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Towards high throughput analysis using 96-well plate solid phase extraction to determine sedatives and β-blocker residues in food control monitoring

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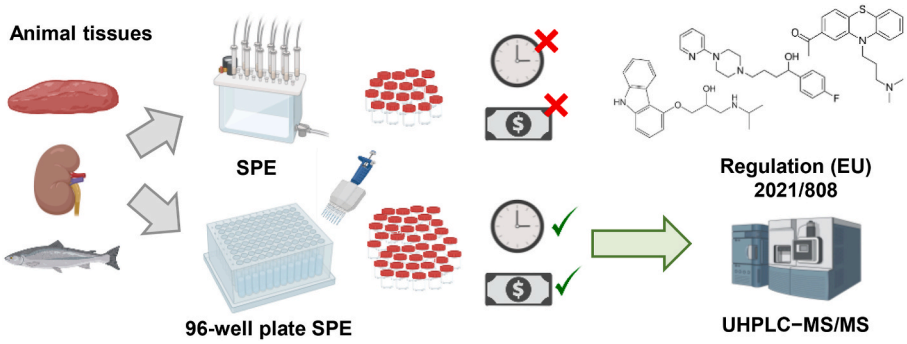
HIGHLIGHTS

- Validated SPE method for kidney analysis of sedatives and β-blocker was developed.
- Single matrix fortified calibration curve to quantify different animal species.
- The developed method has been successfully extended to meat and fish samples.
- 96-well plate format permit future automation of sample preparation.
- Method is implemented in the National Control Monitoring Plan for food residues.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Veterinary drugs are widely used in animal production to prevent infections and treat diseases but, this may cause a risk to consumers. Due to the high number of food samples required to monitor yearly, simple, fast, sensitive and selective analytical methods are needed in control laboratories to ensure consumers safety. Nevertheless, many analytical methodologies available in these laboratories include multiple steps and therefore are time-consuming and hinder the analysis throughput requiring significant amounts of solvents and reagents.
Results: This work developed a 96-well plate solid phase extraction (SPE) method for the extraction of seven sedatives and a β-blocker in animal kidney by ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS). The developed method was validated based on Implementing Regulation (EU) 2021/808 in kidney, meat and fish. The performance characteristics of the validation (1–5 μg kg^{-1}) showed good linearity ($R^2 > 0.998$) while decision limits (CCα) were between 1 and 1.2 μg kg^{-1}. Trueness and precision were determined at three levels (n = 7) and the results showed values ranging from 85 to 103 % and from 1 to 9 %, respectively. The feasibility of the method was demonstrated for the residue control requirement established by EU. Method was applied to 201 samples of kidney, meat and fish.
Significance: This study is the first to present an optimized and validated holistic method for sedatives and β-blocker using 96-well plate SPE and UHPLC–MS/MS. The method showed good performance in kidney, meat

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and fish samples being an universal method for any species along the same type of sample. The fast, easy, efficient, reliable and universal method showed high throughput and so reduced the analysis time by nine-fold and the required solvent amount by four times fulfilling the green chemistry principals.

1. Introduction

The use of veterinary drugs in livestock animals may cause a risk to consumers. Currently, veterinary drugs are widely used in animal production to prevent for example infections or treat diseases and promote increases in feed conversion ratios [1]. To ensure the safety of consumers, farmers must stop the administration of veterinary drugs before animals are slaughtered [1,2]. Therefore, the European Commission established Maximum Residue Levels (MRL) for several veterinary drugs in a broad range of animal tissues [3]. In modern farming, sedatives and β-blockers are frequently used to reduce stress during the transportation of food producing animals. The risk of residues in edible tissues is higher than other veterinary drugs because these sedatives and β-blockers are frequently used just a few hours before slaughter. Therefore, their use can lead considerable residue concentrations in animal tissues and cause possible health hazards to consumers [1,4]. As a result, monitoring the concentrations of sedatives and β-blockers in animal tissues is vital for exposure risk assessment and therefore, to ensure citizens safety. In control laboratories, mainly, the workflow for the analysis of samples include sampling, transportation and sample preparation procedures before the subsequent determination of target compounds using chromatographic separation techniques with different detection systems.

In the literature, target compounds have been determined by liquid chromatography (LC) using reversed-phase chromatography and spectrophotometric detection (LC/UV–Vis) [4–6]. However, the most frequently used technique for their determination is liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) since tandem mass spectrometry allows to overcome limitations observed on other techniques providing high sensitivity, selectivity, and structural information for the identification, confirmation, and characterization of analytes in complex matrices at low concentration levels [7–10]. Most of these LC–MS/MS methods are used to determine target compounds in animal kidney [5,11,12] and/or muscle [6,13,14] with few exceptions in liver [15], milk [14] and eggs [4]. Generally, protocol is based on tissue extraction using organic solvents (mainly acetonitrile) followed by clean-up procedures such as solid phase extraction (SPE) using hydrophilic and hydrophobic balance (HLB) [7], ion exchange NH_2 [10] and hybrid cation exchange reversed-phase sorbent (MCX) [5] cartridges. Other authors, such as Delahaut et al. [16] and Mitrowska et al. [8], avoided the use of SPE and extracted the compounds directly with a solid-liquid extraction with 20 mL of acetonitrile followed by a centrifugation process. Although in the last decade the analytical methods have taken advantage of improvements to simplify sample treatment and separation steps [17,18], many of these procedures continue to be challenging. The extraction methods are time-consuming and hinder the analysis throughput requiring significant amounts of solvents and reagents. Therefore, it is well known that the main bottleneck of the analytical methodologies in control laboratories is still the sample preparation step.

