

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Individual and cyclic estrogenic profile in women: Structure and variability of the data

This is a pre print version of the following article:

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1727839> since 2020-02-16T18:24:47Z

Published version:

DOI:10.1016/j.steroids.2019.108432

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

Manuscript Number: STEROIDS-D-19-00017R2

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 \times days \times 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.

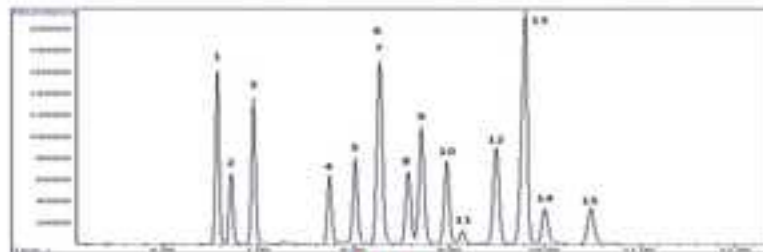
The last paragraph was extensively revised, as follows: (a) the position of second and third sentences of the paragraph was inverted; (b) the central sentence was expanded and split in two to make the inherent conclusion clear.

Other comments:

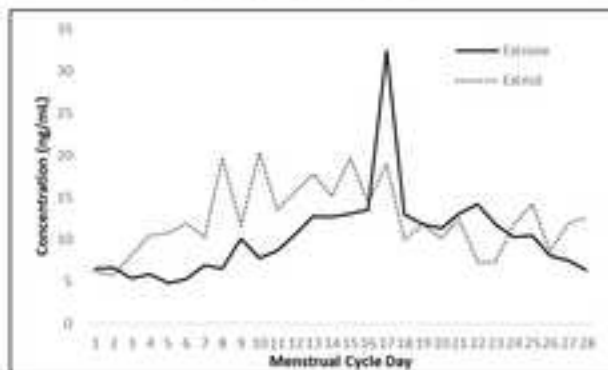
- With respect to limitations, the authors need to clearly describe the limitations of their assay design and specifically reference possible limitations of using only 2 internal standards on identifying independent peaks when more standards are available.

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.”

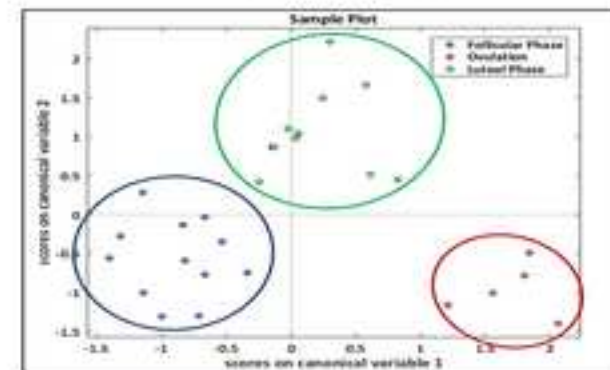
INDIVIDUAL AND CYCLIC ESTROGENIC PROFILE IN WOMEN: STRUCTURE AND VARIABILITY OF THE DATA



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.

1 INTRODUCTION

1
2
3
4 Estrogens play a variety of crucial roles in the menstrual cycle and throughout the entire life of
5
6 women. The menstrual cycle is the cyclic orderly sloughing of the uterine lining, in response to the
7
8 interactions of hormones produced by the hypothalamus, pituitary and ovaries [1]. The duration
9
10 of a complete menstrual cycle spans from 21 to 35 days, with an average of 28 days. The
11
12 menstrual cycle is usually divided into the follicular and the luteal phases. The follicular phase
13
14 begins from the first day of menses until ovulation, which typically occurs around the 14th day.
15
16 After ovulation, the luteal phase starts and lasts 14 further days, on average [1–4]. Lifestyle
17
18 factors, such as smoking, physical activity and alcohol consumption may affect the phases of the
19
20 menstrual cycle [5]. Abnormally high and low values of body mass index (BMI) are frequently
21
22 associated to menstrual dysfunctions, due to the correlation of the estrogens metabolism with the
23
24 nutrition and dietary composition and the role of adipose tissue in aromatase conversion [6]. The
25
26 natural rhythmic fluctuations of the estrogens that control the menstrual cycle influence the
27
28 fertility [7–11] and various physical and psychological conditions [3,12–15].

29 An important methodological issue with the study of estrogens data is how to align the cycles of
30
31 the different women to allow comparisons [9]. In the Nurses' Health Study II, this issue was
32
33 overcome by sampling all the women during their luteal phase [16]. The main problem for this
34
35 approach is the difficult recognition of the menstrual phase in women with irregular periods. To
36
37 date, only the variation of estrone or estradiol levels were evaluated across complete menstrual
38
39 cycles, possibly because these are the main estrogens circulating in the human body, together
40
41 with estriol [17]. A comprehensive evaluation of an extended estrogenic profile was previously
42
43 proposed with the purposes of detecting any possible correlations between estrogens and breast
44
45 cancer risk: the urinary estrogenic profile of 15 free and conjugated estrogens was collected from
46
47 a large cohort of pre-menopausal women and retrospectively interpreted, on the basis of their
48
49 clinical history [16]. Extended estrogenic profiles were also correlated with terminal duct lobular
50
51 unit involution, a marker of increased breast cancer risk [18]. In parallel studies on post-
52
53 menopausal women, the determination of blood estrogens and metabolites revealed a lower risk
54
55 of breast cancer for the subjects with high levels of the hydroxylated 2-pathway metabolites
56
57 [19,20].

58 The estrogen determinations most frequently reported in the literature are conducted on either
59
60 urine or oral fluid, using radioimmunoassay (RIA), enzyme immunoassay (EIA), or enzyme-linked
61
62
63
64
65

33 immunosorbent assay (ELISA) [22]. While these immunoassay methods provide high throughput,
1 efficiency, ease of use, fast turnaround time and low cost, they frequently do not have the
24 necessary specificity and sensitivity to accurately measure low estrogen concentrations, due to
3
45 cross-reactivity with structurally similar substances [21,22]. This limits the chance of estrogen
5
66 profiling during the follicular and late luteal phases, when their concentration level is particularly
7
87 low. In contrast, the hyphenation of chromatographic and mass spectrometric (MS) techniques
9
38 provides the simultaneous dosage of both parent estrogens and their metabolites ensuring at the
11
39 same time extremely low detection limits [21-23]. Liquid chromatography (LC) and gas
13
40 chromatography (GC) coupled with MS are consistently used in multi-analyte profiling, with LC-MS
15
41 increasingly favored for its straightforward applicability, even if GC-MS has traditionally dominated
17
42 the analysis of estrogens and other endogenous steroids for years. Actually, GC-MS provides broad
18
43 steroids profiles after a single derivatization step, achieving high specificity, good sensitivity, and
20
44 limited matrix effects [23]. In general, the advantage of high-resolution separation is increasingly
22
45 valued in targeted and untargeted metabolomics to obtain complete urinary endogenous steroid
24
46 profiles that include estrogens, androgens, corticoids, and progesterone [24-26]. Multi-residual
26
47 GC-MS methods for the detection of wide estrogen profiles have occasionally been developed in
28
48 the past [27-30], even if the laborious sample preparation steps somehow contributed to the
30
49 progressive decline of GC-MS procedures in favor of LC-MS.
32
350

34
351 In the present study, 15 estrogens were monitored in nine women along one menstrual period
36
372 using an optimized and fully validated GC-MS method. The collected data were used to build a
38
393 preliminary multivariate model shaping the menstrual cycle, which may represent a valuable tool
40
414 in the study of fertility issues, as well as in the screening and evaluation of various pathological
42
435 conditions, including endocrinology disorders and hormone-dependent cancers.
44
456

46 MATERIAL AND METHODS

48 *Chemicals and reagents*

49
50
51
52 4-methoxyestrone, 4-methoxyestradiol, 2-methoxyestrone, 16 α -hydroxyestrone, 2-
53
54 methoxyestradiol, 2-hydroxyestradiol, 4-hydroxyestrone, 4-hydroxyestradiol, 17-epiestriol and 16-
55
562 epiestriol were purchased from Toronto Research Chemical Inc. (Toronto, ON, Canada). 17 α -
57
583 estradiol, 17 β -estradiol, hexane, methanol, ethyl acetate, ascorbic acid, ammonium iodide, *tert*-
59
604 butyl methyl ether (TBME), dithioerythritol and N-methyl-N-(trimethylsilyl) trifluoroacetamide
61
62
63
64
65

65 (MSTFA), β -glucuronidase/arylsulfatase (from *Helix pomatia*) mixture, were provided by Sigma-
1 Aldrich (Milan, Italy). Estrone, 2-hydroxyestrone, estrone 3-(β -D-glucuronide) sodium salt, estriol,
2
3
4
5
6
7
8
9
10 C-18 endcapped Solid-Phase Extraction (SPE) cartridges were provided by UCT Technologies
11
12
13
14 USA).

