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Development of a method for the determination of sedatives in bovine and porcine urine and kidneys by liquid chromatography–tandem mass spectrometry

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Abstract

Introduction: Sedatives have been used for a long time as animal tranquillisers to prevent stress and weight loss during their transportation. The proper determination of these substances in food of animal origin is essential for consumer safety. **Material and Methods:** A 1 g portion of pig or cow urine or homogenised kidney was mixed with acetonitrile, sodium chloride was added, and the solution was further mixed and then centrifuged. The supernatant was transferred to a new centrifuge tube with primary and secondary amine, octadecylsilane and ZrO_2, and mixed rapidly. The filtered solution was evaporated under a nitrogen stream. The residue was dissolved in 200 µL of acetonitrile, centrifuged with filters and then transferred to vials. Samples were analysed by high-performance liquid chromatography–tandem mass spectrometry. **Results:** The decision limit for confirmation was calculated at 2.5 µg kg^{-1} for all sedatives with relative standard deviation repeatability and reproducibility below 20%. **Conclusion:** The validation results showed that this method meets the pertinent EU criteria for such methods and is suitable for sedative analysis in urine and kidney matrices.

Keywords: sedatives, tranquillisers, QuEChERS, liquid chromatography–tandem mass spectrometry, Commission Implementing Regulation (EU) 2021/808.

Introduction

Out of concern for consumer safety, the EU requires testing for many substances that should not be present in food of animal origin (5, 10–12, 16, 30, 31, 32–34, 37). In order to best protect consumers, the EU frequently lengthens the list of substances monitored in member states. By virtue of constantly improving analytical capabilities, the limits of content of monitored substances which are permissible are frequently lowered (5, 10–12, 14). Examples of substance groups for which the acceptable limits were reduced and the size of the groups was enlarged are the sedative groups denoted under B1c (Group B – Pharmacologically active substances authorised for use in food-producing animals – sedatives) and A2d (Group A – Prohibited or unauthorised pharmacologically active substances in food-producing animals – other substances) in Commission Delegated Regulation (EU) 2022/1644 (11). In earlier versions of the regulation, these groups included chlorpromazine (ClPN), azaperone (APN), its metabolite azaperol (APL), and the β-blocker carazolol (CAR). As a result of the last amendment to EU law, propionylpromazine (PrPN), haloperidol (HPL) and acepromazine (AcPN) were added (11, 14). The minimum method performance requirement (MMPR) for all these substances was set by the relevant EU reference laboratories at the level of 5.0 µg kg^{-1} (12). Because of their many side effects, such as adverse effects on the central nervous system (induced by APN and APL) or genotoxicity (caused by PrPN, ClPN and AcPN), these sedatives were listed in Regulation 1644 as substances that should be monitored in the production of food of animal origin (specifically pigs and cattle) (11). The grouping of xylazine (XyN) with the sedatives not approved for human use, because it causes many side effects such as drowsiness, hypotension, bradycardia and respiratory depression (24), justifies its inclusion in the method developed in the present research.

To analyse these types of substances it is necessary to develop an analytical extraction and purification procedure that will be cheap, quick, easy to perform, and have good laboratory reproducibility. Therefore, when developing a method, attention should be paid to

whether the proposed modifications affect several critical parameters. These are most notably recovery and repeatability (which should be better when the modification is made), the decision limit (CCα, which should be lower), matrix effect (which should be weaker), the amount of dry residue collected (which should decrease) and the amount of phospholipids not removed (which should decrease). Phospholipids are the main components of cell membranes and body fluids and are material which complicates the analysis of biological samples for the determination of metabolites or xenobiotics by high-performance liquid chromatography, especially in combination with tandem mass spectrometry (LC-MS/MS). Screening methods must also meet the requirements of the applicable EU regulations (10, 11).

