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Development of a liquid chromatography-tandem mass spectrometry with pressurized liquid extraction for determination of glucocorticoid residues in edible tissues

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ABSTRACT

A multi-residues method using pressurized liquid extraction (PLE) and liquid chromatography combined with mass spectrometry (LC–MS/MS) has been developed for determination of eight glucocorticoids (prednisone, prednisolone, hydrocortisone, methylprednisolone, dexamethasone, betamethasone, beclomethasone, fludrocortisone) in muscle of swine, cattle, and sheep. Parameters affecting PLE extraction including extraction solvent, extraction temperature, extraction pressure and extraction cycles were optimized. The optimized method employed 11 ml extraction cells, hexane–ethyl acetate (50:50, v/v) as extraction solvent, 1500 psi of extraction pressure and 50 °C of extraction temperature. The samples were detected by LC-ESI-MS/MS in negative mode with selected reaction monitoring (SRM) mode. The recovery of glucocorticoids spiked at levels of 0.5–6 μg kg^{-1} ranged from 70.1% to 103.1%; the between-day relative standard deviations were no more than 9.6%. The limits of quantification were 0.5–2 μg kg^{-1} in muscle. The results demonstrated that the method is simple, fast, robust, and suitable for identification and quantification of glucocorticoids residues in foods of animal origin.

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1. Introduction

Glucocorticoids (Fig. 1) are important medicine for possessing anti-inflammatory, antipyretic and immunosuppressive effect in food-producing animals, but they are also frequently employed as growth promoters, which is illegal in China and the European Union. Due to their adverse effect on human health, the use of glucocorticoids in food producing animal is controlled in China and the European Union (EU). Consequently, there is an urgent need to develop comprehensive control measures to monitor residues of glucocorticoids in edible tissue samples.

Many analytical methods have been described in the literature for determination of glucocorticoids in biological samples. GC–MS/MS method for the determination of glucocorticoids is very time-consuming, due to non-volatility [1–5]. Liquid chromatography with ultraviolet (UV) method has low sensitivity and specificity [6–8]. Liquid chromatography coupled with mass spectrometry or tandem mass spectrometry has become the most powerful instrument for determination glucocorticoid residues in cow milk [9–13] and tissues [14–21].

In these analytical methods, sample extraction procedures are still perceived as bottlenecks. Different sample preparation methods have been described for the analysis of glucocorticoid residues in tissues, including liquid-liquid extraction and solid phase extraction [14,16–21]. The main limitations of these methods include consuming large volumes of solvents and spending long time.

Pressurized liquid extraction (PLE) is a sample preparation technique that combines elevated temperature and pressure with liquid solvent to achieve fast and efficient extraction of the analytes from the solid matrix. PLE has been used as robust and time saving alternatives that seem to have the potential to enable automated sample handing and because it was possible to avoid some of the health risks caused by both the analytes and solvent. PLE has been applied in recent year for the analysis different analytes in food samples [15,22–26]. Draisci et al. have described the application of the PLE to the analysis of dexamethasone and betamethasone in bovine liver [15]. But, the method included only one matrix and two compounds. So it would be very preferable to be able to detect glucocorticoid multi-residues in different matrices with PLE.

Therefore, the purpose of this work was to develop a simple and sensitive LC–MS/MS confirmatory method with a PLE to analyze simultaneously the residues of glucocorticoids in different matrices. The chromatographic separation of dexamethasone

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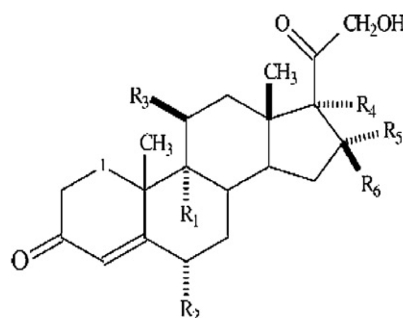

Glucocorticoid	R_1	R_2	R_3	R_4	R_5	R_6
Dexamethasone	-F	-H	-OH	-OH	$-CH_3$	-H
Betamethasone	-F	-H	-OH	-OH	-H	$-CH_3$
Prednisone	-H	-H	=O	-OH	-H	-H
Prednisolone	-H	-H	-OH	-OH	-H	-H
Methylprednisolone	-H	-H	-OH	-OH	-H	-H
Beclomethasone	-Cl	-H	-OH	-OH	-H	$-CH_3$
Hydrocortisone	-H	-H	-OH	-OH	-H	-H
Fludrocortisone	-F	-H	-OH	-OH	-H	-H

