Solvent Selection in Countercurrent Chromatography (CCC)

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1. The Basics - Biphasic solvent systems

The basic requirement for a CCC solvent system is that it consists of two immiscible phases (a biphasic solvent system). A particular compound will have a different relative solubility in each of the phases. Therefore, the compound is effectively distributed between the two phases. The distribution can be quantified by taking the concentration of the compound in the upper phase and dividing it by the concentration of the same compound in the lower phase. This ratio may be termed a "partition coefficient" or "distribution ratio" and symbolized by P, $K_{U/L}$ or K_D.

The distribution ratio determines how the compound will behave during a CCC separation. In general, the more soluble a compound is in the phase that has been chosen as a CCC mobile phase faster (lower volume) it will elute. If the compound is equally distributed in the two phases (K_D = 1) the compound will elute in one column volume no matter which phase has been chosen as the mobile phase.

1.a Octanol & Water

The simplest biphasic system consists of two immiscible solvent systems such as water and "oil." The most famous two solvent biphasic system is 1-octanol and water. The distribution of a compound between 1-octanol and water is a critical property of a compound that predicts its behavior in biological systems. This parameter is often written as the base 10 logarithm of the distribution coefficient $LogK_{ow}$ or LogP. This parameter is considered to affects drug absorption, bioavailability, hydrophobic drug-receptor interactions, metabolism of molecules, as well as their toxicity. LogP has become also a key parameter in studies of the environmental fate of chemicals. Several theoretical calculations of LogP (Log*P*, ClogP, IA LogP, miLogP etc.) are available for any real or imagined organic structure.

Interestingly, CCC has been used to determine experimental LogP values for a wide range of compounds.

Of course, many 2-solvent combinations are possible. It should be noted that even in immiscible solvent systems a small percentage of the upper phase solvent is dissolved in the lower phase and vice versa. This is why biphasic solvent systems must be equilibrated by shaking them together before they can be used.

1.b CCC 2-Phase Systems

A few 2-solvent biphasic solvent systems have been exploited in CCC separations, however, CCC solvent systems tend to be tertiary or quaternary. As the name suggests, tertiary biphasic solvent systems consist of three solvents. Typically, one of the solvents is water that forms an aqueous layer. At least one of the other solvent systems is immiscible with water and forms an "organic" layer. The third solvent may favor the aqueous phase, such as methanol, or the organic phase, such as ethyl acetate. The composition of solvents in each layer is complex because each solvent is found to some extent in each layer. Various attempts have been made to determine the composition of complex biphasic systems.

It should be noted that the aqueous phase may be the upper phase or the lower phase depending on the density of the aqueous phase. Hexane or other hydrocarbons are hydrophobic solvents that are less dense than water while chloroform and dichloromethane are common solvents that are more dense than water. This phenomenon must be taken into account when comparing the distribution coefficient of particular compound in a biphasic system such as hexane-ethyl acetate-water that has an organic upper phase with another biphasic system such as chloroform-methanol-water that has an aqueous upper phase.

Chloroform-methanol-water is a widely used solvent system in CCC. However, the use of chloroform has fallen out of favor and dichloromethane is used a reasonable and somewhat safer substitute for chloroform. An example of a family of solvent systems with consisting of chloroform-methanol-water is termed the ChMWat solvent system family:

ChMat system number	Chloroform	Methanol	Water
-3	10	0	10
-2	10	1	9
-1	10	2	8
0	10	3	7
+1	10	4	6
+2	10	5	5
+3	10	6	4
+4	10	7	3

In this solvent system family the volume of chloroform stays constant and equal to the sum of methanol and water volumes. The proportion of methanol to water increases incrementally from 0/10 to 7/3. The organic layer becomes less polar as the ChMwat system number increases.

Phase diagrams….