15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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 μ g/mL and 1 μ g/mL by appropriate dilution with methanol. Estrone-d₄ and 17 β -estradiol-d₄ were used as isotopically labelled internal standards for quantitation and were added from separate methanol working solutions at the final concentrations of 100 μ g/mL and 50 μ g/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 17 β -estradiol-d₄ and estrone-d₄ 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 μ L 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 μ L of β -glucuronidase/arylsulfatase mixture to the urine samples, which were then incubated at 37 $^{\circ}$ C overnight. The next morning, 100 μ L β -glucuronidase from *Escherichia Coli* was added, together with 50 μ L of ascorbic acid solution and the final enzymatic deconjugation of the remaining glucuronide estrogens was carried out for 1 hour at 58 $^{\circ}$ 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

97 Megafuge 1.0 Heraeus; ASHI, Milan, Italy) at 4000 rpm for 5 min. The extraction process was
1 repeated twice, and the two combined organic phases were transferred into a vial and evaporated
28 to dryness under a gentle stream of nitrogen at 40 °C using a Techne Sample Concentrator
3
49 (Barloworld Scientific, Stone, UK). The dried residue was reconstituted and derivatized for 1 hour
5
100 at 70 °C by adding 50 µL of MSTFA/NH₄I/dithioerythritol (1.000:2:4 v/w/w) solution. A 2 µL aliquot
7
101 was injected into the GC/MS system in the splitless mode.
9
102

13 GC-MS analysis 14

15 All analyses were conducted on an Agilent 6890N Network GC System interfaced to a 5975 inert XL
16
17 Mass Selective Detector (Agilent Technologies, Milan, Italy). The GC was equipped with a J&W
18
19 Scientific HP-1 17.0 m x 200 µm (i.d.) x 0.11 µm (f.t.) capillary column. The helium gas carrier was
20
21 employed at a constant pressure of 23.25 psi and 1.1 mL/min initial flow. The GC oven
22
23 temperature was initially set at 200 °C, held for 2 min, then was raised to 225 °C with an 8 °C/min
24
25 ramp. Then, the temperature was increased to 234 °C with a 3 °C/min heating rate, held for 3 min
26
27 and raised again to 245 °C with a 3 °C/min ramp. The final oven temperature of 315 °C was
28
29 reached with a 40 °C/min heating rate and held for 3 min. The total run time was 19.54 min. The
30
31 GC injector and transfer line were maintained at the temperature of 280 °C. Trimethylsilyl
32
33 derivatives of the analytes were ionized by electron ionization (EI) at 70 eV. Data were acquired in
34
35 the selected ion monitoring (SIM) mode at a dwell time of 20 ms [32,34].
36
37

38 Method validation 39

40 The analytical method was validated according to the Eurachem criteria and recommendations
41
42 [35]: linearity range, selectivity, specificity, limit of detection (LOD), limit of quantitation (LOQ),
43
44 intra-assay precision and accuracy, repeatability, matrix effect, extraction recovery, and carry-over
45
46 were determined. A pool of urines collected from healthy male volunteers (laboratory personnel),
47
48 was negativized by solid-phase extraction using a C-18 end-capped cartridge previously
49
50 conditioned with 2-propanol and ultra-pure water. The absence of any detectable trace of
51
52 estrogens was verified. The resulting sample was used as blank urine and spiked with the standard
53
54 solutions within the validation procedure. Full details about the validation of the analytical
55
56 method are reported in a dedicated publication [32].
57
58

59 Linearity, LOD, LOQ 60 61 62 63 64 65

129 The calibration was performed by internal standardization using the least squares regression
1 method from five replicate analyses for each data-point at six concentration levels in the range 1-
130 50 ng/mL. Linearity was evaluated by lack-of-fit test, analysis of variance (ANOVA) test, Mandel's
3 test, and relative standard deviation (RSD) of the slope, according to the approach described by
131 5
132 Desharnais et al. [36]. Moreover, the residual plots and the deviation from back-calculated
7
133 concentrations were examined. When heteroscedastic distribution of data-points was observed, a
9
134 weighting factor of x^{-1} or x^{-2} was employed, depending on the rate of the variance increase with
11
135 the concentration (linear or quadratic).
13
136
15
137 LOD and LOQ were estimated for all the target analytes using the Hubaux-Vos' algorithm at a
16
138 significant level of 95% [37] from the 30 data-points collected to build the calibration lines. To
17
139 confirm the correct estimation further, the calculated LOD and LOQ values were experimentally
18
140 verified with blank samples spiked at concentrations close to the detectable and quantifiable
19
141 values, respectively. In the operational practice, LOQ values were assumed at the lower level of
20
142 the calibration curves.
21
22
23
24
25
26
27
28

29 *Repeatability and accuracy*

30
31 The retention time repeatability was verified on the chromatographic peak of the target analytes
32 recorded in the 30 overall analyses used to build the five calibration curves (see above). Deviations
33 below 1% from calibrators and controls were considered satisfactory. The repeatability of the
34 relative ion abundance was evaluated on the selected ion chromatograms for each target analyte.
35
36 The variations were considered acceptable within $\pm 20\%$, with respect to the controls.
37
38
39 For all analytes, intra-day repeatability and accuracy were evaluated on 10 blank urine samples
40 spiked with all the target analytes at three concentration levels (1.0 ng/mL, 5.0 ng/mL and 25
41 ng/mL). Precision and accuracy were estimated from the percent variation coefficient (CV%) and
42 percent bias (bias%), respectively. Precision was considered satisfactory when the CV% values
43 were below 15% for the low calibration level and below 10% for the other levels. Satisfactory
44 accuracy was achieved when the experimentally determined average concentration lied within
45 $\pm 10\%$ from the expected value.
46
47
48
49
50
51
52
53

54 *Matrix effect, extraction recovery, enzyme performance, carry over*

55
56 The matrix effect was evaluated at the three concentration levels defined above by comparing the
57 experimental results obtained from blank urine samples (mean value from five replicates) and
58
59
60
61
62
63
64
65

161 blank deionized water solution both spiked after the extraction step at the same concentration.
1
162 The matrix effect for each target analyte was expressed as the percentage ratio between the two
3
163 measured concentrations. Extraction recovery was calculated at the same concentration levels by
5
164 comparing the experimental results from blank urine samples spiked respectively before and after
7
165 the extraction step (5 replicates each) and expressed as the percentage ratio between the two
9
166 data.

11
167 The efficiency of β -glucuronidase and arylsulfatase to achieve exhaustive hydrolysis of the
12
168 conjugated metabolites was tested at three concentration levels (1.0 ng/mL, 5.0 ng/mL and 25
13
169 ng/mL) by measuring the percentage ratio between the recovered concentration of estrone
14
170 glucuronide (and sulfate) spiked into a blank sample and that of free estrone spiked to another
18
171 blank sample at the same molar concentration. All the analyses were performed in duplicate.

