New Methods of Analysis and Investigation of Terpenoid Indole Alkaloids

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Abstract

Terpenoid indole alkaloids are biologically active compounds that have been used as pharmaceuticals among others as anticancer, antimalarial, antihypertensive and hypoglycemic agents for more than 40 years. Many efforts have been focused on their extraction, isolation, separation and structural elucidation by means of different approaches based on methodologies already established since the 1960s. New methodologies in extraction and sample preparation from different matrices include environmentally

friendly techniques such as ionic liquids or supercritical fluid extraction as well as the synthesis of molecularly imprinted polymers. Chromatography combined with spectroscopy is still the preferred analytical tool for alkaloid analysis, but recent improvements in mass spectroscopy and nuclear magnetic resonance-based technologies have also been applied to areas of research such as toxicology, quality control, metabolic fingerprinting and metabolic profiling. This review is intended to provide a more general rather than an exhaustive overview on the new methods for terpenoid indole alkaloid analysis focusing on hyphenated high-performance liquid chromatography and mass spectrometry approaches. We will also discuss extraction methods and the strength and weaknesses of the different analytical tools for their application in targeted or non targeted approaches.

ABBREVIATIONS

APCI atmospheric pressure chemical ionization
CCC counter-current chromatography
CE capillary electrophoresis
CID collision-induced dissociation
CPC centrifugal partition chromatography
CZE capillary zone electrophoresis
DAD photodiode array detection
ESI electrospray ionization
FID flame ionization detection
GC–MS gas chromatography coupled to mass spectroscopy
HPCCC high-performance counter-current chromatography
HPLC high-performance liquid chromatography
HSCCC high-speed counter-current chromatography
IT ion trap
LC–MS liquid chromatography coupled to mass spectroscopy
LOD limit of detection
MEKC micellar electrokinetic chromatography
MIP molecularly imprinted polymer
MS mass spectrometry
MS^n multistage mass (tandem mass) spectroscopy
NACE non-aqueous capillary electrophoresis
NMR nuclear magnetic resonance
qNMR quantitative nuclear magnetic resonance
SFE supercritical fluid extraction
SIM single ion monitoring
TIAs terpenoid indole alkaloids
TOF time of flight
UHPLC ultra high-pressure liquid chromatography
UV ultraviolet

1. INTRODUCTION

Terpenoid indole alkaloids (TIAs) are a group of ca. 3000 natural products among which a large number of compounds are being applied as medicine in pharmacology such as vinblastine, vincristine, strychnine and reserpine. Consequently, over the years, a lot of work has been done in bioprospecting for novel drugs from this group, and to improve the production of these compounds in plants or genetically modified organisms. All these studies require analytical tools to identify and quantify these compounds in various matrices such as plants and biological fluids. These tools are mainly based on chromatography and spectroscopy and different combinations of these. Thin–layer chromatography (TLC), gas chromatography (GC) (both since the 1960s) and high–performance liquid chromatography (HPLC; since the 1970s) offer efficient methods for the separation and identification of alkaloids based on retention behaviour that is further supported by more or less specific detection methods. Specificity in detection for TLC is obtained by colour reactions and ultraviolet (UV) spectroscopy, for GC by the combination with mass spectrometry (MS) and for HPLC by UV, MS and nuclear magnetic resonance (NMR) spectroscopy. Ever since the introduction of these methods, they have been extensively applied for alkaloids; however, in terms of separation principle, not much has changed since the first decade of use of these chromatographic tools (Baerheim Svendsen & Verpoorte, 1983; Verpoorte & Baerheim Svendsen, 1984). Stationary phases have improved, but the principle of retention of alkaloids has not changed, for example, in HPLC this is mainly reversed–phase chromatography with slightly acidic solvents, sometimes containing modifiers to reduce tailing due to adsorption of alkaloids on residual silanol groups in the stationary phase, or using ion–pair chromatography to change the selectivity. Though it is always claimed that the a new stationary phase has many advantages and is much better than the previous ones, the major disadvantage is that a different chromatogram will be obtained, requiring extensive validation, and direct comparison with previous results is not possible anymore.

This is where spectroscopic methods without hyphenation with chromatography have an advantage, as they do not involve a separation step. In the case of UV, infrared and NMR, the detection mechanism is based on pure physical properties, in case of a pure compound showing spectra specific for

the chemical structure, in case of mixtures the sum of the spectra of the compounds present. In case of NMR spectroscopy, two-dimensional (2D) NMR can be applied to deconvolute the spectra of the compounds present. NMR has one major advantage above all methods and that is in ^{1}H NMR, all signals can be directly compared. The total signal intensity of each proton is only dependent on the molar concentration of the compound it is part of, which means that one can, without the need for individual calibration curves, quantify the compounds present in a mixture. In the case of mass spectroscopy, a manipulation is required to ionize the compound(s) present, which is a weakness as the spectra obtained are dependent on the instrumental conditions, as well as on the matrix. The advantage is that the resolution, is on the level of the molecular weight and in high-resolution mode, even the molecular formula can be obtained. The possibility of measuring fragmentation of the compounds adds further information for the identity of the compound.

In recent years combining the spectroscopy with chromatography has become common practice; thus, combining the advantages of both is particularly of great value in the identification of unknown, possibly novel, compounds. However, for the quantitative analysis of known compounds, liquid chromatography coupled to UV (LC–UV) and gas chromatography coupled to flame ionization detector (GC–FID) in many cases are fitting the needs of a quantitative analysis, as they are robust and reproducible methods.

2. EXTRACTION AND PURIFICATION METHODS

An elemental aspect in any analytical methodology is the compatibility of the extraction method with the analytical system, which will be ultimately reflected in the quality and usefulness of the chromatograms or spectra. Conventionally, extraction procedures for alkaloids have been done by different mechanical, physical and chemical processes such as Soxhlet extraction, maceration or percolation using organic solvents and liquid–liquid partitioning including aqueous solutions, although nowadays non-conventional methods which are more friendly to the environment have been successfully applied to alkaloid extraction such as ultrasound, microwave, supercritical fluids and ionic liquids (ILs).

The extraction has several aspects that need to be considered: the stability of compounds, the solubility and dissolution rate and the suitability of the extract for the analytical method. The stability is of concern from the very first moment of sampling, for example, when a plant is harvested, immediately the stress metabolism will start which may affect the alkaloids levels, for

example, catabolism, *de novo* biosynthesis and (enzymatic) hydrolysis. Quenching of all metabolic activities is thus required, which can be achieved by heating or even treating with microwaves or freezing followed by freeze-drying. Moreover, the extraction procedure itself can cause the formation of artefacts (Maltese, van der Kooy, & Verpoorte, 2009; Verpoorte, Choi, Mustafa, & Kim, 2008) through reactivity of solvents or contaminations in solvents or by decomposition by heat (e.g. Soxhlet). Solubility can be good, but the rate of solubilization can be slow. Thus, extraction time is an important factor, as is the temperature; a balance must be found between these two in connection with the stability of the compounds to be extracted. In HPLC, the sample must be injected into a solvent that is similar to the mobile phase, for example, a chloroform extract cannot be directly injected on a reversed-phase column. In GC, the solvent must be volatile; in TLC, dirty extracts are not a problem as long as they do not interfere with the separation as a plate is only used once. Therefore, in every extraction procedure, such considerations must be made and it is important to properly validate the extraction method applied in any analytical protocol.