Fig. 1. Chemical structures of the glucocorticoids.

and betamethasone using HPLC with C_{18} column also has been achieved. Different variables affecting PLE efficiency including extraction solvent, extraction temperature, extraction pressure, and static cycles have been optimized. Extracts were filtered and directly analyzed by HPLC–MS/MS without further clean-up. The LC–MS/MS accompanied with a PLE would be a simpler, faster, more sensitive and economic method which could be used regulatory tool for monitoring the residues of glucocorticoids in edible tissues.

2. Experimental

2.1. Chemicals and reagents

Dexamethasone (Dexa), betamethasone (Beta), prednisone (Pred), prednisolone (Predl), methylprednisolone (MPredl), and hydrocortisone (HC) were purchased from Dr. Ehrenstorfer (Gmbh, Augsburg, Germany). Beclomethasone (Becl) and fludrocortisones (Flud) acetate were purchased from Sigma–Aldrich (St. Louis, MO, USA). Distilled water was further purified by passing it through a Milli-Q Plus apparatus (Millipore, Bedford, MA, USA). Acetonitrile was obtained from Fisher (Bar-Bel, France). Other solvents of analytical reagent grade included ethyl acetate, hexane, formic acid, and diatomaceous earth (white powder, 0.2–0.4 mm) were supplied by Shanghai Guoyao Company (Shanghai, China). Tissue samples were obtained from a local supermarket. After being homogenized in a high-speed food blender, the tissue samples were stored below −20 °C in a freezer.

2.2. Standard solutions

Individual stock standard solutions were made by dissolving each pure standard in methanol to obtain 1000 $\mu g\,ml^{-1}$ concentration, prepared every 3 months and stored at or below −20 °C. Tuning solution of each analyte (1 $\mu g\,ml^{-1}$) was prepared by diluting individual stock solution with methanol. Standard diluted solution was mixed with acetonitrile/water (10/90, v/v). A working solution and a standard mixture used to fortify the samples were prepared by diluting individual stock solution with diluted solution, which were prepared every 1 month and stored at or below 4 °C.

2.3. Sample preparation

The extraction of glucocorticoids from tissues samples was performed by PLE. A ASE 200 system (Dionex, Sunnyvale, CA, USA), equipped with 11 ml stainless steel cells and lined with glass-fiber filters from Dionex was used for PLE.

Two grams of sample and 3 g diatomaceous earth were mixed and grounded into powder using a pestle. The mixtures were weighed into 11 ml stainless steel cells capped with two glass-fiber filters. Optimum conditions for the PLE method are summarized in Table 1. The extract was evaporated to dryness in a water bath at 40 °C and reconstituted in 500 μl of the standard diluted solution. The resulting solutions were vortexed for 1 min and then centrifuged at 10,000 rpm for 10 min. The upper clear solution was transferred to another sample vial for LC–MS/MS analysis.

2.4. LC–MS/MS analysis

Analyses were performed on a Finnigan HPLC module consisting of a quaternary gradient Surveyor LC pump and a Surveyor AS auto-sampler (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separations were obtained under gradient condition using a Hypersil Gold C_{18} (150 mm × 2.1 mm, 5 μm) column. The column was maintained at a temperature of 40 °C. Mobile phase

Table 1
PLE conditions.

Extraction solvent	Hexane–ethyl acetate (1:1, v/v)
Temperature (°C)	50
Pressure (psi)	1000
Flush volume (%)	60
Static time (min)	5
Number of cycles	2
Cell volume (ml)	11

Table 2
Optimum precursor and product ions with the respective collision energy (eV) for MS/MS.

