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ARTICLES

Performance Characteristics of Countercurrent Separation in Analysis of Natural Products of Agricultural Significance

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A standard test mix consisting of 21 commercially available natural products of agricultural significance, termed the GUESSmix, was employed to measure the countercurrent chromatography performance characteristics of a very popular quaternary solvent system family made up of hexane−ethyl acetate−methanol–water (HEMWat). The polarity range of the GUESSmix combined with the elution−extrusion countercurrent chromatography (EECCC) technique and the newly developed reciprocal symmetry (ReS) and reciprocal shifted symmetry (ReSS) plots allow liquid–liquid distribution ratios (K_D) to be plotted for every compound eluted on a scale of zero to infinity. It was demonstrated that 16 of the 21 GUESSmix compounds are found in the optimal range of resolution ($0.25 < K_D < 16$) of at least one HEMWat solvent system. The HEMWat solvent systems represented by the ratios 4:6:5:5, 4:6:4:6, and 3:7:4:6 possess the most densely populated optimal ranges of resolution for this standard mix. ReS plots have been shown to reveal the symmetrical reversibility of the EECCC method in reference to $K_D = 1$. This study lays the groundwork for evaluation and comparison of solvent system families proposed in the literature, as well as the creation of new solvent system families with desired performance characteristics.

KEYWORDS: GUESSmix; HEMWat; high-speed countercurrent chromatography; HSCCC; ReS plots; elution−extrusion countercurrent chromatography; EECCC

INTRODUCTION

The chemical analysis of food demands a wide range of chromatography methods to separate and characterize individual natural products, which are contained in complex mixtures and are embedded in complicated matrices. Because of its ability to achieve high-resolution separations, countercurrent chromatography has been shown to play a significant role in the analysis of food products. In particular, the analysis of secondary natural products of health interest contained in functional foods has been demonstrated, among others, by the work with cranberry phytochemicals (*1*), glucoraphanin from broccoli (*2*), tea catechins (*3, 4*), various components of wine (*5–10*), soy isoflavones (*11*), anthocyanins from fruits (*12–15*), and antioxidants (*16, 17*). Moreover, countercurrent methodologies have played a role in the identification and removal of contaminants and toxins in food in the cases of deoxynivalenol from moldy corn and rice (*18*), olitrem B from endophyte-infected ryegrass (*19*), staphylococcal enterotoxin A from milk (*20, 21*), and GGPL-I and GGPL-III from *Mycoplasma fermentans* (*22*). Further research topics in which countercurrent separations have been useful are the analysis of pigments, flavors, and aromas from various food plant sources (*9, 17, 23–25*).

Countercurrent separation (CS) is a powerful liquid-based method for the isolation of food ingredients and phytochemicals in general. CS technology has been implemented at any level of sample load from analytical to process scale and is often referred to as (high-speed) countercurrent chromatography [(HS)CCC] and (centrifugal) partition chromatography [(C)PC]. There are several advantages of this type of separation: extensive preparation of a solvent, supercritical fluid, or essential oil extract is not necessary; all compounds introduced to the column are recovered; the structural integrity of components is preserved

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Table 1. HEMWat Solvent System Family

HEMWat system no.	relative proportions of solvents			
	hexane	ethyl acetate	methanol	water
−8	10	0	10	0
−7	9	1	9	1
−6	8	2	8	2
−5	7	3	7	3
−4	7	3	6	4
−3	6	4	6	4
−2	7	3	5	5
−1	6	4	5	5
0	5	5	5	5
+1	4	6	5	5
+2	3	7	5	5
+3	4	6	4	6
+4	3	7	4	6
+5	3	7	3	7
+6	2	8	2	8
+7	1	9	1	9
+8	0	10	0	10

in a liquid–liquid environment; maximum surface area interaction between the two phases allows for optimal use of both phases; and, once an appropriate solvent system is selected, separation scale-up is straightforward, because the liquid–liquid distribution ratio (K_D) is independent of column volume, flow rate, stationary phase retention, and length of the chromatographic run. The distribution ratio is defined as the concentration of a particular compound in the stationary phase divided by the concentration of the compound in the mobile phase. Representing a key parameter in countercurrent analysis, knowledge of the K_D of analytes is key to the design of CS methods and allows the arithmetic prediction of the separation based on instrument parameters (*26*).

The choice of the two-phase solvent system is the most critical and often the most time-consuming aspect of CS. Compared to the far more popular solid-support chromatography, the selection of CS solvent systems is equivalent to concurrently choosing both column and eluant. Solvent system choice can be divided into two operations: the choice of a solvent system "family" and the selection of component solvent proportions. A solvent system family is created by combining two or more solvents that form a biphasic system when mixed. The relative proportions of the constituent solvents within a family can be modified almost endlessly; therefore, organized systems of solvent system family members have been developed in the CS literature (*27–30*). Biphasic solvent systems composed of varying concentrations of hexane−ethyl acetate−methanol–water are used extensively to separate and isolate phytochemicals from extracts (*31–40*). The HEMWat family of 17 hexane−ethyl acetate−methanol–water solvent systems has been constructed with a progression of polarity from most polar (+8) to least polar (−8), as shown in **Table 1**. A solvent system family organizes the potentially unlimited number of combinations into a manageable, yet representational series of solvent systems.

A recent innovation in CS methodology allows the continuous elution of all the analytes in a mixture in one chromatographic run without the need for a solvent gradient (*41*). The elution−extrusion method (EECCC) employs the fact that the stationary liquid "column" used in the first two phases of elution may be eluted in its entirety during the last phase of the chromatographic run. As a result, analyte resolution is retained and the K_D may be calculated for each compound on the basis of its elution or exclusion volume. Until now, countercurrent chromatograms have been plotted with time or volume on the *x*-axis. This practice makes it impossible to represent the relative and absolute K_D values corresponding to each eluted peak. Reciprocal symmetry (ReS) and reciprocal shifted symmetry (ReSS) plots have been recently proposed to allow K_D to be plotted for every compound eluted in EECCC on a scale of zero to infinity (*42*). ReS plots clearly demonstrate that the value of K_D for each analyte in a given solvent system is independent of the length of a chromatographic run (*41*). Another application of ReS/ReSS plots is the direct visual comparison of changes in K_D for the same mixture of analytes separated in different solvent systems (*43*). Since K_D is independent of column volume, ReS/ReSS plots may also be used to compare performances of different countercurrent instruments by separating the same mixture of compounds in the same solvent system on instruments of various volumes or geometries.

