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A fractionated combined mixture and factorial design for the modelling of the recovery of five tricyclic amines from plasma after liquid–liquid extraction prior to high-performance liquid chromatography

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ABSTRACT

A general systematic approach is described for the chemometric modelling of liquid–liquid extraction data of drugs from biological fluids. Extraction solvents were selected from Snyder's solvent selectivity triangle: methyl *tert.*-butyl ether, methylene chloride and chloroform. The composition of a mixture of the three extraction solvents was varied and the extraction yield (recovery) of a group of tricyclic amines was measured at all compositions selected. Two process variables, the extraction time and the extraction intensity, were varied simultaneously with the mixture variables to study their influence and their interaction with the mixture composition. The combined mixture and factorial design statistical techniques obtained in this way enabled the recovery to be modelled as a function of both the composition of the extraction liquid and the process variables. The models were assessed with regard to both descriptive and predictive capacities. The results showed that structurally related compounds may demonstrate different partitioning behaviour with regard to both mixture variables and process variables. It was concluded that mixtures of solvents result in higher extraction efficiencies for the amines. A positive effect on the extraction efficiency was demonstrated by the extraction intensity process variable and extraction time. A positive effect on the extraction efficiency was demonstrated by an interaction between extraction intensity and time. Mixture models in which process variables were introduced were recognized as being very suitable for modelling liquid–liquid extraction systems.

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INTRODUCTION

Liquid–liquid extraction is often part of bioanalytical assay methods prior to chromatographic analysis. Although this sample preparation stage is very important from an analytical point of view, less attention has been paid until now to the systematic optimization of liquid–liquid extraction. For method development involving liquid–liquid extraction prior to high-performance liquid chromatography (HPLC), great effort is often spent on the development of a proper sample preparation procedure. Choices have to be made with respect to the extraction solvent or mixture of solvents used, what buffer solution will be used, etc. The choice of all these parameters depends on the matrix that has to be analysed, the equipment available, the method of determination that is used and the amount of labour and time spent. The use of systematic methods for the development of sample preparation procedures may decrease the method development time.

In liquid–liquid extraction, the partition coefficient of individual solutes may be influenced by a number of factors. First, the influence of the pH is an important factor [1–4]. Adjusting the pH to a suitable value converts acidic or basic drugs into non-ionized species, which are more soluble in a non-polar solvent. Second, the ionic strength of the biological sample influences the partition coefficient. The addition of highly water-soluble ionized salts decreases the solubility of drugs in the aqueous phase, followed by an increase in the solubility of the drug in the organic phase. This phenomenon is called the "salting out effect" and is caused by diminished availability of water molecules acting as a solvent for the drug [5,6]. The influence of the ratio of the volume of the organic phase, V_{org}, to the volume of the aqueous phase, V_{aq}, is also known. There is no influence of the ratio of V_{org} to V_{aq} on the magnitude of the partition coefficient. However, it does influence the fraction of the drug extracted into the organic phase (recovery). Of course, V_{org} should not be too high, for economical and practical reasons. The temperature of an extraction system has a minor effect on the partition coefficients. Extractions are generally performed at room temperature. Small fluctuations of room temperature have insignificant effects on partitioning. Also important for the extraction of drugs from body fluids is the sample matrix. However, this is an uncontrollable factor. Finally, a very important factor influencing the partitioning of drugs between two phases is the choice of the organic solvent [4,7]. Specific solvents can be selected with regard to their physical properties. Intuitively, one may have an idea about the extraction behaviour of the analyte in developing new analytical assay methods using liquid–liquid extraction. However, it is difficult to select a proper extraction solvent with a first guess. Contingent selection of a mixture of different solvents is even more difficult. Extraction solvents are often selected by trial and error, and improvement of extraction yields is not considered as long as they are satisfactory, even if they are not optimum or if the reproducibility of the extraction could be improved. Implementation of chemometric optimization procedures in sample preparation procedures using liquid–liquid extraction may be useful for the selection of optimum extraction conditions.

Two general systematic optimization procedures are the simultaneous and sequential methods. In sequential methods (*e.g.*, simplex optimization), results of previously performed experiments are used to calculate conditions for new experiments to be carried out. In this manner, the response surface is sequentially tracked until an optimum has been located, which is not, however, *a priori* a global optimum. Another disadvantage of this located optimum is that it may be dependent on the initial variable settings. Finally, a very important restriction of a sequential simplex optimization may be the complexity of the optimization function. This function is a predefined function, which may be composed of several criteria. Such a composite criterion leads to ambigious results [8]. Other important disadvantages of a simplex optimization method are that often local optima are determined and that the number of experiments needed is not known beforehand.

Simultaneous optimization methods do not suffer from these problems. Experiments to be carried out are previously planned, according to some experimental design, within the factor space. The experimental results are collected and then any response criterion desired can be modelled. Simultaneous methods may provide the global optimum. The experimental designs are used to obtain maximum information out of a series of carefully selected experiments.

Applications of simultaneous methods in chromatography have been described by Glajch *et al.* [9] and Weyland *et al.* [10] and more recently by Mulholland and Waterhouse [11–13] and Coenegracht *et al.* [14].

Applications of systematic optimization of extraction liquid composition in biomedical analysis using sequential or simultaneous methods in liquid–liquid extraction have not been reported. In liquid–liquid extraction, the selective interactions of the extraction solvent and the matrix components to be extracted influence the magnitude of extraction [15]. The extraction liquid composition should be chosen such that there is high selectivity for the matrix component(s) to be extracted. The polarity of the extraction solvent used is very important for the recovery. However, the selective interactions between solvent and solute are also very important. Therefore, the selectivity of the solvent should be adjusted such that interaction between the solvent and solute is optimum. In this way, extraction yields can be maximized. Here, we propose a new method for the systematic optimization of the extraction liquid in liquid–liquid extraction, in which several theories have been incorporated. The solvent selectivity theory developed by Rohrschneider [16] and Snyder [17] was used to select three solvents representing different types of selective interactions. Tricyclic amines were extracted with different mixtures of three extraction solvents according to a mixture experimental design in combination with a factorial design for two extraction process variables. The efficiency of the extraction (recovery) was modelled. The final objective of this investigation was to study the applicability of combined fractional experimental designs for the simultaneous optimum choice of extraction liquid composition (the mixture design part), extraction time and extraction intensity (the factorial design part). The optimization criterion was to maximize tha extraction efficiency of the extraction system and to minimize the time needed for a single extraction. The latter criterion was introduced for future application of a laboratory robot with serialized sample processing. Moreover, the ruggedness of the extraction system was evaluated for pairs of amines.

THEORY

Several studies have attempted to relate the partition coefficient of a solute in a liquid chromatographic system between two phases, one of which has a varying composition [16,18–20]. Schoenmakers and co-workers [21,22] derived a relationship for a binary mobile phase in reversed-phase HPLC from the solubility parameter theory of Hildebrand *et al.* [23]. Similarly, a relationship can be derived for liquid–liquid extraction with extraction liquids composed of three components:

$$\ln P = A\varphi_1^2 + B\varphi_2^2 + C\varphi_3^2 + D\varphi_1\varphi_2 + E\varphi_1\varphi_3 + F\varphi_2\varphi_3 + G\varphi_1 + H\varphi_2 + I\varphi_3 + J \quad (1)$$

where A–J are functions of the solubility parameters of the extraction liquid components and φ_1, φ_2 and φ_3 are the fractions of mixture components 1, 2 and 3, respectively. This equation is a reduced form of a more complex equation with three mixture variables, but this complex equation has been simplified because in mixture designs the sum of the fractions of the extraction liquid components equals 1 ($\varphi_3 = 1 - \varphi_1 - \varphi_2$). Eqn. 1 provides considerable insight into the partitioning of a solute between the aqueous and the organic phase in liquid–liquid extraction. Quadratic effects of fractions and interaction effects between two fractions are indicated. This factorial design-like model can be transformed into a quadratic mixture model when the constraint that the sum of the fractions equals 1 ($\varphi_1 + \varphi_2 + \varphi_3 = 1$) is substituted. The mixture variables (the fractions of the components in the extraction liquid) are now represented by x_i:

$$\ln P = \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \varepsilon \quad (2)$$

This transformation of a physico-chemical model into a chemometric model was also discussed by Weyland *et al.* [9]. A ternary non-linear blending term is often added to improve the descriptive power of eqn. 2. Then, a special cubic mixture model (eqn. 3) is obtained:

$$\ln P = \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{123} x_1 x_2 x_3 + \varepsilon \quad (3)$$

where β_1–β_{123} are the regression coefficients to be estimated after the experimental part and ε is the residual error to be estimated. For estimation of the regression coefficients and the residual error, at least

as many experiments have to be performed as the number of model coefficients plus one. In a mixture design experiment, the response to a mixture of q components depends only on the fractions of mixture components and does not depend on the total amount of the mixture.

