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A. Assoumani, C. Margoum, C. Guillemain, Marina Coquery. Use of experimental designs for the optimization of stir bar sorptive extraction coupled to GC-MS/MS and comprehensive validation for the quantification of pesticides in freshwaters. *Analytical and Bioanalytical Chemistry*, 2014, 406 (11), p. 2559 - p. 2570. 10.1007/s00216-014-7638-6 . hal-00996886

HAL Id: hal-00996886

<https://hal.science/hal-00996886>

Submitted on 27 May 2014

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Use of experimental designs for the optimization of stir bar sorptive extraction coupled to GC-MS/MS and comprehensive validation for the quantification of pesticides in freshwaters

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Abstract

Although experimental design is a powerful tool, it is rarely used for the development of analytical methods for the determination of organic contaminants in the environment. When investigated factors are interdependent, this methodology allows studying efficiently not only their effects on the response, but also the effects of their interactions. A complete and didactic chemometric study is described herein for the optimization of an analytical method involving stir bar sorptive extraction followed by thermal desorption coupled with gas chromatography and tandem mass spectrometry (SBSE-TD-GC-MS/MS) for the rapid quantification of several pesticides in freshwaters. We studied, under controlled conditions, the effects of thermal desorption parameters and the effects of their interactions on the desorption efficiency. The desorption time, temperature, flow, and the injector temperature were optimized through a screening design and a Box Behnken design. The two sequential designs allowed establishing an optimum set of conditions for maximum response. Then, we present the comprehensive validation and the determination of measurement uncertainty of the optimized method. Limits of quantification determined in different natural waters were in the range of 2.5 ng L⁻¹ to 50 ng L⁻¹ and recoveries were between 90 and 104%, depending on the pesticide. The whole method uncertainty, assessed at 3 concentration levels under intra-laboratory reproducibility conditions, was below 25% for all tested pesticides. Hence, we optimized and validated a robust analytical method to quantify the target pesticides at low concentration levels in freshwater samples, with a simple, fast, and solventless desorption step.

Keywords: Experimental designs; SBSE; thermal desorption; GC-MS/MS; measurement uncertainty; natural waters

Introduction

Since its introduction by Baltussen et al. in 1999 [1], stir bar sorptive extraction (SBSE) has become one of the most used miniaturized extraction and enrichment technique of organic contaminants in aqueous samples. This solventless technique is based on the same principles as solid phase microextraction (SPME). In other words, the extraction is governed by diffusion of the analytes and its efficiency relies on equilibrium, *i.e.*, the partitioning of the analytes between the sample and the polydimethylsiloxane (PDMS) extraction phase [2]. But SBSE can achieve better extraction recoveries, especially for moderately hydrophobic analytes, as the volume of the extraction phase of the SBSE stir bars (24 μL to 126 μL) is much larger than the volume of the SPME fibers (up to 0.5 μL) [3, 4]. Stir bar sorptive extraction has been applied in pharmaceutical, biomedical, food, and environmental analysis for a wide range of organic chemicals, from polar endocrine disruptors (EDC) such as estrogens, across moderately hydrophobic pesticides to highly hydrophobic polycyclic aromatic hydrocarbons (PAH) and polychlorobiphenyl (PCB) congeners. Several reviews covered in details the analytical applications of this technique [2, 4–6]. The extraction step is followed by desorption, either with organic solvent (liquid desorption, LD) or with heat (thermal desorption, TD) depending on the hydrophobicity and the thermal stability of the analytes, before analysis by liquid or gas chromatography. The optimization of the extraction conditions of organic chemicals in water samples has been carried out in several studies [7–13]. Desorption conditions need to be investigated as well, in order to achieve optimal back extraction of the analytes sorbed in the PDMS phase and yield maximal sensitivity and

precision for the LC or GC analyses. Thermal desorption coupled with GC is more environmentally-friendly as it requires no organic solvent, and allows to reach lower limits of quantification as the whole amount of extracted analytes is transferred into the GC. For thermal desorption, the desorption time, the desorption temperature, the focusing temperature and the desorption flow are the most influential parameters.

Sanchez-Ortega et al. [11] and Camino-Sánchez et al. [12] performed step-by-step optimizations of the thermal desorption of some pesticides. However, this methodology does not allow to study the effect of the interaction of factors and guarantees to hit optimum conditions only if the studied factors are independent [14]. In case interactions between factors are important, only a multivariate approach allows to reach the real optimum, *i.e.*, the best conditions for thermal desorption in this case [15]. This methodology has been used in very few studies for the optimization of the desorption of pesticides from SBSE stir bars. For instance, in order to determine the optimal liquid desorption conditions before the analysis of moderately hydrophobic to hydrophobic pesticides by liquid chromatography coupled with tandem mass spectrometry, Margoum et al. [13] realized an experimental design, which allowed them to investigate the effect of factors and the effect of their interactions on the selected response. Also, MacNamara et al. [15] used this methodology to optimize the parameters of extraction of organophosphorous pesticides from water, together with the parameters of thermal desorption. The use of such methodology for the optimization of the desorption of pesticides from SBSE stir bars, as well as for the optimization of other steps of analytical methods, might seem complex and time-consuming. In fact, quite the reverse, this approach is much faster than traditional one-variable-at-the-time (OVAT) methodology, as the effect of all the factors and the effect of their interactions are studied at the same time. Moreover, regarding thermal desorption optimization, all experiments of the design can be realized in the same analytical sample sequence. Nonetheless, didactical tutorials are needed

to better describe this way of considering optimization and for a larger use of this powerful methodology.

In the present paper, we describe a complete multivariate optimization with the methodology of experimental designs. The aim of this work is to develop an analytical method consisting in SBSE followed by thermal desorption and gas chromatography coupled with tandem mass spectrometry (SBSE-TD-GC-MS/MS). Then, following comprehensive and thorough protocols, we validated the optimized method and determined the measurement uncertainties for the analysis of pesticides in freshwater samples. To realize this study, we selected a relevant set of 8 relatively hydrophobic to hydrophobic pesticides ($3.08 < \log K_{ow} < 4.96$) usually encountered in freshwaters of agricultural watersheds. The extraction conditions have been developed and validated previously for liquid desorption followed by liquid chromatography coupled with tandem mass-spectrometry (SBSE-LD-LC-MS/MS) analysis [13]. In the present study, two experimental designs were conducted for the optimization of the thermal desorption of the selected pesticides. The effect of the desorption time, the desorption temperature, the desorption flow and the injector temperature, and the effect of their interactions were investigated with a screening experimental design. Then, the three most significant parameters were optimized by a second experimental design in order to reach maximal desorption of all selected pesticides. A third experimental design was realized to determine the best conditions for the thermal decontamination of the SBSE stir bars after the desorption step. Finally, the optimized method was validated regarding linearity, limit of quantification (LOQ), trueness and precision following a comprehensive validation procedure, and the analytical measurement uncertainty was assessed in conditions of intra-laboratory reproducibility.

