# High-Performance Liquid Chromatography–Tandem Mass Specrometry Validation of Medroxyprogesterone Acetate in Products of Pork Origin and Serum

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## Abstract

**Different extraction and purification methods are described here to determine medroxyprogesterone acetate (MPA) in pork meat and serum. Spiked samples are investigated over the concentration range of MPA 0.5–20 ng/g. Pork meat tissues are subjected to extraction using organic solvent, and pork serum is simply diluted with acetate buffer. Clean-up is performed using solid-phase extraction on a C18 cartridge, and MPA is eluted with ethanol. Aliquots are injected into a high-performance liquid chromatography–mass spectrometry system. MPA content is determined on the basis of *m/z* 387–327 and 387–123 transitions.**

## Introduction

Medroxyprogesterone acetate (MPA) is a synthetic analogue of the natural steroid hormone progesterone. In veterinary medicine, MPA is used in the synchronization and induction of oestrus in sheep. After therapeutic treatment, MPA is no longer detectable in plasma; tissue residues are higher in fat. The use of MPA and other anabolic steroids as growth promoters in farm animals is banned under European Union Regulations because of their possible toxic effect on public health (1).

Residues of MPA were found in food products of pork origin, animal feed, and of soft drinks in some European countries during 2002. The source of MPA contamination has been traced to waste water from an Irish pharmaceutical factory. The waste water was exported to a Belgian company and mixed into pig feed (2). As a result of this food scare, the European Union placed the MPA-exposed farms under official control. The European Commission established a minimum required performance limit (MRPL) for MPA at 1 ng/g in the control of kidney fat (3). In order to monitor the presence of MPA residues in biological samples and food products, the development of sensitive and specific analytical methods has been a goal. The result of a contamination episode that occurred in the Netherlands was the development of an analytical method to detect the presence of residues of MPA, which endangers public health, in serums and pork products. The present work describes a confirmatory method for detecting MPA residues in food and biological samples by the use of liquid chromatography (LC)–mass spectrometry (MS)–MS with atmospheric pressure ionization under positive chemical ionization (APICI) in multireaction monitoring mode (MRM).

Different extraction and purification methods of serum and fresh pork meat samples have been checked using spiked samples, both on serum and tissue (fat), at concentration levels of MPA ranging between 0.5 and 20 ng/g (ng/mL).

Many workers have used enzyme-linked immuno assay and enzyme-linked immuno sorbent assay (ELISA) because of their easy and fast application. These techniques have been tested to be very sensitive. Nevertheless, the cross-reactivity of the used antibodies with compounds having analogue structural molecules precludes an unambiguous identification of the compound in the complex matrix of biological sample.

As consequence, more specific analytical techniques are mandatory for confirmation purposes, such as gas chromatography (GC)–MS and LC–MS techniques.

GC–MS-based methods, although very sensitive, are time consuming because they often require one or more derivatization steps to improve the low volatility and specificity of the searched analytes. LC–MS is faster because it requires no chemical treatment of the extract and can be easily performed directly on the raw material (4,5).

Residues of MPA can be analyzed underivatized and detected as a protonated molecular ion $[M+H]^+$ with LC–MS. It is possible to perform very sensitive and selective analyses using LC–MS–MS in MRM mode.

## Experimental

### Chemicals, reagents, and standards

All chemical and chromatographic reagents used were of analytical or high-performance liquid chromatographic grade. Acetonitrile, methanol, ammonium acetate, formic acid, and sodium hydroxide were obtained from J.T. Baker (Deventer, the

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Netherlands) and water from a Milli-Q system (Millipore, Bedford, MA). MPA was from Sigma (St Louis, MO). Tris buffer solution was from Carlo Erba (Milan, Italy).

### Standard solution

Stock standard solutions of MPA and methyltestosterone (MT) were prepared by dissolving 10 mg of pure compound in 10 mL of methanol to obtain a final concentration of 1 mg/mL. These solutions were stored at –20°C and checked to remain stable for at least 4 weeks. Working standard solutions were prepared daily in the range of 5.00–100.00 ng/mL by diluting the standard stock solutions with the mobile phase.

