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REVIEW

Purification of Alkaloids by Countercurrent Chromatography

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Abstract
Alkaloids are nitrogen-containing compounds of the secondary metabolism of plants and microorganisms, which can also be found in animals, insects, and marine organisms. Because of the presence of one or more nitrogen atoms in the molecule, these compounds can form salts in the presence of acids, which are soluble in water and not in organic solvents. This characteristic is useful for their extraction from the matrix source and is explored in liquid–liquid partitioning techniques like countercurrent chromatography. Solvent systems used in classic purifications of alkaloids by this separation technique consist of an organic solvent such as chloroform or dichloromethane and a water buffer where the pH varies along the purification. The addition of other organic solvents like methanol and other alcohols to the solvent system is discussed, as well as the use of less polar systems. Recently developed techniques in countercurrent separations, such as pH-zone-refining countercurrent chromatography, are also presented. This comprehensive review covers the early work on separation of alkaloids with the Craig and Post apparatus and the evolution in the use of modern equipment for the isolation and purification of this class of bioactive natural products.

Keywords Basic natural products · Centrifugal partition chromatography · High-speed countercurrent chromatography · pH adjustment · Quaternary alkaloids · Solvent system

Introduction

Alkaloids are nitrogen-containing compounds of the secondary metabolism found in many different organisms, mostly plants and microorganisms (Aniszewski 2015) with endophytic fungi being an alternative source for the biotechnological production of natural product–based drugs (Uzma et al. 2018). This class of compounds can also be found in insects (Rather et al. 2020), marine organisms (Kuramoto et al. 2004), and animals that sequester defensive chemicals, *e.g.*, amphibians, snakes, and likely birds (Saporito et al. 2012). The potential of alkaloids in veterinary, pharmacology, medicine, and agronomy (plant protection) have been extensively reviewed (Debnath et al. 2018; Adamski et al. 2020).

The classical definition of alkaloids describes these natural products as organic compounds derived from amino acids where nitrogen is part of a heterocyclic ring, thus excluding primary amines and amides, like ephedrine and colchicine, respectively. All true alkaloids have a bitter taste and are white solids with the exception of nicotine, which is a brown liquid. When the nitrogen atom is not part of a heterocyclic ring, the compounds are then classified as protoalkaloids (Aniszewski 2015). Some alkaloids can arise from amination reactions of acetate pathway intermediates, as in (+)-coniin, or the amination can occur in later stages as in terpenoid and steroidal alkaloids (Aniszewski 2015). When the nitrogen-containing compound (with or without a heterocyclic ring) is not originated by an amino acid, then it is classified as a pseudoalkaloid (Aniszewski 2015). The amino acid–derived alkaloids can be therefore classified according to their biosynthetic pathway, as derivatives of ornithine,

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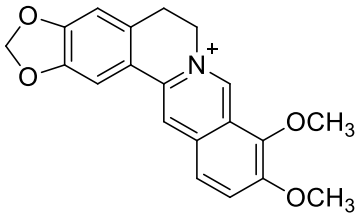
lysine, nicotinic acid, anthranilic acid, tyrosine, tryptophan, and histidine. As in most cases, the carbon skeleton of the precursor amino acid is maintained in the chemical structure of the alkaloid; another classification is possible, according to the typical heterocyclic ring, *e.g*., pyrrolidine, piperidine, pyridine, quinoline, isoquinoline, and indole type alkaloids. Other biosynthetic units can be incorporated along the biosynthetic pathways forming further important skeleton types like tropane, pyrrolizidine, quinolizidine, and indoloterpene alkaloids.

Alkaloids can occur as primary, secondary, or tertiary amines, bearing one or more nitrogen atoms in their molecule. Quaternary ammonium salts are also found, *e.g*., berberine (**1**) found in *Berberis* species, Berberidaceae, and commonly taken orally for diabetes (hyperglycemia), high levels of cholesterol or other lipids in the blood (hyperlipidemia), and high blood pressure (Imenshahidi and Hosseinzadeh 2019; Khashayar et al. 2021).



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The nitrogen atom in the alkaloids confers basicity to these compounds. In fact, the name alkaloid, *alkali* (ashes of plants) + *eidos* (type, appearance), means like an alkali and refers to the ability that these compounds have to form salts in the presence of acids. This ability has been used strategically for the selective acid–base extraction of these compounds from plant parts and/or plant extracts, as the salts formed by the addition of acids are soluble in water and polar protic solvents. This characteristic behavior has also been explored in many analytical techniques employed in the isolation and purification of alkaloids, especially in the liquid–liquid extractions (Verpoorte 2000).

Search Strategy

This review provides the progress achieved all around the world in the use of countercurrent chromatography as a technology for the isolation of alkaloids from plants. The search was carried out in different platforms such as Google Scholar, PubMed, ISI Web of Science, Elsevier, and Springer. Alkaloids, centrifugal partition chromatography, countercurrent distribution, Craig and Post apparatus, countercurrent, countercurrent chromatography, elution mode, high-speed countercurrent chromatography, pH adjustment, pH-zone-refining, and two-phase solvent system were selected as keywords. We revised the plant species with described bioactivity that have been chemically studied by the application of countercurrent chromatography for the isolation and purification of alkaloids. We also discuss the tasks and perspectives that challenge the application of this analytical technology.

General Principle and Advantages

Chromatographic methods have made it possible, in the last 70 years, the separation of complex mixtures of alkaloids, both on an analytical and preparative scale. Column and thin-layer chromatographies (Enyoh et al. 2019), based on adsorptive processes, use solid supports as a stationary phase, while that based on the partition between two liquid phases, the liquid stationary phase is adsorbed on a support. In both cases, the solute–solid interaction can cause irreversible adsorption and/or chemical modifications in the components of the mixtures subjected to separation.

Countercurrent chromatography (CCC) is a form of liquid–liquid partition chromatography in which the stationary liquid phase is retained inside the chromatographic column without the use of a porous or adsorptive matrix (Conway 1990). The solvent systems used in this technique are mixtures of at least two solvents, most generally three or four; even five solvents can be mixed to form a solvent system for CCC, to form a biphasic liquid system where one of the phases will act as stationary phase and the other as the mobile phase. The principle of separation involves the partitioning of a solute between these two immiscible liquid phases, and the distribution of the analyte into each of the two phases is determined by its distribution coefficient (*KD*) or partition coefficient (*K*) if only one solute form is involved (Bojczuk et al. 2017; Vetter et al. 2020).

One of the advantages of CCC is the fact that the technique is useful mainly in preparative separations, due to the nature of its stationary liquid phase that offers a large sample loading capacity. However, it is not restricted to this scale since samples can be separated in analytical and preparative levels, from milligrams to grams of material. More recently, the development of equipment on the kilogram scale provides fractionation on pilot (Kotland et al. 2016) and industrial plants (Kostanyan et al. 2020).

Other important advantages are the speed and efficiency of the method since purifications can last just a few hours, in addition to the good resolution in the separations with a high purity of fractions and the total sample recovery as there are no losses by adsorption. Thus, the recovery of biological activity in fractionations guided by biological tests

is secured (Conway 1990; Alvi et al. 2001). The technique is particularly useful for polar and labile substances, where chromatography with solid supports is completely inadvisable. However, compounds of medium to low polarity can also be separated, since aqueous and non-aqueous systems can be employed (Marston and Hosttetmann 1994).

The versatility of the technique is yet another important advantage that should be highlighted. If we consider the multitude of different solvent systems that can be used in CCC, as well as the different modes of operation of the equipment (elution modes), the possibilities are almost limitless. In this technique, any of the liquid phases can act as a mobile and/or stationary phase, and there is no need for pre-purification of the sample (Conway 1990). Strategies for using the equipment in a rational and targeted manner to obtain the best results in CCC separations have been reported and discussed, many of them related to the isolation and purification of economically or therapeutically important natural products (McAlpine et al. 2012; Friesen et al. 2015), such as volatile oils (de Souza et al. 2010; Leitão et al. 2020), antibiotic (Oka et al. 1998), anthocyanins and natural pigments (Schwarz et al. 2003; Winterhalter 2007), flavonoids (Costa and Leitão 2010), terpenoids (Skalicka-Wozniak and Garrard 2014), and saponins (Song et al. 2016), among others. Not less important in the present days of ecological awareness, it is the "green" compatibility of this technique, since most of the two-phase systems use water as one of the components of the phases, which are the most commonly employed. Faure et al. (2014) discussed how CCC is an inexpensive technique in relation to the consumption of solvents in chromatographic purifications.

Discussion

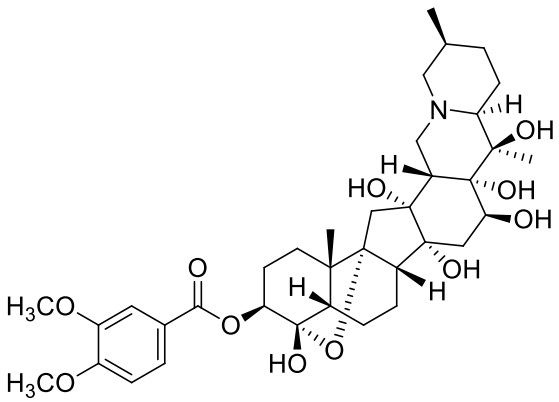
Liquid–Liquid Partition

Considering the basic nature of an alkaloid and its ability to form salts with acids, which are generally soluble in water, allied to the fact that the free base is soluble in organic solvents and not in water, the selective purification of these compounds is possible by liquid–liquid partitioning methods (Verpoorte 2000). The most part or solvent systems used in the classic purifications of alkaloids by liquid–liquid partitioning techniques consist of an organic solvent such as chloroform or dichloromethane and water where the pH varies along the purification. Some other organic solvents like methanol and other alcohols can also be added to the solvent system, as discussed below. A suitable method introduced by Ito in the mid-1990s, called pH-zone-refining countercurrent chromatography (Ito and Ma 1996; Weisz et al. 2007), has been largely used for the preparative purification of polar alkaloids and other ionizable compounds (Weisz et al.1994; Ito 2013).

The Early Days in Countercurrent Separations

The Craig and Post Apparatus

Countercurrent distribution is a solvent extraction partition method developed by Lyman C. Craig in 1943 initially devoted to the study of ergot alkaloids and veratrin, a mixture of steroidal alkaloids from the seeds of *Schoenocaulon officinale* (Schltdl. & Cham.) A.Gray, Melanthiaceae, primarily containing veratridine (**2**). With the onset of World War II, there was an urge to develop a method for the purification of atabrine, a new synthetic antiprotozoal and antirheumatic compound, from its metabolic derivatives in the urine and blood of experimentally treated dogs (Kresge et al. 2005). The commercial machine developed by Lyman Craig and Otto Post consisted of a series of connecting glass tubes (30, 50, 100, 200, or even 1000) where the upper phase of the biphasic solvent system passed through these tubes filled with the stationary lower phase, as a series of separation funnels sequentially connected (Figs. 1 and S1). The number of agitations in each passage as well as the decantation time can be adjusted according to the degree of emulsion generated by the solvent system. This machine was further used in biochemical investigations, especially on the purification of small peptides as insulin, as well as in the study of streptomycins, penicillins, fatty acids, purines, pyrimidines, and polypeptides of the gramicidin type (Craig and Post 1949).



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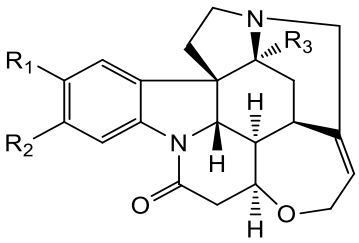
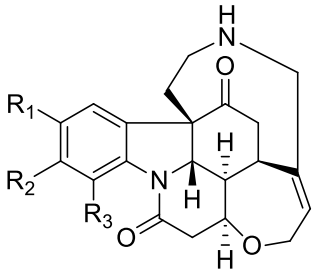
When discussing the purification of alkaloids by countercurrent distribution methods, it is noteworthy to mention the extensive work of Prof. Corrado Galeffi's group. On a paper from 1969, Galeffi and co-workers

proposed a pH gradient method for the purification of these compounds using the Craig and Post apparatus with a biphasic solvent system where the stationary phase was a dense organic solvent ($CHCl_3$) and the mobile phase was a buffer that changed its pH on a discontinuous mode (Galeffi et al. 1969). In liquid–liquid purifications involving pH gradients, two parameters can be modulated: the distribution coefficient, defined as K_r in that publication, and the dissociation constant of a base, K_b, or an acid (Leitão and Costa 2015). The separation depends on the product of these two factors ($K_r \times K_b$), and the method can be applied in two different ways: (1) the use of a buffer as stationary phase and an organic mobile phase which the composition is varied and (2) the use of a heavily dense organic phase, obtained by the use of chlorinated solvents, and a buffer solution as mobile phase, where the pH varies from neutral to acidic. In this early study, the authors investigated the function that controls the double distribution and dissociation equilibria of two weak alkaline indole alkaloids, strychnine (**3**) and brucine (**4**) found in the dried seed of *Strychnos nux-vomica* L., Loganiaceae. Both compounds cause excitation of all parts of the central nervous system as competitive antagonist at the inhibitory neurotransmitter glycine receptors (Lu et al. 2020).



3 $R_1=R_2=R_3=H$
4 $R_1=R_2=OCH_3$; $R_3=H$
5 $R_1=R_3=H$; $R_2=OCH_3$
6 $R_1=OCH_3$; $R_2=R_3=H$
7 $R_1=R_2=H$; $R_3=OH$
8 $R_1=R_2=OCH_3$; $R_3=OH$

By using the second strategy, the authors achieved the preparative purification of nine alkaloids from *S. nux-vomica*, the previously isolated compound **3**, along with a mixture of brucine (**4**) and α- and β-colubrines (**5** and **6**), pseudostrychnine (**7**), pseudobrucine (**8**), icajine (**9**), vomicine (**10**), novacine (**11**), and four unknown compounds, in a 200-stage (tubes) Craig and Post apparatus (Figs. 1 and S1). Chloroform was used as the stationary phase and a phosphate buffer with a pH gradient between 6.5 and 3.3 as the mobile phase, and 8000 transfers were needed for the complete elution and separation of the whole alkaloid mixture. By exploring differences in the distribution coefficient when the dissociation equilibrium of alkaloids was very similar, the authors achieved the purification of the mixture of brucine and α- and β-colubrines by changing the composition of the stationary organic phase to chloroform–ethyl acetate 13:7. The four unknown compounds were later identified as isostrychnine, 3-hydroxy-α-colubrine, 3-hydroxy-β-colubrine (Galeffi 1974), and 15-hydroxystrychnine (Galeffi et al 1979). The method was further modeled for acids and bases with the application of a statistical method for the separation by discontinuous changes of pH depending on whether the mobile phase was organic or aqueous (Galeffi 1974).



