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Determination of tobacco alkaloids in single plant cells by capillary electrophoresis

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Abstract

A new capillary electrophoresis system with direct UV detection for the analysis of the tobacco alkaloids nicotine, nornicotine and anabasine in plant microsamples was developed. An electrolyte containing a high concentration of citric acid to provide good buffer capacity at pH 3.6 was found to be most suitable in terms of sensitivity and separation efficiency. At this low pH the tobacco alkaloids are present in cationic form, showing high mobility and increased UV absorption. This system was used for the analysis of nicotine in single epidermal leaf cells of tobacco plants. Only vacuolar concentrations of nicotine were determined, as the vacuole occupies >95% of the entire volume in epidermal cells. The procedure of sample acquisition and preparation for nicotine analysis of vacuolar samples in the pl range is shown. The results indicate a gradient of nicotine from the leaf base to the tip with higher concentrations present in the cells at the tip. Compared to simultaneously measured bulk leaf samples containing all types of cells, tissues and compartments, the concentrations in epidermal cells are much higher. As nicotine is the major defence substance against insects in tobacco and the epidermis is the most exposed leaf tissue this result is physiologically plausible.

Keywords: Tobacco; Plant materials; Alkaloids

1. Introduction

Plants contain thousands of secondary metabolites which are not involved in the primary metabolic pathways. However, secondary metabolites have very specific functions. In tobacco plants nicotine and related alkaloids are important secondary metabolites which are produced in high concentrations in order to protect the plant from insect attack [1,2]. The wounding of the leaf tissue induces the production of jasmonic acid as signal compound in the leaf, which is transported to the roots and induces the production of nicotine. Consequently, nicotine is synthesized in the roots and transported to the leaves with the transpiration stream in the xylem vessels [3–5]. Though this process is well understood, only little is known about the distribution of nicotine in the different leaf tissues and cells. There is some evidence that alkaloids are inhomogeneously distributed between different cell types [2,6], and they might also show a compartmentation between different cells within the same tissue [6]. In order to further illuminate these questions, the determination

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of the nicotine distribution in leaf tissues is required with high spatial resolution. Analyses on the single cell level are preferable in this respect to obtain a detailed view. Consequently, the analysis of tobacco alkaloids on a single cell level is necessary.

However, a single plant cell is divided into two major compartments, the vacuole and the cytoplasm containing the different organelles. Most of the solutes and metabolites accumulate in the vacuole, where they do not affect the complex metabolic processes located in the highly regulated cytoplasm. This is even more important for poisonous compounds such as nicotine. Thus, one has to compare vacuolar concentrations which means determination of nicotine in the vacuolar sap of individual cells.

In the last few years capillary electrophoresis (CE) has become a powerful analytical technique for many compounds even in complex plant materials [7–13]. Small injection volumes in the nl range opened the possibility of analysing even single cells as shown in various approaches [14–17]. Recently the CE analysis of sap extracted from single plant cell vacuoles was described for the first time [18]. A special procedure which is capable of subsample creation from vacuolar samples in the pl range provided for several analyses of one vacuolar sample and thus, the determination of a large number of components in individual vacuoles. This was shown for inorganic cations and anions [18], carboxylic acids, sugars and amino acids in epidermal and mesophyll cells of wheat plants [19].

A few years ago Yang and Smetena [20] described the CE determination of nicotine with direct UV detection using a phosphate buffer. We have developed a new electrolyte system for the determination of the tobacco alkaloids nicotine, nornicotine and anabasin with capillary electrophoresis. We found a highly concentrated citrate buffer at low pH provided higher efficiency and lower limits of detection (LODs) compared to phosphate buffer.

This buffer was employed for nicotine quantification in vacuoles of individual leaf epidermal cells of *Nicotiana tabaccum*. Special care had to be taken with the sample preparation. Manipulation of pl volumes must be performed under a protective layer of paraffin oil to prevent evaporation and contamination. Consequently, analytes might be partitioned between the sample droplet and the protective paraffin oil, thus leading to analyte loss. As the polarity of nicotine and thus its affinity to the paraffin oil phase is strongly pH dependent, a modification of the previously described sample preparation procedure [18] was developed. Using this modification and the new electrolyte system, the determination of nicotine concentrations in epidermal vacuoles of tobacco leaves is possible. The results of these single cell measurements are compared to measurements of bulk samples and the consequences concerning nicotine distribution in tobacco leaves are discussed.

