Reviews

Countercurrent Separation of Natural Products[#]

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Received March 7, 2008

An assessment of the technology and method development in countercurrent chromatography (CCC) and centrifugal partition chromatography (CPC), collectively referred to as countercurrent separation (CS), is provided. More than six decades of CS theory and applications are critically reviewed and developed into a practical guide to CS for natural products research. The necessary theoretical foundation is given for better use of CS in the separation of biological molecules of any size, small to large, and from any matrix, simple to complex. The three operational fundamentals of CS—instrumentation, biphasic solvent systems, and theory—are covered in a prismatic fashion. The goal of this review is to provide the necessary background and references for an up-to-date perspective of CS and to point out its potential for the natural products scientist for applications in natural products chemistry, metabolome, and proteome research involving organisms from terrestrial and marine sources.

Introduction

Since Lyman Craig's invention of countercurrent distribution (CCD) in the 1940s,[1–3] liquid−liquid separation techniques have come a long way from the room-filling, time-demanding, and high-maintenance equipment to modern benchtop instruments that allow separation in hours or even minutes. In contrast to the more well-known liquid chromatography (LC) methods, namely, those based on solid adsorbents, countercurrent separation (CS) techniques offer high sample capacity and the ability to accommodate crude, unprepared samples. As CS involves the use of only liquids, it became known as an elegant method: Compounds are separated based solely on their differential solubility. There is essentially no loss of sample due to irreversible absorption, no restriction to flow related to solids and/or adsorbent porosity, and no interfering surface chemistry that can potentially alter analytes. All these are major strengths of CS, making it a very attractive technique for natural products research.

Generations of natural products scientists have consistently experienced that there is a particular value to applying a combination of methods to the separation of samples derived from organisms, rather than resorting to only one particular technique. This insight was already shared by Butenandt, a Nobel laureate and discoverer of pheromones and human steroid hormones who utilized CCD.[4,5] In his foreword to Hecker's 1955 monograph on liquid−liquid separation methods, he states "*The researcher well versed in chemical analysis will manage to select and apply the most appropriate separation method at each toehold of the analytical workflow*" [translation].[6] Projected on today's research environment, which is characterized by technologically advanced and computerized equipment, this raises the obvious question of the role of liquid−liquid-based separation methods in the contemporary portfolio of the natural products analyst.

In the 1970s, partition-based GC and adsorption-based HPLC began competing directly with CS techniques. Their superior resolving power sparked a rapid development of GC and HPLC instruments, stationary phases, and turnkey systems. Although continuously developed and driven by the achievements of Yoichiro Ito since the 1970s,[7–10] CS methodology became known as a niche method for specialists, among them numerous natural products chemists who have successfully used the "Ito multilayer separator extractor" centrifuge. Despite having contributed largely to key natural products discoveries (e.g., estrogens[11] and androsterones[12] from urine, corticosteroids from the adrenal cortex,[13,14] tRNA from yeast,[15,16] penicillin[17] and macrolide[18,19] antibiotics, antitumor alkaloids[20–22] as well as camptothecin and taxol,[23] and plant auxins[24]), CS eventually earned the reputation of being difficult to manage in terms of both practical application and theoretical understanding.

Factors that might have contributed to this perception were (a) the relative immaturity of commercially available instrumentation (I) of earlier times; (b) the empirical nature of the formulation and selection of the two-phase solvent systems (S); and (c) the apparent complexity of CS theory (T), involving aspects of engineering, fluid dynamics, and analytical chemistry, among others. Even from today's perspective, the three factors I−S−T, especially when taken together, have considerable potential to present a challenge to the practical implementation of CS, in particular for novice users. At the same time, the CS knowledge base, theory, and technology have advanced to such an extent that successful separations can be achieved on a routine basis, avoiding many of the previous obstacles. It is the goal of this review to provide the necessary background and references for an up-to-date understanding of CS and to point out its potential for the natural products analyst.

Background

The CS Prism and Organization of This Review. Upon closer inspection, the aforementioned three areas of major chal-

[#] Dedicated to the memory of Dr. Edward Chou, Pharma-Tech Research Corporation, Baltimore (MD), a visionary in applied countercurrent separation technology.

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10.1021/np800144q CCC: $40.75
Published on Web 07/31/2008

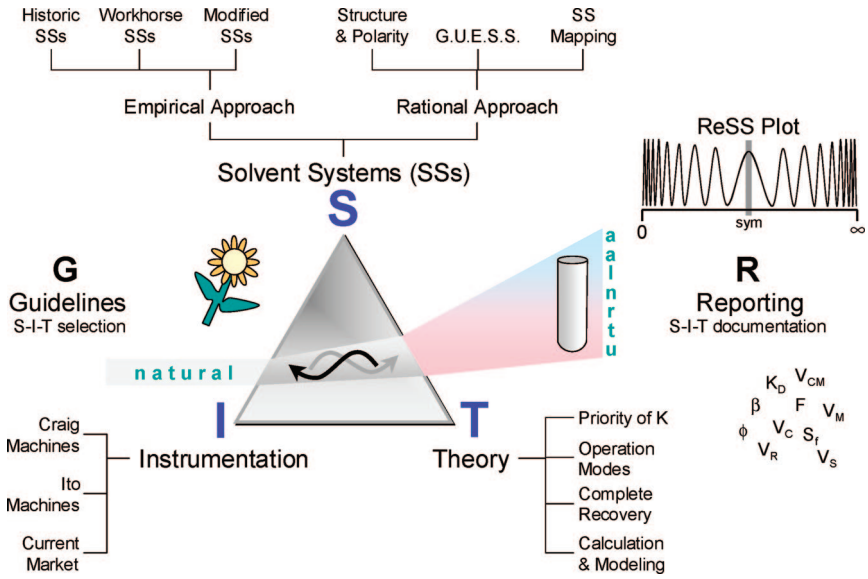

Figure 1. The organization of this review reflects the three main building blocks of CS: Instrumentation (I) enables the immobilization of a liquid stationary phase. Two-phase solvent systems (S) serve as chromatographic phases. Its theory (T) makes CS a unique method, as separations can be fully predicted from instrument (volumes, flow) and analyte parameters (*K* values). The triangular I−S−T model of CS works analogous to a prismatic separation device, capable of resolving complex mixtures into defined chemical entities. The review also provides practical guidelines (G) for optimizing CS performance and for the reporting (R) of CS experiments.

lenges are identical with the three columns on which the entire CS concept rests. Instrumentation (I), solvent systems (S; SSs in the text), and theory (T) span the CS prism space (I−S−T), as shown in Figure 1. The IST prism not only symbolizes how CS is founded, but also reveals the essential knowledge for the analyst to put CS to work for the purpose of natural products analysis. Accordingly, the CS prism has been adopted as the blueprint for the present review of the CS literature. Two sections overarch this structure and assist with the practical implementation of CS: one provides guidelines (G) for establishing experimental protocols; a second deals with aspects of reporting (R), including the construction of CS chromatograms and the efficient documentation of experimental conditions.

Literature Statistics and Technology Development. The CS literature spans nearly seven decades and currently amounts to approximately 3000 journal articles. As can be seen from a graphical representation of the type of instrumentation used in the studies (Figure S1, Supporting Information), the history of CS technology is divided into two main eras: the era of countercurrent distribution (CCD; 1948 to ∼1978, almost 1000 publications), characterized by the key inventions[1−3,25−27] of Lyman C. Craig,[28] and the era of mostly centrifugal force-enhanced countercurrent instrumentation (since ∼1970),[7,9,10,29−32] associated with the name of Yoichiro Ito.[33,34]

In order to explore the influence of natural products research on the historic development of CS, the entire CS literature, as stored in ACS's Chemical Abstracts Service database, was mined by means of Boolean queries involving wildcard search terms such as *plant**, *fung**, and *isolat**. The result (lighter/red-shaded curve area in Figure S1, Supporting Information) uncovers an important fact that matches well with the general perception of experts in the field: natural products research has been a continuous driving force behind CS development through its entire history and continues to play that role today. The existence of such strong ties is not surprising: The extraordinary chemical diversity of nature's metabolomes calls for methods that are capable of solving highly complex separation problems. Similar to thin-layer chromatography (TLC), CS is another example of natural products driving new developments in separation technology. However, as natural products research is a relatively small discipline within the entire field of bioanalytical, biochemical, and biomedical research, modern CS is still in need of asserting itself as an indispensable analytical tool in the more global scientific community.

The meta-analysis of the CS literature also reveals that the majority of natural products studies using CS technology (90%, or 33% of the total CS literature) utilized it for the purpose of preparative isolation. This simultaneously underlines the main strength and weakness of CS at the current stage of development: CS is a scalable, high-capacity and high-resolution preparative tool, but it still has to be fully exploited toward (micro)analytical CS instrumentation and high-sensitivity applications. Its strength as an isolation tool, however, lies in the unique selectivity that CS separations provide, especially when compared to adsorption-based LC methods. While having been experienced by many researchers in the field of natural products, this advantageous property of CS unfortunately remains a perceived rather than a verified asset, as the required chromatographic aspects are rarely documented in the literature in sufficient detail. Noteworthy in this context is the recently increased number of articles that report single-step CS isolation procedures from crude materials, which yield natural products of relatively high purity (>90%; frequently ≥98%, as determined by HPLC). Considering the authors' full CS literature archive, this portion of the CS literature was estimated to hold a significant (∼40%) share of all contemporary CS literature. At the same time, the straightforward achievability of high-purity isolates supports the hypothesis that CS has unique resolution power in the separation of complex samples. Historically, CS has been used primarily in the very early stages of the separation process and, thus, been considered a preseparation rather than a (final) isolation tool. This contrast starts to soften as modern CS instrumentation has demonstrated unique capability for the final purification of natural products from the submicrogram up to the kilogram scale.

Working Definitions and Nomenclature. Throughout both the Craig and the Ito era, liquid−liquid separation techniques have always been associated with the term "countercurrent", which evolved from the early theoretical treatment of the method.[1−3,25−27,35−37] The historical usage of technical terms, however, has not yet become IUPAC-sanctioned terminology that consistently blends past theory and current practice of CS. One example of an obvious discrepancy relates to countercurrent

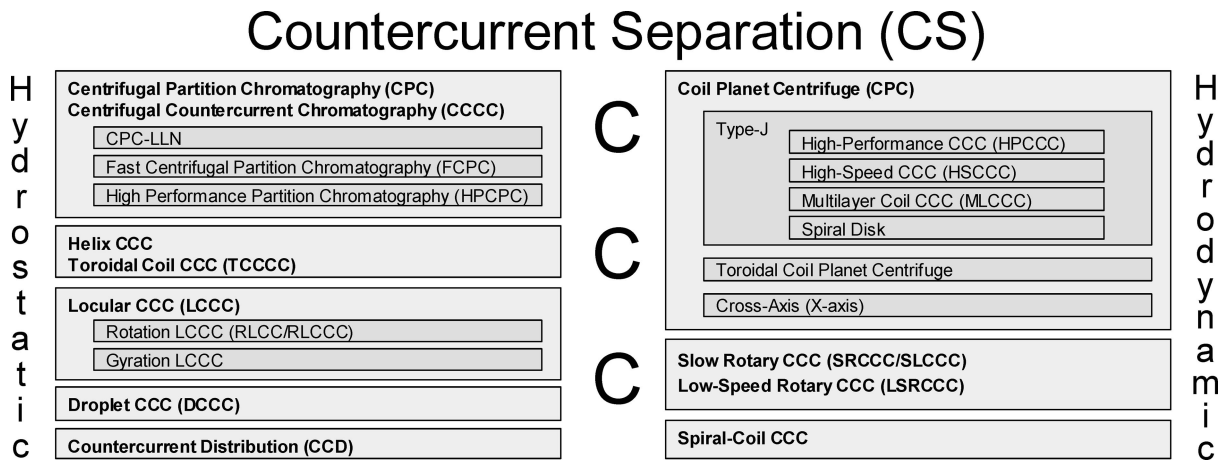

Figure 2. The two main families of countercurrent instrumentation operate on the basis of either the hydrostatic or the hydrodynamic principle. While the coil or partition unit cell is stationary in the former, it rotates about its own axis in hydrodynamic machines, which improves two-phase mixing, but also causes larger variability of stationary phase retention. CCC is an ambiguous term in that it is commonly used to describe all CS instruments, while at the same time indicating a hydrodynamic instrument. The fact that two very separate terms (hydrostatic centrifugal partition chromatography vs hydrodynamic coil planet centrifuge) share the same abbreviation has caused confusion in the literature.

flow itself. As of today, most CS methods, in particular the laboratory scale instruments, rarely apply a true countercurrent flow (e.g., as in closed tube coil planet centrifuge,[29] dual flow CCC,[38] and continuous liquid−liquid extraction[39]), nor did most of the once popular CCD machines, in which one phase was typically kept stationary. The lack of true countercurrent flow is understandable from an engineering point of view, since moving *both* immiscible liquids at the same time and in opposite directions poses considerable challenges on instrument construction. However, for nomenclature purposes it is important to realize that the term "countercurrent" was the initial term used by Craig[2] and later adopted by Ito,[29] and has now been in use for almost 70 years. The term has also branded a series of international conferences (CCC 2000−2008) and the name of an international society (ISCCC).