Herein, the present work aimed to develop a new extraction method with 96-well plate SPE to reduce the amount of solvents and reagents, as well as to improve the high throughput of the analyses. In principle, the extraction method was optimized on both porcine and bovine kidney samples since these are the two main species analyzed in the Dutch National Residue Control Plan. Based on extraction efficiencies, a comparison of different extraction solvents as well as the performance of the ion exchange 96-well plate SPE with different eluents has been carried out in both porcine and bovine kidney. The developed ion exchange 96-well plate SPE-LC–MS/MS method was first validated based on Implementing Regulation (EU) 2021/808 in porcine, bovine, poultry, sheep and goat kidney and at later stage evaluated for its use in bovine, poultry and porcine meat and salmon fish samples.

2. Materials and methods

2.1. Chemicals and reagents

Ultra LC–MS grade water and methanol were purchased from Actu-All Chemicals (Oss, The Netherlands) while acetonitrile from Biosolve (Dieuze, France). Milli-Q water was prepared using a Milli-Q system at a resistance of at least 18.2 MΩ cm (Millipore, Billerica, MA, USA). Ethanol (100 %) and Ammonia (25 %) were purchased from Merck (Darmstadt, Germany) and ammonium acetate from Sigma Aldrich (Misuri, USA). A 0.05 M ammonium acetate solution was prepared by dissolving 0.96 g of ammonium acetate in 250 mL of Milli-Q water, this solution is best to prepare fresh for every analysis. Cation exchange (CX) EVOLUTE® EXPRESS CX 60 mg Fixed Well Plates were purchased from Biotage (Uppsala, Sweden) and Beads 2.3 mm zirconia/silica from BioSpec Products (Bartlesville, Oklahoma, USA).

2.2. Reference standards

Azaperol (APL), Azaperon (APN), Propionylpromazine hydrochloride (PrPN), xylazine (XyN) and carazolol (CZL) were purchased from Dr. Ehrenstorfer™ (LGC Standards GmbH (Wesel, Germany). Acepromazine maleate salt (AcPN), Haloperidol (HPL) and Chlorpromazine hydrochloride (ClPN) were purchased from Sigma Aldrich (St. Louis, MO, USA). Azaperol-d4 (APL-d4), Azaperon-d4 (APN-d4), Propionylpromazine-d6 hydrochloride (PrPN-d6), Acetopromazine-d6 (AcPN-d6), Chlorpromazine-d6 hydrochloride (ClPN-d6) and Carazolol-d7 (CZL-d7) were purchased from Witega (Berlin, Germany) whereas Xylazine-d6 (XyN-d6) from Honeywell Research Chemicals (Morris Plains, New Jersey) and Haloperidol-d4 (HPL-d4) from Cambridge Isotope Laboratories (Tewksbury, Massachusetts, USA). Chemical structure, formula and the acronym of each target compound are represented in Fig. 1.

Individual stock standard solutions of APL, ClPN and HPL were prepared in methanol whereas APN, AcPN, APN, CZL, PrPN and XyN in ethanol at a concentration of 1000 mg L^{-1}. Intermediate standard mixture at 10 mg L^{-1} and 100 μg L^{-1} containing all the target compounds were prepared from stock standard solutions and intermediate standard mixture (10 mg L^{-1}), respectively by appropriate dilution in ethanol. Working standard solutions (1,2,3,4,5 μg L^{-1}) were prepared by corresponding dilution from intermediate standard mixture solutions. All internal standard stock solutions were prepared in ethanol at 1000 mg L^{-1} and intermediate internal standard mixture were prepared at 10 mg L^{-1} and 200 μg L^{-1} by corresponding dilution in ethanol. All these standard solutions were stored at −20 °C until their use.

2.3. Sample extraction

Samples (1 g) were weight in a bead ruptor tube and fortified with 20 μL internal standard mixture (200 μg L^{-1}), vortexed for 1 min and let rest during 5 min. For matrix matched calibration curves (MFS line), in addition to the internal standard mixture, adequate standard mixture solution (100 μg L^{-1}) was added to obtain a calibration line ranging from 1 to 4 μg kg^{-1}. To follow up, 2 mL of acetonitrile were added to all tubes and they were placed in a bead ruptor system (Omni International, Bead Ruptor 24) applying program 10 (Speed 5.65s; number of cycles 2; cycle time 0.45s; break between cycles 0.45s). Samples were centrifuged

during 5 min at 3200 g and the supernatant was extracted in an Eppendorf low bind 96-well plate collection plate (Waters, Milford, Massachusetts, USA). At this stage, 900 μL ammonium acetate (0.05 M pH 5–6.5) were added to each well in the plate and mixed with a 96-well mixer during 5 min at low speed. The pH of the extract were checked to be lower than pH 7. Once the pH was ensured, the samples were extracted by 96-well plate solid phase extraction (SPE, Biotage Evolute Express CX). Prior to extraction, well plates were preconditioned with 1 mL methanol followed by 1 mL ammonium acetate (0.05 M). 1 mL of the prepared sample were transferred to individual wells using a 12-chanel pipet. After loading samples, vacuum was applied to pull the supernatant through (waste) at 3 psi pressure and this procedure was repeated until the rest of the prepared sample is loaded on the SPE. Prior to elution, the wells were washed by 1 mL ammonium acetate (0.05 M) followed by 1 mL methanol. For elution, the SPE plate was placed on a new 2 mL 96-well plate collection plate and samples were eluted with 1 mL methanol containing 2 % of ammonium hydroxide. The eluates were evaporated to dryness under nitrogen at 45 mL min^{-1} flow rate and 55 °C and reconstituted in 150 μL water:acetonitrile (3:2, *v/v*). Collection plate was vortexed to dissolve all the extract and stored at 4 °C until its analysis. Finally, 10 μL of the final extract was injected into the UHPLC−MS/MS system.