2.1. Solvent extraction

Extraction and sample preparation are the first key steps in plant analysis. Any analysis requires the extraction of the desired product from a complex matrix, dissolve it into the appropriate solvent and remove all the undesired compounds that might interfere with the analysis. TIAs have a lipophilic character as free bases at high pH and can be extracted with organic solvents such as alcohols, chloroform or ethyl acetate after plant material is basified with ammonia or sodium carbonate. A too high pH should be avoided as in that case phenolic alkaloids might not be fully extracted. Alternatively, alkaloids can be extracted with polar solvents at low pH, for example, water acidified with phosphoric acid (Moreno, van der Heijden, & Verpoorte, 1993), acetic acid (de Castro et al., 2012; Girardot et al., 2012; Jenks, 2002; Tanaka et al., 2007), tartaric acid (Jenett-Siems, Weigl, Kaloga, Schulz, & Eich, 2003), trifluoroacetic acid (Silvestrini et al., 2002) or hydrochloric acid (Andrade et al., 2005; Verma, Laakso, Seppanen-Lakso, Huhtikangas, & Riekkola, 2007). TIAs have also been extracted without adding acid or base with methanol (Paranhos, Fragoso, da Silveira, Henriques, & Fett-Neto, 2009; Sun & Liu, 2008), dichloromethane (Kumar, Bulumulla, Wimalasiri, & Reisch, 1994), acetone (Zhang, Yu, Liu, & Liu, 2007), ethanol (Wang et al., 2005), methanol–water

(Wang et al., 2012), chloroform or petrol (Etse, Gray, Thomas, & Waterman, 1989).

TIAs can be further purified by liquid–liquid extraction after basification, extraction with an immiscible organic solvent, for example, 10% of ammonia, and then extracted with ethyl acetate (Cao et al., 2012) or from an organic solvent with an aqueous acidic solution. In such liquid–liquid partitioning methods, one should keep in mind that non-polar counter ions like chloride, acetate and trifluoroacetic acids may form ion pairs with alkaloids that are well soluble in the organic solvents (Hermans-Lokkerbol & Verpoorte, 1986). Such approaches obviously separate alkaloids from other compounds with either non-polar (e.g. lipids) or polar character (e.g. sugars) that lack this dual lipophilic/hydrophilic character. It is therefore a targeted analysis for alkaloids.

Solid-phase extraction (SPE) involves selective extraction of TIAs from liquid samples onto a solid support using adsorption or ion exchange materials. For adsorption of TIAs in their free neutral form, reversed-phase materials such as C_8 and C_{18} on silica are widely used. Cation exchangers can be used to selectively bind alkaloids from aqueous extracts (Sheludko, Gerasimenko, Unger, Kostenyuk, & Stoeckgit, 1999). The choice of a SPE method also depends on the type of extract required in the analysis. For GC analysis, the alkaloids should be in the basic form, and for LC, it can be in the basic form or more commonly in the protonated form as in reversed-phase chromatography usually weakly acidic systems are applied. Depending on the method of detection, chlorine- or fluorine-containing solvents should be avoided for GC and for MS, and all components need to be volatile.

Quaternary indole alkaloids can be isolated by precipitation. A crude extract is prepared with an acidic aqueous solution and then they are precipitated with Mayer's reagent at pH 5 (Penelle et al., 2001; Verpoorte & Baerheim Svendsen, 1984) or as Reineckate salts at pH 8 (Ghosal & Srivastava, 1974) or pH 3 (Hu, Zhu, Prewo, & Hesse, 1989). After collection, the precipitate is dissolved into a mixture of organic solvents like acetone–methanol–water 6:2:1 v/v/v (Perera, Samuelson, van Beek, & Verpoorte, 1983; Verpoorte & Baerheim Svendsen, 1984) and exchanged to chlorides by means of an anion exchanger. Nevertheless, because these salts pose serious health risks to humans and to the environment, their use should be discouraged. Quaternary alkaloids can be adsorbed from aqueous extracts using cation exchangers.

2.2. Ionic liquids

ILs have been successfully applied to the extraction of complicated samples such as plant complexes or low-accumulation constituents like TIAs. Since the rate of solubilization in the often viscous IL is a limiting step, they are frequently used in ultrasound- or microwave-assisted extraction. Further steps to make the extract suitable for the analysis include liquid–liquid extraction, liquid-phase microextraction, solid-phase microextraction and aqueous two-phase system extraction. Particularly, microwave-assisted extraction has been the preferred option as it is a rapid, effective and cheap technique for the extraction of TIAs from different matrices (Sparr & Bjorklund, 2000).

In an IL-based ultrasound-assisted approach for the extraction of TIAs from *Catharanthus roseus*, Yang et al. (2011) tested the extraction efficiency of 1-allyl-3-methylimidazolium bromide at a concentration of 0.5 M and a solid–liquid ratio of 1:10 for 2 h of maceration time showing higher extraction efficiency when compared to seven different conventional extraction methods. The same cation and anion combination along with microwave-assisted extraction proved to be effective for isolating alkaloids from *Camptotheca acuminata* (Ma et al., 2012; Wang et al., 2011).

2.3. Molecular imprinted polymers

Molecularly imprinted polymers (MIPs) are an emerging technique that uses polymer materials with high selectivity and affinity towards a particular molecule, the template. MIPs are prepared by crosslinking monomers in a complexation solution in the presence of the template molecules. After the polymerization reaction is finished, the template is removed from the polymer by solvent extraction, leaving behind an imprint with a cavity that sterically and chemically binds the template molecule with high affinity (Xie et al., 2001).

Highly selective MIPs were used in SPE for preparative and analytical separations, using, for example, HPLC to separate vinblastine (Zhu, Huang, Li, & Yin, 2010), vindoline and catharanthine (Lopez et al., 2011) from a commercial extract of *C. roseus*. Methacrylic acid was used as the functional monomer, ethylene glycol dimethacrylate as the crosslinker and toluene or acetone as the porogenic solvent. Thermal polymerization yielded an MIP on which extracts were loaded on a polypropylene SPE cartridge containing the MIP. Analysis of the eluents by HPLC–UV showed high recoveries for vinblastine (93.8%) and catharanthine (101%)

and capacities of 750 and 818 μg/g, respectively, whereas for vindoline, recovery was only 33%. The MIP cavity apparently specifically binds catharanthine as a monomer as well as this moiety as part of the dimeric alkaloids.

2.4. Supercritical CO_2

Supercritical fluid extraction (SFE) has been applied to the isolation of natural products since the late 1970s to a few number of cases like the decafeination of coffee beans and tea leaves (Kaiser, Rompp, & Schmidt, 2001), but since the early 2000s, this technique has experienced an enormous development particularly in the food, toxicological, pharmaceutical and environmental areas. Compared with conventional organic solvents, supercritical fluids, and particularly CO_2, are non-toxic, low cost, environmentally friendly and because of high diffusivity reduce mass-transfer rates. They can be compressed at constant temperature, and therefore, increasing in density and solvent capacity. Their low surface tension facilitates analyte extraction, and since it is gaseous at room temperature and under constant pressure, it can be easily extracted rendering solvent-free analytes (Herrero, Mendiola, Cifuentes, & Ibañez, 2010). The main supercritical solvent used is CO_2 with its low polarity as its main disadvantage, which can be solved with the addition of a polar modifier (co-solvent), for example, 1–10% methanol or ethanol to expand its extraction range to include more polar compounds, which in turns will also reduce the analyte–matrix interactions improving their quantitative extraction. This can be done in two ways, either by mixing the modifier to the CO_2 flow or by mixing it with the raw material in the extraction cell (Mendiola, Herrero, Cifuentes, & Ibañez, 2007).

