

# Reconsideration of sample pH adjustment in bioanalytical liquid–liquid extraction of ionisable compounds

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Received 2 January 2007; accepted 14 March 2007

Available online 24 March 2007

## Abstract

Liquid–liquid extraction (LLE) is widely used as a simple and robust sample preparation technique in bioanalytical sample preparation. When extracting ionisable compounds, most bioanalysts adjust the pH of the sample to achieve fully unionized compounds. Usually, a generally accepted rule is applied to adjust the pH of the aqueous phase, known as the  $pK_a \pm 2$  rule, depending on the acid/base characteristics of the analyte. By taking a closer look at the general equations that describe the extraction behaviour of ionisable compounds, we extended this pH adjustment rule by taking the distribution ratio and the volume of both liquid phases into account. By choosing an extraction pH based on this extended rule, the selectivity of the extraction can be influenced without loss of recovery. As a measure of this selectivity, two equations were proposed to indicate the ability of the extraction system to discriminate between two compounds. Also, milder extraction pH can be used for pH labile analytes. To use this new rule quantitatively, a new calculation method for the determination of the distribution ratio was derived. These calculations were based on normalized recoveries making this method less susceptible to errors in absolute recovery determination. The proposed equations were supported by demonstrating that careful pH adjustment can lead to higher selectivity. The main conclusion was that a closer look at the extraction pH in bioanalytical methods extends the possibilities of obtaining a higher selectivity or the possibilities of extracting pH labile analytes at milder pH conditions without loss of recovery.

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**Keywords:** Liquid–liquid extraction; pH adjustment; Bioanalysis; Modelling; Recovery; Selectivity

## 1. Introduction

Liquid–liquid extraction (LLE) is widely used as a sample preparation technique in the bioanalysis of drugs [1]. Despite the use of selective and sensitive detectors such as mass spectrometers, sample preparation is still necessary to concentrate or to clean-up the sample in order to prevent matrix effects like ionization suppression [1,2].

Modelling of analytical systems can be very useful in understanding the behaviour of that particular system and can be used to optimize the parameters that influence the analytical results. In the past decades, many papers dealing with modelling and optimisation of liquid–liquid extractions have been published.

These papers deal with the selection of extraction solvents [3–6] and the choice of calculation models to determine the optimal mixture from a set of solvents, extraction time and intensity of shaking [4,7,8].

As most drugs and/or their metabolites contain one or more ionisable functional groups, the pH of the aqueous phase influences the extraction recovery, since charged species are assumed to avoid organic solvents. Although possible, the formation of ion-pairs with counter ions from the sample is assumed to be negligible [1,8–10].

Most bioanalytical chemists adjust the pH of the aqueous phase of the extraction system at least two units away from the  $pK_a$  of the ionisable group resulting in a compound that is almost completely uncharged and leads to maximum extraction recovery [1,8]. This widely used rule will be indicated as the “ $pK_a \pm 2$  rule” in the remainder of this paper. Adjustment of the pH to less extreme values could be favourable for unstable

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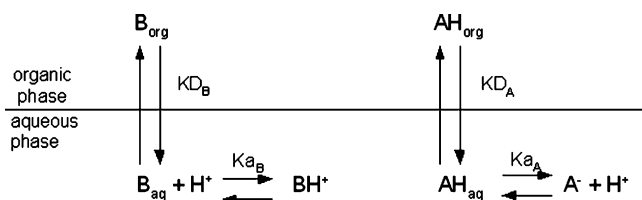


Fig. 1. Extraction equilibria of the extraction of a weak acid, A and a weak base, B. AH and BH<sup>+</sup> indicate the protonated form of the analyte and A<sup>-</sup> and B, the unprotonated form.

compounds under basic or acidic conditions or in order to achieve a different extraction selectivity.

This paper extends the  $pK_a \pm 2$  rule based on conventional theory and equations. A convenient new equation was provided by incorporating the distribution parameters and the volumes used in the LLE system. In routine bioanalytical work, the goal is a one step extraction providing selective and high recovery. Therefore, to model the pH dependency of the analytical recovery of the extraction system, the method should be functional both qualitatively and quantitatively. Conventional methods of modelling the extraction behaviour are based on determining absolute analytical recoveries at a certain pH [10,12,20,21]. Unless detailed precautions are taken, the determination of absolute analytical recovery is very susceptible to systematic error due to non-quantitative liquid handling.

In order to determine the distribution parameters, a new calculation method was also derived from the new pH adjustment rule. This calculation is based on the pH shift of the pH-recovery curve which makes this determination independent of absolute recoveries.