Compound	Precursor ion $[M+HCOO]^-$ *m*/*z*	Product ion I		Product ion II	
		m/*z*	CE (eV)	*m*/*z*	CE (eV)
Pred	403.0	326.9	20	357.2	11
Predl	405.0	329.0	22	358.8	27
HC	407.1	331.1	16	361.1	11
MPredl	419.0	342.9	19	373.1	16
Dexa	437.0	361.0	15	391.5	10
Beta	437.0	361.0	15	391.5	10
Becl	453.0	406.9	13	376.8	13
Flud	467.1	420.8	13	349.2	26

component A was water, and components B and C were acetonitrile and 0.2% formic acid solution. The mobile phase gradient profile was as follows (t in min): $t_{0'}$, A = 65%, B = 30%; $t_{17'}$, A = 65%, B = 30%; $t_{20'}$, A = 45%, B = 50%; $t_{24'}$, A = 45%, B = 50%; $t_{24.1'}$, A = 65%, B = 30%. The mobile phase was delivered to the LC column at a flow rate of 200 μl min^{-1} and the injected volume was 20 μl.

MS/MS analyses were performed on a TSQ Quantum Access triple quadrupole mass spectrometer (Thermo Fisher Scientific) operated in the negative (ESI^-) electrospray ionization modes. Analysis in negative ion mode was done with nitrogen used as aux gas with pressure of 6 arb. Spray voltage was 4.8 kV. Capillary temperature was set at 330 °C. Argon was used as the collision gas for collision-induced dissociation at a pressure of 35 arb. The precursor ion and two product ions for each target compound are listed in Table 2.

2.5. Quantification and method validation

The method was validated according to the EU Commission Decision 2002/657/EC [27]. The performance characteristics including recovery, precision, limits of quantification and limits of detection, calibration curves and specificity were obtained.

The specificity of the method was evaluated by extracting and analyzing 20 blank porcine muscle samples, 20 blank bovine muscle samples, and 20 blank sheep muscle samples, which were collected from the back muscles of animal. The results were evaluated by the presence of interfering substances around the retention time.

The standard mixtures calibration curves were generated on three different days, at concentration levels from 0.2 to 50 μg l^{-1} for Pred, Predl, MPredl, Dexa, Beta, from 0.4 to 100 μg l^{-1} for Becl and Flud, from 0.8 to 200 μg l^{-1} for HC. Peak area was used for quantification. The calibration curves were obtained by plotting the analyte of the peak area against the concentration. To better reflect the sample conditions and to reproduce matrix effects, the matrix-match calibration curves were obtained by spiking blank control samples of tissue with mixtures of glucocorticoids in the same analytical batch.

Muscles of swine, cattle, and sheep known to be noncompliant were served as blank matrices. Accuracy and precision of the analytical method were calculated by the determination of six replicates of tissue blank samples fortified with mixtures of glucocorticoids at three different spiked levels on three different days.

The LOQ was determined using the signal-to-noise (S/N) equal to 10/1 criterion, while fulfilling the criteria for accuracy and precision. The LOD values were considered as the concentrations giving an S/N of 3.

Glucocorticoids were considered as positively identified according to EU Commission Decision 657/2002. Each compound was quantified by SRM, using the two highest characteristic precursor ion/product ion transitions. The analyte must be required to fulfill a minimum total score of 3 identification points. Comparison of the retention times with the corresponding reference standards also helped to identify the compounds. The ratio of retention time of the analyte in the matrix to the same analyte in standard solution was within ±2.5%. Each analyte ion ratio was effectively measured on each chromatography, corresponding to the less intense signal against the most intense one. During the validation the ion ratios measured on the spiked samples, were compared to those obtained from the calibration curve standards.

3. Results and discussion

3.1. Optimization of the PLE method

In the optimization of the PLE procedure, all important parameters affecting extraction efficiency were evaluated: extraction solvent, temperature, pressure and number of the extraction cycles. At first, various solvent mixtures were tested. Once the optimum solvent was found, the impact of varying the temperature, pressure and number of the cycles were also evaluated.

3.1.1. Extraction solvent

The choice of the extraction solvent is probably one of the most critical parameters in PLE procedure. Usually mixtures of organic solvents as hexane, ethyl acetate, methanol and acetonitrile among them have been applied. The ASE conditions used were as follow: 50 °C of extraction temperature, 10 min of static time, 60% flush volume, with one cycles. The results are shown in Fig. 2. The mixture of hexane/ethyl acetate (50:50, v/v) was selected as extraction solvent because it provided high recovery and cleaner extracts. Thus, an extraction solvent of hexane/ethyl acetate (50:50, v/v) was used for subsequent experiment.

3.1.2. Extraction temperature

Since temperature affects strongly the extraction efficiency, a series of experiments at different temperatures (50–90 °C) was performed to determine the best extraction temperatures. Fig. 3 shows the recoveries of the most analytes were decreased with the improvement of the temperature. The extraction temperature of 50 °C was selected as for further experiment.

