ANALYSIS OF CORTICOSTEROIDS IN SAMPLES OF ANIMAL ORIGIN USING QuEChERS AND ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO HIGH RESOLUTION MASS SPECTROMETRY

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Abstract

A rapid and sensitive method for the confirmatory analysis of 8 synthetic corticosteroids (betamethasone, dexamethasone, prednisolone, 6 methyl prednisolone, triamcinolone, flumethasone, beclomethasone, fluocinolone acetonide) is proposed. The method is useful for detecting illegal treatments in different animal species. It consists of an extraction and clean-up using the Quick Easy Cheap Effective Rugged Safe (QuEChERS) strategy. Quantitative determination is achieved by ultra-high performance liquid chromatography coupled to high-resolution mass spectrometry with heated electrospray ionization in negative mode. Quantification is performed using surrogate matrix matched standard calibration curve with dexamethasone-D₄ as the internal standard. The method was validated for analyzing liver samples according to the criteria established by Decision 2002/657/EC. Linearity was assessed in the 1-10 μg kg⁻¹ range and linear correlation coefficients were over 0.99 for all the analytes. CC₀ ranged from 0.04 to 0.16 μg kg⁻¹ for substances without maximum residue limit. The method allows confident quantification and confirmation of corticosteroids in liver samples, and its simplicity makes it suitable for analyzing large numbers of samples.

Keywords: Synthetic Corticosteroids, Liquid Chromatography, High Resolution Mass Spectrometry, Orbitrap, Food analysis, Liver

Introduction

Natural corticosteroids are a family of steroidal hormones derived from cholesterol and produced in the external portion of the adrenal cortex. These compounds have anti-inflammatory properties with analgesic and antipyretic effects. Pharmacological doses are used for their palliative anti-inflammatory or immunosuppressant effects [1]. Large numbers of synthetic steroids with these specific characteristics are produced [2, 3]. Some of these corticosteroids are used for therapeutic purposes in human or veterinary medicine. For instance, dexamethasone (DEX) is twenty times more powerful than cortisol and has a longer period of action [4], and it is used in human and veterinary medicine.
In veterinary practice, when used in low doses for long periods of time corticosteroids are considered to be growth promoters, since they act on lipid metabolism [1,5]. The association between corticosteroids and other growth promoters give them synergetic effect and depletes the urinary concentration of molecules administered for illicit purposes [6-8]. Moreover, corticosteroids negatively affect the organoleptic properties of meat and cause adverse effect on human health [9].

In the European Union, corticosteroids are strictly regulated and classified in Group B2f, as “Other substances that exert a pharmacological activity” [10]. European Regulation 37/2010 [11] only sets maximum residue limits (MRL) in liver matrix for betamethasone (BET) (2 µg kg\(^{-1}\) in bovine and pig), DEX (2 µg kg\(^{-1}\) in ovine and pig), prednisolone (PLN) (10 µg kg\(^{-1}\) in bovine) and 6-methylprednisolone (MPLN) (10 µg kg\(^{-1}\) in bovine). However, these corticosteroids may only be used for therapeutic purpose, with regular prescription by the veterinary [12]. To detect the use of prohibited substances or improper administration of regulated ones, and with the ultimate aim of protecting consumer health from unwary intake of residues, the European authorities have established national residue control plans [10]. These plans require analytical methods with high sensitivity, specificity and accuracy.

In food testing laboratories, veterinary drug residues are normally analyzed using liquid chromatography (LC) coupled to mass spectrometry (MS), mainly using triple quadrupole (QqQ) instruments. However, the resolution of QqQ instruments is not always sufficient for resolving coeluting isobaric interferences in complex matrices, thus affecting the reliability of the results [13-15]. In this sense, it is advantageous to use high-resolution mass spectrometry (HRMS) to analyze this type of compounds [15-17]. HRMS is a powerful tool because of its resolving power and mass accuracy, which allow to discriminate between interference and target ions, thus providing high sensitivity for analyzing low concentrations of analytes [18].

LC/HRMS has greatly improved laboratories’ ability to characterize and confirm detected substances, and official control laboratories are increasingly using HRMS to detect and confirm food contaminants.

