

1 Uptake and degradation of natural and
2 synthetic estrogens by maize seedlings

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10 **Abstract**

11 Runoff from manure-fertilized crop fields constitutes a significant source of natural estrogens
12 (e.g., estradiol [E2] and estrone [E1]) and synthetic estrogen mimics (e.g., zeranol [α -ZAL] and
13 zearalanone [ZAN]) in the environment. However, processes such as sorption to and uptake by
14 plants may inhibit the environmental mobility of hormonally-active compounds. To evaluate the
15 role of plants in the environmental fate of such compounds, we exposed maize seedlings to
16 hydroponic solutions containing E2, E1, α -ZAL, and ZAN. After 12 days of exposure, ZAN
17 concentrations decreased by 78%, E1 and α -ZAL decreased 99.9%, and E2 was undetectable.

1 Exposure to seedlings resulted in both oxidation (i.e., transformations of E2 to E1 and α -ZAL to
2 ZAN) and reduction reactions (i.e., E1 to E2 and ZAN to α -ZAL). Although the oxidation of E2
3 and α -ZAL may be attributed to plant-associated microbes, the reduction of E1 and ZAN is
4 unlikely to be microbially-mediated.

5 **Introduction**

6 It is estimated that two-thirds of beef cattle raised in the U.S. are given hormonally active growth
7 promoters to accelerate muscle gain and increase feed efficiency (1). Livestock growth
8 promoters approved for use in the U.S. include both natural hormones and synthetic mimics (2).
9 Hormonal growth promoters and endogenous hormones are excreted in livestock wastes and may
10 enter the environment via pathways such as spillover from waste lagoons and runoff from
11 manure-fertilized crop fields. These hormonally active compounds may have significant
12 deleterious effects in the environment; for example, estrogens can feminize male fish and reduce
13 fertility and fecundity in wild fish populations at concentrations as low as 10 ng L⁻¹ (3).

14 Among the growth promoters that are approved for use in the U.S. are a natural estrogen, 17 β -
15 estradiol (E2), and its synthetic mimic, zeranol (α -zearalanol, α -ZAL). In mammals, the major
16 metabolites of E2 and α -ZAL are estrone (E1) and zearalanone (ZAN; Fig. 1), respectively (4, 5).
17 Because they are endogenous hormones, E2 and E1 are present in the excreta of all livestock,
18 regardless of whether the animals are treated with E2. α -ZAL and ZAN have been detected in
19 the wastes of animals treated with α -ZAL (e.g., 5, 6). E2, E1, and α -ZAL have been detected in
20 surface waters impacted by livestock agriculture (e.g. 7-9) and E2 has been measured in
21 agricultural runoff at concentrations high enough to impact aquatic organisms three months after
22 application of manure fertilizer (7).

1 The environmental mobility and fate of manure-borne estrogens can be attenuated by processes
2 such as soil sorption (10) and transformation by plants and soil- and plant-associated microbes
3 (11). Field studies have demonstrated that grass buffer strips reduce the concentration of E2 in
4 agricultural runoff (12). Laboratory studies have shown that algae and aquatic macrophytes can
5 reversibly transform the endogenous estrogens E2 and E1 (13, 14). However, previous studies
6 have not addressed the role that crop seedlings, the first plants with which manure-borne
7 contaminants interact, may play in the environmental fate of natural and synthetic estrogens.

8 The aim of this work was to evaluate the role that maize seedlings serve in the environmental
9 fate of E2, E1, α -ZAL, and ZAN. Specifically, we measured the rate at which maize seedlings
10 take up estrogens from hydroponic solutions and identified the transformation of estrogens in the
11 hydroponic solution. These results will contribute to a complete understanding of the
12 environmental fate of manure-borne estrogens and estrogen-mimicking compounds.

13

14 **Experimental Methods and Materials**

15 **Chemicals.** E2 (98%), E1 (99%), zearalanone (98%), and *N, O*-Bis(trimethylsilyl)trifluoro-
16 acetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) were obtained from Sigma-Aldrich,
17 Inc. (St. Louis, MO). α -ZAL was extracted and purified from Ralgro Magnum (Schering-Plough
18 Animal Health Corp., Union, NJ) (10). Other chemicals, including HPLC solvents, were
19 purchased from Fisher Scientific (Pittsburgh, PA).

