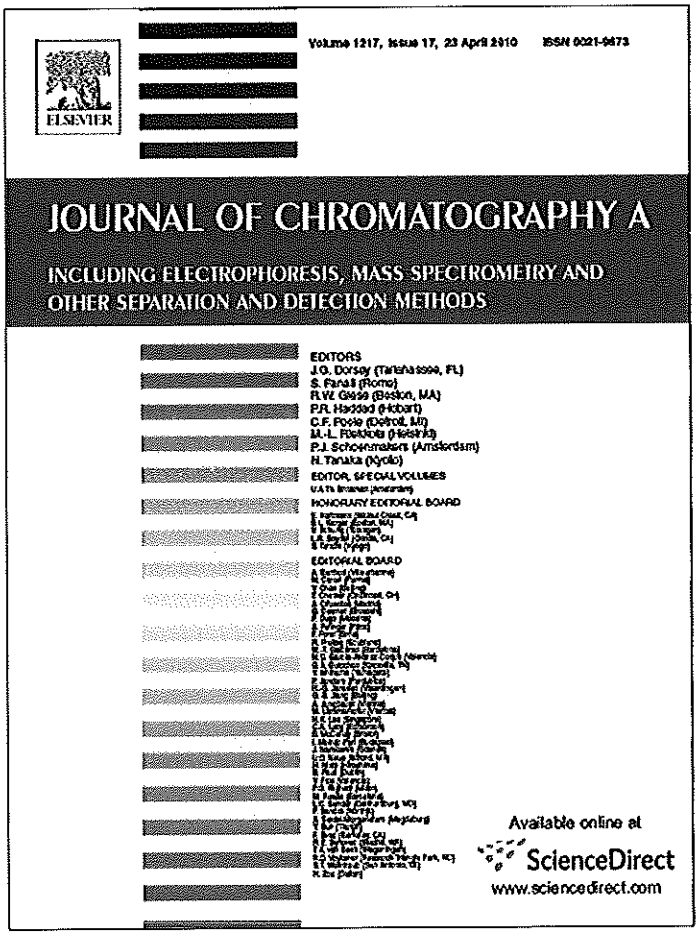

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Confirmatory analysis of non-steroidal anti-inflammatory drugs in bovine milk by high-performance liquid chromatography with fluorescence detection

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ABSTRACT

HPLC with fluorescence detection is considered for confirmatory analysis of group B veterinary drugs by the European Union legislation. A procedure for confirming the presence of anti-inflammatory non-steroidal drug (NSAID) residues in bovine milk by reversed phase high-performance liquid chromatography with fluorescence detection is herein described. The native fluorescence of nine drugs belonging to different NSAID sub-classes, namely flurbiprofen, carprofen, naproxen, vedaprofen, 5-hydroxy-flunixin, niflumic acid, mefenamic acid, meclofenamic acid and tolfenamic acid, allowed for detection in bovine milk down to 0.25–20.0 μg/kg. Confirmation of the nine NSAIDs is attained by fluorescence detection at characteristic excitation and emission wavelengths. The procedure described is simple and selective. Limits of quantification (LOQs) ranging between 0.25 and 20 μg/kg were measured; satisfactory trueness and within-laboratory reproducibility data were calculated at LOQ spiking levels, apart from 5-hydroxy-flunixin. The procedure developed is used in our laboratory for confirmation of each one of the above mentioned NSAIDs in bovine milk, to support results after HPLC quantitative analysis with UV–vis detection.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are worldwide used in veterinary medicine, to treat inflammation, pain, fever and some bovine respiratory diseases, in conjunction with antibiotics [1–3]. Their use can cause toxic side effects, such as gastrointestinal bleeding, intestinal ulceration, aplastic anaemia and inhibition of platelet aggregation; moreover, it has been reported that long-term exposure to phenylbutazone can induce kidney tumours in rats and liver tumours in mice [4].

NSAIDs have been classified as group B substances by the European Council. For many of them, provisional maximum residue limits (MRLs) tolerable in different animal species and target matrices have been set by the Regulation 2377/90/EEC [5] and its amendments [6–10]. In bovine milk, the MRLs for tolfenamic acid [8], meloxicam [9] and 5-hydroxy-flunixin (the marker residue for flunixin) [9] have been set at 50, 15 and 40 μg/kg, respectively. Carprofen has been included in the Annex II of the Regulation only for bovine milk [7], because no MRL is provided. The use of diclofenac is prohibited for milk-producing animals [10]. Ketoprofen, salicylic acid and salicylates, acetylsalicylic acid and acetylsalicylates, are listed in the Annex II of the Regulation, being considered allowed substances only for species not involved in milk and egg production for human consumption. No MRL is established for phenylbutazone, flurbiprofen, ibuprofen, suxibutazone, vedaprofen, meclofenamic acid and mefenamic acid. The widespread use of these substances represents a potential risk to the consumers, because their residues can enter the food chain; therefore, the development of test methods to monitor compliance of animal tissues and food with legislation in the European Union is needed. In Italy, milk is one of the target matrices chosen to monitor the misuse of NSAIDs in animal productions.

Many analytical methods for NSAID determination in milk by high-performance liquid chromatography (HPLC) using UV–vis detection [11–18], liquid chromatography coupled to mass spectrometry (LC/MS) [18–25] and gas chromatography/mass spectrometry (GC/MS) [26,27] have been described in the literature, but most of them focused on just one or a few substances. In the literature, the concentration values reported as limit of quantification (LOQ) or decision limit (CCα) range from 0.46 ng/mL for

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carprofen and 1.29 ng/mL for naproxen, measured by LC–MS/MS on Q-TRAP [25] to 0.59 ng/mL for ibuprofen by GC–MS/MS [27], to 1 ng/mL for 5-hydroxy-flunixin [20] and 6.3 ng/mL for diclofenac [24] by triple quadrupole LC–MS/MS, up to 90 ng/mL for ketoprofen by HPLC-UV [11].