9 $R_1=R_2=R_3=H$
10 $R_1=R_2=H$; $R_3=OH$
11 $R_1=R_2=OCH_3$; $R_3=OH$

Galeffi and co-workers were able to purify several alkaloids by the application of this analytical strategy, many of them with undescribed structures from *Strychnos* species (Marini-Bettolo et al. 1972, 1980; Galeffi and Marini-Bettolo 1981; Martin et al. 1999; Rasoanaivo et al. 2001), as well as from different plant sources including 27 medicinal plants (Galeffi 1980). In addition, a new group of isoquinoline dimers, belonging to the pavine-benzyltetrahydroisoquinoline-type alkaloids and isolated from the stem barks of *Hazomalania voyronii* (Jum.) Capuron (*Hernandia voyronii*), Hernandiaceae, was also purified and their structure elucidation described. From a crude alkaloid mixture (6.3 g), the three new alkaloids, hervelines A–C (**12**–**14**), belonging to this new group of isoquinolines, were isolated, together with other alkaloids using chloroform as the stationary phase and Na_2HPO_4–citric acid buffer solution at discontinuously decreasing pH as the mobile phase (Rasoanaivo et al. 1995). Hervelines A (**12**) and B (**13**) were separated at pH 5.4, whereas herveline C (**14**) was purified at pH 4.0. Hervelines have demonstrated a moderate intrinsic in vitro antimalarial activity (IC_{50} 1.68–3.28 μM) but displayed different effects ranging from synergism for herveline B (**13**) and herveline C (**14**) to simple additive effect for herveline

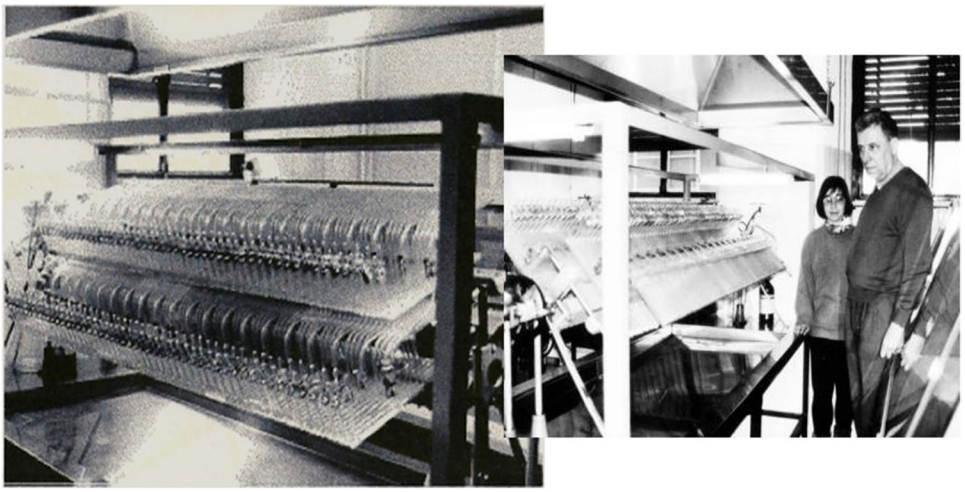
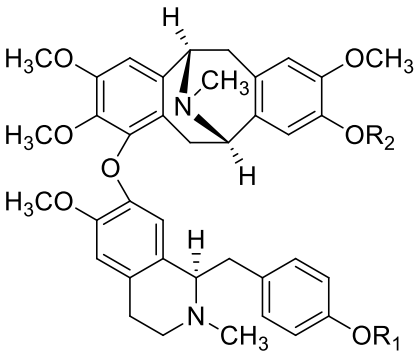


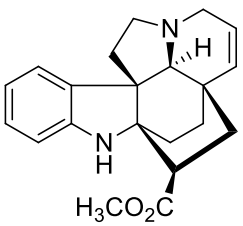
Fig. 1 The 200-tube Craig and Post apparatus at *Istituto Superiore di Sanità*, Rome, Italy. On the right, GG Leitão and C Galeffi (1990)

A (**12**) in a chloroquine combination evaluation, which was further confirmed in vivo for compounds **12** and **13** (Rasoanaivo et al. 1998).



12 R_1=H; R_2=CH_3
13 R_1=CH_3; R_2=H
14 R_1=R_2=CH_3

Indole alkaloids from *Vinca sardoa* (Stearn) Pign., Apocynaceae, were also isolated using a similar approach. By submitting an alkaloid mixture (2.6 g) to countercurrent distribution with dichloromethane as stationary phase and a phosphate–citric acid buffer at a discontinuously decreasing pH as mobile phase, seven indole alkaloids related to venalstonine (**15**) were obtained: at pH 5.4, *N*(1)-methyl-14,15-didehydro-12-hydroxyaspidofractinine ($K_r \times K_b = 1.5 \times 10^{-9}$, 56 mg) was eluted and then at pH 3.4, venalstonine ($K_r \times K_b = 1 \times 10^{-11}$, 87 mg), *N*(1)-methyl-14,15-didehydro-12-methoxyaspidofractinine ($K_r \times K_b = 6 \times 10^{-12}$, 215 mg), and *N*(1)-methyl-14,15- didehydroaspidofractinine ($K_r \times K_b = 4 \times 10^{-12}$, 26 mg). Finally, at pH 2.8, *N*(1)-formyl-14,15-didehydroaspidofractinine ($K_r \times K_b = 2 \times 10^{-12}$, 108 mg), conoflorine ($K_r \times K_b = 1 \times 10^{-12}$, 72 mg), and *N*(1)- formyl-14,15-didehydro-12-hydroxyaspidofractinine ($K_r \times K_b = 8 \times 10^{-13}$, 43 mg) were sequentially eluted (Nicoletti et al. 1998).



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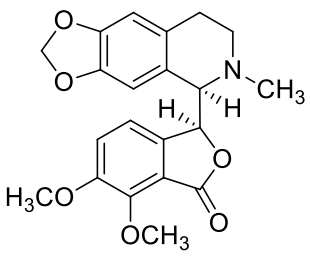
Other groups successively employed the Craig and Post apparatus for the isolation of alkaloids. Some examples follow. A made-up mixture of brucine (**4**) and hydrastine (**16**), an isoquinoline alkaloid used as a uterine hemostatic, antiseptic, and a potent competitive antagonist at mammalian $GABA_A$ receptors with a CD_{50} 0.16 mg kg^{-1}, *i.v.* (Huang and Johnston 1990), was successfully separated with a 25-tube Craig and Post apparatus by using benzene or petroleum ether as mobile phase and the Britton and Robinson universal pH buffer (Britton and Robinson 1931) as stationary phase in a gradient from basic to acid, where the pH changed by 0.3 pH units in each tube (Coch et al. 1965).



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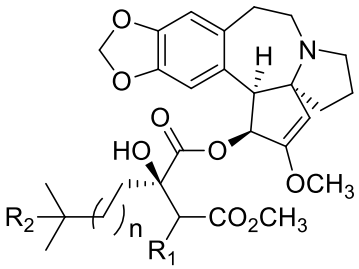
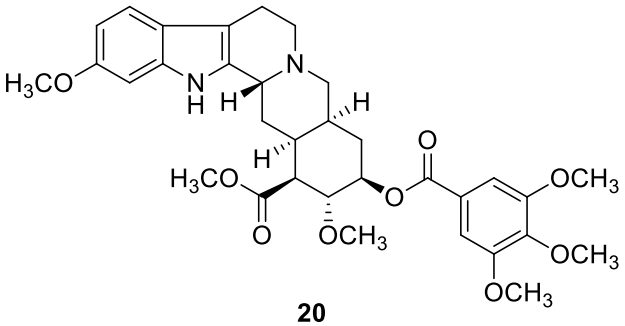
By alternating a 10-tube apparatus for preliminary fractionation of the crude alkaloidal mixture with chloroform and McIlvaine's buffer, column chromatography, and a 200-tube countercurrent distribution apparatus (Fig. 2), Powell and co-workers (1974) were able to obtain 36 g of three purified cephalotaxine esters (**17–19**), the bioactive antitumor alkaloids from *Cephalotaxus harringtonia* (Forbes) K. Koch var. *harringtonia* cv. *fastigiata*, Taxaceae, from 455 kg of plant material. Alkaloids from the roots of *Rauvolfia serpentina* (L.) Benth. ex Kurz, Apocynaceae, were also investigated with this technique (Kidd and Scott 1957; Banerjee et al. 1957); for example, reserpine (**20**), a pharmacologically active compound used in medicine for the treatment of high blood pressure, usually in combination with a thiazide diuretic or vasodilator, acts as a sympatholytic agent and antihypertensive medication by acting as an adrenergic uptake inhibitor (Weir 2020). This drug was quantified in *Rauvolfia* samples by countercurrent distribution: upon studying the distribution of **20** in several two-phase solvent systems, a mixture of ether-chloroform (3:1) and water buffer system was used to establish that a *K* of 1 was obtained with pH 3.1. By using a 24-transfer apparatus (a Gilson-Wright semi-automatic countercurrent apparatus), the authors were also able to estimate the amount of **20** in an alkaloid-rich fraction from *R. vomitoria* (Kidd and Scott 1957). Despite the utility of countercurrent distribution in the separation of many natural products and semi-synthetic compounds, this had the disadvantage of using fragile glass tubes and bulky instrumentation (Ito and Ma 1996). Nevertheless, the important feature of this machine in those pioneer investigations was the fact that the purifications were performed on a gram scale basis as exemplified with the studies described above.



17 R_1=H; R_2=OH; n=1
13 R_1=OH; R_2=H; n=1
14 R_1=H; R_2=OH; n=2



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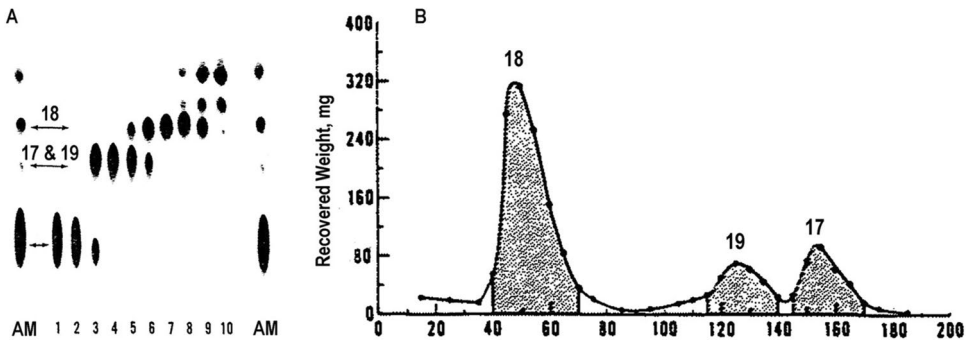


Fig. 2 **A** Thin-layer chromatography of *Cephalotaxus harringtonia* crude alkaloid mixture and fractions after a 10-tube countercurrent distribution of the mixture. AM: crude alkaloid mixture; 1–10 fractions obtained after countercurrent distribution of AM. The spot in fractions 1 and 2 represents the major alkaloid cephalotaxine. **B** The 200-tube countercurrent distribution of a 9.84 g mixture of antitumor esters of cephalotaxine. Solvent system: $CHCl_3$-McIlvaine's buffer (pH 5). Shaded areas represent collected fractions 5–7 combined to obtain alkaloids **17–19** of high purity in a preparative scale. Adapted from Powell et al. 1974

Droplet Countercurrent Chromatography

The appearance of instrumentation like the droplet countercurrent chromatograph (DCCC) in the 1970s, and then the centrifugal partition techniques like centrifugal partition chromatography (CPC) and high-speed countercurrent chromatography (HSCCC) in the 1980s, broadened the versatility of countercurrent chromatography. Now, either phase of the biphasic liquid system could be used as the mobile phase, while with the Craig and Post apparatus, only the upper phase of the system could act as the mobile one. Even with the limitations imposed by DCCC to the use of some solvent systems, *e.g*., the formation of suitable droplets (Hostettmann et al. 1984), this instrument was used in many natural product separations (Marston and Hosttettmann 1994), including alkaloids, as presented below.

Using a DCCC equipment, Verpoorte's group developed important studies on the use of pH gradients and ion-pair formation on the separation of alkaloids (Hermans-Lockkerbol and Verpoorte 1986; Van der Heijden et al. 1987). The separation of a made-up mixture of the alkaloids berberine (**1**), strychnine (**3**), brucine (**4**), quinine (**21**), and dihydroquinine in different pH gradients with the addition of counter-ions to several buffers was described (Hermans-Lockkerbol and Verpoorte 1986). Figure 3 illustrates the experimental settings (Exps. 1–7) with an aqueous mobile phase $CHCl_3$-MeOH-H_2O and various buffers (from neutral to acidic), 5% hydrochloric acid and 0.05 M phosphoric acid in mixtures (in a 5:5:3 ratio). Phosphoric acid in the aqueous phase (Fig. 3, Exp. 5) produced a faster elution of all tested alkaloids and even the tertiary alkaloids elute before the quaternary berberine. In HCl 5% (1.4 M), all alkaloids were well retained and satisfactory separated (Fig. 3, Exp. 7); this result allowed to conclude that this behavior was due to the formation of ion-pairs that would be soluble in the organic stationary phase. To test this hypothesis, the distribution of strychnine (**3**) was measured in the same $CHCl_3$-MeOH-H_2O (5:5:3) system in the presence of several counter-ions (Cl^-, Br^-, and $ClO4^-$, *inter alia*) and several buffers at different pH values. The results showed that the protonated alkaloid could form an ion-pair with the anions present in the buffer, affecting solubility and, consequently, the distribution of the compound in the two phases. In order to avoid decomposition of alkaloids during the course of this type of liquid–liquid separations, the pH will not have to be lower than 4, and, to improve the separations, an anion like perchlorate, chloride, or acetate should be added to the aqueous phase to form an ion-pair with the protonated alkaloid. This strategy was used to separate the alkaloids from suspension cultures of three *Tabernaemontana* species: *T. divaricate* (L.) R.Br. ex Roem. & Schult., *T. elegans* Stapf, and *T. pandacaqui* Lam., Apocynaceae (Van der Heijden et al. 1987). The use of thiocyanate as a counter-ion was also studied. Alkaloid mixtures were separated with the solvent system $CHCl_3$-MeOH-McIlvaine buffer (0.025 M citrate and 0.05 M phosphate) (5:5:3) at pH 4.2 (adjusted with phosphoric acid) in an ascending mode (aqueous phase as mobile) and addition of either perchlorate or thiocyanate counter-ions in a

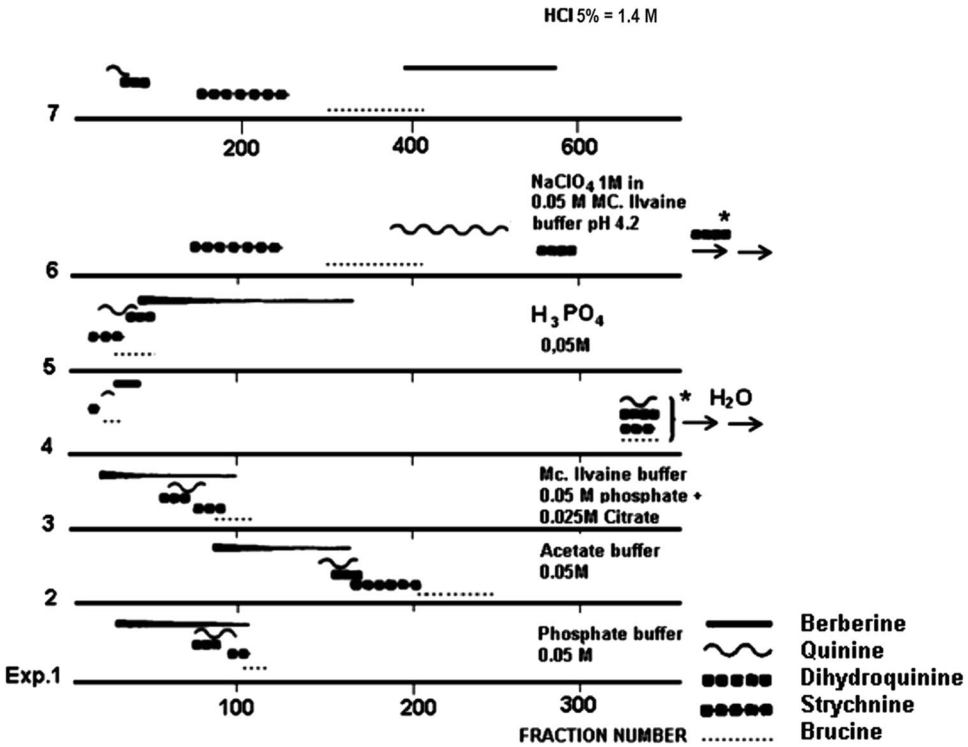
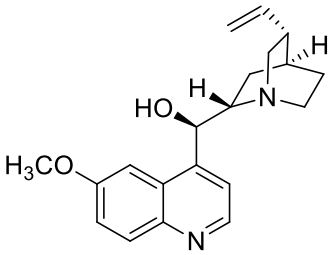


Fig. 3 DCCC separation of the mixture of berberine, quinine, dihydroquinine, strychnine, and brucine with CHCl3-MeOH-H2O (5:5:3), aqueous phase as mobile with various buffers, and with pH gradient elution. (*) Not eluted during the chromatographic run. Adapted from Hermanlokkerbol and Verpoorte 1986

gradient. Comparison of the results obtained with the two counter-ions allowed to conclude that perchlorate gave the best results in terms of selectivity.