Eqn. 3 describes the relationship between the composition of an extraction liquid and the partition coefficient. However, liquid–liquid extraction is often quantified by the recovery R, *i.e.*, the fraction of the total amount of analyte transferred from the aqueous phase into the organic phase ($R = \Phi_{org}$):

$$R = \Phi_{org} = \frac{P\left(\frac{V_{org}}{V_{aq}}\right)}{P\left(\frac{V_{org}}{V_{aq}}\right) + 1} \quad (4)$$

where V_{aq} and V_{org} are the volumes of the aqueous and organic phase, respectively, and P is the partition coefficient of a given analyte under certain conditions (pH, ionic strength, extraction liquid, temperature, sample matrix). This relationship can be transformed into the following:

$$P = \frac{R}{(1-R)} \cdot \frac{V_{aq}}{V_{org}}$$

With a constant ratio of the phase volumes through all the experiments:

$$P \propto \frac{R}{1-R}$$

Thus, when recoveries of analytes are measured, these recoveries can be related to the extraction liquid composition by writing eqns. 2 and 3 as follows:

$$\ln\left(\frac{R}{1-R}\right) = \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 \; (+ \beta_{123} x_1 x_2 x_3) + \varepsilon \quad (5)$$

Summarizing, to optimize the partition coefficient P of a solute i and consequently R_i, P_i and R_i should be maximized by mixing three solvents in the correct proportions. The use of mixture design statistical techniques with the natural logarithm of the partition coefficient as response criterion is a valid way to achieve this.

Introduction of process variables in mixture models

For the selection of factors that influence extraction one has to select as few variables as possible, to avoid models with large numbers of coefficient and, consequently, large numbers of experiments. Factors that influence liquid–liquid extraction are the following: the pH of the aqueous phase of the extraction system; the ionic strength of the aqueous phase; the ratio of the volumes of the organic phase and the aqueous phase, V_{org} and V_{aq}; the temperature of the extraction system; the matrix in which the solute is dissolved; the choice of the solvent; the time in which the extraction is performed (extraction time); and the intensity with which the extraction takes place (extraction intensity) – the rotations per minute of a tumble mixer.

The first six factors were discussed in the Introduction. Arguments for the introduction of the last two factors (extraction intensity and extraction time) are discussed below.

Liquid–liquid extraction is based on partitioning equilibrium of a solute over the two phases. Important factors for this phenomenon may be the time of extraction and the intensity of extraction. The influence of these factors is generally not known. For extraction solvents with different compositions, equilibria may possibly adjust differently. Thus, when different solvents (or mixtures of solvents) are used, different adjustment parameters (process variables) are probably needed. They may be different for several solutes [24]. Often an extraction equilibrium is obtained rapidly. Extraction times are often too long [25], which was also acknowledged by Campbell [26]. However, Lagerström *et al.* [27] found a time-dependent extraction from plasma. They investigated the influence of extraction time on the extraction yield of hydrophobic tertiary amines in diethyl ether, hexane–propanol (95:5) and methylene chloride. They suggested that the amines investigated were probably occluded in protein precipitates and that diffusion out of that environment was very time consuming. Schill *et al.* [28] showed the effect of dilution in protein precipitation: extraction yields of two sulphonamides increased strongly after dilution of plasma with water.

A survey of a number of recent issues of *Journal of Chromatography (Biomedical Applications)* showed that a small number of publications describing liquid–liquid extraction prior to determination did

not report the extraction time. However, most of the publications in these issues did not report the extraction intensity or even the method of shaking. This indicates that the significance of the physical conditions during an extraction is often disregarded and is not considered as being important. However, investigation of the extraction conditions other than pH or solvent used may be interesting, as demonstrated by the examples reported above [24–26].

Of course, neither factor influences partition coefficients, but they may help to accelerate the adjustment of extraction equilibria. Arguments can be put forward to optimize both factors. In most routine laboratories samples to be analysed are prepared simultaneously; the actual shaking of all samples does not last longer than the time needed to extract one sample. In automated liquid–liquid extraction processes (robots), however, in which samples ar processed consecutive [29–31], time is an important variable; the shorter the time of extraction, the higher is the throughput of samples. On the other hand, equilibria may adjust slowly and long periods of extraction may be necessary. Optimization of the extraction time may be required. Higher extraction intensities decrease the size of the droplets of the organic and the aqueous phase in the extraction container. This results in a larger contact surface between the two phases and may influence extraction positively. Vigorous shaking may reduce the extraction time needed. In other words, there may be interaction between extraction time and extraction intensity. Disadvantages of thorough mixing may be the formation of emulsions, which may be difficult to separate in a centrifuge. A disadvantage of both long periods of extraction and high extraction intensities may be the amount of contamination extracted. Also, drawbacks of extended extraction may be irreversible adsorption of associated drug molecules to plasma proteins. Hence for many reasons it is important to select proper values of the process variables.

Three variables were selected: the composition of the extraction liquid (which may be divided into a number of sub-variables, the number of mixture components), the extraction time and the extraction intensity. The extraction liquid components are called mixture variables. Extraction time and extraction intensity are called process variables or factors and their influence can be studied with factorial designs. It is not irrational to presume interaction(s) between mixture and process variables: equilibrium may be achieved faster using a given extraction solvent A than using a given extraction solvent B or a mixture of A and B.

Optimization strategy

Mixture designs. A wide variety of applications of mixture experimental designs were summarized by Cornell [32]. For the optimization of the liquid–liquid extraction of drugs from biological matrices, the use of mixture components as variables seems very reasonable, as discussed previously. The logarithm of the partition coefficient of the analyte is the response to be optimized and the fractions of the different mixture components in the extraction solvent are the control variables. Particularly when more solutes with different structures have to be extracted from a sample, a mixture of solvents may give a better response than a single solvent.

Aspects of mixture designs have been described in detail by Scheffé [33], Snee [34] and Gorman and Hinman [35]. Influences of pure components cannot be investigated in mixture designs, as a change in the fraction of one mixture component always causes a change in the fraction of another component; variables cannot be varied independently. The quadratic and cubic terms in eqns. 2 and 3 are therefore not called interaction terms, but non-linear blending terms.

By mixing more solvents into one extraction solvent, the multivariate solubility parameter δ [22,23] of the extraction liquid can be adjusted to an optimum value.

Factorial designs. Factorial designs are often applied to study the influence of process variables (factors) or to optimize the settings of factors. The application, use and characteristics of factorial designs have been discussed in detail by Box *et al.* [36].

Combined designs. When both mixture and process variables are observed in one design, interaction between these variables can be investigated. Therefore, a combined experimental design has to be selected, which may become large unless efficient fractionation can be accomplished. There are numerous examples in the literature of combined mixture and factorial designs [37–43]. Fractionation may often be necessary: the experimental effort may

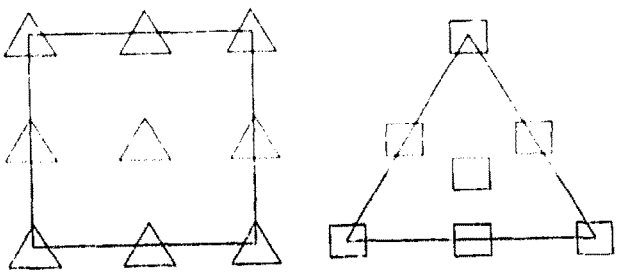


Fig. 1. Two representations of full combined mixture and factorial designs for three mixture variables and two factorial variables at three levels.

increase considerably without fractionation. In our problem, where we have two factors at three levels (3^2 = nine experiments) and three mixture variables with a special cubic mixture model (eqn. 3: seven experiments), $7 \cdot 9 = 63$ model coefficients plus an error term would have to be estimated. Therefore, 64 experiments should be performed according to the designs in Fig. 1, which represents (1) the performance of a mixture design with every combination of process variable settings of the factorial design and (2) the performance of a factorial design with all compositions of the mixture. In fact, these representations correspond to the same experiments. Doornbos *et al.* [42] developed fractionated combined mixture-factorial designs with a concomitant hierarchy of polynomial models. Optimality of the designs was judged with G, V and D optimality criteria. By rotation and contraction they selected a combination design for a 2^{5-1} design (Fig. 2).

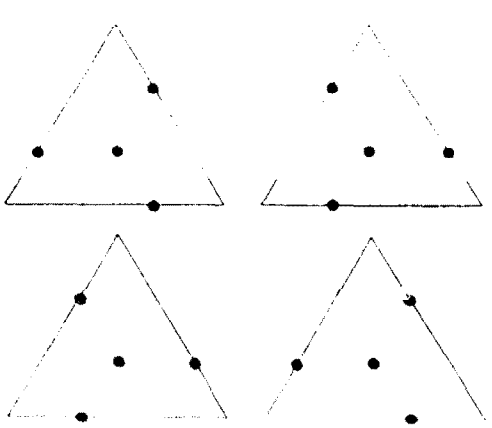


Fig. 2. A fractionated 2^{5-1} combined design after rotation and contraction [42].

EXPERIMENTAL

Selection of extraction solvents

The composition of the extraction liquid is very important for the magnitude of the partition coefficient of a solute. Several quantities have been studied to describe solvent properties [16–18,22,44–46]. Snyder [17,18] grouped the relative selectivities of solvents into solvent selectivity classification groups, each group being characterized by its proton-donating, proton-accepting and dipole interaction properties.

Solvents used here for a general liquid–liquid extraction method were selected from Snyder's solvent selectivity triangle. The goal was to select three solvents that represent a wide variety of selective interactions, so that extraction liquids composed of mixtures of these solvents may enter into maximum interaction with the analyte. Additionally, the solvents should be sufficiently polar to ensure quantitative extraction. In addition to selectivity and polarity requirements, the solvents should also meet a few other criteria, mainly for practical reasons: they should not be miscible with water, have low boiling points (for relatively fast evaporation procedures) and have densities sufficiently different from the density of water, for pure solvents and for selected binary or ternary mixtures of solvents.

