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Jennifer Brennan ORCID iD: 0000-0003-0386-3496

Donald Tillitt ORCID iD: 0000-0002-8278-3955

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VM7Luc4E2 cells and LC/MS for quantifying estrone in water

Comparison of Two Estrogen CALUX Cell Bioassays to Liquid Chromatography-Mass Spectrometry for Quantifying Estrone in Water Samples

Jennifer Brennan[1,2,*], (https://orcid.org/0000-0003-0386-3496) Abigail Henke[1,#], (https://orcid.org/0000-0002-8182-4639) Robert Gale, (https://orcid.org/0000-0002-8533-141X) Diane Nicks, (https://orcid.org/0000-0001-8080-2449) and Donald Tillitt (https://orcid.org/0000-0002-8278-3955)

US Geological Survey Columbia Environmental Research Center, Columbia, Missouri, USA

Abstract: Chemically activated luciferase expression (CALUX) cell bioassays are popular tools for assessing endocrine activity of chemicals such as certain environmental contaminants. Although activity equivalents can be obtained from CALUX analysis, directly comparing these equivalents to those obtained from analytical chemistry methods can be problematic due to the complexity of endocrine active pathways. We explored the suitability of two estrogen CALUX bioassays (the OECD-approved VM7Luc4E2 cell bioassay and the VM7LucERβc9 cell bioassay) for quantitation of estrogen. Quadrupole-time of flight ultraperformance liquid chromatography/mass spectrometry (LC/MS) was selected as a comparative method. Regression analysis of measured estrone (E1) calibration samples showed all three methods as highly predictive of nominal

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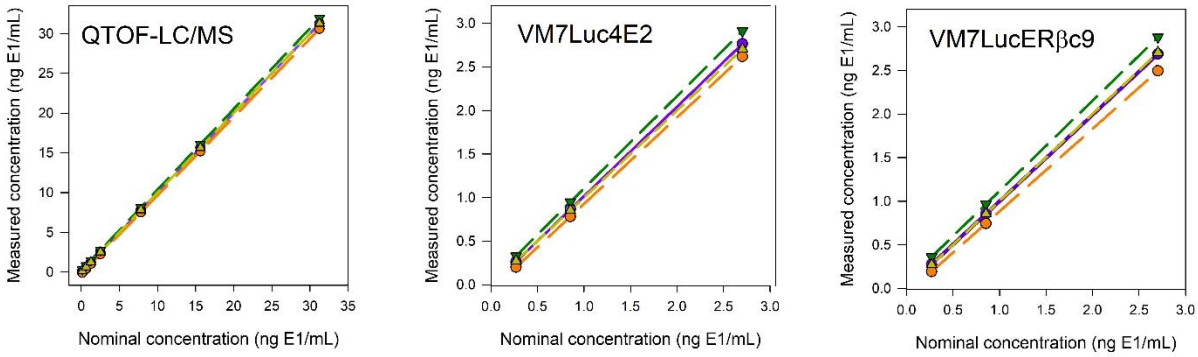
concentrations ($p \leq 7.5 \times 10^{-51}$). Extracts of water sampled from laboratory dilutor tanks containing estrone (E1) at 0, 20, and 200 ng/L alone, and in combination with atrazine (ATZ) were selected to test the quantitative capabilities of the CALUX assays. Process controls (0 and 100 ng E1/L) and a separate E1 standard (10 ng/mL, used to prepare the E1 process control) were also tested. E1 levels determined by LC/MS analysis and bioanalytical equivalents (BEQ, ng E1/L) determined by CALUX analyses were comparable except in certain instances where the samples required dilution prior to CALUX analyses (e.g., the E1 process control and E1 standard). In those instances, measurements by CALUX were slightly but significantly decreased relative to LC/MS. ATZ had no effect on the ability of either LC/MS or the CALUX bioassays to quantify E1. This study illustrates the CALUX bioassays as successful in quantifying an estrogen in simple water samples and further characterizes their utility for screening.

