Published in final edited form as:

Methods Mol Biol. 2010 ; 652: 1–54. doi:10.1007/978-1-60327-325-1_1.

Quantification of Endogenous Retinoids

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Abstract

Numerous physiological processes require retinoids, including development, nervous system function, immune responsiveness, proliferation, differentiation, and all aspects of reproduction. Reliable retinoid quantification requires suitable handling and, in some cases, resolution of geometric isomers that have different biological activities. Here we describe procedures for reliable and accurate quantification of retinoids, including detailed descriptions for handling retinoids, preparing standard solutions, collecting samples and harvesting tissues, extracting samples, resolving isomers, and detecting with high sensitivity. Sample-specific strategies are provided for optimizing quantification. Approaches to evaluate assay performance also are provided. Retinoid assays described here for mice also are applicable to other organisms including zebrafish, rat, rabbit, and human and for cells in culture. Retinoid quantification, especially that of retinoic acid, should provide insight into many diseases, including Alzheimer's disease, type 2 diabetes, obesity, and cancer.

Keywords

Retinoid; retinoic acid; retinal; retinaldehyde; retinol; retinyl ester; mass spectrometry; LC/MS/MS; HPLC

1. Introduction

Retinoid homeostasis involves balance among several retinoids in multiple tissues effected through dietary intake, storage, mobilization, transport, and metabolism (*see* Fig. 1.1) (1–5). Lecithin:retinol acyltransferase (LRAT) and in skin diacylglycerol acyltransferase-2 (DGAT2) and retinyl ester hydrolases (REH) mediate storage and mobilization of vitamin A (retinol), respectively. Metabolism activates retinol into retinoic acid (RA) by a reversible and rate-limiting dehydrogenation of retinol into retinal, catalyzed by short-chain retinol dehydrogenases (SDR), followed by an irreversible dehydrogenation by retinal dehydrogenases (RALDHs) into RA. A number of cytochrome P450 (CYP) enzymes catabolize RA to polar metabolites (6, 7). All-*trans*-RA (atRA) mediates a multitude of systemic effects, including development, nervous system function, immune response, cell proliferation, cell differentiation, and reproduction, by regulating transcription of hundreds of genes through binding to retinoic acid receptors (RAR) α , β , γ and peroxisome proliferator-activated receptor (PPAR) β/δ (8–11). Expression loci of specific retinoid-binding proteins, enzymes, and receptors, which contribute to RA generation, signaling, and

catabolism, indicate that RA concentrations in vivo are temporally/spatially controlled to produce the individual actions of vitamin A (6, 7, 12–15).

Various approaches have been used to determine effectors of retinoid metabolism, including genetic alteration of retinoid-binding proteins (16–21), enzymes (22–25), and receptors (8, 26, 27); dietary manipulation of vitamin A intake (28); and exposure to xenobiotics (29–34). Quantifying how manipulation of retinoid metabolism effects the flux of retinoids through metabolic paths, the availability of substrate for RA production, and/or endogenous RA levels will provide insight into retinoid homeostasis and metabolism and, thereby, function. Dysfunctions in retinoid homeostasis have been linked to dyslipidemia, diabetes, obesity, cancer, and Alzheimer's disease, but these data have not necessarily been accompanied by robust quantification of retinoid concentrations in vivo (35–43). Quantification of RA and/or other retinoids would seem essential to elucidate mechanisms by which retinoids contribute to disease.

