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An isotope dilution ultra high performance liquid chromatography-tandem mass spectrometry method for the simultaneous determination of sugars and humectants in tobacco products

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Abstract

CDC's Division of Laboratory Sciences developed and validated a new method for the simultaneous detection and measurement of 11 sugars, alditols and humectants in tobacco products. The method uses isotope dilution ultra high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) and has demonstrated high sensitivity, selectivity, throughput and accuracy, with recoveries ranging from 90% to 113%, limits of detection ranging from 0.0002 to 0.0045 μg/mL and coefficients of variation (CV %) ranging from 1.4 to 14%. Calibration curves for all analytes were linear with linearity R^2 values greater than 0.995. Quantification of tobacco components is necessary to characterize tobacco product components and their potential effects on consumer appeal, smoke chemistry and toxicology, and to potentially help distinguish tobacco product categories. The researchers analyzed a variety of tobacco products (e.g., cigarettes, little cigars, cigarillos) using the new method and documented differences in the abundance of selected analytes among product categories. Specifically, differences were detected in levels of selected sugars found in little cigars and cigarettes, which could help address appeal potential and have utility when product category is unknown, unclear, or miscategorized.

Keywords

Isotope dilution LC-MS/MS; sugar; humectant; tobacco

1. Introduction

Sugars and humectants are often added in relatively high abundance in selected tobacco products as potential flavor additives or to control moisture. Native sugars are also present in tobacco and these, along with added sugars, can impart palatable sensory attributes and

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make tobacco products more appealing, especially to children and youth [1]. Humectants retain moisture, extend the shelf life of tobacco products and facilitate the manufacturing process [2]. Sugars and humectants are considered safe in food products; however, sugars could serve as precursors for organic acids or harmful chemicals in smoke, such as acetaldehyde, formaldehyde, acrolein, acetone and 2-furfural, among others formed during combustion [3]. Humectants can degrade to oxides during tobacco smoking [4–6]. Propylene oxide from the degradation of humectant propylene glycol has been classified as a Group B2 probable human carcinogen [7]. Glycerol can release acrolein via heat-induced dehydration [8]. While some reports suggest that sugars and humectants have no toxicological effect when added to smoke tobacco [1, 4, 5], combustion/pyrolysis of these additives can form harmful smoke chemicals that may contribute to adverse health effects.

In addition to quantifying sugars and humectants in tobacco, the analytical data generated by the study method may serve as a partial basis helping understanding the appeal of selected products and or characterizing among tobacco types and tobacco products, such as little cigars, cigars, cigarettes, pipe tobacco, smokeless tobacco and roll-your-own-tobacco. Such differentiation is important because federal tax rates in the United States differ across tobacco product categories and sub-categories, and product characteristics could impact regulatory efforts. Moreover, sugar levels are known to differ among certain product types [9].

Differences in sugar profiles could reflect the natural contributions from different tobacco types from the post-harvest processing or sugars added during product manufacturing. Tobacco products can contain many different types of tobacco, which, in turn, have been subjected to varying growing conditions and curing processes, including air-curing, flue-curing, fire-curing, and sun-curing. Air-, fire-, and sun-cured tobaccos typically have lower native sugar content than flue-cured tobacco [10]. Usually, air-cured tobacco undergoes fermentation for the manufacture of cigars, and the fermentation process can change sugar content [10].

Current methods for measuring sugars include capillary electrophoresis (CE) [11], gas chromatography (GC) [12], liquid chromatography (LC) [10, 13–15], GC coupled with mass spectrometry (GC-MS) [16], and LC coupled with mass spectrometry (LC-MS) [17, 18]. Among these, GC-related methods require derivatization of the hydroxyl groups to improve sensitivity and chromatographic resolution, a laborious, time-consuming procedure that may introduce contamination [12, 16]. An ion chromatography (IC) method that does not require derivatization has been applied for sugar detection in tobacco by many researchers [13, 15]. Clarke et al. developed an LC-MS/MS method for the determination of sugars (including glucose, fructose, and sucrose) in tobacco products in which co-eluting sugar compounds from tobacco matrix can be resolved by mass spectrometry, if the compounds form ions with different mass-to-charge ratios [17]. Existing methods for humectant detection range from IC to GC to GC-MS [2, 10]. Tang et al. developed an IC method, and applied it to analyze 12 sugars, alditols, and humectants in tobacco [9]. Rainey, et al. concluded that a GC-MS method can compensate for the inadequate chromatographic resolution between the analyzed humectants [2].

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In the present study, an isotope dilution UHPLC-MS/MS method was developed and validated to measure 11 sugars, and humectants in tobacco products. The target analytes are fructose, glucose, sucrose, mannose, maltose, xylitol, inositol, sorbitol, glycerol, propylene glycol and triethylene glycol. This method has high accuracy, sensitivity, precision, specificity and throughput, with a total run time of 15 min per sample. Compared to LC-MS/MS method described by Clarke et al. [17] for the detection of fructose, glucose and sucrose, our presented LC-MS/MS method uses a hydrophilic interaction liquid chromatography (HILIC) column with 1.7 μm particle size for 11 analytes separation. The small particle size improved peak separation between fructose and glucose compared to existing method [17]. A much higher sensitivity was achieved with our method (1.2 ng/mL vs. 25–50 ng/mL [17]). Sample run time also shortened to 15 min from 20 min [17]. The IC method described by Tang et al. needs 55 min to detected 12 sugars and humectants [9]. To demonstrate utility, this method was used to characterize selected samples of three different types of combustible tobacco products: cigarettes, little cigars and cigarillos.

2. Materials and methods

2.1. Standards and reagents

High purity standards for target compounds and isotopically labeled compounds used as internal standards are listed in Table 1. LC-MS grade acetonitrile was obtained from Sigma-Aldrich (St. Louis, MO). Fisher HPLC grade methanol and J.T. Baker HPLC grade water were obtained from Fisher Scientific (Fairlawn, NJ).

Individual standard stock solution and isotopically labeled internal standard stock solution were prepared in methanol/water (1:1, v/v), and stored at −20 °C. Working standard mixture solutions and internal standard mixture solution were prepared in water and stored at −20 °C. Standard solutions for calibration curve were prepared in water before each analytical run.

2.2. Quality control (QC) sample preparation

University of Kentucky (Lexington, KY) 3R4F research cigarettes were purchased and stored at −20 °C and used to prepare QC samples. The 3R4F cigarettes were removed from the freezer as needed and conditioned in a smoking chamber (ISO 3402:1999, 22 °C, 60% humidity) for 72 h. After conditioning, the tobacco filler from one cigarette was weighed and placed in a 2-oz. Wheaton amber vial (Fisher Scientific, Fairlawn, NJ). The filler was extracted with 35 mL HPLC water on Thermo Scientific MAXQ 2000 digital shaker (Fisher Scientific, Fairlawn, NJ) for 1 h at room temperature and 250 rpm. Then, 500 μL extract solution was transferred in a Costar Spin-X centrifuge unit (Fisher Scientific, Fairlawn, NJ) and centrifuged at 5,000 rpm for 5 min. After centrifuging, 200 μL filtrate was diluted with HPLC water to 100.0 mL to make a low-concentration quality control (QCL) pool. A high-concentration quality control (QCH) pool of 100.0 mL was prepared by spiking 200.0 μL filtrate with analyte standard stocks. QC pools were aliquoted (140 μL) into SUPELCO QSert Vial 300 μL (Sigma-Aldrich, St. Louis, MO) and stored at −20 °C. QC pools were characterized (mean and standard deviation) with 20 independent analyses of each QC pool. The QC samples in the analytical batch were prepared by mixing 50.0 μL QC from aliquoted

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QC pool with 50.0 μL working internal standard mixture solution. Two QCL and two QCH samples were prepared and included in each analytical batch. A modified approach of the Westgard rules was used to evaluate the QC results [19]. If the QC failed, the batch was discarded and a fresh sample set was prepared.

