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Title: Individual and cyclic estrogenic profile in women: structure and variability of the data.

Article Type: Regular Article

Keywords: Estrogens; menstrual cycle; PARAllel FACtor analysis (PARAFAC); multivariate statistics; Gas chromatography-mass spectrometry (GC-MS)

Corresponding Author: Mr. Eugenio Alladio, M.Sc.

Corresponding Author's Institution: Università degli Studi di Torino

First Author: Cristina Bozzolino, M.Sc.

Order of Authors: Cristina Bozzolino, M.Sc.; Sara Vaglio, M.Sc.; Eleonora Amante, M.Sc.; Eugenio Alladio, M.Sc.; Enrico Gerace, PhD; Alberto Salomone, Prof.; Marco Vincenti, Prof.

Abstract: The concentration of estrogens in the body fluids of women is highly variable, due to the menstrual cycle, circadian oscillations, and other physiological and pathological causes. To date, only the cyclic fluctuations of the principal estrogens (estradiol and estrone) have been studied, with limited outcome of general significance. Aim of the present study was to examine in detail the cyclic variability of a wide estrogens' panel and to interpret it by multivariate statistics.
Four estrogens (17α-estradiol, 17β-estradiol, estrone, estriol) and eleven of their metabolites (4-methoxyestrone, 2-methoxyestrone, 16α-hydroxyestrone, 4-hydroxyestrone, 2-hydroxyestrone, 4-methoxyestradiol, 2-methoxyestradiol, 4-hydroxyestradiol, 2-hydroxyestradiol, estriol, 16-epiestriol, and 17-epiestriol) were determined in urine by a gas chromatography - mass spectrometry method, which was developed by design of experiments and fully validated according to ISO 17025 requirements. Then, urine samples collected every morning for a complete menstrual cycle from 9 female volunteers aged 24-35 years (1 parous) were analysed. The resulting three-dimensional data (subjects × days × estrogens) were interpreted using several statistical tools. Parallel Factor Analysis compared the estrogen profiles in order to explore the cyclic and inter-individual variability of each analyte. Principal Component Analysis (PCA) provided clear separation of the sampling days along the cycle, allowing discrimination among the luteal, ovulation, and follicular phases. The scores obtained from PCA were used to build a Linear Discriminant Analysis classification model which enhanced the recognition of the three cycle's phases, yielding an overall classification non-error rate equal to 90%. These statistical models may find prospective application in fertility studies and the investigation of endocrinology disorders and other hormone-dependent diseases.

Response to reviewers

Reviewer #1: The manuscript requires additional careful editing for both content and context.

A major issue is the reference to women between the ages of 25-35 as childbearing or fertile women, this implication is not necessary or helpful. All references to "fertile" or "childbearing" women should be removed from the manuscript and women should simply be described as between female volunteers aged 25-35 years. A reference to proportion of the 9 who have previously given birth may be appropriate for context (e.g. X% parous). Further, it is not accurate to refer to the women as reproductive-age women, as this encompasses all women aged 15-49.

The text was modified in every phrase where either the term “fertile” or “childbearing” was referred to women in order to underline that they were in the pre-menopausal age. More accurate reference to the age was made and the inclusion of one parous woman within the group of nine volunteers was made explicit.

Abstract: the abstract has been satisfactorily revised, however the last sentence is unclear - what is meant by "these models open an outlook"?

The latter sentence was modified as follows: “These statistical models may find prospective application in…”

Highlights: acronyms that are not common need to be avoided in the highlights unless they are defined. As such, the last two highlights need revised/rewritten

The third and fourth highlights were revised by substituting the acronyms with more comprehensible verbal expressions.

Introduction: The level of detail and citations in the introduction are excessive, it is unclear how the references to fluctuations in estrogens and characteristics like sexual behavior, preferences, PSYCHIATRIC (not psychic) disorders are relevant to the assay being developed or the current study. These should be omitted and the introduction shortened.

The Introduction section was shortened and 12 citations were removed (former 13-18, 20-21, 23-25, 27), according to the Reviewer’s suggestion.

Also in the introduction, the reference and discussion of the studies that have employed the LC-MS estrogen metabolite assay need to be corrected. The NHSII citation (28) is correct in line 68 (need to correct pre-menopause to pre-menopausal), but the reference to the STUDIES (not study) conducted using primarily serum in postmenopausal women should be to the recent meta-analysis by Sampson et al. https://www.ncbi.nlm.nih.gov/pubmed/28011624, not reference 30 Falk et al. Further in premenopausal women, the following reference https://www.ncbi.nlm.nih.gov/pubmed/27138982 would be more relevant to the current study than the studies conducted in postmenopausal women.

Citation of the two references suggested by the Reviewer was introduced in the dedicated paragraph and the text was modified accordingly. The two underlined misprints were corrected.

Line 88 in introduction, the references to the methods papers should replace reference 28 with the actual methods paper from that article https://www.ncbi.nlm.nih.gov/pubmed/16223252

The correct citation for the analytical method description was updated.

Line 96 - progestins refers to only synthetic progestin compounds, authors should use progestogens (if they are referencing both naturally occurring progesterone and synthetic progesterone compounds (exogenous)) or progesterone (endogenous only)

The cited papers refer to endogenous substances only. The correct term “progesterone” replaced the wrong one (progestin).

Line 99 - only 12 estrogen metabolites listed, unclear and not reconciled with the list of 15 at line 111, please only list the 15 estrogen/estrogen metabolites once in the paper, (lists are provided at line 99, 111, 117, and elsewhere).

The list of targeted steroids was:
- maintained in the Abstract
- eliminated from the Introduction (former lines 99-101)
- maintained in the Material and Methods – Chemicals and reagents
- transferred to the MethodX file within the Experimental Design section together with most of the Experimental and Discussion sections related to the analytical method.

Urine sample collection, line 291 - unclear what is meant by 'did not assume birth control pills'; line 292 how was creatinine measured? (need to state method).

The phrase was substituted with the following “None of them was taking any pharmaceutical drug or combined oral contraceptive pills in the period of sample collection”.
The method and instrumentation used to measure creatinine was specified in the text.

The chemometrics section is much improved.

Thank you.

Results and discussion - it is suggested to move method development and experimental designs to either the methods or the supplemental material - it does not seem appropriate in the results/discussion section.

Method validation results could be revised to reduce redundancies and report results more concisely, it is overly long as written.

In general, the manuscript contains more details about the assay methods than are necessary, I agree with the other reviewer that much could be moved to supplemental material.

We agree with the Reviewer that “Steroids” should not report detailed description of the analytical method optimization and validation. On the other hand, a previous publication reporting these details does not exist and we are interested that the present analytical method could be clearly described in an easily accessible format. Therefore, rather than transferring this content into the Supplementary Material, we included it into a MethodX file, to be published as an open access material. We hope that our proposal meets the Reviewer’s request and, at the same time, is acceptable by the Editor.

Conclusions: citations are needed at line 522 regarding previously published GC-MS protocols. Last paragraph of the conclusion is not clear, in particular it is not clear what the authors are concluding after reading these three sentences.

Citation of two papers - already mentioned in the Introduction - was added.
The last paragraph was extensively revised, as follows: (a) the position of second and third sentences of the paragraph was inverted; (b) the central sentence was expanded and split in two to make the inherent conclusion clear.

