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Optimization of the derivatization reaction and the solid-phase microextraction conditions using a D-optimal design and three-way calibration in the determination of non-steroidal anti-inflammatory drugs in bovine milk by gas chromatography–mass spectrometry

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ABSTRACT

An experimental design optimization is reported of an analytical procedure used in the simultaneous determination of seven non-steroidal anti-inflammatory drugs (NSAIDs) in bovine milk by gas chromatography with mass spectrometry detection (GC–MS). This analytical procedure involves a solid-phase microextraction (SPME) step and an aqueous derivatization procedure of the NSAIDs to ethyl esters in bovine milk. The following NSAIDs are studied: ibuprofen (IBP), naproxen (NPX), ketoprofen (KPF), diclofenac (DCF), flufenamic acid (FLF), tolfenamic acid (TLF) and meclofenamic acid (MCL). Three kinds of SPME fibers – polyacrylate (PA), polydimethylsiloxane/divinylbenzene (PDMS/DVB) and polydimethylsiloxane (PDMS) – are compared to identify the most suitable one for the extraction process, on the basis of two steps: to determine the equilibrium time of each fiber and to select the fiber that provides the best figures-of-merit values calculated with three-way PARAFAC-based calibration models at the equilibrium time. The best results were obtained with the PDMS fiber. Subsequently, 8 experimental factors (related to the derivatization reaction and the SPME) were optimized by means of a D-optimal design that involves only 14 rather than 512 experiments in the complete factorial design. The responses used in the design are the sample mode loadings of the PARAFAC decomposition which are related to the quantity of each NSAID that is extracted in the experiment. Owing to the fact that each analyte is unequivocally identified in the PARAFAC decomposition, a calibration model is not needed for each experimental condition. The procedure fulfils the performance requirements for a confirmatory method established in European Commission Decision 2002/657/EC.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most frequently used pharmaceutical compounds in human and veterinary medicine. They can be used for suppressing inflammatory processes, treating allergies and reducing pain [1] and are widely in demand, as neither sedation, nor respiratory depression, nor addiction figure among their side-effects. These NSAIDs are acidic compounds with pK_a values between 3.5 and 4.5. The acid group is essential in the inhibition of the cyclooxygenases COX-1 and COX-2, the basic enzymes in the biosynthesis of prostanglandins (responsible for swelling and pain).

NSAIDs are used in the treatment of coliform mastitis [2] in cattle and certain respiratory diseases in conjunction with antibiotics [3]. They may also be used for non-therapeutic purposes because they are administered to cattle to produce pale meats [4] and can reduce edible fat when administered orally [5].

Ibuprofen (IBP), flufenamic acid (FLF), naproxen (NPX), ketoprofen (KPF), tolfenamic acid (TLF), diclofenac (DCF) and meclofenamic acid (MCL) are NSAIDs that can be classified into several subclasses: arylalkanoic acid derivates (DCF), arylpropionic acid derivates or profens (IBP, KPF and NPX) and *N*-arylanthranilic acid derivates or fenamic acids (FLF, TLF and MCL). A prolonged use of NSAIDs in humans can cause adverse health effects such as intestinal ulceration, gastrointestinal disorders, aplastic anaemia and the inhibition of platelet aggregation [6]. The TLF and DCF compounds are among the substances regulated by the European Union [7] in milk. IBP, NPX and KPF are among the most frequently determined in environmental analyses due to the widespread use of NSAIDs and the potential risk to the consumer if their residues enter the food chain.

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The criteria adopted to evaluate the performance of the presently proposed method are external criteria, precisely the recommendations made by the Community Reference Laboratories (CRLs) [8] for the determination of NSAIDs. According to these recommendations, which develop the criteria of the European Decision 2002/657/EC, a procedure for this task should be capable of monitoring IBP, NPX, and mefenamic acid (MCL and FLF are of the same sub-class) for a concentration of 10 $\mu g\,L^{-1}$, DCF (banned in [7]) for 5 $\mu g\,L^{-1}$, and TLF for 50 $\mu g\,L^{-1}$. During the development of the experimental part of the present work, the regulation [7] has been superseded by Regulation [9] published on January 20, 2010 in which DCF is no longer banned but it has a MRL fixed at 0.1 $\mu g\,L^{-1}$.

In the recent past, the analytical determination of NSAIDs in milk has been done by liquid chromatography with UV–vis or fluorescence detection [10], for MS/MS detection [4,11–14] above all. Only one recent work [15] has been found on the determination of NSAIDs in milk by gas chromatography (GC). On the other hand, there are many suitable analytical methods for the determination of NSAIDs in aqueous samples (environmental samples) by GC–MS with solid-phase extraction [16–19] or SPME (solid-phase microextraction) [20–23]. Ref. [24] is a review specifically devoted to NSAID analysis in environmental samples, including sample preparation.

European Commission Decision 2002/657/EC [25] states that the best choice is, for confirmatory purposes, the use of a chromatographic technique coupled with a mass spectrometry detector. Owing to the polarity of the NSAIDs, GC–MS analysis can be applied only after analyte derivatization, but it is not usually preferred for the analysis of NSAIDs in milk samples because of the complexity of the matrix, and the time taken up by NSAID extraction and derivatization reactions. Current LC–MS or LC–MS/MS technology might be more appropriate than GC–MS in the analysis of NSAIDs in milk samples, but both purchase and maintenance costs of LC–MS or LC–MS/MS instrumentation are still excessively high in comparison with GC–MS. Many laboratories are therefore not equipped with LC–MS/MS instrumentation and thus it is of interest to develop a less costly GC–MS analytical methods.

A derivatization step must be performed prior to GC analysis of NSAIDs, in order to increase the volatility and decrease the polarity of these analytes owing to their acidic group. The use of alkylchloroformates as a derivatization reagent is the approach selected, because derivatization occurs in aqueous media at room temperature, within a few minutes and because alkylchloroformates only react with NH_2 and carboxylic OH groups [26]. Thus, this derivatization reaction yields simpler chromatograms in complex matrices that contain large amounts of sugars or other compounds. To our knowledge, this derivatization reaction has not been used for the determination of NSAIDs in milk. In this work, an analytical method is proposed for the determination of the 7 previously mentioned NSAIDs in bovine milk based on GC–MS after the SPME step and an aqueous derivatization step with a mixture of ethanol/pyridine/ethyl chloroformate (EtOH/Py/ECF).

Three kinds of SPME fibers (PDMS, PDMS/DVB and PA) are compared in this paper, in order to establish the most suitable one for the extraction, on the basis of the following two steps: (1) to determine the equilibrium time of the fiber of all the NSAIDs and (2) to select the fiber that provide the best values for several figures of merit (number of outliers, trueness, residual standard deviation, decision limit or CCα, detection capability or CCβ, and the mean of the absolute values of relative errors) calculated with three-way calibration models based on PARAFAC analysis. As far as the authors are aware, the second fiber selection criterion has not been reported in the literature on SPME.

PARAFAC calibration models can simultaneously identify and quantify NSAIDs in a single analysis on the basis of the second-order signals recorded by GC–MS instrumentation. For confirmatory methods, European Commission Decision 2002/657/EC establishes requirements for the unequivocal identification of analytes. These requirements are based on the concept of identification points (IPs) with maximum-permitted tolerances for relative abundances between several m/z ratios and the most abundant m/z ratio (base peak). A minimum of either 3 m/z ratios (identification points) have to be recorded if the analyte is permitted or 4 m/z ratios, if banned. In this work, 6 m/z ratios are recorded for each NSAID and all the NSAIDs are unequivocally identified with PARAFAC calibration models.

Subsequently, 8 experimental factors (5 relate to the SPME process and 3 to the derivatization reaction) were optimized, using the experimental design methodology, in bovine milk samples. This procedure enabled the examination and optimization of each parameter within a predefined range, by performing a series of experiments in each of which the values of the different parameters were changed at the same time. It is common practice to optimize each experimental factor in the literature on SPME (extraction time in the fiber, extraction temperature, derivatization time) separately, increasing the experimental effort. In our experiment, with 7 factors at two levels and 1 factor at four levels, a complete factorial design has 512 experiments, but by means of a D-optimal design all experimental factors studied are optimized with only 14 experiments.