20
172 The carry-over effect was evaluated by injecting in alternate sequence five blank urine samples
22
173 spiked with all the analytes at the highest concentration and five blank deionized water solutions.
24
174 Moreover, the carry-over effect was considered negligible if the S/N ratio was lower than 3 at the
26
175 analytes' retention time for each monitored ion chromatogram obtained from the latter solutions.
28

30

31 177 **Real urine sample collection**

32
33 178 First morning urine samples were collected every day during a complete menstrual cycle (28 days)
34
35 179 from 9 female volunteers aged 24-35 years, average $27.6 \text{ y} \pm 3.4$ (1 parous). The 252 total samples
36
37 180 were maintained at $-20 \text{ }^\circ\text{C}$ and randomly analysed once the monthly collection was completed. All
38
39 181 the women were healthy. None of them was taking any pharmaceutical drug or combined oral
40
41 182 contraceptive pills in the period of sample collection. For all urine samples, the analytical
42
43 183 determinations were normalized against their creatinine concentration to compensate for the
44
45 184 physiological urinary dilution [2,4]. Creatinine was determined by the alkaline picrate photometric
46
47 185 method using the dedicated kit on Architect C800 instrumentation (Abbott srl, Rome). In order to
48
49 186 follow privacy regulations, an anonymous code was attributed to each participant subject who,
50
51 187 anyway, voluntarily donated samples to the present project.

52

53 188 **Chemometrics**

54
55 189 Multivariate data analysis was carried out using Matlab[®] (The MathWorks, MA, USA) version 9.0.0
56
57 190 with PCA Toolbox version 1.2 [38], N-way Toolbox version 2.10 [39] and Classification Toolbox
58
59 191 version 5.0 [40].
60

61
62
63
64
65

193 Data were arranged into a three-dimensional array (3-way), labelled as $\underline{\mathbf{X}}$, with dimensions ($I \times J \times$
194 K) chosen as follows: (I) 9 subjects (representing the female volunteers), (J) 28 days (representing
195 the menstrual cycle duration), (K) 15 variables (representing the studied estrogens). To analyse the
196 three-dimensional data, a PARAllel FACtor analysis (PARAFAC) model [41-43] was applied. The
197 Alternating Least Squares (ALS) algorithm basically decomposes the $\underline{\mathbf{X}}$ 3-way array into three two-
198 dimensional matrices, namely \mathbf{A} ($I \times L$), \mathbf{B} ($J \times L$) and \mathbf{C} ($K \times L$), where the former variables (I, J, K)
199 are expressed as a function of a new multivariate parameter (L) representing the loadings [41-43].
200 The \mathbf{B} and \mathbf{C} matrices show the natural fluctuation of each estrogen concentration throughout the
201 28-day menstrual cycle.

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

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

220 221 **RESULTS AND DISCUSSION**

222 223 **Method optimization and validation**

225 The DoE optimization of sample preparation [32] was aimed to achieve simpler and faster
1
226 extraction conditions than those used in previous studies [27-30]. The best combination of drying
2
227 temperature (found at 40°C) and extraction solvent was found with the ethyl acetate + hexane
3
228 (2:3 v/v) mixture as it corresponded to higher resolution and intensity of the chromatographic
4
229 peaks with respect to TBME [32].
5

6
230 Optimal chromatographic separation among the estrogens of similar chemical structure (for
7
231 example, 2-methoxyestrone and 16 α -hydroxyestrone) was obtained by using a slow increase of
8
232 the oven temperature (3 °C/min) between 225 °C and 245 °C interrupted by a hold time at 234 °C
9
233 for 3 min. Nevertheless, the full chromatographic run was completed in less than 20 minutes and
10
234 the retention times of the target analytes lied between 6.58 min (17 α -estradiol) and 10.50 min
11
235 (16-epiestriol).
12

13
14
15
16
17
18
19
20
21
22

23 24 *Linearity, LOD, LOQ*

25
26 Full validation data are reported elsewhere [32]. The linearity of the calibration curves was tested
27
28 in the concentration range of 1.0 – 50 ng/mL for all the analytes. Lack of fit's, ANOVA, RSD slope
29
30 and Back-calculation tests proved to yield calculated results below the respective critical values for
31
32 all the target analytes. Among the target analytes, only 17 α -estradiol, 4-hydroxyestradiol and 2-
33
34 methoxyestradiol were characterized by a quadratic calibration model. Most of the estrogens'
35
36 models used an x^{-2} weighting correction, except 17 α -estradiol, 2-methoxyestradiol, estrone, 4-
37
38 methoxyestrone and estriol. From the residual plots, the calibration linearity was confirmed by the
39
40 presence of random residuals patterns along the concentration ranges for all the analytes.

41
42 LOD values ranged between 0.2 ng/mL and 0.4 ng/mL. The LOQ values, estimated below 1.0
43
44 ng/mL for all target analytes, were verified experimentally. The first point (1.0 ng/mL) of each
45
46 calibration range was successfully tested for precision and accuracy, as reported below, and was
47
48 subsequently used as operational LOQ.
49

50
51

52 53 *Repeatability and accuracy*

54
55 Ion abundance and retention time repeatability proved experimentally appropriate. Intra-assay
56
57 precision and accuracy satisfied the target criteria, as the CV% are lower than 15% for all the
58
59 analytes at all tested concentration levels, while the percent bias (bias%) lied between –8.2% (2-
60
61 hydroxyestrone) and +12% (2-hydroxyestradiol) at 1.0 ng/mL, –11% (4-hydroxyestradiol) and
62
63
64
65

256 +6.8% (4-hydroxyestrone) at 5.0 ng/mL and -6.2% (17 α -estradiol) and +5.6% (2-hydroxyestradiol)
1
257 at 25 ng/mL [32].
3

258

5
259 *Matrix effect, extraction recovery, enzyme performance, carry over*

7
260 The matrix effect values ranged from -12% for 4-methoxyestrone to +15% for 16 α -hydroxyestrone
9
261 at low level, from -9.2% for 4-hydroxyestrone to +12% for 17-epiestriol at medium level and from
11
262 -5.6% for 2-hydroxyestradiol to +6.3% for 16 α -hydroxyestrone at high level. These scattered
13
263 values are close to the experimental bias and do not evidence any significant matrix effect. The
15
264 average recovery efficiency was 99%, with minima and maxima ranging from 89% for 4-
17
265 hydroxyestrone to 108% for 4-hydroxyestradiol and 17-epiestriol at 1.0 ng/mL; from 87% for 17 α -
19
266 estradiol to 107% for 4-hydroxyestrone at 5.0 ng/mL; from 94% for 17 α -estradiol to 110% for 2-
21
267 hydroxyestradiol at 25 ng/mL. Again, the extraction recovery was virtually complete at all
23
268 concentration levels allowing a correct estimation of the target analytes' concentration.

25
269 The percent hydrolysis achieved by both β -glucuronidase and arylsulfatase on estrone glucuronide
27
270 and estrone sulfate at all concentration levels was close to 100%, supporting the claim that the
29
271 deconjugation efficiency on phase II metabolites could be considered complete. No carry over
31
272 effect was observed.

