Rapid method for analysis of nicotine and nicotine-related substances in chewing gum formulations

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Abstract

Based on environmental requirements and demands for a high throughput a rapid method for the analysis of nicotine and nicotine-related substances in chewing gum formulations was developed. The method is based on sample preparation through liquid–liquid extraction followed by reversed-phase HPLC using gradient elution. It allowed up to nine analytes to be determined within 15 min, including the sample preparation, and was considered as accurate and robust. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Chewing gum; Pharmaceutical analysis; Nicotine

1. Introduction

Stricter health and environmental regulations in the development of new pharmaceuticals has increased the demand for quality control. Among the nicotine formulations for smoking cessation therapy, nicotine chewing gum (Nicorette; Pharmacia & Upjohn, Helsingborg, Sweden) is the most widely used product. The present quality control includes monitoring of the nicotine level (2 or 4 mg per gum) as well as some oxidation products: cotinine, nicotine-*cis-N*-oxide and nicotine-*trans-N*-oxide. In addition to these analytes, other oxidation products and related analytes such as myosmine, β-nicotyrine, nornicotine, anatabine and anabasine (Fig. 1) have also been included in the method development.

The presently used high-performance liquid chromatography (HPLC) methods for this analysis lack the speed required in order to handle an increasing number of samples and is furthermore based on environmentally harmful solvents [1]. Our goal was, therefore, to develop a fast, robust, accurate and environmentally friendly HPLC method in accordance with the producers requirements.

Nicotine is a moderately strong base with $pK_{a1}=3$ and $pK_{a2}=8$ for the corresponding acid, whereas the other analytes are weak bases with pK_a values in the range of 4.5–5.5. The polarity of the non-protonated forms, estimated from reversed-phase HPLC, increases in the order β-nicotyrine$<$nicotine$<$myosmine$<$cotinine$<$nicotine-*N*-oxide. The oxidation of nicotine is catalysed by light. It is more stable

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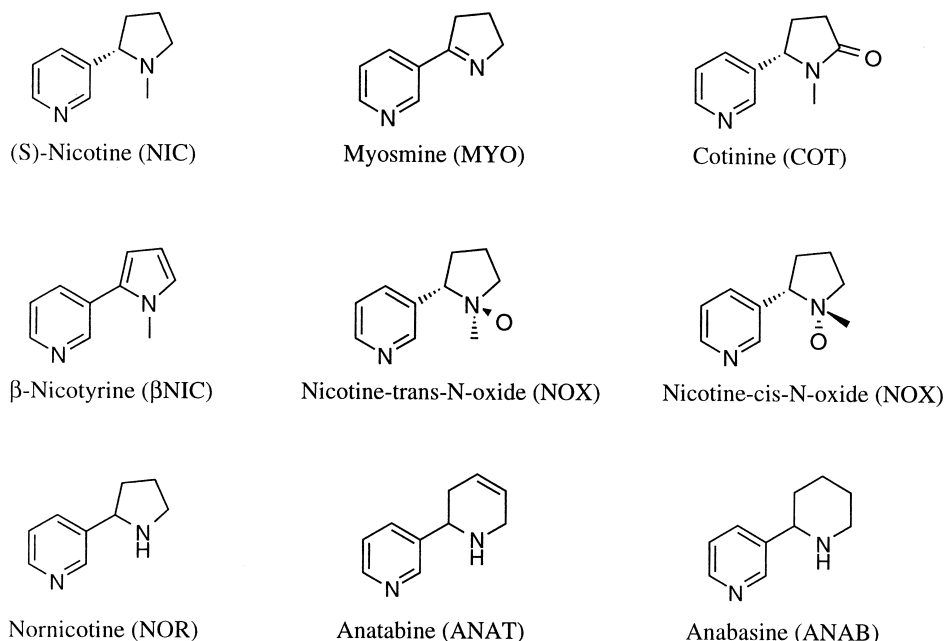[3]Present address: Polypeptide Laboratories AB, Malmö, Sweden.

Fig. 1. Chemical structures of the major analyte, nicotine and the minor nicotine related analytes.

(S)-Nicotine (NIC)

Myosmine (MYO)

Cotinine (COT)

β-Nicotyrine (βNIC)

Nicotine-trans-N-oxide (NOX)

Nicotine-cis-N-oxide (NOX)

Nornicotine (NOR)

Anatabine (ANAT)

Anabasine (ANAB)

in its protonated form than in its unprotonated, more volatile, form [2]. It is chiral and occurs naturally as the *S* isomer. In the gum, nicotine (2 or 4 mg) is bound to polyacrilex, a weak cation exchanger (Amberlite) [1]. The gum, further contains a non-polar polymeric matrix, flavors and sodium hydrogencarbonate. For a fully automated procedure, the HPLC method should ideally be chosen according to the sample preparation, i.e., similar solvents, pH and temperatures. Thus, samples in organic solvent are suited for normal-phase HPLC whereas samples in water are suited for reversed-phase or ion-pair chromatography (Fig. 2). The sample preparation can be divided into different steps. First the gum matrix has to be dissolved in order to allow release of the analytes into solution. In order to accomplish this, a non-polar to weakly polar solvent is required (e.g., hexane, heptane, tetrahydrofuran, ethylacetate, chloroform etc.). Secondly in order to release the analytes from the ion exchanger either acid or base has to be added.

