

Simultaneous Determination of Residues of Non-Steroidal Anti-Inflammatory Drugs and Glucocorticosteroids in Animal Muscle by Liquid Chromatography-Tandem Mass Spectrometry

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Abstract A method for the determination of a wide range residues of anti-inflammatory drugs (16 acidic non-steroidal anti-inflammatory drugs and four metamizole metabolites and five corticosteroids) has been developed. In the first step of sample preparation, acetate buffer was added to minced muscle samples and 15-min ultrasound-assisted enzymatic hydrolysis was performed. Next, the samples were extracted twice with acetonitrile, freeze-dried and analysed. The analytes were separated on a C18 column with a 25-min gradient of methanol/acetonitrile (8:2) and 0.05 M ammonium formate at pH 5.0 and determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The method was validated according to the requirements described in the Commission Decision 2002/657/EC: linearity, precision (repeatability and within-laboratory reproducibility), accuracy, decision limit $CC\alpha$ and detection capability $CC\beta$ were calculated. The method developed fulfilled the performance criteria and can be used in the official control of veterinary drug residues in food of animal origin. The method was positively verified in the proficiency test and in the analysis of incurred material.

Keywords Non-steroidal anti-inflammatory drugs · Corticosteroids · Mass spectrometry · Muscle · Residues

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticosteroids (GCs) are widely used in veterinary medicine as well as in treatment of diseases in food-producing animals. Despite its effectiveness, the important drawback of pharmacotherapy is drug residues in animal tissues. It became an important issue in the food safety. Potential toxicity of medicinal veterinary products has to be evaluated before the drug registration. When necessary, maximum residue limits (MRLs) in food are established. In the case of NSAIDs, MRL values were established in animal muscle for the following: salicylic acid ($400 \mu\text{g kg}^{-1}$ for turkey), flunixin ($10 \mu\text{g kg}^{-1}$ for *Equidae*, $20 \mu\text{g kg}^{-1}$ for bovine, $50 \mu\text{g kg}^{-1}$ for porcine), meloxicam ($20 \mu\text{g kg}^{-1}$ for bovine, caprine, porcine, rabbit, *Equidae*), metamizole (marker residue: 4-methylaminoantipyrine, $100 \mu\text{g kg}^{-1}$ for bovine, porcine, *Equidae*), diclofenac ($5 \mu\text{g kg}^{-1}$ for bovine, porcine), tolfenamic acid ($50 \mu\text{g kg}^{-1}$ for bovine, porcine), carprofen ($500 \mu\text{g kg}^{-1}$ for bovine, *Equidae*) and firocoxib ($10 \mu\text{g kg}^{-1}$, *Equidae*). For GCs, MRLs for muscles are the following: dexamethasone ($0.75 \mu\text{g kg}^{-1}$ for bovine, caprine, porcine, *Equidae*), betamethasone ($0.75 \mu\text{g kg}^{-1}$ for bovine, porcine), methylprednisolone ($10 \mu\text{g kg}^{-1}$ for bovine, *Equidae*) and prednisolone ($4 \mu\text{g kg}^{-1}$ for bovine, *Equidae*) (European Commission 2010). For some other compounds (phenylbutazone, oxyphenbutazone, ibuprofen, naproxen, mefenamic acid), recommended concentration (5 or $10 \mu\text{g kg}^{-1}$ for all species) has been proposed (CRL Guidance Paper 2007). Important features of AI metabolism (like formation of glucuronides) impose the necessity of the application of a hydrolysis step in the sample preparation (Jedziniak et al. 2013a). In some cases, a deconjugation step is necessary, e.g. carprofen, when the marker residue was expressed as a “sum of

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carprofen and carprofen glucuronide conjugate” (European Commission 2010).

In the case of glucocorticosteroids, a significant part of residues are conjugated as glucuronide (e.g. 50 % of dexamethasone residues) (Vree et al. 1999). Some medicines like phenylbutazone and diclofenac have limited stability in samples and during the sample preparation (Reddersen and Heberer 2003; European Food Safety Authority 2013).

Residues of anti-inflammatory drugs (AIs), both NSAIDs and GCs, have been found in food in recent years in relatively low percentage. In the years 2007–2010, the proportion of non-compliant samples in the European Union remained relatively constant (around 0.1–0.2 %) (European Food Safety Authority 2012). Nevertheless, the detection of phenylbutazone residues (NSAID which is not authorised for use in food-producing animals), found in the horse meat, was an important issue in the EU in 2012 (European Food Safety Authority 2013). In the group of GCs, dexamethasone was most commonly detected in bovines (21 non-compliant samples in 2009, representing 0.37 % of total samples) (European Food Safety Authority 2011).

For the wider view on chemical contaminants in food, dozens or even hundreds of analytes were included in multiclass methods based on LC-MS/MS. Due to the variety of chemical properties, only simple sample preparation can be used, which is effective in the terms of analyte coverage but can generate some difficulties. One of them is the matrix effect, phenomena (ion suppression or ion enhancement) caused by matrix constituents present in the sample (Matuszewski 2006).

Most of the published LC-MS/MS methods cover a wide range of NSAIDs or GCs. Recently, thanks to more sensitive and faster mass spectrometers, AIs have been included in the multiclass-multiresidue methods with LC-MS/MS (Geis-Asteggianti et al. 2012; Robert et al. 2013) or LC-TOF (Ortelli et al. 2009; Peters et al. 2009). Multiclass methods cover dozen of analytes, but their sample preparation has to be very generic; such methods cannot cover the analysis of conjugated analytes.

It should be emphasised that only few methods allow to determine both groups of analytes and include hydrolysis of phase II metabolites (e.g. glucuronides) in the sample preparation.

Chrusch et al. published the procedure for the simultaneous determination of 29 veterinary drugs (NSAIDs, GCs and anabolic steroids) in animal tissues based on acidic hydrolysis, multistage solid-phase extraction (SPE) clean-up and different LC conditions for specific groups of analytes (Chrusch et al. 2008). Recently, basic hydrolysis and clean-up with mixed-mode cation-exchange cartridge were used for the simultaneous determination of corticosteroids, anabolic steroids and basic NSAIDs in milk and muscles (Kaufmann et al. 2014). An important drawback of the above methods is multistep sample preparation based on the SPE.

