Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 1166–1171

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Validation of a GC–FID method for rapid quantification of nicotine in fermented extracts prepared from *Nicotiana tabacum* fresh leaves and studies of nicotine metabolites

Agnès Millet[a], Florian Stintzing[b], Irmgard Merfort[a,*]

[a] *Institute for Pharmaceutical Sciences, Department of Pharmaceutical Biology and Biotechnology, Albert-Ludwigs-University Freiburg, Stefan-Meier-Str. 19, 79104 Freiburg, Germany*
[b] *WALA Heilmittel GmbH, 73087 Bad Boll/Eckwälden, Germany*

ARTICLE INFO

Article history:
Received 26 January 2009
Received in revised form 19 February 2009
Accepted 20 February 2009
Available online 9 March 2009

Keywords:
Nicotiana tabacum
GC–FID
Nicotine validated quantification
Amine-deactivated column
GC–MS
Nicotine metabolites

ABSTRACT

A new GC–FID method, which allows rapid and reliable quantitation of nicotine in tobacco leaf extracts, was developed and validated. To avoid nicotine adsorption on the column, an amine-deactivated capillary column was used. The method developed was applied to study the degradation of nicotine in a fermented aqueous extract, and a loss of nearly 20% of nicotine over 12 months was observed. Careful inspection of GC–MS runs from concentrated samples of the same extract revealed the presence of nicotine metabolites such as nornicotine, anatabine, myosmine, 2,3′-bipyridyl, and 2-pyrrolidinone.

1. Introduction

Nicotine is the main alkaloid in tobacco leaves (*Nicotiana tabacum* L., Solanaceae), the latter being best known for their use in cigarettes rather than for their therapeutical applications. Nevertheless, preparations from *N. tabacum* are used alone or in combination with other compounds in homeopathy and in anthroposophic medicine to treat symptoms such as spasms, cramps and asthma [1,2].

Several chromatographic methods have been developed for the quantification of tobacco alkaloids but most of them are time-consuming, limited in use or require expensive equipment: RP-HPLC methods are restricted in their use because the basic properties of the alkaloids require pH-ranges that common RP-columns cannot fulfill. In addition, the respective mobile phases need to be finely adjusted to avoid peak tailing [3]. Hence, most quantification methods described in literature so far are based on gas chromatography combined with mass spectrometry or nitrogen–phosphorus detection. Nicotine has been mostly analyzed in cigarettes [4,5], urine [6], human oral fluid [7], plasma [8] or hair [9], but only a few reports on nicotine quantification in tobacco leaves are available [4,10,11]. In gas chromatography, different columns such as fused silica columns coated with different types of methylpolysiloxane [4,6,7,9,10], a 14%-cyanopropyl-phenyl-methylpolysiloxane column [5,11] or a megabore capillary column [4] have been applied for nicotine studies. Only once the use of an amine-deactivated capillary column was reported [8].

The aim of the present work was to develop and validate a simple and rapid quantification method for nicotine in *N. tabacum* preparations. In contrast to literature reports, GC–MS combined with a classic methylpolysiloxane capillary column was only successful for qualitative analysis. Quantification turned out to be impossible, as the nicotine content data showed a large variability when the same extract sample was studied over a period of time. However, this GC–MS method allowed determination of nicotine and its derivatives which may be formed during processing and storage. Quantification was possible through development of a validated GC–FID method with an amine-deactivated capillary column using anabasine as internal standard.

2. Experimental

2.1. Chemicals

Nicotine was purchased from Roth (Karlsruhe, Germany, >98%, bidest), (±)-nornicotine from Fluka (Seelze, Germany, 98%, GC),

* Corresponding author. Tel.: +49 761 203 8373; fax: +49 761 203 8383.
E-mail address: irmgard.merfort@pharmazie.uni-freiburg.de (I. Merfort).

0731-7085/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2009.02.024

DL-anabasine from Extrasynthèse (Genay, France, ≥98%, determined by GC–MS), 2,6-di-*tert*-butyl-4-methylphenol (BHT) as well as 2-pyrrolidinone from Sigma (Seelze, Germany, both >99%, GC). Methanol p.a. was obtained from Roth (Karlsruhe, Germany).

