ELSEVIER

Journal of Chromatography A, 967 (2002) 85–113

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Review

High-performance liquid chromatographic, capillary electrophoretic and capillary electrophoretic–electrospray ionisation mass spectrometric analysis of selected alkaloid groups

Joachim Stöckigt[a,*], Yuri Sheludko[a,b], Matthias Unger[c], Irina Gerasimenko[a,b], Heribert Warzecha[a], Detlef Stöckigt[d]

[a]*Department of Pharmaceutical Biology, Institute of Pharmacy, Johannes Gutenberg-University, Staudinger Weg 5, D-55099 Mainz, Germany*

[b]*Institute of Cell Biology and Genetic Engineering, National Academy of Sciences of Ukraine, Zabolotnogo str. 148, 252143 Kiev, Ukraine*

[c]*Finzelberg GmbH CoKG, Koblenzerstr. 48-56, 56626 Andernach, Germany*

[d]*AnalytiCon AG, Hermannswerder Haus 17, 14473 Potsdam, Germany*

Abstract

Systems for efficient separation of selected alkaloid groups by high performance liquid chromatography (HPLC), capillary electrophoresis (CE) and capillary electrophoresis coupled with electrospray ionisation mass spectrometry (CE–ESI-MS) are described. The optimized HPLC system was applied for the separation of 23 standard indole alkaloids as well as for qualitative and quantitative analyses of crude alkaloid extracts of *Rauvolfia serpentina*×*Rhazya stricta* hybrid cell cultures. The developed conditions for CE analysis proved to be efficient for separation of mixtures of standard indole and β-carboline alkaloids. The described buffer system is also applicable in the combination of CE with electrospray ionisation mass spectrometry. This analytical technique allowed the separation and identification of components of standard indole alkaloid mixture as well as crude extracts of *R. serpentina* roots, *R. serpentina* cell suspension cultures and cortex of *Aspidosperma quebracho-blanco*. The influence of buffer composition and analyte structures on separation is discussed.
© 2002 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Hybrid plant cell suspension cultures; *Rauvolfia serpentina*; *Rhazya stricta*; *Aspidosperma quebracho-blanco*; Apocynaceae; Monoterpenoid indole alkaloids; Alkaloids; β-Carboline alkaloids

Contents

1. Introduction 86
2. HPLC-analysis of alkaloid profiles in hybrid plant cell cultures 86
   2.1. Optimization of the HPLC-system for the separation of indole type alkaloids 88
   2.2. Alkaloid pattern in non-induced and induced cells 89
      2.2.1. *Rauvolfia serpentina*×*Rhazya stricta* cell suspension cultures 89

*Corresponding author. Tel.: +49-6131-392-5751; fax: +49-6131-392-3752.
E-mail address: stoeckig@mail.uni-mainz.de (J. Stöckigt).

0021-9673/02/$ – see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0021-9673(02)00037-7

2.2.2. Investigation of the *R. serpentina*×*R. stricta* cell suspension cultures (line *R*×*R*17M) 92
2.2.3. Investigation of the *R. serpentina*×*R. stricta* cell suspension cultures (line *R*×*R*17K) 92
2.3. Structures of identified alkaloids 94
2.3.1. Investigation of the *R. serpentina*×*R. stricta* cell suspension cultures (line *R*×*R*17M) 94
2.3.2. Investigation of the *R. serpentina*×*R. stricta* cell suspension cultures (line *R*×*R*17K) 95
2.3.3. Comparison of the phytochemical characteristics of *R*×*R*17M and *R*×*R*17K cell suspension cultures 98
2.4. Influence of induction on the accumulation of single alkaloids 98
3. CE-analysis of alkaloids 99
3.1. Recent CE-analyses of alkaloids 100
3.2. CE-analyses of indole and related alkaloids 100
3.2.1. CE separation of indole alkaloids 100
3.2.2. CE-analysis of β-carboline alkaloids 103
4. CE–ESI-MS analysis of alkaloids 104
4.1. Electrospray ionisation mass spectrometry (ESI-MS) 104
4.2. ESI-MS–MS 105
4.3. CE–ESI-MS system applied to alkaloid standards 106
4.4. CE–ESI-MS analysis of crude alkaloid extracts 108
4.4.1. *Rauvolfia* root alkaloids 108
4.4.2. *Rauvolfia* alkaloids from cell suspension cultures 109
4.4.3. Alkaloids from Cortex Quebracho 110
5. Conclusions and prospects 111
Acknowledgements 111
References 111

1. Introduction

Alkaloids represent a fascinating group of natural products for several reasons. Such compounds belong to one of the largest groups of secondary metabolites comprising ~10 000 different structures often with remarkable complexity. Many exhibit important biological and pharmacological activities and for several decades have been therapeutically applied in the treatment of various diseases. Some of the alkaloids with impressive activities which have prompted the development of broadly applied drugs on the pharmaceutical market, include the well known cytotoxic bisindole alkaloids vincaleucoblastine/vincristine from *Catharanthus*, the diterpenoid alkaloid taxol from *Taxus*, the highly important analgesic morphine, the spasmolytics tubocurarine and papaverine, the vasodilating agents vincamine and ajmalicine, emetine with its emetic activity, the antitussive codeine, galanthamine for the treatment of Alzheimer disease and the antiarrhythmic alkaloids quinidine and ajmaline [1].

The diversity of alkaloid structures forced scientists to concentrate during recent decades on the elucidation of biosynthetic pathways at the enzymatic level. Now the first examples exist for the detection of series of enzymes catalyzing multistep biosynthetic sequences, e.g. in the field of isoquinoline alkaloids [2], indole bases [3] and pyrrolizidine alkaloids [4]. Thus, during the last few years a most successful study of the molecular genetics of alkaloid formation has been undertaken [5] including the first examples of heterologous expression of appropriate enzymes catalyzing alkaloid metabolism [6–10].

Usually all the alkaloids occur in multicomponent mixtures and separation of alkaloids from other groups of natural products is the first requirement for detailed qualitative, quantitative and structural analysis of single alkaloids. In this article we will present an overview on the separation of selected alkaloid groups by high performance liquid chromatography (HPLC), capillary electrophoresis (CE) and capillary electrophoresis coupled with mass spectrometry (CE–ESI-MS), thus indicating the range of useful methods now available for alkaloid analysis.

2. HPLC-analysis of alkaloid profiles in hybrid plant cell cultures

One of the most effective methods for the quali-

tative and quantitative analysis of alkaloids is HPLC because of its high resolution power and automatisation. Different stationary phases were used in the past for normal phase packing, e.g. aluminum oxide [11] and silica gel [12], or reversed-phase materials like RP8 [13,14] or RP18 [15–18].

In some cases ion-exchange columns were used to determine alkaloids such as the highly polar polyhydroxy alkaloids [19] or opium alkaloids [20]. The use of acetonitrile–water mixtures with ammonium formiate as eluent allowed the coupling to MS [19,21]. When diluted hydrochloric acid was used as an eluent for ion-exchange chromatography, the direct measurement of biological activity could be performed after addition of phosphate to the obtained fractions [22].

Liquid chromatography on reversed-phase columns is nowadays ''state of the art'' in the analysis of natural products and especially alkaloids. The most attractive advantage of RP chromatography is the ability to determine simultaneously a wide variety of compounds which differ markedly in molecular structure, molecular mass, polarity and acidity/basicity. Only very polar compounds, like the above mentioned polyhydroxy alkaloids or derivatives lacking a UV absorbing chromophore, are difficult to separate with reversed-phase columns followed by conventional UV detection. To overcome these problems the application of gas chromatography is an excellent alternative [23]. However, for HPLC-analysis of basic alkaloids with reversed-phase material no generally applicable method exists. On the one hand, the basic properties of the substances which lead to a high polarity when dissolved in water result in a decreased affinity to the lipophilic stationary phases applied. On the other hand, the silanophilic properties of the silica gel, e.g. ionic interaction with deprotonated silanol groups or hydrogen bonding, may lead to additional retention mechanisms and eventually tailing peaks. In the latter case the hydrogen bonds between silanol groups and analytes can strongly improve the separation of hydroxylated compounds such as cardiac glycosides or glucoalkaloids. But the major problem is the strong interaction of basic compounds with anionic silanol functions resulting in strong tailing and sometimes total loss of signal.

Some strategies are commonly applied to solve these problems. The adjustment of the pH value of the buffer to neutral or weakly basic conditions is a possible way to avoid the ionic attraction by partially deprotonating the cationic alkaloids. Unfortunately, a fully conversion of the protonated alkaloids to their corresponding bases would require a buffer pH value which lies more than two units above the pK values of the analytes. In detail, this would mean that for a giving compound possessing a pK value of 7.5, the resulting pH value for HPLC has to be higher than 9.5. Reversed-phase silica gel is very unstable at these conditions even if a rigorous endcapping is performed. As a consequence most applications are performed using a low pH value (below 3) where silanol groups are fully protonated. If the alkaloids are weak bases, the use of phosphate, citrate or Tris at neutral to medium basic conditions in some cases can help to manage the problem. Whereas phosphate and citrate are good counterions for the cationic alkaloids, Tris serves as a counterion for the deprotonated silanol groups. Also the buffering capacity of phosphate and Tris is excellent at the above mentioned conditions. It should be noted that the application of moderate basic pH values in the HPLC-analysis of weak bases often strongly improves the selectivity if severe peak broadening does not occur.

Besides the discussed strategies, the use of specifically designed RP material sometimes helps to suppress unwanted retention mechanisms. Blocking of free silanol groups with methyl or ethyl functions after the derivatization of the silica gel with, for example, octadecyl silanes can effectively minimize ionic interaction between deprotonated silanol groups and cationic analytes, but also reduces polar interaction which sometimes improves selectivity.

A highly effective but more expensive method of avoidance of the ionic mechanisms is the use of ion pair reagents such as hexane-, heptane- or octanesulfonic acids [24–27]. They are often used in concentrations up to 50 mM. Their high affinity to protonated bases and their low pK_a value, which provides a quantitative dissociation over a wide pH range, makes them an attractive alternative to the cheaper amines. The biggest disadvantage of the ion pair reagents is their irreversible adsorption to the stationary phase especially when RP18 material is used in combination with long chain acids; additionally ion pair reagents are not as volatile as the basic

amines. The concentration of ion pair reagents can be minimized through the use of buffers with high contents of salts like phosphate or acetate because of the high affinity of these ions to the positively charged alkaloids, which leads to the formation of uncharged ion pairs. Thus ionic interactions between the analytes and deprotonated silanol functions decrease, while the higher lipophilicity of the ion pair improves the retention of the analytes at the lipophilic RP material. Since acetate ions tend to form complexes with iron present in stainless steel columns, phosphate is the best alternative for ion pair chromatography of alkaloids on reversed-phase materials.

2.1. Optimization of the HPLC-system for the separation of indole type alkaloids

As RP18 material provides the highest affinity to lipophilic structures, such as the carbon skeletons of monoterpenoid indole alkaloids, and also shows the best shielding of the silica gel [28–30], we decided to apply an RP18 column for the optimization of the buffer system which we had developed for a more general application to alkaloid analysis. Unless otherwise stated, for all initial experiments we used isocratic conditions with acetonitrile and a 25 m*M* phosphate buffer as eluents.

The formation of uncharged and thus more lipophilic ion pairs depends on (i) the concentration of the counter-ions, (ii) the pH value of the buffer, (iii) the dissociation constant of the ion pair reagents and analytes, and (iv) the dielectric constant of the eluent mixture which favors the ionic mechanisms between the cationic alkaloids and the anionic sulfonic acid [31]. The lower the dielectric constants of the solvents used, the higher are the formation rates of ion pairs. For this reason we chose acetonitrile as eluent and an acidic buffer solution which provided a strong dissociation of the sulfonic acid and a quantitative protonation of the basic alkaloids.

Phosphate proved to be good counter-ion for the cationic alkaloids, so that it was possible to reduce the amount of ion pair reagent to concentrations below 10 m*M*. Since the most crucial point for optimization of the method is the pH value of the buffer, we tested different pH values of a 25 m*M* phosphate buffer containing 10 m*M* hexanesulfonic acid in the range of 2.0–3.5. Besides a small deterioration in the peak shape using a pH value above 3, no significant changes in separation efficiency were observed. Because pH values below 2.5 give rise to problems with stationary phases based on reversed-phase materials and an increased buffering capacity of phosphate at pH value below 3, we decided to use a buffer pH value of 2.5. The next step was to optimize the concentration of the ion pair reagent. The influence of hexanesulfonic acid concentration on the separation capacity of the system has been studied. It was observed that decreasing the hexanesulfonic acid concentration to zero led to reduction of differences in the retention times of compounds (Fig. 1). It was also observed that the shape of peaks became clearly broader (Fig. 1a), and increasing the hexanesulfonic acid concentration (up to 25 m*M*) gave no improvement in the peak separation (data not shown). Thus the optimum concentration of hexanesulfonic acid was found to be 2.5 m*M*. Further manipulations of the gradient sometimes allowed improved separation of some alkaloids with similar retention times. Nevertheless, such modifications did not cause substantial alterations in the general character of the separation.

Finally, we investigated the influence of the electrolyte on the separation of the alkaloids and we observed that a higher concentration of phosphate (39 m*M*) further improved the peak shape of the analytes without a loss of resolution or major changes in retention times. The next investigation revealed that the reduction of the salt concentration in the buffer could significantly affect the separation of alkaloids. Decreasing the salt concentration by a factor of 10 (up to 3.9 m*M*), accompanied by 2-fold reduction of hexanesulfonic acid concentration (up to 1.25 m*M*), led to a dramatic worsening of crude alkaloid extract separation when the pH value was 2.5 (Fig. 2a). Carrying out HPLC-analysis with the above-mentioned concentrations of both compounds and with pH adjusted up to 4.4 (Fig. 2b) and, then, at pH 5.5 (Fig. 2c), we were able to improve the separation considerably although not reaching the best origin level (Fig. 2d). The experiments at pH 4.4 and 5.5 were performed in triplicate. No changes in the retention times of the compounds were observed indicating that the very low buffer capacity was still sufficient under the described conditions.

