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Porcine follicular fluids: Comparison of solid-phase extraction and matrix solid-phase dispersion for the GC–MS determination of hormones during follicular growth

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Abstract

The capabilities of solid-phase extraction (SPE) and matrix solid-phase dispersion (MSPD) for the determination of the hormones 17β-estradiol, 2-hydroxyestradiol, 4-hydroxyestradiol and 2-methoxyestradiol by gas chromatography–mass spectrometry (GC–MS) in a very complex matrix like porcine follicular fluids were compared, thus proving the highest effectiveness of the SPE technique. Validation was carried out in terms of limit of quantitation (LOQ), precision, accuracy, recovery and stability. LOQ values in the low $\mu g\,kg^{-1}$ were achieved, with all the other parameters satisfying the acceptance criteria for the validation of bioanalytical methods. The applicability of the method to the determination of the hormones in porcine follicular fluids was demonstrated, thus allowing to observe an increase of the concentration of the hormones during the follicular growth.

Keywords: Hormones; Follicular fluids; 17β-Estradiol; Gas chromatography–mass spectrometry; Validation

1. Introduction

The follicular fluid, which fills the antrum of ovarian follicles, represents a biological matrix very rich in hormones and growth factors produced by surrounding cells. Among these hormones, 17β-estradiol (17-BE) is the most representative: its concentration changes according to follicle development and reaches a peak approaching ovulation [1].

Several 17-BE effects are mediated by its metabolites, i.e. catecholestradiols (2-hydroxyestradiol, 2-OHE and 4-hydroxyestradiol, 4-OHE) and methylated catecholestradiols, i.e. 2-methoxestradiol, 2-MEOE [2].

In particular, catecholestrogens have been proposed as autocrine or paracrine regulators of ovarian function, possessing stimulatory effects on follicular cell differentiation [3] and steroidogenic activity; these actions are accomplished by means of their interaction with estrogen receptors and with enzymes regulating their biosynthesis and catabolism [4].

Up to now the lack of a simple, specific and sensitive assay capable of quantitating the level of all estradiol metabolites from biological fluids has hindered research aimed at elucidating the role of this substances in health and diseases. On the other hand, the determination of these compounds at trace levels in complex matrices demands the development of selective and sensitive analytical methods. Although immunoassay techniques have been validated for the determination of a few estradiol metabolites in plasma [5,6], their use for the analysis of follicular fluids is inappropriate. In fact, since follicular fluid contains blood plasma constituents that cross the follicular barrier as well as the products of granulosa cell metabolism, the concentration of specific substances reflects this transfer mechanisms and is also influenced by the metabolic activity of the follicular structures. With regard to the transfer of plasma proteins, the blood–follicle barrier was found to be selectively permeable only for proteins with molecular masses <500 kDa. In addition, the protein charge affects plasma protein

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transfer into follicular fluid. The concentration of cholesterol, triacylglicerols or phospholipids in pig ovarian follicular fluid is normally 40% or less than that of pig serum. By contrast, free fatty acid concentration in porcine follicular fluid is similar to or higher than that of pig serum. Therefore cross-reactivities of antiserum, which are negligible or low in plasma, may become substantial in follicular fluid, thus requiring an improvement of antiserum specifity and/or chromatographic separation [7].

Moreover, it has been recently demonstrated that follicular fluid differs from plasma in that it resembles an extracellular matrix [8,9]. It contains proteins and soluble extracelluar matrix molecules including proteoglycans which consists of a core protein with attached glycosaminoglycans. The proteogycans identified in follicular fluid include verscian, inter-α trypsin inhibitor and perlcan. Over decades many authors have analyzed follicular fluid to identify its glycosaminoglycans composition and synthesis by granulosa cells. Among these is of particular interest hyaluronian. It has been suggest that the presence of all these osmotically active molecules might be responsible for the formation of follicular fluid by creating an osmotic gradient across the follicular wall.