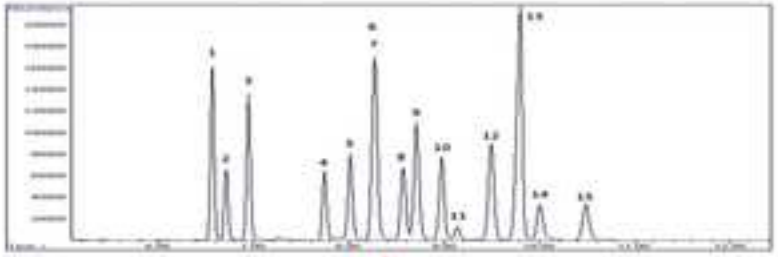
Other comments:

- With respect to limitations, the authors need to clearly describe the limitations of their assay design and specifically reference possible limitations of using only 2 internal standards on identifying independent peaks when more standards are available.

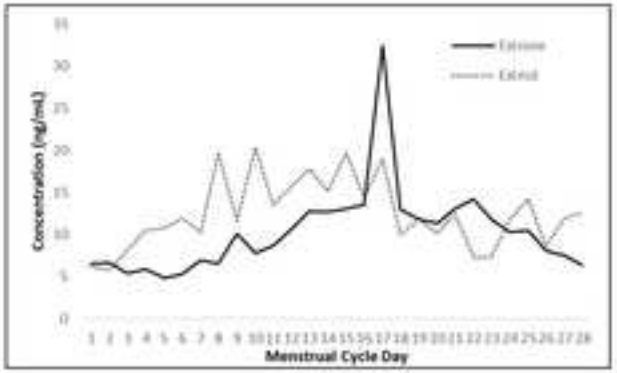
The following sentence was added to the first paragraph of the Conclusions: “In case that the concentration of one specific estrogen has to be determined with high accuracy, the method can be further improved by using a dedicated isotopically-labelled homolog as the internal standard.”

Graphical Abstract

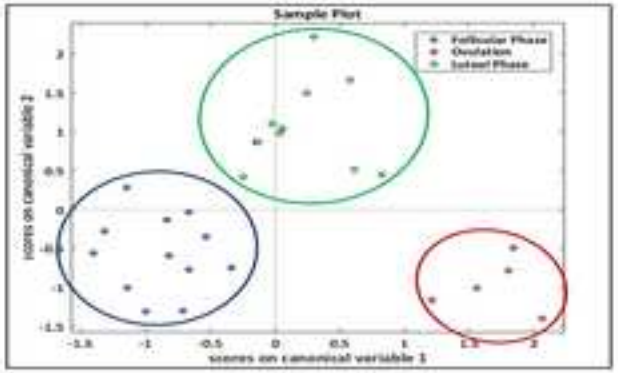
INDIVIDUAL AND CYCLIC ESTROGENIC PROFILE IN WOMEN: STRUCTURE AND VARIABILITY OF THE DATA



PARAFAC



PCA + LDA



Highlights

- A GC-MS method detecting 15 estrogens in urine was developed and fully-validated;
- Urine samples from 9 women were collected daily for a menstrual cycle and analyzed;
- Fluctuations along the cycle were observed using 3-D multivariate statistics;
- A linear discriminant model allowed to single out the different menstrual phases.

INTRODUCTION

Estrogens play a variety of crucial roles in the menstrual cycle and throughout the entire life of women. The menstrual cycle is the cyclic orderly sloughing of the uterine lining, in response to the interactions of hormones produced by the hypothalamus, pituitary and ovaries [1]. The duration of a complete menstrual cycle spans from 21 to 35 days, with an average of 28 days. The menstrual cycle is usually divided into the follicular and the luteal phases. The follicular phase begins from the first day of menses until ovulation, which typically occurs around the 14th day. After ovulation, the luteal phase starts and lasts 14 further days, on average [1–4]. Lifestyle factors, such as smoking, physical activity and alcohol consumption may affect the phases of the menstrual cycle [5]. Abnormally high and low values of body mass index (BMI) are frequently associated to menstrual dysfunctions, due to the correlation of the estrogens metabolism with the nutrition and dietary composition and the role of adipose tissue in aromatase conversion [6]. The natural rhythmic fluctuations of the estrogens that control the menstrual cycle influence the fertility [7–11] and various physical and psychological conditions [3,12–15].

An important methodological issue with the study of estrogens data is how to align the cycles of the different women to allow comparisons [9]. In the Nurses' Health Study II, this issue was overcome by sampling all the women during their luteal phase [16]. The main problem for this approach is the difficult recognition of the menstrual phase in women with irregular periods. To date, only the variation of estrone or estradiol levels were evaluated across complete menstrual cycles, possibly because these are the main estrogens circulating in the human body, together with estriol [17]. A comprehensive evaluation of an extended estrogenic profile was previously proposed with the purposes of detecting any possible correlations between estrogens and breast cancer risk: the urinary estrogenic profile of 15 free and conjugated estrogens was collected from a large cohort of pre-menopausal women and retrospectively interpreted, on the basis of their clinical history [16] Extended estrogenic profiles were also correlated with terminal duct lobular unit involution, a marker of increased breast cancer risk [18]. In parallel studies on post-menopausal women, the determination of blood estrogens and metabolites revealed a lower risk of breast cancer for the subjects with high levels of the hydroxylated 2-pathway metabolites [19,20].

The estrogen determinations most frequently reported in the literature are conducted on either urine or oral fluid, using radioimmunoassay (RIA), enzyme immunoassay (EIA), or enzyme-linked

immunosorbent assay (ELISA) [22]. While these immunoassay methods provide high throughput, efficiency, ease of use, fast turnaround time and low cost, they frequently do not have the necessary specificity and sensitivity to accurately measure low estrogen concentrations, due to cross-reactivity with structurally similar substances [21,22]. This limits the chance of estrogen profiling during the follicular and late luteal phases, when their concentration level is particularly low. In contrast, the hyphenation of chromatographic and mass spectrometric (MS) techniques provides the simultaneous dosage of both parent estrogens and their metabolites ensuring at the same time extremely low detection limits [21-23]. Liquid chromatography (LC) and gas chromatography (GC) coupled with MS are consistently used in multi-analyte profiling, with LC-MS increasingly favored for its straightforward applicability, even if GC-MS has traditionally dominated the analysis of estrogens and other endogenous steroids for years. Actually, GC-MS provides broad steroids profiles after a single derivatization step, achieving high specificity, good sensitivity, and limited matrix effects [23]. In general, the advantage of high-resolution separation is increasingly valued in targeted and untargeted metabolomics to obtain complete urinary endogenous steroid profiles that include estrogens, androgens, corticoids, and progesterone [24-26]. Multi-residual GC-MS methods for the detection of wide estrogen profiles have occasionally been developed in the past [27-30], even if the laborious sample preparation steps somehow contributed to the progressive decline of GC-MS procedures in favor of LC-MS.

In the present study, 15 estrogens were monitored in nine women along one menstrual period using an optimized and fully validated GC-MS method. The collected data were used to build a preliminary multivariate model shaping the menstrual cycle, which may represent a valuable tool in the study of fertility issues, as well as in the screening and evaluation of various pathological conditions, including endocrinology disorders and hormone-dependent cancers.

