Analysis of Certain Nitrogenous Compounds in Tobacco. Part 1: Adenosine, 2,5- and 2,6-Deoxyfructosazines, Mannosamine and Glucosamine*

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SUMMARY

Nitrogenous compounds such as amino acids and proteins are frequently analyzed in tobacco since they are considered precursors of toxicants in cigarette smoke. However, much less attention is given to other nitrogenous compounds such as amino sugars and deoxyfructosazines, although their concentration in tobacco can be equal to or even higher than that of most free amino acids. These nitrogenous compounds may contribute to the formation of toxicants in smoke, or may contribute to the sensory properties of cigarette smoke, reasons for which their analysis is important. This study describes a procedure for the analysis of adenosine, 2,5- and 2,6-deoxyfructosazines (DFs), mannosamine and glucosamine in tobacco. The analysis uses a liquid chromatography-tandem mass spectrometry (LC/MS/MS) technique. Sample preparation for analysis consists of the extraction of the tobacco with a solution of 90% water and 10% methanol, followed by filtration. The separation of the analytes was done on a hydrophilic interaction liquid chromatography HILIC column using an isocratic procedure with a solvent consisting of 78% CH₃CN, 22% H₂O, that also contained 0.1 % HCOOH and 0.143 g/L CH₃COONH₄. The measurements were done using electrospray positive ionization mass spectrometric detection. The analytical procedure was validated and was proven very reliable. A number of tobaccos were analyzed, including several flue-cured and Burley USA tobaccos, off-shore tobaccos, two Oriental tobaccos, two green tobaccos, as well as tobaccos from commercial and Kentucky reference cigarettes. The ranges for the analytes per g tobacco were found between 0.4 and 20.3 µg/g for adenosine, between 0.0 and 608.5 µg/g for 2,5-DF, between 0.0 and 424.5 µg/g for 2,6-DF, between 12.5 and 415.5 µg/g for mannosamine and between 25.9 and 1885.7 µg/g for glucosamine.

The study also indicated that the levels of DFs and that of the amino sugars in tobacco show a very good correlation.

This correlation can be explained by the same source of the two classes of compounds, namely the reaction of (reducing) sugars and ammonia. [Beitr. Tabakforsch. Int. 24 (2011) 233–242]

KEYWORDS

Adenosine, 2,5- and 2,6-deoxyfructosazines, mannosamine, glucosamine, tobacco, HPLC analysis, HILIC column

ZUSAMMENFASSUNG

Stickstoffhaltige Verbindungen im Tabak, wie Aminosäuren und Proteine, werden häufig untersucht, da sie als Vorläufer für toxische Substanzen im Zigarettenrauch betrachtet werden. Dagegen wird anderen stickstoffhaltigen Verbindungen wie Aminosuktern und Deoxyfructosazinen weniger Aufmerksamkeit gewidmet, obwohl deren Konzentration im Tabak genauso groß oder sogar größer ist, als die von freien Aminosäuren. Dass diese stickstoffhaltigen Verbindungen zu der Bildung von toxischen Substanzen im Tabakrauch oder zu den sensorischen Eigenschaften von Zigarettenrauch beitragen können, sind wichtige Gründe für die Untersuchung. Diese Arbeit beschreibt ein Verfahren zur Analyse von Adenosin, 2,5- und 2,6-Deoxyfructosazinen (DFs), Mannosamin und Glucosamin im Tabak. Zur Analyse wurde eine liquid chromatography-tandem mass spectrometry (LC/MS/MS)-Technik verwendet. Die Probenvorbereitung für die Analyse bestand aus der Extraktion des Tabaks mit einer Lösung aus 90% Wasser und 10% Methanol, gefolgt von einer Filtration. Die Analyten wurden mit Hilfe einer hydrophilic interaction liquid chromatography HILIC-Säule nach einem isokratischen Verfahren getrennt. Das Lösungsmittel bestand aus 78% CH₃CN, 22% H₂O mit 0.1% HCOOH und 0.143 g/L CH₃COONH₄. Die Messungen

