Development of an Analytical Method for Quantification of Endocrine-Disrupting Phytoestrogenic Compounds in Estuarine Environment Using UHPLC-MS/MS

by

Jingyi Qi

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Approved by

Dr. Joel Hayworth (Co-Chair), Associate Professor of Civil Engineering (Environmental)
Dr. Clifford Lange (Co-Chair), Associate Professor of Civil Engineering (Environmental)
Dr. Mark Barnett, Professor of Civil Engineering (Environmental) and Associate Chair

Abstract

Endocrine disrupting compounds (EDCs) are nearly ubiquitous in the daily lives of individuals around the globe. Phytoestrogenic polyphenols (PEPPs, also known as phytoestrogens) represent a subgroup of EDCs. They are naturally occurring chemicals that possess estrogen-mimicking effects. Some literature has reported deteriorative influences of PEPPs on reproductive function of many aquatic species. However, the links between detected impacts on living organisms in natural ecosystems and potential sources of PEPP exposure are not fully understood. One reason for this is our inability to quantitate multiple PEPPs at trace concentrations in environmental matrices. In this study, an improved UHPLC-ESI-MS/MS method was developed and validated to simultaneously quantify 15 PEPPs in sediment and surface water. The method was demonstrated by analyzing samples collected from the Perdido Bay estuarine system in coastal Alabama. No compounds were found above detectable levels in sediment samples examined; however, enterolactone was detected and quantitated in the majority of the surface water samples. Highest enterolactone concentrations were found in samples collected from two freshwater tributaries discharging into Perdido Bay. The developed and validated quantitative method can be used to better understand the fate and transport of these chemicals in natural environments and their potential effects on living resources. The method can be further developed in future studies to broaden the number PEPP analytes and method applicability to a variety of matrices, including wastewater, biofilms, algae, and groundwater.

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List of Abbreviations

%R	Percent recoveries
6-PN	6-prenylnaringenin
8-PN	8-prenylnaringenin
AJS-ESI	Agilent jet-stream electrospray ionization
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
APIG	Apigenin
AR	Analyte response
BIO-A	Biochanin A
CE	Collision energy
DAID	Daidzein
DMSO	Dimethyl sulfoxide
DW	Dry weight
E2	17β-estradiol
EDC	Endocrine disrupting compounds
ENTD	Enterodiol
ENTL	Enterolactone

ER	Endocrine receptor
ESI	Electrospray ionization
FORM	Formononetin
FV	Fragmentor voltage
GC	Gas chromatography
GEN	Genistein
GLY	Glycitin
HDPE	High-density polyethylene
HPLC	High-performance liquid chromatography
IS	Internal standard (chrysin)
IXN	Isoxanthohumol
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
M/Z	Mass to charge ratio
MRM	Multiple ion monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MS2	Full scan
NARN	Naringenin
ON	Ononin
PEPP	Phytoestrogenic polyphenols
PI	Product ion

POP	Persistent organic pollutant
QqQ	Triple quadruple
QToF	Quad time of flight
Qtrap	Quadrupole ion trap
RES	Resveratrol
RP	Reverse phase
RT	Retention time
SDG	Secoisolariciresinol diglucoside
SIM	Selected ion monitoring
SPE	Solid phase extraction
SRM	Selected reaction monitoring
TIC	Total ion current
UHPLC	Ultra-high-performance liquid chromatography
Vg	Vitellogenin
Vn	Vitelline
WHO	World health organization
WWTP	Wastewater treatment plant
XN	Xanthohumol

Chapter 1

Introduction

1.1 Scope and objectives of this research effort

The potential impact of phytoestrogenic polyphenolics (PEPPs, also known as phytoestrogens) on aquatic organisms living in estuarine systems has not been extensively studied. One of the main reasons for this is the lack of rapid analytical methods for trace quantitation of PEPPs in environmental matrices. The study presented in this thesis describes the development and validation of a quantitative analytical method that is simple, rapid, and accurate for a select group of PEPPs having different physiochemical properties, in sediment and surface water from an Alabama coastal estuary. The method employs an ultra-high performance liquid chromatography, triple quadrupole mass spectrometer system (UHPLC-MS/MS) to simultaneously detect and quantify 15 PEPPs from different classes in estuarine waters and sediments. The developed method is advantageous because it allows for shorter analysis time without sacrificing chromatographic separation. Additionally, the method includes simple sample extraction and clean-up procedures. The results of this study fill a critical knowledge gap regarding the fate and transport of PEPPs in estuarine environments by providing an advanced analytical method for quantitating the presence and distribution of PEPPs in estuaries. This method can be modified and extended to detect PEPPs in other environmental matrices, such as wastewater, biofilms, and

groundwater, and provide an assessment tool for exploring PEPP remedial technologies and approaches.

1.2 Background

Endocrine systems regulate hormones within organisms, which in turn control the proper function of cells and/or organs. As described by World Health Organization (WHO), an endocrine disrupting compound (EDC) is "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations" (Damstra, Barlow, Bergman, Kavlock, & Kraak, 2002). A wide range of chemicals can be categorized as EDCs, such as natural steroidal hormones, synthetic estrogens/androgens, and industrial chemicals (Z.-h. Liu, Y. Kanjo, & S. Mizutani, 2010). EDCs are nearly ubiquitous in our daily life: they can be found in personal care products, prescribed drugs, synthetic protective coatings, in dietary supplements, and a host of other natural and man-made substances. The increasing use by humans of products containing EDCs elevates their environmental concentrations, which can lead to a variety of adverse impacts to aquatic ecosystems and human well-being (Bergman et al., 2013). One recent estimate is that approximated $340 billion was allocated for medical cost in the U.S. in 2010 resulting from exposure to endocrine disrupting compounds (Attina et al., 2016). One explanation for the increased presence of EDCs in the environment is the accelerating use of EDC-bearing substances in modern human society, coupled with the lack of EDC removal or treatment technologies.

The reproductive hormone-receptor systems in vertebrates are extremely sensitive to EDCs. In aquatic wildlife species, EDCs has been shown to cause irregular reproduction

functions such as changes in sex and abnormal reproductive behavior leading to population declines. Efforts to understand the relationships between the presence of these compounds in aquatic systems and the health impacts on exposed organisms have thus far focused on relatively more potent and easily detected compounds such as steroidal hormones, pesticides and pharmaceuticals (Keith, Jones-Lepp, & Needham, 1999; Mills & Chichester, 2005; Zhou, Cai, & Zhu, 2010). However, EDCs with lower potencies can accumulate in the environment and/or transform into compounds that possess higher endocrine disrupting potential (Depledge & Billinghurst, 1999; Lewis & Ford, 2012).

1.3 Phytoestrogenic polyphenols

Some naturally occurring phytoestrogenic polyphenolics (PEPPs, also known as phytoestrogens) are known to exhibit endocrine disrupting effects (Boberg et al., 2013; Chighizola & Meroni, 2012; Ferreira-Dias et al., 2013; Z. H. Liu, Y. Kanjo, & S. Mizutani, 2010; Waring et al., 2008). These compounds are structurally and/or functionally similar to ovarian and placental estrogens and their active metabolites (Martin, Horwitz, Ryan, & McGUIRE, 1978; Setchell & Adlercreutz, 1988; Verdeal, Brown, Richardson, & Ryan, 1980; Whitten & Patisaul, 2001). The first documented instances of endocrine disruption by PEPPs were in the mid-nineteen century when an infertility syndrome was observed in ewes and other livestock grazing on clover pastures in Australia and New Zealand. These pastures were later identified as having high concentrations of formononetin, a natural chemical within the clover plant (Cederroth, Zimmermann, & Nef, 2012). This group of compounds can bind to estrogen receptors (ERs) and induce either estrogenic or antiestrogenic responses in target tissues sensitive to estrogens (Bacciottini et al., 2007). They can also interfere with estrogenic response or change the total amount of free

estrogens in organisms through alternative mechanisms, in which case they do not show affinity to ERs (Michel, Halabalaki, & Skaltsounis, 2013).

Functions of estrogens include regulation of development, and differentiation and reproduction within animals (Ibarreta, Daxenberger, & Meyer, 2001). Among all the estrogenic compounds, 17β-Estradiol (E2), a female hormone, is most potent. Despite the fact that PEPPs have estrogenic activities with potencies that are tenth to ten-thousandth of the activity of E2, they can be more potent than other anthropogenic EDCs. In addition, PEPPs can exist in large quantities in plant-dense areas, such as farm feed manufacturing industries, and exposure to these compounds may affect humans and wildlife (Jarosova, Javurek, Adamovsky, & Hilscherova, 2015; Mazur, Duke, Wähälä, Rasku, & Adlercreutz, 1998). Thus, attention on PEPPs has grown due to their established and potential endocrine disruption activities (Guerrero-Bosagna, Weeks, & Skinner, 2014).

Many studies support the known or potential health benefits of some PEPPs, such as symptom alleviation of certain illnesses (Chen, Lin, & Liu, 2015; He et al., 2015; Husain, Khanna, Puri, & Haghighizadeh, 2015; Schmidt et al., 2016; Somekawa, Chiguchi, Ishibashi, & Aso, 2001). However, PEPPs are subject to much controversy due to the possibility of the understudy of the long-term effects of these compounds (alone or combined with other compounds) or publication bias. (Eisenbrand, 2007; Grosso et al., 2017; Rietjens, Louisse, & Beekmann, 2017; Sahin, 2014; Soni et al., 2014). Furthermore, some research groups have detected deteriorative influences of PEPPs on reproductive function in many aquatic organisms. Some of these studies were not thoroughly conducted because they failed to link the detected impacts in living organisms in natural ecosystems under real dynamic conditions with the potential sources of PEPP exposures. This failure

could be mainly due to the lack of scientific attention regarding the environmental levels of PEPPs. Thus, there is a demand for analytical methods with improved sensitivity and efficiency to help better understanding of the fate and transport of PEPPs in aquatic systems and provide insight into how environmental PEPPs are affecting aquatic organisms.

1.3.1 Natural sources

Plants produce PEPPs for two primary purposes: to defend against predators and to attract beneficial insects and bacteria for their survival and growth (Fox, 2004). The major groups of PEPPs are isoflavonoids, flavonoids, stilbenes, and lignans, which are categorized based on their different chemical structures and biological functions (Cos et al., 2003). They are predominantly found in the Leguminosae family, in which they mainly exist as aglycones in red clovers, and as glycosidic conjugates in oilseeds. In fermented food, such as tofu and miso, PEPPs can also be deconjugated to aglycones, which may be consumed as a part of regular diets (Clarke, Bailey, & Lloyd, 2008; Dixon, 2004; Eisenbrand, 2007; Foster, Chan, Platt, & Hughes, 2002; Ososki & Kennelly, 2003; Q. Wu, Wang, & Simon, 2004). The salient point is the composition and the concentration of some PEPPS vary prominently in different species and even different structures within a species. (Kuhnle et al., 2009; Mazur et al., 1998; Michel et al., 2013).

Some isoflavonoids (mainly daidzein, genistein) with their glycosylated forms and coumestans have been reported existing in soybeans and clovers at relatively high concentrations. Ononin, formononetin and biochanin A are the prevalent bioactive isoflavonoids in red clover, whereas ononin is the glycoside form of formononetin (Clarke et al., 2008; Zhang et al., 2015). Glycitin, an isoflavone glucoside, is found in lentils, haricot beans, red kidney beans, and chickpeas. Hops, commonly used for beer production,

are the main source of prenylflavonoids, one of the subgroups of isoflavonoids, which includes 6-prenylnaringenin, 8-prenylnaringenin, xanthohumol, isoxanthohumol (Cos et al., 2003; Dhooghe et al., 2010; Quifer-Rada et al., 2013). Flavonoids, such as naringenin and apigenin, can also be found in fruits, cabbages, herbs and some heartwoods of tree species (Guo et al., 2015; Wan et al., 2007). Stilbene resveratrol can be primarily found in grapes, wine, peanuts, and pines (Cornwell, Cohick, & Raskin, 2004). A noticeable amount of lignans (enterolactone and enterodiol), which are the main components of plant cell walls, appear in many fiber-rich foods like flax seeds and sesame seeds (Jarosova et al., 2015; Patisaul & Jefferson, 2010; C.-C. Wang, Prasain, & Barnes, 2002). Flaxseeds and sesame seeds, often used in baking, contain secoisolariciresinol diglucoside in large quantities. These compounds can be digested and transformed into the mammalian lignans enterolactone and enterodiol, which can have weak estrogenic and antiestrogenic effects (Michel et al., 2013; Muir & Westcott, 2000; C.-C. Wang et al., 2002).

Until now, the most studied class of PEPPs is isoflavonoids (especially daidzein and genistein) due to their significant estrogenic potential, which has a fifteen-carbon (C6-C3-C6) skeleton that is differentiated from flavonoids by the position of the B-ring (Figure 1.3.1.1). Aglycones daidzein, genistein, and glycitein mainly exist as their glucoside form (attached to a sugar unit) of daidzin, genistin, and glycitin, or as their methoxylated forms of formononetin and biochanin A (4'-methyl ethers), respectively. Aglycones, which are part of the metabolites of soy isoflavonoids by enteric bacteria in intestines, show higher potencies than corresponding glycosides in general (Clarke et al., 2008; Kinjo et al., 2004). Some studies have demonstrated, however, that flavonoids have higher relative estrogenic activities than lignans (Rocha & Rocha, 2015b; Whitten & Patisaul, 2001). 8-

prenylnaringenin was recently identified showing an ERα agonist activity 100 times more potent than genistein (Quifer-Rada et al., 2013). Prenylflavonoids in hops are mainly present as xanthohumol. In the brewing process of beer production, xanthohumol converts into isoxanthohumol during boiling, which is a precursor of 8-prenylnaringenin (Dhooghe et al., 2010). 6-prenylnaringenin is a positional isomer of 8-prenylnaringenin. Lignans share a structural characteristic of polyphenol linked by a four-carbon bridge.

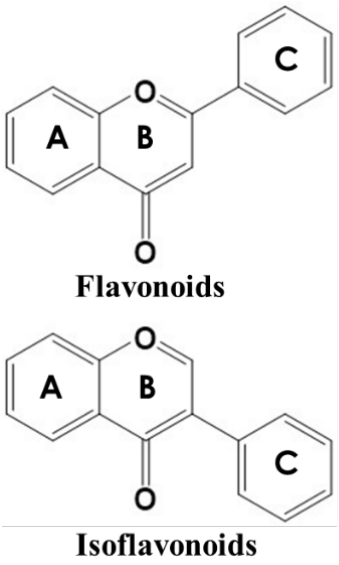


Figure 1.3.1.1: The Structural difference between flavonoids and isoflavonoids.

1.3.2 Increased environmental occurrence

Recently, attention to PEPPs has increased due to their potential human health benefits for alleviating symptoms of particular illnesses, such as cancer, cardiovascular disease, osteoporosis, menopausal symptoms, male infertility, obesity and type 2 diabetes. (A Sobenin, A Myasoedova, & N Orekhov, 2016; Anderson, Cotterchio, Boucher, & Kreiger, 2013; Kuhnle et al., 2009; C.-C. Wang et al., 2002). Consequently, manufacture of dietary supplements claiming to contain PEPPs is increasing. Many of these dietary

supplements, along with plant-based cooking oil, vegetarian diets, and even infant formulas, are available in the U.S. commercial market, making PEPPs abundant in our daily life (Cederroth et al., 2012). Digested and undigested PEPPs are excreted through human urine and have been detected in environmental media (Z. H. Liu et al., 2010).

Recent studies have reported on detection of PEPPs not only in wastewater but also in rivers, lakes, seas, and drinking water, as shown in Table A1. The main pathways for PEPP contamination in aquatic environments are industrial effluents, wastewater treatment plant (WWTP) effluents, runoff from agricultural areas, and runoff from regions that are close to surface water (Jarosova et al., 2015). Most farm animal and fish feeds contain natural PEPPs, which are released by feedlot effluents, together with agricultural runoff entering into natural waters. These compounds then seep and accumulate into sediments with a minimal decomposition rate (Matozzo, Gagné, Marin, Ricciardi, & Blaise, 2008). Dana W. Kolpin et al. studied the spatial and temporal occurrence of six PEPPs in stream sampling sites across Iowa. Formononetin was detected in the majority of the collected samples (80%), while coumestrol was not detected. Significant concentration levels of daidzein and equol were found, and an increasing trend for PEPPs was observed during spring snowmelt (Kolpin et al., 2010). A research group has conducted several studies to study the seasonal and spatial distribution of EDCs of various classes (including some most studied PEPPs) in estuarine environments in Portugal. In one of their studies, they found out a seasonal trend of daidzein and genistein reaching their peak value during summer whereas biochanin-A shown the highest level during winter (C. Ribeiro, Tiritan, Rocha, & Rocha, 2009).

A study was conducted to investigate the occurrence and transformation of

enterolignans, which are mammalian lignans, in plants and natural waters and noted that the occurrence of enterolignans in plants could be due to plant uptake from surrounding water in which these compounds are present. Higher concentrations were detected in influents from a WWTP (mean values range from non-detectable to 548 ng/L) compared to that of effluents and river water (mean values range from not present to 14 ng/L), which demonstrated a relatively high removal efficiency for most PEPPs except formononetin by this WWTP (Bacaloni et al., 2005; Smeds, Willför, Pietarinen, Peltonen-Sainio, & Reunanen, 2007).

1.3.3 PEPPs as endocrine disrupting compounds

Identifying the primary compounds causing estrogenic activities in aquatic ecosystems is challenging given the fact that environmental estrogens often vary in chemical structures. These compounds can impose multiple anthropogenic stress factors, which have additive, antagonistic, or synergistic effects on hormonal regulation that could abnormally influence growth, stress response and reproduction in vertebrates (E. D. Clotfelter & A. C. Rodriguez, 2006; Jarosova et al., 2015; Oberdörster & Cheek, 2001; Teichert, Borja, Chust, Uriarte, & Lepage, 2016; Yamamoto, Garcia, Kupsco, & Ribeiro, 2017). There are a number of studies showing PEPPs can act as inhibitors of various protein kinases which are related to the function of cell proliferation (Ingham, Gesualdi, Toth, & Clotfelter, 2004). In addition to the effects addressed above, PEPPs have also been reported to induce alterations in hormone synthesis, transport, receptor interaction, metabolism, excretion, feedback regulation, hormone disruption during sex differentiation, shift in the sex ratio (more females), and various gonadal abnormalities, which may only be observed after sexual maturation in both freshwater and marine species (Ethan D Clotfelter & Alison

C Rodriguez, 2006; Jarosova et al., 2015; Oberdörster & Cheek, 2001).