32

3273

35 274 **PARAFAC Model**

37
3275 The PARAFAC approach is commonly employed in environmental data analysis, when repeated
39
276 chronological monitoring of sampling sites yields three-dimensional data structures. The same
41
277 statistical tool is suitable for our chronological monitoring of estrogen profiles [39,41-43]. A
43
278 PARAFAC model was built to extract the concentration profile for each estrogen along the 28-day
45
279 menstrual cycle, by smoothing the large individual variability of estrogenic profiles, that proved
47
280 significant for the 9 investigated women. Due to the different duration of the menstrual cycles,
49
281 spanning between 28 and 30 days, the ovulation peak occurred at different days, from the 13th to
51
282 the 17th day, in agreement with the literature [17,45-47]. To comply with this source of variability,
53
283 the extreme sampling days were removed from the series collected from the women with a
55
284 menstrual cycle longer than 28 days. Actually, the extreme days (i.e. the first and the last of the
57
285 menstrual cycle) exhibited comparable results with the subsequent and preceding samples,
59
286 respectively. The final PARAFAC processing allowed the equalize each menstrual cycle within a
61
287 unique scale so as to evaluate and compare the natural variation of the estrogenic levels. The

62

63

64

65

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

290 All the extrapolated estrogenic profiles are reported in Figure 1 and exhibit several remarkable
3
291 features. In particular, 17 β -estradiol (2a) and estrone (2b) show two peaks, the first occurring
5
292 close to the ovulation with a time-shift of 3-4 days between the two hormones, while the second
7
293 smoother peak appears in the period around the 20th-25th day of the cycle. These profiles are
9
294 comparable to those reported in the literature [7–9,12,48]. In contrast, no peak is observed for
11
295 17 α -estradiol (2a) in the central part of the cycle and only a faint increase of its level is detectable
13
296 in the luteal phase of the cycle. The lack of correlation between 17 α - and 17 β -estradiol profiles
15
297 may explain the scarce specificity of the immunoassays methods used for their quantification.
17

298 Several metabolite profiles are characterized by the occurrence of a single concentration peak
19
299 around the ovulation, namely 2-hydroxyestradiol (1c), 4-hydroxyestradiol (1c), and 2-
21
300 methoxyestrone (1f) at the 15th day, but 4-hydroxyestrone (1e) and 2-hydroxyestrone (1e)
23
301 together with estrone (1b) at the 17th day. Surprisingly, 2-methoxyestrone shows a chronological
25
302 correlation with hydroxyestradiol isomers instead of hydroxyestrone isomers, as it would be
27
303 expected. On the other hand, 4-methoxyestrone (1f) show a sharp peak in the follicular phase of
29
304 the cycle, that is not observed for the isomer 2-methoxyestrone. The different behaviour observed
31
305 for 2- and 4-methoxyestrone isomers contrasts with those recorded for the analogous
33
306 hydroxyestrone (1e) and hydroxyestradiol (1c) isomers. All these observations add details on the
35
307 complex regulating system of the estrogen biochemistry active during the ovulation phase which
37
308 can not be explained by straightforward and progressive metabolic pathways [14].
39

40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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 17 α - and 17 β -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

319 before the ovulation and the second during the luteal phase. Noteworthy, the first peak has not
1
320 been observed in our study for 17α -estradiol.

321 The correspondence of our data with literature profiles and the agreement between oral fluid and
3
322 urine data, and between GC-MS and immunoassay methods represent further confirmation of the
5
323 reliability of the present approach to gain general information about the relative concentration of
7
324 the circulating hormones. The multi-residual GC-MS method proposed in this study proved to
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

327 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

351 non-error rate of 90% was achieved, together with an accuracy equal of 93%, as is shown in the
1
352 confusion matrix reported in Table 1. Only two data-points were misclassified, namely the 17th
3
353 day, which was classified in class 3 (luteal phase) instead of class 2 (ovulation), and the 18th day,
5
354 which was classified in class 1 (follicular phase) instead of class 3. The misclassification of the 17th
7
355 and 18th days was not surprising since both data-points belong to the transition period from the
9
356 ovulation to the luteal phase and correspond to a sudden drop of the estrone, 2- and 4-
11
357 hydroxyestrone concentrations (Figure 1b, 1e). The accurate classification of all days belonging to
13
358 the transition from the follicular to the ovulation phase is explained by the smoother
15
359 concentration increment observed for 17 β -estradiol, 2- and 4-hydroxyestradiol from the 11th to the
17
360 15th day of the cycle (Figure 1a, 1c).

19
361 The scores plot reported in Figure 3B shows the good partition of the days in three well-defined
21
362 classes corresponding to the follicular phase, the ovulation and the luteal phase. The loadings plot,
23
363 representing the PC variables in the space of the LDA canonical variables (Figure 3C), indicates the
25
364 correspondence between class discrimination and PCs. In particular, PC2 is high during the
27
365 ovulation and low during the follicular and luteal phases. Hence, it is able to identify the ovulation
29
366 period from the other phases of the cycle. On the other hand, the luteal phase is characterized by
31
367 elevated values of PC1 (and PC7), which is low in the follicular phase and especially low during the
33
368 ovulation, distinguished also by a high value of PC8.

35
369 Studies that use the menstrual cycle phase as a proxy for directly measured ovarian hormone
37
370 levels typically fail to capture their inherent variability. The lack of reliable methods to divide the
39
371 menstrual cycle into its component phases was proved, as divergent outcomes may be produced
41
372 by using different methods [3,10]. The application of the present multivariate statistical model to
43
373 GC-MS data is expected to overcome this limit and allow a correct definition of the phases of the
45
374 menstrual cycle, an important issue in the study of fertility. For example, Barrett et al [7] who
47
375 determined the concentration of urinary estrone alongside a complete menstrual cycle
49
376 established the difference in ovarian function between nulliparous and parous women. In general,
51
377 the menstrual cycle features represent important indicators of the reproductive health and
53
378 endocrine function. For example, Small et al [11] found a connection between the menstrual cycle
55
379 variability and the likelihood of pregnancy, Venners et al [9] discovered that higher estrogen
57
380 concentrations were associated with the occurrence of clinical pregnancy, and Baird et al [8]
59
381 studied the hormonal pattern most appropriate for pre-implantation. All the areas of interest
61
382 linked to reproduction could benefit from a multivariate interpretation of a wide estrogen profile,

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

385

386 **CONCLUSIONS**

387

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

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

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

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

415
61
62
63
64
65

415 Further refinement of the present classification model is underway, as its full validation will
1
416 require a much larger training and test sets than the one used in this proof-of-concept
3
417 contribution based the on the recruitment of nine volunteers.
5
418

419 REFERENCES

- 420
- 421 [1] B.G. Reed, B.R. Carr, *The Normal Menstrual Cycle and the Control of Ovulation*, MDText.com, Inc., 2000.
 - 422 [2] A. Bellem, S. Meiyappan, S. Romans, G. Einstein, Measuring estrogens and progestagens in humans: An
423 overview of methods, *Gend. Med.* 8 (2011) 283–299. doi:10.1016/j.genm.2011.07.001.
 - 424 [3] P.C. Regan, Rhythms of desire: The association between menstrual cycle phases and female sexual desire, *Can.*
425 *J. Hum. Sex.* 5 (1996) 145–156.
 - 426 [4] H.S. Driver, E. Werth, D.-J. Dijk, A.A. Borbély, The Menstrual Cycle Effects on Sleep, *Sleep Med. Clin.* 3 (2008)
427 1–11. doi:10.1016/j.jsmc.2007.10.003.
 - 428 [5] Y. Liu, E.B. Gold, B.L. Lasley, W.O. Johnson, Factors affecting menstrual cycle characteristics, *Am. J. Epidemiol.*
429 160 (2004) 131–140. doi:10.1093/aje/kwh188.
 - 430 [6] P. Montero, C. Bernis, V. Fernandez, S. Castro, Influence of body mass index and slimming habits on menstrual
431 pain and cycle irregularity, *J. Biosoc. Sci.* (1996) 315–323.
 - 432 [7] E.S. Barrett, L.E. Parlett, G.C. Windham, S.H. Swan, Differences in ovarian hormones in relation to parity and
433 time since last birth, *Fertil. Steril.* 101 (2014) 1773–1780.e1. doi:10.1016/j.fertnstert.2014.02.047.
 - 434 [8] D.D. Baird, A.J. Wilcox, C.R. Weinberg, F. Kamel, D.R. McConaughey, P.I. Musey, D.C. Collins, Preimplantation
435 hormonal differences between the conception and non-conception menstrual cycles of 32 normal women,
436 *Hum. Reprod.* 12 (1997) 2607–2613. doi:10.1093/humrep/12.12.2607.
 - 437 [9] S.A. Venners, X. Liu, M.J. Perry, S.A. Korricks, Z. Li, F. Yang, J. Yang, B.L. Lasley, X. Xu, X. Wang, Urinary estrogen
438 and progesterone metabolite concentrations in menstrual cycles of fertile women with non-conception, early
439 pregnancy loss or clinical pregnancy, *Hum. Reprod.* 21 (2006) 2272–2280. doi:10.1093/humrep/del187.
 - 440 [10] J.B. Brown, Types of ovarian activity in women and their significance: the continuum (a reinterpretation of
441 early findings), *Hum. Reprod. Update.* 17 (2011) 141–158. doi:10.1093/humupd/dmq040.
 - 442 [11] C.M. Small, A.K. Manatunga, M. Klein, C.E. Dominguez, H.S. Feigelson, R. McChesney, M. Marcus, Menstrual
443 cycle variability and the likelihood of achieving pregnancy., *Rev. Environ. Health.* 25 (n.d.) 369–78.
 - 444 [12] J.R. Roney, Z.L. Simmons, Hormonal predictors of sexual motivation in natural menstrual cycles, *Horm. Behav.*
445 63 (2013) 636–645. doi:10.1016/j.yhbeh.2013.02.013.
 - 446 [13] D.M. Campagne, G. Campagne, The premenstrual syndrome revisited, *Eur. J. Obstet. Gynecol. Reprod. Biol.*
447 130 (2007) 4–17. doi:10.1016/j.ejogrb.2006.06.020.
 - 448 [14] A. Shechter, D.B. Boivin, Sleep, Hormones, and Circadian Rhythms throughout the Menstrual Cycle in Healthy
449 Women and Women with Premenstrual Dysphoric Disorder, *Int. J. Endocrinol.* 2010 (2010) 1–17.
450 doi:10.1155/2010/259345.
 - 451 [15] D.H. Schwartz, S.E. Romans, S. Meiyappan, M.J. De Souza, G. Einstein, The role of ovarian steroid hormones in
452 mood, *Horm. Behav.* 62 (2012) 448–454. doi:10.1016/j.yhbeh.2012.08.001.
- 63
64
65

