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Development and validation of a LC-MS/MS assay for quantification of serum estradiol using calibrators with values assigned by the CDC reference measurement procedure

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ABSTRACT

Background: Estradiol was historically measured by gas chromatography–mass spectrometry before development of immunoassays. Although immunoassays were fast requiring low sample volume, they had specificity issues. More recently, liquid chromatography-tandem mass spectrometry became the methodology of choice for estradiol quantitation in serum. However, one thing all methods have in common is lack of standardization.
Methods: A LC-MS/MS assay was developed and clinically validated using calibrators with concentrations assigned by a Reference Measurement Procedure in an effort to increase accuracy of calibration. Results: Two hundred microliters of serum was subjected to liquid-liquid extraction using hexane:ethyl acetate followed by derivatization using dansyl chloride. Gradient elution had a run time of 6.5 min. The analytical measurement range was 2 to 1000 pg/mL with between-run imprecision of < 10%. Method comparisons with two reference laboratories showed acceptable bias and so did Reference Materials from the European Commission Joint Research Center, Institute for Reference Materials and Measurements and the College of American Pathologist Accuracy-Based survey samples.
Conclusions: A LC-MS/MS assay for serum estradiol was developed and clinically validated with calibrator concentrations assigned by the CDC RMP to help improve accuracy.

1. Introduction

Estradiol is one of the estrogen steroid hormones that is involved with development of the female characteristics such as breast growth, body shape changes and fat deposition and is mainly produced by the ovaries and the adrenal cortex [1]. Serum estradiol concentrations may be measured at birth in cases of ambiguous genitalia and in late childhood to determine if a child has started puberty too early (precocious) or if puberty is delayed [2]. It may also be measured in women in cases of polycystic ovarian syndrome, hypopituitarism, amenorrhea or menopause [3–5]. Estradiol may also be measured in males if they have gynecomastia or prostate cancer [6,7].

Quantification of estradiol was historically carried out by gas chromatography-mass spectrometry (GC–MS) followed by manual direct radioimmunoassay (RIA) [8]. Subsequently immunoassay was implemented and remains popular, but a number of laboratories have changed back to mass spectrometry [9]. Immunoassays were developed for automated analyzers that reduced the required sample volume and the complexity of the analysis for clinical laboratories, but they suffer from a lack of specificity due to endogenous and exogenous interferences [10]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) became more commonly used clinically due to increased specificity and reproducibility. However, even using this sophisticated technology, it is challenging to ensure that endogenous interferences do

Abbreviations: LC-MS/MS, liquid chromatography-tandem mass spectrometry; GC–MS, gas chromatography–mass spectrometry; RIA, radioimmunoassay; CDC, Centers for Disease Control and Prevention; CDC HoSt, CDC Hormone Standardization Program; RMP, Reference Measurement Procedure; UFLC, ultra-fast liquid chromatography; ESI, electrospray ionization; SRM, selected reaction monitoring; IRMM, European Commission Joint Research Center, Institute for Reference Materials and Measurements; CAP ABS, College of American Pathologist Accuracy-Based survey; 95% CI, 95% confidence intervals; LOQ, limit of quantitation; AMR, analytical measurement range

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Table 1
selected reaction monitoring transitions and voltages for the dansyl chloride derivatives of estradiol and D5-estradiol (internal standard).

Analyte	Q1 (*m/z*)	Q3 (m/z)	DP (V)	EP (V)	CXP (V)	CE (mV)
Estradiol derivative (quantifier transition)	506.1	171.1	130	10	11	44
Estradiol derivative (qualifier transition)	506.1	156.1	130	10	11	52
D5-estradiol derivative (quantifier transition)	511.1	171.1	130	10	11	44
D5-estradiol derivative (qualifier transition)	511.1	156.1	130	10	11	52

Q1: quadrupole 1 mass; m/z: mass to charge ratio; Q3: quadrupole 3 mass; DP: declustering potential; V: volts; EP: entrance potential; CXP: collision cell exit potential; CE: collision energy; mV: millivolts.

not cause false increases in estradiol concentrations. Further, a large sample volume is often required to measure estradiol at clinically relevant concentrations and the assays can be very labor intensive and require specialized equipment. One thing that all estradiol methods have in common is the lack of standardization in measurement [11].

