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► To cite this version:

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

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8 **Abstract**

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

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

30

31 **Introduction**

32

33 Since its introduction by Baltussen et al. in 1999 [1], stir bar sorptive extraction (SBSE) has
34 become one of the most used miniaturized extraction and enrichment technique of organic
35 contaminants in aqueous samples. This solventless technique is based on the same principles
36 as solid phase microextraction (SPME). In other words, the extraction is governed by
37 diffusion of the analytes and its efficiency relies on equilibrium, *i.e.*, the partitioning of the
38 analytes between the sample and the polydimethylsiloxane (PDMS) extraction phase [2]. But
39 SBSE can achieve better extraction recoveries, especially for moderately hydrophobic
40 analytes, as the volume of the extraction phase of the SBSE stir bars (24 μL to 126 μL) is
41 much larger than the volume of the SPME fibers (up to 0.5 μL) [3, 4]. Stir bar sorptive
42 extraction has been applied in pharmaceutical, biomedical, food, and environmental analysis
43 for a wide range of organic chemicals, from polar endocrine disruptors (EDC) such as
44 estrogens, across moderately hydrophobic pesticides to highly hydrophobic polycyclic
45 aromatic hydrocarbons (PAH) and polychlorobiphenyl (PCB) congeners. Several reviews
46 covered in details the analytical applications of this technique [2, 4–6]. The extraction step is
47 followed by desorption, either with organic solvent (liquid desorption, LD) or with heat
48 (thermal desorption, TD) depending on the hydrophobicity and the thermal stability of the
49 analytes, before analysis by liquid or gas chromatography. The optimization of the extraction
50 conditions of organic chemicals in water samples has been carried out in several studies [7–
51 13]. Desorption conditions need to be investigated as well, in order to achieve optimal back
52 extraction of the analytes sorbed in the PDMS phase and yield maximal sensitivity and

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

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

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

80 In the present paper, we describe a complete multivariate optimization with the methodology
81 of experimental designs. The aim of this work is to develop an analytical method consisting in
82 SBSE followed by thermal desorption and gas chromatography coupled with tandem mass
83 spectrometry (SBSE-TD-GC-MS/MS). Then, following comprehensive and thorough
84 protocols, we validated the optimized method and determined the measurement uncertainties
85 for the analysis of pesticides in freshwater samples. To realize this study, we selected a
86 relevant set of 8 relatively hydrophobic to hydrophobic pesticides ($3.08 < \log K_{ow} < 4.96$)
87 usually encountered in freshwaters of agricultural watersheds. The extraction conditions have
88 been developed and validated previously for liquid desorption followed by liquid
89 chromatography coupled with tandem mass-spectrometry (SBSE-LD-LC-MS/MS) analysis
90 [13]. In the present study, two experimental designs were conducted for the optimization of
91 the thermal desorption of the selected pesticides. The effect of the desorption time, the
92 desorption temperature, the desorption flow and the injector temperature, and the effect of
93 their interactions were investigated with a screening experimental design. Then, the three
94 most significant parameters were optimized by a second experimental design in order to reach
95 maximal desorption of all selected pesticides. A third experimental design was realized to
96 determine the best conditions for the thermal decontamination of the SBSE stir bars after the
97 desorption step. Finally, the optimized method was validated regarding linearity, limit of
98 quantification (LOQ), trueness and precision following a comprehensive validation
99 procedure, and the analytical measurement uncertainty was assessed in conditions of intra-
100 laboratory reproducibility.

101

102 **Experimental**

103

104 Reagent and chemicals

105

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

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

116 The individual pesticide stock solutions were prepared in acetone at a concentration of 100
117 mg L⁻¹ and stored at 4 °C for two months. Standard solutions for extraction were prepared
118 daily with ultrapure water by appropriate dilution of the stock solutions. Helium gas (purity =
119 99.999%) used for thermal desorption and chromatographic analysis, nitrogen gas (purity =
120 99.995%) used for thermal decontamination, and carbon dioxide gas (purity = 99.5%) used
121 for focusing the desorbed pesticides in the cooled GC injector were purchased from Air
122 Liquide (Juhem, France).