Experimental

Reagent and chemicals

The 8 selected pesticides (acetochlor (ATC), diflufenican (DFF), metolachlor (MTC), chlorfenvinphos (CFV), chlorpyrifos-ethyl (CPE), chlorpyrifos-methyl (CPM), fenitrothion (FNT), and procymidon (PCM)), the analytical standards, *i.e.*, deuterated surrogates (chlorpyrifos-ethyl d10 (CPE-d10) and fenitrothion d6 (FNT-d6)), and the internal standard (hexabromobenzene (HBB)) were supplied by VWR (Strasbourg, France) and Sigma Aldrich (Saint-Quentin Fallavier, France). Sodium chloride NaCl (purity = 99.5%) was purchased from VWR (Strasbourg, France).

GC grade methanol, acetonitrile, acetone, and dichloromethane were purchased from VWR (Strasbourg, France). Ultrapure water was obtained using a Millipore water purification system equipped with a LC-Pak cartridge (Billerica, MA, USA).

The individual pesticide stock solutions were prepared in acetone at a concentration of 100 mg L⁻¹ and stored at 4 °C for two months. Standard solutions for extraction were prepared daily with ultrapure water by appropriate dilution of the stock solutions. Helium gas (purity = 99.999%) used for thermal desorption and chromatographic analysis, nitrogen gas (purity = 99.995%) used for thermal decontamination, and carbon dioxide gas (purity = 99.5%) used for focusing the desorbed pesticides in the cooled GC injector were purchased from Air Liquide (Juhem, France).

Sample preparation and SBSE procedure

The extraction method was optimized and validated with spiked samples of ultrapure water, Evian® mineral water, and surface water from two rivers in France (Saône River and Morcille

River). Prior to SBSE, the river water samples were filtered under vacuum using 0.7- μ m glass fiber filters (GF-F, Whatman).

The development and the validation of the extraction of the selected pesticides by SBSE have been published elsewhere [13]. Briefly, the extraction of the pesticides was carried out with SBSE stir bars (20 mm length \times 1 mm film thickness with ca. 126 μ L of PDMS phase) from Gerstel (Mülheim an der Ruhr, Germany). Before each use, the stir bars were thermally conditioned at 50 °C for 24 h, then rinsed in ultrasonic bath with 10 mL of methanol/dichloromethane mixture (50/50, v/v) for 30 min, and finally dried at 50 °C for 1 h. The extraction was performed on 20 mL of water samples with 2.0 g of NaCl at 800 rpm for 3 h. After the extraction step, SBSE stir bars were gently rinsed with ultrapure water and dried with Kimwipes® precision paper, then placed overnight at -18 °C before chemical analysis.

Instrumentation and analytical conditions

Thermal desorption of the stir bars was performed with a CTC Analytics CombiPal Autosampler (Zwingen, Switzerland), equipped with a desorption oven. During the desorption phase, the desorption oven was directly connected to a programmable temperature vaporization (PTV) injector, which was installed in a Varian 3800 GC (Bruker, Marne-la-Vallée, France).

Stir bars were placed in empty glass tubes for thermal desorption, spiked with 5 μ L of hexabromobenzene at 20 mg L⁻¹ in acetone as internal standard prior to analysis. The analytes were desorbed from the stir bars in the optimized conditions and focused, by carbon dioxide cooling when needed, in a liner packed with deactivated glass wool at the optimized PTV injector temperature in split mode. The split is located at the bottom of the PTV injector, which means the whole part of pesticides desorbed from the stir bars in the desorption oven is

focused in the liner, whatever the split ratio. The PTV injector was then ramped at 200 °C min⁻¹ to a final temperature of 280 °C (held for 10 min) and the analytes were transferred into a Zebron ZB-5ms capillary column (30 m x 0.25 mm x 0.25 µm) provided by Phenomenex (Le Pecq, France). The carrier gas was helium at a flow rate of 1.0 mL min⁻¹. The column oven was programmed at the same temperature as the injector during 10 min, ramped at 30 °C min⁻¹ to 180 °C, at 5 °C min⁻¹ to 240 °C, at 10 °C min⁻¹ to 280 °C, and finally at 30 °C min⁻¹ to 300 °C and held for 5 min, for a total analysis time of 36 min. After thermal desorption and during the chromatographic separation of the analytes, the SBSE stir bars were kept in the desorption oven for thermal decontamination with nitrogen gas under the optimized conditions, to insure total elimination of pesticide residues. Each SBSE stir bar was desorbed and decontaminated for 60 min.

The mass detector Varian 4000MS (Bruker, Marne-la-Vallée, France) was an ion trap equipped with an external source operated in electronic impact ionization mode at 70 eV, and the analytes were monitored in MS/MS mode except for hexabromobenzene, which was detected in SIS (selected ion storage) mode. The MS/MS settings of the selected pesticides and the surrogates were determined by automatic optimization using MS Workstation software (version 6.9), assisted by manual optimization. Two types of collision-induced dissociation (CID) of the target pesticides were investigated, resonant CID and non-resonant CID. Resonant CID is more compound specific and more energetic than non-resonant CID. It is originally more adapted to stable parent ions such as PAH, PCB, dioxins and furans. For these two types of CID, two types of waveforms were applied to the ion trap (Table 1). In resonant mode, waveforms represent the application of low voltage and high frequency whereas, in non-resonant mode, high voltage and low frequency are applied. The identification of the selected pesticides was performed with the retention time, the quantification transition and the mass spectrum of the fragmentation products (Table 1).

Experimental designs for method optimization

The optimization of the thermal desorption parameters was realized in two sequential experimental designs. Statgraphics software (Centurion XV.II) from SigmaPlus was used for data processing. Firstly, a fractional factorial design $2^{4 \times 3/4}$ was realized for screening purpose (Design n°1). In this design, 4 factors with 2 levels (low level (-1)) and high level (+1)) were investigated, which represent, for a full factorial design, 2^4 experiments. We planned to realize 3 quarters of these experiments, with 2 additional experiments at the center point of all factors (medium level (0)), making a block of 14 experiments. This block was repeated once for a total of 28 experiments and 16 degrees of freedom for the experimental error. The low level, the center point and the high level of each factor investigated in design n°1 are presented in Table 2. We selected this design in order to obtain the same information as from a classical full factorial design, but with a smaller number of experiments. In other words, both designs allow the evaluation of the effect of the factors and the effect of the two-factor interactions, with enough degrees of freedom for the experimental error; but the fractional design comprises 28 experiments, whereas a full factorial design replicated twice (2 blocks) with 2 center points per block needs 36 experiments. The effect of the desorption time, the desorption temperature, the desorption flow, the injector temperature, and the effect of their interactions on the pesticide chromatographic peak areas were evaluated and rated, in order to identify and select the most influential factors.

