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Optimization study for the reversed-phase ion-pair liquid chromatographic determination of nicotine in commercial tobacco products

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Abstract

The availability of published methods for the determination of nicotine in commercial tobacco products based on state-of-the-art chromatographic methods is limited. Nicotine is a diprotic base with pK_a's of 3.12 (pyridine ring) and 8.02 (pyrrolidine ring). Other monoprotic and diprotic bases are also present in commercial tobacco including anatabine, nornicotine, anabasine, and cotinine. In this paper, the chromatography of nicotine and the minor tobacco alkaloids under reversed-phase ion-pairing conditions is thoroughly studied. The results of this study are used to understand the retention mechanisms of the tobacco alkaloids, to examine their observed elution order with respect to fundamental analyte properties (size, functionality, and acid–base strength), and to select optimum chromatographic conditions for the determination of nicotine in commercial tobacco products. Published by Elsevier Science B.V.

Keywords: Tobacco; Nicotine; Alkaloids

1. Introduction

In 1994, our laboratory was searching for a reliable method for the determination of nicotine in commercial tobacco products. As reported in more detail elsewhere [1], the availability of recently published methods based on state-of-the-art chromatographic methods was limited. The most recently published LC methods [2–4] were based on chromatographic conditions (i.e., mobile phase pH's near 7) which are now known to be less than optimal for basic analytes on reversed-phases [5], and were inconvenient due to time-consuming and/or cumbersome sample preparation procedures.

Reversed phase ion-pair liquid chromatography has been established as a viable alternative for the analysis of basic analytes [5]. Over the last seven years, our laboratory has used reversed-phase ion-pair liquid chromatography for the routine screening and determination of alkaloids in cases involving unapproved drugs or suspected tampering/poisoning [6,7]. One of our routine methods uses a citric acid buffer with sodium octane sulfonate as the ion-pairing agent [7]. The method is applicable to a broad range of alkaloids varying in polarity and size including nicotine, cocaine, strychnine, and yohim-

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bine, and has also been applied to a variety of matrices including milk, orange juice, vegetable juice, and processed squid.

Based on our success with the determination of nicotine in these rather complex matrices, we investigated this approach for the determination of nicotine in commercial tobacco products. In order for this approach to be viable, it must be capable of resolving nicotine from the minor tobacco alkaloids (including nornicotine, anatabine, and anabasine), as well as other endogeneous tobacco components and commercial additives. In this paper, we determine the effects of buffer identity, buffer pH, and ion-pairing agent concentration on the retention, peak shape, and peak efficiency of nicotine, as well as determining the selectivity between nicotine and the minor tobacco alkaloids. In addition, we examine the observed elution order of the tobacco alkaloids with respect to their fundamental analyte properties (size, functionality, and acid–base strength). The results of the study are used to understand the retention mechanisms of nicotine and the minor tobacco alkaloids under reversed-phase ion-pairing conditions, and to select optimum chromatographic conditions for the determination of nicotine in commercial tobacco products.

2. Experimental

2.1. Standards and reagents

Nicotine hydrogen tartrate (Catalog No. N-5260), nornicotine (98%), cotinine (98%), and myosmine (Catalog No. M-8765) were obtained from Sigma (St. Louis, MO, USA). Anabasine (85%) and uracil (98%) were obtained from Aldrich (Milwaukee, WI, USA). Anatabine (96.4%) was obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository (Bethesda, MD, USA). The nicotine content of the nicotine hydrogen tartrate salt was determined at 32.6% using CORESTA Recommended Method No. 39 [8]. This corresponds to a purity of 100% (100.3%) as the salt.

Citric acid monohydrate (ACS reagent) and sodium octane sulfonate (98% or Ultra) were obtained from Sigma (St. Louis, MO, USA). Potassium phosphate monobasic (HPLC grade) was obtained from Fisher (Pittsburgh, PA,USA). Sodium formate (reagent) was obtained from Matheson, Coleman, and Bell (Cincinnati, OH, USA).

2.2. Instruments and conditions

A Hewlett Packard 1050 liquid chromatograph with PDA detector and a Zorbax Rx C_{18} column (5 μm, 4.6 mm×150 mm) were used for all experiments. A number of mobile phase buffers varying in buffer type, pH, and ion-pairing agent concentration were tested throughout the study. The composition of the optimized buffer was 50 m*M* citric acid, 10 m*M* sodium octane sulfonate, adjusted to a pH of 3.0 with 1 *M* KOH. The optimized mobile phase conditions were 65:35 buffer:methanol with a flow-rate of 1.25 ml/min. Some work is also presented with a variation of the mobile phase conditions comprising 70:30 buffer:methanol (buffer at pH 3.2) with a flow-rate of 1.00 ml/min. For each experiment, the specific mobile phase conditions are noted in the appropriate sections below. The injection volume was 10 μl. Detection was at 259 nm.

2.3. pH experiment

A large batch of 50 m*M* citric acid, 10 m*M* sodium octane sulfonate was prepared and divided into six portions. The pH of each portion was adjusted with either 1 *M* phosphoric acid or 1 *M* KOH to give pH's of 2.5, 2.75, 3.0, 3.5, 4.5, 5.5, and 6.5. Each portion was used as the buffer in a mobile phase comprising 65:35 buffer:methanol. Two to four replicate injections of individual standard solutions of nicotine, anatabine, nornicotine, and anabasine (nominally 200 μg/ml in corresponding buffer) were made for each of the seven mobile phases. A solution of uracil (50 μg/ml in buffer) was used to determine t_0. Peak efficiencies were calculated by the Hewlett–Packard software (Version A.02.05) as $(M_1)^2/M_2$ where M_1 and M_2 represent the first and second statistical moments, respectively. Peak tailing factors were calculated according to the USP definition [9].

2.4. Buffer type experiment

Individual 50 m*M* batches of citric acid, potassium

phosphate monobasic, and sodium formate were prepared. Sodium octane sulfonate (10 m*M*) was added to each batch, and the pH adjusted to 3.0 using either 1 *M* phosphoric acid or 1 *M* KOH. Each buffer was used in a mobile phase comprising 65:35 buffer:methanol. Triplicate injections of a standard nicotine solution (nominally 200 μg/ml in corresponding buffer) were made for each of the three mobile phases. Peak efficiencies and peak tailing factors were calculated as for the pH study.