Vitellogenin (Vg) is a precursor of the egg yolk protein vitelline (Vn) which is necessary for embryo development in oviparous vertebrates. Endogenous estrogens within female fish can elevate the level of Vg in the bloodstream in order to develop oocytes when they reach sexual maturity. In contrast, the level of Vg in juveniles is much lower, and naturally, there would be no Vg produced in male fish. Estrogens and chemicals that mimic estrogen may trigger the induction and abnormal production of Vg, which would remain in the plasma with an insignificant degradation rate. Thus, plasma Vg levels in immature female and male fish are considered a useful indicator of estrogenic exposure in aquatic environments. However, Vg induction due to estrogenic effects in invertebrates has not been well studied, even though these organisms represent a large group of aquatic organisms. As suggested by Mazozzo et al., Vg induction can be useful as a biomarker of exposure to estrogenic compounds in both vertebrates and invertebrates. Up to now, no comprehensive studies have been conducted to evaluate Vg induction in aquatic organisms (fish and invertebrates specifically) due to PEPP exposure. Further research is necessary, not only to determine whether there are significant differences in plasma Vg levels in aquatic organisms exposed to PEPPs, but also to provide a better understanding of the influence of environmental PEPPs on aquatic animals, especially fish and invertebrates of commercial importance. Table 1.3.3.1 summarizes the observed impacts on aquatic organisms exposed to PEPPs. Because enterolactone and enterodiol have been identified as weakly estrogenic and antiestrogenic chemicals, further research should be conducted to study the potential adverse influences of these compounds on aquatic organisms (L.-Q. Wang, 2002).

Table 1.3.3.1: Observed impacts on aquatic organisms exposed to PEPPs.

Analyte	Observed impacts on aquatic organisms	Reference
8-PN	No estrogenic effects observed in exposed medaka.	(Zierau et al., 2005)
Bio-A	Induction of plasma VTG in brown trout. Increased VTG in homogenate and number of females in zebrafish. Induction of vitellogenin secretion in yearling sturgeon. Competition for estrogen receptor sites in rainbow trout and sturgeon.	(Jarosova et al., 2015; Latonnelle, Le Menn, Kaushik, & Bennetau-Pelissero, 2002; Pelissero, Bennetau, Babin, Le Menn, & Dunogues, 1991)
DAID	Increase of production of eggs, but no effect on survival, length or reaction times to a threatening stimulus, and no changes in anatomy, physiology or behavior was observed in fathead minnow. Induction of vitellogenin secretion in yearling sturgeon. Competition for estrogen receptor sites in rainbow trout and sturgeon.	(Jarosova et al., 2015; Latonnelle et al., 2002; Pelissero et al., 1991)
FORM	Decrease in survival in fathead minnow. No induction of vitellogenin secretion in yearling sturgeon. Competition for estrogen receptor sites in rainbow trout and sturgeon.	(Jarosova et al., 2015; Latonnelle et al., 2002; Pelissero et al., 1991)
NARN	No estrogenic effects in the medaka sex reversal/VTG gene expression assay.	(Zierau et al., 2005)

Table 1.2.2.1: *(continued)*

Analyte	Observed impacts on aquatic organisms	Reference
GEN	Induction of vitellogenin secretion in yearling sturgeon. Triggering of hermaphroditism in fish. Competition for estrogen receptor sites in rainbow trout and sturgeon. Various gonadal abnormalities and induction of VTG in Japanese medaka. Decrease in survival and affected somatic growth, but no effect on the production of eggs, anatomy, physiology or behavior in fathead minnow. Reduced ATP content in channel catfish and walleye, which might influent in vitro fertilization rate. Reduced aggressive behavior, increased tendency to build nests, but no effect on GSI, sperm concentration, motility or fertilization success in fighting fish. Edema, head and tail deformation in zebrafish embryos. Disruption of embryonic development in South African clawed frog and reduced growth rate of fathead minnow in the exposure of genistein at an extreme level.	(Green & Kelly, 2008; Ingham et al., 2004; Jarosova et al., 2015; Latonnelle et al., 2002; Pelissero et al., 1991; Rocha & Rocha, 2015a)

1.3.4 PEPPs in estuarine systems

Estuaries are shallow transitional areas connecting freshwater and marine ecosystems. Highly dynamic and complex, these coastal ecosystems serve as habitats for a wide range of organisms of commercial and recreational importance (Hansen & Rattray, 1966). Estuaries have been exploited for commercial, industrial, and residential use. Around 50% of the world's population live or work in proximity to estuaries (Costanza, Kemp, & Boynton, 1993). Increasing social and economic pressures have led to increased pollution from anthropogenic contaminants, impairing estuarine water and sediment quality and endangering estuarine organisms (Borja et al., 2008).

Estrogenic potency has been detected in environmental samples from estuaries worldwide (Simpson et al., 2000; Verslycke, Vethaak, Arijs, & Janssen, 2005). For examples, three endocrine disruptors (benzo(a)pyrene. 4-nonylphenol, and di(ethyl-hexyl)phthalate) have been linked to adverse naupliar development in estuarine copepods, which is an important species in the estuarine food web (Forget-Leray, Landriau, Minier, & Leboulenger, 2005). Because some PEPPs structurally mimic estrogens, it is possible that these compounds could adversely impact estuarine organisms. Some human population groups consume considerable amounts of PEPPs in their daily diet. Similar to other pharmaceuticals, ingested PEPPs can be excreted as intact chemicals or their metabolites, appear in municipal wastewaters, and eventually in natural water bodies like rivers and estuaries. Environmental concentrations of PEPPs in a selection of estuaries are summarized in Table 1.3.4.1.

Table 1.3.4.1: Summary of environmental concentrations of PEPPs in estuaries. Only reported PEPPs of interest for this research are listed.

Matrix	BIO-A	DAID	ENTD	ENTL	FORM	GEN	GLY	**Detection instrument**	**Reference**
Watershed Sediments	<0.5-19	<0.5-20			n.d.-2.4	n.d.-<0.5	n.d.	LC-ESI-MS/MS	(Levengood, Tam, & Szafoni, 2010)
Coastal surface water		<0.43				<0.61		LC-TIS-MS/MS	(Beck, Bruhn, Gandrass, & Ruck, 2005)
River estuary	<12.4-191	<10.0 - 597				<3.2-184		HPLC-DAD	(C. Ribeiro, Tiritan, et al., 2009)
River estuary		n.d.-130.0	n.d.-93.0	n.d.-<43.7		n.d.-135.0		GC-MS/MS	(A. R. Ribeiro, Maia, Santos, Tiritan, & Ribeiro, 2016)
River estuary	n.d.-170	n.d.-500				n.d.-320		HPLC-DAD	(C. Ribeiro, Pardal, Tiritan, et al., 2009)
River estuary	<8.4-60.2	<3.0-526				<2.6-507.1		HPLC-DAD	(C. Ribeiro, Pardal, Martinho, et al., 2009)

Table 1.3.4.1 (continued)

Matrix	BIO-A	DAID	ENTD	ENTL	FORM	GEN	GLY	**Detection instrument**	**Reference**
River estuary	130.8-844.5	3.4-32.3			423.4-2604.8	24.5-113.4		$GC\text{-}MS^n$	(Rocha, Cruzeiro, Reis, Rocha, & Pardal, 2013)
Urban estuary	50-590	53-11945			26-5494	128-5093		$GC\text{-}MS^n$	(Rocha, Cruzeiro, Reis, Pardal, & Rocha, 2014)
River, estuary, and coastline	23.5-350	2.86-78.5			90 - 801	18.5-120.3		$GC\text{-}MS^n$	(Rocha, Cruzeiro, Peixoto, & Rocha, 2014)
River estuary	17.8-59.7	5.67-12.4			2.9 - 5.8	88-2288		$GC\text{-}MS^n$	(Rocha, Cruzeiro, Reis, Pardal, & Rocha, 2015)
Coastal lagoon	290-675	56-147			300-3416	34.1-90.1		$GC\text{-}MS^n$	(Rocha, Cruzeiro, Reis, Pardal, & Rocha, 2016a)
River estuary	8.8-217.5	13.5-20.0			8.4-75.3	19.7-69.2		$GC\text{-}MS^n$	(Rocha, Cruzeiro, Reis, Pardal, & Rocha, 2016b)

1.3 The advantages of UHPLC-ESI-QqQ-MS/MS

Chromatography has been widely used for effective separation and mass spectrometry for sensitive quantification of PEPPs. Chromatography is a physical method for separating mixtures of chemicals using two distinct phases. Chemical separation is achieved by using either a liquid mobile phase (liquid chromatography (LC)) or a gas mobile phase (gas chromatography (GC)) to carry the chemical mixture through a stationary phase in a fixed direction. The mixture is then separated due to the affinity of each compound between the two phases. In GC, volatile chemicals are heated up to their vapor phase and carried by helium or some other inert gas through a GC column (solid stationary phase) and separated by controlling the oven temperature and gas flow rate. In LC, a polar compound mixture is carried by a combination of organic and inorganic solvents through an LC column (solid stationary phase) and separated by the mechanism of adsorption, size exclusion, ion exchange, affinity, or sorption. Since most PEPPs are polar compounds, LC is more suitable to separate PEPP analytes than GC (Cazes & Scott, 2002; Miller, 2005; Poole, 2003). Mass spectrometry is employed to gather the mass information of each compound previously separated by chromatography. Additional dimensions of isolation by mass are added as more stages of mass analysis are combined in tandem mass spectrometry (MS) to enhance the selectivity of the methods (Gross, 2006).

Among all available technologies, reverse phase (RP) ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) provides optimum separation efficiency and determination accuracy (Raju, Kadian, Taneja, & Wahajuddin, 2015; H. Wu et al., 2013). High-resolution tandem mass spectrometers can provide accurate mass information for each chemical. Therefore, MS/MS is suitable for

chemical structure elucidation of unknown compounds. Atmospheric pressure ionization (API) ionization source is used as an interface in mass spectrometers to vaporize the chromatographically-separated compounds, which are in mobile phase, and ionize them into molecular ions so they can be detected by the instruments (Gross, 2006). There are two main types of API techniques: electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). ESI is suitable for ionizing moderate and polar chemicals such as PEPPs compared to APCI (H. Wu et al., 2013).

Chapter 2

Materials and experimental design

2.1 Study area

The area from which samples were collected for this study is the Perdido Bay estuary in coastal Alabama. The study area and sample collection locations are shown in Figure 2.1.1; additional sample location information is given in Table 2.1.1. Increasing population in the area surrounding Perdido Bay has led to an increase in the amount of anthropogenic pollutants entering the estuary from a variety of sources, including discharge from urban infrastructure (including leakage from septic systems and WWTP infrastructure, and stormwater runoff), and agricultural runoff. These contaminants pose a threat to estuarine water and sediment quality, and the health of a myriad of aquatic organisms (Kim Anh Tran, R. MacFarlane, Yuen Chong Kong, O'Connor, & Yu, 2016; Tran, MacFarlane, Kong, O'Connor, & Yu, 2017).

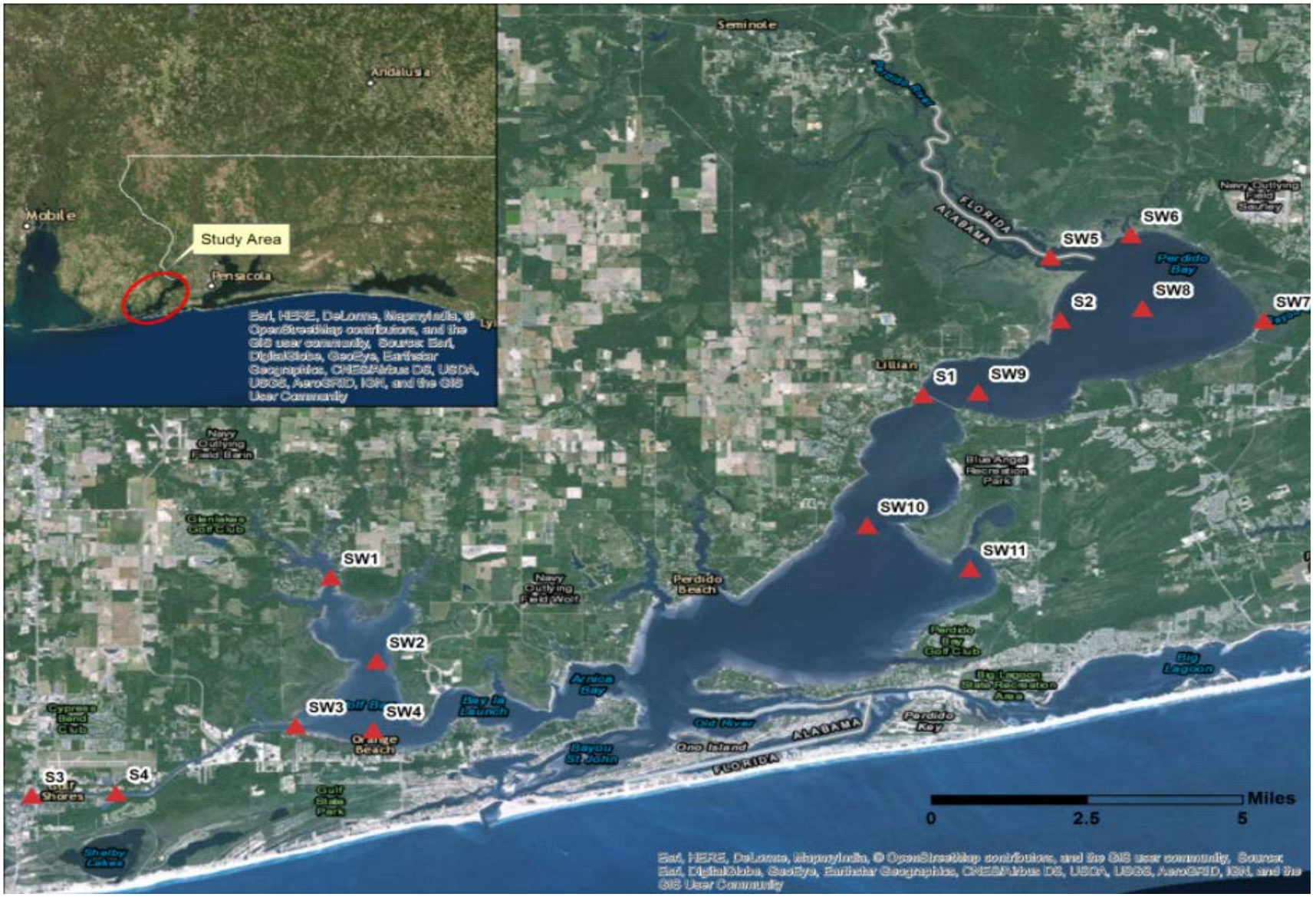


Figure 2.1.1: Sediment and surface water sampling locations, Alabama estuaries.

Table 2.1.1: Sampling details in water samples collected from Perdido Bay estuary.

Water samples	Longitude	Latitude	Sample Location	Date	Time	Depth (ft)		Water Temp (°C)		Specific Conductance (µS/cm)		Practical Salinity (PSU)	
						Total	Sample	Ave	SD	Ave	SD	Ave	SD
SW1A	-87.6011	30.34883	Moccasin Bayou	3/2/2017	15:50	6.0	3.0	20.54	0.01	15633.50	61.94	9.11	0.04
SW1B													
SW2A	-87.5883	30.32215	Middle Wolf	3/2/2017	16:25	8.0	4.0	20.08	0.01	24548.24	63.57	14.88	0.04
SW2B													
SW3A	-87.6108	30.30161	Low Wolf	3/2/2017	17:10	7.0	3.0	19.97	0.06	24988.91	862.71	15.17	0.57
SW3B													
SW4A	-87.5891	30.30012	GIWW to Wolf Bay	3/2/2017	16:46	6.0	3.0	19.87	0.02	26201.90	23.06	15.97	0.01
SW4B													
SW5A	-87.3997	30.45064	Perdido River	3/3/2017	09:50	14.0	7.0	17.80	0.36	5424.39	1895.18	2.93	1.10
SW5B													
SW6A	-87.3772	30.45778	Mouth of Elevenmile Creek	3/3/2017	10:15	3.0	1.5	18.24	0.11	6215.92	667.69	3.38	0.39
SW6B													
SW7A	-87.34	30.43054	Mouth of Bayou Marcus	3/3/2017	-*	-*	-*	18.19	0.04	9780.63	44.57	5.49	0.03
SW7B													
SW8A	-87.374	30.43432	Upper Perdido Bay	3/3/2017	12:00	6.0	3.0	17.78	0.11	7161.50	137.25	3.93	0.08
SW8B													
SW9A	-87.4199	30.40769	Middle Perdido Bay	3/3/2017	14:45	7.0	3.0	18.39	0.07	12991.03	122.46	7.45	0.08
SW9B													
SW10A	-87.4509	30.36529	Lower Perdido Bay	3/3/2017	15:12	11.0	6.0	18.60	0.06	21243.90	65.45	12.69	0.04
SW10B													
SW11 A	-87.4222	30.35155	Tarkiln Bayou	3/3/2017	10:15	3.0	1.5	18.96	0.07	23431.43	71.16	14.13	0.05
SW11B													

*Data was not recorded due to the severe weather condition during sampling.

2.1.1. Chemicals and instruments

The PEPP target analytes used in this study were chosen based on the abundance of their occurrence in the environment, their potential estrogenic activity, and the availability of pure standards in the commercial market. Coumestrol, the most common coumestans, was not included as a target analyte due to a lack of availability of its analytical standard in high purity at the time during which this study was conducted. The analytes of interest are detailed in Table 2.1.1.2.

Analytical phenolic standards (> 98% purity): daidzein (DAID), genistein (GEN), biochanin A(BIO-A), formononetin (FORM), glycitin (GLY), ononin (ON), naringenin (NARN), apigenin (APIG), resveratrol (RES), 8-prenylnaringenin (8-PN), 6-prenylnaringenin (6-PN), xanthohumol (XN), isoxanthohumol (IXN), enterodiol (ENTD), enterolactone (ENTL), secoisolariciresinol diglucoside (SDG), chrysin (IS), magnesium sulfate, dimethyl sulfoxide, and Whatman glass microfiber filters GF/C (1.2 µm, 47 mm) were purchased from Sigma Aldrich (St. Louis, MO). LC-MS grade solvents (methanol, acetonitrile, and water), analytical grade formic acid, and ammonium acetate reagents were obtained from VWR International (Suwanee, GA). Chem Tube-Hydromatrix, ammonium formate, Captiva Nylon/PTFE syringe filters (0.2 µm) were purchased from Agilent Technologies (Wilmington, DE).

Analytical column (Zorbas InfinityLab Poroshell 120 Bonus-RP, 2.1 x 100 mm, 2.7 µm, p/n 861768-901; InfinityLab Poroshell 120 Phenyl-Hexyl, 2.1 x 100 mm, 2.7 µm, p/n 695775-912) and guard column (InfinityLab Poroshell 120 Phenyl-Hexyl guard column, 2.1x 5 mm, p/n 821725-914) were also procured from Agilent Technologies (Wilmington, DE). Oasis PRiME HLB 6cc extraction cartridge, 20-Position vacuum manifold were

supplied by Waters Corporation (Milford, MA, USA).

