Quantification and Profiling of Hepatic Retinoids in Freshwater Fishes by Liquid Chromatography – Tandem Mass Spectrometry

by

Michael William Dunning

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis.

This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Statement of Contributions

I hereby clarify authors' contributions to the manuscript that comprises Chapter 2 of this thesis.

Chapter 2 is titled "Analysis of Retinoids in Fish Hepatic Tissue by LC-MS/MS" and is co-authored by Dr. Mark Hewitt (Environment and Climate Change Canada, Water Science and Technology Directorate, Aquatic Contaminants Research Division) and Dr. Mark Servos (Department of Biology, University of Waterloo). I designed the methodology, collected the data, performed data analysis, and wrote the manuscript. Dr. Mark Hewitt and Dr. Mark Servos provided assistance in research direction, interpretation of the data, and discussion. All authors edited and proofread the manuscript.

Abstract

Retinoids (vitamin A_1) are a class of compounds essential for a number of physiological processes including vision and immune system function. They are stored as biologically inactive retinyl esters which are mobilized and converted to the transcriptionally-active retinoic acids (RAs) according to physiological and nutritional requirements. RAs are key components in gene expression and are required for proper embryonic development in vertebrates. In addition to retinoids, dehydroretinoids (vitamin A_2) appear to be of high importance in some species of freshwater fish. In some cases dehydroretinoids are found to be more abundant than retinoids, which has led to classification of fish species as "retinol-type" or "dehydroretinol-type" based on the major class of retinoids utilized. The exact functions, metabolism, and distribution of dehydroretinoids in fish remain to be elucidated and requires further investigation into how the retinoid and dehydroretinoid systems interact in aquatic species.

Disruptions to the retinoid system have been observed in several species of fish by exposure to xenobiotics and contaminants from various sources including mine tailings and pulp mill effluent. These assessments typically only consider retinyl esters and/or free retinol, however a need has been recognized in the literature for retinoid analysis to establish what linkages exist between depressed levels of retinol/retinyl esters caused by pollutant exposure and how this may impact levels of biologically active vitamin A. Retinol and retinyl esters are most often analyzed by ultraviolet spectrophotometry. While these methods provide acceptable detection limits for quantification, they lack specificity and require authentic standards which can be expensive or impossible to obtain. Retinoic acids are thought to be present in fish at the pmol g^{-1} level and due to analytical limitations have not been adequately quantified to date.

The objective of this thesis was to develop a comprehensive and robust method to measure retinoids and dehydroretinoids in fish, thereby facilitating further research into retinoid biology. A streamlined extraction method was applied to generate separate fractions of non-polar retinoids (retinol/retinyl esters) and active retinoids (retinoic acids) from as low as 5 mg of hepatic tissue. A new approach coupling liquid chromatographic separations to mass selective detection was developed that enabled the quantification of major retinoids by triple quadrupole mass spectrometry. The analysis was validated with standards and found to provide acceptable sensitivity, accuracy, precision, and linearity. The limit of quantification was in the range of 0.12 – 1.10 ng mL^{-1} for retinoic acids, 1.04 ng mL^{-1} for retinol, and 0.82 ng mL^{-1} for retinyl palmitate. This new method was then applied to select archived fish tissues of brown bullhead (*Ameiurus nebulosus*) and shorthead redhorse sucker (*Moxostoma macrolepidotum*) from two North American Great Lakes Areas of Concern (AOCs) to demonstrate the ability of the method to detect differences in hepatic retinoid status in populations of fish exposed to different environmental stressors. The method was capable of detecting significant decreases in free retinol and retinyl esters in shorthead redhorse sucker from a contaminated site. Retinoic acid status could not be adequately assessed in hepatic tissue due to significant matrix interferences resulting in failure to meet QA/QC criteria. The application of high resolution quadrupole time-of-flight instrumentation also allowed tentative identification of retinoids for which standards are not commercially available, and semi-quantitative profiling of vitamin A$_1$/vitamin A$_2$ distribution in hepatic tissue. Profiling of liver samples from shorthead redhorse sucker showed near equal levels of retinyl and dehydroretinyl esters, while dehydroretinoids appeared to be dominant over retinoids in brown bullhead.

These investigations advanced the field of retinoid biology by allowing for a greater range of compounds which can be confidently analyzed without the need for expensive or unavailable standards, and by providing the ability to semi-qualitatively assess retinoid/dehydroretinoid abundances in different fish species. Resolving the persistent matrix issues which complicates the analysis of retinoic acids in hepatic tissue should be the focus of future work. Once resolved, this method may be used to begin answering some of the open questions regarding retinoid metabolism and functions in fish.

Acknowledgements

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List of Abbreviations

13cRA: 13-cis-retinoic acid

9cRA: 9-*cis*-retinoic acid

AOC: Area of concern

APCI: Atmospheric pressure chemical ionization

APPI: Atmospheric pressure photoionization

atRA: All-*trans*-retinoic acid

DAD: Diode array detector

EIC: Extracted ion chromatogram

ESI: Electrospray ionization

GC: Gas chromatography

HPLC: High performance liquid chromatography

MRM: Multiple reaction monitoring

MS: Mass spectrometry

MS/MS: Tandem mass spectrometry

Q-LIT: Quadrupole linear ion trap

QqQ: Triple quadrupole

QToF: Quadrupole time-of-flight

RA: Retinoic acid

RAc: All-*trans*-retinyl acetate

RAL: All-*trans*-retinaldehyde

RAR: Retinoic acid receptor

RARE: Retinoic acid response element

RE: Retinyl ester

ROL: All-*trans*-retinol

RPa: All-*trans*-retinyl palmitate

RXR: Retinoid X receptor

SPE: Solid phase extraction

SPP: Superficially porous packing

VA: Vitamin A

"So long, and thanks for all the fish." —

Douglas Adams, *The Hitchhiker's Guide to the Galaxy* (1979)

Chapter 1 – Introduction

Biomonitoring efforts have made extensive use of fish as sentinels in order to track and identify alterations to the aquatic environment associated with anthropogenic sources of pollution (Munkittrick et al. 2002). Retinoids (vitamin A) are important biomolecules involved in numerous key physiological functions and hepatic retinoid levels have been used as indicators of pollutant exposure for the past several decades (Spear and Moon 1986). Interruptions to vitamin A homeostasis have been observed in various species of fish and mammals following exposure to pollutants such as wastewater effluents, metals, and halogenated organic compounds (Alsop et al. 2003, Inoue et al. 2010, Defo et al. 2012). Such assays are based on spectrophotometric techniques which are used to quantify esterified storage retinoids but lack the sensitivity to quantify biologically active vitamin A. A general need has been recognized in the literature for retinoid analysis to establish potential linkages among pollutant exposure, altered retinoid stores, and levels of biologically active vitamin A (Alsop et al. 2003, Defo et al. 2014). The widespread adoption of mass spectrometry in research laboratories has provided greatly improved analytical capabilities relative to spectrophotometry. These tools now provide the necessary detection limits and specificity to begin probing the retinoid system in fish in greater detail by analyzing tissue samples for biologically active retinoids in addition to characterizing the stored retinoids traditionally analyzed by spectrophotometry. The ability to accurately and precisely quantify retinoids would aid in understanding the ultimate biological consequences of altered vitamin A status in fish exposed to environmental stressors.

1.1 Metabolism and Functions of Retinoids

The term "retinoid" refers to a class of low molecular weight, lipophilic compounds "…consisting of four isoprenoid units joined in a head-to-tail manner" (IUPAC-IUB Joint

Commission on Biochemical Nomenclature 1982). Among the array of natural and synthetic compounds encompassed in this definition, those which qualitatively exhibit the biological activity of all-*trans*-retinol (ROL) are together referred to as vitamin A (Defo et al. 2014). No known vertebrates are capable of *de novo* vitamin A synthesis. Retinoids are assimilated through the diet in the forms of retinol and retinyl esters (REs) from animal sources or as provitamin carotenoids, which are naturally-occurring plant pigments (Gesto et al. 2012). Vitamin A is essential for a number of physiological processes including vision, cellular differentiation, and immune system function. It is also a key component of vertebrate reproduction and embryonic development (Alsop et al. 2008, Gesto et al. 2012). Retinoids are ubiquitous and found throughout the body in blood and tissue. In fish, they are mainly sequestered in the liver as biologically inactive retinyl esters. Retinyl palmitate (RPa) is usually the major esterified form however the ester profile will differ between species and tissue type (Defo et al. 2012). According to physiological and nutritional requirements of the organism REs are mobilized via hydrolysis to free retinol for secretion and transport to fortify the circulating pool of available vitamin A (Fernández and Gisbert 2011). Once in the target cell, ROL is first oxidized to retinaldehyde (RAL), which is an essential component of the visual cycle in vertebrates. This conversion is reversible and serves as a metabolic regulatory point, as the subsequent oxidation of RAL to the transcriptionally-active retinoic acids (RAs) is irreversible (Gesto et al. 2013). The structures of selected retinoids are shown in Figure 1-1 and a summary of these biochemical pathways is presented in Figure 1-2.

all-*trans*-retinol (ROL)

all-*trans*-retinaldehyde (RAL)

all-*trans*-retinyl palmitate (RPa)

all-*trans*-retinoic acid (atRA)

9,13-di-*cis*-retinoic acid (9,13dcRA)

13-*cis*-retinoic acid (13cRA)

all-*trans*-dehydroretinol (dROL)

all-*trans*-dehydroretinyl palmitate (dRPa)

9-*cis*-retinoic acid (9cRA)

all-*trans*-acitretin (ACTR)

all-*trans*-retinoic acid-d5 (atRA-d5)

all-*trans*-retinyl acetate (RAc)
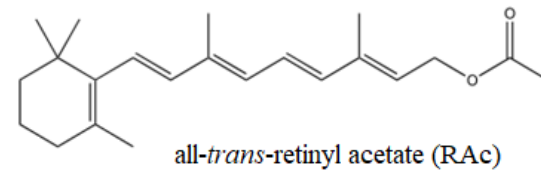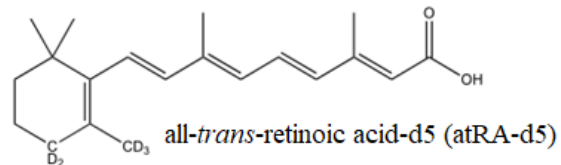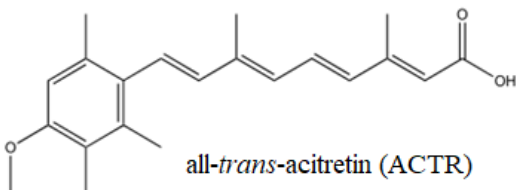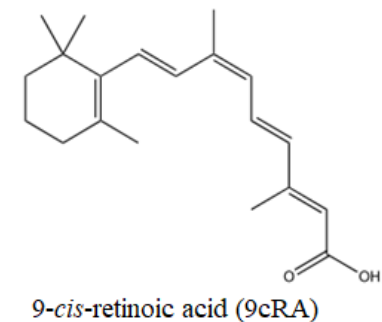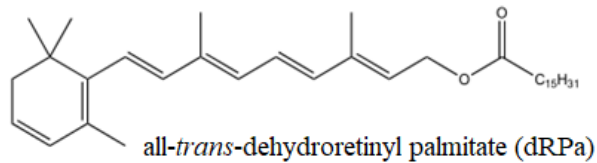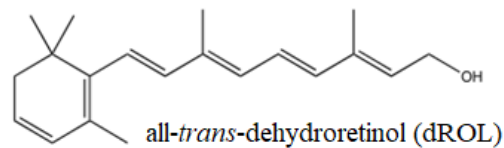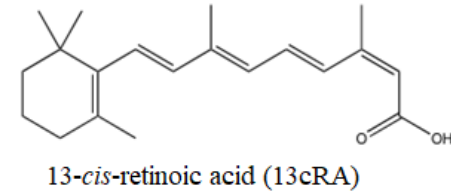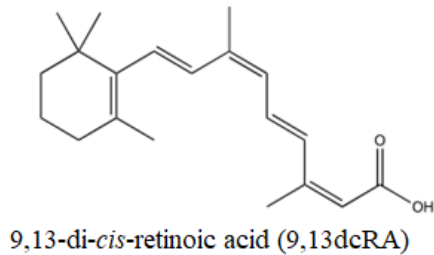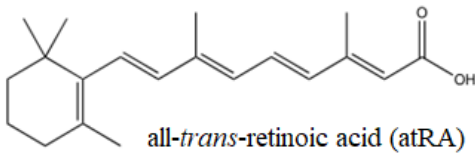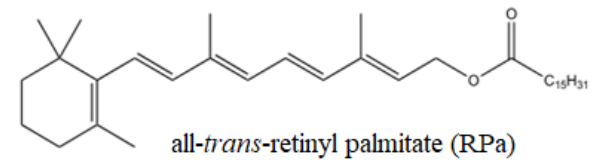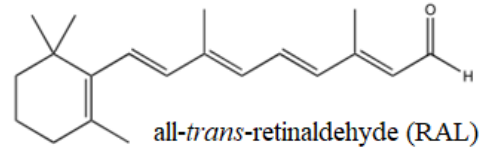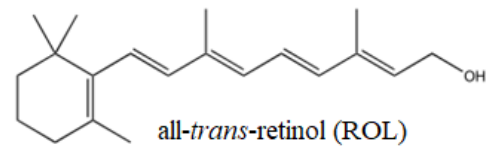
Figure 1-1: Chemical structures of selected retinoids
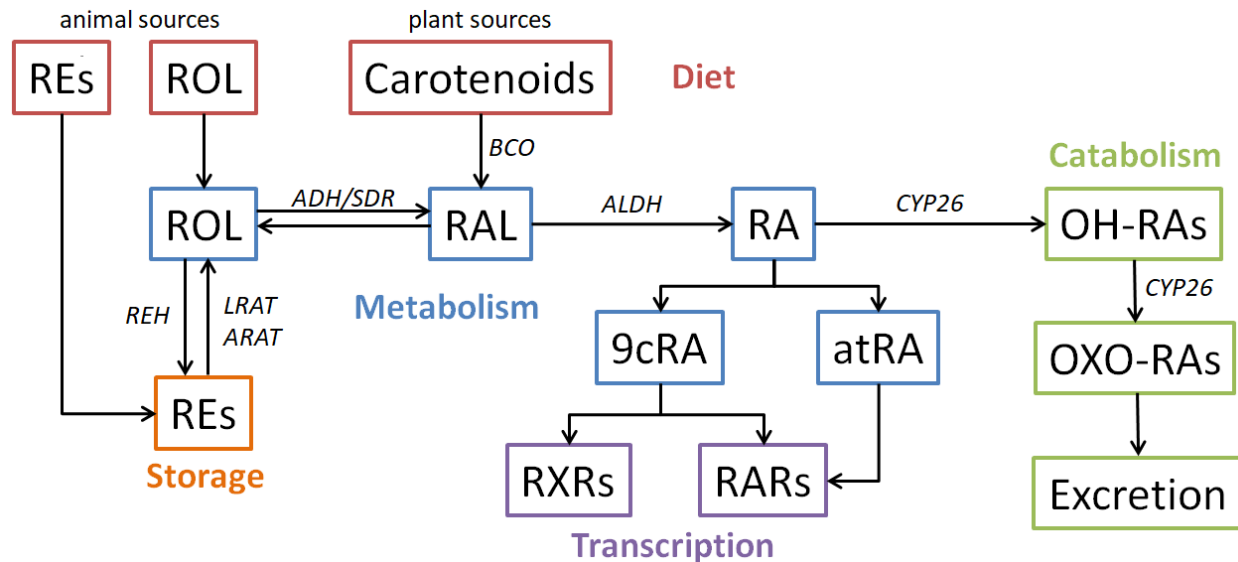
Figure 1-2: Simplified diagram of vitamin A metabolism in vertebrates

ADH: alcohol dehydrogenase. ALDH: aldehyde dehydrogenase. ARAT: acyl-CoA:retinol acyltransferase. BCO: β-carotene oxygenase. CYP26: cytochrome p450 26A1. LRAT: lecithin:retinol acyltransferase. REH: retinyl ester hydrolase. SDR: short chain dehydrogenase/reductase.

Dehydroretinoids (Figure 1-3) are characterized by an additional desaturation at the 3,4 position of the β-ionone ring and have been observed in a variety of species of birds, amphibians, and most notably fish, especially those which spend at least part of their lifecycle in freshwater (Defo et al. 2012, Gesto et al. 2012). Under the classification scheme presented by Goswami and Barua, fish species can be characterized as "retinol-type" (vitamin A_1, VA_1) or "dehydroretinol-type" (vitamin A_2, VA_2) based on the major class of retinoids utilized (Goswami and Barua 1981). Understanding of the occurrence and function of dehydroretinoids is currently limited. They are thought to act in parallel to the retinoid system, however it is unclear to what degree the two systems may interact. Studies have suggested that some freshwater fish possess the ability to convert ROL to dROL although it is unclear whether this process is reversible. Estimates of the biological activity of dehydroretinoids vary widely as conflicting studies estimate the biological

activity of dehydroretinol between 40% and 100% of that of ROL (La Frano and Burri 2014). Conversely, retinoic acid and dehydroretinoic acid are thought to be equally potent in certain biological systems, however a descriptive model for the interactions of retinoic acids and dehydroretinoic acids in vitamin A function remains equivocal (Doyon et al. 1998).
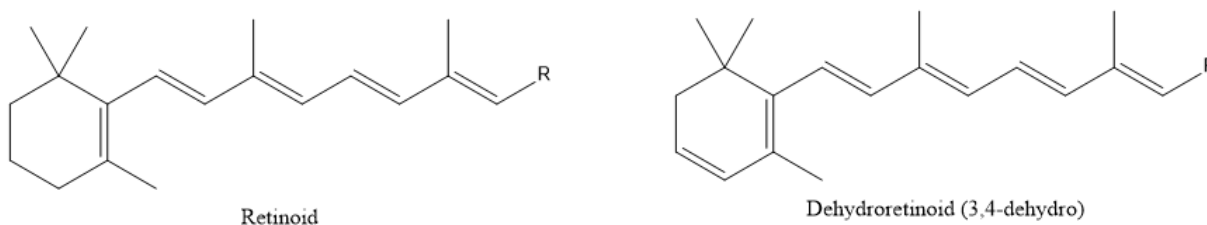
Figure 1-3: Retinoids vs. dehydroretinoids

Retinoic acids have been identified as specific ligands for the retinoic acid receptors (RARα, RARβ, RARγ) and retinoid X receptors (RXRα, RXRβ, RXRγ), which are part of the part of the steroid-thyroid hormone receptor superfamily (Defo et al. 2014) and are common to all vertebrates. RAR/RXR heterodimers bind to DNA in regions known as retinoic acid response elements (RAREs) (Rhee et al. 2012), ultimately promoting or repressing transcription of nearby genes. RXRs also form homodimers and dimerize with several other nuclear receptors including the vitamin D and thyroid hormone receptors (Alsop et al. 2008). RAs exist *in vivo* primarily as 9-*cis* (9cRA), 13-*cis* (13cRA), and all-*trans*-retinoic acid (atRA). atRA is a ligand for the RARs while 9cRA activates both the RARs and RXRs (Alsop et al. 2001). 13cRA shows little affinity for either nuclear receptor and has been suggested to act through rearrangement (as well as the 9,13-di-*cis* isomer) to more active stereoisomers (Rhee et al. 2012). 9cRA is thought to be the least abundant RA isomer based on estimates in rainbow trout (*Oncorhynchus mykiss*) (Gesto et al. 2012). *In vivo* levels of retinoic acids are highly regulated as they are potent teratogens and

both excess and deficiency of RAs lead to severe negative effects on overall organism health (Defo et al. 2012, La Frano and Burri 2014). In rainbow trout, vitamin A-deficient diets induced higher mortality rates, reduced growth, and anemia, among other deleterious effects (Alsop et al. 2001). RA signalling was also shown to play a pivotal role not only in proper embryonic development but also into early developmental stages of post-hatch fry. RAs are involved in directing embryonic development along the cranio-caudal (head-tail) axis in vertebrates and disruptions of this RA-signalling mechanism can lead to skeletal or cardiovascular defects and mortality (Alsop et al. 2001). The key role of retinoic acid in reproduction has been similarly demonstrated in zebrafish (*Danio rerio*) where fish exposed to inhibitors of RA synthesis spawned significantly fewer eggs and a diet deficient in vitamin A induced a reduction of whole body retinoids, fewer spawned eggs, and a significant decrease in egg retinaldehyde content (Alsop et al. 2008).

1.2 Effects of Aquatic Contaminants on Retinoid Status

Disruption of the retinoid system by exposure to xenobiotics and environmental contaminants has been observed in a variety of species of fish. Lake trout (*Salvelinus namaycush*) exposed to iron and manganese from mine tailings exhibited 60 – 150 fold decreases in hepatic stores of retinyl palmitate relative to fish taken from a reference site (Payne et al. 1998). A more recent study on seven tropical teleost fish species revealed a negative correlation in dehydroretinoid stores following exposure to lead, arsenic, and cadmium and positive correlations in fish with higher levels of hepatic iron, copper, and zinc (Pereira et al. 2012). In contrast to these findings, cadmium exposure was shown to increase hepatic retinoid levels in yellow perch (*Perca flavescens*) (Defo et al. 2012). This conflicting evidence further complicates

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our limited understanding of the retinoid system and indicates that retinoid responses of fish to aquatic contaminants may be species specific.