Regarding other literature data, only methods for the determination of hormones in animal and human plasma and other biological fluids, i.e. urine have been published: Zacharia et al. [10] devised a method based on the use of gas chromatography–mass spectrometry (GC–MS) for the determination of these compounds in rat plasma. GC with electron capture detection (ECD) was proposed for the determination of catecholestradiols and methoxyestradiols [11]. A liquid chromatography with ultraviolet detection method for the determination of 2-methoxyestradiol in human plasma was developed by Lakhani et al. [12]. Assays for the determination of estrogens in urine were recently published [13,14] by using an isotope dilution gas chromatographic–mass spectrometric and a high performance liquid chromatographic method, respectively. Some reviews regarding the analysis of estrogens in different samples have been also published [15–18], but no data concerning the determination of hormones in very complex matrices like porcine follicular fluids are available. Under these circumstances, the development of a reliable assay for the determination of catecholestrogens in follicular fluid deserves a major interest. In fact, even if the importance of endocrine signals in the regulation of follicular development has long been recognized, the follicular microenvironment also plays a critical role in determining follicular fate [19]. In particular, the fluid in ovarian follicle has long been suspected of playing a role in the regulation of steroidogenic capacity of granulosa cells. In addition, follicular fluid represents the culture medium for the growth and the differentiation of the oocyte and therefore it has been suggested to influence, either directly or indirectly, oocyte viability and developmental potential. In consequence, the composition of follicular fluid might be of use as an indicator of the maturation and thus of the quality of the follicle. In general, the possibility of a local regulatory role for catecholestrogens generated in situ has been suggested for several estrogen target tissues. Among these, the ovarian follicle seems a particularly promising site to establish such a role [20].

Taking into account that porcine follicular fluid is more viscous with respect to other physiological fluids (i.e. human/animal plasma, serum, etc.) the evaluation of alternative extraction procedures for the determination of the investigated hormones is demanding. Under these circumstances, the aim of this work was the comparison of different extraction procedures based on solid-phase extraction (SPE) and matrix solid-phase dispersion (MSPD) for the development and validation of a simple and rapid GC–MS method able to determinate the investigated estradiols at trace levels in follicular fluids, with the further intent to better elucidate the role of 17-BE metabolites in animal health and disease.

2. Experimental

2.1. Reagents

2-Fluoroestradiol (2-FE, 99.9% purity), 17β-estradiol (17-BE), 2-hydroxyestradiol (2-OHE), 4-hydroxyestradiol (4-OHE) and 2-methoxyestradiol (2-MEOE), all 98% purity, were purchased from Steraloids (London, UK). Trifluoroacetic anhydride (TFA, >99% purity), toluene, acetonitrile, acetone (99% purity), diethyl ether (99.8% purity) and sodium sulphate anhydrous (>99% purity) were from Sigma–Aldrich (Milan, Italy). All the hormones, with the exception of 17-BE, which was maintained at room temperature, were stored at −20 °C, whereas TFA was maintained under nitrogen at 4 °C.

Stock solutions were prepared in acetone at the concentration of 100 mg l^{-1} and stored at −20 °C for up to 2 weeks, whereas standard and working solutions were prepared daily by dilution from the stock solutions.

2.2. GC–MS analysis

A HP 6890 Series Plus gas chromatograph (Agilent Technologies, Milan, Italy) equipped with the MSD 5973 mass spectrometer (Agilent Technologies) was used for GC–MS analysis. Helium was used as the carrier gas at a flow rate of 1 ml min^{-1}; the gas chromatograph was operated in splitless mode (injection volume: 0.5 μl) with the PTV injector (Agilent Technologies) equipped with a PTV multi-baffled liner (i.d. 1.5 mm, Agilent Technologies) and operating under the following conditions: 70 °C for 0.5 min, 700 °C min^{-1} to 280 °C. Chromatographic separation was performed on a 30 m × 0.25 mm, d_f 0.25 μm Factor Four capillary column equipped with a EZ-guard column (Varian Inc., Turin, Italy). The following GC oven temperature program was applied: 70 °C, 30 °C min^{-1} to 230 °C, 230 °C for 8 min, 10 °C min^{-1} to 250 °C, 30 °C min^{-1} to 310 °C, 310 °C for 15 min. Transfer line and source were maintained at the temperature of 250 and 230 °C, respectively. The mass spectrometer was operated in time scheduled-ion monitoring mode (SIM) by recording the current of the following ions: from 6.00 to 10.20 min *m/z* 482, 369, 256 for 2-FE and *m/z* 464, 351, 309 for 17-BE; from 10.20 to 12.60 min *m/z* 576, 463, 421 for 2-OHE and 4-OHE; from