- 453 [16] A.H. Eliassen, D. Spiegelman, X. Xu, L.K. Keefer, T.D. Veenstra, R.L. Barbieri, W.C. Willett, S.E. Hankinson, R.G.
454 Ziegler, Urinary estrogens and estrogen metabolites and subsequent risk of breast cancer among
455 premenopausal women, *Cancer Res.* 72 (2012) 696–706. doi:10.1158/0008-5472.CAN-11-2507.
- 456 [17] H. Samavat, M.S. Kurzer, Estrogen metabolism and breast cancer, *Cancer Lett.* 356 (2015) 231–243.
457 doi:10.1016/j.canlet.2014.04.018.
- 458 [18] H. Oh, Z.G. Khodr, M.E. Sherman, M. Palakal, R.M. Pfeiffer, L. Linville, B.M. Geller, P.M. Vacek, D.L. Weaver, R.E.
459 Chicoine, R.T. Falk, H.N. Horne, D. Papathomas, D.A. Patel, J. Xiang, X. Xu, T. Veenstra, S.M. Hewitt, J.A.
460 Shepherd, L.A. Brinton, J.D. Figueroa, G.L. Gierach, Relation of serum estrogen metabolites with terminal duct
461 lobular unit involution among women undergoing diagnostic image-guided breast biopsy, *Horm. Cancer* 7
462 (2016) 305-315. doi: 10.1007/s12672-016-0265-2.
- 463 [19] R.T. Falk, L.A. Brinton, J.F. Dorgan, B.J. Fuhrman, T.D. Veenstra, X. Xu, G.L. Gierach, Relationship of serum
464 estrogens and estrogen metabolites to postmenopausal breast cancer risk: A nested case-control study, *Breast*
465 *Cancer Res.* 15 (2013). doi:10.1186/bcr3416.
- 466 [20] J.N. Sampson, R.T. Falk, C. Schairer, S.C. Moore, B.J. Fuhrman, C.M. Dallal, D.C. Bauer, J.F. Dorgan, X.-O. Shu, W.
467 Zheng, L.A. Brinton, M.H. Gail, R.G. Ziegler, X. Xu, R.N. Hoover, G.L. Gierach, Association of estrogen metabolism
468 with breast cancer risk in different cohorts of postmenopausal women, *Cancer Res.* 77 (2017) 918-925. doi:
469 10.1158/0008-5472.CAN-16-1717.
- 470 [21] X. Xu X, T.D. Veenstra, S.D. Fox, J.M. Roman, H.J. Issaq, R. Falk, J.E. Saavedra, L.K. Keefer, R.G. Ziegler, Measuring
471 fifteen endogenous estrogens simultaneously in human urine by high-performance liquid chromatography-mass
472 spectrometry, *Anal. Chem.* 77 (2005) 6646–6654. doi: 10.1021/ac050697c.
- 473 [22] J.M. Faupel-Badger, B.J. Fuhrman, X. Xu, R.T. Falk, L.K. Keefer, T.D. Veenstra, R.N. Hoover, R.G. Ziegler,
474 Comparison of liquid chromatography-tandem mass spectrometry, RIA, and ELISA methods for measurement
475 of urinary estrogens, *Cancer Epidemiol. Biomarkers Prev.* 19 (2010) 292–300. doi:10.1158/1055-9965.EPI-09-
476 0643.
- 477 [23] A.A. Franke, L.J. Custer, Y. Morimoto, F.J. Nordt, G. Maskarinec, Analysis of urinary estrogens, their oxidized
478 metabolites, and other endogenous steroids by benchtop orbitrap LCMS versus traditional quadrupole GCMS,
479 *Anal. Bioanal. Chem.* 401 (2011) 1319–1330. doi:10.1007/s00216-011-5164-3.
- 480 [24] S.H. Lee, Y.J. Yang, K.M. Kim, B.C. Chung, Altered urinary profiles of polyamines and endogenous steroids in
481 patients with benign cervical disease and cervical cancer., *Cancer Lett.* 201 (2003) 121–31.
- 482 [25] M.H. Choi, J.-Y. Moon, S.-H. Cho, B.C. Chung, E.J. Lee, Metabolic alteration of urinary steroids in pre- and post-
483 menopausal women, and men with papillary thyroid carcinoma, *BMC Cancer.* 11 (2011) 342.
484 doi:10.1186/1471-2407-11-342.
- 485 [26] J. Lee, H.M. Woo, G. Kong, S.J. Nam, B.C. Chung, Discovery of urinary biomarkers in patients with breast cancer
486 based on metabolomics, *Mass Spectrom. Lett.* 4 (2013) 59–66. doi:10.5478/MSL.2013.4.4.59.
- 487 [27] U. Knust, T. Strowitzki, B. Spiegelhalter, H. Bartsch, R.W. Owen, Optimization of an isotope dilution gas
488 chromatography/mass spectrometry method for the detection of endogenous estrogen metabolites in urine
489 samples, *Rapid Commun. Mass Spectrom.* 21 (2007) 2245–2254. doi:10.1016/j.jcrs.2012.09.017.
- 490 [28] X.Y. Xiao, D. McCalley, Quantitative analysis of estrogens in human urine using gas chromatography/negative
491 chemical ionisation mass spectrometry, *Rapid Commun. Mass Spectrom.* 14 (2000) 1991–2001.

