Review

Theoretical models in LC based bioanalytical method development

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ABSTRACT

Bioanalytical method development largely depends on the experience and the preference of the developer. Mathematical models could help in selecting the proper conditions to develop a selective and robust method, using liquid chromatography, liquid–liquid extraction, solid phase extraction and protein precipitation. This paper reviews the literature providing relevant equations and algorithms to model LC based bianalytical methods for the quantification of small molecules. By using the cited references, it will be possible to build models to describe the analytical methods either as an approximate impression or in a detailed way, incorporating many experimental variables. Special attention has been paid to matrix effects, the most important issues in bioanalysis and possible solutions to handle these issues are discussed.

By proper use of the proposed models a more structured method development is accomplished, resulting in a description of the method that could be used for future use to control the complete bioanalytical method.

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1. Introduction

The field of pharmaceutical bioanalysis concerns with the analysis of drugs in human body fluids. Bioanalytical methods are used within pharmaceutical industries, pharmaceutical contract research organisations, pharmacy laboratories and university laboratories.

With the introduction of LC-MS, sample preparation was thought to be reduced to a minimum but this vision turned out to be obsolete and sample preparation is still necessary to concentrate the sample or to remove unwanted compounds that could interfere with the detector response [1–5].

Nowadays, sample throughput is more and more important and sample preparation should be as efficient as possible [4,6].

Although the method development time should be reduced to a minimum, the result should be a robust and reliable method.

The development of a bioanalytical method is often based on non-structural trial and error methods. The quality and the performance of such a method depend highly on the theoretical skills, the experiences and the preferences of the developer whereas there is no doubt that a structural approach leads to a more efficient and traceable way of method development.

This paper is focussed on bioanalytical methods used for the quantitative determination of drugs and their metabolites as used in therapeutic drug monitoring, clinical toxicology and in the preclinical and clinical development stage of drug development [6].

In this type of work the analysis concerns with the quantitation of a limited number of analytes, mostly a parent drug and one to three metabolites. The analytical method is supposed to be able to determine all analytes, free of interferences with good precision and accuracy. Since the structure of the analytes is often known, the method development can be highly focussed on these compounds by selecting the analytical conditions based on the properties of the functional groups of the analyte and the type of biological matrix.

The main issue in bioanalytical method development is the highly complex matrix in which the analytes reside, such as blood plasma or serum, urine and sometimes oral fluid, liquor, pus saliva or whole blood.

Method development generally starts with the adjustment of the detector settings followed by the development of a chromatographic system which is able to separate the analytes within a runtime as short as possible.

The first sample preparation attempts may lead to extracts containing many interferences. Sequential adjustment of the chromatography and the sample preparation method finally lead to a satisfactory method.

By performing these experiments in a more structured way, their results could be used to set up a suitable model of the system which is able to model and predict the behaviour of the analytes under different analytical conditions.

A model is a simplified mathematical representation of the real system. The outcome of these equations (i.e. retention time or recovery) is referred to as 'response' and the factors that influence the response are called the analytical parameters or variables (i.e. modifier concentration or pH). The term 'simplified' means that not every parameter that influences the result is taken into account. The more detailed should be the model, the more complex the equations will be. It is up to the user to decide which parameters should be incorporated into the model based on significance.

Structured method development, based on the generally available theory, leads to very usable information of the behaviour of the analytical system. Even without the mathematics necessary for complete modelling, it is still worthwhile to develop bioanalytical methods in a more structured way based on the theoretical

behaviour. The proposed mathematics to relate the experimental observations to the response parameters of the model, leads to a detailed description of the behaviour of the method. Using this calibrated model, predictions of the method behaviour under different analytical conditions are possible. Results of additional experiments during method development could be easily incorporated, resulting in a more reliable model. During method development, the resulting model could be used to select the optimal conditions to achieve a certain separation, to position the analyte peaks beyond a suppression zone or to select the optimal extraction conditions. The theoretical models may help to investigate the robustness of the method by evaluating the variation in the predicted response when varying the analytical conditions. Finally, the models could be used for trouble shooting purposes or for the transfer of work to another person. In this paper, a selection from the enormous number of publications was taken to provide the reader with the necessary equations and algorithms to describe the most often used bioanalytical techniques: reversed phase liquid chromatography, protein precipitation, liquid–liquid extraction and solid phase extraction.

Since most papers deal with neat analyte solutions, also the pitfalls are discussed for using theoretical models with real biological samples.

The cited references were chosen based on their practical use in theoretical models during method development.

2. Liquid chromatography

In bioanalysis, reversed-phase liquid chromatography is the preferred analytical method to introduce a sample into a detector. Therefore, this section is focussed on the description of this type of separation. The chromatographic system is used to separate the analytes from all other compounds that could interfere with the detection of the analytes.

Modelling the chromatographic separation involves the prediction of retention times and subsequently the prediction of the resolution between adjacent peaks as a function of the most commonly used separation parameters such as modifier concentration, mobile phase pH and column temperature.

Many theoretical papers have been published concerning the relationship between the mobile phase composition and the retention time.

2.1. Retention vs modifier concentration

Back in 1976, Horváth et al. described the interactions of solutes with a non-polar stationary phase based on the solvophobic theory [7]. This theory reveals that retention in RP-LC is mainly caused by the strong interaction of water, pushing the analyte towards the apolar stationary phase. They also provided the mathematical relationships of the interactions between the solutes with the mobile phase and the stationary phase. Although these equations are hard to be used for modelling purposes, they provide a solid base for understanding the behaviour of the analytes in reversed phase systems.