12.60 to 15 min *m/z* 494, 381, 339 for 2-MEOE. A solvent delay of 6 min was applied. The molecular ions were used for quantitation, whereas the corresponding ion ratios were used to confirm the identification of the analytes. A dwell time of 100 ms was used for all the ions. Preliminarily, full scan EI data were acquired to determine appropriate masses for SIM under the following conditions: ionisation energy: 70 eV, mass range: 50–600 amu, scan time: 3 scans/s. All the analyses were performed setting the electron multiplier voltage at 1700 V.

Signal acquisition and elaboration were performed using the HP Chemstation (Agilent Technologies).

2.3. Samples

Swine ovaries were collected at a local abbatoir, placed into ice-cold phosphate-buffered saline (PBS) supplemented with penicillin (500 IU/ml) and streptomycin (500 μg/ml), maintained in a freezer bag at 4 °C, and transported to the laboratory within 1 h. Ovaries were washed twice with PBS, then with ethanol (70%, v/v) and finally again with PBS at room temperature.

Follicular fluid was harvested by aseptical aspiration from antral follicles classified on the basis of their diameter into: small (<3 mm), medium (3–5 mm) and large (>5 mm). Three samples for each tipology as a function of the follicular diameter were considered.

Prior to analysis, all the samples were stored at −80 °C until analysis for up to 10 days.

2.4. SPE

To 1.0-ml follicular fluid spiked with 10 μg kg^{-1} (μg kg^{-1} is referred to the follicle weight) of 2-FE (internal standard) 1 ml of acetone was added drop by drop to obtain protein precipitation. After centrifugation (1300 rpm, 10 min), the supernatant was applied to the SPE C18 cartridges (Supelco, Bellefonte, PA, USA) previously conditioned with 3-ml diethyl ether. Elution was performed with 4-ml ether which was then evaporated to dryness under a nitrogen stream before the derivatisation process.

2.5. MSPD

1.0 ml of follicular fluid spiked with 2-FE (internal standard) at the final concentration of 10 μg kg^{-1} was placed into a mortar and gently blended with 4 g of ISOLUTE MSPD C18 (Argonaut Technologies, Foster City, CA, USA) using a pestle. The mixture was introduced into an empty SPE cartridge fitted with appropriate frits. The analytes were eluted with 4-ml ether and the eluent was collected and evaporated to dryness under nitrogen before derivatisation.

2.6. Derivatisation

Fifteen microliters of dry toluene (reaction solvent) and 5 μl of trifluoroacetic anhydride (TFA) (derivatising agent) were added to sample extract. The derivatisation procedure was carried out for 10 min at 30 °C. At the end of the reaction, the derivatised samples were submitted to GC–MS analysis.

2.7. Validation

Method validation was carried out to meet the acceptance criteria for bioanalytical method validation [21].

Instrumental detection (LOD) and quantitation (LOQ) limits were calculated according to EURACHEM guidelines [22]. Once calculated, LOQ was tested for accuracy and precision to meet the previously cited international criteria.

The presence of matrix effect was evaluated by comparing the slopes of the regression models (five levels, three replicated measurements for each level) obtained by using the external standard and the standard addition method, respectively.

