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Short communication

Simultaneous analysis of 17α-estradiol and 17β-estradiol in bovine serum by liquid chromatography–tandem mass spectrometry

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ABSTRACT

A new LC–MS/MS method for the separation, identification and quantification of residues of 17α-estradiol (17α-E2) and 17β-estradiol (17β-E2) in bovine serum is reported. Deuterium-labelled 17β-estradiol was used as internal standard. The method was in-house validated in accordance with European Union criteria and adopted in a proficiency study organised by the Community Reference Laboratory (CRL-RIVM, Bilthoven, The Netherlands). The analytes were extracted from serum using acetate buffer, purified by C18 solid-phase extraction (SPE) and chromatographed on a C18 LC column. They were then ionized in a heated nebulizer (HN) interface operating in negative ion mode, where only intact deprotonated molecules, $[M-H]^-$, were generated at m/z 271 and 274 for 17α/17β-E2 and 17β-E2-d_3, respectively. The decision limits obtained (CCα, i.e., critical concentration alpha) were 0.06 ng/mL and 0.03 ng/mL, respectively for 17α-E2 and 17β-E2. Detection capability (CCβ, i.e., critical concentration beta) values were 0.08 ng/mL and 0.04 ng/mL, respectively, for 17α-E2 and 17β-E2. Precision, accuracy and specificity were satisfactory, recovery ranged from 86.3% to 93.2% and the method resulted sensitive for the required purposes. This method is currently in use for Official Control purposes.

1. Introduction

Estradiol (1,3,5(10)-estratrien-3,17β-diol), estrone (1,3,5(10)-estratrien-3-ol-17-one) and estriol (1,3,5(10)-estratrien-3,16a-17β-triol) are endogenous estrogenic steroids (Scheme 1) that modulate the differentiation, growth and physiology of reproductive organs [1,2]. Through the estrogenic receptor α (ERα) and estrogenic receptor β (ERβ), which are members of the nuclear receptor superfamily of transcription factors, estradiol also affects bone tissue, liver, brain and the cardiovascular system. 17β-Estradiol (17β-E2) is used in prophylactic therapy of heart disease, osteoporosis and for alleviation of menopausal symptoms. Antiestrogens are useful in the treatment of advanced breast cancer by targeting it in a different manner with respect to aromatase inhibitors which block the conversion of testosterone to estradiol (Scheme 1). 17α-Estradiol (17α-E2) is endowed with lower activity; nonetheless, recent findings elucidating the complexity of hair growth modulation by estrogens suggested that a key to more effective hair growth manipulation with ER ligands lies in the use of selective ERα or β antagonists/agonists [3].

Furthermore, natural and synthetic steroid hormones are among the compounds involved in endocrine disruption [4]. They may act to interfere with the normal endocrine function causing the development of hormone-related carcinomas in humans [5]. Thus, the potent steroid hormones that are potentially present in foodstuff as residues (either as pharmacologically active substances or as active metabolites) are of particular concern. Among the European Union member states, special attention and highly sensitive analytical methods in various matrices are required [6].

Liquid chromatography coupled with atmospheric pressure chemical ionization (APCI)–tandem mass spectrometry (LC–MS/MS) represents the best choice for the quantification of estrogens at very low concentrations. Our laboratory participated in a CRL-RIVM proficiency study (estradiol in samples of bovine serum) requesting the determination and quantification of 17α/17β-E2 in bovine serum [7]. While the separation of estradiol diastereoisomers has been reported [8], and there are several methods for the analysis of 17β-E2 [9,10], to the best of our knowledge

