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Review

Analysis of tropane and related alkaloids

Birgit Dräger*

Institute of Pharmaceutical Biology, Martin-Luther-University Halle-Wittenberg, Hoher Weg 8, 06120 Halle/Saale, Germany

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Abstract

The current methods for tropane alkaloid chromatographic separation and determination are summarised. The alkaloids included are: the medicinally applied tropic acid esters hyoscyamine and scopolamine and their derivatives, cocaine and derivatives, the metabolites and degradation products of these compounds occurring in plant material, calystegines as nortropane alkaloids, anatoxins as homonortropane alkaloids, pelletierines and pseudopelletierines as alkaloids with isomeric structures. Developments in GC, HPLC, CE and TLC are presented and the advantages of each method for plant analysis are discussed. A summary for each chromatographic method lists the instrumentation and parameters applied for tropane alkaloids.
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Keywords: Reviews; Tropane alkaloids; Alkaloids; Hyoscyamine; Scopolamine; Cocaine; Calystegine; Anatoxin; Toxins

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*Tel.: +49-345-552-5765; fax: +49-345-552-7021.
E-mail address: draeger@pharmazie.uni-halle.de (B. Dräger).

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1. Introduction

The growing interest in secondary metabolites of plants has directed attention to methods of secondary product analysis. Chromatographic procedures for the determination of alkaloids are well established. In view of the improvements and variations published during recent years, an update of the methods for the analysis of tropane alkaloid appears useful. New methods such as capillary electrophoresis (CE) and improved instrumentation in the hyphenated techniques of gas chromatography–mass spectrometry (GC–MS) or high-performance liquid chromatography–mass spectrometry (HPLC–MS), as well as recently detected tropane-alkaloid related compounds like the calystegines and the anatoxins justify a review of the state of the art for their chromatographic analyses.

During recent years, several reviews on tropane alkaloids have been written which include sections on analytical methods. The contributions by Fodor and Dharanipragada [1–4] and by O'Hagan [5] describe the structures of newly detected tropane alkaloids as well as approaches for their synthesis, analysis and also give pharmacological data. A survey on the variety and the distribution of tropane alkaloids in the plant kingdom contains comprehensive data on their extraction, purification and quantification by chromatography [6].

Books and reviews on the chromatography of alkaloids with sections on tropane alkaloids have been published [7–10]. These also contain basic information on the chromatographic techniques. Special aspects of tropane alkaloid analysis are addressed in other reviews, e.g. GC of alkaloids [11] or liquid chromatography of alkaloids coupled with mass spectrometry [12].

Chemical properties of the individual compounds such as dissociation constant, stereochemistry and spectroscopic data are useful characteristics for the development of chromatographic separations. These are listed in reviews focusing on chemical aspects such as synthesis and reactions [13], on occurrence in the plant kingdom [14–16] and on spectroscopic data in carbon-13 and proton nuclear magnetic resonance (NMR) and mass spectrometry (MS) [14,15]. Tropane alkaloids have been reviewed in various other ways, with an emphasis on the bio-

chemical effects of alkaloids and on biogenetic approaches [17,18].

1.1. Applications of tropane alkaloid analysis

Tropane alkaloids are analysed for different purposes, leading to different demands for the specification of methods.

(i) Plant tissues are analysed when investigating, e.g. the organ-dependent or time-dependent accumulation in plant breeding programmes, the alkaloid biosynthesis or the quality of plant raw material for alkaloid extraction, purification and isolation. Chromatographic methods for this purpose have to be highly selective among many similar alkaloid metabolites and highly sensitive for minute quantities.

(ii) In pharmaceutical quality control the composition of the samples usually is simple, but exact reproducibility and automation of a method is required. Sensitivity is necessary as well to ensure the detection of byproducts or product degradation.

(iii) In clinical drug monitoring or in toxicological cases the samples are complex, e.g. blood serum, other body fluids or even hair. In forensic analysis similar samples have to be handled. Sample preparation becomes a major issue here to provide sufficient concentration of analytes. The detection is usually combined with identification methods such as MS.

(iv) Again, different demands for chromatographic methods are to be fulfilled in pharmaceutical drug development, investigations on receptor interactions and pharmacodynamic mechanisms. Very low concentrations have to be monitored when working with isolated receptors. Sometimes radiolabelling has to be applied to provide lower limits of detection. Cocaine as a tool in neurologic research has gained importance since very sensitive methods of detection are available for analysing tissue samples.

This review will centre on applications in plant research. Other applications of tropane analysis are referred to, if additional information is supplied that may be useful for plant analysis.

1.2. Alkaloids included

The number of tropane alkaloids known from natural sources exceeds 200 [15]. They have been isolated from many different plant families, e.g. Solanaceae, Erythroxylaceae, Convolvulaceae, Proteaceae, Rhizophoraceae, Brassicaceae, Euphorbiaceae [16], and they comprise mono-, di- and triesters, carboxylated and benzoylated tropanes. There are many additional semisynthetic or fully synthetic analogues for pharmaceutical purposes. Many tropane alkaloids carry several common names, e.g. hyoscine=scopolamine or 6β-hydroxyhyoscyamine=anisodamine. The racemic mixture of (*R*,*S*)-hyoscyamine has a separate name, atropine. This leads to complications in evaluating reports on tropane alkaloids analysis. For the identification of synonyms, *Chemical Abstracts* may be consulted. Many tropane alkaloids are chiral compounds. Frequently, the chirality is introduced by tropic acid or by the presence of ecgonine. Only the *R* form of tropic acid occurs in natural tropane alkaloids, however, racemisation readily occurs and it is often encountered upon extraction by basic solution. Chromatographic separation of enantiomers is included here, if methods are available.

Most of the tropane alkaloids identified from plant sources are reported as structures in the literature with some chemical properties, but their concentration has usually not been measured. Chromatographic procedures for their determination are not published. The present review will concentrate on the well-known and widely applied alkaloids hyoscyamine, scopolamine and cocaine and further ester alkaloids, if their chromatographic properties are described (Figs. 1 and 2). Metabolites and degradation products are included (Fig. 3). Synthetic derivatives of tropanes are important in pharmaceutical and clinical analysis. Such studies will be mentioned for comparison.

Monocyclic tropane metabolites like hygrine and ring-enlarged tropane homologues like pseudopelletierine and monocyclic pelletierine are included, because of their structural similarity and because they sometimes occur together with tropanes and are formed by similar biosynthetic steps [19]. Anatoxins as homotropanes with an enlarged bicyclic ring system are included because of their interesting

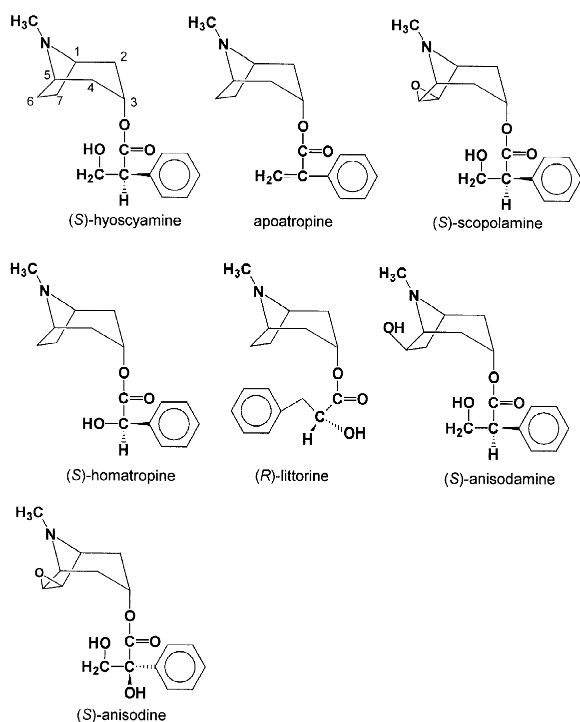


Fig. 1. 3α-Tropane ester alkaloids.

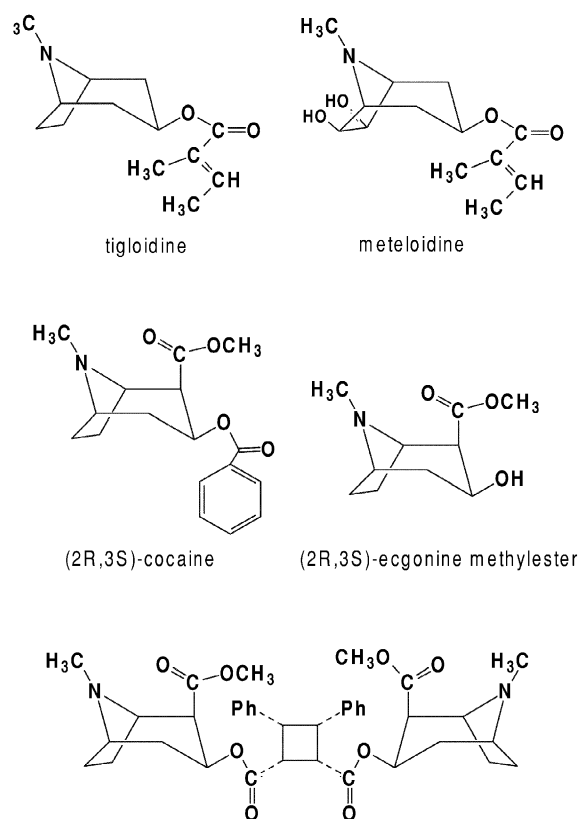


Fig. 2. 3β-Tropane ester alkaloids.

biochemical properties and their toxicological importance (Fig. 4).

Calystegines are a group of nonesterified hydroxylated nortropane alkaloids [20] that carry 3, 4 or 5 hydroxyl groups. Recently, dihydroxynortropanes have also been elucidated [21]. Looking at the structures (Fig. 5) and considering the high polarity of the calystegines it is evident that chromatographic procedures different from those for the well-known tropane alkaloids are required for their analysis.

1.3. Focus on methods

The review will cover GC, HPLC and CE, it will include hyphenated techniques such as GC–MS and HPLC–MS. Thin-layer chromatography (TLC) will be mentioned briefly, because it is still frequently applied for an initial screening. NMR analysis as a method of structural elucidation, rather than of quantification, will not be covered. Special methods for preparative chromatographic purification of alkaloids are not listed.

1.4. Objective

Readers of this section are probably interested in an overview of the chromatographic methods, their

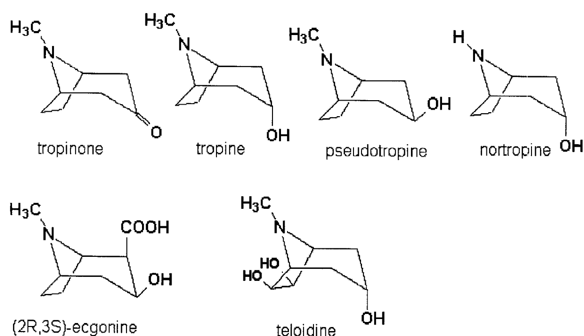


Fig. 3. Tropane alkaloids metabolites and degradation products.

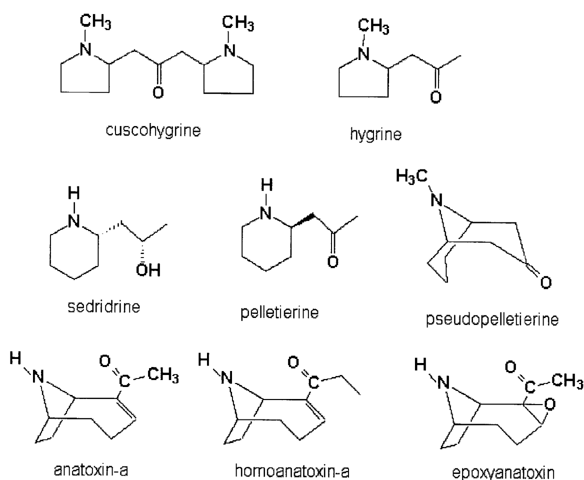
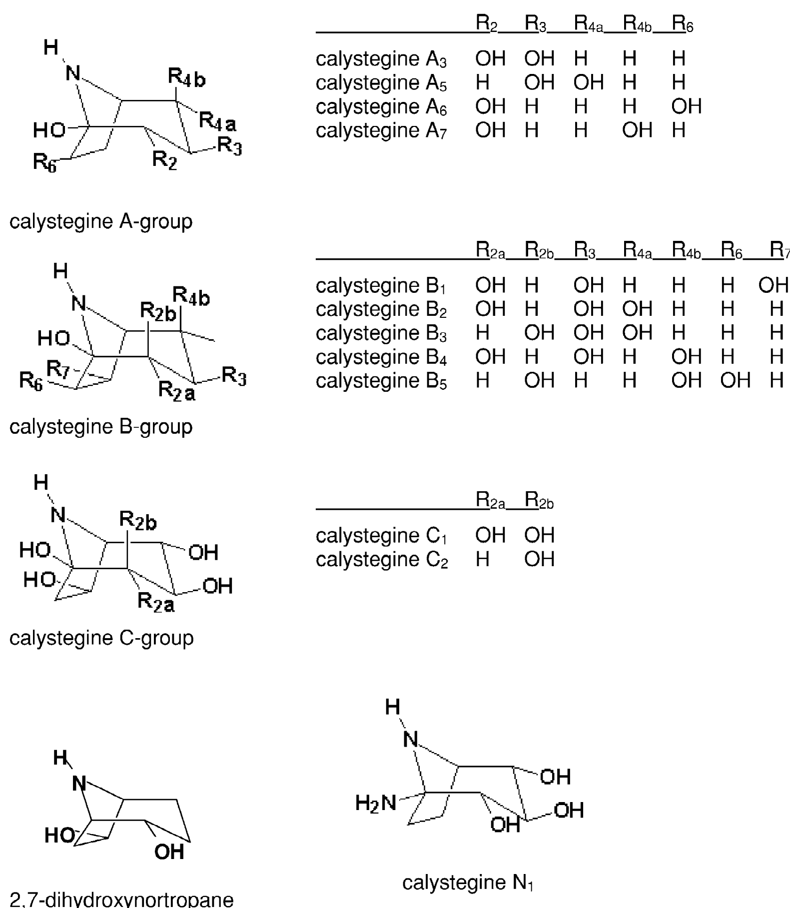


Fig. 4. Monocyclic analogues and ring-enlarged homologues.

advantages and limitations, because they may have an actual problem of tropane alkaloid analysis to solve. I shall therefore try to concentrate on practical approaches and I shall not focus on publications that give a theoretical introduction in the various fields of chromatography or CE. Such publications are cited in the respective chapters.