2.5. Ion-pairing agent concentration experiment

A large batch of 50 m*M* citric acid was prepared and divided into six portions. Sodium octane sulfonate was added to each portion to give concentrations of 0 m*M*, 2.5 m*M*, 5.0 m*M*, 10 m*M*, 25 m*M*, and 50 m*M*. After adding the sodium octane sulfonate, the pH of each portion was adjusted to 3.0 using 1 *M* KOH. Each portion was used as the buffer in a mobile phase comprising 65:35 buffer:methanol. Triplicate injections of a standard nicotine solution (nominally 200 μg/ml in corresponding buffer) were made for each of the six mobile phases.

3. Results and discussion

3.1. Chromatography of nicotine on C_{18} reversed-phase

Nicotine is a diprotic base with pK_a's of 3.12 (pyridine ring) and 8.02 (pyrrolidine ring) [10]. At pH 7.0–7.5, less than 25% of the nicotine is converted to its neutral (free base) form. Under these conditions, there is significant potential for nicotine to interact strongly with residual surface silanols and exhibit poor peak shape and peak tailing. To minimize this problem, the chromatographer can choose a base-deactivated reversed-phase and add triethylamine (TEA) to the mobile phase. We evaluated the peak shape of nicotine on a commercial stationary phase (Zorbax Rx C_{18}) which is considered 'base-friendly' using a buffer comprising 0.2% v/v phosphoric acid titrated to a pH of 7.3 with TEA [3,11]. After titration, the final TEA content of the buffer was ca. 0.4% by weight. The buffer was used in mobile phases with buffer:methanol ratios in the range 40:60 to 30:70. In all cases, the nicotine peak was broad and exhibited significant peak tailing. It's possible that a higher level of TEA (1%) would have improved the peak features [5].

At pH 2.5–3.0, nicotine exists as a mixture of its monoprotonated (+1) and diprotonated (+2) forms with the monoprotonated form representing 19–43% of the total nicotine over the indicated pH range. Under these conditions, nicotine is unretained and the potential for interaction with residual silanols is minimal. The chromatographer can add an ion-pairing agent to the mobile phase in order to increase the retention of nicotine. Our prior experience with this approach [7] confirmed that good peak shapes could be obtained for nicotine. We investigated this approach for the determination of nicotine in commercial tobacco products and conducted a thorough study to determine the effects of pH, buffer type, and ion-pairing agent concentration. It was necessary to develop a separation which resolved nicotine from other tobacco components including the minor tobacco alkaloids.

3.2. Effect of pH on the retention and selectivity of tobacco alkaloids using a citric acid buffer containing an ion-pairing agent

Nicotine is the major tobacco alkaloid in the varieties of *Nicotiana tabacum* used for the production of commercial cigarettes and smokeless tobacco [2,12–14]. Anatabine, nornicotine, anabasine, cotinine, and myosmine are among the minor tobacco alkaloids which have also been identified in these tobacco varieties [2,13–18]. Although the order of prevalence of the minor tobacco alkaloids varies, the three most prevalent are frequently anatabine, nornicotine, and anabasine [13–18].

Citric acid buffers are capable of buffering the pH range 2.5–6.4, allowing the study of a wide pH range without changing the buffer identity. Fig. 1A shows the retention factor of nicotine as a function of pH for mobile phases in which the buffer portion comprised 50 m*M* citric acid/10 m*M* sodium octane sulfonate. Retention of nicotine decreased rapidly in the pH range 2.5 to 3.0, reached a minimum near pH 4.5, and then increased rapidly between pH 5.5 and 6.5.

Nicotine's pH-retention behavior can be under-

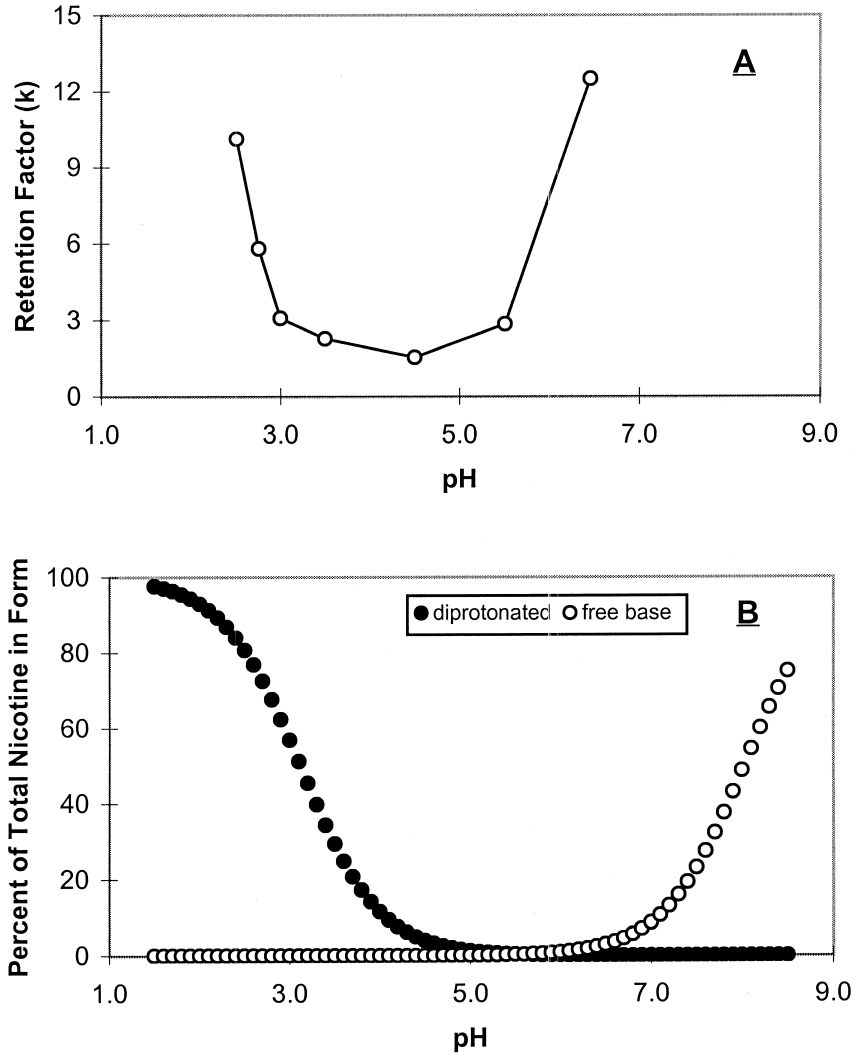

Fig. 1. Correlation between nicotine retention and the relative concentration of its acid–base forms under ion-pairing conditions: (A) nicotine retention factor as a function of pH; (B) percentage of nicotine present in diprotonated and free base forms as a function of pH.

