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Research article

Box Behnken design-based optimized extraction of non-dioxin-like PCBs for GC-ECD and GC-MS analyses in milk samples

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ABSTRACT

A multivariate optimization process of the sample extraction procedure by Box-Behnken design through a global desirability function is described for the determination of six non-dioxin-like polychlorinated biphenyls (NDL-PCBs # 28, 52, 101, 153, 138 and 180) in milk by GC-ECD and mass spectrometry. Three factors were involved in refining the extraction conditions: the acetone percentage in the extraction mixture, the sample/solvent ratio, and the extraction time. The three-factor design required 26 experiments that were carried out in duplicate and in a randomized order to minimize the bias effects of uncontrolled variables. The optimized factors (acetone percentage: 30%; sample-to-solvent ratio: 0.11 g mL^{-1}; extraction time: 45 min) ensured a low solvent consumption and a reduced extraction time, allowing a rapid and simultaneous preparation of multiple sample extracts. The method was validated according to the European directives (Decision 657/2002/EC, SANTE 2017/11813/EC) through the evaluation of linearity, selectivity, LOD, LOQ, recovery, precision, and ruggedness.

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1. Introduction

In recent years, multivariate optimization has been frequently applied to the development of analytical methods, considering its advantages in the reduction of the number of required experiments and consequently resulting in lower reagent consumption and laboratory work [1]. Furthermore, the multivariate approaches allow the assessment of the statistical significance of the factors under investigation as well as the evaluation of the interaction effects between the factors on the response. Then, a large amount of information can be obtained from a minimum number of experimental runs. On the contrary, in the univariate strategies, the effect of each variable is singularly studied, independently of the level of the other factors involved in the optimization process. Therefore, if there are significant interaction effects between factors, the optimal conditions determined by the univariate studies could be very different from the (correct) results found by the multivariate optimization, in which the levels of all the variables are changed simultaneously.

Among the different chemometric tools currently applied to analytical chemistry, the Box Behnken design (BBD) is often used to optimize the most influential parameters involved in the method development. Several applications include the optimization of the extraction process in pesticide residue analysis from food samples [2–4] and the determination of environmental contaminants [5].

Box-Behnken experimental design was described for the first time in 1960 [6]. Considering that the efficiency of experimental design can be defined as the number of parameters divided by the number of experiments BBD, together with the Doehlert matrix, is very efficient if compared to the three-level full factorial designs and slightly more efficient than the central composite design. Furthermore, BBD does not contain combinations for which all factors are simultaneously at their highest or lowest levels, avoiding experiments performed under extreme conditions, for which

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unsatisfactory results might occur. On the other hand, it is not indicated for situations in which it is necessary to know responses in these situations. Multivariate techniques are routinely applied to optimize the different working conditions in various extraction processes to improve their performance. Several reviews and research papers have been published on this subject [2], nevertheless, to date, for the determination of polychlorobiphenyls (PCBs) the advantages of the BBD approach have not been fully exploited. Indeed, among the exponentially increasing number of literature works, only a limited number of applications have been reported on the development of optimized analytical methods by Box Behnken design for the analysis of PCBs in food, biological and environmental samples [7–10].

PCBs are a group of anthropogenic chemicals, in the past widely used as dielectric and coolant fluids in electrical apparatus, carbonless copy paper and in heat transfer fluids. Commercial production of PCBs ended in 1977 because of health effects associated with exposure, but as a consequence of their bioaccumulative character and resistance to metabolic degradation [11,12], PCBs are still extensively widespread along the food chain [13]. In the European Community, Maximum Residue Levels (MRLs) have been established for PCBs in food products (European Commission Regulation 1259/2011/EC). In raw milk and dairy products, an MRL of 40 ng g^{-1} fat has been set for the sum of the six non-dioxin-like polychlorinated biphenyls (NDL-PCBs, congeners # 28, 52, 101, 138, 153, and 180), typically used as indicators to monitor the contamination levels in foodstuffs, due to their higher abundance respect to other congeners [14]. For the determination of PCBs in food samples, screening analytical methods based on gas chromatography (GC) and electron capture detection (ECD) have been proposed [15–18] for high throughput applications in monitoring and risk-assessment studies, whereas for confirmatory analyses, GC coupled with mass spectrometry (MS) is currently used [19–23], as recommended by the European Directives (European Commission Decision 657/2002/EC). The high number of papers dealing with the determination of PCBs confirms that this topic still deserves great attention by the scientific community: furthermore, NDL-PCBs extraction from food samples with high fat content, such as milk, is still a challenging step. Very often, long and tedious extraction procedures are required and the risk of low recoveries and/or chromatograms full of interfering peaks is still high. In particular, for the NDL-PCBs, recognized as food contamination indicators, the development of a suitable analytical method specific for their determination, that can assure high recoveries and performances with an efficient, rapid and easy-to-use extraction procedure is a fundamental issue.

In this work, an analytical method is specifically proposed for the selective determination of six NDL-PCBs (# 28, 52, 101, 153, 138 and 180) in milk samples. A multivariate process by a three-level Box Behnken experimental design (consisting in 26 experiments, carried out in duplicate and with a randomized order) is proposed for the optimization of the sample extraction procedure, followed by detection and quantification by GC-ECD and mass spectrometry. Finally, the method was validated in terms of linearity, selectivity, detection and quantification limits, recovery, precision and ruggedness, following the European directives.

2. Materials and methods

2.1. Chemicals

High purity (≥97%) NDL-PCB mix standards (IUPAC congeners 28, 52, 101, 153, 138, 180) at a concentration of 10 mg L^{-1} in isooctane for each NDL-PCB were provided by Dr Ehrenstorfer (Augsburg, Germany). Working standard solutions were prepared just before injection by dilution in isooctane. NDL-PCB #209 (Dr Ehrenstorfer, > 99.0%, 10 mg L^{-1} in isooctane) was used as internal standard (IS) and added to NDL-PCB standard calibration solutions to a final concentration of 100 μg L^{-1}. Solid-phase extraction Bond Elut-PCB cartridges (1 mg, 3 mL) were supplied by Agilent Technologies (Inc. Folsom, CA, 95,630). Glassware was treated with a sulphochromic mixture (Carlo Erba Reagenti, Milano, Italy) for organic and inorganic residues removal, followed by a washing step with different solvents (water, acetone, and n-hexane of HPLC grade) to eliminate cross-contamination.