The first methods for the determination of these substances were based on liquid chromatography combined with classical detectors (ultraviolet-visible spectroscopy and fluorescence detection) (2, 8, 17, 21), but these substances were also analysed using the ELISA technique (6) and thin-layer chromatography (18). Methods in which classical detectors were used were laborious and required a large amount of reagents (2, 8, 17, 21). They also required confirmation of test results. Liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) made possible working with smaller sample sizes and thus with less solvent during the extraction and purification of the tested samples. Mass spectrometry's specificity made it possible to confirm or possibly exclude the tested substance. This technique accelerated the development of analytical methods and the determination of residues of these substances in biological material (1, 4, 7, 20, 23, 25, 35, 36, 38, 39). Better possibilities for the determination of these substances resulted in the simplification of sample preparation. The most commonly used extraction methods for this group of compounds are liquid–liquid extraction (LLE), solid-phase extraction (SPE), and QuEChERS (quick, easy, cheap, effective, rugged, and safe). Liquid–liquid extraction is extraction with acetonitrile, methanol, trifluoroacetic acid and ethyl acetate; additionally, ammonia, ammonium formate or acetic acid may be used (1, 4, 8, 28, 29, 36, 40). A modification of LLE extraction is QuEChERS, in which the sample is additionally purified using octadecylsilane (C_{18}) and primary and secondary amine (PSA) sorbents (38, 41). A more conservative approach granting better purification of the extract but requiring longer sample preparation time is the SPE technique. In this technique, the most commonly used columns are those containing silica, C_{18}, and the polymeric reversed-phase sorbent (1, 4, 22, 35, 38). The difficulty of determining these substances in kidney tissue prompted some authors to test the possibility of combining the LLE technique with SPE for better purification of the extracts (1, 38). Another solution is hollow-fibre liquid-phase micro-extraction (27), which is an example of a different approach to the determination of chlorpromazine in urine.

This study aimed to develop a method for the determination of sedatives and specifically of ClPN, PrPN, HPL, AcPN, XyN, APN and its metabolite APL, and the β-blocker CAR in porcine and bovine kidneys and urine using the QuEChERS technique followed by LC-MS/MS analysis. The assumption was that it would be a QuEChERS method because it combines the best of LLE and SPE extraction, *i.e.* it is fast, cheap, simple and has good reproducibility. The method was also intended to meet new requirements regarding limits for the tested compounds and be validated under the new EU requirements (9–12).

Material and Methods

Chemicals and reagents. Analytical standards of AcPN, ClPN and their internal standards (AcPN-D_4 and ClPN-D_6), were purchased from LGC Labor (Augsburg, Germany), as were standards of HPL, PrPN and XyN. Standards of CAR, CAR-D_7, APN-D_4 and APL-D_4 were obtained from WITEGA (Berlin, Germany) and those of APL, APN and zirconium (IV) oxide were procured from Sigma-Aldrich (St. Louis, MO, USA). Internal standards of HPL-D_4, PrPN-D_6 and XyN-D_6 were acquired from TRC Canada (North York, ON, Canada). Sodium chloride, acetonitrile and methanol were from Avantor Performance Materials Poland S.A. (Gliwice, Poland). Primary and secondary amine and C_{18} were sourced from Supelco (Bellefonte, PA, USA). Magnesium sulphate ($MgSO_4$) was prepared in our laboratory by pre-heating to 400°C overnight (33). Ammonium formate was ordered from Honeywell (Seelze, Germany). Ultrapure water was filtered through a Millipore Milli-Q system (Burlington, MA, USA). Midi-spin filters with microfiltration PVDF membranes of 0.2 µm pore size were supplied by Frisenette (Knebel, Denmark), and Nanosep MF filters were from Pall (Port Washington, NY, USA). All reagents were of analytical or higher grade.

Individual stock solutions of analytical standards and their internal standards (1,000 µg mL^{-1}) were prepared in methanol and stored in the dark at temperatures below −18°C for no longer than 24 months. The working standard and internal standard solutions at a concentration of 0.1 and 0.3 µg mL^{-1} were prepared in methanol and stored in the dark at 6 ± 4°C for no longer than 12 months.

LC-MS/MS. The LC-MS/MS system consisted of an AB Sciex ExionLC HPLC system connected to an AB Sciex API 5500 Qtrap mass spectrometer (AB Sciex, Concord, ON, Canada). Analyst 1.6.3 software (AB Sciex) controlled the system, and the data was processed by Multiquant 3.0.2 software (AB Sciex). Liquid chromatography was performed as described by Cerkvenik-Flajs (2) in a Luna C_{18} column with 3 µm particle size and with 50 × 2 mm dimensions connected to a C_{18} precolumn of 4 × 2 mm (both Phenomenex, Torrance, CA, USA). The mobile phase was composed

of two reagents: A was 10% methanol in 0.2 M ammonium formate and B was methanol. The composition of the mobile phase was 100% A for 2 min, then 90% B for 1 min, and after that 100% A once more until the end of the sequence. The column was operated at 40°C. The flow rate was 0.4 mL min^{-1}. The MS/MS system was operated at the following parameters: curtain gas was set at 35 psi, collision gas at the high setting, desolvation temperature at 600°C, and ion source gases 1 and 2 at 40 psi. Electrospray in positive mode was used at a voltage of 5,500 V and the electron multiplier was used at a voltage of 2,100 V. The ions were monitored in multiple-reaction monitoring mode (Table 1).

