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Review

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Countercurrent Separation of Natural Products: An Update

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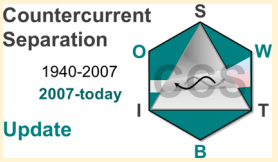
Supporting Information

ABSTRACT: This work assesses the current instrumentation, method development, and applications in countercurrent chromatography (CCC) and centrifugal partition chromatography (CPC), collectively referred to as countercurrent separation (CCS). The article provides a critical review of the CCS literature from 2007 since our last review (*J. Nat. Prod.* **2008**, *71*, 1489−1508), with a special emphasis on the applications of CCS in natural products research. The current state of CCS is reviewed in regard to three continuing topics (instrumentation, solvent system development, theory) and three new topics (optimization of parameters, workflow, bioactivity applications). The goals of this review are to deliver the necessary background with references for an up-to-date perspective of CCS, to point out its potential for the natural product scientist, and thereby to induce new applications in natural product chemistry, metabolome, and drug discovery research involving organisms from terrestrial and marine sources.



INTRODUCTION

Countercurrent separation has a special place in the isolation of natural products. The chromatographic separation of the chemical components in a complex extract is of great concern for natural product researchers and for those who evaluate chemicals of natural origin in biological activity assays and clinical trials. Accordingly, a considerable toolbox of procedures is being developed and popularized for the purpose of extracting and purifying chemicals from natural sources. Among them, liquid−liquid extraction has been in use for decades as a preliminary fractionation technique that precedes chromatography.[1] Desirable characteristics of liquid−liquid separation techniques are (a) the easy scale-up to high-capacity (preparative) scale, (b) the array of solvent choices, which allows for considerable flexibility, (c) the capability of separating ubiquitous matrix materials from target analytes, and (d) frequently observed maximum sample recovery.

Liquid−liquid separation techniques with sufficient resolution to be termed "chromatography" were developed in the 1940s by Martin, Synge, Craig, and Post.[2−4] After more than seven decades of further development, they have matured into modern countercurrent separation (CCS, *syn.* CS; refer to section on nomenclature under Background) techniques that overall share the following 10 advantageous characteristics:[5−8] (1) Automated, relatively user-friendly instruments have been especially designed for preparative CCS with features such as low pressures, high flow rates, and a wide range of solvents. (2) Analytical-scale instruments are now available for method development and small-scale separations. (3) The stationary phase is much less expensive than most solid stationary phases and not so subject to the whims of manufacturers when it comes to resupply, leading to a long-term advantage regarding reproducibility. (4) Targeting of a single analyte may be achieved, or several analytes, representing a wide range of polarities, may be separated provided a suitable method is developed. For this purpose, normal- and reversed-phase modes are available simply by switching between mobile and stationary phases, and they produce strictly mirroring results, which is not achievable with solid media. (5) Solvent system selection has become much more sophisticated and straightforward, allowing for more rapid and more targeted development of CCS conditions. Additionally, a wide range of economical and environmentally friendly solvents may be used. (6) CCS *K* values (partition coefficients) are experimentally reproducible, even in scale-up operations. (7) CCS may be hyphenated with almost any LC-compatible detector. (8) Straightforward mathematical modeling, based on *K* values, which represent physicochemical characteristics of analytes, may aid in retention time (volume) prediction, analyte identification, and separation scale-up. (9) Complete sample recovery is especially helpful for bioactivity-guided isolation schemes and strictly bioactivity-focused methods such as biochemometrics. (10) CCS solvents create a mild chemical environment that minimizes analyte degradation and chemical transformation.

Continuing a previous exhaustive review of the CCS literature,[9] this update article covers the advancements and applications of interest to natural product researchers since 2007.

BACKGROUND

Literature Statistics Reflect the Predominance of Natural Product Related Articles. According to a vetted SciFinder search, a total of 657 articles on countercurrent separation were published from 2008 through 2013. Of these

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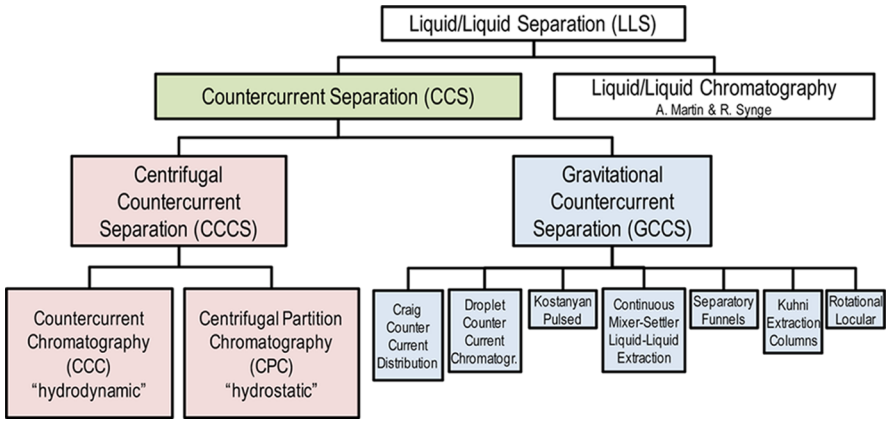

Figure 1. Cladistics tree of liquid–liquid separation techniques showing the position of the widely practiced and contemporary laboratory and scale-up methods, the historical methods such as Craig's counter-current distribution (CCD), as well as the related extraction and chromatography techniques found in natural product and related literature.

Table 1. Solvent Abbreviations Used in this Review to Define Countercurrent Separation Solvent Systems, as Sorted by Approximate Polarity

solvent	abbreviation	solvent	abbreviation	solvent	abbreviation
petroleum ether	Pet	diethyl ether	De	isopropyl alcohol	Iso
hexane	H	ethyl acetate	E	*n*-propanol	Pro
heptane	Hep	chloroform	Ch	*n*-butanol	Bu
cyclohexane	Cy	dichloromethane	Di	acetic acid	Aa
toluene	Tol	acetone	At	ethanol	Et
methyl *tert*-butyl ether	*ter*	DMSO	So	methanol	M
THF	Tet	acetonitrile	Ac	water	Wat

articles, 490 (75%) address the separation of natural product mixtures. Other types of articles may be categorized as new columns and configurations (6%), review articles (3%), protein separations (2%), modeling and theory (2%), chiral separations (2%), synthetic mixtures (2%), microbial transformations (1%), and others (7%).

Working Definitions and Basic Nomenclature. Terminology continues to be an area of concern and contention within the CCS community, and this circumstance is reflected in the literature. First of all, the term "countercurrent" may equally be represented in the literature as the hyphenated "counter-current", historically abbreviated as "CC". To propose a resolution of this linguistic ambivalence, and for a number of additional reasons, including recent developments and insights (S1, Supporting Information), the present article gives preference to the "CCS" abbreviation, with the understanding that it is synonymous with "CS" as used by the authors since 2008. Out of 322 CCS articles published since 2007 and surveyed in this study, 54% use the term "counter-current" in the title, while 17% use the term "countercurrent". Of the same CCS articles that describe the isolation of natural products, 66% used "K" to represent the relative concentration of a given analyte partitioned between the two phases of a biphasic solvent system (SS). A total of 15% used a different symbol such as K_D, K_d, k, K_c, k', k, D, K_{CCC}, or $K_{U/L}$. The remaining articles did not use a symbol for this partitioning. In the same set of articles, 70% used the term "partition coefficient" to describe the partitioning of a given analyte between the two phases of a biphasic SS. However, 9% used a different term, such as distribution constant, partition ratio, distribution coefficient, K value, distribution ratio, or liquid–liquid distribution constant. The remaining articles (21%) did not use a term for partition coefficient.

At least six recent articles addressed the issue of terminology. The IUPAC recommendations were reviewed and suggested for revision in a 2009 publication: the specific terminology of liquid–liquid partition ratio (K_D) in place of partition coefficient (K) was endorsed by the authors.[10] At the same time, two articles authored by Brown et al. made the point that liquid–liquid chromatography processes called "countercurrent" seldom have two liquids flowing in opposition directions.[11,12] A recent article by Y. Ito sought to clarify this point and elaborated on the history and meaning of the term countercurrent chromatography.[13,14] An earlier article by Walter Conway was descriptively entitled "Counter-current chromatography (CCC): Simple process and confusing terminology".[15] In reference to the use of "K" and "partition coefficient," the author states: "It is proposed that the chromatography retention parameter, K_C, be called the distribution coefficient and that a new biphasic distribution parameter, $K_{\Delta(A)}$, be defined for CCC and be called the species partition ratio."[15] In conclusion, there is an established convention of basic CCS terminology as recorded in the literature. At the same time, there is a lack of uniformity that tends to confuse the novice CCS practitioner. The lack of consensus has its roots in the clash between tradition, the academic quest for accuracy, and a streak of independent inventiveness, which has characterized CCS theory and practice from the beginning. The present review seeks to balance widely used terms of historical precedence with insights acquired at the biannual CCC 20xx conferences (i.e., www.CCC2014.com and www.CCC2016.com) in order to communicate the beauty and simplicity of CCS technology to a new generation of users.

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Situating CCS among Liquid−Liquid Separation Techniques. As shown in the cladistics tree of liquid−liquid separation (CCS) technologies (Figure 1), countercurrent separation may be situated as an umbrella term encompassing all liquid−liquid techniques involving two freely available liquid phases. The term "countercurrent" when describing these techniques often does not mean literally that the two phases are flowing in opposite directions. However, "countercurrent" is a general way to describe the mixing and settling process occurring in all of these techniques.[13] A major distinction in CCS is between techniques that involve a centrifuge to assist in the separation of the two phases and those techniques relying on gravity. The two general distinctions of the centrifugal variants of CCS are instruments containing one or more coils that rotate in a planetary fashion (hydrodynamic CCS) vs those instruments composed of a series of cells that rotate around a single axis (hydrostatic CCS).

Solvent System Abbreviations. In this article, a system of solvent abbreviations is employed to facilitate solvent system (SS) representations (Table 1). In order to describe SSs, the solvents are written in order of polarity with whole number volume ratios. These representations are consistent with much, but not all, of the CCS literature. Modifications to the SSs are represented in parentheses following the most polar solvent, as most modifiers are water-soluble acids, bases, and/or inorganic salts. SSs modified after pre-equilibration for ion-exchange CCS are represented with eluter(s) and retainer(s) separated by a hyphen.

Recent Advancements and Current Status of CCS. CCS is an innovative and rapidly developing field in terms of both theory and practice. Advancements in the understanding of solute−solvent and phase interactions lead to better instrument design and the development of new separation methods. Historically, CCS developments have been driven by an empirical problem-solving focus on improving separations.

The present review summarizes the progress in the field under the same major headings as the preceding review.[9] Three key topics of the last review, i.e., instrumentation, solvent systems, and theory, are updated in the present study. As summarized in Figure 2, three new major topics that cover the technologies behind natural product applications of CCS have been added: the optimization of CCS conditions, the role of CCS in the natural products isolation workflow, and the characterization of natural product bioactivity. To avoid repetition, the reader is referred to the previous review[9] with regard to CCS practical guidelines and reporting, which continue to apply. Another very helpful source, especially for the novice and other practitioners who seek practical guidance, has been presented by Yochiro Ito: in his 2005 review, the originator of most modern forms of countercurrent instrumentation summarizes over 40 years of CCS experience and provides references to numerous CCS applications for natural products in table form.[16] The present review focuses on the current practice and recent advancements in the field, and a brief overview of the six key topics (represented by the letters in Figure 2) is as follows.

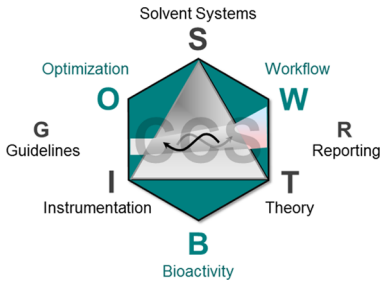

Figure 2. Visual representation of this review article. Shown are the main building blocks of CCS technology addressed in the previous review and updated here, as well as the new components, optimization (O), workflow (W), and bioactivity (B). Guidelines (G) and reporting (R) continue to apply as published earlier.[9]

Instrumentation (I). An increasing number of flexible, reliable, and user-friendly instrument systems are commercially available. The design of new CCS columns continues to be a dynamic and inventive field. New innovations feature new materials and plastic printing technologies to create channels and to fashion supports that accommodate tubing spiral disk technology.[17,18] In addition to centrifuge and column improvements, more attention has been paid to peripherals and operating software that are specifically designed for CCS.[19−21]

Solvents (Ss) and Solvent Systems (SSs). Any solvent and solute combination that forms a biphasic or multiphasic solvent system may be supported by a CCS instrument. The wide range of possible solvent and solute combinations allows for significant creativity in the formulation of SSs with a wide range of polarity and selectivity combinations. Much work has been done on the inclusion of solutes in SS formulations to modify the polarity and/or increase the selectivity of one phase for a particular class of analytes. Modifiers (liquid or solid) may be added either before the pre-equilibration of phases or after the phases have been pre-equilibrated and separated. SS selection continues to be an important topic for the advancement of CCS usage. The large number of possible solvent combinations and the character of CCS as a narrow polarity window analyte targeting technique, rather than a wide polarity window gradient technique typically found in LC, make SS selection a crucial step in every CCS application. SS selection methods range from trial-and-error to theoretical. A substantial semi-empirical middle ground has been established with SS selection based on the predictability within SS families or establishing factorable solvent polarity values.

Optimization (O) of Operation Parameters. Once an SS has been selected, the operation parameters that are most likely to affect, in an interdependent manner, an instrument's ability to perform a desired separation are sample loading, flow rate, rotation speed, and temperature. Sample loading is of particular interest because CCS has a very useful capacity to perform preparative-scale natural product separations.[22] Sample loading is constrained by four factors: (i) the sample concentration in a particular sample loop volume, (ii) the column volume of the instrument, (iii) the desired resolution, and (iv) the instrument design.

Workflow (W). New efforts are being made to include CCS in the overall separation workflow. CCS has come to be employed as part of complex fractionation schemes that involve several steps and different separatory and chromatographic techniques rather than a simple one-step targeting fractionation procedure. The ability of CCS to handle large volumes of crude sample means that minimizing sample preparation such as solvent evaporation and filtration may make the separation process more efficient and economical. A typical sequence may be liquid−liquid separation, open column LC, one or two CCS steps, and preparative HPLC. Creating online chromatography connections is clearly advantageous. Four methods are

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currently employed to couple chromatography columns: recycling CCS, CCS to CCS, LC to CCS, and CCS to LC.[23] The ability of CCS to accommodate relatively large sample volumes and diverse sample solvents is advantageous for chromatographic coupling.

Theory (T) Including Modeling. Modeling CCS separations of analytes having known, or predicted, K values is particularly useful for scalability and predicting resolution by adjusting various parameters such as flow rate, rotation speed, analyte mass, stationary-phase volume retention ratio, and column volume. It is important that modeling methods embrace recently developed pre-equilibrated CCS methods such as flow gradients, elution extrusion CCC (EECCC), back-extrusion CCC, and intermittent countercurrent extraction (ICcE).[24] A few attempts are being made to model methods with phase modification(s) made after pre-equilibration such as linear gradient, step gradient, ion exchange, and pH-zone-refining.[25] The development of separation methods allows the CCS instrument to be used at its full capacity to perform optimal fractionations. Separation methods that are easily automated, such as EECCC, are of particular interest.[26,27] Separation methods such as ICcE, which lead to the continuous or semicontinuous production of target analytes, are of great importance for commercial applications.[28] Well-established methods continue to evolve, e.g., dual-mode into multiple dual-mode.[29,30] In addition, new methods are developed, including back-extrusion[31,32] and back-step[33] CCS, due to both the simplicity and flexibility of CCS technology.

Bioactivity (B) and Nonplant Applications. The biological evaluation of multiple fractions, a key operation in bioassay-guided fractionation, is accessible if high-throughput assays are employed. Again, minimizing sample preparation between separation and bioassay is clearly efficient and economical. Biochemometric methods have been developed to mine data from fractions to streamline the arduous process of purifying individual natural products *before* purifying bioactive compounds.[34−36] The majority of CCS articles that feature the fractionation of natural product mixtures involve plant extracts. However, the interest in purifying bioactive components has led to expanding the application of CCS to include extracts from toad skin,[37] rat brain,[38] pig gallbladder bile,[39] *Lactobacillus* species[40] and other bacteria,[41] *Streptomyces* species,[42,43] marine dinoflagellates,[44] cyanobacteria,[45] algae,[46] mushrooms,[47] endophytic fungi,[48] *Penicillium* species,[49] and yeasts.[50] The variety of organism extracts fractionated with CCS further demonstrates the versatility and adaptability of liquid−liquid chromatography.

INSTRUMENTATION

During the earlier development of CCS technology, the primary focus was on column and centrifuge design. As a result, the CCS columns were interfaced with pumps, valves, operating systems, and fraction collectors that were initially designed for other liquid chromatography applications. In most cases, these peripherals were adequate but not ideal. Recently, comprehensive solvent delivery and operating systems have been introduced, complete with an array of sensors (permittivity-based phase metering apparatus, pressure, temperature, and UV absorption) that allow the operator a visual verification of the separation process. In some systems, phase and volumetric data are converted into a real-time display of stationary-phase retention and partition coefficient values, which are critical to column performance and reproducibility.[19,51,52] When coupled with the EECCC method, partition coefficient determination becomes a powerful tool for metabolomic investigation. Remote access and operation increases the user-friendly aspect of this technology. A recent article described the development of this technology as well as its model application to the separation of green tea catechins.[19]

Modern Hydrodynamic Instrumentation Based on J-Type Centrifuges. These hydrodynamic instruments continue to constitute the majority of instruments being used for natural product separations. From the literature survey that forms the basis of this review, 84% of the articles reported using hydrodynamic instruments. Modifications to hydrodynamic instruments, likewise, make up the majority of instrument innovations reported in the past seven years. The founder of countercurrent chromatography, Yoichiro Ito, continues to be very active in the development of CCS technology. Over a dozen articles referenced in this section have him as a coauthor.

