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RESEARCH ARTICLE

Optimization of the Ion Source-Mass Spectrometry Parameters in Non-Steroidal Anti-Inflammatory and Analgesic Pharmaceuticals Analysis by a Design of Experiments Approach

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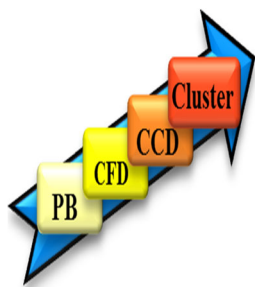
Abstract. The flow rates of drying and nebulizing gas, heat block and desolvation line temperatures and interface voltage are potential electrospray ionization parameters as they may enhance sensitivity of the mass spectrometer. The conditions that give higher sensitivity of 13 pharmaceuticals were explored. First, Plackett-Burman design was implemented to screen significant factors, and it was concluded that interface voltage and nebulizing gas flow were the only factors that influence the intensity signal for all pharmaceuticals. This fractionated factorial design was projected to set a full 2^2 factorial design with center points. The lack-of-fit test proved to be significant. Then, a central composite face-centered design was conducted. Finally, a stepwise multiple linear regression and subsequently an optimization problem solving were carried out. Two main drug clusters were found concerning the signal intensities of all runs of the augmented factorial design. p-Aminophenol, salicylic acid, and nimesulide constitute one cluster as a result of showing much higher sensitivity than the remaining drugs. The other cluster is more homogeneous with some sub-clusters comprising one pharmaceutical and its respective metabolite. It was observed that instrumental signal increased when both significant factors increased with maximum signal occurring when both codified factors are set at level +1. It was also found that, for most of the pharmaceuticals, interface voltage influences the intensity of the instrument more than the nebulizing gas flowrate. The only exceptions refer to nimesulide where the relative importance of the factors is reversed and still salicylic acid where both factors equally influence the instrumental signal.

Keywords: Cluster analysis, Nonlinear constrained optimization, Pharmaceuticals, Plackett-Burman design, Response surface methodology, Stepwise multiple linear regression

Introduction

Pharmaceuticals are an indispensable part of daily life [1]. They are used for the treatment, cure, prevention, or diagnosis of diseases, and for the improvement of human physical and mental well-being [2]. Non-steroidal anti-inflammatory drugs (NSAIDs) and analgesic drugs are two of the most commonly used group of drugs worldwide. They are used to suppress pain and inflammatory states in cases of relevant diseases, such as rheumatoid arthritis [3].

In recent years, increasing attention has been drawn towards the discharge, presence, and potential risks of pharmaceuticals in the environment [4, 5]. Most of these studies investigated locations nearby suspected sources, such as waters affected by large urban centers, raw sewage, or located downstream of wastewater treatment plants [6].

Using the key words "pharmaceuticals" and "environment," a careful browsing through published papers in the scientific community showed that there are no returns on these subjects before 1970. However, after 1970 there is a steady rise in

publications on both subjects. In 1970, seven papers were published. However, this number increased to 211 in the year 2000. By 2010, 1013 papers were published, with that number slightly decreasing to 977 in 2014 (retrieved from ISI Web of Knowledge in September of 2015).

The decrease in the level of detection of pharmaceuticals in the environment is largely due to improvements in analytical methodology and technology, with mass spectrometry (MS) or tandem mass spectrometry (MS/MS) as advanced methods, which allow detection of target compounds at the nanogram per liter level and are commonly applied for the detection of pharmaceutical compounds [6].

It is important to note that researchers are free to either apply an already published methodology (analysis and/or extraction) to their compounds and their samples, or develop their own method. In both cases, the majority of researchers still continue to use conventional optimization, changing one factor at a time (OFAT) by keeping all variables constant except one. Additionally, the statistical designs are not well known among scientists except those who have statistical expertise. As mentioned in the work of Leardi [7], optimizing factors one at a time results in a large number of experiments necessary to carry out research. This leads to an increase in time spent, cost, equipment wear, as well as in the use of reagents and materials for such experiments. This method also fails to consider possible interactions between different variables. This type of optimization does not guarantee that the real optimum will ever be reached. Furthermore, it gives only a local knowledge of the phenomenon and often requires a much larger experimental effort [7].

Owing to an emphasis on quality improvement, there has been an increase in the application of experimental design techniques in the optimization methodologies. By careful choice of factor levels, it is possible to optimize a response that is influenced by the factors detected as significant by Plackett-Burman design [8, 9]. Response surface methodology (RSM) has more advantages than the traditional single parameter optimization because it can save time, space, and raw materials [10, 11].

In particular, it is important to identify the factors that influence analyte signal intensity and quality [12, 13]. In 2010, Dillon et al. [14] developed a methodology for the performance assessment of electrospray ionization systems applied to volatile organic compounds determination. A central composite factorial design combined with exponential dilution was used. Ionization voltage, drying gas flow rate, and nebulizing gas flow rate were the factors studied. The authors concluded that it was evident that no one factor appeared to dominate the response. Drying-gas flow rates were found to be more important than nebulizing gas flow rates. Raji and Schug [12] investigated four factors, spray voltage, ion transfer capillary temperature, ion transfer capillary voltage, and tube lens voltage, in two different ESI-MS instruments. Yates algorithm was used to estimate the effect of each factor as well as interactions. The benefits of using chemometrics in mass spectrometry were highlighted by the authors and tube lens voltage was found to have a significant effect on analytes (amino acids) response on both instruments [12]. Another study was

conducted by Titato et al. [15] for the selection of the best values for the MS system parameters on ESI and APCI interfaces. A variable selection technique was carried out in order to determine the critical factors (cone voltage, source temperature, and drying-gas flow rate). Although cone voltage was the only critical factor found in the APCI ionization mode, cone voltage, source temperature, and drying-gas flow rate were the critical factors when ESI was used.

The current research presents the application of experimental design, with the aim of improving the UHPLC-MS/MS signal of 13 pharmaceuticals from the group of NSAIDs and analgesics drugs, including metabolites and degradation products. To this end, ion source factors with significant effect on each compound MS response were identified through the Plackett-Burman design, and subsequently complete experimental designs were applied to the significant factors. Full factorial and central composite face-centered (CCF) designs were implemented in order to obtain the best ion source conditions that maximize the MS/MS signal. OFAT analysis was also performed and the results were compared with those obtained using the factorial design.

Finally, the CCF results provided the basis for a cluster analysis and signal intensity maximization through stepwise multiple regression.

Experimental

Pharmaceuticals

Thirteen pharmaceuticals, including metabolites and degradation products, from a group of NSAIDs and analgesic drugs were studied. Ibuprofen, hydroxyibuprofen, (ibuprofen metabolite), carboxyibuprofen (ibuprofen metabolite), acetaminophen, *p*-aminophenol (acetaminophen degradation product), *p*-acetamidophenyl β-D-glucuronide (acetaminophen metabolite), acetylsalicylic acid, salicylic acid (degradation product, or metabolite or even by-product of industrial processes), naproxen, ketoprofen, nimesulide, diclofenac sodium salt, and dipyrone sodium salt used in the present work were of high purity grade and were purchased from Sigma-Aldrich (Steinheim, Germany). Chemical structures and physicochemical properties of the selected pharmaceuticals are presented in Supporting Information (Supplementary Table S1).