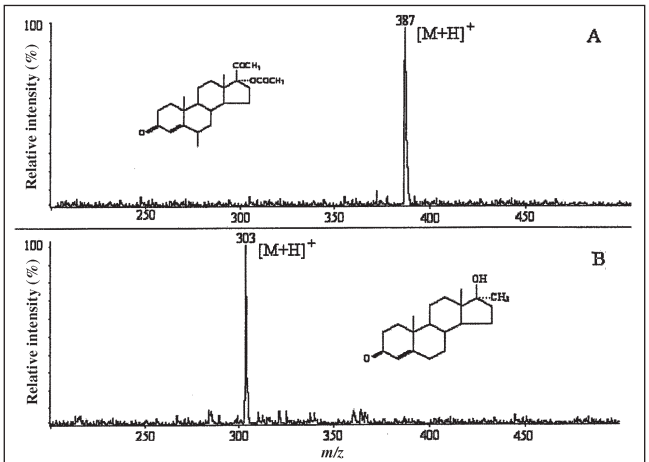


**Figure 1.** LC–MS analysis for product ion mass spectra of MPA (A) and MT (B).

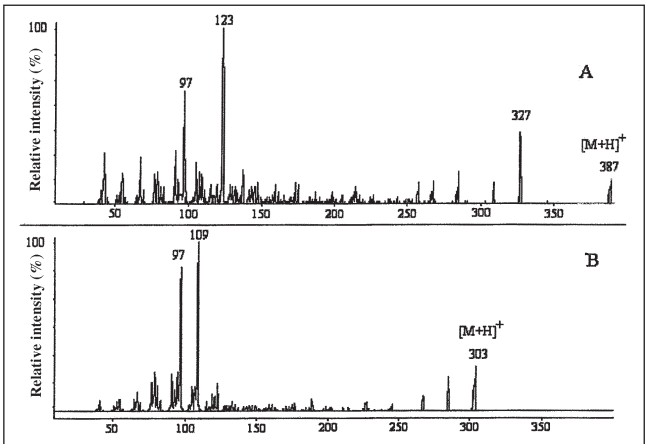


**Figure 2.** LC–MS analysis for product ion mass spectra of MPA (A) and MT (B).

### Sample collection and storage conditions

Samples were collected as part of the Official Control National plan for monitoring drug residues in food of animal origin and they were stored at –20°C. The blank samples were tested with the ELISA method for method validation.

### Serum sample treatment

Serum (3.0 mL) was added to 3.0 mL of 0.15M acetate buffer solutions (ABS), and the mixture was sonicated in an ultrasonic bath for 10 min. Clean-up by solid-phase extraction (SPE) was carried out using a $C_{18}$ cartrige ($C_{18}$, 500 mg, 3 mL) (J.T. Baker) which was previously conditioned with 3.0 mL of methanol and 3.0 mL of water. After sample loading, the cartridge was washed with 5.0 mL of ABS, 5.0 mL of water, and 2.0 mL of methanol–water (40:60 v/v). Finally, the analyte was eluted from the column with 3.0 mL methanol, and the organic phase was evaporated under nitrogen stream at 40°C. The dry residue was dissolved in 300 µL of methanol–water (50:50 v/v), and 5 µL of this solution was injected into the LC–MS for MRM analysis.

### Product of pork origin: sample treatment

Fat (10 g) was obtained from a pork product by heating for 1 h at 50°C until the fat was melted; 1 g of melted fat was weighed into a centrifuge tube, and 15 mL of petroleum ether was added and shaken overnight at 40°C.

The solution was cooled at –20°C for 15 min, transferred to centrifugation tubes, and finally centrifuged at 3500 rpm for 15 min. The cleaned extract was dried under a nitrogen stream at 40°C. The dry residue was dissolved in 2 mL of methanol, then it was strongly shaken for 20 s. The solution was centrifuged and diluted with 5 mL of distilled water.