The properties of co-extracted interferences from a biological matrix are mostly unknown. Hence, the influence of the extraction pH on the recovery of the co-extracted interferences has to be investigated by performing extractions at different pH values. By modelling the extraction behaviour of the analytes, the pH range and volumes of extraction solvents can be optimized more systematically when trying to minimize the (unknown) interferences without loss of recovery.

To demonstrate the use of the proposed method of pH adjustment on the selectivity of the LLE system, a set of simple equations were derived in order to demonstrate the selectivity in a quantitative way. To the best of our knowledge, the quantification of selectivity has not previously been described for bioanalytical extraction.

## 2. Theory

Liquid–liquid extraction is based on partition of an uncharged analyte between an aqueous phase and a theoretically water-immiscible organic phase until equilibrium is reached.

In an extraction system for an ionisable compound the most important equilibria are the dissociation of the ionisable group, with  $K_a$  as the dissociation constant, and partitioning of the unionized species, with KD as equilibrium constant, as depicted in Fig. 1.

The distribution of the analyte, regardless of its chemical form, is described by its distribution ratio,  $D$  in Eq. (1) [11], where  $C_{org}$  and  $C_{aq}$  represent the total concentrations of the analyte in the organic and in the aqueous phase, respectively. This is in fact the observed parameter in practical work, as we cannot distinguish between the charged and uncharged forms during extraction.

$$D = \frac{C_{org}}{C_{aq}} \quad (1)$$

The distribution of the uncharged form of the analyte is described by the partition ratio or distribution constant, KD [11], for an acid and a base represented by Eqs. (2a) and (2b), respectively.

$$KD_A = \frac{[AH]_{org}}{[AH]_{aq}} \quad (2a)$$

$$KD_B = \frac{[B]_{org}}{[B]_{aq}} \quad (2b)$$

The dissociation equilibrium of the analyte is assumed to take place in the aqueous phase only [1,8,11] and is described by Eq. (3a) for a weak acid and Eq. (3b) for a weak base which can be rearranged to the generally known Henderson–Hasselbalch equation, Eq. (4a) for an acid and Eq. (4b) for a base. This equation was used to describe the relationship between the pH and the ratio of the ionized and the unionized form of the analyte.

$$K_{aA} = \frac{[A^-][H^+]}{[HA_{aq}]} \quad (3a)$$

$$K_{aB} = \frac{[BH^+]}{[B_{aq}][H^+]} \quad (3b)$$

$$pH = pK_{aA} + \log \frac{[A^-]}{[HA]} \quad (4a)$$

$$pH = pK_{aB} + \log \frac{[B]}{[BH^+]} \quad (4b)$$

Combination of Eqs. (1)–(4) leads to an equation which describes the relationship between  $D$  and KD as a function of the pH, which can be rearranged to Eqs. (5a) and (5b) for an acid and a base, respectively.

$$D_A = \frac{KD_A}{1 + 10^{pH-pK_{aA}}} \quad (5a)$$

$$D_B = \frac{KD_B}{1 + 10^{pK_{aB}-pH}} \quad (5b)$$

In bioanalytical work, the distribution ratio,  $D$ , can be determined from the analytical recovery,  $R$ , of an extracted analyte [8]. This recovery can be calculated by comparing the response (i.e. peak height or area produced by an LC-system) of an extracted sample with the response of a solution containing the analyte in a concentration corresponding to a recovery of 1. The distribution ratio can now be calculated from Eq. (6) taking the volume

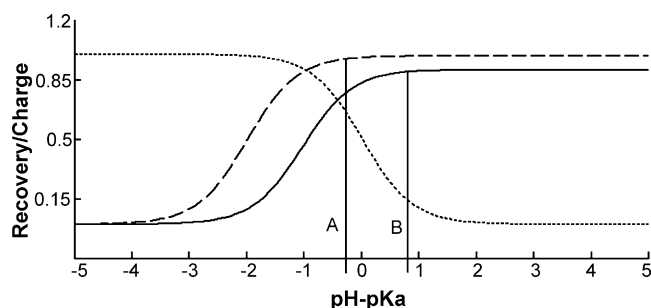


Fig. 2. Charge and recovery of two fictitious basic compounds with the same value of  $pK_a$  in an extraction system with equal volumes of aqueous and organic phases. Lines A and B indicate the pH at which 99% of the maximum recovery can be achieved. (.....) Charge of the functional basic group; (---) compound B1, with a KD-value of 100; (—) compound B2 with a KD-value of 10.

of aqueous phase,  $V_{aq}$ , and the volume of organic phase, the extraction solvent,  $V_{org}$ , into account [8].

$$D = \frac{R}{1-R} \times \left( \frac{V_{aq}}{V_{org}} \right) \quad (6)$$

For our work, Eq. (4) was rearranged to determine the fraction of the ionized (charged) form,  $\alpha$ , of the analyte resulting in Eq. (7a) and (7b) for an acid and a base, respectively.