For the last 10 years, The Centers for Disease Control and Prevention (CDC) have been focused on the standardization of hormone measurements including estradiol [12] due to various publications showing discrepancies in the measurement of estradiol between different methodologies [13,14]. As part of the CDC Hormone Standardization Program (HoSt) [15,16], a reference measurement procedure (RMP) for estradiol was developed [17]. One of the services that the CDC offers is a Reference Measurements Service whereby they will assign values to laboratory-prepared calibrators using the RMP [18]. Therefore, we developed and validated a LC-MS/MS assay to measure total estradiol using calibrators with values assigned by the CDC RMP.

2. Materials and methods

2.1. Reagents

Mass spectrometry grade solvents and water were from VWR International (Brisbane, CA). Dansyl chloride, acetone and sodium bicarbonate were from Sigma-Aldrich (St Louis, MO). Formic acid was from Thermo Fischer Scientific (Fremont, CA). Estradiol, estrone, estriol, testosterone, epitestosterone, dehydroepiandrosterone, androstenedione and D5-estradiol were from Cerilliant Corporation (Round Rock, TX). Double charcoal stripped human male serum was from Golden West Biologicals (Temecula, CA). The low concentration quality control sample was from UTAK Laboratories (Valencia, CA) and the medium and high concentration quality control samples were Lyphocheck® Immunoassay Plus levels 2 and 3 from BioRad Laboratories (Irvine, CA).

2.2. Patient samples

Institutional review board approval was obtained from the University of California, San Francisco Committee on Human Research for this study. One hundred and one remnant patient samples who had estradiol measured by LC-MS/MS at a reference lab were retained and run on this developed assay. A further 20 patient samples with estradiol concentrations determined by LC-MS/MS were obtained from another reference laboratory and run on this developed assay.

2.3. Liquid-liquid extraction and derivatization

Two-hundred microliters of serum was pipetted into a glass tube and 25 μL of internal standard (1 ng/mL D5-estradiol) was added and the sample vortexed and left at room temperature for 5 min. One milliliter of 90:10 (v:v) hexane:ethyl acetate was added and the sample vortexed for 2 min, left at room temperature for 5 min and centrifuged at 3000 rpm (1200 × *g*) for 10 min. The samples were then placed in dry ice until the aqueous bottom layer froze and the top organic layer was poured off into another tube. The organic layer containing the estradiol was then dried down under nitrogen at 45 °C. Fifty microliters of 100 mM sodium bicarbonate at pH 10 was added to the dried extract, vortexed and allowed to sit at room temperature for 1 min. Fifty microliters of 1 mg/mL dansyl chloride in acetone was then added to the same tube, vortexed, allowed to sit at room temperature for 1 min, vortexed again and then and placed in a 60 °C heating block for 3.5 min. Fifty microliters of methanol was then added and the sample transferred to an autosampler vial.

2.4. Liquid chromatography and mass spectrometry

A Shimadzu Prominence UFLC system (Shimadzu Scientific Instruments, Pleasanton, CA) was used with a Kinetex® phenyl-hexyl 100 × 3 mm, 2.6 μm column (Phenomenex, Torrance, CA) maintained at 40 °C. Mobile phases were 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in 70:30 (v:v) methanol: acetonitrile (mobile phase B) and estradiol was eluted via gradient elution over 6.5 min with a flow rate of 0.8 mL/min holding at 20%B for 1 min, ramping to 70% B over 0.2 min, ramping to 95% B over 3.3 min, holding at 95%B for 1 min and back to 20% B for a 1 min for reequilibration. The injection volume was 30 μL. A 6500 QTRAP (SCIEX, Redwood City, CA) was utilized in positive polarity with electrospray ionization (ESI). Selected reaction monitoring (SRM) was used with two transitions (quantifier and qualifier) each for estradiol and D5-estradiol (Table 1). The source parameters were: curtain gas of 30 psi, ion spray voltage of 5500 V, temperature of 500 °C, gas 1 of 70 psi, gas 2 of 80 psi and medium collision gas.

