computer. The data can be acquired, manipulated and displayed in real time and can be stored for record purposes.

Looking to the future, it is reasonable to expect continued evolutionary development: new selective detectors, more complex analysers for automated sample processing, increasing use of coupled techniques, columns with immobilized phases of a wider range of selectivity, etc. It is hoped that further research and development will encourage the use of GC-MS in the areas of alkaloid analysis that still await investigation.

Acknowledgements

The author gratefully acknowledges M. Martin-Pedrosa, T. Ortega, C. Cuadrado and C. Burbano for their helpful comments.

See also: **II/Chromatography: Gas:** Detectors: General (Flame Ionization Detectors and Thermal Conductivity Detectors); Detectors: Mass Spectrometry; Detectors: Selective. **III/Alkaloids:** Liquid Chromatography; Solid-Phase Extraction; Solid-Phase Microextaction; Supercritical Fluid Extraction; Thin-Layer (Planar) Chromatography. Extraction: Analytical Extractions.

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High Speed Countercurrent Chromatography

See **III / MEDICINAL HERB COMPOUNDS: HIGH SPEED COUNTERCURRENT CHROMATOGRAPHY**

Liquid Chromatography

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Definition and Classification of Alkaloids

Alkaloids represent a wide variety of chemical structures (**Figure 1**). More than 16 000 are known and most are derived from higher plants. Alkaloids have also been isolated from microorganisms, marine organisms like algae, dinoflagellates and puffer fish and terrestrial animals like insects, salamanders and toads.

An alkaloid has been defined by Pelletier as a cyclic organic compound containing nitrogen in a negative oxidation state which is of limited distribution among living organisms. From the analytical chemical point of view, the most important trait of alkaloids is their

basicity arising from a heterocyclic tertiary nitrogen atom. Notable exceptions are colchicine and the xanthines (e.g. caffeine), with pK_a values between 1 and 2. Alkaloids are biosynthetically derived from amino acids, such as phenylalanine, tyrosine, tryptophan, ornithine and lysine. The biogenesis of alkaloids is used for their classification, as this is directly linked with their molecular skeleton, e.g. the two

