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A NEW MULTIRESPONSE OPTIMIZATION APPROACH IN COMBINATION WITH A D-OPTIMAL EXPERIMENTAL DESIGN FOR THE DETERMINATION OF BIOGENIC AMINES IN FISH BY HPLC-FLD

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Abstract

A new strategy to approach multiresponse optimization in conjunction to a D-optimal design for simultaneously optimizing a large number of experimental factors is proposed. The procedure is applied to the determination of biogenic amines (histamine, putrescine, cadaverine, tyramine, tryptamine, 2-phenylethylamine, spermine and spermidine) in swordfish by HPLC-FLD after extraction with an acid and subsequent derivatization with dansyl chloride. Firstly, the extraction from a solid matrix and the derivatization of the extract are optimized. Ten experimental factors involved in both stages are studied, seven of them at two levels and the remaining at three levels; the use of a D-optimal design leads to optimize the ten experimental variables, significantly reducing by a factor of 67 the experimental effort needed but guaranteeing the quality of the estimates. A model with 19 coefficients, which includes those corresponding to the main effects and two possible interactions, is fitted to the peak area of each amine. Then, the validated models are used to predict the response (peak area) of the 3456 experiments of the complete factorial design. The variability among peak areas ranges from 13.5 for 2-phenylethylamine to 122.5 for spermine, which shows, to a certain extent, the high and different effect of the pretreatment on the responses. Then the percentiles are calculated from the peak areas of each amine. As the experimental conditions are in conflict, the optimal solution for the multiresponse optimization is chosen from among those which have all the responses greater than a certain percentile for all the amines. The developed procedure reaches decision limits down to 2.5 µg L^{-1} for cadaverine or 497 µg L^{-1} for histamine in solvent and 0.07 mg kg^{-1} and 14.81 mg kg^{-1} in fish (probability of false positive equal to 0.05), respectively.

Keywords: Multiresponse optimization, D-optimal design, biogenic amines, HPLC-FLD, derivatization, fish.

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1. Introduction

Usually, when developing an analytical procedure, several responses have to be simultaneously optimized, and the optimum experimental conditions found for the different analytical responses may be in conflict. In these cases, a decision needs to be made which usually involves choosing a good alternative from several possibilities. In this context, different methods have been established to carry out the simultaneous optimization of multiple responses. For example, applications of the desirability method generalized by Derringer and Suich [1] can be found in multianalyte chemical analysis [2–4] and, more recently, of the Pareto optimal front approach [5,6].

These multiresponse methods are based on fitted mathematical models which relate responses and experimental variables or factors. Although response surface or complete factorial designs would be more suitable designs for estimating the coefficients of these models (usually the variance inflation factors, VIFs, of the coefficients of the models are equal or close to 1 in these cases), the number of experiments required to perform this type of designs may be too large, depending on the number of experimental factors implied. Thus, to use strategies to reduce the experimental effort, such as D-optimal design methodology, is almost mandatory. D-optimal designs [7] make it possible the study of several experimental factors with a reduced number of experiments and enable the adaptation of the design to each analytical problem by independently setting the required levels for each factor as well as the needed interactions. D-optimal designs have already been successfully used for solving this kind of analytical issues [2,8,9].

In this work, a new strategy to approach multiresponse optimization is proposed. The developed method is simpler and more affordable than others in literature since no complex software algorithms are required once the models which relate experimental factors and responses are fitted and suitably validated, and has provided reliable results when applied to the optimization of the determination of biogenic amines (BAs) in swordfish (*Xiphias gladius*) by HPLC-FLD.

BAs are nitrogen compounds of low molecular weight and biological activity coming essentially from decarboxylation of amino acids [10], and may cause toxicological problems

if ingested over relatively high levels [11]. Histamine (HIS), putrescine (PUT), cadaverine (CAD), tyramine (TYR), tryptamine (TRP), 2-phenylethylamine (PHE), spermine (SPM) and spermidine (SPD) are considered to be the most important biogenic amines occurring in foods; they have been found in cheese [12], wine [13], fish [14–17] or meat products [18,19], among others. Their determination is really important in the case of fish and fish products not only from the toxicological point of view but also because BAs are frequently related to the quality of food as a sign of lack of freshness or inadequate hygienic storage conditions or degradation of processed or fermented products [20]. Although many BAs have been found in fish, only histamine has an established legal limit for the human consumption. EU [21] has fixed maximum levels of histamine in fishery products from some particular fish species at 200 mg histamine kg^{-1} (400 mg histamine kg^{-1} in the case of fish sauce produced by fermentation), whereas 50 mg histamine kg^{-1} is the maximum allowable histamine level recommended by the Food and Drug Administration (FDA).

Several methods have been developed to determine BAs in foods [22,23], many of them based on liquid chromatography [24] with various detection techniques, ultraviolet or fluorescent detection being the most frequently used. Due to the lack of chromophores in most of BAs, derivatization is absolutely essential for carrying out their detection in both cases. Furthermore, previous separations are often necessary when complex matrices are analyzed.

In this work, the quantitative determination of the mentioned eight BAs is carried out by HPLC-FLD with a gradient elution program after extraction with an acid aqueous solution and subsequent derivatization with dansyl chloride (Dns-Cl) since these amines do not exhibit native fluorescence. In addition, the study of the matrix effect, avoided in many of the papers found in literature, has been performed. The determination of BAs in this kind of matrices requires stages previous to the analysis (derivatization, extraction, etc.), which usually involve many experimental factors, and interactions among them can be expected, so their optimization usually implies a considerable number of experiments. For that reason, the multiresponse optimization strategy proposed here is performed in conjunction to a D-optimal design for efficiently and simultaneously handling the large number of experimental factors involved, significantly reducing the economic, time and environmental cost of the analysis.

