured tobacco cannot be ruled out with the carbohydrate data.

CONCLUSIONS

The most significant outcome of these experiments is that, overall, the two methods are consistent with respect to the trends in carbohydrate content. A review of the results shown in Figures 1 and 2 and listed in Table 1 demonstrates that relative differences in SFA results between samples are generally reinforced by the results from the IC-PAD experiments. While no direct comparison of results from SFA and IC-PAD is possible, it is clear that each data set can provide similar conclusions regarding relative carbohydrate contents between tobacco samples.

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