Comparison of isotope pattern deconvolution and calibration curve quantification methods for the determination of estrone and 17β-estradiol in human serum

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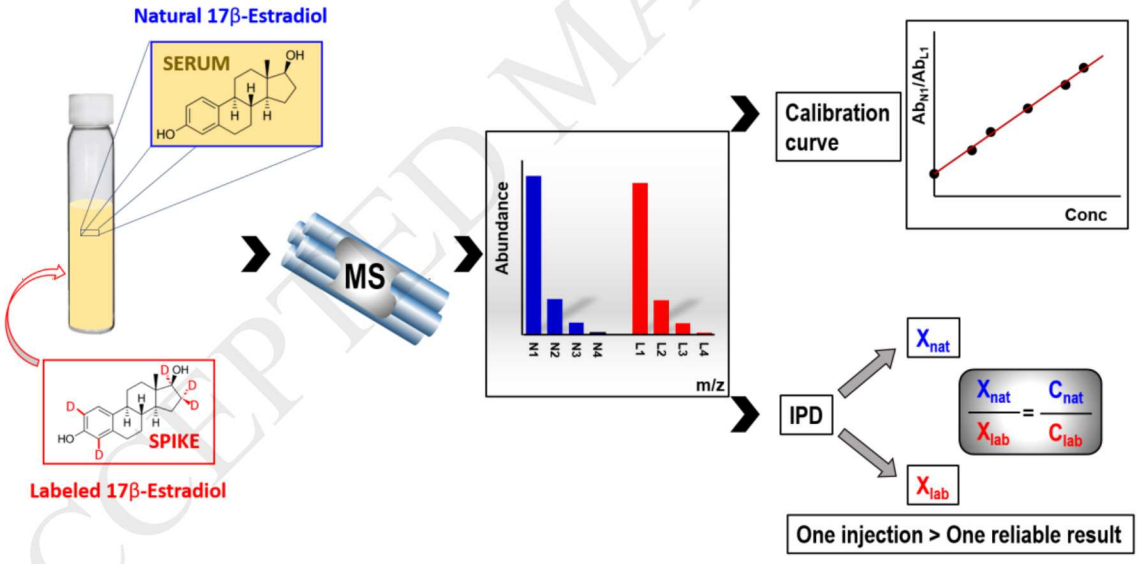
Graphical abstract



Highlights

- LC-MS quantification determination of estrone and 17β-estradiol in human serum

- Classical calibration curve compared to multivariate isotope pattern deconvolution
- Methods successfully validated according to European medicines agency guidelines
- Both methods are suitable for routine analysis within the context of hospital's needs
- IPD has demonstrated to be faster and cost saving

Abstract

A Liquid Chromatography coupled to tandem mass spectrometry (LC-MS/MS) based method have been developed for the determination of the main estrogen compounds –estrone (E1) and 17β-estradiol (E2)– in human serum. Two isotope dilution mass spectrometry (IDMS) quantification procedures have been used: a classical calibration curve-based method were compared to a recently developed isotope pattern deconvolution (IPD) method. IPD is based on isotopic abundance measurements and multiple linear regression. Validation was performed in terms of intra-assay repeatability (n=5), inter-assay reproducibility (n=9) and accuracy using spiked steroid-free serum at 5 concentration levels and 3 certified reference materials. Both methodologies meet EMEA requirements yielding recoveries between 79-106% and coefficient of variations of 1.7-8.3% along all experiments. Limits of quantification as low as 5 ng/L were achieved. 40 real samples were analysed for comparison purposes showing a great correlation between calibration and IPD concentration values. Real samples were also quantified by routine immunoassay analysis, which showed a significant proportional bias of 2.55 for E1 and good correlation for E2. While methods were considered suitable for routine or countercheck analysis within the context of hospital's needs, IPD has demonstrated to be faster and cost saving.

Keywords: estrone; 17β-estradiol; serum; LC-MS/MS; isotope dilution mass spectrometry; isotope pattern deconvolution

1. Introduction

Estrogens are sexual steroidal hormones, derived from cholesterol, which are involved in a great number of gender-specific and non-gender-specific biological processes. The two main active estrogens in humans are estrone (E1) and 17β-estradiol (E2) and they are important in growth, nervous system maturation, bone structure and pregnancy, as well as playing a key role in breast cancer development [1–4].