The  $pK_a \pm 2$  rule is based on these equations, since they show less than 99% of ionisation for an acid at  $pK_a - 2$  for an acid and  $pK_a + 2$  for a base.

$$\alpha_A = \frac{10^{pH-pK_{aA}}}{1 + 10^{pH-pK_{aA}}} \quad (7a)$$

$$\alpha_B = \frac{10^{pK_{aB}-pH}}{1 + 10^{pK_{aB}-pH}} \quad (7b)$$

Eqs. (5) and (6) were combined, resulting in a description of the relationship between the recovery,  $R$ , of an acid (8a) or a base (8b) and the pH of the aqueous phase:

$$R_A = \frac{KD_A \times V}{KD_A \times V + 1 + 10^{pH-pK_{aA}}} \quad (8a)$$

$$R_B = \frac{KD_B \times V}{KD_B \times V + 1 + 10^{pK_{aB}-pH}} \quad (8b)$$

where  $V = V_{org}/V_{aq}$ .

A graphical representation of Eq. (8b) is given in Fig. 2, where the recovery of two fictitious basic compounds, B1 and B2, are plotted against the pH of the aqueous phase. A plot according to Eq. (8) will be called a ‘‘recovery curve’’ in the remainder of this paper. These compounds are similar in acidity ( $pK_{aB1} = pK_{aB2}$ ) but differ in their distribution constants, KD. The pH is expressed as the difference between the pH and the  $pK_a$  to make the graph independent of the actual value of the  $pK_a$ . The volumes of the aqueous phase and the organic phase are kept equal ( $V = 1$ ).

From Fig. 2 it is obvious that in order to obtain a ‘robust’ (flat) recovery:

- (1) The pH should not necessarily be adjusted to at least  $pK_a + 2$  in the case of a basic compound ( $pK_a - 2$  for acids). Extraction recovery can be high even though the compound is

largely charged. This is because the acid–base dissociation shifts to the left, Fig. 1, as the uncharged part of the compound is extracted into the organic phase.

- (2) Higher distribution constants permit lower extraction pH for a basic compound without loss of recovery and can lead to different selectivity with respect to compounds differing in distribution constant. This is shown in Fig. 2 where lines A and B indicate the pH at which at least 99% of the maximum achievable recovery can be obtained.

From the theory mentioned above, we can conclude that the recovery of a particular analyte depends on the  $pK_a$  value of the ionisable group which can be found in literature, estimated by means of dedicated computer programs or determined experimentally. A good approximation of KD depends on the nature of the analyte and the particular extraction system and must therefore be determined experimentally for each combination of matrix to be extracted and extraction solvent.

### 2.1. Extraction selectivity

To measure differences in selectivity of an LLE system we have defined a selectivity parameter,  $S_D$ , based on the distribution of two analytes  $X_1$  and  $X_2$  in an extraction system, as the absolute difference in analytical recovery:

$$S_D(X_1, X_2) = |R_{X_1} - R_{X_2}| \quad (9)$$

Higher values of  $S_D$  indicate that the extraction system is able to discriminate between two compounds to a higher extent.

An  $S_D$  value of 1 indicates complete discrimination of the two analytes, meaning that one compound still resides completely in the aqueous phase whereas the other is completely extracted into the extraction solvent. An  $S_D$  value of 0 indicates a similar extraction recovery of both analytes which can be of any value between 0 and 1.

The example in Fig. 2 shows that for these specific compounds, low values of  $S_D$  can be achieved at  $pH - pK_a > 1$  but also at  $pH - pK_a < -3$ .

The difference is that the relative concentration of compound B1 in the extract, is much higher at  $pH - pK_a < -3$ . Therefore, we have also defined the relative extraction selectivity,  $S_{rel}$ , which defines the relative purity of the extract. Although the absolute recovery is low,  $S_{rel}$  is much higher at  $pH - pK_a = -3$  in Fig. 2 than at  $pH - pK_a > 1$ .  $S_{rel}$  is defined as  $S_D$  divided by the highest recovery of the two analytes over which the  $S_{rel}$  is calculated resulting in Eq. (10).

$$S_{rel} = \frac{|R_{X_1} - R_{X_2}|}{\max(R_{X_1}, R_{X_2})} \quad (10)$$

$S_D$  and  $S_{rel}$  should always be mentioned together to quantify the selectivity of the extraction system at a given pH.

### 2.2. pH dependent shift of the recovery curve

From Eq. (8) and Fig. 2, we can conclude that the recovery curve of a basic compound shifts to the left (i.e. lower pH) at

higher values of  $K_D$ . To quantify this shift, its magnitude will be compared to the  $pK_a$  value.