2.2. Sample collection

Milk samples of different brands were bought in local markets. Other (pasteurized) milk samples were collected from local farms regularly inspected by veterinary services. Samples with different fat content were analyzed to test matrix interferences. For each sample the fat content determination was performed as described in the following paragraphs.

2.3. Sample preparation

For the extraction-cleanup process, 100 mL of a hexane/acetone mixture (70/30, v:v) were added to a whole milk aliquot of 11 g. The extraction was carried out for 45 min by a magnetic stirrer; after sonication for 30 min, the clear supernatant was evaporated to 4 mL under a nitrogen stream at 45 °C in a Turbovap system (Caliper Mod. LV, Hopkinton, MA, USA). Then, 4 mL of sulphuric acid (98%) was added and the mixture was kept overnight at room temperature. After centrifugation at 4 °C for 20 min at 3500 rpm, the upper clear phase was transferred into a tube and evaporated to dryness under a nitrogen stream at 45 °C. Then, the residue was dissolved in 2 mL of n-hexane and loaded into a Bond Elut-PCB cartridge (1 mg, 3 mL), previously conditioned with 3 mL of n-hexane. The elution was obtained using 10 mL of n-hexane. Finally, after evaporation to dryness under a stream of nitrogen at 45 °C, the residue was dissolved in 0.5 mL of isooctane containing 100 μg L^{-1} of NDL-PCB #209, used as an internal standard, before GC-ECD analysis.

2.4. Fat content determination and extraction

The Thermo Scientific™ Dionex™ ASE™ 350 Accelerated Solvent Extraction (ASE) system (Thermo Fisher Scientific, Waltham, MA USA) was used for the fat content determination and extraction. The ASE process was performed at an oven temperature of 120 °C and pressure of 1500 psi; Other ASE conditions: three 10-min cycles, 6 min and 3 min of heat and static time, respectively; flush volume 100%; 60 s purge time; carrier gas nitrogen; stainless steel extraction cells 10 mL; collection vials 60 mL. An amount of 1 g of milk was mixed with Extrelut® (Merck, Darmstadt, Germany) in a ratio of 1:2, and air-dried in the oven at 100 °C for 15 min. An extraction mixture of petroleum ether/isopropanol (2:1) was used (30 mL g^{-1} of sample); finally, the extracts were evaporated to dryness at 40 °C by a rotavapor system.

2.5. Gas chromatography/electron capture detection

Chromatographic separations were performed by an AutoSystem XL GC (PerkinElmer, Waltham, MA, USA) coupled with an Electron Capture Detector (ECD). The glass liner Siltek deactivated (PerkinElmer) was used in splitless mode at 250 °C. The chromatographic separations were carried out using an analytical column TG-5SILMS (VF-5 ms, 30 × 0.25 mm inner diameter, 5% diphenyl-95% dimethylsiloxane liquid phase, film thickness:

0.25 μm; Thermo Fisher Scientific, Waltham, MA USA) coupled to the corresponding 5 m safeguard (Thermo Fisher Scientific). A volume of 1.0 μL of the final extract was injected into the chromatographic system. The flow rate of the carrier gas (Helium, 99.999%, pressure-pulse mode: 30 psi for 1 min) was 1.0 mL min^{-1}. The ECD temperature was 375 °C. The oven temperature was initially set at 130 °C, then increased to 190 °C in 3 min at a rate 20 °C min^{-1} and to 280 °C in 9 min at 10 °C min^{-1}. The final temperature of 280 °C was kept for 15 min, with a total run time of 27.0 min. Acquisition and data processing were performed by the TotalChrom Workstation (PerkinElmer).

2.6. Box–behnken experimental design

The Box Behnken design was used for the evaluation of the effects of the extraction variables on the recovery of NDL-PCBs from milk samples, before GC-ECD determination. Three most influential factors including the acetone percentage in the extraction mixture (X_1), the sample/solvent ratio (X_2) and the extraction time (X_3) were selected as independent variables for optimization at three levels: X_1: 5% (−1), 17.5% (0), 30% (+1); X_2: 0.075 g mL^{-1} (−1), 0.100 g mL^{-1} (0), 0.125 g mL^{-1} (+1); X_3: 45 min (−1), 90 min (0), 135 min (+1). The responses were: the recovery percentage (Y_1) and its standard deviation (Y_2). A total of 26 experiments were performed in random order with two replicates at the center point to estimate the pure error. A multi-response optimization was accomplished using a desirability function provided by XLSTAT (Statistical Software for Excel, option Quality). Experimental data were fitted to the quadratic model using a second-order polynomial model. Coefficients (linear, quadratic, and interaction) were determined by the least squares regression. Analysis of variance (ANOVA) was used to determine the significance and interactions of the factors ($p < 0.05$). Desirability analysis was employed to assess if a combination of variables satisfies the goal that was defined for the response, using a scale ranging from 0.0 (undesirable) to 1.0 (highly desirable).

2.7. Confirmatory analyses by GC-MS

GC-MS analyses were performed on a Thermo Scientific TSQ EVO 8000 GC system equipped with a triple quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA USA). Gas chromatographic analysis was carried out in the monitoring reaction mode. The presence of at least two significant MS/MS transitions was used to identify the analytes. For each PCB, the *m*/*z* values for the ms/ms transitions have been fixed based on what reported in the official European documents (SANTE 2017/11813/EC and Dec 2002/657/EC). The ion selection was performed choosing characteristic isotopic ions, especially Cl and Br clusters, not exclusively originating from the same part of the analyte molecule. The selected diagnostic ions were: 186.1, 256.0 and 258.0 for PCB#28; 220.0, 255.0 and 290.0 for PCB#52; 323.9 and 326.0 for PCB#101; 360.0, 362.0 and 290.0 for PCB#153; 360.0, 362.0 and 290.0 for PCB#138; 393.9 and 395.9 for PCB#180.

The chromatographic separations were performed using the capillary column Rxl (30 m × 0.25 mm x 0.25 μm) from RESTEK Pure Chromatography (Bellefonte, PA, USA). A sample volume of 1.5 μL was injected by Programmed Temperature Vaporizing (PTV) in splitless mode. The injector temperature started at 100 °C and ramped to 260 °C in 1 min; finally, a cleaning time of 5 min at 320 °C was applied. The oven temperature was initially set at 70 °C for 1.0 min and then increased to 150 °C at a rate 30 °C min^{-1} and to 260 °C at 6 °C min^{-1}; a final temperature of 290 °C was kept for 5.5 min with a total run time of 28.5 min. Acquisition and data processing were performed by the Trace Finder and Xcalibur workstations (Thermo Fisher Scientific).