When an acid is added the ion exchanger and the analytes will be protonated and the analyte thus released from the ion exchanger in a more stable form. The analytes can here be directly extracted into

an aqueous phase which can then be directly analysed by ion-pair [1] or reversed-phase [3] HPLC. However this procedure requires 100% extraction efficiency which can be difficult to achieve in one extraction, particularly for the less polar analytes (see below). For this reason, releasing the analytes by addition of base followed by direct injection may be

Nicorette

1-phase system:
Analytes in organic phase

2-phase system:
Analytes in aqueous phase

Normal phase HPLC
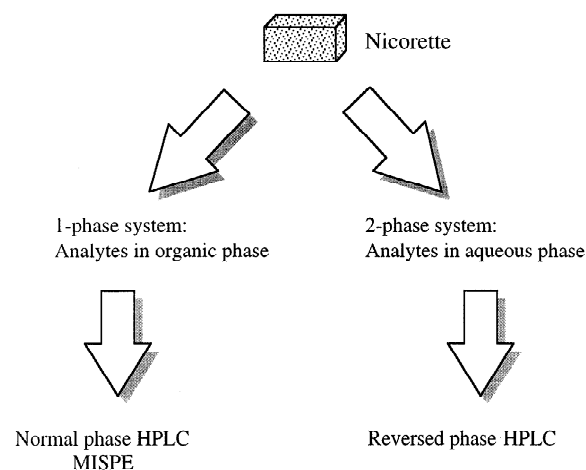MISPE

Reversed phase HPLC

Fig. 2. General strategies in the analytical method development. MISPE: Molecular imprinted solid phase extraction [4].

preferable when high recoveries of the less polar analytes is important [4].

In the two-phase alternative, the pH and the buffering capacity has to be adjusted in order to neutralise the hydrogencarbonate and fully protonate the analytes. The extraction procedure has been studied regarding the influence of the type of acid, pH and the addition of salt on the recovery of nicotine, on the separation time and on the amount of precipitates. After an initial optimisation, spiked placebo gums were prepared and the recovery for all analytes determined. This was followed by a comparison of different HPLC formats. It was found that reversed-phase HPLC methods were superior to ion-pair chromatographic methods in terms of robustness, sensitivity and speed of analysis.

2. Experimental

2.1. Chemicals

2.1.1. Analytes

Nicotine hydrogen (+)-tartrate dihydrate, BDH Chemicals, CAS: 128758-70-3, nicotine-(*cis*-, *trans*-)-*N*-oxides CAS: 491-26-9, (±)-anabasine, CAS: 13078-04-1, anatabine, myosmine, CAS: 532-12-7 and β-nicotyrine, CAS: 525-75-7, were obtained from Pharmacia and Upjohn Research Labs., Helsingborg, Sweden. (±)-Nornicotine, CAS: 5746-86-1, was purchased from Sigma whereas (−)-cotinine, CAS: 486-56-6, came from Aldrich.

2.1.2. Other chemicals

The following quality and sources of the solvents and bulk chemicals were used: acetonitrile 99.9+%, HPLC grade, Sigma–Aldrich, CAS: 75-05-8; methanol 99.9+%, HPLC grade, Sigma–Aldrich, CAS: 67-56-1; *n*-heptane >99%, for chromatography, LiChrosolv, Merck, CAS: 00142-82-5; water, purified by Milli-Q; dipotassium hydrogenphosphate trihydrate buffer substance, for chromatography, LiChropur, Merck; orthophosphoric acid ≥85%, Fluka, CAS: 7664-38-2; sodium hydroxide ≥98%, Fluka, CAS: 1310-73-2; acetic acid ≥99.5%, Fluka, CAS: 64-19-7.

2.2. Equipment

The following equipment was used: balance: PJ Precisa junior 500c, PAG Oerlikon; magnetic stirrer: MR 2000, Heidolph; digital pH meter: pH 525, WTW; electrode: SenTix 97T, WTW; sonicator: Sonorex TK 52H, Bandelin electronic; mobile phase filtration unit: filtration unit 1, Supelco; filters: PTFE, pore size: 0.45 μm, Sartorius; single-use syringes: Injekt 10 ml, B. Braun Melsungen; disposable filter holders: white rim, 0.45 μm, FP 030/2, Schleicher & Schuell.

2.2.1. HPLC

In the HPLC method development a Hewlett-Packard instrument (HP1100 or HP1050) equipped with a binary pump (HP1100) or a quaternary pump (HP1050), a thermostatted column compartment (HP1100) an auto-sampler, a diode array detector (HP1100) and an HP work station was used.