As a Ph.D. student or researcher you may have a certain plant tissue or enzyme to analyse or you may want to determine the identity and concentration of tropane alkaloids. How should you begin? This certainly depends on what equipment you have in your laboratory and on which methods you have experience in. The ideal case, in which you can choose between GC, HPLC, CE, have a range of detectors for each instrument, a range of separation columns and solvents for each approach, and have



	R_2	R_3	R_{4a}	R_{4b}	R_6
calystegine A_3	OH	OH	H	H	H
calystegine A_5	H	OH	OH	H	H
calystegine A_6	OH	H	H	H	OH
calystegine A_7	OH	H	H	OH	H

	R_{2a}	R_{2b}	R_3	R_{4a}	R_{4b}	R_6	R_7
calystegine B_1	OH	H	OH	H	H	H	OH
calystegine B_2	OH	H	OH	OH	H	H	H
calystegine B_3	H	OH	OH	OH	H	H	H
calystegine B_4	OH	H	OH	H	OH	H	H
calystegine B_5	H	OH	H	H	OH	OH	H

	R_{2a}	R_{2b}
calystegine C_1	OH	OH
calystegine C_2	H	OH

Fig. 5. Calystegines.

full access to methods such as GC–MS, HPLC–MS is unlikely! Rather, you might have worked with HPLC with UV detection up to now, and you wonder whether it is worth the effort to develop a GC analysis.

The detailed answers to the question on how to begin depend on

(i) the composition of the mixture of tropane alkaloids and the structures that are to be determined
(ii) the number and composition of the whole samples
(iii) the volume of sample available
(iv) the concentration of the analytes
(v) the demands for preciseness and sensitivity, and on several other criteria.

Each section is introduced by a summary to help guide the reader towards the most appropriate methods for their specific needs.

2. Extraction and sample preparation

2.1. Extraction

Extraction and sample preparation are of major importance in plant analysis. Clinic and forensic analyses also rely on appropriate sample preparation to achieve a sufficient limit of detection. The procedure before the chromatographic measurement must ensure exhaustive extraction of the material to be analysed, separation of matrix compounds that interfere with analysis, sufficient concentration of the analytes.

Information on the chemical properties of the alkaloids are essential for the achievement of these goals (Table 1).

The solubility is decisive for the choice extraction method. All tropane alkaloids, except apoatropine, cocaine and the truxillines, are fairly water soluble, at least at acidic pH. This is different from many other alkaloids that have higher calculated octanol–water partition coefficients, e.g. quinine 3.44 ± 0.43 and reserpine 4.37 ± 0.88. The water solubility of tropane alkaloids enables a selective extraction with aqueous acid, excluding lipophilic compounds. By extraction of leaf material for tropane alkaloids with water most of the chlorophyll will remain in the tissue, and thus provide extracts with fewer contaminants. For large scale extraction of hyoscyamine, scopolamine, or cocaine supercritical fluid extraction with CO_2 modified by the addition of methanol was shown to be an interesting alternative, because it is versatile, reproducible and saves organic solvent [22]. For calystegine extraction, water or methanol are suitable. Depending on the type of plant tissues methanol may be preferable, especially if a lot of starch and water-soluble mono- and oligosaccharides are present in roots or tubers. The saccharides are the most interfering compounds with the subsequent analysis, and they will not dissolve in alcohol as easily as in water.

A selective extraction is highly recommended in general, because it can speed up all further operations with the extract, i.e. simplify sample preparation and allow direct chromatographic analysis in many cases. The appropriate extracting solvent can be chosen for each single alkaloid theoretically considering the pK_a and pK_b, the solubility and the octanol–water partition coefficient. In practise, one will always examine the extraction method chosen by eluting the material several times and testing the extracts individually in order to learn after how many repetitions an extraction is exhaustive. Sufficient homogenisation of tissues is a prerequisite for exhaustive extraction.

In spite of selective extraction, the extract will sometimes carry interfering compounds or need concentration of analytes before chromatography.

Sample preparation reported for tropane alkaloids is usually done by liquid–liquid extraction (LLE) with nonmiscible solvents or by solid-phase extraction (SPE).

2.2. Sample preparation

2.2.1. Ester alkaloids

Sample preparation without losses is important, if a quantitative assessment of all alkaloids in a given plant sample is required. For many alkaloids, the sample preparation procedure is based on the different solubility of the alkaloids salt in comparison to the free bases. An acidic aqueous extract must be basified to transfer alkaloids into the free base form for extraction into organic solvents nonmiscible with water. Excessive use of alkali, however, is detrimen-

Table 1

Alkaloids-esters	CAS number	Molecular mass	Dissociation constant[a]	Solubility[b]	Log P_{OW}[c]	Melting point (°C)	Boiling point (°C)	λ_{max}
(*S*)-Hyoscyamine	101-31-5	289.37	pK_b 4.3	H_2O vs pH 4, s pH 8, sps pH 10 EtOH, $CHCl_3$ vs	1.528±0.371	108.5		$A^{1\%}_{1\ cm}=8.2$ (258 nm, dilute acid)
(*S*)-Hyoscyamine sulphate	620-61-1		pK_a 9.7	H_2O vs, EtOH s, Ether, $CHCl_3$ sps		203 decompos. (water-free salt)		
Apoatropine	500-55-0	271.35	pK_b 4.0±0.20	H_2O s pH 4, sls pH 7, sps pH 8; EtOH, $CHCl_3$ s	3.218±0.329	61.5–62.5[d]		
Homatropine	87-00-3	275.34	pK_b 3.9–4.1	EtOH, acetone, ether, benzene, $CHCl_3$ s	1.567±0.390	100		$A^{1\%}_{1\ cm}=5.0$ (258 nm, EtOH)
Homatropine hydrobromide	51-52-9		pK_a 9.9	H_2O, EtOH (60°) s		215 decompos.		$A^{1\%}_{1\ cm}=7.5$ (258 nm, MeOH)
(*R*)-Littorine	21956-47-8	289.37	pK_b 4.0±0.20 pK_a 12.9±0.20	H_2O vs pH 4, s pH 8, sps pH 10, EtOH, $CHCl_3$ vs	1.567±0.390			
6β-Hydroxyhyoscy-amine = anisodamine	55869-99-3	305.37	pK_b 5.1±0.60	H_2O vs pH 7, s pH 8, sls pH 10	0.250±0.385	92–95.5[d]		
(*S*)-Scopolamine = hyoscine	51-34-3	303.35	pK_b 6.4	H_2O vs pH 4, sls pH 7, sps pH 10, EtOH, ether, $CHCl_3$ vs	1.336±0.461	59 (monohydrate)		$A^{1\%}_{1\ cm}=14$ (257 nm, dilute acid)
(*S*)-Scopolamine hydrobromide	6533-68-2		pK_a 7.6	H_2O, EtOH vs		197 (trihydrate)		$A^{1\%}_{1\ cm}=4.5$ (258 nm, MeOH)
(*S*)-Anisodine	52646-92-1	319.35	pK_b 6.0±0.40 pK_a 11.3±0.29	H_2O vs pH 4, sls pH 7, sps pH 8	0.778±0.519			
Tigloidine	495-83-0	223.31	pK_b 4.0±0.20	H_2O s pH 7, sls pH 8, sps pH 10	2.881±0.326			
Meteloidine	526-13-6	255.31	pK_b 6.1±0.20 pK_a 13.6±0.20	H_2O vs pH 4, sls pH 8, sps pH 10	1.182±0.417	141–142[d]		
(2*R*, 3*S*)-Cocaine	50-36-2	303.35	pK_b 5.4	$CHCl_3$, ether, EtOH vs, H_2O sls pH 4, sps pH 10	3.079±0.384	96–98	187–188	$A^{1\%}_{1\ cm}=430$ (233 nm, dilute acid)
(2*R*, 3*S*)-Cocaine hydrochloride	53-21-4		pK_a 8.6	H_2O, hot EtOH vs		195–197	(0.01 kPa)	$\epsilon=7136$ (230 nm, MeOH)
(2*R*, 3*S*)-Ecgonin-methylester	7143-09-1	199.25	pK_b 4.4±0.20	H_2O vs pH 8, s pH 10, MeOH vs	−0.232±0.371	81–83[d]	83–85 (0.2 Torr)	
epi-Truxilline	113351-64-7	658.78	pK_a 9.05±0.20		4.449±0.485			
ζ-Truxilline	113350-58-6				4.306±0.482			
μ-Truxilline	113350-57-5				4.306±0.482			
γ-Truxilline	113350-56-4				4.449±0.485			
ϵ-Truxilline	113350-55-3				4.449±0.485			
neo-Truxilline	113350-54-2				4.306±0.482			
peri-Truxilline	113350-53-1				4.449±0.485			
δ-Truxilline	113350-52-0				4.306±0.482			
ω-Truxilline	113297-77-1				4.306±0.482			
α-Truxilline	490-17-5				4.449±0.485			
β-Truxilline	490-15-3				4.306±0.482			
Alcohols and ketones								
Tropine	120-29-6	141.21	pK_b 3.2±0.20	H_2O vs pH 10	−0.102±0.261	63	229–233	
Pseudotropine	135-97-7	141.21	pK_b 3.2±0.20	H_2O vs pH 10	−0.102±0.261	108–109	240–241	
Teloidine	575-62-2	173.21	pK_b 5.3±0.70 pK_a 13.8±0.70	H_2O vs pH 10	−1.524±0.391			
Nortropine	538-09-0	127.18.	pK_b 3.1±0.20	H_2O vs pH 10	−0.098±0.265			
Norpseudotropine	501-33-7	127.18	pK_b 3.1±0.20	H_2O vs pH 10	−0.098±0.265			
Tropinone	532-24-1	139.19	pK_b 5.1±0.20	H_2O vs pH 8, s pH 10, MeOH vs	0.068±0.346	42	103–104 (13 Torr)	
Nortropinone	5632-84-8	125.17	pK_b 4.8±0.20	H_2O vs pH 10	−0.325±0.340			
(2*R*,3*S*)-Ecgonine	481-37-8	185.22	pK_b 2.6±0.40 pK_a 3.7±0.60	H_2O vs pH 10	−0.596±0.366	205 (×1 H_2O) 198 water free		

Table 1. Continued

Alkaloids-esters	CAS number	Molecular mass	Dissociation constant[a]	Solubility[b]	Log P_{OW}[c]	Melting point (°C)	Boiling point (°C)	λ_{max}
Hygrine	496-49-1	141.21	pK_b 4.2±0.20	H_2O vs pH 8, s pH 10	0.282±0.269	Liquid at RT	77 (11 Torr)	
Hygroline	496-47-9	143.21	pK_b 3.6±0.20	H_2O vs pH 8, s pH 10	0.423±0.261			
Sedridine	501-83-7	143.23	pK_b 2.95±0.20	H_2O vs pH 8, s pH 10	1.307±0.501			
Pseudopelletierine	552-70-5	153.22	pK_b 5.0±0.20	H_2O vs pH 8, s pH 10, MeOH vs	0.632±0.346			
Pelletierine	2858-66-4	141.23	pK_b 3.6±0.20	H_2O vs pH 10	0.454±0.265			
Cuscohygrine	454-14-8	224.34	pK_b 4.0±0.20	H_2O vs pH 8, sls pH 10	0.722±0.347	Liquid at RT	118–125 (2 Torr)	
Anatoxin-a	64285-06-9	165.23	pK_b 4.5±0.20	H_2O vs pH 8, sls pH 10	0.910±0.279	Liquid		
Homoanatoxin-a	142926-86-1	179.26	pK_b 4.6±0.20	H_2O vs pH 7, s pH 8, sls pH 10	1.441±0.279			
Epoxyanatoxin	152406-37-6	181.23	pK_b 4.7±0.20	H_2O vs pH 8, s pH 10	0.141±0.410			
Calystegine A3	131580-36-4	159.18	pK_b 5.7±0.70 pK_a 13.8±0.60	H_2O vs pH 10	−1.091±0.427			
Calystegine A5	165905-26-0	159.18	pK_b 5.7±0.70 pK_a 14.0±0.60	H_2O vs pH 10	−1.162±0.415			
Calystegine A6	177794-04-6	159.18	pK_b 5.0±0.70	H_2O vs pH 10	−1.213±0.429			
Calystegine A7	197565-90-5	159.18	pK_b 5.9±0.70 pK_a 14.0±0.60	H_2O vs pH 10	−1.284±0.416			
Calystegine B1	127414-86-2	175.18	pK_b 6.8±0.70 pK_a 13.6±0.70	H_2O vs pH 10	−1.715±0.462			
Calystegine B2	127414-85-1	175.18	pK_b 6.7±0.70 pK_a 13.5±0.70	H_2O vs pH 10	−1.072±0.488			
Calystegine B3	178231-95-3	175.18	pK_b 6.7±0.70 pK_a 13.5±0.70	H_2O vs pH 10	−1.072±0.488			
Calystegine B4	184046-85-3	175.18	pK_b 6.7±0.70 pK_a 13.5±0.70	H_2O vs pH 10	−1.072±0.488			
Calystegine B5	197565-91-6	175.18	pK_b 6.9±0.70 pK_a 13.7±0.70	H_2O vs pH 10	−1.580±0.469			
Calystegine C1	156705-04-3	191.18	pK_b 7.7±0.70 pK_a 13.2±0.70	H_2O vs pH 10	−1.585±0.525			
Calystegine C2	190957-44-9	191.18	pK_b 7.7±0.70 pK_a 13.2±0.70	H_2O vs pH 10	−1.585±0.525			
Calystegine N1	177794-03-5	174.20	pK_b 4.3±0.70 pK_a 13.6±0.70	H_2O vs pH 10	−1.496±0.471			

Data in the Table were obtained from Chemical Abstracts by ACS (American Chemical Society) and [260–263]. RT, room temperature. 1 Torr = 133.322 Pa.

[a] The dissociation constants were determined at room temperature, if they are given as definite number and taken from [257]. Those that appear with a deviation, e.g. ±0. 40, result from calculation by using Advanced Chemistry Development (ACD) software SOLARIS V4.67 ((C) 1994-2002 ACD), published in Chemical Abstracts.

[b] vs, very soluble, >1 *M*; s, soluble, 0.1 *M* >1 *M*; sls, soluble, 0.01 *M* >0.1 *M*; sps, soluble, <0.01 *M*.

