



# High-throughput screening of chemicals for estrogenic and androgenic activity using mass spectrometry

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## Abstract

SELDI-TOF mass spectrometry coupled with a short-term fish assay was used to investigate changes in plasma protein expression as a means to screen chemicals for endocrine activity. Adult male sheepshead minnows were treated with model estrogens and adult female fish treated with model androgenic chemicals. Negative control compounds and solvent control treatments were conducted simultaneously with each exposure. Fish were sampled, plasma diluted, processed on weak cation exchange arrays and analyzed. Spectral processing resulted in 58 and 376 individual m/z peak clusters for the estrogen and androgen exposures, respectively. Partial least-squares discriminant analysis (PLSDA) was used to develop separate estrogen and androgen-responsive models using sample spectra from exposures with 17 $\beta$ -estradiol (E2) and dihydrotestosterone (DHT) as the training sets. The E2 model classified the estrogen agonists and control samples with 100% specificity (% true negative) and 100% sensitivity (% true positive). The androgen classification model performed with >90% specificity,  $\geq$ 78% sensitivity for non-aromatizable androgens and  $\leq$  79% for aromatizable androgens. The reduction in sensitivity observed with aromatizable androgens is thought to be caused by their metabolic conversion to estrogens by aromatase. These results show MOA specific plasma protein biomarkers can be reproducibly detected at low levels using HTP mass spectrometry.

## Introduction

The increasing number and diversity of chemicals requiring regulatory oversight and the high cost associated with their evaluation using current testing strategies has provided an incentive to develop more efficient assays to screen chemicals for potential adverse effects. Ideally, these "next generation" methodologies must be reliable and cost effective, have easily interpretable results, be capable of detecting multiple chemical modes of action and reduce dependency on animal testing without sacrificing accuracy and sensitivity.

A relatively new technique, Surface Enhanced Laser Desorption and Ionization Time of Flight Mass Spectrometry (SELDI-TOF-MS) can determine peptide and protein molecular mass, as indicated by their mass to charge ratio (m/z). For SELDI, sample preparation, fractionation and TOF analysis can be performed directly on the selective chromatographic chip surface and requires no separate preparative and fractionation steps prior to sample application. ProteinChip arrays have 8 or 16 sample formats thus providing a rapid method to detect low abundance proteins and peptides. SELDI-MS is used to examine protein expression profiling as a means to screen chemicals based on previously identified modes of action. Using short-term exposures of fish, plasma samples from control and exposed treatments are applied to ProteinChip arrays to produce protein expression profiles unique to each treatment. Using multivariate approaches, a classification model is constructed from control and treatment protein profiles to identify differentially expressed proteins that predict the MOA of interest. These profiles can be used to develop chemical category-specific protein expression libraries. Chemicals can then be tested and evaluated for toxicity based on comparison of protein profiles to those identified in the library. Although the profiles generated will be specific to a species, this profiling approach appears to be transferable to any species or chemical toxicity mechanism of interest. Such assays can assist in prioritizing chemicals before more costly definitive tests are initiated.

## Materials and Methods

### Exposure and Sample Preparation

- Adult sheepshead minnows (*Cyprinodon variegatus*) were exposed to test compounds using an intermittent flow-through dosing apparatus. Male fish were used in the estrogen exposures (Table 1) and gravid females for exposure to androgens (Table 2).
- Following 7 days of exposure, blood was collected from the caudal artery/vein and plasma separated by centrifugation. Sex was verified by direct observation of the gonad.
- Individual plasma samples were processed and applied to the sample surfaces of weak cation exchange (CM10) ProteinChip arrays.
- Samples were processed in duplicate and randomized across all arrays within an experiment.
- Continuing calibration and protein mass standards were included in each experiment to assess reproducibility of intensity and mass accuracy over time.

Treatment	Abbreviation	Concentration ( $\mu$ g/L)	Inclusion Rationale	Set Type
17 $\beta$ -estradiol	E2	0.2	Natural estrogen	Training
17 $\beta$ -estradiol	E2-200	0.2	Natural estrogen	Prediction
Bisphenol-A	BPA	1000	Xenoestrogen	Prediction
4-tert-pentylphenol	TPP	100	Xenoestrogen	Prediction
Methoxychlor	MXC	6	Xenoestrogen	Prediction
Endosulfan	ES	0.6	Stressor control	Prediction
Chlorpyrifos	CP	80	Stressor control	Prediction
Triethylene glycol	TEG	12 <sup>a</sup>	Solvent control	Training
Seawater Control (Females)	SWF	NA	Female negative control	Prediction
Seawater Control (Male)	SWM	NA	Male positive control	Prediction

Table 1. Compounds and treatments, nominal chemical concentrations examined and treatment rationale used for discovery of estrogen-specific biomarkers using male fish (N: E2, TEG=10, E2-200= 5, remaining treatments=1). (<sup>a</sup> $\mu$ L/L)

Treatment	Abbreviation	Concentration ( $\mu$ g/L)	Inclusion Rationale	Set Type
Dihydrotestosterone - A	DHT-A	7.1 <sup>a</sup>	Natural androgen	Training
Dihydrotestosterone - B	DHT-B	5.9 <sup>a</sup>	Natural androgen	Prediction
Methyl-dihydrotestosterone	MDHT	5.0	Synthetic androgen	Prediction
Trenbolone	TB	5.0	Xenoandrogen	Prediction
Testosterone	T	14.8 <sup>a</sup>	Aromatizable natural androgen	Prediction
Methyltestosterone	MT	5.0	Aromatizable synthetic androgen	Prediction
Endosulfan	ES	0.6	Stressor control	Prediction
Chlorpyrifos	CP	80	Stressor control	Prediction
Ethanol	EIOH	12 <sup>b</sup>	Solvent control	Training

