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Review

Research Progress in the Separation of Chemical Components from Essential Oils by High-Speed Countercurrent Chromatography

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Abstract: Essential oils (EOs) are vital secondary metabolites in plants. They have garnered substantial attention owing to their distinct flavors and desirable attributes, including potent antioxidant, antibacterial, and antitumor properties. Nevertheless, the active constituents of EOs exhibit intricate chemical structures, and conventional separation techniques are inadequate for purifying the individual chemical components from EOs. High-speed countercurrent chromatography, based on the principles of a hydrodynamic equilibrium system, has emerged as a liquid–liquid chromatographic separation method renowned for its ability to handle substantial single injection volumes and the absence of irreversible adsorption. Consequently, in recent years, this technique has been widely employed in the isolation and refinement of natural products. In this review, a comprehensive analysis is conducted, contrasting the merits and demerits of high-speed countercurrent chromatography with conventional separation methods. The solvent systems, elution modes, commonly employed detectors, and practical applications are reviewed in the context of high-speed countercurrent chromatography for essential oil separation and purification. Furthermore, this review offers a glimpse into the potential prospects of applying this technique, with the intention of serving as a valuable reference for the use of high-speed countercurrent chromatography in the purification of EOs.

Keywords: high-speed countercurrent chromatography; essential oils; separation; application

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1. Introduction

In recent years, with the promotion of the concept of 'green consumerism,' consumer interest in natural products has increased. In particular, there has been a substantial increase in scientific research on the use of aromatic and medicinal plants as alternatives to synthetic drugs, which has led to the widespread application of essential oils (EOs) in various fields.

EOs are secondary plant metabolites that are mainly synthesized and stored by the oil-producing cells of the different organs of aromatic plants [1], such as the flowers (roses), buds (lilac), leaves (eucalyptus, tea tree), barks (cinnamon), peels (lemon, citrus), fruits (cypress), and grasses (lemongrass, rosehips) of woody and herbaceous plants. Based on the distribution of plant EOs in different parts of the plants, a range of EO extraction methods are required, including steam distillation, enzyme extraction, supercritical fluid extraction, ultrasonic-assisted extraction, microwave-assisted extraction, and ohmic heating [2]. Notably, these methods are both green and environmentally friendly. EOs are generally classified as volatile aromatic microdroplets with intricate compositions, typically comprising tens or hundreds of compounds. The constituents of EOs are traditionally categorized into volatile and non-volatile compounds, with the volatile elements constituting approximately 90–95% of the total EO mass. These volatile compounds include fatty alcohols, aliphatic aldehydes, esters, terpenes, and their oxygenated derivatives, all of which exert different biological activities. In addition, the EOs are renowned for their distinct flavors and desirable biological activities, such as antioxidant, antibacterial, and

antitumor properties [3–5]. For example, the terpene linalool, which is the main component of the *Laurus nobilis* (L.) EO (10.2%) [6] has been reported to exhibit a dose-dependent sedative effect in the central nervous system, affecting the expression of Adenylate Cyclase 1 (ADCY1) and ERK (Extracellular signal-regulated kinase).

EOs are natural antimicrobials, and so based on current safety concerns and the ongoing search for healthier, more natural products, natural antimicrobials are becoming more readily accepted by consumers than synthetic antimicrobials [7]. Numerous literature reports [8,9] have confirmed the antimicrobial properties of EOs, in addition to describing their application in food packaging [10], cosmetic preparations [11], and traditional medicines [12]. However, owing to the structural similarity among the active components of EOs and their predisposition to oxidation, the high-purity separation of these constituents has emerged as a prominent focal point of research in the above fields. Considering that natural products contain many different chemical components that exhibit a range of pharmacological effects, it is necessary to separate and purify these compounds to clarify their individual effects. Furthermore, following their successful purification, the chemical structures of the individual components must be deduced prior to subjecting them to activity evaluations. Such investigations are conducive to the in-depth study of the pharmacological and action mechanisms of these natural product components.

Current and traditional methods employed for the separation of active ingredients from EOs include molecular distillation, low-temperature crystallization, column chromatography, and osmotic evaporation. A comparison of the principles, advantages, disadvantages, and scopes of application of these techniques is presented in Table 1. From the data presented in this table, it is evident that although the purification of chemical components from EOs can be achieved using traditional separation techniques, these approaches tend to suffer from low separation purities and high equipment requirements.

Table 1. Comparison of current EO separation techniques.

Method	Separation Principle	Advantages	Disadvantages	Scope of Application	Ref
Molecular distillation	Separation based on the different mean free ranges of motion of the components.	Preservation of physiological activity under low-temperature conditions and a high separation efficiency.	High equipment requirements and high energy consumption in vacuum environments.	Suitable for alcohols, aldehydes, sesquiterpenes, and other small-molecule compounds with high boiling points.	[13]
Low-temperature crystallization	Separation of EOs and their metal salts based on their solubility differences at different temperatures and in different solvents.	Low equipment requirements and preservation of the product's physiological activity at low temperatures.	High energy consumption, low sample recovery, and low separation purity under low-temperature conditions.	Suitable for components of EOs that are poorly soluble at low temperatures.	[14]
Column chromatography	Separation of EOs based on their different partition coefficients between the stationary and mobile phases.	Fast and efficient, easy to operate, and can be used for large-scale production.	Difficulties in separate structurally similar components; large-scale injection leads to a reduced resolution.	Suitable for structurally differentiated high-content EO fractions.	[15]

Table 1. *Cont.*

Method	Separation Principle	Advantages	Disadvantages	Scope of Application	Ref
Osmotic evaporation	Separation of EOs based on the different diffusion rates of the components across the permeable membrane.	High selectivity, low energy consumption, no other reagents required.	High costs of the permeable membranes; limited availability of different permeable membrane types.	Suitable for EO components that are highly soluble with high diffusion rates on the permeable membrane surface.	[16]
High-speed countercurrent chromatography	Separation of EOs based on different partition coefficients in the solvent system.	Retained product activity, high recovery, and easy-to-scale production.	Difficulty in performing solvent system screening for separation.	Suitable for the separation of almost all EOs, especially structurally similar compounds.	[17]

High-speed countercurrent chromatography (HSCCC) involves the application of special hydrodynamic methods and the combination of spiral tube directivity with a high-speed planetary motion to produce a unique fluid dynamics phenomenon. As a result, the relative movement of the two phases (one fixed, one mobile phase) differs in the spiral tube, leading to efficient contact, mixing, distribution, and transfer [18]. Separation using this approach consists of a continuous and highly efficient liquid–liquid partitioning separation technique based on differences in the partition coefficients (K) of the compounds in the two solvent phases [19]. Compared to traditional separation methods, this method is characterized by a high sample recovery, a lack of irreversible adsorption, and flexible separation procedures [20], which can meet the separation requirements of various types of EOs. In recent years, research in related fields has led to great progress in the application of HSCCC for the separation of EOs. For example, Gu et al. [21] established the fingerprint of *Salvia miltiorrhiza* using the non-aqueous capillary electrophoresis (NACE) analytical method and the HSCCC approach, ultimately obtaining the non-polar compounds from the mixture. Compared with the NACE method, the HSCCC approach led to the isolation of 12 components and demonstrated superior performance in identifying tanshinones, which made its fingerprint containing more chemical information than that of NACE. Both the NACE and HSCCC methods can effectively provide the overall concentration distributions of the various components; however, the principles of the two methods are extremely different, resulting in different elution sequences and relative peak contents. For example, HSCCC was more effective in the analysis of tanshinones, thereby providing superior chemical information regarding its fingerprint compared to that obtained using the NACE method. Moreover, it has been demonstrated that HSCCC is a feasible and economical method for the identification of the fingerprint of traditional Chinese medicines (TCMs). Additionally, Ren et al. [22] mainly focused on improving and innovating the instruments employed for HSCCC, whereas Krystyna et al. [23] focused on the separation of highly active compounds from the EOs of TCM, along with the examination of their physiological activities.