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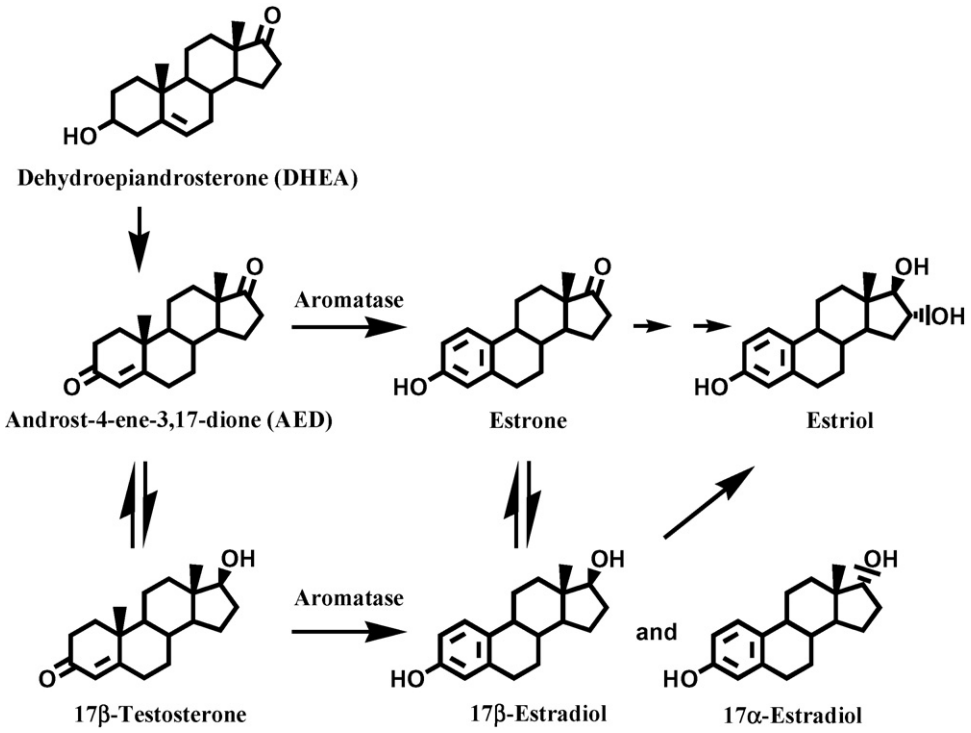

Scheme 1. Endogenous estrogen steroids with their biosynthetic relationships.

there have been no reports on their chromatographic separation and quantification in bovine serum.

The aim of the present work was to develop a rapid, suitable and simple LC–MS/MS method for the quantification of naturally occurring 17α/17β-E2 in bovine serum [11]. The optimized analytical method was applied in the CRL-RIVM proficiency study [7], and resulted reliable for Official Control purposes.

2. Experimental

2.1. Chemicals and reagents

All solvents were HPLC or analytical grade and were purchased from Riedel-de Haen (Seelze, Germany). Ultrapure water was dispensed from a Milli-Q purification system (Millipore, Bedford, MA, USA). 17α-E2 and 17β-E2 were purchased from Sigma–Aldrich (St. Louis, MO, USA) and the internal standard (I.S.) 17β-E2-d_3 from the CRL-RIVM (Bilthoven, The Netherlands). Individual standard stock solutions of 17α-E2 and 17β-E2 at 1 mg/mL were prepared in methanol and stored at −20 °C. Standard calibration solutions in the range 0.06–1.0 ng/mL for 17α-E2 and 0.03–1.0 ng/mL for 17β-E2 were prepared by dilution of the individual standard stock solutions with methanol.

2.2. Sample collection and extraction

Serum samples were collected both from cattle at slaughterhouses within the National Residue Control Plan (NRCP) for Italy and from a proficiency study organised by RIVM and stored at −20 °C until the time of analysis.

The sample preparation was performed as previously described by the same authors [10]. Serum samples were extracted by acetate buffer solution, purified by solid-phase extraction (SPE) using a C18 cartridge (SPE, Bakerbond C18, 500 mg, 3 mL, J.T. Baker, Holland) and eluted with methanol. The extracts were reconstituted with 50 μL of methanol and 5 μL were automatically injected into the LC–MS/MS system.