The authors want to share their experience in the determination of NSAIDs in animal tissues (Jedziniak et al. 2010, Jedziniak et al. 2013b, Olejnik et al. 2013) and develop a new method with wider range of analytes (both metamizole metabolites and “acidic” NSAIDs and GC), enzymatic hydrolysis step and simple sample preparation. According to the best authors’ knowledge, methods for the simultaneous determination of NSAIDs and GCs with enzymatic hydrolysis were not published.

Such approach ensures faster and cheaper analysis and allows to determine conjugated analytes, what is not possible with previously published procedures.

Material and Methods

Reagents and Sorbents

Acetonitrile (ACN) and methanol (MeOH), both LC-MS grade, were obtained from J.T. Baker, Germany. Ammonium formate, HPLC grade, was from LGC Standard, UK. Sodium acetate, puriss p.a. was purchased from POCH, Poland. Acetic acid, formic acid (all LC-MS grade), β -glucuronidase from *Helix pomatia* (HP-2) and ascorbic acid (puriss p.a.) were from Sigma-Aldrich, Germany. Ultrapure water (resistance >18 m Ω) was obtained from Milli-Q system (Millipore, France).

The analytical standards were supplied by the following manufacturers: diclofenac (DC), flunixin meglumine (FLU), carprofen (CPF), mefenamic acid (MEF), tolfenamic acid (TOL), meloxicam sodium (MEL), naproxen (NAP), celecoxib (CELE), flufenamic acid (FLUF), ibuprofen (IBU), ibuprofen glucuronide (IBU-GLU), dexamethasone (DEX), betamethasone (BMT), flumethasone (FLUM), prednisolone (PRED), carprofen-d3 (CPF-d3), meloxicam-d3 (MEL-d3)—Sigma-Aldrich, Germany; 4-methylaminoantipyrine (4-MAA), 4-formylaminoantipyrine (4-FAA), 4-aminoantipyrine (4-AA), 4-acetylaminoantipyrine (4-AcAA), phenylbutazone (PBZ) and oxyphenbutazone monohydrate (OPB)—LGC Standard, UK; flurbiprofen (FLURB), flurbiprofen-d3 (FLURB-d3), indomethacin (IND), indomethacin-d4 (IND-d4), methylprednisolone (MPRED), dexamethasone-d4 (DEX-d4), ibuprofen-¹³C3 (IBU-13C3)—Cambridge Isotopes Laboratories, USA; and firocoxib (FIRO), firocoxib-d6 (FIRO-d6), phenylbutazone-¹³C12 (PBZ-13C12), oxyphenbutazone-¹³C6 (OPB-13C6) flunixin-d3 (FLU-d3) and 4-methylaminofenazone-d3 (4-MAA-d3)—Witega, Germany. Niflumic acid (NIF), diclofenac-¹³C6 (DC-13C6), mefenamic acid-d4 (MEF-d4) and tolfenamic acid-¹³C6 (TOL-13C6) were gifted by the European Union Reference Laboratory for Drug Residues in Berlin.

Cartridges and sorbents used for method development were the following: C18 (500 mg, 3 mL, Avantor, USA); C18 bulk sorbent (Avantor, USA); C8 (500 mg, 3 mL, Avantor, USA); Oasis HLB (60 mg, 3 mL, Waters, USA); Strata X, Strata X-C,

Strata XL and Strata XL-A (all 60 mg, 3 mL, Phenomenex, USA); HybridSPE (100 mg, 3 mL, Supelco, USA); and primary secondary amine (PSA, Supelco, USA).

Standard Solutions, Buffers and Samples

Stock standard solutions ($1000 \mu\text{g mL}^{-1}$) were prepared by weighing of 10.0 mg of each analyte and dissolving in 10.0 mL of methanol (stable for 12 months). Working standard solutions ($100 \mu\text{g mL}^{-1}$) were prepared by the dilution of the proper solutions with methanol and were stable for 6 months.

Three mixed standard solutions were prepared by the dilution of suitable aliquots of working standard solutions ($100 \mu\text{g mL}^{-1}$) and used for a muscle sample spiked on three levels (Table 2). The first one contained $0.25 \mu\text{g mL}^{-1}$ of PBZ and OPB and was stable for 1 month. The second one contained $0.25 \mu\text{g mL}^{-1}$ of DC; $2.5 \mu\text{g mL}^{-1}$ of TOL; $1 \mu\text{g mL}^{-1}$ of MEL; $0.2 \mu\text{g mL}^{-1}$ of PRED; $0.0375 \mu\text{g mL}^{-1}$ of DEX; BMT; $5 \mu\text{g mL}^{-1}$ of 4-MAA; and $0.5 \mu\text{g mL}^{-1}$ of FLU, MEF, NAP, IBU, FIRO, CELE, FLUF, FLURB, IND, NIF, FLUM, MPRED, TRIAM, 4-FAA, 4-AA and 4-AcAA and was stable up to 6 months. The third standard (the mixed solution of internal standards (ISs)) contained $2.0 \mu\text{g mL}^{-1}$ of each IS (DEX-d4, DC-13C6, PBZ-13C12, FIRO-d6, FLURB-d3, FLU-d3, IBU-d3, IND-d4, CPF-d3, MEF-d4, TOL-13C6, MEL-d3, 4-MMA-d3, OXPBZ-13C6) and was stable for 6 months. All standards solutions were stored at $2\text{--}10^\circ\text{C}$ and used for spiking muscle samples at three levels.

Acetate buffer used in the enzymatic hydrolysis was prepared by dissolving sodium acetate (2.7 g) and ascorbic acid (0.17 g) in water (100 mL). The pH was adjusted to 4.5 with acetic acid.

A blank muscle samples (bovine, swine, horse) were collected from different regions of Poland by veterinary inspection.

Equipment

In the sample preparation, vortex mixer (IKA, Germany), nitrogen evaporator (VLM, Germany), freezer (Liebherr, Germany), laboratory centrifuge (Sigma, Germany) and laboratory glassware and pipettes were used. Syringe filters (Puradisc PTFE $0.45 \mu\text{m}$) were from Whatman, UK. The analysis was performed using Agilent 1200 liquid chromatograph (Agilent Technologies, Germany) connected to ABSciex QTRAP 5500 mass spectrometer (ABSciex, Canada), controlled by Analyst 1.5 software.

