Evaluation of Several Minor Alkaloid Levels in Tobacco with Separation of R- and S-Nornicotine *

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SUMMARY

The chiral separation of minor alkaloids from tobacco is of interest because R and S isomers of these compounds have differences in their physiological activity. This difference is also reflected in the physiological properties of tobacco-specific *N*'-nitrosamines (TSNAs), in particular that of *N*'-nitrosonornicotine. This compound results mainly from nornicotine nitrosation. The previously reported analytical techniques for the enantiomer separation of minor alkaloids have various shortcomings, such as the need for bidimensional chromatography or poor enantiomer separation. A new method for the analysis of nornicotine, anabasine and anatabine has been developed, based on an original derivatization and a simple gas chromatography/mass spectrometry (GC/MS) analysis. The method allows separate quantitation of S-nornicotine and R-nornicotine, and the analysis of anabasine and anatabine (without isomer separation). It was found that the proportion of S-nornicotine in the total nornicotine present in tobacco varies, depending on the tobacco type, between 52.6% for a flue-cured tobacco to 91.4% for a Burley. Green tobaccos (freeze dried) showed lower levels of minor alkaloids and S-nornicotine accounted for between 31.6% to 43.8% of the total nornicotine (in the analyzed samples). [Beitr. Tabakforsch. Int. 25 (2013) 649–659]

ZUSAMMENFASSUNG

Die chirale Trennung von Nebenalkaloiden von Tabak ist von Interesse, da die R- und S-Isomere dieser Verbindungen Unterschiede in der physiologischen Aktivität aufweisen. Diese Unterschiede spiegeln sich auch in den physiologischen Eigenschaften von tabakspezifischen *N*-Nitrosaminen (TSNA) insbesondere bei *N*-Nitrosonornikotin wider. Diese Verbindung entsteht im Wesentlichen bei der Nitrosierung von Nornikotin. Die bisher beschriebenen Analysetechniken zur Enantiomerentrennung von Nebenalkaloiden haben diverse Mängel wie die Notwendigkeit

der zweidimensionalen Chromatographie oder eine schlechte Enantiomerentrennung. Es wurde eine neue Methode zur Analyse von Nitrosonornikotin, Anabasin und Anatabin entwickelt, die auf einer herkömmlichen Derivatisierung und einer einfachen Analyse mittels Gaschromatographie-Massenspektrometrie (GC/MS) beruht. Die Methode ermöglicht die separate Quantifizierung von S-Nornikotin und R-Nornikotin sowie die Analyse von Anabasin und Anatabin (ohne Isomerentrennung). Hierbei wurde festgestellt, dass der Anteil von S-Nornikotin am Gesamtnornikotin im Tabak je nach Tabaksorte zwischen 52,6% bei heißluftgetrocknetem Tabak und 91,4% bei Burleytabak variiert. In grünen Tabaksorten (gefriergetrocknet) fanden sich geringere Mengen an Nebenalkaloiden und der Anteil von S-Nornikotin am Gesamtnornikotin lag (bei den analysierten Proben) zwischen 31,6% und 43,8%. [Beitr. Tabakforsch. Int. 25 (2013) 649–659]

RESUME

La séparation chirale des alcaloïdes mineurs du tabac présente un intérêt car les isomères R et S de ces composés présentent des différences dans leurs activités physiologiques. Ces différences se reflètent également dans les propriétés physiologiques des *N*-nitrosamines spécifiques du tabac (TSNA), en particulier celles de la *N*-nitrosonornicotine. Ce composé est principalement issu de la nitrosation de la nornicotine. Les techniques analytiques précédemment rapportées pour la séparation des énantiomères d'alcaloïdes mineurs présentent divers manques, comme le besoin d'une chromatographie bidimensionnelle ou une mauvaise séparation des énantiomères. Une nouvelle méthode d'analyse de la nornicotine, de l'anabasine et de l'anatabine a été mise au point, basée sur une dérivation originale et une simple analyse par chromatographie en phase gazeuse/spectrométrie de masse. La méthode permet une quantification séparée de la (S)-nornicotine et de la (R)-nornicotine ainsi que l'analyse de l'anabasine et de l'anatabine (sans séparation des isomères). Les résultats

obtenus montrent que la proportion de (S)-nornicotine dans la nornicotine totale présente dans le tabac varie selon le type de tabac entre 52,6% pour un tabac blond et 91,4% pour un Burley. Les tabacs verts (lyophilisés) ont montré des teneurs inférieures en alcaloïdes mineurs et que la (S)-nornicotine constitue entre 31,6% et 43,8% de la nornicotine totale (dans les échantillons analysés). [Beitr. Tabakforsch. Int. 25 (2013) 649–659]