wurden mit Hilfe einer Elektrospray-Positiv-Ionisierungsmassenspektrometrie detektiert. Das analytische Verfahren wurde validiert und hat sich als sehr zuverlässig erwiesen. Eine Vielzahl an Tabaken wurde untersucht, darunter einige Flue-cured und US-amerikanische Burley-Tabake, Offshore-Tabake, zwei Orient-Tabake, zwei grüne Tabake, sowie Tabak von kommerziellen Zigaretten und Kentucky Referenzzigaretten. Die gemessenen Analyten pro g Tabak lagen für Adenosin in einem Bereich zwischen 0,4 und 20,3 µg/g, für 2,5-DF zwischen 0,0 und 608,5 µg/g, für 2,6-DF zwischen 0,0 und 424,5 µg/g, für Mannosamin zwischen 12,5 und 415,5 µg/g und für Glucosamin zwischen 25,9 und 1885,7 µg/g.

Die Untersuchung zeigt, dass der Gehalt von DFs und der Gehalt von Aminosukern im Tabak sehr gut miteinander korrelieren. Diese Korrelation kann durch die gleiche Quelle dieser beiden Komponentenklassen, nämlich der Reaktion von (reduzierenden) Zuckern und Ammoniak, erklärt werden. [Beitr. Tabakforsch. Int. 24 (2011) 233–242]

RESUME

Les composés azotés tels que les aminoacides et les protéines sont fréquemment analysés dans le tabac car ils sont considérés comme des précurseurs des produits nocifs dans la fumée de cigarette. Beaucoup moins d'attention est toutefois portée à d'autres composés azotés tels que les sucres aminés et les déoxyfructosazines, bien que leur concentration dans le tabac puisse être aussi élevée, voire même supérieure à celle de la majorité des aminoacides libres. Ces composés azotés pourraient contribuer à la formation de produits nocifs dans la fumée, ou pourraient contribuer aux propriétés sensorielles de la fumée de cigarette. Leur analyse est donc importante. Cette étude décrit une procédure d'analyse de l'adénosine, des 2,5- et 2,6-déoxyfructosazines (DFs), de la mannosamine et de la glucosamine dans le tabac. Cette analyse utilise une technique liquid chromatography-tandem mass spectrometry (LC/MS/MS). La préparation des échantillons pour l'analyse consiste à extraire le tabac à l'aide d'une solution à 90% d'eau et 10% de méthanol, suivie d'une filtration. La séparation des analytes a été réalisée dans une colonne hydrophil interaction liquid chromatography HILIC en employant une méthode isocratique avec un solvant constitué à 78% de CH₃CN, à 22% de H₂O, il contenait également 0,1 % de HCOOH et 0,143 g/L de CH₃COONH₄. Les mesures ont été réalisées par électro-nébulisation ionique positive - spectrométrie de masse. La procédure analytique a été validée et elle s'est avérée très fiable. Plusieurs tabacs ont été analysés, y compris plusieurs tabacs séchés à l'air chaud et des tabacs burley américains, des tabacs off-shore, deux tabacs orientaux, deux tabacs verts ainsi que des tabacs de cigarettes commerciales et Kentucky de référence. Les fourchettes des analytes pro g tabac se situaient entre 0,4 et 20,3 µg/g pour l'adénosine, entre 0,0 et 608,5 µg/g pour 2,5-DF, entre 0,0 et 424,5 µg/g pour 2,6-DF, entre 12,5 et 415,5 µg/g pour la mannosamine et entre 25,9 et 1885,7 µg/g pour la glucosamine.

L'étude a également indiqué une très bonne corrélation entre les taux de DF et de sucres aminés dans le tabac. Cette corrélation peut s'expliquer par la source commune des deux

classes de composés, à savoir la réaction des sucres réduits et l'ammoniaque. [Beitr. Tabakforsch. Int. 24 (2011) 233–242]

INTRODUCTION

Nitrogenous compounds such as amino acids and proteins are frequently analyzed in tobacco since they are considered precursors of toxicants in cigarette smoke, and higher response in the Ames tests have been correlated with higher level of nitrogenous compounds in tobacco (1–4). However, besides amino acids and proteins, much less attention is paid to other nitrogenous compounds such as amino sugars and deoxyfructosazines (DFs).