Thus, in this paper, research into the separation and purification of EOs by HSCCC is reviewed in terms of the solvent system, elution mode, detector, and practical sample applications. In addition, relevant recent work is examined to provide reference guidelines for the optimization of the separation conditions, with the aim of maximizing component separation from EOs using HSCCC. Table 2 lists the applications of HSCCC in the separation of EOs over the past five years, in addition to providing the corresponding solvent systems, elution modes, and detector-related information.

Table 2. Application of HSCCC in the separation and purification of chemical components from different EOs.

No	EO Source	Type	Solvent System Composition	Compound and Purity	K-Value	Elution Mode	Flow Rate (mL/min)	Detector	Ref.
1	*Eugenia caryophyllata* L.	Arizona	n-hexane/ethyl acetate/methanol/water (1:0.5:1:0.5, *v*/*v*)	Eugenol (98.5%)	0.92	tail-to-head	2.0	HPLC-DAD, GC-MS, ^{1}H-NMR, and ^{13}C-NMR	[24]
2	*Cyperus rotundus* L.	Arizona	n-hexane/ethyl acetate/methanol/water (1:0.2:1.1:0.2, *v*/*v*)	α-Cyperone (98.8%)	1.20	head-to-tail	2.0	HPLC-DAD and MS	[25]
3	*Angelica sinensis* (oliv.) Diels.	Arizona	n-hexane/ethyl acetate/ethanol/water (1:1:1:1, *v*/*v*)	Ligustilide (98.5%)	1.16	head-to-tail	1.5	HPLC-UV and GC-MS	[26]
4	*Illicium verum* Hook. f.	Arizona	n-hexane/ethyl acetate/methanol/water (1:0.2:1:0.1, *v*/*v*)	Anisaldehyde (98.9%)	1.42	tail-to-head	2.0	HPLC-DAD and MS	[27]
5	*Mentha piperita* L.	Arizona	n-hexane/ethyl acetate/methanol/water (4:1:4:1, *v*/*v*)	(*Z*)-Methyl isoeugenol (96.8%) (*E*)-Anethol (99.7%) Menthol (99%) Isomenthone (99%) Menthone (98%) Terpinen-4-ol (96.5%) Neomenthol (94.8%)	1.95 6.30 0.78 3.59 2.61 0.92 1.29	tail-to-head	6.0	GC-MS and UV	[28]
6	*Pimpinella anisum* L.	Ito + Arizona	n-hexane/methanol (1:1, *v*/*v*) n-heptane/ethyl acetate/methanol/water (5:5:2:2, *v*/*v*) n-heptane/methanol (1:1, *v*/*v*)	Pulegone (94%) (*Z*)-Anethole (93%) (*E*)-Foeniculin (93.6%) Linalool (99%) Terpinen-4-ol (98%) α-Terpineol (94%) p-Anisaldehyde (93.54%)	0.93 1.27 2.30 1.70 2.01 1.12 0.46	gradient elution	6.0	UV and GC-MS	[29]
7	*Alpiniaoxyphylla Miquel*	Ito	n-hexane/methanol/water (5:4:1, *v*/*v*)	Nootkatone (92.3%)	1.25	head-to-tail	1.5	UV, GC-MS, and ^{1}H-NMR	[30]
8	*Baccharis dracunculifolia* L.	Ito	n-hexane/methanol/water (5:4:1, *v*/*v*)	(*E*)-Nerolidol (93.7%)	\	tail-to-head	2.0	HPLC and GC-MS	[31]
9	*Cinnamomum camphora* (L.) Presl.	Ito	n-heptane/methanol/water (10:7:3, *v*/*v*)	Borneol (99.9%) Camphor (99.9%)	1.01 2.64	continuous injections	4.0	GC-MS	[32]
10	*Curcuma wenyujin* L.	Ito	petroleum ether/ethanol/ether/water (5:4:0.5:1, *v*/*v*)	Germacrone (97.0%) Curdione (95.0%)	\ \	tail-to-head	1.0	HPLC-UV, MS, and ^{1}H-NMR	[33]
11	*Cuminum cyminum* L.	Ito	n-hexane/methanol/water (5:4:1, *v*/*v*)	Cuminaldehyde (95.42%) p-Menta-1,4-dien-7-al (97.21%)	1.29 1.63	head-to-tail	2.0	UV, GC-MS, ^{1}H-NMR, and ^{1}H-^{1}H COSY	[34]
12	*Pimenta pseudocaryophyllus* L.	Ito	n-hexane/butanol/methanol/water (12:4:4:3, *v*/*v*)	Chavibetol (98%)	1.22	tail-to-head	2.0	HPLC-DAD and GC-MS	[35]