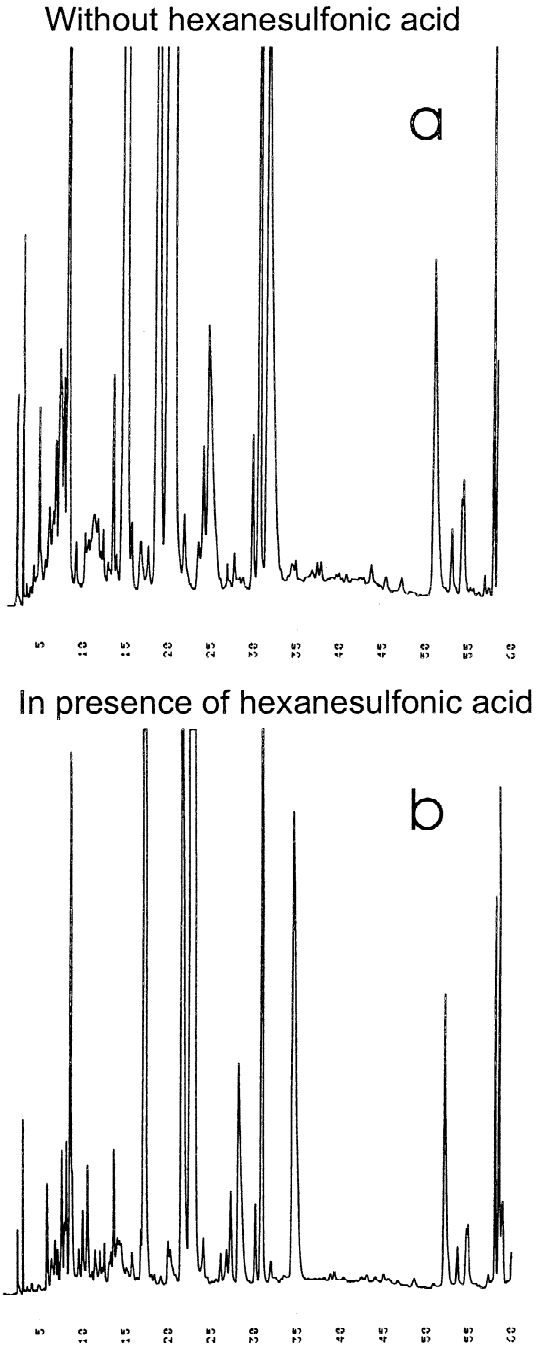

Fig. 1. HPLC separation of the extracts of *Rauvolfia serpentina*×*Rhazya stricta* hybrid cell cultures (*R*×*R*17M, 5th day after methyl jasmonate treatment); (a) acetonitrile:39 m*M* NaH_2PO_4 buffer (pH 2.5) (no hexanesulfonic acid); (b) acetonitrile:39 m*M* NaH_2PO_4/2.5 m*M* hexanesulfonic acid (pH 2.5) (standard buffer); gradient 15:85 to 20:80 within 5 min, 40:60 within 40 min, 80:20 within 15 min, 1 ml/min flow rate.

Otherwise such systems with much reduced salt concentration could be useful in cases where the presence of large amounts of chemicals in the buffer is not desirable. For example, it is valuable for analytical procedures coupled with liquid chromatography (LC–MS, LC–NMR).

Thus HPLC was performed with a Merck/Hitachi system connected to a CC 250/4 Nucleosil 100-5 C_{18} column (Macherey-Nagel, Düren, Germany) with the solvent system acetonitrile:39 m*M* NaH_2PO_4/2.5 m*M* hexanesulfonic acid buffer (pH 2.5), gradient 15:85→20:80 within 5 min→40:60 within 40 min→80:20 within 15 min, 1 ml/min flow rate and detection at 255 nm [32].

The HPLC system finally optimized as described above was successfully applied to the separation of 23 standard indole alkaloids (Fig. 3). It is noteworthy that the application of this HPLC system allowed separation and identification not only of substances belonging to different classes of indole alkaloids, but also enabled the separation of some isomers [32]. Fig. 3 displays the separation of three epimers of ajmaline, i.e. ajmaline, sandwicine and isosandwicine. The retention times of these alkaloids are clearly distinguishable and might be used for qualitative determination of the alkaloids in a crude extract. Nevertheless, one should note that the attempts to separate other alkaloids (for example, vomilenine and yohimbine) did not lead to good separations. This does not however contradict the possibilities for further improvement of this system in order to find conditions suitable for the separation of compounds under investigation.

2.2. Alkaloid pattern in non-induced and induced cells

2.2.1. Rauvolfia serpentina×Rhazya stricta cell suspension cultures

It has been reported over the last two decades that jasmonate and methyl jasmonate (MJ) can not only act as plant hormones but are also involved in tendril coiling [33], in the induction of proteinase inhibitors [34] and conjugates of cinnamic acids, like coumaric acid [35]. Both substances can also induce the biosynthesis of benzophenanthridine and monoterpenoid indole alkaloids in plant cell suspension cultures of, for example, *Eschscholtzia californica*

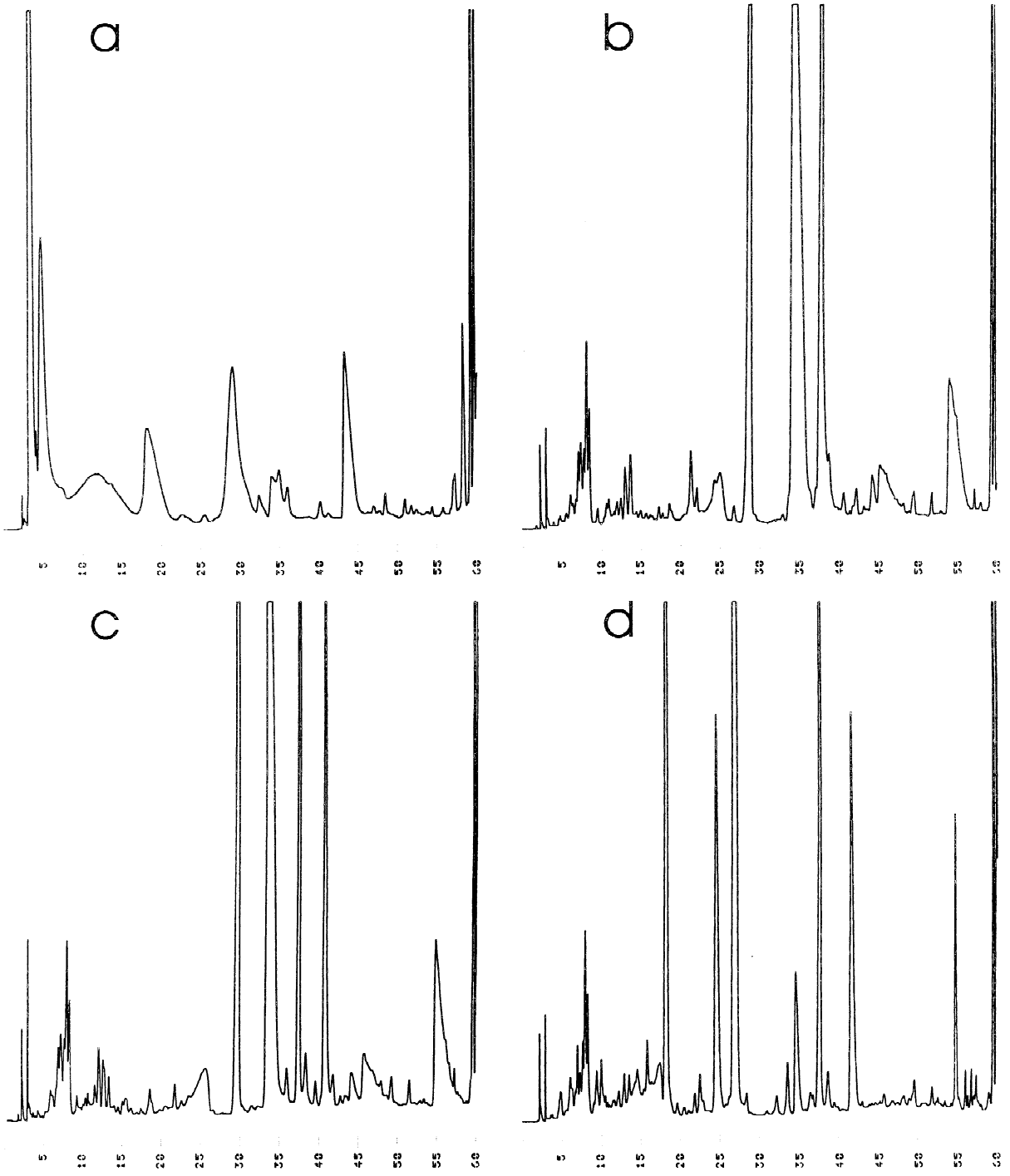

Fig. 2. HPLC separation of the extracts of *R. serpentina*×*R. stricta* hybrid cell cultures (*R*×*R*17M, 5th day after methyl jasmonate treatment): (a) acetonitrile:3.9 m*M* NaH_2PO_4/1.25 m*M* hexanesulfonic acid buffer (pH 2.5); (b) acetonitrile:3.9 m*M* NaH_2PO_4/1.25 m*M* hexanesulfonic acid buffer (pH 4.4); (c) acetonitrile:3.9 m*M* NaH_2PO_4/1.25 m*M* hexanesulfonic acid buffer (pH 5.5); (d) acetonitrile:39 m*M* NaH_2PO_4/2.5 m*M* hexanesulfonic acid buffer (pH 2.5); gradient 15:85 to 20:80 within 5 min, 25:75 within 20 min, 40:60 within 20 min, 80:20 within 15 min, 1 ml/min flow rate [32].

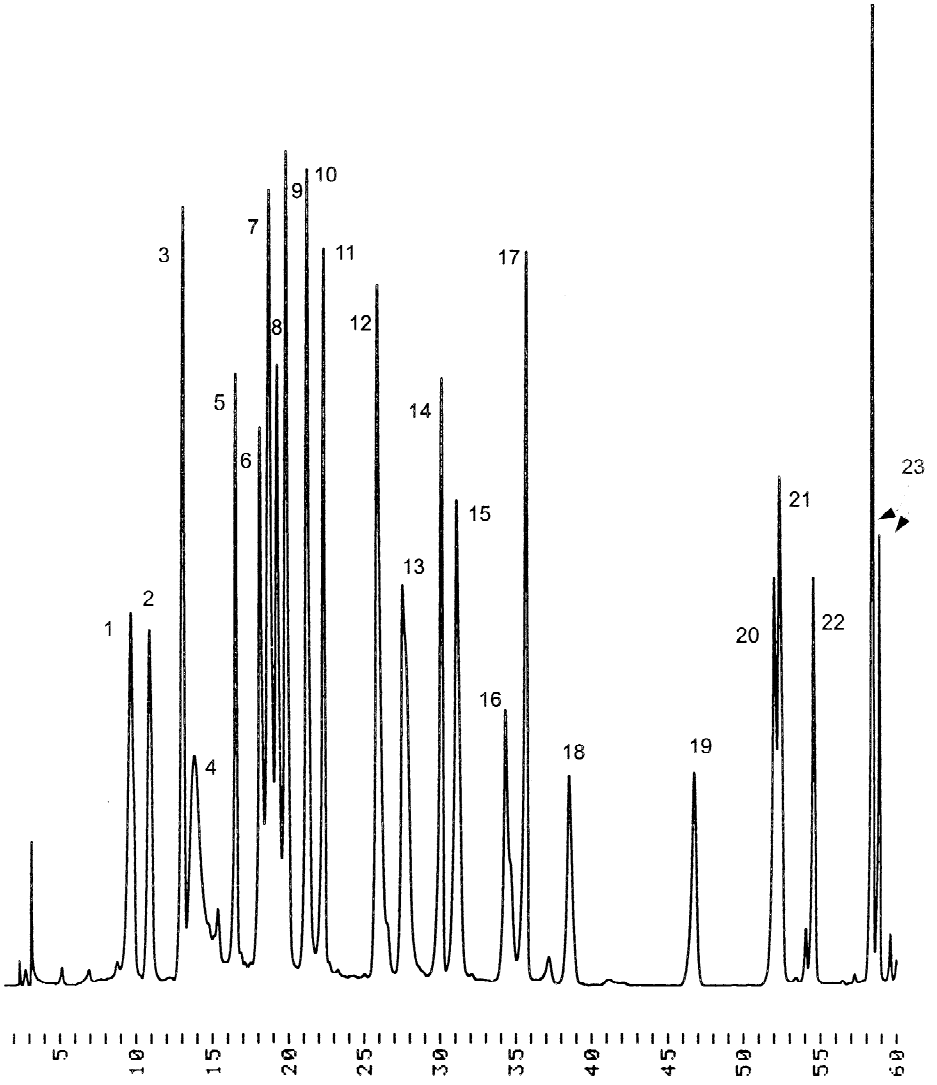

Fig. 3. HPLC separation of a standard mixture of indole alkaloids: (1) sarpagine; (2) tryptamine; (3) 19(*S*),20(*R*)-dihydroperaksine; (4) perakine; (5) raucaffricine; (6) stemmadenine; (7) isosandwicine; (8) sandwicine; (9) ajmaline; (10) vomilenine; (11) strictosidine; (12) (−)eburnamonine; (13) 17-*O*-acetyl-norajmaline; (14) strictosidine lactam; (15) ajmalicine; (16) 17-*O*-acetyl-ajmaline; (17) vincoside lactam; (18) 17,21-*O*-diacetylajmaline; (19) 21-*O*-acetylvomilenine; (20) reserpine; (21) deserpidine; (22) rescinnamine; and (23) isomers of vallesiachotamine [32].

and *Rauvolfia canescens* [36], as well as taxanes in *Taxus* cell suspension cultures [37,38]. These latter effects are interesting from several points of view, for instance to enhance the accumulation of certain alkaloids or to increase the appropriate enzyme amounts which could probably simplify the isolation and purification of these proteins. And, if this induction is well pronounced, it can also be used for the establishment of cell cultures with high levels of cytochrome P450 reductase activities of alkaloid formation [39,40]. Such cultures would then be efficient materials for cloning and heterologous expression of these reductases as it has already been shown for such expression in yeast and insect cells [41,42]. On the other hand stimulation of alkaloid biosynthesis would be desired in cases where cell

culture systems provide only trace amounts of alkaloids and do not allow simple isolation and identification.

This is especially true for extraordinary systems like hybrid cells. In recent years, we have generated such hybrid cultures by somatic hybridization techniques from two apocynaceous medicinal plant-derived cell cultures, *R. serpentina* Benth. ex Kurz and *R. stricta* Decaisne [43]. Both single cell systems had been well investigated earlier concerning their alkaloid pattern [44,45]. After optimization of growth parameters, the hybrid cells could be generated routinely in the kilogram range but in general showed only low alkaloid production, especially as many trace alkaloids were formed. A detailed phytochemical investigation of this hybrid system proved the biosynthetic formation of some alkaloids of both parent cell lines and also of alkaloids which were previously not known from the parents, e.g. β-carboline, 1-acetyl-β-carboline, 1-methoxycarbonyl-β-carboline and 5(*S*)-5-carbomethoxystrictosidine [46,47]. All these investigations were hampered by the small alkaloid yields. Therefore we applied MJ to induce the alkaloid diversity in this hybrid cell culture system and analyzed the resultant mixture by the above developed HPLC system. We compared induced and non-induced cells from hybrid lines cultivated independently in two places, at the Institute of Pharmacy in Mainz (line *R*×*R*17M maintained as cell suspension cultures) and at the Institute of Cell Biology and Genetic Engineering in Kiev (line *R*×*R*17K maintained as callus cultures).

