Analysis of selected alkaloids and sugars in tobacco extract

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Abstract

A new approach for the determination of selected alkaloids (nicotine and nornicotine) and sugars (fructose, glucose, sucrose, xylose and maltose) in tobacco is described. The analytical method is based on the simultaneous extraction of alkaloids and sugars from tobacco samples with an acetic acid–methanol solution, followed by parallel isolation of target analytes from extract aliquots by solid-phase extraction, using octadecyl silica (C_{18}) for alkaloids and aminopropyl silica (NH_2) for sugars. The extracts were analysed by liquid chromatography using UV absorbance detection for alkaloids and refractive index detection for sugars. The recoveries from spiked extracts were high and reproducible, ranging from 94 to 97% for alkaloids, and from 62 to 97% for sugars, at concentration levels that were as expected following extraction from average quality tobacco material. The method was tested in the analysis of a Virginia tobacco sample. The results obtained for individual compounds were in good agreement with those obtained by spectroscopic measurements of total alkaloids and total sugars in the same tobacco sample.   © 1997 Elsevier Science B.V.

Keywords: Tobacco; Nicotine; Nornicotine; Fructose; Glucose; Sucrose; Xylose; Maltose

1. Introduction

Certain sugars and tobacco alkaloids are inevitable constituents in all types of tobacco material, and both their quantities and proportions strongly affect not only the quality of tobacco as a raw material, but the properties of the final tobacco products as well. Therefore, reliable determination of these compounds in tobacco appears to be essential for the classification of tobacco material according to its quality. Quantitative determination of alkaloids and sugars can be achieved using different analytical methods. Alkaloids can be determined gravimetrically, e.g. nicotine as nicotine–silicotungstate [1] or dipicrate [2], volumetrically using the Cundiff-Markunas method [3], or using the spectrophotometric method developed in CORESTA [4] (Co-operation Centre for Scientific Research Relative to Tobacco, Paris, France). In these methods, total alkaloid content is expressed as nicotine. Determination of tobacco sugars is also carried out by the spectrophotometric method, which allows a distinction to be made between total sugars and the total reducing sugar content [5,6].

Simultaneous extraction of alkaloids and sugars

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from tobacco material has been performed so far only in combination with colorimetric analysis, enabling determination of the contents of the total alkaloids and the total reducing sugars as cumulative parameters [5]. However, the modern technology involved in tobacco processing demands a more detailed insight into tobacco compounds. High resolution chromatographic methods should enable the quantitative determination of individual components in tobacco material to be obtained, instead of just measuring specific compound classes [7–10], provided the target compounds are efficiently extracted from the tobacco sample.

Efficient extraction of alkaloids from a tobacco sample is accomplished only with acidic extracting solutions, which, in turn, cause hydrolysis of higher sugars and sucrose. Harvey at al. [5,11] have found that the aqueous solution used for the extraction of alkaloids, which contained 2% hydrochloric acid and 20% methanol, hydrolysed sugars readily, even at room temperature. If 5% acetic acid was used instead of a stronger mineral acid, hydrolysis of sugars was not observed and extraction was completed after only 5 min of sonication. However, tobacco contains many different organic compounds that are easily co-extracted with target compounds and are likely to interfere in subsequent chromatographic analysis. Therefore, a selective clean-up procedure is necessary, allowing efficient isolation of target compounds from tobacco extract, with removal of possible interferences and preconcentration of analytes in a single step.

As a clean-up step, solid phase extraction (SPE) is chosen, because it is a rapid, reproducible and time- and money-saving method. It allows immediate injection of SPE eluent into the high-performance liquid chromatography (HPLC) system. In the last few years, it has been used in many different analyses, such as analyses of carbohydrates in plant material [10,12,13] and of alkaloids in plant material [14] and biological samples [15–17].

In this work, a new approach to the determination of alkaloids and sugars in tobacco is described. The method is based on the simultaneous extraction of compounds from tobacco material, isolation of analytes from tobacco extracts by SPE, and the HPLC analysis of isolated compounds.