Their concentration can be equal or even higher than that of most free amino acids in tobacco (5). These compounds could be precursors of toxicants in smoke. Also, the sensory properties of cigarettes may be influenced by these compounds.

The chemical formulas of the compounds analyzed in this study are given in Figure 1. Adenosine is a nucleoside widely distributed in nature, and is also present in tobacco. The formation of DFs is mainly related to reactions between reducing sugars and NH₃, both present in tobacco. Fructose (in open cycle form), for example, reacts with NH₃ to form 2,5-DF as shown in Figure 2. Glucose also reacts with NH₃ to form 2,6-DF as shown in Figure 3. Both reactions are associated with the formation of relatively high yields of browning polymers in which reactive molecules formed as intermediates between the sugar and NH₃ condense to produce macromolecules (6). For this reason, the concentration of DFs in tobacco is likely to be correlated with the level of nitrogenous browning polymers. D-Glucosamine is the biochemical precursor of most nitrogen-containing sugars in biota and humans and is found naturally in the form of glucosamine-6-phosphate (7). Glucosamine-6-phosphate is synthesized from fructose-6-phosphate and glutamine (8). Other sources of amino sugars are also possible.

The analysis of the above indicated nitrogenous compounds in various matrices and using various procedures has been reported in the literature. Adenosine, for example, was analyzed using high-performance liquid chromatography HPLC (9) or capillary electrophoresis (10). Analysis of deoxyfructosazines (DFs) was less frequently reported in the peer reviewed literature (11, 12). Descriptions of analytical procedures for glucosamine are more common because of the health-related implications of this compound. Glucosamine was analyzed by HPLC (13), by HPLC following some type of derivatization (14–19), by HPLC using refractive index detection (20), using LC/MS (21–24), by electrophoresis (25, 26), using high-performance thin-layer chromatography (27), or even by gas chromatography (28). Some of these methods allow the analysis of glucosamine and of mannosamine together (29–32).

The development of a unique method for the analysis of all five analytes with application to a tobacco matrix was the purpose of this study. Results for the analysis of these five analytes in tobacco were not previously published in peer reviewed literature.

Figure 1. Chemical formulas for the compounds analyzed in this study.

Figure 2. Reaction of fructose (in open chain form) with ammonia to form 2,5-DF.

Figure 3. Reaction of glucose (in open chain form) with ammonia to form 2,6-DF.

Table 1. Some tandem mass spectrometry MS/MS conditions used in this analysis.

Compound	Transition from m/z	Transition to m/z	Retention time (min)	Declustering potential (V)	Collision energy (eV)
DHP (I.S.)	113	85	4.98	200	26
Adenosine	268.3	136.2	5.86	60	24
2,6-DF	305	227.3	8.16	60	26
2,5-DF	305	227.3	8.51	60	26
Mannosamine	180	162.2	10.42	60	13
Glucosamine	180	162.2	11.45	60	13

EXPERIMENTAL

Reagents and solvents

Adenosine, mannosamine HCl, glucosamine, HCl and 2,5-dihydroxypyrazine standards as well as glucose, fructose and diammonium phosphate were obtained from Aldrich (Sigma-Aldrich, St.Louis, MO, 63168, USA). Acetonitrile was obtained from Fisher (Fisher-Scientific, Suwanee, GA 30024, USA). 2,5-DF and 2,6-DF are commercially available, but they were synthesized in this study using a reaction between fructose or glucose, respectively, and diammonium phosphate. For this purpose, 1.8 g reducing sugar (glucose or fructose) and 1.32 g $(\text{NH}_4)_2\text{HPO}_4$ were each dissolved in 20 mL water. The solutions were mixed together in a round bottom flask adapted with an ascending condenser. The solutions were boiled for about 3 hours and the solution turned to a yellowish-brown color. The ascending condenser was replaced to a descending one which was also connected to vacuum (around -50 mm Hg) and the reaction mixture was heated at a temperature not exceeding 90 °C until complete elimination of water. The solid dark brown residue was extracted three times with about 10 mL of boiling ethanol. In a few hours and upon cooling, 2,5-DF crystallized from the combined ethanol solutions if fructose was the initial reducing sugar. 2,6-DF was formed if glucose was the reducing sugar. The purity of the product was verified by HPLC using the same separation further discussed for the analysis of samples, but using UV detection at 276 nm. From the peak areas in the chromatograms and assuming the same response factor for the two isomers, it was found that the 2,5-isomer was present at a level of about 5% in the 2,6-isomer, and even a lower level of 2,6-isomer was present in the 2,5-isomer. The two LC chromatograms presented in merge format of synthesized 2,6-DF (ret. time 8.16 min) and of 2,5-DF (ret. time 8.51 min) are given in Figure 4. The use of a refractive

