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Gas-liquid chromatographic determination of major tobacco alkaloids

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Abstract

Polar and apolar columns were tested for the chromatographic analysis of tobacco alkaloids. Disadvantages of polar-based polyethylene glycol phases are reported, such as the adsorption of the nornicotine alkaloid and the occurrence of errors on quantitation of alkaloids due to the presence of interfering compounds in tobacco extracts. A chromatographic method using a new apolar base-modified phase was evaluated for the analysis of alkaloids in tobacco products. The nicotine and other major alkaloids were quantitated using two internal standards simultaneously. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Tobaccos; Alkaloids

1. Introduction

The most used technique for quantitation of tobacco alkaloids has been gas–liquid chromatography, the first studies having been developed with packed columns in 1962, when it was proposed a method [1] for the determination of alkaloids in tobacco. In 1965, a study comparing packed and capillary column results for the analysis of various alkaloids was presented [2]. Gas chromatographic methods for the analysis of this kind of compound were reviewed in 1984 [3]. In the last decades, many studies have been performed for the determination of alkaloids in tobacco and tobacco smoke, and various new alkaloids were isolated. However, new analytical chromatographic approaches have not been developed for quantitation of the major tobacco alkaloids.

In gas chromatographic studies of tobacco alkaloids, different stationary phases have been applied, both polar [4–7]and non-polar [8–10], and the polar base-modified polyethylene glycol phases have been the most used. Burns and Collin [11] presented a method for the quantitation of alkaloids in tobacco using a packed polar Carbowax 20 M–potassium hydroxide column. Later, Severson et al. [12] reported problems of adsorption and tailing of the more basic alkaloids in this type of phases, and proposed a method on a new liquid phase of the Superox-4 capillary column. With this new liquid phase the column became more efficient, thermally stable and low in surface activity [13]. However, studies performed in our laboratory [14–16] have

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shown that adsorption of the more basic alkaloids remains on the polar columns designed for amine analysis. It was also verified that chromatographic mobility of some interfering compounds relatively to the alkaloids may originate errors on the identification and quantitation of tobacco alkaloids.

Recent technology [17] has provided new apolar-based polysiloxane phases which were recommended for the analysis of basic analytes, and it was considered of interest to test the performance of these phases for the quantitation of tobacco alkaloids. The aim of the present study was to evaluate a method for the determination of alkaloids in tobacco products using a PTA-5 column and to report the advantages of this apolar base-modified phase in comparison with the polar phases.

2. Experimental

2.1. Materials

Flue-cured (low level alkaloids) tobaccos from France, and Burley (medium and high level alkaloids) tobaccos from Argentine and Guatemala were provided directly from Tabaqueira, Portugal. The cured tobaccos were dried overnight (15–18 h) in an oven at 50°C, and ground in a Wiley mill (0.4 mm).

All reagents were of analytical grade. Nicotine, nornicotine and anabasine were obtained from Sigma (St. Louis, MO, USA); myosmine from Carl Roth (Karlsruhe, Germany); nicotyrine, 2,3′-bipyridyl, quinoline and quinaldine from Fluka Chemie (Bucks, Switzerland); cotinine and 2,4′-bipyridyl from Aldrich (Milwaukee, USA).

2.1.1. Apparatus

Laboratory mill, Wiley no. 4 (Fort Wayne, IN, USA); ultrasonic cleaning bath, Transsonic T 660/H, Elma (Germany) with a thermostatic heater. Clifton NE 4-T, Nickel-Electro (North Somerset, UK). Rotavapor, Büchi (Flavil, Switzerland). Gas Chromatograph Hewlett-Packard, HP 5890 SII (Wilmington, DE, USA) equipped with a flame ionisation detector (FID), a nitrogen–phosphorus detector (NPD), a split/splitless injector, an automatic sampler HP 7673 and an integrator HP 3396 SII. The gas chromatography–mass spectrometry (GC–MS) system consisted of an HP 5890 SII gas chromatograph coupled to an HP 5970 mass detector equipped with an electron impact source and direct interface.