Hydrodynamic Nonsynchronous Column Rotation. Ignatova et al. reported on results from a specially manufactured hydrodynamic CCS instrument that had independent control of the "sun" and "planet" axes. Planetary motion involves rotation around the sun axis, called the axis of revolution, and the planet axes, called the axes of rotation. In the "J-type" instrument, the axes all rotate in the same direction. The two axes may also experience synchronous or nonsynchronous rotation. In synchronous rotation, the axes turn at a fixed angular momentum relative to the axis of revolution as the planetary axes are connected to the sun axis by cogs or pulleys. In nonsynchronous rotation, the angular momentum of the axes of rotation may be adjusted independently of the axis of revolution. The instrument permitted observation of the stationary-phase retention, K value determination, and resolution of a test mixture of three analytes in an SS consisting of *n*-heptane, ethyl acetate, methanol, and water (HepEMWat; see Table 1 for the generally used abbreviations of solvents and SSs). The traditional 1:1 J-type ratio of sun and planet rotation was outperformed by at least one nonsynchronous condition. The K values of the analytes shift somewhat as the parameters are changed in nonsynchronous rotation, suggesting that the mixing efficiency of the instrument affects K values.[53]

Hydrodynamic I-Type Centrifuges. The I-type synchronous planetary motion is characterized by the central (sun) axis rotation in the opposite direction of the column holder (planet) rotation. Yang and co-workers have published a series of articles that explore the practical applications of I-type centrifuges. In a recent article, biphasic SSs of HEMWat (0.1 M HCl) 5:5:5:5 and BuAaWat 19:1:20 were used to separate dipeptide and DNP-amino acid samples, respectively. In this study, four different column configurations were created by winding and connecting two short bobbins. The best configurations were obtained by connecting a left-handed column with a right-handed column tail-to-head or by connecting a left-handed column with another left-handed column tail-to-head.[54−57]

Hydrodynamic Bobbin Design. The most intense development work has involved the design of the planetary bobbins that make up the fluid path of the CCS instrument. These innovations demonstrate the dynamism of CCS technology. The configuration of the tubing or channels associated with the rotating bobbins has a profound effect on the mixing and settling dynamics and, therefore, the separation characteristics of the CCS instrument. In one case, CCS coils created by winding tubing on a conical bobbin were shown to increase efficiency with higher retention of stationary phase in a

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hydrodynamic instrument. The features of these new columns were demonstrated with the HEMWat CCS fractionation of four tanshinones from *Salvia miltiorrhiza* rhizomes.[58]

In addition, the CCS hydrodynamic column cannot be considered as an inert frictionless tube through which the liquids are pumped. Studies have determined the ways that tubing or a liquid channel may participate in the separation process to a greater extent, thus getting more separation (mixing and settling) with less tubing (channel) length. For example, spiral disk manufacturing techniques have made it possible to explore channel characteristics that were difficult to produce with simple PTFE tubing wound around a bobbin. One approach has been to simply change the cross-section shape of the tubing by employing flat-twisted, spiral-grooved, or triangular tubing. If the solvents encounter an irregular flow passage instead of a smooth cylindrical channel, the mixing and settling of the two immiscible phases may be enhanced.[59−61]

Spiral tubing columns are created by fitting tubing into spiral grooves that have been cut into a plastic spiral disk. The disk may then be mounted on a conventional hydrodynamic instrument. In a recent article, Knight et al. reported that spiral disk CCS exhibited higher retention of stationary-phase volume, the adaptation to a wider range of SS formulations including aqueous two-phase systems (ATPSs) and non-aqueous SSs, higher column loading, more reproducible performance, and more robust columns and fittings. This research created the possibility of custom-designed columns for specialized techniques such as separation of active enzymes at low temperatures, polymer separation, and continuous (dual) flow configurations.[17,62−64] Cao et al. investigated spiral tubing column performance for the separation of four theaflavins from black tea with a HEMWat SS and the separation of anthocyanidins from *Lycium ruthenicum* fruit with a *ter*BuAcWat SS.[65]

A visualization study of a 2-D spiral column placed in a hydrodynamic instrument was undertaken to observe the factors influencing bilateral phase distribution and stationary-phase retention. In particular, the authors wanted to understand why the traditional 3-D helical columns have difficulty in retaining a stationary phase for ATPSs. The density difference and interfacial tension between the two phases seem to be key SS parameters. However, the frictional force between the phases and the tubing walls also plays a crucial role in stationary-phase volume retention.[66]

Machined disk technology may also be used to create different flow paths by cutting the channels in zigzag, sawtooth, or figure-8 configurations. The intention is that an irregular flow path will promote more mixing and settling over a smaller volume than conventionally wrapped columns. The designs have been applied to the separation of two dipeptides with BuAaWat and three DNP-amino acids with HEMWat (0.01 M HCl). Protein separations have also been attempted with a figure-8 configuration disk.[67−70]

Aqueous two-phase systems have specialized CCS applications, such as protein separation, but stationary-phase volume retention (Sf) is often poor on J-type instruments due to the relatively small differences in density between the two phases. Therefore, toroidal columns, where a continuous length of tubing is wound on a flexible nylon support, have been exploited to increase Sf values for ATPSs. Toroidal columns are considered to be hydrostatic columns, even though they may be operated in a centrifuge designed for hydrodynamic columns.[71,72] A recent review addressed the application of centrifugal liquid−liquid chromatography using ATPSs.[73]

Multichannel CCC. Wu et al. introduced a new CCS method termed "multi-channel CCC" in 2008. A traditional three-bobbin hydrodynamic centrifuge was wrapped with three parallel tubes instead of just one. Each bobbin may be run as a separate column, each with its own solvent reservoir, pump, detector, and fraction collector. Each column is practically identical, so the results should be the same as doing three different experiments on the same instrument. The authors simultaneously separated ethyl acetate extracts of *Soldidago canadensis* flowers and buds, *Suillus placidus* fruit bodies, and *Trichosanthes kirilowii* roots using the same HEtWat SS. After subsequent HPLC purification, four natural products were tested for cytotoxicity against a panel of six human cancer cell lines.[74]

Hydrodynamic Vortex CCS. Ito et al. described a novel CCS instrument that was created by mounting a high-density polyethylene disk vortex column on a J-type coil planet centrifuge. The column is specially designed so that the shaking movement of the vortex will allow the mobile phase to move through the column while the stationary phase is retained. Three model systems were employed to test this technology: separation of two Sudan dyes with HAc, of two DNP-amino acids with HEMWat (0.1 M HCl) 5:5:5:5, and of a pair of dipeptides in BuAaWat 4:1:5. The effect of flow rate and cell diameter on stationary-phase volume retention and resolution was studied.[75−77] The vortex CCS instrument was featured in an article by Weisz et al. They applied CCS to the separation of dye mixtures with HEMWat (TFA). Both conventional and pH-zone refining techniques were employed.[78]

Comparison of Hydrodynamic Instruments. Guzlek et al. compared the separation performance between different instruments using selected analytes from the generally useful estimation of SSs (GUESS) mixture and HEMWat SSs. The variety of operator-determined parameters renders a fair comparison of different instruments problematic. The article compared the separation of GUESS mixtures in the same SS on a 320 mL Pharmatech CCC-1000 and a 136 mL Dynamic Extractions Spectrum model. A significant difference between the two instruments is the ability of the Spectrum to generate higher *g* forces than the CCC-1000 (243 m/s^2 compared with 54 m/s^2), which, in turn, allowed for reasonable stationary-phase volume retention at higher flow rates.[79]

Modern Hydrostatic Instrumentation. Modern hydrostatic instruments continue to evolve with the design of new flow cell configurations. Visualization of the interactions between mobile and stationary phase during centrifugal partition chromatography (CPC) operation may inform the optimal parameters for CPC operation as well as the influence of cell features on phase mixing and settling. Two articles employed an optical measurement system to visualize the hydrodynamics of biphasic SSs in a CPC chamber. Their findings from the analysis of five different solvent formulations show that SSs with higher Sf are characterized by a higher interfacial tension and significant density differences. However, these highly retained SSs tend to display less dispersion during operation, characterized by reduced interfacial area. EWat is an example of a highly stable and slightly dispersed SS. HepEMWat 6:4:5:5 is an example of a less stable and highly dispersed SS. Higher flow rates tend to increase dispersion but also lower Sf. Therefore, the optimization of instrument parameters is a matter of give-and-take and tends to vary

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with different SS characteristics.[80,81] Application of the phase-mixing optimization in a CPC instrument was performed for the isolation of nybomycin n3 from *Streptomyces* sp. BA809 with a HEMWat SS and the EECCC method. The separation was prefaced with the selection of operating parameters based on the phase mixing observed in the CPC cells.[42]

Centrifugal Partition Extraction. A redesigned CPC column with larger channels has been named centrifugal partition extraction (CPE). The advantages of employing larger channels in CPC are (1) increased flow rates may be achieved with reasonable stationary-phase retention volume ratios and (2) in most cases higher sample loading as a percentage of total column volume may be achieved. These advantages are particularly useful in ion-exchange applications. Hamzaoui et al. described the isolation of glycyrrhizin from *Glycyrrhiza glabra* roots with a modified EBuWat SS to which trioctylmethylammonium chloride (Al336) was added to the organic stationary phase as a retainer and potassium iodide was added to the mobile aqueous phase as an eluter.[82] It was reported that 2.21 g of glycyrrhizin was purified from 20 g of crude extract with 86.5% recovery in a 303.5 mL CPE column. In a similar study, Hamzaoui et al. described the isolation of sinalbin from *Sinapis alba* seeds with a modified EBuWat SS with the same retainer and eluter as previously reported.[83] In this case, 4.6 g of sinalbin was isolated from 25 g of crude extract with a 92% recovery. A comparison of the same isolation performed with a conventional CPC column revealed that the higher flow rates and increased column loading of the CPE instrument resulted in a significant increase in productivity measured in grams of sinalbin per hour per column volume. Another application for CPE was reported by Ungureanu et al., who recovered torularhodin from *Rhodotorula rubra* culture broth with a HAtWat SS.[84] The lipophilic natural product was extracted into the organic stationary phase as the diluted culture broth was pumped through it. The stationary phase was then extruded out of the column to obtain the torularhodin.

Small-Volume Instruments. The first report of a small-scale instrument was made in 1987 by Ito and Lee on a 38 mL hydrodynamic instrument that was evaluated with a HEMWat SS CCS of a test mixture of four indole plant hormones.[85] However, commercial small-volume instruments are a relatively new phenomenon. These instruments provide a much-needed analytical aspect to CCS instrumentation. They are ideal for the method development phase of CCS projects in order to minimize time and solvent consumption. In general, the availability of small instruments allows the efficient separation of small sample sizes. Berthod et al. investigated the critical parameters of small-volume hydrodynamic CCS instruments.[86] A 12-component GUESS mixture was used to compare the performance of four different columns in the same HEMWat 4:6:4:6 SS. Narrow bore columns that may be rotated at high speeds provide both high stationary-phase retention and resolution power. However, this is a difficult combination to realize in a practical engineering sense because high rotation speeds tend to engender equipment failure and narrow-bore columns have to be operated at higher pressures to create reasonable flow rates. Larger bore columns that may handle higher flow rates are often deemed as more efficient columns in terms of throughput capabilities.[10] Small-scale hydrodynamic CCS instruments currently available include the 16−20 mL Tauto TBE 20A, the 18 mL Dynamic Extractions "Mini", and the 35−40 mL model GS 20 from the Beijing Institute of New Technology Application. Small-scale CPC instruments are also being marketed. Commercial 25 mL FCPC (Kromaton) instruments giving rise to the term "ultrafast centrifugal partition chromatography" are now commercially available. Armen-Gilson produces a 50 mL SCPC-50 instrument.[87]

Hydrostatic vs Hydrodynamic Instruments. Friesen, followed by Faure et al., explored the possibility of substituting limonene for heptane in CCS SSs.[88−91] In addition to the proposed environmental friendliness of using a natural extract in place of a petroleum product, this study offered an illustration of the advantages of hydrostatic instruments. The 24% higher density of limonene over heptane significantly decreased the retention of the stationary phase for SSs using limonene instead of heptane in hydrodynamic instruments. On the other hand, hydrostatic instruments were able to maintain over 50% stationary-phase retention with a limonene/methanol/water 10:9:1 SS at a reasonable flow rate.[90]

DCCC. Droplet countercurrent chromatography was introduced in 1974, and its development and commercialization occurred about the same time as centrifugal CCS instruments were also being designed.[92] As a result, the popularity of DCCC has never approached that of centrifugal-based CCS instruments. However, the method is still quite effective at separating complex natural product extracts, as has been shown by three articles published from 2009 to 2011. Three terpenoids were separated and isolated from *Trichilia quadrijuga* stems with a DCCC apparatus employing a HEMWat SS.[93] Imbricatolic acid, a novel dihydrobenzofuran lignan glycoside (juniperoside A), and 16 other natural products were isolated from *Juniperus communis* berries. The aqueous methanolic extract was partitioned with hexane, chloroform, and butanol. The three nonaqueous partitions were treated differently. The hexane fraction was applied to a silica gel column. The chloroform fraction was separated with DCCC employing a ChMWat SS. The butanol fraction was separated with DCCC employing a BuAtWat SS.[94] Three dihydrokaempferol glycosides and gallic acid were separated and characterized from *Pouteria obovata* fruit flour with a BuAtWat SS. The separation was performed on a Büchi DCCC equipped with an array of 300 2.2 mm i.d. tubes, each with a 2.0 mL volume.[95]

Pulsed CCS. In pulsed CCS, a cylinder has been divided into partitions with membrane disks (horizontal sieve plates). The solvent motion created by a pulsed pumping action creates the mixing and settling that is a result of rotation in hydrodynamic CCS. In one method, the lower phase of a two-phase SS is retained in the partition, while the upper phase is pumped from the bottom. Alternatively, the upper phase may be retained and the lower phase pumped from the top of the column. The volume and flow rate of the pulse as well as the wait time between pulses are critical parameters. Kostanyan published an initial article on the process that focused on the theory and modeling of the apparatus.[96] A follow-up article described a series of these columns connected in order to increase the number of theoretical plates over a single column. OctWat and octane/octanol/water SSs were employed with a series of simple carboxylic acids (acetic, propionic, butyric, and valeric) to demonstrate the separation performance.[97]

Another method of using pulsed solvent delivery to create mixing and settling of phases was described by Kostanyan and Voshkin. The mobile phase was pumped through a series of coils, much like hydrodynamic CCS. Instead of centrifugal motion retaining the stationary phase in the column, a pulsing device situated at the mobile phase entrance created a continuous back-and-forth movement of the solvents in the

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column. The pulsing created a localized countercurrent movement in the coils. The coils were fashioned from glass tubing so that the phase mixing could be directly observed. Tests with caproic and valeric acid in a kerosene/water (10% aqueous ammonium sulfate) SS showed that the amplitude and frequency of the pulse play the role of rotation speed in hydrodynamic CCS. The more and greater the pulsing, the more stationary-phase volume is retained. The flow rate played the same role in pulsed CCS as in hydrodynamic CS: a higher flow rate decreases Sf.[98]

Considered Rational Choices in CCS Instrument Selection. Survey results indicate that the hydrodynamic CCC instruments are the most frequently employed, comprising 84% of the articles. Hydrostatic CPC instruments are featured in most of the remaining articles, with one article describing a slow rotation countercurrent chromatograph (SRCCC)[99] and three articles using a droplet countercurrent chromatograph (DCCC).[93−95] Reported column volumes ranging from 16 mL to 15 L reflect the wide choice of columns currently available. Indeed, many instruments may be configured to change column volumes, and/or columns may be removed and replaced by columns of different volumes. As shown in Figure 3, the most popular column volumes for all CCS instruments fall in the 220 to 300 mL range, which may be loaded with gram quantities of sample.

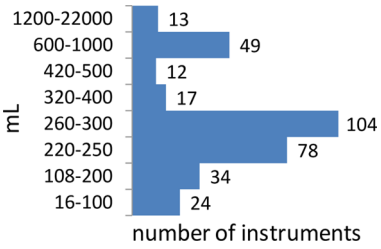

Figure 3. Reported column volumes of all countercurrent separation instruments used in natural product separations since 2007.

SOLVENT SYSTEMS

Solvent System Formulation. The use of multiphasic SSs to effect separations is the unifying feature of liquid−liquid separation techniques. The number of formulations that may be made to produce biphasic SSs is virtually limitless. Two or more solvents may be mixed in an infinite number of proportions. In most cases, the biphasic SS is pre-equilibrated in a separatory funnel before the two phases are separated and equilibrated in the CCS column. In addition, modifying solutes may be added to the mixture either before or after pre-equilibration. Reporting of SS formulation gives the solvent combinations followed by their volume ratios. Generally, solvents are reported in the order of least polar to most polar, but this is not always the case. There is no current consensus in how to report volume ratios. In many cases, the volume ratios are given in whole number ratios, but decimals are sometimes used. Due to a considerable amount of confusion in the literature, the following two sections seek to clarify two SS formulation issues: ratios of binary SSs and unified SS notation.

Binary Solvent Systems. While most two-phase SSs are mixtures of three, four, or even more solvents, there are several combinations of just two liquids that are immiscible and suitable for CCS. Examples are HepM, EtWat, BuWat, ChWat, and finally OctWat, the logP system that spans the widest polarity range but mostly plays a theoretical role.[100] Notably, the proportion of the two solvents is irrelevant for the proper description of these systems: regardless in what ratio they are mixed, each phase will always represent one solvent saturated with the other. However, as ratios of such binary mixtures are still described frequently in the literature, it should be pointed out that it can be helpful to use certain proportions of two solvents that deviate from 1:1 when trying to produce a certain amount of upper and lower phases for CCS use. For example, in an equilibrated BuWat system, more water will be in the saturated organic phase than *n*-butanol in the aqueous phase. Depending on how much mobile relative to stationary phase the CCS experiment requires (run time corresponding to the target *K* value for elution, plus potential extrusion), it can be helpful to mix largely disproportionate amounts (e.g., 5:1) of the two solvents. However, it should still be noted that this does not affect the equilibrium composition of the two-phase system.

