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New practical algorithm for modelling analyte recovery in bioanalytical reversed phase and mixed-mode solid phase extraction

G. Hendriks[a,*], D.R.A. Uges[b], J.P. Franke[c]

[a] PRA International, Early Development Services, P.O. Box 200, 9407AE Zuidlaren, The Netherlands
[b] Laboratory for Drug Analysis and Toxicology, Department of Pharmacy, Groningen University Hospital, P.O. Box 30.000, 9700 RB Groningen, The Netherlands
[c] Department of Pharmaceutical Analysis, University Centre of Pharmacy, P.O. Box 196, 9700 AD Groningen, The Netherlands

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ABSTRACT

Solid phase extraction (SPE) is a widely used method for sample cleanup and sample concentration in bioanalytical sample preparation. A few methods to model the retention behaviour on SPE cartridges have been described previously but they are either not applicable to ionised species or are not suitable when using multiple wash and elution steps with solvents differing in volume, modifier concentration and acidity. Furthermore, these models were not applied to mixed mode SPE sorbents.

In order to overcome these limitations a new SPE modelling algorithm was proposed. The retention behaviour was determined directly on the SPE cartridge by connecting the cartridge online with an HPLC system using a simple but suitable device that was developed and described.

The results from these online experiments were used to model the elution behaviour using a quadratic retention function combined with an exponentially modified Gaussian peak shape model to predict analyte recovery under different wash and elution conditions.

The validity of the proposed algorithm was tested using practical SPE experiments with an aqueous test mixture as well as with spiked human plasma. Different sequential wash and elution steps were performed using solvents differing in volume and composition.

The predicted band shape and recoveries in each collected step were in good agreement with the results obtained from practical experiments.

The proposed algorithm is very useful for the description of the SPE behaviour of the analytes on the actual used SPE cartridge and can be used in structural and automated SPE method development.

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1. Introduction

Solid phase extraction (SPE) is one of the most widely used methods for sample preparation prior to HPLC or LC–MS analysis in the bioanalysis of small molecules [1,2]. A variety of column dimensions and stationary phase sorbents have been developed and the evolution of SPE is still continuing [3]. Especially, flexibility and the ease of miniaturising and automating the extraction process are the reasons that SPE still gaining popularity [4,5].

The theory of the extraction mechanisms and the use of the different SPE formats are well described in the literature [6,7].

The development of an SPE method is still mainly based on experimental trial-and-error procedures. However, some methods have been developed to characterise the SPE system and to model the sample extraction process. An overview of these methods was well discussed by Poole et al. [8]. The disadvantages of these methods are the need to calculate and to determine a reasonable amount of system- and solute parameters as with the solvation parameter model [6,7]. The main disadvantage of the latter model is that it is strictly applicable to neutral compounds or to ionisable compounds in the neutral form [6]. Some methods involve the packing of HPLC columns with SPE sorbent to determine the retention characteristics of the sorbent by means of an LC system [9–12]. However, the availability of such a large amount of sorbent and the time consuming preparation of a column may be a problem. Also the effects of the cartridge geometry, the frits, the dead volume and the packing density of the cartridges were not taken into account [9]. None of these methods model the elution profile of the actually used SPE cartridge and symmetrical analyte bands are assumed by keeping only the theoretical plate number into account without a correction for peak asymmetry. Neither the use of a combination of both acidic and basic wash and elution steps for ionisable compounds, nor the use of such solvents in combination with mixed-mode sorbents was previously described using these methods.

* Corresponding author. Tel.: +31 592 303477; fax: +31 592303223.
E-mail address: HendriksGert@PRAintl.com (G. Hendriks).

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Another approach in method development method proposed by Waters (Milford, MA, USA) and was described by Cheng et al. [13]. This method involves the use of an aqueous solution containing the analytes, which was applied to a number of SPE cartridges of the same kind. Subsequently, each cartridge was eluted with a fixed volume of an aqueous solvent with a different modifier concentration. The effluents were then analysed by a suitable chromatographic method and the analyte responses were plotted against the modifier concentration. From this plot, the maximum modifier concentration for the wash steps could be derived as well as the minimum modifier concentration needed for the elution of the analytes. Although the performance of the actual SPE cartridge was taken into account, this method is restricted to a fixed volume and does not deal with multiple sequential wash steps causing partial elution.

Also chemometrically methods have been successfully applied to SPE [14,15] in which the analytical recovery was taken as the response to be modelled. However, to describe a response surface as a function of the modifier concentration, the pH and volume, many experiments are needed. Then this method still lacks the possibility of recovery prediction when acidic and basic solvents are used in the same SPE procedure concerning ionisable analytes.

Since far most of the analytes in the bioanalysis of pharmaceutical compounds contain ionisable groups and mixed mode SPE sorbents gain more and more popularity, the previously described models cannot be successfully applied to a bioanalytical SPE method development.

To overcome these problems an algorithm was developed to model the recovery of a particular analyte, either neutral or ionisable, under basic and acidic conditions on the actual SPE cartridge to be used. The algorithm should be able to predict the recovery of the analyte after the application of a solvent at a given composition and volume. The method should also be time saving by the possibility of automating and even be suitable if no structural information of the analyte is available.

2. Theory

Given an SPE cartridge based on syringe barrel format, the modelling of the recovery of the analytes on a particular SPE cartridge was divided into three stages:

- Selecting a suitable sorbent and the determination of the elution behaviour of the analytes on the chosen SPE sorbent by connecting the cartridge on line with an HPLC system. A suitable adapter had to be developed to connect the cartridge between a regular autosampler and a detector (i.e. mass spectrometer). The peak profile provided by this system should closely match the profile of a manually eluted SPE cartridge.
- Modelling of the retention behaviour of the cartridge when used off line using either acidic or basic solvents, differing in modifier concentration and in volume.
- Modelling the elution profile of the analyte bands as they elute from the cartridge (i.e. peak shape) and prediction of the offline analyte recovery.

2.1. Online cartridge connection

To develop a device for the connection of a syringe barrel shaped SPE cartridge, some requirements had to be met. The connections should be leak free and not result in a significant extra dead volume, which could disturb the shape of the analyte band. Also the adapter should be reusable and easily connected to the LC autosampler, the detector as well as to the SPE cartridge.

2.2. Modelling of the retention behaviour

Although different approaches can be found in the literature to relate the fraction of organic modifier to the retention time of the compound, a widely used model to relate the logarithm of the retention factor, $\log K'$ to the modifier concentration by means of a second order polynomial, Eq. (1) [6,8–11,16].

$$\log K' = a\varphi^2 + b\varphi + c \tag{1}$$

In this retention function, φ represents the volume fraction of the used modifier and a, b and c are the parameters to be calculated. Retention data, as obtained by the online experiments, can be easily fit by means of least squares regression. This equation was used as the basis of the elution profile of the analytes.

Care must be taken, especially for polar compounds, that a quadratic fit can result in an up going curve at higher modifier concentrations. In that case, the lowest possible $\log K'$, i.e. at the valley of the fitted parabola, should be used at each modifier concentration beyond that valley.

The elution volume can now be calculated directly at a given solvent composition [6,9,12,16], which makes this method especially useful for trace enrichment of large sample volumes followed directly by the elution with a suitable solvent [16]. In bioanalytical sample preparation, however, the application of the sample is often followed by one or more wash steps to remove possible interferences and analytes may be wanted to be eluted separately by multiple elution steps. Each wash and elution step has its influence on the migration of the analytes on the sorbent bed. Therefore, the proposed procedure was treated as the elution of the analytes using a multiple step gradient.

The retention factor, K', was related to the migration of the analyte band on the sorbent bed by means of the band tracking model [17].

The position of the solute (band) in the cartridge is expressed as a relative distance between the top of the sorbent and the spout of the cartridge, X. If $0 < X < 1$ the top of the solute band resides in the cartridge. With $X > 1$, the top of the solute band is eluted from the cartridge.

Per unit of volume the displacement on the sorbent bed is given by the relative velocity, vr.

A value for vr of 0.50 means that per mL of solvent, the solute is displaced over a distance of 50% of the total sorbent bed length.