2. Experimental

2.1 Reagents

Hystamine dihydrochloride (CAS no. 56-92-8; 99% minimum purity), putrescine dihydrochloride (CAS no. 333-93-7; 98% minimum purity), spermidine trihydrochloride (CAS no. 334-50-9; 98% minimum purity), Dansyl chloride (CAS no. 605-65-2; 99% minimum purity) were purchased from Sigma (Steinheim, Germany). Tyramine (CAS no. 51-67-2; 99% purity), tryptamine hydrochloride (CAS no. 343-94-2; 99% purity), cadaverine (CAS no. 462-94-2; 95% purity), 2-phenylethylamine (CAS no. 64-04-0; 98% minimum purity), 1,7-diaminoheptane (CAS no. 646-19-5; 98% purity), were supplied by Aldrich. Spermine dehydrate (CAS no. 403982-64-9; 99.5% minimum purity) was obtained from Fluka (Barcelona, Spain).

Acetonitrile (CAS no. 75-05-8; LiChrosolv® isocratic grade for liquid chromatography) was supplied by Merck (Darmstadt, Germany). All other reagents used were of analytical grade. Deionised water was obtained by using the Milli-Q gradient A10 water purification system from Millipore (Bedford, MA, USA).

2.2 Standards and samples

Stock solutions of each biogenic amine and 1,7-diaminoheptane (IS, internal standard) were individually prepared in 0.1 M hydrochloric acid at a concentration around 1000 mg L^{-1}. Standards were prepared in 0.4 M perchloric acid from the stock solutions. All these solutions were stored at low temperature (4 ºC) and protected from light. The Dns-Cl solution was prepared daily in acetone.

Fish samples were purchased from local food stores.

2.3 Instrumental

Analyses were carried out on an Agilent 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) consisted of the quaternary pump VL (G1311C), a standard

autosampler (G1329B), a thermostatted column compartment (G1316A) and a fluorescence detector (G1321B). A Kinetex® C18 100A (150 mm length × 4.6 mm i.d., 5.0 µm particle diameter) analytical column (Phenomenex, Torrance, CA, USA) was used for the separation of the derivatized amines. A Velp Scientifica RX3 Vortex shaker (Milan, Italy), a water bath equipped with a Digiterm 200 immersion thermostat (JP Selecta S.A., Barcelona, Spain), and a Sigma 2-16K refrigerated centrifuge (Osterode, Germany) were used.

2.4 Experimental procedure

Fish samples were homogenized using a commercial blender. 5 g of the sample were transferred to a centrifuge tube and extracted with V_{Extr} mL of *Extractant* with concentration C_{Extr} by vortex mixing for t_{vortex} min, next the supernatant was centrifuged at a rotational speed (s_{centr}) for a time (t_{centr}) at 4ºC and filtered through Whatman No. 1 filter paper. The residue was extracted again in equal conditions and both supernatants were combined and made up to 25 mL with the extraction solution. Then 10 mL of the extract were neutralized with 10 M sodium hydroxide followed by addition of 2 mL of 0.5 M carbonate-hydrogencarbonate buffer (*pH*). 2 mL of Dns-Cl solution (*%Dansyl*, prepared in acetone) were added to 1 mL of the buffered solution and the reaction mixture was left for t_{deriv} min in the darkness at T_{deriv} ºC for derivatization of amines.

The levels of the experimental variables involved in the optimization (in italics in the text above) are in Table 1, and the details of the optimization procedure will be given in the Results and discussion Section.

The procedure after optimization was as follows: each sample was extracted twice with 10 mL of 0.4 M perchloric acid by vortex mixing for 2 min, being the combined extracts centrifuged at 5000 g for 10 min; whereas derivatization was performed on the buffered extracts (pH=10.5) with 0.5% Dns-Cl for 30 min at 40 ºC.

Before the addition of 100 µL of 25% ammonium hydroxide to remove the surplus dansyl chloride, the mixture was cooled to room temperature for 30 min in the darkness and made up to 5 mL with acetonitrile, centrifuged at 3000 g for 5 min and the supernatant filtered through 0.22 µm-pore-size filters. Derivatized standard solutions may be stored in the dark at 4ºC for

several months except in the case of SPM which may be stored in these conditions only for a little over a month.

After derivatization, standards (buffered and derivatized as above) and extracts were injected into the HPLC-FLD system. The extraction procedure gave a final solution representing 33 mg of the commodity per mL of extract. The injection volume was 10 µL. The mobile phases were water (A) and acetonitrile (B). The gradient conditions were as follows: B was increased from 40 to 70% over 12.5 min and held at 70% for 1.5 min, after which B was increased to 100% over 2 min and then decreased to 40% over 4 min (20 min run time). The flow rate was set to 1 mL min^{-1} and the column compartment to 40ºC. The excitation and emission wavelengths of the fluorescence detector were set at 350 nm and 520 nm, respectively.

2.5 Software

Experimental designs were built and analysed with NEMRODW [25]. MATLAB version 7.10 (The MathWorks) was used to perform the multiresponse optimization [26]. The least squares regression models were fitted with STATGRAPHICS Centurion XVI [27] and the least median of squares (LMS) regression models were fitted with PROGRESS [28]. Decision limit, CCα, and detection capability, CCβ, were determined using the DETARCHI program [29].

