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Gas chromatographic determination of catecholestrogens following isolation by solid-phase extraction

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Abstract

A sensitive and specific assay for the determination of the catecholestrogens 2-hydroxyestradiol (2-OHE2) and 4-hydroxyestradiol (4-OHE2) using gas chromatography with electron-capture detection (GC–ECD) is described. The formation of 2- and 4-OHE2 was assessed following activation of 17β-estradiol in the microsomal fraction of female rat livers. The analytes were isolated by solid-phase extraction, derivatized to their heptafluorobutyryl esters with heptafluorobutyric acid anhydride, and subjected to solvent exchange prior to analysis; this resulted in minimal chromatographic interference, long column life, and stable derivatized analytes. Derivatized catechols were separated and confirmed with dual column chromatography (DB-5 and DB-608) and quantitated using GC–ECD. The DB-608 column was preferred for quantitation as it provided better 4-OHE2 resolution from interference. Key validation parameters for the assay include sensitivity, intra- and inter-assay precision, and accuracy. Instrument sensitivity and limits of detection (LOD) and quantitation (LOQ) were determined statistically from fortification data approaching expected limits. For 2-OHE2 and 4-OHE2, respective values for these parameters were; instrument sensitivities of 0.4 and 0.7 pg, LODs of 0.8 and 1.3 ng/mg, and LOQs of 2.6 and 4.3 ng/mg.

Keywords: Catecholestrogens

1. Introduction

Catecholestrogen formation is a major route of metabolism of endogenous as well as exogenous estrogens [1,2]. The cytochrome P450-mediated aromatic hydroxylation of estrogens results in the formation of the catecholestrogens 2-hydroxyestradiol (2-OHE2) and 4-hydroxyestradiol (4-OHE2) (Fig. 1) [3]. The liver is the primary site of catecholestrogen formation but a number of other sites of metabolism exist including brain [4–6], kidney [4,7], and breast [8–10]. In addition to their physiological roles, these biologically active metabolites possess unique properties and have been impli-

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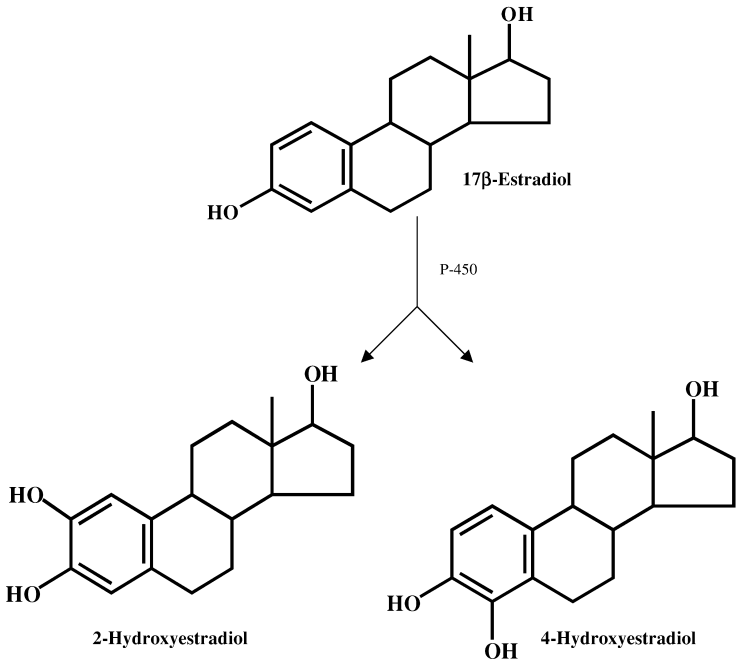

Fig. 1. Formation of catecholestrogens from 17β-estradiol. 17β-Estradiol undergoes cytochrome P-450-catalyzed oxidation to form the catecholestrogens, 2-OHE2 and 4-OHE2.

cated in pathological processes, including carcinogenesis [11–13].

The catecholestrogens are biologically and chemically labile molecules that may spontaneously autooxidize, dimerize, or bind to macromolecules [1,14]. Conventional methods of analysis for catecholestrogens include both indirect [15–18] and direct product isolation techniques [19–24]. Indirect methods can circumvent many of the problems associated with the labile nature of these compounds. However, such methods have been shown to generate erroneous catechol levels [25]. Direct product isolation methods have gained favor due to their increased sensitivity, versatility, and relative simplicity.