Clean-up of the solution was performed using an SPE $C_{18}$ cartridge (J.T. Baker $C_{18}$, 500 mg, 3 mL), which was conditioned with 3.0 mL of methanol and 3.0 mL of tris buffer. After sample

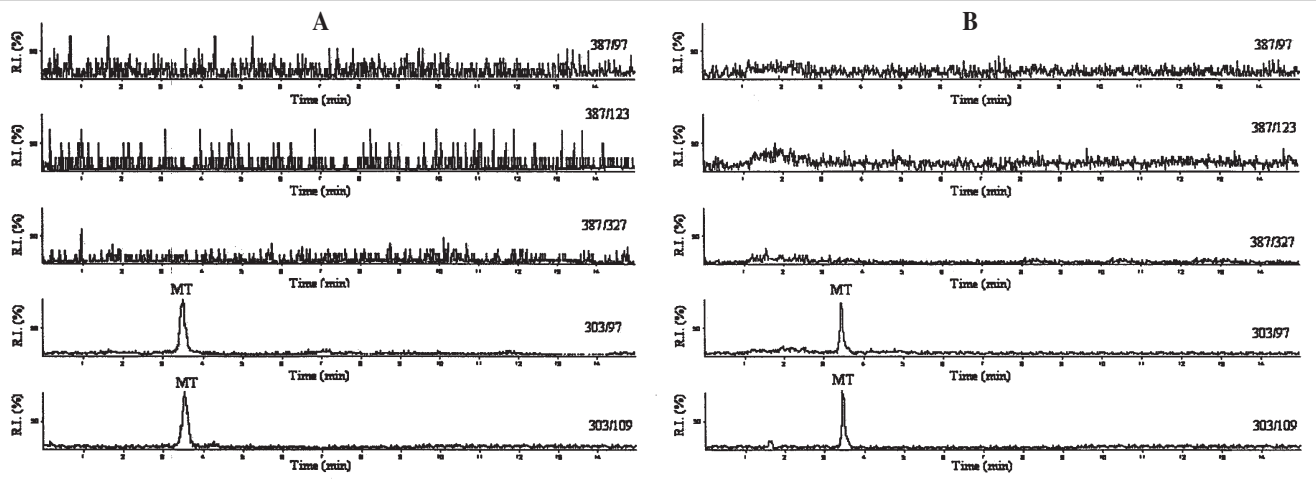


**Figure 3.** LC–MS–MS analysis for blank pork serum sample (A) and blank pork food product sample (B).

loading, the cartridge was washed with 3.0 mL of tris buffer and 2.0 mL of 40% methanol. MPA residues were eluted from the column with 3.0 mL methanol and dried under nitrogen stream at 40°C. The dry residue was dissolved in 0.1 mL of methanol–water (50:50 v/v), and 5 µL of this solution were injected into the LC–MS for MRM analysis.

### HPLC–MS system and operating conditions

The HPLC system consisted of an isocratic solvent delivery pump (PerkinElmer, Shelton, CT). A series 200 pump LC equipped with a Valco (Houston, TX) injection valve fitted with a 5-µL sample loop was used. Separation of MPA was carried out on a column packed with $C_{18}$ Zorbax Bonus-RP (150 × 2.1 mm, 5 µm) from Hewlett-Packard (Palo Alto, CA) at room temperature, operated under isocratic conditions with a mobile phase of 1% formic acid–methanol–acetonitrile (30:10:60, v/v/v), at a flow rate of 0.250 mL/min.

Analyses were performed on an API 2000 tandem MS (Applied Biosystems, Foster City, CA) equipped with an APCI source and a heated nebulizer interface. The LC–MS interface was set at 380°C, discharge current was set at 3 µA, and orifice potential voltage was set at 70 V.

## Results and Discussion

In the present work, the LC–MS technique was used to achieve the unambiguous identification of medroxiprogesterone in serum and food of pork origin. Flow injection mode (FIA) was used to optimize tandem MS operative condition of ionization. The best sensitivity was achieved using a mobile phase containing 1% formic acid, 70 V orifice potential, and heated nebulizer temperature of 380°C.