Sample Preparation

The muscle samples ($2\pm 0.01 \text{ g}$) were weighed into a 50-mL polypropylene centrifuge tube. The volume $20 \mu\text{L}$ of IS solution was added, and the sample was vortex-mixed and allowed to rest for 10 min. Then, acetate buffer (4 mL) and β -glucuronidase ($50 \mu\text{L}$) were added. The sample was vigorously mixed and sonicated (15 min, 37°C). Next, the sample was

extracted with two portions of ACN (5 mL) by vortex mixing (1 min) and centrifuged ($4120\times g$, 20 min, -5°C). The combined extracts (10 mL) were transferred into a 10-mL glass tube and placed in the freezer (-20°C) for 60 min. The upper layer of the extract (2 mL) was collected and evaporated to dryness in the nitrogen stream (at 40°C) and reconstituted in 1 mL of mobile phase (MeOH/ACN/ H_2O , 0.5:0.5:9, v/v/v). The sample was filtered through syringe filters, transferred into the autosampler vial and analysed by LC-MS/MS.

LC-MS/MS Analysis

The chromatographic separation was carried out on a Luna C18 column ($3 \mu\text{m}$, $4.6\times 150 \text{ mm}$, Phenomenex, USA) connected with a C18 guard column ($2.0\times 4 \text{ mm}$, Phenomenex, USA). The gradient was applied with MeOH/ACN (8+2, v+v, containing 0.1 % formic acid—phase A) and 0.01 M ammonium formate, pH 5.0 (phase B). The initial conditions of the gradient kept until 5 min were 15 % A and 85 % B. The phase A content was increased to 32 % at 5.5 min, and such conditions were maintained until 12 min. Next, phase A content was increased to 48 % at 12.5 min. From 12.5 to 16 min, the content of phase A was increased to 70 %, and such conditions were maintained until 20 min. From 20.5 min, the system was re-equilibrated with the initial composition of the mobile phase. The total run time of the method was 24 min. The flow rate was 0.8 mL min^{-1} , the injection volume was $50 \mu\text{L}$, and the column temperature was 30°C .

Mass spectrometry analysis was performed using electrospray (electrospray ionization (ESI), polarity switching) in multiple reaction monitoring (MRM) mode. The ESI interface conditions were optimised as follows: the source temperature 600°C , capillary voltage -4.5 kV in negative mode and $+5.5 \text{ kV}$ in positive mode, curtain gas 30 psi, nebuliser gas 60 psi and turbo heater gas 20 psi. For each analyte, two fragmentation reactions were monitored whereas one was monitored for ISs. Detailed MS conditions are described in Table 1.

Verification of Enzymatic Hydrolysis Efficiency

The blank muscle samples (bovine, swine, horse muscle, $n=3$) were spiked with IBU-GLU standard at the level of $50 \mu\text{g/kg}$. The samples were determined with a developed procedure, and IBU concentration was measured.

Development of Extract Clean-Up (SPE, Dispersive SPE, Freezing)

The extracts ($n=3$) of spiked samples were passed through SPE cartridges (C18, C8, HLB, Strata X, Strata X-C, Hybrid) and eluate was collected. The cartridges were vacuum dried for 1 min, and eluate was evaporated to dryness (N_2 ,

40 °C). The dry residue was reconstituted in 1 mL of mobile phase (MeOH/ACN/H₂O, 0.5:0.5:9, *v/v/v*) and analysed by LC-MS/MS. For verification of dispersive SPE, the following sorbents: C18 (100 mg) and C18 with PSA (both 100 mg) were added to 2 mL of extracts, vortex-mixed (1 min) and centrifuged (14,000 rpm). The 1 mL of extract was evaporated to dryness (N₂, 40 °C), reconstituted in 1 mL of mobile phase and analysed by LC-MS/MS. For verification of freezing effectiveness, the spiked extracts (10 mL) were and placed in the freezer (−20 °C) for 60 min. The upper layer of the extract (2 mL) was collected and evaporated to dryness in the nitrogen stream (at 40 °C) and reconstituted in 1 mL of mobile phase and analysed by LC-MS/MS.

Validation

The method was validated as a confirmatory procedure according to Commission Decision 2002/657/EC (European Commission 2002), and all criteria for confirmation (requirements for retention time and criteria for ion ratios) were taken from the above document. Standard calibration curves were prepared by the injection of mixed standard solutions on five concentration levels and plotting ratios of recorded peak areas of each analyte and adequate ISs versus concentration. In the selectivity study, 20 blank muscle samples from animals (bovine, swine, horse) from different regions of Poland were analysed. The matrix effect was

Table 1 MS/MS parameters for determination of AIs in muscle (ABSciex QTRAP 5500)

Analyte	Parent ion (<i>m/z</i>)	Declustering potential (eV)	Daughter ions (<i>m/z</i>)	Collision energy (eV)	Internal standard
ESI+					
4-Acetylaminoantipyrine	246	192	104, 83 ^a	31, 42	4-MAA-d3
4-Aminoantipyrine	204	185	83, 56 ^a	42, 19	4-MAA-d3
4-Formylaminoantipyrine	232	191	104, 83 ^a	29, 27	4-MAA-d3
4-Methylaminoantipyrine	218	170	97, 56 ^a	17, 44	4-MAA-d3
Celecoxib	382	290	362 ^a , 282	40, 45	FIRO-d6
Diclofenac	296	50	250 ^a , 215	17, 27	DC-13C6
Firocoxib	337	100	283 ^a , 237	14, 23	FIRO-d6
ESI−					
5-Hydroxyflunixin	311	−51	267, 227	−22, −33	FLU-d3
Betamethasone	437	−80	361, 307	−25, −40	DEX-d4
Carprofen	272	−40	228, 226 ^a	−17, −37	CPF-d3
Dexamethasone	437	−65	361 ^a , 307	−25, −42	DEX-d4
Diclofenac	294	−80	250 ^a , 214	−28, −16	DC-13C6
Flufenamic acid	280	−157	236 ^a , 216	−24, −26	FLU-d3
Flumethasone	455	−75	379 ^a , 325	−25, −31	DEX-d4
Flunixin	295	−51	251 ^a , 191	−25, −41	FLU-d3
Flurbiprofen	243	−70	199 ^a , 175	−10, −12	FLURB-d3
Ibuprofen	205	−20	161, 159	−9, −11	IBU-13C3
Ibuprofen glucuronide	381	−20	193, 205	−8, −12	IBU-13C3
Indomethacin	356	−97	312 ^a , 297	−12, −27	IND-d4
Mefenamic acid	240	−60	196 ^a , 192	−24, −40,	MEF-d4
Meloxicam	350	−55	286 ^a , 146	−17, −25	MEL-d3
Methylprednisolone	419	−79	343 ^a , 309	−25, −43	DEX-d4
Naproxen	229	−50	185 ^a , 170	−14, −20	FLU-d3
Niflumic acid	281	−155	237 ^a , 217	−23, −30	FLU-d3
Oxyphenbutazone	323	−56	295 ^a , 134	−25, −35	OPB-13C6
Phenylbutazone	307	−70	279 ^a , 131	−26, −28	PBZ-13C12
Prednisolone	405	−80	329 ^a , 295 ^a	−23, −25	DEX-d4
	329	−200			
Tolfenamic acid	260	−74	227, 216 ^a	−14, −22	TOL-13C6
Triamcinolone acetoneide	479	−70	413 ^a , 337	−30, −20	DEX-d4