Table 2.1.1.2: Chemical information of analytes of interest.

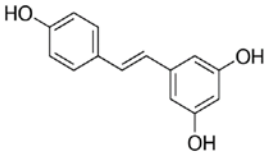
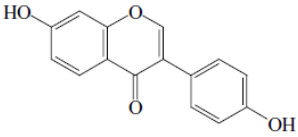
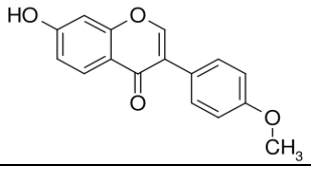
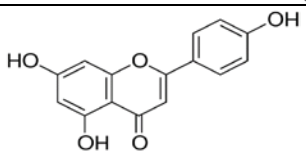
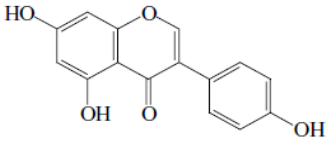
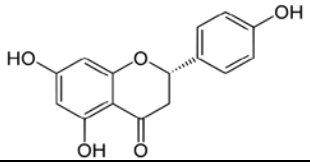
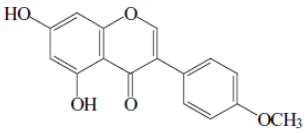
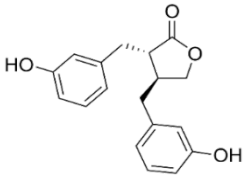
Analyte	Chemical structures	CAS	Class	Molecular weight
RES $C_{14}H_{12}O_3$		501-36-0	Stilbene	228.2
DAID $C_{15}H_{10}O_4$		486-66-8	Isoflavonoid	254.2
FORM $C_{16}H_{12}O_4$		485-72-3	Isoflavonoid	268.3
APIG $C_{15}H_{10}O_5$		520-36-5	Flavonoid	270.2
GEN $C_{15}H_{10}O_5$		446-72-0	Isoflavonoid	270.2
NARN $C_{15}H_{12}O_5$		67604-48-2	Flavonoid	272.3
BIO-A $C_{16}H_{12}O_5$		491-80-5	Isoflavonoid	284.3
ENTL $C_{18}H_{22}O_4$		80226-00-2	Lignan	298.3

Table 2.1.1.2. *(Continued)*

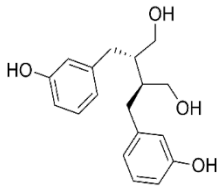
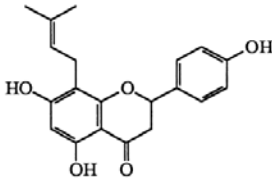
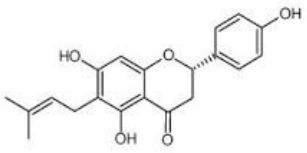
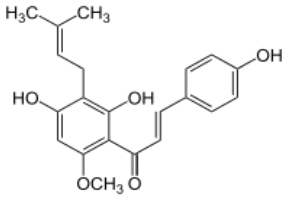
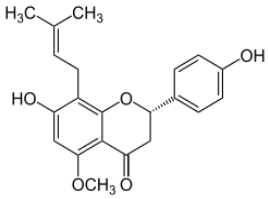
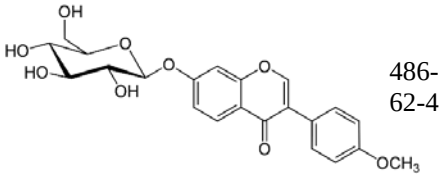
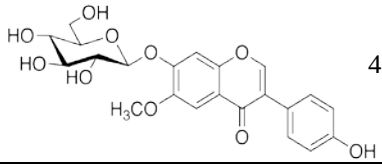
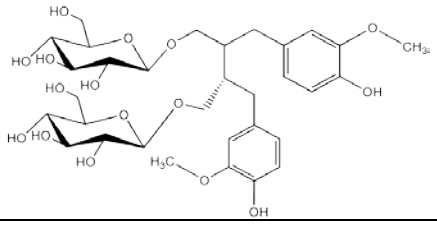
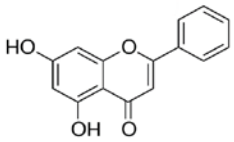
Analyte	Chemical structures	CAS	Class	Molecular weight
ENTD $C_{18}H_{18}O_4$		78473-71-9	Lignan	302.4
8-PN $C_{20}H_{20}O_5$		53846-50-7	Flavonoid (prenylflavonoid)	340.4
6-PN $C_{20}H_{20}O_5$		68236-13-5	Flavonoid (prenylflavonoid)	340.4
XN $C_{21}H_{22}O_5$		6754-58-1	Flavonoid (prenylflavonoid)	354.4
IXN $C_{21}H_{22}O_5$		521-48-2	Flavonoid (prenylflavonoid)	354.4
ON $C_{22}H_{22}O_9$		486-62-4	Isoflavonoid	430.4
GLY $C_{22}H_{22}O_{10}$		40246-10-4	Isoflavonoid	446.4

Table 2.1.1.2. *(Continued)*

Analyte	Chemical structures	CAS	Class	Molecular weight
SDG $C_{32}H_{46}O_{16}$		148244-82-0	Lignan	686.7
IS $C_{15}H_{10}O_4$		480-40-0	Flavonoid	254.2

2.2.2 Sample collection

Sediment samples S1 to S4 were collected using a vibracore system designed for collecting relatively long cores (4 inches in diameter and up to 6 feet in length) in high energy shallow coastal environments according to the procedure reported in a previous study and illustrated in Figure 2.2.2.1 (Mulabagal, Wilson, & Hayworth, 2017). Surface water samples were collected in duplicate into high-density polyethylene (HDPE) containers (8L total per sample) using a 1L stainless steel Kemmerer Bottle water sampler. Water samples were collected at one-half of the total water depth at each sampling location. All sediment and water samples were transported in coolers on ice (4 ºC) to the laboratory and stored at -20 ºC until analyzed.

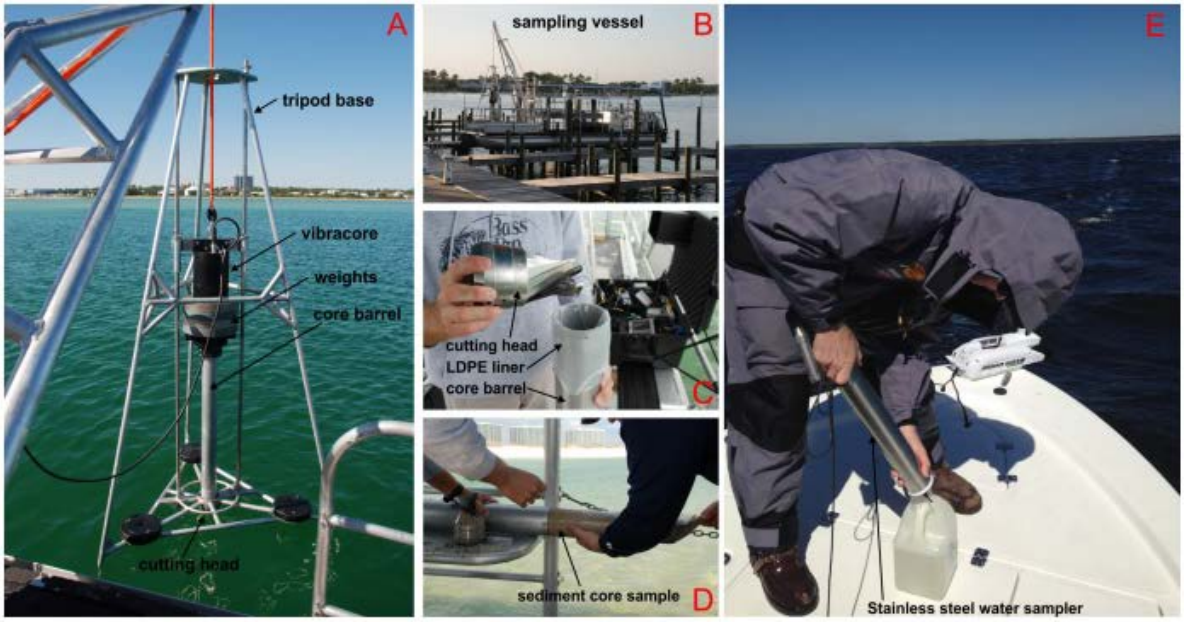


Figure 2.2.2.1: Detailed illustration of sampling instruments.

2.2.3 Sample preparation and cleanup procedure

Sediment core samples were thawed at room temperature and divided into two portions (each 3ft in length) and labeled as the top (T, sediment close to the surface) and bottom part (B, sediment close to the bottom of collection point). Samples S1 to S4 were extracted according to the published procedure using a mixture of methanol (80%) and water (20%) as extraction solvents (Mulabagal et al., 2017). A schematic of the sample preparation procedures is shown in Figure 2.2.3.1.

Estuarine surface water samples were defrosted to room temperature before extraction. Each sample (4L) was pulled through GE Whatman glass microfiber 1.2 µm filters (GE, Boston, MA, USA) using the micro-filtration assembly under vacuum to remove all suspended particulates. Filtrates were processed with solid phase extraction (SPE) using a Waters Oasis PRiME HLB 6cc extraction cartridges (Waters Corporation, Milford, MA, USA) using a vacuum manifold system. Samples were loaded onto SPE

cartridges and then washed with LC grade water (10 mL) to remove any salt-based matrices. The cartridges were vacuum-dried, and the retained target analytes on the sorbent were eluted with methanol (10 mL). The eluent was filtered through 0.2µm membrane syringe filters and spiked with an internal standard (chrysin, 5 ng/mL) prior to UHPLC-MS/MS analysis. The sample preparation procedures are illustrated in Figure 2.2.3.1.

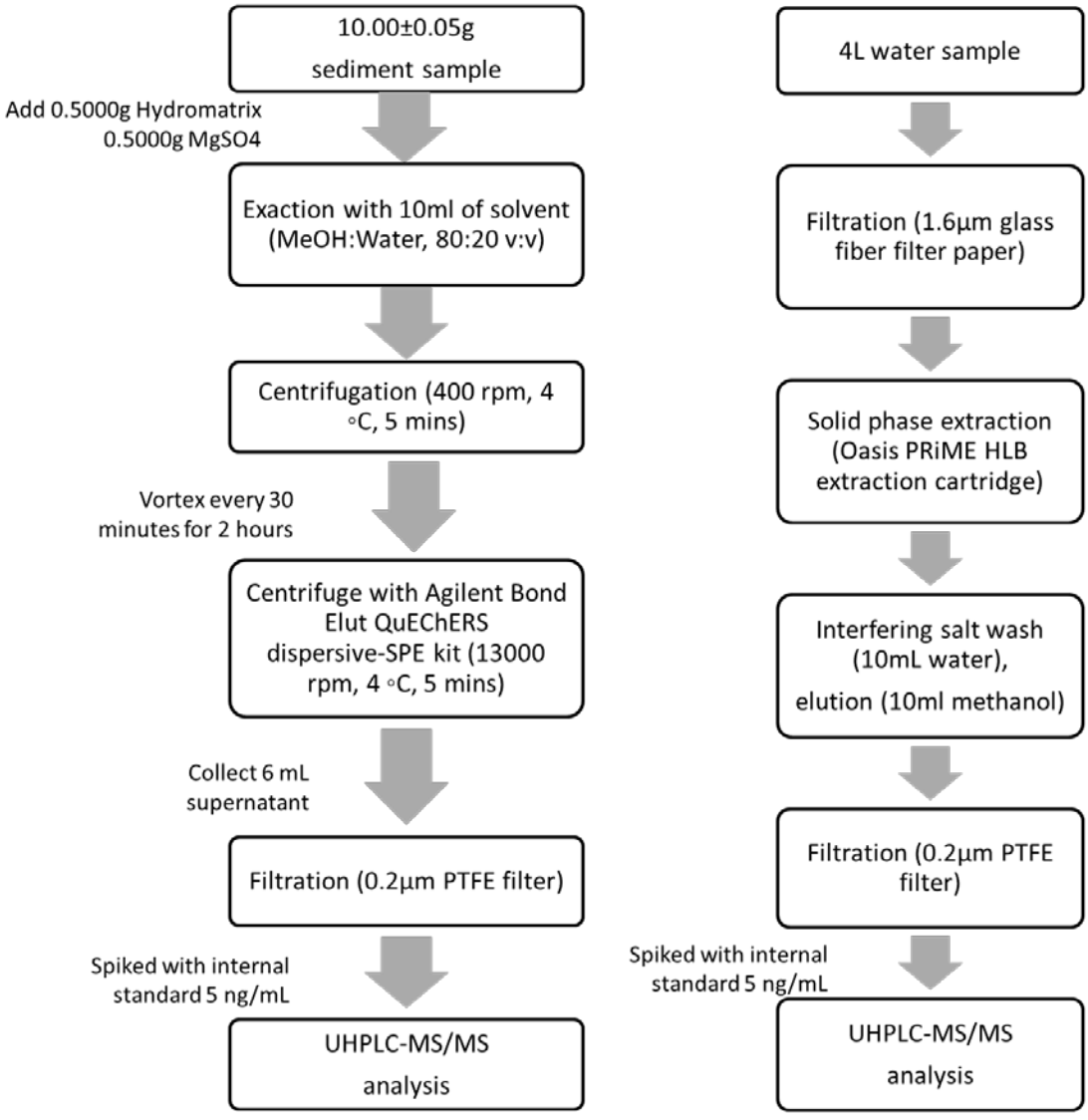


Figure 2.2.3.1: Scheme of the sample preparation procedures for sediment samples (left) and surface water samples (right).

2.2.4. Analytical standards preparation

Analytical grade PEPP standards (purity >98%) were accurately weighed and dissolved in methanol to obtain stock solutions at a concentration of mg/mL, except apigenin which was prepared in dimethyl sulfoxide (DMSO) due to its low solubility in methanol. The ratio of the solvent mixture adopted for standard solution dilution was determined based on the overall solubility of all analytes of interest. Stock solutions were diluted into working standards (0.1 and 0.01 µg/mL) with methanol/water (90:10, v/v), and used in full scan (MS2), selected ion monitoring (SIM) and product ion (PI) scan experiments. Multiple reaction monitoring (MRM) optimization experiments were conducted with PEPP standard mixture at 0.01 µg/mL. Calibration levels were prepared by diluting stock solutions using methanol/water (90:10, v/v) to obtain the desired concentration range of 0.1 to 50 ng/mL.

Chrysin was selected as the internal standard because of the structural similarity to those of the target analytes. In addition, application of chrysin as an internal standard for PEPPs determination has been reported in the literature (Magiera, Baranowska, & Kusa, 2012; Prasain et al., 2010; Soucy, Parkinson, Sochaski, & Borghoff, 2006). The stock solution of chrysin was prepared with a solvent mixture (ethanol: methanol, 2:1, v/v) into 1µg/mL concentration based on its solubility. The stock solutions were then further diluted using methanol/water (90:10, v/v) to get desired concentrations used in the recovery study and quantitative analysis.

2.3 Method development and optimization

UHPL-MS/MS analysis of target PEPPs was performed using an Agilent 1290

high-speed pump (model G7120A) connected to a triple quadrupole mass spectrometer (model G6460C) with an Agilent Jet-Stream Electrospray Ionization source (AJS-ESI, Agilent Technologies Inc., Santa Clara, CA, USA). Chromatography separation was tested using narrow bore UHPLC columns (InfinityLab Poroshell 120 Bonus-RP, 2.1 x 100 mm, 2.7 µm, Part No. 861768-901; and InfinityLab Poroshell 120 Phenyl-Hexyl, 2.1 x 100 mm, 2.7 µm, Part No. 695775-912, Agilent Technologies Inc., Santa Clara, CA, USA). Data analysis was conducted using Agilent MassHunter Workstation Software Qualitative Analysis Version B.07.00. The starting parameters of the instruments recommended by Agilent were applied as the initial condition and the column used was EclipsePlusC18 RRHD (1.8 µm, 2.1×50 mm, Agilent Technologies Inc., Santa Clara, CA, USA). The preferred ionization mode and therefore the precursor ion of each compound was determined by Full (MS2) scan experiment. Preliminary retention times were acquired to confirm the identity of each compound and were referenced for fragmentor voltage (FV) optimization to enhance chromatogram signals in the Single Ion Monitoring (SIM) scan experiment. Product Ion (PI) scan experiments were then carried out to determine suitable transitions from every precursor ion, which were used as the quantitative ion (quantifier) and the qualitative ion (qualifier). Subsequently, collision cell energies (CE) were optimized to obtain the highest chromatogram abundancy for all analytes. Lastly, the experiment was set up in multiple reaction monitoring (MRM) mode so that instrumental conditions were further improved to monitor optimal response for each target compound. MRM parameters of IS were optimized based on the reported literature (Magiera et al., 2012; Prasain et al., 2010).

2.3.1. Full (MS2) scan analysis

The MS2 scan is the basic structural identification experiment, set up to individually scan the molecular ion between mass to charge ratio (m/z) of 50 and 80 plus the molecular weight of each compound. In the MS2 scan, the compound is either protonated (M+H) or deprotonated (M-H) with a possibility of adduct formation due to the potential existence of salts in the standard solution or fragmentation into smaller mass pieces. Full scan mode was applied to determine the favorable ionization mode (positive or negative), and thus the precursor ion for each individual compound. The preferred ionization mode was determined by comparing the abundance of the peaks in chromatograms. The mass to charge ratios were obtained in the corresponding mass spectrum at the center of the peak (Figure A1). Because the scanning range was broad, individual standards in high concentrations were used for this experiment (0.1 µg/mL).

2.3.2. SIM scan analysis with FV optimization

SIM scan selectively looks for the specific analytes with increased sensitivity compared to MS2 scan. The sequence of elution in the chromatogram under initial conditions was estimated using SIM scan analysis on individual standards. The number above each peak was the reference retention time (RT) of the compound under initial conditions, which was then used for compound identification for FV optimization. Experiments for SDG were carried out separately after the complete optimization of the method due to the ionization limit of ESI for the target analytes. FV optimization was conducted to enhance instrument responses to each compound as indicated by the magnitude of the peak area detected by the instrument. FV optimization was performed on

individual analytes in the pure solvent using a series of fragmentor voltages from 80 V to 190 V with 10 V steps in either positive or negative mode according to the favorable ionization behaviors observed in the full scan experiment addressed above (Figure A2).

2.3.3. PI scan analysis with CE optimization

Product ion (PI) scan was used to further confirm analytes of interest by identifying their product ions associated with the optimal CE generating the maximum product ion signals obtained in peak spectrums. The PI scan was performed on individual analytes in the pure solvent using a series of collision energies ranging from 0 eV to 60 eV with 5 eV steps in the mode (positive or negative) most suitable for the analysis (Figure A3).