2.3. Equipment LC–MS/MS and chromatographic conditions

Mass spectral analysis was carried out using an API 3000 triple quadrupole mass spectrometer (AB Sciex Instruments, Foster City, CA, USA), equipped with an atmospheric pressure chemical ionization and heated nebulizer (HN) source set in negative ionization mode and an LC system PerkinElmer Series 200 Micro Pump (PerkinElmer, USA) with a PE Series 200 autosampler.

Nitrogen was used for the curtain (set at 12, an arbitrary unit for analysis, −60 psi) and collision gases (set at 7–60 psi), and air was used for the nebulizer (8.0 L/min–80 psi) and auxiliary gases (4.0 L/min–80 psi). The source block temperature was set at 400 °C and the needle current was set at −3 μA.

The declustering potential and the dwell time for each transition reaction were set at −48 V and 330 ms, respectively. Data acquisition was performed using the AB Sciex Analyst 1.4.1 software in negative multiple reaction monitoring (MRM) alternating three transition reactions (m/z 271 → 145 and m/z 271 → 183 for both 17α-E2 and 17β-E2 and m/z 274 → 145 – the most abundant ion – for 17β-E2-d_3). Identification of the analytes was achieved according to the criteria of the Commission Decision 2002/657/EC [12].

The chromatographic separation of the analytes was performed by gradient elution (26 min) on a Gemini column (Phenomenex, USA) (150 mm × 2.0 mm i.d., 5 μm) equipped with a C_{18} guard column (4 mm × 2 mm i.d.) (Security Guard, Phenomenex, USA). Mobile phases were 0.21 mM NH_4OH (pH 9.8) (A) and acetonitrile (B). Flow rate throughout the chromatographic analysis was 0.2 mL/min and the following gradient was applied: 0 min, 90% A; 2.0 min, 90% A; 20 min, 95% B; 21 min, 90% A; and

26 min, 90% A. The column was regenerated for 5 min before injections.

2.4. Calibration and quantitation

Matrix calibration curves were prepared daily by spiking blank bovine serum samples (2 mL) with 0.2 ng/mL of I.S. followed by mixtures of 17α-E2, 17β-E2 and 17β-E2-d_3 to obtain concentrations in the range of critical concentration alpha (CCα), 1 ng/mL (i.e., 0.03–1 ng/mL for 17β-E2 and 0.06–1 ng/mL for 17α-E2). The curves were constructed by plotting peak area ratios of the analyte to I.S. versus hormone concentrations using a least-squares linear regression model. Estimations of the amounts of analyte in fortified