3. Results and discussion

3.1. PCB extraction and sample clean-up

The sample extraction process is an essential and critical step in the pesticide analysis, representing the base for the determination of residues at the trace level. The major drawbacks are the complexity of food matrices and the low analyte levels to be quantified, therefore the process could be tedious, time-consuming, and labour-intensive. Recently, innovative sample extraction processes based on hollow-fiber liquid phase microextraction [24], QuEChERS [25] or functionalized sol–gel aluminum strip microextraction [26] have been proposed. The extraction efficiency is dependent on several physical and chemical parameters that, whatever is the adopted extraction method, should be further refined to obtain efficient recoveries and reproducible results. The use of the experimental design in the optimization process of the extraction procedure proves to be effective with a minimum of experiments, time, and costs [2].

Preliminary experiments on spiked milk samples at a fortification level of 40 ng g^{-1} fat were carried out to evaluate existing extraction procedures for the determination of pesticides and other persistent organic pollutants in milk, based on QuEChERS [21] and SPE [27]: unfortunately, NDL-PCBs analyses were compromised by the presence of matrix interfering peaks in the relevant retention time-window. In our previous work, a high sample throughput and low solvent consumption extraction/clean-up method has been proposed and successfully applied for the detection of NDL-PCBs analysis in eggs [28]. Starting from these results, a new procedure has been set up and optimized by BBD for the analysis of NDL-PCBs in milk samples by GC-ECD.

3.1.1. Sample extraction optimization by Box Behnken design

On the base of preliminary results (both performed on egg samples, as reported in our previous work [28], and further exploratory investigations on the milk matrix), three significant factors (namely, the acetone percentage in the extraction mixture based on acetone/hexane, the sample/solvent ratio, and the extraction time) were considered as the most influencing input variables. Then, their effect on the recovery and the reproducibility of the extraction process was studied by BBD at three levels. The experimental domain for each experimental variable was chosen, according to preliminary results, as follows: (i) acetone percentage in the mixture acetone/hexane between 5 and 30% (v:v); (ii) sample/solvent ratio from 0.075 to 0.125 g mL^{-1}; (iii) extraction time from 45 to 135 min. The three-factor BBD consisted in 26 experiments (13 different experimental sets: 12 at factorial points and 1 at the center) that were performed in duplicate and in a randomized manner to minimize the bias effects of uncontrolled variables. Each of the 26 extracts was injected two times. All the analyses were performed on milk samples fortified with PCBs at a spiking level (as the sum of the 6 NDL-PCBs) of 40 ng g^{-1} fat, corresponding to 1.5 ng g^{-1} fresh sample. The results were evaluated using the extraction recovery percentage (calculated from the ratio between the concentration measured in spiked samples and the nominal fortification level) and the standard deviation of the replicate analyses (associated to each of the 26 BBD experiments), as shown in the bar-chart of Fig. 1.

The influence of each variable and the possible effects on the responses (recovery % and relative standard deviation) were studies through response surface methodology. Hypersurfaces were constructed from each response (Y_i) as a function of the variable factors (X_i) using a quadratic polynomial model as shown below [29]:

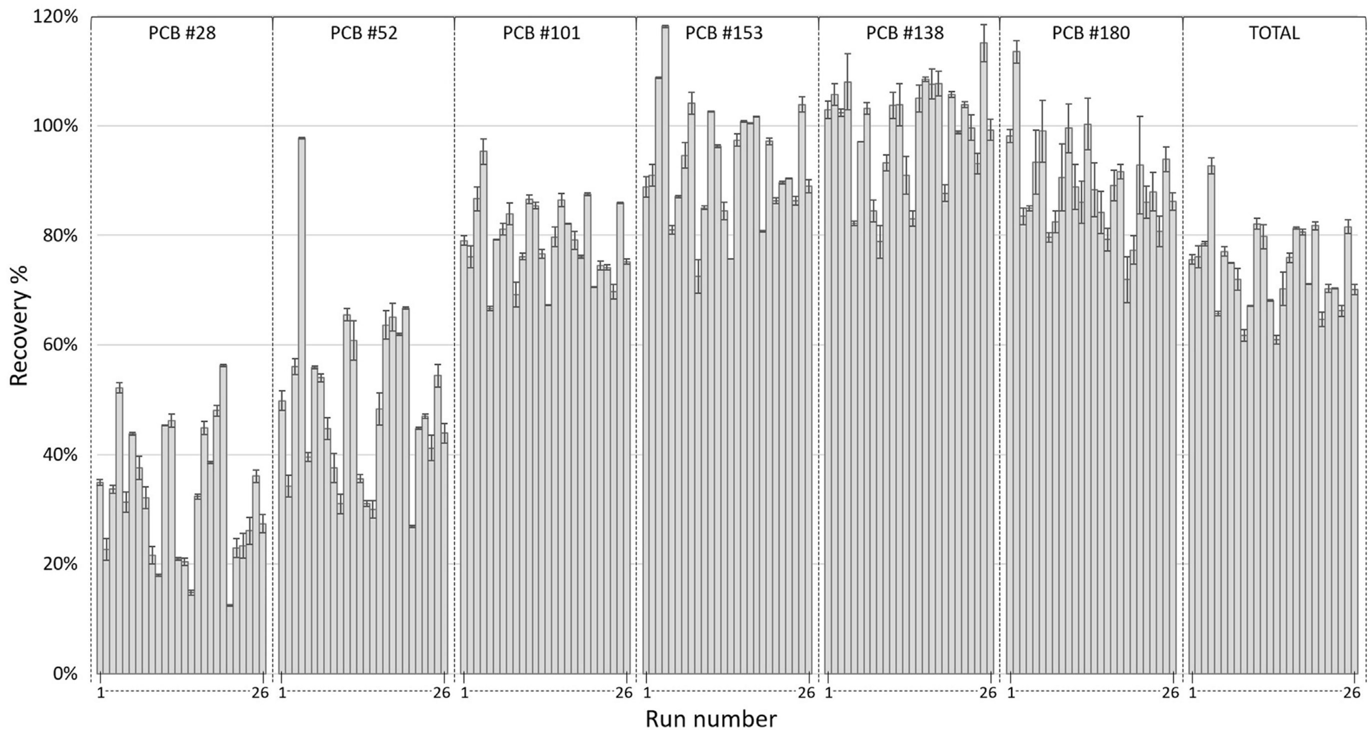

Fig. 1. Recovery values obtained for the sum of NDL-PCBs by BBD. The error bars represent the standard deviations associated to the replicate analyses of the 26 BBD experiments.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{J=i+1}^{3} \beta_{ij} X_i X_j \quad \text{(Eq.1)}$$