In our laboratory for official control of animal productions, the analysis of NSAIDs belonging to different sub-classes in bovine milk is performed by a multi-residue HPLC-DAD quantitative method. The availability of test methods for further confirmation is helpful to support legal action in the case of misuse of prohibited substances or undeclared treatments. The Decision 2002/657/EC from the European Council [28] established that fluorescence detection can be employed for confirmation of group B substances in the official control of foods. In this framework, herein, a procedure for NSAID analysis in bovine milk by HPLC with fluorescence detection (HPLC-FLD) is described. To this purpose, the study of native fluorescence of 18 NSAIDs routinely analysed in our laboratory was carried out. Selective excitation and emission wavelengths were tested for each drug, to set the best experimental conditions for confirmation by fluorescence detection. As a result, only 9 NSAIDs out of the 18 studied were detectable down to the concentrations of interest for confirmatory purposes within official control activity: carprofen, flurbiprofen, naproxen and vedaprofen (arylpropionic acid derivatives); mefenamic acid, meclofenamic acid and tolfenamic acid (anthranilic acid derivatives); niflumic acid and 5-hydroxy-flunixin (nicotinic acid derivatives). On this basis, the presence of NSAID residues in milk can be confirmed by HPLC-FLD if the analysis of UV–vis spectrum is not sufficient, or if mass spectrometry based methods and/or instrumentation are not available in the laboratory.

In our routine laboratory, HPLC-FLD is used for confirmation of the above mentioned nine NSAIDs in bovine milk to support HPLC-DAD result, or when identification based on UV–vis spectrum fails. Trueness and within-laboratory reproducibility, as well as the limits of quantification (LOQs) for HPLC-FLD analysis of these nine NSAIDs were calculated. The results are discussed evaluating the reliability of HPLC-FLD analysis for confirmatory purposes in bovine milk, according to the European Union law, in comparison to HPLC with UV–vis detection and ion trap mass spectrometry methods developed in our laboratory.

2. Experimental

2.1. Materials and reagents

Carprofen (CPF), ketoprofen (KPF), phenylbutazone (PBZ), oxyphenbutazone (OBZ), naproxen (NPX), niflumic acid (NFL), diclofenac (DCF), flurbiprofen (FLB), ibuprofen (IBP), flunixin (FLU), meloxicam (MLX), mefenamic acid (MFN), meclofenamic acid (MCL), suxibutazone (SBZ) and tolfenamic acid (TLF) were supplied by Sigma–Aldrich (Milan, Italy); salicylic acid (SA), 5-hydroxy-flunixin (5OH-FLU) and vedaprofen (VDP) were obtained from the European Reference Laboratory for Residues of Veterinary Drugs in Berlin (CRL-BVL). All the reference materials were of analytical grade purity.

Phosphoric acid (85%, w/v), hydrochloric acid (37%, w/v), diethyl ether, glacial acetic acid (Carlo Erba, Milan, Italy) and ascorbic acid (Merck, Darmstadt, Germany) were all analytical grade reagents. HPLC grade acetonitrile, methanol and *n*-hexane were supplied by J.T. Baker (Mallinckrodt Baker B.V., Deventer, The Netherlands). HPLC grade water was in-house produced using a MilliQ system (Millipore, Bedford, MA, USA).

C18 end-capped (EC) solid-phase extraction (SPE) cartridges with 1 g sorbent bed and 6 mL reservoir volume were purchased from Isolute (Step-Bio, Bologna, Italy).

Table 1
The experimental conditions to study the native fluorescence of 18 NSAIDs.

Drug	Excitation wavelength (nm)	Emission wavelength ranges (nm)	Gain set
Salicylic acid	220	230–500	High
Meloxicam	360	370–570	High
Naproxen	245	280–465	Medium
Flurbiprofen	245	258–458	Low
Vedaprofen	245	250–430	Medium
Ketoprofen	254	265–465	High
Carprofen	260	275–465	Medium
Ibuprofen	220	230–430	High
Flunixin	360	370–570	High
5-Hydroxy flunixin	360	378–578	High
Niflumic acid	360	378–578	High
Mefenamic acid	360	378–578	High
Meclofenamic acid	360	388–588	High
Tolfenamic acid	360	378–578	High
Diclofenac	375	385–585	High
Oxyphenbutazone	375	385–585	High
Phenylbutazone	375	385–585	High
Suxibutazone	375	385–585	High

2.2. Milk samples

Bovine milk samples were collected from farm animals not treated with NSAIDs.

2.3. NSAIDs standard solutions

Standard stock solutions at 1 mg/mL were prepared by dissolving 10.0 ± 0.1 mg of each drug in 10 mL of acetonitrile/methanol (90/10, v/v). Standard solutions at 10 μg/mL of each NSAID studied were prepared by dilution with methanol; a mix standard solution at 10 μg/mL containing all the 18 drugs was prepared by diluting 0.100 mL of each standard stock solution to a final volume of 10 mL with methanol. All the standard solutions were stable for at least 6 months if stored at −20 °C. NSAID mix working standard solutions at 2.5, 5.0, 10.0, 20.0, 50.0, 100.0, 200.0, and 400.0 ng/mL were prepared before each working session from the mix standard solution at 10 μg/mL, by dilution with methanol.

2.4. Study of NSAIDs fluorescence emission spectra

Eighteen NSAIDs routinely screened in our laboratory were tested to evaluate the applicability of the approach based on fluorescence detection for the purposes of the official control. A preliminary study of the native fluorescence of the 18 NSAIDs was carried out, by testing specific excitation wavelengths and recording the emission spectra of each drug. The wavelengths corresponding to the maxima in the UV–vis absorbance spectra of each drug were chosen for fluorescence excitation. The fluorescence emission spectra and the wavelengths corresponding to the maxima were recorded for each drug, using a CARY Eclipse Fluorescence Spectrophotometer (VARIAN, Inc., Palo Alto, CA, USA). The results were elaborated by the Excel™ 2003 software (Windows). The study was performed by flow injection of standard solutions of each NSAID at 10 μg/mL in methanol, apart from NPX and CPF, which were at 1 μg/mL, because of higher fluorophore emission intensity. The excitation and emission wavelengths are reported in Table 1, as well as the gain of the PMT Detector voltage, which was set in the low, medium or high mode (corresponding to 400, 600 and 800 V, respectively).