2.3. Tobacco sample preparation

Samples examined in this study included 20 brands of cigarettes, 16 brands of little cigars, and 8 brands of cigarillos sold in the US market. The cigarettes were conditioned, weighed, extracted, centrifuged in the same manner as the reference cigarette described in section 2.2. Then, 20.0 μL filtrate was diluted to 10 mL. The little cigars and cigarillos were ground with a commercial coffee grinder before conditioning. Approximately 1 g ground little cigar or cigarillo filler was weighed out and extracted with 35 mL water, followed by centrifugation and dilution—the same protocol used for the cigarettes. Subsequently, 50.0 μL diluted filtrate of cigarette, little cigar, or cigarillo and 50.0 μL working internal standard mixture solution was added and mixed in a QSert Vial to make a prepared tobacco product sample of 100.0 μL for LC-MS/MS measurement.

2.4. Instrumentation

Chemical separation was performed on an Agilent 1260 Infinity Quaternary liquid chromatography (LC) system (Agilent Technology Inc., Wilmington, DE), using an Acquity UPLC BEH Amide column (1.7 μm particle size, 2.1 mm I.D. × 100 mm, Waters Inc., Milford, MA). Acetonitrile (ACN)/water (98:2, v/v) with 0.1% (v/v) triethylamine (TEA) (solvent A) and ACN/water (30:70, v/v) with 0.1% TEA (solvent B) were used as mobile phases. Analyses were conducted within optimum chromatographic parameters for LC operation, as follows. Mobile phase flowed at a rate of 0.3 mL/min. The gradient elution was 0–9 min, 85–40% solvent A; 9–9.1min, 40–85% solvent A; 9.1–15 min, 85% solvent A. The column was maintained at 40 °C. The sample volume injected was 2 μL. During the first 0.8 min and the last 5 min of the gradient, the mobile phase was redirected to waste and not to the mass spectrometer. The autosampler injection needle was flushed for 10 s between each sample. The LC sample compartment was kept constant at 4 °C.

The LC system was coupled with an API 5500 Triple Quad mass spectrometer (Applied Biosystems, Foster City, CA) with an electrospray interface. All analytes were ionized in negative mode as $[M\text{-}H]^-$. The mass spectrometer was operated under multiple-reaction monitoring (MRM) mode. The MS parameters for each analyte and analog were optimized separately by direct infusion of individual standard solution. Table 2 presents the MRM transitions used for analyte quantitation and confirmation, and for internal standards (ISTD). Source parameters were determined by Flow Injection Analysis (FIA) and were identical for all analytes: ion spray voltage −4500 V, source temperature 350 °C, curtain gas (CUR) 30 psi, nitrogen collision gas (CAD) 7 psi, ion source gas 1 (GS1) 50 psi, ion source gas 2 (GS2) 50 psi. The dwell times were optimized and kept at 80 ms for all analytes. AB Sciex Analyst® Software Version 1.6.1 (Applied Biosystems, Foster City, CA) was used for data acquisition.

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2.5. Quantitation

Analyst® Software Version 1.6.1 (Applied Biosystems, Foster City, CA) was used for data analysis, including peak integration, calibration and quantitation. Each peak was manually inspected to confirm correct integration. Relative response factor was calculated based on the ratio of the peak area of the analyte quantitation transition to that of the isotopically labeled internal standard transition. The peak area ratio of the analyte quantitation transition to the isotopically labeled internal standard transition was used to quantify the unknowns through comparison with the calibration curve. Sample results are reported as % (w/w).

2.6. Recovery

Recoveries for all analytes were assessed by spiking known amounts of analytes at low and high levels into the 3R4F cigarette filler. Spiking solution was prepared by adding and mixing analytes in water. The calculated volume of spiking solution for low or high level recovery experiment was aspirated with pipette and dispensed on the 3R4F filler, followed by addition of 35 mL water. The cigarette samples for recovery measurements were prepared in the same way as cigarette samples in section 2.2 and 2.3. Control blank samples without spiking analytes were prepared as regular unknown samples. Five replicates were prepared and analyzed for each set of recovery experiment samples (control blank, low level spiked, and high level spiked). Replicate concentrations for each analyte were averaged, and the analyte concentration in control blank was subtracted from the measured analyte concentration in prepared low or high level spiked samples. The recovery was calculated as the percentage ratio of calculated spiked concentration to the theoretically spiked concentration.

2.7. Freeze-thaw and stability experiments

Spiked 3R4F research cigarette samples were frozen (−20 °C) and thawed (room temperature) 10 times to check the stability of analytes relative to the number of freeze-thaw cycles. The same spiked samples were also kept at different storage temperatures (22 °C, −4 °C, and −20 °C) to ascertain the stability of analytes under these storage conditions for 7 days.

2.8. Robustness testing

Robustness of method accuracy with respect to key extraction parameters (water volume, time, speed and temperature) was assessed. The extraction parameters in the final method are: extraction solvent water 35 mL, time 60 min, speed 250 rpm, and temperature 22 °C (room temperature). Low and high level parameter values were chosen so that they were 20–50% lower or higher than the parameter values in the final method. Prepared QCL samples were used for this test.

3. Results and Discussion

3.1. LC-MS/MS

Organic base (triethylamine) was added in the mobile phase to improve analyte ionization in negative mode and to assist in analyte separation. A full scan chromatogram of the analytes

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in a spiked QC sample is shown in Fig. 1. Eleven analytes were separated within 10 min with a total run time of 15 min, including equilibration. The overlap between propylene glycol and triethylene glycol (peaks 1 & 2, RT 1.23 min), between xylitol (peak 4, RT 3.51 min) and D-(−)-fructose (peak 5, RT 4.06 min), between D-(+)-mannose (peak 6, RT 4.90 min) and D-sorbitol (peak 7, RT 5.13 min), between D-sorbitol and D-(+)-glucose, and between myo-inositol (peak 10, RT 8.86 min) and maltose (peak 11, RT 9.01 min) were completely resolved in the reconstructed ion chromatograms by different MRM transitions with MS. The humectants (propylene glycol, triethylene glycol, and glycerol) were eluded at the beginning of the LC separation. The employed HILIC column could not retain them very well, especially propylene glycol and triethylene glycol. A higher ratio (90%) of mobile phase A (90%) was tried at the initial stage of gradient separation, but it could not help the separation and improve the retaining of these two analytes significantly. Since there were no analyte peaks in the adjacent regions around retention time 2.7 min and 7.0 min, the MS scan was divided into 3 periods: 0–2.7 min, 2.7–7.0 min, and 7.0–10 min. In each period, only the MRM transitions for the analytes eluted in that period were monitored. More data points were generated for the analyte peaks in the MRM mode with multiple periods than for the undivided MRM mode. In order to detect D-(+)-mannose, which had a relatively low concentration in tobacco samples, sucrose ion transition signal was detuned by increasing its collision energy (CE) from the optimum value of −24 v to −35 v (Table 2).

