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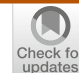
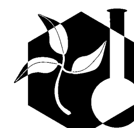


An overview of the two-phase solvent systems used in the countercurrent separation of phenylethanoid glycosides and iridoids and their biological relevance

Simon Vlad Luca · Anca Miron · Svetlana Ignatova · Krystyna Skalicka-Woźniak



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Abstract Phenylethanoid glycosides (PhGs) and iridoids are two vast groups of water soluble secondary metabolites widely distributed in plant kingdom and well known for their important biological activities. Their purification by conventional chromatography is time consuming, uses large amounts of organic solvents and requires repeated steps. Moreover, the sample recovery is low, because the hydroxyl groups of PhGs and iridoids make them strongly adsorbed onto the solid support (silica gel, Sephadex LH-20) during separation. Being a liquid–liquid based technology, countercurrent separation (CCS) comes as an alternative tool to overcome the issues associated with solid-phase adsorbents. It has been successfully used for the separation of many groups of specialized plant metabolites. This is the first extensive review describing the application of CCS for purification of iridoids and PhGs, based on the research papers from the last 20 years (1998–2018) that used countercurrent chromatography and centrifugal partition chromatography technologies. In total, 65 papers described the isolation of 84 different phytochemicals (28 PhGs and 56 iridoids) from 42 plant species belonging to 16 distinct families that were separated with 59 different biphasic solvent systems. Since PhGs and iridoids are highly polar molecules, more than half of the employed systems (56%) were composed of various ratios of ethyl acetate–alcohol–water, as well as binary systems, such as ethyl acetate–water and *n*-butanol–water. The current review may be used as starting point for CCS users on their sinuous road of isolating known or waiting to be discovered PhGs and iridoids.

Keywords Iridoids · Phenylethanoid glycosides · Centrifugal partition chromatography · Countercurrent chromatography · Counter-current chromatography · Natural products · Purification

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S. V. Luca · A. Miron
Department of Pharmacognosy, Grigore T. Popa University of Medicine and Pharmacy Iasi, 16 Universitatii Street, 700115 Iasi, Romania

S. V. Luca · K. Skalicka-Woźniak (✉)
Department of Pharmacognosy with Medicinal Plant Unit, Medical University of Lublin, 1 Chodzki Street, 20-093 Lublin, Poland
e-mail: kskalicka@pharmacognosy.org

S. Ignatova
Department of Chemical Engineering, Advanced Bioprocessing Centre, CEDPS, Brunel University London, Kingston Lane, Uxbridge UB8 3PH, UK

Introduction

Countercurrent separation (CCS) is widely used for the purification or analysis of bioactive molecules. It is a support-free liquid–liquid extraction technique, where both stationary and mobile phases are two immiscible liquids, with stationary phase being retained in the column due to gravitational and centrifugal forces (Ito 2005). CCS can be divided into countercurrent chromatography (CCC) and centrifugal partition chromatography (CPC) (Friesen et al. 2015). CCC mainly involves machines with a variable gravity field produced by a double axis planetary motion and a seal-free arrangement of the column (generally tubing wrapped around bobbins) and so-called "flying leads", tubing connecting the column(s) with ancillary equipment. CPC employs a constant gravity field produced by a single axis of rotation, together with rotatory seals for supply of solvent. CPC separation takes place in cells or chambers which are engraved in cartridges or disks tightly bolted together (Marston and Hostettmann 2006).

A series of favorable characteristics such as high loading capacity, lack of irreversible adsorption, low solvent consumption and ability to handle crude extracts, make CCS a frequently used technique for natural product separations (Ito 2005). Additionally, there are other irrefutable advantages: scale-up can be easily achieved; analytical grade solvents can be used, ensuring thus a high flexibility; the costs of purification is lower, as lower grade and more environmentally friendly solvents can be used; maximum sample recovery is observed (which is especially important in bioactivity-guided separation) and bioactivity can be preserved as solvent systems could be designed for a particular target molecule. Available instruments nowadays are automated, relatively user-friendly and designed to work at low pressures and high flow rates (Friesen et al. 2015).

Selection of a solvent system is the most crucial step in any CCS method development in order to achieve an optimum separation based on different partitioning of compounds between two liquid phases. Most of the available solvent and solute combinations that form a biphasic or multiphasic solvent system may be used in a CCS instrument. The wide range of possible combinations allows creating mixtures with a wide range of polarity and selectivity (Friesen et al. 2015). Two or more solvents may be mixed in an infinite number of proportions. The most important is to find the proper solvent system that provides the appropriate partition coefficient (K) value(s) to deliver the target compounds into the K value "sweet spot" of optimal resolution, which has been defined as $0.4 < K < 2.5$ in regular elution mode (Friesen and Pauli 2007). A smaller K value results in a loss of peak resolution, whilst a higher value produces excessive band broadening.

The experimental procedure normally includes the determination of the partition coefficient of the target solute and related impurities in pre-selected biphasic solvent systems with the shake flask method. The concentration of the compound in each upper and lower phase is commonly determined by gas chromatography (GC) or high-performance liquid chromatography (HPLC) analysis (Hopmann et al. 2012).

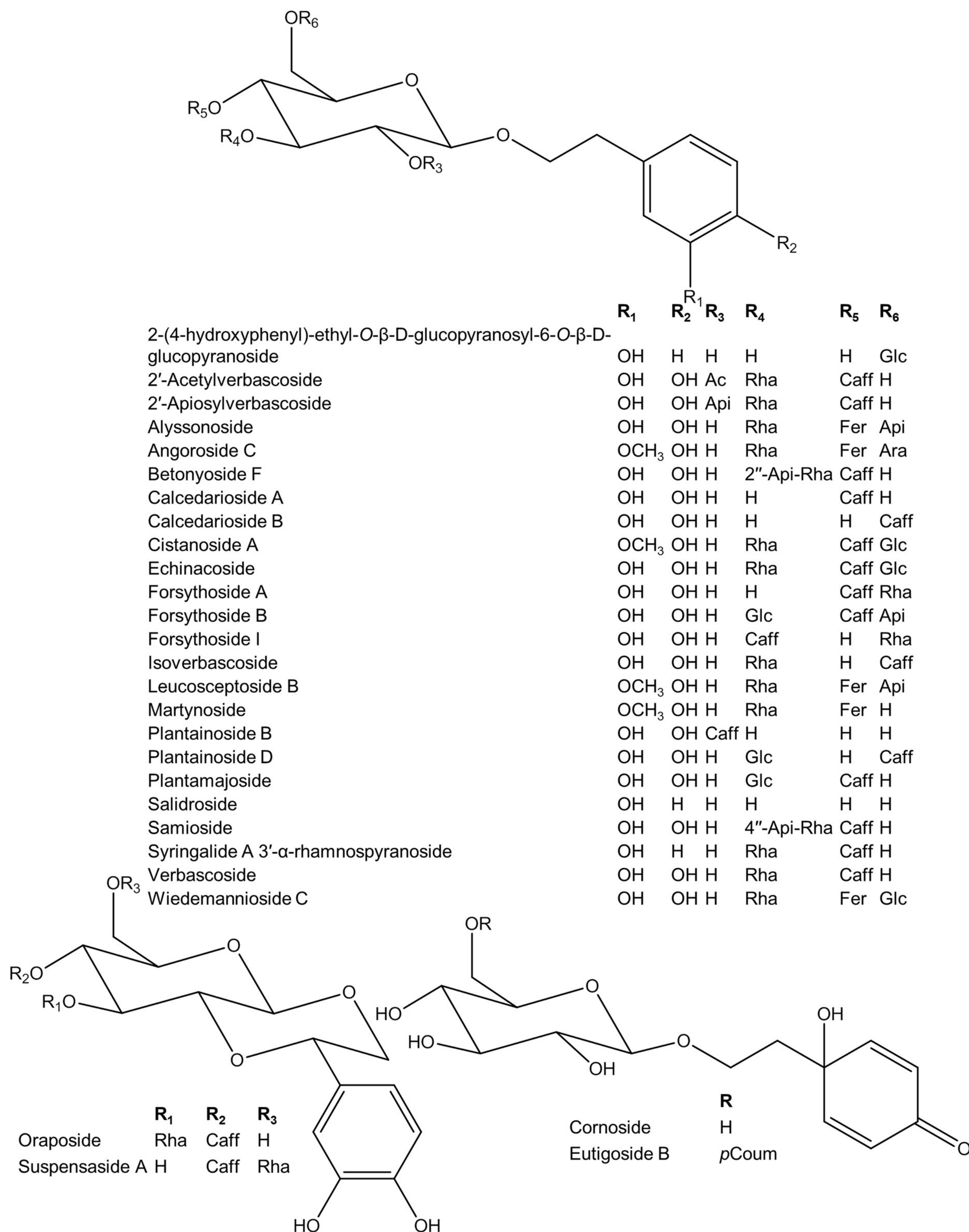
Phenylethanoid glycosides (PhGs) and iridoids are two vast groups of water soluble secondary metabolites widely distributed in plant kingdom. Despite the fact that they have different structures and biosynthetic pathways, their co-existence in several plant families is a well-known fact. *PhGs* are structurally characterized by a β-glucopyranose core unit to which a hydroxyphenethyl (C6–C2) moiety is attached through a glycosidic bond. Phenylpropanoid (C6–C3) residues, acetyl groups and other monosaccharides (rhamnose, apiose, galactose, xylose, etc.) are frequently linked to the glucose center of the molecule (Jimenez and Riguera 1994; Alipieva et al. 2014). Since most PhGs contain caffeic acid as aromatic acid moiety, they are also termed sometimes as caffeic acid glycoside esters, phenylpropanoid glycosides or caffeoyl phenylethanoid glycosides (Calis 2002). These compounds are not specific to any plant organ, being isolated from roots, barks, leaves, aerial parts as well as from callus and suspension cultures. The majority of the PhGs reported to date were found in the Scrophulariaceae, Oleaceae, Plantaginaceae, Lamiaceae and Orobanchaceae families. For example, verbascoside, the most prevalent representative, has been identified in more than 200 plant species belonging to 23 families (Schaluer et al. 2004). The early steps of the biogenetic pathways of PhGs are known, but several key enzymes and their corresponding genes remain to be discovered. However, the hydroxyphenethyl and phenylpropanoid moieties of a typical PhG (such as verbascoside) are synthetized

from different types of precursors: tyrosine, tyramine and/or dopamine precursors by the shikimate pathway and phenylalanine via a cinnamate pathway. Tyramine is incorporated into PhGs through oxidation to the corresponding aldehyde, reduction to alcohol and, finally, β-glycosylation (Alipieva et al. 2014). In the case of dihydroxy derivatives, it was observed that the dihydroxy precursors (dopamine/L-DOPA) are less efficiently incorporated than their monohydroxy equivalents, suggesting that decarboxylation and deamination precede the hydroxylation at C-3 of the aromatic residues (Jimenez and Riguera 1994). Based on the number of sugar moieties, PhGs can be classified into monoglycosides (e.g. calcedariosides A, B, salidroside, plantainosides C and D), diglycosides (verbascoside, isoverbascoside, martynoside, forsythosides A and B) and triglycosides (alyssonoside, angoroside C, cistanoside A, echinacoside, wiedemannioside C, leucosceptoside B). PhGs have been increasingly brought to researchers' attention in the past few decades, as they were found to possess promising antioxidant, neuroprotective, antitumor, antiproliferative, hepatoprotective, antimicrobial, antiprotozoal, anti-inflammatory, immunomodulating and cardioprotective properties (Fu et al. 2008; Xue and Yang 2016). Some PhGs (verbascoside, oraposide, jionoside C) have already been included in several clinical trials (Jimenez and Riguera 1994). "General" verbascoside from *Rehmaniae* leaves (which is a mixture of PhGs extracted from *Rehmania glutinosa* Libosch.) reduced proteinuria and erythrocyturia in patients diagnosed with primary chronic glomerulonephritis after 8 weeks of administration (200 mg tablets, twice/day) alone or in combination with irbesartan (Qiu et al. 2013, 2014).

Iridoids form a perpetually expanding group of cyclopentano[*c*]pyran monoterpenoids. Before the 1990s, there were 827 iridoid glycosides, secoiridoids, iridoid aglycones, iridoid derivatives and bisiridoids reported in literature (El-Naggar and Beal 1980; Boros and Stermitz 1990, 1991) and further 877 new iridoids were isolated from 1994 to 2010 (Dinda et al. 2007a, b, 2009, 2011). The bicyclic H-5/H-9*β* β-*cis*-fused cyclopentano[*c*]pyran ring system is the most common structural feature of these compounds; cleavage of cyclopentane ring produces secoiridoids, while cleavage of pyran ring produces cyclopentane derivatives (Dinda et al. 2007a). It was initially believed that iridoids are biogenetically derived from geranyl pyrophosphate through the action of terpene synthase, which generates cationic species that are subsequently cyclized and rearranged in hundreds of possible structures (Sampaio-Santos and Kaplan 2001). Later, when iridoid synthase was discovered by Geu-Flores et al. (2012), it was shown that its substrate is represented by 8-oxogeranial which is reduced to an enol intermediate using nicotinamide adenine dinucleotide phosphate (NADPH) that is further cyclized via a step-wise Michael addition to form the characteristic bicyclic 5–6 ring iridoid framework of nepetalactol; nepetalactol can easily give rise to any known iridoid (Lindner et al. 2014). The last steps in the biosynthesis of iridoids are considered to be *O*-glycosylation and *O*-alkylation. Iridoid synthesis is often regarded as an alternative route to the typical monoterpene biosynthesis. For instance, in Lamiaceae family, an inverse relationship between the production of monoterpenes and iridoids was observed (Sampaio-Santos and Kaplan 2001).

El-Naggar and Beal (1980) divided iridoids into several groups: iridoid glycosides with C-8, C-9 (e.g. catalpol, catalposide, harpagoside) and C-10 (e.g. gardenoside, geniposide, loganin) carbon skeleton, secoiridoids (e.g. gentiopicroside, morronoside, oleuropein, sweroside, swertiamarin), bisiridoids, bis-secoiridoids and non-glycosidic derivatives. Iridoids are present in numerous medicinal plants endowed with bitter, sedative, antipyretic, antitussive, wound healing and hypotensive properties. This fact encouraged the investigation of their bioactivities, leading to documented neuroprotective, anti-inflammatory, immunomodulating, hepatoprotective, cardioprotective, antitumor, antioxidant, antimicrobial, hypoglycemic, hypolipidemic, choleretic and spasmolytic effects (Tundis et al. 2008; Viljoen et al. 2012).

The conventional purification of PhGs and iridoids by semi-preparative HPLC and other types of column chromatography is time consuming, uses large amounts of organic solvents and requires repeated steps. Moreover, the sample recovery is low, because the hydroxyl groups of PhGs and iridoids make them strongly adsorbed onto the solid support (silica gel, Sephadex LH-20) during separation (Li et al. 2005). Therefore, CCS comes as an alternative tool to overcome the issues associated with solid-phase adsorbents and it has been successfully used for the separation of various natural products (Marston and Hostettmann 2006).