2.5. Milk sample clean up

The sample clean up procedure, previously described [18], in this study was applied also to meloxicam and vedaprofen

purification: 5.00 ± 0.01 g of milk were added with 10 mL acetonitrile/methanol (90/10, v/v), mixed by vortex for 1 min, then centrifuged at 1267 × *g* for 10 min at 4 °C. The supernatant was separated and added with 20 mL ascorbic acid buffer 0.010 mol/L pH 3.0 (AA buffer) and 0.2 mL HCl 1.0 mol/L; after hydrolysis for 10 min at 25 °C, the sample was loaded at atmospheric pressure onto a C18 (EC) 1 g SPE cartridge, previously rinsed with 3 mL *n*-hexane/diethyl ether 1/1 (v/v), 3 mL methanol and 5 mL AA buffer. After loading, the SPE cartridge was washed with 3 mL AA buffer and 3 mL MilliQ water/methanol (90/10, v/v), then dried under vacuum for 30 min. Finally, the SPE cartridge was eluted with 3 mL *n*-hexane/diethyl ether 1/1 (v/v), and the eluate was evaporated to dryness under a nitrogen stream at room temperature. The residue was dissolved in 500 μL of HPLC grade methanol.

2.6. Reversed phase HPLC analysis with fluorescence detection (HPLC-FLD)

HPLC analysis was performed using a Waters system, equipped with 600E quaternary pump, 717 Plus autosampler and 2475 fluorescence detector (Waters Corp., Milford, MA, USA). Chromatographic separation was attained injecting 50 μL sample volume onto a 4 μm particle 250 mm × 4.6 mm 80 Å Max-RP Synergi stainless steel column (Phenomenex, Torrance, CA, USA), at 1.2 mL/min flow rate, using 0.010 mol/L *o*-phosphoric acid pH 2.1 as mobile phase A, and acetonitrile as mobile phase B. The chromatography was carried out by linear gradient at room temperature, according to the following programme: from 35% B at time 0 to 65% B in 25 min, then to 100% B in 5 min, holding on for 5 min, finally to 35% B in 2 min; the equilibrium time between analyses was 15 min.

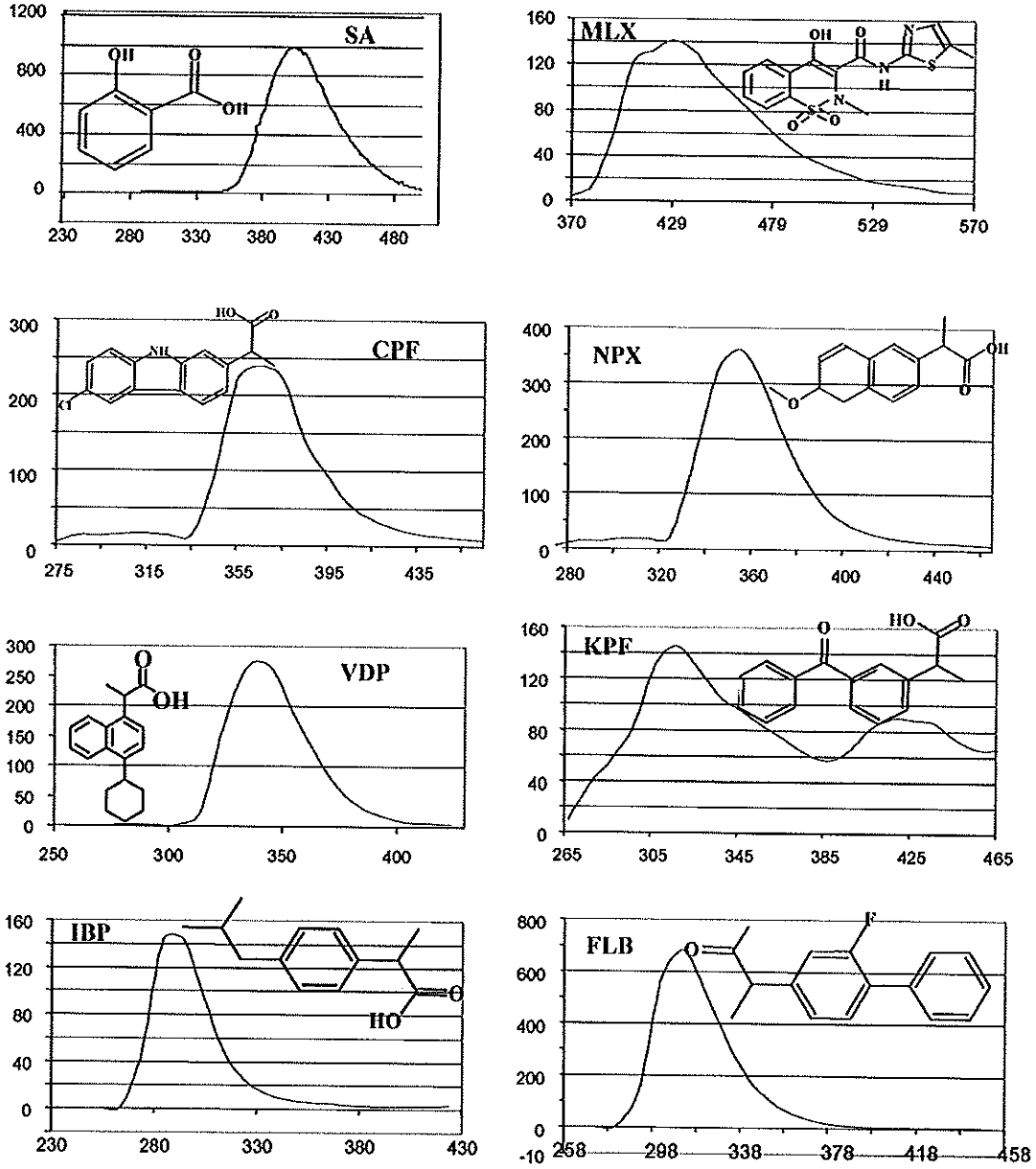

Fig. 1. The fluorescence emission spectra of SA, MLX and arylpropionic derivatives (CPF, NPX, VDP, KPF, IBP and FLB) at 10 μg/mL in methanol, apart from NPX and CPF at 1 μg/mL.