20 **Hydroponic uptake experiments.** Uptake of E2, E1, α -ZAL, and ZAN was evaluated with
21 maize seedlings (*Zea mays*, Golden Cross Bantam [Hybrid]; Ferry-Morse Seed Co, Fulton, KY).
22 Seeds were surface-sterilized by a 5 min soak and two rinses in Milli-Q water (Millipore,
23 Billerica, MA), 1 min soak in hydrogen peroxide (30%), and three rinses in Milli-Q water. The

1 seeds were then placed onto dampened filter paper in glass Petri dishes which had been sterilized
2 by autoclave (Tuttnauer USA, Hauppauge, NY), and germinated in the dark for 4 d.

3 Uptake experiments were conducted by growing maize seedlings in hydroponic solutions of
4 estrogens. 2 μM solutions of target estrogens were prepared in $\frac{1}{2}$ -strength Hoagland's nutrient
5 solution (pH 6.8) (15). Autoclaved 9 mL glass vials were filled with estrogen or control
6 solutions and capped with plastic open-top caps containing Teflon septa (National Scientific,
7 Rockwood, TN) with 3.5 mm holes. One seedling was then placed into the hole in each cap such
8 that the roots were in the solution and the kernel was situated on top of the septum. There were
9 four replicates per treatment, including blank controls containing no estrogens and glassware
10 controls containing no plant. All vials were wrapped in foil, with the cap and plant exposed, and
11 placed under plant growth lights (Ecolux Plant & Aquarium, GE, Louisville, KY) with a 16 h
12 photoperiod. To maintain water levels, vials were refilled daily with Milli-Q water.

13 Plants were destructively sampled at given time points. The aqueous phases were filtered
14 using Gelman type A/E glass fiber filters with 0.6 μm pore size (Pall Corp.) and collected for
15 analysis by reverse-phase high-pressure liquid chromatography (RP-HPLC).

16 **RP-HPLC analysis of estrogen concentrations.** Estrogen concentrations were quantified
17 using RP-HPLC (1515 isocratic pump and 717plus autosampler, Waters Corp., Milford, MA)
18 with UV-Vis detection (Waters 2487 dual λ absorbance detector) and a Waters Sunfire C18
19 column. Injection volumes were 150 μL and the mobile phase was 50:50 v/v acetonitrile:water
20 with 1 mL min^{-1} flow rate. Detection wavelengths and limits of quantification (LOQ) are shown
21 in Table 1.

22 **Transformation product identification.** Transformation products were qualitatively
23 identified using gas chromatography coupled with mass spectrometry (GC-MS). To prepare for

1 identification by GC-MS, five replicates of each treatment and control were prepared as
2 described above and destructively sampled after 1 d (E2 and E1), 3 d (α -ZAL), or 5 d (ZAN) of
3 exposure. Analytes from the filtered hydroponic solutions were concentrated onto Oasis HLB
4 Plus solid phase extraction (SPE) packs (Waters Corp.), which had been preconditioned with
5 methanol and Milli-Q water. The SPE packs were eluted with 2 mL methanol over 5 min. The
6 methanol was allowed to evaporate, and the residue was redissolved in 50 μ L hexane followed
7 by derivatization with 50 μ L BSTFA (1% TMCS) at 60° C for 45 min.

8 Derivatized solutions were then analyzed by GC-MS (HP 6890 series GC system and 5973
9 mass selective detector; Hewlett-Packard Company), with 1.5 μ L injections in splitless mode.
10 Chromatographic separation was conducted on a 15 m Restek RTX-5MS column (Restek
11 Corporation, Bellefonte, PA). The carrier gas was helium (99.999% purity) with a constant flow
12 of 1.2 mL min⁻¹. The inlet temperature was 260° C, interface was 250° C, MS source was 230°
13 C, and MS quad was 150° C. The GC oven was programmed to begin at 130° C (hold 1 min),
14 increase 20° C min⁻¹ to 230° C, and finally increase 5° C min⁻¹ to 300° C, for a total run time of
15 20 min. The MS detected ions in the range m/z 50-650 in scan mode. The major ion peaks of all
16 analytes under these conditions are shown in Table 1.

