Beiträge zur Tabakforschung International/*Contributions to Tobacco Research* Volume 21 · No. 7 · October 2005
DOI: 10.2478/cttr-2013-0803

Analysis of Minor Alkaloids in Tobacco: A Collaborative Study*

by

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SUMMARY

A collaborative study regarding the analysis of minor alkaloids in tobacco and tobacco products was initiated by the Analytical Methods Committee of the Tobacco Science Research Conference (TSRC). One purpose of the study was to assess the reliability of methods used for the analysis of minor alkaloids and nicotine in tobacco and tobacco products, as practiced in different laboratories. A second purpose was to select a preferred method or to develop a hybrid method based on the best elements from the procedures currently used for the analysis of minor alkaloids. A hybrid method was developed and its results compared with the values from existing methods. The hybrid method proves to be reliable, has good repeatability, and is easy to implement. This new method can be used as a reference procedure for minor alkaloids and nicotine analysis using either flame ionization (FID) or mass spectrometric (MS) detection. A comparison with the older techniques shows that the hybrid method considerably improved the repeatability, but only marginally increased the reproducibility for different laboratories. This may be, in part, because any new method requires some time to become fully standardized and reliable. Also, the contribution of other sources of variation such as different standards and different equipment may have limited the improvement in reproducibility. [Beitr. Tabakforsch. Int. 21 (2005) 369–379]

ZUSAMMENFASSUNG

Der für analytische Methoden verantwortliche Ausschuss der „Tobacco Science Research Conference" (TSRC) hat einen Ringversuch zur Untersuchung von sekundären Alkaloiden im Tabak und Tabakrauch initiiert. Ein Ziel dieser Untersuchung war es, die Zuverlässigkeit der in den verschiedenen Labors zur Analyse von sekundären Alkaloiden und Nikotin im Tabak und in Tabakprodukten verwendeten Methoden zu bestimmen. Ein zweites Ziel bestand darin, eine bevorzugte Methode auszuwählen oder eine Hybridmethode zu entwickeln, in der die besten Elemente der gegenwärtig benutzten Verfahren zur Analyse von Sekundäralkaloiden Verwendung finden. Eine Hybridmethode wurde entwickelt und die damit erzielten Ergebnisse mit denen etablierter Methoden verglichen. Die Hybridmethode erwies sich als verlässlich, sie zeigte gute Wiederholbarkeit und die Durchführung war einfach. Diese neue Methode kann für die Analytik von Sekundäralkaloiden und Nikotin sowohl bei Verwendung eines Flammenionisationsdetektors (FID) als auch mit massenspektrometrischer (MS) Detektion als Referenzmethode dienen. Ein Vergleich mit älteren Techniken zeigt, dass die Wiederholbarkeit durch die Hybridmethode beträchtlich, die Reproduzierbarkeit in verschiedenen Labors aber lediglich geringfügig verbessert wurde. Dies könnte zum Teil daran liegen, dass sich jede neue Methode erst im Laufe der Zeit als Standard etablieren und als verlässlich gelten kann. Außerdem könnten auch andere Variationsfaktoren, wie unterschiedliche Standards und Ausstattungen eine bessere Reproduzierbarkeit erschwert haben. [Beitr. Tabakforsch. Int. 21 (2005) 369–379]

RESUME

Une étude collective sur l'analyse des alcaloïdes secondaires du tabac et des produits de tabac a été réalisée par le comité de méthodes analytiques de la « Tobacco Science Research Conference » (TSRC). Un premier objectif de l'étude était de déterminer la fiabilité des méthodes utilisées par les différents laboratoires pour l'analyse des alcaloïdes secondaires et de la nicotine dans le tabac et les produits de tabacs. Un

* Received: 7th March 2005 – accepted: 1st July 2005

Table 1. List of participating laboratories

Participating institution	Contact person
Altadis/Institut du Tabac de Bergerac, France	Dr. Catherine Poisson
Arista Laboratories, USA	Dr. Karl Wagner
Austria Tabak, Austria	Dr. Jutta Müller
BAT Souza Cruz, Brazil	Dr. Ana Hovell, Dr. Horacio M. Oliveira
Brown & Williamson Tobacco Corp., USA	Dr. Serban Moldoveanu, Mrs. Nancy Qian
Cia Colombiana de Tabaco S.A., Colombia	Dr. Jorge Ivan Castano
Filtrona Technology Centre, England	Dr. Mick Dunn
Japan Tobacco Inc., Japan	Dr. Hitoshi Saito
Labstat International Inc., Canada	Mr. Terry Field
Lancaster Laboratories, USA	Dr. John S. Kauffman
Lorillard Tobacco Co., USA	Dr. Jim Morgan
LTR Industries, France	Dr. Thierry Joyeux
R.J. Reynolds Tobacco Company, USA	Dr. Bert Gordon, Mr. S. Mark DeBusk, Mrs. Karen Kilby
Southern Testing and Research Laboratories, USA	Dr. Deepa Goli
Swedish Match North Europe, Sweden	Dr. Eva Norlén Moritz
Swedish Match North America, USA	Dr. David M. Johnson
U.S. Smokeless Tobacco Mfg. LP, USA	Dr. Cliff B. Bennett, Dr. Shawn Shanmugan
United Chemical Technologies, Inc., USA	Dr. John A. D'Asaro, Dr. Michael Telepchak
University of Kentucky, Dept. of Agronomy, USA	Prof. Harold Burton
University of Kentucky, Dept. of Agronomy, USA	Prof. Lowell Bush

deuxième objectif était de choisir une méthode sélectionnée ou de développer une méthode hybride basée sur les meilleurs éléments des procédures actuellement utilisées pour l'analyse des alcaloïdes secondaires. Une méthode hybride a été développée et les résultats ont été comparés avec les valeurs obtenues avec des méthodes établies. La méthode hybride s'est avérée fiable, la répétabilité est bonne et la mise au point facile. Cette nouvelle méthode peut être utilisée comme procédure de référence pour des alcaloïdes secondaires et l'analyse de la nicotine par détection par ionisation de flamme (FID) ou par spectrométrie de masse (MS). Une comparaison avec les techniques plus anciennes montre que la méthode hybride améliore considérablement la répétabilité mais n'accroît que marginalement seulement la reproductibilité dans des laboratoires différents. Ceci peut être partiellement dû au fait que chaque nouvelle méthode nécessite un certain temps avant d'être normalisée et fiable. De plus, la contribution d'autres sources de variation comme des standards et des équipements différents pourraient avoir limité l'amélioration de la reproductibilité. [Beitr. Tabakforsch. Int. 21 (2005) 369–379]