The relative speed of an unretained compound, vr_0, can be expressed as:

$$\mathrm{vr}_0 = \frac{1}{\mathrm{Vm}} \tag{2}$$

in which Vm is the volume of the mobile phase in the sorbent bed and is also referred as the cartridge hold up volume. Vm can be derived from the top of the solute band from online chromatographic experiments by measuring the dead time, t_0:

$$\mathrm{Vm} = t_0 F \tag{3}$$

in which F is the flow rate of the LC system.

The retention factor as calculated by Eq. (1) at a given solvent composition can now be related to the relative velocity of the analyte, vr, by:

$$\mathrm{vr} = \frac{\mathrm{vr}_0}{(K' + 1)} \tag{4}$$

And the relative migration distance, X, during the particular wash or elution step with a volume, V, is now:

$$X = \mathrm{vr}V \tag{5}$$

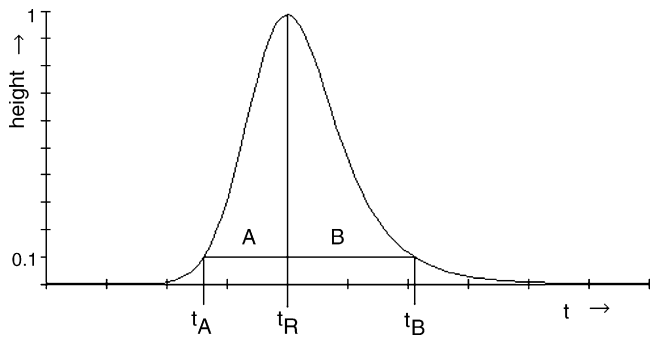

Fig. 1. Example of an EMG peak with measurements for calculating τ and σ_G as explained in the text.

Combination of Eqs. (2–5) results to

$$X = \frac{V}{V_m}\frac{1}{K'+1} \tag{6}$$

Given the chromatographic behaviour (K') of the analyte at a given modifier concentration, for each wash or elution step ($j = 1, \ldots, k$), X_j can now be calculated. The summation of each step results in the total migration distance, X_m, indicating the relative position of the analyte band on the sorbent bed.

$$X_m = \sum_{j=1}^{k} X_j = \sum_{j=1}^{k} \frac{V_j}{V_m}\frac{1}{K'_j+1} \tag{7}$$

As soon as $X_m \geq 1$, the top of the band is eluted. When no retention on the particular column was obtained at any modifier concentration, the retention factor can be assumed as 0. At very strong retention, the retention factor can be assumed to be infinitely high.

Since each wash and elution step in SPE involves the application of a certain volume which is completely pushed through the sorbent bed, the mixing with a previously applied solvent is not excluded but reduced to a minimum. Therefore, each application of solvent can be interpreted as a discrete step. Hence, even acidic and basic solvents, differing in modifier content as well, can be used alternately, provided that a retention function according to Eq. (1) is established for a basic composition as well as for an acidic composition and the conforming function is to be used for each type of solution, either acidic or basic, to be applied onto the SPE column.

2.3. Modelling the elution profile

Analyte bands in SPE may elute as a more or less tailing peak.

A general accepted and used model to describe a non-symmetric peak is the Exponentially Modified Gaussian (EMG) peak [18,19–21] which can be interpreted as the combination of a Gaussian peak and an exponential decay.

The EMG peak can be notated in some different forms. We used the one given by Foley and Dorsey [20], Eq. (8), as it turned out to be the most stable in calculations when used in the reversed way as will be discussed later. Fig. 1 shows an example of an EMG peak as generated by using Eq. (8).

$$f(t) = \frac{A\sigma_G}{\tau\sqrt{2}} \exp\left[\left(\frac{\sigma_G}{\tau}\right)^2 \frac{1}{2} - \frac{(t-t_G)}{\tau}\right] \int_{-\infty}^{Z} \exp[-x^2]\, dx \tag{8}$$

where

$$Z = \frac{\left[(t-t_G)/\sigma_G - \sigma_G/\tau\right]}{\sqrt{2}}$$

In these equations, the height of the peak is calculated at time, t, A is the peak amplitude of the Gaussian component, τ is the exponential modifier, t_G and σ_G are the retention time and the standard deviation of the Gaussian part of the peak, respectively, and x is a dummy variable for integration.

Estimated values of σ_G and τ can be obtained from measurements from the chromatographic online experiments according to the Equations for Calculating Chromatographic Figures of Merit for Ideal and Skewed Peaks [20]. The obtained chromatogram resembles the one as depicted in Fig. 1. In this figure, A and B are measured at 0.1 times the total peak height.

The calculated values for τ and σ_G have to be transformed from a time domain in the chromatogram, to a relative position domain on the SPE cartridge.

As soon as the top of the band elutes from the column, the total travelled relative distance is 1. Therefore, the retention time, t_R, corresponds to 100% of the relative migration distance of the top of the peak. The origin of the chromatogram corresponds to a relative travelled distance of 0.

The proposed equations are transformed to the relative distance domain, X, by division by t_R, resulting in Eqs. (9–11). For convenience, the relative distances can be expressed as a percentage of the total distance. These equations should then be multiplied with 100%.

$$\bar{M}_2 = \frac{W_{0.1}^2}{1.764(B/A)^2 - 11.15(B/A) + 28} \tag{9}$$

$$\sigma_G = \frac{W_{0.1}}{3.27(B/A) + 1.2}\frac{100\%}{t_R} \tag{10}$$

$$\tau = \sqrt{\bar{M}_2 - \sigma_G^2}\frac{100\%}{t_R} \tag{11}$$

where M_2 is called the second statistical moment, (i.e. the variance) and W corresponds to the sum of A and B.

Eq. (8) is written in the form with the tail on the right hand side towards higher values of retention time (Fig. 1). However, on a relative position scale the peak tail is on the left hand side towards lower migration distances. Also, the position of the Gaussian part of the band has to be calculated in order to position the maximum of the band on the right place. Therefore, Eq. (8) was slightly adapted to use it in the reversed order. Retention time, t, was replaced with X, the fractional migration distance on the sorbent bed and t_G was replaced with X_G, the total migrated distance of the maximum of the Gaussian part of the EMG peak.

Due to the nature of the EMG model, the top of the peak, X_m, shifts towards higher retention times with respect to the Gaussian component, depending on the values for σ and τ. The equation to calculate the position of the maximum of the Gaussian component was proposed by Björn et al. [21]. This equation was also adapted to use it in the reversed order resulting in Eq. (12):

$$X_G = X_m + \left(0.72 + 0.46 \ln\left(\frac{\tau}{\sigma_G}\right)\right) \tag{12}$$

The numerical constants were directly taken from ref. [21].

The final function to describe the eluted peak is can now be represented as Eq. (13):

$$f(X) = \frac{A\sigma_G}{\tau\sqrt{2}} \exp\left[\left(\frac{\sigma_G}{\tau}\right)^2 \frac{1}{2} - \frac{(X_G - X)}{\tau}\right] \int_{-\infty}^{Z} \exp[-x^2]\, dx \tag{13}$$

with

$$Z = \frac{[(X_G - X)/\sigma_G - \sigma_G/\tau]}{\sqrt{2}}$$

The area, A, of the portion of the band eluted from the column can be represented as the integral of Eq. (13) from $X = 1$ to infinity:

$$A_{\text{eluted}} = \int_{1}^{\infty} f(X)\ \mathrm{d}X \tag{14}$$

The total area of the peak can be obtained by integrating from $-\infty$ to ∞ and the recovery, R, expressed as fraction of the eluted portion of the band is now:

$$R = \frac{100A_{\text{eluted}}}{A_{\text{total}}(\%)} \tag{15}$$

2.4. Use of the SPE model

By using the SPE cartridge connector, the cartridge to be tested is connected to an LC system where it acts as the column.

Although it is not common in SPE to run a linear gradient, an initial scouting gradient, as is common in HPLC method development [22], can provide a lot of information about retention behaviour and selectivity. Based on a scouting gradient, a choice for the sorbent type can be made by scanning different types of stationary phases.

Once a suitable sorbent was chosen, the elution behaviour of the analytes under isocratic conditions can be determined.