Unified Solvent System Notation. Currently, no standards exist in the notation of the mixtures used to formulate two-phase SSs. The most straightforward approach may be to apply the basic mathematic principle of the lowest common denominator in cases where solvent proportions are represented by whole numbers such as HEMWat 2:3:2:3. In this case, the sum of 2/10, 3/10, 2/10, and 3/10 equals unity. However, in the formulation of SS families it is helpful to establish a common denominator between several SSs. For example, HEMWat 5:5:5:5 and HEMWat 4:6:4:6 have the common denominator of 20. This practice provides an accessible method of comparing SS proportions and their relative polarities within a family. Solvent system families such as the "Arizona" family do not have a common denominator and, therefore, obscure the comparison of two or more formulations with different common denominators ("Arizona F" 1:5:1:5 and "Arizona W" 6:1:6:1). Considering that the percent system is ubiquitous in everyday life, and in order to accommodate SSs with different common denominators without the need to use decimal fractions, the base of 100 is an attractive alternative for standardizing SS notation. Section S2 of the Supporting Information provides synoptic tables for the conversion of the notation of the most widely used SSs.

Documented Use of Solvent Systems for Natural Product Separation. Typically, the CCS practitioner will select a solvent system based on the available solvents. In the surveyed articles, a total of 18 different solvents were employed. This demonstrates the capacity of CCS columns to accommodate a large variety of different solvents. However, as shown in Figure 4A, the most common solvent components were water (97% of all SSs contained water), ethyl acetate (64%), methanol (49%), and hexane (36%). The halogenated solvents chloroform, dichloromethane, and carbon tetrachloride were present in only 9% of the SSs employed, with a strong emphasis on the former two. "Hexane" may be purchased as *n*-hexane or a mixture of isomeric hexanes. Many articles do not clearly identify if they are using *n*-hexane or a mixture of hexanes. In CCS practice, the differences between hydrocarbon solvents such as *n*-hexane, hexanes, *n*-heptane, and petroleum ether are very small to negligible.[101]

The next practical consideration is how many solvents are needed to formulate a desirable SS. According to the survey, and as shown in Figure 4B, SSs used to separate natural products were most often formulated with three (34%) and

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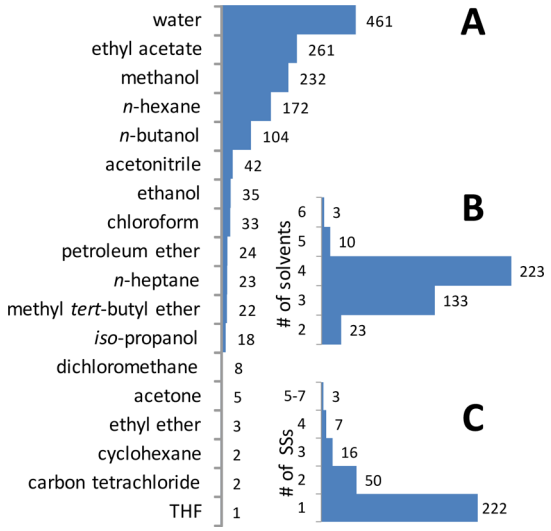

Figure 4. Abundance and diversity of solvents and solvent systems (SSs) in reported natural product countercurrent separations since 2007: (A) frequency of particular solvents as solvent system components in all articles; (B) number of solvents per SS employed; (C) number of SSs per published study.

four (57%) solvent combinations. While several two-solvent combinations form biphasic SSs (see subsection Binary Solvent Systems above), their separation efficacy is generally less than those employing more solvents. The HEMWat SS contains two solvents of extremely different polarities, hexane and water, with ethyl acetate as lipophilic modifier and methanol as a polar modifier. The greatest number of solvents in a documented biphasic SS formulation was six.[102−104] As shown in Figure 4C, over three-fourths of the articles surveyed reported the use of a single SS for the separation. The highest number of SSs used in a single publication was seven.[105] Considering that the chemistry of the SS determines the selectivity of the separation, the combinatorial exploration of SS composition generates information for future studies, especially when metabolomic mixtures require additional resolution power.

HEMWat Family of SSs. The popularity of the SS consisting of hexane, EtOAc, MeOH, and water (HEMWat; Table 1) for the isolation of natural products suggests that it is the "go-to" SS for natural product CCS. Its widespread use may be attributed to the facility of creating stable biphasic SSs that have a volumetric ratio of nearly 1:1 with combinations of hexane, ethyl acetate, methanol, and water that also cover a wide range of polarities. Of the articles surveyed, over 35% employed a HEMWat SS. Another 16% employed a HEMWat system with one of the solvents replaced such as HepEMWat. Replacing hexane with heptane is a common substitution since heptane may be easily obtained as a single isomer and it has less toxicity than hexane. A recent review by Wu and Wu highlights the importance of HEMWat SSs for the separation of flavonoids and cites eight articles that employ HEMWat SSs for the separation of bioactive phenols.[106] Another review by Costa and Leitao also emphasizes the importance of the HEMWat SSs for the separation of flavonoids, concluding that HEMWat is the SS of choice in more than 60% of the publications featured in their review. The authors also observed that flavonoid aglycones are readily separated by HEMWat SSs, whereas the glycosylated flavonoids often need a more polar SS such as those in the EBuWat SS family.[107]

Nonaqueous SSs. Useful biphasic SSs formulated without water are sparse in the CCS literature, constituting only 3% of the articles surveyed. However, these nonaqueous SSs may be called upon to isolate nonpolar natural products such as hydrocarbons from natural product extracts. Table 2 summarizes the relevant literature[46,108−118] and illustrates current examples of nonaqueous SSs employed in CCS separations of natural products. Notably, they all contain a hydrocarbon solvent such as hexane. The most frequently used polar solvent in these formulations is acetonitrile.

Table 2. Nonaqueous Solvent Systems Used for the Countercurrent Separation of Natural Products since 2007

natural product(s) class	source	solvent system	ref
three retinal isomers	photoisomerization of all-*trans*-retinal	HAc	108
three thienyl natural products	*Flaveria bidentis*	HAc	109
cycloartenyl ferulate and 24,25-dihydro-24-methylenecycloartenyl ferulate	rice bran oil	HAc	110
hyperforin	*Hypericum perforatum* aerial parts	HepM(Ac)	111
three essential oil components	*Flaveria bidentis* leaves	HAcEt	112
all-*trans*-lycopene	tomato paste	HDiAc	113
ferulic acid esters of phytosterols and triterpene alcohols called "γ-oryzanol"	rice bran oil	HepBuAc	114
β-caryophyllene	*Vitex negundo* leaf oil	HChAc	115
two diterpene acids	*Copaifera glycycarpa* oleoresin	HEAc	116
six volatile natural products	essential oil of *Curcuma wenyujin* rhizomes	PetAtAc	117
two chlorophyll derivatives	*Amaranthus tricolor* aerial parts	HM	118
fucosterol	*Pelvetia siliquosa* marine algae	HepM	46

Chlorinated Solvents. Chlorinated solvents, in particular chloroform, have a long history of use in countercurrent separations. One of the original test mixtures for the development of modern CCS instruments developed by Y. Ito was a mixture of *N*-dinitrophenyl-amino acids separated in ChAaWat (0.1 M HCl).[119] The ChMWat SS was introduced in 1977 as chloroform/methanol/aqueous borate buffer (pH 8.2) 4:4:3 for the separation of candicidin and related polyene antibiotics extracted from *Streptomyces griseus*.[120] The ChMWat SS was later used to isolate flavonoids from a sea buckthorn.[121] This combination proved to be useful in practice and was employed in many subsequent separations. However, while chloroform has fallen from favor as a viable solvent since 2000, its replacement with dichloromethane has not been widely adopted for CCS applications. In fact, dichloromethane is seldom used as a biphasic SS component even though it is a common laboratory solvent. The literature since 2007 contains 28 examples (S3, Supporting Information) of halogenated solvents used in CCS fractionation of natural product mixtures.[39,47,94,108,115,122−144]

On-Demand SS Formulation. One drawback of CCS SS formulation occurs when both phases are prepared and pre-equilibrated in advance so that the relative phase volumes cannot be controlled independently. This inevitably leads to an excess volume of one of the phases being prepared. One way of

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addressing this situation is to prepare phases independently ("on demand") according to the relative quantities of their post-equilibration constituents. These quantities may be determined experimentally or calculated using thermodynamics-based models. In some cases, these values are available in the literature.[101]

On-Demand SS Formulation with the UNIFAC Model. The SS selection for the isolation of dioscin from *Dioscorea nipponica* was guided by the application of the UNIFAC (universal quasi-chemical functional-group activity coefficients) model to prepare the two phases separately. A comparison was done with *K* values determined by equilibrated SSs and SSs formulated according to UNIFAC calculations for HEMWat, HEEtWat, and EBuWat. While the measured *K* values differed somewhat between the pairs of differently formulated SSs, other key parameters such a stationary-phase retention volume ratio, separation time, purity, yield, and percent recovery were not significantly different.[145] These first comparative results may also indicate that unless the volumetrics of a CCS experiments are controlled rigorously and known, experimental *K* values from CCS runs can deviate from those measured by a partitioning experiment or another method.

On-Demand SS Formulation with Simple Ratios. Wagenaar et al. explored the role of CCS instruments in an industrial setting where synthetic mixtures are routinely processed. The authors chose the HEMWat SS family as their entry-level SS due to its usefulness in natural product research and robust versatility. On-demand solvent mixing was employed to reduce waste and solvent preparation time and confirmed the viability of using simple ratios for on-demand SS formulation. The study also resulted in an approximate correlation between the retention time of a target analyte on a RP-HPLC column and the best HEMWat SS for CCS chromatography.[146]

On-Demand SS Formulation with Gas Chromatography. On-demand mixing requires that the chemical composition of the equilibrated SS phases be determined. Gas chromatography with a flame-ionization detector is useful for determining the levels of organic solvents in each phase. Published examples of natural product separations performed with this process[25,127,142,147−149] are summarized in Table 3. The water content may be determined directly by a Karl Fischer titration or indirectly with a difference method.

Table 3. On-Demand Solvent System Formulations for the Countercurrent Separation of Natural Products since 2007

natural products (no. of cpds)	source	solvent system(s)	ref
alkaloids (5)	*Coptis chinensis*	ChMWat	127
ginsenosides (13)	*Panax ginseng* (Korean red ginseng)	DiIsoMWat	142
ginsenosides (8)	*Panax ginseng* (white ginseng)	DiIsoMWat	142
andrographolides (3)	*Andrographis paniculata* aerial parts	HEMWat	147
ginsenosides (12)	*Panax quinquefolium* roots	EBuWat, EBuMWat, HEBuWat	148
tanshinones I and IIA	*Salvia miltiorrhiza* rhizome	HEEtWat	149
tanshinones (5)	*Salvia miltiorrhiza* rhizome	HEMWat 8:2:5:5 and 8:2:3:7 gradient	25

Modifications of SSs. As commonly observed in LC, the use of additives is an important means of engineering the selectivity of liquid phases to achieve the desired separation. In CCS, depending on their nature and distribution, (an) additive(s) affect(s) one or both of the two phases. The following summarizes examples of such modifications reported in the literature.

Phase Modification before Pre-equilibration. In addition to the practically limitless combination of different solvents to create biphasic SSs, there is also the option of modifying the SSs with various additives such as inorganic ions or small amounts of liquids. According to this survey, in about 20% of CCS natural product separations, SSs were modified with a water-soluble acid, base, or inorganic salt. Trifluoracetic acid and acetic acid were the most common modifiers. In addition, hydrochloric acid, formic acid, ammonium hydroxide, ammonium acetate, sodium hydroxide, silver nitrate, copper nitrate, sodium chloride, potassium phosphate buffers, and ionic liquids such as $[C_{4mim}][PF_6]$ were used.

Phase Modification before Pre-equilibration with Inorganic Salts. Three 5-hydroxyisoflavone isomers were isolated from *Belamcanda chinensis* rhizomes by adding copper(II) nitrate to the SS. Addition of this inorganic salt to a PetEMWat SS increased the affinity of the three 5-hydroxyisoflavone isomers for the aqueous phase. The partition coefficient (*K*[aqueous/organic]) more than doubled between the SS without and the SS with 0.05 M copper(II) nitrate. Further increasing the salt concentration led to a slight increase in the *K* values. Therefore, it is likely that the copper ions act as chelating agents to modify the elution of phenolic species such as isoflavones.[150]

An article by Romero-Gonzales et al. described the isolation of organic acids from green coffee bean extracts. The ability of salts to modify the solubility characteristics of the aqueous (mobile) phase was investigated extensively. First, a series of six salts at various concentrations was used to determine the partition coefficient of 5-caffeoylquinic acid in an EWat SS. In general, as the salt concentration increased, the solubility of the organic acid in the aqueous phase decreased. Apparently, the salt made the water more polar and drove out more of the organic acid toward the organic phase. Moreover, the nature of the salt made a significant difference in the corresponding partition coefficient. This effect was explained by noticing that the ability of the salt to force the organic acid from the aqueous phase generally follows the Hofmeister series that describes the ability of an ion to attract water molecules.[151] Second, lithium chloride was chosen as a reasonable salt and then tested in six different concentrations in three different SSs. The partition coefficients for 10 different chlorogenic acids were recorded for each combination of SS and salt concentration.[152]

Silver ion chromatography has been used for decades in the separation of lipids.[153] In liquid chromatography and TLC, the solid (silica gel or cation-exchange media) stationary phase is impregnated with silver ions before the chromatography is performed. In CCS, it is a simple matter to add a silver salt to the aqueous stationary phase. A silver ion SS formulation was used in the CCS separation of olefins from marine *Micromonospora* species. Silver nitrate, added to the aqueous stationary phase of the PetAtWat SS, was used by Zeng et al. to separate tacrolimus from dihydrotacrolimus.[154] The same two macrolides and ascomycin, obtained from *Streptomyces tsukubaensis*, were similarly separated with an H*ter*Wat (0.1 M $AgNO_3$) SS by Wen et al., who tested silver nitrate

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concentrations from 0.05 to 0.2 M. The addition of this salt made a significant difference in the resolution of these three closely related macrolides. Silver nitrate could be recovered at the end of the separation.[155] Schröder et al. also described a silver nitrate aided fractionation.[43]

Phase Modification before Pre-equilibration with DMSO. The inclusion of DMSO in CCS SSs was reported to lead to beneficial effects on selectivity. An article by Qiu et al. described the CCS of the *Ginkgo biloba* terpene lactones, bilobalide and ginkgolides A, B, C, and J, via successive CCS operations. A two-step CCS experiment was performed first with ChMWat SS followed by HEMWat (0.5% DMSO) to separate three fractions collected from the first run. The addition of DMSO to the HEMWat SS imparted some beneficial separation characteristics.[123] Ignatova et al. investigated introducing samples dissolved in DMSO into a HEMWat CCS linear gradient elution. Samples dissolved in DMSO exhibited slightly different K values than samples dissolved in HEMWat, but DMSO was not detrimental to separation efficiency.[156] In addition to on-demand mixing, Wagenaar et al. also used DMSO to dissolve samples for injection without any deleterious effects on CCS chromatograms.[146]

A tetrahydrofuran, dimethyl sulfoxide, and water SS (TetSoWat) was used to separate a series of xylose oligomers from hydrolyzed birch wood. The monomeric xylose eluted first with K = 4.0 in the chosen formulation, while the longer chain xylose oligomers eluted in order of their increasing molecular mass. According to the authors, the "production of fractionated oligomers, which are significantly more economical than purchasing directly from the supplier will be utilized for our subsequent elucidation of the depolymerization kinetics of oligomers."[157] There are relatively few CCS applications for carbohydrates of this type. In a follow-up article, the same authors developed a more economical BuEtWat SS that had similar separations characteristics as the original TetSoWat SS.[158]

Phase Modification before Pre-equilibration with Ionic Liquids. The proof of principle that ionic liquids may be suitable for CCS was established in two articles authored by Berthod and Ruiz-Angel.[159] Xu et al. described the isolation of neomangiferin and mangiferin from *Anemarrhena asphodeloides* by CCS using ionic liquids as a SS modifier.[160] Liu et al. described the separation of two flavonoid glycosides from the plant *Oroxylum indicum* using ionic liquids.[161] The literature suggests that *Oroxylum indicum* flavonoids and *Anemarrhena asphodeloides* mangiferins are popular CCS targets.[162−171] The same ionic liquid-containing SS was used in both articles: ethyl acetate/water/[C(4)mim][PF(6)] 25:25:1. In the Liu et al. article, a total of 12 different ionic liquid modified SSs were studied using three different ionic liquids.[161] While the ionic liquid represents about only 2% of the total SS volume, it significantly influences the separation characteristics. Modifiers such as ionic liquids need to be removed by an additional process after the CCS separation, as they are not volatile. A macroporous resin column may be used to remove the ionic liquid from the fractions containing the target natural products.