To this end, this report describes a new chemical assay for the direct isolation and analysis of catecholestrogens generated through microsomal activation of 17β-estradiol (E2) using solid-phase extraction (SPE), microchemical synthesis (derivatization), and solvent exchange prior to analysis by gas chromatography with electron-capture detection (GC–ECD). Validation of this method was accomplished by determining assay sensitivity, precision, and accuracy.

2. Experimental

2.1. Chemicals

17β-Estradiol (E2), 2-hydroxyestradiol (2-OHE2), 4-hydroxyestradiol (4-OHE2), NADPH, and L-ascorbic acid were obtained from Sigma (St. Louis, MO, USA). 17β-Estradiol had purity no less than 98.0%, while both catecholestrogens had purities of no less than 95.0%. The surrogate, 4-bromoestradiol (4-BrE2), was obtained from Steraloids (Wilton, NH, USA), and had a minimum purity of 98.0%. Heptafluorobutyric acid anhydride (HFAA) and anhydrous triethylamine (TEA), used as derivatizing agent and catalyst, respectively, were obtained from Pierce Chemical (Rockford, IL, USA). Reagent-grade anhydrous sodium sulfate (Na_2SO_4) was obtained from Mallinckrodt (Paris, KY, USA). All reagents

used in the assay were reagent grade or better and all organic solvents were pesticide grade or better.

2.2. Microsomal preparation

These studies were carried out using tissues provided to us by the National Toxicology Program (http://ntp-server.niehs.nih.gov) as part of an ongoing series of chronic 2-year rat bioassays examining the relative potencies for carcinogenicity of individual, and mixtures, of dioxin-like compounds. Female, Sprague Dawley rats (Harlan Sprague–Dawley®, Indianapolis, IN, USA) receiving corn oil with 1% acetone vehicle from the control group of an ongoing study were used. Eight-week-old rats were administered vehicle via oral gavage 5 days per week for 13 weeks and sacrificed at the end of the period. Livers (1 mg) were homogenized in 2 ml of 250 m*M* sucrose and 0.1 m*M* EDTA, pH 7.4. The microsomal subfraction was prepared by centrifugation at 150 000 *g* and the pellet washed once with a 20 m*M* potassium phosphate buffer, pH 7.4, containing 10 m*M* EDTA. The pellet was resuspended and stored in 20 m*M* Tris containing 1 m*M* EDTA, and 20% (v/v) glycerol, pH 7.4. Microsomal protein concentrations were determined by a commercially available assay (Micro BCA Protein Assay, Pierce, Rockford, IL, USA) and microsomes subsequently stored at −80°C. Microsomes were stored up to 1 year without appreciable loss in activity. Therefore, frozen microsomes were used in these studies.

2.3. Incubation conditions

The incubation conditions were adapted from those previously outlined by Roy et al. [19] with some modification. The incubation mixture consisted of 1 mg/ml pooled microsomal protein and 50 m*M* Tris, pH 7.5, containing 2 m*M* L-ascorbic acid, 5 m*M* NADPH, and 50 μ*M* E2 (in ethanol) in a final volume of 250 μl. Samples were incubated for 10 min at 37°C in an American Optical Shaker Bath (Chicago, IL, USA) set at 60 oscillations per minute. The incubation reaction was quenched by immediately placing samples on ice (4°C), followed by the addition of 1 ml ice cold (4°C) Tris, pH 3.0, containing 2 m*M* L-ascorbic acid and 50 ng 4-BrE2. Spiked samples were prepared as above, except in the absence of NADPH, whereas control blanks were prepared in the absence of substrate.

2.4. Solid-phase extraction

Analytes were isolated by SPE using Isolute® Phenyl, end-capped cartridges (Jones Chromatography, Lakewood, CO, USA). A Gast® vacuum pump (St. Louis, MO, USA) was used to maintain a flow-rate of 1–2 drops per second (i.e., approximately 2–4 ml/min). The solid-phase was conditioned with 1 ml of Pesticide Grade® methanol (Mallinckrodt, Paris, KY, USA) followed by 1 ml of pH 3.0, 50 m*M* Tris containing 2 m*M* ascorbate and 0.1% (v/v) methanol. The quenched sample was loaded onto the solid-phase followed by an additional 1 ml of the acidified buffer solution, described above, which was used to rinse the sample tube for quantitative transfer. The cartridge was then rinsed with 20% (v/v) acetone in de-ionized water. Each cartridge was aspirated on an N-Evap® nitrogen evaporator (Organomation Associates, Berlin, MA, USA) under positive pressure (i.e., approximately 20 p.s.i.; 1 p.s.i.=6894.76 Pa) for 10 min. Dried cartridges were then positioned in the vacuum manifold to drain into solvent-rinsed 15 ml Kimax® (Chicago, IL, USA) conical centrifuge tubes. The cartridges were eluted with 1 ml of Nanograde® acetone (Mallinckrodt, Paris, KY, USA) at a flow-rate of 1–2 drops per second. The extracts were then concentrated under nitrogen to 0.5 ml.