2.4. Optimization of sample extraction and study of matrix effects

Method has been developed and optimized based on extraction recoveries (EE%) in three replicates. EE% has been calculated by comparing the mean measured peak areas with a spiked blank kidney of porcine and bovine and that obtained from standard mixtures prepared in the same solvent and at the same concentration level. Additionally, matrix effect (ME, %) in the ionization process was estimated for each compound from the relative difference between the peak area observed in the analysis of the spiked blank extract and that obtained from standard mixtures prepared in the mobile phase at the same concentration level.

2.5. LC−MS instrumentation and working conditions

The chromatographic separation of target compounds was performed on an ACQUIRY UHPLC I-Class System equipped with an Ebara EV-SA30-2 pump, an Acquity autosampler and a column oven (Waters, Milford, Massachusetts, USA). An Acquity UPLC BEH C18 (100 mm × 1.0 mm i.d., 1.7 μm particle size) was used as analytical column in the developed method. The UHPLC system was coupled to Xevo TQS (Waters) mass spectrometer equipped with a triple quadrupole mass analyzer and an electrospray ionization (ESI) source.

The chromatographic separation was carried out with 0.1 % ammonia in Milli-Q (solvent A) and acetonitrile (solvent B) as mobile phase components. The gradient elution program was as follows: 0–5.00, linear gradient elution from 30 to 80 % solvent B; 5–5.1, linear gradient elution from 80 to 100 % solvent B; 5.1–8.9 isocratic conditions at 100 % solvent B and 8.9–9 linear gradient elution to return to initial conditions at 30 % Solvent B. The flow-rate of the mobile phase was 500 μL min^{-1}, injection volume was 10 μL, and column oven and sample track temperature were held at 65 and 12 °C, respectively during the chromatographic run of 10 min.

Ionization source working conditions were as follows: Source and desolvation temperature were set at 130 °C and 550 °C, respectively; ESI spray voltage at 3.2 kV and source offset at 35V for all compounds except for ClPN at 50V. Nitrogen used as desolvation and cone gas at a flow rate of 800 and 150 L h^{-1}, respectively. The mass spectral data were acquired in selected reaction monitoring mode (SRM) in positive ion mode and both quadrupoles (Q1 and Q3) operated at 1 *m/z* full width half maximum (FWHM) with a scan width of 1 *m/z*. Argon (≥99.995 %) was used as a collision induced dissociation (CID) gas at a pressure of 0.18 mL min^{-1} in the collision cell (Q2). Table S1 summarizes the multiple reaction monitoring (SRM) working conditions used: the selected precursor-product ion transitions for quantitation and confirmation purposes, relatively, the optimum collision energies (CEs, eV) for the selected transitions and optimal cone voltages.

2.6. Method validation

Regulation (EU) (2021/808) has been implemented to validate the developed method concerning the performance of the analytical methods and interpretation of results [19]. Method quantification was based on peak area and was performed using internal standard calibration curve obtained from analyzing matrix matched calibration of porcine, bovine, equine, sheep and goat kidneys. Following Regulation (EU) (2021/808) [19], complete validation (three days) has been carried out in porcine kidney while partial validation (one day) in bovine and minor species which involve equine, sheep and goat kidney, bovine, porcine and poultry meat and flex scope in salmon fish.

For identification of the target compounds, 5 identification points are used: (1) in LC, the relative retention time (RRT) expressed as a ratio between the retention time of the analyte and the retention time of the internal standard; (2) SRM quantitation transition; (3) SRM confirmation transition; (4) the relative deviation of the relative retention time,

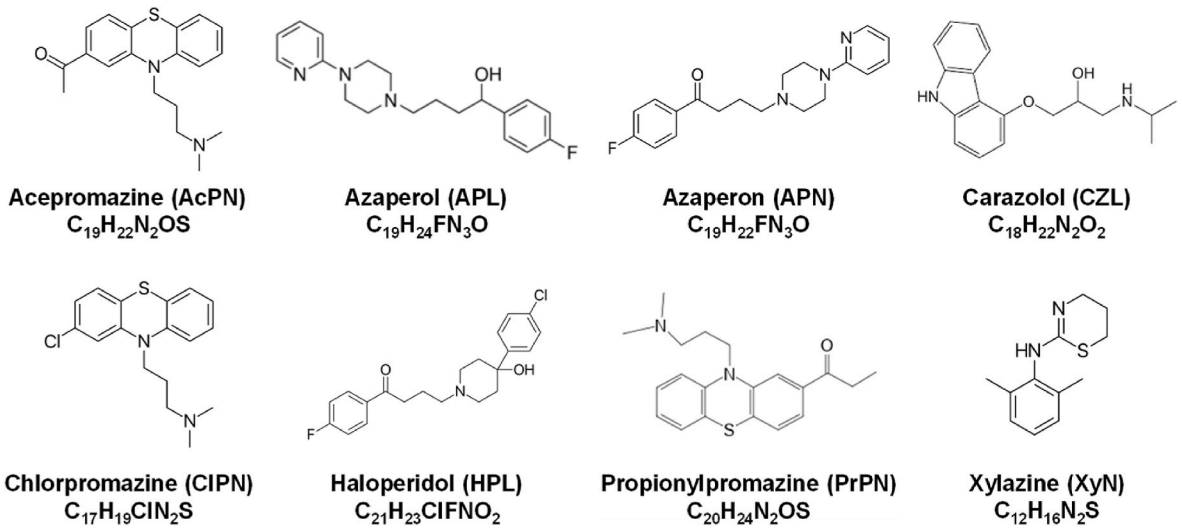

Fig. 1. Chemical structures, acronyms and chemical formula of the studied sedatives and β-blocker.

based on an average of the MFS standards, which should be ≤ 1 % and (5) the relative deviation of the ion ratio based on the ion ratio of the spikes of the MFS calibration line shall be ≤ 40 %.