2.3.3. MRM analysis and method optimization

The MRM experiment, which is a quantitative target analyte scan, was carried out to detect the transitions of all compounds to each product ion simultaneously using the optimized FV and CE for the individual compounds. Cell accelerator voltage was reduced to 4V to reduce the over-breakdown of compounds before fragmentation and the dwell time was shortened to 40 msec to confine the peak width. Further optimization was performed by adjusting the dissolving solvent mixture used in the preparation of working standards, as the solubility of each compound in the different solvents was variable. The dissolving solvents tested were methanol: water (90:10, v/v) and methanol: water (80:20, v/v). Methanol: water (90:10, v/v) yielded optimal results.

2.4 Method Validation

Data quantification analysis was conducted using Agilent MassHunter Workstation

Software Quantitative Analysis for QQQ Version B.07.00. All the target analytes were determined by chromatographic and mass spectral parameters including retention time, specific qualifier and quantifier ion ratio with those of pure standards. The developed optimized method was applied to target analyte analysis in surface water and sediment samples collected from the Perdido Bay estuary. Quantitative analysis was performed using the internal standard method to improve the precision of the method by correcting the possible variation caused by ionization and matrix effects in the samples. The method was then validated to demonstrate its efficacy, selectivity, and robustness for routine analysis. Method linearity was tested by triplicate analysis of PEPP standards mixture at concentrations ranging from 0.1 to 50 ng/mL. Recovery experiments were conducted by spiking sediment and surface water samples at the concentration of 20 and 50 ng/mL. Quantitation was performed with a seven-point calibration curve for each compound. Method specificity was tested by analyzing solvent blanks between sample runs to test any interference due to chromatography carryover effects. Limit of detection (LOD) and limit of quantification (LOQ) values were calculated as the analyte peaks with a signal-to-noise ratio of 3 and 10, respectively.

2.4.1 Calibration curve, LOD, and LOQ

Quantitation was performed with a seven-point calibration curve using working standard mixture with a series of dilution described in Section 2.2.4, where an acceptable linearity with r^2 value ≥ 0.99 is required. LODs and LOQs for each analyte were defined as the concentration, prepared in standard solution (methanol/water: 90:10, v/v), detected by the instrument showing signal to noise ratio no less than 3 and 10, respectively using a series of fifteen concentrations analyzed for four times each (n=4).

2.4.2 Recovery study

Method specificity was tested by analyzing solvent blanks between sample runs to test any interference due to chromatography carryover effects. Repeatability of the method was determined by evaluating the variance of results among replicate analyses (Moreira, Pinto, Gomes, Goicoechea, & Araújo, 2015). Duplicate samples were prepared for each experiment and analyzed five times each. Matrix effects were investigated by determining the variation between known spiking concentrations of each compound and the detected concentration of spiked water and sediment samples. Method accuracy was assessed by spiking sediment and water samples with a standard mixture of two known concentrations, 20 ng/mL and 50 ng/mL, respectively. Percent recoveries (%R) were calculated using analyte response (AR) in spiked and un-spiked sediment and surface water samples according to the following formula:

%R = ((AR spiked sample - AR un-spiked sample)/ spiked standard concentration) * 100.

Chapter 3

Results and discussion

3.1. Method development

Although initially not included in our list of analytes, ononin was of interest due to its existence in chickpeas and soybeans, which are often consumed in human diets. When this analyte was added to the list of target compounds at a later stage of our method development, we adapted the MRM parameters of ononin from the literature. Experiments for SDG were terminated during the MRM method development due to an undesirable chromatography result. An extreme concentration above 500 ng/mL had to be used to in order to quantify SDG. This was problematic because long-term loading of any compound in high concentration could cause carryover of the compound which in turn could interfere with quantification accuracy. Additionally, SDG has never been reported at elevated concentration levels in natural aquatic systems and was unlikely to be present above detection levels in the Perdido Bay estuary water and sediment samples.

Several mobile phase combinations with different modifiers used in the binary pump system were assessed in this study to generate overall narrower peaks and fewer ion suppressions: A: 5mM ammonium formate in water and B: 5mM ammonium formate in methanol; A: 99% water w/ 0.1% formic acid, 1% organic (methanol: acetonitrile, 90:10, v/v) and B: 99% organic (methanol: acetonitrile, 90:10, v/v) and 1% water with 0.1%

formic acid; A: 1mM ammonium formate in water and B: 1mM ammonium formate in methanol; A: 5mM ammonium acetate in water and B: 5mM ammonium acetate in methanol. Among these combinations, better overall separation and less ion suppression were observed using a solvent combination of A: 1mM ammonium formate in water and B: 1mM ammonium formate in methanol. A comparison of the MRM results in an overlaid mode using 10 ng/mL standard mixture using different mobile phases is presented in Figure A5.

Due to the cluster of retention times of some compounds, a UHPLC column with different packed materials and length (Agilent Poroshell 120 Phenyl Hexyl column, 2.7 µm, 2.1×100 mm) was also examined to differentiate retention times among closely eluted compounds, so that the separation could be achieved in shorter run time. Various gradient programs of mobile phases were then tested to maximize baseline separation. Higher responses and narrower peaks were noted by adjusting the column compartment temperature, sample injection volume, and the flow rate to 40 °C, 5 µL and 0.25 mL/min, respectively. For the purpose of trace level detection of selected PEPPs, the MS/MS parameters were optimized by conducting a series of MRM experiments with changes of single or two parameters each time. The sheath gas temperature and flow were reduced to 350 °C and 10 L/min, respectively, to optimize ionization. Negative capillary voltage and negative nozzle voltage were modified to 4000 V and 1000 V, respectively, to moderate the ionization of compounds analyzed in negative mode. The optimal mobile phase gradient program and MS/MS instrumental conditions are summarized in Table 3.1.1. The final MRM acquisition information is shown in Table 3.1.2. Chromatographic separation achieved for all the target analytes is presented in Figure 3.1.1.

Table 3.1.1: Optimized instrumental conditions.

UHPLC system	Agilent 1290 infinity II model G7120A	
Column	Agilent Poroshell 120 Phenyl Hexyl column, 2.7 µm, 2.1×100 mm	
Mobile phase	A: 1mM ammonium formate in water	
	B: 1mM ammonium formate in methanol	
Gradient method conditions	Time (min)	B %
	0	30
	1	50
	4	50
	4.5	55
	6.2	55
	7	68
	11.5	75
	13	85
	13.2	99
	13.9	99
	14	30
Post run:	3 min	
Flowrate	0.25 mL/min	
Total run time analysis	13 min	
Column temperature	40 °C	
Injection volume	5 µL	
Injection wash solvent	Methanol/Water (70:30, v/v)	
Mass spectrometry	Agilent 6400 Series Triple Quadrupole LC/MS model G6460C	
Gas temperature	300 °C	
Gas flow	10 L/min	
Nebulizer	40 psi	
Sheath gas temperature	350 °C	
Sheath gas flow	10 L/min	
Capillary voltage	+4000 V/-4000 V	
Nozzle voltage	+500 V/-1000 V	
Delta EMV	+200 V/-200 V	
Cell acceleration voltage	4V	
MS1/MS2 resolution	Unit	

Table 3.1.2: Optimized quantitative UHPLC-MRM parameters for target analytes.

	Target analyte	Retention time (R_t, min)	Precursor Ion	Product Ions	Fragmentor voltage (V)	Collision cell energy (eV)	Polarity
1	RES	2.943	227	143	130	25	N
				185	130	15	
2	DAID	4.064	253	132	160	40	N
				208	160	30	
3	FORM	7.814	267	252	130	20	N
				222.9	130	30	
4	APIG	6.388	269	116.9	150	30	N
				151	150	25	
5	GEN	5.649	269	132.9	150	30	N
				63.1	150	35	
6	NARN	5.959	271	151	120	10	N
				118.9	120	25	
7	BIO-A	9.187	285.1	213.1	150	40	P
				152	150	25	
8	ENTL	5.879	297	253.1	140	15	N
				106.9	140	25	
9	ENTD	3.94	301.1	253	140	20	N
				106	140	30	
10	8-PN	9.833	339.1	218.9	140	15	N
				118.9	140	25	
11	6-PN	11.933	339.1	219.1	150	15	N
				118.9	150	25	
12	XN	12.158	353.1	118.9	150	20	N
				233	150	15	
13	IXN	8.911	355.1	179	130	25	P
				299	130	15	
14	ON	4.19	431.1	269.1	100	10	P
15	GLY	2.476	447.1	285	80	5	P
	IS	8.994	253	143	150	30	N

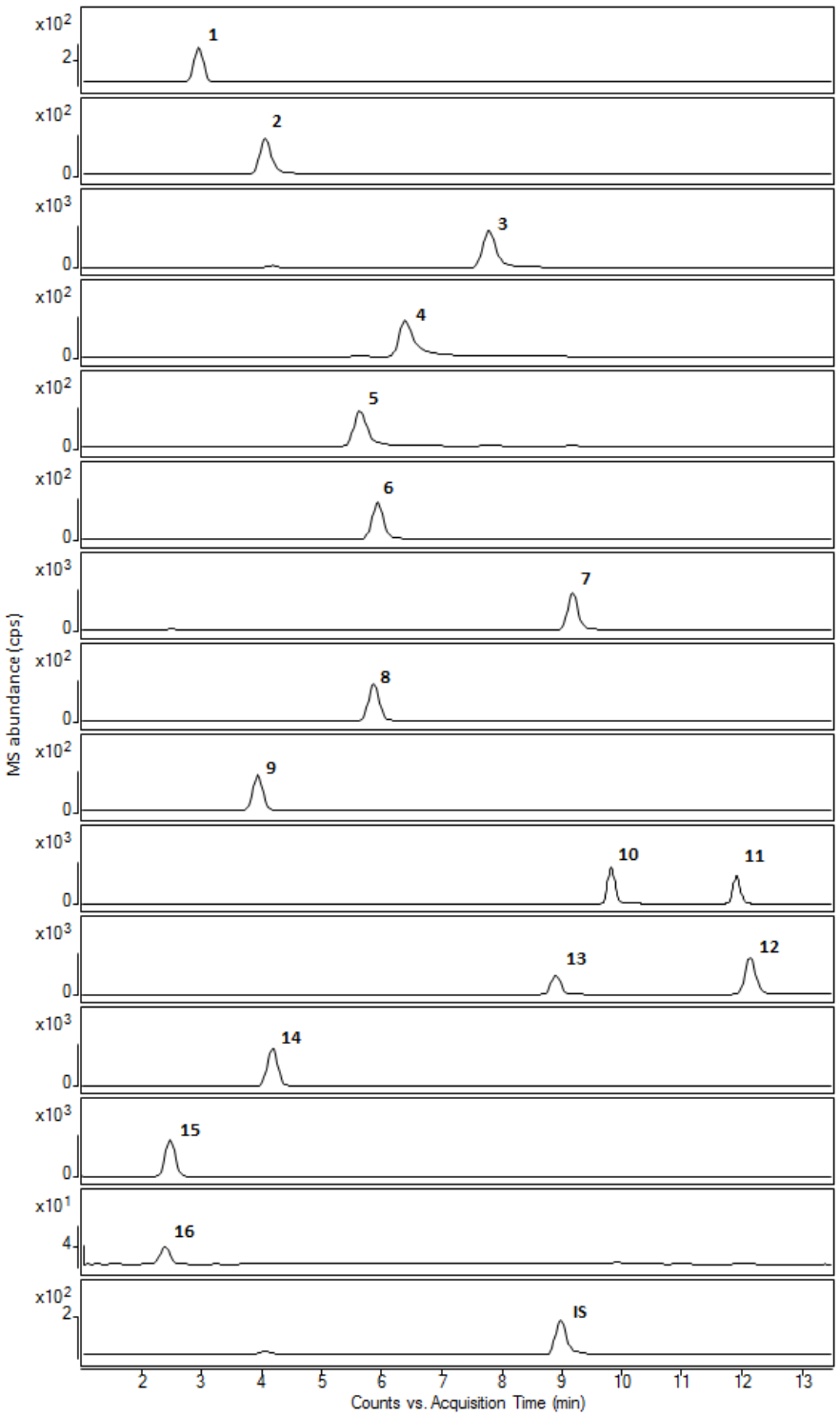


Figure 3.1.1: Extracted UHPLC-MRM chromatogram of target PEPP standards 1-15 (10 ng/mL), and IS (chrysin, 2ng/mL), respectively. Compound numbering as listed in Table 3.1.2.

3.2. Method validation

Environmental samples are complex matrices; therefore care must be taken to ensure proper extraction and pre-concentration of real environmental samples. Water extraction procedures using Whatman GF/C filters and Oasis HLB cartridges to perform the SPE were adopted based on previous studies (Bacaloni et al., 2005; Jinguo Kang, Hick, & Price, 2007; Laganà et al., 2004; Levengood et al., 2010). To enhance extraction efficiency, we adapted a previously published environmental sample extraction protocol and optimized the extraction solvent, composition, and extraction time parameters (Mulabagal et al., 2017). Matrix effects were investigated by determining the variation between known spiking concentrations of each compound and the detected concentrations of spiked water and sediment samples. All seven-point calibration curves developed for quantitation analysis showed strong linearity with $r^2 > 0.998$ (Figure A6). LODs and LOQs for all target analytes were in the range of 0.03 to 0.98 pg/inj and 1.95 to 7.81 pg/inj as shown in Table 3.2.1, respectively. Recovery experiments were performed by post-extraction spiking experiments, and the percent recovery result is displayed in Table 3.2.2. The recoveries of the majority of the analytes of interest were between 70 to 120% in sediment samples.

Table 3.2.1: LODs and LOQs for all target PEPP analytes.

	Target analyte	**LOD pg/inj.**	**LOQ pg/inj.**
1	Resveratrol	0.98	7.81
2	Daidzein	0.24	3.91
3	Formononetin	0.06	1.95
4	Apigenin	0.12	7.81
5	Genistein	0.24	3.91
6	Naringenin	0.12	1.95
7	Biochanin A	0.12	1.95
8	Enterolactone	0.24	1.95
9	Enterodiol	0.12	1.95
10	8-Prenylnaringenin	0.06	1.95
11	6-Prenylnaringenin	0.03	1.95
12	Xanthohumol	0.03	1.95
13	Isoxanthohumol	0.06	1.95
14	Ononin	0.03	0.98
15	Glycitin	0.06	0.98
	Chrysin (IS)		

Table 3.2.2: Target analyte percent recoveries (n = 6) for sediment and water samples spiked with standard analyte mixture at 20ng/mL and 50 ng/mL, respectively.

Analyte	**S1 T**	**S1 B**	**S2 T**	**S2 B**	**S3 T**	**S3 B**
RES	79.7 ± 4.0	83.4 ± 5.2	83.0 ± 4.0	75.0 ± 4.0	64.2 ± 4.6	50.6 ± 4.0
DAID	110.5 ± 3.1	107.8 ± 5.3	102.7 ± 7.7	105.2 ± 3.5	105.4 ± 8.3	95.8 ± 3.7
FORM	111.4 ± 4.2	110.0 ± 5.4	109.0 ± 6.7	111.0 ± 4.9	106.2 ± 3.0	110.2 ± 4.4
APIG	108.3 ± 9.5	102.9 ± 6.4	102.4 ± 4.4	90.8 ± 5.9	107.0 ± 4.1	112.1 ± 3.6
GEN	110.9 ± 3.2	111.5 ± 2.0	108.5 ± 6.7	103.1 ± 2.3	111.0 ± 2.1	103.4 ± 6.7
NARN	105.0 ± 6.5	103.7 ± 2.3	101.5 ± 2.4	101.6 ± 2.8	96.1 ± 4.8	88.7 ± 3.6
BIO-A	109.2 ± 3.5	112.0± 5.1	110.1 ± 6.4	105.2 ± 1.7	115.0 ± 4.8	109.2 ± 3.3
ENTL	108.4 ± 7.0	112.9 ± 1.8	114.5± 3.5	114.4 ± 3.6	113.8 ± 5.3	113.6 ± 1.2
ENTD	86.0 ± 3.0	87.3 ± 6.0	79.6 ± 5.2	70.9 ± 4.5	77.2 ± 5.3	66.4 ± 3.1
8-PN	115.3 ± 3.6	117.2 ± 3.0	112.1 ± 4.8	107.3 ± 6.9	115.2 ± 5.8	102.4 ± 5.2
6-PN	114.1 ± 2.2	114.8 ± 2.8	110.4 ± 4.3	107.3 ± 6.6	120.5 ± 4.3	112.3 ± 2.0
XN	108.0 ± 6.4	108.8 ± 6.5	113.7 ± 2.5	123.7 ± 8.2	111.7 ± 3.0	115.1 ± 2.4
IXN	114.0 ± 4.0	116.1 ± 1.8	114.3 ± 3.5	116.8 ± 4.9	106.2 ± 3.0	113.4 ± 1.4
ON	110.2 ± 6.0	108.5 ± 3.3	108.4 ± 4.2	110.2 ± 4.6	112.5 ± 3.6	115.6 ± 4.9
GLY	106.2 ± 3.0	103.1 ± 4.1	96.4 ± 3.7	103.0 ± 2.7	103.4 ± 3.3	102.5 ± 2.9
Analyte	**S4 T**	**S4 B**	**SW 1**	**SW 2**	**SW 3**	**SW 4**
RES	53.2 ± 5.2	45.5 ± 2.2	97.3 ± 3.5	101.4 ±1.3	93.1 ± 2.3	93.4 ± 1.5
DAID	97.4 ± 7.4	99.0 ± 6.0	103.6 ± 5.0	101.4 ± 2.5	99.9 ± 2.5	100.1 ± 1.9
FORM	106.6 ± 4.3	108.0 ± 3.5	105.8 ± 3.0	100.4 ± 1.4	90.7 ± 1.7	98.6 ± 2.2
APIG	103.1 ± 2.9	108.8 ± 6.3	95.8 ± 2.4	104.6 ± 1.6	100.9 ± 5.9	107.9 ± 2.3
GEN	106.0 ± 3.8	103.4 ± 5.4	104.2 ± 7.6	101.0 ± 1.3	99.0 ± 2.5	102.3 ±1.3
NARN	87.7 ± 6.8	85.1 ± 7.0	116.8 ± 4.1	103.8 ± 1.6	97.3 ± 2.8	101.2 ± 1.4
BIO-A	110.4 ± 3.8	113.6 ± 7.1	92.3± 2.2	99.7 ± 5.7	88.8 ± 1.5	89.7 ± 1.6
ENTL	109.6 ± 5.1	112.2 ± 8.1	107.4 ± 1.9	104.2 ± 2.9	101.1 ± 2.5	103.0 ± 2.5
ENTD	73.2 ± 4.7	61.3 ± 4.4	102.2 ± 1.1	106.6 ± 1.1	103.3 ± 2.4	106.9 ± 1.6
8-PN	106.1 ± 4.7	101.1 ± 6.6	108.3 ± 1.4	105.5 ± 3.3	106.0 ± 1.4	113.0 ± 1.1
6-PN	110.9 ± 5.6	111.8 ± 7.7	97.6 ± 1.3	101.1 ± 1.9	102.9 ± 2.1	107.7 ± 1.2
XN	108.7 ± 4.5	110.5 ± 7.1	100.2 ± 1.5	99.2 ± 0.2	97.2 ± 1.1	99.7 ± 1.6
IXN	112.0 ± 6.2	116.9 ± 6.2	93.5 ± 1.9	97.5 ± 1.9	92.4 ± 2.3	92.1 ± 1.6
ON	112.8 ± 2.8	120.5 ± 7.5	79.3 ± 2.6	106.0 ± 1.8	101.4 ± 3.6	102.1 ± 2.4
GLY	97.2 ± 2.4	108.1 ± 7.9	108.1 ± 2.5	109.3 ± 3.6	108.2 ± 1.6	108.4 ± 2.8

3.3. Application to real samples in estuarine media

The validated method was applied to sediment and water samples collected from the Perdido Bay estuary. The equation used to calculate the concentrations in real surface water samples is:

$$C_{real} = \frac{C_{sample} \times V_{sample}}{V_{total}} \quad \text{(Eq. 1)}$$

where C_{real} = concentration in real water sample, ng/L,

C_{sample} = concentration quantified by the instrument, ng/mL,

V_{total} = total sample volume, L,

V_{sample} = total sample extract volume, mL.

