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## Highlights

- A vortex assisted MSPD method for ibuprofen enantiomer extraction from breast milk
- Factorial design with desirability functions was used for optimization of extraction
- Experimental conditions to keep enantiomeric fraction and resolution were achieved
- Enantiomeric fractions between 0.3 and 0.5 were estimated in breast milk samples

# Determination of ibuprofen enantiomers in breast milk using vortex-assisted matrix solid-phase dispersion and direct chiral liquid chromatography

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## Abstract

A mixture of β-cyclodextrin (β-CD) and primary and secondary amine (PSA) sorbents was employed for the extraction and quantification of ibuprofen enantiomers from human breast milk, combining a vortex-assisted matrix solid-phase dispersion method (MSPD) and direct chiral liquid chromatography (CLC) with ultraviolet detection (UV). The MSPD sample preparation procedure was optimized focusing on both the type and amount of dispersion/sorption sorbents and the nature of the elution solvent, in order to obtain acceptable recoveries and avoiding enantiomer conversion. These MSPD parameters were optimized with the aid of an experimental design approach. Hence, a factorial design was used for identification of the main variables affecting the extraction process of ibuprofen enantiomers. Under optimum selected conditions, MSPD combined with direct CLC-UV was successfully applied for ibuprofen enantiomeric determination in breast milk at enantiomer levels between 0.15 and 6.0 $\mu g \cdot g^{-1}$. The proposed analytical method also provided good repeatability, with relative standard deviations of 6.4 % and 8.3 % for the intra-day and inter-day precision, respectively.

**Keywords**: matrix solid-phase dispersion, vortex, ibuprofen, enantiomers, breast milk**,** direct chiral liquid chromatography.

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## 1. Introduction

Ibuprofen (IB), (*R*,*S*)-2-(4-isobutylphenyl)propionic acid, is a non-steroidal anti-inflammatory drug (NSAID) commercialized as racemic mixture with an annual global production reported in gigagrams [1]. However, its anti-inflammatory action is mainly associated with the (+)-(*S*)-enantiomer. Ibuprofen undergoes stereoselective metabolism, resulting in stereoselective pharmacokinetics parameters, with higher plasma and urinary concentrations for the (+)-(*S*)-isomer [2].

The main difficulties in analyzing non-steroidal anti-inflammatory drugs, such as ibuprofen, in food and biological samples are due to the tight non-covalent interactions established with matrix proteins and the amount of occurring fatty material. Several strategies have been used to extract NSAIDs depending on determination technique; for liquid chromatography separation of NSAIDs in bovine milk and muscle tissue the extraction procedure included deproteinization/extraction with organic solvent and solid phase extraction (SPE) clean-up on OASIS® [poly(N-vinylpyrrolidone-divinylbenzene] cartridges [3]. By gas chromatography (GC) separation technique, solid phase microextraction (SPME) and derivatization to ethyl esters was used to determine NSAIDs in bovine milk [4]. Azzouz et al. have also determined pharmacologically active substances, including IB, in cow, goat and human breast milk by GC with mass spectrometry (MS) detection. After milk deproteinization, sample enrichment and clean-up was done by continuous SPE using the OASIS® sorbent [5].

Like other analytical methods, traditional SPME and SPE have some drawbacks. SPME includes high time consumption, fiber breakage, stripping of coatings, low operating temperature and instability. On the other hand, SPE is one of the most commonly used clean-up techniques, but it involves several steps and requires longer time and higher organic solvent volumes than other modern techniques recently reported. To overcome these drawbacks, methods such as dispersive solid-phase extraction (DSPE) have been developed. This rapid and simple technique was proposed by Anastassiades et al. for food and environmental samples, which was included as a novel clean-up procedure for the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) technique [6]. DSPE is based on the addition of the sorbent material into an extract aliquot to remove the matrix interferences, which is then separated from the extract bulk by centrifugation. Thus, DSPE avoids passing the extract through SPE columns or cartridges, using smaller quantities of both sorbent and solvents and saving time and labor.

Other extraction methods such as magnetic solid phase extraction (MSPE) and matrix solid phase dispersion (MSPD) have also been described to avoid mentioned drawbacks. MSPE has received considerable attention because of its rapid, effective and unique mechanism of solid-liquid separation under a magnetic field. This means that the magnetic supports tagged with the analytes can be isolated from large volumes of sample solutions by a magnet, avoiding other steps such as centrifugation or filtration [7, 8]. Thus, Ghorbani et al. have employed ultrasound-assisted MSPE with LC for microextraction of ibuprofen and naproxen from cow milk, human urine, river water and well water [9]. Four NSAIDs, included ibuprofen, have been determined too by MSPE followed by LC with UV detection in wastewater effluents [8]. Other authors have reported the excellent extraction performance of a β-cyclodextrin polymer for MSPE, due to its ability to bind selectively organic molecules into its hydrophobic cavity and to form stable host-guest inclusion complexes [7].

On the other hand, MSPD is an extraction process based on the dispersion of solid adsorbent in the sample. It was developed by Barker et al. for the isolation of drugs from bovine muscle [10], and resulted in a useful tool for the residue extraction from solid, semi-solid, viscous, and mainly proteinaceous and fatty biological matrices [11-16]. Although MSPD was initially developed for extracting solid samples, now it is applied to viscous and complex samples such as milk [16]. MSPD is simple, faster than SPE and it does not require specific equipment and extraction conditions (room temperature and atmospheric pressure) to preserve analytes from degradation and denaturation [2, 15-16].

Despite the cited advantages, MSPE and; specially, MSPD sample pretreatment are not commonly used for determination of isomers in general or enantiomers in particular. Characterization and determination of isomers from different honeysuckle samples was carried out using trace MSPD with a β-cyclodextrin [17], and Zhan et al. determined enantiomers of organochlorine pollutants in oil seeds using n-hexane/dichloromethane as the eluting solvent (the eluent was evaporated with nitrogen at 30 ºC and the residue was re-dissolved in isooctane before CG determination with electron capture detection using a chiral column) [11]. In general, special attention was paid to compatibility between sample preparation and chiral separation in order to get appropriate enantioresolution [18-20].