Moreover, control laboratories need high throughput analysis methods. In this sense, sample treatment is the bottleneck for analyzing corticosteroids in food samples. Regarding the extraction of
corticosteroids, relatively polar solvents, such as ethyl acetate, acetonitrile, methanol or hydro-organic mixtures have been proposed, in most cases using conventional shaking, or ultrasonic bath. Extracts are further cleaned up, mostly by solid phase extraction (SPE), especially in the reverse mode. Thus, some authors describe the use of C₁₈ [17, 19] or polymeric cartridges [20,21], and less frequently the combination of two SPE steps, with C₁₈ and SiO₂ cartridges [22], or the use of mixed mode strong cation exchange cartridges [23].

The QuEChERS methodology [24] allows to markedly speed up the sample treatment and is a very attractive approach for control laboratories dealing with large numbers of samples. Recent reports describe a QuEChERS based method for analyzing flumethasone (FLU) in beef muscle [25], and multifamily residue methods that include some corticosteroids [16].

The aim of this work is to develop a high throughput and sensitive method for simultaneously determining 8 synthetic corticoids in liver samples, namely DEX, BET, PLN, MPLN, FLU, triamcinolone (TRI), beclomethasone (BCL) and fluocinolone acetonide (FCN). The QuEChERS strategy is applied for the extraction and clean up steps, and the quantitative determination is performed by ultra-high performance liquid chromatography (UHPLC) coupled to high-resolution mass spectrometry, using a hybrid quadrupole Orbitrap mass spectrometer, with heated electrospray ionization (HESI).

**Materials and Methods**

**Chemicals and Reagents**

BCL, BET, DEX, FLU, FCN, MPLN, PLN and TRI with a minimum purity of 95% each, were purchased from Sigma-Aldrich (Seelze, Germany); Dexamethasone-D₄ (DEX D₄) 95%, was purchased from CDN Isotopes Inc. (Canada).

Purified Water Type I was obtained from a Milli-Q System (Millipore, Billerica, MA, USA); methanol HPLC was obtained from Fisher Scientific (UK); acetonitrile HPLC hypergrade (ACN) was obtained
from LiChrosolv (Darmstadt, Germany); formic acid and anhydrous sodium acetate were obtained
from Sigma-Aldrich (Seelze, Germany); chloroform, hexane, sodium hydroxide and glacial acetic acid
99% were obtained from Panreac (Barcelona, Spain); QuEChERS salts (6 g of magnesium sulfate and
1.5 g of sodium acetate) and dispersive tubes (0.4 g of Primary Secondary Amines (PSA), 0.4 g of C18
and 1.2 g of magnesium sulfate) were obtained from Agilent Technologies (New Castle, DE, USA).
All other chemicals were analytical reagent grade.

Individual stock solutions (1000 mg L\(^{-1}\)) of BCL, BET, DEX, FLU and TRI, were prepared by
weighing 25 mg separately, and dissolving it into 25 mL of methanol in dark volumetric flasks.
Individual stock solutions (1000 mg L\(^{-1}\)) of FCN, MPLN and PLN were prepared by weighing 25 mg
separately and dissolving it into 25 mL of ACN:chloroform (50:50) mixture in dark volumetric flasks.
These solutions were stable at least for 2 years at -20±3°C. Intermediate solutions (20 mg L\(^{-1}\)) were
prepared by taking 0.5 mL of each stock solution with automatic pipette, bringing it to a 25 mL dark
volumetric flask and wiping with ACN. These solutions were stable at least for 1 year at -20±3°C.
Working solutions (0.4 mg L\(^{-1}\)) were prepared by taking 0.5 mL of 20 mg L\(^{-1}\) solution and diluting to
25 mL with mobile phase. These solutions were stable for 3 months at +4±1°C.

Internal standard (DEX-D4) stock solution (1000 mg L\(^{-1}\)) was prepared by weighing 10 mg of
commercial standard and dissolving it in a dark flask with 10 mL of methanol. This solution was
stable at least for 2 years at -20±3°C. Internal standard intermediate solution (20 mg L\(^{-1}\)) was prepared
by taking 0.5 mL of DEX-D4 1000 mg L\(^{-1}\) solution and diluting to 25 mL with ACN. This solution was
stable for 1 year at -20±3°C. Internal standard working solution (0.4 mg L\(^{-1}\)) was prepared taking 0.5
mL of intermediate solution of DEX-D4, putting it to a 25 mL volumetric dark flask and mixing with
re-suspension solution (water:ACN (80:20) mixture). This solution was stable for 3 months at +4±1°C.
Formic acid 0.1% (mobile phase A) was prepared daily by measuring 1 mL of formic acid with an
automatic pipette and transferring it into a one liter flask with water type I, to obtain 1 liter of solution,
and filtering through 0.22 μm polyvinylidene fluoride (PVDV) filter.