The responses in the D-optimal design are the sample mode loadings obtained by means PARAFAC calibration models of each NSAID. These loadings are related to the quantity of each NSAID that is extracted in each experiment of the D-optimal design. Owing to the unequivocal identification of each analyte with PARAFAC decomposition (second order property), a calibration model is no longer necessary for each experimental condition. Consequently, in practice it is highly efficient the joint use of a PARAFAC decomposition and a D-optimal design. This has been used by our research group in the determination of sulfonamides by using excitation-emission molecular fluorescence with solid phase extraction [27]. However, it is the first time that a D-optimal design coupled to a PARAFAC decomposition is used in GC–MS for jointly selecting the most favourable experimental conditions in the steps of solid phase microextraction and derivatization.

2. Experimental

2.1. Chemicals and materials

All organic solvents were of HPLC-grade and the rest of chemicals were of analytical-reagent grade. Ethanol (EtOH), acetonitrile (ACN), n-hexane, sodium chloride and pyridine (Py) were supplied by Merck (Darmstadt, Germany). Ethyl chloroformate (97%) was purchased from Aldrich (Steinheim, Germany) and deionized water was obtained by using the Milli-Q gradient A10 water purification system of Millipore (Bedford, MA, USA).

Analytical standards of tolfenamic acid, meclofenamic acid sodium salt, ibuprofen (98%), ketoprofen, diclofenac sodium salt, flufenamic acid and naproxen (98%) were obtained from Sigma–Aldrich (Steinheim, Germany). $^{13}C_6$-tolfenamic acid (98%, TLF-$^{13}C_6$) was supplied by WITEGA Laboratorien (Berlin, Germany) and was used as internal standard for all NSAIDs.

An autosampler SPME fiber holder and SPME fibers with different coatings: poly(dimethylsiloxane) (PDMS, 100 μm film thickness), polyacrilate (PA, 85 μm) and poly(dimethylsiloxane-divinilbenzene) (PDMS/DVB, 65 μm) were purchased from Supelco (Bellefonte, PA, USA). The fibers were conditioned, before their first use, as recommended by the manufacturer.

2.2. Standard solutions and aqueous derivatization reaction

Individual stock solutions at 5000 mg L^{-1} of all NSAIDs were prepared in ethanol. A combined diluted NSAID solution of all analytes (50 mg L^{-1}) was prepared by dilution in the same solvent. Working NSAID solutions at 5 mg L^{-1} were prepared every day by dilution with ethanol.

The internal standard stock solution of TLF-$^{13}C_6$ at 1000 mg L^{-1} was prepared in ethanol and an internal standard working solution (10 mg L^{-1}) was prepared daily by dilution in the same solvent. All standard solutions were stored at 4 °C, in amber bottles and protected from light for less than 12 months (stock solutions) and for less than 1 month (combined solution).

The aqueous derivatization reaction of the acid groups of the NSAIDs to their ethyl esters was performed with a mixture of EtOH/Py/ECF at room temperature. This procedure can therefore be applied to aqueous matrices such as milk, plasma or urine. Subsequently, the derivatized analytes were extracted from the aqueous matrices by SPME direct immersion and finally determined by GC–MS.

2.3. Treatment of the test solutions for SPME fiber selection

The test solutions were prepared as follows: to 4 mL dissolution of EtOH:H_2O (1:1, v/v), were added 200 μL of the working solution of the NSAIDs (which corresponds to a final concentration of 100 μg kg^{-1} of all the analytes) and 600 μL of the derivatizing mixture EtOH/Py/ECF (200 μL of each) being adjusted to a final volume of 10 mL with deionized water. The total volume was transferred to a 10 mL vial to perform the automatic SPME process and was encapsulated. The vials were placed in an ultrasonic bath at room temperature and sonicated for 15 min to generate the derivatization reaction. Subsequently, the vial was placed in the automatic equipment and the SPME stage and its subsequent chromatographic analysis were performed, at an SPME extraction temperature of 60 °C. Extraction was carried out with agitation and subsequent conditioning of the fiber took place for 5 min at 250 °C.

2.4. Milk sample treatment

Blank bovine milk samples were used. Ten grams of bovine milk were placed into a 50 mL polypropylene centrifuge tube. Fortified samples were prepared by the addition of 50 and 100 μL of the internal standard solution and the NSAID working solution, respectively (corresponding to final concentrations at 50.0 μg kg^{-1} for all analytes). Fortified samples were then homogenized in a vortex-mixer (Velp Scientifica, Milan, Italy) for 30 s and left to stand for 10 min.

Subsequently, 10 mL of ACN and 2 g of NaCl were added. The tubes were shaken in a vortex-mixer for 60 s and centrifuged at 5000 rpm for 15 min, at 5 °C. The acetonitrile (top layer) was then removed and placed into 15 mL polypropylene tubes and 4 mL of n-hexane were added. The tubes were shaken and centrifuged under the same conditions. The n-hexane (top layer) was removed and discarded.

The acetonitrile extracts were then evaporated to dryness with a rotary evaporator at 50 °C. The residues were finally reconstituted with 4 mL of EtOH:H_2O (1:1, v/v). After addition of V μL (V_{DER}) of the derivatization mixture of EtOH/Py/ECF, the final volume was adjusted at 10 mL with deionized water and placed in a 10 mL screw-cap glass autosampler vial and sealed.

The vial was then immersed in an ultrasonic bath at room temperature, and the derivatization reaction was performed during t min (t_{DER}). Subsequently, the SPME stage was performed (the fiber was immersed into the aqueous sample) and following extraction, the fiber was directly exposed to the hot injector of the GC and the chromatogram was recorded.

Table 1
Retention time, RT, and m/z ratios recorded in SIM mode for each analyte.

Analyte	RT (min)	m/z ratios
IBP	11.31	105, 117,119, 161[a], 191, 234[b]
FLF	13.36	92, 166, 235, 243, 263[a], 309[b]
NPX	14.12	115, 141, 154, 170, 185[a], 258[b]
KPF	14.95	77, 105, 165, 194, 209[a], 282[b]
TLF	15.41	180, 208[a], 243, 245, 289[b], 291
TLF-$^{13}C_6$	15.41	214[a], 295[b], 297
DCF	15.65	214[a], 216, 242, 244, 323[b], 325
MCL	16.58	242[a], 244, 277, 279, 323[b], 325

The m/z ratios recorded represent the derivatized analytes that are the ethyl esters of their corresponding NSAIDs.
[a] Base peak.
[b] Molecular ion.

The experimental factors under study in the D-optimal design were related to the derivatization reaction, namely derivatization time (t_{DER}), EtOH/Py/ECF volume (V_{DER}) and EtOH/Py/ECF proportion (P_{DER}); and for the SPME, namely extraction temperature (T_{EXT}), extraction time (t_{EXT}), sample agitation (A_{GT}), fiber-conditioned temperature (T_{CON}) and fiber-conditioned time (t_{CON}).

2.5. SPME and chromatographic instruments

The SPME process was performed using a Triplus autosampler (Thermo Scientific, Milan, Italy), equipped with an SPME module, coupled to a GC–MS. Chromatographic analyses were performed with the Agilent 6890N gas chromatograph equipped with a split–splitless injector, coupled to an Agilent 5975 quadrupole mass selective detector (Agilent Technologies, Palo Alto, CA, USA). Separation was achieved with the J&W DB-5MS capillary column with dimensions of 30 m × 0.25 mm i.d., 0.25 μm film thickness (J&W Scientific, Folsom, CA, USA). A silanized narrow-bore injector liner (0.75 mm i.d.) for the SPME injections was installed in the split/splitless injector. This configuration automatizes the microextraction process and the chromatographic analysis.

Injections were performed in splitless mode with the split valve closed for 3 min and a solvent delay of 10 min. Helium was used as the carrier gas at a constant flow rate of 1.0 mL min^{-1}. The injector was kept at a temperature of 250 °C, the transfer line temperature was 250 °C, the ion source temperature was 230 °C and the quadrupole temperature was 150 °C. The oven temperature was programmed at an initial temperature of 50 °C for 3 min, and then increased to 250 °C at steps of 20 °C min^{-1}, and held at this temperature for 7.0 min. The oven equilibration time was set at 0.25 min.