Data handling was performed by the software Empower Pro™ (Waters). The experimental conditions for fluorescence detection were set selecting for each drug the emission wavelength that showed the best response in the preliminary study; a compromise between chromatographic background and the fluorescence signal of the drug was attained for each NSAID. Only 9 out of 18 NSAIDs exhibited fluorescence emission that can be exploited for residue analysis in milk at low concentration levels. The fluorescence excitation and emission wavelengths for each NSAID are reported in Table 2, specifying also the drugs detectable. Quantitative calculations were performed by interpolation of the external standard calibration curves, calculated daily by linear regression of peak areas versus standard solution concentrations, in the range from 2.0 to 400 ng/mL for the NSAIDs tested. During each working session, a blank reagent, blank and spiked samples of bovine milk were analysed.

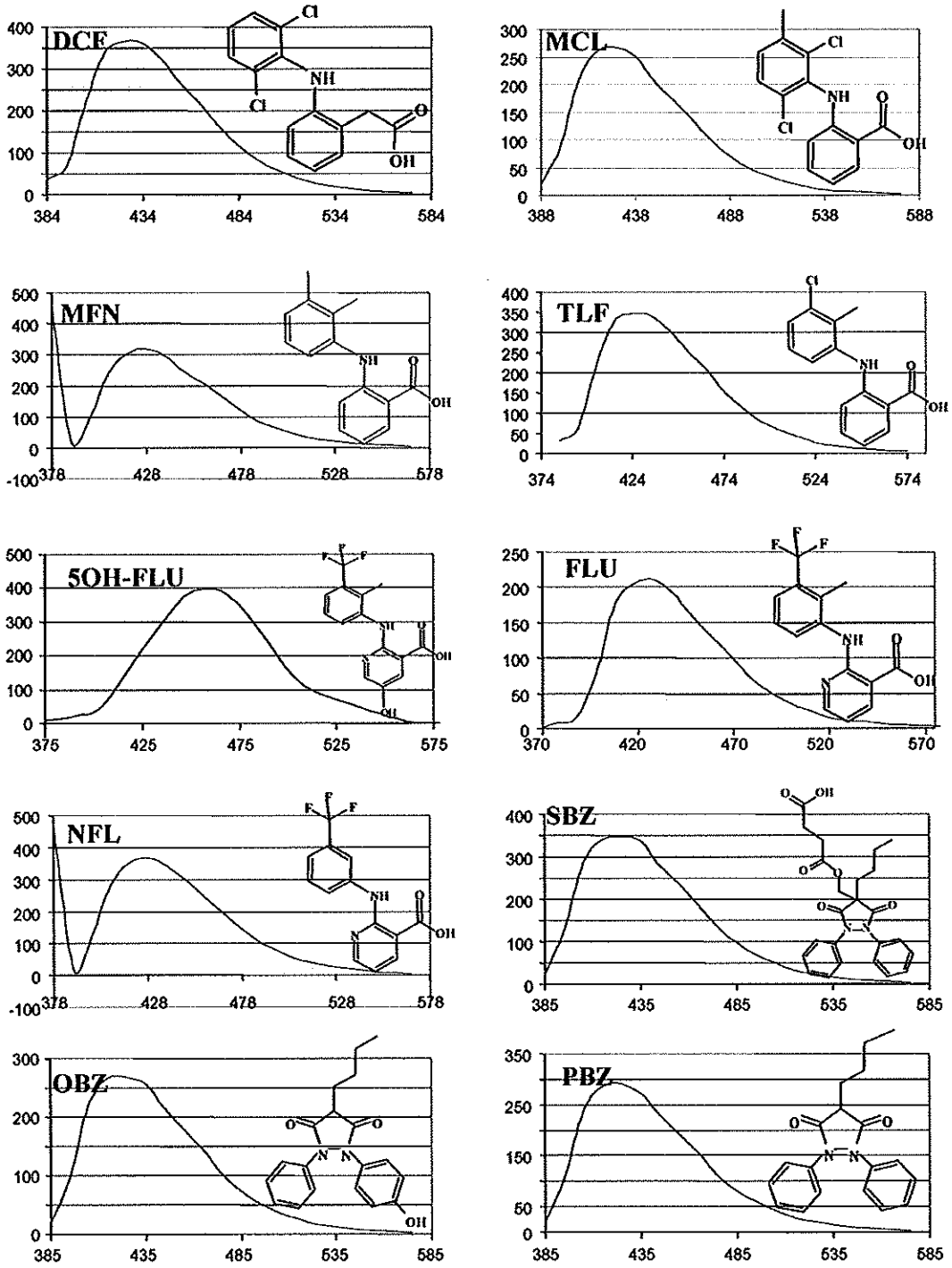

Fig. 2. The fluorescence emission spectra of anthranilic acid derivatives (DCF, MCL, MFN and TLF), nicotinic acid derivatives (5OH-FLU, FLU and NFL) and pyrazolidinedione derivatives (SBZ, PBZ and OBZ) at 10 μg/mL in methanol.

Table 2
The experimental conditions for fluorescence detection of nine NSAIDs using different excitation and emission wavelengths.

Excitation wavelength (nm)	Emission wavelength (nm)	Detected NSAIDs
360	495	5OH-FLU, NFL, MFN, MCL, TLF
260	370	CPF
245	330	NPX, FLB, VDP

2.7. Specificity of the HPLC-FLD method

During this study, 20 uncontaminated bovine milk samples, both raw and pasteurised, were divided in aliquots, then analysed to test the presence of matrix components interfering with detection of the drugs studied.

