



Distribution ratio, distribution constant and partition coefficient. Countercurrent chromatography retention of benzoic acid

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ABSTRACT

There is some confusion in chromatography between terms such as solute distribution ratio, distribution constant and partition coefficient. These terms are very precisely defined in the field of liquid–liquid systems and liquid–liquid extraction as well as in the field of chromatography with sometimes conflicting definitions. Countercurrent chromatography (CCC) is a chromatographic technique in which the stationary phase is a support-free liquid. Since the mobile phase is also liquid, biphasic liquid systems are used. This work focuses on the exact meaning of the terms since there are consequences on experimental results. The retention volumes of solutes in CCC are linearly related to their distribution ratios. The partition coefficient that should be termed (IUPAC recommendation) distribution constant is linked to a single definite species. Using benzoic acid that can dimerize in heptane and ionize in aqueous phase and an 18 mL hydrodynamic CCC column, the role and relationships between parameters and the consequences on experimental peak position and shape are discussed. If the heptane/water distribution constant (marginally accepted to be called partition coefficient) of benzoic acid is 0.2 at 20 °C and can be tabulated in books, its CCC measured distribution ratio or distribution coefficient can change between zero (basic aqueous mobile phase) and more than 25 (acidic aqueous mobile phase and elevated concentration). Benzoic acid distribution ratio and partition coefficient coincide only when both dimerization and ionization are quenched, i.e. at very low concentration and pH 2. It is possible to quench dimerization adding butanol in the heptane/water system. However, butanol additions also affect the partition coefficient of benzoic acid greatly by increasing it.

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1. Introduction

Countercurrent chromatography (CCC) is a purification technique that uses a biphasic liquid system to separate the compounds in a mixture. The liquid stationary phase is constrained in a CCC column by centrifugal fields without any solid support. The mobile phase is the other phase of the biphasic liquid system. The CCC technique was abundantly described in the literature [1–3]. CCC is a chromatographic technique. As such, it must use all nomenclature defined for chromatography even if the official International Union for Pure and Applied Chemistry (IUPAC) definitions are almost two decades old [4]. A more recent however non official list of terms for chromatography was proposed by Majors and Carr, two experts in the field [5].

Biphasic liquid systems are used in CCC. So, the liquid–liquid equilibrium nomenclature also defined by IUPAC must be used as much as possible [6]. This dual affiliation of CCC is source of conflicting definitions especially regarding the solute distribution between the two liquid mobile and stationary phases. In

classical chromatography, the solute distribution is essentially a solid–liquid partitioning mixed with other physico-chemical phenomena such as pore size exclusion, moving versus stagnant mobile phase exchanges and/or silanol interaction. In CCC, the solute interaction with the mobile and stationary phases is exclusively liquid–liquid partitioning.

The aim of this work is to delineate and to point out possible definition conflicts in the notion of solute distribution and the terms: distribution ratio, distribution constant and partition coefficient. Benzoic acid, an ionizable compound, will be used as a model compound to follow its behavior in a typical CCC biphasic liquid system and so, to illustrate practically as well as theoretically the difference between these solute distribution parameters.

2. Definitions

First it is worth reminding the literal meaning of terms. A *ratio* is the result of one value divided by another. The two values or parameters must be of the same nature meaning that both values must have the same dimension and units. Consequently a ratio is always a dimensionless value without unit but whose numerical value can change. This is not the case of a *constant* that may or may not have a dimension or unit. However, in a given unit

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system, the value of a constant is by definition permanent. It can be listed in tables; e.g. the perfect gas constant, R , has the permanent value of $8.31 \text{ J mol}^{-1} \text{ K}^{-1}$ in the international unit system. The R value is $2 \text{ cal mol}^{-1} \text{ K}^{-1}$ if energy is expressed in calories and even $40.7 \text{ psi floz mol}^{-1} \text{ K}^{-1}$ if the pound per square inch ($1 \text{ psi} = 6894 \text{ Pa}$ or J m^{-2}) and fluid ounce ($1 \text{ floz} = 29.6 \text{ cm}^3$ or mL) of the old French “avoirdupois” unit system are used [7].

A *coefficient* is a numerical measure of a parameter or a physico-chemical property. Depending on the property, it may be either a dimensionless ratio (with possible changing value) or a constant with or without a unit; e.g. a diffusion coefficient is usually expressed in $\text{cm}^2 \text{ s}^{-1}$; a correlation coefficient is dimensionless.

IUPAC defines the *distribution ratio* with the symbol D as the ratio of the total concentration of a solute in the liquid phase 1, regardless of its chemical forms, to its total concentration in the other liquid phase referred as 2. It is specified that the distribution ratio of a solute is an experimental parameter whose value does not necessarily imply that distribution equilibrium between the two liquid phases has been achieved [6]. Here begins a possible source of confusion. The symbol D is reserved in chromatography for solute diffusion coefficient, never for solute distribution ratio [3,5]. Several authors proposed to use the symbol K_C [3,5,8]. This latter symbol is certainly much better than D since the K letter with various subscripts is associated to phase exchange equilibrium in chromatography. K_C will be used in this work as the symbol of the solute distribution ratio (all forms). It is clearly proved that serious discussions are needed to harmonize definitions and symbols.