2.2. Tobacco extracts

Several samples of a fermented aqueous *N. tabacum* extract were obtained from WALA GmbH (Bad Boll/Eckwälden, Germany). The extract was prepared according to an official specification [12] using fresh tobacco leaves, water, lactose and honey as carbon sources in a ratio of 100:75:0.75:0.75 (w/w/w/w). Making use of the natural microbial flora of the plant, spontaneous fermentation of this blend was performed over 3.5 days at room temperature before separation of the tobacco leaves from the extract. The resulting turbid filtrate was again fermented for 3.5 days at room temperature and before further storage for 12 months at 15 °C. Aliquots were taken after 24 h (I), 3.5 days (II) and 7 days (III) of extraction as well as after 2 (IV) and 12 months (V) of storage at 15 °C in the dark. These samples were immediately frozen at −20 °C until analyses. For quantification 30 μl of the fermented aqueous extract allowed to reach room temperature and 10 μg anabasine were combined, shortly vortexted and diluted with methanol up to a final volume of 1 ml. This solution was prepared just before analysis.

2.3. GC conditions

2.3.1. GC–MS for identity and purity analyses

GC analyses were carried out with a Hewlett-Packard 6890 series GC–system (Wilmington, USA) equipped with an Agilent 5973 Network Mass Selective Detector (Agilent Technologies, Palo Alto, USA) with electron ionization (ionization energy of 70 eV) and an autosampler. A fused silica capillary column (25 m × 0.25 mm i.d.) coated with 0.25 μm dimethylsiloxane (RTX-1 MS, Restek, Bad Homburg, Germany) and helium 5.0 as carrier gas at a flow rate of 1.0 ml/min were used. Two temperature profiles were applied. The first one, T-I [13] started at 120 °C for 2 min followed by a rate of 10 °C/min to 270 °C, which was held for 20 min. The second one, T-II (modified after [8], developed for the search of metabolites) started at 60 °C, followed by a rate of 10 °C/min to 120 °C, which was held for 10 min. The injector and detector temperatures were set to 290 °C, the injection volume was 1.0 μl (20 μl of the fermented aqueous extract was diluted to a final volume of 1.0 ml MeOH) and the split was 10:1. Mass spectra were recorded over the *m*/*z*-range from 40 to 400. A SIM mode was used for the detection of anabasine at *m*/*z* 84, for nornicotine at *m*/*z* 119, for nicotine at *m*/*z* 84 and 119 and for anatabine at *m*/*z* 160, respectively. For first experiments to quantify nicotine, a SIM mode was used at *m*/*z* 133.

2.3.2. GC–FID for quantitative analyses

Gas chromatography analyses were carried out with a Hewlett-Packard 6890 series instrument (Wilmington, USA) equipped with a flame-ionization detector (FID). An amine-deactivated capillary column (30 m × 0.25 mm i.d. × 0.50 μm d.f.) was used (RTX-35 Amine, Restek, Bad Homburg, Germany). The carrier gas was nitrogen at a constant flow of 1.0 ml/min. The temperature profile T-II (see GC–MS) was used. The injector and detector temperatures were set at 290 °C, the injection volume was 1.0 μl (by manual injection) and the split was 50:1.

2.4. Validation

The quantification method was validated according to the FDA [14] and ICH (International Conference on Harmonization) guidelines [15,16]. The guidelines were compared according to [17]. The validation included tests on specificity, linearity, precision, accuracy and robustness.

2.4.1. Linearity

Stock solutions of 0.1 mg/ml nicotine and anabasine in methanol were separately prepared in 10 ml volumetric flasks. Calibration solutions were made by mixing 100 μl of the anabasine stock solution and 50–600 μl of the nicotine stock solution and finally adding methanol to reach a final volume of 1000 μl. Each concentration was analyzed twice.

2.4.2. Precision

The precision of the system was established by injecting one sample (30 μg/ml nicotine and 10 μg/ml anabasine) six times on the same day (Table 2). The long-term stability of the system was checked by analyzing the fermented aqueous extract (II) stored frozen at −20 °C again after 5 months.

The repeatability and the inter-day intermediate precision were determined by analyzing three samples of different concentrations covering the range of the calibration curve (5, 30 and 60 μg/ml nicotine, prepared from the same stock solutions, and 10 μg/ml anabasine) three times a day at 3 different days (Table 3).