Overall, it is mostly for historical reasons that scientists almost inevitably connected the term "countercurrent" with analytical methods that involve liquid−liquid partitioning processes and employ solvents but no solid phases to achieve chemical separations. Therefore, *countercurrent separation* (CS) has been chosen in the present work as an overarching term that integrates the historic "countercurrent" background with ongoing developments in the "separation"-related sciences. The term countercurrent chromatography (CCC) was coined in the early 1970s,[10] to describe both hydrostatic and hydrodynamic systems. Today, the term is used ambiguously, referring only to hydrodynamic systems, or referring to all liquid−liquid separation techniques or subsets thereof. Thus, in order to eliminate confusion, this article uses CS to describe *all* multistage liquid−liquid separation techniques. Figure 2 provides a basic guide to common terms used for CS instruments, organizing them into hydrostatic and hydrodynamic categories. This also clarifies ambiguities of literature terms, such as the term "CPC", which has been equally used for hydrostatic centrifugal partition chromatography as well as for the hydrodynamic coil planet centrifuge used in HSCCC. While official IUPAC definitions for the whole field of CS are not yet in place, a committee of experts is in the process of addressing this.[40]

Recent Advancements and Current Status of CS. As CS uses pairs of immiscible liquids as stationary and mobile phase, respectively, the choice of a "column" and "eluant" are linked inescapably. Moreover, because the two-phase solvent system defines the physicochemistry of the milieu in which the separation takes place, the appropriate choice of the solvent system (SS) is key to success in CS. SS selection is currently evolving from an empirical hit-or-miss procedure to a rationally designed process, in which natural products play an important role as structurally diverse standard analytes. For example, routine thin-layer chromatography, as widely used in fraction monitoring, can be employed to augment SS selection using the GUESS method.[41] In addition, it has been recently shown that SS family mapping can be used to compare both polarity range and selectivity of SSs. Addressing one of the major problems associated with the successful first attempt, the recently established "sweet spot" model demonstrates that CS targets a relatively narrow band of polarity when compared with the wide gradient coverage typically utilized in HPLC.

Taking advantage of the liquid nature of the stationary phase in CS, recent evidence shows that the usable "sweet spot" of CS can be extended to the highly retained analytes.[42] This can be achieved by elution extrusion (EECCC)[43–45] and back-extrusion CCC (BECCC),[46] methods that are generally amenable to a range of CS instruments. The practical implementation and development of the full theory of EECCC and BECCC represents very recent progress in advancing the capability of CS. Further new technology relates to the graphical representation of CS:[45,47,48] Reciprocal symmetry (ReS) and shifted reciprocal symmetry (ReSS) plots are reflective of the symmetric nature of the partition process plots and are capable of capturing the high-resolution "sweet spot" of CS in the center of the chromatograms. In addition, ReS[S] plots cover the whole polarity range, i.e., zero to infinite retention, and lead to chromatograms that are reflective of the high-resolution potential of CS.

Recent research has also demonstrated the linear scale-up capabilities of CS.[49] This has led to the development of laboratory, technical, and production scale equipment that covers a throughput range from (sub)milligrams per day to tons per annum without the need for method development at each step. Finally, although in its infancy, hyphenation of CS separation modules with spectroscopy represents an emerging field[50,51] and shows much promise for the coupling of countercurrent chromatographs with mass spectrometers (CCC-MS).[52–54]

Countercurrent Instrumentation

Origin. All CS instrumentation can trace their roots back to one very simple and elegant technique (Figure S2, Supporting Information): single-stage liquid−liquid extraction best represented by the separatory funnel. A valued tool of the natural products laboratory, this simple apparatus has been used for centuries, providing partitioning of solutes based on differential solubility. These

separations are easy to understand, predict, tune, and control, but provide the efficiency of only a single theoretical plate. Although this crude ability to separate is of great value, a logical step in its evolution was improving efficiency by increasing the number of stages. Driven mainly by industry and apparatus, continuous, multichamber liquid−liquid extractions were being used regularly and were generally well understood in the 1930s.[39] Unfortunately, most industrial applications at the time were satisfied by large bulky systems of only a few theoretical plates. However, progress was being made toward more efficient systems,[55] most notably by Nobel Laureates Archer Martin and Richard Synge. They developed more compact and efficient extraction instrumentation[56] and made an important breakthrough with their invention of solid-support-based liquid−liquid chromatography.[57] Martin and Synge continued their work with what became popularly know as partition chromatography, leading further to the significant development of paper chromatography.[58,59] However, at the same time Lyman Craig[28] was developing an apparatus in the United States that did not rely on a solid support.

The Countercurrent Distribution. Craig and his colleague, Otto Post, can be credited with inventing and constructing the first apparatus[2] to streamline stepwise, multichamber, liquid−liquid extractions, into so-called countercurrent distribution (CCD, Figure S2, Supporting Information). Created for analytical work, the development of the Craig−Post apparatus was primarily focused on increasing efficiency. However, its inherent sample loading capacity as a liquid-only technique made it known primarily for its preparative abilities. The apparatus took several mechanical forms throughout its evolution,[26] but all had three main similarities: (i) Each apparatus had series of separatory funnels (tubes or cells) mounted together on a common support structure. (ii) The tube support was in turn mounted on a rotary bearing, allowing all funnels to be rotated or shaken for simultaneous mixing. (iii) Each apparatus also had means to transfer the lighter (mobile) phase from one tube, to its adjacent tube, an action that happened simultaneously with every tube of the instrument. The first, and lesser known, of the two basic types of CCD instruments was the "tube distribution apparatus",[26] a cylindrical assembly of stainless steel tubes (Figure S2, Supporting Information). By 1950, the development of this instrument had given way to the preferred "glass distribution train",[3] providing an instrument that was easier and faster to operate, while offering superior scalability. Automated early on, these instruments were capable of hundreds of thousands of extractions within a 24 h period and provided efficiency approaching that of a solid adsorption column.[3] The Craig−Post apparatus became widely used through the 1950s and 1960s, propelled by its simple design and high loading capacity.

Origin of Modern CS Instrumentation. Yoichiro Ito is widely identified as the father of modern CS.[34] This legacy started with his work on the coil planet centrifuge (Figure S2, Supporting Information).[29] Successfully designed to separate particles according to size and relative density, these instruments rotated a length of helically coiled tubing (closed on each end) in a planetary motion. He also discovered that the same instrument could be used to separate solutes based on their partition coefficients in a biphasic SS.

The coil planet centrifuge was relatively small in size compared to the CCD instruments, which had grown to large dimensions. This "microscale" instrument[7] provided true, rapid, countercurrent partitioning with the efficiency of several hundred plates and further allowed for use of more viscous SSs required for work with biomolecules. While improving flow rates and eliminating maintenance of a rotating seal, the closed tubing, however, did not allow for continuous elution. In the pursuit of flow-through designs, a particularly simple solution was found without requiring any movement of the coil.[31] By placing a coil of tubing perpendicular to the force of gravity, filling the tubing with one phase of a biphasic SS, and then slowly pumping through it the other, a portion of the first phase gets trapped in each turn of the tubing. In this way, each turn of the coil effectively provides zones of mixing and settling between the two phases. These zones impart repeated partitioning of solutes introduced with the mobile phase. This simple design is the basis for all modern hydrostatic CS instrumentation.

By rotating the same coil through its central axis, and effectively imparting an Archimedean screw force, the entire contents of the coil tend toward one end of the column (head).[31] By pumping the mobile phase in the opposing direction (into the head), better retention of the stationary phases was achieved, as well as increased mixing, and therefore efficiency. This is the basis for hydrodynamic systems. Development of hydrostatic CS continued with the development of toroidal coil countercurrent chromatography,[10,60] which used the centrifugal force of a centrifuge instead of gravity to increase retention of the stationary phase. Advancement of hydrodynamic systems focused on the original coil planet centrifuge, adapting the design to allow for a flow-through system. It was found that, with appropriate gearing, coupled with careful routing of the tubing, an uninterrupted flow path to and from a rotating column could be achieved without the use of any rotary seals.[61] In this way, the flexible (usually Teflon) tubing can bend, but not twist or bind. There are actually several ways in which this could be done, and many ways in which a column could be situated with respect to the central axis.[31] This work lent itself to heightening the performance and throughput of CS, most notably by improving flow rates.

Modern Hydrostatic Instrumentation. Droplet countercurrent chromatography (DCCC),[9] though an older technique, is included in this section because of its continued use and commercial availability.[62,63] These instruments consist of many inert vertical tubes ("columns"), connected top to bottom, in series, by small-bore tubing (Figure S2, Supporting Information). Operating in a manner similar to the stationary coil previously described, a mobile phase is percolated through a stationary phase, which is trapped in each column by gravity. Turbulence within the droplets of rising or falling mobile phase provides mixing and therefore partitioning of solutes introduced to the system. The scalability, reliability, and simplicity of these instruments made them popular (refs 64−66 and literature cited therein). However, the days or even weeks required for separations and the limitations of usable SSs continued to drive the development of more efficient CS instrumentation. In response, several forms of locular countercurrent chromatography[10,66] were introduced to allow operation with a more diverse range of SSs.

By far the most popular of the modern hydrostatic type instruments is the centrifugal partition chromatograph (CPC, Figure S2, Supporting Information).[67] These instruments were first introduced in the early 1980s[68] by the same company that collaborated with Ito in building the first coil planet centrifuge, used in many hospitals in Japan.[34] CPC uses centrifugal force generated by a single-axis centrifuge to hold a stationary phase in place, drastically improving mobile phase flow rates and mixing between the phases. This is accomplished through a series of channels and ducts etched into inert plates, mimicking the function of the columns and tubes in DCCC. The past decade has attracted the interest of global manufacturers, further driving CPC's development. Today's instruments benefit over their predecessors with improved rotary seals that lengthen maintenance intervals and increase pressure/rotational speed limitations, which translates to improved resolution and throughput. Additionally, significant improvement of channel geometry has also had a positive effect on resolution and stationary phase retention.[69,70]

Modern Hydrodynamic Instrumentation. By 1981, with the arrival of the coil planet centrifuge-based high-speed countercurrent chromatograph (HSCCC, Figure S2, Supporting Information) design, separations now took hours instead of days.[71] Shortly thereafter, the first commercial instruments became available. Still,

development of the technique brought significant improvements such as multiple column holders to allow self-balancing[72,73] and continuing development of new coil configurations.[74] This innovation provided fuel for continued commercial instrument development based on Ito's designs. Figure S3 (Supporting Information) provides an example of a complete HSCCC separation workstation. Today, there exists a large variety of commercially available hydrodynamic instruments, all of which are based on Ito's various coil planet centrifuge designs, most notably the type-J instruments. Over the past decade, several new manufacturers have become established,[75] using modern manufacturing techniques to provide a new range of CS instruments with quality, reliability, and reduced maintenance. Also, noncommercial developmental CS research continues to thrive, particularly with respect to designing new coil geometries to improve retention of aqueous/aqueous systems such as the spiral disk,[76–78] slow rotary,[79–82] and cross-axis configurations.[83–85]

Hydrostatic vs Hydrodynamic Instruments. Although distinctions can be made between hydrodynamic and hydrostatic instruments, it is important to remember that both types (a) provide mechanical retention of an abundant liquid stationary phase, (b) allow the uninhibited flow of a mobile phase through the system, and (c) provide a mechanism for repeated mixing and settling steps between the two phases. Because of these core similarities, CS instrumentation can generally be considered functionally equivalent. Operationally speaking, a user should understand the particulars of each; however, the basic theory and concepts, as discussed in further sections, are the same. It is important, nonetheless, to note that the two types do have their own respective advantages. The differences between the two most popular hydrostatic and hydrodynamic instruments available today, centrifugal partition chromatographs (CPCs) and type-J coil planet centrifuges, respectively, will be addressed below.

CPC instruments are generally more effective at retaining a wider range of solvents systems,[67,86,87] particularly those that have little density difference between the phases, most notably aqueous/aqueous systems[88–90] used to focus on highly polar compounds.[91] They are further resilient to stationary phase bleed caused by disruption of the SS equilibrium due to high loading and complex sample matrixes. Conversely, the smooth and continuous flow paths of type-J instruments eliminate special cleaning steps between runs, are able to accommodate crude extracts or otherwise unprepared samples, and can even tolerate direct injection of raw material in suspension. The feasibility of the latter approach was recently documented by isolating quaternary protoberberine alkaloids from powdered plant material of *Coptis chinensis*.[92] Further, the type-J instrument lends itself to a broader range of the various operation modes such as EECCC, as later described, providing more options for method optimization. Finally, type-J instruments are available in analytical volumes below 50 mL, a barrier that CPC has yet to overcome. However, advancement of both techniques continues to reduce these limitations.

Rational Choices in CS Instrument Selection. An important part of CS instrument selection is to first understand how the instrument fits into the overall experimental configuration and integrates with peripherals such as pump, injector, and detector. In most cases, a CS instrument can be installed as an alternative column in a working LC setup, providing a new level of versatility and selectivity to systems normally restricted to solid phase separations. A recent trend in manufacturing of CS instruments is to provide some level of peripheral integration, analogous to how standard (HP)LC systems are equipped.

