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Capillary zone electrophoresis of alkaloids Influence of structure on electrophoretic mobility

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Abstract

A comprehensive discussion of important aspects for the analysis of alkaloids by capillary zone electrophoresis (CZE) is given. The influence of structure on the electrophoretic mobility (EM) of indole alkaloids was investigated using a running buffer which is generally applicable to the CZE analysis of alkaloids. The EM, which at the applied conditions was mostly dependent on the size and shape of the solvated analyte ions, was additionally affected by the presence of hydrogen bonds or ion–dipole interactions between protonated and unprotonated alkaloids of the same species. This could be derived from the existence of alkaloidal dimer cluster ions $[2M+H]^+$ when mass spectrometry was used for detection. © 1998 Elsevier Science B.V.

Keywords: Electrophoretic mobility; Alkaloids

1. Introduction

Alkaloids represent an important group of natural products because of their widespread use and therapeutical value in phytomedicine. Since this group contains a high number of substances (about 10 000 compounds have been identified up to now) which differ strongly in their structures, molecular masses and basic properties, the qualitative and quantitative analysis of this compounds is still a challenge. The analytical methods used in alkaloid determination are thin-layer chromatography (TLC) [1], gas chromatography (GC) [2], high-performance liquid chromatography (HPLC) [3,4] and combined techniques such as GC–mass spectrometry (MS) [5,6] or HPLC–MS [7,8].

Nowadays capillary electrophoresis (CE) is a useful tool for phytochemical analysis [9] because of many advantages including small sample amounts, speed and excellent separation efficiency. Like HPLC, the method is also suitable for use with MS [10–16]. Capillary zone electrophoresis (CZE) for the analysis of basic substances, especially natural compounds, was applied in the past for crude drugs [9] but also for pharmaceutical preparations [17,18]. For strong bases like alkaloids CZE was frequently applied because of the alkaloids positive charge over a broad pH range and in addition the possibility to perform CE–MS if volatile buffer salts are used [12]. Another principal method for the CE analysis of low-molecular-mass compounds is micellar electrokinetic chromatography (MEKC) where also uncharged molecules can be analysed [19]. This method was preferentially applied for natural compounds containing nitrogen atoms which are practically not basic, e.g., capsacinoids or purine alkaloids [9]. The rare application of MEKC for basic alkaloids is probably due to a low solubility of weak bases under

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neutral or alkaline conditions and a possible interaction of strong bases with anionic micelles of the most often used sodium dodecyl sulfate [20].

Whereas for flavonoid analysis with CZE a comprehensive discussion on the effect of structure on electrophoretic mobility (EM) is given in [21], a similar approach for alkaloids does not exist. Although a number of publications deal with CZE of alkaloids [9,22–27] many of them lack of information about the influence of structure on electrophoretic mobility.

Previously we described the CZE and CE–MS analysis of four different alkaloid classes with a broadly applicable buffer system consisting of 100 mmol/l ammonium acetate pH 3.1–acetonitrile (50:50, v/v) [12]. Because these alkaloid classes varied strongly with regard to the molecular masses, structures and basic properties, the structurally related factors responsible for the EM of alkaloids in CZE are of general interest. In order to evaluate these mechanisms we used a model mixture of fifteen monoterpenoid indole alkaloids and biogenic amines which was analysed with CZE using UV and MS detection. Several theoretical aspects could be derived from the migration order of this structurally very different substances but also from mass spectra obtained after on-line combined CE–MS [12]. In addition, important considerations for method development in alkaloid separation with CZE are given and discussed in detail.

2. Experimental

2.1. Chemicals

Acetonitrile (LiChrosolv), methanol (LiChrosolv), sodium hydroxide and acetic acid (analytical-reagent) where obtained from Merck (Darmstadt, Germany). Ammonium acetate (microselect grade) was obtained from Fluka (Buchs, Switzerland).

2.2. Sample preparation

All standards used were dissolved in MeOH and diluted to a final concentration of ca. 0.1 mg/ml.