The best known models used in RP-LC were primarily introduced and discussed by two different groups.

Both Snyder et al. and Schoenmakers et al. introduced relationships between the modifier concentration, φ, and the capacity factor, k'.

Their theories are suitable for isocratic elutions as well as for gradient based separations.

The theory of Snyder et al. was summarised and discussed in ref. [8] which can be used as a good starting point in chromatographic

modelling. Their theory was based on gradient LC systems in which the mobile phase follows an ideal linear gradient, known as the linear solvent strength theory (LSS), resulting in a linear relationship between φ and $\log k'$.

To use the basic LSS theory, three assumptions were made:

1. the sample is injected in a very small volume, which is generally the case in HPLC.
2. there is no dead volume between the gradient generator and the injector.
3. sorption of mobile phase components to the stationary phase does not occur.

Based on the LSS theory, equations concerning prediction of retention time, bandwidth, resolution and selectivity were presented. The use of the proposed equations was clarified and demonstrated in ref. [9].

A different approach was given by Schoenmakers at al. Their model to describe the relationship between the organic modifier concentration and the retention was based on the solubility parameter theory [10] and has the form of a quadratic curve rather than a straight line.

The curvature depends on the nature of the analyte and the used organic modifier [10,11]. Although the validity of both functions was judged by both authors, one can conclude that the quadratic curve describes the true shape in more detail compared to the solvophobic theory [7]. Both authors agreed that the quadratic curvature could be replaced by a straight line as long as $1 < k' < 10$ [9].

Schoenmakers gradient elution theory also assumes the gradient profile to be unchanged by the equipment but corrects for the gradient delay from the top of the column to arrive at the analyte band on the column.

The resulting equations according to Schoenmakers for the prediction of retention times in gradient systems are harder to work with in practice and can only be used in a dedicated software program. The LSS theory, however, is easier to work with and gives a better general overview of the retention behaviour under gradient conditions.

The suggested $\log k'$–φ relationship by Schoenmakers was further extended for use with low organic modifier content in the mobile phase (<10%) which takes the sorption of organic modifier to the stationary phase into account [12] resulting in an extra curvature in this modifier region. This modification was also supported by the curvature as predicted by the solvophobic theory [7].

The model parameters have to be calculated from experimentally obtained data.

LSS parameters can be obtained from at least two gradient elution runs whereas a quadratic relationship needs at least three chromatographic runs to fit the obtained retention data to the corresponding modifier concentration by means of a regression technique [13].

Both Snyder and Schoenmakers suggested the use of a gradient elution as a starting point in experimental method development in order to select the best separation conditions. Based on their equations, the choice of using a gradient elution or isocratic elution can be deliberately made [8,14–16]. For method development purposes, Schoenmakers et al. also now use the straight line relationship as a good approximation [14]. For this early stage of method development, they also suggested simple mathematical equations and rules for changing the type of modifier (ACN, methanol or tetrahydrofurane) that may lead to a change in selectivity while maintaining retention approximately constant. From a single gradient run, a choice can be made concerning gradient or isocratic separation mode and the type of organic modifier used in the mobile phase.

2.2. Equipment constraints and refinements to the models.

As mentioned before, the prediction of retention times during gradient elution depends highly on the quality of the used equipment. The main issue is the volume from the point of gradient mixing to the inlet of the column which is called the dwell volume. This dwell volume causes a delay in the gradient profile as well as a distortion of this profile also known as dispersion. The dwell volume also may result in analyte pre-elution, the elution of compounds before the actual gradient reaches the column or in erroneous retention time predictions. These issues were discussed in refs. [17,18]. Although the effect of the dwell volume on the dispersion of the gradient curve was explained and discussed, the conclusion was that the effect of gradient dispersion could be compensated for or could be best omitted by taking some chromatographic constraints into account to minimize the dispersion effects such as using a less steeper gradient and taking precautions to let all analytes elute during the gradient.

When using small i.d. LC columns with fast gradients, which is a common practice nowadays, the effect of the dwell time on retention time prediction is hard to avoid. Hendriks et al. provided a method to incorporate the effect of the dwell volume on the gradient profile dispersion [19]. The actual distorted gradient was used to calculate retention times more accurately compared to the LSS model. This model was also able to calculate retention times originating from multi step gradient profiles.

A refinement to the retention time prediction model is given in ref. [20] where even non-ideal processes in the column are discussed such as solvent demixing, non-linear plots of $\log k'$ vs gradient time and changes in dead volume due to changes in mobile phase compositions and flow rate. Although these considerations can be used to fine-tune the models, these refinements are of limited value in bioanalytical method development since the effects of these phenomenons do not always judge the effort in building more complex models.

Due to differences in LC equipment, mainly the dwell volume, care must be taken in transferring methods between different LC systems since retention times and even selectivity may vary between different types of equipment while running the same gradient program. Also, the adaptation of the gradient when changing column geometry, is of importance to retain band spacing. The methods and equations to transfer existing gradient methods between different types of equipment or when changing column dimensions is well described in refs [21,22].

One must realise that the dwell volume of each system used should be carefully measured as described in refs [17–19]. Once measured, these results can be used in future calculations as long as the system configuration is left unchanged.