Phase Modification before Pre-equilibration with Acid and/or Base. Dahlberg et al. reported on the separation of three tetrahydroiso-alpha acids from hops (*Humulus lupulus*). The authors employed a buffered aqueous phase in a HEMWat SS for the EECCC separation. Ammonium acetate and ammonium phosphate were used as buffers. Buffer concentration was found to influence peak shape and K values. As expected, decreasing pH of the lower aqueous phase containing organic acids increased their solubility in the upper organic phase.[172,173] The isolation of DL-tetrahydropalmatine from *Corydalis yanhusuo* with a modified HEMWat CCS was reported by Zhang et al., who added acetic acid and triethylamine to the SS and measured the pH of the upper and lower phases. An increase in pH increased K due to the increased solubility of the alkaloid in the upper (stationary) phase.[174] A similar approach was adopted by Dai et al., who added NaOH to a BuMWat SS to isolate corydine and stepharine from *Stephania yunnanensis*.[175] The isolation of safflomins A and B from *Carthamus tinctorius* was achieved with a *ter*BuAcWat SS modified with TFA. During the SS development, it was noted that adding increasing amounts of TFA or formic acid to a HEBuMWat or *ter*BuAcWat SS increases solubility of the phenolic safflomins in the upper organic phase.[176] The effect of pH was studied for the following three different test mixtures in octanol/water SS: (i) methyl ethyl ketone, acetone, and methyl isobutyl ketone; (ii) three beta-blockers, and (iii) three sulfonamides. Generally, when pH conditions create charged ionized forms, the octanol/water partition coefficient decreases, and under pH conditions that create a neutral form, the octanol/water partition coefficient increases.[177]

Berthod et al. performed an extensive investigation of the CCS retention of benzoic acid in a variety of common SSs, paying special attention to pH effects. Benzoic acid may be found in three forms as a solute: neutral, basic, and dimerized. In one series of experiments, the authors observed the behavior of benzoic acid in a HepBuWat biphasic solvent system at different pHs. The pH of the solvent system may be controlled by buffering the water used to formulate them. Experiments were done with pHs lower than, equal to, and higher than the dissociation constant of benzoic acid. As the pH increases, the observed K values also increase. This may because more acidic solutions contain more neutral benzoic acid species that are more soluble in the stationary (organic) phase. In another series of experiments, the authors observed the behavior of benzoic acid in a HepBuWat biphasic solvent system at different ionic strengths. At pH 2, the observed K values of benzoic acid in HepBuWat increase as the ionic strength of the water used to formulate the SS increases. This may be due to at least two factors described by the authors. First, the higher salt concentration forces benzoic acid out of the mobile (aqueous) phase into the stationary (organic) phase. Second, the higher salt concentration pushes butanol out of the mobile (aqueous) phase into the stationary (organic) phase. These two salting out effects work together to produce longer retention times at higher salt (NaCl) concentrations at a constant pH.[178]

Phase Modification before Pre-equilibration with Ion-Pairing Factors. An application of acid/base modification of CCS SSs is the addition of TFA to form ion pairs with target analytes. Jerz et al. reported the separation of betalain pigments by adding TFA to a BuAcWat CCS SS.[179] Ion pairing instigated with TFA addition to BuAcWat and *ter*BuAcWat SSs allowed polar beta-cyanins from the cactus fruits of *Hylocereus polyrhizus* to be soluble in the organic phase. In this study, fractions termed "polarity windows" were analyzed by LC-ESIMS/MS to determine their chemical composition.[180] High molecular weight acyl-oligosaccharide-linked beta-cyanins were separated from the purple bracts of *Bougainvillea glabra* with a *ter*BuAcWat SS modified with 0.7% TFA. CCS made it possible

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to separate and identify six highly unstable and polar betalains with a range of K values from 0.78 to 1.23.[181,182]

Phase Modification before Pre-equilibration with Salts to Create Hydroalcoholic SSs. Hydrophilic alcohol−water biphasic SSs for CCS were proposed in 2006, but very few applications have been reported.[183] Shibusawa et al. described the use of a ProWat (0.8 M potassium phosphate buffer pH 7.4) SS to separate nucleobases, nucleosides, and nucleotides. A mixture of eight nucleic acid constituents was separated as a proof of principle.[184] In a related report, Shibusawa et al. used a test mixture of nucleic acid bases including adenine, adenosine, AMP, ADP, and ATP with a BuEtWat (50% saturated ammonium sulfate) CCS on a spiral multilayer coil.[185] Zeng et al. reported the separation of a test mixture made up of methyl green, tartrazine, tyrosine, and epinephrine with a series of BuEtWat (50% saturated ammonium sulfate) SSs on a spiral column. A test mixture of three catecholamines was separated subsequently with an optimized BuEtWat (50% saturated ammonium sulfate) SS.[186]

Modifications after Pre-equilibration. Another approach to solvent system modification is the addition of compounds after the phases have been pre-equilibrated in a separatory funnel and separated. The term "eluter" is used for the compound modifying the mobile phase, whereas the "retainer" is the compound added to the stationary phase. With these methods, the column is not equilibrated with mobile phase before elution. Typically, the sample is injected at the same time as the mobile phase is introduced to the column filled with stationary phase.

Modifications after Pre-equilibration with Ion Exchange. Ion-exchange CCS creates an isotachic train in the column that is characteristic of techniques where the phases are modified after pre-equilibration.[187] In pH-zone refining, acids and bases are used as retainers and displacers to separate solutes that display a dramatic difference in their relative solubilities in upper and lower phases between their neutral and ionized forms. The same principle is applied in ion-exchange CCS, although it is not strictly pH that is being modulated. For example, when di(2-ethylhexyl)phosphoric acid (DEHPA) and triethyl amine (TEA) were used as retainers in the *ter*BuAcWat SS organic stationary phase and $CaCl_2$ and/or HCl are used as eluters in the aqueous mobile phase, a model mixture of five dipeptides was separated. The ion-exchange CCS method was further refined by filling the column so that the first 1/4 column length closest to the inlet in descending mode had an upper stationary phase with a DEHPA/TEA ratio of 3.33, and the second 3/4 column length had a stationary phase with a DEHPA/TEA ratio of 46.5.[188] The isolation of a glucosinolate natural pesticide, sinalbin, from white mustard seeds was achieved with strong ion-exchange CPC. An equilibrated EBuWat SS was first prepared and separated. The upper stationary phase was modified with 80 mM Aliquat 336 as a retainer. The lower phase was modified with 80 mM NaI as an eluter.[189,190] A similar approach was used to isolate gram quantities of seven glucosinolates from four different plant seeds.[191]

Hamzaoui et al. reported on the isolation of saponins from *Glycyrrhiza glabra* roots with BuWat, modified with trioctylmethylammonium chloride as the retainer in the organic stationary phase and potassium iodide as the eluter in the aqueous mobile phase. The sample was dissolved in mobile phase and introduced to the column. The sample was then eluted with one column volume of unmodified mobile phase. After this, a potassium iodide modified mobile phase was introduced that eluted the target natural products. Flow rate, sample loop volume, and mass loading were optimized for this study.[82]

Modifications after Pre-equilibration with pH-Adjusted Methods. The tendency of organic acids and bases to form different species with very different solubility characteristics has been exploited with pH-zone-refining CCS separation methods, a−c, as follows.

a. Modifications after Pre-equilibration with pH-Zone-Refining. A recent review article by Y. Ito, the inventor of pH-zone-refining, offers a comprehensive look at pH-zone-refining origins, mechanism, and procedures.[192] The article features a table of 66 pH-zone-refining articles. Of these, 29 are articles describing the isolation of natural products, of which 12 were natural product articles published since 2007. The principal advantages of this method are very high sample loading and highly resolved ionizable analyte fractions. The disadvantages are (1) the technique has not been modeled, (2) it is applicable only to ionizable analytes, and (3) SS selection involves the optimization of biphasic SS, retainer, and eluter. Twenty-three pH-zone-refining separations are included in the sample loading Table S4 of the Supporting Information. In addition to pH-zone-refining, several other CCS methods have been developed that create ion-exchange environments by adding an eluter to the mobile phase and a retainer to the stationary phase. The modified mobile and stationary phases are typically not equilibrated prior to introducing the sample into the column.

b. Modifications after Pre-equilibration with pH Gradient. As in classical CCS elution, the pH gradient method begins with an equilibrated CCS column. The mobile phase, however, is introduced as a continuous or stepwise pH gradient. The isolation of chlorogenic acid from *Lonicera japonica* flowers and buds was achieved with a pH-gradient elution method. The CCS column was first filled and equilibrated with the unmodified EBuWat SS, with the aqueous phase used as mobile phase. After introducing the sample into the column, a continuous gradient between neutral and basified (10 mM NH_4OH) mobile phase was pumped through the column. The chromatogram peak shape resembles classic CCS elutions.[193] The isolation of six cephalotaxine-type alkaloids (all tertiary amines) from *Cephalotaxus fortunei* was performed with a step-pH-gradient CCS method. A HEWat SS was equilibrated, and the phases were separated. The upper organic (stationary) phase was modified with a 0.05% trifluoroacetic acid retainer. The lower aqueous (mobile) phase was separated into three portions, and each portion was modified with a different eluter: 2% ammonium hydroxide, 0.2% ammonium hydroxide, and 0.01% TFA. The column was filled with stationary phase, and the sample was injected. Then, the column was eluted with the 2% ammonium hydroxide first, 0.2% ammonium hydroxide second, and 0.01% TFA last. The chromatogram has the "square" peaks characteristic of pH-zone-refining. It was noted that the order of elution was the same as a reversed-phase HPLC column with methanol and aqueous ammonium carbonate elution.[194]

The isolation of three alkaloids from *Nelumbo nucifera* seed embryos was also performed with a pH-gradient elution method. In this study, the authors prepared and pre-equilibrated a DeWat (pH 7.5 sodium phosphate buffer) SS. The CCS column was filled with a 50/50 mixture of the two phases. The column was then equilibrated by pumping the aqueous mobile phase through the column prior to sample

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injection. After the sample was injected, the pH 7.5 buffer mobile phase was gradually replaced by a pH 7.2 buffer. According to partitioning experiments, reducing the pH of the mobile phase by 0.3 pH unit reduced the *K* values of the target natural products by a factor of 4. The chromatogram resembles the classical CCS Gaussian elution peaks rather than pH-zone-refining signature square peaks.[195]

c. Modifications after Pre-equilibration with Dynamic pH Junction. A dynamic pH-junction method was proposed to isolate five alkaloids from the microwave-assisted extract of *Stephania cepharantha* with a HEMWat SS that had HCl added to the lower (mobile) phase. The column was equilibrated with the lower phase, and triethylamine was added to the sample dissolved in the lower phase in order to create a dynamic pH-junction effect. Increasing the concentration of the TEA retainer in the sample solution increased the free base population of the alkaloids and their affinity for the organic stationary phase and, thus, their retention times.[196]

Solvent System Selection Strategies. Selecting the SS plays a major role in the application of CCS technology to the separation of natural products and other analytes. In fact, the overall productivity of a CCS-based separation strategy may depend heavily on the speed, efficiency, and success of the methods used to determine the most suitable SS. The following four principal methods are well-established tools for this process: (a) the use of small-volume ("analytical") CCS instruments for trial runs; (b) partitioning experiments aimed at the determination of partitioning behavior and/or *K* values, employing various detection methods such as UV, HPLC, GC-MS, and quantitative NMR;[123] (c) TLC-based methods such as the GUESS method, which have the advantage of working in tandem with fraction monitoring, representing a ubiquitous method in the natural products isolation laboratory; and (d) computational screening models that take into account the thermodynamic properties of both solvents and analytes to predict analyte behavior and matching SSs. A detailed overview of these tools and their documentation in the up-to-date literature has been published.[197]

Most reports on CCS-based natural product isolations include information about the SS selection process. About 70% of CCS reports describe a SS selection process, and the results are summarized typically in a table of SSs and the corresponding *K* value(s) of the target analyte(s). However, the breadth of the exploration of the "chemical diversity" space of SSs in the literature varies substantially: the average number of SS families investigated per article was 2.5, with a low of 1 and a high of 26 (Figure 5A).[122] Indeed, almost half (43%) of the articles test only a single SS family, which implies that a more comprehensive probing of the CCS selectivity space likely offers opportunities for enhanced separation performance. The total number of SSs used for SS selection (Figure 5B) ranges from 2 to 63 with an average of 7.6, which completes the picture with an average of 4.2 SS per SS family tested, with a low of 1 and a high of 28.

■ OPTIMIZATION OF CCS OPERATION PARAMETERS

After the solvent system has been selected to provide the necessary polarity and selectivity conditions for the separation to occur, there remains the task of optimizing the CCS operating conditions to make the best use of the technology. These adjustments may be made in a predictable and routine fashion for most separations. Of particular interest to natural product researchers is the maximum sample loading capacity of

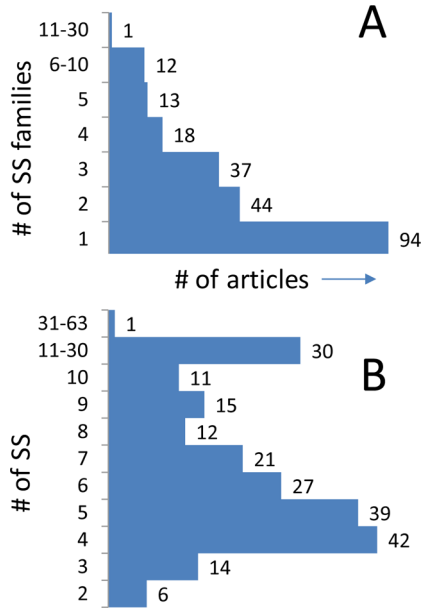

Figure 5. Solvent system (SS) selection in the CCS literature with regard to the number of SS families (A) and the diversity of SSs evaluated per individual study (B) in the literature since 2007.

the chromatographic method, which provides a reasonable resolution of target analytes. With a given column volume, the parameters of flow rate, rotational speed, and temperature may be adjusted to give an optimal separation of a particular quantity of sample in a given SS.

Optimization of Sample Loading. Although the sample mass separated was reported for 92% of the surveyed articles, column loading was not always optimized. Columns are often loaded with less than the possible loading capacity due to the scarcity of the sample or because of the enhanced resolution of a smaller sample compared with a larger sample. Separating a too-small sample is simply inefficient on the basis of time spent and solvent used per weight of product. Generally, resolution decreases as sample loading increases.[198] In addition, the optimal column loading varies from sample to sample. Figure 6 shows that a column loading of 0.5 to 5 mg of sample per column mL was commonly used to achieve adequate natural product resolution. Generally, Dynamic Extractions' high *g*-force hydrodynamic series (7.7 mg/column mL average) and hydrostatic CPC (7.3 mg/column mL average) instruments have a higher sample loading capacity than other hydrodynamic instruments (2.1 mg/column mL average). Comparing low *g*-force hydrodynamic instruments in Figure 6A with all CCS instruments in Figure 6B shows that instances of column loading above 5 mg sample per mL column volume increases substantially when high *g*-force hydrodynamic and CPC instruments are factored in.

Sample loading may be increased by increasing the sample concentration in the same sample loop or increasing the sample volume. Optimization of sample loading is performed to maximize the amount of sample that may be chromatographed with the desired resolution. As in other forms of chromatography, sample loading capacity in CCS depends on the solubility of the sample in the SS. The CCS cell model theory was applied to the topic of sample loading and the resulting resolution of a separation. Previously published data from multiple dual-mode centrifugal partition chromatography were analyzed.[199]

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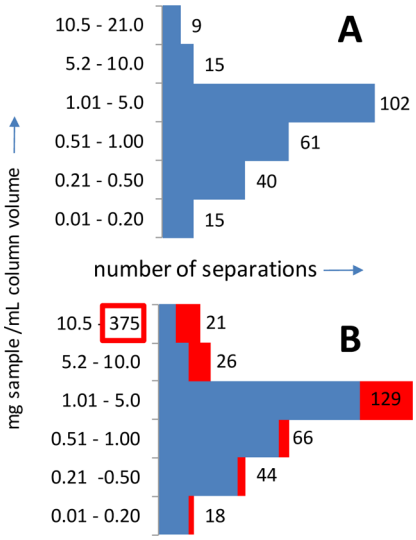

Figure 6. Sample loading in the countercurrent separation (CCS) of natural products in mg sample per mL of column volume reported since 2007: (A) sample loadings for separations with low *g*-force hydrodynamic instruments; (B) sample loadings reported for all CCS instruments. The red boxes indicate changes that were a result of including CPC and high *g*-force hydrodynamic instruments to the totals.