[c] Calculated octanol–water partition coefficient.

[d] Melting point from [258]; other melting points from [259].

tal for the ester alkaloids, e.g. hyoscyamine, scopolamine and the acetyl and tigloyl esters, as they will hydrolyse. If LLE is used, it is advisable to be done either very quickly or from only a moderately alkaline aqueous solution, basified by sodium carbonate or ammonia. Hyoscyamine production was monitored in transformed root cultures of *Datura stramonium* after rapid sample preparation by LLE from the aqueous extract at pH 12 [23]. A similar method was used for atropine measurement in human serum. The samples were prepared by LLE with diethyl ether from serum brought to pH 12. Purification and, in this case more importantly, a concentration of 10- to 20-fold was achieved [24]. Scopolamine in human serum was determined after fast serum extraction by methylenechloride; hydrol-

ysis was not observed [25]. Atropine was determined in biological fluids such as blood and gastric contents by micellar electrokinetic capillary electrophoresis (MECC). The samples were prepared from borate-buffered (pH 10) biological fluids by LLE with butylchloride, which is polar, and lighter than water [26].

An alternative to LLE is SPE. A wide choice of matrices is available in ready-made cartridges of several sizes. SPE on reversed-phase (RP) C_{18} phases uses the same principle as LLE; the aqueous extract is alkalinised and applied to the extraction column, where all sufficiently lipophilic compounds are retained. Elution is usually done with acidic methanol. This method is applicable to plant extracts [27], and laboratory-made RP18 extraction columns were proved to be equivalent [28]. The SPE purification on RP18 was also applied to blood serum samples, urine samples and egg yolk [29]. The determination of *N*-butylscopolamine bromide in serum and blood samples required concentration of analytes, which was done by SPE on RP18 columns [30]. Instead of RP cartridges many authors use especially prepared diatomaceous earth, kieselguhr (Extrelut) [31]. The basified aqueous extract is applied to the dry column, and the liquid is completely absorbed by the kieselguhr. The free alkaloid bases, like any lipophilic compound, are exposed on the surface of the kieselguhr particles and are eluted by organic solvents such as chloroform. A survey on alkaloid appearance in *Atropa belldonna* plant and root culture was given after extraction of plant tissue in aqueous sulphuric acid or in methanol and purification by SPE on diatomaceous earth (Extrelut) [32]. When subsequent HPLC analysis is planned, samples may be prepared using laboratory-made cartridges with pure silica gel and straight elution by the HPLC mobile phase [33]. Sample work-up by SPE is quite common in clinical and forensic analysis of alkaloids in serum and urine, because the method is rapid, reliable and yields sufficient concentration of analytes [34,35]. Rapid screening for 100 basic drugs and metabolites, among them atropine and cocaine, was done in urine using cation-exchange SPE and HPLC with a diode-array detection system. Here the additional advantages of cation-exchange SPE were appreciable: elimination of time-consuming shaking and centrifugation steps and reduced sample–operator contact with potentially infectious samples detection [35]. Although LLE is often used, it is tedious compared to SPE and prone to losses by alkaline hydrolysis or insufficient extraction due to good water solubility of the free bases. Saponins often contained in plant material give rise to emulsion during shaking of the liquids, and the phases are then difficult to separate even after centrifugation. SPE is faster, it uses less solvent and it is more versatile as several matrices are suitable for tropane alkaloids. Using refillable or laboratory-made cartridges makes the process less costly and, in addition, a lot of solvents can be saved.

2.2.2. Biosynthetic metabolites and degradation products

When performing biosynthetic studies, the tropane alkaloid metabolites require special attention, because usually their concentration is low and their water solubility is high. They tend to partially stay in the aqueous phase even as free bases. Their octanol–water partition coefficient is around 0 or less, which means equal distribution between the aqueous and the organic phase or even better water solubility (Table 1). LLE fails to give satisfactory results. It is the general practice to concentrate metabolites by SPE. Often Extrelut is applied and needs a modified elution with a larger solvent volume per gram Extrelut (2×4 ml chloroform, then 1×4 ml chloroform–methanol (9:1, v/v) [36], or Extrelut with the usual volume of 3 ml per gram is used with a more polar solvent mixture of dichloromethane–isopropanol (17:3, v/v) [37]. The plant extracts are made alkaline with carbonate or ammonia up to pH 10–11 before application to the column. These procedures ensure a complete elution of the alcohols. An alternative sample preparation for alkaloid alcohols with low molecular masses may be found in their volatility (Table 1). Hygroline and the ring-enlarged homomer *N*-methylsedridine were steam-distilled from the plant material quite efficiently [38].

2.2.3. Calystegines

Due to their hydrophilic nature calystegines are not extracted from Extrelut columns, because at any pH they will dissolve much better in water than in organic solvent (Table 1). In fact they have been overlooked for a long time in well-known drugs like

belladonna leaf, because with they were always discarded with the aqueous phase. For calystegine analysis, plant tissues are extracted by aqueous methanol or by water, and the alkaloids are purified by a strong cation-exchange column where they are retained by the charge of the secondary amino group [39,40].

2.2.4. Anatoxines

When analysing anatoxins from water samples, the major difficulty is the concentration of the analytes. After alkalisation of the water to pH ~10, anatoxin-a can be extracted by LLE into dichloromethane, which is thereafter evaporated [41]. With SPE using a weak cation-exchange material, the water samples can be applied at pH 7 and eluted by methanol containing 0.2% trifluoroacetic acid [42]. For subsequent HPLC–MS analysis, a tandem SPE sample work-up procedure is useful. A RP18 cartridge was used first followed by a cation-exchange one [43].

3. Gas chromatography

3.1. Development of the method

Atropine was one of the first alkaloids to be analysed by GC [44]. At that time only packed columns, mostly self-made, with a wide inner diameter (3–6 mm I.D.) were applied. The newly developed method for the separation of pure tropane alkaloids was soon adopted for plant extracts [45]. The alkaloids were prepared by solvent extraction, converted into salts, and separated directly as such. A 1–2 m long column with 3 mm I.D. was used; SE30 (silicon elastomer) was the coating of the stationary phase. The alkaloid salts decomposed in the hot injector and migrated as free bases. The first attempts also showed a limitation of GC which is still to be faced today: decomposition of compounds which are not sufficiently volatile. Atropine is dehydrated to apoatropine, the degree being dependent on the amount of glass wool in the injector and on the temperature of the injector [45].

Systematic tests of different GC column fillings [8] and tubing material for GC columns [46] as well as quantitative measurements of atropine and scopolamine by GC followed the first publications [47].

Soon attempts were made to derivatise those alkaloids that tended to decompose due to insufficient volatility, e.g. ecgonine, pseudoecgonine (=2*S*,3*S*-ecgonine) and benzoylecgonine [48]. In the following years numerous articles on the quantification of tropane alkaloids by GC were published. Atropine was measured in pharmaceutical preparations e.g. in liquids containing Valerian extract [49], or in the presence of cholinesterase reactivators [50] or together with theophylline, phenobarbital and aminophenazone in tablets [51].

The history of GC (Fig. 6), however, seems to reach much further back.

Plant tissue analysis for tropane alkaloids has always been a challenge. Various plant organs and

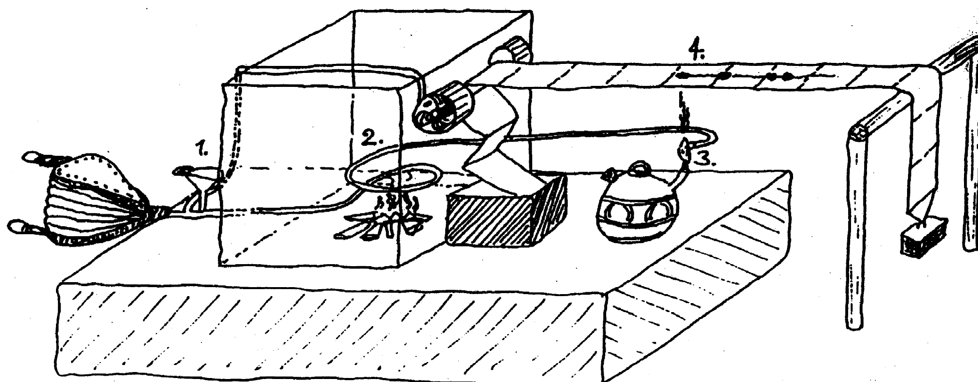


Fig. 6. Design for a gas chromatograph by Leonardo da Vinci (reprint permitted by Chrompack International, Middelburg, The Netherlands). 1, sample inlet; 2, oven; 3, signal amplifier; 4, recorder.

developmental stages of *Atropa belladonna* were examined for atropine, scopolamine, apoatropine and cuscohygrine [52]. It was shown that quantitative measurements by GC were faster and more precise than other methods used previously, i.e. mostly colorimetric measurements after tedious sample preparations and alkaloid isolation. GC soon became the method of choice for analysing plant tissues, e.g. monitoring the alkaloid content in *Datura innoxia* plants under salt stress [53]. The development of capillary columns with chemically bonded coatings greatly improved the GC analysis of tropane alkaloids as they are suitable for alkaloid analysis at higher temperatures. The resolution by capillary columns is better due to a higher number of theoretical plates. Capillary columns reach up to several 10 000 theoretical plates (N_{th}), the effective plate number depending on the individual compound and application. Capillary columns are easily installed compared with packed column with glass tubing which may break during installation. Packed columns are more versatile and can be filled with every stationary phase desired, but the variety of capillary column coatings in addition to various lengths, I.D.s and film thicknesses make the choice wide and the application easy.

Cell and tissue cultures of tropane-alkaloid containing plants, e.g. *Hyoscyamus niger* and *Duboisia leichhardtii* were established and alkaloid analysis by GC on capillary columns became routine. The identity of the alkaloids was assessed by GC–MS [54–56]. The mass spectra of several tropane alkaloids and their sequential fragmentation had been investigated previously using deuterium labelled compounds [57]. Further tropane alkaloid fragmentation patterns may be deduced considering the published patterns and applying the rules of preferred bond cleavage [58]. In most cases nonderivatised alkaloids were analysed. Attempts to enhance the detection sensitivity for atropine were made by using electron capture detection (ECD) [59]. The state of the art of tropane alkaloid GC as well as on GC of other alkaloids was comprehensively reviewed in 1984 by Baerheim-Svendsen and Verpoorte [8].

Researchers analysing plant extracts or pharmaceutical samples are usually interested in the results and do not operate GC as an object of interest on its own right. Still, a few guidelines on GC operation, column choice and influential parameters are useful to have [60]. Longer columns for example give better resolution, and N_{th} corresponds to the length, but simultaneously the time of analysis is directly proportional to the length as well. Longer migration times lead to peak broadening, so the effective resolution is increased by approximately 40% only, when doubling the column length. Choosing a smaller I.D. is more effective for better resolution. The usual film thickness of the chemically bonded silanol derivatives is 0.25-μm and higher film thickness enhances the capacity of the column. Thicker films, however, require higher temperatures for elution which may be critical for barely volatile alkaloids (Table 1). The injection technique has an important impact on the quality of the separation, and on the array of compounds that can be analysed at all [61]. For tropane alkaloids the common split injection is mostly used, but more sophisticated techniques, e.g. on-column injection may be of advantage for heat-labile compounds [62].

3.2. Applications in plant and tissue culture analysis

Several plants used for the production of atropine and scopolamine were reinvestigated by GC with capillary columns and by GC–MS, e.g. *Atropa belladonna* and *Datura innoxia*, and several new alkaloids were identified [32,63]. Flame ionisation detection (FID) and phosphorus–nitrogen detection (NPD) were used simultaneously for detection. The authors used Kovats retention indices to indicate alkaloid retention instead of giving the retention times only. The retention indices were given for each separated alkaloid according to the methods of Kovats, which allows a better transfer of published retention data to other separation systems by using an array of hydrocarbons for calibration [64]. It is regrettable that the Kovats system is not applied more often. Further, investigations are more comparable and alkaloids can be identified more reliably. *Atropa baetica* plants were investigated by GC–MS and shown to contain a similar alkaloid mixture like *A. belladonna*, but with higher alkaloid concentration in the main roots [65]. For Egyptian *Hyoscyamus* species, among them *H. muticus* and *H. albus*, the alkaloid profile was compared with the retention of

known compounds and confirmed by GC–MS. MS was operated with electron impact (EI) at 40 eV as ionisation method, and typical fragments were monitored for identification. The alkaloids were quantified in roots and aerial parts [31]. Quantification and recovery by a similar method was found sufficiently accurate between 10 and 100 μg/ml for extracts from *Atropa belladonna* [27]. *Nicotiana tabacum* was transformed with cDNA sequences from *Hyoscyamus niger* coding for two enzymes of the tropane alkaloid pathway. Both genes were expressed, and after metabolite application both enzyme proteins showed in situ activity by accumulation of the respective metabolic products. The tropane alkaloids together with those of the nicotine type were identified by GC–MS [66]. Alkaloids of the piperidine type, pelletierine and the like, and of the pyrrolidine type, hygrine as typical compound, were found to have chemotaxonomic significance for the genus *Sedum*. The alkaloids were identified and separated by GC–MS [67–69].

Currently, tropane alkaloid analysis by GC on capillary columns is a routine method, and packed columns have been mostly abandoned for plant alkaloid analysis. Retention indices were determined for many pharmaceutical compounds and alkaloids, and the capillary columns, methyl silicone coated, were found to have a high reproducibility [70]. GC–EI-MS is mostly applied for structure confirmation even if alkaloid quantification is performed by HPLC [71].

Many articles have been published on the GC analysis of plant tissue cultures producing tropane alkaloids, mostly transformed or nontransformed root cultures of Solanaceae. Both types of root cultures have been shown to accumulate considerable amounts of hyoscyamine and scopolamine. The analyses largely have focused on the quantification of hyoscyamine and scopolamine, on the calculation of the alkaloid yields of the cultures and on the minor alkaloids in order to compare the biosynthetic spectrum with that of the parent plants (Table 2).

In many of these reports, alkaloid quantification by GC and FID was combined with additional identification by GC–EI-MS at 24–70 eV. This is a fast and sensitive method of confirmation of known alkaloids and leads to tentative identification of new alkaloids with related structures by combination of retention properties and fragmentation patterns. The range of voltage chosen for ionisation shows that the conditions must be adjusted for each single instrument to obtain optimal fragmentation patterns of the alkaloids.