The solvents selected were similar to those which Glajch *et al.* [9] used for normal-phase liquid chromatography. Methyl *tert.*-butyl ether (a proton acceptor) was selected instead of diethyl ether, as the former is less volatile. The other two solvents selected were methylene chloride (dipole interactions) and chloroform (proton donor). These three solvents meet all practical requirements. The polarities P' [17] of the solvents are 2.5, 3.1 and 4.1, respectively. The solvents were used in pure form: no supporting solvent was used.

Process variables

The extraction process variable adjustments were a rotating speed of 20, 40 and 60 rotations per minute (rpm) during 5, 15 and 25 min of shaking.

Three levels of a process variable justify investigation of quadratic effects of this process variable. Also, interaction between mixture variables (fractions of solvents in the extraction liquid) and both process variables can be examined.

Regression models and experimental design

The fractions of the three solvents in the extraction liquid are mixture variables. Details of mixture variables were given by Cornell [47]. The process variables extraction intensity and extraction time have to be optimized using a factorial design. Here we propose a combined design for the simultaneous optimization of mixture and process variables, which reduces the number of experiments needed. All variable interactions are given in Fig. 3.

Previous experiments led to the choice of a special cubic model for mixture and process variables (eqn. 6). To estimate the 22 coefficients and the error term at least 23 experiments are required.

variable	1	t	v	t*t	v*v	t*v
x_1	■	■	■	■	■	■
x_2	■	■	■	■	■	■
x_3	■	■	■	■	■	■
x_1x_2	■					
x_1x_3	■					
x_2x_3	■					
$x_1x_2x_3$	■					

Fig. 3. Schematic representation of interaction terms in eqn. 6 (t = extraction time; v = extraction intensity; x_i = mixture variables).

$$E(y) = \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{123} x_1 x_2 x_3 + \gamma_1^1 x_1 t + \gamma_2^1 x_2 t + \gamma_3^1 x_3 t + \gamma_1^{11} x_1 t^2 + \gamma_2^{11} x_2 t^2 + \gamma_3^{11} x_3 t^2 + \gamma_1^2 x_1 v + \gamma_2^2 x_2 v + \gamma_3^2 x_3 v + \gamma_1^{22} x_1 v^2 + \gamma_2^{22} x_2 v^2 + \gamma_3^{22} x_3 v^2 + \gamma_1^{12} x_1 tv + \gamma_2^{12} x_2 tv + \gamma_3^{12} x_3 tv + \varepsilon \quad (6)$$

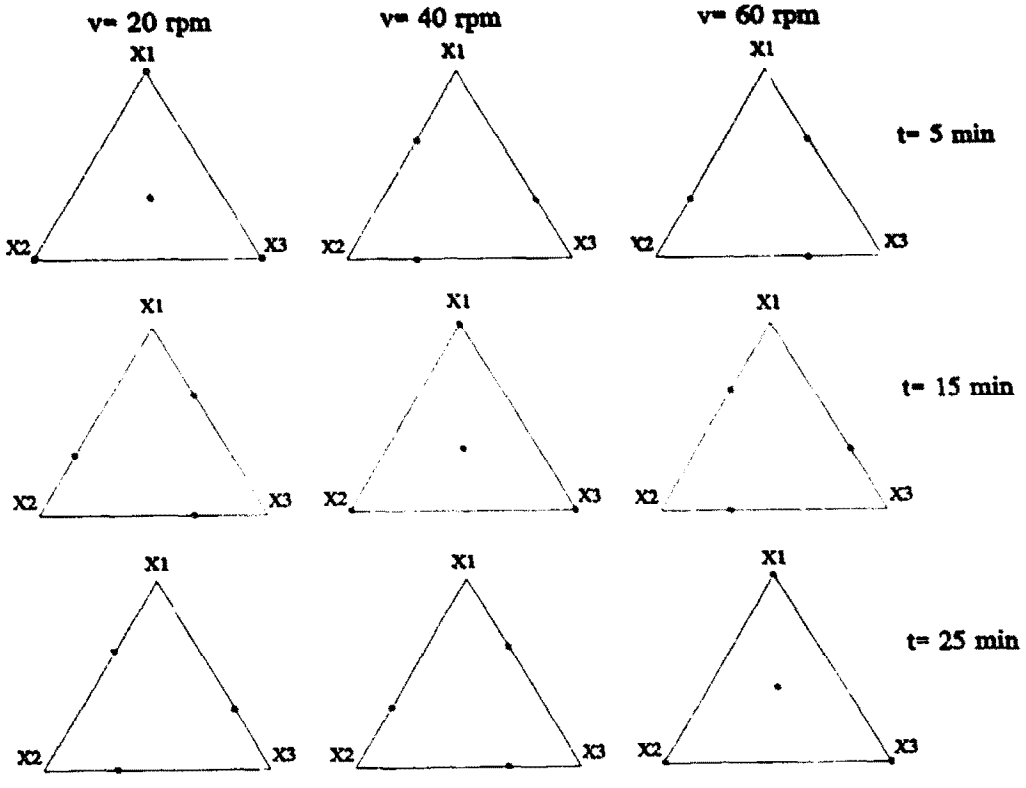


Fig. 4. Experimental design for the extraction of tricyclic amines.

Eqn. 6 is a special cubic mixture model expanded with combinations of mixture variables with linear and squared process variables and with an interaction term between the two process variables with one mixture variable; t is the extraction time and v is the extraction intensity. The β-terms are parameters of the mixture models included. The γ-terms are the parameters for the interaction terms between mixture and process variables. Superscripts in the parameters refer to the process variable(s) involved and subscripts in the parameters refer to mixture variables involved.

Each of the three solvents selected (the mixture variables x_i) is located at a vertex of a triangle, here called factor space. Each point within the factor space is a combination of the fractions of the solvents in the extraction liquid. Several mixture triangles are plotted which symbolize the levels of the process variable(s). Experiments selected should be well distributed over the factor space. With the method described here, no restrictions were made to maximum or minimum fractions of mixture components. The entire factor space (the mixture triangle) is used for design of experiments. In total, 30 experiments were selected, which is seven more than needed for the model (eqn. 6). The extra experiments were used for statistical evaluation of the model. Fig. 4 depicts the factor space and the experiments in the fractional experimental design selected for the amines. Corresponding values of the fractions of methyl *tert.*-butyl ether, chloroform and methylene chloride and the process variables t (extraction time) and v (extraction intensity) are given in Table I.

The criterion modelled in this study was the extraction efficiency (recovery, R). Owing to the introduction of the two process variables the physico-chemical model of the partition coefficient introduced in the theory section was of less value.

Instruments and instrumental conditions

Analyses were performed with an HPLC system consisting of a Waters (Milford, MA, USA) Model 45 HPLC pump used at a flow-rate of 1.2 ml min^{-1} and a Kratos (Ramsey, NJ, USA) Model 757 UV detector (wavelength 250 nm, range 0.01 a.u.f.s., rise time 1 s). Injections of extracts into a Zymark (Hopkinton, MA, USA) Z 310 HPLC injection station, equipped with an electrically controlled Rheodyne valve with a 10-μl sample loop, were performed by a Zymate II robot system. The Zymark Z 310 analytical instrument interface was used to control the HPLC injection station. Data analysis was performed by means of a Spectra-Physics SP4270 computing integrator. The analytical column was a Chrompack (100 × 3.0 mm I.D.) Spherisorb, 5-μm CN cartridge system, which was maintained at 35°C by a thermostatic bath. The injection volume was 10 μl. Mixing was performed on a vortex mixer type FV2 (Janke und Kunkel, Staufen, Germany), shaking of the extraction container was performed on a Heidolph (Kelheim, Germany) Reax-2s shaker for tumble mixing and a Heraeus (Osterrode am

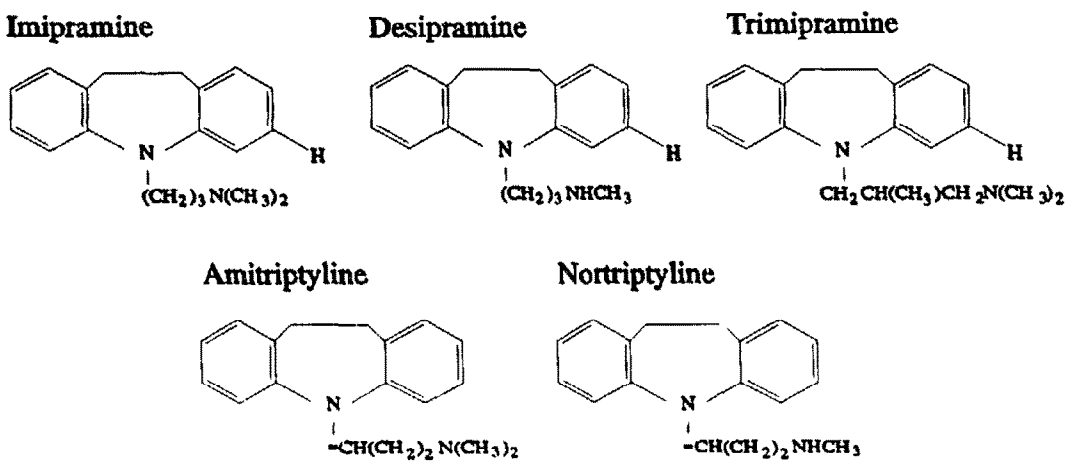


Fig. 5. Structures of the tricyclic amines.

Harz, Germany) Labofuge GL was used for centrifuging.

