Evaluation of the Content of Free Amino Acids in Tobacco by a New Liquid Chromatography-Tandem Mass Spectrometry Technique*

by

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SUMMARY

The present study describes a reliable technique for the analysis of free amino acids in tobacco leaf. The levels of amino acids in tobacco are important since they are related to both tobacco quality and the potential generation in cigarette smoke of toxicants having amino acid precursors. Other techniques used in the past for amino acid analysis have various shortcomings that were avoided in the present method. The new method uses HPLC separation and a tandem mass spectrometer for detection with no derivatization step as sample preparation. The separation has been obtained using ion pair HPLC on a reversed phase column that offers excellent chromatographic resolution. The MS/MS detection procedure offers very good sensitivity and positive identification of the analytes. The procedure was fully validated and can be used for the analysis of 24 amino acids. It was applied for the quantitation of amino acids from 16 types of tobacco including flue-cured and Burley, some domestic and some not grown in the USA, two types of Oriental tobacco, and from tobacco of a 3R4F Kentucky reference and a common commercial cigarette. It was shown that the analysis provides useful information regarding the amino acid level variation between tobacco types, between tobacco stalk positions, and between the growing locations of different tobaccos. [Beitr. Tabakforsch. Int. 26 (2015) 334–343]

ZUSAMMENFASSUNG

Die vorliegende Studie beschreibt ein zuverlässiges Verfahren zur Analyse der freien Aminosäuren in Tabakblättern. Dabei ist der Gehalt an Aminosäuren im Tabak wichtig, da er sowohl mit der Qualität des Tabaks als auch mit der möglichen Entstehung von Giftstoffen im Zigarettenrauch, deren Vorläufer Aminosäuren sind, im Zusammenhang steht. Andere in der Vergangenheit eingesetzte Verfahren zur Analyse von Aminosäuren haben verschiedene Nachteile, die in dem vorliegenden Verfahren umgangen wurden. Bei der neuen Methode kommen die HPLC-Trennung und ein Tandem-Massenspektrometer ohne Derivatisierungsschritt zur Probenvorbereitung für den Nachweis zum Einsatz. Die Trennung wurde mittels Ionenpaar-HPLC an einer Umkehrphasensäule mit hervorragender chromatographischer Auflösung erreicht. Das MS/MS-Nachweisverfahren bietet eine sehr gute Sensitivität und positive Identifizierung der Analyten. Das Verfahren wurde vollständig validiert und kann für die Analyse von 24 Aminosäuren verwendet werden. Es wurde für die Quantifizierung von Aminosäuren von 16 Tabaksorten eingesetzt, darunter Flue-cured und Burley, einige in den USA einheimische und einige nicht in den USA wachsende Sorten, zwei Arten von Orienttabak sowie Tabak einer Kentucky-Referenzzigarette 3R4F und einer handelsüblichen Zigarette. Dabei wurde gezeigt, dass die Analyse nützliche Informationen zur Variation des Aminosäuregehalts zwischen Tabaksorten, Tabakstängel-Positionen und den Anbaubedingungen verschiedener Tabaksorten ergibt. [Beitr. Tabakforsch. Int. 26 (2015) 334–343]

RESUME

La présente étude décrit une technique fiable d'analyse des acides aminés libres présents dans la feuille de tabac. Les teneurs en acides aminés sont d'importance dans le tabac puisqu'elles sont liées tant à la qualité du tabac qu'à la production potentielle, dans la fumée de tabac, de substances toxiques ayant des acides aminés précurseurs. D'autres techniques utilisées par le passé lors de l'analyse des acides aminés souffrent de diverses lacunes que la présente méthode évite. Cette nouvelle approche fait appel à la séparation en CLHP ainsi qu'à un spectromètre de masse en tandem (MS/MS) pour la détection sans passer par l'étape de dérivation pour préparer l'échantillon. La séparation est obtenue grâce à une colonne CLHP en phase inverse avec appariement d'ions, qui offre une excellente résolution chromatographique. La procédure de détection MS/MS offre une très bonne sensibilité et une identification positive des analytes. La procédure est pleinement validée et peut être utilisée pour l'analyse de 24 acides aminés. Elle a été appliquée à l'analyse chimique quantitative d'acides aminés provenant de 16 types de tabac, notamment du tabac jaune et du tabac Burley, certains tabacs cultivés aux USA et d'autres cultivés ailleurs, deux types de tabac d'Orient, du tabac de cigarettes de référence Kentucky 3R4F et une cigarette ordinaire vendue dans le commerce. Il a été démontré que l'analyse apporte des informations utiles quant à la variation de la teneur en acides aminés selon le type de tabac, la position des tiges de tabac et le lieu de culture des différents tabacs. [Beitr. Tabakforsch. Int. 26 (2015) 334–343]

