Journal of Chromatography A, 1218 (2011) 6292–6301

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Development and validation of a confirmatory method for the determination of 12 non steroidal anti-inflammatory drugs in milk using liquid chromatography–tandem mass spectrometry

Estelle Dubreil-Chéneau*, Yvette Pirotais, Mélaine Bessiral, Brigitte Roudaut, Eric Verdon

Veterinary Drug Residues Unit, Laboratory of Fougeres, ANSES (National Agency for Food, Environment and Occupational Health Safety), BP 90203, La Haute Marche, 35133 Javené, France

ARTICLE INFO

Article history:
Received 22 September 2010
Received in revised form 25 April 2011
Accepted 5 June 2011
Available online 12 June 2011

Keywords:
Non steroidal anti-inflammatory drugs
NSAIDs
Residues
Milk
Validation
LC–MS/MS

ABSTRACT

A rapid and reliable LC–MS/MS method for the simultaneous confirmation of twelve non steroidal anti-inflammatory drugs (NSAIDs) in bovine milk was developed and fully validated in accordance with the European Commission Decision 2002/657/EC. The validation scheme was built in accordance with the MRLs or target analytical levels (EU-CRL recommended concentrations and detection capabilities) of the analytes, except for diclofenac for which the lower level of validation achieved was 0.5 $\mu g\,kg^{-1}$ whereas its MRL is 0.1 $\mu g\,kg^{-1}$. The NSAIDs investigated were as follows: phenylbutazone (PBZ), oxyphenylbutazone (OPB), naproxen (NP), mefenamic acid (MF), vedaprofen (VDP), flunixin (FLU), 5-hydroxyflunixin (FLU-OH), tolfenamic acid (TLF), meloxicam (MLX), diclofenac (DC), carprofen (CPF) and ketoprofen (KTP). Several extraction procedures had been investigated during the development phase. Finally, the best results were obtained with a procedure using only methanol as the extraction solvent, with an evaporation step included and no further purification. Chromatographic separation was achieved on a C18 analytical column and the run was split in 2 segments. Matrix effects were also investigated. Data acquisition implemented for the confirmatory purpose was performed by monitoring 2 MRM transitions per analyte under the negative electrospray mode. Mean relative recoveries ranged from 94.7% to 110.0%, with their coefficients of variation lying between 2.9% and 14.7%. Analytical limits expressed in terms of decision limits (CCα) were evaluated between 0.69 $\mu g\,kg^{-1}$ (FLU) and 27.54 $\mu g\,kg^{-1}$ (VDP) for non-MRL compounds, and at 0.10 (DC), 15.37 (MLX), 45.08 (FLU-OH), and 62.96 $\mu g\,kg^{-1}$ (TLF) for MRL compounds. The validation results proved that the method is suitable for the screening and confirmatory steps as implemented for the French monitoring plan for NSAID residue control in bovine milk.

1. Introduction

Among the veterinary drugs commonly used in dairy cattle, non-steroidal anti-inflammatory drugs (NSAIDs) are widely employed for their multiple beneficial effects. These compounds are prescribed for antipyretic, analgesic and anti-inflammatory properties. Their common action to reduce pain and inflammation is due to the inhibition of the prostaglandin synthesis. But beside these therapeutic effects, toxicity and side effects such as gastro-intestinal effects or renal problems, can occur.

NSAIDs can be divided into several groups according to their chemical structure (Fig. 1) such as the propionic acid derivatives (KTP, CPF, VDP, NP); the anthranilic acid derivatives (TLF, MF); the nicotinic acid derivatives (FLU); the pyrazolones (PBZ): the acetic acid derivatives (DC); the class of oxicams (MLX). Due to the fate that they may enter in the human food chain, NSAIDs are legally controlled and classified. The European Union has set Maximum Residue Limits (MRLs) for some NSAIDs to minimize the risk to human health associated with their residue consumption. Consequently, there are four NSAIDs authorised for dairy cows: TLF with a MRL at 50 $\mu g\,kg^{-1}$, FLU with a MRL at 40 $\mu g\,kg^{-1}$ for its metabolite FLU-OH, MLX with a MRL at 15 $\mu g\,kg^{-1}$, and DC with a MRL recently set at the very low level of 0.1 $\mu g\,kg^{-1}$ [1]. CPF and KTP have no MRL required for their residues in milk. The levels chosen to validate were 50 $\mu g\,kg^{-1}$ for CPF and 5 $\mu g\,kg^{-1}$ for KTP. And the other NSAIDs, MF, VDP, NP, PBZ and its metabolite OPB do not have any MRL set within the EU, and therefore should not be found in milk. Analysis at low concentration levels is required for NSAIDs, especially for DC (MRL at 0.1 $\mu g\,kg^{-1}$), and for non-authorised NSAIDs that should be analysed at a level of 5 $\mu g\,kg^{-1}$ (PBZ, OPB) and 10 $\mu g\,kg^{-1}$ (NP, MF) which are the levels recommended by the EU-CRL [2]. No level is recommended for VDP which is difficult to analyse, so the level set for validation was 50 $\mu g\,kg^{-1}$.For DC, the EU-CRL informed the National Reference Laboratories to

* Corresponding author. Tel.: +33 2 99 94 78 78; fax: +33 2 99 94 78 80.
E-mail address: estelle.dubreil@anses.fr (E. Dubreil-Chéneau).

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doi:10.1016/j.chroma.2011.06.006