2.8. Limits of quantification, mean recoveries and within-laboratory reproducibility of the HPLC-FLD method

The limits of quantification (LOQs) of the NSAIDs detectable by the fluorescence methods were determined by analysing different aliquots of bovine milk samples not treated with NSAIDs, spiked at various concentrations (0.25, 0.50, 1.0, 4.0, 5.0, 10.0 and 20.0 μg/kg). To evaluate method reliability, both within-laboratory reproducibility and trueness were calculated spiking blank milk samples at the LOQ of each NSAID. The within-laboratory reproducibility, defined by the Decision 2002/657/EC as the precision obtained in the same laboratory under predetermined conditions over justified long time intervals, was calculated in terms of relative standard deviation (RSD) analysing six different spiked samples over two working days, by two different analysts;

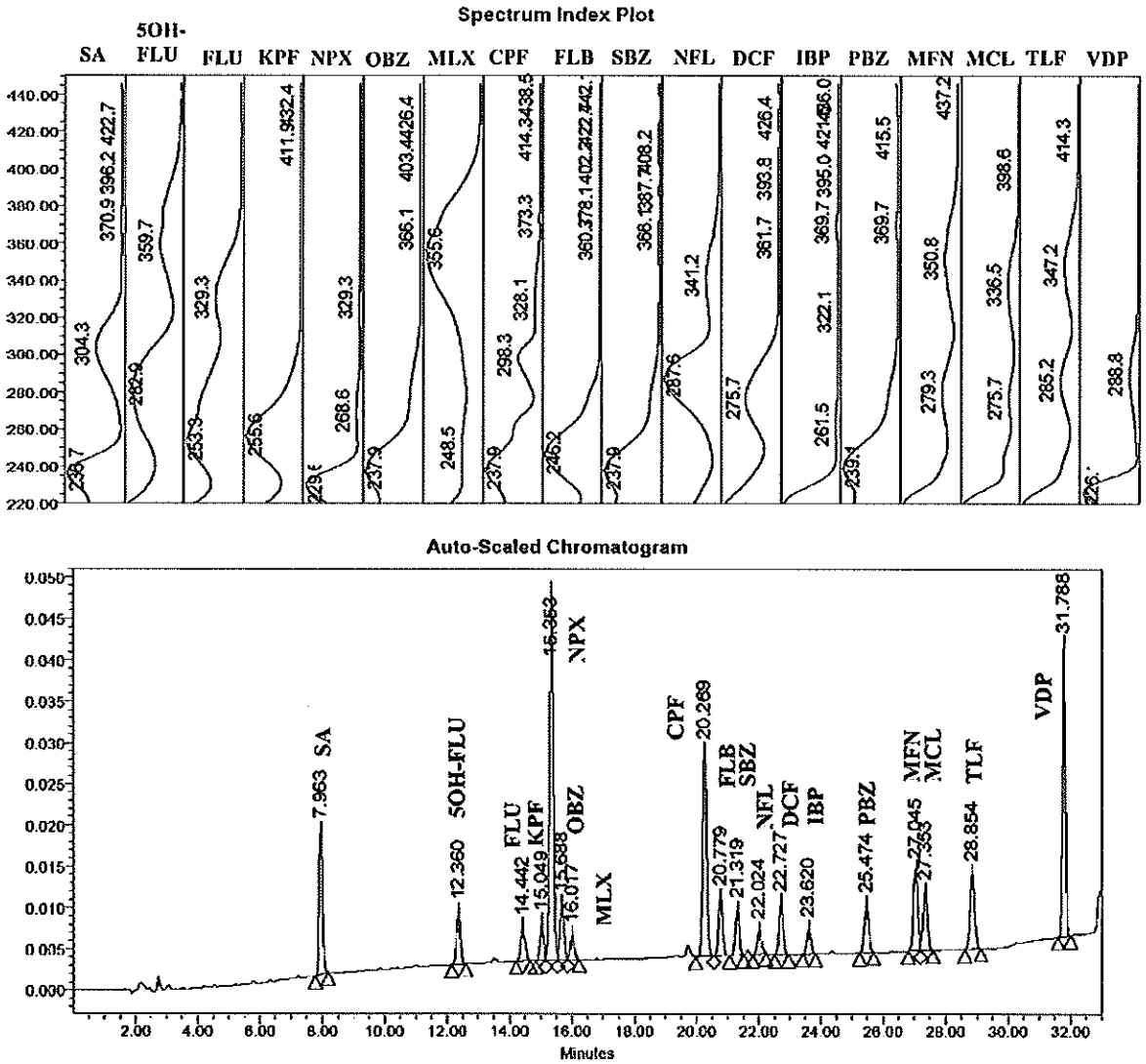

Fig. 3. The chromatogram at 230 nm of a NSAIDs standard mix at 0.400 μg/mL, and their respective UV–vis spectra (220–440 nm). The standard mix contains salicylic acid (SA), 5-hydroxy-flunixin (5OH-FLU), flunixin (FLU), naproxen (NPX), ketoprofen (KPF), oxyphenbutazone (OBZ), meloxicam (MLX), carprofen (CPF), flurbiprofen (FLB), suxibutazone (SBZ), niflumic acid (NFL), diclofenac (DCF), ibuprofen (IBP), phenylbutazone (PBZ), mefenamic acid (MFN), meclofenamic acid (MCL), tolfenamic acid (TLF) and vedaprofen (VDP).

trueness was calculated by mean recoveries from all spiked samples.