Nowadays, there is an increasing interest in the simultaneous high-sensitivity and high throughput measurement of these estrogens for both clinical research and routine analysis. The main samples of interest include paediatric, pre-pubertal, post-menopausal and male serum samples. Unfortunately, most of immunoassays are not able to reach the very low levels needed in those cases. Only competitive radioimmunoassay (RIA) [5], which is associated with the health and safety risks of using radioactive materials, shows suitable limits of quantification.

In general, immunoassay-based analysis often suffer from cross-reactivity with interfering endogenous components of the matrix, causing poor correlation with mass spectrometry-based methods, lacks of precision and accuracy, and only one estrogen is determined at a time. Thus, there is an increasing interest to shift towards stable isotope dilution (ID) in combination with Gas Chromatography and Liquid Chromatography coupled to mass spectrometry (GC-MS and LC-MS) or tandem mass spectrometry (MS/MS) methods for the determination of estrogens and other steroids [5–8].

Despite the known advantages of estrogen measurement by LC-MS/MS, which include increased sensitivity and specificity, and multiple determinations in one run their analysis is still challenging since these compounds do not contain highly ionisable functional groups. Thus, due to the extremely low concentration levels normally present in serum (often in the range of a few nanograms per liter), several approaches have been explored in the last years to tackle this problem.

Some work has been made in terms of extraction efficiency or chromatographic separation optimization. Two dimensional (2D) LC-MS/MS [9,10] or automated exhaustive sample treatments [11,12] that allow large sample volume injection while

reducing background noise have been tested. Sensitivity is also enhanced sometimes by the addition of fluoride salts to the mobile phase. All those approaches need some complex instrumental equipment.

On the other side, derivatization of analytes is a widely-spread methodology in clinical analysis for the enhancement of sensibility by introducing a highly ionisable moiety in the structure. Recently, different derivatization agents have been tested [13-16]. Nevertheless, dansyl chloride is still the most used one because the derivative shows high ionisation efficiency, is obtained in aqueous solution with mild conditions, preventing unexpected hydrolysis processes, and can be directly injected in LC columns [17–21]. However, reduced selectivity is expected since charge remains in the dansyl moiety after collision-induced fragmentation and the measured MS transitions are not compound specific.

After derivatization, rigorously validated ID-LC-MS/MS methods provide reliable results for estrogen determination in serum. Classically, isotope dilution mass spectrometry (IDMS) is used along with calibration curve to avoid any matrix effect or instrumental drift, in a procedure that is time consuming. An alternative calculation in IDMS based on the measurement of isotopic abundances, isotope pattern deconvolution (IPD), can be used [22]. When the labelled internal standard (IS) is added to the sample, the altered isotopic abundances in the blend are a linear combination of those from the natural and from the labelled analogues. A deconvolution calculation using multiple linear regression provides the concentration of the natural compound in the sample. IDMS with IPD is a reliable, fast, and less expensive method. IPD provides one result per injection with accuracy, enhances the throughput of the whole method and it has already been successfully applied to different compounds and complex matrices. Namely, endocrine disrupting compound in wastewater [23,24], or endogenous steroids in human urine [25–27].

In this work, we present the development and validation of a method for the determination of E1 and E2 in serum by LC-MS/MS according to the European Medicines Agency's (EMEA) guidelines [28]. Conventional calibration curve with internal standard quantification (the MS based method routinely applied in the laboratory) is thoroughly compared with the newly developed IPD calculations. With

this new IPD quantification methodology, used for the first time to estrogen determination in serum, accurate, precise and less expensive analysis are expected.

2. Materials and Methods

2.1. Materials

Standard solutions of 17β-estradiol (E2) and 17α-estradiol were purchased from LGC Standards (Luckenwalde, Germany). Standards solutions of estrone (E1), $^{13}C_3$-estrone (E1-$^{13}C_3$) and $^{2}H_5$-17β -estradiol (E2-D_5) were purchased from Cerilliant (Round Rock, TX, USA). All stock solutions were stored at -20ºC and employed to prepare gravimetrically diluted working standard solutions in acetonitrile. DC Mass Spect Gold MSG4000 ultra-low steroids and hormones human serum was purchased from Golden West Biological (Temecula, CA, USA). Certified reference materials BCR 576, BCR 577 and BCR 578 consisting in lyophilized serum with different E2 concentrations where obtained from the Institute for Reference Materials and Measurements (Geel, Belgium).