With the cartridge connected to the LC system, different isocratic chromatograms should be recorded at different modifier concentrations. For each type of mobile phase (acidic or basic) the obtained retention times will be related to the modifier concentration by Eq. (1). Also, from the isocratic chromatograms, information about the peak shape can be obtained and by performing a few measurements as described in Section 2.3, this peak shape can be approximated. It was assumed that the peak shape parameters can be kept constant, regardless of the used solvent. Variations in these parameters may result in a discrepancy between the predicted shape of the eluted band and the real shape resulting in a variable recovery prediction. However, SPE cartridges were not expected to perform as analytical columns and therefore some variation in recovery was considered acceptable.

The SPE model can now be used as a simulator for the real system.

The strategy to use this model is to start with the sample application and to find the conditions that favour retention. Care must be taken that the highest possible retention conditions should be gained in the sample application step, since the sorbent has to compete with a lot of endogenous matrix components such as proteins and fatty acids in plasma samples. To eliminate most of the plasma proteins and salts, the first wash step consists of a solvent without organic modifier. When only one analyte is involved or multiple analytes that should be eluted in the same fraction, a suitable wash solvent can be searched for, with the highest possible concentration of organic modifier that does not elute the analyte. Finally, the elution solvent can be determined using the lowest possible modifier concentration that completely elutes the analyte and leaving strongly retained matter on the sorbent. All wash elution steps may vary in volume acidity and modifier concentration.

When the aim is to elute the analytes separately, the choice of solvent composition can be based on the elution sequence in the scouting chromatograms, starting with the earliest eluting analyte and finishing with the most retained analyte. The acidity of each step is based on the maximum selectivity between the analyte to be eluted and the remainder of the analytes. The modifier content and elution volume will be chosen to elute the analyte of interest and leave the other analytes on the sorbent.

3. Experimental

3.1. Materials

Acetanilide, pseudoephedrine hydrochloride, metoprolol and amitriptyline hydrochloride were purchased from Sigma (St. Louis, MO, USA). A test solution was prepared at 1000 ng/ml in water as well as in human EDTA plasma. This concentration was chosen at a high level to result in a sufficiently high response, even for low recoveries, after diluting the SPE effluent. Ultra pure water was obtained by using a Milli-Q water purification system (Millipore, Bedford, MA, USA). Methanol, acetonitrile, HPLC grade, formic acid and Ammonia (25%), analytical grade, were obtained from Merck (Darmstadt, Germany).

Solutions of 1% of formic acid in water were prepared by mixing 10 ml of formic acid with 990 ml of water. In the same way, 1% of formic acid in methanol, 1% of ammonia (25%) in water and 1% of ammonia (25%) in methanol were prepared. These solvents were used either as mobile phase A and B, respectively, in the online SPE chromatographic experiments. A solution of 0.1% formic acid in water was used as mobile phase A in the analytical LC–MS mode. In this case acetonitrile was used as mobile phase B. Different solutions containing either 1% of formic acid or 1% of ammonia, varying in methanol content, were used for the manually performed SPE experiments to evaluate the model.

Oasis HLB and Oasis MCX solid phase extraction cartridges, both 1 ml and 30 mg sorbent, were purchased from Waters (Milford, MA, USA). EDTA plasma was provided by a healthy volunteer.

3.2. Equipment

A Shimadzu binary gradient LC system consisting of two LC10ADvp pumps, an SCL 10Avp controller and a column heater CTO10ACvp (Shimadzu, Kyoto, Japan) were used. A CTC PAL (CTC analytics, Basel, Switzerland) was used as the injector. For the online SPE chromatography, the mobile phase flow was set to 1.00 ml/min and the mobile phase composition was mixed by the gradient system. After every mobile phase change, the system was allowed to equilibrate for 2 min.

The analytical mode of the system was used for the analysis of the obtained elution fractions from the practical experiments.

In the analytical mode, a Waters XbridgeShield RP18 column, 50 mm × 2.1 mm, 3.5 μm (Waters, Milford, MA, USA) was used as the analytical column which was maintained at 40 °C. The mobile phase gradient table is represented in Table 1.

The detector was a Sciex API 4000 mass spectrometer and the software to control the mass spectrometer and the LC system, Analyst 1.4.2, were from MDSSciex (MDSSciex, Ontario, Canada). The MS was optimised in positive MRM mode for all four analytes resulting in the most important operational parameters as outlined below and in Table 2.

Table 1
LC gradient profile as used in the analytical LC–MS mode

Time (min)	Flow (ml/min)	% A	% B
0.00	0.400	90	10
3.00	0.400	40	60
3.10	0.400	90	10
5.00	0.400	90	10

Table 2
Compound dependent MS parameters

Compound	Q1 mass	Q3 mass	Dwell time (ms)	DP (V)	CE (V)	CXP (V)	EP (V)
Amitriptyline	278.2	117.2	100	60	35	10	10
Pseudoephedrine	166.0	148.2	100	40	40	10	10
Acetanilide	135.9	94.2	100	45	45	10	10
Metoprolol	268.1	116.1	100	65	65	10	10

Source	Turbo ion spray
Temperature	700 °C
Ion spray voltage	4500 V
GS1 (nebuliser gas)	50
GS2 (turbo gas)	50
Cad gas	4
Resolution	Unit resolution for Q1 and Q3

All calculations concerning SPE modelling were performed on a Microsoft Windows based personal computer using MathCAD 2001i Professional as calculation software (Mathsoft, Cambridge, MA, USA)

3.2.1. SPE cartridge connector

The SPE cartridge connector consists of a stainless steel adapter rod with a 0.1 mm i.d. which is placed directly on the top frit of the cartridge.

The spout of the barrel was placed in a stainless steel collection unit. 0-Rings seal the connections between the rod and the frit and the connection between the spout and the collection unit.

The rod and the collection unit were placed into a clamping device in which the clamp force can be adjusted by means of a nut on the thread on the adapter rod, to result in a leak free connection between the top-frit and the collection unit Fig. 2.

Using a suitable dilution of the analytes, preferably in an aqueous solution, injections can be made using different mobile phase compositions.

By using a gradient pump system, these injection sequences can be programmed and can be ran automatically using the same cartridge.

3.3. Experiments

To demonstrate the use of the above-mentioned theory and to evaluate its accuracy, extensive practical experiments were setup. These tests were designed to evaluate the model under a wide range of experimental conditions, using model compounds varying over a wide range in polarity. Since most analytes in the bioanalysis of drugs, exhibit basic characteristics, three basic analytes were selected as model compounds. Amitriptyline, metoprolol and pseudoephedrine, exhibit these basic characteristics with pK_a values of 9.8, 9.7 and 9.5, respectively, and cover a wide range in polarity with log P values of 4.55, 1.49 and 1.31, respectively. The fourth compound, acetanilide, behaves as a neutral compound in the tested solvents and is moderately polar, log P = 1.37. Although different values of pK_a and log P can be found in literature the given values were calculated as estimates by the software program MarvinSketch (ChemAxon Kft, Budapest, Hungary) and were suitable for the purpose of our work. In order to investigate the predictability in a real sample matrix the test compounds were spiked at a 1000 ng/ml concentration level in water as well as in human EDTA plasma. A reversed phase sorbent as well as a mixed mode cation exchange sorbent were used to test the proposed algorithm. Polymer based sorbents were used because of their stability over a wide pH range and the lack of free silanol interactions.

3.3.1. Online SPE experiments

For the online SPE experiments, an SPE cartridge was connected to the LC system by means of the SPE cartridge connector.

All experiments were performed with mobile phase compositions of water (solvent A) and methanol (solvent B) each containing either 1% of formic acid for the acidic modelling or 1% of ammonia (25%) for the basic modelling.

Each cartridge was initially flushed with 100% of solvent B, and subsequently conditioned to pure aqueous conditions. A scouting gradient was run from 0% B to 100% B in 5 min.

Different percentages of modifier, varying from 0% to 100% in steps of 10%, either acidic or basic were used on both types of sorbent. At each modifier combination, 5 μl of the aqueous test mixture was injected and the chromatogram was recorded for 10 min at a flow rate of 1.00 ml/min. The dead time (t_0) was estimated, by measuring the retention time for pseudoephedrine, the most polar analyte, at 100% of acidic modifier concentration on a HLB cartridge, since no suitable t_0 marker for LC–MS experiments was available. Equal t_0 values were assumed for both types of sorbents.