Studies regarding SFE have been performed for the major TIA's from *Catharanthus*, *Tabernaemontana* and *Evodia* species (Table 9.1). Lee et al. (1992) extracted vindoline and catharanthine from the leaves of *C. roseus* without the addition of a modifier. About 67.2% (w/w) of vindoline was recovered using a CO_2 flow rate of 150 ml/min at 40 °C, whereas in the case of catharanthine, a higher flow rate of 400 ml/min at the same temperature had a yield of 52% (w/w). Song et al. (1992) compared the addition or absence of ethanol as the modifier for vindoline extraction. The largest amount (58 wt%) was obtained without the addition of the modifier showing that vindoline solubility is more sensitive to pressure than to temperature. However, a mixture of CO_2–methanol–triethylamine (80:12:8) proved to be more effective than methanolic extraction of vinblastine and

Table 9.1 Experimental conditions for supercritical fluid extraction of TIAs

Plant material	Matrix	Target compound	Aim	Extraction conditions	Analytical method	References
Catharanthus roseus	Leaves	Vindoline, catharanthine	To selectively extract vindoline	CO_2, 150 bar, 40 °C, 10 h	HPLC, LC–MS	Lee et al. (1992)
	Leaves	Vindoline	To compare extraction with and without co-solvent	CO_2 + 3% ethanol, 300 bar, 35 °C, 5 h	HPLC, LC–MS	Song et al. (1992)
	Aerial parts and roots	Vinblastine, vincristine	To apply a basified SFE solvent	CO_2–methanol–triethylamine (80:12:8), 340 bar, 80 °C	LC–MS	Choi et al. (2002)
	Leaves	Catharanthine, vindoline, 3′,4′-anhydrovinblastine	To optimize the method and to compare it to conventional extraction methods	CO_2 + 6.6% methanol, 200–300 bar, 80 °C, 40 min	HPLC, LC–MS	Verma et al. (2008)
Evodia rutaecarpa	Fruits	Evodiamine, rutaecarpine	To test the use of methanol as co-solvent	CO_2 + 50% methanol, 280 bar, 62 °C, 1.3 h	HPLC	Liu et al. (2010)
Tabernaemontana catharinensis	Aerial parts	Coronaridine, voacangine, voacristine, voacangine hydroxylindolenine, voacristine hydroxylindolenine, 3-hydroxylcoronaridine	To evaluate temperature, pressure and co-solvent	CO_2 + 4.6% ethanol, 250 bar, 45 °C, 2 h	GC–MS, GC–FID, ^{1}H and ^{13}C NMR	Pereira et al. (2004)
Uncaria tomentosa	Root bark	Isopteropodine, pteropodine, isomitraphylline, uncarine F, mitraphylline, speciophylline, rhynchophylline, isorhynchophylline	To compare extraction with and without co-solvent	CO_2 + 10% methanol, 253 bar, 60 °C, 30 min	HPLC–MS and GC–MS	Lopez-Avila, Benedicto, and Robaugh (1997)

Abbreviations: FID, flame ionization detector.

vincristine (Choi, Yoo, & Kim, 2002) and CO_2 with 6.6% methanol for catharanthine (Verma, Hartonen, & Riekkola, 2008).

Alkaloids from *Tabernaemontana catharinensis* were extracted using a mixture of supercritical CO_2 and 4.6% ethanol (Pereira et al., 2004). The flow of CO_2 was held at 4 bar/min with a depressurization step of 70 bar (12 bar/min). Liu, Guo, Chang, Jiang, and Wang (2010) extracted the main alkaloids from unripe fruits of *E. rutaecarpa* using 50% methanol as the modifier in a flow rate of 0.4 ml/min with a static extraction for 5 min and then dynamic up to 90 min.

3. ANALYTICAL METHODS

3.1. HPLC and hyphenated techniques

HPLC has been the method of choice for the analysis of alkaloids. It has been recognized since the 1970s as the most versatile and most widely applied technique for efficient separation and analysis of alkaloids (Verpoorte & Baerheim Svendsen, 1984). Most of the separations are done on reversed-phase materials such as C_8, C_{18} and phenyl-bonded phases on silica. McCalley (2002) extensively described the importance of the characteristics of the stationary phases for the separation of alkaloids. The most common eluents for the separation of alkaloids are methanol–water and acetonitrile–water buffered at pH 2–4 (buffer strength > 25 mM) in order to keep alkaloids in their more polar protonated form to reduce tailing due to interaction of the basic nitrogen with the residual acidic silanol groups of the stationary phase (Kingston, 1979; Verpoorte & Baerheim Svendsen, 1984). Ion–pair chromatography with, for example, long alkyl chain sulfonic acids is also used in alkaloid separations. Various amines such as triethylamine are sometimes added to reduce tailing. For detection, UV absorption is the most widely used since indole alkaloids have strong and specific UV chromophores that can be easily used to identify them, for example, using HPLC with photodiode array detection (DAD). Nevertheless, MS has been a major tool in the identification and structure elucidation of alkaloids, as it not only allows determination of the chemical structure of known and unknown compounds but also offers high sensitivity, and hence the combination of DAD and mass spectroscopy coupled with liquid chromatography, liquid chromatography coupled to mass spectroscopy (LC–MS) is the most selective detection for alkaloids (Verpoorte & Niessen, 1994). Mobile phases for LC–MS systems using isocratic separations on reversed-phase silica gel type stationary phases are fully volatile acidic eluents containing e.g. formic acid,

acetic acid, trifluoroacetic acid, ammonium carbonate or ammonium formate (Table 9.2).

Typically, mass spectroscopy data for TIA analysis are acquired in the positive mode and based on the combination of retention time, UV and mass spectra, known compounds are rapidly dereplicated and new structures are identified from different plant matrices such as crude extracts from intact plants and organs, hairy roots, cell suspension cultures and from biological matrices. In multistage MS detection experiments, the use of deuterium-labelled internal standards has proved to be sensitive enough for the accurate quantification of yohimbine from commercially available aphrodisiacs and bark from *Pausinystalia yohimbe* by using yohimbine-d_3 (Zanolari, Ndjoko, Ioset, Marston, & Hostettmann, 2003) or clonazepam-d_4 for ibogaine and noribogaine determination from human tissues (Chèze, Lenoan, Deveaux, & Pépin, 2008). Recent approaches using direct-injection electrospray ionization (ESI)–MS/MS (Chen, Zhang, Zhang, Chen, & Chen, 2013; Zhou, Tai, Sun, & Pan, 2005) or flow-injection ESI–MS/MS (Favretto, Piovan, Filippini, & Caniato, 2001) can omit the sample preparation step and then be used to confirm the presence of alkaloids in different matrices. In addition, these techniques can provide characteristic structural information such as precursor and product ion information, which is useful for multicomponent screening purposes.

3.2. Ultra high-pressure LC–MS

One of the latest developments in LC–MS has been the introduction of very pH-stable stationary phases, sub-2-μm particles and monolith columns. This requires high pressures (>400 bar) and is now known as ultra high-pressure liquid chromatography (UHPLC). May achieve up to 100,000 number of plates per time unit (N/t_0) and peak capacities of 900 and reducing the analysis time by a factor of 20. With the same column length, a three-fold efficiency improvement can be observed compared to 5 μm supports (Nguyen, Guillarme, Rudaz, & Veuthey, 2006).