Dansyl chloride (Dns-Cl) and carbonate-bicarbonate buffer were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid for LC was from Fischer Chemicals (Loughborough, UK). All solvents used were LC-MS grade, water, acetonitrile, ethyl acetate and methyl-tert-butyl ether (MTBE) were from Biosolve BV (Valkenswaard, The Netherlands).

Standard stock solutions were prepared by gravimetrical dilution of certified 1 mg/mL solutions to approximately 50 µg/mL, writing down the exact concentrations. Subsequent solutions were also prepared by gravimetrical dilution. Dansyl chloride 1 mg/mL solution was prepared by dissolving exactly 25 mg in 25 mL of acetonitrile while the carbonate/bicarbonate buffer 0.05 M was prepared by dissolving one capsule in 100 mL of water LCMS and adjusting the pH to 10.5 by addition of a NaOH solution.

2.2. LC-MS/MS Conditions

Identification, characterization and quantification of the derivatized compounds was carried out with an UHPLC system using a Nexera x2 UPLC (Shimadzu, Kyoto, Japan) coupled with a quadrupole-linear ion trap QTRAP 6500 system (AB Sciex, Foster City, CA, USA) equipped with an IonDrive™ Turbo V interface. Chromatographic separation

was tested with two different core-shell columns: Kinetex F5 100A (1.7 µm, 100 x 2.1 mm) and Kinetex C18 100A (2.6 µm, 100 x 3 mm). Separation was finally achieved with the C18 column fitted with a C18 AJ0-8775 guard column (all from Phenomenex, Torrance, CA, USA). The column oven was kept at 40ºC, injection volume was 30 µL and samples were kept at 8ºC in the autosampler. Mobile phases consisted in H_2O containing 0.01% HCOOH as phase A, and ACN containing 0.01% HCOOH as phase B. Gradient conditions were applied as follows: 50% B (0-0.5 min), linear increase to 70% B in 0.1 min, linear increase to 95% B in 4.4 min, 95% B (5-6 min), 50% B (6.1-7.5 min). In order to protect the instrument from matrix contaminations, two cuts controlled by a divert valve were programmed at the beginning and at the end of the chromatographic run, allowing only the 2.5 to 6.5 min section to reach the mass spectrometer.

Analytes were ionized by electrospray in positive mode (ESI+) and detected by MS/MS in selected reaction monitoring (SRM). Optimum signal was obtained with the following parameters: 500ºC as source temperature, curtain gas at 30 psi, collision assisted dissociation at 12 psi, IonSpray at 5.5 kV, nebulizer gas (GS1) at 30 psi and 60 psi of hot air through the Turbo V™ heaters (GS2). A dwell time of 25 ms was selected. Component-dependent parameters are shown in Table 1.

Analyst software version 1.6.2 (Sciex) was used for data acquisition. MultiQuant version 3.0.2 (Sciex) was used for data treatment and quantification.

2.3. Sample preparation

Micro-solid phase extraction (µSPE) and supported liquid extraction (SLE), both with 96-well plates platform, and Liquid-liquid extraction (LLE) methods were tested for estrogen extraction from serum at different concentrations. Ethyl acetate and methyl *tert*-butyl ether (MTBE) as eluent or acceptor phase were used. LLE was the extraction method definitely chosen. To 250 µL of calibrators, controls or serum samples in 12x75 mm 5-mL glass tubes, 10 µL of labelled compound mix in acetonitrile were added, then samples were vortexed for 10 s. Liquid-liquid extraction (LLE) was applied using 2 mL of MTBE, 10 s of vortex mixing and 15 min of mechanical agitation. In order to help in phase separation, samples were centrifuged at 3000 rpm for 5 min. 1.7 mL of

supernatant were transferred to clean 2-mL Eppendorf vials and evaporated under vacuum at 35ºC.