- 492 doi:10.1002/1097-0231(20001115)14:21<1991::AID-RCM125>3.0.CO;2-H.
- 493 [29] J.-Y. Moon, K.J. Kim, M.H. Moon, B.C. Chung, M.H. Choi, A novel GC-MS method in urinary estrogen analysis
494 from postmenopausal women with osteoporosis, *J. Lipid Res.* 52 (2011) 1595–1603. doi:10.1194/jlr.D016113.
- 495 [30] P. Hoffmann, M.F. Hartmann, T. Remer, K.P. Zimmer, S.A. Wudy, Profiling oestrogens and testosterone in
496 human urine by stable isotope dilution/benchmark gas chromatography-mass spectrometry, *Steroids.* 75 (2010)
497 1067–1074. doi:10.1016/j.steroids.2010.06.014.
- 498 [31] R. Leardi, Experimental design in chemistry : A tutorial, *Anal. Chim. Acta.* 652 (2009) 161–172.
499 doi:10.1016/j.aca.2009.06.015.
- 500 [32] C. Bozzolino, S. Vaglio, E. Amante, E. Alladio, E. Gerace, A. Salomone, M. Vincenti, Optimization and validation
501 of a GC-MS quantitative method for the determination of an extended estrogenic profile in human urine,
502 *MethodsX* (submitted contextually).
- 503 [33] R.G. Ziegler, B.J. Fuhrman, S.C. Moore, C.E. Matthews, Epidemiologic studies of estrogen metabolism and
504 breast cancer, *Steroids.* 99 (2015) 67–75. doi:10.1016/j.steroids.2015.02.015.
- 505 [34] E. Alladio, R. Caruso, E. Gerace, E. Amante, A. Salomone, M. Vincenti, Application of multivariate statistics to
506 the Steroidal Module of the Athlete Biological Passport: A proof of concept study, *Anal. Chim. Acta.* 922
507 (2016). doi:10.1016/j.aca.2016.03.051.
- 508 [35] Eurachem, *The Fitness for Purpose of Analytical Methods - A Laboratory Guide to Method Validation and
509 Related Topics*, 2014. doi:10.1016/S0014-2999(99)00500-2.
- 510 [36] B. Desharnais, F. Camirand-Lemyre, P. Mireault, C.D. Skinner, Procedure for the Selection and Validation of a
511 Calibration Model I—Description and Application, *Journal of Analytical Toxicology.* 41 (2017) 261–268. doi:
512 10.1093/jat/bkx001.
- 513 [37] A. Hubaux, G. Vos, Decision and Detection limits for linear Calibration Curves, *Analytical Chemistry.* 42 (1970)
514 849–855. doi: 10.1021/ac60290a013.
- 515 [38] D. Ballabio, A MATLAB toolbox for Principal Component Analysis and unsupervised exploration of data
516 structure, *Chemom. Intell. Lab. Syst.* 149 (2015) 1–9. doi:10.1016/j.chemolab.2015.10.003.
- 517 [39] C.A. Andersson, R. Bro, The N-way Toolbox for MATLAB, *Chemom. Intell. Lab. Syst.* 52 (2000) 1–4.
518 doi:10.1016/S0169-7439(00)00071-X.
- 519 [40] D. Ballabio, V. Consonni, Classification tools in chemistry. Part 1: Linear models. PLS-DA, *Anal. Methods.* 5
520 (2013) 3790–3798. doi:10.1039/c3ay40582f.
- 521 [41] R. Bro, PARAFAC. Tutorial and applications, *Chemom. Intell. Lab. Syst.* 38 (1997) 149–171. doi:10.1016/S0169-
522 7439(97)00032-4.
- 523 [42] P. Geladi, Analysis of multi-way (multi-mode) data, *Chemom. Intell. Lab. Syst.* 7 (1989) 11–30.
524 doi:10.1016/0169-7439(89)80108-X.
- 525 [43] A.K. Smilde, R. Bro, P. Geladi, *Multi-way analysis with applications in the chemical sciences*, First Edit, John
526 Wiley & Sons, Ltd., Chichester, UK, 2004.
- 527 [44] R. Bro, A.K. Smilde, Principal component analysis, *Anal. Methods.* 6 (2014) 2812–2831.
528 doi:10.1039/c3ay41907j.
- 529 [45] S.B. Brown, S.E. Hankinson, Endogenous estrogens and the risk of breast, endometrial, and ovarian cancers,
530 *Steroids.* 99 (2015) 8–10. doi:10.1016/j.steroids.2014.12.013.

531 [46] L.G. de A. Chuffa, L.A. Lupi-Júnior, A.B. Costa, J.P. de A. Amorim, F.R.F. Seiva, The role of sex hormones and
steroid receptors on female reproductive cancers, *Steroids*. 118 (2017) 93–108.
doi:10.1016/j.steroids.2016.12.011.

532
533

534 [47] E. Folkerd, M. Dowsett, Sex hormones and breast cancer risk and prognosis, *Breast*. 22 (2013) S38–S43.
doi:10.1016/j.breast.2013.07.007.

535
536 [48] E.S. Barrett, I. Thune, S.F. Lipson, A.-S. Furberg, P.T. Ellison, A factor analysis approach to examining
relationships among ovarian steroid concentrations, gonadotrophin concentrations and menstrual cycle length
characteristics in healthy, cycling women, *Hum. Reprod*. 28 (2013) 801–811. doi:10.1093/humrep/des429.

537
538
539
540
541
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

542 **Figure captions**

1
543 **Figure 1.** Concentration profile (normalized for the creatinine value) of the target analytes along the 28-day
2
544 menstrual cycle achieved by applying PARAFAC approach: (a) 17 α -estradiol and 17 β -estradiol, (b) estrone
3
545 and estriol, (c) 2-hydroxyestradiol and 4-hydroxyestradiol, (d) 2-methoxyestradiol and 4-methoxyestradiol,
4
546 (e) 2-hydroxyestrone and 4-hydroxyestrone, (f) 2-methoxyestrone and 4-methoxyestrone, (g) 16 α -
5
547 hydroxyestrone and 16-epiestriol, and (h) 17-epiestriol.
6
7
8

9
548
10
11
549 **Figure 2.** Results provided by the PCA model: (a) Score plot relevant to PC1 (Var. = 14.91 %) vs PC2 (Var. =
12
550 13.31%) showing the occurrence of three different clusters corresponding to the three phases of the
13
551 menstrual cycle, i.e. follicular phase (blue dots), ovulation (red dots) and luteal phase (green dots). (b) PC1
14
552 vs menstrual cycle day, representing the transition from the follicular phase to the ovulation phase. (c) PC2
15
553 vs menstrual cycle day, representing the transition from the ovulation phase to the luteal phase.
16
17
18

19
554
20
21
555 **Figure 3.** Results achieved by building the LDA model. (a) Multinormality test graph. (b) Score Plot relevant
22
556 to the first two latent variables, showing the partition of the data in three well-defined classes: follicular
23
557 phase (blue dots), ovulation (red dots) and luteal phase (green dots). (c) Loading Plot relevant to the first
24
558 two latent variables, showing the PCs that mainly characterize the three classes of samples.
25
26
27

28
559
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Figure 1
[Click here to download high resolution image](#)