KEYWORDS

Amino acids, tobacco, LC-MS/MS, HPLC-fluorescence

INTRODUCTION

The level of amino acids in tobacco is an important parameter for leaf characterization, both related to tobacco quality (1–3) and also related to the generation of toxicants in cigarette smoke (4–5). The smoke sensory properties are affected by the amino acids either directly, or through the further formation of Amadori compounds (1). Among the main toxicants having amino acids as a precursor are hydrogen cyanide (6), acrylamide (7, 8), and heterocyclic amines (6). For these reasons, research regarding amino acid analysis in tobacco leaf has been active over a long period of time (9, 10). Also, a considerable number of analytical methods are reported in the literature for the analysis of either free amino acids (12, 13) or of amino acids in proteins after protein hydrolysis (13). Analysis of amino acids is a challenging subject since the GC (GC-MS) analysis is not possible without derivatization, and for HPLC, the conventional ultra violet (UV) or fluorescence detection of underivatized amino acids is not applicable. The amino acid molecules do not contain chromophores or fluorophores (except for a few amino acids containing aromatic groups that have strong UV absorption) and their detection cannot be done by UV or fluorescence. For this

reason HPLC is also frequently performed after derivatization. Among the most common procedures of derivatization for HPLC analysis is the reaction with *o*-phthalaldehyde (OPA) for amino acids containing a primary amino group and with fluorenylmethyl chloroformate (FMOC) for amino acids containing a secondary amino group (14, 15). However, derivatization adds an important source of errors since it implies an additional processing step for the samples, and typically the derivatized amino acids are not stable in time. For this reason, present work had the goal of developing an analytical technique for amino acid analysis without involving derivatization. An additional problem with the analysis of amino acids without derivatization is related with their separation. Hydrophilic interaction chromatography (HILIC) type columns and ion-exchange columns can be used for free amino acid separation, but the peak resolution in these cases is typically lower than that for reversed phase (RP) (16, 17). For allowing a separation of amino acids on a C18 column, the use of ion pairing was considered the best solution. The present study describes a new method for free amino acid analysis by a novel HPLC separation using ion pairing and a reversed-phase (RP) chromatographic column, followed by mass spectrometric detection (MS/MS detection in multiple reaction monitoring, MRM mode). The method offers a number of advantages such as no need for derivatization, excellent peak shape in the chromatogram, good separation, and positive identification of each amino acid in the tobacco complex matrix.

EXPERIMENTAL

Materials

Various amino acids including asparagine, glutamine, tryptophan and 3,3,3-*d*₃-alanine, trifluoroacetic acid, hydrochloric acid (37%), and acetonitrile (CHROMASOLV® Plus) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Heptafluorobutyric acid was purchased from TCI America (Portland, OR, USA). Pure water (18.2 mΩ/cm) from a Barnsted Nanopure unit (Thermo Scientific, Waltham, MA, USA) was used for the preparation of solutions. The "Amino Acid Standard H" with 2.5 μmol/mL concentration of several amino acids was purchased from Thermo Fisher Scientific (Rockford, IL, USA). Autovial 0.45 μm pore PVDF filters (Whatman, Clifton, NJ) were used for sample filtration.

Instrumentation

The extraction of the samples was performed on a wrist action shaker (Burrell Co., Pittsburgh, PA, USA). The separation was performed on an Agilent 1200 HPLC binary system (Agilent Technologies Inc., Wilmington, DE, USA). The HPLC system consisted of a binary pump, an autosampler with cooling capability, and a column thermostatted compartment. The chromatographic column was a Dionex Acclaim™ RSLC Polar Advantage II, 2.1 × 250 mm with 2.2 μm particles (from Thermo Scientific). This is a silica based C18 column with a patented bonding chemistry that provides enhanced hydrolytic stability from pH 1.5–10

and allows resolution of a wide variety of polar and nonpolar analytes. The detection of the analytes was performed using an API-5000 triple quadrupole mass spectrometer (AB Sciex, Framingham, MA, USA).

Extraction of amino acids from tobacco leaf

About 5 g of tobacco (cut filler) was finely ground using a coffee grinder. The samples were kept before analysis for 48 h in a conditioned room at 21 ± 0.5 °C and $60 \pm 2\%$ relative humidity. This procedure brings samples to a common value for water activity. The amino acids were extracted using an aqueous solution of 0.1 M HCl. The solution also contained 25 $\mu\text{mol/L}$ (0.2286 $\mu\text{g/mL}$) d_3 -alanine as an internal standard. Another internal standard used optionally was 25 $\mu\text{mol/L}$ phenylglycine (0.3807 $\mu\text{g/mL}$). Phenylglycine is not present in tobacco. For the extraction, 50 mg of finely ground tobacco (conditioned as previously indicated) were precisely weighed (± 0.1 mg) into a 50 mL vial. To this tobacco, 25 mL extracting solution was added. The vial with the tobacco and the extracting solution was then subject to mechanical agitation for 30 min, e.g., on a wrist action shaker. A portion of the resulting extract was filtered through a 0.45 μm PVDF filter. The samples were stable for at least 48 h at 10 °C. An alternative extraction procedure uses only 5 mL extracting solution and 50 mg tobacco in a 20 mL vial and mechanical agitation. This solution can be used for the analysis of amino acids in tobacco, except for proline. Proline is at a high level in flue-cured and Oriental tobaccos, and the proline peak in the chromatogram is overloaded and not possible to quantitate. However, a modification in the MS/MS acquisition conditions (decrease of the collision cell potential for proline measurement from 15 V to 5 V) allowed the use of more concentrated solution for analysis.