3. Results and discussion

3.1. Study of NSAIDs fluorescence emission spectra

All the NSAIDs studied showed native emission fluorescence when excited at the selective wavelengths reported in Table 1; the gain set for each compound to get output signals in the emission spectra ranging between 0 and 1000 fluorescence units is also reported. The fluorescence emission was particularly intense for some drugs: NPX, FLB, CPF and VDP among arylpropionic acid derivatives; NFL among nicotinic acid derivatives; SBZ, PBZ and OBZ among pyrazolidinedione derivatives; DCF, MFN, MCL, TLF among anthranilic acid derivatives; and SA (Figs. 1 and 2). From a general point of view, the availability of resonance structures of the molecule, due to the presence of aromatic rings condensed (NPX and VDP) or linked by a nitrogen atom (NFL, DCF, MFN, MCL, TLF, SBZ, PBZ and OBZ) seems to improve fluorescence emission. The drugs MLX, KPF and IBP do not show these characteristics in their own molecular structure, and exhibited relatively low fluorescence intensity.

3.2. Milk sample clean up

The effectiveness of the clean up procedure of NSAIDs from bovine milk, including meloxicam and vedaprofen, introduced within the study herein presented, was evaluated by mean recovery data. This clean up procedure is simple and rapid, allowing to process many samples in a relatively short time.

3.3. Reversed phase HPLC analysis with fluorescence detection (HPLC-FLD)

The 18 NSAIDs, showing apolar structures with acidic functional groups, were separated by reversed phase HPLC method on C12 stationary phase HPLC column, with non-polar end-capping, characterised by high selectivity for non-polar hydrophobic compounds. A chromatogram at 230 nm of a standard mix at 0.400 μg/mL is reported in Fig. 3. All the drugs are separated on the baseline, apart from the group including NPX, KPF, OBZ and MLX; although these compounds are eluted within 1 min in the chromatogram, they are well distinguishable. Selective wavelengths can be used for UV–vis detection, like already described in our previous

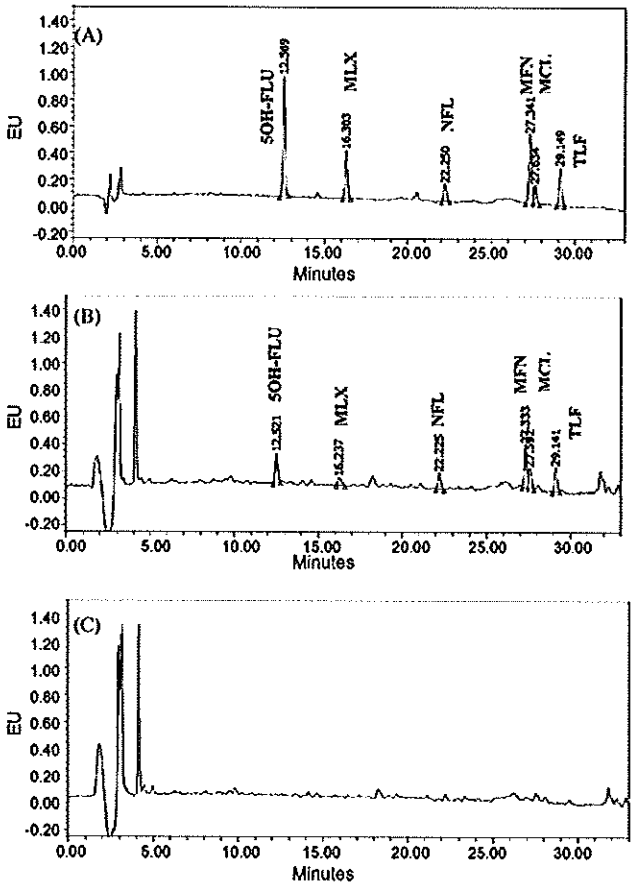

Fig. 4. Comparison between the chromatograms of a mix standard containing 5OH-FLU, MLX, NFL, MFN, MCL and TLF at 0.400 μg/mL (A), a bovine milk sample spiked at 20 μg/kg (B) and a blank bovine milk sample (C).

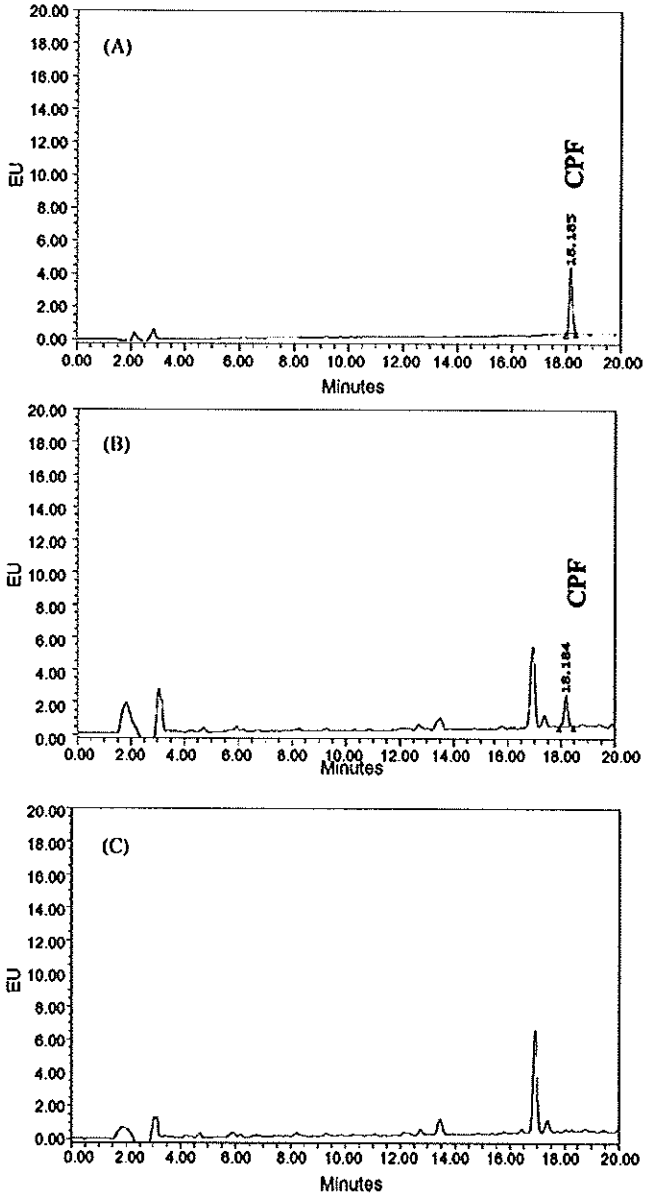

Fig. 5. Comparison between the chromatograms of carprofen (CPF) standard at 0.005 μg/mL (A), a bovine milk sample spiked with CPF at 0.50 μg/kg (B) and a blank bovine milk (C).

work [18], to obtain characteristic chromatograms. In this study MLX, representative of oxicam derivatives, and VDP were introduced; the first is eluted close to OBZ, the latter is well separated on the baseline at the end of the chromatogram.