MATERIAL AND METHODS

Chemicals and reagents

4-methoxyestrone, 4-methoxyestradiol, 2-methoxyestrone, 16α-hydroxyestrone, 2-methoxyestradiol, 2-hydroxyestradiol, 4-hydroxyestrone, 4-hydroxyestradiol, 17-epiestriol and 16-epiestriol were purchased from Toronto Research Chemical Inc. (Toronto, ON, Canada). 17α-estradiol, 17β-estradiol, hexane, methanol, ethyl acetate, ascorbic acid, ammonium iodide, *tert*-butyl methyl ether (TBME), dithioerythritol and N-methyl-N-(trimethylsilyl) trifluoroacetamide

(MSTFA), β-glucuronidase/arylsulfatase (from *Helix pomatia*) mixture, were provided by Sigma-Aldrich (Milan, Italy). Estrone, 2-hydroxyestrone, estrone 3-(β-D-glucuronide) sodium salt, estriol, estrone-d_4 and 17β-estradiol-d_4 were supplied by LGC Promochem SRL (Milan, Italy). β-glucuronidase from *Escherichia coli* was purchased from Roche Life Science (Indianapolis, IN, USA). Ultra-pure water was obtained from a Milli-Q® UF-Plus apparatus (Millipore, Bedford, MA, USA). C-18 endcapped Solid-Phase Extraction (SPE) cartridges were provided by UCT Technologies (Bristol, PA, USA) and estrone 3-sulfate sodium salt was supplied by Steraloids Inc. (Newport, RI, USA).

All stock standard solutions were prepared in methanol at 1 mg/mL and stored at −20° C until use. Working solutions containing a mixture of all analytes were prepared at the final concentrations of 20 µg/mL and 1 µg/mL by appropriate dilution with methanol. Estrone-d_4 and 17β-estradiol-d_4 were used as isotopically labelled internal standards for quantitation and were added from separate methanol working solutions at the final concentrations of 100 µg/mL and 50 µg/mL, respectively.

Sample preparation

The sample preparation conditions were optimized after design of experiments [31], described elsewhere [32]. The urine sample (6 mL) was fortified with both 17β-estradiol-d_4 and estrone-d_4 internal standard solutions at the final concentrations of 50 ng/mL and 25 ng/mL. After that, the pH was checked and, if necessary, some drops of HCl were added to attain a final pH of 5.5. 2 mL acetate buffer 1.1 M (pH 5.5) and 50 µL ascorbic acid 1 M were added, too. Ascorbic acid was necessary to protect the labile catechol groups and prevent their degradation [27,29]. A deconjugation step, useful to transform the glucuronide and sulphate conjugated estrogens [2,17,33] into the free form, was executed by adding 20 µL of β-glucuronidase/arylsulfatase mixture to the urine samples, which were then incubated at 37 °C overnight. The next morning, 100 µL β-glucuronidase from *Escherichia Coli* was added, together with 50 µL of ascorbic acid solution and the final enzymatic deconjugation of the remaining glucuronide estrogens was carried out for 1 hour at 58 °C. Once the hydrolysis was completed, the mixture was cooled to room temperature and 2 mL of 0.1 M carbonate buffer (pH 9) with some drops of NaOH 1 M were added, to obtain a final pH > 9. Liquid-liquid extraction (LLE) was performed by adding 5 mL of ethyl acetate and hexane (2:3 v/v) mixture to the sample, which was subsequently shaken in a vortex multimixer (Tecnovetro, Monza, Italy) for 5 min and subjected to centrifugation (model

Megafuge 1.0 Heraeus; ASHI, Milan, Italy) at 4000 rpm for 5 min. The extraction process was repeated twice, and the two combined organic phases were transferred into a vial and evaporated to dryness under a gentle stream of nitrogen at 40 °C using a Techne Sample Concentrator (Barloworld Scientific, Stone, UK). The dried residue was reconstituted and derivatized for 1 hour at 70 °C by adding 50 µL of MSTFA/NH_4I/dithioerythritol (1.000:2:4 v/w/w) solution. A 2 µL aliquot was injected into the GC/MS system in the splitless mode.

GC-MS analysis

All analyses were conducted on an Agilent 6890N Network GC System interfaced to a 5975 inert XL Mass Selective Detector (Agilent Technologies, Milan, Italy). The GC was equipped with a J&W Scientific HP-1 17.0 m x 200 µm (i.d.) x 0.11 µm (f.t.) capillary column. The helium gas carrier was employed at a constant pressure of 23.25 psi and 1.1 mL/min initial flow. The GC oven temperature was initially set at 200 °C, held for 2 min, then was raised to 225 °C with an 8 °C/min ramp. Then, the temperature was increased to 234 °C with a 3 °C/min heating rate, held for 3 min and raised again to 245 °C with a 3 °C/min ramp. The final oven temperature of 315 °C was reached with a 40 °C/min heating rate and held for 3 min. The total run time was 19.54 min. The GC injector and transfer line were maintained at the temperature of 280 °C. Trimethylsilyl derivatives of the analytes were ionized by electron ionization (EI) at 70 eV. Data were acquired in the selected ion monitoring (SIM) mode at a dwell time of 20 ms [32,34].

Method validation

The analytical method was validated according to the Eurachem criteria and recommendations [35]: linearity range, selectivity, specificity, limit of detection (LOD), limit of quantitation (LOQ), intra-assay precision and accuracy, repeatability, matrix effect, extraction recovery, and carry-over were determined. A pool of urines collected from healthy male volunteers (laboratory personnel), was negativized by solid-phase extraction using a C-18 end-capped cartridge previously conditioned with 2-propanol and ultra-pure water. The absence of any detectable trace of estrogens was verified. The resulting sample was used as blank urine and spiked with the standard solutions within the validation procedure. Full details about the validation of the analytical method are reported in a dedicated publication [32].

Linearity, LOD, LOQ

The calibration was performed by internal standardization using the least squares regression method from five replicate analyses for each data-point at six concentration levels in the range 1-50 ng/mL. Linearity was evaluated by lack-of-fit test, analysis of variance (ANOVA) test, Mandel's test, and relative standard deviation (RSD) of the slope, according to the approach described by Desharnais et al. [36]. Moreover, the residual plots and the deviation from back-calculated concentrations were examined. When heteroscedastic distribution of data-points was observed, a weighting factor of x^{-1} or x^{-2} was employed, depending on the rate of the variance increase with the concentration (linear or quadratic).

LOD and LOQ were estimated for all the target analytes using the Hubaux-Vos' algorithm at a significant level of 95% [37] from the 30 data-points collected to build the calibration lines. To confirm the correct estimation further, the calculated LOD and LOQ values were experimentally verified with blank samples spiked at concentrations close to the detectable and quantifiable values, respectively. In the operational practice, LOQ values were assumed at the lower level of the calibration curves.

Repeatability and accuracy

The retention time repeatability was verified on the chromatographic peak of the target analytes recorded in the 30 overall analyses used to build the five calibration curves (see above). Deviations below 1% from calibrators and controls were considered satisfactory. The repeatability of the relative ion abundance was evaluated on the selected ion chromatograms for each target analyte. The variations were considered acceptable within ±20%, with respect to the controls.

For all analytes, intra-day repeatability and accuracy were evaluated on 10 blank urine samples spiked with all the target analytes at three concentration levels (1.0 ng/mL, 5.0 ng/mL and 25 ng/mL). Precision and accuracy were estimated from the percent variation coefficient (CV%) and percent bias (bias%), respectively. Precision was considered satisfactory when the CV% values were below 15% for the low calibration level and below 10% for the other levels. Satisfactory accuracy was achieved when the experimentally determined average concentration lied within ±10% from the expected value.