2. Experimental

2.1. Chemicals

Analytical grade nornicotine (Sigma, St. Louis, MO, USA) and nicotine, purity >98% (Merck, Schuchardt, Germany); analytical grade D-(+)-glucose, D-(−)-fructose and sucrose (Merck, Darmstadt, Germany); analytical grade pyridine, D-(+)-xylose, maltose monohydrate and lactose monohydrate (Kemika, Zagreb, Croatia), which were used as reference compounds. Analytical grade diethylamine, phosphoric acid, acetic acid, methanol, ethanol and acetonitrile (Kemika), which were used for extraction, sample clean-up and chromatography.

2.2. Solutions

The solution used for the extraction of tobacco material was prepared by mixing 50 ml of acetic acid with 200 ml of methanol and adding distilled water to a volume of 1000 ml [5]. A solution of 0.08 M diethylaminophosphate buffer was prepared by dissolving 15 ml of diethylamine in 1.74 l of distilled water, followed by adjusting the pH to 7.56 with a few drops of o-phosphoric acid [7].

2.3. Instruments

The HPLC system used was from LDC-Analytical (Riviera Beach, FL, USA) and was equipped with a ConstaMetric 3000 pump, an SM 3000 UV detector and a RefractoMonitor IV refractive index (RI) detector, a 20-μl injector loop (Rheodyne, Cotati, CA, USA) and a column heater. The separation of alkaloids was performed on a ResElut C_{18} column (250×4.6 mm I.D., 5 μm particle size, Varian, Harbor City, CA, USA), using gradient elution with diethylaminophosphate buffer, pH 7.56 (solvent A) and a methanol–acetonitrile (1:1, v/v) mixture (solvent B). The elution was performed at a column temperature of 40°C and with a flow-rate of 0.7 ml min^{-1}, starting with 100% of A for the first 2 min; increasing the percentage of B to 40% over the next 4 min, and then increasing the percentage of B up to 70% in the last 9 min. The compounds were quantified by measuring the UV absorbance at 254 nm

using pyridine as the internal standard. The sugars were separated on a Bondesil NH₂ column (250×4.6 mm I.D., 5 μm particle size, Varian) using an acetonitrile–water mixture (81:19, v/v) at a flow-rate of 1.0 ml min^{-1}. The temperature of the column was maintained at 25°C. The eluted compounds were detected by the RI detector and quantified using lactose as the internal standard.

The compounds were isolated from tobacco extract by SPE using Mega BondElut C$_{18}$ for alkaloids and Mega BondElut NH$_2$ for sugars, both in 1 g/6 ml cartridges, in combination with vacuum manifold VacElut 20 (Varian).

2.4. Procedure

A 10-ml volume of the extracting solution was poured over 500 mg of flue-cured and ground tobacco sample, and extraction was carried out in an ultrasonic bath for 10 min at room temperature. The suspension was filtered under vacuum using a büchner funnel, the tobacco residue was rinsed a few times with 2–3 ml of the extracting solution and the combined filtrate was transferred into a 25-ml volumetric flask. Alkaloids and sugars were analysed separately in 5 ml aliquots of extract.

For the isolation of alkaloids, C$_{18}$ cartridges were activated by passing 3 ml of ethanol and 5 ml of water through them. Seven drops of a 40% NaOH solution were added to 5 ml of tobacco extract, and the alkaline sample (pH~12) was passed through the activated sorbent. The sorbent was washed with 2 ml of water and dried under vacuum. Alkaloids were eluted from the sorbent with 3 ml of methanol, and four drops of 0.5 M acetic acid were added to neutralise the eluate. An internal standard solution was added before chromatographic analysis.

For the determination of sugars, a 5-ml volume of tobacco extract was evaporated under vacuum at 50°C to a volume of 1.5 ml, 7 ml of acetonitrile were added and the sample was passed through the NH$_2$ cartridge, preceded by activation of the sorbent with 3 ml of methanol and 5 ml of acetonitrile–water (4:1, v/v). Sugars were eluted from the cartridge with 3 ml of water, and a solution of lactose was added as the internal standard for chromatographic analysis.