3. Results and discussion

3.1 Optimization of the experimental procedure

3.1.1 Experimental design

On the basis of some methods found in bibliography [18,30,31] and previous experience, a procedure of analysis was raised. Firstly, the simultaneous optimization of the extraction and the derivatization stages was performed. The 10 experimental variables in Table 1, which were involved in both stages, were included in the optimization analysis. Seven of the factors (*Extractant*, C_{Extr}, V_{Extr}, t_{vortex}, s_{centr}, t_{centr} and *%Dansyl*) were studied at 2 levels, the remaining

three factors (*pH*, T_{deriv} and t_{deriv}) at 3 levels, and possible interactions between C_{Extr} and V_{Extr} and between T_{deriv} and t_{deriv} were also studied. The levels of these factors and their codification are shown in Table 1.

A mathematical model with 19 coefficients, Eq. (1), which includes those corresponding to the main effects and the two interactions was fitted for each amine.

$$\begin{aligned} y = {} & \beta_0' + \beta_{1A}' x_{1A} + \beta_{2A}' x_{2A} + \beta_{3A}' x_{3A} + \beta_{4A}' x_{4A} + \beta_{5A}' x_{5A} + \beta_{6A}' x_{6A} + \beta_{7A}' x_{7A} + \\ & \beta_{8A}' x_{8A} + \beta_{8B}' x_{8B} + \beta_{9A}' x_{9A} + \beta_{9B}' x_{9B} + \beta_{10A}' x_{10A} + \beta_{10B}' x_{10B} + \beta_{2A3A}' x_{2A} x_{3A} \\ & + \beta_{9A10A}' x_{9A} x_{10A} + \beta_{9A10B}' x_{9A} x_{10B} + \beta_{9B10A}' x_{9B} x_{10A} + \beta_{9B10B}' x_{9B} x_{10B} + \varepsilon \end{aligned} \quad (1)$$

The binary variables x_{iA}, for the factors at two levels i=1,…, 7, have the value of 1 when the i-th factor is at level A and of -1 for level B. In the case of a 3-level factor (i=8, 9, 10), the variables x_{iA} and x_{iB} have respectively the values 1 and 0 when the i-th factor is at level A, 0 and 1 when the factor is at level B and -1 and -1 when the factor is at level C.

Therefore, the interpretation of coefficients in Eq. (1) depends on the levels of all factors. For example, only the term $\beta_{1A}' x_{1A}$ is related to factor 1, which is at 2 levels A and B. Taking into account the sum of all other terms in Eq. (1), say K (corresponding to the fixed levels of the remaining factors), the interpretation is as follows: when factor 1 is at level A, the response *y* will be $K + \beta_{1A}'$, and when factor 1 is at level B, *y* will be $K - \beta_{1A}'$. That is, if β_{1A}' is positive, the response decreases $2 \times \beta_{1A}'$ when factor 1 moves from level A to B, and it increases accordingly if β_{1A}' is negative.

For a factor at three levels, such as factor 8, its effect is modeled through the two addends $\beta_{8A}' x_{8A} + \beta_{8B}' x_{8B}$. If K denotes, like in the previous paragraph, the sum of all the remaining factors, at their corresponding fixed levels, when factor 8 is at level A, considering the values of variables x_{8A} and x_{8B}, the response *y* will be $K + \beta_{8A}' x_{8A} + \beta_{8B}' x_{8B} = K + \beta_{8A}' \times 1 + \beta_{8B}' \times 0 = K + \beta_{8A}'$; similarly, when the factor is at level B, *y* will be $K + \beta_{8B}'$, and $K - \left(\beta_{8A}' + \beta_{8B}'\right)$ when it is at level C. Thus, depending on the size and sign of these two coefficients, the model in Eq. (1) can fit non-linear effects. For example, if

$\beta'_{8A} = -2.5$ and $\beta'_{8B} = 2$, the responses, when changing the factor levels, would be K–2.5, K+2 and K+0.5, which shows a quadratic effect. The interpretation of the coefficients of the interaction terms are described in Table S1 of the supplementary material.

A complete factorial design would have required 3456 experiments, whereas the selected D-optimal design has only 23. The quality of the estimates was guaranteed since the VIFs of the coefficients of the model in this last case ranged from 1.2 to 2.1 (1.8 was the largest VIF of the model coefficients for the complete factorial design), which means precise estimates of the coefficients. Table 2 shows the experimental plan (5 replicates of experiment 6 were also conducted to validate the fit of the model).

Twenty-eight fish samples were fortified with 44.4 mg kg^{-1} of the BAs (444.4 mg kg^{-1} of HIS since the analytical procedure is considerably less sensitive in this case) and 44.4 mg kg^{-1} of IS. Next the samples were treated, following the experimental procedure described in Section 2.4, according to the experimental plan in Table 2 (in random order), and a chromatogram was obtained for each experiment. Figure 1 shows the chromatogram obtained for one of the replicates of experiment 6.

The peak areas of the chromatograms were used as response to fit a model for each amine. Table 3 shows the coefficients and statistics of the models fitted. All the models were significant at 0.05 significance level (except for IS, with a p-value equal to 0.06) and did not have significant lack of fit at 95% confidence level (p-values > 0.05). Residuals were randomly distributed and followed a normal distribution. Therefore, the models were valid and suitably explained the variability of the responses (the nine peak areas) since the coefficients of determination ranged from 0.85 to 0.97.

3.1.2 Multiresponse optimization

Once the models fitted were validated, they were used to estimate the responses, i.e. the peak areas for each amine, of the 3456 experiments of the complete factorial design. This is a different method of carrying out the optimization by considering not only the effect of factors and possible interactions on the responses through the significant coefficients, which is the usual way when using D-optimal designs, but taking into account the contributions of the

factors to the models through all the coefficients for the subsequent multiresponse optimization.