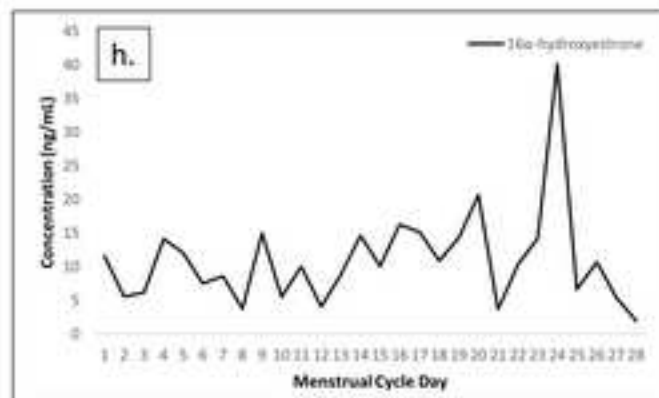
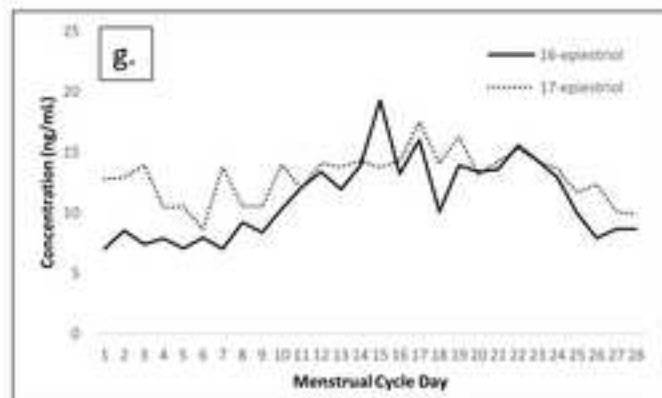
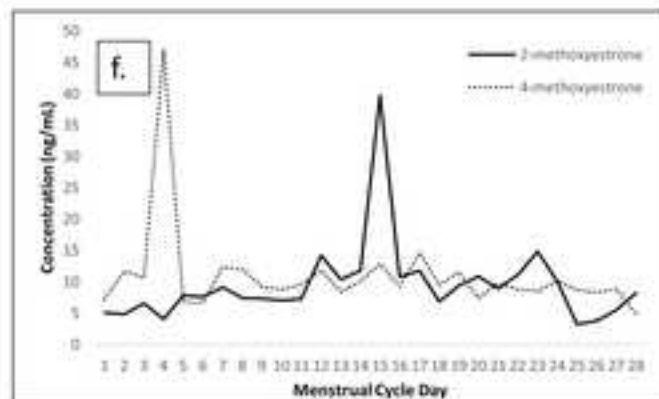
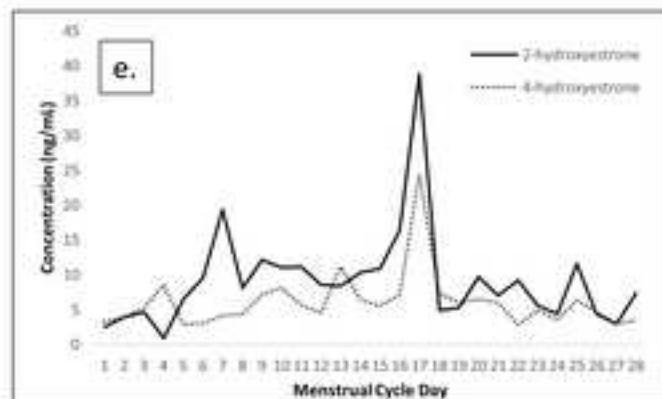
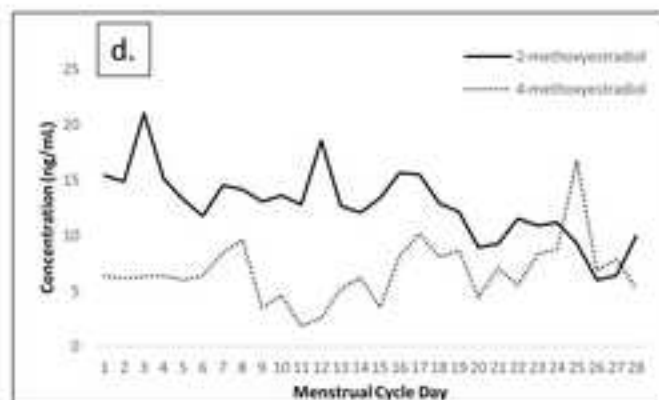
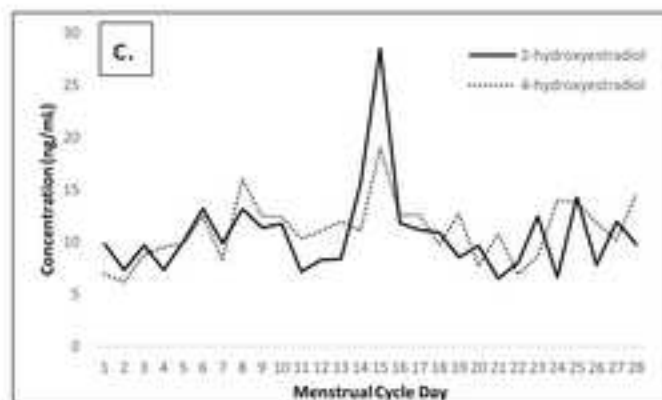
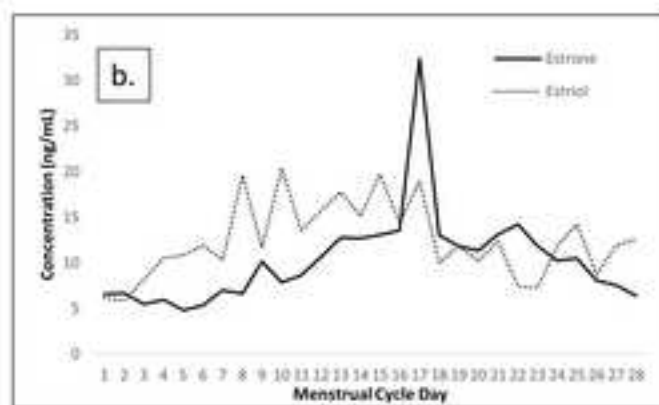
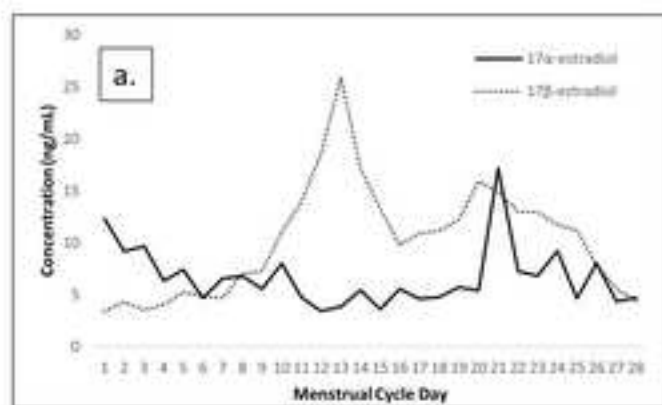


Figure 2

[Click here to download high resolution image](#)