123

124 Sample preparation and SBSE procedure

125

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

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

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

139

140 Instrumentation and analytical conditions

141

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

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

153 focused in the liner, whatever the split ratio. The PTV injector was then ramped at 200 °C
154 min⁻¹ to a final temperature of 280 °C (held for 10 min) and the analytes were transferred into
155 a Zebron ZB-5ms capillary column (30 m x 0.25 mm x 0.25 µm) provided by Phenomenex
156 (Le Pecq, France). The carrier gas was helium at a flow rate of 1.0 mL min⁻¹. The column
157 oven was programmed at the same temperature as the injector during 10 min, ramped at 30 °C
158 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
159 300 °C and held for 5 min, for a total analysis time of 36 min. After thermal desorption and
160 during the chromatographic separation of the analytes, the SBSE stir bars were kept in the
161 desorption oven for thermal decontamination with nitrogen gas under the optimized
162 conditions, to insure total elimination of pesticide residues. Each SBSE stir bar was desorbed
163 and decontaminated for 60 min.

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

178

179 Experimental designs for method optimization

180

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

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

203 (medium level (0)) were added, making a block of 15 experiments. This block was repeated
204 once for a total of 30 experiments and 19 degrees of freedom for the experimental error. This
205 design allows the determination of response surfaces with fewer experiments than a 3^3 full
206 factorial design (30 herein versus 54 in a full factorial design replicated twice, *i.e.*, 2 blocks),
207 but provides enough degrees of freedom to evaluate the experimental error. Fig. 1a and Fig.
208 1b show respectively graphical representations of the Box Behnken design and the 3^3 full
209 factorial design as cubes. The three axes of the cubes represent the 3 thermal desorption
210 factors to be optimized, with their levels (low level (-1), medium level (0), and high level
211 (+1)), and the dots represent the experiments of the designs. For the graphical representation
212 of the Box Behnken design, the experiments are located at the middle of each edge of the
213 cube, so 12 experiments. The dots at the center of the cube represent the 3 experiments added
214 at the center point of all 3 factors, so a total of 15 dots. For the graphical representation of the
215 3^3 full factorial design, the experiments are located at each corner (8 experiments), at the
216 middle of each edge (12 experiments), at the middle of each face (6 experiments) and at the
217 center of the cube (1 experiment), so a total of 27 experiments. These 27 experiments
218 represent all 27 combinations of the 3 levels of 3 factors.

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

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

229 In addition, the optimal conditions for the thermal decontamination of the SBSE stir bars after
230 the desorption step were investigated with a third experimental design (Design n°3). Another
231 Box Behnken design was carried out (1 block, 3 center points, and 3 factors with 3 levels for a
232 total of 15 experiments and 9 degrees of freedom for the experimental error). The 3 levels of
233 each factor investigated in design n°3 are presented in Table 3. In this experiment, we
234 investigated the effect of the decontamination temperature, the decontamination time, and the
235 amount of pesticides in the SBSE stir bar on the decontamination efficiency. The amount of
236 pesticides in the stir bars was controlled by extraction of water samples of different pesticide
237 concentrations. Depending on the pesticide, the concentrations in water samples varied, but
238 were set for each pesticide as 50 times the respective LOQ ($50 \times \text{LOQ}$) for the low level,
239 $1025 \times \text{LOQ}$ for the center point, and $2000 \times \text{LOQ}$ for the high level. For design n°3, the
240 responses were the pesticide chromatographic peak areas of the subsequent analysis of the
241 SBSE stir bars. In other words, the SBSE stir bars used for the extraction of water samples
242 spiked with all the pesticides at different concentrations were thermally desorbed for analysis
243 and decontaminated under each set of the conditions established in the experimental design;
244 then, they were thermally desorbed a second time for analysis in order to determine the
245 amounts of pesticides remaining after the thermal decontamination step of the first analysis.