INTRODUCTION

The name "tobacco minor alkaloids" is used for several β-substituted pyridines that are present in tobacco in addition to nicotine. Among the minor tobacco alkaloids are compounds such as anabasine or 3-(2-piperidinyl)pyridine, anatabine or 3-(2-1,2,3,6-tetrahydropyridyl)pyridine, cotinine or 1-methyl-5-(3-pyridyl)-2-pyrrolidinone), 2,3'-dipyridyl or isonicoteine, *N*-formylnornicotine or 2-(3-pyridyl)pyrrolidinecarbaldehyde, myosmine or 3-(1-pyrrolin-2-yl)pyridine, nornicotine or 3-(pyrrolidin-2-yl)pyridine, and β-nicotyrine or 3-(1-methylpyrrol-2-yl)pyridine. These compounds are found at various levels in tobacco and tobacco products, nornicotine and anatabine being the two most abundant minor alkaloids, each typically accounting for 2% to 6% of the total alkaloid content of tobacco. (Nornicotine content is typically higher in burley). Anabasine, myosmine, and the other compounds previously listed are present at lower levels, each one accounting for about 0.1–0.5% of total alkaloids. Other alkaloids with even lower levels also are present in tobacco, e.g. (1).

The role of minor alkaloids in tobacco products has been widely discussed in the literature. These compounds are important, either in connection with the taste of different tobacco products (2,3) or with health (4–7) and environmental issues (8,9), thus interest in their analysis remains active. A number of methods for the analysis of minor alkaloids and nicotine is published in the literature (9–19). These methods have been applied to a variety of tobaccos and tobacco products, thus a comparison of the advantages and disadvantages of each method is rather difficult to make from the literature alone. For this reason, a collaborative study regarding the analysis of minor alkaloids in tobacco and tobacco products was initiated by the Analytical Methods Committee of the Tobacco Science Research Conference (TSRC). The list of participating laboratories is given in Table 1. Each laboratory was randomly assigned a separate number between 1 and 20 for blinded identification purposes.

The study was organized somewhat differently from a typical collaborative study (20) in that it had two sample analysis phases. The first phase assessed a number of existing methods currently applied in the laboratories for the analysis of minor alkaloids and nicotine. A "between two phases" process was incorporated to select a best method or to develop a hybrid method, which would be based on the best elements from the existing methods, for the analysis of minor alkaloids. The second analytical phase evaluated the resulting hybrid method, as applied in the participating laboratories, and compared the variability of data between the hybrid method and the data generated by

Table 2. GC conditions for the analysis of minor alkaloids in tobacco

Parameter	Setting
Injection temperature	275 ˚C
Injection type	Splitless
Injection volume	1.0 mL
Carrier gas	Helium
Gas velocity (constant flow)	1.0 mL/min
Purge valve on time	0.5 min
Initial oven temperature	50 ˚C
Initial hold time	1.0 min
Rate of first ramp	5 ˚C/min
Final temperature	185 ˚C
Hold time	0 min
Rate of second ramp	10 ˚C/min
Final temperature	320 ˚C
Final hold time	0 min
Detection temperature (FID)	320 ˚C
Transfer line temperature for the MS detection	300 ˚C

the different methods previously practiced. The use of a common method compared to different methods in each laboratory was not *a priori* considered a better alternative because, in an ideal situation in which analytical techniques provide accurate results, it should not matter which technique is used.

EXPERIMENTAL

For the first phase of this study, each laboratory used its own method on two control tobaccos (one flue-cured tobacco and one burley tobacco). The tobaccos were homogenized and samples were sent to each participating laboratory from the Department of Agronomy of the University of Kentucky. The analytes required to be measured included nicotine and the following minor alkaloids: anabasine, anatabine, nornicotine, and myosmine. Measurements of cotinine, *N*-formylnornicotine, 2,3'-dipyridyl and nicotyrine levels were optional. The analytical protocol required that each sample be analyzed on four different days within two weeks. Furthermore, on each day, four sub-samples of each homogenized tobacco sample were to be analyzed [not just four gas chromatography-liquid chromatography (GC-LC) injections from the same preparation]. The results consisted of raw data from only one injection, with no elimination of suspected outliers. Only analyses known to be faulty could be repeated. This protocol generated sixteen analyses for each tobacco sample: results were reported on a dry-weight basis. The water content was measured in each laboratory on a separate portion of the sample. Chemical standards were purchased individually by each laboratory, but a preferred list of suppliers with the specified lot number of each chemical was provided.

The results from Phase 1 led to the development of a hybrid method as an optimum procedure for the analysis of minor alkaloids in tobacco and tobacco products. This hybrid method contains experimental elements from procedures applied in different laboratories that were rated highly from the analysis of the data generated in Phase 1 of the study. The hybrid method, as described below, was evaluated in Phase 2 of the study.

The hybrid method uses a GC separation with flame ionization detection (FID) for the analysis of nicotine, anabasine, anatabine, nornicotine, and myosmine. An alternative procedure uses the same hybrid method except for the detection, which is done using mass spectrometry (MS), and allows the analysis of a wider range of compounds.

The analysis employing the hybrid method starts with 1.0 g of tobacco sample (as is), which is weighed with precision of 0.1 mg. To this sample, 5.0 mL of aqueous 2 *N* NaOH is added to achieve complete wetting, and the sample is allowed to stand at room temperature for 15 min. The purpose of this step is to transform into base form any minor alkaloids present as salts. For the GC-FID technique, 50.0 mL of an extracting solution are added to the wet sample. For the alternative GC-MS technique, 250 µL of a methanol solution containing 1000 µg/mL $[^2H]_8$-2,2'-dipyridyl (available from C/D/N/ Isotopes Inc, Pointe-Claire, Canada) is added, and then 50.0 mL of the extracting solution is added to the wet sample.

The extracting solution is prepared using 2 mL of a stock solution which is diluted to 200 mL with *tert*-butyl methyl ether (TBME). The extraction stock solution is prepared by dissolving 0.2 g of 7-methylquinoline (I.S. for FID determination) in 50 mL of TBME. The final concentration of the extraction working solution should be 40 µg/mL of 7-methylquinoline (7-methylquinoline can be purchased from Aldrich, www.sigma-aldrich.com).

The sample is extracted by shaking for 1 h on a mechanical shaker. After extraction, the phases in the extracting container are allowed to separate: 30 min were typically sufficient for this step. The organic phase can be used directly for GC-FID analysis, typically with a 1-µL injection. For GC-MS analysis, an aliquot of 10 mL from the organic layer is separated and reduced to 1 mL by evaporation under nitrogen. From this solution, 1 µL is injected into a GC-MS system for analysis. A separate tobacco sample is used for moisture determination. Each laboratory used its own procedure for this measurement.