Matrix effect, extraction recovery, enzyme performance, carry over

The matrix effect was evaluated at the three concentration levels defined above by comparing the experimental results obtained from blank urine samples (mean value from five replicates) and

blank deionized water solution both spiked after the extraction step at the same concentration. The matrix effect for each target analyte was expressed as the percentage ratio between the two measured concentrations. Extraction recovery was calculated at the same concentration levels by comparing the experimental results from blank urine samples spiked respectively before and after the extraction step (5 replicates each) and expressed as the percentage ratio between the two data.

The efficiency of β-glucuronidase and arylsulfatase to achieve exhaustive hydrolysis of the conjugated metabolites was tested at three concentration levels (1.0 ng/mL, 5.0 ng/mL and 25 ng/mL) by measuring the percentage ratio between the recovered concentration of estrone glucuronide (and sulfate) spiked into a blank sample and that of free estrone spiked to another blank sample at the same molar concentration. All the analyses were performed in duplicate.

The carry-over effect was evaluated by injecting in alternate sequence five blank urine samples spiked with all the analytes at the highest concentration and five blank deionized water solutions. Moreover, the carry-over effect was considered negligible if the S/N ratio was lower than 3 at the analytes' retention time for each monitored ion chromatogram obtained from the latter solutions.

Real urine sample collection

First morning urine samples were collected every day during a complete menstrual cycle (28 days) from 9 female volunteers aged 24-35 years, average 27.6 y ± 3.4 (1 parous). The 252 total samples were maintained at −20 °C and randomly analysed once the monthly collection was completed. All the women were healthy. None of them was taking any pharmaceutical drug or combined oral contraceptive pills in the period of sample collection. For all urine samples, the analytical determinations were normalized against their creatinine concentration to compensate for the physiological urinary dilution [2,4]. Creatinine was determined by the alkaline picrate photometric method using the dedicated kit on Architect C800 instrumentation (Abbott srl, Rome). In order to follow privacy regulations, an anonymous code was attributed to each participant subject who, anyway, voluntarily donated samples to the present project.

Chemometrics

Multivariate data analysis was carried out using Matlab® (The MathWorks, MA, USA) version 9.0.0 with PCA Toolbox version 1.2 [38], N-way Toolbox version 2.10 [39] and Classification Toolbox version 5.0 [40].

Data were arranged into a three-dimensional array (3-way), labelled as **X**, with dimensions ($I \times J \times K$) chosen as follows: (I) 9 subjects (representing the female volunteers), (J) 28 days (representing the menstrual cycle duration), (K) 15 variables (representing the studied estrogens). To analyse the three-dimensional data, a PARAllel FACtor analysis (PARAFAC) model [41-43] was applied. The Alternating Least Squares (ALS) algorithm basically decomposes the **X** 3-way array into three two-dimensional matrices , namely **A** ($I \times L$), **B** ($J \times L$) and **C** ($K \times L$), where the former variables (I, J, K) are expressed as a function of a new multivariate parameter (L) representing the loadings [41-43]. The **B** and **C** matrices show the natural fluctuation of each estrogen concentration throughout the 28-day menstrual cycle.

In order to separate the different phases of the menstrual cycle (i.e., follicular phase, ovulation and luteal phase), the Principal Component Analysis (PCA) [44] was carried out, as an exploratory method for multivariate data analysis. Since PCA works on two dimensional data matrices, the 3-way matrix **X** was unfolded in a J×IK matrix (i.e., 28×135), after autoscaling. The PCA model was built employingfollowing a venetian blinds cross-validation procedure, with a number of data splits equals to 5. The optimal number of principal components (PCs) was chosen from the predicted residual sums of squares (PRESS), root mean squared error of cross-validation (RMSECV) and the scree plot. Further parameters, including eigenvalues, percentage variance captured by each PC (Var%) and percentage cumulative variance captured by the model (CumVar%) were also evaluated [44].

Lastly, a linear discriminant analysis (LDA) model was built to verify the classification power of the multivariate estrogenic profile with respect to the phase of the menstrual cycle (e.g., luteal phase, ovulation and follicular phase). The variables used to build the LDA model were the first 10 PCs scores, obtained as linear combinations of the original estrogen concentrations. This approach has the advantage of removing noise from the dataset and improving the classification performances. The data multi-normality was verified and again a cross-validation procedure was performed by applying the venetian blinds design technique with 5 data splits. The classification criterion based on the Bayes' rule assigned each sampled day to the category showing the highest probability [40].

RESULTS AND DISCUSSION

Method optimization and validation

The DoE optimization of sample preparation [32] was aimed to achieve simpler and faster extraction conditions than those used in previous studies [27-30]. The best combination of drying temperature (found at 40°C) and extraction solvent was found with the ethyl acetate + hexane (2:3 v/v) mixture as it corresponded to higher resolution and intensity of the chromatographic peaks with respect to TBME [32].

Optimal chromatographic separation among the estrogens of similar chemical structure (for example, 2-methoxyestrone and 16α-hydroxyestrone) was obtained by using a slow increase of the oven temperature (3 °C/min) between 225 °C and 245 °C interrupted by a hold time at 234 °C for 3 min. Nevertheless, the full chromatographic run was completed in less than 20 minutes and the retention times of the target analytes lied between 6.58 min (17α-estradiol) and 10.50 min (16-epiestriol).

Linearity, LOD, LOQ

Full validation data are reported elsewhere [32]. The linearity of the calibration curves was tested in the concentration range of 1.0 – 50 ng/mL for all the analytes. Lack of fit's, ANOVA, RSD slope and Back-calculation tests proved to yield calculated results below the respective critical values for all the target analytes. Among the target analytes, only 17α-estradiol, 4-hydroxyestradiol and 2-methoxyestradiol were characterized by a quadratic calibration model. Most of the estrogens' models used an x^{-2} weighting correction, except 17α-estradiol, 2-methoxyestradiol, estrone, 4-methoxyestrone and estriol. From the residual plots, the calibration linearity was confirmed by the presence of random residuals patterns along the concentration ranges for all the analytes.

LOD values ranged between 0.2 ng/mL and 0.4 ng/mL. The LOQ values, estimated below 1.0 ng/mL for all target analytes, were verified experimentally. The first point (1.0 ng/mL) of each calibration range was successfully tested for precision and accuracy, as reported below, and was subsequently used as operational LOQ.

Repeatability and accuracy

Ion abundance and retention time repeatability proved experimentally appropriate. Intra-assay precision and accuracy satisfied the target criteria, as the CV% are lower than 15% for all the analytes at all tested concentration levels, while the percent bias (bias%) lied between −8.2% (2-hydroxyestrone) and +12% (2-hydroxyestradiol) at 1.0 ng/mL, −11% (4-hydroxyestradiol) and

+6.8% (4-hydroxyestrone) at 5.0 ng/mL and −6.2% (17α-estradiol) and +5.6% (2-hydroxyestradiol) at 25 ng/mL [32].

Matrix effect, extraction recovery, enzyme performance, carry over

The matrix effect values ranged from −12% for 4-methoxyestrone to +15% for 16α-hydroxyestrone at low level, from −9.2% for 4-hydroxyestrone to +12% for 17-epiestriol at medium level and from −5.6% for 2-hydroxyestradiol to +6.3% for 16α-hydroxyestrone at high level. These scattered values are close to the experimental bias and do not evidence any significant matrix effect. The average recovery efficiency was 99%, with minima and maxima ranging from 89% for 4-hydroxyestrone to 108% for 4-hydroxyestradiol and 17-epiestriol at 1.0 ng/mL; from 87% for 17α-estradiol to 107% for 4-hydroxyestrone at 5.0 ng/mL; from 94% for 17α-estradiol to 110% for 2-hydroxyestradiol at 25 ng/mL. Again, the extraction recovery was virtually complete at all concentration levels allowing a correct estimation of the target analytes' concentration.