Then, derivatization of analytes was carried out. To the dried extracts, 20 µL of bicarbonate/carbonate buffer and 30 µL of 1 mg/mL dansyl chloride in acetonitrile were added. Incubation was performed in a ThermoMixer (Eppendorf) at 60ºC and 1000 rpm for 10 min. Derivatized extracts were diluted with 50 µL of H_2O, transferred to LC vials with 0.2-mL inserts and injected in the LC-MS system.

2.4. Isotope Pattern Deconvolution

Isotope pattern deconvolution (IPD) is an alternative approach of classical isotope dilution mass spectrometry methods, which are based on natural-to-labelled area ratios to build the calibration curve. In IPD, the natural distribution of abundances of the analyte of interest are altered by adding the corresponding isotope-labelled analogue and then multiple linear regression is used to mathematically deconvolute the resulting combined distribution of abundances to obtain the molar fractions [22]. Briefly, the abundance in the mix ($A_{mix}^{SRM_i}$), measured after the spike of labelled analogues to the natural occurring analytes, is a linear combination of the natural ($A_{nat}^{SRM_i}$) and labelled ($A_{lab}^{SRM_i}$) compound abundances. In the case of *n* measured transitions in the tandem mass spectrometry and using a single spike of labelled compound, this can be expressed in matrix form as follows:

$$\begin{bmatrix} A_{mix}^{SRM_1} \\ A_{mix}^{SRM_2} \\ \vdots \\ A_{mix}^{SRM_n} \end{bmatrix} = \begin{bmatrix} A_{nat}^{SRM_1} & A_{lab}^{SRM_1} \\ A_{nat}^{SRM_2} & A_{lab}^{SRM_2} \\ \vdots & \vdots \\ A_{nat}^{SRM_n} & A_{lab}^{SRM_n} \end{bmatrix} \begin{bmatrix} X_{nat} \\ X_{lab} \end{bmatrix} + \begin{bmatrix} e^{SRM_1} \\ e^{SRM_2} \\ \vdots \\ e^{SRM_n} \end{bmatrix} \quad \text{(Eq. 1)}$$

Where an error vector (e^{SRM_i}) has to be included in order to solve the equation system. Then, multiple linear regression is applied to minimize the error vector and to obtain the molar fractions (X_{nat} and X_{lab} respectively) that fit better the measured abundances in the mix. Calculations can be conducted easily and automatically by any spreadsheet software. Finally, as the amount of labelled compound (N_{lab}) added to the sample is known, the amount of natural analyte (N_{nat}) is obtained:

$$N_{nat} = N_{lab} \frac{X_{nat}}{X_{lab}} \quad \text{(Eq. 2)}$$

As it can be seen, no methodological calibration is needed for the quantification and one injection produces one result. It requires, though, performing a full characterization of both compounds in terms of their isotopomer distribution of abundances and the certification by reverse isotope dilution of the labelled standard solutions.

3. Results and Discussion

3.1. Method development

Initial tests were performed to try if the quantification could be carried out without the need of derivatization. Individual 500 µg/L standard solutions in methanol/water (1:1, v/v) of each compound were infused in the ESI-MS/MS system in both positive (0.01% of formic acid was added) and negative modes. Instrumental conditions were optimized and the results indicated that sensitivity was insufficient for the desired lower limits of quantification. Thus, derivatization was deemed necessary. Compound-dependent instrumental conditions were optimized by means of the Automated Compound Optimization tool from Analyst software. Individual 500 µg/L dansylated standard solutions in acetonitrile/water (30:70, v/v) were infused in the ESI-MS/MS system in positive mode at a rate of 7 µL/min. Firstly, MS full-scans were obtained to select and optimize the precursor ion ($[M+H]^+$ in all cases), followed by MS/MS scans to determine the transitions to acquire and to optimize the fragmentation parameters. Ionization source conditions were automatically optimized using Automated Source Optimization by injecting the same standards in Flow Injection Analysis (FIA) mode. The results obtained for each compound were compared and compromise values were selected in the cases where optimal values were different between analytes.