To evaluate the peak characteristics, for each compound, chromatograms were selected in which the complete band was eluted within the duration of the chromatogram. Both σ_G and τ were calculated as explained and were used in further calculations.

3.3.2. Evaluation of peak profile

To compare the predicted elution profile with the profile of the peak as eluted from manually performed SPE experiments, a preconditioned OASIS HLB cartridge was loaded with 0.500 ml of the aqueous test solution, mixed with 0.500 ml of a 1% formic acid solution. The cartridge was eluted 15 times with 0.500 ml of a solution containing 30% of methanol and 1% of formic acid in water. Each fraction was collected, diluted with 2.50 ml of 0.1% formic acid and analysed directly on the LC–MS system in the analytical mode. The peak areas of acetanilide as fraction of the total area of all fractions, were plotted against the elution volume and compared to the predicted recoveries under corresponding simulated conditions, for each fraction.

3.3.3. Recovery prediction

To demonstrate the use the proposed algorithm in SPE method development and to evaluate the model for accuracy, various experiments were performed. Different combinations of wash and elute steps were used, varying in volume, modifier concentration and in acid or base content. Both SPE sorbents were used and each experiment was performed with the aqueous test mixture as well as with the spiked plasma. To evaluate the effect of residue solvent between the various wash and elute steps, the cartridges were flushed between acid and a basic wash with 0.500 ml of a basic solution without modifier. The same procedure was applied with an acidic solution when changing from a basic to acidic solution. These fractions are referred to as transition fractions.

An aliquot of 500 μl of test sample was conditioned with either 500 μl of a 1% formic acid solution or with a 1% ammonia (25%) solution. The SPE cartridge was conditioned by the subsequent addition of 1.00 ml of methanol followed by 1.00 ml of the same solution as the sample was conditioned with. The sample was applied to the

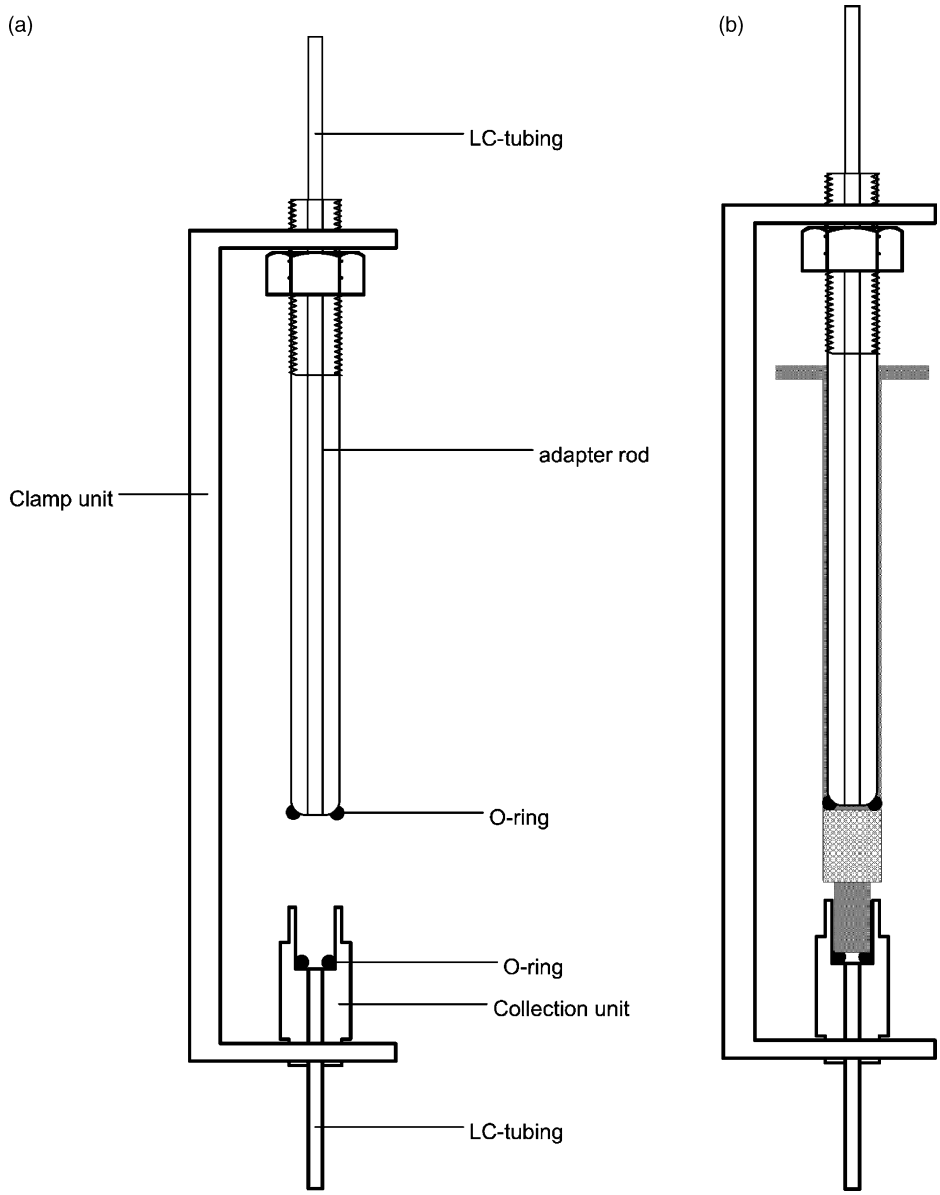

Fig. 2. Profile of the SPE cartridge connector (a) and connector with SPE cartridge inserted (b).

cartridge and pushed through the column in approximately 1 min by positive air pressure. The cartridges were washed and eluted with a variety of solvent compositions, as will be indicated in Section 4. Each fraction was collected, except for the first wash step of the plasma samples as it contained too much endogenous material which could lead to problems in the LC–MS system. All collected fractions were diluted to a total volume of 4.00 ml with a 0.1% of a formic acid solution. Of this mixture, 5 μl was injected into the LC–MS system in the analytical mode.

For each fraction, the eluted part of the analyte band was expressed as the quotient of the peak area and the total peak area of all fractions within the particular experiment.

4. Results and discussion

4.1. Results of the online SPE experiments

The results of the initial scouting gradient chromatograms are represented in Fig. 3.

From these figures, the differences in retention behaviour and selectivity are obvious.

The internal volume of the used LC system, the dwell volume, [17] was 0.35 ml and the dead time, t_0, of the chromatogram was 0.15 min at a total flow rate of 1.0 mL/min. The delay in the gradient profile with respect to *X*-axis in the chromatograms was 0.50 min.

All analytes could be trapped on both types of sorbent, which is important in view of the isolation of the analytes from the sample matrix. On the HLB column the retention is much higher in a basic solvent than in acidic environment since reversed phase interaction is the dominant retention mechanism. Although all analytes were eluted within the gradient time at both types of solvent, elution is preferred in an acidic solvent. The scouting gradients also reveal the possibility of a separating the different analytes.