For quantification of alkaloids the FID response is often used, but more sensitive detection and quantification is enabled by a NPD [32,63,74,94]. The

Table 2

Parent plant	Tissue culture	Reference
Datura candida hybrid	Transformed root culture	[72,73]
Datura quercifolia	Transformed root culture	[74]
Datura innoxia	Anther culture derived plantlets	[75]
Hyoscyamus albus	Transformed root culture, plants	[76,77]
Datura candida × *D. aurea* hybrid	Transformed root culture	[78]
Several *Datura* and *Hyoscyamus* species and *Scopolia stramonifolia* petioles	Transformed root culture	[79]
Hyoscyamus niger	Root culture	[80]
Datura stramonium	Transformed root culture	[81–84]
Atropa belladonna	Transformed root culture in airlift fermenter	[85]
Atropa belladonna	Transformed root culture, regenerated plants	[86–88]
Atropa belladonna	Genetically engineered root culture over-expressing hyoscyamine-6-hydroxylase	[89]
Atropa baetica	Transformed root culture	[90]
Duboisia myoporoides	Root culture	[91–93]

sensitivity was found to be enhanced approximately fivefold compared with the FID signal, leading to the following limits of detection (LOD): for tropine and pseudotropine 5 μg/ml [37,95], for hyoscyamine and scopolamine 1 μg/ml, for apoatropine 0.5 μg/ml and for tropinone 0.2 μg/ml [96]. The analysis of pelletierine and pseudopelletierine alkaloids in *Punica granatum* is also performed by GC and NPD detection, identification was done by GC–MS [97]. The advantages of a NPD clearly lie in the better sensitivity and selectivity for nitrogen and phosphorus containing compounds. However, the NPD does not show other compounds with carbon, oxygen sulphur and hydrogen only, so that the purity of an extract cannot be determined. A further disadvantage is the corrosion of the alkali salt source that occurs with usage. The response of the NPD therefore tends to change during the life-time of the alkali source which is slowly consumed. For quantification, frequent recalibration and the use of an internal standard are indispensable. FID sensitivity has continuously been improved, and therefore many researchers find it unnecessary to deal with the difficulties inherent in using NPD. But, if higher selectivity is required besides better sensitivity a simultaneous detection by FID and NPD may be achieved by postcolumn outlet splitting and it is recommended. An example of detection of tropine, pseudotropine and tropinone by NPD and FID in an extract from root cultures of *Atropa belladonna* is given in Fig. 7. The temperature programme is set to a slight increase only, so that tropine and pseudotropine are

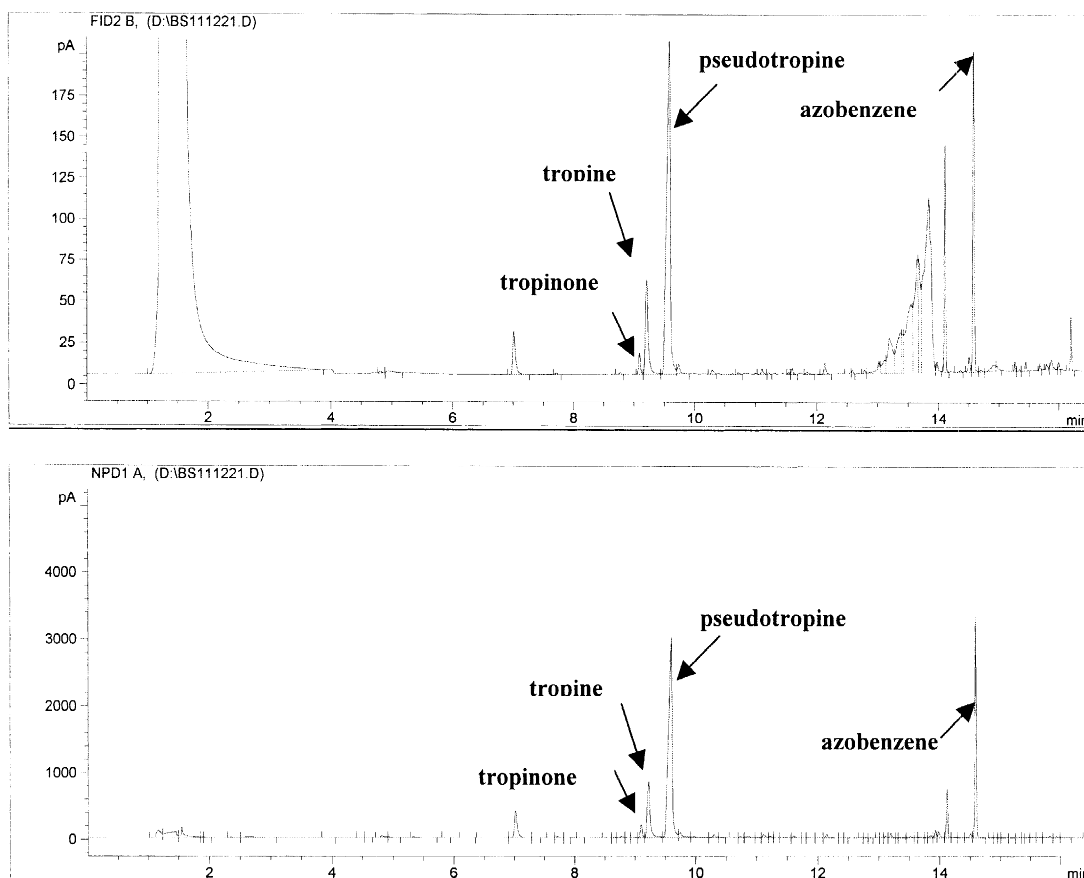


Fig. 7. Gas chromatogram of tropane alkaloid metabolites from a root culture of *Atropa belladonna*, simultaneous FID- and NPD detection. The detector response (*y* axes) were scaled to match. Retention times: tropinone, 9.092 min; tropine, 9.216 min; pseudotropine, 9.596 min; azobenzene (internal standard), 14.113 min.

well separated. In this temperature programme, hyoscyamine would be seen much later. The NPD is 14 times more sensitive for tropine and pseudotropine than the FID (the *y* axes of both chromatograms have different scaling), and the ratio of FID to NPD response is typical for each group of alkaloids. When examining the extract for further alkaloids, the NPD trace contains important information. It is evident that the group of signals at 13 min and the signal at 15.1 min can be ignored, because they do not have any nitrogen response, and the NPD:FID ratio of the signal 14.1 min is ~5 and therefore no alkaloid of the group of interest. The signal at 7 min turned out to be hygrine.

Reliable quantification of atropine and scopolamine is mostly achieved without derivatisation [6], but for precise results some authors have found it necessary to make trimethylsilyl (TMS) derivatives using silylating agents before GC analysis to prevent loss of water and formation of apoatropine and aposcopolamine during injection [37]. Derivatisation of atropine and scopolamine is also preferred for minute quantities in clinical analysis (see below).

While plants containing atropine and scopolamine have been thoroughly investigated in order to find good sources for pharmaceutical drug production, the interest in the production of cocaine by Erythroxylum plants is a result of the widespread abuse of cocaine preparations. Many analyses have been undertaken on Erythroxylum species other than *E. coca* and *E. novogranatense*; for reviews on the structures and species see [6,14,15]. The root bark of *Erythroxylum monogynum* and the stem bark of *Erythroxylum lucidum* and of *Erythroxylum zambesiacum* were shown to contain tropane alkaloids. This was confirmed by GC–EI-MS and by synthesis of the novel compounds. No cocaine was found in these samples [98–100]. Analytical methods for minor alkaloids can serve to identify the origin of illicit cocaine [101]. A comprehensive review shows that GC with various detection methods (FID, NPD, ECD, MS) is the major technique used in this field [102]. Sometimes elaborate sample preparation is necessary to enable the analysis of minor compounds. Ion-pair chromatography was used for sample clean-up to facilitate detection and determination of small amounts of pseudococaine [=(2*S*)-benzoyloxy ecgonine methylester, cocaine has (2*R*)-configuration] in Coca leaves and illicit cocaine samples [103]. The GC and GC–MS analyses of cocaine and related compounds are performed mostly without derivatisation, as there is no free hydroxyl group (as in hyoscyamine and scopolamine) that tends to cleave off as water [104,105]. However, the metabolites ecgonine, pseudoecgonine and benzoylecgonine are too unstable to be separated without derivatisation [106]. The truxillines, dimeric derivatives of cinnamoylcocaine, due to their high molecular masses, must be derivatised. A method using lithium aluminum hydride reduction and then acylation with heptafluorobutyric anhydride was published [107] and later replaced by silylation with *N,O*-bis(trimethylsilyl) acetamide to obtain TMS derivatives, which can be analysed by GC [108]. For the detection and identification of trace levels of new alkaloids in cocaine-containing Erythroxylum species, derivatisation before GC analysis was found beneficial. *N*-Methyl-*N*-(trimethylsilyl)fluoroacetamide (MSTFA) was used for silylation, and after GC separation, the mass spectra of the derivatives could be interpreted by comparison with known compounds [104,105,109].

3.3. Metabolite monitoring in biosynthesis investigations

Better means of analysis opened the way for investigations of alkaloid metabolites, biosynthetic pathways and the products of in vitro enzymatic reactions. The analyses were done by GC or GC–MS in most cases because some metabolites and minor alkaloids of the tropane alkaloid pathway, e.g. tropinone, tropine, pseudotropine, acetyl tropine and acetyl pseudotropine show very low UV light absorption, making this method unsuitable for detection. The work on tropane alkaloid biosynthesis has been thoroughly reviewed [110,111]. Enzyme assays for putrescine-*N*-methyltransferase, tropinone reductase and hyoscyamine-6β-hydroxylase using GC for evaluation have been summarised [112]. Enzymes esterifying tropine and pseudotropine with acetic acid or tigloic acid were found in tropane alkaloid producing root cultures and measured by GC–MS [113–115].

GC–MS has become an important means of

following labelled precursors fed to plant tissues in order to trace their metabolic fate. Nonradioactive stable isotopes can be used for feeding and no purification of compounds is necessary to prove precursor incorporation data. In a series of feeding experiments with differentially labelled precursors the hypothesis was proven that littorine, the phenyllactic ester of tropine, is formed first and subsequently isomerised to give hyoscyamine. The turnover of littorine to hyoscyamine was proved by feeding ^{13}C-labelled littorine to transformed root cultured of *Datura stramonium*, and no backward reaction from hyoscyamine to littorine was observed [116]. Deuterium- and ^{13}C-labelled 3-phenyllactate was applied as precursor, and the incorporation of label into was monitored by GC–EI-MS and NMR spectroscopy [117–121]. The separation of the positional isomers hyoscyamine and littorine was achieved by a DB 17 column (50% phenylpolysiloxane 50% dimethylpolysiloxane) and was confirmed by using synthetic standards in GC–MS analysis, where only littorine forms a fragment of m/z 91 that is not found in the spectrum of hyoscyamine [78]. This work has been summarised [122]. Phenyllactic acid was also labelled with ^{18}O and fed to root cultures of *Datura stramonium* in continuation of the attempt to understand the mechanism of rearrangement from littorine to hyoscyamine [123]. The results were evaluated by GC–MS. It was shown further that fluorinated tropane alkaloids were generated when phenyllactic acid was applied, that carried a fluor atom in either the 2′, 3′ or 4′ position, proving that the conversion of flurinated littorine was not inhibited by the fluor atom on the aromatic ring [124].

The intermediates between *N*-methylpyrrolinium salt and tropinone have been investigated by feeding [^{13}C]-acetate and labelled potential intermediates and detection with ^{13}C-NMR and GC–MS. It was shown that hygrine is not a direct precursor of tropinone [125]. Hygrine, several analogues and products were separated by GC.

For understanding tropane alkaloid biosynthesis, the substrate specificity of the individual enzymatic steps is of interest. That can be tested by feeding structural analogues of putrescine, cadaverine, tropinone or pseudopelletierine and measuring the products by GC–MS [94,126]. The enzyme specificities in the tropane alkaloid pathway were described by measuring the turnover of the substrate analogues by the isolated enzymes in vitro [36,127]. In these analyses, tropinone, tropine, pseudotropine, their acetyl and tigloyl esters, hyoscyamine and scopolamine were separated by GC [94] as well as the reaction products after feeding the respective analogous compounds [126].

GC-supported investigations have also been undertaken on the metabolites of cocaine in plants. Histochemical localisation and quantification of pseudotropane alkaloids in *Erythroxylum coca*, var. coca and in *E. novogranatense*, var. novogranatense by GC showed the alkaloids to be accumulated in vacuoles of photosynthetic and vascular parenchyma [128]. Hygrine and cuscohygrine were analysed in Coca leaves using GC and HPLC, and both procedures were found to give concordant results. The detection limits are 100 μg/ml by GC–FID for hygrine and cuscohygrine. By HPLC with UV detection at 220 nm the LOD for cuscohygrine was the same and the LOD for hygrine is even lower at 50 μg/ml [129]. The recovery of added hygrine and cuscohygrine from plant samples was significantly better by GC, indicating that the method has better reliability.

3.4. Clinical and forensic analysis for comparison

Sensitive means of analysis have not only enabled a deeper insight into the metabolism and biosynthesis in the plant, but also into the alkaloid metabolism in humans. The number of publications that describe the GC and GC–MS analysis of tropane alkaloids and their degradation products in human serum is too high to be presented here. The usual single dose of scopolamine is below 1 mg for humans. This illustrates the demands for sensitivity of the analytical method employed.

With GC and ion trap tandem MS the limit of detection (LOD) is 50 pg/ml, the limit of quntification (LOQ) is 200 pg/ml which is sufficient to enable pharmacokinetic analysis of single doses of scopolamine after appropriate concentration of serum samples by LLE [25]. Scopolamine was derivatised by MSTFA–toluene (1:4), and the internal standard was atropine [130]. Even lower concentrations of scopolamine or atropine can be measured by radioreceptor assays, but this method requires the use

of radiolabelled alkaloids and isolated muscarinic receptors from e.g. calf brain. The determination uses the quantification of radioligand displacement from the receptor by scopolamine or atropine in the serum sample after sample preparation by SPE on reversed-phase silica [131,132].