21

Modern Countercurrent Chromatography

Modern countercurrent chromatography makes use of several instruments that operate in a centrifugal manner, with column movement being of two types — the column can rotate along a single axis of rotation, generating a hydrostatic equilibrium between the two immiscible liquid phases, also called centrifugal partition chromatography (CPC), or rotate around two axes, a central one and its own axis, often called the planetary axis, generating an elliptical motion and the hydrodynamic equilibrium between the two immiscible liquid phases, also called high-speed countercurrent chromatography (HSCCC) (Berthod et al. 2009; Vetter et al., 2020). The centrifugal fields generated upon rotation of the column in the two types of instruments are different: while this field is constant in hydrostatic columns, it is highly variable in hydrodynamic columns, forming zones of mixing and settling of the two liquid phases (Berthod et al. 2009). Most solvent systems are very well retained in hydrodynamic columns except for the aqueous two-phase solvent systems (ATPS), which are well retained though in CPC instruments.

The chromatographic columns in CPC machines are formed by channels interconnected by small ducts, engraved on a disk, which, when pilled, form chambers (Fig. S2). The most recent design of these channels is the the so-called twin-cells. The comparison of the design of these cells based of flow pattern and separation experiments has been investigated (Schwienheer et al. 2015). The stationary phase is held inside these chambers, while the mobile phase percolates through them (Berthod et al. 2009). Chromatographic columns in HSCCC are formed by a tubing coiled around a bobbin (Fig. S3). As above-mentioned, in modern CCC, the mobile phase can be either one of the solvent systems (upper or lower). Thus, in hydrostatic instruments, when the heavier (lower) phase is used as mobile phase, the instrument is operating in the descending mode (analogous to a DCCC instrument), whereas when the lighter (upper) phase is the mobile phase, the instrument is operating in the ascending mode. In hydrodynamic instruments, due to Archimedean forces, zones move to the high-pressure side of the coil, called the "head" (Berthod et al. 2009). The terminology used for operations in HSCCC machines is "head-to-tail" (H → T) when the denser phase is used as the mobile and "tail-to-head" (T → H) when the lighter phase is employed.

Solvent Systems

Different solvent systems have been used for the purification of alkaloids by either CPC or HSCCC. An overview of recent progress in solvent systems, additives, and modifiers of countercurrent chromatography was recently published (Liu et al. 2018). Fang and co-workers (2011) described a compilation of several solvent systems for the purification of alkaloids from plants by both HSCCC and pH-zone-refining CCC. This review compiles data on the separation of 94 alkaloids from more than 30 different sources by conventional HSCCC using 13 different solvent systems (Table S1). More than 67% of the alkaloids were purified with hexane–ethyl acetate–methanol-water (the so-called HEMWat system) and $CHCl_3$-MeOH-H_2O (Fang et al. 2011). In fact, these solvent systems represent two versatile families that can be used in countercurrent chromatography. The first, HEMWat covers a large range of polarities, while the second can be used for the separation of more polar compounds. The amount of MeOH in $CHCl_3$-MeOH-H_2O can easily modulate the polarity of the system.

The addition of acids to the aqueous phase of these solvent systems is a common feature in the purification of alkaloids, exploring their ability to form salts, thus favoring their extraction to the water-rich phase according to adjustments in the pH. A good example of this pH modulation according to the basicity of the alkaloid is shown in the work of Yuan and co-workers (Yuan et al. 2001), where $CHCl_3$-MeOH-H_2O (5:5:3) is used as a versatile solvent system for the pre-fractionation of the crude extracts of five Chinese traditional medicine herb: *cortex phellodendri* (the cortex of *Phellodendron chinense* Schneid and *P. amurense* Rupr), *semen strychnine* (seeds of *Strychnos nux-vomica* L.), green tea (leaves of *Camellia sinensis* (L.) Kuntze), *Sophora flavescens* Aiton, and *Datura metel* L. The aqueous phase in each fractionation had their acidity adjusted (with the addition of NaH_2PO_4 or HCl) to the optimal pH for the separation of alkaloids. In all experiments, the lower phase was the mobile phase.

The use of bases in the aqueous phase, instead of acids, for the purification of alkaloids has been reported (Shikanga et al. 2011; Atlabachew et al. 2016). In a paper by Wang and co-workers (2015), a series of solvent systems (ethyl acetate-butanol-water, HEMWat, and HEMWat with aqueous NH_3,

all tested in several ratios) were examined, and the *K* values for five diterpene alkaloids of *Aconitum duclouxii* were calculated. The authors reported that, in the first investigated solvent system, ethyl acetate-butanol-water (EBuWat 4:1:5; 4:1.5:5 and 4:2:5), most part of the target alkaloids remained in the upper phase (infinite *K* values). By changing to the HEMWat system (1:1:1:1; 1.5:0.5:1:0.5; and 1.5:0.2:1:0.2), the group obtained better results, but the *K* values still remained high. When aqueous NH_3 was introduced in the aqueous phase of the HEMWat 1:1:1:1:1 (in the ratios of 0.5; 0.2, and 0.1), the *K* values of the five alkaloids decreased and remained in the range 0.5–2.0. The preparative purification of 1 g of the alkaloidal extract with *n*-hexane–ethyl acetate–methanol-water-25% NH_3 (1:1:1:1:0.1, v/v) afforded five alkaloids in a single run within 4 h. The flow rate of the mobile phase, the temperature, and the revolution speed were also evaluated (Wang et al. 2015).

Liu et al. (2015) described the use of a three-phase solvent system for the comprehensive separation of a variety of compounds, among these a series of alkaloids, from *Dicranostigma leptopodum* (Maxim.) Fedde (Papaveraceae) by high-speed countercurrent chromatography. Three-phase solvent systems, as reported by the authors, are potentially used for the separation of compounds with a large range of polarity. Twelve solvent systems including hexane-M*t*BE-ACN-water and hexane-MeOAc-ACN-water in several different ratios were analyzed; five gave two phases instead of three and were discarded. The solvent system composed of hexane-M*t*BE-ACN-water (2:2:3:2) was chosen. Following, the conditions for the composition of the stationary phase and flow rate were optimized. In contrast to conventional HSCCC, the stationary phase in a three-phase solvent system is a binary combination of an intermediate phase (IP) and the lower phase (LP) of the system. The volume ratio of these two phases retained in the rotating column was carefully chosen since this experimental variable affects directly the HSCCC separation. The investigation of the flow rate showed that S_f values decreased with the increase of the flow rate, but the ratio IP/LP was poorly affected. However, with high flow rates (7 ml/min), the hydrodynamic equilibrium of the three phases was compromised, and the flow rate of 5 ml/min was established. The separation of compounds on the extract of *D. leptopodium* was performed by adding 0.5% triethylamine (TEA) in the system. In the first 25 min run, one steroidal compound (sitosterol) and two alkaloids, protopine and allocryptopine, eluted with the upper phase. Then the mobile phase was changed to the intermediate phase (IP), at a flow rate of 4 ml/min and seven compounds eluted, among them the alkaloids isocorydione, isocorydine, and coptisine (a quaternary alkaloid), along with other compounds. Finally, by switching to the lower phase as mobile, two further quaternary alkaloids, berberrubine and berberine (**1**), eluted along with compounds retained in the column. This study showed the ability of a three-phase solvent system to separate on the same run compounds from medium to high polarity, such as quaternary alkaloids.

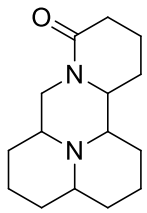
The use of ionic liquids as modifiers of solvent systems in both conventional and pH-zone-refining CCC separations was established for the effective purification of alkaloids from lotus (*Nelumbo nucifera* Gaertn., Nelumbonaceae) (Fang et al. 2017). Ionic liquids can shorten analytical times and affect distribution of compounds between the two phases, thus affecting resolution (Wu et al. 2018). Fang and co-workers (2017) investigated the conditions for the purification of six alkaloids from the whole plant extract of lotus, optimizing parameters such as solvent systems for pH-zone-refining CCC, concentrations of eluter and retainer, types and content of ionic liquids added, and the post-treatment of samples. The alkaloids *N*-nornuciferine, liensinine, nuciferine, isoliensinine, roemerine, and neferine were isolated with a purity higher than 90% with the solvent system hexane–ethyl acetate–methanol-water-[C4mim][PF6] at ratios of 5:2:2:8:0.1 with 10 mM TEA added at the organic stationary phase as retainer and 3 mM HCl as eluter in the aqueous mobile phase. The types and amount of added ionic liquids to several ratios of the solvent system petroleum ether-ethyl acetate–methanol-water for the separation by conventional HSCCC of four alkaloids, nuciferine, *N*-nornuciferine, pronuciferine, and roemerine, from 100 mg of the alkaloidal extract from leaves of lotus, were studied by Wu and co-workers (2018). The best results (purities > 90%) were achieved with petroleum ether-ethyl acetate–methanol-water-[C4mim][BF4] (1:5:1:5:0.15).

Purification Strategies

Purification strategies in countercurrent chromatography involve choosing both the correct solvent system, which is crucial for a successful fractionation, and the elution/operation mode, which can shorten elution times and efficiency of the process (Huang et al. 2016). Some basic requirements such as settling times, partition coefficient (*K*), and separation factor (α) of target compounds, among others, (Marston and Hostettmann 1994) should be considered. According to Ito (2005), 90% of the time spent on a separation process by countercurrent chromatography corresponds to the solvent system selection. Elution can be performed in the normal mode, when the mobile phase is less polar than the stationary phase, or in the reversed-phase mode, where the stationary phase is less polar than the mobile phase.

The mobile phase in CCC can elute in either isocratic or gradient modes. In the isocratic elutions, which are more common in CCC, the composition of the mobile phase is kept unchanged during the whole process. In gradient elution, the composition of the mobile phase is changed along the separation process to enhance its elution strength, while

the stationary phase is kept constant. Performing gradients in CCC, however, is not an easy task as changes in the composition of the mobile phase will, necessarily, alter the composition of the stationary phase. In this case, a suitable solvent system should be chosen so that ideally, the stationary phase undergoes minimum changes on its composition, while the mobile phase could experience notable changes (Wu et al. 2012). Gradients in CCC can be linear or step gradient and are performed in different ways: (a) by increasing the mobile phase strength by changing/modifying/adding a solvent; (b) by modifying the flow rate (the flow rate is increased during the run); (c) by performing a pH gradient, where the pH of the mobile phase is increased/decreased to elute compounds based on their acid/basic characteristics; and (d) by salting out gradients, where the stationary phase is saturated with a salt solution (Leitão and Costa 2015). Changes in the composition of the mobile phase are the most efficient method for enhancing separation among the eluted compounds which cannot be separated through conventional isocratic solvent system (Zou et al. 2021). Matrine (**22**), *N*-oxymatrine, *N*-formyl cytisine (**23**), and *N*-acetyl cytisine (**24**) were isolated with a purity higher than 93% by HSCCC using stepwise elution from the stem of *Euchresta tubulosa* Dunn., Fabaceae. A liquid–liquid extraction (methylene chloride-methanol–water, 5:1:4, *v*/*v*) was used for the preliminary enrichment. The final resolution was achieved with carbon tetrachloride-methylene chloride-methanol–water (2:3:3:2, v/v) and methylene chloride-methanol–water (5:3:2, v/v) for the HSCCC using stepwise elution (Li et al. 2019).



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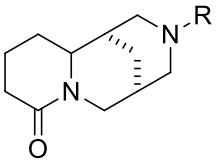
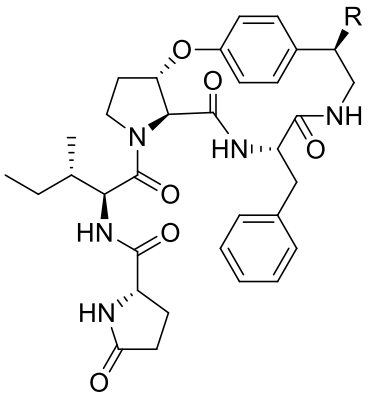


23 R=CHO
24 R=$COCH_3$

Different operation modes have been established for the effective isolation of compounds from complex mixtures of natural products highly retained in the stationary phase (high K values, > 5), *e.g.*, elusion-extrusion and back-extrusion, dual mode, multi-dual CCC, recycling mode, co-current mode, gradient mode, two-dimensional CCC, and pH-zone-refining CCC (Fig. 4). Recent overviews of solvent systems and elution modes in CCC have been published (Huang et al. 2016; Khan and Liu 2018).

Elusion-extrusion is performed by pumping the stationary phase instead of the mobile one through the coil once compounds with suitable retention times are eluted (Lu et al. 2008; Sun et al. 2020). Thus, compounds still in the CCC system can be extruded using the liquid nature of the stationary phase to extend the hydrophobicity window (Berthod et al. 2003). Recently, the high-performance countercurrent chromatography (HPCCC) separation of a cyclopeptide fraction from *Opuntia dillenii* (Ker Gawl.) Haw, Cactaceae, was carried out with the solvent system EtOAc-*i*-PrOH-H_2O 25:1:25 on a reversed-phase mode (Surup et al. 2021) to isolate the novel cyclopeptides opuntisine A (**25**) and B (**26**). A variety of biological activities has been described for this class of cyclopeptides, which include antibacterial, antifungal, anti-plasmodial activities, as well as central nervous system (CNS) effects as sedative and anti-depressant effects (Tuenter et al. 2017a,b).