2.3. Temperature and pH effects on retention

The temperature dependency on the separation can be described as discussed in refs. [23–25]. Here, it was shown that two chromatographic runs, recorded at different temperatures would provide sufficient information to model the gradient retention at different temperatures with sufficient precision [24]. However, the effect of temperature changes on the resolution of "regular" samples, i.e. homologs or molecules with repeating identical units, was of less importance. A change in temperature will only be beneficial in combination with a steeper gradient in order to achieve a faster separation. For "irregular" samples however, a change in temperature may lead to different selectivities and may cause a change in elution order [25]. In bioanalytical work, the extracts to be analysed can be treated as irregular samples when interfering peaks are also incorporated. These endogenous compounds may respond differ-

ently to temperature changes than the analytes. Hence, the column temperature could be used as a tool to separate the analytes from these interferences.

Since most pharmaceuticals or their metabolites contain ionisable groups, the choice of the mobile phase pH is very important as the pH affects the degree of ionisation and hence the retention. Lopes-Marques and Schoenmakers provided the equations to model the effect of mobile phase pH and modifier concentration on the retention time for monoprotic acids or bases [26]. Later, Schoenmaker and Thijssen discussed the usability and limitations of the proposed models. In fact, at least three different chromatographic runs were necessary to describe the pH dependency of the retention time preferably performed with mobile phase pH near the pKa of the analytes. However, when more data points are involved in calculation the reliability of the model greatly enhances [27].

Later studies by Heinisch and Rocca, demonstrated the influence of the temperature and the organic modifier content on the pKa of the analytes and the used buffer species. Fluctuations or changes in these parameters may cause large deviations in the predictive power of the models, mainly depending on the nature of the analyte and the buffer species [28]. Because of these effects for ionisable compounds, they advised the use of a quadratic form of the $\log k' - \varphi$ relationship at a fixed pH. The pH–retention relationships were also well explained and demonstrated by Pous-Torres et al. [29,30] for monoprotic solutes as well as for polyprotic solutes.

From a practical point of view, it is not recommendable to model the complete pH dependency near the pKa of the analyte since this will not increase the robustness of the method unless a critical separation could only be achieved in this pH region. Instead, by investigating a relatively low pH and a relatively high pH (pH < pKa − 2 and pH > pKa +2) the most extreme differences in selectivity are covered, leading to more robust and stable retention times.

2.4. Column selectivity

So far, only the mobile phase chemistry was considered in the models. However, different types of column sorbent may reveal different selectivities due to interactions other than the hydrophobic interactions. These selectivity differences between columns were extensively modelled by the hydrophobic subtraction model which relates non-hydrophobic column interactions such as ion exchange interactions, steric hindrance, column hydrogen bond basicity and column hydrogen bond acidity to the selectivity of many columns tested. By using this model, columns of different or comparable selectivities can be selected. The model to describe the selectivity parameters which is still under construction by incorporating more columns was reviewed by Snyder et al. [32]. More detailed information concerning the development and background of the hydrophobic subtraction model can be found in refs [33–39].

2.5. Liquid chromatography models in bioanalysis

A great deal of the available theory was used to develop software, dedicated for modelling chromatographic systems. As far as we know of these, DryLab is considered to be the best known and best described software in literature. This program is based on the LSS model and is able to predict retention times and resolution as a function of mobile phase modifier content, pH and temperature. The use and capabilities are described in many papers and well reviewed by Molnar [31]. The disadvantage of DryLab is that it is unable to model gradient elution when the system is calibrated with isocratic data and because of the use of the LSS model, reten-

tion predictions of solutes eluting before or after the gradient are less accurate. The band tracking model as described by Hendriks compensates for these inaccuracies and this model is calibrated with isocratic runs to predict retention times under isocratic elution as well as under gradient conditions [19].

The main problem in the bioanalytical separations is to separate the analytes from co-extracted endogenous compounds of which the nature is unknown. Also, for these interferences, the retention times could be modelled in order to get a complete overview of the chromatographic method. Another solution to remove these substances could be realized by adjusting the sample preparation procedure. In the case of LC-MS, the co-eluting interferences are mostly invisible, but may cause severe ionisation suppression. Therefore, a modelled chromatographic system could be used to find LC conditions resulting in co-eluting analyte and internal standard peaks in order to compensate for unwanted effects in the ionisation source provided that this internal standard and the analyte exhibit comparable ionisation behaviour.

The value of using theoretical models in method development lies primarily in understanding the effects and the impact of the theoretical relationships when varying the separation parameters systematically in order to affect the retention times to achieve different selectivities.

In bioanalytical LC method development, the first aim is to find the relationship between the modifier concentration and the analyte retention time at a fixed column temperature and mobile phase pH, away from the solutes pKa values. When selectivity is not appropriate, a change in modifier type is the first choice followed by a change in mobile phase pH. Finally, the temperature could be used to fine tune the separation or to decrease the mobile phase viscosity to allow higher flow rates.

If separation selectivity is still insufficient, a column with a different selectivity could be chosen. With this column the mobile phase dependency, as well as pH and temperature dependency on the separation should be investigated again.

An overview of the cited references is given in Fig. 1.

3. Liquid–liquid extraction

Although more and more replaced by solid phase extraction, liquid–liquid extraction (LLE) is still a widely used method for sample cleanup in bioanalysis [6].

LLE is based on the distribution of solutes between an aqueous phase, the sample to be extracted, and a water immiscible organic solvent.

The response parameter of interest is the recovery of the analyte. Secondly, the aim is to extract potentially interfering substances as less as possible.

The distribution of the analyte depends mainly on the affinity of the uncharged analyte species for the extraction solvent and the pH of the aqueous phase.

The first consideration to be made is the choice of the extraction solvent, either a pure solvent or a mixture of solvents.