Quantification of retinoids requires attention to proper handling and, in some cases, isomeric distribution. The susceptibility of retinoids to isomerization and oxidation is well documented and necessitates care during sample collection, handling, and storage (44–51). Resolution of isomers is important in RA analyses, as isomers of RA have different affinities for nuclear receptors and, therefore, may afford different biological actions. *atRA* activates retinoic acid receptors (RAR) (8, 52) and peroxisome proliferator-activated receptor, type β/δ (9, 10). *9-cis-RA* (9cRA) activates both RAR and retinoid X receptors (RXR) (26). Retinoids may exert additional biological effects through dimerization of RXR with an array of type II nuclear receptors, such as thyroid hormone, peroxisome proliferator-activated, vitamin D, liver X, farnesoid X, pregnane, constitutively activated, and the small nerve growth factor-induced clone B subfamily of nuclear receptors (26). *13-cis-RA* (13cRA) does not activate RAR or RXR directly but induces dyslipidemia and insulin resistance, most likely through conversion into *atRA* (35, 53–56). Tissues and serum contain 9,13-di-*cis-RA* (9,13dcRA), which may reflect conversion from 13cRA and/or 9cRA, and does not activate RAR or RXR (57–59).

The disparity in endogenous abundance often demands attention to analytical methodology. RE storage levels (~high micromolar) can differ by as much as six orders of magnitude from endogenous RA levels (~low nanomolar) (49–51). Previously, analytical limitations of direct RA quantification have hindered the complete investigation of retinoid metabolism essential for understanding retinoid function and its relationship to disease risk. A number of recent analytical efforts have provided assays with the necessary sensitivity, specificity, and/or isomeric resolution to quantify endogenous RA levels (and other retinoids) in tissue and serum, and thus, indirect methods of quantification should be avoided (48–51, 60, 61).

Indirect methods used as substitutes for direct RA measurement and analytically robust assays, such as in vitro reporter assays or transgenic RA reporter mouse strains, lack specificity, lack means of quantification, and/or have produced contradictory results (62–64). These non-instrumental methods, based on reporter gene expression, have not been developed into analytically rigorous assays, are not specific for all-*trans-RA* (e.g., 3,4-didehydro-RA, 9cRA, 4-oxo-RA, 4-hydroxy-RA, and 4-hydroxy-retinol all produce signals),

are not quantitative, and can give both false-positive and false-negative results (63–65). Additionally, because reporter detection systems reflect RAR activation, they cannot evaluate retinoid presence in real time and may reflect the longer term consequences of receptor activation, after the retinoid has been catabolized. Administration of a super-physiological dose of retinol to raise RA levels to a level detectable by UV absorbance is also problematic. An example of this approach dosed as much as 50 mg/kg, ~300-fold greater than the recommended daily intake of retinol for a mouse (30, 66, 67). Super-physiological doses such as this induce an artificial environment where serum atRA levels are raised ~1600-fold higher than typical steady-state values of ~2.5 pmol/ml, likely overwhelming normal metabolism and eliciting retinoid toxicity responses (30, 49, 50, 66, 67).

Each of the retinoid detection methods described in the literature, including LC-MS/MS, LC-MS, HPLC-UV, GC-MS, and ECD, has different sensitivities, effectiveness with various biological matrices, benefits, and limitations. A comparison of these methodologies is provided (*see* Table 1.1). HPLC with UV detection has the benefit of ease and economics, but has LOQ ~0.4–1 pmol and does not provide mass identification (45, 51, 68). Recent advances in column technology and column switching capabilities have assisted in lowering detection limits (48, 69). HPLC with electrochemical detection has sensitivity in the femtomolar range, but lacks the definite mass identification of analytes of MS, is subject to interference from other analytes, and has solvent/electrode/flow-dependent sensitivity (70–73). GC/MS affords sensitivity, with a lower limit of detection <250 fmol, but GC/MS requires derivatization for RA detection (74, 75). LC/MS-based assays offer mass identification of analytes, but do not have the potential sensitivity or the enhanced specificity of selected reaction monitoring (SRM) (76–79). Triple-quadrupole LC/MS/MS offers the most effective RA detection at this time with sensitivity, specificity, no requirement for derivatization, and definite mass identification (49, 50). Future directions for retinoid analysis may include utilization of greater sensitivity MS/MS instrumentation, incorporation of high-throughput methodology (61), quantification of localized areas and/or individual cellular populations (e.g., areas isolated by laser capture microdissection or subcellular fractionation) (80, 81), and simultaneous quantification of retinoids with other biologically significant metabolites to enable retinoid pathway analysis in concert with the evaluation of other metabolic pathways (82).