246

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

248

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

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

261 The method validation was inspired from standard methods ISO/IEC 17025:2005 and
262 AFNOR NF T90-210:2009 [17, 18], and previous validations of LC-MS/MS methods [13,
263 19]. Measurement uncertainty of the analytical method was assessed according to standard
264 methods ISO 11352:2012 and AFNOR XP T 90-220:2003 [20, 21], and previous validation of
265 an LC-MS/MS method [13].

266

267 *Study of the linearity range*

268

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

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

281

282 *Verification of the limits of quantification*

283

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

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

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

291

292 *Trueness and precision*

293

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

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

303

304 *Measurement uncertainty*

305

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

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

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

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

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

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

320

321

322 **Results and discussion**

323

324 Experimental designs

325

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

327

328 Table 4 shows the experimental design and the experimental matrix composed of 8 columns

329 as responses (pesticide areas) and 28 rows for the experiments, obtained from the analyses of

330 stir bars under the thermal desorption conditions set by the experimental design. A Principal

331 Component Analysis (PCA) was applied to this data matrix (previously mean-centered) with

332 Statgraphics software. The first vector explained 74% of the total variance of the experimental

333 results; and all variables (the chromatographic peak areas of the studied pesticides) had

334 positive loadings on this vector (Fig. 2a). This means that all studied pesticides had similar

335 behaviors regarding the thermal desorption. Therefore, we considered the score of each

336 experiment on this vector (Fig. 2b) as a global response (to be maximized). This methodology

337 for determination of a global response has been previously used in the successful optimization

338 of a SBSE-TD-GC analysis of 7 pesticides in water [15]. On Fig. 2b, each experiment has a

339 score on the first vector and on the second vector, and is represented by a “+” symbol. The

340 two experiments which obtained the best scores on the first vector are experiments n°2 and

341 n°16 (Table 4). For both experiments, the thermal desorption conditions were desorption

342 temperature at 280°C, desorption flow at 75 mL.min⁻¹ for 10 min with the injector

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

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

$$349 \quad Y = 20.3 - 0.42A^{***} - 0.06B^{**} - 1.00C^{***} - 0.15D^{**} + 0.001AB^{***} + 0.02AC^{***} + \\ 350 \quad 0.002AD^{***} + 0.0003BC + 0.00003BD + 0.009CD^{***}$$

351
352 where A = Desorption flow, B = Desorption temperature, C = Desorption time, and D =
353 Injector temperature, and AB, AC, AD, BC, BD, CD are their two-factor interactions. The
354 significant terms in the model are marked with stars: (*) means p-values < 0.05, (**) means p-
355 values < 0.01, and (***) means p-values < 0.001. So, in this case, a very high level of
356 significance was obtained. Not only the effects of all 4 factors were significant, but also the
357 effects of 4 interactions were significant, which could not have been observed with an OVAT
358 optimization methodology. Moreover, two of these interactions were even quite unexpected.
359 Indeed, one can note the significance of AD and CD, two interactions that involve factors
360 from different parts of the analytical system (desorption oven and PTV injector). The
361 statistical analysis of the design showed that all factors and interactions had positive effect on
362 the response. This means that the desorption flow, temperature and time, and the injector
363 temperature have to be set at their higher levels for maximum response. Moreover, regarding
364 the interactions, the effect of an increase of desorption temperature, for instance, is greater
365 when desorption flow is at higher level.

366

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

369

370 On the basis of the results of design n°1, we decided to set the desorption time at 10 min, the
371 maximal level tested, without performing further optimization for higher values, in order to
372 keep the total analysis time at 60 min maximum. Thus, the optimization of the thermal
373 desorption was realized with the injector temperature (Factor A), the desorption flow (Factor
374 B), and the desorption temperature (Factor C). Table 5 shows the Box Behnken experimental
375 design and the resulting experimental matrix. Similarly to the results of the design n°1, a PCA
376 was applied to the experimental results of the design n°2 (previously mean-centered) and
377 showed that all the variables have positive loading on the first vector (Fig. 3a), which
378 explained 77% of the variance. Therefore, we considered the score of each experiment on the
379 same vector (Fig. 3b) as a global response (to be maximized). On Fig. 3b, the two
380 experiments which obtained the best scores are experiments n°12 and n°27 (Table 5). For
381 both experiments, the optimal thermal desorption conditions were desorption temperature at
382 260 °C, desorption flow at 75 mL.min⁻¹ for 10 min with the injector temperature at 60 °C,
383 these values confirm the results of the PCA realized with the data of design n°1.