For visualizing the high dimensional space of the estimated responses, Figure 2 shows the parallel coordinates plot of the responses predicted for all the experiments of the complete factorial design. The coordinates are used for the amines, so that height in each coordinate is the predicted peak area of the corresponding amine. The lines join the values obtained under the same experimental conditions (so 3456 broken lines representing 9-dimensional points).

It is obvious from Figure 2 that differences in the size of peak areas occur, as chromatogram in Figure 1 also shows, due to the different sensitivity of the analytical method to the different amines. It is more sensitive for aliphatic amines (CAD, PUT, SPM, SPD and IS) which have more amine groups and/or less steric hindrance to be labeled with dansyl. But despite these differences, Figure 2 and Table 4, where ranges for the different amines are shown, clearly highlight a larger dispersion in the responses estimated for SPD and SPM than for the remaining amines (peak areas ranged from 0 to 110 for both amines). This means that the peak areas of these two amines, and therefore the concentration of their dansyl derivatives, depend to a greater extent than for the rest of amines on the conditions in which extraction and derivatization took place. Lines in green in Figure 2 show the solutions that reach the maximum value in at least one of the amines and make evident the conflict caused by the change in the experimental conditions.

The next step of the developed approach is to calculate the percentiles of the predicted peak areas of each amine. The aim is to set a certain percentile (like a threshold) and then to select those solutions that surpass this percentile in all the amines. Continuous light red line segments in Figure 2 indicate the solutions which have all the responses greater than the 80th percentile, seven in this case.

Table 5 shows the experimental conditions for extraction and derivatization of these 7 solutions. Four of them (in bold in Table 5) also provide estimated peak areas above 83th percentile (no experimental conditions were found with larger percentiles for the 9 responses simultaneously). As there were no distinct differences (in areas) between these last four solutions (the highest light red line segments in Figure 2), the optimal solution was then chosen to be the one with lowest volume of extractant (level A of x_2) and the shortest time of

derivatization (level A in x_{10}). That is, the multiresponse optimization approach led to choose experiment 702 in Table 5, which corresponds to the following conditions: x_1 (*Extractant*) = PCA, x_2 (C_{Extr}) = 0.4 M, x_3 (V_{Extr}) = 10 mL, x_4 (t_{vortex}) = 2 min, x_5 (s_{centr}) = 5000 g, x_6 (t_{centr}) = 10 min, x_7 (*%Dansyl*) = 0.5%, x_8 (*pH*) = 10.5, x_9 (T_{deriv}) = 40 ºC, and x_{10} (t_{deriv}) = 30 min.

3.2 Performance criteria

3.2.1 Matrix effect and recovery

Two calibration lines were performed with standards and matrix-matched standards (standards in blank matrix subjected to the entire treatment) to study the possible existence of matrix effect and to calculate the recovery of the analytical procedure, relating standardized areas and concentrations in the first case and increase of standardized area and added concentrations in the second one. For both analysis, 15 solutions, with concentrations ranged 0 to 0.44 mg L^{-1} of PHE, PUT, CAD, SPD and SPM, from 0 to 1.77 mg L^{-1} of TRP and TYR, from 0 to 17.73 mg L^{-1} of HIS, and 0.13 mg L^{-1} of IS, were prepared.

Robust regression models [28], based on the LMS regression, were previously fitted to detect outliers (data with absolute value of standardized residual above 2.5). The least squares (LS) models fitted once outliers were removed explained significantly at a 95% confidence level the experimental responses (p-values < 0.05). The coefficients of correlation ranged from 0.98 to 1.00.

The slopes and the intercepts of the standard and matrix-matched calibration models were statistically compared, Table 6 shows these results. Slopes were significantly different at a 95% confidence level for all the amines, clearly showing the matrix effect of the swordfish components on the analytical responses. On the other hand, for, biogenic amines present in the analysed swordfish samples, intercepts were also significantly different at the same confidence level.

The recovery of the optimized procedure was calculated by properly comparing the slopes of the fitted models. Since the slopes of the calibration lines obtained are exactly the same as those that would have been obtained if the regression models “increase of standardized peak area ($y–y_0$) *vs.* amount of analyte spiked” had been estimated, the recovery rate can be

calculated as the ratio of the slopes of the calibration lines in matrix and in solvent [32]. Table 6 shows also the recovery rates found, that were over 70% in all the cases except for TRP, for which a 58% of recovery rate was reached.

3.2.2 Accuracy

Trueness and precision were determined from the accuracy lines, i.e. regression models between calculated and true concentrations in solvent [33]. The joint hypotheses "the slope is 1 and the intercept is 0" was checked in order to determine trueness. Figure 3 shows the joint confidence estimated regions (confidence ellipse) for slope and intercept. All the ellipses in the plot include the point (1,0) so that the trueness is guaranteed for the determination of all the BAs.

The precision of the optimized method can be estimated from the residual standard deviation of the accuracy line. Table 6 shows the standard deviation of the regression models, which may be considered an estimation of the intermediate repeatability in the concentration ranges [34]. The lowest precision corresponds to the determination of HIS since the area of the joint confidence ellipse, Figure 3, is the highest, and the highest precision to PHE.

3.2.3 Decision limit and detection capability

As well as EU defines the decision limit (CCα) in the Commission Decision 2002/657/EC, ISO 11483-2 expresses the critical value of the net concentration as 'the value of the net concentration the exceeding of which leads, for a given error probability α, to the decision that the concentration of the analyte in the analysed material is larger than that in the blank material'. And the detection capability (CCβ) for a given probability of false positive, α, as 'the true net concentration of the analyte in the material to be analyzed, which will lead, with probability 1-β, to the correct conclusion that the concentration in the analyzed material is larger than that in the blank material'.