Vitamin A is thought to provide protection from cancer due to its antioxidant properties. Decreased retinoid stores have been associated with exposure to reactive oxygen species and may indicate mobilization of retinoids to combat oxidative stress, maintain tissue function, and detoxify metals (Defo et al. 2014). Some metal-exposed fish with increased hepatic retinoids show decreased whole-body vitamin A, suggesting that retinoids may be mobilized from other tissues to fortify liver antioxidant levels. Fish with decreased whole-body retinoids were not necessarily observed to be in poor health or physical condition which suggests dietary intake is still sufficient to maintain normal vitamin A function in the absence of retinyl esters (Alsop et al. 2007, Pereira et al. 2012).

Severely reduced levels of hepatic retinol, retinyl esters, and corresponding dehydroretinoids have also been observed in white sucker (*Catostomus commersoni*) inhabiting a pulp mill effluent receiver (Alsop et al. 2003). Fish exposed to pulp mill effluent have been shown to display a myriad of other biological effects including delayed sexual maturity, reduced sex steroid concentrations, and decreased egg production (Bowron et al. 2009). It remains unclear whether any of the altered reproductive parameters not directly linked to sex steroid production are linked to retinoid perturbations. Beyond the potential impacts on retinoid storage, pulp mill effluent has also been demonstrated to contain xenobiotic components which block binding of all-*trans*-retinoic acid to RARs and interrupt RA-mediated gene transcription (Schoff and Ankley 2002). A study on pulp mill effluent sampled from several mills across Canada revealed that some effluents contained compounds capable of displacing receptor-bound atRA and 9cRA from gill RARs and liver RXRs of rainbow trout (Alsop et al. 2003). Furthermore,

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exposure to pulp mill effluent has also been linked to increased induction of cytochrome P450 enzymes including P450RAI (*cyp26*), the enzyme responsible for RA catabolism (Alsop et al. 2003). In addition, RAR agonists have been identified in sewage treatment plants and other aquatic environments in North America, Japan, and China, suggesting that pollution of aquatic ecosystems with RAR agonists is widespread (Inoue et al. 2010).

1.3 Physical Properties and Handling of Retinoids

Retinoids are a diverse class of compounds spanning a broad range of polarity from the ionizable RAs to the highly non-polar retinyl esters. The octanol-water partition coefficients (log K_{ow}) of retinoids are estimated to be ~ 5 or greater, making them nearly insoluble in water. Retinyl esters readily dissolve in hexane, dichloromethane or ether while the more polar retinoids are soluble in the above solvents and also water-miscible organic solvents such as alcohol, acetonitrile (ACN), or acetone (Kane and Napoli 2010). In general, solubility of retinoids in these solvents ranges from 10 - 40 g L^{-1}. Select physical properties of the major retinoids have been summarized in Table 1-1.

Table 1-1: Physical properties of selected retinoids

Compound	Formula	Mass (g mol^{-1})	pKa	log D_{ow} (pH 2)	log D_{ow} (pH 10)	log S (pH 2)	log S (pH 10)	Solubility (µg L^{-1}) (pH ~ 7)
Retinoic acid	$C_{20}H_{28}O_2$	300.4351	5	5.0	1.5	-6.7	-1.9	60
Acitretin	$C_{21}H_{26}O_3$	326.4301	5	5.6	2.1	-6.4	-1.6	n.d.
Retinaldehyde	$C_{20}H_{28}O$	284.4357	n.d.	4.9		-7.0		30
Retinol	$C_{20}H_{30}O$	286.4516	n.d.	4.7		-6.8		17
Retinyl acetate	$C_{22}H_{32}O_2$	328.4883	n.d.	5.1		-7.4		n.d.
Retinyl palmitate	$C_{36}H_{60}O_2$	524.8604	n.d.	12		-14		n.d.

pKa: acid dissociation constant; log D_{ow}: pH-dependant octanol-water partition coefficient; log S: log_{10} water solubility (mol L^{-1}, calculated); solubility: water solubility (µg L^{-1}, measured); n.d.: no data available. Compounds with only one log D_{ow}/log S value indicates no dependence on pH.

[1] Physicochemical properties (pKa, log D_{ow}, log S) calculated by MarvinSketch version 17.29.0 (ChemAxon, 2017)

[2] Water solubility measured by Szuts and Harosi (1991)

Retinoids are notoriously sensitive to light, heat, oxygen, as well as pH extremes (Gundersen 2006) and will rapidly degrade or isomerize under unfavourable conditions, corrupting information on the distribution of important stereoisomers such as all-*trans* and 9-*cis*-retinoic acids (Kane and Napoli 2010). To preserve sample integrity and obtain the highest quality data, every step of handling must be performed under low-intensity red or yellow light (Gundersen 2006). Excised tissues should be immediately snap-frozen in liquid nitrogen and stored at -80°C until extraction. Samples must be handled frozen and should be homogenized on ice using the gentlest method available to avoid introducing air into the homogenate or generating excessive heat. Antioxidants such as butylatedhydroxytoluene (BHT) are sometimes added to solvents at ~ 1 g L^{-1} to mitigate oxidative degradation during handling and extraction. Only whole tissues can be stored for any amount of time – homogenates must be extracted and analyzed immediately (Kane and Napoli 2010). Retinoids also adhere strongly to some plastics, which can cause losses as high as 40% and should therefore only be handled using new or base-washed borosilicate glassware, amber type wherever possible (Kane and Napoli 2010). Studies have indicated that retinoids can be stable for periods of months to several years depending on storage conditions (Comstock et al. 1995). In general retinol and its esters are more stable than retinoic acids but are not immune to degradation.

1.4 Extraction of Retinoids from Biological Matrices

Numerous methods for extracting retinoids from biological samples have been reported. The choice of method ultimately depends upon several factors including target analyte(s), degree of sample clean-up required, and the capabilities of the analytical method. Extracting retinoids from a complex matrix such as tissue is more laborious than from serum due to the large number of potentially interfering co-extractives present. The majority of work accomplished to date on

quantitative retinoic acid determinations in the mammalian literature has been focused on clinical applications for serum due to the simplified matrix and amenability to high-throughput analyses. Nevertheless, tissues including liver, gonad, brain, eye, heart, and muscle from rats or mice have all previously been analyzed for retinoids (Kane and Napoli 2010). A disadvantage of analyzing serum is that retinyl esters are converted to retinol for mobilization and therefore gives no indication as to the retinoid storage status of the individual. As storage and active retinoids exist at vastly different endogenous ranges, analyzing for multiple compounds in a single sample becomes highly complex. Total (free + esterified) ROL and dROL levels in rainbow trout liver on the order of 200 nmol g^{-1} have been reported. When detected, retinoic acids were estimated at $0.2 - 2.8$ pmol g^{-1}, approximately five orders of magnitude lower than ROL and nearly two orders of magnitude lower than RA levels observed in mammalian tissues (Gesto et al. 2012). For this reason most methods do not report simultaneous extraction of RAs and non-polar retinoids without a fractionation procedure, as the endogenous ranges of these compounds are too far apart for reliable quantitative analysis.

Liquid-liquid exactions with hexane have successfully been applied for extraction of all major retinoids from tissues and serum with recoveries often in the $70 - 100$ % range (Arnold et al. 2012, La Frano and Burri 2014). These methods are rapid and provide high recoveries with minimal handling, however virtually no sample clean-up is achieved and necessitates sensitive and selective quantification methods. It is possible to capitalize on the acid-base properties of retinoic acids to isolate them from the abundant non-polar retinoids using this technique, at the expense of time and large volumes of solvent used (Kane and Napoli 2010). Due to matrix interferences the best results for RAs are obtained from serum extracts rather than from tissues. Extraction of tissues can introduce proteins and fats into the final extract, which can result in

unnecessarily complicated chromatograms. Mono-phase liquid extraction can also be used to extract retinoids with high recovery (>90%) by combining homogenization, protein precipitation, and retinoid extraction into a single step by using a large volume of water-miscible organic solvent such as alcohol or acetonitrile (McCaffery et al. 2002, Suh et al. 2006). Following centrifugation the supernatant can be directly analyzed or pre-concentrated prior to analysis. Solid phase extraction (SPE) provides a more robust option to extract multiple retinoids from complex matrices and is capable of isolating all major retinoids across the polarity scale with recoveries exceeding 90% (Gesto et al. 2012). While more time-consuming than simple liquid extractions, SPE produces much cleaner extracts by reducing matrix interferences, which greatly improves quality of data when analyzing complex samples for trace constituents.

1.5 Chromatography of Retinoids

Over the last 30 years high performance liquid chromatography (HPLC) has emerged as the tool of choice for separating retinoids in biological extracts. Other techniques including gas chromatography (GC) have been employed with varying degrees of success (Napoli et al. 1985) however the high temperatures required for analysis are not ideal for such thermally labile compounds. Retinoids can exist in 3 – 5 *cis/trans* isomers, making separation of many retinoids in a single extract a challenging task. Achieving baseline resolution may not be possible depending on sample complexity and the calibre of HPLC hardware available.

While no longer the dominant method, normal phase HPLC has been applied to a number of retinoid separations and has proven particularly useful in resolving retinoid isomers. Separation of seven ROL isomers, RA isomers, and RA metabolites have all been achieved on bare-silica columns using hexane/dioxane/isopropanol gradients (Gundersen and Blomhoff 2001, McCaffery et al. 2002). Normal phase separations are capable of separating retinyl esters from

other retinoids, however most silica columns do not have the selectivity to separate a mixture of esters due to their minimal interactions with the polar stationary phase. The use of reversed phase techniques in retinoid analysis has greatly increased in recent years and is now the preferred method of separation. A wide variety of non-polar stationary phases including C18, C30, fluorophenyl, and alkylamide packings offer orthogonal selectivity to optimize analyses specifically for the target compounds present in the sample (Snyder et al. 2011). Most retinoids can be separated with adequate runtimes (< 30 minutes) using simple water/methanol or water/acetonitrile gradients on a C18 column. Other separations with less common mobile phases and column chemistries are employed to decrease separation time and/or resolve numerous retinyl esters (Defo et al. 2012, Rocchi et al. 2016). Methods for the separation of retinoic acids are often reported using alkylamide columns (Kane and Napoli 2010, Gesto et al. 2012) as the increased polarity of the stationary phase enhances retention and therefore resolution of the various isomers (Gundersen 2006).

1.6 Methods of Detection

Detection of retinoids has most commonly been accomplished using ultraviolet (UV) spectrophotometry by taking advantage of their naturally strong UV absorbance. The electron-rich diterpene backbone common to all retinoids absorbs in the near ultraviolet region (300 – 400 nm) with a high degree of specificity as there are few interfering matrix components which absorb light at these wavelengths (Gundersen 2006). Prior to the early-2000s UV detection had dominated for its low cost and adequate detection limits, however its analytical limitations had hindered RA analyses up to this point (Kane and Napoli 2010). Absolute detection limits in the high picogram range have been recently achieved using UV detection, which are more than suitable for quantification of retinol and retinyl esters but are still insufficient for RA

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measurements (Gesto et al. 2012). Pairing mass spectrometry (MS) with liquid chromatography provides a powerful combination tool that pairs the resolving power of HPLC and the superior sensitivity and specificity of mass analyzers. HPLC-MS techniques have rapidly become the tool of choice for analysts performing trace and ultra-trace analyses in clinical and research laboratories.

1.6.1 Ion Source Selection

Soft atmospheric pressure techniques, namely electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), are the two most common ion sources used today in HPLC-MS (de Hoffmann and Stroobant 2013). APCI is applicable to compounds of intermediate molecular weight and polarity such as retinoids. In this method, liquid sample is vaporized in a heated nebulizer and passed through a region of electrical discharge generated by a steel needle held at high voltage. Usually the vaporized HPLC mobile phase is initially ionized, ultimately generating analyte ions and adducts through charge-transfer reactions. APCI (+) usually produces only singly-charged ions, often the protonated molecular species $[M+H]^+$ (de Hoffmann and Stroobant 2013). It is generally recognized that ESI results in more ion suppression, higher background, and greater in-source fragmentation than APCI (Gundersen 2006, Kane and Napoli 2010). Comparison of positive and negative ionization modes of ESI and APCI for retinoic acids has shown that APCI (+) provides the strongest net signal with the greatest dynamic range (Wang et al. 2001). ESI (+/-) has been successfully applied to analyses for retinoic acids and their polar metabolites however APCI (+) currently remains the dominant method.

1.6.2 Tandem Mass Spectrometry

Tandem mass spectrometry (MS/MS) provides vastly superior detection limits with a high degree of selectivity compared to methods employing UV or single-stage MS detection, which can both suffer from susceptibility to matrix interferences. The most common type of MS/MS analyzers are triple-quadrupole (QqQ) instruments, in which two mass-fixed quadrupoles operate with a multipole collision cell between them (de Hoffmann and Stroobant 2013). Q1 acts as a mass filter to isolate precursor ions from the source, which are then transmitted to Q2 and fragmented by collisions with neutral gas molecules (usually nitrogen). The Q3 mass filter then selects a characteristic product ion fragment(s) for detection (de Hoffmann and Stroobant 2013). This type of quantitative analysis is usually referred to as multiple reaction monitoring (MRM). While fewer net ions reach the detector versus single-stage MS detection, MRM techniques drastically improve detection limits and specificity. Imposing restrictions on both precursor and product ions for detection results in up to a 1000-fold reduction of background noise and thereby dramatically increases the signal-to-noise ratio (Kane and Napoli 2010). Single-quadrupole MS has been used to quantify RAs in rat tissues with absolute detection limits of 210 pg (Wang et al. 2001), which has since been surpassed by UV detection (Gesto et al. 2012). More recent applications of MS/MS detection for RA analysis in serum have achieved detection limits in the femtogram range, corresponding to 1000-fold increases in sensitivity versus single-stage techniques (Arnold et al. 2012, Jones et al. 2015).

1.6.3 QToF Mass Analyzers

Quadrupole time-of-flight (QToF) is a type of hybrid mass analyzer that has traditionally found applications in proteomics, metabolomics, and impurity screening in the food and pharmaceutical industries. Similar to QqQ instruments, this technique uses a front-end

quadrupole to isolate a precursor ion from the ion source, followed by precursor fragmentation in a collision cell. Rather than monitoring one or two characteristic product ions, a time-of-flight analyzer is used to collect all the product ions at the collision cell exit and accelerate them through an electric potential into a flight tube. With constant applied voltage, flight time increases with molecular weight and results in a spatial/temporal separation of ions (de Hoffmann and Stroobant 2013). The mass-to-charge ratio is then calculated for each species as it reaches the detector. Compared to QqQ analyzers, QToF instruments offer a higher degree of selectivity by better distinguishing analyte signal from the background due to its high mass resolution (de Hoffmann and Stroobant 2013). Ultimately QToF can simultaneously provide full-spectrum, accurate mass MS and MS/MS data that aids in analyte identification and allows for screening of target compounds for which commercially available standards are not available or are prohibitively expensive.

1.7 Great Lakes Areas of Concern

The Great Lakes Water Quality Agreement (GLWQA) is an agreement between Canada and the United States to restore and protect the chemical, physical, and biological integrity of the Great Lakes within a science-based management framework (Munawar et al. 2012). Areas of Concern (AOCs) are regions identified under the GLWQA to be suffering from severe pollution and/or degradation from anthropogenic stressors resulting in at least one "Beneficial Use Impairment" (BUI), which include "degraded fish and wildlife populations" and "fish tumours or other deformities" among others (Munawar et al. 2012). Located on the northeastern shore of Lake Ontario, the Bay of Quinte AOC was designated with 10 of 14 BUIs related to eutrophication, urban development and historical sediment contamination (Environment and Climate Change Canada 2017). The St. Clair River is a bi-national AOC on the boundary of

Ontario and Michigan, with connections to Lakes Huron and Erie. This AOC was designated with 12 of 14 BUIs due to extreme degradation of water quality and environmental health from industrialization, urbanization, and agricultural activities (Environment and Climate Change Canada 2017). Many groups have tracked general fish health, endocrine disruption, and various other biological endpoints over time in effort to monitor the progress of on-going remediation and restoration efforts in AOCs across the Great Lakes Basin.

1.8 Scope and Objectives

Establishing linkages between retinoid perturbations and the potential impacts on physiological functions will lead to a better understanding of how environmental stressors impact ecosystems. Disruptions to retinoid metabolism, particularly those which appear to alter RA synthesis and function are important to fish health. Monitoring hepatic retinoid concentrations has been suggested to be a key indicator of pollutant and other stressor effects on vitamin A status (Defo et al. 2014). Unfortunately, sensitive and robust methods for the measurement of stored and active retinoids in fish are not fully developed. Current analytical methods for retinoic acid determinations have mostly focused on clinical studies for mammalian applications. Although assessments on retinol and retinyl esters have been made in fish, they are almost always analyzed by ultraviolet spectrophotometry, which lacks specificity and requires authentic standards for quantification which can be expensive or unavailable.

This study is intended to promote ongoing research in aquatic toxicology for the measurement of retinoids in fish at endogenously relevant concentrations from samples of biological origin. To achieve this goal, the following objectives were set:

1. Develop methods for the routine extraction, quantification, and profiling of selected biologically active and stored hepatic retinoids using LC-MS/MS techniques;

2. Validate the methods for achieving established QA/QC parameters (e.g. precision, accuracy, and limits of quantitation/detection);

3. Demonstrate the feasibility for application by analyzing select archived fish tissues to assess retinoid status of fish populations exposed to different environmental stressors.

This thesis outlines the development, validation, and application of a new method for the quantification of stored and biologically active retinoids by LC-QqQ as well as profiling of retinol and retinyl esters by LC-QToF. The method was applied to fish from the Bay of Quinte and St. Clair River AOCs to demonstrate the ability of the method to detect differences in hepatic retinoid status in populations sampled at near and far-field sites at these AOCs.

Chapter 2 – Analysis of Retinoids in Fish Hepatic Tissue by LC-MS/MS

2.1 Summary

This chapter details the development, validation, and application of a new method for the quantification and profiling of stored and biologically active retinoids (vitamin A_1) and dehydroretinoids (vitamin A_2) in fish hepatic tissues. The current research on measurement of biologically active retinoids has mostly been focused on mammalian applications and consequently, sensitive and robust methods for assessing retinoid status in fish are not fully developed. A streamlined solid phase extraction (SPE) procedure was applied to generate separate fractions of stored (retinol, retinyl esters, and corresponding dehydroretinoids) and biologically active retinoids (retinoic acids) from as low as 5 mg of hepatic tissue. An approach coupling liquid chromatographic separations to mass selective detection was developed and validated for limits of detection and quantification, linearity, precision, and accuracy using analytical standards. The limit of detection for retinoic acids was in the range of $0.07 - 0.25$ ng mL^{-1} for retinoic acids, corresponding to a mass limit of detection (mLOD) of $1.79 - 6.31$ pg. The limits of detection for the quantification of retinol and retinyl palmitate were 0.54 and 0.25 ng mL^{-1} respectively, corresponding to a mLOD of 10.4 pg retinol and 8.2 pg retinyl palmitate. The combined approach of triple quadrupole and high resolution quadrupole time-of-flight instrumentation allowed for semi-quantitative measurements of dehydroretinol and dehydroretinyl palmitate through characterization by accurate-mass MS and MS/MS spectra. The method was then applied to assess retinoid status in wild brown bullhead (*Ameiurus nebulosus*) and shorthead redhorse sucker (*Moxostoma macrolepidotum*) populations from two North American Great Lakes Areas of Concern. The results indicate this method is able to demonstrate measurable differences in stored retinoid concentrations and through qualitative profiling allows

tentative identification of unknown analytes and assessment of vitamin A_1/vitamin A_2 distribution in fish hepatic tissue. Persistent matrix interferences hindered analyses for the biologically active retinoids and further work is required to resolve these issues.

2.2 Introduction

Retinoids are a diverse class of diterpenes which are structurally related to all-*trans*-retinol (vitamin A) and qualitatively exhibit similar biological activity (Gundersen and Blomhoff 2001). They are responsible for numerous physiological functions including vision, cell and tissue maintenance, reproduction, and embryonic development (Alsop et al. 2008). The majority of total vitamin A is stored in the liver as biologically inactive retinyl esters (REs), and retinyl palmitate (RPa) is usually the major ester form in most vertebrate species (Gesto et al. 2012). According to nutritional and physiological requirements REs are hydrolyzed to free retinol (ROL) to fortify circulating vitamin A levels. In the target cell, ROL is oxidized through retinaldehyde to transcriptionally-active retinoic acids (RAs) (Fernández and Gisbert 2011). As specific ligands for the retinoic acid and retinoid X nuclear receptors (RARs/RXRs), several RA isomers including all-*trans* (at), 9-*cis* (9c), and 13-*cis* (13c) play pivotal roles in gene expression and proper morphological development of the embryo (Defo et al. 2014). Retinoic acids are also potent teratogens and imbalances can result in skeletal and cardiovascular defects or mortality (Alsop et al. 2001). As retinoids cannot be synthesized *de novo* and must be obtained through the diet, they have been long considered good biomarkers for pollutant exposure (Doyon et al. 1998, Rodríguez-Estival et al. 2011, Defo et al. 2014).