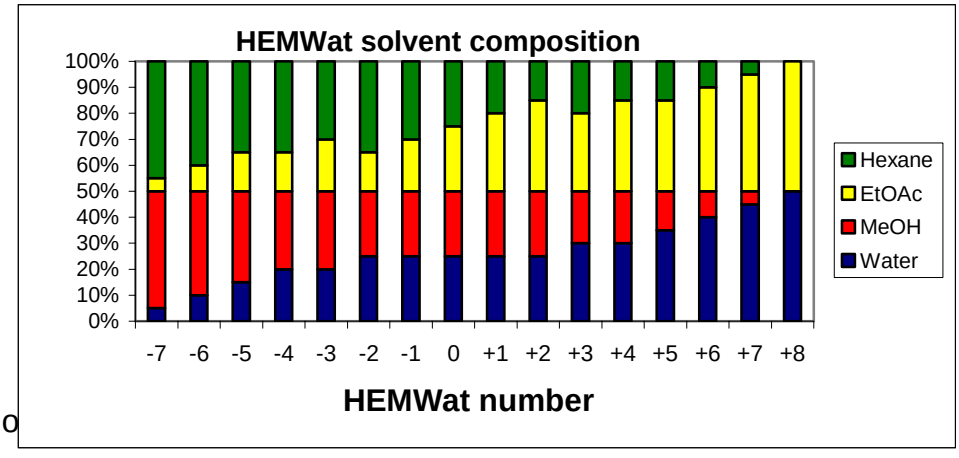
1.c. The HEMWat System

One popular method of concocting a solvent system involves the mixing of a hydrocarbon solvent such as hexane with ethyl acetate, methanol and water.

Since the relative proportions of 3 and 4-solvent biphasic systems can be varied, these solvent systems are usually presented a solvent system families. For example the hexane-ethyl acetate-methanol-water solvent system family termed the HEMWat system may be represented by the following family:

HEMWat system number	Hexane	Ethyl Acetate	Methanol	Water
-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

The HEMWat method was designed to provide a systematic process of choosing a CCC solvent system for separating a wide range of organic compounds of low and medium polarity. In the proposed method the volume of hexane and ethyl acetate is constant and equal to the volume of methanol and water. The polarity of the system increases as the numbers (–7 to +8) designated for each solvent system become more positive. In the HEMWat solvent system family the organic phase is mainly composed of hexane and ethyl acetate in the upper phase of biphasic mixture, while the aqueous phase is mainly composed of methanol and water in the lower phase of biphasic mixture.



The HEMWat system was inspired from a paper by F. Oka: “Oka F.; Oka H.; Ito Systematic Search for Suitable 2-Phase Solvent Systems for High-Speed Countercurrent Chromatography. J. Chromatogr. **1991**, *538*(1), 99-108.” Oka combined a hexane-ethyl acetate-methanol-water solvent system family with a more polar ethyl acetate-*n*butanol-methanol-water family.

Oka number	Hexane	Ethyl Acetate	1-Butanol	Methanol	Water
1	10	0		5	5
2	9	1		5	5
3	8	2		5	5
4	7	3		5	5
5	6	4		5	5
6	5	5		5	5
7	4	5		4	5
8	3	5		3	5
9	2	5		2	5
10	1	5		1	5
11		5			5
12		4	1		5
13		3	2		5
14		2	3		5
15		1	4		5
16		0	5		5

A solvent system family similar to the HEMWat family is called the “Arizona” family from “Counter-current chromatography: instrumentation, solvent selection and some recent applications to natural product purification” Foucault & Chevolot.

Arizona	Heptane	Ethyl acetate	Methanol	Wat
A	0	1	0	1
B	1	19	1	19
C	1	9	1	9
D	1	6	1	6
E				
F	1	5	1	5
G	1	4	1	4
H	1	3	1	3
I				
J	2	5	2	5
K	1	2	1	2
L	2	3	2	3
M	5	6	5	6
N	1	1	1	1
O				
P	6	5	6	5

Q	3	2	3	2
R	2	1	2	1
S	5	2	5	2
T	3	1	3	1
U	4	1	4	1
V	5	1	5	1
W	6	1	6	1
X	9	1	9	1
Y	19	1	19	1
Z	1	0	1	0

The best way to explain why several closely-related solvent system families have been described is because they are designed to give a rational method of looking for optimal solvent systems with which to separate compounds by CCC.

A recent paper by Yoichiro Ito describes a methyl *tert*-butyl ether-*n*butanol-acetonitrile-water solvent system family.

Methyl *tert*-butyl ether	1-butanol	Acetonitrile	Water
1	0	0	1
4	0	1	5
6	0	3	8
2	0	2	3
6	4	5	5
2	2	1	5

Since CCC is by been widely practiced as an empirical separation science little has been done to understand the relative effectiveness or even relative polarity different solvent systems and different solvent system families.