3.2. Method validation

Ten calibration standard mixtures were used to construct the calibration curves. A 1/x weighted least-square model was fit to all the calibration curves. The limit of detection (LOD) was three times the standard deviation at zero concentration ($3S_0$), based on Taylor analysis of twenty sets of calibrators [20]. Calibration curves for all analytes were linear, with linear correlation coefficients (R^2) values greater than 0.999. Because the concentration of individual analyte in the tobacco samples varied, the concentration range for each analyte in the calibration standard set was different. The linear range spans 3–4 orders of magnitude, depending on the analyte. The method LOD, calibration range and linearity for each analyte are shown in Table 3.

Method accuracy and repeatability were evaluated using spike recovery results (Table 4). Recoveries were determined for all analytes, at two concentrations, through five independent sample preparations and analyses. The mean recovery across all analytes ranged from 90 to 113%, with an overall average of 100%. The coefficient of variation across all analytes ranged from 1 to 15%, with an overall average of 5.5%.

Method precision was evaluated by calculating the coefficient of variation of 20 sets of measurements on two sets of QC samples (QCL and QCH) over a period of 40 days. The inter-day variability of QCs ranged from 1.4 to 11% (Table 5). Relatively greater variability was observed for QCL (5.8%, on average) compared with QCH (2.4%, on average). Bias results for QCH ranged from −3.8 to 1.4%, demonstrating a high degree of accuracy for the method.

Analyte stability in spiked tobacco samples was investigated by storing spiked tobacco samples under different temperatures (22 °C, −4 °C, and −20 °C) and by subjecting the

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spiked tobacco samples to 10 freeze-thaw cycles. All analytes were stable under the different storage temperatures for a week and through the 10 freeze-thaw cycles.

Method robustness was demonstrated by the consistency of final calculated analyte results under a number of extraction conditions, including varying water levels (25 mL, 35 mL, 45 mL), run times (45 min, 60 min, 90 min), temperatures (17 °C, 22 °C, 27 °C) and speeds (200 rpm, 250 rpm, 300 rpm).

3.3. Application to tobacco product samples

The developed method was used to characterize three types of tobacco products - cigarillo, little cigar and cigarette - in terms of sugar, alditol and humectant content. Little cigars resemble cigarettes in size and shape; cigarillos are essentially mini-cigars. Our purpose was to investigate the applicability of this method for distinguishing among these types of tobacco products. Samples were prepared and analyzed in triplicates. Measurement results are summarized in Table 6. Table 7 presents the mean and range of each analytes in three types of tobacco products.

The main sugar components were D-(−)-fructose, sucrose, and D-(+)-glucose. Humectant triethylene glycol was detected only in several brands of cigarillos. The total sugar content for cigarillos, little cigars, and cigarettes ranged from 0.07 to 15.1%, 0.06 to 1.11%, and 3.37 to 9.09% (w/w), respectively. Figure 2 shows mean total sugar content and measured alcohol content (including alditols and humectants) for these three types of tobacco products, based on measurements taken on 44 randomly selected brands. Cigarettes had a significantly higher level of sugar than little cigars, with mean concentrations of 5.83% versus 0.60%, respectively. Cigars, including little cigars, are typically made from air-cured and fermented tobacco, which contains little sugar. American blended cigarettes, used in this study, predominantly contain flue-cured, fire-cured, and air-cured tobacco. Domestic cigarette tobaccos typically have relatively high natural sugar content compared with tobaccos used to make cigar [6, 8]. The measured total sugar level for little cigars and cigarettes is consistent with the values reported elsewhere [12, 15, 16]. Cigarettes' mean total alcohol level (3.03%) was relatively higher than that for little cigars (1.91%). Only one little cigar brand (Little Cigar #8) had a total alcohol level comparable to that of a cigarette, at 3.34%. Similarly, only one cigarette brand (Cigarette #1) had a total alcohol level comparable to that of a little cigar, at 1.05%. Based on these results, total sugar content seems promising to help distinguish product categories between cigarettes and little cigars (manufactured filtered cigars having similar size and shape of cigarettes). While this is true for the brands tested, additional work would be valuable for product characterization. Some examined cigarillos and little cigars had similar levels of sugars and alcohols. Results suggest that total sugar content and alcohol levels cannot be used to directly differentiate cigarillos from little cigars or cigarettes (Fig.3).

Multivariate data analysis techniques - principal component analysis (PCA) and linear discriminant analysis (LDA) were applied for the classification of three types of tobacco products by using 11 measured analytes as variables. JMP software (version 11.1.1, SAS Institute INC, Cary, NC, USA) was used for the multidimensional data analysis purpose. Figure 4 (a) & (b) illustrate the score and loading plots of PCA respectively. The score plot

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(Fig. 4 (a)) shows that cigarettes and little cigars were clearly separated on PC1 (explaining 49.4% of the total variance). The loading plot (Fig. 1 (b)) shows the influence of the 11 analytes on PC1 & PC2 and on each tobacco product type in conjunction with score plot (Fig. 1 (a)). Humectant glycerol together with sugars sucrose, glucose, and fructose etc. had a strong influence on the PC1 towards positive values. Triethylene glycol and xylitol had influence on the PC1 toward the negative values. Cigarettes usually containing higher sugar contents appear on the positive PC1 side. Little cigars usually containing low sugar contents and no triethylene glycol appear on the negative PC1 side. Cigarillos with high triethylene glycol level and low sugar level appear on the negative PC1 side close to the little cigars, however, those with high sugar levels on positive PC1 side. Propylene glycol had a significantly strong influence on the PC2 towards the positive values. Cigarillos with high propylene glycol and sugar levels appear on the upper right of the score plot. However, cigarillos still could not be completely differentiated from cigarettes or little cigars by applied PCA. Another multivariate data analysis technique – LDA was applied on the same dataset, and the biplot of LDA is presented in Fig.5. Figure 5 shows that three types of tobacco products were well separated in the canonical space. Cigarettes and little cigars were able to be clearly separated on Canonical1. Minor sugars mannose, maltose and alditol myo-inositol had strong influence on the separation of cigarettes and little cigars. Cigarillos were able to be clearly separated from cigarettes on Canonical2, which was influenced by mannose strongly, as well as by triethylene glycol and maltose towards the negative values, and by all other analyte towards the positive values. Cigarillos were distint from little cigars on either canonical of the two dimensional canonical space. All the separations by LDA are based on the measured analytes and analyte values of three types of tobacco products, and the influence direction and strength of each analyte in the canonical space. 100% correct classification rate was obtained with LDA. LDA is a supervised multivariate data analysis technique, which has both classification and identification capability. If an appropriate model is built by LDA with sufficient training samples, unknown samples could be identified by the LDA model.