2.3. Preparation of spiked placebo chewing gum

A placebo gum was sonicated for 40 min in 20 ml diethyl ether. Nicotine-containing polyacrilex resin corresponding to 2 mg nicotine was added to a flask followed by 1 ml of a standard solution containing the oxidation products (100 μg/ml). The solubilised chewing gum was then added together with 5 ml diethyl ether for rinsing. The solvent was thereafter allowed to evaporate at 25°C for 12 h.

2.4. Procedure for method validation

2.4.1. Extraction

2.4.1.1. Preparation of extraction solution

Ten ml of 85% phosphoric acid was dissolved in Milli-Q water and filled up with water to a volume of 1000 ml (volumetric flask). The pH of the extraction solution was 1.4. The solution contained 1.47 *M* phosphoric acid and was therefore 0.442 *N*.

2.4.1.2. Preparation of neutralization solution

6.0 g sodium hydroxide was dissolved in water. One hundred and fifty ml methanol was added to the sodium hydroxide solution and the resulting solution

was filled up to a volume of 1000 ml with water (volumetric flask).

2.4.1.3. Extraction and neutralisation of analytes from Nicotine chewing gum

A Nicorette chewing gum was dissolved in 40 ml heptane and liquid–liquid extracted with 40 ml of the extraction solution. One ml of the lower phase was taken with an Eppendorf pipette to a 2.5-ml glass vial. One ml of the neutralisation solution was added, and the vial was shaken for about 30 s. Afterwards the solution was filtered using a single-use syringe and a disposable filter holder to remove the formed precipitation.

2.4.2. Gradient methods

2.4.2.1. Mobile phases

Methods 1 and 2: Mobile phase A: 50 ml 0.4 M K_2HPO_4, 800 ml water, 150 ml methanol. The complete phase (apparent pH 9.1) was adjusted to an apparent pH of 8.5 using phosphoric acid. Mobile phase B: 50 ml water, 950 ml acetonitrile.

Method 3: Mobile phase A: 50 ml 0.4 M K_2HPO_4, 800 ml water, 150 ml methanol. The complete phase was adjusted to pH 8.5 using acetic acid. Mobile phase B: 100 ml water, 900 ml methanol.

2.4.2.2. Instrumental conditions

Method 1: Instrument: HP 1050, gradient: 0–1 min: 0% B, 1–8.5 min: 0–50% B, 8.5–9.5 min: 50% B, 9.5–10 min: 50–0% B, 10–15 min: 0% B, flow-rate: 1.2 ml/min, injection volume: 20 µl; detection wavelength: 254 nm; run time: 12 min; delay time: 3 min; temperature: ambient.

Method 2: Instrument: HP 1100; gradient: 0–1 min: 0% B, 1–8.5 min: 0–50% B, 8.5–9 min: 50% B, 9–9.5 min: 50–0% B, 9.5–15 min: 0% B, flow-rate: 0.6 ml/min, injection volume: 10 µl, detection wavelength: 254 nm, run time: 15 min; temperature: 22°C.

Method 3: Instrument: HP 1100; gradient: 0–0.8 min: 0% B, 0.8–2 min: 0–35% B, 2–7 min: 35–50% B, 7–8.5 min: 50% B, 8.5–11.5 min: 50–0% B, 11.5–15 min: 0% B, flow-rate: 0.6 ml/min, injection volume: 15 µl; detection wavelength: 260 nm; run time: 15 min; temperature: 22°C.

2.4.2.3. Analytical columns

Method 1: Prodigy 5 µm ODS(3), 125×4.6 mm I.D., Phenomenex, P/NO 00E-4097-EO, S/NO 169946.

Methods 2 and 3: Prodigy 5 µm ODS(3), 100× 3.2 mm I.D., Phenomenex, P/NO 00D-4097-RO, S/NO 185370, with guard column Prodigy 5 µm ODS(3), 30×3.2 mm I.D., Phenomenex, P/NO 03A-4097-RO, S/NO 173547G.

The ion-pair chromatographic and isocratic runs: Purosphere RD-18 endcapped (5µm) 125×4 mm I.D., Merck Darmstadt S/NO FE 105237.

2.4.2.4. Preparation of standard solutions

Stock solutions Nicotine: about 60 mg of nicotine hydrogen (+)-tartrate dihydrate was put into a 100-ml measuring flask. It was dissolved in acetonitrile, and the flask was filled up to the mark with acetonitrile.

Decomposition products: About 5 mg of the product was put into a 5-ml measuring flask. It was dissolved in acetonitrile and the flask was filled up to the mark with acetonitrile. In the case of anabasine however, the product was dissolved in water and the flask was filled up to the mark with water.

Standard solutions The eight standard solutions were made in 25-ml measuring flasks. A calculated volume of the stock solution was diluted with mobile phase A up to the mark, so that the final concentration in the flasks would be 50 µg/ml for nicotine and 5 µg/ml for the decomposition products. One mixture of all the analytes was made in one 25 ml measuring flask. Calculated volumes of the stock solutions were diluted with mobile phase A up to the mark, so that the final concentration in the flask would be 50 µg/ml for nicotine and 5 µg/ml for the decomposition products.