The decision limits and capabilities of detection of the optimized procedure were calculated through the regression curves fitted with the first 9 standards in all the cases. The values estimated are shown in the four last lines of Table 6 and are expressed both in concentration in vial and in the foodstuff. The procedure enables to determine with probabilities of false

positive (α) and false negative (β) equal to 0.05, down to around 150 µg of CAD or PHE per kg of fish whereas, in the case of HIS, 28.7 mg kg^{-1} are reached. This means that the procedure is less sensitive for HIS than for the rest of BAs analysed, as is has been pointed out above. However, the detection capability found is far below the limit established by EU [21] for HIS in some fresh fishery products, 200 mg kg^{-1} in fish.

3.3 Analysis of fish samples

The quantitative determination of the BAs under consideration was performed applying the experimental procedure described in Section 2.4 to swordfish samples. All the analytical procedure was carried out in sextuplicate, taking into account the standardized peak areas. In the fish samples, no chromatographic peaks were obtained for TRP, PHE, HIS and TYR, but the following contents for the rest of BAs were found: PUT, 0.27 ± 0.09 mg kg^{-1}; CAD, 1.41 ± 0.04 mg kg^{-1}; SPD, 3.02 ± 0.17 mg kg^{-1}; and SPM, 7.15 ± 0.93 mg kg^{-1} (semi-intervals were calculated at 95% confidence level).

4. Conclusions

The use of the D-optimal design methodology has led to significantly reduce, by a factor of 67, the experimental effort required to optimize 10 experimental variables implied in two steps of the analytical procedure.

The multiresponse optimization approach developed for optimizing simultaneously the 9 responses (peak areas) is very efficient and easier to perform over other methods. It has enabled one to consider all contributions to the models, i.e. not only the significant coefficients but the complete models.

The validated procedure has allowed to reach detection capabilities down to 70 µg L^{-1} for CAD (which means 130 µg kg^{-1} in fish) and 14.8 mg L^{-1} for HIS (which means 28.7 mg kg^{-1} in fish) for $\alpha = \beta = 0.05$.

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FIGURE CAPTIONS

Figure 1 Chromatogram of experiment 6 in Table 2. In the extract injected, the procedure gives a final added concentration of 14.8 mg L^{-1} of HIS, 1.5 mg L^{-1} of the rest of BAs, and 1.5 mg L^{-1} of IS.

Figure 2 Parallel coordinates plot of the solutions predicted for each amine for the 3456 experiments of the complete factorial design. The 7 solutions which have all the responses greater than the 80th percentile are in light red, solutions which reach the maximum in at least one of the amines; and the rest of solutions are in dark blue.

Figure 3 (a) Joint confidence ellipses, at a 95% significance level, for slope and intercept of the accuracy lines. (b) Enlarged view. HIS: black long dash-double dot line, PUT: red long dash line, CAD: yellow long dash-dot line, TYR: magenta medium dash-dot line, TRP: blue short dash line, PHE: cyan solid line, SPM: green dotted line and SPD: purple dashed line.

Table 1 Factors, codified variables and experimental domain of interest for optimization.

Factors	Codified variable	Level A	Level B	Level C
Extractant	x_1	TCA	PCA	–
C_{Extr}	x_2	TCA 5% or PCA 0.4 M	TCA 10% or PCA 0.6 M	–
V_{Extr} (mL)	x_3	7.5	10	–
t_{vortex} (min)	x_4	1	2	–
s_{centr} (g)	x_5	3000	5000	–
t_{centr} (min)	x_6	5	10	–
%Dansyl (%)	x_7	0.5	1	–
pH	x_8	8.5	9.5	10.5
T_{deriv} (ºC)	x_9	20	40	60
$t_{deriv.}$ (min)	x_{10}	30	45	60

Table 2 Experimental plan of the D-optimal design (5 replicates of experiment 6, in bold, has also been performed).

No. Exp.	*Extractant*	C_{Extr}	V_{Extr} (mL)	t_{vortex} (min)	s_{centr} (g)	t_{centr} (min)	*%Dansyl* (%)	*pH*	T_{deriv} (°C)	t_{deriv} (min)
1	TCA	High	10	1	3000	10	0.5	10.5	40	30
2	PCA	Low	10	1	5000	5	0.5	10.5	20	30
3	TCA	Low	7.5	1	3000	5	1	9.5	20	30
4	PCA	High	10	2	3000	5	0.5	10.5	40	45
5	PCA	High	10	1	3000	5	0.5	8.5	40	60
6	**PCA**	**High**	**7.5**	**1**	**5000**	**10**	**0.5**	**9.5**	**40**	**45**
7	PCA	High	10	2	5000	10	1	8.5	60	45
8	PCA	Low	7.5	2	5000	5	0.5	10.5	60	30
9	TCA	Low	7.5	1	5000	10	0.5	8.5	60	60
10	PCA	High	7.5	2	5000	5	1	8.5	40	30
11	PCA	High	7.5	1	3000	10	1	10.5	20	60
12	TCA	Low	10	1	3000	5	1	8.5	60	30
13	TCA	Low	7.5	2	3000	10	1	8.5	40	45
14	TCA	High	7.5	1	3000	5	0.5	9.5	60	45
15	TCA	High	10	1	5000	5	1	9.5	20	45
16	TCA	High	7.5	2	3000	5	0.5	10.5	20	45
17	PCA	Low	10	2	3000	5	1	9.5	60	60
18	TCA	High	7.5	2	3000	10	0.5	8.5	20	30
19	TCA	Low	10	2	5000	5	0.5	8.5	20	60
20	PCA	Low	7.5	2	3000	10	0.5	9.5	40	30
21	PCA	Low	10	1	3000	10	0.5	8.5	20	45
22	TCA	Low	10	2	5000	10	1	10.5	40	60
23	TCA	High	10	2	5000	10	0.5	9.5	60	30

Table 3 Parameters and statistics of the D-optimal models fitted for each BA: coefficients of the models (coefficients b_0 to b_{9B10B}), coefficient of determination (R^2), and *p*-values of the significance of the regression and lack of fit.