**Instrumentation**
A Vanquish UHPLC Ultimate 3000 binary pump, with a CORTECS column (Waters) C18 (50 x 2.1) mm, 1.6 µm, was used. Oven temperature was +40°C and flow rate was 400 µL min⁻¹. The mobile phases were aqueous 0.1% formic acid (A) and ACN Hypergrade (B), and the gradient program was (time min, % B): (0, 20); (6, 30); (6.1, 100); (7, 100); (7.5, 20); (9.0, 20). Total run time was 9 minutes.

The chromatographic system was coupled to a hybrid quadrupole-Orbitrap HRMS instrument (Q-Exactive, Thermo Scientific, Bremen, Germany), with a heated electrospray ionization (HESI) in negative ionization mode. The Product Reaction Monitoring (PRM) acquisition mode was applied. In the PRM mode the precursor ions are isolated by the quadrupole and fragmented at the high energy collisional dissociation (HCD) cell. The product ions are analyzed at the Orbitrap mass analyzer, and the traces for the selected quantification and confirmation ions are acquired, using time windows, with high resolution.

The following conditions were used: heater temperature 310°C, capillary temperature 350°C, sheat flow gas (N₂) 40 L h⁻¹, auxiliary gas 10 L h⁻¹ and spray voltage 3.5 kV. The mass spectrometer was calibrated daily to ensure a mass accuracy better than 5 ppm, using Pierce LTQ Velos ESI Positive ion and Pierce LTQ Velos ESI Negative ion calibration solutions (Thermo Fisher Scientific, Rockford, IL, USA). The isolation window in the quadrupole was 2 Da. We set a collision energy (NCE) of 10% for all compounds, except for FCN (15%), a resolution of 70,000 (m/z 200, FWHM), scanning rate 2 Hz and automatic gain control of 5e⁶ with a maximum injection time of 100 ms. The information about precursor, quantification and confirmation ions for each analyte is shown in Table 1.

To control the UHPLC-HRMS and data processing, TraceFinder 4.1 software (Thermo Fisher Scientific, San Jose, CA, USA) was used.

Nitrogen was obtained from a Zephyr generator (Clantecnologica, Seville, Spain).

A Retsch GM200 blender (Haan, Germany) was used for sample homogenization. A vertical agitation system (AGYtax SR1 CP57, Spain), a refrigerated centrifuge (Hettich Rotanta 460R, Germany), a nitrogen evaporation system with controlled temperature (Turbo-VAP LV, Biotage, Sweden), an
ultrasonic bath (Selecta, Barcelona, Spain), a multiple vortex agitator (Genius 3, Germany) were used in the extraction steps, Whatman 2 mL vials with 0.45 µm PVDF filter and class A volumetric glass material were used.

Samples
Liver samples from various animal species (bovine, porcine, ovine, aviary and rabbit) were stored immediately on reception at -20°C in a properly numbered plastic jar until analysis. Liver samples were properly homogenized before analysis using a laboratory blender.
All blank samples used to validate the procedure were analyzed to confirm the absence of corticosteroids.

Extraction and clean up procedure
For extraction, 50 mL tubes to weigh 5g of liver sample were used, and 100 µL of internal standard 0.4 mgL⁻¹ was added. After shaking for 30 seconds, 10 mL of water type I was added. Then 15 ml of ACN was added, and this mixture was shaken in a vortex. QUECHERS salts (6 g MgSO₄, 1.5 g CH₃COONa) were added, and the sample was shaken using vertical shaker, sonicated, and centrifuged at 3500 rpm for 5 minutes at + 5°C. One 10 ml aliquot was transferred to a dispersive tube containing 0.4 g PSA, 0.4 g C18 and 2 g MgSO₄, shaken using a multivortex and a vertical shaker, sonicated, and centrifuged at 3500 rpm for 5 minutes at + 5°C. The supernatant was evaporated to dryness at +45°C for 45 minutes. The dry residue was re-dissolved with 500 µL of re-suspension solution, shaken in vortex, sonicated and filtered using vials with an incorporated filter. Finally, 5µL of the extract was injected into UHPLC-HRMS system.