Graphical abstract



Keywords: Analytical chemistry; Estrogenic compounds; In vitro toxicology; CALUX; Bioassay

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* Correspondence brennan.jennifer@epa.gov

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[1]These authors contributed equally to this manuscript

[2]Present affiliation Office of Pollution Prevention and Toxics, US Environmental Protection Agency, Research Triangle Park, North Carolina, USA

[3]Present affiliation Biology Department, Baylor University, Waco, Texas, USA

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INTRODUCTION

High throughput cell bioassays have become popular in regulatory, industrial, and environmental laboratories for assessing biological activity in a large number of samples quickly, inexpensively, and with relative ease. Typically, these bioassays are used as screening tools and, in the case of mixtures such as sample extracts, generate bioanalytical equivalency (BEQ) factors to relate a compound or mixture's relative bioactive potency to the concentration of an established reference chemical in order to estimate potential level of concern (Safe 1998; Zhou et al. 2021; Escher et al. 2018; Neale et al. 2015). Chemically activated luciferase expression (CALUX) cell bioassays are one such method for providing sample BEQs. Determination of BEQs via the CALUX assay for samples containing dioxin-like chemicals [i.e. EPA test method 4435] is accepted as comparable to the corresponding toxic equivalencies (TEQs) determined by analytical methods, such as gas chromatography/high resolution mass spectrometry (GC/HR-MS) (Garrison et al. 1996). However, for samples containing endocrine-active substances (EAS), direct comparison of CALUX BEQs with those derived from analytical chemistry is more challenging due to the complexity of endocrine active pathways present in biological systems, including CALUX and CALUX-type assays

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(Rogers and Denison 2002; Hashmi et al. 2018; Denison et al. 2011). Nonetheless, the CALUX bioassay may be a reasonable approach for quantitation of EAS under more controlled situations, such as laboratory exposure studies, and would have the advantages of less expensive instrumentation and higher throughput than methods such as mass spectrometry (Cross and Hornshaw 2016).

The purpose of this study was to explore the quantitative accuracy of the estrogen CALUX bioassays. Laboratory dilutor tank water samples from a fish (largemouth bass, *Micropterus salmoides*) estrone (E1)/atrazine (ATZ) co-exposure study were quantified by two CALUX estrogen bioassays as well as quadrupole-time of flight liquid chromatography/ mass spectrometer (Q-TOF LC/MS). We used the internationally validated VM7Luc4E2 cell line that is utilized in OECD TG 455, the Tox21 Program, and the ToxCast estrogen receptor (ER) pathway model and the further modified cell line VM7LucERβc9 (which contains both ERα and ERβ) (OECD 2021; Huang et al. 2014; Judson et al. 2015; Brennan et al. 2016) for supporting an in vivo laboratory study. E1 is a commonly detected EAS in surface waters (detected in 30% of surface waters within the USA with average levels approximately 3 ng E1/L) as well as estuaries and treated sewage effluents (detected in 85% of USA wastewaters with average concentration approximately 370 ng/L) (Brennan et al. 2020). ATZ, a herbicide used to control the growth of broadleaf plants, is commonly found in surface waters as well as groundwater samples in both urban and agricultural settings (Bradley et al. 2017; Wang et al. 2020). A recent survey of U.S. streams found ATZ at approximately 70% of sample sites with median concentration of 34 ng/L (Bradley et al. 2017). Similarly, ATZ median concentration was 63 ng/L in a survey of surface waters within the Yangtze River Delta

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in China (Peng et al. 2018; Bradley et al. 2017). Recently, ATZ was investigated for effects on developing gonads of largemouth bass and was found to have both similar and differential gene expression as a model estrogen, 17α-ethinylestradiol (EE2) (Leet et al. 2020). Objectives of the present study were to 1) compare quantitation by the CALUX assays and Q-TOF LC/MS using dilutor water samples containing E1 and ATZ, and 2) To determine whether the LC/MS and CALUX bioassays were consistent in detecting subtle changes in E1 levels in smaller data sets (e.g., dilutor water samples collected prior to and post-fish addition to the dilutor tanks).

METHODS

Fish culture, husbandry, and exposure

Largemouth bass (*Micropterus salmoides*) were reared and maintained as previously described in accordance to IACUC protocols (Leet et al. 2020). As part of a larger study and utilizing the experiment design described in a previous study (Leet et al. 2020), largemouth bass fry were exposed to solvent control (0.0001% EtOH), E1 (nominally 20 or 200 ng/L), or E1 + ATZ in the following nominal combinations: 20 ng/L E1 + 10 micro-g/L ATZ, 20 ng/L E1 + 100 micro-g/L ATZ, 200 ng/L E1 + 100 micro-g/L ATZ. Final exposure concentrations were 0, 10, and 100 micro-g/L ATZ and 0, 20, and 200 ng/L E1 with solvent concentration 0.0001% EtOH. Chemical exposure in the proportional, flow-through dilutors was conducted as described previously (Leet et al. 2020). Details of the dilutor set-up and dosing of juvenile largemouth bass with E1 alone or in combination with ATZ is described in Supplemental Information.