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

$$386 \quad Y = 35.1 - 0.48A^{***} - 0.37B^{***} - 0.18C^{***} + 0.002AA^{***} + 0.004AB^{***} +$$
$$387 \quad 0.0007AC^{**} + 0.002BB + 0.0004BC + 0.0003CC^{*}$$

388

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

391 during 10 min under a helium flow of 75 mL min⁻¹ in split mode (1/75 ratio), while
392 maintaining a focusing temperature of 60 °C in the PTV injector of the GC-MS/MS system.
393 Another Box Behnken design was realized to optimize the conditions of thermal
394 decontamination (Design n°3) of the SBSE stir bars after desorption (Table 3). Only traces of
395 chlorpyrifos-methyl, with area equivalent to the area of blank samples, were detected in the
396 second GC-MS/MS analyses of the stir bars corresponding to the highest concentration level
397 tested during the experimental design. Therefore, the optimized conditions for the thermal
398 decontamination of the SBSE stir bars were the highest point in the experimental space, *i.e.*,
399 300 °C for 50 min.

400

401 *Advantages of the experimental design approach*

402

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

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

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

423

424 *Performances and validation of the analytical method*

425

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

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

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

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

448 Results on measurement uncertainty are reported in Table 6. For all selected pesticides at the
449 three concentration levels, satisfactory measurement uncertainty for SBSE-TD-GC-MS/MS
450 was obtained ($U \leq 25\%$). To our knowledge, very few validations of SBSE-TD-GC-MS/MS
451 methods have included studies of the uncertainty of the analytical results [12, 23]. Regarding
452 the pesticides studied herein, Camino-Sánchez et al. [12] reported uncertainties ranging from
453 40 to 46% for chlorfenvinphos, chlorpyrifos-ethyl, chlorpyrifos-methyl, fenitrothion, and
454 metolachlor; but the uncertainties were determined with another approach on which very few
455 details were presented. In our method, 90 water samples were used for the determination of
456 realistic uncertainties under intra-laboratory reproducibility conditions, *i.e.*, with 3 types of
457 water, at 3 concentration levels, prepared by 3 operators and analyzed at different days.
458 Results showed for all target pesticides homogeneous uncertainty across the concentration
459 range. Margoum et al. [13] used the same approach for the evaluation of uncertainties on the
460 analysis of 15 pesticides by SBSE-LD-LC-MS/MS. We obtained similar uncertainty values
461 for the analysis of pesticides targeted by both methods, *i.e.*, chlorfenvinphos, chlorpyrifos-
462 ethyl, diflufenican, fenitrothion, and procymidon.

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

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

472

473 **Conclusion**

474

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

489 In this study, a set of 8 moderately hydrophobic to hydrophobic pesticides ($3.08 < \log K_{ow} <$
490 4.96) was used for the optimization of the analytical method by means of experimental
491 designs. As this interesting approach should be applied for other method optimizations,
492 further work will focus on adding other hydrophobic micropollutants in this SBSE-TD-GC-
493 MS/MS method.