17

18 **Results and Discussion**

19 **Uptake from hydroponic media.** It was predicted that E2, E1, α -ZAL, or ZAN would be
20 bioavailable to plants because they are moderately hydrophobic (log K_{OW} values between 3 and
21 4) (11). Indeed, these natural and synthetic estrogens were rapidly removed from hydroponic
22 solutions exposed to maize seedlings (Fig. 2). After 12 d of exposure, E2 was undetectable, E1
23 and α -ZAL concentrations decreased 99.9%, and ZAN decreased 78%. Glassware controls

1 showed little loss of estrogens, and estrogens were not detected in any blank controls. Because
2 these uptake rates were measured with hydroponic solutions, they are anticipated to be higher
3 than similar uptake in natural systems where sorption to manure and soil particles will inhibit
4 uptake by plants. However, sorption will also inhibit the bioavailability to other organisms and
5 environmental mobility of these estrogens.

6 **Transformation of estrogens.** Transformation products were identified in all hydroponic
7 solutions following exposure to maize seedlings. The extracted ion GC-MS chromatograms of
8 plant-exposed and glassware control samples demonstrate that exposure to maize resulted in the
9 oxidation of E2 to E1 and α -ZAL to ZAN and the reduction of E1 to E2 and ZAN to α -ZAL (Fig.
10 3). The reversible transformation between E2 and E1 has previously been reported in algae (13)
11 and duckweed (14) but not in terrestrial plants. Although the concentrations at which
12 transformation products were produced varied among parent estrogens, there were similar
13 temporal patterns in product production between E2 and E1 and between α -ZAL and ZAN (Fig.
14 4).

15 E1 and E2 were produced at only low concentrations in plant-exposed solutions of E2 and E1,
16 respectively, and were quickly removed from the solutions. After 8 d, E1 was undetectable in E2
17 solutions and E2 was at a quantifiable concentration in only one of four E1 samples. Although
18 E2 was measured in E1 glassware controls, perhaps due to microbial contamination of the
19 hydroponic solution, E2 was produced more quickly and at greater concentrations in plant-
20 exposed samples. The transformation of E1 to E2 is important because E2 is a significantly
21 more potent estrogen than E1. In natural systems where uptake of estrogens by plants is
22 incomplete, plant metabolism of E1 may contribute to the E2 load, and therefore overall
23 estrogenicity, in runoff.

1 Although the initial concentrations of α -ZAL and ZAN were similar to those of E2 and E1, the
2 products of the synthetic estrogens were produced at much higher concentrations than those of
3 the natural estrogens. Further, the product concentrations increased more slowly in plant-
4 exposed α -ZAL and ZAN than in E2 and E1, and in the 8 d experiment showed no indication of
5 leveling off or decreasing. These data reflect the slower rates of α -ZAL and ZAN uptake by
6 maize seedlings.

7 ZAN is a very weak estrogen, with only approximately 13% the estrogenic activity of α -ZAL
8 (18). Thus, the conversion of α -ZAL to ZAN will decrease the overall estrogenicity of the
9 solution. However, the observed reduction of ZAN to α -ZAL does not necessarily indicate a
10 significant increase in estrogenic activity. In studies of mammalian degradation of the
11 zearalenone family of compounds, it has been found that ZAN is reduced to a racemic mixture of
12 α -ZAL and β -ZAL, where β -ZAL is only slightly more estrogenic than ZAN (approximately
13 19% the estrogenicity of α -ZAL) (19). We did not separate α -ZAL and β -ZAL, so it is possible
14 that the transformation product observed in maize-exposed ZAN solutions is, in fact, a racemic
15 mixture. As a product, the racemic mixture of α - and β -ZAL is only four times as estrogenic as
16 ZAN, as compared to α -ZAL, which is more than seven times as estrogenically active as ZAN.

17 The observed transformations of plant-exposed E2, E1, α -ZAL, and ZAN may be attributed to
18 metabolism by the maize seedlings or to plant-associated microbes. All equipment and
19 experimental components were carefully sterilized, so any microbes present in plant-exposed
20 solutions were likely endophytic or otherwise closely associated with the plants. Because these
21 plants were grown in soilless media, soil and rhizosphere microbes did not contribute to
22 transformations as they may in natural systems. Although mammals can reduce E1 to E2,
23 microbial processes can perform this transformation only under strongly reducing conditions

1 (20). Based on pathways known to occur in aerobic or slightly anaerobic conditions, microbial
2 oxidation of E2 to E1 and α -ZAL to ZAN can be predicted *a priori* while microbial reduction of
3 E1 to E2 and ZAN to α -ZAL cannot (21). Thus, our observations of alcohol-to-ketone
4 transformations in plant-exposed solutions may be caused (in whole or in part) by plant-
5 associated microbes, but it is *unlikely* that the reverse reactions are taking place via microbially-
6 mediated transformations.