Secondly, the most 3 influential factors (the desorption temperature, the desorption flow, the injector temperature) were selected and optimized with a Box Behnken design (Design n°2). In this design, the 3 factors with 3 levels (low level (-1)), medium level (0) and high level (+1)) were investigated in 12 experiments. Three experiments at the center point of all factors

(medium level (0)) were added, making a block of 15 experiments. This block was repeated once for a total of 30 experiments and 19 degrees of freedom for the experimental error. This design allows the determination of response surfaces with fewer experiments than a 3^3 full factorial design (30 herein versus 54 in a full factorial design replicated twice, *i.e.*, 2 blocks), but provides enough degrees of freedom to evaluate the experimental error. Fig. 1a and Fig. 1b show respectively graphical representations of the Box Behnken design and the 3^3 full factorial design as cubes. The three axes of the cubes represent the 3 thermal desorption factors to be optimized, with their levels (low level (-1), medium level (0), and high level (+1)), and the dots represent the experiments of the designs. For the graphical representation of the Box Behnken design, the experiments are located at the middle of each edge of the cube, so 12 experiments. The dots at the center of the cube represent the 3 experiments added at the center point of all 3 factors, so a total of 15 dots. For the graphical representation of the 3^3 full factorial design, the experiments are located at each corner (8 experiments), at the middle of each edge (12 experiments), at the middle of each face (6 experiments) and at the center of the cube (1 experiment), so a total of 27 experiments. These 27 experiments represent all 27 combinations of the 3 levels of 3 factors.

The 3 levels of each factor investigated in design n°2 were the same as those for design n°1 (Table 2), except for the desorption temperature, which were 220, 260 and 300 °C. Design n°1 revealed that the desorption temperature had a positive effect on the desorption of the studied pesticides. Therefore, we decided to investigate the effect of this factor at higher temperatures. Changing the levels of the factors had no impact on the interpretation of design n°2 as the data of the two designs were processed separately. Also, according to the results of the design n°1, this might result in higher desorption of all studied pesticides. The high level for this factor is the maximum temperature for thermal desorption of the SBSE stir bars as

advised by Gerstel. For both experimental designs, the blocks represented two different days for chemical analysis.

In addition, the optimal conditions for the thermal decontamination of the SBSE stir bars after the desorption step were investigated with a third experimental design (Design n°3). Another Box Behnken design was carried out (1 block, 3 center points, and 3 factors with 3 levels for a total of 15 experiments and 9 degrees of freedom for the experimental error). The 3 levels of each factor investigated in design n°3 are presented in Table 3. In this experiment, we investigated the effect of the decontamination temperature, the decontamination time, and the amount of pesticides in the SBSE stir bar on the decontamination efficiency. The amount of pesticides in the stir bars was controlled by extraction of water samples of different pesticide concentrations. Depending on the pesticide, the concentrations in water samples varied, but were set for each pesticide as 50 times the respective LOQ ($50 \times \text{LOQ}$) for the low level, $1025 \times \text{LOQ}$ for the center point, and $2000 \times \text{LOQ}$ for the high level. For design n°3, the responses were the pesticide chromatographic peak areas of the subsequent analysis of the SBSE stir bars. In other words, the SBSE stir bars used for the extraction of water samples spiked with all the pesticides at different concentrations were thermally desorbed for analysis and decontaminated under each set of the conditions established in the experimental design; then, they were thermally desorbed a second time for analysis in order to determine the amounts of pesticides remaining after the thermal decontamination step of the first analysis.

Validation of the optimized SBSE-TD-GC-MS/MS method

Although it is essential to ensure robustness and reliability of an analytical method, the validation is often performed only partially or in non-representative conditions, so performance results may be inapplicable to real samples. For instance, limits of detection

(LOD) and LOQ may be underestimated when determined in standard solutions because matrix effects are not considered. These limits may also be underestimated when evaluated with signal-to-noise (S/N) ratios, as they only represent instrumental limits and repeatability is usually not evaluated [16]. Moreover, trueness and precision of the extraction recoveries are usually determined at a single level of spiking, although they may vary along the concentration range. Measurement uncertainty is an estimation of the error on the analytical result taking into account sources of variability such as the preparation of the samples, the sample matrix, and the day of the analysis. It represents the interval in which the true concentration value of a real sample can be expected [13].

The method validation was inspired from standard methods ISO/IEC 17025:2005 and AFNOR NF T90-210:2009 [17, 18], and previous validations of LC-MS/MS methods [13, 19]. Measurement uncertainty of the analytical method was assessed according to standard methods ISO 11352:2012 and AFNOR XP T 90-220:2003 [20, 21], and previous validation of an LC-MS/MS method [13].

Study of the linearity range

For each selected pesticide, two adjacent calibration ranges (a low concentration range and a high concentration range, with one common concentration level) were investigated. Each calibration curve was constituted with pesticide standard solutions prepared in ultrapure water and extracted by SBSE. Each calibration range was composed of 6 concentration levels, which could be different depending on the pesticide. The linearity of the method was statistically tested with 5 calibration curves. All these curves were obtained through extraction of ultrapure water samples spiked with the selected pesticides. Linearity was validated when the correlation coefficient (R^2) of each calibration curve (5 x 2 curves per pesticide) and their

mean value were higher than 0.990 and the deviation between theoretical and calculated standard concentration values were lower than the maximal acceptable deviation (MAD). These MAD were fixed for each concentration level from 10% to 60% (60% for the lowest concentration level, at LOQ level).

Verification of the limits of quantification

The LOQ was first estimated, then it was verified experimentally for each pesticide. First, the instrumental LOQ were determined with an S/N of 10. Then, to take matrix effects into account, this instrumental LOQ were corrected with a security factor ($k = 5$). For each compound, this theoretical limit of quantification (LOQ_{th}) was verified by spiking and analyzing water samples ($n = 10$). Mean concentration (\overline{LOQ}) and standard deviation (s_{LOQ}) were calculated and compared to an MAD fixed at 60% of the LOQ_{th} . For each pesticide, the two following equations had to be verified:

$$\overline{LOQ} - 2 \times s_{LOQ} > LOQ_{th} - 60\% \times LOQ_{th} \quad (1)$$

$$\overline{LOQ} + 2 \times s_{LOQ} < LOQ_{th} + 60\% \times LOQ_{th} \quad (2)$$

Trueness and precision

The trueness and precision of the method were assessed at LOQ level, at a medium concentration level, and at a high concentration level of the calibration curves. For each pesticide, the LOQ level and the medium concentration level were on the low concentration range curve, whereas the high concentration level was on the high concentration range curve. Five duplicates of water samples were spiked at each concentration level, and mean recoveries and the corresponding relative standard deviations (RSD) were determined under

within-laboratory reproducibility conditions [20, 21]. Mean recoveries were determined with calibration curves constituted with pesticide standard solutions prepared in water and extracted by SBSE.