2.3. Instrumentation

2.3.1. CE–UV

A Bio-Rad BioFocus 3000 apparatus (Munich, Germany) equipped with a liquid cooling system and a fast scanning detector was applied for the CZE analyses with UV detection. The fused-silica capillary used had an internal diameter of 50 μm and an effective length of 50 cm (Polymicro Technologies, Phoenix, AZ, USA). The applied voltage was 15 kV and the temperature of the capillary was set to 25°C. For sample injection a pressure of 345 mbar was applied for 1 s. Between runs the capillary was purged for 2 min with 1 *M* sodium hydroxide, 2 min with water and finally 3 min with running buffer. The electrolyte consisted of a 100 mmol/l solution of ammonium acetate (adjusted to pH 3.1 with acetic acid)–acetonitrile (50:50, v/v).

2.3.2. CE–MS

For on-line CE–MS separations a BioFocus 2000 apparatus (Bio-Rad) coupled to a Finnigan MAT Model 95 forward-geometry sector-field mass spectrometer (Finnigan MAT, Bremen, Germany) upgraded with an API-II ion source (Finnigan MAT) was used. For details of the CE–electrospray ionisation (ESI) device and further experimental data see Refs. [12,28,29].

3. Results and discussion

3.1. Analysis of alkaloids with CZE

Most of the alkaloids possess a basic nitrogen which is heterocyclic bound. An exception may be alkaloids with an amide function where the nitrogen is not basic but also biogenic amines which contain an unsubstituted nitrogen as part of a primary amine. Due to their basic properties the molecules exist mainly in the protonated water soluble form if the pH value of the solution lies below the pK_a values as can be derived from the Henderson–Hasselbalch equation [30]. In contrast to MEKC the application of CZE is limited to charged substances. This charge can be produced by protonation–deprotonation via an acidic–basic pH value of the applied buffer but can be also introduced by complexation [31]. Since

nearly all alkaloids have pK_a values above 6 many electrolytes with pH values in the range of ca. 2–8 are applied for CZE. But even buffers with pH values above 9 were used in the past [22] (see Table 1).

3.1.1. Considerations for method development

Prior to method development some important aspects have to be taken into account: (i) type and number of charges per molecule; (ii) stability of the analytes in acidic or basic media; (iii) control of the electroendoosmotic flow (EOF); (iv) solubility of analytes in the solvent used for sample preparation; (v) low ionic strength of the sample.

Whereas additional basic functions lead to a higher EM as can be seen for the bisindoles vinblastine (7) and vincristine (9) (Figs. 1 and 2), the presence of an additional acidic function leads to a reduced EM as shown for yohimbinic acid (12) (see Fig. 2). The longer migration time of (12) is due to the partially dissociated carboxylic group at C (16) and thus the resulting pK_a value (=isoelectric point, pI) of (12) is determined by the pK_a value of the basic nitrogen (pK_{a1}) and the pK_a value of the carboxylic group (pK_{a2}) as expressed in Eq. (1) [30].

$$pI = \frac{pK_{a1} + pK_{a2}}{2} \quad (1)$$

It should be mentioned that the dissociation of the carboxylic group of (12) led also to a significantly lower signal-to-noise ratio when MS was used for detection as can be clearly seen in the MS spectrum of (12) (Fig. 3). Substructures like phenolic hydroxyl groups or glucuronic acids also influence the EM depending on the pH value of the electrolyte as shown for the glucuronides of morphine [32].

Since many alkaloid separations are performed in acidic or basic solutions (Table 1) the presence of an unstable functional group can influence the EM. For example the presence of a lactone group which is not stable at the present pH value can be hydrolysed to the anionic form thus leading to a higher migration time as expected from its molecular mass and pK_a value. This was observed for the antineoplastic alkaloid camptothecin when analysed with CZE at pH values above 5 (data not shown) [33].