2.5. Assay calibration and quality control

Calibrators were prepared by spiking double charcoal stripped male serum with estradiol aiming for concentrations of 10, 100, 250, 500 and 1000 pg/mL. Aliquots of the calibrators were sent to the CDC to have concentrations assigned to them by the Reference Measurement Service and the assigned concentrations were 10, 98, 249, 500 and 1001 pg/mL. During an analytical run, the peak areas for estradiol were normalized to the peak areas for D5-estradiol and the concentration calculated using a zero calibrator (double charcoal stripped serum) and 5 non-zero calibrators as described above. Three quality control samples were used in every batch run on the developed assay and had mean concentrations of 7, 246 and 574 pg/mL. The laboratory QC procedure, which is based on the Westgard rules, was applied to each batch run to determine if it was acceptable. Ion ratios (the peak area of the qualifier ion divided by the peak area of the quantifier ion) were calculated for the 5 non-zero calibrators in each run and a range of ± 20% was established. Any unknown sample had to have an ion ratio within the range for both estradiol and D5-estradiol in order to be included in the validation data. No unknown samples had an ion ratio > 20% from the mean ion ratio.

2.6. LC-MS/MS assay validation

Assay validation was carried out based upon published recommendations [19,20]. Linearity, reportable range, between-day and within-day precision, sensitivity, interferences, ion suppression [21], carryover and stability were all determined using standard methods and

are described in more detail in the Supplementary Material.

To determine accuracy, the 6 calibrators used for this assay had their values assigned by the CDC RMP. Further accuracy studies were undertaken using three Reference Materials from the European Commission Joint Research Center, Institute for Reference Materials and Measurements (IRMM). Lastly, the College of American Pathologist Accuracy-Based survey (CAP ABS) samples from Survey A in 2018 were also run on the estradiol assay. Method comparisons were also carried out running patient samples on this LC-MS/MS method and LC-MS/MS assays from 2 reference laboratories.

3. Results

Results from the imprecision, sensitivity, ion suppression, interferences, carryover and stability studies can be found in the Supplementary Material.

3.1. Linearity, reportable range and accuracy

The LC-MS/MS calibrators had concentrations assigned by the CDC RMP and the assay was linear for estradiol between 2 and 1001 pg/mL (slope: 1.032, intercept: 0.2, R^2:0.99) with a mean total error of 0.4 pg/mL or 5.4% across the linear range of the assay (Fig. 1). The reportable range of the assay was determined to be 2–4000 pg/mL based upon a 1:5 dilution. The CAP ABS survey samples from Survey A in 2018 were run on the LC-MS/MS assay and they were found to have concentrations of 6, 49 and 23 pg/mL compared to the CDC-assigned target concentrations of 6, 51 and 24 pg/mL. Therefore, the bias of the LC-MS/MS assay was 0, −4 and −4% for each sample respectively. The IRMM standards were also run on the LC-MS/MS assay and were found to have concentrations of 29, 171 and 334 pg/mL compared to the reference concentrations of 31, 188 and 365 pg/mL. Therefore the bias of the LC-MS/MS assay was −6, −9 and −8% for each standard respectively.

3.2. Method comparisons

One hundred and one remnant samples were run on the developed LC-MS/MS assay and a LC-MS/MS assay at a reference laboratory. The estradiol concentrations of the samples ranged from < 2 to 446 pg/mL with a mean concentration of 60 pg/mL and a median concentration of 40 pg/mL based upon the reference laboratory results. Deming regression comparing the estradiol concentrations from both methods yielded a slope of 1.008 (95% confidence intervals (CI): 0.970 to 1.046), an intercept of −4.3 (95% CI: -7.8 to −0.8) and a correlation coefficient of 0.9833 (Fig. 2a). The mean bias was −6.4% indicating that the developed assay had a negative bias compared to the reference laboratory method (Fig. 2b). When samples with estradiol concentrations of < 100 pg/mL were compared (n = 87), Deming regression yielded a slope of 0.954 (95% CI: 0.891 to 1.017), an intercept of −2.1 (95% CI: -4.9 to 0.7) and a correlation coefficient of 0.9560 (Fig. 3a). The mean bias was −10.7% indicating that the developed assay had a negative bias compared to the reference laboratory (Fig. 3b).