where Y is the response variable; β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients for intercept, linearity, square, and interaction, respectively; and X_i and X_j represent the independent variables, coded according to the equation [30]:

$$x_i = \frac{(X_1 - X_0)}{\Delta X_i} \quad \text{(Eq.2)}$$

where x_i is the coded value of the variable X_i; X_0 is the real value of X_i at the center point; ΔX_i is the change in the real value of the variable corresponding to a variation of a unit for the dimensionless value of the variable. The actual and coded levels of the independent variables used in the experimental design are shown in Table S1 (Supplementary Data).

Response surface analysis was carried out using three-dimensional response surface plots, which graphically explained the presence of interactions among the independent variables and their influence on the response variables. In Fig. 2, the response surfaces were drawn as 3D plots of two factors while the other factor was kept constant at the central point. In Fig. 2A the regions in red correspond to maximum values for the total recovery, where the percentages were close to 85–90% while the regions in blue correspond to minimum values when level factors are not suitable to be chosen. On the contrary, for the RSD% response surface, the ideal conditions associated with minimum values were the blue/green zones (Fig. 2B). ANOVA was used to determine the significance and interactions of the independent variables; the three factors (both the linear and the quadratic terms) were found to have a statistically significant impact on the recovery percentage ($p = 0.05$). In particular, the acetone content was the more influencing effect on the extraction efficiency, reaching maximum recovery and low RSD values at high acetone percentages, while extraction time showed the lower impact factor.

3.1.2. Partial and global desirability functions

To obtain a global response that could find the best solution in terms of extraction efficiency and analysis precision, the optimum conditions were determined by the Derringer's desirability function [31]. Two desirability functions (total recovery calculated for the sum of each PCB and relative standard deviation) were built assigning a specific desirability value in the 0–1 range, with the following targets: maximization for the total recovery (response Y_1) and minimization for RSD% (response Y_2). The partial desirability function d_1, for the response Y_1, was calculated according to Eq. (3), taking into consideration that an expected recovery range of 60–140% is officially reported in the European Commission document SANTE 2017/11813/EC for pesticide residues analysis.

$$d_1 = \begin{cases} 0 & \text{if } Y_i < 60\% \\ \left(\frac{Y_i - 60}{85 - 60}\right) & \text{if } 60\% \le Y_i < 85\% \\ 1 & \text{if } 85\% \le Y_i \le 95\% \\ \left(\frac{120 - Y_i}{120 - 95}\right) & \text{if } 95\% < Y_i \le 140\% \\ 0 & \text{if } Y_i > 140\% \end{cases} \quad \text{(Eq. 3)}$$

For the response Y_2 the partial desirability function d_2 was calculated according to Eq. (4), to obtain RSD values lower than 5%:

$$d_2 = \begin{cases} 0 & \text{if } Y_i > 5\% \\ \left(\frac{5 - Y_i}{5 - 1}\right) & \text{if } 1\% < Y_i \le 5\% \\ 1 & \text{if } Y_i < 1\% \end{cases} \quad \text{(Eq. 4)}$$