	R1	R2	R3	R4	R5	R6
2-(4-hydroxyphenyl)-ethyl-*O*-β-D-glucopyranosyl-6-*O*-β-D-glucopyranoside	OH	H	H	H	H	Glc
2′-Acetylverbascoside	OH	OH	Ac	Rha	Caff	H
2′-Apiosylverbascoside	OH	OH	Api	Rha	Caff	H
Alyssonoside	OH	OH	H	Rha	Fer	Api
Angoroside C	OCH_3	OH	H	Rha	Fer	Ara
Betonyoside F	OH	OH	H	2″-Api-Rha	Caff	H
Calcedarioside A	OH	OH	H	H	Caff	H
Calcedarioside B	OH	OH	H	H	H	Caff
Cistanoside A	OCH_3	OH	H	Rha	Caff	Glc
Echinacoside	OH	OH	H	Rha	Caff	Glc
Forsythoside A	OH	OH	H	H	Caff	Rha
Forsythoside B	OH	OH	H	Glc	Caff	Api
Forsythoside I	OH	OH	H	Caff	H	Rha
Isoverbascoside	OH	OH	H	Rha	H	Caff
Leucosceptoside B	OCH_3	OH	H	Rha	Fer	Api
Martynoside	OCH_3	OH	H	Rha	Fer	H
Plantainoside B	OH	OH	Caff	H	H	H
Plantainoside D	OH	OH	H	Glc	H	Caff
Plantamajoside	OH	OH	H	Glc	Caff	H
Salidroside	OH	H	H	H	H	H
Samioside	OH	OH	H	4″-Api-Rha	Caff	H
Syringalide A 3′-α-rhamnospyranoside	OH	H	H	Rha	Caff	H
Verbascoside	OH	OH	H	Rha	Caff	H
Wiedemannioside C	OH	OH	H	Rha	Fer	Glc

	R1	R2	R3
Oraposide	Rha	Caff	H
Suspensaside A	H	Caff	Rha

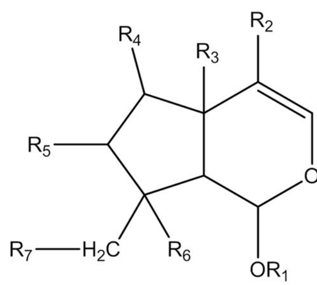
	R
Cornoside	H
Eutigoside B	*p*Coum

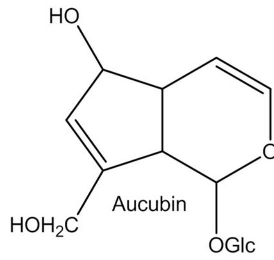
Fig. 1 Phenylethanoid glycosides isolated through countercurrent separation. *Ac* acetyl, *Api* apiosyl, *Ara* arabinosyl, *Caff* caffeoyl, *Fer* feruloyl, *Glc* glucosyl, *pCoum p*-coumaroyl, *Rha* rhamnosyl

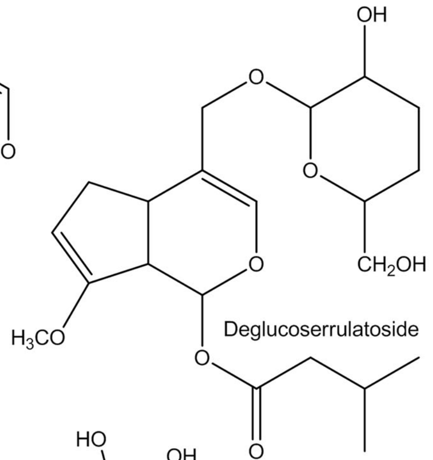
Methodology

In order to assess the use of two phase solvent systems in isolation of PhGs and iridoids, the search for papers describing all aspects concerning their CCS was carefully performed in online databases (Scopus, PubMed). In the first step, all possible terms denoting the technique ("counter-current chromatography", "countercurrent chromatography", "CCC", "HSCCC", "HPCCC", "centrifugal partition chromatography", "CPC") were paired one by one with terms designating the class of interest compounds ("iridoid", "bisiridoid", "secoiridoid", "phenylethanoid glycosides", "phenylpropanoid glycosides"). However, when the search was performed with the latter combination, some of the accessed results were not taken into consideration as not all phenylpropanoid glycosides are structurally included in the phytochemical class of PhGs (syringine, eleutheroside D). Next, each listed compound was searched in the above mentioned databases in combination with previously described terminologies related to CCS techniques. Some compounds, especially phenylethanoid mono- and di-glycosides, raised confusions regarding their nomenclature. For example, verbascoside, acteoside, kusaginin and orabanchin have the same chemical structure of 2-(3,4-dihydroxyphenyl)-ethyl-1-*O*-α-L-rhamnopyranosyl-(1 → 3)-(4-*O*-*E*-caffeoyl)-β-D-glucopyranoside. Other examples are isoverbascoside often named as isoacteoside, oraposide as orabanchoside or crenatoside, and calcedarioside often referred to as calceolarioside. Thus, when the database searches were conducted, all designations were used as keywords, but in order to avoid confusion, only "verbascoside", "isoverbascoside", "oraposide" and "calcedarioside" were subsequently employed as terminology in this paper.

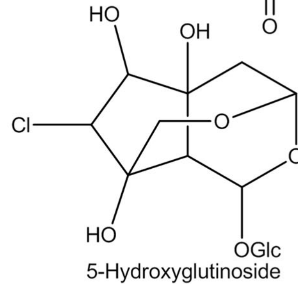
Finally, the authors collected 65 research papers from the last 20 years (1998–2018) describing CCC and CPC isolation of PhGs and iridoids. In total, 84 different phytochemicals (Figs. 1, 2) were separated from 42 plant species belonging to 16 distinct families. The results are presented hereafter in form of two tables (Tables 1, 2) that sum up the most important features of the CCS of PhGs and iridoids. Undoubtedly, the information about the source of the target compounds (plant name, family, plant parts) is primarily given. As extraction is the first step that dictates the type and complexity of a matrix, the extraction solvent is further specified. Since yield and purity are important for method development, data about the sample loading, yield and purity of isolated compounds were provided. Of course, the operating parameters for CCS, such as type of technique (CCC/CPC), elution mode (isocratic/step-gradient/step-flow gradient, dual mode, NP, RP) as well as the composition of the solvent systems and K values were also given in these tables. Moreover, in order to give the readers an idea about the number of isolation steps required, up- and down-stream complementary chromatographic techniques (column chromatography, thin-layer chromatography, semi-preparative HPLC) were included. Further insights are provided in the Electronic Supplementary Material. The data in the Excel table are arranged in alphabetical order of the plant species, with additional subclassification of each compound, sample loading details (sample loading per injection, sample volume, sample concentration, column volume, sample loaded per each 100 mL of column volume, sample solvent, and sample solvent ratio), CCC/CPC column details (internal diameters or number of cells), operating parameters (column's revolution speed, mobile phase flow-rate, eluant detection wavelength), instrument model and manufacturer. One of the main differences as compared to Tables 1 and 2 comes from the fact that normalized solvent ratios were given in this file, in order to make solvent systems easily comparable among each other. Another aspect that was taken into consideration regarding the organization of this file was that compounds within the same plant species were placed according to their elution order from the CCS column. Moreover, the logP values, a parameter important for estimating the distribution of a compound between two immiscible phases, were calculated using ChemDraw Ultra 12.0 software. As we are not aware of the existence of a similar work, this study can act as a starting point for CCS users that target the isolation of compounds belonging to these two phytochemical classes. In the next section, an extensive discussion on the use of CCS in isolating PhGs and iridoids with focus on employed solvent systems is presented. Finally, the last part is devoted to analyzing the biological relevance of PhGs and iridoids isolated through CCS.



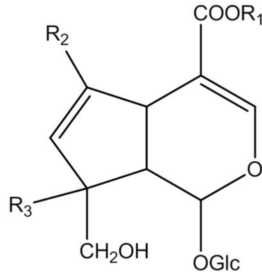




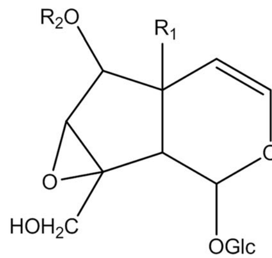
	R_1	R_2	R_3	R_4	R_5	R_6	R_7
6-β-hydroxyantirrhide	Glc	H	H	OH	OH	$=CH_2$	
7-*O*-(4-maloyl)loganin	Glc	$COOCH_3$	H	H	OMal	H	H
7-Dehydrologanin	Glc	$COOCH_3$	H	H	=O	H	H
8-O-acetylharpagide	Glc	H	OH	OH	H	OAc	H
10-Hydroxyhastatoside	Glc	$COOCH_3$	OH	=O	H	H	OH
10-Hydroxycornin	Glc	$COOCH_3$	H	=O	H	H	OH
Caprarioside	Glc	H	OH	OH	H	OBz	H
Chlorotuberside	Glc	$COOCH_3$	H	OH	Cl	OH	H
Harpagoside	Glc	H	OH	OH	H	OCinn	H
Ipolamiide	Glc	$COOCH_3$	OH	H	H	OH	H
Loganetin	H	$COOCH_3$	H	H	OH	H	H
Loganic acid	Glc	COOH	H	H	OH	H	H
Loganin	Glc	$COOCH_3$	H	H	OH	H	H
Penstemonoside	Glc	$COOCH_3$	OH	OH	H	H	H
Phloyoside II	Glc	$COOCH_3$	OH	OH	Cl	OH	H
Shanzhiside methyl ester	Glc	$COOCH_3$	H	OH	H	OH	H



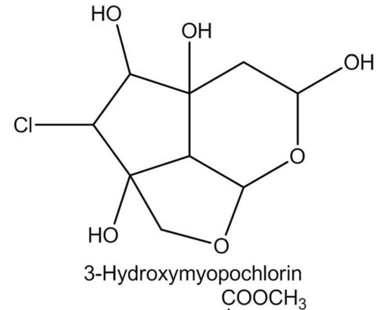
5-Hydroxyglutinoside



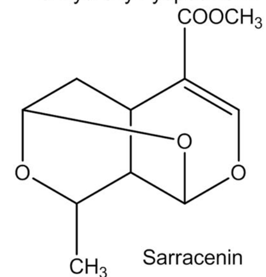
	R_1	R_2	R_3
6-β-Hydroxy geniposide	CH_3	OH	H
Gardenoside	CH_3	H	OH
Geniposide	CH_3	H	H
Geniposidic acid	H	H	H



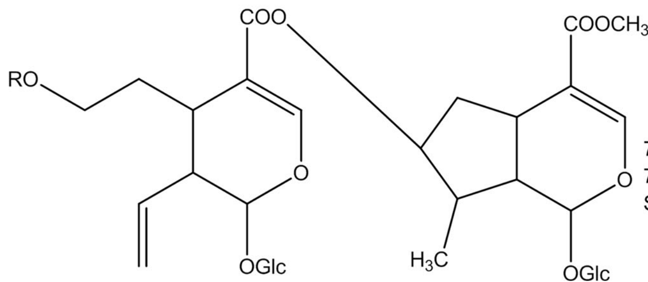
	R_1	R_2
Catalpol	H	H
Catalposide	H	*p*-OH-Bz
Macfadyenoside	OH	H
Picroside I	H	Cinn
Picroside II	H	Van
Picroside III	H	Fer
Verproside	H	3,4diOHBz



3-Hydroxymyopochlorin



Sarracenin



	R
7-*O*-(*E*-caffeoyl)-sylvestroside I	Caff
7-*O*-(*E*-*p*-coumaroyl)-sylvestroside I	*p*Coum
Sylvestroside I	H

◀**Fig. 2** Iridoids isolated through countercurrent separation. *Ac* acetyl, *Bz* benzyl, *Caff* caffeoyl, *Cinn* cinnamoyl, *Fer* feruloyl, *Glc* glucosyl, *Mal* maloyl, *pCoum p*-coumaroyl, *Van* vanilloyl

CCS application to phenylethanoid glycosides and iridoids purification

A total of 28 distinct PhGs and 56 iridoids were isolated using 59 different biphasic solvent systems. Since PhGs and iridoids are highly polar molecules, 66% of all employed solvent systems contained a minimum of 45% water. About 56% of the solvent systems were composed of various ternary ratios of ethyl acetate–alcohol–water or binary systems, such as ethyl acetate–water and *n*-butanol–water. Chlorinated solvents (chloroform, dichloromethane) were present in 18% of the solvent systems and mainly employed for iridoids separations using CCC only. One of these solvent systems contained acetic acid as pH modifier. Chlorinated solvents provide very good solubility for natural products extracts but are mostly avoided nowadays for safety restrictions. Three alkane based systems (5%) employed for purification of secoiridoids and C-9 iridoids included combinations, such as *n*-hexane–ethyl acetate–*n*-butanol–water, *n*-hexane–*n*-butanol–water and *n*-hexane–*n*–butanol–methanol–water–acetic acid. These systems were mostly exceptions for this polarity level of target compounds. From another point of view, the solvent systems used to separate PhGs and iridoids were most often formulated with three (65%) or four (31%) solvent combinations using short-chain alcohols (methanol, ethanol, 1- or 2-propanol) as modifiers to reduce the polarity difference between the two liquid phases.

CCC [also referred as high-speed CCC (HSCCC) or high-performance CCC (HPCCC)] was by far the most used technique, being reported in 83% of the papers, followed by centrifugal partition chromatography (CPC). This can be simply explained by a wider distribution of CCC instruments across the international research community, especially in China, and their lower cost and ease of manufacture. There is almost equal number of CPC and CCC instruments used in industry these days but access to their applications is rather limited (Ignatova and Sutherland 2015). Out of 22 common solvent systems for PhGs separations with CCC, 64% were ethyl acetate–*n*-butanol–water family with ratios depending on anatomical part of a plant (Fig. 3). 73% of CPC papers were about iridoids purifications with ethyl acetate–*n*-propanol–water (35–30:15–20:50) as the most employed solvent systems. The content of ethyl acetate and *n*-butanol varied in moving from iridoids C-9 to C-10, followed by secoiridoids and bis-secoiridoids, according to their decreasing polarity order. The least polar system *t*-butyl methyl ether–acetonitrile–water (30:30:40) was used for the isolation of non-glycosidic iridoids. Some variations in the mode of operation of the CCC and CPC columns were observed during the survey, including step gradient elution (de Juliao et al. 2010; Leitao et al. 2015), enhancement of flow rate (Liang et al. 2013b; Rho et al. 2016), two-step CCS (Lei et al. 2001b; Lemus et al. 2015; Li et al. 2008), elution–extrusion CCS (Chen et al. 2011; Liang et al. 2018) and recycling CCS (Chen et al. 2011).

CCS and phenylethanoid glycosides' isolation

The 28 PhGs isolated through CCS belonged to eight different plant families: Verbenaceae, Orobonchaceae, Scrophulariaceae, Plantaginaceae, Oleaceae, Lamiaceae, Crassulaceae and Gesneriaceae. Verbascoside and isoverbascoside were by far the most frequently isolated PhGs (23 and 8 times, respectively), either intentionally or as part of a larger group (Table 1). Possessing a wider polarity range as compared to monoglycosides and triglycosides, phenylethanoid diglycosides are the most distributed class of PhGs. Consequently, a high number of CCS applications focused on their isolations. Nevertheless, they were also separated altogether with mono-/triglycosides from various plant matrices [mono + di: *Abeliophyllum distichum* (Li et al. 2013), *Chirita longgangensis* (Duan et al. 2014); di + tri: *Cistanche deserticola* (Li et al. 2008); *Lamiophlomis rotata* (Yue et al. 2013a), *Lantana trifolia* (de Juliao et al. 2010), *Pedicularis longiflora* var. *tubiformis* (Zhang et al. 2015), *Penstemon barbatus* (Wang et al. 2013)].