Commercially available CS instruments exist in a variety of types and can differ in column volume, revolution speed, pressure limitations, stationary phase retention, resolution capabilities, and throughput potential. Literature comparing instruments and CS performance is scarce.[93,94] Therefore, methods for the systematic evaluation of contemporary CS equipment and CS methodologies require future research. Although attempts have been made,[94] the lack of standardized methods and comprehensive data for performance comparison hampers instrument selection for both the novice and experienced user alike. The following provides basic considerations for comparing and selecting an instrument. Noting the differences stated in the previous section, selection of an instrument type (e.g., hydrostatic/CPC vs hydrodynamic/type-J) should take into account the purification level or refinement stage and the polarity of the samples. Examining the literature for compound types similar to those of interest and noting the SS(s) used can also be of help. A particular focus on highly polar compounds suggests a hydrostatic instrument that can facilitate aqueous/aqueous SSs.

Analytical instruments are available in volumes as low as a few milliliters (4.6 mL),[54] and production scale instruments can be purchased with capacities of up to tens of liters (5 to 25 L).[95,96] While larger instruments have a clear advantage in terms of loading, smaller equipment is generally less expensive, involves a lower solvent consumption, and requires shorter run times when running small samples. The survey of recent literature presented in the following section found that over half the CS instruments used were in the 200−400 mL range. This trend illustrates a compromise where method development and semipreparative scale separations are comfortably performed on the same instrument. Time, solvent consumption, and the increased amount of sample needed for detection reduce the appeal of method development on larger capacity instruments, especially those in the multiliter range. In these cases, an analytical instrument is recommended for preliminary method development.

When considering scale, the user should bear in mind that loading capacity in CS is dependent on the solubility of a sample in and its influence on the settling time of the SS being used. Poor solubility, or samples that cause severe disruption to the settling time of the SS, can negatively affect resolution and cause problems with stationary phase retention.[33] Many of the "classical" rules applied in the natural products laboratory that stem from adsorption-based LC do not transfer to CS, mostly to CS's favor. As opposed to the common 1:100 loading rule in silica LC, loadings of 5%[33] and up to 20% of the total column volume are seen in the CS literature,[97] with both loading volume and concentration having an influence on resolution.[98] Another example of lack of congruence between adsorption-based LC and CS is the number of theoretical plates necessary for separation efficiency (see section on Theory and Concepts and Section S3, Supporting Information, in particular eq 6).

The time needed for completion of a CS run and loading capacity determine the throughput capabilities of an instrument. Run time is dependent on the column volume, stationary phase retention, mobile phase flow rate, and the partition coefficient of the target solute(s). Attainable flow rates relate to an instrument's ability to retain the stationary phase against the flowing mobile phase. Higher rotational speed can mean better stationary phase retention, higher flows, and shorter run times, provided pressure limits are not exceeded.

Resolution increases with stationary phase retention and efficiency. Efficiency is dictated by several factors,[94] most notably the number of mixing/settling zones and the quality of mixing and mass transfer within those zones. In CPC instruments this is dictated by the number of channels providing the distinct zones, in addition to the flow rate, revolution speed, and cell geometry, which influence mixing. Efficiency in type-J instruments relies on column geometry, its position with respect to the center axis, the number of turns of the coiled column (i.e., column length), revolution speed, and flow rate. With both instruments, increasing flow rates beyond instrument recommendations with the goal to achieve faster run times can be at the cost of resolution due to the decrease of stationary phase retention volume ratio (S_f).

Solvent Systems: The Empirical Approach

Documented Use of Solvent Systems for Natural Product Separation. Over the years, several excellent reviews[33,99] and chapters[32,65] have appeared that list commonly used CS solvent systems (SSs) from the perspective of general classes of natural products. One aim of the present work was to perform an in-depth evaluation of documented use of SSs in the recent literature and gain insight into those tools that have proven to be most valuable for the separation of natural product samples. A survey conducted from some 150 articles, published from 2000 to 2007, forms the basis for much of this section. Peer-reviewed journal articles chosen for meta-analysis have described the isolation and characterization of natural products with a CS experiment as one or more of the chromatographic steps. The majority of articles are from two journals: *Journal of Chromatography A*, 29%, and *Journal of Liquid Chromatography & Related Technologies*, 26%. The remaining articles are from 22 other journals. This cross-section of the literature is representative of both journals that regularly publish and those that only occasionally publish CS-related articles. Articles that describe natural product isolation rather than instrument design, method development, operational design, or the separation of synthetic or inorganic chemicals comprise almost half (43%) of this literature.

The choice of an appropriate SS is fundamental to CS and can require a significant time investment, which can occupy up to 90% of the time devoted to CS experiment design.[100] The process begins with choosing one or more appropriate SS families, which represent particular combinations of solvents that form biphasic systems. Usually SS families contain three or four different solvents, but may range from a simple two-solvent family to any number of constituent solvents. In this survey, 56% of SSs consisted of four and 37% of three liquids, while 5% and 2% employed two or five liquids, respectively. Typically, SS constituents are listed in order of increasing polarity. For example, one SS family is described as *n*-hexane−EtOAc−MeOH−water.

The role of individual solvents used to constitute CS SSs in the natural products literature may be illustrated by considering the number of times a particular solvent is used as a percentage of total solvent systems described (Figure S4, Supporting Information). In 187 SSs reported in the separation and purification of natural products, a total of 20 different solvents were used. The resulting percentages show that water, EtOAc, MeOH, and *n*-hexane are by far the most utilitarian solvents in CS. In fact, the results suggest that CS chromatographers have a quite limited range of solvents that they are currently using in natural products separation. This may be due to the fact that the mixture of *n*-hexane, EtOAc, MeOH, and water (frequently termed the "HEMWat" or "Arizona SS" family) works so well, suggesting that there might be no need to look further. For the practical chromatographer the cost, accessibility, safety considerations, environmental impact, and/or physical properties of the dominant solvents make them an obvious choice for CS work. On the other hand, it is possible that not enough SS evaluation and development is being done in order to promote those solvents and those SSs that are truly optimal for CS. Once a SS family is selected, one or more SSs of that family are tested in, for example, a shake-flask partition study (Section S1, Supporting Information) with the mixture to be separated. A SS is a particular combination of solvents in fixed ratios. For example, the proportions tested may be *n*-hexane−EtOAc−MeOH−water 6:4:6:4, 5:5:5:5, and 4:6:4:6. There is no widely accepted convention on how proportions are listed. Often, but not always, the ratios of solvents are represented by the lowest combination of whole numbers: 3:2:3:2 rather than 9:6:9:6 or 1.5:1:1.5:1. The results of shake-flask experiments are expressed in terms of the partition coefficient for each analyte of interest in each SS tested. The partition coefficient (K) is the concentration of an analyte in the upper phase divided by its concentration in the lower phase. The shake-flask value of K can be used to predict the retention time of the analyte in the CS run.

In chromatography journals, the SS selection for a particular separation is often documented in great detail. In this survey, half of the articles describe, in some depth, how the employed SSs were selected. Many of these articles include a table detailing the list of SSs used in shake-flask experiments and the corresponding K values of the target analyte(s). For example, Seger et al. created a rather extensive table showing the partition coefficients of destruxins A, B, and E in 20 different SSs from 12 different SS families.[101] Of those articles that gave a detailed description of SS selection, there were an average of 3.0 SS families investigated and 8.5 SSs per article. In some cases, the chromatographers may have had in mind the SS family they wanted to use for a particular separation, but needed to develop the optimal proportion of solvents. In other cases, a wider search with a variety of SS families was performed to find the best SS. In most cases, authors drew upon their previous experience and/or literature reports instead of attempting new SS combinations. Thus, there is a clear preference for SSs that had given successful separations in the past or had been reported to be successful in separating the target class of compounds. In the tradition of the literature, SS selection so far has been an empirical process involving significant elements of experience.

On the other hand, the simplicity of the shake-flask experiment makes it an uncomplicated though tedious method to test several SSs in the quest for an optimal separation. One elegant solution to overcome the burden of labor is the use of a liquid-handling robot.[102] There are clear advantages to this rational approach of SS selection, since partition coefficient values derived from shake-flask experiments are a reliable predictor of countercurrent chromatographic behavior. In fact, the partition coefficient of an analyte in any biphasic mixture is equivalent to the liquid−liquid distribution ratio, K_D, of the same analyte in a CS experiment as calculated by $K_D = (V_R - V_M)/V_S$. In practice, the retention volume of the analyte (V_R) is calculated from the retention time and flow rate. The mobile phase volume (V_M) and stationary phase volume (V_S) are observed for each chromatographic experiment. According to countercurrent separation theory, K and K_D are equivalent provided that three conditions are met. First, it is assumed that both processes have reached equilibrium. Second, the upper phase is the stationary phase for the CS experiment. If the lower phase is the stationary phase, then $1/K \equiv K_D$, since the K_D in CS theory is defined as the concentration of an analyte in the stationary phase divided by its concentration in the mobile phase. Third, the compound is present in the same chemical form in each process. To be sure, CS has been used to determine $K_{octanol/water}$ of compounds in certain cases.[103−106] Considering the potential confusion (see Supporting Information in ref 47) and the lack of IUPAC-sanctioned definitions, the present article, in keeping with the majority of the literature, uses the simple term K to designate both K and K_D in CS.

As CS instruments with a greater analytical capacity become available, it will become more common to abandon shake-flask experimentation in favor of analytical CS experiments to adjust the SS composition and proportions to optimal values.[107−113] For example, a 40 mL column was used to develop a separation method that was subsequently used on a larger, 230 mL, column to separate 300 mg of crude extract.[112] Using analytical CS to select an appropriate SS is related to CS scale-up since they both rely on the fact that the K value of an analyte in a particular SS is independent of column, volume, flow rate, rotational speed, and stationary phase retention ratio.

The choice of SSs goes beyond simple separation optimization. The solvents used in the SS must be compatible with the detector. For example, solvents that absorb strongly in the UV are normally avoided when UV−vis detectors are employed. As other detectors such as ELSD,[114−117] pH meters,[118] and MS[51,119] are employed to

a greater extent, the solvent choice may also expand. Since solvents for preparative scale applications are used in relatively large quantities, solvent economy, disposal requirements, and safety considerations may also play a role in solvent choice. In this survey, a total of 659 individual solvents were used to design 187 SSs. There was a surprising consistency of solvents used. Four solvents, i.e., *n*-hexane, EtOAc, MeOH, and water, comprised 74% of individual solvents used to create various SSs. The overwhelming popularity of these four solvents was not entirely unanticipated since the quaternary SS, *n*-hexane−EtOAc−MeOH−water, can be considered the most utilitarian SS in CS today. Water was used in over 96% of SSs. The solvents *n*-BuOH, $CHCl_3$, MeCN, EtOH, *tert*-butyl methyl ether, and *n*-heptane round out the top 10 choices in order of their occurrence. Also employed to a smaller extent are CH_2Cl_2, *i*-PrOH, petroleum ether, *n*-PrOH, carbon tetrachloride, acetone, ethyl ether, toluene, and sunflower oil. Obviously, CS methods allow for the choice of a wide variety of solvents per se. However, only relatively few are used extensively in biphasic SS formulation.

The relative rarity of nonaqueous SSs merits special attention to their usage in the CS of natural products. As may be expected, nonaqueous SSs are used for the separation of strongly lipophilic compounds such as terpenes. Wei et al. isolated lycopene from tomato paste with *n*-hexane−CH_2Cl_2−MeCN (20:7:13).[120] Lu et al. used *n*-hexane−MeOH to purify squalene from microalgae in one chromatographic step.[121] Most recently, Du et al. isolated solanesol from tobacco leaf extract in petroleum ether−EtOH−MeOH (200:1:200).[122] In a similar application, the nonaqueous mixture EtOH−vegetable oil was employed in a high-capacity slow-rotation countercurrent chromatography (SRCCC) instrument.[123,124]

In addition to SSs selection, the CS operator must decide which phase will function as the mobile phase. The choice of mobile phase will determine the relative retention time of an analyte if its K is not equal to 1. For example, an analyte with $K = 2$ will move through the column 4 times as fast if the upper phase is mobile rather than if the lower phase is mobile. It may seem that a faster separation is most desirable at first. However, the longer the duration an analyte dwells in the column, the greater the resolution between it and other analytes. The choice of mobile phase is therefore part of a balance between minimizing retention time and maximizing resolution. According to this survey, 74% of chromatographic runs were done under reversed phase conditions; that is, the hydrophilic phase was mobile phase. The reasons for the consistent choice of reversed phase elution are not entirely clear. It may be that reversed phase conditions give better stationary phase volume retention, which is seen to be desirable in CS.

The CS Workhorse: The "Arizona" or "HEMWat" Family of SSs. By far the most popular SS family consists of *n*-hexane−EtOAc−MeOH−water solvent combinations. Of the articles surveyed, 29% use a combination of these four solvents to perform the featured liquid−liquid separation. This SS family has been used in CS since 1982.[125] Even within a SS family, the individual solvents may be combined in an infinite number of relative proportions. Therefore, an organized method of SS selection is necessary even within a SS family.