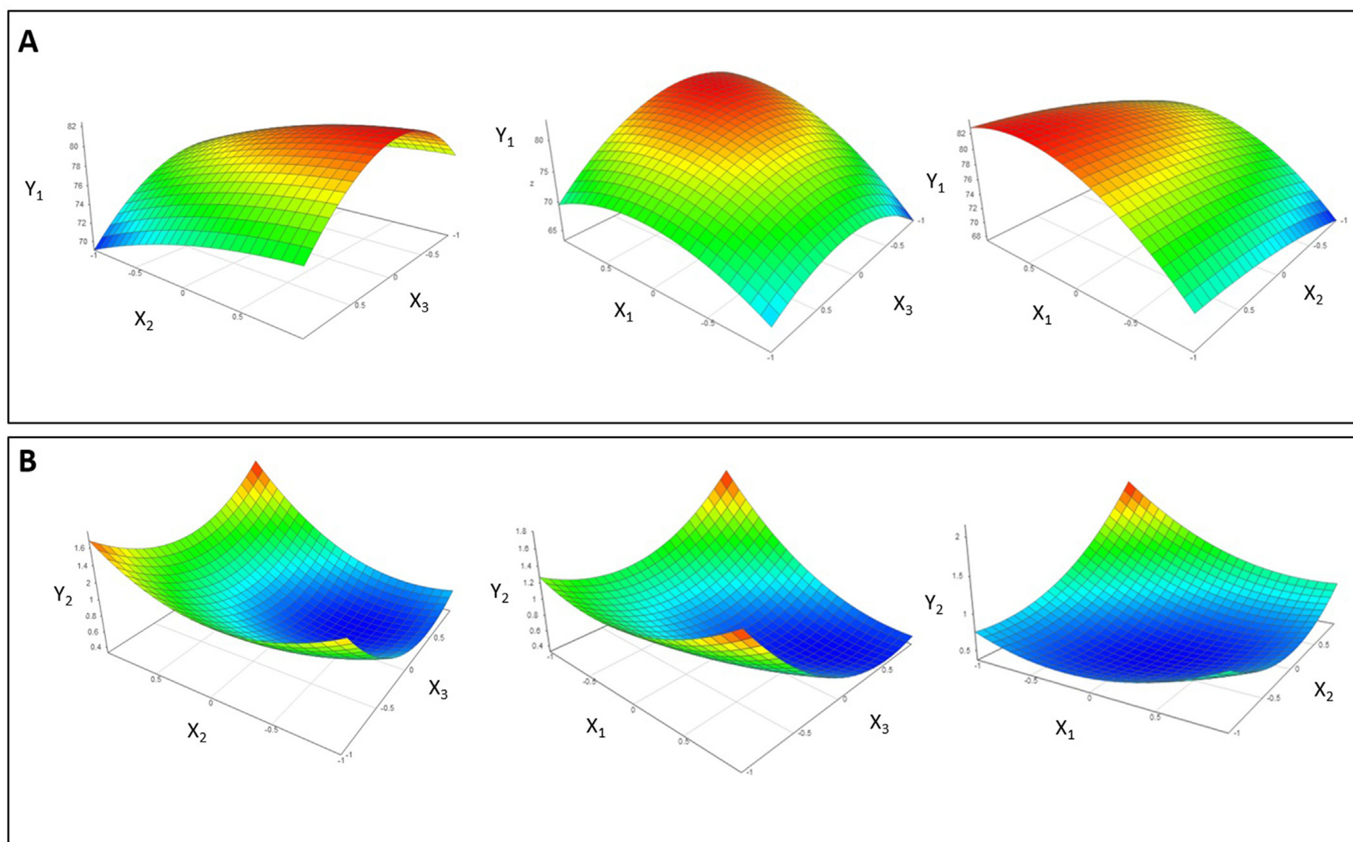

Fig. 2. Response surface plots of the total recovery (response Y1, panel A) and relative standard deviation for the sum of the 6 NDL-PCBs (response Y2, panel B), showing the effect of % acetone in the solvent extraction (X_1), sample/solvent ratio (X_2) and extraction time (X_3) Each response surface refers to couples of factors while the other factor was kept constant at the central point.

Then, the overall desirability function was obtained by the geometric mean of specific desirability assigned to each factor, to simultaneously optimize the recovery percentage and improve repeatability data. A key aspect of a screening method is the overall analysis time, that should assure high throughput applications in monitoring and risk-assessment studies. Taking into account this aspect and considering the relatively small dependence of the response surfaces from extraction times, the model parameters of the global desirability function together with the 3D-plot have been obtained by fixing the factor x_3 at -1 (45 min). At this extraction time, the ideal extraction conditions are associated with $x_1 = x_2 = 1$ (i.e. acetone percentage of 30% and the sample-to-solvent ratio of 0.125 g mL^{-1}). Nevertheless, a deeper investigation was necessary to verify if these conditions were also acceptable for the individual recovery of PCB #28 and #52, which are critical congeners during the extraction process. Indeed, PCB #28 and PCB#52 were characterized by very low recoveries from milk samples (see Table 1), with a high risk of a complete loss of these compounds. The desirability functions were then determined for PCB #28 and #52, as reported in the Supplementary Data. A negative impact on the recovery of PCB #28 was observed for $x_2 = 1$, being also sub-optimal for PCB #52. To find the optimal conditions taking into account this critical issue on PCB #28 and #52, a new desirability function was obtained as a single composite function, by considering the individual recovery percentage for PCB #28 and PCB #52 and the total recovery for the sum of all PCBs (see Supplementary Data for details, Figs. S2 and S3). The 3D-plot of the desirability function (at $x_3 = -1$) showed maximum values around $x_1 = 1$ and $x_2 = 0.44$. As a result, the ideal conditions were: acetone percentage of 30%, sample-to-solvent ratio of 0.11 g mL^{-1} and extraction time of 45 min. With this optimized extraction conditions, a high throughput can be obtained if multiple extracts are prepared in parallel and progressively injected in the chromatographic system With this procedure is then possible to perform a total of 9–10 runs for a working day session, considering a total analysis time of ca. 50 min.

3.2. Optimization of the fat content determination process

To express the NDL-PCB contents in the samples as ng g^{-1} of fat, as specified by European Commission Regulation 1259/2011/EC, the ASE procedure was used to automate the fat extraction process from milk samples, starting from the optimized procedure recently published in our previous work for the PCB analysis in chicken eggs [32]. Several factors affecting the efficiency of the ASE process (sample weight, extraction solvent volume and composition, temperature, and the number of extraction cycles) were tested on lyophilized milk samples. The fat recovery was evaluated by comparison with the labelled values.

The oven temperature for the drying process was explored in the range 100–125 °C (5 °C steps) and the optimal value of 120 °C was set for 10 min. Then, binary and ternary mixtures of apolar solvents, generally used for the fat extraction from food samples (hexane, petroleum ether, isopropanol, chloroform and methanol) at different combination percentages were compared for the liquid-liquid extraction. The best results in terms of recovery (84–113%) were obtained by using the binary mixture petroleum ether/isopropanol (2:1). 3 extraction cycles of 10 min were sufficient to recover the fat amount reported in the label (RSD% lower than 9.4%,

Table 1
Box–Behnken design with recovery percentage and standard deviation for each NDL-PCB at a concentration level equal to 0.25 ng g^{-1} fresh sample. The standard deviations are associated to the replicate analyses of the 26 BBD experiments.