2.2.2. Investigation of the R. serpentina×R. stricta cell suspension cultures (line R×R17M)

Line *R*×*R*17M was grown as a fine suspension in LS-nutrition medium in 1-l Erlenmeyer flasks for 7 days with shaking (100 rpm) and constant light (~ 600 lx). For alkaloid induction MJ was added in 70% ethanol to a final concentration of 100 μ*M* at the 5th day of growth. Control cultures were treated with the corresponding amount of ethanol. Isolation of the crude alkaloid mixture from cells and from the nutrition medium was performed on the 1st and the 5th day after MJ treatment by a procedure described recently [27]. The HPLC-analysis of these mixtures was then carried out as mentioned in Section 2.1.

Fig. 4 exhibits HPLC separations of alkaloids of *R*×*R*17M cell suspension culture on the 5th day after MJ treatment. The major alkaloids which were found in extracts are represented by perakine, norajmaline, raucaffricine, ajmaline, vomilenine, strictosidine, strictosidine lactam, 17-*O*-acetylajmaline, reserpine, ajmalicine and isomers of vallesiachotamine (Fig. 4). As can be easily recognized, the treatment of the cell suspension with MJ led to a general increase of alkaloid content. The enormous induction of alkaloid biosynthesis is obvious. Based on HPLC signal intensities, the induction rates were, for example, for compound 3 (raucaffricine) 5-fold, for compound 6 (strictosidine) 47-fold and for compound 8 (17-*O*-acetyl-norajmaline) 43-fold. The contents of the compounds mentioned above as well as of some other induced alkaloids in the cells and in the nutrition medium are displayed in Table 1, indicating that except for the concentration of the alkaloid ajmalicine, all others are enhanced. It can therefore be concluded that MJ treatment in the case of the here described hybrid cell systems should be an efficient method of generation of sufficient alkaloid amounts for isolation, purification and structure elucidation. As illustrated in Table 1, the amounts of alkaloids found in the nutrition medium of treated and non-treated cells are low compared to the alkaloids isolated from cells. For this reason the excretion (or accumulation of alkaloids by cell rupture) into the surrounding nutrition medium is not interesting from the phytochemical point of view for these cultures. It can be concluded that application of the induction of alkaloid biosynthesis under these conditions has allowed for the first time isolation and structural identification of a great range of alkaloids from cultivated hybrid cells.

2.2.3. Investigation of the R. serpentina×R. stricta cell suspension cultures (line R×R17K)

The hybrid cell subcultures *R*×*R*17K have been cultivated since 1989 [43], but cells were maintained as suspension cultures only for 6 months before phytochemical analysis. These *R*×*R*17K cell suspensions were treated with MJ as described above on the 10th day after cell inoculation. Biomass was harvested on the 5th day after MJ treatment. Crude alkaloid extracts of the hybrid cells were investigated with the above described HPLC technique (Fig. 5).

The glucoalkaloids strictosidine and strictosidine

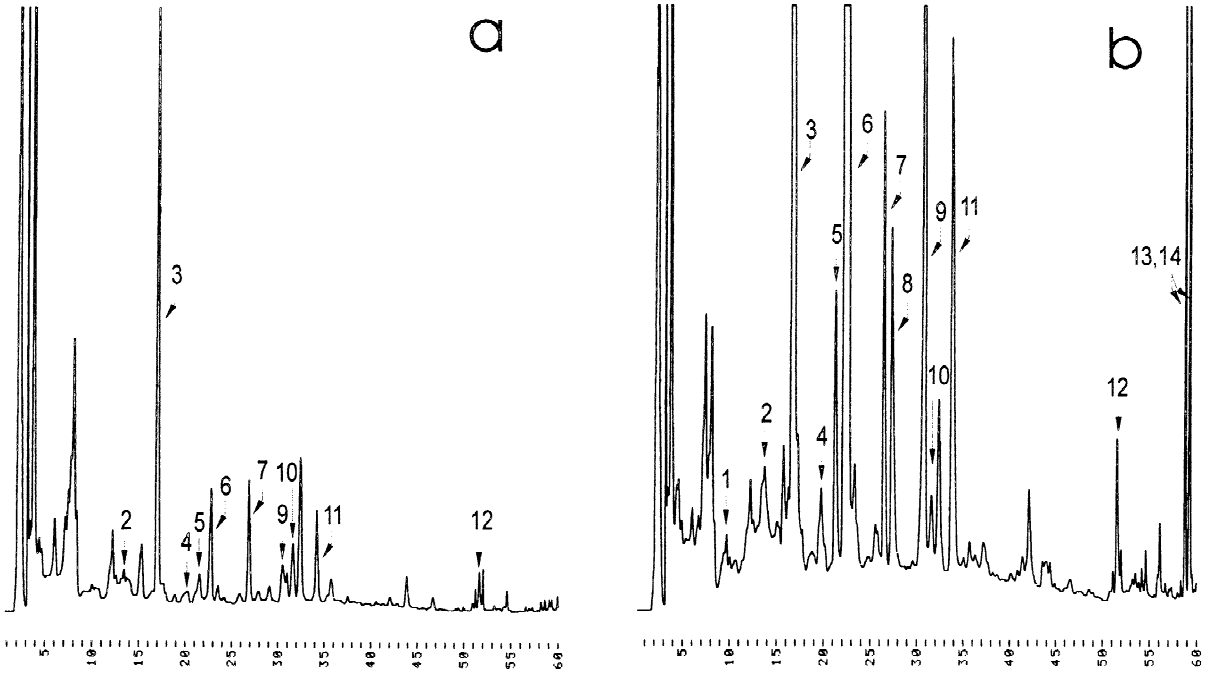

Fig. 4. HPLC separation of the extracts of *R. serpentina*×*R. stricta* hybrid cell cultures (line *R*×*R*17M) (control (a) and MJ treated cultures (b)) on the 5th day after treatment. The major alkaloids were identified: (1) sarpagine; (2) perakine, norajmaline; (3) raucaffricine; (4) ajmaline; (5) vomilenine; (6) strictosidine; (7) unidentified alkaloid with MW 410; (8) 17-*O*-acetyl-norajmaline; (9) strictosidine lactam; (10) ajmalicine; (11) 17-*O*-acetylajmaline; (12) reserpine; and (13,14) isomers of vallesiachotamine.

lactam were identified as the major components in the *R*×*R*17K hybrid cell culture. In addition stemmadenine, tryptamine, tubotaiwine, and isomers of vallesiachotamine have been identified in extracts of these cells.

As distinct from *R*×*R*17M hybrid cell suspen-

Table 1
The average alkaloid contents[a] in *R*×*R*17M hybrid culture medium and cells on the 5th day after MJ treatment

Alkaloid	Control		MJ-treated	
	Cells[b] (mg/100 g D.W.)	Medium[c] (mg/l)	Cells[b] (mg/100 g D.W.)	Medium[c] (mg/l)
17-*O*-Acetylajmaline	8.6	1.0	46.3	5.6
17-*O*-Acetyl-norajmaline	2.6	0.1	111.6	4.8
Ajmaline	2.2	–	7.8	0.4
Ajmalicine	3.7	–	4.3	0.4
Raucaffricine	76.4	–	341.6	–
Reserpine	2.0	–	5.5	0.1
Strictosidine	11.1	–	513.1	–

–, Alkaloid concentration less than 0.1 mg/l.

[a] The differences between all respective means for MJ-treated and control cultures are significant ($P=0.95$) except for the ajmalicine concentration means in the hybrid tissue.

[b] 100 g of dry weight corresponds to ~7 l of cell suspension.

[c] 1 l of filtered medium corresponds to ~2.5 l of cell suspension.

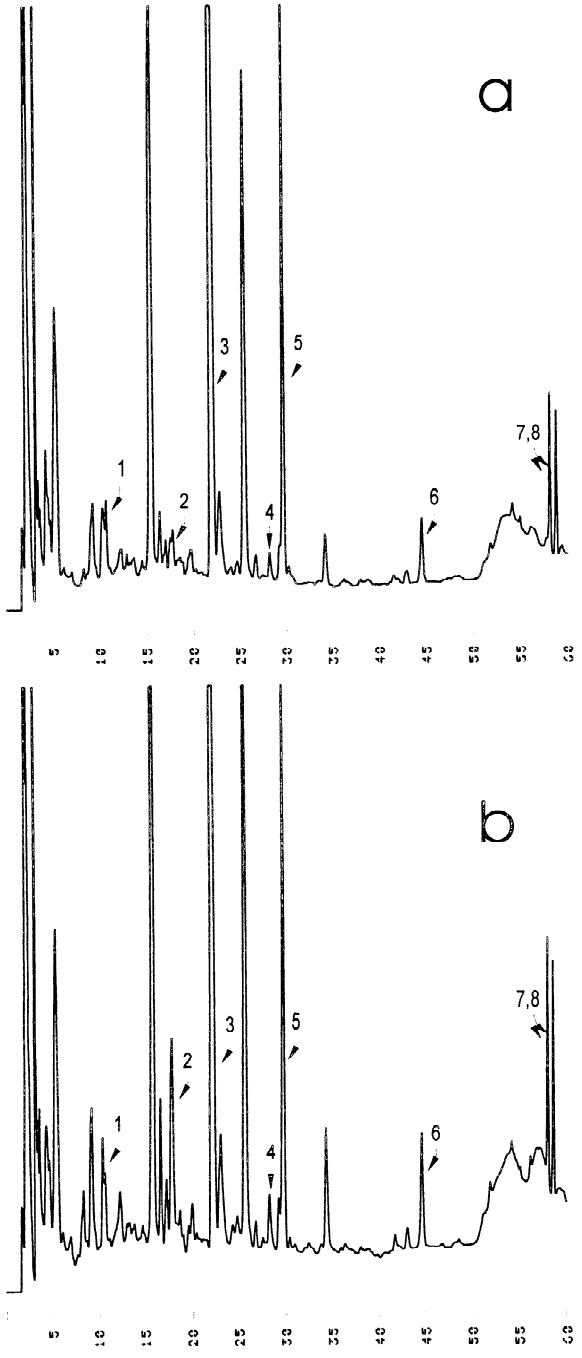

Fig. 5. HPLC separation of the extracts of *R. serpentina*×*R. stricta* hybrid cell cultures (line *R*×*R*17K) (control (a) and MJ treated cultures (b)) on the 5th day after treatment. The major components were identified: (1) tryptamine; (2) stemmadenine; (3) strictosidine; (4) tubotaiwine; (5) strictosidine lactam; (6) unidentified alkaloid with MW 308; and (7,8) isomers of vallesiachotamine.

sions, *R*×*R*17K hybrid subcultures treated with MJ (Fig. 5b) did not exhibit any pronounced alteration of alkaloid production when compared with the control samples (Fig. 5a). One of the possible explanations of this phenomenon could be based on the assumption that some MJ controllable genes (for instance, strictosidine synthase [48]) in *R*×*R*17K hybrid subcultures had originally a high level of expression and could not be increased by MJ treatment. This assumption is supported by the fact that the original concentration of strictosidine in the untreated *R*×*R*17K cultures was much higher than in *R*×*R*17M hybrid cells (0.37 g/100 g of dry weight and 0.01 g/100 g of dry weight, respectively). The limitation of the biosynthesis due to the deficiency of one of the substrates for strictosidine synthase, e.g. secologanin, might also be a reason for the presence of tryptamine in the culture extracts. The results described above present a graphic illustration of genetic and phytochemical divergence between two hybrid subcultures, which were maintained differently as callus (*R*×*R*17K) or suspension (*R*×*R*17M) cell cultures for ~8 years.

2.3. Structures of identified alkaloids

2.3.1. Investigation of the R. serpentina×R. stricta cell suspension cultures (line R×R17M)

Because of the good growth characteristics of the hybrid cells, the cell material for alkaloid isolation was not a limiting factor. A 200-g sample of dry material of the induced cells was extracted and the alkaloids pre-purified as described earlier [27]. Using common techniques of alkaloid separation (TLC, HPLC, flash chromatography) and a number of alkaloid identification methods (HPLC, UV, MS, NMR, derivatization and comparison with reference compounds), 26 alkaloids were characterized (Table 2, Fig. 6) consisting of five ajmaline-, four indolenine-, four yohimbine-, two oxindole-, two sarpagine-, two tetraphyllicine-, one heteroyohimbine-, two macroline-type alkaloids and five alkaloids of miscellaneous structures, which demonstrates the great diversity of alkaloid biosynthesis in these cultivated hybrid cells. Compared with earlier results on the analysis of the alkaloid pattern of this hybrid cell line, this was the first time that a detailed analysis was performed with most of the major

Table 2
Alkaloids identified in *R. serpentina*×*R. stricta* cell suspension culture (line *R*×*R*17M) after MJ treatment

Compound	Analytical method
Ajmalicine	MS, UV, co-TLC, HPLC, CAS
Isomer of yohimbine	MS, co-TLC, CAS
Rescinnamine	MS, co-TLC, CAS
Reserpine	MS, UV, co-TLC, CAS, HPLC, ^{1}H-NMR
Deserpidine	MS, UV, CAS
Sarpagine	MS, co-TLC, CAS
21-Hydroxysarpagan-glucoside	MS, CAS
Vinorine	MS, co-TLC, CAS
Vomilenine isomers	MS, UV, co-TLC, CAS, HPLC, ^{1}H-NMR
Raucaffricine	MS, UV, co-TLC, CAS, HPLC, ^{1}H-NMR
Perakine	MS, UV, co-TLC, HPLC, CAS
17-*O*-Acetyl-norajmaline	MS, UV, co-TLC, CAS, ^{1}H-NMR
Norajmaline	MS, UV, co-TLC, CAS
Ajmaline	MS, UV, co-TLC, CAS, HPLC, AC
17-*O*-Acetylajmaline	MS, UV, co-TLC, CAS, HPLC, ^{1}H-NMR, AC
17-*O*-Acetylrauglucine	MS, CAS
17-*O*-Acetyl-nortetraphyllicine	MS, CAS
17-*O*-Acetyltetraphyllicine	MS, CAS
Suaveoline	MS, CAS
Macrophylline	MS, CAS
Isomer of yohimbine oxindole	MS, CAS
Isomer of yohimbine pseudoindoxyl	MS, CAS
Strictosidine	MS, UV, co-TLC, CAS, HPLC, AC
Vallesiachotamine isomers	MS, UV, co-TLC, HPLC, CAS
Strictosidine lactam	MS, UV, co-TLC, HPLC, CAS
Isomer of isositsirikine	MS, CAS

AC, acetylated alkaloid.

alkaloids identified—a result which was reached by MJ induction only. Although most of the identified alkaloids have been found in cultivated *R. serpentina* cells [3,44,49], several were not previously isolated from cell systems or are rather unusual for *R. serpentina*. For instance, strictosidine lactam (although relatively easily formed from strictosidine spontaneously by hydrolysis under alkaline conditions) is not typical for *Rauvolfia*, but has been identified in cell suspensions of *R. stricta* [45]. In fact, the 18-hydroxy-yohimbine type alkaloids rescinnamine and deserpidine are well known from extracts of *R. serpentina* roots [50], but here they are found for the first time in the hybrid cells and have not so far been characterized from other *Rauvolfia* in vitro cell systems. Moreover, the alkaloids macrophylline, yohimbine oxindole and yohimbine pseudoindoxyl were never reported to occur in *R. serpentina* or *R. stricta* plants or in cell and tissue cultures derived from them, but structurally similar alkaloids were obtained in the past from other *Rauvolfia* species (plants of *Rauvolfia vomitoria*, *Rauvolfia macrophylla* [51–53]).