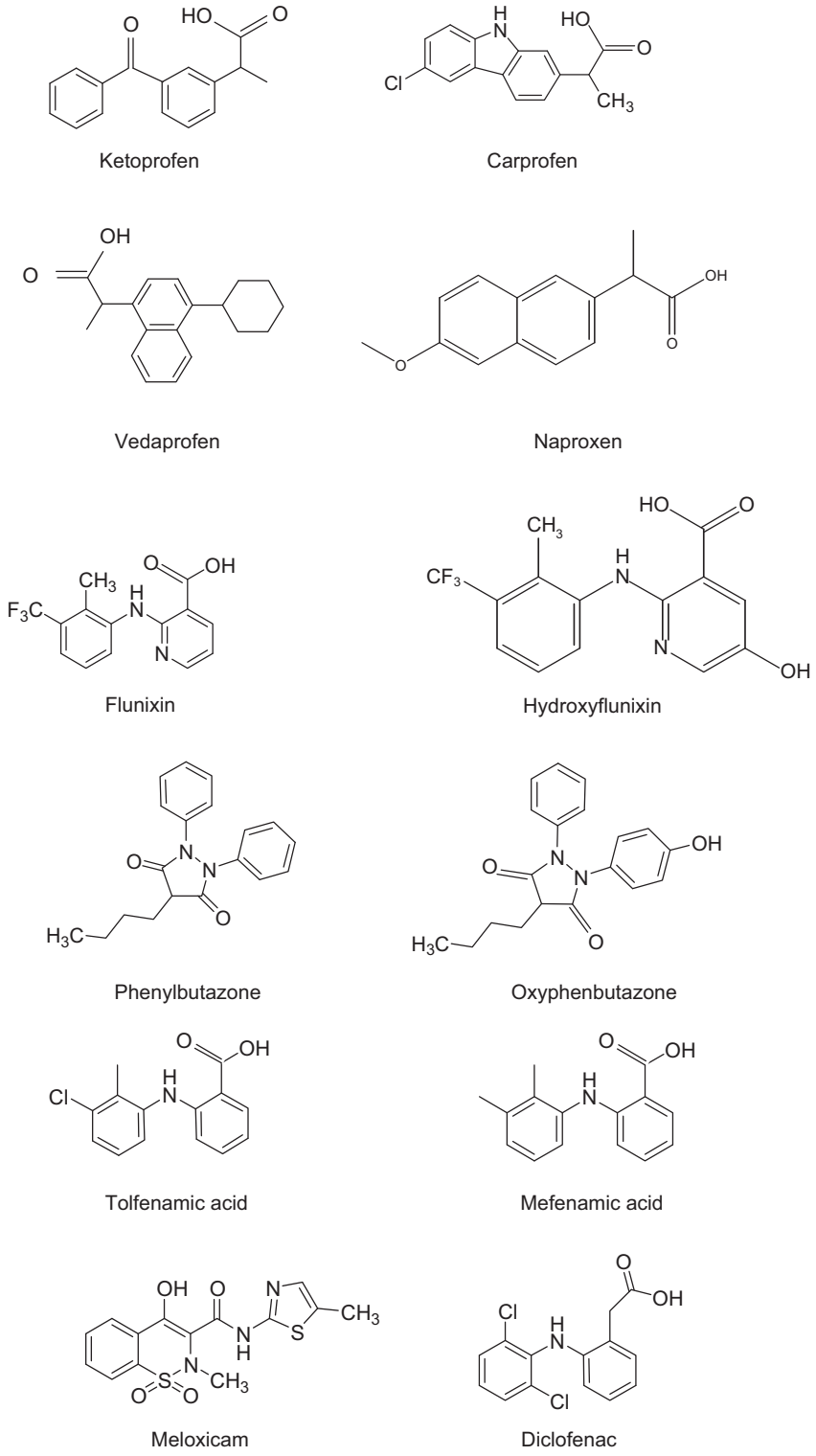

Fig. 1. Chemical structures of the 12 NSAIDs analysed.

analyse this residue at least at 1 μg kg^{-1} until a specific method is developed. These low concentration levels have triggered the development of analytical methods based on technologies sensitive enough to allow monitoring and accurately quantifying these compounds in bovine milk. For the other NSAIDs without any recommended limit, the levels set to validate were 50 μg kg^{-1} for CPF and VDP, and 5 μg kg^{-1} for KTP.

Several analytical methods have been reported in the literature to determine one or more NSAIDs in biological matrices. Most of these methods report the determination of NSAIDs in biological fluids like serum or plasma [3–6] or tissues [7–10]. But very few methods have been reported for the analysis of NSAIDs in milk. Moreover, some of them include the reduced analysis of one or two compounds [11–14]. Recently, a few published multi-residue

methods deal with the determination of the selected NSAIDs in milk [15–21]. In 2010, Dowling et al. [18] proposed an acetonitrile extraction followed by an improved SPE purification for the determination of 10 NSAIDs in milk by LC–MS/MS. This method featured some advantages in comparison with the others; the number of NSAID substances able to be confirmed and the full validation conducted at regulatory levels are the two main issues to be stressed.

In this paper, we display a study we carried out to develop a multi-residue method straightforward and fast enough for the routine regulatory analysis of 12 NSAIDs in milk. The use of two chromatographic columns with runtimes of 23 min and 12 min, respectively, leads to this reliable method. Compared to the method of Dowling et al., the proposed method additionally includes OPB, the marker residue of PBZ, NP and VDP, but ibuprofen is not considered. Several extraction and purification procedures were compared in order to choose the most efficient ones to be validated according to the EU Commission Decision 2002/657/EC guidelines [22].

2. Experimental

2.1. Chemicals and reagents

The commercial NSAID standards (tolfenamic acid, diclofenac sodium, meloxicam sodium, phenylbutazone, ketoprofen, naproxen, carprofen, mefenamic acid) were purchased from Sigma-Aldrich Co. (St-Quentin Fallavier, France); flunixin meglumine and d_4-diclofenac were obtained from Cluzeau (Ste-Foy-La-Grande, France); d_{10}-phenylbutazone and oxyphenbutazone monohydrate were purchased from LGC standards (Teddington; UK); vedaprofen was supplied by Intervet (Igoville; France); 5-hydroxyflunixin, d_3-flunixin and d_3-meloxicam was provided by Witega (Berlin; Germany). Acetonitrile, methanol and glacial acetic acid were of HPLC grade and were purchased from Fisher Scientific (Leicestershire, UK). Formic acid 98–100% was supplied by Merck (Darmstadt, Germany). Filters for final filtration of the biological extracts before chromatographic injection were purchased from Millipore (Millex GV, 0.45 μm).

2.2. Standard solutions

The individual stock standard solutions were prepared in a mixture of acetonitrile and methanol (90:10 v/v) at a concentration of 1000 μg mL^{-1} for each of the 12 NSAID standards and at a concentration of 500 μg mL^{-1} for the 4 deuterated internal standards, d_3-flunixin (FLU-d_3), d_4-diclofenac (DC-d_4), d_{10}-phenylbutazone (PBZ-d_{10}), d_3-meloxicam (MLX-d_3). The stock solutions were stored at −20 °C in volumetric amber flasks. The solutions were stable for 6 months except solutions of PBZ and OPB with a reduced stability of 3 months especially because they tend to become oxidized and OPB is light sensitive. Working standard solutions were prepared by appropriate dilution of the stock solutions using the same mixture of acetonitrile and methanol.

2.3. Instrumentation

Chromatography was performed using a Thermo Fisher Surveyor instrument (San Jose, CA, USA) and separations were achieved using an Uptisphere Strategy C18 column (150 mm × 2 mm; 5 μm particle size) from Interchim (Montluçon; France). Chromatographic separation was carried out using a mobile phase consisting of 1 mM acetic acid-acetonitrile (90:10 v/v) (eluent A) and acetonitrile (eluent B). In case of confirmation of ketoprofen only, eluent A was replaced by a mixture of 0.2% formic acid and acetonitrile (90:10 v/v). The gradient conditions were as follows: from 0 to 15 min ramp linearly from 20 to 80% of eluent B; hold for 2 min; then ramp again linearly over 2 min to reach back 20% of eluent B; and hold for 4 min to re-equilibrate the system before moving to the next injection. The high flow rate, 0.4 mL min^{-1}, was set to get thin peaks and the oven temperature was maintained at 40 °C to reduce the pressure of the column.