3. Results and discussion

3.1. Determination of alkaloids

Most of the difficulties encountered in the analysis of tobacco alkaloids arise from their acid–base properties. Depending on the acidity of the solution, the nicotine molecule can be present in three different forms, as shown in Fig. 1.

To facilitate the extraction of alkaloids from a tobacco sample, the solution for extraction has to be acidic (e.g. pH 2.7 in this work) in order to convert the basic alkaloid compounds into water-soluble protonated forms. In this form, their isolation by selective SPE from aqueous sample by reversed-phase adsorption on C$_{18}$ is likely to be very poor. By adding a small volume of a sodium hydroxide solution to the extract immediately before SPE, the alkalinity of the sample should be increased, by at least one pH unit above the pK_2 of nicotine. Under these conditions (e.g. pH~12 in this work), most alkaloid molecules are converted into the non-protonated form, which should facilitate their isolation from the tobacco extract before HPLC analysis. However, such a high sample alkalinity is not compatible with chromatographic conditions. To avoid deterioration of the octadecyl silica stationary phase, a mobile phase with pH<8 is strongly recommended. Diethylaminophosphate buffer, pH 7.56, was chosen as the mobile phase in this work. The use of a buffered mobile phase seems to be crucial in the alkaloid analysis on non-polar stationary phases, because on this type of column, basic compounds

$K_1 = [H^+][NicH_2^{2+}]/[NicH^+]$

p$K_1 = 3.12$[18]

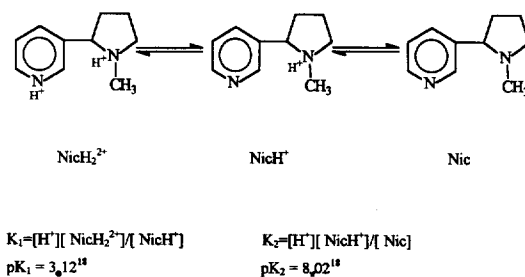$K_2 = [H^+][NicH^+]/[Nic]$

p$K_2 = 8.02$[18]

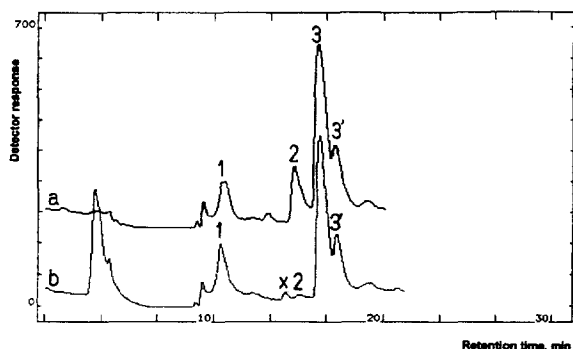Fig. 1. Different forms of the nicotine molecule, depending on the acidity of the solution.

Fig. 2. Chromatogram of (a) alkaloid standard solution contained 0.79 mg ml^{-1} of nicotine, 0.32 mg ml^{-1} of nornicotine and 0.11 mg ml^{-1} of pyridine, and (b) sample solution after SPE of alkaloids from an extract of a Virginia tobacco sample: 1 = Pyridine, internal standard; 2=nornicotine; 3=nicotine, mono-protonated form; 3′=nicotine, non-protonated form; X=unknown, possibly alkaloid, not included in the analysis.

tend to elute as asymmetrical peaks with peak-tailing increasing with the pK_a value of the analyte [8,19].

Fig. 2 shows a typical chromatogram of alkaloid standard solution used for the quantitation of nicotine and nornicotine in tobacco extracts. Two overlapping peaks of nicotine apparently correspond to mono-protonated and non-protonated forms of the molecule (see Fig. 1). Tailing or even splitting of the nicotine peak cannot be avoided since the pK_a value of the pyrrolidine moiety is 8.02 [18]. Thus, for the determination of the nicotine content, the total area of both peaks has to be taken into account. Despite this difficulty, the reliability of the chromatographic analysis of alkaloids was checked using model solutions containing 0.05–1.3 mg ml^{-1} of nicotine and 0.06–1.6 mg ml^{-1} of nornicotine. These concentration ranges were chosen on considering the expected quantities of alkaloids in average quality tobacco material, which would correspond to ~0.8 mg ml^{-1} of nicotine and ~0.06 mg ml^{-1} of nor-

nicotine, after extraction of the real tobacco sample (the nornicotine content may be higher in some tobaccos of poor quality). The detector response was found to be reproducible and linear for both compounds in the tested concentration ranges with high correlation coefficients (0.9988 for nicotine and 0.9974 for nornicotine). Detection limits, using a 4:1 S/N ratio, were estimated at approximately 0.01 mg ml^{-1} for each compound.