Sampling and Analyses

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Water samples from dilutor tanks were collected twice weekly and extracted as described in Supplemental Information. There were six sampling dates prior to fish addition and eleven sampling dates post-fish addition. Samples were obtained by collecting 100 mL of water from high level dilutors (i.e., tanks dosed with 200 ng E1/L, ± 100 micro-g ATZ) and 250 mL of water from control (0 ng/L E1) and low-level dilutors (i.e., tanks dosed with 20 ng E1/L ± 10 or 100 micro-g ATZ). Water samples were then filtered through Gelman type A/E glass fiber filter using an SPE vacuum manifold, loaded onto a preconditioned SPE cartridge, eluted from the SPE cartridges with 10 mL dichloromethane, processed as described in supplemental methods, and finally reconstituted in 1 mL of 10/90 MeOH/H_2O. Full extraction methodology is described in the Supplemental Methods. Bioactivity analyses were carried out using the human breast carcinoma chemically activated luciferase expression (CALUX) VM7Luc4E2 and VM7LucERβc9 cell lines (Rogers and Denison 2000; Brennan et al. 2016) according to a modified version of a previously published method (Brennan and Tillitt 2018), with modifications described in Supplemental Methods. Cell type authenticity was verified as described previously (Brennan and Tillitt 2018). Relative luminescence values were normalized to protein in cell lysates as described previously and in Supplemental Information (Brennan and Tillitt 2018). Percent coefficient of variance (% CV), Z' factor, signal to noise, limit of detection (LOD), limit of quantitation (LOQ), and quarter-, half-, and three quarter-maximal effective concentrations (EC_{25}, EC_{50}, EC_{75}) were calculated for each CALUX bioassay as described previously, with the modification of LOD as three times the standard deviation of the blank (Brennan and Tillitt 2018). Individual water samples were analyzed in three to four independent experiments in each CALUX

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bioassay with four analytical replicates per experiment. Activity of water samples (0, 20, and 200 ng/L E1 ± 10 or 100 micro-g ATZ as described in Supplemental Methods) and process controls (0 or 100 ng/L E1) in the CALUX assays were expressed as BEQ relative to the activity of estrone (E1, ng E1/L). To determine BEQs in the CALUX assays, water sample and E1 calibration standard activities were first normalized to a maximally inducing activity achieved by 7 pg E2 (1 nM). Then, values for BEQs were generated from single-point activities using the Solve function in SigmaPlot (v14.0, from Systat Software, Inc., San Jose, CA USA) for an E1 standard curve fitted with a sigmoidal Hill's 4-parameter equation. In certain samples, the activity of the water sample was too low or too high to quantify within the E1 calibration curve, and the Solve Function was unable to generate a BEQ in those instances. If the Solve function was unable to generate a BEQ in more than 20% of samples within a particular sample type (e.g. 100 ng/L E1 Process Control, 200 ng/L E1 tank), those extracts were further diluted in 10% MeOH in H_2O for reanalysis in the CALUX bioassays (1:8 for extracts generated from 200 ng/L E1 water samples and 1:4 for the Reference Check 10 ng E1/mL standard as well as extracts generated from the 100 ng/L E1 Process Control). All handling and disposal of cell culture materials was in accordance with guidelines set forth by the U.S. Department of Labor Occupational Safety and Health Administration 29 CFR 1910.1030 (OSHA 1992).

Samples were also analyzed by UPLC-MS/MS (Acquity UPLC; Synapt G2-Si Q-TOF Ion Mobility Mass Spectrometer (Waters, Corp.)), operated with MassLynx software (v4.1, Waters) as described in Supplemental Methods. TargetLynx software (v4.1, Waters) was used for quantitation. Results presented for LC/MS and CALUX

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assay performance metrics (Table 1, Figure 1) are expressed as E1 concentration in the calibration standards (ng E1/mL) whereas results presented for activity of water samples (Figure 2, SI Figure 2) are expressed as E1 concentration in the original water sample (ng E1/L or BEQ). Validation results of the E1/ATZ co-treatment in the CALUX bioassays (Figure 1) are presented as amount of chemical in the microtiter well.