Considering these results and bearing in mind that a number of isolated alkaloids have yet to be structurally determined, it is quite clear that MJ treatment is an important method for efficiently harnessing the high potential of cell culture systems to generate a great diversity of natural products; the method could be very useful for the screening of plant cell systems employing HPLC fractionation combined with high throughput screening (HTS) systems. The current HPLC system should be very helpful in performing such experiments.

2.3.2. Investigation of the R. serpentina×R. stricta cell suspension cultures (line R×R17K)

The known alkaloids 1,2-dehydroaspidospermidine, rhazinilam, sarpagine, stemmadenine, strictosidine, strictosidine lactam, tabersonine, tubotai-

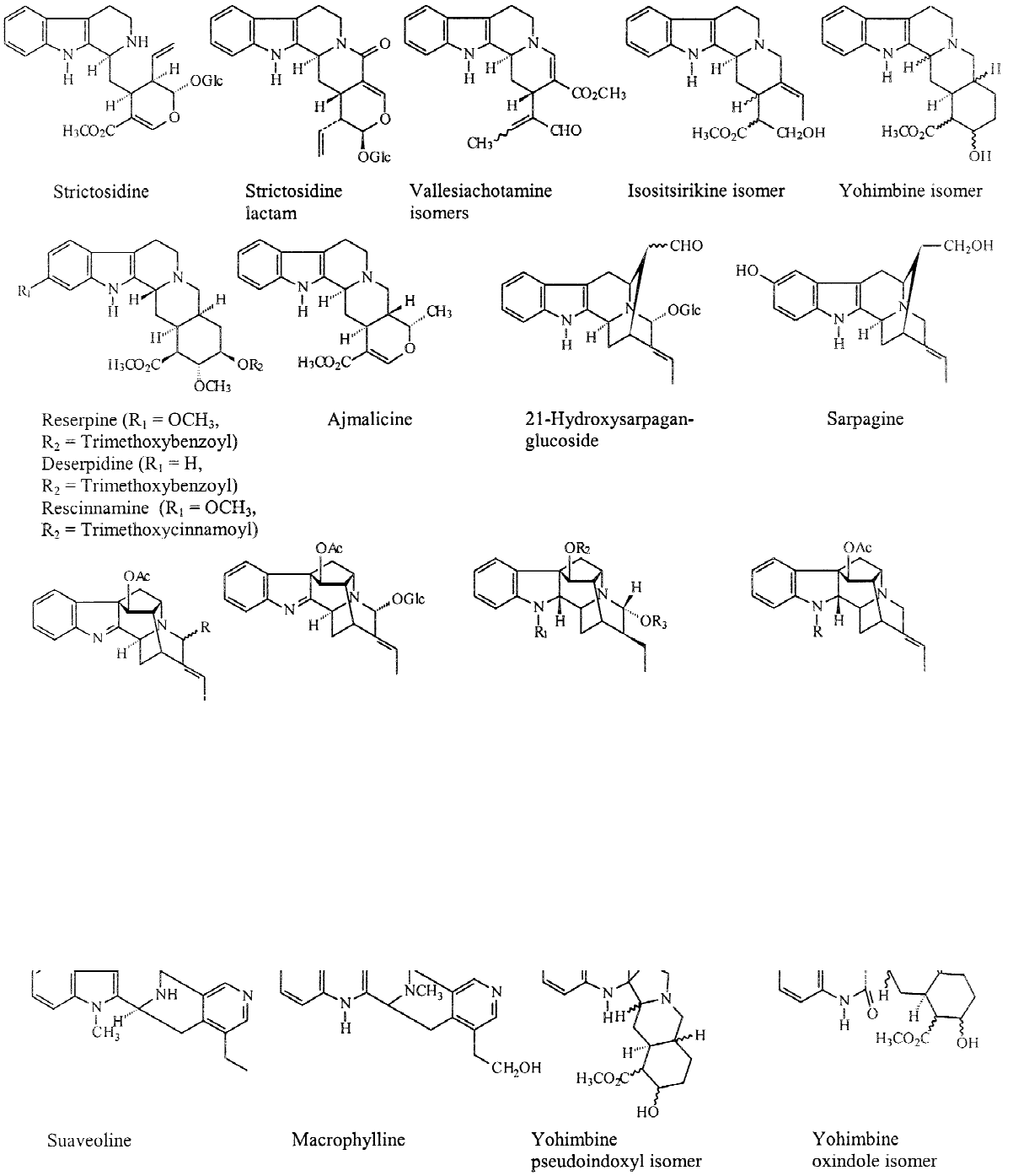

Fig. 6. Indole alkaloids synthesized in hybrid cell suspension culture of *R. serpentina*×*R. stricta* (line *R*×*R*17M) after treatment with methyl jasmonate.

Table 3
Alkaloids identified in *R. serpentina*×*R. stricta* cell suspension culture (line *R*×*R*17K)

Compound	Analytical method
Isomer of 1,2-dehydroaspidospermidine	MS, UV, co-TLC, CAS, HPLC
Rhazinilam	MS, UV, CAS, ^{1}H-NMR
Stemmadenine	MS, UV, co-TLC, CAS, HPLC, ^{1}H-NMR
Strictosidine	MS, UV, co-TLC, CAS, AC, HPLC
Strictosidine lactam	MS, UV, co-TLC, CAS, AC, ^{1}H-NMR, HPLC
Sarpagine	MS, UV, co-TLC, CAS
Isomer of tabersonine	MS, UV, co-TLC, CAS, HPLC
Tryptamine	MS, UV, co-TLC, CAS, HPLC
Tubotaiwine	MS, UV, co-TLC, CAS, ^{1}H-NMR, HPLC
Vomilenine isomers	MS, UV, co-TLC, CAS
Vallesiachotamine isomers	MS, UV, co-TLC, CAS, ^{1}H-NMR, HPLC

AC, acetylated alkaloid.

wine, vomilenine, isomers of vallesiachotamine and the indole alkaloid biogenetic precursor tryptamine were identified in *R*×*R*17K hybrid cell suspensions after applying analytical methods described above (Table 3, Fig. 7) [54].

Unlike *R*×*R*17M cell suspensions, where major alkaloids were described previously as characteristic for *R. serpentina*, the alkaloid pattern of *R*×*R*17K hybrid cell subcultures indicates the alkaloid metabolism features characteristic of *Rhazya* species. 1,2-Dehydroaspidospermidine, rhazinilam, stemmadenine, strictosidine, strictosidine lactam, and isomers of vallesiachotamine were reported in the past as isolated from different parts of *R. stricta* [55–60]. Tabersonine was isolated from plants of *Rhazya orientalis* [61]. All these alkaloids, except strictosidine and stemmadenine, were found also in the cultivated cells of *R. stricta* [45]. Earlier work on

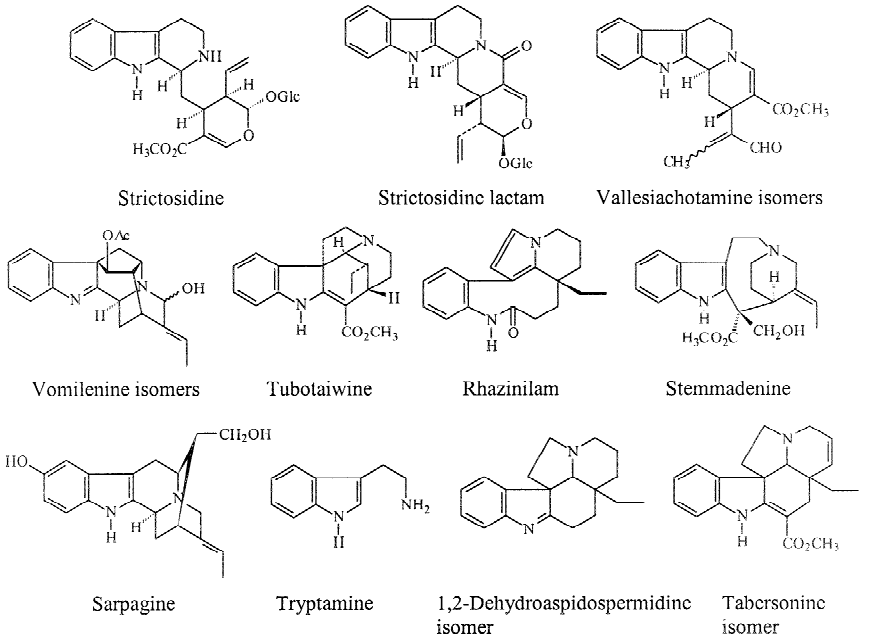

Fig. 7. Indole alkaloids synthesized in *R*×*R*17K hybrid cell suspension cultures.

alkaloids of *R*×*R*17M hybrid cell suspensions clearly indicated the occurrence of tubotaiwine in the hybrid cultures [46]. Trace amounts of sarpagine and vomilenine (alkaloids which are known to be characteristic compounds for *Rauvolfia* species) were detected in the cells suggesting that not all *Rauvolfia* alkaloid pathways are reduced in the hybrid subcultures. The detection of tubotaiwine in *R*×*R*17K hybrid cells could be evidence that the hybrid subcultures continue to display some specific features of alkaloid biosynthesis which are the consequences of their hybrid nature.

2.3.3. Comparison of the phytochemical characteristics of R×R17M and R×R17K cell suspension cultures

Summarizing and comparing the phytochemical characteristics of the hybrid cell subcultures under investigation, we can conclude that the major alkaloids of *R*×*R*17M cell line present substances described as typical for *Rauvolfia* species. Detailed investigation of the alkaloid pattern of these cell subcultures revealed no alkaloid which could be considered for a biochemical marker of the genus *Rhazya*. On the other hand, identification of alkaloids that have not yet been isolated from *Rauvolfia* plants or cell systems (e.g. isomer of yohimbine oxindole, yohimbine pseudoindoxyl) indicates that some specific attributes of secondary metabolism occur in the hybrid subcultures. In contrast to *R*×*R*17M cell line, isolation from *R*×*R*17K cell subcultures of *Aspidosperma*-type alkaloids such as 1,2-dehydroaspidospermidine, tabersonine and stemmadenine, which were detected previously in *R. stricta* plants and/or cell cultures [45,55,60], confirms that *Rhazya* biosynthetic pathways are expressed in the hybrid cells. Moreover, isolation of small amounts of sarpagine and vomilenine suggests that at least a part of the ajmaline biosynthetic pathway *Rauvolfia* genes are also still active in *R*×*R*17K hybrid subcultures. One more illustration of phytochemical divergence between the subcultures discussed here could be their different reactions with methyl jasmonate. The absence of apparent induction of alkaloid accumulation in *R*×*R*17K cell subcultures after treatment with methyl jasmonate in conditions, which caused pronounced increase of alkaloid production and/or accumulation in *R*×*R*17M hybrid cells, demonstrates the distinction of secondary metabolism regulation in the investigated hybrid cell strains.

The data presented could be evidence that both hybrid cell lines have the tendency for segregation of alkaloid biosynthetic pathways. However, despite this, they retain apparent features of alkaloid metabolism reconstruction when compared with the original parental species even after a long time of cultivation as undifferentiated cells. The occurrence of still unidentified compounds in the hybrid subcultures indicates their high potential as the source of rare or new potentially pharmacologically-active substances from plant cell cultures.

2.4. Influence of induction on the accumulation of single alkaloids

The above mentioned HPLC system was also excellent for quantitative determination of the accumulation of single alkaloids induced by methyl jasmonate.

Quantitative determinations of the seven major alkaloids of *R. serpentina*×*R. stricta* hybrid cell suspension culture (line *R*x*R*17M), e.g. 17-*O*-acetylajmaline, 17-*O*-acetyl-norajmaline, ajmaline, ajmalicine, raucaffricine, reserpine and strictosidine, were made by HPLC on the basis of peak areas compared with standard curves. The respective alkaloid concentrations in the culture tissue have been recorded and are presented in Fig. 8. The differences between all respective means for MJ treated and control cultures are significant ($P=0.95$) except for the ajmalicine concentration means in the hybrid tissue on the 5th day after treatment.

Fig. 8 displays that induction of alkaloid biosynthesis after treatment with methyl jasmonate has not been equal for all investigated compounds. The most pronounced stimulation was observed for strictosidine and 17-*O*-acetyl-norajmaline. Accumulation of strictosidine and 17-*O*-acetyl-norajmaline in MJ elicited cells on the 5th day after treatment surpassed 47 and 43 times, respectively, the content of these alkaloids in the control cultures. In fact, such level of induction means qualitative change of alkaloid pattern.