The instrument used for the development of the GC separation was an Agilent 6890 gas chromatograph (equivalent instrumentation can be used in other labs.). The gas chromatograph was equipped with a 30 m × 0.25 mm i.d. ZB-50 column, 0.25 µm film thickness, with a 4-mm split/splitless glass liner with glass wool. (An equivalent DB-17 column gives similar separation.) The GC separation conditions are given in Table 2.

By FID detection, only nicotine, nornicotine, anabasine and anatabine can be detected with high accuracy, while myosmine can be detected but with a larger error. MS detection is necessary for the analysis of the lower levels minor alkaloids including myosmine, nicotyrine, 2,3'-dipyridyl, cotinine, *N*-formylnornicotine. The instrument used for the development of the MS alternative was an Agilent 5973 MSD (equivalent instrumentation can be used in other labs). The total ion chromatogram is acquired with MS, but the quantitation is done with extracted ion peaks. A number of 2.85 scans per second was used for the acquisition of a mass range between 33 and 450 amu. The extracted ions used for quantitation are listed in Table 3. A typical total

Table 3. Ions used as extracted ions for quantitation using MS detection

Compound	Ion	Remark
Nornicotine	147, 119	Analyte
Myosmine	146, 118	Analyte
$[^2H]_8$-2,2'-dipyridyl	164	Internal standard
Anabasine	162, 84	Analyte
Nicotyrine	158	Analyte
Anatabine	160	Analyte
2,3'-Dipyridyl (isonicoteine)	156	Analyte
Cotinine	176, 98	Analyte
N-Formylnornicotine	176, 147	Analyte

ion chromatogram for a sample of a flue-cured tobacco, obtained following the previously described procedure with MS detection, is shown in Figure 1 (nicotine peak eluting at 18.5 min is not shown in the chromatogram).
Calibration curves were obtained for the quantitation by plotting Response of a pure compound vs. Concentration, where

$$\text{Response} = \frac{\text{Peak Area for Pure Compound}}{\text{Peak Area for Internal Standard}}, \quad \text{and}$$

$$\text{Concentration} = \text{Concentration of each Pure Compound}.$$

The concentration of a compound in the sample can be obtained from the calibration curve using the Response of Unknown calculated as

$$\text{Response of Unknown} = \frac{\text{Peak Area for Compound in Sample}}{\text{Peak Area for Internal Standard}}.$$

The Peak Area is either the FID response, or in the case of MS detection the area for the extracted ion peak.
The calibration curve for nornicotine with MS detection is shown as an example in Figure 2. The calibration for

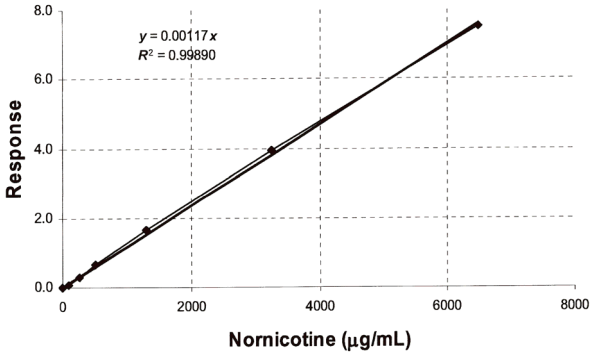

Figure 2. Calibration curve for nornicotine (R^2 = 0.9989) with MS detection

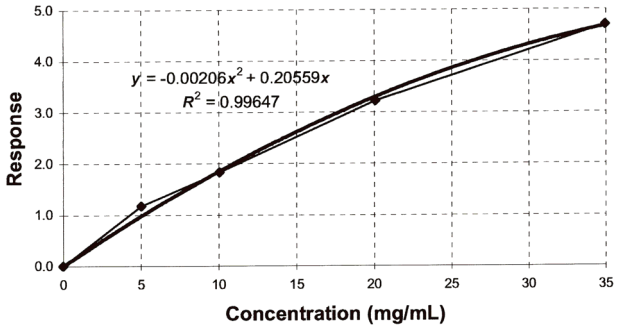

Figure 3. Calibration curve for nicotine (R^2 = 0.9965) with MS detection

nicotine is linear for the GC-FID alternative. However, for MS quantitation of nicotine, a quadratic calibration curve is more appropriate, as shown in Figure 3.
One step which required evaluation in the hybrid analytical technique was the sample extraction time. For the determination of the optimum extraction time, a flue-cured tobacco sample was analyzed for minor alkaloids. After the analysis, 1 mL of a stock solution inTBME containing 20 mg/mL

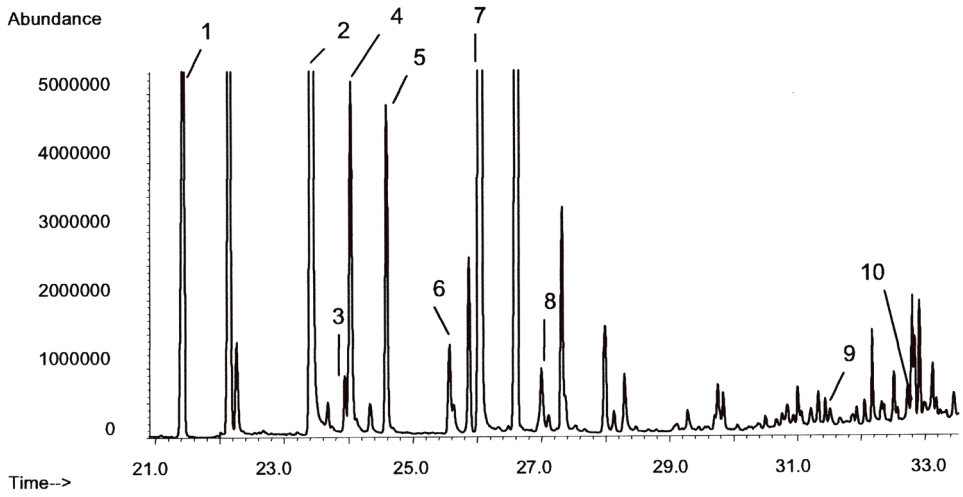

Figure 1. Total ion chromatogram for a flue-cured tobacco using MS detection: 1 = 7-Methylquinoline; 2 = Nornicotine; 3 = Myosmine; 4 = $[^2H]_8$-2,2'-Dipyridyl (I.S.); 5 = Anabasine; 6 = Nicotyrine; 7 = Anatabine; 8 = 2,3'-Dipyridyl; 9 = Cotinine; 10 = *N*-Formylnornicotine. (Nicotine peak eluting at 18.5 min is not shown in the chromatogram.)