KEYWORDS: Minor alkaloids, tobacco, S-nornicotine, R-nornicotine

INTRODUCTION

Analysis of minor alkaloids in tobacco has been the subject of numerous studies (1–7). These compounds are of particular interest for various reasons including potency toward nicotinic cholinergic receptors (8), propensity of formation of tobacco-specific nitrosamines (TSNAs) (9–12), and sensory properties (8, 13). The chiral separation of minor alkaloids from tobacco has also been of interest since the physiological activity of the enantiomers of these compounds have been shown to be different (14, 15). This difference in enantiomer activity is also reflected in the physiological properties of TSNAs, in particular that of *N*'-nitrosoanatabine (NNN) (16, 17). NNN results mainly from the nitrosation of nornicotine present in tobacco and the R/S configuration is not changed during nitrosation. This compound and 4-(methyl-*N*'-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are the most abundant suspected carcinogens in tobacco. Although tobacco contains *N*'-nitrosoanatabine (NAT) and smaller quantities of *N*'-nitrosoanabasine (NAB), these two compounds are not classifiable as to their carcinogenicity to humans (IARC Group 3) and are of lower interest (18, 19). For this reason, the analysis of minor alkaloids with particular interest in the enantiomer separation of nornicotine has been the subject of several published studies (20–24). However, the previously reported analytical techniques for the enantiomer separation of minor alkaloids have various shortcomings. An early study concerning the separation of enantiomers of nornicotine, anatabine and anabasine in tobacco (21) was based on fractional crystallization of S-6,6-dinitro-2,2'-diphenic acid and R-6,6-dinitro-2,2'-diphenic acid derivatives of the minor alkaloids. More modern methods use direct phase high-performance liquid chro-

matography (HPLC) with modest separation of nornicotine enantiomers (22), or two GC chiral columns without achieving baseline separation of R/S nornicotine isomers (23), or more complex procedures such as derivatization plus bidimensional GC separation (24). A new technique for analysis of minor alkaloids in tobacco with the separation of R/S enantiomers of nornicotine has been developed and is described in detail.

EXPERIMENTAL

Chemicals

The solvents methyl *tert*-butyl ether, pyridine, and the reagent isobutyl chloroformate were obtained from Aldrich (Aldrich, Milwaukee, WI 53201, USA). The minor alkaloid standards were obtained from TRC (Toronto Research Chemicals, Toronto, ON M3J 2J8, Canada).

Minor alkaloid extraction from the tobacco sample

The extraction of minor alkaloids from the tobacco sample was performed following a common procedure recommended in the literature (6, 25). The extraction started with 0.2 g of a tobacco sample (as is), which was weighed with precision of 0.1 mg. To this sample, 0.5 mL of aqueous 1 N NaOH was added to achieve sample wetting, and the sample was allowed to stand at room temperature for 15 min. The purpose of this step was to transform any minor alkaloids present as salts into their base forms. The alkaline tobacco slurry was extracted with 4.0 mL of methyl *tert*-butyl ether (MTBE) containing 125 µg mL⁻¹ ethyl quinoline-3-carboxylate as an internal standard. The extraction was performed on a mechanical shaker for 30 min. After the extraction, the MTBE solution was filtered into a 4 mL vial through a 0.45 mm PVDF Whatman autovial (Whatman, Clifton, NJ, 07014, USA) in which was placed about 1 g of anhydrous Na₂SO₄. The solution of the extracted samples could be kept in a refrigerator for several weeks for further utilization, providing that the vial was well capped and no MTBE evaporation took place. The extraction efficiency using this procedure was not verified in this study, since it has been proven for the extraction of minor alkaloids in other published reports (see *e.g.*, (6)).