The percent hydrolysis achieved by both β-glucuronidase and arylsulfatase on estrone glucuronide and estrone sulfate at all concentration levels was close to 100%, supporting the claim that the deconjugation efficiency on phase II metabolites could be considered complete. No carry over effect was observed.

PARAFAC Model

The PARAFAC approach is commonly employed in environmental data analysis, when repeated chronological monitoring of sampling sites yields three-dimensional data structures. The same statistical tool is suitable for our chronological monitoring of estrogen profiles [39,41-43]. A PARAFAC model was built to extract the concentration profile for each estrogen along the 28-day menstrual cycle, by smoothing the large individual variability of estrogenic profiles, that proved significant for the 9 investigated women. Due to the different duration of the menstrual cycles, spanning between 28 and 30 days, the ovulation peak occurred at different days, from the 13^{th} to the 17^{th} day, in agreement with the literature [17,45-47]. To comply with this source of variability, the extreme sampling days were removed from the series collected from the women with a menstrual cycle longer than 28 days. Actually, the extreme days (i.e. the first and the last of the menstrual cycle) exhibited comparable results with the subsequent and preceding samples, respectively. The final PARAFAC processing allowed the equalize each menstrual cycle within a unique scale so as to evaluate and compare the natural variation of the estrogenic levels. The

number of significant factors for the PARAFAC model was two, that explain a CumVar% of 86.98%, relative to Var%=75.17% and Var%=10.81% for factor 1 and factor 2, respectively.

All the extrapolated estrogenic profiles are reported in Figure 1 and exhibit several remarkable features. In particular, 17β-estradiol (2a) and estrone (2b) show two peaks, the first occurring close to the ovulation with a time-shift of 3-4 days between the two hormones, while the second smoother peak appears in the period around the 20^{th}-25^{th} day of the cycle. These profiles are comparable to those reported in the literature [7–9,12,48]. In contrast, no peak is observed for 17α-estradiol (2a) in the central part of the cycle and only a faint increase of its level is detectable in the luteal phase of the cycle. The lack of correlation between 17α- and 17β-estradiol profiles may explain the scarce specificity of the immunoassays methods used for their quantification.

Several metabolite profiles are characterized by the occurrence of a single concentration peak around the ovulation, namely 2-hydroxyestradiol (1c), 4-hydroxyestradiol (1c), and 2-methoxyestrone (1f) at the 15^{th} day, but 4-hydroxyestrone (1e) and 2-hydroxyestrone (1e) together with estrone (1b) at the 17^{th} day. Surprisingly, 2-methoxyestrone shows a chronological correlation with hydroxyestradiol isomers instead of hydroxyestrone isomers, as it would be expected. On the other hand, 4-methoxyestrone (1f) show a sharp peak in the follicular phase of the cycle, that is not observed for the isomer 2-methoxyestrone. The different behaviour observed for 2- and 4-methoxyestrone isomers contrasts with those recorded for the analogous hydroxyestrone (1e) and hydroxyestradiol (1c) isomers. All these observations add details on the complex regulating system of the estrogen biochemistry active during the ovulation phase which can not be explained by straightforward and progressive metabolic pathways [14].

4-methoxyestradiol (1d), 16-epiestriol, and 17-epiestriol (1g) display a profile in which the concentration increases around the ovulation and remains quite stable for the subsequent 10 days, whereas 2-methoxyestradiol (1d) shows a constant decrease along the cycle.

Barrett et al [7] and Venners et al [9] determined the urinary concentration of estrone alongside the entire menstrual cycle by immunoassay: the resulting profiles showed the same pattern that we observed in the PARAFAC profile, even if the analytical methods were different. A comparable profile was also observed by Baird et al [8] who used a radio-immunoassay method. Likewise, the profiles of 17α- and 17β-estradiol that we observed substantially overlaps the ones reported by Roney et al [12] and Barrett et al [48], although in these studies the concentrations were measured by immunoassay in the oral fluid. Basically, two peaks are observed, the first one just

before the ovulation and the second during the luteal phase. Noteworthy, the first peak has not been observed in our study for 17α-estradiol.

The correspondence of our data with literature profiles and the agreement between oral fluid and urine data, and between GC-MS and immunoassay methods represent further confirmation of the reliability of the present approach to gain general information about the relative concentration of the circulating hormones. The multi-residual GC-MS method proposed in this study proved to represent a fast, cheap, practical, and reliable analytical tool for the monitoring of an extended estrogenic profile in young women (24-35 years), overcoming the lack of specificity typical of immunoassay methods.

PCA results and LDA model

Principal component analysis (PCA) was performed on the complete 28 × 135 data matrix with the purpose of discovering any underlying structure in the data The optimal number of principal components (PC) to be considered was two representing a CumVar% of 28.22% and a RMSECV% of 16.13%. The limited percentage of total variance explained by PC1+PC2 (only 28.22%) is coherent with the large variability of the data. In practice, the new PC variables, as linear combination of the old ones (concentration of estrogens), emphasize the information content present in the data while reducing the contribution of their random fluctuation. The scores plot of PC1 (Var% 14.91%) vs PC2 (Var% 13.31%) is reported in Figure 2A and shows the occurrence of three broad clusters corresponding to the three phases of the menstrual cycle: follicular, ovulation, and luteal. The follicular and ovulation phase data are separated along PC2, while the ovulation and luteal phase data along PC1.

By plotting PC1 and PC2 as a function of the menstrual cycle day in two separate diagrams (Figure 2B-2C), the phase transitions become visible and the starting point of both the ovulation and luteal phase can be clearly identified.

A preliminary LDA model was built using the information extracted by the PCA scores. While the PARAFAC technique demonstrated that the original data were affected by large internal variability which prevented the construction of a reliable and stable classification model based on them, the PC scores are free from correlation and noisy pattern. Therefore, the PCA scores were used instead of the original estrogen data to build the LDA model.

The multi-normality of the PC scores was successfully checked (Figure 3A) and then a cross-validating procedure was applied to the 28×10 matrix (10 PCs were considered). A cross-validated

non-error rate of 90% was achieved, together with an accuracy equal of 93%, as is shown in the confusion matrix reported in Table 1. Only two data-points were misclassified, namely the 17th day, which was classified in class 3 (luteal phase) instead of class 2 (ovulation), and the 18th day, which was classified in class 1 (follicular phase) instead of class 3. The misclassification of the 17th and 18th days was not surprising since both data-points belong to the transition period from the ovulation to the luteal phase and correspond to a sudden drop of the estrone, 2- and 4-hydroxyestrone concentrations (Figure 1b, 1e). The accurate classification of all days belonging to the transition from the follicular to the ovulation phase is explained by the smoother concentration increment observed for 17β-estradiol, 2- and 4-hydroyestradiol from the 11th to the 15th day of the cycle (Figure 1a, 1c).

The scores plot reported in Figure 3B shows the good partition of the days in three well-defined classes corresponding to the follicular phase, the ovulation and the luteal phase. The loadings plot, representing the PC variables in the space of the LDA canonical variables (Figure 3C), indicates the correspondence between class discrimination and PCs. In particular, PC2 is high during the ovulation and low during the follicular and luteal phases. Hence, it is able to identify the ovulation period from the other phases of the cycle. On the other hand, the luteal phase is characterized by elevated values of PC1 (and PC7), which is low in the follicular phase and especially low during the ovulation, distinguished also by a high value of PC8.