The selectivity/specificity of the developed method was assessed by analyzing 7 blank samples of different porcine, bovine, equine, sheep and goat kidneys directly in the chromatograms obtained from the blank and spiked kidney samples. The occurrence of possible extra-peaks in the retention time window expected for the analyte elution was tested by monitoring the selected two SRM transitions for each target compound onto the blank matrix chromatograms. For MFS line, blank kidney samples have been spiked with working standard solutions at five levels corresponding to 0, 1, 2, 3 and 4 μg kg^{-1}. The spikes of the MFS line are injected both at the beginning and at the end of the series. Based on these injections, linearity is calculated using the least squares method. To fullfill the established criteria, the correlation coefficient (R^2) of the MFS line before and after must be ≥ 0.9800.

The accuracy and repeatability were determined by fortifying 7 blank kidney samples at three validation levels: 1,2,3 μg kg^{-1}. Samples were analyzed on the same day with the same instruments and same operators. The validation study shall be conducted under intra-laboratory reproducibility conditions at LCL* 1,2,3 levels. Therefore, for full validation in porcine, intra-laboratory reproducibility was calculated based on the mean repeatability obtained during three days in different instruments and different operators while for partial validation, the repeatability was multiplied by 1.5. The decision limit (CCα) was calculated by fortifying 21 samples at the LCL level and according to section 2.6 in Regulation (EU) (2021/808) [19].

The stability of target compounds in the extract is determined by storing the MFS line and the 7 samples at 1 xLCL level in the freezer after the first validation day. On the second validation day, these samples are re-evaluated and the following criteria must be fulfilled: (1) the criteria for accuracy and repeatability; (2) the identity of 6 of the 7 samples must be confirmed; (3) the signal-to-noise ratio of the product ions shall be ≥ 3.

3. Results and discussion

3.1. Optimization of Liquid chromatography-tandem mass spectrometry

In this study, the chromatographic separation of seven sedatives and a β-blocker (Fig. 1) was carried out in a reversed-phase UHPLC (BEH C18) column, since it offers the most universal column choice due to its trifunctional BEH particles that enable to work at a range of pH 1–12. The target compounds have pKa values ranging from 6.8 to 9.6, and therefore these characteristics indicate that mobile phase components with pH values at least close to 11 should be used to favor the formation of neutral species of the target compound and therefore increase the interaction with the stationary phase. Some authors have proposed the use of phosphate buffer and acetonitrile [6] as mobile phase components. However, it is well-known that these low volatile mobile phases can produce the contamination of the ion source in mass spectrometry and hinder the ionization of analytes, so this strategy was discarded in this study. As alternative, following the work published by Kaufmann et al. [13] the use of ammonium hydroxide (NH_4OH) was evaluated because ammonia containing mobile phases produce stronger positive ESI response of analytes than acidic mobile phases like formic acid owing to their high basicity properties and the proton transfer possibilities from the ammonia. In this work authors used a 150 mm length and 5 μm particle size column whereas in the present work, the length of the chosen analytical column was 10 mm and the particle size 1.7 μm and therefore it was intended to take advantage of the ultra-high performance provided by this column technology that should allow a highly efficient chromatographic separation and short analysis time.

Several mobile phases and grandient elution programs were tested to optimize the chromatographic separation of sedatives and β-blocker. The use of ammonium hydroxide and ammonia in mobile phase component A were evaluated, and best results in terms of chromatographic resolution were observed when using 0.1 % ammonia. Therefore, 0.1 % of ammonia in water (A) and acetonitrile (B) were selected as mobile phase components in gradient elution mode as described in section 2.4. The indicated conditions allowed to separate the seven sedatives and a β-blocker in less than 9 min, obtaining good peak shapes and baseline resolution except for XyN and CZL, which partially coeluted. Despite the possibility to separate these two compounds by mass spectrometry based on their *m/z* value differences, the signal enhancement/suppression of them was evaluated by injecting individual solutions and a mixture of both at the same concentration. Results showed that the difference of the peak areas were lower than 10 % which were similar to the repeatability (RSD%) values indicating that the co-elution of XyN and CZL did not affect on their response.

The ultra-high performance liquid chromatography system was coupled to a triple quadrupole mass spectrometer with H-ESI source. In order to optimize the ionization behaviour of target compounds in ESI, infusion of working standard solutions of target compounds was carried out. All target compounds ionized in positive ion mode generating the protonated molecule $[M+H]^+$ as the base peak of the mass spectra in full scan mode. To improve the selectivity and sensitivity of the method and to ensure the identification and quantitative determination of target compounds, tandem mass spectrometry was evaluated by acquiring their product ion scan at collision energies between 0 and 50 eV. The corresponding product ions of each target compound were characterized and the two most selective and abundant ones were selected for quantitative and confirmatory purposes when working in multiple reaction monitoring mode. The collision energies and the selected precursor and product ions are given in Table S1.