In order to achieve the chromatographic separation of all analytes and isobaric interferences, two chromatographic columns were tested under different gradient conditions and compositions, including methanol and acetonitrile with different combinations and concentrations of NH_4COOH and HCOOH. A Kinetex F5 pentafluorophenyl column (multiple interactions) was initially tested and optimized, which lead to a 9-min water/methanol gradient modified with 0.1% HCOOH plus 0.2 mM NH_4F. Although the resulting chromatographic method was able to separate

properly all analytes, including 17α-estradiol (tested as isobaric interference of E2), the peaks presented bad chromatographic shape due to an excessive tailing in all analytes. Thus, the Kinetex C18 was tested, following the same optimization procedure, and the acetonitrile-based method described in the experimental section was found to be optimal. Although not being able to separate 17α-estradiol from E2, as its occurrence in human serum has not been reported, the resulting 7.5-min gradient was used.

For the extraction of analytes from the serum matrix, several approaches were tentatively assayed using standard solutions in water as well as spiked steroid-free serum at 5, 50 and 500 ng/L of E1 and E2. LLE, µSPE and SLE methods using MTBE and ethyl acetate as acceptor phase or eluent were tested. For µSPE and SLE methods, both as 96-well plates, either resulted in bad recoveries or poor matrix elimination, with the increased difficulty of carrying out the subsequent derivatization step in the plates. Although being more time-consuming and laborious, LLE was chosen as it allowed near complete extraction (>90% recovery by absolute area when comparing the area in the extract versus the area of a standard of equivalent concentration) of the analytes and presented no problems in the following steps.

3.2. Method validation

The validation of the developed method was performed according to EMEA's "Guideline on bioanalytical method validation"[28].

For calibration-based quantification, calibration curves were prepared in steroid-free serum between 5 and 1000 ng/L of E1 and E2 (6 point plus blank –not spiked- and zero –spiked with IS only- samples), which showed deviations in the back-calculated concentrations below 15% respect the nominal value in at least 4 points in all cases (data not shown).

Validation standards were prepared in steroid-free serum at approximately 5, 10, 200, 400 and 800 ng/L of E1 and E2. In addition, additional validation assays were performed for E2 using three CRM: BCR 576, BCR 577 and BCR 578, with 31, 188 and 365 ng/L of E2 respectively (no certified concentrations of E1 were provided). Accuracy and precision were evaluated in terms of intra-assay repeatability (n=5) and inter-assay reproducibility (n=9) for all validation standards and CRM, by both calibration and IPD quantification approaches. All experiments with validation standards yielded

recoveries between 79-118% and coefficient of variations of 1.2-14% for both methodologies (Tables 2 and 3). Regarding the CRM determination, both methods gave concentrations in the range of 81-101% respect the certified values and CV below 5% in all cases, except in the inter-assay experiment for BCR576 by IPD were it resulted in 17% CV (Table 4).

The method's LOQ was established as the minimum concentration value that was successfully validated, i.e., 5 ng/L for both analytes and quantification methods. That decision was made based on the impossibility to obtain true blank samples to which spike controlled amounts of analytes, since the theoretically steroid-free serum materials did contain some small quantities. The blank concentrations were estimated from the quantification of the zero point of the calibration curve -which is spiked only with internal standard- by IPD. MSG4000 commercial serum was found to contain on average 1.69 ng/L of E1 and 2.78 ng/L of E2. Concentrations found are clearly below our methods' LOQ, however chromatographic peaks corresponding to the analytes were clearly differentiable from background. (Figure S1, supplementary material). Thus, after calculating the mean value for all the calibration blank points used during the validation, concentrations of E1 and E2 in the commercial serum were estimated. Subsequent quantifications of validation standards and nominal values of calibration curves were adjusted accordingly.

The occurrence of possible matrix effects was checked for hemolyzed, renal, icteric and lipemic serum samples, as well as for two random normal serum samples. Samples were spiked at 3 times the lower limit of quantification (3xLLOQ) and near the upper limit of quantification (nearULOQ), and then subjected to the proposed sample treatment along with non-spiked samples of each type. Ratios between areas corresponding to natural and labelled compound were calculated, subtracting the non-spiked value (blank with internal standard), and then compared with a reference standard prepared in methanol and subjected to the same treatment. Acceptance ranges were set at 85-115% of the reference ratio for nearULOQ experiments and 80-120% for 3xLLOQ. Results are shown in Table 5 for all the different matrices and spike levels tested. Renal serum samples produced the greatest deviations from the reference ratios and the method was deemed unfit for such samples. Hemolyzed serum sample spiked at 3xLLOQ also produced an abnormal recovery for E2, although

the method performed well for E2 at a higher level and E1 at both levels. Similarly, performance in icteric samples was acceptable for E1 at high concentration and E2 at both levels, but unacceptable for E1 at low level. Finally, the method was suitable for E2 in lipemic samples but not for E1. Overall, no significant matrix effects were found in untreated samples, which were of main interest for the study.

