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Optimization of solid-phase extraction for a liquid chromatographic–tandem mass spectrometric general unknown screening procedure by means of computational techniques

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Abstract

A solid-phase extraction (SPE) method was optimized to suit the particular demands of an information-dependent acquisition LC–MS–MS procedure for general unknown screening in a forensic toxicology setting. In a first phase, a Plackett–Burman screening design with fold-over was carried out to distinguish the significant factors affecting the extraction procedure. This part eventuated in the determination of only three statistically relevant parameters, requiring consecutive optimization. To that end, in phase II of this study, a rotatable central composite design was applied to define the response surface as a function of the significant parameters and to choose the optimal conditions for the SPE.

Keywords: General unknown screening; Screening; Experimental design; Solid-phase extraction; Information-dependent acquisition

1. Introduction

Statistical experimental design, also known as design of experiments (DoE) is the methodology of how to conduct and plan experiments in order to extract the maximum amount of information in the fewest number of analyses. The application of mathematical, statistical and logical principles to chemistry, i.e. chemometrics, offers a sound alternative for optimization of chemical systems and processes; it is applied to determine in an efficient way the set of conditions that are required to obtain a product or process with desirable, often optimal characteristics [1]. It provides information about how factors interact in a way that one-factor-at-a-time (OFAT) cannot determine. OFAT's major drawback is that it holds all factors constant while testing only one-at-a-time [2]. Chemometrical applications in analytical chemistry are without any doubt becoming more widespread every day [3–5].

In the field of forensic toxicology, DoE could be of use for the development of a solid-phase extraction (SPE) for general unknown screening (GUS) procedures, since the extraction cannot be directed to a given substance. Especially when using information-dependent acquisition (IDA), the SPE procedure has to be a general procedure where a compromise must be reached. The substances of interest, unknown beforehand in number or composition, are all to be isolated at a yield as high as possible while those interfering substances from the biological matrix are removed. After all, IDA is a technique based on the automatic "on-the-fly" MS to MS–MS switching abilities of, in our case, a quadrupole time-of-flight (Q-TOF) system [6]. Precursor ions, observed in a MS survey scan, are automatically selected for interrogation by MS–MS, once a predefined intensity threshold is exceeded. A major criterion which governs the applicability of IDA in systematic toxicological analysis (STA) is the lack of interferents which initiate and thus temporarily "occupy" the MS–MS channels, effectively blinding the method to the compounds of real toxicological interest [6]. Consequently, the applicability of such an IDA method largely depends on the quality of the extraction procedure for a biological sample. Due to the large number

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of factors that could exert a significant influence on such a SPE procedure and the three types of response variables that have to be considered, namely the overall extraction yield (EY), and both the number of relevant compounds effectively retrieved and the total number of ions detected by IDA, it would be impossible to perform an optimization study in the OFAT way. Moreover, such an experimental study would not take two-or three way interactions between factors into account. A chemometrical optimization approach clearly is the method of choice, and here we report the results of such a DoE based SPE optimization within the framework of MS based IDA in forensic toxicology.

Based on the results of an earlier performed study on the suitability testing of SPE sorbents for the sample clean-up in STA, an apolar C_8 SPE column, shown to perform best for this purpose in terms of extraction yield and clean-up potential, was used for the entire experiment [7]. Furthermore, 17 diverse basic and neutral (i.e. benzodiazepines and methaqualone) compounds were chosen in order to represent a wide variety of compound classes as well as a broad spectrum of physicochemical characteristics (pK_a, molecular mass and functional group characteristics). Blood was preferred as biological matrix, because of its relevant toxicological characteristics. The first step in our experimental design was to screen the candidate factors within a defined experimental domain, i.e. the extreme levels at which the factors will be studied. A correct definition of the extreme experimental boundaries is critically important. At the same time a selection of the responses to be investigated has to be made. To determine whether the candidate factors have a significant effect, a Plackett–Burman design with fold-over was carried out. The second phase of this study then consisted of further optimization of the relevant factors using a multi-level design. A rotatable central composite design (CCD) was applied to define the response surface as a function of the significant parameters and to choose the optimal conditions for the SPE. By interpretation of the several response surfaces, the optimum value of three significant parameters was defined.