Table 2. *Cont.*

No	EO Source	Type	Solvent System Composition	Compound and Purity	K-Value	Elution Mode	Flow Rate (mL/min)	Detector	Ref.
13	*Zingiber officinale* L.	Ito	n-hexane/ethyl acetate/methanol/water (7:3:5:5, *v*/*v*)	Methyleugenol (96%)	0.57	head-to-tail	2.0	HPLC-DAD, UV, and GC-MS	[36]
				6-Gingerol (98.6%)	0.89				
		Ito	n-hexane/methanol/water (3:2:1, *v*/*v*)	Zingerone (99.4%)	0.76				
14	*Curcuma longa* L.	HBAW	n-hexane/chloroform/acetonitrile (6:2:5, *v*/*v*)	Sesquiterpenes (99.2%)	0.59	head-to-tail	6.0	HPLC-DAD, ^{1}H-NMR, and ^{13}C NMR	[37]
		HBAW	n-heptane/ethyl acetate/acetonitrile/water (9.5:0.5:9:1, *v*/*v*)	ar-Turmerone (99.39%)	0.78				
				β-Turmerone (99.53%)	1.66				
				α-Turmerone (99.25%)	1.92				
				α-Atlantone (98.56%)	2.77				
15	*Flaveria bidentis* L.	HBAW	n-hexane/acetonitrile/ethanol (5:4:3, *v*/*v*)	Caryophyllene oxide (92.6%)	1.15	head-to-tail	1.5	HPLC-DAD, GC-MS, ^{1}H-NMR, and ^{13}C NMR	[38]
16	*Piper mollicomum* Kunth.	HBAW	n-hexane/acetonitrile/ethyl acetate (1:1:0.4, *v*/*v*)	7,11-Dimethyl-3-methylene-1,6,10-dodecatriene (99.1%)	2.49	tail-to-head	2.0	GC-FID, GC-MS, ^{1}H-NMR, and ^{13}C NMR	[39]
				Caryophyllene (98.9%)	2.93				
				Camphene (82.0%)	0.37				
				Camphor (98.5%)	1.47				
				Bornyl acetate (91.2%)	0.73				
				(*E*)-Nerolidol (92.8%)	2.06				
17	*Nigella damascena* L.	HBAW	petroleum ether/acetonitrile/acetone (2:1.5:0.5, *v*/*v*)	β-Elemene (96%)	2.58	tail-to-head	6.0	GC-MS, ^{1}H-NMR, and ^{13}C NMR	[40]
18	*Vitex negundo* L. var. *heterophylla* Franch. Rehd.	HBAW	n-hexane/dichloromethane/acetonitrile (10:3:7, *v*/*v*)	β-Caryophyllene (95.0%)	2.56	tail-to-head	2.0	ELSD, GC-FID, and GC-MS,	[41]
			n-hexane/chloroform/acetonitrile (6:2:5, *v*/*v*)	β-Caryophyllene (95.3%)	1.84		1.5		
19	*Eugenia uniflora* L.	HBAW	n-hexane/acetonitrile (1:1, *v*/*v*)	Selina-1,3,7(11)-trien-8-one (92.5%)	0.91	head-to-tail	2.0	GC-FID, GC-MS, ^{1}H-NMR, and ^{13}C NMR	[42]
				Selina-1,3,7(11)-trien-8-one epoxide (93.1%)	1.55				
				Selina-1,3,7(11)-trien-8-one(92%)	1.09	tail-to-head			
				Selina-1,3,7(11)-trien-8-one epoxide (97.5%)	0.65				
20	*Pectis brevipedunculata* L.	HBAW	n-hexane/acetonitrile (1:1, *v*/*v*)	Citral (98.7%)	\	tail-to-head	2.0	GC-FID, GC-MS, ^{1}H-NMR, and ^{13}C NMR	[43]
				Geraniol (86.0%)	\				
				Neral (87.5%)	\				
				Geranial (91.0%)	\				
				Citral (100.0%)	\		1.0		

Table 2. *Cont.*

No	EO Source	Type	Solvent System Composition	Compound and Purity	K-Value	Elution Mode	Flow Rate (mL/min)	Detector	Ref.
21	*Daucus carota* L. ssp. carota	HBAW	n-hexane/acetonitrile/ methyl tert-butyl ether (1:1:0.1, *v*/*v*)	Daucol (80.0%)	0.78	head-to-tail	6.0	GC-MS and UV	[44]
				Geranyl acetate (84.0%)	0.93				
				Caryophyllene oxide (85.0%)	1.44				
				Carotol (95.0%)	2.00				
				Sabinene (97.0%)	4.20				
				D-Limonene (84.0%)	6.17				
				α-Pinene (91.0%)	9.70				
		HBAW	n-hexane/acetonitrile/ methyl tert-butyl ether (2:1:0.1, *v*/*v*)	Daucol (90.0%)	0.67				
				Geranyl acetate (82.0%)	0.84				
				Caryophyllene oxide (85.0%)	1.27				
				Carotol (99.0%)	1.50				
				Sabinene (99.0%)	3.99				
				D-Limonene (82.0%)	5.29				
				α-Pinene (89.0%)	8.07				
22	*Artemisia argyi*	HBAW	n-hexane/acetonitrile/methanol (2:2:1, *v*/*v*/*v*)	Eucalyptol (81.93%)		head-tail		GC-MS and UV	[45]

Compound K-value not provided in the literature reference; Liquid chromatography (HPLC); gas chromatography (GC); nuclear magnetic resonance (NMR) spectroscopy; hydrogen diode array detection (DAD); evaporative light scattering detector (ELSD); and ultraviolet–visible (UV) spectrophotometry; Purity was calculated using area normalization according to the literature.

2. HSCCC Solvent System for EO Separation

Although HSCCC has many advantages over traditional separation methods, its widespread application in essential oil separation is hampered by some disadvantages. Although similar systems can be screened in the literature, there are still many uncertainties affecting the separation efficiency for actual samples, such as elution mode, stationary phase, mobile phase flow rate, separation temperature, centrifuge speed, etc. Due to the immaturity of the theoretical system, HSCCC cannot be applied to an industrial scale. This is also a shortcoming that the researchers need to strive to improve. Since HSCCC is a liquid–liquid partition-based chromatography system, solvent selection is extremely important, and an appropriate K-value is a prerequisite for efficient separation. To ensure that the samples exhibit high solubility in the solvent system and to avoid issues related to denaturation and decomposition, it is usually necessary to control the K-value of the target product in the range of 0.5–2 [46]. If the K-value is too small, the sample will elute rapidly, leading to poor separation. In contrast, if the K-value is too large, large amounts of solvent will be required over a long run time, and the separation efficiency will be reduced.

2.1. Classical Solvent Systems

The three classical solvent systems currently used for HSCCC include the Arizona system (n-heptane/ethyl acetate/methanol/water) [47], the Ito system (n-hexane/ethyl acetate/methanol/n-butanol/water) [48], and the HBAW system (n-heptane/n-butanol/acetonitrile/water) [49]. The solvent systems used for isolation of the various chemical components from EOs over the last 5 years are also listed in Table 2.

The above three classic solvent systems consist of weakly and strongly polar solvents that form two-phase solvent systems, and subsequently, medium polarity solvents are selectively added for optimization. More specifically, weakly polar solvents include n-hexane, n-heptane, and petroleum ether, and these are used as the solvent base to dissolve the least polar components [48]. An appropriate increase in the proportion of weakly polar solvents can effectively improve the separation efficiency. In contrast, strongly polar solvents include methanol, ethanol, acetonitrile, and water, which are used to widen the range of separation polarities of the solvent system [49]. Commonly used medium polarity solvents, such as ethyl acetate, n-butanol, and ethyl ether, are used to selectively modify the polarity of the system for separation of the target products [50]. Such optimization can tune the solvent polarity to render the target components more soluble and achieve the most effective separation conditions. Although the Ito and Arizona systems use similar basic solvents, they employ different procedures for screening the K-values of the compounds [29]. Only a few of the optimized solvent ratios of the Arizona system meet the suitable polarity requirements (ethyl acetate/water = 1:1 or n-hexane/methanol = 1:1), and so there is no obvious boundary between these two Arizona solvent systems [25,26].

When selecting a classical solvent system, it is necessary to consider its scope of application. To obtain the ideal separation effect, the solvent system must match the polarity of the target product to meet the "principle of similar solubility". The separation polarities of the above three classical solvent systems vary based on the polarity-adjusting agents employed in the different systems. The Arizona system [24–28] mainly adopts the medium polarity ethyl acetate as the adjusting agent to rapidly determine the optimal separation system upon varying the solvent ratios; however, the separation polarity range of this system is narrow. The Ito system mainly adopts high polarity methanol, ethanol, and water as its adjusting agents to broaden the separation polarity range and separate EOs containing both strongly and weakly polar compounds. The HBAW system mainly adopts the slightly weaker acetonitrile as the strongly polar solvent. Therefore, its applicable polarity is slightly smaller, rendering it suitable for application in the separation of weakly polar terpenoids from EOs.