The following examples feature articles where sample loading has been intentionally optimized. For the isolation of honokiol and magnolol from *Magnolia officinalis* (Houpu) sample loading studies were performed by increasing both sample concentration and volume. Increases in sample concentration or volume exhibited a decrease in resolution. A sample concentration of 80 mg/mL in a 0.05 mL volume was considered optimal for an 18 mL hydrodynamic column.[200] Three alkylamides were isolated from *Zanthoxylum bungeanum* pericarp with a HEMWat SS. An optimal sample loading capacity of 1.0 g of extract with a flow rate of 1.5 mL/min was established for a 300 mL hydrodynamic column.[201] Three acetophenone derivatives were isolated from *Cynanchum bungei* roots with a BuMWat SS. An optimal sample loading capacity of 750 mg/mL in 2 mL was established for a 230 mL hydrodynamic column.[202] Five diterpenoids were isolated from *Tripterygium wilfordii* with a HEMWat SS. An 18 mL hydrodynamic analytical column was used to study four different sample concentrations in a 0.5 mL sample loop and three different volumes at a 20 mg/mL sample concentration. The optimal sample concentration and loading were 20 mg/mL and 1.0 mL, respectively.[203] Capsaicin and dihydrocapsaicin were isolated from *Capsicum frutescens* fruits with a HEMWat (10% acetic acid) SS. Adding acetic acid increases the solubility of the target natural products in the lower aqueous phase. Optimization of four sample concentrations and four sample volumes was performed on an 18 mL hydrodynamic instrument. The optimal sample concentration and volume were 25 mg/mL and 1 mL, respectively.[204]

Optimization of Flow Rate. The flow rate is an important CCS operating parameter simply because the flow rate most influences the separation time once the appropriate SS has been selected. It has been well established that an inverse relationship exists between flow rate and resolution in hydrodynamic instruments. Therefore, a balance must be achieved between a minimum separation time and a desirable analyte resolution with a given sample size. Three alkylamides were isolated from *Zanthoxylum bungeanum* pericarp with a HEMWat SS. An optimal flow rate of 1.5 mL/min was established for a 1.0 g extract loading on a 300 mL hydrodynamic column.[201] A flow optimization study was performed for the isolation of honokiol and magnolol from *Magnolia officinalis* (Houpu). A series of flow rates from 1 to 2.5 mL/min in steps of 0.5 mL/min were evaluated. The flow rate of 2.5 mL/min was considered to be the best.[200] Another flow rate optimization study was performed for the isolation of five diterpenoids from *Tripterygium wilfordii* with a HEMWat SS. Four different flow rates were evaluated on an 18 mL hydrodynamic analytical column. As a result, the optimal performance was exhibited when the flow rate was changed from 1 to 1.5 and then to 2.0 mL/min during the separation.[203] A model sample system consisting of benzyl alcohol and *p*-cresol in a HEMWat SS was used to optimize flow rate and rpm. Optimum conditions for resolution were a flow rate of 20 mL/min and a rotation speed of 1200 rpm on a 912.5 mL hydrodynamic column. The optimized parameters were then employed to separate three rotenoids and one isoflavone from a *Millettia pachycarpa* preparation with an emphasis on maximizing throughput.[205] Four flavonoids were isolated from black currant leaves with a HEMWat SS followed by C_{18} prep-HPLC. A flow rate optimization study was performed with five different flow rates. A sample loading study was also performed with five different sample loads. A flow rate of 1.5 mL/min with a sample loading of 100 mg was deemed optimal on a 290 mL hydrodynamic column.[206]

Sample loading and flow rate were optimized for the isolation of five flavonol glycosides from *Ginkgo biloba* leaves with a HEBuMWat (0.5% acetic acid) SS. A study of three different sample concentrations and three different flow rates was performed. The optimal conditions were determined as follows: 80 mg/mL sample concentration, 1.5 mL/min flow rate, 2 mL sample loop, 40 mL column volume, 1600 rpm, and 25 °C.[207] A flow rate optimization study was undertaken during the isolation of three xanthones from *Swertia mussotii* plants with a HEMWat SS. The optimal flow rate was 1.5 mL/min for a 150 mg sample separated at 800 rpm and 25 °C on a 280 mL hydrodynamic column.[208] Du et al. described a "chemometric approach" to simultaneously optimize rpm, temperature, and flow rate for the HEEtWat CCS separation of phenol and resorcinol by employing the Box-Behnken response surface model and Derringer's desirability function. An optimal flow rate of 1.30 mL/min, rotation speed of 1,800 rpm, and temperature of 31 °C were established for a 20 mL hydrodynamic CCS instrument.[209] Depending on instrument design (hydrodynamic vs hydrostatic) the flow rate also impacts phase mixing—an important consideration in hydrostatic instruments where the flow rate directly impacts achievable resolution.

Optimization of Rotational Speed and Temperature. Ideally, optimization of sample loading, flow rate, rotational speed, and temperature must all be coordinated. In hydrodynamic instruments, resolution increases as sample loading and flow decrease and rotational speed increases. The effect of temperature on stationary-phase retention volume and resolution of analytes has been systematically studied in only a few cases. Only 27% of the articles surveyed reported the operating temperature, which ranges from 10 to 60 °C.[210,211] Generally, biphasic SSs tend toward coalescence as the temperature increases which translates in the lower sta-

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Table 4. Reporting of Separation Parameters: Process Throughput (PT in Grams of Sample Processed per Hour of Separation Time), Process Efficiency (PE in Grams of Sample Processed per Hour of Separation Time), Process Environmental Risk Factor (ER in Liters of Solvent per Gram of Product), and the Process's General Evaluation Factor (GE in Grams of Sample Times Grams of Product per Hour of Separation Time per Liter of Solvent) in Countercurrent Separations of Natural Products since 2007

natural product(s) (no. of cpds)	source	solvent system	PT [g/h]	PE [g/h]	ER [l/g]	GE [g²/(h·l)]	ref
ginsenosides (4)	*Panax ginseng* roots	DiMWat and HBuWat (0.1% formic acid)	5.14	0.21−0.54	0.065−0.18	1.17−8.27	140
salvianolic acid B	*Salvia miltiorrhiza* rhizomes	HEMWat (0.1% acetic acid)	2.23	0.77	4.0	0.192	250
ginsenosides (4)	*Panax ginseng* roots	DiIsoWat (ammonium acetate)	0.23	6.73−14.6	0.06−0.71	9.5−243	251
geniposide	*Gardenia jasminoides* fruits	EBuWat	5.0	0.55	4.9	0.113	252

tionary-phase retention. On the other hand, the tendency toward coalescence may actually improve analyte resolution. It is also important to note that the temperature setting of the heating/cooling apparatus may not be the same as the temperature of the solvent passing through the coils.[198]

Baldermann et al. paid close attention to temperature effects for the isolation of all-*trans*-lycopene from tomato paste with a HDiAc SS temperature difference phase diagram. The SS coalesced at 25 °C but was stable at 20, 15, and 10 °C. Lycopene *K* values, SS settling times, and the % volume of the lower phase decreased as the temperature decreased in partitioning experiments. The optimal temperature was determined to be 15 °C for this separation.[113] An optimization strategy featuring temperature studies was described by Schröder et al. during their purification of beta-sitosterol and sitostanol from a commercial crude beta-sitosterol Merck standard. A total of 29 SSs composed of either HEAc or HMWat were evaluated for their stability at 20, 35, 40, and 45 °C. Some SSs did not separate into two phases at higher temperatures.[43]

Phenolic compounds were isolated from hawthorn (*Crataegus laevigata*) with a response surface methodology that featured an empirical/statistical model for finding the best combination of temperature, flow rate, and rotation speed to purify a particular natural product by CCS. A hawthorn extract was first subjected to a series of 12 partition experiments to determine the best SS for the separation of chlorogenic acid, epicatechin, procyanidin B2, and procyanidin C1. An EBuWat SS was chosen. At that point, the sample was separated in 13 different combinations of three temperatures (15, 25, and 35 °C), flow rates (1.0, 1.5, and 2.0 mL/min), and rotational speeds (500, 750, and 1000 rpm). The purity of the recovered chlorogenic acid was determined for each separation. With this information, a surface response plot between two of the parameters and the chlorogenic acid purity was determined. Combining the information on three resulting surface response plots gave the optimal parameters as 25 °C, 1.5 mL/min, and 850 rpm for a 280 mL hydrodynamic column.[212]

A scale-up investigation for the isolation of five biflavones and one monomeric flavone from *Selaginella tamariscina* with a HepEMWat SS was undertaken. Optimal sample concentration, sample loading volume, flow rate, and rotation speed of 20 mg/mL, 0.1 mL, 1.0 mL/min, and 2000 rpm, respectively, were established for a 4.7 mL column volume at 25 °C.[213] The relationship between column length and resolution was investigated during the isolation of 10 flavonols from *Gossypium hirsutum*. A 50 mg sample was chromatographed on 125, 250, 375, and 500 mL columns by using one, two, three, or four coils of a four-coil instrument. It was reported that analyte resolution and separation volume increase as column volume increases.[214]

A study of the effect of injection volume on retention time, peak width, peak height, and analyte resolution was undertaken by modeling and an experimental CCS procedure. It was found that the retention time of the analytes increased in a linear fashion as injection volume increases. Peak width and peak height increased as the injection volume increased. The resolution decreased as the injection volume increased, as was shown with a mixture of 3,4,5-trihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, and 2-mercaptobenzimidazole in a HEMWat SS.[215] Altogether, 276 g of cyanidin-3-glucoside was separated from 1.5 kg of crude *Myrica rubra* berry extract with a 40 L slow rotary CCC. A *ter*BuAcWat (0.02% TFA) SS was employed. Rotation rate optimization was performed by measuring the stationary-phase volume retention ratio at six different rpm's.[216] Three flavonoids were isolated from *Citrus aurantium* fruits with EBuWat and ChBuMWat SSs. Optimization of flow rate, temperature, and rotation speed revealed that a flow rate of 2 mL/min, a revolution speed of 800 rpm, and a temperature of 30 °C were optimal for separations on a 300 mL hydrodynamic instrument.[125]

Scale-up of Operation. The scale-up of CCS natural product isolation procedures is of great significance to commercial applications. CCS may be optimized using small instruments and then directly scaled up based on simple *K* value calculations. In particular, Dynamic Extractions markets a line of three instruments with different size columns (from 4.7 mL to 18 L) and has performed extensive research on the loading capacity and throughput of these instruments.[217] For example, Yuan et al. described the isolation of five biflavones and one simple flavone from *Selaginella tamariscina* by scaling up the process from a 2 mg sample on a 4.7 mL column, to a 8.6 mg sample on a 17.2 mL column, to a 400 mg sample on a 916 mL column with nearly the same purities, resolutions, and elution times while increasing throughput by 50-fold.[213] The performance of an 18 L Dynamic Extractions process-scale hydrodynamic CCS instrument was demonstrated by the separation of a four-component GUESS mixture in a HEMWat SS. The higher capacity instrument showed improved resolution over lower capacity counterparts. Higher *g* forces generated by the larger instrument showed a marked increase in sample throughput over smaller hydrodynamic instruments.[218] The same instrument was used to prepare 627 g of spinetoram-J and 146 g of spinetoram-L from 1 kg of spinetoram.[219]

The literature survey performed revealed over 50 publications that reported a sample loading of one or more grams,[47,82,83,99,132,140−142,189,191,200−202,204,205,216,219−249] as

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summarized in S4, Supporting Information. Ion-exchange methods have been shown to increase the loading capacity of CCS instruments. Ionizable analytes are eluted in separate bands rather than the (potentially overlapping) Gaussian peaks found in CCS methods with no modification of phases after pre-equilibration. A total of 23 entries in the preparative sample loading Table S4, Supporting Information, are pH-zone-refining and related techniques.

Measuring Important Parameters. The survey concluded that most natural product isolation articles that feature CCS have reported the mass of sample applied to the CCS column (92%), the mass of natural products recovered (87%), and the natural product purity (82%). However, the percentage recovery of target analytes was only reported in 14% of the articles. In addition to these well-known parameters, there was an increasing interest in reporting parameters that refer to the efficiency of the CCS chromatographic process,[140,250−252] as summarized in Table 4.

For example, the isolation of salvianolic acid B from *Salvia miltiorrhiza* was monitored for sample loading, mass recovery, product purity, percent recovery, process throughput (PT), process efficiency (PE), process environmental risk factor (ER), and general process evaluation factor (GE). The authors reported sample mass loading, mass recovery, product purity, and percent recovery as 1500 mg, 475 mg, 96.1%, and 42.8%, respectively. PT and PE are very generally used in various business applications; the second two factors (ER and GE) seem to be fairly novel parameters. Process throughput (in mass per time) reporting may accentuate the high-throughput nature of CCS under optimized sample loading conditions. Process environmental risk factor (in volume of solvent needed per gram of recovered analyte) reporting may emphasize the fact that CCS is a low organic solvent consumption method, especially under optimized sample loading conditions.[250]

WORKFLOW

Considering the metabolomic complexity of natural product mixtures, it is advantageous to consider CCS as an integral part of the total separation workflow. Sample extraction, prefractionation, and/or primary fractionation typically precede CCS chromatography. In addition, if a CCS step does not achieve the desired target analyte purity, other chromatographic methods can be applied. It is also important to understand that CCS is one out of many chromatographic techniques that could be performed to accomplish the desired separation. In many cases, success is realized when a combination of orthogonal methods is employed in a rational fashion. Notably, CCS is very efficient in providing analytical orthogonality relative to virtually all separation techniques that are used abundantly in natural products research.[253] However, the researcher must be aware of the strengths and weaknesses of each technique in order to select and position them appropriately in the overall separation protocol. The following provides a summary of experience documented in the recent literature regarding the efficient use of CCS in separation workflows.

Sample Preparation for CCS. The ability of CCS columns to accommodate crude samples has been recognized as especially applicable to natural product isolation protocols. Solvent extraction of finely divided solid organic material is the norm for natural product isolations. The crude extract may be further prepared for chromatography by evaporation of solvents and/or separatory funnel liquid−liquid extraction. Survey results indicated that in most separation protocols involving CCS (82%) the first chromatography separation was indeed CCS. For the remainder of cases, a variety of open or flash column liquid chromatography media were employed as a preparatory step for CCS chromatography. Silica gel columns were employed in about a third (29%) of LC applications, while Diol, RP-18, and Sephadex LH-20 media were also frequently used. A large selection of styrene-divinylbenzene-based organic polymeric adsorbents is also available for LC. There is a balance between performing CCS with crude samples, which may reduce the amount of sample that may be separated effectively, and accomplishing the same CCS fractionation with LC-prepared samples, which will likely have a larger loading capacity and better separation characteristics. While the survey concluded that CCS might be viewed frequently as a high-resolution variant of liquid−liquid extraction, its single-point application and/or positioning prior to solid-phase LC in the workflow often does not convey its full separation potential.

Phase as an Extraction Solvent ("Direct Injection"). Very often the transformation of a crude extract to a chromatography-ready sample involves a solvent evaporation step that requires extra time, instrumentation, and energy. As CCS columns may be loaded with sample loops that may contain 6% to 10% of the total column volume, extracts may be loaded directly onto the instrument without intermediary solvent evaporation. For example, curcumin, demethoxycurcumin, and bisdemethoxycurcumin were purified from *Curcuma longa* rhizomes (turmeric) with an HChMWat SS. Sample preparation consisted of vigorously mixing curcumin powder with both upper and lower phases of the SS. The resulting solution was centrifuged, and the supernatant directly introduced into the sample loop. In this case, the sample volume was less than 1% of the column volume.[122] A group of three articles has described another direct injection approach. For sample preparation, the upper phase of the SS was used as both accelerated solvent extraction solvent and CCS stationary phase. Using this approach, three caffeoylquinic acids were isolated from *Hypericum perforatum* with an EMWat SS.[254] Six ginsenosides and three notogenosides were isolated from *Panax notoginseng* roots with a series of EBuWat, EBuMWat, and HEBuWat SSs.[211] Twelve ginsenosides were isolated from *Panax quinquefolium* roots with a series of EBuWat, EBuMWat, and HEBuWat SSs. In all three of these articles, the sample loop volume was 8% of the instrument column volume.[148]

Several modern extraction techniques have been employed to prepare samples for CCS. Sun et al. compared five extraction methods for the CCS isolation of paeonol from *Cynanchum paniculatum* roots, namely, supercritical fluid extraction, ultrasonic extraction, microwave-assisted extraction, steam distillation, and Soxhlet extraction. Supercritical fluid extraction was found to be the optimal process after adjustment of pressure, temperature, time, and sample particle size.[228] Table S5, Supporting Information, summarizes the modern extraction methods used to prepare samples of CCS during the survey period.[50,103,111,125,129,228,255−272] Accelerated solvent extraction, microwave-assisted extraction, and supercritical fluid extraction were used in several cases.

Separation Strategies with Multiple CCS Fractionation Steps. In addition to CCS being employed as one fractionation step in a complex multistep fractionation scheme, it is also possible to use CCS in two or more successive fractionation steps. Repeated CCS has the effect of lengthening the effective CCS column in order to increase resolution with

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no change in polarity or selectivity of the chromatographic environment. If the SS's mobile and/or stationary phases are changed from the original run, changes in both polarity and selectivity can aid the separation of closely eluting analytes.

Successive CCS. The most straightforward way to run successive CCS fractionation is to rechromatograph the target analytes in the same SS. This method is particularly effective if the target natural products with similar *K* values are recycled through the system. This has the effect of lengthening the CCS column until a baseline separation may be achieved. Another approach is to rechromatograph the target natural products with a polarity-adjusted SS of the same composition. Of course, the second SS may also have a different solvent composition and, thus, can engender an orthogonal separation of the target natural products.[23] There have also been a few cases where a fraction from one CCS column is introduced directly in the sample loop for a subsequent (hyphenated) CCS step.