In this study, no sediment samples showed peaks related to the target analytes. Water samples WE1 and WE8 contained enterolactone, a lignan, at 3.90 ± 0.39 ng/L and 5.32 ± 0.73 ng/L, respectively (Figure 3.3.1, Figure 3.3.2, and Table A2). The high concentration of enterolactone detected at site SW7 may be related a nearby landfill facility or to a paper mill upstream of SW5. It has been shown that some PEPPs can be discharged at high concentrations into the environment by pulp and paper mills (Ingham et al., 2004). Although only enterolactone was detected in the waters samples collected from the Perdido Bay estuary, PEPPs have been detected in other estuaries by LC-ESI-MS/MS, LC-DAD, GS-MS/MS, and GC-MS^n. Among which, Biochanin A, daidzein, formononetin, and genistein were studied most often. Additionally, formononetin and genistein were reported at relatively higher levels as shown in Table 1.2.4.1. PEPPs in environmental matrices have also been analyzed using LC-QTOF, LC-LTQ MS, LC-DAD (Cahill, Logrippo, Dineen, James, & Caprioli, 2015; Farré et al., 2008; Lundgren & Novak, 2009; Moreira et al., 2015;

C. Ribeiro, Pardal, Martinho, et al., 2009; C. Ribeiro, Pardal, Tiritan, et al., 2009; C. Ribeiro, Tiritan, et al., 2009).

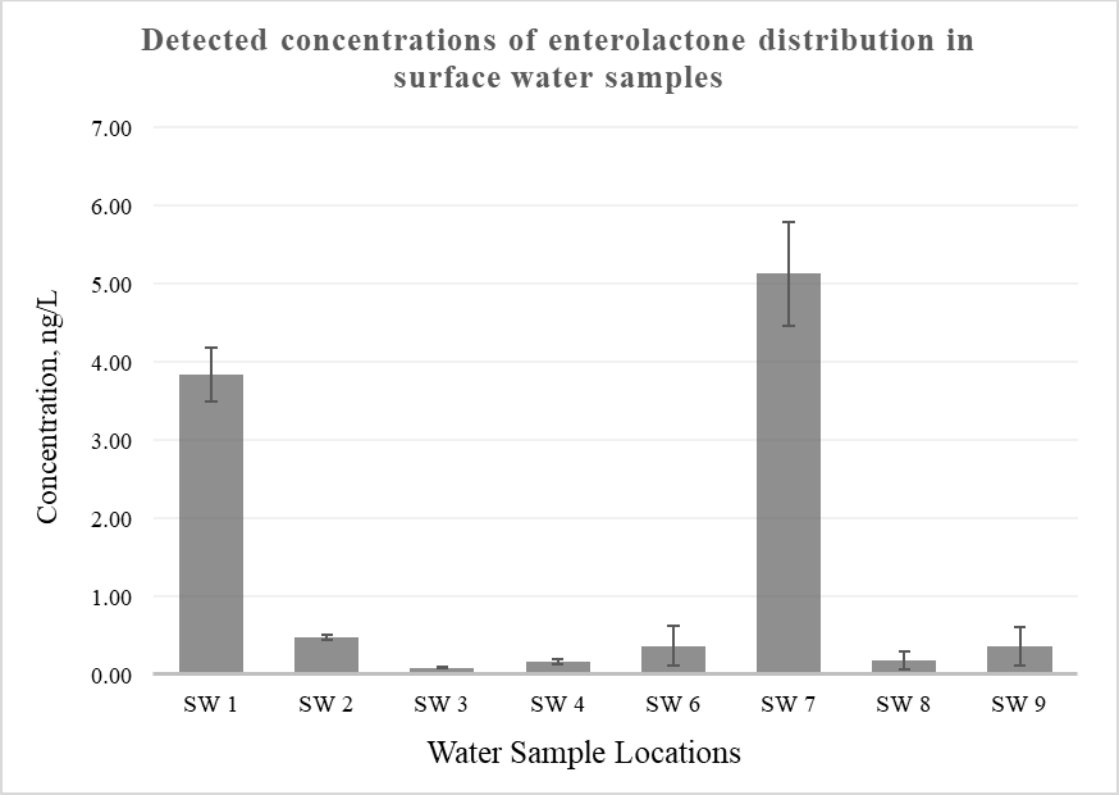


Figure 3.3.1: Detected concentrations of enterolactone in surface water samples in mean ± SD (n=10).

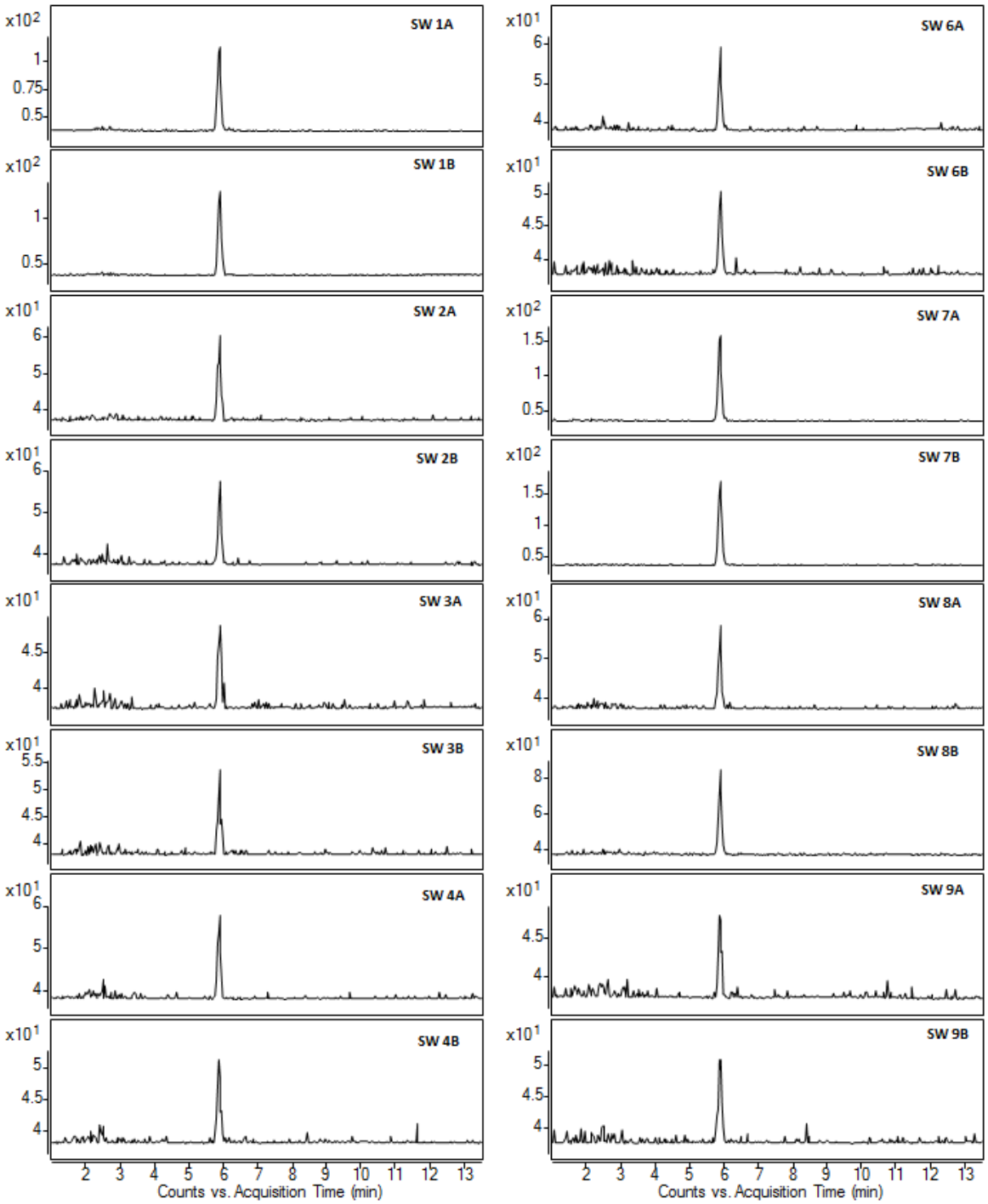


Figure 3.3.2: Extracted UHPLC-MRM chromatogram of enterolactone (m/z = 297.0 ≥ 106.9) detected in Alabama estuarine surface water samples.

3.4. Discussion

Although analytical determination for PEPPs by HPLC- MS/MS in environmental samples have been widely reported, published methods either detected fewer PEPPs simultaneously or had considerably longer instrumental run times than the UHPLC-MS/MS method developed in the present study. For example, a method for determining ten isoflavonoids in seawater, freshwater algae and cyanobacteria using HPLC-ESI-MS/MS and ultrasound-assisted supercritical fluid extraction with a 10 minute run time was developed. (Klejdus, Lojkova, Plaza, Snoblova, & Sterbova, 2010). Published UHPLC-ESI-MS/MS for PEPPs have mainly targeted isoflavonoids in legumes and plant extracts (Delgado-Zamarreño, Pérez-Martín, Bustamante-Rangel, & Carabias-Martínez, 2012; Kiss, Popa, Paltinean, & Loghin, 2012; Vila-Donat et al., 2015). For instance, a method to analyze 12 isoflavonoids (three aglycones and their three corresponding glycosides, and six esterified glycosides) in soymilk within 2.5 minutes using UHPLC-MS/MS was recently developed. Selected reaction monitoring (SRM) was used in this method to achieve baseline separation of analytes with instrumental LODs ranging from 1 to 30 pg and LOQs from 4 to 99 pg (Park & Jung, 2017). This method uses very short runtime because the target analyte list is limited (only isoflavonoids). In addition, SRM can acquire only one mass transition, which is less selective to analytes compared to MRM that utilizes both quantifier and qualifier to confirm the analyte to minimize false positive results. Another recent UHPLC-MS/MS method for the detection of nine PEPPs in milk and yogurt have a run time of 10 minutes. The LOQs of selected PEPPs in this method were 0.02-0.08 μg/L (0.2–0.8 μg/kg dw) and 0.02-0.10 μg/kg (0.2–0.8 μg/kg dw) in milk and yogurt, respectively (Socas-Rodríguez, González-Sálamo, Herrera-Herrera,

Hernández-Borges, & Rodríguez-Delgado, 2017).

Environmental samples, however, are more complex matrices compared to biological or food samples. Few methods for quantification of PEPPs in estuarine samples exist in the literature; the majority of the published methods come from researchers studying estuaries in Portugal using GC-MS or HPLC-DAD for analysis. The method developed and described in this thesis is the first to use UHPLC-MS/MS to detect and quantitate trace concentrations of a broad list of target PEPPs (15 analytes including flavonoids, lignans, isoflavonoids, and stilbenes) in estuarine samples. This novel method has both shorter instrument time and excellent chromatographic separation, along with an efficient and simple sample preparation protocol. The outcomes of this research could significantly contribute to the scientific understanding of how environmental PEPPs are affecting aquatic organisms and humans, and provide a convenient assessment tool for exploring the efficacy of PEPP remediation technologies.

Despite the fact that PEPPs are not regulated by the EPA due to their low estrogenic potencies, PEPP mixtures and/or transformation products existing in natural surface water are capable of exceeding the estrogenic potencies of E2 by orders of magnitude. Thus, it is possible that mixtures of PEPPs or their transformation products can induce estrogenic effects in aquatic organisms comparable to E2. For example, concentrations of prenylflavonoids detected in beer are below levels which can affect human health; however, digested prenylflavonoids can be transformed into a more potent compound (8-PN) with a ten-fold increase in estrogenic potency (Possemiers, Bolca et al. 2006). Furthermore, most EDCs can interfere with normal endocrine function at very low levels, and the link between EDC dose and toxic activity often is nonlinear. Thus, trace levels of

EDCs have the potential to induce adverse effects, while higher concentrations may not (Vandenberg, Colborn et al. 2012).

Chapter 4

Conclusions and recommendations

4.1 Conclusions

PEPPs are naturally occurring compounds synthesized by plants that individually possess weak estrogenic activities, but as mixtures or transformation products can have greatly increased estrogenic potency. The potential health benefits of many PEPPs have increased the production and human and animal consumption of these compounds as food products and dietary supplements. Thus, it is likely that PEPP concentrations in natural aquatic systems will continue to increase in the future. Thus, there is a need for simple, robust and economic trace-level analytical methods to monitor these compounds and to study their possible estrogenic effect on aquatic organisms. The validated quantitative method using UHPLS-MS/MS developed in this study is a simple, fast and sensitive approach for analysis of 15 PEPPs in environmental samples with a total analysis time of 12.5 minutes. The method developed in this study was successfully applied to sediment and surface water samples collected from the Perdido Bay estuary in coastal Alabama. Surface water samples detected one target analyte, enterolactone, with a concentration ranging from 0.08 to 5.69 ng/L.

4.2 Recommendations

The validated method can be employed for analysis of target analytes in complex environmental samples. In addition, the method can be modified and expanded to detect PEPPs in other environmental matrices, such as wastewater, biofilms, and groundwater. Additional, this method can be used to determine the transport and environmental fate of target PEPPs in aquatic environments and the possible influences of these compounds on aquatic organisms (Feifarek, Shappell, & Schoenfuss, 2018; Shappell, Feifarek, Rearick, Bartell, & Schoenfuss, 2018). The method developed in this study can also provide a convenient assessment tool for examining the efficacy of potential PAPP remediation technologies and strategies.

Appendix

Table A1: Summary of reported environmental concentrations of PEPPs, ng/L for water samples and ng/g for solid samples (except for the study done by Smeds et al., 2009, the concentrations of which are in nM). Only reported PEPPs of interest for this research are listed.

Matrix	DAID	GEN	BIO-A	FORM	ENTD	ENTL	RES	Reference
Sewage	7-120	15-384	3-18					(Laganà et al., 2004)
River	2-3	4-7	1-2					
River	2-4	3-5	1-3	n.d.				(Bacaloni et al., 2005)
Wastewater	5-1685	7-954	n.d.-76	n.d.-10				
River	36.2-276	3.96-366	n.d.				n.d.	(Kuster, Azevedo, De Alda, Neto, & Barceló, 2009)
Streams	41	8	<lod-5.6	5.3-13.5				(Kolpin et al., 2010)
Water	5-30	<loq-14	7-22	44-157				(Erbs, Hoerger, Hartmann, & Bucheli, 2007)
Creek water	7	n.d.	n.d.	1	0.2	5		(J. Kang, Price, & Hick, 2006)
Sewage	390	80	2	2	70	600		
Wastewater		50						(Kiparissis, Hughes, Metcalfe, & Ternes, 2001)
Wastewater	5.7-15000	n.d.						(Liu, Ito, Kanjo, & Yamamoto, 2009)
Agricultural soil	36.4-74.2	n.d.-80.5	10.3-258	55.4-3350				(Hoerger et al., 2011)
Ultrapure water					n.d.	0.036		(Smeds et al., 2007)
Tap water					n.d.	0.044		
Humic water					n.d.	0.041		
Sea Water					n.d.	0.086		
Wastewater influent					0.097	4.10		

Table A1: *(Continued)*

Matrix	DAID	GEN	BIO-A	FORM	ENTD	ENTL	RES	Reference
Wastewater effluent					0.092	2.21		
Wastewater		<0.1-<0.6						(Furuichi et al., 2006)
Wastewater	<20-1656	<35-353.3	0-376.72					(Farré et al., 2008)
Metro Plant Effluent	1.8	1.6	n.d.	2				(C. Ribeiro, Pardal, Martinho, et al., 2009)
Industrial Wastewater	<lod-108000	<lod-151000	<lod-300	<lod-299				(Lundgren & Novak, 2009)

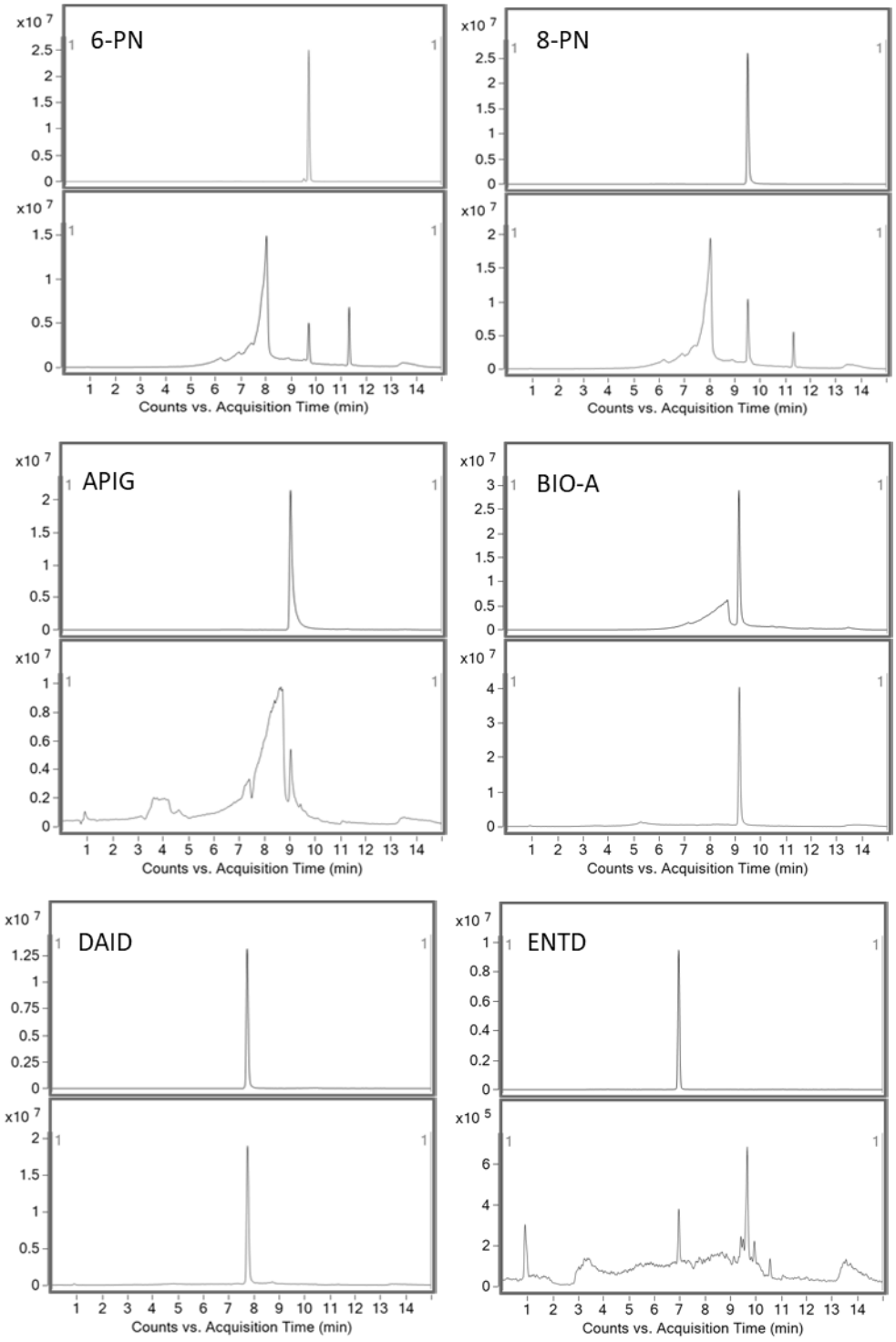


Figure A1: Full scan result of target analytes.