Described here are all procedures needed for accurate and reliable quantification of retinoids, including detailed descriptions of how to properly handle retinoids, prepare standard solutions, harvest tissues, collect samples, homogenize, extract, separate, and detect retinoids. Strategies for optimizing extraction of retinoids from biomatrices, chromatographic resolution, and detection sensitivity are provided, as are the steps necessary for evaluating the performance of retinoid assays. Retinoid assays described here for mice also have proven applicable to other organisms including zebrafish, rat, rabbit, and human.

2. Materials

2.1. Proper Handling and Transfer of Retinoids, Retinoid Solution Preparation

1. Disposable glass containers (vials, etc.) for solutions (Fisher Scientific)

2. Disposable glass pipettes (Pasteur and graduated) for transfer (Fisher Scientific)
3. Calibrated glass syringes (Hamilton) for measuring small volumes of retinoid solutions
4. -20°C freezer for short-term (less than 1–2 weeks) storage of solutions and prepared samples
5. UV–Vis spectrometer
6. Quartz cuvettes
7. Disposable glass vials/containers
8. Disposable glass Pasteur pipettes
9. Calibrated glass syringes for measuring retinoid volumes (Hamilton)

2.2. Sample Collection

1. Animals should be of similar strain, size, age, diet, and/or fasting state
2. Yellow room lights or darkened room with desk lamp with yellow bulb
3. Dissecting tools
4. Eppendorf tubes or similar to collect tissue
5. Balance to weigh tissue
6. Liquid nitrogen to flash freeze samples
7. -80°C freezer for storage of samples (tissue samples can be stored 1–2 weeks with no degradation)

2.3. Homogenization

1. Saline (0.9% NaCl)
2. Ground glass hand homogenizers (Duell, size 22 or similar, Kontes) and/or motorized homogenizer (Heidolph or similar) with ground glass mortar/pestle (Kontes) and/or Polytron stainless steel motorized homogenizer
3. Disposable 1 ml graduated glass pipettes to measure homogenate
4. 16×150 mm disposable glass culture tubes
5. UV–Vis spectrometer, disposable polystyrene cuvettes, Bradford reagent (Bio-Rad) for protein determination

2.4. Internal Standard

1. Internal standard solution at concentration approximately similar to analyte detection amount to be delivered to each sample in $\sim 5\text{--}10\ \mu\text{l}$ DMSO, ethanol, acetonitrile, or other compatible solvent

2.5. Conversion of Retinal into Retinal Oxime and Extraction

1. 0.1 M *O*-ethylhydroxylamine in 0.1 M HEPES, pH 6.5
2. Hexane
3. 16 × 150 mm disposable glass culture tubes
4. Vortex mixer
5. Disposable 10 ml glass pipettes
6. Nitrogen gas solvent evaporator (Organomation Associates Inc. model N-EVAP 112, Berlin, MA) or similar with disposable gas delivery tips
7. Disposable 5¾" glass Pasteur pipettes for nitrogen gas solvent evaporator gas delivery tips

2.6. RA/Retinol/RE/Polar Metabolite Extraction and Resuspension

1. 0.025 M KOH in 100% ethanol
2. 4 M HCl
3. Internal standard solutions (make approximately same concentration as analyte of interest)
4. Hexane
5. 16 × 150 mm disposable glass culture tubes
6. Disposable 10 ml glass pipettes
7. Vortex mixer
8. Dynac centrifuge (Becton Dickinson) or similar for 16 × 150 mm culture tubes
9. Nitrogen gas solvent evaporator (Organomation Associates Inc. model N-EVAP 112, Berlin, MA) or similar with disposable gas delivery tips
10. Disposable 5¾" glass Pasteur pipettes for nitrogen gas solvent evaporator gas delivery tips
11. Ice bucket(s) and ice
12. Resuspension solvent(s): acetonitrile, hexane with 0.4% IPA, and/or retinal isomer mobile phase
13. Disposable 9" glass Pasteur pipettes for transfer of resuspended samples to inserts
14. Limited volume glass inserts for HPLC vials (available from Agilent, Waters, etc.)