Table 4. Recovery efficiency of various minor alkaloids and nicotine at different extraction times

Compound	Extraction time		
	30 min (%)	60 min (%)	2 h (%)
Anabasine	99	99	95
Anatabine	98	96	87
Cotinine	55	55	55
2,3'-Dipyridyl	110	97	97
Myosmine	92	96	90
N-Formylnornicotine	53	48	40
Nicotine	90	85	65
Nicotyrine	>110	99	99
Nornicotine	90	90	90

nicotine, 1000 µg/mL of nornicotine and anatabine, and 100 µg/mL of myosmine, anabasine, 2,3'-dipyridyl, nicotyrine, cotinine and *N*-formylnornicotine was added to a 1.0 g tobacco sample. The samples were reanalyzed, the background minor alkaloids subtracted, and the extraction efficiency was determined from the amount of recovered compounds. The results obtained by GC-MS analysis are reported in Table 4.

As seen from Table 4, the extraction efficiencies are good for most minor alkaloids after 30 min extraction time and only minor increases are seen for myosmine after 60 min extraction time. Some compounds, such as nicotine, even showed a slight decrease in the recovery for the longer extraction times. Because no significant benefits were found for 2 h extraction, and only minor differences were seen between 30 min and 60 min of extraction, a 60-minute extraction time was selected for the hybrid method. This was in agreement with the extraction time practiced by most laboratories in Phase 1 of the study.

The protocol for Phase 2 of the study required the analysis of five samples. These samples covered a wide range of minor alkaloids levels and included three single grade tobaccos, i.e., a burley, a flue-cured and an Oriental, and two tobacco blends, i.e., one from the 2R4F Kentucky reference cigarette and the other from the 2S3 Kentucky reference moist snuff, indicated below as the "Smokeless" sample. In Phase 2, four separate analyses of a sample were performed within one day and different samples were analyzed on different days. As in Phase 1, the results included raw data from only one injection with no elimination of suspected outliers (only analyses known to be faulty were allowed to be repeated), results were reported on a dry-weight basis, water content was measured in each laboratory on a separate portion of the sample, and chemical standards were purchased individually by each laboratory.

For statistical analysis, the set of data for each analyte was formed into a matrix $\{x_{i,j}\}$, where index "*i*" indicated the laboratory ($i = 1, \ldots p$) and index "*j*" indicated the replicate ($j = 1, \ldots n$). The within-laboratory average, $\overline{X_1}$, and within-laboratory standard deviation (STD), S_i, were calculated for each laboratory. These were obtained using expressions:

$$\overline{X_i} = \frac{1}{n}\sum_{j=1}^{n} x_{i,j} \text{ for } i = 1,2\ldots p \text{ and}$$
$$S_i = \sqrt{\frac{1}{n-1}\sum_{j=1}^{n}(x_{i,j} - \overline{X_i})^2} \quad [1]$$

The among-laboratories average, $\overline{X}$, and the standard deviation between-laboratories average value, $S_{\overline{X}}$, were calculated using expressions:

$$\overline{X} = \frac{1}{p}\sum_{i=1}^{p}\overline{X_i} = \frac{1}{pn}\sum_{i=1}^{p}\sum_{j=1}^{n} x_{i,j} \text{ and}$$
$$S_{\overline{X}} = \sqrt{\frac{1}{p-1}\sum_{i=1}^{p}(\overline{X_i} - \overline{X})^2} \quad [2]$$

The repeatability STD S_{-r} and reproducibility STD S_{-R} were calculated using expressions:

$$S_{-r} = \sqrt{\frac{1}{p}\sum_{i=1}^{p} S_i^2} \text{ and}$$
$$S_{-R} = \sqrt{S_{\overline{X}}^2 + \frac{n-1}{n}S_{-r}^2} \quad [3]$$

The coefficients of variation CV_{-r} characterizing repeatability and CV_{-R} characterizing reproducibility were calculated as:

$$CV_{-r} = \frac{S_{-r}}{\overline{X}}100\% \text{ and}$$
$$CV_{-R} = \frac{S_{-R}}{\overline{X}}100\% \quad [4]$$

A two way-ANOVA was used for statistical comparisons and elimination of outliers (21,22).

The limits of detection (LOD) and quantitation (LOQ) for the hybrid method were not explicitly given in this report. The typical procedure applied for the calculation of LOD as 3 S_i and that of LOQ as 10 S_i for a very low content sample were not applicable because no tobacco standards with very low content of minor alkaloids were available. On the other hand, the calculation of these values for a mixture of pure compounds was not considered appropriate because in this case the interferences from a tobacco matrix are not accounted for. However, for a mixture of pure compounds all LOD values were below 15 µg/mL for both FID and MS detection.

RESULTS AND DISCUSSION

The results from Phase 1 of the study were used for two purposes. The first purpose was the assessment of accuracy and precision of the analysis of minor alkaloids in various laboratories. The second purpose was to select a best method, or to lead to the development of a hybrid method based on the best elements from the procedures currently used for the analysis of minor tobacco alkaloids. The average levels of several minor alkaloids and the STD S_{-R} values for these measurements, as measured in different laboratories in Phase 1, are shown in Table 5. The values for the coefficients of variation CV_{-r} and CV_{-R} obtained from Phase 1 of the study are shown in Table 6.

The between-phases method selection/development process began with the selection of the "best" analytical procedure among those evaluated in Phase 1. An overall score was calculated for each laboratory's procedure that considered accuracy, precision, and the number of minor alkaloids

Table 5. Averages (in μg/g, dry basis) and STD S_{-R} values for several minor alkaloids and nicotine as measured in different laboratories for Phase 1

Compound	No. of labs.	Values	Burley	Flue-cured
Anabasine [a]	18	Average	226.5	268.3
		S_{-R}	47.6	46.2
Anatabine [a]	18	Average	1979.5	2057.9
		S_{-R}	289.5	288.5
2,3'-Dipyridyl	7	Average	30.9	37.5
		S_{-R}	18.1	19.7
N-Formyl-nornicotine	6	Average	185.7	85.9
		S_{-R}	98.9	64.8
Myosmine [a]	12	Average	138.5	74.1
		S_{-R}	129.7	54.5
Nicotine [a]	17	Average	44198.8	40971.4
		S_{-R}	2875.5	2922.6
Nornicotine	18	Average	5743.2	1593.7
		S_{-R}	934.4	370.9

[a] One result was eliminated from the calculation of average and STD S_{-R} because it was detected as an outlier.