3.2. Development of extraction technique

For the analysis of sedatives and β-blockers in animal tissue, SPE is the extraction technique mostly used [5,13,15,20]. However, 5–10 g of sample is usually needed while the amount of solvent used for the extraction and clean-up process can reach up to 80 mL and the time needed for homogenization and the sample preparation process is estimated to be around 8h for a batch of 40 sample analysis serie. This fact causes the slow down of the control laboratory throughput and the cost of the needed material and personal hour can be also high. To overcome this throughbacks, the miniaturized SPEs in 96-well plate format that allows rapid extraction and clean up in a single step with lower solvent conμsumption could be very useful. Therefore, the transition from the available SPE methodology based on cation exchange (CX) cartridges used in the food control laboratory of Wageningen to 96-well plate SPE CX has been studied in the present work. To this end, the type and quantity of extraction solvent, the homogenization strategy and the washing solvent employed in the SPE have been optimized on both porcine and bovine samples because these are the two main analyzed species in the Dutch National Residue Control Plan.

Because the standards and internal standards are prepared in ethanol, and larger quantities are used in the initial methodology, it is suspected that the presence of ethanol may influence the signal response of the analytes in the sample. Therefore, the effect of the addition of a small amount (10 %) of ethanol in the extraction solvent (acetonitrile) was evaluated. As can be seen in Fig. 2A, the recovery of the target compounds is greater when there is no presence of ethanol except for PrPN where the recovery is 5 % lower. Therefore, as a compromise for the best performance of target compounds, acetonitrile is selected as the extraction solvent in the initial phase of the extraction protocol. These findings were in accordance with other authors that use also pure acetonitrile to extract some of the present analytes in muscle [7,13], kidney [14,16] and liver [15] matrices. Once the extracting solvent was selected, it was of great importance to evaluate the applied homogenization system since the amount of extraction solvent used in the biological sample was reduced from 5 mL to 1 mL. It must be mentioned

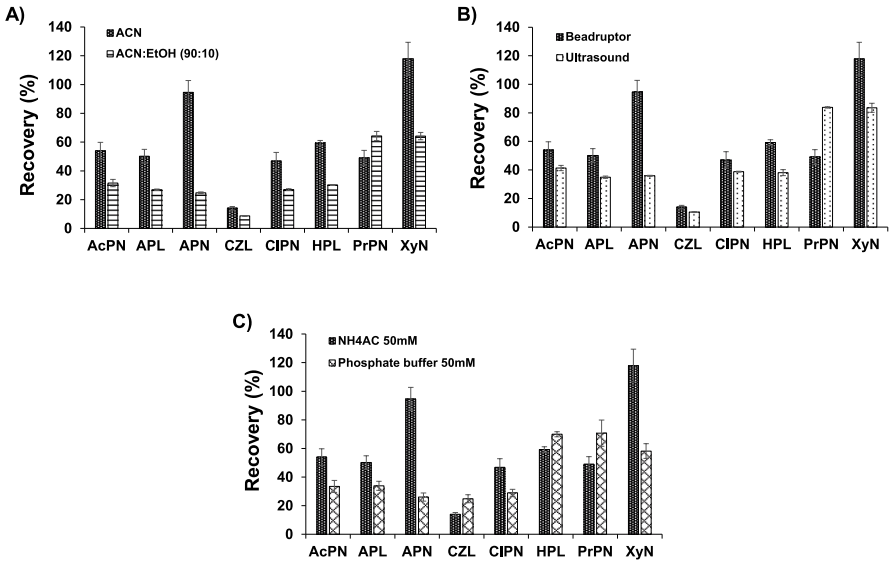

Fig. 2. Recoveries of target compound using different (A) extraction solvent: acetonitrile *vs.* acetonitrile:ethanol (90:10, *v/v*); (B) homogeneization: beadruptor *vs.* ultrasound; (C) washing solvent: NH_4Ac 50 mM *vs.* phosphate buffer 50 mM.

that ultrasound homogenization is efficient for low-throughput applications involving liquid samples with large volumes, while if you are looking for a high throughput and aim to work with small amounts of sample, the bead ruptor is recommended. In addition, if the estimated time with each process is considered, in the first case a minimum time of 5–10 min is necessary, while with the bead ruptor within 2 min, a good homogenization of the sample is achieved. Therefore, both homogenization systems were compared for the present case and as can be observed in Fig. 2B, in most cases, the obtained recoveries were different in each case and better when using the beadruptor technique. Thus, considering the better performance and its advantages as described above, the beadruptor system was selected as homogenization step for further studies. Finally, to improve the evaporation step and avoid any LC-MS system contamination due to the use of phosphate buffer as washing solvent in the clean up stage, the use of a 50 mM ammonium acetate solution was studied as alternative. In this case, as can be seen in Fig. 2C, five compounds out of eight resulted in better recoveries when using ammonium acetate (50 mM), although it should be noted that in the remaining 3 compounds (CZL, HPL and PrPN) the use of phophate buffer differs by less than 10 % on the recovery values. Thus, to simplify the method and avoid any possible instrument contamination, ammonium acetate is selected as washing solvent in the clean up step on the 96-well plate SPE. For the elution of target compounds from the 96-well plate SPE, the solvent used (methanol containing 2 % of ammonium hydroxide) on the initial methodology is maintained, although the required amount is reduced from 5 to 1 mL.

If the performance characteristics of both the SPE and 96-well plate SPE methods in terms of recoveries and matrix effect are compared, it is evident that the high recovery values (up to 230 %) in some cases with the SPE method is due to the large matrix effect that can be observed (up to 290 % in the case of PrPN). However, the results obtained with the 96-well plate method have recoveries values between 10 and 60 % with relative standard deviation values lower than 20 %. In this case, despite the presence of the matrix effect in all compounds, this value is reduced up to 75 % as it is the case of azaperol which is reduced from 130 % to 20 %. This difference can be attributed to the larger amount of sample used with the conventional SPE method, with the final extract being equally pre-concentrated to 100 μl. Thus, in the case of the SPE method, the concentration of the analyte is preconcentrated 30 times, while in the 96-well plate 6 times. Nevertheless, in order to cope the matrix effect observed in the proposed methodology with 96 wel-plate SPE format, the signal of each target compound was corrected by the corresponding internal standard and the quantification of the analytes were carried out by means of matrix matched calibration curve.