3. Results and discussion

3.1. Gum dissolution and extraction of analytes

In these studies, 2 mg gums or spiked placebo gums were dissolved in a non-polar solvent and the analytes extracted into an aqueous phase and analysed using ion-pair chromatography. Some general observations were made in these studies. First,
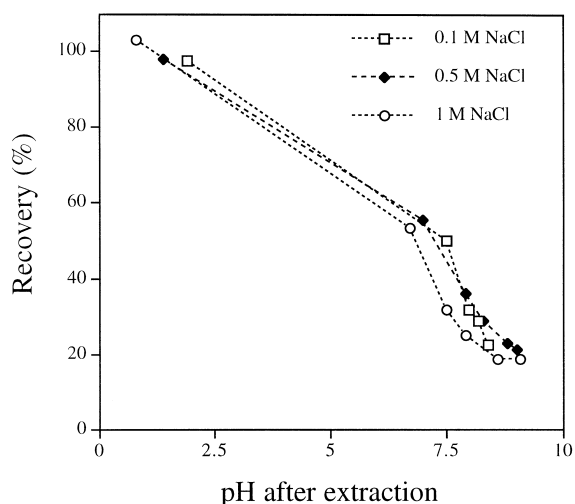
Fig. 3. Extraction of chewing gum standards at different pH values and salt concentrations. Extraction solutions containing NaCl at 0.1, 0.5 or 1.0 M were prepared in order to speed up the phase separation and clarify the solutions. The pH was adjusted with HCl to the desired pH. pH was measured before and after extraction. Extraction conditions: chewing gum standard was extracted in 40 ml hexane+40 ml acid followed by 0.5 min sonication. The aqueous phase was analyzed by ion-pair chromatography using a mobile phase consisting of: acetonitrile–0.1 M KH$_2$PO$_4$ (25:75, v/v) with the addition of 0.008 M SDS. Flow-rate: 0.52 ml/min.

enough acid has to be added to neutralize the sodium hydrogencarbonate in the gum and to give a pH of less than 1 after extraction. Only at this low pH, a quantitative extraction of nicotine is achieved (Fig. 3) satisfying the requirements concerning recovery. A simple equilibrium calculation using the pK_a of nicotine shows that a pH two units below the pK_a is necessary in order to double protonate more than
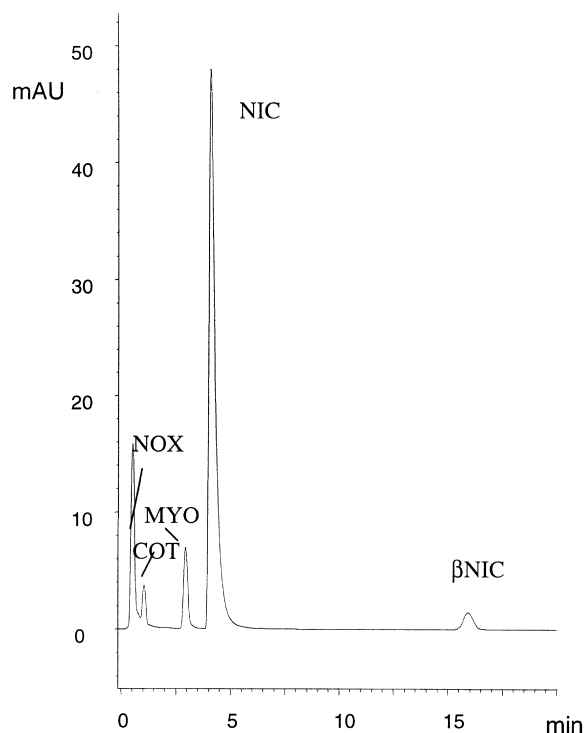

Fig. 4. Separation of nicotine (NIC) (0.2 mg/ml), β-nicotyrine (βNIC), cotinine (COT), myosmine (MYO) and nicotine-*cis-N*-oxide and nicotine-*trans-N*-oxide (NOX), (0.005 mg/ml each) on a reversed-phase column. After neutralisation (NaOH), sharp peaks of all analytes were obtained using a mobile phase consisting of acetonitrile–0.02 M potassium phosphate, pH 7.8 (20:80, v/v). Column: Purosphere C$_{18}$e (125×4 mm) 5 μm; detection: UV 254 nm; injection volume: 10 μl; temperature: room temperature; flow-rate: 0.7 ml/min.

99%. Thus using the validated extraction procedure, where a slightly higher pH is used [1], the recoveries are not quantitative although they may still be

Table 1
Recovery of nicotine and some nicotine-related compounds in spiked placebo chewing gums

Compound	Flavour 1		Flavour 2		Flavour 3	
	Recovery (%)	R.S.D. (%) (n=3)	Recovery (%)	R.S.D. (%) (n=3)	Recovery (%)	R.S.D. (%) (n=3)
Cotinine	110	4	136	9	118	5
cis-Nicotine-*N*-oxide	85	3	148	18	154	20
trans-Nicotine-*N*-oxide	115	7	100	12	104	7
Myosmine	64	2	59	4	112	1
Nicotine	112	3	103	2	110	1
β-Nicotyrine	87	1	81	10	87	1

See Fig. 3 for conditions.

acceptable. Use of trivalent acids like citric and phosphoric acid seemed to give a higher recovery of nicotine at a given pH after extraction, possibly due to complexation of the anions with the analytes. However the amount of residual solids after dissolution was higher in these cases.