25 R=OH
26 R=H

In the dual mode, the role of the mobile and stationary phases as well as the flow direction is reversed during the separation process by using a switching valve between normal-phase and reversed-phase modes. Both normal-phase mode (usually upper nonpolar phase pumped as the mobile phase) and reversed-phase mode (usually lower polar phase pumped as the mobile phase) are used followed by a switch between these two at any point during the operation. This mode has been applied in bioassay-guided isolation of bioactive compounds from crude extracts (Alvi 2001). The multiple dual mode elution, also referred as intermittent dual CCC, is a modification of dual mode elution involving a succession of sequential dual mode phases. This mode of elution enhances the resolution and efficiently separates analytes with similar partition coefficients. Such higher separation efficiency is achieved through successive switching between the normal and reverse elution which leads to an artificial increase of coil length and increases the number of liquid–liquid partitions (Delannay et al. 2006). In the recycling mode, the detector's outlet is simply connected,

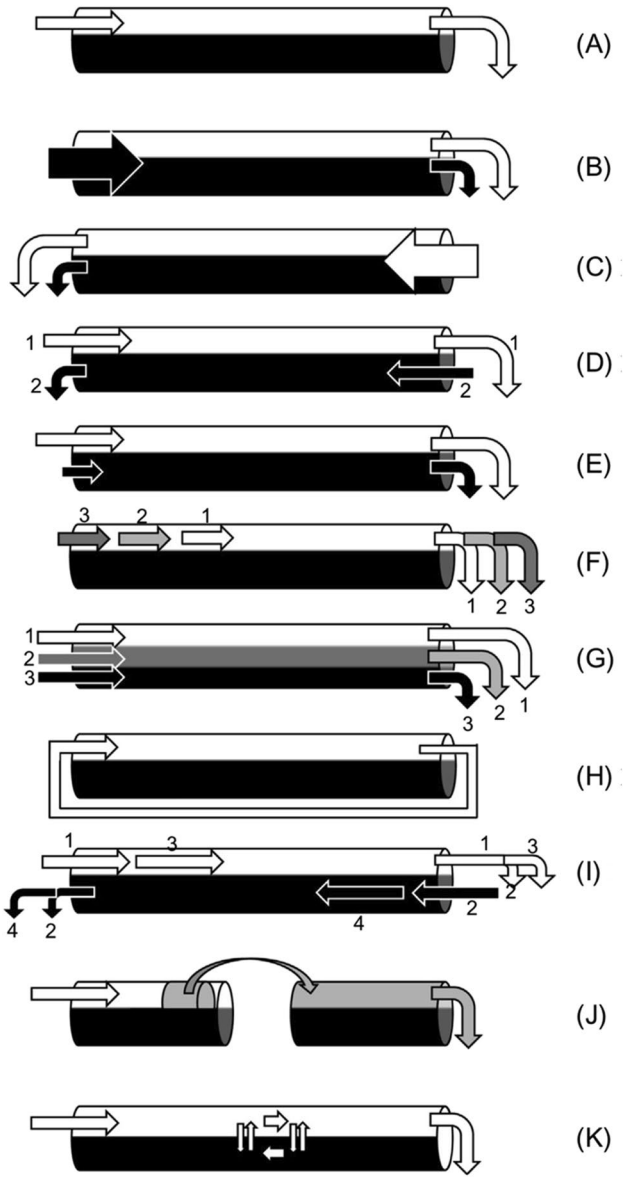
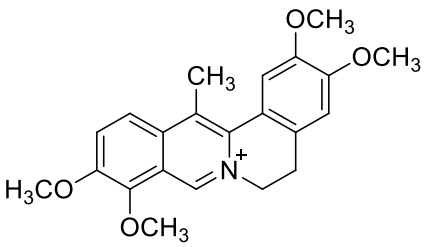


Fig. 4 Different elution modes of CCC. Numbers refer to different steps, and arrows (and their color) denote which phase is moved. **A** Classic elution; **B** elusion-extrusion; **C** back-extrusion; **D** dual mode; **E** co-current mode; **F** gradient mode; **G** three-phase system; **H** recycling mode; **I** multiple dual mode; **J** two-dimensional CCC; **K** pH-zone-refining. Reproduced from Vetter et al. 2020

through a pipe and regulator, to the mobile phase pump's inlet for generating the recycling mode of elution. Recycling is a simple and efficient strategy for improving the peak resolution of natural products (Du et al. 1998) and enantiomers by using chiral solvent systems (Tong 2020). Multi-channel recycling CCC systems require the combination of two CCC centrifuges to allow the application of two-dimensional heart-cutting where a freely selectable distinct part of the chromatogram (first dimension) is transferred to the second dimension with the goal to separate (partly) co-eluting compounds (Meng et al. 2014). Lin and co-workers (2020) developed a strategy for the precise separation and isolation of lysine-specific demethylase 1 inhibitors from the rhizomes of *Corydalis yanhusuo* W.T.Wang ex Z.Y.Su & C.Y.Wu, Papaveraceae, by using multi-mode CCC guided by liquid chromatography coupled to mass spectrometry/mass spectrometry (LC–MS/MS). This enzyme is overexpressed in many tumors and, according to the authors, has been used in antitumor therapy to regulate cell growth. The authors first virtually screened representative alkaloids from this plant by molecular docking with lysine-specific demethylase 1 to select active skeleton types. Then, the purification of the crude alkaloid extract by pH-zone-refining CCC and analysis of fractions by LC–MS/MS was simultaneously performed, showing three potential active fractions, which were further purified by online-storage and recycling CCC separation (Lin et al. 2020). Finally, three high-purity target quaternary alkaloids, dehydrocorydaline (**27**), coptisine, and columbamine, were successfully isolated as a new class of potential natural LSD1 inhibitors.



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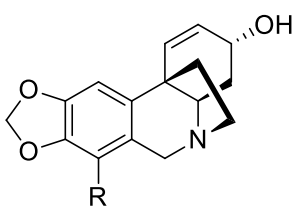
In co-current elution, both the mobile and stationary phases move inside the column in the same direction but at different flow rate to accelerate the elution of compounds with long elution volumes. This procedure constitutes a form of continuous CCC (Sutherland et al. 1984). Thus, the total distance covered by the analytes becomes way too shorter than the length of the column. Separation speed, hence, is increased, while solvent consumption and band-broadening are reduced (Berthod 2006).

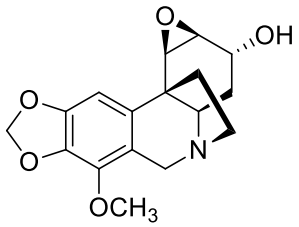
pH-Zone-Refining

pH-zone-refining CCC (PZRCCC) is a special large-scale, preparative technique for the purification of ionizable compounds such as weak acids and bases, according to their *pKa* or pK_b values and hydrophobicities (Ito and Ma 1996; Ito 2013). In this technique, the chromatograms resemble those of displacement chromatography, where compounds elute as rectangular peaks (Ma et al. 1994) and the impurities form sharp peaks at the vicinity of these peaks (Ito 1996). A retainer is used to keep compounds in the stationary phase, while an eluter is added to the mobile phase, to displace the

desired compounds. Depending on the polarity of the mobile phase, separations can be performed by either of the following modes: in the normal displacement, the retainer is used to keep the analyte in the aqueous stationary phase, while the eluter (or displacer) transfers the analytes into the organic mobile phase; whereas in the reverse displacement, the eluter, now in the aqueous mobile phase, elutes the analytes retained in the organic stationary phase (Ma et al. 1994). By means of both elution modes, Ma and co-workers (1994) isolated three Amaryllidaceae alkaloids, crinine (**28**), powelline (**29**), and crinamidine (**30**), from the crude alkaloidal extract (3 g) of *Crinum moorei* Hook.f., using TEA and HCl as retainer/eluter. An efficient strategy based on acid or base modified liquid–liquid extraction and pH-zone-refining CCC was recently described for selective enrichment, separation, and purification of alkaloids and organic acids from natural products (Yu et al. 2020).



28 R=H
29 R=OCH_3



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When the organic phase is used as mobile, alkaloids elute as free bases; therefore, when dealing with labile alkaloids, it is preferred to perform reverse displacement mode, where the alkaloids elute as salts, being therefore, more stable (Ito and Ma 1996). In fact, most of the papers reporting the separation of alkaloids by PZRCCC describe the use of reverse displacement mode (Table S1), except for the purification of some quaternary alkaloids where TEA is used as eluter in the organic mobile phase (Table S3). When working with this special CCC technique, the choice of the appropriate solvent system involves optimization of additional parameters other than a suitable *K* for target compounds in a solvent system free of acid or base, which are the concentration of both retainer and eluter in the stationary and mobile phases, respectively. Usually, equimolar concentrations of eluter and retainer are used, and this parameter can influence the retention of stationary phase as well as the concentration and retention time of eluting analytes (Zou et al. 2018). Ito (2005), in his review article on golden rules for selecting optimal conditions for HSCCC, describes a step-by-step procedure for the selection of a proper solvent system for performing pH-zone-refining CCC of either an acidic or a basic analyte. A list of solvent systems based on different ratios of two to four-solvent composition was suggested (Table 1), basically hexane–ethyl acetate–methanol-water (less polar) and methyl-*t*-butyl-ether (M*t*BE)-BuOH-acetonitrile–water (starting from M*t*BE–water). The concentration of the analytes is measured in basic and acidic media. In the case of acid analytes, if K_{acid} is $>>1$ and $K_{base\ is} << 1$, then the solvent system is suitable for the separation. For a basic analyte such as an alkaloid, K_{acid} should be $<<1$ and $K_{base} >> 1$. If those results are not achieved, the suggestion is to move to a more polar solvent system. Most separations by pH-zone-refining CCC are performed using M*t*BE-acetonitrile–water and HEMWat as solvent systems (Table 1 and S1). For example, an isolation protocol using this approach provided six akuamma alkaloids from *Picralima nitida* (Stapf) T.Durand & H.Durand (Apocynaceae), in high purity and quantities for an extensive in vitro investigation as kappa opioid receptors (Creed et al. 2021). A good discussion on solvent systems used for pH-zone-refining separation of alkaloids can be found in the review of Fang and co-workers (2011) where, among other findings, the authors report the increment in the sample loading capacity by using HEMWat, as the solubility of many alkaloids is improved by this solvent system family. The method formerly described by Ito involves seven steps, while Fang and co-workers (2020) developed a faster screening method using a crude alkaloidal extract from the Chinese medicinal plant *Gelsemium elegans* (Gardner & Chapm.) Benth., Gelsemiaceae, as an example, where the partition coefficient $K_{base} >> 1$ was replaced by the value $K > 1$ in neutral conditions. Several solvent compositions of hexane–ethyl acetate–methanol-water and M*t*BE-acetonitrile–water were tested in a simplified manner.

One of the most important advantages of pH-zone-refining CCC is the increased sample loading capacity that can be > 10 times higher than in classic CCC separations (Weisz et al. 1994; Fang et al. 2013). Fang and co-workers (2011) nicely illustrated this tendency for enlarged sample loading capacity with the purification of *Nelumbo* alkaloids. By comparing the separation results, the authors report that only 120 mg crude extract were successfully separated by conventional HSCCC with the optimized solvent system, whereas 4 g could be purified by PZRCCC. One example of industrial-scale purification of alkaloids with this technique is the case study of Kotland and co-workers (2016) on the purification of the pharmaceutical ingredients (anticancer agents) catharanthine (**31**) and vindoline (**32**) from a crude extract of the aerial parts of *Catharanthus roseus* G. Don, Apocynaceae. These two monomers are components of two natural dimers, vinblastine and vincristine, and two hemisynthetic ones, vinorelbine and vinflunine, which are used in anticancer chemotherapy, as microtubule-targeting agents (Martino et al. 2018). The purification of alkaloids **31** and **32** from an industrial scale was studied by centrifugal partition chromatography with a biphasic solvent system of toluene–acetonitrile-water 4:1:5 (v/v). The scale-up

Table 1 Ito's suggested solvent systems for pH-zone-refining countercurrent chromatography

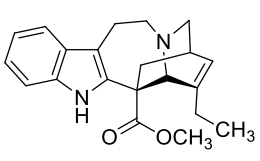
Solvent 1	Hexane/EtOAc/MeOH/H_2O	
Solvent 2	10:0:5:5	Hydrophobic
	9:1:5:5	
	8:2:5:5	
	7:3:5:5	
	6:4:5:5	
	5:5:5:5	
	M*t*BE/*n*-BuOH/ACN/H_2O	
	1:0:0:1	Hydrophilic
	4:0:1:5	
	6:0:3:8	
	2:0:2:3	
	4:2:3:8	
	2:2:1:5	

Reproduced from Ito and Ma (1996)

Abbreviations: *CAN*, acetonitrile; *BuOH*, butanol; *EtOAc*, ethyl acetate; *MeOH*, methanol;

MtBE, methyl-*tert*-butyl-ether

methodology was optimized in terms of column capacity and mass transfer efficiency. The optimized parameters included concentration of retainer and eluter (TEA in the organic stationary phase and sulfuric acid in the aqueous mobile phase), flow rate, and rotation speed. The study was first carried out in a 36 ml column with 832 partition twin-cells with the aim of maximizing the recovery of target compounds and productivity. The experiments were then scaled up to two larger columns of 305 ml and 1950 ml, equipped with 231 and 238 partition cells. The efficiency of the column design and the retention of stationary phase (column capacity) were important invariants for the scale-up study. The results further revealed that the validated methodology could afford the injection of up to 150 g of crude *Catharanthus* extract on the 1950 ml column, highlighting the possibility of reaching a productivity of about 4 kg extract processed per day. Recoveries for both catharanthine and vindoline were 78% and 91%, respectively. Although the concentration of this alkaloids is low in the plant, 60.2–329.9 and 114.8–659.7 µg/g for **31** and **32**, respectively (Koel et al. 2020), the productivity calculated for catharanthine + vindoline in this scale-up study reaches a maximum of 225 mmol/l/h (Kotland et al. 2016).



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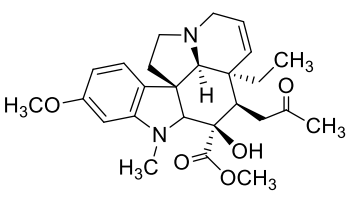

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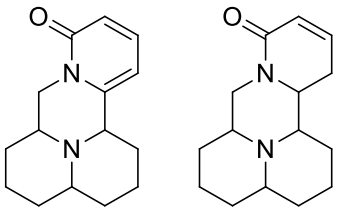
pH-zone-refining CCC has been successfully and extensively applied for the isolation of many structurally diverse types of alkaloids, as well as in the purification of other classes of natural products such as phenolics, cannabinoids, terpenoids (including ginkgolic acids from *Ginkgo biloba*), fatty acids, and natural and synthetic pigments (Table S1). This technique has also been applied for the purification of amino acid derivatives, peptides, dyes, and enantiomers by using chiral selectors (Ito and Ma 1996). Chiral selectors based on cellulose and amylose arylcarbamates have been developed for chiral separations in CCC (Pérez and Minguillón 2006; Pérez et al. 2006). Cinchona-derived alkaloids have been developed as ion-exchange-type chiral selectors for the separation of enantiomeric *N*-derivatized amino acids and 2-aryloxypropionic acids by countercurrent chromatography (Franco et al. 2002). Due to its importance in the field of alkaloid purification, the application of pH-zone-refining countercurrent chromatography will be exemplified with selected examples below.

Applications: Selected Examples

Since the review of Fang and co-workers (2011), 17 papers describing the isolation of alkaloids by conventional HSCCC have been published (Table S2). Some publications report the use of combined pH-zone-refining CCC and conventional HSCCC (Zhenjia et al. 2010; Dong et al. 2011; Zhang et al. 2014; Li et al. 2015; Sun et al. 2015b; Zuo et al. 2021), as well as hyphenation of CCC with other techniques (Dai et al. 2012; Yuan et al. 2013), mainly with preparative HPLC (Sun et al. 2015a; Han et al. 2017; Tang et al. 2017; Zhang et al. 2016; Zhou et al. 2019). Combination of conventional silica gel column chromatography (CC) and HSCCC was also reported (Shikanga et al. 2011; Guimarães et al. 2013).