Successive CCS by Recycling with the Same SS. A very basic form of recycling CCS was developed by Liu et al. for the isolation of the γ-oryzanol components, cycloartenyl ferulate and 24,25-dihydro-24-methylenecycloartenyl ferulate, from rice bran oil, with the nonaqueous HAc SS. A fraction collected from the first separation was rechromatographed with the same SS for further purification.[110] In the same manner, Schröder et al. reported the purification of eight phytosterols from rapeseed, olive, and linseed oils via a complex fractionation scheme featuring two successive HMWat (1% silver[I] nitrate) 170:120:5 CCS operations.[43,273] Shi et al. reported the separation of 3′-hydroxydaidzein and tectorigenin by a four-step recycling process with a PetEMWat SS. The two natural products were in a fraction previously obtained from a PetEMWat EECCC separation.[274] The separation of nine anthroquinones from *Cassia obtusifolia* seeds was accomplished by an initial step gradient CCS with two polarity-adjusted HEMWat SSs (1:1:1:1 and 4:3:4:3). Three fractions from the first CCS fractionation were further separated by recycling CCS (two to four cycles), also with HEMWat SSs.[275] The separation of apocynin, androsin, and three picrosides from *Picrorhiza scrophulariiflora* was accomplished with two successive HEMWat (1:2:1:2) and EBuWat (0.1% formic acid) EECCC separations. Finally, picrosides I and III were resolved by six recycling steps with an EWat SS.[276]

Successive CCS with Polarity-Adjusted SSs. The second method of successive CCS fractionation is the use of two or more polarity-adjusted SSs of the same composition. Inui et al. employed a strategy they termed "gradient array" to systematically separate five natural products from *Oplopanax horridus*. The fractionation scheme involved a gradient array of four polarity-adjusted HEMWat SS runs. For the first four CCS runs, the low *K* value lipophilic fractions were recovered and rechromatographed on less polar SSs in the following sequence: HEMWat 5:5:5:5, 7:3:5:5, 7:3:6:4, and 8:2:8:2.[139]

Two successive two-step nonaqueous polarity-adjusted SS gradients were employed for the separation of hyperforin from the aerial parts of *Hypericum perforatum*. In parallel, two successive two-step polarity-adjusted HEMWat gradients were used to purify hypericin from *H. perforatum* aerial parts. In this case, the first separation was in a reversed-phase mode, while the second was normal phase. Lutin and hyperoside were separated from *H. perforatum* aerial parts with three successive polarity-adjusted EBuWat SSs. The first separation was performed in back-extrusion elution mode.[111] Table S6, Supporting Information, summarizes 17 fractionation protocols inclusive of two to four polarity-adjusted SSs.[49,111,139,214,247,277−285]

Successive CCS with Orthogonal SSs. The use of orthogonal chromatographic methods is a mainstay of natural products separation. As CCS may be performed with a wide variety of SS families, CCS is highly capable of matching one SS with another of similar polarity but different composition and selectively characteristics, i.e., establishing orthogonality. The CCS of *Ginkgo biloba* terpene lactones by successive CCS runs was described by Qui et al. SS selection was aided by quantitative ^{1}H NMR analysis (qHNMR) of the upper and lower layers of a partition experiment. A two-step successive CCS operation was performed first with a ChMWat SS to produce three target fractions. This was followed by a HEMWat with the addition of 0.5% DMSO ("HEMSoWat") to complete the purification. Fraction analysis with qHNMR spectroscopy provided structural data for target natural products and impurities as well as quantitative purity information.[123] Table S7, Supporting Information, gives an overview of the use of two orthogonal SSs by summarizing 21 such fractionation protocols published recently.[42,47,123−125,140,164,285−297]

Successive CCS with Hyphenated CCS-CCS (2-D CCS). A recent review article describes eight examples of two-dimensional CCS where the effluent from the first column was introduced directly to a second CCS column or a solid-phase LC column.[23] The propensity of CCS to produce concentrated fractions facilitates the direct coupling of its columns without an intervening solvent evaporation operation.

Gradients. Gradient elution is practiced to a lesser extent in CCS than it is in LC. However, there are dozens of examples of gradient elution applications in the CCS literature. The most common form is the step gradient. The CCS column is equilibrated with the first SS and the sample injected. If the process is in reversed-phase mode, a less polar mobile phase is pumped into the column at a certain point to elute the lower polarity natural products. At the point when the new mobile phase is introduced, the mobile and stationary phases are no longer pre-equilibrated, so some loss of stationary phase may occur. Also, the calculation of *K* values becomes problematic for analytes eluting with the second mobile phase. There are a few examples of linear gradients as well. Typically, the second mobile phase has the same components as the first mobile phase, but there are examples where this is not the case. The following provides a summary of documented CCS gradient methodologies.

Step Gradient Elution with Polarity-Adjusted SSs. The majority of articles employing step gradients perform a two-step gradient in the reversed-phase mode. However, the Romero-Gonzales et al. article cited earlier employed a three-step salt concentration gradient.[152] Table S8, Supporting Information, summarizes 22 fractionation protocols including the use of two or three polarity-adjusted step gradients.[37,47,111,126,152,229,267,275,277,298−308]

Step Gradient Elution with Differently Formulated SSs. Altogether, 16 natural products (from adhyperforin to a quercetin glycoside) were separated from *Apocynum venetum* leaves in a single CCS run employing a four-step gradient elution scheme. The lower phase of HEAcWat 3:7:4:9 SS was used as the stationary phase. The upper phases of pre-equilibrated HEAcWat 3:7:4:9, EAcWat 5:3:7, EMWat 5:2:5, and BuMWat 5:1:5 were introduced successively to perform this normal-phase gradient-based separation.[309] A total of 12

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Table 5. Downstream Methods Used since 2007 to Rechromatograph Fractions Produced by Countercurrent Separation of Natural Products and Comparison with Solid-Phase-Based LC

natural products (no. of cpds)	source	solvent system	downstream chromatography	ref
flavonoids (4)	black currant leaves	HEMWat	C_{18} HPLC	206
flavonoids (7)	*Belamcanda chinensis* roots	*ter*EBuAcWat (0.1% TFA)	C_{18} HPLC	311
flavone C-glycosides (4)	*Ficus microcarpa* leaves	*ter*EBuWat (0.1% TFA)	C_{18} HPLC	312
epigallocatechin and avicularin	*Hypericum perforatum* aerial parts	EMWat	C_{18} HPLC	313
glycosyl flavonoids (3)	*Crataegus* sp. leaves	BuWat	C_{18} HPLC	314
mangiferin and isomangiferin	*Cyclopia subternata* shoots	*ter*AcWat (formic acid/ NH_4OH)	C_{18} HPLC	164
pyranoanthocyanins (2)	*Prunus cerasus* juice	*ter*BuAcWat (0.1% TFA)	C_{18} HPLC	315
15*S*- and 15*R*-betanin	*Phytolacca americana* fruits	BuAcWat (0.7% TFA)	C_{18} HPLC	179
phthalides (4)	*Ligusticum wallichii* roots	HEMAcWat	C_{18} HPLC	316
flavonoid glycosides (4) and *para*-hydroxybenzoic acid	*Halimodendron halodendron* aerial parts	ChMWat (2.5% acetic acid)	C_{18} HPLC and Sephadex LH-20	130
polyphenolics (30)	*Aspalathus linearis* leaves (rooibos tea)	EBuWat and EWat	C_{18} HPLC and silica gel prep-TLC	317
theaflavins (4)	*Camellia sinensis* leaves (black tea)	HEMWat	Sephadex LH-20	318
benzofuranones (2) and saponins (3)	*Nigella glandulifera* seeds	HEMWat	Sephadex LH-20	319
oligiostibenes (3)	*Vitis thunbergii* roots	ChMWat	Sephadex LH-20	131
galactolipids	*Cucurbita pepo* seeds	PetDiProMWat	C_{18} HPLC	320
saponins	*Tiechemella heckelii* seeds	*ter*BuAcWat (0.5% TFA)	preparative TLC and flash chromatography	321
arctiin	*Arctium lappa* aerial parts	EBuWat (1% NaCl)	macroporous resin column	322
anthraquinones (5)	*Morinda officinalis* roots	HEMWat	C_{18} HPLC	323
kuraridin	*Sophora flavescens* roots	HEMWat	C_{18} MPLC	324
naphthylisoquinoline alkaloids (10)	*Ancistrocladus tectorius* twigs	HepEMWat	silica gel CC and C_{18} HPLC	325
arylalkanes (9)	*Zingiber officinale* rhizomes	HepEMWat	silica gel flash chromatography and C_{18} HPLC	326
flavone glycosides (2), flavone celtidifoline (1), other classes (11)	*Lantana trifolia* leaves	HEBuWat step gradient	C_{18} HPLC and Sephadex LH-20 and silica gel LC	229
brevetoxins (4)	marine dinoflagellate *Karenia brevis*	(not given)	phenyl-hexyl HPLC and C_{18} HPLC	44

flavonoids and two phenolic acids were obtained with CCS followed by preparative HPLC from *Mucuna sempervirens* leaves. In this case, the stationary phase was water saturated with *n*-butanol, while the following single-phase SSs were used as successive mobile phases: HE (1:1), HE (1:2), HE (1:4), ethyl acetate, EBu (4:1), EBu (2:1), EBu (1:1), and EBu (1:2).[105]

Linear Gradient Elution. Linear gradient elution is another form of gradient elution possible with CCS instruments. In the same way as stepwise elution, the column is equilibrated with an initial "matched" biphasic SS. The gradient gradually introduces the "nonmatched" mobile phase as the elution progresses. It is assumed that changing the solvent ratios of the mobile phase will modify the solvent ratios in the stationary phase progressively. Ignatova et al. correlated a gradient elution profile of a mixture of standards with their partition coefficients.[156] For the gradient elution studies, the HEMWAt SSs were prepared in three ways: (1) mixing followed by equilibration in a separatory funnel, (2) mixing each phase separately according to their experimentally determined compositions, and (3) mixing the phases on-demand using a quaternary pump. Two model analyte mixtures were created from synthetic and pharmaceutical analytes. The upper phase of HEMWat 5:5:5:5 was used as the stationary phase, and the lower phase was run on a linear reversed-phase gradient from HEMWat 5:5:5:5 to HEMWat 9:1:9:1. The correlation was accomplished by predicting which HEMWat system number would give a partition coefficient of 1 for the analyte in question. *K* values for each analyte were measured in six different HEMWat SSs by the partitioning method to provide correlation data.[156]

In another study, five tanshinones were isolated from *Salvia miltiorrihiza* with an on-demand solvent mixing process that was developed by determining the composition of upper and lower phases of nine HEEtWat SSs. For the separation, a two-SS linear reversed-phase gradient was employed beginning with HEEtWat 8:2:5:5 and ending with HEEtWat 8:2:7:3. Linear gradient modeling was applied to the separation of tanshinones from *Salvia miltiorrhiza*.[25] Another example was the isolation of three podophyllotoxin derivatives from *Dysosma versipellis* with both linear and step gradients of two polarity-adjusted HEMWat SSs 4:6:3:7 and 4:6:4:6.[307]

Downstream Combination of CCS with Complementary Techniques. Management of the CCS effluent is a matter of practical concern. The goal of chromatography is separation, and, therefore, in the typical preparative CCS setting, a fraction collector is employed to fractionate the effluent. If a large number of test tubes are collected, they may be combined to constitute a reasonable number of fractions. Fraction control with an online detector and/or offline analysis methods such as TLC allow the practitioner to predict the best strategy for combining the eluent in the collected test tubes. Interestingly, CCS was the final isolation step in 82% of the articles surveyed. In the remaining articles, CCS fractions were further purified with preparative HPLC, open column LC, or preparative TLC. Subsequent rechromatography, or crystallization, of selected fractions may be attempted based on the various chromatog-

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raphy techniques available, whereupon the collection, detection, analysis, and combination process begins anew.

Downstream Hyphenation. Downstream hyphenation and coupling of CCS involve one or more detection techniques that are either placed online with eluent or applied to the collected fractions. In principle, CCS may be hyphenated with any detection technique that has been adapted to LC such as UV−vis, PDA, MS, Corona, and evaporative light scattering detectors (ELSD). In practice, UV−vis detection was most common. The literature survey conducted revealed that 85% of CCS articles describing the isolation of natural products included at least one CCS chromatogram. Of these, 87% employed UV−vis detection. The next most common detection method was ELSD, with 9%, followed by pH and MS monitoring. Offline methods of fraction control include TLC, NMR, GC-MS, various HPLC methods, gustatory evaluation (with safety considerations applied), and mass (weighing). As flow rates of at least 1 mL/min were typically employed, a preparative cell or flow splitting arrangement must be used. Direct HPLC analysis of CCS fractions has been reported. A recent review article featured 21 articles between 1990 and 2011 that coupled CCS with online MS detection. The same article reported 10 studies between 2006 and 2013 that featured online HPLC monitoring of CCS eluents.[23]

It is worth noting that 96% of articles that present CCS chromatograms use time as the *x*-axis unit. In a few cases volume, coil volumes, tube numbers, or combined fractions were represented on the *x*-axis. The use of the analyte-specific CCS parameter, the partition coefficient (K), offers a more instrument-independent and reproducible means of representing CCS chromatograms. For this purpose, a universal scheme capable of covering zero to infinity K values, the ReS and ReSS methods,[310] exists, and the authors have demonstrated recently its versatility for the description of SS orthogonality ($ReSS^2$ plots).[52]

Downstream Orthogonal Chromatography. The observation that CCS was reported to be the final chromatographic step for 82% of the surveyed articles leads to several (different) conclusions. On one hand, this statistic attests to the fitness of CCS as a high-resolution chromatography technique, capable of producing high-purity isolates. In the remaining articles, a variety of orthogonal chromatography methods were employed such as RP-(preparative)-HPLC, Sephadex LH-20 LC, silica gel LC, macroporous resin LC, and preparative TLC. The use of mechanistically different techniques confirms that separation schemes adequate for complex natural product mixtures inevitably require repeated separation steps involving various chromatographic media. One advantage of the use of CCS in upstream steps of fractionation is the minimization of sample preparation efforts, as only simple organic solvents are used to formulate CCS SSs. Table 5 summarizes the downstream chromatography methods used to rechromatograph fractions produced by the CCS of natural products since 2007.[44,130,131,164,179,206,229,311−326]

Comparison between CCS and Solid-Phase-Based LC. Moreover, CCS is often seen as an alternative method to solid-phase-based LC in the sense that if other LC methods are problematic, then CCS (notably also a form of LC) is explored as an alternative means of fractionating difficult-to-separate natural products. However, the true value of CCS technology will not be fully realized until unbiased comparisons are made between optimized LC and CCS procedures for the separation of the same extracts or mixtures. To date, only a few such comparisons are available in the literature.

A useful systematic comparison between CCS and LC was reported by DeAmicis et al. Spinetoram-J and spinetoram-L were prepared from 1 kg of spinetoram, a commercial insecticide containing both spinosyn J and spinosyn L. These congeneric natural products differ only by a double bond and a methyl group. CCS was performed on a 912.5 mL hydrodynamic instrument, while HPLC separation was achieved on an 11 × 25 cm preparative C_8 column. An extensive comparison was made between the two methods. The most evident advantages for CCS compared with HPLC were a lower total fraction volume containing pure spinosyns (86 vs 1196 L) and lower total mobile phase used (490 vs 2560 L). While each single CCS fractionation experiment required much more (17.5×) time than a single HPLC separation, fewer separations were necessary due to the higher loading capacity of the CCS instrument. In total, the sample throughput of CCS was 2.4 times greater than that of HPLC (0.045 vs 0.019 kg/h).[219]

In another comparative study, three flavanolignans were isolated from silymarin derived from *Silybum marianum* with an HChMWat (0.5% acetic acid) CCS procedure. A comparison between a 260 mL CCS instrument and 19 × 300 mm semipreparative C_{18} HPLC separation reported that CCS had higher sample loading (1465 vs 300 mg), higher recoveries (90% vs 75%), and lower cost. On the other hand, HPLC afforded a larger number of purified natural products (seven vs three compounds) with slightly higher purities.[133] Moreover, isolation of hypericin and pseudohypericin from *Hypericum perforatum* led to a comparison of Si gel flash LC, Amberlite XAD-16 LC, SephadexLH-20 LC, and ChMWat CCS. While CCS gave the best recovery of pseudohypericin (87%), the Sephadex procedure yielded a higher recovery of hypericin than CCS (91 vs 76%).[134] Five triterpene saponins were isolated from *Gypsophila paniculata* with an HBuMWat(0.02% TFA) SS. A comparison between the 300 mL CCS instrument and 20 × 250 mm C_{18} HPLC reported that CCS had larger sample loading (220 vs 30 mg), longer separation times, shorter preprocessing times, better reproducibility, and lower cost.[327] Another example was sulforaphane as isolated from broccoli seed meal with an HEMWat CCS procedure. Comparison of a CCS 250 mL column and a LC protocol featuring a 19 × 300 mm C_{18} HPLC separation indicated that CCS had higher sample loading (850 vs 300 mg) and higher recovery (98.5% vs 87.4%). The total separation time was longer with HPLC due to a required SPE (solid-phase extraction) sample cleanup step.[328]

Cajaflavanone was isolated from *Derris ferruginea* stems. The employed FCPC purification with a 275 mL column was compared to an LC protocol starting with a 7 × 45 cm silica gel MPLC column and followed by a 2.4 × 35 cm Sephadex LH-20 column. The resulting cajaflavanone was of nearly equal purity. However, CCS due to its higher loading capacity required 40 times less overall time and 36 times less solvent than solid-phase-based LC. In addition, the percent recovery was 10 times higher with CCS than with LC.[329] Alkannin, shikonin, and their esters were isolated from *Alkanna tinctoria* roots with HEEtWat and HAcM SSs. A comparison of natural product purities from a 305 mL CCS column and an LC procedure starting with silica gel and followed by Sephadex LH-20 column chromatography resulted in target natural products of greater purity with CCS.[330] Finally, two flavones and one novel coumarin were isolated from *Spiranthes australis* roots with an HEEtWat SS. A

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comparison between CCS and C_{18} HPLC indicated that CCS had a higher recovery (~90 vs ~70%). In addition, CCS was accomplished in one 4 h chromatographic run in a 300 mL column, whereas HPLC purification with a 10 × 200 mm column required 20 runs over the time span of 26 h.[210]

THEORETICAL AND SEMIEMPIRICAL MODELING

In addition to *K* value prediction, theoretical modeling of CCS chromatograms is of great importance in developing CCS strategies. Once an analyte *K* value is predicted or measured, the next step is to predict the behavior of the analyte during CCS chromatography under conditions determined by column volume, stationary-phase retention, flow rate, direction of flow, rotational speed, and sample loading. Theoretical modeling allows the CCS practitioner to develop a separation strategy that maximizes resolution, while at the same time minimizing separation time and solvent consumption. Modeling is especially important in the development of elution extrusion, cocurrent flow, dual-flow, and solvent gradient CCS.