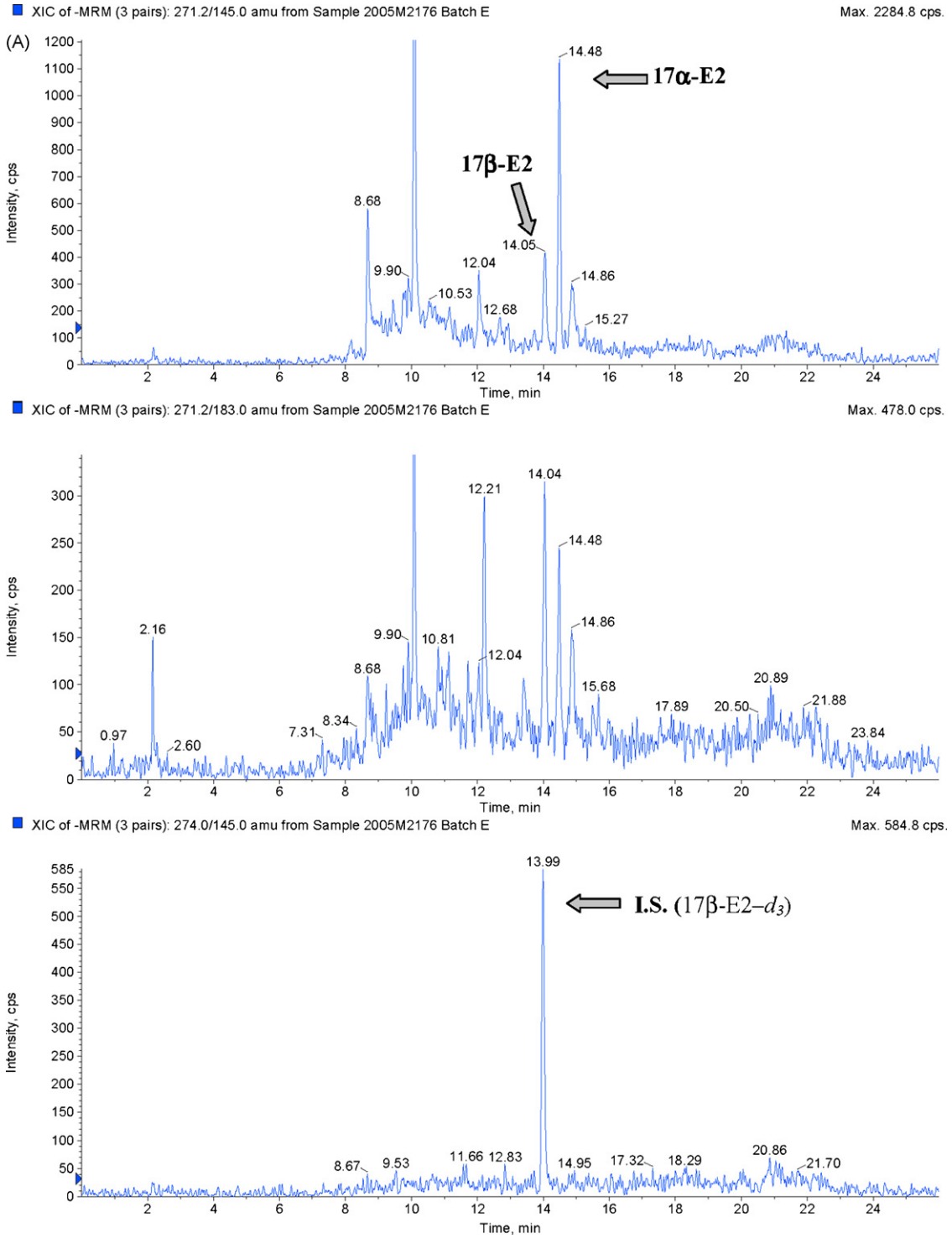

Fig. 1. (A) Extracted ion current (XIC) chromatogram in MRM mode of a real sample: 17α-E2 0.16 ng/mL, and 17β-E2 0.10 ng/mL. Internal standard (I.S.), 17β-E2–d_3. (B) MRM chromatogram of a blank sample. Internal standard (I.S.), 17β-E2-d_3. (C) Negative ion mass spectra and product ion mass spectra of 17α-E2 and 17β-E2-d_3.