HPLC separation

The amino acids were separated using ion pairing on the Acclaim™ RSLC Polar Advantage II column 2.1×250 mm with 2.2 μm particles. The samples were kept in the auto-sampler at 10 °C. The separation was performed at a flow rate of 0.2 mL/min using gradient. Solution A was water containing 1% acetonitrile, 0.5% heptafluorobutyric acid, 0.02% trifluoroacetic acid (by volume), and solution B was 0.1% trifluoroacetic acid in acetonitrile (by volume). The gradient conditions started with 0% solution B, linear increase to 5% solution B from 5 min to 7 min, linear increase to 60% B from 7 min to 18 min and kept at 60% B for 3 more min. The column is reset to 5% B and then to 0% B. An equilibration time of 4 min was necessary before each run. The rather long equilibration time was necessary in the separation since the flow was relatively low, and the column is 250 mm long. The column temperature was kept at 20 °C. The injection volume was 3 μL . Needle wash with methanol was performed before every injection.

Mass spectral detection

The detection of the analytes was performed using a triple quadrupole mass spectrometer, working in multiple reaction monitoring (MRM) mode. The parameters for the detection

Table 1. Parameters for the MS detection.

Parameter	Value	Units
Polarity	positive	—
Collision gas (CAD)	5	mL/min
Curtain gas (CUR)	10	mL/min
Ion source gas 1 (GS1)	55	mL/min
Ion source gas 2 (GS2)	55	mL/min
Ion spray voltage (IS)	5500	V
Temperature (TEM)	450	°C
Interface heater (ihe)	off	—
Declustering potential (DP)	60.0	V
Entrance potential (EP)	4.0	V
Collision energy (CE) for all amino acids	15.0	eV
Collision energy (CE) for proline when using extraction of 50 mg tobacco in 5 mL solution	5.0	eV
Collision cell exit potential (CXP)	13.0	V
Acquisition time per ion	50	ms
Total acquisition time	25	min

Table 2. Transitions and retention times for the amino acids.

No.	Amino Acid	Abbr.	Precursor ion	Product ion	Retention time (min)
1	Valine	Val	72	55	17.21
2	Glycine	Gly	76	30	4.53
3	Alanine	Ala	90	44	5.52
4	Sarcosine	Sar	90	30	5.97
5	d_3 -Alanine	d_3 -Ala	93	47	5.48
6	Serine	Ser	106	88	4.50
7	Proline	Pro	116	70	8.52
8	Threonine	Thr	120	56	5.19
9	Cysteine	Cys	122	87	12.01
10	Isoleucine	Ile	132	86	19.93
11	Leucine	Leu	132	86	20.25
12	Hydroxyproline	Hyp	132	86	4.63
13	Asparagine	Asn	133	74	4.46
14	Ornithine	Orn	133	70	19.90
15	Aspartic acid	Asa	134	88	4.88
16	Glutamine	Gln	147	101	4.81
17	Lysine	Lys	147	84	16.17
18	Glutamic acid	Glu	148	84	5.77
19	Methionine	Met	150	133	18.19
20	Phenylglycine	Phg	152	106	19.20
21	Arginine	Arg	175	70	19.50
22	Histidine	His	156	110	17.60
23	Phenylalanine	Phe	166	120	21.09
24	Citrulline	Cit	176	70	19.25
25	Tyrosine	Tyr	182	165	19.56
26	Tryptophan	Trp	205	188	22.18

of amino acids are given in Table 1. The transitions for MRM mode of analysis (mass of precursor ion and product ion), and the retention time for 26 amino acids that were evaluated in this study are given in Table 2. A typical chromatogram obtained for a solution of standards containing 25 $\mu\text{mol/L}$ standards is shown in Figure 1. The separation of the amino acids was good, and since each amino acid has a specific transition, some overlapping of the chromatographic peaks poses no problem.

Figure 1. Chromatogram for a mixture of amino acids containing 25 µmol/L standards.

Quantitation

For the quantitation of the amino acids, five standards were prepared, containing all the amino acids at concentrations in the range 0.25 to 50 µmol/L of each amino acid. From the list of amino acids indicated in Table 2, the standards for citrulline, ornithine, and sarcosine were absent (d_3 -alanine and phenylglycine were added as internal standards). The calibration for some amino acids was linear, but for other amino acids a quadratic calibration was necessary. The equations of the form

$$Y = aX^2 + bX$$

where Y is the concentration of the amino acid in mg/mL and X is the peak area for the calibration lines are listed in Table 3. The R^2 of the trendlines are also shown in the table (the equations of trendlines and R^2 values were obtained using Microsoft Excel capabilities). Most R^2 values in Table 3 are higher than 0.99. Only isoleucine showed $R^2 = 0.97580$, leucine showed $R^2 = 0.98168$ and phenylalanine (including a high concentration point) showed $R^2 = 0.94280$. The calibration curves were obtained by plotting the levels of standards versus the corresponding peak areas for each analyte without normalization by an internal standard. The internal standards have been used only for verifying the reproducibility of the chromatograms. The two internal standards showed small variations not larger than $\pm 5\%$. Since no sample preparation (except for extraction) has been applied to the sample, the internal standard was just one other analyte with its own variability, and it was not desirable to impose its variability to all other compounds. In addition to the equations of the calibrations, Table 3 also shows the signal to noise S/N of the peaks of

individual amino acids for their lowest standard (0.25 µmol/L). As shown in Table 3, the R^2 values for the calibrations are very good, except for phenylalanine where the range was limited between 0.25 µmol/L and 25 µmol/L for achieving better correlation.