In recent years, the HBAW system has been explored in greater detail, including the implementation of an anhydrous solvent system, which is more suitable for the separation of weakly polar terpenes [51]. Based on these developments, a variety of products can be

obtained by selecting the HBAW system for the separation and purification of EOs. To generate a two-phase solvent system, this system uses only weakly polar n-hexane and n-heptane combined with the strongly polar acetonitrile [52]. Notably, the use of such a limited solvent has the advantages of simple proportioning and convenient recycling. The anhydrous system also does not require the removal of water by rotary evaporation at the end of the separation process, and product separation can be performed directly. Ultimately, this prevents the inactivation and denaturation of the separated products, rendering this approach particularly desirable in the separation of EO components [53].

2.2. Selective Reagent Solvent Systems

Given the low number of theoretical plates exhibited by HSCCC and the poor separation of structurally similar compounds using only the basic two-phase solvent system, selective reagents are added to improve the system partition equilibrium, to increase the compound K-value [54], to shorten the separation time, and to enhance the separation efficiency. Currently, the selective reagents commonly employed in the HSCCC separation of EOs are metal ion-selective reagents, ionic liquids, and cyclodextrins [55].

2.2.1. Metal Ion Solvent Systems

Metal ion-selective reagents are solutions containing metal ions, such as Ag^+ and Cu^{2+}, which contain empty d orbitals and can receive π-electrons from the double bonds of EOs, reversibly forming weakly coordinating compounds. This lowers the K-values of the target compounds and enhances the efficiency and selectivity of separation. For example, elemene is a ternary polyunsaturated sesquiterpene that can exist as four different isomers (i.e., the α-, β-, γ-, and δ-isomers). β-Elemene is known to block the cell cycle and induce apoptosis, thereby providing a good broad-spectrum anticancer activity [55]. Indeed, it has now been approved for use as an anticancer adjuvant drug by the State Food and Drug Administration of China [56], highlighting the requirement for an efficient and gentle purification route. In this context, Lu et al. [17] employed silica gel chromatography to co-dry the β-elemene fraction of the *Curcumae rhizoma* (L.) EO, followed by purification using an Ito system of n-hexane/methanol/water (2:1.5:0.5, *v*/*v*) in the head-to-tail mode. The separation efficiency was systematically evaluated by adding varying concentrations of silver nitrate, and it was found that the addition of 0.15 mol/L silver nitrate to the stationary phase was optimal. Through gas chromatography–mass spectrometry (GC-MS) offline analysis, 145 mg of β-elemene with a purity of 99% was successfully separated from 445 mg of the *Curcumae rhizoma* (L.) EO. Compared with traditional distillation processes, there were improvements in both the purity and yield, demonstrating that HSCCC is an efficient purification method. Additionally, the authors found that silver ions effectively increased the retention time of β-elemene, preventing its co-elution with other impurities. These results appear to confirm that the addition of an appropriate amount of a metal ion reagent to the solvent system can effectively enhance the separation efficiencies of specific compounds. As another example, *Acorus tatarinowii* (L.) Schott, one of the common adjuvants used in traditional Chinese medicine for treating cognitive impairments and cerebral infarction [57], contains α- and β-asarone as its primary active constituents. α-Asarone exhibits lower toxicity and is better suited for the development of novel neuroprotective drugs. However, α-asarone is present in lower quantities than its β isomer, is prone to degradation, and shares both structural isomerism and a similar polarity with β-asarone. These factors present substantial co-elution challenges during large-scale separation and preparation using conventional solvent systems owing. To address this issue, Zhu et al. [58] used an HBAW system consisting of n-hexane/ethyl acetate/ethanol/water (2:1:2:1, *v*/*v*) for the separation and purification of *Acorus tatarinowii* (L.) Schott extract via the tail-to-head mode. Silver nitrate was used as the selective reagent, and optimization experiments revealed that the addition of 0.30 mol/L silver nitrate to the aqueous phase resulted in a superior separation performance compared to the control group (i.e., without silver nitrate). However, the separation efficiency for α- and β-asarone

was suboptimal, so the silver ion concentration was increased to 0.50 mol/L, and the sample injection volume was increased to 2.0 g to facilitate high-throughput separation. According to the high-performance liquid chromatography (HPLC) elution profiles, 1.4 g of β-asarone and 0.09 g of α-asarone were successfully isolated, with both compounds exhibiting purities of >98%. To elucidate the mechanism by which the silver ions promote separation, density functional theory calculations were employed to compute the metal complexes. A comparative analysis revealed that the addition of silver ions led to changes in the dihedral angles of the target compounds, resulting in greater stability for the β-isomer complex. Consequently, the separation factor between the α- and β-isomers was increased 1.36-fold, leading to their successful separation. This indicates that the addition of metal ion-selective reagents can significantly enhance the efficiency of HSCCC separation. However, the introduction of metal ions can inevitably lead to contamination and the presence of residues in the purified products, thereby reducing product yields and limiting their subsequent applications in areas such as drug development.

2.2.2. Ionic Liquid Solvent Systems

Ionic liquids (ILs) are a class of organic salts composed entirely of ions. They are considered "green alternatives" to traditional solvents because of their low vapor pressures, ease of removal and recycling, and high stabilities. ILs have widespread applications in the separation of natural products [59], which is achieved by modulating the polarities of the upper and lower phases through a rich combination of cations and anions. Consequently, the K-values of the target compounds can be optimized to enhance the selectivity of the solvent system [60]. *Chrysopogon zizanioides* (L.) is currently an important raw material in the fragrance processing industry. It is composed of over 300 different compounds, including α-cadinene, humulene, and α-selinene, which are of particular interest to the fragrance industry. However, separating these compounds is impractical despite the fact that such separation could be instrumental in enhancing the fragrance profile and advancing the fragrance industry. In this context, Brown et al. [61] employed a weakly polar solvent, n-hexane, as the base solvent to separate the components of *Chrysopogon zizanioides* (L.). They added 1-dodecyl-3-methylimidazolium bis{(trifluoromethyl)sulfonyl}amide ([C12mim][NTf2]) as an IL in a solvent ratio of 1:3 (v/v). In this system, [C12mim][NTf2] acted as the stationary phase, and n-hexane served as the mobile phase. Using GC-MS/FID, 106 fractions were obtained, wherein the compounds in fractions T6–T25 included sesquiterpenes (e.g., α-cadinene, humulene, and α-selinene), whereas the compounds in fractions T26 upwards mainly contained oxygenated sesquiterpenes, alcohols, ethers, and other components. These results suggest that the addition of appropriate ILs to a base solvent system can effectively classify and separate complex compounds.