stood both in terms of the typical pH effect observed for basic analytes under ion-pair conditions [5] and the acid/base properties of nicotine [19]. If the ion-pairing agent concentration is high enough, the retention of a basic analyte will decrease with increasing pH essentially tracking the diminishing fraction of the ionized form of the analyte as pH increases [5]. Under these conditions, retention of the neutral form of the analyte would be suppressed throughout the entire pH range. The pH-retention behavior of nicotine in the present work displays the typical pH effect for a basic analyte under ion-pair conditions over the lower pH range only (pH 2.5–4.5). Nicotine retention increases over the higher pH range (4.5–6.5) consistent with the typical pH effect for a basic analyte in the absence of an ion-pairing agent. In the present work, the ion-pairing agent concentration is 10 m*M* in the buffer which is used in conjunction with 35% methanol in the mobile phase. Thus, it is likely that the concentration of

ion-pairing agent is not sufficient to suppress retention of the nicotine free base form over the entire pH range [5].

This interpretation is confirmed by considering the distribution of nicotine among its three acid/base forms (diprotonated, monoprotonated, and free base) as a function of pH as shown in Fig. 1B. The monoprotonated form of nicotine accounts for greater than 50% of the total nicotine over the pH range 3.2–8.0, and reaches a sustained maximum proportion over the pH range 4.9 to 6.3. The proportion of diprotonated nicotine nicotine decreases rapidly with increasing pH and represents 1% or less of the total nicotine above pH 5.1. The proportion of free base nicotine does not reach 1% until about pH 6, and then increases rapidly. Inspection of Figs. 1A and B together shows that the rapid decrease in nicotine retention observed over the pH range tracks the rapid decrease in the proportion of diprotonated nicotine which occurs over the same pH range. Both the diprotonated (+2) and monoprotonated (+1) forms of nicotine are capable of ion-pairing with octane sulfonate. However, the diprotonated form can ion-pair with two octane sulfonate molecules and would be expected to exhibit higher retention than the monoprotonated form.

Likewise, the rapid increase in nicotine retention over the pH range 5.5–6.5 tracks the increase in the proportion of free base nicotine which occurs over this pH range although the free base still only accounts for about 3% of the total nicotine at pH 6.5. The contribution of the diprotonated and free base forms reaches a minimum at intermediate pH values, and retention is dominated by the monoprotonated form. Under the present conditions, the monoprotonated form exhibits the least retention of the three acid/base forms.

Plate counts and tailing factors were calculated for the nicotine peaks obtained in the pH study. The highest plate count was observed at a pH of 2.5 (2400 plates); the next highest plate count was observed at a pH of 3.0 (2200 plates). Above pH 3.0, the plate count decreased with increasing pH (Fig. 2A). In order to optimize plate count with respect to analysis time, the plate counts were normalized by dividing by the corresponding retention times (N/t_R), and plotted as a function of pH (Fig. 2A). This plot shows an obvious optimum region near pH 3.0, where the plate count per unit retention time (i.e. per unit 'analysis time') is the highest. A plot of the tailing factor versus pH (Fig. 2B) also shows pH 3.0 to be optimum with minimum tailing. The optimum results obtained at pH 3.0 are not strictly a function of retention on the stationary phase because minimum retention occurred over the pH range 3.5–5.5. At pH 5.5 and 6.5, the nicotine peak was extremely broad and exhibited significant peak tailing; peak splitting occurred at pH 6.5, making these higher pH 'ion-pair' conditions unacceptable for analytical use.

The same general pH-retention behavior which was observed for nicotine was also observed for the minor tobacco alkaloids anatabine, nornicotine, and anabasine (Figs. 3A–D). Similar pH-retention behavior may be expected for nicotine and these minor alkaloids because each is a diprotic base containing a pyridine nitrogen and a secondary or tertiary amine nitrogen. The selectivity of nicotine relative to each of these minor alkaloids was calculated and plotted as a function of pH (Fig. 4). Good selectivity (α= 1.2–2.2) was obtained at all pH's except pH 4.5 where nicotine and nornicotine coeluted (α=1.01). Nicotine eluted prior to all of the minor alkaloids over the pH range 2.5 to 3.5 (selectivity calculated as $k_{\text{minor alkaloid}}/k_{\text{nicotine}}$) and after all of the minor alkaloids above pH 4.5 (selectivity calculated as $k_{\text{nicotine}}/k_{\text{minor alkaloid}}$). The change in the relative elution order of nicotine and the minor alkaloids in going from lower to higher pH provides further evidence that a change in the dominant retention mechanism has occurred, i.e. retention dominated by the ion-pair forms of nicotine and the minor alkaloids at lower pH and retention dominated by the respective free base forms at higher pH.