ESI+ positive ionization, ESI negative ionization)

^a Transition used for quantification

Table 2 Validation results of multiresidue method for determination of anti-inflammatory drugs in the muscle

Analyte	Linearity	Range ($\mu\text{g kg}^{-1}$)	Spiking level ($\mu\text{g kg}^{-1}$)			Accuracy (%) ^c	Repeatability, CV (%)	Within-laboratory reproducibility, CV (%)	CC α ($\mu\text{g kg}^{-1}$)	CC β ($\mu\text{g kg}^{-1}$)	LOQ ($\mu\text{g kg}^{-1}$)	U (%)						
			I	II	III													
NSAIDs																		
4-Acetylaminoantipyrine	0.995	2.5–50	5.0	10.0	15.0	100	124	123	10.3	16.6	14.1	13.4	19.9	18.3	6.63	8.27	2.5	40
4-Aminoantipyrine	0.994	2.5–50	5.0	10.0	15.0	87.7	106	100	7.7	5.8	10.2	11.6	7.0	15.3	5.57	6.14	2.5	14
4-Formylaminoantipyrine	0.997	2.5–50	5.0	10.0	15.0	105	96	107	9.9	11.6	13.5	16.8	19.7	23.0	6.62	8.23	2.5	39
4-Methylaminoantipyrine	0.992	2.5–500	50	100 ^a	150	118	112	105	9.7	9.5	7.7	15.5	15.2	12.3	11.2	124	25	30
Carprofen	0.991	12.5–1500	250	500 ^a	750	109	106	104	9.4	8.7	7.3	12.2	11.3	8.8	546	592	12.5	23
Celecoxib	0.997	2.5–50	5.0	10.0	15.0	89.5	81.2	90.3	18.2	15.6	19.7	23.7	20.3	25.6	6.66	8.33	2.5	41
Diclofenac	0.992	1.25–20	2.5	5.0 ^a	7.5	91.4	89.3	87.4	7.0	5.7	7.3	9.1	7.4	9.5	5.30	5.61	1.25	15
Firocoxib	0.999	2.5–50	5.0	10.0 ^a	15.0	103	105	109	10.8	9.3	10.1	14.0	12.1	13.1	10.99	11.98	2.5	24
Flufenamic acid	0.990	2.5–50	5.0	10.0	15.0	36.1	48.3	46.1	18.0	16.5	12.1	23.4	21.5	15.7	6.76	8.52	2.5	43
Flunixin	0.994	2.5–50	5.0	10.0 ^a	15.0	96.2	98.3	103	9.3	6.3	3.2	12.1	8.2	4.2	10.6	11.3	2.5	16
Flurbiprofen	0.994	2.5–50	5.0	10.0	15.0	87.2	98.3	105	4.4	6.2	3.6	5.7	7.4	7.1	5.61	6.22	2.5	15
Ibuprofen	0.991	2.5–50	5.0	10.0	15.0	87.5	95.1	97.5	8.3	4.2	6.5	10.8	5.5	11.1	5.45	5.90	2.5	11
Indomethacin	0.992	2.5–50	5.0	10.0	15.0	84.5	87.5	94.6	6.2	10.4	7.4	8.1	13.5	9.6	6.11	7.22	2.5	27
Mefenamic acid	0.996	2.5–50	5.0	10.0 ^b	15.0	84.1	91.7	93.4	12.5	5.9	8.1	16.3	9.4	10.5	5.77	6.55	2.5	19
Meloxicam	0.999	5–100	10	20 ^a	30	90.7	100	99.4	6.5	7.8	5.4	8.5	10.1	9.7	21.6	23.3	5	20
Naproxen	0.995	2.5–50	5.0	10.0 ^b	15.0	102	86.5	87.5	6.2	6.7	5.3	8.1	8.7	6.9	5.71	6.43	2.5	17
Niflumic acid	0.992	2.5–50	5.0	10.0 ^b	15.0	61.1	67.5	66.7	6.9	7.0	8.9	11.7	9.8	11.6	5.80	6.61	2.5	20
Oxyphenbutazone	0.991	1.25–20	2.5	5.0 ^b	7.5	93.4	96.5	84.7	19.6	12.7	9.0	25.5	19.1	11.7	3.28	4.06	1.25	38
Phenylbutazone	0.994	1.25–20	2.5	5.0 ^b	7.5	96.6	92.5	91.7	7.1	14.7	10.3	14.2	20.6	13.4	3.34	4.19	1.25	41
Tolfenamic acid	0.998	12.5–200	25	50 ^a	75	85.5	82.5	87.7	5.5	5.5	9.6	7.7	8.8	13.4	53.6	57.2	12.5	17
Corticosteroids																		
Betamethasone	0.992	0.18–3.0	0.37	0.75 ^a	1.12	85.6	88.9	100	8.4	4.4	5.3	11.8	8.8	7.4	0.803	0.86	0.18	18
Dexamethasone	0.991	0.18–3.0	0.37	0.75 ^a	1.12	87.6	88.9	98.5	6.7	5.2	6.4	11.3	10.4	11.9	0.813	0.88	0.18	21
Flumethasone	0.996	0.18–3	5.0	10.0	15.0	93.3	103	107	9.1	11.1	14.0	11.8	22.2	19.6	11.82	13.64	2.5	44
Methylprednisolone	0.998	2.5–50	5.0	10.0 ^a	15.0	87.2	88.6	97.5	6.2	6.5	6.7	8.1	11.7	11.4	10.96	11.92	2.5	23
Prednisolone	0.999	1.0–10	2	4.0 ^a	6	76.5	75.6	82.7	7.4	9.4	8.4	9.6	16.5	12.6	4.54	5.09	1.0	33