Equating the second derivative of Eq. (8) to zero and solving it for pH gave us the pH of the inflection point of the recovery curve, indicating the pH at which half of the maximum achievable recovery of a basic compound can be expected,  $pH_{0.5}$ :

$$\frac{d^2}{dpH^2} R_B(pH) = 0 \Rightarrow pH_{0.5} = pK_{aB} - {}^{10} \log(KD_B \times V + 1) \quad (11a)$$

And for an acid:

$$\frac{d^2}{dpH^2} R_A(pH) = 0 \Rightarrow pH_{0.5} = pK_{aA} + {}^{10} \log(KD_A \times V + 1) \quad (11b)$$

At an infinitely low value of  $KD$ , the values of  $pH_{0.5}$  and  $pK_a$  are similar and half of the maximum achievable recovery is obtained at the pH where the degree of charge,  $\alpha$ , equals 0.5.

This is in fact the situation on which the  $pH = pK_a \pm 2$  rule is based.

The second term in the right side of Eq. (11) actually indicates the shift of the recovery curve.

Every  $KD > 0$  value leads to a shift of this curve.

This also means that the pH at which 99% of the maximum recovery can be obtained decreases in the case of a basic analyte and increases in the case of an acid. Since the shape of the recovery curve remains constant, the conventional rule can now be expanded by Eq. (11) to calculate the pH at which at least 99% of the maximum recovery can be obtained,  $pH_{0.99}$ , by adding 2 at the right side for Eq. (11a) or subtracting 2 for Eq. (11b).

As a new rule, we can state that for every order of magnitude of  $KD$ , the extraction pH can be lowered by a value of 1 for a base and raised by a value of 1 for an acid without loss of recovery. This also goes for the volume ratio,  $V$ . Increasing the volume of extraction solvent also leads to a shift of the curve allowing further decrease or increase of the pH in case of a base or acid, respectively.

For a qualitative use of Eq. (11), suitable values for the distribution constant and of the  $pK_a$  are needed.

### 2.3. Determination of the distribution constant ( $KD$ )

The determination of  $KD$  is described in detail [10,12,20,21] and leads to Eq. (12a) for an acid and Eq. (12b) for a base.

$$KD_A = \frac{R_A}{1 - R_A} \times \frac{(1 + 10^{pK_{aA} - pH})}{V} \quad (12a)$$

$$KD_B = \frac{R_B}{1 - R_B} \times \frac{(1 + 10^{pH - pK_{aB}})}{V} \quad (12b)$$

The determination of  $KD$  is mainly based on recovery values that have been obtained experimentally and is therefore subjected to errors affecting this recovery [15–17]. These errors can result in a high variability for the calculated  $KD$  value.

Low recovery values are very susceptible to these experimental errors while high recovery values can lead to huge errors in

$KD$ , due to the nature of Eqs. (8) and (12) [21]. Due to measurement errors, a recovery of 0.99 cannot be distinguished from a recovery of 0.999 but this leads to  $KD$  values differing by a factor of 10. Therefore, the determination should preferably be based on recovery values near 50%. Multiple determinations at different values of pH can be fitted to Eq. (8) by a regression technique [20,21]

### 2.4. The $pK_a$ value and pseudo $KD$ , $KD'$

To use Eqs. (11) and (12), we need a suitable value of the  $pK_a$ . An estimate of the  $pK_a$  of the ionisable group can often be found in appropriate literature or can be estimated by dedicated software programs. For such  $pK_a$  values we have to realize that the  $pK_a$  may refer to the thermodynamic  $pK_a$  at infinite dilution and at zero ionic strength [12–14] or that the circumstances at which the  $pK_a$  is determined is not given at all.

Also, the analytes reside in a mixture of a biological matrix and a buffer with a certain (unknown) ionic strength. Every change in  $pK_a$  due to ionic strength effects leads to shift of the recovery curve equal to the change in  $pK_a$ .

Such a changed  $pK_a$  value or a biased pH due to a systematic error in pH measurement may lead to a supposed shift in the observed recovery curve. This may lead to an over- or under estimation of the calculated  $KD$  value resulting in a pseudo  $KD$ ,  $KD'$ , as a compensation for the errors in the measured pH or in changes in  $pK_a$  with respect to the used  $pK_a$  estimate when using Eqs. (8) or (12). For modelling the extraction system this is not necessarily an issue as long as it concerns relatively high values of  $KD$ . However, when recovery predictions according to Eq. (8) are based on this over- or under estimated  $KD$  value, this will also lead to a change in the predicted maximum achievable recovery, i.e. recovery obtained at high pH for a base and recovery at low pH for an acid. This goes for lower values of  $KD$  in particular. For this reason we can state that the calculation of  $KD'$  in order to compensate for a biased  $pK_a$  or pH is less suitable for describing an extraction system of analytes with relatively low  $KD$  values. In such a case the volume ratio can be increased to increase the recovery or greater effort should be made to find a more suitable extraction solvent.