3.3. Method comparison

Once the developed method was proved to meet the requirements of EMEA guidelines for both quantification procedures a thorough comparison was conducted.

To this end, the methods were applied to the analysis of 40 real serum samples, including men and women of age comprised between 7 and 81 years old, randomly and anonymously selected. Samples were previously analysed by RIA (E1) or electro-chemiluminiscence immunoassay (ECLIA) (E2) methods using the established procedures of the hospital's routine laboratories (LOQ around 17 ng/L for both approaches).

Passing-Bablok regression analysis was performed with open-source software, cp-R [29]. The correlation between the newly developed LC-MS/MS method based on calibration curve versus the Hospital's reference RIA determination for E1 resulted in a proportional bias (slope) of 2.55 and constant bias (intercept) of -9.41 ng/L (Figure 1A). Meanwhile, E2 LC-MS/MS calibration determinations suitably correlate against ECLIA, resulting in a proportional bias of 1.04 and constant bias of 1.44 ng/L (Figure 1B).

The differences observed in the analysis of E1 by LC-MS respect the routine analysis performed by RIA are not really unexpected. They could be caused by interferences competing in the bonding phase of the immunoassay procedure. Therefore, the implementation of a new quantification method for routine analysis would require a complete characterization of the population's normal ranges of estrone in order to be able to properly assess future diagnosis and carry out steroidal monitoring tasks.

On the other hand, the two MS-based methodologies were also compared. The proposed IPD method correlates properly with calibration-based quantification. Regression analyses presented R^2 values between 0.98 and 0.99. However, IPD provided slightly lower concentration values with proportional biases of 0.83 and 0.73 for E1 and E2 respectively, while constant biases were -3.19 and 0.47 ng/L (Figure 2A

and B). These results agree with the previously observed trends in recovery values obtained with validation standards and CRMs (Tables 2-4).

The seemingly better values provided by calibration curve quantification respect those of IPD may be caused by either the process of certification of standards and the presence of analytes in the theoretically steroid-free serum employed for this study. Existence of estrogens in the blank serum do not affect calibration curve procedure as calibration and validation standards are prepared in the same way. However, if not properly quantified and taken into account, the effect on measured abundances and IPD calculations can be significant. Thus, real free-steroid or accurately quantified ultra-low steroids matrix must be used. Regarding unsuitable certification of standards, the re-certification of labeled standards by IPD and reverse isotope dilution (RID) using suitable certified reference materials, has been proved as a correct way to improve accuracy [30]. Moreover, higher bias for E2 and IPD when compared with calibration can also be affected by deuterium scrambling problems. Losses of labile deuterium atoms during sample treatment or ionisation that can produce severe quantification problems in IPD isotopic abundance based calculations can occur for the labelled IS E2-D_5.

Finally it should be emphasized that both quantification methods meets the EMEA guidelines but IPD entail substantial savings in time and money (quantity of standards used), since one injection provides one result.

4. Conclusions

In this work we present the development of two LC-MS quantification methods for the simultaneous determination of estrone (E1) and 17β-estradiol (E2) in human serum, sharing the same sample treatment.

Validation with laboratory prepared standard solutions in free-steroid serum and with CRM BCR 576, 577 and 578 (human serum matrix of different concentrations) showed that both methods meet EMEA guidelines requirements in terms of precision, accuracy and matrix effect compensation. The methods were applied to real samples and compared between them and against the individual reference routine analysis based on immunoassay (RIA for E1 and ECLIA for E2) by Passing-Bablok regression analysis.