The *n*-hexane−EtOAc−MeOH−water combination has been organized into two important SS family tables. A SS family table organizes a single SS family into a manageable number of representative SSs arranged by increasing or decreasing relative polarity. The first table was proposed in 1991, where *n*-hexane−EtOAc−MeOH−water and EtOAc−*n*-BuOH−water SS families were merged into a single table.[126] A second table focusing on *n*-hexane−EtOAc−MeOH−water was proposed in 2004 in the form of an array of 16 SSs that spanned the range from *n*-hexane−EtOAc−MeOH−water ("HEMWat") 9:1:9:1 to 0:10:0:10.[41] In each table, the SS family members are numbered to simplify the references and to situate them by relative polarity. The "HEMWat" SS family is also included in a systematic treatment of CS SSs published in 2005.[102,127]

Of the articles surveyed, 18% used a SS that resembles the *n*-hexane−EtOAc−MeOH−water with a single substitution. For example, the most common substitutions are heptane in place of *n*-hexane or EtOH in place of MeOH. Recently, a study has been done to assess the effects of varying the hydrocarbon member of "HEMWat.[128] Results of this study showed that there were minimal changes in the CS chromatogram (K values). A popular table has been conceived that arranges a series of 23 *n*-heptane−EtOAc−MeOH−water solvents systems in order of polarity. Instead of numbers, they are indexed by letter, and the SS table is called the "Arizona" table.[129]

Chlorinated solvents, in particular $CHCl_3$, have a long history of use in countercurrent separations. The original test mixture for the development of modern countercurrent chromatography instruments developed by Ito was a mixture of *N*-dinitrophenyl−amino acids separated in $CHCl_3$−acetic acid−0.l N aqueous HCl in a 2:2:1 ratio.[10] Chloroform was also extensively used in countercurrent distribution[130] and droplet countercurrent chromatography (DCCC). In fact, the first documented use of DCCC was in the separation of a DNP−amino acid mixture with $CHCl_3$−acetic acid−0.1 N aqueous HCl (2:2:1).[9] Separation of gramicidins with $CHCl_3$−benzene−MeOH−water was performed in 1982[71] and of bacitracin with $CHCl_3$−95% EtOH−water in 1989.[131] The combination of $CHCl_3$−MeOH−water was first reported in 1985. Siderochelin A and pentalenolactone were isolated using $CHCl_3$−MeOH−water (7:13:8 and 1:1:1, respectively) by Brill et al.[132] This combination proved to be very useful and was used in many subsequent separations throughout the 1980s and 1990s.[133−141]

Modification of Empirical Solvent Systems. Modification of SSs with organic or inorganic solutes occurred in 20% of the SSs surveyed. Most commonly, an acid is added to lower the pH of the aqueous phase in order to, presumably, minimize the ionization of organic acids in the sample. Trifluoroacetic acid, acetic acid, or hydrochloric acid were used in at least three-fourths of all solute-based SS modifications. Trifluoroacetic acid has the advantage of being easily evaporated away from the sample after the chromatography has taken place. Phosphate buffers, formic acid, sodium hydroxide, sodium iodide, benzalkonium chloride, potassium perchlorate, and ammonium sulfate were also used as solute modifiers of CS SSs. Bourdat-Deschamps et al. tested six different solutes in a DCM−MeOH−water biphasic SS in order to devise a two-step isolation of four protoberberine quaternary alkaloids from a crude extract of *Enantia chlorantha* by CPC.[142]

Modification of either or both phases of a two-phase SS adds another dimension to the combinations of solvents. Two very interesting modification have been reported recently that have the potential of being applied to natural product separations. In the first case, the aqueous phase was modified with a buffer to the extent that it created two phases when mixed with the normally water-miscible alcohols EtOH and *n*-PrOH.[143] These alcohol−aqueous buffer SSs were used to extract and purify salvianolic acid B from crude extracts of *Salvia miltiorrhiza*. The second case proposes the addition of surfactants (sodium 1-heptanesulfonate or sodium 1-hexanesulfonate) to the lipophilic *n*-hexane mobile phase. The resulting SSs were used to separate model mixtures of three steroids or four esters. Clearly, the separations were affected by the formation of micelles under countercurrent chromatography conditions.[144]

A special case of solute modification of SS is the use of "chiral selectors" to effectuate the separation of enantiomers in CS. Several articles have appeared where cinchona derivatives have been added to the lipophilic stationary phase that were capable of complexing with acidic chiral compounds.[145−147] This is an interesting application of CS technology, but these types of modifications are not

currently used in compound purification from natural sources. Maçiuk et al. describe a strong-ion exchange CS experiment in the purification of rosmarinic acid from plant cell culture extracts. This successful separation entailed adding benzalkonium chloride to the organic stationary phase and sodium iodide to the aqueous mobile phase of a $CHCl_3$−*n*-BuOH−water (9:2:9) SS.[148] Another special application of solute modification of SSs has been termed "pH zone-refining" by its creator, Yoichiro Ito. A recent article by Chin et al. describes the SS necessary to characterize four tropane aromatic ester alkaloids from the stem bark of *Erythroxylum pervillei.*[149] The aqueous mobile phase of the *tert*-butyl methyl ether−water SS was modified with hydrochloric acid, while the lipophilic stationary phase was modified with triethylamine. The pH zone-refining method is an intriguing CS application, but has been used sparingly in natural products work since it was introduced in 1994.[150–152]

Recently, three-phase SSs such as those formed with certain proportions of *n*-hexane, *tert*-butyl methyl ether, MeCN, and water have been used to separate mixtures of natural products.[153–155] The use of all three phases in a single CS experiment expands the covered range of polarities. To date, there has not been an application directed at the isolation of natural products. However, Shibusawa et al. used two of three phases to separate catechin oligomers from unripe apples.[156]

Successive CS. More than three-quarters of the surveyed articles describe a single CS SS used in a key, and often final, purification step. Apparently, the most often exploited advantage of CS is to target the purification of a single component as the final step of a one or two-step isolation, which results in the quantitative recovery of the desired natural product in high purity. Almost a third of the surveyed articles report the isolation of a single natural product. In fact, CS is considered to be a novel method for the isolation of known compounds of biological interest in high-purity preparative quantities. Depending on the particular sample and the goal of the separation, the requirements for sample preparation in CS can be minimal. In fact, crude extracts may be directly injected on the CS column. Preparative steps may include separatory-funnel liquid−liquid partitioning or a large-volume flash chromatography column. Almost a third of the surveyed articles perform a preliminary column chromatography step prior to the CS experiment. Schäfer and Winterhalter compared direct injection of crude kava root extract with injection of an extract that was fractionated on a Sephadex LH-20 column. In this case, prefractionation allowed for higher loading capacity and better resolution of target kava-lactones in the CS step.[157] In almost a quarter of the surveyed articles, more than one SS was used to fractionate the target compounds. The multi-SS separations may be categorized in four fractionation strategies, a−d, described in the paragraphs below.

(a) The polarity-adjusted strategy. Different polarity fractions from a prefractionated crude extract may be chromatographed with different SSs as part of a fractionation scheme that targets several compounds of varying polarity. Yao et al. reported the systematic separation and purification of nine chemical components from the Chinese medicinal herb *Adenophora tetraphylla*. Four different SSs were employed as part of an extensive fractionation scheme that began with the partitioning of the crude extract between water and diethyl ether.[158]

(b) The SS combination strategy. Successive CS fractionation of the same extract may be used to resolve target analytes in a rather narrow polarity range.[137,159–163] For example, Maier et al. isolated three hydroxycinnamoyl tartaric acids from grape pomace by first using a system of *n*-hexane−EtOAc−MeOH−water (3:7:3:7) with 0.5% TFA in the head-to-tail elution mode. Next, the target compounds were separated from the coextracted polyphenolics and subsequently isolated in a second run in *tert*-butyl methyl ether−MeCN−*n*-BuOH−water (2:2:1:5) with 0.5% TFA in tail-to-head elution mode.[164] The two-step purification of scutellarin by Gao et al. involves an initial fractionation with *n*-hexane−EtOAc−MeOH−HOAc−water (1:6:1.5:1:4 followed by EtOAc−*n*-BuOH−MeCN−0.1% HCl (5:2:5:10) to isolated the target compound in 96.5% purity.[165] In fact, successive CS separations may be arranged in a "multidimensional" or "two-dimensional" CS experiment.[109,111,166] For example, three furanocoumarins were isolated from the traditional Chinese herb "Bai Zhi", *Angelica dahurica*, using CS with a pair of SSs composed of *n*-hexane−EtOAc−MeOH−water (1:1:1:1 and 10:10:9:11) by connecting two centrifuges with a column switching valve.[108]

(c) The recycling strategy. The same SS that was used in the first CS experiment may be employed again to recycle primary fractions by repeated CS.[157] Han et al. used a typical recycling arrangement to facilitate this operation.[167] A representative example of the use of successive CS separations that form part of a larger isolation scheme, involving multiple chromatographic techniques, is described in an article by Chadwick et al. reporting on a "sample cutting" technique. The authors used a combination of postfractionation CS and successive CS, resulting in the isolation of 21 compounds from hops including estrogenic prenylated flavones.[168]

(d) The gradient elution strategy. In "gradient" CS, the composition of the mobile phase is adjusted during the CS run to help hasten the elution of highly retained compounds.[161,169–171] In summary, of those literature reports that use more than one SS as part of the CS fractionation procedure, the average number of CS steps is 2.24.

Optimization of Operational Parameters. In addition to SS selection, the optimization of operational parameters is a prerequisite for effective CS. Most often, flow rate and rpm speed are the parameters to be optimized. In general, slower flow rates may give better resolution, but they may also lengthen the time of experiment excessively. Fast flow rates shorten experimental times but are more likely to be the cause of insufficient resolution and loss of stationary phase.[122,172,173] Therefore, the flow rate should be fast enough to ensure a sufficient resolution in a reasonable amount of time. Typically, larger capacity instruments require and/or support faster flow rates than smaller capacity instruments. When the flow rates (*y*-axis) and volumes (*x*-axis) collected from the present survey are plotted, they give an overall positive slope. However, there is much variation due to the different types of instruments and their design. For example, CPC instruments are typically run at higher rotational speeds per volume than type-J instruments. Recently, a new line of instruments designed to support faster flow rates in order to maximize the high-throughput capabilities of the technique have been introduced.[174]

In general, a faster rotational speed increases the stationary phase retention volume ratio, which is seen as a desirable operational condition. When the rotational speeds (*y*-axis) and volumes (*x*-axis) collected from the present survey are plotted, they give an overall slightly negative slope. This may be simply the result of mechanical limitations: it is more difficult to stabilize a large-capacity instrument. The rpm should be consistent with the instrument design and not put too much of a strain on the bearings, seals, and tubing. Rotational speed also has an influence on pressure: higher pressures are required to maintain a constant flow rate as rpm increases.[175] Even so, there is much variation due to the different types of instruments and their design. Optimal rotation speed is often studied in tandem with flow rate for any given separation. Fisher et al. described the scale-up and optimization of the extraction of the potential anticancer natural product glucoraphanin from broccoli. The CS experiment was carried out under varying conditions of three different flow rates and two different rotational speeds in order to select the optimal combination.[176] Ha et al. studied five different flow rate−rpm combinations in the preparative isolation of four ginsenosides from Korean red ginseng.[177] Chen et al. tried four different flow rates with a fixed rotation speed.[107] Influence of both flow rate and rotational speed was studied by Marchal et al. in a CPC instrument.[67] It was shown that peak resolution may be

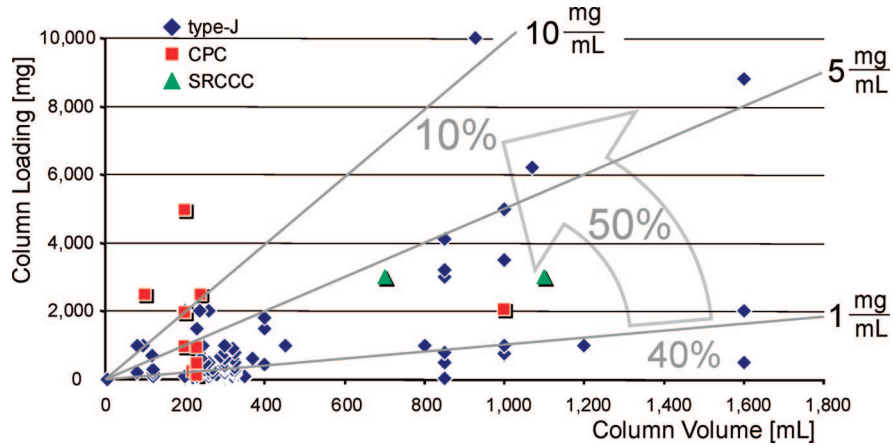

Figure 3. Depicting the mass of reported crude natural product sample as a function of column volume illustrates current CS loading practice. A total of 108, 30, and 2 data points exist for type-J, CPC, and SRCCC instruments, respectively, and the average loading is 2.2, 7.6, and 2.6 mg/mL, respectively. Two data points not included are 22.5 g loaded on a 5.16 L CPC[96] and 3 g on a 3.4 L SRCCC instrument.[79] The actual column loading is below 1 mg/mL for 40% of reported separations and between 1 and 5 mg/mL for another 50% of cases. Only 10% utilize the full CS loading capacity above 5 or even 10 mg/mL. Loading capacity is a strong yet not fully used advantage of CS, especially when compared to preparative solid phase techniques such as HPLC ($\ll$1 mg/mL).

improved with an increase in the rotational speed with a concomitant decrease in the flow rate,[178] and a comprehensive study developed a mathematical model of flow rate and rpm.[179] Experimental data have supported the mathematical relationship that the retention of the stationary phase decreases proportionally to the ratio of the square root of the mobile phase flow rate to the rotational speed ($F^{1/2}/\omega$).