Mass spectrometry analysis was carried out using a Thermo Fisher TSQ Quantum Ultra tandem quadrupole mass spectrometer (San Jose, CA, USA). The instrument was operated using electrospray (ESI) ionization in negative mode, except for the confirmation of ketoprofen. In case of ketoprofen confirmation, analysis was performed using ESI in positive mode. Data acquisition was performed using the Xcalibur software. The following MS/MS parameters were set: sample tube or desolvatation temperature, 350 °C; capillary voltage, 4000 V; sheath gas pressure (air), 35 (arbitrary unit); auxiliary gas pressure (air), 10 (arbitrary unit); ion sweep gas pressure (air), 25 (arbitrary unit); collision gas pressure, 1 mTorr. Dwell times were set at 50 ms. Standard solutions of each compound (10 μg mL^{-1} in acetonitrile/methanol 90:10 v/v) were infused through a syringe pump at 5 μL min^{-1} and introduced into the LC flow (mobile phase at 80% A, 20% B at 0.4 mL min^{-1}) via a T-piece before it entered the detector. A display of the specific MRM parameters (2 transitions) for each NSAIDs are shown in Table 1.

2.4. Sample preparation

The raw bovine milk was thawed at ambient temperature and mixed. Then 2 g of the homogeneous milk sample were weighed into a centrifuge tube. The internal standard solution (IS_{deut}) was added giving rise to a concentration of 10 μg L^{-1} for MLX-d_3, FLU-d_3, CAR-d_3, DC-d_4 and 40 μg L^{-1} for PBZ-d_{10}. The milk sample was vortex-mixed and allowed to stand for 10 min in a dark place. Eight mL of methanol were added and the milk sample was again vortex-mixed to homogenize the milk with the extracting solvent. The sample was further placed on a mechanical rotary shaker for 10 min at 100 rpm and then centrifuged for 5 min at 14000 g in refrigerated conditions at +4 °C. A 5 mL aliquot of the supernatant was transferred to 12-mL disposable plastic tubes and evaporated to dryness under gentle stream of nitrogen at +40 °C during about 1 h and a half. The residual sample was then redissolved in 500 μL of a 1 mM acetic acid/acetonitrile mixture (80:20 v/v). The concentrated extract was vortex-mixed briefly and again centrifuged for 5 min at 14000 g (+4 °C). The final extract was filtered through a 0.45 μm syringe filter, and transferred to a capped vial adapted to the HPLC autosampler ready for injecting 20 μL into the LC–MS/MS instrument.

2.5. Method validation according to the Commission Decision 2002/657/EC

The validation study was built using spiked milk samples, named SC when they stood for the matrix calibration standards and named SV when simulating the matrix validation samples. The objective of the study was to give an estimation of the qualitative and quantitative parameters of the method and to compare them against the criteria of performance from the Decision 2002/657/EC. Blank raw milk materials found to contain no detectable NSAIDs prior to the spiking procedure were subjected to the analytical process to serve as SC and SV.

2.5.1. Qualitative criteria

The specificity of the method is tested for the two MRM transitions of each analyte by comparing chromatograms obtained from standard solutions, from different blank milk samples and from spiked milk samples. No interferences were observed at the retention times of the 12 NSAIDs. Only the deuterated internal standards

Table 1
LC–MS/MS parameters for the analytes.

Compound	Retention time (min)	Ionisation mode	*m/z* precursor ion	Tube lens offset	*m/z* product ions	Collision energy (eV)	IS used	Mean ion ratio (%)
KTP	10.3	ES−	253.0	40	209.0	10	FLU-d3	/
					/	/		
NP	10.5	ES−	229.0	43	169.0	39	FLU-d3	6.1
					141.0	51		
CPF	12.6	ES−	272.0	49	228.0	18	FLU-d3	12.1
					226.0	22		
VDP	17.2	ES−	281.0	48	237.0	15	FLU-d3	0.9
					235.0	21		
FLU	10.9	ES−	295.0	48	251.0	20	FLU-d3	13.9
					209.2	34		
FLU-OH	9.2	ES−	311.0	67	267.0	20	FLU-d3	13.6
					227.0	29		
MLX	9.7	ES−	350.0	55	286.0	17	MLX-d3	72.4
					146.0	23		
PBZ	14.4	ES−	307.0	72	279.0	20	PBZ-d10	48.1
					131.0	26		
OPB	10.7	ES−	323.0	67	295.0	20	PBZ-d10	35.0
					134.0	22		
TLF	15.7	ES−	260.0	47	216.0	18	FLU-d3	0.2
					180.3	24		
DC	13.2	ES−	294.0	50	250.0	14	FLU-d3	2.8
					214.0	20		
MF	14.9	ES−	240.0	54	196.0	20	FLU-d3	5.5
					192.0	27		
FLU-d3 (IS)	10.9	ES−	298.0	48	254.0	20	/	/
MLX-d3 (IS)	9.7	ES−	353.0	55	289.0	17	/	/
PBZ-d10 (IS)	14.4	ES−	317.0	72	289.0	20	/	/
DCF-d4 (IS)	13.2	ES−	298.0	50	254.0	14	/	/
KTP	10.3	ES+	255.0	91	209.0	20	FLU-d3	52.3
					105.0	30		
FLU-d3 (IS)	10.9	ES+	300.0	83	282.0	24	/	/

FLU-d_3 and DC-d_4 share the same transitions but their retention times are different.

Following the EC Decision, the signal-to-noise ratios, the relative retention times and the ion ratios corresponding to the less intense MRM signals of each NSAID against the most intense one were all evaluated for the SV.

2.5.2. Calibration curves

The SC consisting of samples built with blank milk matrix materials spiked at 4 concentrations (0.5 MRL or 0.5 TAL, 1 MRL or 1 TAL, 1.5 MRL or 1.5 TAL, 2 MRL or 2 TAL) were processed to evaluate the linearity of the calibration curves. The levels of concentrations set for the spiking samples (SC and SV) are reported in Table 2. They were chosen in accordance with the existing MRLs or with the recommended concentrations (TAL). Only DC was not validated in line with its MRL level of 0.1 $\mu g\,kg^{-1}$ because it was found not possible with this procedure to reach 0.5 MRL(0.05 $\mu g\,kg^{-1}$), so the acceptable level chosen to validate the DC was set at 1 $\mu g\,kg^{-1}$ (1 TAL).

Table 2
levels of concentrations for the spiking samples (SC and SV).