CV% coefficient of variation expressed in percent, CC α detection limit, CC β detection capability, U expanded uncertainty

^a Maximum residue limit (for bovines)

^b Recommended concentration (EURL requirements)

^c Accuracy was calculated with internal standard correction

verified by the post-extraction addition method. Blank muscle samples (bovine, swine, horse muscles, each of $n=6$) were spiked with standard solution at the spiking level II (Table 2) at the end of the analytical procedure (just before final evaporation) and determined. The ratios of analytes' peak areas in the post-extraction samples and injected working standard solutions were calculated and expressed as "percents" (relative matrix effect). The above calculation was performed using correction for IS (IS-corrected relative matrix effect).

In the accuracy and precision study, muscle samples (previously determined as blank ones) were spiked with AIs on three levels: 0.5, 1 and 1.5 times the MRL for 4-MAA, FLU, MEL, TOL, DC, DEX, BET, PRED, CPF and MPRED. For other compounds, spiking levels were chosen by the authors considering the concentration proposed by European Union Reference Laboratories (Table 2) or provisional sensitivity of the method. For the repeatability study, three series of swine muscle were analysed under the identical conditions (six samples for each spiking level). Standard deviation (SD) and coefficient of variation (CV, %) were calculated for each level. The within-laboratory reproducibility was obtained by the analysis of two additional series (on three levels) in reproducibility conditions (another technician, two different days) of bovine and horse muscles, and overall SD and CV were calculated. The overall mean concentrations obtained in the reproducibility study were used to calculate accuracy (%). Decision limits ($CC\alpha$) and detection capability ($CC\beta$) were calculated using within-laboratory reproducibility results, according to the procedure described in the Commission Decision 2002/657/EC (European Commission 2002) and the guidance paper (CRL Guidance Paper 2007). $CC\alpha$ and $CC\beta$ were calculated according to the following equation:

$$CC\alpha = \text{MRL (or 1st spiking level)} + 1.64 \times SD_{\text{WLR}}$$

$$CC\beta = CC\alpha + 1.64 \times SD_{\text{WLR}}$$

MRL (or 1st spiking level)

– concentration equal to MRL value or 1st spiking level

SD_{WLR} – standard deviation of within

–laboratory reproducibility at MRL/1st spiking level

Limit of quantitation was established as the lowest point of matrix calibration curve. The uncertainty (U) was calculated as the ratio of coverage factor ($k=2$) and standard deviation (SD) of within-laboratory reproducibility:

$$U = k \cdot SD_{\text{within-laboratory reproducibility}}$$

and expressed in percent.

Fig. 1 Ion chromatogram (transitions used for quantification) obtained from bovine muscle sample spiked with anti-inflammatory drugs at level II (4-AA 4-aminofenazone, 4-AcAA acetylaminofenazone, 4-FAA 4-formylaminofenazone, 4-MAA 4-methylaminofenazone, PRED prednisolone, MEL meloxicam, MPRED methylprednisolone, BMT betamethasone, DEX dexamethasone, FLUM flumethasone, NAP naproxen, OPB oxyphenbutazone, FLU flunixin, NIF niflumic acid, FIRO firocoxib, FLURB flurbiprofen, CPF carprofen, DC diclofenac, IND indomethacin, PBZ phenylbutazone, FLUF flufenamic acid, IBU ibuprofen, MEF mefenamic acid, TOL tolfenamic acid, CELE celecoxib)

Methods' Comparison

The incurred material (muscle samples: pork chop, pork neck, pork shoulder) was collected from pig euthanised 24 h after administration of Dexafort™ (DEX, dose 0.6 mg kg⁻¹, b.w., I.V.) The experiment was carried out in compliance with the Decision No 2/2012 of the Local Animal Experimentation Ethics Committee in Lublin, Poland.

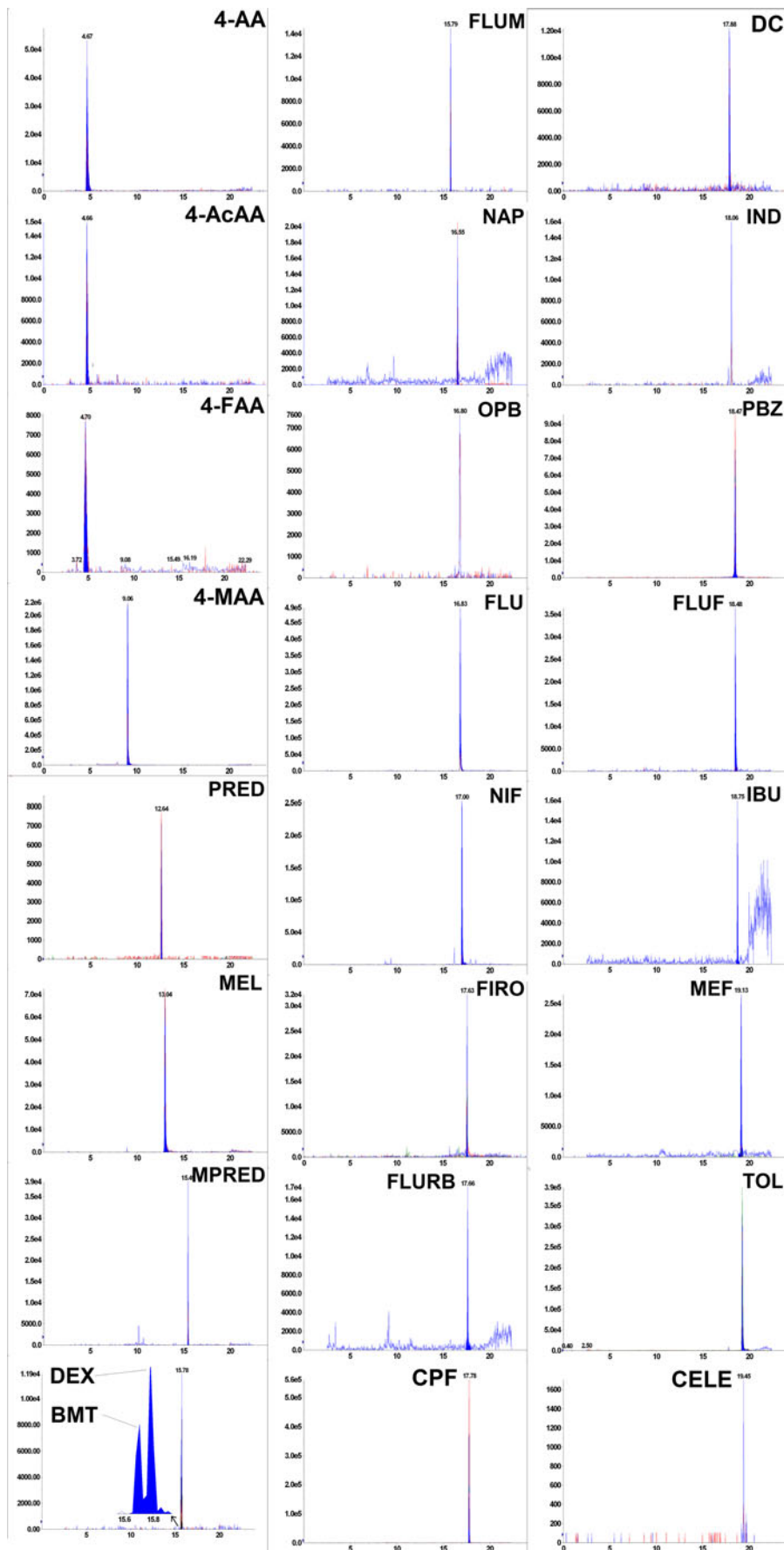
The collected muscle samples (pork chop, pork neck, pork shoulder, each of $n=3$) were analysed with the described procedure. The muscle samples were also analysed according to the procedure for determination of corticosteroids (Van den Hauwe et al. 2002).