Out of the 21 plant species, PhGs were mainly isolated by CCC from extracts of roots (8), stems (4), whole plants (5) and upper parts [aerial parts (3), leaves (5)], with 14 solvent systems based on ethyl acetate–*n*-butanol–water family (Fig. 3). The matrix of a plant extract and its complexity (including additional purification steps before CCS) has obvious effects on the solvent system polarity. For the

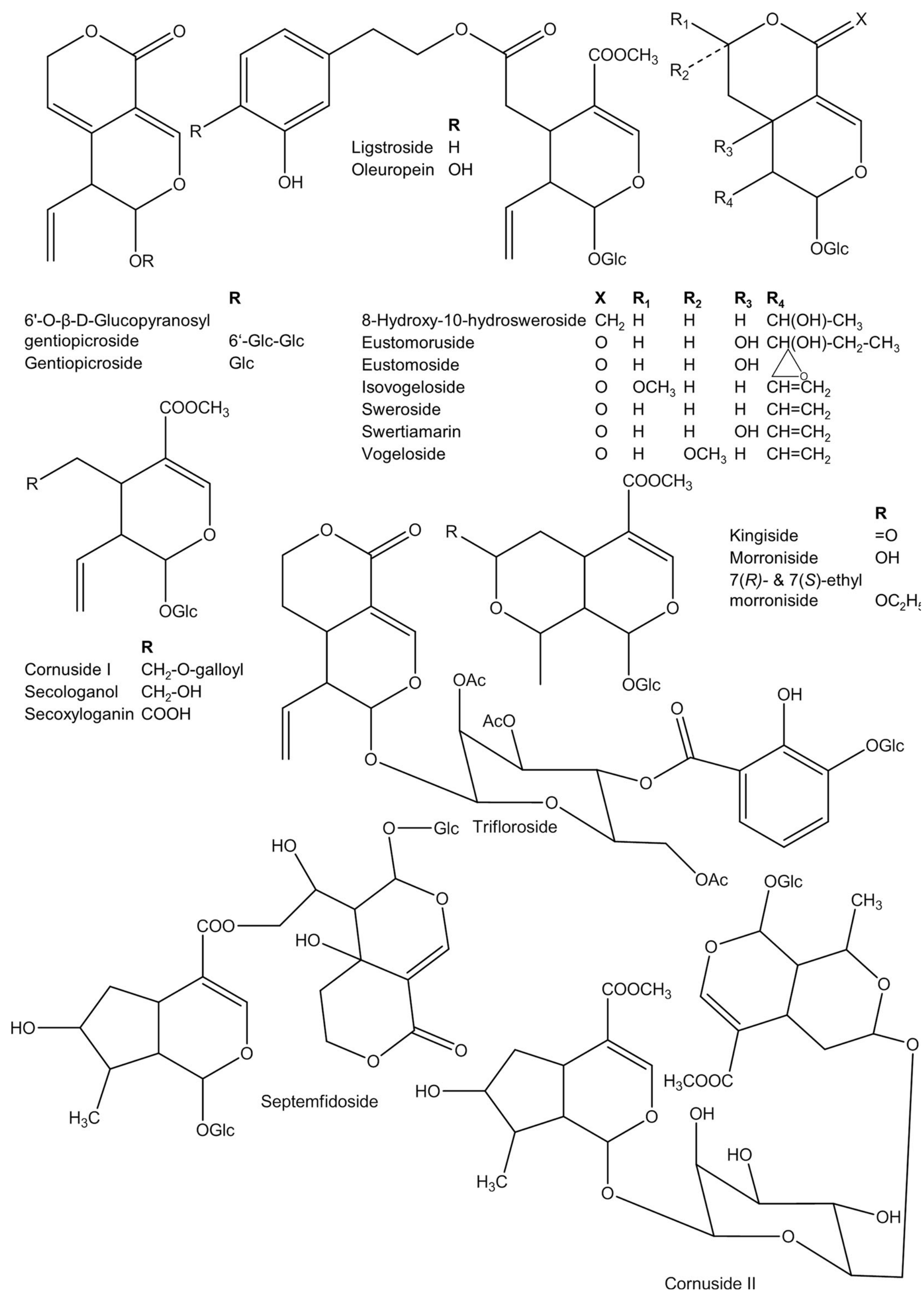


Fig. 2 continued

Table 1 List of phenylethanoid glycosides isolated through countercurrent separation

Compound	Species	Extraction	Sample amount	Isolated amount	Purity	Upstream	CCS technique	CCS solvent system	Downstream	K value	References
2-(4-hydroxyphenyl)-ethyl-*O*-β-D-glucopyranosyl-6-*O*-β-D-glucopyranoside	*Rhodiola crenulata* Fish et Mey (Crassulaceae) roots	80% Et	150 mg	20.9 mg	99.6%	LLE+ MR D101	CCC, isocratic, RP	EBuWat (0.5:4.5:5)	spHPLC	0.29	Chen et al. 2012
2′-Acetyl verbascoside	*Lippia alba* f. *intermedia* Moldenke (Verbenaceae) aerial parts	96% Et	3 g	182 mg	73.9%	LLE	CCC, step-gradient, NP	HEBuWat (4:10:x:10, x=1-7)		–	Leitao et al. 2015
	Cistanche deserticola Y.C. Ma (Orobanchaceae) stems	80% Et	1412 mg	25.2 mg	92.5%	LLE	CCC, isocratic, RP	EEtWat (5:0.5:4:5)		1.30	Li et al. 2008
	Cistanche salsa (C.A. Mey.) G. Beck (Orobanchaceae) stems	75% Et	228 mg	ns	98.0%	LLE	CCC, isocratic, two-steps, RP	EBuEtWat (4:0.6:0.6:5)		–	Lei et al. 2001a
	Cistanche deserticola Y.C. Ma (Orobanchaceae) stems	60% Et	297 mg	7.2 mg	98.0%	LLE + SG	CCC, isocratic, RP	EBuEtWat (4:0.6:0.6:5)		3.66	Han et al. 2012
2′-Apiosyl verbascoside	*Lantana trifolia* L. (Verbenaceae) aerial parts	96% Et	500 mg	39.3 mg	81.8%	LLE	CCC, isocratic, NP	EBuWat (10:2:10)		–	Leitao et al. 2015
Alyssonoside	*Lamiophlomis rotata* (Benth.) Kudo (Lamiaceae) whole plant	65% Et	150 mg	29 mg	99.5%	LLE + MR	CCC, isocratic, RP	EBuWat (13:3:10)		0.61	Yue et al. 2013a
Angoroside C	*Scrophularia ningpoensis* Hemsl. (Scrophulariaceae) ns	90% Et	311 mg	49 mg	98.5%	LLE	CCC, dual mode, RP/NP	EBuWatAa (1:8:10:1)	SG	0.51	Tong et al. 2009
	Scrophularia ningpoensis Hemsl. (Scrophulariaceae)	70% Et	200 mg	31 mg	98.5%	MR D101	CCC, isocratic, NP	ChBuMWat (4:1:3:2)		1.81	Tian et al. 2012
Betonyoside F	*Lantana trifolia* L. (Verbenaceae) leaves	Et	3 g	118.2 mg	ns	LLE	CCC, step-gradient, NP	HEBuWat (4:10:x:10, x=1-7)		–	Julio et al. 2010
Calcedariosides A and B	*Chirita longgangensis* W.T.Wang (Gesneriaceae) aerial parts	95% Et	200 mg	16.7 mg 18.1 mg	98.8% 97.9%	MR D101	CCC, isocratic, RP	EBuMWat(5:0.1:0.5:4.5)		3.68 1.95	Duan et al. 2014
Cistanoside A	*Cistanche deserticola* Y.C. Ma (Orobanchaceae) stems	80% Et	1412 mg	18.4 mg	92.5%	LLE + CCC with EBuWat (5:0.5:4.5)	CCC, isocratic, two-steps, RP	EBuEtWat (0.5:0.5:0.1:1)		0.52	Li et al. 2008
Cornoside	*Abeliophyllum distichum* Nakai (Oleaceae) leaves	M	1 g	45 mg	93.6%		CCC, isocratic, RP	EBuWat (8:0.7:5)		0.31	Li et al. 2013
Echinacoside	*Pedicularis longiflora* Rudolph. var. tubiformis (Klotz) Tsong (Scrophulariaceae)	70% Et	120 mg	3.5 mg	93.6%	LLE + MR	CCC, isocratic, RP	EBuWat (10:6:15)		0.22	Zhang et al. 2015
	Cistanche deserticola Y.C. Ma (Orobanchaceae) stems	80% Et	1412 mg	28.5 mg	92.5%	LLE + CCC with EBuWat (5:0.5:4.5)	CCC, isocratic, two-steps, RP	EBuEtWat (0.5:0.5:0.1:1)		0.92	Li et al. 2008
	Penstemon barbatus (Cav.) Roth (Plantaginaceae) leaves	50% M	ns	42 mg	96.3%	MR AB8	CCC, CECCC, RP	BuWat (1:1)		0.78	Xie et al. 2010
	Cistanche tubulosa (Schrenk) Wight (Orobanchaceae) ns	80% M	220 mg	16.9 mg	99.1%		CCC, isocratic, NP	EBuWatAa (1:1.2:2:0.2)		0.55	Xie et al. 2012b
	Penstemon digitalis Nutt. ex Sims (Plantaginaceae) leaves	50% M	ns	3.96 mg	98.9%	MR AB8	CCC, isocratic, RP	BuWat (1:1)	spHPLC	0.75	Wang et al. 2013
Eutigoside B	*Abeliophyllum distichum* Nakai (Oleaceae) leaves	M	1 g	13.9 mg	95.4%		CCC, isocratic, RP	EBuWat (8:0.7:5)		2.14	Li et al. 2013
Forsythoside A	*Forsythia suspensa* (Thunb.) Vahl (Oleaceae) fruits	70% Et	250 mg	24.5 mg	98.2%	MR D101	CCC, isocratic, RP	EBuMWat(4:0.5:0.5:5)		0.89	Yang et al. 2013
	Forsythia suspensa (Thunb.) Vahl (Oleaceae) leaves	0.6 M [C_6MIM]Br	120 mg	59.7 mg	97.9%	PA	CCC, isocratic, RP	EEtWatAa (4:1:6:0.25)		0.88	Sun et al. 2016
Forsythoside B	*Pedicularis longiflora* Rudolph. var. tubiformis (Klotz) Tsong (Scrophulariaceae) whole plant	70% Et	120 mg	22.7 mg	97.8%	LLE + MR	CCC, isocratic, RP	EBuWat (10:6:15)		1.16	Zhang et al. 2015
	Lamiophlomis rotata (Benth.) Kudo (Lamiaceae) whole plant	65% Et	150 mg	27 mg	97.7%	LLE + MR	CCC, isocratic, RP	EBuWat (13:3:10)		0.33	Yue et al. 2013a
Forsythoside I	*Forsythia suspensa* (Thunb.) Vahl (Oleaceae) leaves	0.6 M [C_6MIM]Br	120 mg	5.4 mg	96.1%	LLE + MR	CCC, isocratic, RP	EBuWat (10:6:15)		0.86	Sun et al. 2016
Isoverbascoside	*Stachytarpheta cayennensis* (Rich.) Vahl. (Verbenaceae) roots	Et	1 g	37 mg	ns	LLE	CCC, step-gradient, NP	EBuWat (1:x:1, x=0.05-1)		–	Leitao et al. 2005
	Abeliophyllum distichum	M	1 g	10.2 mg	93.1%		CCC, isocratic,	EBuWat (8:0.7:5)		1.44	Li et al.

Table 1 continued

Compound	Species	Extraction	Sample amount	Isolated amount	Purity	Upstream	CCS technique	CCS solvent system	Downstream	K value	References
	Nakai (Oleaceae) leaves						RP				2013
	Pedicularis longiflora Rudolph. var. *tubiformis* (Klotz) Tsong (Scrophulariaceae) whole plant	70% Et	40 mg	18 mg	98.0%	LLE + SG	CCC, isocratic, RP	ChBuMWat (4:3:4:5)		0.83	Chen et al. 2014
	Cistanche deserticola Y.C. Ma (Orobanchaceae) ns	80% Et	1412 mg	30.1 mg	92.5%	LLE	CCC, isocratic, two-steps, RP	EEtWat (5:0.5:4.5)		1.11	Li et al. 2008
	Plantago psyllium L. (Plantaginaceae) seeds	80% M	978 mg	17.5 mg	94.0%	LLE	CCC, isocratic, RP	EWat (1:1)		0.92	Li et al. 2005
	Cistanche tubulosa (Schrenk) Wight (Orobanchaceae) stems	M*	214 mg	ns	95%	Sephadex LH-20	CCC, isocratic, two-steps, RP	EBuEtWat (35:6:6:50 and 30:10:6:50)		–	Lei et al. 2001b
	Cistanche deserticola Y.C. Ma (Orobanchaceae) stems	60% Et	297 mg	13 mg	95.0%	LLE	CCC, isocratic, RP	EBuEtWat (4:0.6:0.6:5)		1.29	Han et al. 2012
	Lamiophlomis rotata (Benth.) Kudo (Lamiaceae) whole plant	65% Et	150 mg	23 mg	99.3%	LLE	CCC, isocratic, RP	EBuWat (13:3:10)		3.16	Yue et al. 2013a
Leucosceptoside B	*Lamiophlomis rotata* (Benth.) Kudo (Lamiaceae) whole plant	65% Et	150 mg	13 mg	97.3%	LLE	CCC, isocratic, RP	EBuWat (13:3:10)		1.19	Yue et al. 2013a
Martynoside	*Stachytarpheta cayennensis* (Rich.) Vahl. (Verbenaceae) roots	Et	1 g	55 mg	ns	LLE	CCC, step-gradient, NP	EBuWat (1:x:1, x=0.05-1)		–	Leitao et al. 2005
	Lantana trifolia L. (Verbenaceae) leaves	Et	3 g	5.8 mg	ns	LLE	CCC, step-gradient, NP	HEBuWat (4:10:1:10, x=1-7)	Sephadex LH-20	–	Juliao et al. 2010
Oraposide	*Orobanche rapum-genistae* Thuill. (Orobanchaceae) whole plant	70% Et	300 mg	30 mg	82%		CCC, isocratic, RP/NP	EAcWat RP: 2.6:2.6:4.8 NP: +2% M		1.15	Viron et al. 1998
	Orobanche rapum genistae Thuill. (Orobanchaceae) whole plant	70% Et	6 g	450 mg	100%		CPC, isocratic, RP	EAtWat (10.6:1:9.7)		–	Viron et al. 2000
Plantainosides B and D	*Chirita longgangensis* W.T.Wang (Gesneriaceae) aerial parts	95% Et	200 mg	9.5 mg 28.4 mg	94.4% 97.6%	MR D101	CCC, isocratic, RP	EBuMWat(5:0.1:0.5:4.5)		0.82 0.35	Duan et al. 2014
Plantamajoside	*Plantago asiatica* L. (Plantaginaceae) aerial parts	80% Et	1.341 g	45.6 mg	93.3%	LLE	CPC, isocratic, RP	EBuEtWat (0.5:0.5:0.1:1)		2.69	Li et al. 2009
Salidroside	*Rhodiola crenulata* Fish et Mey (Crassulaceae) roots	AtWat (70:30)	1216 mg	21.9 mg	98.0%	LLE	CCC, iscratic, two-steps, RP	EBuWat (1:4:5) and ChMIsoWat (5:6:1:4)		–	Han et al. 2002
	Rhodiola sachalinensis A. Bor (Crassulaceae) roots	M	250 mg	32 mg	98.0%		CCC, isocratic, RP	EBuWat (3:2:5)		0.52	Li and Chen 2001
	Rhodiola rosea L. (Crassulaceae) roots	M	100 mg	0.5 mg	90.0%	LLE	CCC, isocratic, RP	EBuWat (3:2:5)		0.65	Mudge et al. 2012
	Rhodiola crenulata Fish et Mey (Crassulaceae) roots	80% Et	150 mg	120.2 mg	99.1%	LLE	CCC, isocratic, RP	EBuWat (0.5:4.5:5)	spHPLC	0.85	Chen et al. 2012
Samioside	*Lantana trifolia* L. (Verbenaceae) leaves	Et	3 g	5.9 mg	ns	LLE	CCC, step-gradient, NP	HEBuWat (4:10:1:10, x=1-7)	Sephadex LH-20 and spHPLC	–	Juliao et al. 2010
Suspensaside A	*Forsythia suspensa* (Thunb.) Vahl (Oleaceae) fruits	70% Et	250 mg	8.8 mg	98.0%	MR D101	CCC, isocratic, RP	EBuMWat (4:0.5:0.5:5)		1.94	Yang et al. 2013
Syringalide A 3′-α-rhamnospyranoside	*Cistanche deserticola* Y.C. Ma (Orobanchaceae) stems	60% Et	297 mg	12.5 mg	99.0%	LLE	CCC, isocratic, RP	EBuEtWat (4:0.6:0.6:5)		2.56	Han et al. 2012
Verbascoside	*Stachytarpheta cayennensis* (Rich.) Vahl. (Verbenaceae) roots	Et	1 g	300 mg	ns	LLE	CCC, step-gradient, NP	EBuWat (1:x:1, x=0.05-1)		–	Leitao et al. 2005
	Abeliophyllum distichum Nakai (Oleaceae) leaves	M	1 g	36.2 mg	94.0%		CCC, isocratic, RP	EBuWat (8:0.7:5)		1.06	Li et al. 2013
	Lippia alba f. *intermedia* Moldenke (Verbenaceae) aerial parts	96% Et	3 g	589 mg	57.9%	LLE	CCC, step-gradient, NP	HEBuWat (4:10:x:10, x=1-7)		–	Leitao et al. 2015
	Lippia javanica Spreng. (Verbenaceae) leaves	50% M	1247 mg	ns	71.0%	SG	CCC, dual mode, NP/RP	EBuWat (10:1:10)		–	Oyourou et al. 2013
	Lantana trifolia L. (Verbenaceae) aerial parts	96% Et	500 mg	29.5 mg	80.5%	LLE	CCC, isocratic, NP	EBuWat (10:2:10)		–	Leitao et al. 2015
	Lantana trifolia L. (Verbenaceae) leaves	Et	3 g	418.5 mg	ns	LLE	CCC, two-steps, step-gradient and isocratic, RP	HEBuWat (4:10:x:10, x=1-7) and HEBuWat (4:10:6:10)		–	Juliao et al. 2010
	Pedicularis longiflora Rudolph. var. *tubiformis* (Klotz) Tsong (Scrophulariaceae) whole plant	70% Et	40 mg	20 mg	97.0%	LLE	CCC, isocratic, RP	ChBuMWat (4:3:4:5)		0.55	Chen et al. 2014
	Pedicularis longiflora Rudolph. var. tubiformis (Klotz) Tsong (Scrophulariaceae) whole plant	70% Et	120 mg	48.7 mg	98.1%	LLE	CCC, isocratic, RP	EBuWat (10:6:15)		4.11	Zhang et al. 2015
	Plantago psyllium L.	80% M	978 mg	165 mg	98.0%	LLE	CCC, isocratic,	EWat (1:1)		0.82	Li et al.