Repeatability and inter-day intermediate precision were also monitored for the fermented aqueous extract (II, see Table 3).

2.4.3. Accuracy of the mean

Accuracy was determined by spiking a sample of the fermented aqueous extract (sample II, mean concentration of nicotine: 7.1 μg/ml) with three concentrations of the reference compound. For this purpose, 50, 300 and 600 μl of a nicotine and 100 μl of an anabasine stock solution were added to 20 μl of the aqueous extract (II). Each sample was then diluted with methanol to reach a final volume of 1000 μl.

2.4.4. Robustness

The freeze and thaw stability of the reference solutions were evaluated by analyzing one sample (30 μg/ml nicotine) three times a day on 3 different days (Table 5). This sample was kept at −20 °C and was unfrozen before each series of injection.

2.5. Nicotine quantification in the fermented aqueous extract

All aliquots of the fermented aqueous extract (solutions I–V) were analyzed in triplicate.

2.6. Detection of nicotine metabolites in the fermented aqueous extract

For the GC–MS analyses of compounds occurring in traces, 1 ml of each original sample was freeze-dried, 1 ml MeOH was added, mixed for 10 min in an ultrasonic bath and centrifuged before the supernatant was injected in the GC–MS system (temperature program T-II).

3. Results and discussion

3.1. Method development

Using the GC–MS method (temperature program T-I) with a fused silica capillary column (coated with dimethylsiloxane) and BHT (=2,6-di-*tert*-butyl-4-methylphenol) [9] as internal standard to quantify nicotine (SIM mode, with *m*/*z* 133 for nicotine and *m*/*z* 220 for BHT, calibration data not shown), did not allow adequate quantification. Several measurements on the aliquot II from the fermented aqueous extract, carried out within 2 months yielded different results with a standard deviation of up to 69% (Fig. 1).

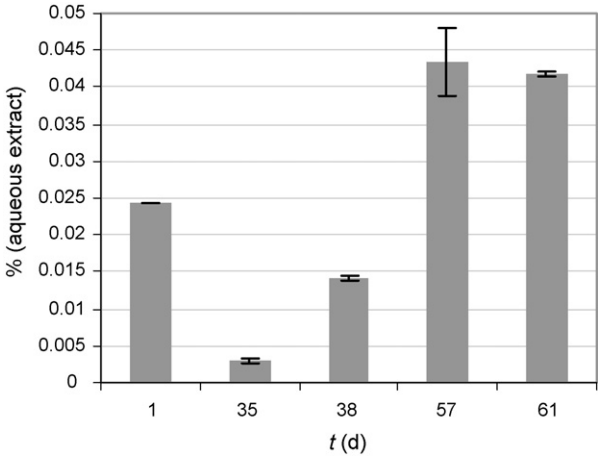

Fig. 1. Determination of the nicotine content (%) in a sample of fermented aqueous extract (aliquot II: sample after 3.5 days extraction) over 2 months, using the GC–MS method (temperature program T-I) with a fused silica capillary column (coated with dimethylsiloxane) and BHT as internal standard. Samples were prepared just before each determination and injected twice.

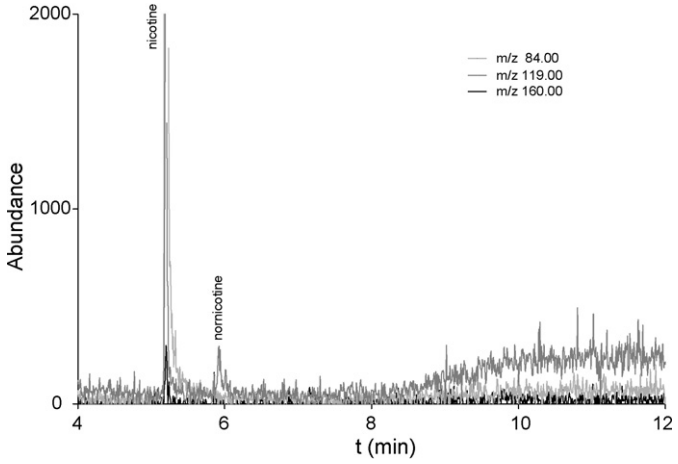

Fig. 2. Ion chromatograms obtained from the fermented aqueous extract from tobacco leaves (aliquot II: sample after 3.5 days of the extraction) after GC–MS analysis using the ions at *m*/*z* 84, 119 and 160 (temperature program T-I).