	TRP	PHE	PUT	CAD	HIS	IS	TYR	SPD	SPM
b_0	11.20	36.15	90.44	73.50	29.91	117.87	10.40	65.87	53.06
b_{1A}	1.21	-1.58	-4.23	-3.11	-1.04	-5.08	0.05	-3.86	-2.38
b_{2A}	0.65	-0.45	-1.69	-1.54	-0.39	-3.91	0.24	0.23	1.26
b_{3A}	-0.44	-1.06	-2.83	-2.21	-0.69	-3.61	0.00	-2.63	-1.03
b_{4A}	-0.03	-0.05	-0.59	-0.50	0.02	-1.13	0.52	0.99	2.06
b_{5A}	-0.23	-0.29	-1.08	-0.79	-0.54	-1.67	-0.01	0.56	0.95
b_{6A}	0.53	-0.61	-3.71	-2.64	-1.22	-2.80	-0.32	-3.95	-3.71
b_{7A}	1.17	0.07	0.40	0.60	-0.40	2.79	-0.45	-4.36	-5.41
b_{8A}	-1.64	0.74	-3.83	-2.65	0.39	3.49	-5.24	-25.21	-35.50
b_{8B}	-1.29	-0.03	3.21	2.07	0.60	0.46	1.44	8.70	7.31
b_{9A}	-2.39	-0.33	3.65	1.87	1.04	0.52	-0.51	6.91	8.28
b_{9B}	2.03	0.13	-0.14	0.36	0.52	-0.19	0.25	-0.44	0.59
b_{10A}	1.67	0.08	-0.61	-0.38	-0.13	-0.45	0.71	2.11	2.92
b_{10B}	-0.31	-1.24	-4.74	-3.49	-1.85	-6.14	-0.33	-5.11	-7.09
b_{2A3A}	-0.88	-0.30	-0.54	-0.35	-0.56	-0.24	-0.30	70.30	-0.62
b_{9A10A}	0.68	0.26	1.45	0.77	0.34	1.49	-0.53	96.60	1.06
b_{9A10B}	-0.06	0.28	3.07	2.28	1.25	2.51	0.56	11.40	3.32
b_{9B10A}	0.05	0.47	2.62	2.30	0.72	2.05	0.63	33.60	3.41
b_{9B10B}	-0.01	0.09	-2.99	-2.15	-0.76	-2.39	0.38	6.00	-6.47
Significance of regression[a] (p-value)	$< 10^{-4}$	$< 10^{-4}$	0.03	0.02	0.03	0.06	$< 10^{-4}$	$< 10^{-4}$	$< 10^{-4}$
Lack of fit[b] (p-value)	0.05	0.05	0.43	0.33	0.33	0.43	0.03	0.39	0.07
R^2	0.88	0.92	0.88	0.89	0.88	0.85	0.97	0.95	0.95

(a) Null hypothesis: the linear model is not significant

(b) Null hypothesis: the regression model adequately fits the data

Table 4 Ranks, maximum and minimum values of the peak areas estimated for the 3456 experiments of the experimental design.

	TRP	PHE	PUT	CAD	HIS	IS	TYR	SPD	SPM
rank	22.08	13.52	50.41	38.18	16.96	68.04	15.53	97.42	122.50
max	23.01	43.15	113.64	91.44	37.14	152.01	17.67	108.53	110.92
min	0.93	29.63	63.23	53.27	20.19	83.98	2.14	11.12	-11.59

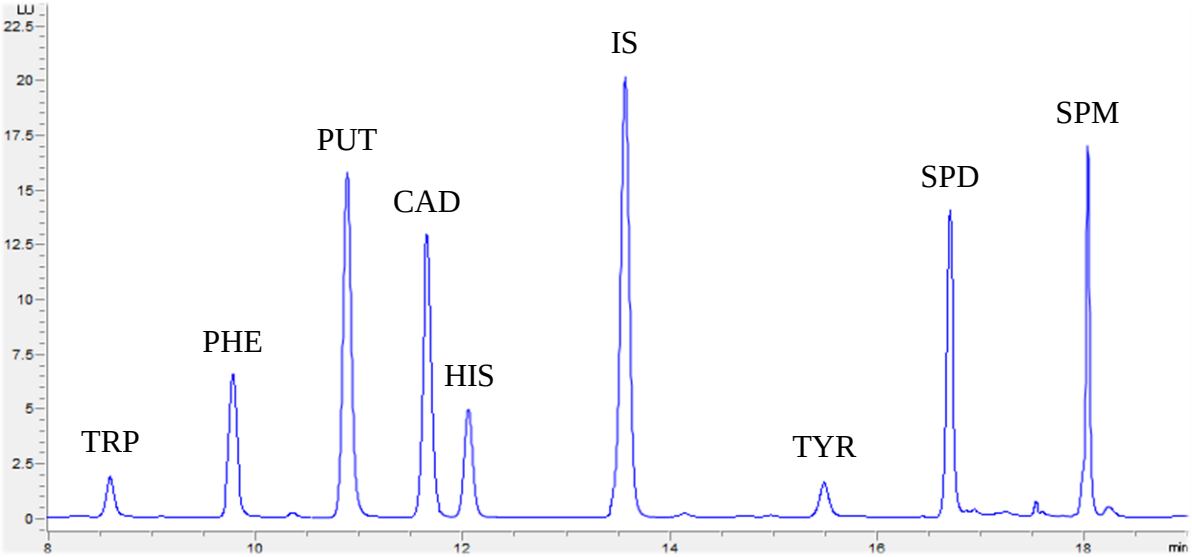
Table 5 Experiments of the complete factorial design and experimental conditions of the solutions which have all the responses greater than the 80th percentile. Solutions which have all responses greater than the 83th percentile are in bold.