Table 1. Parameters for the GC/MS analysis (Method 1).

Parameter	Description	Parameter	Description
Initial oven temperature	50 °C	Flow mode	Constant flow
Initial time	1.0 min	Flow rate	1.1 mL/min
Oven ramp rate	10 °C/min	Nominal initial pressure	7.75 psi
Oven temperature	230 °C	GC outlet	MSD
Final time	10 min	Outlet pressure	Vacuum
Total run time	29.0 min	MSD transfer line temperature	250 °C
Inlet temperature	250 °C	Ion source temperature	230 °C
Inlet mode	Splitless	Quadrupole temperature	150 °C
Purge off time	0.5 min	MSD EM gain	1.75
Carrier gas	Helium	MSD solvent delay	10.0 min
Injection volume	1.0 µL	Mass range	29–550 a.u.

Figure 1. Typical chromatogram for a Burley tobacco extract without isomers separation. Peak identification is shown in Table 2.

Table 2. List of compounds of interest shown in the chromatogram for a Burley tobacco extract.

No.	Ret. time min	Compound name	CAS No.	MW
1	12.49	Nicotine	54-11-5	162
2	14.09	Nornicotine	494-97-3	148
3	14.36	Myosmine	532-12-7	146
4	14.70	Anabasine	494-52-0	162
5	14.97	Hydrocarbon		
6	15.15	β -Nicotyrine	487-19-4	158
7	15.44	Anatabine	581-49-7	160
8	15.54	Neophytadiene	504-96-1	278
9	15.93	2,3'-Bipyridine	581-50-0	156
10	17.77	3-Quinolinic acid ethyl ester (I.S.)	50741-46-3	201
11	18.52	Cotinine	486-56-6	176
12	22.90	4,8,14-Duvatriene-1,3-diol	7220-78-2	306

GC/MS analysis without enantiomer separation (Method 1)

The analysis of the level of minor alkaloids in the tobacco extract, without the separation of enantiomers can be carried out following a procedure adapted from the literature (6). The instrument used for the analysis was a GC/MS 5973/6890 system from Agilent (Agilent, Wilmington, DE 19808, USA).

The separation was performed on a ZB-50 capillary column 30 m $\times$ 0.25 mm i.d., with 0.25 mm film thickness from Phenomenex (Torrance, CA 905010, USA). The separation conditions are given in Table 1. In the conditions described in Table 1, a typical Burley tobacco extract generates the chromatogram shown in Figure 1. The peak identification is shown in Table 2.

The simultaneous quantitation of nicotine and of minor alkaloids is not feasible by this method, because the resulting nicotine chromatographic peak is overloaded. Among the minor alkaloids, only nornicotine, anabasine and anatabine were measured. The measurement was performed using calibration curves for specific extracted ion areas. The levels of compounds in the injected solutions were obtained from six standards in the range of 7.8 $\mu\text{g mL}^{-1}$ to 250 $\mu\text{g mL}^{-1}$. The internal standard was used only for the verification of the repeatability of the analysis and not for normalization. The ions used for quantitation, the equations used for the calibration, and the regression coefficient R^2 values for the trendline are given in Table 3.

GC/MS analysis with nornicotine enantiomer separation (Method 2)

For the separation of minor alkaloids nornicotine, anabasine and anatabine and simultaneously of R- and S-nornico-

Table 3. The ions used for quantitation, the equations used for the calibration, and R^2 values for the trendline in Method 1.