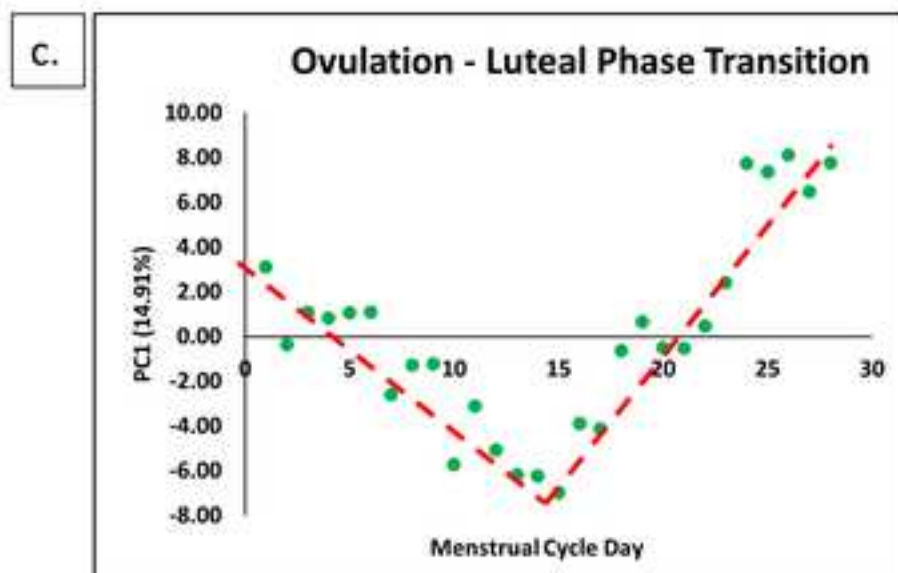
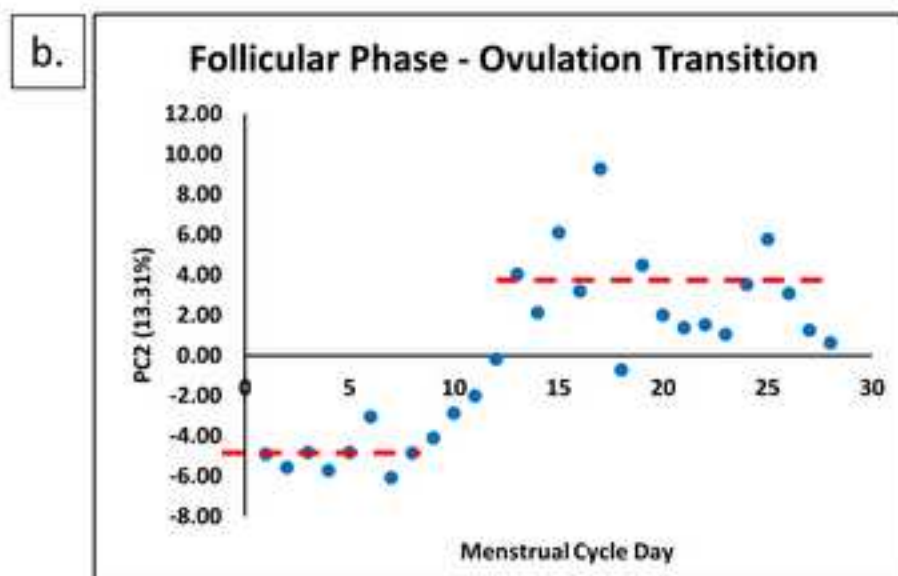
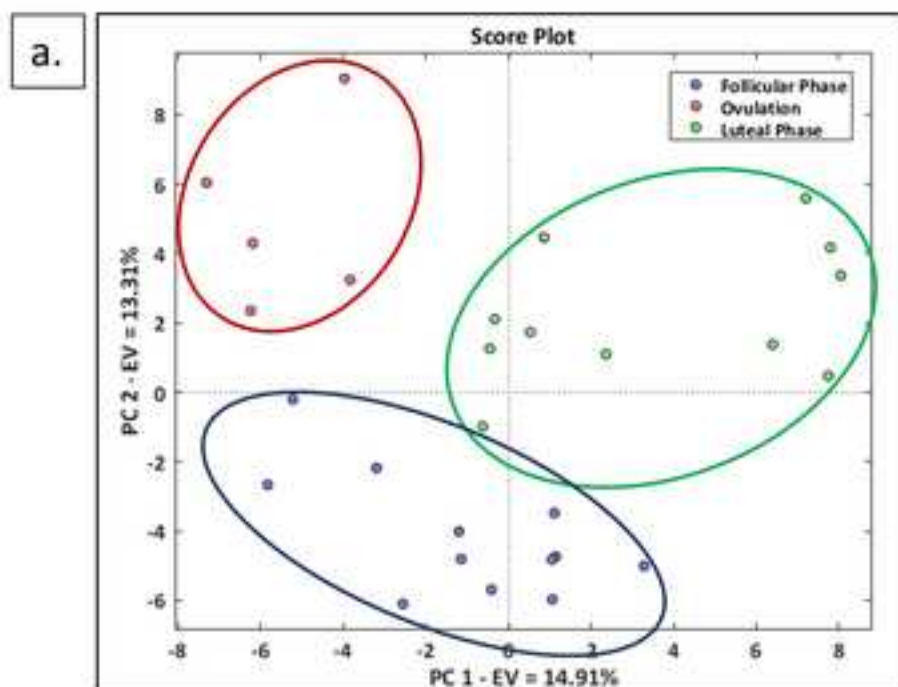


Figure 3

[Click here to download high resolution image](#)

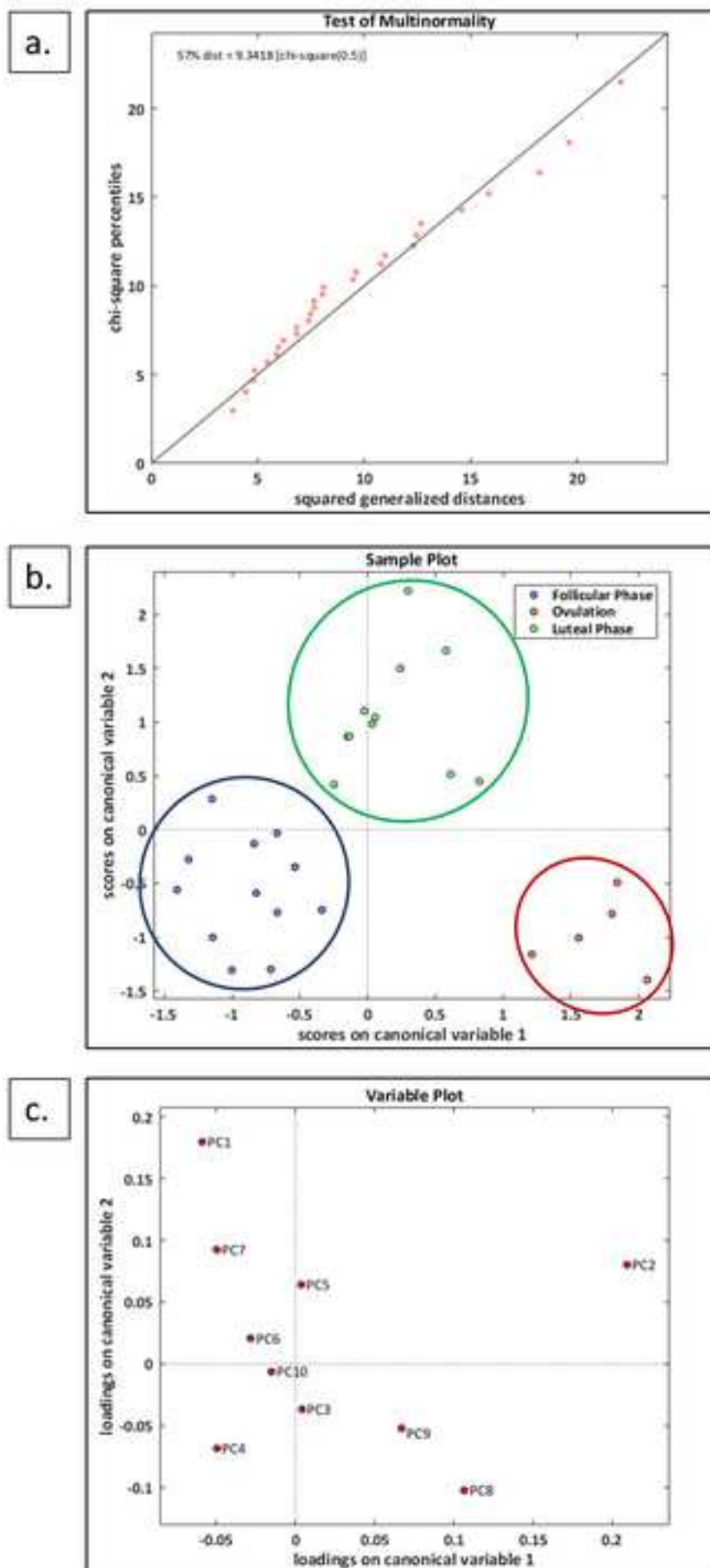


Table 1

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
17 α -estradiol	1.0 – 50.0	0.9977	0.33	0.67	2.31	1.62	2.37	17	Quadratic	x^{-1}
17 β -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}
16 α -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
17 α -estradiol	13	9.7	7.3	+7.9	+2.7	-6.2	+8.1	+10	+4.4	106	87	94
17 β -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
16 α -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

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%		

Cover Letter

[Click here to download Supplementary Material: Cover letter - Rev2.docx](#)

Manuscript with modifications

[Click here to download Supplementary Material: Manuscript reviewed - Rev2.docx](#)

Figure Captions

[Click here to download Supplementary Material: Figure_captions_Rev2.docx](#)

Supplementary Material

[Click here to download Supplementary Material: Supplementary Material Rev.docx](#)

Method Details (MethodsX)

[Click here to download Method Details \(MethodsX\): MethodsX.zip](#)