The variation of the calculated nicotine concentration was too high, failing in the determination of a true nicotine content.

After some injection series of nicotine standard solutions during several months (data not shown), two important facts were noticed: first, nicotine appeared to be partly adsorbed on the column, making the detected nicotine content dependent on the adsorbed nicotine on column. This can apparently often be observed by alkaloids on non-amine-deactivated capillary columns (pers. communication, Agilent Company). Second, the physical and chemical properties of BHT were too different from those of nicotine, failing to make a satisfactory correction. Another possible option using labeled nicotine as described elsewhere [5,7] was not considered for cost reasons. Finally, the alkaloid anabasine was taken as internal standard, as it could only be detected in small amounts by GC–MS analysis in a methanolic extract (data not shown), and in traces in a fermented aqueous extract from *N. tabacum* leaves (Fig. 2).

Therefore, a new GC–FID method was established and validated, using an amine-deactivated capillary column with anabasine as internal standard. This method yielded specific and robust nicotine contents.

3.2. Validation procedure

3.2.1. Specificity

The specificity of the developed method was shown by spiking the fermented aqueous tobacco leaf extract with reference substances (nicotine, anabasine and nornicotine, see Fig. 3). Only nicotine was detected in the tobacco leaf extract, but no other alkaloids known from tobacco leaves. The peak purity of nicotine was checked by GC–MS analyses, comparing the mass spectrum of the

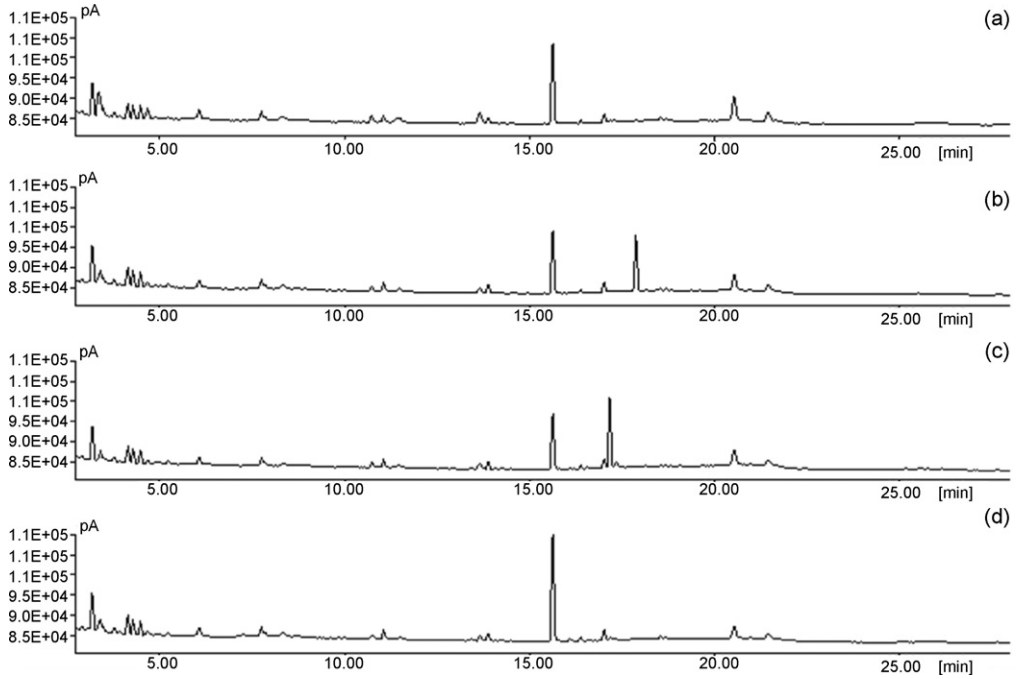

Fig. 3. GC–FID-chromatogram from the fermented aqueous extract prepared from tobacco leaves (aliquot II: sample after 3.5 days of the extraction; temperature program T-II): 25 μl fermented aqueous extract per ml methanol (a); extract 20 μl/ml spiked with 5 μg anabasine (b); 20 μl/ml spiked with 5 μg nornicotine (c); 20 μl/ml spiked with 5 μg nicotine (d).

Table 1
Overview of the linearity data for nicotine.