Run	Independent Variables[a]			Measured Responses: % Recovery (Y_1) ± SD (Y_2)						
	X_1 (%)	X_2 (g mL^{-1})	X_3 (min)	#28	#52	#101	#153	#138	#180	Total
1	5	0.075	90	34.94 ± 0.54	49.80 ± 1.81	79.04 ± 0.85	88.84 ± 1.87	102.94 ± 1.56	98.14 ± 1.16	75.61 ± 0.91
2	5	0.075	90	22.64 ± 2.00	34.20 ± 2.00	76.04 ± 2.00	90.92 ± 2.00	105.76 ± 2.00	113.48 ± 2.00	76.03 ± 2.00
3	30	0.075	90	33.66 ± 0.71	55.98 ± 1.44	86.64 ± 2.15	108.80 ± 0.11	102.36 ± 0.68	83.48 ± 1.53	78.49 ± 0.36
4	30	0.075	90	52.20 ± 0.91	97.72 ± 0.17	95.30 ± 2.29	118.16 ± 0.23	108.00 ± 5.09	84.92 ± 0.51	92.72 ± 1.48
5	5	0.125	90	31.26 ± 1.84	39.50 ± 0.82	66.60 ± 0.40	80.98 ± 0.76	82.24 ± 0.40	93.36 ± 5.83	65.65 ± 0.40
6	5	0.125	90	43.76 ± 0.28	55.90 ± 0.31	79.26 ± 0.03	87.04 ± 0.17	97.08 ± 0.01	99.00 ± 5.66	77.03 ± 0.88
7	30	0.125	90	37.56 ± 2.09	53.98 ± 0.71	81.16 ± 1.07	94.60 ± 2.38	103.18 ± 1.10	79.64 ± 0.85	75.03 ± 0.03
8	30	0.125	90	32.04 ± 2.00	44.72 ± 2.00	83.88 ± 2.00	104.08 ± 2.00	84.40 ± 2.00	82.48 ± 2.00	71.93 ± 2.00
9	5	0.100	45	21.62 ± 1.61	37.56 ± 2.66	69.20 ± 2.26	72.50 ± 3.03	78.84 ± 3.00	90.58 ± 6.08	61.72 ± 1.09
10	5	0.100	45	17.92 ± 0.17	31.00 ± 1.81	76.14 ± 0.59	85.04 ± 0.28	93.24 ± 1.47	99.58 ± 4.44	67.16 ± 0.07
11	30	0.100	45	45.34 ± 0.03	65.50 ± 1.16	86.54 ± 0.82	102.62 ± 0.03	103.72 ± 2.43	88.88 ± 4.13	82.10 ± 1.03
12	30	0.100	45	46.16 ± 1.19	60.74 ± 3.59	85.42 ± 0.59	96.28 ± 0.28	103.86 ± 3.87	86.06 ± 3.82	79.75 ± 2.21
13	5	0.100	135	20.96 ± 0.28	35.58 ± 0.71	76.56 ± 0.79	84.44 ± 1.64	90.98 ± 3.42	100.30 ± 4.72	68.13 ± 0.11
14	5	0.100	135	20.40 ± 0.68	31.00 ± 0.51	67.22 ± 0.03	75.64 ± 0.01	83.02 ± 1.39	88.26 ± 4.89	60.92 ± 0.78
15	30	0.100	135	14.76 ± 0.51	30.02 ± 1.61	79.72 ± 1.75	97.36 ± 1.13	105.00 ± 2.49	84.14 ± 3.87	70.23 ± 3.05
16	30	0.100	135	32.33 ± 0.47	48.30 ± 2.91	86.48 ± 1.19	100.80 ± 0.11	108.50 ± 0.42	79.20 ± 2.09	75.93 ± 0.87
17	17.5	0.075	45	44.80 ± 1.19	63.60 ± 2.60	82.14 ± 0.08	100.50 ± 0.08	107.62 ± 2.69	89.08 ± 2.77	81.29 ± 0.20
18	17.5	0.075	45	38.56 ± 0.23	65.02 ± 2.52	79.08 ± 1.70	101.72 ± 0.06	107.66 ± 2.23	91.60 ± 1.30	80.61 ± 0.50
19	17.5	0.125	45	47.98 ± 0.99	61.96 ± 0.17	76.12 ± 0.28	80.74 ± 0.08	87.64 ± 1.53	71.90 ± 4.16	71.14 ± 0.06
20	17.5	0.125	45	56.22 ± 0.14	66.70 ± 0.25	87.48 ± 0.28	97.14 ± 0.59	105.70 ± 0.54	77.32 ± 2.55	81.76 ± 0.73
21	17.5	0.075	135	12.48 ± 0.11	26.86 ± 0.20	70.54 ± 0.03	86.30 ± 0.48	98.80 ± 0.28	92.84 ± 8.94	64.64 ± 1.38
22	17.5	0.075	135	22.98 ± 1.73	44.74 ± 0.20	74.50 ± 0.76	89.64 ± 0.23	103.88 ± 0.51	86.00 ± 2.94	70.29 ± 0.72
23	17.5	0.125	135	23.30 ± 2.23	46.92 ± 0.40	74.14 ± 48	90.38 ± 0.03	99.64 ± 2.32	87.92 ± 3.51	70.38 ± 0.02
24	17.5	0.125	135	26.08 ± 2.49	41.16 ± 2.32	69.74 ± 1.33	86.30 ± 0.76	93.12 ± 1.87	80.74 ± 2.80	66.20 ± 0.99
25	17.5	0.100	90	36.02 ± 1.10	54.36 ± 2.04	85.94 ± 0.14	103.90 ± 1.39	115.12 ± 3.39	93.90 ± 2.29	81.54 ± 1.25
26	17.5	0.100	90	27.38 ± 1.73	43.86 ± 1.84	75.16 ± 0.45	88.98 ± 1.16	99.20 ± 1.92	86.14 ± 1.61	70.11 ± 0.92

[a] X_1: % acetone in the solvent extraction; X_2: sample/solvent ratio; X_3 extraction time.

n = 10, under reproducibility conditions in different working days, with different operators and reagent lots).

3.3. Optimization of the chromatographic conditions by GC-ECD

In the development of a multi-residue method, the optimization of the chromatographic conditions is a critical stage, in particular when ECD is used for screening analysis. Indeed, ECD could suffer from sample matrix interferences, due to potential co-eluting compounds and then particular attention has been devoted to the optimization of the experimental chromatographic conditions, aimed at ensuring a high-throughput analysis and good peak resolutions. Details of the optimized temperature gradient program are summarized in the experimental section. A satisfactory separation with symmetrical and narrow peaks was obtained in a time window from 12 to 23 min, with a total run time of 27 min.

3.4. Method validation

As recommended by the European regulations (European Commission SANTE 2017/11813/EC, European Commission Regulation 2017/644/EC and European Commission Decision 657/2002/EC), the validation of the analytical methods is essential to provide reliable results in risk-assessment studies, as well as in official controls for pesticide determinations. Therefore, the screening GC-ECD analytical method was extensively validated through the evaluation of selectivity, linearity, detection and quantification limits, precision, recovery, and ruggedness. Details on selectivity, precision and recovery tests are described in the Supplementary Data, paragraph GC-ECD method validation.