Homoschedasticity was verified by applying the Bartlett test. Lack-of-fit and Mandel's fitting test were also performed to check the goodness of fit and linearity [23]. The significance of the intercept (significance level 5%) was established by running a *t*-test.

Repeatability and inter-day precision were calculated in terms of R.S.D. (%) on three concentration levels (at the LOQ level for each analyte, at the final concentrations of: 20 μg kg^{-1} and 100 μg kg^{-1} for 17-BE; 5 μg kg^{-1} and 20 μg kg^{-1} for 2-OHE, 4-OHE and 2-MEOE) performing five replicates at each level.

The same concentration levels were used to assess accuracy, which was calculated in terms of recovery rate (RR (%)) as follows:

$$\mathrm{RR}\,(\%) = \frac{c_1 - c_2}{c_3} \times 100$$

where c_1 is the concentration of the fortified sample, c_2 the concentration of the sample before fortification and c_3 is the concentration of fortification. Three different concentration levels (low, medium and high) with five replicated measurements were analysed. The extraction yield in terms of percent recovery was calculated by comparing the results obtained from the injection of pure standards ($n = 3$) with those related to the analysis of follicular fluids containing the same amount of analytes ($n = 3$).

Stability, expressed as percentage of the initial concentration of the hormones in the follicular fluid samples analyzed the day after the sampling, was evaluated in terms of freeze–thaw stability (storage at −80 °C), short-term stability and long-term stability.

3. Results and discussion

In order to study the variability of the hormones content in porcine follicular fluid samples during the follicular growth, the investigated hormones were transformed into volatile derivatives prior to the gas chromatographic determination, since underivatised analytes are not amenable for GC analysis. Although many studies dealing with the detection of estrogens by using both GC–MS/MS and LC–MS/MS capabilities have been published [15,18,24–26] we chose to use a more simple and widespread technique like GC–SIM–MS, very useful for routine applica-