Slope ± standard error	4.842 ± 0.037
Slope: confidence interval (95%)	4.763–4.920
Intercept ± standard error	0.4 ± 1.3
Intercept: confidence interval (95%)	−2.261–3.123
Correlation coefficient, r^2	0.9992
Range (C_N in μg/ml)	5–60
Number of standards	8
Number of replicates	2

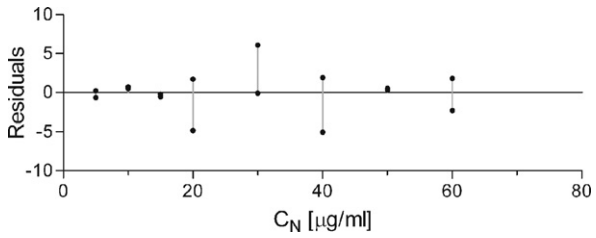

Fig. 4. Residual plot of the nicotine reference solutions at eight different concentrations injected in duplicate. The residuals are randomly scattered, justifying the linear model.

nicotine peak in the fermented aqueous extract with that obtained from the reference (data not shown).

GC–MS studies were carried out with the fermented aqueous extract from *N. tabacum* (sample after 3.5 days, aliquot II) and ion chromatograms were taken using the selected signals at *m/z* 84, 119 and 160 as characteristic fragment ions for the main tobacco alkaloids (Fig. 2). These analyses were in agreement with the GC–FID studies, as again only nicotine besides very small amounts of nornicotine were detected in the fermented aqueous extract of tobacco leaves.

3.2.2. Limit of quantification (LOQ)

The limit of quantification for nicotine was determined to be 5 μg/ml. The signal/noise ratio was 60:1 (ICH requires a value >10:1) and the maximum recovery (see Table 3) was 117.8% (FDA requires a recovery <120%). 5 μg/ml was therefore set as the lowest concentration for the calibration.

3.2.3. Linearity

The calibration was performed by analyzing nicotine reference solutions at eight concentration levels (5–60 μg/ml) in duplicate. A linear response from the corrected area to the concentration was obtained. The calibration curve was tested on slope and intercept (Table 1). The residuals were graphically examined (Fig. 4).

3.2.4. Precision

The system precision was investigated by injecting six times the same standard solution (30 μg/ml nicotine) in the GC–FID. The standard deviation was <5% and the recovery in the range of 100 ± 5%. Results are shown in Table 2.

The long-term stability of the system was checked by comparing the concentration obtained for the fermented aqueous extract on

Table 2
Validation data: system precision for a 30 μg/ml nicotine solution.

Parameter	Nicotine
Number of replicates	6
Mean concentration (μg/ml)	29.09
R.S.D. (%)	3.19
Recovery (%)	96.42

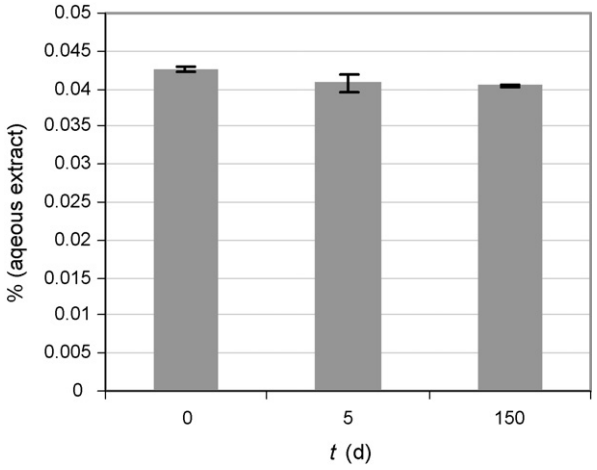

Fig. 5. Determination of the nicotine content (%) in the fermented aqueous extract over 5 months by GC–FID (temperature program T-II). Samples were prepared just before each analysis and injected three times.

different days, at first on day 0, then after 5 days and finally after 5 months. Each analysis was done in triplicate. The results (Fig. 5) showed a small deviation over the 5-month period (3.8% standard deviation).

Repeatability and intermediate precision were monitored by analyzing three standard solutions with different concentrations on 3 days (Table 3). The standard deviations were <5% for the higher concentrations (30 and 60 μg/ml) and <10% for the LOQ-value (5 μg/ml).