7 These results demonstrate that maize plants may have a significant impact on the
8 environmental fate of natural and synthetic estrogens in manure fertilizer, by rapidly removing
9 estrogens from runoff and causing chemical transformations that do not occur by aerobic
10 microbial pathways. Given these findings, we suggest that crop plants may have unique effects
11 on the environmental fate of many pharmaceuticals and endogenous biomolecules present in
12 manure fertilizer, and these effects may be exploited to prevent environmental contamination
13 with such compounds.

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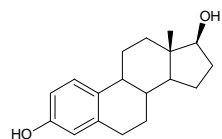
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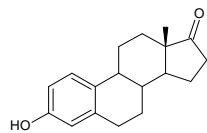
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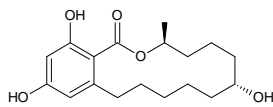
Figure 1. Estrogens and estrogen mimics used in this study.



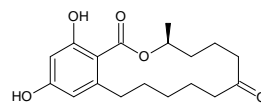
Estradiol (E2)



Estrone (E1)

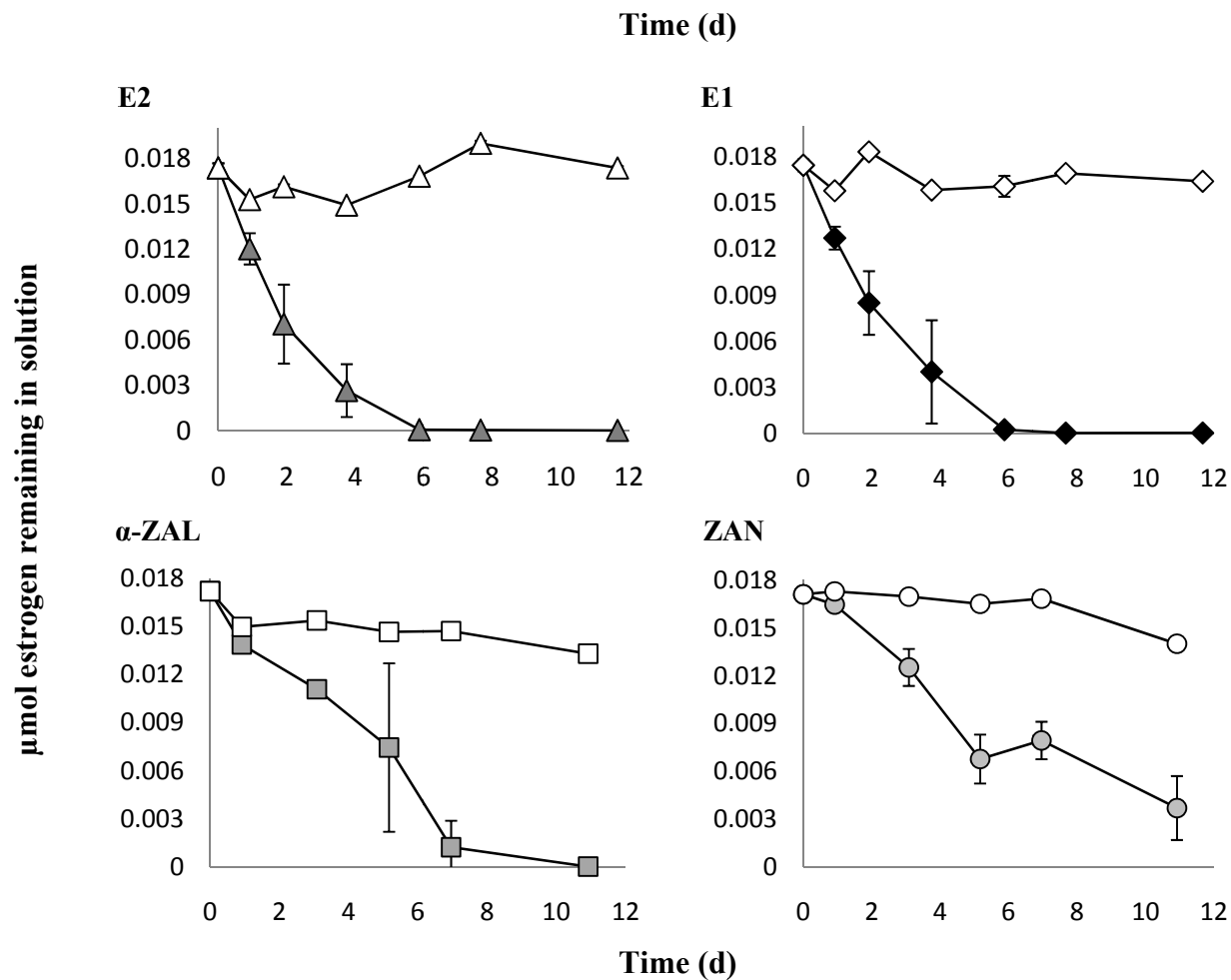


Zeranol (α -ZAL)



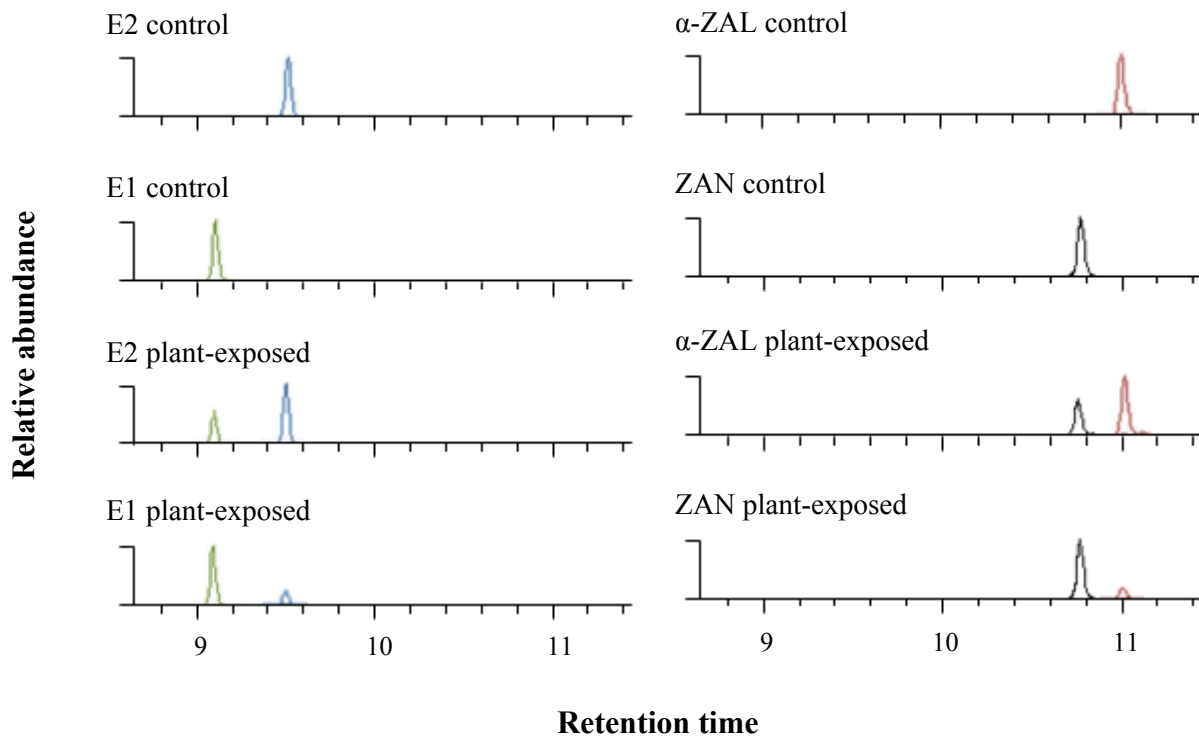
Zearalanone (ZAN)

Figure 2. Decrease of aqueous estrogen concentrations with exposure to maize seedlings.