When working with the isolation of alkaloids from plant sources, it is usual to perform a sample pre-treatment with the aim of obtaining an alkaloid-rich extract for further purification (Yang et al. 2013). Several classic acid–base alkaloid extraction protocols have been extensively described in pharmacognosy books and review articles (Adejoke et al. 2019; Jian et al., 2019). According to Fang and co-workers (2011), the concentration of target compounds in a sample to be submitted to PZRCCC should not be lower than 1 mM; otherwise, the resolution of peaks will be very poor. Yang and co-workers (2013) developed a sample pre-treatment strategy for PZRCCC based on a further acid–base extraction with the aim of increasing the target compound concentration. The authors used the crude alkaloidal extract from seeds of *Sophora alopecuroides* L., Fabaceae, first obtained by acid–base extraction (the first basic extract), as an example to illustrate and develop the method which consisted of partitioning 20 g

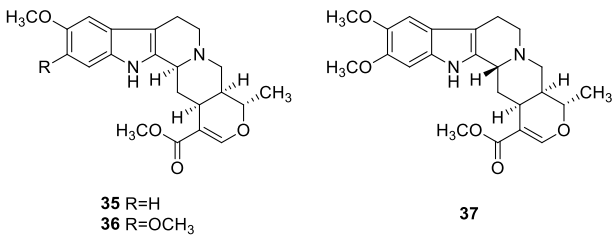
of this first basic crude extract on the optimized solvent system M*t*BE-CH_3CN-water (2:2:3, v/v) where the upper organic phase was basified to pH 10 with TEA. Thus, the second basic alkaloid-rich extract was further purified by PZRCCC with the same solvent system (with TEA as retainer and HCl as eluter in the aqueous phase) affording 169 mg of sophoramin (**33**) and 696 mg of sophocarpin (**34**). This strategy doubled the yield of the target compounds when compared to the results obtained with the un-treated sample (crude alkaloidal extract).



33 **34**

A similar strategy was adopted by Feng and co-workers (2015) for the target-oriented separation of minor indole alkaloids from the medicinal plant *Uncaria rhynchophylla* (Miq.) Miq. ex Havil, Rubiaceae. The two-phase solvent system was first optimized, and then, following the flow chart illustrated in Fig. 5, the crude alkaloid extract was dissolved in the basified upper phase and extracted twice with the basified lower phase to remove polar impurities. Then, the upper phase was extracted twice with the acidified lower phase to obtain the enriched alkaloid fraction and to remove nonpolar impurities which remained in the upper phase. In the sequence, a desalinization step followed where the lower aqueous phase was basified to pH 10 and evaporated and redissolved in water, followed by extraction with ethyl acetate, rendering the enriched alkaloid extract ready for pH-zone-refining purification.

When performing PZRCCC, usually bases such as TEA are added to the organic phase as retainer, while hydrochloric acid is used as eluter in the aqueous phase. Vieira and co-workers (2013) have investigated the use of organic acids such as formic acid in several concentrations for the separation of indole alkaloids from the dichloromethane extract from the bark of *Aspidosperma rigidum* Rusby, Apocynaceae. By using the solvent system methyl-*t*-butyl-ether and water with different concentrations of TEA as retainer in the organic stationary phase and formic or hydrochloric acids as eluter in the aqueous phase (Table 2), three major alkaloids, 3α-aricine (**35**), isoreserpiline (**36**), and 3β-reserpiline (**37**), were isolated. 3α-Aricine is eluted as a pure compound in the experiments performed with (10–15 mM) formic acid as eluter, while with HCl, only 3β-reserpiline is eluted as a pure compound (Fig. 6). By enhancing the concentration of formic acid from 10 to 15 mM, alkaloids **35** and **36** were obtained as pure compounds. Sulfuric acid instead was used by Kotland and co-workers (Kotland et al. 2016) as an eluter acid for the purification of indole alkaloids from *Catharanthus roseus*.



35 R=H
36 R=OCH_3

37

A novel solvent system for PZRCCC of polar alkaloids was developed by Zou and co-workers (Zou et al. 2018) using the indole alkaloids from toad venom as an example. According to the authors, the high polarity of some alkaloids makes it difficult to separate by PZRCCC with classical solvent systems on a large-scale basis. The study describes a hydrophilic organic/salt-containing two-phase solvent system composed of acetonitrile, sodium chloride, and water. According to the authors, the developed solvent system provided a wider range of polarity for polar components than classical aqueous two-phase system (ATPS), without the effect of free hydrogen ion (which would affect the formation of sharp retainer border), strong ionic strength, or high viscosity that these ATPS solvent systems display, thus rendering it particularly suitable for PZRCCC of polar alkaloids. The study also describes the phase diagram for this new solvent system, showing the mass binodal curve and tie lines, as well as the areas where the system forms one-phase, two-phase (useful for CCC), and a three-phase area, the latter originated by precipitation of the salt. The indole alkaloid-enriched extract was obtained by extracting 1 kg powdered toad venom with 50% ethanol in an ultrasound-assisted process and concentration on a microporous resin. By using the strategy of solvent system selection for PZRCCC described by Ito and after testing eight different solvent compositions, the authors chose the optimized acetonitrile–sodium chloride–water (54:5:43, w%) that gave suitable K_{acid}, K_{base}, and settling times. The following step was the establishment of concentrations of retainer TEA in organic stationary phase and eluter HCl in the aqueous mobile phase. Figure 7 shows

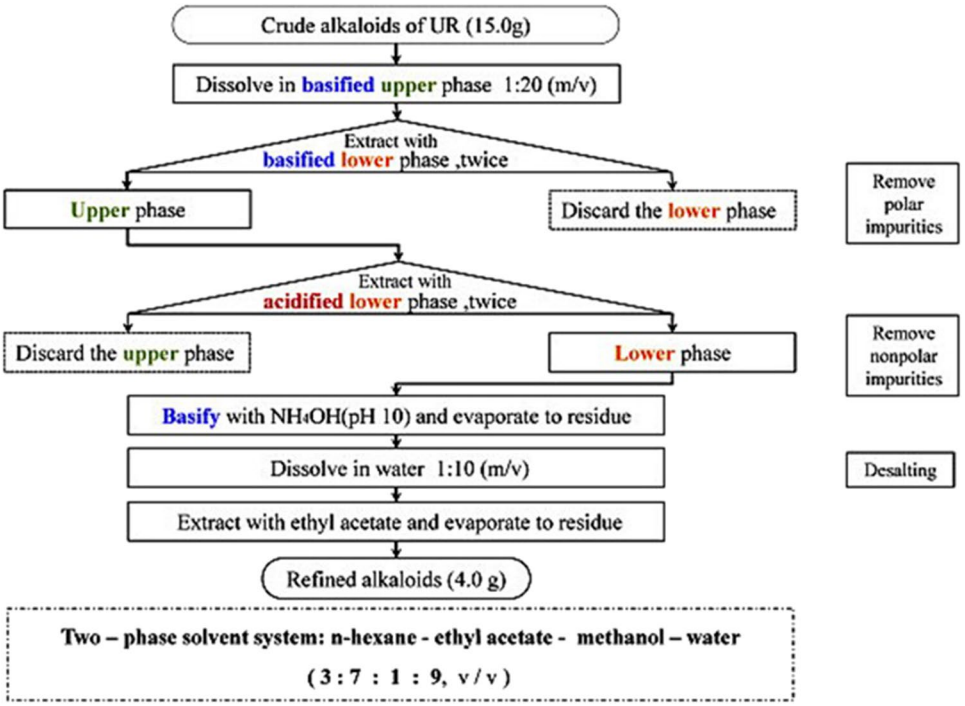
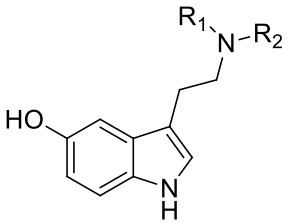


Fig. 5 Flow chart of enrichment steps of refined alkaloids by the two-phase solvent system. Reproduced from Feng et al. 2015

Table 2 pH-zone-refining experiments for determining the concentrations of retainer triethylamine (TEA) and eluter acids for the purification of indole alkaloids **32**–**34** from *Aspidosperma rigidum* Rusby dichloromethane extract (adapted from Vieira et al. 2013)

Experiment	Retainer TES (mM)	Eluter acid concentration (mM)
A	15	10 formic acid
B	10	10 formic acid
C	10	15 formic acid
D	5	5 HCl



38 R_1=R_2=H
39 R_1=H; R_2=CH_3
40 R_1=R_2=CH_3

the results for the purification of 5-hydroxy-tryptamine (**38**, serotonin), 5-hydroxy-*N*'-methyl-tryptamine (**39**), and 5-hydroxy-*N*', *N*'-dimethyl-tryptamine (**40**, bufotenine). When equal amounts of TEA and HCl were used (10 mM), only bufotenine (**40**) eluted as a pure fraction and as a mixture eluted serotonin (**38**) and 5-hydroxy-*N*'-methyl-tryptamine (**39**) eluted (Fig. 7A). When the concentration of HCl was adjusted to 15 mM, the three alkaloids eluted as pure compounds (Fig. 7B). Interestingly, serotonin (**38**) is widely found in Mediterranean foodstuffs, fruits, vegetables, and medicinal herbs and attenuates, or even prevents, stress oxidative-related disorders, such as diabetes, inflammatory, and neurological pathologies (Gonçalves et al. 2021).

Quaternary Alkaloids

Most of alkaloid purifications by pH-zone-refining CCC deal with free bases of several skeleton types. According to the review of Fang and co-workers (2011), 94% of the alkaloids purified by PZRCCC are tertiary amines, and 6% are secondary. However, some remarkably interesting examples report the isolation of quaternary alkaloids by this technique. Interestingly, most part of these examples use $CHCl_3$-MeOH-H_2O as solvent system instead of those proposed by Ito for PZRCCC (Tables 1 and S3), supposedly

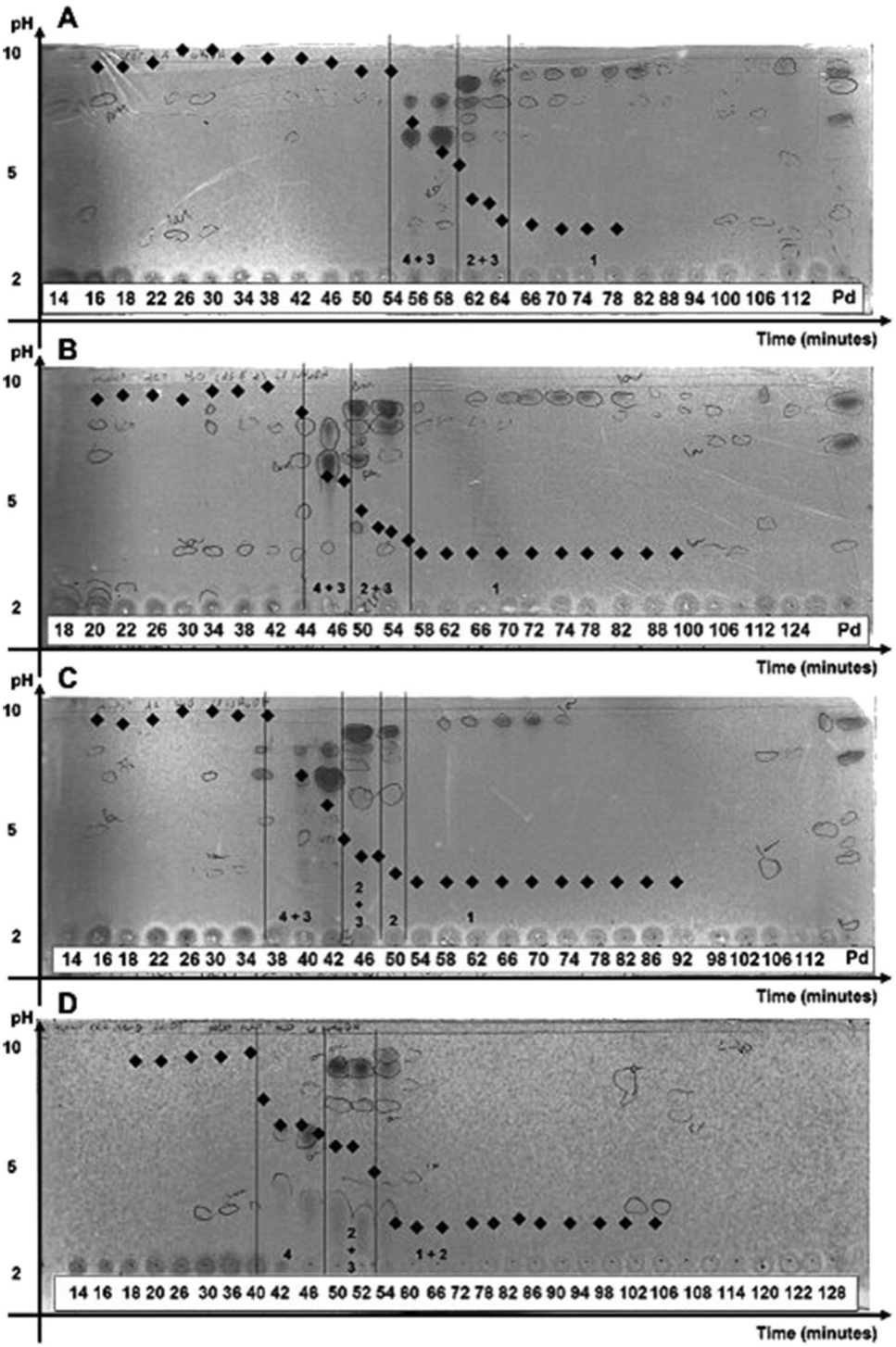


Fig. 6 TLC plates with the results of the pH-zone-refining CCC. Experimental conditions, TLC—silica gel plates developed with ethyl acetate:acetone:water (25:8:2) and a drop of concentrated $N^{+}H4OH$ solution; detection, Dragendorff's reagent; CCC—coil volume, 80 ml; flow rate, 2 ml/min; rotation speed, 850 rpm; sample loading, 200 mg; fraction size, 4 ml; solvent system, MtBE–water; **A** 15 mM TEA in the organic stationary phase (OSP) and 10 mM formic acid in the aqueous mobile phase (AMP); **B** 10 mM TEA in the OSP and 10 mM formic acid in the AMP; **C** 10 mM TEA in the OSP and 15 mM formic acid in the AMP; **D** 5 mM TEA in the OSP and 5 mM hydrochloric acid in the AMP. Reproduced from Vieira et al. 2013

because this system is a polar solvent mixture more adequate for the purification of polar quaternary alkaloids. Compared to tertiary alkaloids, quaternary alkaloids generally show lower alkalinity and high polarity, which might lead to further difficulty for their separation by PZRCCC. In fact, several of the papers describing the purification of quaternary alkaloids (Sun et al. 2014; Zhu et al. 2016; Liu et al. 2019) used high concentrations of HCl as retainer in aqueous stationary phases (as high as 60 mM) and lower concentrations of TEA as eluter in the mobile organic phase (5–10 mM), performing normal displacement mode instead of reverse displacement mode, frequently used for the purification of free base alkaloids. Quaternary protoberberine alkaloids have been isolated from several Chinese medicinal plants by using hydrochloric acid as retainer in the aqueous stationary phase (Table S3). These protoberberine alkaloids

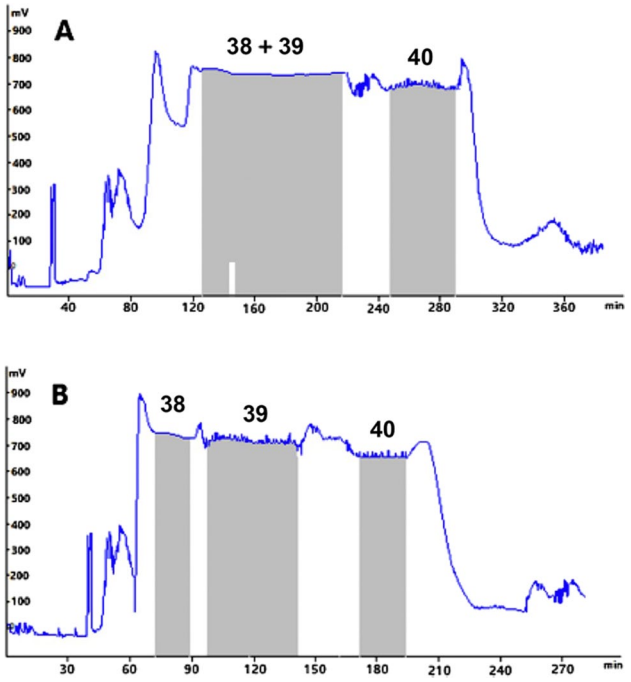
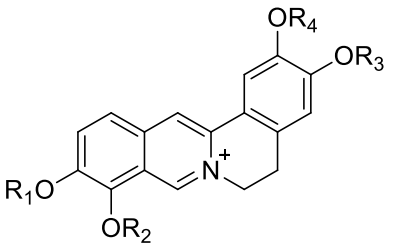


Fig. 7 pH-zone-refining CCC chromatograms for separation of polar indole alkaloids from Toad Venom (38–40). Experimental conditions: flow rate, 1.5 mL/min; revolution speed, 800 rpm; separation temperature, 25 °C; solvent system, acetonitrile–sodium chloride–water (54:5:421%, w%); **A** 10 mM TEA in upper organic phase and 10 mM HCl in lower aqueous phase; **B** 10 mM TEA in upper organic phase and 15 mM HCl in lower aqueous phase. Reproduced from Zou et al. 2018

have been shown to possess diverse biochemical and pharmacological actions while being nontoxic to humans even at high dosages. Needless to say, protoberberine alkaloids that are widely distributed in several botanical families have a long history of use worldwide in folk medicine with several important biological activities used for the treatment of cancer, inflammation, diabetes, depression, hypertension, and various infectious areas. Quaternary protoberberine alkaloids selectively bind to G-quadruplex DNA, commonly present in several protooncogenic-DNA promoters (Jarošová et al. 2019).