Statistics

Significant difference in E1-induced luciferase activity in preliminary experiments with E1 and ATZ chemical standards (described in Supplemental Results, SI Figure 1) was determined with Student's t-test (2 tailed, Type 2) in Microsoft Excel (α = 0.01). Regression analysis, including determination of 95% confidence intervals, was performed in Microsoft Excel. Significant differences in sample estrone levels determined by LC/MS from those determined by CALUX bioassays were analyzed with One-Way ANOVA (SigmaPlot, v14.0). Significant differences in BEQ values with and without ATZ were also determined by One-Way ANOVA. Normality and Equal Variance of data were determined by Shapiro-Wilkes and Brown-Forsythe tests, respectively (α = 0.05). Kruskal-Wallis One-Way ANOVA on Ranks was performed on data that failed the Shapiro-Wilkes test. Multiple comparisons were performed with Dunn's test when significant difference was identified ($p < 0.01$). Differences in water sample estrone concentrations pre- and post-fish addition were also determined using these same statistical methods with the exception that Tukey's test was used in one instance where Dunn's test was not provided as an option.

RESULTS AND DISCUSSION

Validation steps for CALUX quantitation

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The CALUX assays and LC/MS had comparable limits for E1 detection and quantitation (Table 1). LOQ values 0.089-0.19 ng E1/mL indicate similar ability of the CALUX assays and LC/MS to quantify low levels of E1 in the water samples. LOD and LOQ values for the VM7Luc4E2 cell bioassay (0.046 ± 0.0048 and 0.089 ± 0.0085 ng E1/mL, respectively) aligned more closely with LC/MS LOD and LOQ (0.05 and 0.125 ng E1/mL, respectively) than with the VM7LucERβc9 cell bioassay LOD and LOQ (Table 1). We observed excellent agreement between nominal (yellow dashed line) and measured (purple solid line) concentrations for the calibration standards within the linear portion of the E1 calibration curves for LC/MS (p-value = 2.27×10^{-97}) and both CALUX assays (p-values = 1.94×10^{-60} and 7.47×10^{-51} for VM7Luc4E2 cells and VM7LucERβc9 cells, respectively, Figure 1, SI Tables 2-4). Dynamic range represented for LC/MS was between 0.125 and 31.25 ng E1/mL (nominal, $R^2 = 0.995$), whereas the dynamic range for the CALUX assays was between 0.27 and 2.7 ng E1/mL (nominal, $R^2 = 0.982$ and 0.968 for VM7Luc4E2 and VM7LucERβ9 cells, respectively). In retrospect, more points within the linear portion of the E1 calibration curve (such as concentrations above LOQ and below 0.27 ng E1/mL) would better represent the dynamic range of the CALUX assay. On the upper portion of the E1 calibration curve, additional calibration standard concentrations between 2.7 ng E1/mL and 8.5 ng E1/mL would better define the upper portion of the linear range in the CALUX assays. Greater signal to noise ratio (S/N, based on ratio of maximal E2 induction to solvent control) was observed in the VM7Luc4E2 bioassay (125 ± 12) than in the VM7LucERβc9 bioassay (63 ± 7.5) and was similar to the S/N previously obtained in our laboratory (Brennan and Tillitt 2018). S/N was also assessed in the CALUX assays for a low concentration E1 standard (0.270

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ng E1/mL) and compared to S/N in LC/MS for the 0.125 ng E1/mL calibration standard (Table 1). Greater S/N was observed in LC/MS (13-30) compared to ERα VM7Luc4E2 (10.2) and VM7LucERβc9 cells (5.9). In both CALUX bioassays, the Z′ and CV were within acceptable range (> 0.6 and < 15%, respectively) (OECD 2021; ICCVAM 2011), and the VM7Luc4E2 values were similar to those previously obtained in our laboratory (Brennan and Tillitt 2018). Half-maximal effective E1 concentrations in the VM7Luc4E2 and VM7LucERβc9 bioassays (Table 1), expressed as molarity in the medium, ($6.3 \pm 0.4 \times 10^{-11}$ and $5.5 \pm 0.4 \times 10^{-11}$ M E1, respectively) indicate slightly increased sensitivity (approximately a half-order of magnitude) when compared to previously published values in VM7Luc4E2 cells (OECD 2016; ICCVAM 2011).