In the cases where HCl was used as acid, addition of 0.1 M NaCl gave shorter clearance time and time for phase separation. The phases were clearer than without addition of salt and clearer at lower pH after

extraction i.e., pH<1.5 corresponding to a start pH of around 0.5. At higher salt concentrations more precipitate is formed and filtration has to be carried out. 0.1 M NaCl was therefore used in the subsequent experiments. Sonication was carried out in order to further speed up the phase separation and make the extraction more efficient.

A major drawback of using pH below 1 is the limited stability of nicotine and its oxidation products. This problem can be solved by introducing a
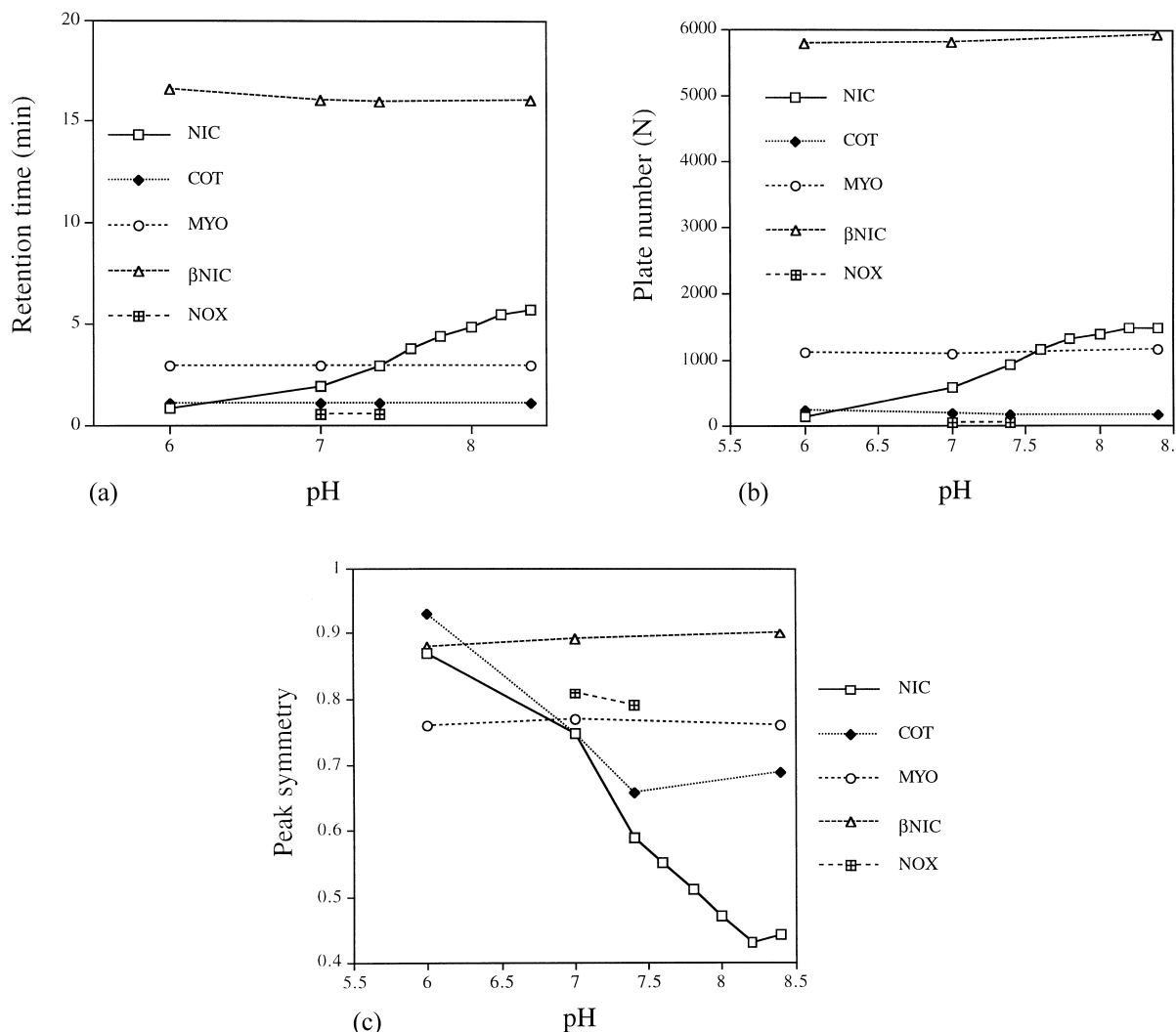

Fig. 5. Influence of mobile phase pH on (a) retention time (t_R), (b) plate number (N) and (c) peak symmetry for the nicotine analytes separated on a reversed-phase column (Purosphere C_{18}e) using as mobile phase: acetonitrile–0.02 M potassium phosphate (20:80, v/v). Injection volume: 10 μl.

neutralization step just after the separation of the phases.