Table 1 continued

Compound	Species	Extraction	Sample amount	Isolated amount	Purity	Upstream	CCS technique	CCS solvent system	Downstream	K value	References
	(Plantaginaceae) seeds						RP				2005
	Cistanche deserticola Y.C. Ma (Orobanchaceae) stems	80% Et	1412 mg	14.6 mg	92.5%	LLE	CCC, isocratic, two-steps, RP	EEtWat (5:0.5:4:5)		0.87	Li et al. 2008
	Cistanche salsa (C.A. Mey.) G. Beck (Orobanchaceae) stems	75% Et	228 mg	ns	98.0%	BuOH L-L fraction	CCC, isocratic, two-steps, RP	EBuEtWat (4:0.6:0.6:5)		–	Lei et al. 2001a
	Cistanche tubulosa (Schrenk) Wight (Orobanchaceae) stems	M*	214 mg	ns	95%	Sephadex LH-20	CCC, isocratic, two-steps, RP	EBuEtWat (35:6:6:50 and 30:10:6:50)		–	Lei et al. 2001b
	Cistanche deserticola Y.C. Ma (Orobanchaceae) stems	60% Et	297 mg	30.9 mg	99.0%	LLE + SG	CCC, isocratic, RP	EBuEtWat (4:0.6:0.6:5)		1.20	Han et al. 2012
	Lamiophlomis rotata (Benth.) Kudo (Lamiaceae) whole plant	65% Et	150 mg	41 mg	99.2%	LLE + MR	CCC, isocratic, RP	EBuWat (13:3:10)		1.96	Yue et al. 2013a
	Penstemon barbatus (Cav.) Roth (Plantaginaceae) leaves	50% M	ns	79.6 mg	96.4%	MR AB8	CCC, isocratic, RP	BuWat (1:1)		3.37	Xie et al. 2012a
	Cistanche tubulosa (Schrenk) Wight (Orobanchaceae) stens	80% M	220 mg	5.1 mg	95.0%	-	CCC, isocratic, NP	EBuWatAa (1:1.2:2:0.2)		1.11	Xie et al. 2012b
	Penstemon digitalis Nutt. ex Sims (Plantaginaceae) leaves	50% M	ns	67.2 mg	92.6%	MR AB8	CCC, isocratic, RP	BuWat (1:1)		3.37	Wang et al. 2013
	Orobanche rapum-genistae Thuill. (Orobanchaceae) whole plant	70% Et	300 mg	30 mg	75%		CCC, isocratic, RP/NP	EAcWat RP: 2.6:2.6:4.8 NP: +2% M		0.55	Viron et al. 1998
	Rehmannia glutinosa Libosch. (Orobanchaceae) roots	70% Et	165 mg	45 mg	96%	MR D101	CCC, isocratic, RP	EBuWat (1:4:5)		3.10	Bu et al. 2017
	Orobanche rapum-genistae Thuill. (Orobanchaceae) whole plant	70% Et	6 g	1200 mg	80%		CPC, isocratic, RP	EAtWat (10.6:1:9.7)		–	Viron et al. 2000
	Capraria biflora L. (Scrophulariaceae) aerial parts	M	15 g	598 mg	ns		CPC, isocratic, NP	TetEWat (4:9:12)		–	Lemus et al. 2015
	Plantago asiatica L. (Plantaginaceae) aerial parts	80% Et	1341 mg	293.8 mg	93.3%	LLE	CPC, isocratic, RP	EBuEtWat (0.5:0.5:0.1:1)		2.69	Li et al. 2009
	Lippia citriodora Kunth (Verbenaceae) leaves	M	10 g	2.1 g	90.0%	Di-M (98:2)	CPC, dual mode, NP/RP	EEtWat (5:0.5:4.5)		–	Cheimonidi et al. 2018
Wiedemannioside C	*Pedicularis longiflora* Rudolph. var. tubiformis (Klotz) Tong (Scrophulariaceae) whole plant	70% Et	120 mg	12.6 mg	97.9%	LLE + MR	CCC, isocratic, RP	EBuWat (10:6:15)		0.39	Zhang et al. 2015

[C$_6$MIM]Br 1-hexyl-3-methylimidazolium bromide, *Aa* acetic acid, *Ac* acetonitrile, *At* acetone, *Bu* *n*-butanol, *CCS* countercurrent separation, *CECCC* recycling countercurrent chromatography, *Ch* chloroform, *CPC* centrifugal partition chromatography, *Di* dichloromethane, *E* ethyl acetate, *Et* ethanol, *CCC* high-speed countercurrent chromatography, *H* *n*-hexane, *Iso* isopropanol, *LLE* liquid–liquid extraction, *M* methanol, *MR* macroporous resin, *NP*, normal-phase, *ns* not specified, *PA* polyamide, *RP*, reversed-phase, *SG* silica gel, *spHPLC* semi-preparative high-performance liquid chromatography, *Tet* tetrahydrofuran, *Wat* water

[a]Methanol extract from faeces of Beagle dogs at 24 h after feeding with 3 g/kg PhGs

purification of root extracts, solvent systems had 50% water, 40% (on average) *n*-butanol and the rest was ethyl acetate, with only one or two PhGs isolated in a single run. Separation of up to five PhGs requires more non-polar solvent systems based on 45% water, 25-0% *n*-butanol, 25–50% ethyl acetate and 5% ethanol. The polarity of these systems is controlled by changing the ethyl acetate/*n*-butanol ratio. The large number of PhGs were isolated from extracts of "above ground" plant material with typical solvent ratios of 45–50% water, 30–50% ethyl acetate and the rest is either *n*-butanol or ethanol/methanol. There was no clear correlation between solvent system polarity and PhGs classes based on the number of sugar moieties, partly because plants generally contain a range of PhGs of different types. However, the number of sugar units affect the elution order when present in the same extract, with triglycosides eluting first with more polar phase followed by diglycosides and then monoglycosides.

Table 2 List of iridoids isolated through countercurrent separation

Compound	Species	Extrac-tion	Sample amount	Isolated amount	Purity	Upstream	CCS technique	CCS solvent system	Downstream	K value	References
10-Hydroxy hastatoside 10-Hydroxycornin	*Cornus officinalis* Sieb. Et Zucc. (Cornaceae) fruits	80% Et	1 g	0.77 mg 0.61 mg	88.4% 83.6%	MR D101	CCC, isocratic and EECCC, RP	BuWatAa (5:5:1)		4.52 11.49	Liang et al.2018
3-Hydroxy myopochlorin 5-hydroxy glutinoside	*Capraria biflora* L. (Scrophulariaceae) aerial parts	M	15 g	ns	ns		CPC, isocratic, two-steps, NP	TetEWat (4:9:12) and EProWat (8:4:12)	SG	–	Lemus et al. 2015
6'-*O*-β-D-Glucopyranosyl gentiopicroside	*Gentiana crassicaulis* Duthie ex Burk. (Gentianaceae) roots	57.5% Et	500 mg	12.8 mg	98.1%		CCC, isocratic, step-flow gradient, NP	EtBuMAa(1%) (0.5:7.5:0.5:3.5)		1.75	Liang et al. 2013b
6-β-Hydroxy geniposide	*Gardenia jasminoides* Ellis (Rubiaceae) fruits	40% Et	800 mg	52.2 mg	93.4%	MR HPD100	CCC, isocratic, RP	BuEtWat (10:1:10)		1.02	Wang et al. 2015
6-β-Hydroxy antirrhide	*Capraria biflora* L. (Scrophulariaceae) aerial parts	M	15 g	ns	ns	-	CPC, isocratic, two-steps, NP	TetEtWat (4:9:12) and EProWat (8:4:12)	SG	–	Lemus et al. 2015
7-(*S*)- and 7-(*R*)-*O*-ethylmorroniside	*Cornus officinalis* Sieb. Et Zucc. (Cornaceae) fruits	80% Et	1 g	2.51 mg 5.09 mg	89.5% 95.7%	MR D101	CCC, isocratic, RP	EMWatAa(5:0.5:5:0.5)		0.89 1.32	Liang et al. 2018
7-Dehydrologanin	*Cornus officinalis* Sieb. Et Zucc. (Cornaceae) fruits	80% Et	1 g	4.51 mg	93.7%	MR D101	CCC, isocratic, RP	BuWatAa (5:5:1)		6.17	Liang et al. 2018
7-*O*-(4-maloyl) loganin	*Cornus officinalis* Sieb. Et Zucc. (Cornaceae) fruits	80% Et	1 g	2.22 mg	90.6%	MR D101	CCC, isocratic, RP	EMWatAa(5:0.5:5:0.5)		0.22	Liang et al. 2018
7-*O*-(*E*-*p*-coumaroyl)- and 7-*O*-(*E*-caffeoyl)-sylvestroside I	*Scabiosa stellata* Cav. (Caprifoliaceae) whole plant	70% Et	3g	23 mg 7 mg	ns	MR HP20	CPC, isocratic, RP	TerAcWat(3:3:4)	RP-SG	–	Lehbili et al. 2018
8-Hydroxy-10-hydrosweroside	*Gentiana scabra* Bunge (Gentianaceae) roots	95% Et	50 mg	8 mg	96.7%	LLE	CCC, isocratic, RP	HBuMAa(0.4%)(1.4:8: 3:15.5)		0.29	Liang et al. 2007
8-*O*-acetylharpagide	*Capraria biflora* L. (Scrophulariaceae) aerial parts	M	15 g	ns	ns		CPC, isocratic, two-steps, NP	TetEWat(4:9:12) and EProWat(8:4:12)		–	Lemus et al. 2015
	Oxera coronata de Kok (Lamiaceae) aerial parts	Wat	347.3 g	63.9 g	80-90%		CPC, isocratic, NP	EProWat(3:2:5)		–	Remeur et al. 2017
Aucubin	*Aucuba japonica* Thunb. (Garryaceae) leaves	Wat	105 g	23.9 g	ns		CPC, isocratic, NP	EProWat(10:3:7)		17.6	Markovic et al. 2014
Caprarioside	*Capraria biflora* L. (Scrophulariaceae) aerial parts	M	15 g	16 mg	ns		CPC, isocratic, NP	TetEWat(4:9:12)		–	Lemus et al. 2015
Catalpol	*Rehmannia glutinosa* Libosch. (Scrophulariaceae) roots	75% Et	105 mg	35 mg	95.6%	LLE + SG	CCC, isocratic, NP	EBuWat (2:1:3)		0.51	Tong et al. 2015
	Capraria biflora L. (Scrophulariaceae) aerial parts	M	15 g	ns	ns		CPC, isocratic, two-steps, NP	TetEWat(4:9:12) and EProWat(8:4:12)	SG	–	Lemus et al. 2015
Catalposide	*Veronica ciliata* Fisch. (Scrophulariaceae) aerial parts	95% Et	200 mg	10 mg	99.0%	LLE	CCC, isocratic, NP	HBuWat (1.5:5:5)		0.52	Lu et al. 2016
Chlorotuberside	*Lamiophlomis rotata* (Benth.) Kudo (Lamiaceae) ns	65% Et	150 mg	27 mg	97.3%	LLE + MR D101	CCC, isocratic, RP	EBuWat (5:14:12)		0.63	Yue et al. 2013b
Cornusides I and II	*Cornus officinalis* Sieb. Et Zucc. (Cornaceae) fruits	80% Et	1 g	4.78 mg 29.4 mg	92.3% 97.4%	MR D101	CCC, EECCC, RP	EMWatAa (5:0.5:5:0.5)		0.54 1.99	Liang et al. 2018
Deglucoserrulatoside	*Gentiana macrophylla* Pall (Gentianaceae) ns	Wat	ns	37 mg	95.3%		CCC, isocratic, RP	EBuWatAa (2:3:5:0.6)	spHPLC	–	Wu et al. 2012
Eustomorusside Eustomoside	*Scabiosa stellata* Cav. (Caprifoliaceae) whole plant	70% Et	3g	6 mg 5 mg	ns	MR HP20	CPC, isocratic, RP	TerAcWat(3:3:4)	SG	–	Lehbili et al. 2018
Gardenoside	*Gardenia jasminoides* Ellis (Rubiaceae) fruits	40% Et	800 mg	151.1 mg	91.7% ,	MR HPD100	CCC, isocratic, RP	BuEtWat (10:1:10)		0.75	Wang et al. 2015
Geniposide	*Gardenia jasminoides* Ellis (Rubiaceae) fruits	40% Et	800 mg	587.2 mg	98.2%	MR HPD100	CCC, isocratic, RP	EBuWat (2:1.5:3)		0.70	Wang et al. 2015
	Gardenia jasminoides Ellis (Rubiaceae) fruits	50% Et	1 g	389 mg	98.0%	MR D101	CCC, isocratic, RP	EBuWat (2:1.5:3)		0.40	Zhou et al. 2005
	Gardenia jasminoides Ellis (Rubiaceae) fruits	80% Et	150 mg	43 mg	98.7%	LLE	CCC, isocratic, RP	EBuWat (1:4:5)		0.31	Liang et al. 2014
	Gardenia jasminoides Ellis (Rubiaceae) fruits	Wat	4 g	445 mg	95.2%	MR HPD100	CCC, isocratic, RP	EBuWat (1:4:5)		0.93	Zhang et al. 2012
	Gardenia jasminoides Ellis (Rubiaceae) fruits	80% M	500 mg	56.2 mg	95%		CPC, isocratic, RP	EIsoWat (3:2:5)		–	Kim and Kim 2007
Geniposidic acid	*Gardenia jasminoides* Ellis (Rubiaceae) fruits	40% Et	800 mg	24.5 mg	92.5%	MR HPD100	CCC, isocratic, RP	BuEtWat (10:1:10)		1.84	Wang et al. 2015
	Gardenia jasminoides Ellis (Rubiaceae) fruits	80% Et	150 mg	4 mg	90.4%	LLE	CCC, isocratic, RP	EBuWat (1:4:5)		2.41	Liang et al. 2014
	Eucommia ulmoides Oliv. (Eucommiaceae) leaves	Wat	80 mg	41.2 mg	99.2%	LLE + MR D101 + carbon	CCC, isocratic, RP	EBuWat (1:2:3)		0.76	Dai et al. 2013
Gentiopicroside	*Gentiana scabra* Bunge (Gentianaceae) roots	Et	300 mg	136 mg	99.6%	MR	CCC, isocratic, RP	EBuWat (2:1:3)		–	Xu et al. 2007