The control of the EOF is important especially when electrolytes with pH values between 3 and 7 are applied [30]. In this case the EOF enhances the migration velocity of the alkaloids because this bulk flow is normally directed towards the cathode. In fact, this reduces the run time but since every alkaloid is influenced in the same way the higher migration velocities level the differences in the effective EM. Thus, a diminished resolution is obtained [30]. The application of a high salt concentration effectively reduces the EOF, but also enhances Joule heating. Organic solvents like alcohols or acetonitrile as buffer additives are another possibility to control the EOF mainly by changes in the viscosity of the medium [34].

The preparation of the sample with running buffer may cause problems if the pH value of the electrolyte is neutral or even higher especially when relatively weak bases are analysed [27]. Because most of the unprotonated alkaloid bases are highly lipophilic the water solubility is drastically reduced at neutral or alkaline conditions. An elegant way to avoid these problems is the use of organic solvents for sample preparation [12,24]. This additionally improves the stacking effect and leads to a better sensitivity and resolution because sharper peaks are obtained. Furthermore, the low ionic strength of the sample which provides a higher electric field causes a strong preconcentration of substances during elec-

Table 1
Selected examples for the variation of buffer composition in CZE of alkaloids

Sample	Buffer	Ref.
Ephedra alkaloids	20 m*M* isoleucine, 5 m*M* barium hydroxide, pH 10.0	[22]
Oxindole alkaloids	20 m*M* phosphate, pH 5.6	[23]
Quaternary alkaloids	500 m*M* acetate–acetonitrile (1:1), pH 4.6	[24]
Isoquinoline alkaloids	66 m*M* phosphate–methanol (60:40), pH 2.4	[25]
Brucine and strychnine	10 m*M* phosphate–methanol (9:1), pH 2.5	[26]
Various alkaloid classes	100 m*M* acetate–acetonitrile (1:1), pH 3.1	[12]