Validation

Several aspects related to the validation of the LC-MS/MS method were evaluated following recommendation provided in the literature (18). The selectivity of the method is very good, the combination of HPLC separation and MS/MS detection being sufficient for the elimination of interferences.

The precision of the method is also very good, as further shown for a set of tobaccos analyzed by this technique (see section Results and Discussion). The analysis in triplicate of 16 tobacco types for 21 amino acids, led to RSD% typically below 5% and for some amino acids at low levels to RSD between 5% and 10%. From 336 different averages (different samples and different amino acids) one RSD% value was 10.3% for isoleucine in a flue-cured sample, and one was 10.6% (for cysteine in a Burley sample).

The evaluation of linearity in the LC-MS/MS method showed that a number of amino acids generate quadratic responses in the technique and only quadratic curves displayed R^2 values higher than 0.90 as shown in Table 3. However, all amino acids used for the calibrations were at equal concentration. This is not the case for tobacco samples that may contain large levels of asparagine, aspartic acid, and proline, and much lower levels of other amino acids. In addition to that, the MS/MS response for different analytes is very different and may differ for the

Table 3. Equations for the calibration curves. $Y = aX^2 + bX$, where Y is the concentration of the amino acid in mg/mL and X is the peak area. The R^2 of the trendlines for the calibrations are also shown, as well as the signal to noise S/N for the lowest standard (0.25 $\mu\text{mol/mL}$).

No.	Amino acid	a	b	R^2	S/N
1	Alanine	— ^a	5.7399 e-8	0.99129	23.8
2	Arginine	—	1.8576 e-7	0.99594	11.2
3	Asparagine	1.2443 e-15	5.6202 e-8	0.99940	94.4
4	Aspartic acid	2.1454 e-15	7.0439 e-8	0.99870	152.4
5	Citrulline	—	—	—	11.5
6	Cysteine	—	1.2308 e-5	0.99727	7.1
7	d3-Alanine	—	—	—	—
8	Glutamic acid	—	4.0667 e-8	0.99743	137.3
9	Glutamine	1.5253 e-14	1.4082 e-7	0.99838	81.8
10	Glycine	1.8604 e-13	3.5670 e-7	0.99552	16.2
11	Histidine	2.6872 e-16	1.5928 e-8	0.99625	338.6
12	Hydroxyproline	1.7397 e-16	4.1807 e-9	0.99742	590.8
13	Isoleucine	3.7915 e-16	2.4138 e-9	0.97580	552.6
14	Leucine	3.7538 e-16	1.0648 e-8	0.98168	458.5
15	Lysine	—	4.5148 e-8	0.99911	106.8
16	Methionine	—	3.3102 e-8	0.99330	1435.1
17	Ornithine	—	—	—	—
18	Phenylalanine	5.3655 e-16	-7.2185 e-9	0.94280	—
18a	Phenylalanine (low range)	2.9297 e-16	-7.3718 e-10	0.99288	609
19	Phenylglycine	—	—	—	—
20	Proline	1.7842 e-17	5.9270 e-9	0.99883	128.1
20a	Proline (low range)	—	1.4997 e-8	0.99784	—
21	Sarcosine	—	—	—	—
22	Serine	2.6751 e-13	4.3417 e-9	0.99890	11.1
23	Threonine	—	9.9900 e-8	0.99881	79.1
24	Tryptophan	2.8858 e-16	3.7112 e-9	0.99055	2611.1
25	Tyrosine	7.8556 e-16	4.2847 e-8	0.99840	477.4
26	Valine	—	6.6510 e-8	0.99600	14.9

^a — indicates not applicable, since these amino acids were not quantitated.

same analyte concentration, by two orders of magnitude. For these reasons, an appropriate choice of concentration for the standards may further improve R^2 values for the calibrations.

The extraction with 0.1 N HCl for the free amino acids has been demonstrated in other methods for amino acid analysis (14) and data from long experience in the laboratory indicated that 30 min extraction time are sufficient for the complete extraction of many soluble analytes. For this reason only limited experiments were performed to evaluate extraction efficiency, which was performed in the study only for one tobacco type (lower stalk flue-cured). The variation in the level of three amino acids (asparagine, aspartic acid, and proline) as a function of extraction time is shown in Figure 2 (triplicate measurements). As expected, a time of extraction of about 30 min was sufficient for a complete extraction for the analyzed amino acids. A longer-than-one-hour extraction time indicated a slight decrease in the measured level of asparagine and an increase in aspartic acid, very likely due to hydrolysis of a small proportion of asparagine to aspartic acid. The same effect was noticed for the glutamine/glutamic acid pair. The recovery of the analytical procedure was also evaluated in the study. The % recovery of the amino acids for a flue-cured tobacco sample was evaluated using two spiking levels. For this purpose, three samples of 50 mg tobacco were analyzed in duplicate. The first sample did not have

any addition of amino acids. The second sample was extracted with 45 mL extracting solution, and 5 mL of a standard containing 2.5 $\mu\text{mol/L}$ of each amino acid was added. The third sample was extracted with 45 mL extracting solution and 5 mL of a standard containing 25 $\mu\text{mol/L}$ of each amino acid was added. The three extracts were analyzed and the extraction efficiency was calculated as the ratio of analyzed amino acid level vs.