Sample preparation. A 1 ± 0.05 g portion of pig or cow urine or homogenised kidney was mixed with internal standards at a concentration of 7.5 µg kg^{-1} and with 2.5 mL of acetonitrile using a vortex mixer for approximately 30 s. Then 0.25 g of sodium chloride was added and mixed. After centrifugation for 10 min at 4,500 rpm at approximately 4°C, the supernatant was transferred to a new centrifuge tube with 0.05 g of PSA, 0.1 g of C_{18} and 0.05 g of ZrO_2, and mixed rapidly. Zirconium dioxide was tested along with TiO_2 for their effect on the purity of the extracts, and the selection of C_{18} and PSA sorbents was based on findings in previous experiments (33). A 0.15 g mass of $MgSO_4$ was added to each sample, and the solution was processed with the vortex mixer for 30 s, transferred to a Midi-spin filter, then centrifuged for 10 min at 2,000 × *g*. The filtered solution was evaporated under a gentle nitrogen stream at 45 ± 5°C. The residue was dissolved in 200 µL of acetonitrile, centrifuged with Nanosep MF 0.22 µm filters for 2 min at 13,400 rpm at room temperature, and then transferred to vials.

Validation. The method was validated according to Commission Implementing Regulation (EU) 2021/808 (10). The following validation parameters were estimated: selectivity, working range, repeatability, within-laboratory reproducibility, apparent recovery, ruggedness, CCα, relative matrix effect, and uncertainty of the method. Selectivity was verified by analysing 20 blank samples for each matrix (kidney and urine) and for each species. In addition, blank samples were spiked at 5.0 µg kg^{-1} to investigate whether the matrix caused any interference at the time of analysis for any analyte. The working range of the method was 2.5–25.0 µg kg^{-1}. Repeatability was tested at three levels (2.5, 5.0 and 7.5 µg kg^{-1}). Seven samples were tested at each level. For within-laboratory reproducibility, the samples were fortified in another two sets at the same levels. The analyses were performed by different people, different lots of reagents were used, and the samples were analysed using a different mass spectrometer. In addition, apparent recovery was calculated at these levels. The method was also tested for ruggedness (at 5.0 µg kg^{-1}). Seven factors were selected: sample size (0.9–1.1 g), amount of acetonitrile (2.25–2.75 mL), centrifugation (1,800–2,200 × *g*), filters (PVDF or nylon), evaporation time (to dryness or to dryness +5 min), final volume (180–220 µL) and column (new or old).

Table 1. Mass spectrometry parameters for precursor ions and product ion transitions of neuroleptics and internal standards

Analyte	Retention time (min)	Precursor ion (m/z)	Ion transition (m/z)	Declustering potential (eV)	Collision energy (eV)
Acepromazine	2.76	326.9	86.1 58.2	100	24 72
Azaperol	2.61	330.1	121.0 312.0	96	29 21
Azaperone	2.65	328.1	165.0 122.9	91	27 43
Carazolol	2.60	299.1	116.0 222.0	96	27 27
Chlorpromazine	2.85	319.0	86.0 245.9	96	25 31
Haloperidol	2.74	376.0	165.1 123.0	110	30 43
Propionylpromazine	2.82	341.0	58.0 86.0	95	70 25
Xylazine	2.52	221.0	90.0 164.1	100	27 33
Acepromazine - D_6	2.76	333.0	92.0 64.0	110	25 69
Azaperol - D_4	2.61	334.1	121.0 153.0	96	31 39
Azaperone - D_4	2.65	332.1	169.0 121.0	101	29 29
Carazolol - D_7	2.60	306.1	123.1 222.0	101	27 27
Chlorpromazine - D_6	2.85	325.0	92.1 245.9	81	25 33
Propionylpromazine - D_6	2.82	347.0	92.1 64.1	100	24 70
Xylazine - D_6	2.52	226.9	90.0 170.0	120	29 34

The matrix effect was checked for each matrix and each substance at the second concentration level using the same method as previously described (33). Phospholipid content remaining in the sample after clean-up was determined with the method described by Flieger *et al.* (15). The uncertainty of the method was calculated for each matrix and for each analyte at the level of 5.0 µg kg^{-1}, applying a coverage factor of 2.