The recoveries of all analytes from the chewing gum standards are high (>90%) except for β-nicotyrine, the most hydrophobic analyte that is extracted with only maximum 87% recovery (Table 1). This is one limitation of this procedure. The excessive recoveries obtained for some analytes and some flavours are attributed to coelution of matrix components in this system. In the following method development an extraction procedure based on phosphoric acid was used (see Section 2.4.1).

3.2. Reversed-phase HPLC method development

This work aimed at finding a method that would be superior to the presently used ion-pair HPLC method in terms of robustness and speed of analysis. For the system to be suitable the following requirements had to be fulfilled [1]: number of theoretical plates $N > 3000$, the injection repeatability: <1.0% (R.S.D. of nicotine peak area), variation of slope (nicotine): <2% and variation of retention time: <5% compared to the retention time of the standard.

We used three columns belonging to a new generation of HPLC columns based on high purity silica or aluminium oxide and a dense C_{18} modification. These are claimed to be particularly suited for the separation of *N*-basic compounds and are claimed to be stable at pH values up to 9.5 allowing polar *N*-basic compounds to be retained in their neutral form [5]. We chose Phenomenex Prodigy ODS 3 due to its superior performance among the tested columns. The initial mobile phase optimisations were done starting with acetonitrile–potassium phosphate systems. First, we observed that direct injection of the extract without pH adjustment was not possible. Although most analytes eluted as sharp peaks, myosmine eluted as two peaks due to ring opening. After neutralisation, sharp peaks of all analytes were obtained using a mobile phase consisting of acetonitrile–0.02 *M* potassium phosphate, pH 7.8 (20:80, v/v) (Fig. 4).

3.2.1. Mobile phase pH

Using this composition, a pH optimisation was carried out (Fig. 5). Due to the problems associated with myosmine at lower pH values (ring opening) only pH values above 6 were initially studied. As expected from the pK_a values of the analytes, only nicotine responded to pH changes in the interval 6–8.5. In this interval, nicotine loses its charge and the retention time increases with pH and stabilizes above 8. To increase robustness in an isocratic method, a pH above 8 or below 6 should therefore be chosen. The plate number follows closely the increase in retention time but is still below the required plate number. The peak asymmetry increases with pH as expected based on the increasing amount of ionized silanol groups.

3.2.2. Acetonitrile or methanol

As seen in Figs. 5 and 6, acetonitrile is a stronger eluent than methanol i.e., for the same organic solvent content the retention time using methanol is higher for all analytes in agreement with the known elution strength. At 50% organic solvent content the retention is insufficient when using acetonitrile as solvent and all analytes eluted under 3 min. When using methanol, all analytes were resolved except for the nicotine-*N*-oxides whereas at lower methanol contents, *cis*- and *trans*-nicotine-*N*-oxide were resolved.
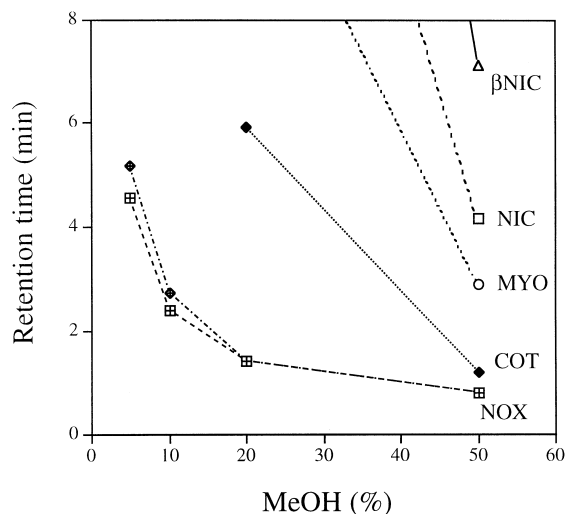

Fig. 6. Influence of organic modifyer on the capacity factor at a mobile phase pH of 7.8. Other conditions, see Fig. 5.

3.2.3. β-Cyclodextrin

Addition of β-cyclodextrin to the mobile phase was attempted in order to selectively reduce the retention of the more hydrophobic analytes such as β-nicotyrine. This should be feasible in view of the elution order of nicotine alkaloids on a cyclodextrin bonded phase [6]. β-Nicotyrine will thus be preferentially complexed in the mobile phase which reduces its interaction with the stationary phase leading to reduced retention times. As seen in Fig. 7, the expected trend was observed, although even a relatively high concentration of β-cyclodextrin in the mobile phase did not reduce the retention time of β-nicotyrine to an extent that would satisfy the method requirements. It is clear that one isocratic system capable of resolving all analytes within 10 min is not possible using reversed-phase HPLC.

3.2.4. Peak identity and purity

As seen in Fig. 8, nicotine, cotinine and the nicotine-*N*-oxides exhibit similar UV spectra introducing an uncertainty in the identification of these peaks on the basis of UV spectral comparisons only. In the case of poorly resolved peaks, the reliability of the peak purity calculations may also be reduced. Myosmine and β-nicotyrine, on the other hand, show characteristic spectra different from the former group which makes identification more reliable.