494

495

496 **Acknowledgements**

497

498 The authors thank the French National Research Agency (ANR) through PoToMAC project
499 (ANR 2011 CESA 022 02) and the French National Agency for Water and Aquatic
500 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	DFE	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	-	-	-

502 ^a: deuterated surrogates

503 ^b: internal standard

504 Table 2. Factor levels of the experimental design for the screening of the desorption
505 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

506

507

508 Table 3. Factor levels of the Box Behnken design for the optimization of the decontamination
509 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

510 LOQ: limit of quantification

511

512

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	DFP 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

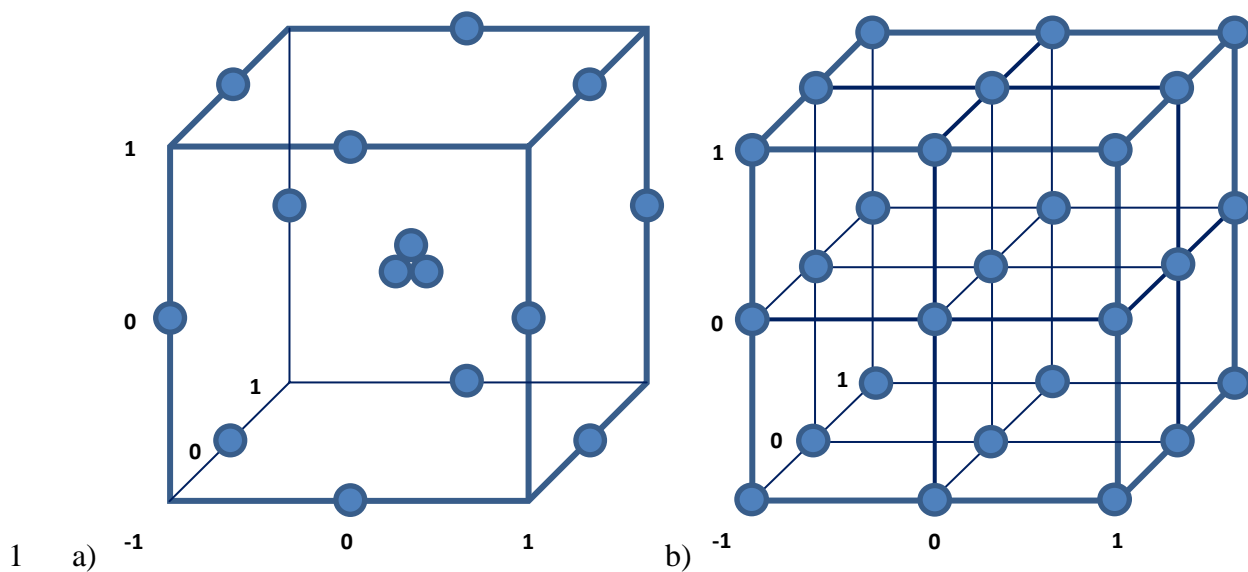
Table 5. Experimental matrix and experimental results of the Box Behnken design (Design n°2), with desorption time set at 10 min

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

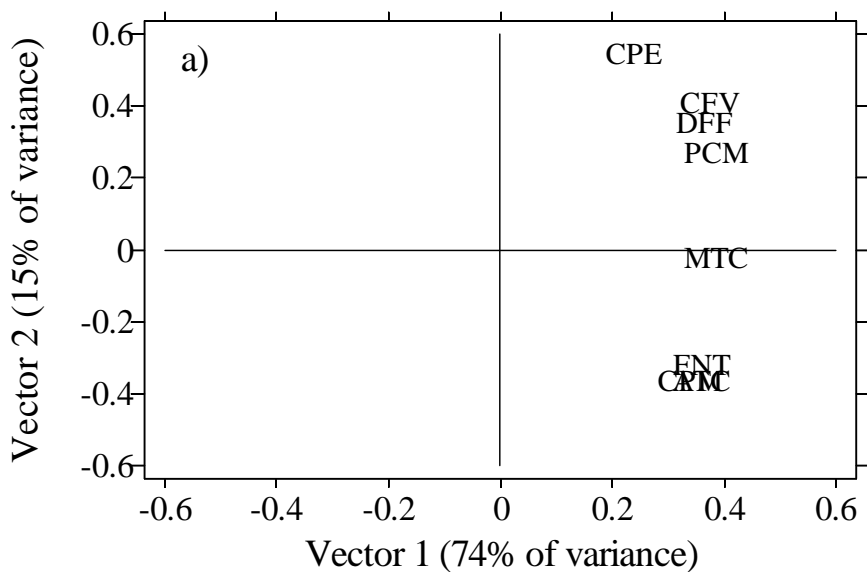
Compound	Concentration range (µg L ⁻¹)	Regression coefficient (R ²)	LOQ level			Medium level			High level		
			Spiked concentration (µg L ⁻¹)	Mean recovery (RSD) (%) (n = 10)	U (%) (k = 2) (n×p= 30)	Spiked concentration (µg L ⁻¹)	Mean recovery (RSD) (%) (n = 10)	U (%) (k = 2) (n×p = 30)	Spiked concentration (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