2.5. Derivatization and cleanup

The concentrated extracts received 100 μl of 100 m*M* TEA in Nanograde® acetonitrile (Mallinckrodt, Paris, KY, USA) followed by the addition of 50 μl of HFAA. Tubes were capped tightly and agitated lightly after each addition. All vessels containing derivatization reagents were then placed into a Pierce Reacti-Therm™ heating block (Rockford, IL, USA) with machined holes containing approximately 0.5 cm of sea sand to distribute heat evenly. The heating block was maintained at 30°C, and samples were incubated for 15 min. Derivatization was ceased by the addition of 10 ml of a 2% solution of Na_2SO_4, and then mixed at 20 rpm for 1 min on a

Fisher Rotorack® rotary mixer (Fisher Scientific, Pittsburgh, PA, USA). The analytes of interest were extracted from the aqueous phase with three, 2 ml Nanograde® *n*-hexane (Mallinckrodt, Paris, KY, USA) extractions. The hexane extracts were concentrated using a N-Evap® nitrogen evaporator to a final volume of 0.5 ml and a 1-μl aliquot was injected onto the GC.

2.6. Gas chromatography

Samples were analyzed using a Hewlett-Packard 5890 Series II® Gas Chromatograph equipped with two ^{63}Ni pulsed electron capture detectors, and interfaced to HPChem® Instrument Software for integration and sample analysis. A two-column confirmation was utilized using two unique phase, bonded fused-silica columns: DB-5 (30 m×0.25 mm×0.25 μm) and DB-608 (30 m×0.32 mm×0.5 μm) (J&W Scientific, Rancho Cordova, CA, USA). The DB-608 column was selected as the primary column due to superior separation of the analytes (Figs. 2 and 3). The GC conditions were: 13.5 and 5.5 p.s.i. nitrogen carrier gas pressure for DB-5 and DB-608, respectively, splitless injection, 270°C inlet temperature, 320°C detector temperature, and 100°C initial oven temperature. The temperature program used a 1-min hold period followed by a 30°C/min temperature ramp to 245°C, and a 10-min hold period. A second temperature gradient of 2°C/min from 245 to 265°C was followed by a 4-min hold period and a final 30°C/min temperature ramp to 270°C. A final hold period of 8 min followed.