2. Experimental

2.1. Apparatus

The experiments were carried out on a laboratory-built CE system, equipped with a Lambda 1000 UV-detector (Bischoff, Leonberg, Germany) and a high voltage power supply type HCN 6 M - 30000 from FUG (Rosenheim, Germany). Untreated fused-silica capillaries (Chromatographie Service, Langerwehe, Germany) with 50-μm I.D. and 360-μm O.D. were used. The total lengths of the capillaries were either 70 or 80 cm. For injection of the picoliter samples 2 cm of the polyimide coating was removed on the injection side, and the outer diameter of the capillary tip was conically reduced to ~150 μm by manual grinding on a fast rotating corundum plate.

Two types of microcapillaries were used for sampling and for measuring identical aliquots of sample and internal standard. Capillaries for cell sap sampling were pulled from borosilicate glass capillaries with 1.0 mm O.D.×860 μm I.D. (Clark Electromedical Instruments, Pangbourne, UK) to a tip diameter of ~1 mm using a vertical pipette puller Model 700C (David Kopf Instruments, Tujunga, CA, USA). Constriction capillaries were prepared from normal sampling capillaries with a microforge.

Droplets of volumes in the nanoliter range were produced using an ultra micro pump (World Precision Instruments, Berlin, Germany) equipped with a modified microsyringe (ILS, Stützerbach, Germany) with luer connected glass microcapillary. Sampling injection was carried out by moving the end of the separation capillary with a micromanipulator (Leitz, Wetzlar, Germany) towards the sample droplet. All

operations were observed with a stereoscopic microscope (Nikon SMZ-1B, Düsseldorf, Germany).

2.2. Chemicals

For sample storage paraffin oil (type 76235; Fluka, Buchs, Switzerland) was used. It was ultrasonically extracted several times with Milli-Q water to remove ionic impurities and centrifugated. Sodium citrate was purchased from Fluka. Nicotine was purchased from Aldrich (Milwaukee, WI, USA), nornicotine and anabasin from ICN (Costa Mesa, USA), and pyridine and 4-amino-pyridine from Fluka.

2.3. Plant material

N. tabacum was grown in a 12-m*M* $NaNO_3$ medium and 150 μmol/m^2 s light intensity for 9 weeks. To obtain comparable results with different plants the sixth leaf was always chosen for analysis.

2.4. Sample acquisition and handling of vacuolar sap

The basic procedures of sampling and handling of sap from individual vacuoles have been reported in a previous work [19]. Briefly, 20–100 pl of vacuolar sap are extracted with a silicone oil filled microcapillary. The sample droplet is transfered onto the bottom of a Petri dish, filled with paraffin oil. Subsamples, each ~10 pl, are created from the vacuolar sample using a constriction capillary. The subsamples are injected together with an identical volume of internal standard (prepared with the same constriction capillary) into several 5-nl water droplets, also present in the Petri dish. The water droplets, containing identical volumes of vacuolar sap and internal standard are injected into the CE capillary and analysed.

3. Results and discussion

3.1. Determination of tobacco alkaloids

As previously described by Yang and Smetena [20] the UV absorption of nicotine is strongly pH dependent showing the largest absorptivity at low pH. In addition, at low pH nicotine exists in its diprotonated form and shows high electrophoretic mobility. Thus, an electrolyte system with high buffer capacity in a low pH range is advantageous. Citric acid in high concentration was chosen for this purpose, as it has suitable buffering properties in the range of pH 2–6 with a first p*K* value of 3.13. Higher plate numbers and lower LODs were achieved with this buffer compared to phosphate. Plate numbers with a phosphate buffer were 60 000–70 000 and the LOD was 10 μ*M*, whereas, with the citrate buffer plate numbers of 110 000 for nicotine, 122 000 for anabasin and 126 000 for nornicotine were obtained. The LOD was 2 μ*M*.

Fig. 1 shows a typical electropherogram of the tobacco alkaloids using this electrolyte. As the analytes are cationic at low pH, they migrate coelectroosmotically and the run only takes 7 min. A buffer concentration of 150 m*M* was found to reach the highest possible plate numbers. Large differences in resolution of nicotine and anabasine were observed with only small changes of the field strength. The maximum resolution was found at 23 kV with a capillary length of 70 cm and an inner diameter of 50 μm.