4. Conclusions

A rapid, isotope dilution LC-MS/MS method was developed for the simultaneous analysis of 11 sugars, alditols and humectants in tobacco and tobacco products. The method was validated and characterized and demonstrated high sensitivity, accuracy, repeatability and precision. The method was used to measure the 11 analytes in 44 brands of tobacco products, including cigarillos, little cigars, and cigarettes. Tested cigarettes typically had higher total sugar content and higher levels of humectants than the little cigars subjected to our analysis, and these differences were statistically significant. In contrast, the tested cigarillos had a wide range of sugar levels - overlapping with both cigarettes and little cigars - demonstrating that sugar content may not be a complete characteristic for differentiating cigarillos from cigarettes or little cigars. Principal component analysis proved a clear distinction between cigarettes and little cigars based on measured 11 analytes. Linear discriminant analysis provided a better statistical technique to completely distinguish three types of tested tobacco products without misclassification on tested samples. More work, on a wider array of products and tobacco constituents (e.g. nicotine, minor alkaloids, tobacco

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specific amines, menthol, and pH) could be exploited to fully explore the utility of multivariate statistical data analysis techniques to more clearly differentiate among product types and identify mis-categorized products. However, our initial work on the presented sugars and humectants method alone combined with multivariate data analysis shows the potential to distinguish among tobacco product categories.

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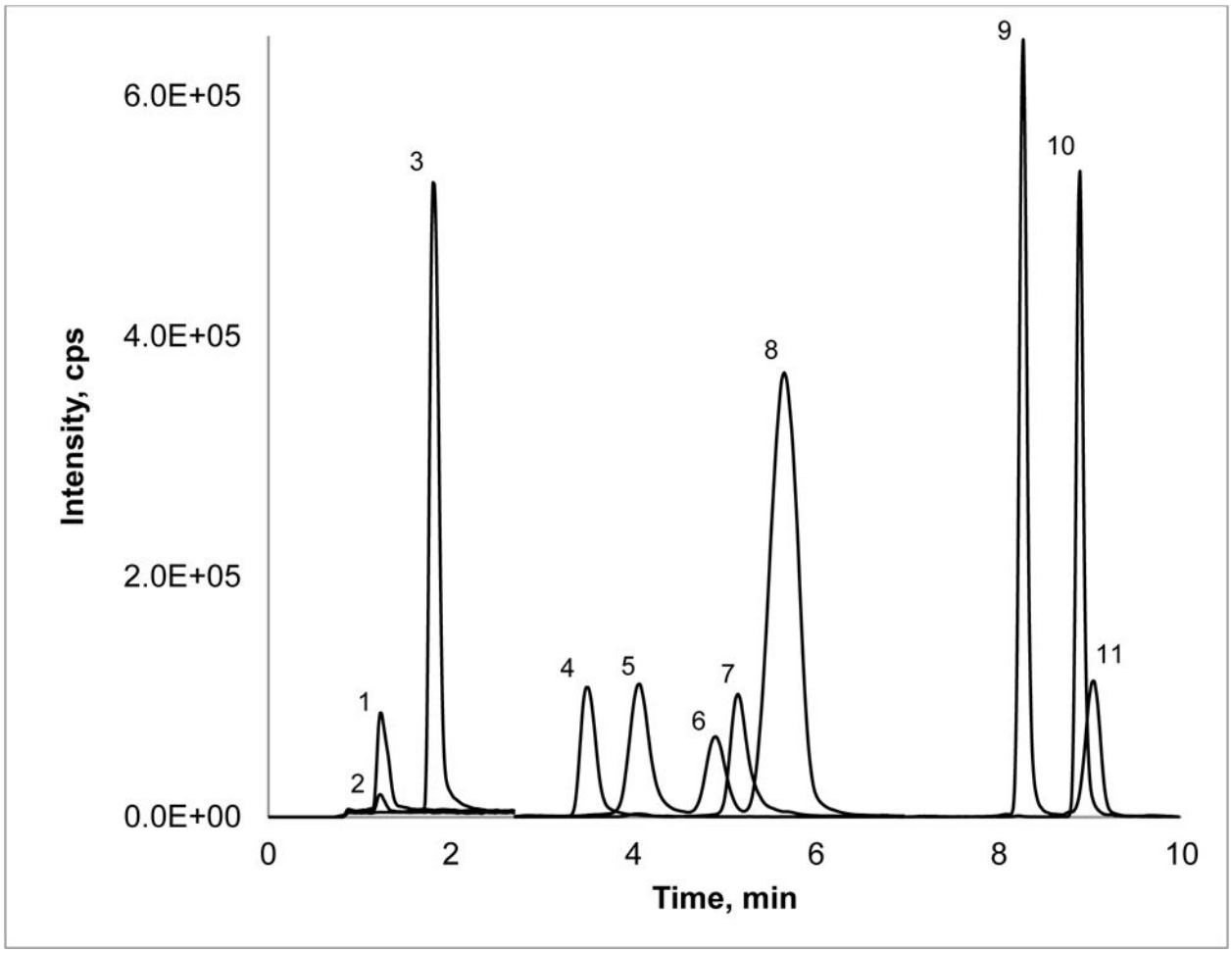

Fig. 1.

LC-MS/MS chromatogram of sugars, alditols, and humectants in QC high – spiked research cigarette 3R4F sample. 1 – propylene glycol (1.23 min); 2 – triethylene glycol (1.23 min); 3 – glycerol (1.81 min); 4 – xylitol (3.51 min); 5 – D-(−)-fructose (4.06 min); 6 – D-(+)-mannose (4.90 min); 7 – D-sorbitol (5.13 min); 8 – D-(+)-glucose (5.67 min); 9 – sucrose (8.26 min); 10 – myo-inositol (8.86 min); 11 – maltose (9.01 min).

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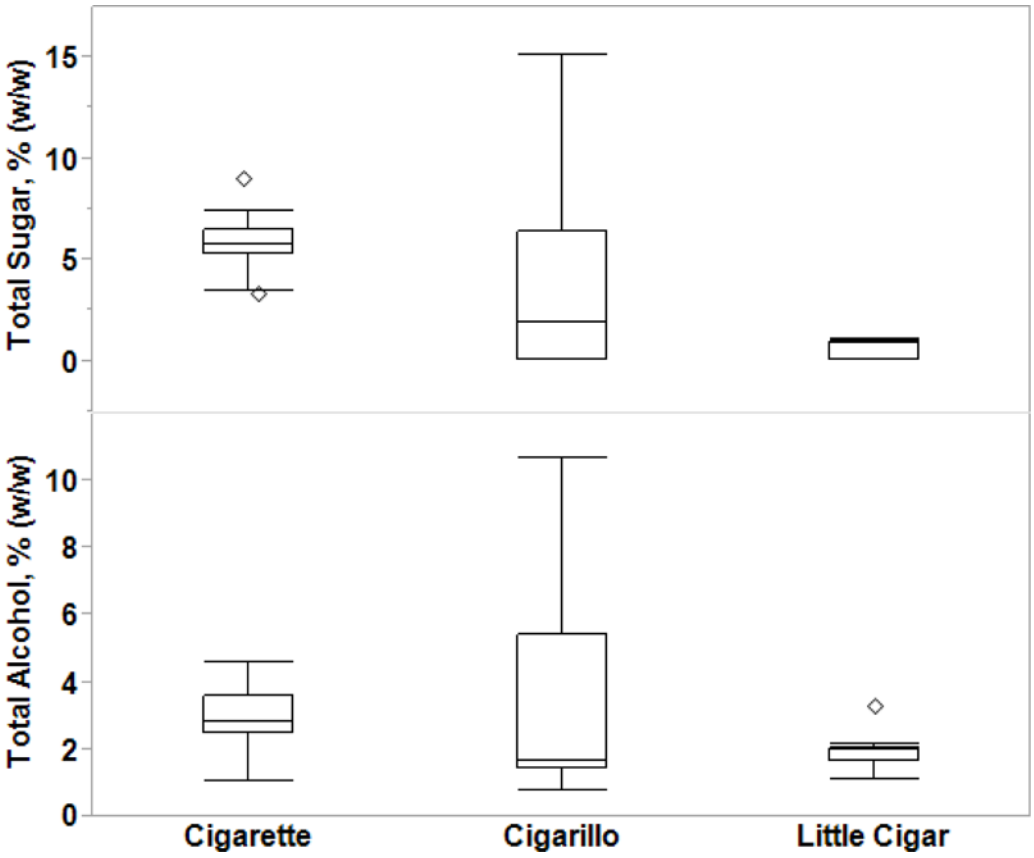

Fig. 2.