Several chiral stationary phases (CSPs) are available for ibuprofen enantioseparation by liquid chromatography (LC). Stationary phases of phenylcarbamates of amylose have

been employed in normal phase mode using hexane-isopropanol-trifluoroacetic acid mixtures as the mobile phase, modified with a solution of ammonium acetate in methanol before entering into the electrospray interface of the mass spectrometry system [21]. Vancomycin antibiotic stationary phases have also been used for the separation of ibuprofen enantiomers using methanol modified with 4 mM ammonium acetate ($NH_4Ac$) as the mobile phase [22]. A stationary phase based on *(R)*-1-naphthyl-glycine 3,5-dinitrobenzoic acid was used for separation of ibuprofen, ketoprofen and naproxen enantiomers in wastewater using a mobile phase based on 90% (v) tetrahydrofuran and 10% (v) ammonium acetate in methanol [23]. In addition, the enantiomeric separation of ibuprofen has been achieved using a protein-based α-acid glycoprotein (AGP) stationary phase [24, 25], which offers some advantages such as the use of aqueous mobile phases.

In this work, we report the development of a vortex assisted MSPD method for the extraction of ibuprofen enantiomers from breast milk, and its determination by direct chiral LC using an α-acid glycoprotein based CSP. The effect of several experimental parameters, such as sorbent type, dispersant amount and nature of extraction solvent, were optimized to obtain the maximum recovery for each enantiomer while maintaining the enantioresolution and the enantiomeric fraction (EF) value at 0.5, typical for racemic mixtures. Different sorbents were selected from preliminary experiments, and the adequate sorbent amounts for the MSPD extraction of ibuprofen enantiomers were optimized by experimental design.

To the best of our knowledge, the combination of vortex enhanced MSPD and chiral LC is introduced for the first time in this work as an efficient method for extraction of ibuprofen enantiomers from breast milk samples. Only two studies reported elsewhere have determined IB in human milk from lactating women, but (*R*)- and (*S*)-IB are not distinguished. The first one is a pharmacokinetic study [26] and it has been taken as a reference for establishing sample spiked levels. The other one has determined IB by continuous SPE followed by GC-MS (5).

## 2. Material and methods

### 2.1. Reagents and materials

All reagents and solvents were of analytical grade, and purified water from a Milli-Q system was used (Millipore, Bedford, MA, USA). Methanol (MeOH) and 2-propanol of gradient-HPLC grade were supplied by Scharlab (Barcelona, Spain). Sodium sulphate

was supplied by Probus (Badalona, Spain), Florisil® (magnesium silicate, 60-100 mesh) was purchased from Carlo Erba Reagents (Sabadell, Spain), Discovery® DSC-18 polymerically bonded octadecyl endcapped (70 Å pore size), Discovery® DSC-SAX polymerically bonded quaternary amine, Discovery® DSC-WCX polymerically bonded ethylenediamine triacetic acid, diatomaceous earth, primary and secondary amine (PSA)-bonded silica were provided by Supelco (Bellfonte, PA, USA). Chirobiotic™ T (teicoplanin) (5 μm) was purchased from Advanced Separation Technologies Inc. (Whippany, NJ, USA); α- and β- cyclodextrin were obtained from Serva Feinbiochemica (Heidelberg, Germany).

The target analyte (*R,S*)-2-(4-(2-methylpropyl)phenyl)propanoic acid (*rac*-IB, 98% pure) and (*S*)-2-(4-(2-methylpropyl)phenyl)propanoic acid (*S*-IB, 99% pure) were supplied by Sigma-Aldrich (St. Louis, MO, USA).

Analyte stock solutions (300 mg $L^{-1}$) were prepared in methanol and stored in the dark at 4 ºC for three months maximum. Fresh working standard solutions were prepared by suitable dilution of stock solutions as required. In order to prevent the possible analyte degradation, working solutions were daily prepared.

### 2.2. Equipment

The chiral chromatographic separation was made using $\alpha_1$-acid glycoprotein Chiral-AGP™ column (100 x 3.0 mm, 5 μm) supplied by Chrom-Tech (Cheshire, U.K.) in a HPLC chromatograph consisting of an injection valve with a 20 μL sample loop (Rheodyne, Cotati, CA, USA), a gradient pump 125 S Solvent Module System Gold (Beckman, Fullerton, CA, USA) and a Beckman UV 166 Detector System Gold (11 μL, 1 cm pathlength), both interfaced to a computer equipped with a Gold Nouveau Chromatography Workstation Software (v 1.6) for chromatographic data processing.

MSPD of ibuprofen from milk samples was carried out by using an ultrasound bath provided by P-Selecta and a vortex mixer from VELP Scientifica (Usmate, Italy). An Unicen centrifuge model 21 supplied by Ortoalresa (Madrid, Spain) was used for centrifugation of milk extracts.

### 2.3. Assisted MSPD procedure

Half a gram of milk, 0.30 g diatomaceous earth, 0.30 g $Na_2SO_4$, 0.26 g (PSA)-bonded silica and 0.021 g β-cyclodextrin were thoroughly grinded by triplicate in a porcelain

mortar for 5 min. The resulting homogeneous paste was transferred to a centrifuge glass tube, and 2 mL of MeOH were added. The tube was shaken by vortex for 1 min and the obtained suspension was centrifuged during 15 min at 2963 *g*. The extract was then evaporated to dryness under gentle nitrogen stream, and reconstituted with 0.5 mL of the LC mobile phase before chromatographic determination.