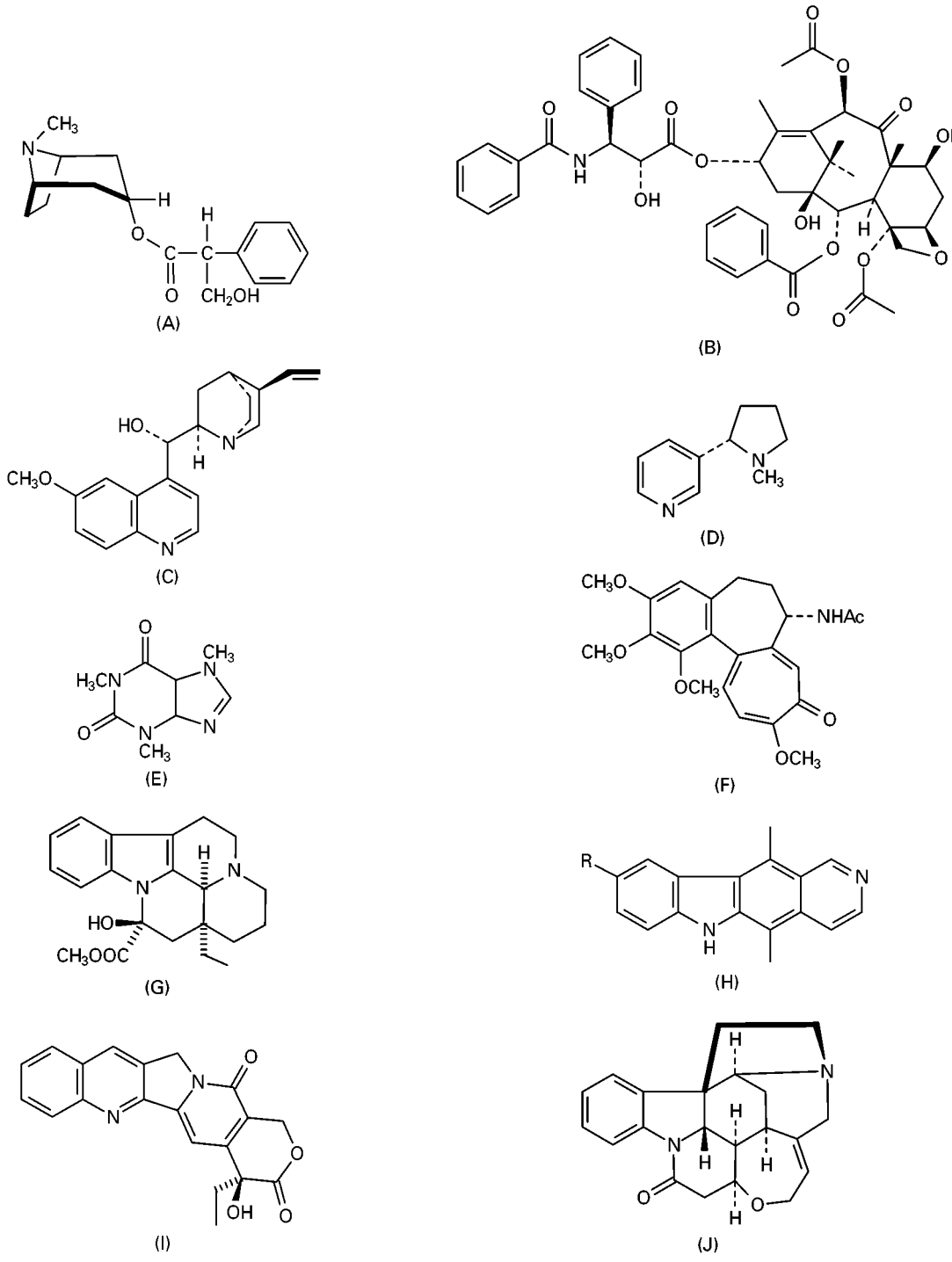
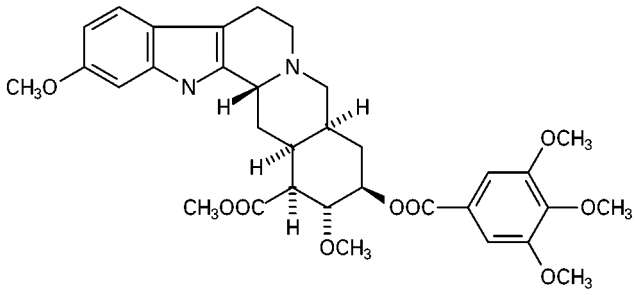


Figure 1 Structures of alkaloids. (A) L-hyoscyamine; (B) taxol; (C) quinine; (D) nicotine; (E) caffeine; (F) colchicine; (G) vincamine; (H) R = H ellipticine; R = OH 10-hydroxyellipticine; (I) camptothecin; (J) strychnine; (K) reserpine; (L) H-20α tetrahydro alstonine; H-20β ajmalicine; (M) emetine; (N) berberine; (O) galanthamine; (P) sanguinarine; (Q) R = H morphine; R = CH_3 codeine; (R) R = H *d*-tubocurarine; R = CH_3 *d*-chondrocurarine; (S) solasodine.



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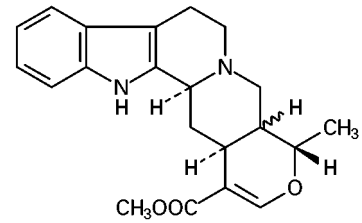

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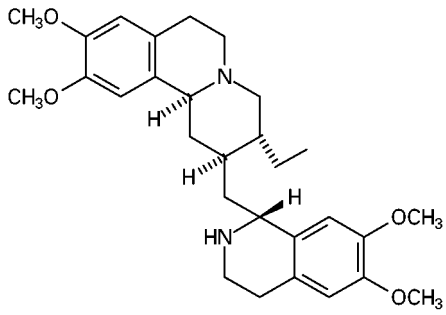

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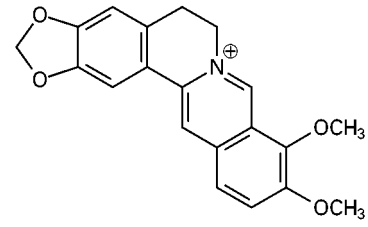