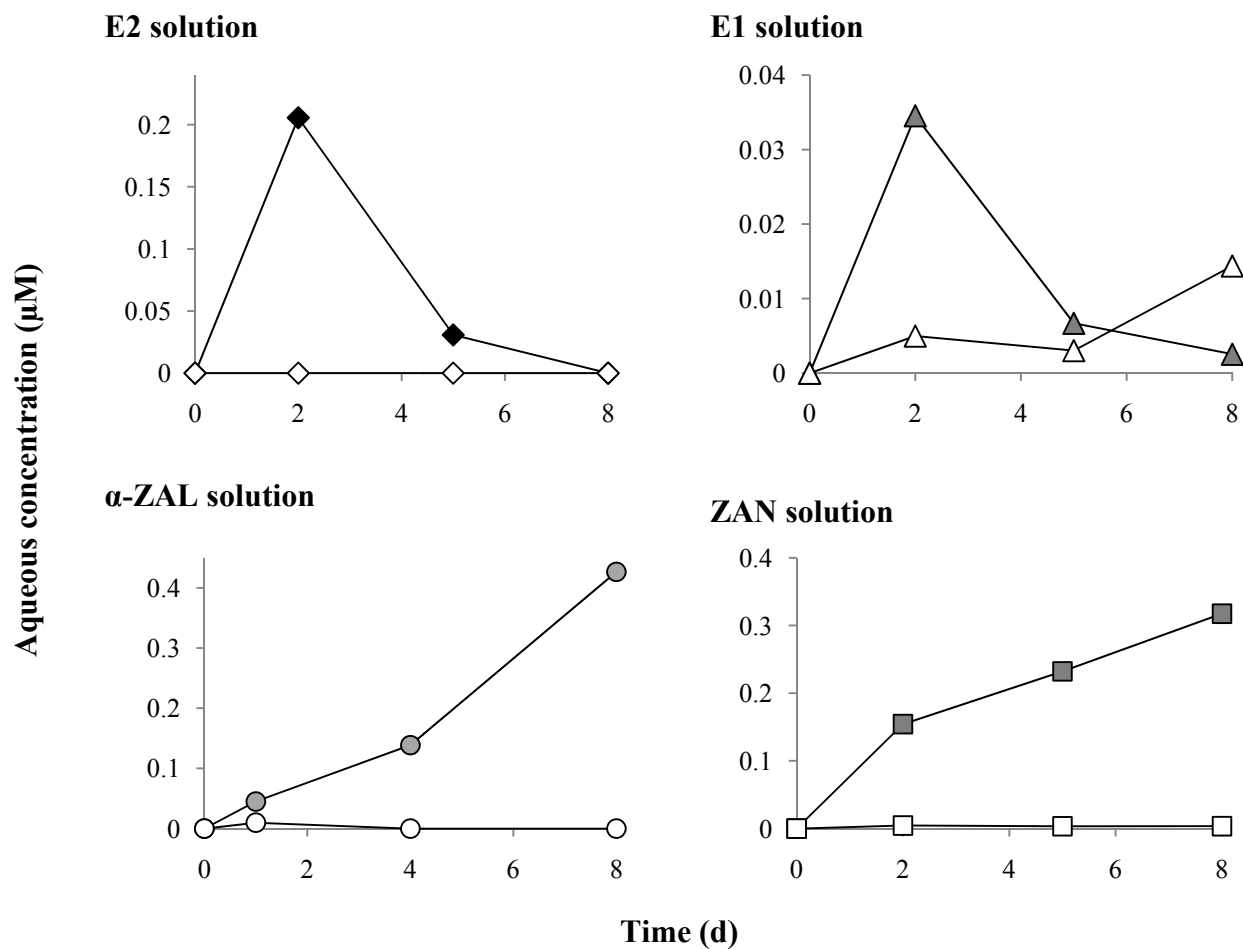
^aMaize-exposed (dark symbols) and glassware control (open symbols) samples of E2 (▲, △), E1 (◆, ◇), α-ZAL (■, □) and ZAN (●, ○)

Figure 3. Extracted ion GC-MS spectra of control and maize-exposed estrogen solutions. Blue: E2 (m/z 416), green: E1 (m/z 342), red: α -ZAL (m/z 433), black: ZAN (m/z 449).



1

Figure 4. Transformation products in glassware control and maize-exposed hydroponic solutions of estrogens.



E1 (◆, ◇), E2 (▲, △), ZAN (●, ○), and α-ZAL (■, □) as transformation products in plant-exposed (dark symbols) and glassware control (open symbols) samples.

1
2

Table 1. RP-HPLC parameters and MS fragments used to identify target compounds.

	E2	E1	α-ZAL	ZAN
Detection λ (nm)	280	265	220	220
HPLC LOQ^a (μM)	0.006	0.007	0.002	0.001
Major ion peaks (m/z)^a	416 ^b 285 129 73	342 ^b 257 218 73	538 ^c 433 307 73	464 ^c 449 335 73

^aLOQ, limit of quantification ^b16 ^c17