The optimization study of MSPD was done by using a factorial experimental design; four factors and two levels with two blocks with a total of 32 experiments. To obtain the response function, $Na_2SO_4$ (0.3-0.7 g), diatomaceous earth (0.15-0.35 g), (PSA)-bonded silica (0.15-0.35 g) and β-cyclodextrin (15-35 mg) were studied at two levels each. All the experiments were carried out using 0.50 g of milk spiked with 100 µL of 3.5 $mg \cdot L^{-1}$ *rac*-IB solution. The order of the experiment was fully randomized. Responses studied were expressed as enantiomer resolution ($R_s$), enantiomeric fraction (*EF*) and chromatographic peak area of each enantiomer. The chemometric method used to explore data was response surface analysis. Multiple response analyses (MRA) were performed to determine the combination of experimental factors which simultaneously optimize experimental responses. Data analysis was done by employing the software package Statgraphics Centurion XVI (StatPoint Technologies Inc., Warrenton, VA, USA).

### 2.4. Enantiomeric determination of ibuprofen by LC-UV

Direct chiral separation of ibuprofen was accomplished by isocratic elution using a mixture of 2-propanol and 20 mM phosphate buffer pH 5.5 (0.2:99.8 v/v) as mobile phase. Flow rate was set to 0.50 mL $min^{-1}$ and UV detection was done at 230 nm. The optimization study of ibuprofen chiral separation is described elsewhere [25].

### 2.5. Analysis of milk samples

Human female milk samples were collected from an informed volunteer between September 16, 2014 and April 25, 2015. Samples were frozen at -20 ºC and stored in the dark until analysis. Before spiked experiments, breast milk was analysed in order to verify any detectable amount of ibuprofen enantiomers. Milk samples (0.50 g) were spiked with racemic ibuprofen in the concentration range found in other studies [26], between 0.20 and 2.0 $\mu g \cdot g^{-1}$ (*rac*)-IB. To study the conversion of (*R*)-IB to (*S*)-IB [27], several concentrations of both enantiomers were taking into account. However, only chemical standards of pure (*S*)-IB were commercially available. Consequently, the IB

racemate was spiked with the (*S*)-isomer. Mixtures of (*rac)*-IB/(*S*)-IB were analyzed by direct chiral LC at two levels of concentration (0.20/0.18 $\mu g \cdot g^{-1}$ and 0.35/0.20 $\mu g \cdot g^{-1}$). Samples were prepared in triplicate in all studied conditions.

## 3. Results and discussion

### 3.1. MSPD optimization

With the aim to obtain the maximum extraction efficiency for each ibuprofen enantiomer, several parameters were taking into account during optimization studies. When *rac*-IB mixtures were analysed, EF values were maintained around 0.47±0.02 [25].

The materials used as sorbents for retention or for clean-up purposes included non-retentive supporting materials as diatomaceous earth, classical dispersants in MSPD such as Florisil, or chemically bonded polymeric phases such as DSC-weak cation exchanger (WCX) or DSC-strong anion exchanger (SAX). In order to improve the adsorption/clean-up step of breast milk samples spiked with racemic ibuprofen, chiral selectors (teicoplanin Chirobiotic™ T, α- and β- cyclodextrin) or materials used in QuEChERS extraction methods to remove lipids, such as (PSA)-bonded silica, were evaluated in the MSPD optimization study.

Sonication or vortex shaking followed by centrifugation were evaluated as alternative to the conventional solid phase extraction elution step, which implies the filling and packing of a SPE cartridge with the mixture of sorbents.

*3.1.1. Effect of sorbent and elution solvent*

All experimental studies were carried out by adding 100 µL of the 300 $mg \cdot L^{-1}$ *rac*-IB solution to 0.50 g of breast milk. Spiked samples were blended with amounts between 0.2 and 0.6 of $Na_2SO_4$, used as desiccant, 0.2-0.3 g of Florisil, DSC18, DSC-WCX, DSC-SAX or diatomaceous earth, used as dispersants, 0.2-0.3 g of (PSA)-bonded silica and/or 20-30 mg of chiral selectors, teicoplanin Chirobiotic™ T, α- and β- cyclodextrin, used for clean-up and selective adsorption. During the study, it was observed that the absence of $Na_2SO_4$ caused an adverse effect on the extraction efficiency, probably due to the absorption of the sample water into the solid phase (dispersant/sorbent).

The obtained results in terms of recovery and enantiomeric fraction are shown in Figure 1 . The highest recovery for both IB enantiomers were obtained when sodium sulphate, PSA-bonded silica, diatomaceous earth and cyclodextrin, were used. It was observed that the presence of small amounts of the β-cyclodextrin chiral selector promoted

diverse interactions and hence the adsorption of both enantiomers, with the resulting improvement in recoveries. Cyclodextrins (CDs) are naturally chiral molecules based on cyclic oligosaccharides composed of D-glucose units that are linked by α(1,4)-glucosidic bonds. CDs have the shape of a torus with a hydrophobic interior cavity and a hydrophilic outside, the cavity size being small for α-CD and large for γ-CD. As a consequence, in addition to secondary interactions, hydrogen bonding or dipole-dipole interactions with the hydroxyl groups, β-CD, compared to α-CD and teicoplanin chiral selectors, has the specific shape and the apropiate cavity size to form inclusion complexes with the IB enantiomers.

Regarding EF (Figure 1B), values in the range 0.43-0.54 were observed in all experimental conditions, except when DSC-weak cation exchanger (WCX) combined with PSA-bonded silica and sodium sulphate were used (Figure 1B(e)). As can be seen in Figure 1A(e), very low recoveries were obtained for the *(S)*-IB enantiomer under these conditions, producing a great deviation of the theoretical EF value specified for racemic mixtures.