The growing abuse of cocaine and of addiction to the compound has stimulated intensive research into its metabolism in the human body. Again, the literature is too extensive to be included here, but in a brief overview, it may be stated that for the study of cocaine receptors (they are dopamine receptors) in the brain, alternatives to chromatographic methods are generally used. Receptor assays with tritiated or other radiolabelled ligands [133] or radioimmunoassay (RIA) or single photon emission computed tomography (SPECT) studies [134] have been used to obtain very sensitive measurement techniques. The pharmacokinetics of cocaine, i.e. transport and metabolism in the body, in contrast, are often measured by GC and GC–MS. Urine or serum samples were tested by an enzyme multiplied immunoassay technique, and the result was confirmed by GC–NPD [135]. In particular, cocaine metabolism was found in placenta tissue leading to a toxic metabolite, norcocaine, that was analysed by GC and detected simultaneously by FID and NPD [136]. The results were confirmed by GC–MS. The power of the GC methods has been emphasised recently by the sensitive measurement of cocaine deposited in the hair of consumers [137]. The identification was achieved by GC–MS with chemical ionisation [138,139]. Here the race-dependent incorporation of cocaine in hair was investigated. Analysis was done by GC–MS with an ion trap and by GC–NPD [140]. The LOQs are amazing: several authors report LOQs of 0.1–0.4 ng/mg hair [140,141]. As little as 10 mg hair may be used per analysis [142]. The identification of cocaine in hair is of forensic interest, and a court decision usually requires GC–MS data as indicators of a person's cocaine intake, whereas receptor assays alone are not considered sufficiently reliable. The dose dependency and the kintetics of cocaine incorporation into hair was determined by incorporation of deuterium-labelled cocaine. Volunteers showed a high inter-individual difference in cocaine deposition in hair, but the traces of a single dose of more than 35 mg could reliably be detected for 2–6 months afterwards [143]. The deposits and metabolites of numerous other drugs besides cocaine can be traced in hair, mostly by GC–MS techniques [144]. For police investigations and court cases, the source of cocaine that has been confiscated or found in a person's body fluid is important to know. Cocaine batches and sources can be distinguished by their content of minor alkaloids, such as hygrine, pseudococaine and methylecgonine esterified with other carboxylic acids than benzoic aid, even if they appear in trace levels only [145–147]. If the cocaine had undergone a certain chemical treatment, this can be detected by traces of reaction products, e.g. chlorine substitutions in the aromatic ring arising from treatment with sodium hypochlorite [148].

3.5. Calystegines and anatoxin-a

In 1990 the structures of a new group of hydrophilic nortropane alkaloids, the calystegines were published for the first time [149]. Calystegines were isolated from transformed root cultures of *Calystegia sepium*, Convolvulaceae. They carry three to five hydroxyl groups, which are not esterified. The calystegines rapidly became of interest because of their glycosidase inhibitory activity [150,151]. They are of toxicological interest because they are contained in edible fruits and vegetables [152]. The state of the art on calystegine research was reviewed in 1996 [153].

Due to their hydrophilic properties calystegines need methods for sample preparation that differ from those usually applied in tropane alkaloid analysis (Section 2). The subsequent analysis by GC is only possible after derivatisation, i.e. silylation. For GC–MS investigation, the compounds were treated with MSTFA in pyridine for 12 h at 60 °C. Under these conditions, every OH group and the secondary nitrogen group is derivatised [150]. However, as these aggressive conditions lead to some decomposition of calystegines, the method cannot be used for quantification. Derivatisation by hexamethyldisilazane containing 10% trichlorosilane leads to complete derivatisation of the hydroxyl groups; but the nitrogen is not attacked nor is any calystegine degradation observed [154]. After derivatisation, the calystegines were separated on a capillary GC column and their identities in the plant extracts were

confirmed by GC–MS by monitoring characteristic fragments [154]. With this method the calystegines were measured in transformed root cultures of *Atropa belladonna*, in root cultures and in intact plants of several other tropane-alkaloid containing Solanaceae [40,155–157]. Further investigations focused on plant extracts of numerous Convolvulaceae, and GC–MS after silylation was applied as the routine method [158]. Recently, calystegines have been determined in many developmental stages of potato (*Solanum tuberosum* cult. Liu) tubers and plants [39]. The biosynthesis of calystegines in potato tubers appears to proceed via the classical tropane alkaloid pathway, metabolites and enzymatic products in potato were measured by GC and GC–MS [159,160]. Application of ^{15}N-labelled tropinone to *Calystegia sepium* root cultures leads to labelled pseudotropine and calystegines in a time-dependent manner. Calystegines and pseudotropine were identified and quantified by GC–FID NPD as TMS derivatives (Fig. 8). The incorporation of label was followed by GC–MS. By these experiments the biosynthesis of calystegines was proved to include tropinone and pseudotropine as metabolites. 2,7-Dihydroxynortropane (Fig. 5) was labelled by [^{15}N]-tropinone application, however, the hydroxylation pattern in this metabolite does not match the proposed biosynthetic pathway that always includes a hydoxyl group at C3 deriving from the reduction of tropinone [161].

For several years, anatoxin-a and homologous compounds have been of major interest to the public because they are responsible for fresh water poisoning of humans and animals caused by so-called ‘‘toxic algae’’. Anatoxin-a is a homotropane derivative (Fig. 4) with an enlarged ring (2-acetyl-9-azabicylo[4.2.1]non-2-ene). The toxicity is high. It was determined to be 200 μg/kg in mice when applied by intraperitoneal injection [162]. In order to prevent excessive extraction of high volumes of fresh water, sensitive means of analysis are necessary. Mostly anatoxins are analysed by HPLC, but an effective method using GC–ECD after derivatisation is available. Pentafluorobenzyl bromide was used to substitute the secondary nitrogen of anatoxin-a, and detection by ECD of the product was very sensitive due to the high halogen content. The LOD was 5 ng/ml sample [41]. HPLC analysis of anatoxins was combined with GC–MS after derivatisation for confirmation of the identity of the analytes [42,163]. The derivatisation of anatoxins is not necessary to enable separation by GC, but it leads to enhanced sensitivity [163].

3.6. Summary

3.6.1. Columns: fused-silica with chemical bonded silanol phases

(i) 100% dimethyl polysiloxane (e.g. DB1), this type of column is used most often, most compounds are retained in the order of their molecular mass
(ii) 5% phenyl polysiloxane, 95% dimethyl polysiloxane (e.g. DB5®)
(iii) 14% cyanopropyl phenyl polysiloxane, 86% dimethyl polysiloxane (e.g. DB1701)
(iv) 50% phenyl polysiloxane, 50% dimethyl polysiloxane (e.g. DB17), this column is particularly useful for the separation of littorine and hyoscyamine which otherwise needs derivatisation [76] or GC–MS [78].

Column length: 15–50 m, mostly 25 m or 30 m.
I.D.: 0.32 or 0.25 mm
Film thickness: 0.1–0.5 μm, mostly 0.25 μm[1]

3.6.2. Injection and separation

(i) Split injection, split ratio 1:10–1:50
(ii) for very dilute samples: splitless for 10–30 s, then high split ratio (1:50:1–70) for some minutes to prevent a broad solvent signal [61]
(iii) on-column for compounds that tend to decompose or be retained in the split injector [62]
(iv) programmed temperature vaporiser (PTV) for compounds that tend to decompose or be retained in the split injector [61]

Injection volume: 0.5–2 μl, independent from the type of injection.

Carrier gas: nitrogen (not with NPD) or helium.

[1]Not indicated by many authors, but influential on column capacity and on the temperature necessary for elution.

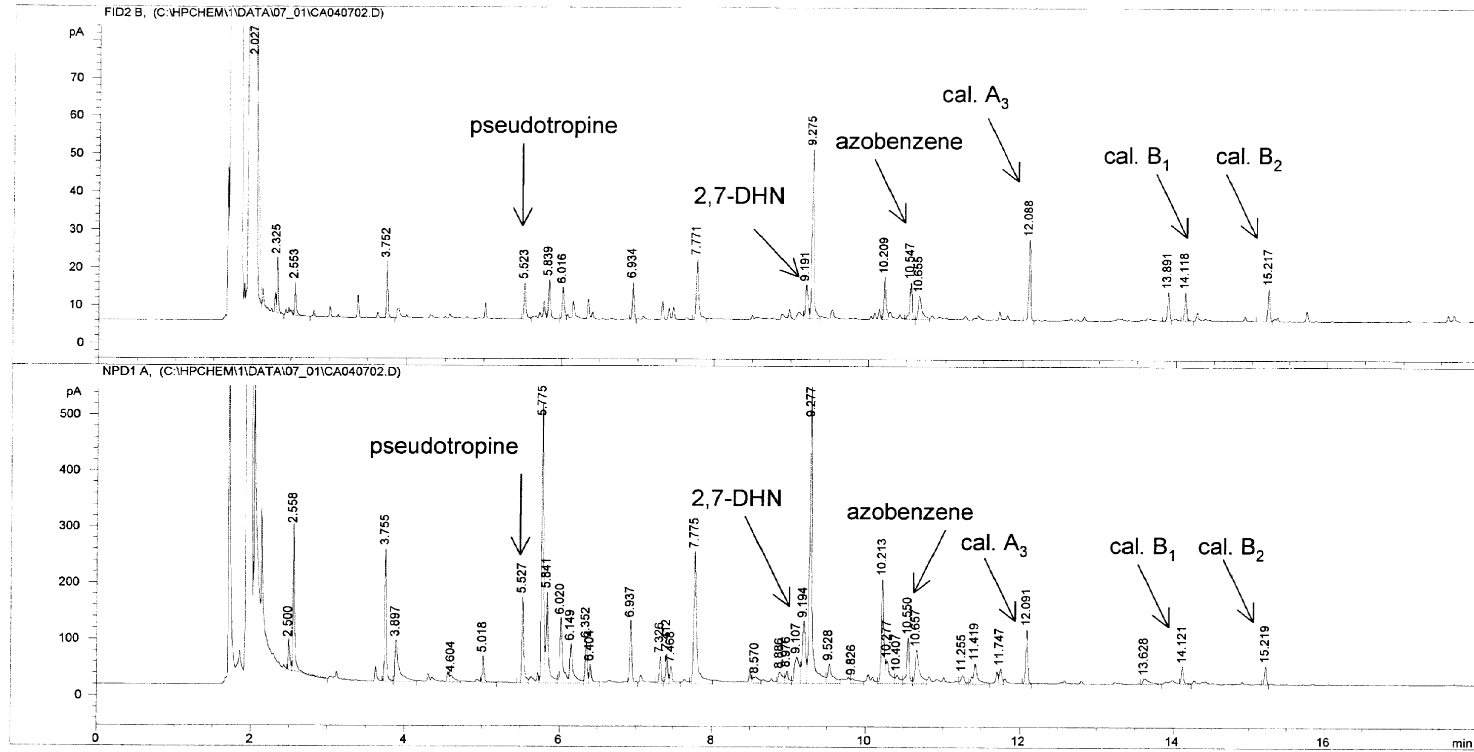


Fig. 8. Gas chromatogram of calystegines and their metabolites from root cultures of *Calystegia sepium* after silylation, simultaneous FID and NPD. Quantification was done by the FID response.

Carrier gas flow: approximately 15–30 cm/s[2].

Temperature programme: varies depending on the type and dimension of the column, the type of injection and the groups of compound to be analysed.

3.6.3. Detection

Limit of detection[3]

(i) with FID 1 μg/ml for hyoscyamine, 100 μg/ml for hygrine
(ii) with NPD: 1 μg/ml for hyoscyamine, 0.2 μg/ml for tropinone, 5 μg/ml for tropine
(iii) with GC–MS: 50 pg/ml for scopolamine.

Limit of quantification[3]

(i) with FID: 3 μg/ml hyoscyamine
(ii) with NPD 3 μg/ml for hyoscyamine, 1 μg/ml for tropinone, 10 μg/ml for tropine
(iii) with GC–MS 200 pg/ml for scopolamine.

Time per analysis: 15 min–60 min.

3.7. Conclusion

GC is the method of choice for biosynthetic or catabolic studies on the metabolites of tropane alkaloids that have hardly any UV absorption.

The advantages of GC clearly lie in the equally high sensitivity of detection for all tropane alkaloid metabolites. This is especially true for GC–MS but also holds for the usual FID detection. With GC–MS reliable information on the identity of the compounds analysed is available as well. Furthermore, the high selectivity of capillary columns enables separation of many alkaloids simultaneously within comparatively short times.

The major disadvantages is the sometimes tedious sample work-up which may include derivatisation.

4. High-performance liquid chromatography

HPLC for pharmaceutical preparations of alkaloids was started about 30 years ago. In 1973 the separation of atropine, homatropine, scopolamine and apoatropine on a silica gel column with tetrahydrofuran containing 1% ammonia as solvent was reported. The column was 1 m long×4.5 mm I.D. [164]. Smaller particle sizes of the adsorbent enabled the use of shorter columns. On a 5-μm silica gel column with diethyl ether–methanol–diethylamide (90:10:1), baseline separation of atropine, apoatropine and scopolamine was achieved [165]. RP columns were successfully applied to the analysis of atropine and tropic acid using a solvent containing heptane sulphonic acid as ion-pair reagent. At the same time, cocaine and related pseudotropine alkaloids were analysed by HPLC. Because of the frequent abuse of this alkaloid, methods of analysis from biological samples (e.g. urine) were soon developed [166]. A RP18 (reversed-phase, octadecyl-derivatised silica) column was used with a solvent containing acidic potassium phosphate buffer (pH 2.7) with 17% acetonitrile. The limit of detection was given as 0.1 μg/ml sample. The state of the art of HPLC for tropane and pseudotropane alkaloids was comprehensively reviewed by Baerheim Svendson and Verpoorte [167].

4.1. Applications in plant and tissue culture analysis

The first report on quantification of tropane alkaloids in plant samples by HPLC was published in 1985. A microparticular (5 μm) silica-gel column was employed using with the same solvent mixture as in the first separation published: tetrahydrofuran containing 1% ammonia. The contents of atropine and scopolamine were given for *Atropa belladonna* and *Datura innoxia* roots, twigs, leaves, and fruits, and a survey of the alkaloid contents of many samples of *Datura stramonium* leaves from different parts of Serbia was given [168]. The interest in tropane alkaloid biosynthesis prompted the develop-

[2] In most publications the carrier gas flow-rate is not given although this is of decisive influence for the retention time. Instead, the pressure is often indicated which is used to pump the carrier gas into the column. The pressure, however, does not correspond directly to the flow-rate of the gas because column length and diameter, film thickness, and temperature also influence the carrier gas flow. It is highly recommended to measure and indicate the gas flow-rate by injecting compounds with no retention at defined temperatures.