To describe an extraction system, the calculated  $KD'$  should always be accompanied by the  $pK_a$  value it was based on.

We developed a calculation method to estimate a usable  $KD'$  which is independent of the absolute recovery.

When using normalized recoveries, calculations are only based on responses from real sample extracts and are not compared to the response of a pure solution containing all analytes at the 100% level. The responses from the sample extracts are compared to each other. This makes the calculation method less susceptible for systematic (non quantitative) liquid handling and makes the practical work easier to perform.

### 2.5. The curve shift method

To develop an algorithm to calculate  $KD'$  the experimentally determined recovery values were normalized with respect to the highest recovery found at a pH where no further increase of

recovery is observed (see Fig. 2). The normalized recovery ( $R_n$ ) is expressed as the fraction of the maximal realisable recovery and hence is scaled between 0 and 1.

A closer look at Eq. (11) shows that the shift of the recovery curve is actually a shift with respect to the charge curve of Eq. (7). The only difference is that Eq. (7) explains the fraction of charged compound. When swapping Eq. (7) for an acid and a base respectively they reveal the fraction of uncharged compound with respect to the pH. When the KD is infinitely low, the normalized recovery curve follows this charge curve.

For a base, the fraction of uncharged compound,  $\beta$ , is given by Eq. (13).

$$\beta_B = \frac{10^{\text{pH}-\text{p}K_{\text{aB}}}}{1 + 10^{\text{pH}-\text{p}K_{\text{aB}}}} \quad (13)$$

Solving Eq. (13) for pH results in Eq. (14), where pH is calculated for each fraction of uncharged compound.

$$\text{pH}(\beta) = \text{p}K_{\text{aB}} + \log\left(\frac{-\beta}{\beta - 1}\right) \quad (14)$$

Although  $\beta$  is the fraction of uncharged compound, we substituted the normalized recovery,  $R_n$ , for  $\beta$  in Eq. (14) as both fractions are supposed to be equal at infinitely low KD. At higher KD values, the difference between the pH at which  $R_n$  is determined,  $\text{pH}(R_n)$ , and the calculated pH according to Eq. (14), at which this fraction is expected, based on the charge curve, is equal to the right hand term of Eq. (11a) and leads to Eq. (15).

$$\text{pH} - \text{pH}(R_n) = -\log(\text{KD} \times V + 1) \quad (15)$$

Solving Eq. (15) for  $R_n$  leads to Eq. (16a) and shows the normalized recovery curve as a function of the pH for a base. The determination of KD based on Eq. (16) is now only based on the curve shift due to KD and  $V$ .

$$R_n(\text{pH}) = 10^{(\text{pH}-\text{p}K_{\text{aB}})} \times \left( \frac{\text{KD} \times V + 1}{\text{KD} \times V \times 10^{(\text{pH}-\text{p}K_{\text{aB}})} + 10^{(\text{pH}-\text{p}K_{\text{aB}})} + 1} \right) \quad (16a)$$

For an acid, Eq. (16b) can be derived in the same way.

$$R_n(\text{pH}) = 10^{(\text{p}K_{\text{aA}}-\text{pH})} \times \left( \frac{\text{KD} \times V + 1}{\text{KD} \times V \times 10^{(\text{p}K_{\text{aA}}-\text{pH})} + 10^{(\text{p}K_{\text{aA}}-\text{pH})} + 1} \right) \quad (16b)$$

## 2.6. Experimental determination of $\text{KD}'$

$\text{KD}'$  values should be based on experimentally obtained recoveries which are more reliable when its values are far from the extremes of 0 and 1. From a practical point of view, this means that we should base the  $\text{KD}'$  determinations on normalized recoveries near 0.5.

Multiple extraction experiments should be performed at different pH values around the  $\text{p}K_{\text{a}}$  of the compound and especially

at lower pH for a basic compound and higher pH for an acidic compound. By plotting the normalized recovery against the extraction pH according to Eq. (16),  $\text{KD}'$  can be estimated by a non-linear regression technique. A least squares regression method [18,21] can be used to fit Eq. (16) to the experimentally obtained data with  $\text{KD}'$  as the only regression parameter to be calculated with an estimated value of  $\text{p}K_{\text{a}}$ . The benefit of a regression technique would be that the largest differences to be minimized are observed in the steepest regions of the recovery curve (at a  $R_n$  of 0.5), making this regression technique very sensitive for the most reliable  $R_n$  values.