Building a bridge between complex natural samples, such as foods and agricultural products, and theoretical models of CS, a mixture of natural products that represents the diverse polarities, structural characteristics, and functional groups found in natural product extracts has been developed as a means of modeling the behavior of diverse analytes in CS (**Figure 1**) (*44*). Experiments with this mixture have clearly shown the value and necessity of optimizing chromatographic conditions in order that the analytes of interest occupy a region of optimal resolution determined by their K_D values. Combined with ReSS plots, this mixture of natural products has very recently been shown to also be a powerful tool in evaluating solvent systems and solvent system families (*43*).

Evaluating solvent systems continues to be a major challenge of CS. Not only is there a wide choice of solvent system families with a particular combination of solvents (e.g., hexane−ethyl acetate−methanol–water), but the relative volumes of the solvent components can be varied in an infinite manner. There is, therefore, a great need for methodologies by which to evaluate and predict solvent system behavior for a variety of analytes to determine the selection of both composition and volume ratio of solvents so they can successfully be used in a separation procedure. Thus, methodology by which solvent system performance can be measured is in demand as it is crucial for the systematic exploration of the varied chemical constituents of foods and agricultural products using CS.

MATERIALS AND METHODS

Instrumentation. High-speed countercurrent chromatography (HSCCC) was performed on a Model CCC-1000 J-type instrument (Pharma-Tech Research Corp., Baltimore, MD). It consisted of a self-balancing three-coil centrifuge rotor and coils wrapped with 1.6 mm internal diameter of PTFE (Teflon) tubing to a volume of 40 mL each. The distance between the coil holder axis and central axis of the centrifuge (R) was 7.5 cm. The β ratio (β_r) varied from 0.73 at the head to 0.47 at the tail ($\beta_r = r/R$, where r is the spool radius and R is the rotor radius). The rotation of the coil assembly relative to the coil winding situated the head at the periphery position. Furthermore, the HSCCC system included a Laboratory-Alliance Series III digital single-piston solvent pump, a Shimadzu SPD-10A UV/vis detector with preparative flow cell, and a fraction collector. Chromatogram data were collected with a Cole-Parmer 80807-00 modular paperless recorder and transferred in digital form to an Excel spreadsheet.

Thin-Layer Chromatography. Collected fractions were reduced in volume and analyzed with TLC at room temperature. Alugram 20 × 20 cm precoated 0.20 mm thick silica gel G/UV_{254} aluminum plates (Macherey-Nagel, Germany) were cut to 9.5 cm × 20 cm before spotting. Plates were dipped in the general purpose reagent (4% *p*-anisaldehyde, 4% sulfuric acid, 92% acetic acid), drained, and heated on a Camag TLC plate heater III at 95 °C for about 5 min. Digital preservation of all TLC chromatograms was achieved with a Canon CanoScan LiDE20 scanner.