Table 2 continued

Compound	Species	Extraction	Sample amount	Isolated amount	Purity	Upstream	CCS technique	CCS solvent system	Downstream	K value	References
	Gentiana scabra Bunge (Gentianaceae) roots		ns	ns	97.0%		CCC, isocratic, NP	ChTerMWat (5:2:4:4)		–	Wang et al. 2007
	Gentiana scabra Bunge (Gentianaceae) roots	M	100 mg	35.66 mg	98.6%	LLE	CCC, isocratic, RP	EBuWat (2:1:3)		0.70	Chen et al. 2017
	Swertia mussotii Franch. (Gentianaceae) whole plant	75% Et	300 mg	75 mg	99.6%	LLE	CCC, isocratic, RP	ChBuMWat (4:2:3:3)	Sephadex LH-20	0.72	Chen et al. 2015
	Swertia franchetiana H. Smith (Gentianaceae) ns	ns	100 mg	14 mg	ns		CCC, isocratic	ChMWat (4:4:2)		–	Cheng et al. 2010
	Gentiana macrophylla Pall. (Gentianaceae) aerial parts	95% Et	300 mg	150 mg	94%		CCC, isocratic	ChMWat (4:4:2)		–	Huangfu et al. 2007
	Gentiana crassicaulis Duthie ex Burk. (Gentianaceae) roots	57.5% Et	500 mg	40.7 mg	99.4%		CCC, step-flow gradient, NP	EBuMAa(1%) (0.5:7.5:0.5:3.5)		1.06	Liang et al. 2013b
	Gentiana macrophylla Pall. (*Gentianaceae*) aerial parts	Wat	ns	52.3 mg	99.2%		CCC, isocratic	EBuWatAa (2:3:5:0.6)	spHPLC	–	Wu et al. 2012
	Gentiana macrophylla Pall. (Gentianaceae) roots	M	750 mg	348 mg	97%	LLE	CCC, step-flow gradient, NP	BuTfa(0.1%) (1:1)		1.56	Rho et al. 2014
	Centaurium erythraea Rafn. (Gentianaceae) aerial parts	M	4 g	13 mg	ns		CPC, isocratic, NP	EEtWat (7.5:3:5)	spHPLC	–	Mandova et al. 2017
Harpagoside	*Scrophularia ningpoensis* Hemsl. (Scrophulariaceae) roots	90% Et	276 mg	11 mg	97.0%	LLE	CCC, isocratic, RP	EBuWat (9:1:10)		1.01	Tong et al. 2006
	Scrophularia ningpoensis Hemsl. (Scrophulariaceae) roots	70% Et	200 mg	22 mg	98.0%	MR D101	CCC, isocratic, NP	ChBuMWat (4:1:3:2)		1.05	Tian et al. 2012
	Scrophularia ningpoensis Hemsl. (Scrophulariaceae) roots	90% Et	311 mg	14 mg	96.5%	LLE	CCC, dual mode, RP/NP	EBuWatAa (1:8:10:1)		6.20	Tong et al. 2009
Ipolamiide	*Stachytarpheta cayennensis* (Rich.) Vahl. (Verbenaceae) roots	Et	1 g	73 mg	ns	LLE	CCC, step-gradient, NP	EBuWat (1:x:1, x=0.05-1)		–	Leitao et al. 2005
Isovogeloside	*Halenia campanulata* Cuatrec. (Gentianaceae) ns	M	240 mg	24 mg	ns	SG	CCC, isocratic, NP	ChMWat (9:12:8)		–	Hostettman and Marston 2001
Kingiside	*Cornus officinalis* Sieb. et Zucc. (Cornaceae) fruits	80% Et	1 g	5.2 mg	91.6%	MR D101	CCC, isocratic, RP	BuWatAa (5:5:1)		0.38	Liang et al. 2018
Ligstroside	*Olea europea* L. (Oleaceae) leaves	60% Et	120 mg	ns	93.0%	MR AB8	CCC, isocratic, RP	EWat (1:1)	spHPLC	0.74	Zhang et al. 2014
Loganetin	*Alstonia scholaris* (L.) R.Br. (Apocynaceae) stem bark	M	1.5 g	48 mg	94.4%	LLE	CPC, isocratic, NP	TerAcWat (3:1.5:3) + HCl + TEA		–	Maurya et al. 2014
Loganic acid	*Gentiana scabra* Bunge (Gentianaceae) roots	M	100 mg	4.36	98.1%	LLE		EBuWat (2:1:3)		0.41	Chen et al. 2017
	Gentiana crassicaulis Duthie ex Burk. (Gentianaceae) roots	57.5% Et	500 mg	16.3 mg	98.6%	-	CCC, isocratic, RP	EBuMAa(1%) (0.5:7.5:0.5:3.5)		2.82	Liang et al. 2013b
	Gentiana macrophylla Pall (Gentianaceae) aerial parts	95% Et	600 mg	22.4 mg	90.2%		CCC, isocratic	EBuWatAa (2:3:5:0.6)	spHPLC	0.24	Wu et al. 2012
	Gentiana macrophylla Pallas (Gentianaceae) roots	M	750 mg	54 mg	97.0%	LLE	CCC, step-flow gradient, NP	BuTfa(0.1%) (1:1)		4.76	Rho et al. 2014
	Cornus officinalis Sieb. et Zucc. (Cornaceae) fruits	80% Et	1 g	5.8 mg	91.1%	MR D101	CCC, isocratic, RP	BuWatAa(5:5:1)		1.36	Liang et al. 2018
Loganin	*Cornus officinalis* Sieb. et Zucc. (Cornaceae) fruits	75% Et	100 mg	12.6 mg	98.6%	D101 MR	CCC, isocratic, NP	DiBuMWatAa (5:2:5:4:0.1)		0.76	Liang et al. 2013a
	Cornus officinalis Sieb. et Zucc. (Cornaceae) fruits	50% M	50 mg	11.5 mg	98.6	D101 MR	CCC, isocratic, RP	BuMAa(1%) (4:1:6)		1.30	Liu et al. 2009
	Cornus officinalis Sieb. et Zucc. (Cornaceae) fruits	80% Et	1 g	55.8 mg	97.2%	MR D101	CCC, isocratic, EECCC, RP	BuWatAa (5:5:1)		15.17	Liang et al. 2018
Macfadyenoside	*Capraria biflora* L. (Scrophulariaceae) aerial parts	M	15 g	109 mg	ns		CCC, isocratic, two-steps, NP	TetEWat (4:9:12) and EProWat (8:4:12)		–	Lemus et al. 2015
Morroniside	*Cornus officinalis* Sieb. et Zucc. (Cornaceae) fruits	75% Et	28.5 mg	28.5 mg	99.1%	D101 MR	CCC, isocratic, NP	DiBuMWatAa (5:2:5:4:0.1)		1.57	Liang et al. 2013a
	Cornus officinalis Sieb. et Zucc. (Cornaceae) fruits	50% M	50 mg	28.7 mg	97.8%	D101 MR	CCC, isocratic, RP	BuMAa(1%) (4:1:6)		0.74	Liu et al. 2009
	Cornus officinalis Sieb. et Zucc. (Cornaceae) fruits	80% Et	1 g	68.8 mg	98.7%	MR D101	CCC, isocratic, RP	BuWatAa (5:5:1)		0.84	Liang et al. 2018
Oleuropein	*Olea europea* L. (Oleaceae) leaves	60% Et	120 mg	ns	91%	MR AB8	CCC, isocratic, RP	EWat (1:1)	spHPLC	0.66	Zhang et al. 2014
	Cynomorium songaricum Rupr. (Cynomoriaceae) stems	Wat	600 mg	11.3 mg	95.6%	MR	CCC, isocratic, RP	EWat (1:1)		1.17	Wang et al. 2016
	Olea europea L. (Oleaceae) leaves	Wat	ns	25.4 mg	95%		CPC, isocratic, RP	HEEtWat (1:9:1:9)		0.71	Boka et al. 2015
Penstemonoside	*Lamiophlomis rotata*	65% Et	150 mg	21 mg	99.3%	LLE +	CCC, isocratic,	EBuWat (5:14:12)		1.03	Yue et al.

Table 2 continued

Compound	Species	Extraction	Sample amount	Isolated amount	Purity	Upstream	CCS technique	CCS solvent system	Downstream	K value	References
Phloyoside II	(Benth.) Kudo (Lamiaceae) whole plant			29 mg	98.5%	MR D101	RP			0.38	2013b
Picrosides I-III	*Picrorhiza scrophulariiflora* Pennel (Plantaginaceae) fruits	95% Et	ns	7.2 mg 15.6 mg 1.8 mg	94.6% 96.0% 96.3%		CCC, multi-steps (isocratic + EECCC + CECCC), RP	HEMWat (1:2:1:2), EBuWatFa (4:1:5:0.005) and EWat (1:1)		–	Chen et al. 2011
Sarracenin	*Cornus officinalis* Sieb. Et Zucc. (Cornaceae) fruits	80% Et	1 g	4.89 mg	85.4%	MR D101	CCC, isocratic, RP	EMWatAa (5:0.5:5:0.5)		9.08	Liang et al. 2018
Secologanol Secoxyloganin	*Centaurium erythraea* Rafn. (Gentianaceae) aerial parts	M	4 g	9 mg 5 mg	ns		CPC, isocratic, NP	EEtWat (7.5:3:5)		–	Mandova et al. 2017
Septemfidoside	*Scabiosa stellata* Cav. (Caprifoliaceae) whole plant	70% Et	3 g	3 mg	ns	MR HP20	CPC, isocratic, RP	TerAcWat (3:3:4)	SG	–	Lehbili et al. 2018
Shanzhiside methyl ester	*Lamiophlomis rotata* (Benth.) Kudo (Lamiaceae) whole plant	65% Et	150 mg	37 mg	99.2%	LLE + MR D101	CCC, isocratic, RP	EBuWat (5:14:12)		0.23	Yue et al. 2013b
Sweroside	*Gentiana crassicaulis* Duthie ex Burk. (Gentianaceae) roots	57.5% Et	500 mg	21.8 mg	98.7%		CCC, step-flow gradient, RP	EBuMAa(1%) (0.5:7.5:0.5:3.5)		0.77	Liang et al. 2013b
	Swertia franchetiana H. Smith (Gentianaceae) ns		100 mg	27 mg			CCC, isocratic	ChMWat (4:4:2)		–	Cheng et al. 2010
	Swertia mussotii Franch. (Gentianaceae) whole plant	75% Et	300 mg	74 mg	99.2%	BuOH L-L fraction	CCC, isocratic	ChBuMWat (4:2:3:3)	Sephadex LH-20	0.28	Chen et al. 2015
	Gentiana macrophylla Pall (Gentianaceae) roots	Wat	ns	27.5 mg	98.8%		CCC, isocratic	EBuWatAa (2:3:5:0.6)	spHPLC	–	Wu et al. 2012
	Cornus officinalis Sieb. et Zucc. (Cornaceae) fruits	75% Et	100 mg	5.9 mg	97.3%	MR D101	CCC, isocratic, NP	DiBuMWatAa (5:2:5:4:0.1)		1.04	Liang et al. 2013a
	Cornus officinalis Sieb. et Zucc. (Cornaceae) fruits	80% Et	1 g	14.5 mg	96.9%	MR D101	CCC, isocratic, RP	BuWatAa (5:5:1)		8.91	Liang et al. 2018
	Centaurium erythraea Rafn. (Gentianaceae) aerial parts	M	4 g	33 mg	ns		CPC, isocratic, NP	EEtWat (7.5:3:5)	spHPLC	–	Mandova et al. 2017
	Scabiosa stellata Cav. (Caprifoliaceae) whole plant	70% Et	3 g	4 mg	ns	MR HP20	CPC, isocratic, RP	TerAcWat (3:3:4)	SG	–	Lehbili et al. 2018
Swertiamarin	*Gentiana scabra* Bunge (Gentianaceae) roots	M	100 mg	3.05 mg	97.2%	LLE	CCC, isocratic, RP	EBuWat (2:1:3)		0.56	Chen et al. 2017
	Gentiana crassicaulis Duthie ex Burk. (Gentianaceae) roots	57.5% Et	500 mg	25.1 mg	99.1%		CCC, step-flow gradient, NP	EBuMAa(1%) (0.5:7.5:0.5:3.5)		1.48	Liang et al. 2013b
	Gentiana scabra Bunge (*Gentianaceae*) roots	95% Et	50 mg	18 mg	98.4%	LLE	CCC, isocratic, RP	HBuMAa(0.4%) (1.4:8:3:15.5)		0.54	Liang et al. 2007
	Gentiana macrophylla Pall (Gentianaceae) whole plant	Wat	ns	22.4 mg	98.0%		CCC, isocratic	EBuWatAa (2:3:5:0.6)	spHPLC	–	Wu et al. 2012
	Gentiana macrophylla Pallas (Gentianaceae) roots	M	750 mg	41 mg	97.0%	LLE	CCC, step-flow gradient, NP	BuTfa(0.1%) (1:1)		3.03	Rho et al. 2014
	Cornus officinalis Sieb. et Zucc. (Cornaceae) fruits	80% Et	1 g	0.52 mg	85.1%	MR D101	CCC, isocratic, RP	BuWatAa (5:5:1)		2.23	Liang et al. 2018
	Centaurium erythraea Rafn. (Gentianaceae) aerial parts	M	4 g	36 mg	98.2%		CPC, isocratic, NP	EEtWat (7.5:3:5)	spHPLC	–	Mandova et al. 2017
Sylvestroside I	*Scabiosa stellata* Cav. (Caprifoliaceae) whole plant	70% Et	3g	3	ns	MR HP20	CPC, isocratic	TerAcWat (3:3:4)	SG	–	Lehbili et al. 2018
Trifloroside	*Gentiana scabra* Bunge (Gentianaceae) roots	95% Et	50 mg	11 mg	97.1%	LLE	CCC, isocratic, RP	HBuMAa(0.4%) (1.4:8:3:15.5)		3.46	Liang et al. 2007
Trifloroside	*Gentiana scabra* Bunge (*Gentianaceae*) roots	M	100 mg	31.15 mg	98.9%	EtOAc L-L fraction	CCC, isocratic, RP	HEMWat (1:3:1:3)		1.89	Chen et al. 2017
Verproside	*Veronica ciliata* Fisch. (Scrophulariaceae) aerial parts	95% Et	200 mg	2 mg	99.8%	LLE	CCC, isocratic, NP	HBuWat (1.5:5:5)		1.00	Lu et al. 2016
Vogeloside	*Halenia campanulata* Cuatrec. (Gentianaceae) ns	M	240 mg	32 mg	ns	SG	CCC, isocratic, NP	ChMWat (9:12:8)		–	Hostettman and Marston 2001