## 3. Experimental

### 3.1. Experimental design

The tri-cyclic antidepressants amitriptyline and imipramine and their respective metabolites nortriptyline and desipramine were chosen as model compounds. The analyses were performed using a normal phase HPLC system, which allows direct injections of the organic extraction solvent. In this way, potential problems during solvent evaporation or reconstitution which could introduce errors in the determination of recovery [16] were circumvented. The choice of extraction solvent could be investigated as described in [8], but we chose a mixture of 1.5% of iso-amyl alcohol in hexane based on past experience with this in house developed method.

Literature  $\text{p}K_{\text{a}}$  values [19] of each analyte were used in calculations. Buffers were prepared at similar molar concentrations covering a wide pH range about the  $\text{p}K_{\text{a}}$  values of the analytes. After extraction, as described in Section 3.5, for each analyte, the peak areas were compared to the highest area of all experiments. All areas for the same analyte were normalized against the highest area by stating this result as the reference with a recovery of 1.  $R_n$  values were plotted against the pH of the aqueous phase. The pseudo KD was calculated by use of the Microsoft Excel 2002 solver option by minimizing the sum of the squared differences between the obtained recovery values and the predicted recovery values at the corresponding pH values according to Eq. (8b).  $\text{KD}'$  was set to be the parameter to be optimized.

### 3.2. Chemicals and reagents

Amitriptyline (ami), nortriptyline (nor), imipramine (imi) and desipramine (desi) were purchased from Sigma (St. Louis, MO, USA). Methanol, hexane, dichloromethane, iso-amyl alcohol, ammonium acetate, sodium acetate, citric acid, sodium di-hydrogen phosphate mono hydrate, Tris (hydroxymethylaminomethane), hydrochloric acid (36%), sodium hydroxide, diethyl amine and acetic acid were all of analytical grade and obtained from Merck (Darmstadt, Germany). Water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). EDTA plasma was obtained from a healthy female volunteer.

Stock solutions of ami, nor, imi and desi were prepared in methanol at a final concentration of 0.100 mg/ml.

Table 1

Mean results ( $n = 3$ ) of the normalized recovery,  $R_n$ , of the extraction recovery experiments of amitriptyline, imipramine, nortriptyline and desipramine

Buffer		Aqueous phase	$R_n/R.S.D.$ (%)			
Type	pH	pH measured	Ami	Nor	Imi	Desi
1 M HCl	*	0.50	0.00/0.0	0.00/0.0	0.00/0.0	0.00/0.0
Acetate	4.00	4.65	0.00/0.0	0.00/0.0	0.00/0.0	0.00/0.0
Acetate	5.00	5.55	0.01/16.5	0.00/0.0	0.25/4.6	0.00/0.0
Citrate	5.50	5.84	0.26/2.4	0.04/23.9	0.76/3.1	0.00/0.0
Phosphate	6.00	6.29	0.45/1.6	0.10/8.4	0.86/0.9	0.00/0.0
Phosphate	6.40	6.58	0.62/0.7	0.14/7.0	0.96/0.8	0.02/173
Phosphate	7.00	7.07	0.72/2.7	0.31/3.5	0.93/1.0	0.19/9.5
Phosphate	8.00	7.93	0.97/0.3	0.72/1.4	0.99/0.2	0.57/2.9
TRIS	8.50	8.48	1.00/0.4	0.89/2.2	0.99/1.5	0.79/0.8
TRIS	9.00	8.90	1.00/0.6	0.95/1.1	1.00/1.0	0.91/2.0
Phosphate	11.50	11.05	1.00/0.9	1.00/8.8	0.99/1.1	1.00/11.5
Phosphate	12.00	11.47	0.99/0.1	0.99/0.9	0.99/0.3	1.00/2.6
Phosphate	12.50	11.80	0.98/0.5	0.93/2.5	0.98/1.6	0.86/2.4

\* Not measured.

Plasma samples were prepared by spiking analyte free plasma with ami, nor, imi and desi to a final concentration of 200 ng/ml.

Acetate, citrate, phosphate and Tris buffers were prepared at a concentration of 0.200 M and adjusted with either undiluted hydrochloric acid or with a 10 M sodium hydroxide solution to their final pH. Acetate buffers were adjusted to pH 4.0 and 5.0. Citrate buffer was adjusted to pH 5.5. The phosphate buffer was adjusted to pH 6.0, 6.4, 7.0, 8.0, 11.5, 12.0 and 12.5. The Tris buffer was set to pH 8.5 and 9.0.

The extraction solvent was prepared by mixing 985 ml of *n*-hexane with 15.0 ml of iso-amyl alcohol.