3.3. Effect of buffer type at pH 3.0 in the presence of 10 mM ion-pairing agent

pH 3.0 was shown to be an optimum pH with respect to the peak shape and peak efficiency per unit analysis time for nicotine under ion-pair conditions using a 50 m*M* citrate buffer. Phosphate and formate are also capable of buffering at pH 3.0. We substituted phosphate or formate for citrate in a mobile phase comprising 65:35 buffer:methanol (pH 3.0, 10 m*M* sodium octane sulfonate). Fig. 5 shows chromatograms obtained using the citrate, phosphate, or

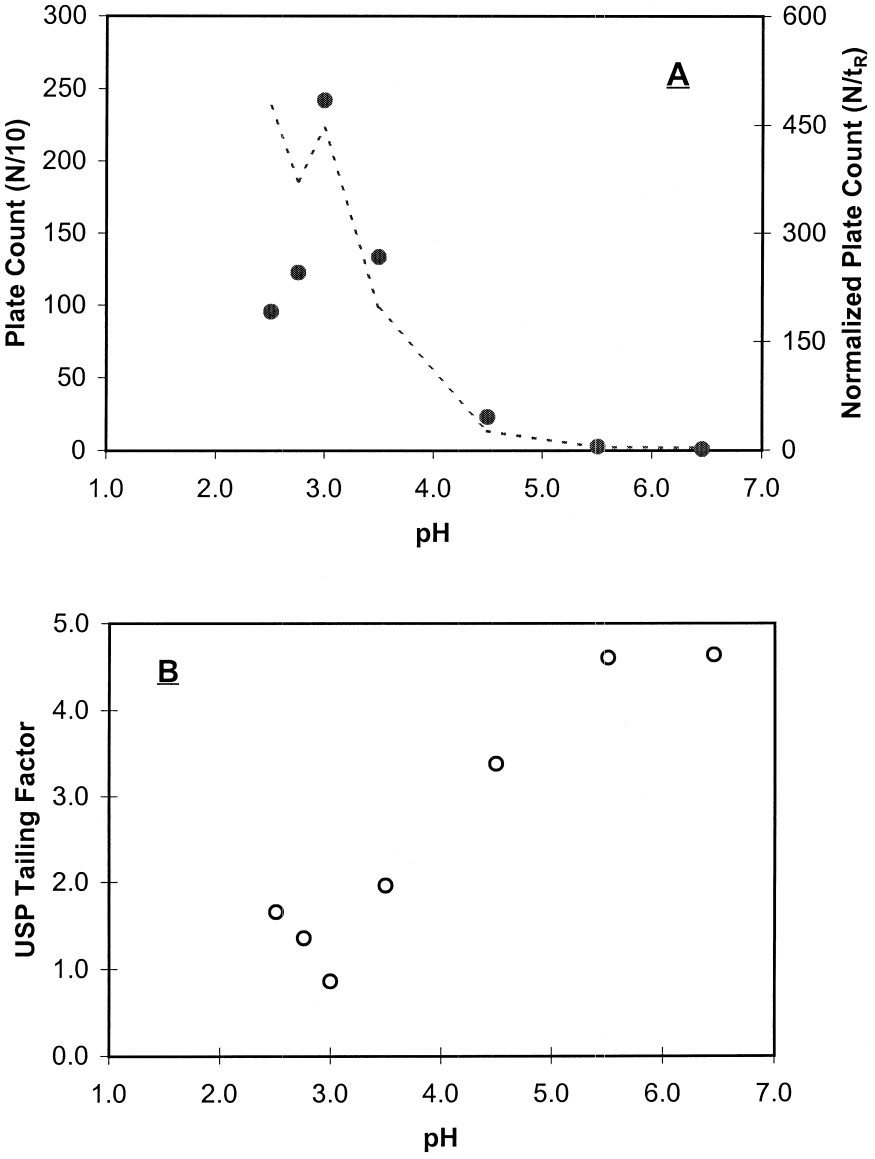

Fig. 2. Nicotine peak parameters as function of pH: (A) plate count (N; dotted line) and plate count 'normalized' for analysis time (N/t_R; solid circles). Note that the plate count (N) was divided by 10 for plotting purposes. (B) USP tailing factor. See text for discussion.

formate buffer for a standard solution of nicotine in the corresponding buffer. Retention increased in the order phosphate, formate, citrate. The fact that the highest retention was obtained using citrate may indicate an ion-pairing interaction between nicotine and citrate, because citrate is the largest anion. The nicotine peak obtained using formate was distorted with partial splitting (Fig. 5B). The nicotine peak obtained using phosphate (Fig. 5A) exhibited tailing (tailing factor 1.9) and provided moderate efficiency (1000 plates). The best peak shape and efficiency were obtained using citrate (Fig. 5C, tailing factor