Following Conway's proposition, the *distribution constant* will be given the symbol K_Δ [9] instead of the IUPAC symbol K_D . Conway defines K_Δ as $[A]_S/[A]_M$ where A is a solute in its single definite form in the liquid phase 1, the stationary phase, to its concentration in the very same form in the other phase 2, the mobile phase, at equilibrium. Reading the IUPAC recommendations, good synonyms for the defined K_Δ parameter could be *distribution coefficient*, *partition ratio* or *partition coefficient* with the possible symbol P , although IUPAC clearly indicates that the use of this latter term is not recommended [6]. If the term *partition coefficient* is not recommended, it is not forbidden either. It is however clearly defined as a constant for a single definite species. Conway also suggests the denomination *partition constant* for the K_Δ constant [9].

There are more possible conflicting situations. When water is part of the biphasic liquid system, IUPAC imposes that Liquid 1 be the organic phase and Liquid 2 the aqueous phase (e.g. octanol/water distribution constants). Similarly, in chromatography, the concentration ratios used to form K_C must be [stationary phase] over [mobile phase]. If in most chromatographic systems this arrangement is no problem since the solid phase is always and necessarily the stationary phase, in CCC either phase of the two aqueous and/or organic liquid phases, can be the stationary phase.

Confusion is easy since the Majors and Carr definition for *distribution constant* is incorrect in both the published text [5] and web site [8] being that of the *distribution ratio* term. Both terms coincide for non-ionizable compounds only. These expert authors saw the problem and suggested to call the K_C term the *distribution coefficient* [5,8]. The symbols used in Refs. [4–6,8] also differ from symbols defined and used in this work. It is recalled that we just want to point out the problem. Official *ad hoc* institutions will have the responsibility to clarify the situation establishing the standards in definitions, terms and symbols.

3. Experimental

3.1. Chemicals

Heptane and methanol were obtained from Fisher (Loughborough, Leicestershire, UK), 1-butanol was from Riedel de Haën

(Seelze, Germany). Phosphoric acid, sodium hydroxide and chloride, hydrochloric acid and benzoic acid were provided by Sigma–Aldrich (L'Isles d'Abeau Chesne, France). Water was distilled and purified by a ELGA purelab UHQ-MK3 system (Veolia Water System, Bucks, UK). The biphasic liquid systems were prepared by mixing the indicated solvent volumes, shaking vigorously and letting stand for at least 15 min. The aqueous mobile phase pH was adjusted using different acids and/or bases and keeping the ionic strength constant at 0.1 M by NaCl additions.

3.2. Countercurrent chromatograph

The CCC apparatus was a MilliTM Dynamic Extraction 18 mL hydrodynamic CCC instrument. The Milli hydrodynamic CCC “column” has a 20 cm diameter rotor holding a single 7.5 cm diameter coil equilibrated by a counterweight. The coil contains 36 m of 0.8 mm 1/16 in. (1.57 mm) o.d. Teflon[®] tubing coiled in approximately 220 turns in the 7.5 cm bobbin. The beta ratio was varying from 0.5 (internal first layer) to 0.85 (external outer tubing layer). This modern hydrodynamic CCC column is able to generate a high centrifugal field ($440 \times g$ at 2000 rpm) [10,11].

Classical liquid chromatography equipment surrounds the hydrodynamic CCC “column”. A single pump Shimadzu LC6A (Kyoto, Japan) is used for both liquid phases. This pump is sturdy enough to accept one liquid phase and immediately after the other immiscible phase without losing its priming or clogging its check valves. A Rheodyne 7010 six-port valve (Cotati, CA, USA) was used with a 0.5 mL loop to inject the samples. A Shimadzu SPD-6A UV detector allowed to locate the solutes. The detector signal was processed by an A/D converter (ULYS, Datalys, Saint Martin d'Herès, France) and displayed on a PC computer running the AZUR version 4.0 software (Datalys).

All CCC experiments were run in the reversed phase mode. The stationary phase was the upper organic phase. The mobile phase was the lower aqueous phase flowing in the head-to-tail direction at a constant flow rate of 0.8 mL/min. The rotor rotation speed was always 2000 rpm generating a high centrifugal field of about $440 \times g$. The temperature was regulated at 30°C . For a typical experiment, the MilliTM CCC column was first filled by the upper organic phase in 5 min at 5 mL/min without rotation. Next, the pump was rinsed by the aqueous mobile phase, the rotation as started and stabilized at 2000 rpm and the mobile phase was flown at 0.8 mL/min in the descending or head-to-tail direction. The exiting eluent was initially the lighter organic phase collected in a graduated cylinder. When the lower aqueous phase was seen in the graduated cylinder, the collected volume of lighter organic phase could be measured. It corresponded to the V_M volume of mobile phase contained in the CCC column. The V_S volume of organic phase remaining inside the CCC column is simply $V_C - V_M$. A great accuracy on the measurement of these volumes is needed for correct computation of distribution ratios.