Analyses were performed in the electron impact ionization mode at 70 eV operating in single ion monitoring (SIM) mode; the electron multiplier was set at 1482 V and the source vacuum at 10^{-5} Torr. According to European Commission Decision 2002/657/EC, a minimum of either 3 or 4 identification points are necessary to confirm the presence of a permitted and a banned veterinary drug, respectively. In this work, 6 m/z ratios (6 identification points) were recorded per compound (only 3 for the internal standard). Table 1 shows the m/z ratios and the retention time of each compound. The dwell time per m/z ratio was 100 ms.

Decision 2002/657/EC establishes that for the detection using mass spectrometry in SIM mode, the molecular ion would preferably be one of the diagnostic ion selected (provided it is stable) together with some other characteristic fragments and all its isotopic ions.

Superscript "b" in Table 1 indicates the molecular ions of all derivatized ethyl esters of each of the studied NSAIDs. For example, for DCF (penultimate row in Table 1), the molecular ion of its derivatized ethyl ester is at m/z ratio 323 and it has two atoms of

chlorine in its structure. Therefore, it has a characteristic isotope in m/z ratio 325, which should be recorded according to Decision 2002/657/EC.

To determine the characteristic fragments of the derivatized DCF, spectra from the NIST library [28] were used. There we found the mass spectra of the original DCF and, not of the derivatized ethyl ester, but the similar enough derivatized methyl ester. The most characteristic fragments for both DCF and the derivatized methyl ester are the 214 m/z ratio (base peak) and 242 m/z ratio; each one containing a characteristic isotopic fragment due to the chlorine atoms in m/z ratios 216 and 244, respectively. Nevertheless, the final selection of the described m/z ratios is made after checking experimentally that these m/z ratios were the most characteristic of the derivatized ethyl ester obtained for DCF with the proposed derivatization process.

The selection of m/z ratios for derivatized ethyl ester of the rest of NSAIDs is made similarly.

2.6. Software

Data acquisition was performed using the Triplus Sampler version 1.6.9 SPME management software (Thermo) and MSD ChemStation version D.02.00.275 chromatographic management software (Agilent).

NEMRODW [29] was used to build and to analyze the D-optimal design for finding the optimal conditions of the SPME procedure and the derivatization reaction. PARAFAC (or PARAFAC2 when needed) three-way decomposition techniques were built with PLS-Toolbox [30] for use with MATLAB version 7 (The MathWorks Inc.). The regression models (estimated concentration *vs.* true concentration) in the three-way (PARAFAC or PARAFAC2) calibrations were performed with the PROGRESS program [31] that applies the least median of squares (LMS) regression, a robust technique for detecting outliers in linear regression. The statistical tests for the validation of the regressions models were performed with STATGRAPHICS [32].

Decision limit, detection capability and multivariate sensitivity for the three-way calibration models were determined using the NWAYDET program. This program displays the detection capability for any given false-positive (or false non-compliance), α, and false negative (or false compliance), β; probabilities that are specified in European Commission Decision 2002/657/EC [25] and ISO 11843 [33].

3. Theory

GC/MS data are arranged in a three-way array, $\underline{\mathbf{X}}$, and analyzed with PARAFAC or PARAFAC2 (three-way decomposition techniques). In this section, the parallelism that exists between the PARAFAC decomposition and a physical model of GC/MS data, under commonly held assumptions, is discussed. In a recent work of our research group [34], the same parallelism between the PARAFAC decomposition and LC–MS/MS data is shown, and a review on three-way techniques which are used for calibration in chromatography can be seen in Ref. [35].

Considering an analyte, its signal (abundance) x_k recorded by a MS spectrometer at the kth m/z ratio is

$$x_k = \chi\varepsilon_k, \quad k = 1, 2, \ldots, K \tag{1}$$

where ε_k is a coefficient of proportionality between the analyte concentration and the abundance. ε_k depends on the kth m/z ratio; the vector of these coefficients constitutes the spectral profile (the mass spectrum).

As the mass spectrometer is coupled to a chromatograph, the signal (abundance) not only depends on the m/z ratio and the concentration of the analyte but also on the elution time, because the fraction of analyte that is eluting from the chromatographic column to the mass spectrometer, changes over time. So at the jth elution time, the recorded abundance becomes

$$x_{jk} = \chi\tau_j\varepsilon_k, \quad j = 1, 2, \ldots, J; \quad k = 1, 2, \ldots, K \tag{2}$$

where τ_j can be considered as the fraction of analyte that is going through the mass spectrometer at time j. The vector of all τ_js forms the chromatographic profile (chromatographic peak).

If F spectrally active substances coelute, the recorded abundance is the sum of the contributions of these F different compounds

$$x_{jk} \cong \sum_{f=1}^{F} \chi_f\tau_{jf}\varepsilon_{kf}, \quad j = 1, 2, \ldots, J; \quad k = 1, 2, \ldots, K \tag{3}$$

where F is the total number of compounds that are coeluting. Finally, assuming that analyte fth has the same retention time in all chromatographic runs, the abundance measured at the kth m/z ratio, the jth retention time and the ith sample can be expressed as

$$x_{ijk} \cong \sum_{f=1}^{F} \chi_{if}\tau_{jf}\varepsilon_{kf}, \quad i=1, 2, \ldots, I; \quad j=1, 2, \ldots, J; \quad k = 1, 2, \ldots, K \tag{4}$$

The set of all x_{ijk} data forms the three-way array $\underline{\mathbf{X}}$. A PARAFAC model of rank F can be expressed [36,37] as

$$x_{ijk} = \sum_{f=1}^{F} a_{if}b_{jf}c_{kf} + e_{ijk}, \quad i = 1, 2, \ldots, I; \quad j = 1, 2, \ldots, J; \quad k = 1, 2, \ldots, K \tag{5}$$

where e_{ijk} are the residual errors of the model. As can be observed, the PARAFAC model of Eq. (5) corresponds to the physical model of Eq. (4). Under the above-mentioned conditions, a PARAFAC model of F components can be used to estimate the coefficients of proportionality for each analyte at all m/z ratios recorded (i.e. the mass spectral profile of each analyte) by means of vector $\mathbf{c}_f = (c_{1f}, c_{2f}, \ldots, c_{Kf})$; the fraction of analyte that leaves the chromatographic column along the chromatographic run (i.e. the chromatographic profile or chromatogram of the analyte) by means of the vector $\mathbf{b}_f = (b_{1f}, b_{2f}, \ldots, b_{Jf})$; and the relative concentration of every analyte in all I samples (i.e. the sample profile of each analyte) by means of the vector $\mathbf{a}_f = (a_{1f}, a_{2f}, \ldots, a_{If})$. The coordinates of vectors $\mathbf{c}_f$, $\mathbf{b}_f$ and $\mathbf{a}_f$ are respectively referred to as spectral, chromatographic and sample loadings.

The PARAFAC model is highly affected by deviations from the trilinear structure of the data. Slight changes in the retention time of an analyte between runs are habitual in chromatography. In addition, the use of the narrow-bore injector liner of only 0.75 mm i.d. (highly recommended in SPME), means that narrow peaks are obtained and therefore, the compounds are recorded in very few scans. The sum of these effects can invalidate the assumption of invariant chromatographic profiles in each sample and, therefore, in the PARAFAC model too. The PARAFAC2 decomposition technique [38] overcomes this difficulty and allows some deviation in the chromatographic profiles. References are made in the literature to ways in which PARAFAC2 can solve this type of problem when GC/MS is used [39,40,35,41].

The PARAFAC (or PARAFAC2) model has been fitted by means of the alternative least square (ALS) algorithm, which works sequentially in each way and at every step of the iteration, in order to fit the data to the model of the Eq. (5). In addition, it is possible to impose realistic and appropriate restrictions on the model with PARAFAC (or PARAFAC2) decompositions, such as non-negativity (which is appropriate for the chromatographic or spectral profiles) or the uni-

modality property (which is appropriate for the chromatographic profile).

The detection capability (CCβ) can be calculated, according to ISO 11843, with second order signals modeled with three-way calibrations [35,40,42].

4. Results and discussion

4.1. Selection of the fiber type in the solid-phase microextraction

The first part of this work is focused on the determination of the most appropriate fiber type from among those that are available to perform the extraction of the NSAIDs (PDMS, PA and PDMS-DVB). The selection is made on two steps: (1) To determine the equilibrium time of the fiber. (2) To select the fiber that provides the best results for various figures of merit obtained from calibrations taken in spiked aqueous samples using, for each fiber, the extraction time obtained in step (1).