Perturbations to the retinoid system have been observed in several species of fish following exposure to xenobiotics in municipal wastewater and pulp mill effluents (Schoff and Ankley 2002, Alsop et al. 2003, Inoue et al. 2010) and other environmental contaminants such as

mine tailings (Payne et al. 1998) and heavy metals (Defo et al. 2012, Pereira et al. 2012). Organic pollutants such as pesticides and organohalogens have also shown adverse effects on retinoid status in other vertebrates (Defo et al. 2012). These responses are usually manifested as marked reductions to hepatic retinoid levels. In some cases opposing responses have been observed between species exposed to the same stressor which suggests that responses of the retinoid system to environmental stressors may be species specific (Defo et al. 2014).

In addition to retinoids, some species of freshwater fish have been observed to utilize dehydroretinol (dROL) and dehydroretinyl esters (dREs) as their major form of vitamin A (Defo et al. 2012, Gesto et al. 2012). Dehydroretinoids are characterized by an additional unsaturation at the 3,4-position of the β-ionone ring. Fish can be characterized as "retinol-type" (vitamin A_1) or "dehydroretinol-type" (vitamin A_2) species based on the major type of retinoids utilized (Goswami and Barua 1981). Little is known about the functions and distribution of dehydroretinoids and how they interact with the retinoid system. Due to analytical limitations there has until recently been a lack of quantitative information on retinoic acid levels in fish, which are estimated to be present in liver at the low-picomole per gram range (Gesto et al. 2012). Retinoids, especially the biologically active forms, are also notoriously sensitive to light and heat which makes their handling and analysis challenging. The more abundant retinol/retinyl esters are often analyzed by high performance liquid chromatography (HPLC) coupled to diode array detection (DAD), which can provide sufficient detection limits for their quantification however these methods lack the specificity that mass analyzers can provide (Gundersen 2006, Kane and Napoli 2010). Through high resolution mass spectrometry it should be possible to tentatively identify retinoids for which commercial standards are unavailable by using accurate mass MS and MS/MS spectra.

The objective of this study was to develop and optimize a method to allow streamlined extraction and analysis of multiple retinoids in fish hepatic tissues for assessment of retinoid status in relation to environmental stressors. Triple quadrupole (QqQ) and quadrupole time-of-flight (QToF) approaches were combined to quantify the major active retinoids and profile stored retinoids for relative abundance and distribution. These methods were applied to fish tissues collected from two of the North American Great Lakes Areas of Concern (AOCs: the Bay of Quinte in Lake Ontario and the St. Clair River between Lake Huron and Lake Erie) to determine if differences in storage and biologically active retinoids could be detected using this method.

2.3 Materials and Methods

2.3.1 Chemicals and Standards

Analytical standards were purchased from Toronto Research Chemicals (North York, ON, Canada). Solvents (HPLC grade or better) were purchased from Fisher Scientific (Toronto, ON, Canada) and solid reagents and chemicals were purchased from Sigma Aldrich (Oakville, ON, Canada). Standard solutions were prepared by weight in ethanol and corrected by spectrophotometry using known ϵ values (Barua and Furr 1998). Stock solutions were stored at -20°C for a maximum of one month on the basis of previous stability studies (Rocchi et al. 2016). Working standards were prepared fresh daily from the stock solutions.

2.3.2 Extraction

Retinoids were extracted from frozen liver samples (~ 200 mg) by homogenization in methanol followed by solid phase extraction (SPE) fractionation/clean-up as described previously (Gesto et al. 2012) to produce two fractions: a RA fraction containing retinoic acids and a non-polar fraction containing retinol and the storage fatty acid esters. Homogenates were spiked with an internal standard mixture containing 4 μM acitretin, 4 μM atRA-d5 and 20 μM

retinyl acetate (RAc). The resulting extracts were dried, reconstituted in 100 µL ACN (RA fraction) or 200 µL 9:1 methanol:diethyl ether (non-polar fraction), and transferred to amber autosampler vials with volume-reducing glass inserts. Dilutions of the final extracts were made as described below to yield equivalent tissue masses of 5 mg for quantitation of retinol/retinyl esters, 50 mg for RA analysis, and 50 mg for profiling of non-polar retinoids by liquid chromatography (LC)-QToF. A simple schematic demonstrating the preparation and analysis of the two retinoid fractions is shown in the Supporting Information (SI, Figure S-1).

2.3.3 Liquid Chromatography

Separations were performed on an Agilent Technologies (Santa Clara, CA, USA) 1200-series HPLC system comprised of a vacuum degasser, binary pump, thermostatted autosampler, and thermostatted column compartment. The autosampler was cooled to 4°C for both polar and non-polar separations. A 5 µM caffeine standard was prepared as an instrument performance standard (IPS). The autosampler was programmed to overlap a 1 µL IPS injection with each sample in order to assess precision over a large number of injections. Samples were flagged for review if drastic shifts in ion ratio (± 10%) or retention time (± 0.2 minutes) of the IPS were observed.

RA isomers were separated on a Supelco RP-Amide analytical column (2.1 x 150 mm, 3 µm) protected by a Supelco ABZ+Plus guard cartridge (2.1 x 20 mm, 5 µm) as described previously (Kane and Napoli 2010). Injection volumes were 25 µL. Non-polar retinoids were separated on an Agilent Poroshell-120 EC-C18 column (3.0 x 100 mm, 2.7 µm) protected by a matched guard column (3.0 x 5 mm, 2.7 µm). The column compartment was heated to 40°C. Mobile phase A was methanol/tetrahydrofuran/water/formate (75:18:7:0.1) and mobile phase B was composed of the same solvents at different ratios (57:36:7:0.1). Solvent was delivered at 430

μL min^{-1} using the following gradient expressed as %B: 0 – 0.4 min, 0%; 0.4 – 1.2 min, 0 – 100%; 1.2 – 9 min, 100%; 9 – 10 min, 100 – 0%. Injection volumes were 10 μL.

2.3.4 Mass Spectrometry

Retinoid quantifications were performed on an AB Sciex (Concord, ON, Canada) 5500 quadrupole-linear ion trap (Q-LIT) mass spectrometer fitted with a Turbo V ion source operating in positive-mode atmospheric pressure chemical ionization (APCI). Two multiple reaction monitoring (MRM) transitions per analyte were selected for analysis. Q1 and Q3 were operated at high and unit resolution, respectively. Optimal source and compound-dependent parameters were determined using flow injection analysis (FIA) of 3 μM standards in mobile phase and are summarized in Table S-1. Data acquisition and processing were controlled by Analyst version 1.6.2.

High resolution profiling of retinoids was performed with an Agilent 6520 quadrupole time-of-flight (QToF) mass spectrometer operating in positive-mode APCI. Full-scan MS spectra (*m/z* 100 – 1000) were acquired at 3 Hz to screen for precursor ions characteristic of retinol/retinyl esters (*m/z* 269.2264) and dehydroretinol/dehydroretinyl esters (*m/z* 267.2107). Precursor detection triggered MS/MS acquisition (*m/z* 50 – 300) at 2 Hz using collision energies of 18 V. Optimal ion source parameters were determined using FIA of 20 μM standards in mobile phase and are summarized in the SI. An Agilent 1100-series isocratic pump was used to introduce a reference mass solution into the column eluent at a rate of 100 μL min^{-1} via tee infusion. Lock masses at *m/z* 121.050873 and *m/z* 922.009798 were used to correct for drift and ensure acceptable mass accuracy was maintained. Data acquisition and processing was controlled by MassHunter version B.05.02.

2.3.5 Method Validation

Linearity, limits of detection and quantification (LOD/LOQ), precision, and accuracy were investigated as validation parameters for both analyses. The LOD and LOQ were calculated as the amount of analyte required to produce a signal of three and ten times the standard deviation of the blank, respectively (n = 5).

RA calibration curves were generated with standard concentrations of 0, 1, 4, 15, 60, 250, and 1000 ng mL^{-1} (n = 5). Lines of best fit were calculated using 1/x weighted linear least squares regression. ROL and RPa concentrations of 2, 10, 60, 280, and 1400 ng mL^{-1} (n = 5) were used to generate calibration curves and lines of best fit were calculated using linear least squares regression with 1/x^2 weighting.

2.3.5 Statistics

Statistical analyses were performed in SigmaPlot version 13.0 (Systat Software, San Jose, CA, USA). Two-way ANOVAs were performed after data had been log$_{10}$-transformed to ensure normality (Shapiro-Wilk test) and equal variance (Brown-Forsythe test), followed by Tukey's Honestly Significant Difference post-hoc tests. Hepatic retinoid concentrations were compared within each site, and between sites for each individual analyte. Concentrations of differing analytes were not compared between sites. Pairwise comparisons considered a 95% confidence interval ($\alpha = 0.05$). Error bars represent the standard error of the mean (SEM) and retinoid concentrations are reported as mean ± SEM ($3 \leq n \leq 8$).

2.4 Results

2.4.1 LC-MS/MS Optimization

Chromatographic conditions and mass analyzer conditions were optimized separately for RA isomers and non-polar retinoids. Several chromatographic separations were evaluated on

conventional and superficially porous packing (SPP) columns to rapidly resolve RA isomers (Figure 2-1). Only the porous C18 column allowed for reduced separation time while maintaining acceptable resolution. The RP-Amide column was ultimately selected for its superior resolving power at the expense of increased analysis time, resulting in complete separation of all RA isomers and internal standards within 15 minutes (Figure S-2). Accounting for washing and re-equilibration, samples were injected every 25 minutes. The diagnostic 301 → 205 and 301 → 255 MRM transitions were selected for RAs based on literature review (Kane and Napoli 2010, Saha et al. 2014). A Q3 product ion scan from the deuterated analogue atRA-d5 revealed the 306 → 260 transition at high intensity, however 306 → 206 was observed rather than the anticipated 306 → 210 transition (Figure S-3). Product ions for acitretin were selected based on literature review (Sharma et al. 2012, Doyle and Kline 2017) followed by a Q3 scan to confirm those products were present at sufficient intensities.

In the separation of non-polar retinoids a two-fold improvement in sample throughput was achieved by adapting a previously described separation (Defo et al. 2012) for use on a SPP C18 column, allowing for the separation of ROL, RAc, and RPa in under 10 minutes (Figure S-4). With equilibration, samples could be injected every 15 minutes. The full-scan APCI-MS spectra of retinol and retinyl palmitate (Figure S-5) share a common base peak at nominal m/z 269, resulting from the loss of water and palmitic acid, respectively. Diagnostic molecular ions including $[M^-]^+$, $[M+H]^+$, and $[M-H]^+$ can also be observed, however not at abundances reliable for quantitation. Thus, two MRM transitions characteristic of dehydrated retinol (269 → 119 and 269 → 199) were chosen based on literature review to maximize sensitivity for quantitative analysis of all non-polar retinoids (Rocchi et al. 2016).
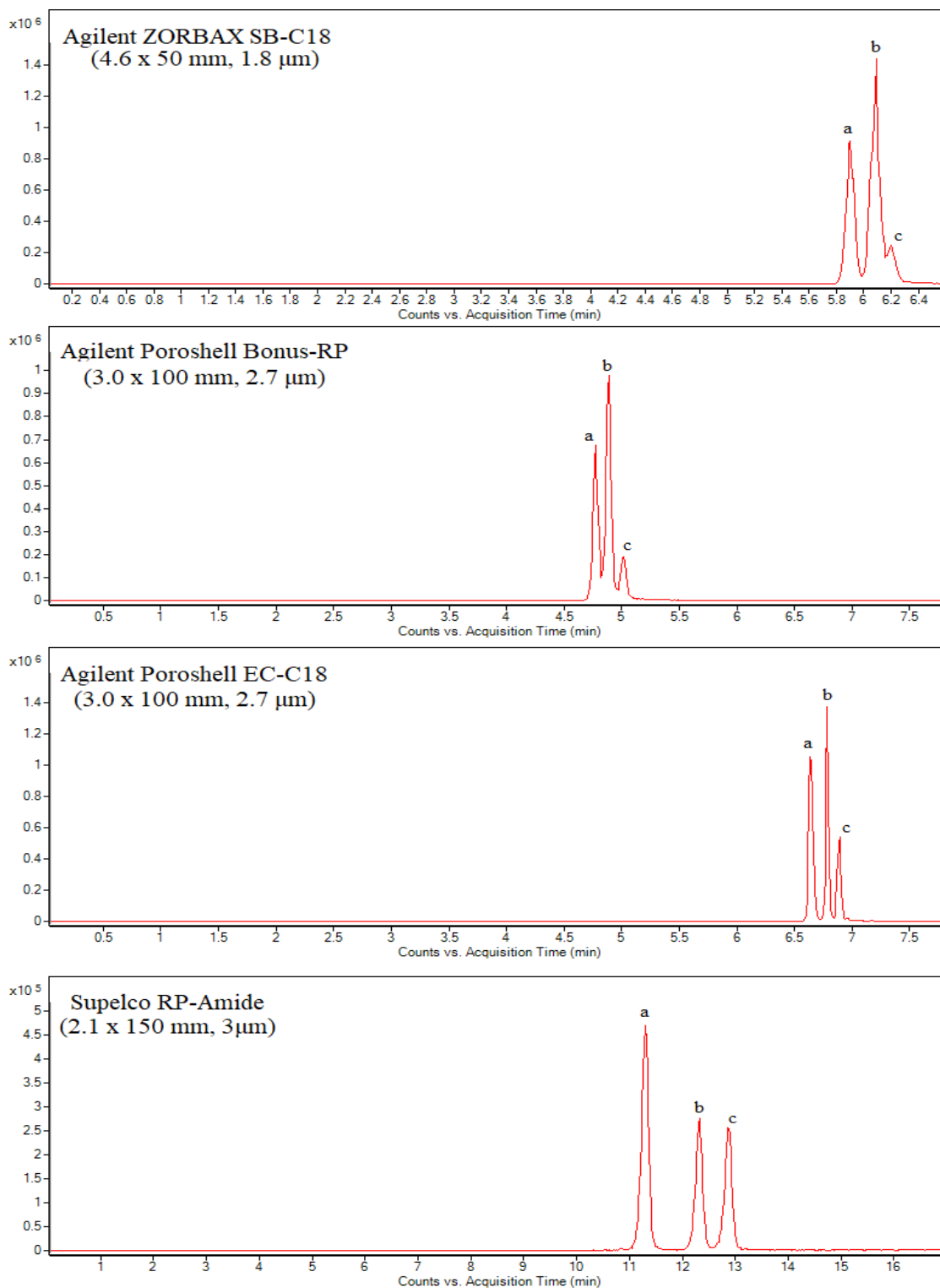
Figure 2-1: Chromatography of RA isomers on various analytical columns

13-*cis*-RA (a), 9-*cis*-RA (b), and all-*trans*-RA (c) were separated on several different analytical columns. Mobile phase A was water + 0.1% formic acid and mobile phase B was acetonitrile + 0.1% formic acid. RA concentrations were approximately 100 ng mL^{-1}.

2.4.2 Method Validation

Limits of detection < 1 ng mL^{-1} were achieved for retinoic acids, retinol, and retinyl palmitate (Table 2-1). RA calibration curves (Figure S-6) demonstrated excellent linearity (R^2 = 0.9999) over a wide concentration range. Including the LOQ, accuracy was 91 – 114% and coefficients of variation (CV) at each injection level ranged from 0.24 – 7.7% (n = 5). The calibration curves for retinol and retinyl palmitate (Figures S-7 and S-8) were skewed by the highest calibration points, however they still demonstrated acceptable linearity ($R^2 \geq 0.994$). For ROL, accuracy was 83 – 117% and % CV at each injection level (n = 5) ranged from 3.2 – 9.3%. For RPa, accuracy and % CV (n = 5) ranged from 86 – 112% and 1.7 – 5.5%, respectively. As shown in Table 2-2, retention times were consistent for all analytes across multiple injections (n = 30) and the ion ratios were highly reproducible for the RA analysis. More variable ion ratios were observed for the non-polar retinoids, however these RSDs are reflective of other MS/MS analyses for retinol and retinyl esters (Rocchi et al. 2016).

Table 2-1: Limits of detection and quantification of retinoids by LC-MS/MS

Analyte	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	mLOD (pg)
Separation I: Retinoic acids			
13-*cis*-RA	0.07	0.12	1.79
9-*cis*-RA	0.08	0.54	2.06
all-*trans*-RA	0.25	1.1	6.31
Separation II: Non-polar retinoids			
ROL	0.54	1.04	10.4
RPa	0.25	0.82	8.2

LOD: limit of detection; LOQ: limit of quantification; mLOD: mass limit of detection

Table 2-2: LC-MS/MS parameters for analyte identification

Analyte	RT ± SD (min)	Q (*m/z*)	q (*m/z*)	Ratio (% RSD)
Separation I: Retinoic acids				
Acitretin	10.57 ± 0.02	327 → 177	327 → 267	21 (6.2)
13-*cis*-RA	13.06 ± 0.02	301 → 205	301 → 255	65 (5.1)
9-*cis*-RA	14.23 ± 0.01	301 → 205	301 → 255	63 (7.9)
all-*trans*-RA	14.85 ± 0.02	301 → 205	301 → 255	64 (3.8)
all-*trans*-RA-d5	14.73 ± 0.02	306 → 206	306 → 260	42 (5.1)
Separation II: Non-polar retinoids				
ROL	2.13 ± 0.01	269 → 119	269 → 199	59 (24)
RAc	2.76 ± 0.01	269 → 119	269 → 199	56 (18)
RPa	8.35 ± 0.01	269 → 119	269 → 199	57 (22)

RT: Mean retention time ± standard deviation (n = 30); Q: quantifier transition; q: qualifier transition. Ion ratio calculated as mean relative abundance of qualifier and quantifier transitions, q/Q x 100% (n = 30).

2.4.3 Extraction Optimization

The SPE method used to isolate retinoids from tissue (Gesto et al. 2012) calls for ~ 200 mg of wet tissue to be extracted for subsequent LC-DAD analysis, however it was found that reduced sample amounts were required for MS/MS analysis. For non-polar retinoids, 5 mg of tissue was sufficient for MRM analysis while 50 mg was adequate for QToF profiling. Matrix effects in the RA analysis could not be eliminated by adjusting amounts of sample extracted, however a sample of 50 mg was found to suffer the least from interferences.

Detector saturation in the non-polar MRM analysis was evident at 200, 100, and 50 mg amounts of liver extracted by peak deformation and drastically altered ion ratios (data not shown). A 1:40 dilution of the 200 mg extracts (5 mg equivalent) was evaluated which yielded adequately quantifiable peaks with appropriate ion ratios (Figure S-9). The QToF instrument was operated in the "extended dynamic range" mode and in 200, 100, and 50 mg extracts had adequate MS/MS sensitivity with the ability to detect molecular ions in the full-scan MS spectra.

Matrix interferences were substantial in the RA isomer separation and were not markedly improved at any of the three levels tested. General trends of increased baseline noise and more intense interferences were observed with greater amounts of tissue extracted (Figure S-10). A high MRM background was also observed for the 301 → 255 RA qualifier ion (Figure S-11), which was approximately ten times higher in matrix than in blanks. Ion ratios and retention time of the internal standards were not affected. Other MRM candidate ions including m/z 123 and m/z 159 were also evaluated and found to produce equally complex chromatograms (data not shown). 9cRA and atRA remained above their respective LOQs at all three levels tested, however ion ratios for both compounds were outside of tolerance ranges ($\pm$ 20%) and the data is therefore suspect. 13cRA was not detected in any of the samples analyzed.

2.4.4 Analyte Degradation

Pooled liver tissues from shorthead redhorse sucker (*Moxostoma macrolepidotum*) from a reference site were artificially degraded by exposure to ambient light and room temperatures (rather than under yellow light on ice) during handling to investigate if matrix interferences in the RA separation could be a result of degradation during storage. Measured 9cRA and 13cRA levels were altered following exposure to heat and light while atRA levels and non-polar retinoids were unaffected (Figure S-12). 13cRA concentrations increased from a non-detect to 5.04 ± 1.03 ng mL^{-1} and 9cRA was significantly increased from 5.22 ± 0.21 to 9.51 ± 0.37 ng mL^{-1} ($p < 0.05$). MRM quantitation of non-polar retinoids showed no differences in ROL or RPa concentrations however the calculated values were far beyond the calibration range as samples were not diluted prior to analysis. The non-polar fractions were also profiled for degradation by LC-QToF and no differences were observed in the total ion or MS/MS chromatograms (data not shown). Exposure to heat and light induced measurable changes in the concentrations of two RA

isomers, which was anticipated given the lability of active retinoid compounds. It is unknown why atRA levels were unaffected, which suggests other interfering matrix components may be contributing to the measured response for RA compounds. It is still not clear whether these interferences are naturally present in the matrix or are products of analyte degradation during storage. The non-polar retinoids appeared relatively insensitive to harsh handling, which is consistent with observations that stored retinoids are generally more robust than the biologically active retinoic acids (Kane and Napoli 2010).