1.d Modifications of the aqueous phase.

The aqueous phase of a biphasic solvent system may be modified by the addition of a water-soluble salt, acid, or base. For example, one of the very first applications of CCC separation was the separation of amino acids by a solvent system consisting of chloroform, acetic acid and 0.1 M aqueous HCl in a 2:2:1 ratio. Water soluble salts may include NaCl, Na_2HPO_4, or NH_4OAc. Water soluble acid additives include trifluoroacetic acid (TFA), HCl, dichloroacetic acid, or acetic acid. A typical base additive is ammonium hydroxide (NH_4OH).

Two important methods of determining the fitness of solvent systems is to determine the volume ratios and settling time. The following table describes the solvent composition, volume ratios and settling times for HEMWat solvent system family. For practical purposes, the volume ratio (upper phase volume divided by lower phase volume) of a CCC biphasic solvent system should be as close to 1 as possible. This means that nearly equal amounts of upper phase and lower phase would be available for use in mobile and stationary phases as needed. A rapid settling time of about 30 seconds or less would allow the phases to mix and separate suitably under the conditions presented by the CCC instrument. The settling time influences the retention of the

stationary phase – a very important CCC parameter. The settling time is simply measured by observing the time required for the two phases to completely separate in a shaken test tube. HSCCC stationary phase retention data, for HEMWat –6, –3, 0, +3, +6 and +7 at different flow rates, has been previously published.

HEMWat system number	Hexane	Ethyl Acetate	Methanol	Water	Volume ratio (U/L)	Settling time in seconds
-7	9	1	9	1	0.72	11
-6	8	2	8	2	0.73	13
-5	7	3	7	3	0.69	13
-4	7	3	6	4	0.76	10
-3	6	4	6	4	0.68	14
-2	7	3	5	5	0.83	18
-1	6	4	5	5	0.76	22
0	5	5	5	5	0.71	27
+1	4	6	5	5	0.68	21
+2	3	7	5	5	0.67	28
+3	4	6	4	6	0.83	20
+4	3	7	4	6	0.83	18
+5	3	7	3	7	0.91	30
+6	2	8	2	8	0.93	33
+7	1	9	1	9	0.91	15
+8	0	10	0	10	0.95	10

Generally, the settling time increases as the percentage of water in the lower phase and/or hexane in the upper layer increases.

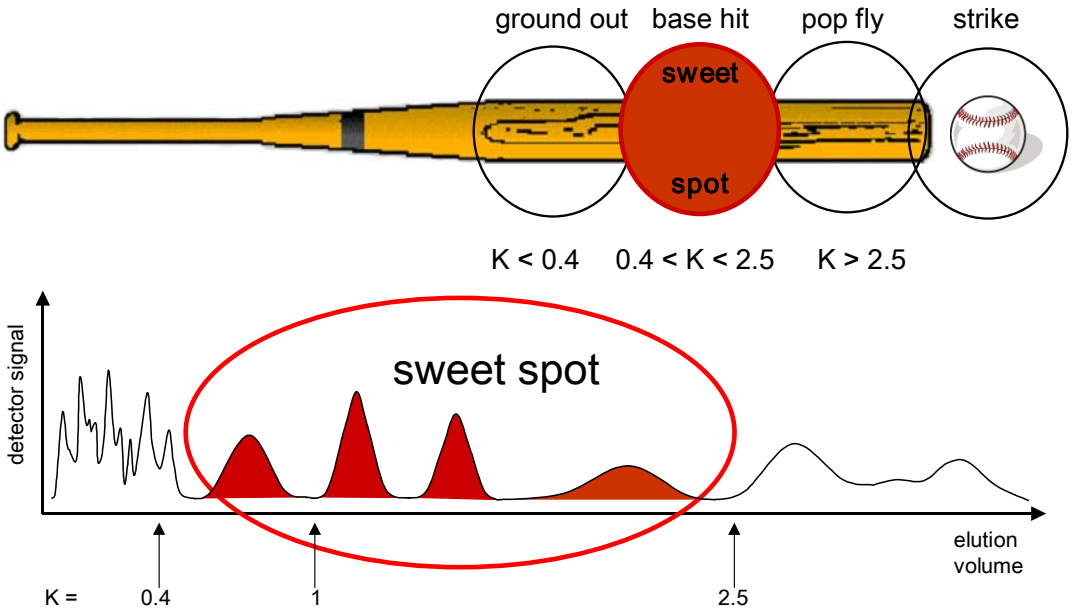
Other considerations for the solvent system are:

- The stability or “shelf-life” of the solvent system. For example, ethyl acetate may decompose into acetic acid and water over time. Typically, solvent systems are mixed and equilibrated fresh for each HSCCC run.
- The quality of solvents. Solvents are available in different grades depending on their purity and addition of stabilizers. Typically, HPLC grade solvents are used.
- The stability of the crude preparation in the solvent system. Biological compounds are prone to degradation and rearrangement in certain solvent systems.
- The solubility of the crude preparation in the solvent system. Compounds that precipitate in certain solvent combinations may cause problems for the machinery as well as the separation.

B. Solvent System Selection for CCC Separation.

While CCC does not retain any compounds on the "column," it may not separate many of them in any appreciable way unless the solvent system has been chosen very carefully. There is a "window of opportunity" present in CCC separations that is related to the K value of a given compound in a particular solvent system. The distribution constant, K, can be expressed as the concentration of the compound in the stationary phase divided by the concentration of the compound in the mobile phase. A solvent system, where the K value of a particular compound is close to one, is considered to be the ideal system for separating the compound. Small K values result in a loss of peak resolution, while large K values tend to produce excessive sample band broadening and long run times. In addition, the decision of which phase (upper or lower) will be the mobile phase is less important if K = 1, since the retention volume of the target compound will be very similar in either mode.

The window of opportunity presented by CCC separations may be compared to the "sweet spot" of bat and racket sports. The sweet spot is the area of the racket or bat that offers the optimum return for effort invested. Missing the sweet spot may result in missing the ball altogether. Hitting the ball outside of the sweet spot may "get the job done" in some cases, but not with the elegance and power of hitting it in the sweet spot. A working definition of the sweet spot in CCC is the interval of K values between 0.4 and 2.5



Over the years, several methods of solvent system selection for CCC have been proposed, studied and utilized. An accepted method of predicting CCC behavior is to perform a partitioning study of a compound by measuring the relative concentrations of the compound in the upper and lower layers of a biphasic solvent system. The P value can be expressed as the concentration of the compound in the upper phase divided by the concentration of the compound in the lower phase. P values obtained by partitioning studies predict the retention

time of a particular compound, e.g., in an HSCCC instrument, when the proper consideration is made for the mobile and stationary phase of the HSCCC run.

The most common form of partition study is descriptively called the “shake-flask” method. This method involves dissolving a small amount of a compound or mixture in a biphasic system, shaking them together, and allowing the system to equilibrate before measuring the concentration of the target compound(s) in each layer. The concentration in each layer can be measured by three principle methods:

1. The two phases may be separated and the solvents evaporated in order to obtain the mass of the residues. This gravimetric method requires relatively large amounts of compound to get a reliable result. It is also not very useful for mixtures, which may contain large amounts of extraneous compounds.
2. The relative concentrations can be measured by measuring the UV-vis absorption of each layer. This spectroscopic method works well for targeting a particular chromophore by itself, or in a mixture of non-absorbing compounds. It can be done with small amounts of compounds. However, the spectroscopic method does not work for compounds that do not absorb in UV-vis and for mixtures where compounds’ absorptions interfere with each other. Also, since the compound is being measured in two different solvents, steps must be taken to minimize solvent interference with spectroscopic measurements.
3. In the case of mixtures, each phase can be analyzed by high pressure liquid chromatography (HPLC) or gas chromatography (GC) and the relative amounts compounds present in each layer can be determined. This chromatographic method requires the development of a reliable HPLC or GC protocol that gives a reasonable separation of the compounds of interest. The chromatographic method is relatively time consuming when several solvent systems must be tried. In addition, for many natural product samples the target analyte may not even be known, such as is always the case in bioassay-guided fractionation.