Measurement uncertainty

Three operators prepared spiked water samples with 3 types of water (Evian® mineral water, and surface waters from 2 rivers in France); then the extractions of the water samples and the analyses were performed on different days. The measurement uncertainty was assessed for the same 3 concentration levels as the determination of precision of the method, *i.e.*, at LOQ level, at a medium concentration level, and at a high concentration level. Ten triplicates for each concentration level ($n \times p = 30$) were realized under intra-laboratory reproducibility conditions [20, 21]. For each concentration level, a Cochran test and a Grubbs test were performed on the experimental results; then the repeatability variance (s_{repeat}^2) and the reproducibility variance (s_{repro}^2) were determined as follows:

$$s_{\text{repeat}}^2 = \frac{\sum_{i=1}^p \sum_{j=1}^n (x_{ij} - \bar{x}_i)^2}{(n-1)p} \quad (3)$$

$$s_{\text{repro}}^2 = \frac{\sum_{i=1}^p n(\bar{x}_i - \bar{\bar{x}})^2}{n(p-1)} + \frac{(n-1)s_{\text{repeat}}^2}{n} \quad (4)$$

where $p = 10$, $n = 3$, x_{ij} is the calculated concentration for a sample, \bar{x}_i is the average calculated concentration for one triplicate, and $\bar{\bar{x}}$ is the average of all 30 calculated concentrations.

Finally, the measurement uncertainty (U) was calculated as follows with an expanded coverage factor (k) of 2:

$$U(\%) = \frac{k \times s_{\text{repro}}}{x} \times 100 \quad (5)$$

Results and discussion

Experimental designs

Screening of the desorption parameters by a fractional factorial design (Design n°1)

Table 4 shows the experimental design and the experimental matrix composed of 8 columns as responses (pesticide areas) and 28 rows for the experiments, obtained from the analyses of stir bars under the thermal desorption conditions set by the experimental design. A Principal Component Analysis (PCA) was applied to this data matrix (previously mean-centered) with Statgraphics software. The first vector explained 74% of the total variance of the experimental results; and all variables (the chromatographic peak areas of the studied pesticides) had positive loadings on this vector (Fig. 2a). This means that all studied pesticides had similar behaviors regarding the thermal desorption. Therefore, we considered the score of each experiment on this vector (Fig. 2b) as a global response (to be maximized). This methodology for determination of a global response has been previously used in the successful optimization of a SBSE-TD-GC analysis of 7 pesticides in water [15]. On Fig. 2b, each experiment has a score on the first vector and on the second vector, and is represented by a “+” symbol. The two experiments which obtained the best scores on the first vector are experiments n°2 and n°16 (Table 4). For both experiments, the thermal desorption conditions were desorption temperature at 280°C, desorption flow at 75 mL.min⁻¹ for 10 min with the injector

temperature at 60 °C. Therefore, the results of the PCA could give an indication on the conditions which result in high desorption of the pesticide.

The statistical analysis of this screening design, with the score on the first vector as response, showed that there was no significant experimental error and no significant difference in the results of the experiments of the two blocks. The model obtained, with $R^2 = 0.97$, is the following:

$$Y = 20.3 - 0.42A^{***} - 0.06B^{**} - 1.00C^{***} - 0.15D^{**} + 0.001AB^{***} + 0.02AC^{***} + 0.002AD^{***} + 0.0003BC + 0.00003BD + 0.009CD^{***}$$

where A = Desorption flow, B = Desorption temperature, C = Desorption time, and D = Injector temperature, and AB, AC, AD, BC, BD, CD are their two-factor interactions. The significant terms in the model are marked with stars: (*) means p-values < 0.05, (**) means p-values < 0.01, and (***) means p-values < 0.001. So, in this case, a very high level of significance was obtained. Not only the effects of all 4 factors were significant, but also the effects of 4 interactions were significant, which could not have been observed with an OVAT optimization methodology. Moreover, two of these interactions were even quite unexpected. Indeed, one can note the significance of AD and CD, two interactions that involve factors from different parts of the analytical system (desorption oven and PTV injector). The statistical analysis of the design showed that all factors and interactions had positive effect on the response. This means that the desorption flow, temperature and time, and the injector temperature have to be set at their higher levels for maximum response. Moreover, regarding the interactions, the effect of an increase of desorption temperature, for instance, is greater when desorption flow is at higher level.

Optimization of thermal desorption (Design n°2) and decontamination (Design n°3) by Box Behnken designs

On the basis of the results of design n°1, we decided to set the desorption time at 10 min, the maximal level tested, without performing further optimization for higher values, in order to keep the total analysis time at 60 min maximum. Thus, the optimization of the thermal desorption was realized with the injector temperature (Factor A), the desorption flow (Factor B), and the desorption temperature (Factor C). Table 5 shows the Box Behnken experimental design and the resulting experimental matrix. Similarly to the results of the design n°1, a PCA was applied to the experimental results of the design n°2 (previously mean-centered) and showed that all the variables have positive loading on the first vector (Fig. 3a), which explained 77% of the variance. Therefore, we considered the score of each experiment on the same vector (Fig. 3b) as a global response (to be maximized). On Fig. 3b, the two experiments which obtained the best scores are experiments n°12 and n°27 (Table 5). For both experiments, the optimal thermal desorption conditions were desorption temperature at 260 °C, desorption flow at 75 mL.min⁻¹ for 10 min with the injector temperature at 60 °C, these values confirm the results of the PCA realized with the data of design n°1.

The model, obtained with $R^2 = 0.98$ and leading to the response surface shown in Fig. 4, is the following:

$$Y = 35.1 - 0.48A^{***} - 0.37B^{***} - 0.18C^{***} + 0.002AA^{***} + 0.004AB^{***} + 0.0007AC^{**} + 0.002BB + 0.0004BC + 0.0003CC^{*}$$

The evaluation of the effects of the main factors and the effect of their interactions led to the following optimized conditions of the thermal desorption of the selected pesticides: 300 °C

during 10 min under a helium flow of 75 mL min⁻¹ in split mode (1/75 ratio), while maintaining a focusing temperature of 60 °C in the PTV injector of the GC-MS/MS system. Another Box Behnken design was realized to optimize the conditions of thermal decontamination (Design n°3) of the SBSE stir bars after desorption (Table 3). Only traces of chlorpyrifos-methyl, with area equivalent to the area of blank samples, were detected in the second GC-MS/MS analyses of the stir bars corresponding to the highest concentration level tested during the experimental design. Therefore, the optimized conditions for the thermal decontamination of the SBSE stir bars were the highest point in the experimental space, *i.e.*, 300 °C for 50 min.

Advantages of the experimental design approach

The first advantage of a multivariate approach over an OVAT approach is the time saved for the whole experimentation process. Indeed, for screening purposes, building an experimental design implies defining all the factors that can have an effect on the response studied before realizing any experiment. Whether in OVAT approach, usually, only the factors that the operator believes will have an effect are tested [14]. Moreover, contrary to what is commonly thought, fewer experiments are needed in an experimental design to obtain the same -or more information- as for an OVAT approach. For instance, in our case, 28 experiments were performed to study at the same time the effect of 4 factors and the effect of their interactions, at 2 levels, in two blocks and to assess the experimental error. If the same study is performed with an OVAT approach, triplicates are usually needed to assess the experimental error. Therefore, to obtain the same level of information (excluding the effect of the interaction of the factors), 48 experiments would be necessary.