Solutions, Reagents, Solvents, and Materials

Individual stock standards (at a concentration of 1 g L^{-1}) were prepared on a weight basis in acetonitrile, with the exception of *p*-aminophenol, acetaminophen glucuronide, naproxen, diclofenac, and dipyrone, which were prepared in acetonitrile-methanol (1:1, v/v), since these substances are slightly soluble in pure acetonitrile and freely soluble in methanol. Almost all pharmaceutical stock standard solutions were prepared every 6 mo and were stored at −20 °C. In the case of *p*-aminophenol, special attention had to be taken into account,

and fresh stock standard solutions were prepared every month because of its limited stability [16].

Working standard solutions, containing all pharmaceuticals, were prepared in the mobile phase by mixing appropriate amounts of the stock solutions. These solutions were prepared before each analytical run.

Deionized water was produced using a Elix apparatus (Millipore, Molsheim, France) and purified water (resistivity of 18.2 MΩ.cm) using a Simplicity 185 system (Millipore). Purified Milli-Q water was used for mobile phase in the UHPLC. All chromatographic solvents were filtered through a 0.22 μm nylon membrane filter, 47 mm (Supelco, Bellefonte, PA, USA) using a vacuum pump (Dinko D-95; Barcelona, Spain) and degassed for 15 min in an ultrasonic bath (Sonorex Digital 10P; Bandelin DK 255P; Germany).

Acetonitrile LC-MS (assay ≥ 99.95%) was supplied by Biosolve (Valkenswaard, The Netherlands) and methanol LC-MS Ultra CHROMASOLV (assay ≥ 99.9%) and 2-propanol LC-MS CHROMASOLV (assay ≥ 99.9%) were supplied by Sigma-Aldrich.

UHPLC−MS/MS System

The analyses were performed on a Shimadzu Nexera UHPLC−MS/MS system triple-quadrupole mass spectrometer (LCMS−8030; Shimadzu Corporation, Kyoto, Japan) and operated in the electrospray ionization (ESI) mode. The LCMS−8030 are equipped with a LC−30 AD pump (two solvent delivery modules), a CTO−20 AC column oven, a DGU-30A3 degasser, a SIL−30 AC auto injector, and a CBM−20A system controller. LabSolutions software (Shimadzu Corporation) was used for control and data processing. The injection volume was 5 μL. The autosampler was operated at 4 °C and the autosampler needle was rinsed before and after aspiration of the sample using acetonitrile-methanol-propanol (1:1:1, v/v/v).

MS settings were analyte-specific and were optimized by direct injection of individual 10 mg L^{-1} standard solutions. All the pharmaceutical compounds were analyzed in the negative ESI mode with the exception for the degradation product *p*-aminophenol, which was analyzed in the positive ESI mode. Mass spectrometer was operated in multiple reaction monitoring mode (MRM) and two product ions were selected when possible. In the case of acetaminophen, hydroxyibuprofen, ketoprofen, and ibuprofen, only one transition could be recorded because of their poor fragmentation [16]. The most sensitive MRM transition was used for quantitation (quantifier) whereas the second one was used for confirmation (qualifier). Ratios of the measured area counts for both MRM transitions were then monitored. A dwell time of 25 ms was used for all compounds.

The transitions used for each pharmaceutical along with the observed ion ratio are described in (Supplementary Table S1).

The flow rates of drying and nebulizing gas, heat block and desolvation line temperatures, interface voltage, ESI sprayer position, and ESI protrusion are potential parameters to increase instrumental sensitivity [17, 18]. The influence of the ESI sprayer position and protrusion alignments were not evaluated in this study. The two alignments were done manually without any precision on the position previously set and consequently not reproducible. Concerning the ESI sprayer position, a scale from 0 to 3 mm with a 1 mm resolution were marked. With regard to the ESI protrusion, only two positions (0 and 1 mm) were marked with a 90° angle distance between them. As the change of those two parameters is very small, the operator assigned to do this work can unintentionally introduce an error. Thus, ESI position and ESI protrusion were set according to the results obtained in the work of Paíga et al. [19]. There were, therefore, five parameters [interface voltage (IV, kV), drying gas flow rate (DGF, L·min^{-1}), nebulizing gas flow rate (NGF, L·min^{-1}), heat block temperature (HBT, °C), and desolvation line temperature (DGF, °C)] still to be tested in this work.

Statistical Design

Recent research emphasizes the importance of using statistics in the optimization [7, 19]. The present study employs a design of experiment (DoE) strategy in order to find the optimum conditions for the MS ion source parameters reducing significantly the number of runs in the optimization step. The flow-chart of the DoE algorithm implemented in this work is presented in Figure 1. The results obtained at each step of the optimization process allow performing the experiments that follow in such a way that it does not compromise obtaining the global maximum response of the MS ion source parameters for the studied compounds. The results obtained using the OFAT and the statistic experiments were compared. Additional information of the statistics tools used in this study are included as the Supporting Information (see Supplementary Data).

Results and Discussion

Considerations and Preliminary Results Using OFAT Experiments

Among the majority of the published works, the use of statistics in the optimization process is not commonly applied because of the difficulty of interpreting the results and, therefore, failure to acquire optimal conditions.

In a previous publication of the authors [19], the MS ion source parameters were optimized using OFAT approach. As a result the NGF and DGF flow rates of 2.6 and 12.5 L·min^{-1}, the IV of 5.0 kV and the DLT and HBT temperatures of 250 °C and 300 °C were obtained, respectively. The maximum values that LCMS-8030 can operate are limited to 5 kV for IV, 20 L·min^{-1} for DGF, 3 L·min^{-1} for NGF, 300 °C for DLT, and 500 °C for HBT. Thus, the minimum, maximum, and the increment of the variable parameter in each set of experiments were fixed in accordance with the technical support information from Shimadzu and the limitations of the LCMS−8030. A total of 55 runs were carried out distributed over 26 experiments to study the IV (between 0 and 5 kV, with a step of 0.2 kV), six experiments to study the NGF (range from 0.5 to