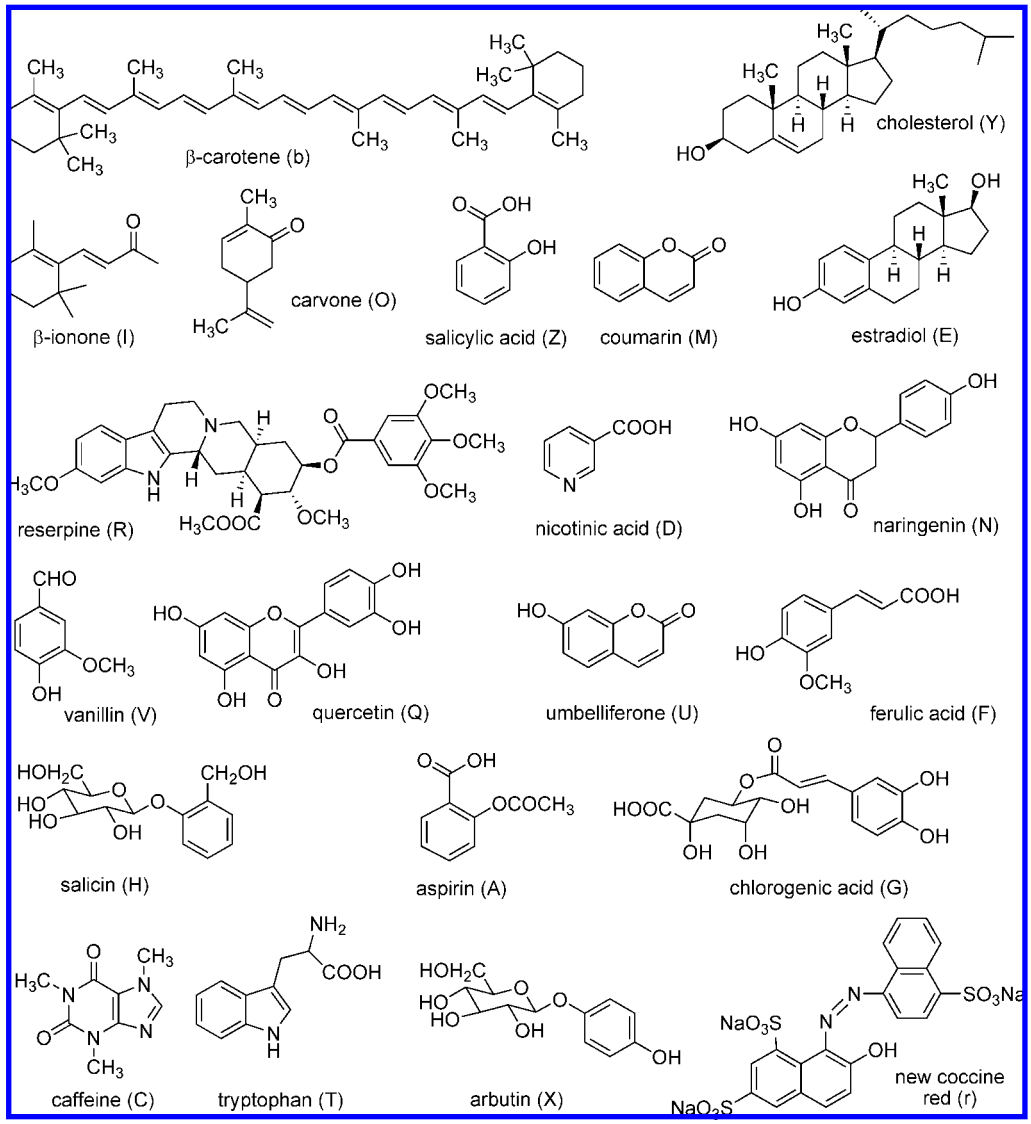

Figure 1. The GUESSmix compounds and their single letter abbreviations.

Chemicals. HPLC grade solvents were purchased from Fisher Scientific or Sigma-Aldrich. GUESSmix component chemicals were purchased from the Sigma Aldrich Fluka group (St. Louis, MO, and Milwaukee, WI). **Figure 1** lists the 21 GUESSmix compounds employed in this study. The biphasic liquid system selected is the mixture of hexane−ethyl acetate−methanol–water in various volume ratios as defined in **Table 1**. The stationary phase was the lighter organic phase, and the mobile phase was the denser aqueous phase.

HSCCC Procedures. Samples of GUESSmix compounds were prepared as previously described (*43*), using a stock solution with a final concentration of approximately 0.1 g/mL of combined compounds. The stock solution was stored at −30 °C and warmed to room temperature before use. The GUESSmix compounds were prepared for chromatography by drying 2.2 mL of the stock solution under forced air, and the resulting residue was then suspended in equal volumes of upper and lower phase of the solvent system. The biphasic mixture of GUESSmix compounds was then filtered and loaded into a 2 mL sample loop.

The solvent system was thoroughly mixed, vented, and allowed to separate into two distinct phases before use. The lipophilic lighter stationary phase was initially pumped into the column with no rotation. Then the coils were rotated at 1200 rpm as the hydrophilic denser mobile phase was pumped at a flow rate of 1 mL/min entering the column head. In order to observe the volume of stationary phase eluted from the column, the resulting effluent was collected in a graduated cylinder. The hydrodynamic equilibrium was considered to be established when the volumes of the two phases of the eluant were approximately equal. The standard compound mixture was injected on the column, the fraction collector started, and the recorder turned on. All fractions were collected at 3 min per tube. After a predetermined volume (V_{CM}, also called the switch volume) of aqueous mobile phase had eluted from the column, the organic phase was pumped into the column, marking the beginning of sweep elution and subsequent extrusion, and also entering through the column head. After the lipophilic marker, β-carotene, eluted from the column (120 mL after V_{CM}), the run was discontinued. The collected fractions were reduced in volume, and TLC was performed to corroborate the UV/vis data.

RESULTS AND DISCUSSION

Correlating Shake Flask Partition Coefficients with CS Distribution Ratios. One of the best-known ways to determine the polarity of a molecule is to measure or calculate the partition coefficient of the molecule in a biphasic mixture of octanol and water ($K_{octanol/water}$) (*45*). The partition coefficient is the concentration of an analyte in the upper phase divided by the concentration of the same analyte in the lower phase of an equilibrated biphasic solvent system. The partition coefficient of an analyte in any biphasic mixture is related to the liquid–liquid distribution ratio, K_D, of the same analyte in a countercurrent separation experiment as calculated by $K_D = (V_R$