No.	Compound	Quantitation ion	Confirmation ions	Equation*	R^2
1	Nornicotine	119	147, 148, 70	$Y = 2.4569\text{e-}5 X$	0.9897
2	Anabasine	162	133, 161, 84	$Y = 2.9099\text{e-}5 X$	0.9977
3	Anatabine	160	105, 159, 131	$Y = 2.9596\text{e-}5 X$	0.9903
4	3-Quinolinic acid ethyl ester (I.S.)	156	201, 128, 173		

* Note: Y represents the amount in mg mL^{-1} and X the peak area.

tine, a derivatization step was necessary. For this purpose, the minor alkaloids containing active hydrogens in their molecules were derivatized with isobutyl chloroformate (isobutyl chlorocarbonate). With nornicotine, for example, isobutyl chloroformate reacts as follows:

The separation of the derivatized compounds and of those not derivatized (since they do not have active hydrogens) was performed on a chiral column. Only the separation of R- and S-nornicotine was of interest in the study. The separation of R- and S-anabasine and R- and S-anatabine was not pursued.

For the derivatization, 0.6 mL tobacco extract in MTBE was placed in a 1.5 mL GC vial and was treated with 20 μ L pyridine and 10 μ L isobutyl chloroformate. The solution became turbid, but after being kept at room temperature for about 15 min, the solution cleared and could directly be used for injection into the GC/MS system. The column used for the separation was a Rt-BDEXsm 30 m $\times$ 0.25 mm i.d., with 0.25 mm film thickness from Restek (Restek, Bellefonte, PA 16823, USA). The instrument used for the analysis of the minor alkaloids was a GC/MS 5973/6890 system from Agilent. The conditions for the GC/MS analysis are described in Table 4 and a typical Burley tobacco extract chromatogram is shown in Figure 2. The peak identification is shown on the figure. The separation of R- and S-nornicotine derivatives was good (resolution $R \approx 1$), and allowed for the integration of the R and S peaks separately. Figure 3 shows an expanded portion of the chromatogram from Figure 2, with separated nornicotine isomers.

The spectra of isobutyl chloroformate derivatives of nornicotine, anabasine and anatabine have not been reported previously in the literature. These spectra are shown in Figures 4, 5, and 6, respectively. The structures of some of the main fragments corresponding to EI⁺ mass spectra are also shown in each figure.

Table 4. Parameters for the GC/MS analysis (Method 2).

Parameter	Description	Parameter	Description
Initial oven temperature	50 °C	Purge off time	0.5 min
Initial time	2.0 min	Carrier gas	Helium
Oven ramp rate	30 °C/min	Injection volume	1 μ L
Oven temperature	160 °C	Flow mode	Constant flow
Final time first ramp	9 min	Flow rate	1.0 mL/min
Oven ramp rate	1 °C/min	Nominal initial pressure	7.75 psi
Oven temperature	205 °C	GC outlet	MSD
Final time	0 min	Outlet pressure	Vacuum
Oven ramp rate	20 °C/min	MSD transfer line temperature	250 °C
Oven final temperature	225 °C	Ion source temperature	230 °C
Final time	4.33 min	Quadrupole temperature	150 °C
Total run time	70.0 min	MSD EM gain	1.75
Inlet temperature	230 °C	MSD solvent delay	10.0 min
Inlet mode	Splitless	Mass range	29–350 a.u.

The quantitation of R-nornicotine, S-nornicotine, R/S-anabasine, and R/S-anatabine was performed using calibration with a set of 6 derivatized standards with concentrations between 250 μ g mL⁻¹ and 7.8 μ g mL⁻¹ R/S-nornicotine, R/S-anabasine, and R/S-anatabine. The internal standard was used only for the verification of the repeatability of the analysis but not for normalization. The ions used for quantitation, the equations used for the calibration, and the R² values for the trendline are given in Table 5.

The analytical procedure was further tested for stability of the derivatized analytes over time, and for the analysis repeatability. For this purpose, two samples, a Burley and a flue-cured tobacco were analyzed in duplicate over a period of 24 h after derivatization. The results are shown in Table 6, which gives the average of measurements and their relative standard deviations (RSD%). The RSD% for the total average was taken between the averages for different waiting periods. As shown in Table 6, the samples are stable for about 24 h and can be analyzed in this interval of time with good precision. However, a slight decrease in the results is still noticed as a general trend. For this reason, after the derivatization is performed, it is not recommended to analyze the samples after 24 h.

