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Enantiomeric analysis of anatabine, nornicotine and anabasine in commercial tobacco by multi-dimensional gas chromatography and mass spectrometry

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Abstract

A fully automated multi-dimensional gas chromatography (MDGC) system with a megabore precolumn and cyclodextrin-based analytical column was developed to analyze the enantiomeric compositions of anatabine, nornicotine and anabasine in commercial tobacco. The enantiomer abundances of anatabine and nornicotine varied among different tobacco. *S*-(−)-anatabine, as a proportion of total anatabine, was 86.6% for flue-cured, 86.0% for burley and 77.5% for oriental tobacco. *S*-(−)-nornicotine, as a proportion of total nornicotine, was 90.8% in oriental tobacco and higher than in burley (69.4%) and flue-cured (58.7%) tobacco. *S*-(−)-anabasine, as a proportion of total anabasine, was relatively constant for flue-cured (60.1%), burley (65.1%) and oriental (61.7%) tobacco. A simple solvent extraction with dichloromethane followed by derivatisation with trifluoroacetic anhydride gave relative standard deviations of less than 1.5% for the determination of the *S*-(−)-isomers of all three alkaloids. The study also indicated that, a higher proportion of *S*-(−)-nornicotine is related to the more active nicotine demethylation in the leaf.
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Keywords: Anabasine; Anatabine; Enantiomeric composition; GC/MS; MDGC; Nornicotine; Tobacco

1. Introduction

Nicotine, anatabine, nornicotine, and anabasine are the most common alkaloids of the *Nicotiana* species [1]. In commercial tobacco, nicotine accounts for approximately 95% of the total alkaloid fraction. Anatabine and nornicotine are the next two most abundant alkaloids, each accounting for about 2–6% of the total alkaloid content. Nornicotine content is usually higher in burley tobacco while anabasine is less prevalent and makes up 0.1–0.5% of the total alkaloid pool [2]. All four alkaloids are chiral. Naturally occurring nicotine is predominately the *S*-(−)-isomer while the minor alkaloids (anatabine, nornicotine, and anabasine) are enantiomeric mixtures [3].

Determining the enantiomeric distributions of these minor compounds is useful in understanding the *in planta* metabolic fate of the alkaloids, particularly during their transformation to compounds of interest such as the tobacco specific nitrosoamines (TSNAs) [4]. Because of the much higher concentration of nicotine and the presence of other matrix-related compounds, the enantiomeric distribution of the minor *Nicotiana* alkaloids has not been well studied. Two prominent papers that described alkaloids in the plant were inconsistent in their findings, with Perfetti and Coleman [5] reporting that the enantiomeric distribution for nornicotine varied between different tobacco while Armstrong et al. [3] found that the enantiomeric ratios for the three minor alkaloids were similar in different tobacco.

GC columns with chiral stationary phases based on modified cyclodextrin have been effective for the enantiomeric separation of the *Nicotiana* alkaloids in either derivatised [3,6] or underivatised form [5]. In this study, a multidimensional GC approach with a megabore precolumn and chiral analytical column provided a facile method for the enrichment of the low-level alkaloids anatabine, nornicotine and anabasine and the determination of their enantiomeric compositions in different commercial tobacco.

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2. Experimental

2.1. Reagents and materials

Analytical regents were used unless otherwise indicated. Chemical standards of (±)-nornicotine and (±)-anabasine were purchased from Sigma (St Louis, Mo, USA). Anatabine was isolated from tobacco by preparative GC in this laboratory [7]. Trifluoroacetic anhydride (TFAA) was purchased from Shanghai Chemical Co. (Shanghai, China). Samples of 18 flue-cured, 12 burley and 11 oriental tobacco were obtained from Shanghai Tobacco (Group) Corp. (Shanghai, China).

2.2. Tobacco extraction

Tobacco was ground into 40–60 mesh powder using a mortar and pestle and a 0.025 g sample was placed into 20 ml test tube. After adding 5 ml of dichloromethane and 1 ml of 10% sodium hydroxide, the mixture was shaken by hand for 1 min to mix thoroughly. The tube was centrifuged at 1500 rpm for 5 min and then 1.0 ml of the dichloromethane layer was transferred into a 2 ml autosampler vial.