The efficacy of C_{18} reversed-phase adsorption for the isolation of alkaloids from tobacco extracts was checked with extracting solutions spiked with 0.6 mg ml^{-1} of nicotine and 0.07 mg ml^{-1} of nornicotine. After passing the alkalised sample through the C_{18} cartridge, no alkaloids were detected in the collected solution, indicating that they were completely trapped on the adsorbent. The cartridge had to be washed with no more than 2 ml of distilled water to remove sodium hydroxide and polar interfering compounds that may have been on the sorbent after passing through the sample. Larger volumes of water were found to reduce the recovery of nornicotine (e.g. 6 ml of water removed ~20% of nornicotine from the cartridge).

In the preliminary SPE experiments, the elution of alkaloids with solvents of different polarity was tested. Nicotine and nornicotine are very soluble in chloroform, which is used in some procedures to extract alkaloids from tobacco [20]. Elution with 3 ml of chloroform permitted the almost complete recovery of nicotine (95±3%), but nornicotine was not detected at all. A much more polar solvent was needed for the elution of nornicotine. The best results were obtained with methanol (Table 1). However, despite rinsing the cartridge with water, the methanolic eluate was still too alkaline and caused the sample to be turbid. Thus, the eluate had to be neutralised with a few drops of 0.5 M acetic acid. Neutralisation seemed to improve the chro-

Table 1
Recovery of alkaloids by C_{18} reversed-phase SPE from spiked extracting solution (0.6 mg ml^{-1} of nicotine and 0.07 mg ml^{-1} of nornicotine) with and without the addition of glucose, fructose, sucrose and xylose at 2 mg ml^{-1} each

	Nicotine recovery, %±SD (n)	Nornicotine recovery, %±SD (n)
Without sugars	97±4 (10)	94±6 (10)
With sugars	95±5 (10)	91±3 (10)

SD=standard deviation.
n=number of samples.

matographic behaviour of the eluted compounds on the C_{18} stationary phase, since the slightly increased recoveries of 98±3% for nicotine and 99±2% for nornicotine were observed.

Possible interferences caused by the presence of sugars during the isolation of alkaloids were checked using a spiked extracting solution containing glucose, fructose, sucrose and xylose (2 mg ml^{-1} each). The high and reproducible recoveries shown in Table 1 indicate that the presence of sugars did not affect the sorption or elution of alkaloids.

3.2. Determination of sugars

A typical chromatogram of a standard sugar mixture is shown in Fig. 3. All peaks, except those of glucose and fructose, are baseline separated, including the maltose–lactose disaccharide pair, which is difficult to separate according to literature data [21]. In order to improve the separation between glucose and fructose, acetonitrile is added both to the sample solution and to the mobile phase. The addition of acetonitrile increases the retention times of sugar on the aminopropyl silica stationary phase, thus improving the separation.

The linearity of response using the RI detector was tested for all investigated sugars in the concentration range from 0.3 to 7.0 mg ml^{-1} of each, based on the anticipated concentrations of compounds to be extracted from tobacco material with an average sugar
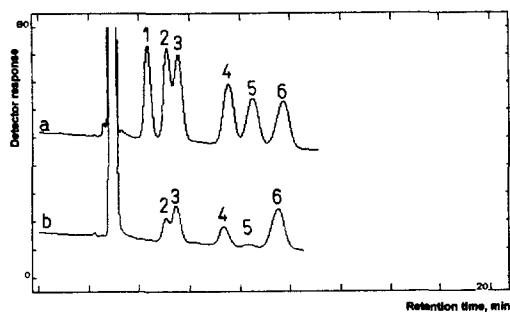