2. Experimental

2.1. Chemicals

The compounds studied were morphine, benzoylecgonine, XTC, codeine, strychnine, ethylmorphine, nalorphine, cocaine, lidocaine, bromazepam, methaqualone, diazepam, triazolam, methadone, trazodone, haloperidol, oxazepam and butorphanol (internal standard). This representative test set was made from drug standards from different sources, available from our laboratories collection. Methanol and acetonitrile were all of HPLC grade (Biosolve, Valkenswaard, The Netherlands). Acetic acid (purity minimum 99.7%) and ammonium acetate (purity minimum 98%) were supplied by Sigma–Aldrich (Steinheim, Germany), while ammonia solution 25% was purchased from Merck-Eurolab (Leuven, Belgium). The apolar C_8 columns (sorbent mass 100 mg, 1 mL) were provided by International Sorbent Technology (IST, Hengoed, UK).

2.2. Biological optimization samples

A mixture of the drug standards was prepared in acetonitrile in a concentration of 4 μg/mL of each compound, except for bromazepam and oxazepam, which were present in a concentration of 12 μg/mL. The concentration of butorphanol was 1 μg/mL. Prior to the SPE clean-up procedure, the whole blood was pretreated as follows. After fortifying 1 mL of the blood sample with 50 μL of the standards mix, it was mixed on a vortex-mixer for 30 s, equilibrated and ultrasonicated for 15 min. The blood sample was then diluted with 1 mL ammonium acetate buffer, whereupon a fixed period of mixing, ultrasonicating and centrifugation (3000 rpm) followed. The resulting supernatant was then applied on the SPE extraction columns.

2.3. Instrumentation

Reproducible, automated SPE was performed on a Zymark RapidTrace Solid Phase Extraction Workstation (Zymark, Hopkington, MA, USA) equipped with one single extraction module. The IDA experiments were performed on a Waters Alliance 2790 separation module integrated with a Q-TOF instrument (Waters, Manchester, UK).

2.4. Statistical software

The generation of the Plackett–Burman design and the central composite design was performed using the statistical software package Design Expert 6.0 (Stat-Ease, Minneapolis, MN, USA). This software was used because of its straightforward capability to design a multivariable experimental protocol.

2.5. Chromatographic conditions

Chromatography was conducted on a Xterra MS C_{18} column (3.5 μm particle size, 100 × 2.1 mm; Waters, Milford, MA, USA). The flow rate was set to 0.3 mL/min. Gradient elution was performed, starting at 100% of a mixture of water–methanol–acetonitrile (80:10:10, v/v) containing 5 mM ammonium acetate (solvent A), programmed linearly, within 7 min, to 50% of a mixture of water–methanol–acetonitrile (20:40:40, v/v), again containing 5 mM ammonium acetate (solvent B), holding for 7 min. In order to remove late eluting substances, a step gradient to 100% solvent B was included for 1.5 min. Subsequently, the system was programmed to regain its initial conditions over 0.5 min, followed by 8 min re-equilibration prior to the next injection. The injection volume was 25 μL and the entire column effluent was directed into the mass spectrometer.

Table 1
Overview of the experimental factors and their corresponding boundaries

		Symbol	Low	High	Unit
1	Flow first washing step	*A*	0.5	5	mL/min
2	Volume first washing step	*B*	1	6	mL
3	Molarity of the buffer (first washing step)	*C*	10	200	mM
4	Percentage MeOH in first washing step	*F*	10	60	%
5	Flow second washing step (hexane)	*E*	0.5	5	mL/min
6	Volume second washing step (hexane)	*H*	0	3	mL
7	Flow water washing step	*D*	0.5	5	mL/min
8	Volume water washing step	*G*	1	6	mL
9	Drying time	*K*	0	6	min
10	Flow elution step	*J*	0.5	5	mL/min
11	Volume elution step	*L*	1	6	mL

2.6. Mass spectrometric analysis and IDA

Detection of the compounds was performed in ESI+ mode using IDA, generating a survey scan, single MS spectra with molecular mass information, product-ion spectra and extracted ion fragmentograms. The latter are used to evaluate extraction yield when compared to the trace obtained for a supplemented blank extract. Ref. [6] details the whole IDA procedure and corresponding experimental settings.

2.7. SPE procedure

Experimental design was used to optimize the following generic SPE procedure. One milliliter ammonium acetate buffer (pH 9.0) was added to the blood samples (1 mL). Before application of the samples (2 mL), the SPE columns were conditioned with 3 mL methanol and 3 mL ammonium acetate buffer (pH 9.0). The columns were washed with ammonium acetate buffer containing methanol, hexane and water and were subsequently dried. Elution was performed with methanol–acetic acid (99:1, v/v). The internal standard was added and the eluate was evaporated to dryness under a gentle stream of nitrogen, and the dry residue was redissolved in 200 μL of solvent A, 25 μL of which was injected into the LC–MS system. In this generic procedure, we identified in total 11 factors which were believed to possibly affect the extraction process.