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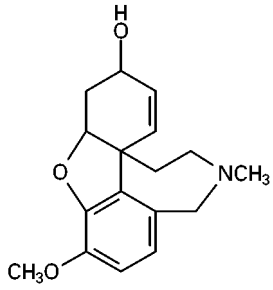

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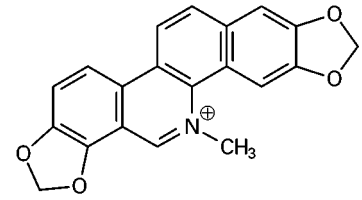

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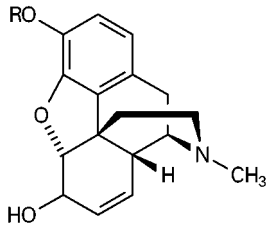

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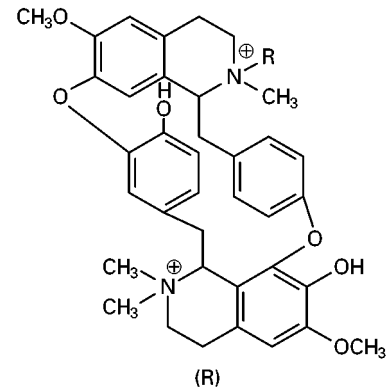

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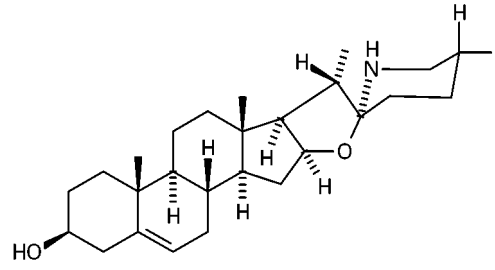

(S)

Figure 1 *Continued*

Table 1 Alkaloids of pharmaceutical interest

Indole alkaloids	*Tropane alkaloids*
Ajmalicine	Cocaine
Ajmaline	Scopolamine
Camptothecin	Atropine (*d*/*l*-hyoscyamine)
Ergocornine	
Ergocristine	*Terpenoid alkaloids*
Ergocryptine	Aconitine
Ergonovine	Solasodine
Ergosine	Taxol
Ergotamine	Tomatidine
Harmane	Veratrine
9-Hydroxyellipticine	
Lysergic acid	*Miscellaneous*
Physostigmine	Caffeine
Psilocybin	Colchicine
Reserpine	Ephedrine
Rescinnamine	Lobeline
Serotonin	Mescaline
Strychnine	Muscarine
Yohimbine	Nicotine
Vincamine	Pilocarpine
Vinblastine	Saxitoxin
Vincristine	Sparteine
	Tetrodotoxin
Quinoline alkaloids	Theobromine
Quinine	Theophylline
Quinidine	
Isoquinoline alkaloids	
Apomorphine	
Berberine	
Boldine	
Chelerythrine	
Codeine	
Emetine	
Galanthamine	
Heroin	
Morphine	
Narceine	
Noscapine	
Papaverine	
Sanguinarine	
Thebaine	
Tubocurarine	

largest groups are indole alkaloids (more than 4100 compounds) and isoquinoline alkaloids (more than 4000 compounds). Other important groups are tropane alkaloids (*c*. 300 compounds), steroidal alkaloids (*c*. 450 compounds), pyridine and pyrrolizidine alkaloids (about 250 and 570 compounds, respectively).