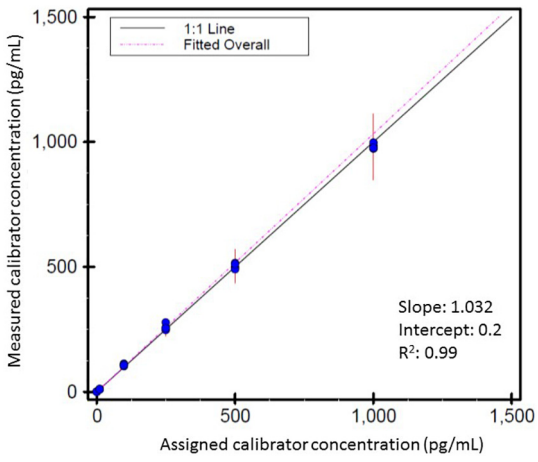

Fig. 1. Measured calibrator concentration back calculated by the LC-MS/MS software on 5 different runs plotted against the assigned calibrator concentration.

Twenty patient samples and the estradiol concentrations were obtained from another reference laboratory and run on the developed LC-MS/MS assay. The estradiol concentrations of the samples ranged from < 1 to 848 pg/mL with a mean concentration of 204 pg/mL and a median concentration of 96 pg/mL based upon the reference laboratory results. Deming regression comparing the estradiol concentrations from both methods yielded a slope of 0.912 (95% CI: 0.864 to 0.961), an intercept of 8.7 (95% CI: −7.5 to 24.8) and a correlation coefficient of 0.9950 (Fig. 4a). The mean bias was −5.1% indicating that the developed assay had a negative bias compared to the reference laboratory method (Fig. 4b).

4. Discussion

It has been shown that there is large variation in the measurement of serum estradiol concentrations using different methodologies [13]. One study compared 11 immunoassays and 6 mass spectrometry assays in measuring estradiol in 40 single donor serum samples with concentrations ranging from 2.5 to 285 pg/mL, to a reference measurement procedure (RMP) [14]. They found a mean bias ranging from −2.4 to 235% and concluded that calibration bias was the major contributor to this variation.

In 2014, the CDC launched the Hormone Standardization Program (HoSt) for Estradiol with the aim of standardizing estradiol measurements in serum through method comparison to a RMP in single donor fresh frozen serum samples and estimating the bias of the participant assays [16]. Phase I of this program can be used to perform a bias assessment of your assay, and to adjust calibration to achieve desirable performance and Phase II is to ensure the performance is consistent over time [16]. The HoSt program for testosterone was implemented in 2007 and has proven successful in reducing the mean absolute bias between mass spectrometry assays by 50% between 2007 and 2011 [22]. However for many laboratories, it would be cost prohibitive to participate in this program. An alternative option is the Reference Measurements Service from the CDC that will assign concentrations to laboratory prepared calibrators using the estradiol RMP [18]. While this is still costly, it is of lower cost than participating in the HoSt program and since there is no National Institute of Standards and Technology Standard Reference Material that contains estradiol currently, our laboratory decided to use the Reference Measurements Service to assign concentrations to calibrators prepared in-house.

Laboratories can also use the CAP ABS survey samples to check the calibration bias of their assay to the CDC RMP for estradiol. The results presented herein show a maximum bias of −4% for the survey A samples from 2018. The criteria the CDC has for passing the HoSt program is as follows: for target concentrations > 20 pg/mL, a bias of ± 12.5% and for target concentrations ≤20 pg/mL, a bias of +/− 2.5 pg/mL [16]. One of the CAP ABS survey samples had a concentration of 6 pg/mL, and our result was 6 pg/mL. The other two CAP ABS survey samples had concentrations of 51 and 24 pg/mL and our bias was −4% for both samples which is well within the HoSt program criteria. The IRMM samples were also run on the newly developed LC-MS/MS method with all 3 concentrations being > 20 pg/mL. Our maximum bias was −9% which is again within the HoSt program criteria. The bias for the IRMM samples may be larger than seen for the CAP ABS survey samples because they are provided lyophilized and so have to be reconstituted by the laboratory before use, introducing more potential error and bias into the measurement.