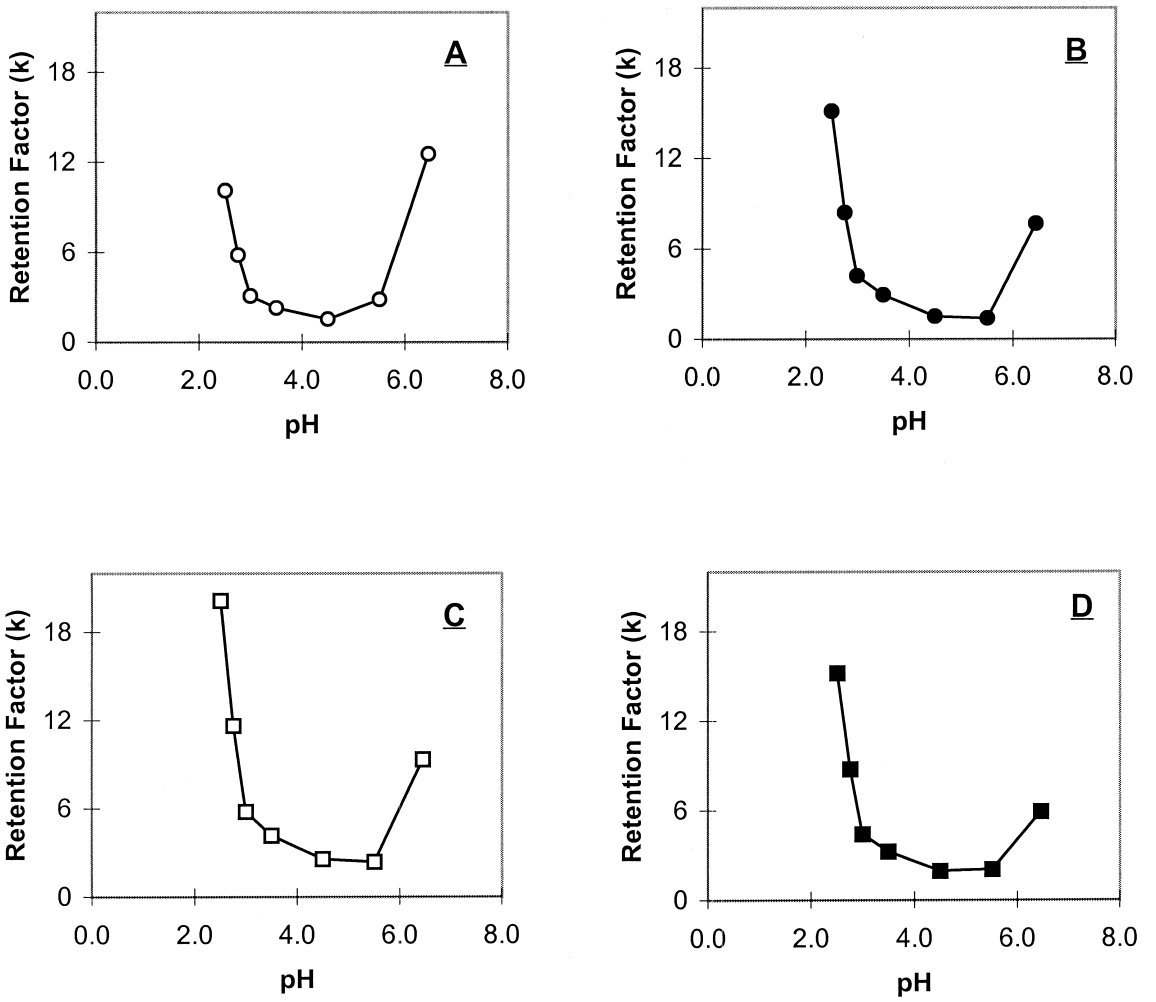

Fig. 3. Retention of nicotine and the minor tobacco alkaloids as a function of pH: (A) nicotine; (B) nornicotine; (C) anabasine; (D) anatabine.

1.2, 1400 plates). The effect of buffer concentration was not studied. Note that plate counts were calculated as $(M_1)^2/M_2$(statistical moments).

3.4. Effect of IPR concentration using a pH 3.0 citric acid buffer

The effect of ion-pairing agent concentration (sodium octane sulfonate) was studied over the range 0–50 m*M* using a pH 3.0 citric acid buffer (50 m*M*). In the absence of ion-pairing agent, nicotine eluted with the column void volume. This was expected because practically 100% of the nicotine is present in the charged form (monoprotonated or diprotonated) at pH 3.0. Nicotine retention increased with increasing ion-pairing agent concentration over the entire range (Fig. 6). The greatest increase occurred over the concentration range 2.5–25 m*M* with a ca. six-fold increase in the retention factor. Good peak shapes were observed over the entire range. The addition of 10 m*M* ion-pairing agent increased the

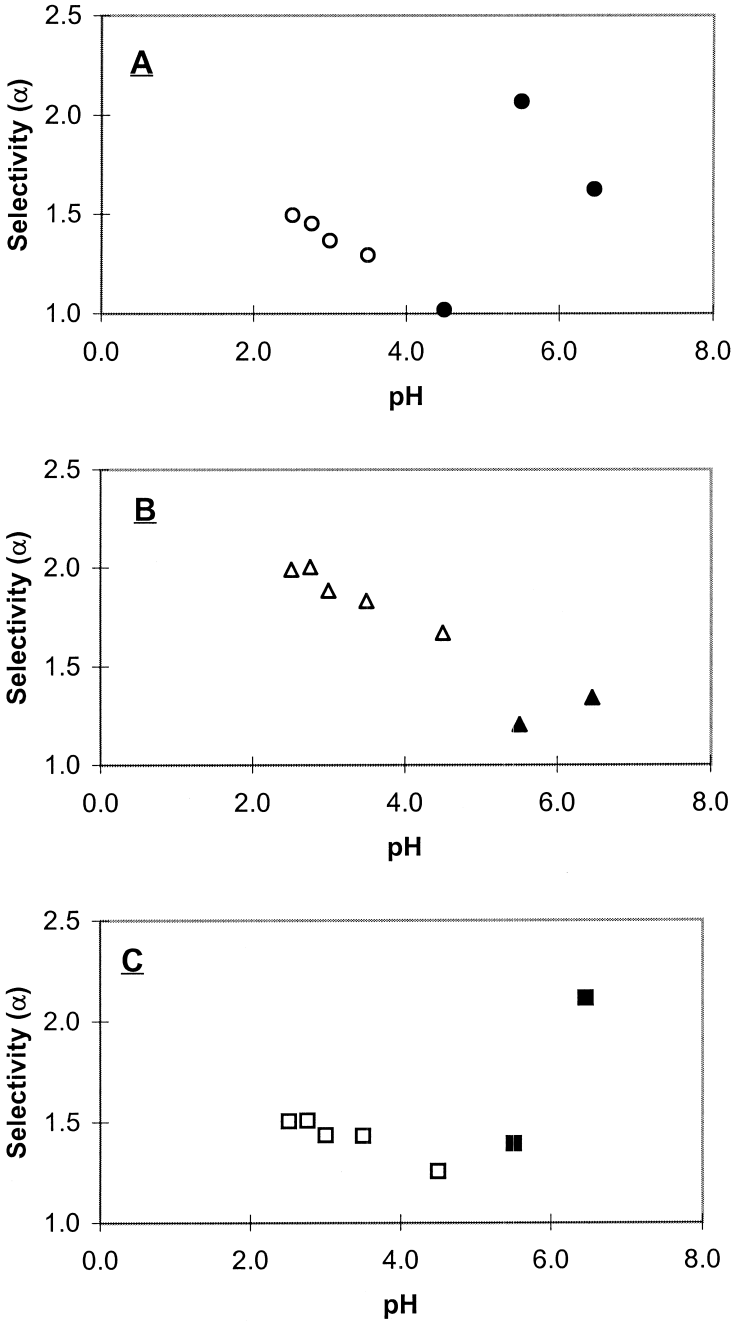

Fig. 4. Selectivity of nicotine versus the minor tobacco alkaloids as a function of pH: (A) nornicotine; (B) anabasine; (C) anatabine. Selectivity calculated as $k_{\text{minor alkaloid}}/k_{\text{nicotine}}$ (open symbols) or $k_{\text{nicotine}}/k_{\text{minor alkaloid}}$ (solid symbols).