Compound	Levels ($\mu g\,kg^{-1}$)			
	0.5 MRL or TAL	1 MRL or TAL	1.5 MRL or TAL	2 MRL or TAL
KTP	2.5	5	7.5	10
NPX	5	10	15	20
CPF	25	50	75	100
VDP	25	50	75	100
FLU	5	10	15	20
FLU-OH	20	40	60	80
MLX	7.5	15	22.5	30
PBZ	2.5	5	7.5	10
OPB	2.5	5	7.5	10
TLF	25	50	75	100
DC	0.5	1	1.5	2
MFAS	5	10	15	20

A specific method dedicated to DC at 0.1 $\mu g\,kg^{-1}$ in milk will have to be developed in the future. One calibrating SC serie including a blank milk sample and injected twice (before and after the SV series in the sequence order) was replicated on 3 different days. The linearity of the response was determined by using a linear regression model. For this purpose, the peak area ratios (analyte to internal standard) of the respective NSAID were plotted against the NSAID spiked concentrations expressed in $\mu g\,kg^{-1}$.

2.5.3. Accuracy considered in terms of trueness and precision

Trueness was assessed through the estimation of the recovery. The recovery was obtained by back-calculating each day from the matrix calibration curve of the day (SC series) the concentrations of the SVs at each level of concentration. The SV are samples reconstituted with milk matrix materials. They contain known concentrations of the analytes of interest. In the validation study, the SV are supposed to simulate the future routine samples that the analytical procedure will have to monitor. Thus, 6 different batches of blank raw milk materials from various origins were selected in order to extend the representativity of the SV, and to include in the quantitative data more variability due to possible matrix effects. The concentration levels for the SV were selected at the same levels as those for the SC. Six replicates were used at each concentration level and for 3 days, that means 18 SV were analysed per level of concentration. The mean of the back-calculated concentrations for each level of concentration divided by the theoretical spiked concentration finally gave the percentage of recovery.

The precision of the method was evaluated at each level of concentration by calculating the relative standard deviation (RSD in percent) in repeatability conditions (RSD calculated as the mean of the 6 replicates SV of the day) and in intra-laboratory reproducibility conditions (RSD calculated from the 18 replicates SV during the 3 days of validation study).

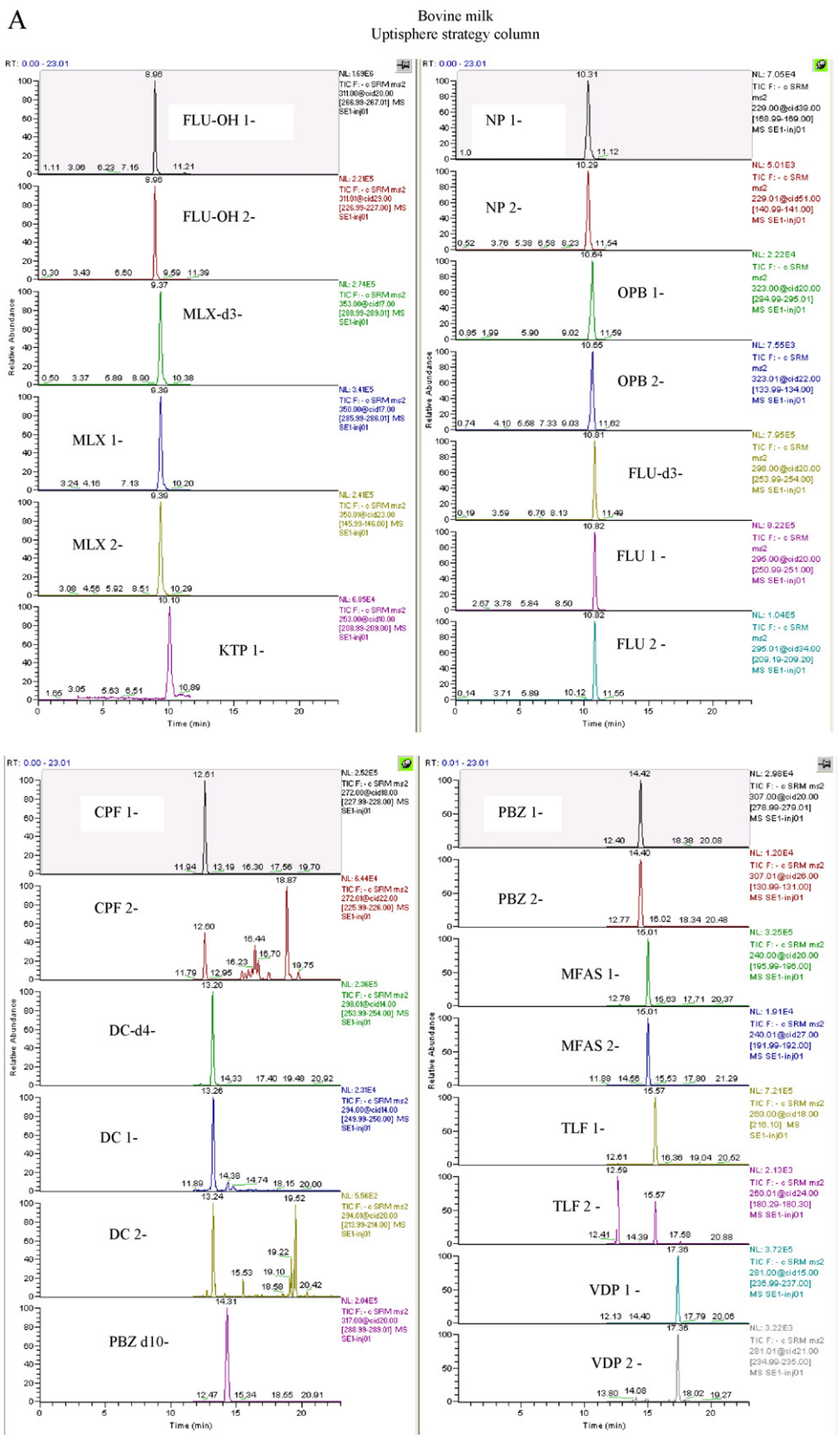

Fig. 2. Chromatograms of the 12 NSAIDs of spiked raw milk at 1 MRL or 1 TAL, equivalent to 1 $\mu g\,kg^{-1}$ for DC, 5 $\mu g\,kg^{-1}$ for KTP, PBZ, OPB, 10 $\mu g\,kg^{-1}$ for NP, FLU, MF, 15 $\mu g\,kg^{-1}$ for MLX, 40 $\mu g\,kg^{-1}$ for FLU-OH, and 50 $\mu g\,kg^{-1}$ for CPF, VDP, TLF, (A) bovine milk extract injected with the uptisphere strategy column method, (B) ewe milk extract injected with the ascentis express column method and (C) KTP in ESI positive mode, in bovine milk with the uptisphere strategy column method (on the left), in ewe milk with the ascentis express column method (on the right).