UHPLC interfaces with high-resolution tandem mass spectrometers and NMR can greatly improve analysis in terms of resolution, speed, reproducibility, sensitivity and unequivocal identification of trace compounds providing confirmative information for studies in e.g. quality control, fingerprinting, authentication, standardization or identification of biomarkers. Other approaches include microfractionation bioactivity-based analysis (Hou et al., 2012), chromatographic profiling (Xu et al., 2012), monitoring alkaloid production in cell suspension cultures (He, Yang, Tan, Zhao, & Hu, 2011;

Table 9.2 LC systems for quantitative and qualitative analysis of alkaloids

Plant species	Target compound(s)	Matrix	LC conditions column/ particle size (μm)/mobile phase A and B/internal standard (IS)	Analytical method	References
Catharanthus roseus	Profiling approach	Roots	Luna C_{18}/5/A: ACN and B: 1% acetic acid in water/ No IS	HPLC– DAD–ESI– MS/MS	Ferreres et al. (2010)
	Vindoline, vindolidine, vincristine, vinblastine, catharanthine, 19*S*-vindolinine, vindolinine	Commercial extract	Zorbax Eclipse XDB-C_8/ 5/A: 0.1% triethylamine and B: methanol/No IS	HPLC–ESI– MS/MS	Zhou et al. (2005)
	Vincristine	Human plasma	Luna C_8/3/A: 1% acetic acid in water and B: ACN/vincristine and vinblastine	LC–MS/MS	Guilhaumou et al. (2010)
	Vinblastine, vindoline, ajmalicine, catharanthine, vinleurosine	Stems	DL C_{18}/5/A: ACN and B: 10 mM ammonium acetate/No IS	LC–MS/MS	Chen et al. (2013)
Claviceps sp.	Ergometrine, ergosine, ergotamine, ergocornine, ergocryptine, ergocristine	Cereal and cereal products	XBridge MS C_{18}/3.5/A: water–0.2 M ammonium bicarbonate–methanol (85:5:10 v/v/v) and B: water–0.2 M ammonium bicarbonate–methanol (5:5:90 v/v/v)/ methylergometrine and dihydroergotamine	LC–MS/MS	Di Mavungu et al. (2012)

Evodiae fructus	Evodiamine, rutaecarpine	Human serum	Venusil XBP C_{18}/5/A: 5 mM ammonium formate–methanol–water (85:15 v/v)/evodiamine and rutaecarpine	LC–ESI–MS/MS and LC–APCI–MS/MS	Wen, Li, Liu, Liao, and Liu (2006)
Mitragyna inermis	Uncarine D	Leaves	Waters C_{18} Symmetry/5/A: phosphate–methanol and B: methanol/naphthalene	HPLC–DAD	Fiot et al. (2005)
M. speciosa	Mitragynine	Urine	Zorbax C_{18}/5/A: 10 mM ammonium formate in water and B: 0.1% acetic acid in ACN/No IS	LC–IT–MS	Philipp et al. (2009)
Pausinystalia yohimbe	Yohimbine	Bark and commercial aphrodisiacs	Nucleosil 100-5 C_{18} AB/5/A: 2 mM triethylamine in water and B: 2 mM in ACN/Yohimbine-d_3 (for MS) and codeine (for UV)	HPLC–UV; HPLC–APCI–MS; HPLC–ESI–MS	Zanolari et al. (2003)
Rauvolfia serpentina	Reserpine, ajmaline, ajmalicine	Roots	Chromolith Performance C_{18}/4.6/A: 0.01 M sodium phosphate and B: 0.5% acetic acid in ACN/reserpine, ajmaline and ajmalicine	HPLC–DAD	Srivastava, Tripathi, Pandey, Verma, and Gupta (2006)

Table 9.2 LC systems for quantitative and qualitative analysis of alkaloids—cont'd

Plant species	Target compound(s)	Matrix	LC conditions column/ particle size (μm)/mobile phase A and B/internal standard (IS)	Analytical method	References
R. serpentina × Rhazya stricta	Screening approach	Hybrid cell cultures	Nucleosil 100-5 C_{18}/A: 39 mM sodium phosphate in ACN and B: 3 mM sodium phosphate– 2.5 mM hexanesulfonic acid in ACN/No IS	HPLC	Stöckigt et al. (2002)
R. verticillata	Fingerprint analysis	Roots and rhizomes	Diamonsil C_{18}/5/A: water and B: 0.1% formic acid/No IS	LC–Q– TOF–MS	Hong, Cheng, Wu, and Zhao (2010)
Strychnos nux-vomica	Strychnine	Urine	Chrompack cyanopropyl/ 3/A: ACN and B: 1% acetic acid in water/ nalorphine	LC– APCI–MS/ MS	Van Eenoo, Deventer, Roels, and Delbeke (2006)
		Blood	Hypurity C18/5/A: ACN and B: 20 mM sodium dihydrogen phosphate/ chloroquine	LC–DAD	Duverneuil, de la Grandmaison, de Mazancourt, and Alvarez (2004)

Tabernanthe iboga	Ibogaine, noribogaine	Human plasma and blood	Zorbax eclipse XD8 C_8/5/A: 0.02% trimethylamine in ACN and B: 2 mM ammonium formate/fluorescein	LC–ESI–MS	Kontrimaviciute, Breton, Mathieu, Mathieu-Daudé, and Bressollee (2006)
		Biological fluids and hair	ODB Uptisphere C_{18}/5/A: 20% ACN and B: 2 mM formate/clonazepam-d_4	LC–ESI–MS/MS	Chèze et al. (2008)
Uncaria tomentosa	Pteropodine, isopteropodine, speciophylline, uncarine, mytrapylline, isomytraphylline, ryncophyllin, isoryncophyllin, corynoxeine, isocorynoxeine	Bark and leaves	Lichrosorb C_{18}/5/A: 30 mM ammonium acetate and B: methanol–ACN (1:1 v/v)/tryptophol	HPLC–ESI–MS	Montoro, Carbone, Zuniga-Quiroz, De Simone, and Pizza (2004)
		Bark	Zorbax XDB C_{18}/5/A: 35 mM triethylammonium acetate and B: ACN/Mytraphylline	HPLC–ESI–MS	Bertol, Franco, and de Oliveira (2012)
Vinca minor	Vinblastine, desacetylvinblastine, vincristine	Human plasma	Ultrasphere C_{18}/5/A: 15 mM ammonium acetate in methanol or ACN and B: ACN or methanol/Vinorelbine	LC–APCI–MS	Ramírez, Ogan, and Ratainn (1997)

Abbreviations: ACN, acetonitrile; APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization; FID, flame ionization; IT, ion trap; MS/MS, tandem mass spectroscopy; Q, quadrupole; TOF, time of flight.

He, Yang, Xiong, et al., 2011), metabonomic approaches (Wang et al., 2010), toxicological studies (Liu, Zhu, Li, Yan, & Lei, 2011) or in functional studies (Lorenz, Olsovska, Sulc, & Tudzynski, 2010) using UHPLC–MS where alkaloids are include identified by their retention time, UV spectra, fragmentation pattern data and high-resolution MS data and in some cases confirmed by NMR experiments.

3.3. Gas chromatography coupled to mass spectroscopy

Most of the TIAs are polar compounds and not volatile due to their indolyl and tertiary amino group that is not amenable for derivatization, although some of them have been successfully analysed by capillary GC using high temperatures of injection (200–300 °C) and temperature gradients from 100 to 250 °C (Verpoorte, 2005). The combination of GC with MS is an efficient tool in the preliminary or even complete identification of alkaloids. This approach is used in fingerprinting and bioactivity–guided approaches and even applying hyphenation with capillary electrophoresis (CE) (Table 9.3). For complete identification, fragmentation of the molecular ion is important, which can be achieved by tandem mass spectroscopy (MS^n). For quantitative analysis, GC–FID or the specific Nitrogen detector have an advantage over gas chromatography coupled to mass spectroscopy (GC–MS) in the detection and quantitation. In GC–MS, each compound will have a different detector response, which means that absolute quantitation requires calibration curves of each single compound, whereas in the other detection methods, the detector response is more or less similar for all compounds, thus allowing comparison of the peaks within a chromatogram without the need for calibration compounds. That thus allows the analysis of rare alkaloids of which not sufficient material is available for making calibration curves.