Figure 2. Variation with extraction time for the measured level of three amino acids in a flue-cured tobacco (triplicate measurement).

Table 4. Recovery (%) for amino acids spiked at two levels on a cured tobacco sample.

Compound	Tobacco A	
	Low spiked	High spiked
Alanine	105.7	107.7
Arginine	100.0	107.2
Asparagine	82.8	101.1
Aspartic acid	96.8	96.3
Glutamic acid	108.9	100.5
Glutamine	103.4	111.4
Glycine	86.8	86.0
Histidine	101.2	108.0
Isoleucine	100.9	97.1
Leucine	79.1	83.7
Lysine	102.2	106.1
Methionine	97.7	100.4
Phenylalanine	102.7	89.4
Proline	120.6	97.3
Serine	91.3	91.3
Threonine	106.6	108.6
Tryptophan	103.5	103.0
Tyrosine	98.2	99.2
Valine	100.8	98.8

calculated level. The recovery (%) values for each amino acid are shown in Table 4.

The accuracy of the present method (LC-MS/MS) has been compared with that of a HPLC-fluorescence method (LC-FLD) with derivatization using *o*-phthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC) described in the literature (11) that was previously implemented in the laboratory. However, the comparison was not possible for all amino acids analyzed by LC-MS/MS. This was caused by the fact that the LC-FLD method is less sensitive than the LC-MS/MS method and showed for some amino acids results below detection limit (e.g., for leucine and iso-

leucine) or because specific amino acids were not analyzed in the LC-FLD method (e.g., tryptophan and hydroxyproline). The comparison showed that proline and lysine have large differences between the two methods. Proline levels indicated by the LC-MS/MS method were significantly larger (up to 50%) than those detected by the LC-FLD. Proline having a secondary amine group, does not react with OPA and was detected based on its derivatized form with FMOC. The peak of proline elutes late in the LC-FLD separation and its measurement may encounter difficulties. For this reason, the LC-MS/MS procedure was considered to have significantly more reliable results for proline. In the case of lysine, the results for LC-FLD were larger than those obtained by the LC-MS/MS method (with RSD% of 30%–50%). Lysine having two amine groups may react differently than other amino acids during derivatization, and its peak in LC-FLD procedure is known to show larger variability than for other amino acids. For the other amino acids where data were available by both methods, the agreement was very good, with RSD% differences (from the average of results from both methods) being usually less than 10%. An example of comparison between the LC-FLD and LC-MS/MS method for two different tobaccos is illustrated in Table 5.

The limit of quantitation LOQ (and implicitly the limit of detection LOD) were both very different from one amino acid to another. The S/N values for the lowest standard (0.25 $\mu\text{mol/L}$) were shown in Table 3. Considering that the LOQ corresponds to $S/N \approx 10$ (see, e.g. (19)), it can be seen that for all amino acids the condition $S/N > 10$ is fulfilled at 0.25 $\mu\text{mol/L}$ concentration (lowest standard). Some amino acids have a much larger S/N values than 10 at this concentration and therefore they can be measured at lower concentrations. In addition, the sample preparation requires a dilution of 1/500 from sample weight to volume of solvent. This dilution factor can be decreased significantly if a better sensitivity is necessary.

Table 5. Example of comparison for the levels of amino acids in mg/g between the LC-FLD and LC-MS/MS methods for two different tobaccos.

Amino acid	Tobacco A (mg/g)		Difference % FLD - MS	Tobacco B (mg/g)		Difference % FLD - MS
	LC-FLD	LC-MS/MS		LC-FLD	LC-MS/MS	
Alanine	0.578	0.575	0.5	0.528	0.530	-0.4
Arginine	0.150	0.130	14.3	0.150	0.147	2.0
Asparagine	0.431	0.446	-3.4	0.493	0.471	4.6
Aspartic acid	4.948	4.490	9.7	9.830	8.474	14.8
Glutamic acid	3.996	3.732	6.8	4.223	3.972	6.1
Glutamine	0.337	0.320	5.2	0.393	0.350	11.6
Glycine	0.130	0.142	-8.8	0.156	0.165	-5.6
Histidine	0.453	0.415	8.8	0.613	0.543	12.1
Isoleucine	0.113	0.099	13.2	0.113	0.095	17.3
Leucine	0.108	0.106	1.9	0.108	0.106	1.9
Lysine	0.724	0.427	51.6	0.725	0.448	47.2
Methionine	0.012	0.014	-12.9	0.012	0.013	-5.5
Phenylalanine	0.142	0.150	-5.5	0.362	0.364	-0.6
Proline	3.139	5.311	-51.4	1.426	2.524	-55.6
Serine	0.212	0.194	8.9	0.312	0.331	-5.9
Threonine	0.099	0.087	12.9	0.122	0.116	5.0
Tyrosine	0.056	0.051	9.3	0.055	0.049	11.5
Valine	0.100	0.094	6.2	0.139	0.137	1.4

Table 6. Types of tobacco analyzed in this study.