Boxplots of total sugar content and total alcohol content (including alditols and humectants) for three types of tobacco products. Error bars at the top of each column indicate the standard deviation of the measurement.

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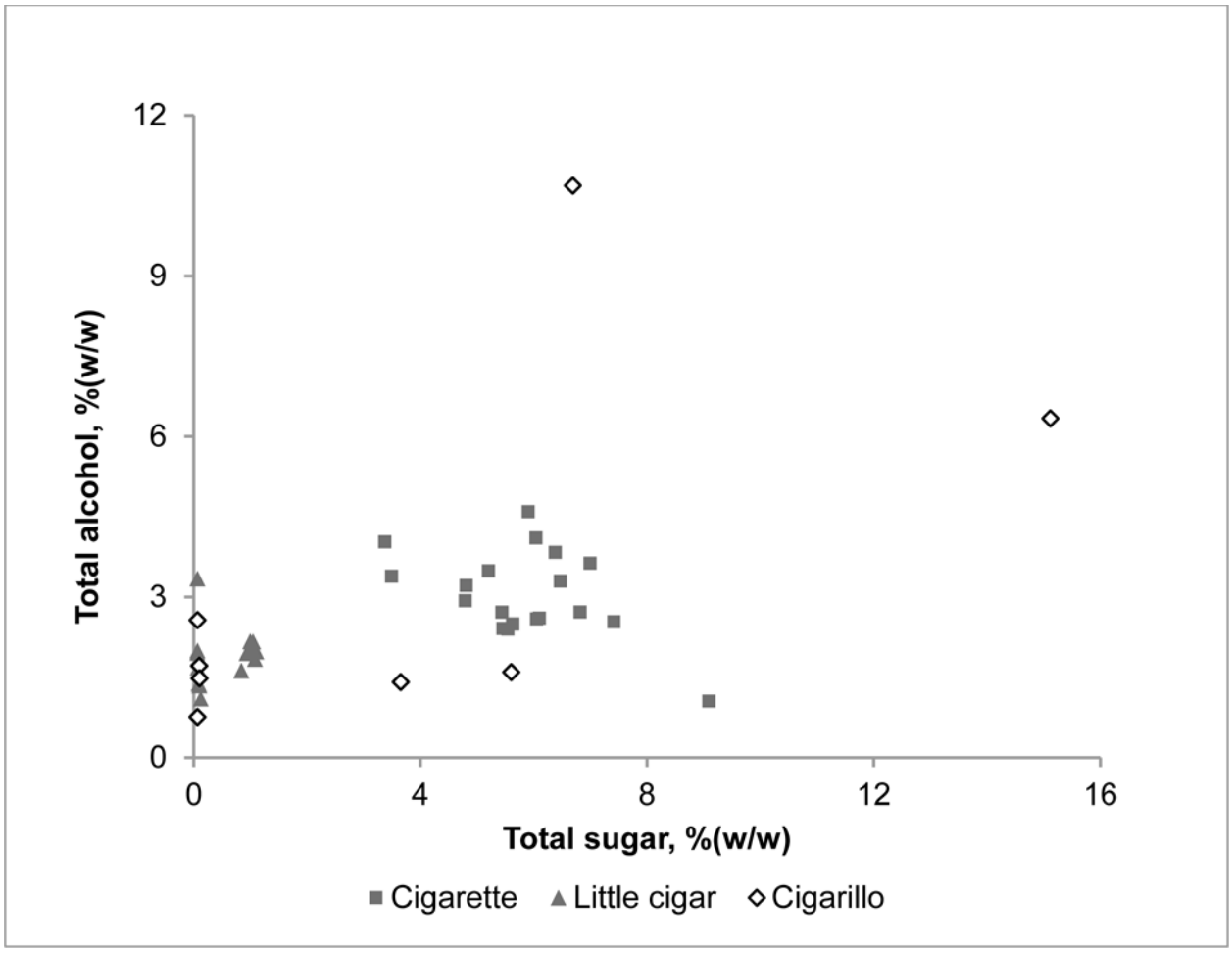

Fig. 3.
Plot of total alcohol vs. total sugar content of tobacco products.

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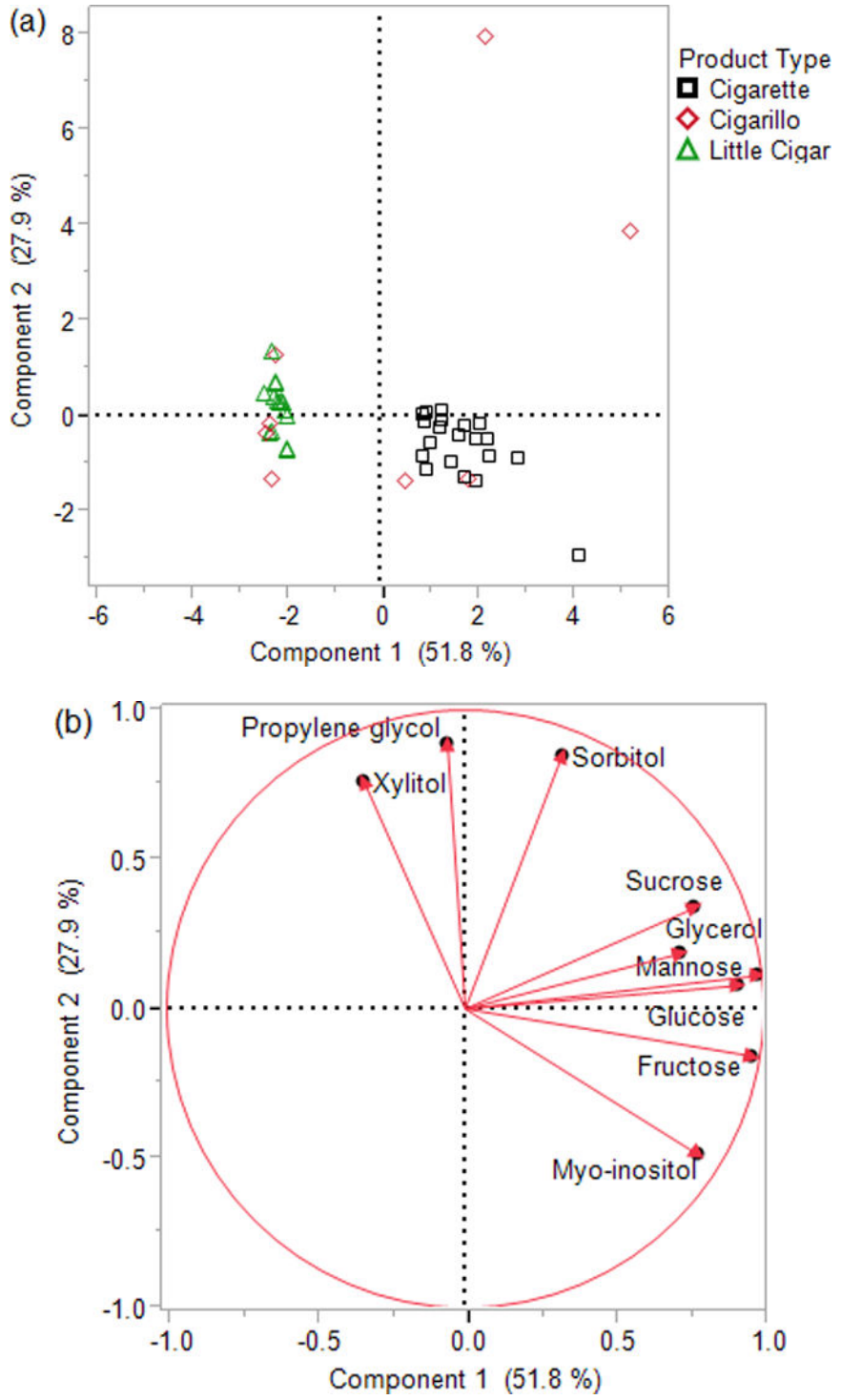