As discussed above, the concentration of both the retainer and eluter can affect peak resolution and retention times. Sun and co-workers (2014) were able to successfully purify berberine (**1**), columbamine (**41**), jatrorrhizine (**42**), palmatine (**43**), and coptisine (**44**) from an extract (1 g) of *Coptis chinensis* by adjusting the concentrations of hydrochloric acid as retainer in the stationary phase and TEA as eluter in the mobile phase. By using the optimized solvent system $CHCl_3$-MeOH-H_2O (4:3:3, v/v), the first preparative purification was performed with equal amounts of retainer and eluter: 10 mM HCl in the aqueous phase and 10 mM TEA in the organic phase, yielding four main fractions, where only jatrorrhizine (**42**) and berberine (**1**) were successfully purified. Then, in order to enhance peak resolution, the concentration of the eluter base was reduced, and that of the retainer acid was increased. Thus, the concentration of HCl was raised to 40 mM in a second experiment, improving the resolution of the separation, resulting in five main fractions, where three of them, peaks A (**41**), D (**44**), and E (**1**), eluted as pure compounds, although compound B (**42**) and C (**43**) still came out as a mixture. The rectangular peaks formed in this separation were too narrow, indicating that the alkaloids eluted too fast. Finally, in the third experiment, the concentrations of both HCl and TEA were optimized to 60 mM and 5 mM, respectively. These experimental conditions afforded five-flat pH-zones (Sun et al. 2014), as measured by the pH value of the collected fractions (Fig. 8), which demonstrated the complete resolution of the alkaloids with a purity over 96.6%, as confirmed by HPLC: 5.4 mg of columbamine (**41**, peak A), 6.1 mg of jatrorrhizine (**42**, peak B), 58.3 mg of coptisine (**43**, peak C), 25.6 mg of palmatine (**44**, peak D), and 503.9 mg of berberine (**1**, peak E).



41 R_1=R_2=R_3=CH_3; R_4=H
42 R_1=R_2=R_4=CH_3; R_3=H
43 R_1=R_2= $-CH_2-$; R_3=R_4= $-CH_2-$
44 R_1=R_2=R_3=R_4=CH_3
45 R_1=R_2=R_3=R_4=H

Li and co-workers (Li et al. 2015) described the purification of five protoberberine alkaloids from *Coptis rhizome* in two stages, where, in the first stage, the strategy of a reactive extraction was used. The modulation effect of seven carboxylic acids (acetic, propionic, difluoroacetic, dichloroacetic, trifluoroacetic, trichloroacetic, and pentafluoropropionic acids) on the five target compounds was studied by adding them separately to the optimized CCC solvent system (M*t*BE-BuOH-EtOAc-H_2O 2:2:1:8, v/v) and measuring *K* values for each compound. After analyzing the relationships between log *P* (hydrophobicity) and pK_a of the acids and the *K* values of the alkaloids, it was concluded that the values of *K* for the targets increased when the chain of alkyl and the number of halogen substituent in the acids are increased, and so, TFA was chosen as modulator as the *K* value of the five alkaloids was above 2 with this acid in the selected solvent system.

Several concentrations of the modulators trifluoroacetic acid (TFA) and ammonium ($NH_3.H_2O$) were tested for the reactive extraction step. After measuring the partition coefficient of the five compounds in each concentration of the modulating agents, the first stage of reactive extraction was performed with 40 mM TFA in the upper stationary phase and 20 mM ammonium in the lower mobile phase. Epi-berberine, (**45**), was purified (over 97% purity) from the rest of the alkaloid mixture in this first stage. Alkaloids **1** and **42–44** were purified in the second stage with the same solvent system and TEA at 25 mM serving as retainer in the organic phase and 10 mM HCl as eluter in the aqueous phase. The mechanism of reactive extraction involves high hydrophobicities of the formed salts which allowed the target quaternary alkaloids to be transferred from the aqueous phase to the organic stationary phase.

Kamto and co-workers (2021) described the use of EtOAc-BuOH-H_2O (EBuWat) as a polar solvent system for the separation of three aporphine alkaloids, lirioferine (**46**), cocsarmine (**47**), and biscocsarmine (**48**), from *Triclisia dictyophylla* Diels, Menispermaceae. The first attempt to use HEMWat as solvent system family at several ratios and concentrations of TEA and HCl as retainer and eluter, respectively, failed to obtain the desired $K_{base} >> 1$ and $K_{acid} << 1$ even at the highest polarity ratios of 1:6:1:6. EBuWat was then screened. This solvent system is composed of two immiscible solvents (ethyl acetate and water) and a third one, butanol, which is manly an organic modifier. So, the composition ethyl acetate-BuOH-water *x*:*y*:10 was tested where *x*:*y* varied from 9:1 to 5:5. After choosing the very polar composition EBuWat 5:5:10, the concentration of retainer TEA and eluter HCl was adjusted. HCl was kept at 5 mM, whereas TEA concentration was screened from 5 to 80 mM to retain all alkaloids in the organic stationary phase, being 60 mM the one chosen for the purification of the crude extract. Even at 80 mM TEA, not all quaternary alkaloids, cocsarmine (**47**) and biscocsarmine (**45**), would completely remain in the basic organic phase. Their quaternary nature was further confirmed after structural elucidation (Kamto et al. 2021).

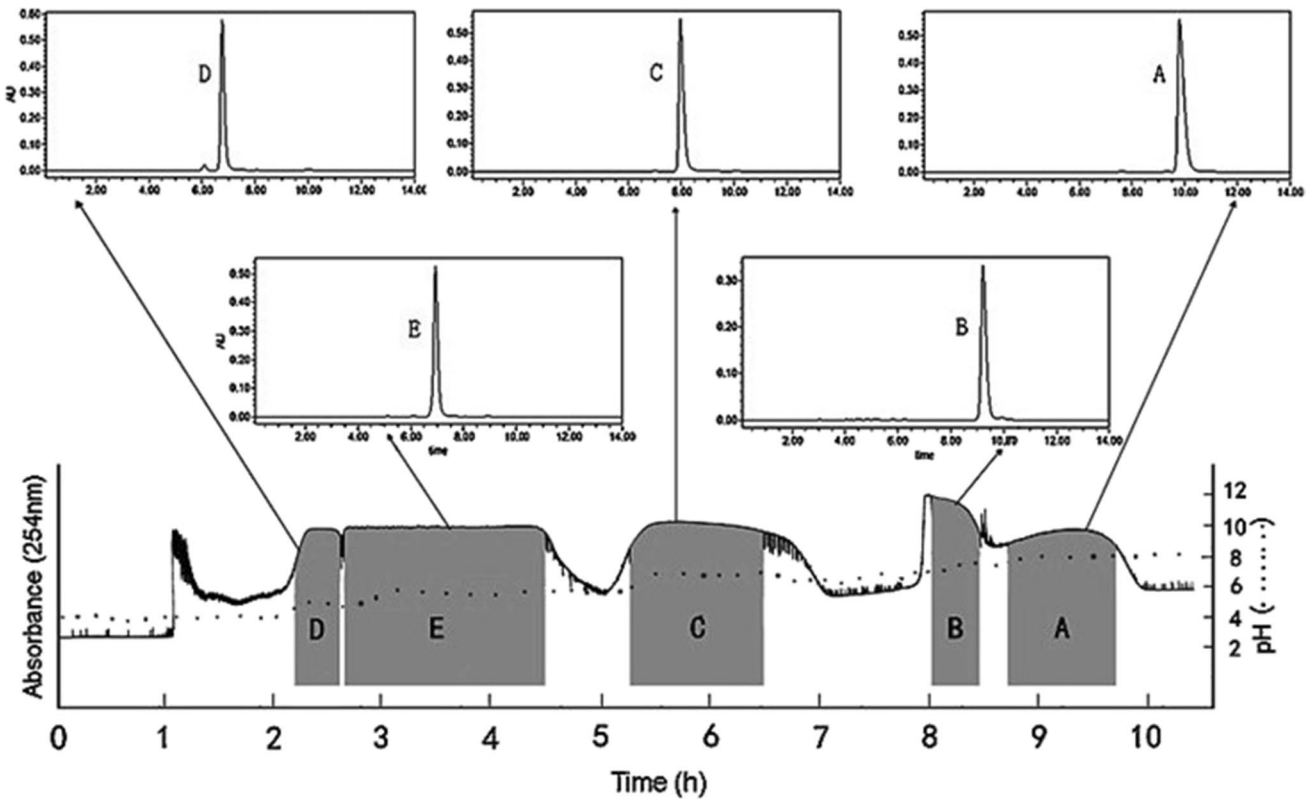
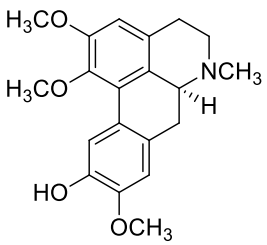


Fig. 8 pH-zone-refining CCC and HPLC control for the separation of the crude extract from *Coptis chinensis* Franch. Experimental conditions: solvent system, $CHCl_3$–MeOH–H_2O (4:3:3, v/v); 60 mM HCL in upper aqueous phase and 5 mM TEA in lower organic phase; revolution speed, 850 rpm; flow rate, 2 mL/min; sample size, 1.0 g; HPLC–UV detection wavelength, 254 nm. Alkaloid assignments: columbamine (**41**, peak A), jatrorrhizine (**42**, peak B), coptisine (**43**, peak C), palmatine (**40**, peak D), and berberine (**1**, peak E). Reproduced from Sun et al. 2014



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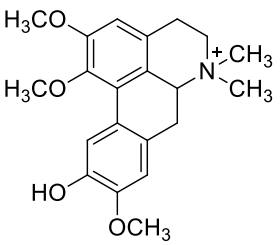

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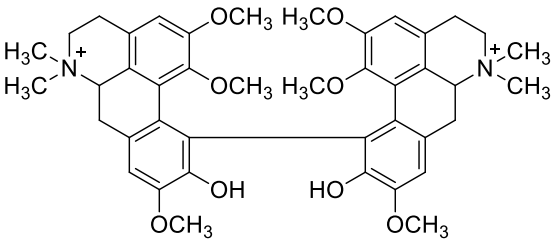

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Perspective on Future Directions

Conventional methods for alkaloid purification still remain a challenging task, due to the multiple steps to be achieved, especially when dealing with quaternary alkaloids, which require the implementation of polar solvent systems. pH-zone-refining CCC has shown itself to be a valuable technique for the preparative separation of alkaloids by the optimization of the concentrations of both retainer in the stationary phase and eluter in the mobile phase in order to obtain suitable flat pH-zones with enhanced resolution between peaks and improved retention times.

Hyphenation of CCC to other analytical techniques has been increasingly used, especially liquid chromatography–tandem mass spectrometry (LC–MS/MS), to rapidly annotate bioactive compounds in complex natural product-derived mixtures. Recently, the additive combination of in silico virtual screening by multi-target drug approach turns CCC into one of the most powerful tools for the fast and efficient preparative isolation of natural products in bioassay-guided fractionations and isolation of lead compounds for further biological testing and drug discovery.

Finally, the considerable preparative capacity of CCC with low cost makes it exceptionally attractive for the development of complementary methods for chiral separation of pure compounds from racemic mixtures at low cost for industrial synthesis of pharmaceuticals based on natural product scaffolds. In the future, the application of liquid–liquid chromatography in chiral separation would certainly be popular if the efficiency and retention of the samples through the number of theoretical plates of the separation column are greatly improved. Developing and screening new varieties of chiral selectors are also of enormous importance for improving chiral separations.

Conclusions

The selected examples discussed here show that the basic nature of alkaloids conferred by the nitrogen atom in their chemical structure is a characteristic feature that can be explored for the purification of this class of compounds by liquid–liquid partitioning techniques. Most of the solvent systems employed in the purifications by conventional CCC resemble the biphasic systems used for liquid–liquid extraction modulated by pH, *e.g.*, $CHCl_3$-H_2O; the solvent system $CHCl_3$-MeOH-H_2O is frequently used, especially for quaternary alkaloids separation by pH-zone-refining CCC, and its selectivity can be modified by the substitution of the alcohol; hexane–ethyl acetate-MeOH-H_2O is a common system. M*t*BE-acetonitrile-BuOH-water is frequently used in PZRCCC separations. The use of aqueous acid buffers which pH varies according to the nature of the alkaloid has been employed in most of the described isolations, but the addition of bases in the aqueous phase is also reported. The evolution of equipment used for alkaloid separation, from the Craig and Post apparatus to the modern centrifugal techniques like CPC and HSCCC, represents a significant step forward as hydrostatic and hydrodynamic equipment can be operated in several different ways, opening new horizon for the art of separation such as the use of three-phase solvent systems.

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Author Contribution GGL was responsible for the conceptualization of the review, the literature search, and the preparation of the manuscript first draft. CML and SCM performed literature search, compiled tables for the Supporting Information material, and helped on the manuscript draft. RPM contributed to contextualize the information in the field of pharmacognosy and provided critical reading and insightful recommendations for the final manuscript. All of the authors have read the final manuscript and approved the submission.

References

Adamski Z, Blythe LL, Milella L, Bufo SA (2020) Biological activities of alkaloids: from toxicology to pharmacology. Toxins 12:210. https://doi.org/10.3390/toxins12040210

Adejoke HT, Louis H, Amusan OO, Apebende G (2019) A review on classes, extraction, purification and pharmaceutical importance of plants alkaloid. J Med Chem Sci 2:130–139. https://doi.org/10.26655/JMCHEMSCI.2019.8.2

Alvi KA (2001) Screening natural products: bioassay-directed isolation of active components by dual-mode CCC. J Liq Chrom Relat Tech 24:1765–1773. https://doi.org/10.1081/JLC-100104377

Aniszewski T (2015) Alkaloids: chemistry, biology, ecology, and applications. Elsevier. 2nd Ed., pp. 1–98.