2.2.3. Cyclodextrin Solvent Systems

Cyclodextrins (CDs) are cyclic oligosaccharides bearing hydrophilic and lipophilic interior cavities [62]. These cavities can encapsulate and complex specific straight-chain compounds, thereby improving the solubility and stability of the target product. These interactions also tune the K-value of the target compound in the solvent system [63], imparting it with a different retention time to the other compounds to achieve separation. Currently, the most commonly used CDs can be categorized into three types, namely α-, β-, and γ-CDs, based on their different degrees of polymerization. The α- and γ-CDs are more soluble in water and exhibit higher toxicities, thereby rendering them less suitable for product separation. In contrast, the β-CDs are less soluble in water, are structurally stable, and are non-toxic. They can, therefore, be derivatized and modified to achieve the desired separation effects. Herbal EOs contain a range of complex components and typically require repeated separations using column chromatography. This process is time-consuming and results in a significant waste of solvents, thereby limiting progress in the pharmaceutical industry. To address this issue, Tong et al. [64] employed HSCCC in the head-to-tail mode and incorporated an Ito system of n-hexane/water (1:1, v/v) for

the separation of major compounds from seven commonly used herbal EOs. To further enhance the separation of the target compounds, β-CD and its derivatives, methyl-β-cyclodextrin (Me-β-CD) and hydroxypropyl-β-cyclodextrin (HP-β-CD), were added as selective reagents at a concentration of 0.1 mol/L. The K-values of various compounds were then estimated using HPLC to screen for the most selective reagents. According to the measured K-values, β-CD was only suitable for the separation of *Myristicae semen* (L.) and *Chuanxiong rhizoma* (L.). In contrast, HP-β-CD and Me-β-CD were not suitable for the separation of *Myristicae semen* (L.), and HP-β-CD was also unsuitable for the separation of *Curcumae rhizoma* (L.). Based on these results, the authors used β-CD as the selective reagent to separate 18 mg of α-terpineol from 556 mg of *Myristicae semen* (L.). In addition, they used Me-β-CD as the selective reagent to separate 11 mg of germacrone from 536 mg of *Curcumae rhizoma* (L.), 55 mg of senkyunolide A from 522 mg of *Chuanxiong rhizoma* (L.), and 49 mg of ligustilide from 530 mg of *Angelicae sinensis radix* (L.). Furthermore, using HP-β-CD as the selective reagent, the separation of 11 mg of germacrone from 507 mg of *Chuanxiong rhizoma* (L.) and the separation of 15 mg of trans-α-ionone from 500 mg of *Aucklandiae radix* (L.) was achieved. Their results [64] demonstrated that HSCCC is an efficient method for separating the major components of EOs. The potential for performing derivative modifications of β-CD to improve the separation efficiencies of EO components was also demonstrated.

3. HSCCC Elution Mode for EO Separation

Various elution modes suitable for EO purification have been developed based on the fluid characteristics of the HSCCC stationary phase. Currently, the most common elution modes for separating chemical components from EOs by HSCCC include displacement and gradient elution [65]. Given the complexity of the EO components, in addition to their structural similarities, a combination of multiple elution modes can be employed to enhance the purification efficiency.

3.1. Elution–Extrusion Mode

The elution–extrusion mode fully exploits the characteristics of both liquid phases in HSCCC. After the elution of the compounds with smaller K-values, the flow path is switched to a pump in the stationary phase, and the components with larger K-values are extruded into the coiled tube. Once the operation is complete, the stationary phase inside the coiled tube is replaced, allowing the next equilibrium and separation to proceed directly. This enables continuous high-throughput separation [66]. Given the complexity of the EO components, along with their structural similarities, it can be challenging to determine the optimal solvent system to achieve high separation efficiencies for all components. Theoretically, the elution–extrusion mode can sequentially elute all components in the order of their K-values, avoiding the issue of band broadening associated with larger K-values. This partially compensates for the limitations of the HSCCC solvent systems, which have relatively narrow polarity ranges. In this context, Wang et al. [67] employed an HBAW system with a relatively narrow polarity range (n-hexane/acetonitrile/ethanol; 5:3:2, *v*/*v*) for the purification of 420 mg of the *Curcuma wenyujin* (L.) EO based on the elution–extrusion mode. Due to the relatively high K-values of α- and β-elemene in this solvent system (3.42 and 2.55, respectively), the authors adopted a two-phase elution strategy. During the initial 300 min, the conventional reversed-phase elution mode was employed at a flow rate of 2 mL/min to reduce the retention time and avoid any purity reduction attributed to band broadening. Subsequently, they switched to the elution–extrusion mode at a flow rate of 20 mL/min to rapidly introduce a new stationary phase and extrude the residual mobile phase from the coiled tube. According to the GC chromatogram, they successfully isolated 1.18 mg of δ-elemene and 2.76 mg of β-elemene from the residual mobile phase. However, considering that eucalyptol and curzerene have similar K-values during elution (1.51 and 1.30, respectively), their co-elution in the initial separation did not yield the desired results. Therefore, the authors switched to an HBAW system based

on n-hexane/acetonitrile/acetone (4:3:1, *v*/*v*) for a second separation, and GC analysis revealed the isolation of 3.13 mg of eucalyptol with a purity of 97%. This illustrates that the elution–extrusion mode can be employed for the initial separation of complex compounds, followed by a combination of multiple elution modes for the further purification of specific products. Importantly, this approach provides key insights into the HSCCC separation of complex mixtures. In another study, with the aim of screening for active inhibitory components against natural *Mycobacterium tuberculosis*, Ramos et al. [68] employed an HBAW system with a relatively narrow polarity range (n-hexane/acetonitrile/methyl tert-butyl ether; 10:10:1, *v*/*v*). They used the elution–extrusion mode to separate the polar and complex *Eucalyptus citriodora* (L.) EO, which possessed compound K-values ranging from 0.69 to 2.98. A total of 60 separated fractions were obtained and screened against *Mycobacterium tuberculosis* using an aerosol contact inhibition activity assessment to construct a bioactivity chromatogram. GC-MS analysis revealed the separation of 32 compounds, including highly active compounds such as α-eudesmol, β-eudesmol, trans-farnesol, and 6-methyl-2,4-ditert-butylphenol, with purities of 96.5, 95.4, 95.4, and 90.4%, respectively. These studies collectively demonstrate that the elution–extrusion mode not only effectively broadens the K-value range for solvent separation but it also avoids the requirement for time-consuming screening procedures and optimization processes. Moreover, it yields high-purity separation products and offers a viable pathway for the separation of complex components.

3.2. Gradient Elution Mode

One of the characteristics of HSCCC is the fact that it commonly employs fixed-ratio solvent systems; however, achieving the desired separation of compounds with a wide range of polarities can be challenging. Gradient elution typically involves altering the solvent composition of the mobile phase during elution, causing changes in the distribution coefficients of various sample components and allowing their sequential elution [20]. Two common gradient elution methods exist, namely isocratic and stepwise gradient elution. During isocratic gradient elution, the solvent composition changes continuously over time, whereas stepwise gradient elution can be viewed as a combination of multiple isocratic elution steps. Notably, the isocratic gradient mode only alters the solvent composition, limiting its polarity range, whereas the stepwise gradient mode can change the solvent type by directly switching from non-polar to polar solvents, thereby rendering it more suitable for the separation of compounds with a broader polarity range. For example, to separate the antifungal chemical component α-cedrol from the EO of *Platycladus orientalis* (L.) leaf, Rehman et al. [69] employed the isocratic gradient elution mode. They used the Arizona system, initially consisting of n-hexane/ethyl acetate/methanol/water (1:1:1:1, *v*/*v*); after 120 min, they gradually modified the solvent system composition to 19:1:19:1 (*v*/*v*). According to their GC-MS results, 29.8 mg of α-cedrol was successfully isolated from 200 mg of the *Platycladus orientalis* (L.) leaf EO, representing a recovery rate of 86.6%. To further enhance the separation and purification efficiency, the authors adjusted the initial solvent composition of the non-polar components to 4:1:4:1 (*v*/*v*). Without altering any other conditions, they repeated the separation steps to isolate 65.72 mg of α-cedrol from 400 mg of the *Platycladus orientalis* (L.) leaf EO in 60 min, representing a recovery rate of 94.58%. This approach not only provided an effective method for the efficient extraction and isolation of α-cedrol, but it also demonstrated specific application prospects for use in the fields of drug discovery and flavor chemistry. As another example, *Pimpinella anisum* (L.) is a common herbaceous plant found in Asia and Europe. It is known for its digestive and diuretic properties and is widely used in both traditional medicine and food processing [70]. Krystyna et al. [29] used a stepwise gradient elution approach to isolate terpene components from *Pimpinella anisum* (L.) for further development and utilization. Given that the components anethole and folliculin have K-values of 4.01 and 31.76 in the Ito system, an Ito system based on n-heptane/ethyl acetate/methanol/water (5:5:2:2, *v*/*v*) was used over a 40 min run. Subsequently, the system was switched to an HBAW system