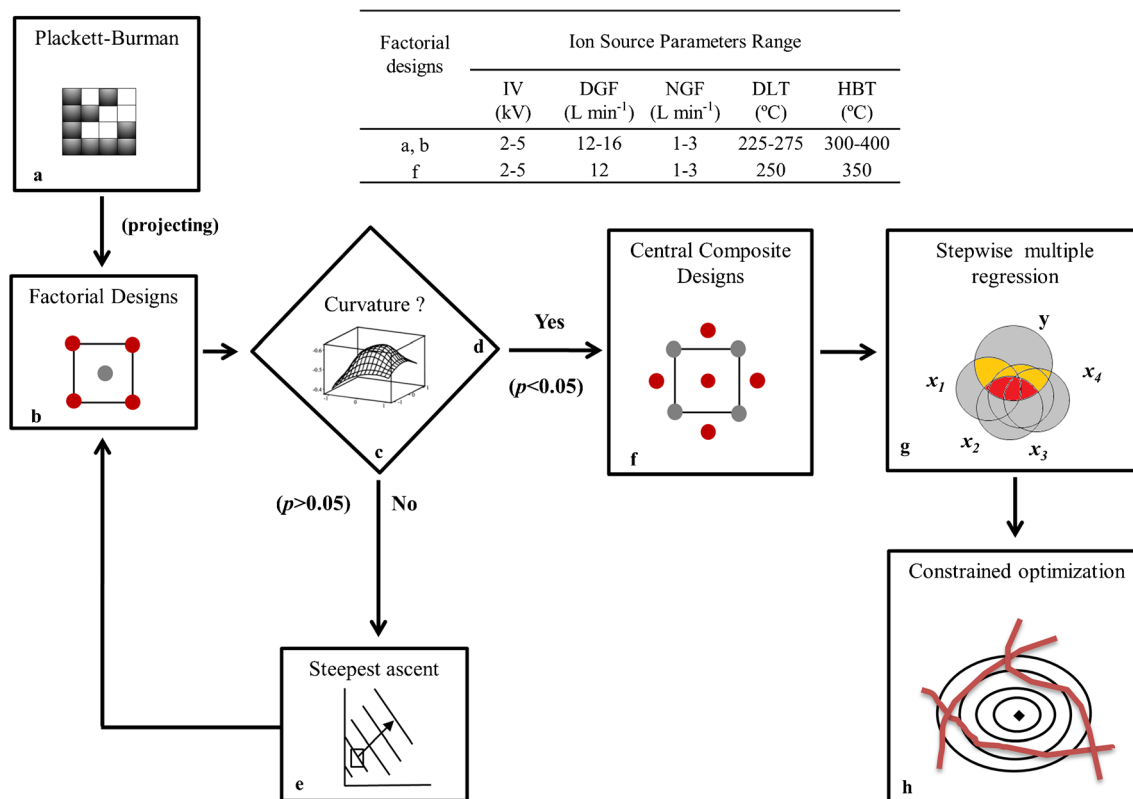| Factorial designs	Ion Source Parameters Range				
	IV (kV)	DGF (L min⁻¹)	NGF (L min⁻¹)	DLT (ºC)	HBT (ºC)
a, b	2-5	12-16	1-3	225-275	300-400
f	2-5	12	1-3	250	350

Figure 1. Flow chart of the algorithm designed for MS ion source parameters optimization

3.0 L min⁻¹ by a step of 0.5 L min⁻¹), five experiments to study the DGF (between 10 and 20 L min⁻¹, with a step of 2.5 L min⁻¹), five experiments to study the DLT (between 200 and 300 °C, with a step of 25 °C), and 13 experiments to study the HBT (range from 200 to 500 °C spaced by 25 °C).

Recent papers underline the importance of using statistics in the optimization [7, 20]. In this regard, the present work employs a DoE strategy (Figure 1) in order to find the optimum conditions for the ESI-MS ion source parameters, reducing significantly the number of runs in the optimization step. The results obtained by OFAT and DoE were thereafter compared.

Plackett-Burman Used as a Screening Design

Screening designs are used to highlight the significant factors from those potentially influencing the response at stake [7]. Plackett-Burman design confounds significance between main effects and two-factor interaction terms; nevertheless, it considerably reduces the number of experiments necessary for a design structure that no main effects are aliased with each other.

Temperature and gas flow rate are parameters that affect the desolvation efficiency. Improper settings may result in loss of signal [21]. In this study, a 12-run Plackett-Burman design was built to identify the main factors affecting the response. For the effect, five factors, (IV, DGF, NGF, DLT, and HBT) were chosen (Supplementary Table S2). Each experiment contains one of the two level values −1 and +1 of each factor. Furthermore, for any pair of factors, each combination of levels (− −, −

+, + −, and + +) appears three times, ensuring orthogonality between columns. None of the 12 experiments is similar to each other. Three injections for each experiment were performed and three concentrations for each pharmaceutical (0.1, 1.0, and 10 mg L⁻¹) were injected. Nine replicates for the center point were conducted to estimate the experimental error (pure error).

In an attempt to rule out significant factors wrongly rejected, three Plackett-Burman designs (PB1, PB2, and PB3) were implemented, each one with different parameter variation range and center point positions (Supplementary Table S3). The experiments were carried out randomly. The sequence of the injections was programmed in the batch of the LabSolutions software LC-MS/MS. Twelve chromatographic methods were introduced with the conditions of each experiment of the Plackett-Burman design (Supplementary Tables S2 and S3), and one more method was programmed for the center point conditions. The procedure was repeated for the other two Plackett-Burman designs. In the end of the batch sequence, a method with the optimum conditions obtained in OFAT experiments was set. The results were compared.

The chromatographic areas of the studied pharmaceuticals were measured (data not shown) and the variability between injections was checked for all experiments by relative standard deviation (RSD) calculation.

For the PB3 design (Supplementary Table S3), lower area and higher RSD results (>10%) were obtained in the experiments 5, 8, and 12 (Supplementary Table S2) when both NGF and IV factors were fixed at level −1, independent of the level

values set for the other factors. For the PB1 and PB2 designs (Supplementary Table S3), more experiments (runs 1, 5, 8, 10, 11, and 12, Supplementary Table S2) with higher variability were observed. In all of these experiments, the only common factor level was level –1 for NGF. For these latter designs, the value of the NGF at level –1 was of 0.4 and 0.5 L min^{-1}, respectively for PB1 and PB2, whereas for thePB3 design it was 1.0 L min^{-1} (Supplementary Table S3). The loss of the signal is more pronounced for the lowest values of the NGF factor and therefore the PB1 and PB2 designs had more experiments with lower area and thereafter higher values of RSD. The highest signal was obtained in experiment 3 on PB2 and PB3 designs and experiment 6 on PB1 design.

The ratio of the chromatographic areas of the previous experiments with the chromatographic area of the OFAT experiment with maximum response was computed and the results are shown in Supplementary Table S4. For each pharmaceutical, ratios higher, equal, or lower than 1, indicate that the maximum area of the instrument (experiment 3 on PB2 and PB3 designs and experiment 6 on PB1 design) for the studied Plackett-Burman design are higher, equal, or lower than the maximum area obtained by the OFAT approach. For the analytes concentration of 1 mg L^{-1}, ratios from 0.42 (*p*-aminophenol and acetylsalicylic acid) to 1.67 (acetaminophen) on PB1 design, 0.74 (ibuprofen) to 2.87 (*p*-aminophenol) on PB2 design, and 1.10 (hydroxyibuprofen) to 1.45 (acetaminophen glucuronide) on PB3 design were obtained. Higher average ratio (Supplementary Table S4) was obtained on PB3 design (average ratio of 1.27), followed by PB2 design (average ratio of 1.21), and ending with PB1 design (average ratio of 1.05). A similar behavior was observed in all concentrations tested. As an example, for the PB3 design the average ratios were 1.32, 1.27, and 1.30 for the concentrations of 0.1, 1.0, and 10 mg L^{-1}, respectively. Supplementary Figure S1 (Supporting Material) presents a table into a scheme of ESI conditions with the comparison between the PB3 design and OFAT approach. It was observed that higher signal for each pharmaceutical with lower number of experiments was obtained using Plackett-Burman design (PB3). It is worth mentioning that the number of injections needed using Plackett-Burman design was 4.58 times lower than by OFAT approach, with considerable cost savings as well as being environmentally friendly, reducing the use of organic solvents for LC-MS/MS analysis and extending the life of the equipment. Microsoft Excel flowsheet was created, and the conclusions drawn about the important factors are presented in Supplementary Table S5. Data is shown for only one pharmaceutical (acetaminophen) since the instrument performance for the other compounds was similar. For all pharmaceuticals a negative effect was observed for DGF, which indicates a decrease of the chromatographic areas from level –1 to level +1 with the exception for *p*-aminophenol. For the remaining factors (IV, NGF, HBT, and DLT) and for all pharmaceuticals the results were positive and an increase of the chromatographic areas from level –1 to level +1 was observed.