Results and Discussion

LC-MS/MS Conditions

During the development of the method, we used our previous experiences with the determination of NSAIDs in milk, where negative ionisation was more suitable for most of acidic NSAIDs and positive ionisation showed better signal to noise values for metamizole metabolites, coxibs and some NSAIDs like DC (Jedziniak et al. 2012). GCs also can be detected in both polarities, but we decided to determine stable negatively ionised formic adducts of the analytes $[M+\text{formate}]^-$ according to the method published by Van de Hauwe et al. (2002). Although formic acid seems to be a proper choice as an additive in mobile phase for acidic analytes, it was not suitable in the case of metamizole metabolites (wide and tailing peaks). Thus, a composition of the mobile phase (MeOH/ACN and 0.05 M ammonium formate solution at pH 5.0) was a compromise between peak shape, sensitivity and time of analysis.

The separation of DEX and BMT (two isomers with the same MRM transitions) was an additional challenge. In recent publications, two strategies were observed: first, separation with carbon-loaded column (Li et al. 2010) or C18 column with relatively long runtimes (Chen et al. 2010). Shorter separation time can be achieved with UHPLC systems (Herrero et al. 2012; Tölgyesi et al. 2012; Deceuninck et al. 2013). Our early experiences show that C18 or C8 columns (Intersil from GLScience, Luna and Kinetex from Phenomenex, Halo from



AMT) in classic MS format (150×2.1 mm, $3 \mu\text{m}$) and different gradient profiles separate both compounds insufficiently with described mobile phase and gradient. For this reason, we adopted wider column (150×4.6 mm, $3 \mu\text{m}$) with higher flow rate (0.8 mL min^{-1}) according to the experience of Malone et al. (2009) (Fig. 1).

Sample Preparation Development—Enzymatic Hydrolysis

The inclusion of deconjugation step, i.e. enzymatic or chemical hydrolysis, was an important assumption of the method.

Although some authors use acidic (Chrusch et al. 2008) or alkaline hydrolysis (Chen et al. 2010; Kaufmann et al. 2014), in most published methods, enzymatic hydrolysis with beta-glucuronidase was used (Jedziniak et al. 2010; Van den Hauwe et al. 2002). An important advantage of using chemical hydrolysis is the effectiveness of the reaction, whereas enzymatic hydrolysis is more selective and ensures milder conditions than chemical one, what results in cleaner extracts (Wang et al. 2011). Optimisation of this step of sample preparation requires incurred material and was performed in some previous studies (Van den Hauwe et al. 2002). For DEX deconjugation step in liver samples, 4-h enzymatic hydrolysis