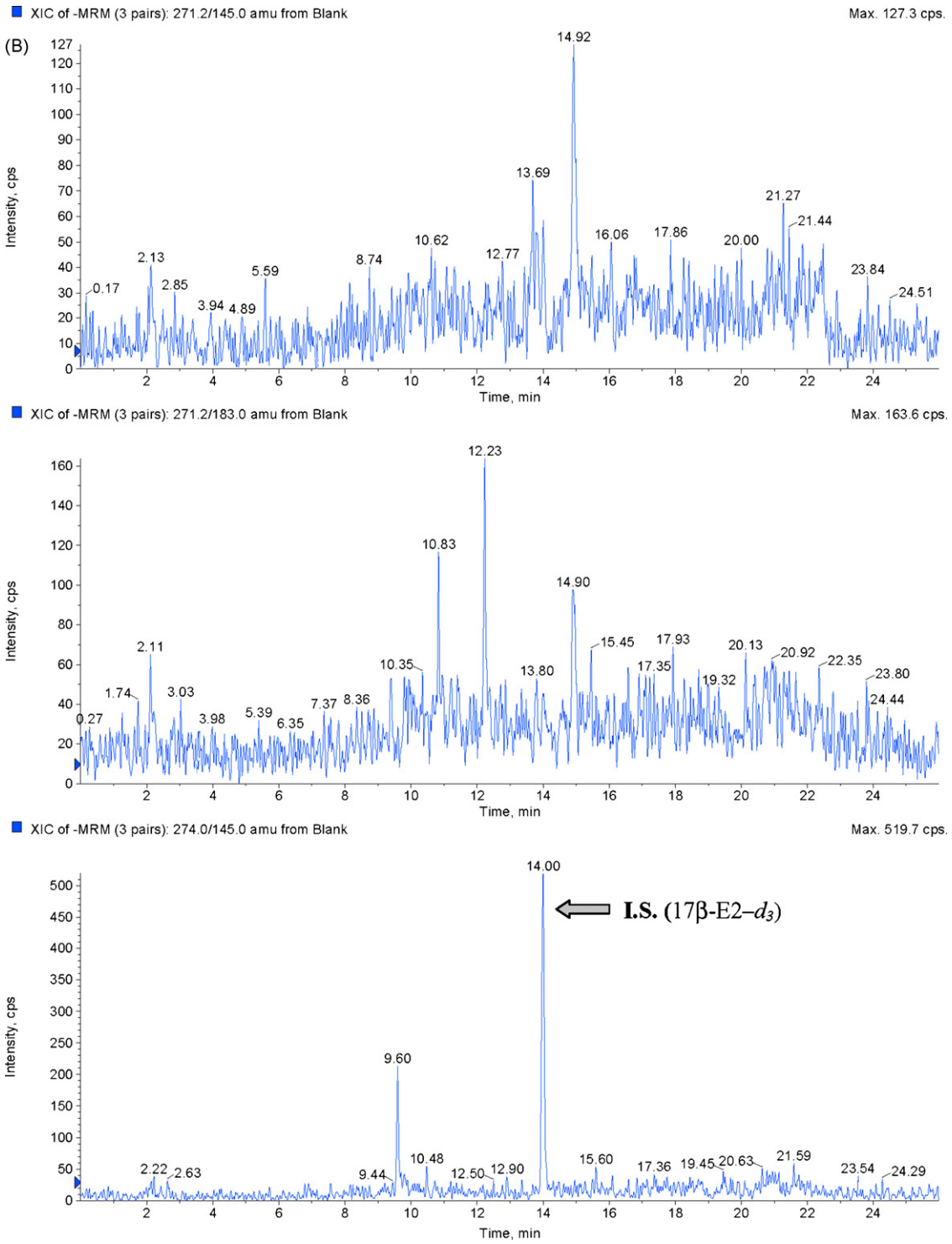

Fig. 1. (*Continued*)

and real samples were interpolated from these calibration graphs, while analyte peak areas were computed using Analyst 1.4.1.

3. Results and discussion

The present method was developed primarily for regulatory purposes and to confirm illegal hormone administration. Residues of 17α-E2/17β-E2 in serum samples were considered confirmed only if: (a) the signal-to-noise ratio, at the less intense transition reaction for 17α-E2 and 17β-E2, was at least ≥3; (b) the relative retention time matched with that of the calibration standard within a margin of ±2.5%; (c) the relative intensities of the detected ions, expressed as a percentage of the intensity of the most intense ion, corresponded to those of the calibration standard within the maximum permitted tolerances.