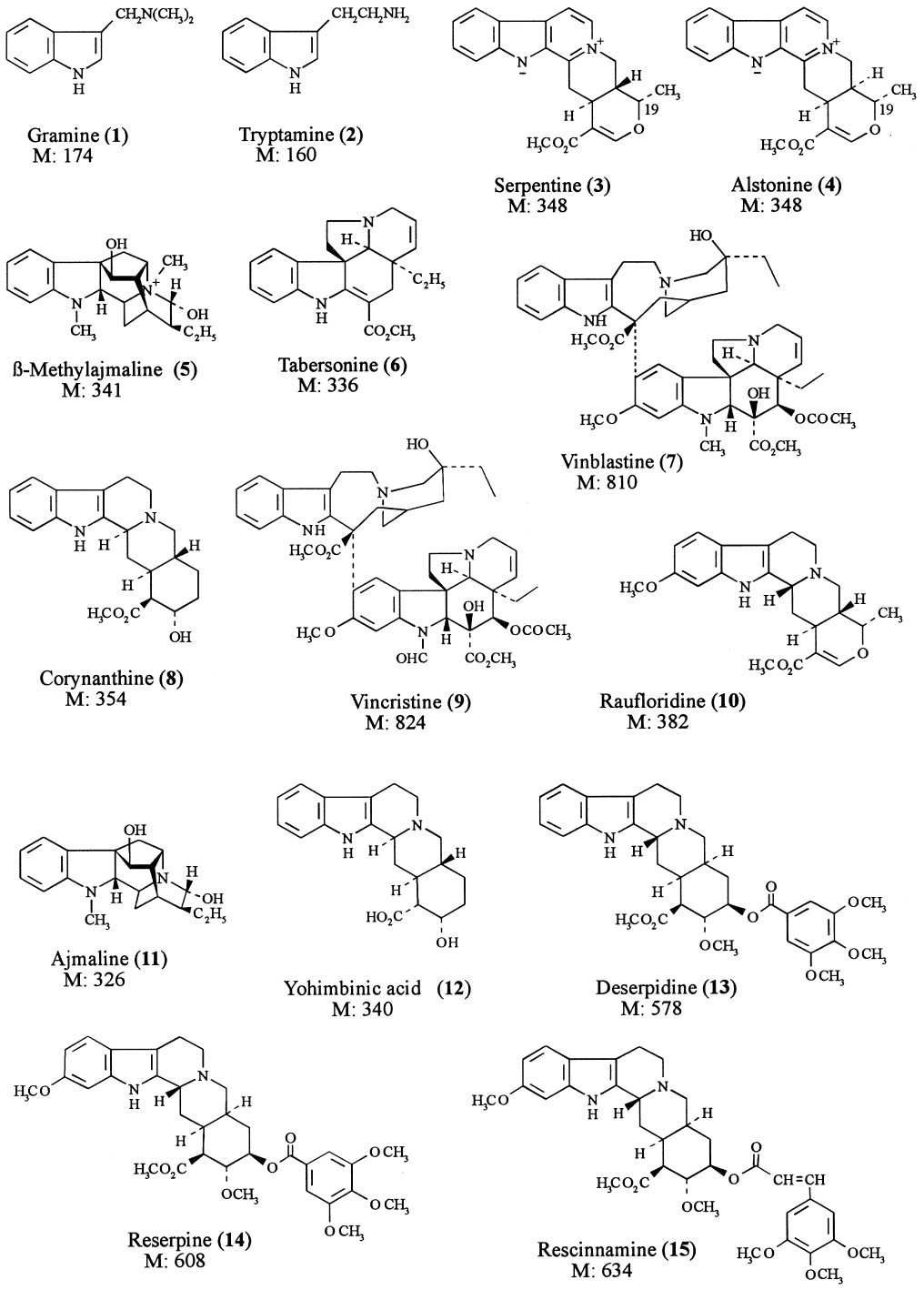

Fig. 1. Names, molecular masses (M) and chemical formulas of 15 indole alkaloids.

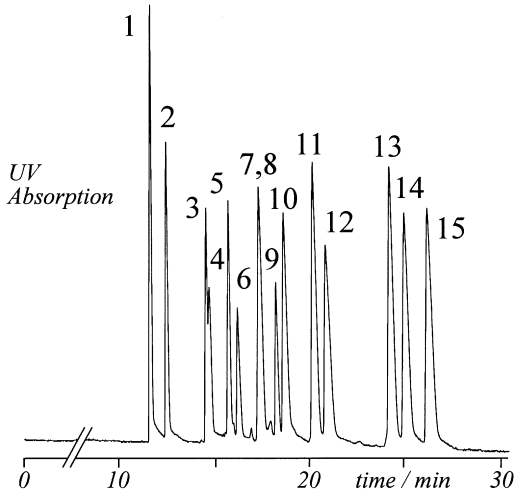

Fig. 2. Electropherogram of the CZE separation of 15 indole alkaloids. Conditions: voltage 15 kV, capillary temperature 25°C, UV detection at 200 nm; buffer: 100 mmol l^{-1} ammonium acetate pH 3.1–acetonitrile (1:1, v/v); (for capillary dimensions and sample preparation see Section 2.3.1).

trokinetic injection [35]. With a modified electrokinetic injection technique called field amplified sample injection (FASI) [35] the detectability of alkaloids in crude extracts was a thousand-times higher compared to conventional pressure injection [36,37].