2.4.5 Profiling of Retinoids by LC-QToF

LC-QToF was used to compare standards and endogenous retinoids in order to demonstrate feasibility of assessing dehydroretinoids in liver samples without authentic standards. Analogous transitions of dehydrated dROL as determined by LC-QToF (267 → 119 and 267 → 197) were included in the MRM method to semi-quantitatively estimate dROL and dRPa concentrations by comparing to the 269 → 199 calibration responses for ROL and RPa, respectively. Good agreement between the accurate mass extracted ion chromatogram (EIC) obtained by LC-QToF and the MRM chromatogram suggests that the 267 → 119 MRM transition is indeed specific for dehydroretinoids (Figure 2-2).
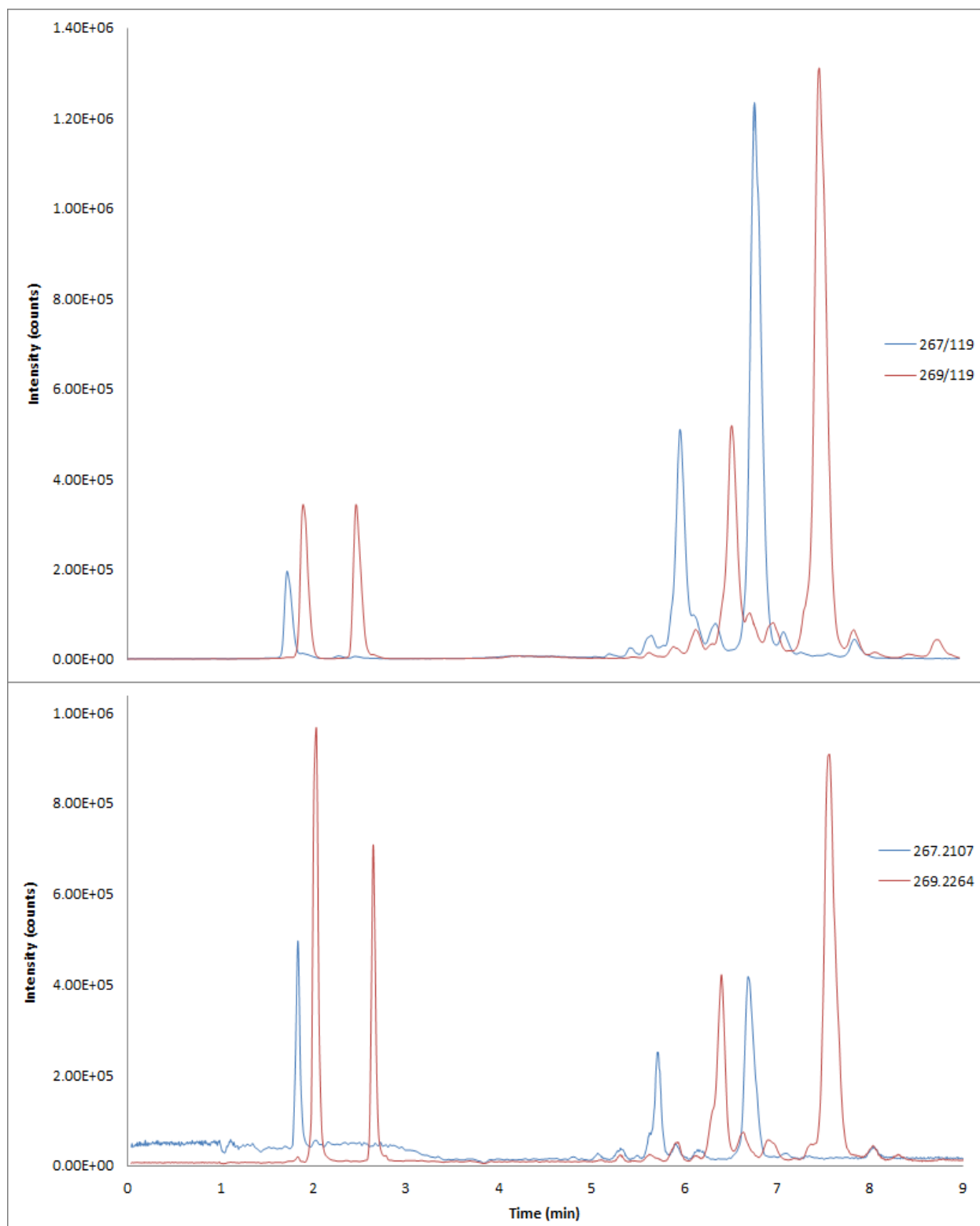
Figure 2-2: Comparison of Q-LIT MRM (top) and QToF EIC (bottom) instrument response

Analyzed sample was 50 mg liver from female shorthead redhorse sucker. The extract was diluted 1:10 for MRM analysis (5 mg equivalent). EIC signal is *m/z* ± 50 ppm.

Using ROL and RPa standards, the MS/MS product ion spectra from *m/z* 269.2264 are identical (Figure 2-3a), yielding the expected characteristic products at *m/z* 93.0699, 119.0855, and 199.1481 with better than 10 ppm mass accuracy. The product ion spectra from ROL and RPa in liver samples match those of the standards in terms of retention time, product abundance, and ion ratio (Figure 2-3b). A representative product ion spectrum of a suspected dehydroretinoid (*m/z* 267.2107, Figure 2-3c) is shown to be highly similar to the standards and endogenous retinoids based on product ion abundance and numerous shared products including *m/z* 93.0699 and 119.0855. This suggests these fragments originate from common structural features. It is interesting to note that most product ions above nominal *m/z* 145 differ from the retinoids by approximately two proton masses (2.0156 amu), suggesting these fragments retain the additional double bond moiety of dehydroretinoids.
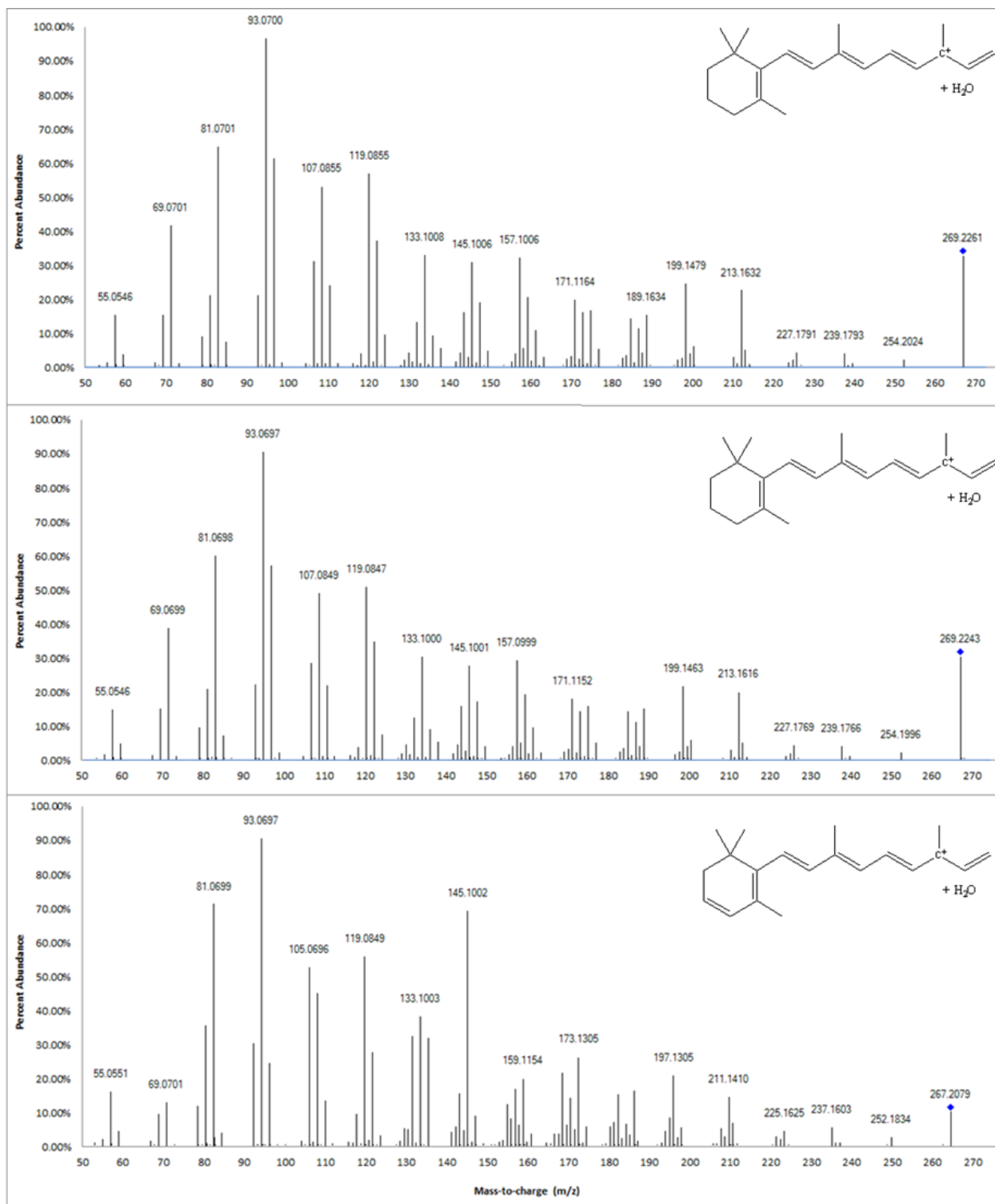
Figure 2-3: QToF MS/MS product ion spectra of standards and endogenous retinoids

Top: retinoid standard; middle: endogenous retinoid in liver; bottom: suspected dehydroretinoid in liver.

2.4.6 Application of the Method to Fish from Great Lakes AOCs

Wild fish sampling and site locations

Brown bullhead (*Ameiurus nebulosus*) were collected in mid-October of 2014 from the Bay of Quinte AOC (Figure 2-4) by electrofishing from the near-field (Trenton) and far-field (Deseronto, ~40 km removed) sites. The Bay of Quinte was listed as an AOC from impairments related to eutrophication, urban development and historical sediment contamination (Environment and Climate Change Canada 2017). Shorthead redhorse sucker were collected in late October of 2014 from the St. Clair River AOC (Figure 2-4). Fish from the near-field site (Stag Island) were captured by electrofishing, and from the far-field site (Lake Huron, ~30 km upstream) by overnight hoop nets. The St. Clair River is a bi-national AOC on the boundary of Ontario and Michigan, designated as such due to industrialization, urbanization, and agricultural activities (Environment and Climate Change Canada 2017).

For all sites, sampling was performed the same day as capture (nets lifted the following morning after overnight sets). Fish were anaesthetized in a water bath containing clove oil and ethanol and euthanized by spinal severance. A subsample (~1 g) of liver was placed in a cryovial and immediately snap-frozen in liquid nitrogen before being transferred to a freezer at -80°C for storage until extraction. Eight samples from each site were selected for analysis. As retinoid levels can often be correlated to sex, only female specimens were selected to eliminate gender as an explanatory variable and enable comparisons between sites.
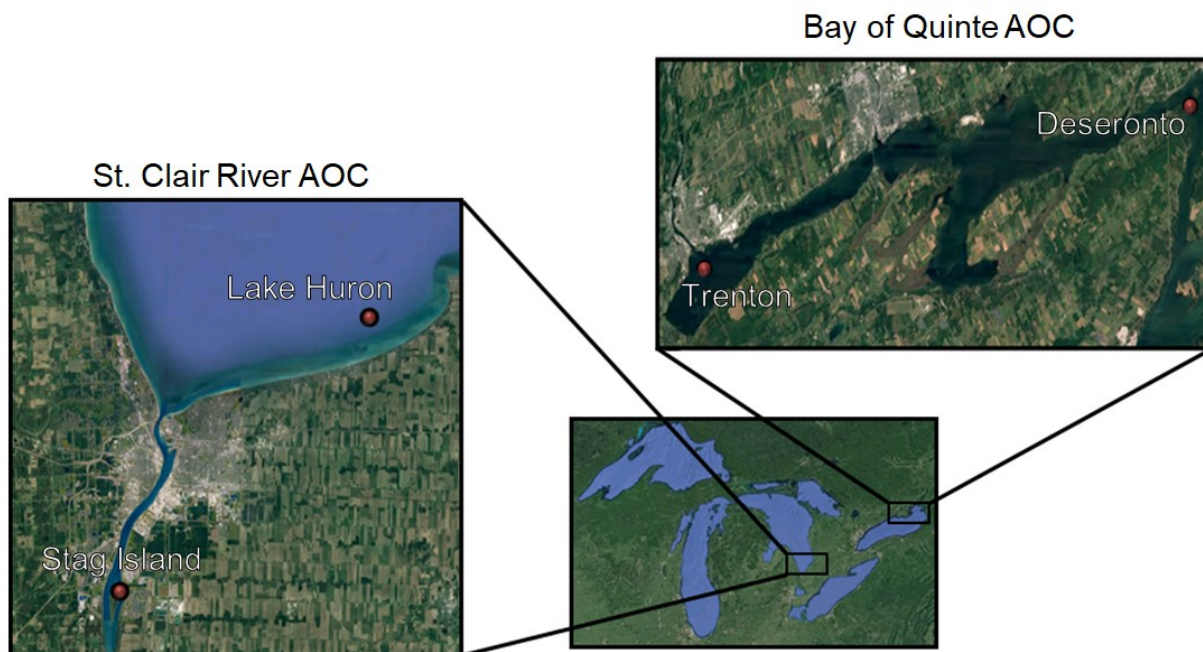
Figure 2-4: Locations of sampling sites from St. Clair River and Bay of Quinte AOCs.

Lake Huron and Stag Island were the respective far and near-field sites for the St. Clair River AOC. Deseronto and Trenton were the respective far and near-field sites for the Bay of Quinte AOC. Google Maps.

Non-polar retinoids

Significant differences between near and far-field populations were observed for all measured retinoids from the St. Clair River ($p < 0.05$) despite high variability as % CVs ranged from 50 - 75% at Lake Huron and 68 - 123% at Stag Island (Figure 2-5). dROL, ROL, RPa, and dRPa were markedly reduced in fish collected at Stag Island. Representative EICs from the near and far-field sites are in good agreement with the MRM analysis and indicate that these samples contain retinoids as the major form of vitamin A, however relatively high levels of dehydroretinoids were also observed (Figure 2-5, insets). Lower variability was observed at the Bay of Quinte sites as % CVs range from 27 - 51% at Deseronto and 40 - 69% at Trenton (Figure 2-6), suggesting the high variance observed in the St. Clair River data may be at least in part

biological. No differences between near and far-field populations were observed for any analyte at this AOC. In contrast to the St. Clair River samples, the EICs from both sites (Figure 2-6, insets) show that dehydroretinoids dominate in these samples and only a fraction of the retinoids are vitamin A_1 type. There are also very few differences between sites in terms of the ester profile and abundance.

Several of the major peaks identified as retinoids based on LC-QToF fragment patterns were assigned by relative retention times and full-scan MS data. RT comparisons were based on the originally reported retinoid separation (Defo et al. 2012). dROL (a) and dRPa (f) (Figure 2-5, insets) were assigned by relative retention time and by identifying molecular species in the full-scan MS data. The $[M+H]^+$ ions for dROL (*m/z* 285.2188) and dRPa (*m/z* 523.4388) were observed at δ 9 and δ 23 ppm mass error, respectively. The leading shoulder on the retinyl palmitate peak (g) is likely retinyl oleate based on retention time (Doyon et al. 1998) and the molecular ion $[M+H]^+$ at *m/z* 551.4616 (δ 37 ppm). The late eluting peak (h) is proposed to be retinyl stearate, however no molecular ions were detected for this compound. The prominent unknown esters are proposed to be dehydroretinyl palmitoleate (d) and retinyl palmitoleate (e) based on assumed molecular ions of *m/z* 521.4306 (δ 9 ppm) and *m/z* 523.4371 (δ 26 ppm), respectively. Co-elution with the linoleate and myristate esters has been documented (Rocchi et al. 2016) however no molecular ions for these esters were observed. Peaks (i) and (j) could not be identified by molecular ions or by MS/MS fragment spectra.
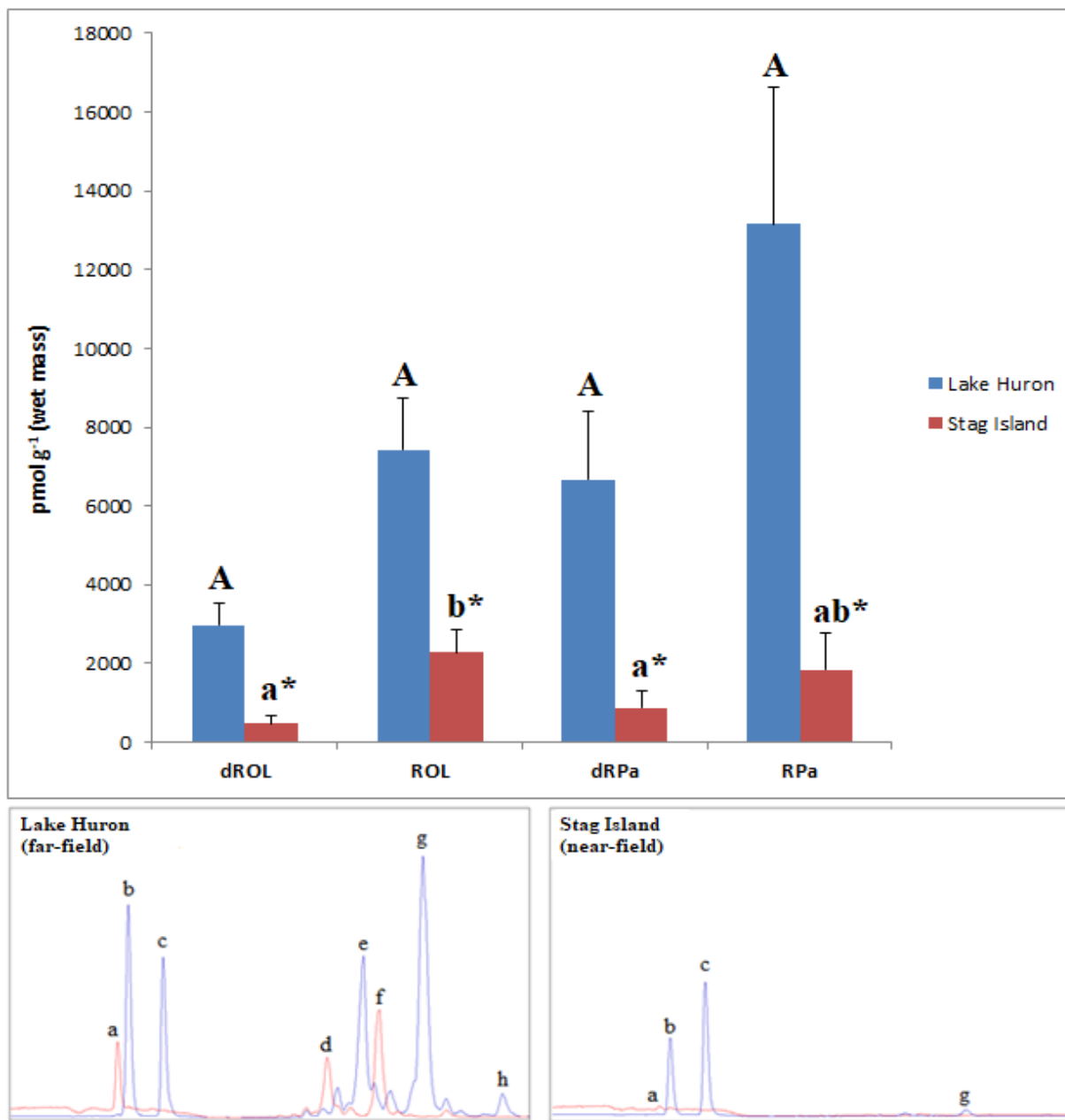
Figure 2-5: Measured concentrations of non-polar retinoids in female shorthead redhorse sucker at the St. Clair River AOC reference site (Lake Huron) and near-field site (Stag Island; top)

Values are mean ± SEM (n = 8). Capitalized and lower-case letters indicate comparisons within the reference and near-field sites, respectively. Means with dissimilar letters are significantly different from one another (p < 0.05). Asterisks (*) indicate a significant difference from the reference site for each compound (p < 0.05).