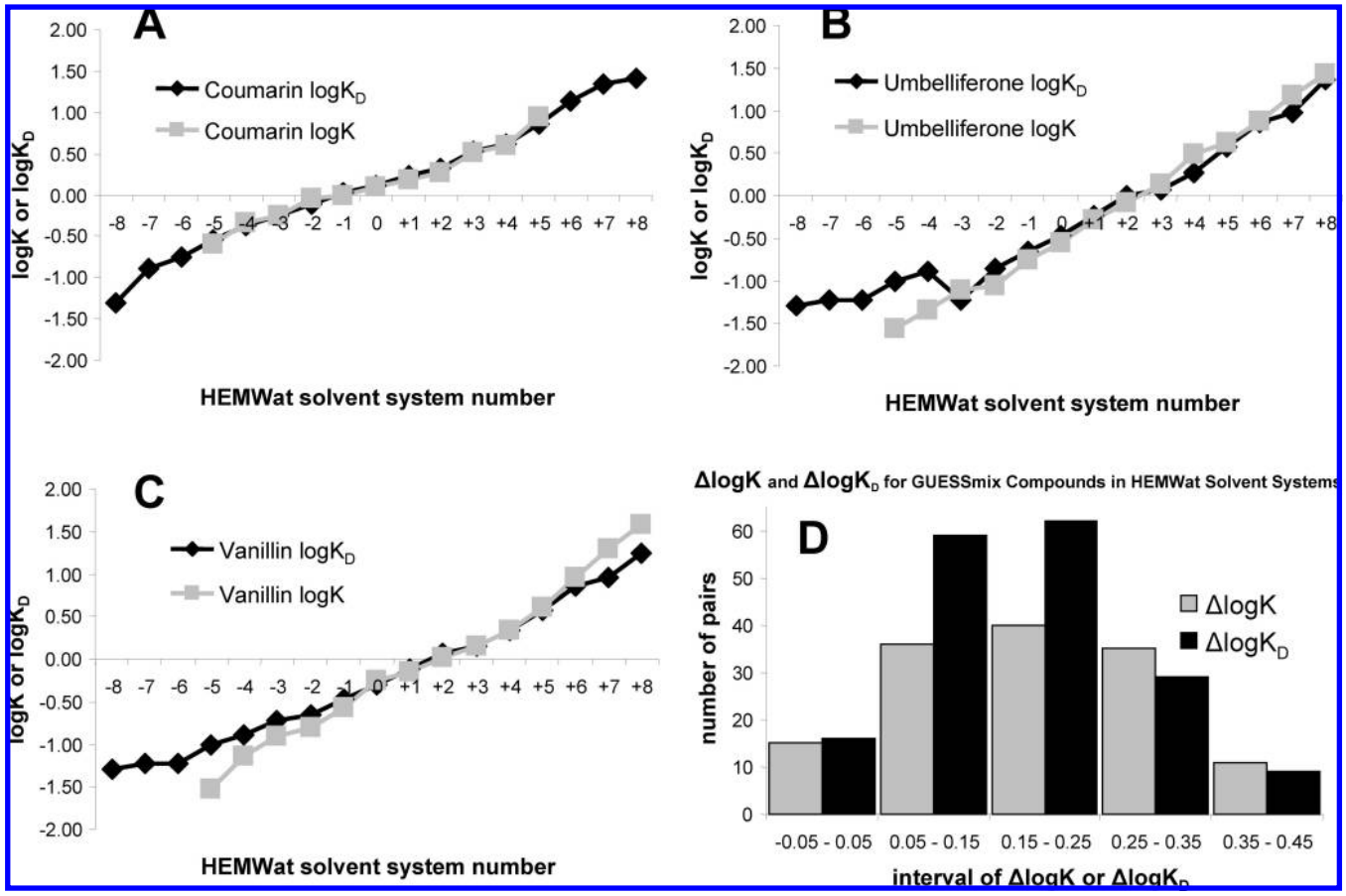

Figure 2. Comparison of shake flask partition coefficients (K) and liquid–liquid distribution ratios (K_D) for three compounds in HEMWat solvent systems: (**A**) coumarin; (**B**) umbelliferone; (**C**) vanillin. (**D**) Frequency of Δ log K_D values for 14 GUESSmix compounds.

$- V_M)/V_S$ (*26*). 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 four conditions are met. First, it is assumed that both processes have reached equilibrium. The shake flask experiment represents a static equilibrium whereas the countercurrent process is a dynamic equilibrium. Second, a 1-to-1 volume ratio in the shake flask experiment corresponds to the dynamic equilibrium conditions of the countercurrent process. Third, 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. Fourth, the compound is present in the same chemical form in each process. Ionization and solute–solute interactions, such as dimerization of carboxylic acids, would change the chemical form of an analyte in solution. Indeed, despite the exigencies of these conditions, CCC has been used to determine $K_{octanol/water}$ of compounds in certain cases (*46–49*).

There are at least three practical considerations that come into play, however, when comparing shake flask K values with K_D values calculated from a CS run. First, it must be kept in mind that the two values are measured by different means. Shake flask K values are typically determined by UV absorption ratios either directly or after HPLC separation. On the other hand, the value of K_D is determined by selecting a peak position for the retention time of the analyte in order to determine the retention volume, V_R, of the analyte. Second, the influence of other compounds present in the mixture may be significant. Shake flask partition coefficients were determined with a single compound in a biphasic system in this study. By contrast, distribution ratios were determined as part of the GUESSmix separated over the course of a CS experiment. Third, proximity to one is a factor for both methods. Experimental calculations of both K and K_D above 10 and below 0.1 lose some precision since the measurements for numerator and denominator differ by a factor of 10 or more.

In **Figure 2A−C** shake flask partition coefficients are compared with liquid–liquid distribution ratios for three different analytes. As expected, the correlation for K and K_D is closest for values between 10 ($\log_{10} = 1$) and 0.1 ($\log_{0.1} = -1$). Overall, the correlations are quite close considering the theoretical conditions for equivalence and practical experimental considerations described in the preceding paragraphs.

Linear Behavior of Both log K and log K_D Plots. The linearity of the resultant log K/log K_D plots in **Figure 2A−C** was not an expected outcome of this study. The solvent combinations chosen to make up the HEMWat family do not necessarily indicate that the log K_D plots will be linear. As seen from **Figure 2D**, the majority of the slopes of the log K_D plots fall in a narrow range. This provides a way to predict the K_D of an analyte in any HEMWat solvent system given its K_D in one HEMWat solvent system by employing the average slope of $\triangle\log K_D = 0.16$.

Solvent System Family Mapping. The shaded area of **Figure 3** corresponds to the region of optimal resolution for this particular series of solvent systems. The region of optimal resolution between $0.25 \leq K_D < 16$ was chosen by consideration of literature reports of optimal separation (*26, 27*) as well as the chromatograms generated by this particular series of

K_D intervals	0 ≤ K_D < 0.0625	0.0625 ≤ K_D < 0.125	0.125 ≤ K_D < 0.25	0.25 ≤ K_D < 0.5	0.5 ≤ K_D < 1	1 ≤ K_D < 2	2 ≤ K_D < 4	4 ≤ K_D < 8	8 ≤ K_D < 16	16 ≤ K_D < 32	32 ≤ K_D < ∞
-8	rXHTDC GRFQUA VNMEZ				OI	Y					b
-7	rXHTDC GRFQUA VNE		MZ		O	I		Y			b
-6	rXHTDC GRFQUA VNE		MZ			O	I				Yb
-5	rXHTDC GRAV	FQUN E		MZ			O	I			Yb
-4	rXHTDC GR	AVFQ U	NE	M	Z		O		I		Yb
-3	rXHTDC GR	FQU	AVN	E	MZ		O		I		Yb
-2	rXHTDC GR	FQU	AVN		ME	Z	O		I		Yb
-1	rXHTDC G		RFQ U	AVN		MEZ		O		I	Yb
0	rXHTG	DR	CF	QUAV	N	ME	Z	O		I	Yb
+1	rXHT		GRD C	F	QUAV	NM	EZ	O		I	Yb
+2	r	XHT		DCG	RF	QUAV	NMEZ	O		I	Yb
+3	r	XHT		DCG	RF	QUAV	M	NEZ		O	IYb
+4	rXHT			DCG		RFU	QAV	NMEZ		O	IYb
+5	r	XHT		DC	G		RFUA V	M		QZ	NEOI Yb
+6	r	XHT		D	CG	F		RUAV	M	Z	QNEO IYb
+7	r	XHT		D	CG	F			RUAV	MZ	QNEO IYb
+8	rXHT			D	CG					FRUA VM	Z QNEO IYb