Dagnino, Schripsema, Peltenburg, and Verpoorte (1991) showed the feasibility of capillary GC for the analysis of a wide range of TIAs, mainly found in the genus *Tabernaemontana*. Gallagher et al. (1995) developed a derivatization method to estimate ibogaine levels in biological samples by GC–MS using ibogaine-d_3 as internal standard. Several derivatizing agents were compared, for example, trifluoroacetyl, heptafluorobutyric anhydride, and trifluoroacetic anhydride in order to determine the best derivatization conditions in terms of choice of chemical reagent, conditions and detection parameters for the reliable quantitation of this alkaloid in different tissues after oral administration. The derivatization of noribogaine and ibogaine by ethylation (Hearn, Pablo,

Table 9.3 Methods for the detection of alkaloids in different samples using GC–MS techniques

Compound	Matrix	Work-up	Internal standard	Derivatization	Stationary phase	Detection mode	References
Ibogaine	Brain	LLE (*n*-hexane)	Ibogaine-d_3	Trifluoroacetic anhydride	DB-5MS (30 m × 0.25 mm; 0.1 μm)	EI, MS	Gallagher et al. (1995)
Strychnine	Liver, lung, brain, spleen, skeletal muscle, bile, urine, blood	LLE (butyl chloride)	Methapyrilene	–	DB-5MS (15 m × 0.25 mm; 0.25 μm)	EI, MS/ MS, full scan	Rosano, Hubbard, Meola, and Swift (2000)
	Blood, liver, kidney, small intestine, urine	LLE (toluene– heptane– isoamyl alcohol 67:20:4 v/ v/v)	Papaverine	–	HP1 (12.5 m × 0.2 mm; 0.33 μm)	EI, SIM	Marques et al. (2000)
	Blood	SPE	Papaverine	–	Ultra 2 (12 m × 0.25 mm; 0.25 μm)	EI–MS, SIM	Barroso et al. (2005)
	Slimming foods	SPE	Leucomalachite green	–	VF-5MS (30 m × 0.25 mm; 0.25 μm)	EI–MS/ MS, full scan, SIM	Li et al. (2012)

Continued

Table 9.3 Methods for the detection of alkaloids in different samples using GC–MS techniques—cont'd

Compound	Matrix	Work-up	Internal standard	Derivatization	Stationary phase	Detection mode	References
Uleine, demethoxyaspidormine	Bark of *Himatanthus lancifolius*	SLE (1% HCl)	–	–	HP (30 m × 0.25 mm; 0.25 μm)	MS	Baggio et al. (2005)
Affinisine, voachalotine	Root bark of *Tabernaemontana laeta* and *T. hystrix*	SLE	Isatin	–	DB1 (30 m × 25 mm; 0.3 μm)	EI, FID	Vieira et al. (2008)
Aspidospermidine, demethoxypalosine, aspidocarpine, aspidolimine, fendlerine, aspidolimidine	Stem bark of *Aspidosperma spruceaunm*	SLE (methanol)	–	–	DB5-MS (30 m × 0.25 mm; 0.25 μm)	EI–MS/ MS	Aguiar et al. (2010)
Yohimbine	Bark of *Pausinystalia yohimbe*	SLE (methanol)	Diazepam	–	DB5-MS (30 m × 0.32 mm; 0.25 μm)	EI, MS, SIM	Chen et al. (2008)
Voacangine, dregamine	Root extract from *T. elegans*	SLE (ethanol)	–	–	DB5-MS (30 m × 32 μm; 0.25 mm)	MS, Scan mode	Pallant et al. (2012)

Abbreviations: EI, electron impact; FID, flame ionization detection; LLE, liquid–liquid extraction; SIM, selected ion monitoring; SLE, solid–liquid extraction.

Hime, & Mash, 1995) or silylation (Alburges, Foltz, & Moody, 1995) after sample clean-up procedures with SPE has also proved to be effective in the determination of these alkaloids from blood, plasma and urine samples.

In bioactivity-guided experiments, a number of alkaloids have been identified from plant extracts or fractions by means of GC–MS (Cardoso, Vilegas, & Honda, 1998) or GC–FID (Cardoso, Vilegas, & Pozetti, 1997). Particularly, alkaloids from different *Tabernaemontana* species (Andrade et al., 2005; Pallant, Cromarty, & Steenkamp, 2012; Vieira et al., 2008) and *Himatanthus lancifolius* (Baggio et al., 2005) are amenable for analysis without any derivatization step.

Adulterants such as strychnine along with other compounds present in different commercially available slimming products were determined by GC–MS/MS. Optimization of the method included a sample clean-up step. Since the target compounds are weak bases, the extraction included SPE using a strong cation exchange cartridge which was washed with 2% formic acid, 30% methanol–water and 2% ammoniated methanol (Li et al., 2012).

3.4. High-speed counter-current chromatography

High-speed counter-current chromatography (HSCCC) is a two-phase solvent system, without solid phases, instead with liquid stationery phase, to resolve target compounds relying on the different partitioning of solutes between two immiscible solvents which makes it a very effective tool for the preparative separation and purification of natural products (Zhao & He, 2006).

The preparative isolation of alkaloids can be achieved by means of HSCCC. Because of the ionic nature of alkaloids, systems with a controlled pH are preferred for their separation. For example, pH-zone-refining counter-current chromatography (CCC) has been quite successful in separating alkaloids based on the pK_a values, showing the typically characteristic rectangular peaks for the analytes as common in displacement chromatography (Ito & Ma, 1996). Improved efficiency can be obtained by using ion-pairing gradients, for example, solvent two-phase systems consisting on methanol–chloroform–aqueous phosphate or citrate buffer (pH 4) containing perchlorate, acetate or chloride as the ion-pairing agent (Fang, Liu, Yang, Wang, & Huang, 2011; van der Heijden Hermans-Lokkerbol, Verpoorte, & Baerheim Svendsen, 1987). Important was the observation that ion pairs of alkaloids with chloride and perchlorate are quite well soluble in chloroform–methanol, something which is important information to keep in mind in liquid–liquid partitioning procedures for the isolation of

alkaloids, as at acidic pH considerable amounts of alkaloids may pass into an organic solvent due to ion pairing (van der Heijden et al., 1987).

There are few studies on the separation and isolation of TIAs using CCC. They report on the separation of alkaloids from *C. roseus* (Renault et al., 1999), *Strychnos guaianensis* (Quetin-Leclercq et al., 1995), *S. nux-vomica* (Miao Cai, Xiang, An, & Ito, 1998), *Hortia oreadica* (Severino et al., 2009), *Geissospermum vellosi* (Mbeunkui, Grace, & Lila, 2012) and *Tabernaemontana* (van der Heijden et al., 1987), and *T. catharinensis* (Gonçalves, Curcino, Oliveira, & Braz-Filho, 2011).

Alkaloids from a crude extract of *C. roseus* as well as an artificial mixture of vinblastine, vincristine and catharanthine were successfully separated as monomers and dimers by means of centrifugal partition chromatography (CPC), a variation of CCC, in the pH-zone refining mode (Renault et al., 1999). The solvent phases used were methyl *tert*-butyl ether–acetonitrile–water (4:1:5 v/v/v). The upper organic phase was basified with 8 mM of triethylamine and used as mobile phase (ascending mode) or with 10 mM when used as the stationary phase (descending mode). The lower aqueous phase was acidified with 10 mM HCl (as a retainer stationary phase) or 8 mM (as a displacer mobile phase).