3.1.3. Extraction pressure

Three different pressures, 1000, 1800, 2000 psi, were assayed. Recoveries did not improve when values other than the initial one (1000 psi) were used (Fig. 4). The extraction pressure was set 1000 psi for further experiment.

3.1.4. Number of extraction cycles

The number of the extraction cycles was tested to assure a rapid extraction as well as high recovery. The number of extraction cycles was varied between one and three. In general, an increment of the number of extraction cycles allows the exposure of the matrix to fresh solvent and favors the solvent/sample equilibrium, improving partition into the liquid phase and increasing the analytes recoveries. Fig. 5 showed that recoveries increased with the number of the extraction cycles until two. When three cycles were used, the recovery was a bit lower. Two cycles was selected as optimum.

3.1.5. Extraction time

To evaluate if extraction time could influence extraction efficiency, different extraction times (5, 7, 10, 12 min) were performed. The results showed that recoveries did not increase with the static time improvement. So, the static time was set at 5 min.

3.1.6. Flush percentage

The flush percentage refers to the amount of solvent flushed through the cell following the static heating step, expressed as

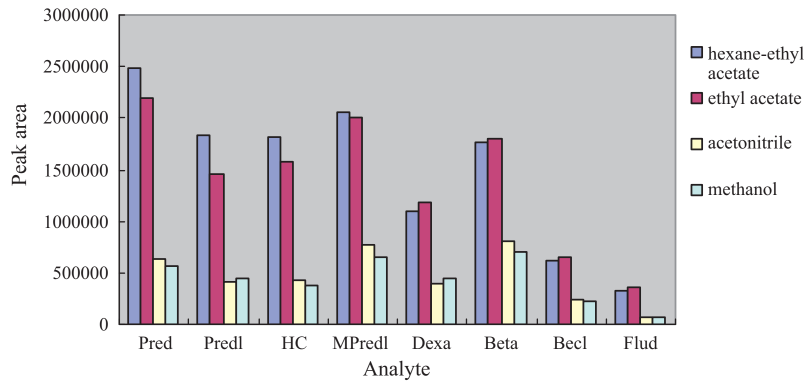

Fig. 2. Effect of extraction solvent on the PLE extraction of glucocorticoids from porcine muscle. Temperature: 50 °C; pressure: 1500 psi; 1 cycle of 5 min.

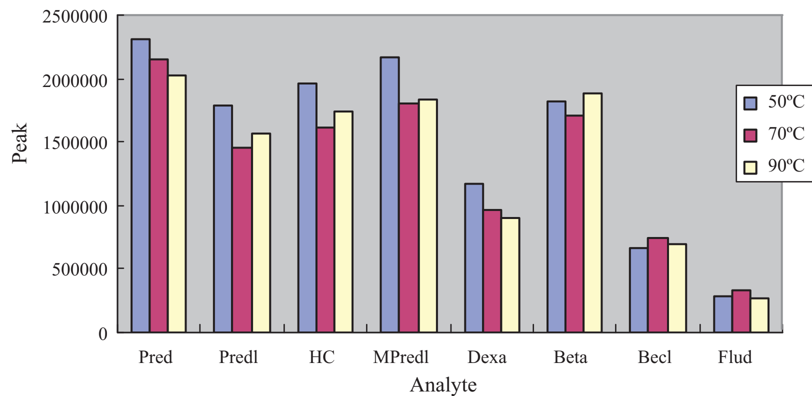

Fig. 3. Effect of extraction temperature on the PLE extraction of glucocorticoids from porcine muscle. Solvent: hexane–ethyl acetate (50:50, v/v,); pressure: 1500 psi; 1 cycle of 5 min.

a percentage of the cell volume. Increasing the flush volume allows more solvent to pass through the sample, bust it also increase the final volume for the extract. The flush volume (20, 40, 60, 80%) did not significant affect the extraction efficiencies of the analytes. So the flush volume was set at their default values (60%).