UHPLC/HMRS conditions for confirmatory analysis
Compounds were identified by retention time (RT) and accurate mass. Maximum permitted deviation of the retention time was set at 1%, related to the RT of the analytes in surrogate matrix matched standards (SMMS) extracts. Ions selected in the quadrupole were the formate adducts for all compounds, due to the ability of corticosteroids to form adducts, except for TRI. These ions were partially fragmented in the collision cell and afterwards analyzed using HRMS.

We used as quantification ion a fragment of the molecule in the majority of cases, except for BCL and FCN (Table 1): in these cases we used the formate adduct of the molecule. In addition to the quantification ion, two confirming ions were monitored (when possible) in the same experiment using a mass accuracy better than 5 ppm. Table 1 shows the confirmation ions for each analyte. The ion ratio (ratio between the quantification ion and the confirmation ion) was used as an additional criterion for confirming positive results. The ion ratio of the compounds in the sample should be in agreement with that of the surrogate matrix matched standards (SMMS) with a maximum tolerance of ± 20%.

**Calibration**

Calibration was performed using SMMSs in the range 1-10 µg kg⁻¹ and adding Dexamethasone-D₄ as internal standard according to 657/2002/CE Decision and its interpretation [26, 27]. SMMSs were prepared as follows: blank samples were spiked with a mixture of analytes and internal standard, and were left during 30 min before carrying out the analytical procedure. The SMMSs were then submitted to the complete analytical procedure (*i.e.* QuEChERS+UHPLC-MS/HRMS).

The calibration curve for each analyte was obtained by linear regression analysis of the ratio between the peak area of the analyte and that of the internal standard plotted against the analyte concentration. This type of calibration provides results already corrected for matrix effect and recovery.

Linearity was assessed by calculating the correlation coefficient ($r^2$) and point-to-point deviation. The value of $r^2$ was > 0.999 for all analytes except DEXA, for which $r^2$ was 0.998. Residuals requirements were set as follows: < 25% at the first point of the calibration curve and < 15% for higher calibration concentrations.


Results and Discussion

Optimization of UHPLC/HRMS

The chromatographic separation was performed at +40°C with a CORTECS C18 core shell column using a mobile phase based on water with 0.1% formic acid and ACN. The chromatographic conditions, in terms of gradient and flow rate, are described in section 2.2. Separation of the 8 compounds was achieved within 6 minutes, and DEX and BET stereoisomers were completely resolved (Figure 1). Total chromatographic run time, including re-equilibration, was 9 minutes. MS detection is generally based on corticosteroids’ ability for adduct formation. Addition of low concentration (0.1%) of acetic or formic acid in the mobile phase induces the formation of acetate or formate adducts [18, 28-30]. For HRMS detection with the Q-Orbitrap spectrometer, negative ionization and the PRM mode were used; PRM is similar to Product Ion Scan in triple quadrupole instruments, but generates a HRMS product ion spectrum. Thus, in PRM, the precursor ion is selected in the quadrupole (isolation window 2 Da), then fragmented in the HCD cell, and the product ions are analyzed at the Orbitrap mass analyzer. For all the analytes the formate adduct was selected as precursor ion, except for TRI, for which the [M-H]⁻ ion was chosen (Table 1). Fragmentation experiments, varying the collision energy, were performed to select suitable quantification and confirmation ions. It was observed that formate adducts for BCL and FCN underwent lower fragmentation, with [M+HCOO]⁻ ions showing intense signals in the HRMS product ions spectra, and thus they were selected as quantification ions. The formate adduct was selected as confirmation ion for all analytes except for TRI, BCL and FCN. Information about quantification and confirmation ions for all the analytes is given in Table 1.