2.1.2. Capillary columns

Polar Columns: Superox II, cross-linked, base-modified polyethylene glycol (30 m×0.32 mm×0.25 μm) Alltech (Deerfield, IL, USA); Supelcowax-10, cross-linked, base-modified polyethylene glycol (30 m×0.25 mm×0.25 μm) Supelco (Bellefont, PA, USA); CP Wax-51CB, base-deactivation for analysis of amine (30 m×0.32 mm×0.25 μm) Chrompack (Middelburg, The Netherlands). Apolar columns: DB-1, bonded and cross-linked, dimethyl polysiloxane (30 m×0.25 mm×0.25 μm) J&W Scientific (Folsom, CA, USA); PTA-5, bonded, base-modified 5% diphenyl–95% dimethylpolysiloxane (30 m×0.25 mm×0.5 μm), Supelco (Bellefont, PA, USA).

2.2. Alkaloid extraction procedure

Based on a previous study [18] an ultrasonic extraction method was implemented. The method consists of adding 1 g of ground-cured tobacco, 1 ml of internal standard (I.S.) quinaldine (10 mg/ml in methanol), 1 ml of I.S. quinoline or I.S. 2,4′-bipyridyl (0.2 mg/ml in methanol) and 40 ml of methanol alkalised with 0.05 mol/l of potassium hydroxide, into a round flask with a vertical condenser. The extraction was performed in an ultrasonic bath with thermostatic heater at 82°C during 40 min. For the quantitation of the alkaloids (except nicotine) the extracted solutions were concentrated at 40°C to an approximately volume of 4 ml in a Rotavapor.

2.3. Gas chromatography analysis

Different chromatographic conditions were used with the various polar and apolar columns mentioned. The injector and detector temperatures were tested from 225 to 300°C and from 230 to 370°C, respectively. The selected conditions for the PTA-5 column were the following: injector temperature, 250°C; FID and NPD detector temperatures, 300°C; linear velocity of helium, 30 cm s^{-1}; split 1:20;

injection volumes of 1–2.5 μl. Oven programs: for the nicotine analysis, an initial temperature of 160°C during 9 min, 160–280°C at 30°C min^{-1} and finally 280°C during 5 min; for the other alkaloids, an initial temperature of 150°C during 20 min, 150–280°C at 25°C min^{-1} and finally 280°C during 5 min (I.S. 2,4′-bipyridyl) or an initial temperature of 160°C during 16 min, 160–280°C at 25°C min^{-1} and finally 280°C during 5 min (I.S. quinoline).

The quantitation was made with calibration curves using two internal standards simultaneously. Six calibration solutions of standard alkaloids dissolved in methanol were prepared from 0.20 to 2.00 μg/μl of nicotine and from 0.02 to 0.40 μg/μl of the other alkaloids. For the nicotine analysis the concentration of the I.S. quinaldine was 0.5 μg/μl and for the other alkaloids the concentration of the I.S. 2,4′-bipyridyl or of the I.S. quinoline was 0.04 μg/μl. Since anatabine was not available, it was assumed that it has a response factor similar to the response factor of anabasine.

3. Results and discussion

To select the most convenient detector for the analysis of tobacco alkaloids, experiments were carried out with FID and NPD detectors. It was found that the NPD response is higher than the FID response, the nitrogen-selective detector giving rise to more clean chromatograms. However, taking into account the dependence of the NPD detector on the experimental conditions and the instability of the NP bead with ageing [15], for routine analysis the FID detector is considered more suitable.

3.1. Alkaloid analysis on polar columns

Tobacco extracts with low and high levels of alkaloids were analysed on polar and apolar phases widely used for alkaloids analysis, and on a new apolar base-modified phase (PTA-5 column). The data summarised in Table 1 show that the results were obtained with different degrees of precision. On polar columns (Superox II, Supelcowax-10 and CP Wax-51CB) less precise results were obtained, namely for nornicotine. Severson et al. [12] emphasised that acidic sites formed on polar phases origin column activity with considerable adsorption of nornicotine. On base-modified polyethylene glycol phases, the adsorption of alkaloids can effectively be reduced by injections of ethylenediamine [12,14,15]. Nevertheless, the adsorption of nornicotine persists leading to less precise quantitative determinations of alkaloids (Table 1) and tailings in the chromatographic peaks can be observed (Fig. 1a,b). On CP Wax-51CB column adsorption of different alkaloids was observed and an effective resolution was not possible.