Temperature-controlled CS instruments have been introduced recently, producing more consistent and reproducible CS results. In this survey, 36% of the CS articles report on the temperature. Temperature control may be achieved through water circulating in a box surrounding the centrifuge or by air being blown through the centrifuge compartment. Operating temperatures for optimized CS experiments have been reported in the modest range of 20 to 35 °C. Temperature may be varied for optimal separation of target analytes. Peng et al. and Ma et al. reported trying six temperatures between and including 15 and 40 °C in the isolation of three flavonoids from *Patrinia villosa*[180,181] and scoparone from *Artemisia scoparia*,[182] respectively.

When the CS instrument is used as a preparative method, the sample loading capacity is an important factor. In general, the loading capacity of an instrument increases as the volume of the column increases. From the present survey, 92% of the articles gave sufficient information to determine the column loading in mg of sample mixture introduced to the instrument per mL of column volume. Loading values ranged from 0.02 to 12.5 mg/mL in the articles surveyed. The average loading was 2.2 mg/mL. The range from 0.5 to 2 mg/mL accounted for the majority of reported loading values. Zhao and He proposed that the sample load increases exponentially with column volume.[98] While this bodes well on future CS scale-up efforts, it will require experimental corroboration. The authors extensively studied three phenolics (hydroquinone, pyrocatechol, and phenol) in *n*-hexane−EtOAc−EtOH−water (1:1:1:1) in regard to the effect of sample concentration and volume on peak resolution and stationary phase retention. In addition, Maryutina et al. reported that peak resolution decreases with an increase in sample volume.[178]

Figure 3 illustrates the column loading that is being currently being practiced by CS chromatographers by showing the mass of crude natural product sample reported as a function of column volume. In many cases, CS investigators might not have access to several different CS columns. Therefore, underloading a column is practiced as a conservative means of saving time and/or optimizing resolution and is apparently preferred over multiple runs with smaller volume columns. In addition, CS chromatographers may purposefully underestimate loading capacity of their instruments. If the column is underloaded, they will still obtain the desired separation. If the column is overloaded, the separation may not take place satisfactorily and will need to be repeated. A third consideration may be that loading capacity varies widely with SS and sample composition. It may not be a priority for the CS chromatographer to optimize each sample for loading capacity. In any case, even at a modest sample loading, CS is superior to solid-adsorbent liquid chromatography for solvent consumption and loading capacity ($\ll$1 mg/mL column in solid phase [HP]LC).

Solvent Systems: The Rational Approach

Correlation of Structure and Polarity. One of the most well-known ways of characterizing a compound is to determine its partition coefficient in an *n*-octanol−water biphasic system. In fact, the actual partition experiment is rarely done, since instead there are a variety of algorithms available with which to calculate log *P* or log $K_{o/w}$ based on the structural characteristics of the compound. Since the value of $K_{o/w}$ directly predicts the behavior of a compound in the CS experiment (i.e., *K* being predictive of the CS partition coefficient, *K*, of an analyte), why is octanol−water not more widely used to isolate compounds of known structure and therefore of known log $K_{o/w}$? The reason is that just knowing when the compound will elute is insufficient in the design of a CS experiment to resolve a target compound from other components in a complex mixture. It has been demonstrated abundantly that the resolving power of CS is best within a certain interval of *K* values. Our literature survey (Figure 4) shows that over half of the *K* values of isolated natural products lie in the interval of $0.5 < K < 2$. If the interval is extended to $0.25 < K < 4$, then the percentage rises to 85% of isolated and purified natural products. Therefore, CS chromatographers design their separation experiment to focus the *K* of the compound(s) of interest within a definite interval of values. This interval of optimal separation has been named the "sweet spot" of CS in reference to the sweet spot of bat and racket sports (Figure 4).[41] Therefore, the chromatographer's task is not so much to determine the retention time of an analyte in a particular SS, but to find a SS that will focus the target analyte(s) in a "sweet spot" of *K* values.

Octanol−water is recognized as a critical SS because of its relationship to the potential bioavailability of a pharmaceutical candidate. However, it is entirely possible that an algorithm for determining *K* could be written for any biphasic SS once a certain

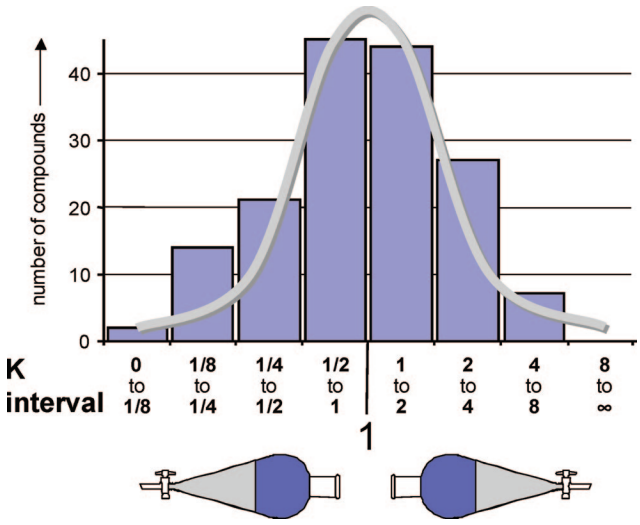

Figure 4. Meta-analysis of the CS literature for the distribution of partition coefficients (*K* values) as an indicator of optimum partition behavior. Most successfully separated compounds fall into the range of 0.125 to 8. The great majority of the *K* values represented in this graph (160, from 79 articles) were calculated from the chromatograms. While optimum *K* values may vary with CS instrument types, the findings support the "sweet spot" concept that designates a certain region of optimal resolution. In classical elution CS, the "sweet spot" is centered at $K = 1$, and $0.4 < K < 2.5$ represents a conservative range.[41] In extrusion modes such as EECCC,[45] the "sweet spot" can be significantly extended toward higher-*K* ranges ($0.25 < K < 16$).[48] The Gaussian-like *K* distribution also supports the symmetric (Figure S6, Supporting Information) and reversible nature of CS as a liquid−liquid partition method, indicated by the separatory funnels.

number of known partition coefficients are available. Surprisingly, this has not been done for any SS other than octanol−water. There are at least two reasons why there is no dominant "one size fits all" SS in CS. First, the variety of polarity in natural products is much wider than any one SS and/or SS family can manage. Second, even if the SS has the appropriate polarity, the target analyte may not be adequately resolved from other compounds in the mixture. Therefore, there is a body of literature in CS that attempts to characterize SSs in such a way that the chromatographer can make an efficient search to determine an optimal SS for a particular separation.

A good example of an endeavor to index SSs by their ability to focus a given compound at a particular *K* value is a paper by Walter Conway where he establishes an equipollent index using homologous series of *N*-alkylbenzamides, *N*-acylcytosines, and *N*-acylcytidines.[183] The equipollent of a SS is the length of the carbon chain of the particular homologous series that would give a $K = 1$. For example, as the proportion of EtOAc in a heptane−EtOAc−water SS increases, the *N*-alkylbenzamide equipollent index for the SS decreases. This means that as the SS becomes more polar due to the addition of EtOAc, it will focus shorter-chain *N*-alkylbenzamides in the "sweet spot" around $K = 1$. Once the equipollent index is known, the *N*-alkylbenzamides can then be equated with a target compound by log $K_{o/w}$ or some other factor such as reversed phase HPLC retention times. For example, heptane−EtOAc−water (4:1:4) has an *N*-alkylbenzamide equipollent index of 1.9, corresponding to *N*-ethylbenzamide with a log $K_{o/w}$ of 1.51. A natural product such as 3,4-dimethoxycinnamic acid with a log $K_{o/w}$ of 1.56 may be a good candidate to be separated in this SS. The reliability of these correlations has not been extensively studied to date.

A way to enhance the usefulness of shake-flask and/or analytical CS experiments may be to develop algorithms to "fill in the blanks" (extrapolate) between tested SSs and others. In this way, the chromatographer may be able to generate a large amount of predictive data from a relatively few *K* value determinations. For example, it has been proposed that the *K* for a compound tested in one "HEMWat" SS may be predicted for another by the simple formula $\log K_b = \log K_a + 0.16(b - a)$, where the variable *a* is the number of the SS in which *K* is known, and *b* the number of the SS to be predicted.[48]

SSs are typically selected in a sequential rather than an arbitrary fashion. The process followed by Li et al. to design a SS to isolate phillyrin gives insight into the decision-making process.[184] Their starting point was the tertiary *n*-BuOH−EtOAc−water SS family, because it had been used in the literature for a similar separation. The SS *n*-BuOH−EtOAc−water (2:3:5) gave a shake-flask partition coefficient of 6.25 for phillyrin. This was undesirable in terms of retention time if performed with a hydrophilic mobile phase and undesirable in terms of resolution if done with a lipophilic mobile phase. The next step was to reduce the polarity of the upper phase in order to make it less soluble to phillyrin: The SS *n*-BuOH−EtOAc−water (1:4:5) gave a partition coefficient of 3.87. While this was going in the right direction, toward unity, a limit was reached using *n*-BuOH in the upper phase. Instead of continuing with this SS family, the authors switched to a much more lipophilic organic phase with the *n*-hexane−EtOH−water SS family: The SS *n*-hexane−EtOH−water (5:1:4) gave a *K* value of 0.011 for phillyrin. The analyte clearly was not soluble in the strongly lipophilic hexane-rich upper phase. Adding EtOAc modified the effect of hexane, as was evident from the mixture *n*-hexane−EtOAc−EtOH−water (5:5:3:7), which gave a partition coefficient of 0.099. While going in the right direction, the compound was still not sufficiently soluble in the lipophilic phase. From the experiments with *n*-BuOH−EtOAc−water, it was evident that the compound is fairly soluble in EtOAc relative to water so the proportion of EtOAc was increased. They followed with *n*-hexane−EtOAc−EtOH−water (3:7:3:7), which gave a partition coefficient of 0.246 for phillyrin. At this point, the experiments were closing in on the "sweet spot", as it became clear that increasing the proportion of water would make the hydrophilic phase less soluble to the compound. Continuing with *n*-hexane−EtOAc−EtOH−water (3:7:1:9), a partition coefficient of 0.312 was observed for phillyrin. This again was going in the right direction, but did not represent a very big shift in relative solubility. More EtOAc had to be used to entice the compound into the upper phase. This finally led to the SS *n*-hexane−EtOAc−EtOH−water (1:9:1:9) and a partition coefficient of 0.799, which was close enough to unity to afford a reasonable separation in an adequate amount of time with the hydrophilic phase mobile.