Theoretical Modeling: A Numerical Model. Yang et al. used a numerical model to create a table of concentration distributions to describe the dynamic equilibrium of CCS. Proof of principle was demonstrated with a mixture of 3,4,5-trihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 2-mercaptoethanobenzimidazole, 8-quinolinol, and 2-(4-chlorophenoxy)-2-methylpropionic acid. A HEMWat SS was used in the model separation. Experimental parameters were placed in equations to produce a chromatogram that reflected the mathematical relationships of CCS.[331]

Theoretical Modeling: Continuous-Stirred Tank Reactors. Guzlek et al. described a modeling system that predicted the elution profile of a CCS experiment using instrument and operational parameters such as column length, internal diameter of tubing, β-value, number of column loops, flow rate, stationary-phase volume retention, analyte partition coefficient, and solute mass. The model considers the CCS column as a series of identical continuous-stirred tank reactors. Selected GUESSmix analytes[332] were used as one of the methods for validation. Different column volumes and instruments with different β-values were also compared. Solute retention times were predicted as well as the effects of changing flow rate and rotational speed.[333]

Theoretical Modeling: Equilibrium Cell Model. Kostanyan et al. developed a perfect replacement-based approach with an equilibrium cell model to describe the separation process in EECCC. Input values included the number of equilibrium cells, the stationary-phase volume retention, the analyte concentration, the column volume, the switch volume, the flow rate, and analyte *K* values.[334]

Theoretical Modeling: Countercurrent Distribution. Kostanyan and Voshkin modeled dual-mode CCS in terms of countercurrent distribution (CCD) interphase mass transfer and axial eddy diffusion in a closed channel. It was shown that resolution is higher with alternating mobile and stationary phases than with both phases flowing simultaneously.[5,335] Diverse modeling approaches and links to those modeling programs that are available online are posted on theliquidphase.org Web site. A CCS modeling software application that will produce a simulated chromatogram of a CCS experiment with the appropriate input variables was described by de Folter and Sutherland. The mechanics of the program are based on consideration of the CCS experiment as a CCD instrument with a discrete number of cells that correlate to the theoretical plates. Input values include column volume, flow rate, stationary-phase volume retention ratio, rotation speed, and partition coefficient(s) of the analyte(s). One feature of this software was that it may be used to predict elution-extrusion, cocurrent, and dual-flow operational modes.[336]

Theoretical Modeling: Probabilistic Model. A probabilistic model for immiscible phase separations and extractions (ProMISE) was developed that uses diffusion theory instead of compartments and theoretical plates. Input of the analyte *K* values, mobile phase flow rate, coil volume, rotational speed, stationary-phase volume retention, and analyte concentration allows the software to predict the chromatogram featuring retention times, peak shape, and peak width. An efficiency factor that represents the mixing/settling was used in place of theoretical plates to provide a more comprehensive description of instrument performance.[337]

Theoretical Modeling: Gradient Elution. Modeling gradient elution has been a challenge to CCS practitioners and theoreticians, because gradient elution is a technique with phases that are not pre-equilibrated. The pre-equilibrated column is perturbed as a new mobile-phase composition is introduced. However, three attempts have been made to model gradient elution in the well-established HEMWat and HEEtWat SS families. For HEMWat SSs, the approach used an empirically based modeling method that was demonstrated with the estimation of *K* values in two model mixtures containing diverse pharmaceuticals. Linear gradients of HEMWat composition were used. First of all, the correlation between stationary-phase volume retention and the elution volume of the analytes was established as the stationary phase is lost continually during a linear gradient run. An adjusted stationary-phase volume was used to calculate the partition coefficient with classical CCS equations. The gradient *K* value was then correlated to a specific HEMWat formulation using empirically generated plots.[156] A gradient elution model was proposed by Wu et al. for the isolation of five tanshinones from *Salvia miltiorrhiza* rhizome. First, an HEEtWat family of nine SSs was created with the formula 8:2:*x*:10−*x* where *x* was an integer between 1 and 9. Then, the compositions of the phases were determined, *K* values of all five tanshinones were assessed by partitioning in these SSs, and a series of isocratic elution EECCC experiments were performed. With these data, gradient elution *K* values were predicted with a series of equations. Finally, a linear gradient of HEEtWat 8:2:5:5 to 8:2:7:3 was used to isolate the tanshinones.[25] Wu et al. proposed two different methods of modeling gradient elution CCS. In order to predict retention times, the partition coefficient of the analyte in both the initial and final SS must be known. The gradient may be linear where the composition of the mobile phase is varied in a constant manner or a simple stepwise gradient. Allowances were made for isocratic elution both before and after the gradient was performed. Volume factors were based on the experimental flow rate, time, column volume, and stationary-phase volume retention ratio. The methods were tested with two tanshinones in a HEMWat SS.[338]

Model Mixtures and the GUESS Method. Model mixtures of analytes can be employed to develop CCS instrumentation, assess SS polarity characteristics, evaluate SS selectivity, and develop elution modes. In some cases, extracts with well-known characteristics are utilized as model mixtures, but typically model mixtures are composed of commercially available analytes. The GUESSmix model mixture, composed of 20 commercially available natural products with a wide polarity

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range, was introduced in 2005.[332] It was initially used to develop a TLC-based method for SS selection. However, the utility of the GUESSmix expanded to evaluate SS polarity and selectivity characteristics, as well as to develop separation methods such as EECCC.[26,198] Since 2007, the GUESSmix, either in entirety or in part, has continued to be used for various purposes, as reflected by the nine entries in Table 6.

Table 6. Use of GUESSmix Components for the Development of Countercurrent Separation Instruments, Theory, and Methodology since 2007

no. of GUESSmix components	solvent system(s)	application	ref
4	HEMWat 4:5:4:5	demonstration of the scale-up of ICcE methodology	28
3	HEMWat 2:3:2:3	performance test of a nonsynchronous CCS instrument	53
8	HEMWat 2:3:2:3	comparison of the separation performance between different CCS instruments	79
4	HEMWat 2:3:2:3	evaluation of the performance of an 18 L process-scale hydrodynamic CCS instrument	218
4	HEMWat 4:5:4:5	assistance in a feasibility study of ICcE method development	221
7	HEMWat 2:3:2:3	validation of a modeling system	333
12	HepEMWat	development of a method for rapid SS screening	339
12	HepEMWat 5:5:5:5	comparison of the performance of four different small-volume CCS instruments	86
4	HEMWat family	evaluation of a set of mathematical models for describing ICcE separations	340
10	HEMWat family	development of a computer-assisted SS selection protocol	341

The features of a useful CCS model system are (1) easily obtainable, high-purity, stable analytes; (2) well-known K values of the analytes in a stable SS such as HEMWat 5:5:5:5 or 4:6:4:6; (3) UV-active analytes that may be readily detected by UV−vis detectors; and (4) at least one analyte pair eluting close together so that resolution may be studied and compared. Importantly, the usage of consistent analytes and corresponding SSs allows meta-analyses based on previous literature to be performed and enables meaningful comparisons between instruments and/or methods. Table 6 summarizes the use of the GUESSmix components for the development of CCS instruments, theory, and methodology in publications since 2007.[28,53,79,86,218,221,333,339−341]

Separation Methods. In the classical CCS elution method, a biphasic SS is formulated and equilibrated before the two phases are separated. The column is then filled with stationary phase and equilibrated subsequently with rotation, and the mobile phase is introduced. After sample introduction, elution is continued until all the analytes of interest have been eluted. The main downside of classical elution is that highly retained analytes will take a long time to elute and require a large volume of solvent. The gradient elution method described previously is one procedure that has been devised to accelerate the elution of highly retained analytes. Another setback with classical elution is that poorly retained analytes are not well resolved. Therefore, several methods have been created that address the problems associated with poorly and/or highly retained analytes. Table 7 summarizes the methods described in more detail in the following categories. The various methods

Table 7. Summary of Countercurrent Separation Elution Methods: Elution-Extrusion Countercurrent Chromatography (EECCC), Back-Extrusion Countercurrent Chromatography (BECCC), and Intermittent Countercurrent Extraction (ICcE)

elution method	phase change[a]	flow direction change	rotation direction change	comments
classical				highly retained analytes remain in stationary phase
EECCC	single			analytes elute in order of K values
BECCC		single		elution order reverses; some analytes may elute at separate volumes
back-step CCC	twice			a plug of aqueous phase introduced to elute highly retained analytes
dual-mode	single	single		elution order reverses
dual-rotation	single		single	elution order reverses
multiple dual-mode	multiple	multiple		elution order reverses each cycle
ICcE	multiple	multiple		sample loop is in the middle of a single column or between two separate columns

[a]Phase change refers to switching mobile and stationary phase.

such as EECCC, BECCC, back-step, and dual-mode CCC illustrate the unique capabilities of CCS that result from the liquid nature of its two chromatographic phases: because they are both liquids, even the "stationary" phase can be moved as part of the chromatographic protocol. This allows manipulations and targeting that is impossible with solid-phase-based LC.

Elution-Extrusion CCC. Elution-extrusion countercurrent chromatography (equally suitable for both CCC and CPC instruments) was developed as a practical method of accelerating the elution of highly retained analytes in 2003, while the full theory was described later in 2007.[26,27] EECCC is a variation of the column pump-out method proposed by Conway.[8] In effect, EECCC is a method whereby all the analytes are first eluted and then extruded from the column, with the total volume determined by the operator. The analyte K values may serve to predict analyte elution/extrusion volumes, or they may be calculated as a function of their retention volumes. Table 8 provides an overview of the use and versatility of EECCC in the separation of natural products since 2007.[32,140,147,172,224,276,289,295,339,342−345] An example of theory and practice working together was the sequential "overlapping" EECCC separations proposed by Wu et al.[147] This study exploits the fact that EECCC is amenable to repeated separation runs whereby the column is recharged with the original stationary phase at the end of each run. Model equations were employed to predict the most efficient method of separating three andrographolides from *Andrographis paniculata* aerial parts using multiple EECCC runs with the same HEMWat SS. It was shown that a new sample could be injected before the previous sample had completed the EEECC extrusion step. Thus, multiple EECCC separation runs can be a viable alternative to scaling up a separation to a larger volume column.

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Table 8. Use of Elution-Extrusion Countercurrent Chromatography in the Separation of Natural Products since 2007

natural products (no. of cpds)	source	solvent system	ref
ginsenosides (4)	*Panax ginseng* roots	HBuWat (0.1% formic acid)	140
andrographolides (3)	*Andrographis paniculata* aerial parts	HEMWat	147
tetrahydroiso-alpha bitter acids (3)	*Humulus lupulus* inflorescences	HEMWat with a buffered aqueous phase	172
flavonoids (5)	*Flaveria bidentis* aerial parts	EMWat	224
picrosides (3) and acetovanillones (2)	*Picrorhiza scrophulariiflora* fruits	HEMWat EBuWat (0.1% formic acid) EWat	276
phenylbutenoids (6)	*Zingiber cassumunar* rhizomes	HEMWat & HAcWat	289
cimicifugic acids	*Cimicifuga racemosa* roots	EBuWat (NH_4OH/TFA) & HEMWat	295
antioxidant natural products	*Piper longum* fruit	HepEMWat	339
antioxidant natural products	*Polygonum cuspidatum* root	HepEMWat	339
phenolic natural products (5)	*Dendrobium chrysotoxum* stems	HEMWat(2-step)	342
diterpene (1) and alkaloids (3)	*Tripterygium wilfordii* aerial parts	HEMWat	343
antioxidant phenolics	*Rheum rabarbarum* (rhubarb) roots and stems	HEMWat	344
parnafungins (4)	*Fusarium larvarum* fungus	HEMWat	345
quinolones (5) and other alkaloids (3)	*Evodia rutaecarpa* fruits	HEMWat	32

A particular application of EECCC is the two-column (2VC) mode, in which the mobile phase is switched when the elution volume is equal to the column volume ($V_{CM} = V_C$).[26] The 2VC technique was applied to the isolation of six phenylbutenoids from *Zingiber cassumunar* rhizomes.[289] The 2VC EECCC method was also employed for a series of SS selection experiments in order to screen plant extracts with three strategic HepEMWat SS formulations. The resulting protocol was used for the fractionation of antioxidant natural products from *Piper longum* fruit and *Polygonum cuspidatum* root extracts.[339] Li et al. compared EECCC with the classical elution mode to report that EECCC required less time and solvent consumption for the isolation of five phenolic natural products from *Dendrobium chrysotoxum* stems.[342] However, Ye et al. reported that simply eluting the column at a high flow rate gave better resolution and was 8 times faster than EECCC or dual-mode elution for the separation of one diterpene and three alkaloids from *Tripterygium wilfordii* extract.[343] The success of the pump-out method may depend on the instrument design and the need to calculate *K* values of highly retained analytes.

The EECCC method is of particular importance in bioassay-guided fractionation studies, because this method ensures the complete recovery of all metabolites introduced into the CCS column. A rapid biological screening method for discovering antioxidant natural products was developed using EECCC methodology.[344] Four parnafungins from the fungus *Fusarium larvarum* exhibited antifungal activity through inhibition of mRNA polyadenylation. Bioassay-guided fractionation by EECCC in a HEMWat SS was followed by C_{18} HPLC.[345]

Back-Extrusion CCS. EECCC is a continuous technique in the sense that analytes elute in the order of their *K* values. However, in back-extrusion CCC (BECCC), the column direction (head-to-tail or tail-to-head) is switched at a predetermined point to initiate the extrusion of the stationary phase that reverses the order of elution. Column direction switching in the BECCC technique creates a mixing of phases and could result in the same analyte eluting at two different volumes ("echo" peaks).[31] Lu et al. used both EECCC and BECCC in the fractionation of an *Evodia rutaecarpa* extract with a HEMWat SS. EECCC was deemed suitable for high-throughput separation as it is a continuous technique that recharges the column with stationary phase. On the other hand, BECCC may be useful for the rapid elution of highly retained natural products once the appropriate analytes of interest have been collected.[32]

Back-Step CCS. Three hydroxylstilbenes were isolated from *Vitis riparia-berlandieri* SO4 (Selektion Oppenheim 4) roots with a HepEMWat CCS-ESIMS hyphenation procedure that included a novel "back-step" technique. The SS phases were prepared individually according to composition values documented in the literature. Simulation software was employed to assist with SS selection based on partitioning study *K* values. After a half column volume of aqueous mobile phase was eluted from a pre-equilibrated column, a half column volume of water that had been previously equilibrated with the organic phase was pumped in as the mobile phase, without change in the flow direction, to elute water-soluble natural products. The water "back-step" was followed by reintroducing the original mobile phase to complete the separation. This technique removed unretained compounds that would have coeluted with the first fractions of interest and reduced the risk of in-column precipitation.[33]

Dual-Mode CCS. The dual-mode CCS method occurs when the phase being pumped into the column is changed with a simultaneous change in the direction of flow. For example, a column is equilibrated and eluted with the lower phase mobile in a head-to-tail direction and then, at a certain point, the upper (stationary) phase is pumped into the column and the direction changed concurrently to tail-to-head. Similar to the back-extrusion method, the switch reverses the order of elution so that the most highly retained solutes from the first mode become the ones that are eluted first in the second mode. This dual-mode elution method effectively lessens the solvent volume necessary to elute all the analytes introduced. However, in contrast to EECCC, the time it takes for the last analyte to elute is dependent on its *K* value in the second mode of the procedure. The retained analyte with the lowest *K* value at the time of the mode switch will be the last to elute in the second mode. Moreover, it is important to time the dual-mode switch so that an analyte of interest is not partially eluted in both modes. Table S9, Supporting Information, summarizes the use of dual-mode elution in the CCS literature concerning natural products since 2007.[128,230,279,346−349]

Dual-Rotation CCS. In the "dual-rotation" method, the phase being pumped into the column is changed while concurrently reversing the direction of rotation in hydrodynamic CCS instruments. In this case, analytes continue to elute in the order of the *K* values relative to the initial column conditions. The dual-rotation method was demonstrated with the separation of five synthetic dihydroxybenzoic acid isomers using a HEMWat SS.[350] Atropine and scopolamine were isolated from *Datura metelis* with conventional, dual-mode, and

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dual-rotation methods using pH-zone-refining CCS. While the authors report three different pH-zone-refining experiments, the counter-rotation dual-mode elution method was reported to give the best results.[244]

Multiple Dual-Mode CCS, Successive CCS, and Intermittent Countercurrent Extraction. Multiple dual-mode (MDM) CCS involves two or more dual-mode switches over the course of the countercurrent separation (see explanation in Figure 7). Following the first dual-mode switch

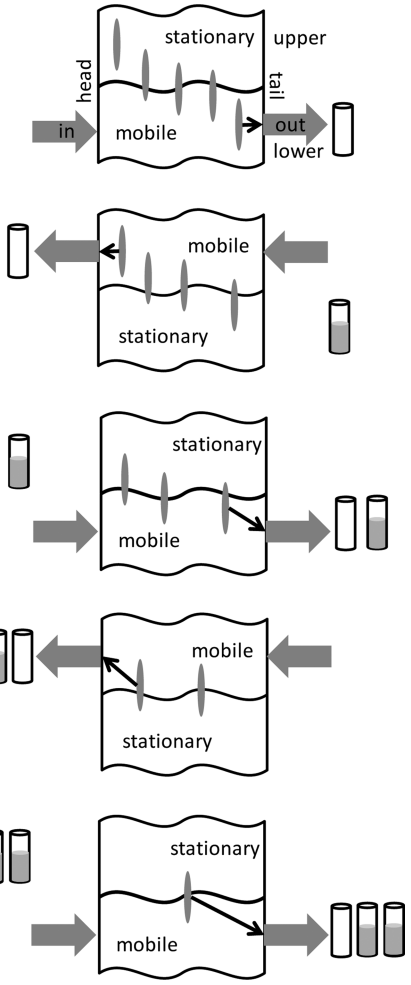

Figure 7. Scheme explaining the process of multi-dual-mode operation in CCS.