By way of comparison between the conventional SPE method and the use of 96-well plates, in terms of analysis time, it should be noted that with a regular SPE in a working day (8h) up to 40 samples can be extracted, while the later one allows 96 samples to be extracted in 2 h, so in addition to the speed and efficiency, the cost of the employee is also reduced. Besides, in terms of analysis cost with each method, it can be estimated that about 30 individual SPE cartridges have a similar cost to a 96-well plate, while the amount of solvent used also varies significantly, with 32 mL per sample required for the conventional method and 8 mL per sample when using the 96-well plate format. Therefore, this study has demonstrated how the possibility of using the 96-well plates for the extraction of target compounds in the kidney would reduce the amount of solvents and material required as well as the analysis time, improving the throughput of the control laboratory.

3.3. Method validation in kidney samples

ACPN, ClPN, HPL and PrPN are prohibited substances without a Reference Point of Action (RPA). According to the EURL Guidance Paper [3], the Minimum Method Performance Requirement (MMPR) for target compounds is set at 5 μg kg^{-1}. The other sedatives are allowed with a Maximum Residue Limit (MRL) value for APL, APN and CZL whereas XyN does not require an MRL. Therefore, the Lowest Concentration Levels (LCL) of the target compounds to be determined in this validation has been stablished as 1 μg kg^{-1} in all cases. See Table 1 for an overview of the selected target compounds in this work and their corresponding MMPR, MRL and LCL values.

3.3.1. Porcine kidney

The developed SPE-LC−MS/MS method has been fully validated as a quantitative confirmatory method in porcine kidney because it is the most analyzed animal in the National Residue Control Plan (NRCP). The following performance characteristics have been evaluated in accordance with Regulation (EU) (2021/808) [19] and ISO17025: selectivity/specificity, linearity, accuracy, repeatability, within-lab reproducibility, CCα, robustness, stability and traceability (section 2.5).

Table 1
Overview of the studies sedatives and β−blocker with their acronyms and corresponding Maximum Residue Limit (MRL), Minimum method performance requirements (MMPR) and Lowest concentration levels (LCL) values.

Compound	Acronym	MRL (μg kg^{-1})		MMPR (μg kg^{-1})	LCL (μg kg^{-1})
		porcine	bovine		
Acepromazine	AcPN			5	1
Azaperol	APL	100[a]			1
Azaperon	APN	100[a]			1
Carazolol	CZL	25	15	5	1
Chlorpromazine	ClPN				1
Haloperidol	HPL			5	1
Propionylpromazine	PrPN			5	1
Xylazine	XyN				1

[a] Is the sum of azaperon and azaperol.

The analysis of porcine kidney blank extracts did not show any interfering peaks in the retention time window expected for the analyte elution with the selected two SRM transitions for each target compound (Fig. S1). Hence, the method is selective and specific for quantification and/or confirmation of target compounds in porcine kidney tissues. The linearity of the analytical response of target compounds within the matrix fortified line (MFS) fulfilled the stablished criteria obtaining correlation coefficients (R^2) higher than 0.998 for all the compounds. Besides, the accuracy expressed as percentage (%), was determined using the corresponding blank sample spiked at three validation levels LCL x 1, x2, and x3 (described in section 2.5) and resulted always between 95 and 101 (n = 21). Furthermore, repeatability and within lab reproducibility were also studied at the three same concentration levels. The repeatability showed relative standard deviation (RSD, %) values ranging from 1.6 % to 9 % (n = 21), while within lab reproducibility results were always lower than 20 %, 24 % and 19 % (n = 21) in low, medium and high concentration levels, respectively, in all cases. The CCα of target compounds was assessed between 1.1 and 1.5 μg kg^{-1} for all target compounds, resulting always below the Minimum Method Performance Requirement (5 μg kg^{-1}) established by EURL Guidance Paper [3]. Table 2 summarizes the accuracy, repeatability, within lab reproducibility and the decision limit (CCα) of all target compounds in porcine kidney at the corresponding validation level. Additionally, the stability of the target compounds was also evaluated as described in section 2.5. All samples meet the stablished criteria and therefore it can be concluded that the analytes are stable for at least 11 days when stored in the refrigerator.

3.3.2. Bovine, horse, sheep and goat kidney

Partial validation (one day) of bovine, and minor species including caprine, equine and ovine kidney has been carried out since these species are also less frequently monitored in the NRCP. In these cases, linearity, CCα value, accuracy, repeatability, within-lab reproducibility, and specificity have been evaluated according to Regulation (EU) (2021/808) [19] and ISO17025. As in the case of porcine kidney, the performance characteristics criteria established in the legislation has also been met in the case of bovine kidney (n = 7) and caprine, equine and ovine kidney (n = 9). Considering the percentage of kidney samples of each of these species that are usually analyzed (less than 15 % for each serie), the fact of having to prepare a matrix matched calibration line for each species that is included on the control slows down the work of the laboratory. Therefore, to increase the laboratory's throughput, the possibility of applying the matrix matched calibration line of the major species (in this case porcine) for the quantitation of the target compounds was evaluated. To this end, the respond factor of each target compound in porcine kidney and in bovine and minor species was compared. As can be observed in Fig. 3 where the response factor of azaperol and carazolol at different concentration in bovine (A and B) and minor species (C and D) is represented, the regression coefficient (R^2) is greater than 0.99 in al cases, showing that the use of the matrix calibration line in porcine kidney enables to quantify the target compounds in bovine and minor species kidney samples.