Chemicals and reagents

The tricyclic amines imipramine (IMI), amitriptyline (AMI), nortriptyline (NOR), desipramine (DES) and trimipramine (TRI) (Fig. 5) were supplied by Sigma (St. Louis, MO, USA). These amines were used because Lagerström *et al.* [27] found a time-dependent recovery for these compounds in several extraction solvents. Acetonitrile (ACN), methylene chloride (DCM) and methanol (MeOH) were supplied by Labscan (Dublin, Ireland) and were of HPLC grade. Chloroform (Clf) was of analytical-reagent grade and supplied by Malinckrodt (Promochem, Wesel, Germany). Phosphoric acid (85%), sodium hydroxide (NaOH), disodium phosphate ($Na_2HPO_4 \cdot 2H_2O$) were all of analytical-reagent grade and supplied by Merck (Darmstadt, Germany). Methyl *tert.*-butyl ether (Uvasol) (tBME) was also supplied by Merck. Water was purified by using a Milli-RO-4 and a Milli-Q water purification system (Millipore, Bedford, MA, USA). Unless stated otherwise, Milli-Q quality water was used throughout. All blank plasma samples used in this study were obtained from a single pool of blank plasma. This was done in order to eliminate the effect that may be present through the use of different plasma samples.

A phosphate buffer (pH 6.5) was prepared by dissolving 450 mg of $Na_2HPO_4 \cdot 2H_2O$ in 500 ml of water. pH adjustment was performed using concentrated phosphoric acid. The mobile phase was prepared by mixing 200 ml of phosphate buffer, 600 ml of ACN and 200 ml of MeOH. NaOH solution (0.03 *M*; pH 12.5) was prepared by dissolving 1.20 g of NaOH in 1 l of water. Stock solutions of the tricyclic amines were prepared by dissolving 100 mg of the compounds in 50 ml of water. These solutions were stored at 4°C and were used to prepare a standard solution of 2 mg l^{-1} of all amines in water. This standard solution contained all five amines and was used for the extraction studies and stored at 4°C.

Analytical procedure

A 250-μl aliquot of plasma to be analysed and 250 μl of the standard solution were pipetted into a 11.5-ml glass tube, then 250 μl of NaOH solution (pH 12.5) were added and mixed for 10 s on a vortex mixer. A 9-ml volume of extraction liquid was added and the tubes were extracted on a Heidolph tumble mixer according to the experimental design in Fig. 4 and Table 1. A potential problem arises if the solvents used are mixed in different compositions: a composition can possibly be selected that has a density equal to the density of the aqueous layer, which may give rise to problems with the phase separation.

After centrifugation at 4000 rpm (2755 *g*) for 10 min, the organic layer was transferred into another glass tube of 11.5 ml and evaporated to dryness under a gentle stream of nitrogen at 55°C. The residue was reconstituted in 1 ml of MeOH; 10 μl of the solution were injected into the HPLC system.

For the determination of the absolute analytical recovery ($R = \Phi_{org}$), the peak heights of the prepared samples were compared with the mean peak height of seven direct injections of the standard solution into the HPLC system.

For the correct determination of the recoveries and the partition coefficients (for use in future studies), each tube was weighed separately before and after the extraction solvent dispensing step (to give w_{in}) and before and after phase separation (to give w_{out}). The volume of the extraction liquid used for extraction, V_{in}, was calculated from w_{in} and the density of the extraction liquid considered. In this way the exact volumes used could be measured, which were used to calculate the recoveries and the partition coefficients:

$$\Phi_{org} = R = \frac{\text{peak height extractions}}{\text{peak height direct injection}} \cdot \frac{w_{in}}{w_{out}}$$

$$P = \frac{R}{1 - R} \cdot \frac{0.75}{V_{in}}$$

where 0.75 is the volume of the aqueous layer (ml), V_{in} is the volume of the organic phase (*ca.* 9 ml) used for extraction ($V_{in} = w_{in}/\rho_i$; ρ_i is the density of the extraction liquid involved).

As the volumes of both the organic and the aqueous phase influence the recovery of a solute, experiments were done to study if there was an effect of mixing on the volume of the resulting extraction liquid: volumes of 10 ml of methyl *tert.*-butyl ether, chloroform and methylene chloride were mixed to give binary and ternary solvents. These experiments

TABLE I

RECOVERIES OF THE TRICYCLIC AMINES AFTER EXTRACTION ACCORDING TO THE EXPERIMENTAL DESIGN IN FIG. 3

Experimental design					Recovery				
x_{DCM}	x_{CH}	x_{HMIC}	t^a	v^b	TRI	AMI	IMI	NOR	DES
1.0000	0.0000	0.0000	0.0000	0.0000	0.1462	0.2260	0.2726	0.4377	0.5216
0.0000	1.0000	0.0000	0.0000	0.0000	0.1111	0.1897	0.2669	0.4730	0.5780
0.0000	0.0000	1.0000	0.0000	0.0000	0.2875	0.3541	0.4267	0.6428	0.7709
0.3333	0.3333	0.3333	0.0000	0.0000	0.1780	0.2640	0.3370	0.5300	0.6400
0.6667	0.3333	0.0000	0.0000	0.5000	0.2225	0.3151	0.3976	0.6230	0.7294
0.3333	0.0000	0.6667	0.0000	0.5000	0.3732	0.4575	0.5418	0.7950	0.9123
0.0000	0.6667	0.3333	0.0000	0.5000	0.3063	0.4098	0.5227	0.7985	0.9383
0.3333	0.6667	0.0000	0.0000	1.0000	0.2262	0.3191	0.4162	0.6638	0.7943
0.6667	0.0000	0.3333	0.0000	1.0000	0.3487	0.4847	0.5981	0.8195	0.9463
0.0000	0.3333	0.6667	0.0000	1.0000	0.4723	0.5626	0.6695	0.8646	0.9649
0.3333	0.6667	0.0000	0.5000	0.0000	0.1244	0.2367	0.2980	0.4184	0.5670
0.6667	0.0000	0.3333	0.5000	0.0000	0.2323	0.3320	0.4140	0.7193	0.8502
0.0000	0.3333	0.6667	0.5000	0.0000	0.3012	0.3828	0.4665	0.6984	0.8076
1.0000	0.0000	0.0000	0.5000	0.5000	0.2262	0.3318	0.4372	0.7074	0.7811
0.0000	1.0000	0.0000	0.5000	0.5000	0.1551	0.2559	0.3859	0.6897	0.8021
0.0000	0.0000	1.0000	0.5000	0.5000	0.3854	0.4596	0.5621	0.7595	0.8319
0.3333	0.3333	0.3333	0.5000	0.5000	0.2744	0.3930	0.5125	0.7170	0.8090
0.6667	0.3333	0.0000	0.5000	1.0000	0.1928	0.3034	0.4203	0.6944	0.7793
0.3333	0.0000	0.6667	0.5000	1.0000	0.3774	0.4673	0.5742	0.7725	0.8606
0.0000	0.6667	0.3333	0.5000	1.0000	0.2696	0.3971	0.5335	0.7395	0.8189
0.6667	0.3333	0.0000	1.0000	0.0000	0.1907	0.3047	0.4346	0.7587	0.8844
0.3333	0.0000	0.6667	1.0000	0.0000	0.3525	0.4462	0.5653	0.7993	0.9484
0.0000	0.6667	0.3333	1.0000	0.0000	0.2889	0.4188	0.5635	0.7871	0.9055
0.3333	0.6667	0.0000	1.0000	0.5000	0.1782	0.2975	0.4641	0.8630	1.0093
0.6667	0.0000	0.3333	1.0000	0.5000	0.2937	0.4269	0.5859	0.8792	0.9724
0.0000	0.3333	0.6667	1.0000	0.5000	0.4262	0.5341	0.6836	0.9292	1.0206
1.0000	0.0000	0.0000	1.0000	1.0000	0.2660	0.4010	0.5190	0.9074	1.0173
0.0000	1.0000	0.0000	1.0000	1.0000	0.1822	0.3145	0.4850	0.9244	1.0404
0.0000	0.0000	1.0000	1.0000	1.0000	0.4645	0.5485	0.6836	0.9444	1.0143
0.3333	0.3333	0.3333	1.0000	1.0000	0.3485	0.4918	0.6562	0.9538	1.0570

[a] Extraction time: 0.0 = 5 min, 0.5 = 15 min and 1.0 = 25 min.
[b] Extraction intensity: 0.0 = 20 rpm, 0.5 = 40 rpm and 1.0 = 60 rpm.

were done prior to the actual extraction experiments. The densities and volumes of the outcoming liquids were measured to investigate the effect of mixing on the density and volume of the outcoming liquid.

Models of the recoveries were calculated with multiple ordinary least-squares regression for each amine on an IBM PS/2 Model 60 computer using the laboratory-made software package SOLEX (systematic optimization of liquid extraction) written in Pascal.

RESULTS AND DISCUSSION

Effect of mixing on the density and volume

The mixture compositions in Table I did not give rise to any problems with phase separation due to equal densities of the organic and the aqueous layers; all compositions used could be separated from the aqueous layer.

The density and volume measurements after mixing different solvents showed that there was no influence of mixing on the density and volume of the

outcoming liquid. The outcoming volume (V_o) of the mixed liquid was equal to the sum of the individual volumes of the different extraction solvents. For a liquid composed of arbitrary volumes of methylene chloride (V_{DCM}), chloroform (V_{CH}) and methyl *tert.*-butyl ether (V_{tBME}),

$$V_o = V_{DCM} + V_{CH} + V_{tBME}$$

A linear relationship ($r = 0.9999$) was found to describe the density of the outcoming (ρ_o) liquid as a function of the particular densities (ρ_i) and fractions of the different solvents in the liquid (x_i). For a liquid composed of n extraction solvents:

$$\rho_o = \sum_{i=1}^{n} x_i \rho_i \quad \left(0 \leqslant x_i \leqslant 1; \sum_{i=1}^{n} x_i = 1\right)$$

Owing to the linearity of these properties it was unnecessary to correct for mixing effects on density and volume.