Table 1
Repeatability of chromatographic tobacco alkaloid analysis ($n=5$) using different columns[a]

Columns	Flue-cured tobaccos		Burley tobaccos	
	Average (mg/g)	RSD	Average (mg/g)	RSD
Nicotine				
Superox II	11.24	0.02	34.97	0.02
Supelcowax 10	13.75	0.01	36.59	0.03
CPWax-51 CB	9.09	0.06	45.14	0.01
DB-1	12.21	0.00	33.75	0.00
PTA-5	7.40	0.00	40.43	0.00
Nornicotine				
Superox II	0.10	0.20	0.44	0.61
Supelcowax 10	0.18	0.33	0.73	0.41
CPWax-51 CB	–	–	1.86	0.19
DB-1	0.10	0.28	0.70	0.06
PTA-5	0.24	0.06	4.34	0.03
Anabasine				
Superox II	1.26	0.01	0.62	0.06
Supelcowax 10	1.61	0.05	0.59	0.08
CPWax-51 CB	–	–	0.25	0.29
DB-1	–	–	0.04	0.20
PTA-5	0.08	0.06	0.24	0.07
Anatabine				
Superox II	0.36	0.03	1.24	0.03
Supelcowax 10	0.41	0.06	1.83	0.04
CPWax-51 CB	–	–	0.65	0.20
DB-1	0.17	0.04	0.84	0.04
PTA-5	0.63	0.03	1.91	0.03

[a] Nicotine quantitation with the I.S. quinaldine and for the other alkaloids with the I.S. 2,4′-bipyridyl. RSD, relative standard deviation.

3.1.1. Interfering compounds

Although the polar columns may provide reliable quantitative analysis of tobacco alkaloids, they do not resolve the overlapping of the myosmine and

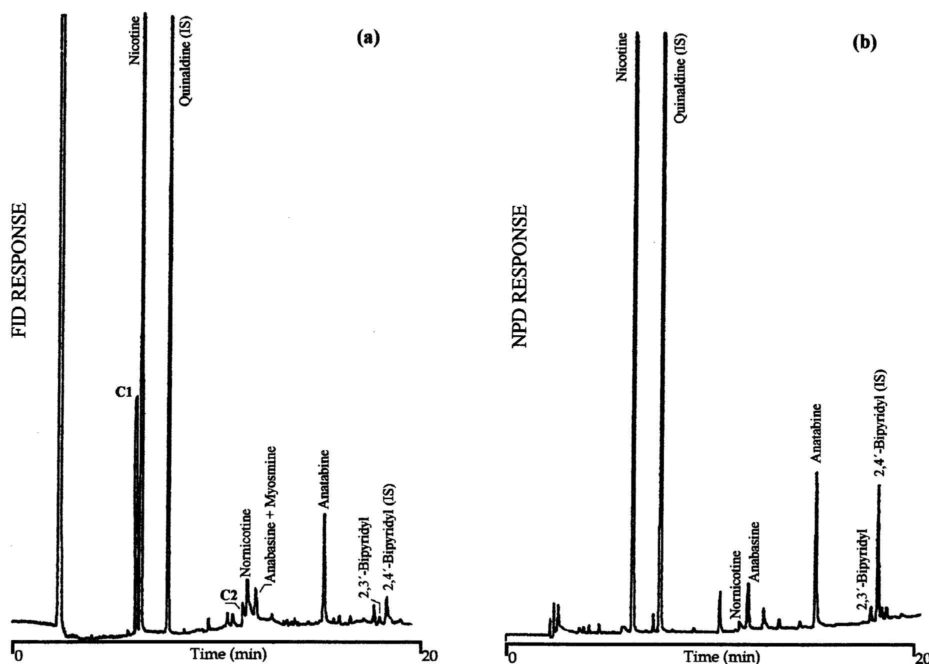

Fig. 1. Chromatograms of extracts of tobacco alkaloids on a Superox II column using FID and NPD detectors. (a) Burley extract (high level alkaloids); (b) flue-cured extract (low level alkaloids). Oven temperature program: 160–240°C at 4°C min^{-1}; linear velocity of helium, 20 cm s^{-1}.