Studies that use the menstrual cycle phase as a proxy for directly measured ovarian hormone levels typically fail to capture their inherent variability. The lack of reliable methods to divide the menstrual cycle into its component phases was proved, as divergent outcomes may be produced by using different methods [3,10]. The application of the present multivariate statistical model to GC-MS data is expected to overcome this limit and allow a correct definition of the phases of the menstrual cycle, an important issue in the study of fertility. For example, Barrett et al [7] who determined the concentration of urinary estrone alongside a complete menstrual cycle established the difference in ovarian function between nulliparous and parous women. In general, the menstrual cycle features represent important indicators of the reproductive health and endocrine function. For example, Small et al [11] found a connection between the menstrual cycle variability and the likelihood of pregnancy, Venners et al [9] discovered that higher estrogen concentrations were associated with the occurrence of clinical pregnancy, and Baird et al [8] studied the hormonal pattern most appropriate for pre-implantation. All the areas of interest linked to reproduction could benefit from a multivariate interpretation of a wide estrogen profile,

such as the one proposed in the present study, which may find application also in the investigation of a variety of physical and psychological disorders.

CONCLUSIONS

In the present study, a GC-MS method is proposed for the simultaneous detection of 15 estrogens in the urine of a group of young women, that involves easy sample pretreatment, overcoming some of the limitations of previously published GC-MS protocols [28,29]. The reliability of the procedure was validated following a rigorous protocol and good performances were obtained, particularly in terms of efficient extraction recovery and adequate sensitivity, making the GC-MS approach competitive with the more demanding LC-MS/MS technique. In case that the concentration of one specific estrogen has to be determined with high accuracy, the method can be further improved by using a dedicated isotopically-labelled homolog as the internal standard.

Despite the large variability of the experimental data, the use of multivariate statistics on urine sample sequences collected from nine women – in particular the application of the PARAFAC approach - proved capable to extract the typical concentration profile for each analyte along the menstrual cycle, including estriol and the eleven metabolites not previously investigated in women. As a matter of fact, most of the existing literature only reports the variations of estrone and estradiol concentrations across the complete menstrual cycle, whereas in the present case a generalized picture for a broad urinary estrogen panel along the whole menstrual period has been described for the first time.

The advantages of using multivariate data analysis was made evident by the application of PCA, which yielded an easier visualization and efficient partition of the data into three groups, corresponding to the three phases of the menstrual cycle, namely the follicular phase, ovulation, and the luteal phase, together with the transitions between the phases.

The preliminary LDA model built on the PCA scores produced a reliable classification of each day along the cycle series, with a satisfactory cross-validated non-error rate of 90%. Therefore, the multivariate comparison of the estrogen profile collected from a single urine sample with the proposed model is likely to provide a trustworthy classification of this sample in terms of phase of the menstrual cycle (follicular, ovulation, luteal). Possible applications of the model include the detection of the fertile days along the cycle, the screening of pathological conditions, and the identification of particular stressing or psychological conditions of the investigated subjects.

Further refinement of the present classification model is underway, as its full validation will require a much larger training and test sets than the one used in this proof-of-concept contribution based the on the recruitment of nine volunteers.

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Figure captions

Figure 1. Concentration profile (normalized for the creatinine value) of the target analytes along the 28-day menstrual cycle achieved by applying PARAFAC approach: (a) 17α-estradiol and 17β-estradiol, (b) estrone and estriol, (c) 2-hydroxyestradiol and 4-hydroxyestradiol, (d) 2-methoxyestradiol and 4-methoxyestradiol, (e) 2-hydroxyestrone and 4-hydroxyestrone, (f) 2-methoxyestrone and 4-methoxyestrone, (g) 16α-hydroxyestrone and 16-epiestriol, and (h) 17-epiestriol.

Figure 2. Results provided by the PCA model: (a) Score plot relevant to PC1 (Var. = 14.91 %) vs PC2 (Var. = 13.31%) showing the occurrence of three different clusters corresponding to the three phases of the menstrual cycle, i.e. follicular phase (blue dots), ovulation (red dots) and luteal phase (green dots). (b) PC1 vs menstrual cycle day, representing the transition from the follicular phase to the ovulation phase. (c) PC2 vs menstrual cycle day, representing the transition from the ovulation phase to the luteal phase.

Figure 3. Results achieved by building the LDA model. (a) Multinormality test graph. (b) Score Plot relevant to the first two latent variables, showing the partition of the data in three well-defined classes: follicular phase (blue dots), ovulation (red dots) and luteal phase (green dots). (c) Loading Plot relevant to the first two latent variables, showing the PCs that mainly characterize the three classes of samples.

Figure 1

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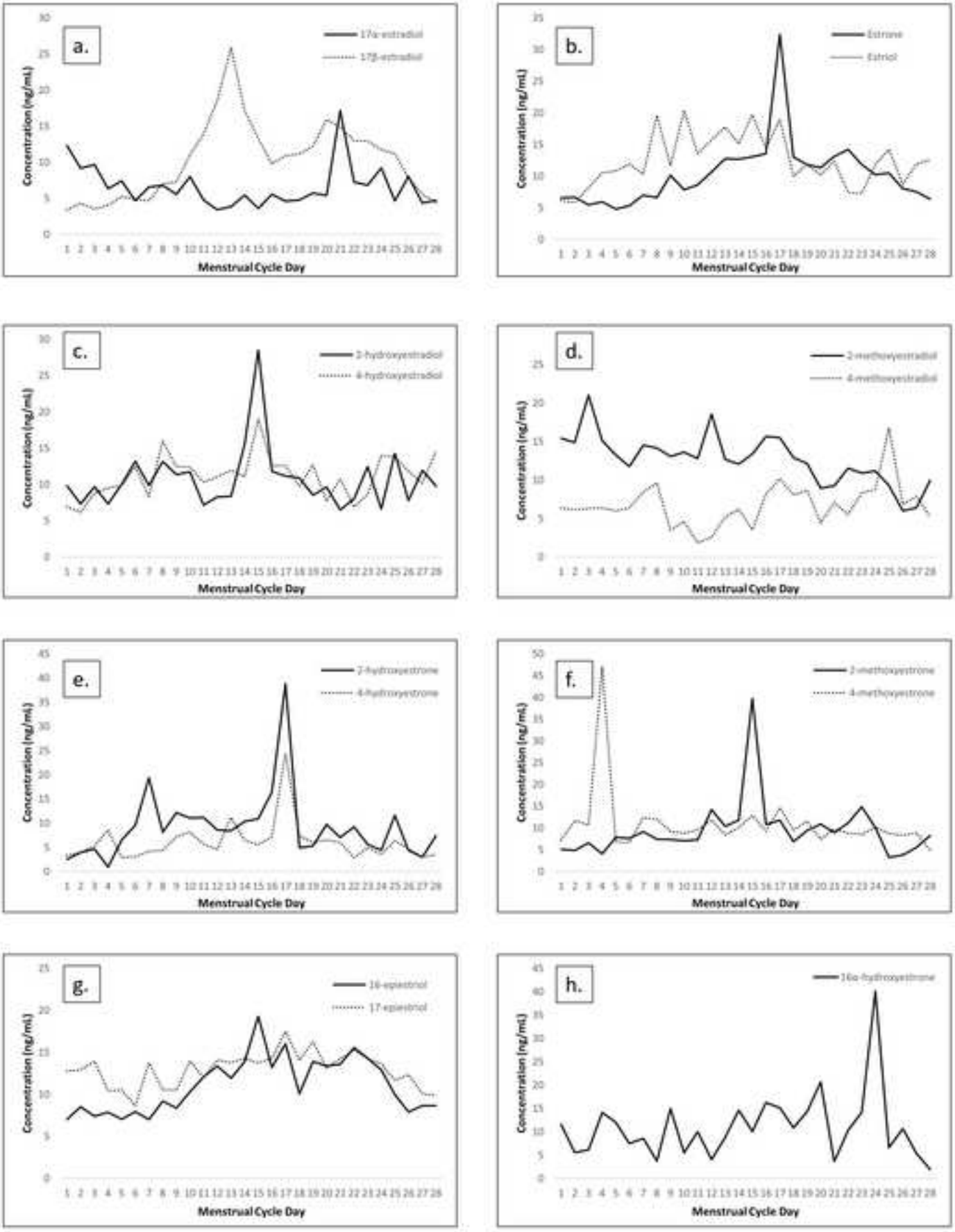