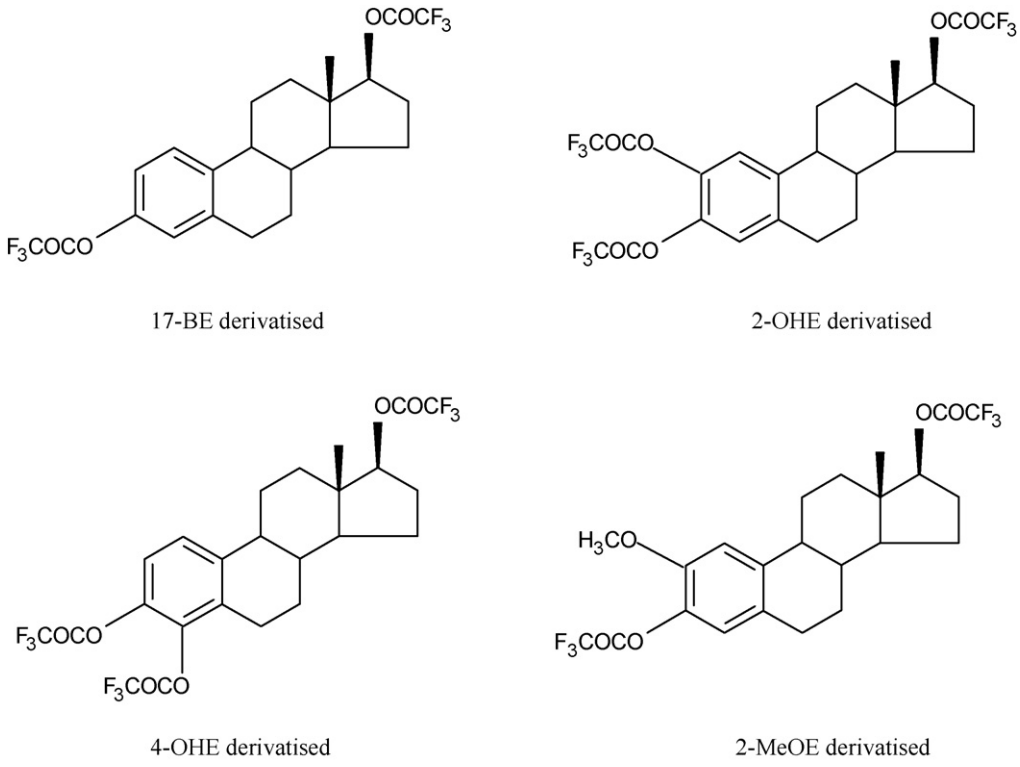

Fig. 1. Derivatised hormones (derivatising agent: TFA; derivatising solvent: toluene).

tions. As suggested by some studies [27–30] TFA was chosen owing to its capabilities to readily derivatise both the phenolic and the alcoholic hydroxyl groups of the analytes without the production of not desirable by-products (Fig. 1). In addition TFA could be very useful taking into account that other detectors like the sensitive electron capture detector (ECD) could be used for hormones determination.

Since different derivatising solvents have been proposed for derivatisation purposes [8,28,31], three different aprotic solvents were tested: acetonitrile, toluene and acetone. Contrary to literature data in which acetonitrile is reported as suitable solvent when TFA is used as derivatising agent [8,31], toluene allowed to reach the highest chromatographic responses (Table 1). These results are in agreement with those achieved by Lerch and Zinn [28] and the obtained behaviour could be explained taking into account that both acetone and acetonitrile have a higher moisture content and a higher solvating effect with respect to toluene, thus reducing the derivatisation process. In a subsequent step, the derivatisation time was optimised and for this purpose solutions containing the analytes at the concentration of 50 $\mu g\,l^{-1}$ were derivatised for 10, 30 and 60 min (Fig. 2). ANOVA did not show significant differences ($p > 0.05$) among the mean responses obtained varying the time, thus a time of 10 min was chosen for fast analysis. Rapid analyses were obtained also in terms of instrumental conditions, since the use of a proper GC oven program allowed to obtain the baseline separation of all the analytes in a very short time, i.e. 15 min (Fig. 3).

Table 1
Study of the derivatising solvent: Bonferroni test results for the hormone 17-BE

Solvent	Chromatographic response mean ± S.D.
Toluene	240000 ± 19400 a
Acetonitrile	140000 ± 10100 b
Acetone	156000 ± 6000 b

Different letters on the mean values denote significant differences ($p < 0.05$). Data with the same letters are not significantly different.

Taking into account that both the complexity and the viscosity of the follicular fluid samples did not allow the direct derivatisation of the analytes in the matrix as reported by Zacharia et al. [10], the evaluation of the capabilities of different extraction/purification procedures like SPE and MSPD was required.

3.1. SPE versus MSPD

In order to evaluate the best extraction conditions in terms of sorbent material, elution solvent, elution volume and elution flow, preliminary SPE experiments were performed using standard solutions. Taking into account the chemical–physical properties of the investigated hormones, two kinds of sorbents were evaluated: divinylbenzene-polystyrene (DVB-PS) and C18.

The elution flow was found a crucial parameter: analytes were eluted by using only the gravity force without the introduction of supplementary aspirations to avoid their loss during the extraction step. Regarding the choice of the elution solvent, two aprotic compounds like diethyl ether and toluene were considered. In the case of DVB-PS, the best results were obtained using toluene (4 ml) (Fig. 4A). The observed behaviour could be explained taking into account that π–π interactions are able to retain the