Less clear stimulation of alkaloid biosynthesis was registered for raucaffricine and 17-*O*-acetyl-ajmaline. Alkaloid formation on the 5th day after MJ

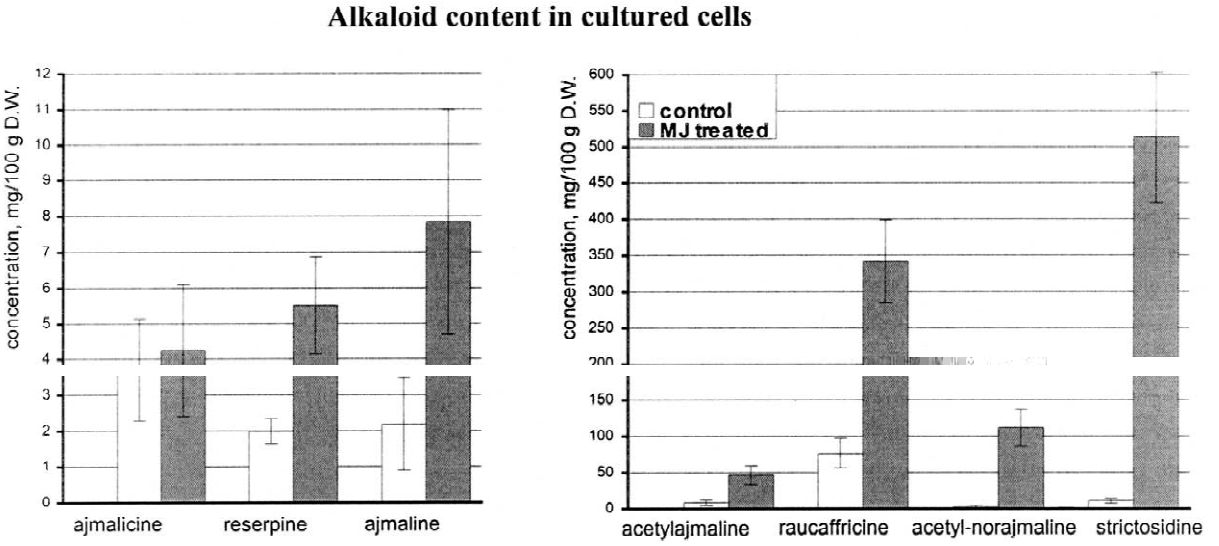

Fig. 8. Alkaloid content (mg/100 g dry weight) in the *R. serpentina*×*R. stricta* hybrid cultured cells (line *R*×*R*17M) on the 5th day after methyl jasmonate treatment. Bars represent confidence intervals ($n=4$). The differences between all respective means for MJ treated and control cultures are significant ($P=0.95$) except for the ajmalicine concentration means.

treatment led to a 4.5-fold accumulation of raucaffricine and a 5.4-fold higher yield of 17-*O*-acetylajmaline compared with the control cultures. For ajmaline and reserpine, however, only a slight increase of ~2–3-fold was observed. It is, therefore, obvious that the biosynthesis of the different alkaloids was induced to varying extents.

The results obtained also clearly indicate that the HPLC solvent system developed is excellent for the analyses of crude alkaloid mixtures. However, present and future developments in alkaloid analysis will also concentrate on other than the HPLC methodology discussed. It is evident that enormous progress has been made during recent years in the application of capillary electrophoresis (CE) for the separation of complex alkaloid mixtures and its combination with mass spectrometry (CE–MS) for the identification of alkaloidal plant constituents. The next section will discuss these points in more detail.

3. CE-analysis of alkaloids

After its introduction capillary electrophoresis soon proved to be a powerful tool for the analysis of high molecular mass molecules such as peptides and nucleic acids [62,63] and also for the analysis of small molecules such as pharmaceuticals [64] and natural products [65,66]. Two different methods were shown to be effective for the capillary electrophoresis of low molecular mass products namely micellar electrokinetic chromatography (MEKC) and capillary zone electrophoresis (CZE). Micellar electrokinetic chromatography was introduced by Terabe in 1984 and is nowadays of widespread interest because of the presence of SDS micelles in the buffer medium allowing the separation of uncharged substances [67]. This method was preferentially applied for alkaloids containing nitrogen atoms which are almost non-basic, e.g. capsaicinoids and purine alkaloids [65,66]. The rare application of MEKC for basic compounds is probably due to the low solubility of weak bases under neutral or alkaline conditions and also a possible interaction of strong bases with anionic micelles of the most often used sodium dodecyl sulfate [68]. 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 running buffer but can be also introduced by complexation [69].

Most known alkaloids represent strongly basic

molecules with a heterocyclic-bound nitrogen and exhibit pK_a values above 5. Thus in acidic electrolytes the alkaloids are protonated at the basic nitrogen atom and migrate towards the cathode. The electroendoosmotic flow enhances the migration velocity of the alkaloids because this bulk flow is normally also directed towards the cathode. The separation mechanism depends on the mass-to-charge ratio but is also influenced by the molecular structure as outlined in Sections 3.2.1 and 3.2.2.

Capillary zone electrophoresis as a highly effective method for the analysis of charged substances was used to determine alkaloids in standard solutions (Sections 3.2 and 4.3) and also in crude extracts (Section 4.4).

3.1. Recent CE-analyses of alkaloids

Alkaloid determination with capillary electrophoresis was performed several times using MEKC and CZE. Since all our experiments were performed in the capillary zone electrophoresis mode the literature given in this section will focus on citations dealing with CZE.

Among the alkaloid groups analyzed by CZE are isoquinolines [70–73], opium alkaloids [74–76], tobacco alkaloids [77,78], tropane alkaloids [79] and indole alkaloids [80–85]. The qualitative and quantitative determination of quaternary alkaloids from Phellodendri cortex was performed by Liu and Sheu who applied a 1:1 mixture of a 500 m*M* sodium acetate solution, pH 4.6, and acetonitrile as the running buffer [70]. Pietta et al. analyzed six isoquinoline alkaloids from *Chelidonium majus* using a 66 m*M* phosphate buffer at pH 2.4 containing 40% methanol [72]. Capillary zone electrophoresis was also found to be very useful for the quantification of opiates in urine [74] and opium alkaloids in poppy [75]. Nicotine and related alkaloids were quantified in tobacco products by Lu and Ralapati [78] and the main tropane alkaloids of transformed *Hyoscyamus muticus* plants were determined with a 40 m*M* phosphate buffer at pH 7.8 within less than 13 min [79]. A detailed discussion of the CZE-analysis of indole alkaloids will be given in Section 3.2.

3.2. CE-analyses of indole and related alkaloids

Capillary zone electrophoresis was applied to the analysis of oxindole alkaloids in *Uncaria tomentosa*, a slightly acidic phosphate buffer (pH 5.6) being used. The applied method was found to be useful for the quantitation of the alkaloids in crude methanolic extracts of the root bark [80]. Phosphate as an electrolyte was used for the determination of strychnine and brucine in seeds of *Strychnos nux-vomica* and also for the quantification of these alkaloids in human plasma. The applied buffer system consisted of a 9:1 mixture of 10 m*M* phosphate, pH 2.5, and methanol [81]. Lee et al. used both MEKC and CZE for the analysis of indole alkaloids in Evodiae fructus, the unripe fruits of *Evodia rutaecarpa*. For the CZE-analysis a buffer solution consisting of 40 m*M* sodium dihydrogen-phosphate–acetonitrile (9:1) was chosen and applied to the quantitative determination of the *Evodia* alkaloids in methanolic (70%, v/v) extracts of the fruits [82]. Vinblastine and vincristine were quantified in *Catharanthus roseus* leaves by Chu et al. who applied a solution of 200 m*M* ammonium acetate, pH 6.2, as an electrolyte. When methanol was added to the buffer the column efficiency increased but the resolution for vinblastine and vincristine was reduced [83]. Blaschke and co-workers identified ergot alkaloids in sclerotia of *Claviceps purpurea* by CZE with a buffer medium consisting of 20 m*M* β-cyclodextrin, 8 m*M* γ-cyclodextrin, 2 *M* urea and 0.3% polyvinyl alcohol in a phosphate buffer at a pH value of 2.5. The addition of cyclodextrin derivatives led to the separation of epimeric substances which are difficult to separate using conventional electrolytes. In addition, the limit of detection was improved 30-fold when laser-induced fluorescence detection was applied [84].

3.2.1. CE separation of indole alkaloids

In order to develop a buffer system which is capable of separating a high number of structurally different alkaloid classes, we used a standard mixture of 15 indole alkaloids varying strongly with regard to the molecule structure, basic properties, solubility and molecular mass (Fig. 9).

On account of its good buffering capacity at low pH values and its low UV absorbance, phosphate was chosen as a promising counter-ion for the alkaloids. We prepared aqueous electrolytes containing 25, 50 or 100 m*M* sodium dihydrogen-phosphate at pH values ranging from 2.0 to 3.5, but also solutions of

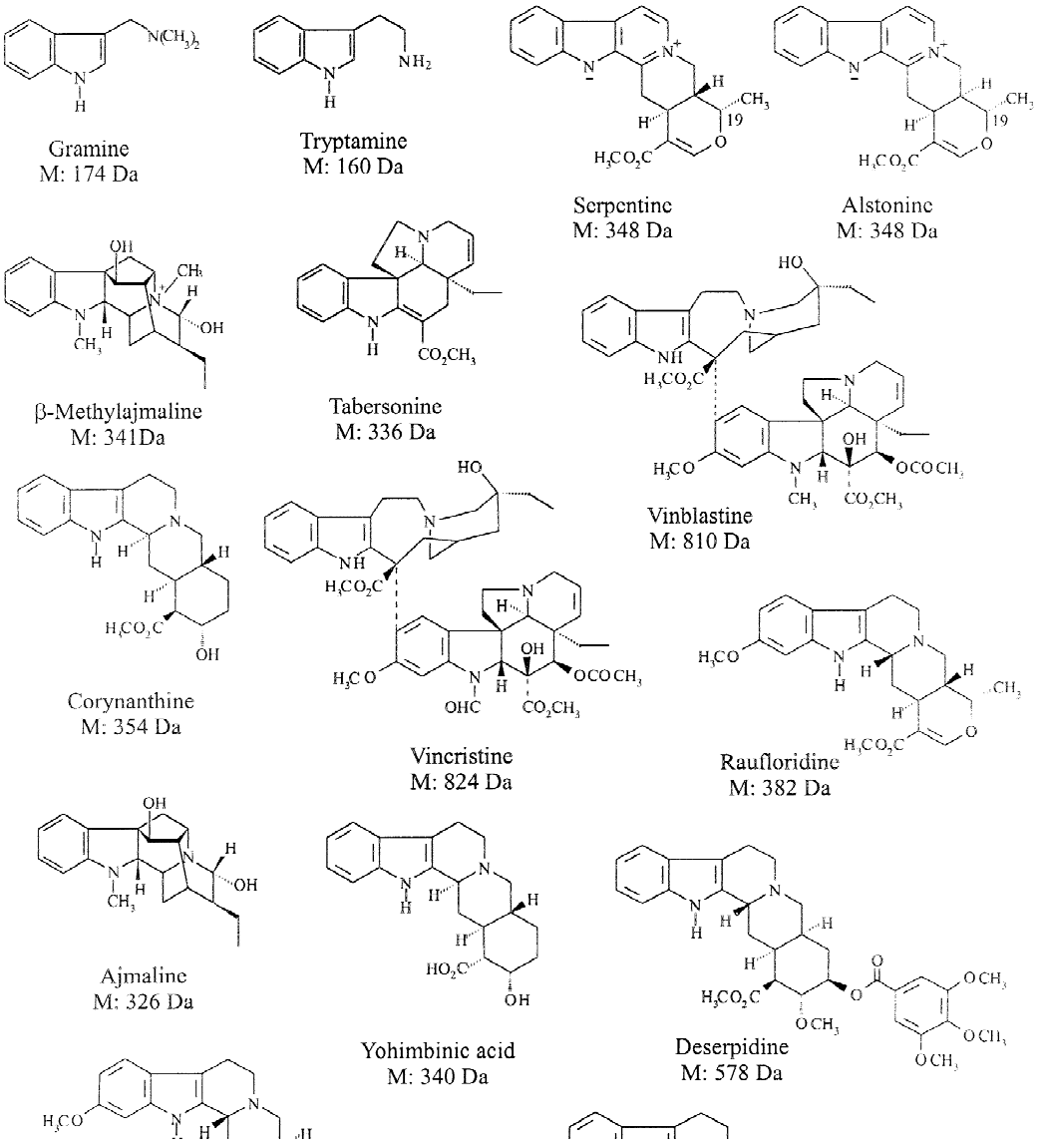

Fig. 9. The chemical formulae and nominal molecular masses of the 15 indole derivatives.

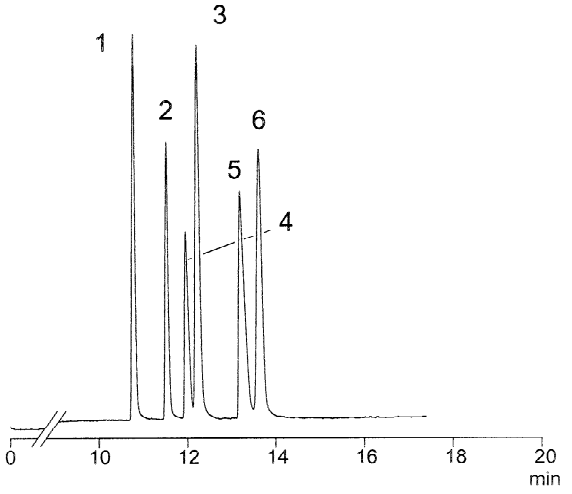

Fig. 12. CZE separation of the six β-carboline alkaloids:. (1) norharmane; (2) harman; (3) harmaline; (4) harmine; (5) harmalol; and (6) harmol. Conditions: voltage 15 kV; temperature 15 °C; UV-detection at 260 nm; buffer: 100 m*M* ammonium acetate, pH 3.1–acetonitrile 1:1; capillary: fused silica, 50 μm I.D.; total length 55 cm; effective length 50 cm.

mine and harmol. It can be speculated that the increased basicity of the resulting dihydropyridine substructure leads to the higher electrophoretic mobility of harmaline and harmalol because the mass-to-charge ratio is reduced.

Because the electrolyte described here was also successfully used for the CZE separation of quite other alkaloid groups like protoberberines, benzophenanthridines and poppy alkaloids, it can be concluded that this buffer system based on a 1:1 mixture of a 100 m*M* ammonium acetate solution, pH 3.1, and acetonitrile can be generally applied for the CZE-analysis of basic compounds and especially alkaloids [85].

As will be discussed in the following section, this buffer system is also applicable for the combination of CE with electrospray mass spectrometry allowing advanced identification of alkaloid standard mixtures and crude alkaloid extracts from plants and cell suspension cultures.