Optimization of the extraction method

Liver samples were used to optimize various parameters of the extraction method. The QuEChERS approach was first proposed for the analysis of pesticides in fruits [24], but it is currently applied in different fields of analysis [31, 32]. Commercial QuEChERS Kits for fatty
samples were assayed. ACN was used as extraction solvent, sodium acetate and magnesium sulphate as a salting-out medium and a mixture of PSA and C18 as a dispersive media for clean-up. The standard QuEChERS procedure uses 10 g of sample and 15 mL of ACN. During the assays, it became necessary to add water to the samples to prevent them from becoming too viscous and to allow proper extraction. Thus, in the preliminary tests, the ratio of sample to the amount of water added was evaluated, and proportions of 2, 1, 0.5 and 0.3 were tested using samples spiked at 1 µg kg\(^{-1}\) concentration. The ratio 0.5 gave the extracted sample an appropriate density and an effective separation of the mixture, with a clean upper layer after centrifugation, suitable for chromatographic analysis.

In order to avoid matrix overload in the chromatographic system, the sample weight was investigated (3, 5 and 10 g). 5 g was enough to detect and confirm concentrations of at least 1 µg kg\(^{-1}\), and thus it was not necessary to analyze a larger amount of sample. Therefore, for the extraction step the established conditions were: 5 g of sample, 10 mL of water, 15 mL of ACN, and 6 g of magnesium sulfate and 1.5 g of sodium acetate. Afterwards, the organic layer was cleaned-up by dispersive solid phase extraction with PSA, C18 and magnesium sulfate, the extract was evaporated to dryness, and the solid residue dissolved in water:ACN (80:20). Finally, a commercial QuEChERS kit for drug residues in meat was also tested. This kit uses sodium chloride in the extraction step and C18 as the dispersive medium for clean up; the results were not successful, since the clean-up step was not effective enough and the extracts were too fatty, and thus this kit was discarded.

The efficiency of the QuEChERS extraction method using the fatty samples kit was evaluated by recovery studies. Extraction recoveries were determined using spiked blank porcine liver samples, previously analyzed and confirmed to be free of analytes. We followed this approach, that can lead to some overestimation of recoveries, because there were no certified reference materials available. Thus, aliquots of a blank liver sample were fortified at 1, 2 and 10 µg kg\(^{-1}\). For each concentration level, two sets of samples (n=6 each) were prepared, the first one spiked before the extraction step and the second one spiked after extraction and clean-up, and the results of both sets were compared. The procedure was repeated on three different days, for a total of 18 replicates at each concentration level. Absolute recoveries ranged from 35 to 49%, with RSD values below 15%. Although absolute
recoveries lie in the low range, values were reproducible. In addition, calibration by SMMS corrects for extraction recoveries.

The proposed method is very convenient for routine analysis of corticosteroids in food samples, in laboratories with high workload. Compared to methods based on a conventional extraction followed by a clean-up by SPE, the new method reduces the time spent in sample treatment in about 50%.

**Method validation**

The method was validated according to 657/2002/EC Decision [26], and the results are collected in Table 2. The validation study was performed using porcine liver as the basic matrix.

**Blank studies**

The analytical method was tested in blank liver samples from different species (bovine, ovine, swine, avian and rabbit) with satisfactory results. No signal in the retention time of the analytes could be measured, demonstrating the method’s selectivity. The results were also evaluated in terms of internal standard response: RSD% among different species was 7.8%, proving that the matrix effect was very limited. These results evidenced that the different liver matrixes were equivalent in terms of the method performance.

**Linearity**

The criteria for evaluating linearity are described in calibration section. We found satisfactory results for all analytes in the validation range (1-10 µg kg\(^{-1}\)), with correlation coefficients > 0.99. Response factor as a second linearity criterion was also determined. Response factor was calculated as the ratio between the response (peak area) of each calibration point and its theoretical value of concentration. A good linearity implies the parameter to be equal for all calibration points. The parameter was evaluated in terms of RSD between all calibration points. In all cases was below 20%, demonstrating the good linearity of the method.

**Precision**

Precision was determined, in terms of reproducibility, from experiments conducted on three different days and extracting 6 spiked samples per day. Samples were spiked at 3 different concentrations,
namely 1, 2 and 10 µg kg\(^{-1}\), and were extracted using different reagent batches and by different
analysts. The method’s precision, expressed as the RSD, ranged from 2.9 to 13.5%.