On the MCX sorbent, all basic analytes are trapped in acidic mobile phase, since ionic interactions are the dominant retention mechanism. These analytes can be elutes in a basic solvent but a separation of the three analytes will be impossible. As expected,

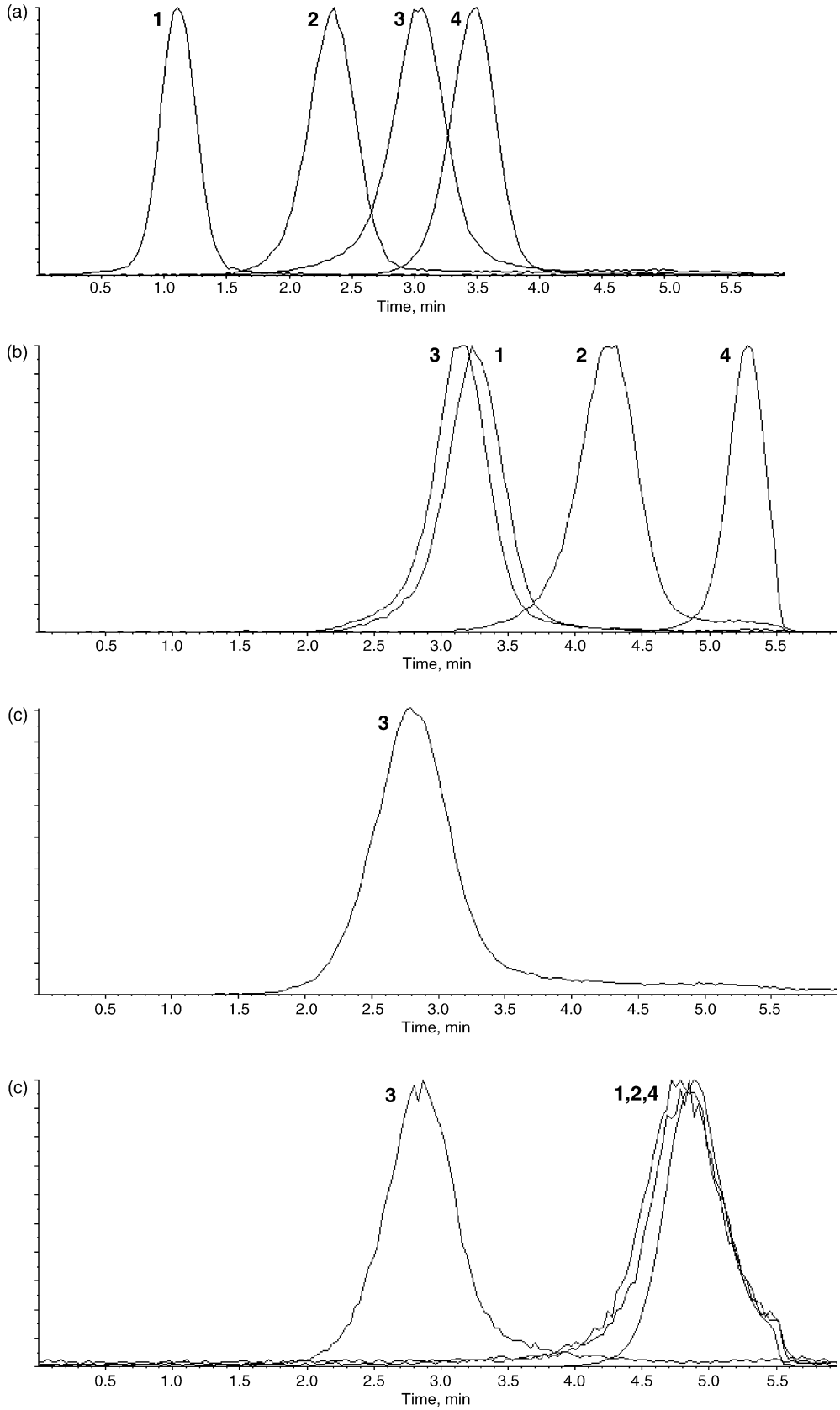

Fig. 3. Scouting gradient chromatograms on different types of SPE sorbents using acidic or basic mobile phases. (a) HLB sorbent, acidic mobile phase. (b) HLB sorbent, basic mobile phase. (c) MCX sorbent, acidic mobile phase. (d) MCX sorbent, basic mobile phase. Compounds 1: pseudoephedrine, 2: metoprolol, 3: acetanilide, 4: amitriptyline.

the neutral analyte acetanilide can be separated from the basic analytes and a sorbent flush with 100% of modifier will be possible without loss of basic analytes. Based on this information, a decision can be made concerning the sorbent type to proceed with.

For each sorbent and mobile phase combination, a retention function was calculated from the isocratic experiments according to Eq. (1). These results are graphically presented in Fig. 4a–h.

Only chromatograms in which the retention time was within the duration of the chromatogram were used for the determination of the retention models. The dead time, t_0, was found to be 0.15 min. The retention of amitriptyline, metoprolol and pseudoephedrine on the MCX column at acidic mobile phase, was treated as infinitely high and hence no curve was calculated.

Fig. 4 shows that the quadratic model fits the experimentally obtained points very well. The differences in retention behaviour under acidic and basic conditions are clearly visible. For pseudoephedrine on the HLB column, retention was very poor, even at low modifier concentrations. None of the analytes showed increased retention at higher modifier concentrations.

4.2. Evaluation of the peak profile

From the results of the online SPE chromatographic experiments it was seen that the peaks do not appear as symmetrical bands. Moreover, smooth EMG peaks were not always obtained as shown in Fig. 5, where a representative online chromatogram is shown of the aqueous test mixture on an HLB cartridge eluted with an acidic mobile phase containing 40% of modifier. Due to some irregularity, it can be difficult to determine the points *A* and *B* as depicted in Fig. 1. Narrow peaks at high modifier concentrations may also result in larger measurement errors. Therefore, errors in the determination of t_R, *A* and *B* can be expected resulting in varying values of σ_G and τ when determined at different modifier concentrations as shown in Table 3.

However, an SPE cartridge cannot be expected to be as reproducible and to produce the same peak shapes as an analytical HPLC column does. Therefore, for σ_G and τ overall mean values of 30 and 50, respectively, were taken as estimates for the parameters to describe the shape of all analyte bands at any modifier composition by assuming that band broadening is only affected by diffusion and the exponential decay is only due to extra column effects [23]. The impact of the high variation in the value of τ is relatively low. Although not demonstrated here, a difference in this value of 10 will result in a difference of less than 5% in recovery prediction, which we considered to be acceptable for a sample preparation procedure.

The flow dependency of the band broadening [8,10] will be kept fairly constant by keeping all flow rates as constant as possible at 1 ml/min during manual elution.

Using the calculated retention functions and the overall mean parameters for σ_G and τ, the recoveries were predicted for the fractional elution of the test mixture.

Table 3
Mean results of the calculated band shape parameters expressed as percentages

Compound	HLB				MCX			
	Acidic		Basic		Acidic		Basic	
	σ_G	τ	σ_G	τ	σ_G	τ	σ_G	τ
Acetanilide	33	27	27	27	30	37	29	39
Amitriptyline	31	45	37	51			28	86
Metoprolol	30	50	31	50			28	76
Pseudoephedrine	33	32	33	31			27	44

To demonstrate the use of the proposed model, Fig. 6 shows the calculated band profile, according to Eq. (13), for acetanilide after elution with 4 ml (8 fractions of 0.5 ml) of an acidic solution containing 30% of modifier. The *X*-axis presents the relative migration distance, *X* (%). The origin of this graph represents the top of the sorbent bed and the mark at $X = 100\%$ represents the outlet of the column. The amplitude of the band is arbitrary. The recovery curve is given according to Eqs. (14) and (15).

X_m was calculated by Eq. (7) to be 186%. Using this value, X_G was calculated to be 214% according to Eq. (12). Since the total migrated relative distance, X_m, exceeds 100%, the top of the acetanilide band has left the cartridge. By calculating a virtual band shape beyond the elution point, the remainder of the band profile on the column can be calculated. The graph of the recovery curve represents the portion of the band that has been eluted ($X > 100\%$) and the portion still residing on the column ($X < 100\%$).

After application of 4 ml of elution solvent, the recovery was calculated to be 89%.

This was in good accordance with the practical experiments in which the eluted fraction was experimentally determined to be 87%, after eluting with the same volume of solvent.

The predicted results for acetanilide, for each fraction, were compared to the experimental results and presented in Fig. 7a and b. The maximum standard deviation in the triplicate recovery measurements was 0.6%.

Fig. 7a and b show that for acetanilide the elution profile of the analyte band as measured in the different fractions is in good agreement with the predicted values and that the EMG model can be used in SPE prediction as a good approximation for the description of the profile of the analyte band. It must be noted that SPE cartridges may vary in their performance, since they are not intended to be used as and behave as an analytical column.

Although not demonstrated here, different injection volumes or different analyte concentrations resulted in equal band shapes.

Similar experiments with the other compounds revealed that pseudoephedrine was eluted almost completely in the first fraction and metoprolol after the second fraction. Amitriptyline, however, was eluted in total for less than 10%. These results were also in good agreement with to the calculated, predicted results.