Regarding the statistical significance of the effects, an error estimate was obtained from the dummy factor effects, *s.e.*, and a statistic *t* was computed as Equation 1:

$$t = \frac{|E|}{s.e.} = \frac{|E|}{\sqrt{\sum\limits_{dummy} E_i^2 \Big/ n_{dummy}}} \tag{1}$$

where *E* stands for a factor effect. This statistic is then compared with *t* critical value for a level of significance of 0.05 and n_{dummy} degrees of freedom. The more degrees of freedom the test has, the more powerful it will be. The effects of the studied factors in the Plackett-Burman design were then presented in standardized Pareto charts (Supplementary Figure S2), which indicate that the NGF and the IV were the only two significant factors for all pharmaceuticals. The charts also show that the IV factor influences the ESI-MS signal more than NGF except for nimesulide and salicylic acid.

Complete Factorial Design

After detecting the significant factors, the optimum operation conditions can only be achieved by implementing a more complex experimental design [22]. A full factorial design is the next step following the algorithm in Figure 1b. It is important to enhance the benefit of using the previous 12-run Plackett-Burman design because it already contains the set of combinations necessary to project into a full 2^2 factorial design with three replicates at the vertices. Additionally, in the last section, the authors also presented runs at the center point; thus, in this stage, no further experiments need to be performed. For the effect experiments of the PB3 design concerning injections of 1 mg·L^{-1} of the analytes were used (Supplementary Table S3) because of the higher signal obtained among the three Plackett-Burman designs studied. Although these experiments include the combination of levels of five factors, only the IV (kV) (x_1) and the NGF (L min^{-1}) (x_2) remain as the main effects for regression model purposes. The chromatographic area was taken as the dependent variable and the results were processed in a spreadsheet. The nine replicates at the center point and the four sets of experiments extracted from the previous Plackett-Burman design (set 1: $x_1 = -1$ and $x_2 = -1$ (Supplementary Table S2, experiments 5, 8, and 12), set 2: $x_1 = -1$ and $x_2 = +1$ (Supplementary Table S2, experiments 2, 7, and 9), set 3: $x_1 = +1$ and $x_2 = -1$ (Supplementary Table S2, experiments 1, 10, and 11), and set 4: $x_1 = +1$ and $x_2 = +1$ (Supplementary Table S2, experiments 1, 10, and 11)) were analyzed.

The data was fitted to a first order model with interaction term (Equation 2 [8]) and its adequacy was checked. To be correctly applied it is necessary that the responses obtained may be well fitted to the Equation 2 for the 2^k factorial design:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \sum_{j>i}^{k} \beta_{ij} x_i x_j + \varepsilon \tag{2}$$

where y is the experimental response, k is the number of factors, x_i are the studied factors, β_0 is the intercept and computed as the grand average of all responses, β_i are one-half of the corresponding factor effect estimates and represent the coefficients of the linear parameters, β_{ij} stands for the interaction term coefficients and are calculated the same as the previous ones, and ε is the residual associated to the errors committed in the experiments or even because of the lack of fit of the regression model.

The values of β_0, β_1, β_2, and β_{12} were predicted by least squares method and substituted in Equation 2. The equations obtained for each pharmaceutical are listed in Supplementary Table S6.

Appropriate analysis of variance (ANOVA) was also carried out. The mean sum of squares of the effects and interaction as well as the curvature is compared against the mean sum of squares of pure error and their significance tested in the model (Supplementary Table S7). The total sum of squares of the responses comprises the sum of squares due to the regression (SS model) and the residual sum of squares (SS residual). The latter can then be further divided into pure error and lack-of-fit error, and the ratio between the means sum of squares of the latter by the former was used to test the adequacy of the model (Table 1).

If the lack of fit of the proposed model is not significant, steepest ascent method may be applied in order to move rapidly towards the optimum region (Figure 1c). Conversely, if there is lack of fit, probably due to a missing pure quadratic effect in the model, additional runs must be performed to improve model adequacy (second order model) (Figure 1d) [8]. It can be observed in Table 1 and Supplementary Table S7, that p-values of the statistic F corresponding to the lack-of-fit error (curvature term) are smaller than 0.05 for all pharmaceuticals except for p-aminophenol (>0.05). In this case, there is no evidence of curvature in the response over the region of exploration. The response of p-aminophenol increases linearly with the increase of levels of the two factors (IV and NGF). In this sense, the highest signal was obtained for the experiments in which both factors were set at level +1. For the other 12 pharmaceuticals there is evidence of curvature in the response over the region of

exploration because p-value is lower than 0.05. Thus, to evaluate possible pure quadratic terms, a central composite face-centered design was then implemented.

Central Composite Design

In order to determine the optimal conditions of the key ESI-MS operational parameters and the effects of their interactions and quadratic terms, a second order model must be applied (Figure 1f). Looking at the conditions used in the previous section (Supplementary Table S3), level +1 already corresponds to the maximum value of IV and NGF parameters allowed by MS ion source interface. Thus, a circumscribed central composite design could not be implemented because the equipment does not allow setting the above factors at levels $-\alpha$ and $+\alpha$ when $|\alpha|>1$. Owing of the aforementioned, a central composite face-centered design, CCF, was chosen. In this design, the treatment combinations are at the midpoints of edges of the space domain and at the center, and are useful in avoiding experiments performed under extreme conditions wherein unsatisfactory results might occur [22]. It is a cubic design plus center points with $2k$ axial points that are situated at a distance ± 1 from the center of the design [23].

When a factor is not significant, it means that regardless of the level, the response remains constant. Before applying the CCF design to this study, it is important to define the numerical values for the non-significant factors, and some considerations must be observed. All the non-significant factors were set at the center level of the PB3 design except the DGF factor, which was set at level -1 corresponding to 12 L·min^{-1}. Although DGF factor does not significantly influence the area of the MS signal it was observed by the previous results that, in general, the lower is the DGF, the higher is the response.

In all runs of CCF design, the non-significant factors remain constant (described in the last paragraph) and NGF and IV were the two factors of the target of study. Once again the chromatographic area was used as the dependent variable, whereas the IV (x_1) and NGF (x_2) were the independent variables. The batch of experiments implemented and the values set for each factor level are presented in Supplementary Table S8.

Table 1. Lack of Fit Test for the Full 2^2 Factorial Design

Pharmaceutical	SS Lack of fit	df Lack of fit	MS Lack of fit	SS Pure Err	df Pure Err	MS Pure Err	F Lack of fit	p-value Lack of fit
Acetaminophen	8.60E+09	1	8.60E+09	9.28E+09	16	5.80E+08	14.8	**1.42E-03**
p-Aminophenol	2.63E+12	1	2.63E+12	2.57E+13	16	1.60E+12	1.64	0.219
Acetaminophen glucuronide	5.17E+09	1	5.17E+09	3.49E+09	16	2.18E+08	23.7	**1.72E-04**
Ibuprofen	3.48E+11	1	3.48E+11	1.73E+11	16	1.08E+10	32.3	**3.42E-05**
Hydroxyibuprofen	5.15E+11	1	5.15E+11	2.36E+11	16	1.47E+10	34.9	**2.20E-05**
Carboxyibuprofen	2.31E+11	1	2.31E+11	9.63E+10	16	6.02E+09	38.4	**1.28E-05**
Acetylsalicylic acid	5.91E+11	1	5.91E+11	1.05E+11	16	6.58E+09	89.8	**5.76E-08**
Salicylic acid	1.36E+14	1	1.36E+14	7.75E+12	16	4.84E+11	281.6	**1.41E-11**
Diclofenac	2.33E+12	1	2.33E+12	3.60E+11	16	2.25E+10	103.7	**2.14E-08**
Dipyrone	3.00E+11	1	3.00E+11	6.43E+10	16	4.02E+09	74.8	**1.99E-07**
Nimesulide	1.42E+14	1	1.42E+14	7.37E+12	16	4.61E+11	307.1	**7.26E-12**
Naproxen	8.06E+11	1	8.06E+11	3.02E+11	16	1.89E+10	42.7	**6.85E-06**
Ketoprofen	1.31E+12	1	1.31E+12	3.07E+11	16	1.92E+10	68.5	**3.56E-07**