Model Mixtures and the GUESS Method. Model analyte mixtures have had a long use in countercurrent separations. For example, almost all instrument design of HSCCC was carried out with a mixture of up to nine different *N*-dinitrophenyl−amino acids separated in a $CHCl_3$−acetic acid−0.1 N aqueous HCl SS.[10] A mixture of four auxins was introduced to instrument design testing in 1982 that was separated with a *n*-hexane−EtOAc−MeOH−water solvent system.[125] A five-component steroid mixture has been used by Berthod et al. in several publications to explore SS characteristics and CS methodology.[43,128,185] An alkylbenzene mixture in a *n*-heptane−MeOH−water SS was used to evaluate CS instruments for their stationary phase retention and chromatographic resolution at various operational parameters.[42,94,186] Some reports favor a benzyl alcohol and *p*-cresol combination in heptane−EtOAc−MeOH−water (7:3:5:5).[97,178]

Historically, natural products have played an important role in the continued development of CS technology. For example, the flavonoid extract from *Hippophaea rhamnoides* has been used for testing of CS instruments and operational parameters.[134,187–192] Generally, the separation of at least three sea buckthorn isoflavones is observed in a $CHCl_3$−MeOH−water SS. In

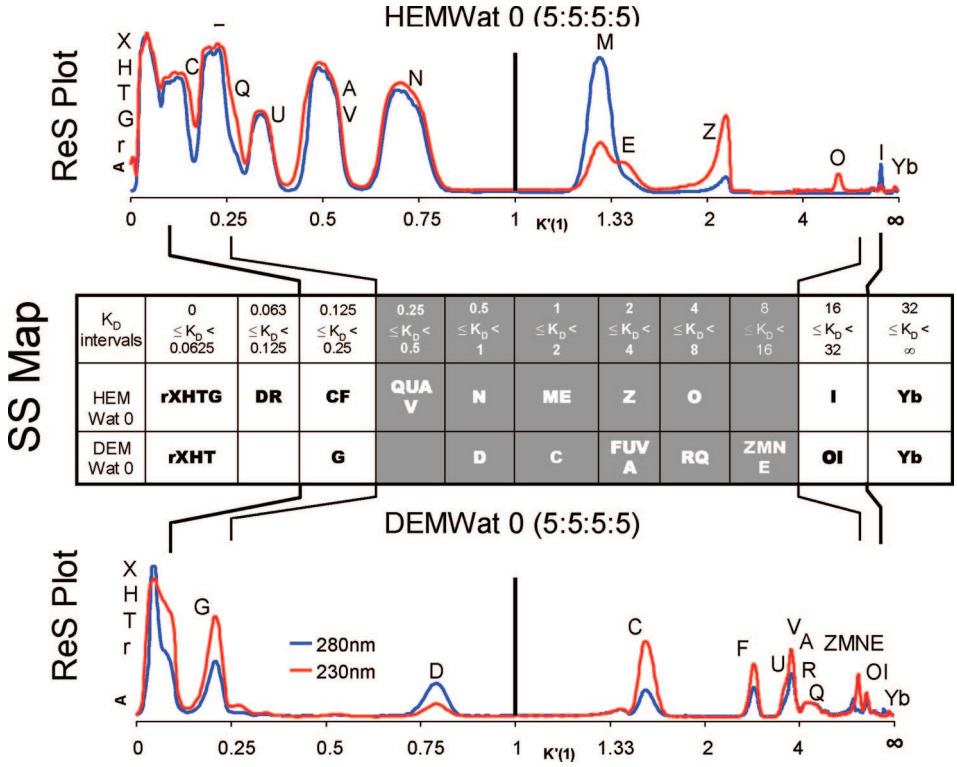

Figure 5. Comparison of different forms of CS chromatograms that allow a universal description of CS performance. The ReS plots (Figure S6, Supporting Information), which reflect parameters such as peak shape and resolution, can be converted into SS maps that describe the order of elution, resolution of analytes, and optimum range of resolution ("sweet spot"; indicated by brackets) by binning *K* values. SS maps allow systematic studies of SS performance, because they can visualize separations of a large number of compounds in various SSs.[48] While any chromatogram including ReS plots is intrinsically limited by the detection method, the SS map can show all analytes by combing results from different detections. The example shows results from the CS of GUESSmix analytes in equal-volume mixtures of EtOAc, MeOH, and H_2O with *n*-hexane ("HEMWat") or DCM ("DEMWat").[48]

addition, the separation of four flavonoids from *Oroxylum indicum* seed extracts form the basis for three different studies of various CS parameters.[53,107,119,193] The flavonoid constituents were first separated with $CHCl_3$−MeOH−water (8:10:5).[194] Subsequently, an *n*-hexane−EtOAc−MeOH−water (5:6:5:5) SS was developed to perform a similar separation.[195] Noteworthy is the direct use of *Camellia sinensis* extract in the development of SRCCC as a low-cost, high-capacity method for large-scale CS.[79] The use of slow rotation and spiral-coil tubing has recently been expanded to the laboratory scale in the form of a low-speed rotary CCC (LSRCCC).[82]

An effort to satisfy both the need for standard mixtures of known composition and the separation of a variety of natural products was made by introducing a multicomponent mixture of natural products of varying polarity. The formulation was driven by the need to emulate the diverse polarities, molecular mass, and functional groups seen in natural product extracts. As a result, a mixture of 22 commercially available natural products was initially used to develop a TLC-based SS selection protocol for CS described as a generally useful estimation of solvent systems (GUESS).[41] The GUESS method of selecting an appropriate SS for a CS experiment aimed to satisfy the following criteria: (a) systematic in its approach; (b) time efficiency; (c) versatile for a wide range of natural products; (d) flexible enough to allow some margin of error in making a judgment; (e) adaptable to rational fine-tuning; and (f) applicable to mixtures of unknown composition as well samples of known composition.

Since thin-layer chromatography (TLC) plays a central the role as a SS selection method in solid-support liquid chromatography, the GUESS method involves the estimation of SS choice based on TLC behavior. The GUESS method provides a TLC-based method for CS, allowing a good first "GUESS", and being able to replace conventional shake-flask procedures. TLC is a common denominator of all natural products separations. Samples ranging from crude extracts to purified compounds are routinely subjected to TLC as a quick and easy way to assess their composition, identity, and purity. In fact, the GUESS method has been done in reverse for decades. It is customary to separate an extract or column fraction by CS and then perform TLC on the collected CS fractions in order to ascertain their composition and purity. If TLC can be used routinely to analyze CS fractions, then it should be possible to use TLC to predict CS elution performance. However, relating TLC and CS is fundamentally challenging since their respective physicochemical means of separating compounds is quite different. At least one method of predicting droplet countercurrent chromatography (DCCC) behavior based on TLC observations has been proposed.[196] With this method, silica gel TLC was done with the organic layer of a $CHCl_3$−MeOH−water biphasic solvent system in order to predict the best mobile phase for optimal DCCC performance in that solvent system.

A recent study explored the possibility of using the GUESS method to develop a TLC-based method of predicting CS behavior in the *n*-hexane−EtOAc−MeOH−water ("HEMWat") SS family.[41] It was found that, instead of mixing a biphasic SS for each TLC SS, it is sufficient to observe the R_f of a compound in a *n*-hexane−EtOAc mixture whose proportions correspond to a "HEMWat" family member. In order to relate the two chromatography methods, it is proposed that the TLC R_f value 0.5 corresponds to a CS *K* value of 1. In this way, a reasonable first try SS can be proposed without extensive shake-flask or analytical CS work. Fine-tuning of the first try SS is almost always advantageous, but the GUESS method allows the effort of the chromatographer to be focused on SS optimization rather than SS discovery. To date, this method has only been systematically investigated with "HEMWat"

and $CHCl_3$−MeOH−water, and it is not known if it can be extended to other popular SS families.

Systematic Mapping of SSs. As a result of the GUESS method development, it was recognized that the mixture of compounds, subsequently termed the GUESSmix, may be used to compare and contrast SS performance. Since the span of polarities in the GUESSmix, unlike any other model system prior to its development, is on the order of 20 log $K_{o/w}$ units, it was advantageous to employ CS as a method of determining the K value of each analyte in one experiment. Fortunately, elution-extrusion CS emerged on the scene during the same period as the GUESSmix was being developed.[42,43,45] The elution-extrusion technique not only allows the elution, and therefore separation, of every compound of a mixture in a reasonable amount of time to be determined by the chromatographer, it also provides the chromatographer the means to determine the K value of each analyte based on its retention volume. This was an ideal technique to compare the K values of the GUESSmix compounds as they were run in different SSs.

The coupling of the GUESSmix and elution-extrusion CS was first employed as a means to compare the behavior of the 22 GUESSmix compounds as the proportions of individual solvents varied within a SS family.[197] In this way the polarity range of a SS family could be studied at the same time as its selectivity applied to compounds of similar CS retention. The CS properties of two ternary SS families (for nomenclature, see Section S7, Supporting Information), EtOAc−*n*-BuOH−water (EBuWat) and *tert*-butyl methyl ether−MeCN−water (*ter*AcWat), as well as a quaternary SS, *n*-hexane−*tert*-butyl methyl ether−MeCN−water (H*ter*AcWat), were explored in order to contrast and compare their CS potential. In the past, similar studies had generated line graphs with K as the *y*-axis and a progression of SSs as coordinates on the *x*-axis. However, when graphing the K values of over 20 compounds, these graphs get fairly cluttered. Additionally, since the separation of CS is not linear, a linear treatment of K values did not sufficiently reveal the "sweet spot" behavior of compounds of particular interest in CS. As a result, a SS mapping was developed.[48,197] Using this method, compounds are represented by letters and their K values are binned in an arrangement that centers around $K = 1$ and magnifies the portion of the range corresponding to the "sweet spot" of high resolution in CS. In this way, the absolute and relative migration of individual compounds can be observed as the SS composition changes within a family. The study also reinforced the concept of the portal SS:[197] It has been mentioned in the literature that it is sufficient to test a mixture in one representative SS of each SS family.[198] Whenever this portal SS appears to have the potential to perform the desired separation, the separation can likely be fined-tuned within the same SS family.

Another reinforced concept is that different SS families are fit to cover different polarity ranges. For example, if relatively polar glycosides are to be separated, a correspondingly polar SS family such as EtOAc−*n*-BuOH−water may be appropriate. Indeed, the SS families tested displayed the ability to separate a rather narrow range of relative polarities.[197] One reason that SS families have relatively narrow polarity coverage is that miscibility limitations exist among the suitable solvent components. Finally, it has been shown that not all SSs are equal in their ability to resolve a mixture of compounds.[197] Certain combinations within a SS family yield better resolution of the GUESSmix than others. At this point, differences between SSs in terms of achievable analyte resolution remains an area of CS that is not well understood. Apparently, tertiary and quaternary SSs allow for certain flexibility in SS selection that binary SSs do not. While, in principle, the use of more than four SS components is possible, the advantage and particular rationale of this approach remain to be proven.

Very recently, the particular value of standardized mixtures for CS evaluation has been recognized, as a result of a multicomponent mixture of 15 compounds ranging in polarity from carotene to tryptophan used by Shibusawa et al. to demonstrate three-phase SS effectiveness.[155] Separations of nine different standard compounds as well as those from commercial *Camellia sinensis* extracts were performed with *tert*-butyl methyl ether−MeCN−0.1% aqueous TFA (2:2:3) to study separation profiles of tea and food products.[199]

Theory and Concepts in CS

The Priority of *K*. Much work has gone into the understanding of CS and the pursuit of accurate and sometimes complex modeling methods.[200–202] However, CS theory and concepts required from a user's perspective are relatively straightforward and entail only rudimentary knowledge of partitioning and the fundamental equations (S2 and S3, Supporting Information). The CS theory, as briefly summarized in the Supporting Information, explains that K is the variable most independant of operational and instrumental parameters. Because K directly characterizes an analyte, its elution in a CS instrument can be easily and accurately predicted.

Modes of Operation. Like solid-support techniques, CS has the ability to run in a variety of modes and variations thereof. However, the liquid nature of both the mobile and stationary phases gives CS an intrinsic flexibility, far surpassing techniques limited by permanent immobilization of their stationary phases. CS gives users the ability to manipulate both phases in order to facilitate a desired separation and eliminate the risk of losing solutes to permanent adsorption. Over the decades several modes of operation have been studied and standardized, clarifying the effect of these different methods of using CS.

By far, the most popular and straightforward modes of operation are elution modes with upper or lower phase mobile (syn. normal and reversed mode, respectively; not to be confused with "normal phase mode" and "reversed phase mode", respectively). Up to this point, we have generally described CS, and its theory, within the context of these basic modes. In elution mode, the stationary phase is held in place while the mobile phase is pumped through it. The decision to use normal or reverse mode is often determined by the solute's K value, which becomes the inverse when switching from normal to reverse mode or vice versa. Other considerations are the fast drying of fractions using a mobile organic phase and the higher stationary phase retention in hydrodynamic systems using a mobile heavy (or lower) phase.[33]

Elution-extrusion countercurrent chromatography (EECCC, Figure 6)[42,43,45] is a simple and increasingly popular mode of operation for CS and is targeted at shortening run time and suppressing peak broadening for solutes with higher K values. EECCC is an extension of normal and reverse modes and involves switching the phase being pumped after some amount of elution mode operation. Switching the phases effectively pushes out (extrudes) the contents of the column, as they exist within. This is advantageous for retrieving compounds of high K values, which normally take a long time to elute, but may be already separated within the column (Figure 6). EECCC is ideal for concluding almost any elution mode run, providing a column refilled with fresh stationary phase at the conclusion of the extrusion process.

Cocurrent CCC[44,185] is something of a cross between elution mode and EECCC.[45] In this mode, some portion of "stationary" phase is concurrently introduced with the mobile phase during a run. This causes the stationary phase to slowly move in the same direction as the faster moving mobile phase, increasing the speed, but lowering the efficiency of a run.

Dual-mode CCC describes the switching from reverse to normal mode, or vice versa, in mid run.[203,204] After some amount of elution, both the directions of flow and the phase being pumped are switched. This has similar advantages to EECCC described above, but causes a change in elution order, which can make the resulting chromatogram and fractions more difficult to follow. However, dual-

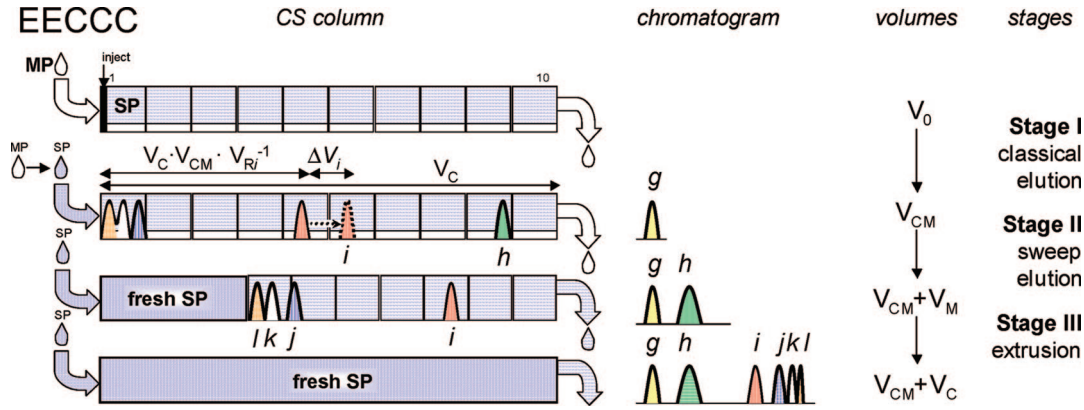

Figure 6. The basic concept of elution-extrusion countercurrent chromatography (EECCC) provides access to *all* analytes, including those that are highly retained in the column. While in adsorption-based (HP)LC such analytes would be irrecoverable, the liquid nature of the stationary phase opens unique possibilities of handling the "stationary" phase.

mode does offer the possibility of switching directions/phases several times, allowing it to be used as a tool to effectively increase the efficiency of the column.