[3] The LOD is usually defined as three times the height of the baseline noise. The LOQ is 10 times the baseline noise. The values listed here are taken from different publications and are not directly comparable.

ment of sensitive HPLC methods. For the separation of hyoscyamine and scopolamine from roots and leaves of *Datura innoxia*, Plank and Wagner used a RP18 (5 μm) HPLC column (25 cm×4 mm) eluted by buffered methanol–water as solvent (40:60, pH 7.25, triethyl ammonium phosphate 0.2%). Elution was monitored at 204 nm, and the alkaloids were measured with a LOD of 1 μg/ml sample [28]. Absolute detection limits of 2.5 ng for scopolamine and 5 ng for hyoscyamine were reported for a solvent system that was buffered at pH 3.5 with triethylamine and formic acid, and detection was done at 254 nm [169]. This became a standard method and is still often applied [170]. Hyoscyamine production was also measured in transformed root cultures of *Datura stramonium*. The HPLC method consisted of a RP18 column (30 cm×3.9 mm) eluted with a solvent system composed of acetonitrile–water–acetic acid–tetrahydrofuran (50:50:5:2) which was used isocratically. Detection was at 230 nm [23]. Further publications on the determination of tropane alkaloids in plant materials and pharmaceutical preparation appeared in the following years. Using a RP18 column with a buffered (triethylamine–phosphoric acid, 30 m*M*, pH 2.2) aqueous acetonitrile mixture (12.5% acetonitrile) hyoscyamine, scopolamine and tropic acid were measured with LODs of 1 μg/ml for the alkaloids and 0.25 μg/ml for tropic acid. The detection wavelength was 204 nm [171]. The method was applied to micropropagated plants of *Datura innoxia* [172] and for cell suspension cultures of *Datura innoxia* immobilised on calcium alginate [33].

Ion-pair chromatography on RP columns was found advantageous because tropane ester alkaloids are quite strong bases (Table 1) and tend to deprotonate in neutral buffers, leading to excessive tailing. Alkaline buffers are not tolerated by most HPLC stationary phases, especially if the support is silica gel. With detection at 210 nm hyoscyamine, scopolamine, anisodamine and anisodine were determined in Chinese Solanaceous plants. Sodium dodecyl sulphate (SDS) was the counter ion and a LOD was approximately 1 μg/ml for all four alkaloids. The calibration for quantification was done between 20 and 200 μg/ml [173]. Similar detection limits are given for a HPLC method measuring alkaloids from a transformed root culture of *Duboisia leichhardtii* [174]. SDS was also used as counter ion when measuring hyoscyamine and scopolamine in several solanaceous crude drugs [175]. The chromatography of tropane alkaloids as SDS ion pairs is sufficiently selective to allow the baseline separation of 6β-hydroxyhyoscyamine, 7β-hydroxyhoyscyamine, scopolamine, hyoscyamine and littorine in this elution order [87,176]. In complex mixtures of powdered plants applied against gastrointestinal disorders, hyoscyamine could be determined using a column-switching technique. Both columns were filled with a chemically bonded ODS (octadecyl silica, identical with RP18) gel (TSK Gel 120 A). The first column was eluted with 67 m*M* sodium phosphate buffer pH 3.5–methanol (48:52), and after the first column the SDS-containing (17.5 m*M*) second solvent system was added by a valve to elute the second column. With this configuration, many other secondary plant products e.g. from ginseng, ginger, glycyrrhiza, gentain and geranium could be eliminated or separated far enough to achieve a quantification of hyoscyamine, scopolamine, anisodamine and anisodine with 0.3–0.6 μg/ml as LOQ at 210 nm [177]. Another column switching technique made use of two different columns eluted by the same solvent (acetonitrile–water–triethylamine 35:65:0.5, v/v) A RP cyano-column was used first and was followed by an RP18 column [178]. The detection at 254 nm was linear between 100 μg/ml and 5 mg/ml atropine and scopolamine. A major inconvenience of ion-pair chromatography is the difficulty or impossibility of recovering sample compounds for further analysis due to the ion-pair reagent. Reliable resolution of alkaloids in alkaline solvents systems is offered by RP18-modified polysterene columns, which tolerate high pH, but are more expensive [26].

Investigations by HPLC have also been undertaken to measure cocaine and its typical metabolites in plants. Hygrine and cuscohygrine were analysed in Coca leaves using GC and HPLC, and both procedures were found to give concordant results. By HPLC and UV detection at 220 nm the LOD for hygrine was 50 μg/ml and 100 μg/ml for cuscohygrine [129]. Putrescine is a precursor for tropane alkaloids and yields *N*-methylputrescine as product of putrescine-*N*-methyltransferase. These metabolites can only be measured by HPLC, if dansylation adds

a UV-absorbing moiety to the molecules. Using fluorescence detection, the method becomes very sensitive [179].

As most of the alkaloid separations by HPLC are done on RP18 as stationary phase, a data base of retention indices of toxicologically relevant substances on this type of column published in 1994 [180] is of interest. Gradient elution with a mixture of acetonitrile and triethylammonium phosphate buffer (25 m*M*, pH 3.0) was applied. The gradient profile was 10–70% acetonitrile in 30 min and 5 min 70% acetonitrile with a flow-rate of 1 ml/min. The RP18-column was 125×4 mm I.D. and contained 4 μm material. The detection was done by diode array and most of the UV spectra and the UV maxima are contained in the publication as well.

4.2. HPLC with other than UV detectors

Because of the low UV absorption of tropane alkaloids [180] many attempts have been made to combine HPLC separation with other than UV detectors. In 1987, atropine determination in pharmaceutical preparations containing powdered belladonna leaf and further compounds was achieved by electrochemical detection [181]. A polarographic analyser was used in combination with a three-electrode station. The working electrode was a rotary glassy carbon or platinum unit with 3 mm diameter. The saturated calomel reference electrode was placed in a compartment separated by a porous bridge from the measuring cell and filled with the same solution. A platinum wire was the auxiliary electrode. The products of the electrochemical reaction were identified as secondary amines and formaldehyde. The optimum amperometric detection was obtained at 1.2 V. The LOD was 2.9 ng per analysis, with a sample size of 20 μl that is 145 ng/ml, more than 10-fold the sensitivity of HPLC with UV detection at 220 nm. In a screening of alkaloid drugs in food, a comparison between electrochemical and fluorescence detection was made [182]. It was shown that the tropane alkaloids atropine, scopolamine and cocaine showed, as expected from their UV absorption, no response in the fluorescence detector with an excitation wavelength of 254 nm and emission measurement at 408 nm. With amperometric electrochemical detection they were responsive, but their reaction was not so strong as that of other alkaloids e.g. the ergot alkaloids. Scopolamine was found to react 10-fold more strongly to a 1.2 V working potential than atropine. This shows that with ECD the LOD can be lowered, but again the tropane alkaloids are only moderately responsive compounds. Conductometric detection was shown to be possible for atropine, homatropine and scopolamine [183]. Peaks were detected as negative changes in conductance. It was shown that the method is easy to operate and does not need complicated sample preparation. The LOQ was 500 ng/ml for each alkaloid. If fluorescence detection is applied after precolumn derivatisation of atropine, the LOD was lowered to 10 ng/ml sample [184], with a LOQ of 50 ng/ml. The derivatisation reagent used was 1-anthroylnitrile (1-AN) and the separation was accomplished on a RP18 column.

Microbore-HPLC columns with I.D. of typically 2 mm are advantageous in having low solvent consumption with a flow-rate of 200 μl/min or less. Of course, the demands for a sensitive detection are even higher than with ordinary HPLC. The detection of atropine and scopolamine after separation on a RP8 column and a PRP-1 polymeric column was possible with postcolumn derivatisation with tris(2,2′-bipyridine)ruthenium and chemiluminescence detection [185]. With the development of CE an obvious enhancement of UV detector sensitivity was achieved. This, in combination with the advantages of microbore columns, i.e. low solvent and low sample consumption, opens a promising future for microbore-column-HPLC, even with UV detection. For most chromatographers, however, it will be necessary to purchase a complete new system with pumps that deliver precisely low solvent quantities and with a highly sensitive detector, a drawback to this method.

Microbore columns may also be of advantage in HPLC–MS. Coupling the sensitivity of detection of a mass spectrometer to an HPLC appears most promising for alkaloids otherwise difficult to detect at low concentrations, but the problems lie in the evaporation of the mobile phase eluting from the HPLC, which in the case of tropane alkaloids contains mostly buffers. The usual buffers are scarcely volatile or nonvolatile and the alkaloids elute as salts due to the acidic pH. After separation

on a polymeric RP column in an alkaline ammonium acetate buffer (pH 10.4, 0.1 *M*, mixed with 30% acetonitrile) it was possible to analyse hyoscyamine, homatropine and scopolamine by thermospray MS. A loading of 5 ng/ml could be detected with a signal-to-noise ratio of 10:1 [26]. The choice of the interface for LC–MS coupling (electrospray, thermospray, ion spray etc.) as well as the resulting ionisation method are of crucial importance for their application to the analysis of natural products. In a comprehensive review of the literature on LC–MS coupling for analysis of alkaloids, Verpoorte and Niessen also discuss solvent compatibilities and stationary phases which are useful in LC–MS [12]. If the liquid solvent can be minimised, this will be a definite advantage for coupling liquid chromatography to MS. Supercritical fluid chromatography (SFC) uses carbon dioxide with organic modifiers as such as methanol, trifluoroacetic acid or triethyl amine as solvent systems. The SFC column was connected to a quadropole mass spectrometer by an atmospheric pressure chemical ionisation interface. *Atropa belladonna* plant extracts were measured, and the sensitivity for hyoscyamine was below 1 ng per signal [186].

4.3. Pharmaceutical, clinical and forensic analysis for comparison

For quality assessment in pharmacy, HPLC is the routine method although many pharmacopoeias still advise other methods for tropane alkaloid analysis. The alkaloids are either titrated as bases or determined photometrically as ion pairs with an acidic dye anion. Purity is mostly examined by TLC. These methods are time consuming and not very accurate. Atropine and scopolamine should be analysed by a precise and robust HPLC method [187]. Szasz et al. suggested various HPLC methods for compendial quality testing for Solanceaeous alkaloids. They used, among others, a solvent system consisting of Chiralcel OD with *n*-hexane–isopropanol–methanol–triethylamine, that separated the enantiomers (see 4.3.1) of the alkaloids [188]. HPLC methods for quality testing are usually more sturdy than GC methods, although a combination of GC and HPLC assays can be advantageous if different compounds have to be measured. Obidoxime and atropine in automatic injection devices were determined separately, obidoxime by HPLC and atropine by GC, and this was found more effective than a single chromatographic procedure for both components [189].

In clinical and forensic analysis, HPLC is the method of first choice to analyse blood serum samples. For tropane alkaloids, improved UV and diode array detectors and better solvents have enhanced the sensitivity so that these alkaloids are monitored routinely around 210 nm after separation, usually on RP columns. A LOD of 8.5 ng/ml was reported for atropine after separation on a RP18 column in an acidic solvent (pH 2.8, acetonitrile–6 m*M* phosphoric acid, 8:2). The detection wavelength was 215 nm and the low LOD per ml was achieved by injecting 200 μl of sample [190]. When serum and urine samples from volunteers who had received oral administration of *N*-butylscopolamine were tested, a very low oral absorption from the intestine was determined [30]. The detection limit at 212 nm after appropriate sample concentration was given as <10 ng/ml body fluid, which corresponds to 200 ng/ml chromatographic sample. A similar method was used for atropine measurement in protein solutions like human serum. The samples were concentrated 10- to 20-fold and atropine was measured at 210 nm after separation on an RP18 column with a mobile phase containing 10 m*M* heptane sulphonic acid as ion-pair reagent [24].

Forensic examinations for drugs of abuse are performed on serum and urine samples. In this field many applications are published for the analysis of cocaine and its metabolites. For example, a column switching technique was applied to the direct analysis of cocaine in urine [191]. The applications of HPLC and GC–MS for identification of drugs of abuse in blood have been reviewed, including columns, mobile phases, methods of sample preparation and validation for each individual analyte [34]. Another review lists methods for the rapid screening of basic drugs and their metabolites in urine. Sample work-up was done by cation-exchange SPE columns, and analysis was by HPLC–DAD [35]. Recently, hair analysis has become an additional means of surveillance of drug intake. A review on testing drugs in hair includes GC–MS as well as HPLC–DAD methods and the efficacy of the separation and quantification is compared [192].

4.3.1. Enantiomer separation

The enantioselective action of (*S*)-hyoscyamine was reported in 1904 [193], and today it is well known that only the (*S*)-enantiomers of scopolamine and hyoscyamine exert a strong effect on cholinergic receptors. They exclusively appear in plants, too. Still, a lot of drugs containing racemic mixtures of the alkaloids are marketed today and this may be due to tradition and insufficient means of analysis. For HPLC, a number of columns are now available containing chiral selective stationary phases.

An α_1-acid glycoprotein-containing column separates atropine and homatropine racemic mixtures [194]. The pH, the temperature and the concentration of propanol modified the separation. Anions of octanoic acid and (*S*)- and (*R*)-2-phenylbutyric acid were found to improve the chiral separation of atropine and suppress that of homatropine. (*S*)-Hyoscyamine eluted before (*R*)-hyoscyamine, and that may be a disadvantage when testing for impurities of the (*R*)-enantiomer in (*S*)-hyoscyamine. Rapid and complete chiral chromatographic separation of quarternary tropane alkaloids was achieved on a cellulose-based chiral stationary phase in reversed-phase and normal-phase modes [195]. The test alkaloids had a hyoscyamine and scopolamine skeleton and carried additional methyl-, ethyl-, butyl-, pentyl-, hexyl- and heptyl-alkyl chains on the nitrogen, leading to a permanent positive charge. Separation was completed on cellulose tris(3,5-dimethylphenyl-)carbamate (Chiralcel OD). Alkylsulphonic acids, halogen salts and halogen alkylammonium halogen salts were all found to have very variable effects, depending on their concentrations and possibly on the inclusion of bases into the cellulose cavities. Another separation of chiral model compounds, including homatropine on cellulose tris(4-methylbenzoate) was also improved by organic modifiers such as alcohols, trifluoroacetic acid and triethylamine added to the mobile phase.