Fig. 1 shows the extraction time profiles obtained for all the analytes and with the three fibers, Fig. 1(A) for PA, Fig. 1(B) for PDMS, and Fig. 1(C) for PDMS/DVB. In each figure (so for each fiber), the area of the m/z ratios of the base peak of each extracted analyte is depicted, after the fiber has been immersed in the sample for 5, 20, 35, 50 and 65 min.

As may be seen in Fig. 1, the PDMS/DVB fiber extracts the largest quantity for all the analytes, followed by the PDMS fiber and the PA fiber. On the contrary, regarding the equilibrium time it can be stated that:

(i) Equilibrium still has not been reached for FLF, MCL and TLF in the PDMS/DVB fiber after 65 min. It was reached at some point in between 50 and 65 min for DCF. The behavior was anomalous for IBP: maximum extraction was obtained at 20 min and later as time elapsed, instead of remaining stable, IBP extraction in the fiber was reduced.
(ii) In the PDMS fiber, equilibrium was reached for FLF, MCL and TLF at around 30 min. Equilibrium was reached for the other NSAIDs in a short enough time. In addition, in the IBP, and in the rest of analytes, after the equilibrium time is reached, the extracted quantity remains very stable as the extraction time increases.
(iii) Equilibrium was reached for all the NSAIDs in the PA fiber before the 5 min extraction time had elapsed.

Taking these results into account, in order to apply step (2) for the selection of the fiber, extraction times of 5, 30 and 60 min were respectively set for the PA, the PDMS and the PDMS/DVB fibers.

The calibration models needed to evaluate the figures of merit in step (2) were conducted by least-squares fitting of the standardized loadings of the sample mode of each analyte, obtained in the PARAFAC (or PARAFAC2) decomposition *vs.* the added concentration of each analyte. The calibration samples were distributed throughout 6 levels of concentration (0, 10, 25, 50, 75 and 90 $\mu g\,kg^{-1}$), with two replicates at 10 and 90 $\mu g\,kg^{-1}$. The internal standard, TLF-$^{13}C_6$, was added to all the samples at a concentration of 50 $\mu g\,kg^{-1}$. In all samples, 6 m/z ratios were recorded for each analyte (see Table 1) during 14 elution times (scans) around the retention time of each analyte. In this way, the dimensions of the tensor $\underline{\mathbf{X}}$ for each substance were $14 \times 6 \times 8$ ($14 \times 3 \times 8$ for TLF-$^{13}C_6$), in which the first dimension refers to the chromatographic profile, the second dimension to the profile of the m/z ratios (mass spectrum) and the third dimension refers to the sample profile (calibration samples).

The calibration stages based on PARAFAC (or PARAFAC2) are shown in Refs. [34,35]. A diagram may be seen in reference [43] that shows how to perform a PARAFAC-type multiway calibration when the data is recorded with LC–MS/MS. The schema is equally applicable when data are recorded with GC–MS.

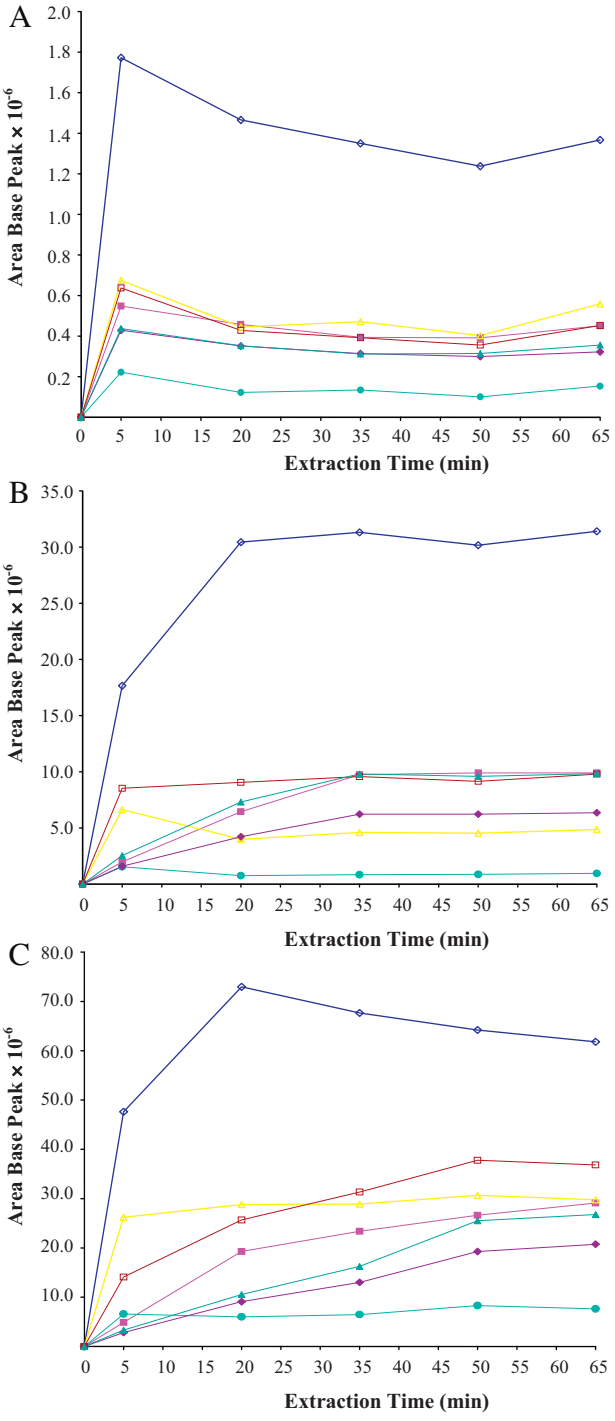

Fig. 1. Sorption time profiles for the NSAIDs studied: (A) PA fiber, (B) PDMS fiber and (C) PDMS/DVB fiber. Spiked water samples (10 mL) containing the NSAIDs at 100 $\mu g\,L^{-1}$: IBP (empty dark blue diamond), FLF (filled pink square), NPX (empty yellow triangle), KPF (filled blue circle), TLF (filled purple diamond), DCF (empty brown square) and MCL (filled green triangle). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 2 shows the number of necessary factors and the variance explained with the PARAFAC (or PARAFAC2) decomposition for each analyte in each fiber. In all the cases, the decomposition is made, with the calibration samples so that figures of merit can be evaluated. All the decompositions are performed by applying the

Table 2
Number of factors needed (NF) and explained variance of the three-way data, in %, with the PARAFAC decomposition at each fiber and by analyte. The number in brackets is the variance explained with a PARAFAC2 decomposition with the same number of factors.

Analyte	PA fiber		PDMS/DVB fiber		PDMS fiber	
	NF	Explained variance (%)	NF	Explained variance (%)	NF	Explained variance (%)
IBP	1	98.6 (99.3)	1	80.0 (99.3)	1	99.0 (99.3)
FLF	1	98.4 (98.9)	1	95.0 (99.3)	1	98.9 (99.4)
NPX	2	98.0	1	92.0 (99.5)	1	99.0 (99.4)
KPF	2	96.4 (97.4)	1	78.7 (96.7)	1	95.0 (95.8)
TLF	2	97.6 (99.5)	1	89.5 (97.0)	1	96.4 (96.8)
TLF-$^{13}C_6$	2	97.3 (99.9)	1	88.5 (95.5)	1	95.0 (95.5)
DCF	1	98.0 (98.6)	1	94.4 (98.2)	1	97.7 (98.2)
MCL	1	98.5 (99.0)	1	93.7 (98.8)	1	92.6 (98.8)

alternative least-squares (ALS) algorithm, placing non-negativity restrictions on the chromatographic mode and on the mass spectrum mode (MS). As may be seen from Table 2, only 1 factor is needed for all the analytes in the PDMS and PDMS/DVB fibers, but 2 factors are needed for the NPX, KPF, TLF, and TLF-$^{13}C_6$ in the PA fiber.

In general, the explained variance in the PA and PDMS fibers increases by less than 1% when performing a PARAFAC2 composition, except with the PDMS fiber in MCL and with fiber PA for KPF, TLF and TLF-$^{13}C_6$. On the contrary, there are very significant increases in the explained variance in the PDMS/DVB fiber when using the PARAFAC2 decomposition, in all the analytes. In those cases where the increase of the explained variance is important, the sample mode loadings obtained with the PARAFAC2 decomposition are used; in all other cases, the sample mode loadings obtained with the PARAFAC decomposition are used. Thus, the explained variance of the experimental data in all the multivariate calibrations is above 95% and mostly near 99%.