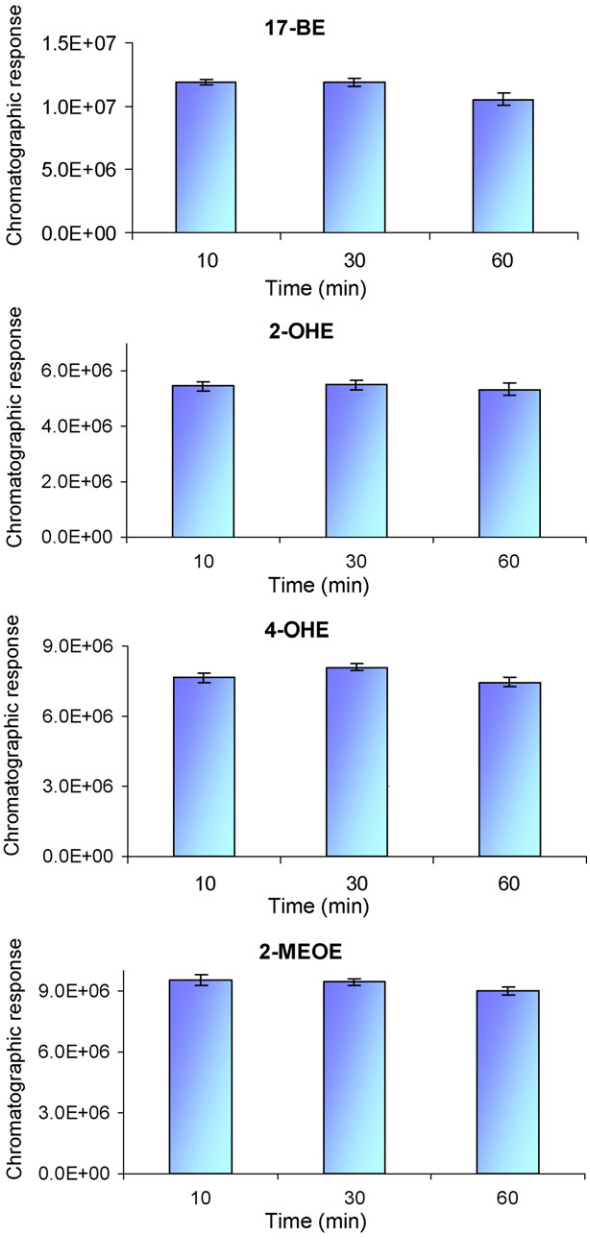

Fig. 2. Study of the effect of the derivatisation time on the response of the hormones investigated. For each analysis six replicated measurements were performed.

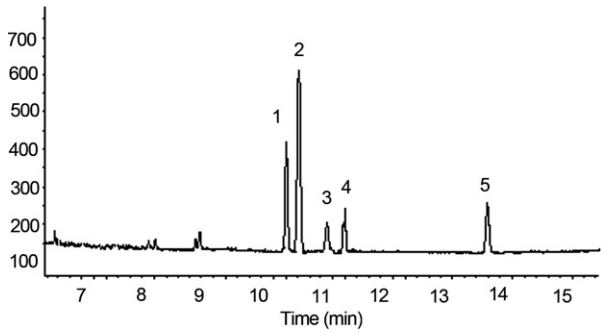

Fig. 3. GC–MS–SIM chromatogram of a follicular fluid sample containing: (1) 2-FE (IS), (2) 17-BE, (3) 4-OHE, (4) 2-OHE, and (5) 2-MEOE derivatised with TFA.

analytes on the sorbent and that toluene is able to compete for their removal. By contrast, when the C18 sorbent material was used, optimal results were obtained by using diethyl ether (4 ml) (Fig. 4B). In this case, the behaviour could be ascribed to the highest polarity of ether with respect to toluene, thus allowing the elution of the analytes from the cartridge. The use of diethyl ether proved also to be very useful before the derivatisation step, since its evaporation was favoured by the highest volatility with respect to that of toluene.

As reported in Fig. 4, using the C18 cartridges higher chromatographic responses were obtained for all the analytes with extraction yields ($n = 3$) in the 93(±4)–111(±7)% range versus 73(±4)–91(±5)% range obtained using the DVB-PS sorbent, thus suggesting the use of the C18 cartridges for the SPE procedure.

Owing to the high viscosity of the porcine follicular fluids, samples could not be directly loaded onto the cartridges [12], so an additional pre-treatment consisting in the precipitation of proteins prior to the SPE process was always carried out. For this purpose acetone was added drop by drop.