3.1. Solvent selection

Solvent selection is properly reviewed by Barwick [40] where the main selectivity differences of organic solvents were discussed. The mostly used approach is the solvent classification by Snyder [41,42] which is based on the ability of hydrogen bonding acidity (H-donor), the hydrogen bonding bacisity (H-acceptor) and dipole interaction of the solvent with the solutes. Although many organic solvents are available, the increasing regulations, either governmental or local, restrict the use of chlorinated or carcinogenic solvents [40].
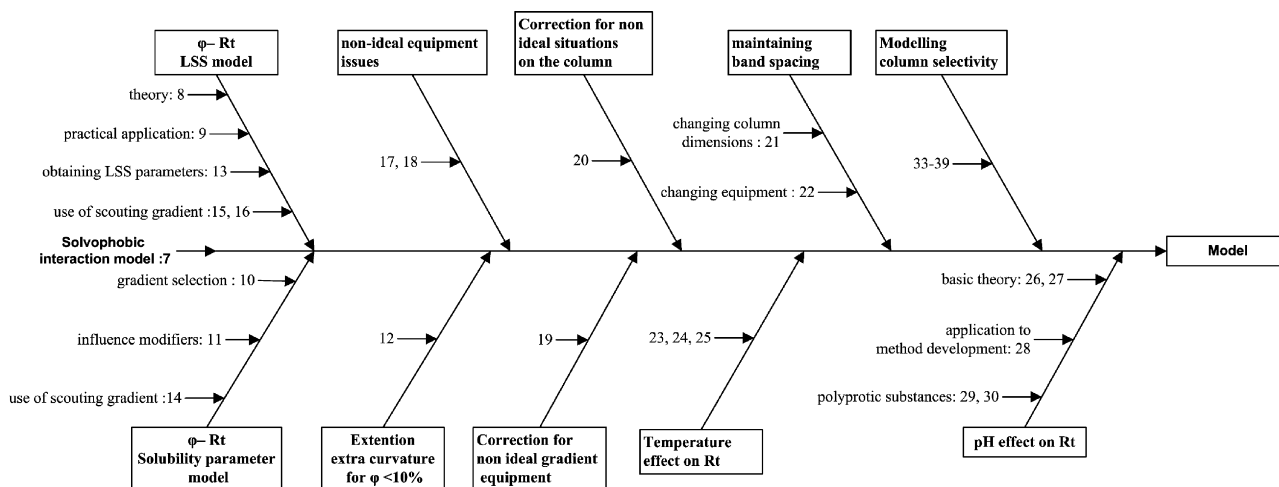
Fig. 1. Overview of the cited literature references (numbers in figure) for LC modelling.

The selection of a suitable extraction solvent or combination of solvents was well described by Wieling et al. [43]. This paper can be used as the basis of bioanalytical LLE since it is one of the few papers dealing with the theoretical aspects of the optimisation of bioanalytical LLE.

Their model was based on the determination of the analyte recovery at the predefined solvent compositions under non-ionised conditions and the subsequent calculation of the distribution constant for each solvent mixture. A chemometric approach was used to calculate the response surface of a solvent triangle consisting of three different organic solvents at each corner and several mixtures of the pure solvents, equally distributed over the triangle. The selection of the three pure solvents was based on Snyder's solvent classification using solvents with different selectivity characteristics.

This response surface describes the partition coefficient as a function of the solvent composition within the solvent triangle. Based on these results, the recoveries can now be modelled as a function of extraction solvent composition, sample volume and extraction solvent volume.

3.2. pH dependency

In the paper by Wieling et al. [43] the pH was not incorporated. Just as in LC methods, the pH has an enormous effect on the extraction selectivity when ionisable compounds are involved.

Hendriks et al. proposed a procedure which takes the pH of the aqueous phase into account, resulting in a potential powerful tool to improve the extraction selectivity when ionisable solutes are involved [44]. Here it was demonstrated that, the effect on the recovery is not necessarily noticeable as the pH approaches the pKa of the analyte but strongly depends on the hydrophobicity of the uncharged analyte.

By extracting samples at different pH values, the analyte recovery could be described as a function of the pH, the sample volume and the volume of extraction solvent. Based on this relationship, an extension to the generally accepted pKa ±2 rule was demonstrated. This rule stated that the aqueous pH should be adjusted at least two units away from the solute pKa in order to obtain the solutes in a highly unionised state. By this extension, it is obvious to see that pH values nearer to the pKa value or even beyond the pKa, to the ionised state, could still result in high analyte recoveries depending on the distribution ratio of the uncharged solute species.

3.3. Sample pH adjustment

Performing LLE at different sample pH values is often done by mixing the sample with a proper buffer which is adjusted at the target pH. However, one has to be aware that the pH of the resulting mixture does not necessarily match this target pH. The resulting pH may differ, depending on the buffer species, the buffer concentration, the pH of the buffer and the volumes of the sample and the buffer. Therefore, the pH of the resulting mixture should be taken into calculations, rather than the pH of the used buffer.

Alternatively, the pH adjustment of the sample could be performed by proper planning and calculation of these pH adjustment buffers based on ref. [45].

Here, the buffer capacity of human plasma was determined by acid-base titration experiments. By means of a calculation model, the pH of the resulting mixture could be calculated using a predefined buffer solution or, alternatively, the buffer pH could be calculated in order to obtain the target pH after mixing.

Refs. [43] and [44] together can now be used to describe the complete behaviour of the extraction system taking the pH adjustment method from ref. [45] into account.

These LLE models from ref. [44] also show the minimum or maximum pH for a back extraction in case of a base or an acid, respectively. This back extraction to an aqueous phase can serve as an extra selective cleanup by extracting only the ionisable analytes, leaving the neutrals in the organic solvent.