2.7. Separations

2.7.1. Reverse-Phase Retinoic Acid Separations (See Sections 3.13.1 and 3.13.2)

1. Ascentis alkyl amide C16 column (Supelco): 2.1×100 mm, 3 μ m (gradient 1), and/or Ascentis alkyl amide C16 column (Supelco): 2.1×150 mm, 3 μ m (gradient 2)
2. Supelcosil ABZ+PLUS Supelguard cartridge column (Supelco, 2.1×20 mm, 5 μ m)
3. Pre-column filter
4. Acetonitrile with 0.1% formic acid
5. Water with 0.1% formic acid
6. Methanol with 0.1% formic acid
7. Acetonitrile (for column storage)

2.7.2. Normal-Phase Retinoic Acid Separation (See Section 3.13.3)

1. Zorbax SIL column (Agilent) 4.6×250 mm, 5 μ m
2. Hexane with 0.4% isopropyl alcohol

2.7.3. Total Retinal Separation (See Section 3.14.1)

1. Zorbax SB-C18 column (Agilent) 4.6×100 , 3.5 mm
2. Acetonitrile
3. Water
4. Water with 10% formic acid
5. 1,2-Dichloroethane

2.7.4. Retinal Isomer Separation (See Section 3.14.2)

1. Two Zorbax SIL column (Agilent) 4.6×250 mm, 5 μ m
2. 11.2% ethyl acetate, 2% dioxane, 1.4% 1-octanol, 85.4% hexane

2.7.5. Total Retinol, Total Retinyl Ester Separation (See Section 3.15.1)

1. Zorbax SB-C18 column (Agilent) 4.6×100 , 3.5 mm
2. Acetonitrile
3. Water
4. Water with 10% formic acid
5. 1,2-Dichloroethane

2.7.6. Retinol Isomer Separation (See Section 3.15.2)

1. Zorbax SIL column (Agilent) 4.6×250 mm, 5 μ m

2. Hexane with 0.4% isopropyl alcohol

2.7.7. Polar Metabolite Separation (See Section 3.16)

1. Zorbax SB-C18 column (Agilent) 4.6 × 100, 3.5 mm
2. Acetonitrile
3. Water
4. Water with 10% formic acid

2.8. Analysis Instrumentation

1. HPLC with autosampler (Agilent 1200 series or comparable) and triple-quadrupole mass spectrometer (API-3000/API-4000, Applied Biosystems, or comparable) for endogenous RA analysis
2. HPLC with autosampler and UV detector (Agilent 1200 series or comparable) for endogenous retinol, retinal, and RE analysis

3. Methods

3.1. Retinoid Handling

1. Handle retinoids under yellow (or red) light ONLY (*see* Note 1). Retinoid degradation due to light exposure is illustrated in Fig. 1.2. Handling under yellow light includes all procedures of the following:
 - Standard solution preparation (*see* Note 2)
 - Tissue collection/dissection(*see* Note 3)
 - Tissue dissection requiring a microscope (*see* Note 4)
 - Homogenization
 - Extraction
 - Resuspension
2. Use only glass containers, pipettes, and calibrated syringes to handle retinoids (*see* Note 5)
3. Clean calibrated syringes before and after use by flushing 15–20 times with acetone and/or ethanol (*see* Notes 6 and ⁷)

¹If you do not have a room with overhead yellow lights, a desk lamp outfitted with a yellow light bulb can be used in a darkened room. “Bug-light” type yellow bulbs (which block lower wavelength light that attracts bugs) work well and can be purchased at any hardware store. Do NOT use yellow “party bulb”-type lights.

²Exposure to full spectrum (white) light (regular room lights) should be avoided, even for brief periods of time. (Noticeable degradation takes place in ~10 min!)