Aa acetic acid, *Ac* acetronitrile, *Bu* *n*-butanol, *CCS* countercurrent separation, *CECCC* recycling countercurrent chromatography, *Ch* chloroform, *CPC* centrifugal partition chromatography, *Di* dichloromethane, *EECCC* elution-extrusion countercurrent chromatography, *E* ethyl acetate, *Et* ethanol, *Fa* formic acid, *CCC* high-speed countercurrent chromatography, *H* *n*-hexane, *Iso* isopropanol, *LLE* liquid–liquid extraction, *M* methanol, *MR* macroporous resin, *Ter* methyl *t*-buthyl ether, *NP* normal-phase *Pro* *n*-propanol, *ns* not specified, *RP* reversed-phase, *SG* silica gel, *spHPLC* semi-preparative high-performance liquid chromatography, *Tfa* trifluoroacetic acid, *Tet* tetrahydrofuran, *Wat* water

The first paper about CCC purification of PhGs from a whole plant crude extract proposed a two-phase solvent system composed of ethyl acetate–acetonitrile–water (2.6:2.6:4.8) in the reversed phase mode. When the system was run in the normal phase mode, addition of 2% methanol was required to adjust partition coefficients. Verbascoside and oraposide were successfully separated from *Orobanche rapum-genistae* Thuill. However, in both elution modes, the purities of the isolated PhGs did not exceed 82% (Viron et al. 1998). A subsequent report focused on CCC separation of verbascoside and 2′-acetylverbascoside from the stem extract of *Cistanche salsa* (C.A. Mey.) G. Beck (Lei et al. 2001a). As PhGs easily dissolve in *n*-butanol during liquid–liquid partitioning step before CCC, the authors decided to choose a biphasic solvent system containing equal amounts of *n*-butanol as organic phase modifier and ethanol as aqueous phase modifier (ethyl acetate–*n*-butanol–ethanol–water 4:0.6:0.6:5). Since the purity of verbascoside and 2′-acetylverbascoside isolated in the first run was not satisfactory, they were submitted to a second separation using the same solvent system (two-step CCC); consequently, the resulting PhGs had high purities (> 98%). As part of a more extensive study concerning the metabolic regulation of PhGs in the gastrointestinal tract, the same group of authors (Lei et al. 2001b) used a two-step CCC separation procedure to isolate verbascoside and isoverbascoside (purities higher than 95%) from the methanolic extract of Beagle dogs faeces at 24 h after feeding them with 3 g/kg PhGs from *Cistanche tubulosa* (Schrenk) Wight stems. The solvent systems used for this separation consisted of two different mixtures of ethyl acetate–*n*-butanol–ethanol–water (35:6:6:50 and 30:10:6:50). Injection of the 214 mg sample into the first more non-polar system (36% ethyl acetate–6% *n*-butanol) caused a vast stripping of the stationary phase leaving only 16% of it in the column and resulting in co-elution of both target compounds. The second CCC run with a more polar solvent system (30% ethyl acetate–10% *n*-butanol) allowed a better separation. Similar systems (containing the same four solvents, but in different ratios) were employed by Li et al. (2008) with the aim to isolate verbascoside and other PhGs (2′-acetylverbascoside, cistanoside A, echinacoside, and isoverbascoside) from *Cistanche deserticola* Y.C. Ma. Han et al. (2012) used the same solvent system as in Lei et al. (2001a), but on a different CCC instrument and by adding a silica gel chromatography step before CCS. This allowed isolation of four PhGs from stem extract of *Cistanche deserticola* Y.C. Ma. When ethanol was replaced with methanol, new biphasic solvent systems were created, leading to CCC separation of rare PhGs, such as: calcedariosides A and B, as well as plantainosides B and D from *Chirita longgangensis* W.T. Wang, forsythoside A and suspensaside A from *Forsythia suspensa* (Thunb.) Vahl. (Duan et al. 2014; Yang et al. 2013). For the purification of verbascoside and isoverbascoside from *Plantago psyllium* L. seed extract, Li et al. (2005) successfully used the two phases of the mixture composed only of equal amounts of ethyl acetate and water; such a solvent system has the advantages of being easy to handle and having a quick settling time.

In most of the papers, single injections under isocratic conditions were enough to achieve PhGs in a purified form. However, in the case of salidroside from *R. crenulata* root extract, the fractions resulting from the first run with ethyl acetate–*n*-butanol–water (1:4:5) were subsequently eluted with an atypical solvent system composed of chloroform–methanol–isopropanol–water (5:6:1:4) (Han et al. 2002). This type of chlorinated solvent systems is often used for the separation of root extracts in CCC, particularly ginsenosides (Skalicka-Wozniak and Garrard 2014). Working with chlorinated solvent systems is not a common practice in isolating PhGs by CCS. One reason is that the polarity of chloroform (or its replacement dichloromethane) does not provide a suitable range of partition coefficients for target PhGs. Also, with a move towards greener solvents, chlorinated solvent systems are less used due to health and safety restrictions. However, two examples of successful PhG separations have been reported. Chen et al. (2014) purified verbascoside and isoverbascoside from *Pedicularis longiflora* Rudolph var *tubiformis* (Klotz) Tsong with chloroform–*n*-butanol–methanol–water (4:3:4:5), whereas angoroside C was isolated from *Scrophularia ningpoensis* Hemsl. with chloroform–*n*-butanol–methanol–water (4:1:3:2) (Tian et al. 2012).

In order to isolate PhGs, some authors added acetic acid to an ethyl acetate–*n*-butanol–water system. CCC separation is driven by partitioning of compounds between two liquid phases, similar to liquid–liquid extraction. The addition of a small amount of an organic acid can significantly improve the separation

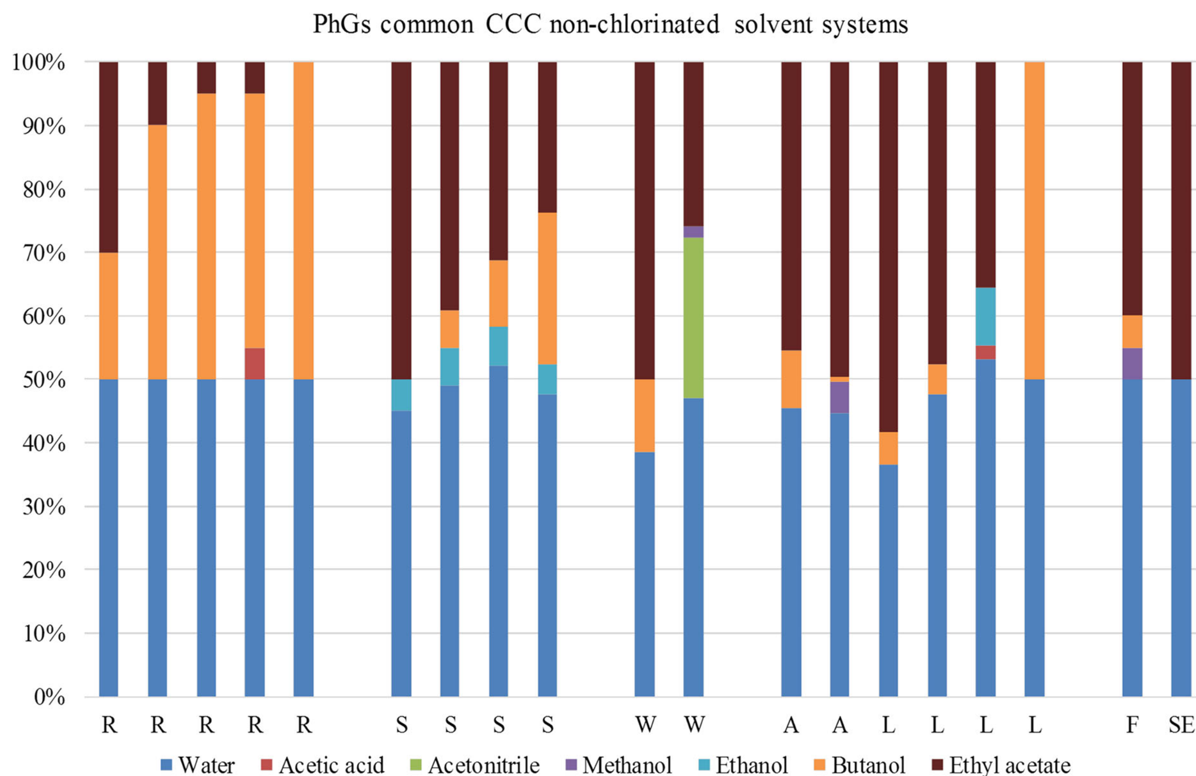


Fig. 3 Phenylethanoid glycosides—common CCC non-chlorinated solvent systems. *A* aerial parts, *F* fruits, *L* leaves, *R* roots, *S* stem, *SE* seeds, *W* whole plant

by neutralizing the ionizable acidic functional groups and breaking hydrogen bonds (Berthod and Mekaoui 2011; Skalicka-Wozniak and Garrard 2014). Therefore, Xie et al. (2012b) obtained pure verbascoside and martynoside from *Cistanche tubulosa* (Schrenk) Wight with the help of ethyl acetate–*n*-butanol–water–glacial acetic acid (1:1.2:2:0.2), whereas Tong et al. (2009) isolated angoroside C from *Scrophularia ningpoensis* Hemsl. with ethyl acetate–*n*-butanol–water–glacial acetic acid (1:8:10:1). Another acid-based solvent system is represented by ethyl acetate–*n*-butanol–water–glacial acetic acid (4:1:6:0.25) that led to the successful purification of forsythosides A and I from *Forsythia suspensa* (Thunb.) Vahl. (Sun et al. 2016). However, any addition of pH modifier should be carefully considered as it might lead to an additional processing step after CCC separation as well as compromise stability of target compounds.

Not all CCC separations required quaternary solvent mixtures. *n*-Butanol–water (1:1) system was successfully employed in the purification of verbascoside from *Penstemon barbatus* (Cav.) Roth and *P. digitalis* Nutt. ex Sims root and leaves extracts (Xie et al. 2010, 2012a; Wang et al. 2013). Additionally, this solvent system also led to the isolation of echinacoside from the same two species, but subsequent purification processes were needed. For the leaf extract, semi-preparative HPLC separation was performed (Wang et al. 2013), whilst for the root extract, the authors employed another CCC technique, known as recycling CCC; echinacoside, with an advanced purity, was obtained from the 4th cycle (Xie et al. 2010). Recycling CCC is a repetitive (multiple) isocratic elution mode. It involves re-directing the eluent from the detector outlet back into the CCC column inlet (via pump inlet) at a set point of a separation. The mobile phase, which contains the target compounds, is introduced into the column inlet for the next separation cycle (Chen et al. 2011). Although recycling CCC methods require a prolonged time, the resolution of peaks is significantly improved, especially for compounds with similar K values.

It is worth mentioning, that as compared to other categories of natural constituents (e.g. coumarins)

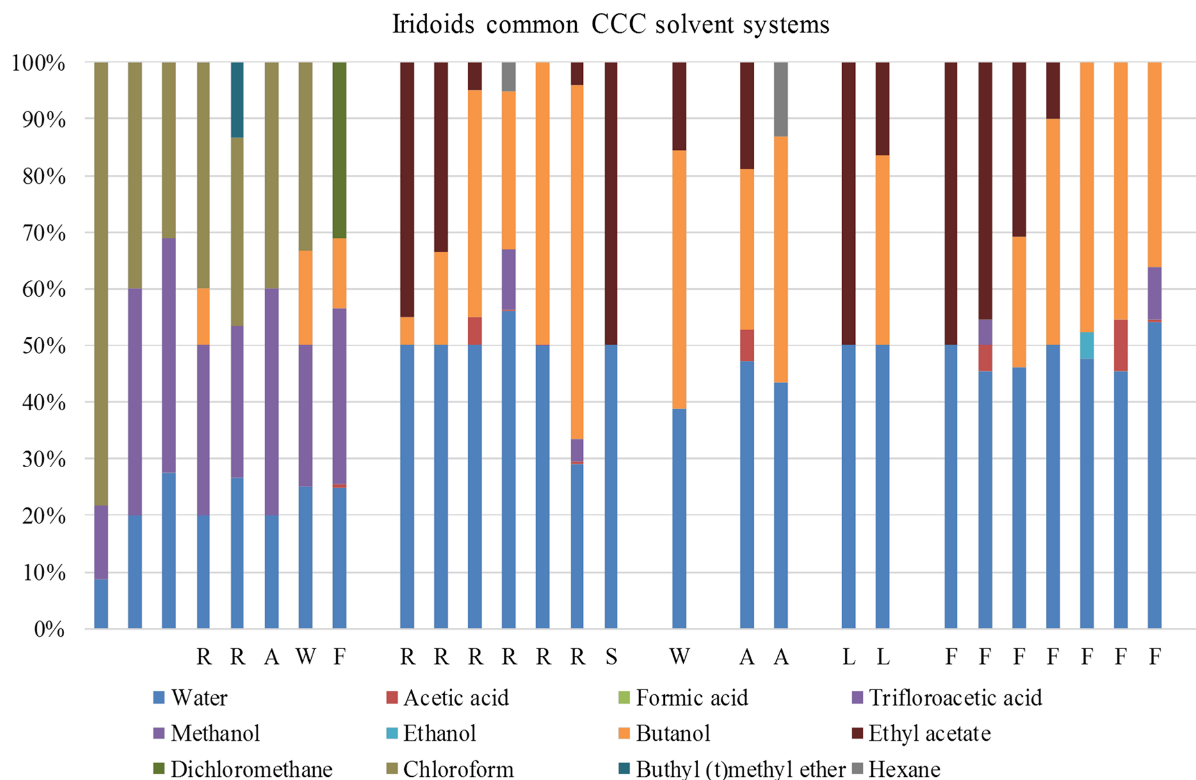


Fig. 4 Iridoids—common CCC solvent systems. *A* aerial parts, *F* fruits, *L* leaves, *R* roots, *S* stem, *W* whole plant

(Skalicka-Wozniak et al. 2014; Walasek et al. 2015) where crude extracts easily yielded pure compounds in a single CCS run, PhGs were rarely isolated directly from the raw extracts. Generally, after extraction with ethanol, methanol, ethanol–water or methanol–water mixtures, liquid–liquid partitioning extractions were conducted, and the ethyl acetate or *n*-butanol fractions were submitted to CCS directly or after column chromatography with appropriate solvents over silica gel or macroporous resins. These preliminary operations are needed to eliminate other undesired hydrophilic constituents and to make CCS more efficient. However, there are some examples when various PhGs were purified by CCS directly from the crude extracts. In the case of the methanolic extract of A*beliophyllum distichum* Nakai leaves yielded high purity (> 92.5%) verbascoside, isoverbascoside, eutigoside A and cornoside (Li et al. 2013). In addition, 70% ethanolic extract of a whole plant of *Orobanche rapum-genistae* Thuill. yielded verbascoside and oraposide having purities of 75% and 82%, respectively (Viron et al. 1998).