anabasine peaks, and the chromatographic conditions must be optimised to obtain an effective separation of nornicotine, anabasine and of an interfering compound C2. Moreover, another compound C1 can also interfere with nicotine as shown in Fig. 1a. The two interfering compounds are eliminated by using the NPD detector (Fig. 1b). The interfering compounds (C1 and C2) in the tobacco extracts were identified by GC–MS. As foreseen the compound C1 is the neophytadiene [19,20]. With regards to compound C2, it has been previously referenced in other studies [3,11,12], but without conclusive results. A mass spectrometry library search suggested the hexadecanoic methyl ester. This assumption was confirmed by standard addition chromatographic experiments and by GC–MS. It should be mentioned that previous studies [14,16] verified that the concentration of the ester varies significantly as a function of the tobacco type, the flue-cured tobaccos having the highest quantities of this compound.

In the present study, chromatographic mobility of the interfering compounds, C1 and C2 versus alkaloids was observed (Table 2 and Fig. 2), which may originate errors in the identification and the quantitation of the tobacco alkaloids. The life time of the columns also influences the relative position of the peaks.

In conclusion, it seems that the use of polar columns with the FID detector requires GC–MS confirmation to avoid wrong determinations of tobacco alkaloids.

3.2. Alkaloid analysis on apolar columns

3.2.1. Chromatographic method

Considering the problems arising on using polar columns and taking into account the more precise results obtained with a new apolar base-modified diphenyl dimethylpolysiloxane phase (Table 1), a chromatographic method using a PTA-5 column was implemented. Chromatograms of a standard alkaloid solution and of a tobacco alkaloid extract are presented in Fig. 3a,b.

In order to select the internal standards, blank

Table 2
Relative retention times to nicotine of alkaloids and interfering compounds as a function of oven programs

Compounds	Columns								
	Supelcowax 10[a]			Superox II[b]			CPWax-51 CB[b]		
Oven programs:	160°C	Prog.[c]	200°C	160°C	Prog.[c]	200°C	160°C	Prog.[d]	200°C
Nicotine	1	1	1	1	1	1	1	1	1
C1 (neophytadiene)	1.06	1.00	0.95	1.04	1.00	0.95	1.18	1.23	1.00
Nornicotine	2.41	1.73	1.72	2.31	1.70	1.61	2.15	1.77	1.52
Anabasine	2.60	1.81	1.82	2.49	1.78	1.69	2.34	1.83	1.61
C2 (hexadecanoic methyl ester)	2.80	1.76	1.67	2.64	1.74	1.56	2.91	1.96	1.65

[a] Linear velocity of helium, 30 cm s^{-1}.
[b] Linear velocity of helium, 20 cm s^{-1}.
[c] Program: 160–240°C (4°C min^{-1}).
[d] Program: 150°C (10 min), 150–240°C (7°C min^{-1}).

experiments were carried out and it was verified that the I.S. quinaldine, from some suppliers, had impurities that could interfere in the analysis of tobacco alkaloids. The quinaldine from Fluka had also some detectable impurities, but these did not interfere with the different alkaloids (Fig. 2).

The internal standard commonly used for the quantitation of alkaloids (except nicotine) is 2,4′-bipyridyl with a retention time close to that of 2,3′-bipyridyl. Both compounds are eluted in the final part of the chromatogram having a less regular base line. Experiments were performed using another