Results

All required validation parameters, namely repeatability, within-laboratory reproducibility, trueness (apparent recovery), and uncertainty of the method, are presented as ascertained at a concentration of 5.0 µg kg^{-1} in Table 2. The correlation coefficient of the standard curves prepared with the fortified samples for each analyte was ≥0.95. Depending on the substance and matrix assayed, the repeatability relative standard deviation for fortified samples was in a 6.4–12.0% range, while the range for the within-laboratory reproducibility relative standard deviation was 5.0–18.0%. The average apparent recovery was 97.1–102.1%. The CCα was established at the level of 2.5µg kg^{-1} for all analytes. The expanded uncertainty was calculated for each compound and each of the matrices at the second concentration level applying a coverage factor of 2, which provided a level of confidence of approximately 95% (Table 2).

The blank samples analysed for each matrix (kidney and urine) and for each species indicated that the developed method was selective. The satisfactory selectivity of the LC-MS/MS protocol is shown in the chromatograms presented in Fig. 1.

Processing blank samples spiked at 5.0 µg kg^{-1} gave results showing that the matrix caused no interference at the time of analysis for any analyte. The visualisation of the chromatography conducted on these samples is in Fig. 2.

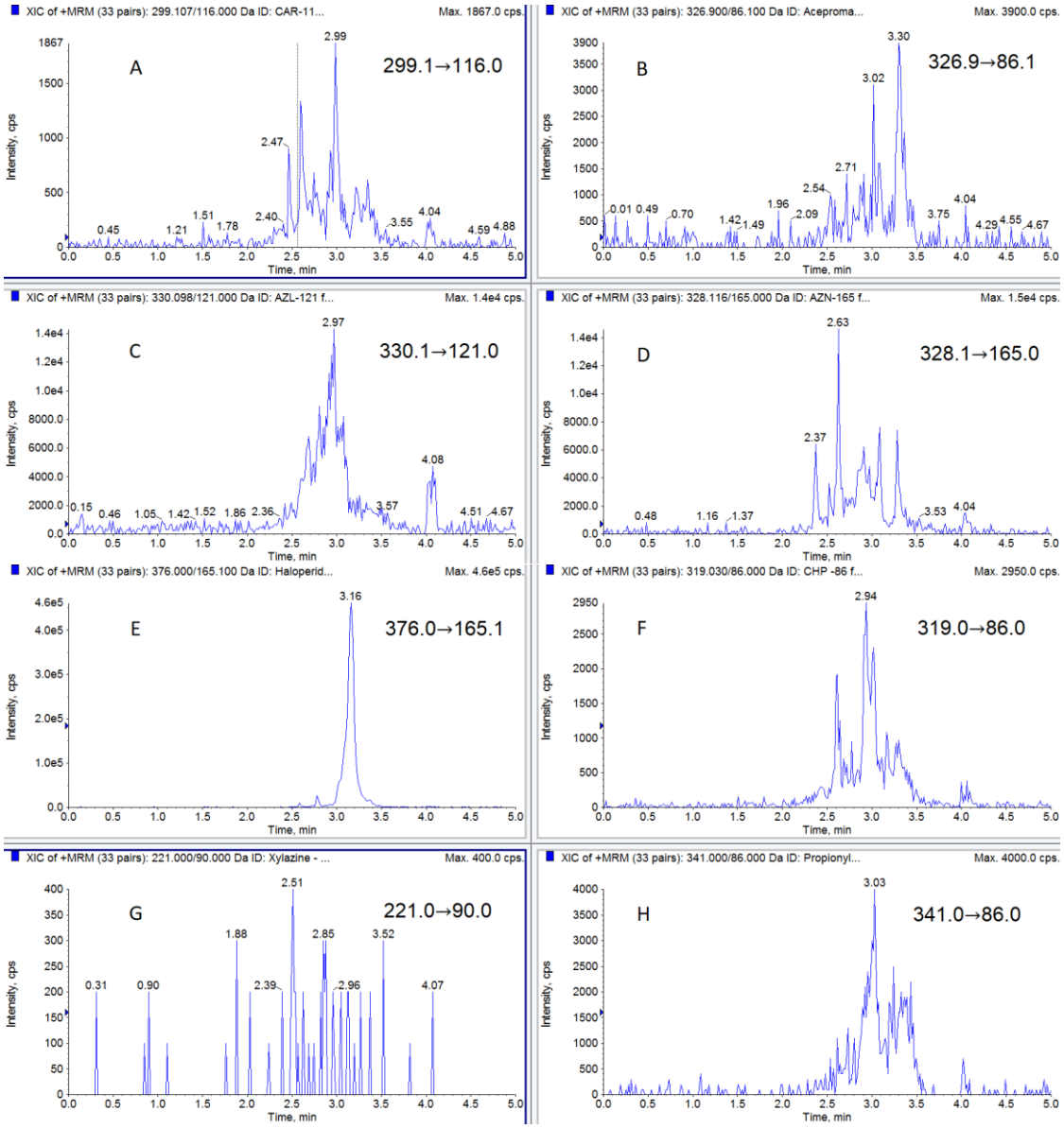