Inset: QToF MS EIC of *m/z* 267.2107 (red) and *m/z* 269.2264 (blue), ± 50 ppm. a = dehydroretinol; b = retinol; c = retinyl acetate (IS); d = dehydroretinyl palmitoleate; e = retinyl palmitoleate; f = dehydroretinyl palmtiate; g = retinyl palmitate; h = retinyl stearate.
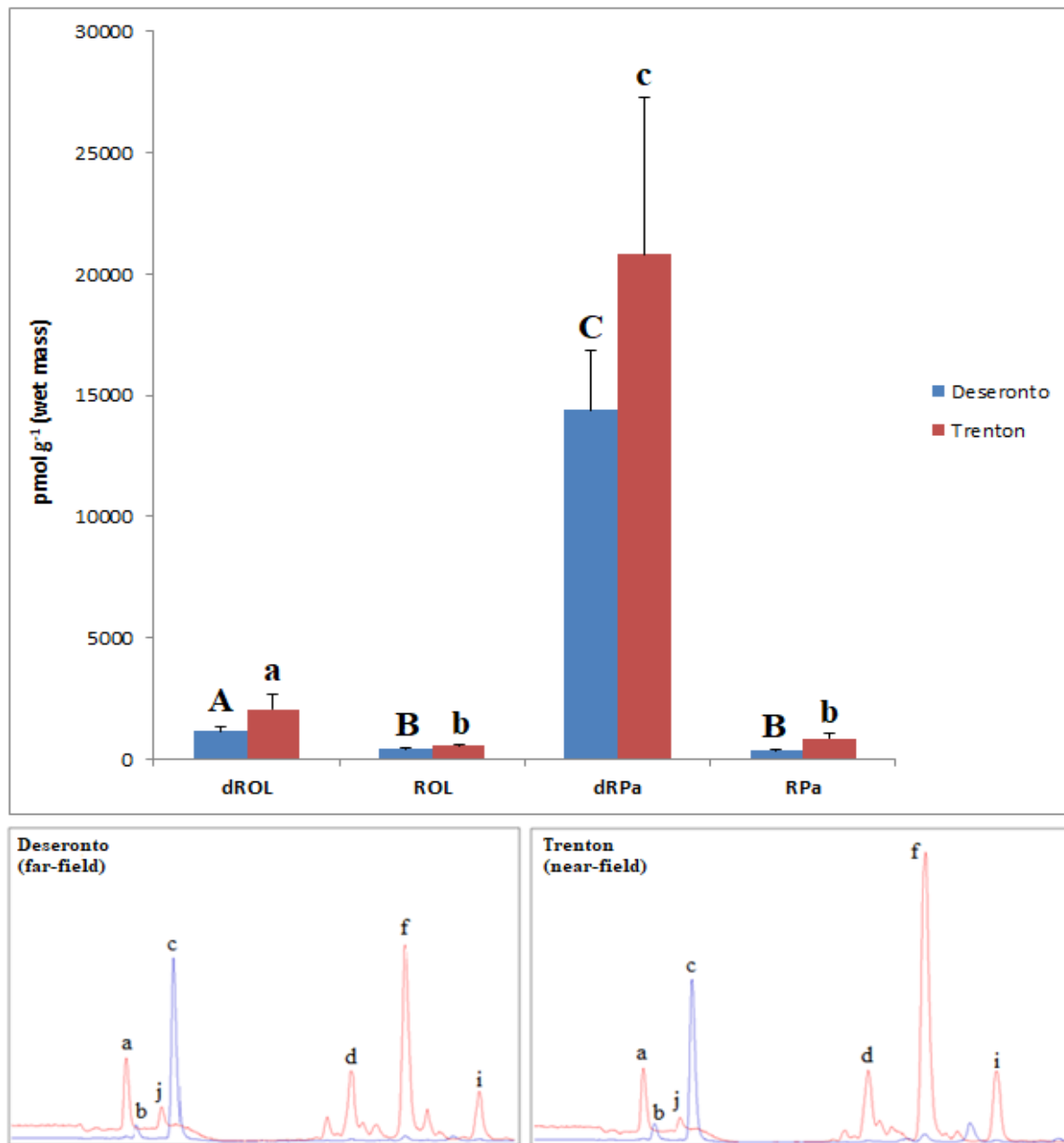
Figure 2-6: Measured concentrations of non-polar retinoids in female brown bullhead at the Bay of Quinte AOC reference site (Deseronto) and near-field site (Trenton; top)

Values are mean ± SEM ($7 \leq n \leq 8$). Capitalized and lower-case letters indicate comparisons within the reference and near-field sites, respectively. Means with dissimilar letters are significantly different from one another ($p < 0.05$). Asterisks (*) indicate a significant difference from the reference site for each compound ($p < 0.05$).

Inset: QToF MS EIC of m/z 267.2107 (red) and m/z 269.2264 (blue), ± 50 ppm. a = dehydroretinol; b = retinol; c = retinyl acetate (IS); d = dehydroretinyl palmitoleate; f = dehydroretinyl palmtiate. Peaks i and j were not identified.

Retinoic acids

At the St. Clair River AOC, 9cRA was observed to be significantly higher ($p < 0.05$) at Stag Island (Figure 2-7). atRA was found to be significantly lower ($p < 0.05$) at the near-field site at the Bay of Quinte AOC (Figure 2-8). Matrix effects were again persistent in the 301 → 205 MRM chromatograms as evidenced by loss of peak shape and unidentified interferences (Figures 2-7 and 2-8, insets). At both AOCs, atRA appeared to be the most abundant isomer and 13cRA was the least abundant isomer at the far-field sites. At the near-field sites for AOCs, atRA and 9cRA levels did not appear to be different. Due to the magnitude of the matrix effects and resultant poor chromatography, interferences, and ghost peaks, the method does not meet data quality standards and therefore cannot be applied or reported. Further work is needed to resolve the matrix effects and develop a reliable method for retinoic acid quantifications.

Figure 2-7: Measured RA concentrations in female shorthead redhorse sucker at the St. Clair River AOC reference site (Lake Huron) and near-field site (Stag Island; top)

Values are mean ± SEM ($6 \leq n \leq 8$). Capitalized and lower-case letters indicate comparisons within the reference and exposed sites, respectively. Means with dissimilar letters are significantly different from one another ($p < 0.05$). Asterisks (*) indicate a significant difference from the reference site for each compound ($p < 0.05$).
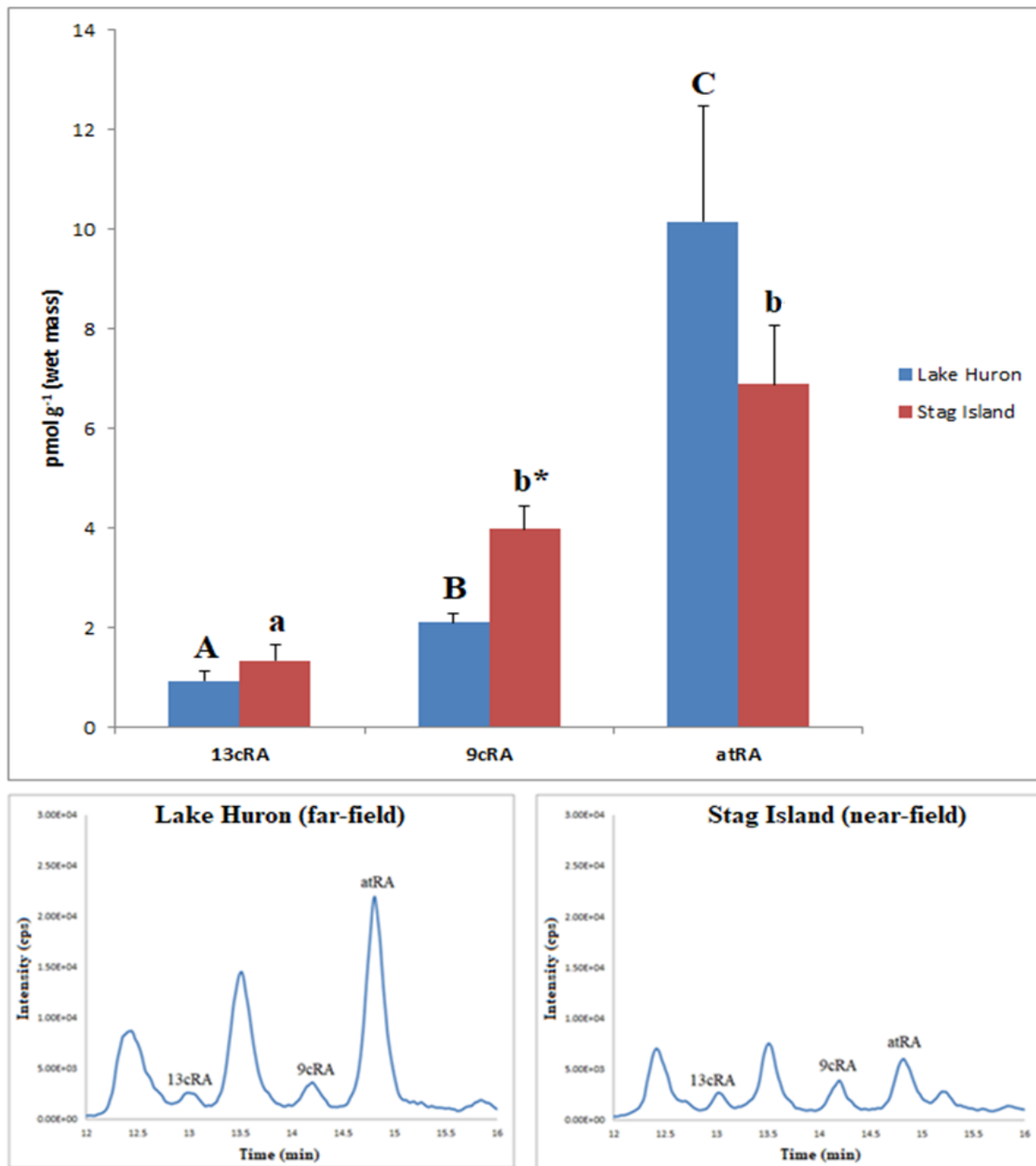
Inset: $301 \rightarrow 205$ MRM for retinoic acids.
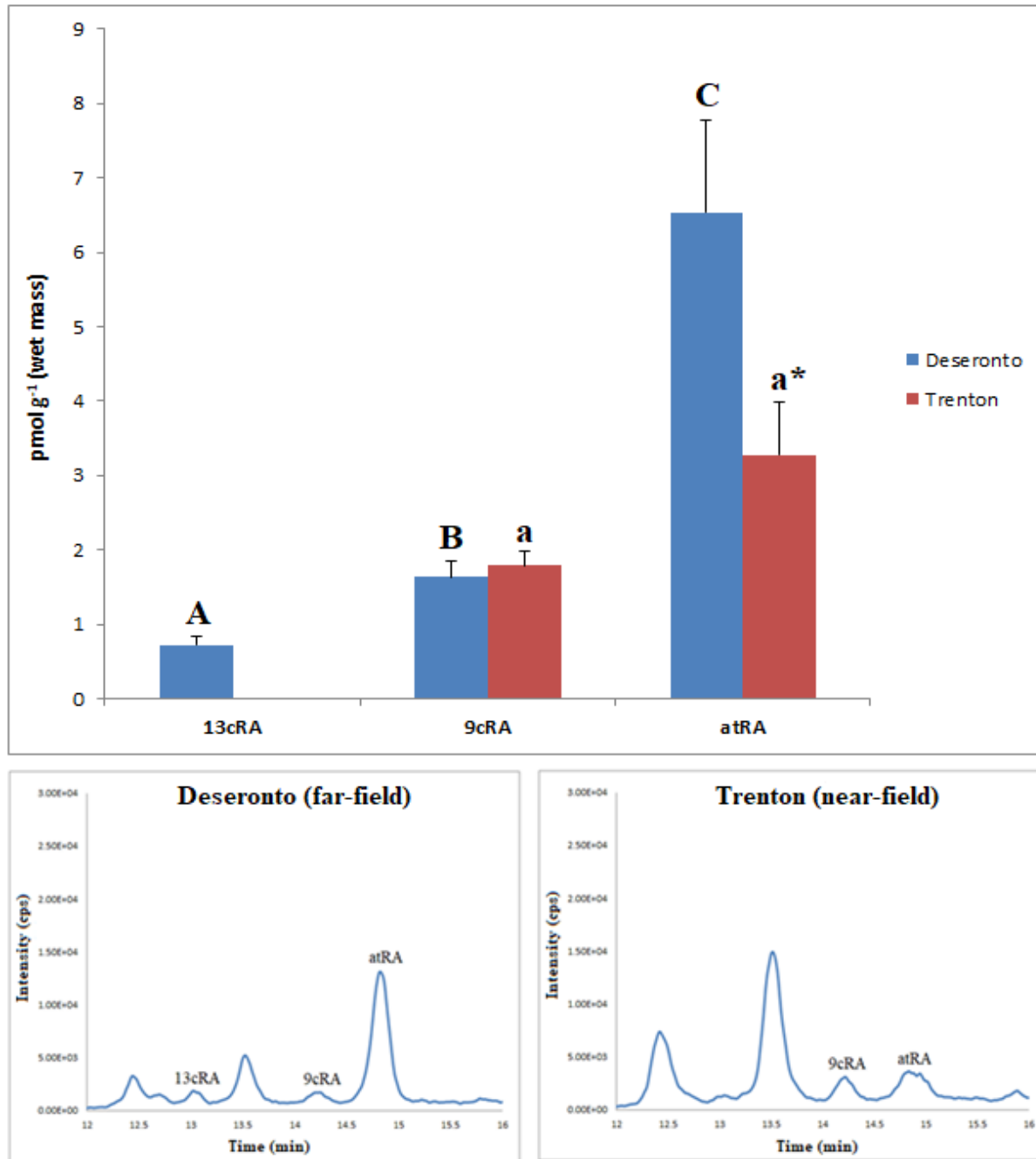
Figure 2-8: Measured RA concentrations in female brown bullhead at the Bay of Quinte AOC reference site (Deseronto) and near-field site (Trenton; top)

Values are mean ± SEM ($3 \leq n \leq 8$). Capitalized and lower-case letters indicate comparisons within the reference and exposed sites, respectively. Means with dissimilar letters are significantly different from one another ($p < 0.05$). Asterisks (*) indicate a significant difference from the reference site for each compound ($p < 0.05$).

Inset: 301 → 205 MRM for retinoic acids

2.5 Discussion

In this study, a multi-instrument LC-MS/MS method for the measurement of multiple retinoids was developed and applied to fish collected from two Great Lakes AOCs. The method was capable of rapid, quantitative assessment of non-polar retinoid status (free and esterified retinols) including tentatively identifying retinoids for which commercial standards are unavailable through high resolution QToF profiling. Measureable differences in retinoid status between shorthead redhorse sucker collected from the near and far-field sites at the St. Clair River AOC were demonstrated. No differences were observed in brown bullhead populations sampled at the Bay of Quinte AOC, however high levels of dehydroretinoids were detected and demonstrates the ability to profile both classes of retinoids in fish hepatic tissues. Unresolved matrix issues prevented confident quantitation of the biologically active retinoic acids due to unacceptable chromatography and prominent MS/MS interferences, resulting in failure to meet QA/QC criteria (i.e. ion ratio). As presented, the method is suitable for assessing non-polar retinoid status in extracts of hepatic tissue. The target of future work should be to resolve the matrix interferences in the RA analysis to provide a more complete approach to retinoid analysis in fish health studies.

Method development and optimization

Approaches including liquid-liquid extraction with hexane, mono-phase liquid extraction with methanol followed by SPE clean-up, and mono-phase liquid extraction with acetonitrile/*tert*-butanol were evaluated as options for extraction of retinoids from hepatic tissues. Methanol extraction followed by SPE was chosen based on the unique dual advantages of fractionation and sample clean-up offered by this approach, which other methods could not provide. The SPE cartridges used were Waters Oasis™ MAX (Mississauga, ON, Canada) which

contains a mixed-mode sorbent with both reversed-phase and anion exchange capabilities and allows for fractionation of retinoids (neutral and polar compounds) by exploiting the acid/base properties of retinoic acids. During sample loading all retinoids are initially retained by reversed-phase interactions as retinoic acids are neutral. Subsequent washing of the sorbent with a weak base (5% NH_4OH in water) effectively partitions the sample as the ionizable RAs are locked to the sorbent by the anion exchange functionality while retinol and retinyl esters remain bound by reversed-phase interactions. Two distinct sample fractions can then be obtained through selective elution. The non-polar retinoids are first removed with pure methanol which disrupts reversed-phase binding. The retinoic acids are then eluted with acidified methanol (containing 2% formic acid) to neutralize ionizable compounds and thus break the anion exchange interaction. This SPE approach improves upon simple hexane extraction by reducing matrix interferences and generating cleaner sample extracts, while reconciling the vastly different endogenous ranges of retinyl esters and retinoic acids through partitioning which mono-phase liquid extractions alone cannot provide.

When separating non-polar retinoids on C18 columns, retinyl esters will often co-elute as pairs/trios as they differ only in length and degree of unsaturation in the fatty acid chains. In general, dehydroretinoids will elute before their retinoid counterparts, and retention decreases as degree of unsaturation increases (Rocchi et al. 2016). As the palmitate forms (RPa/dRPa) are often the most abundant REs, they are also often the primary targets for retinoid assessments in fish (Doyon et al. 1998, Defo et al. 2012). It is generally accepted that there will be signal contribution from the less abundant retinyl oleate unless extraordinary lengths are taken to resolve them. The objective in this application was to rapidly separate several major retinoids (i.e. ROL, dROL, RPa, and dRPa) under conditions suitable for MS analysis. While complete

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resolution of all retinyl esters is ideal, the long separation time required is not conducive to routine analysis, particularly when dealing with labile compounds. Additionally, application of LC-QToF demonstrated the ability to identify retinoids by molecular ions, which places less emphasis on chromatographically resolving retinyl esters as closely or co-eluting compounds could be differentiated by mass-accurate molecular ions.

While several new separations were evaluated on various stationary phases to separate the retinoic acids, the Supelco alkylamide column described in the literature (Kane and Napoli 2010) was ultimately found to be able to completely resolve RA isomers. Relative to traditional C18 columns, alkylamide stationary phases are more polar due to the embedded amide group and thus better retain organic acids, leading to enhanced resolution (Gundersen 2006). The increase in stationary phase polarity is evidently essential to fully resolving RA isomers, however carbon loading and/or column dimensions also appears to be critical as the separation could not be reproduced on the porous Bonus-RP column which shares a similar stationary phase. As retinoic acids are particularly unstable it would be worthwhile to explore other SPP alkylamide columns in the future to increase sample throughput and minimize any potential analyte degradation arising from long analysis times.

In determinations of retinoic acids and retinol/retinyl esters by mass spectrometry, ionization techniques including ESI (+/-), APCI (+/-), and several others have been evaluated (Rocchi et al. 2016). APCI is well-suited to relatively non-polar compounds of intermediate molecular weight, and positive ionization usually offers the greatest sensitivity and dynamic range (Wang et al. 2000, Wang et al. 2001). Thus, APCI (+) was selected for this analysis. Most MRM product ions were selected based on a review of available literature of MS/MS analyses for retinoids in biological matrices. atRA-d5 (internal standard) required a full optimization to

determine product ions as acceptable response could not be achieved with the MRM transitions described in the literature. In determining MRM candidate ions for atRA-d5, the analogous 306 → 210 transition was not observed and 306 → 206 was instead observed at high intensity, which was unanticipated. Examination of a recently proposed mechanism for the 301 → 205 transition characteristic of retinoic acids (Jones et al. 2015) appears to fit the observed loss of deuterium from atRA-d5 and therefore 306 → 206 was chosen based on signal response.

Precision and accuracy targets were respectively set at ± 15% CV (± 20% CV at the LOQ) and ± 15% of the nominal value (± 20% at the LOQ) in accordance with guidelines for bioanalytical method validation (Food and Drug Administration 2001) and were met for all analytes across the calibration range. There are conflicting opinions on the use of internal standards (IS) in retinoid analysis. It has been suggested that if extraction efficiency is sufficiently high, internal standards are not required (McCaffery et al. 2002) as long equilibration times between endogenous protein-bound retinoids and the IS compounds may skew quantitative results. Others suggest analyte losses can be better controlled for by use of deuterated or chemical analogues (Kane and Napoli 2010, Arnold et al. 2012). The SPE protocol used was validated by Gesto et al. (2012) to provide recoveries up to 94% for retinoids in serum. Preliminary testing showed consistently high recovery in blanks, however IS recoveries in liver samples were highly variable (26 - 84%). Internal standards were therefore included in the method for qualitative purposes only (e.g. monitoring of isomerization or retention time shifts) and analytes quantified using the external standards method. Further work into investigating IS recoveries from tissue extracts is recommended in order to include these compounds in future analyses and improve data quality.

Measurement of non-polar retinoids at Great Lakes AOCs

Two populations of fish were selected in order to assess method applicability to samples of different species with wide geographical separation and exposure to different environmental stressors. Due to the limited scope of the study direct comparisons cannot be made between AOCs, however general trends can still be elucidated. Retinoid distribution in shorthead redhorse sucker collected from the St. Clair River AOC is particularly interesting as no differences were observed between analytes at the far-field site (Table 2-3), however the majority of vitamin A is expected to be sequestered as retinyl esters and thus palmitate ester concentrations should be much greater than those of free retinol (Doyon et al. 1998, Gesto et al. 2012). Reductions in hepatic retinoid stores and increases in free retinol have been associated with oxidative stress (Defo et al. 2014), although the similar trends observed at both sites suggests other factors may be responsible. Retinoid concentrations in brown bullhead collected from the Bay of Quinte AOC more closely resemble the expected distribution of retinoids, showing the majority of vitamin A stored as retinyl esters with dRPa significantly more abundant ($p < 0.05$) than ROL or dROL at both sites.

In this application, brown bullhead liver tissues from the Bay of Quinte were found to be abundant in dehydroretinoids. Other work on retinoid analysis in brown bullhead collected from Lake Erie and Lake Ontario (including the Bay of Quinte) found high levels of dehydroretinyl esters in liver (Arcand-Hoy and Metcalfe 1999), supporting this assessment that brown bullhead may be a VA_2-utilizing species. Liver samples of shorthead redhorse sucker did not appear to favour one retinoid form over the other. A review of available literature yielded no information on retinoid assessments in this species and thus no comparisons can be made to the literature. Differences in dietary vitamin A sources may also play a role in these observed ratios of

retinoids to dehydroretinoids, meaning further work would be required to definitively classify shorthead redhorse sucker as VA_1 or VA_2 utilizing species. Profiling of retinoids by LC-QToF demonstrated that the palmitate esters were the most abundant (largest) peaks in all samples and detected numerous other esters present in these extracts, some of which could be identified. The results of this application suggest that quantitative assessment of retinoid status between populations is possible using only the major retinoids (i.e. palmitate esters and free retinols) and does not require full identification of all other peaks. Non-quantitative profiling of other esters can however still yield valuable information on how species metabolize and store vitamin A.