4. CE–ESI-MS analysis of alkaloids

The application of HPLC and CE separation methods with UV detection to the analysis of alkaloids from distinct classes has been described in Sections 2 and 3, respectively. The present section starts with a humble attempt to introduce some of the basics of capillary electrophoresis on-line coupled with mass spectrometry (CE–MS). Electrospray ionisation-mass spectrometry (ESI-MS) is discussed (Section 4.1), followed by short comments on electrospray ionisation-mass spectrometry–mass spectrometry (ESI-MS–MS). A few examples of analysing alkaloid standards with CE–ESI-MS will be given in Section 4.3. The finding of alkaloids in crude mixtures, such as root extracts or cell suspension cultures, will be outlined in Section 4.4.

The first alkaloid analyses employing CE–MS have been reported by Henion and co-workers [86,87]. They used an ion trap as a mass spectrometric detection device. Recently, Sturm and Stuppner reported the successful CE–MS analysis of isoquinoline alkaloids in plant material with a quadrupole mass spectrometer [88]. Bjoernsdottir et al. demonstrated that electrolytes containing organic modifiers could be applied to analyse crude opium [89]. Mentioning these selected early reports, the next section deals with a short introduction of theirs, ours and the most frequent ionisation method used in CE–MS: electrospray.

4.1. Electrospray ionisation mass spectrometry (ESI-MS)

Electrospray ionisation combined with mass spectrometry was introduced in 1984 by Fenn and co-workers [90,91]. However, a few years elapsed before the importance of this method was recognised [92–94]. Today ESI represents a ready-to-use ionisation method, which is applied to the analysis of a variety of substances including proteins, peptides, nucleic acids, dendrimers and metal complexes. With regard to alkaloid analysis, ESI-MS was employed for the first time a few years ago [13,95–98]. The on-line coupling of CE and ESI-MS in order to separate *and* identify alkaloids in tablets [86], opium tincture [76], barks [76,86], root extracts [49] as well as in artificial mixtures [85,87], has been reported recently. CE–MS techniques employing atmospheric pressure chemical ionisation (APCI), thermospray (TSP), fast-atom bombardment (FAB) or other

methods have been applied successfully but will not be covered.

The principle of ESI consists of spraying electrostatically a liquid [98–103] which contains electrolyte molecules, e.g. analytes. A number of spray modes can be distinguished according to the physical properties of the liquid or the spray-inducing conditions (see for example Refs. [104,105]). The electric field gradient between the capillary for liquid delivering and the ''entrance'' to the mass spectrometer of ~1 kV/cm and electrolyte flow rates ranging from 10 nl/min to 500 μl/min appear to be suited for analytical ESI-MS.

Increasing electrolyte concentration in the liquid to be sprayed leads to decreasing ESI-MS performance. Non-volatile buffers tend to spoil the mass spectrometer, because of crystallizing on surfaces inside the machine. Therefore, the use of ammonium acetate, formiate or hydrogen-carbonate is recommended as buffer system.

Four subsequent steps are identified in the ESI process [106]. (i) Charged droplets are formed from a liquid containing dissolved electrolytes. (ii) The charged droplets shrink by solvent evaporation and repeated droplet disintegration (fission). This leads to small and highly charged droplets. (iii) Ions are produced in the gas phase. (iv) In an additional process, gas-phase ions may be modified in the atmospheric or ion-sampling region of the mass spectrometer, e.g. nozzle-skimmer dissociation (NSD [107]). A schematic view of these four major processes is provided in Fig. 13. The transfer from ions in solution to ions in the gas phase is strongly endoergic because it is a desolvation process.

In ESI-MS the dominating signals result from the so-called ''quasi-molecular'' ions: (i) protonated or

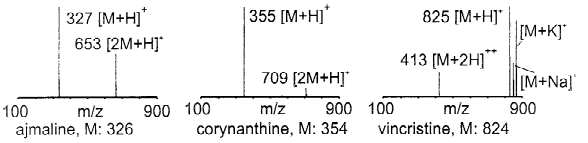

Fig. 14. ESI mass spectra of selected alkaloids.

sodiated molecules, i.e. $[M+H]^+$ or $[M+Na]^+$, (ii) clusters, e.g. $[2M+H]^+$, $[3M+Na]^+$ or $[M+H+solvent]^+$, or (iii) multiple charged molecules like $[M+2H]^{2+}$ (Fig. 14). Furthermore, protons can be exchanged by alkali ions, e.g. $[RCO_2Na+Na]^+$. Similarly to FAB or MALDI and in contrast to electron ionisation (EI), ESI is regarded as a ''soft'' ionisation technique because fragmentation of intact quasi-molecular ions is a minor process. However, structure-specific fragmentation can be induced from electrosprayed ions during additional processes mentioned above [95,107] or in a collision chamber of an MS–MS system (see below).

Despite serving as a coupling device between capillary electrophoresis and MS, ESI can link MS to supercritical fluid chromatography (SFC), liquid chromatography (LC) and electrophoresis on a chip or related techniques.

4.2. ESI-MS–MS

In order to identify an analyte in ESI-MS, collision-induced dissociation (CID), nozzle-skimmer dissociation (also called skimmer CID or in-source fragmentation), or photodissociation (PD) can be performed. In any of these cases structure-specific fragments must be produced and ESI-MS–MS offers this possibility.

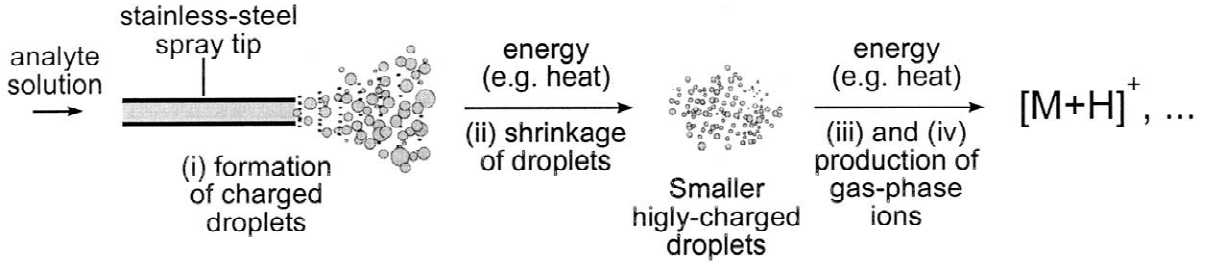

Fig. 13. Pictorial representation of the four major processes in electrospray ionisation (ESI).

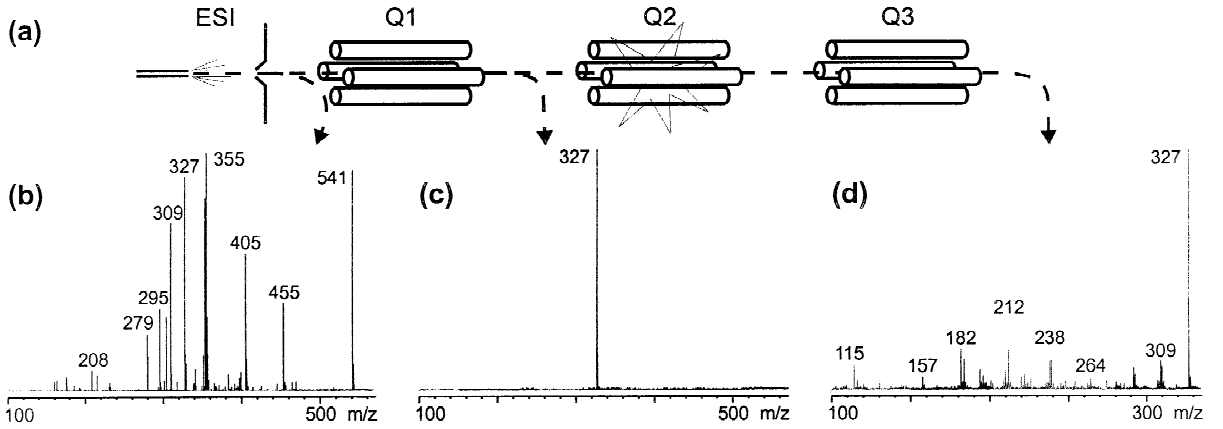

Fig. 15. (a) Scheme of an ESI-MS–MS experiment with a triple quadrupole instrument. (b) ESI-MS spectrum as obtained from an alkaloid mixture. (c) Isolation of m/z 327 by Q1, i.e. only ions with m/z 327 are allowed to pass Q1. (d) Ions with m/z 327 were fragmented in Q2 and analyzed by Q3.

The abbreviation ESI-MS–MS means that an ion is formed by ESI, selected in a primary MS experiment and on-line analysed by a second MS experiment[1]. The actual set-up of ESI-MS–MS can be described with the aid of pictograms [108–110]. For example, a triple-stage quadrupole mass spectrometer consisting of Q1, Q2, and Q3 can be used to perform ESI-MS–MS experiments (cf. Fig. 15): The ion to be dissociated is selected in Q1 (Fig. 15b,c), it fragments in Q2 after collisions with gas-phase molecules, e.g. argon, the fragments are separated by scanning Q3 (Fig. 15d) and eventually detected. Furthermore, ion traps (Penning or Paul types), sector instruments, time-of-flight (TOF) analysers or combinations of them can be used for MS–MS studies. An introductory discussion on mass analysers is given inter alia in Refs. [111,112].

Experimental conditions of EI are standardised and readily reproducible. Even with different types of m/z analysers or ion sources from different manufacturers, the characteristic EI-mass spectrum of an analyte remains the same. Thus, an EI-mass spectrum recorded with a sector-type instrument can be retrieved from a MS library which has been built from data obtained on a quadrupole MS. EI mass spectra of alkaloids in particular are well documented [113] and computer-assisted search algorithms can aid in their interpretation (see for example Refs. [114,115]). However, efforts in interpreting specific ESI-MS–MS or ESI-NSD-MS of alkaloids have been reported only recently [116,117].

In Fig. 16, a comparison between ESI-MS–MS, ESI-NSD-MS, and EI-MS data of ajmaline, corynanthine, and reserpine is presented [116]. A few structure-specific fragments can be readily extracted from the ESI-NSD-MS and ESI-MS–MS data of corynanthine and reserpine, but not in the case of ajmaline [117]. In any case, the three data sets can be searched for in a library of mass spectra and the corresponding structure will be retrieved if respective data are present in the library. However, structure determination based exclusively on a mass spectrum which is not present in a library is up to now almost impossible.

4.3. CE–ESI-MS system applied to alkaloid standards

Some basics of the CE–ESI-MS technique have been mentioned in the beginning of this section. Experimental results concerning the use of this technique for analysing alkaloids in mixtures are now presented. The analysis of indole alkaloid standards and a comparison between electrophorograms obtained by UV and MS detection constitute the first step thereof [118].

A mixture of 15 monoterpenoid indole alkaloids

[1]The term ''MS'' is used for the method mass spectrometry *and* for the mass spectrometer itself.

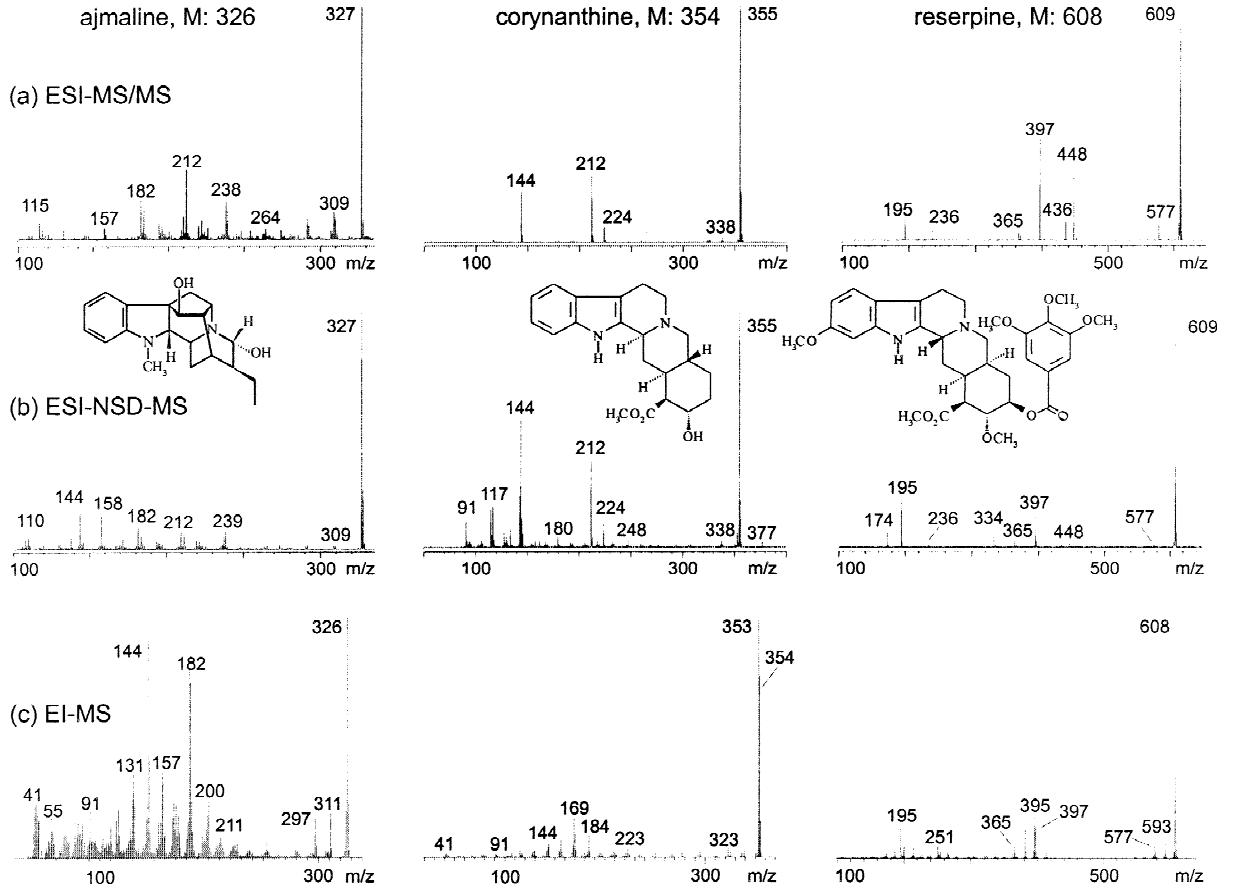

Fig. 16. Comparison of (a) ESI-MS–MS, (b) ESI-NSD-MS, and (c) EI-MS data of ajmaline (left row), corynanthine (middle), and reserpine (right).

was analysed by CE–ESI-MS [85]. In Fig. 17, the respective electrophorograms monitored by UV (Fig. 17a) and by the corresponding reconstructed total ion current (RIC, Fig. 17b) are presented. The CE–UV electrophorogram indicates reduced migration times as compared to the CE–MS electrophorogram. These differences are due to the instrumental set-up. In the given CE–MS coupling, the UV detection occurs on the capillary at a distance of 21 cm, whereas the ESI-MS detector is situated at the end of the capillary, i.e. after 78 cm.