3.1.2. Factors which determine the selectivity

The selectivity of a separation in CZE is determined by the different migration velocities of the analytes. Further, the migration velocity of a charged molecule in an electric field results from its size (or molecular mass), shape and net charge. The size and shape of a molecule cannot be influenced directly but the molecules net charge is dependent on its pK_a value and can be controlled via the pH value of the electrolyte. Since only the protonated alkaloids migrate in an electric field and since the pK_a values of alkaloids differ strongly depending on their structure the migration velocities and the resulting selectivity are mainly influenced by the pH value of the buffer. Theoretically, the optimum selectivity is obtained if the pH value is close to the pK_a values of the analytes [38]. For alkaloid separations with CZE this explanation is often not sufficient, because excellent selectivities for strong bases are also obtained using acidic electrolyte systems (Table 1). In order to give a reasonable explanation additional facts have to be considered. It is obvious that the counterions like phosphate or acetate applied in this applications (Table 1) show a high affinity to the ammonium form of the protonated alkaloids. Because the cationic alkaloids are surrounded by oppositely charged counterions which migrate to the anode, the EM of the analytes is reduced [30]. Since this effects are also dependent on the size and shape of the molecules the selectivity at low pH values may be mainly controlled by ionic interactions between the protonated alkaloids and the negatively charged counterions. The reduced dielectricity constant in buffers with a high content of organic solvents (see Table 1)

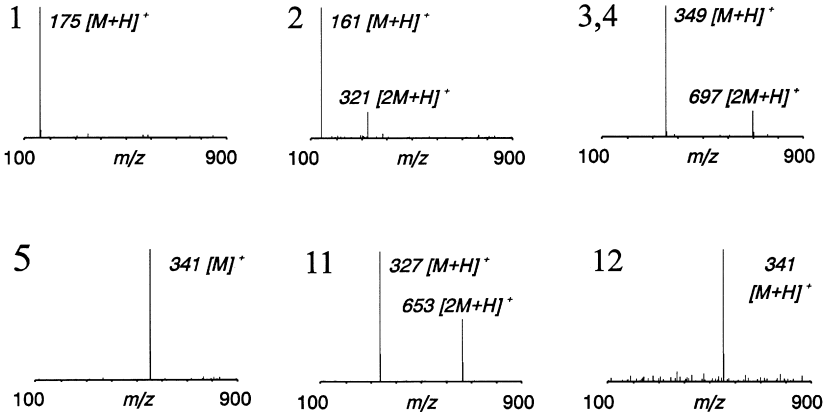

Fig. 3. Selected ESI mass spectra of indole alkaloids as obtained from the CE–MS analysis. The numbers refer to the indole alkaloids as given in Fig. 1 and the numbers in front of the parentheses refer to the m/z values.

especially acetic acid which is needed for the adjustment of pH values, may favour this ionic mechanisms.

3.2. Influence of structure on electrophoretic mobility

As mentioned above for our applied buffer system, the main reasons for the differences in EM are the size and shape of the molecules. The size of an ionic species in solution depends on its molecular mass and the resulting degree of solvation but the shape is determined by the molecular geometry. Thus, the EM of an alkaloid is also dependent on its configuration and conformation as can be seen for the epimeric indoles serpentine (3) and alstonine (4) in Figs. 1 and 2. The *trans* configuration of (3) at C (15) and C (20) (Fig. 1) provides a stretched form of the molecule and results in a reduced friction. In contrast to the *cis*-configured (4) this leads to a higher EM for (3) as clearly shown in Fig. 2.