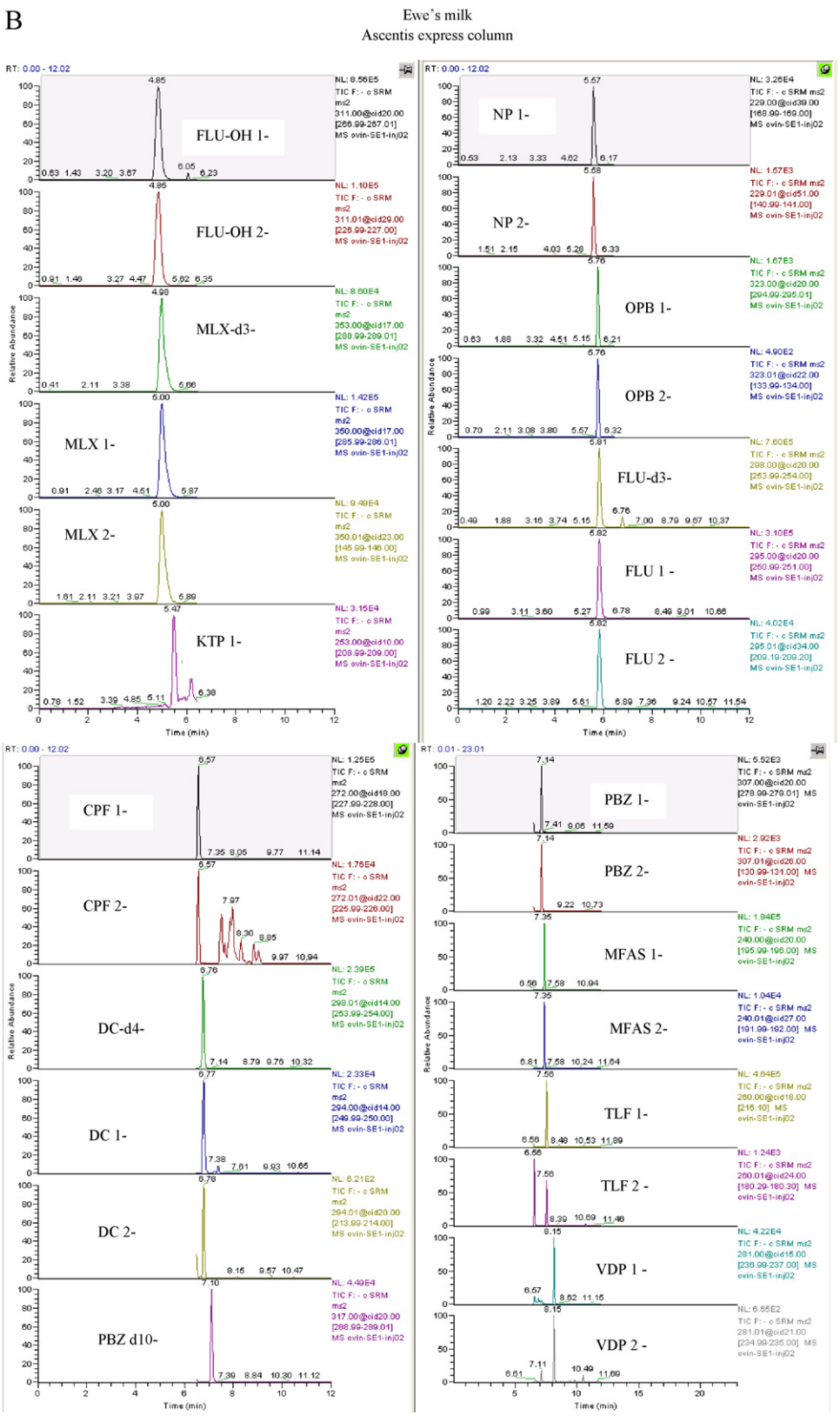

Fig. 2. (*Continued*)

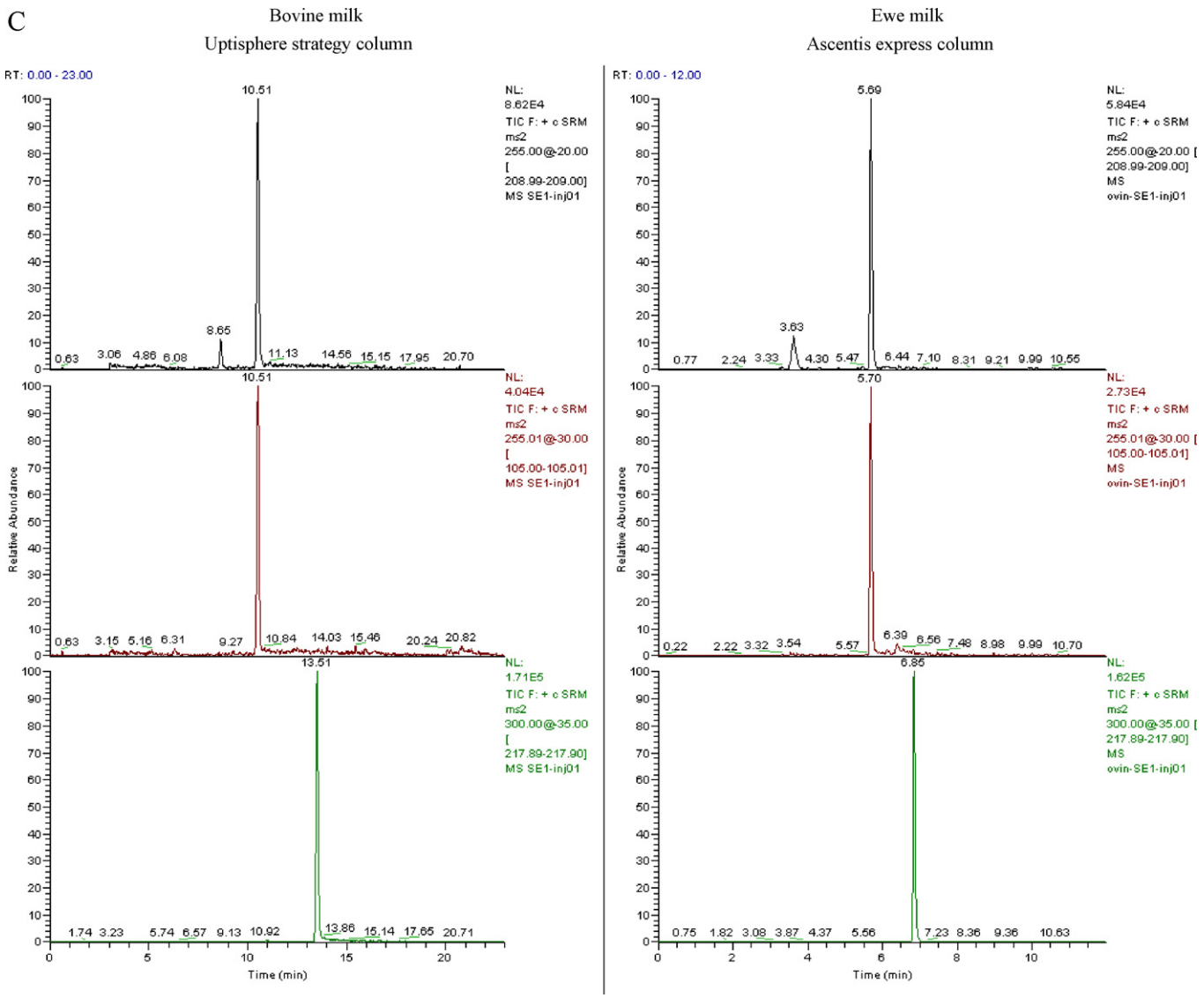

Fig. 2. (*Continued*).