Figure 3. Solvent system family map illustrating the whole range of K_D values for 21 compounds in 17 different HEMWat solvent systems.

experiments. At this point, there is no established interval, or formula for deriving the interval, corresponding to the region of optimal resolution. However, the traditional understanding of the region of optimal separation as centered around $K_D = 1$ has been modified due to the EECCC method, which has the effect of extending the region of optimal resolution to K_D values represented by retention volumes greater than one column volume (*50*).

The HEMWat family of solvent systems has the capacity to separate a varied range of phytochemicals as exemplified by the observation that 16 of the 21 representative compounds are found in the region of optimal resolution in at least one HEMWat solvent system. In fact, compounds occupying the designated region of optimal resolution represent 115 (32%) out of 357 data entries in **Figure 3**. The polarity range of those GUESSmix compounds found in the region of optimal resolution possess log $K_{octanol/water}$ values from −1.88 (chlorogenic acid) to 9.52 (cholesterol). The GUESSmix compounds themselves represent a wide range of polarities, most of which have log $K_{octanol/water}$ values that suggest good absorption and permeability drug characteristics. Certainly, there is an interest in separating compounds which exhibit polarities outside of this range;

nevertheless, the HEMWat solvent system family cuts a wide swath through the range of compound polarities that are likely to be of interest to the agricultural chemist.

When compared to previously evaluated solvent system families, such as chloroform−methanol–water, ethyl acetate−butanol−water, and *tert*-butyl methyl ether−acetonitrile−water, the HEMWat family has a wider continuous polarity range than other solvent system families (*43, 44*). In other words, the HEMWat solvent system family is a good first try when targeting the separation of a compound or series of compounds that have no precedence in CS. Unless the polarity of the target compound lies outside of the fairly wide range of HEMWat-compatible values, the compound(s) will likely be separated with acceptable resolution in one or more of the HEMWat solvent systems. This proposition is affirmed by the overwhelming popularity of solvent systems composed of hexane−ethyl acetate−methanol–water, and closely related mixtures thereof, in CS. For example, in a recent review article, hexane−ethyl acetate−methanol–water solvent systems were employed in one-third of all reported (20 out of 60 reviewed) CS applications (*51*).

The distribution of K_D values in a given solvent system is affected, certainly, by the choice of compounds for this study. The GUESSmix was conceived as an instrument to allow comparison between solvent systems and not as an absolute measure of solvent system polarity or performance. Useful information on the comparative polarity and selectivity between solvents may be gained by such an approach, particularly when the compounds in the test mix represent a range of molecular weights, functional groups, and polarities as determined by their log $K_{octanol/water}$ values (*43*).

Another feature of the HEMWat solvent system comparison may be ascertained from **Figure 3**. The solvent systems termed HEMWat +2, +3, and +4 have the highest population of compounds in their respective regions of optimal resolution while the populations decline steadily on both the more polar and less polar sides. Therefore, HEMWat +3 likely represents a portal with which to enter this solvent system family. If a compound mixture is tested with HEMWat +3, and the target compound(s) is (are) not present in the region of optimal resolution, it is unlikely that other HEMWat solvent systems will be able to resolve these compounds; thus the portal designation of HEMWat +3. However, if a compound mixture is tested with the HEMWat +3 and the target compound(s) is (are) present in the region of optimal resolution but not well resolved, it may be useful to try other members of the HEMWat solvent system family to better resolve these compounds.

Polarity Comparison. The relative polarities of solvent systems may be compared by dividing compounds into those with $K_D \leq 1$ and those with $K_D > 1$ for a particular solvent system. In **Figure 4**, the values describing the number of compounds with $K_D \leq 1$ for the HEMWat solvent system family cross the midpoint of the *y*-axis between +1 and +2. This overlaps with the series of solvent systems with the most highly populated regions of optimal resolution. This method of measuring relative polarities may be used to compare the HEMWat solvent system family to previously published solvent systems (*43*). It can be seen from **Figure 4** that HEMWat covers a much larger polarity range than ethyl acetate−butanol−water (EBuWat) and *tert*-butyl methyl ether−acetonitrile−water (*ter*-AcWat). The polarity range of HEMWat and hexane−*tert*-butyl methyl ether−acetonitrile−water (H*ter*AcWat) is similar, but HEMWat covers it continuously, whereas H*ter*AcWat does not.

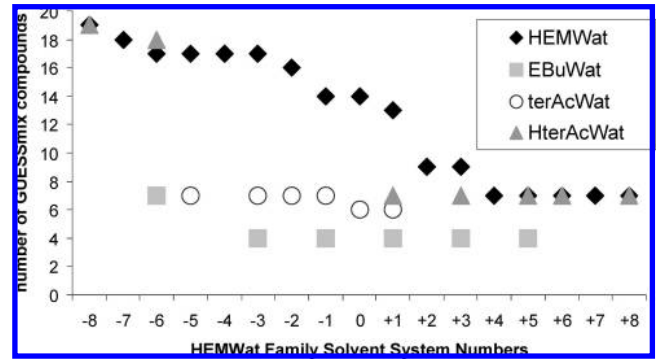

Figure 4. Number of GUESSmix compounds (out of 21) which have K_D values less than or equal to 1 in a variety of solvent systems. Data for ethyl acetate−1-butanol−water (EBuWat), *tert*-butyl methyl ether−acetonitrile−water (*ter*AcWat), and hexane−*tert*-butyl methyl ether−acetonitrile−water (H*ter*AcWat) are found in a previous work (*43*).