Besides the linearity and stability, a further validation of Method 2 was performed to establish the limit of detection (LOD) and limit of quantitation (LOQ) for the method. For this purpose, five repeated injections of the lowest standard (7.8 μ g mL⁻¹) were carried out. LOD was estimated from 3 $\times$ standard deviation (SD) and LOQ from 10 $\times$ SD. The results are shown in Table 7. It can be concluded that Method 2 showed good selectivity, stability, linearity, and appropriate LOQ values for the quantitation of R- and S-nornicotine, anabasine and anatabine in tobacco.

RESULTS AND DISCUSSION

The two methods for minor alkaloids analysis have been applied for the measurement of nornicotine (one method with R and S separation), anabasine and anatabine in a series of tobacco samples. These tobaccos are described in Table 8, and they represent a wide range of samples, including different types of tobacco.

Figure 2. Typical chromatogram for a Burley tobacco extract derivatized with isobutyl chloroformate and the separation of R- and S-nornicotine.

Figure 3. Portion of the chromatogram displayed in Figure 2, showing the separation of R- and S-nornicotine derivatives.

Figure 4. Mass spectrum of isobutyl chloroformate derivative of nornicotine or 2-methylpropyl 2-(pyridin-3-yl)pyrrolidine-1-carboxylate.

Figure 5. Mass spectrum of isobutyl chloroformate derivative of anabasine or 2-methylpropyl -2-(pyridin-3-yl)piperidine-1-carboxylate.

Figure 6. Mass spectrum of isobutyl chloroformate derivative of anatabine or 2-methylpropyl 2-(pyridin-3-yl)-3,6-dihydro-2H-pyridine-1-carboxylate.

Table 5. The ions used for quantitation, the equations used for the calibration, and R^2 values for the trendline in Method 2.

No.	Compound	Quantitation ion	Confirmation ions	Equation*	R^2
1	R-nornicotine	191	193, 249, 247	$Y = 1.0939e-5 X$	0.9892
2	S-nornicotine	191	193, 248, 247	$Y = 1.0999e-5 X$	0.9919
3	Anabasine	161	205, 262	$Y = 3.8700e-6 X$	0.9883
4	Anatabine	203	260, 159	$Y = 1.14342e-5 X$	0.9855
5	3-Quinolinic acid ethyl ester (I.S.)	201	156, 173, 128		

* Note: Y represents the amount in mg mL^{-1} and X the peak area.

Table 6. Averages for the level of nornicotine (total), anabasine and anatabine with samples analyzed in an interval of 48 h.

No.	Sample	Time after derivatization h	Average R-nornicot. $\mu\text{g g}^{-1}$	RSD% R-nornicot.	Average S-nornicot. $\mu\text{g g}^{-1}$	RSD% S-nornicot.	Average anabasine $\mu\text{g g}^{-1}$	RSD% anabasine	Average anatabine $\mu\text{g g}^{-1}$	RSD% anatabine
1	Burley	0 – 2	777	(2.32)	1750	(1.24)	238	(2.34)	1901	(2.14)
2	Burley	~ 12	765	(3.21)	1731	(3.33)	233	(4.21)	1897	(3.12)
3	Burley	~ 24	766	(0.21)	1744	(1.32)	211	(3.33)	1876	(4.16)
	Total average		769	(0.87)	1742	(0.56)	227	(6.32)	1891	(0.71)
4	Flue-cured	0 – 2	276	(4.23)	577	(2.33)	229	(4.21)	1440	(2.11)
5	Flue-cured	~ 12	275	(3.21)	568	(2.12)	222	(1.23)	1421	(3.11)
6	Flue-cured	~ 24	254	(2.11)	559	(1.55)	234	(2.12)	1409	(2.45)
	Total average		268	(4.63)	568	(1.58)	228	(2.64)	1423	(1.10)

Table 7. Estimated limit of detection (LOD), limit of quantitation (LOQ) for standard solutions and the corresponding LOQ for sample.