For difficult extractable compounds such as zwitterionic analytes or pH susceptible compounds, ion-pair LLE can be a solution [46–48]. Although satisfactory results could be obtained, the development of such a method takes a lot of effort in searching for the best counter ion and its concentration, the optimal pH and a suitable extraction solvent. For this reason a switch to solid phase extraction would be a better choice.

3.4. LLE models in bioanalytical method development

In the preceding papers, the recovery was modelled in real samples at ambient temperature. The extraction time and the intensity of shaking influence the equilibration time [43]. For modelling, it is assumed that full extraction equilibrium is achieved.

LLE in bioanalysis can be a very well focussed cleanup method, especially when ionisable analytes are involved. A good strategy to develop an LLE method is to start with the selection of a suitable extraction solvent using a spiked sample in the actual matrix to be
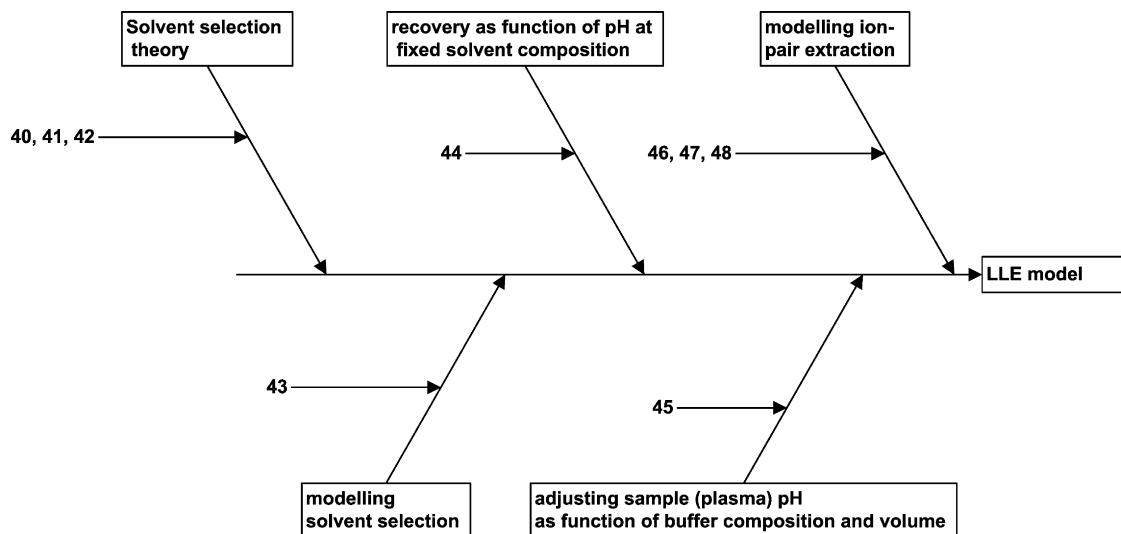
Fig. 2. Overview of the cited references (numbers in figure) for LLE modelling.

used at a relatively high concentration. The pure solvents should be different in selectivity and a full combined mixture design [43] should be sufficient to screen for the extraction solvents leading to the highest recoveries. This design is composed of three pure solvents, three binary mixtures of two of the individual solvents and one mixture composed of equal amounts of all three solvents. Since the response surface of these mixture designs is relatively smooth, it is not likely that a high and sharp maximum will exist in between the individual model compositions. Therefore, even when it is not the purpose to calculate the response surface of the solvent triangle, this structural experimental approach still leads to a good solvent combination.

From these results, the solvent composition giving the highest recovery with minimal interferences can be selected. Potentially interfering substances still present in the extract can be separated by changing chromatographic conditions or by changing the pH of the aqueous phase until recovery significantly drops. The optimum pH is the pH at which the interference is extracted to a minimum while the recovery does not significantly decrease. Finally, a back extraction to a small volume of an aqueous phase can be performed at a pH at which no recovery would be obtained in the extraction to the organic phase. Interferences still present in the chromatogram

are now reduced to a minimum and a final fine tuning of the chromatographic conditions should lead to interference free analyte peaks (Figs. 2 and 3).

4. Solid phase extraction

Solid phase extraction (SPE) is gaining more and more popularity in bioanalytical sample preparation [6].

An overview concerning SPE method development and the available sorbents and their formats, was given by Hennion et al. and Poole et al. [49,50]. One of their observations was that the development of a SPE method was still a matter of trial and error, partly due to the lack of published methods for SPE development so far [49].