In Table 3, the results obtained in several figures of merit (number of outliers, linear model $C_{CALCULATED}$ *vs.* C_{TRUE} fitted by least squares, residual standard deviation of the fitted model, coefficient of determination, R^2, the values of the decision limit and the detection capability and the mean of absolute values of relative error) for all the analytes and in each type of fiber.

The best results in almost all the figures of merit and in almost all the analytes were obtained with the PDMS fiber, above all in IBP. The model obtained with the PDMS/DVB fiber is not linear for this analyte.

In the microextraction, both the extracted quantity and its variability are important. Some figures of merit, especially CCα and CCβ, gather both properties together. Concretely CCα and CCβ depend on the quotient between the residual standard deviation of the calibration line (precision) and the slope of the same line (analytical sensitivity). Consequently, they are a good measure of the quantity extracted through the sensitivity, and of its variability through the residual standard deviation. Additionally, CCα and CCβ have an important analytical value as the decision limit and the capability of detection, respectively, to evaluate the performance of the method according to ISO 11843-2 [33] and 2002/657/EC Commission Decision [25]. The time needed to reach equilibrium is not properly a selection criterion but an operational parameter that must be initially fixed to be able to apply the priority criterion, that is, the analytical fit for purpose of the method evaluated by means of CCα and CCβ. It was therefore concluded that the best fiber of the three types under study, in order to perform the determination of the NSAIDs, is the PDMS fiber.

The decision limits (CCα) obtained are 7.75, 3.36, 5.47, and 3.38 $\mu g\,kg^{-1}$ for IBP, NPX, MCL and FLF, all of them less than the recommended concentration of 10 $\mu g\,L^{-1}$ stated in CRL Guidelines [8]. For DCF the resulting CCα is 5.07 $\mu g\,kg^{-1}$, similar to the value 5 $\mu g\,L^{-1}$ indicated in CRL Guidelines. Finally, for TLF the found CCα is 3.38 $\mu g\,kg^{-1}$ which is less than the 50 $\mu g\,L^{-1}$ in the 2010/37/EC

Table 3
Equation of the model ($C_{CALCULATED}$ *vs.* C_{TRUE}) and figures of merit calculated with multiway calibration models (PARAFAC or PARAFAC2) in spiked water samples with extraction times of 5, 30 and 60 min for PA, PDMS and PDMS/DVB fibers, respectively.

		IBP	FLF	NPX	KPF	TLF	DCF	MCL
PA fiber	Outliers	2 of 8	2 of 8	2 of 8	2 of 8	1 of 8	2 of 8	0 of 8
	C_{CALC} *vs.* C_{TRUE}	8.5E−7 + 1.0*x*	−6.9E−6 + 1.0*x*	−4.0E−7 + 1.0*x*	1.4E−3 + 1.0*x*	9.3E−7 + 1.0*x*	5.9E−7 + 1.0*x*	6.6E−6 + 1.0*x*
	s_{yx}	4.942	3.583	2.323	3.258	2.314	2.720	1.413
	R^2	0.985	0.987	0.997	0.992	0.996	0.992	0.998
	CCα ($\mu g\,kg^{-1}$)	12.61	8.91	5.72	8.01	5.55	11.89	3.15
	CCβ ($\mu g\,kg^{-1}$)	24.06	17.00	10.92	15.28	10.67	19.94	6.07
	MAE (%)	14.23	15.00	9.39	14.87	2.77	8.448	2.61
PDMS/DVB fiber	Outliers	Non linear	1 of 8	2 of 8	1 of 8	1 of 8	1 of 8	0 of 8
	C_{CALC} *vs.* C_{TRUE}		4.7E−3 + 1.0*x*	3.4E−5 + 1.0*x*	3.1E−3 + 1.0*x*	2.2E−5 + 1.0*x*	5.6E−5 + 1.0*x*	−3.8E−4 + 1.0*x*
	s_{yx}		4.885	2.313	3.104	1.152	1.954	2.46
	R^2		0.987	0.994	0.994	0.999	0.997	0.996
	CCα ($\mu g\,kg^{-1}$)		11.52	5.75	7.18	2.66	7.55	5.48
	CCβ ($\mu g\,kg^{-1}$)		22.13	10.97	13.80	5.12	12.62	10.60
	MAE (%)		8.16	8.63	8.28	2.52	5.35	3.55
PDMS fiber	Outliers	2 of 8	0 of 8	0 of 8	0 of 8	0 of 8	1 of 8	1 of 8
	C_{CALC} *vs.* C_{TRUE}	5.8E−4 + 1.0*x*	2.5E−5 + 1.0*x*	4.0E−4 + 1.0*x*	8.8E−4 + 1.0*x*	1.7E−4 + 1.0*x*	2.0E−4 + 1.0*x*	1.9E−4 + 1.0*x*
	s_{yx}	3.128	1.514	1.509	2.145	1.518	1.306	2.355
	R^2	0.990	0.998	0.998	0.997	0.998	0.999	0.996
	CCα ($\mu g\,kg^{-1}$)	7.75	3.38	3.36	4.77	3.38	5.07	5.47
	CCβ ($\mu g\,kg^{-1}$)	14.80	6.52	6.50	9.21	6.54	8.47	10.51
	MAE (%)	9.24	3.39	5.11	5.68	3.12	4.21	4.96

MAE, mean of absolute value of relative errors; s_{yx} is the residual standard deviation of the $C_{CALCULATED}$ *vs.* C_{TRUE} regression; CCα is the limit of decision, and CCβ is the detection capability.
False-positive, α, and false-negative, β, probabilities fixed at 5% (as DCF is a banned substance in milk, α is fixed at 1% for this analyte).

Table 4
Accuracy and sensitivity of the method, in μg kg^{-1},developed with PDMS fiber.

Analyte	Confidence interval*		Analytical sensitivity†
	Intercept	Slope	
IBP	[−5.21,5.21]	[0.86,1.13]	17.12
FLF	[−2.09,2.09]	[0.96,1.04]	7.01
NPX	[−2.08,2.08]	[0.96,1.04]	6.99
KPF	[−2.96,2.96]	[0.94,1.06]	9.93
TLF	[−2.09,2.09]	[0.97,1.03]	7.03
DCF	[−1.93,1.93]	[0.96,1.04]	6.39
MCL	[−3.47,3.47]	[0.93,1.07]	11.55

* At 5% of significance.
† Evaluated at 45 μg kg^{-1}, with probabilities of false non-compliance, α, and false compliance, β, fixed at 0.05.

Regulation. In the new version of the regulation, DCF is not banned any more and now has a MRL equal to 0.1 μg L^{-1}, which is well bellow the decision limit obtained in the proposed procedure. Ref. [15] reports values of CCα equal to 0.59, 2.69 and 0.9 μg kg^{-1} for IBP, KPF and DCF by using GC–MS/MS, SPE and posterior derivatization of the dry extract.

In the studied range of concentrations, from 0 to 90 μg kg^{-1}, the response is linear. For all the analytes, the method fulfils trueness because the intercept and the slope of the regression line $C_{MEASURED}$ *vs.* C_{TRUE} are significantly equal to zero and one, respectively, at 5% significance level. This is seen in Table 4 in the fact that the corresponding 95% confidence intervals contain zero (intervals for the intercept) and one (intervals for the slope). Intermediate reproducibility in the range of concentrations goes from 1.31 μg kg^{-1} for DCF to 3.13 μg kg^{-1} for IBP (Table 3).

In Ref. [44] a procedure is proposed for evaluating the analytical sensitivity when a multivariate calibration curve is needed. The procedure is also valid for multi-way calibration models. The relation to other concepts such as decision limit and capability of detection at the maximum residue limit (MRL) can be consulted in the tutorial by Ortiz et al. [45]. In short, it is based on determining the quantity that the method is able to discriminate from a reference value, with established probabilities of false noncompliance, α, and of false compliance, β. With the PDMS fiber and taking as a reference the central value of the calibration range, 45 μg kg^{-1}, the sensibility varies from 6.39 μg kg^{-1} for DCF to 17.12 μg kg^{-1} for IBP (when $\alpha = \beta = 0.05$).