**Trueness**

Since no certified reference material was available, trueness was calculated in terms of recovery of
spiked samples. Results were satisfactory, in the 78-101% range, in agreement with 657/2002/EU
recommendations. Slopes obtained in regression analysis of spiked and calculated concentration data
were close to 1, with regression coefficients higher than 0.99.

**CC\(\alpha\) and CC\(\beta\)**

On one hand, for compounds with a published MRL (namely DEX, BET, PLN, MPLN in liver of
some particular species) [11], CC\(\alpha\) and CC\(\beta\) were calculated from blank samples spiked at the MRL
level, according to the guidelines in Decision 657/2002/CE. On the other hand, for compounds with no
MRL, these parameters were calculated using the ISO 11843 extrapolation approach [33]. CC\(\alpha\) ranged
from 0.04 to 0.16 µg kg\(^{-1}\), depending on the analyte. CC\(\beta\) results ranged from 0.08 to 0.35 µg kg\(^{-1}\).
These values point out that the method is highly sensitive. Results are shown in Table 2.

The laboratory has participated in proficiency tests using the new method and very satisfactory results
have been achieved; Fapas 02339 Food Chemistry exercise consisted of a bovine liver containing BET
(5 µg kg\(^{-1}\)). Results were excellent, with a Z-score of 0.0, proving the suitability of the proposed
method. In the 4\(^{th}\) run of Progetto Trieste interlaboratory exercises, the method was applied to the
analysis of a lyophilized bovine liver sample. DEX was detected and quantified (2.49 µg kg\(^{-1}\)), and the
obtained z-score was 0.41.

The new method is currently used at the laboratory for the analysis of liver samples from various
species from different slaughterhouses in Catalonia, as a routine method of analysis. Figure 2 shows
the chromatogram of a porcine liver sample that contained 1.5 µg kg\(^{-1}\) of DEX. In addition to the
DEX peak, some small peaks can be observed at the trace chromatograms of PLN, BCL and FCN, at
their corresponding retention time windows. However, these peaks are below the limit of
quantification and do not fulfill the ion ratio criteria.
Conclusions

The new method combines the QuEChERS strategy for sample treatment with the confirmatory analysis by UHPLC-MS/HRMS of 8 synthetic corticosteroids. On the one hand the QuEChERS approach allows to notably increasing the method throughput, since time for sample treatment is reduced by about 50% in comparison with methods using conventional extraction and SPE clean up. In addition, there is a remarkable waste reduction. On the other hand, the use of HRMS provides excellent performance in terms of selectivity and also sensitivity, which are critical issues in the analysis of low concentration levels in complex samples. The method is very simple and rapid, and up to 30 samples can be daily processed at the laboratory by a single operator. These characteristics are essential for an effective and reliable food safety control. In addition, they facilitate the market withdrawal of non-compliant products, containing illicit synthetic compounds used as growth promoters in livestock farming.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest

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Table 1. Compounds information. Precursor, quantification and confirmation ions
| Compound       | Acronym | Structure | Molecular formula | Molecular mass | RT (min) | Precursor ion [m/z] | Quantification ion [m/z] | Confirmation ions [m/z] |
|----------------|---------|-----------|-------------------|----------------|----------|--------------------|--------------------------|-------------------------|
| Betamethasone  | BET     | ![Betamethasone Structure](image1.png) | C_{22}H_{29}FO_{5} | 392.46        | 3.80     | 437.2              | 359.1666                 | 437.1993, 361.1824       |
| Dexamethasone  | DEX     | ![Dexamethasone Structure](image2.png) | C_{22}H_{29}FO_{5} | 392.46        | 3.94     | 437.2              | 359.1666                 | 437.1993, 361.1824       |
| Methylprednisolone | MPLN | ![Methylprednisolone Structure](image3.png) | C_{22}H_{30}O_{5} | 374.47        | 3.65     | 419.22             | 341.1758                 | 419.2019, 313.1812       |
| Prednisolone   | PLN     | ![Prednisolone Structure](image4.png) | C_{21}H_{28}O_{5} | 360.44        | 2.22     | 405.2              | 327.1604                 | 405.1931, 299.1653       |
| Beclomethasone | BCL     | ![Beclomethasone Structure](image5.png) | C_{22}H_{29}ClO_{5} | 408.92        | 4.40     | 453.2              | 453.1689                 | 407.1632, 377.1527       |
| Flumethasone   | FLU     | ![Flumethasone Structure](image6.png) | C_{22}H_{28}F_{2}O_{5} | 410.45        | 3.93     | 455.2              | 379.1726                 | 455.1893, 409.1828       |
| Compound                  | Abbreviation | Molecular Formula | MW | MW0 | MW0a |
|--------------------------|--------------|-------------------|----|-----|------|
| Fluocinolone acetonide   | FCN          | C_{24}H_{30}F_2O_6| 452.48 | 497.2 | 497.2007 | 431.1875 |
| Triamcinolone            | TRI          | C_{21}H_{27}FO_6  | 394.43 | 393.2 | 345.1727 | 325.1444 |
| Dexamethasone-D4         | DEX-D4       | C_{22}H_{25}D_4FO_5| 396.46 | 441.2 | 363.1948 | ---- |
Table 2. Results of the validation study in porcine liver matrix