Fig. 1. Chromatograms of blank kidney samples. A) carazolol; B) acepromazine; C) azaperol; D) azaperone; E) haloperidol; F) chlorpromazine; G) xylazine; H) propionylpromazine

Table 2. Validation results for determination of neuroleptics in kidney and urine

Analyte	Matrix	Repeatability (RSD_r, %)	Within-lab reproducibility (RSD_{wR}, %)	Apparent recovery (%)	Expanded uncertainty (µg kg^{-1})	CCα (µg kg^{-1})
Acepromazine	Kidney	9.3	7.1	97.1	0.82	2.5
	Urine	9.8	8.3	101.6	1.04	2.5
Azaperol	Kidney	9.5	6.7	100.6	0.85	2.5
	Urine	7.7	8.1	101.0	1.10	2.5
Azaperone	Kidney	7.0	5.5	100.5	0.80	2.5
	Urine	6.0	5.2	101.2	0.67	2.5
Carazolol	Kidney	6.4	5.0	99.9	0.70	2.5
	Urine	7.4	9.8	101.6	1.43	2.5
Chlorpromazine	Kidney	7.4	9.2	100.5	1.43	2.5
	Urine	6.6	7.3	101.3	0.96	2.5
Haloperidol	Kidney	8.0	10.4	97.2	1.49	2.5
	Urine	12.0	18.0	100.6	2.03	2.5
Propionylpromazine	Kidney	8.5	8.3	97.3	1.08	2.5
	Urine	9.5	8.2	102.1	1.28	2.5
Xylazine	Kidney	9.2	10.5	99.3	1.45	2.5
	Urine	9.4	8.7	100.0	1.13	2.5

RSD_r – repeatability relative standard deviation
RSD_{wR} – within-laboratory reproducibility relative standard deviation

The calculated ion suppression of the matrix effects for all substances in all matrices did not exceed 20%. Neither matrix suppressed or enhanced the signal obtained from any of the eight analytes. Results between 80–120% indicated that this technique did not encounter a matrix effect problem and such results are evident in Fig. 3.