Extraction of tricyclic amines

No difficulties in any of the settings of the variables in Table I were observed with respect to emulsion formation due to vigorously shaking the extraction container. The recoveries of the five tricyclic amines were measured with the extraction

TABLE II

CALCULATED REGRESSION COEFFICIENTS AND MODEL VALIDATION CRITERIA OF THE COMBINED SPECIAL CUBIC MIXTURE MODEL AND THE QUADRATIC FACTORIAL MODEL (EQN. 6) WITH THE RESULT OF ALL EXPERIMENTS FOR THE AMINES

Models fitted with relative values of extraction time (5 min = 0; 15 min = 0.5; 25 min = 1) and extraction intensity (20 rpm = 0; 40 rpm = 0.5; 60 rpm = 1).

Parameter	Solute				
	TRI	AMI	IMI	NOR	DES
β_1	0.154210	0.234054	0.274570	0.459544	0.549154
β_2	0.113260	0.193460	0.264623	0.473312	0.588316
β_3	0.290544	0.351359	0.420103	0.675951	0.808063
β_{12}	−0.033326	−0.070526	−0.129337	−0.250048	−0.156492
β_{13}	−0.016629	0.044897	0.067622	0.115694	0.234026
β_{23}	0.195647	0.291028	0.337651	0.318056	0.309693
β_{123}	0.099205	0.300874	0.608050	−0.200810	−0.678243
	0.027051	0.034034	0.084671	0.257396	0.258674
	−0.121329	−0.031847	−0.038799	−0.425237	−0.484925
	−0.066026	−0.107685	−0.108416	−0.152534	−0.242696
	0.152585	0.206947	0.341010	0.334618	0.230069
	0.192261	0.162094	0.293614	0.772193	0.865737
	0.304983	0.346528	0.387500	0.237280	0.148014
	0.026032	0.070831	0.125472	0.152000	0.168939
	0.166929	0.127651	0.206621	0.648396	0.721530
	0.213938	0.248285	0.280305	0.230019	0.311115
	−0.034391	−0.028792	−0.086476	−0.008965	0.090038
	−0.101252	−0.063534	−0.151102	−0.557608	−0.653206
	−0.062259	−0.104108	−0.128425	−0.093811	−0.032648
	−0.067718	−0.124277	−0.218690	−0.298908	−0.300969
	−0.073636	−0.078822	−0.087763	0.009673	−0.006080
	−0.214948	−0.183735	−0.162549	[illegible]651	−0.011766
r	0.9900	0.9888	0.9940	0.9835	0.9783
r^2	0.9802	0.9778	0.9880	0.9673	0.9571
RSD	0.005	0.006	0.006	0.007	0.007
SEP	0.047	0.041	0.036	0.103	0.115
SEP-adj	0.047	0.045	0.038	0.088	0.094
mPRESS	0.002	0.002	0.001	0.010	0.013
mPRESS-adj	0.002	0.002	0.001	0.007	0.008

liquid compositions, extraction times and extraction intensities in Table I, which also gives the results. For adequate sensitivity of detection and analytical reproducibility, it is essential that extraction recovery of the analyte is at least 50%. Desipramine and nortriptyline have recoveries higher than 50% for all extraction liquid compositions using all process variables. The other amines, trimipramine, amitriptyline and imipramine, show relatively low recoveries. These recoveries can be improved by selecting another composition and/or another value for the process variables, as can be seen in Table I.

For desipramine, the recovery in some cases exceeds 100%, which is mainly a result of small variations in the HPLC analysis and the evaporation step (1.5–4%). However, the variation of the recovery of one analyte relative to another is smaller, as variations in HPLC analysis and evaporation affect all compounds similarly.

Polynomial regression was performed on the data in Table I using eqn. 6. Regression models and descriptive capacities of the combined regression models in accordance with eqn. 6 are given in Table II.

Generally, it can be concluded that the recovery of the tricyclic amines is best in methyl *tert.*-butyl ether and worst in chloroform. Hence the recovery increases with decreasing solvent polarity. This is unexpected, as it is often assumed that the more polar the extraction solvent, the higher is the recovery of the analytes in the solvent. It seems appropriate to assume an important role of the selectivity of the extraction liquid in the extraction. Methyl *tert.*-butyl ether is capable of having stronger interactions with the amines than chloroform or methylene chloride, probably because of its proton-accepting properties.

The model presented in eqn. 6 describes well the results obtained according to the experimental design in Fig. 4. Correlation coefficients vary from 0.978 for desipramine to 0.994 for imipramine, values which correspond to explained variance percentages of 95.7–98.8. The standard deviation of the residuals (RSD) after the regression analysis was very small, 0.0053 for trimipramine to 0.0070 for imipramine. The predictive power of the regression models can be judged by two criteria. First, the mean predictive error sum of squares (mPRESS):

$$\text{mPRESS} = \frac{1}{n}\sum_{i=1}^{n}(y_i - \hat{y}_i)^2$$

where y_i is the experimental value for the recovery

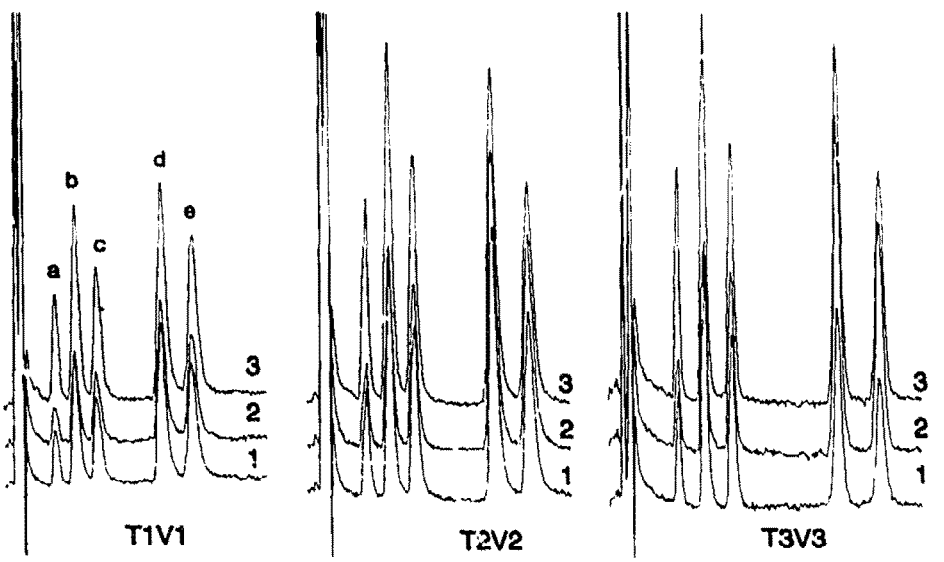
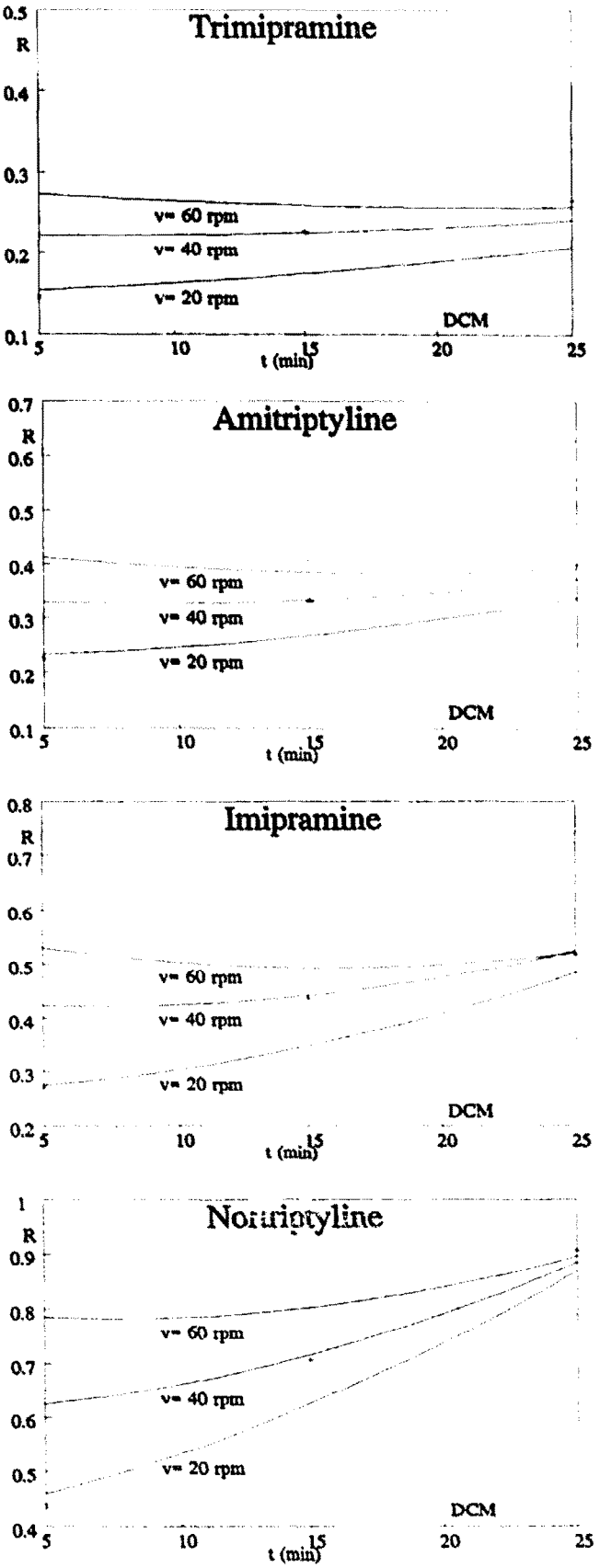


Fig. 6. Chromatograms of the five tricyclic amines after liquid–liquid extraction with pure methylene chloride (1), chloroform (2) and methyl *tert.*-butyl ether (3). Three settings of the process variables were used: T1V1, t = 5 min, v = 20 rpm; T2V2, t = 15 min, v = 40 rpm; T3V3, t = 25 min, v = 60 rpm.