3.2. Comparison of PLE and shaking extraction

The PLE process takes advantage of the increasing solubility of analyte at temperatures and pressures well above the common, raising the diffusion rate and decreasing the viscosity and surface tension, so the kinetic processes for analytes desorbing from the matrix are accelerated. The extraction efficiency of PLE for glucocorticoids was compared with those obtained by shaking extractions. The relative yields of the glucocorticoids extracted from edible tissues samples were compared (Table 3). PLE extraction was done within about 30 min, while shaking extraction required about 2 h. The extraction efficiency of PLE was slightly higher than those of shaking extraction, and PLE has the advantage of shorter extraction time than the other, which is the main advantage of PLE over shaking extraction. PLE was conducted automatically, using less solvent. In addition, the defatting step was simultaneously to the extraction of target drugs in PLE, whereas, in the shaking the steps were posterior to the extraction of target drugs using liquid-liquid partition.

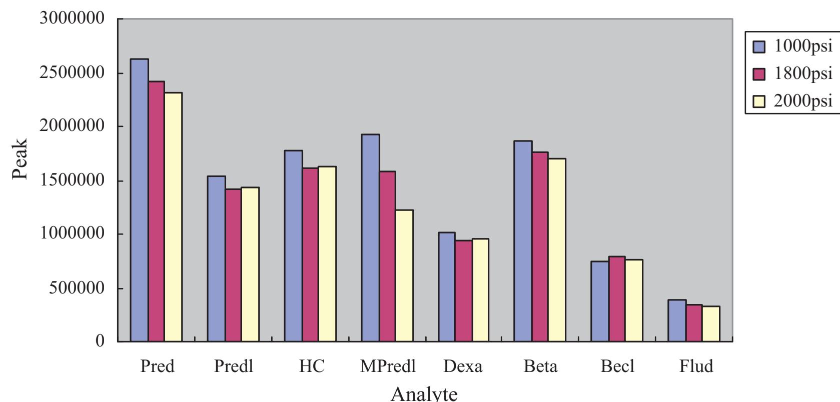

Fig. 4. Effect of extraction pressure on the PLE extraction of glucocorticoids from porcine muscle. Solvent: hexane–ethyl acetate (50:50, v/v); temperature: 50 °C; 1 cycle of 5 min.

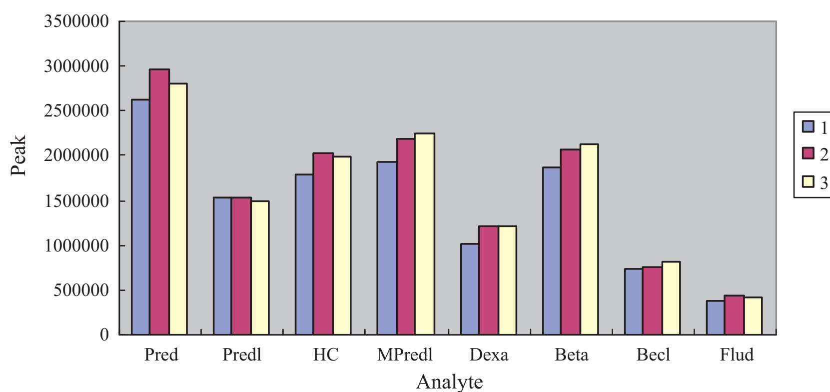

Fig. 5. Effect of extraction cycles on the PLE extraction of glucocorticoids from porcine muscle. Solvent: hexane–ethyl acetate (50:50, v/v); temperature: 50 °C; pressure: 1500 psi.

Table 3
Relative amounts of glucocorticoids in edible tissues by ASE and shaking extraction methods.

Parameters	ASE	Shaking extraction
Extraction solvent	Hexane–ethyl acetate (1:1, v/v)	ethyl acetate or acetonitrile
Extraction time	30 min	2 h
Extraction solvent volume	20 ml	20–40 ml
Defatting method	ASE	LLE
Defatting solvent	Hexane	Hexane
Automatic/manual	Automatic	Manual
Inter-day RSD	Low	High
Capital investment	High	Low

3.3. Matrix effect

An investigation of possible matrix effects on the ionization of analytes was carried out by comparing standard and matrix-matched calibration curves for each analytes. Calibrated solutions for standard calibration curves were prepared in the LC mobile phase and calibrate solutions for matrix-matched calibration curves were prepared in blank matrix extracts. The *t*-test was applied to statistically evaluate the slope differences between standard and matrix-matched calibration curves, giving a significance alpha level of 0.05 with 6 degrees of freedom. Under these conditions, the critical *t*-value is 2.4. For *t*-values higher than 2.4 the slope difference is considered statistically significant, i.e. considerable matrix effects occur. The obtained *t*-values did not reveal significant ion suppression for glucocorticoids in tissues. Therefore, standard calibration curves were used for all the glucocorticoids tested.