Atlabachew M, Combrinck S, Viljoen AM, Hamman JH, Gouws C (2016) Isolation and *in vitro* permeation of phenylpropylamino alkaloids from Khat (*Catha edulis*) across oral and intestinal mucosal tissues. J Ethnopharmacol 194:307–315. https://doi.org/10.1016/j.jep.2016.09.012

Banerjee RP, Chatterjee ML, Häusler HF (1957) *Rauwolfia* alkaloids: fractionation by countercurrent distribution. J Pharm Pharmacol 9:120–127. https://doi.org/10.1111/j.2042-7158.1957.tb12264.x

Berthod A, Ruiz-Angel MJ, Carda-Broch S (2003) Elution−extrusion countercurrent chromatography. Use of the liquid nature of the stationary phase to extend the hydrophobicity window. Anal Chem 75:5886–5894. https://doi.org/10.1021/ac030208d

Berthod A (2006) Band broadening inside the chromatographic column: the interest of a liquid stationary phase. J Chromatogr A 1126:347–356. https://doi.org/10.1016/j.chroma.2006.05.027

Berthod A, Maryutina T, Spivakov B, Shpicun O, Sutherland IA (2009) Countercurrent chromatography in analytical chemistry (IUPAC technical report). Pure Appl Chem 81:355–387. https://doi.org/10.1351/PAC-REP-08-06-05

Bojczuk M, Żyżelewicz D, Hodurek P (2017) Centrifugal partition chromatography. A review of recent applications and some classic references. J Sep Sci 40:1597–1609. https://doi.org/10.1002/jssc.201601221

Britton HTS, Robinson RA (1931) CXCVIII. Universal buffers solutions and the dissociation constant of veronal. J Chem Soc 1456–1462. https://doi.org/10.1039/JR9310001456

Coch JA, de Ferrari EC, Delbene U (1965) Counter current distribution of alkaloids with a pH gradient. J Chromatogr A 17:193–195. https://doi.org/10.1016/S0021-9673(00)99854-6

Conway WD (1990) Countercurrent chromatography, apparatus, theory and applications. VCH Publishers, Weinheim

Costa FN, Leitão GG (2010) Strategies of solvent system selection for the isolation of flavonoids by countercurrent chromatography. J Sep Sci 33:336–347. https://doi.org/10.1002/jssc.200900632

Craig LC, Post O (1949) Apparatus for countercurrent distribution. Anal Chem 21:500–504. https://doi.org/10.1021/ac60028a013

Creed SM, Gutridge AM, Argade MD, Henessy MR, Friesen JB, Pauli GF, van Rijn RM, Riley AP (2021) Isolation and pharmacological characterization of six opioidergic *Picralima nitida* alkaloids. J Nat Prod 84:71–80. https://doi.org/10.1021/acs.jnatprod.0c01036

Dai X, Hu R, Sun C, Pan Y (2012) Comprehensive separation and analysis of alkaloids from *Stephania yunnanensis* by countercurrent chromatography coupled with liquid chromatography tandem mass spectrometry analysis. J Chromatogr A 1226:18–23. https://doi.org/10.1016/j.chroma.2011.10.022

Debnath B, Singh WS, Das M, Goswami S, Singh MK, Maiti D, Manna K (2018) Role of plant alkaloids on human health: a review of biological activities. Mater Today Chem 9:56–72. https://doi.org/10.1016/j.mtchem.2018.05.001

Delannay E, Toribio A, Boudesocque L, Nuzillard JM, Zèches-Hanrot M, Dardennes E, Le Dour G, Sapi J (2006) Renault JH (2006) Multiple dual-mode centrifugal partition chromatography, a semi-continuous development mode for routine laboratory-scale purifications. J Chromatogr A 1127:45–51. https://doi.org/10.1016/j.chroma.2006.05.069

Du Q-Z, Ke C-Q, Ito Y (1998) Recycling high-speed countercurrent chromatography for separation of taxol and cephalomannine. J Liq Chromatogr Relat Technol 21:157–162. https://doi.org/10.1080/10826079808001944

Dong H, Zhang Y, Fang L, Duan W, Wang X, Huang L (2011) Combinative application of pH-zone refining and conventional high-speed counter-current chromatography for preparative separation of alkaloids from *Stephania kwangsiensis*. J Chromatogr B 879:945–949. https://doi.org/10.1016/j/chromb.2011.02.051

Enyoh CE, Isiuku BO, Verla AW (2019) Applications of column, paper, thin layer and ion exchange chromatography in purifying samples: mini review. SF J Pharm Anal Chem 2:1018

Fang L, Liu Y, Yang B, Wang X, Huang L (2011) Separation of alkaloids from herbs using high-speed counter-current chromatography. J Sep Sci 34:2545–2558. https://doi.org/10.1002/jssc.201000857

Fang L, Zhou J, Lin YL, Wang X, Sun Q, Li JL, Huang L (2013) Large-scale separation of alkaloids from *Gelsemium elegans* by pH-zone refining counter-current chromatography with a new solvent system screening method. J Chromatogr A 1387:80–85. https://doi.org/10.1016/j.chroma.2013.07.069

Fang Y, Li Q, Shao Q, Wang B, Wei Y (2017) A general ionic liquid pH-zone refining counter-current chromatography method for separation of alkaloids from *Nelumbo nucifera* Gaertn. J Chromatogr A 1507:63–71. https://doi.org/10.1016/j.chroma.2017.05.048

Faure K, Mekaoui N, Berthod A (2014) Saving solvents in chromatographic purifications: the counter-current chromatography technique. In: Inamuddin, Mohammad A (eds) Green chromatographic techniques. Separation and purification of organic and inorganic analytes. Springer, Dordrecht, pp 1–18. https://doi.org/10.1007/978-94-007-7735-4_1

Feng RH, Hou JJ, Zhang YB, Pan HQ, Yang W, Qi P, Yao S, Cai LY, Yang M, Jiang BH, Liu X (2015) An efficient and target-oriented sample enrichment method for preparative separation of minor alkaloids by pH-zone-refining counter-current chromatography. J Chromatogr A 1409:159–165. https://doi.org/10.1016/j.chroma.2015.07.066

Franco P, Blanc J, Oberleitner WR, Lindner W, Minguillón C (2002) Enantiomer separation by countercurrent chromatography using *Cinchona* alkaloid derivatives as chiral selectors. Anal Chem 74:4175–4183. https://doi.org/10.1021/ac020209q

Friesen JB, McAlpine JB, Chen SN, Pauli GF (2015) Countercurrent separation of natural products: an update. J Nat Prod 78:1765–1796. https://doi.org/10.1021/np501065h

Galeffi C (1974) Application of a statistical method to counter-current distribution of acids and bases and their separation by discontinuous change of pH. J Chromatogr 92:1–7. https://doi.org/10.1016/S0021-9673(00)97183-8

Galeffi C (1980) New trends in the separation of active principles from plants. J Ethnopharmacol 2:129–134. https://doi.org/10.1016/0378-8741(80)90006-9

Galeffi C, Ciasca-Rendina MA, Delle Monache EM, Del Fresno AV, Marini-Bettolo GB (1969) A gradient method for the counter-current separation of alkaloids using a heavy organic phase. J Chromatogr 45:407–414. https://doi.org/10.1016/S0021-9673(01)86237-3

Galeffi C, Nicoletti M, Messana I, Marini-Bettolo GB (1979) On the alkaloids of *Strychnos*–XXXI. 15-hydroxystrychnine, a new

alkaloid from *Strychnos nux-vomica* L. Tetrahedron 35:2545–2549. https://doi.org/10.1016/0040-4020(79)88018-7

Galeffi C, Marini-Bettolo GB (1981) On the alkaloids of *Strychnos* XXXVI: the alkaloids of *Strychnos hirsuta* Spruce ex Bentham; two new β-carbolinic alkaloids, strychnohirsutine and tetradehydrostrychnohirsutine. Tetrahedron 37:3167–3170. https://doi.org/10.1016/S0040-4020(01)98849-0

Gonçalves AC, Nunes AR, Alves G, Silva LR (2021) Serotonin and melatonin: plant sources, analytical methods, and human health benefits. Rev Bras Farmacogn 31:162–175. https://doi.org/10.1007/s43450-021-00141-w

Guimarães HA, Vieira IJ, Braz-Filho R, Crotti AE, dos Almeida VS, de Paula RC (2013) Two novel plumeran indole alkaloids isolated from *Aspidosperma cylindrocarpon* (Apocynaceae). Helv Chim Acta 26:1793–1800. https://doi.org/10.1002/hlca.201200522

Han T, Cao X, Xu J, Pei H, Zhang H, Tang Y (2017) Separation of the potential G-quadruplex ligands from the butanol extract of *Zanthoxylum ailanthoides* Sieb. & Zucc. by countercurrent chromatography and preparative high performance liquid chromatography. J Chromatogr A 1507:104–114. https://doi.org/10.1016/j.chroma.2017.05.058

Hermans-Lockkerbol A, Verpoorte R (1986) Droplet counter-current chromatography of alkaloids. The influence of pH-gradients and ion-pair formation on the retention of alkaloids. Planta Med 52:299–302. https://doi.org/10.1055/s-2007-969158

Hostettmann K, Hostettmann M, Marston A (1984) Isolation of natural products by droplet counter-current chromatography and related methods. Nat Prod Rep 1:471–481. https://doi.org/10.1039/NP9840100471

Huang XY, Ignatova S, Hewitson P, Di DL (2016) An overview of recent progress in elution mode of counter current chromatography. TrAC Trends Anal Chem 77:214–225. https://doi.org/10.1016/j.trac.2015.08.006

Huang JH, Johnston GA (1990) (+)-Hydrastine, a potent competitive antagonist at mammalian GABAA receptors. Br J Pharmacol 99:727–730. https://doi.org/10.1111/j.1476-5381.1990.tb12997.x

Imenshahidi M, Hosseinzadeh H (2019) Berberine and barberry (*Berberis vulgaris*): a clinical review. Phytother Res 33:504–523. https://doi.org/10.1002/ptr.6252

Ito Y, Ma Y (1996) pH-zone-refining countercurrent chromatography. J Chromatogr A 753:1–36. https://doi.org/10.1016/S0021-9673(96)00565-1

Ito Y (2005) Golden rules and pitfalls in selecting optimum conditions for high-speed counter-current chromatography. J Chromatogr A 1065:145–168. https://doi.org/10.1016/j.chroma.2004.12.044

Ito Y (2013) pH-zone-refining counter-current chromatography: origin, mechanism, procedure and applications. J Chromatogr A 1271:71–85. https://doi.org/10.1016/j.chroma.2012.11.024

Jarošová P, Sándor R, Slaninková A, Vido M, Peš O, Táborský P (2019) Quaternary protoberberine alkaloids and their interactions with DNA. Chem Pap 73:2965–2973. https://doi.org/10.1007/s11696-019-00857-z

Kamto ELD, Mendonça SC, Zintchem AAA, Tiam ER, Pegnyemb DE, Leitão GG (2021) Aporphine alkaloids from *Triclisia dictyophylla* Diels by pH-zone refining countercurrent chromatography. Chromatographia 84:13–20. https://doi.org/10.1007/s10337-020-03977-x

Khan BM, Liu Y (2018) High speed counter current chromatography: overview of solvent-system and elution-mode. J Liq Chrom Relat Tech 41:629–636. https://doi.org/10.1080/10826076.2018.1499528

Khashayar A, Bahari Z, Elliyeh M, Ghasemi M (2021) Therapeutic effects of berberine in metabolic diseases and diabetes mellitus. Rev Bras Farmacogn. https://doi.org/10.1007/s43450-021-00159-0

Kidd DAA, Scott PGW (1957) Alkaloids of *Rauwolfia* species: Part II. The estimation of reserpine in samples of *Rauwolfia* by means of countercurrent distribution. J Pharm Pharmacol 9:176–180. https://doi.org/10.1111/j.2042-7158.1957.tb12270.x

Koel M, Kuhtinskaja M, Vaher M (2020) Extraction of bioactive compounds from *Catharanthus roseus* and *Vinca minor*. Sep Purif Technol 252:117438. https://doi.org/10.1016/j.seppur.2020.117438

Kotland A, Chollet S, Diard C, Autret JM, Meucci J, Renault JH, Marchal L (2016) Industrial case study on alkaloids purification by pH-zone refining centrifugal partition chromatography. J Chromatogr A 1474:59–70. https://doi.org/10.1016/j.chroma.2016.10.039

Kostanyan AA, Voshkin AA, Belova VV (2020) Analytical, preparative, and industrial-scale separation of substances by methods of countercurrent liquid-liquid chromatography. Molecules 25:6020. https://doi.org/10.3390/molecules25246020

Kresge N, Simoni RD, Hill RL (2005) Lyman Creighton Craig: developer of the counter-current distribution method. J Biol Chem 280:127–129. https://doi.org/10.1016/S0021-9258(19)63136-4

Kuramoto M, Arimoto H, Uemura D (2004) Bioactive alkaloids from the sea: a review. Mar Drugs 2:39–54. https://doi.org/10.3390/md201039

Leitão GG, Costa FN (2015) Gradient elution in countercurrent chromatography. Planta Med 81:1592–1596. https://doi.org/10.1055/s-0035-1546267

Leitão SG, Martins GR, Martínez-Fructuoso L, de Sousa SD, da Fonseca TS, Castilho CV, Baratto LC, Alviano DS, Alviano CS, Leitão GG, Pereda-Miranda R (2020) Absolute stereochemistry of antifungal limonene-1,2-diols from *Lippia rubella*. Rev Bras Farmacogn 30:537–543. https://doi.org/10.1007/s43450-020-00081-x

Li Y, Cai F, Zhang M, Zhang H, Wang Y, Hu P (2015) Two-stage fractionation of polar alkaloids from *Rhizoma coptidis* by countercurrent chromatography considering the strategy of reactive extraction. J Chromatogr A 1378:58–64. https://doi.org/10.1016/j.chroma.2014.12.028

Li W, Wang H, Dong A (2019) Preparative separation of alkaloids from stem of *Euchresta tubulosa* Dunn by high-speed counter-current chromatography using stepwise elution. Molecules 24:4602. https://doi.org/10.3390/molecules24244602

Lin Y, Luo J, Li L, Liu X, Wang W, Zhu L, Han C, Kong L (2020) Precise separation of lysine-specific demethylase 1 inhibitors from *Corydalis yanhusuo* using multi-mode counter-current chromatography with virtual screening. J Chromatogr A 1625:461294. https://doi.org/10.1016/j.chroma.2020.461294

Liu Y, Chen X, Liu J, Di D (2015) Three-phase solvent systems for the comprehensive separation of a wide variety of compounds from *Dicranostigma leptopodum* by high-speed counter-current chromatography. J Sep Sci 38:2038–2045. https://doi.org/10.1002/jssc.201401466

Liu Y, Kuang P, Guo S, Sun Q, Xue T, Li H (2018) An overview of recent progress in solvent systems, additives and modifiers of counter current chromatography. New J Chem 42:6584–6600. https://doi.org/10.1039/C7NJ04747A

Liu M, Liu Q, Chen M, Huang X, Chen X (2019) Large scale separation of acetylcholinesterase inhibitors from *Zanthoxylum nitidum* by pH-zone-refining counter-current chromatography target-oriented by ultrafiltration high-performance chromatography with ultraviolet and mass spectrometry screening. J Sep Sci 42:1194–1201. https://doi.org/10.1002/jssc.201801238

Lu Y, Pan Y, Berthod A (2008) Using the liquid nature of the stationary phase in counter-current chromatography V. The Back-Extrusion Method J Chromatogr A 1189:10–18. https://doi.org/10.1016/j.chroma.2007.11.010

Lu L, Huang R, Wu Y, Jin JM, Chen HZ, Zang LJ, Luan X (2020) Brucine: a review of phytochemistry, pharmacology, and toxicology. Front Pharmacol 11:377. https://doi.org/10.3389/fphar.2020.00377

Ma Y, Ito Y, Sokolosky E, Fales HM (1994) Separation of alkaloids by pH-zone-refining counter-current chromatography. J Chromatogr A 685:259–262. https://doi.org/10.1016/0021-9673(94)00667-9