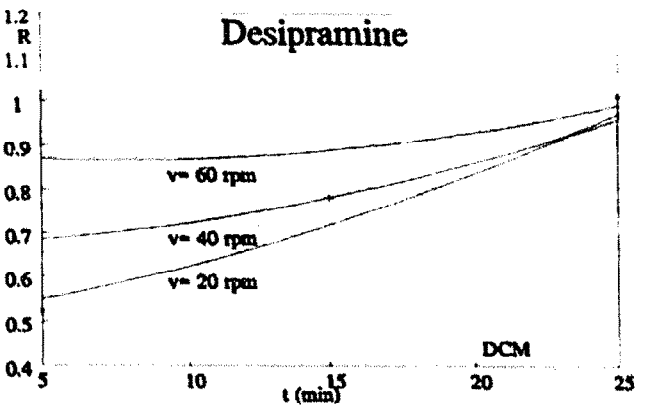


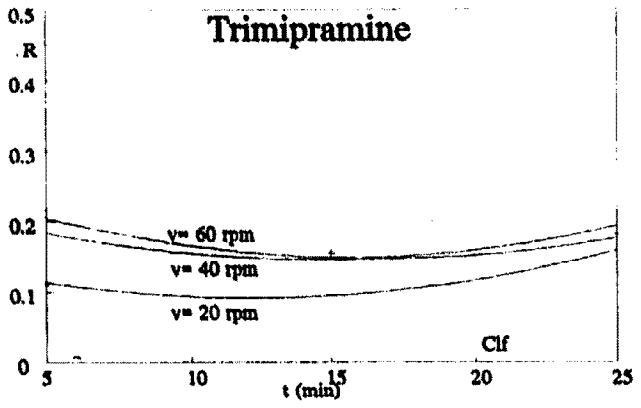
Fig. 7. Recoveries of five tricyclic amines in pure methylene chloride vs. extraction time t for three extraction intensities (20, 40 and 60 rpm).

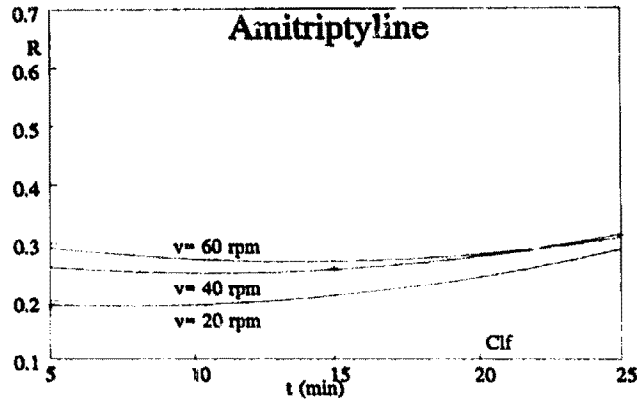
and $\hat{y}_i$ is the value predicted by the regression model, in which experiment i was not incorporated. The mPRESS value is very good for trimipramine, amitriptyline and imipramine and acceptable for nortriptyline and desipramine. Second, the standard error of prediction (SEP) was calculated:

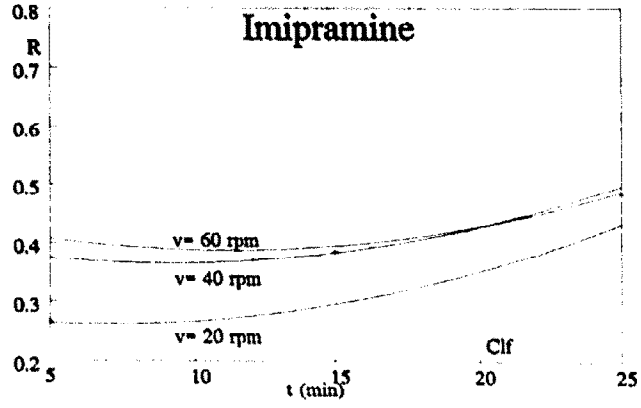
$$\mathrm{SEP} = \sqrt{\frac{\sum_{i=1}^{n} (d_i - \bar{d})^2}{n-1}}$$

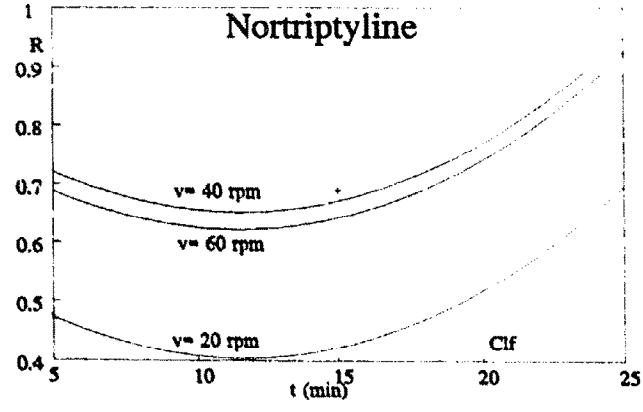
where d_i is the predicted error calculated from y_i (the experimental value) and $\hat{y}_i$ (the value predicted by the regression model, in which experiment i was not incorporated), $d_i = y_i - \hat{y}_i$; $\bar{d}$ is the mean of d_i. The SEP value shows the same characteristics for the amines as the mPRESS criterion, *i.e.*, good for trimipramine, amitriptyline and imipramine and acceptable for nortriptyline and desipramine. However, the adjusted value for PRESS and SEP, which are the values calculated without the use of extrapolated design points in the cross-validation, are significantly better for nortriptyline and desipramine.

Fig. 6 shows nine chromatograms of the five amines using three different extraction liquids [(1) DCM, (2) Clf and (3) tBME] with three different settings of the process variables (T1V1, $t = 5$ min, $v = 20$ rpm; T2V2, $t = 15$ min, $v = 40$ rpm; T3V3, $t = 25$ min, $v = 60$ rpm). It can be seen from these chromatograms that the peak heights of the amines increase considerably from T1V1 to T2V2 owing to an increase in the recovery and that there is also a small increase from T2V2 to T3V3. From these









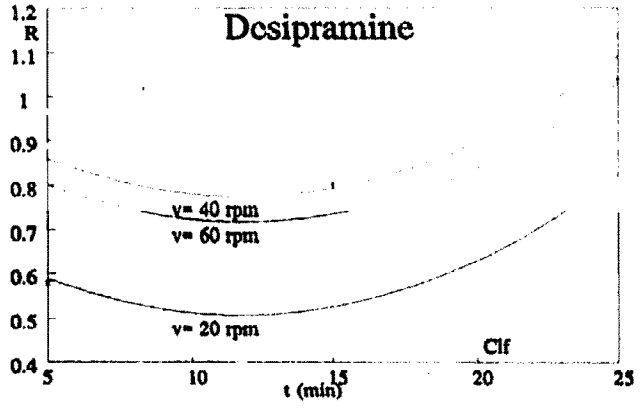
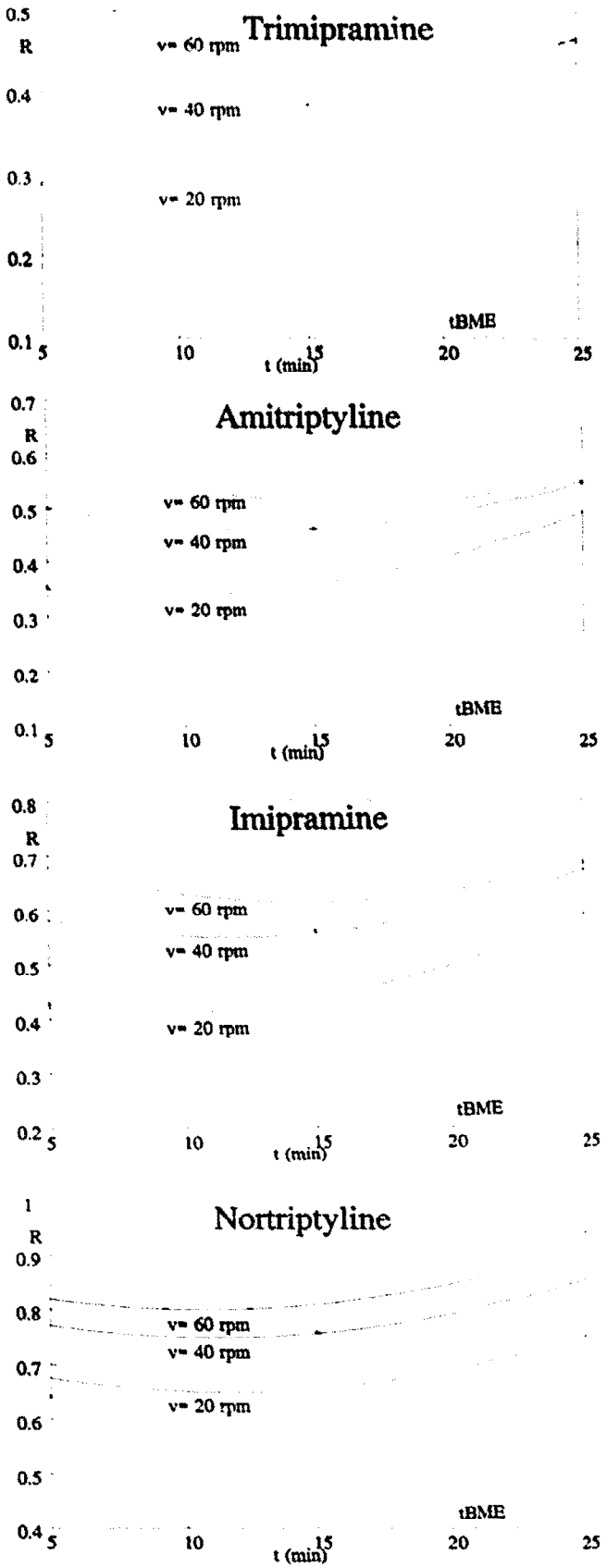