(change of both mobile phase and flow direction), a subsequent dual-mode switch is made, which has the effect of lengthening the column volume. Increasing the length of the flow path for a selected group of analytes increases their resolution along with their peak volumes. This is similar to what is occurring in the recycling CCS method, only in MDM CCS the target analytes do not leave the column until the end of the run. The MDM method was proposed by Delannay et al. and demonstrated with a test mixture of acenaphthylene and naphthalene in a HepAc SS with seven cycles. A mixture of two synthetic diastereomeric pairs was also separated in the same SS with seven cycles.[351] Table 9 summarizes the use of multiple dual-mode and intermittent countercurrent extraction in the separation of both mixtures of synthetic compounds and natural products since 2007.[29,45,221,223,340,341,351−357]

Two target natural products were isolated from *Lichina pygmaea*, a cyanobacterial lichen, with BuAaWat SS by means of an MDM method. First, the equilibrated column was eluted with the upper phase mobile, and nonpolar natural products were collected. Second, with the lower phase mobile, the most polar natural products were collected. Then, with the upper phase mobile, the intermediate polarity target compounds were eluted, followed by a fourth cycle with the lower phase mobile to collect the remaining natural products.[45]

Intermittent countercurrent extraction is similar to MDM CCS. MDM may be used on any CCS instrument; however, ICcE may be used only on hydrodynamic CCS instruments where the tubing comes out of the instrument to allow sample injection between the bobbins. In ICcE the sample is introduced in the middle of a CCS column or between two physically separate CCS columns (instruments with dual capability are currently available only as prototypes). The arrangement allows the operator greater latitude to "wash out" analytes of higher and lower polarity than the target analytes before their elution from the column. Hewitson et al. investigated this method with both a test mixture and a plant extract. Initially, the authors performed a feasibility study with four selected analytes from the GUESSmix.[332] The separation of caffeine, vanillin, naringenin, and carvone by ICcE was optimized in a HEMWat 4:5:4:5 SS. Carvone ($K_{(U/L)}$ = 7.4) and naringenin (K = 1.25) were eluted by the upper phase (normal-phase mode), while vanillin (K = 0.55) and caffeine (K = 0.09) were eluted in the lower phase (reversed-phase mode). The practical application of ICcE was demonstrated by the enrichment of triptolide from *Tripterygium wilfordii*. HEMWat 2:3:2:3 was used to separate triptolide (K = 1.07) from both higher and lower K value components by retaining it in the column, while the other natural products were "washed out" of the column by intermittently switching the flow direction. As a result, 118 mg of triptolide was separated from 9.2 g of crude extract. The features of this ICcE separation were high loading capacity, low solvent consumption (10 L), and shortened separation time (3 h).[221] A practical application of ICcE methodology was described by Goll et al. for the isolation of capsaicin and dihydrocapsaicin from a commercial mixture. The K values provided by the COSMO-RS predictions were used to design an ICcE method with a continuous feed. Six operating parameters were adjusted to elute capsaicin with the aqueous phase mobile of a HepEMWat SS and dihydrocapsaicin with the organic phase mobile.[357] Development of modeling calculations is useful because both the number of cycles and length of intervals between the cycles are important MDM and ICcE variables that are not encountered in other CCS methods.

Dual-Flow CCS or Continuous Countercurrent Extraction. In dual-flow CCS, both phases in the column are flowing in opposite directions. Typically the column is filled with one phase before equilibrating the column by pumping both phases simultaneously. Interestingly, the 50% retention of both phases cannot be achieved by pumping both phases at equal flow rates. However, once a flow rate combination that gives 50% retention is found, this value is quite reproducible and robust. In fact, the observation that the phase retention is stable in dual-flow CCS is a significant advantage of this method over classical CCS, where one phase is held stationary in the column.[358]

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Table 9. Use of Multiple Dual-Mode and Intermittent Countercurrent Extraction in the Separation of Mixtures since 2007

objective	mixture	solvent system	ref
feasibility study	four-component GUESSmix	HEMWat	221
enrichment of triptolide	*Tripterygium wilfordii* extract	HEMWat	221
isolation of honokiol and magnolol	commercial botanical mixture	HEMWat	223
mathematical models for ICcE separations	four-component GUESSmix	HEMWat	340
feasibility study	acenaphthylene and naphthalene	HepAc	351
feasibility study	two synthetic diastereomer pairs	HepAc	351
isolation of two natural products	*Lichina pygmaea* extract	BuAaWat	45
testing a mathematical model of computer-programmed ICcE	DNP-amino acid samples	HEMWat (0.1 M HCl)	352
investigation of sample loading capacity and throughput	four-component GUESSmix	HEMWat	353
development of theoretical framework to predict MDM	two DNP amino acids	HepEMWat (0.1% HCl)	29
study the effect of key ICcE operating parameters on selectivity and throughput	eight-component modified GUESSmix	HEMWat	354
optimizing parameters and instrument configurations for a continuous feed ICcE separation	eight-component modified GUESSmix	HEMWat	355
mathematical modeling	pyrocatechol and hydroquinone	HepMWat	356, 428
capsaicin and dihydrocapsaicin	commercial botanical mixture	HepEMWat	357

BIOACTIVITY AND NONPLANT APPLICATIONS

CCS is an especially powerful method for the isolation of bioactive natural products by means of bioactivity-guided fractionation and related procedures. The loss-free separation characteristic of CCS ensure that potentially bioactive natural products are not irreversibly adsorbed by the chromatographic medium. The ability of CCS to accommodate crude biological extracts is another advantage of CCS in this context. The use of preparative-scale CCS is also important, as bioactive natural products may be present as minor metabolites in an organism's metabolome. Table S10, Supporting Information, summarizes the use of CCS to prepare fractions for bioassay screening of natural products in articles published since 2007.[20,35,41,44,74,94,102,105,116,122,128,135−137,139,229,230,279,284,285,295,298,307,308,324−326,344,345,359−402]

CCS Advantages in Bioactivity-Guided Fractionation. An example of an extensive bioactivity-guided fractionation scheme that features the advantages of CCS chromatography was presented by Inui et al. The authors described the bioassay-guided fractionation leading to the isolation and identification of five anti-TB-active natural products from *Oplopanax horridus* inner stem bark. Initially, the authors used two polarity-adjusted HEMWat biphasic SSs for separatory funnel separations. After the anti-TB activity of each fraction was assessed, the active fraction was fractionated by four polarity-adjusted HEMWat SS runs. The 13 fractions from the final CCS run along with the fractions from the first (7), second (8), and third (8) runs were tested for anti-TB activity. The three most active fractions from the first gradient array separation were combined and chromatographed with a C_{18} MPLC column. One fraction yielded 3,10-epoxy-11-hydroxynerolid-6-ene. A second fraction was chromatographed with Sephadex LH-20, and 6,11-dihydroxy-7,10-epoxynerolidol was recovered. Three highly active fractions from the third gradient battery run were combined and rechromatographed with an HDiMWat SS. One fraction yielded oplopandiol. Another fraction was chromatographed on an MPLC CN column, and falcarindiol was recovered. A third fraction was rechromatographed on a C_{18} HPLC column, and sesamin was recovered. Of the five purified natural products, falcarindiol showed the highest anti-TB activity.[139]

CCS-Based Alternatives to Bioassay-Guided Fractionation. A follow-up article by Inui et al. offered a CCS-driven alternative to the traditional bioassay-guided fractionation process where a crude sample containing hundreds of natural products was repeatedly fractionated to produce a small number of purified natural products with potential biological activity. In this case, an *Oplopanax horridus* inner stem bark extract was fractionated with CCS, and 64 fractions were obtained. These fractions were tested with a high-throughput MABA anti-TB assay, which produced a biochromatogram that revealed 19 distinct biopeaks with significant activity. The individual components of each fraction were mapped with high-resolution CG-MS. The resulting chromatographic and biological data were mined via three-dimensional biochemometric analysis to identify highly active natural products before further fractionation was necessary. The ability of CCS to generate a reproducible, wide-polarity, loss-free chromatographic separation with good resolution was crucial to the effectiveness of this method of obtaining biological activity information from a complex matrix.[35] The feasibility of this biochemometric approach was confirmed recently in a study of the anti-TB-active volatile constituents of *Eucalyptus citriodora*.[34]

CCS-Based Assessment of Purity and Bioactivity. Another role that CCS may play in the investigation of bioactivity is the establishment of correlations between compound purity and bioactivity, i.e., a purity−activity relationship, as described by Jaki et al. In this case, the purity−activity relationship of ursolic acid toward its $H_{37}Rv$ anti-TB activity and Vero cytotoxicity was investigated. Nine commercial samples of ursolic acid were assessed for purity with qHNMR spectroscopy and for bioactivity so that the relationship between ursolic acid purity and bioactivity could be correlated. For example, one commercial ursolic acid sample with an initial purity of 69.7% was purified by seven separate CCS runs. Representing an unexpected result, it was determined that the anti-TB activity of ursolic acid-containing fractions had an *inverse* correlation to purity, while the Vero cytotoxicity remained the same.[359] Hence, it was concluded that ursolic acid per se does not have the previously ascribed anti-TB activity and was not a viable lead compound for this type of biological activity.

CCS-Based Assessment of "Residual Complexity". CCS has been instrumental in revealing the "residual complexity" of natural product preparations, in which this factor influences biological activity. "Residual complexity" may

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be attributed to the presence of small amounts of (mixtures of) chemicals present in even purified natural product preparations.[253] For example, benzoic acid, a major constituent of a bioactive fraction, was subtracted chemically from cranberry extract with ChMWat CCS to test its antiadhesion bioactivity against uropathogenic *Escherichia coli*. Upon benzoic acid removal, the antiadherent activity of the fraction was fully retained, demonstrating the presence of the active principles in a relatively minor ("residual") but still relatively complex portion of the "residually complex" active fraction. This study highlighted the likely fallacy inherent in ascribing the bioactivity to the major natural product in that extract. Moreover, upon examination, the residual presence of scopoletin was detected in the subtracted benzoic acid, establishing two aspects of "residual complexity" resolved by CCS.[135]

Applying the same concept, Powell et al. used both pH-zone-refining with EBuWat (ammonium hydroxide/TFA) and EECCC with a HEMWat SS as part of a bioassay-guided fractionation scheme that identified the presence of a very minor (40 ppm) constituent of black cohosh, *Nω*-methylserotonin, as the active principle that was responsible for the in vitro serotonergic activity of *Cimicifuga racemosa* extracts. The study also showed that although actein triterpenes and cimicifugic acid phenols are suitable markers for chemical standardization, they do not correlate with the serotonergic activity.[361,403]

Another form of residual complexity is the transformation of natural products into other chemical entities in situ. For example, Chen et al. employed CCS to obtain high-purity desmethylxanthohumol (DMX) for bioactivity studies involving the purported estrogenic activity of DMX. It was demonstrated by qNMR that DMX rearranges to 6- and 8-prenylnaringenin under assay conditions and compromises the assay results.[360] Interestingly, CCS was capable of resolving these analytes.[404]

The bioassay-guided fractionation of the volatile oil of *Angelica sinensis* roots was performed with an endothelial cell proliferation inhibition assay. The bioactivity of both upper and lower phases was measured to determine the SS that gave the most equal distribution of activities. CCS with a HepEMWat SS was collected into 45 fractions. The nine fractions that exhibited endothelial cell proliferation inhibition all contained *Z*-ligustilide and/or *n*-butylidenephthalide. The resulting biochromatogram featured fraction numbers on the *x*-axis and the inhibitory potency on the *y*-axis and showed three peaks associated with bioactivity. As the crude volatile oil preparation exhibited higher activity than either *Z*-ligustilide or *n*-butylidenephthalide at the same concentrations, the authors proposed the presence of "synergistic factors" in the crude volatile oil preparation.[136] Thus, this study marks another instance of "residual complexity" unraveled by means of CCS.

A final example was eriocalyxin B, which was isolated from *Isodon eriocalyx* by a bioassay-guided fractionation scheme. CCS fractions of the crude medicinal plant extract were separated with HEMWat 3:5:3:5 on a 260 mL hydrodynamic column and analyzed by a caspase-3 activation assay. The active fraction turned out to be the "column wash" fraction made up of lipophilic natural products. This was surprising, as this plant is normally prepared as a tea for its medicinal qualities. The lipophilic fraction was rechromatographed on a 16 mL CCS column with HEMWat 1:1:1:1. The active fractions were now found in the "sweet spot" ($K \approx 0.7$) of optimal resolution. A third CCS purification with HEMWat 21:20:21:20 gave eriocalyxin B ($K \approx 1.6$), which induced apoptosis in cancer cells. Low-volume CCS was used to its full advantage to isolate a small amount (~5 mg) from a 500 mg original sample.[284]

Applications to Nonplant Organisms. The growth of CCS as a mainstream separation method for bioactive natural products may be seen in the wide diversity of organisms that have been studied. Different classes of living organisms present different challenges primarily due to their difference in the areas of matrix materials and groupings of metabolites. The recent literature contains numerous examples for CCS applications of preparations from animals, bacteria, and fungi.

Animals. While much of traditional medicine is plant-based, there are some important animal preparations, such as the TCM "ChanSu", which is made from the dried white secretion of the auricular and skin glands of toads. Six structurally related bufadienolides were isolated from "ChanSu" with a polarity-adjusted HEMWat step gradient.[37] Table S11, Supporting Information, summarizes CCS applied to the isolation and/or characterization of natural products from nonplant organisms.[37−42,44−50,155,300,308,345,405−410]

Bacteria. Bacterial natural products are of extreme importance for pharmaceutical applications, and, therefore, they have been a common focus of CCS studies. For example, four congeneric gentamicins were isolated from *Micromonospora purpurea* with a BuWat (10% NH_4OH) SS. The presence of base in the SS maintained the strongly basic aminoglycosides in the un-ionized form and increased the *K* value by favoring the upper organic stationary phase. The subsequent antibacterial assays revealed that all isolated natural products inhibited bacterial growth with about the same potency.[41]

Fungi. Fungal metabolites are of importance in both traditional medicine and drug development. For example, inotodiol and trametenolic acid were isolated from *Inonotus obliquus* by HEMWat hydrodynamic CCS with evaporative light scattering detection. Triterpenoids present a particular challenge because they are difficult to detect with UV spectroscopy. The authors tested 10 HEMWat formulations before selecting one giving *K* values of 0.60 and 0.91 for inotodiol and trametenolic acid, respectively. The CCS separation was performed by injecting the crude extract of the fungus without an intervening column chromatography step. The authors reported recoveries of 85.5% and 85.7%.[410]

■ CONCLUSIONS AND OUTLOOK

One Natural Product with One Step. When planned and optimized properly, the selectivity and separation potential of CCS may yield impressive results. This can be exemplified by the relatively large number of recent articles reporting on the purification of one natural product in a single CCS step (Table S12, Supporting Information).[50,115,259,262,267,271,291,324,329,398,409,411−426] While rigorous purity evaluations remain the exception in these studies, they still demonstrate the growing role of CCS in natural product research laboratories and how researchers have explored successfully the potential of CSS as a high-resolution preparative separation method.

Fully Integrated CCS for the Natural Products Separation Workflow. This review has provided a perspective as to why CCS has the capability to be integrated into the natural products separation workflow as a high-capacity, high-resolution chromatography technique to contribute its unique advantages to the integrity of natural product isolation procedures. CCS still retains the possibility of creating

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a one-step targeted isolation of an analyte of interest. However, it is more likely to be employed as part of a multistep fractionation procedure to result in the isolation and characterization of several potentially bioactive and novel analytes. The four authors of this review article have practiced and taught for a collective total of over 100 years and found it to be accessible to the novice user once a few basic principles have been learned. Introductory theoretical and practical guidance may be found in our previous review,[9] in Ito's "Golden Rules" review,[16] and in the most recent report on the Cherry One instrumentation.[19] Combined with this review, all three sources provide introductory, advanced, and state-of-the-art information on how to get started, optimize existing protocols, and explore new applications, respectively. Their ~1000 references serve as entry points into all aspects of basic and applied CCS.

The Dynamic Nature of CCS Technology. From the point of view of instrumentation, SS selection, and method development, CCS is a dynamic and highly innovative field. CCS technology continues to adapt to advancements in related fields of chromatography in terms of automation and hyphenation. In addition, there are advancements that innovate beyond what comparable technologies have done and/or are capable of, such as development of the phase metering apparatus and intermittent countercurrent extraction. Much CCS innovation is directed at capitalizing on the inherent advantages of CCS technology, such as the direct injection of extracts, ion-exchange methods, and the unique potential of "manipulating" both chromatographic phases as being liquids.

Areas of Future Growth. This review presents an update of current CCS theory and practice in a format that attempts to make connections between the various aspects of CCS technology. One enduring challenge of CCS technology is to "tame" the many options that are available to CCS operators into a rational approach that may be learned and practiced by a growing number of users.[427] The advances in automation and the theoretical prediction of SSs are encouraging in this regard. Standardization of SS finding and evaluation procedures can be seen as another important means of enhancing the usability of CCS. On the other hand, it is advantageous to have many choices available to increase the effectiveness of the natural products separation toolbox, in order to address the analytical challenges posed by the ubiquitous metabolomic samples in natural products research.

■ ASSOCIATED CONTENT

Supporting Information

Additional content and tables that summarize the literature on the following topics, respectively: (S1) Working Definitions and Basic Nomenclature: Additional Information; (S2) Translation of Common Solvent Compositions into Percentages; (S3) Halogenated Solvents in CCS Applications; (S4) Sample Loading in CCS of Natural Products; (S5) Modern Extraction Methods for CCS Sample Preparation; (S6) Polarity-Adjusted Successive CS; (S7) Orthogonal Successive CS; (S8) Step-Gradient Elution in CS; (S9) Dual-Mode Elution in CCS of Natural Products; (S10) Bioassay-Oriented Natural Product Research Involving CS; (S11) Natural Products Purified from Nonplant Organisms by CCS; (S12) Reports on the Successful Isolation of a Single Natural Product Using One-Step Countercurrent Separation; (S13) References of the SI. Further Supporting Information is distributed via the corresponding author's dedicated CCS Web pages at http://go.uic.edu/countercurrent. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/np501065h.

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Notes

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