4.1. Break through curve based models

The mostly discussed SPE method development is based on the establishment of breakthrough curves for each analyte. A breakthrough curve is the resulting detector response curve when an analyte containing solution is pumped through the SPE column while monitoring the effluent by a suitable detector [49–53]. The
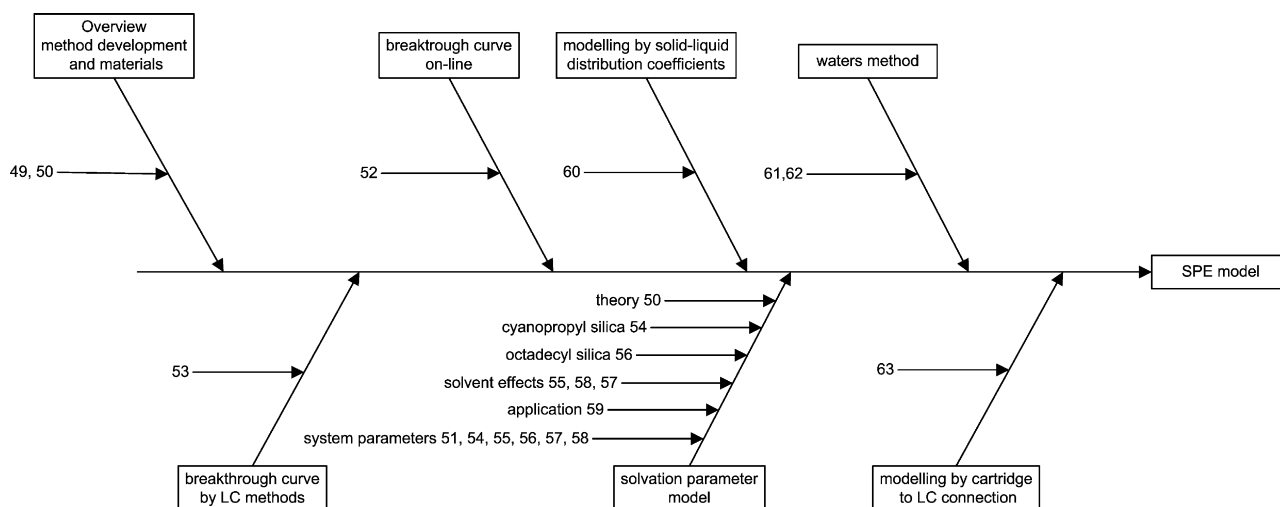

Fig. 3. Overview of the cited references (numbers in figure) for SPE modelling.

most interesting point in this sigmoidal curve is the breakthrough volume. That is the volume at which the analyte starts eluting from the sorbent [50]. These breakthrough curves could be recorded directly by means of an on-line connected cartridge as used in an on-line SPE system [52] or by an indirect method by passing the analyte containing solution through the sorbent followed by analysis of the effluent. Since this is a time consuming and laborious method, different approaches have been developed for estimating the breakthrough volumes.

Hennion et al. proposed a method for measuring the breakthrough volume based on HPLC experiments in which an analytical column housing was packed with the used SPE sorbent [53]. From the resulting graphs retention factors could be determined at a particular mobile phase composition and with some estimates concerning the theoretical plate number in the original cartridge, the breakthrough volumes could be calculated.

Poole et al. presented a more general model to establish the retention factor of the analyte under certain mobile phase compositions based on the solvation parameter model [50,51,54–59]. This model is based on a combination of system constants and solute properties. Some of the solute properties (descriptors) can be calculated from their physical properties and some others have to be determined experimentally by GLC methods or from water-solvent distribution constants [50]. The system parameters, which describe the properties of the sorbent under the specified solvent compositions, can be determined by analysing a suitable set of compounds with known solute descriptors. This results in a system map which can be used for SPE method development using the same type of sorbent and solvents.

Many sorbents have been classified [51,54–58] and the effect of different organic solvents was demonstrated in refs. [55,57,58]. An example of the use of this model is given in ref. [59] for the isolation and concentration of estrogens from urine.

4.2. Equilibrium method

A different way of determining the SPE conditions was proposed by Ferreira et al. [60]

The SPE system dimensions and the volumes of solvents to be used for washing and eluting were based on distribution coefficients of solid–liquid systems. To determine this parameter, the SPE sorbent powder was allowed to equilibrate with either a matrix solution containing the analyte or a wash or elute solvent, also containing the analyte. Then, at equilibrium, the liquid phase was analysed using a suitable method and the distribution coefficients were calculated. With this parameter, the breakthrough volumes could be determined. This is actually the only proposed method where the SPE characteristics were determined in the actual sample matrix and therefore omitting possible matrix effects. The disadvantage of use in bioanalytical methods is that the analyte concentration of the matrix should be determined with a secondary method, either by protein precipitation or a liquid extraction method.

4.3. Models based on actually used SPE cartridges

None of the above mentioned methods used the actual sampling system. Instead, the sorbent was removed from the cartridges and was either used to fill a HPLC column with, or used to determine the distribution coefficients in a vessel. Also, for the solvation parameter model the solute descriptors have to be determined from additional experimental work since these parameters are not always available or easy to compute. This is especially an issue when the chemical structure of the analyte is not available or when method development time should be reduced to a minimum as

in commercial laboratories. The main disadvantage of the solvation parameter model is that it is strictly applicable to neutral compounds or to ionisable analytes in the unionised form [50] while most drugs or their metabolites contain ionisable groups. Also, this method is not applicable to mixed mode sorbents, where the sorbent exhibits multiple interaction mechanisms such as a hydrophobic and an ion exchange mechanism.

To overcome these issues, the SPE method development method proposed by Waters (Milford, MA) could be used [61,62]. This method uses the actual SPE cartridges and is suitable for ionisable analytes and could also be used in combination with mixed mode sorbents. Multiple SPE columns were loaded with an aqueous analyte solution. Each column was eluted with either an acidic or a basic solution differing in organic solvent (methanol) concentration and the eluates were analysed. A plot of the analytical response against the concentration of the organic solvent was used to estimate the best conditions for washing and eluting the analytes. This approach is very suitable since it is a relative quick method and no structure information is needed for modelling the SPE behaviour. A disadvantage is that this method used a fixed volume of wash or elution solvent so that for a complete picture of the method, the procedure should be repeated for a range of volumes. Also in this method, as in all other mentioned methods, the effect of multiple wash steps were not taken into account and the migration of the analyte band on the sorbent was considered to act as an on/off procedure where the point of switching is determined by the elution strength of the used wash or elution solvent.