Table 2-3: Levels of free retinols and palmitate esters in female shorthead redhorse sucker from the St. Clair River AOC and female brown bullhead from the Bay of Quinte AOC

Analyte	St. Clair River		Bay of Quinte	
	Lake Huron	**Stag Island**	**Deseronto**	**Trenton**
dROL	2965 ± 569	468 ± 199*	1153 ± 223	2068 ± 592
ROL	7414 ± 1318	2281 ± 579*	434 ± 66	551 ± 79
dRPa	6665 ± 1724	873 ± 412*	14383 ± 2474	20804 ± 6470
RPa	13158 ± 3466	1835 ± 958*	370 ± 38	872 ± 211

Retinoid levels reported as mean ± SEM ($7 \le n \le 8$) in pmol g^{-1} wet mass. Asterisked values (*) represent statistically significant differences in the near-field sites ($P < 0.05$).

Matrix interferences in RA analysis

Substantial matrix effects hindered quantitative analysis for retinoic acids in hepatic tissue. In hope of providing additional confidence in analyte identification, two characteristic transitions for RAs ($301 \rightarrow 205$ and $301 \rightarrow 255$) were selected for this application. The inclusion of qualifier ions is rarely reported in the literature, although most RA analyses are typically applied to serum extracts and it is unlikely that matrix effects are as prevalent as in tissue. Evidently, the *m/z* 255 product was a sub-optimal choice of qualifier for this matrix as ion ratios were up to 25 times higher than demonstrated with RA standards during method development.

Given the poor data quality, the results obtained for RA measurements in the AOC samples (Table 2-4) are highly suspect. It is likely that the age of the samples also contributed to the observed interferences as analytes will degrade over time even when stored at -80°C (Kane and Napoli 2010) and the samples analyzed in this study were approximately three years old. Information on the kinetics of RA degradation in tissue is presently unavailable. The high levels of 9cRA measured in tissue samples may be artifacts of degradation during storage or sampling as tissues may not have been exposed to ambient temperatures for too long during harvesting. Low levels/absence of 13cRA is particularly puzzling as RA isomer distributions in fish are thought to be comparable to those in mammals, with 9cRA being the least abundant and atRA being the most abundant (Ørnsrud et al. 2009, Gesto et al. 2012). It is unclear as this point if environmental factors such as stress or fasting may affect RA isomer distributions in wild fish. These are questions that future method applications could be directed toward answering.

Table 2-4: Levels of retinoic acids in female shorthead redhorse sucker from the St. Clair River AOC and female brown bullhead from the Bay of Quinte AOC

Analyte	St. Clair River		Bay of Quinte	
	Lake Huron	Stag Island	Deseronto	Trenton
13cRA	0.94 ± 0.20	1.34 ± 0.33	0.72 ± 0.12	non-detect
9cRA	2.11 ± 0.17	$3.97 \pm 0.46^*$	1.63 ± 0.23	1.79 ± 0.19
atRA	10.15 ± 2.32	6.90 ± 1.17	6.53 ± 1.24	$3.27 \pm 0.72^*$

Retinoid levels reported as mean $\pm$ SEM ($3 \leq n \leq 8$) in pmol g^{-1} wet mass. Asterisked values (*) represent statistically significant differences in the near-field sites ($P < 0.05$).

Further work is required to assess the magnitude of matrix effects on RA analysis and resolve these analytical issues. While non-polar retinoids are generally considered more robust (Kane and Napoli 2010) and can evidently be stored for some period of time. A dedicated study is needed to determine how to best harvest, handle, and store samples for retinoic acid analysis to

eliminate concerns over sample integrity and produce higher quality data. Analyte degradation may also be accelerated in whole tissues, as studies have indicated serum retinoids can be stabilized with ascorbic acid and stored frozen for several years (Driskell et al. 1985a, Driskell et al. 1985b, Comstock et al. 1995). The analysis of serum samples for RAs in fish also presents an interesting potential avenue of exploration. It is possible that the low RA levels in tissue are masked by interfering co-extractives which cannot be sufficiently reduced by even sophisticated extraction approaches such as SPE. The much cleaner matrix in serum samples may circumvent this issue entirely. The current impressive sensitivity of triple quadrupole instruments may make this approach a feasible alternative.

Chapter 3 – Conclusions and Recommendations

The work presented in this thesis represents the crucial first steps in developing and implementing LC-MS/MS techniques for the routine analysis of endogenous retinoids in fish tissues. The presented method is capable of rapid quantification and profiling of non-polar retinoids (retinol and retinyl esters) with a high degree of sensitivity and specificity. This LC-MS/MS approach advances the area of retinoid biology by expanding the range of compounds which can be analyzed without the need for authentic standards, most of which are unavailable or prohibitively expensive. The measurement of biologically active retinoids (retinoic acids) in hepatic tissues was limited by substantial matrix interferences which could not be resolved through selective MS/MS approaches or sophisticated sample clean-up procedures (i.e. SPE). The method meets QA/QC criteria (linearity, limits of detection, precision, and accuracy) for standards, and thus further work into eliminating these matrix interferences is required in order to accurately assess retinoic acid levels in fish from complex biological matrices. Application of the method to analysis of liver samples of shorthead redhorse sucker collected in 2014 from the Great Lakes St. Clair River Area of Concern (AOC) demonstrated the ability to detect statistically significant differences in non-polar retinoid concentrations between the far-field and near-field populations. Liver samples of brown bullhead collected in 2014 from the Great Lakes Bay of Quinte AOC showed no differences between near and far-field populations. Qualitative profiling by LC-QToF determined the fish sampled were abundant in dehydroretinoids, thus demonstrating the ability to assess relative VA_1/VA_2 distribution in fish tissues. The following sub-sections detail avenues of exploration for improving the LC-MS/MS methodology for retinoic acid determinations and potential applications of the revised method in answering additional questions regarding retinoid metabolism and functions in fish.

The matrix interferences complicating the RA analysis are likely due to a combination of factors stemming from age of the samples selected, the low endogenous levels of RAs, and the challenges associated with analyzing a complex matrix for trace constituents. In order to develop a reliable method for RA determinations in fish, the primary objective should be to resolve these analytical issues by simplifying the matrix through improved sample preparation techniques or selection of a less complex sample type. To build on the streamlined approach of generating two retinoid fractions (non-polars and RAs) from one tissue sample, investigation into additional sample clean-up steps could be explored. Various SPE approaches such as stacked cartridges (two-stage SPE) and dispersive SPE (QuEChERS) are available. Dispersive liquid-liquid microextraction (DLLME) is a novel technique for enriching analyte concentrations from low sample volumes and has been applied to the isolation of non-polar retinoids from fruit juices, however to date has not been used for extraction of retinoic acids (Viñas et al. 2013). An entirely new extraction approach or secondary clean-up of the RA extracts may help to remove interfering matrix components, however the extraction protocol would need to be re-validated and may require a considerable amount of method development to optimize.

Given that many RA analyses are conducted for serum samples in mammalian literature (Gundersen et al. 2007, Arnold et al. 2012, Saha et al. 2014), it would be worth investigating whether such samples could be used in place of liver for retinoic acid assessments in fish while utilizing the extraction method developed for non-polar retinoids. In both tissues and serum, RA levels in fish are thought to be approximately two orders of magnitude lower than those in humans, which would place serum RA concentrations in the fmol per mL range (Gesto et al. 2012). The impressive sensitivity of triple quadrupole instruments has recently allowed for limits

of detection in the sub-fmol range for retinoic acids in serum (Jones et al. 2015), making a transition to serum analysis an attractive option for reducing the severe matrix interferences encountered in liver tissues and thus improve data quality. Additionally, the SPE method used by Gesto et al. (2012) has already been validated for application to serum samples with high recovery of retinoic acids and so avoids the lengthy process of developing new sample extraction approaches.

In addition to the atmospheric ionization methods typically used in retinoid analysis (ESI and APCI), a less often employed technique called atmospheric pressure photoionization (APPI) has been brought to market. APPI is similar to APCI, however the corona discharge needle is replaced with an ultraviolet krypton lamp and ultimately ionizes analyte molecules through radiation rather than electric current (Himmelsbach 2012). APPI is applicable to a similar range of compounds as APCI in terms of molecular weight and polarity, although for certain analyses can offer up to eight-fold sensitivity increases relative to methods traditionally employing APCI (Robb et al. 2000). The use of APPI in retinoid analysis has not been reported to date, however its applications should be explored. Any potential opportunities to increase sensitivity should be seized in the analysis of retinoic acids given their low endogenous concentrations in biological matrices. Additionally, APPI produces relatively simple mass spectra (Robb et al. 2000) and may help reduce the matrix interferences which presently limit RA measurements simply by not ionizing non-target compounds. In the analysis of non-polar retinoids, whether the in-source fragmentation of retinyl esters can be mitigated with APPI techniques should be explored, as combination with the high mass resolution of QToF instruments would provide a more powerful tool for retinoid profiling and identification by directly measuring intact molecular species rather than shared pseudo-molecular fragments.

St. Clair River AOC

Despite major improvements made under Remedial Action Plans, the St. Clair River remains a listed AOC with three outstanding beneficial use impairments including "restrictions on fish and wildlife consumption" (Environment and Climate Change Canada 2017). The preliminary results obtained in this method application demonstrated statistically significant reductions in free retinols and palmitate esters in shorthead redhorse sucker populations from the near-field site in 2014. The data provides rationale for returning to this AOC for additional investigations to determine if stressors which appear to impact retinoid status still persist. An expanded study considering increased numbers of both male and female fish, and spanning multiple seasons (spring/fall) would be the next step in assessing retinoid status of shorthead redhorse sucker populations at the St. Clair River. A broader study would also allow for additional profiling by LC-QToF of VA_1/VA_2 abundances in shorthead redhorse sucker and help to elucidate patterns in retinoid metabolism for this species. Considering the biological relevance of retinoic acids and the current state of method development for their measurement, it would be prudent to resolve the RA isomer analysis issues before returning to the St. Clair River AOC in order to obtain a clearer picture of overall retinoid status in these fish populations.

Bay of Quinte AOC

The Bay of Quinte currently remains a listed AOC, however drastic improvements have been made in restoring a number of beneficial use impairments and clean-up is estimated to be finalized in 2019 (Environment and Climate Change Canada 2017). No differences in non-polar retinoid concentrations were observed for brown bullhead populations between the near and far-field sites at this AOC in 2014. This initial assessment suggests there is not an impact on retinoid status at the Bay of Quinte, however a more comprehensive study would be required to fully

demonstrate this as the samples selected here represent only one sampling season for a single sex. Qualitative profiling by LC-QToF suggests brown bullhead preferentially utilize dehydroretinoids as their main vitamin A form, which is consistent with other retinoid measurements in brown bullhead at this AOC (Arcand-Hoy and Metcalfe 1999). Additional retinoid profiling in brown bullhead sampled from other locations would provide additional evidence to help confirm this species as a VA$_2$-type organism.

Potential future applications

There are a number of unresolved questions regarding retinoid metabolism in fish, the exact functions and distributions of dehydroretinoids, and how retinoids/dehydroretinoids interact within the overall retinoid system. Deeper understandings of these interactions are critical, particularly when interested in assessing environmental impacts to species which might preferentially utilize dehydroretinoids (e.g. brown bullhead) as their main vitamin A form. In the future, the LC-MS/MS method developed here could be directed toward investigating some of these questions.

As demonstrated by feeding a diet rich in ROL, some species of fish may have the ability to convert retinol to dehydroretinol as evidenced by enriched dROL levels in hepatic tissue (La Frano and Burri 2014). In conjunction with these types of feeding studies, high resolution QToF profiling could be used to assess retinoid distributions in a variety of species of fish considering the low amount of tissue (50 mg) required for analysis. This application would help to establish how species of interest metabolize retinoids and potentially allow for classification of species as VA$_1$ or VA$_2$ utilizers. As wild brown bullhead appear to use dehydroretinoids (Arcand-Hoy and Metcalfe 1999), repetition of such a study would help to strengthen the case of classifying this species as VA$_2$-type if it can be shown that dietary retinoids are metabolized to dehydroretinoids.

With a refined method for the measurement of retinoic acids, further investigation into retinoid metabolism can be undertaken by incorporating the analysis of RAs to discern potential effects of dietary retinoids on the distribution of retinoic acid isomers.

Considering that dehydroretinoids appear to be of high importance in some species of fish, a logical extension of the revised RA method would be incorporate the assessment of dehydroretinoic acid (dRA), which is presently problematic. Analytical standards are not cost-effective to purchase from commercial suppliers as large-batch custom synthesis is required, and long-term compound stability remains an issue. The latest generation of QToF instruments are up to five times more sensitive relative to earlier iterations and can now provide detection limits in the low ng mL^{-1} range (Anumol et al. 2016). Newer instrumentation may be able to provide a way forward for non-quantitative or semi-quantitative profiling of these compounds in the absence of authentic standards. Dehydroretinoic acid is thought to be the prominent active retinoid in the embryonic stages of some species including zebrafish (McCaffery et al. 2002) and thus warrants further investigation in other fish species. If the biological relevance of these compounds can be sufficiently demonstrated, this may also motivate chemical suppliers to bring dehydroretinoic acid analytical standards to market, which would be required for quantitative analyses.

In transitioning to serum samples for RA analysis, interesting *in vivo* applications become possible for retinoid studies in fish. As blood can be sampled non-lethally, serum analysis would enable time-point sampling of fish in various exposure treatments or environmental stressor scenarios that may affect retinoids. A drawback to this approach is that larger fish would be required in order to obtain sufficient amounts of sample which is more logistically involved than working with small-bodied fish, and frequent blood sampling is also labour intensive. As an

example, zebrafish exposed to an inhibitor of RA synthesis (diethylaminobenzaldehyde, DEAB) spawned 95% fewer eggs, suggesting impacts on retinoic acid levels through alteration of retinoid metabolism (Alsop et al. 2008). This experiment could be repeated with larger-bodied fish and through an LC-MS/MS approach serum retinoids could be tracked throughout an exposure and provide additional insight on rates of inhibition of RA synthesis. Serum has been analyzed in the mammalian literature for circulating retinol levels (Gundersen et al. 2007) which could also be incorporated into this analysis through SPE fractionation to determine if there are effects on free ROL concentrations when RA synthesis is altered. At defined endpoints, fish could then be lethally sampled and further retinoid analyses (e.g. ester profiling) conducted on tissues in addition to serum. This experimental setup would achieve the most utility from each specimen while providing rich insight into retinoid metabolism in fish through LC-MS/MS.

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Appendix A: Supporting Information for Chapter 2

Optimized mass spectrometer ion source conditions (APCI):

Retinoic Acids (Q-LIT) - curtain gas: 30; collision gas: low; needle current: 3; probe temperature: 325; source gas: 80; probe: 5 mm vertical, 5 mm horizontal, 0.5 mm tip extension.

Non-polars (Q-LIT) - curtain gas: 30; collision gas: low; needle current: 5; probe temperature: 325; source gas: 70; probe: 5 mm vertical, 5 mm horizontal, 0.5 mm tip extension.

Non-polars (QToF) - capillary voltage: 2000; fragmentor voltage: 150; corona current: 4; drying gas: 5; nebulizer pressure: 40; vaporizer temperature: 300; drying gas temperature: 350.

Table S-1: Q-LIT compound-dependent parameters

Analyte	Q1 (*m/z*)	Q3 (*m/z*)	DP (V)	EP (V)	CE (V)	CXP (V)	Dwell Time (ms)
Separation I: Retinoic acids							
Caffeine	195	138	70	9	25	7	25
	195	110	70	9	28	6	25
Acitretin	327	177	60	5	17	10	80
	327	267	60	5	20	8	80
RAs (all)	301	205	70	10	11	13	200
	301	255	70	10	16	8	200
atRA-d5	306	206	60	12	20	12	80
	306	260	60	12	17	8	80
Separation II: Non-polar retinoids							
Caffeine	195	138	80	6	25	16	50
	195	110	80	6	30	12	50
ROL/REs	269	119	65	10	25	14	150
	269	199	65	10	20	6	150
dROL/dREs	269	119	65	10	25	14	150
	269	199	65	10	20	6	150

DP: declustering potential; EP: entrance potential: CE: collision energy; CXP: collision cell exit
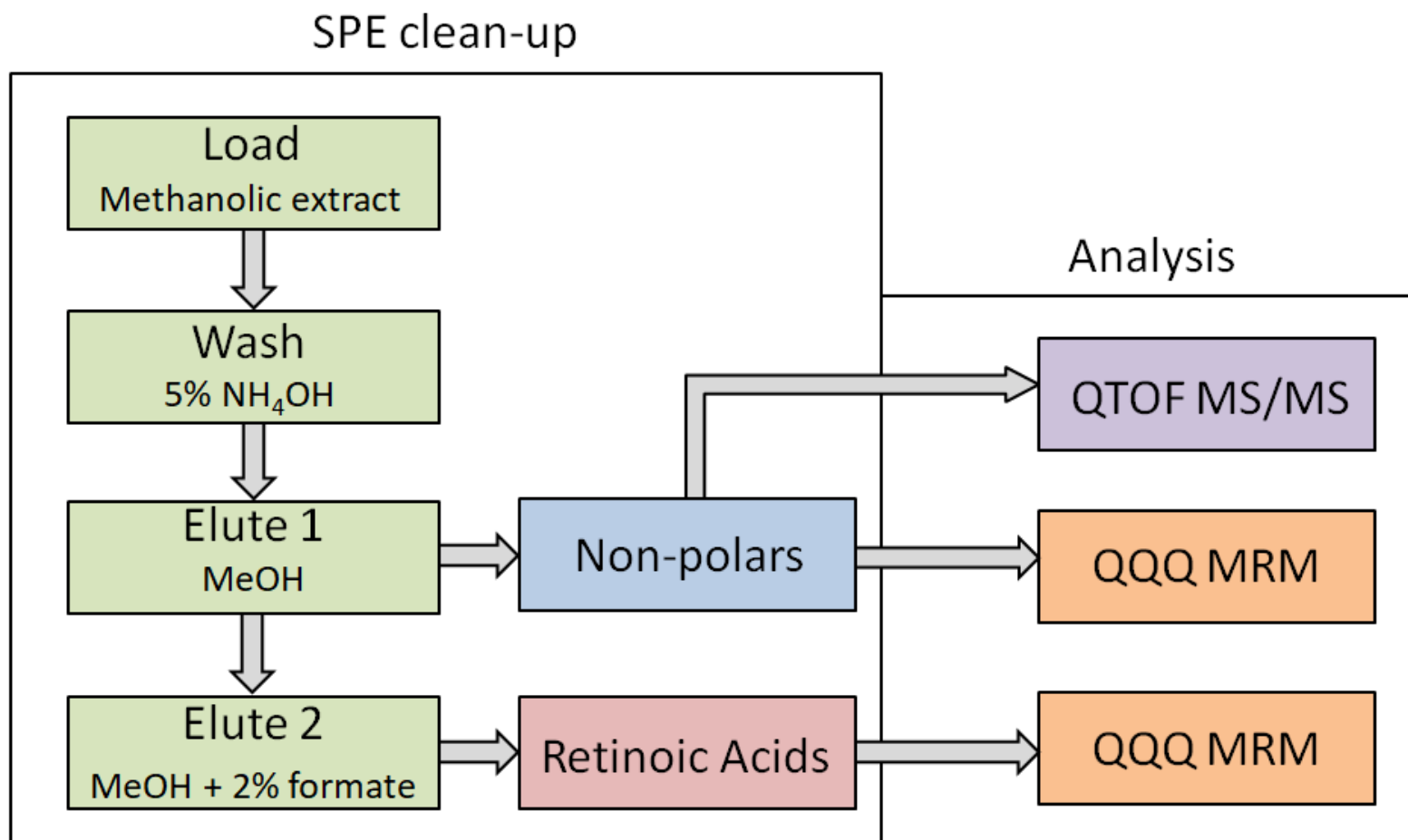
Figure S-1: Workflow for sample fractionation by SPE and analysis by LC-MS/MS
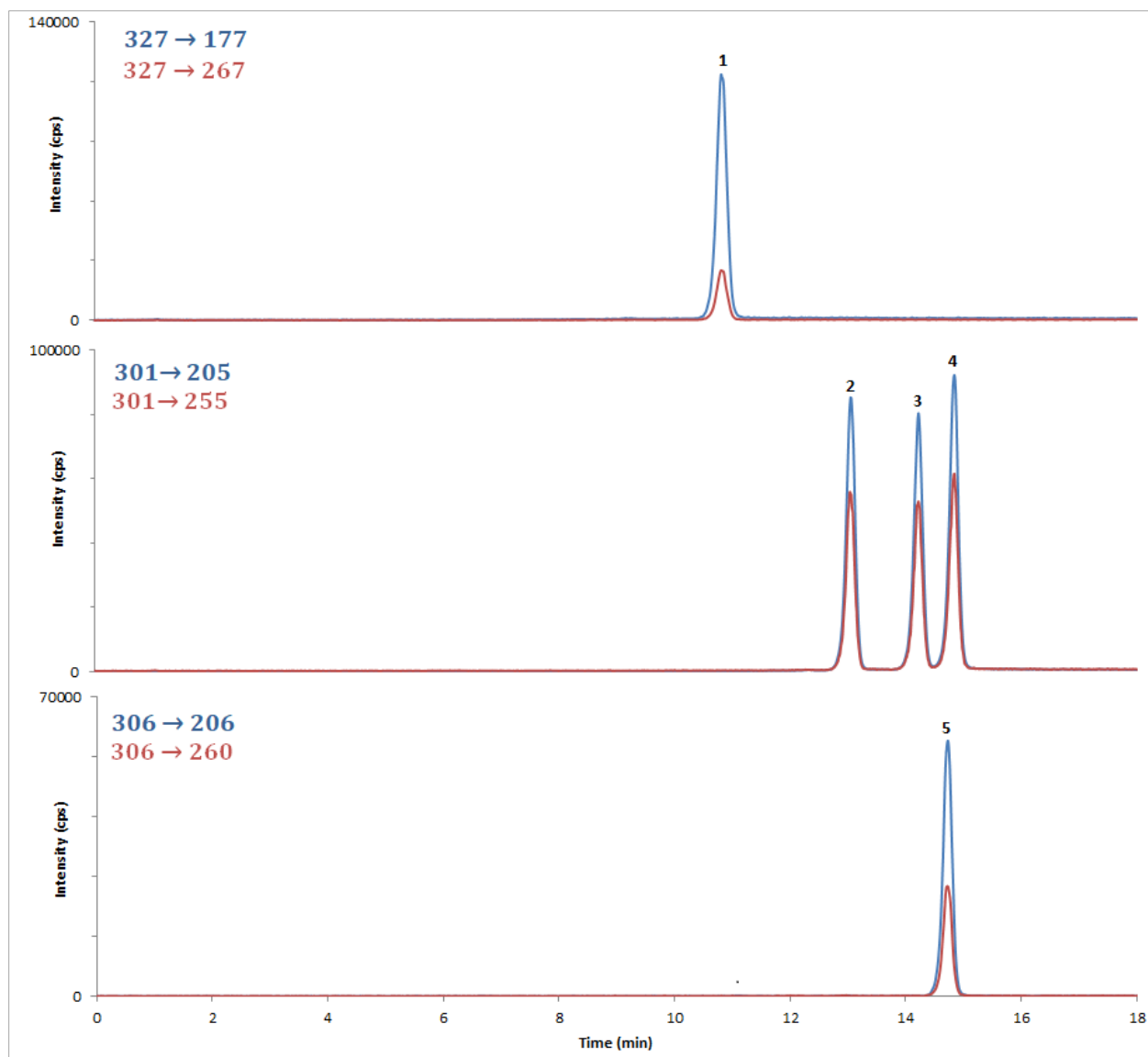
Figure S-2: Chromatographic separation of polar retinoids