In an adaptation of a published method [3], 1.0 ml of TFAA was added to the tobacco extract in the autosampler vial. The vial was capped, mixed thoroughly and acylation was allowed to continue at room temperature for 30 min. The sample was analysed by MDGC/MS, using the method described below, without further isolation.

Repeatability of extraction and analysis was tested for each tobacco type using five separate samples.

2.3. MDGC/MS

Analysis was performed on an Agilent 6890 GC–5973 MSD (Agilent Technologies, Palo Alto, CA, USA) that had been fitted with a MDS 6890 kit (SGE Analytical Science, Ringwood, Australia) [8]. To allow the connection of the megabore column and maintenance of the resultant gas flows, the GC was fitted with accessory MDS components as described in Fig. 1. While available as separate items, in this case the accessories were sourced from a MDS 2000 (SGE Analytical Science) [9].

The needle valve used for mid-split adjustment of the MDS 2000 was replaced by a fused silica restrictor (3b in Fig. 1). Because restrictors 3a and 3b have the same length and internal diameter and both of them are in same oven, a stable gas flow through the pre-column is maintained during by-pass or heart-cutting operations.

The solenoid valve used for mid-split control of the MDS 2000 was replaced by the optional gas actuated on/off valve (18 in Fig. 1). In this arrangement, the actuating gas is shared with the heart-cutting valve (13 in Fig. 2). During heart-cutting, the on/off valve is open and excessive carrier gas from the pre-column passes through restrictor 3b to vent via the on/off valve. Chromatographic components from the pre-column are condensed in a section of fused silica tubing by the cold trap (6 in Fig. 2). When heart-cutting is finished, the on/off valve is also closed. When the coolant flow is stopped, the trap reheats and the focused components are released onto to the analytical column (2 in Fig. 1) for analysis.