On the other hand, methanol (MeOH), methanol acidified with formic acid (0.8%, v) and acetonitrile were used as elution solvent. The elution pH is one of the most critical parameters that could affect the desorption process. Poor recoveries (lower than 30%) were obtained when mixtures of methanol with formic acid were used. Extraction with methanol produced higher recoveries than acetonitrile due to its higher polarity. Regarding methanol elution volumes, recoveries achieved with 2.0 or 5.0 mL were very similar and thus, 2.0 mL of MeOH were used for further studies.

Therefore, sodium sulphate, β-cyclodextrin, PSA-bonded silica, diatomaceous earth and methanol were selected taking into account recovery and EF values, and were used in the following experiments.

*3.1.2. Grinding time*

The grinding time has influence on the interaction between the dispersant and the sample matrix and then could affect the extraction efficiency. The grinding time was evaluated in the range 1-10 min while keeping the rest of experimental conditions constant. Grinding for 1 or 2 min led to an apparently homogeneous mixture, but a poor extraction yield for both IB enantiomers was obtained. Prolonged grinding times, higher than 5 min, did not increase the recoveries significantly. Therefore, 5 min was selected as the optimum time for the grinding step.

271

272 *3.1.3. Optimization of assisted MSPD*

273 Sonication or vortex-assisted shaking were studied as an alternative to the conventional
274 SPE elution step. After dispersion of the breast milk sample in a porcelain mortar, the
275 resulting homogeneous paste was placed in a centrifuge glass tube with 2 mL of
276 methanol. The tube containing the methanolic suspension was subjected to sonication or
277 vortex shaking for a period of time between 1 and 5 min. Recoveries in the range 15-
278 17% were obtained when an ultrasound bath was used. By contrast, the vortex assisted
279 MSPD procedure produced the highest recoveries. No differences were observed on the
280 recoveries when vortex shaking was done for 1 or 5 min, and consequently, a vortex
281 time of 1 min was used in the following studies.

282

283 *3.1.4. Experimental design optimization*

284 Once the nature of the dispersant/sorbent and the elution solvent were selected, a
285 factorial experimental design was planned to optimize the amounts of both desiccant
286 and solid sorbents (section 2.3). This study was performed to obtain the best conditions
287 that yielded high recoveries for each enantiomer, and to evaluate the chromatographic
288 chiral separation quality in terms of enantioresolution (Rs) and enantiomeric fraction
289 (EF). Breast milk samples were spiked with *rac*-IB at a concentration level of 0.7 µg
290 $g^{-1}$. Since to co-extraction of endogenous matter might produce losses of resolution
291 affecting the efficiency of chromatography separation [20], two responses variables, Rs
292 and EF, were evaluated. Enantioresolution could indicate the chromatographic chiral
293 separation quality, while enantiomeric fraction could show the integrity of the IB
294 racemic mixture when it is subjected to the MSPD procedure (due to chiral interactions
295 changes in enantiomeric composition may occur).
296 After thirty two MSPD experiments were done, analysis of the factorial design allowed
297 estimating the most important factors from those potentially influencing the considered
298 responses, as well as the interaction effects between the experimental factors. As can be
299 seen in the Pareto´s diagram (Figure 2), the most important effects to maintain EF
300 around 0.50 (theoretical value for a racemic mixture) were PSA, sodium sulphate and
301 the interaction of sodium sulphate with both diatomaceous earth and β-cyclodextrin.
302 High amounts of sodium sulphate decreased EF values, while the interaction between
303 sodium sulphate and β-cyclodextrin maintained EF at a value around 0.50. On the other
304 hand, it should be noticed that the highest amount of PSA helps to reduce amount of fat

available for extraction into methanol (the human fat breast milk concentration is around 3.2 g per 100 mL [26]).

As can be observed in the estimated normalized response surface (Figure 3), variation of enantioresolution was more strongly influenced by diatomaceous earth and PSA than by sodium sulphate and β-cyclodextrin. For multiple response analysis, the optimization criterion was maintaining EF in 0.47 ± 0.02 with the maximum recovery of each enantiomer and an acceptable resolution. In table 1, the desirability function for each studied response, as well as the predicted and experimental obtained values under optimized conditions, are presented. The statistical analysis showed that there were no significant differences between experimental and predicted results at the confidence level of 95%. Accordingly, the optimum conditions selected for the vortex assisted MSPD were 0.30 g diatomaceous earth, 0.30 g $Na_2SO_4$, 0.26 g (PSA)-bonded silica and 21 mg β-cyclodextrin.

### 3.2. Performance of the MSPD-chiral LC method

Feasibility of the chiral direct LC method combined with vortex assisted-MSPD extraction method was established by spiking breast milk extracts free of *rac*-IB, studying limit of detection (LOD) and limit of quantitation (LOQ), linearity range and precision. The main quality parameters of the developed analytical method are summarized in Table 2.

#### *3.2.1. Limits of detection and quantitation*

LODs and LOQs were estimated for each enantiomer as 3.3 SD/S and 10 SD/S respectively, where the standard deviation (SD) of the response was determined based on the residual SD of a regression line built around LOQ, and S is the slope of this calibration.

#### *3.2.2. Linearity range*

Calibration curves (n=8) were obtained by performing a linear least squares regression based on the spiked breast milk extracts, by plotting the peak area values against the IB enantiomer concentrations, which were varied from 0.14 to 6.0 $\mu g \cdot g^{-1}$. Excellent linearity was achieved within the studied concentration range. Correlation coefficients were between 0.995 and 0.993. Square values of the Pearson correlation coefficient were 0.987 for (*S*)-IB and 0.991 for (R)-IB. These R-squared values indicated that the

residuals of the regression had a variance 98.7% or 99.1% smaller than the variance of the response. Accordingly, 98.7% and 99.1% of the variance of the response was explained by the regression, and 1.3% or 0.9 % of this variance was left over in the residuals.