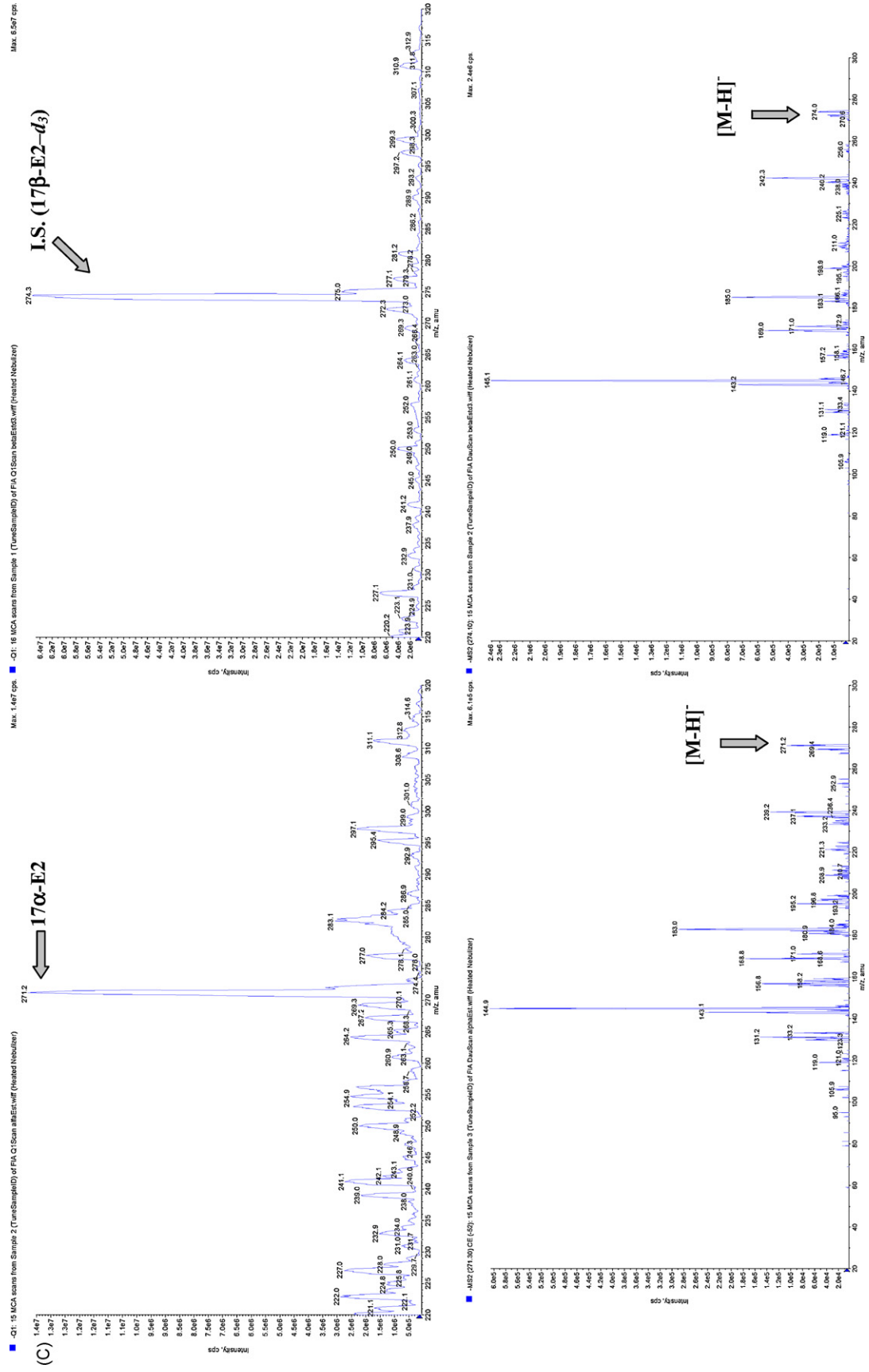

Fig. 1. (*Continued*)

Table 1
Intra-day validation statistics for 17α/17β-E2 and 17β-E2-d_3 in bovine serum samples

Day	Parameter (mean values)[a]	Validation sample level (ng/mL)						
		17α-E2			17β-E2			17β-E2-d_3
		0.08	0.12	0.16	0.04	0.06	0.08	0.5
1	Recovery (%)	73.1	76.4	80.5	82.4	84.7	84.9	91.9
	Precision (CV %)	9.8	8.8	5.1	7.3	7.2	7.0	6.4
2	Recovery (%)	96.3	96.8	102.7	95.9	97.5	99.2	102.6
	Precision (CV %)	10.6	6.3	4.7	7.0	6.9	5.0	5.2
3	Recovery (%)	89.6	95.0	97.1	90.3	91.3	92.1	96.8
	Precision (CV %)	9.4	8.6	8.1	9.1	8.1	3.7	3.5

[a] $n = 6$ replicates.