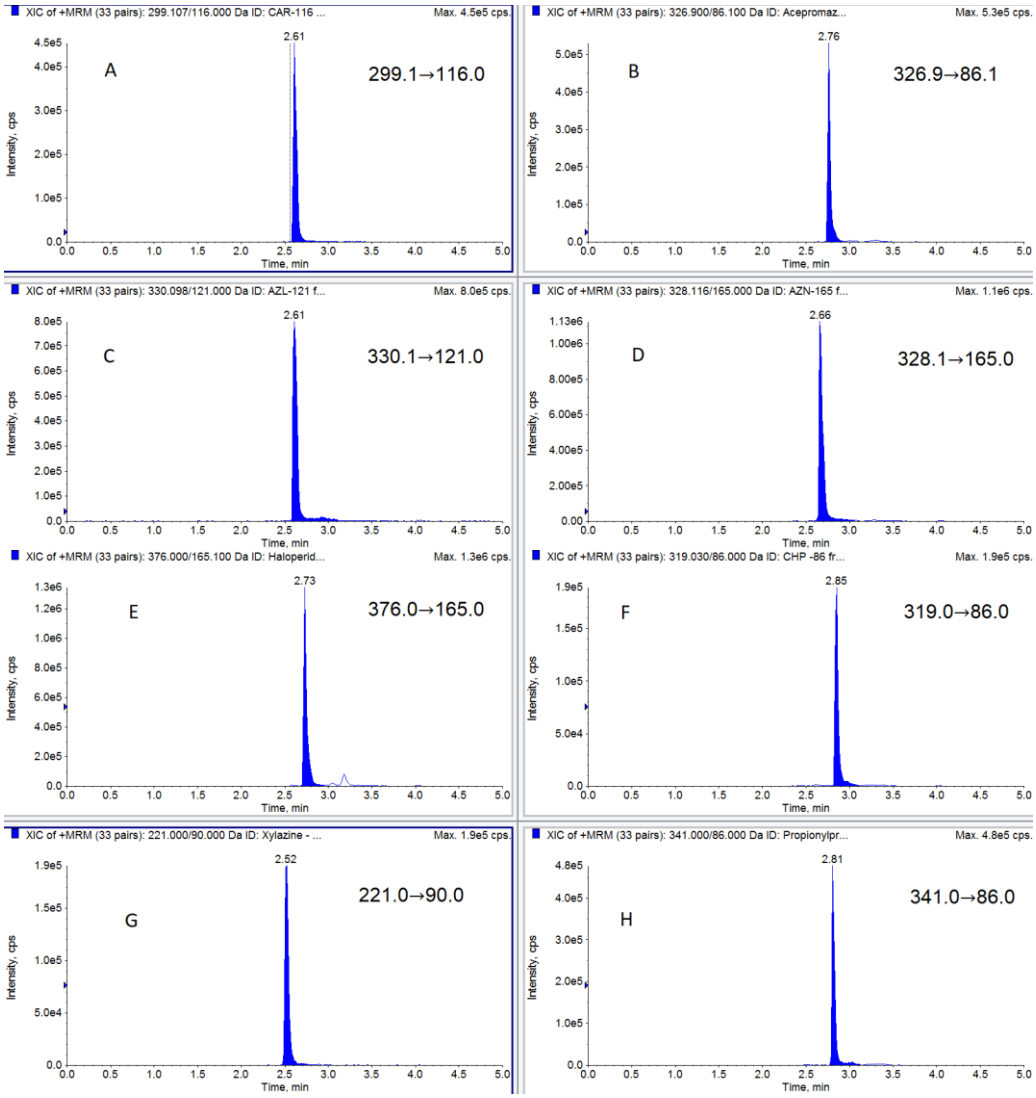

Fig. 2. Chromatograms of kidney tissue samples spiked at 5.0 µg kg^{-1} for A) carazolol (t_R = 2.61); B) acepromazine (t_R = 2.76); C) azaperol (t_R = 2.61); D) azaperone (t_R = 2.66); E) haloperidol (t_R = 2.73); F) chlorpromazine (t_R = 2.85); G) xylazine (t_R = 2.52); H) propionylpromazine (t_R = 2.81)

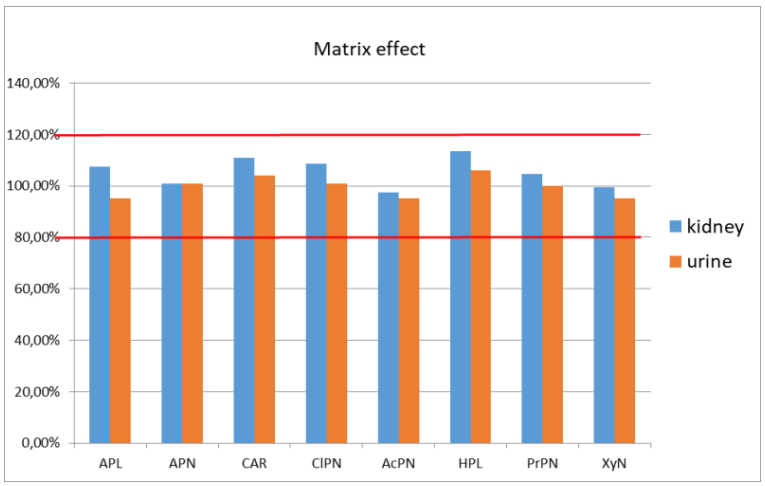

Fig. 3. Matrix effect in liquid chromatography–tandem mass spectrometry to detect sedatives in pig and cattle kidney tissue and urine. APN – azaperone; APL – azaperol; CAR – carazolol; ClPN – chlorpromazine; AcPN – acepromazine; HPL – haloperidol; PrPN – propionylpromazine; XyN – xylazine. Red lines – boundaries of suppressive (lower) or enhancing (upper) matrix effect