4.2. Optimization of the solid phase microextraction process and the derivatization reaction in aqueous phase with a D-optimal design

Having chosen the fiber type, the following part of the study is focused on the optimization of 8 experimental factors (5 SPME process factors and 3 derivatization reaction factors in an aqueous medium) in samples of bovine milk. Table 5 shows the factors under consideration and their levels which were described in Section 2.4.

As may be seen in Table 5, there are 7 factors at two levels and one factor at 4 levels, such that $2^7 \times 4 = 512$ experiments would be necessary in a complete factorial design. This number of experiments is unviable, hence the experimental effort is reduced by means of a D-optimal design [46]. This procedure for making 'ad hoc' designs for a problem is deeply developed both theoretically and computationally. The objective is to reduce experimental effort to the strictly necessary to estimate with suitable precision the effects and interactions that the research needs to study, these interactions should be previously established.

A search space is needed, in our case is formed by 512 possible experiments coming from the complete factorial design. Briefly the procedure is as follows: for each "n" (number of experiments to be done) the algorithm searches among the 512 the "n" experiments that give the confidence region for the coefficients of the model with the smallest volume. Then, the value of "n" is chosen in such a way that the maximum of the variance function of the corresponding design is near 1 (better if it is less than 1). In that way, there is guarantee that the selected design will give estimations of the coefficients and the predicted response with the smallest possible variance.

Table 5
SPME factors and derivatization procedure for the study of milk samples in the D-optimal design.

Factors (units)	Associated variable	Levels			
		L1 (A)	L2 (B)	L3 (C)	L4 (D)
t_{EXT} (min)	X_1	20	30	–	–
T_{EXT} (°C)	X_2	60	70	–	–
A_{GT}	X_3	No	Yes	–	–
t_{CON} (min)	X_4	5	7	–	–
T_{CON} (°C)	X_5	200	250	–	–
t_{DER} (min)	X_6	15	25	–	–
V_{DER} (μL)	X_7	300	600	–	–
P_{DER}EtOH/Py/ECF	X_8	4/1/1	1/4/1	1/1/4	2/2/2

To relate the experimental response with the variation of factors, a mathematical model was proposed, in which one of the levels (level L2 in the 2-level factors and level L4 in the 4-level factors) is established as a reference level in Eq. (6). The effect on the experimental response may be established with this "state of reference" method, when the factor changes from the reference level to the other levels. Hence, the proposed mathematical model will have the following coefficients: the intercept, one coefficient for each of the factors at 2 levels (seven in total) and 3 coefficients for the factor at 4 levels, so the model has 11 coefficients. Moreover, foreseeing the presence of interaction between t_{EXT} and T_{EXT} factors and also between t_{CON} and T_{CON} factors, two additional coefficients were added.

Thus, the proposed model has 13 coefficients, such that at least 13 experiments are necessary to fit the model. Table 6 shows the D-optimal design chosen with 14 experiments selected from among the complete 512 factorial design. Table 6 also contains the experimental responses that are the sample mode loadings for each analyte after performing a PARAFAC (or PARAFAC2) decomposition of the tensor formed by the samples of bovine milk that correspond to the D-optimal design together with the calibration samples.

The second-order property of the PARAFAC (or PARAFAC2) decomposition model guarantees a direct relation between the value of the sample mode loading and the quantity of each analyte extracted from each of the design samples, provided that the chromatographic profile and the mass spectral profile of each analyte are unequivocally identified in the samples belonging to the D-optimal design. A practical advantage here is that it is not necessary to perform a calibration model for each of the design conditions to determine the quantity of analyte in each of the design samples [27,47]. Thus, if N denotes the number of calibration standards, the experimental effort is reduced from $512 \times N$ experiments to only $14 + N$ experiments, by combining a D-optimal design with the sample mode loadings obtained with PARAFAC. This is only possible if the tensor decomposition technique has the second-order property and the experimental data are tri-linear.

The preparation of the fortified deionized water samples that are used in the calibration stage is analogous to what is described in Section 2.3 with an extraction time in the PDMS fiber of 30 min. The calibration is performed at 7 levels of concentration: 0, 10, 20, 30, 40, 50 and 60 μg kg^{-1}, with the levels of 10 and 50 μg kg^{-1} replicated twice and the levels of 20, 30 and 40 μg kg^{-1} replicated once (14 samples in total). The internal standard is set at 50 μg kg^{-1} in all samples.

Table 6
D-optimal design and the response, the loadings of the sample mode calculated with a PARAFAC2 decomposition for each analyte. For the codification of factors (X_i, $i = 1, \ldots, 8$) see Table 5. In bold, the experiments measured on the first day.

Levels of the factors								Experimental responses: sample mode loadings ($L_{ANALYTE}$)[a]						
X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	L_{IBP}	L_{FLF}	L_{NPX}	L_{TLF}	$L_{TLF\text{-}^{13}C_6}$	L_{DCF}	L_{MCL}
1	**1**	**1**	**2**	**2**	**1**	**1**	**1**	**0.042**	**0.016**	**0.200**	**0.036**	**0.030**	**0.078**	**0.027**
2	**2**	**2**	**1**	**2**	**2**	**1**	**1**	**0.167**	**0.067**	**0.312**	**0.080**	**0.080**	**0.243**	**0.125**
1	**2**	**2**	**1**	**1**	**1**	**2**	**1**	**0.151**	**0.090**	**0.265**	**0.106**	**0.103**	**0.204**	**0.132**
2	1	1	2	1	2	2	1	0.075	0.016	0.197	0.035	0.030	0.135	0.032
1	**2**	**1**	**1**	**2**	**1**	**1**	**2**	**0.054**	**0.018**	**0.189**	**0.024**	**0.022**	**0.087**	**0.033**
2	**1**	**2**	**1**	**1**	**2**	**1**	**2**	**0.190**	**0.109**	**0.366**	**0.104**	**0.100**	**0.278**	**0.201**
1	**1**	**2**	**2**	**1**	**2**	**2**	**2**	**0.143**	**0.138**	**0.252**	**0.148**	**0.138**	**0.177**	**0.187**
2	**2**	**1**	**2**	**2**	**2**	**2**	**2**	**0.083**	**0.038**	**0.199**	**0.051**	**0.041**	**0.120**	**0.054**
2	2	2	2	1	1	1	3	0.155	0.019	0.288	0.146	0.146	0.220	0.174
1	2	1	1	1	2	1	3	0.024	0.001	0.108	0.024	0.021	0.050	0.026
2	1	2	2	2	1	2	3	0.197	0.042	0.346	0.182	0.185	0.277	0.204
1	1	1	1	2	2	2	3	0.033	0.003	0.177	0.026	0.024	0.060	0.024
1	2	2	2	2	2	1	4	0.183	0.074	0.361	0.107	0.107	0.281	0.148
2	2	1	1	1	1	2	4	0.098	0.036	0.204	0.042	0.040	0.142	0.050

[a] The PARAFAC2 decomposition of KPF is incorrect and this analyte was removed in the estimation and interpretation of the coefficients calculated with the design.

It was decided to prepare the design samples individually, in order to avoid the influence of other possible effects on the results of the design. Thus, for the first sample, the chromatographic record was obtained after pretreatment, derivatization and extraction on the fiber (under the conditions indicated in the design). While recording the chromatogram, the pretreatment of the second sample began and so on. In this way, it was only possible to finish a maximum of 7 experiments in a working day and for this reason, the total design was measured over two working days. Table 6 shows the experiments, which were carried out on a random basis on each working day to avoid other possible effects linked to the time.

The bovine milk used to perform the D-optimal design samples was spiked at 50 μg kg^{-1} with all the analytes (including the internal standard), and processed with the procedure described in Section 2.4. Fig. 2 shows the chromatogram of one milk sample of the D-optimal design.

Once finished all the analyses (for the samples in the design and for the calibration samples) the corresponding data matrices are joined throughout the sample mode giving rise to the three-way tensors. The dimension of the tensors created in this way is $18 \times 6 \times 21$ (chromatographic profile mode, mass spectral mode and sample mode, 14 + 7, respectively) for each one of the analytes and on each day, except for the internal standard, the dimension of which is $18 \times 3 \times 21$. Subsequently, the PARAFAC and PARAFAC2 decompositions were performed in each tensor. The multiway decomposition did not lead to a model that clearly identified KPF in the milk samples, reason why this analyte was eliminated when analyzing the D-optimal design.