Table 2. Compounds and treatments, nominal chemical concentrations examined and treatment rationale used for discovery of androgen-specific biomarkers using gravid female fish. (<sup>a</sup> Measured by ELISA; <sup>b</sup>  $\mu$ L/L)

### Data Collection and Processing

- CM10 (weak cation exchange) arrays were analyzed using high throughput, low resolution Bio-Rad PBS-Ic TOF-MS (estrogens) and an Enterprise 4000 TOF-MS (androgens). Peaks were detected in the range of 1-15 kDa for estrogens and 1-200 kDa for androgens
- Data were processed using Bio-Rad ProteinChip data manager software 3.0.6. Upon importation, raw spectra were baseline subtracted and mass calibrated. Spectra were normalized to the total ion current and spectra with normalization factors <0.5 or >2.0 were eliminated.
- Peaks of similar m/z across spectra were detected using a S/N ratio of >5 and >2 on the first and second pass, adding estimated peaks, and a 0.3% mass window. Clustered peak (protein mass) intensity values were centered and Pareto scaled prior to analysis.
- Partial least squares-discriminant analysis (PLS-DA) was used to develop separate models for the estrogen and androgen exposure studies. The training sets were comprised of 10 TEG solvent control and 10 E2 treatments for the estrogen study and 22 EIOH solvent control and 25 DHT treatments for the androgen study.
- Significant PLS components for each model were determined using the default software setting where 1/7<sup>th</sup> of the data is excluded during each round. Observations with a Hotelling's T<sup>2</sup> value greater than the model 99% CI was considered a serious outlier and excluded. All analyses were conducted using SIMCA-P+12 software (Umetrics Inc.).
- The estrogen and androgen models were applied to their respective predictions sets listed in Tables 1 & 2. Samples were classified as a positive response if the value for the predicted variable in the predictions classification list was  $\geq$ 0.5.



## Results and Discussion

### Estrogen Study

- SELDI analysis detected 58 m/z total peak clusters (protein masses) in male plasma.
- Analysis of the TEG solvent control and E2 samples produced 2 significant PLS components with an R<sup>2</sup> of 0.99 and a cross-validated R<sup>2</sup> (Q<sup>2</sup>) of 0.93 (Figure 1).
- Application of the estrogen-responsive model to the prediction data set listed in Table 1 resulted in correct classification of the samples with 100% sensitivity (% true positive) and 100% specificity (true negative control) (Figure 2).
- One of the significantly upregulated proteins possessing a mass-to-charge (m/z) of 3025.5 was identified by high-resolution tandem mass spectrometry as sheepshead minnow zona radiata protein, fragment 2 further supporting the estrogen-responsive nature and biological relevance of the model.

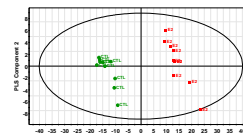


Figure 1. Score plot of the estrogen classification model developed with E2 (red) and TEG (green) treatments. Eclipse denotes Hotelling's T<sup>2</sup> for 95% confidence region.

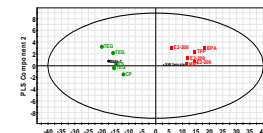


Figure 2. PLS-DA score plot of estrogen classification model illustrating the discrimination between estrogen and non-estrogen treatments. "SW female" denotes plasma from a gravid female minnow.

### Androgen Study

- SELDI analysis detected 376 m/z total peak clusters (protein masses) in female plasma.
- Analysis of the EIOH solvent control and DHT-A samples produced 2 significant PLS components with an R<sup>2</sup> of 0.67 and a cross-validated R<sup>2</sup> (Q<sup>2</sup>) of 0.35 (Figure 3). Self validation of the DHT-A and EIOH training samples resulted in correct classifications of 100 and 91%, respectively (Table 3).
- Performance of the androgen-responsive model in correctly classifying non-aromatizable androgens was  $\geq$  78% and 38 and 79% for the aromatizable androgens T and MT, respectively. The methylation may slow the conversion of MT to estrogen by aromatase resulting in the higher numbers of MT samples classified as androgenic as compared to T.

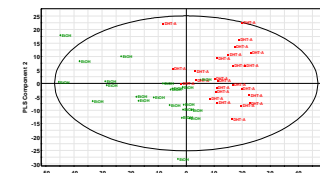


Figure 3. Score plot of the androgen classification model developed with DHT-A (red) and EIOH (green). Eclipse denotes Hotelling's T<sup>2</sup> for 95% confidence region.

Abbreviation	Sensitivity (%)	Specificity (%)	Total N
DHT-A	100	---	25
DHT-B	78	---	23
MDHT	83	---	24
TB	88	---	25
T	38	---	24
MT	79	---	24
ES	---	87	30
CP	---	96	25
EIOH	---	91	22

Table 3. Performance of the androgen classification model developed with DHT-A and EIOH as the training set.

## Conclusions

- Plasma protein profiling using a small fish model and SELDI-TOF-MS provided a sensitive and specific high-throughput method to screen chemicals for estrogenic and androgenic activity.
- Although the diagnostic proteins expressed may be specific to the species tested, the general technique can be transferred to any species of interest.