3.2. Modification of the sample handling procedure

The principal procedure of vacuolar sample acquisition and sample preparation is described in the

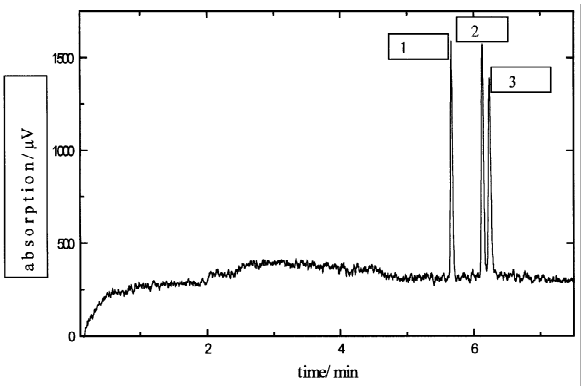

Fig. 1. Electropherogram of tobacco alkaloids: 1, nornicotine; 2, nicotine and 3, anabasine; electrolyte: 150 m*M* citric acid, pH 3.6; capillary: 70 cm (50 cm to detector)×50 μm I.D.; conditions: voltage 23 kV, current: 114 μA; detection: UV, 260 nm.

Experimental section. For the analysis of nicotine in single cells the sample handling procedure had to be modified with respect to a potential extraction of nicotine by the protective paraffin oil. The polarity of nicotine is strongly dependent on the pH. At a pH range lower than 2.7 nicotine exists in its diprotonated form, in the range of 4.5–7.0 the monoprotonated species predominates and above a pH of 7.0 nicotine is neutral [20].

Consequently, as expected, the partitioning of nicotine into the paraffin oil phase increases with increasing pH. In order to determine the critical pH range, at which extraction of nicotine by paraffin oil takes place, aqueous nicotine droplets of different pH were positioned into a layer of paraffin oil and the nicotine concentration in the droplets was determined after different time intervals. Fig. 2 shows the recovery of nicotine in identical aqueous droplets of different pH as a function of time. At a pH of 5.5, 45% of the nicotine is extracted by the paraffin oil within 1.5 h. Decreasing the pH of the sample droplets, the nicotine recovery increases. At a pH of 3.5 the loss of nicotine is lower in comparison to a pH of 5.5. After 1.5 h 32% of the nicotine was extracted into the paraffin oil. Using droplets of pH 2.5 or lower the nicotine recovery remained 100% for several hours, providing enough time for sample preparation and analysis.

Therefore the sample preparation procedure, which was developed for highly polar solutes, was modified. The sample obtained by extraction of sap from the vacuole was immediately divided into subsamples. The latter were transfered into 5-nl droplets of diluted HCl at pH 2.5 instead of pure water droplets. Using this modification analyte loss due to partitioning into paraffin oil was successfully