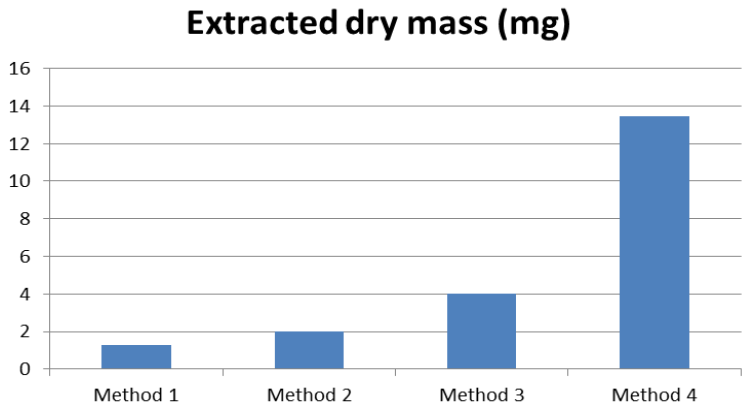

Fig. 4. Comparison of extracted dry mass (g) from 1 g kidney samples using different methods. Method 1 – standard method; Method 2 – standard method with modification (nylon filter); Method 3 – standard method with modification (TiO_2); Method 4 – standard method with modification (ethyl acetate)

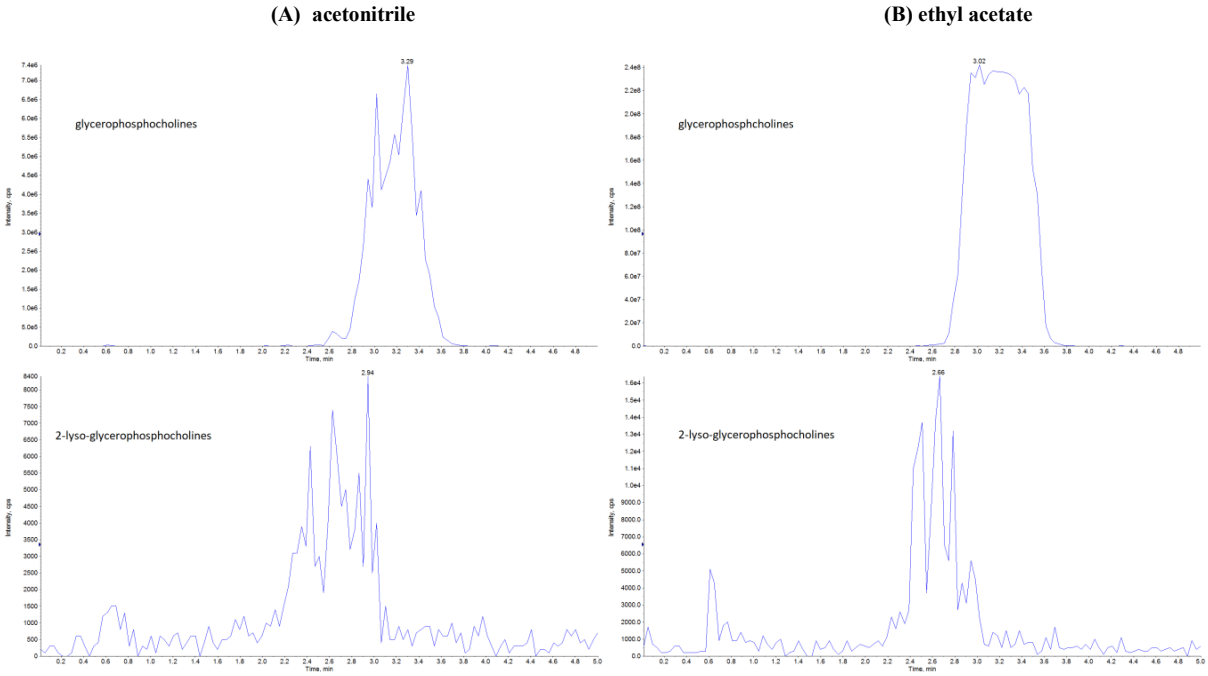

Fig. 5. Chromatograms of glycerophosphocholines (184 m/z) and 2-lyso-glycerophosphocholines (104 m/z) (A) after acetonitrile extraction, (B) after ethyl acetate extraction from kidney