In a subsequent step of the study, MSPD was evaluated as an alternative technique in order to develop a more rapid extraction procedure that could permit the possibility of avoiding the precipitation of the proteins required before the SPE process. To our knowledge no data regarding the use of matrix solid-phase dispersion for the determination of hormones in follicular fluid samples have been published. On the basis of the results obtained by the SPE procedure, ISOLUTE MSPD C18 silica and diethyl ether were used as sorbent material and elution solvent, respectively. The results obtained were not satisfactory: in fact, difficulties related to the achievement of a homogeneous packing of silica inside the SPE tube and consequently problems

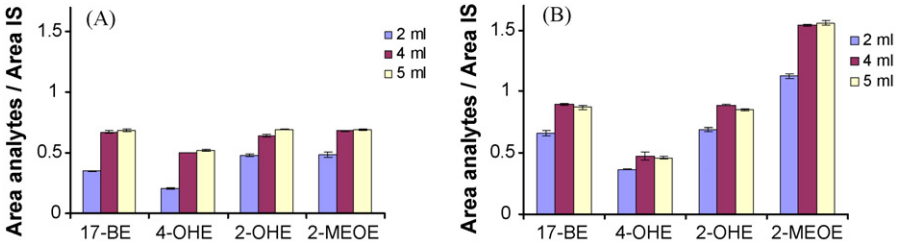

Fig. 4. SPE extraction: comparison between (A) DVB-PS (toluene) and (B) C18 (diethyl ether) cartridges.

Table 2
Comparison between the external standard and the standard addition method for matrix effect assessment

Analytes	External standard method		Standard addition method		t_{calc} [a]
	$a \pm \sigma_a$ [b]	$b \pm \sigma_b$ [c]	$a \pm \sigma_a$ [b]	$b \pm \sigma_b$	
17-BE	0.822 ± 0.015	–	0.46 ± 0.02	$0.02 \pm 5 \times 10^{-3}$	−15.1
2-OHE	0.886 ± 0.037	–	0.58 ± 0.01	$0.01 \pm 2 \times 10^{-3}$	−33.4
4-OHE	0.224 ± 0.052	–	0.19 ± 0.01	$0.01 \pm 1 \times 10^{-3}$	−3.7
2-MeOE	1.690 ± 0.034	–	0.50 ± 0.03	$0.07 \pm 6 \times 10^{-3}$	−27.6

[a] t_{tab} ($\alpha = 0.05$, 27) = 2.05.
[b] Calibration curve $y = ax + b$, CI 95%.
[c] Not significant.

associated to the stability of the elution flow were observed. The overall repeatability of the MSPD procedure was influenced by all these phenomena. In addition, lower recoveries, i.e. 56(±12)–79(±16)%, $n = 3$, with respect to the SPE process were observed, thus evidencing the highest capabilities of the SPE process.

3.2. Validation

The best performances obtained using SPE suggested the use of this technique for the extraction of the hormones under investigation in the follicular fluids. Although this extraction procedure has been already used for the determination of hormones in other physiological fluids, the absence of data regarding follicular fluids required a complete validation of the proposed method.

Taking into account that no blank matrix was available and that the viscosity of the follicular fluid samples did not allow the removal of the investigated analytes by a cleaning procedure, i.e. by stripping the matrix on charcoal, instrumental detection and quantitation limits were calculated by using standard solutions. Very low LOD and LOQ values were calculated, being LOQ equal to 0.2 $\mu g\,kg^{-1}$ for 17-BE and 2-OHE, 0.8 $\mu g\,kg^{-1}$ for 4-OHE and 0.3 $\mu g\,kg^{-1}$ for 2-MEOE, with LOD values of about three times lower. These findings attest the capability of the developed method of quantifying estrogens at trace levels.