4. Solute liquid–liquid partitioning

4.1. Relationships between parameters

The IUPAC definitions differentiate the solute *distribution ratio* K_C involving the solute concentration regardless of its chemical form and the solute *distribution constant* K_Δ involving a single definite form of the solute [4,6]. Using benzoic acid as the model solute, it can be written as AH for its φ -COOH molecular form and A^- for its ionized φ -COO⁻ benzoate form. The two forms are related by the aqueous dissociation constant, K_A :

$$K_A = \frac{[\varphi\text{-COO}^-][\text{H}^+]}{[\varphi\text{-COOH}]} = \frac{[A^-][\text{H}^+]}{[AH]} \quad (1)$$

Table 1
Calculated distribution ratios, K_C (all forms) of benzoic acid for different aqueous pH values and different initial concentrations.

pH	Total concentration (mM)	K_C distribution ratio	AH molecule ^a	A ⁻ anion ^a	AH ₂ dimer ^a
2	500	24.8	4.7%	0	95.3%
	100	10.8	10.1%	0	89.9%
	10	3.2	28.6%	0.01%	71.3%
	2.5	1.5	48.6%	0.03%	51.3%
4.2	500	12.1	4.6%	3.8%	91.6%
	100	5.3	9.5%	7.9%	82.5%
	10	1.4	24.7%	20.6%	54.7%
	2.5	0.6	37.5%	31.2%	31.3%
6	500	0.14	1.6%	86.4%	12.0%
	100	0.03	1.8%	95.3%	2.9%
	10	0.006	1.9%	97.8%	0.3%
	2.5	0.004	1.9%	98.0%	0.1%
7	500	0.002	0.2%	99.7%	0.2%
	100	0.0006	0.2%	99.8%	0.1%
	10	0.0004	0.2%	99.8%	0
	2.5	0.0003	0.2%	99.8%	0

Benzoic acid K_2 dimerization constant = 15,900 M⁻¹.

Benzoic acid K_{Δ}^{AH} heptane/water partition coefficient or distribution constant = 0.2.

Benzoic acid K_A acid/base dissociation constant = 6.3×10^{-5} or $pK_A = 4.2$.

K_C is the benzoic acid distribution ratio (all forms) calculated using Eq. (7).

^a Amount of benzoic acid in the molecular (in both aqueous and heptane phases), ionic (in aqueous phase only) or dimer form (in heptane phase only) expressed in percentage of the total introduced concentration in the 1/1 (v/v) heptane/water biphasic liquid system.

In the most general case, both species, the molecular and the ionized forms can partition between the two liquid phases referred as liquid phase *a* and liquid phase *b*. Since there are two forms, there are two distribution constants, one for the AH benzoic acid molecule:

$$K_{\Delta}^{AH} = \frac{[AH]_b}{[AH]_a} \quad (2)$$

Another one for the A⁻ benzoate anionic form:

$$K_{\Delta}^{A^-} = \frac{[A^-]_b}{[A^-]_a} \quad (3)$$

In organic heptane phase, two benzoic acid molecules can form dimers by H-bond association according to [12]:



The dimerization constant, K_2 , is expressed as:

$$K_2 = \frac{[(\varphi\text{-COOH})_2]}{[\varphi\text{-COOH}]^2} \quad (5)$$

The K_2 numerical value in pure heptane at 30 °C was determined as 15,900 M⁻¹ [12]. For example, a 10⁻³ M benzoic acid heptane solution contains only 0.162 mM of free benzoic acid, or only 16.2% of all molecules, and 0.419 mM of dimers immobilizing 0.838 mM of AH molecules associated two by two.

In the Liquid *a*/Liquid *b* biphasic system, the benzoic acid distribution ratio, K_C , involves all chemical forms of benzoic acid including benzoate anions and dimers. K_C can be expressed by:

$$K_C = \frac{[AH]_b + [A^-]_b + 2[(AH)_2]_b}{[AH]_a + [A^-]_a + 2[(AH)_2]_a} \quad (6)$$

In the case of the heptane/water system and benzoic acid molecule, simplification of Eq. (6) can be made considering that dimerization cannot occur in the aqueous phase (i.e. $[(AH)_2]_a = 0$) with water molecules blocking the benzoic acid H-bonds responsible for dimerization in the apolar aprotic heptane phase. Using Eqs. (1)–(5), the benzoic acid distribution ratio, K_C , in the heptane/water system can be expressed by the complex equation:

$$K_C = \frac{K_{\Delta}^{AH}(1 + 2K_{\Delta}^{AH}K_2[AH]_a) + K_{\Delta}^{A^-}(K_A/[H^+])}{1 + (K_A/[H^+])} \quad (7)$$

The distribution ratio K_C of benzoic acid depends on the four constants: K_{Δ}^{AH} , $K_{\Delta}^{A^-}$, K_2 and K_A being respectively the benzoic acid heptane/water partition coefficient (IUPAC recommended denomination being *distribution constant* [6]), the benzoate anion heptane/water partition coefficient or distribution constant, the benzoic acid heptane dimerization constant and the benzoic acid acidity (or dissociation) constant. The distribution ratio K_C varies with $[AH]_a$, the benzoic acid concentration in the aqueous phase, and with the aqueous phase pH (Eq. (7)).