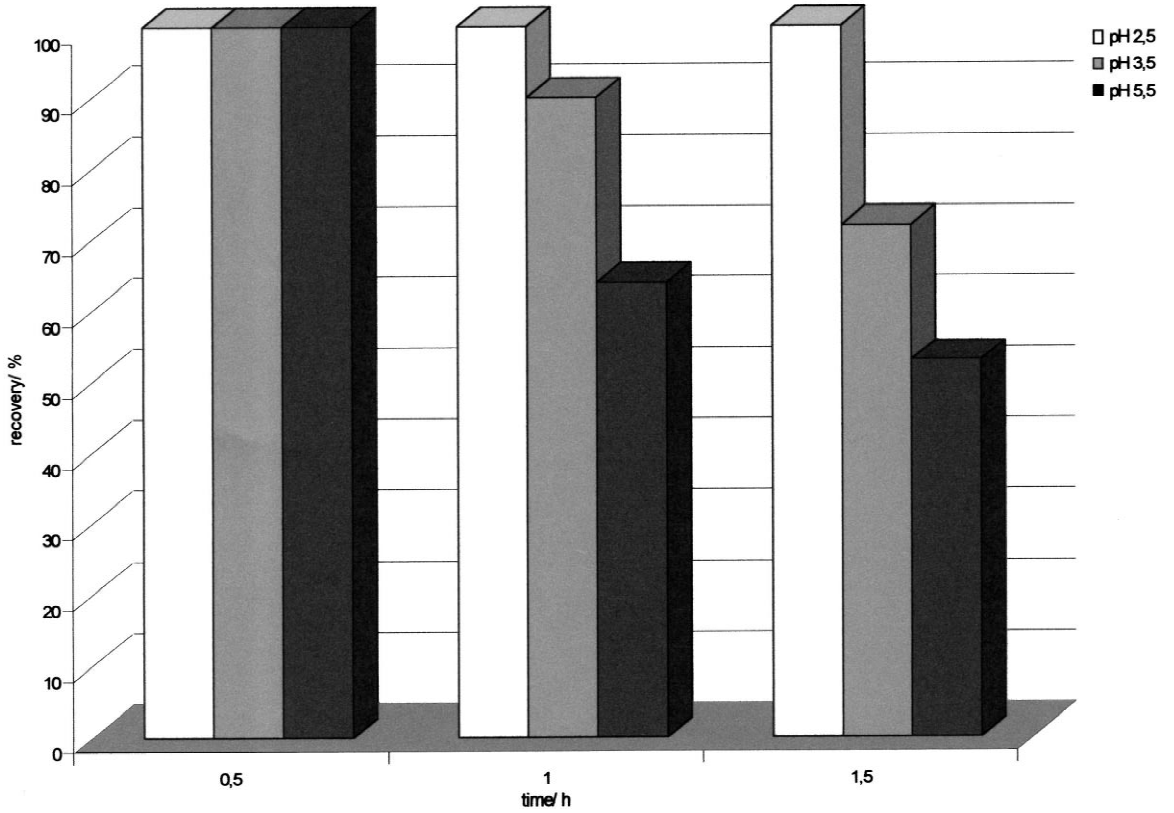

Fig. 2. The recovery of nicotine at the aqueous sample droplet surrounded by paraffin shown for a time series (0.5, 1, 1.5 h) and at different pH values: a pH of 2.5 or lower must be chosen to prevent the extraction of analyte during the operation time of the experiment.

prevented. Even storage of the samples at −20°C was possible for several days without a decrease in nicotine recovery. 4-Amino-pyridine was used as internal standard because it shows a strong absorption at 260 nm and is not extracted into the paraffin oil under the applied experimental conditions.

3.3. Determination of nicotine in vacuolar sap of epidermal cells

The described techniques were subsequently used to determine nicotine in vacuolar samples from individual epidermal leaf cells. All experiments were performed with 9-week-old plants of *N. tabaccum*. In order to investigate potential differences between epidermal cells located at different areas of the leaf, samples were taken from cells at the leaf base and the leaf tip. As leaf growth takes place at the base, this region corresponds to younger cells (and tissues) compared to the tip.

Fig. 3 shows the electropherogram of a vacuolar sample extracted from an epidermal cell at the leaf tip. In this vacuolar sample the concentration of nicotine was 20 μ*M*, and the concentration of the internal standard 4-amino-pyridine was 109 μ*M*. The absolute amount of nicotine can be calculated to 0.3 pmol. This has to be seen in comparison to the LOD of 2 μ*M* which means under the experimental conditions an absolute LOD of 0.013 pmol.

The concentration of the used internal standard was 100 m*M*. The tobacco alkaloids anabasine and nornicotine could not be observed, presumably due to the low concentration compared to nicotine. This example demonstrates that CE is capable of nicotine analysis in extremely small tissue samples of tobacco.

The measured nicotine concentrations in epidermal cells at the leaf base were significantly lower (approximately half) compared to the cells at the tip. This result is shown in Fig. 4A. The concentrations were ~60 m*M* (single cell vacuoles at leaf tip) and 35 m*M* (single cell vacuoles at leaf base), respectively. This concentration gradient corresponds to the different age of the cells at the base and tip and may indicate that nicotine is continuously transported into the epidermal cells and accumulated in the vacuoles.

In order to examine, whether the nicotine concentrations in the epidermis are characteristic for the