No. Exp.	x_1	x_2	x_3	x_4	x_5	x_6	x_7	x_8	x_9	x_{10}
696	**B**	**B**	**B**	**A**	**B**	**B**	**A**	**C**	**B**	**A**
700	B	B	A	B	B	B	A	C	B	A
702[a]	**B**	**A**	**B**	**B**	**B**	**B**	**A**	**C**	**B**	**A**
704	B	B	B	B	B	B	A	C	B	A
3366	**B**	**A**	**B**	**A**	**A**	**B**	**A**	**C**	**C**	**C**
3374	B	A	B	B	A	B	A	C	C	C
3382	**B**	**A**	**B**	**A**	**B**	**B**	**A**	**C**	**C**	**C**

([a]) Experimental conditions chosen

Table 6 Performance criteria of the analytical method optimized: parameters of calibration curves in solvent and fish and of accuracy line (s_{yx} is the standard deviation of regression), recovery rates and decision limit and detection capability (for $\alpha = \beta = 0.05$).

		TRP	PHEN	PUT	CAD	HIS	TYR	SPD	SPM
Calibration curve (solvent)	Intercept	-0.0108	0.0027	0.0159	0.0090	-0.0126	0.0016	-0.0180	-0.0590
	Slope	1.2871	2.0206	5.3343	4.1998	0.1563	0.8030	5.3519	3.3718
	Correlation coefficient	0.9998	0.9998	0.9991	0.9995	0.9966	0.9999	0.9983	0.9957
	s_{yx}	0.0169	0.0072	0.0422	0.0234	0.0888	0.0075	0.0532	0.0477
Calibration curve (fish)	Intercept	0.0506	-0.0062	0.1200	0.0612	0.0489	0.0446	0.5587	0.8643