2.5.4. Matrix effects

To evaluate the matrix effect (ME) expressed as either the signal suppression or the signal enhancement of the analytes due to the other components extracted from the milk matrix, two approaches were applied. First, the global matrix effect of the raw milk materials was assessed by calculating the ratio in %: ME = (peak area of standard analyte spiked in matrix/peak area of analyte spiked in solvent) × 100. This was calculated for each series on the 3 days, but using the same batch of milk. Finally, the applicability was evaluated by testing the inter-batch effect. This was operated during the validation by testing different sets of raw milk materials from 6 different origins.

2.5.5. Decision limit and detection capability

The decision limit (CCα) is the analytical limit at and above which it can be concluded with an error probability of α that a sample is non- compliant. Detection capability (CCβ) means the smallest content of a substance that may be detected, identified and/or quantified in a sample with an error probability of β. The two critical limits CCα and CCβ were determined in accordance with the ISO Standard no. 11843, "Capability of detection – Methodology in the linear calibration case", proposed in the European Decision 2002/657/EC.

3. Results and discussion

3.1. Method development

The acidic NSAIDs are either authorised veterinary drugs with MRLs established in bovine milk (and caprine milk only for MLX), or either non authorised veterinary drugs since no MRLs have currently been established. They are all, those authorized, regulated through the Table 1 of the Commission Regulation (EU) No 37/2010, so the minimum required number of identification points (IP) is set to three according to Decision 657/2002/EC. In fact, two MRM transitions were monitored for each of the analytes leading to a earn of four IP which is enough for confirmatory purpose. In a previous work (data not published), NSAIDs were analysed by LC–MS/MS using ESI in the positive mode. But not all the requested MRM transitions were correctly defined and some were not sensitive enough. These facts induced variations in signals leading to strong variations in the quantitation. So optimisation was performed using ESI in the negative mode. Only ibuprofen was still not found to have two

correct and sensitive transitions in our tune and chromatography conditions and thus was not included in the method. All the considered NSAIDs gave two suitable MRM transitions in the ESI negative mode, except ketoprofen. Indeed, only one major transition was found for KTP, and the selective minor transition of ketoprofen was not detected in any of the chromatograms. So a compromise was chosen to include this major transition in the ESI negative mode method for screening purpose, and to analyse ketoprofen in the ESI positive mode only in case of a confirmatory need, since two correct MRM transitions were found in this positive mode. Moreover, it has to be stressed that ketoprofen is not assigned any MRL as this veterinary drug was not considered at any risk for human consumption during its evaluation process by the European competent veterinary medicinal product committee. During the optimisation of the gradient and the choice of the column, we tried to isolate ketoprofen in a specific negative ESI segment but after a few set of tests, the chromatographic separation was still not efficient enough. Then, the compromise described here above was agreed to be the best solution.

Except this chromatographic work implemented for ketoprofen, the liquid chromatographic optimisation for the other NSAIDs was quite satisfactory. The mobile phase already described by Dowling et al. [18] was finally chosen, which consists of a gradient between a low acidic aqueous phase and acetonitrile. No addition of base was found necessary to deprotonate the acidic NSAIDs in the ESI negative mode. Adding different concentrations of ammonium formiate (10 mM, 5 mM and 1 mM) in the mobile phase during chromatographic trials did not improve any of the signals. So, to this very low acidic mobile phase, a simple gradient was applied. Then, the chromatogram was advantageously segmented in two parts to get the maximum sensitivity. For this work of development and validation, a classic reversed phase analytical column (Uptisphere C18 Strategy: 150 mm × 2 mm; 5 μm) was used. Several months later, the development was extended to another food matrix, the meat. Upon this occasion, it was proposed to replace this classical column by another one featuring a lower particle size diameter (i.e. Supelco C18 Ascentis Express: 100 mm × 2.1 mm; 2.7 μm) and supposed to provide narrower peaks, with good separation and faster analysis finally leading in our hands to runs lasting 12 min against 23 min with the classical column. The gradient conditions were as follows: from 0 to 4 min ramp linearly from 20 to 80% of eluent B; hold for 0.5 min; then ramp again linearly over 0.5 min back to 20% of eluent B; and hold for 7 min to re-equilibrate the system before the next injection. This new column was finally chosen for the further developments on different other matrices. Chromatograms obtained for the two different conditions of columns are displayed in Fig. 2.

The final step in this development study was to optimize the sample preparation with the aim to keep it rapid, practicable and robust in such a way to allow an efficient transfer of the method toward the network of French routine laboratories implementing the monitoring plans for the screening and the confirmation of NSAIDs. The different stages of sample preparation often cited in the literature [6,9,10,18] were tested: hydrolysis, ascorbic acid addition, organic solvents extraction, purification by SPE. First, different extractive solvent (acetonitrile, methanol, ethyl acetate) were assessed and the analytes recovery obtained from each of the extractant was calculated. Furthermore, an acidic hydrolysis was tested as residues of CPF in particular can form bounds with glucuronic or sulphuric acid. The hydrolysis (1 N HCl) was combined with another ethyl acetate extraction. Results are displayed in Fig. 3. Eventually, acetonitrile and methanol led to similar recoveries for the 12 NSAIDs tested but acetonitrile gave some even higher recoveries for OPB and PBZ (>120%). The same observation came with the acidic hydrolysis, which was finally not kept in the method because it affected sensitivity and recoveries for some of the analytes (CPF, TLF, and VDP). To complete these results, matrix fortified calibration standards were prepared for the methanol extractant, for the acetonitrile extractant, and for extraction by methanol but with also defattening with 1 mL of isooctane. The coefficients of determination (R^2) of the calibration curves and the intensities of the signals were compared for the three series of extractions. Finally, the methanol extractant was found to give the most satisfactory results.