3.2.5. Accuracy

The accuracy of the mean was at first taken from the repeatability data at different concentrations. The percent recovery was calculated in each case (Table 3). For the lowest concentration, the maximum recovery rate was 117.8%, which is still in the required range for a LOQ-value according to the FDA. The recovery values for the higher concentrations were all in the range of 100 ± 5%. Some recovery experiments were also done by spiking a fermented aqueous extract with different concentrations of the standard. The results, shown in Table 4, were all in the range of 100 ± 5%.

3.2.6. Robustness

The robustness was studied by means of a "freeze and thaw" stability test. Even though the standard deviation remained below 5% in the "within" as well as in the "inter-day" precision, the recovery

Table 3
Validation data: repeatability and intermediate precision for nicotine in standard solutions with different concentrations and in a fermented aqueous extract.

Concentration of nicotine (true value) (μg/ml)	Mean conc. exp. determined (μg/ml)	R.S.D. (%) of repeatability (within-day precision)	R.S.D. (%) of intermediate precision (3 days)	Recovery (%)
5	5.6	0.55–4.01	6.67	103.71–117.81
30	29.7	0.96–4.20	1.35	98.17–100.53
60	60.9	0.27–1.02	1.89	99.88–103.56
Aqueous extract	7.1	1.11–1.97	0.70	–

Table 4
Validation data: repeatability and recovery by spiking a fermented aqueous extract with different nicotine concentrations.

Concentration spiked (μg/ml)	Mean conc. (exp. determined) (μg/ml)	R.S.D. (%) of repeatability (three replicates)	Recovery (%)
5	11.8	0.15	96.97
30	36.0	2.47	96.77
60	63.7	0.09	94.84

Table 5
Validation data: robustness—freeze and thaw stability of a reference solution (30 μg/ml nicotine).

Day	Mean conc. (exp. determined) (μg/ml)	R.S.D. (%) of repeatability (within-day precision)	R.S.D. (%) of intermediate precision (3 days)	Recovery (%)
1	28.8	0.24		96.02
2	27.7	2.09	3.59	92.25
3	26.8	0.43		89.40

Table 6
Nicotine content (C in %) in a fermented aqueous tobacco leaf extract after different time periods of extraction (samples I–III) and following storage at 15 °C in the dark (samples IV-V).

Sample	Time (days)	C (%) aqueous extract	R.S.D. (%)	% of initial concentration
I	1	0.043	7.66	100
II	3.5	0.042	3.19	96.0
III	7	0.035	4.72	86.2
IV	60	0.036	4.42	83.6
V	360	0.034	9.70	78.4

decreased to values <90% after 3 days (Table 5). Hence, the reference solutions should not be frozen and thawed more than two times. It is therefore recommended to freeze aliquots at least at −20 °C and use them only once.

3.3. Stability of nicotine content in the fermented aqueous tobacco extracts

Concentrations in percent of the different aliquots of the fermented aqueous extract (batch from 2006) are given in Table 6. After 12 months, only 78% of the initial concentration of nicotine was remaining in the fermented aqueous extract. This decrease of nicotine in a fermented aqueous extract was also observed in a batch from 2007 (results not shown).

3.4. Metabolism of nicotine in fermented aqueous extracts

The degradation of nicotine observed, within 1 week up to 12 months, prompted us to look for metabolites in these samples (Fig. 6).

Nornicotine, which was not detected in the sample after 24 h, appeared after 3.5 days and increased over 12 months. This agrees with the observation that nornicotine can be produced from nicotine by fermentation processes [18] and that this metabolite is not only genuinely biosynthesized by the plant.

In addition, a new peak appeared in the chromatogram at 8.1 min after 3.5 days and increased over 12 months. Based on literature data [19,20] and the specific fragment ions at *m/z* 85, 56, 42, and 28, the peak was identified as 2-pyrrolidinone. Further unambiguous confirmation was achieved by direct comparison with a reference using GC/MS.

Traces of myosmine, known as metabolite of nicotine and nornicotine generated enzymatically or under exclusion of oxygen [18,21], were detected in the extract in the period from 24 h until 2 months. During the same time period, the amount decreased perpetually. This can be explained by its spontaneous reaction in water to form poikiline through ring opening [22] (Fig. 7). However, poikiline was not detected. Therefore, it is likely that it further reacted to 2-pyrrolidinone (Fig. 7).