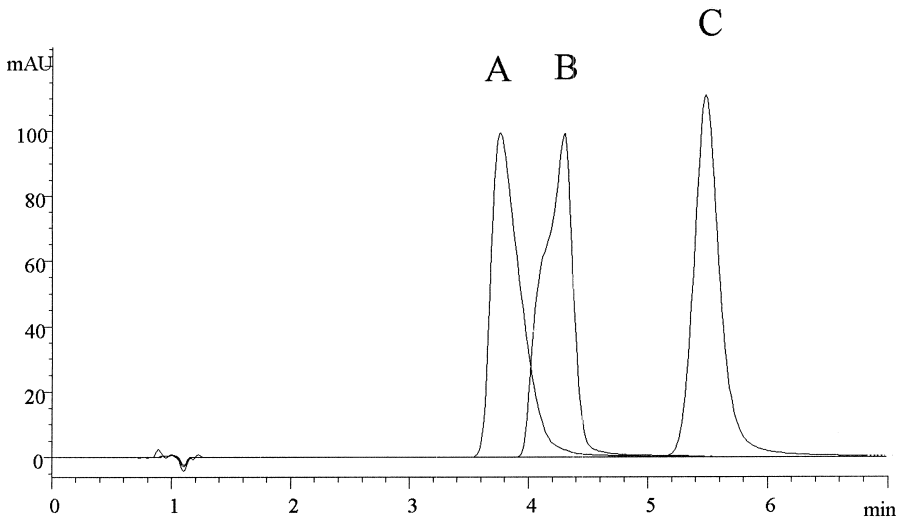

Fig. 5. Effect of buffer identity on nicotine retention and peak shape at pH 3.0: (A) phosphate; (B) formate; (C) citrate.

retention time of nicotine to about 5 min (k 3–4) which was sufficient to resolve nicotine from a host of relatively polar tobacco components which elute near the column void volume for both cigarettes and smokeless tobacco (Fig. 7A and B).

3.5. Chromatography of nicotine and the minor tobacco alkaloids using optimized reversed-phase ion-pair conditions

Based on the previous experiments, an optimal

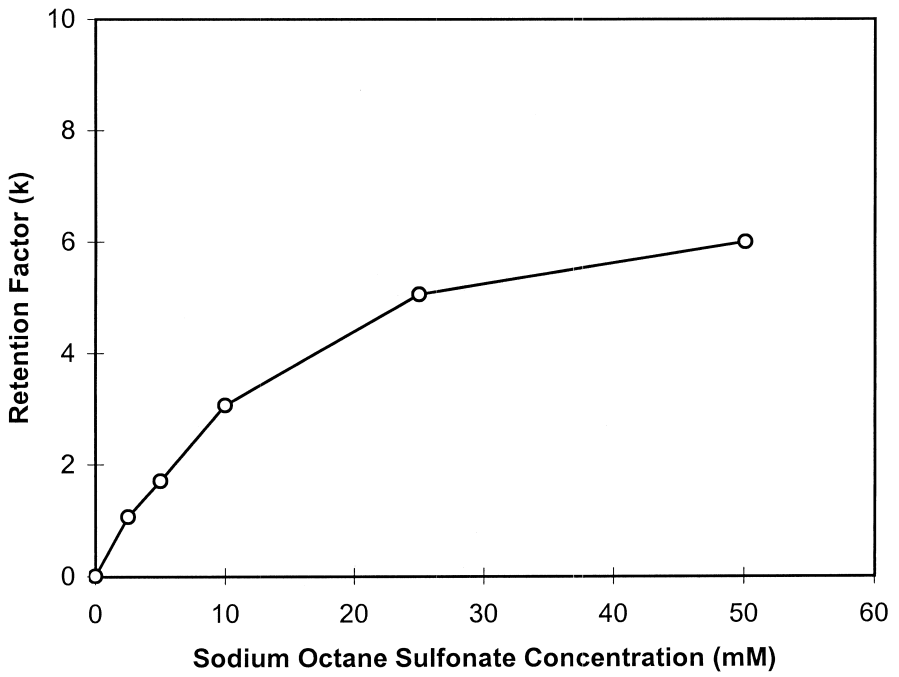

Fig. 6. Effect of ion-pairing agent concentration on nicotine retention at pH 3.0.

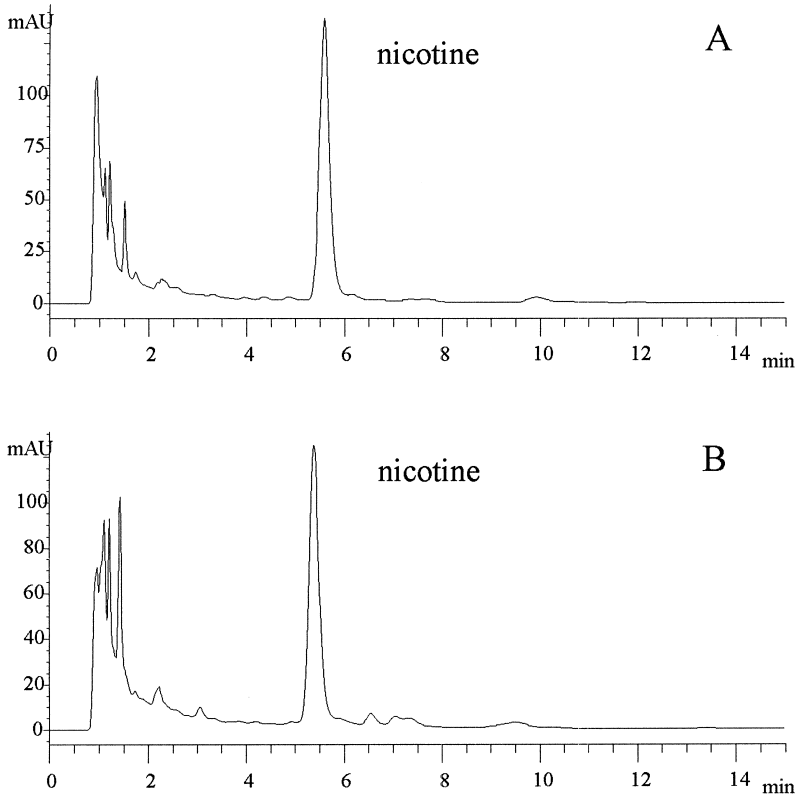

Fig. 7. Chromatograms of commercial tobacco products obtained using optimized reversed-phase ion-pairing conditions: (A) commercial cigarette; (B) commercial moist snuff brand.

buffer for the reversed-phase ion-pair separation of nicotine was 50 m*M* citrate (pH 3.0) with 10 m*M* sodium octane sulfonate. The use of this buffer in a mobile phase comprising 65:35 buffer:methanol resulted in a k_{nicotine} of about 3.5 within the optimal range for HPLC (k 1–10). Fig. 8A shows the separation of a standard mixture of nicotine and four minor tobacco alkaloids (anatabine, nornicotine, anabasine, and cotinine) using these conditions. The observed elution order was cotinine, nicotine, nornicotine, antabine, and anabasine. Nicotine was well resolved from all of the minor alkaloids; however, nornicotine and anatabine were only partially resolved from one another.