*3.2.3. Precision*

Instrumental precision was verified as intra- and inter-day repeatability. It was expressed as relative standard deviation (RSD, %) and evaluated in terms of peak area of each enantiomer at a fixed enantiomer concentration. Intra-day precision was calculated from three consecutive injections on the same day (n=3) and inter-day variation from the injections done during three successive days (three replicates each day, N=9). Acceptable and similar RSD values were estimated for intra- and inter-day injections, showing the performance and satisfactory repeatability of the proposed method in spiked breast milk.

*3.2.4. Accuracy*

The accuracy of the MSPD-chiral LC method was reported as percent recovery. Previous analysis demonstrated that analysed samples did not contain any IB enantiomers at the concentration levels of the method LODs. (*rac*)-IB (amounts between 0.20 $\mu g \cdot g^{-1}$ and 2.0 $\mu g \cdot g^{-1}$) were added to 0.50 g of sample to obtain a final concentration of those found in pharmacokinetics studies of ibuprofen in human mature milk [26]. In order to obtain mixtures where concentration of the active IB enantiomer, (*S)*-IB, is higher than (*R*)-IB, solutions containing known amounts of (*rac*)-IB were spiked with pure (*S*)-IB (0.20/0.18 $\mu g \cdot g^{-1}$ and 0.35/0.20 $\mu g \cdot g^{-1}$, for *rac*-IB/(*S*)-IB). Recoveries and EF values were determined by analysis of the spiked milk samples and the results are included in Table 3. In general, high recoveries between 71 and 88% with good precision, between 4 and 6%, were obtained all over the spiked ranges and (*R*)/(*S*) enantiomeric ratios evaluated, demonstrating the applicability of the developed analytical method for a complicated matrix like human breast milk, with minimal sample preparation. As can be observed in Figure 4, the peak of endogenous matter at the beginning of the chromatogram did not overlap the first enantiomer eluted.

**3.3. Comparison with different methods for milk analysis**

As discussed in introduction section, the proposed methods for the IB determination in milk from lactating women are very scarce. Other studies reported elsewhere have determined NSAIDs, including ibuprofen, in cow and bovine milk samples using GC or LC (3-5, 9). However, these estudies have not distinguished between (*S*)-IB and (*R*)-IB. As a result, analytical parameters such as LOD and LOQ are very difficult to compare if they are not referred to IB enantiomers, and especially, if the determination technique is not the same and the matrix is not breast milk in all of them.

Azzouz et al. have determined ibuprofen in human breast milk by continuous SPE followed GC-MS. After deproteinization of the milk (5 g) with acetonitrile, sample enrichment and clean-up was done by SPE using the Oasis® sorbent. However, the high content of acetonitrile in the aqueous sample dramatically reduced the sorption of analytes. Thus, evaporation and reconstitution of the sample before introduction into the continuous SPE system was required. In addition, a derivatization step was necessary prior to GC determination increasing the experimental effort (5).

Table 4 includes the comparison among the proposed method and other published ones for various milk matrices. The shown values are not referred to IB enantiomers and some of them have used different determination techniques. However, they evidence that the developed vortex-assisted MSPD method fits other extraction procedures, exhibiting the same order of precision and recovery. Compared to these methodologies, the proposed method has the advantages of simplicity of operation, high speed, and very low sample (0.5 g) and solid support consumption. Moreover, this extraction procedure offers additional benefits such as the detection and quantitation of the pharmacologically active (*S*)-IB without previous treatments (deproteinization and/or derivatization).

## Conclusions

A sensitive, selective and accurate method for extraction of IB enantiomers from breast milk has been developed and fully validated. The optimized vortex-assisted MSPD procedure, combined with direct chiral LC-UV detection, has provided a simple, effective and reproducible method for rapid determination of (*S*)-IB and (*R*)-IB at the low concentration levels usually found in human breast milk. A mixture of solid sorbents was employed for the retention and clean-up by MSPD, where the β-cyclodextrin chiral selector showed great adsorptive ability toward IB enantiomers.

Compared with other SPE extraction procedures described for milk samples, this approach offers clear advantages such as lower consumption of sample, sorbents and solvents. It also avoids the common problem of the clogging of the SPE columns or cartridges as well as the problems related to breakthrough volumes. In addition, the use of this methodology avoids previous steps in the sample preparation (eg. milk deproteinization and/or removal of fatty material). Therefore, the method described herein proposes a reliable, inexpensive and easy way to perform methodology that provides satisfactory recoveries for both IB enantiomers at different concentrations and different enantiomeric fractions.

On the other hand, a multivariate strategy was used to optimize the main variables affecting IB extraction process. Experimental design resulted to be an effective tool for modelling the raw extraction data and hence establishing or predicting changes in EF values and recoveries at different experimental conditions applied in assisted MSPD, with no need to perform additional laboratory experiments.