Trueness was assessed by comparison of the calibration lines calculated by using the external standard and the standard addition method. The demonstration of the presence of matrix effect for all the analytes suggested the use of the standard addition method for the determination of the analytes in the follicular fluids samples (Table 2).

Excellent precision was proved with R.S.D. (%) lower than 7%, thus satisfying the requirements of the guidelines for the validation of bioanalytical methods [21].

Extraction yields higher than 78% were obtained for all the analytes, whereas recoveries in the 81(±8)–98(±3)% ($n = 3$) proved the accuracy of the developed method.

Owing to the presence of labile hydroxyl groups, the stability of the investigated hormones in the time was also evaluated. Data obtained after going through three freeze and thaw cycles proved that no degradation of the investigated analytes occurs when both the matrix (storage at −80 °C) and the stock solutions (storage at −20 °C) are maintained at room temperature just for the thawing time. Under these circumstances, no significant differences ($p > 0.05$) among the chromatographic responses obtained before, during and after the freeze and thaw cycles at room temperature were observed.

As for the short-term storage, a relevant degradation of the hormones was evidenced by maintaining both the stock solutions and the matrix at room temperature for more than 4 h after thawing, thus observing an evident decrease in the chromatographic responses, with differences from the initial concentration more than 30%.

Concerning the long-term stability in the case of stock solutions, it was proved that the recommended storage temperature (−20 °C) could be maintained for up to 2 weeks, whereas shorter times and lower temperatures were required for the preservation of the follicular fluids. More precisely, samples had to be maintained at −80 °C for a maximum of 10 days, with differences from the initial values always lower than 5%.

3.3. Application to porcine follicular fluid samples

Finally, applicability of the validated method for the determination of hormones in follicular fluids was demonstrated by analysing different kinds of samples of porcine follicular fluids (Table 3). The obtained results revealed an increase of the hormone concentrations during follicular growth. In fact, follicular fluid 17-BE levels were significantly higher in large

Table 3
Hormone content in follicular fluid samples

Follicular class	Hormones ($\mu g\,kg^{-1}$)			
	17-BE	2-OHE	4-OHE	2-MeOE
Small	23.40 ± 1.03	0.29 ± 0.04	–	0.882 ± 0.032
Medium	39.23 ± 0.27	1.24 ± 0.39	1.760 ± 0.036	3.91 ± 0.16
Large	51.98 ± 0.34	5.73 ± 0.11	7.39 ± 0.030	10.018 ± 0.0041

Each data represent mean ± S.D. of nine measurements.

follicles than in medium and small ones. This is in agreement with a previous report [32] and could derive from the increased granulosa cell aromatase activity which accomplishes follicle growth and development [33]. In the same way, 2-OHE and 4-OHE levels significantly increased during follicle development as a result of the enhanced 2/4-hydroxylase activity as a consequence of a direct effect of the increased 17-BE levels. The parallel rise of 2/4-hydroxylase activity and 17-BE, its substrate, would be consistent with a precisely timed regulatory function for these catecholestrogens in the preovulatory follicle. As for 2-MEOE, in accordance with the previous data a progressive rise in concentrations was observed during follicle growth. In addition, 2-MEOE appeared to be the predominant catecholestrogen found in follicular fluid of each follicle class. It has been demonstrated that catecholestrogens can stimulate granulosa cell steroidogenesis while inhibiting their proliferation: these findings indicate the potential for an intraovarian autocrine/paracrine functions for these hormones by favouring the differentiation of granulosa cell [34].

4. Conclusions

The capabilities of two different extraction procedure like SPE and MSPD for the GC–SIM–MS determination of the hormones 17-BE, 2-OHE, 4-OHE and 2-MEOE at trace levels in a very viscous and complex matrix like porcine follicular fluids were evaluated. The best performances obtained by the SPE-based method in terms both of repeatability and recoveries allowed to validate a very fast and simple method, very useful for routine applications. Finally, the analysis of different follicular fluid samples during follicle growth allowed to investigate the role of 17-BE and its metabolites in animal health.

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