The EM was not only determined by monomeric alkaloids, but also by dimeric alkaloid complexes as could be derived from the obtained mass spectra (Fig. 3) using CE–MS connected via an ESI interface. Surprisingly the ESI–MS spectra of alkaloids measured in Ref. [12] showed not only the characteristic $[M+H]^+$ signals but also additional signals of dimer cluster ions $[2M+H]^+$. Since the mass-to-charge ratio of such clusters is doubled compared to the protonated monomers the EM of the analyte ion is slowed down. However, this fact may be also related to the migration order of substances because the formation of this cluster ions was not generally observed. For example tryptamine (2) migrates after gramine (1) although its molecular mass is lower (Fig. 1). The existence of the $[2M+H]^+$ signal for tryptamine (2) (ca. 20% of the intensity of the $[M+H]^+$ signal) (Fig. 3) may lead to the higher migration time of (2). For gramine (1) the existence of a tertiary amine with additional methyl groups at the nitrogen may hinder the formation of $[2M+H]^+$ ions. But the most prominent example is ajmaline (11) where the signal intensity of the $[2M+H]^+$ cluster reaches 50% of the signal intensity for the protonated alkaloid monomer. Consequently the migration time of (11) (M_r 326) compared to tabersonine (6) (M_r 336), corynanthine (8) (M_r 354) or raufloridine (10) (M_r 382) is much longer. However, the existence of such cluster ions may originate from strong intermolecular forces between the protonated and unprotonated alkaloids because their signal intensity was relatively high (up to 50%) (Fig. 3) related to the dominating $[M+H]^+$ signal [12]. The absence of a $[2M+H]^+$ signal for β-methylajmaline (5) (Fig. 3) which differs from ajmaline (11) only by an additional methyl group at the N_β nitrogen (Fig. 1) points to a possible intermolecular interaction between the hydrogen of the protonated ammonium nitrogen and the nitrogen of the unprotonated alkaloid via hydrogen bonds as shown in Fig. 4a. An exception may be the formation of dimer cluster ions for serpentine (3) and alstonine (4) (Fig. 3). Because these zwitterionic molecules possess a quaternary N_β-nitrogen and an anionic N_α-nitrogen which is highly nucleophilic (the so called quaternary anhydronium form), the protonation in solution occurs at the N_α-nitrogen. In this case an ion–dipole interaction between the anionic N_α-nitrogen of the unprotonated alkaloid and the N_α-hydrogen of the protonated alkaloid exists (Fig. 4b). It can be speculated that the high amounts of acetonitrile and acetic acid which lower the dielectricity constant of the applied buffer solution, contribute to the described mechanisms because these mechanisms are in principle electrostatic interactions (refer to Section 3.1.2). Furthermore, the formation of hydrogen bonds between charged and uncharged alkaloid species might

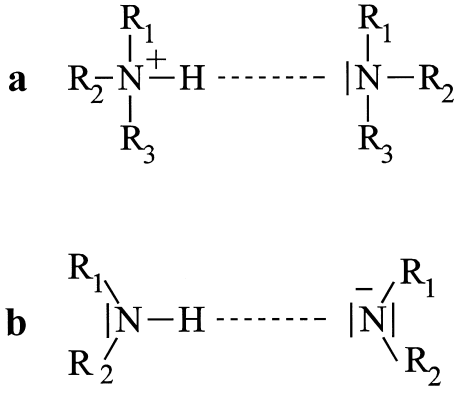

Fig. 4. Schematic drawing of the mechanisms in cluster formation between protonated and unprotonated indole alkaloids. (a) Hydrogen bonds; (b) ion–dipole interactions.

be also an explanation for the existence of unprotonated strong basic alkaloids at a pH value of 3.1.

4. Conclusions

The factors which determine the influence of structure on the EM of 15 indole alkaloids are deduced from the selectivity and migration order of their CZE separation. For the applied buffer the main influence on the EM is caused by the molecular mass and the shape of the solvated alkaloid cations. Additional information for the effect of structure on EM could be deduced from the presence of dimer cluster ions $[2M+H]^+$ which increase the mass-to-charge ratio. The discussed mechanisms herein may be helpful for the interpretation of results from CZE and CE–MS analyses of unknown alkaloid extracts using the recently described generally applicable buffer system [12].