No matter how efficient or reliable the shake-flask method may be, the problem of “where to start” still needs to be addressed. CCC users take usually take one of two possible paths to solvent system selection;

1. A literature search can be done for a successful CCC separation of a compound related to the target compound(s) of the current project. In addition to individual research papers, an assortment of books chapters, monographs and review articles also present solvent systems that have been successfully employed to separate a wide variety of compounds.
2. A suitable two-phase solvent system may be chosen by starting with a particular family of solvent systems that the researcher feels are appropriate to the class of compounds that are desired to be separated. Usually, the researcher already has an idea of the relative polarity of the target compound(s) in relation to its (their) behavior in TLC.

Since thin layer chromatography (TLC) has traditionally played the role as solvent system selection method in solid-support chromatography, a method that involves the estimation of CCC solvent system choice based on TLC behavior has been suggested. A Generally Useful Estimation of Solvent Systems in CCC, allowing a good first "G.U.E.S.S.", and being able to replace conventional procedures has been proposed by Friesen et al. Without a doubt, TLC is a common denominator of all natural products separations. Samples ranging from crude extracts to purified compounds are subjected to TLC as a quick and easy way to assess their composition, identity and purity. Many useful TLC solvent systems are known and routinely used in laboratories all over the world. In fact, the G.U.E.S.S. method has been done in reverse for decades. It is customary to separate an extract or column fraction by CCC, and then perform

TLC on the collected CCC fractions in order to ascertain their composition and purity as seen. Since TLC can be routinely used to analyze CCC fractions,it should be possible to use TLC to predict CCC elution performance. However, relating TLC and CCC 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. In this method, silica gel TLC was done with the organic layer of a chloroform/methanol/water biphasic solvent system in order to predict the best mobile phase for optimal DCCC performance in that solvent system.

Many functional solvent systems have been proposed, studied and successfully employed over the years.

Sample	Amount (mg)	Solvent system	Year	Reference
mevinolinic acid	79.2	HEMWat 5:5:5:5	2003	[1]
osthol	88.3	HEMWat 5:5:5:5	2004	[2]
(+)-dihydromyricetin	11.3	Hexane – Ethyl acetate – Methanol – Water 1:3:2:4	2002	[3]
ivermectin B1a	18.7	Hexane – Ethyl acetate – Methanol – Water 1:1:10:10	1996	[4]
honokiol	80	Hexane – Ethyl acetate – Methanol – Water 5:2:5:2	2004	[5]
Spiramycin I	13.4	Hexane – Ethyl acetate – Methanol – Water 3:6:5:5	2000	[6]
Chrysin baicalein	4.2 6.8	Hexane – Ethyl acetate – Methanol – Water 5:6:5:5	2003	[7]
Epigallocatechin-3-O-gallate (EGCG)	45	Hexane – Ethyl acetate – Methanol – Water 3:10:3:10	2000	[8]
5,7-dihydroxy-3',4'-trihydroxyflavone-3-O-6"-rhamose	28	Hexane – Ethyl acetate – Methanol – Water 3:10:3:10	2004	[9]
Theaflavins		Hexane – Ethyl acetate – Methanol – Water 1:3:1:6	2001	[10]
Theaflavins		Hexane – Ethyl acetate – Methanol – Water 6:25:6:25	2004	[11]
andrographolide neoandrographolide	189 9.5	Hexane – Ethyl acetate – Methanol – Water 2:8:5:5	2003	[12]
notopterol isoimperatorin		Light petroleum – Ethyl acetate – Methanol – Water 25:25:24:25	2000	[13]
darlingine darlingine N-oxide	75 17	ChMWat 13:7:8	1999	[14]
Resveratrol piceid	72.5 35.45	ChMWat 4:3:2	2001	[15]
Resveratrol Anthraglycoside A Anthraglycoside B	600 50 20	ChMWat 4:3:2	2001	[16]
Squalene	0.2	Hexane-Methanol 2:1	2003	[17]
Shikonin	19.6	Hexane-Ethyl acetate-Ethanol-Water 16:14:14:5	2004	[18]
Salvianolic acid B	342	Hexane-Ethyl acetate-Ethanol-Water 3:7:1:9	2002	[19]
Przewaquinone A	15.3	Carbon tetrachloride-methanol-water-hexane 3:3:2:1	2003	[20]
epigallocatechin	1,300	Hexane-ethyl acetate-water 1:9:10	2004	[21]
Astaxanthin		Hexane-Ethyl acetate-Ethanol-Water 10:10:13:6	2001	[22]

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