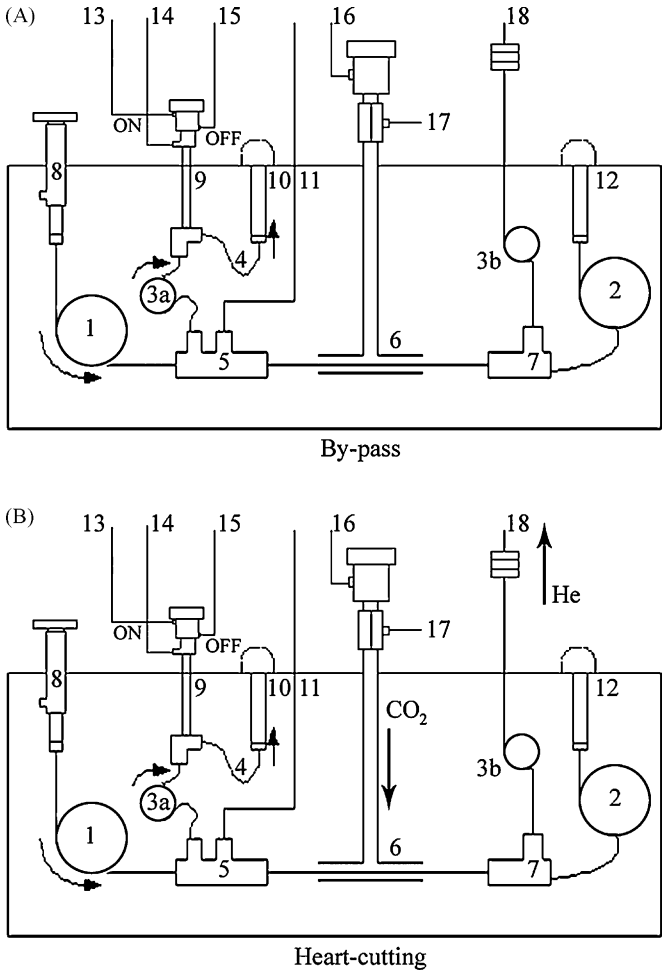

Fig. 1. MDGC system from megabore to capillary column with online enrichment: (A) by-pass and (B) heart-cutting. In this figure: (1) pre-column, (2) analytical column, (3a) and (3b) fused silica restrictors, (4) transfer-line, (5) mid-point restrictor, (6) cold trap (DB-5, 120 mm × 0.53 mm id × 1.0 μm film thickness), (7) mid-point splitter, (8) injection port, (9) heart-cutting valve, (10) monitoring detector, (11) mid-point pressure module, (12) analytical detector, (13) and (15) actuating gas of heart-cutting valve, (14) make up gas of heart-cutting valve, (16) actuating gas of coolant valve, (17) coolant inlet and (18) on/off valve.

The carrier gas was high purity helium (99.999%). The pre-column was a DB-5 (15 m × 0.53 mm i.d., 0.25 μm film thickness, Agilent, USA) operated with an inlet pressure of 114 kPa. The chiral column was a R_t-β-DEX_{sm} (30 m × 0.25 mm i.d., 0.25 μm film thickness, Restek, USA) with an inlet pressure of 103 kPa. The GC oven temperature was programmed for the 1st dimensional separation from an initial temperature of 80 °C at 3 °C/min to a final temperature of 180 °C which was held for 5 min. The oven temperature for the chiral separation was programmed from an initial temperature of 80 °C at 1 °C/min to a final temperature of 180 °C which was maintained for a further 5 min. The heart-cutting retention time range was 16.0–19.0 min. Liquid CO_2 was used as coolant with the valve opened at 14 min and closed at 21 min. The temperature of the split/splitless injection port was 250 °C. The injection

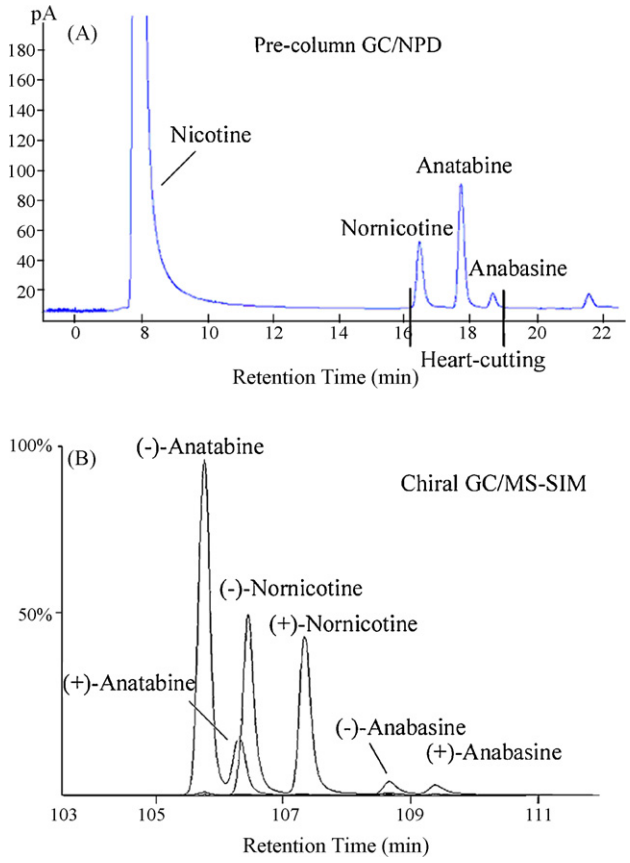

Fig. 2. Chromatograms of TFA-derivative of the minor alkaloids in flue-cured tobacco: (A) pre-column GC/NPD and (B) chiral GC/MS-SIM

volume was 2 μl. Injection was splitless with the inlet purge on at 0.2 min and a purge flow of 40 ml/min. A nitrogen and phosphorous detector (NPD) was used for monitoring the 1st dimensional separation. The NPD temperature was 300 °C and gas flows were 2 ml/min hydrogen, 60 ml/min air and 10 ml/min helium make-up gas.