3.4. Method validation

The linearity of the standard mixtures was good for all analytes in the whole range of tested concentrations, as proved by the correlation coefficients being greater than 0.999 for all curves. For each compound three matrix-match calibration of different tissues standard curves were calculated. There were no significant differences between the curves. The correlations (*r*) with each calibration curves are higher than 0.99.

After comparing with the background noise in various matrices, the results showed that, there were no interference peaks that could be detected on the expected retention time for these target analytes (Fig. 6).

Table 4
The validation results of glucocorticoids in tissues.

Analyte	Fortified concentration (μg kg^{-1})	Average recovery (%, $n = 18$)			Inter-day RSD (%, $n = 18$)		
		Porcine muscle	Bovine muscle	Sheep muscle	Porcine muscle	Bovine muscle	Sheep muscle
Pred	0.5	84.7	81.1	82.8	8.2	8.5	9.2
	1	90.6	87.2	86.5	7.8	8.0	8.3
	2	89.5	91.1	86.3	6.5	7.2	7.8
Predl	0.5	74.1	70.1	72.6	9.1	8.3	8.8
	1	78.6	75.7	72.5	7.2	7.5	7.9
	2	79.2	77.5	73.9	8.3	7.4	8.3
HC	2	90.6	88.5	92.7	9.6	8.7	9.1
	4	92.3	90.2	89.4	9.1	8.9	8.5
	8	103.1	94.6	97.6	8.4	8.2	8.2
MPredl	0.5	82.0	84.2	79.3	7.3	7.6	7.0
	1	85.6	81.8	82.9	7.5	7.5	7.2
	2	88.9	85.6	84.5	6.6	6.9	7.3
Dexa	0.5	73.7	75.9	71.3	8.4	8.7	8.1
	1	75.6	77.4	73.6	7.5	7.2	7.9
	2	77.8	74.4	73.2	7.0	6.9	7.4
Beta	0.5	75.0	71.4	72.1	8.0	7.5	7.9
	1	78.2	72.5	74.8	7.3	7.3	7.5
	2	79.3	73.9	75.7	7.1	6.9	7.4
Becl	1	71.4	75.8	73.4	8.9	9.1	8.2
	2	72.3	74.0	77.2	8.1	8.3	8.5
	4	75.6	78.1	75.3	7.5	7.7	7.3
Flud	1	72.1	70.3	73.2	7.8	7.2	8.1
	2	75.6	75.6	70.8	7.1	6.8	7.5
	4	76.2	73.2	74.6	7.4	7.6	7.3