Free and derivatised α-, β- and γ-cyclodextrins were tested for inhibition of racemisation of (*S*)-hyoscyamine and (*S*)-scopolamine [196]. All cyclodextrins except α-cyclodextrin (6 glucose units, β-cyclodextrins = 7 glucose units, γ-cyclodextrins = 8 glucose units) could retard racemisation. This shows that cyclodextrins can effectively interact with tropane alkaloids and can be used for chiral separation as well. Native and acetylated β-cyclodextrins were used to determine the enantiomeric purity of scopolamine isolated from *Datura sanguinea* extracts [197]. An achiral–chiral column coupling (RP18; σ-cyclodextrin, Cyclobond) was used, and the eluates were monitored at 254 nm [198]. It was shown that the extraction process from the plant material had a strong influence on the enantiomeric purity of the alkaloids obtained, alkali and heat being most destructive. Enantioseparation of atropine is also possible on achiral RP18-material alone using 0.25 m*M* (2*S*,3*S*)-dicyclohexyl tartrate in phosphate buffer, pH 2.8, as mobile phase [193]. Detection was at 214 nm, and 0.2 ng/ml (*R*)-hyoscyamine could be detected in a solution of 7.2 mg/ml (*S*)-hyoscyamine. Methods for HPLC separation of enantiomers on polysaccharide type chiral stationary phases have been summarised [199].

4.4. Calystegines and anatoxin-a

The structural elucidation of the calystegines was possible only after chromatographic isolation by HPLC with refractometric detection (RI) from *Calystegia sepium* root culture extracts [149].

An RI detector is not sufficiently sensitive to allow quantification from plant material. In complex mixtures lack of selectivity may also pose a problem by peak interference with other compounds. The biggest drawback, however, is the incompatibility with gradient elution, which is indispensable for sufficiently satisfying separations. ECD is possible for compounds that are structurally similar to calystegines, i.e. castanospermine and related alkaloids [200]. These also carry several hydroxyl groups, have a ring-bound secondary nitrogen and are oxidised by pulsed electrode potentials. Similar methods can be applied to measure calystegines [201]. An amino column, common in carbohydrate separation, is used. Amperometric detection is possible by pulsed potential cycles, which have to be adjusted to the individual buffer composition used for elution.

Anatoxins, in contrast to calystegines, contain an α,β-unsaturated keto group and have a λ_{max} at 227 nm that allows UV monitoring. A base-deactivated RP18-silica column was used with acetonitrile–phosphate buffer, 5–33 m*M*, pH 3.0, and sodium dodecyl sulphate, 0.5 m*M*, as ion pair reagent. The LOD was

50–100 ng/ml [163]. An RP18 column (Ultrocarb) with an extraordinarily high load of carbon (30% carbon, 5-μm particles, the usual carbon content on RP18 phases is 16–19%) was found useful for the analysis of anatoxins in a solvent gradient of acetonitrile–water, both containing 0.1% trifluoroacetic acid [202]. Detection at 227 nm allowed a LOD of 25 ng/ml.

Even though detection of most anatoxins is possible at 227 nm, derivatisation with 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F) and fluorimetric detection greatly enhances sensitivity [203]. The LODs were better than 10 ng/l for all six anatoxins included in the study [203]. The derivatisation reagent selectively couples to the secondary nitrogen of anatoxin-a and homoanatoxin-a. In this method the degradation products of the toxins, in which the double bond in the heterocyclic ring is removed, can also be detected [42].

Anatoxin-a and its degradation product epoxyanatoxin-a were analysed by LC–MS using a thermospray interface. Acetyltropine was used as internal standard. The separation of the analytes on an RP column requires buffers to prevent excessive tailing, and the thermospray detection requires volatile solvents. Thus a volatile buffer consisting of 0.1 *M* ammonium acetate, pH 5.0, was chosen. A LOD of 500 pg/injection was found for both analytes which gives a LOD of 25 ng/ml sample when a 20-μl sample is injected [43].

4.5. Summary

4.5.1. Column stationary phases

(i) RP18, sometimes RP8, mostly on silica gel as support. A wide range of columns is available, however, in practice few other types than RP columns have been used.
(ii) Shielded or ''end-capped'' silica materials are base-deactivated RP18-silica. They are increasingly used to prevent tailing of the alkaloids due to their interaction with free acidic silanol groups, if the solvent system does not contain ion-pair reagents.
(iii) RP18 on polymeric support without free acidic groups has also been applied.
(iv) RP-cyano columns or other exceptional phases, especially for column switching, in combination with RP columns have been reported.

Enantiomeric separation
(i) cyclodextrin columns and derivatised cyclodextrins
(ii) derivatised cellulose
(iii) α_1-acid glycoprotein

Special columns for unusual tropane alkaloids
(i) anatoxins on a RP column with a high carbon load (30%)
(ii) calystegines on an amino column

Particle size[4]: 3–10 μm Particles: irregular (seldom) spherical (mostly), porous or nonporous. Mostly, particles are 4–5 μm, spherical, porous silica based.

Column dimensions: 10–25 cm length, 4–5 mm I.D. microbore: 5–15 cm length, 1–2 mm I.D.

Injection volume: 10–20 μl for normal columns, <1 μl for microbore columns.

Solvent systems: dependent on the column. For RP columns ion pair reagents in an acetonitrile–water mixture are widely applied to counteract the alkaline properties of tropane alkaloids.

Limits of detection[5]
(i) with UV 220 nm: hygrine 50 μg/ml, cuscohygrine 100 μg/ml, hyoscyamine and scopolamine 1 μg/ml
(ii) with UV 210 nm: hyoscyamine and scopolamine 1 μg/ml
(iii) with UV 215 nm: hyoscyamine 8.5 ng/ml (200 μl injection)
(iv) with UV 204 nm: tropic acid 250 ng/ml
(v) with ECD: 145 ng/ml hyoscyamine
(vi) with UV 227 nm: 25 ng/ml anatoxin
(vii) with LC–MS: 25 ng/ml anatoxin.

Limits of quantification[5]
(i) with UV 210 nm: hyoscyamine and scopolamine 300–600 ng/ml (column switching)

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[4]The particle size has a crucial influence on the number of theoretical plates (N_{th}), which further depends e.g. on the I.D., the solvent mixture and on the compounds to be separated. Common HPLC columns (125 mm, 5 μm particles) reach 7000–10 000 N_{th} while a column with 3 μm spherical material, 4 mm I.D. and 215 cm length may have 20 000 N_{th} under the same conditions. Further details and basic information on HPLC separation parameters have been updated recently [204].

[5]The values listed here are taken from different publications, they are not directly comparable.

(ii) with conductometric detection: hyoscyamine, homatropine and scopolamine 500 ng/ml
(iii) with fluorometric detection after derivatisation: hyoscyamine 50 ng/ml.

Time per analysis: 15–60 min.

4.6. Conclusion

HPLC with UV detection is the method of choice for routine analysis of plant material, only if the intact ester alkaloids with an UV absorbing aromatic acid or other UV absorbing functionality are to be measured. The advantages of HPLC are: robust method with widely available instrumentation and high sensitivity due to improved UV detectors. A wide range of other detectors is available for specific problems and more sensitive determination. Usually, sample preparation for HPLC is simple, if necessary at all. For the sensitive measurement of tropane alkaloids at low UV wavelengths, however, sample purification is required. HPLC, in contrast to GC or CE, can be scaled up to allow the preparative separation of alkaloids.

LC–MS for tropane alkaloid analysis is a promising approach which will be increasingly used in the future, as improved interfaces and volatile but highly selective solvent systems become increasingly available. The metabolites and catabolites of tropane alkaloids which show no UV absorption can be measured by LC–MS or with other specialised detectors or after derivatisation and UV detection. In practice, however, these compounds are usually determined by GC.

5. Capillary electrophoresis

When capillary electrophoresis (CE) was in the establishment phase, pharmaceuticals—among them tropane alkaloids—were the first types of compounds to be separated [205]. CE appears well suited to alkaloid analysis because these compounds are natural cations, if the appropriate acidic buffer pH is chosen. Migration of the analytes in the usual cationic mode (sample introduction at the anode and detection and outlet at the cathode) is caused by the charged nitrogen atom. The sample volume in CE is very low, a few nanolitres, and therefore very sensitive detection is required. Detection in CE is usually achieved by a DAD system, a drawback for CE separation of tropane alkaloids due to their low UV light absorption [180]. While the tropic acid esters and others esters with aromatic carboxylic acids may be measured, the free amino alcohols like tropine and pseudotropine and other metabolites like hygrine and cuscohygrine are not detected at all.

5.1. Enantiomer separation

Enantiomer separation by CE turned out to be very efficient and was soon considered as one of the major advantages of the CE technique [206]. Cyclodextrins often serve as additives and chiral selectors in free-solution CE [207,208] and in micellar electrokinetic chromatography [209], the first reports dating back about 10 years. In capillary gel electrophoresis, cyclodextrins were incorporated into the gel to separate dansylated amino acid enantiomers [210].

α-, β-and γ-Cyclodextrins and derivatives thereof have all been used for this application. Cyclodextrins have been derivatised in numerous ways leading to better selectivity. For example carbamylated β-cyclodextrin was co-polymerised with acrylamide–bisacrylamide and used for the capillary gel electrophoresis (cGE) separation of homatropine and atropine [211]. In this application, homatropine enantiomers could be baseline separated, but no separation was achieved for atropine, which has a closely related structure. This example indicates that the chiral resolving power of cyclodextrins must be tailored for each compound to provide optimal resolution. Atropine enantiomer resolution was achieved by 50 m*M* hydroxypropyl-β-cyclodextrin in a counter-ion balanced background electrolyte with 50 m*M* tris(hydroxymethyl)methyl-3-aminopropane sulphonic acid (TAPS) [212]. The resolution and selectivity of nonderivatised β-cyclodextrin in CE for chiral separations was tested systematically; pH, β-cyclodextrin concentration and several other parameters being varied and, again, homatropine served as model compound [213]. Sulphobutyl ether-β-cyclodextrins, in comparison with dimethyl-β-cyclodextrins and hydroxypropyl-β-cyclodextrins, were shown to have particularly high selectivity for atropine enantiomers. The advantages of the sulphobutyl derivatisation clearly lies in the negative

charge of the substituents, affording longer and more effective interaction with the basic chiral analyte [214]. Sulphated β-cyclodextrins (with an average degree of 8–11 substitutions per glucose unit) and sulphobutyl ether-β-cyclodextrins (average degree of 4 substitutions per glucose unit), both with the sulphate groups randomly distributed over all hydroxyl positions, and the single isomeric hepatakis(2,3-*O*-diacetyl-6-sulphato)-β-cyclodextrin were compared systematically for the enantiomer separation of atropine, homatropine, ipratropium (=*N*-isopropylhyoscyamine), scopolamine and *N*-butylscopolamine. Separation was achieved for atropine, homatropine and ipratropium in an alkaline medium with 5–15 m*M* cyclodextrin derivative [215]. The method was shown to be applicable to plant extracts [216]. Further cyclodextrin derivatives were tested for separation of atropine, homatropine and eight synthetic tropinyl and piperidinyl esters by capillary zone electrophoresis (CZE). β-Cyclodextrin derivatives proved to have better resolution than γ-cyclodextrins, and heptakis (2,3,6-tri-*O*-methyl)-β-cyclodextrin was found superior to hydroxymethyl-β-cyclodextrin and nonderivatised β-cyclodextrin [217]. The enatiomeric separation of atropine was applied for the measurement of pharmaceuticals and *Scopolia japonica* plant extracts, using trimethyl-β-cyclodextrin as chiral selector. The relation between the enantiomers of hyoscyamine and scopolamine could be quantified reliably. The results were alarming because this relation appeared variable in pharmaceutical preparations, but the pharmacodynamic effect of (*S*)-hyoscyamine is known to be twentyfold stronger than of (*R*)-hyoscyamine [218]. Truxillines are dimeric cinnamoyl methylecgonins (Fig. 2) that have indicative value for cocaine batches of various provenances. Separation of truxillines by CE was achieved with a anionic β-cyclodextrin sulfobutyl ether IV as a run buffer additive at pH 8.6. The measurement of these minor constituents of cocaine was only possible after LLE and size-exclusion HPLC [219]. Up to this state of the art, enantiomer separation could only be tested with alkaloids that contain both a chiral acid moiety and an aromatic ring for UV detection. The combination of CE with EI-MS is a major step forward [220]. When charged cyclodextrins were used in addition as chiral separators, the sensitive measurement of atropine and homatropine enantiomers was achieved [221].

5.2. Quantification from diverse matrices

In publications on chiral separation, the capacity and selectivity of the CE method is at the centre of interest rather than the measurement of tropane alkaloids. Nevertheless there has been considerable progress in the practical application of CE for natural product analysis [222].

For several years atropine and related tropane alkaloids have been measured both in pharmaceutical preparations and extracts of plants by CE as one method of determination.

Buffer concentrations were varied and optimum separation of atropine, homatropine and scopolamine from pharmaceutical preparations was achieved in a 50 μm I.D. fused-silica capillary with 100 m*M* tris buffer pH 7.0 [223]. The LOD was 1 μg/ml for all three alkaloids and the LOQ was given as 3 μg/ml [223]. In a variation of the method the authors added hydroxypropyl-β-cyclodextrin to the running buffer and separated the alkaloids at acidic pH. The endoosmotic flow was reduced and better resolution was achieved [224]. The LOD was lowered to 0.5–0.8 μg/ml and the LOQ was 1.5–2.4 μg/ml, depending on the individual compound. The selectivity could be manipulated by the hydroxypropyl-β-cyclodextrin concentration, but enantiomers were not separated.

The analysis of tropane alkaloids from plant extracts poses more difficulties. Plant extracts not only contain several tropane alkaloids with closely related structures, e.g. hyoscyamine and scopolamine, but also metabolites such as littorine and degradation products like apoatropine in minute quantities. With a CZE method atropine, scopolamine and tropic acid could be separated with 5, 7.5 and 2.5 μg/ml detection minimum, respectively. The alkaloids were extracted from freeze-dried plant material by methanol, and the authors concluded that further clean-up would be necessary to ensure better consistency [225]. For the measurement of hyoscyamine and scopolamine in plant extracts, CE is becoming a routine method [226]. Calystegines can be separated by CE, but UV detection of the compounds is not possible. Two methods were published to circumvent the problem. Calystegines can be

complexed with borate like sugars. The complexes can be separated by CE and detected by UV light at 191 nm [227]. Alternatively, calystegines can be detected by electrochemical oxidation, 2 μg/ml are visible in a sample of 1 nl [228].