During quantitative analysis, selected ion monitoring (SIM) mode was used. Targeted ions for anatabine, nornicotine and anabasine were *m/z* 159, 147 and 161, respectively. Dwell time for each ion was 80 ms. The temperatures of transfer line, ion source and mass analyzer were 250, 230 and 150 °C, respectively.

3. Results and discussion

The heart-cutting multidimensional GC is especially desirable for chiral analysis [10–12]. Because only the targeted portions on pre-column are transferred to chiral column, matrix interference to chiral compounds and contamination of heavy constituents to chiral column are greatly reduced. Therefore, accuracy of enantiomeric composition could be enhanced. At the same time, lifetime of the labile chiral column could be prolonged. The pre-column could also be viewed as a clean-up tool, procedure of sample pretreatment could be simplified.

In this study, sample pretreatment was very simple compared to an off-line LC–GC method [3]. After addition of derivative agent (TFAA), solvent extract of tobacco was directly injected into MDGC. In this way, large number of tobacco samples can be analyzed with low cost of labor and chemical reagents.

During our preliminary analyses that did not include the derivatisation step, the enantiomers of anatabine and anabasine were baseline resolved on the chiral column but the resolution of nornicotine enantiomers was poor. Derivatisation with TFAA allowed the complete resolution of the three enantiomer pairs (Fig. 2).

The mass spectra of the TFAA derivatised alkaloids in a flue-cured tobacco extract are shown in Fig. 3. Derivatised anatabine, nornicotine and anabasine gave molecular ions of *m/z* 255, 243 and 258, respectively. The corresponding base peaks at *m/z* 159, 147 and 161 are attributable to detrifluoroacylation in each case. Because *m/z* 159, 147 and 161 are specific and abundant ions in MS spectra, these ions were chosen for SIM analysis.

The robustness of the method in the determination of enantiomeric ratios is shown for the three alkaloids in Table 1. The excellent repeatability is attributable to three factors: a simple

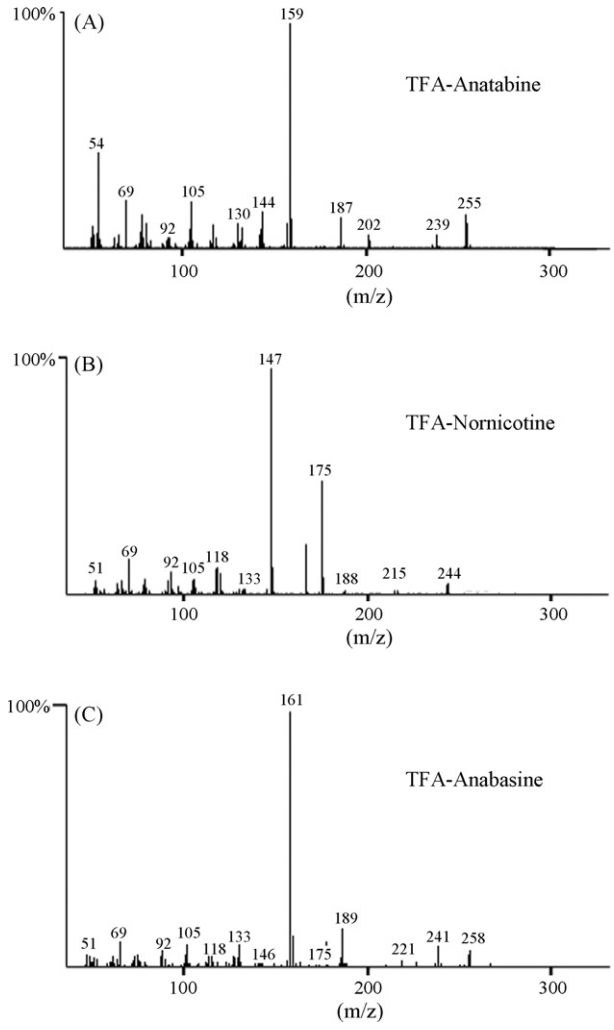

Fig. 3. MS spectra of TFA-derivative of the alkaloids in flue-cured tobacco: (A) TFA-anatabine, (B) TFA-nornicotine and (C) TFA-anabasine.