When studying the variance of the data explained by each type of decomposition, a remarkable increase with PARAFAC2 was observed for all analytes (in the case of DCF and NPX the increase was higher than 20%). For that reason, it was decided to use the loadings of the PARAFAC2 decomposition as experimental responses of the D-optimal design (see Table 6). This increase in the explained variance when using PARAFAC2 can be attributable to slight displacements in the retention time of the observed analytes in some of the milk samples, as may be seen in Fig. 3.

Fig. 3 shows a superposition of the chromatograms of the three calibration samples fortified to 50 μg kg^{-1} and the 7 D-optimal design samples (performed with bovine milk and also spiked to 50 μg kg^{-1}) recorded on the first day for DCF.

Each chromatogram is depicted by joining the abundances recorded in 18 elution times, which is the dimension of the chromatographic profile mode. Taking into account that some of the chromatograms have several points in which the abundance is very small (for example, the three spiked water samples that appear from 15.702 min) the number of points that defines the

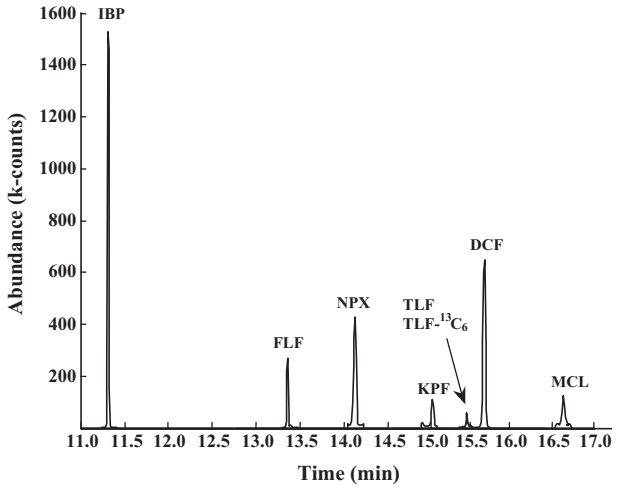

Fig. 2. Chromatogram of a fortified milk sample at 50 μg kg^{-1} (experiment number 6 of the D-optimal design in Table 6) recorded at the m/z base peak of each NSAID (showed in Table 1).

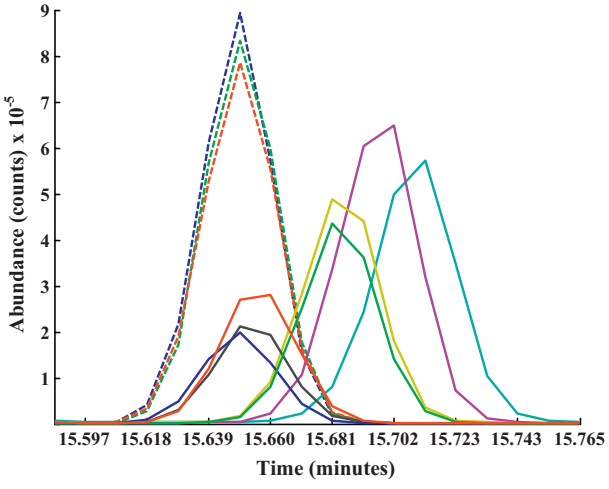

Fig. 3. Overlapped chromatograms of the three calibration samples at 50 μg kg^{-1} (dashed lines) and the 7 bovine milk samples (spiked at 50 μg kg^{-1}) of the D-optimal design measured on the first day (solid lines) recorded for DCF at an m/z ratio of 214 (base peak).

chromatogram in Fig. 3 can be less than 15, which are the usual recommendation to determine the area under a chromatographic peak. However, a PARAFAC model does not make use of the area but the abundance in each elution time of all the recorded ions. Eqs. (1) and (2), that theoretically describe the recorded abundance for each m/z ratio in each elution time, are not integrated in the chromatographic profile to obtain the area. On the contrary, PARAFAC decomposes in unique factors, related to each analyte, the recorded abundances. If it were about quantifying by using the peak areas and monitoring 6 ions in SIM mode, the dwell time would be 80 ms, while 100 ms would be enough to monitor 4 or 3 ions.

It should be pointed out that only 1 factor was necessary in all the PARAFAC2 decompositions, and they were carried out with the non-negativity restriction in all modes. Moreover, analyte identification, with regard to retention time and the relative abundance of the m/z ratios that were recorded, were satisfied for all the analytes and in all the D-optimal design samples, in accordance with the contents of Decision 2002/657/EC (see Table 7).

The two last columns of Table 7 show the relative abundances (with regard to the base peak) of the m/z ratios that were recorded for each analyte in all of the D-optimal design samples. These relative abundances were calculated from the loadings of the spectral mode estimated with the PARAFAC2 decomposition of the tensors formed by the calibration samples together with the spiked milk samples of the D-optimal design. In this way, a single mass spectral profile was obtained in all the design samples measured on the same day for each analyte. When comparing these values with the intervals described in column 4 of Table 7 (calculated from the spectral mode loadings estimated with the PARAFAC2 decomposition of tensors formed solely by the calibration samples and applying the tolerances specified in European Commission Decision 2002/657/EC), it was confirmed that all the values were within the intervals except for a small difference in the m/z 115 of NPX when considering the design samples measured on the first day. In consequence, all the analytes were identified in all the D-optimal design samples and there were at least 3 (for IBP, FLF, NPX, TLF, TLF-$^{13}C_6$ and MCL) or 4 (for DCF) identification points.

Thus, the sample mode loadings of the PARAFAC2 decomposition represent a satisfactory estimation of the quantity of each analyte that was extracted, in each of the experiments described in the D-optimal design.

The model with 13 coefficients fails to describe the sample loadings as function of the change in the experimental factors. However, if the two interactions between t_{EXT} and T_{EXT} and between t_{CON} and T_{CON} are not considered, the reduced model with 11 coefficients (Eq. (6)) was satisfactorily fitted to the experimental data describing them significantly as a function of the change in the levels of the factors under study:

$$Y = b_0 + b_{1A}X_{1A} + b_{2A}X_{2A} + b_{3A}X_{3A} + b_{4A}X_{4A} + b_{5A}X_{5A} + b_{6A}X_{6A} + b_{7A}X_{7A} + b_{8A}X_{8A} + b_{8B}X_{8B} + b_{8C}X_{8C} \quad (6)$$

where X_{ij} ($i = 1, \ldots, 8$; $j = A, B, C$) are binary variables that take a value of 1 when the ith factor is at the jth level, and 0 in any other case; b_{ij} are the coefficients of the model estimated by least squares and b_0 is the intercept. These coefficients indicate the effect on the response, when the factor changes from the reference level to the rest of the levels.

As the model in Eq. (6) significantly fit the experimental results, the interpretation is as follows: the ith factor is active, if the coefficient that estimates the change in the level of this factor is statistically significant. For example, if the coefficient b_{3A} is significant, it may be concluded that the changes to the SPME if a sample is or is not agitated will influence the experimental responses (quantity of each analyte extracted). By analogy, if at least one of the coefficients b_{8A}, b_{8B} or b_{8C} is statistically significant, the conclusion is that the change in the proportions of the derivatizing mixture has an effect on the experimental responses and the P_{DER} factor is active.

The significance of the factors and therefore their influence on the experimental responses is tested by means of the hypothesis test: null hypothesis, "the coefficient is 0, the factor is not active" against the two-sided alternative hypothesis, "the coefficient is different from 0, the factor is active". Fixing the significance level at 5%, the null hypothesis will be rejected if the p-values of the coefficients are below 0.05.

As the proposed mathematical model has 11 coefficients that are estimated with 14 experiments, there are degrees of freedom that allow evaluating the significance of the model and of the coefficients. The hypothesis test that confirms the significance of the model is: null hypothesis, "the least-squares fitted model does not explain the experimental variance"; alternative hypothesis, "the fitted model explains the experimental variance". As in the earlier test, setting at 5% the significance level, the null hypothesis will be rejected if the corresponding p-value is below 0.05.

The fitted models for the 7 experimental responses are statistically significant (p-values between 0.003 and 0.05), with coefficients of determination, R^2, between 0.964 and 0.995. All the fitted models therefore reproduce the experimental data satisfactorily.