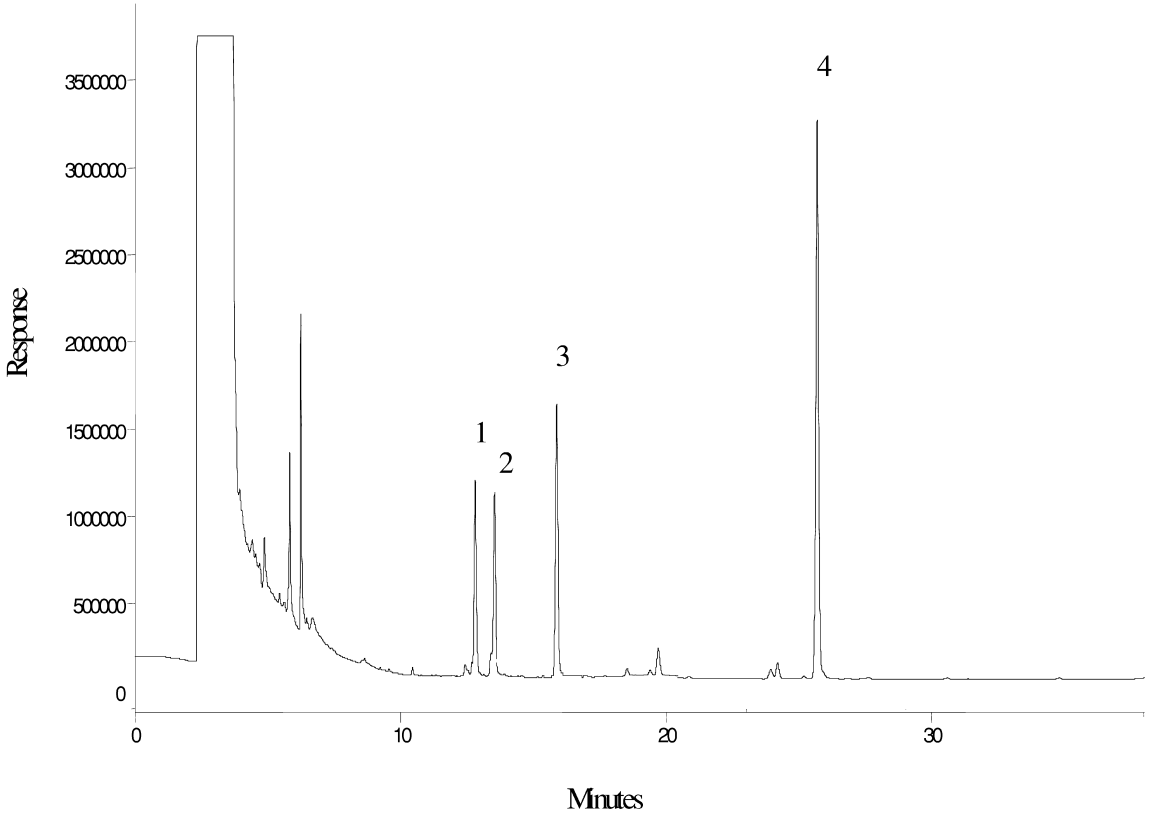

Fig. 2. The separation of estrogens by capillary gas chromatography. The DB-608 separation of 200 ng/ml standards of 2-OHE2 (1), 4-OHE2 (2), E2 (3) and the surrogate, 800 ng/ml 4-BrE2 (4) following derivatization and analysis by GC–ECD.