2.8. Experimental design and evaluation

2.8.1. Screening design: Plackett–Burman design

2.8.1.1. Determination of the factors and the experimental domain. For screening purposes, one has to choose between a full factorial design and a (reduced) fractional factorial design. As in our case, a screening design for 11 factors was needed, we opted for a Plackett–Burman design. A full factorial design would be impracticable due to the large amount of experiments to be carried out (e.g. 2048 or 2^{11} for 11 factors). Via fold-over, the resolution of the design was enhanced from III to IV to eliminate confounding between main effects and two-factor interactions; in this way pure estimation of the main effects, clear from any interaction is possible [8–10]. In total, 24 experiments have to be performed, automatically randomized by the DoE software to protect against lurking factors such as temperature, humidity or the like. The 11 factors, that possibly affect the extraction procedure and that will in the following be referred to as factors *A*–*L*, are shown in Table 1. The extraction pH, the column conditioning settings and the elution mix composition were optimized in an earlier stage [7] and thus not included in the screening design. The experiments were performed at two levels for each of the investigated factors, coded as "−1" (low) and "+1" (high), the so called boundaries of the experimental domain.

2.8.1.2. Determination of the responses. The biological samples were extracted according to the different variations to the generic method as directed by the design. After analysis, the response variables were calculated. In order to evaluate the influence of the factors, three types of response variables were evaluated, namely the overall extraction yield (response 1, R 1), and both the number of compounds effectively retrieved (response 2, R 2) and the total number of ions detected by IDA (response 3, R 3). Because the analysis is a multicompound screening analysis, optimum extraction yield (EY) is always a compromise. It is better to have moderate EYs for many compounds than to have 100% EY for some and almost nothing for other compounds. To evaluate the overall EY (i.e. 17 different compounds as a whole), a transformation function *F* was used to transform the EY of each compound to a new value; calculation of the geometric mean of these new values resulted then in the first response value, referred to as R 1 [7]. The transformation function *F* has the following characteristics: for EY's below 50% [$y = (1/2)w(2x-1)+(1/2)\sqrt{1+(w^2-1)(2x-1)^2}$, x is EY and w is the weighing factor = 15] and above 100% [$y = 1+w(x-(1/2))-(1/2)\sqrt{1+4(w^2-1)(x-(1/2))^2}$, $w = 5$] a more than linear penalty is assigned, while for EY's between 50% and 100% [$y = 1 + w((3/2) - x) - (1/2)\sqrt{1+4(w^2-1)((3/2)-x)^2}$, $w = 5$] a more than linear reward is assigned.

Table 2
Overview of the experimental factors at their corresponding investigated levels

		Symbol	$-\alpha$	-1	0	$+1$	$+\alpha$	Unit
1	Volume first washing step	*A*	1	2	3.5	5	6	mL
2	Percent MeOH in first washing step	*B*	10	20	35	50	200	%
3	Molarity of the buffer	*C*	10	48	105	162	60	mM

2.8.2. Optimization design: central composite design

2.8.2.1. Determination of the factors and the experimental domain. The screening data revealed three factors significantly influencing the SPE procedure. Namely, the volume of the first washing step (referred to as factor *B* in the screening design and now factor *A* in the optimization design), the percentage of methanol in the first washing step (now factor *B*, previously referred to as factor *F*) and the molarity of the used buffer (factor *C*). To define the optimum settings of these factor levels, i.e. the combination of factor values yielding the best results for the three responses, a multi-level design is needed. To solve the problem of economy, i.e. the amount of experiments to be performed, an orthogonal, circumscribed, rotatable central composite response surface design was applied. These designs are typically used for quantitative factors and designed to estimate the main effects, plus the quadratics and two-way interactions. A central composite design can be divided in three parts: a two-level factorial design (cube points with levels of "-1" and "$+1$"), a star design (star or axial points with levels of "$-\alpha$" and "$+\alpha$") and a centre point that is replicated several times (all levels equal 0). For a three factor CCD, eight cube points, six star points and at least one centre point are required. Each factor is encountered at five levels ($-\alpha$, -1, 0, $+1$, $+\alpha$). The investigated factors and their numerical values are shown in Table 2. To achieve a rotatable and orthogonal design, α has to be set to 1.68 and 9 replicates are needed for the centre point. A design is called orthogonal, when the variance of the prediction does not depend on the direction in which one looks starting from the centre point, but only on the distance from the centre point. Replication of the cube and star points, together with nine replicates of the centre points eventuated in a total of 37 experimental runs.