Fig. 8. Recoveries of five tricyclic amines in pure chloroform *vs.* extraction time *t* for three extraction intensities (20, 40 and 60 rpm).

chromatograms the selectivity of the different extraction liquids used can also be seen: the ratio of the peak heights of each combination of two peaks varies with the extraction liquid used.

The plots of the extraction efficiency *versus* the extraction time of the extraction system in Figs. 7, 8 and 9 (DCM, Clf and tBME, respectively) demonstrate an obvious effect of both extraction intensity and extraction time. The importance of the extraction time decreases if the extraction intensity increases. This is especially demonstrated by the recovery of nortriptyline in methylene chloride (Fig. 7), which increases from less than 60% at 20 rpm to almost 90% at 60 rpm using an extraction time of 5 min. The latter is almost the same as the recovery at 20 rpm for 25 min. This is important information for extraction performed by laboratory robotic systems. Such systems often are programmed for scheduled sample preparation (*i.e.*, processing samples sequentially; within the procedure several samples may be processed in different modules at the same time [29–31]). A significant decrease in the duration of the often time-consuming extraction phase in sample preparation may dramatically decrease the total duration of the analysis of a series of samples or may dramatically increase sample throughput (productivity) in the laboratory.

The initial decrease in recovery as a function of extraction time is not significant. This is a result of small variations in recovery due to HPLC variations and variations in evaporation. Fig. 10 shows the contour plots of the recovery of nortriptyline for



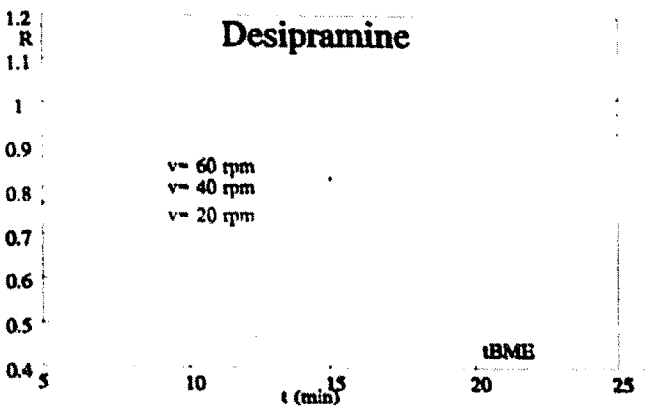
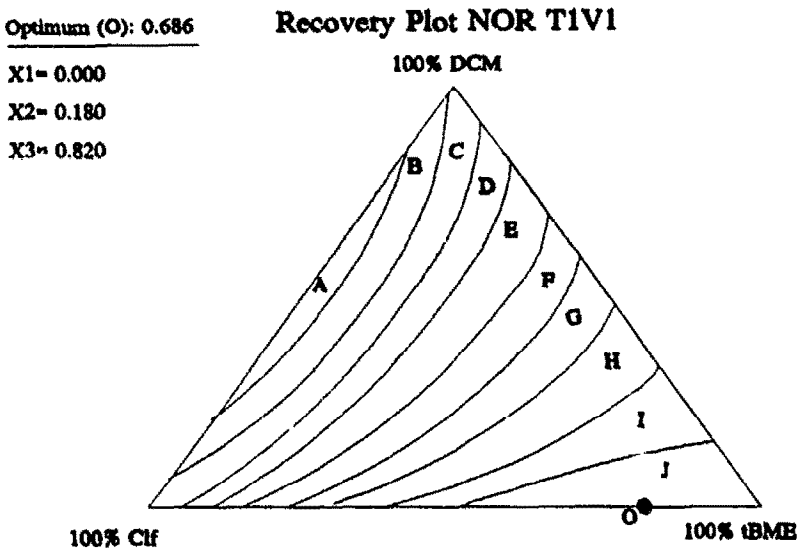
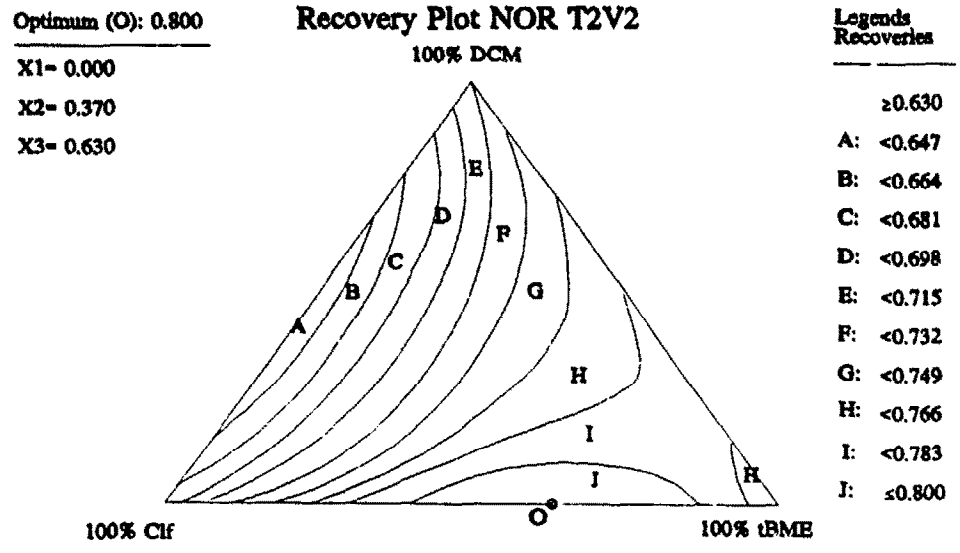


Fig. 9. Recoveries of five tricyclic amines in pure methyl *tert.*-butyl ether vs. extraction time *t* for three extraction intensities *v* (20, 40 and 60 rpm).

three different settings of the process variables. It demonstrates a change in the extraction behaviour with respect to the composition of the extraction liquid when the settings of the process variables are altered: the optimum moves from 18% Clf in tBME (T1V1) to 51% Clf in tBME (T3V3). The conclusion for nortriptyline from Fig. 10 may be the following: the optimum selectivity of the extraction liquid within the experimental design applied here may be reached by mixing chloroform and methyl *tert.*-butyl ether. However, the partitioning equilibrium may adjust more slowly with such a mixture as compared with a extraction liquid consisting of pure methyl *tert.*-butyl ether. Methyl *tert.*-butyl ether has a lower partition coefficient but the equilibrium is reached faster.

Figs. 11 and 12 show the ratio of the recovery of trimipramine to those of amitriptyline, imipramine, nortriptyline and desipramine after extraction with methylene chloride, chloroform and methyl *tert.*-butyl ether with varying extraction time (Fig. 11) and extraction intensity (Fig. 12). It can be seen that the extraction time and the extraction intensity can be optimized in a proper extraction liquid composition with respect to the ratio of the recoveries. The ratios can be optimized towards unity or the robustness of the extraction conditions can be optimized to the best values. For the extraction of compounds it is important to have high recoveries; the higher the recovery, the better is the precision. In analyses where two or more solutes have to be extracted simultaneously (*e.g.*, compounds with an internal