Percent recovery was calculated for E1 in the spiked process control by dividing measured E1 concentration (or BEQ in the case of the CALUX assays) by nominal concentration. Percent accuracy of the E1 reference check, the source of E1 for the spiked process control, was also calculated as measured E1 concentration (or BEQ) by nominal. Good agreement in percent recovery of the E1-spiked process control and percent accuracy of the E1 reference check as determined by LC/MS and both CALUX assays indicates high extraction efficiency for E1 (SI Table 6). For example, 98 ± 12% recovery and 104 ± 3% accuracy were determined by the VM7Luc4E2 cell bioassay for the spiked process control and reference check, respectively (SI Table 6). By comparison, measured E1 concentrations in water samples were approximately 60% of nominal concentrations (49-64%, 56-68%, and 46-65% of nominal concentrations as determined by LC/MS, VM7Luc4E2 bioassay, and VM7LucERβc9 bioassay, respectively, SI Table 6), indicating some loss of E1 in the flow-through dilutor system. These values are slightly

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lower than previously determined dilutor concentrations of another estrogen, EE2, tested in the same dilutor set up in a previous study (73-84% of nominal) and E2 tested in a similar 21-day dilutor study (approximately 70% of nominal) (Leet et al. 2020; Owens 2007).

Comparison of LC/MS to CALUX bioassays for estrone quantitation in water samples

Estrone concentrations and BEQs determined by LC/MS and CALUX, respectively, were almost identical for water samples prepared from dilutor tanks (Figure 2). No significant differences were observed between concentrations determined by LC/MS and VM7Luc4E2 cells for any of these samples or for the negative process control sample (p-values between 0.036-0.30, Figure 2, SI Table 5). BEQ values (expressed in ng/L E1) generated by VM7LucERβc9 cells were slightly but significantly lower ($p < 0.001$) than LC/MS-determined E1 levels for 200 ng/L E1 water samples with ATZ (92 ± 2.3 BEQ vs 120 ± 4.7 ng/L E1, for the VM7LucERβc9 cell bioassay and LC/MS, respectively) and without ATZ (94 ± 3.4 BEQ vs 130 ± 7.0 ng/L E1, for the VM7LucERβc9 cell bioassay and LC/MS, respectively) (Figure 2, SI Table 5). The VM7LucERβc9 cell line responds to a greater range of estrogenic chemicals than the VM7Luc4E2 cell line (Brennan et al. 2016) and this preferential response potentially includes antagonists (Compton et al. 2004) which could be inadvertently present in the water samples and not detected by LC/MS. However, significantly lower BEQ values were not consistently observed in the VM7LucERβc9 bioassay ($0.001 < p \leq 1.000$, Figure 2, SI Table 5), suggesting a reason other than ERβ-selective antagonism from an unknown chemical. A consistent trend was observed in the LC/MS analysis and CALUX bioassays for slightly elevated E1 concentrations in the 20 ng/L E1 tanks cotreated with

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100 micro-g/L ATZ (p = 0.194, 0.098, and 0.027, for LC/MS, VM7Luc4E2 and VM7LucERβc9, respectively) but this trend was not observed in the 20 ng/E1 tanks co-treated with 10 micro-g/L ATZ (p = 0.194, 1.000, and 1,000, respectively) or the 200 ng/L E1 tanks co-treated with 100 micro-g/ATZ (p = 0.630, 0.741, and 0.902, respectively).

Estrone levels in the spiked process control, as well as the E1 reference check, which was used to generate the spiked process control, were slightly but significantly elevated ($p < 0.001$) in LC/MS analysis when compared to BEQ values generated by CALUX analyses, indicating consistency within each method (Figure 2). Average E1 concentration in the reference check and spiked process control (nominal concentrations 10 ng E1/mL and 100 ng/L E1, respectively) was 14 ± 0.29 ng E1/mL and 120 ± 8.7 ng/L E1, respectively, as determined by LC/MS and 10 ± 0.3 ng E1/mL and 100 ± 7.3 ng/L E1, respectively, in the form of BEQs as determined by the VM7Luc4E2 bioassay (SI Table 5).

The logic to dilute extracts with nominal concentrations 10-20 ng E1/mL for CALUX analyses was supported based on the relatively narrow dynamic range of the CALUX assays and approximate upper bend-points mentioned above. In the case of the unspiked (blank) process control and control tanks (nominal concentration 0 ng/L E1), a portion of those samples (9% and 14-15% in VM7Luc4E2 and VM7LucERβc9 cells, respectively) were below the LOQ and could not be quantitated (see associated data release, https://doi.org/10.5066/P98PC526 (Brennan 2022)).

To determine whether the LC/MS and CALUX bioassays were consistent in the ability to determine subtle differences in smaller data sets, E1 levels in samples collected

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from dilutor tanks prior to adding fish were compared to those after fish were added. Addition of juvenile largemouth bass to dilutor tanks decreased E1 levels in tanks dosed with 200 ng/L E1 according to LC/MS and CALUX analyses with significant differences noted in the LC/MS and VM7Luc4E2 analyses ($p = 0.002$, 0.004, and 0.021 for LC/MS, VM7Luc4E2, and VM7LucERβc9, respectively); whereas no significant E1 decrease was observed by LC/MS, VM7Luc4E2, or VM7LucERβc9 following fish addition in 200 ng/L E1 tanks co-treated with ATZ ($p = 0.717$, 0.422, and 0.693, respectively, SI Figure 2). These results demonstrate the consistency between and sensitivity of the LC/MS and CALUX analyses.