Dual-flow CCC, not to be confused with "dual-mode CCC" above, is a mode that allows for true moving bed chromatography (TMB).[38] This technique requires an instrument that has a feed port in the middle of the column or between two adjacent columns, for sample injection. The two phases of the SS are pumped against each other, one from each end of the column, and collected from the opposite end. This causes solutes with $K = 1$ to remain in equilibrium near the injection point, while solutes with $K < 1$ values elute in order of increasing values from one end, and those with $K > 1$ elute in order of decreasing values from the other end of the column.[74]

The method known as pH zone-refining CCC,[33] though referred to here as an operating mode, is also accurately described as the use of a special class of SSs for the separation of solutes that are ionizable by the change of pH. Although highly effective, and providing a 10-fold increase in column loading capacity,[33] the way pH zone-refining CCC works is fundamentally different from the repeated partitioning of prior methods. One major difference is that it is a nonequilibrium method in that there is no constant hydrodynamic equilibrium to be established at the beginning of the run. While the two phases still need to be immiscible, the gradual change of pH in the column creates additional dynamics within the system. Applications of pH zone-refining CCC concern the separation of alkaloids such as those from *Sophora flavescens*,[205] *Catharanthus roseus*,[206] *Aconitum sinomontanum*,[151] *Camellia sinensis*,[207] *Huperzia serrata*,[150] and *Hydrastis canadensis*.[208] Other areas of application are the separation of curcuminoids from turmeric (*Curcuma xanthorrhiza*),[209] antifungal fermentation broths,[210] and food additives.[211–213]

Other variations of CS operation with limited documentation in the literature include solvent and flow gradients,[170] multidimensional CCC (MDCCC),[109,181,214] and chiral CCC.[145,146,215–217]

Scale-up of Operation. Ease of scale-up and high-throughput capabilities[193] have always been advanced as a distinct advantage of CS over solid-support LC methods. The advantage is twofold and comprises both a linear scalability and an economical benefit. Several scale-up studies have emerged in recent literature to underscore the practicality of scale-up in CS technology. In 2003, Booth et al. described the separation of erythromycin from a commercial erythromycin base preparation. Pilot studies were performed with a type-J CS instrument containing a 100 mL coil. The separation was initially optimized for rpm, flow rate, sample concentration, and loading volume in *n*-hexane−EtOAc−MeOH−water (7:10:10:5). The sample was then partitioned on a 1 L type-J CS instrument. A maximum estimated product throughput of 410 g/day, with a yield and purity of 100% and 92%, respectively, was obtained.[173,218] Further studies with broth-derived antibiotics realized a throughput of up to 330 g/day with a greater than 97% purity.[219] Flow rate and sample loading capacity are critical parameters in increasing throughput. At a certain, experimentally determined, point, increasing flow rate and/or loading capacity engenders a decrease in resolution. In 2005, Fisher et al. reported optimizing a CS method originally developed by Fahey et al.[220] in order to isolate glucoraphanin from broccoli to maximize throughput. A highly polar SS, *n*-PrOH−MeCN−saturated ammonium sulfate−water (10:5:12:10), was employed to separate crude extracts on 5, 1000, and 5000 mL instruments. The culmination of this study realized the production of 52.6 g of 98% pure glucoraphanin from 589 g of extract over the course of three days.[176] In 2007, Pinel et al. described the one-step multigram scale purification of xanthathin, 4-epi-xanthanol, and 4-epi-isoxanthanol from the leaves of *Xanthium macrocarpum* with a 5000 mL CS instrument.[96] In the same article, the comparison of CS to traditional preparative silica gel-based chromatography revealed that CS not only increases purity of the fractions obtained but also decreases solvent consumption necessary to perform the desired separation.

A Cookbook Recipe to CS of Natural Products

The following section provides an overview of the key considerations and practical workflow involved in the CS of a natural products sample. Summarizing the text, Figure 7 provides a genuine CS flowchart from sample to CS separation, whereas Section S5 (Supporting Information) gives an overview of basic CS troubleshooting.

From Sample to Separation. When considering how to approach the LC of a natural product sample, the main considerations are its complexity, the presence of matrix compounds, solubility characteristics, and quantity. All in all, the liquid−liquid nature of CS power is less vulnerable to sample complexity and the presence of impurities than solid-adsorbent LC. Since CS is a high-capacity technique that works well with crude samples, it has often been characterized as being "preparative".[221] In fact, CS has qualities of both preparative and analytical high-resolution chromatography. The key feature of the sample in terms of CS is its solubility characteristics, which will, in turn, influence the optimal SS.

CS has the capacity to separate crude samples, which are obtained by solvent extraction of natural material. The characteristics of such crude extracts include a wide range of compounds varying in size, polarity, and chemical functionality, the presence of matrix compounds, and inconsistent solubility. The number and type of extraction solvents determine the range of polarity of the resulting

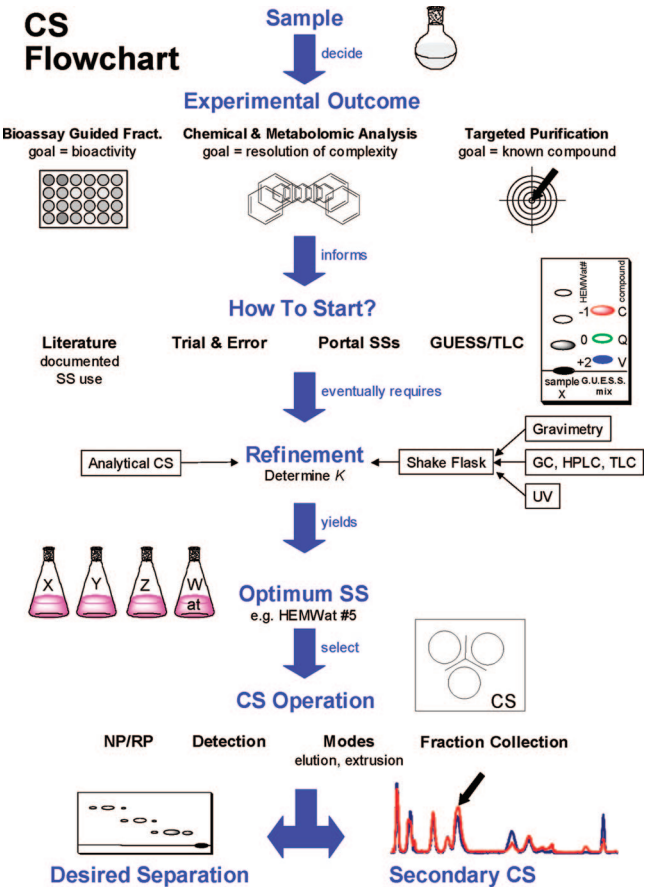

Figure 7. A generic practical approach to the CS of natural products.

crude extract, which will determine its chromatographic behavior in CS. In the EECCC experiment, for example, those compounds at the polarity extremes are concentrated at either end of the CS run with no additional solvent expenditure. This pattern may be clearly seen in the CS of the GUESSmix standard (Figure S6, Supporting Information), which contains natural products of varying polarity. These extreme polarity fractions can be collected and rechromatographed in an appropriate SS.

Adaptation to Final Experimental Outcome and SS Selection. The versatile nature of CS means that the choice of CS experiment can be adapted to the many experimental outcomes. In a bioassay-guided fractionation experiment, the goal is to assay a wide range of sample constituents for a particular biological activity. Not only will CS expose the whole range of analytes present in an extract, it is also loss-free and highly reproducible. In the first CS step of discovering bioactive analytes, it is desirable to separate the extract as efficiently as possible in terms of mass distribution (e.g., by utilizing EECCC[45] and BECCC[46] extrusion techniques) so that further separations can target specific regions of polarity and/or bioactivity. Natural products chemical and metabolomic investigations are geared toward discovering new natural products with novel structures and resolving highly complex mixtures. Dereplication is a key feature in these chemical and metabolomic studies since those natural products present in greater amounts are often known compounds. Initial CS steps are designed to divide the sample in such a way that known major compounds can be eliminated (e.g., by chemical subtraction)[222] in order to purify minor analytes. Since target compounds, such as those used to produce natural product reference materials, are typically present at low abundance, early CS steps must necessarily be able to handle large amounts of solute, constituting the need for large-capacity CS columns and enforcing the high loading capacity advantage of CS (Figure 3). As in bioassay-guided fractionation, access to the whole range of natural products in an extract is essential. In the targeted isolation of known or closely related compounds of similar polarity, CS has the advantage of being able to utilize results from shake-flask experiments. CS has proven to be a very effective technique in the isolation of relatively large amounts of high-purity compounds once a SS has been identified where the target analyte has a partition coefficient (K) in the "sweet spot" (Figure 4). The selection of the SS is another crucial step in the design of a CS protocol. While the basic theory has been covered in the section on correlation of structure and polarity, S4 of the Supporting Information provides a practical approach to SS selection.

Choosing CS Operation Parameters. Operation parameters come into play only after a SS has been selected. The first decision is whether to perform the separation with the *hydrophilic phase mobile or stationary*. As reported previously, the majority of CS experiments are performed with the hydrophilic phase mobile due to advantages with UV−vis detection (see section on empirical SSs). However, both normal and reversed phase elution is an equally viable choice for most SSs and solutes. Two guiding principles of CS must be taken into account: (i) increased solubility of an analyte in the mobile phase indicates lower elution volumes, and (ii) increased retention of analyte in the column indicates improved resolution relative to other analytes in the sample. The CS experiment, therefore, is a balancing act between collecting compounds with the most efficient volume of solvent and increasing resolution through greater retention volumes. When it is desirable to analyze the whole spectrum of natural product polarities within a sample, extrusion CCC techniques such as EECCC and BECCC are optimal. Compounds elute in order of their relative affinity for the mobile phase, the experiment length can be easily adjusted to optimize resolution, and the methods avoid any loss of sample. Two additional advantages to the extrusion techniques are that all K values can be calculated and the chromatogram represented in a universal plot format such as ReS[S]. The EECCC and BECCC techniques can be understood as CS technology's counterpart to the gradient elution popularly used in solid-adsorbent LC.

As previously demonstrated, UV−vis monitoring of the eluant continues to be popular in CS primarily due to its accessibility and ease of use. MS and ELSD detection so far have received limited application due to their (semi)destructive nature and the requirement to apply split valves in order to accommodate the relatively large flow in CS. As with any form of LC, TLC and monitoring of fractions is also routinely done in CS. Off-line GC-MS or HPLC monitoring of fractions is also an option that generates large amounts of information concerning the sample composition and CS performance. Determination of fraction mass is rarely reported, but is a relatively straightforward way of obtaining information on chromatographic mass distribution. When biological activity is the guiding principle, monitoring typically has to be done off-line.

In a purely preparative manner, CS may be used to perform a rudimentary separation of a complex sample into as few as 3 to 5 nonoverlapping fractions of different polarities. This process has been described as the "ABC method"[140,223] or "sample cutting".[168] The advantages of using a CS instrument instead of a separatory funnel or a preparative solid-adsorbent column are that CS involves less solvent usage and yields superior resolution between fractions, even when large amounts of matrix substances are present and used in a preparative scale. The "sweet spot" of optimal resolution (see above, Figure 4, and Figure 5) will contain well-resolved fractions even at large loading capacity (Figure 3). The two fractions of opposite polarities eluting on either side of the "sweet spot" can be collected and rechromatographed without the loss of compound from the original sample.

Secondary Chromatographic Separation. Any specific strategy will depend on the overall outcome goal: For bioassay-guided fractionation, the most active fractions will be combined in a rational way and rechromatographed to further resolve active

compounds. For chemical/metabolomic analysis, the hydrophilic and lipophilic ends of an extrusion experiment such as EECCC can be run in a SS from the same SS family by adjusting the polarity of the secondary separation accordingly. TLC, the traditional shake-flask experiment, or analytical CS can predict chromatographic behavior in the subsequent experiment. Third, for targeted CS, it is desirable to rechromatograph fractions from the "sweet spot" of a particular SS in a complementary SS of similar polarity but containing different constituent solvents (different chemistry).[197] This is comparable to "orthogonal" chromatography employed to purify analytes.