³All tissue harvest and dissections for retinoid analysis should be performed under yellow light. Sample collection under white lights will result in isomerization and degradation of retinoids.

⁴Dissections should be performed with a yellow filter on the light source like a Volpi (Auburn, NY, USA) NCL 150 light source with a red or yellow filter.

⁵Retinoids stick to many plastics and variable loss of up to 40% occurs when pipetting retinoid solutions with regular (plastic) pipette tips.

⁶To flush, draw up clean solvent and expel to waste.

4. Use fresh pipettes for transfer of materials to prevent cross-contamination among samples. This includes transfer of
 - Standard solutions
 - Homogenate
 - Hexane extracts
 - Resuspended samples
5. Use fresh pipettes for nitrogen delivery to each sample when evaporating solvent (*see* Note 8)
6. Clean homogenizers with water followed by ethanol and/or acetone before and after use to remove retinoid residue (*see* Note 9)

3.2. Retinoid Standard Preparation and Quantification

It is inappropriate to prepare and quantify retinoids by weight. Absorption spectroscopy is a simple and rapid way to accurately quantify retinoid concentrations (83). Sample absorbance spectra for several retinoids are shown in Fig. 1.3.

1. Dissolve a small amount of retinoid in the appropriate solvent (*see* Table 1.2, Note 10).
2. Collect the full absorbance spectrum (250–450 nm) (*see* Notes 11 and ¹²).
3. Calculate the concentration using Beer's law, $A = \epsilon bc$ (*see* Note 13).
 - A , absorbance at maximum (*see* maxima for selected retinoids listed in Table 1.2).
 - ϵ , molar absorptivity ($M^{-1} \text{ cm}^{-1}$).
 - b , path length (cm). Path length is 1 cm in a standard (1×1 cm) cuvette.
 - c , concentration (M).
4. Dilute or evaporate an appropriate aliquot and re-dissolve in the desired solvent (if necessary).

⁷Retinoid residue within calibrated syringes can contaminate samples without proper cleaning. It is not an exaggeration that 15–20 flushes are required.

⁸An evaporator with the capability to use disposable nitrogen delivery elements (such as glass pipettes) is highly desirable.

Evaporators with permanent nitrogen delivery elements will cross-contaminate samples.

⁹An acid bath can be used to periodically remove tissue (and/or retinoid) residue. Soak overnight in ~1 M HCl or HNO₃ followed by neutralization and flushing with copious amounts of water to remove all residue.

¹⁰Use of a disposable instrument, like the tip of a Pasteur pipette, can help prevent contamination by retinoid residue remaining on reusable tools.

¹¹Cuvettes should be cleaned thoroughly before and after use with ethanol and/or acetone and then dried completely. If acetone is used, complete removal is particularly important as acetone will absorb significantly at low wavelengths. For a more stringent cleaning rinse cuvettes with concentrated nitric acid followed by water followed by 100% ethanol and complete drying.

¹²Solutions with extra peaks, maxima at the wrong wavelength, or a large peak at low wavelength (200–300 nm) are either contaminated and/or degraded and should not be used.

¹³Molar absorptivity (ϵ) is wavelength (λ) and solvent dependent. Measure absorbance according to the wavelength and solvent for a given ϵ as described in Table 1.2.

3.3. Purification of Retinoid Reagents

Commercially available retinol can have retinal contamination (as high as 1–10%) that can influence experimental results when treating cells or subcellular fractions.

3.3.1. Chromatographic Purification of Retinol

1. A concentrated solution of retinol should be prepared in hexane with 5% acetone.
2. Inject retinol and purify using a Zorbax Sil 9.4 × 250 mm column (Agilent) and 5% acetone in hexane at 5 ml/min.
3. Collect retinol which should elute at ~20 min ($\pm$ depending on acetone concentration).
4. Evaporate solvent (*see* Note 14) and resuspend in a solvent of choice (*see* Table 1.2).
5. Determine the concentration of the solution by measuring its absorbance and using Beer's law (*see* Section 3.2).
6. Dilute and/or evaporate with nitrogen and resuspend in another solvent (if necessary).