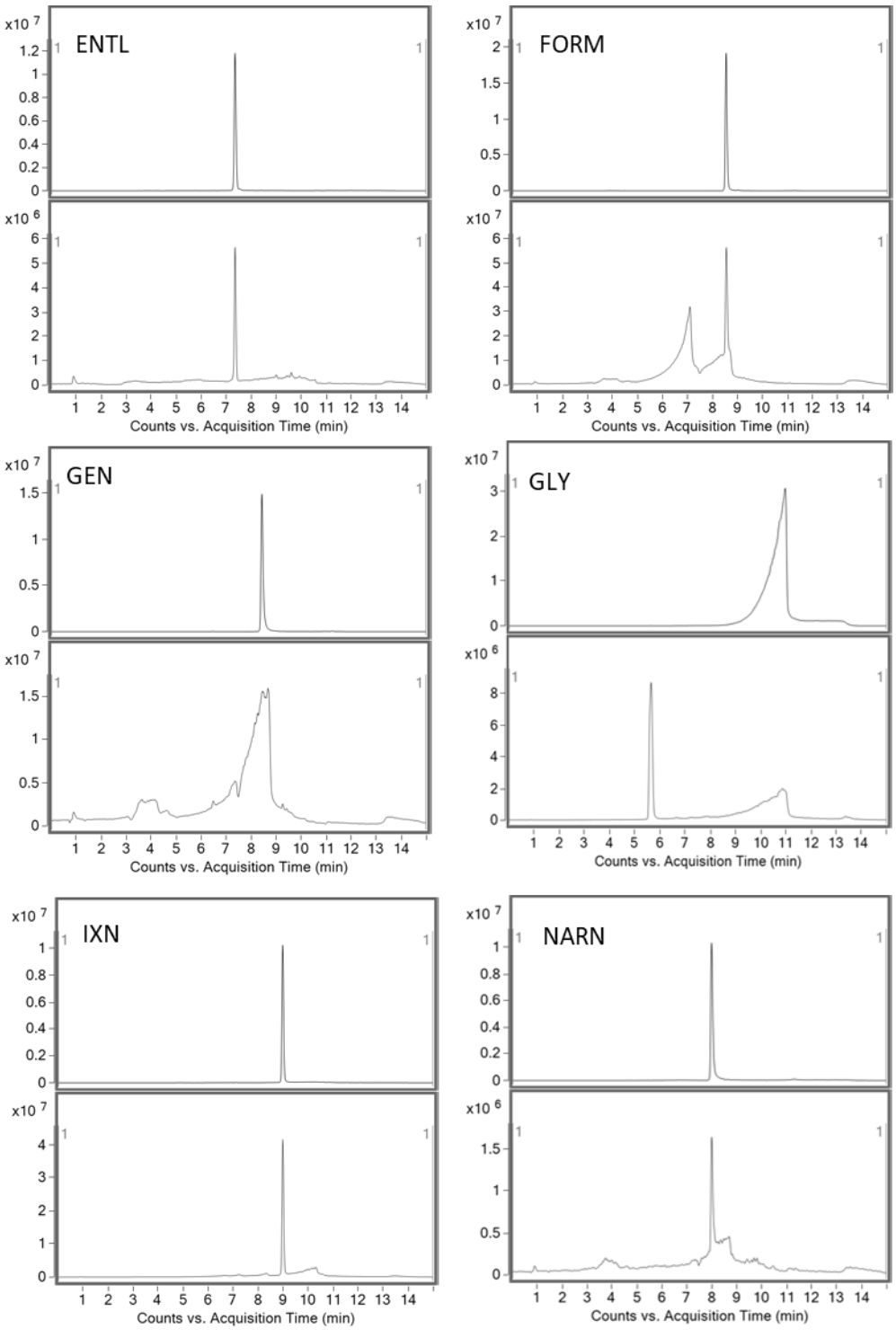


Figure A1: *(Continued)*

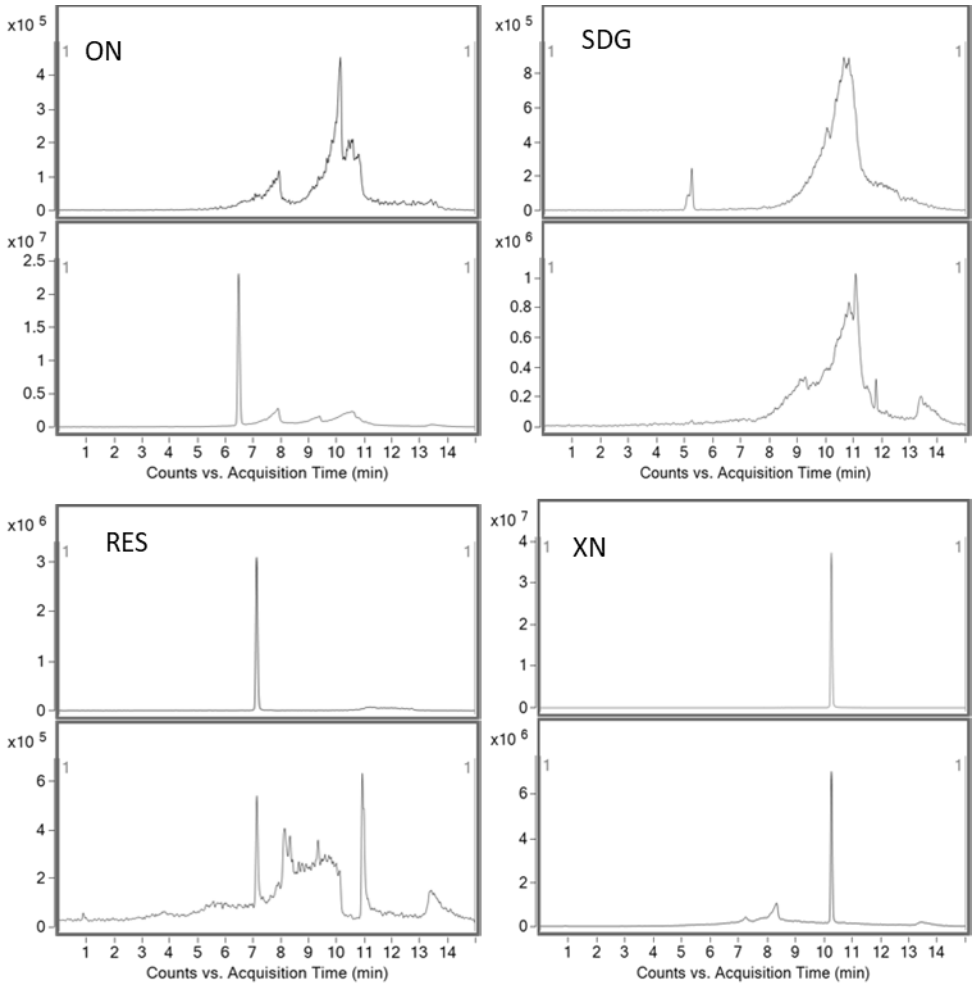


Figure A1: *(Continued)*

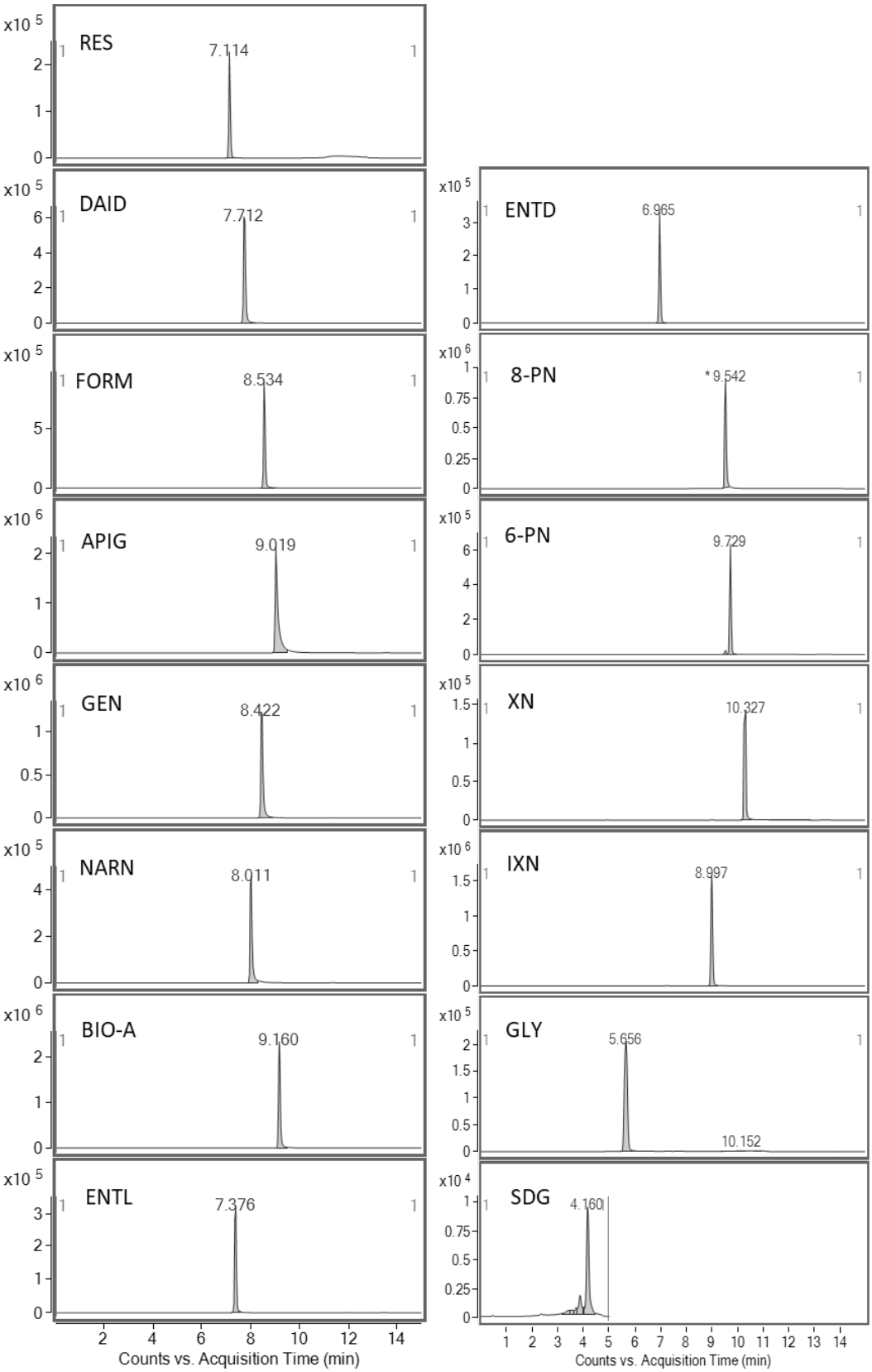


Figure A2: Preliminary retention time results from SIM scan experiment.

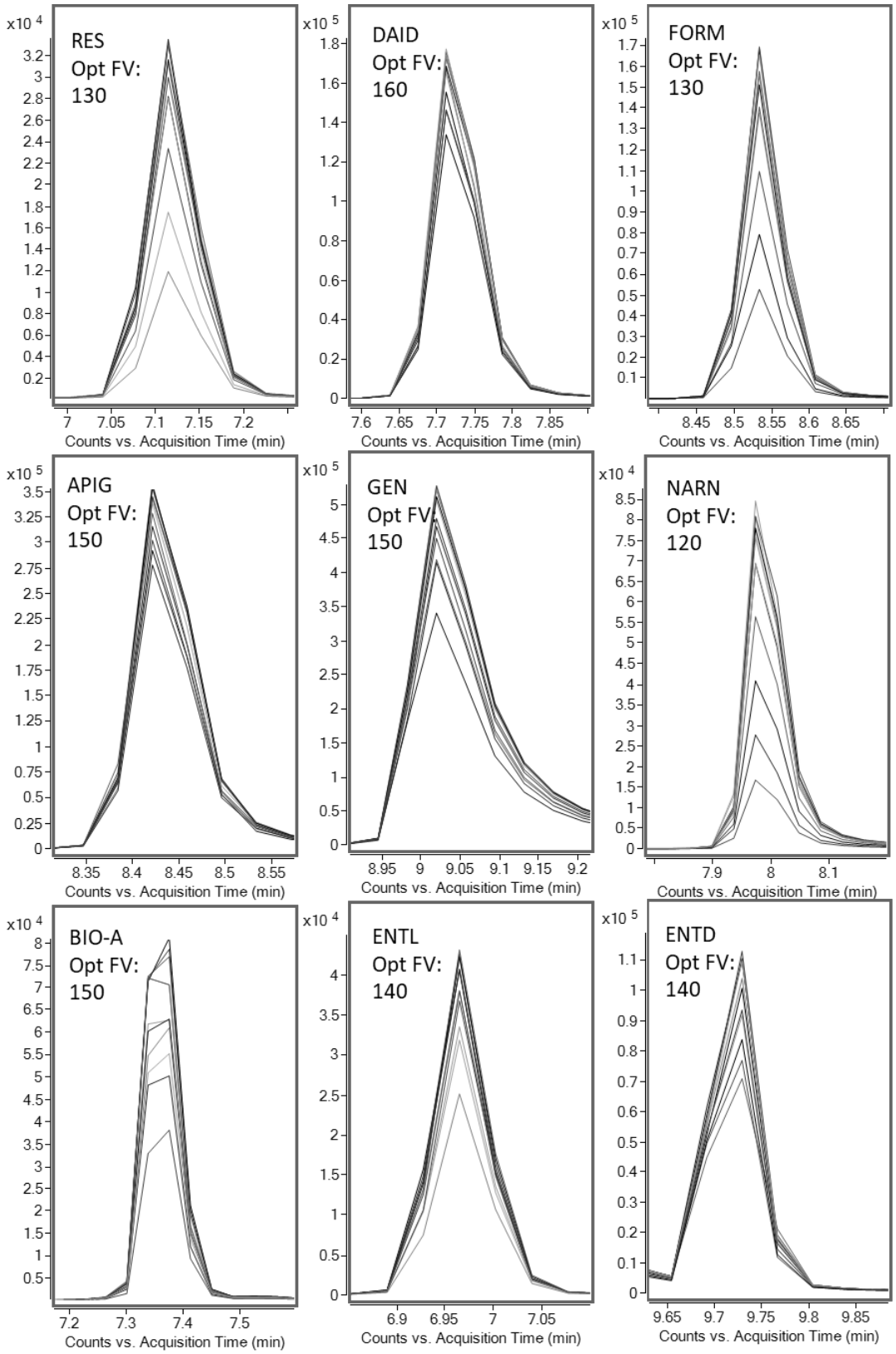


Figure A3: FV optimization results from SIM scan experiment.