Table 1
Repeatability of enantiomeric composition of alkaloids in tobacco (R.S.D.%, $n = 5$)

Tobacco	*S*-(−)-Anatabine	*S*-(−)-Nornicotine	*S*-(−)-Anabasine
Flue-cured	0.2	0.1	1.1
Burley	0.5	0.1	1.4
Oriental	0.2	0.6	0.6

procedure for sample pretreatment which avoids any opportunity for sample degradation or loss of analyte, the separation power of MDGC which allows analysis in the presence of major matrix co-extractants (in this case nicotine) and the enhanced selectivity of MS-SIM detection.

Unlike the previously cited studies [3,5] where only one sample for each tobacco type was analyzed, in this study 41 tobacco samples were analyzed. The samples included 18 kinds of flue-cured, 12 kinds of burley and 11 kinds of oriental tobacco. Enantiomeric compositions for the three alkaloids in the tobacco samples are given in Table 2.

The oriental, burley and flue-cured tobacco showed differences in the average proportion of *S*-(−)-nornicotine in the nornicotine pool. In contrast, the proportions of *S*-(−)-anabasine in the anabasine pool and, to a lesser extent, *S*-(−)-anatabine in the anatabine pool were relatively constant across all tobacco types.

Among different kinds of tobacco, the most striking difference is the variability in the proportion of nornicotine present as the *S*-(−)-enantiomer. The oriental tobacco had the highest proportion of *S*-(−)-nornicotine while the flue-cured tobacco had the lowest proportion.

In three flue-cured tobacco samples (China Yunnan C_2F, China Henan C_3F and China Yunnan B_2F), the proportion of the nornicotine present as the *S*-(−)-enantiomer was 36.77, 42.32 and 49.29%, respectively. That the amount of *S*-(−)-nornicotine was less than the *R*-(+)-isomer is unusual and the reasons for this result are unclear. However, studies on burley tobacco have previously demonstrated that root tissues often contained in excess of 50% nornicotine as the *R*-(+) enantiomer [13].

Previous studies have also found that leaf samples with high proportions of (+)-nornicotine were from plants exhibiting low

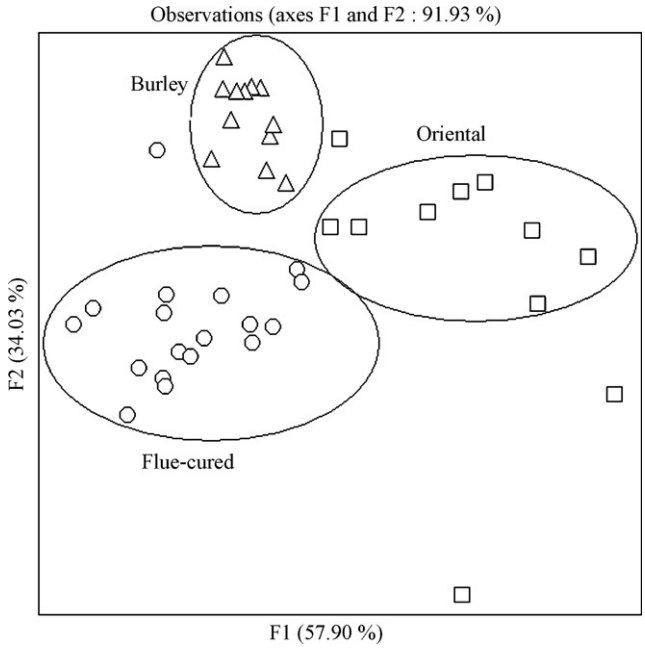

Fig. 4. Scores plot of the 41 tobacco samples plotted for the first and second principal components (PCA of percent *S*-enantiomers of the alkaloids against tobacco type).