To develop the HPLC-FLD method we set different excitation and emission wavelengths for each NSAID, to obtain the best signal-to-noise response for each fluorophore. This procedure was tested for fluorescence detection of the NSAIDs in spiked bovine milk. Only for 9 out of 18 NSAIDs, i.e. 5OH-FLU, NPX, CPF, FLB, NFL, MFN, MCL, TLF and VDP, we observed no interference peaks from matrix in the fluorescence chromatograms, at the spiking levels tested; these nine drugs can be selectively detected setting their respective fluorescence excitation and emission wavelengths (Table 2). In the case of MLX, although the fluorescence intensity was not high, the drug was eluted in a part of the chromatogram where no matrix interference affected its detection (Fig. 4A and C). Unfortunately, MLX was hardly distinguishable at 20 μg/kg spiking level (Fig. 4B), i.e. above its MRL at 15 μg/kg; for this reason, fluorescence detection is not satisfactorily applicable to MLX, and was not further studied.

The results demonstrate fluorescence detection is satisfactorily applicable to the analysis of TLF, considering its MRL at 50 μg/kg; MFN and MCL elute close in the chromatogram, and are not completely separated on the baseline, although they are clearly distinguishable (Fig. 4). The NSAIDs belonging to the group of arylpropionic acid derivatives were analysed using different excitation and emission wavelengths; as expected, the fluorescence emission of both KPF and IBP was too low for detecting these drugs at the spiking levels studied. On the contrary, the fluorescence detection of the other arylpropionic acid derivatives was attained also at very low concentrations. CPF was clearly detectable in a milk sample spiked at 0.50 μg/kg (Fig. 5); the analysis was not affected by matrix components.

NPX, FLB and VDP were again clearly detectable at 0.50, 0.25 and 1.0 μg/kg, respectively (Fig. 6); the presence of some peaks from matrix components did not affect detection of drugs. The linearity of fluorescence response was verified for 5OH-FLU, NFL, MFN, MCL, TLF, CPF, NPX, FLB and VDP within the concentration range from their respective LOQs up to 0.400 μg/mL in methanol ($0.9920 < R^2 < 0.9992$, Table 3).

From a general point of view, the HPLC-FLD method developed can be applied to a single NSAID to be confirmed, or can be performed for multi-residue analysis, using the same experimental conditions (e.g. for 5OH-FLU, NFL, MFN, MCL, TLF), or even switching the fluorescence detector between different wavelengths within the same chromatographic run.

3.4. Specificity of the HPLC-FLD method

The HPLC-FLD method was specific for the analysis of NFL, 5OH-FLU, MFN, MCL, TLF, CPF, NPX, FLB and VDP in bovine milk, because no interference from matrix was observed in blank samples analysed, at the spiking levels tested.

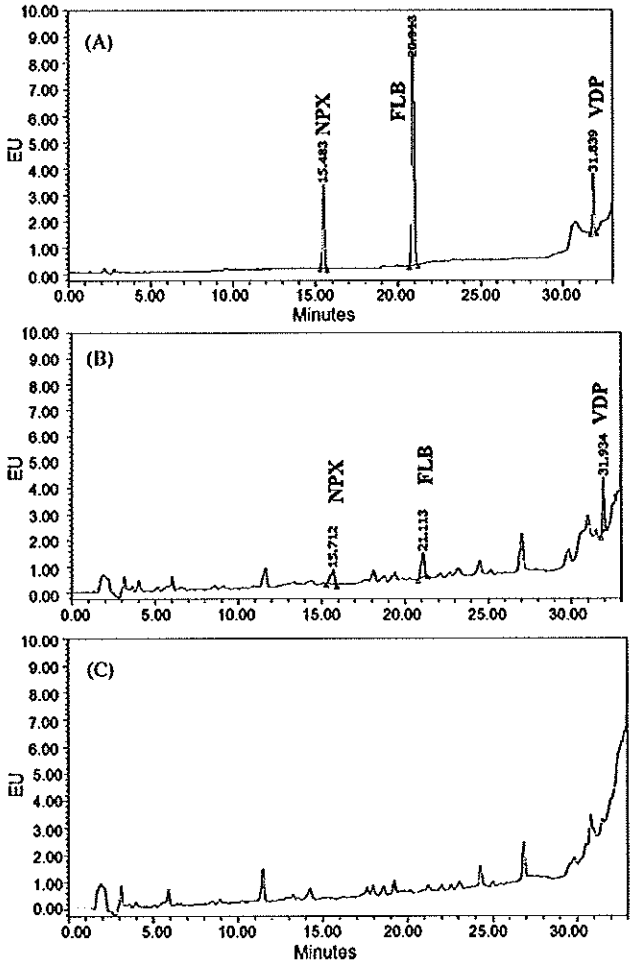

Fig. 6. Comparison between the chromatograms of a mix standard at 0.010 μg/mL (A), a bovine milk sample spiked with naproxen (NPX) at 0.50 μg/kg, flurbiprofen (FLB) at 0.25 μg/kg, vedaprofen (VDP) at 1.0 μg/kg (B) and a blank bovine milk (C).