3.4.1. Calibration curves and limits of detection and quantification

The linearity test was performed by three series of analyses on three different days, by injecting six mixed standard solutions of NDL-PCBs, at concentrations of 1.0, 2.5, 5, 10 and 20 μg L^{-1}, corresponding to 0.05, 0.125, 0.25, 0.50 and 1.00 ng g^{-1} in the matrix. Calibration curves were obtained by plotting the ratio between analyte peak area and IS peak area against the NDL-PCB concentration. Analogously, the identification of the target compounds was accomplished by calculating the relative retention time as the ratio between the analyte and IS retention times. For all NDL-PCBs, a good fitting was observed in the range 1–20 μg L^{-1} with correlation coefficients higher than 0.9990. The signal-to-concentration ratio (y/x) was calculated for each experimental point to evaluate the goodness-of-fit of the data to the calibration curve). Then, the x_i/y_i ratios were checked to ensure that their deviation from the mean value of signal-to-concentration ratio did not exceed ±10%. The absence of systematic instrumental bias was confirmed by the confidence interval for the intercept including the zero value at 95% confidence level. By Mandel's fitting test [33], the residual variances, resulting from the linear and the quadratic calibration function, were compared by an F-test and the hypothesis H_0 (no significant difference between the residual variances) was accepted for all the NDL-PCBs. Therefore, calibration straight-lines rather than over curvilinear or non-linear models well fitted the experimental data. The calibration parameters, evaluated for each analyte, are reported in Table 2. The instrumental limits of detection (LOD) and quantification (LOQ) were estimated by the chromatograms of the NDL-PCB standard solutions obtained for the lowest calibration level (1.0 μg L^{-1}), at a signal-to-noise ratio of 3 and 10, respectively. The noise level was evaluated as peak-to-peak value, i.e. the difference between the maximum positive and the maximum negative amplitudes of baseline in the time window around the analyte retention time. LODs and LOQs were in the range 0.13–0.39 ng μL^{-1} and 0.44–1.3 ng μL^{-1}, respectively (corresponding to 0.18–0.52 ng g^{-1} fat and 0.59–1.7 ng g^{-1} fat in the matrix). These values are considerably lower than the legal limits of 40 ng g^{-1} fat (established for the sum of the six NDL-PCBs), allowing NDL-PCB determination at trace levels and reducing the risk of false-negative results. LODs and LOQs in the matrix, obtained

Table 2
Performance and chromatographic parameters of PCBs analyzed by the proposed GC-ECD method.

Analyte	t_R^a (min)	Linear Range (R) (μg L^{-1})	Sensitivity (10^{-5} μV μg $^{-1}$ L)	Instrumental				Method	
				LOD (μg L^{-1})	LOQ (μg L^{-1})	LOD (ng g^{-1} fat)	LOQ (ng g^{-1} fat)	LOD (ng g^{-1} fat)	LOQ (ng g^{-1} fat)
				Solvent[b]		Matrix[c]		Bovine milk[d]	
PCB#28	8.46	0.98–20 (0.9990)	417 ± 11	0.29	0.98	0.39	1.30	0.86	2.86
PCB#52	9.02	1.3–20 (0.9991)	260 ± 9	0.39	1.29	0.52	1.72	1.09	3.62
PCB#101	10.51	0.87–20 (0.9991)	433 ± 12	0.26	0.87	0.35	1.16	0.97	3.23
PCB#153	11.99	0.48–20 (0.9992)	775 ± 18	0.14	0.48	0.19	0.64	0.26	0.85
PCB#138	12.52	0.56–20 (0.9990)	745 ± 19	0.17	0.56	0.22	0.74	0.27	0.91
PCB#180	13.87	0.44–20 (0.9997)	1169 ± 15	0.13	0.44	0.18	0.59	0.39	1.30

[a] Retention time; tolerance range ± 0.5%. Instrumental LOD and LOQ values referred to standard solutions prepared in solvent[b] and their estimation in matrix[c]. Method Detection and Quantification Limits calculated on spiked bovine milk samples at 20 ng g^{-1} fat.

by chromatograms of spiked milk samples at a concentration level of 20 ng g^{-1} fat, ranged from 0.26 to 1.1 ng g^{-1} fat and 0.85–3.6 ng g^{-1} fat, respectively. These values prove that, even in the case of real sample analyses, the proposed method returns LOQs considerably lower than legal limits.

3.4.2. Precision and recovery

As reported in European Commission Decision 657/2002/EC and SANTE 2017/11813/EC, in absence of official and certified reference material (CRM), the trueness of measurements was assessed through the analysis of spiked samples, prepared starting from blank material with a known fat amount. After homogenization, proper known amounts of PCBs were added to obtain the desired spiking level, thus the same PCB concentration was obtained in all the aliquoted sample portions. Precision and recovery data have been previously processed by the Shapiro-Wilk test [34] to verify normal distribution. Afterwards, a one-way ANOVA test was performed to verify the homogeneity of the mean concentration values evaluated among the validation sessions at each fortification level. Results from ANOVA were used to calculate intra-laboratory repeatability relative standard deviations (RSD_r) following the Decision 2002/657/EC. Recovery percentages were calculated by comparing the concentration of spiked samples, determined by the external calibration regression line, with the nominal fortification level. For the sum of NDL-PCBs, it was verified that the calculated mean recovery at each spiking level complied with the recovery range of 60–140%, reported in the official documents (European Commission SANTE 2017/11813/EC) dealing with the method validation and quality control procedures for Pesticide Residues Analysis in Food and Feed. Total recovery values of 74.9 ± 1.6%, 75.7 ± 4.4% and 85.4 ± 3.6 (n = 6) were obtained at the fortification level of 20, 40 and 80 ng g^{-1} fat, respectively. The intra-day RSD_r values (ranging from 2.1% to 5.8%) were well below the reference values of 15%, derived by Horwitz equation [35] for a mass fraction ≤ 0.1 mg kg^{-1}, under repeatability conditions, demonstrating a good method precision.

3.4.3. Method ruggedness

As described in Decision 2002/657/EC, the method ruggedness (defined as the capacity to reproduce results when the method is applied under small changes in the nominal values of the experimental factors established in the optimization step) for the extraction process of NDL-PCBs from milk was confirmed by using the Youden experimental design. The seven factors chosen as variables that could influence the results were: extraction volume, vortex agitation time, extraction time under ultrasonication, centrifugation time, centrifugation speed, the volume of sulphuric acid and final extract evaporation temperature. For the selected seven factors, alternative lower and higher levels than the nominal mean value are denoted with the upper case letters A, B, C, D, E, F, G, and the corresponding lower case letters a, b, c, d, e, f, and g, respectively, as shown in Table 3. Then, among the 128 (i.e. 2^7) different combinations resulting from the Youden design, a subset of eight experiments was chosen as a balance between capital and small letters. Therefore, only eight determinations are enough to study the influence of the seven Youden factors. The ruggedness test was performed on spiked milk samples at 40 ng g^{-1} fat and the results were determined by the sum of the observed NDL-PCB amounts as ng g^{-1} fat. Then, the standard deviation of the differences (S_{Di}) between the averages of the results associated with the capital letter experiments and the averages of their corresponding small letter experiments were calculated. The statistical comparison between the obtained S_{Di} value with the method standard deviation, determined under within-laboratory reproducibility

Table 3
Youden experiment design for ruggedness studies (Dec 657/2002/EC).