activity towards nicotine demethylation in the leaf [13]. In this study, the absolute amounts of alkaloids in tobacco were also determined by a GC method as reported previously [14]. The contents of total alkaloids and their distributions in different tobacco were given in Table 2. The results were consistent with the published data [2]. Burley has the highest total amount of alkaloid and oriental tobacco has the lowest. As for the relative proportion of each alkaloid to total alkaloids, nornicotine is the highest minor alkaloid in burley and oriental tobacco, and anatabine is the highest alkaloid in flue-cured tobacco. Anabasine has the lowest level in all kinds of tobacco. By comparing the enantiomeric composition with the corresponding amount, a positive correlation was revealed between *S*-(−)-nornicotine (%) and nornicotine level in tobacco. Generally, a higher percentage of *S*-(−)-enantiomer in tobacco comes with a higher percent nornicotine to total alkaloids, which means a higher

Table 2
Enantiomeric composition of anatabine, nornicotine and anabasine in tobacco samples

Tobacco samples	Total alkaloids (%)	Percentage composition of alkaloids (%)				*S*-Anatabine (%)	*S*-Nornicotine (%)	*S*-Anabasine (%)
		Nicotine	Anatabine	Nornicotine	Anabasine			
Flue-cured ($n = 18$)[a]	1.8 ± 0.5	95.4 ± 0.8	2.9 ± 0.6	1.2 ± 0.3	0.5 ± 0.1	86.6 ± 1.5	58.7 ± 11.5	60.0 ± 1.5
Burley ($n = 12$)[a]	4.0 ± 0.8	89.3 ± 3.8	4.0 ± 0.9	6.4 ± 3.2	0.4 ± 0.1	86.0 ± 1.5	69.4 ± 5.1	65.4 ± 1.1
Oriental ($n = 11$)[a]	1.1 ± 0.3	93.6 ± 2.0	1.9 ± 0.4	4.2 ± 1.7	0.3 ± 0.1	77.5 ± 5.5	90.8 ± 5.3	61.5 ± 3.3
Flue-cured ($n = 1$)[b]	–	–	–	–	–	82.4	74.4	59.2
Burley ($n = 1$)[b]	–	–	–	–	–	85.9	79.5	58.7
Oriental ($n = 1$)[b]	–	–	–	–	–	83.7	86.1	59.4
Flue-cured ($n = 1$)[c]	–	–	–	–	–	85	57	57
Burley ($n = 1$)[c]	–	–	–	–	–	85	82	57
Oriental ($n = 1$)[c]	–	–	–	–	–	85	79	61

[a] Results from this study, the data were presented as mean value ± S.D.
[b] Data from Armstrong et al. [3].
[c] Data from Perfetti and Coleman [5].

proportion of *S*-(−)-nornicotine is related to the more active nicotine demethylation in the leaf [13].

The possibilities of classifying the tobacco samples by variety based on the enantiomeric data were evaluated. The percentage of *S*-(−)-enantiomers in anatabine, nornicotine and anabasine from 41 different tobacco samples (Table 2) were analyzed using principal component analysis (PCA). The PCA was performed using Excel 2003 (Microsoft Co., USA) and the results are shown in Fig. 4. In the chart of the principal component scores, the combination of the first and second principal components successfully identified 41 tobacco samples into three groups (burley, oriental and flue-cured). The factor loadings generated by PCA showed that, percentage of *S*-(−)-nornicotine contributed most to the first principal component, whereas percentage of *S*-(−)-anabasine contributed most to the second principal component. The factor loadings of *S*-nornicotine in F1 and *S*-anabasine in F2 were 0.934 and 0.996, respectively.

4. Conclusions

A fully automated MDGC system from megabore to capillary column with on-line enrichment was established. Enantiomeric composition of anatabine, nornicotine and anabasine in tobacco was determined by MDGC/MS. Through composite analysis of commercial tobacco, regular patterns of the enantiomeric composition in flue-cured, burley and oriental tobacco were elucidated.

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