Fig. 3. Chromatogram of (a) a sugar standard solution containing 3.69 mg ml^{-1} of xylose, 4.12 mg ml^{-1} of fructose, 3.92 mg ml^{-1} of glucose, 3.53 mg ml^{-1} of sucrose, 3.50 mg ml^{-1} of maltose and 3.46 mg ml^{-1} of lactose, and (b) a sample solution after SPE of sugars from an extract of a Virginia tobacco sample: 1=xylose; 2=fructose; 3=glucose; 4=sucrose; 5=maltose; 6=lactose, internal standard.

content. The detector response was found to be reproducible and linear for all compounds with high correlation coefficients (from 0.9990 for glucose and maltose to 0.9995 for sucrose), and the detection limits were estimated to be 0.1 mg ml^{-1} for each compound at an S/N ratio of 4:1.

All attempts to isolate both alkaloids and sugars using a consecutive SPE procedure with the same sample solution, by passing the sample through a non-polar C_{18} sorbent (to retain alkaloids) and then through the polar NH_2 sorbent (to retain sugars) did not work out. The strong alkaline conditions needed for isolation of alkaloids disabled the adsorption of sugars on the polar NH_2 sorbent. No suitable neutralisation procedure was found, which would have enabled the efficient isolation of sugars without introducing interferences in later chromatographic analysis. Therefore, isolation of sugars had to be carried out on a separate aliquot of tobacco extract.

Since sugars are very polar compounds, their adsorption is likely to be efficient only on an adsorbent of similar polarity, such as aminopropyl silica, provided the sample solution is less polar than the sorbent. However, the solubility of sugars in an aqueous extract solution after extraction of tobacco is too high for efficient trapping on the NH_2 sorbent, and the sample solution has to be made less polar. Analogously to the chromatographic conditions applied later to the sugar mixture on the aminopropyl silica stationary phase, where separation is achieved by adding acetonitrile to the aqueous mobile phase, the adsorption of sugars on the NH_2 cartridge was based on the same principle. The best retention results were thus obtained by evaporating the extract to reduce its volume and mixing it with up to 80% acetonitrile. It was important that the final volume of sample before passing it through the cartridge did not exceed 10 ml, since the water content (still comprising 20% of the sample solution) could elute sugars from the sorbent in the same order as in chromatography. The results obtained by the adsorption of glucose, fructose, sucrose and xylose from the extracting solution containing 2 mg ml^{-1} of each, and by their elution from the cartridge with 3 ml of distilled water, are presented in Table 2. High and reproducible recoveries were observed for all of the investigated sugars, except for xylose. Considering the expected concentrations of xylose to be extracted

Table 2
Recovery of sugars by SPE on aminopropyl silica sorbent from spiked extracting solution containing glucose, fructose, sucrose, xylose and maltose (2 mg ml^{-1} of each) with and without the addition of alkaloids (0.56 mg ml^{-1} of nicotine and 0.06 mg ml^{-1} of nornicotine)

	Recovery, %±SD (n)				
	Xylose	Fructose	Glucose	Sucrose	Maltose
Without alkaloids:					
Retained on NH$_2$	62±5 (10)	81±4 (10)	89±4 (10)	97±2 (10)	NM
Passed through	35±5 (10)	14±4 (10)	4±1 (10)	ND	NM
With alkaloids:	53±4 (10)	71±7 (10)	86±5 (10)	93±2 (10)	89±2 (10)

SD=standard deviation; n=number of samples; NM=not measured; ND=not detected.

from tobacco and the detection limit of HPLC analysis, even this lower recovery was considered satisfactory for reliably determining xylose in tobacco extract.

The efficacy of sugar isolation by selective SPE was tested by checking for the presence of analytes in the sample solution after passing it through the sorbent cartridge. These results (Table 2) also indicate that retention on the NH$_2$ cartridge is, in fact, column chromatography. Xylose, eluting after the shortest retention time from the HPLC column with the same stationary phase, is less efficiently trapped on the sorbent cartridge. One third of its total amount is washed out from the sorbent with the sample solution itself. The sucrose with the longest retention time in HPLC analysis is practically completely retained on the sorbent. After passing through the cartridge, it cannot be detected in the sample solution.