We successfully quantitated the response of a single-concentration water sample using the CALUX bioassays with less effort than an alternative approach requiring dilution series of both the sample and reference chemical (Neale, Leusch, and Escher 2021). The latter approach is appropriate when the active substance is unknown and may not exhibit a parallel dose-response relationship to the reference chemical or in cases where there are multiple active substances where antagonism or synergism is possible.

CONCLUSIONS

We determined that both CALUX cell bioassays are capable of quantifying estrogen agonists in dilutor water extracts with comparable accuracy to that of QTOF LC-MS. These findings support use of the estrogen CALUX bioassays for quantifying laboratory exposures to a single estrogenic component and as a complementary tool to analytical chemistry. In addition, cell bioassays possess the unique ability to evaluate agonistic, antagonistic, or cytotoxic effects of chemicals and chemical mixtures on biological systems. Quantitation via the VM7Luc4E2 and VM7LucERβc9 cell lines is a

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feasible alternative for simple laboratory samples containing a single estrogenic component without the expense and expertise required for LC-MS instrumentation. However, in more complex matrices such as environmental (field) samples, LC-MS has the advantage of being able to individually quantify multiple estrogens within the same sample. The complementary uses of CALUX and LC-MS are evident in studies using effects directed analysis workflows or those aiming to increase efficiency by screening for sample activity prior to chemical identification and quantitative testing.

Supporting Information—The Supporting Information are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

Author Contributions

Jennifer Brennan: Conceptualization; Data curation; Formal analysis; Methodology; Project administration; Supervision; Validation; Visualization; Writing–original draft; Writing–review & editing. **Abigail Henke:** Data curation; Formal analysis; Investigation; Methodology; Visualization; Writing–original draft; Writing–review & editing. **Robert Gale:** Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Resources; Software; Validation; Visualization; Writing–original draft; Writing–review & editing. **Diane Nicks:** Data curation; Formal analysis; Investigation; Methodology; Resources; Supervision. **Donald Tillitt:** Conceptualization; Funding acquisition; Methodology; Project administration; Resources; Supervision; Visualization; Writing–original draft; Writing–review & editing.

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Data Availability

Relevant data are within the article and the Supplemental File. Raw data can be found through the US Geological Survey and is publicly available at https://doi.org/10.5066/P98PC526.

Competing Interests

The authors declare no conflicts of interest.