This offers yet another explanation for the popularity of the HEMWat solvent system family.

ReSS Plots. ReSS plots are particularly useful in comparing the behavior of the GUESSmix in HEMWat solvent systems. Since the *x*-axis of the ReSS plot is in terms of K_D, and not in volume, it offers a direct way to compare solvent systems with much more richness than a solvent system map. A reasonable approach to decide where to situate the midline of the ReSS plot is that it should be approximately at the volumetric center of the run. The volumetric center is the point where half of the volume of a chromatographic run has been eluted. If readings are taken, or fractions collected, at regular volume intervals, the volumetric center is also the center of data points. For example, the volumetric center of the HEMWat –2 experiment in **Figure 5** is at $V_R = 210$ mL, which corresponds to $K_D = 2.15$. Thus, placing the midline at $K_D = 2$ is most appropriate as it balances the number of data points on each side of the *x*-axis and yields peak shape symmetry along the midline that is compatible with the other chromatograms.

The ReSS plot chromatograms in **Figure 5** show the chromatograms of four selected solvent systems represented in **Figure 3**. ReSS plots of chromatograms reveal the high-resolution capabilities of CS as well as the displacement of individual analytes to higher K_D values as the polarity of the solvent system increases. The ReSS plot chromatograms also show the shape and resolution of the compound peaks that are not well represented in the solvent system map in **Figure 3**. For HEMWat –6, the majority of the GUESSmix compounds are gathered in the unresolved polar region with $0 \leq K_D < 0.25$. As the HEMWat numbers increase, most of these compounds migrate across the region of optimal resolution, $0.25 \leq K_D < 16$, and end up gathered together in the unresolved nonpolar region with $16 \leq K_D \leq \infty$ of HEMWat +6.

Symmetrical Nature of Countercurrent Separations. ReSS plots make it possible, for the first time, to experimentally demonstrate the full symmetrical reversibility of the CS method between normal phase and reversed-phase modes, as shown in **Figure 6**. When the lower phase is the mobile phase of the CS experiment, then $K_D = K$. However, if the upper phase is the mobile phase, then $K_D = 1/K$. Therefore, the normal phase is plotted backward (and upside down) in **Figure 6**. Generally, the extrusion peaks in stage III of the EECCC run are narrower and better resolved than the peaks in classical elution, EECCC stage I. This is because peak resolution is directly proportional to the time the compound spends in the column (*41*).