Also, screening designs allow to assess the significance of factors and to select the most significant ones regarding the response in a non-arbitrary way. Another advantage of our experimental design is the study of interactions between factors. In case factors are interdependent, an OVAT approach does not guarantee to reach the real optimum. Moreover, unexpected significant interactions might be detected [14].

Finally, in the present study, the data matrix of 8 columns as responses and 28 rows for the experiments allowed to realize a robust PCA and to obtain reliable information regarding the behaviors of the tested pesticides during the thermal desorption step.

Performances and validation of the analytical method

The results of the validation of the analytical method are reported in Table 6. Excellent linearity was observed for all calibration curves ($R^2 \geq 0.9972$) and the deviation between theoretical and calculated standard concentrations was between 10% for the highest concentration level and 60% for the lowest one.

The LOQ in water were validated in accordance with Eq. 1 and Eq. 2; they ranged from 2.5 ng L⁻¹ for chlorpyrifos-methyl to 50 ng L⁻¹ for diflufenican. Based only on S/N ratios of 10, instrumental LOQ ranged from 0.05 to 10 ng L⁻¹. But, we validated LOQ in different water matrices while ensuring required trueness and repeatability. Our method allowed to obtain LOQ which are more relevant for the routine analysis of unknown freshwater samples. These LOQ are in agreement or up to 5-fold higher than those reached in previous SBSE-TD-GC-MS/MS analytical methods for chlorpyrifos-methyl, chlorpyrifos-ethyl, chlorfenvinphos, fenitrothion, and metolachlor in water samples [12, 22]. In those methods, however, either the type of water used for the determination of the LOQ was not specified or the LOQ were instrumental (*i.e.*, an S/N ratio of 10). To our knowledge, the SBSE-TD-GC-MS/MS analysis

of moderately hydrophobic to hydrophobic pesticides ($3.80 < \log K_{ow} < 4.20$) with different physical chemical properties such as procymidon, acetochlor, and diflufenican in water samples has not been reported in the literature.

For all selected pesticides and all concentration levels, mean recoveries, determined with calibration curves constituted with pesticide standard solutions prepared in water samples and extracted by SBSE, ranged from 90 to 104% (Table 6). Satisfactory global inter-day precision, estimated at each concentration level as RSD of 10 determinations on spiked water samples, was between 5.2 and 17.4%.

Results on measurement uncertainty are reported in Table 6. For all selected pesticides at the three concentration levels, satisfactory measurement uncertainty for SBSE-TD-GC-MS/MS was obtained ($U \leq 25\%$). To our knowledge, very few validations of SBSE-TD-GC-MS/MS methods have included studies of the uncertainty of the analytical results [12, 23]. Regarding the pesticides studied herein, Camino-Sánchez et al. [12] reported uncertainties ranging from 40 to 46% for chlorfenvinphos, chlorpyrifos-ethyl, chlorpyrifos-methyl, fenitrothion, and metolachlor; but the uncertainties were determined with another approach on which very few details were presented. In our method, 90 water samples were used for the determination of realistic uncertainties under intra-laboratory reproducibility conditions, *i.e.*, with 3 types of water, at 3 concentration levels, prepared by 3 operators and analyzed at different days. Results showed for all target pesticides homogeneous uncertainty across the concentration range. Margoum et al. [13] used the same approach for the evaluation of uncertainties on the analysis of 15 pesticides by SBSE-LD-LC-MS/MS. We obtained similar uncertainty values for the analysis of pesticides targeted by both methods, *i.e.*, chlorfenvinphos, chlorpyrifos-ethyl, diflufenican, fenitrothion, and procymidon.

In comparison with the SBSE-LD-LC-MS/MS method developed and validated by Margoum et al. [13] for the analysis of pesticides, 2 to 100-fold lower LOQ were obtained by our SBSE-

TD-GC-MS/MS method, partly because the whole amount of pesticides sorbed in the SBSE stir bar is transferred into the GC for chemical analysis. Moreover, thermal desorption allows much faster and easier sample treatment. After the extraction step, the SBSE stir bar is simply placed into desorption tube for online thermal desorption and GC-MS/MS analysis, instead of substantial extra time for offline liquid desorption and preparation of the sample to be injected into the LC. Finally, thermal desorption and decontamination allow dramatic reduction of organic solvent consumption.

Conclusion

Experimental design is a powerful approach for optimization of analytical method and the larger is the number of factors to be evaluated, the more this approach is necessary for a comprehensive, simple and fast study. As the use of such methodology is mistakenly thought complex and time-consuming, didactic tutorials are needed to better describe it. In this study, we used two sequential experimental designs for the optimization of the desorption conditions of an SBSE-TD-GC-MS/MS method for the determination of 8 selected pesticides in freshwaters. Design n°1 allowed a screening of the effect of the desorption time, the desorption temperature, the desorption flow, the injector temperature, and the effect of their interactions on the peak areas of the target pesticides as responses. Design n°2 resulted in the optimization of the 3 desorption parameters that proved to be influential by the screening design to obtain maximal responses (*i.e.*, the desorption temperature, the desorption flow and the injector temperature). We developed and validated a robust and reliable analytical method for simple, fast, and environmentally-friendly analysis of agricultural pesticides in freshwaters.

In this study, a set of 8 moderately hydrophobic to hydrophobic pesticides ($3.08 < \log K_{ow} < 4.96$) was used for the optimization of the analytical method by means of experimental designs. As this interesting approach should be applied for other method optimizations, further work will focus on adding other hydrophobic micropollutants in this SBSE-TD-GC-MS/MS method.

Acknowledgements

The authors thank the French National Research Agency (ANR) through PoToMAC project (ANR 2011 CESA 022 02) and the French National Agency for Water and Aquatic Environments (ONEMA) via the AQUAREF programme for financial support.

501 Table 1. Retention time and mass spectrometry detection conditions of the selected pesticides

Compound	Abbreviation	Retention time (min)	Scan type	Quantification transition (m/z)	Waveform type	Excitation amplitude (V)	Excitation storage level (m/z)
Acetochlor	ATC	19.1	MS/MS	223 > 146	non-resonant	65	98.2
Chlorpyrifos-methyl	CPM	19.1	MS/MS	286 > 208	resonant	1.06	126
Fenitrothion d6 ^a	FNT-d6	19.8	MS/MS	283 > 266	non resonant	44	77.9
Fenitrothion	FNT	19.9	MS/MS	277 > 260	non resonant	63	122.0
Metolachlor	MTC	20.2	MS/MS	238 > 162	non resonant	73	104.8
Chlorpyrifos-ethyl	CPE	20.3	MS/MS	314 > 258	non resonant	82	138.3
Chlorpyrifos-ethyl d10 ^a	CPE-d10	20.2	MS/MS	324 > 260	non resonant	87	142.7
Chlorfenvinphos	CFV	21.5	MS/MS	323 > 267	non resonant	85	142.3
Procymidon	PCM	21.7	MS/MS	283 > 255	non resonant	64	93.5
Diflufenican	DFF	26.3	MS/MS	394 > 266	non resonant	98	173.6
		Retention time (min)	Scan type	Low mass (m/z)	High mass (m/z)	-	-
Hexabromobenzene ^b	HBB	27.2	SIS	548	558	-	-