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**Figure 1**

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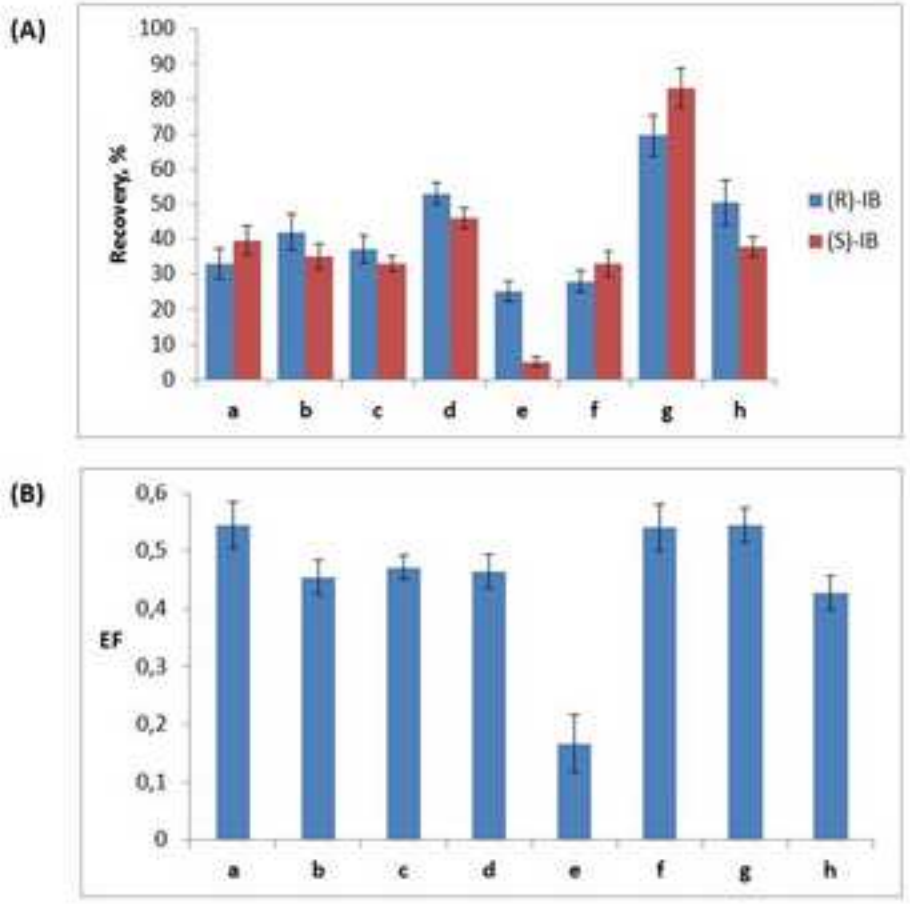

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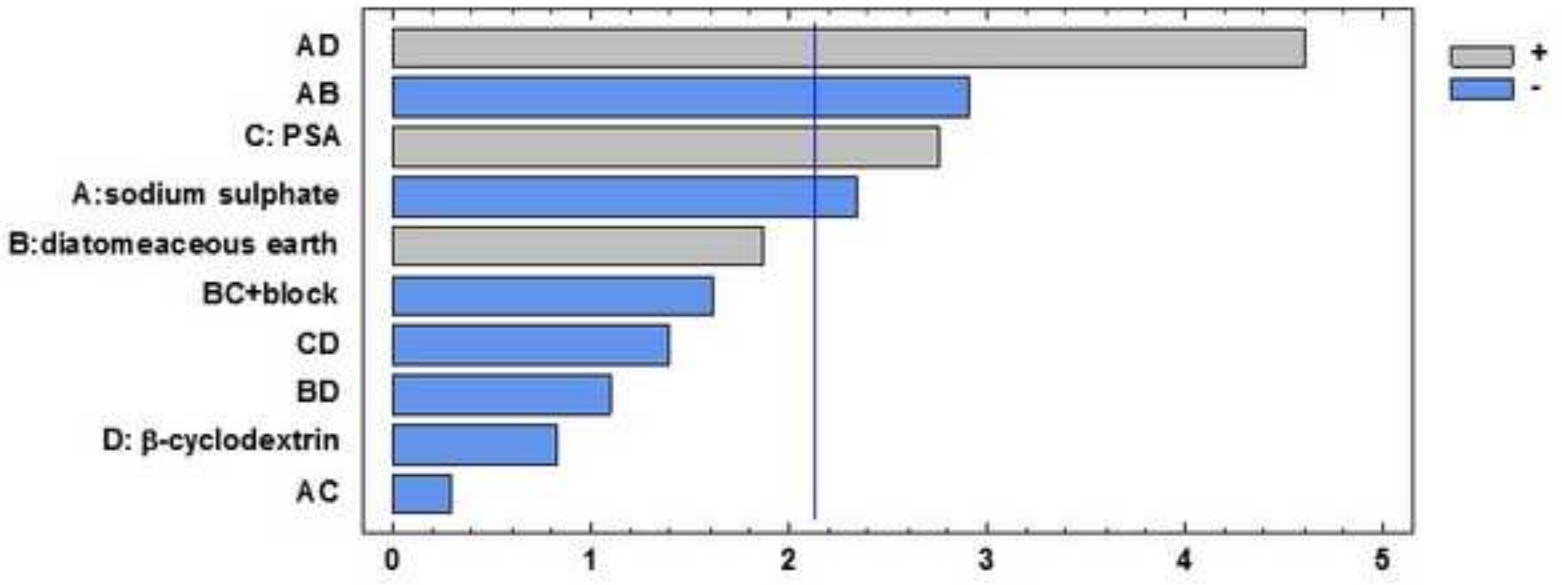