Quetin-Leclercq et al. (1995) briefly mentioned the fractionation of a chloroform residue by HSCCC when they isolated for the first time guianensine, an alkaloid from the stem bark of *Strychnos guianensis* using a multilayer–coil separator–extractor and a solvent system of ethyl acetate–methanol–water (4:1:3 v/v/v) where the lower aqueous phase was used as a stationary phase and the upper organic phase was pumped from the bottom to the upper part of the column which was also applied for strychnine and brucine separation from seeds of *S. nux-vomica* using a two-phase solvent system consisting of chloroform and 0.07 M sodium phosphate in a buffer solution of 0.04 M citric acid (1:1 v/v) (Miao et al., 1998).

Ingkaninan, Hazekamp, Hoek, Balconi, & Verpoorte (2000), Ingkaninan, Hermans-Lokkerbol, & Verpoorte (1999), reported the use of CPC for the pre-separation of crude extracts for rapid dereplication of known biological active compounds in plant materials. This included the analysis of several TIAs producing *Tabernaemontana* plants, allowing, for example, the rapid identification of two active TIAs (tubotaiwine and apparicine).

Severino et al. (2009) demonstrated the advantage of HSCCC in the isolation of the alkaloids rutaecarpin and dictamine from dichloromethane extract of *H. oreadica* leaves. They used the two-phase solvent system composed of *n*-hexane–ethanol–acetonitrile–water (10:8:1:1 v/v/v/v), where

the upper phase was used as the mobile phase and the lower phase was used as the stationary phase in a tail-to-head elution mode. Further conventional methods of column chromatography yielded rutaecarpin and dictamnine with excellent recoveries compared to the concentration of the compounds quantified simultaneously by LC–APCI–MS/MS analysis of the same extract (93.1% and 84.9%, respectively). In a similar study, the combination of high-performance counter-current chromatography (HPCCC) and LC–MS/MS was successfully established to isolate indole alkaloids from the methanol extract from the stem bark of *G. vellosi* (Mbeunkui et al., 2012). Extract separation was achieved with the solvent system ethyl acetate–butanol–water (2:3:5 v/v/v) in an elution–extrusion with the upper phase as stationary phase and the combination of flash column chromatography. Identification of five different indole alkaloids was carried out with ESI multistage mass spectrometry (MS^n) data and confirmed by NMR methods.

Voachalotine and 12-methoxy-N_b-methylvoachalotine were resolved from the methanolic extract from the roots of *T. catharinensisin* by HSCCC in 4 h with a solvent system consisting of chloroform–methanol–water (5:10:6 v/v/v) with a 95% and 97% purity, respectively, and their identity was confirmed by ^1H and ^{13}C NMR experiments (Gonçalves et al., 2011).

Five indole alkaloids from the stem bark of *G. vellosii* were isolated with a combination of HPCCC and flash chromatography. To further analyse them, ESI–IT–TOF–MS and NMR experiments were conducted (Mbeunkui et al., 2012). In order to study the fragmentation pattern of these alkaloids, multiple tandem mass spectrometric data were produced by CID of the protonated molecule ion based on the most abundant ions $[M+2H]^{2+}$ and $[M+H]^+$ and a fragmentation pathway geissolosimine, geissospermine, geissoschizoline, geissoschizone and vellosiminol was proposed.

3.5. Capillary electrophoresis

CE represents an attractive analytical technique for the rapid qualitative and quantitative analysis of molecules with a wide range of polarity and molecular weight, including small molecules such as drugs but also macromolecules such as proteins or nucleic acids (Unger, 2009). Because of its versatility and high separation efficiency, CE is an alternative to the widely used RP-HPLC. CE has gained much interest for the analysis of natural products in plant extracts, quality control of herbal medicines, pharmaceutical formulations and food supplements (Ganzera, 2008; Verardo, Gomez-Caravaca, Seura-Carretero, Caboni, & Fernández-Gutiérrez, 2011).

The relatively poor sensitivity of CE, resulting from the small loading volumes, can be circumvented by the incorporation of pre-concentration strategies, while the advantages of MS detection are embodied in the improvement of detection sensitivity as well as the capability of both determining the exact mass of analytes and providing structural information, including the possibility to identify and determine co-migrating species in overlapping peaks (Niessen, Tjaden, & van der Greef, 1993; Ramautar, Somsen, & de Jong, 2011).

The ideal candidates for CZE are permanently charged molecules such as quaternary alkaloids and electrokinetic chromatography, but in fact, all acidic, basic and neutral compounds can be analysed by CE (Gotti, 2011). For TIA analysis, non-aqueous capillary electrophoresis (NACE) has been the most widely used since electrolytes such as ammonium acetate and ammonium formate can be used, allowing the hyphenation of CE and MS (Scriba, 2007). Buffer systems and CE methods used for TIA analysis are listed in Table 9.4.

NACE was useful for the separation of 11 *Vinca* alkaloids from an artificial mixture. Results were compared to those of HPLC using UV traces of both methods at 214 nm showing that although HPLC is more sensitive than CE in terms of limit of detection (LOD) and limit of quantification (LOQ), CE can be a good alternative by reducing analysis time and giving better resolution (Barthe et al., 2002).

Posch, Martin, et al. (2012) described an NACE–MS method to screen the psychoactive alkaloids present in two commercial preparations from *Mitragyna speciosa* as a quality control for added active compounds like the opioid *O*-desmethyltramadol, which can be fatal for humans. The use of a non-aqueous buffer system allowed the separation of diastereomers of mytraginine. The same methodology proved to have a high resolving power for the separation of iboga alkaloids from *Voacanga africana* although the choice of detector was not enough to discriminate between analytes with similar masses and migration times. In a similar study with preparations from *M. speciosa*, a higher selectivity and resolution were observed when BGE was switched to ammonium formate (Posch, Müller, et al., 2012). For the analysis of indole alkaloids from the root bark of *P. yohimbe* by NACE and GC–MS, the latter proved to be more sensitive (LOD 0.6 and 1.0 µg/ml, respectively) in terms of identification (Fig. 9.1; Chen et al., 2008).

An aqueous CE system using α, β or γ cyclodextrins (CD) was tested for the enantiomeric separation of vincamine, vinpocetine and vincadifformine. The best separations were achieved with β-CD and γ-CD. The proposed structures for the inclusion complexes were based on rotating-frame nuclear

Table 9.4 Buffer systems and CE methods for the analysis of terpenoid indole alkaloids from different plant species

Plant species	Target compound	CE method	Electrolyte	Analytical method	References
Catharanthus roseus	Vinblastine, vincristine	CZE	0.2 M ammonium acetate, pH 6.2	UV	Chu, Bodnar, White, and Bowman (1996)
	Vinblastine, vindoline, catharanthine	CE	20 mM ammonium acetate in 1.5% acetic acid	MS	Chen, Li, Zhang, Chen, and Chen (2011)
Claviceps purpurea	Ergonovinine, ergonovine, ergocornine, ergocryptine, ergocornine, ergocristine, ergosine, ergocristinine, ergotamine	CE	20 mM β-CD, 8 mM γ-CD, 2 M urea, 0.3% PVA in phosphate buffer, pH 2.5	UV	Frach and Blaschke (1998)
Evodiae fructus	Evodiamine, rutaecarpine, carboxyevodiamine, 1-methyl-2-nonyl-4(1*H*)-quinolone, 1-mehtyl-2-[(*Z*)-6-undecenyl]-4(1*H*)-quinolone, 1-methyl-2-undecyl-4(1*H*)-quinolone, evocarpine, 1-methyl-2-[(6*Z*,9*Z*)-6,9-pentadecadienyl]-4(1*H*)-quinolone, dihydroevodiamine	CZE, MEKC	CZE: 40 mM sodium dihydrogen phosphate–ACN (9:1 v/v) MEKC: 20 mM phosphate, 40 mM SDS, 9 mM sodium borate, pH 7.31	UV	Lee, Chuang, and Sheuu (1996)