Under ion-pairing conditions, the elution order should reflect the stoichiometry and degree of ion-pair formation between the analyte and the ion-pairing agent, as well as the analyte size. Cotinine (Fig. 9A), which eluted first, has only one basic nitrogen (pyridine) and is expected to ion-pair with octane sulfonate with 1:1 stoichiometry. Nicotine, nornicotine, anatabine, and anabasine (Fig. 9B–E) each have two basic nitrogens: a pyridine nitrogen which is common to the four alkaloids, and a secondary or tertiary amine nitrogen which varies among the four alkaloids. Each of these alkaloids is able to ion-pair with octane sulfonate with 1:2 stoichiometry, which explains their higher retention relative to cotinine.

Nicotine and nornicotine each have five-membered pyrrolidine rings while anatabine and anabasine each have six-membered heterocyclic rings. The smaller size of nicotine and nornicotine explains their elution prior to anatabine and anabasine. Finally, the elution of nicotine prior to nornicotine, and anatabine prior to anabasine can be explained by the relative degree of ion-pair formation. Because the analytes must be in their acidic forms in order to ion-pair, the degree

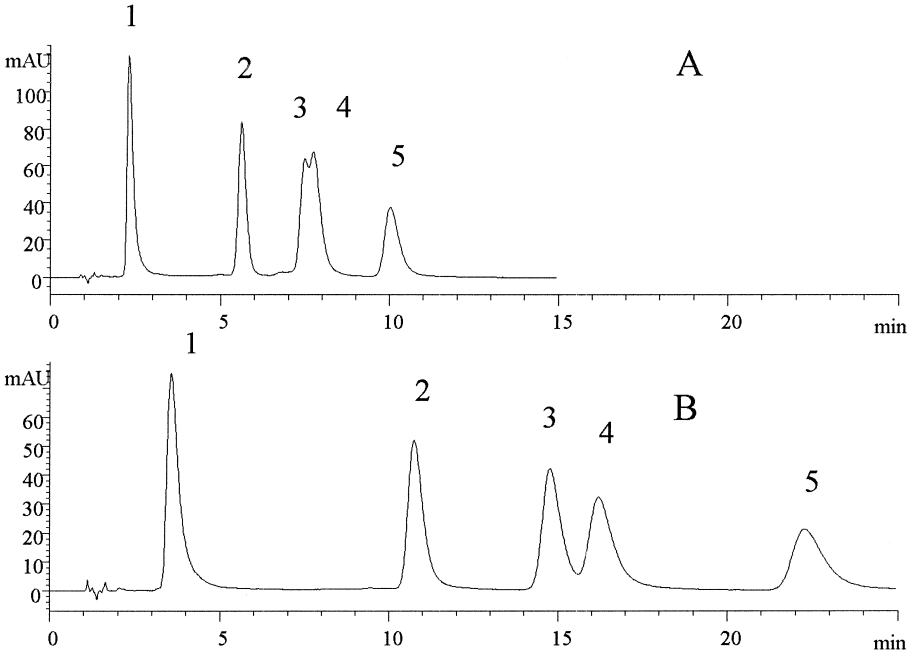

Fig. 8. Chromatograms of nicotine and the minor tobacco alkaloids obtained using: (A) optimized reversed-phase ion-pairing conditions; (B) modified conditions used to resolve a coeluting component. Peak identification: (1) cotinine; (2) nicotine; (3) nornicotine; (4) anatabine; (5) anabasine.

of ion-pair formation should be directly proportional to their acid–base dissociation constants. The lower the pK_a, the less of the acidic form which will be available for ion-pair formation, and the lower retention which is expected. Because the buffer pH is 3.0, the relevant pK_a is that of the pyridine nitrogen which determines the ratio of diprotonated to monoprotonated alkaloid. The pK_a of the pyridine nitrogen in nicotine is 3.1 [12,20]. The pK_a of the pyridine nitrogen in nornicotine is 3.3 [20]. The lower pK_a for nicotine relative to nornicotine is consistent with the observed elution order of nicotine prior to nornicotine. Likewise, it is predicted that the relative values of the pyridine nitrogen pK_a's in anatabine and anabasine have an influence on the relative degree of ion-pair formation and retention for the two alkaloids. However, this could not be examined as a literature pK_a value [20] was found for anabasine only (pK_a 3.1) and not anatabine. Structural differences must also be considered. The structure of anatabine and anabasine differ in the presence of an additional double bond in anatabine which would also normally result in decreased retention for anatabine relative to anabasine.

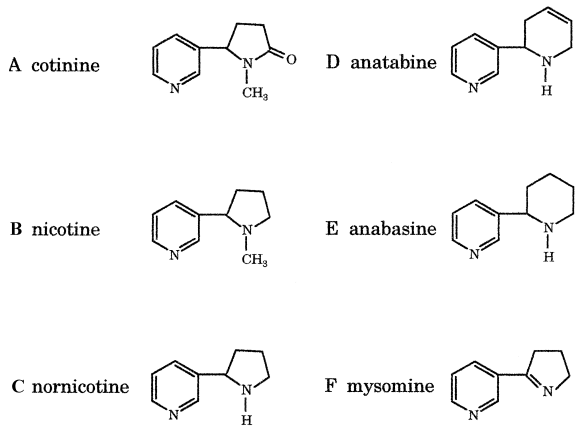

Fig. 9. Structures of nicotine and the minor tobacco alkaloids: (A) cotinine; (B) nicotine; (C) nornicotine; (D) anatabine; (E) anabasine; (F) myosmine.