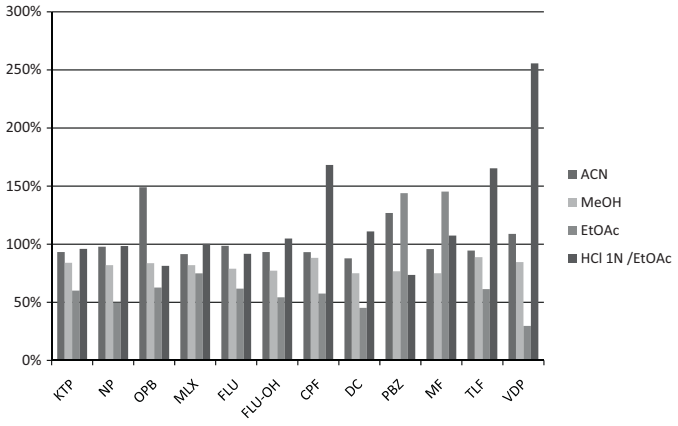

Fig. 3. Results of recoveries (%) between different extraction solvents.

A simple methanol extraction (8 mL of methanol for extraction and 5 mL evaporated) was then compared to the same methanol extraction but with addition of ascorbic acid prior to the extraction (1 mL of 0.1 M ascorbic acid). Ascorbic acid addition is often employed to avoid oxidation of PBZ. The addition led to a loss of sensitivity for most of the analytes (KTP, VDP, CAR, NP, FLU) and improved the signal only for OPB. As the signals were the same for PBZ, we discarded the ascorbic acid addition. To the simple methanol extraction was further added a purification step by SPE with a polymeric sorbent; prior to the SPE process, the evaporated extract obtained from the simple MeOH extraction was redissolved in 12 mL of water; then for the SPE purification several sorbents were compared: Strata X, Focus, Oasis HLB, Oasis MAX, Bond Elut Plexa. The best recoveries were obtained by using the Strata-X cartridge but results were found not to be reproducible over different assays. Finally, a comparison between the single methanol extraction and a complete 3-step procedure comprising acid hydrolysis, addition of ascorbic acid and purification by SPE was performed. Results are given in Table 3. Again, the single methanol extraction was still found better than the thorough 3-step procedure and so was kept for the validation stage. At the end, the best internal standards for all analytes were determined by comparing regression coefficients of the calibrations applying each internal standard and especially when no labelled isotopic internal standards were available. For instance, FLU-d_3 was found to fit with most of analytes (KTP, NP, MF) even though it would be better to have deuterated internal standards for some analytes like VDP or TLF. For CAR was used at the beginning of the trials the CAR-d_3 but CV % were high and CAR-d_3 was not found stable during our analyses. FLU-d_3 was found to be even more adapted for CAR. However, for OPB, PBZ-d_{10} was found to correct the signals better than FLU-d_3, even though the retention times and the chemical properties between OPB and PBZ-d_{10} are quite different.

3.2. Validation study

The validation study was performed on bovine raw milk, as the majority of samples collected for the national control plans are directed toward the cow milk. But after this cow milk validation, major parameters of the method were also checked on caprine and

Table 3
Comparison of extraction efficacy (A: simple MeOH extraction, B: acid hydrolysis (HCl 3 N), ascorbic acid, ACN and C18 cartridge).

Compounds	IS	R^2 extraction A (without hydrolysis)	R^2 extraction B (with hydrolysis)
KTP 1	FLU d3	0.9891	0.7284
NP 1	FLU d3	0.9674	0.9679
NP 2	FLU d3	0.9463	0.9575
OPB 1	PBZ d10	0.9988	0.8493
OPB 2	PBZ d10	0.9923	0.8295
MLX 1	MLX d3	0.9997	0.9988
MLX 2	MLX d3	0.9995	0.9921
MLX d3		%RSD = 2.8	%RSD = 77.6
FLU 1	FLU d3	0.9994	0.9995
FLU 2	FLU d3	0.9989	0.9986
FLU d3		%RSD = 3.4	%RSD = 31.9
FLU-OH 1	FLU d3	0.9986	0.7605
FLU-OH 2	FLU d3	0.9985	0.7620
CPF 1	FLU d3	0.9968	0.9570
CPF 2	FLU d3	0.9986	0.9639
DC 1	DC-d4	0.9992	0.5518
DC 2	DC-d4	0.9946	0.8553
DC-d4		%RSD = 3.4	%RSD = 13.6
PBZ 1	PBZ d10	0.9994	0.1270
PBZ 2	PBZ d10	0.9989	0.9182
PBZ d10		%RSD = 6.7	%RSD = 66.8
MF 1	FLU d3	0.9981	0.8406
MF 2	FLU d3	0.9978	0.3399
TLF 1	FLU d3	0.9966	0.9549
TLF 2	FLU d3	0.9973	0.9496
VDP-1	FLU d3	0.9955	0.8583
VDP-2	FLU d3	0.9669	ND

ewe milks. Results were found similar to those obtained on cow milk. However, the work undertaken during the phase of method development taught us the importance to use a matrix similar to the unknown controlled samples when preparing the matrix spiked calibration curve. This fact came to be needed because this method does not require a thorough purification to be performed during the procedure.

Regarding the results of the validation, the qualitative parameters were fully in line with the criteria of the Decision 2002/657/EC (signal-to-noise ratios, relative retention times and ion ratios). Mean ion ratios are presented in Table 1. Concerning quantitative parameters, the 36 matrix spiked calibration curves operated during the validation process (3 curves per analyte) were examined to evaluate the linearity of the response. The coefficients of determination of the linear regression (R^2) were all above 0.97 except 4 random curves over the 36 for which regression coefficients were around 0.94 (2 for VDP, 1 for TLF, 1 for CAR). For DC, the regression coefficients were all >0.995 despite the low levels investigated. This is probably partly due to the efficient use of its own deuterated internal standard, DC-d_4.

Regarding the accuracy of the method in terms of trueness and precision, results were all satisfactory. For samples spiked at concentrations between 1 and 10 $\mu g\,kg^{-1}$, the Commission Decision 2002/657/EC recommends relative recoveries between 70% and 110% (or −30% and +10% in terms of bias), and between 80% and 110% for concentrations over 10 $\mu g\,kg^{-1}$ (or −30% and +10% in terms of bias). All the values obtained fell within these ranges, with the lower value for FLU-OH (94.7%) and the higher value for VDP (110%).

The precision of the method is expressed through the RSD values in %. For intra-day assays (r %), values were less than 12.3% obtained for OPB and 11.1% obtained for VDP. These 2 NSAIDs are known to be the most difficult to analyse with signals giving rise to more variation than for the other NSAIDs. Moreover, no deuterated internal standards were found available for those 2 compounds at the time of the development and validation of this method. But these RSDs are still perfectly acceptable since they are in complete accordance with the repeatability values considered "between one half and two thirds" of the reproducibility values as recommended by Commission Decision 2002/657/EC at a level of 100 $\mu g\,kg^{-1}$, that means RSD shall be ranging between 11% and 15%. For the within-laboratory reproducibility (or R inter-series), the RSDs are not greater than the RSDs for repeatability. Results of trueness, repeatability (r %) and intra-laboratory reproducibility (R %) are displayed in Table 4.