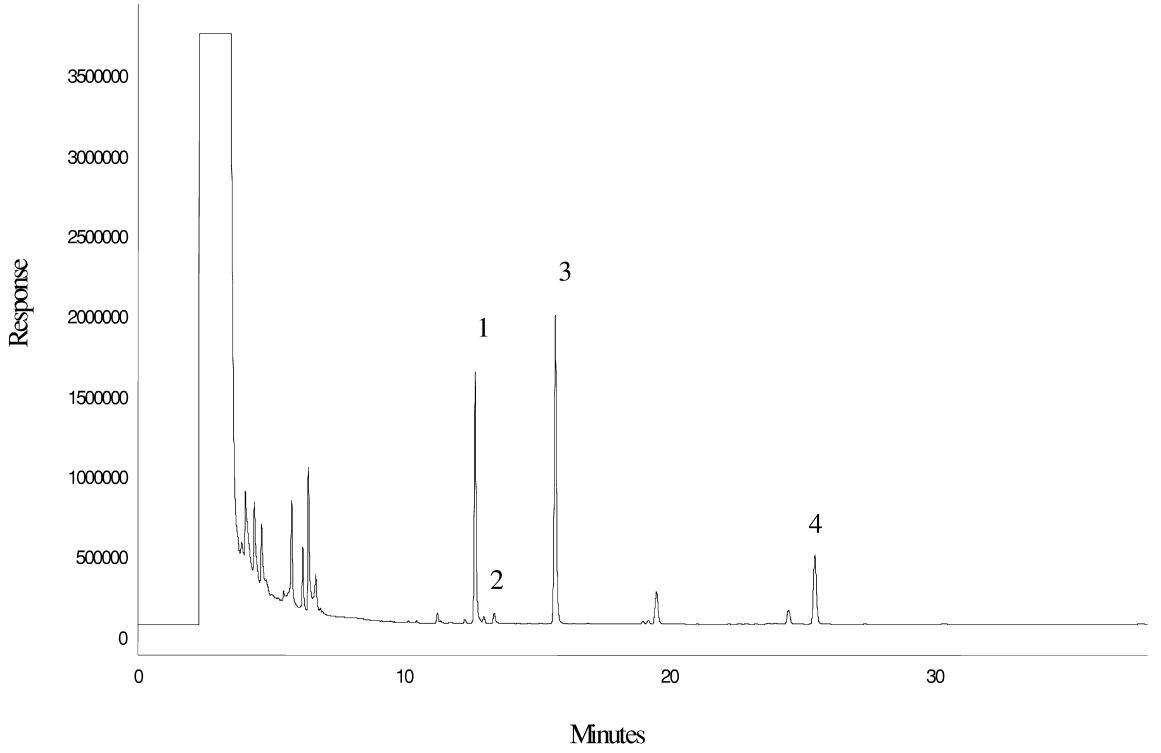

Fig. 3. DB-608 separation of estrogens by capillary gas chromatography following incubation with microsomes and NADPH, SPE, derivatization, and analysis by GC–ECD. The concentrations and elution order is 300 ng/ml 2-OHE2 (1), 40 ng/ml 4-OHE2 (2), 300 ng/ml E2 (3) and 100 ng/ml 4-BrE2 (4).

3. Results

3.1. Assay characteristics

3.1.1. Chromatography

Standards of E2, 2-OHE2, 4-OHE2, and the surrogate, 4-BrE2, were derivatized and their heptafluorobutyryl derivatives effectively separated by capillary gas chromatography (Fig. 2). Separation of the analytes was achieved on both the DB-5 (not shown) and DB-608 columns. However, some interference with 4-OHE2 was seen on the DB-5 column. This interference was not observed in the E2-free blanks, NADPH-free spikes, or the standards, but it was apparent in samples undergoing microsomal activation of E2. For this reason the DB-608 was chosen as the primary column and the DB-5 column served as the secondary, confirmation column. Chromatographic separation of catecholestrogens generated by microsomal activation of E2 is shown in Fig. 3. Under the outlined chromatographic conditions the retention times of 2-OHE2, 4-OHE2, E2, and 4-BrE2 were 13.4, 14.2, 16.7, and 26.8 min, respectively.

The method sensitivity, and limits of detection (LOD) and quantitation (LOQ) were established for this method using limit calculations as previously described [26,27]. Method sensitivity refers to the amount of analyte, in the presence of matrix, that can be distinguished from the baseline noise. Limit of detection is defined as the lowest concentration of analyte that can be detected but not necessarily quantitated, and the LOQ is defined as the lowest concentration of analyte that can be detected with sufficient accuracy and precision under the standard operational conditions of the method. Injection of

matrix-fortified standards (i.e., spikes) ranging in concentration from 1.6 to 60 ng/mg was used to construct regression curves from which limit calculations were performed. Method sensitivities were established for 2-OHE2 and 4-OHE2 at 0.4 and 0.7 pg. The LODs for 2-OHE2 and 4-OHE2 were 0.8 and 1.3 ng/mg, respectively. The corresponding LOQs for 2-OHE2 and 4-OHE2 were 2.6 and 4.3 ng/mg, respectively.

3.2. Linear regression analysis

17β-Estradiol, 2-OHE2 and 4-OHE2 were spiked into a NADPH-free incubation mixture containing 1 mg/ml of microsomes, then, processed and analyzed as previously outlined. Three-point standard curves were constructed for each of the analytes: E2 curves were constructed from 500 to 1500 ng/ml, 2-OHE2 from 100 to 800 ng/ml, and 4-OHE2 from 10 to 100 ng/ml. The dose–response curves for each analyte were linear (i.e., in the form $y = mx + b$) over relatively wide ranges and the *y*-intercepts were close to the origin. The linear regression parameters, calculated from peak areas, are presented in Table 1.

3.3. Precision and accuracy

Inter-assay variation was evaluated first by examining the reproducibility of the derivatization reaction, and second, by measuring the extraction efficiencies and accuracies of the overall assay. Intra-assay variation was determined by measuring the repeatability of the detector response following repeated injections of a single standard containing E2, 2-OHE2, and 4-OHE2. In all cases, precision was expressed in terms of relative standard deviation (RSD).

Table 1
Linear regression parameters calculated from standard curves of E2, 2-OHE2, and 4-OHE2 in the presence of microsomal protein

Analyte	Slope[a]	*y*-Intercept	R^2
E2	19726±494	23931±6995	0.99
2-OHE2	22282±614	35173±8686	0.99
4-OHE2	17912±570	27801±8070	0.98

[a] Both the slope and the *y*-intercept are expressed as the mean±standard deviation of six individual derivatizations within the same assay, $n = 18$.