Fig. 4.

(a) Score and (b) loading plots of the principal component analysis for the three types of tobacco products by using 11 measured analytes.

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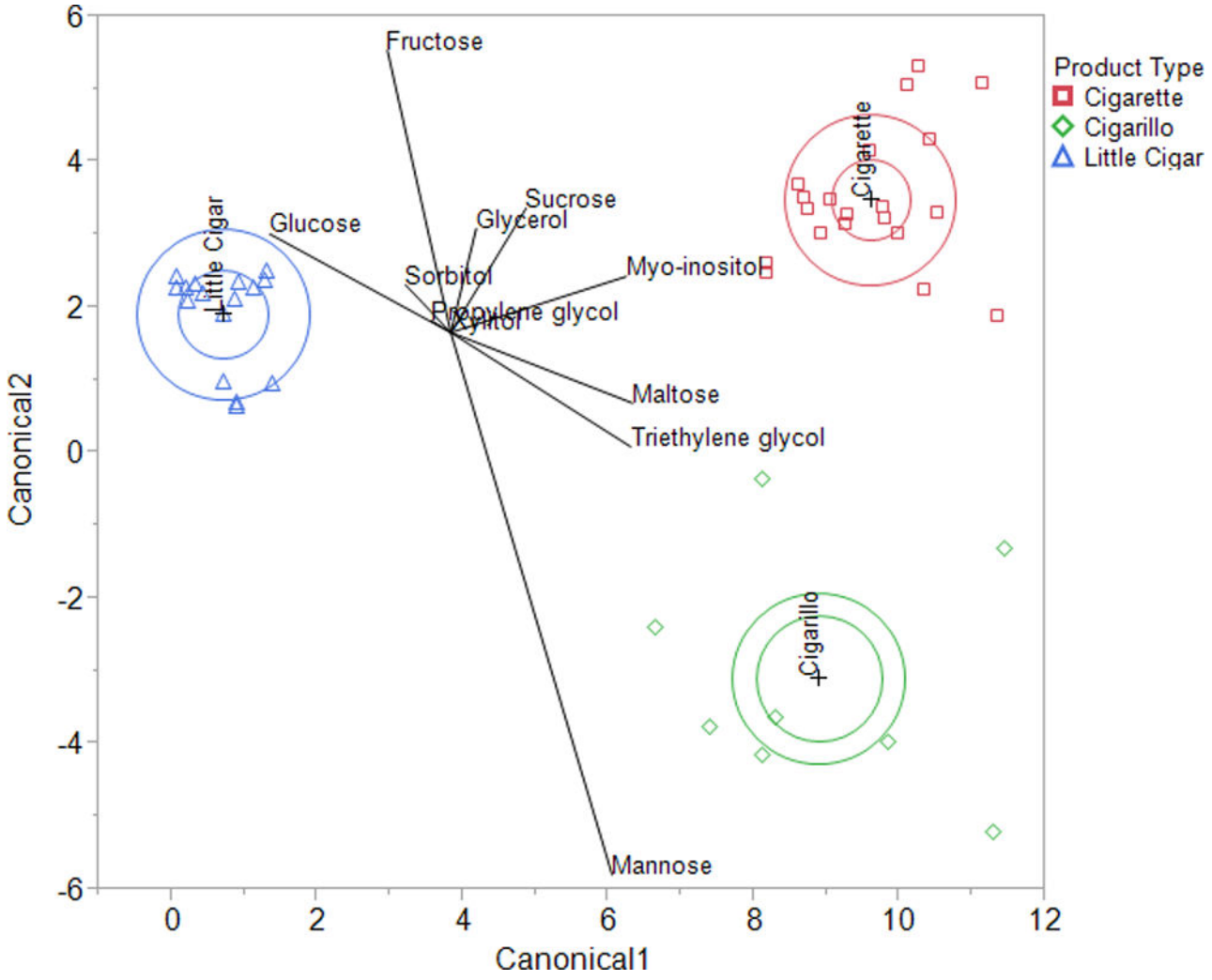

Fig. 5.

Biplot of linear discriminant analysis for the three types of tobacco products by using 11 measured analytes. Color-labeled inner circle for each group represents the multivariate mean, the size of which corresponding to a 95% confidence limit for the mean. Color-labeled outer circle for each group shows area that contains roughly 50% of the group samples. Propylene glycol ray (between sorbitol and glucose rays) and xylitol ray (between myo-inositol and sucrose rays) are not visible due to the biplot scale.

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Table 1

Sugars, alditols, humectants, and the internal standards measured in the method.

Compound	Internal standard
D-(−)-Fructose[a]	D-Fructose-$^{13}C_6$[b]
D-(+)-Glucose[a]	D-Glucose-$^{13}C_6$[b]
D-(+)-Maltose[a]	D-Sucrose-Glucose-$^{13}C_6$[b]
D-(+)-Mannose[a]	D-Glucose-$^{13}C_6$[b]
D-Sorbitol[a]	D-Sorbitol-$^{13}C_6$[c]
myo-Inositol[a]	D-Sucrose-Glucose-$^{13}C_6$[b]
Xylitol[a]	D-Fructose-$^{13}C_6$[b]
Sucrose[a]	D-Sucrose-Glucose-$^{13}C_6$[b]
Glycerol[a]	Glycerol-$^{13}C_3$[b]
Propylene glycol[a]	1,2-propanediol-$^{13}C_3$[b]
Triethylene glycol[a]	1,2-propanediol-$^{13}C_3$[b]

[a] Sigma, St. Louis, MO

[b] Cambridge Isotope Inc., Andover, MA

[c] Aldrich, St. Louis, MO

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Table 2

MRM transitions and compound dependent MS parameters for target analytes.