index (RI) detector in the HPLC separation was used to verify that no un-reacted sugar remained in the mixture.

Sample analysis

An amount of 100 ± 0.1 mg finely ground tobacco was weighed in a flask of at least 25 mL to start the analysis. To the sample was added 20 mL extracting solution consisting of H_2O 90% + CH_3OH 10% with an internal standard. The internal standard (I.S.) was 2,5-dihydroxypyrazine (DHP) and its concentration in the extracting solution was 100 $\mu\text{g/mL}$. This compound was selected since it is not present in tobacco, elutes close to the analytes, and has a good MS response. The extraction was performed on a VWR VX-2500 Multi-tube vortexer for 30 min, at room temperature (VWR Scientific Inc., Bristol, PA 19007, USA). A few mL of the sample extract were filtered through a 0.45 μm PVDF filter (Whatman Inc., Florham Park, NJ 07932, USA). Only 0.2 mL of this solution was taken and diluted to 1 mL with a solution that also makes the mobile phase for the HPLC separation. This solution consisted of 78% CH_3CN and 22% H_2O that also contained 0.1 % HCOOH and 0.143 g/L $\text{CH}_3\text{COONH}_4$. The amount of tobacco taken in this final solution was therefore equivalent with 1 mg/mL. The extraction of tobacco first with 20 mL solution followed by further dilution was performed to have a tobacco sample of a size that allows a homogeneous sample, and to save some solvent. A volume of 2 μL diluted extract was injected in a 1200 Agilent HPLC system (Agilent, Wilmington, DE 19808, USA). The system consisted of a degasser, a binary pump, and a WPALS autosampler and was equipped with a Luna 3u HILIC 200A column, 160×4.6 mm from Phenomenex (Phenomenex, Torrance CA 90501, USA). The separation was performed in isocratic mode at a flow rate of 0.5 mL/min. The detector was a MS/MS system API 5000 from AB Sciex (AB Sciex, Foster City, CA 94404, USA). The acquisition was performed in scheduled multiple reaction monitoring (scheduled MRM) mode.

Table 2. Comparison between the results obtained using calibration and standard addition for a flue-cured tobacco sample.

	Adenosine ($\mu\text{g/mL}$)	2,6-DF ($\mu\text{g/mL}$)	2,5-DF ($\mu\text{g/mL}$)	Mannosamine HCl ($\mu\text{g/mL}$)	Glucosamine HCl ($\mu\text{g/mL}$)
Result from calibration	0.00148	0.10563	0.12465	0.27442	0.97701
Addition	0.008	0.0	0.08	0.08	0.08
Expected result	0.00948	0.10563	0.20465	0.35442	1.05701
Result from std. addition	0.00926	0.10694	0.20311	0.35311	1.05732
Difference (%)	-2.34	1.23	-0.76	-0.37	0.029

Figure 4. LC chromatograms of synthesized 2,6-DF (ret. time 8.16 min) and of 2,5-DF (ret. time 8.51 min) presented in merge format, as measured by UV detection at 276 nm.

Figure 5. Typical LC/MS/MS chromatogram obtained for a standard solution containing 2.0 $\mu\text{g/mL}$ I.S., 0.025 $\mu\text{g/mL}$ adenosine, and 0.25 $\mu\text{g/mL}$ of 2,6-DF, 2,5-DF, mannosamine HCl and glucosamine HCl (retention times related with the name of the compound given in Table 1).

Figure 6. Typical LC/MS/MS chromatogram obtained for a tobacco sample from 3R4F Kentucky reference cigarette (retention times related with the name of the compound given in Table 1).