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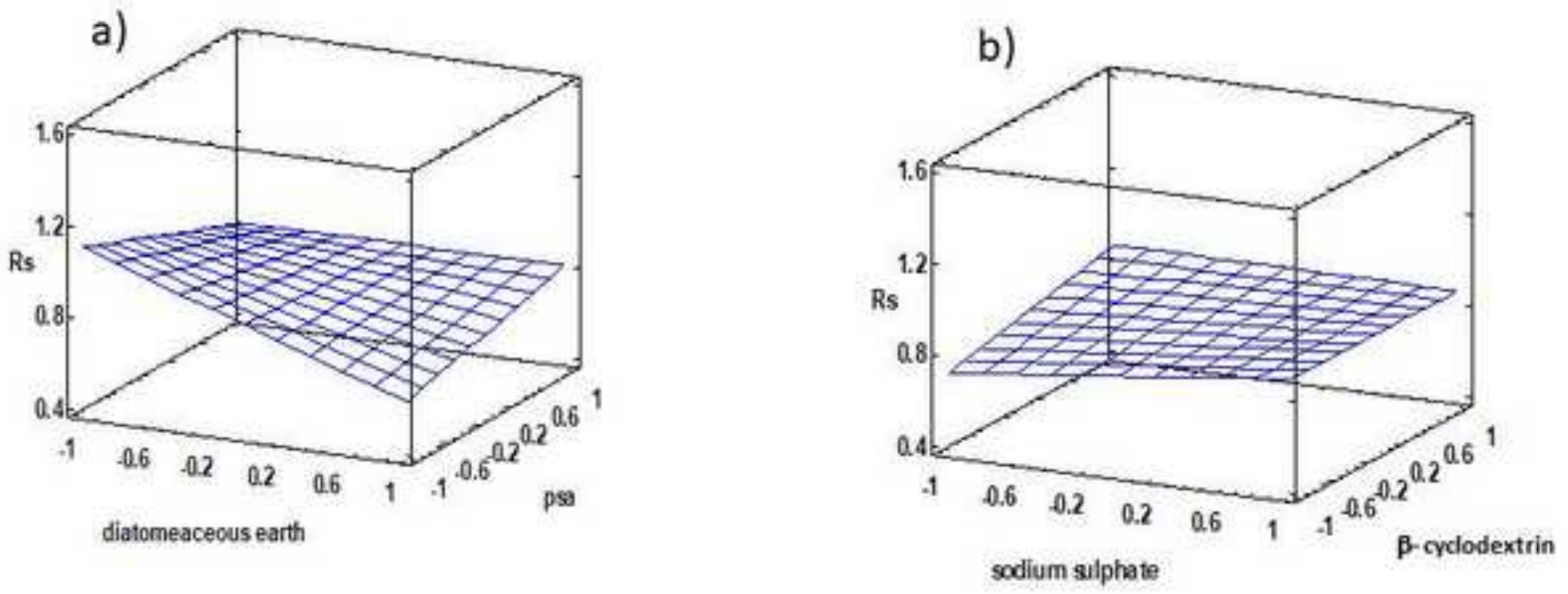

**Figure 4**

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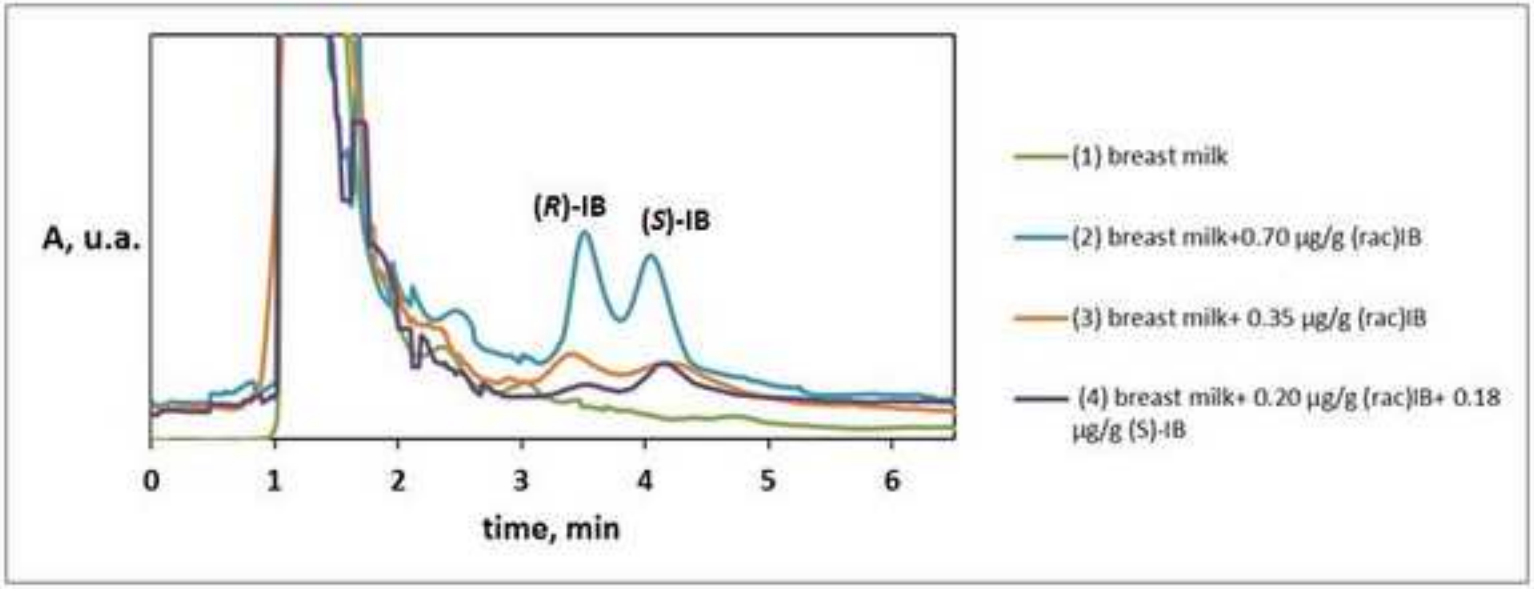

## Figure captions

**Figure 1**. Enantiomer recoveries (A) and enantiomeric fractions obtained (B) using different solids in the dispersion/clean-up step of MSPD.

Extraction conditions: 0.5 g sample, 1 g solid support.

(a) 0.5 g sodium sulphate and 0.5 g diatomaceous earth
(b) 0.5 g sodium sulphate, 0.25 g diatomaceous earth and 0.25 g DSC-WCX
(c) 0.5 g sodium sulphate, 0.25 g diatomaceous earth and 0.25 g DSC-SAX
(d) 0.5 g sodium sulphate, 0.25 g diatomaceous earth and 0.25 g PSA-bonded silica
(e) 0.5 g sodium sulphate, 0.25 g PSA-bonded silica and 0.25 g DSC-WCX
(f) 0.5 g sodium sulphate, 0.25 g diatomaceous earth , 0.25 g PSA-bonded silica and 30 mg teicoplanin
(g) 0.5 g sodium sulphate, 0.25 g diatomaceous earth , 0.25 g PSA-bonded silica and 30 mg β-cyclodextrin
(h) 0.5 g sodium sulphate 0.25 g diatomaceous earth , 0.25 g PSA-bonded silica and 30 mg α-cyclodextrin

Error bars indicates RSD (%) (n=3)

**Figure 2**.-Pareto chart: Standardized effects of each factor studied over enantiomeric fraction. An effect that extends beyond the reference line is potentially important.