4.3. SPE recovery prediction

The predicted values, as well as the mean results of the duplicate measurements of the manually performed SPE experiments are presented in Tables 4–7.

The different experiments are outlined and discussed below.

Although extensive fractionating of the SPE effluent is not very meaningful in real SPE work, it was only used to evaluate the analyte recovery (or break through) to test the proposed model.

Experiments 1 and 2 were performed with the reversed phase sorbent OASIS HLB and the experiments 3 and 4 with the OASIS MCX cartridges.

4.3.1. HLB experiments

The sample was applied to the cartridge in a basic solution for maximal retention of all analytes.

The scouting gradients in Fig. 3a and b, show clear differences in selectivity for the analytes.

When amitriptyline would be the only analyte of interest and when the goal would be to isolate this most non-polar analyte from the sample with minor interference of the other compounds, a matrix cleanup could be performed by washing the sorbent with 1 mL of a basic solution containing modifier to a maximum of 70%. Using the model, it was determined that

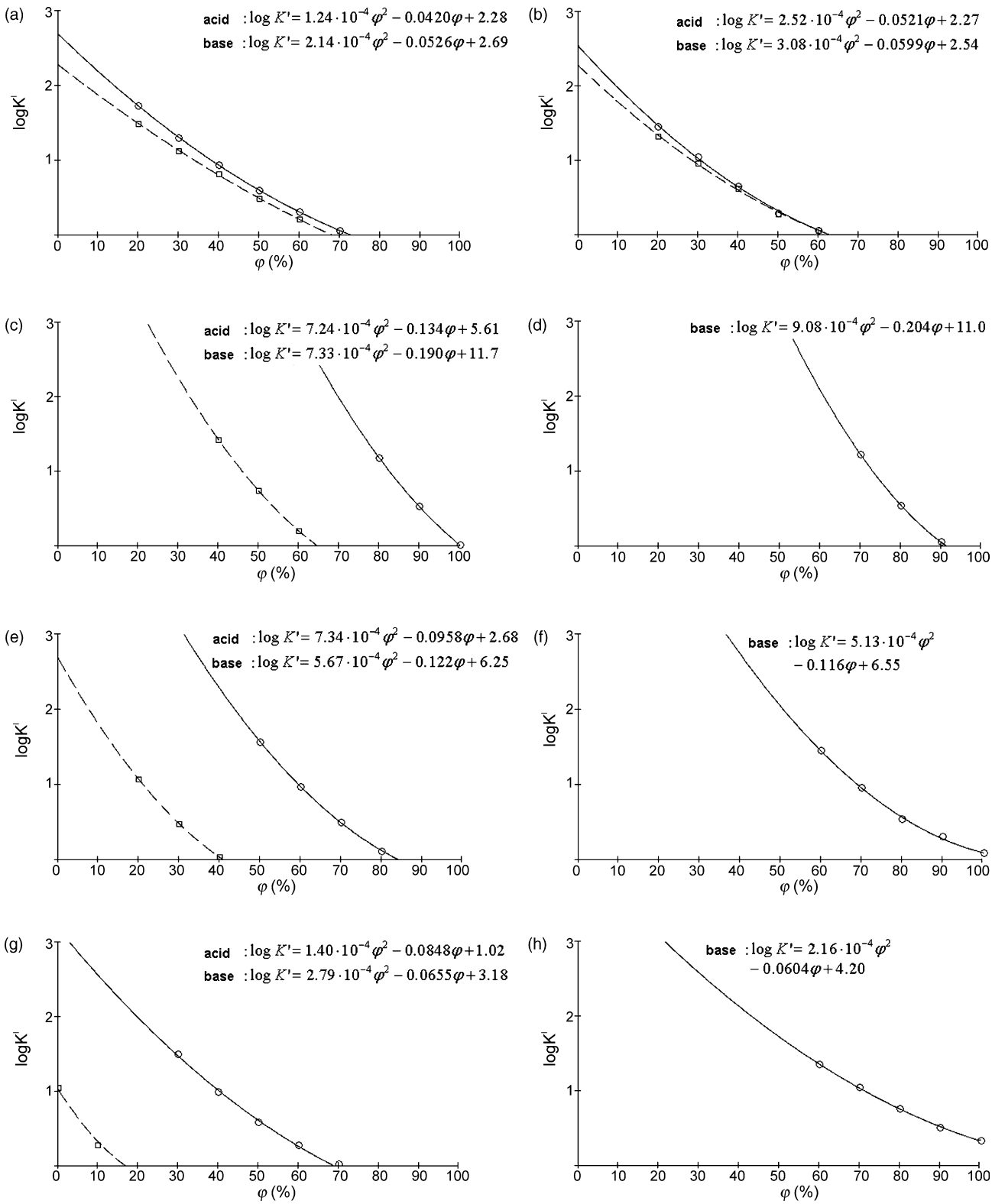

Fig. 4. Retention functions of the model compounds on two types of SPE sorbent. Solid lines and ○s represent the calculated curve and the individual results of the basic solution, respectively, and the dashed line and the boxes represent the calculated curve and the individually measured points of the acidic solutions. (a) Acetanilide on HLB column. (b) Acetanilide on MCX column. (c) Amitriptyline on HLB column. (d) Amitriptyline on MCX column. (e) Metoprolol on HLB column. (f) Metoprolol on MCX column. (g) Pseudoephedrine on HLB column. (h) Pseudoephedrine on MCX column.

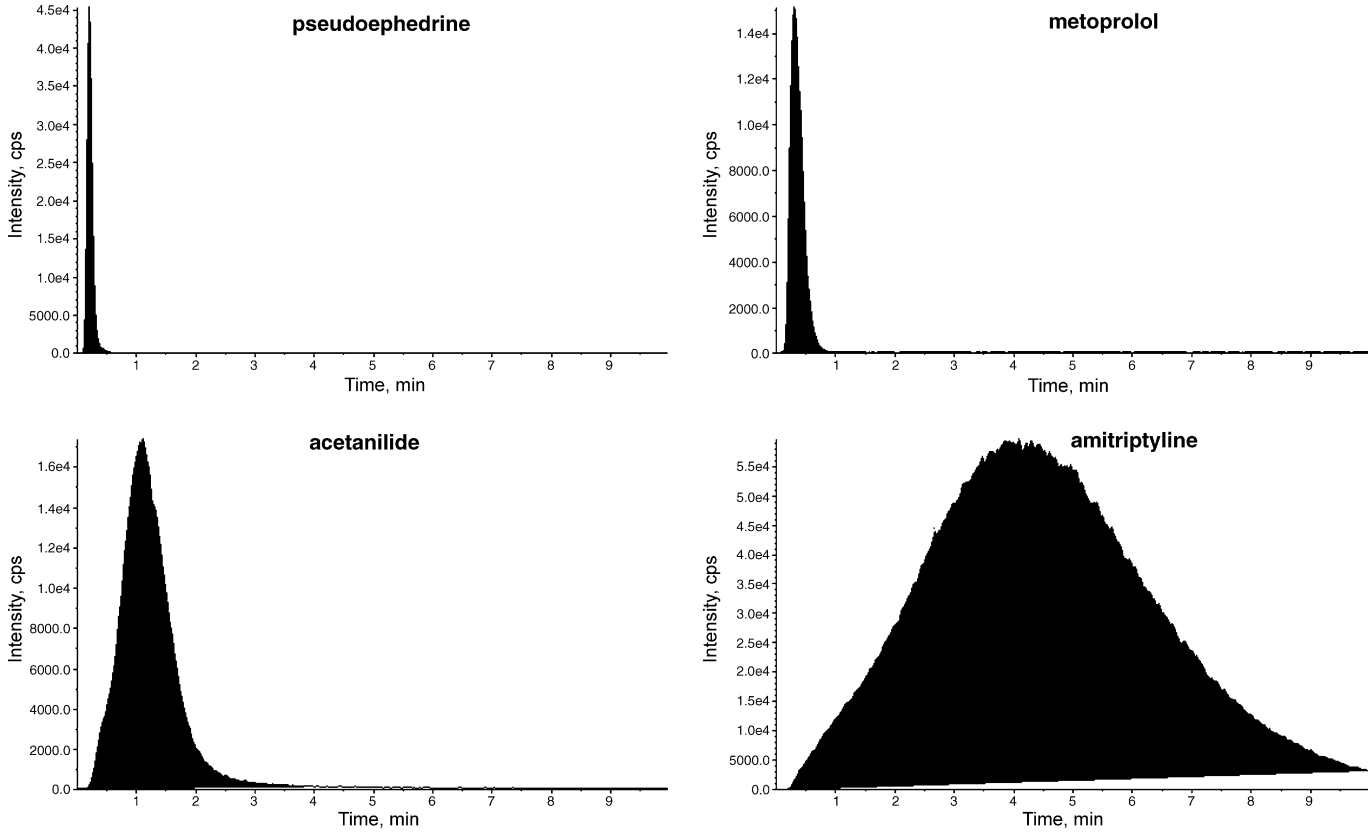

Fig. 5. Representative online SPE chromatograms of an OASIS HLB cartridge eluted with an acidic mobile phase composition of 40% B.