4.2. Theoretical study of benzoic acid distribution

The benzoic acid partition coefficient or, to follow IUPAC instructions, distribution constant, K_{Δ}^{AH} is equal to 0.2 or its log K_{Δ}^{AH} is equal to -0.7 at 25 °C, a value listed in a literature table [13]. The benzoate anion is so poorly partitioning in the heptane phase that its distribution constant, $K_{\Delta}^{A^-}$ could not be found in the literature being almost nil. For computation purpose, it will be arbitrarily taken as $K_{\Delta}^{A^-} = 0.00001$ or $\log K_{\Delta}^{A^-} = -5$ at 25 °C. The benzoic acid dissociation constant in water is well documented as $K_A = 6.3 \times 10^{-5}$ or $pK_A = 4.2$ [7]. The heptane/water dimerization constant was recently determined as $K_2 = 15,900 \text{ M}^{-1}$ [12].

Eq. (7) shows that the benzoic distribution ratio depends on the constants just cited along with the pH of the aqueous phase as the $[H^+]$ concentration, responsible for the benzoic acid ionization as well as on the amount of benzoic acid in its molecular form $[AH]_a$, linked to possible dimerization in the organic phase. Clearly the benzoic acid distribution ratio, K_C , could be termed a distribution coefficient but certainly not a distribution constant [5,8].

Table 1 lists the benzoic acid distribution ratio, K_C , calculated for different pH values and different initial concentrations introduced in a one to one volume ratio of heptane/buffer biphasic liquid system. The proportions of the three species, molecule, anion and dimer, are also indicated expressed in percentage of the introduced benzoic acid concentration. Table 1 shows the significant effect of the chemical reactions, ionization and dimerization, on the species repartition in the heptane and aqueous phase. At low pH value, pH 1, benzoic acid is essentially in its molecular form. Most of the molecular form that partitions in the heptane phase dimerizes. When the maximum 0.5 M benzoic acid is introduced in the biphasic system

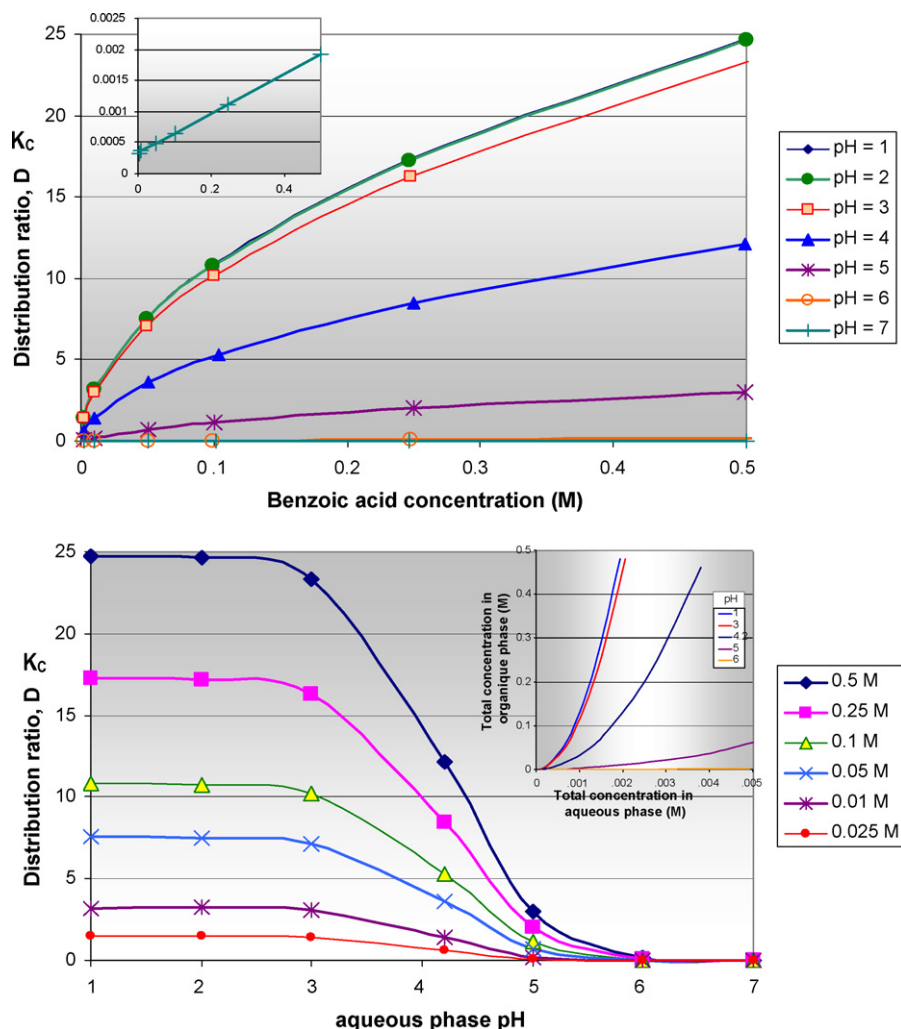


Fig. 1. Effect of the total benzoic acid concentration (top) and aqueous phase pH (bottom) on the benzoic acid distribution ratio, K_C . Heptane to buffer volume ratio is one to one. The inset in the top figure shows the linear change of the benzoic acid distribution ratio at pH 7. The inset in the bottom figure shows the benzoic acid adsorption isotherms (concentration in the aqueous phase versus concentration in the heptane phase) at different mobile phase pH values (1, 3, 4.2, 5 and 6).

at pH 1, 95.3% of it is located in the heptane phase as dimers. Adding the amount in monomeric form (0.8%), 96.1% of the introduced benzoic acid is located in the heptane phase and the remaining 3.9% is in the aqueous phase entirely in the molecular form. The distribution ratio, K_C , is $96.1/3.9 = 24.8$, two orders of magnitude higher than the benzoic acid distribution constant, $K_{\Delta}^{\text{AH}} = 0.2$.