Discussion

The first step in the optimisation of the method was to establish the best mass spectrometer conditions. Because most of the published protocols for determination of these substances used electrospray ionisation in positive ionisation mode (1, 4, 7, 20, 23, 25, 35, 36, 38, 39), we also used this type of ionisation for its high sensitivity and reproducibility. After optimisation of the analytes and selection of appropriate characteristic ions, we optimised the chromatographic conditions. Chromatographic separation for these substances is usually performed using C_{18}- or octylsilane (C_8)-filled columns of different lengths (1, 3, 4, 8, 25).

In our study, we used C_{18} columns for chromatographic separation using a gradient method. Methanol, acetonitrile, formic acid and acetic acid are commonly chromatographically separated in buffers such as sodium acetate, ammonium formate or ammonium acetate (2, 6, 8, 17, 18, 21). According to our experience, methanol generally gives a higher signal than acetonitrile (33), so we decided to use it; additionally, the mobile phase was modified with ammonium formate. Such a mobile phase composition allowed good chromatographic separations without materially impairing interferences. The proposed method is very fast (5 min) and gives good separation (symmetric peak shape and minimal matrix effect).

The clean-up is one of the crucial steps in method development. For the determination of these substances, methods can be divided into LLE , SPE (2, 6, 8, 17, 18, 21), QuEChERS extraction (38, 41) and a combination of LLE and SPE (1, 3). The fast, cheap and simple character of the QuEChERS technique and its good reproducibility recommended this method for the analysis of these compounds. The most commonly used reagents to extract the tested sedatives are acetonitrile, ethyl acetate, trifluoroacetic acid and methanol (1, 4, 8, 28, 29, 36, 40). Acetonitrile and ethyl acetate gave the best recoveries; therefore, these two solvents were considered during the development of the method. The suitability of these solvents was assessed by the number of recoveries, the purity of the extracts, and the weight of the amount of dry residue after evaporation (Fig. 4). The analysis of recoveries shows an advantage to extraction with ethyl acetate, which is also confirmed by the results obtained by Bock *et al.* (1). In comparison, analysis of the dry residue clearly shows a better result with acetonitrile. The dry residue after extraction with ethyl acetate is an amount ten times higher than the amount after extraction with acetonitrile (Fig. 4). Additional confirmation of the advantages of acetonitrile is provided by chromatograms showing the amounts of phospholipids in the sample after extraction with acetonitrile and ethyl acetate (Fig. 5). Phospholipids being difficult components to eliminate (15), they make the matrix effect significantly stronger and thus downgrade the final determination result. They can also significantly affect the sensitivity of the method.

The next optimisation step concerned sample purification. Analysis of the results obtained clearly shows that better results and purer extracts were obtained when ZrO_2 was added along with the sorbents (Method 1 in Fig. 4). The filters used during pre-purification (PVDF or nylon) were also evaluated for which was superior. Better purification was achieved through the use of PVDF filters (Method 1 in Fig. 4).

We did not observe any differences during validation either in terms of species (porcine or bovine) or in terms of sample (urine or kidney tissue).

The novelties in this method were the comparison of the extraction reagents in various aspects, the testing of the usefulness of the effect of ZrO_2 and TiO_2 on the purity of the extracts, and the use of additional filters to increase the purity of the extracts. Another difference from previous sedative screening protocols in the present research was that, in addition to the analysis of substances that are required by the EU, XyN was added to the method. In our opinion, this substance should also be included in the monitoring conducted by all EU countries.

The validation parameters indicate that the method meets the relevant acceptance criteria set by the EU (10). The use of the method was assessed in a proficiency test performed by the European Union Reference Laboratory at Wageningen (z-score = 0.88 for Horwitz precision limit, z-score = 1.59 for acceptable precision limit and z-score = 1.18 for analytical performance number) with satisfactory z-scores between −2.0 and 2.0.

Conclusion

A study comparing different methods for the extraction of analytes from biological material showed that the developed method provides good recovery of the tested substances and consumes little labour and time for sample processing, which prompted us to validate it.

These validation results confirm the suitability of the method for routine monitoring of residues of these compounds in all presented matrices in the National Monitoring Plan in Poland.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: None required.

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