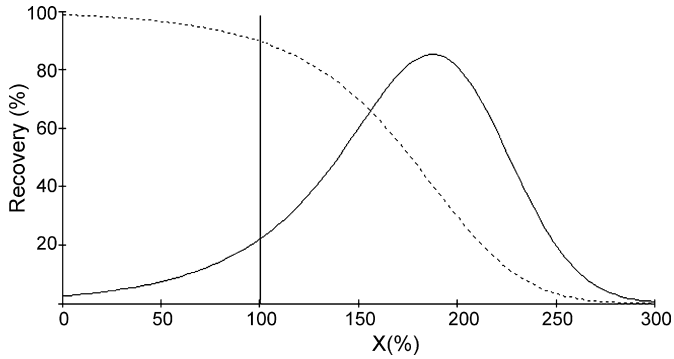

Fig. 6. Predicted band profile (solid line) and the recovery curve (dotted line) of acetanilide as a function of the fractional migration distance on the SPE cartridge after 4.0 ml of elution solvent. The vertical line indicates the point of outlet of the column.

amitriptyline could now be selectively eluted in only 0.5 mL of an acidic solution containing 70% of modifier. The complete procedure (experiment 1) was tested in practice and the results are presented in Table 4. By this approach it is shown that, as expected, amitriptyline could be eluted completely (>95%) in fraction d. However, due to incomplete elution of metoprolol in fraction b, the remainder of metoprolol also appeared in fraction d. Hence, both substances were not separated completely. The transition fraction, c, did not contain a significant amount of analyte.

Furthermore, Table 4 shows that for metoprolol a significant matrix effect was observed as can be seen from the differences in the recovery values between the aqueous sample and the plasma sample.

To demonstrate the elution of the analytes individually in different fractions, initially all analytes should be retained on the column and therefore, the sample should be applied to the sor-

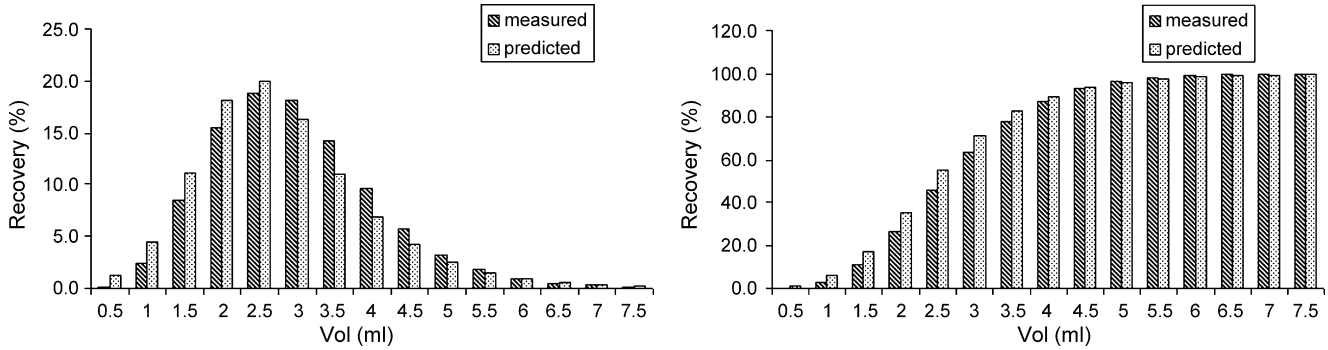

Fig. 7. Recoveries (a) and cumulative recoveries (b) for the fractional elution of acetanilide with an acidic solution containing 30% of modifier.

Table 4
Experiment 1: predicted and experimentally obtained recoveries with the OASIS HLB SPE cartridge

Elution sequence				Recovery (%)											
Step #	Volume (ml)	φ (%)	Acid/base	Acetanilide			Amitriptyline			Metoprolol			Pseudoephedrine		
				Pred.	Exp. aq.	Exp. pl.	Pred.	Exp. aq.	Exp. pl.	Pred.	Exp. aq.	Exp. pl.	Pred.	Exp. aq.	Exp. pl.
*	1.0	0	B	0.2			0.1			0.1			0.2		
a	1.0	0	B	0.0	0.1		0.0	0.1		0.0	0.0		0.0	0.1	
b	1.0	70	B	98.9	99.3	98.1	0.1	0.1	3.6	81.2	79.4	69.8	99.4	93.6	91.0
c	0.50	0	A	0.0	0.1	0.3	0.0	0.0	0.2	0.3	2.3	3.1	0.2	0.5	1.3
d	0.50	70	A	0.9	0.5	1.6	97.8	99.8	96.2	18.2	18.2	27.2	0.3	5.8	7.7

* Application of the sample.

Table 5
Experiment 2: predicted and experimentally obtained recoveries with the OASIS HLB SPE cartridge

Elution sequence				Recovery (%)											
Step #	Volume (ml)	φ (%)	Acid/base	Acetanilide			Amitriptyline			Metoprolol			Pseudoephedrine		
				Pred.	Exp. aq.	Exp. pl.	Pred.	Exp. aq.	Exp. pl.	Pred.	Exp. aq.	Exp. pl.	Pred.	Exp. aq.	Exp. pl.
*	1	0	B	0.2			0.1			0.1			0.2		
a	1	0	B	0.0	0.1		0.0	0.1		0.0	0.0		0.0	0.0	
b	0.5	0	A	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	1.7	4.4	4.2
c	1	10	A	0.3	0.0	0.1	0.0	0.0	0.6	0.3	0.0	0.5	94.6	82.2	66.6
d	0.5	0	B	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.6	1.3
e	2	50	B	97.8	98.9	89.0	0.0	0.0	0.3	4.9	4.1	3.4	3.5	10.0	17.9
f	0.5	0	A	0.1	0.1	0.5	0.0	0.0	0.0	0.2	0.2	0.2	0.0	0.2	1.1
g	1	40	A	1.4	0.5	4.1	1.0	0.7	16.1	94.3	89.9	75.7	0.0	2.0	7.9
h	1	70	A	0.3	0.3	6.3	98.9	99.1	82.5	0.3	5.6	20.2	0.0	0.5	1.0

* Application of the sample.

bent as a basic solution. The scouting gradient in Fig. 3a shows maximum selectivity between pseudoephedrine and the other analytes in an acidic solution. A low modifier content of 10% was sufficient to elute pseudoephedrine while retaining the other analytes on the sorbent. The second analyte to be eluted should be acetanilide. Since pseudoephedrine was already removed, the maximum selectivity was expected to be obtained under basic conditions (Fig. 3b). Metoprolol and amitriptyline should be separately eluted by switching back to acidic conditions where maximum selectivity for these two analytes was observed. A separation between these analytes was found to be accomplished by elution with two acidic solvents, differing in modifier content. The results of the practical experiments (experiment 2) are presented in Table 5.