At pH 7, more than 99% of benzoic acid is ionized in the benzoate anionic form whatever concentration is added to the biphasic system (Table 1). Since the benzoate form does not partition with the heptane phase, the dimer concentration is also very low. When 0.5 M benzoic acid is introduced in the heptane/pH 7 buffer 1/1 system, 0.2% is located in the heptane phase essentially as dimers and the vast majority, 99.8%, is in the benzoate anionic form in the aqueous phase. The experimentally observed benzoic acid distribution ratio is then $K_C = 0.2/99.8 = 0.002$ two orders of magnitude lower than the benzoic acid $K_{\Delta}^{\text{AH}} = 0.2$ partition coefficient or distribution constant.

Fig. 1 shows the set of data presented in Table 1 focusing on the total introduced concentration (top figure) or on the aqueous phase pH (bottom figure). As Eq. (7) shows, introducing more benzoic acid in the system produces an increase in the distribution ratio, K_C . This is due to the increase of benzoic acid molecules, factor $[\text{AH}]_a$, in the aqueous phase. At low pH, most of the introduced benzoic acid maintains its molecular form. The competition between

liquid–liquid distribution and dimerization in the heptane phase is responsible for the curvature of the K_C versus $[\text{AH}]$ initial concentration curves. At low concentrations, there are less dimers, so less benzoic acid in the heptane phase producing a lower K_C ratio. Conversely, at high benzoic concentrations, more dimers form meaning more benzoic acid in the heptane phase and a significantly higher K_C ratio is experimentally observed. At elevated pH, most of the introduced benzoic acid ionizes in benzoate anions leaving a constant proportion of molecules in the aqueous phase (0.2% at pH 7), this produces a linear increase of K_C at pH 7 (inset in top Fig. 1).

All Fig. 1, bottom, curves show the same thing: as the pH of the aqueous phase increases, benzoic acid ionizes and becomes located in the aqueous phase in its benzoate anion form. Classical sigmoid titration curves are obtained. The K_C ratio obtained at the pK_A value (pH 4.2) is half that obtained in acidic solutions (Fig. 1, bottom). At neutral pH and higher pH, the anionic form is the only form. Since there are practically no molecular forms, there are also no dimers explaining the extremely low concentration of benzoic acid in the heptane phase and, consequently, the very low benzoic acid distribution ratio. The inset shows the adsorption isotherms obtained plotting the total benzoic acid concentration in the heptane phase (monomers and dimers) versus the total benzoic acid concentration in the aqueous phase (monomers and anions) at different aqueous phase pHs. All isotherms are convex: as the concentration increases

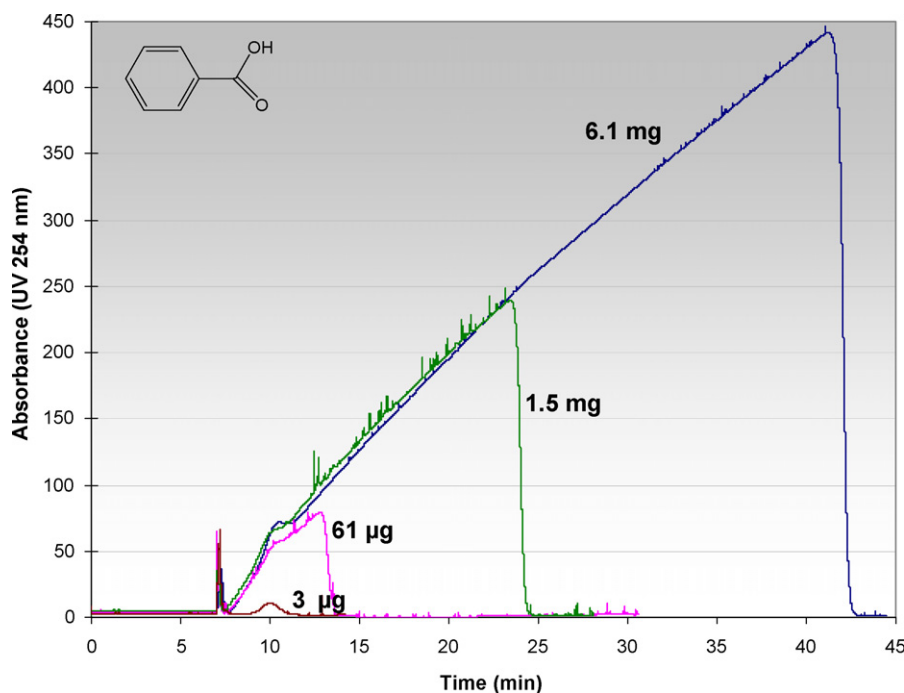


Fig. 2. Increasing amounts of benzoic acid injected in a pH 2 aqueous mobile phase. The benzoic acid mass injected is indicated on the chromatogram. Hydrodynamic CCC column Dynamic Extractions Milli, 18 mL; rotor speed: 2000 rpm; stationary phase: heptane ($V_S = 12.4$ mL; $S_f = 69\%$); mobile phase: water pH 2 (HCl 0.01 M); flow rate 0.8 mL in the descending head to tail direction. Injection volume: 500 μ L of benzoic acid in 50/50 methanol/water solution. Injected concentrations (mass): 0.05 mM (3 μ g); 1 mM (61 μ g); 25 mM (1.5 mg) and 0.1 M (6.1 mg). UV detection at 254 nm.