^a: deuterated surrogates

^b: internal standard

Table 2. Factor levels of the experimental design for the screening of the desorption conditions (Design n°1)

Factors	Low level (-1)	Center point (0)	High level (+1)
A = injector temperature (°C)	20	40	60
B = desorption flow (mL min ⁻¹)	50	62	75
C = desorption temperature (°C)	200	240	280
D = desorption time (min)	2	6	10

Table 3. Factor levels of the Box Behnken design for the optimization of the decontamination conditions (Design n°3)

Factors	Low level (-1)	Center point (0)	High level (+1)
A = concentration (µg L ⁻¹)	50 × LOQ	1025 × LOQ	2000 × LOQ
B = decontamination temperature (°C)	250	275	300
C = decontamination time (min)	10	30	50

LOQ: limit of quantification

Table 4. Experimental matrix and experimental results of the fractional factorial design (Design n°1)

Experiment	Block	Injector temperature (°C)	Desorption flow (mL min ⁻¹)	Desorption temperature (°C)	Desorption time (min)	ATC peak area	CPM peak area	FNT peak area	MTC peak area	CPE peak area	CFV peak area	PCM peak area	DFF peak area
1	1	20	75	280	2	3006	19700	2112	10874	3740	1367	205	821
2	1	60	75	280	10	962055	5214000	105104	3470000	130000000	182270	45335	23446
3	1	20	50	200	2	268	1865	143	885	394	101	0	0
4	1	60	50	200	2	6930	71808	488	6110	2745	0	0	0
5	1	60	50	200	10	1080000	4311000	115693	2404000	872149	7124	3901	489
6	1	20	50	200	10	7504	66923	3747	20879	8027	2682	356	572
7	1	40	62	240	6	165808	1388000	7194	227874	71975	8794	1740	3270
8	1	60	50	280	2	14916	116536	1398	18364	5899	558	43	173
9	1	20	50	280	2	345	2664	294	1793	777	461	41	130
10	1	40	62	240	6	134764	979366	5998	206702	65104	8893	1707	3021
11	1	20	50	280	10	9305	84364	5238	33076	11233	4698	808	1960
12	1	20	75	200	10	48521	338867	19963	169133	58556	21124	4656	7761
13	1	60	75	200	2	36344	215214	2281	62032	19776	1475	192	313
14	1	60	50	280	10	860073	5431000	112792	2283000	774930	15099	9718	3150
15	2	20	75	280	2	6884	38811	5422	42140	11193	4477	724	2858
16	2	60	75	280	10	1413000	4568000	228150	5266000	2238000	179632	67296	33093
17	2	20	50	200	2	259	1397	138	1333	370	135	20	0
18	2	60	50	200	2	1522	10747	336	3437	958	0	17	32
19	2	60	50	200	10	991152	5719000	71488	2341000	668062	4558	1996	873
20	2	20	50	200	10	10300	84100	5101	37308	11362	2517	495	889
21	2	40	62	240	6	113284	763604	10430	206159	48236	6575	1676	4815
22	2	60	50	280	2	10645	80398	1239	20310	5321	391	63	248
23	2	20	50	280	2	605	3896	502	3510	1135	557	46	171
24	2	40	62	240	6	105681	801857	9745	197716	46892	6806	1595	3525
25	2	20	50	280	10	16040	120690	7356	57314	17259	3590	846	2600
26	2	20	75	200	10	44737	312874	20824	194372	54717	13411	3230	6227
27	2	60	75	200	2	22736	128949	2332	52309	10789	1184	219	533
28	2	60	50	280	10	1219000	4678000	190144	1944	999080	10338	7416	3294

Table 5. Experimental matrix and experimental results of the Box Behnken design (Design n°2), with desorption time set at 10 min

Experiment	Block	Injector temperature (°C)	Desorption flow (mL min ⁻¹)	Desorption temperature (°C)	ATC peak area	CPM peak area	FNT peak area	MTC peak area	CPE peak area	CFV peak area	PCM peak area	DFP peak area
1	1	40	62	260	402432	2305000	26532	1189000	359449	25332	6008	10328
2	1	40	62	260	338157	2196000	30290	1142000	328761	22691	5991	10155
3	1	40	75	220	816051	4766000	88298	3254000	831925	52561	14291	19155
4	1	40	50	300	351868	2916000	16715	620746	174898	14285	3569	5677
5	1	60	62	300	1389000	5196000	222121	5369000	2551000	147391	66787	17832
6	1	40	75	300	1219000	5082000	104682	4186000	1293000	70306	19599	49130
7	1	20	62	220	35272	304485	15084	175043	65034	15374	4976	7013
8	1	40	50	220	182666	1536000	7483	278088	85803	5643	1466	1524
9	1	20	50	260	15264	137156	8585	74295	25303	6108	1603	3009
10	1	40	62	260	639403	4262000	31484	1427000	360825	26009	6525	10140
11	1	20	62	300	42701	353515	16389	215010	72104	24180	6475	19714
12	1	60	75	260	1155000	6337000	240588	5362000	2581000	341391	101966	32259
13	1	20	75	260	66632	474705	32864	348295	117358	37021	10041	23101
14	1	60	50	260	1121000	4700000	96157	3851000	1409000	15784	9755	3005
15	1	60	62	220	1360000	5155000	163109	5335000	2434000	61109	40279	6724
16	2	40	62	260	680290	3924000	34797	1620000	403961	24843	6714	13616
17	2	40	62	260	716657	4670000	42254	1705000	441991	26891	8460	13508
18	2	40	75	220	1071000	5503000	83920	3280000	867038	46513	14183	21676
19	2	40	50	300	332811	2365000	14301	629835	162180	14449	3146	7124
20	2	60	62	300	1472000	5171000	228192	5787000	2752000	130074	64149	23875
21	2	40	75	300	1337000	4956000	130785	4624000	1427000	73377	23657	59909
22	2	20	62	220	40271	320914	14911	191722	74888	16002	4360	8217
23	2	40	50	220	71532	608192	3880	105372	35862	2310	523	605
24	2	20	50	260	7441	78472	3619	31827	12827	1992	494	1241
25	2	40	62	260	360639	2823000	16597	665452	198594	11518	3198	5354
26	2	20	62	300	24792	181799	11360	116406	41647	12668	2709	7672
27	2	60	75	260	1405000	4924000	2332	6045000	3031000	162260	77801	21696
28	2	20	75	260	46787	347662	190144	251807	96070	23367	6604	17834
29	2	60	50	260	1086000	6691000	73336	2831000	1011000	5106	2675	1054
30	2	60	62	220	977287	6391000	161530	3691000	1568000	15483	10521	2514

Table 6. Linear dynamic range, regression coefficient, mean recovery and relative standard deviation (n = 10), and measurement uncertainty (n×p = 30) at 3 concentration levels