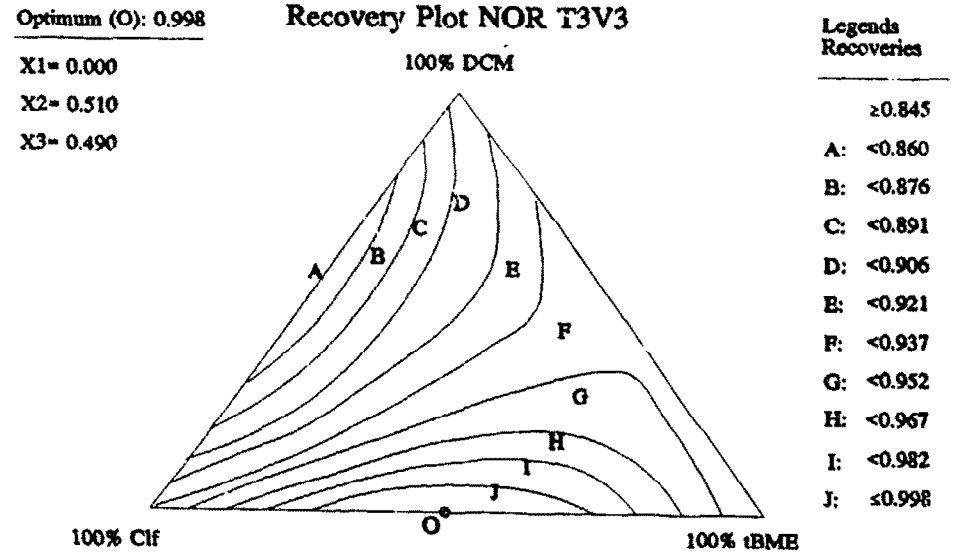
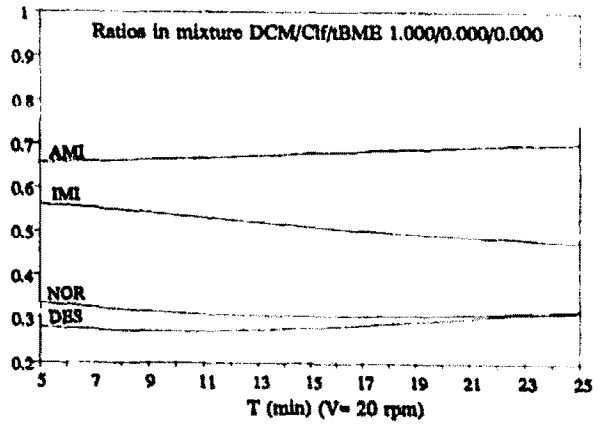
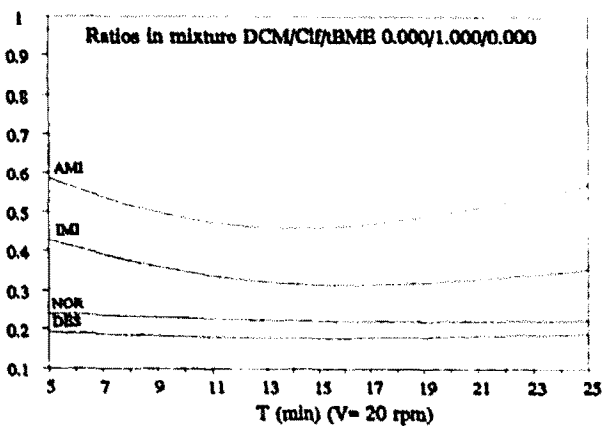


Fig. 10. Contour plots of the recovery of nortriptyline made with three settings of the process variables: T1V1, $t = 5$ min, $v = 20$ rpm; T2V2, $t = 15$ min, $v = 40$ rpm; T3V3, $t = 25$ min, $v = 60$ rpm.





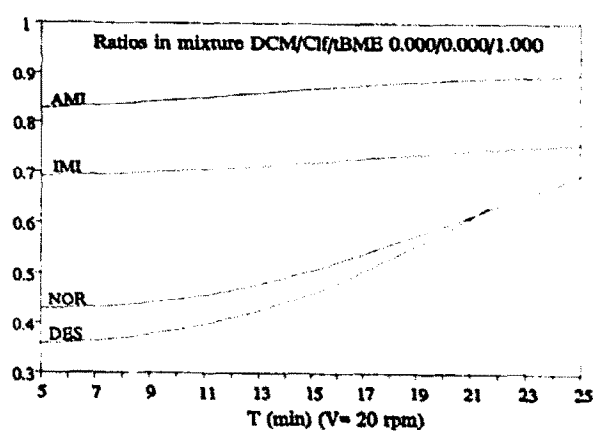
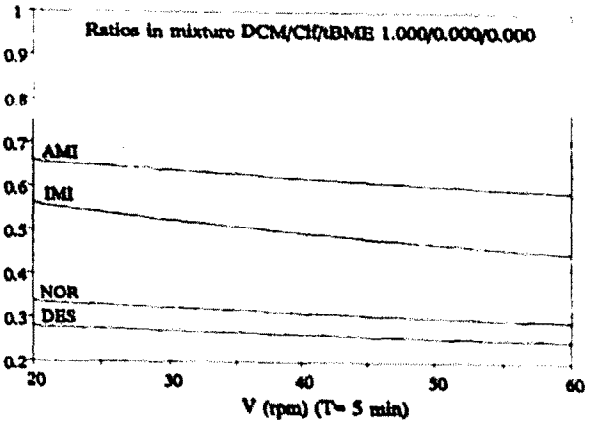
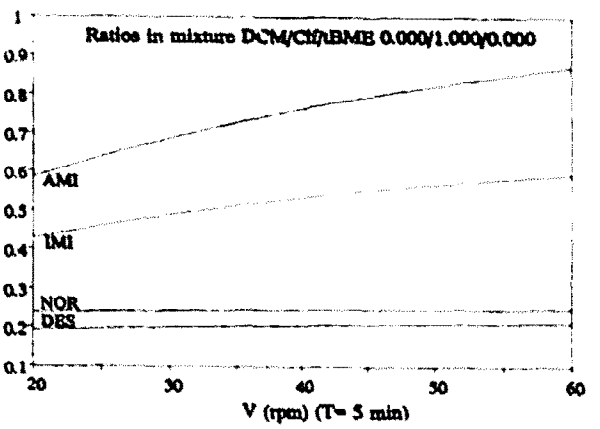


Fig. 11. Ratios of the recovery of trimipramine to the recoveries of amipramine, imipramine, nortriptyline and desipramine after extraction with methylene chloride (DCM), chloroform (Clf) and methyl *tert.*-butyl ether (tBME) *versus* the extraction time *T*.





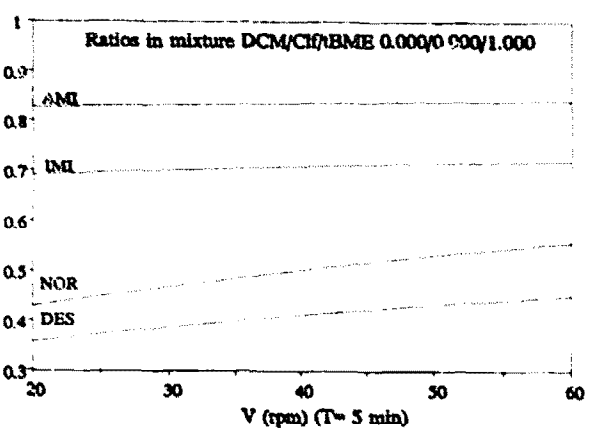


Fig. 12. Ratios of the recovery of trimipramine to the recoveries of amipramine, imipramine, nortriptyline and desipramine after extraction with methylene chloride (DCM), chloroform (Clf) and methyl *tert.*-butyl ether (tBME) *versus* the extraction intensity *V*.

standard), it is also important to have an equal affinity of the extraction liquid for all compounds to be extracted ($R_i/R_j = 1$) and robust extraction ratios: small variations in the extraction conditions (extraction liquid composition, extraction time, extraction intensity) should therefore not affect the

ratio of the recoveries of two compounds. Conditions should be selected where the variation in the ratio, due to variation in the conditions, is small, *i.e.*, the ratio is robust. The robustness criterion can be used to select values of the extraction intensity and the extraction time where the conditions are robust to small variances. This robustness is very important in routine analysis [48].

A typical case is the extraction of trimipramine and desipramine in tBME with varying extraction time (Fig. 11): the extraction ratio of these analytes is optimum (0.75) using an extraction time of 25 min. However, using this extraction time, the robustness of these conditions for the ratio is not very good. The best extraction time for this pair of analytes with respect to the robustness of the extraction conditions for the ratio is 5 min. However, using this extraction time, the ratio is worse (0.28).

Generally, it can be seen from the results in this paper that interactions between extraction liquid composition and process variables do not equally affect the recovery of all amines; small differences can be seen. Therefore, even when the differences between compounds to be extracted are very small, as in this instance, a significant difference in extraction behaviour can be seen. This observation may lead to new extraction experiments where compounds are used that have larger differences. Then the differences in extraction behaviour may be even more dramatic.

CONCLUSIONS

Mixtures of preselected organic solvents result in higher recoveries of the tricyclic amines than pure solvents. The application of a factorial design incorporated in a mixture experimental design in the optimization liquid–liquid extraction of drugs from biological matrices gives good results for the extraction of a number of tricyclic amines from plasma. Optimization of recoveries is reasonable by mixing three solvents with different selective interactions. The introduction of two process variables and the simultaneous variation of these variables with the mixture variables makes possible the simultaneous modelling of combined experimental designs. A preselected limited number of design points permits the use of fractional designs for the optimization of liquid–liquid extraction of drugs from biological matrices; the applicability of combined fractional experimental designs for the simultaneous optimization of extraction liquid composition, extraction time and extraction intensity has been demonstrated. For a group of tricyclic amines there is interaction between the two process variables (extraction intensity and extraction time): a higher extraction intensity justifies a shorter extraction time. For future research, using optimization of liquid–liquid extraction of drug with a laboratory robot and subsequent use of the optimized extraction liquid composition and process variable adjustment under routine conditions may result in a distinct decrease in the time needed for an analytical run.

An interaction exists between the composition of the extraction liquid and the process variables; the extraction behaviour changes when these variables are varied simultaneously. Structurally related compounds demonstrate different extraction behaviours in a ternary liquid–liquid extraction system composed of methylene chloride, chloroform and methyl *tert.*-butyl ether. In investigations for finding proper internal standards, one should take into account the extraction liquid used for extraction of both analyte and internal standard.

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