Table 2
Reproducibility of the derivatization reaction for E2, 2-OHE2, and 4-OHE2

Analyte	Slope[a]	Relative standard deviation (%)	R^2
E2	6987±638	9.1	0.90
2-OHE2	7417±672	9.1	0.90
4-OHE2	5731±574	10.0	0.89

[a] The slope is expressed as the mean±SD of five separate assays, $n = 15$.

Conversion of the estrogens to their heptafluorobutyryl derivatives was relatively rapid (i.e., 15 min) and required minimal sample manipulation. To determine inter-assay variation of the derivatization reaction five separate assays were performed. In each assay multiple levels of E2, 2-OHE2, and 4-OHE2 underwent derivatization and were analyzed by GC–ECD under the conditions previously outlined. The variability in the slope of the mean linear regression equation of the derivatized estrogens was evaluated and its mean, standard deviation, and RSD determined (Table 2). These values were indicative of low variability among successive derivatizations.

Extraction efficiencies were determined for each of the analytes by evaluating the quantitative recovery of a single level spike of each. The spikes were prepared as outlined previously in the presence of microsomes and processed accordingly. The recoveries of the catechols and E2 were maintained above 90% and all of the corresponding accuracies of determination (i.e., the mean percent deviation of concentrations from the mean theoretical value) were determined to be less than 20% (Table 3). Intra-assay variability was also evaluated and was found to be low for all estrogens sought (Table 4).

4. Discussion

The difficulties realized in the analysis of estrogens are primarily due to the low concentrations of estrogens in biological matrices and the labile nature of these compounds. Isolation of catecholestrogens, and other estrogens, by SPE minimizes

Table 3
Extraction efficiency and accuracy of measurements of E2, 2- and 4-OHE2, and 4-BrE2 in the presence of microsomes

Analyte	Amount fortified[a] (ng/mg)	Amount recovered[b] (%)	Relative standard deviation (%)	Accuracy[c] (%)
E2	600	94.1±11.9	12.7	13.4
2-OHE2	600	98.8±8.8	8.9	8.9
4-OHE2	600	93.3±10.3	11.1	13.4
4-BrE2	600	97.7±12.5	14.4	18.3

[a] Expressed as nanograms of analyte per milligram of microsomal protein.
[b] Mean recovery±SD of $n=16$.
[c] Mean percent deviation of the concentrations from the theoretical, fortified amount.

sample handling and hence, exposure of these labile metabolites to conditions that favor their oxidation. Phenyl solid-phase media was chosen over C_{18}, C_2, florisil and silica based on superior removal of matrix interference that allowed acceptable, reproducible recovery of analytes. C_{18} media allowed coelution of matrix interference with analytes, while C_2, florisil and silica reduced recoveries by retaining analytes too strongly. In addition, the phenyl SPE system allowed for the use of low solvent volumes and elution by a solvent with a relatively low boiling point, making the SPE and subsequent evaporation steps quick and efficient.

Instrument sensitivity and limits of detection for the catecholestrogen chemical assay are representative of a sensitive assay that can detect catecholestrogens, and other estrogens, in the low picogram range, as demonstrated previously [19]. The LODs and LOQs for both catecholestrogen analytes were well below the concentrations found following hepatic microsomal activation of E2. Tissues such as liver, with a high metabolic potential, provide a sample matrix where catechols may be generated in higher amounts relative to other tissues. Consequently, such sensitive results provide a basis for detecting and quantitating catecholestrogens directly in tissues with comparatively low metabolic potential, such as breast and brain tissue.

Table 4
Intra-assay variation evaluated by assessing the repeatability of multiple analyses of the same standard preparation

Analyte	Slope[a]	Relative	R^2
E2	11317±595	5.3	0.93
2-OHE2	10747±749	6.3	0.90
4-OHE2	8747±607	6.9	0.89

[a] Slope is the mean of six replicate analyses.

Chromatography of HFAA derivatives is generally troublesome due to the presence of the relatively involatile excesses of derivatizing agent and other reaction by-products [28]. Excess HFAA leads to high levels of background noise, column contamination, and premature degradation of the stationary phase, and ultimately the ECD. Liquid–liquid extraction of excess HFAA and associated by-products into 2% sodium sulfate provides an efficient means of clean-up of derivatizing agent, providing for improved chromatography and longer column life. Concomitantly, back-extraction of the derivatized analytes into hexane affords an improved solvent system, increasing the stability of the analytes from a matter of hours [19] to one of weeks (data not shown).

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