SS = Sum of squares; DF = Degree of freedom; MS = Mean square; p-values less than 0.05 (bold type) are statistically significant

A total of nine experimental combination levels were designed and four injections were performed for each combination for a total of 36 injections. The experiments were done randomly and the results (chromatographic areas) were treated as a mean of the four replicates. The mean and relative standard deviation, RSD, were calculated using a spreadsheet. The pharmaceuticals p-aminophenol, nimesulide, and salicylic acid were the compounds with higher area and, conversely, acetaminophen and acetaminophen glucuronide were those whose signal was the lowest. RSDs between 0.44% (run no. 8, diclofenac) and 6.5% (run no. 9, nimesulide) were obtained (Supplementary Figure S3).

The maximum sum of the chromatographic areas obtained for the 13 pharmaceuticals (Supplementary Figure S3) was achieved in run no. 4 using level +1 for both factors (IV and NGF), followed by run no. 6 with level +1 for IV and center point for NGF. The lowest value of the sum of the chromatographic areas for all pharmaceuticals was found in run no. 1 where both factors (IV and NGF) were set at the lower level (level –1).

In order to correlate between factors and the response, a second-order model with interaction was fitted to the experimental data. The general form of the fitting second-order function is presented as Equation 3:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j>i}^{k} \beta_{ij} x_i x_j + \varepsilon \qquad (3)$$

where y is the response, β_0 is the constant, β_i are the first order terms, β_{ii} are the quadratic coefficients, and β_{ij} are the interactive coefficients. The coefficient values obtained by least squares minimization of the model to the experimental data for the studied pharmaceuticals are presented in Supplementary Table S6, where x_1 and x_2 are the IV and NGF factors on the original units, respectively, and y is the corresponding chromatographic area obtained. Also, the model adequacy test was carried out by ANOVA analysis implemented with Statistica package ver. 7.0 (StatSoft, Tulsa, OK, USA) and shown in Table 2.

Replacing the variables x_1 and x_2 with the values of each level [levels –1 (IV = 2.0 kV, NGF = 1 L min^{-1}), 0 (IV = 3.5 kV, NGF = 2 L min^{-1}), +1 (IV = 5.0 kV, NGF = 3 L min^{-1})], it was observed that the signal increased when x_1 and x_2 were increased from –1 to +1, which shows that maximum signal occurs when both IV and NGF take the level value +1 for all pharmaceuticals.

The quality of the regression was statistically checked by calculating the coefficient of determination R^2 and the statistical significance of the model was tested, where the p-value of the statistic F as the ratio of the mean square of the model divided by the mean square of residual is compared against the level of significance of 0.05 (Table 2). The fitted model is considered adequate when the P-value is lower than 0.05 [8]. According to the P-values in Table 2, the second-order model fits well the experimental data for all compounds.

The R^2 value provides a measure of how much variability of the total observed response values can be explained by the model. The closer the R^2 values are to 1, the better the model predicts the response. In this work, the R^2 ranged from 0.8341 for nimesulide to 0.9898 for p-aminophenol indicating that between 83.41% and 98.98% of the total area variance is ascribed to the experimental factors studied (Table 2) and so a good agreement was obtained between observed and predicted values.

However, a t-test made to the significance of individual regression coeficients (data not shown) that are part of Equation 3 prove that each variable is unimportant when a full model is considered containing all remaining variables in stake. Thus, a full second order model with interaction is inadequate. Alternatively a stepwise multiple linear regression was then implemented.

Cluster Analysis

In an attempt to correlate a possible physicochemical property of the analytes with the ESI-MS signal, a hierarchical clustering was carried out using for the effect the CCF design responses. Supplementary Table S9 presents the cluster solution based on Ward's method. The goal was to minimize the within-cluster

Table 2. ANOVA Results for Central Composite Face-Centered Design

Pharmaceutical	SS Model	dof Model	MS Model	SS Residual	dof Residual	MS Residual	F Model	p-value Model	Multiple R^2	Adjusted R^2
Acetaminophen	7.90E+09	5	1.58E+09	6.49E+08	6	1.08E+08	14.6	**0.002629**	0.924093	0.860836
p-Aminophenol	5.91E+13	5	1.18E+13	6.09E+11	6	1.02E+11	116.4	**0.000007**	0.989793	0.981287
Acetaminophen glucuronide	6.39E+09	5	1.28E+09	1.06E+08	6	1.77E+07	72.2	**0.000028**	0.983662	0.970046
Ibuprofen	1.85E+11	5	3.70E+10	1.21E+10	6	2.02E+09	18.3	**0.001421**	0.938524	0.887293
Hydroxyibuprofen	4.50E+11	5	9.01E+10	2.48E+10	6	4.14E+09	21.8	**0.000883**	0.947732	0.904175
Carboyibuprofen	1.77E+11	5	3.55E+10	5.21E+09	6	8.68E+08	40.8	**0.000148**	0.971455	0.947667
Acetylsalicylic acid	9.53E+11	5	1.91E+11	2.05E+10	6	3.41E+09	55.8	**0.000060**	0.978957	0.961420
Salicylic acid	4.19E+13	5	8.38E+12	2.87E+12	6	4.78E+11	17.5	**0.001602**	0.935951	0.882577
Diclofenac	1.14E+12	5	2.28E+11	6.48E+10	6	1.08E+10	21.1	**0.000961**	0.946200	0.901366
Dipyrone	5.13E+11	5	1.03E+11	1.31E+10	6	2.19E+09	47.0	**0.000099**	0.975083	0.954320
Nimesulide	2.29E+13	5	4.58E+12	4.55E+12	6	7.59E+11	6.0	**0.024564**	0.834096	0.695842
Naproxen	4.55E+11	5	9.10E+10	1.76E+10	6	2.93E+09	31.0	**0.000325**	0.962772	0.931749
Ketoprofen	1.04E+12	5	2.09E+11	5.91E+10	6	9.86E+09	21.2	**0.000952**	0.946364	0.901667

SS = sum of squares; DF = degree of freedom; MS = mean square; p-values less than 0.05 (bold type) are statistically significant
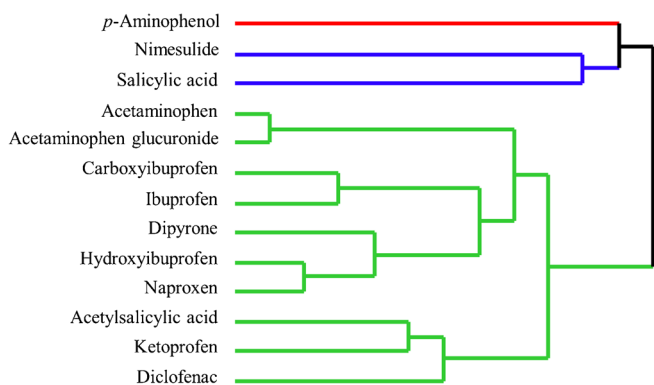
Figure 2. Dendrogram showing affiliations between the pharmaceuticals tested

sum of squares of the responses obtained on the nine combinations of factor levels of the CCF. To facilitate the reading of the clustering process, a dendrogram is represented in Figure 2. It is worth noting that two out of the three most homogeneous clusters are formed by the drug and its metabolite. These are the cases of the clusters CL12 and CL10. It is also noteworthy that CL3 is composed of the two single analytes, the MS signal of which is more influenced by NGF than IV. However, unlike what was found in previous works [24, 25], it was not possible to establish a trend between ESI-MS signal intensity and the pKa or even the molecular volume of the compound.