Acitretin (1), 13cRA (2), 9cRA (3), atRA (4), and atRA-d5 (5) were separated on a Supelco RP-Amide analytical column (2.1 x 150 mm, 3 μm). Initial conditions were 30:70 water/acetonitrile. Final conditions were 5:95 water/acetonitrile. Mobile phases were modified with 0.1% formic acid. Detection by +APCI-MS/MS (MRM). Blue trace is primary (quantifier) ion, red trace is secondary (qualifier) ion.
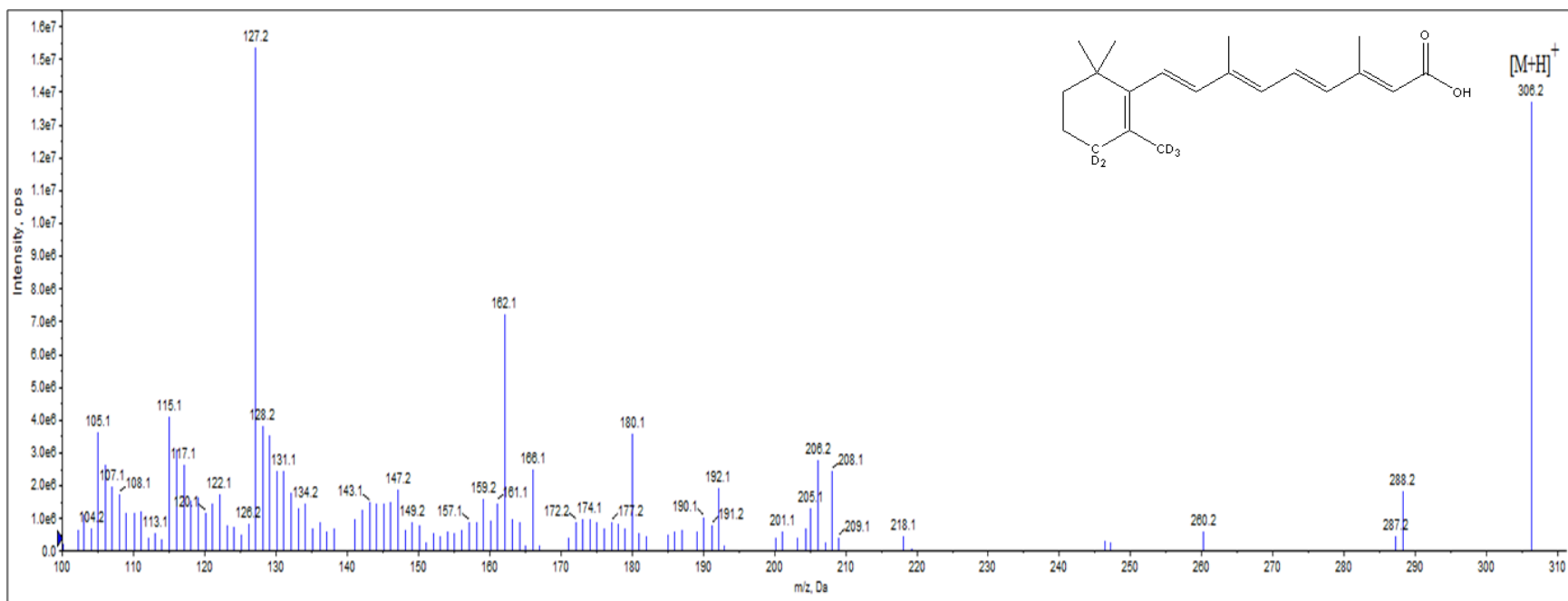
Figure S-3: Q-LIT +APCI-MS/MS product ion spectrum of atRA-d5
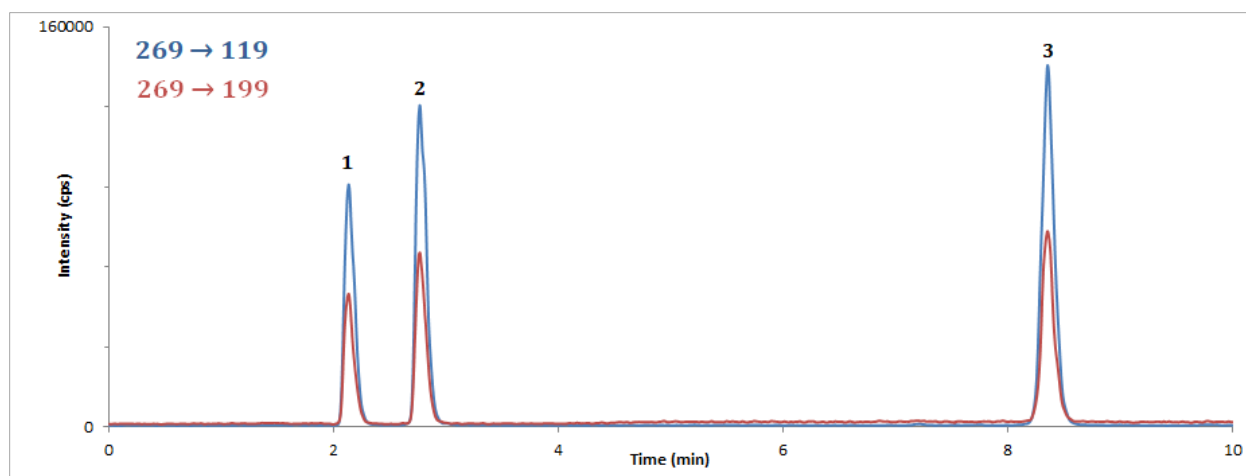
Figure S-4: Chromatographic separation of non-polar retinoids

ROL (1), RAc (2), and RPa (3) were separated on an Agilent Poroshell 120 EC-C18 analytical column (3.0 x 100 mm, 2.7 μm). Initial conditions were 75:18:7 methanol/tetrahydrofuran/water. Final conditions 57:36:7 methanol/tetrahydrofuran/water. Mobile phases were modified with 0.1% formic acid. Detection by +APCI-MS/MS (MRM). Blue trace is primary (quantifier) ion, red trace is secondary (qualifier) ion.
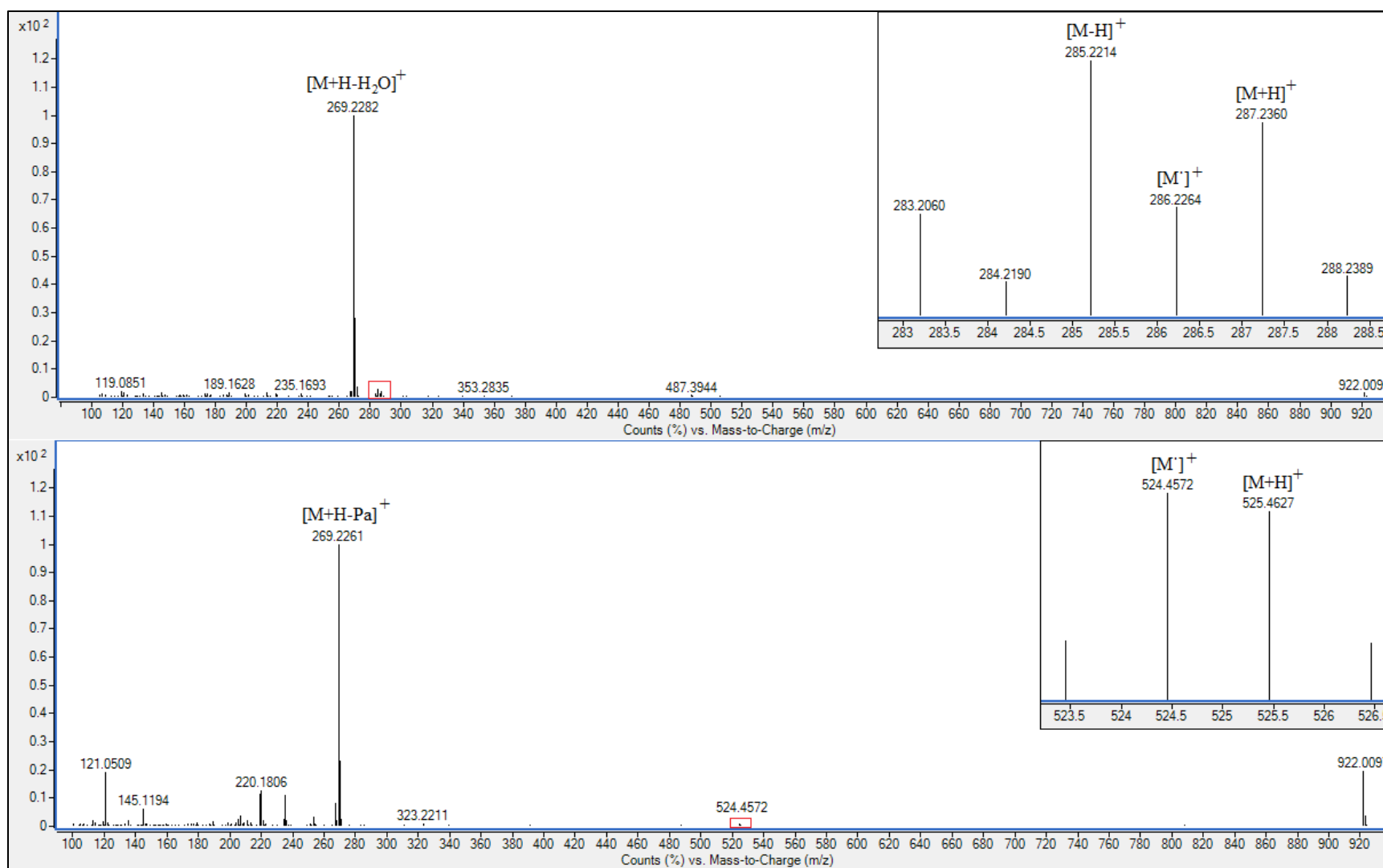
Figure S-5: QToF +APCI-MS spectra of ROL (top) and RPa (bottom)

Molecular ions (insets) and pseudo-molecular ions have been identified.
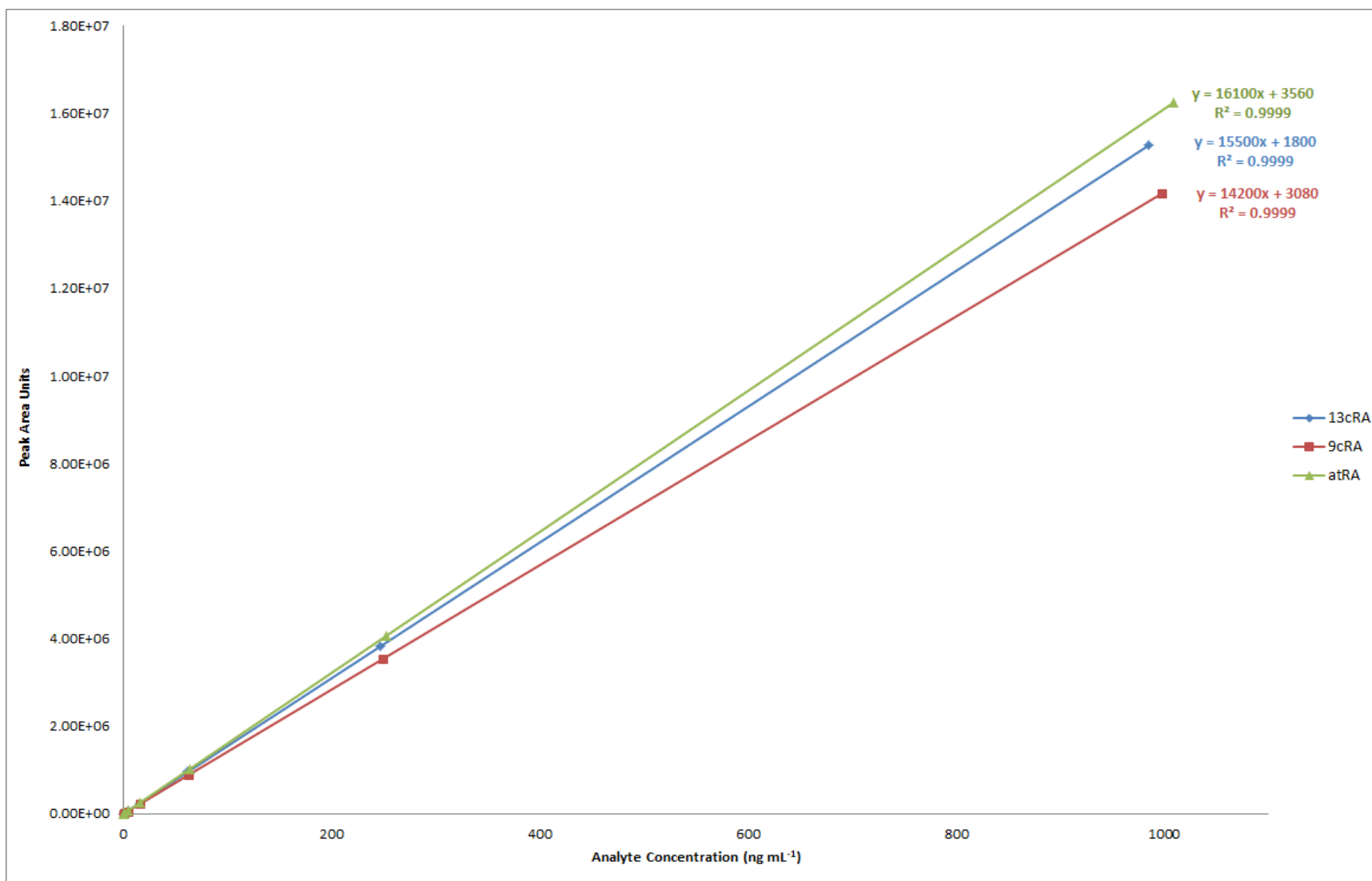
Figure S-6: Calibration curve for 13cRA, 9cRA, and atRA

Lines of best fit determined by linear regression with 1/x weighting. The linear range is from 1 – 1000 ng mL^{-1}.
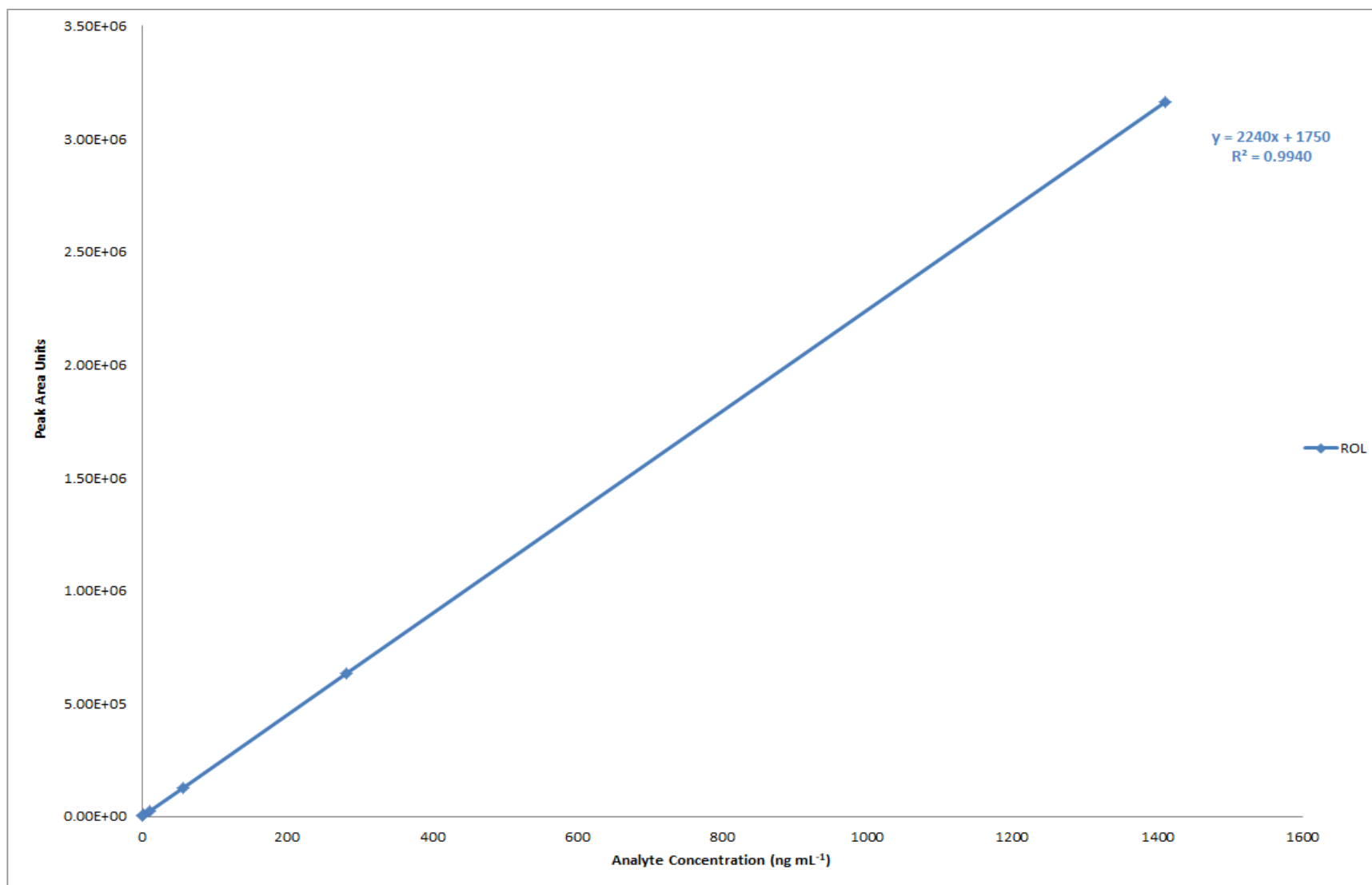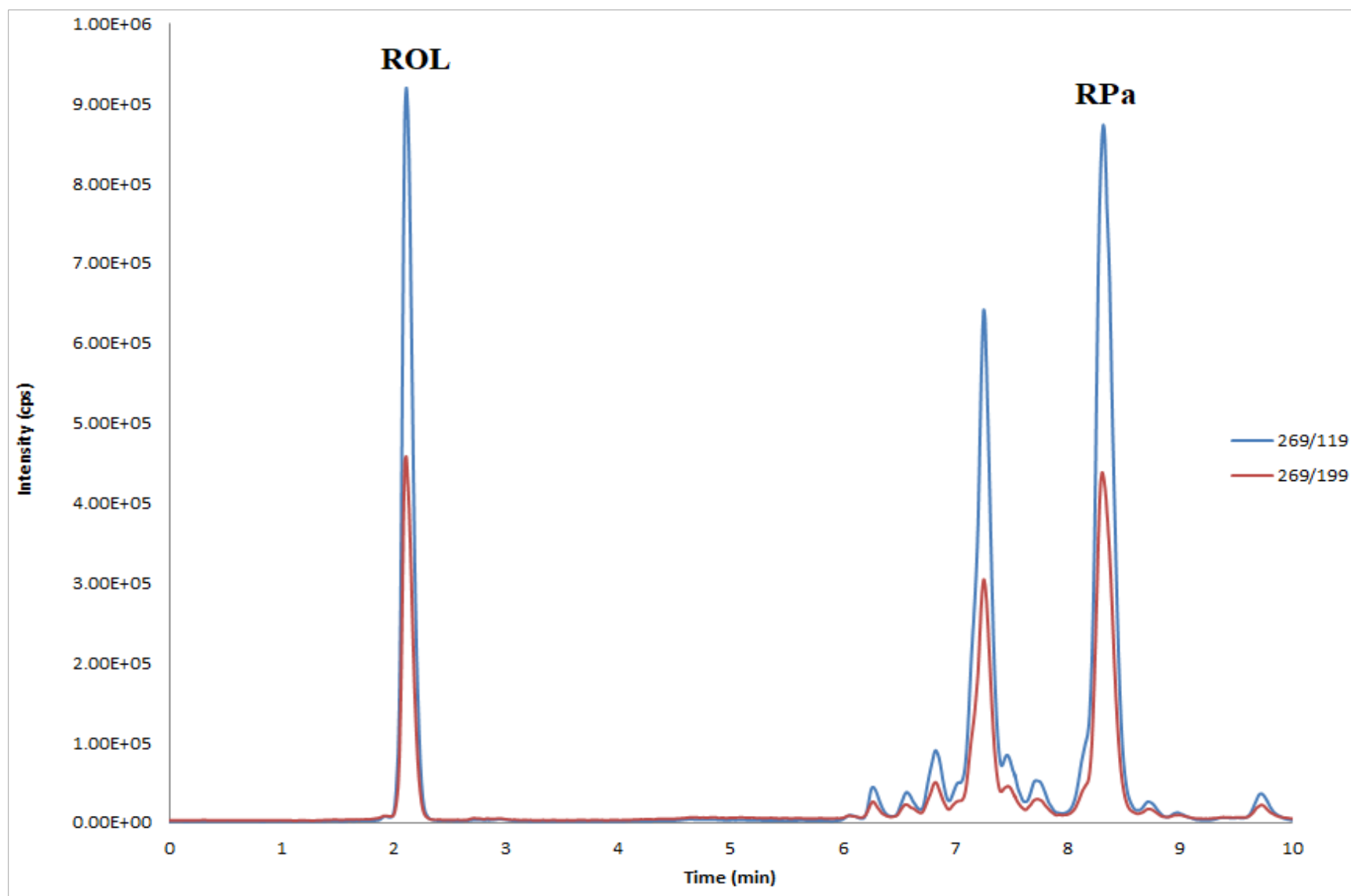
Figure S-7: Calibration curve for ROL