2.8.2.2. Determination of the responses. By analogy with the screening design, the extraction efficiency was evaluated using three response variables (see above).

3. Results and discussion

3.1. Plackett–Burman design

Per response variable, a half-normal probability plot is created, depicting the absolute value of all effects. To detect the significant variables, Lenth's individual contrast method (ME) was applied. Lenth proposed a method that has good power to detect significant effects [11]. It provides an excellent quantitative augmentation to the graphical normal probability plot analysis employed in the analysis of screening experiments [12]. An example of such a plot is given in Fig. 1. Complimentary to the above graphical data interpretation, the evaluation of the numerical interpretation of the data set was performed, i.e. all estimable effects for the coded levels of the factors, together with the sum of squares and the contribution of each factor, in terms of percentage. Subsequently, analysis of variance (ANOVA) analysis was performed to determine those relevant factors affecting each dependent variable of interest. ANOVA is based upon a model which can give some idea of the changes to be expected in the different responses when factor effects become more important. ANOVA was performed on each response separately. Estimating the effects of the 11 extraction parameters and their standard errors enables the parameter effects in the response variables to be distinguished as being significant or not [13]. For all three models, probability values of less than 0.05 are obtained, implying these models are significant, and none of them have lack of fit ($P > 0.05$). Analogously, the several model terms can be checked for their significance. The interpretation of the associated *F*-test indicated that the factors *B*, *C*, *F*, *H* for R 1, the factors *A*, *B*, *C*, *F*, *H* for R 2 and the factors *B* and *F* for R 3 are significant ($P < 0.05$). Validation of the three presented models

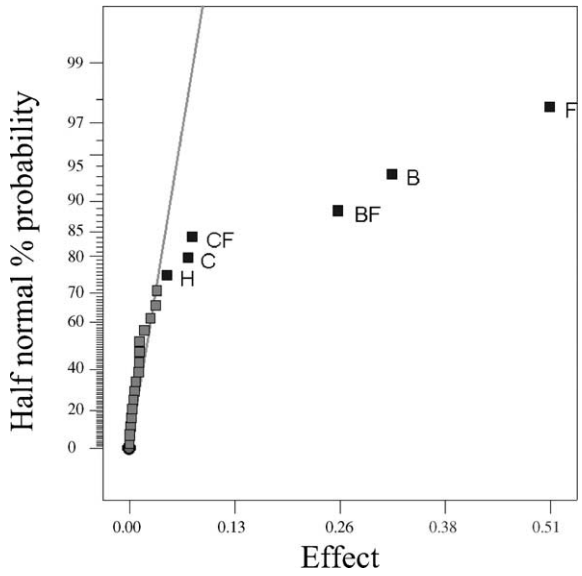

Fig. 1. Half-normal probability plot for R 1 with all major effects selected by Lenth's method.

Table 3
Overview of the results of the screening design

		Symbol	Desired outcome		
			R 1 ↑	R 2 ↑	R 3 ↓
1	Flow first washing step	*A*		+1	
2	Volume first washing step	*B*	−1	−1	+1
3	Molarity of the buffer (first washing step)	*C*	−1	−1	
4	Percentage MeOH in first washing step	*F*	−1	−1	+1
5	Flow second washing step (hexane)	*E*			
6	Volume second washing step (hexane)	*H*	−1	−1	
7	Flow water washing step	*D*			
8	Volume water washing step	*G*			
9	Flow elution step	*J*			
10	Volume elution step	*L*			
11	Drying time	*K*			

Significantly relevant factors are marked with "+1" if the high boundary value yields the best result, or with "−1" if the low boundary value yields the best result.