Another approach to purify a crude plant extract is gradient elution. In CCC it can be a change in the mobile phase composition or flow rate, in a linear or step wise manner (Leitao et al. 2005; Ignatova et al. 2011). It also can be run in both reversed and normal phase mode. The main difference from HPLC is that in CCC any change of a mobile phase composition leads to a change in a stationary phase. Therefore, it is better to select a solvent system family where one of the phases' composition does not change much. This phase should be used as a stationary one (Berthod et al. 2002). Leitao et al. (2005) applied a step-gradient CCC method in normal phase mode with ethyl acetate–*n*-butanol–water (1:x:1, x = 0.05–1) as solvent system for the fractionation of *Stachytarpheta cayennensis* (Rich.) Vahl. root extract. It produced three PhGs with a rhamnose unit in their structures (verbascoside, isoverbascoside and martynoside) and three iridoids, one of which was ipolamiide, recovered from the aqueous stationary phase at the extrusion stage. By applying the same gradient method, the separation of aerial part and leaf extracts of *Lantana trifolia* L. and *Lippia alba* f. *intermedia* Moldenke (de

Juliao et al. 2010; Leitao et al. 2015) required a modification of the solvent system by expanding its polarity range via addition of *n*-hexane, resulting thus in an *n*-hexane–ethyl acetate–*n*-butanol–water system (0.4:1:x:1, x = 0.1–0.7). The ethyl acetate and water content range increased from 40–32 to 49–33%, with *n*-butanol range reduced from 2–34 to 4–23%. The presence of *n*-hexane made the polarity difference between phases larger, as the organic phase became more non-polar. Once a method is set up, the gradient methodology can be repeated with other extracts, providing a reproducible elution of the same target compound under the same conditions (Leitao et al. 2015). It is the case for verbascoside invariably eluting at a *n*-butanol ratio of 0.5 (de Juliao et al. 2010). This step-gradient CCC system (0.4:1:x:1, x = 0.1–0.7) was used for the separation of verbascoside and/or other PhGs, such as 2-(4-hydroxyphenyl)-ethyl-*O*-β-D-glucopyranosyl-6-*O*-β-D-glucopyranoside, 2′-apiosylverbascoside, isoverbascoside, allyssonoside, echinacoside, forsythoside B, leucosceptoside B, salidroside or wiedemannioside C from *Lantana trifolia* L. and *Lippia alba* f. *intermedia* Moldenke. In all these examples, the sample loading was in average 1 g per 100 mL column volume. Therefore, gradient elution in CCC might be used as a standard approach for fractionation of natural product extracts at all scales.

Several PhGs have been purified by CPC techniques in isocratic mode. CPC, due to its cascade mixing in consequtively connected cells (forming a separation column) and a single rotational axis, can retain solvent systems with a smaller density difference between liquid phases and can cope with emulsification. Therefore, much more polar additives like ethanol or acetone can be used in larger proportions, which causes longer settling times but brings the polarity difference between phases closer, resulting in the separation of compounds within a smaller polarity range. For example, Viron et al. (2000) repeated the separation of verbascoside and oraposide from *Orobanche rapum-genistae* Thuill. (Viron et al. 1998), by replacing acetonitrile with less toxic acetone and changing ratios of organic solvents resulting in an ethyl acetate–acetone–water (5:0.5:4.5) solvent system that allowed a higher loading of the crude extract. It seems the ethyl acetate–alcohol–water-based solvent systems, with 45–48% ethyl acetate and water, can provide isolation of verbascoside from different extracts of upper part of a plant. Verbascoside was purified from *Lippia citriodora* Kunth with ethyl acetate–ethanol–water (5:0.5:4.5) (Cheimonidi et al. 2018) and *Plantago asiatica* L. with ethyl acetate–*n*-butanol–ethanol–water (5:5:1:10) (Li et al. 2009); the polarity of organic phase was adjusted by adding *n*-butanol. In the latter example, another PhG (plantamajoside) was isolated. Using 16% of tetrahydrofuran, the ethyl acetate–tetrahydrofuran–water (4.5:2:6) system provided the isolation of verbascoside in a single step, together with a large number of iridoids eluting from *Capraria biflora* L. (Lemus et al. 2015). Tetrahydrofuran and acetone have not been previously employed in CCC separations of PhGs.

CCS and iridoids' isolation

Of the 56 iridoids isolated through various CCS techniques from plants belonging mostly to Gentianaceae, Rubiaceae, Scrophulariaceae, Verbenaceae, Lamiaceae and Plantaginaceae families, 29 were iridoid glycosides, 19 secoiridoid glycosides, 5 bisiridoids and 3 non-glycosylated iridoids. Iridoid glycosides with a C-10 carbon skeleton and secoiridoids were present in crude extracts either together or separate, regardless the anatomical part of a plant used, whereas iridoid glycosides with a C-9 carbon skeleton were generally present in extracts on their own. Fruit extracts provided the largest number of iridoids isolated in one application when separated by CCC, followed by aerial parts, whole plants and roots. Gentiopicroside (10 times), sweroside (8 times) and swertiamarin (7 times) were the most frequently isolated compounds. Taking into consideration that iridoids are also water soluble compounds, ethyl acetate–alcohol–water solvent systems similar to those employed for the isolation of PhGs were used in the separation of this category of secondary metabolites, but generally with a higher content of *n*-butanol or other short-chain alcohols (Fig. 4). Ten out of 15 ethyl acetate–butanol–water systems had 35% butanol (or higher) and 40-50% water.

Almost the same range of solvent systems were employed for root extracts separation to isolate iridoids as in the case of PhGs. Interestingly, eight chlorinated solvent systems were suggested mainly for secoiridoids purification. The polarity of the chloroform–methanol–water systems with 24% water in average and equal ratios of two organic solvent was

adjusted by addition of 13% *n*-butanol to make it more polar or 13% *t*-butyl methyl ether to make it more apolar.

Mixtures of ethyl acetate–*n*-butanol–water (widely encountered in CCS of PhGs) led to the successful CCC purification of catalpol from *Rehmannia glutinosa* Libosch. (Tong et al. 2015), chlorotuberside, penstemonoside, phloyoside II and shanzhiside methyl ester from *Lamiophlomis rotata* (Benth.) Kudo (Yue et al. 2013b), geniposide and geniposidic acid from *Gardenia jasminoides* Ellis and *Eucommia ulmoides* Oliv. (Dai et al. 2013; Liang et al. 2014; Wang et al. 2015; Zhang et al. 2012; Zhou et al. 2005), gentiopicroside, loganic acid and swertiamarin from *Gentiana scabra* Bunge (Chen et al. 2017; Xu et al. 2007), harpagoside from *Scrophularia ningpoensis* Hemsl. (Tong et al. 2006) and ipolamiide from *Stachytarpheta cayennensis* (Rich.) Vahl. (Leitao et al. 2005). All previous compounds were isolated under isocratic conditions, except ipolamiide as a part of a large group for which a step-gradient elution was performed with ethyl acetate–*n*-butanol–water (1:x:1, x = 0.05–1); this method allowed not only the isolation of ipolamiide and two other unidentified iridoid glycosides, but also of three PhGs mentioned above (verbascoside, isoverbascoside and martynoside). Addition of apolar (*n*-hexane) or polar (ethanol) solvent to *n*-butanol–water system was also taken into consideration, depending on the structure of the target compound and matrix complexity. For instance, 6-β-hydroxygeniposide, gardenoside and geniposidic acid (C-10 iridoids) were isolated from *Gardenia jasminoides* Ellis fruit extract with the aid of *n*-butanol–ethanol–water (10:1:10) (Wang et al. 2015), whereas catalposide and verproside (C-9 iridoids) were purified from *Veronica ciliata* Fisch. aerial part extract with *n*-hexane–*n*-butanol–water (1.5:5:5) (Lu et al. 2016). In the former case, ethanol reduced the polarity difference between phases as it mainly goes into the aqueous phase making it more apolar; whereas the addition of *n*-hexane gives the opposite effect as it is not water miscible and it will be distributed in the organic phase only, making it more apolar. The simple solvent system ethyl acetate–water (1:1) was used for the CCC separation of two secoiridoids, ligstroside and oleuropein from *Olea europea* L. leaf extract. However, both compounds needed further semi-preparative HPLC purification (Zhang et al. 2014). Using the same solvent system, oleuropein was also isolated from *Cynomorium songaricum* Rupr. in a single step, with purity higher than 95% (Wang et al. 2016). The explanation might lie in the difference of the extract matrix as it was aqueous-ethanolic extract of leaves in Zhang et al. (2014) and aqueous stem extract in Wang et al. (2016). Systems from traditional *n*-hexane–ethyl acetate–methanol–water family (called HEMWat) have been rarely used for the CCC separation of water soluble compounds such as iridoids due to systems polarity range. However, there is one example when *n*-hexane–ethyl acetate–methanol–water (1:3:1:3) was used for purification of trifloroside, a less polar secoiridoid, from the ethyl acetate root extract of *Gentiana scabra* Bunge (Chen et al. 2017). Some similar compounds were isolated using solvent systems containing chloroform. For example, gentiopicroside was separated from *Gentiana scabra* Bunge with chloroform-*tert*-butyl methyl ether–methanol–water (5:2:4:4) (Wang et al. 2007), *Gentiana macrophylla* Pallas and *Swertia franchetiana* H. Smith with chloroform–methanol–water (4:4:2) (Cheng et al. 2010; Huangfu et al. 2007) and *Swertia mussotii* Franch with chloroform–*n*-butanol-methanol–water (Chen et al. 2015). Moreover, harpagoside was isolated from *Scrophularia ningpoensis* Hemsl. with chloroform–*n*-butanol–methanol–water (4:1:3:2) (Tian et al. 2012) and isovogeloside and vogeloside from *Halenia campanulata* Cuatrec. with chloroform–methanol–water (9:12:8) (Hostettmann and Marston 2001).

Optimizing partitioning of ionized compounds or depressing emulsification of a solvent system with the help of small amounts of organic acids is a very common practice in the CCC purification of iridoids. Acidic modifiers can substantially shorten the settling time of the chosen biphasic solvent system, improve the retention of the stationary phase and therefore improve separation, especially when the sample solution contains acidic impurities. Moreover, due to protonation, the molecules that contain ionizing groups (carboxylic, phenolic) become more hydrophobic and therefore their affinity for the organic phase is increased (Ito 2005). For example, mixtures of ethyl acetate–*n*-butanol–water containing acetic acid led to the isolation of harpagoside from *Scrophularia ningpoensis* Hemsl. (Tong et al. 2009), deglucoserrulatoside, gentiopicroside, loganic acid, sweroside and swertiamarin from *Gentiana macrophylla* Pall. (Wu et al. 2012). Ethyl acetate–*n*-butanol–

methanol-1% (aq) acetic acid (0.5:7.5:0.5:3.5) was successfully used as solvent system for the separation of five iridoid glycosides (6′-*O*-β-D-glucopyranosyl gentiopicroside, gentiopicroside, loganic acid, sweroside and swertiamarin) from the traditional Tibetan medicine *Gentiana crassicaulis* Duthie ex Burk in a single run (Liang et al. 2013b). Moreover, some acidified systems with nonpolar solvents (*n*-hexane or dichloromethane) were also employed with the purpose to isolate similar plant secondary metabolites. *n*-Hexane–*n*-butanol–methanol-0.4% (aq) acetic acid (1.4:8:3:15.5) led to the one-step purification of 8-hydroxy-10-hydrosweroside, swertiamarin and trifloroside from *Gentiana scabra* Bunge (Liang et al. 2007), whereas dichloromethane–*n*-butanol–methanol–water–glacial acetic acid (5:2:5:4:0.1) to that of sweroside, morronoside and loganin from *Cornus officinalis* Sieb. et Zucc. (Liang et al. 2013a). Another variation of an acidified solvent system tested for the separation of the latter two compounds from the same plant species consisted of *n*-butanol–methanol-1% (aq) acetic acid (4:1:6) (Liu et al. 2009). A mixture of *n*-butanol-0.1% (aq) trifluoroacetic acid (1:1) was successfully employed for the isolation of loganic acid, gentiopicroside and swertiamarin from *Gentiana macrophylla* Pall. under consecutive flow rate gradient conditions (from 1.5 to 5 mL/min) (Rho et al. 2016).

A very peculiar example is represented by the isolation of picrosides I-III from the crude ethanolic extract of *Picrorhiza scrophulariiflora* Pennel. when three different CCC techniques were applied. First, the crude extract was eluted under isocratic conditions with *n*-hexane–ethyl acetate–methanol–water (1:2:1:2). Then the main fraction was subjected to elution–extrusion CCC (EECCC) with ethyl acetate–*n*-butanol–water-concentrated formic acid (4:1:5:0.005) when picroside II and another fraction resulted. EECCC was implemented by Berthod et al. (2003) to extend the hydrophobicity window of the classical CCC, resulting in the extrusion of the most retained solutes out of the column with acceptable peak resolution. The method has two steps: the first step is a classical CCC elution, whereas in the next one, the stationary phase containing the partially separated hydrophobic solutes is extruded out of the column in a continuous way using the liquid stationary phase. EECCC can be time and solvent sparing, being advantageous for retrieving compounds with high K values which normally take a long time to elute but may be already separated within the column. Eventually, the impure fraction previously obtained by EECCC was submitted to CECCC with ethyl acetate–water (1:1) when pure picrosides I and III were achieved in the 6th cycle (Chen et al. 2011).

In the study of Liang et al. (2018), 18 compounds were isolated from *Cornus officinalis* Sieb. et Zucc. fruit extract, of which 15 iridoids C-10 and secoiridoids. Kingiside, morroniside, loganic acid, swertiamarin, 10-hydroxyhastatoside, 10-hydroxycornin, 7-dehydrologanin, sweroside were separated with *n*-butanol–water–glacial acetic acid (5:5:1), whereas for the isolation of 10-hydroxycornin and loganin, the elution was changed to EECCC mode. 7-*O*-(4-maloyl)loganin, cornusides I and II, 7(*S*) and 7(*R*)-*O*-ethylmorroniside and sarracenin were purified with the help of ethyl acetate–methanol–water–glacial acetic acid (5:0.5:5:0.5). Such a high concentration of glacial acetic acid in the *n*-butanol–water system was required due to a high loading of the crude, which was 1 g on 300 mL CCC column, to ensure constant pH. Also, for such separations, CCC often run in reversed elution mode for better pH control. Beside the great number of compounds isolated practically with only two different solvent systems, it is also noticeable that the CCC equipment was not coupled with a classical UV detector, but with a mass spectrometer (that has a higher sensitivity). Moreover, most of these compounds are very rare iridoids, while 7-*O*-(4-maloyl)loganin was a new characterized constituent.

As compared to PhGs, when very few compounds were separated with CPC, this technique was more often employed for isolating iridoids (8 papers out of 11 found for the CPC part of the review). In three applications, CPC was practically used for the production of iridoids from large quantities of extracts isolating seven C-9 iridoids from the aerial part extract (Lemus et al. 2015), five secoiridoids from another aerial part extract (Mandova et al.2017) and four bis-secoiridoids and two secoiridoids from a whole plant extract (Lehbili et al. 2018) with an average loading of 1.3 g per every 100 mL of column volume in normal phase elution mode.