Furthermore, the signal-to-noise ratio (S/N) in the RIC is lower, because in ESI-MS background signals are present originating from electrolyte and sheath liquid ions. Consequently, the S/N in CE–MS strongly depends on the qualitative and quantitative composition of analyte, electrolyte and sheath liquid [85,119]. For example, the S/N ratio of the RIC can be reduced by a factor of ~2 if the rate of the sheath flow is reduced from 2 to 1 μl min^{-1}.

Comparing the signal intensities of the analytes in Fig. 17a and b, the differences result (i) from the alkaloid's distinct absorptivities at a selected UV wavelength and (ii) from the alkaloid's distinct proton affinities as it is important in the ESI-MS detection. As an illustrating example, the signals originating from 1 and 2 versus 3 and 4 will be discussed in brief. The intensity of the UV absorption is similar within ~±10% for the four components as expected at 200 nm. The ESI-MS response, however, differs by a factor of ten, probably being due to the different pK_a values of 1 and 2 versus 3 and 4.

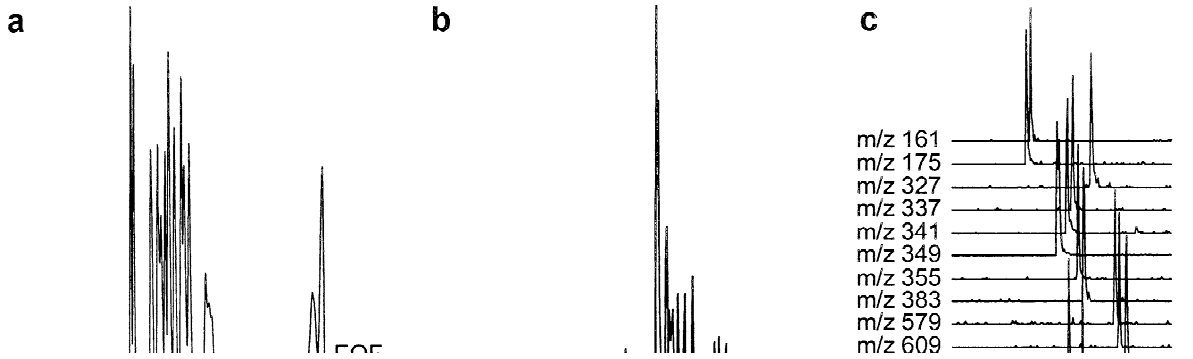

Fig. 17. UV, RIC, and single m/z electrophorograms of the CE–MS analysis of 15 indole alkaloids and biogenic amines. (a) UV electrophorogram at 200 nm obtained after ~20 cm. (b) Corresponding RIC electrophorogram (after ~75 cm) of the same run characterized by a lower S/N and a different detector response compared to part a. (c) Single m/z electrophorograms extracted from the RIC. Note the different time scales in a, b, and c.

The single m/z electrophorograms in Fig. 17 have been extracted from the RIC electrophorogram [85,119]. They are characterised by a similar S/N ratio as for the UV electrophorograms. For each of the 15 components (Fig. 9), the protonated molecule $[M+H]^+$ leads to the dominating signal in the mass spectrum, e.g. m/z 175 for gramine or m/z 825 for the dimeric alkaloid vincristine [85]. Only β-methylajmaline is detected as M^+ m/z 341 since it is already bearing one positive charge on the quaternary nitrogen atom. It is worth noting that mass spectra have been recorded with S/N ratios of at least 100:1. Three to five scans have been accumulated in each mass spectrum. In addition to the molecular ions (cf. Fig. 14), most of the spectra gave further signals arising from the sodium or potassium adducts, $[M+Na]^+$ and $[M+K]^+$ with relative signal intensities up to 20%. In some cases solvent clusters with methanol, acetonitrile or water can also be observed ($[M+Na+S]^+$, S: solvent), but the relative intensities of these ions are less than 3%. Further evidence of each compound can be obtained from alkaloid cluster ions, $[2M+H]^+$, and doubly protonated molecular ions like $[M+2H]^{2+}$, cf. ajmaline and vincristine. This additional MS information can be important for the identification of unknown alkaloids in complex mixtures (see below).

4.4. CE–ESI-MS analysis of crude alkaloid extracts

Following the successful analysis of standard mixtures by CE–ESI-MS, alkaloid detection from crude mixtures was performed and will be outlined in the current section. Compared with the experimental parameters established before, instrumental parameters had to be changed only modestly. In the following three examples, the UV and m/z electrophorograms of two distinct experiments will be compared. One was obtained with a CE–UV system and the other resulted from a CE–ESI-MS configuration.

4.4.1. Rauvolfia root alkaloids

The chemical analysis of the root material of *R. serpentina* Benth. ex Kurz has been carried out several times (see for example Refs. [120,121]), because this plant is a common old medicine used for the treatment of various diseases in India in former times. The roots constitute the industrial source for the isolation of prominent alkaloids like reserpine, rescinnamine, yohimbine, ajmalicine or ajmaline which are still of pharmacological-therapeutic interest. Using a crude extract of commercially available *Rauvolfia* roots, the CE–MS method de-

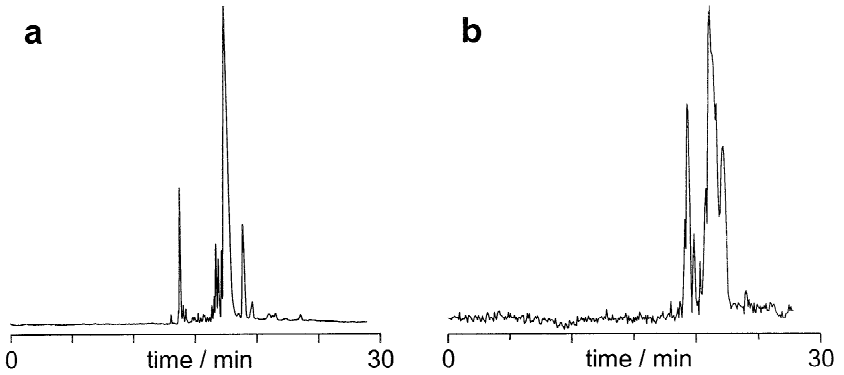

Fig. 19. Electrophorograms of the CE–UV only (a, detection at 224 nm) and the CE–ESI-MS analysis (b, RIC m/z 100–1000) of an alkaloid extract obtained from cell suspension cultures of *Rauvolfia serpentina*.

alkaloids amount to 19–23 min. As deduced from the mass spectra, at least 20 constituents were present in the cell extract. Most of the detected quasi-molecular ions, M^+ or $[M+H]^+$, result from typical alkaloids of cultivated *Rauvolfia* cell suspensions (cf. Refs. [118,125,126]). The major alkaloids of the cells are known to be nortetraphyllicine, tetraphyllicine, ajmaline and 17-*O*-acetylajmaline (given in the order of increasing molecular mass) [125–127]. Indeed, respective signals have been detected as the most intense peaks in the CE–MS analysis. Furthermore, the CE–MS data point to other characteristic ''cell culture alkaloids'' [125–127], e.g. norajmaline and vomilenine, acetyltetraphyllicine or perakine (m/z 351). In addition, m/z data of alkaloids occurring in significantly lower amounts have been detected, e.g. of ajmalicine or 17-*O*-acetyl-N_α-norajmaline. Because of a lack of additional MS data on the structure of these components, a straightforward identification of such compounds has not been accomplished by CE–MS.

4.4.3. Alkaloids from Cortex Quebracho

In a further example concerning CE–MS analyses of crude alkaloid mixtures, commercially available samples from the cortex of *Aspidosperma quebracho-blanco* Schlecht were investigated [76]. The samples were extracted with methanol and directly subjected to CE–UV only and CE–MS analysis under conditions as described above.

As depicted in Fig. 20a, the CE–UV electrophorogram indicates the presence of 20 components in less than 30-min migration time. Besides three major signals, several other constituents can be

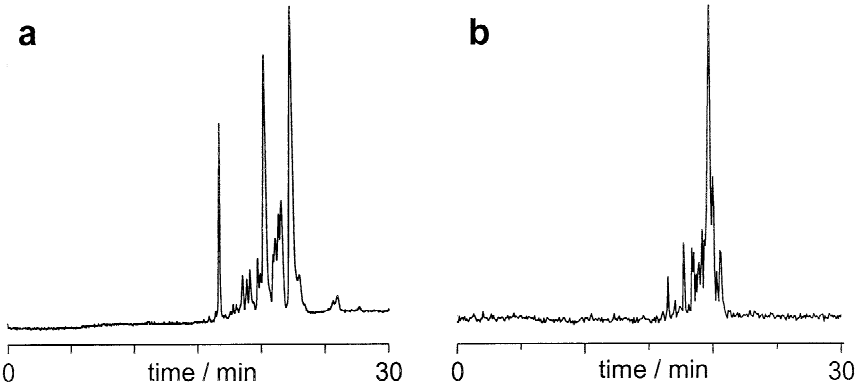

Fig. 20. Electrophorograms of the CE–UV only (a, at 224 nm) and the CE–ESI-MS analysis (b, RIC m/z 100–1000) of a crude methanol extract from Cortex Quebracho.

detected. When the same sample was measured in the CE–MS mode, ~16 different peaks were observed in the RIC electrophorogram (Fig. 20b). As already mentioned in the preceding sections, the S/N ratio of the latter is reduced by a factor of ~5 compared with that of the CE–UV only electrophorogram.

Based on the data of the CE–MS analysis, 28 different quasi-molecular ions, $[M+H]^+$, have been recognised. More than one component appears with m/z 295, 329, and 341, indicating distinct alkaloids with probable molecular masses of 294, 328, and 340, respectively. With regard to these results, the investigated crude extract of Cortex Quebracho may consist of ~50 alkaloids, many of which have not yet been identified [76].

5. Conclusions and prospects

The development of the HPLC solvent system described at the beginning of this article clearly shows that HPLC as a routine and classical method in alkaloid separation techniques can still be optimised. However, the pre-purification of crude alkaloid extract will be a crucial step for the entire analytical procedure. This is not necessarily true for alkaloid determination by CE, because even with very crude methanol extracts, excellent electrophorograms were obtained. In contrast to that, quantitation of alkaloid contents is at the present time clearly simpler with HPLC.

The here described CE system using a volatile buffer might provide a ready-to-use and generally applicable electrolyte which allows both excellent separation and successful application to the hybrid method CE–MS [85].

The recently established CE–ESI-MS method [49,76,85,116–118] can be successfully applied to the analysis of alkaloids. These results indicate that alkaloids can be detected in artificial mixtures of pure substances, and crude extracts from acid–base or methanol extraction procedures. Thus, the operational parameters described above facilitate a rapid and efficient method for alkaloid screening in unpurified plant matrices. In our experience, CE–MS provides a valuable alternative to current LC–MS methods for the analyses of secondary plant metabolites. A still significant drawback in CE–MS with electrospray interface results from the low structural information concerning the obtained mass spectra, since all major signals arise from quasi-molecular and adduct ions. This limitation might be overcome by CE–MS–MS in future.

Unfortunately, spectra obtained from MS–MS cannot be compared directly with 70-eV electron impact mass spectra and this does not allow automated searches in available MS libraries. The need for collection of MS–MS data and the establishment of appropriate spectra libraries will therefore be a necessary task leading to simpler and faster identification of natural products, and not only alkaloids.

Acknowledgements

The continuous support of our research in the field of alkaloids by the Deutsche Forschungsgemeinschaft (Bonn, Bad-Godesberg, Germany) is highly appreciated. We are thankful to the Fonds der Chemischen Industrie (Frankfurt/Main, Germany) and to the German Federal Ministry for Research and Technology (Bonn) for financial support. Our thanks are also due to Professor Dr. W.E. Court (Mold, Wales) for linguistic advice. D.S. is grateful for the kind support provided by Bio-Rad Laboratories GmbH (Munich, Germany). The contributions by D. Belder (Max-Planck-Institut für Kohlenforschung, Mülheim an der Ruhr) are highly appreciated.

References

[1] B. Fugmann, S. Lang-Fugmann, W. Steglich (Eds.), RÖMPP Lexicon Naturstoffe, Georg Thieme, New York, 1997.
[2] M.H. Zenk, Pure Appl. Chem. 66 (1994) 2023.
[3] J. Stöckigt, in: G.A. Cordell (Ed.), The Alkaloids, Vol. 47, Academic Press, New York, 1995, p. 115, (and literature cited therein).
[4] T. Hartmann, L. Witte, in: S.W. Pelletier (Ed.), Alkaloids: Chemical and Biological Perspectives, Elsevier Science, Oxford, 1995, p. 9.
[5] T.M. Kutchan, in: G.A. Cordell (Ed.), The Alkaloids, Vol. 50, Academic Press, San Diego, 1998, p. 257.
[6] H. Dittrich, T.M. Kutchan, Proc. Natl. Acad. Sci. USA 88 (1991) 9969.
[7] T.M. Kutchan, A. Bock, H. Dittrich, Phytochemistry 35 (1994) 353.