The botanical origin of the alkaloids is also used as a classification method, e.g. *Papaver* (opium) alkaloids, *Cinchona* alkaloids, *Rauvolfia* alkaloids, *Catharanthus* alkaloids, *Strychnos* alkaloids, ergot alkaloids, cactus alkaloids and *Solanum* alkaloids. As secondary metabolites, alkaloids probably play a role in defending organisms against pests and diseases. For example, for some types of alkaloids, insect antifeedant activity has been established. Thus, many alkaloids have strong biological activities. Their effect in humans can be explained by structural relationships with important signal compounds (neuro-transmitters) like dopamine, acetylcholine, noradrenaline and serotonin. Consequently, some alkaloids are used as medicines or in pharmacological studies (**Table 1**). In addition to pure compounds, crude plant extracts containing alkaloids are used (phytotherapy). Another area where alkaloids play a major role is in drugs of abuse, e.g. mescaline, cocaine, morphine and its semisynthetic derivative, heroin. Alkaloids are also of interest in the analysis of doping (e.g. strychnine, ephedrine, caffeine) and poisons (e.g. strychnine, pyrrolizidine alkaloids, coniine, nicotine, aconitine, tetrodotoxin).

Due to their various applications, the analysis of alkaloids is of great importance. The very different types of (ab)use of the alkaloids mean that the type of analyses also varies. Alkaloids must be analysed in a broad variety of matrices, such as plant material, tablets, drug seizures, urine and blood. Each requires different sample clean-up methods and chromatographic selectivities. Liquid chromatography is the most commonly used method since the instability and low volatility of alkaloids mean that gas chromatography has a limited applicability. Because the extracts are often complex and 'dirty', thin-layer chromatography is useful in analysing alkaloid-containing plant extracts.

Chemical Properties and Artefact Formation

Most alkaloids have basic properties with pK_a values of about 6 to 12, but usually 7–9. The free base is soluble in organic solvents and not in water. Protonation of the nitrogen in the free base usually results in a water-soluble compound. This behaviour is the basis of the selective isolation of alkaloids by liquid/liquid partitioning processes. Quaternary alkaloids are poorly soluble in organic solvents but soluble in water at any pH.

Many alkaloids are difficult to crystallize in the form of the free base, but do crystallize as a salt. Alkaloids are usually colourless; only some highly conjugated compounds are coloured or show strong fluorescence (e.g. berberine and serpentine).

Alkaloids are not very stable; in particular, *N*-oxidation is quite common. Stability is influenced by solvents, as well as heat and light. Halogen-containing organic solvents such as chloroform and dichloromethane are widely used in alkaloid research.

Chloroform in particular is a very suitable solvent, because of its relatively strong proton donor character. However, this solvent easily causes the formation of artefacts, e.g. (*N*-)oxidation occurs easily. Dichloromethane may result in the formation of quaternary *N*-dichlorometho-compounds. Similar compounds are formed with the minor impurities present in chloroform. Peroxides in ethers may also cause *N*-oxidation.

Alkaloids are more stable in toluene, ethyl acetate and alcoholic solutions. Carbinolamine functions are often found in alkaloids, either formed during the coupling of a carbonyl group and an amine in the biosynthesis, or as products formed from rearrangements of *N*-oxides. Carbinolamines readily react with alcohols (e.g. *O*-methyl pseudostrychnine formed from pseudostrychnine with methanol). Ketones such as acetone and methylethylketone are well-known artefact formers. Berberine, for example, may react with acetone. Ammonia and acetone may react during column chromatography, yielding condensates that give a Dragendorff-positive reaction. Ammonia may also react with aldehydes present in plant materials, giving rise to artificial alkaloids, e.g. the pyridine-type alkaloid gentianine is formed from sweroside during extraction.

Extraction

Due to the more lipophylic character of alkaloids as free bases, they can be extracted under neutral or basic conditions (e.g. after basification of the plant material or biofluid to pH 7–9 with ammonia, sodium carbonate or sodium bicarbonate) with organic solvents (such as dichloromethane, chloroform, ethers, ethyl acetate and alcohols). Strongly basic alkaloids can only be completely extracted at higher pH (>10), e.g. tryptamine. As a general rule of thumb, for the extraction of an alkaloid one should choose a pH of $pK_a + 2$. On the other hand, alkaloids containing phenolic groups are protonated at higher pH, and thus not extracted by organic solvents under such conditions (e.g. morphine).

Alkaloids can be extracted in protonated form (after acidification to pH 2–4 with diluted acids like phosphoric acid, sulfuric acid, citric acid) with water or alcohols (e.g. methanol).