Lines of best fit determined by linear regression with $1/x^2$ weighting. The linear range is from $2 - 1400$ ng mL^{-1}.
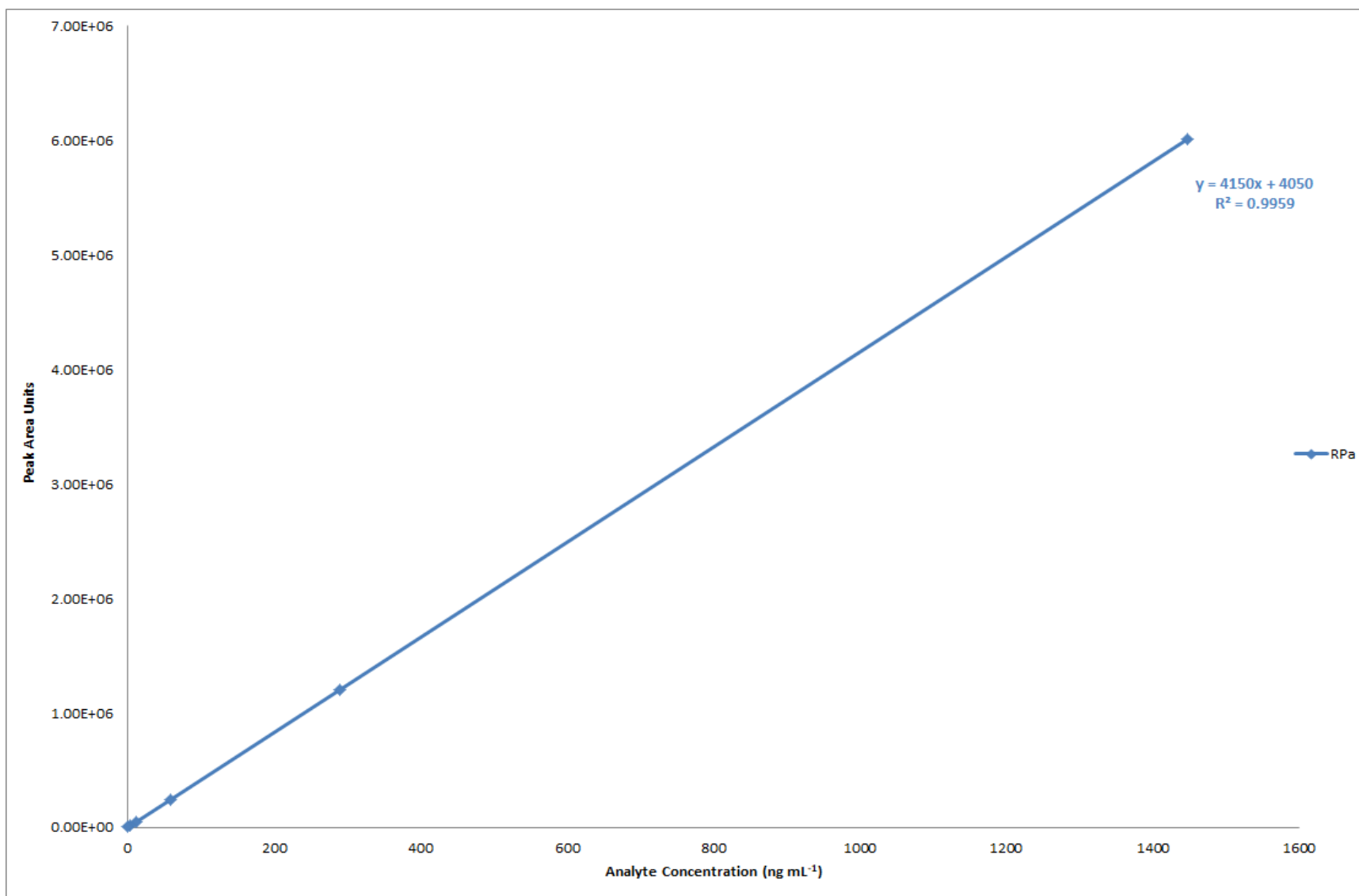
Figure S-8: Calibration curve for RPa

Lines of best fit determined by linear regression with $1/x^2$ weighting. The linear range is from $2 - 1400$ ng mL^{-1}.

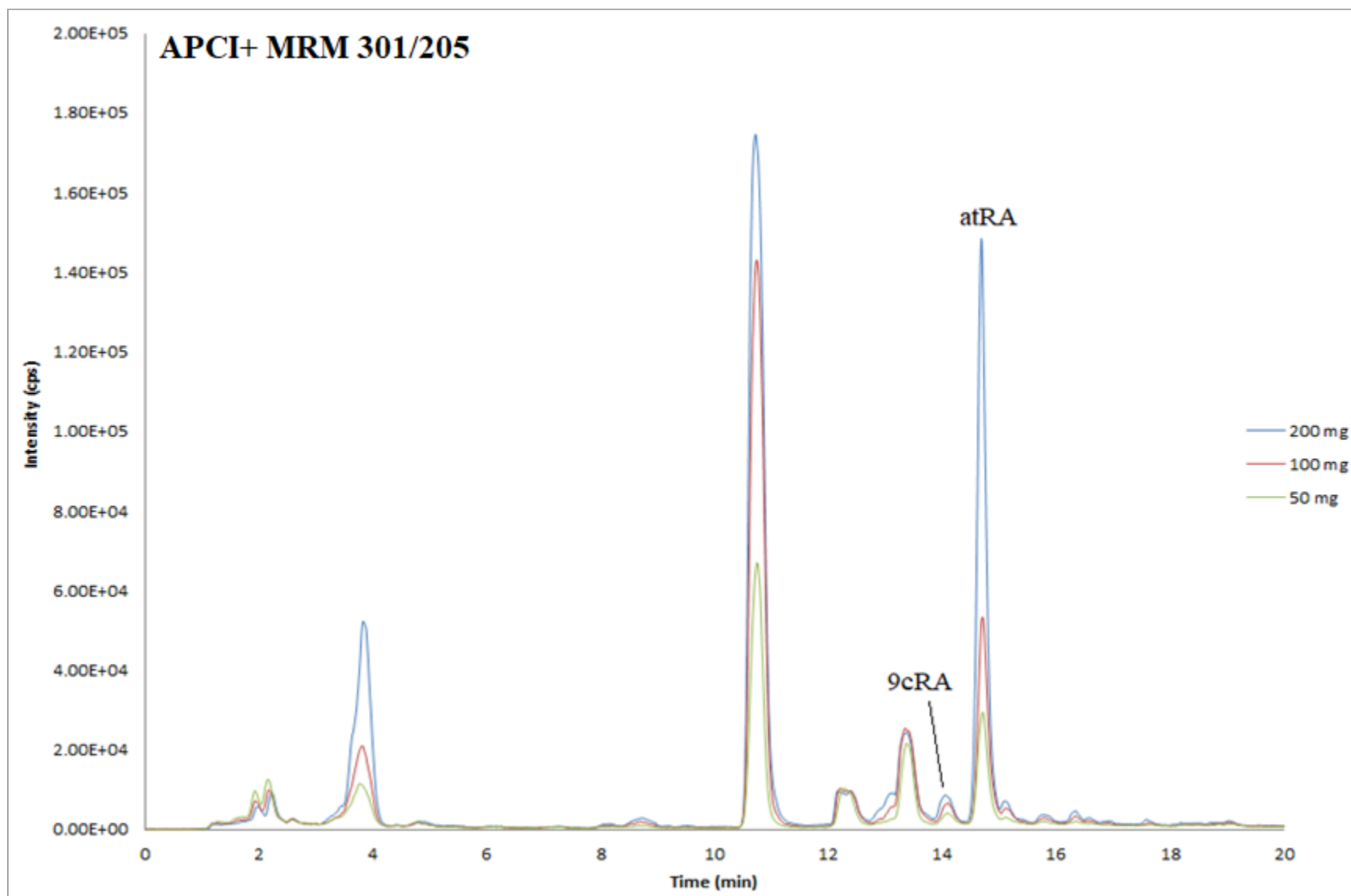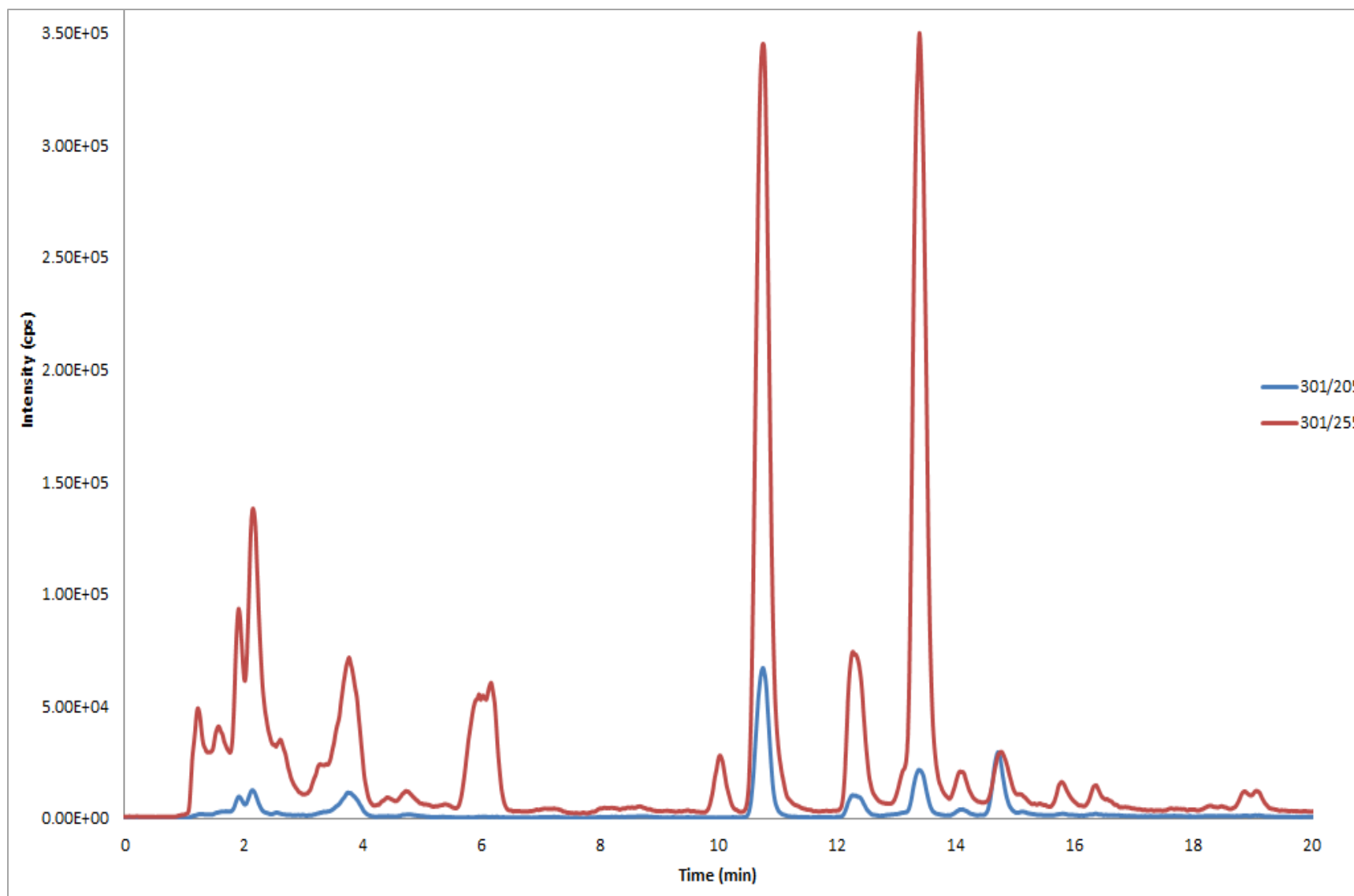**Figure S-9:** Q-LIT MRM response for non-polar retinoids in 5 mg wet tissue
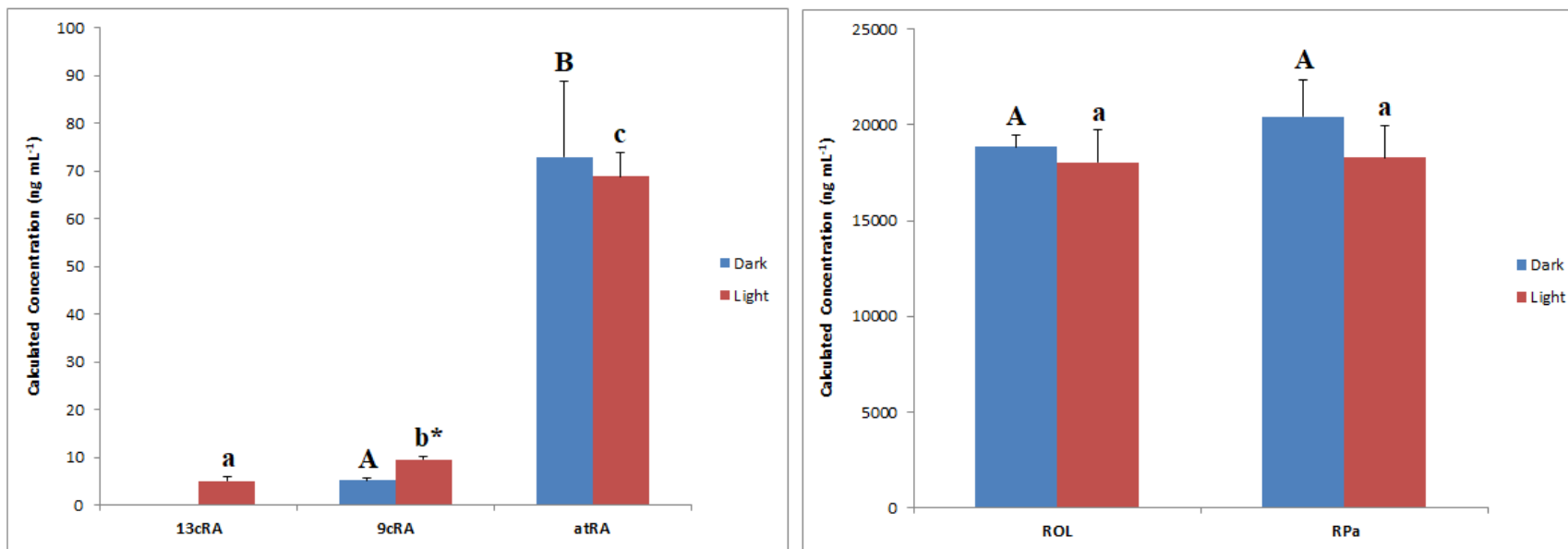
**Figure S-10:** RA MRM response for varied amounts of extracted tissue

Figure S-11: Inversion of ion ratio in RA analysis (50 mg extract)

Figure S-12: Measured retinoid concentrations following exposure to heat and light

Values are mean ± SEM (n = 3). Means with dissimilar letters are significantly different from one another (p < 0.05). Asterisks (*) indicate a significant difference from handling in the dark and on ice for each compound (p < 0.05).
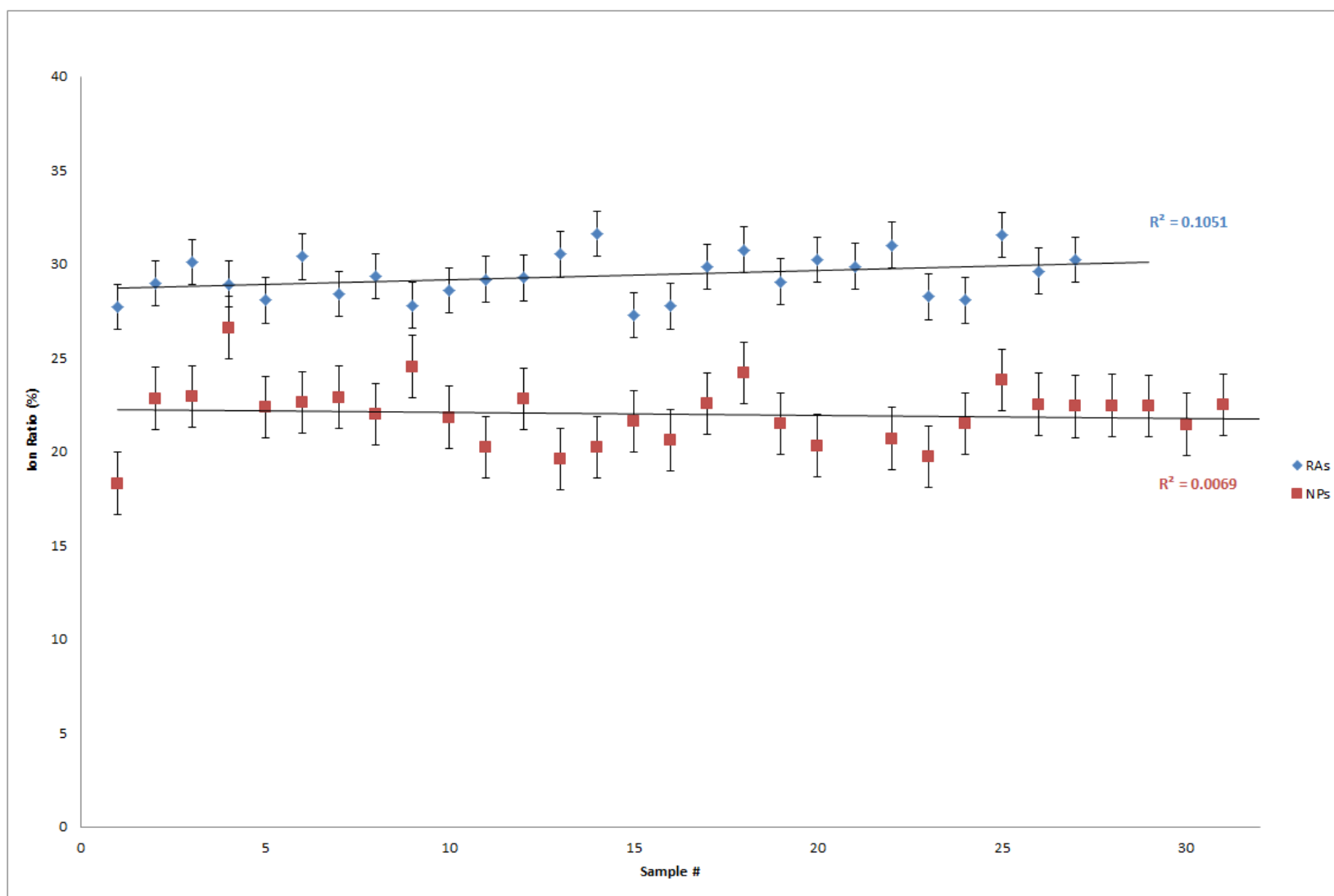
Figure S-13: Ion ratio for caffeine IPS over multiple sample injections

Ion ratio calculated as relative abundance of qualifier and quantifier transitions, q/Q x 100%.