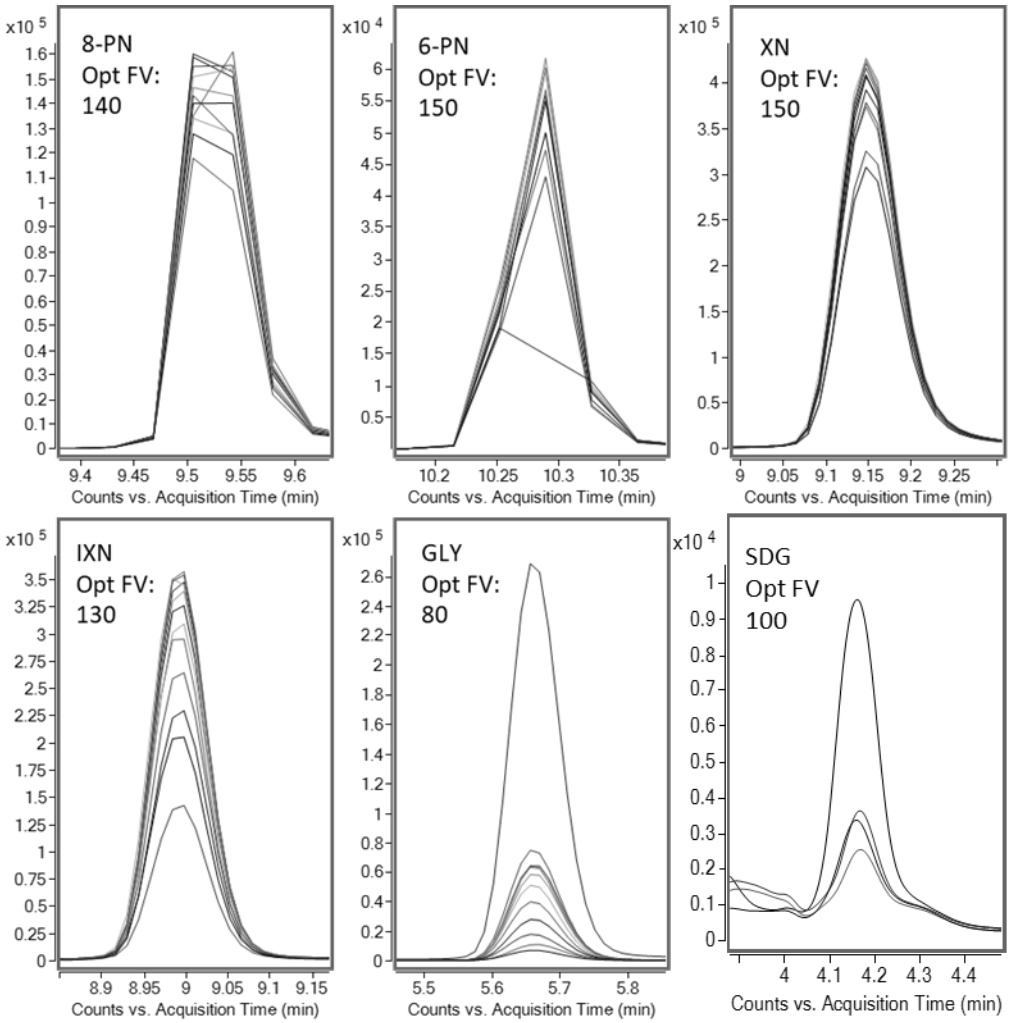


Figure A3: *(Continued)*

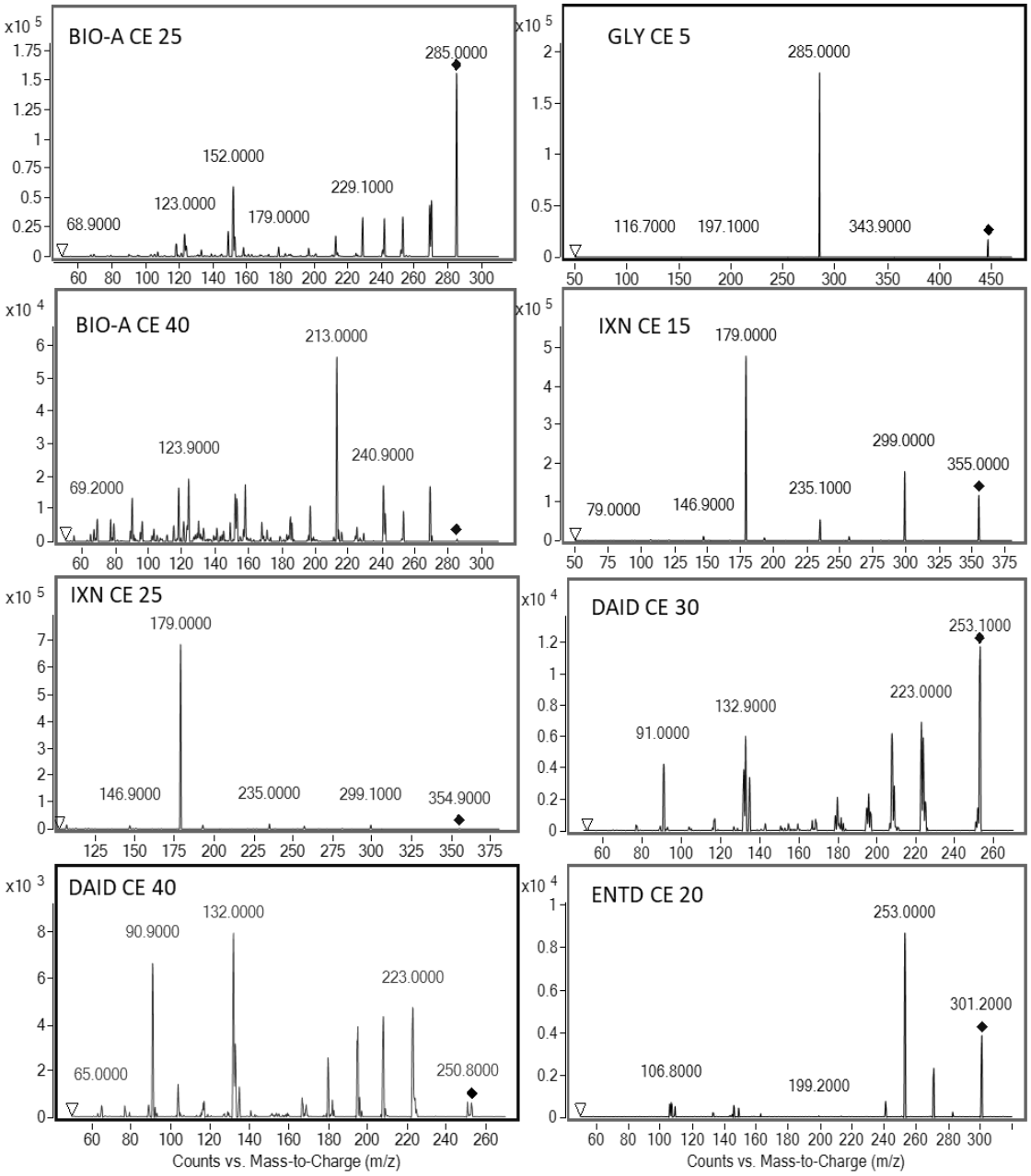


Figure A4: Product ion scan results.

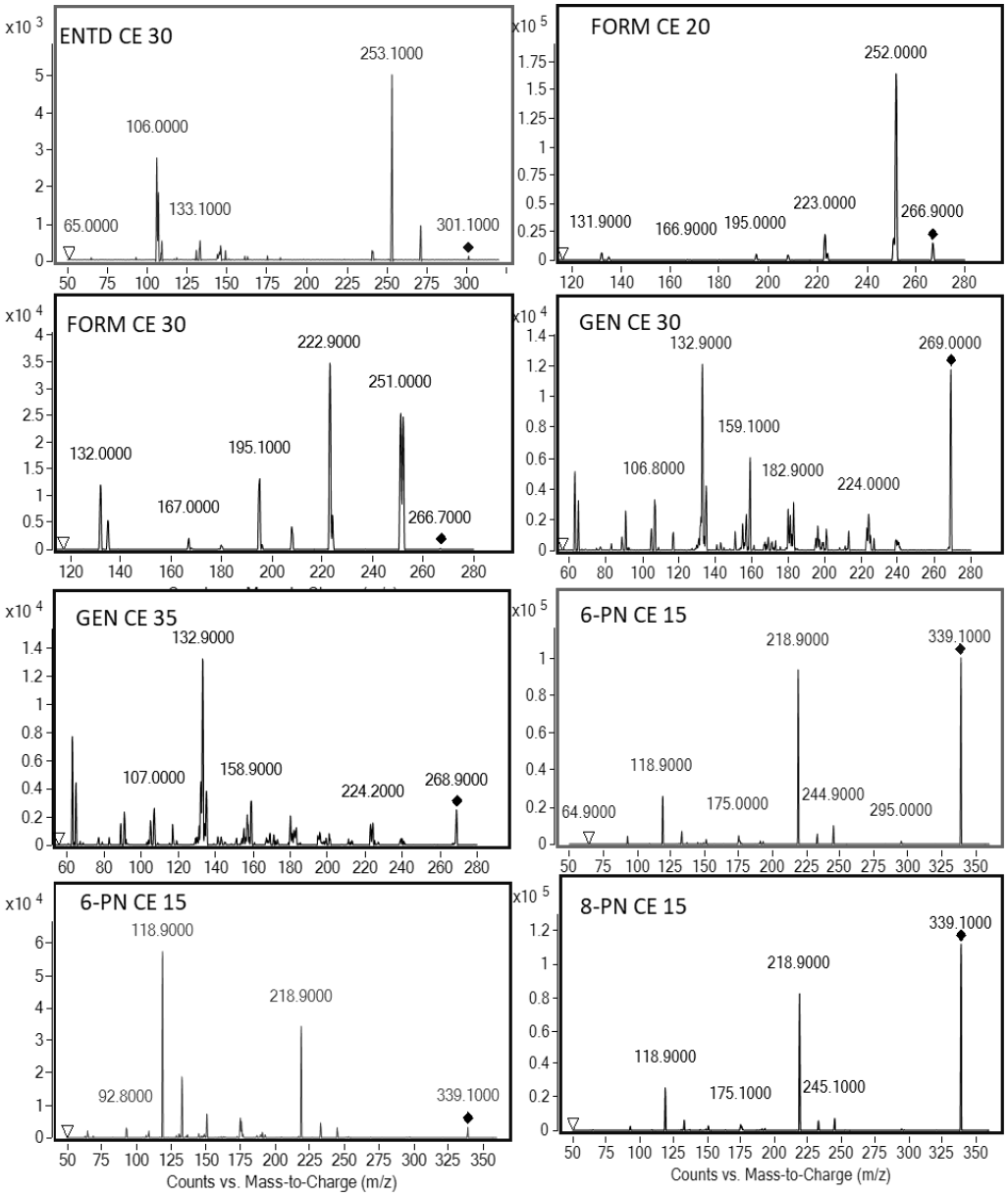


Figure A4: *(Continued)*

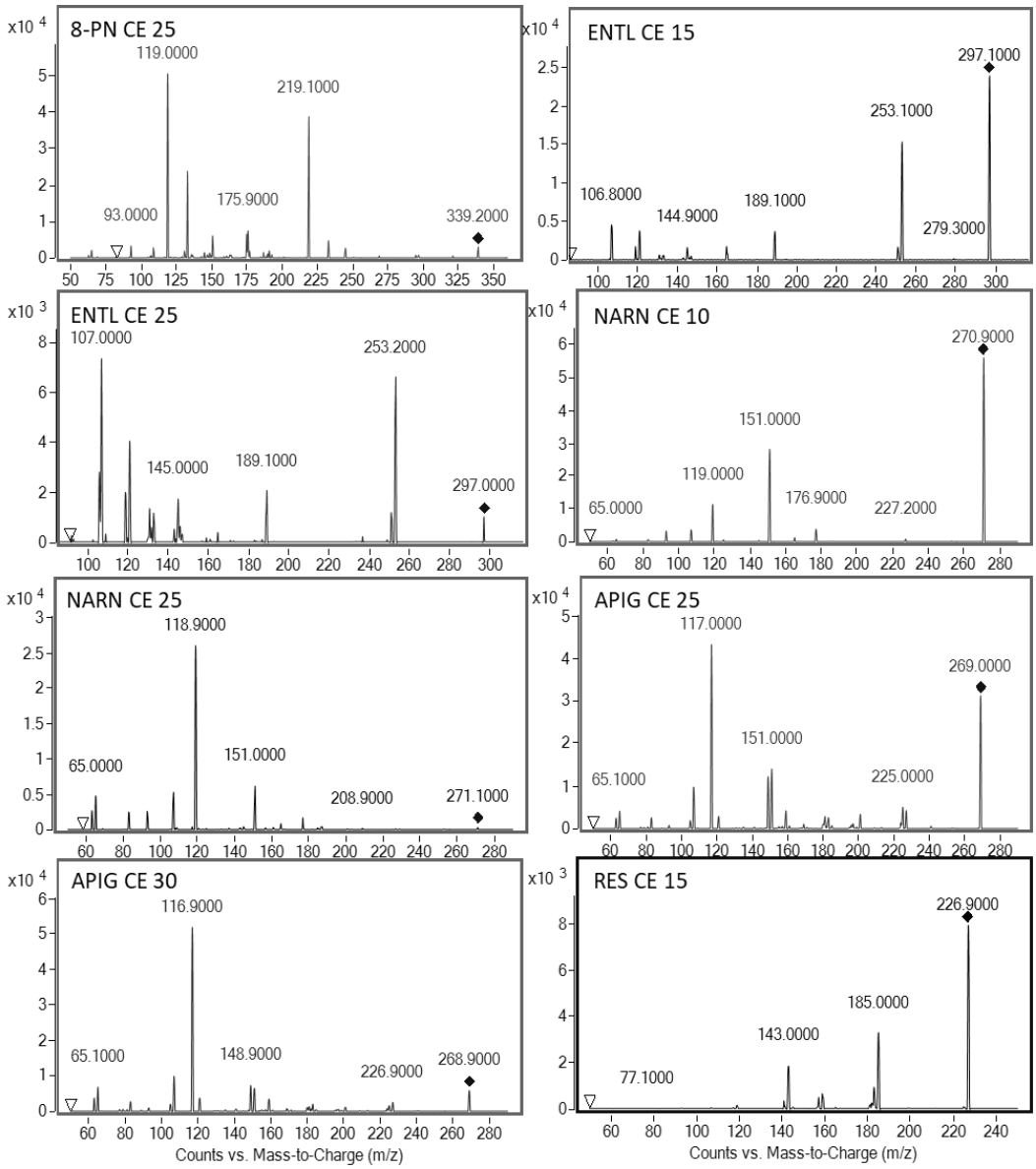


Figure A4: *(Continued)*

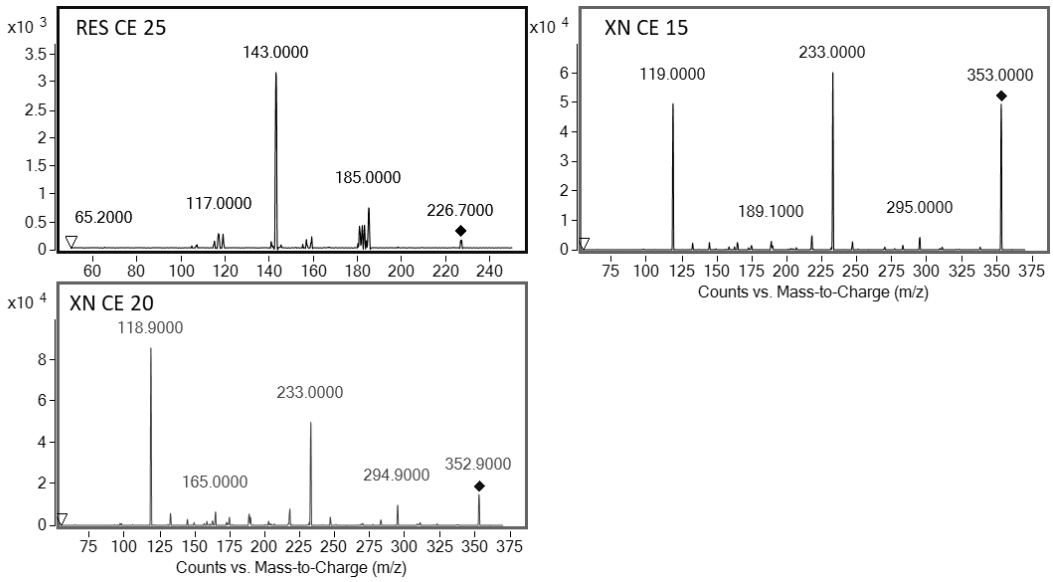


Figure A4: *(Continued)*

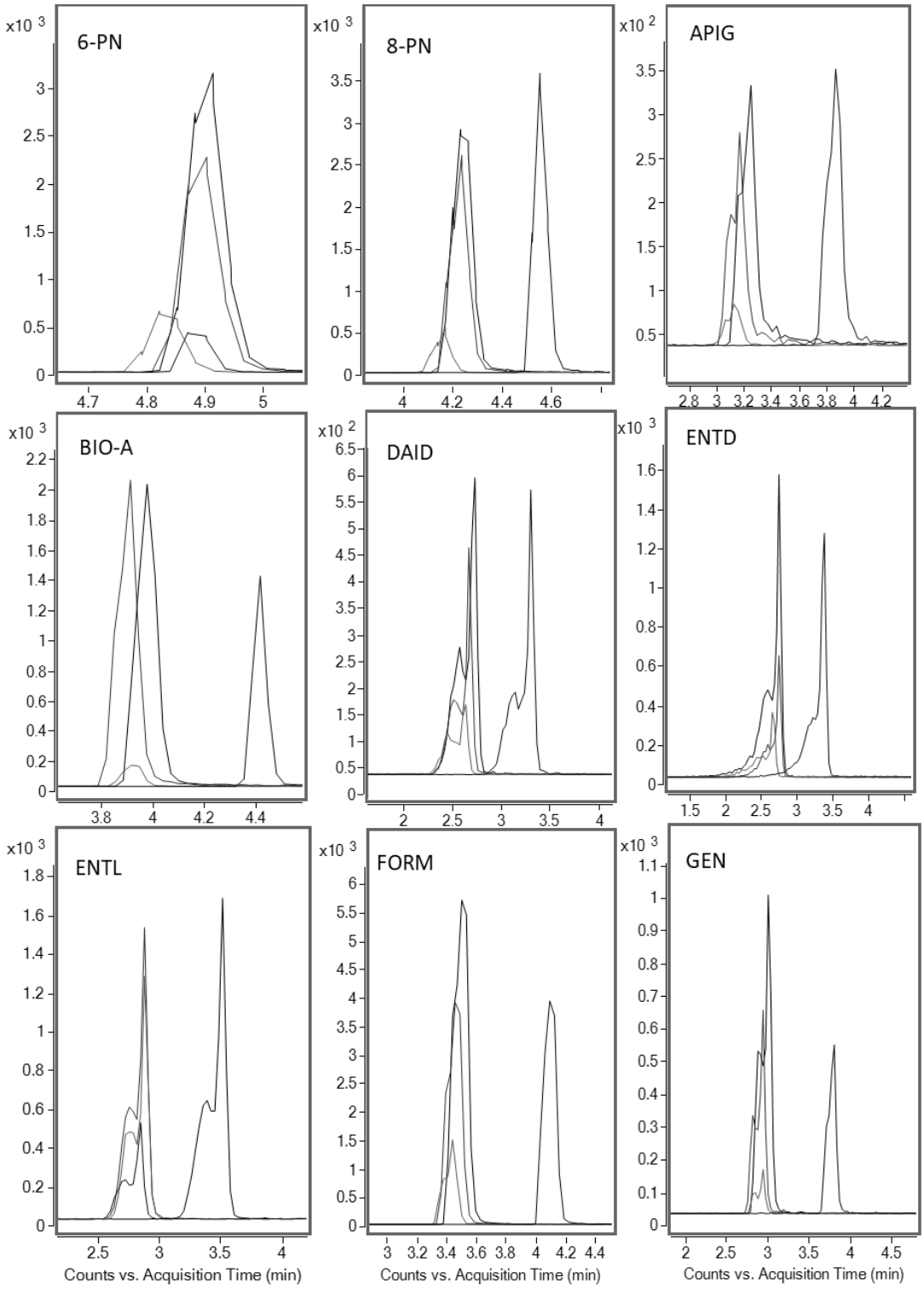


Figure A5: Comparison of the peak response in chromatograms using four different solvent combinations for each analyte in MRM mode. The optimal mobile phase was determined by the highest instrument response for all analytes.