Hendriks et al. [63] proposed a method that uses the actual SPE column to model the retention behaviour by connecting the SPE cartridge to an HPLC system using a simple SPE cartridge connector. Multiple injections of neat analyte solutions can be performed using different mobile phase compositions. The retention behaviour was modelled by general chromatographic modelling techniques (see Section 2) and an equation of a non-symmetric peak to predict the analyte recovery after each wash or elution step. This method is able to describe the analyte retention behaviour under ionised as well as under neutral analyte conditions. This method is also easy to automate the method development process and provides a way for a rapid screening of multiple types of SPE cartridges and sorbents. The proposed cartridge adapter should also be suitable for determining breakthrough curves as discussed in Section 4.1 and omit the need to fill LC columns with SPE sorbent in the above mentioned methods.

4.4. SPE models in bioanalytical method development

A pitfall of all discussed methods is the matrix effect since all of these methods, except one, used pure neat analyte solutions to model the SPE characteristics for use with real matrix samples [63]. Since plasma, serum and urine are complex matrices, containing many compounds that could interfere with analyte retention, care must be taken in applying the models to real samples. To diminish the effect of the matrix, it can be diluted as far as possible before applying it to the sorbent. Also, it is wise to take some margin into account in selecting organic modifier concentrations used in wash and elution steps. The matrix effect is compound dependent and rather unpredictable. Therefore, the recovery of a proposed SPE method should always be verified by using a spiked matrix sample.

5. Protein precipitation

Protein precipitation is the method of choice when high throughput of plasma or serum samples is desired [6,64]. Proteins are precipitated by the addition of a suitable organic solvent, strong
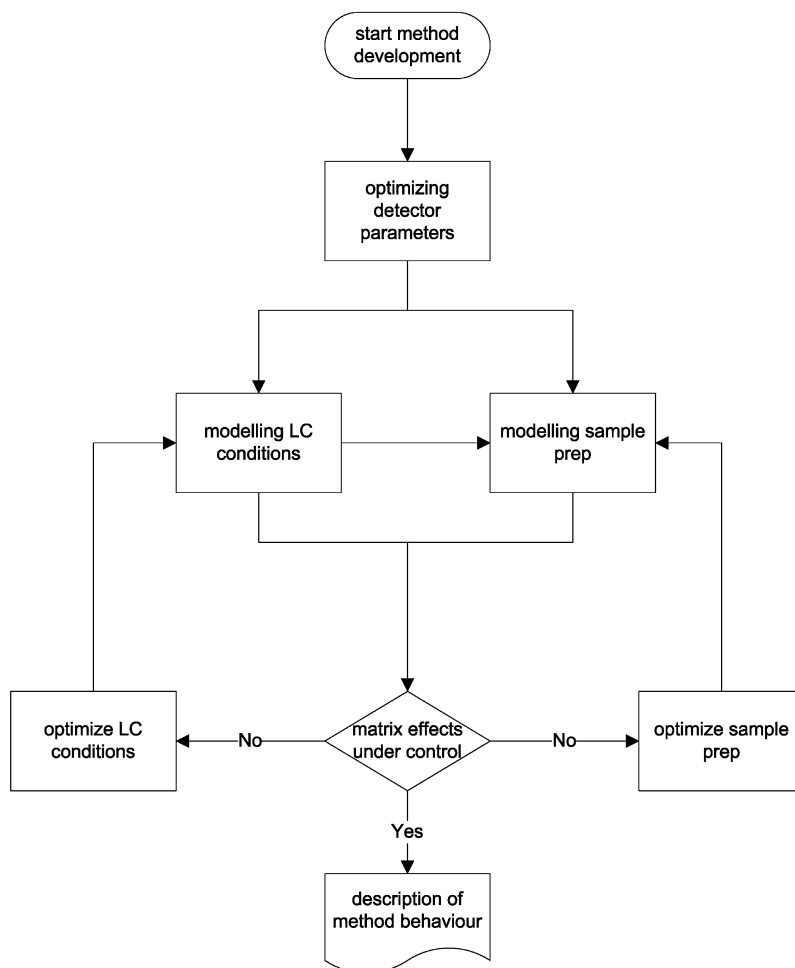
Fig. 4. Schematic representation of the method development process.

acid or some salts of heavy metals to the sample. After centrifugating the sample the clear supernatant can be used for analysis, either directly or after diluting it to match with the initial LC conditions. The need for high throughput is especially the case in clinical toxicology, the drug discovery process and the therapeutic drug monitoring process in hospital laboratories. In these cases analyte concentrations are, in general, high enough allowing the dilution of the samples to be analysed at a high signal to noise ratio.

Since protein precipitation is one of the most crude and non-selective preparation methods, analyte concentrations should be high enough to achieve a signal that dominates the signal of the endogenous material for accurate determination. In LC-MS applications, matrix effects or ionisation suppression is the main pitfall since many matrix components could interfere with the ionisation of the analyte with a high variation between different matrix batches [65].

There are two papers very useful in determining the proper amount of precipitating agent.

Blanchard [66] investigated the use of precipitating agents by their effectiveness of protein removal at different matrix-precipitant ratios. He also published the final pH of the precipitate. Polson et al. [67] also investigated several precipitating agents and their precipitation efficiencies were comparable with those of Blanchard. Polson also compared the results of the different precipitants to the ionisation effects in LC-MS both in intensity as well as in duration and compared the effects with different mobile phase compositions. He demonstrated variation in precip-

itation efficiencies between different matrix species, matrix lots, and differences in ionisation suppression effects for various precipitants and mobile phase compositions. Their results are very useful in selecting the proper precipitant based upon their effectiveness in protein removal and their compatibility with the remainder of the assay. Souverain et al. demonstrated that analyte recovery in protein precipitation is not always self-evident especially when strong acids were used as precipitant, probably due to analyte co-precipitation [68].