Analyte	RT (min)	Quantitation ion transition	DP (V)	CE (V)	EP (V)	CXP (V)	Confirmation ion transition	DP (V)	CE (V)	EP (V)	CXP (V)
D-(−)-Fructose	4.06	178.9/89.0	−25	−12	−9	−9	178.9/59.0	−25	−22	−9	−9
D-(+)-Glucose	5.67	178.9/89.0	−30	−10	−10	−13	178.9/119.0	−30	−12	−10	−10
D-(+)-Maltose	9.01	341.1/161.0	−45	−10	−5	−15	341.1/101.0	−45	−22	−5	−9
D-(+)-Mannose	4.90	178.9/119.0	−30	−10	−10	−11	178.9/89.0	−30	−12	−10	−9
D-Sorbitol	5.13	180.9/89.2	−40	−20	−10	−9	180.9/70.9	−40	−26	−10	−9
myo-Inositol	8.86	178.9/87.0	−40	−22	−9	−9	178.9/161.0	−40	−16	−9	−15
Xylitol	3.51	151.0/71.0	−30	−22	−10	−7	151.0/59.0	−30	−22	−10	−7
Sucrose	8.26	341.1/89.0	−50	−35	−6	−9	341.1/179.0	−50	−25	−6	−15
Glycerol	1.81	90.9/59.0	−35	−14	−7	−9	90.9/89.0	−35	−10	−7	−10
Propylene glycol	1.23	74.9/73.0	−30	−8	−11	−9	74.9/59.0	−30	−12	−11	−10
Triethylene glycol	1.23	149.0/60.9	−30	−10	−10	−7	149.0/59.0	−30	−22	−10	−9
D-Fructose-$^{13}C_6$	4.06	184.9/92.1	−25	−12	−10	−9					
D-Glucose-$^{13}C_6$	5.67	184.9/61.0	−35	−20	−10	−7					
D-Sorbitol-$^{13}C_6$	5.13	187.0/92.0	−40	−20	−10	−9					
D-Sucrose-Glucose-$^{13}C_6$	8.26	347.1/92.0	−50	−24	−3	−9					
Glycerol-$^{13}C_3$	1.81	93.9/61.0	−25	−14	−11	−9					
1,2-propanediol-$^{13}C_3$	1.23	78.0/76.0	−45	−9	−12	−16					

RT: retention time; DP: dissociation energy; CE: collision energy; CXP: collision cell exit energy: EP: entrance energy.

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Table 3

Method limit of detection (LOD), calibration range and linearity for each analyte.

Compound	LOD (ng/mL)	Calibration range (μg/mL)	Linearity R^2
D-(−)-Fructose	1.2	0.0075 – 4.5	0.9998
D-(+)-Glucose	1.2	0.010 – 6.0	0.9999
D-(+)-Maltose	0.2	0.0010 – 0.48	0.9997
D-(+)-Mannose	0.6	0.0020 – 1.2	0.9997
D-Sorbitol	0.3	0.0010 – 0.60	0.9998
myo-Inositol	1.2	0.0030 – 1.8	0.9990
Xylitol	0.2	0.0015 – 0.90	0.9997
Sucrose	1.2	0.0020 – 1.2	0.9997
Glycerol	3.6	0.0050 – 3.0	0.9999
Propylene glycol	3.9	0.013 – 7.5	0.9995
Triethylene glycol	4.5	0.025 – 15	0.9998

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Table 4

Analyte recovery for fortified 3R4F research tobacco at two spike levels (based on 5 replicates for each spike level).

Analyte	Spike level	Spike concentration (μg/mL)	Spike recovery (%)	CV, %
D-(−)-Fructose	low	0.153	90	12
	high	0.765	100	1.8
D-(+)-Glucose	low	0.182	97	8.4
	high	0.912	102	1.6
D-(+)-Mannose	low	0.0420	94	5.0
	high	0.209	98	0.8
D-Sorbitol	low	0.0210	99	4.8
	high	0.107	107	15
Maltose	low	0.0170	103	7.7
	high	0.0860	106	1.5
myo-Inositol	low	0.0600	112	10
	high	0.300	113	1.1
Sucrose	low	0.0400	107	15
	high	0.200	101	3.0
Xylitol	low	0.0310	95	7.4
	high	0.157	99	2.0
Glycerol	low	0.100	95	12
	high	0.499	100	5.5
Triethylene glycol	low	0.534	100	0.9
	high	2.67	98	1.4
Propylene glycol	low	0.262	97	2.8
	high	1.31	100	0.5

CV: coefficient of variation

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Table 5

Precision and accuracy of QC samples (based on 20 independent measurements for each QC pool).

Compound	QCL		QCH			
	Measured mean, μg/mL	CV, %	Expected μg/mL	Measured mean, μg/mL	CV, %	Bias, %
D-(−)-Fructose	1.44	1.8	5.44	5.32	2.6	−2.2
D-(+)-Glucose	1.09	1.8	7.09	6.82	1.7	−3.8
D-(+)-Maltose	0.0274	8.8	0.267	0.264	2.8	−1.3
D-(+)-Mannose	0.0368	9.5	0.437	0.416	3.6	−4.8
D-Sorbitol	0.0210	11	0.301	0.298	1.9	−1.0
myo-Inositol	0.164	2.0	1.36	1.36	2.5	−0.6
Xylitol	0.0380	14	0.438	0.444	2.3	1.4
Sucrose	0.108	2.4	1.11	1.09	1.8	−2.0
Glycerol	0.994	1.4	3.99	3.96	1.5	−0.9
Propylene glycol	ND	ND	1.00	0.962	3.9	−3.8
Triethylene glycol	ND	ND	2.00	1.97	2.3	−1.5

ND: Not detectable.

Expected analyte concentration in QH = spiked analyte concentration in QH + analyte concentration in QL.

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Table 6

Levels of sugars, alditols and humectants in different tobacco products. Mean of 3 measurements. Unit: % (w/w).