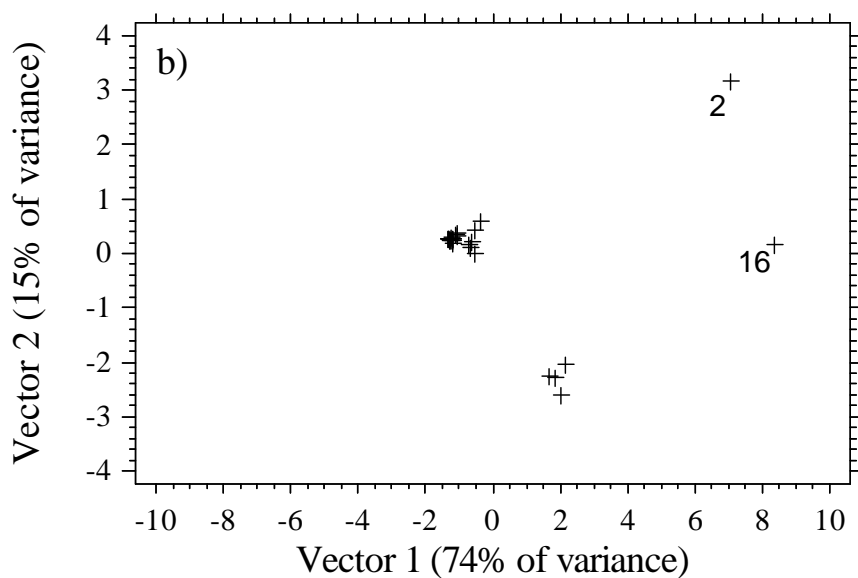


1 a) -1 0 1 b) -1 0 1

2 Fig. 1 Graphical representations of a Box Behnken design (a) and a full factorial 3^3 design (b).
3 The three axes of the cubes represent the three thermal desorption factors to be optimized,
4 with their levels (-1 = low level, 0 = medium level, and 1 = high level), and the dots represent
5 the experiments of the designs
6