In the study of Markovic et al. (2014), 23.9 g of aucubin was purified from 105 g of leaf extract from *Aucuba japonica* Thunb. using ethyl acetate–*n*-propanol–water (7:3:10) in three repetitive runs. This solvent system, but with slightly higher *n*-propanol

content, was later successfully used by the same research group twice more to separate C-9 iridoids. In Lemus et al. (2015), two new (3-hydroxymyopchlorin and 5-hydroxyglutinoside) and five known C-9 iridoids (caprarioside, macfadyenoside, 8-*O*-acetylharpagide, catalpol and 6-β-hydroxyantirrhide) were separated from *Capraria biflora* L. The methanolic extract of aerial parts was submitted to CPC, first with tetrahydrofuran–ethyl acetate–water (4:9:12) and second with ethyl acetate–*n*-propanol–water (8:4:12). Adjusting the solvent ratios to ethyl acetate–*n*-propanol–water (3:2:5) allowed the purification of 8-*O*-acetylharpagide from the aqueous leaf extract of *Oxera coronata* Kok in one run (Remeur et al. 2017). Replacing *n*-propanol with its isomer, as in ethyl acetate-2-propanol–water (3:2:5), led to the isolation of C-10 iridoid geniposide from *Gardenia jasminoides* Ellis (Kim and Kim 2007). Increasing the ethyl acetate content from 30 to 48% and using ethanol (19%) instead of 2-propanol (20%) made this solvent system less polar. The polarity of the final solvent system ethyl acetate–ethanol–water (7.5:3:5) was enough to enrich the main target, swertiamarin, and separate four more secoiridoids (gentiopicroside, secologanol, secoxyloganin, sweroside) from the aqueous extract of *Centaurium erythraea* Rafn. aerial parts (Mandova et al. 2017).

Two new bis-secoiridoids (7-*O*-(*E*-p-coumaroyl)-sylvestroside I, 7-*O*-(*E*-caffeoyl)-sylvestroside I) together with two other known bis-secoiridoids and three secoiridoids (sylvestroside I, septemfidoside, sweroside, eustomoside and eustomoruside) were isolated from *Scabiosa stellata* Cav. with less polar solvents of *tert*-butyl methyl ether–acetonitrile–water (3:3:4) (Lehbili et al. 2018). This type of solvent systems seems working well for less polar iridoids. Loganetin, a non-glycosidic iridoid, was separated from the stem bark extract of *Alstonia scholaris* (L.) R.Br. with *tert*-butyl methyl ether–acetonitrile–water (3:1.5:3) in which pH modifiers were added to both phases (HCl in the lower aqueous phase and triethylamine in the upper organic phase) (Maurya et al. 2014). The conditions are typical for pH-zone refining elution mode, which is often used for separation of ionized compounds (Ito 2005). However, the solvent system was equilibrated before sample injection resulting in running the CPC separation in isocratic normal phase mode with rather unusual approach to pH control. Another example of a less polar solvent system was used for purification of oleuropein from *Olea europea* L. with *n*-hexane–ethyl acetate–ethanol–water (1:9:1:9) (Boka et al. 2015). In all large scale separations described above, the CPC fractions needed further purification steps with the help of conventional chromatographic methods.

Similar to PhGs, isolation of pure iridoids directly from the crude extract after a single CCC run is unusual, but not impossible. Gentiopicroside, 6′-*O*-β-D-glucopyranosyl gentiopicroside, loganic acid, sweroside and swertiamarin were isolated in pure form after one CCC experiment directly from the 57.5% ethanolic extract of *Gentiana crassicaulis* Duthie ex Burk. obtained by microwave-assisted extraction. As it can be easily deduced, the composition of the microwave-assisted extraction solvent was not chosen randomly, but after optimization experiments in which parameters such as the effect of ethanol concentration, irradiation time and microwave power on the yield of target compounds were statistically analyzed. Consequently, it is understandable why only one purification step was needed for the isolation of pure iridoids (Liang et al. 2013b).

Biological relevance of phenylethanoid glycosides and iridoids isolated through CCC

PhGs and iridoids have never ceased to surprise researchers in terms of their potential medicinal uses, as they were found to possess a broad spectrum of bioactivities (antioxidant, neuroprotective, antitumor, antimicrobial, anti-inflammatory, immunomodulating, cardioprotective, etc.). Numerous review articles have focused on summarizing the pharmacological activities of these two classes of phytochemicals (Dinda et al. 2007b, 2009, 2011; Fu et al. 2008; Tundis et al. 2008; Xue and Yang 2016). However, in this section the emphasis will be put only on those few papers dealing with both CCS and biological evaluation of isolated compounds.

It was interesting to notice that, although the first PhGs/iridoids isolated through CCS date from 1998, the earliest reports that included at least one biological assay were published in 2010, with an increased frequency of papers describing bioactivity screenings in the last few years. This tendency might have its reasoning in the fact that researchers were initially engaged mostly in proving that CCS is a powerful alternative tool to conventional chromatographic

techniques. Once more and more papers showed that CCS is indeed fast and efficient in isolating PhGs and iridoids, the efforts were readily focused on showing the pharmacological utility of the purified compounds, which is practically the purpose of any isolation in natural product research.

In the case of PhGs, only 6 papers addressed not only the CCS process, but also testing the antioxidant, sedative, aldose reductase inhibition and cytotoxic properties of the isolated compounds. Several PhGs were assessed in various antioxidant tests. Verbascoside and isoverbascoside from *Pedicularis longiflora* Rudolph var. *tubiformis* (Klotz.) Tsoong exhibited strong 2,2-diphenyl-1-pycrylhydrazyl (DPPH) radical scavenging activity, with IC_{50} values of 15.6 and 18.9 μg/mL, respectively; the activity was slightly lower than that of a very well-known natural antioxidant, gallocatechin (IC_{50} = 7.2 μg/mL) (Chen et al. 2014). Salidroside, together with the new PhG (2-(4-hydroxyphenyl)-ethyl-*O*-β-D-glucopyranosyl-6-*O*-β-D-glucopyranoside) from *Rhodiola crenulata* Fish et Mey, showed moderate DPPH scavenging effects with IC_{50} values of 10.97 μM and 12.13 μM, respectively (Chen et al. 2012). Duan et al. (2014) investigated the antioxidant capacity of PhGs from *Chirita longgangensis* W.T.Wang. All compounds showed scavenging effects in different systems, but the potency was different, depending on the reactive oxygen species to be quenched. In the hydroxyl radical-, superoxide anion- and hydrogen peroxide-luminol chemiluminescence assays, the sequence of scavenging effects was as follows: plantanoiside D > plantainoside B > calcedarioside A > calcedarioside B; plantanoiside B > calcedarioside B > plantainoside D > calcedarioside A; and calcedarioside B > plantainoside B > plantainoside D > calcedarioside A. The PhGs isolated from *Abeliophyllum distichum* Nakai (verbascoside, isoverbascoside, eutigoside B and cornoside) were tested in a 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) scavenging assay. The compounds exerted their antioxidant activity in the following order: verbascoside > isoverbascoside ≫ eutigoside B > cornoside; verbascoside was by far the most active constituent, with IC_{50} value lower even than that of the positive control quercetin (Li et al. 2013). Moreover, in the same study, the four PhGs were submitted to an aldose reductase inhibition assay. The inhibition of enzyme decreased in the order verbascoside > isoverbascoside > eutigoside B ≫ cornoside (Li et al. 2013).

de Juliao et al. 2010 investigated the sedative effects of two PhGs from *Lantana trifolia* L. (verbascoside and betonyoside F). The reason why only these two PhGs were submitted to further biological analyses was probably dictated by the yielded amounts: a few mg of samioside and martynoside vs. hundreds of mg of betonyoside F and verbascoside. However, as the yield of verbascoside was around four times higher than that of betonyoside F, only verbascoside was included in an in vivo experiment. It was shown that the ethanolic extract, ethyl acetate extract and verbascoside produced an intense reduction of the walked squares at 1 and 10 mg/kg (extracts) and 1 and 3 mg/kg (verbascoside) in one and 2 h after the administration in mice, respectively. However, verbascoside at 300 μM had no affinity for the [^{3}H] flunitrazepam binding sites, whereas betonyoside F showed an IC_{50} of 550 μM. These values were found to be very high compared to the activity of classical benzodiazepines (IC_{50} values in nM range), suggesting that this is probably not the mechanism by which verbascoside exerts its sedative properties.

The cytotoxic potential of verbascoside purified from *Lippia citriodora* Kunth by CPC was investigated by Cheimonidi et al. (2018). It was shown that verbascoside produced a significant toxicity in metastatic mouse skin carcinoma A5 cells, mouse melanoma B16.F1 and B16.F10 cells, osteosarcoma U2 OS, Sa OS and KH OS cells; moreover, it exhibited synergistic effects with hydrogen peroxide, doxorubicin and epoxomicin and re-sensitized doxorubicin resistant osteosarcoma cell lines. It was suggested that verbascoside exerted its cytotoxicity by inhibiting protein kinase C (enzyme that affects the signaling status of many oncogenic pathways) and modulating antioxidant responses, proteostatic modules and immune responses. Additionally, intraperitoneal administration of verbascoside decreased the tumor growth in an in vivo melanoma mouse model, by activating anti-tumor-reactive immune responses; however, oral administration was not efficient.

There are only eight papers dealing with both CCS and evaluation of biological activity of isolated iridoids. Chen et al. (2017) investigated the anti-nitric oxide production effects of four iridoids isolated from *Gentiana scabra* Bunge. The results showed that all compounds were able to effectively inhibit nitric oxide

production induced by lipopolysaccharide (200 ng/mL) in a dose-dependent manner in murine microglial BV-2 cells. At 250 μM, gentiopicroside, loganic acid, swertiamarin and trifloroside produced an inhibition of 73.3%, 58.1%, 40.8% and 41.0%, respectively.

Some of the CCS isolated iridoids have been proven to possess potent antioxidant activity in various assays. For instance, oleuropein from *Cynomorium songaricum* Rupr. exerted promising DPPH radical scavenging activity (Wang et al. 2016), whereas catalposide and verproside from *Veronica ciliata* Fisch. showed good ferric reducing antioxidant power, DPPH and ABTS cation radicals scavenging effects (Lu et al. 2016). Moreover, the latter two iridoids were evaluated in the same study for their anti-hepatocarcinoma activity using HepG2 cells. These compounds inhibited the proliferation of tumor cells in a concentration-dependent manner, with IC_{50} values of 184.59 μg/mL for catalposide and 177.147 μg/mL for verproside. All 15 iridoids isolated from *Cornus officinalis* Sieb. et Zucc. were investigated for their cytotoxic activity against glioma U87MG and LN229 cells, but no effects were detected at 100 μM after 5 days of incubation (Liang et al. 2018).

Of the seven iridoids isolated from *Capraria biflora* L. through CPC, the two new compounds (3-hydroxymyopochlorin and 5-hydroxyglutinoside) and caprarioside were subjected to in vitro screening against a panel of 37 bacterial strains; however, no activity was detected at 50 mg/L (Lemus et al. 2015). Nevertheless, even if some iridoids are considered inefficient as antibacterial agents, Maurya et al. (2014) investigated the synergistic action of loganetin and nalidixic acid against *Escherichia coli* nalidixic acid resistant and sensitive strains. When tested alone, loganetin was found to be inactive as it displayed a MIC value of 500 μg/mL. But when 10 μg/mL of loganetin was mixed with nalidixic acid, the MIC of nalidixic acid was reduced by 4–8 times. Therefore, the suggested bioenhancing potential of loganetin can be useful in lowering the dose of antibiotics, reducing the drug resistance development frequency and increasing the efficacy of antibiotics against the multidrug resistant *E. coli* strains.

It is worth to note that among these papers, only two described the bioactivity-guided isolation of various iridoids. The 70% ethanolic extract of *Scabiosa stellata* Cav. was initially fractionated over Diaion HP-20 and the five yielded fractions were then screened for antimicrobial activity against 22 microorganisms and anti-tyrosinase activity. As two fractions (B and C) were proven to have superior biological properties, they were both subsequently submitted to CPC experiments. Twelve compounds were purified, of which seven iridoids (two new and five known). 7-*O*-(*E*-*p*-Coumaroyl)- and 7-*O*-(*E*-caffeoyl)-sylvestroside I showed good inhibitory effects against *Enterococcus faecalis* (MIC = 31.2 μg/mL), *Staphylococcus epidermidis* (MIC = 31.2 μg/mL) and *S. aureus* (MIC = 62.5 μg/mL). Eustomoruside exhibited good inhibitory effects against *E. faecalis* and *S. aureus* (MIC = 62.5 μg/mL). Only sylvestroside I showed antibacterial activity against *E. coli* in addition to *S. aureus* (MIC = 62.5 μg/mL). All isolated iridoids were also evaluated for their DPPH radical scavenging effect, but only eustomoruside and eustomoside were active, exhibiting IC_{50} values of 7.1 and 7.2 μg/mL, respectively, whereas for the other compounds the 50% DPPH inhibition could not be reached even at 200 μg/mL. In the anti-tyrosinase assay, none of the compounds was active at the concentration of 665 μg/mL. The cytotoxic activity of the previously unknown iridoids was tested in vitro against HT1080 fibrosarcoma cell line and only 7-*O*-(*E*-caffeoyl)-sylvestroside I was active, with an IC_{50} value of 35.9 ± 0.06 μg/mL (Lehbili et al. 2018).

Zhang et al. (2014) developed a bioassay-guided isolation of pure constituents from *Olea europea* L. using an on-line HSCCC method coupled with a post-column α-amylase anti-diabetic assay. The six fractions purified from olive leaves on resin AB-8 were initially screened for their anti-diabetic effects. As fraction IV showed the strongest inhibitory activity, it was further submitted to HSCCC separation with ethyl acetate–water (1:1) and then α-amylase was added to each sample collection tube behind the HSCCC column. Finally, the fractions showing anti-diabetic activity were subsequently purified by semi-preparative HPLC, yielding oleuropein and ligstroside. The anti-diabetic activity of oleuropein and ligstroside was not further investigated.

Conclusions

All the above presented examples show the enormous potential of CCS in isolating and purifying PhGs and iridoids from different plant sources with various two-

phase solvent systems. In most of the cases, CCS was efficiently integrated into the separation workflow of these secondary metabolites. Up-stream (liquid–liquid extraction, column chromatography over macroporous resins, polyamide, Sephadex LH-20 or silica gel) and down-stream (semi-preparative HPLC, column chromatography over silica gel or Sephadex LH-20) purification techniques were sometimes needed. Nevertheless, CCS offers removal of a complex matrix of natural extracts making further processing easier and more effective, as well as the possibility of creating single-step isolation of a target constituent (e.g. verbascoside from *Abeliophyllum distichum* Nakai, gentiopicroside from *Gentiana crassicaulis* Duthie ex Burk).

Different solvent systems can be successfully used for the separation of the same compounds (e.g. verbascoside), while a proper selected solvent system can be modulated to target similar constituents from the same extract (e.g. iridoids from *Cornus officinalis* Sieb. et Zucc., phenylethanoids from *Cistanche deserticola* Y.C. Ma). It is also important to notice that CCS functions as a highly versatile preparative method, as different operation (normal and reversed-phase) and elution (isocratic, step-gradient, elution-extrusion, cycling-elution, flow-enhanced) modes can be readily employed. The examples thoughtfully described in the current review may be used as starting point for CCS users on their sinuous road of isolating known or waiting to be discovered PhGs and iridoids.

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