Table 2
Inter-day validation statistics for 17α/17β-E2 and 17β-E2-d_3 in bovine serum samples

Parameter (mean values)[a]	Validation sample level (ng/mL)						
	17α-E2			17β-E2			17β-E2-d_3
	0.08	0.12	0.16	0.04	0.06	0.08	0.5
Recovery (%)	86.3	89.4	93.2	89.5	91.2	92.1	97.1
Precision (CV %)	15.0	13.0	12.1	9.8	9.1	8.4	6.7

[a] $n = 6$ replicates.

3.1. LC–MS/MS analysis

17α-E2 and 17β-E2 and their I.S. were first analysed and optimized in negative ion mode. The full mass spectra displays the deprotonated molecular ions $[M-H]^-$ at m/z 271 for 17β-E2, in accordance with the fragmentation pattern reported previously by the same authors [10]. MRM LC–MS/MS chromatogram (or extracted ion current chromatogram) for studied compounds in a typical positive bovine serum sample, blank sample and representative product ion mass spectra are shown in Fig. 1A–C.

Negative ion mass spectra and product ion mass spectra of 17α-E2 and 17β-E2-d_3 are shown in Fig. 1. Collision-induced dissociation (CID) experiments of molecular ions m/z 271 and 274 allowed the identification of the diagnostic product ions m/z 145, 183 and 145, for 17α-E2, 17β-E2 and 17β-E2-d_3, respectively. MRM analyses were performed by using precursor–product transitions m/z 271 → 145 and 271 → 183 for 17α-E2 and 17β-E2, and m/z 274 → 145 for 17β-E2-d_3.

Good linearity was obtained for matrix calibration curves, with a correlation coefficient $r > 0.995$. Linearity was checked in the range of 0.06–1.0 ng/mL for 17α-E2 and 0.03–1.0 ng/mL for 17β-E2. Thus, the analytes were detected at concentration ranges of 0.15–0.18 and 0.10–0.96 respectively for 17α-E2 and 17β-E2. These ranges are commonly found within the National Residue Control Plan of Italy.

3.2. Method validation

The validation statistics are reported in Tables 1 and 2: chosen validation levels are 0.08 ng/mL, 0.12 ng/mL and 0.16 ng/mL for 17α-E2, and 0.04 ng/mL, 0.06 ng/mL and 0.08 ng/mL for 17β-E2, in accordance with the EU criteria [12].

Inter-day recovery data ranged from 86.3% to 93.2% for 17α-E2 and from 89.5% to 92.1% for 17β-E2, whereas for 17β-E2-d_3 it was 97.1%. The coefficients of variation were also satisfactory, with values for intra-day repeatability varying from 4.7% to 10.6% for 17α-E2 and from 3.7% to 9.1% for 17β-E2. The within-laboratory reproducibility percentage was from 12.1% to 15.0% for 17α-E2 and from 8.4% to 9.8% for 17β-E2.

A system of identification points (IPs) is used to define the number of ions and their corresponding ratios that must be measured when using MS techniques. For the LC–MS/MS analysis of 17α-E2 and 17β-E2, which belong to Group A substances [13], a minimum of four IPs are required. In this case, measurement of one precursor ion (i.e., m/z 271 or 274) earns 1 IP plus the two transition reactions (m/z 271 → 145, m/z 271 → 183 and m/z 274 → 183; giving 3 IPs, i.e., 1.5 IPs each) leading to a total of 4 IPs.

4. Conclusions

The paper described an in-house validated LC–MS/MS method that allows easy and fast separation, identification and quantification of the analytes. The retention times, the simple and fast preparative steps represent significant advantages that make this method very well suited for analytical purposes such as for high-throughput determination and confirmation of 17α/17β-E2 in bovine serum samples.

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