was performed, but data are not given, since the models obtained in the optimization design are much more relevant. The overall results of our screening investigation are summarized in Table 3: only statistically significant parameters are marked with "+1" if the high boundary yielded the best result, with "−1" if the low boundary yielded the best result. Examination of this table revealed the insignificance of factors *D*, *E*, *G*, *J*, *K*, and *L*, which are in no way involved in the overall extraction efficiency, and, thus, can be chosen arbitrarily. They were chosen in such a way that the obtained extraction procedure was as short as possible. Therefore, the factors *D*, *G*, *J*, and *L* were, respectively, set to 5 mL/min, 1 mL, 5 mL/min, and 1 mL. The low boundary level of factor *K*, i.e. the drying time, was originally set to 0 min, and as such drying of the sorbents was excluded from the SPE procedure. Whenever the results were conflicting (factors *B* and *F*) the factors were subject to further optimization in the next stage of our experimental design study. In case of the other three factors (factors *A*, *C*, and *H*), where no conflicting outcome was observed, they can, in principle, be set at their best-results-value and discarded for the remainder of the optimization procedure. However, from the overall results it is clear that the first washing step is mostly significant in the overall extraction procedure. In that respect, we chose to include the molarity of the buffer (i.e. factor *C*), as an extra factor, in the optimization design, despite the fact it theoretically could be fixed on the best setting. The flow of the first washing step (factor *A*) at the contrary was not withheld for further optimization, it was fixed on the best condition, as the overall picture equally shows in every step of the procedure flow is of limited significance, i.e. the flow of the second washing step, as well as of the water washing step, and of the elution step are not significant, while the flow of the first washing step is only for the response significant. As the best-results-value of factor *H* (volume second washing step) is 0 mL, this step was eliminated in the extraction method, together with factor *E* (flow second washing step).

3.2. Optimization design: central composite design

Analysis of the data set allows construction of a "sequential model sum of squares" summary table for every response, indicating how terms of increasing complexity contribute to the total model. Examination of the probability ("Probability > *F*") revealed that for R 1, R 2, and R 3 the quadratic models fitted best. The cubic models were aliased, which was not unexpected, since the central composite matrix provided too few unique design points to determine all of the terms in the cubic model. Subsequently, an in-depth statistical study was performed for the three responses, based on best fitting model suggestions. By analogy with the screening design, ANOVA analysis was performed to check the adequacy of the suggested models and identify the significant factors. These results are described in Table 4. For response 1, the factors *A*, *B*, *C*, B^2, *AB*, and *BC* were identified to be significant, while for response 2 the factors *A*, *B*, A^2, B^2, C^2, and *AB* were included in the model, and for response 3 the factors *A*, *B*, A^2, and B^2 were significant. All models passed the "lack-of-fit" tests that compare the residual error to the "pure error" from replicated design points. A probability value of more than 0.05 means that the model can be used as response predictor. Next, the three presented models needed validation (i) to verify that the chosen model adequately describes the relationship between the *x* and *y* variables, or in other words that there is no lack of fit, and (ii) to check the assumptions of normality and constant variance of the residuals. The validation occurred by analysing the residuals using the following constructed plots: a normal probability plot of the studentized residuals, a distribution plot of the studentized residuals versus the predicted values, an outlier T plot and a Box Cox plot. An overview of the above described plots for R 1 is given in Fig. 2. Under ideal conditions, for the normal probability plots of the studentized residuals, i.e. the number of standard deviations of the actual values from their respective predicted values, a straight line is created, indicating no abnormalities. No significant deviation from

Table 4
ANOVA results (optimization design) for the three investigated response variables

	Factor	Sum of squares	DF	Mean square	F value	Probability > F
Analysis of variance for R 1						
Model terms		2.270	6	0.380	301.04	<0.0001
	A	0.200	1	0.200	159.02	<0.0001
	B	1.760	1	1.760	1403.37	<0.0001
	C	0.013	1	0.013	10.51	0.0029
	B^2	0.160	1	0.160	125.50	<0.0001
	AB	0.130	1	0.130	102.67	<0.0001
	BC	6.511E−03	1	6.511E−03	5.18	0.0302
Residual		0.038	30	1.2570E−03		
	Lack-of-fit	5.59E−03	8	6.9820E−04	0.48	0.8586
	Pure error	0.032	22	1.4610E−03		
Cor total		2.31	36			
S.D.		0.035				
Mean		0.68				
R.S.D. (%)		5.25				
Analysis of variance for R 2						
Model terms		49291.32	7	7041.62	155.65	<0.0001
	A	5652.77	1	5652.77	124.95	<0.0001
	B	31867.07	1	31867.07	704.39	<0.0001
	C	69.33	1	69.33	1.53	0.2257
	A^2	1121.85	1	1121.85	24.80	<0.0001
	B^2	7486.85	1	7486.85	165.49	<0.0001
	C^2	222.46	1	222.46	4.92	0.0346
	AB	2730.48	1	2730.48	60.35	<0.0001
Residual		1311.98	29	45.24		
	Lack-of-fit	466.94	7	66.71	1.74	0.1521
	Pure error	845.04	22	38.41		
Cor total		50603.30	36			
S.D.		6.73				
Mean		111.66				
R.S.D. (%)		6.02				
Analysis of variance for R 3						
Model terms		9.39E+07	4	2.35E+07	48.02	< 0.0001
	A	8.96E+06	1	8.96E+06	18.32	0.0002
	B	6.58E+07	1	6.58E+07	134.64	< 0.0001
	A^2	5.45E+06	1	5.45E+06	11.14	0.0021
	B^2	1.61E+07	1	1.61E+07	32.81	<0.0001
Residual		1.57E+07	32	4.89E+05		
	Lack-of-fit	5.71E+06	10	5.71E+05	1.26	0.3088
	Pure error	9.94E+06	22	4.52E+05		
Cor total		1.10E+08	36			
S.D.		699.31				
Mean		5221.26				
R.S.D. (%)		13.39				