In MECC the analytes are included in the micelles that are formed by a surfactant [229], mostly SDS. At 50 m*M* SDS all alkaloids from a plant extract that were detectable at 195 nm could be separated. The addition of organic modifiers such as acetonitrile and methanol enabled the separation of the positional isomers hyoscyamine and littorine [230,231]. The use of MECC was found advantageous for the separation of structurally similar scopolamine derivatives, scopolamine methyl bromide, scopolamine butyl bromide, scopolamine-*N*-oxide, from pharmaceutical preparations. Relative standard deviation was approximately 5% for inter-day analyses and between 2.2% for 280 μg/ml and 4.1% for 80 μg/μl for intra-day analyses [232]. The Doehlert design was used to optimise the parameters pH, concentration of SDS and organic modifier in MECC. At optimum conditions the LOD was 0.6 μg/ml for hyoscyamine and 1.0 μg/ml for scopolamine [233]. After sample purification by LLE, MECC can be applied to the determination of atropine in biological fluids such as blood and gastric contents. In a forensic-case study, atropine was found with a LOD of 1.2 μg/ml and a LOQ of 4 μg/ml. The sample preparation by LLE introduced an concentration step of 20-fold [234]. Root cultures of *Hyoscyamus muticus* contain littorine besides hyoscyamine and scopolamine and this was detected for the first time by MECC [235].

5.3. Nonaqueous capillary electrophoresis

Another variation of the CE technique is nonaqueous CE. The capillary and the instrumentation remain the same, but the choice of solvents increases, and solvent mixtures often provide better selectivities than aqueous solutions alone. For separation of several tropane alkaloids an acetonitrile–methanol mixture containing ammonium acetate was chosen. This latter buffer compound dissolves readily in nonaqueous solutions [236]. The selectivity of the method for the respective alkaloids could be fine-tuned by the acetonitrile–methanol ratio. The detection limit remained unchanged compared with CZE in aqueous solution. Nonaqueous CE can be applied for the analysis of plant extracts [237]. The advantage of the method lies in its versatility. Furthermore, the use of water-free solvents facilitates the direct coupling of the CE instrument to a mass spectrometer, while MECC cannot be coupled to MS.

5.4. Summary

Electric field: 15–30 kV.

Column: fused-silica, 36–72 cm length, 50–100 μm I.D.

Injection: hydrostatic or by pressure (0.35–250 Pa), 4–10 s, resulting in an injected volume of 4–30 nl.

Buffer: 10–100 m*M*, sodium- or potassium phosphate, sodium borate, Tris; pH 2.8–9.8.

SDS for MECC: 30–80 m*M*.

Chiral selectors: β-cyclodextrins, free or derivatised, 5–40 m*M*.

Temperature 20–30 °C.

Detection: diode array or fixed wavelength usually at low UV (190–240 nm).

LOD: 0.6–5 μg/ml for hyoscyamine and scopolamine[6].

LOQ: 1.8–15 μg/ml for hyoscyamine and scopolamine[6].

Time per analysis: 5–45 min.

5.5. Conclusion

CE has high selectivity and can provide fast separations. The LOD expressed as concentration in the sample is higher compared with GC and HPLC because of the small sample volumes and the relatively insensitive UV detection for tropane alkaloids. The method requires limited sample work up and is particularly useful for chiral separations. Using nonaqueous solvents, CE can be coupled to MS and will probably provide a powerful means of alkaloid separation and identification in the near future.

[6]The LOD is usually defined as three times the height of the baseline noise. The limit of quantification is 10 times the baseline noise. The LOD and LOQ values given here relate to atropine scopolamine and *N*-alkylated derivatives.

6. Thin-layer chromatography—a summary

TLC was the method of choice for alkaloid analysis before instrumental chromatography methods like GC and HPLC were established. A general introduction into the chromatography of alkaloids giving basic techniques and applications of TLC is contained in [10].

Nowadays TLC is still frequently used for tropane alkaloids although few publications appear with innovative techniques. Rather, TLC is used as a method of initial screening with a semiquantitative evaluation. As there has relatively less change in the simple TLC separation of tropane alkaloids than with instrumental chromatography, only a brief summary is given here, and for further details the comprehensive review of Baerheim Svendsen and Verpoorte should be consulted [7]. These authors assembled numerous data on stationary phases, solvent systems, and detection reagents for tropane alkaloids and other alkaloids [7]. They give R_f values of compounds and LODs for individual alkaloids. An update with further details especially on cocaine and metabolites was published in 1989 [9]. In a comprehensive review on separation systems for tropane alkaloids Woolley showed how to develop the optimal solvent composition systematically [6]. This approach is also useful for preparative chromatography and isolation of alkaloids. For further fundamentals of TLC, selection of stationary phases, parameters influencing retention and many more readers may refer to the comprehensive monograph by Geiss [238]. Systematic development of solvent systems by the PRISMA optimisation model [239] was also done for tropane alkaloid separation [240].

TLC is often used for initial screening, and also for confirmation of the identity of alkaloids determined by GC–FID or HPLC with UV detection. These means of detection are not specific, and in a complex chromatogram the identification may be erroneous. Thus, confirmation by TLC and specific staining is advantageous. For alkaloid-*N*-oxides TLC was used in addition to GC and GC–MS [32]. The occurrence of piperidine alkaloids of the pelletierine type was examined in Sedum species and found to be in accordance to the established infrageneric classification. TLC for these alkaloids can be developed by undiluted Dragendorff's reagent which gave a detection limit of 10 mg/spot [241]. Solvent systems and detection (Dragendorff's reagent) were similar to those for tropane alkloids [67,69]. The identification of products from in vitro assays of isolated enzymes of tropane alkaloid metabolism and separation of tropine and pseudotropine were done by GC and TLC [242].

Calystegines were initially analysed by high-voltage paper electrophoresis, but the procedure is time-consuming and requires specific instrumentation [243]. TLC methods for calystegines are faster and easier to handle. Detection was found to be sensitive with silver nitrate followed by alkali solution, with the LOD of 5 μg/ml [154]. Further detection reagents for calystegines have been summarised [153]. Screening for calystegines is often done by TLC using the silver nitrate reagent for detection [40,158].

A further field of frequent application of TLC is the monitoring of chromatographic fractions or synthesis products. Synthesis of labelled tropane alkaloid precursors for feeding experiments sometimes require elaborate reaction conditions. The progress of the reaction and the reaction products are often monitored by TLC [118]. TLC plates are sometimes used as a catalyst for chemical reactions. Tropinone can be reduced after application on silica-gel plates by spraying with sodium borohydride to give tropine and pseudotropine. The products are subsequently separated chromatographically [244]. The purification of calystegines by ion-exchange chromatography is monitored by TLC. Here the chlorine-*o*-toluidine reagent is preferred, because of the high sensitivity for amino acids which are the major contaminants in calystegine fractions [245]. For calystegines the chlorine-*o*-toluidine reagent has a LOD of 100 ng [246]. Variations of TLC methods using e.g. overpressured solvents can be used for fast and efficient separations and also for systematic development and testing of solvent systems of a simple composition that are preferred for preparative column chromatography [247,248]. Separation of alkaloids is normally done on a hydrophilic stationary phases, silica gel or on alumina and with nonpolar solvents systems, but reversed-phases can also be used. Extracts from leaves of *Erythroxylum coca* were separated on RP18 silica gel with methanol as mobile phase. Detection is possible by spraying with Dragendorff's reagent [128].

Quantification of analytes by TLC is also possible,

though not often applied, since it usually requires instrumental densiometric evaluation of the TLC plates. For hyoscyamine and scopolamine in an extract of *Datura stramonium*, the LOD was 35–50 μg/ml [249]. Cocaine and the degradation products ecgonine, ecgonine methyl ester and benzoylecgonine were separated by TLC, detection being done with the widely-used Dragendorff's reagent for alkaloids [7] or alternatively, by heating developed plates to 280 °C. It was found that heating produced fluorescent derivatives which could be evaluated densitometrically with light of 313 nm for excitation and 390 nm for emittance [250]. The LOQ was 1 μg/ml. For TLC with subsequent densitometric quantification, high-performance TLC (HPTLC) plates that have smaller and more homogeneous particles and a higher selectivity due to more theoretical plates per centimetre are preferred. Automatic sample application and automatic development, many-fold if required, are the best conditions for exact quantification by densitometry.

If normal quality (Si 60) TLC plates are used, the theoretical plate number is lower than with equivalent separations performed by HPLC. However, this can be partially compensated for by using two-dimensional development in different solvents. Calystegine quantification is possible by TLC and densiometric evaluation, if automated multiple development is used [251]. The separation of many structurally related calystegines and their biosynthetic precursors, nortropanols, can be directed by the choice of solvent mixtures and development steps [246].

6.1. Conclusions

The advantages of TLC are
(i) simplicity
(ii) versatility due to many stationary phases and nearly unlimited solvent compositions
(iii) high velocity per analysis, considering that normally a plate is developed with several samples at a time
(iv) high sensitivity with appropriate detection procedures
(v) reliable identification of analytes due to the combination of R_f values and specific detection reagents
(vi) simple sample preparation.

If the method is kept simple, however, only semiquantitative evaluation is possible.

Quantitative evaluation by densitometry requires additional instrumentation and extensive and repeated calibration.

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Appendix A

A.1. GC detector glossary

A.1.1. Flame ionisation detection (FID)

The volatile compounds leaving the GC column are directed into a chamber where a flame is sustained by defined flows of hydrogen and synthetic air that are directed by a small jet. Synthetic air is composed of nitrogen and oxygen and is thus free of carbon dioxide or other contaminants that would disturb the steadiness of the flame and thus reduce the sensitivity by increasing the baseline noise. The compounds are oxidised in the flame. The jet is negatively charged and forms the cathode. The oxidation of the organic compounds generates anions proportional to the mass of compound, which are captured by a collector electrode, an anode. The signal is amplified and displayed on a mV trace. The FID is most sensitive for carbon containing compounds. The response is dependent on the carbon contents of a molecule and on the chemical environment of the carbon. Hydrocarbons usually give higher responses than oxidised carbons like esters or ketones.

A.1.2. Phosphorus nitrogen detection (NPD)

The detector uses a principle similar to FID. The

compounds leaving the column are directed into a hydrogen–oxygen atmosphere. However, due to a reduced hydrogen proportion there is no real flame, but just a plasma. This extends the life-time of the alkali salt pellet which is the heart of the NPD system. The alkali salt source mostly contains a rubidium mixture and has the shape of a bead, a ring or a small rod. It is negatively charged and may be heated (depending on the specific instrument) and it converts N- and P-containing compounds to anions which, as in the FID system, are captured by a collector anode, again connected to an amplifier. The NPD system, when correctly tuned, is insensitive for carbon, but the sensitivity for nitrogen- and phosphorus-containing compounds is higher than with a FID system, even if there are fewer nitrogen and phosphorus atoms per molecule than carbon atoms.

A.1.3. Electron capture detection (ECD)

Compounds that are able to react with thermal electrons in a reaction chamber are measured. The detection of compounds is indirect. The concentration of the electrons in the reaction chamber and their reduction by compounds entering the chamber is determined. The range of compounds giving ECD responses depends on the temperature of the reaction chamber. ECD is selective and very sensitive at the same time, but prone to giving erroneous results [252].

A.1.4. Mass spectrometry (MS) with electron impact ionisation (EI)

The molecules are exposed to an electron beam that is generated by a cathode in a low vacuum chamber (ion source). Fragmentation of the analyte molecules results from the electrons abstracting further electrons from the compounds. The molecules are destabilised and decompose in a compound-specific manner. Usually cations are measured by the analyser which is a combination of strong magnets.

A.1.5. Mass spectrometry (MS) with chemical ionisation (CI)

Chemical ionisation in MS needs the addition of a reactant gas into the ion source. Gases used for this purpose are methane, isobutane, ammonia, nitrogen oxides, methanol and recently water [253]. The reactant gas molecules ionise first, and in the second part of the reaction positively charged analyte ions are generated by the reactant gas ions. The analyte molecules are usually not fragmented.

A.1.6. Mass spectrometry (MS) with ion trap

The ion trap principle was invented in 1953 [254], but it took about 3 decades until it was established as an MS mode for sensitive detection. Ions representing the complete charged analyte molecule or typical fragments of the analyte are accumulated in the ion trap for a certain time and then measured. The response is linear to the concentration and can be used for quantification. If single ions are selected for quantification the method usually is more sensitive due to lower background (single ion mode). The ion trap can be used with chemical ionisation (see above). Daughter ions generated by tandem MS from a main ion of the first CI reaction can be used for selective and sensitive quantification as well.

A.2. CE glossary

A.2.1. Free solution CE

The analytes are separated in a capillary, 30–70 cm long and made of fused silica that is filled with buffer only. The migration velocity of the analytes is determined by their own charge, by the charge and the migration of the buffer ions and by the endo-osmotic flow.

A.2.2. Capillary zone electrophoresis (CZE)

There are several methods of separating analytes in free solution CE: capillary zone electrophoresis, capillary isoelectric focusing (cIEF) and capillary isotachophoresis (cITP). While in cIEF and cITP chemical gradients in the buffer are used to achieve sharp bands in CZE a homogeneous background electrolyte is provided. CZE is the most often applied technique for CE with free solution mobility, and CZE often is referred to as "free solution CE".

A.2.3. Micellar electrokinetic capillary chromatography (MECC or MEKC)

MECC is a variation of free solution CE, that is characterised by addition of surfactants. The concentration of the surfactants is above the critical micellar constant (CRC), and they form micelles in

the buffer solution which include the analytes. The inclusion of the analytes is dependent on their structure and may be selective. In the case of chiral surfactants racemic analytes may be separated into enantiomers [255].

A.2.4. Capillary gel electrophoresis (CGE)

The capillary column is filled with a gel, often polyacrylamide, providing a stationary phase with additional size-exclusion properties.

A.2.5. Capillary electrochromatography (CEC)

A hybrid technique of HPLC and CE, in which capillaries of approximately 100 μm I.D. and of 10–50 cm length are filled with silica particles of different sizes and porosities [256]. The most common stationary phase is RP18 silica gel with a particle size of 3 μm or less.

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