Additionally, to compare the obtained results at the three validation levels with the different matrix matched calibration lines, statistical treatment of the data was performed using a two-way analysis variances (ANOVA) test. The pvalues obtained were always higher than the significance level of 0.05 (p values ranging from 0.26 to 0.94), which indicated that there were no statistically significant differences between the use of a matrix matched calibration line in porcine and bovine/minor species for the quantification of target compounds in bovine/minor species kidney samples. After the satisfactory result, a one day validation of bovine (n = 7) and minor species (n = 9) was carried out with the use of matrix matched calibration in porcine kidney obtaining results that comply with the legislation in both cases (Table S2). Therefore, the possibility of being able to quantify target compounds in kidney samples of any species based on the major species (porcine) would reduce analysis time, improving laboratory performance.

Table 2
Performance characteristics of the developed LC−MS/MS method per analyte in porcine kidney (n = 21).

	Concentration (μg kg^{-1})	Accuracy (%)	Repeatability (RSD, %)	Within lab reproducibility (RSD, %)	CCα (μg kg^{-1})
AcPN	1	101	7.9	20	1.5
	2	95	6.9	23	
	3	97	6.6	20	
APL	1	102	3.3	4.9	1.1
	2	100	2.4	2.9	
	3	100	2.1	3.7	
APN	1	101	2.9	5.0	1.1
	2	100	1.6	3.7	
	3	100	1.9	4.0	
CZL	1	102	3.7	4.3	1.1
	2	100	4.1	5.1	
	3	101	3.1	5.1	
ClPN	1	101	9.0	18	1.4
	2	97	7.4	20	
	3	101	7.5	16	
HPL	1	101	3.6	5.0	1.1
	2	100	1.6	2.4	
	3	100	1.6	1.6	
PrPN	1	101	7.5	20	1.5
	2	95	7.1	24	
	3	97	7.2	21	
XyN	1	99	3.3	5.1	1.1
	2	98	1.8	2.2	
	3	99	1.7	2.5	

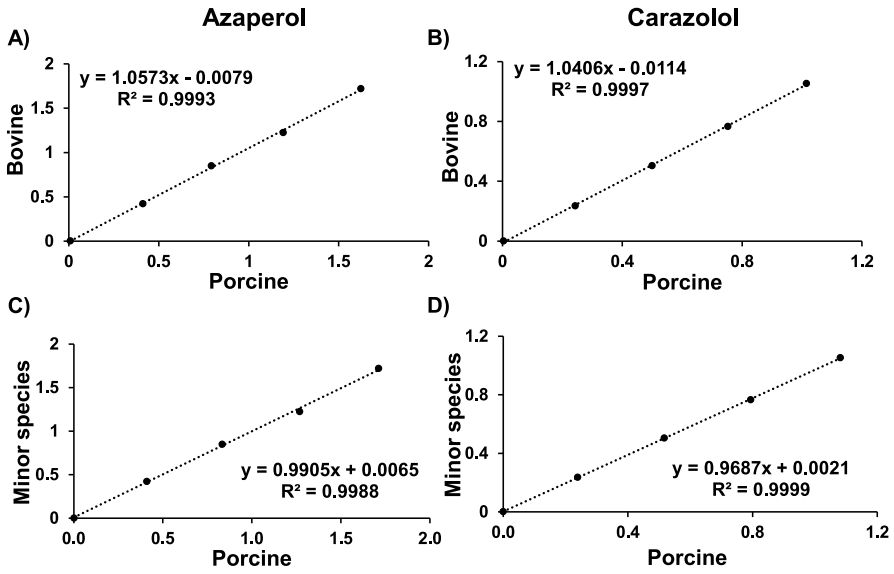

Fig. 3. Matrix matched plot of Azaperol (A, C) and Carazolol (B, D) representing the response factor in porcine matrix *vs*. response factor in bovine matrix (A and B) and in minor species (C and D).

3.4. Extension of the method to meat and fish

The developed method based on an extraction using 96-well plate SPE compared to regular SPE protocols, as shown previously has resulted in advantages in regard to analysis time and costs. Therefore, the application of this method to other different matrices that are also analyzed to monitor the target compounds in food control would further increase the efficiency of the laboratory. In this case, to increase the scope of the developed method, its performance has also been evaluated in matrices such as meat and fish. In the case of meat, since it is the second matrix most frequently analyzed, bovine and poultry spices have been studied performing a scope extension of one-day partial validation protocol at the three validation levels LCL x 1, x2, and x3 as described in Regulation (EU) (2021/808) [19]. As in the case of porcine kidney, the use of bovine meat for matrix matched calibration line has resulted in similar results for all target species allowing the application of a single matrix fortified calibration curve for the quantification of all meat species. In the case of fish, since it's a matrix that is less frequently analyzed in the NRCP a flex scope at 1 μg kg^{-1} was performed in salmon fish following Regulation (EU) (2021/808) [19]. The results showed that the developed analytical method to determine setdatives and β-blocker was adequate for the inclusion of two additional matrices to