Product type	Product Brand	Fru	Glu	Suc	Man	Xyl	Sor	Mal	Ino	Gly	PPG	TEG	Total Sugar	Total Alcohol
Cigarillo	1	2.35	0.89	0.30	0.08	0.05	0.04	0.02	0.53	0.12	0.58	0.10	3.65	1.41
	2	3.05	1.93	0.47	0.11	0.04	0.05	0.04	0.53	0.26	0.72	ND	5.60	1.60
	3	1.91	1.94	2.57	0.13	0.11	0.21	0.15	0.07	3.13	7.17	ND	6.69	10.7
	4	3.73	2.93	8.15	0.21	0.08	0.10	0.10	0.06	3.38	2.72	ND	15.1	6.34
	5	0.02	0.03	0.02	0.005	0.04	0.03	ND	0.07	0.10	0.27	0.25	0.07	0.76
	6	0.04	0.04	0.005	0.007	0.06	0.03	ND	0.09	0.08	1.18	0.27	0.10	1.71
	7	0.05	0.04	0.003	0.005	0.07	0.04	ND	0.08	0.54	0.53	0.22	0.10	1.48
	8	0.03	0.03	0.007	0.005	0.07	0.09	ND	0.11	0.09	1.80	0.42	0.07	2.57
Little Cigar	1	0.65	0.39	0.02	0.01	0.08	0.03	0.02	0.07	0.35	1.32	ND	1.08	1.84
	2	0.61	0.39	0.004	0.01	0.07	0.03	0.02	0.08	0.56	1.30	ND	1.03	2.05
	3	0.52	0.29	0.005	0.02	0.11	0.05	0.01	0.08	0.34	1.06	ND	0.84	1.63
	4	0.06	0.05	0.01	0.007	0.06	0.04	ND	0.09	0.13	0.78	ND	0.12	1.10
	5	0.58	0.39	0.004	0.01	0.09	0.03	0.01	0.06	0.48	1.50	ND	1.00	2.17
	6	0.54	0.36	0.006	0.01	0.08	0.03	0.01	0.06	0.49	1.30	ND	0.93	1.95
	7	0.05	0.03	0.003	0.006	0.06	0.04	ND	0.12	0.19	0.98	ND	0.09	1.38
	8	0.03	0.02	0.007	0.005	0.06	0.03	ND	0.09	0.24	2.92	ND	0.06	3.34
	9	0.59	0.37	0.004	0.01	0.08	0.03	0.01	0.07	0.51	1.33	ND	0.99	2.02
	10	0.61	0.39	0.01	0.01	0.07	0.03	0.01	0.06	0.55	1.46	ND	1.05	2.17
	11	0.03	0.02	0.009	0.004	0.06	0.03	ND	0.07	1.79	0.04	ND	0.06	2.00
	12	0.02	0.02	0.01	0.004	0.06	0.03	ND	0.07	1.70	0.10	ND	0.06	1.96
	13	0.03	0.03	0.004	0.004	0.08	0.07	ND	0.08	0.81	0.63	ND	0.07	1.68
	14	0.04	0.03	0.02	0.005	0.08	0.06	ND	0.08	0.84	0.28	ND	0.10	1.35
	15	0.58	0.41	0.005	0.01	0.08	0.03	0.01	0.07	0.43	1.38	ND	1.01	1.98
	16	0.64	0.41	0.02	0.01	0.08	0.03	0.02	0.07	0.57	1.23	ND	1.11	1.98
Cigarette	1	4.80	3.35	0.75	0.15	0.02	0.03	0.05	0.80	0.16	0.03	ND	9.09	1.05
	2	1.82	0.84	2.68	0.07	0.07	0.05	0.02	0.42	1.58	0.60	ND	5.44	2.72
	3	1.85	0.86	2.66	0.07	0.07	0.06	0.03	0.41	1.63	0.24	ND	5.46	2.41

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Product type	Product Brand	Fru	Glu	Suc	Man	Xyl	Sor	Mal	Ino	Gly	PPG	TEG	Total Sugar	Total Alcohol
	4	1.87	0.84	2.75	0.07	0.07	0.05	0.02	0.41	1.62	0.25	ND	5.55	2.40
	5	2.28	1.19	2.89	0.08	0.06	0.04	0.04	0.47	2.07	0.65	ND	6.47	3.30
	6	2.67	1.28	1.55	0.09	0.05	0.04	0.03	0.43	1.57	0.40	ND	5.63	2.49
	7	2.31	0.86	2.76	0.08	0.06	0.05	0.03	0.44	3.16	0.39	ND	6.04	4.10
	8	2.48	1.08	2.22	0.09	0.05	0.04	0.03	0.51	3.74	0.26	ND	5.90	4.59
	9	3.06	1.77	2.45	0.10	0.07	0.04	0.04	0.51	1.79	0.14	ND	7.42	2.54
	10	2.10	0.99	2.43	0.08	0.07	0.05	0.03	0.47	1.71	0.20	ND	5.63	2.50
	11	2.92	1.57	2.19	0.10	0.07	0.04	0.04	0.49	1.93	0.18	ND	6.82	2.72
	12	1.99	1.00	3.00	0.08	0.08	0.05	0.03	0.48	1.77	0.23	ND	6.10	2.60
	13	2.09	1.04	2.81	0.08	0.07	0.05	0.03	0.42	1.83	0.22	ND	6.05	2.59
	14	2.81	1.11	0.76	0.09	0.04	0.05	0.03	0.46	2.54	0.13	ND	4.81	3.22
	15	2.62	1.06	2.58	0.09	0.06	0.06	0.03	0.47	2.96	0.29	ND	6.38	3.83
	16	2.95	1.24	0.84	0.10	0.04	0.04	0.06	0.48	2.66	0.28	ND	5.20	3.49
	17	2.40	1.10	1.18	0.08	0.06	0.04	0.03	0.43	1.63	0.77	ND	4.79	2.93
	18	3.76	1.70	1.37	0.12	0.05	0.05	0.05	0.47	2.95	0.10	ND	7.00	3.63
	19	1.87	0.65	0.76	0.07	0.05	0.04	0.02	0.44	2.81	0.70	ND	3.37	4.03
	20	1.91	0.67	0.82	0.07	0.05	0.04	0.02	0.46	2.70	0.14	ND	3.49	3.39

ND: Not detectable; Fru: fructose; Glu: glucose; Suc: sucrose; Man: mannose; Xyl: xylose; Sor: sorbitol; Mal: maltose; Ino: myo-inositol; Gly: glycerol; PPG: propylene glycol; TEG: triethylene glycol.

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Table 7

Mean values and ranges of measured sugars and humectants in different tobacco categories. Unit: % (w/w).

Product type		Fru	Glu	Suc	Man	Xyl	Sor	Mal	Ino	Gly	PPG	TEG
Cigarillo	Mean	1.40	0.98	1.44	0.07	0.06	0.07	0.04	0.19	0.96	1.87	0.18
	Range	0.02–3.73	0.03–2.93	0.003–8.15	0.005–0.21	0.04–0.11	0.03–0.21	ND–0.42	0.06–0.53	0.08–3.38	0.27–7.17	ND–0.42
	SD	1.55	1.15	2.85	0.08	0.02	0.06	0.06	0.21	1.42	2.29	0.15
Little Cigar	Mean	0.35	0.23	0.01	0.01	0.08	0.04	0.01	0.08	0.62	1.10	ND
	Range	0.02–0.65	0.02–0.41	0.003–0.01	0.004–0.01	0.06–0.11	0.03–0.07	ND–0.016	0.06–0.12	0.13–1.79	0.10–2.92	
	SD	0.29	0.29	0.01	0.004	0.01	0.01	0.01	0.02	0.48	0.68	
Cigarette	Mean	2.53	1.21	1.97	0.09	0.06	0.04	0.03	0.47	2.14	0.31	ND
	Range	1.82–4.80	0.65–3.35	0.75–3.00	0.07–0.15	0.02–0.08	0.03–0.06	0.02–0.06	0.41–0.80	0.16–3.74	0.03–0.77	
	SD	0.74	0.59	0.86	0.02	0.01	0.01	0.01	0.08	0.80	0.21	

SD: standard deviation; ND: Not detectable (a value of zero was assigned to ND when calculating the mean); Fru: fructose; Glu: glucose; Suc: sucrose; Man: mannose; Xyl: xylose; Sor: sorbitol; Mal: maltose; Ino: myo-inositol; Gly: glycerol; PPG: propylene glycol; TEG: triethylene glycol.

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