### 3.3. Equipment

Chromatographic analysis was performed on a HPLC system consisting of a GyncoTec model 300 isocratic pump (GyncoTec, Germering, Germany) operating at a flow rate of 1.00 ml/min, a Merck/Hitachi LaChrom L-7200 autosampler, and a Merck/Hitachi LaChrom UV-detector (Merck, Darmstadt, Germany) operating at  $\lambda = 239$  nm. EZChrom Elite client/server version 2.8.3 (Chromtech, Idstein Germany) was used to acquire and analyse the chromatographic data. pH measurements were performed with a Metrohm model 713 pH meter equipped with a combined glass electrode which was calibrated at pH 4.0, pH 7.0 and 9.0 according to the manufacturers instructions (Metrohm Herisau, Switzerland).

### 3.4. Chromatographic conditions

Chromatographic separation was performed at ambient temperature on a Chromspher Si column (100 mm  $\times$  4.6 mm; 3  $\mu$ m) (Varian, Palo Alto, CA, USA). The mobile phase was prepared by mixing 100 ml of methanol, 900 ml of dichloromethane and 15.0 ml of a buffer solution. This buffer solution was prepared by mixing 200 ml of a 30% v/v acetic acid solution in water and 10.0 ml of diethyl amine. The pH of the buffer was adjusted to pH 3.2 with a 30% v/v acetic acid solution. The injection volume was 100  $\mu$ l in all cases.

### 3.5. Method

Plasma samples were analysed in triplicate. For spiked plasma, 1.00 ml was mixed with 1.00 ml of the appropriate buffer as indicated in Section 3.2. Another 1.00 ml of blank plasma was mixed with the same buffer for pH measurement. For the extraction solvent, 2.00 ml was added and the tubes were capped and extracted by tumble mixing for 30 min at 60 rpm. The aqueous volume,  $V_{aq}$ , and the volume of extraction solvent,  $V_{org}$ , were equal, resulting in a volume ratio of 1. Phase separation was performed at ambient temperature by centrifuging the samples for 5 min at ca. 2000  $\times$  g. The extraction solvent was transferred to autosampler vials and injected into the HPLC system. The peak area was used in calculations as the analytical response. The pH of the sample/buffer mixture was measured in separate mixtures.

## 4. Results and discussion

The above mentioned chromatographic conditions lead to retention times of 4.79, 5.28, 9.29 and 10.08 min for amitriptyline, imipramine, nortriptyline and desipramine respectively.

The mean results of the triplicate measurements and their corresponding relative standard deviation (R.S.D.) of these experiments are presented in Table 1.

From the results in Table 1, and the  $pK_a$  values from [19], the  $KD'$  values were calculated as explained in Section 2.5 by fitting the experimental results to Eq. (16a). The  $pK_a$  values and the calculated results are presented in Table 2.

Table 2  
Found  $KD'$  values and  $pK_a$  values

Component	$pK_a$	$KD'$
Amitriptyline	9.4	918
Nortriptyline	9.7	178
Imipramine	9.5	6870
Desipramine	10.2	246

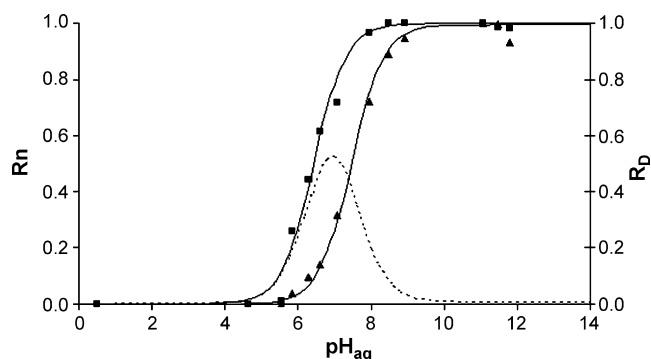


Fig. 3. Graphical representation of the extraction recovery results. (■) Experimental points of amitriptyline; (▲) experimental points of nortriptyline; solid line: fitted curve; dashed line: calculated  $S_D$  from fitted curves.

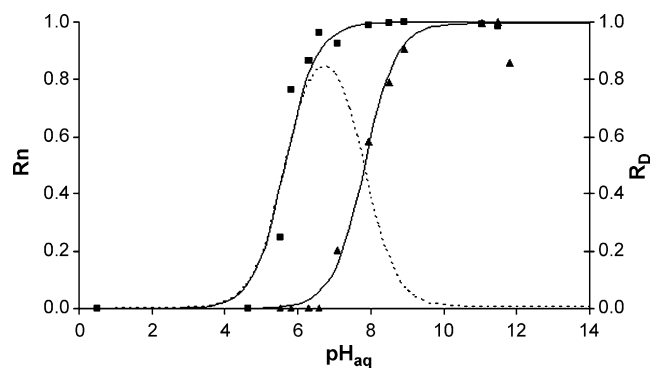


Fig. 4. Graphical representation of the extraction recovery results. (■) Experimental points of imipramine; (▲) experimental points desipramine; solid line: fitted curve; dashed line: calculated  $S_D$  from fitted curves.