using n-heptane/methanol (1:1, v/v), and separation was continued for a further 40 min. The GC-MS results revealed that six main compounds, namely p-anisaldehyde, α-terpineol, linalool, terpinen-4-ol, anethole, and foeniculin, were sequentially separated with purities of 93.54, 94, 99, 98, 93, and 93.6%, respectively. These results indicate that compared to the conventional elution mode, the gradient elution mode can efficiently separate a wider range of substances with varying polarities. In addition, it allows for an increased sample injection volume while maintaining satisfactory recovery rates. Therefore, this mode has considerable potential for use in various industrial applications.

4. Common HSCCC Detectors for EO Separation

To render HSCCC an efficient means of purification and separation, various high-sensitivity detectors have been employed to meet the detection requirements of EO compounds. As shown in Table 2, HPLC and GC are commonly used as offline detectors for the initial identification and purity assessments of separated products [67]. They are often equipped with ultraviolet (UV) detectors and diode array detectors (DAD) for scanning and screening the separated fractions, thereby reducing sample loss during detection and enhancing product identification efficiency [31]. In addition, an evaporative light scattering detector (ELSD) serves as an alternative to UV and DAD for products that lack or exhibit weak UV absorption properties. Its key advantage lies in the fact that it does not rely on the optical characteristics of the products, thereby enabling the swift and efficient identification of compounds that cannot be readily detected using UV and DAD approaches [36]. For precise product identification, MS can be employed as an offline detector to acquire crucial information, such as the molecular weight of a compound. In this context, Tong et al. [45] used a solvent system composed of n-hexane/acetonitrile/methanol (2:2:1, $v/v/v$) for the first-dimensional separation of the *Artemisia argyi* EO. All CCC fractions were subsequently analyzed by GC to obtain a wealth of information related to the EO composition. All the compounds were identified by GC-MS, and the chemical compositions were visualized following the comprehensive 2D CCC × GC separation achieved by creating a two-dimensional contour plot map. For newly structured products, NMR can also be used as an offline detector to determine specific structures [71], providing substantial assistance in the qualitative analysis of EO compounds. For example, Ni et al. [72] used an n-hexane-ethyl acetate/methanol/water (10:2:5:7, $v/v/v/v$) system to purify ginger EO via the HSCCC head-to-tail mode and analyzed the purified compounds by HPLC. Their results showed that 90.38 ± 0.53 mg of 6-gingerol (purity 99.6%) was obtained from 600 mg of the molecular distillation residue. The structure of 6-gingerol was identified using the EI/MS, ^{1}H NMR, and ^{13}H NMR spectroscopic approaches.

5. Use of HSCCC to Separate the Active Compounds of EOs

In recent years, ongoing research into EOs has led to recognition of the remarkable physiological activities of their bioactive compounds, such as antioxidant, antimicrobial, and anticancer properties. Importantly, HSCCC is a gentle and efficient separation technique that maximizes the preservation of the physiological activities of the separated components. Thus, currently, two HSCCC approaches exist for separating bioactive compounds from EOs [73]. The first approach involves non-targeted separation, in which the EOs are initially subjected to HSCCC for purification and separation of their individual compounds. Subsequently, activity screening is performed. The second approach adopts a targeted separation strategy, wherein modern chromatographic techniques are initially used for screening the compound activities. Subsequently, HSCCC is used to isolate the active components. Notably, this approach significantly enhanced the screening and isolation efficiencies of the active compounds.

5.1. Separation of Antioxidant Active Ingredients by HSCCC

The excessive production of free radicals and reactive oxygen species during normal metabolic processes in the body can cause oxidative damage to cells or organs, leading to

chronic diseases such as atherosclerosis and cardiovascular diseases. Therefore, the removal of excess free radicals or reactive oxygen species from the body is beneficial for human health. Antioxidants, which are also known as free-radical scavengers, can terminate chain reactions by binding with free radicals or reactive oxygen species, thereby preventing the destruction of essential molecules in the body. Currently, the most commonly used antioxidants are produced synthetically, with examples including propyl gallate, butylated hydroxyanisole, butylated hydroxytoluene, and tert-butylhydroquinone. Although these synthetically produced antioxidants efficiently bind to free radicals and reactive oxygen species generated in the body, the majority of synthetic antioxidants are associated with potential health risks, including liver damage and carcinogenic effects, which restrict their use. Therefore, the search for highly active natural antioxidants is of growing importance. Recently, HSCCC has been widely used to separate natural antioxidants from plant secondary metabolites, including EOs. These natural antioxidants have the potential for use as alternatives to synthetic antioxidants in food packaging and other fields [73]. Current research into the separation of natural antioxidants using HSCCC can be categorized into three types, namely, the non-targeted separation of natural antioxidants, the targeted separation of known antioxidants, and the separation of antioxidants based on their activities. For example, Jaramillo et al. [74] employed an HBAW system consisting of acetonitrile, methyl tert-butyl ether, and n-hexane to perform non-targeted separation of the chemical components in EOs obtained from four plants in Colombia. They evaluated the antioxidant activities of the EOs and components using two in vitro assays, namely the $DPPH^{\cdot}$ (radical α,α-diphenyl-β-picrylhydracil) and $ABTS^{+\cdot}$ (2,2′-azino-bis-(3-ethylthiazoline-benzenesulfonicacid-6)) assays. More specifically, the EOs from *Aloysia citriodora* (L.), *Lippia abla* (L.), and *Xylopia aromatica* (L.) were successfully separated using an HBAW system composed of n-hexane/acetonitrile/methyl tert-butyl ether (1:1.5:0.2, *v*/*v*) at a flow rate of 1.5 mL/min. In addition, the EO from *Lantana armata* (L.) was separated using an HBAW system based on n-hexane/acetonitrile/methyl tert-butyl ether (1.5:1:0.2, *v*/*v*) at a flow rate of 2.0 mL/min. By observing the UV separation curves, 7, 8, 10, and 12 major fractions were obtained from the EOs of these four plants. Through subsequent in vitro DPPH and ABTS experiments, it was found that the *Aloysia citriodora* (L.) EO exhibited the strongest antioxidant activity, although overall, the activities were low. In another study, Krystyna et al. [23] conducted a component analysis of the *Heracleum mantegazzianum* (L.) fruit EO using GC-MS and employed an HBAW system consisting of n-hexane/acetonitrile/methyl tert-butyl ether (1:1:0.1, *v*/*v*) for the targeted separation of its known active components. Five highly pure fractions were isolated based on UV and GC-MS chromatographic curves. Over a 74 min run, these fractions yielded n-octanol (1.89 mg, 95% purity), n-octyl acetate (2.53 mg, 95% purity), hexyl-2-methylbutanoate (1.14 mg, 94% purity), n-octyl isobutanoate (1.20 mg, 98% purity), and n-octyl-2-methylbutanoate (1.90 mg, 99% purity); the remaining 10 fractions consisted of mixtures. Additionally, the authors employed DPPH and the β-carotene/linoleic acid auto-oxidation system to evaluate the antioxidant activities of the *Heracleum mantegazzianum* (L.) fruit EO and its fractions. Their results revealed that the *Heracleum mantegazzianum* (L.) fruit EO exhibited strong antioxidant activity, whereas its fractions had weaker (or no) antioxidant activities. This observation suggests that the antioxidant activity of the EO may be due to the synergistic effects between its various chemical components. However, it was found that the EO did not exhibit inhibitory activity against bacteria, and only the octyl acetate component demonstrated a strong antifungal activity, thereby rendering it a potential lead compound for the development of natural biocides. Furthermore, Wang et al. [67] employed a GC-MS-DPPH offline detection system to conduct an initial screening of the active components present in the *Curcuma wenyujin* (L.) EO, with a focus on the antioxidant activity. By comparing the reduction in peak areas of various compounds before and after the DPPH reaction, they preliminary identified eucalyptol, camphor, δ-elemene, β-elemene, and curzerene as having strong antioxidant activities. These compounds were subsequently separated using an HBAW system consisting of n-hexane/acetonitrile/ethanol (5:3:2, *v*/*v*). However,