	Slope	0.7424	1.8989	4.4500	3.8979	0.1313	0.6140	3.8141	2.7409
	Correlation coefficient	0.9976	0.9980	0.9978	0.9973	0.9978	0.9990	0.9908	0.9816
	s_{yx}	0.0403	0.0185	0.0557	0.0439	0.0651	0.0213	0.0998	0.1044
Recovery rates (%)		57.7	94.0	83.4	92.8	84.0	76.5	71.3	83.8
Accuracy line	Intercept	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	Slope	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
	Correlation coefficient	0.9998	0.9998	0.9991	0.9995	0.9966	0.9999	0.9983	0.9957
	s_{yx}(mg L^{-1})	0.0109	0.0029	0.0066	0.0047	0.4735	0.0078	0.0083	0.0118
CCα (µg L^{-1})		24.2	2.8	4.1	2.5	497.4	7.0	8.5	27.9
CCβ (µg L^{-1})		47.1	5.5	7.9	4.8	964.2	13.7	16.5	53.8
CCα (mg kg^{-1} in fish)		1.05	0.08	0.12	0.07	14.81	0.23	0.30	0.83
CCβ (mg kg^{-1} in fish)		2.04	0.15	0.24	0.13	28.70	0.45	0.58	1.61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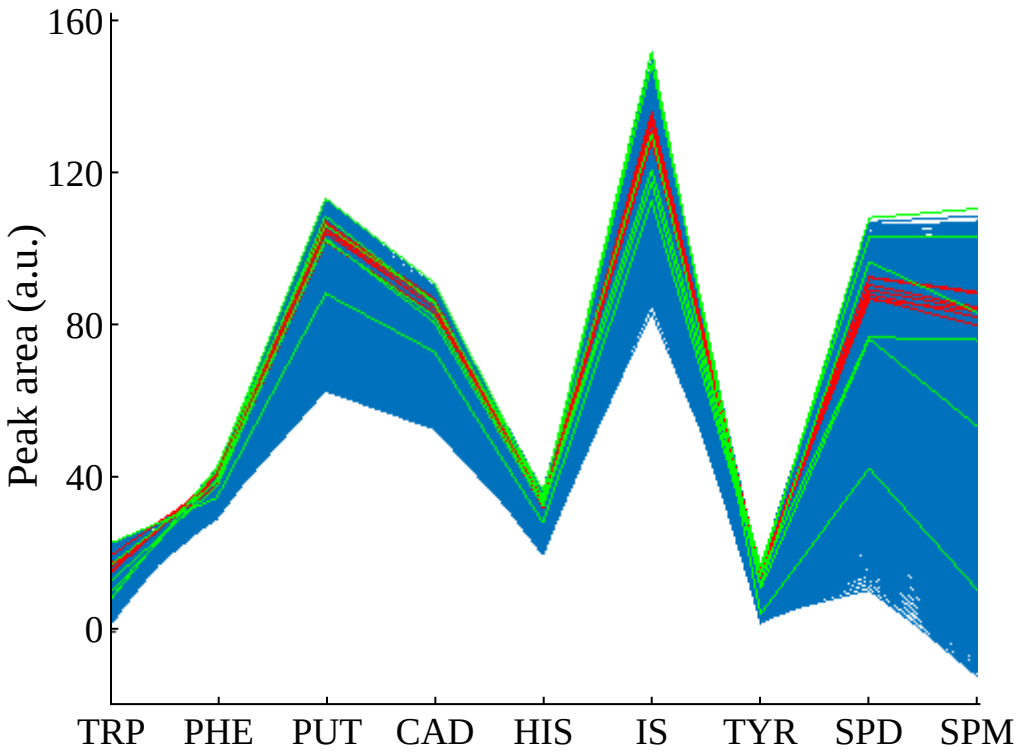
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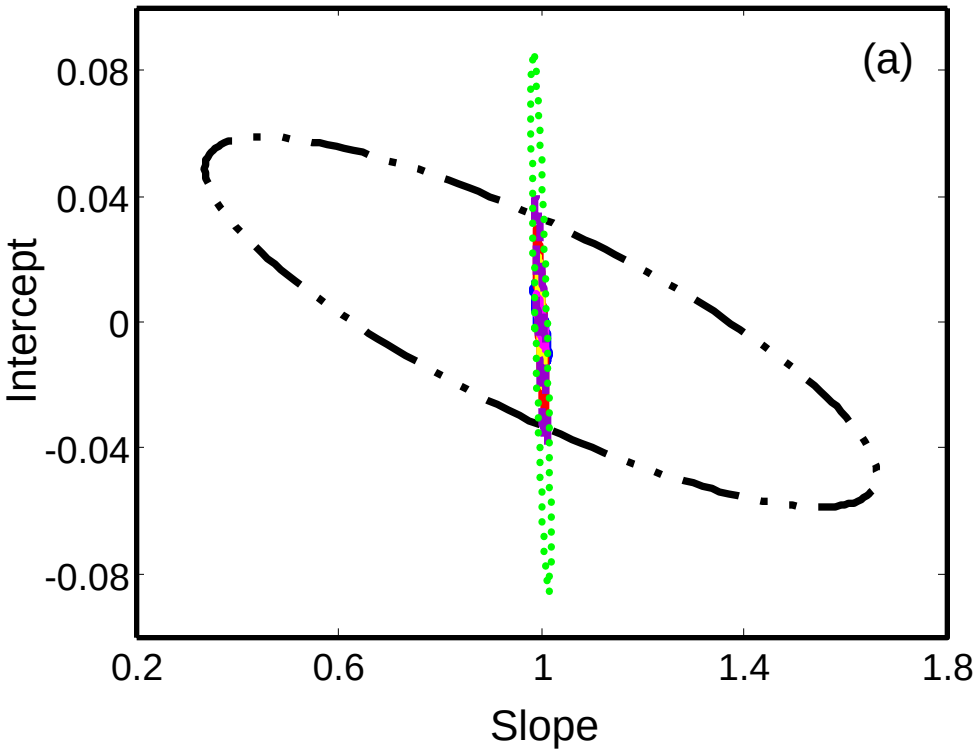
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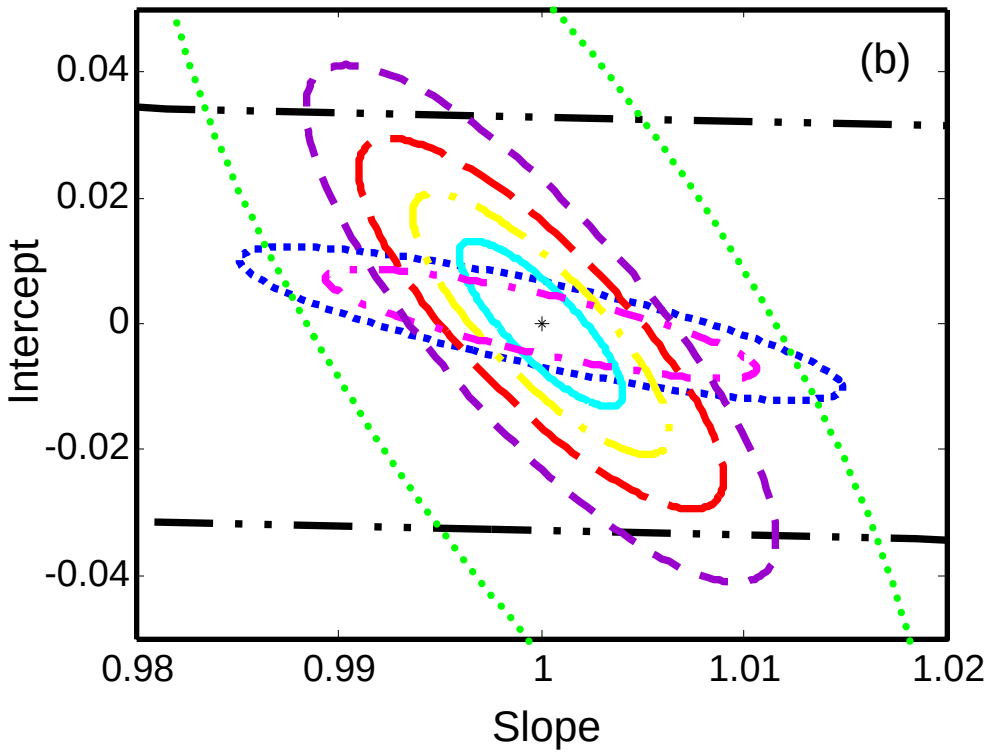
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