From these data, we can now describe the extraction behaviour of all four analytes. The results of the regression are shown in Figs. 3 and 4.

Using the data from Table 2, the recovery curves can be plotted according to Eq. (8) and from these curves we can now determine the selectivities and the relative selectivities at each pH of each set of analytes according to Eqs. (9) and (10), respectively. Because of the relatively high  $KD'$  values, the plotted curves according to Eqs. (8) and (16) are approximately equal. The selectivities  $S_D$  are therefore also plotted in Figs. 3 and 4.

The results of the maximum achievable selectivity are presented in Table 3.

The data in Figs. 3 and 4 demonstrate extraction of all our analytes at maximum recovery at an aqueous phase pH of at least 9.8, where all analytes are theoretically extracted at a recovery of at least 99%. Despite some scatter of the data points around the fitted curve (largely due to the absence of temperature control), the

calculation method was able to model the extraction behaviour with good precision as we were performing the extraction of the analytes routinely at pH 9.8 with a recovery approaching 1 for all analytes.

The data also support Eq. (11) and show that the original  $pK_a \pm 2$  rule was not applicable to this extraction system, as the pH of 9.8 is even 0.4 units lower than the highest  $pK_a$  value (10.2 for desipramine). At this pH, no selectivity was achieved, nor was this intended, when extracting all analytes quantitatively. However, using our analytes as a model compound, we demonstrated that lowering the pH of the aqueous phase leads to a significant change in selectivity of the analytes. Based on the results from Table 3, imipramine and desipramine were almost completely resolved in the extraction solvent at a pH of 6.7, as indicated by a  $S_{rel}$  of 0.92. When these parameters were calculated from the experimentally obtained data from Table 2 at pH 6.58 values of 0.94 and 0.98 for  $S_D$  and  $S_{rel}$ , respectively were found.

Changes in volume of extraction solvent would also lead to changes in selectivity at constant pH due to a shift of the recovery curve according to Eq. (11). Hence, when changing the volume ratio, the pH should be adjusted in order to keep the selectivity constant.

## 5. Conclusion

We revised the frequently used rule of adjusting the extraction pH as often used in bio-analytical chemistry. In order to achieve a stable recovery of 99% of the maximum achievable recovery, the  $pK_a \pm 2$  rule can be extended by the rule that the pH can be lowered by one unit for basic compounds for each order of magnitude of  $KD$ . For acidic analytes, the pH can be increased as  $KD$  increases.

To quantify the pseudo distribution constant ( $KD'$ ), we set up a method based on the shift of the recovery curve with respect to the charge versus pH curve. This pH shift method is less susceptible to errors in recovery determination.

The calculated  $KD'$  leads, together with a literature  $pK_a$  value, to a reliable model of the extraction behaviour of the analytes.

We proposed a pair of equations to quantify a measure of the selectivity of an extraction system.

It is worthwhile to take a closer look at the pH of the aqueous phase in bioanalytical sample extraction concerning ionisable compounds in order to achieve a different selectivity. It is also worthwhile to change the extraction pH to less extreme values in case of extracting analytes which are unstable at extreme pH.

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Table 3

Maximum achievable selectivity and relative selectivity at indicated pH

Analyte	Imipramine			Nortriptyline			Desipramine		
	pH	$S_D$	$S_{rel}$	pH	$S_D$	$S_{rel}$	pH	$S_D$	$S_{rel}$
Amitriptyline	6.1	0.42	0.57	7.0	0.52	0.66	7.1	0.66	0.82
Imipramine				6.6	0.77	0.90	6.7	0.84	0.92
Nortriptyline							7.7	0.20	0.32

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## Glossary

- A*: acid  
*aq*: aqueous phase  
*B*: base  
*C*: concentration  
*D*: distribution ratio  
*K<sub>a</sub>*: dissociation constant  
*KD*: distribution constant  
*KD'*: pseudo KD  
*LLE*: liquid–liquid extraction  
*org*: organic phase  
*pK<sub>a</sub>*:  $-\log(K_a)$   
*R*: recovery  
*R<sub>n</sub>*: normalized recovery  
*R.S.D.*: relative standard deviation  
*S.D.*: standard deviation  
*S<sub>D</sub>*: distribution selectivity  
*S<sub>rel</sub>*: relative selectivity  
*V*: volume  
 $\alpha$ : fraction of charged species  
 $\beta$ : fraction of uncharged species