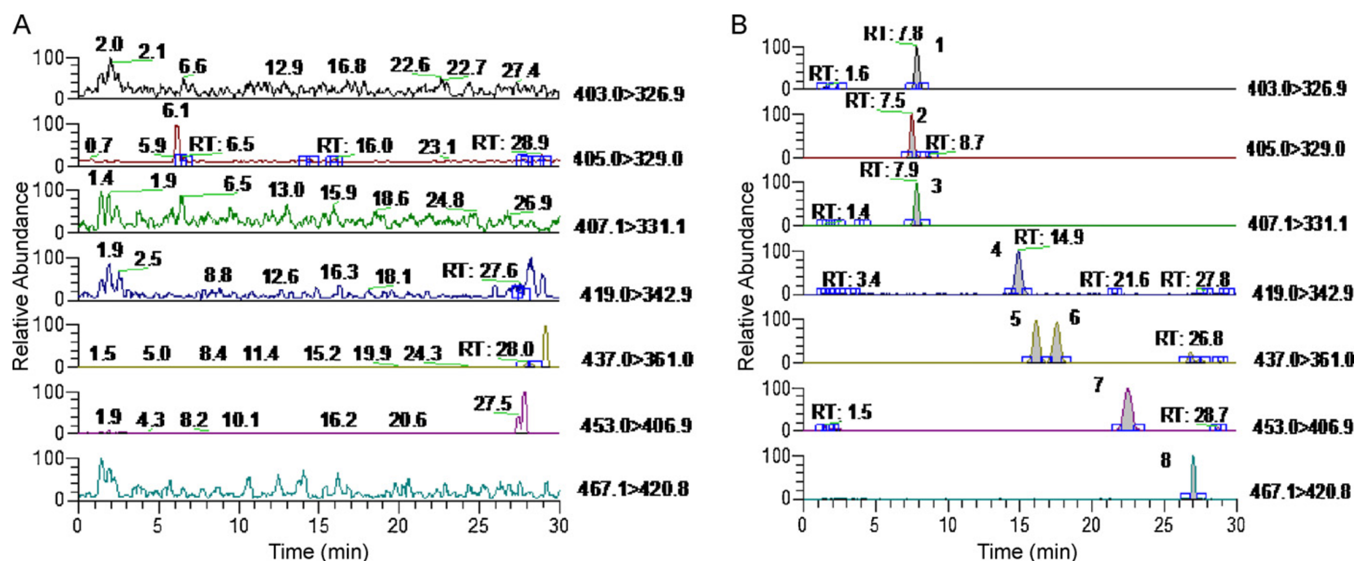

Fig. 6. Selected reaction monitoring LC–MS/MS chromatograms of blank porcine muscle (A) and spiked porcine muscle (B) (0.5 μg kg^{-1} for Pred, Predl, Mpredl, Dexa, Beta; 1 μg kg^{-1} for Becl and Flud; 2 μg kg^{-1} for HC). Peak number: 1 Pred; 2 Predl; 3 HC; 4 Mpredl; 5 Dexa; 6 Beta; 7 Becl; 8 Flud.

Table 5
The ion ratios of glucocorticoids in different tissues.

Compound	Product ions	Standard solution Ion ratio	Spiked muscle samples Ion ratio
Pred	326.9/357.2	0.291 ± 0.004	0.289 ± 0.006
Predl	329.0/358.8	0.091 ± 0.007	0.075 ± 0.006
HC	331.1/361.1	0.165 ± 0.005	0.143 ± 0.008
MPredl	342.9/373.1	0.086 ± 0.006	0.071 ± 0.010
Dexa	361.0/391.5	0.075 ± 0.006	0.055 ± 0.008
Beta	361.0/391.5	0.105 ± 0.008	0.082 ± 0.007
Becl	376.8/406.9	0.996 ± 0.005	0.923 ± 0.008
Flud	420.8/349.2	0. 215 ± 0.006	0.183 ± 0.008

Recovery experiments were performed by comparing the analytical results of extracted veterinary drug from fortified whole tissue samples with unextracted standards prepared at the same concentrations in blank extract representing 100% recovery. The results are summarized in Table 4.The overall recoveries ranged from 70.1% to 103.1%; and the RSD values were all below 9.6%, demonstrating the good recovery and precision of the method.

The LOQ of the examined glucocorticoids extracted from edible tissues ranged from 0.5 to 2 μg kg^{-1} in muscle. The LOD of glucocorticoids ranged from 0.2 to 1 μg kg^{-1} in muscle.

For the confirmation of the presence of glucocorticoids residues their identity could be determined according to EU Commission Decision 657/2002 with a minimum total score of 3 identification points. Since one Precursor ion and two product ions were monitored this requirement is fulfilled. Each analyte ion ratio was shown in Table 5. The calculated results from this work are in compliance with EU Commission Decision 657/2002.

3.5. Analysis of incurred tissue samples

This method has been applied for daily analysis of 40 real samples collected from markets including 20 porcine muscles and 20 bovine muscles. Only HC has been detected in 2 bovine muscles samples, the concentrations of which were 5.26 μg kg^{-1} and 3.29 μg kg^{-1}. Results indicated that this method has proved to be suitable for identifying violate samples of glucocorticoids in tissues.

4. Conclusions

The major goal of this research was to investigate for the first time the suitability of ASE for the extraction of eight glucocorticoids from edible tissues. This method allows the extraction for the analytes without purification, and it has the main advantage of reducing analytes losses during sample handling, time required for the analytical procedure and costs for material and manpower. The identification and quantification of multiple glucocorticoid residues in the edible tissues were successfully achieved using LC–MS/MS. The method has satisfactory validation characteristics with respect to specificity, accuracy, precision, and sensitivity. Therefore, we conclude that this LC–MS/MS method is suitable for the routine determination of glucocorticoid residues in edible tissues.

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