in the aqueous phase, it increases even more in the organic phase due to the dimerization occurring in the heptane phase whatever the pH is. Adsorption isotherms and chromatographic peak shapes are linked. The convex isotherms allow predicting fronting deformations of the chromatographic benzoic acid peaks with an aqueous mobile phase and a heptane stationary phase.

5. Benzoic acid distribution ratio and chromatographic peak position

In countercurrent chromatography (CCC), the heptane phase can be the liquid stationary phase and the aqueous phase can be the mobile phase. If benzoic acid is injected as a solute in a CCC equilibrated column containing these two phases, it is retained if it goes in the stationary phase in any chemical form. So its peak retention volume, V_R , is related to the *distribution ratio*, K_C , for all chemical forms and not to the partition coefficient or the distribution constant for a single chemical form. The CCC retention equation is [1–3]:

$$V_R = V_M + K_C V_S \quad (8)$$

in which V_M and V_S are respectively the mobile and stationary phase volume inside the column of internal volume $V_C (=V_M + V_S)$. The measured retention volume of the solute, V_R , allows to calculate its distribution ratio, K_C , using:

$$K_C = \frac{V_R - V_M}{V_S} \quad (9)$$

5.1. The heptane/water system

The solubility of heptane in water was estimated to be about 0.0007% (v/v) or 0.7 μ M at 25 $^{\circ}$ C [14]. Such a low value will be considered as nil, so that the heptane/buffer system is really easy to prepare just mixing any proportion so that the heptane

phase becomes “wet” (solubility of water in heptane 2.7 mg L^{-1} or 0.15 mM at 20 $^{\circ}$ C [7]).

The CCC column was loaded with the heptane phase as the stationary phase and equilibrated at 2000 rpm and 0.8 mL min^{-1} of aqueous buffer phase flown in the head to tail direction. 5.5 mL (± 0.1 mL) of heptane were displaced allowing to calculate the V_M volume as 5.8 mL, hence the V_S volume is $V_C - V_M = 18 - 5.5 = 12.5$ mL (± 0.1 mL) and the stationary phase retention parameter, S_f , is 69% ($\pm 1\%$). This parameter was roughly the same when different buffer solutions were used.

Fig. 2 shows the chromatograms obtained injecting increasing amounts of benzoic acid in the CCC column containing the heptane stationary phase with a pH 2 mobile phase (0.01 M HCl solution). The first injection of 3 μ g (0.5 mL of 0.05 mM benzoic acid solution or 25 nmole injected) produced a quasi Gaussian peak exactly centered on 10 min or 8 mL. This allows for the calculation of the benzoic acid distribution ratio as $K_C = (8 - 5.5)/12.5 = 0.2$ (Eq. (9)). This K_C value is exactly the listed IUPAC K_{Δ}^{AH} of benzoic acid [13]. It means that, at this low injected concentration (5×10^{-5} M) and low pH (0.01 M HCl), both dimerization and ionization are negligible as shown in Table 1. In this case, the CCC measured benzoic acid distribution ratio K_C value and the partition coefficient or distribution constant K_{Δ}^{AH} of benzoic acid coincide (Fig. 2, bottom chromatogram).

This coincidence does not hold as soon as more concentrated benzoic acid solutions are injected. As calculated in Table 1 and experimentally shown in Fig. 2, at pH 2, the measured benzoic acid distribution ratio, K_C , increases with the injected concentration. It is pointed out that Table 1 lists data obtained in a perfectly equilibrated heptane/buffer system giving $K_C = 10.8$ at pH 2 and a benzoic acid concentration of 0.1 M. A K_C ratio of 10.8 should produce a retention volume of 140 mL (Eq. (8)) or 174 min with our CCC column ($V_M = 5.5$ mL, $V_S = 12.5$ mL) and conditions (pH 2 and 0.8 mL/min). When 0.5 mL of a 0.1 M benzoic acid solution is experimentally injected (6.1 mg), the peak maximum is located at 42 min

Table 2
Heptane/1-butanol/water biphasic liquid systems tested for benzoic acid retention in a hydrodynamic CCC column.

	Initial% (v/v)	Upper phase		Lower phase	
		% (v/v)	M	% (v/v)	M
Composition 0^a					
Butanol	0	0	0	0	0
Heptane	200	100		0	
Water	400	0		100	
Composition 1					
Butanol	15	0.57	0.063	3.47	0.39
Heptane	200	99.43		0	
Water	385	0		96.53	
Composition 2					
Butanol	20	0.77	0.085	4.62	0.530
Heptane	200	99.23		0	
Water	380	0		95.38	
Composition 3					
Butanol	25	0.97	0.107	5.79	0.672
Heptane	200	99.03		0	
Water	375	0		94.21	

The distribution constant of 1-butanol in the heptane–water system is 0.16 [13]. No salt is added in the aqueous phase.