Table 6
Experiment 3: predicted and experimentally obtained recoveries with the OASIS MCX SPE cartridge

Elution sequence				Recovery (%)											
Step #	Volume (ml)	φ (%)	Acid/base	Acetanilide			Amitriptyline			Metoprolol			Pseudoephedrine		
				Pred.	Exp. aq.	Exp. pl.	Pred.	Exp. aq.	Exp. pl.	Pred.	Exp. aq.	Exp. pl.	Pred.	Exp. aq.	Exp. pl.
*	1.0	0	A	0.2			0.1			0.1			0.1		
a	1.0	0	A	0.1	0.1		0.0	0.1		0.0	0.0		0.0	0.1	
b	1.0	10	A	0.5	0.1	0.1	0.0	0.0	0.2	1.0	0.0	0.0	0.0	0.0	0.2
c	1.0	100	A	99.2	97.7	97.7	0.0	0.1	0.7	0.0	0.0	0.6	0.0	0.0	0.5
d	0.50	0	B	0.0	0.2	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.2
e	1.0	60	B	0.0	1.8	1.8	0.1	0.0	0.1	0.0	0.8	1.6	1.5	1.4	2.7
f	1.0	100	B	0.0	0.2	0.2	99.8	99.7	99.0	98.2	99.1	97.6	94.5	98.4	96.3

* Application of the sample.

Table 7
Experiment 4: predicted and experimentally obtained recoveries with the OASIS MCX SPE cartridge

Elution sequence				Recovery (%)											
Step #	Volume (ml)	φ (%)	Acid/base	Acetanilide			Amitriptyline			Metoprolol			Pseudoephedrine		
				Pred.	Exp. aq.	Exp. pl.	Pred.	Exp. aq.	Exp. pl.	Pred.	Exp. aq.	Exp. pl.	Pred.	Exp. aq.	Exp. pl.
*	1.0	0	B	0.2			0.1			0.1			0.1		
a	1.0	0	B	0.0	0.2		0.0	0.3		0.0	0.1		0.0	0.1	
b	1.0	60	B	98.9	96.8	97.6	0.1	0.2	10.1	1.0	2.5	11.3	1.5	4.1	16.0
c	1.0	0	A	0.1	0.3	0.3	0.0	0.0	0.6	0.0	0.1	0.6	0.0	0.5	1.3
d	1.0	100	A	0.8	2.5	1.9	0.0	0.0	0.3	0.0	0.1	0.2	0.0	0.2	0.3
e	0.50	0	B	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0
f	1.0	100	B	0.0	0.2	0.2	99.8	99.4	89.1	98.2	97.1	87.8	94.5	95.1	82.3

* Application of the sample.

In this experiment, the use of different volumes, different modifier concentrations and a double change in acidity was demonstrated. Although, it is not a common choice in practice, this procedure was used as a stress test to investigate the predictability of the proposed algorithm. The analytes eluted in the expected fractions: pseudoephedrine in fraction c, acetanilide in fraction e, metoprolol in fraction g and finally, amitriptyline in fraction h. Moreover, a good similarity between the predicted results and the results from the aqueous sample were found in all cases. It must be noted, however, that matrix effects were observed. Amitriptyline seemed to elute earlier and metoprolol and pseudoephedrine were more retained. The transition fractions b, d and f did not contain a significant amount of analyte.

4.3.2. MCX experiments

The following experiments were performed on the mixed mode cationic exchange sorbent MCX. A mixed mode sorbent can be used as an ion exchange sorbent to separate the basic analytes from neutral and acidic matrix components. For this purpose the sample is applied in an acidic solution enabling the analytes to ionize and interact with the cationic exchange groups of the sorbent. Since neutral analytes and non-polar acids are also expected to be retained on the hydrophobic backbone of the sorbent, a wash step with high modifier content will elute these compounds. From Fig. 3c, it can be seen that in the scouting gradient acetanilide was retained in some extent and that the basic analytes were not eluted at all, even at high modifier concentrations. To prevent the possible precipitation of retained proteins, an acidic wash with low organic modifier content can be used to remove these proteins without loss of recovery (Table 6, fraction b). A flush with a 100% organic solution should remove all retained neutrals and acids (fraction c). Finally, the basic analytes will be removed simultaneously in a basic solution. Since the analytes are very non-polar in a basic solution, 100% of organic solvent was needed for a complete elution as also shown in Fig. 3d. These chromatograms also reveal the lack of selectivity between the basic analytes and hence it will not be possible to achieve a separation between any of these analytes.

The experimental results of this approach (experiment 3) are presented in Table 6. All predicted values are in good accordance with the experimentally obtained values. The neutral analyte acetanilide eluted in fraction c. All the other basic analytes were found in fraction f. The transition fraction, d, did not contain any of the analytes. No matrix effects were observed.

Both retention mechanisms of the mixed mode sorbent can also be used in an alternate way. Then the sample should be applied under basic conditions so that the reversed phase mechanism on the MCX cartridge dominates. Fig. 3d shows the possibility of retaining the analytes. A cleanup step in order to elute matrix components with a relatively high concentration of modifier without loss of basic analyte can be performed. A basics solution with a modifier concentration up to 60% will elute acetanilide completely as well as very polar matrix compounds, polar neutrals and acids without eluting the basic analytes. Switching to acidic conditions will result in charged analytes which caused ionic interaction. Now a flush with 100% of an organic solvent will remove strongly retained neutral and acidic compounds. All basic analytes could now be eluted in a 100% basic modifier solution.

The results of these experiments (experiment 4) as presented in Table 5, indicate that there is a good accordance between data found compared to the predicted recoveries. Acetanilide, being a moderately polar compound, was eluted as expected in fraction b. All other analytes being basic analytes were eluted in fraction f.

However, serious matrix effects were observed for all basic analytes in step b. From the analytes spiked to plasma 10–16% eluted in the fraction b compared to 0–4% for the aqueous solution.

4.3.3. General discussion

Despite the variations in the values for σ_G and τ (Table 3), the mean values for these parameters were suitable estimates for the purpose of the model. The predicted recoveries were in good accordance with the experimentally obtained results for all analytes.

The results also show a negligible recovery in the transition fractions, confirming that the various wash and elution steps could be considered as discrete steps without mixing up with any residual solvent from previously applied solvents. Transition fractions were only used to confirm this hypothesis and unnecessary in further work.

As shown by the results, the elution behaviour of the analytes may be affected by the plasma matrix. The effect of the sample matrix is unpredictable and can either result in earlier elution or in a delay in elution. For the investigation of the proposed algorithm, the modifier concentrations and elution volumes were chosen in a very narrow range. A maximal modifier content was chosen to wash the cartridge without eluting the analytes and a minimum of modifier content was used for the elution of the analytes. In daily practice, however, a reasonable margin of 5–10% of modifier can be incorporated to assure analyte retention or elution. The matrix effect will then be less pronounced.

It is always recommended to test a theoretically predicted SPE procedure with a real sample matrix in the final stage of method development.

From the above-mentioned experiments it can be stated that although cationic exchange interactions are very specific and selective in separating basic analytes from neutral and acidic compounds, a much higher and amendable selectivity could be obtained on the reversed phase sorbent to separate the basic analytes mutually.

These potential characteristics were easily unveiled by the online gradient experiments and would be hard to discover by the conventional manual SPE development procedures.

5. Conclusions

A new model to predict recovery in solid phase extraction was developed.

By using an SPE cartridge connector online with conventional LC–MS equipment, the retention properties of the analytes on the actually used SPE cartridge could be determined easily. By using a scouting gradient on the SPE cartridge, the elution characteristics and selectivity of the analytes on a particular SPE cartridge could be visualised. The use of the proposed design of the adapter leads to chromatograms with analyte band profiles corresponding to the band profiles as found in manual fractional elution.

The quadratic retention function combined with the EMG peak model were used to describe the elution profile of the analytes resulted in good estimates for the real elution profile. Due to the modular character of the model also other retention functions and peak shape models can be easily incorporated.

The results of the proposed model were in good agreement with the results of the practical experiments although care has to be taken for possible matrix effects affecting analyte retention. The model was able to predict the behaviour of the analytes using multiple wash and elute steps, varying in volume, modifier concentration and acidity on both reversed phase sorbents as well as on mixed-mode sorbents. Since the proposed method

takes only one SPE cartridge to model the extraction behaviour for each type of sorbent, it is also cost effective and the on line experiments can be easily automated using generally available LC equipment.

The proposed algorithm is suitable for modelling the SPE behaviour of the analytes and can be used for the structural method development of solid phase extractions.

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