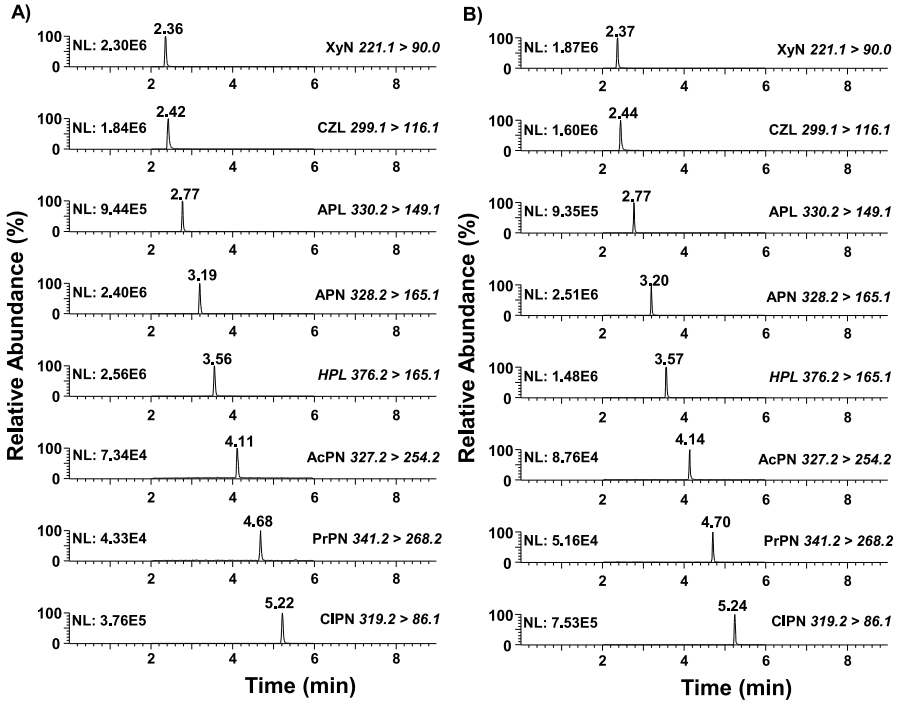

Fig. 4. UHPLC–ESI–MS/MS extracted ion chromatograms of quantitation transitions a blank (A) meat and (B) fish sample spiked at LCL level (1 μg kg^{-1}) for all target compounds.

the National Monitoring Control Plan, with validation performance characteristics very similar to the performance achieved for the different kidney samples (Table S3 and Table S4).

Fig. 4 shows as an example the UHPLC−ESI−MS/MS extracted ion chromatogram obtained by spiking a blank meat (A) and fish (B) sample at LCL level (1 μg kg^{-1}) and as can be seen, the target compounds can be easily detected at this concentration level. On the base of these findings, the feasibility of the developed 96-well plate SPE UHPLC−MS/MS method has been demonstrated to determine sedatives and β-blocker in meat and fish for the residue control requirement established in EURL Guidance Paper [3].

3.5. Analysis of real samples

The developed ion exchange 96-well plate SPE-LC−MS/MS method has been applied to monitor targeted sedatives and β-blocker in kidney, meat and fish samples at the National Reference Laboratory in Wageningen. So far, 201 kidney samples (porcine, calve, bovine and goat), 85 meat samples (bovine and broiler) and 15 fish samples (salmon) have been analyzed and according to EURL Guidance Paper [3] all of them were found to be compliant.

4. Conclusions

At present, in many food control laboratories, the main bottleneck of the analytical methodologies used is the sample preparation steps since many of them are time-consuming and hinder the analysis throughput requiring significant amounts of solvents and reagents. The aim of this study was based upon improving the throughput of the sample preparation procedure for sedatives and β-blocker in kidney samples while achieving the performance characteristics established by the European Union legislation stated in the EURL Guidance Paper [3]. To this end, a 96-well plate SPE UHPLC−MS/MS method has been developed for the reliable and accurate determination of seven sedatives and β-blocker in kidney, meat and fish. The transition from conventional SPE method to 96-well plate SPE method resulted in a reduction of the analysis time and required solvent volume by nine-fold and four times respectively, showing a great advantage on the laboratory throughtput and costs.

The developed 96-well plate SPE UHPLC-MS/MS method for sedatives and β-blocker in kidney samples has been validated according to the Regulation EU 2021/808. The obtained performance characteristics demonstrated that the method is specific, robust, accurate, reproducible and sensitive, with CCα values between 1.1 and 1.5 μg kg^{-1} for all target compounds, resulting always below the Minimum Method Performance Requirement (5 μg kg^{-1}) established by EURL Guidance Paper. Moreover, the results obtained for the analysis of selected kidney and meat species showed that there were no statistically significant differences between the use of the corresponding animal species matrix matched calibration line and the use of porcine kidney or bovine meat matrix matched calibration line in terms of both analyte concentration and the method performance characteristics, allowing the application of a single matrix fortified calibration curve for the quantification of all animal species in each matrix type.

Due to the great advantages that this method has presented compared to the previous one used in kidney samples, its scope was extended to meat and fish samples since at a lower percentage these are also usually analyzed. The validation of the method in these matrices showed similar results to those obtained in kidney samples allowing to include them on the National Monitoring Program. The present method has already been implemented in the Dutch National Residue Control Plan by analyzing up to 201 samples of kidney, meat and fish. It must be pointed that none of the analyzed samples resulted in positive finding above the legislated MRL levels byu the European Union and therefore, to date, no risk has been found in the monitored samples.

The good performance of the developed 96-well plate SPE UHPLC–MS/MS method and the relevant results obtained in the analysis of kidney, meat and fish samples have demonstrated its applicability to determine sedatives and β-blocker under the residue control requirement established in EURL Guidance Paper. To the best of our knowledge, this is the first time that a 96-well plate SPE has been implemented for the determination of food residues in national monitoring program, showing important advantages over the conventional SPE extraction technique.

CRediT authorship contribution statement

Ane Arrizabalaga-Larrañaga: Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization. **Dieke van Doorn:** Writing – review & editing, Validation, Methodology, Formal analysis. **Saskia S. Sterk:** Writing – review & editing, Project administration, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2024.343335.

Data availability

Data will be made available on request.

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