Figure 2

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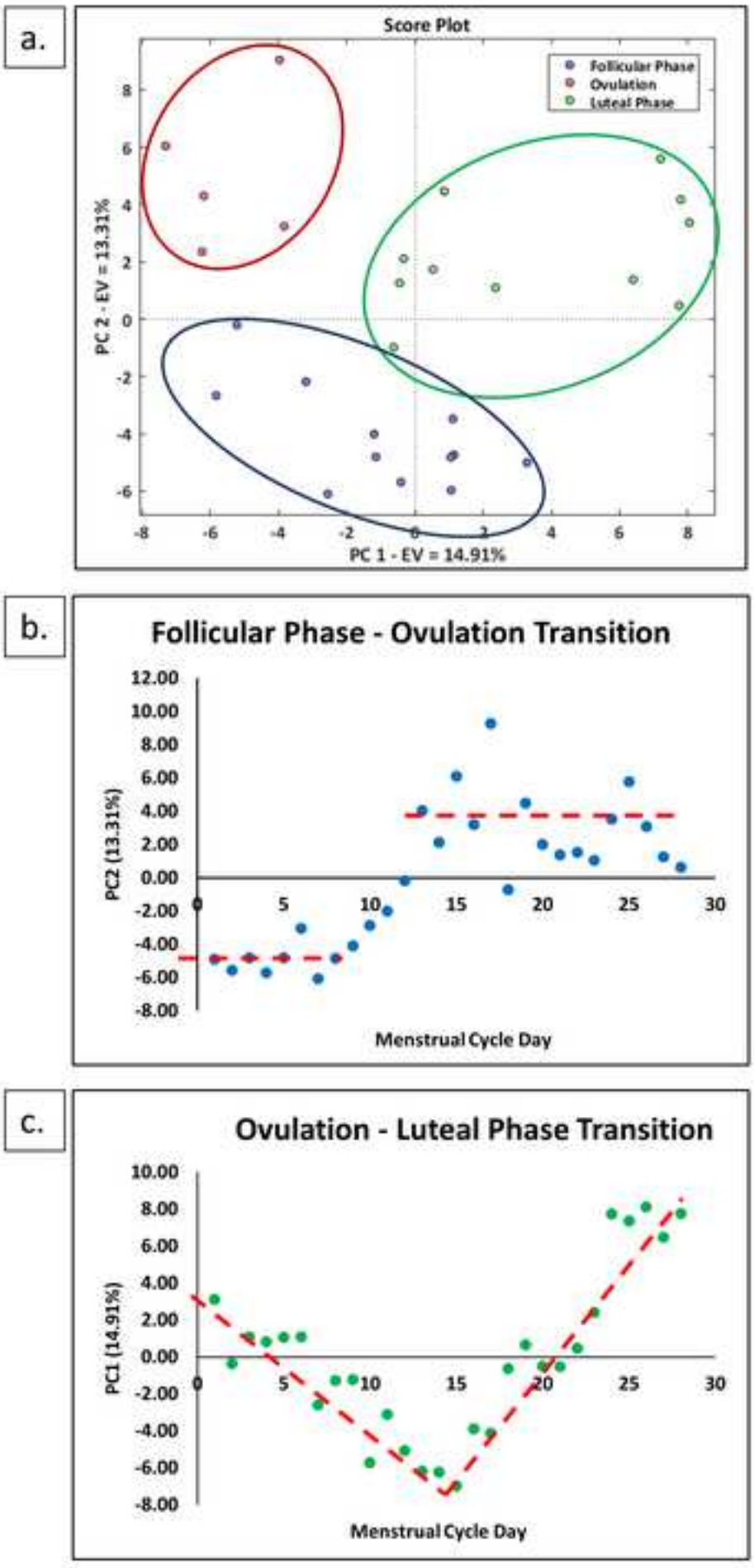

Figure 3

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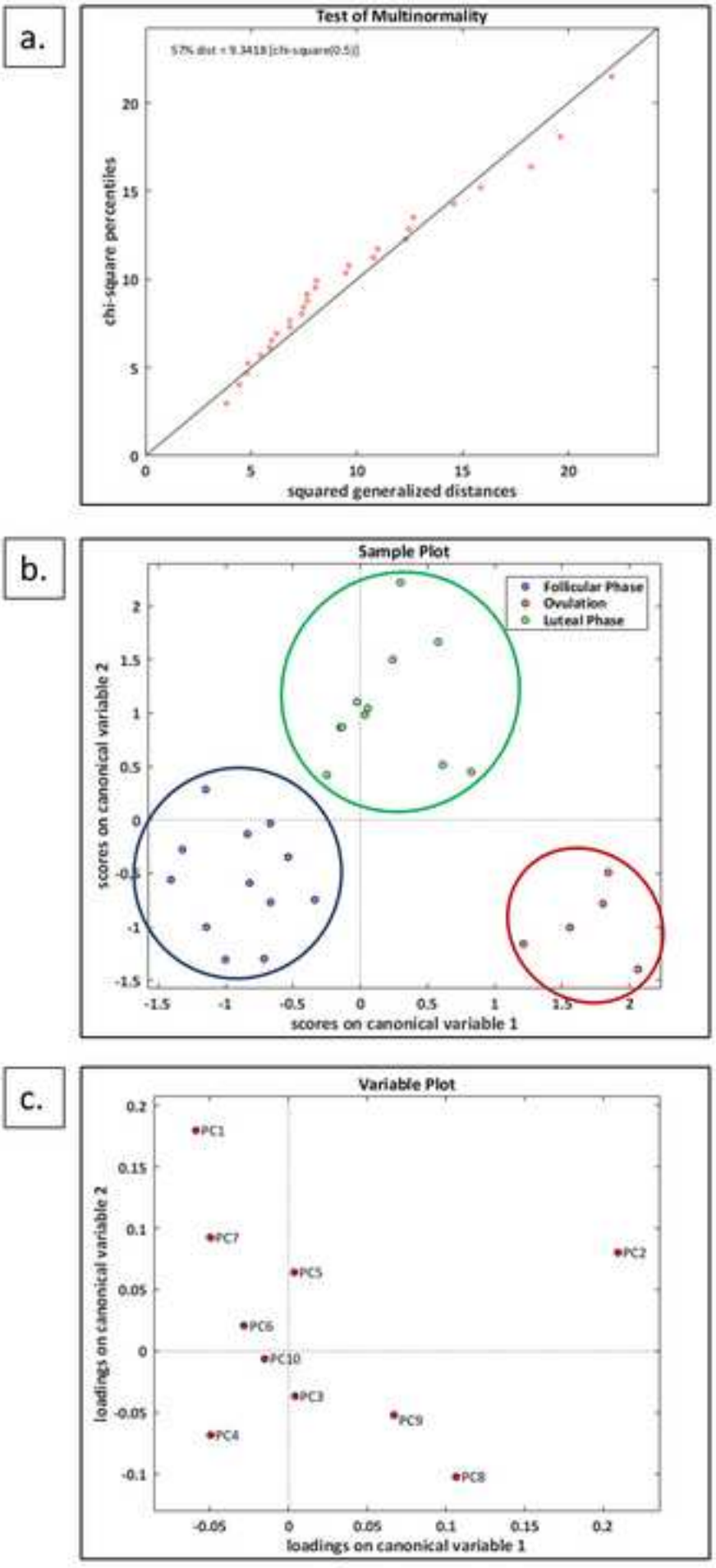