owing to the similar K-values of eucalyptol and curzerene in this solvent system (1.51 and 1.30, respectively), it was necessary to optimize the solvent ratio to 4:3:1 (v/v) prior to performing a secondary separation of this mixed fraction using the elution–extrusion mode. Ultimately, this process yielded 3.13 mg of eucalyptol with a purity of 97%. The authors also evaluated the antioxidant activities of the five separated compounds and found that camphor, δ-elemene, and β-elemene exhibited weaker antioxidant activities, whereas curzerene and eucalyptol demonstrated stronger antioxidant activities. When the latter two compounds were mixed at different ratios, they showed good synergistic antioxidant activities, rendering them potential candidates for the development and utilization of natural antioxidants. It was therefore confirmed that offline GC-MS-DPPH detection is an efficient and rapid method for activity screening and could serve as a front-end activity screening technique for HSCCC.

5.2. Separation of Antimicrobial Active Components in the EOs by HSCCC

In recent years, the overuse of synthetic antibiotics has led to the emergence of bacterial resistance, which poses a substantial threat to human health. Therefore, the identification of alternatives to traditional synthetic antibiotics is an urgent and challenging task. Because natural products are rich in antimicrobial compounds, researchers have increasingly focused on antimicrobial compounds from plant sources. Among these, EOs have been found to demonstrate broad-spectrum antimicrobial activities [75], indicating their potential to serve as natural alternatives to traditional antibiotics. Furthermore, the focus of current research has shifted toward the separation and purification of antimicrobial components from EOs using HSCCC. For example, Elwira et al. [40] employed an HSCCC system with a petroleum ether/acetonitrile/acetone (2:1:0.5, v/v) solvent system to separate 100 mg of the *Nigella damascena* (L.) EO. Upon combination with GC-MS and UV chromatographic approaches, they obtained 22 mg of β-elemene with a purity of 96% after 70 min of separation. To further validate the activity of the isolated product, the authors tested the antibacterial activity of β-elemene against common pathogenic bacteria, such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Escherichia coli*. Their results showed that β-elemene exhibited moderate antibacterial activity against *Mycobacterium tuberculosis* strain H37Ra (ATCC 25177), indicating its potential for use as an antibacterial agent. In another study, Ramos et al. [68] used HSCCC with a solvent system consisting of n-hexane/acetonitrile/methyl tert-butyl ether (10:10:1, v/v) to separate the *Eucalyptus citriodora* (L.) EO. They evaluated the aerosol contact inhibition activities of 160 separated fractions against *Mycobacterium tuberculosis*, and a bioactivity chromatogram was constructed. Additionally, GC-MS was used to analyze the chemical composition of each fraction and to identify the *Mycobacterium tuberculosis*-inhibiting active compounds present in the *Eucalyptus citriodora* (L.) EO. These compounds included citronellol, linalool, isopulegol, α-terpineol, sesquiterpenoids spathulenol, β-eudesmol, and τ-cadinol, thereby demonstrating that HSCCC is a useful method for isolating antibacterial components from EOs.

5.3. Separation of the Anti-Inflammatory and Antitumor Active Components in EOs by HSCCC

According to recent pharmacological studies [58], some EOs have shown promising anti-inflammatory and antitumor activities. Therefore, the isolation and screening of individual EO components based on their anti-inflammatory and antitumor activities are crucial for elucidating the basis of their bioactive properties. Indeed, the anti-inflammatory and antitumor components present in EOs have the potential for use as promising candidates for anti-inflammatory and antitumor drug screening. In this context, *Nigella damascena* (L.), a traditional Chinese medicine used to treat conditions such as high fever, colic pain, and edema, has received particular interest due to its desirable pharmacological effects. For example, Krystyna et al. [76] conducted the HSCCC-based isolation and purification of 200 mg of the *Nigella damascena* (L.) EO using the reversed-phase mode with an HBAW system consisting of petroleum ether/acetonitrile/acetone (2:1.5:0.5, v/v). This separation yielded 13.5 mg of damascenine with a purity of 99.5% and 4.6 mg of β-elemene with