Table 3. Results of five measurements of a flue-cured tobacco sample.

Replicate	Adenosine (µg/g)	2,6-DF (µg/g)	2,5-DF (µg/g)	Mannosamine HCl (µg/g)	Glucosamine HCl (µg/g)
1	0.69	54.08	62.88	135.68	468.43
2	0.79	53.55	61.78	138.74	508.59
3	0.75	54.95	57.12	136.60	498.56
4	0.71	58.84	61.50	134.64	463.18
5	0.64	59.21	58.43	130.42	484.23
Average	0.72	56.12	60.34	135.22	484.60
RSD (%)	6.69	4.30	3.62	2.03	3.56

Some of the conditions used for the MS/MS system are shown in Table 1. Other conditions for the MS/MS included source temperature 700 °C, collision gas 10 mL/min, curtain gas 14 mL/min, ion source gas [1] 45 mL/min, ion source gas [2] 55 mL/min, entrance potential 10 eV, collision cell exit potential 10 eV. Under these conditions, a typical chromatogram for a solution of standards containing 0.025 µg/mL adenosine, and 0.25 µg/mL of 2,6-DF, 2,5-DF, mannosamine HCl and glucosamine HCl is shown in Figure 5 (the compounds identification can be obtained from the associated retention

time shown in Table 1).

The quantitation for each analyte was obtained using calibration with six standard concentrations and using linear equations of the form:

$$\text{Conc}_i (\mu\text{g/mL}) = A_i (\text{Analyte}_i \text{ Peak Area/I.S. Peak Area})$$

(where different A_i values were obtained for each analyte). The standards had a concentration between 0.00125 µg/mL and 0.4 µg/mL for adenosine, and between 0.025 µg/mL and 4.0 µg/mL for the other analytes. The R^2 values for the calibration lines were between 0.9968 (for adenosine) and 0.9994 (for glucosamine). This indicated excellent linearity in a wide range of concentrations.

Table 4. List of analyzed samples.

Sample	Acronym	Description
1	FC low (A)	Lower stalk (lug) flue-cured A
2	FC up (A)	Upper stalk (leaf & some tips) flue-cured A
3	FC low (B)	Lower stalk (lug) flue-cured B
4	FC up (B)	Upper stalk (leaf & some tips) flue-cured B
5	FC low off	Off-shore lower stalk (lugs & primings) flue-cured
6	FC up off	Off-shore upper stalk (leaf & tips) flue-cured
7	Bur. low (A)	Lower stalk (flyings & cutters) burley A
8	Bur. up (A)	Upper stalk (leaf) burley A
9	Bur. low (B)	Lower stalk (flyings & cutters) burley B
10	Bur. up (B)	Upper stalk (leaf) burley B
11	Bur. low off	Off-shore lower stalk (flyings & cutters) burley
12	Bur. up off	Off-shore upper stalk (leaf) burley
13	Samsun	Middle to upper stalk, Samsun Oriental
14	Izmir	Middle to upper stalk, Izmir Oriental
15	Camel	Camel Blue
16	M. Red old	Old Marlboro Red (2 years sample)
17	Galpao green	Galpao green tobacco
18	FC green	Flue-cured green tobacco
19	Newport	Newport
20	2R4F	2R4F (Kentucky reference)
21	M. Red	Marlboro Red
22	1R4F	1R5F (Kentucky reference)
23	3R4F	3R4F (Kentucky reference)
24	1R4F	1R4F (Kentucky reference)

Validation of the analytical procedure

Several steps were taken in order to provide information regarding the selectivity, precision, repeatability, limit of detection and quantitation, and recovery of the proposed analytical procedure (33). The linearity in a specified range of concentrations was already indicated previously. The selectivity of the separation was determined using standard addition on a lower stalk (lug) flue-cured tobacco sample. The analytes in the sample were measured using the previously established calibration, and then in 9.8 mL sample was added 0.2 mL solution containing 0.4 µg/mL adenosine and 4 µg/mL 2,5-DF, mannosamine and glucosamine, respectively. The results were calculated by extrapolation to zero intercept and are shown in Table 2. As seen from this table, the agreement between the results obtained using the calibration and those using standard addition were very good.