The conclusion in modelling protein precipitation is that the efficiencies in precipitation efficiency are well described but that the matrix effects can be an issue depending on the type of matrix, the precipitating agent, the used LC conditions and the analyte. When analyte concentrations are high enough and when these issues have been investigated and have been overcome, protein precipitation is a very suitable method for high throughput analysis.

6. Matrix effects

When modelling bioanalytical methods, matrix effects are probably the main issue in causing erroneous results since most models are established using aqueous or neat analyte solutions.

A definition of matrix effect was given as the influence of materials from the used matrix on the analyte ionisation in the mass spectrometer [6]. However, we have to realise that matrix materials could also affect the sample preparation procedure when compared to aqueous solutions as demonstrated in ref. [63].

Since the responsible matrix substances are mostly unknown, the effects of these compounds on the individual parts of an analytical method are rather unpredictable.

In conventional HPLC, a matrix effect on the detector response could be evaluated as an interfering (co-eluting) peak by analysing an analyte free matrix and compare the response of the interference with the response of the intended limit of quantitation according to the Guidance for Industry [69].

In LC-MS assays, the matrix effects, as co-eluting matter, form a bigger issue as they are not actually detected due to the high selectivity of the MS. Therefore, the matrix effect should be measured indirectly. Matuszewski et al. [2] provided strategies to assess the effect of co-eluting substances on the analyte response by analysing and comparing spiked matrix samples, spiked extracts of analyte free matrix samples and spiked neat solvents (i.e. reconstitution solvent or mobile phase). They also mentioned the importance of differences in matrix effects using different lots of matrices of the same species. In LC-MS, the effect of co-eluting substances can be visualised by injecting blank matrix samples while post column infusing an analyte solution [5,70,71].

From the resulting detector traces we can conclude that matrix suppression (or enhancing) zones appear mostly in the early part of the chromatogram and that suppression zones not always appear as narrow peaks.

To solve these issues several strategies are possible. At the MS side, the ionisation suppression is strongly dependent on the ionisation type. APCI tends to be less susceptible to suppression effects than an ESI source and a switch to APCI should be considered [5,70]. A second approach is the chromatographic separation of the analytes from the suppression zones. In case of a suppression zone in the front of the chromatogram [5], analyte bands can be positioned beyond that zone by selecting the appropriate chromatographic conditions using a model of the retention behaviour as modelled with neat analyte solutions.

A special type of suppression is auto-suppression in which the analyte response factor decreases with concentration resulting in a concave standard curve. A suitable internal standard for instance, may compensate for a curved calibration line when this compound co-elutes with the analyte. Using a proper modelled chromatographic system, the LC conditions at which both compounds elute simultanously, can be found easily.

Also in conventional HPLC, a chromatographic model can be continuously updated by adding retention time data of possible interfering peaks hereby providing a more detailed model.

When chromatographic separation is not an option because of large interferences or many interfering peaks, the sample preparation could be adapted by performing LLE at a different pH as discussed in Section 3.2.

For ionisation suppression issues it was demonstrated that these effects were significantly reduced by proper sample preparation [5] and by the choice of SPE sorbent [3,70].

For use in MS applications the use of a stable isotopically labelled internal standard may compensate for most of the matrix effects as long as co-elution is assured.

A schematic representation of the workflow of the modelling in bioanalytical method development is given in Fig. 4.

7. Conclusion/discussion

In this paper, we provided a selection of literature references from which most of the bioanalytical techniques could be modelled and described. These references provide a proper selection of algorithms and equations, making a deliberate choice possible depending on the degree of detail. With these references, course modelling is possible using simplified equations as well as very detailed models by incorporating all the parameters that may influence the analyte response. The rules and equations could be used individually or could be combined and incorporated in a computer program or spreadsheet to act as a simulation of the actual system.

Although it is a good starting point, modelling analytical techniques in bioanalytical method development should not be restricted to pure and neat analyte solutions. Unknown compounds originating from the biological matrix could interfere with the analyte response and should also be taken into account in the modelling process.

Carefully built models provide a good description of the behaviour of the complete analytical system under different conditions. These results could be considered as a summary of the results of the performed experiments. Also, method development could be performed much more structured and the models could be helpful for future use for trouble shooting purposes. The number of practical experiments depends highly on the degree of desired detail and a compromise has to be made between the extent of labour and the proposed degree of detail.

Care should be taken to include responses into the models that are not affected by unexpected effects. Besides matrix effects affecting the analytical response, other effects could also blur the results. Solvent evaporation of extracts may lead to incomplete reconstitution due to the loss of analyte or adsorption effects and a decrease in the amount of thermal, oxidation sensitive or pH labile compounds may be interpreted as a low recovery. Also, enzymatic activity in biological matrices may decrease the analyte concentration resulting in misinterpreted recovery values.

Moreover, one has to be sure that a state of equilibrium has been reached and that reactions have been complete as in LLE or protein precipitation.

The use of modelling techniques should be available close to the lab floor either by dedicated software or by spreadsheets programs, ensuring a more consistent method development.

A bioanalytical method which was set up using theoretical models and where matrix effects are taken into account could be considered as a controlled method since the effect of changes in analytical parameters can be predicted quantitatively.

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