Table 4
Results of trueness (recovery in %), repeatability and intermediate precision (RSD in %) in spiked milk samples (n = 18).

Levels of SVs	Recovery (%)				r intra-series (%)				R inter-series (%)			
	0.5	1	1.5	2	0.5	1	1.5	2	0.5	1	1.5	2
KTP	100.4	99.1	102.9	102.7	3.9	2.0	3.2	3.5	6.8	7.3	7.3	4.7
NP	105.6	100.6	100.2	99.8	9.6	5.4	7.0	5.5	10.5	7.3	7.1	6.9
CPF	109.6	102.4	99.6	98.5	6.6	6.5	5.1	3.7	7.8	8.5	11.7	10.8
VDP	106.3	110.0	101.1	97.7	5.4	11.1	9.9	4.4	11.4	12.1	10.1	4.4
FLU	102.5	102.8	101.9	101.0	1.3	1.7	2.1	1.4	4.6	3.8	3.7	3.2
FLU-OH	95.7	94.7	94.9	95.8	5.9	6.2	6.9	5.3	7.3	7.1	7.7	7.6
MLX	104.3	103.3	102.4	101.5	1.9	1.0	2.3	1.4	4.7	3.9	4.5	2.9
PBZ	104.5	103.5	97.9	100.4	4.4	3.5	7.3	5.5	8.1	4.8	10.2	5.1
OPB	98.4	103.1	107.5	106.6	12.3	8.2	8.3	4.0	11.7	10.9	9.6	6.4
TLF	107.0	107.3	100.7	100.8	6.2	6.6	7.1	3.9	12.2	5.6	9.7	7.6
DC	105.9	104.4	102.9	101.7	4.7	2.9	4.1	2.5	7.5	4.0	4.6	3.2
MF	105.9	104.8	99.2	98.0	4.3	5.5	5.4	3.0	9.8	6.6	8.0	5.9

Table 5
Results of CCα and CCβ.

Compound	Concentration range ($\mu g\,kg^{-1}$)	CCα ($\mu g\,kg^{-1}$)	CCβ ($\mu g\,kg^{-1}$)	MRLs ($\mu g\,kg^{-1}$)	EU-CRL recommended concentrations
KTP (ESI pos)	2.5–10	1.29	1.61	–	–
NP	5–20	3.43	3.78	–	10
CPF	25–100	19.61	25.05	–	–
VDP	25–100	27.54	31.54	–	–
FLU	5–20	0.69	0.87	–	–
FLU-OH	20–80	45.08	50.15	40	–
MLX	7.5–30	15.37	15.74	15	–
PBZ	2.5–10	0.90	1.14	–	5
OPB	2.5–10	2.42	3.23	–	5
DC	0.5–2	0.10	0.13	0.1	1[a]
TLF	25–100	62.96	75.92	50	–
MF	5–20	2.51	3.27	–	10

[a] Level to reach until a specific method is developed to confirm at the MRL level.

As it was explained in the method validation part (see Section 2.5.4), two approaches were applied to test the global matrix effects and the applicability of the method. The first approach was to calculate the ratio ME (%). ME was ranging between 36.6% for NP and 140.9% for MLX, and was found variable day to day for some analytes (FLU-OH, NP, PBZ). It was difficult to conclude on these results, but it confirmed the need to use matrix calibration curves instead of solvent standard curves. Then, the second approach was aimed at taking into account the inter batch effect. This factor was thus integrated in the repeatability and in the intra-laboratory reproducibility estimations as it is the case in real-life routine control. Applicability was not examined specifically nor extracted from the results. The precision results (r% and R%) were all found acceptable (see Section here above) with this factor of "matrix effect" included in the calculation through matrix calibrations.

The CCα and CCβ (see Table 5) were calculated according to the calibration curve procedure for the non-permitted and for the authorized NSAIDs [23]. The CCα and CCβ were evaluated from the first/major MRM transitions of quantification for the authorised NSAIDs having an MRL set (i.e. FLU-OH, MLX, TLF and except DC not validated at its MRL level) and for the authorised NSAIDs having no MRL set (CPF and KTP). But for the non-authorised NSAIDs, CCα were evaluated from the second/minor MRM transitions, always detected with a S/N = 3 at least. Obviously, for these non-authorized compounds, the results for the critical concentrations were depending on the levels of the calibration curves. For instance, results for CPF and VDP validated at the same levels (levels ranging from 25 to 100 $\mu g\,kg^{-1}$) gave higher values for VDP (CCα = 27.54 $\mu g\,kg^{-1}$) than for CPF (CCα = 19.61 $\mu g\,kg^{-1}$), because of the well-known difficulty to analyse VDP. On another example, the limits calculated for FLU were very low (CCα = 0.69 $\mu g\,kg^{-1}$ with a calibration range of 5–20 $\mu g\,kg^{-1}$) because it is a sensitive, and repeatable analyte, with a good correction by the internal standard FLU-d_3. The same demonstration occurred for PBZ (CCα = 1.53 $\mu g\,kg^{-1}$) and OPB (CCα = 2–42 $\mu g\,kg^{-1}$), limits are lower for PBZ because of the correction by its deuterated internal standard. But results are even found much lower for KTP (CCα = 1.29 $\mu g\,kg^{-1}$) with the same calibration range (but in ESI+). For DC, CCα and CCβ values are very low but show only that the method is able to identify clearly DC at 0.13 $\mu g\,kg^{-1}$ and confirm its presence at 0.10 $\mu g\,kg^{-1}$, but confirm with a good quantification only after 0,5 $\mu g\,kg^{-1}$. So the method is insufficient to quantify at the MRL (0.10 $\mu g\,kg^{-1}$1). However, the choice was made to keep DC in the method because it is still useful for national control plans to screen DC at its MRL. But there is an urgent need to develop a specific method able to confirm and quantify DC at its MRL (0.1 $\mu g\,kg^{-1}$).

4. Conclusion

The LC–MS/MS method developed at out laboratory was found very sensitive, accurate and simple for the detection and the confirmation of 12 NSAIDs in milk. The extraction method is based on simple liquid extraction with methanol and no further clean up step is necessary; so this reliable method is faster than the current procedures proposed in the literature. Furthermore, the method enables the use of two different RP-LC columns (5 μm and 2.7 μm) leading to runtimes of 23 min and 12 min respectively. Validation parameters based on Commission Decision 2002/657/EC were all checked and found correct. The proposed method is a satisfactory compromise between the prerequisite of the Regulation and the routine application in field laboratories at screening and confirmation steps.

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