Table 6. Coefficients of variation CV_{-r} and CV_{-R} obtained from Phase 1 of the study

Compound	No. of labs.	CV	Burley (%)	Flue-cured (%)	Pooled (%)
Anabasine	17	CV_{-r}	11	8	9
		CV_{-R}	21	17	19
Anatabine	17	CV_{-r}	10	8	9
		CV_{-R}	15	14	14
2,3'-Dipyridyl	7	CV_{-r}	15	12	14
		CV_{-R}	59	53	56
N-Formyl-nornicotine	6	CV_{-r}	11	21	16
		CV_{-R}	53	75	64
Myosmine	11	CV_{-r}	37	21	29
		CV_{-R}	94	74	84
Nicotine	16	CV_{-r}	5	5	5
		CV_{-R}	7	7	7
Nornicotine	18	CV_{-r}	11	7	9
		CV_{-R}	16	23	20

analyzed by the procedure. First, a partial score for accuracy was calculated for each analyte by assigning a number from 0 to 10, with 10 assigned to the result closest to the all-laboratories average and 0 assigned to the result farthest away. The averages of these partial scores generated the procedure's score for accuracy. Similarly, a score for precision was calculated based on the lowest STD S_{-r}. Lastly, the number of minor alkaloids analyzed by the procedure was counted. These scores and their totals, which allowed a ranking of the analytical procedures used in the laboratories, are shown in Table 7.

Other details for each method in Table 7 were provided by individual laboratories and are summarized in this paragraph. The amount of sample used for analysis within laboratories ranged from 100 mg to 2 g. Two general types of extraction were reported for the analysis. One type, which changes alkaloids to salt form, uses an initial extraction with a low-pH aqueous solution followed by reextraction of the basified low-pH wet extract with an

Table 7. Type of analytical method and scores for ranking individual laboratory procedures (methods) in Phase 1

Lab. No. [a]	Type of the Method [b]	No. of Analytes	Accuracy Score	Precision Score	Total Score
1	GC-MS	6	5	9	20
2	GC-FID	7	1	7	15
3	GC-FID	5	10	9	24
4	GC-FID	4	8	7	19
6	GC-FID	7	8	10	25
7	GC-MS	4	7	3	14
8	GC-NPD	6	8	5	19
9	No method indicated	5	5	8	18
10	HPLC-MS-MS	7	8	6	21
11	GC-FID	5	8	6	19
13	GC-FID	6	9	7	22
14	GC-MS	7	7	7	21
15	GC-TCD	6	9	7	22
16	GC-MS	5	4	1	10
17	GC-FID	4	5	4	13
18	HPLC-UV	5	8	6	19

[a] Lab No. is the number randomly assigned to each laboratory (4 laboratories did not participate).

[b] Abbreviations: GC = gas chromatography, MS = mass spectrometry, FID = flame ionization detection, NPD = nitrogen phosphorus detector, HPLC = liquid chromatography, TCD = thermal conductivity detection, UV = spectrophotometric detection.

organic solvent, typically *tert*-butyl methyl ether (TBME). The other type, which changes alkaloids to base form, uses the addition of a basic solution to the tobacco followed by extraction with an organic solvent. Various organic solvents were used in this second type of analysis, including methylene chloride, benzene-chloroform mixture, and TBME. The extraction time varied between 30 min and 2 h. Based on the results shown in Table 7, it was concluded that GC-FID was the most common method for the analysis of minor alkaloids, and it gave the best results for the reported analytes. Some good results also were obtained by GC-MS. Only one laboratory used nitrogen phosphorus detection (NPD) and one used thermal conductivity detection (TCD). Only two laboratories used liquid chromatography (LC) separations, one with MS-MS detection. The GC separations used different columns, such as DB-5, DB-17 (or ZB-50, which is equivalent), and Carbowax (Stabilwax®). However, DB-5 columns did not show good separation for nornicotine, which is an important minor alkaloid. One of the best separations was obtained on a DB-17 column (50% phenyl, 50% methyl silicone).

After reviewing these Phase 1 results it was decided, primarily due to restrictions in the experimental procedures of each highly ranked method, to develop a hybrid method. The hybrid method was based on characteristics and details of the best methods listed in Table 7, as previously described in the experimental section. This hybrid method was developed and collaborative field results were gathered in Phase 2 of this study. The average levels (in μg/g, dry basis) for five alkaloids measured in Phase 2 are shown in Figures 4 to 8.

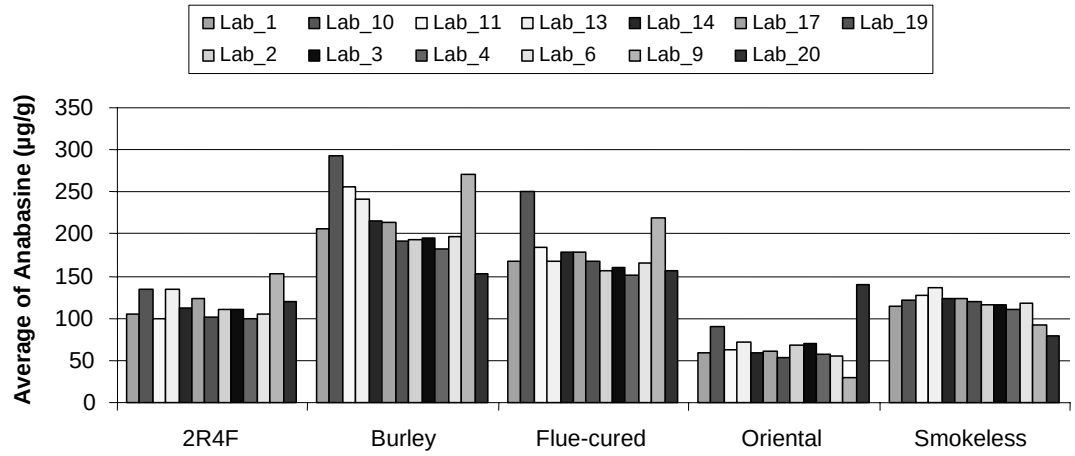

Figure 4. Average results for anabasine analysis in µg/g (dry-weight basis)

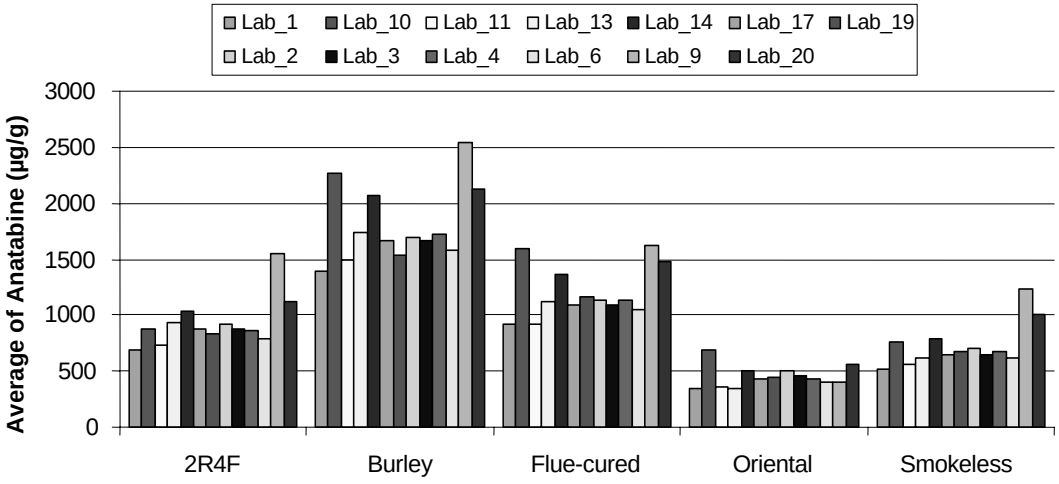

Figure 5. Average results for anatabine analysis in µg/g (dry-weight basis)