The novel isotope dilution quantification method, namely isotope pattern

deconvolution (IPD), provided very similar results to those of calibration-based method. However, some bias related with the presence of low concentrations of estrogens in the theoretical steroid-free serum and the low accurately certified standards is shown. This low bias is regarded as not significant since both methods meet validation requirements. LC-MS/MS calibration-based method presented good correlation against immunoassay for E2 but not for E1, were a proportional bias of 2.55 was found respect immunoassay analysis. These findings agree with the raising awareness of the need to re-evaluate the validity and suitability of bioassay methods for the analysis of estrogens due to their tendency to suffer from interferences and cross-reactivity. In addition, lower limits of quantification were achieved (5 ng/L versus 17 ng/L) while needing a relatively small volume of sample of only 250 µL.
Finally, IPD is shown as a rapid, less expensive, precise and exact method suitable for its application to estrogens in serum as a way to countercheck adverse or disagreeing results between conventional calibration-based and immunoassay methodologies, as well as being posed as a fully functional quantification method.

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Figure captions

Figure 1.- Passing-Bablok regression analysis. Correlation between LC-MS/MS method based on calibration curve and RIA determination of E1 (1A) and ECLIA determinations of E2 (1B).

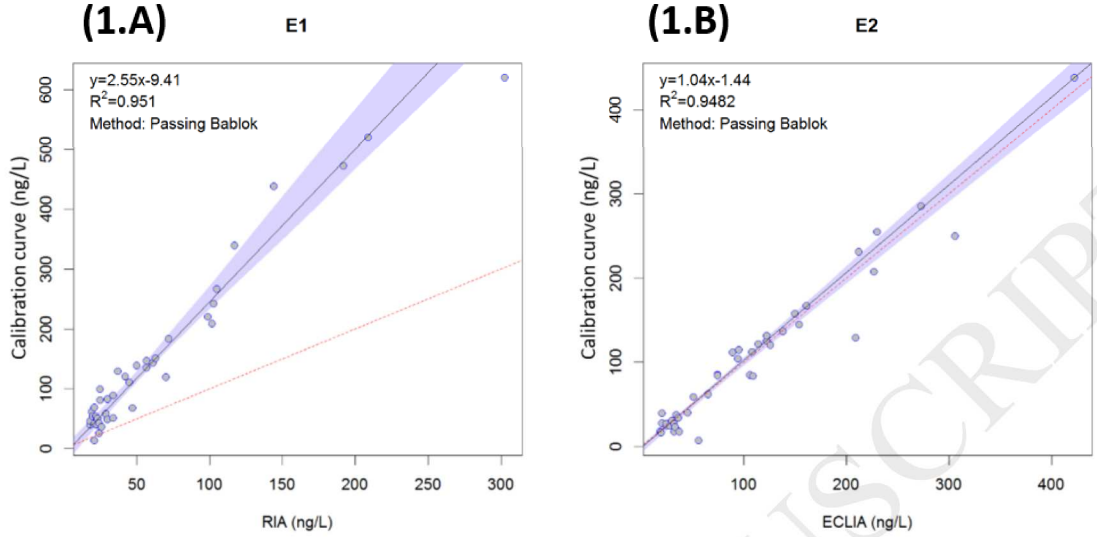

Figure 2.- Passing-Bablok regression analysis. Correlation between IPD and LC-MS/MS method based on calibration curve determination of E1 (2A) and E2 (2B).