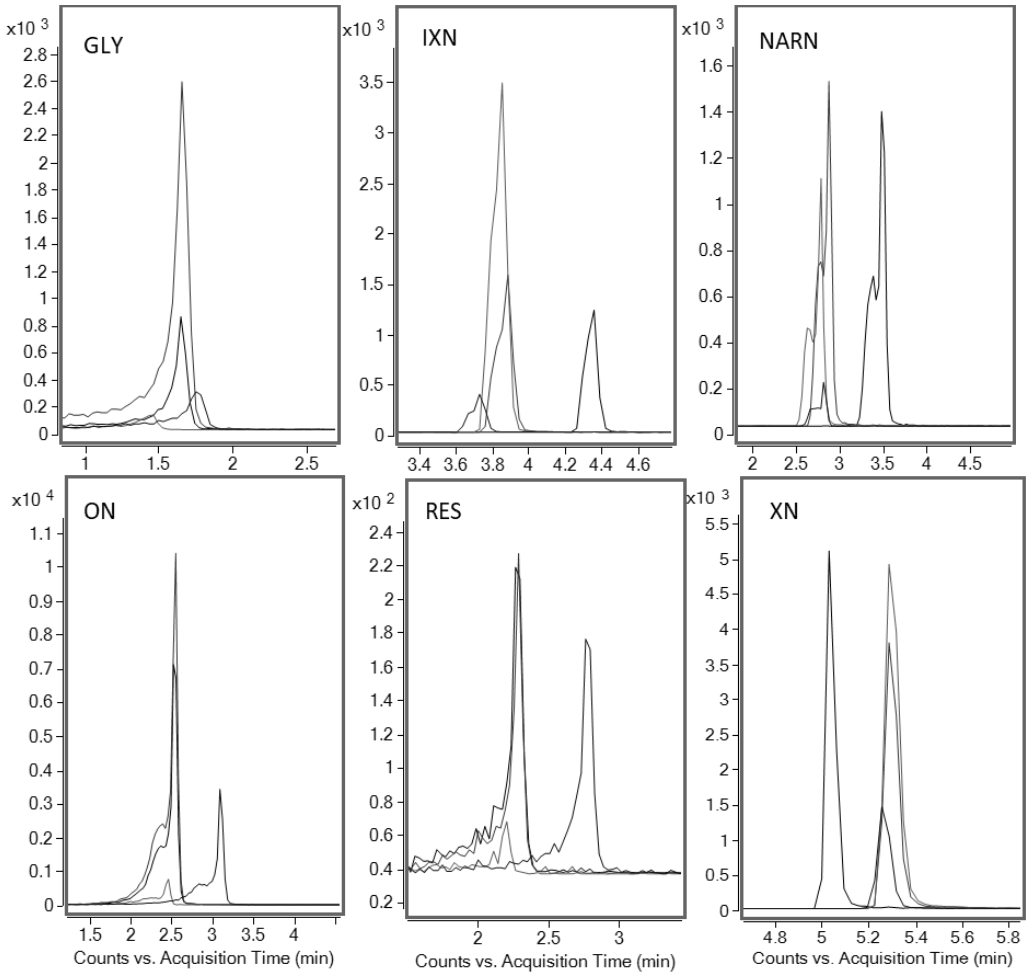


Figure A5: *(Continued)*

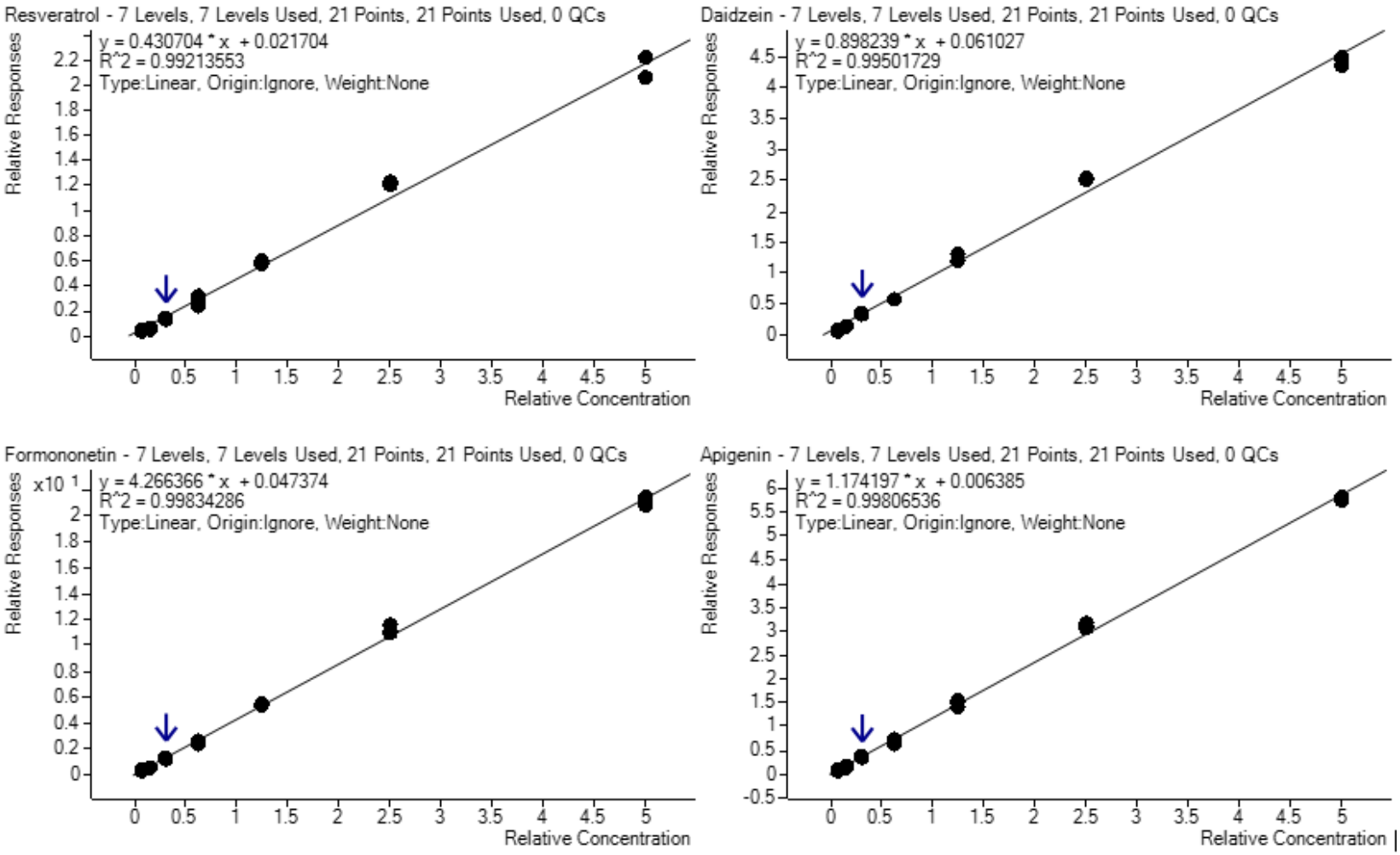


Figure A6: Seven-point calibration curves for target analytes.

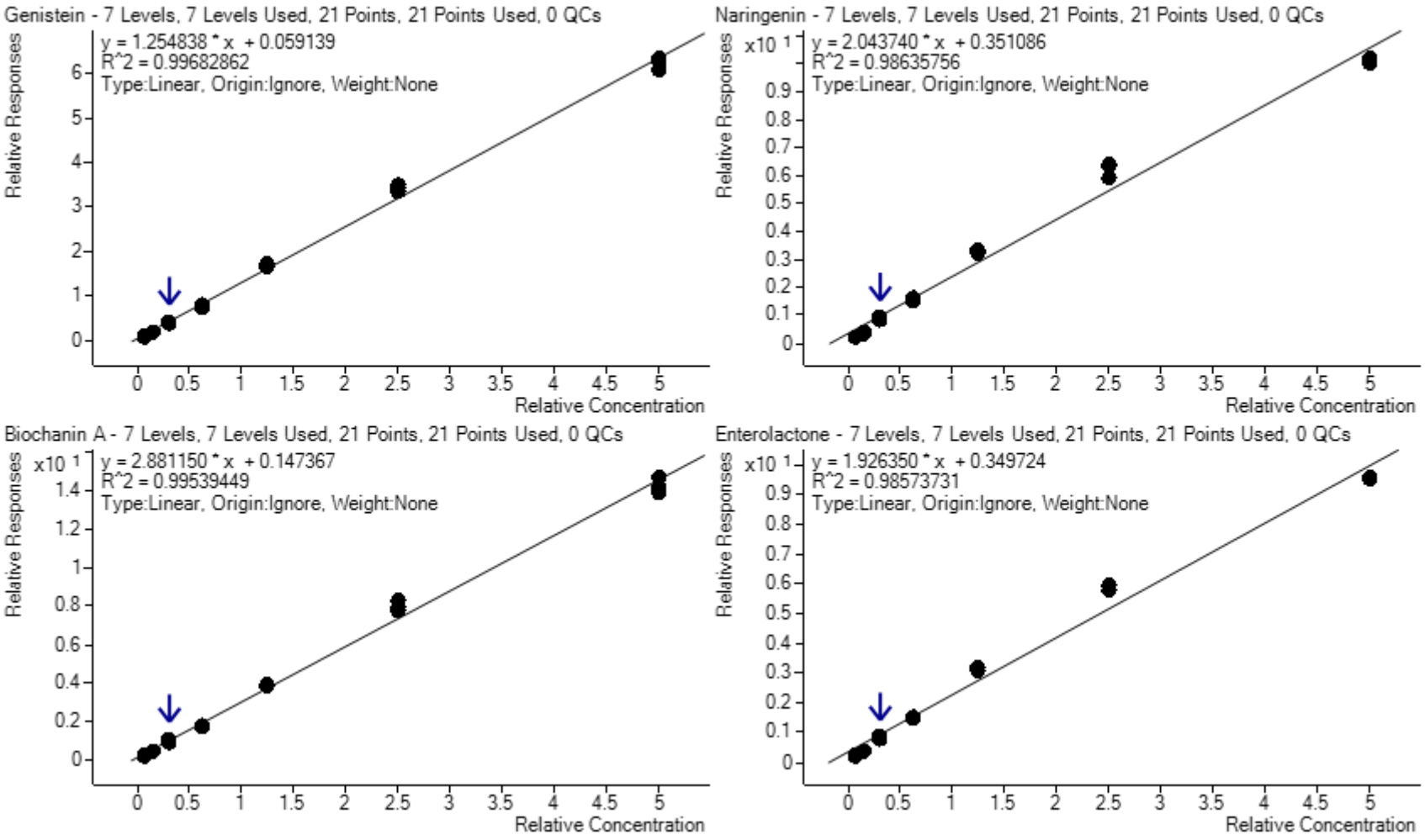


Figure A6: (*continued*)

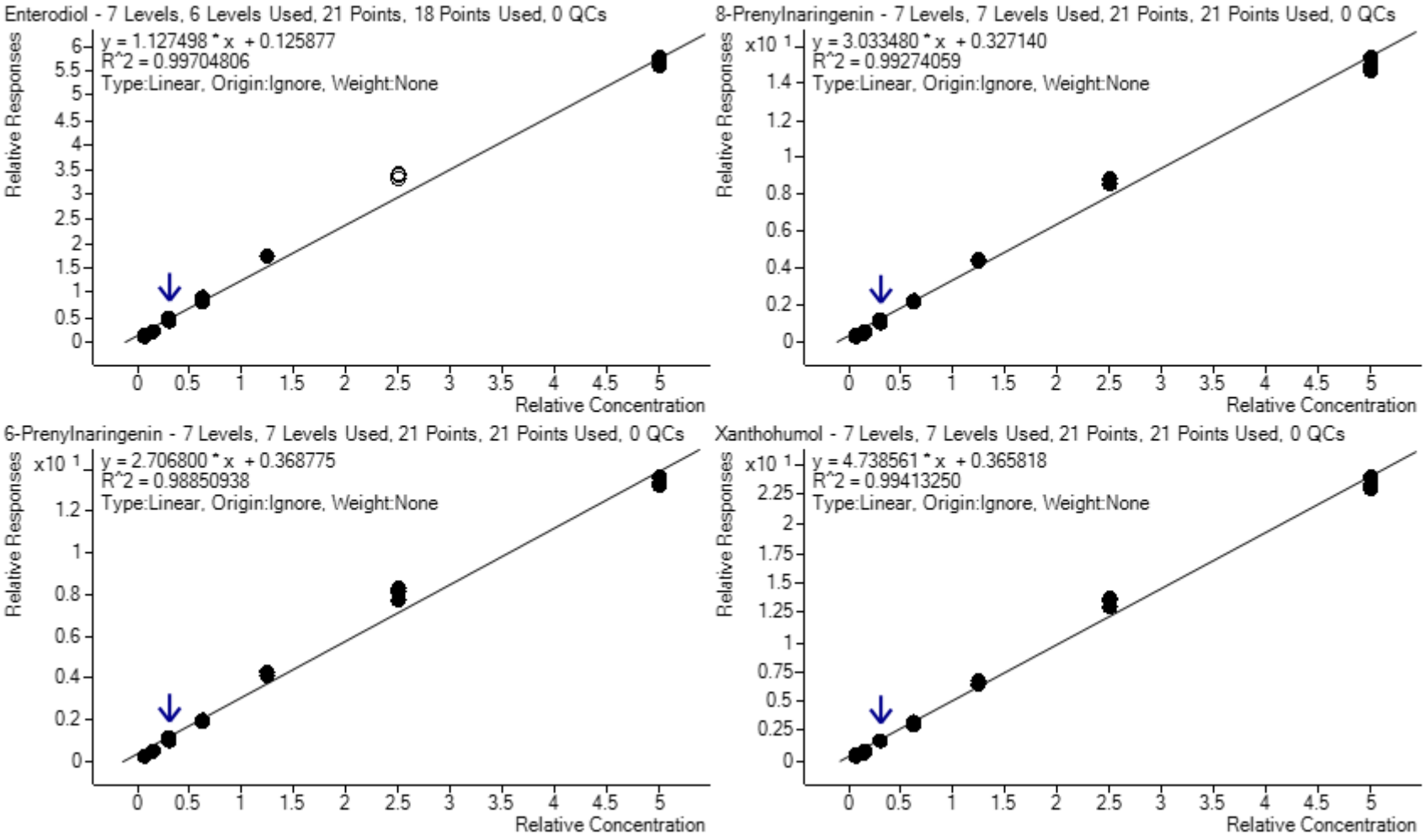


Figure A6: (*continued*)

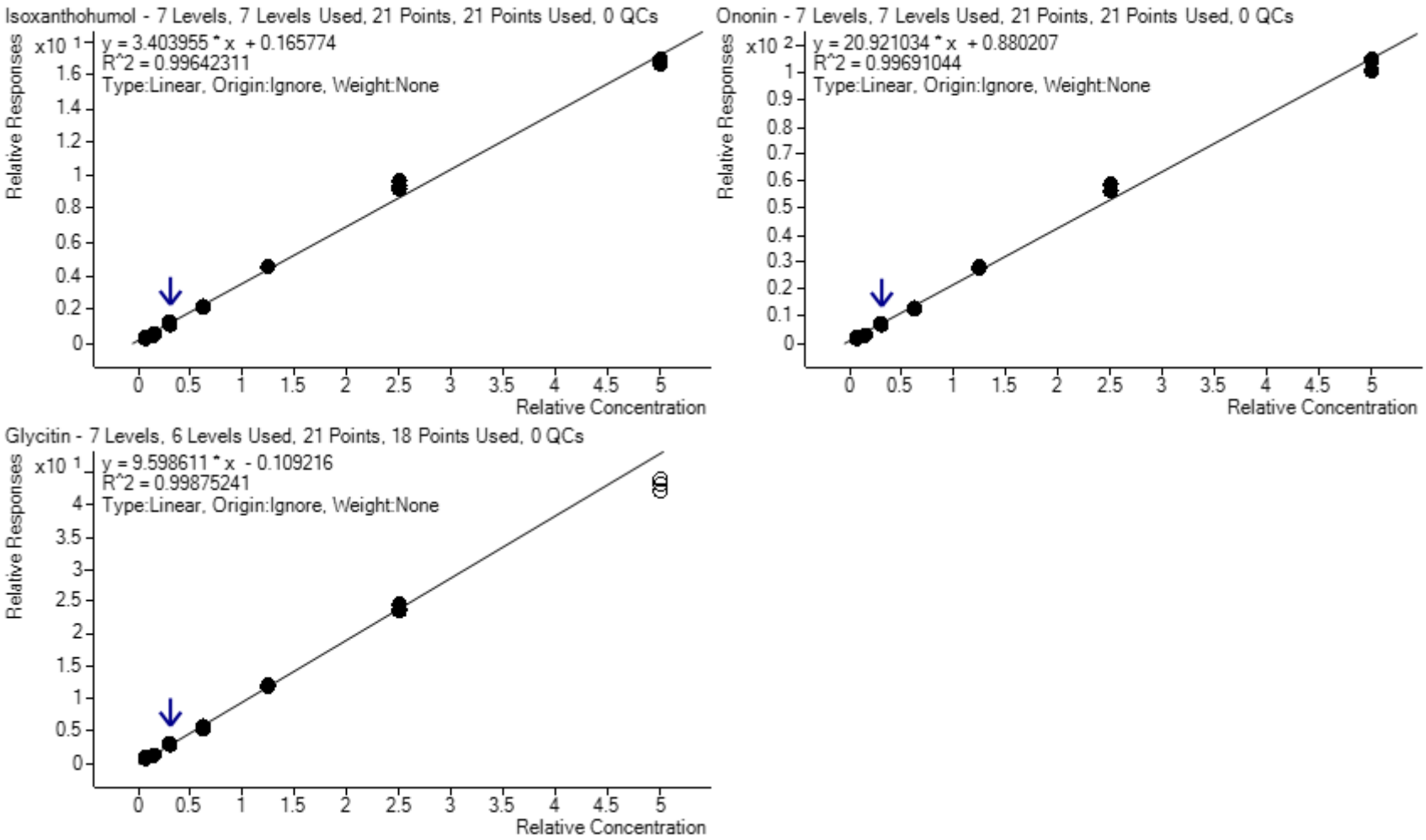


Figure A6: (*continued*)

Table A2: Detected concentrations of enterolactone in surface water samples collected from the Perdido Bay estuary in mean ± SD (n=5). Samples were collected in duplicates (A and B) at each location.

Water samples	**Enterolactone ng/L ± SD**	**Water samples**	**Enterolactone ng/L ± SD**
SW1A	3.66 ± 0.27	SW2A	0.47 ± 0.02
SW1B	4.01 ± 0.33	SW2B	0.48 ± 0.03
SW3A	0.08 ± 0.01	SW4A	0.19 ± 0.03
SW3B	0.09 ± 0.01	SW4B	0.14 ± 0.01
SW5A	ND	SW6A	0.61 ± 0.05
SW5B	ND	SW6B	0.12 ± 0.06
SW7A	4.56 ± 0.24	SW8A	0.29 ± 0.04
SW7B	5.69 ± 0.43	SW8B	0.07 ± 0.01
SW9A	0.11 ± 0.01	SW10A	ND
SW9B	0.61 ± 0.05	SW10B	ND
SW11A	ND		
SW11B	ND		

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