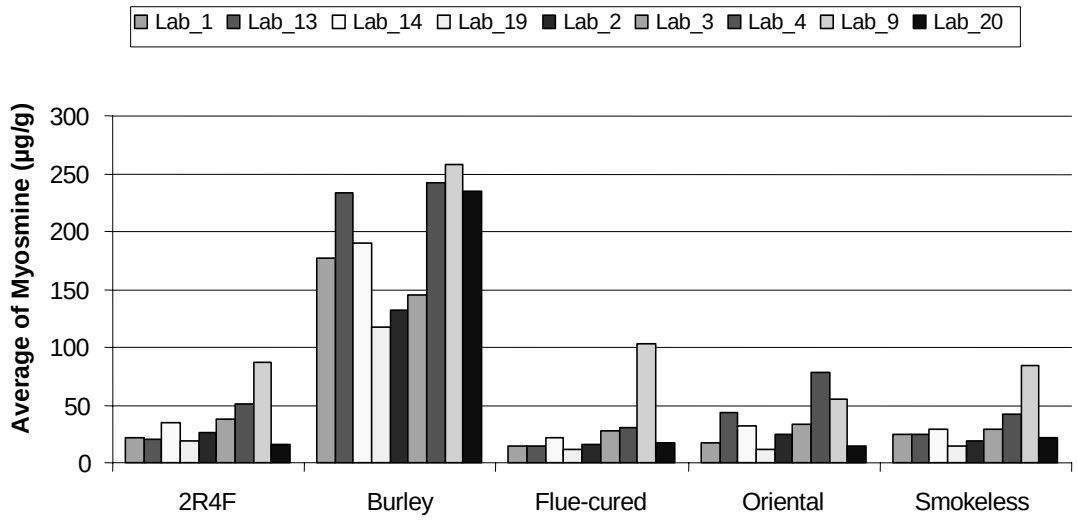

Figure 6. Average results for myosmine analysis in µg/g (dry-weight basis)

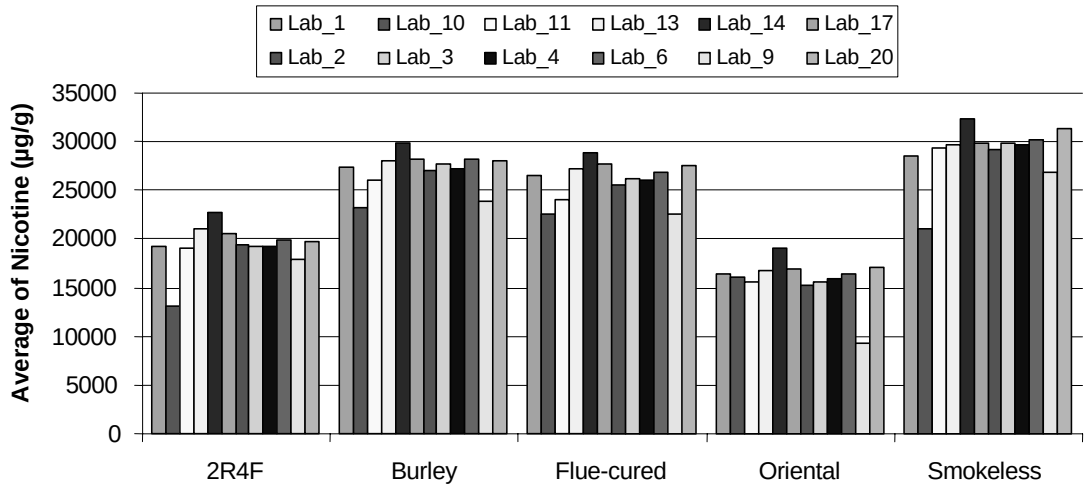

Figure 7. Average results for nicotine analysis in µg/g (dry-weight basis)

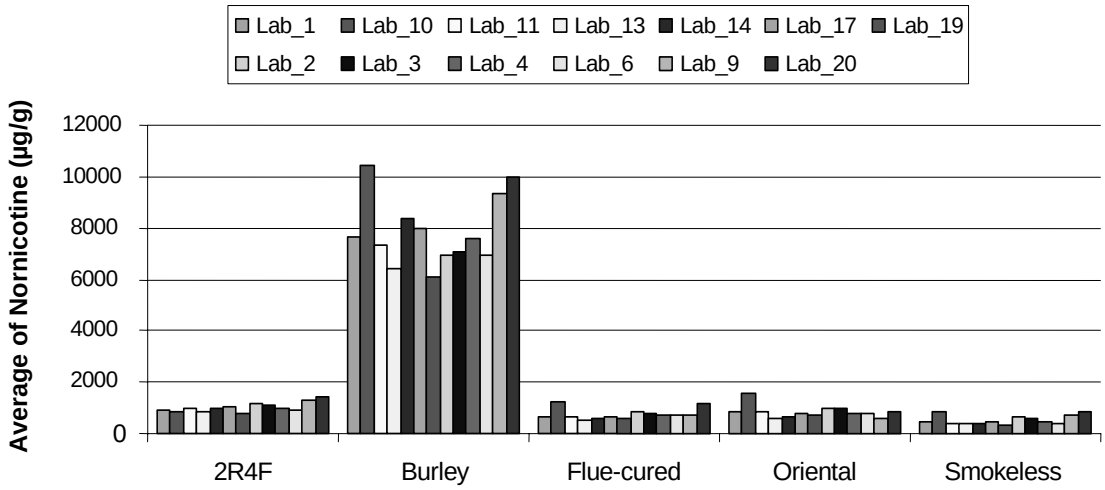

Figure 8. Average results for nornicotine analysis in µg/g (dry-weight basis)

Variation in the alkaloid measurements reported in both phases of this study is, in part, a function of the variation in the absolute and measured water content of the samples. An example of the variation in reported water content is shown in Figure 9. Although only a small variation in the water content is seen for burley, flue-cured, Oriental, and smokeless sample, the water content measured for 2R4F varied from 11% to 14%.