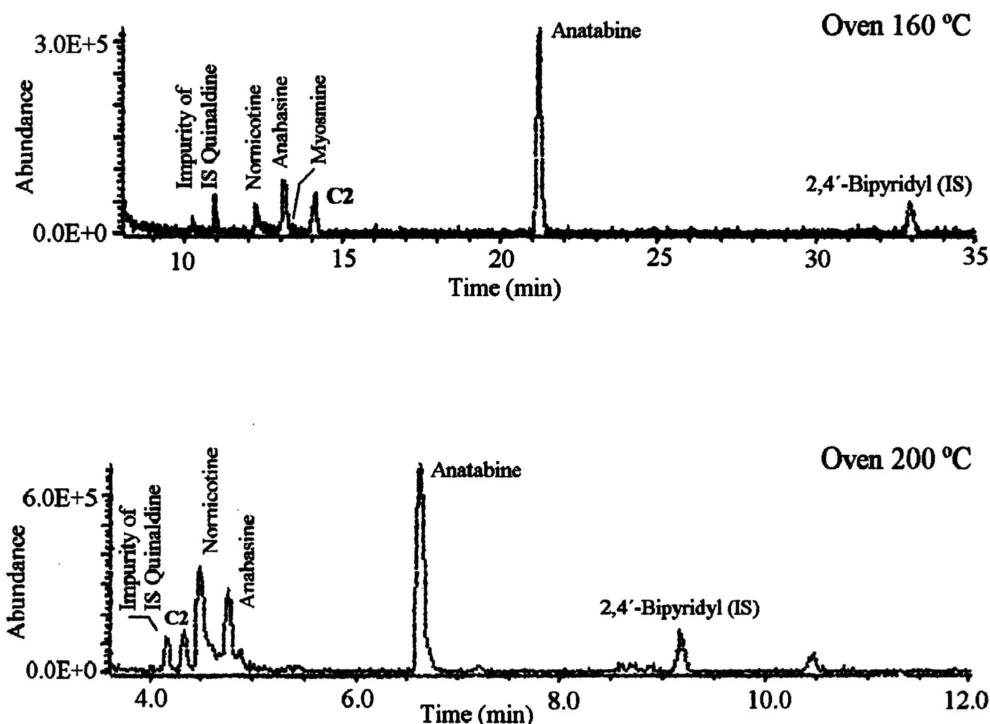

Fig. 2. Total ion currents of a Burley alkaloid extract on a Supelcowax 10 column using different oven temperature programs.