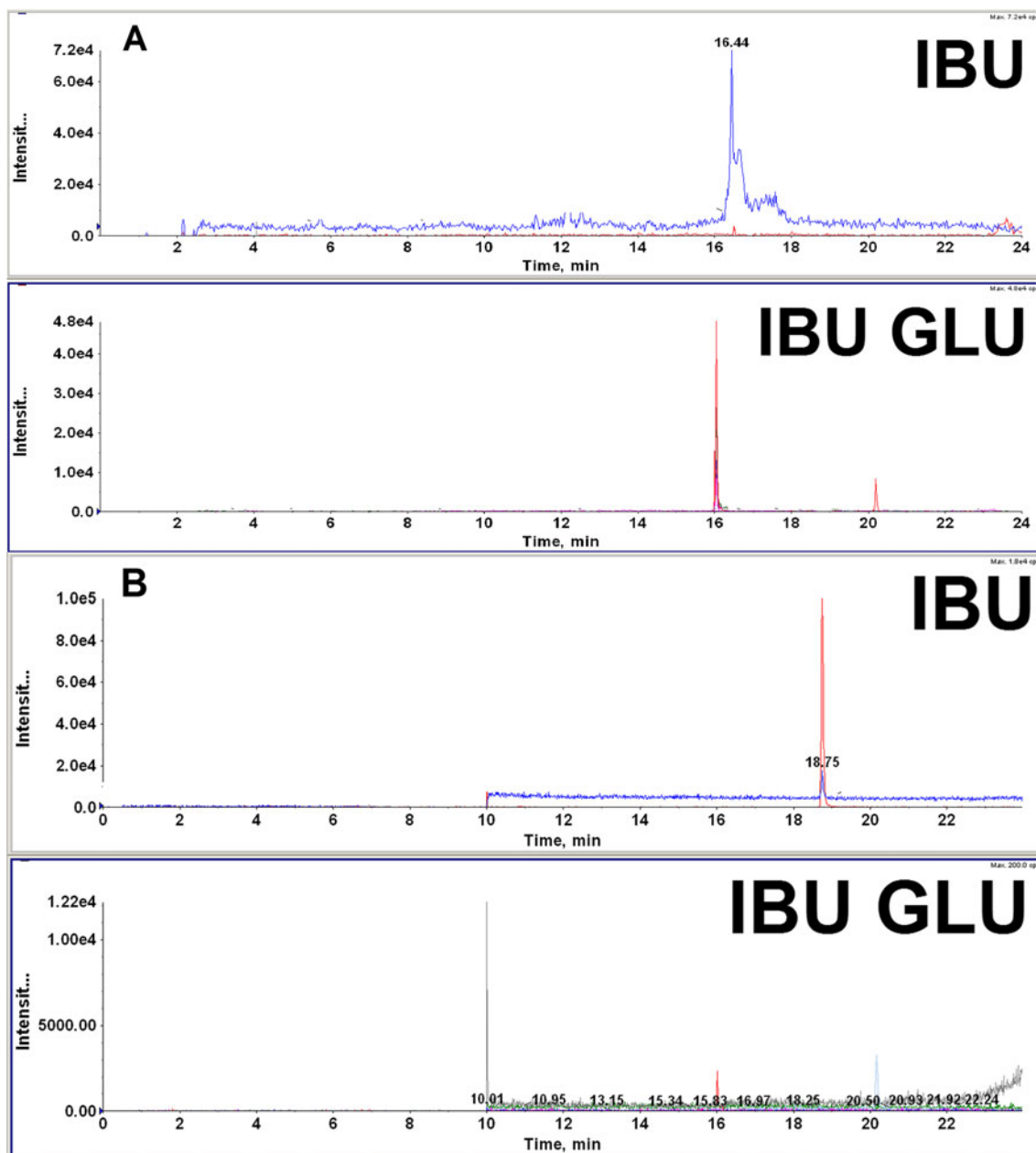
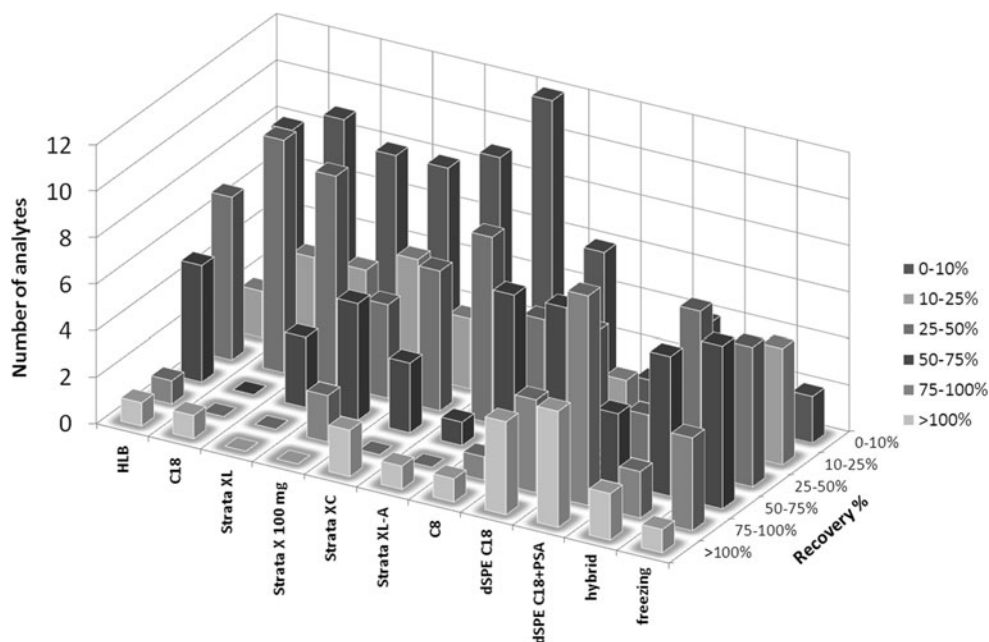


Fig. 2 Ion chromatogram obtained from swine muscle samples spiked with ibuprofen glucuronide (IBU-GLU): **a** sample preparation without enzymatic hydrolysis and **b** sample preparation with enzymatic hydrolysis

Fig. 3 Analytes' extraction recoveries using different sample preparations (different sorbents and freezing used as clean-up step). Recovery rate was calculated without internal standard correction



at pH of 5.2 and at 40 °C was necessary. In our procedure, the time of hydrolysis was shortened to 15 min at 37 °C using ultrasonication (Alvarez-Sánchez et al. 2009). Verification of hydrolysis efficiency was performed with the analysis of blank samples spiked with IBU-GLU. As a result, IBU presence was confirmed and reduction of IBU-GLU peak area (99 %) was observed (Fig. 2).

Sample Preparation Development

An important drawback of enzymatic hydrolysis is the increase in matrix constituents that can interfere with analytes,

requiring a more effective clean-up (Wang et al. 2011). Although SPE was widely used in the NSAID and GC analysis (Antignac et al. 2001; Gentili et al. 2012), this kind of clean-up (loading sample, cleaning solvent and elution) was not verified in this study due to longer time of sample preparation and poor retention of metamizole metabolites on the reversed-phase sorbents (Jedziniak et al. 2012). SPE cartridges (C18, C8, Oasis HLB, Strata X, Strata X-C, Strata XL, Strata XL-A, HybridSPE) were used only as the filter of ACN extract (the extract was passed through the cartridge). Additionally, the effectiveness of dispersive SPE with C18 and PSA sorbent was evaluated. The results (Fig. 3) show relatively wide range