This result indicates that no interference is present in the separation. Based on the results obtained using the standard addition procedure for analysis the method described in this study was also considered to be accurate. However, no results in the peer reviewed literature were available for comparison and accuracy assessment.

The precision of the method was estimated by analyzing the same sample 5 times. The analyzed sample was that of a lower stalk (lug) flue-cured tobacco, and the relative standard deviations (RSD%) for the results are given in Table 3. As seen from this table, the RSD% for adenosine is around 6.7%, and for the other analytes is below 5%.

The repeatability of the method was verified over a period of one month. The repeated injection of the standard containing 0.025 µg/mL adenosine, and 0.25 µg/mL of 2,6-DF, 2,5-DF, mannosamine HCl and glucosamine HCl

Table 5. Results (average of duplicates) for the analyzed samples expressed in mg/g tobacco (as is).

Sample #	Tobacco type (acronym)	Adenosine ($\mu\text{g/g}$)	2,6-DF ($\mu\text{g/g}$)	2,5-DF ($\mu\text{g/g}$)	Mannosamine, free ($\mu\text{g/g}$)	Glucosamine, free ($\mu\text{g/g}$)
1	FC low (A)	0.74	52.82	62.33	114.63	408.13
2	FC up (A)	1.10	45.70	54.81	112.52	459.64
3	FC low (B)	2.90	41.87	54.64	90.26	332.25
4	FC up (B)	1.06	34.09	39.74	87.46	354.40
5	FC low off	2.05	86.12	97.37	88.74	570.88
6	FC up off	3.55	87.23	87.86	60.33	398.73
7	Bur. low (A)	0.35	12.64	36.34	30.50	121.64
8	Bur. up (A)	0.43	27.60	79.53	52.59	207.36
9	Bur. low (B)	0.71	16.13	53.54	25.33	133.95
10	Bur. up (B)	0.47	10.91	40.15	16.10	67.50
11	Bur. low off	1.32	14.12	56.24	24.02	128.75
12	Bur. up off	1.28	31.82	108.26	43.57	215.76
13	Samsun	0.75	209.90	324.64	298.60	1185.77
14	Izmir	2.08	36.97	35.10	59.79	255.03
15	Camel	11.31	140.99	227.51	308.22	992.83
16	M. Red old	20.26	179.14	288.86	415.46	1401.82
17	Galpao green	0.58	0.00	0.02	12.55	25.94
18	FC green	0.66	0.00	0.22	34.85	97.69
19	Newport	13.71	225.24	355.05	293.07	1093.27
20	2R4F	8.33	117.58	174.74	259.17	882.07
21	M. Red	16.69	167.22	286.32	304.69	1008.97
22	1R4F	10.46	424.46	608.49	404.27	1885.75
23	3R4F	15.01	78.67	121.04	214.01	673.18
24	1R4F	14.80	303.17	342.91	256.96	1388.85

showed less than 8% variability for a number of 15 injections for all five analytes. The sensitivity of the method was estimated based on the signal to noise (S/N) values. For the lowest standard (0.00125 $\mu\text{g/mL}$ adenosine, and 0.025 $\mu\text{g/mL}$ of 2,6-DF, 2,5-DF, mannosamine HCl and glucosamine HCl) the values were S/N = 30.4 for adenosine, S/N = 22.8 for 2,6-DF, S/N = 38.2 for 2,5-DF, S/N = 12.4 for mannosamine and S/N = 8.5 for glucosamine. These values indicated a very good sensitivity of the method.

Except for glucosamine with a S/N slightly below 10, the values for the concentrations of the lowest standard were well suited for quantitation. However, the lowest standard for glucosamine was kept in the calibration curve since it did not affect the linearity. Since, except for the sample extraction, no sample preparation was performed in the method described in this study, the recovery was evaluated only from the point of view of the optimum solvent used for sample extraction. All analytes being readily soluble in water, this was considered the ideal solvent for extraction. However, water also dissolves some polymeric materials from tobacco, and these polymers may affect in time the chromatographic column. For this reason, a solution 90% water with 10% methanol was selected as solvent. The comparison of the results obtained by using the two solvents showed no significant difference in the analyte extraction.