[8] K.T. Junghans, R.E. Kneusel, A. Baumert, W. Maier, D. Gröder, U. Matern, Plant. Mol. Biol. 27 (1995) 681.
[9] J. Stöckigt, Lecture presented at the International Symposium of the Phytochemical Society of Europe on Structure and Biosynthesis of Isoquinoline, Indole and Related Alkaloids, April 19–22, 1998, Istanbul, Turkey.
[10] A. Geerlings, J. Memelink, R. van der Heijden, R. Verpoorte, Poster presented at the International Symposium of the Phytochemical Society of Europe on Structure and Biosynthesis of Isoquinoline, Indole and Related Alkaloids, April 19–22, 1998, Istanbul, Turkey.
[11] G.D. Manners, J.A. Pfister, Phytochem. Anal. 4 (1993) 14.
[12] M. Huntin, A. Cave, J.P. Foucher, J. Chromatogr. 268 (1983) 125.
[13] H. Falkenhagen, I.N. Kuzovkina, I.E. Alterman, L.A. Nikolaeva, J. Stöckigt, Nat. Prod. Lett. 3 (1993) 107.
[14] M. Frederich, M. Tits, L. Angenot, Phytochem. Anal. 9 (1998) 63.
[15] W. Kohl, B. Witte, G. Höfle, Planta Med. 47 (1983) 177.
[16] N.R. Ayyangar, S.R. Bhide, J. Chromatogr. 436 (1988) 455.
[17] P. Gomez-Serranillos, E. Carretero, A. Villar, Phytochem. Anal. 5 (1994) 15.
[18] A. Rehwald, O. Sticher, Phytochem. Anal. 6 (1995) 96.
[19] T.-M. Chen, R.C. George, J.L. Weir, T. Leapheart, J. Nat. Prod. 53 (1990) 359.
[20] L.W. Doner, A.-F. Hsu, J. Chromatogr. 253 (1982) 120.
[21] R.J. Molyneux, Phytochem. Anal. 4 (1993) 193, (and literature cited therein).
[22] M.J. Donaldson, H. Broby, M.W. Adlard, C. Bucke, Phytochem. Anal. 1 (1990) 18.
[23] B. Dräger, Phytochem. Anal. 6 (1995) 31.
[24] K. Yoshimatsu, K. Shimomura, Phytochem. Anal. 4 (1993) 217.
[25] A. Kato, M. Moriyasu, Y. Nishiyama, M. Ichimaru, F.D. Juma, J.O. Ogeto, Phytochem. Anal. 4 (1993) 72.
[26] A. Kato, M. Moriyasu, M. Ichimaru, Y. Nishiyama, F.D. Juma, J.N. Nganga, S.G. Mathenge, J.O. Ogeto, Phytochem. Anal. 6 (1995) 89.
[27] Yu. Sheludko, I. Gerasimenko, M. Unger, I. Kostenyuk, J. Stöckigt, Plant Cell Rep. 18 (1999) 911.
[28] N. Tanaka, K. Kimata, K. Hosoya, H. Miyanishi, T. Araki, J. Chromatogr. A 656 (1993) 265.
[29] P. Kuronen, T. Väänänen, E. Pehu, J. Chromatogr. A 863 (1999) 25.
[30] L.A. Ciolino, J.A. Turner, H.A. McCauley, A.W. Smallwood, T.Y. Yi, J. Chromatogr. A 852 (1999) 451.
[31] M. Unger, J. Chromatogr. A 807 (1998) 81.
[32] I. Gerasimenko, Y. Sheludko, M. Unger, J. Stöckigt, Phytochem. Anal. 12 (2001) 96.
[33] E. Falkenstein, B. Groth, A. Mithöfer, E.W. Weiler, Planta 185 (1991) 316.
[34] E.E. Farmer, C.A. Ryan, Plant Cell 4 (1992) 129.
[35] J. Lee, T. Vogt, J. Schmidt, B. Parthier, M. Löbler, Phytochemistry 44 (1997) 589.
[36] B. Gundlach, M.J. Müller, T.M. Kutchan, M.H. Zenk, Proc. Natl. Acad. Sci. USA 89 (1992) 2389.
[37] Y. Yukimune, H. Tobata, Y. Higashi, Y. Hara, Nat. Biotechnol. 14 (1996) 1129.
[38] N. Mirjalili, J.C. Linden, Biotechnol. Prog. 12 (1996) 110.
[39] G. Suzuki, H. Ohta, T. Kato, T. Igarashi, F. Sakai, D. Shibata, A. Takano, T. Masuda, Y. Shioi, K.-I. Takamiya, FEBS Lett. 383 (1996) 83.
[40] H. Ohta, G. Suzuki, K. Awai, T. Masuda, T. Kato, D. Shibata, K.-I. Takamiya, Physiol. Plant. 100 (1997) 647.
[41] A. Rosco, H.H. Pauli, W. Priesner, T.M. Kutchan, Arch. Biochem. Biophys. 348 (1997) 369.
[42] H.H. Pauli, T.M. Kutchan, Plant J. 13 (1998) 793.
[43] I. Kostenyuk, O. Lubaretz, N. Borisyuk, V. Voronin, J. Stöckigt, Y. Gleba, Theor. Appl. Genet. 82 (1991) 713.
[44] J. Stöckigt, A. Pfitzner, J. Firl, Plant Cell Rep. 1 (1981) 36.
[45] K.-H. Pawelka, J. Stöckigt, Z. Naturforsch. 41c (1986) 385.
[46] I.A. Kostenyuk, O.F. Lyubarets, S. Endreß, Y.Y. Gleba, J. Stöckigt, Nat. Prod. Lett. 5 (1995) 303.
[47] N. Aimi, M. Kitajima, N. Oya, W. Nitta, H. Takayama, S. Sakai, I. Kostenyuk, Y. Gleba, S. Endreß, J. Stöckigt, Chem. Pharm. Bull. 44 (1996) 1637.
[48] R.J. Aerts, D. Gisi, E. De Carolis, V. De Luca, T.W. Baumann, Plant J. 5 (1994) 635.
[49] D. Stöckigt, M. Unger, D. Belder, J. Stöckigt, Nat. Prod. Lett. 9 (1997) 265.
[50] D. Banes, A.E.H. Houk, J. Wolff, J. Am. Pharm. Assoc. Sci. Ed. 47 (1958) 625.
[51] N. Finch, W.I. Taylor, P.R. Ulshafer, Experientia 19 (1963) 296.
[52] M.M.A. Amer, W.E. Court, Phytochemistry 19 (1980) 1833.
[53] M.M.A. Amer, W.E. Court, Planta Med. 40 (Suppl.) (1980) 8.
[54] Y. Sheludko, I. Gerasimenko, O. Platonova, Planta Med. 66 (2000) 656.
[55] H.K. Schnoes, A.L. Burlingame, K. Biemann, Tetrahedron Lett. (1962) 993.
[56] G.N. Smith, Chem. Commun. (1968) 912.
[57] D.A. Evans, J.A. Joule, G.F. Smith, Phytochemistry 7 (1968) 1429.
[58] K.T. De Silva, G.N. Smith, K.E.H. Warren, J. Chem. Soc. Chem. Commun. (1971) 905.
[59] K.T. De Silva, A.H. Ratcliffe, G.F. Smith, G.N. Smith, Tetrahedron Lett. (1972) 913.
[60] N.K. Mariee, A.A. Khalil, A.A. Nasser, M.M. Al-Hiti, W.M. Ali, J. Nat. Prod. 51 (1988) 186.
[61] B. Zsadon, A. Vegh, R. Hubay, Mogy. Kem. Foly. 77 (1971) 52.
[62] P.D. Grossman, K.J. Wilson, G. Petrie, H.H. Lauer, Anal. Biochem. 173 (1988) 265.
[63] W.M. Sunada, H.W. Blanch, Electrophoresis 18 (1997) 2243.
[64] J. Sadecka, J. Polonsky, H. Shintani, Pharmazie 49 (1994) 631.
[65] F.A. Tomás Barberán, Phytochem. Anal. 6 (1995) 177.
[66] H.J. Issaq, Electrophoresis 18 (1997) 2438.
[67] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya, T. Ando, Anal. Chem. 56 (1984) 111.
[68] K.-J. Lee, J.J. Lee, D.C. Moon, J. Chromatogr. 616 (1993) 135.
[69] S. Hoffstetter-Kuhn, A. Paulus, E. Gassmann, H. Widmer, Anal. Chem. 63 (1991) 1541.

[70] Y.-M. Liu, S.-J. Sheu, J. Chromatogr. 634 (1993) 329.
[71] Y.-M. Liu, S.-J. Sheu, J. Chromatogr. 623 (1992) 196.
[72] P.G. Pietta, P.L. Mauri, C. Gardana, M.L. Colombo, F. Tomè, Phytochem. Anal. 6 (1995) 196.
[73] M. Unger, J. Stöckigt, J. Chromatogr. A 791 (1997) 323.
[74] R.B. Taylor, A.S. Low, R.G. Reid, J. Chromatogr. B 675 (1996) 213.
[75] I. Bjoernsdottir, S.H. Hansen, J. Pharm. Biomed. Anal. 13 (1995) 687.
[76] M. Unger, D. Stöckigt, D. Belder, J. Stöckigt, Pharmazie 52 (1997) 691.
[77] H.J. Issaq, K.C. Chan, G.M. Janini, G.M. Muschik, J. Liq. Chromatogr. 18 (1995) 1273.
[78] G.H. Lu, S. Ralapati, Electrophoresis 19 (1998) 19.
[79] M. Eeva, J.P. Salo, K.M. Oksman-Caldentey, J. Pharm. Biomed. Anal. 16 (1998) 717.
[80] H. Stuppner, S. Sturm, G. Konwalinka, J. Chromatogr. 609 (1992) 375.
[81] Y.-Y. Zong, C.-T. Che, Planta Med. 61 (1995) 456.
[82] M.C. Lee, W.C. Chuang, S.-J. Sheu, J. Chromatogr. A 755 (1996) 113.
[83] I. Chu, J.A. Bodnar, E.L. White, R.N. Bowman, J. Chromatogr. A 755 (1996) 281.
[84] K. Frach, G. Blaschke, J. Chromatogr. A 808 (1998) 247.
[85] M. Unger, D. Stöckigt, D. Belder, J. Stöckigt, J. Chromatogr. A 767 (1997) 263.
[86] J.D. Henion, A.V. Mordehai, J. Cai, Anal. Chem. 66 (1994) 2103.
[87] F.Y.L. Hsieh, J. Cai, J. Henion, J. Chromatogr. A 679 (1994) 206.
[88] S. Sturm, H. Stuppner, Electrophoresis 19 (1998) 3026.
[89] I. Bjoernsdottir, J. Tjoernelund, S.H. Hansen, Electrophoresis 19 (1998) 2179.
[90] M. Yamashita, J.B. Fenn, J. Phys. Chem. 88 (1984) 4451.
[91] M. Yamashita, J.B. Fenn, J. Phys. Chem. 88 (1984) 4671.
[92] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong, C.M. Whitehouse, Science 246 (1989) 64.
[93] T.R. Covey, R.F. Bonner, B.I. Shushan, J. Henion, Rapid Commun. Mass Spectrom. 2 (1988) 249.
[94] R.D. Smith, J.A. Olivares, N.T. Nguyen, H.R. Udseth, Anal. Chem. 60 (1988) 436.
[95] S.R. Wilson, J. Perez, Y. Wu, Nat. Prod. Lett. 1 (1992) 103.
[96] R.A. Edrada, P. Proksch, V. Wray, L. Witte, W.E.G. Müller, R.W.M. Van Soest, J. Nat. Prod. 59 (1996) 1056.
[97] W.-C. Chuang, D.-S. Young, L.K. Liu, S.-J. Sheu, J. Chromatogr. A 755 (1996) 19.
[98] S. Zhou, M. Hamburger, J. Chromatogr. A 755 (1996) 189.
[99] J. Zeleny, Phys. Rev. 3 (1914) 69.
[100] D.S. Swatik, C.D. Hendricks, AIAA J. 6 (1968) 1569.
[101] C.A. Evans Jr., C.D. Hendricks, Rev. Sci. Instrum. 43 (1972) 1527.
[102] G. Bailey, The Electrostatic Spraying of Liquids, Wiley, New York, 1988.
[103] T. Dülcks, F.W. Röllgen, Int. J. Mass Spectrom. Ion Processes 148 (1995) 123.
[104] Z. Olumee, J.H. Callahan, A. Vertes, J. Phys. Chem. A 102 (1998) 9154, (and references cited therein).
[105] S. Zhou, A.G. Edwards, K.D. Cook, G.J. Van Berkel, Anal. Chem. 71 (1999) 769.
[106] P. Kebarle, L. Tang, Anal. Chem. 15 (1993) 972A.
[107] J.A. Loo, H.R. Udseth, R.D. Smith, Rapid Commun. Mass Spectrom. 2 (1988) 207.
[108] J.N. Louris, L.G. Wright, R.G. Cooks, A.E. Schoen, Anal. Chem. 57 (1985) 2918.
[109] J.C. Schwartz, A.P. Wade, C.G. Enke, R.G. Cooks, Anal. Chem. 62 (1990) 1809.
[110] W.D. Lehmann, J. Am. Soc. Mass Spectrom. 8 (1997) 756.
[111] J.R. Chapman, Practical Organic Mass Spectrometry, Wiley, New York, 1993.
[112] W.D. Lehmann, Massenspektrometrie in der Biochemie, Spektrum der Wissenschaft, Heidelberg, 1997.
[113] F.W. McLafferty (Ed.), Wiley Registry of Mass Spectral Data, Palisade Corp., Newfield, NY, USA.
[114] H. Damen, D. Henneberg, B. Weimann, Anal. Chim. Acta 103 (1978) 289.
[115] D. Henneberg, Adv. Mass Spectrom. 8 (1980) 1511.
[116] D. Stöckigt, D. Belder, Poster presentation at the 30. Arbeitstagung der Arbeitsgemeinschaft Massenspektrometrie, May 20–23, 1997, Konstanz, Germany.
[117] D. Stöckigt, in: P. Schreier, M. Herderich, H.-U. Humpf, W. Schwab (Eds.), Natural Product Analysis, Chromatography-Spectroscopy-Biological Testing, Vieweg, Wiesbaden, 1998, p. 177.
[118] J. Stöckigt, M. Unger, D. Stöckigt, D. Belder, in: S.W. Pelletier (Ed.), Alkaloids: Chemical and Biological Perspectives, Vol. 12, Pergamon, New York, 1998, p. 289.
[119] D. Belder, D. Stöckigt, J. Chromatogr. A 752 (1996) 271.
[120] A. Koskinen, M. Lounasmaa, Fortschr. Chem. Org. Naturst. 43 (1983) 267, (and literature cited therein).
[121] E. Schlittler, in: R.H.F. Manske (Ed.), The Alkaloids, Vol. 8, Academic Press, New York, 1965, p. 287, and literature cited therein.
[122] W.M. Mück, J.D. Henion, J. Chromatogr. 495 (1989) 41.
[123] M.K. Tukhtasinov, N.P. Kir'yalov, R.G. Butenko, I. Grushvitskii, I.Y. Gurevich, I.K. Nikitina, Khim. Prir. Soedin. 1 (1977) 122.
[124] S. Ohta, M. Yatazawa, Agric. Biol. Chem. 43 (1979) 2297.
[125] C.M. Ruyter, H. Schübel, J. Stöckigt, Z. Naturforsch. 43c (1988) 479.
[126] H. Schübel, C.M. Ruyter, J. Stöckigt, Phytochemistry 28 (1989) 491.
[127] V.E. Klyushnichenko, S.A. Yakimov, T.P. Tuzova, Y.V. Syagailo, I.N. Kuzovkina, A.N. Wulfson, A.I. Miroshnikov, J. Chromatogr. A 704 (1995) 357.