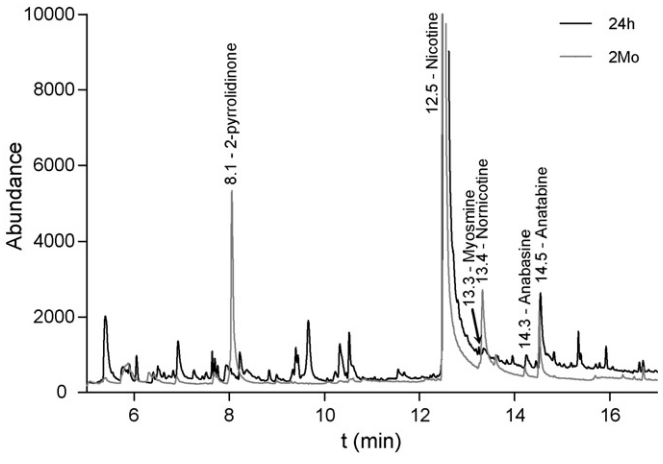

Fig. 6. Superposed chromatograms (SIM mode, *m/z* 84, 119 and 160; temperature program T-II) of the aliquots of the fermented aqueous extract after 24 h and 2 months (occurrence of myosmine in traces after 24 h).

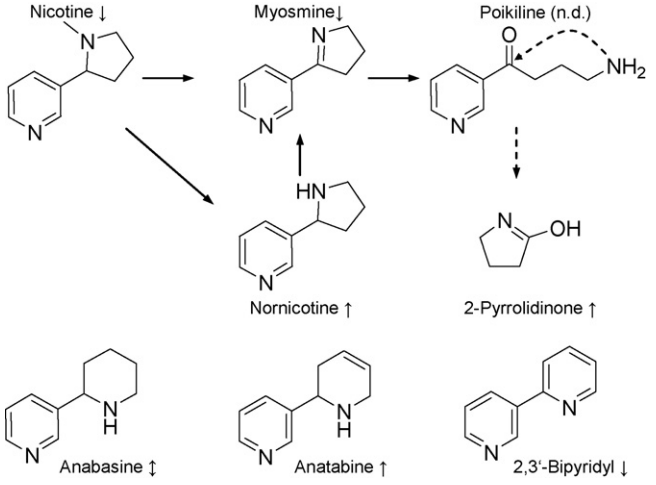

Fig. 7. Possible metabolism of nicotine in a fermented aqueous extract (arrows indicate either an increase or decrease).

2,3′-Bipyridyl could be detected in the first samples up to 2 months by its specific m/z value of 156. Afterwards, the amount of this compound decreased (data not shown). The anatabine peak showed a slight increase. The small peak of anabasine remained within the same range. This again justifies the use of anabasine as reference for nicotine quantification.

Other reported nicotine metabolites like nicotinic acid, cotinine, nicotine oxides, nitrosamines, N-formylnornicotine and derivatives [18,21,23–25] could not be detected in the fermented aqueous extract samples. These results were all verified with another batch of fermented aqueous extract (batch 2007).

4. Conclusion

Nicotine and its derivatives have mostly been studied in dried and fermented tobacco leaves or in cigarette smoke [4,5,10,11]. In the present paper, a new GC–FID method has been developed and validated to quantify nicotine in fermented aqueous extracts from tobacco leaves. The use of an amine-deactivated capillary column avoids adsorption of nicotine on the column and guarantees reproducible results after several GC runs. This affordable method allows to obtain reliable results in a short time and may be used both for quantitative analysis in fresh or dried tobacco material as well as for stability studies and is thus suitable for quality control of nicotine-containing preparations.

The application of the newly established method to a fermented aqueous tobacco leaf extract showed that the content of nicotine decreases in this extract to 78% over 12 months and primarily resulted in the accumulation of nornicotine, the *N*′-demethylation product. This nicotine metabolite was also found earlier in *Nicotiana* cell suspension cultures and an oxidative elimination of the N′-methyl group was suggested [18]. A similar metabolism may occur during fermentation of tobacco leaves in aqueous solutions. Interestingly, myosmine, discussed as the main degradation product in an aqueous solution when exposed to air and light [23], was only detected in traces, but 2-pyrrolidinone occurred in detectable amounts. This may be explained by the fact that myosmine is rapidly transformed into 2-pyrrolidinone under the prevailing fermentation and storage conditions.