As part of the method validation, a comparison between the newly

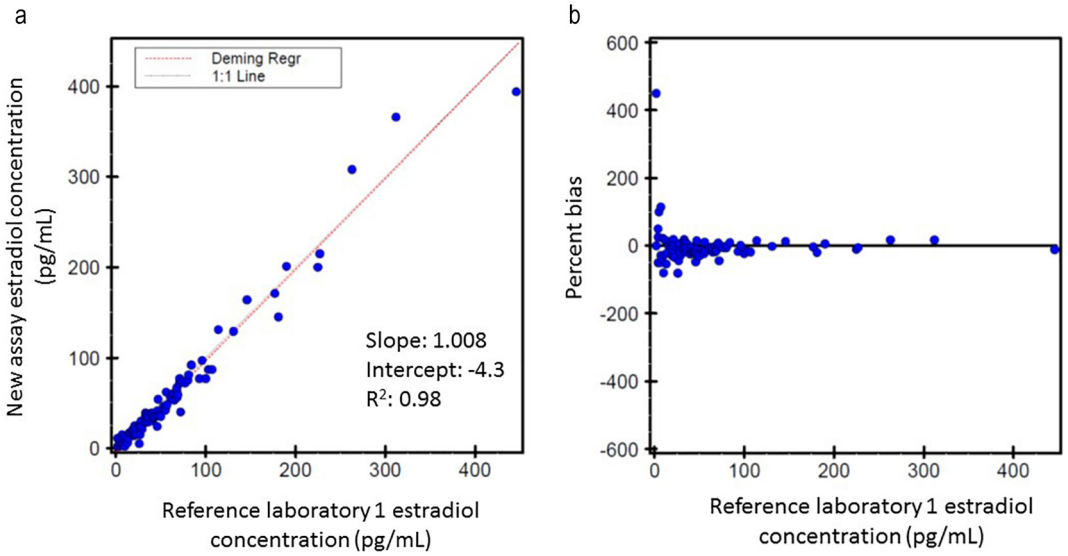

Fig. 2. Comparison of 101 patient samples run by LC-MS/MS at reference laboratory 1 and on the newly developed LC-MS/MS assay. a) linear regression plot; b) percent bias plot.

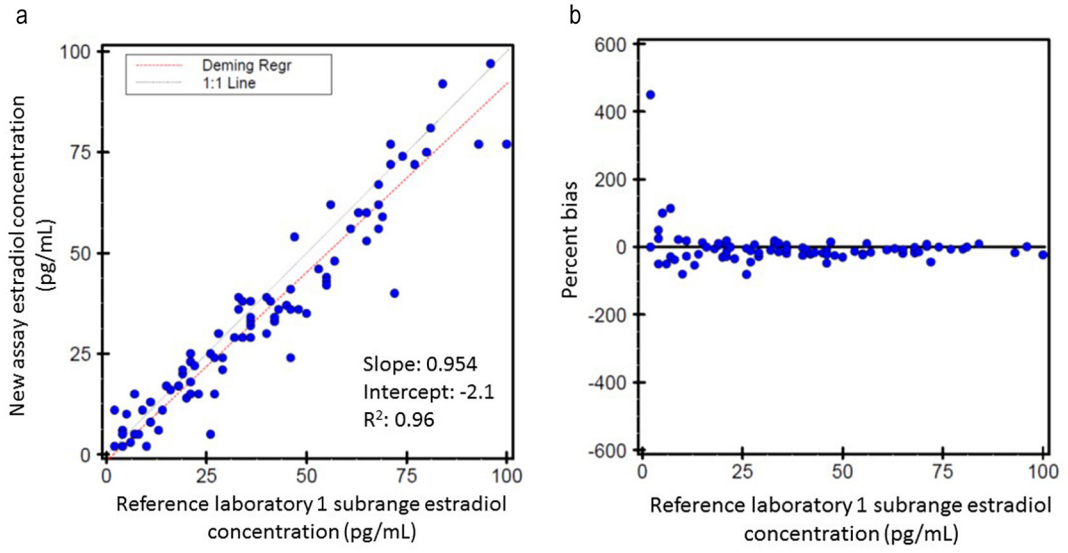

Fig. 3. Comparison of 87 patient samples with concentrations of < 100 pg/mL run by LC-MS/MS at reference laboratory 1 and on the newly developed LC-MS/MS assay. a) linear regression plot; b) percent bias plot.