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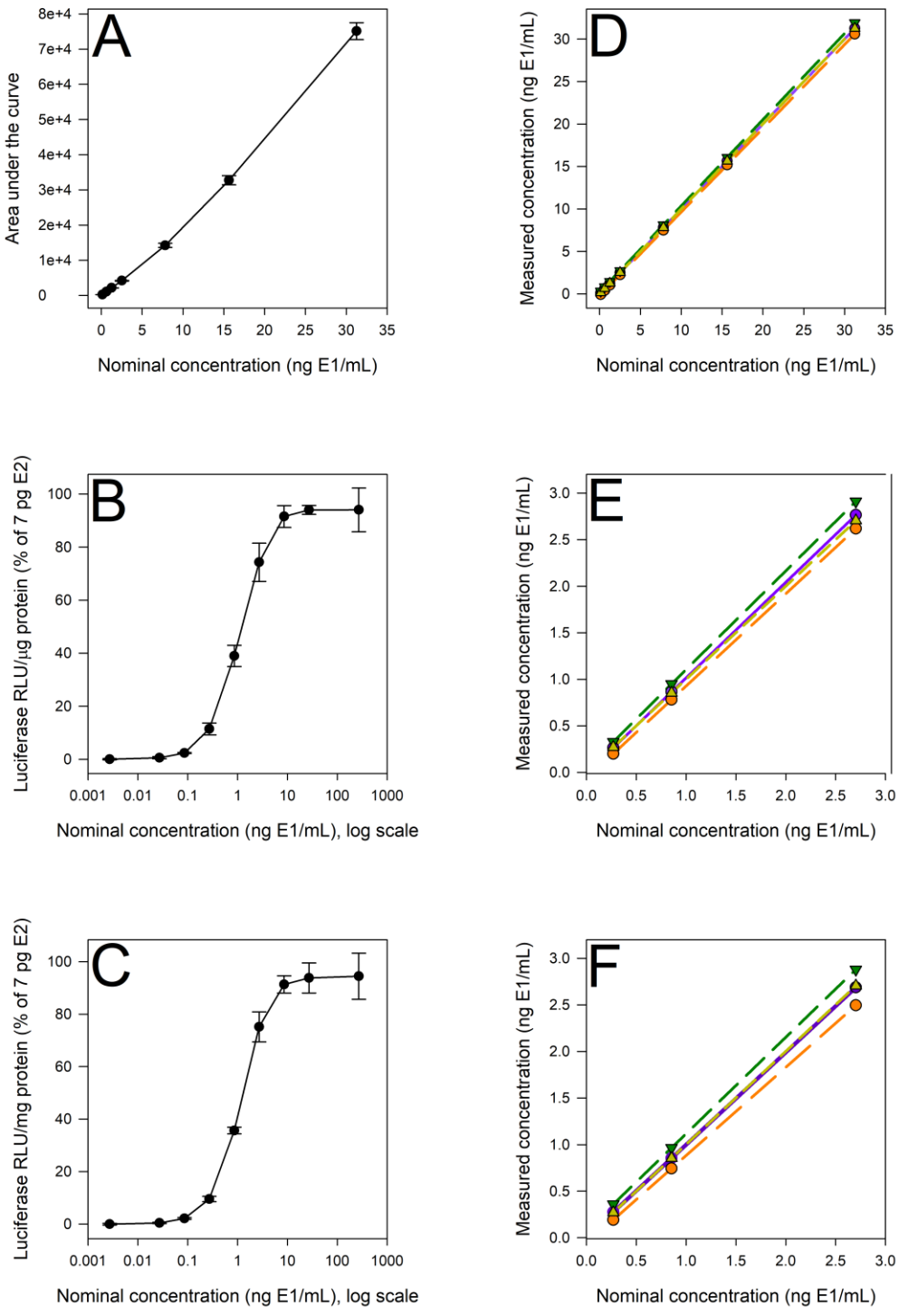

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Figure 1. (A-C) Representative estrone calibration curves for QTOF-LC/MS (A), VM7Luc4E2 cells (B), and VM7LucERβc9 cells (C), and (D-F) linear fit of measured E1 concentrations within the dynamic range to nominal concentrations (solid purple line), including lower 95% confidence interval (orange dashed lined) and upper 95% confidence intervals (green dashed line) in QTOF-LC/MS (D), VM7Luc4E2 cells (E), and VM7LucERβc9 cells (F). A 1:1 regression (measured/nominal = 1) in panels D-F is represented by a yellow dashed line. Results in panels D-F were compiled from calibration curves across multiple experiments (12 independent experiments for QTOF-LC/MS and 23 independent experiments for CALUX bioassays) and include concentrations within the linear portion of the calibration curves. Concentrations below LOQ or within nonlinear portions of the calibration curves shown in A-C were not included in the regressions shown in D-F. Data points in (A) represent the area under the curve and indicate the average ± standard error of 2-5 replicate injections and in (B) and (C) represent luciferase relative light units (normalized to protein) expressed as a percent of maximal activity induced by E2 and indicate the average ± standard error of 4 replicate wells.