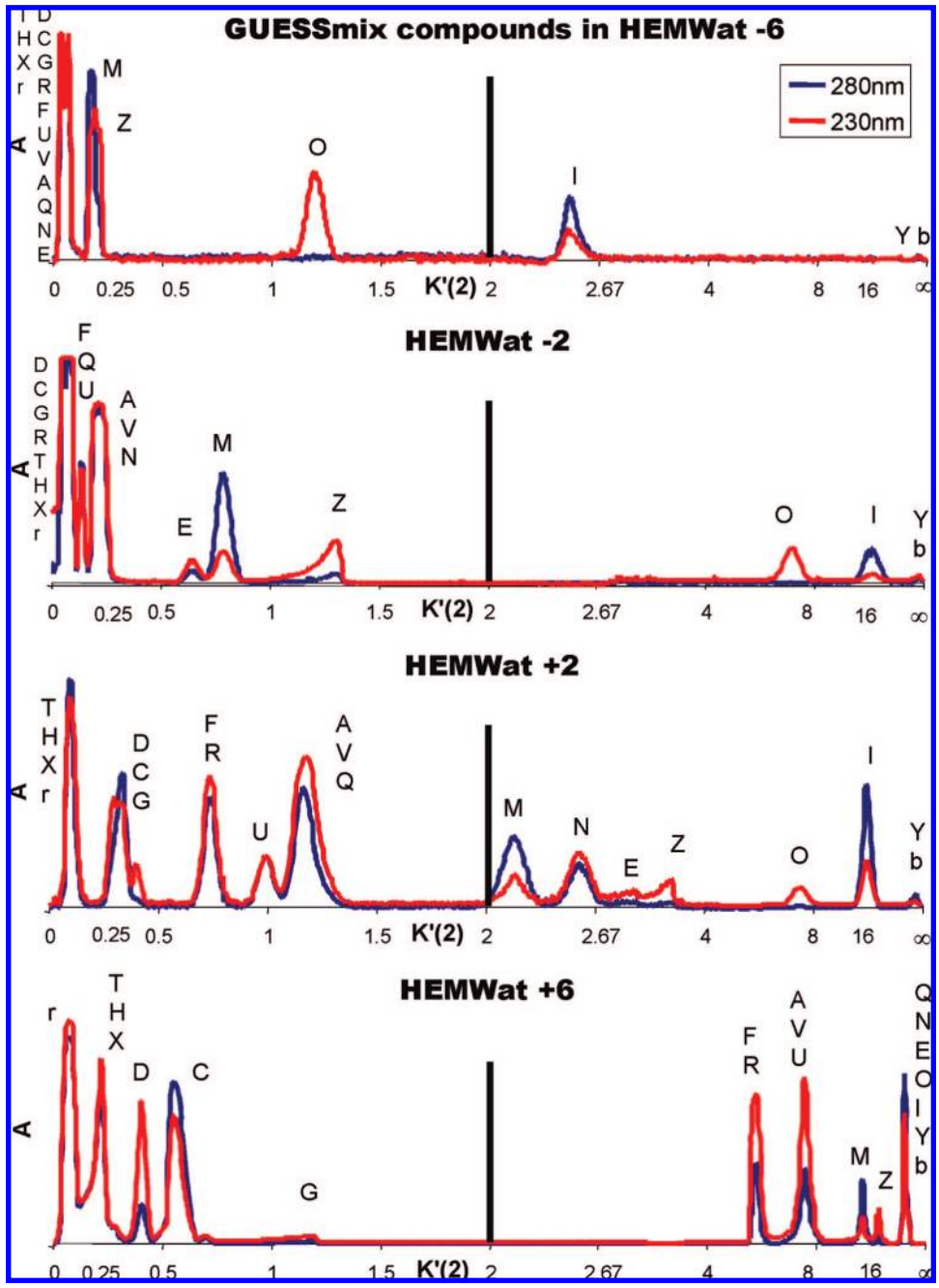

Figure 5. ReSS plots centered at $K_D = 2$ for the chromatography of the GUESSmix compounds in four HEMWat solvent systems.

This study establishes EECCC paired with ReSS plots and HEMWat +3 as the biphasic solvent system as a portal method to develop CS methods for the separation of food constituents and other complex natural mixtures. Utilizing an array of K_D values of the standardized GUESSmix, performance of CS separations can be predicted for unknown analytes that fall into the range of HEMWat polarities. The full symmetry of normal and reversed-phase CS, the simplicity of the GUESSmix-based polarity matching, and the general advantages of CS as a liquid–liquid separation technology are added advantages. The present study also lays the groundwork for evaluation and comparison of other solvent system families proposed in the literature, as well as for the creation of new families with desired performance characteristics. Experiments to expand the current framework of CS solvent systems are underway in our laboratory.

ABBREVIATIONS USED

Centrifugal Partition Chromatography (CPC). Historically, CPC refers to the hydrostatic methods of countercurrent chromatography, which use centrifugal force (counter)current flow emerging from revolution around only one axis. CPC instruments use rotating seals and generally operate at higher flow rates and higher back-pressures than hydrodynamic CCC instruments.

Classical Elution. Elution of analytes with the mobile phase being pumped through the column while the stationary phase is being held in the column through, e.g., centrifugal force. See also EECCC.

Column Volume. One of the principal instrumental parameters in countercurrent separation is the total volume of the column, which is important when understanding analyte elution

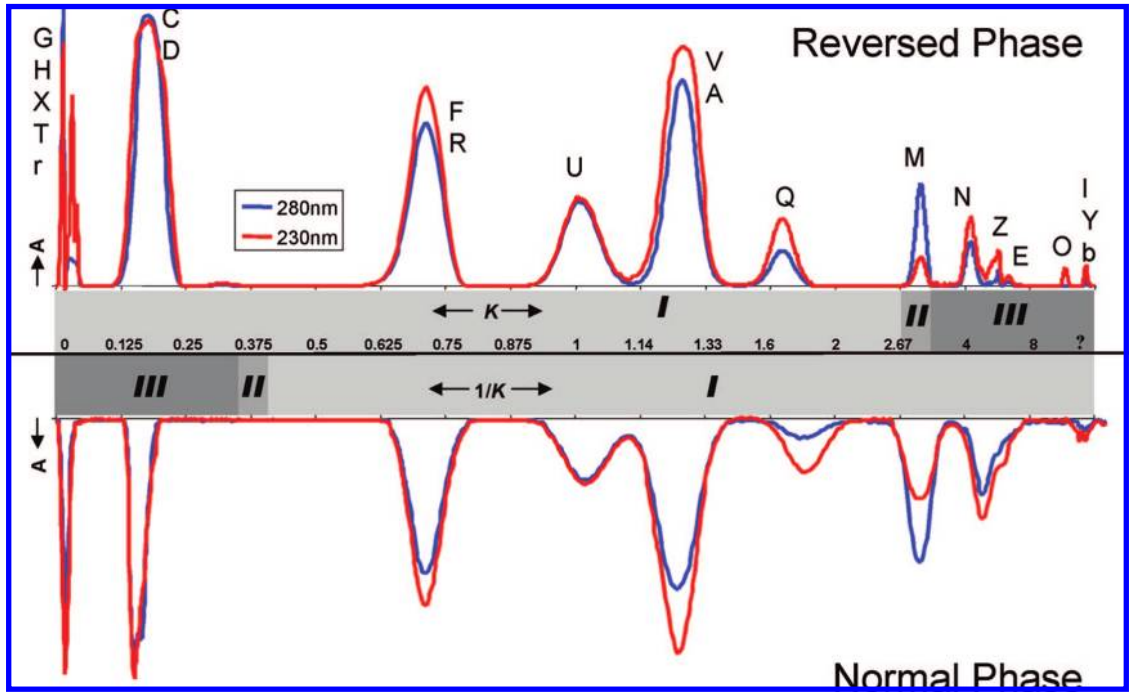

Figure 6. ReS plots for GUESSmix in HEMWat +3 in both reversed-phase and normal phase modes. The normal phase chromatogram is plotted backward and upside down to share the same *x*-axis with the reversed-phase plots. The Roman numerals I, II, and III represent the three stages of EECCC: classical elution, sweep elution, and extrusion, respectively.

and when calculating countercurrent chromatograms. Total column volume (V_C) also determines the load capacity of a particular machine.

Countercurrent Chromatography (CCC). A continuous liquid–liquid partition separation where one liquid phase is immobilized by gravitational or centrifugal force, not by a solid support. The term "CCC" has been coined for equipment developed in the laboratory of Dr. Yoichiro Ito of the NIH (Bethesda, MD), ranging from the early droplet (DCCC) and rotation locular (RLCCC) to the centrifugal multilayer (MLCCC) and high-speed (HSCCC) instruments. Commercialized HSCCC instruments that are based on the coil planet centrifuge principle, lack a rotating seal and make use of centrifugal force for both phase mixing and phase separation. Historically, the acronym CCC refers to the hydrodynamic method of CCC.