^a The data was calculated assuming a nil mutual heptane and water solubility, i.e. any proportion of heptane and water will give pure heptane upper phase and water lower phase for Composition 0.

(Fig. 2, top chromatogram) or 33.6 mL of retention volume V_R that gives a K_C ratio of 2.3 instead of the calculated 10.8. This discrepancy is due to the dynamic nature of the chromatographic process that causes dilution. As soon as benzoic acid equilibrates between heptane and water in a given theoretical “plate” inside the CCC column, the aqueous mobile phase moves into the next plate inducing dilution and creating a new equilibrium in this next plate and so on, according to the adsorption isotherm (Fig. 1, bottom inset). The chromatographic process and the non-linear convex isotherms explain the fronting triangle shape of the concentrated benzoic acid peak shown in Fig. 2.

5.2. The heptane/1-butanol/water system

An easy way to suppress benzoic acid dimerization in the heptane phase is to add an H-bond donor molecule in the apolar phase. Octanol was found to quench completely benzoic acid dimerization at concentration higher than 0.05 M in heptane [15]. 1-Butanol is another excellent candidate for dimerization quenching. It was selected for this purpose because it distributes between the two liquid phases better than octanol. Benzoic acid molecules will no longer form dimers but rather associate through H-bond with heptane solubilized butanol molecules. Obviously, butanol addition to the heptane phase will change the benzoic acid heptane/water K_{Δ} distribution constant since the benzoic acid concentration in heptane will increase. Furthermore butanol is also soluble in water, it will partition between the two phases changing the benzoic acid solubility in water. 1-Butanol partitions between heptane and water with a distribution constant K_{Δ}^{BuOH} of 0.16 at 25 °C ($\log K_{\Delta}^{\text{BuOH}} = -0.80$ [13]). Three different heptane/1-butanol/water compositions as listed in Table 2 were selected to produce a butanol concentration in heptane higher than 0.05 M [15]. All three compositions were tested in CCC.

Fig. 3 shows four chromatograms obtained at four different pH values with the same Composition 2 biphasic liquid system (Table 2). The calculated K_C values (Eq. (9)) corresponding to the benzoic acid peak retention times form a sigmoid curve similar to those shown in Fig. 1, bottom. Similar results were obtained with the two other compositions containing different amounts of

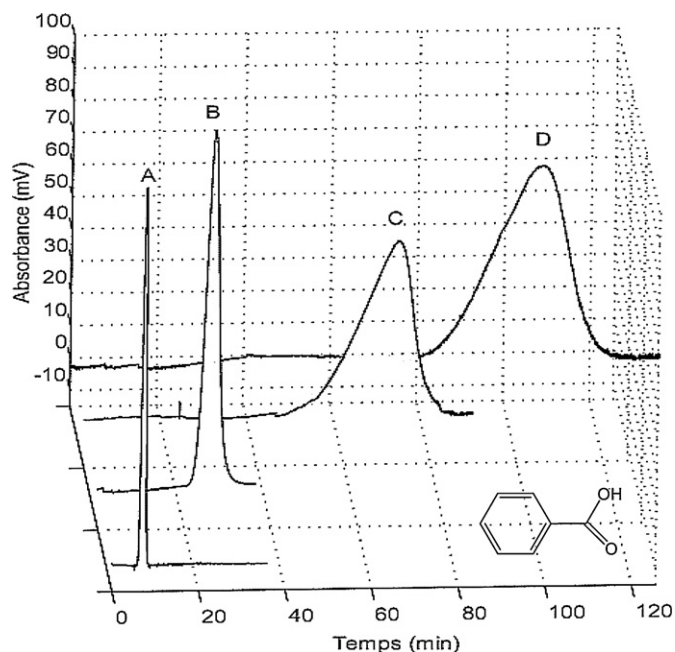


Fig. 3. Retention of benzoic acid at different pH values. Liquid system Composition 2, heptane/butanol/buffer 200/20/380 (Table 2). Mobile aqueous lower phase at 0.8 mL/min in the descending head-to-tail direction. Hydrodynamic CCC column Milli, 18 mL, rotor rotation speed 2000 rpm, 30 °C. Benzoic acid injected mass: 0.5 mg, injected volume: 0.5 mL. UV detection at 254 nm. Aqueous buffer pHs are: A – 6.96; B – 4.61; C – 3.62; and D – 2.19.

butanol (Table 2). In all cases, there was no measurable retention of the benzoic acid peak at mobile phase pH higher than 6. In this case benzoic acid was eluted in the form of benzoate anion. If not nil, the benzoate anion distribution constant $K_{\Delta}^{\text{A}^-}$ is so small that it is not measurable in our experimental conditions.