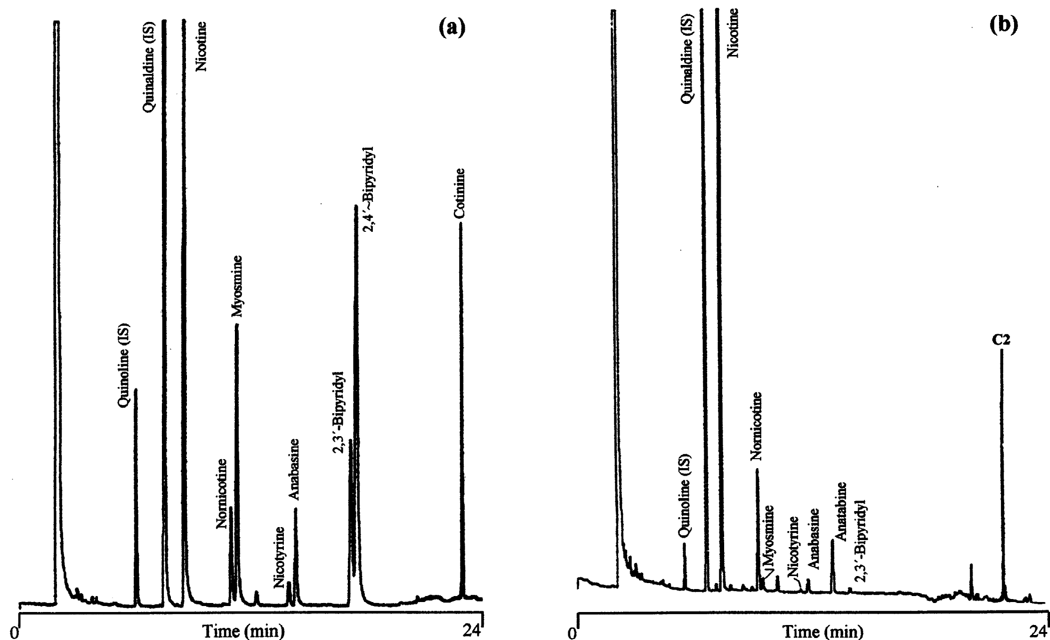

Fig. 3. Chromatograms of tobacco alkaloids on a PTA-5 column using the FID detector. (a) Standard alkaloids; (b) Burley alkaloid extract. Oven temperature programs: (a) 150°C (20 min), 150–180°C at 25°C min^{-1}, 280°C (5 min); (b) 160°C (16 min), 160–280°C at 25°C min^{-1}, 280°C (5 min). Linear velocity of helium, 20 cm s^{-1}.

internal standard, quinoline, which has a smaller retention time than the nicotine and with no interferences. To compare the repeatability of both procedures, two extracts of tobacco alkaloids, each of them with one internal standard were injected five times, and the spread of results was compared using an F-test. It was concluded that both internal standards gave equally precise results.

Calibration curves were established with quinaldine as internal standard for the nicotine quantitation, and 2,4′-bipyridyl or quinoline for the other alkaloids. The variation of the peak areas ratios of the alkaloids and the internal standards as a function of the respective concentration ratios was evaluated within a range previously chosen (Table 3). Before establishing the calibration curves, a test of homogeneity of variances was applied. Calibration solutions with the upper and the lower level concentrations were injected 10 times and the squares of the respective variances (S_1^2 and S_6^2) were calculated. An F-test indicated that the ratios (S_1^2/S_6^2 or S_6^2/S_1^2) for the different alkaloids were lower than the $F_{9,9}$ critical value which verifies the homogeneity of the variances between the limits of established concentration levels (0.20 and 2.0 μg/μl for nicotine and 0.02 and 0.40 μg/μl for other alkaloids).

3.2.2. Evaluation of the method

For each alkaloid three calibration curves were established over the interval of a month and total data are presented in Table 3. The limit of detection (LOD) and the limit of quantitation (LOQ) for each alkaloid were calculated through the standard deviation of the response and the slope of the calibration curves. Regression analysis of data for the different alkaloids showed linearity within the ranges studied. The calibration curves are stable at least over a month according to the relative standard deviation of the parameters of the three curves (Table 4).

The selectivity of the method was verified by standard and tobacco alkaloid chromatograms and it was confirmed by a hyphenated GC–MS technique. As observed in Fig. 3b, the hexadecanoic methyl ester, one of the interfering compound on polar columns, has a retention time longer than the alkaloids and consequently does not interfere in their

Table 3
Calibration curves[a], detection and quantitation limits on a PTA-5 column using different internal standards

I.S.[b]	Slope±SD	Intercept±SD	Standard error	R^2	LOD (μg/μl)	LOQ (μg/μl)
Nicotine						
(a)	1.485±0.002	0.040±0.003	0.012	0.9999	0.025	0.083
Nornicotine						
(b)	13.972±0.124	−0.120±0.028	0.127	0.9959	0.027	0.091
(c)	11.291±0.093	−0.011±0.021	0.094	0.9965	0.025	0.084
Anabasine						
(b)	12.154±0.089	−0.043±0.020	0.073	0.9982	0.018	0.060
(c)	10.057±0.081	0.046±0.018	0.082	0.9967	0.024	0.081
2,3′-Bipyridyl						
(b)	33.509±0.267	−0.149±0.059	0.264	0.9970	0.024	0.080
(c)	27.257±0.221	0.104±0.050	0.224	0.9966	0.025	0.082
Myosmine						
(b)	28.244±0.236	−0.132±0.052	0.234	0.9964	0.025	0.083
(c)	22.709±0.175	0.105±0.039	0.177	0.9970	0.023	0.078
Nicotyrine						
(b)	1.541±0.016	0.013±0.004	0.015	0.9951	0.028	0.095

[a] Calibration curves established with data of three calibration curves determined over the interval of a month. Each of the three calibration curves represents six data points with three replicates per data point.

[b] Internal standards: (a) quinaldine; (b) quinoline; and (c) 2,4′-bipyridyl.