Figure 1 shows a full-scan (mass range *m/z* 200–500) FIA–MS spectra of MPA and MT, used as internal standard (IS). The analytes show an intense signal of $[M+H]^+$ ion at *m/z* 387 for MPA and 303 for MT. Tandem MS was therefore used in order to obtain additional structural information about detecting diagnostic product ions by performing collision-induced dissociation of the precursor ion [M+H]+.

Figure 2 shows the positive product ion mass spectra of MPA and MT. A precursor-product ion combination of *m/z* 387/327, 387/123, and 387/97 for MPA and *m/z* 303/109 and 303/97 for MT were used, according to the MRM technique.

In order to achieve the targeted analyses and maximum sensitivity, as well as for quantitative purposes, MRM LC–MS–MS analyses were performed using an analytical chromatographic column $C_{18}$ Zorbax Bonus-RP (150 × 2.1 mm, 5 µm) from Hewlett-Packard at room temperature, operated under isocratic conditions with a mobile phase of 1% formic acid–methanol–acetonitrile (30:10:60, v/v/v) at a flow rate of 0.250 mL/min. Under these conditions, the retention time of MT and MPA were 3.4 and 5.2 min, respectively.

Specificity of the MRM LC–MS–MS method was checked by processing 20 independent serum samples and 20 independent fat blank control samples. No interference was observed around the retention time of MPA in both matrices (as shown in Figure 3).

Figure 4 shows chromatograms of MPA-contaminated serum and food product samples and the relative retention time of the analyte with results obtained with standard samples; also, the corresponding MRM mass spectra agrees with the spectrometric detection criteria of Commission Decision 2002/657/EC (6).

Table I shows the decision limit (CCα) and the detection capability (CCβ) values calculated in accordance with European Commission Decision 2002/657/EC. The decision limit is, in the case of banned substances, the lowest concentration at which the method can discriminate with a statistical certainty of 1-α whether the identified analyte is present. For these substances, the alpha (α) error shall be 1% or lower (α error means the probability that the tested sample is compliant, even though a noncompliant measurement has been obtained: false noncompliant decision). CCα was determined by analyzing 20 independent samples and calculating the signal in the time window in which the analyte is expected. Three times the signal-to-noise ratio was used for the determination of CCα. The CCβ is the lowest concentration at which the method is able to detect truly contaminated samples with a statistical certainty of 1-β. For banned substances, the beta (β) error shall be 5% or lower, and it means the probability that the tested sample is truly noncompliant, even though a compliant measurement has been obtained (false