Because it was not possible to determine if this occurred because of analytical variability or because the samples in different laboratories, indeed, had different water contents depending on their storage conditions, the variability in water measurement was not considered as a separate parameter and the data on each minor alkaloid were compared in Phase 2 on an as-reported dry basis (as also was done for Phase 1).

Averages (in µg/g, dry basis) and STD S_{-R} values for several minor alkaloids and nicotine as measured in different laboratories for Phase 2 of this study are summarized in Table 8. The results in Table 8 provide guidance regarding the average levels of several minor alkaloids and nicotine in the analyzed samples. The S_{-R} values provide information on the reproducibility of these results.

The statistical analyses of the results generated in Phase 2 of this study are summarized in Table 9. Because only a few laboratories analyzed 2,3'-dipyridyl, cotinine, *N*-formylnornicotine, and nicotyrine, almost no statistical significance for CV_{-r} and CV_{-R} values can be given. However, for the sake of comparison, the calculated CV_{-r} and CV_{-R} values are included in Table 9.

The results from Table 9 show that the hybrid method is appropriate for the analysis of several minor alkaloids and nicotine in tobacco samples. The errors within each laboratory were quite small for the minor alkaloids, especially considering that the method was new for every participating laboratory. The CV_{-r} values were uniformly below 5% for nicotine, anatabine, and nornicotine, and all but one remaining CV_{-r} value (for myosmine at 15%) was 10% or less. Overall, 27 of the 45 CV_{-r} values (60%) were 5% or less and only 1 of 45 (2%) exceeded 10%. This is clearly better performance than what was observed in

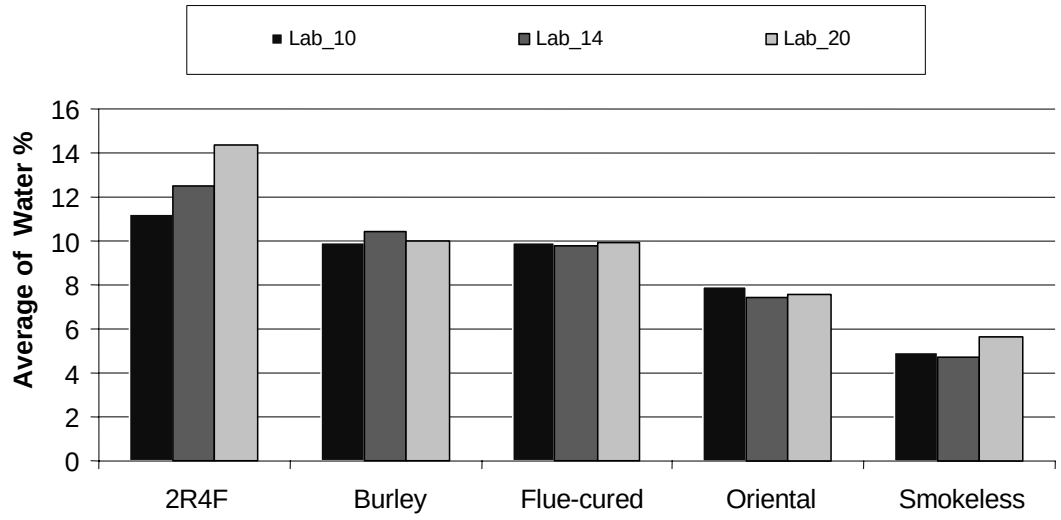

Figure 9. Results of water measurements from three different laboratories

Table 8. Averages (µg/g, dry basis) and STD S_{-R} values for several minor alkaloids and nicotine as measured in different laboratories for Phase 2

Compound	No of labs.	Values	2R4F	Burley	Flue-cured	Oriental	Smokeless
Anabasine [a]	13	Average	116.1	216.2	177.4	61.5	115.2
		S_{-R}	18.2	41.0	29.3	14.8	16.5
Anatabine [a]	13	Average	928.7	1805.1	1205.5	443.5	726.4
		S_{-R}	222.3	341.4	235.1	93.9	195.1
Cotinine	2	Average	93.7	178.7	47.3	117.8	121.8
		S_{-R}	34.5	117.6	8.5	35.9	41.3
2,3'-Dipyridyl	3	Average	25.7	43.5	34.9	9.5	77.2
		S_{-R}	3.8	5.4	6.9	1.6	23.7
N-Formylnornicotine	6	Average	89.2	353.9	63.4	95.7	112.7
		S_{-R}	33.9	155.4	37.5	26.5	44.3
Myosmine [a]	9	Average	34.7	186.4	28.7	28.6	31.6
		S_{-R}	22.9	54.0	29.1	15.2	21.1
Nicotine [a]	13	Average	19833.5	27064.9	25988.7	16487.1	29033.6
		S_{-R}	1280.7	1876.6	2044.3	1076.8	2880.6
Nornicotine [a]	13	Average	1026.9	7862.1	753.5	828.2	529.9
		S_{-R}	185.5	1347.1	220.3	256.3	172.2
Nicotyrine	2	Average	13.5	28.8	18.4	22.1	37.7
		S_{-R}	10.5	15.8	20.0	26.7	9.4

[a] Some results were eliminated from the calculation of averages and STD S_{-R} because they were detected as outliers: for anabasine – Lab 20 on Oriental (too high average); for anatabine – Lab 20 on Oriental (too large within-laboratory variation); for myosmine – Lab 4 on Oriental (too high average) and Lab 20 on burley (too large within-laboratory variation); for nicotine – Lab 9 on Oriental and Lab 10 on 2R4F (too low average); for nornicotine – Lab 20 on Oriental (too large within-laboratory variations). Also, Lab 11 had only three replicates for all samples.

Phase 1, where only 2 of 14 CV_{-r} values (15%) were 5% or less and 4 of 14 (30%) exceeded 10%. On the other hand, CV_{-R} values were still relatively high in Phase 2. Overall, 18 of the 45 CV_{-R} values (40%) were 20% or less and 10 of 45 (22%) exceeded 50%. This is not much better performance than what was observed in Phase 1, where 6 of 14 CV_{-R} values (43%) were 20% or less and 6 of 14 (43%) exceeded 50%. The minor alkaloids, such as myosmine, 2,3'-dipyridyl, nicotyrine, etc., present at low levels showed a considerably larger variability than the other analytes.