Table 4
Relative standard deviations of calibration curve parameters[a]

I.S.[b]	Slope	Intercept	LOD (μg/μl)	LOQ (μg/μl)
Nicotine				
(a)	0.00	0.03	0.19	0.19
Nornicotine				
(b)	0.01	0.12	0.15	0.15
(c)	0.01	0.78	0.06	0.06
Anabasine				
(c)	0.01	0.40	0.06	0.07
2,3′-Bipyridyl				
(b)	0.01	0.31	0.18	0.16
(c)	0.02	0.57	0.15	0.15
Myosmine				
(b)	0.02	0.46	0.09	0.07
(c)	0.01	0.28	0.04	0.06
Nicotyrine				
(b)	0.03	0.41	0.23	0.25

[a] Relative standard deviations of parameters of three calibration curves determined over the interval of a month.

[b] Internal standards: (a) quinaldine; (b) quinoline; and (c) 2,4′-bipyridyl.

quantitation. The presence of this compound was confirmed by standard addition chromatographic experiments and by GC–MS.

The accuracy of the method was evaluated through the precision and trueness (Tables 5 and 6).

The repeatability of the chromatographic method was evaluated by injecting the same tobacco alkaloid extract five times, and the repeatability of the whole procedure was determined by extracting the tobacco alkaloids five times over a short time interval under the same conditions. The precision of the chromatographic and global method was evaluated for tobaccos with low and high levels of alkaloids through the relative standard deviations of the results (Table 5). The relative standard deviations for both methods are similar, showing that the extraction procedure does not increased the variability of the results. Lower precision was found for the tobacco with low level of alkaloids (flue-cured tobacco).

The trueness of the method was assessed by extracting the alkaloids three times from tobacco samples spiked with two concentration levels of alkaloids (Table 6).

In summary, we conclude that on an apolar base-

Table 5
Relative standard deviations of chromatographic and global method results on a PTA 5 column[a]

Tobacco alkaloids	Tobaccos	Average (mg/g)	RSD[b]	RSD[c]
Nicotine	Burley	20.37	0.00	0.02
	Flue-cured	7.42	0.00	0.01
Nornicotine	Burley	1.60	0.03	0.02
	Flue-cured	0.30	0.08	0.11
Anabasine	Burley	0.17	0.05	0.05
	Flue-cured	0.08	0.06	0.07
Anatabine	Burley	0.69	0.03	0.04
	Flue-cured	0.50	0.03	0.01

[a] The results are average of five replications. Nicotine quantitation with the I.S. quinaldine and for the other alkaloids with the I.S. quinoline.
[b] RSD of chromatographic analysis.
[c] RSD of global method.

modified diphenyl dimethylpolysiloxane chromatographic column an effective separation of alkaloids without interferences is possible, and for the nornicotine alkaloid more precise results were obtained. Furthermore, the quantitation of alkaloids in an extract are more reliable when two internal standards are simultaneously used, that is, the I.S. quinaldine for determination of nicotine and I.S. quinoline for the other alkaloids. The accuracy of the improved method is acceptable for quality control of alkaloids in tobacco products.

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Table 6
Recovery of alkaloids on a PTA-5 column[a]

Tobacco alkaloids	Concentration			Recovery	
	Initial±SD (mg/g)[b]	Added (mg/g)	Found (mg/g)	(%)	RSD
Nicotine	20.37±0.50	4.71	24.56	97.9	0.02
		9.41	30.39	102.0	0.01
Nornicotine	1.60±0.04	0.22	1.83	100.5	–
		0.50	2.11	100.5	0.04
Anabasine	0.17±0.01	0.13	0.29	96.7	0.06
		0.65	0.84	102.4	0.08
2,3′-Bipyridyl	0.06±0.01	0.19	0.19	76.0	0.03
		0.86	0.83	90.2	0.07
Myosmine	0.09±0.01	0.17	0.25	96.2	0.04
		0.77	0.85	98.8	0.05

[a] The results are average of three replications. Nicotine quantitation with the I.S. quinaldine and for the other alkaloids with the I.S. quinoline.
[b] Average of five replications.

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