Reporting of CS

Generation of Chromatograms. Chromatograms generated from a CS experiment play an important role in the visualization and interpretation of separation effectiveness. In the present survey, 70% of the articles include at least one CS chromatogram. CS chromatograms are routinely plotted in the traditional chromatographic format with the retention time being represented on the *x*-axis. This is simply a result of chromatograms being generated by strip-chart type recorders. As in many forms of chromatography, retention times are very experiment specific in the sense that they depend on the exact reproducibility of several chromatographic parameters such as flow rate, column pressure, rpm, column volume, length of the run, and stationary phase retention volume ratio (S_f). In particular, it is very difficult to reproduce the S_f from run to run. Unfortunately, traditional chromatograms all but conceal the one parameter that distinguished CS from solid-support liquid chromatography. Clearly, a graphing technique that allows for compounds to be represented in terms of the *K* values would be vastly superior to the customary chromatograms. Since the elution-extrusion technique provides for the calculation of *K* for each compound in a mixture, a means to represent these values on the chromatogram is very advantageous. Reciprocal symmetry (ReS) and reciprocal shifted symmetry (ReSS) plots have been recently proposed to allow *K* to be plotted for every compound eluted in EECCC on a scale of zero to infinity.[47]

ReS plots allow the direct visual comparison of *K* values from any number of CS experiments. This method of representing chromatograms goes well beyond the ability to show reproducibility of chromatographic behavior in parallel runs. For example, ReS plots clearly demonstrate that the value of *K* for each analyte in a given SS is independent of the length of a chromatographic run.[45] This is a concept foreign to solid-support liquid chromatography, where elution is simply extended until the compound(s) of interest are eluted. The fact that the *K* of an analyte is independent of the length of the chromatographic run in CS means that chromatographic runs can be optimized to elute the compound of interest in either the classical elution or extrusion step. The design of a CS experiment, therefore, must take into account the balance between maximizing resolution and minimizing retention time. This is important because resolution is directly proportional to the amount of time that a compound spends on the column. The ReS plots are represented in a nonlinear scale that corresponds to the symmetrical nature of CS. In practice, the symmetry midline (M_s) of the chromatogram may be adjusted to emphasize the "sweet spot" of optimal resolution. For example if M_s is chosen to be 1, the middle half of the chromatogram represents the interval from $0.5 < K < 2$. If M_s is adjusted to 2, the middle half of the chromatogram is the interval from $1 < K < 4$. The general expression for the middle half of a ReSS plot is $M_s/2 < K < 2M_s$. This is important in CS because the "sweet spot" is typically the area of interest. It also imparts a rather pleasing esthetic to the chromatogram since the region of optimal resolution is prominently displayed.

Another application of ReS/ReSS plots is the direct visual comparison of changes in *K* for the same mixture of analytes separated in different SSs.[48,197] This process of SS mapping (Figure 5) allows for a comparison of GUESSmix *K* values in SSs within a family. However, it is also instructive to follow these same trends with chromatograms because peak shape and resolution may be observed in a way not apparent in the generated SS maps.[197] For example, peaks within the extrusion portion of the chromatogram tend to be sharper than those after $K = 0.5$ in the classical elution mode. This is a result of the fact that these analytes, though they remain in the column until the end of the experiment, are not subjected to the same diffusion as those analytes that traverse the column in classical elution mode.

The GUESSmix experiments displayed in ReSS plot form may be used to test and calibrate instruments such as is the case with GC and HPLC test systems.[224] The running of test mixtures under specified conditions is an accepted way of determining if a particular instrument has been configured correctly and is working properly. In the same vein, an instrument's optimal operating conditions such as flow rate, rotational speed, and temperature do not necessarily have to be optimized for each individual sample, but can be predetermined with the help of a test mix such as the GUESSmix. Since *K* is independent of column volume, flow rate, and rotational speed, ReSS plots may also be used to compare performances of different CS instruments by separating the same mixture of compounds (e.g., the GUESSmix).

Elution-extrusion chromatograms plotted in reciprocal symmetry plots will orient the CS field to expand toward more whole sample separations. About a third of CS articles that treat natural products separation are targeting the separation of one (usually a known) compound. There are an average number of 3.5 separated compounds per natural product-oriented CS publication. This illustrates that CS is currently being used for separations targeting a small number of analytes of similar polarity. As a recent CS article suggests,[225] the wide range of not just polarity, but also structural characteristics and molecular size, makes CS an ideal technique to systematically separate complex mixtures of biomolecules. This, of course, is the potential entrée of CS into the modern field of metabolomics.[226] As the field of metabolomics research gains momentum, countercurrent separation technology is poised to make a significant contribution to the toolbox of separation technologies necessary to undertake this ambitious endeavor. Besides the potential to separate a wide assortment of molecules, the lack of irreversible adsorption of analytes in the CS column ensures that metabolites are not irrevocably lost during the separation process. In addition, CS is applicable to both high-capacity preparative scale requirements and analytical high-throughput applications required by metabolomics analysis.

The Symmetry Advantage of CS. One of the most notable features of CS is its symmetry with respect to normal phase versus reversed phase mode separations. Its ability to perform so close to the theoretical ideal of what historically has been considered "normal" (in silica gel-based LC: lipophilic to hydrophilic) and "reversed" (in silica gel-based LC: hydrophilic to lipophilic) distinguished CS from classical solid phase LC separation methods. Symmetry can be achieved whenever separations are performed in the same SS by simply reversing the mobile and stationary phases, because the normal phase is switched to the reversed phase by changing the mobile phase from lipophilic in normal phase to hydrophilic in reversed phase. Not only is the order of elution reversed, but the *K* values of the analytes are numerically inverted. The practical use of this phenomenon is that, once the *K* values of an analyte are determined, the mode can be chosen that is most advantageous for the desired separation. For example, if the shake-flask partition coefficient determined in a "HEMWat" SS is 4, then the *K* in a reversed phase CS run with that SS will be 4. If the normal phase mode is selected, the *K* of the same analyte will be $1/4 = 0.25$. At first, it may seem that a shorter elution time would be advantageous; however, the chromatographer must also consider that the longer an analyte stays in the column, the better it is

Table 1. Recommendations for the Reporting of CS Experiments

parameter type	parameter	experimental report		
		essential	important	optional
instrumental (all)	instrument make, model, and type			
	column volume[a]	E		
	sample loop volume[a]		I	
	extra-column dead volume			O
	back-pressure regulator setting			O
instrumental (type-J specific)	rotor radius (R)		I	
	range of spool radius (r) values		I	
	β ratio range (β_r)	E		
	tubing inside diameter (bore)		I	
	tubing composition		I	
	head center/peripheral relative to flow	E		
	length of tubing			O
	number of turns per spool			O
	direction of winding relative to rotation			O
instrumental (CPC specific)	rotor radius		I	
	channel number			O
	channel volume			O
instrumental (detector)	detector make, model, type	E		
	detector setting (e.g., UV wavelength(s))	E		
	flow cell details		I	
operational	flow rate	E		
	rpm	E		
	solvent system solvent and volume ratios	E		
	mobile phase identity	E		
	flow direction (head-to-tail, tail-to-head)	E		
	stationary phase volume ratio (S_f)	E		
	switch volume (V_{ex}) of elution extrusion if used	E		
	column equilibration and sample injection method		I	
	temperature, if controlled		I	
	pH of aqueous phase, if buffered		I	
	gravitational field generated by rotation			O
	solvent system phase composition			O
	SS interfacial tension			O
	SS density difference of phases			O
	viscosity of each phase			O
	pressure variation during experiment			O
sample	loading mass of sample	E		
	loading volume	E		
	recovery mass of individual compounds		I	
	enrichment		I	
	composition of active fractions and analytical method		I	
	purity of target analytes and determination method		I	
	partition coefficient (K) of target analytes		I	
	percent recovery of target analytes			O

[a] Although column and sample loop volumes may be given by the manufacturer, they can vary from instrument to instrument and should be measured experimentally and reported as such.

resolved from other analytes. The presence of large quantities of lipophilic or hydrophilic matrix compounds in the sample may also influence the decision about which phase is mobile.

On a philosophical note, the CS technique holds to a theorem developed by the celebrated 20th century mathematician Emmy Noether. The theorem states that for every occurrence of symmetry in the laws of physics, there must exist a conservation law, and for every conservation law, there must exist a continuous symmetry.[227] In CS all of the analytes introduced into the column are conserved, while the symmetry properties of CS predict that elution in reversed phase will be the mirror image of the normal phase elution.

Reporting of Experimental Data. The routine reporting of CS experimental data encompasses three areas: instrumental parameters, operational conditions, and sample information. Instrumental parameters refer to factors that describe the type of machine, its dimensions, and column capacity. Operations conditions are those parameters that are determined or observed by the operator for each CS experiment such as flow rate, rotational speed, SS composition, S_f volume ratio, and sample-related information. The results of the present CS literature survey with regard to reporting of CS data are compiled in Section S6 (Supporting Information). Based on a summary of the entirety of CS parameters relevant to the method, Table 1 provides recommendations regarding which of the parameters are most important to ensure the repeatability of CS experiments when reporting the conditions in experimental sections. A systematic nomenclature of CS SSs is detailed in S7 (Supporting Information).

Conclusions and Outlook

Biological Activity and the Loss-Free Advantage of CS. Natural products are most valued as structurally diverse and phylogenetically evolved single *chemical* entities (SCEs) on one hand and as *biologically* active (re)agents capable of acting as endogenous or exogenous ligands or modifiers on the other. Excellent recent reviews have documented the importance of natural products in drug discovery and as pharmacological and toxicological tools.[228–231] The single most emphasized advantage of CS results from its restriction to the straight use of (evaporable) solvents and its ability to operate loss-free. While there are practical limitations in achieving 100% recovery, these constraints can be neglected from the perspective of biomedical research: There is no hidden loss of analytes, and observed loss cannot be the result of *selective* adsorption, which is widely known to occur when using solid stationary phases in LC.

The lack of absorption, paired with the absence of potential catalytic degradation chemistry common to solid adsorbents, secures

CS a unique niche in high-resolution separation methodology. It also confers a distinctive meaning on the loss-free advantage, in that CS enables unique studies from a biological perspective. Examples are (a) the study of the complexity of composition and elucidation of underlying structures; the evaluation of the deviation of natural products from (b) single chemical entity (SCE) and (c) single biological target (SBT) characters; and (d) the frequent limitation of source material. Putting CS to work and enhancing its further development has much potential to foster the rational separation of multiple chemical species from complex matrixes (a), to provide access to well-characterized biological agents with well-defined (stereo)chemistry and purity (b and c), and to save valuable resources in terms of both source material and research supplies (d), respectively. Two recent studies serve as examples of the potentially unique application of CS to address SCE/SBT problems: the investigation of synergy in (ethno)botanicals[223,232] and the concept of chemical subtraction.[222,233]

In summary, what can the natural products researcher expect from CS technology? From an analytical chemistry point of view, CS offers very high-resolution separations in the "sweet spot", side-by-side with (apparent) poor resolution in the regions where retention is close to zero or infinity. This situation is expected to improve soon, as have shown most recent developments that utilize the high-resolution inside the column by applying extrusion methods (EECCC, BECCC). Mechanistically, CS also offers a unique mechanism, i.e., pure partition chromatography, paired with the challenges and opportunities of a liquid stationary phase that is available for dynamic reaction chemistry. Examples for the latter are the pH zone-refinement method (ref 234 and see above) and precipitation.[235,236] Generally, CS's combination of attributes offers unmatched potential to address important challenges in the integrated evaluation of biologically active natural products. Recent examples are in the evaluation of synergy in complex mixtures and the quantitative assessment of the pharmacological parameters of fractions and isolates.

Areas of Future Growth. Considering both recent developments and practical experience with existing CS technology, an expected major area of expansion is the development of new CS instrumentation. In order to bring the numerous benefits of CS to an increasing number of research and application laboratories, factors such as dependability and simplicity, paired with a healthy degree of automation, can be considered key criteria of successful progression. The establishment of ISCCC as a professional organization for global exchange of CS knowledge and advancement marks an important milestone in the concerted development of CS technology. This professional platform is well supplemented by the biannual CCC conferences (http://www.ccc20[xx].org) and online resources (http://www.countercurrent.org and http://www.theliquidphase.org).

Acknowledgment. The authors are grateful to the following individuals for stimulating discussions of selected topics of this work, which have profoundly shaped the author's view of the countercurrent subject and its perspective for bioactive natural products research: Dr. A. Berthod (University of Lyon, Villeurbanne, France), Dr. N. Farnsworth (UIC, Chicago, IL), Dr. H. Fong (UIC, Chicago, IL), Dr. S. Franzblau (UIC, Chicago, IL), Dr. G. Harris (Merck Research Laboratories, Rahway, NJ), Dr. Y. Ito (NHLBI/NIH, Bethesda, MD), Dr. D. Lankin (UIC, Chicago, IL), and Dr. I. Sutherland (Brunel University, Uxbridge, UK).

Supporting Information Available: Supporting text, figures, and tables. This information is available free of charge via the Internet at http://pubs.acs.org.

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NP800144Q