CONCLUSIONS

Only a slight improvement can be seen when comparing CV_{-R} values for the same analyte between Phase 1 and Phase 2 of the study. The rather similar ranges for CV_{-R} values between the two phases of the study indicate that the use of different analytical methods between laboratories is neither the major nor the only source of between-laboratory variability. Nevertheless, additional sources of variability can be pointed out that may have masked the benefits of a common analytical method. It is possible that implementa-

Table 9. Statistical analysis of the results generated in Phase 2

Compound	No of labs.	CV	2R4F (%)	Burley (%)	Flue-cured (%)	Oriental (%)	Smokeless (%)	Pooled (%)
Anabasine	13	CV_{-r}	8	6	5	7	7	7
		CV_{-R}	16	19	17	24	14	18
Anatabine	13	CV_{-r}	4	3	2	3	3	3
		CV_{-R}	24	19	19	21	27	22
Cotinine	2	CV_{-r}	3	3	4	1	6	3
		CV_{-R}	37	66	18	31	34	37
2,3'-Dipyridyl	3	CV_{-r}	3	5	4	6	2	4
		CV_{-R}	15	13	20	16	31	19
N-Formylnornicotine	6	CV_{-r}	7	6	6	6	5	6
		CV_{-R}	38	44	59	28	39	42
Myosmine	9	CV_{-r}	9	5	15	9	7	9
		CV_{-R}	66	29	101	53	67	63
Nicotine	13	CV_{-r}	1	1	1	1	1	1
		CV_{-R}	6	7	8	7	10	8
Nornicotine	13	CV_{-r}	3	1	3	4	2	3
		CV_{-R}	18	17	29	31	32	25
Nicotyrine	2	CV_{-r}	10	8	7	10	5	8
		CV_{-R}	78	55	109	121	25	78

tion variations may have led to higher than expected between-laboratory variability for the hybrid method. Furthermore, the study did not tightly control sample moisture; thus variations in moisture were included in the variation for minor alkaloids. Also, the study required each laboratory to purchase its own standards as part of a typical operating procedure; the differences in the purchased chemicals may have added to the variations between laboratories. Lastly, we note that because the number of participating laboratories is not the same for the two phases and the number of replicates in Phase 1 is different from that in Phase 2, the corresponding CV_{-R} values have different reliability.

Although the new hybrid technique improved only marginally the reproducibility (CV_{-R} values), the new method is simple enough to be easily implemented in modern analytical laboratories. Also, the better repeatability of the method indicated by lower CV_{-r} values shows that the hybrid method can serve as a reliable reference procedure for the analysis of minor alkaloids.

REFERENCES

1. Crooks, P.A.: Chemical properties of nicotine and other tobacco-related compounds; *in*: Analytical determination of nicotine and related compounds and their metabolites, edited by J.W. Gorrod and J. Peyton III, Elsevier, Amsterdam, 1999, pp 69–147.
2. Burton, H.R., R.A. Andersen, P.D. Fleming, and L.R. Walton: Changes in chemical composition of burley tobacco during senescence and curing; J. Agr. Food Chem. 36 (1988) 579–584.
3. Pakhale, S.S. and G.B. Maru: Distribution of major and minor alkaloids in tobacco mainstream and sidestream smoke of popular Indian smoking products; Food Chem. Toxicol. 36 (1998) 1131–1138.
4. Aislaitner, G., S. Yang, and J.W. Gorrod: Preliminary evidence for the formation of reactive intermediates during the in vitro metabolism of minor tobacco alkaloids; Med. Sci. Res. 21 (1993) 819–821.
5. Gorrod, J.W. and J. Wahren (Eds.): Nicotine and related alkaloids: absorption, distribution, metabolism and excretion, Chapman Hall Ltd., London, 1993.
6. Dwoskin, L.P., L. Teng, S.T. Buxton, A. Ravard, N. Deo, and P.A. Crooks: Minor alkaloids of tobacco release (3H) dopamine from superfused rat striatal slices; Eur. J. Pharmacol., 276 (1995) 195–199.
7. Hoffmann, D., K.D. Brunnemann, B. Prokipczyk, and M.V. Djordjevic: Tobacco-specific *N*-nitrosamines and areca-derived *N*-nitrosamines: chemistry, biochemistry, carcinogenicity, and relevance to humans; J. Toxicol. Env. Health 41 (1994) 1–52.
8. Cousino Klein, L. and T. Eissenberg: Recent advances in nicotine and tobacco research; Ninth SRNT Annual Meeting, New Orleans, Louisiana, USA, 19–22 February 2003; Nicotine & Tobacco Res. 5 (2003) 761–794.
9. Henningfield, J.E. and M.V. Djordjevic: Menthol cigarettes: research needs and challenges; Nicotine & Tobacco Res. 6 (2004) Suppl. 1, S11–S16.
10. Yang, S.S. and I. Smetena: Evaluation of capillary electrophoresis for the analysis of nicotine and selected minor alkaloids from tobacco; Chromatographia 40 (1995) 375–378.
11. Brunnemann, K.: Determination of nicotine and minor tobacco alkaloids in indoor air by absorption and gas chromatography; IARC Sci. Publ. 12 (1993) 275–280.
12. Yang, S.S. and I. Smetena: Determination of tobacco alkaloids using solid phase microextraction and GC-NPD; Chromatographia, 47 (1998) 443–448.
13. Nyiredy, S., G. Gross, and O. Sticher: Minor alkaloids from *Nicotiana tabacum*; J. Nat. Prod. 49 (1986) 1156–1157.
14. Jones, N.M., M. Bernardo-Gil, and M.G. Lourenco: Agricultural materials comparison of methods for extraction of tobacco alkaloids; J. AOAC Int. 84 (2001) 309–316.
15. Cai, J., B. Liu, P. Lin, and Q. Su: Fast analysis of nicotine and related alkaloids in tobacco and cigarette

smoke by megabore capillary gas chromatography; J. Chromatogr. A 1017 (2003) 187–193.
16. Troje, Z.S., Z. Frobe, and D. Perovic: Analysis of selected alkaloids and sugars in tobacco extract; J. Chromatogr. A 775 (1997) 101–107.
17. Peyton, J. III, L. Yu, G. Liang, A.T. Shulgin, and N.L. Benowitz: Gas chromatographic-mass spectrometric method for determination of anabasine, anatabine and other tobacco alkaloids in urine of smokers and smokeless tobacco users; J. Chromatogr. 619 (1993) 49–61.
18. Wu, W., D.L. Ashley, and C.H. Watson: Determination of nicotine and other minor alkaloids in international cigarettes by solid-phase microextraction and gas chromatography/mass spectrometry; Anal. Chem. 74 (2002) 4878–4884.
19. Zhang, Y., J. Peyton III, and N.L. Benowitz: Determination of nornicotine in smoker's urine by gas chromatography following reductive alkylation to *N*'-propylnornicotine; J. Chromatogr. 525 (1990) 349–357.
20. Guidelines for collaborative study procedures to validate characteristics of a method of analysis; J. Assoc. Off. Anal. Chem. 72 (1989) 694–704.
21. Scheffé, H.: The analysis of variance; J. Wiley, New York, 1959.
22. 1998 Annual book of ASTM Standards Designation: E691 – 92 (1998); ASTM, West, Conshohocken.

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