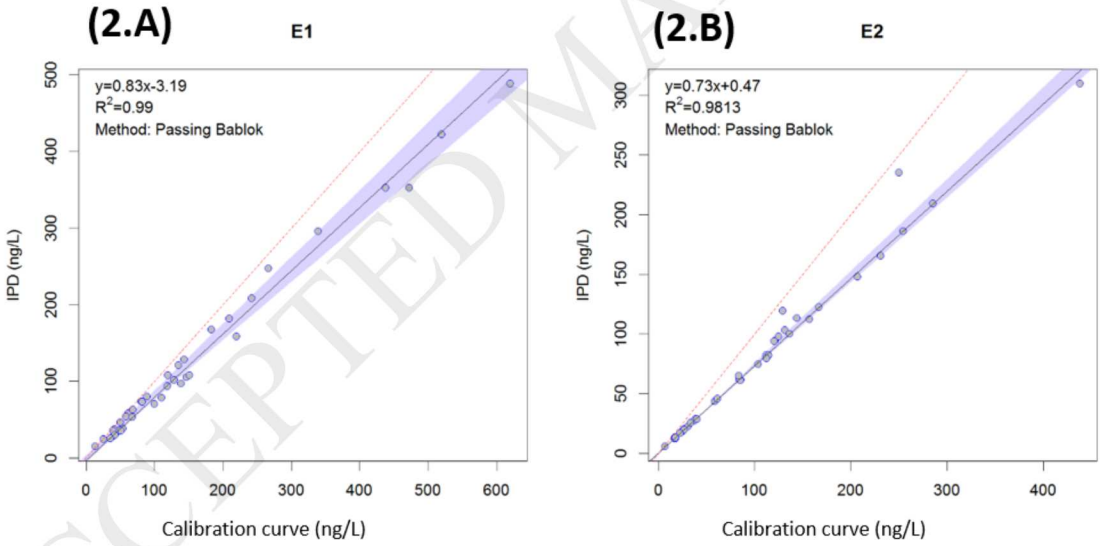

Table 1. Component-dependent MS parameters.

Compound	DP[1]	EP[2]	CE[3]	CXP[4]	Transitions
E1	166	10	43	10	504 > 171
					505 > 171
					504 > 115
E2	151	10	43	12	506 > 171
					507 > 171
					506 > 115
E1-13C3	166	10	43	10	507 > 171
					508 >171
E2-D5	151	10	43	12	511 >171
					512 >171

[1] Declustering Potential (V)

[2] Entrance Potential (V)

[3] Collision Energy (V)

[4] Collision Cell Exit Potential (V)

Table 2. Validation results for E1 in intra- and inter-assay experiments.

E1	Intra-assay (n=5)				Inter-assay (n=9)			
	Cal		IPD		Cal		IPD	
Conc. (ng/L)	Recovery (%)	CV	Recovery (%)	CV	Recovery (%)	CV	Recovery (%)	CV
5	92	13.9%	118	9.5%	99	6.3%	102	4.7%
10	96	6.5%	116	6.0%	102	5.4%	113	5.9%
200	113	1.2%	103	1.3%	112	1.4%	103	1.7%
400	106	2.0%	100	2.1%	107	4.6%	102	4.9%
800	97	6.3%	98	7.0%	96	3.8%	98	4.3%

Table 3. Validation results for E2 in intra- and inter-assay experiments.

E2	Intra-assay (n=5)				Inter-assay (n=9)			
	Cal		IPD		Cal		IPD	
Conc. (ng/L)	Recovery (%)	CV	Recovery (%)	CV	Recovery (%)	CV	Recovery (%)	CV
5	103	8.3%	98	5.0%	106	5.3%	84	5.2%
10	102	4.4%	95	5.3%	105	2.5%	88	2.7%
200	90	4.6%	80	4.6%	104	2.6%	94	2.6%
400	92	3.1%	79	2.9%	105	3.3%	94	3.4%
800	99	2.2%	86	1.7%	108	2.1%	96	1.9%

Table 4. Validation results for E2 determination experiments in three certified reference materials.

CRM		Intra-assay (n=5)				Inter-assay (n=9)			
		Cal		IPD		Cal		IPD	
Code	Conc. E2 (ng/L)	Recovery (%)	CV	Recovery (%)	CV	Recovery (%)	CV	Recovery (%)	CV
BCR576	31.05	97	5.2%	85	5.4%	101	3.6%	84	17.0%
BCR577	187.94	87	4.0%	81	4.2%	93	1.6%	83	1.6%
BCR578	364.99	94	3.8%	88	4.0%	101	3.7%	91	4.2%

Table 5. Matrix effect in different types of serum, obtained by comparison with standards in solvent at the same concentration levels.

Sample type	Matrix Effect (%)			
	E1 at 3xLLOQ	E1 at nearULOQ	E2 at 3xLLOQ	E2 at nearULOQ
Serum A	88	88	80	91
Serum B	95	103	98	104
Hemolized	99	94	171	106
Renal	354	94	118	77
Icteric	170	99	97	99
Lipemic	121	81	97	92