Compound	LOD mg mL^{-1}	LOQ mg mL^{-1}	Equivalent LOQ sample mg g^{-1}
R-nornicotine	0.26	0.84	16.8
S-nornicotine	0.24	0.82	16.4
Anabasine	0.30	1.02	20.4
Anatabine	0.20	0.66	13.2

The results for the level of nornicotine, anabasine, and anatabine in the analyzed tobaccos (as is, *i.e.* wet weight basis) using GC/MS method without enantiomer separation (Method 1) are given in Table 9. All samples were analyzed in duplicate. The results for the level of total nornicotine (R-nornicotine plus S-nornicotine), as well as the levels of anabasine, and anatabine in the analyzed tobaccos using GC/MS method with enantiomer separation (Method 2) are shown in Table 10. These samples were also analyzed in duplicate.

Table 8. Tobaccos analyzed in this study.

No	Tobacco identification	Year	Description
1	By A	2012	Burley blend
2	FC A	2012	Flue-cured blend
3	L-FC B	2008	Lower stalk (lug) flue-cured B
4	U-FC B	2008	Upper stalk (leaf & some tips) flue-cured B
5	L-FC C	2009	Lower stalk (lug) flue-cured C
6	U-FC C	2009	Upper stalk (leaf & some tips) flue-cured C
7	Off L-FC	2008	Offshore*, lower stalk (lugs & primings) flue-cured
8	Off U-FC	2008	Offshore, upper stalk (leaf & tips) flue-cured
9	L-By B	2007	Lower stalk (flyings & cutters) Burley B
10	U-By B	2007	Upper stalk (leaf) Burley B
11	L-By C	2008	Lower stalk (flyings & cutters) Burley C
12	U-By C	2008	Upper stalk (leaf) Burley C
13	Off L-By	2008	Offshore, lower stalk (flyings & cutters) Burley
14	Off U-By	2008	Offshore, upper stalk (leaf) Burley
15	Perique	2008	Perique tobacco whole stalk
16	Dark Air Cured	2012	Middle stalk
17	Fire Cured	?	Upper two thirds of plant
18	Or A	2007	Oriental (middle to upper stalk) A
19	Or B	2005	Oriental (middle to upper stalk) B
20	Cigarette blend	2010	Commercial cigarette
21	2R4Fn	2001	Tobacco from 2R4F cigarettes kept in freezer
22	2R4Fo	2001	Tobacco from 2R4F cigarettes kept at room temperature
23	3R4F	2007	Tobacco from 3R4F cigarettes kept in freezer
24	1R4F	1983	Tobacco from 1R4F cigarettes kept in freezer
25	Green FC	2011	Freeze dried green flue-cured tobacco
26	Green Bu	2011	Freeze dried green Burley tobacco
27	Green Or	2011	Freeze dried green Oriental tobacco

Note: Offshore tobacco indicates tobaccos from outside US. The Oriental tobacco is also "offshore".

Table 9. The level of total nornicotine, anabasine, and anatabine analyzed by Method 1.

No.	Sample Oriental	Average nornicotine $\mu\text{g g}^{-1}$	RSD% nornicotine	Average anabasine $\mu\text{g g}^{-1}$	RSD% anabasine	Average anatabine $\mu\text{g g}^{-1}$	RSD% anatabine
1	By A	2509	1.11	196	0.84	1880	2.35
2	FC A	633	0.82	184	0.91	1438	1.88
3	L-FC B	297	1.11	49	1.89	567	2.01
4	U-FC B	2213	1.21	205	0.83	1572	1.98
5	L-FC C	892	1.71	94	0.89	836	1.76
6	U-FC C	1666	1.87	181	2.55	1273	2.24
7	Off L-FC	914	0.38	124	0.40	1015	0.19
8	Off U-FC	1760	4.59	231	0.79	1937	3.95
9	L-By B	3580	2.38	169	0.63	1825	2.54
10	U-By B	3994	1.28	211	1.00	2452	1.68
11	L-By C	3201	1.98	168	1.35	1516	2.23
12	U-By C	3581	3.29	212	1.13	1774	2.19
13	Off L-By	2603	2.01	88	0.30	597	2.23
14	Off U-By	2414	1.21	91	0.02	652	1.66
15	Perique	2362	3.86	169	4.79	1669	4.44
16	Dark Air Cured	1790	1.18	257	1.87	1694	1.52
17	Fire Cured	1001	0.17	143	0.93	1549	0.37
18	Or A	2403	0.91	49	1.08	861	0.97
19	Or B	839	1.40	28	1.57	160	0.10
20	Cigarette blend	1402	0.92	118	1.73	910	2.39
21	2R4Fn	2401	2.67	107	0.77	905	2.40
22	2R4Fo	1822	1.33	104	1.54	841	1.28
23	3R4F	1696	2.32	114	0.09	964	2.91
24	1R4F	1391	0.20	117	0.14	889	0.53
25	Green FC	78	1.08	14	6.91	94	0.61
26	Green Bu	47	2.71	8	5.90	67	0.66
27	Green Or	86	2.38	7	4.65	98	0.27