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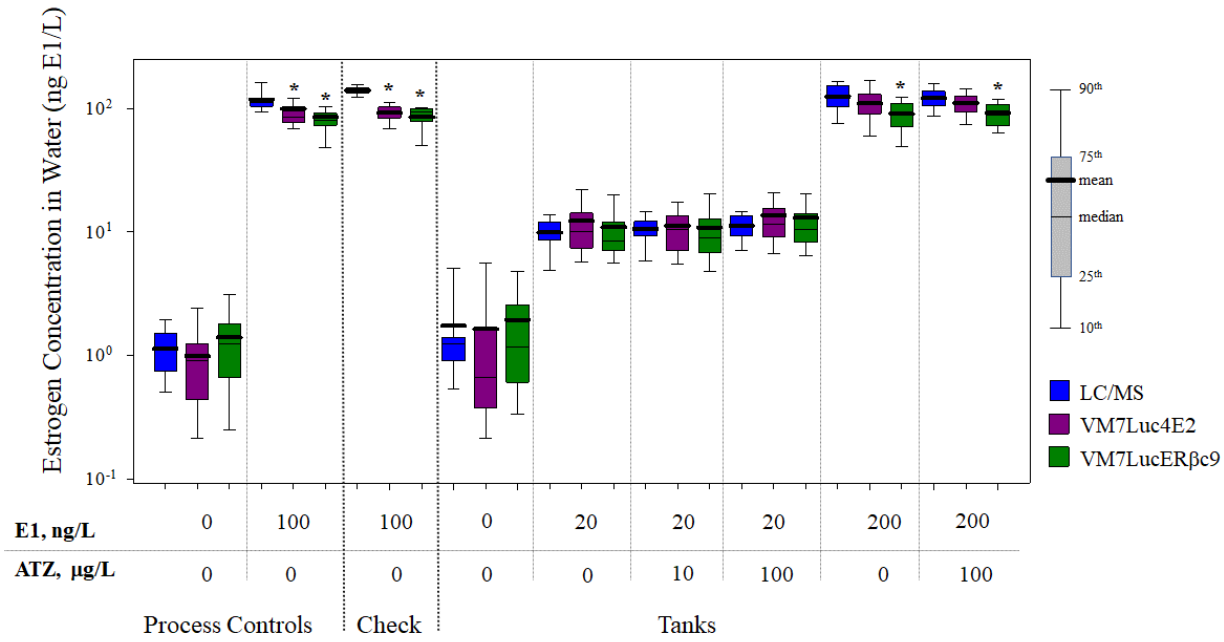

Figure 2. Estrone levels determined by LC/MS or bioanalytical equivalents (BEQ, ng E1/L) determined by CALUX (VM7Luc4E2 and VM7LucERβc9) bioassays in water samples (process control [n = 17] and tanks [n = 31-32]) and reference check[a] samples (n = 14). The lower and upper whiskers of the boxplot correspond to the 10th and 90th percentiles, respectively, while the interquartile range between the 25th and 75th quartiles is denoted by the colored boxplot body. The mean and median for each sample type is represented by the bolded and un-bolded horizontal lines, respectively. Significant difference ($\alpha \leq 0.01$) between LC/MS-determined E1 concentration and CALUX-determined BEQ for a given sample is denoted by an asterisk*.

[a] Nominal concentration for the reference check (10 ng E1/mL standard) is represented as 1 mL of standard added to 99 mL water.

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Table 1. Performance metrics of the CALUX bioassays relative to QTOF-LC/MS

System	LOD (ng E1/mL) [a]	LOQ (ng E1/mL) [a]	r^2 [b]	S/N [c]	CV (%) [d]	EC_{50} (ng E1/mL)	Z′ [d]
VM7Luc4E2	0.046 ± 0.0048 [e]	0.089 ± 0.0085 [e]	0.992-1.000	10.2 ± 1.2	6.34 ± 0.22	1.70 ± 0.12	0.62 ± 0.05
VM7LucERβc9	0.083 ± 0.016 [f]	0.19 ± 0.038	0.989-1.000	5.9 ± 0.9	6.78 ± 0.17	1.48 ± 0.10	0.63 ± 0.03
LC/MS	0.05	0.125	0.975-0.998	13-30	--	--	--

[a] The limit of detection (LOD) for E1 was determined as three times the standard deviation of the blank (CALUX assays) or of lowest-level calibration standard (LC/MS), and limit of quantitation (LOQ) was determined as 10 times the standard deviation of the blank (CALUX assays) or was determined as the lowest-level calibration standard (LC/MS).

[b] Coefficient of determination (r^2) was determined over the 0.00270-270 ng/mL and 0.125-250 ng/mL E1 calibration ranges for CALUX and LC/MS, respectively.

[c] Signal to noise (S/N) was based on the lowest E1 calibration standard (0.125 ng/mL) in LC/MS analyses and on a comparable E1 standard (0.270 ng/mL) in CALUX analyses.

[d] Coefficient of variance (CV, %) is expressed as a percentage of the standard deviation of the activity induced by treatment divided by the square root of analytical replicates further divided by the average activity induced by treatment; and Z′ factor (Z′) is defined in the methods with E2 (7 pg) and DMSO as the positive and negative controls, respectively.

[e] Values shown in the table above for VM7Luc4E2 cells represent the average ± standard error of 24 independent experiments. In certain instances (noted), LOD and LOQ could not be calculated, and values represent the average ± standard error of 23 independent experiments.

[f] Values shown in the table above for VM7LucERβc9 cells represent the average ± standard error of 26 independent experiments. In certain instances (noted), LOD could not be calculated, and values represent the average ± standard error of 22 independent experiments.

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