linearity is observed for all of the three models. For the second plot, the spread of the studentized residuals versus the predicted values is observed. The dispersal should be approximately the same across all levels of the predicted values, in other words the size of the studentized residual should be independent of its predicted value. Analysis of the outlier T plot revealed no constant errors or influential values, i.e. outliers, for R 1, as well as for R 2 and R 3. Finally, the last plot presented, the Box Cox plot, intends to find the raw data transformation that best approaches normality. For the first response, no transformation was needed, meaning normality was obtained as such, while for R 2 and R 3 a power transformation was applied to obtain normality. “Lambda” symbolizes the power applied to the response values; a lambda of 1 indicates no transformation. In all cases, no statistical problems were revealed. Next, a perturbation plot was created to provide silhouette views of the response surface. For an optimization design, this graph shows how the response changes as each factor moves from a chosen reference point, with all other factors held constant at the reference value. A

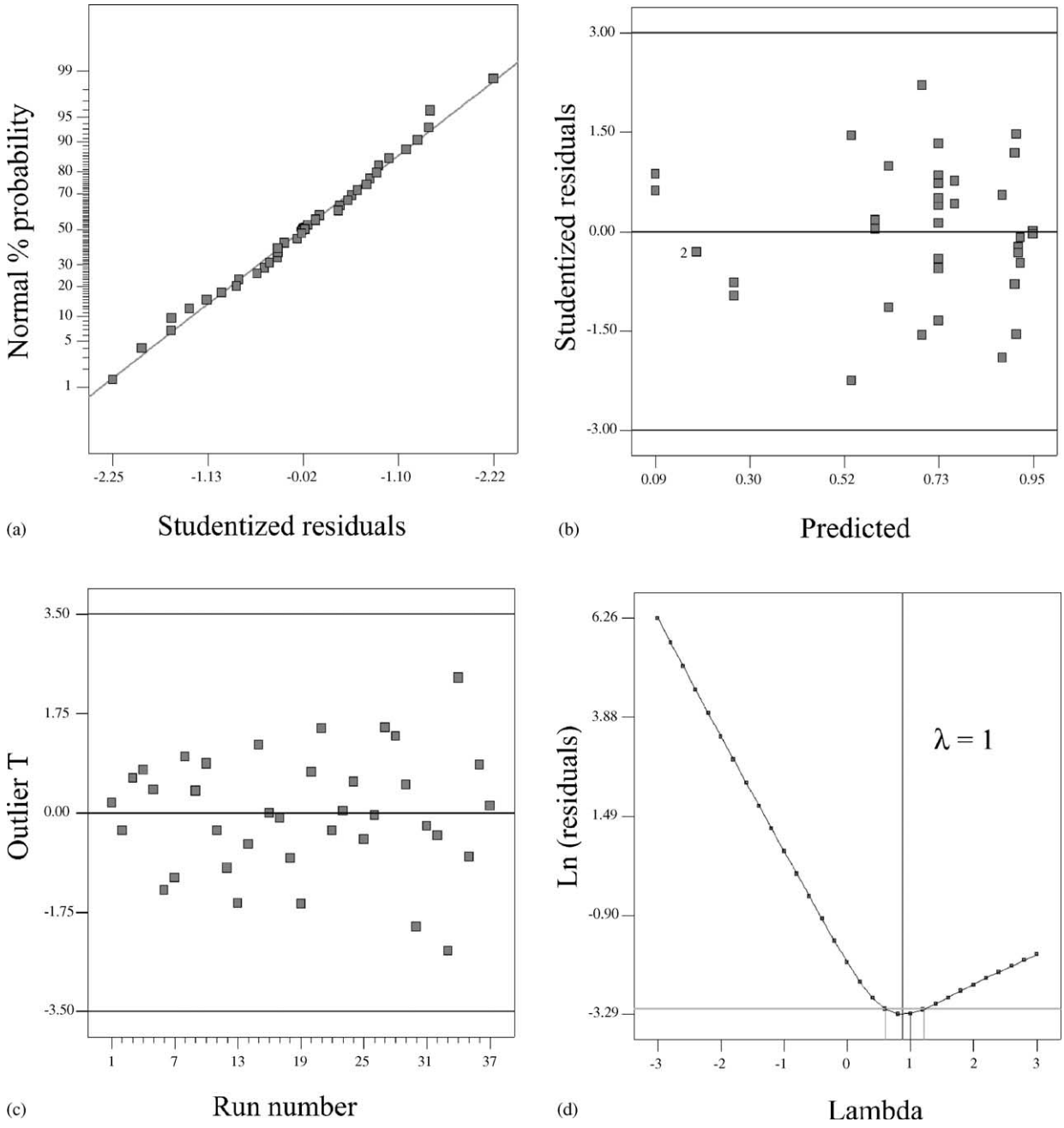

Fig. 2. Normal probability graph of studentized residuals (a), studentized residuals vs. predicted values plot (b), outlier T plot (c) and Box Cox plot for power transformations (d) for the first response variable.

steep slope or curvature in a factor shows that the response is sensitive to that factor. As can be seen for example in Fig. 3, factor *B* mostly affected response 1. Another diagnostic plot that can be constructed as a graphical aid in the interpretation of the obtained data is the response surface, again relating the response to the effect of the factors. Such a plot is depicted in Fig. 4. At a value of 60 mM for factor *C*, the influences of factors *A* and *B* are represented on response 1. All these three-dimensional plots were beneficial to gain an overall understanding of the influence of the parameters on the SPE procedure. Moreover, as in our case only three factors are optimized, the three dimensions provide an interpretable three-dimensional plot which already allows visual determination of the optimum settings for