A comparison of the data from Table 10 showing the levels of nornicotine, anabasine and anatabine obtained by the (new) Method 2 with the data from Table 9 indicates a very good agreement. The equations for the trendlines and the corresponding R^2 values for the linear regressions for individual minor alkaloids between the results obtained with Method 1 versus the results obtained with Method 2 are given in Table 11. The agreement shown in this table indicates that Method 2 provides reliable results for the analysis of the three minor alkaloids evaluated in this study. The RSD% results (for duplicate samples) indicate the good repeatability of the method.

The results for the level of R-nornicotine and S-nornicotine, shown separately, and the proportion of S-nornicotine relative to total nornicotine are given in Table 12. These results indicate that the proportion of S-nornicotine versus the total level of nornicotine in different tobaccos varies considerably. For the analyzed domestic flue-cured tobaccos, a range from 62.2% to 71.0% of S-nornicotine was measured (Note: In this report, US tobaccos are indicated as “domestic” and non-US tobaccos are indicated as “off-shore”). Offshore flue-cured tobaccos, however, have a lower proportion of S-nornicotine, between 52% and

57%. A wider range of proportions of S-nornicotine was noticed among the analyzed Burley tobacco samples, which ranged between 54% and 72%. However, offshore Burley had a high level of S-isomer, around 90%. Perique and fire-cured tobaccos showed a proportion of S-nornicotine similar to some analyzed Burley tobaccos. The dark air-cured tobacco (2012 crop) showed an approximately equal proportion of R- and S-nornicotine. Relatively high levels of the S-isomer were seen in Oriental tobaccos (between 81% and 84%). Cigarette blends (a commercial cigarette and several Kentucky reference cigarettes) had S-nornicotine in the range 69% to 77%. Freeze-dried green tobaccos analyzed in this study had very low levels of nornicotine, and the proportion of S-nornicotine versus total nornicotine was between 31% and 44%. These tobaccos had slightly more R-nornicotine than S-nornicotine. The results for Burley, flue-cured and Oriental tobaccos analyzed in this study are in agreement with the data reported in the literature (21). Also, the data reported for R- and S-nornicotine in this study show the same R/S ratio as the results reported in the literature for R/S isomers of *N*’-nitrosonornicotine in several tobacco blends (16).

Table 10. The level of total nornicotine, anabasine, and anatabine analyzed by Method 2.

No.	Sample	Ave. total nornicotine	RSD% total nornicotine	Ave. anabasine mg/g	RSD% anabasine	Ave. anatabine mg/g	RSD% anatabine
1	By A	2527	1.24	238	2.34	1901	2.14
2	FC A	626	3.21	229	4.21	1440	2.11
3	L-FC B	288	2.01	65	2.21	576	0.89
4	U-FC B	2234	1.08	205	0.80	1527	0.00
5	L-FC C	914	2.56	96	1.10	836	1.67
6	U-FC C	1612	5.42	174	6.71	1265	4.06
7	Off L-FC	840	3.91	106	6.97	935	8.10
8	Off U-FC	1776	5.01	242	4.11	1972	3.58
9	L-By B	3609	0.69	177	3.96	1839	4.30
10	U-By B	4106	5.23	229	3.59	2411	4.81
11	L-By C	3227	2.00	175	1.25	1511	2.28
12	U-By C	3623	5.08	204	3.78	1730	3.16
13	Off L-By	2638	1.70	83	0.73	578	1.74
14	Off U-By	2352	3.76	81	1.95	645	1.58
15	Perique	2387	6.03	159	3.18	1545	6.08
16	Dark Air Cured	1822	1.63	243	2.82	1637	0.34
17	Fire Cured	1037	3.71	176	2.23	1694	2.33
18	Or A	2462	2.38	84	1.90	829	4.48
19	Or B	895	1.45	34	3.63	157	5.21
20	Cigarette blend	1457	1.95	115	1.37	867	1.73
21	2R4Fn	2411	5.18	108	5.85	878	4.09
22	2R4Fo	1889	0.99	104	0.10	826	1.70
23	3R4F	1672	2.34	108	1.27	922	0.65
24	1R4F	1410	3.58	108	1.59	858	2.83
25	Green FC	91	3.06	~14*	2.93	101	4.28
26	Green Bu	49	5.46	~8*	3.36	58	1.69
27	Green Or	93	0.03	~9*	3.84	86	0.78