3.5. Limits of quantification, mean recoveries and within-laboratory reproducibility of the HPLC-FLD method

The LOQs for 5OH-FLU, NFL, MFN, MCL, TLF, CPF, NPX, FLB and VDP were experimentally verified by analysing milk samples spiked at decreasing drug concentrations. It is noteworthy fluorescence detection was effective for all drugs, but especially for naproxen, carprofen, flurbiprofen and vedaprofen. For tolfenamic acid and 5-hydroxy-flunixin the LOQs were quite lower than their MRLs set at 50 and 40 μg/kg, respectively; therefore, the method fits for the purposes of the official control. Trueness and precision data were calculated for these nine NSAIDs at LOQs spiking

Table 3
Method linearity, trueness and within-laboratory reproducibility of the HPLC-fluorescence method, expressed as mean percentage recoveries ($n = 6$), standard deviations (±SD) and relative standard deviations (RSD, %), calculated spiking bovine milk samples at the LOQs determined for each NSAID.

Drug	Linearity in methanol, R^2	LOQ (μg/kg)	Mean percentage recovery (%)	±SD (μg/kg)	RSD (%)
Naproxen	0.9928	0.50	116.6	0.083	14.3
Carprofen	0.9990	0.50	89.3	0.056	12.6
Flurbiprofen	0.9954	0.25	101.0	0.032	6.4
5-Hydroxy-flunixin	0.9992	10.0	38.2	0.51	13.3
Niflumic acid	0.9981	20.0	81.3	0.58	3.6
Mefenamic acid	0.9939	10.0	107.1	2.28	21.3
Meclofenamic acid	0.9966	10.0	114.0	1.18	10.3
Tolfenamic acid	0.9960	10.0	108.9	1.39	12.8
Vedaprofen	0.9920	1.0	64.0	0.083	13.0

Table 4
A comparison between the LOQs of nine NSAIDs by both fluorescence (FLD) and UV–vis (DAD) detection. The capability to identify and confirm each drug by UV–vis spectrum at 5.0 μg/kg spiking levels is also reported.

Drug	LOQ (μg/kg)		Identification at 5.0 μg/kg spiking level by UV–vis spectrum
	FLD detection	DAD detection	
5-Hydroxy-flunixin	10.0	5.0	Not confirmed
Naproxen	0.50	2.0	Not confirmed
Carprofen	0.50	2.0	Confirmed
Flurbiprofen	0.25	4.0	Confirmed
Niflumic acid	20.0	2.0	Confirmed
Mefenamic acid	10.0	4.0	Confirmed
Meclofenamic acid	10.0	4.0	Not confirmed
Tolfenamic acid	10.0	4.0	Confirmed
Vedaprofen	1.0	4.0	Confirmed

levels (Table 3). Method mean recoveries were satisfactory for all drugs, apart from 5OH-FLU. The within-laboratory reproducibility was calculated over two working sessions, by different analysts; RSD values ranging between 3.6% and 21.3% account for appreciable method precision, even at low concentrations.

In a previous work [18] the LOQs of 16 NSAIDs in milk analysed by HPLC-DAD method were determined, evaluating the capability of confirmation of each drug by its own UV–vis spectrum; moreover, the relative merits of identification by both DAD detection and ion trap LC/ESI-MS/MS were compared at 5.0 μg/kg spiking level. The LOQs of 5OH-FLU, NFL, MFN, MCL, TLF, CPF, NPX, FLB and VDP measured by both HPLC-FLD and HPLC-DAD methods are reported for comparison in Table 4; the capability to identify each drug at 5.0 μg/kg spiking level for confirmation by UV–vis spectrum is also shown. It is particularly interesting to note that fluorescence detection of CPF, NPX, FLB and VDP can attain LOQs lower than DAD detection; NPX can be confirmed down to 0.50 μg/kg, whereas UV–vis spectrum identification fails at 5.0 μg/kg. Moreover, FLB and VDP are not identified by our ion trap LC/ESI-MS/MS method at 5.0 μg/kg, but can be confirmed by HPLC-FLD method down to 0.25 and 1.0 μg/kg, respectively.

According to these results, HPLC with fluorescence detection is an interesting alternative to UV–vis spectrum identification for confirmation of some NSAIDs in bovine milk. In particular, for 5OH-FLU and TLF the method allows confirmation below their MRLs in milk; for CPF, the HPLC-FLD method exhibits a LOQ lower than HPLC-DAD method. Regarding FLB and VDP, the method is successful in confirmatory analysis at very low concentrations, and in our laboratory is even an effective alternative to identification by ion trap LC–MS/MS. Likely, 5OH-FLU, NPX and MCL can be confirmed whereas DAD detection does not allow identification by UV–vis spectrum. Although MFN and NFL are detectable by fluorescence down to 10.0 and 20.0 μg/kg, respectively, DAD spectrophotometry showed better performances at lower concentrations.

4. Conclusions

HPLC with fluorescence detection showed to be an effective technique for confirmatory analysis of 5-hydroxy-flunixin, tolfenamic acid, carprofen, flurbiprofen, vedaprofen, naproxen, niflumic acid, mefenamic acid and meclofenamic acid in bovine milk. Apart from niflumic acid and mefenamic acid, HPLC-FLD represents a valid alternative to both UV–vis spectrophotometry and ion trap LC–MS/MS for confirmation in our laboratory; moreover, the LOQs of arylpropionic acid derivatives such as naproxen and carprofen are close or lower than the values reported in the literature using different mass spectrometry techniques, as in the case of LC–MS/MS on Q-TRAP [25]. The specificity of the method and the low LOQs make it a noteworthy choice for naproxen, carprofen, flurbiprofen and vedaprofen analysis down to very low concentration levels.

According to the Decision 2002/657/EC, fluorescence detection for confirmation of group B substances, like NSAIDs, can be applied for both qualitative and quantitative confirmatory analysis. In our laboratory, the HPLC-FLD method is used for further identification of NSAIDs determined by HPLC-DAD, but even for confirmation when the analysis of the UV–vis spectrum and/or ion trap mass spectrometry fails or cannot be employed; this can be a helpful chance for routine laboratory not equipped with MS instrumentation or skilled personnel.

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