Table 9.4 Buffer systems and CE methods for the analysis of terpenoid indole alkaloids from different plant species—cont'd

| Plant species	Target compound	CE method	Electrolyte	Analytical method	References
Mitragyna speciosa	Mitragynine, paynantheine, 7-hydroxy-mitragynine	NACE	60 mM ammonium formate, 5% acetic acid in ACN	qTOF–MS	Posch, Müller, Schulz, Pütz, and Huhn (2012)
	Mitragynine, speciogynine, speciociliatine, mitraciliatine	NACE	58 mM ammonium formate, 1 M acetic acid in ACN	qTOF–MS	Posch, Martin, Pütz, and Huhn (2012)
Pausinystalia yohimbe	Yohimbine	NACE	20 mM ammonium acetate in 0.5% acetic acid	UV	Chen et al. (2008)
Phellodendron wilsonii	Berberine, palmatine, jatrorrhizine, phellondendrine, tetrahydropalmatine, magnoflorine, thalphenine	CE	60 mM ammonium acetate in 40% methanol, pH 4.5	UV, MS	Henion, Mordehai, and Cai (1994)
Psilocybe semilanceata	Psilocybin, baeocystin	CZE	10 mM borate, 10 mM phosphate, 25 mM SDS, pH 11.5	UV	Pedersen-Bjergaard, Rasmussen, and Sannes (1998)
Rauvolfia serpentina, Rauvolfia serpentina × Rhazya stricta,	Gramine, tryptamine, serpentine, alstonine, β-methylajmaline,	CZE	100 mM ammonium acetate in ACN (1:1 v/v), pH 3.1	UV, MS	Stöckigt et al. (2002), Stöckigt, Unger, Belder, and Stöckigt (1997) and

Aspidosperma quebracho-blanco	tabersonine, vinblastine, corynanthine, vincristine, raufloridine, ajmaline, yohimbic acid, deserpidine, reserpine, rescinnamine				Unger, Stöckigt, Belder, and Stöckigtt (1997)
Strychnos nux-vomica	Strychnine, brucine	MEKC	50 mM phosphate, 100 mM SDS in ACN (4:1 v/v), pH 2.0		Wang, Han, Wang, Zang, and Wu (2006)
		CZE	10 mM phosphate buffer in methanol (9:1 v/v), pH 2.5	UV	Zong and Che (1995)
		NACE	25 mM Tris boric acid, methanol–ACN (6:2 v/v), pH 4.0	UV	Gu, Li, Zhu, and Zou (2006)
			30 mM ammonium acetate, acetic acid–ACN (1:1.5 v/v) in methanol	UV	Li et al. (2006)
S. pierrian	Strychnine, brucine, novacine, icajine		80 mM ammonium acetate, 0.1% acetic acid in water–methanol (4:6 v/v)	UV, MS	Feng, Yuan, and Lii (2003)
Uncaria tomentosa	Oxindole alkaloids	CZE	20 mM phosphate buffer, pH 5.6	UV	Stuppner, Sturn, and Konwalinkaa (1992)

Table 9.4 Buffer systems and CE methods for the analysis of terpenoid indole alkaloids from different plant species—cont'd

Plant species	Target compound	CE method	Electrolyte	Analytical method	References
Vinca	Catharanthine, vinorelbine, anhydrovinblastine, vinflunine, vindoline, 4-*O*-deacetylvinorelbine, 4-*O*-deaceylvinflunine, vindesine, 4′-deoxy-20′,20′-difluorovinblastine, vincristine	NACE	50 mM ammonium acetate, 25 mM SDS, 0.6 M acetic acid in methanol–ACN (75:25 v/v), pH 7.7	HPLC–DAD	Barthe et al. (2002)
	Vincamine, vinpocetine, vincadiformine	CE–CD	0.25–50 mM CD, 15 mM NaOH, pH 2.5	UV, NMR	Sohajda et al. (2010)
Voacanga africana	Voacamine, ibogaine, voacangine, 3-oxovoacangin	NACE	58 mM ammonium formate, 1 M acetic acid in ACN	qTOF–MS	Posch, Martin, et al. (2012)

Abbreviations: ACN, acetonitrile; CD, cyclodextrin; CE, capillary electrophoresis; CZE, capillary zone electrophoresis; MEKC, micellar electrokinetic chromatography; NACE, non-aqueous capillary electrophoresis; PVA, polyvinyl alcohol; SDS, sodium dodecyl sulphate.
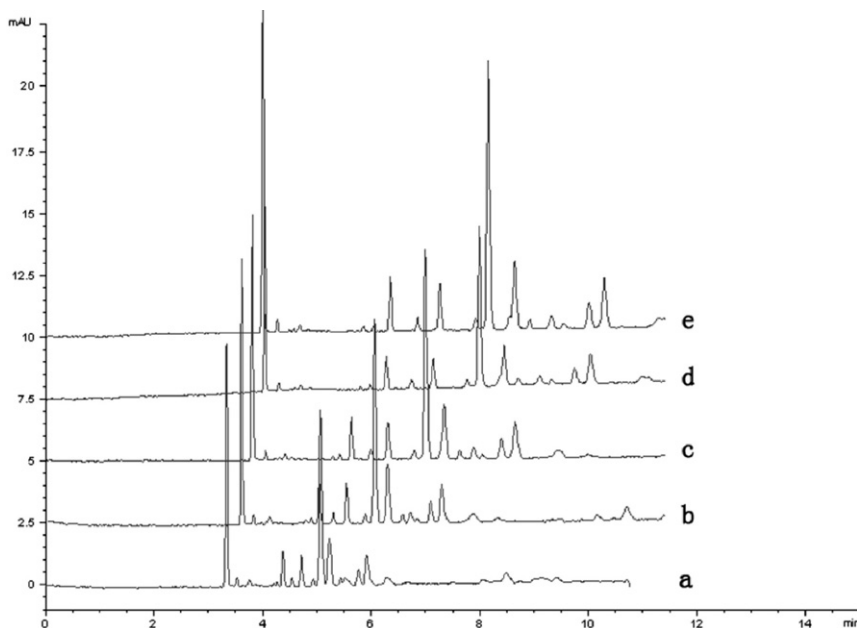
Figure 9.1 Electropherograms of several concentrations of ammonium acetate on the mobility of yohimbe, diazepam and berberine. Buffer solutions (a) 10 mM, (b) 15 mM, (c) 20 mM, (d) 25 mM, and (e) 30 mM ammonium acetate in 0.5% acetic acid. *Adapted with permission from Chen et al. (2008).*

Overhauser effect correlation spectroscopy (ROESY) experiments and their stability constants were determined by ^{1}H NMR chemical shift titrations for the three alkaloids (Sohajda et al., 2010). Using the same CDs, ergot alkaloids were successfully resolved in 12 min analysis time and 30-fold increased sensitivity when a laser-induced fluorescence detector was used (Frach & Blaschke, 1998).

3.6. Quantitative nuclear magnetic resonance

One of the major advantages of quantitative nuclear magnetic resonance (qNMR) is its primary analytical characteristic, because of which it can be applied in the quantitative estimation of purity of compounds without using any specific reference standard (Lindon & Nicholson, 2008). NMR–based metabolomics provides absolute and relative quantification of several metabolites in biological samples without separation of individual components in normal or modulated metabolism, so qNMR spectroscopy

has been widely applied in environmental toxicity, drug toxicity, disease diagnosis, cancer metabolism, pathophysiology of disease, stress, nutrition, drug metabolism, plant metabolism, bacterial metabolism and cell–virus interactions (Bharti & Roy, 2012).