No.	Type ^a	Year	Description
1	FC L (1)	2008	Eastern NC belt, lower stalk (lug) flue-cured
2	FC U (1)	2008	Eastern NC belt, upper stalk (leaf & some tips) flue-cured
3	FC L (2)	2009	South Carolina belt, lower stalk (lug) flue-cured
4	FC U (2)	2009	South Carolina belt, upper stalk (leaf & some tips) flue-cured
5	FC off L	2006	Brazil, lower stalk (lugs & primings) flue-cured
6	FC off U	2006	Brazil, upper stalk (leaf & tips) flue-cured
7	Bu L (1)	2007	Kentucky & Tennessee, lower stalk (flyings & cutters) Burley
8	Bu U (1)	2007	Kentucky & Tennessee, upper stalk (leaf) Burley
9	Bu L (2)	2008	North Carolina & Virginia, lower stalk (flyings & cutters) Burley
10	Bu U (2)	2008	North Carolina & Virginia, upper stalk (leaf) Burley
11	Bu off L	2008	Malawi, lower stalk (flyings & cutters) Burley
12	Bu off U	2008	Malawi, upper stalk (leaf) Burley
13	O Sa U	2007	Turkey, good quality middle to upper stalk, Samsun Oriental
14	O Iz U	2005	Turkey, good quality middle to upper stalk, Izmir Oriental
15	3R4F	2006	Tobacco blend from 3R4F Kentucky reference cigarette
16	Commercial	2012	Common commercial cigarette blend

^a FC = Flue-cured; Bu = Burley; O = Oriental; L = Lower stalk; U = Upper stalk; off = off shore / not USA tobacco

The method robustness evaluation showed that the amino acids solutions (either standards or tobacco extracts) are stable for 24 h at room temperature. After 48 h of storage at room temperature, some amino acids (asparagine, glutamine) tended to hydrolyze and displayed lower levels. The HPLC instrument used in this study had a cooling capability for the autosampler, and the sample temperature was set at 10 °C to improve sample stability. The samples kept in the refrigerator at 2–8 °C showed less than 10% variation after 12 days of storage, except for leucine that showed about 16% variation.

RESULTS AND DISCUSSION

Types of tobaccos evaluated

The types of tobaccos analyzed are listed in Table 6. They include flue-cured and Burley, domestic and off-shore (not USA grown tobaccos), as well as two types of Oriental tobaccos. Tobacco leaf was initially made in cut filler form. Experimental cigarettes containing no humectants or additives were made with each tobacco. The material used for analysis was obtained from the experimental cigarettes kept in the freezer after manufacturing. Also tobacco from a 3R4F cigarette, and one commercial cigarette blend were analyzed.

Figure 3. The level of proline (mg/g) in the analyzed tobacco types. (See Table 6 for tobacco No.) **Figure 4. The level of asparagine (mg/g) in the analyzed tobacco types.** (See Table 6 for tobacco No.)

Results of amino acids analysis in tobacco leaf

The results of LC-MS/MS analysis for 15 tobacco types are given in Table 7. The table indicates the averages of triplicate measurements in mg/g of tobacco, and RSD% for the measurements. The results from Table 7 show that flue-cured tobaccos are characterized by a higher level of proline, while the Burley tobaccos have mainly high levels of asparagine and aspartic acid, in agreement with the information from typical tobacco literature (20). However, a wide range of levels of these amino acids can be seen across various tobaccos. For example, the levels of proline listed in Table 7 show that the largest levels were found in the Oriental tobaccos. Proline levels in the analyzed tobaccos are presented in graph form in Figure 3.

Asparagine levels also vary significantly as a function of tobacco type as shown in Figure 4 (with data from Table 7). This amino acid is of particular interest since it is a precursor for the formation of acrylamide (8) in cigarette smoke. The variation of acrylamide level in smoke (as µg/mg TPM) (for two smoking regimens: 1) ISO and 2) using 60 mL puff volume, 2 s puff, and 30 s puff interval, no vent blocking, indicated as Intense) as a function of the level of asparagine in tobacco is exemplified in Figure 5 (21). For this reason, the selection of tobaccos low in asparagine is recommended in order to avoid high acrylamide levels in smoke. As shown in Figure 4, asparagine is high in two types of Burley and in Samsun Oriental tobacco as compared to other tobacco types. The levels of aspartic acid (mg/g) in the analyzed tobacco types are shown in Figure 6. This

amino acid is high in all Burley tobaccos, relative to other tobacco types.

The data presented in this study is limited to a small set of tobacco samples and a larger set would be necessary for more detailed conclusions. However, even with this limited evaluated set of samples, it can be seen that the level of amino acids between lower stalk (lug) and upper stalk (leaf and some tips) as described in Table 6, differ mainly in the level (with the lower stalk position tobacco having lower levels of amino acids), but not in the amino acid profile.

Also, the study shows that the composition of amino acids in the same type of tobacco (e.g., flue-cured, or Burley) may differ significantly from region to region.

CONCLUSIONS

A new method using RP-HPLC coupled with MS/MS detection for the analysis of free amino acids in tobacco has been developed. The new method uses separation on an

Table 7. Results of amino acid levels in mg/g in the analyzed tobaccos (conditioned as indicated).