3.3. Isocratic systems

One possible alternative would be to set up two isocratic systems. This possibility was investigated by developing two isocratic systems: system 1 using low methanol content at pH 6 and system 2 using
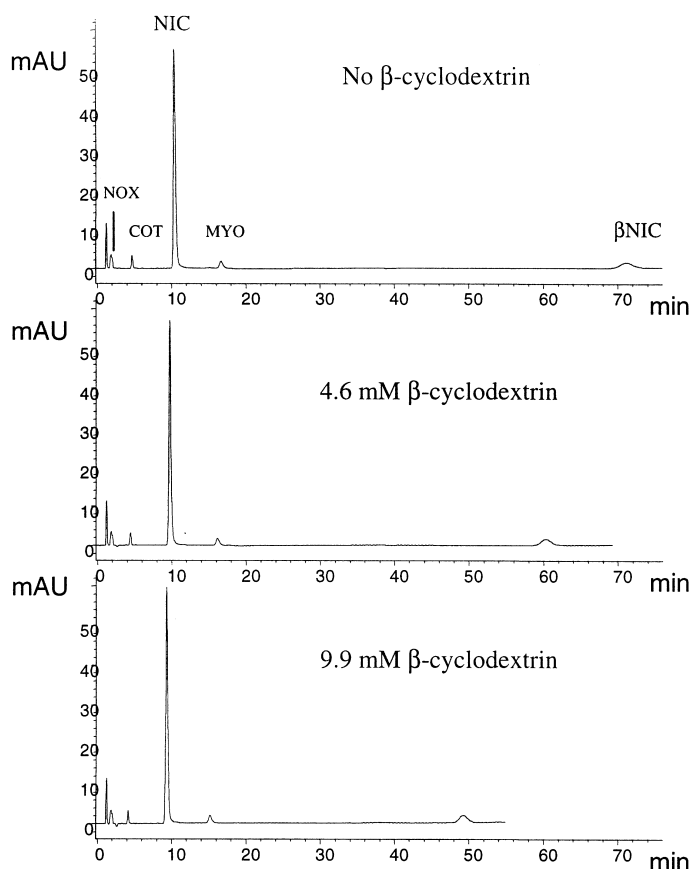

Fig. 7. Influence of the concentration of β-cyclodextrin in the mobile phase on the retention of the nicotine analytes on a Prodigy ODS 3 (150×4.6 mm) reversed-phase column. The mobile phase was methanol–0.02 *M* potassium phosphate, pH 6.8 (25:75, v/v) and the flow-rate 0.9 ml/min.

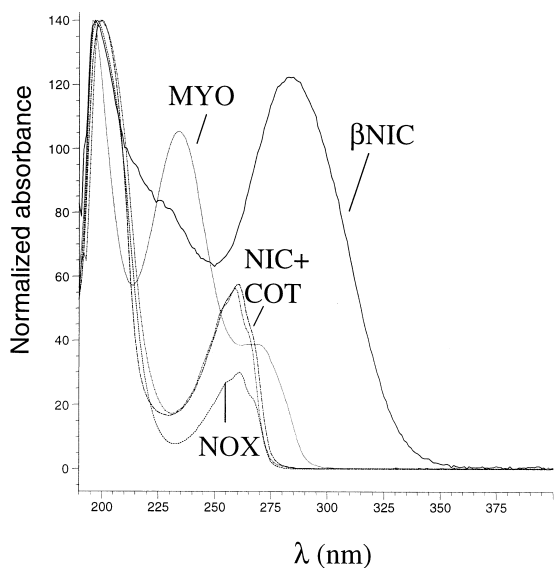Fig. 8. Spectra of the analytes from the chromatogram in Fig. 4.
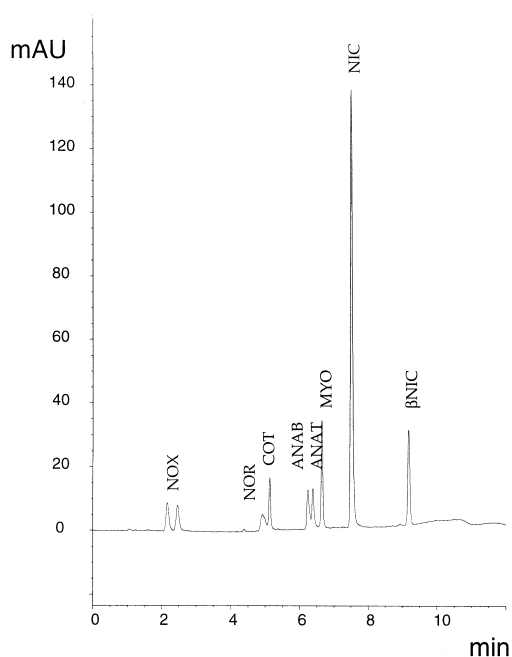
acetonitrile at pH 7.8. Injection of the neutralised extracts from a doped and undoped placebo chewing gum using these systems, allowed *cis-* and *trans-*nicotine-*N*-oxide and nicotine to be determined in System 1 and the remaining analytes in system 2. In both cases no interfering peaks were observed.