Camptothecin, 9-methoxycamptothecin, pumiloside and trigonelline were quantified by ^{1}H NMR analysis in root, stems and leaves from *Nothapodytes foetida* using DMSO-d_6 as solvent and 3,4,5-trimethoxybenzaldehyde as internal standard (Li, Lin, & Wu, 2005). The signals of H-7, H-10, H-19 and H-2 were selected as target signals for quantification of each alkaloid, respectively. Quantitation data were compared and confirmed to that of HPLC.

4. APPLICATIONS IN FINGERPRINT ANALYSIS

4.1. NMR and LC–MS-based metabolic fingerprinting on TIAs

NMR spectroscopy has a long-standing tradition to be applied to the characterization of pure compounds as it has been the case for the structural elucidation of TIAs. The continuous development of more and more sophisticated one-dimensional (1D) and 2D pulse sequences in NMR, various structure elucidation strategies have been developed and in the early 2000s, the unambiguous NMR-based structure elucidation of bisindoles is now inconceivable without using a 'holistic' NMR approach, that is, a full ^{1}H and ^{13}C NMR assignment in conjunction with the establishment of all spin–spin connectivities by a broad range of 2D-NMR methods, that is, homonuclear correlated spectroscopy (COSY), total correlated spectroscopy (TOCSY), heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC), heteronuclear multiple quantum coherence (HMQC), nuclear Overhauser effect spectroscopy (NOESY) or ROESY experiments (Beni, Hada, Dubrovay, & Szantay, 2012).

There are excellent reviews covering the structural elucidation of the alkaloids in *Catharanthus* (Beni et al., 2012; Blaskó & Cordell, 1990; Dubrovay, Háda, Béni, & Szántay, 2012; Háda et al., 2012), *Aspidosperma* (Guimaraes, Braz-Filho, & Vieira, 2012), *Tabernaemontana* (Danieli & Palmisano, 1987; Nielsen, Hazell, Hazell, Ghia, & Torssell, 1994; Talapatra, Patra, & Talapatra, 1975), and *Strychnos* (Penelle et al., 2001, 2000; Rasoanaivo, Martin, Guittet, & Frappier, 2002) already published. However, the

description of these experiments is beyond the scope of this discussion and interested readers are advised to consult the aforementioned publications.

Even though NMR is a crucial tool for identification and structure elucidation of pure samples, it can also make important contributions to the metabolic profiling by complementing MS–based approaches (Forseth & Schroeder, 2011). NMR–based metabolomics can be effectively applied to characterize and distinguish plants on species and genotype levels, different plant tissues within the same plant as well as for the detection of adulterants in foods and in health supplements for quality control (Holmes, Tang, Wang, & Seger, 2006). NMR can be integrated with chromatography to analyse herbal products to generate standardized 'metabolic fingerprints' which contains markers for activity (Heyman & Meyer, 2012).

One of the most common adulterants in *Strychnos* preparations is the 'false angostura bark', *Galipea officinalis*, whose bark closely resembles that of *S. nux-vomica*. An ^{1}H NMR method was developed for the quantitative analysis of strychnine and brucine in seeds and stem bark from *S. nux-vomica* (Frederich, Choi, & Verpoorte, 2003) along with a multivariate analysis which was useful for the metabolic profiling of *S. nux-vomica*, *S. ignatii* and *S. icaja* (Frederich et al., 2004). With this study, it was possible to discriminate the three species according to the composition in different organs, that is, seeds, leaves, stem bark and root bark. The compounds responsible for this discrimination were strychnine, brucine, loganin, fatty acids, icajine and sungucine. *Strychnos nux-vomica* and *S. icaja* stem bark could be distinguished by their content of brucine, but it was not possible to discriminate between the stem from *S. nux-vomica* and its adulterant arguing that the original material must have come from either stem bark or root bark.

Another interesting example concerns NMR–based profiling of *Cinchona* alkaloids in museum samples dating from 1850 to 1950 in order to determine the variation in contents from 117 different bark samples (Yilmaz, Nyberg, & Jaroszewski, 2012). An extraction system was developed using chloroform–d_1, methanol–d_4, D_2O and aqueous 70% perchloric acid (5:5:1:1 v/v/v/v). With an initial principal component analysis (PCA), it was possible to rule out four mislabelled samples that did not correspond at all to the *Cinchona* materials. With STOCSY-CA (statistical total correlation spectroscopy component analysis), it was possible to draw the conclusion that the variation methods in extracted alkaloids is not due to decomposition of quinine but an effect of the different cultivation methods of *Cinchona* trees over time.

Using 1D and 2D NMR, a comparison between the metabolic profile of healthy and phytoplasma–infected *C. roseus* plants was conducted along with multivariate data analysis in order to characterize and identify the metabolites responsible for the discrimination of the samples (Choi et al., 2004). Infected leaves showed an increase in signals of H–9 at δ 6.89 corresponding to vindoline showing a twofold increase than in healthy plants. The TIA precursors secologanin and loganic acid as well as chlorogenic acid and sugars, were four times higher than in healthy plants.

An MS-based fingerprint analysis was reported for yohimbe bark, and 18 different commercial dietary supplements, in order to determine the presence of yohimbine in the samples as well as to assess the quality of these supplements in the form of tablet, capsule or liquid (Sun & Chen, 2012). MS data were only used to confirm the identities of yohimbine, corynanthine and some other alkaloids, but the fingerprint analysis was conducted using the characteristic peaks in a chromatographic approach. In this case, all peaks are normalized against the area of yohimbine. With this method, the authors unambiguously demonstrated that 10 of the tested commercial preparations did not contain the amount of yohimbine claimed in the label of the product.

Mass spectroscopy does not only give the molecular weight, but each compound has also a characteristic fragmentation pattern, which is very useful for identification in GC-MS and LC-MS. Hesse (1974) has brought together all the information on mass spectroscopy of indole alkaloids, a very useful tool for identifying the identity of indole alkaloids.

5. CONCLUSIONS

The analysis of TIAs is a challenging task because of their complex chemical structures, usual low abundance and their difficult and time-consuming extraction procedures from different plant materials as well as from biological fluids. Only highly selective and sensitive methods will be suitable for such analyses. Sensitivity is the disadvantage of ^{1}H NMR when it comes to low contents of alkaloids in plant extracts, yet it is the only technique, which produces signals directly correlated with the amount of analytes in the sample. Even though CE offers some potential improvements in TIAs separation, it often faces sensitivity problems and in the case of GC, although a powerful tool, it is only suitable for a limited number of alkaloids which are volatile or amenable for derivatization. Consequently, liquid chromatographic or electrophoretic techniques in combination with

different detectors have been mostly employed for TIAs analysis. Thus, HPLC in the reversed–phase mode has been and is the preferred separation technique for the analysis of TIAs.

The UV and DAD detection are robust detectors for targeted analysis. LC-MS offers further resolution and are of interest for more in depth analyses. Metabolomics as a novel approach is based on the different methods discussed here. But it requires a strict standardization to be able to store the results of the analyses with other laboratories. It should thus be based on standard protocols and public databases where the data are stored. Considering the extensive data presented in this review, it is clear that there is still a very long way to go to come to a chromatography based metabolomics in which all alkaloids can be analyzed. NMR despite its disadvantages of not being sensitive enough, seems closest to become a metabolomic platform in which also alkaloids can be analyzed.

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