the three factors. Analysis of perturbation plots, three-dimensional plots and the optimization models revealed that factor *C*, i.e. the molarity of the buffer, is of little significance. By visual optimization this factor was set to 60 mM. Since factors *A* and *B* exert a significant influence on all responses, a multi-criteria decision making method was essential and a total desirability function *D* was used to detect the optimum settings of both factors with respect to all responses [14]. The desirability function is

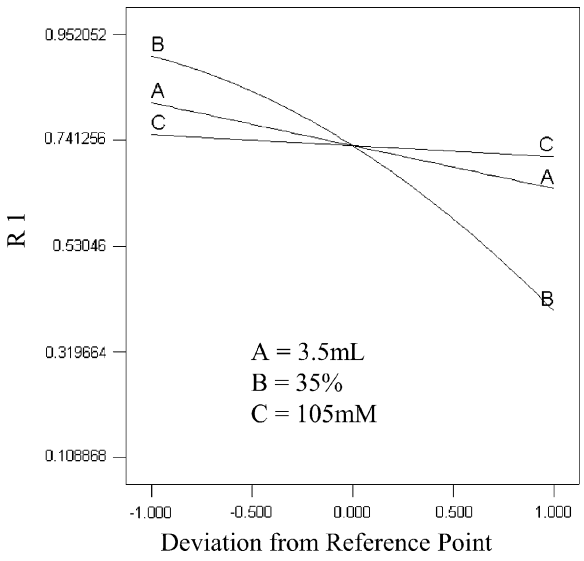

Fig. 3. Perturbation plot for R 1.

a measure of overall quality and provides convenient means to compare several responses and to select the optimum with the most desirable properties; it reflects the desirable ranges for each response (d_i). The measured responses are transformed to a dimensionless desirability scale, that ranges between $d = 0$, for a completely undesired response, to $d = 1$ for a fully desired response. The simultaneous objective function, i.e. the overall quality D, is calculated by combining the desirability values obtained for different criteria by means of the geometric mean [$D = (d_1 \times d_2 \times \cdots \times d_n)^{1/n}$; n is the number of responses]. An algorithm of calculation (iterative) is then applied to the D function in order to determine the set of variable values that maximizes it. The value D is the highest at conditions where combination of the different criteria is globally optimal [15]. The three-dimensional plot of global desirability D, maintaining buffer molarity at 60 mM, is shown in Fig. 5. The D value is maximum for a high volume of the first washing step and percentage methanol of about 15%, while in the other regions of experimental domain the D value decreases. When the first washing volume was set to 5 mL and the percentage methanol to 15%, a D value of 0.89 was obtained. Based on these results, the optimized settings for factors A, B, and C were 5 mL, 15% MeOH and 60 mM. Combined with the conclusions from the screening design and the earlier conducted suitability study [7], the final SPE method as described in Table 5 is obtained. Applying the optimized SPE procedure resulted in an overall better extraction efficiency, as presented in Fig. 6.