a purity of 98.0%. Furthermore, the authors examined the anti-inflammatory activities of the EO, damascenine, and β-elemene specimens using an ex vivo lipopolysaccharide model. They found that these samples exhibited substantial anti-inflammatory activities by suppressing the secretion levels of Interleukin-1β (IL-1β), Interleukin-8 (IL-8), Matrix metalloproteinase-9 (MMP-9), and tumor necrosis factor-α (TNF-α). This suggests that the anti-inflammatory mechanism of the EO is multifaceted. In another study, Wang et al. [36] used three different solvent systems for the separation of sesquiterpenes from ginger EO. They employed the Arizona system consisting of n-hexane/ethyl acetate/methanol/water (7:3:5:5, *v*/*v*), the Ito system consisting of n-hexane/methanol/water (3:2:1, *v*/*v*), and the HBAW system with n-hexane/acetonitrile/chloroform (6:5:2, *v*/*v*). These separations yielded high-purity compounds, including 35 mg of 6-gingerol (98.6% purity), 23 mg of zingerone (99.4% purity), and 105 mg of sesquiterpenes (99.2% purity). The antitumor activities of the isolated compounds were subsequently evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The results indicated that all three compounds significantly inhibited the proliferation of human lung cancer A549, human liver cancer HepG2, and human breast cancer MDA-MB-231 cells. Among the isolated compounds, 6-gingerol demonstrated a particularly strong antitumor activity. To investigate the mechanism of action of this compound, the Hoechst 33342/PI and Annexin V-FITC/PI double staining methods were used for flow cytometry. The results revealed that 6-gingerol exhibited a strong pro-apoptotic effect on human lung cancer cells (A549) and that there was a significant correlation between the apoptosis rate and the 6-gingerol concentration. Notably, all of the above studies employed HSCCC to precisely and efficiently separate the specific chemical components from the EOs prior to the evaluation of their biological activities. However, these methods do not allow high-throughput screening of the biological activities of all fractions to be performed during the separation process. To address this, Ren et al. [22] added a six-port valve to the end of the spiral column in the HSCCC instrument. Initially, a bioactivity-based CCC separation step was performed using the selected solvent system (Figure 1), and the effluent from the CCC was divided into two streams using a two-way valve. This modification allowed for the simultaneous pumping of the separated fractions into a UV detector and a 96-well plate, enabling high-throughput screening of the biological activities of the separated products during the separation process. Additionally, another six-port valve was installed at the exit of the UV detector to store specific fractions online for final elution or recycling to enhance their purities. In this same study, they separated the *Curcuma zedoaria* (Christm.) Rosc EO using an Ito system consisting of n-hexane/ethanol/water (6:3.5:2.5, *v*/*v*). Using the added 96-well plate, they performed real-time screening (MTT assay) for lysine-specific histone demethylase 1 (LSD1) inhibitors in the separated products. The screening results demonstrated that the fractions obtained during the four time periods (i.e., 120–175, 240–290, 290–335, and 440–505 min) exhibited LSD1 inhibition rates > 50%, and fractions 1 (120-175 min), 3 (290–335 min), and 4 (440–505 min) exhibited purities > 90%. After solvent evaporation, they obtained 2.4 mg of the sesquiterpene curcumenone (98.6% purity), 1.38 mg of neocurdione (92.5% purity), and 1.2 mg of curcumol (93.9% purity). Given the lower purity of fraction (2), the authors employed an online storage and recycling elution method for this fraction, which was repeated three times. Eventually, isogermafurenolide (0.86 mg) was successfully separated with a purity of 97.3%. The IC_{50} values of compounds 1–4 were 6.61, 3.97, 9.81, and 21.22 μM, respectively. Notably, isogermafurenolide exhibited the strongest inhibitory activity against (LSD1), with an IC_{50} of 3.97 ± 0.02 μM, which was 4.7 times more potent than the positive control drug, phenelzine. Their work, therefore, not only provides a strategy for the high-throughput bioactivity screening of all isolated fractions using HSCCC, but it could also assist in the design of new LSD1 inhibitors based on specific molecular frameworks.

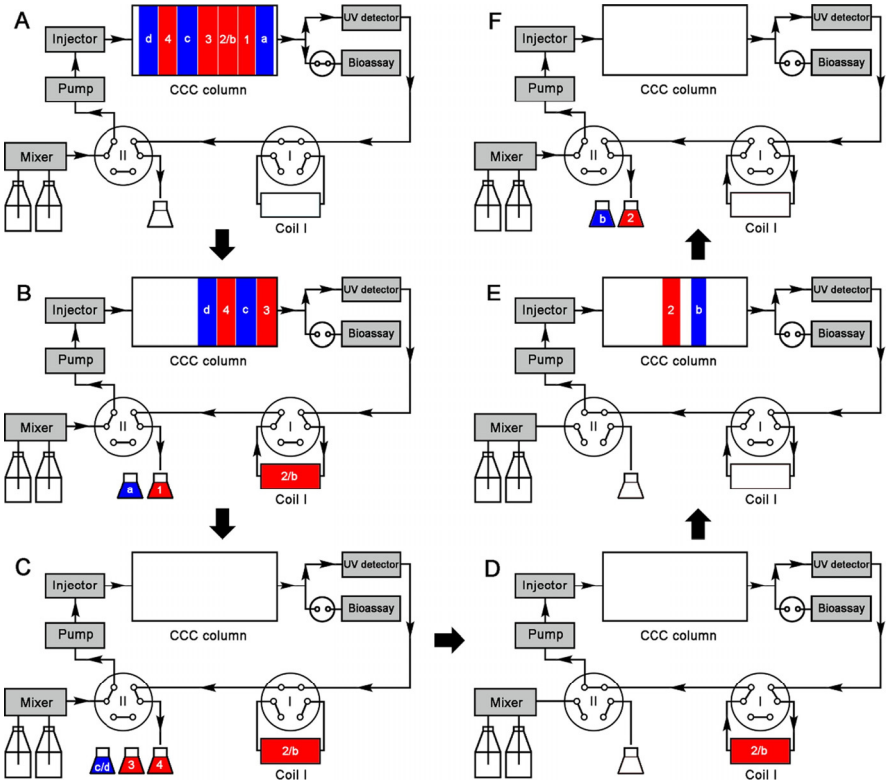

Figure 1. Schematic representation of the target CCC separation technique. (**A**) Bioactivity-based CCC separation. (**B**–**F**) Online storage and recycling of CCC separation.

6. Perspectives

High-speed countercurrent chromatography (HSCCC), a liquid–liquid chromatographic separation technique based on a fluid dynamic equilibrium system, is characterized by its high reproducibility and lack of irreversible adsorption. This technology can be employed to establish fingerprint profiles for various essential oils (EOs) that are valuable for quality control and authentication. Indeed, HSCCC has emerged as a potent tool for separating the chemical components of EOs and holds a promising outlook for applications in this field. This article provides an overview of HSCCC solvent systems, elution modes, detectors, and practical applications for the separation of EOs. The selection of a suitable solvent system is crucial for the separation of EO components by HSCCC. More specifically, it is advisable to choose a solvent system with a lower polarity, such as the HBAW (heptane/butanol/acetonitrile/water) system or a non-aqueous solvent system, to facilitate the subsequent separation and concentration of the fractions. For target compounds bearing conjugated double bonds, selective reagents such as silver ions or ionic liquids can be added to achieve targeted separation and enhance the separation efficiency. HSCCC offers various separation modes that are generally applicable for the separation of natural products, wherein combining multiple modes can lead to more favorable separation results. Additionally, the HSCCC set-up can be equipped with various detectors that can aid in the identification of the separated compounds, thereby enhancing the accuracy of the separation process. To date, numerous studies [77] have described the use of HSCCC to separate and purify EOs, followed by the rapid screening of their active substances and the generation of their corresponding bioactivity chromatograms. Consequently, the active chemical compositions of various EOs have been elucidated, with HSCCC demonstrating significant potential for use in further explorations in the field of EO separation and purification. However, due to the low resolution of HSCCC, it is necessary to employ coupling to other instruments. For example, rapid advancements in mass spectrometry have led to its integration with HSCCC, which can expand the detection range of HSCCC and offer new avenues for bioactivity research [78]. Furthermore, computer-aided drug design tech-

niques, such as molecular docking based on the biological activities of target sites, could be employed to guide the HSCCC-based separation and purification of active components from EOs [79]. Moreover, the gas chromatography–electronic nose technology has been combined with DPPH (α,α-diphenyl-β-picrylhydracil) assays to screen for antioxidant components in various EOs [80–82]. This approach can guide HSCCC for the targeted separation of active components from EOs, ultimately enabling the high-throughput screening and separation of these active ingredients.

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