FACTOR	NOMINAL VALUE	DESCRIPTION		COMBINATION OF DETERMINATIONS NUMBER							
				1	2	3	4	5	6	7	8
Extraction Volume	100 mL	A/a	105/95 mL	A	A	A	A	a	a	a	a
Vortex Agitation Time	45 min	B/b	50/40 min	B	B	b	b	B	B	b	b
Sonication Time	30 min	C/c	40/20 min	C	c	C	c	C	c	C	c
Centrifugation Time	15 min	D/d	20/10 min	D	D	d	d	d	d	D	D
Centrifugation Speed	3000 rpm	E/e	3500/2800 rpm	E	e	E	e	e	E	e	E
Volume of Sulphuric Acid	4 mL	F/f	4.5/3.5 mL	F	f	f	F	F	f	f	F
Evaporation Temperature	45 °C	G/g	50/40 °C	G	g	g	G	g	G	G	g
OBSERVED RESULTS: SUM of PCBs (ng g^{-1} fat)				30.4	30.7	30.9	28.0	30.7	31.5	27.5	29.6
Recovery %				75.8	76.5	77.0	70.2	76.6	78.8	68.7	73.9

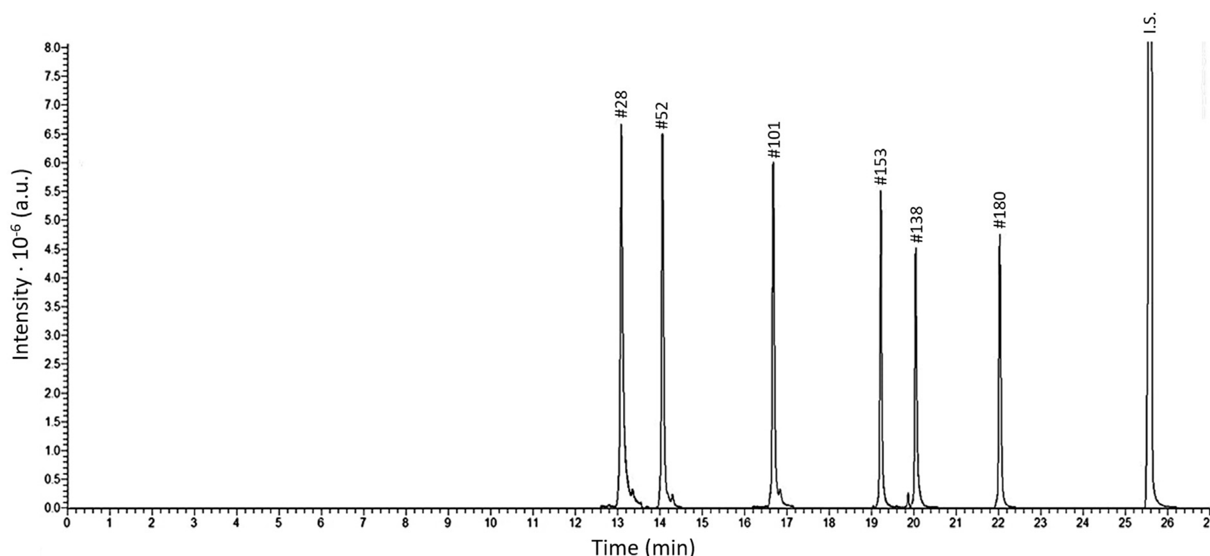

Fig. 3. Total Ion Current chromatogram of a spiked bovine milk sample at 40 ng g^{-1} fat by GC-MS. IS: internal standard (NDL-PCB 209).

conditions (S_{Dr}) at the same fortification level, demonstrated that none of the factors affects the result. Therefore, the proposed method can be considered robust against the chosen modifications in the extraction process.

3.5. Analyses of milk samples by GC coupled with ECD and mass spectrometry

Method feasibility has been demonstrated by the GC-ECD analyses of several milk samples of different animal origin. In each batch of milk samples, a matrix blank was also analyzed to reduce the risk of false-positive results due to the potential chemical contamination in the extraction process. Therefore, a reagent blank was processed according to the complete analytical procedure using an equivalent amount of suitable solvent in place of the test portion, and a sample blank (i.e. a compliant control sample) was prepared from a test portion taken from a sample from which the analyte is absent. For every batch of 10 samples, a reagent blank and a sample blank were included in the sample list to be analyzed. Finally, the optimized extraction and chromatographic conditions were also applied to the NDL-PCB analytical determination by mass spectrometry, confirming the method potential in confirmatory analyses. Then, the same extraction procedure optimized by Box Behnken design for the PCB determination by GC-ECD, has been applied also to the confirmatory analysis by GC-MS. Indeed, when in official check analyses doubtful or non-compliant results are obtained by the first, screening evaluations by GC-ECD, a confirmatory analysis has to be carried out by using an independent instrumental line based on GC-MS. As an example, in Fig. 3 the GC-MS profile obtained for a spiked bovine milk sample (with a fat content of 3.7%) at a fortification level of at 40 ng g^{-1} fat is displayed.

4. Conclusions

The Box–Behnken experimental design and the global desirability functions were successfully applied for the first time to determine the optimal extraction conditions for NDL-PCBs determination in milk by GC-ECD and confirmation analysis by MS. The effect of three dependent variables (acetone percentage in the extraction mixture, the sample-to-solvent ratio, and the extraction time) was studied at three different levels on the recovery percentage and its standard deviation. The optimized sample extraction/clean-up procedure allowed to perform the simultaneous extraction and clean-up of more than 9 samples in an 8-h single day working session. Finally, the method was validated through the evaluation of linearity, detection and quantification limits, selectivity, recovery, precision, and ruggedness, demonstrating its conformity with provisions of the European directives for the accurate screening of NDL-PCBs in complex food matrices.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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