In order to determine the minimum number of homogeneous clusters in the data set, two statistics were computed viz., R-squared, RS, and semipartial R squared, SPR. The plots of RS and SPR as functions of the number of clusters are shown in Figure 3. These statistics provide information about cluster solution and complement each other. Thus, while RS measures the extent to which clusters are different from each other and, consequently, the higher is RS the more homogeneous each cluster is, the SPR measures the loss of homogeneity when two clusters merge at any given step. It means that a small value of SPR would imply that the merged clusters are homogeneous. By the analysis in Figure 3, it may be observed that a big jump
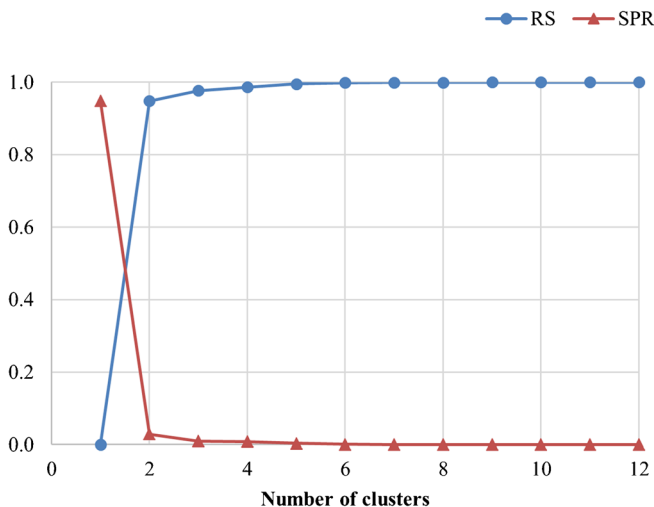

Figure 3. RS and SPR versus the number of clusters

Table 3. Multiple Linear Regression Using the Stepwise Method

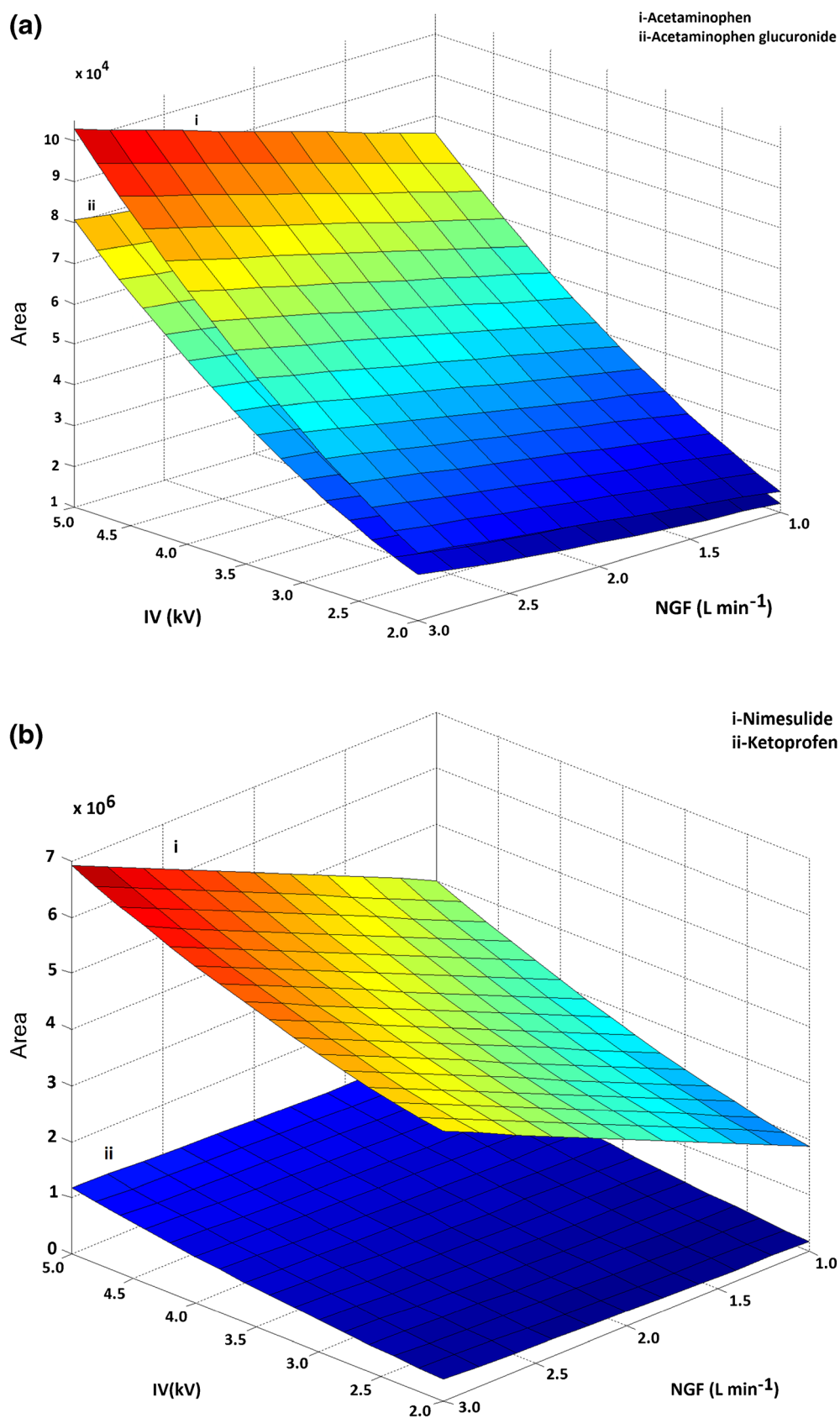| Pharmaceutical	IV			NGF			IV×NGF			IV²			NGF²			$R^2_{adjusted}$	$F_{regression}$
	x_1	s.e.	p-value	x_2	s.e.	p-value	x_1x_2	s.e.	p-value	x_1^2	s.e.	p-value	x_2^2	s.e.	p-value		
Acetaminophen							2733	758	0.005	2476	384	0.000				0.981	307.9
p-Aminophenol	−485666	128183	0.004				238138	33829	0.000	286650	25958	0.000				0.995	823.2
Acetaminophen glucuronide										2834	116	0.000	1079	332	0.009	0.993	796.8
Ibuprofen										16088	985	0.000	8874	2829	0.011	0.985	386.0
Hydroxyibuprofen							14305	4715	0.013	21670	2388	0.000				0.987	450.0
Carboxyibuprofen							6792	2194	0.011	13674	1112	0.000				0.992	731.1
Acetylsalicylic acid							24378	4656	0.000	28826	2359	0.000				0.994	932.0
Salicylic acid	561670	152977	0.004				376993	72002	0.000							0.983	338.5
Diclofenac							40475	8566	0.001	30520	4339	0.000	11872	3008	0.003	0.986	420.7
Dipyrone										26465	1047	0.000				0.993	878.5
Nimesulide				1459559	164476	0.000				101879	22747	0.001				0.977	253.2
Naproxen										25435	1180	0.000	10304	3390	0.012	0.990	625.0
Ketoprofen										38446	2176	0.000	24299	6251	0.003	0.987	469.6

Figure 4. Response surface plots of the MS signal versus IV and NGF for **(a)** acetaminophen glucuronide and acetaminophen; and **(b)** ketoprofen and nimesulide

occurs when, in the clustering process, a step is given from a two-cluster to one-cluster solution. This means that grouping the CCF data in two clusters is the best solution. One of the clusters comprises the compounds that show the highest signal intensities, namely nimesulide, salicylic acid, and p-aminophenol. The other cluster is composed of the remaining 10 compounds.