Acknowledgment

We are grateful to WALA GmbH (Germany) for financial support.

References

[1] WALA Drug Directory, 29th ed., WALA Heilmittel GmbH, Bad Boll/Eckwälden, 2007, p.121.
[2] Vademecum Anthroposophische Arzneimittel, Der Merkurstab/J. Anthroposophic Med. 61 (Suppl.) (2008) 237–238.
[3] L.A. Ciolino, J.A. Turner, H.A. McCauley, A.W. Smallwood, T.Y. Yi, J. Chromatogr. A 852 (1999) 451–463.
[4] J.B. Cai, B.Z. Liu, P. Lin, Q.D. Su, J. Chromatogr. A 1017 (2003) 187–193.
[5] W. Wu, D.L. Ashley, C.H. Watson, Anal. Chem. 74 (2002) 4878–4884.
[6] C.N. Man, L.H. Gam, S. Ismail, R. Lajis, R. Awang, J. Chromatogr. B 844 (2006) 322–327.
[7] I. Kim, W.D. Darwin, M.A. Huestis, J. Chromatogr. B 814 (2005) 233–240.
[8] R.A. Davis, J. Chromatogr. Sci. 24 (1986) 134–141.
[9] K. Zahlsen, O.G. Nilsen, Pharmacol. Toxicol. 75 (1994) 143–149.
[10] F. Saitoh, M. Noma, N. Kawashima, Phytochemistry 24 (1985) 477–480.
[11] S.S. Yang, I. Smetena, C.B. Huang, Anal. Bioanal. Chem. 373 (2002) 839–843.
[12] Homöopathisches Arzneibuch, Spezielle Herstellungsvorschriften, Vorschrift 33c (2003) H5.4 3.
[13] X. Wei, S.P. Sumithran, A.G. Deaciuc, H.R. Burton, L.P. Bush, L.P. Dwoskin, P.A. Crooks, Life Sci. 78 (2005) 495–505.
[14] US Food and Drug Administration Guidance for Industry: Bioanalytical Method Validation 2001, http://www.fda.gov/cder/guidance/4252fnl.pdf (accessed 9/2007).
[15] ICH-Q2A International Conference of Harmonization (ICH): Text on Validation of Analytical Procedures 1995, http://www.fda.gov/cder/guidance/ichq2a.pdf (accessed 9/2007).
[16] ICH-Q2B International Conference of Harmonization (ICH): Validation of analytical procedures: methodology 1996, http://www.fda.gov/cder/guidance/1320fnl.pdf (accessed 9/2007).
[17] S. Chandran, R.S.P. Singh, Pharmazie 62 (2007) 4–14.
[18] T. Bartholomeusz, R.K. Bhogal, R. Molinie, F.X. Felpin, M. Mathe-Allainmat, A.C. Meier, B. Drager, J. Lebreton, A. Roscher, R.J. Richard, F. Mesnard, Phytochemistry 66 (2005) 2432–2440.
[19] A.E.M. Crotti, T. Fonseca, H. Hong, J. Staunton, S.E. Galembeck, N.P. Lopes, P.J. Gates, Int. J. Mass Spectrom. 232 (2004) 271–276.
[20] T. Pathak, N.F. Thomas, M. Akhtar, D. Gani, Tetrahedron 46 (1990) 1733–1744.
[21] E. Wada, T. Kisaki, K. Saito, Arch. Biochem. Biophys. 79 (1959) 124–130.
[22] P.G. Haines, A. Eisner, C.F. Woodward, J. Am. Chem. Soc. 67 (1945) 1258–1262.
[23] T.J. Smyth, V.N. Ramachandran, A. McGuigan, J. Hopps, W.F. Smyth, Rapid Commun. Mass. Spectrom. 21 (2007) 557–566.
[24] I. Wahlberg, K. Karlsson, D.J. Austin, N. Junker, J. Roeraade, C.R. Enzell, W.H. Johnson, Phytochemistry 16 (1977) 1233–1235.
[25] J. Zhou, R. Bai, Y. Zhu, Rapid Commun. Mass. Spectrom. 21 (2007) 4086–4092.