Countercurrent Separation (CS). A general term that encompasses all modern forms of liquid–liquid separation techniques, including (HS)CCC and (F)CPC.

Elution Extrusion CCC (EECCC). A recently developed and fully parametrized CCC method (*41*) that takes advantage of the liquid nature of the stationary phase by combining classical elution and extrusion in a single run. EECCC allows coverage of the whole polarity range of analytes from $K_D = 0$ to ∞. After an initial elution stage, extrusion of the stationary phase is achieved by switching the supply of flowing liquid from the mobile phase to the originally stationary phase, while maintaining the centrifugal force through continued rotation. The point at which extrusion is begun (switch volume, V_{CM}) can be adjusted to optimize the resolution of target analytes and minimize the run time. When V_R is equal to $V_{CM} + V_C$ (V_C being the total volume of the column), all analytes will have exited the column.

Extrusion. The process of pushing out the stationary phase portion of the CCC column. After performing classical elution for a certain period of time, the non-eluted analytes have migrated inside the column. Extrusion provides access to these analytes without the need to reach the classical elution volume and can be achieved by pumping stationary phase into the column. The third stage of EECCC is called the extrusion stage.

GUESSmix. A mixture of commercially available natural products of certain size, polarity, and functional group composition, initially developed to provide a TLC-based method for the generally useful estimation of solvent systems (GUESS) in CCC (*44*). In subsequent studies it has been used to evaluate solvent system performance.

Head. In hydrodynamic columns, the head is the end of the coil where the liquid is pushed by the Archimedean screw force when the machine rotor is spun, i.e., the higher pressure column side.

HEMWat. An array of biphasic solvent systems created by mixing various proportions of hexane, ethyl acetate, methanol, and water, one example of a solvent system family.

High-Speed Countercurrent Chromatography (HSCCC). A hydrodynamic CCC system that uses a multilayer coil separation column and undergoes a type J synchronous planetary motion.

Mobile Phase. In order to equilibrate a CCC system, the column is first filled with stationary phase, then the column is rotated, and mobile phase is pumped into the column until mobile phase starts to elute. The mobile phase volume (V_M) remains constant throughout the classical elution stage of EECCC.

Multiple-Layer Countercurrent Chromatography (ML-CCC). Early variant of modern HSCCC instruments with columns that consist of multiples layers of coiled tubing.

Reciprocal Symmetry and Shifted Reciprocal Symmetry (ReS and ReSS) Plots. Graphical representations of CCC chromatograms capable of representing all K values, zero to infinity. In ReS(S) plots, K_D and $1/K_D$ are positioned on either side of a line of symmetry on the *x*-axis (K_D). ReS(S) plots demonstrate both the invertible and "symmetric" nature of CCC,

a consequence of the exchange of the mobile and stationary phases by reversing the direction of the flow and the symmetry of the liquid–liquid partitioning process between two immiscible phases, respectively.

Liquid–Liquid Distribution Ratio (K_D). The ratio of the concentration of an analyte in the stationary phase to its concentration in the mobile phase at equilibrium [$(K_D)_A = [A]_{stationary}/[A]_{mobile}$]. K_D may also be represented by K or K_c. The retention volume (V_R) of an analyte follows the classical elution equation $V_R = V_M + K_D V_S$. The equation shows the relationship between K_D and the experimentally measurable parameters of retention volume, mobile phase volume (V_M), and stationary phase volume (V_S). If a column is eluted only with mobile phase, it will theoretically take an infinite amount of time for an analyte that is exclusively soluble in the stationary phase ($K_D = \infty$) to exit the column. It shall be noted that there is currently no generally accepted definition for the parameter that describes the partition/distribution behavior in CCC; an IUPAC definition is pending.

Partition Coefficient (K). The partition coefficient is the concentration of an analyte in the upper phase divided by the concentration of the same analyte in the lower phase of an equilibrated biphasic solvent system ($K_A = [A]_{upper}/[A]_{lower}$).

Retention Volume (V_R). The volume at which a particular analyte elutes. Retention volumes are often calculated by multiplying the retention time and the flow rate and are a necessary component of the liquid–liquid distribution ratio calculation.

Region of Optimal Resolution. See Sweet Spot section.

Solvent System. A mixture of liquids in defined proportions that forms two (or three) phases and can be used for CCC.

Solvent System Family. Biphasic solvent systems for CS applications have traditionally been organized as families that are comprised of the same solvents mixed in varying proportions. Common families are hexane−ethyl acetate−methanol–water (HEMWat, **Table 1**), chloroform−methanol–water (ChMWat), and heptane−ethyl acetate−methanol–water (the "Arizona" family). Solvent system families provide a methodical means of searching for a particular solvent system that predicts a reasonable K_D value for the target compound(s) in CS.

Stationary Phase. Mobile phase is being pumped into the column while the stationary phase is held in the column, typically by centrifugal force. In order to equilibrate a countercurrent column, it is first filled with stationary phase, then the column is rotated, and mobile phase is pumped into the column until mobile phase starts to elute. The stationary phase volume (V_S) remains constant throughout the classical elution stage of EECCC.

Stationary Phase Retention. The volume of stationary phase retained in the CCC column, V_S, is experimentally measured and compared with other columns using the dimensionless stationary phase volume ratio or stationary phase fraction parameter, S_F: $S_F = V_S/V_C$ (V_C = total column volume).

Sweep Elution. In EECCC, sweep elution is the second, intermediate stage of elution. After the switch volume (V_{CM}), the original stationary phase is pumped into the column while the original mobile phase is eluting until depleted ("swept").

Sweet Spot. Region of a countercurrent chromatogram or working area of a countercurrent separation that exhibits optimal resolution of the analytes.

Switch Volume (V_{CM}). Volume at which a CCC separation is switched from elution (CM = classical mode) to extrusion; see EECCC.

Tail. In hydrodynamic columns, the tail is the end of the coil opposite to the head. It is also the lower pressure column side; the pressure may even be negative inducing suction.

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