Stepwise Multiple Regression

The multiple linear regression is one of linear regression analyses that is used to set up the relationship between a single response variable (dependent variable) and two or more predictor variables (independent variables) [26]. Stepwise multiple linear regression was used in this work to determine which factors, interaction, and quadratic terms contribute significantly to explain the variability of the dependent variable. The stepwise mode was used with the criteria of $F < 0.05$ to include terms to the model and $F > 0.10$ to exclude terms beginning with the most significant one-variable model. The forward and backward techniques were then carried out to confirm the results. All data were analyzed by Statistical Package for the Social Science 20.0 (SPSS 20.0) software. Multiple coefficient of determination (R^2), multiple correlation coefficient (R), adjusted multiple coefficient of determination (adjusted R^2), coefficients, excluded variables, and ANOVA results were determined by the SPSS package for each pharmaceutical.

In the first attempt, the constant variable (β_0, variable 1) was included in the regression model. After applying the stepwise method, the p-value of the significance of β_0 of all but two pharmaceuticals (p-aminophenol and nimesulide) showed a value higher than 0.05. Moreover, higher adjusted R^2 were obtained for regressions without constant term. Subsequent multiple regressions were conducted excluding β_0. For models with a different number of adjustable parameters, the higher the value of the adjusted R^2, the better the model that fits the data. For example, the value of adjusted R^2 of the acetaminophen full quadratic model was 0.861 (Table 2), whereas the value of adjusted R^2 of the equivalent partial model (Table 3) is 0.981. Therefore, the fitted equations obtained by stepwise method justify better the data than the full second order model. The values of adjusted R^2 obtained for the studied compounds were in the range of 0.977 (nimesulide) to 0.995 (p-aminophenol).

In Table 3, it can be observed that IV factor (x_1) is excluded for all pharmaceutical regressions except for p-aminophenol and salicylic acid; NGF factor (x_2) was excluded for all pharmaceuticals except nimesulide; IV×NGF interaction (x_1x_2) was excluded for dipyrone, acetaminophen glucuronide, ibuprofen, naproxen, nimesulide, and ketoprofen; IV^2 term (x_1^2) was excluded only for salicylic acid, and NGF^2 term (x_2^2) was excluded for p-aminophenol, acetaminophen, acetylsalicylic acid, carboxyibuprofen, diclofenac, hydroxyibuprofen, nimesulide, and salicylic acid. The quadratic term IV^2 is the one that influences more pharmaceuticals MS signal with 12 out of 13 pharmaceuticals being significant, followed by IV × NGF and before NGF^2. The p-value of the significance of the

regression parameters is always lower than 0.05 and the ANOVA test conducted to the best partial model reveals adequacy.

In order to check the homogeneity of the clusters formed from CCF data, two coupled response surface plots were drawn, one representing the partial regression equation of the two pharmaceuticals, acetaminophen and acetaminophen glucuronide, with the most similar responses (Figure 4a) and, conversely, another pair that belongs to different clusters such as ketoprofen and nimesulide (Figure 4b) with clearly distinct surface responses. From these surface plots it can be concluded that the equation models obtained by the stepwise method are in good agreement with the clusters previously formed.

Optimum MS Ion Source Conditions

The second order partial model obtained by stepwise multiple regression foresees the existence of curvature in the response surface. This property is the necessary condition for the existence of stationary points.

In all regression equations except the one for nimesulide response have one stationary point outside the CCF design including minimum and saddle points. However, none of the response surfaces shows a maximum point. For this reason, a constrained optimization exercise was carried out for all pharmaceuticals.

The same combination of both levels +1 for IV and NGF that maximize the MS area of all pharmaceuticals were obtained by the generalized reduced gradient method embedded in Solver add-in of Microsoft Excel. In Figure 5a, comparison between the experimental areas of the MS signal of all pharmaceuticals and the ones predicted by the multiple stepwise regression equations at the constrained maximum is presented. At the worst case (diclofenac) the relative deviation between the two values is only 6.8%. In addition to the identity solid line depicted in the figure that sets the region where the experimental signal values are equal to the predicted ones, closed dotted lines delimit the points belonging to each cluster. As can be
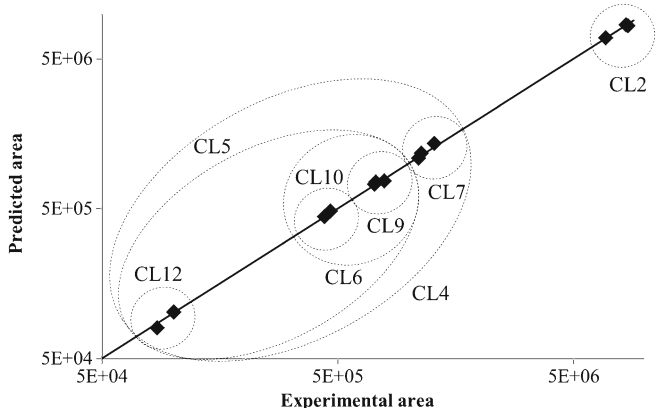

Figure 5. Comparison between predicted MS signal and experimental signal for the constrained maximum. The identity line represents perfect correspondence between the two values. Dashed closed circles represent the clusters

seen in Figure 5, the clustering process was, as a general trend, constructed by the drugs with smaller maximum MS signal towards the higher signal. The cluster CL2, which comprises the three compounds with the highest MS signal, is also the most heterogeneous.

Comparing these results with the best obtained by OFAT approach, an increase in the signal between 34% (dipyrone) to 61% (acetylsalicylic acid) was obtained.