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References

[1] N.R. Farnsworth, R.N. Blomster, D. Damratoski, W.A. Meer, L.V. Cammarato, Lloydia 27 (1964) 302.
[2] H. Wiedenfeld, R. Lebada, B. Kopp, Dtsch. Apoth. Ztg. 135 (1995) 1037.
[3] K.H. Pawelka, J. Stöckigt, Z. Naturforschung C 41 (1986) 385.
[4] T. Tanahashi, M.H. Zenk, J. Nat. Prod. 53 (1990) 579.
[5] W.F. Bayne, F.T. Tao, N. Crisologo, J. Pharm. Sci. 64 (1975) 288.
[6] J. Fehn, G. Megges, J. Anal. Toxicol. 9 (1985) 124.
[7] H. Falkenhagen, I.N. Kuzovkina, I.E. Alterman, L.A. Nikolaeva, J. Stöckigt, Nat. Prod. Lett. 3 (1993) 107.
[8] R. Verpoorte, W.M.A. Niessen, Phytochem. Anal. 5 (1994) 217.
[9] F.A. Tomás Barberán, Phytochem. Anal. 6 (1995) 177.
[10] F.Y.L. Hsieh, J. Cai, J. Henion, J. Chromatogr. A 679 (1994) 206.
[11] J.D. Henion, A.V. Mordehai, J. Cai, Anal. Chem. 66 (1994) 2103.
[12] M. Unger, D. Stöckigt, D. Belder, J. Stöckigt, J. Chromatogr. A 767 (1997) 263.
[13] D. Stöckigt, M. Unger, D. Belder, J. Stöckigt, Nat. Prod. Lett. 9 (1997) 265.
[14] M. Unger, D. Stöckigt, D. Belder, J. Stöckigt, Pharmazie 52 (1997) 691.
[15] J. Stöckigt, M. Unger, D. Stöckigt and D. Belder, in S.W. Pelletier (Editor), Alkaloids: Chemical And Biological Perspectives, Vol. 12, Pergamon Press, in press.
[16] S. Sturm and H. Stuppner, poster presented at the 45th Annual Congress of the Society for Medicinal Plant Research, Regensburg, 7–12 September 1997.
[17] H. Wätzig, C. Dette, Pharmazie 49 (1994) 83.
[18] J. Sadecka, J. Polonsky, H. Shintani, Pharmazie 49 (1994) 631.
[19] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya, T. Ando, Anal. Chem. 56 (1984) 111.
[20] K.-J. Lee, J.J. Lee, D.C. Moon, J. Chromatogr. 616 (1993) 135.
[21] T.K. McGhie, K.R. Markham, Phytochem. Anal. 5 (1994) 121.
[22] Y.-M. Liu, S.-J. Sheu, J. Chromatogr. 600 (1992) 370.
[23] H. Stuppner, S. Sturm, G. Konwalinka, J. Chromatogr. 609 (1992) 375.
[24] Y.-M. Liu, S.-J. Sheu, J. Chromatogr. 634 (1993) 329.
[25] P.G. Pietta, P.L. Mauri, C. Gardana, M.L. Colombo, F. Tomè, Phytochem. Anal. 6 (1995) 196.
[26] Y.-Y. Zong, C.-T. Che, Planta Med. 61 (1995) 456.
[27] R.B. Taylor, A.S. Low, R.G. Reid, J. Chromatogr. B 675 (1996) 213.
[28] D. Belder, D. Stöckigt, J. Chromatogr. A 752 (1996) 271.
[29] D. Stöckigt, G. Lohmer, D. Belder, Rapid Commun. Mass Spectrom. 10 (1996) 521.
[30] R. Kuhn and S. Hoffstetter-Kuhn, Capillary Electrophoresis: Principles and Practice, Springer-Verlag, 1993.
[31] S. Hoffstetter-Kuhn, A. Paulus, E. Gassmann, H. Widmer, Anal. Chem. 63 (1991) 1541.
[32] C.-X. Zhang, W. Thormann, J. Chromatogr. A 764 (1997) 157.
[33] J. Fassberg, V.J. Stella, J. Pharm. Sci. 81 (1992) 676.
[34] T.K. McGhie, J. Chromatogr. 634 (1993) 107.
[35] R.-L. Chien, D.S. Burgi, J. Chromatogr. 559 (1991) 141.
[36] M. Unger and J. Stöckigt, poster presented at the 45th Annual Congress of the Society for Medicinal Plant Research, Regensburg, 7–12 September 1997.
[37] M. Unger, J. Stöckigt, J. Chromatogr. A 791 (1997) 323.
[38] S. Terabe, T. Yashima, N. Tanaka, M. Araki, Anal. Chem. 60 (1988) 1673.