Compound	Concentration range (µg L ⁻¹)	Regression coefficient (R ²)	LOQ level			Medium level			High level		
			Spiked concentration (µg L ⁻¹)	Mean recovery (RSD) (%) (n = 10)	U (%) (k = 2) (n×p = 30)	Spiked concentration (µg L ⁻¹)	Mean recovery (RSD) (%) (n = 10)	U (%) (k = 2) (n×p = 30)	Spiked concentration (µg L ⁻¹)	Mean recovery (RSD) (%) (n = 10)	U (%) (k = 2) (n×p = 30)
Acetochlor	0.005 - 10	0.9985	0.005	100.8 (8.8)	16.9	0.2	93.7 (8.7)	16.7	0.8	90.3 (9.3)	20.4
Chlorfenvinphos	0.025 - 50	0.9972	0.025	104.2 (17.4)	20.3	1.0	94.2 (5.2)	12.8	4.0	101.7 (9.0)	14.4
Chlorpyrifos-ethyl	0.025 - 50	0.9989	0.025	96.3 (9.4)	18.7	1.0	94.9 (7.6)	15.5	4.0	97.4 (6.6)	9.5
Chlorpyrifos-methyl	0.0025 - 5	0.9980	0.0025	94.3 (11.9)	20.8	0.1	96.5 (8.2)	14.5	0.4	96.5 (6.6)	11.5
Diflufenican	0.05 - 100	0.9980	0.05	98.5 (12.0)	25.0	2.0	102.7 (7.3)	14.3	8.0	97.4 (8.2)	16.6
Fenitrothion	0.005 - 10	0.9985	0.005	98.3 (12.5)	23.0	0.2	96.8 (8.2)	14.4	0.8	93.2 (8.5)	16.1
Metolachlor	0.005 - 10	0.9992	0.005	95.3 (7.6)	14.7	0.2	97.4 (7.5)	14.0	0.8	97.5 (8.0)	12.6
Procymidon	0.005 - 10	0.9992	0.005	98.9 (9.3)	18.6	0.2	101.5 (7.0)	12.9	0.8	97.8 (9.4)	15.3

LOQ: limit of quantification; RSD: relative standard deviation; U: measurement uncertainty; k: expanded coverage factor

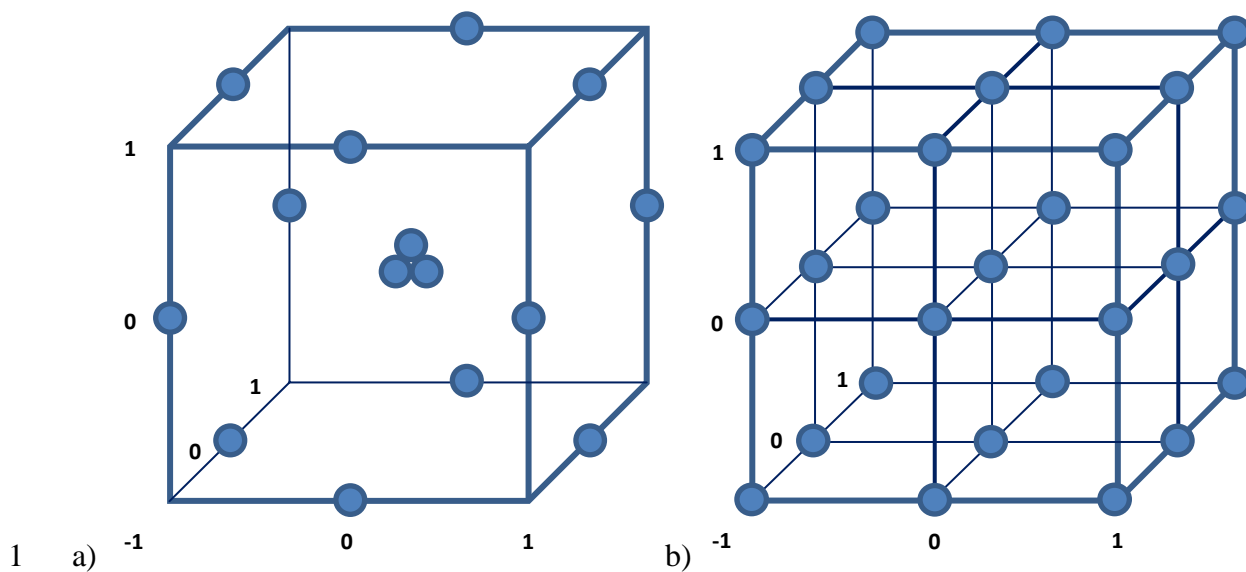
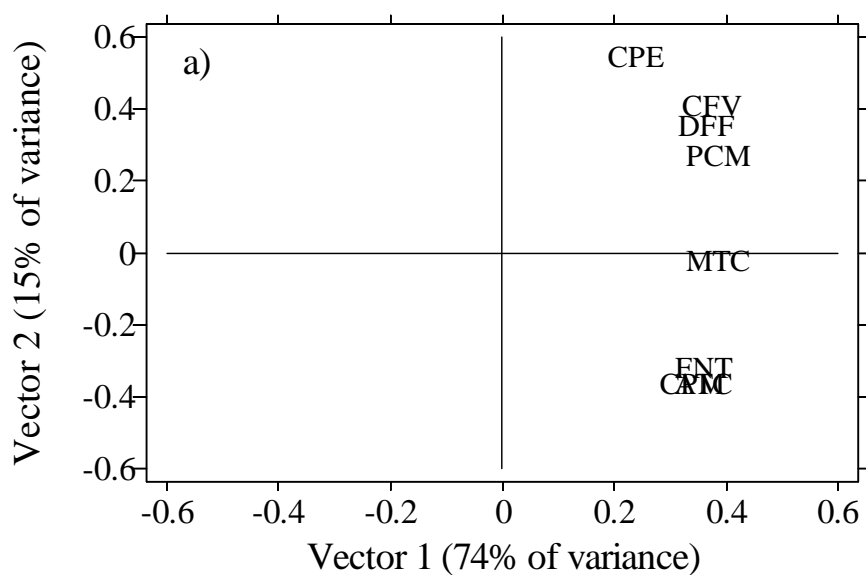
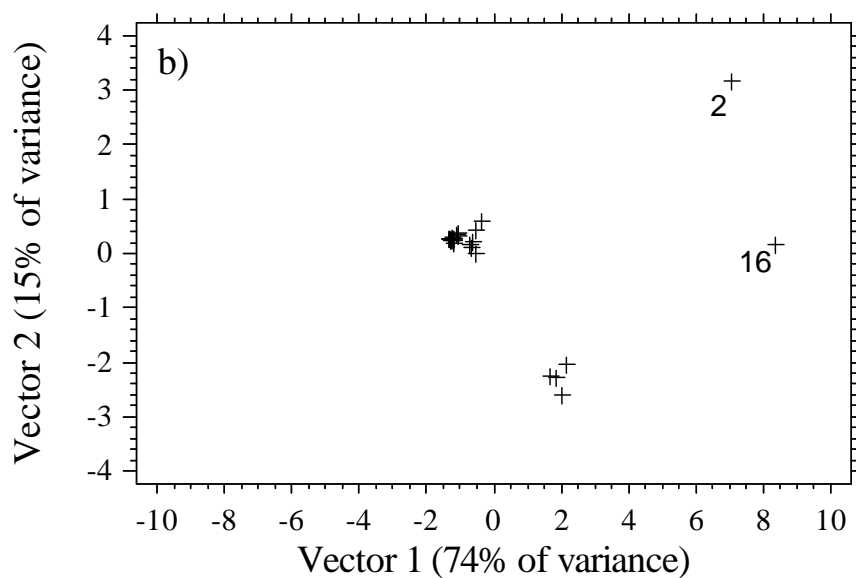