Table 1

Table 1. Validation parameters and results relative to the evaluation of the calibration curves for all the target analytes, as follows: dynamic range of calibration, coefficient of determination (R^2), limit of detection (LOD), limit of quantitation (LOQ), lack-of-fit, ANOVA and RSD slope tests, back calculation results, type of model and relative weights. The critical values of the significance tests are reported, too.

Analyte	Linearity range (ng/mL)	Correlation coefficient (R^2)	LOD (ng/mL)	LOQ (ng/mL)	Lack of fit's test (F_{exp})	ANOVA (F_{exp})	RSD slope test (%)	Back calculation test (%)	Model	Weight
17α-estradiol	1.0 – 50.0	0.9977	0.33	0.67	2.31	1.62	2.37	17	Quadratic	x^{-1}
17β-estradiol	1.0 – 50.0	0.9985	0.27	0.55	2.73	1.39	1.94	15	Linear	x^{-2}
2-hydroxyestradiol	1.0 – 50.0	0.9968	0.40	0.80	1.93	1.85	2.84	19	Linear	x^{-2}
4-hydroxyestradiol	1.0 – 50.0	0.9976	0.35	0.69	2.26	1.18	2.45	19	Quadratic	x^{-2}
2-methoxyestradiol	1.0 – 50.0	0.9985	0.28	0.55	1.12	1.02	1.97	16	Quadratic	x^{-1}
4-methoxyestradiol	1.0 – 50.0	0.9988	0.25	0.49	1.23	1.03	1.75	13	Linear	x^{-2}
Estrone	1.0 – 50.0	0.9980	0.31	0.63	0.77	0.97	2.23	14	Linear	x^{-1}
2-hydroxyestrone	1.0 – 50.0	0.9985	0.27	0.54	1.07	1.01	1.92	15	Linear	x^{-2}
4-hydroxyestrone	1.0 – 50.0	0.9966	0.41	0.83	1.37	1.05	2.94	19	Linear	x^{-2}
16α-hydroxyestrone	1.0 – 50.0	0.9988	0.24	0.49	0.17	0.88	1.74	8	Linear	x^{-2}
2-methoxyestrone	1.0 – 50.0	0.9993	0.19	0.38	0.10	0.87	1.34	16	Linear	x^{-2}
4-methoxyestrone	1.0 – 50.0	0.9993	0.19	0.38	0.29	0.90	1.36	15	Linear	x^{-1}
Estriol	1.0 – 50.0	0.9990	0.22	0.44	0.20	0.89	1.56	11	Linear	x^{-1}
16-epiestriol	1.0 – 50.0	0.9983	0.29	0.58	2.09	1.30	2.04	15	Linear	x^{-2}
17-epiestriol	1.0 – 50.0	0.9987	0.25	0.50	1.58	1.08	1.77	12	Linear	x^{-2}

Lack of fit's test – F_{crit} = 2.776 (n_1 = 4 and n_2 = 24 degrees of freedom)
ANOVA – F_{tab} = 3.842 (n_1 = 1 and n_2 = 28 degrees of freedom)
RSD slope test - %RSD threshold = 5.00%
Back calculation test - % threshold = 20%

Table 2

Table 2. Intra-day precision (CV%), accuracy (bias%), matrix effect and recovery for each analyte tested, together with hydrolysis efficiency of the enzyme. Levels I, II and III represent the concentration levels at which the selected parameters were evaluated, i.e. 1.0 ng/mL, 5.0 ng/mL and 25 ng/mL, respectively.

Analyte	Precision (CV%)			Accuracy (bias%)			Matrix effect (%)			Recovery (%)		
	Level I	Level II	Level III	Level I	Level II	Level III	Level I	Level II	Level III	Level I	Level II	Level III
17α-estradiol	13	9.7	7.3	+7.9	+2.7	-6.2	+8.1	+10	+4.4	106	87	94
17β-estradiol	7.5	1.4	4.1	+7.6	+5.3	-1.1	+3.1	+1.8	+4.4	101	98	100
2-hydroxyestradiol	13	9.4	3.5	+12	-2.6	+5.6	-6.4	-2.1	-5.6	105	97	110
4-hydroxyestradiol	7.3	8.0	1.4	+6.5	-11	+2.3	-1.8	-1.1	-0.3	108	90	101
2-methoxyestradiol	8.1	6.6	1.5	+10	+6.1	-0.5	+2.2	+0.1	+2.0	104	99	98
4-methoxyestradiol	4.9	0.4	1.4	+2.0	+3.5	-0.05	+1.8	+0.6	+3.7	100	99	98
Estrone	13	5.6	1.2	+7.3	+1.8	+3.6	-3.9	-7.3	-2.1	107	97	102
2-hydroxyestrone	12	3.4	1.7	-8.2	+5.0	-2.1	-9.9	-8.6	-1.2	93	100	99
4-hydroxyestrone	9.7	4.1	3.0	-7.8	+6.8	-1.0	-5.0	-9.2	-4.8	89	107	99
16α-hydroxyestrone	13	4.4	5.0	-6.6	-2.0	-2.2	+15	+8.1	+6.3	95	99	101
2-methoxyestrone	11	7.0	3.8	+8.9	-2.1	+1.7	+8.5	+10	+3.1	93	100	101
4-methoxyestrone	12	9.3	7.1	+3.6	+4.8	-4.1	-12	-2.4	-0.1	99	104	100
Estriol	13	9.2	2.9	+9.6	+4.6	+1.5	-9.5	-0.6	-1.5	95	102	96
16-epiestriol	1.3	1.9	2.0	+3.7	+5.1	-0.5	-0.2	-1.1	-3.7	102	95	97
17-epiestriol	4.3	2.2	0.4	+5.8	+5.4	-0.4	+9.8	+12	+2.9	108	92	99

Enzymes	Deglucuronidation (%)			Desulphatation (%)		
	Level I	Level II	Level III	Level I	Level II	Level III
β-glucuronidase, arylsulfatase	99	101	98	102	97	94

Table 3. Confusion matrix provided by the LDA model. The rows represent the real classes, while the columns represent the predicted ones; the correctly classified samples are reported on the diagonal. Overall non-error rate is reported, too.

Confusion matrix	Follicular Phase	Ovulation	Luteal Phase
Follicular Phase	12	0	0
Ovulation	0	4	1
Luteal Phase	1	0	10
Non-error rate	90%		

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