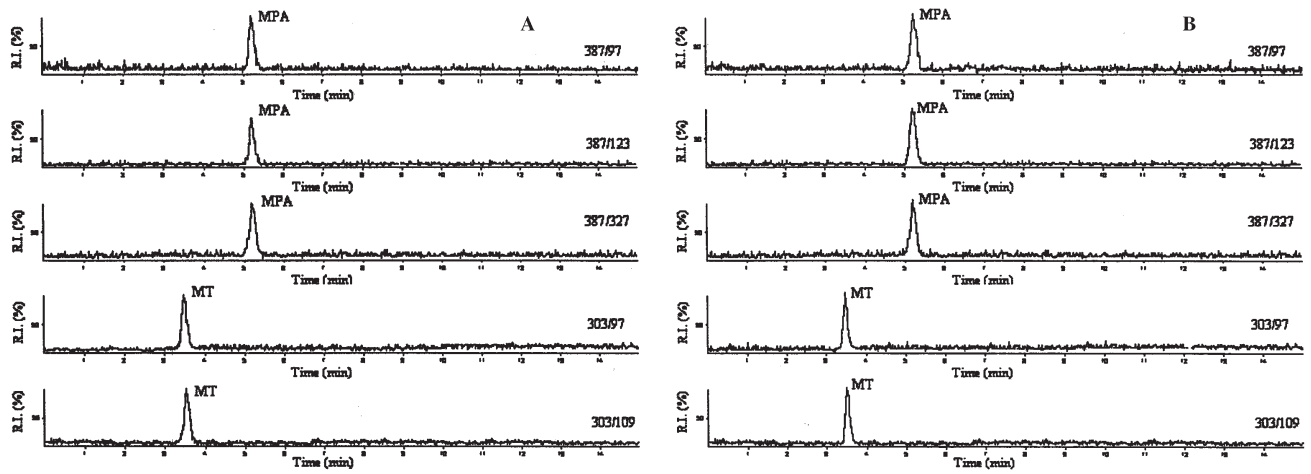


**Figure 4.** LC–MS–MS analysis for positive pork serum sample (A) and positive pork tissue (fat) sample (B).

**Table I. Values of CCα and CCβ**

| Matrix | CCα | CCβ |
|---|---|---|
| Serum | 0.5 ng/g | 0.7 ng/g |
| Tissue (fat) | 0.6 ng/g | 0.9 ng/g |

**Table II. Recovery and Within-Laboratory Reproducibility for MPA in Serum and Fat of Pork Food Product Samples**

| | | Spiked level (ng/g) | | |
|---|---|---|---|---|
| Matrix | Parameter | 1.0 | 1.5 | 2.0 |
| Serum | Recovery (%) | 75.3 | 77.9 | 78.4 |
| | Reproducibility (RSD, %) | 12.3 | 10.2 | 10.7 |
| | $n = 18$ | | | |
| Fat | Recovery (%) | 71.2 | 73.6 | 72.5 |
| | Reproducibility (RSD, %) | 14.4 | 13.6 | 11.9 |
| | $n = 18$ | | | |

**Table III. MPA Concentration in Pork Food Product Samples Tested by LC–MS–MS**

| Samples | Concentration of MPA (ng/g) |
|---|---|
| 1 | 1.2 |
| 2 | 3.6 |
| 3 | 4.3 |
| 4 | 6.1 |
| 5 | 2.2 |
| 6 | 1.5 |
| 7 | 3.7 |
| 8 | 3.9 |
| 9 | 1.3 |
| 10 | 2.4 |
| 11 | 3.1 |
| 12 | 2.8 |

compliant decision). Twenty independent blank samples were spiked at the CCα level for both matrices and analyzed for the determination of CCβ.

Table II shows the data of recoveries and reproducibility of the developed method, calculated within 1 day for 3 different days by analyzing blank samples spiked at three different concentrations (1.0, 1.5, and 2.0 ng/g), and six replicates at each concentration were analyzed each day. The mean recovery value of the method was 77% for MPA in serum and 72% for MPA in pork tissue (fat). The relative standard deviation (RSD%) at each fortification level for MPA in serum was less than 12% and, in pork tissue (fat), less than 14%, showing an excellent method precision.

Table III shows the measured concentration of MPA, ranging between 1.2 and 6.1 ng/g, of the positive samples detected in approximately 3% of the investigated samples (up to 400). This means that the developed method was qualitatively and quantitatively suitable for the analysis of MPA and also close to the MRPL limit. The stability assessment of MPA residues in the matrix was carried out by comparing their resulting concentration up to 4 weeks, storing selected matrices at –20°C. No degradation phenomena were observed during identification or quantitation analysis (or both).

Methods have been validated in agreement with Decision 2002/657/EC measuring the decision limit CCα < 0.5 ng/g (ng/mL) and the detection capability CCβ < 1 ng/g (ng/mL) for the different matrices under consideration.

## Conclusion

The present aim was to develop a confirmatory method to detect the presence of illegal and dangerous anabolic residues in food products of pork origin and in animal breeding according to Commission Decision 2002/657/EC (6). CCα and CCβ lower than 1 ng/g (ng/mL) were possible because of the sample clean-up. This method was validated in accordance with the criteria of Commission Decision 2002/657/EC and is used in routine analysis in our laboratory. Because of the ease of the method and its high sensitivity and specificity for quantitative and qualitative confirmation, it is applicable in official control laboratories in order to protect consumer health.

## References


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