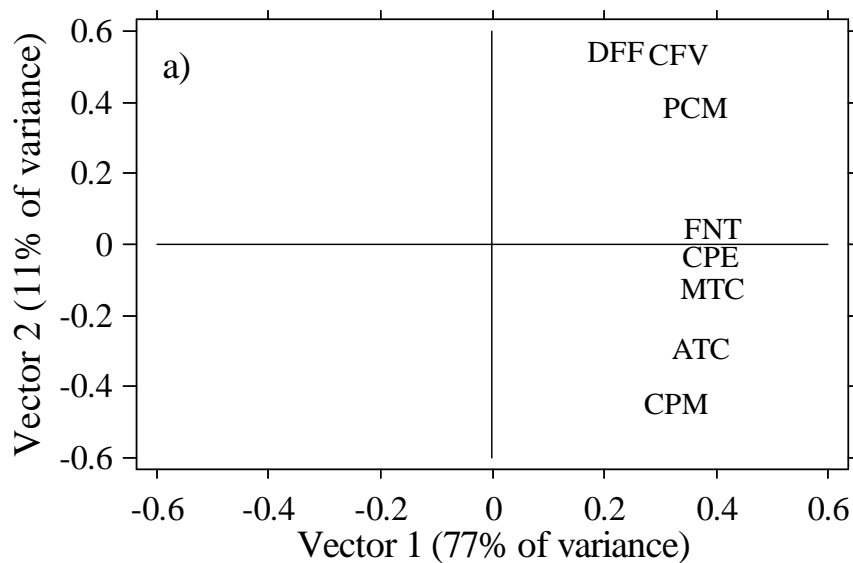


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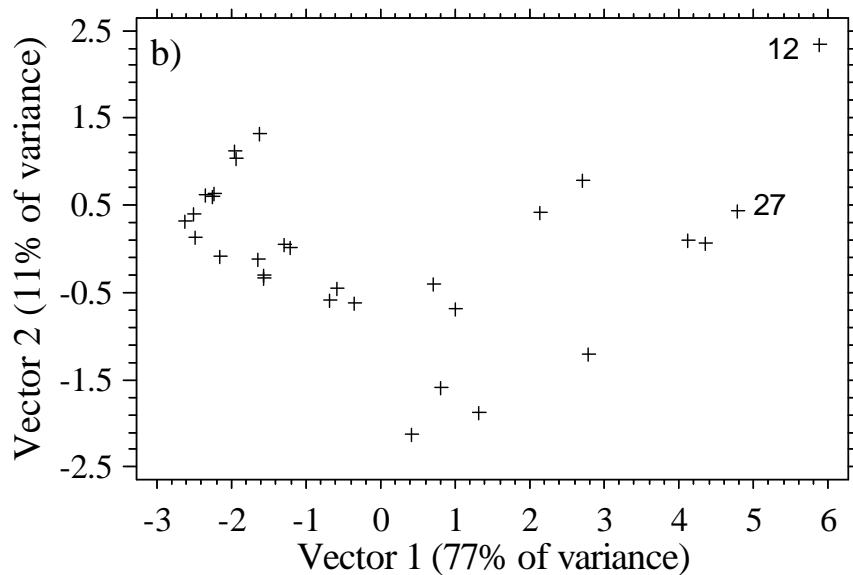


8

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

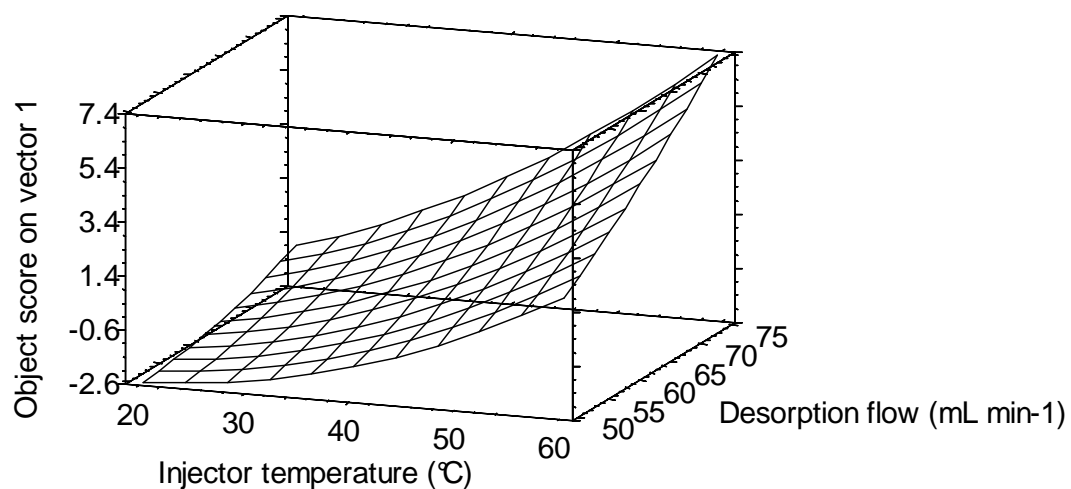


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13

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



17
18 Fig. 4 Response surface of the Box Behnken design for the optimization of the desorption
19 conditions (Design n°2). The chart plots the effect of the injector temperature and the
20 desorption flow on the object score on vector 1 (*i.e.*, global response for the 8 studied
21 pesticides), with the desorption temperature set at 300 °C and the desorption time set at 10
22 min
23

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