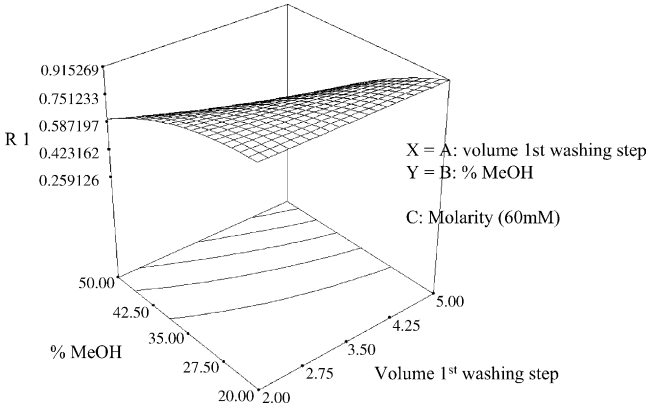

Fig. 4. Three-dimensional response surface plot for response 1 with factor C held constant at 60 mM.

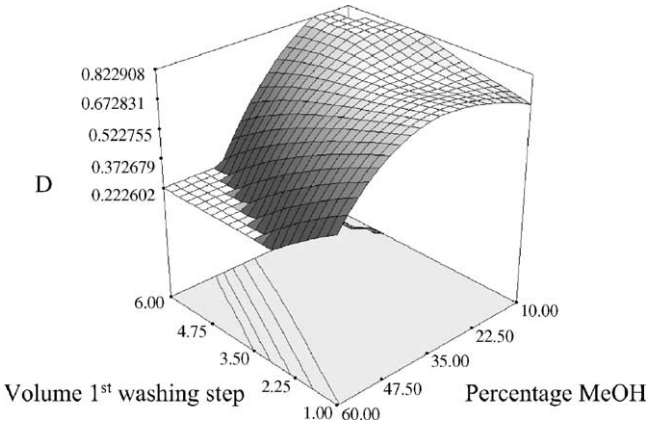

Fig. 5. Graphical representation of the overall desirability function D. Volume of the first washing step is plotted against the percentage methanol in the first washing step maintaining buffer molarity at 60 mM.

Table 5
Overview of the final SPE procedure

		Volume (mL)	Flow (mL/min)
Conditioning	MeOH	3 × 1	1
	AA[a] buffer 60 mM (pH 9.0)	3 × 1	1
Sample loading	Pretreated blood	2	0.5
Washing steps	AA[a] buffer 60 mM (pH 9.0) + 15% MeOH	5	5
	Water	1	5
Elution	MeOH + 1% acetic acid	1	0.5

[a] Ammonium acetate buffer.

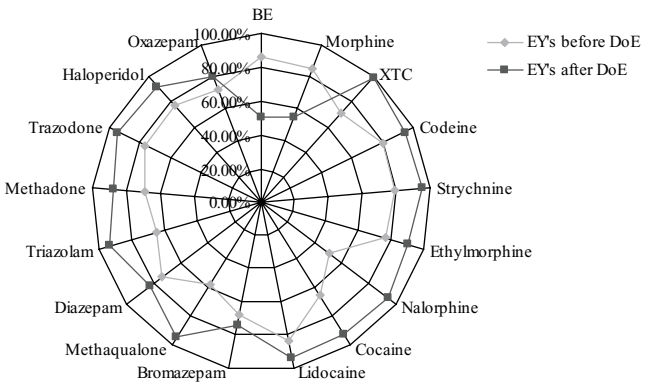

Fig. 6. EYs obtained before and after SPE optimization.

This optimized extraction procedure has now been integrated with our IDA based LC–MS–MS general unknown screening procedure. It is being used for real forensic toxicological blood samples, the results of which advantageously confirm that the procedure perfectly suits its intended purpose.

4. Conclusion

Computational techniques have provided great assistance in gaining an understanding of the dependence of the different parameters in SPE. The results of this study yielded a final SPE procedure, which provided very clean blood extracts while maintaining high analyte recovery. From a total of 11 factors, 3 factors proved to be significant. By using a desirability function optimum conditions for the SPE process were assigned. As such, an optimum SPE procedure was obtained, that fully meets the criteria of a LC–MS based GUS procedure using IDA, an artificial intelligence based product-ion scan mode providing automatic "on-the-fly" MS to MS–MS switching.

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