developed LC-MS/MS assay and two reference laboratories was undertaken. For reference laboratory 1, 101 samples were compared and showed an overall mean bias of −6.4% showing that the new method runs lower than the reference laboratory method. For reference laboratory 2, 20 samples were compared and showed a mean overall bias of −10.7% again showing that the new method runs lower than the reference laboratory method. Differences in calibration are likely to blame for these observed biases, however since none of the 3 laboratories are certified by the CDC HoSt program, it is unknown which laboratory is most accurate. Given that the calibrators used herein had the concentrations assigned by the CDC RMP, it gives the laboratory confidence that the measurements have increased accuracy than if they did not.

The developed LC-MS/MS estradiol method uses 200 µL of serum, has a 6.5 min run time and a limit of quantitation (LOQ) of 2 pg/mL. It has an analytical measurement range (AMR) of 2–1001 pg/mL with acceptable imprecision across the AMR and a reportable range of 2–4000 pg/mL based upon dilution. In comparison to other recently published clinically validated methods, it uses minimal sample volume to achieve a low LOQ, has a comparable run time and a wider AMR [23–26]. A disadvantage of the method is that derivatization is used which adds reagent costs and time to the sample preparation procedure. However, since it is only a 3.5 min derivatization procedure, the time that is added is minimal.

5. Conclusions

A LC-MS/MS assay for serum estradiol was developed and clinically validated with calibrator concentrations assigned by the CDC RMP to help improve accuracy. Two hundred microliters of serum was used and a LOQ of 2 pg/mL was achieved in a run time of 6.5 min. The assay had acceptable imprecision across the measurement range of 2–1001 pg/mL. Method comparisons with two reference laboratories showed acceptable bias as did Reference Materials from the European Commission Joint Research Center, Institute for Reference Materials and Measurements and the College of American Pathologist Accuracy-Based survey samples. Common interferences had no significant effect on the method and neither did the steroid hormones tested. Estradiol was

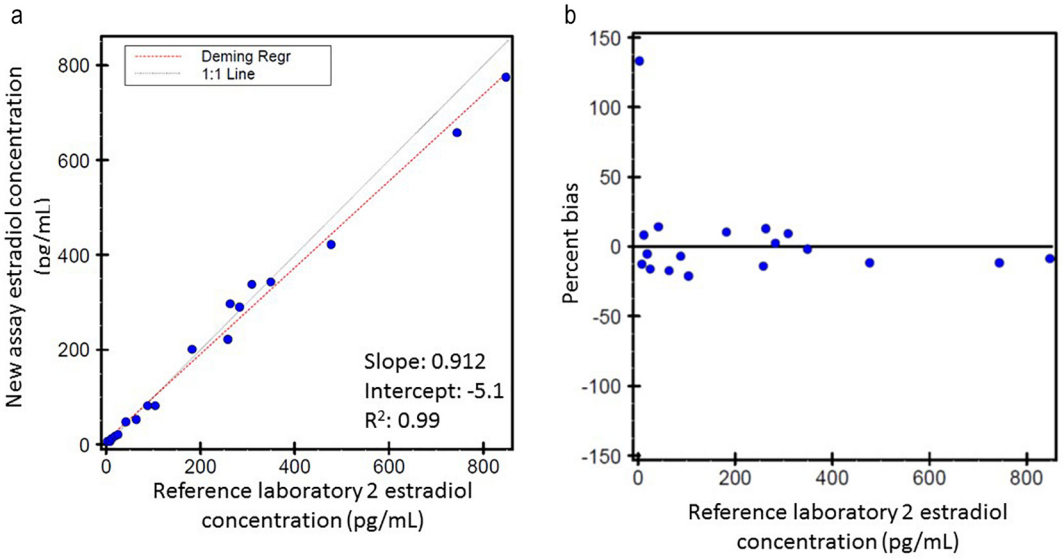

Fig. 4. Comparison of 20 patient samples run by LC-MS/MS at reference laboratory 2 and on the newly developed LC-MS/MS assay. a) linear regression plot; b) percent bias plot.

stable under the conditions tested.

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Declaration of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2019.02.003.

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