Alkaloids can be further purified by liquid–liquid extraction or liquid/solid extraction. In liquid–liquid extraction the alkaloids are, after basification, extracted form an aqueous solution with an immiscible organic solvent or from an organic solvent with an aqueous acid solution. To avoid the formation of lipophylic ion pairs, phosphoric acid, sulfuric acid and citric acid are preferred over acetic acid and hydrochloric acid. By using a back-extraction from aqueous solution to organic and back to aqueous, or from organic to aqueous and back to organic solution, alkaloids can easily be separated from neutral and acidic compounds.

Alkaloids can be extracted from acidic aqueous solutions with organic solvents by using ion-pairing reagents (e.g. alkylsulfonic acids). It should be noted that common anions such as Cl^-, Br^-, I^- and acetate also form ion pairs which are readily soluble in organic solvents.

Solid-phase extraction using adsorption or ion exchange can also be used. For adsorption of the alkaloids in the free form, reversed-phase materials, such as chemically bonded C_8 and C_{18} on silica, are widely used. A suitable solvent system is a mixture of methanol and water; the crude extract is fractionated by stepwise elution of the adsorbent with a solvent of decreasing polarity. XAD-2 is also used for the concentration of alkaloids, e.g. from biological fluids. Various cation exchange materials can be used for the selective extraction of alkaloids.

For preparative purposes purifications based on the precipitation of alkaloids are employed. A crude extract of the alkaloids is made with aqueous acid; subsequently the alkaloids are precipitated with reagents such as Mayer's reagent (1 mol L^{-1} mercury chloride in 5% aqueous potassium iodide) or Reinecke's salt (5% ammonium reineckate in 30% acetic acid) at pH 2, or picric acid (saturated aqueous solution) at pH 5–6. After collection by filtration or centrifugation, the precipitate is dissolved in an organic solvent (acetone : methanol : water; 6 : 2 : 1). The complexing group is then removed by means of an anion exchanger. Quaternary alkaloids cannot be purified by means of liquid–liquid extraction, therefore precipitation is particularly suited for their purification.

Thin-layer Chromatography

Thin-layer chromatography (TLC) is widely used as a versatile method in the analysis of alkaloids. It offers the advantage of a broad range of polarities being separated in one single analysis, which is of interest in plant materials and metabolism studies. The most widely used stationary phase is silica; alumina plates are rarely employed nowadays. Reversed-phase materials, such as chemically bonded C_{18} on silica, are also applied but silica is still used most widely.

Strongly basic alkaloids will show severe tailing on silica gel plates, due to the acidic properties of silica. The use of mobile phases which contain

Table 2 Some common thin-layer chromatography systems for the analysis of alkaloids

Solvent system (*all with silica plates*)	*Commonly used ratios*	*Polarity range*
Cyclohexane–chloroform–diethylamine	5 : 4 : 1–(0) : 9 : 1	lp-mp
Chloroform–acetone–diethylamine	5 : 4 : 1	mp
Chloroform–methanol–ammonia	8 : 1 : 1	mp
Chloroform–methanol/ethanol	99 : 1–1 : 1	lp-mp, wb
Ethyl acetate–isopropanol–25% ammonia	100 : 2 : 1, 80 : 15 : 5, 45 : 35 : 5	lp-mp
Ethyl acetate–methanol	9 : 1–1 : 1	lp-mp, wb
Toluene–ethyl acetate–diethylamine	7 : 2 : 1	lp-mp
Toluene–acetone–ethanol–25% ammonia	20 : 20 : 3 : 1	mp
Dicholoromethane–diethyl ether–diethylamine	20 : 15 : 5	mp
Acetone–methanol–25% ammonia	40 : 10 : 2, 95 : (0) : 5	mp-p
Methanol–25% ammonia	95 : 5	lp-p
n-Butanol–acetic acid–water	4 : 1 : 1	lp-p
Methanol–1 mol L^{-1} aq. M NH_4NO_3–2 mol L^{-1} aq. ammonia	7 : 1 : 2	lp-p
Methanol–0.2 mol L^{-1} aq. M NH_4NO_3	3 : 2	lp-p

lp, Low polarity compounds; mp, medium polarity compounds; p, polar compounds; wb, weakly basic compounds.

a base such as ammonia or diethylamine will overcome this problem. A more elaborate method is the use of TLC plates impregnated with a basic solution.