Fig. 1 Graphical representations of a Box Behnken design (a) and a full factorial 3³ design (b). The three axes of the cubes represent the three thermal desorption factors to be optimized, with their levels (-1 = low level, 0 = medium level, and 1 = high level), and the dots represent the experiments of the designs

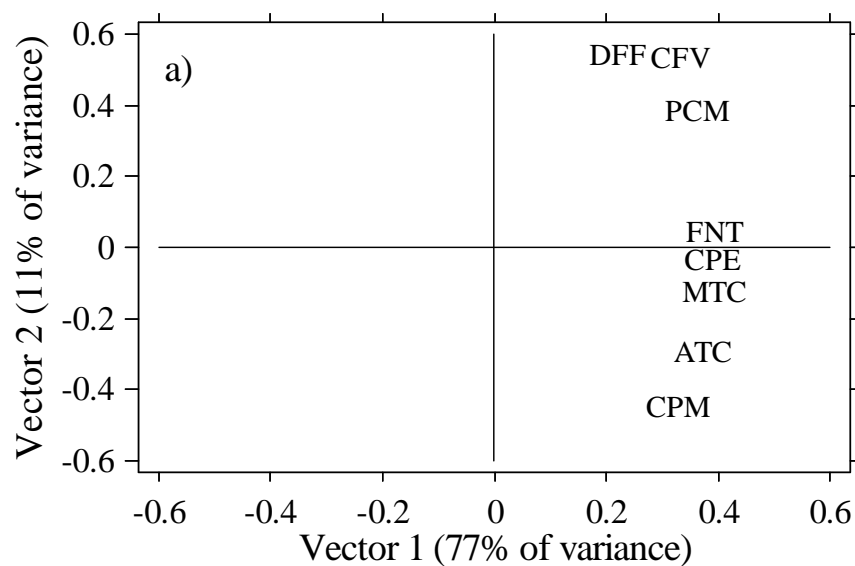


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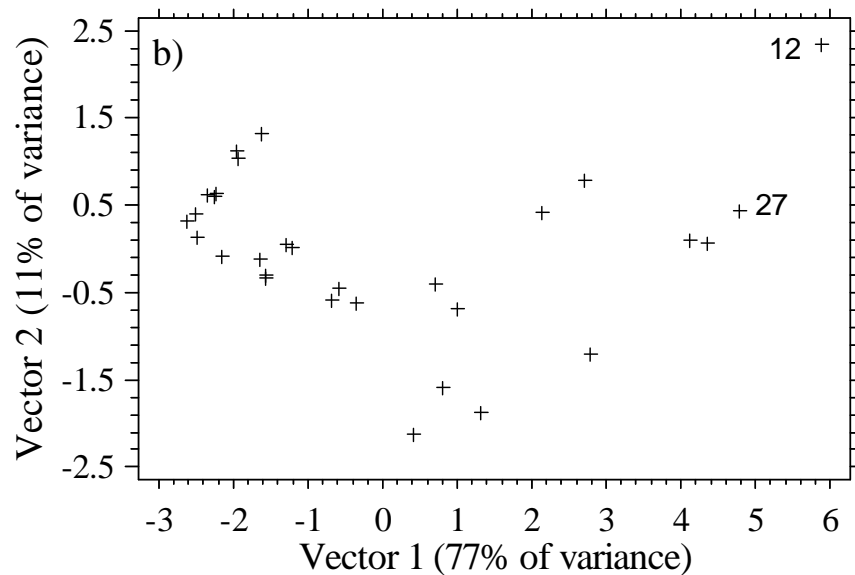


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9 Fig. 2 Loading plot (a) of the studied pesticides and score plot (b) of the experiments of the
10 PCA on the results of design n°1. On the score plot, the numbers next to the “+” symbols are
11 the numbers of the experiments which obtained the best scores on vector 1



12



13

14 Fig. 3 Loading plot (a) of the studied pesticides and score plot (b) of the experiments of the
15 PCA on the results of design n°2. On the score plot, the numbers next to the “+” symbols are
16 the numbers of the experiments which obtained the best scores on vector 1

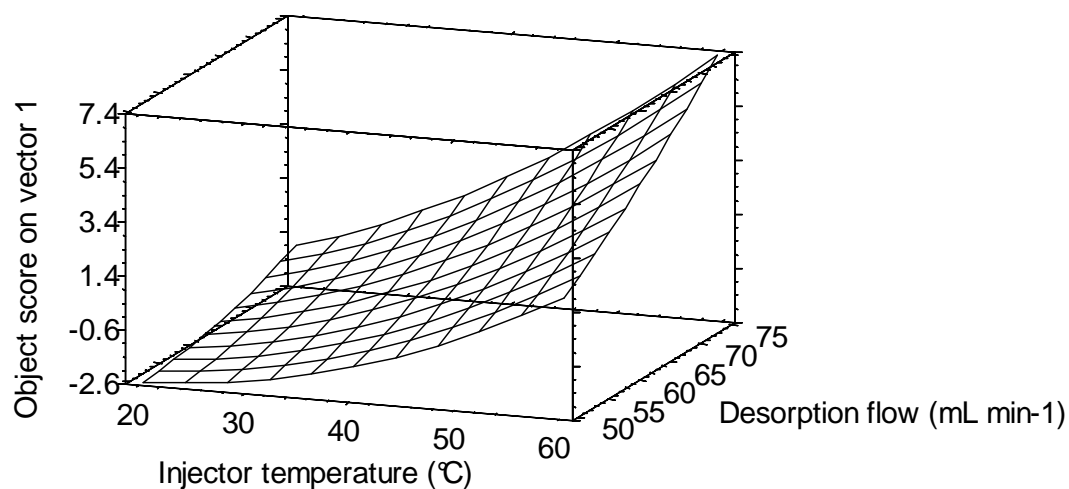


Fig. 4 Response surface of the Box Behnken design for the optimization of the desorption conditions (Design n°2). The chart plots the effect of the injector temperature and the desorption flow on the object score on vector 1 (*i.e.*, global response for the 8 studied pesticides), with the desorption temperature set at 300 °C and the desorption time set at 10 min

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