3.4. Gradient methods

With a binary gradient, all analytes can be resolved as sharp peaks within 10 min (Fig. 9). Systems with acetonitrile and methanol as solvent B has been validated using a Phenomenex Prodigy ODS 3 column. Generally it can be said that in both cases, good stability in the system suitability test was obtained. The retention time repeatability (averaged over 1000 runs) and the limits of detection (LODs) for the nine analytes are given in Table 2. When injecting the chewing gum extracts, no matrix peaks were seen to interfere with the analytes (Fig. 10). Furthermore, using the gradient has the advantage of repeatedly regenerating the column with high organic content thus washing out eventual retained non-polar

Fig. 9. Gradient elution profile of the nicotine analytes using method 1 described in Section 2.4.2. Mobile phase A: 50 ml 0.4 *M* K$_2$HPO$_4$, 800 ml water, 150 ml methanol. The complete phase was adjusted to pH$_{app}$ 8.5 using phosphoric acid. Mobile phase B: 50 ml water, 950 ml acetonitrile. Gradient: 0–1 min: 0% B, 1–8.5 min: 0–50% B, 8.5–9.5 min: 50% B, 9.5–10 min: 50–0% B, 10–15 min: 0% B, flow-rate: 1.2 ml/min, injection volume: 20 µl; detection wavelength: 254 nm; run-time: 12 min; delay time: 3 min; temperature: room temperature.
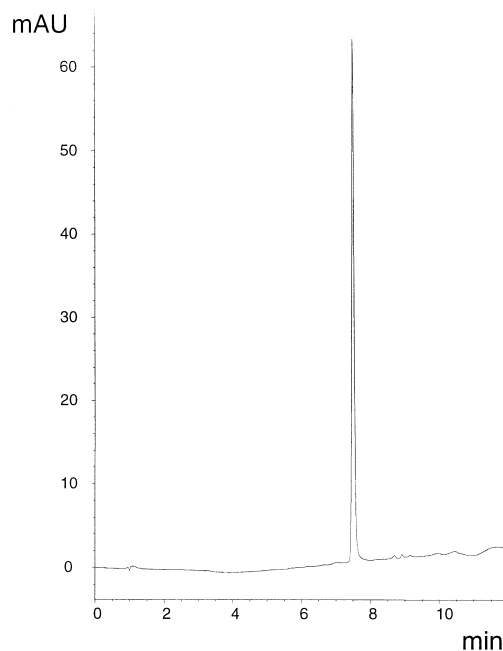

Fig. 10. Injection of a chewing gum extract prepared as described in Section 2.3. The recovery of nicotine was calculated based on the labelled amount of nicotine specified by the producers. Triplicate injections of extracts of three gums thus gave an average recovery of 101.8 (±1.8)%.

Table 2
Retention times (t_R) and the limits of detection (LODs) for the nicotine-related analytes using the gradient methods

Analyte	Method 1			Method 2		Method 3		
	t_R (min) ($n=1000$)	R.S.D. (%)	LOD (μg/ml)	t_R (min) ($n=17$)	R.S.D. (%)	t_R (min) ($n=36$)	R.S.D. (%)	LOD (μg/ml)
cis-Nicotine-*N*-oxide	2.167	1.4	0.12	2.395	0.4	2.308	0.9	0.18
trans-Nicotine-*N*-oxide	2.462	1.5	0.09	2.740	0.4	2.640	0.8	0.18
Nornicotine	4.897	1.6	0.18	5.588	0.2			
Cotinine	5.169	1.1	0.17	6.230	0.2	4.736	0.3	0.26
Anabasine	6.238	1.1	–	7.146	0.2			
Anatabine	6.390	0.2	0.09	7.450	0.1			
Myosmine	6.690	0.8	0.08	7.793	0.2	6.576	0.2	0.13
Nicotine	7.534	0.6	0.77	8.852	0.1	8.249	0.2	0.58
β-Nicotyrine	9.213	0.5	0.08	10.400	0.1	10.355	0.3	0.07

Linear calibration curves were achieved for nicotine (LOD – 50 mg/ml, $n=10$) and for the decomposition products (LOD – 5 mg/ml, $n=10$), respectively.

matrix components. This leads to increased column lifetime. The column dimension was reduced at the expense of resolution but with a significantly reduced solvent consumption (0.6 ml/min) (method 2). The methanol system (method 3) is associated with slightly longer retention times compared to the acetonitrile system leading to longer run times but with the benefit of a less toxic solvent. The high pH was chosen in order to increase method robustness.

4. Conclusions

In summary, a method comprising a liquid–liquid extraction step followed by direct injection and analysis by gradient elution in a reversed-phase HPLC system allowed nine nicotine related analytes to be determined within 15 min. All steps can be automated. This together with its high robustness, sensitivity and accuracy should make this method suitable for routine product analysis.

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