Assuming that dimerization is quenched in the heptane phase, the benzoic acid distribution ratio K_C reduces to:

$$K_C = \frac{K_{\Delta}^{\text{AH}} + K_{\Delta}^{\text{A}^-} (K_A / [\text{H}^+])}{1 + (K_A / [\text{H}^+])} \quad (10)$$

At pH values of two or lower, two units below the benzoic acid pK_A , the $K_A / [\text{H}^+]$ term is small lower than 0.0063. The non retention of the benzoate anion indicates that its distribution constant $K_{\Delta}^{\text{A}^-}$ is small so that in that case and in that case only, the benzoic acid distribution ratio can be taken as the benzoic acid partition coefficient or distribution constant:

$$K_C \approx K_{\Delta}^{\text{AH}} \quad (\text{low pH and dimerization quenched}) \quad (11)$$

Using the Eq. (11) approximation, K_{Δ}^{AH} , the benzoic acid distribution constant was plotted versus the butanol concentration in the heptane phase (Fig. 4). $\log K_{\Delta}^{\text{AH}}$ is linearly related to the butanol concentration as often observed in similar conditions [3]. The intercept of the straight line is 0.08 giving a theoretical benzoic acid K_{Δ}^{AH} value of 1.2 in the heptane/water biphasic system without butanol (possible dimerization) but with buffer salts to produce the pH 2 value (ionic strength 0.1 M). Table 1 lists a very close $K_C = 1.5$ value for a 2.5 mM benzoic acid concentration without butanol at pH 1. More than half (51.3%) of the benzoic acid molecules are located in the heptane phase in dimer form. At infinite dilution and salt free aqueous phase, the true benzoic acid K_{Δ}^{AH} constant was measured as 0.2 [13]. It is interesting to study the ionic strength effect on the benzoic acid K_{Δ}^{AH} value.

Three different Composition 2 solvent systems were prepared at pH 2.1 by HCl and adjusted to ionic strengths of 0.1, 0.5 and 1 M by increasing the added amount of sodium chloride. The benzoic

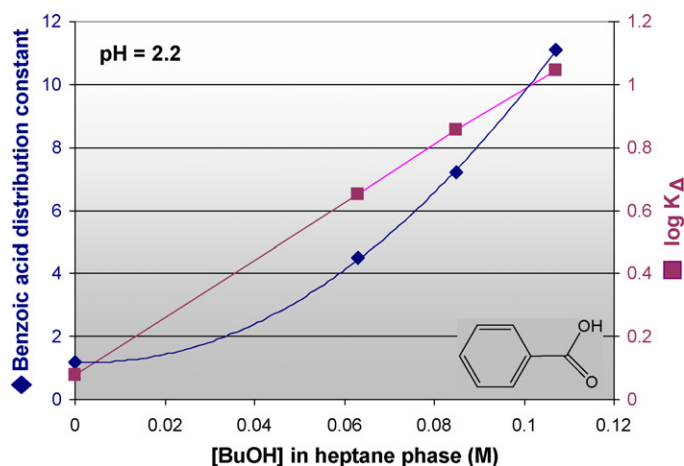


Fig. 4. Changes in the benzoic acid distribution constant, K_D , versus the butanol concentration in the heptane phase. Lozenges and left axis: K_D values; squares and right axis: $\log K_D$ values. Aqueous mobile phase pH: 2.2. Ionic strength 0.1 M.

acid distribution ratios were respectively 7.2, 12 and 17.3. These K_C values are linearly related to the ionic strength μ . The regression line is:

$$K_C = 11.2\mu + 6.2 \quad (r^2 = 0.999, n = 3) \quad (12)$$

This result is a direct observation of the salting out effect. In this case, the observed benzoic acid K_C increases is a combination of the reduced benzoic acid as well as butanol solubilities in the aqueous phase and the increased benzoic concentration in heptane induced by the higher butanol concentration. All these concentration changes are due to the higher salt content in the aqueous phase (salting out effect).

6. Conclusion

In CCC, it is critical to know that the experimental retention volume of a solute (its peak position) depends on the solute distribution ratio between the two liquid phases. In this work, the distribution ratio is defined as the total concentration of a solute in the stationary phase in all possible chemical forms over the total concentration of the solute in the mobile phase in all its possible chemical forms. This ratio is not constant depending on the change occurring between chemical forms. It means that the solute peak retention volume depends on the experimental conditions. In a limited number of cases, the distribution ratio can be equal to the distribution constant or partition coefficient. In the particular case of benzoic acid in the heptane/water system, the benzoic acid

peak position depends on the injected concentration as well as the aqueous mobile phase pH. The concentration dependence is due to benzoic acid dimerization in the heptane phase. The pH dependence is due to ionization in the aqueous phase. Quenching benzoic acid dimerization by adding a small amount of butanol and working at acidic pH to suppress ionization, it is possible to obtain a single benzoic acid molecular form in the aqueous as well as organic liquid phases. In these conditions, distribution ratio and distribution constant are identical. The CCC benzoic acid retention volume is related to the distribution constant but the liquid system is the heptane/butanol/water system. Since butanol is needed to quench dimerization in the heptane phase, it is not possible to determine directly the heptane/water benzoic acid distribution constant or partition coefficient by CCC. The value of the dimerization constant must be known to extract the distribution constant (partition coefficient) from the experimentally obtained distribution ratios.

Acknowledgments

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