No.	Compound	Tobacco 1, FC L (1)		Tobacco 2, FC U (1)		Tobacco 3, FC L (2)		Tobacco 4, FC U (2)	
		mg/g	RSD%	mg/g	RSD%	mg/g	RSD%	mg/g	RSD%
1	Alanine	0.604	2.3	0.680	2.1	0.621	2.7	0.708	1.8
2	Arginine	0.131	1.5	0.117	1.1	0.139	6.0	0.117	0.6
3	Asparagine	3.752	0.3	5.926	0.4	3.480	0.2	3.053	0.8
4	Aspartic acid	0.955	0.2	0.919	1.3	0.881	2.6	0.683	0.3
5	Cysteine	0.009	2.6	0.012	1.9	0.011	5.2	0.013	7.1
6	Glutamic acid	0.496	0.3	0.737	0.5	0.430	0.9	0.635	0.7
7	Glutamine	0.671	0.2	1.117	0.3	0.718	0.4	0.847	0.6
8	Glycine	0.065	0.3	0.032	0.9	0.027	2.8	0.030	0.5
9	Histidine	0.213	1.6	0.286	2.6	0.178	1.7	0.193	5.2
10	Hydroxyproline	0.004	0.9	0.002	0.7	0.001	7.2	0.002	0.0
11	Isoleucine	0.057	2.3	0.046	0.5	0.067	10.3	0.041	1.7
12	Leucine	0.051	4.8	0.044	2.7	0.055	0.2	0.059	1.8
13	Lysine	0.219	0.7	0.180	0.0	0.155	0.0	0.460	0.0
14	Methionine	0.001	0.1	0.001	1.1	0.001	6.1	0.001	5.9
15	Phenylalanine	0.140	0.8	0.214	0.5	0.257	0.4	0.115	0.6
16	Proline	3.273	0.4	3.999	0.3	3.817	1.8	5.257	1.4
17	Serine	0.253	0.1	0.188	0.9	0.059	4.8	0.050	4.9
18	Threonine	0.207	0.4	0.476	0.6	0.224	1.1	0.478	1.1
19	Tryptophan	0.067	2.9	0.051	2.3	0.068	1.9	0.066	0.8
20	Tyrosine	0.120	1.7	0.124	1.2	0.133	0.7	0.076	1.1
21	Valine	0.218	4.4	0.680	2.1	0.141	2.6	0.247	6.8

No.	Compound	Tobacco 5, FC off L		Tobacco 6, FC off U		Tobacco 7, Bu L (1)		Tobacco 8, Bu U (1)	
		mg/g	RSD%	mg/g	RSD%	mg/g	RSD%	mg/g	RSD%
1	Alanine	0.526	2.2	0.474	1.4	0.637	1.6	1.389	2.0
2	Arginine	0.104	1.8	0.114	2.3	0.264	5.6	0.503	4.3
3	Asparagine	2.226	0.4	1.605	0.2	8.365	0.2	17.696	1.2
4	Aspartic acid	0.847	2.0	0.509	1.2	5.323	0.5	7.921	1.5
5	Cysteine	0.013	2.7	0.008	5.7	0.012	5.7	0.007	8.3
6	Glutamic acid	0.727	0.4	0.325	1.2	1.615	1.0	2.070	1.4
7	Glutamine	0.667	0.4	0.147	0.1	0.103	6.4	0.184	0.4
8	Glycine	0.023	3.9	0.017	0.9	0.070	0.0	0.076	0.8
9	Histidine	0.272	1.3	0.160	0.3	0.358	1.9	0.868	2.7
10	Hydroxyproline	0.002	8.3	0.003	1.3	0.002	0.1	0.002	8.2
11	Isoleucine	0.047	1.7	0.036	0.5	0.087	5.4	0.071	3.2
12	Leucine	0.050	8.1	0.041	9.2	0.109	2.0	0.138	0.8
13	Lysine	0.398	0.4	0.251	1.7	0.699	0.0	0.548	0.1
14	Methionine	0.001	1.5	0.001	1.0	0.001	2.0	0.001	5.0
15	Phenylalanine	0.244	1.4	0.255	2.0	0.706	0.7	0.861	1.1
16	Proline	5.187	1.6	4.804	1.1	1.737	1.3	1.904	0.0
17	Serine	0.163	0.2	0.029	2.1	0.075	3.5	0.118	1.1
18	Threonine	0.327	0.8	0.442	0.2	0.206	1.2	0.340	2.0
19	Tryptophan	0.070	1.7	0.031	1.2	0.134	3.4	0.285	1.7
20	Tyrosine	0.093	1.2	0.083	2.4	0.082	0.3	0.179	3.9
21	Valine	0.152	4.7	0.111	5.0	0.143	6.0	0.241	1.0

^a FC = Flue-cured; Bu = Burley; O = Oriental; L = Lower stalk; U = Upper stalk; off = off shore / not USA tobacco

Table 7. Contd.