After estimating the coefficients of Eq. (6) one can determine the effect on the responses, of passing from high level (L2 or B) to low level (L1 or A) in the seven factors at two levels and the effect of passing from a mixture (EtOH/Py/ECF) to another one, that is, to pass from level i (Li) to level j (Lj) for $i > j$. Fig. 4 shows the value of these effects, with the corresponding signs. The significant effects (at 5% significance level) are those that are outside the interval depicted by the discontinuous lines.

Factor t_{EXT} is significant at 5% for IBP with a positive effect. Also it has a positive effect for DCF and MCL (actually, the effects are significant at 6% significance level). In fact, it is seen that for the analytes for which the factor is significant (or nearly significant) the effect is always positive. Therefore the maximum response is achieved for level L2 (extraction time fixed at 30 min). Also factors A_{GT} and t_{CON} have always a positive effect, when they are significant, so the extraction must be done with agitation and fiber conditioning during 7 min.

On the contrary, again when they are significant, T_{EXT} and t_{DER} have a negative effect. Consequently, the temperature of the extraction should be 60 °C and 15 min of the derivatization time.

Factors T_{CON} and V_{DER} are not significant for any analyte, they therefore have no influence within the experimental domain under study.

When it is about factor P_{DER} the effect of changing its level on the different analytes is significant for some of them but not always with the same sign. As consequence, for TLF and TLF-$^{13}C_6$ the response would be maximum for the mixture (1/1/4). For MCL, where the effects L2–L1, L3–L1, and L4–L1 are all significant with a positive sign, the maximum response will never for the mixture (4/1/1). Overall, it may be established that the best option for this factor is an excess of ECF as opposed to EtOH and Py in the derivatizing mixture (EtOH/Py/ECF).

With the model in Eq. (6) the interactions between factors cannot be estimated.

A further aspect to highlight is that the two analytes TLF and TLF-$^{13}C_6$, which are chemically identical, give rise to practically identically D-optimal design models, both in the significance of the fit, and R^2 value, as well as in significant factors and the levels at which they should be set. However, both analytes were recorded with different m/z ratios and their loadings were estimated with different PARAFAC2 models. In fact, their samples were not in the same data tensor. This corroborates the accuracy of the PARAFAC2 mod-

Table 7
Identification of the analytes in the mass spectrum, according to European Union Decision 2002/657/EC in the D-optimal design samples. BP: Base Peak.

Analyte	*m/z*	Calibration samples		D-optimal design samples (milk)	
		Relative[a] abundance (%)	Interval[b] permitted (%)	Relative abundance (%) first day[c]	Relative abundance (%) second day[c]
IBP	105	5.53	[2.76–8.29]	5.55	5.53
	117	13.72	[10.97–16.46]	13.80	13.71
	119	14.50	[11.60–17.40]	14.59	14.46
	161	BP	–	BP	BP
	191	11.00	[8.80–13.19]	11.42	11.03
	234	17.28	[13.82–20.73]	17.12	17.50
FLF	92	3.49	[1.74–5.23]	3.97	3.06
	166	6.81	[3.40–10.20]	8.43	5.19
	235	10.40	[8.32–12.48]	10.71	9.89
	243	5.52	[2.76–8.28]	5.66	5.38
	263	BP	–	BP	BP
	309	39.67	[33.71–45.61]	39.71	39.79
NPX	115	12.12	[9.69–14.54]	15.13	14.02
	141	10.40	[8.32–12.47]	10.89	11.06
	154	5.65	[2.82–8.47]	6.20	6.37
	170	10.35	[8.28–12.42]	9.53	9.80
	185	BP	–	BP	BP
	258	29.59	[25.15–34.03]	26.31	27.00
TLF	180	24.58	[2089–28.26]	25.05	25.09
	208	BP	–	BP	BP
	243	61.79	[55.61–67.97]	61.80	61.74
	245	20.91	[17.77–24.05]	20.85	21.42
	289	54.61	[49.14–60.07]	53.16	54.88
	291	17.59	[14.07–21.11]	17.13	17.74
TLF-$^{13}C_6$	214	BP	–	BP	BP
	295	45.92	[39.03–52.81]	45.22	46.48
	297	15.67	[12.28–18.43]	15.11	15.66
DCF	214	BP	–	BP	BP
	216	35.45	[30.13–40.76]	35.35	35.43
	242	42.55	[36.17–48.94]	42.62	42.61
	244	13.89	[11.11–16.87]	13.94	13.90
	323	29.10	[24.72–33.45]	29.07	29.17
	325	18.41	[14.72–22.09]	18.41	18.50
MCL	242	BP	–	BP	BP
	244	35.52	[30.19–40.85]	34.61	34.35
	277	11.41	[9.12–13.69]	10.42	10.35
	279	7.64	[3.82–11.46]	7.43	7.15
	323	30.76	[26.15–35.37]	26.36	26.22
	325	19.90	[15.92–23.88]	17.06	16.92

[a] Calculated with the loadings (PARAFAC2) of the spectral profile estimated only with the calibration samples ($18 \times 6 \times 14$).
[b] The permitted tolerances are based on the relative ion abundance (% base peak): greater than 50% (±10%), between 20–50% (±15%), between 10–20% (±20%) and lower than or equal to 10% (±50%), see European Commission Decision 2002/657/EC.
[c] Calculated with the loadings (PARAFAC2) of the spectral profile estimated with the calibration samples and D-optimal design samples on each day ($18 \times 6 \times 21$).

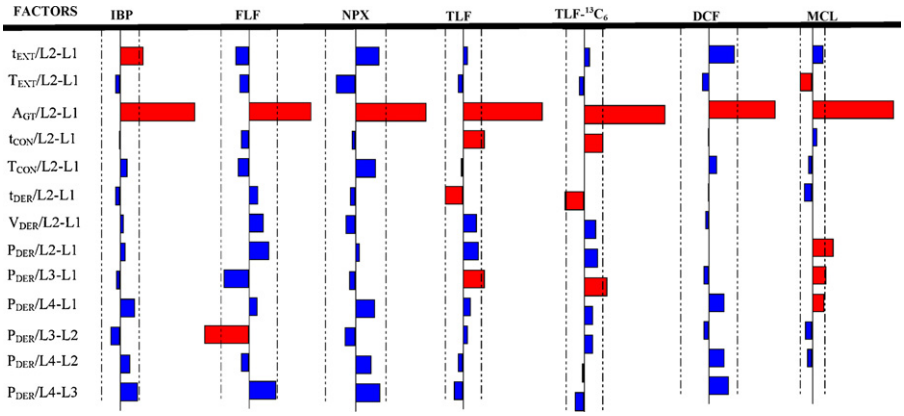

Fig. 4. Effects of the experimental factors on the experimental responses (quantity extracted of each analyte) and their significance (in red significant factors) with a significance level fixed at 5% (dash-dotted lines). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

els estimated with the multiway decomposition, which responds to the physico-chemical structure of the NSAIDs and which have been identified by the PARAFAC (or PARAFAC2) model.

5. Conclusions

This study has shown how D-optimal design makes it possible to evaluate which factors influence the determination of seven non-steroidal anti-inflammatory drugs (NSAIDs) by GC–MS. The determination method is studied in samples of bovine milk and includes a novel derivatization process of the NSAIDs in milk and a subsequent solid-phase microextraction stage prior to its injection in the GC.

Prior to the study of optimization, the most appropriate fiber type to perform the analyte extraction was studied. The choice of the fiber was not solely based on the standard idea of a fiber that extracts the largest quantity of analyte, but also on the fiber which reaches the equilibrium conditions first and which provides the best results for the figures of merit specified in the European regulations on veterinary residues. The calibrations use the sample loadings of the PARAFAC or PARAFAC2 decomposition in relation to the spiked concentrations. According to these two criteria, the PDMS fiber provided the best global results.

Subsequently, the optimization of 8 experimental factors (5 from the SPME process and 3 from the derivatization reaction) was performed by means of a D-optimal design of only 14 experiments (instead of 512). The extracted quantity of each analyte was used as the experimental responses, which was determined through a calibration PARAFAC2 analysis. This type of calibration possesses the second-order property, which unequivocally identifies each analyte. It is no longer necessary therefore to perform a calibration for each experimental condition to determine the extracted quantity of each analyte.

The analytical method proposed fulfils the requirements of European Commission Decision 2002/657/EC for the unequivocal identification of NSAIDs (with regard to the relative abundances of the m/z relations recorded) in bovine milk samples.

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