RESULTS AND DISCUSSION

A number of 24 tobacco samples was analyzed in duplicate using the procedure previously described. A typical LC chromatogram obtained for tobacco from a 3R4F Kentucky reference cigarette is shown in Figure 6 (retention times from Table 1 indicate the nature of compounds).

The analyzed samples consisted of a number of single grade tobaccos, two green tobaccos (dried in air) and a number of tobaccos from commercial and Kentucky reference cigarettes. The list of the analyzed samples with a short description of the material is given in Table 4. The results of analytes content (on a “as is basis”) is given in Table 5 (free base mannosamine and glucosamine are reported). All the samples were analyzed in duplicate. The relative standard deviations (RSD%) were typically around 5% or lower for all samples and all the analytes. Only a few results for adenosine showed RSD% values between 5% and 8%. Although obtained from only two replicates which provides a limited level of confidence, the low values of RSD% for all samples are an additional indication of the good precision of the analysis.

A large range of levels of the analytes was noticed among samples. Except for an oriental tobacco sample (Sample 13) most analytes were found in general at higher levels in cigarette tobaccos compared to single grade tobaccos. The

Figure 7. Linear correlation between the levels of glucosamine and that of 2,5-Df in the analyzed samples.

Figure 8. Reaction of fructose (in open chain form) with ammonia to form glucosamine and mannosamine.

Figure 9. Reaction of glucose (in open chain form) with ammonia to form glucosamine (mannosamine can be formed by the same mechanism).

green tobaccos were very low in all nitrogenous compounds analyzed in this study.

The levels of 2,5-DF and those of 2,6-DF correlate well, as expected. The linear correlation of their levels in the analyzed samples showed a $R^2 = 0.9556$. As expected, adenosine levels do not correlate well either with the DFs or with the amino sugars (e.g. $R^2 = 0.584$ for adenosine vs. glucosamine). Glucosamine and mannosamine also showed a good linear correlation, with a $R^2 = 0.9198$. Not expected was that the correlation between the DFs and amino sugars levels was also very good. For example, glucosamine and 2,5-DF levels showed a linear correlation with $R^2 = 0.906$. The graph of this correlation is shown in Figure 7.

This good correlation may indicate that the source of glucosamine in tobacco may be related to a source similar to that responsible for producing DFs, in addition to a potential synthesis from fructose 6-phosphate and glutamine (8). One such path is the reaction of a reducing sugar and ammonia. The reaction starting with an open cycle form of fructose for the formation of 2-amino-2-deoxy glucose and of 2-amino-2-deoxy mannose may take place as shown in Figure 8. This reaction can explain both the good correlation in the levels of glucosamine and mannosamine, as well as the correlation between the DFs and the amino sugars.

The formation of glucosamine and mannosamine starting with glucose as the originating reducing sugar, may also take place following reactions as shown in Figure 9 for glucosamine (mannosamine is formed by the same reaction). Other reaction paths are also possible for the formation of amino sugars from reducing sugars and ammonia.

CONCLUSIONS

A new LC/MS/MS technique using a HILIC column for separation was developed and validated for the analysis of adenosine, deoxyfructosazines, mannosamine and glucosamine in tobacco. The method is simple, very sensitive and with excellent repeatability. A number of tobacco samples containing single grades tobaccos, two green tobaccos and a number of tobaccos from commercial and Kentucky reference cigarettes were analyzed. The RSD% for the data were typically around 5% or lower for all samples and all the analytes. Only a few results for adenosine showed RSD% values between 5% and 8%.

A large range of levels of the analytes was noticed among the sample. Except for an oriental tobacco sample most analytes were found in general at higher levels in cigarette tobaccos compared to single grade tobaccos. The green tobaccos were very low in all nitrogenous compounds analyzed in this study.

The study also indicated that the levels of DFs and that of the amino sugars in tobacco show a very good correlation. This correlation can be explained by the presence of the same source of the two classes of compounds, namely the reaction of (reducing) sugars and ammonia. The good correlation between glucosamine and mannosamine can also be explained by the formation of the two compounds from fructose.

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