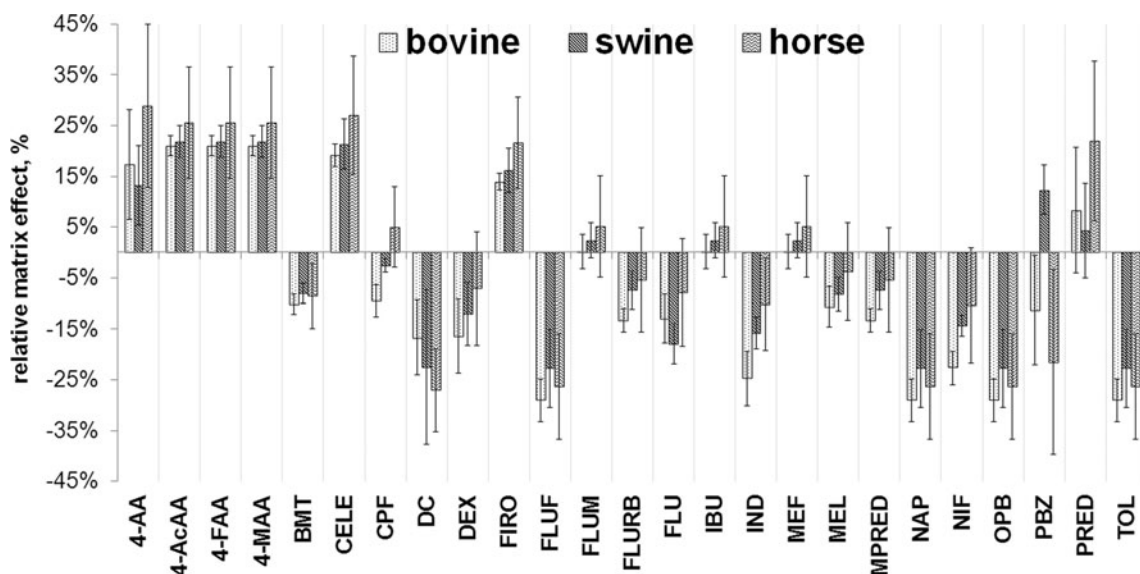


Fig. 4 Internal standard-corrected matrix effect in bovine, swine and horse muscle samples (mean and standard deviation)

Table 3 Results of the muscle sample analysis collected from the pigs treated with anti-inflammatory drugs

Medicine used	Analyte	Developed procedure ($\mu\text{g kg}^{-1}$, $n=2$, mean \pm SD)			Van den Hauwe et al. (2002) ($\mu\text{g kg}^{-1}$, $n=2$, mean \pm SD)		
		Pork chop	Pork neck	Pork shoulder	Pork chop	Pork neck	Pork shoulder
Dexafort™ (dexamethasone, dose 0.6 mg/kg, b.w., I.V.)	Dexamethasone	0.24 \pm 0.07	0.21 \pm 0.03	0.37 \pm 0.03	0.39 \pm 0.03	0.29 \pm 0.04	0.36 \pm 0.02

of analytes' recoveries (extraction recoveries) for different cartridge/sorbent combinations. This observation confirms our experience that SPE or dSPE clean-up did not guarantee high recoveries for most analytes. Moreover, we also observed that a freezing step (1 h, $-18\text{ }^{\circ}\text{C}$) gives comparable recoveries to procedures with clean-up based on SPE or dispersive SPE (Fig. 3). Due to the simplicity of the sample preparation and its effectiveness, we decided to involve this step into the final procedure.

Validation

During verification of the selectivity of the method, we did not observe interference peaks in the retention time of analytes in analysed muscle samples. Nowadays, calculation of a matrix effect became the important part of validation in the method based on LC-MS/MS technique. Although performance criteria for this parameter were not established, many authors present ion suppression (or rarely ion enhancement) of developed methods. The results of the matrix effect study are presented in Fig. 4.

The obtained results show significant reduction of all analytes' peak areas (ion suppression). The calculated relative matrix effect was in the range of 28–77 %. The use of isotope-labelled ISs reduced the influence of this factor in quantitation: IS-corrected relative matrix effect was in the range -29 – 30 % for all analytes with CV in the range 1–20 %.

The linearity of the method (correlation coefficient) and range of the method were on the acceptable level. The validation results show high accuracy for most analytes, in the range of 75–18 %. The lower accuracy was noticed for NIF (61–67 %) and FLUF (36–46 %) and slightly higher AcAA (100–124 %). The reason for this could be the lack of the proper labelled IS. The results of repeatability (CV=3.2–19.6 %) and within-laboratory reproducibility (CV=4.2–25.6 %) study show acceptable precision of determination of most compounds. Calculated $\text{CC}\alpha$ and $\text{CC}\beta$ values were slightly higher for MRL compounds and lower than recommended concentrations for those NSAIDs which are non-registered for food animals (Table 2). The results of validation suggest that, in the routine analyses, the use of matrix-matched calibration curves is preferable.

Method's Verification

Muscle samples taken from pig (pork chop, pork neck, pork shoulder) were analysed by the developed procedure with ultrasonic-assisted enzymatic hydrolysis with beta-glucuronidase (15 min, $37\text{ }^{\circ}\text{C}$), and the results were compared with those obtained by analysing the muscle samples with procedure making use of 4-h enzymatic hydrolysis at $40\text{ }^{\circ}\text{C}$ (Table 3). The results of analyses were comparable (t test, $P>0.05$) and show efficiency of the developed procedure with determination of AI residues in incurred material.

The developed procedure was successfully verified in the FAPAS proficiency test (PBZ in equine muscle, nr 02231). The calculated z -score for results ($4.67\text{ }\mu\text{g kg}^{-1}$ of PBZ) was in the range $-2<z<2$ (z -score=0.0).

Conclusions

The authors presented fast multiresidue method for the determination of AIs in animal muscles. It allows to determine a wide range (25) of NSAIDs and GCs in muscles on the levels required by the law and recommendation of European Union Reference Laboratory. An important advantage of the developed method is short time of enzymatic hydrolysis step and a simple sample clean-up. The results of method validation show its applicability as a screening and confirmatory procedure.

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Compliance with Ethics Requirements

Conflict of Interest Piotr Jedziniak declares that he has no conflict of interest.

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Małgorzata Olejnik declares that she has no conflict of interest.

Konrad Pietruk declares that he has no conflict of interest.

Edyta Protasiuk declares that she has no conflict of interest.

Teresa Szprengier-Juskiewicz declares that she has no conflict of interest.

Jan Żmudzki declares that he has no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed. The animal study described in the paper was carried out in compliance with the Decision No 2/2012 of the Local Animal Experimentation Ethics Committee in Lublin, Poland.

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