For the analysis of highly polar quaternary alkaloids and *N*-oxides, solvent systems consisting of methanol and aqueous salt solutions are useful. In **Table 2** some widely used TLC systems are summarized. For the detection of alkaloids a large number of methods have been reported. Besides quenching ultraviolet (UV) light on fluorescent plates and fluorescence, general reagents for selectively detecting alkaloids are Dragendorff's reagent (orange-brown spots) and potassium iodoplatinate (brown-violet-purple spots; **Table 3**). Dragendorff's reagent may cause false-positive reactions with, for example, compounds containing conjugated carbonyl or lactone functions. The iodoplatinate reagent has less risk of false-positive reactions and is more selective due to a broader spectrum of colours observed for individual alkaloids.

Highly selective reagents have been reported for the visualization of various classes alkaloids (**Table 4**). These are based on different colorations under strongly oxidative conditions.

Liquid Chromatography

High performance liquid chromatography (HPLC) is a major tool for the analysis of alkaloids. Most separations are done on reversed-phase (RP) materials (C_8-, C_{18}- and phenyl-bonded phases on silica). Although extensive tailing due to the interaction of the basic nitrogen and residual acidic silanol groups may occur on the RP materials. In particular, strong bases show this problem. Several solutions have been found to circumvent this. First, special RP materials have been developed for basic compounds. These materials have an altered silica surface, a high load of the alkyl groups or they have undergone a rigorous endcapping treatment to reduce the number of free silanol groups. Often the plate numbers observed for alkaloids on an HPLC column are considerably lower than those measured with the usual neutral test compounds. Polymeric (e.g. polystyrene-based) stationary phases do not have the problem of residual silanol groups; however, plate numbers observed with such columns are not usually better than those found with specially made RP silica materials. Phenyl-type RP columns are also successful in the separation of alkaloids.

Another way of reducing the tailing is through modification of the eluent. By adding long chain alkylamines (e.g. hexylamine) in low concentrations

Table 3 Detection reagents for alkaloids on thin-layer chromatography plates

Dragendorff's reagent (*modification according to Munier*)
(A) 1.7% bismuth subnitrate in 20% aq. tartaric acid solution
(B) 40% potassium iodide in water
A and B are mixed (5 : 2) and the spray reagent is prepared by mixing 50 mL of the stock solution with 100 g tartaric acid and 500 mL water.
Colours observed after spraying: orange-brown spots for alkaloids

Potassium iodoplatinate reagent
The reagent is prepared freshly by mixing 3 mL of 10% aq. hexachloroplatinic acid solution with 97 mL water and 100 mL of 6% aqueous potassium iodide solution.
Colours observed after spraying: brown-violet-purple spots for alkaloids

Table 4 Selective colour reactions for the detection of alkaloids on thin-layer chromatography plates

Spray reagent	*Commonly used for the detection of*
0.2 mol L^{-1} ferric chloride in 35% perchloric acid and heat	Indole alkaloids, isoquinoline alkaloids
1% ceric sulfate in 10% sulfuric acid	Indole alkaloids
1% *p*-dimethylaminobenzaldehyde in ethanol, followed by exposure to hydrochloric acid vapour	Ergot alkaloids
Sulfuric acid and heat	Various alkaloids

(typically 1 mmol L^{-1}) to the mobile phase, tailing can be considerably reduced. Also, addition of amines like triethylamine or tetramethylammonium can be helpful in reducing tailing. Moreover, alkaloids have been analysed on aminopropyl- and cyanopropyl-type of columns, in both normal and reversed-phase modes.

In liquid chromatography of alkaloids, the pH of the mobile phase must be strictly controlled, as stationary phases are unstable at a pH above 8, usually a pH between 2 and 4 is used, i.e. the alkaloids are present in the protonated form. Ion suppression systems are quite common. Because of the preference for the lower pH range of the eluent, ion pairing is used with C_4–C_8 alkylsulfonates at a concentration of 25–100 mmol L^{-1} for the analysis of alkaloids. Increasing length of the alkyl chain causes longer retention.

Some general features of RP HPLC systems for the analysis of alkaloids are given in **Table 5**.