As reported elsewhere [1,21], we have applied the optimized conditions to the determination of nicotine in several commercial brands of cigarettes and moist snuff. As the method was applied to more and more

products, we monitored the spectral purity of the nicotine peak in order to check for coelution of any tobacco components (endogenous or manufacturing additives). We did encounter a minor interference in some of the cigarette brands we tested and the chromatographic conditions were modified to 70:30 buffer–methanol (buffer at pH 3.2) in order to resolve the coeluting component [21]. The effect of the modified conditions on resolution of nicotine from the minor tobacco alkaloids was determined by injecting the standard alkaloid mixture. Resolution between nicotine and each of the minor alkaloids was maintained, and resolution between nornicotine and anatabine was significantly improved (Fig. 8B). The overall elution order was also maintained.

3.6. Instability of myosmine under method conditions

A standard solution of another minor tobacco alkaloid, myosmine, was also injected under the optimized conditions (65:35 buffer–methanol, pH 3.0). Myosmine was observed to elute as a combination of two distinct sharp peaks coeluting with a third broad peak (Fig. 10A). The possibility that the myosmine standard had degraded was tested by obtaining a gas chromatogram of the standard prepared in chloroform:hexane. The result was a single sharp peak, providing no indication of any volatile degradation products. Another possibility was that myosmine was unstable in the buffer and/or mobile phase. This possibility was confirmed in some early literature [22] which reported that myosmine (Fig. 9F) reacts in aqueous solution with scission of the pyrroline ring to give 3-pyridyl ω-aminopropyl ketone. The presence of three peaks in the myosmine chromatogram probably indicates the presence of three distinct compounds in the myosmine/water reaction mixture.

The later eluting of the two myosmine sharp peaks coeluted with the nicotine peak (Fig. 10B). This peak accounted for less than 20% of the total myosmine peak area. Experiments were conducted to determine the degree of interference which could be expected from myosmine for the determination of nicotine in commercial tobacco products. It was

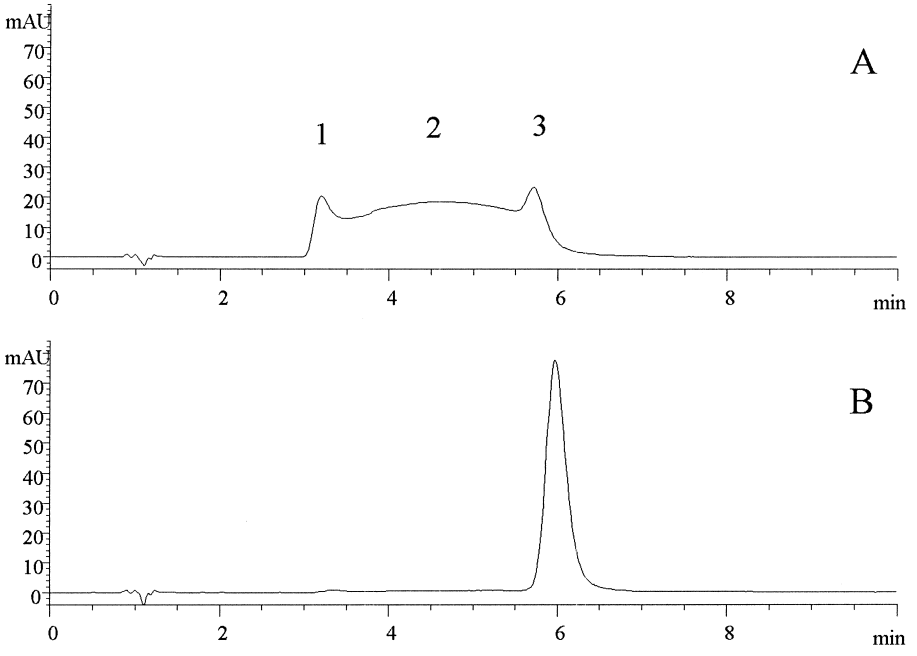

Fig. 10. Instability of myosmine under method conditions: (A) chromatogram of a myosmine standard obtained using optimized reversed-phase ion-pairing conditions; (B) chromatogram of nicotine obtained under the same conditions. The three peaks observed for myosmine (labeled 1, 2, 3) may represent three distinct compounds. See text for discussion.

determined that no interference (i.e., no measurable contribution to the nicotine peak area from myosmine) occurred for myosmine levels of 1% or less relative to nicotine (i.e. myosmine:nicotine ratio of 1:99). The interference was ca. 2% at a myosmine level of 4% relative to nicotine (i.e., a 2% increase in nicotine peak area from the partial coelution of myosmine). The actual range of myosmine levels relative to nicotine is reported at 0–0.5% [2,13,16] in the tobacco varieties used for commercial cigarettes and smokeless tobacco (myosmine was reported as not detected in some cases). Thus, no interference from myosmine is anticipated under the present conditions for the determination of nicotine in commercial tobacco products.

4. Summary

The conditions for the reversed-phase ion-pair liquid chromatographic determination of nicotine were studied with respect to buffer identity, buffer pH, and ion-pairing agent concentration using a commercial C_{18} column. A citrate buffer with a pH of 3.0 provided the best overall nicotine peak efficiency and peak shape with respect to analysis time. An ion-pairing agent concentration of 10 m*M* was sufficient for resolving nicotine from a host of compounds which eluted with the column void volume. Retention of nicotine and the minor tobacco alkaloids was dominated by their ion-pair forms at lower pH (pH 2.5–4.5) and by their free base forms at higher pH (4.5–6.5). The observed elution order was related to the functionality (monoprotic or diprotic base), size, and acid–base strength of the tobacco alkaloids.

Using the optimized conditions (65:35 buffer–methanol; buffer is 50 m*M* citrate, 10 m*M* sodium octane sulfonate, pH 3.0), nicotine was well resolved from the minor tobacco alkaloids nornicotine, anatabine, anabasine, and cotinine. The performance of the optimized method, both with and without some minor variations, has been tested extensively in subsequent studies using both commercial moist snuff [1] and cigarettes [21], and has been shown to be reliable.

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