| Compound | MRL (µg kg⁻¹) | Concentration level (µg kg⁻¹) | Trueness (%) | Precision (RSD%) | CCα (µg kg⁻¹) | CCβ (µg kg⁻¹) | MRL | No MRL | MRL | No MRL |
|----------|---------------|-------------------------------|--------------|-----------------|---------------|---------------|-----|--------|-----|--------|
| BET      | 2ᵃ            | 1                            | 80.1         | 8.3             | 2.3           | 0.16          | 2.6 | 0.27   |
|          |               | 2                            | 78.5         | 11.0            |               |               |     |        |     |        |
|          |               | 10                           | 87.0         | 9.4             |               |               |     |        |     |        |
| DEX      | 2ᵇ            | 1                            | 84.6         | 13.9            | 2.4           | 0.11          | 2.7 | 0.21   |
|          |               | 2                            | 78.4         | 13.5            |               |               |     |        |     |        |
|          |               | 10                           | 85.9         | 10.0            |               |               |     |        |     |        |
| MPLN     | 10ᶜ           | 1                            | 94.0         | 4.4             | 11.1          | 0.04          | 12.2| 0.08   |
|          |               | 2                            | 89.2         | 4.6             |               |               |     |        |     |        |
|          |               | 10                           | 91.9         | 7.4             |               |               |     |        |     |        |
| PLN      | 10ᶜ           | 1                            | 87.2         | 7.2             | 11.2          | 0.04          | 12.4| 0.13   |
|          |               | 2                            | 83.1         | 9.3             |               |               |     |        |     |        |
|          |               | 10                           | 88.9         | 8.4             |               |               |     |        |     |        |
| BCL      | ---           | 1                            | 98.6         | 5.5             | ---           | 0.15          |     |        |     |        |
|          |               | 2                            | 94.0         | 2.9             |               |               |     |        |     |        |
|          |               | 10                           | 94.5         | 8.1             |               |               |     |        |     |        |
| FLU      | ---           | 1                            | 92.6         | 4.6             | ---           | 0.06          |     |        |     |        |
|          |               | 2                            | 92.2         | 4.6             |               |               |     |        |     |        |
|          |               | 10                           | 92.6         | 7.1             |               |               |     |        |     |        |
| FCN      | ---           | 1                            | 93.1         | 5.0             | ---           | 0.10          |     |        |     |        |
|          |               | 2                            | 89.5         | 6.4             |               |               |     |        |     |        |
|          |               | 10                           | 92.0         | 7.4             |               |               |     |        |     |        |
| TRI      | ---           | 1                            | 101.2        | 4.8             | ---           | 0.05          |     |        |     |        |
|          |               | 2                            | 90.9         | 6.2             |               |               |     |        |     |        |
|          |               | 10                           | 89.9         | 6.8             |               |               |     |        |     |        |

a: bovine and porcine liver; b: ovine and porcine liver; c: bovine liver
Fig. 1 LC-MS/HRMS ion chromatograms of an extract from a porcine liver sample spiked with corticosteroids at 1 µg kg⁻¹ level
Fig. 2 LC-MS/HRMS ion chromatograms of a porcine liver sample containing DEX (1.5 µg kg⁻¹)