The number of applications of ion exchange chromatography for the separation of alkaloids is limited. In general, cation exchange columns will also affect the selectivity of the separation through nonionic interactions, e.g. through the stationary phase matrix. Usually an elevated temperature is used to improve peak shape.

A large number of liquid–solid separations on silica have been reported (**Table 6**). The systems applied are similar to those reported for TLC.

UV is most widely used for detection. Particularly for the groups of indole and isoquinoline alkaloids, strong and specific UV chromophores are found. These can greatly assist in identifying compounds, e.g. in using HPLC with diode array detection. The pH of the solvent as well as the solvent itself may have an effect on the UV spectra, e.g. causing shifts of maxima and minima. Some alkaloids can be detected by means of their fluorescence. Some type of alkaloids have poor UV absorption properties, e.g. tropane alkaloids, pyrrolizidine alkaloids and steroidal alkaloids require detection at lower wavelengths (200–220 nm). Electrochemical detection has been applied, enabling the selective attenuation of interfering compounds. Mass spectrometry is a major tool in the identification and structure elucidation of alkaloids. In combination with gas chromatography and liquid chromatography, it is very useful in the qualitative and quantitative analysis of complex mixtures of alkaloids. Solvent systems suited for liquid chromatography–mass spectrometry should only contain volatile compounds (e.g. ammonium acetate, ammonium formate).

Countercurrent Chromatography

The preparative isolation of alkaloids can be achieved by means of modern countercurrent chromatography. Because of the ionic nature of the alkaloid systems with a controlled pH are preferred for the separation. Improved efficiency can be obtained by using ion pair gradients, e.g. solvent two-phase systems consisting of chloroform–methanol–aqueous

Table 5 General outline of reversed-phase high performance liquid chromatography systems for the separation of alkaloids

Stationary phase	*Mobile phase*
C_8, C_{18} or phenyl-bonded phase with low percentage of free silanol groups	*Ion supression mode* Methanol (acetonitrile)–water containing *c.* 0.01–0.1 mol L^{-1} phosphate buffer, ammonium carbonate or sodium acetate (pH 4–7) *Ion pair mode* Methanol (acetonitrile)–water containing *c.* 25–100 mmol L^{-1} alkylsulfonate and 1% acid (e.g. acetic acid), pH 2–4

Table 6 General outlines of normal-phase high performance liquid chromatography systems for the separation of alkaloids

Stationary phase	*Mobile phase*		
Silica gel	Dichloromethane, Chloroform, Diethyl/isopropyl ether, Tetrahydrofuran, or Ethyl acetate	Methanol or Isopropanol	Ammonia, Diethylamine or Triethylamine (*c.* 1% of the mobile phase)

phosphate or citrate buffer, pH *c.* 4, containing perchlorate, acetate or chloride as the ion pairing agent. High loadability and different selectivity compared with column chromatography are important features of countercurrent chromatography.

See also: **III/Alkaloids:** Gas Chromatography; Thin Layer (Planar) Chromatography. **Natural Products:** High-Speed Countercurrent Chromatography.

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Thin-Layer (Planar) Chromatography

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Introduction

In 1938, Izmailow and Schraiber pioneered the thin-layer chromatography (TLC) method for the analysis of plant material containing alkaloids. The subject matter of their scientific research was an extract of a plant rich in tropane alkaloids. Later on, the method was developed by Bekesy, who applied it to the separation of ergot alkaloids. Since then, numerous papers exploring the detection, isolation and quantitative determination of alkaloids by TLC have been published. It has been stated that no other method has delivered so much information on alkaloids.

From the chemical point of view, alkaloids form a very diverse group of organic nitrogen compounds of a basic character (with the exception of some derivatives of purine and colchicine). They have tertiary or quaternary amino groups in their molecules and only a few contain secondary amino groups. Considering the fact that analytical problems connected with alkaloids are mostly concerned with their physicochemical properties, they are commonly divided according to the type of chemical structure into tropane, quinoline, indole, diterpene and others. Another useful classification is based on botanical groups (e.g. tobacco, lupine, ergot, strychnos, vinca and catharanthus alkaloids), and this is