No.	Compound	Tobacco 9, Bu L (2)		Tobacco 10, Bu U (2)		Tobacco 11, Bu off L		Tobacco 12, Bu off U	
		mg/g	RSD%	mg/g	RSD%	mg/g	RSD%	mg/g	RSD%
1	Alanine	0.348	0.2	0.475	0.2	0.669	1.6	0.969	2.2
2	Arginine	0.223	1.8	0.339	1.2	0.189	1.0	0.242	0.7
3	Asparagine	2.098	0.3	3.755	0.7	4.916	0.8	4.923	0.6
4	Aspartic acid	4.830	1.3	10.135	0.4	8.208	0.0	7.693	0.3
5	Cysteine	0.013	10.6	0.007	8.3	0.013	8.2	0.007	3.1
6	Glutamic acid	1.124	0.7	2.118	0.0	1.150	1.4	1.492	0.3
7	Glutamine	0.103	7.3	0.087	5.8	0.109	0.3	0.167	2.4
8	Glycine	0.058	0.4	0.070	2.7	0.061	1.5	0.062	2.1
9	Histidine	0.216	0.4	0.295	0.2	0.170	0.8	0.157	2.4
10	Hydroxyproline	0.003	2.0	0.004	1.3	0.006	1.9	0.006	2.3
11	Isoleucine	0.097	9.5	0.053	2.5	0.050	0.0	0.062	0.2
12	Leucine	0.111	0.0	0.112	0.7	0.086	1.1	0.123	0.1
13	Lysine	0.411	0.2	0.473	0.9	0.462	0.5	0.440	0.0
14	Methionine	0.001	5.3	0.001	2.9	0.001	2.7	0.001	3.3
15	Phenylalanine	0.325	0.1	0.558	0.9	0.542	0.4	0.559	0.9
16	Proline	1.115	0.0	0.958	1.2	1.892	1.4	3.425	2.2
17	Serine	0.123	0.8	0.175	0.4	0.255	0.0	0.318	0.6
18	Threonine	0.257	1.3	0.283	1.4	0.342	1.2	0.415	2.0
19	Tryptophan	0.100	1.3	0.131	0.1	0.104	1.2	0.083	3.3
20	Tyrosine	0.065	9.1	0.073	3.1	0.092	1.5	0.095	3.0
21	Valine	0.141	1.3	0.134	1.0	0.174	3.0	0.308	2.8

No.	Compound	Tobacco 13, O Sa U		Tobacco 14, O Iz U		Tobacco 15, 3R4F		Tobacco 16, Commer.	
		mg/g	RSD%	mg/g	RSD%	mg/g	RSD%	mg/g	RSD%
1	Alanine	1.494	2.9	0.868	1.4	0.308	2.0	0.712	1.9
2	Arginine	0.260	3.0	0.125	1.0	0.253	2.8	0.124	3.2
3	Asparagine	13.934	0.6	1.461	0.5	2.626	3.4	4.641	0.2
4	Aspartic acid	3.499	0.3	0.324	1.7	2.538	2.1	2.513	0.1
5	Cysteine	0.013	7.7	0.007	0.0	0.004	6.1	0.007	5.0
6	Glutamic acid	1.798	1.4	0.303	0.8	1.341	1.7	0.898	1.3
7	Glutamine	2.854	0.1	0.494	0.1	2.304	1.7	0.564	0.4
8	Glycine	0.070	2.3	0.018	0.3	0.122	3.6	0.037	3.6
9	Histidine	0.627	0.9	0.095	3.5	0.660	1.1	0.192	0.5
10	Hydroxyproline	0.004	5.5	0.003	0.6	0.002	2.1	0.002	5.9
11	Isoleucine	0.063	3.8	0.048	0.9	0.027	1.4	0.057	1.0
12	Leucine	0.112	2.4	0.053	2.8	0.040	2.3	0.066	5.7
13	Lysine	0.319	0.1	0.475	0.1	0.485	5.3	0.345	0.0
14	Methionine	0.001	1.7	0.001	3.3	0.001	3.1	0.001	3.3
15	Phenylalanine	0.970	0.4	0.236	1.1	0.175	0.8	0.552	0.1
16	Proline	9.002	1.4	9.194	1.2	3.138	6.1	5.205	0.1
17	Serine	0.386	0.1	0.027	2.0	0.458	2.5	0.173	0.0
18	Threonine	0.323	0.6	0.080	2.3	0.593	1.7	0.212	0.7
19	Tryptophan	0.195	1.3	0.036	2.7	0.049	1.1	0.063	2.0
20	Tyrosine	0.175	2.4	0.067	2.1	0.166	2.2	0.100	0.7
21	Valine	0.227	1.0	0.130	3.8	0.088	3.1	0.135	5.8

^a FC = Flue-cured; Bu = Burley; O = Oriental; L = Lower stalk; U = Upper stalk; off = off shore / not USA tobacco

Acclaim™ RSLC Polar Advantage II column, with gradient separation of amino acids. The accuracy of the new method compares well with a HPLC-fluorescence with derivatization method, previously utilized for amino acids analysis, but has a lower limit of quantitation allowing the measurement of amino acids not possible to analyze by the fluorescence method. The method offers a number of advantages such as no need for derivatization, excellent peak shape in the chromatogram, good separation, and positive identification of each amino acid in the tobacco matrix.

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Figure 5. Variation of the level of acrylamide in cigarette smoke (in µg/mg TPM), as a function of asparagine level in tobacco (mg/g).

Figure 6. The level of aspartic acid (mg/g) in the analyzed tobacco types. (See Table 6 for tobacco No.)

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