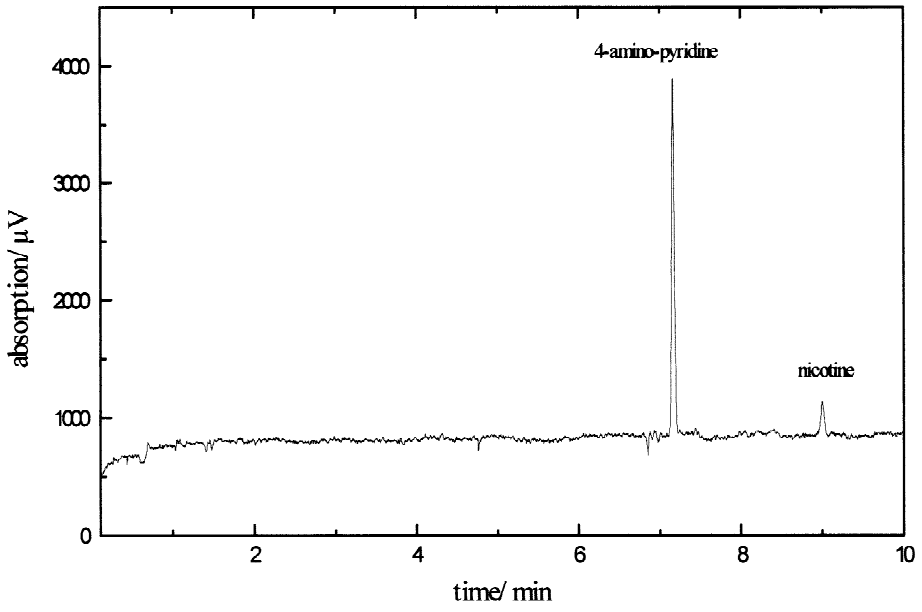

Fig. 3. Electropherogram of nicotine in a single vacuole of an epidermal cell. 4-Amino-pyridine acts as internal standard to recalculate the nicotine concentration inside the vacuole. Electrolyte: 150 m*M* citric acid, pH 3.6; capillary: 80 cm (50 cm to detector)×50 μm I.D.; conditions: voltage 23 kV, current: 110 μA; detection: UV, 260 nm.

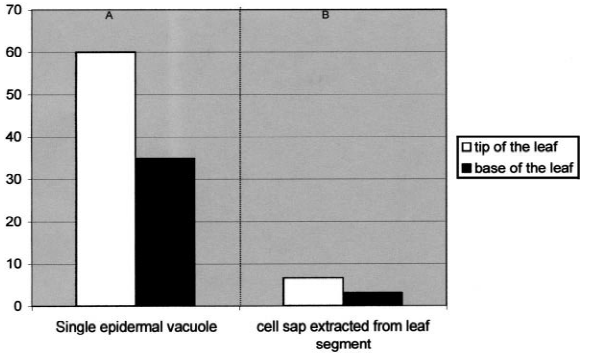

Fig. 4. Concentration gradient of nicotine between two different areas of a leaf. The bars in (A) show the vacuolar nicotine concentration at the epidermis cell in the leaf tip (blue/grey), and at the base of a leaf (red/black). The total amount of the nicotine (=average value over all cell types) in these areas is shown in (B) where cell sap was extracted after cutting 1-cm areas of the same leaf after single cell sampling.

entire leaf, or whether strong differences between the different tissues of the leaf have to be considered, the nicotine concentrations in epidermal cells were compared with values observed from bulk leaf material. Now it is of great interest to know whether nicotine is only found and accumulated in the epidermis and whether the concentration gradient towards the tip of the leaf is specific for the epidermis. Thus, nicotine concentrations in extracts of plant tissue were determined. Circular leaf segments were cut from the leaf base and tip. These segments were frozen and the sap from the vacuoles was isolated by centrifugation. The cell sap was directly analysed in order to get an average nicotine concentration of all cell types present in the respective leaf segment. The measured nicotine concentrations at the base and tip are also presented in Fig. 4B, together with the results from the epidermal vacuoles.

The most obvious result is that the nicotine concentrations in the bulk extracts were approximtely ten times lower than in the epidermal cells with 6–7 m*M* at the tip and 2–3 m*M* at the base. Analogous to the single cell measurements, considerably higher concentrations of nicotine were found in the tip. The nicotine concentrations in bulk extracts were measured by HPLC–GC [21].

From these preliminary results it can be stated that nicotine is accumulated at significant higher levels in the epidermis. As the epidermis is the leaf tissue which is directly exposed to the environment and thus the first target of a potential insect attack, a strong accumulation of defence substances in the epidermis seems reasonable. Of course further measurements of nicotine in individual cells of the different leaf tissues are necessary to confirm these first results presented in this paper.

4. Conclusion

Capillary electrophoresis is a powerful method for the analysis of nicotine in individual plant cell vacuoles. A fast method for separation and sensitive detection of tobacco alkaloids has been presented. Modifications in the sample handling procedure for vacuolar samples provided the trapping of nicotine in an acidic droplet, thus extraction of nicotine into the protective paraffin oil layer can be successfully prevented. The described method allows the quantitative determination of nicotine in single vacuoles and opens the possibility of measuring concentration distributions within plant tissues with very high spatial resolution.

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