Martino E, Casamassima G, Castiglione S, Cellupica E, Pantalone S, Papagni F, Rui M, Siciliano AM, Collina S (2018) Vinca alkaloids and analogues as anti-cancer agents: looking back, peering ahead. Bioorg Med Chem Lett 28:2816–2826. https://doi.org/10.1016/j.bmcl.2018.06.044

Meng J, Yang Z, Liang J, Guo M, Wu S (2014) Multi-channel recycling counter-current chromatography for natural product isolation: tanshinones as examples. J Chromatogr A 1327:27–38. https://doi.org/10.1016/j.chroma.2013.12.069

McAlpine JB, Friesen JB, Pauli GF (2012) Separation of natural products by countercurrent chromatography. In Sarker SD, Nahar L (Eds.) Natural products isolation. Methods in molecular biology (Methods and protocols) vol. 864, Humana Press, pp. 221–254. https://doi.org/10.1007/978-1-61779-624-1_9

Marini-Bettolo GB, Ciasca MA, Galeffi C, Bisset NG, Krukoff BA (1972) The occurrence of strychnine and brucine in an American species of *Strychnos*. Phytochemistry 11:381–384. https://doi.org/10.1016/S0031-9422(00)90018-9

Marini-Bettolo GB, Messana I, Nicoletti M, Patamia M, Galeffi C (1980) On the alkaloids of *Strychnos*. XXXV. The occurrence of akagerine in South American *Strychnos*. J Nat Prod 43:717–720. https://doi.org/10.1021/np50012a003

Marston A, Hostettmann K (1994) Counter-current chromatography as a preparative tool, applications and perspectives. J Chromatog A 658:315–341. https://doi.org/10.1016/0021-9673(94)80023-5

Martin MT, Rasoanaivo P, Palazzino G, Galeffi C, Nicoletti M, Trigalo F, Frappier F (1999) Minor Nb, C (21)-secocuran alkaloids of *Strychnos myrtoides*. Phytochemistry 51:479–486. https://doi.org/10.1016/S0031-9422(99)00033-3

Nicoletti M, Serafini M, Federici E, Galeffi C, Poli F (1998) Indole alkaloids from aerial parts of *Vinca sardoa*. Phytochemistry 47:149–151. https://doi.org/10.1016/S0031-9422(97)00533-5

Oka H, Harada KI, Ito Y, Ito Y (1998) Separation of antibiotics by counter-current chromatography. J Chromatogr A 812:35–52. https://doi.org/10.1016/S0021-9673(97)01277-6

Powell RG, Rogovin SP, Smith CR Jr (1974) Isolation of antitumor alkaloids from *Cephalotaxus harringtonia*. Ind Eng Chem Prod Res Dev 13:129–132. https://doi.org/10.1021/i360050a010

Pérez E, Minguillón C (2006) Optimization of the derivatization in cellulose-type chiral selectors for enantioseparations by centrifugal partition chromatography. J Sep Sci 29:1379–1389. https://doi.org/10.1002/jssc.200600058

Pérez E, Santos MJ, Minguillón C (2006) Application of cellulose and amylose arylcarbamates as chiral selectors in counter-current chromatography. J Chromatogr A 1107:165–174. https://doi.org/10.1016/j.chroma.2005.12.061

Rasoanaivo P, Palazzino G, Nicoletti M, Galeffi C (2001) The co-occurrence of C(3) epimer Nb, C(21)-secocuran alkaloids in *Strychnos diplotricha* and *Strychnos myrtoides*. Phytochemistry 56:863–867. https://doi.org/10.1016/S0031-9422(00)00475-1

Rasoanaivo P, Ratsimamanga-Urverg S, Galeffi C, Nicoletti M, Frappier F, Martin MT (1995) A new group of isoquinoline dimers from *Hernandia voyronii*. Tetrahedron 51:1221–1228. https://doi.org/10.1016/0040-4020(94)00992-4

Rasoanaivo P, Ratsimamanga-Urverg S, Rafatro H, Ramanitrahasimbola D, Palazzino G, Galeffi C, Nicoletti M (1998) Alkaloids of *Hernandia voyronii*: chloroquine-potentiating activity and structure elucidation of herveline D. Planta Med 64:58–62. https://doi.org/10.1055/s-2006-957367

Rather LJ, Ansari MF, Li Q (2020) Recent advances in the insect natural product chemistry: structural diversity and their applications. In: Kumar D, Shahid M (eds) Natural materials and products from insects: chemistry and applications. Springer, Cham, Switzerland, pp. 67–94. https://doi.org/10.1007/978-3-030-36610-0_5

Saporito RA, Donnelly MA, Spande TF, Garrafo H (2012) A review of chemical ecology in poison frogs. Chemoecology 22:159–168. https://doi.org/10.1007/s00049-011-0088-0

Schwarz M, Hillebrand S, Habben S, Degenhardt A, Winterhalter P (2003) Application of high-speed countercurrent chromatography to the large-scale isolation of anthocyanins. Bioch Eng J 14:179–189. https://doi.org/10.1016/S1369-703X(02)00219-X

Schwienheer C, Merz J, Schembecker G (2015) Investigation, comparison and design of chambers used in centrifugal partition chromatography on the basis of flow patterns and separation experiments. J Chromatogr A 1390:39–49. https://doi.org/10.1016/j.chroma.2015.01.085

Skalicka-Wozniak K, Garrard I (2014) Counter-current chromatography for the separation of terpenoids: a comprehensive review with respect to the solvent systems employed. Phytochem Rev 13:547–572. https://doi.org/10.1007/s11101-014-9348-2

Shikanga EA, Viljoen A, Combrinck S, Marston A (2011) Isolation of *Sceletium* alkaloids by high-speed countercurrent chromatography. Phytochem Lett 4:190–193. https://doi.org/10.1016/j.phytol.2011.03.003

Song H, Lin J, Zhu X, Chen Q (2016) Developments in high-speed countercurrent chromatography and its applications in the separation of terpenoids and saponins. J Sep Sci 39:1574–1591. https://doi.org/10.1002/jssc.201501199

Sun C, Li J, Wang X, Duan W, Zhang T, Ito Y (2014) Preparative separation of quaternary ammonium alkaloids from *Coptis chinensis* by pH-zone refining counter-current chromatography. J Chromatogr A 1370:156–161. https://doi.org/10.1016/j.chroma.2014.10.043

Sun C, Duan W, Wang X, Geng Y, Li J (2015) Combinative application of pH-zone refining counter-current chromatography and preparative HPLC for the separation of alkaloids from *Lycoris radiata*. J Liq Chrom Rel Technol 38:1031–1036. https://doi.org/10.1080/10826076.2014.982868

Sun C, Li J, Wang D et al (2015) Preparative separation of alkaloids from *Litsea cubeba* using combined applications of pH-zone-refining and high-speed counter-current chromatography. RSC Adv 5:75831–75837

Sun X, Pei D, Duan WD, Liu JF, Di DL, Huang XY (2020) Full use of the liquid nature of the stationary phase: the development of elution-extrusion counter current chromatography. J Sep Sci 43:3573–3584. https://doi.org/10.1002/jssc.202000341

Surup F, Tran TM, Pfütze S, Budde J, Moussa-Ayoub TE, Rohn S, Jerz G (2021) Opuntisines, 14-membered cyclopeptide alkaloids from fruits of *Opuntia stricta* var. *dillenii* isolated by high-performance countercurrent chromatography. Food Chem 334:127552. https://doi.org/10.1016/j.foodchem.2020.127552

Sutherland IA, Heywood-Waddington D, Peters TJ (1984) Toroidal coil countercurrent chromatography: a fast simple alternative to countercurrent distribution using aqueous two-phase partition: principles, theory, and apparatus. J Liq Chromatogr 7:363–384. https://doi.org/10.1080/01483918408073973

Tang Y, Li S, Li S et al (2017) Screening and isolating potential a-glucosidase inhibitors from *Rhizoma coptidis* by ultrafiltration LC-PDA-ESI/MS combined with high-speed countercurrent chromatography and reverse-phase medium-pressure liquid chromatography. Med Chem Res 26:3384–3394. https://doi.org/10.1007/s00044-017-2031-6

Tong S (2020) Liquid-liquid chromatography in enantioseparations. J Chromatogr A 14:461345. https://doi.org/10.1016/j.chroma.2020.461345

Tuenter E, Segers K, Kang KB, Viaene J, Sung SH, Cos P, Maes L, Heyden YV, Pieters L (2017a) Antiplasmodial activity, cytotoxicity and structure-activity relationship study of cyclopeptide alkaloids. Molecules 22:224. https://doi.org/10.3390/molecules22020224

Tuenter E, Exarchou V, Apers S, Pieters L (2017b) Cyclopeptide Alkaloids Phytochem Rev 16:623–637. https://doi.org/10.1007/s11101-016-9484-y

Uzma F, Mohan CD, Hashem A, Konappa NM, Rangappa S, Kamath PV, Singh BP, Mudili V, Gupta VK, Siddaiah CN, Chowdappa S, Alqarawi AA, Abdallah EF (2018) Endophytic fungi-alternative sources of cytotoxic compounds: a review. Front Pharmacol 9:309. https://doi.org/10.3389/fphar.2018.00309

Van Der Heijden R, Hermans-Lokkerbol A, Verpoorte R, Svendsen AB (1987) Pharmacognostical studies of *Tabernaemontana* species: XX. Ion-pair droplet counter-current chromatography of indole alkaloids from suspension cultures. J Chromatog A 396:410–415. https://doi.org/10.1016/S0021-9673(01)94084-1

Verpoorte R (2000) Liquid chromatography of alkaloids. In: Poole CF, Cook M (eds) Wilson ID, Adlard TR. Academic Press, London, Encyclopedia of Separation Sciences, pp 1949–1956

Vetter W, Müller M, Englert M, Hammann S (2020) Countercurrent chromatography when liquid-liquid extraction meets chromatography. In Poole CF (ed) Liquid-phase extraction. Handbooks in separation science, Elsevier, Frankfurt, pp. 289–325. https://doi.org/10.1016/B978-0-12-816911-7.00010-4

Vieira MN, Leitão SG, Porto PCC, Oliveira DR, Pinto SC, Braz-Filho R, Leitão GG (2013) Application of pH-zone-refining countercurrent chromatography for the separation of indole alkaloids from *Aspidosperma rigidum* Rusby. J Chromatogr A 1319:166–117. https://doi.org/10.1016/j.chroma.2013.10.044

Wang Y, Cai S, Chen Y, Deng L, Zhou X, Liu J, Xu X, Xia Q, Lin M, Zhang J, Huang W (2015) Separation and purification of five alkaloids from *Aconitum duclouxii* by counter-current chromatography. J Sep Sci 38:2320–2326. https://doi.org/10.1002/jssc.201500059

Weisz A, Scher AL, Shinomiya K, Fales HM, Ito Y (1994) A new preparative-scale purification technique: pH-zone-refining countercurrent chromatography. J Am Chem Soc 116:704–708. https://doi.org/10.1021/ja00081a035

Weisz A, Idina A, Ben-Ari J, Karni M, Mandelbaum A, Ito Y (2007) Preparative separation of isomeric and stereoisomeric dicarboxylic acids by pH-zone-refining counter-current chromatography. J Chromatogr A 1151:82–90. https://doi.org/10.1016/j.chroma.2007.03.085

Weir MR (2020) Reserpine: a new consideration of an old drug for hypertension? Am J Hypertens 33:708–710. https://doi.org/10.1093/ajh/hpaa069

Winterhalter P (2007) Application of countercurrent chromatography (CCC) to the analysis of natural pigments. Trends Food Sci Technology 18:507–513. https://doi.org/10.1016/j.tifs.2007.04.013

Wu S, Wu D, Liang J, Berthod A (2012) Modelling gradient elution in countercurrent chromatography: efficient separation of tanshinones from *Salvia miltiorrhiza* Bunge. J Sep Sci 35:964–976. https://doi.org/10.1002/jssc.201100993

Wu N, Xie H, Fang Y, Liu Y, Xi X, Chu Q, Dong G, Lan T, Wei Y (2018) Isolation and purification of alkaloids from lotus leaves by ionic-liquid-modified countercurrent chromatography. J Sep Sci 41:571–577. https://doi.org/10.1002/jssc.201700851

Yang Y, Gu D, Chen Q, Xin X, Yili A, Aisa HA (2013) A sample pre-treatment strategy for pH-zone refining counter-current chromatography. J Chromatogr A 1273:44–48. https://doi.org/10.1016/j.chroma.2012.11.049

Yu P, Ma J, Du Y, Wang Q, Chen C, Bai X, He L, Zou D (2020) An efficient strategy based on liquid-liquid extraction and pH-zone refining counter-current chromatography for selective enrichment, separation, and purification of alkaloids and organic acids from natural products. J Sep Sci 43:3607–3614. https://doi.org/10.1002/jssc.202000528

Yuan LM, Zi M, Ai P, Chen XX, Li ZY, Fu RN, Zhang TY (2001) Versatile two-phase solvent system for alkaloid separation by high-speed counter-current chromatography. J Chromatog A 927:91–96. https://doi.org/10.1016/S0021-9673(01)01057-3

Yuan Z, Xiao X, Li G (2013) Dynamic pH-junction high-speed counter-current chromatography coupled with microwave-assisted extraction for online isolation and purification of alkaloids from Stephania cepharantha. J Chromatogr A 1317:203–210. https://doi.org/10.1016/j.chroma.2013.07.063

Zhang Q, Ma T, Hu N, Ding C, Liang Y, Li W, Suo Y, Ding C (2016) One step to separate five alkaloids from *Hypecoum leptocarpum* by high-speed countercurrent chromatography. J Chromatog Sci 54:466–471. https://doi.org/10.1093/chromsci/bmv153

Zhang Q, Shu X, Jing F, Wang X, Lin C, Luo A (2014) Preparative separation of alkaloids from *Picrasma quassioides* (D. Don) Benn by conventional and pH-zone refining countercurrent chromatography. Molecules 19:8752–8761. https://doi.org/10.3390/molecules19078752

Zhenjia Z, Minglin W, Daijie W, Wenjuan D, Xiao W, Chengchao Z (2010) Preparative separation of alkaloids from *Nelumbo nucifera* leaves by conventional and pH-zone refining counter-current chromatography. J Chromatogr B 878:1647–1651. https://doi.org/10.1016/j.chromb.2010.04.020

Zhou J, Du SY, Dong HJ, Fang L, Feng JH (2019) Preparative separation of monoterpenoid indole alkaloid epimers from *Ervatamia yunnanensis* Tsiang by pH-zone refining counter-current chromatography combined with preparative high-performance liquid chromatography. Molecules 24:1316. https://doi.org/10.3390/molecules24071316

Zhu H, Wang D, Wen L, Yu J, Geng Y, Zhao H, Zhao R, Wang X (2016) Preparative separation of quaternary ammonium alkaloids from *Caulis mahoniae* by conventional and pH-zone-refining counter-current chromatography. RSC Adv 6:83343–83349. https://doi.org/10.1039/C6RA14056D

Zou D, Du Y, Kuang J, Sun S, Ma J, Jiang R (2018) pH-zone refining counter-current chromatography with a hydrophilic organic/salt-containing two-phase solvent system for preparative separation of poplar alkaloids from natural products. J Chrmatogr A 1553:1–6. https://doi.org/10.1016/j.chroma.2018.04.007

Zuo GL, Kim HY, Quispe YNG, Wang ZQ, Hwang SW, Shin KO, Lim SS. (2021) Efficient separation of phytochemicals from *Muehlenbeckia volcanica* (Benth.) Endl. by polarity stepwise elution counter-current chromatography and their antioxidant, antiglycation and aldose reductase inhibition potentials. Molecules 26:224. https://doi.org/10.3390/molecules26010224