Conclusions

The factors used in the design of experiments were the MS ion source parameters (DGF, NGF, HBT, DLT, and IV). The Plackett-Burman design was used to screen the important factors. Instead of 55 experiments used in OFAT approach, only 12 were necessary for PB design, and increase of the analyte signal between 1.22 (ibuprofen) and 1.58 (acetylsalicylic acid) times higher was obtained. The PB design allowed concluding that the same two factors (IV and NGF) influence MS signal of all 13 pharmaceuticals. The Pareto charts reveal that IV is more significant than NGF, except for nimesulide and salicylic acid, and it was confirmed by the stepwise regression modeling. After screening, a complete factorial design was applied and for all pharmaceuticals except p-aminophenol there is evidence of curvature in the response over the region of exploration. Thus, a central composite face-centered design was carried out in order to find the optimum MS conditions that maximize the signal of the selected pharmaceuticals. The R^2 ranged from 0.8341 for nimesulide to 0.9898 for p-aminophenol, and a good agreement between observed and predicted values was obtained. The ANOVA test of the full second order model indicates that the model is satisfactory but when the t-test for the significance of individual terms is undertaken, neither is important. So, a stepwise multiple linear regression was implemented. Meanwhile, a cluster analysis made to CCF data reveals two principal clusters. One, with the most homogeneous results, corresponds to the analytes with minor MS signal, and another comprising nimesulide, salicylic acid, and p-aminophenol with higher MS signal and simultaneously the most heterogeneous cluster. It was observed that the signal increased when IV and NGF were increased from level -1 to $+1$, which was confirmed by solving a constrained nonlinear optimization problem where the maximum signal occurs when both IV and NGF were set at level $+1$ for all pharmaceuticals.

It was proven that the statistical approach presented in this work provides an increase of the NSAIDs MS areas compared with the best results obtained by OFAT approach and constitute a valuable framework for other chemical analytical optimization studies.

Acknowledgments

Compliance with Ethical Standards

Conflict of Interest   The authors declare no conflict of interest.

References

1. Dohle, S., Campbell, V.E.A., Arvai, J.L.: Consumer-perceived risks and choices about pharmaceuticals in the environment: across-sectional study. Environ. Health **12**, 45–58 (2013)
2. Pinhancos, R., Maass, S., Ramanathan, D.M.: High-resolution mass spectrometry method for the detection, characterization and quantitation of pharmaceuticals in water. J. Mass Spectrom. **46**, 1175–1181 (2011)
3. Cleuvers, M.: Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid. Ecotoxicol. Environ. Saf. **59**, 309–315 (2004)
4. Christensen, F.M.: Pharmaceuticals in the environment—a human risk? Regul. Toxicol. Pharmacol. **28**, 212–221 (1998)
5. Daughton, C.G.: Emerging pollutants, and communicating the science of environmental chemistry and mass spectrometry: pharmaceuticals in the environment. J. Am. Soc. Mass Spectrom. **12**, 1067–1076 (2001)
6. Buxton, H.T.: Pharmaceuticals in the Environment: What we know, and need to know, Available at: http://environmentalhealthcollaborative.org/images/Buxton_Health_Summit_final.pdf. Accessed January 27 (2014)
7. Leardi, R.: Experimental design in chemistry: a tutorial. Anal. Chim. Acta **652**, 161–172 (2009)
8. Montgomery, D.C.: Design and analysis of experiments (8th ed.). John Wiley & Sons, Inc., New York (2013)
9. Box, G.E.P., Wilson, K.B.: On the experimental attainment of optimum conditions. J Royal Stat Soc B **13**, 1–45 (1951)
10. Gu, T., Chen, Z., Jiang, X., Zhou, L., Liao, Y., Duan, M., Wang, H., Pu, Q.: Synthesis and inhibition of N-alkyl-2-(4-hydroxybut-2-ynyl) pyridinium bromide for mild steel in acid solution: Box–Behnken design optimization and mechanism probe. Corros. Sci. **90**, 118–132 (2015)
11. Paíga, P., Delerue-Matos, C.: Response surface methodology applied to SPE for the determination of ibuprofen in various types of water samples. J. Sep. Sci. **36**, 3220–3225 (2013)
12. Raji, M.A., Schug, K.A.: Chemometric study of the influence of instrumental parameters on ESI-MS analyte response using full factorial design. Int. J. Mass Spectrom. **279**, 100–106 (2009)
13. Fernandes, V.C., Vera, J.L., Domingues, V.F., Silva, L.M.S., Mateus, N., Cristina, D.-M.: Mass Spectrometry parameters optimization for the 46 multiclass pesticides determination in strawberries with gas chromatography ion-trap tandem mass spectrometry. J. Am. Soc. Mass Spectrom. **23**, 2187–2197 (2012)
14. Dillon, L.A., Stone, V.N., Croasdell, L.A., Fielden, P.R., Goddard, N.J., Thomas, C.L.: Optimisation of secondary electrospray ionisation (SESI) for the trace determination of gas-phase volatile organic compounds. Analyst **135**, 306–314 (2010)
15. Titato, G.M., Bicudo, R.C., Lanças, F.M.: Optimization of the ESI and APCI experimental variables for the LC/MS determination of s-triazines, methylcarbamates, organophosphorous, benzimidazoles, carboxamide, and phenylurea compounds in orange samples. J. Mass Spectrom. **42**, 1348–1357 (2007)
16. Gros, M., Petrovic, M., Barcelo, D.: Development of a multi-residue analytical methodology based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) for screening and trace level determination of pharmaceuticals in surface and wastewaters. Talanta **70**, 678–690 (2006)
17. Marengo, E., Robotti, E., Gosetti, F., Zerbinati, O., Gennaro, M.C.: Evaluation of signal and noise and identification of a suitable target function the tuning of an ESI ion trap mass spectrometer by multivariate pattern recognition tools. J. Am. Soc. Mass Spectrom. **20**, 1859–1867 (2009)
18. Kruve, A., Herodes, K., Leito, I.: Optimization of electrospray interface and quadrupole ion trap mass spectrometer parameters in pesticides liquid chromatography/electrospray ionization mass spectrometry analysis. Rapid Commun. Mass Spectrom. **24**, 919–926 (2010)

19. Paíga, P., Lolić, A., Hellebuyck, F., Santos, L., Correia, M., Delerue-Matos, C.: Development of a SPE-UHPLC-MS/MS methodology for the determination of nonsteroidal anti-inflammatory and analgesic pharmaceuticals in seawater. J. Pharm. Biomed. Anal. **106**, 61–70 (2015)

20. Lemonakis, N., Skaltsounis, A.-L., Tsarbopoulos, A., Gikas, E.: Optimization of parameters affecting signal intensity in an LTQ-Orbitrap in negative ion mode: A design of experiments approach. Talanta **147**, 402–409 (2016)

21. Mallet, C.: Throublesshooting Common MS Problems. Waters Corporation. Available at: http://vijayaanalyticals.com/pdf/waters%20LCMS%20Troubleshooting.pdf. Accessed February 14 (2015)

22. Ferreira, S.L.C., Bruns, R.E., Ferreira, H.S., Matos, G.D., David, J.M., Brandão, G.C., da Silva, E.G.P., Portugal, L.A., dos Reis, P.S., Souza, A.S., dos Santos, W.N.L.: Box-Behnken design: an alternative for theoptimization of analytical methods–a review. Anal. Chim. Acta **597**, 179–186 (2007)

23. Çam, M., Aaby, K.: Optimization of extraction of apple pomace phenolics with water by response surface methodology. J. Agric. Food Chem. **58**, 9103–9111 (2010)

24. Leito, I., Herodes, K., Huopolainen, M., Virro, K., Kunnapas, A., Kruve, A., Tanner, R.: Towards the electrospray ionization mass spectrometry ionization efficiency scale of organic compounds. Rapid Commun. Mass Spectrom. **22**, 379–384 (2008)

25. Oss, M., Kruve, A., Herodes, K., Leito, I.: Electrospray Ionization efficiency scale of organic compounds. Anal. Chem. **82**, 2865 (2010)

26. Ghani, I.M.M., Ahmad, S.: Stepwise multiple regression method to forecast fish landing. Procedia Soc. Behav. Sci. **8**, 549–554 (2010)