**Figure 3**. a) Estimated normalized response surface obtained for resolution (Rs) at different amounts of PSA-bonded silica and diatomaceous earth. (sodium sulphate = 0 and β-cyclodextrin = 0) b) Estimated normalized response surface obtained for resolution (Rs) at different amounts of sodium sulphate and β-cyclodextrin (PSA-bonded silica=0 diatomaceous earth=0)

**Figure 4**. Representative liquid chromatography (LC) chromatograms of: (1) non-spiked breast milk (2) chiral separation of ibuprofen (IB) enantiomers in breast milk after addition of 0.70 µg $g^{-1}$ (*rac*)-IB (3) chiral separation of ibuprofen (IB) enantiomers in breast milk after addition of 0.35µg $g^{-1}$ (*rac*)-IB (4) chiral separation of ibuprofen (IB) enantiomers in breast milk after addition of 0.20 µg $g^{-1}$ (*rac*)-IB and 0.18 µg $g^{-1}$ (*S*)-IB.

**Table 1**. Optimization of the response variables evaluated by factorial experimental design.

| *Response* | *Predicted* | *Desirability function* | *Obtained* |
|---|---|---|---|
| *(S)*-IB | 85.7* | 0.7258 | 85.3±5.9*[a] |
| (*R*)-IB | 82.7* | 0.8394 | 83.1±4.1*[a] |
| Rs | 0.89 | 0.8660 | 0.91±0.05[a] |
| EF | 0.47 | 1.000 | 0.47±0.02[a] |

Optimized desirability = 0.8989

* *recovery* for a *(rac)*-IB concentration of 0.7 µg·g$^{-1}$, *%* [a]

[a] *n=3*

**Table 2.** Analytical characteristics obtained for ibuprofen enantiomers in breast milk extract spiked with (*rac)*-IB.

| | | ***(R)*-IB** | ***(S)*-IB** |
|---|---|---|---|
| Linearity range (n=8), $\mu g \cdot g^{-1}$ | | 0.14 - 6.0 | 0.15 - 6.0 |
| R-squared | | 0.991 | 0.987 |
| Correlation coefficient | | 0.995 | 0.993 |
| LOD, $\mu g \cdot g^{-1}$ | | 0.042 | 0.045 |
| LOQ, $\mu g \cdot g^{-1}$ | | 0.14 | 0.15 |
| Precision [a] (RSD, %) | Intra-day (n=3) | 6.1 | 6.4 |
| | Inter-day (N=9) | 8.1 | 8.3 |

[a] for a (*rac)*-IB concentration of 0.35 $\mu g \cdot g^{-1}$

Table3

**Table 3.** Recoveries and enantiomeric fraction determined for ibuprofen enantiomers in spiked human breast milk.

| Analyte | Added, $\mu g \cdot g^{-1}$ | Recovery*, % | | EF | |
|---|---|---|---|---|---|
| | | (*R*)-IB | (*S*)-IB | Experimental value* | Theoretical value |
| (*rac*)-IB | 0.35 | 78.1 ± 5.9 | 82.4 ± 6.1 | 0.48 ± 0.02 | 0.50 |
| (*rac*)-IB | 0.70 | 83.2 ± 4.4 | 86.1 ± 5.8 | 0.47 ± 0.02 | 0.50 |
| (*rac*)-IB + (*S*)-IB | 0.20+0.18 | 71.0 ± 5.1 | 88.2 ± 4.4 | 0.25 ± 0.02 | 0.26 |
| (*rac*)-IB + (*S*)-IB | 0.35+0.20 | 80.3 ± 4.7 | 84.2 ± 5.0 | 0.30 ± 0.02 | 0.32 |

* mean ± standard deviation (n=3)

**Table 4.** Comparison between the proposed vortex MSPD method and other methods for ibuprofen extraction from milk samples.

| **Author [Reference]** | **Milk sample (amount)** | **Extraction method** | **Previous treatments** | **Determination** | **Validation parameters** | | |
|---|---|---|---|---|---|---|---|
| | | | | | Intra-day precision (%) | Detectability | Recovery (%) |
| Azzouz et al. [5] | Human breast (5 g) | Continuous SPE | Deproteination/derivatization | GC-MS | 4.9 | 0.20 $ng \cdot kg^{-1}$ (LOD) | 93 - 108 |
| Ghorbani et al. [9] | Cow (10 mL) | Ultrasound assisted MSPE | Deproteination | LC-DAD | 3.3 | 0.1 $ng \cdot mL^{-1}$ (LOD) | 92 - 95 |
| Gentili et al. [3] | Bovine (5 mL) | SPE | Deproteination/extraction | LC-MS | 6 | 3.55 $\mu g \cdot kg^{-1}$ (CCα) | 95 - 103 |
| Arroyo et al. [4] | Bovine (10 g) | SPME | Deproteination/derivatization | GC-MS | - | 7.75 $\mu g \cdot kg^{-1}$ (CCα) * | - |
| Present work | Human breast (0.5 g) | MSPD | - | Direct chiral LC-UV | 6.1 (*R*)-IB<br>6.4 (*S*)-IB | 0.042 $\mu g \cdot g^{-1}$ (*R*)-IB<br>0.045 $\mu g \cdot g^{-1}$ (*S*)-IB<br>(LOD) | 78 - 86 |

* CCα (decisión limit)