* Note: The result is below the LOQ level, and therefore only approximated.

Table 11. The equations of trendlines and R² values for the dependence between the results obtained by the two analytical methods for minor alkaloids analysis.

No.	Compound	Equation*	R ²
1	Nornicotine	Y = 1.0108 X	0.9989
2	Anabasine	Y = 1.0336 X	0.9492
3	Anatabine	Y = 0.9892 X	0.9947

*Note: X represents the results of Method 1, and Y the results of Method 2.

CONCLUSIONS

A new method for the analysis of nornicotine, anabasine and anatabine has been developed and partially validated. The method allows separate quantitation for S-nornicotine and R-nornicotine. In terms of accuracy, this procedure compares very favorably with a technique commonly used for minor alkaloids analysis (6). The proportion of S-nornicotine in the total nornicotine present in tobacco varies depending on the tobacco type between 52.6% for an offshore flue-cured tobacco to 91.4% for an offshore Burley. Green tobaccos (freeze-dried) showed lower levels of S-nornicotine, between 31.6% and 43.8% of the total nornicotine (in the analyzed samples).

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Table 12. The level of R-nornicotine, S-nornicotine and the proportion of S-nornicotine vs. total.

No.	Sample	Ave. R-nornicotine $\mu\text{g g}^{-1}$	RSD% R-nornicotine	Ave. S-nornicotine $\mu\text{g g}^{-1}$	RSD% S-nornicotine	S-nornicotine/ nornicotine total %
1	By A	777	2.32	1750	1.24	69.3
2	FC A	202	4.23	422	2.33	67.6
3	L-FC B	99	2.41	189	3.22	65.6
4	U-FC B	649	2.22	1585	1.86	71.0
5	L-FC C	347	2.34	567	2.86	62.2
6	U-FC C	523	7.29	1089	6.00	67.6
7	Off L-FC	398	5.14	442	9.50	52.6
8	Off U-FC	758	3.79	1018	5.92	57.3
9	L-By B	1478	0.58	2132	0.77	59.1
10	U-By B	1856	4.40	2250	5.92	54.8
11	L-By C	916	3.37	2311	1.46	71.6
12	U-By C	1013	3.68	2610	5.63	72.0
13	Off L-By	227	3.05	2411	1.57	91.4
14	Off U-By	238	0.56	2114	4.12	89.9
15	Perique	1016	1.84	1371	1.47	57.5
16	Dark Air Cured	914	4.44	908	2.96	49.8
17	Fire Cured	430	3.74	607	1.42	58.6
18	Or A	403	9.00	2059	5.45	83.6
19	Or B	166	1.93	729	2.22	81.4
20	Cigarette blend	454	2.03	1003	1.92	68.8
21	2R4Fn	622	4.21	1789	5.52	74.2
22	2R4Fo	460	1.58	1429	0.80	75.7
23	3R4F	508	4.61	1163	1.36	69.6
24	1R4F	329	4.06	1081	3.44	76.7
25	Green FC	53	5.61	38	0.50	41.7
26	Green Bu	34	2.32	~16*	4.81	31.6
27	Green Or	52	2.76	41	3.62	43.8

* Note: The result is very close to the LOQ level, and therefore only approximated.

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