The possible influence of alkaloids on the SPE isolation of sugars was checked using a spiked extracting solution containing glucose, fructose, sucrose, xylose and maltose at 2 mg ml^{-1} of each, and nicotine and nornicotine at 0.56 and 0.06 mg ml^{-1}, respectively. The recoveries obtained (Table 2) were equal to, or slightly lower than, those expected, indicating that there was no significant interference effect.

3.3. Analysis of a tobacco sample

The developed SPE procedure was applied to the analysis of a Virginia tobacco sample (variety DH10, harvested in 1995). Quantitative determination of alkaloids and sugars after their isolation by selective SPE from a tobacco extract was performed using the

standard addition method. Aliquots of tobacco extract were spiked with increasing concentrations of target compounds. The spiked samples were processed as described and analysed by HPLC after the addition of internal standards, pyridine for alkaloids and lactose for sugars. For both groups of compounds, a series of five samples (in duplicate) with increasing concentrations of analytes were prepared, along with a series of samples with no addition of standards at all. Linear correlations of peak area vs. concentration of compounds added were found for all target analytes in the spiked samples and unknown concentrations in tobacco sample were obtained by extrapolating the correlations to zero addition. Table 3 shows a good agreement between the standard addition method (sample 1) and the results obtained with non-spiked samples by the common internal standard method (samples 2–8) for nicotine and nornicotine. Considering the sugar analysis, a lower quantity for sucrose and a higher one for glucose and fructose was obtained by the standard addition method in comparison to samples 2–8. This could be ascribed to partial degradation of disaccharide in sample 1 to the corresponding monosaccharides during the preparation of other samples.

The same tobacco sample was extracted and analysed by spectrophotometric methods for the determination of total alkaloid and total sugar contents. According to these cumulative methods, the sample contained 3.12% of total alkaloids and 30.25% of total sugars (22.58% of reducing sugars). The values of nicotine and nornicotine in Table 3 together make up 83% of the total alkaloids measured by the CORESTA spectrophotometric method, and the sum of glucose, fructose, sucrose, xylose and maltose represents 82% of the total sugar content.

Table 3
Content of alkaloid and sugar (%) in the Virginia tobacco sample (variety DH10, harvested 1995), determined by the standard addition method (sample 1) and by the internal standard method (samples 2–8)

Sample no.	Nicotine (%)	Nornicotine (%)	Xylose (%)	Fructose (%)	Glucose (%)	Sucrose (%)	Maltose (%)
1	2.58	0.08	0.13	10.01	12.63	1.47	NM
2	2.57	0.08	ND	7.59	10.67	5.43	0.76
3	2.57	0.06	0.08	7.48	11.06	5.83	0.84
4	2.53	0.05	ND	6.19	8.96	5.52	0.72
5	2.52	0.05	ND	8.15	11.33	6.73	0.61
6	2.54	0.06	ND	6.54	9.67	6.81	0.66
7	2.47	0.07	ND	8.55	10.83	7.01	0.70
8	2.49	0.07	ND	7.46	9.96	5.74	0.71
Mean±SD (no. 2–8)	2.53±0.04	0.07±0.01	ND	7.4±0.9	10.4±0.9	5.8±0.6	0.71±0.07

SD=standard deviation; NM=not measured; ND=not detected.

This can be considered to be a very good agreement between the two methods, since only the two most important alkaloids, and not all of the sugars present in the sample, have been included in the analysis described in this work.

4. Conclusion

An analytical method for the determination of tobacco alkaloids and sugars has been developed, which is based primarily on selective SPE isolation of target compounds from tobacco extract followed by their HPLC analysis. The results are in good agreement with those obtained by currently used spectrophotometric methods for the determination of total alkaloid and total sugar contents. The main advantage of the new method is the possibility of determining individual components from two different groups of compounds in the same extract obtained by single-step extraction from tobacco material. The method is suitable for routine analysis because it offers reliable and not too time-consuming evaluation of tobacco quality.

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