3.3.2. Retinol Purification by Reduction of Retinal—Retinal can be readily reduced to retinol by NaBH₄ and the excess NaBH₄ can be neutralized by reaction with acetone to form 2-propanol.

1. Mix retinol (with retinal contamination) and NaBH₄ in THF with 5% MeOH (*see* Note 15) with NaBH₄ in excess of retinal (at least ~10-fold excess of estimated retinal level).
2. After 15–30 min of reaction time, add an excess of acetone to react with the added amount of NaBH₄ (forming 2-propanol).
2. Blow off solvents with nitrogen or another inert gas.
3. Wash the hexane layer by resuspending in H₂O/hexane (~1:1), vortex – mixing, and removing the (upper) hexane layer. Keep the hexane layer.
4. Add more H₂O to the hexane layer and repeat washing of hexane layer at least three times.
5. Quantify retinol concentration by absorbance spectroscopy (*see* Section 3.2).
6. Dilute and/or evaporate with nitrogen and resuspend in another solvent (if necessary).

3.4. Tissue Sample Preparation

There are a number of factors which can affect endogenous retinoid levels that should be controlled when assaying animals: (1) dietary vitamin A, (2) age, and (3) fasting. (1) *Dietary vitamin A*. Dietary vitamin A affects retinoid levels. Standard lab chow has a copious

¹⁴Acetone will interfere with the absorbance spectrum (absorbing highly at low wavelength) and must be removed.

¹⁵5% MeOH results in a faster reaction than THF alone.

amount of vitamin A (~15–30 IU vitamin A/g). Copious dietary vitamin A mutes or eliminates changes in endogenous retinoids due to genetic manipulation (or other treatment). Diet with a sufficient level of vitamin A (4IU vitamin A/g) is more appropriate for studies of endogenous retinoids (66). Mice often need to be bred more than one generation on a diet of 4IU vitamin A/g to stabilize endogenous retinoid levels. (2) *Age*. Mice should be age matched within a group and similar between compared groups. Age $\pm$ 0.5–1 month is usually acceptable. (3) *Fasting*. Metabolic state can affect retinoid levels. Groups of compared mice should be treated identically in terms of fasting/not fasting.

3.4.1. Tissue Harvest/Sample Collection

1. Harvest tissue under yellow lights (*see* Notes 1 and 3 and **Section 3.1**). If a dissecting microscope is used, a yellow filter should be used with the light source (*see* Note 4).
2. Euthanize mice according to institutional guidelines.
3. Collected blood by cardiac puncture using a 28½-gauge needle or similar. Allow blood to clot on ice for ~30 min and then centrifuge at 10,000 $\times$ g for 10 min (preferably at 4°C) to isolate serum. Draw off serum using a 1 ml calibrated glass pipette to measure the volume. Serum can be flash frozen in liquid nitrogen and stored at –80°C until assay.
4. Dissect and weigh tissue, place in a microcentrifuge tube, and flash freeze in liquid nitrogen. Store tissues at –80°C until assay.
5. If tissue sample is too small to be weighed accurately (localized region, embryo, cells), protein content can be determined and retinoid expressed per gram protein instead of per gram tissue (*see* **Section 3.5.3**).
6. Typical tissue harvest amounts from adult mice are listed in Table 1.3. Amounts provide enough tissue in most cases for multiple analyses. Small sample sizes may require pooling. Amount of tissue needed for an analysis should be tested and optimized (*see* **Section 3.11**).
7. Measurements from approximately 10–20 mg of tissue produce rigorous data from limited samples (e.g., embryo, localized areas, cells), whereas routine tissue assay amounts from adult mice range from approximately 40–115 mg.

3.4.2. Tissue Storage and Retinoid Stability During Storage—RA is susceptible to degradation even if stored at –80°C. Precautions must be taken to assure sample handling prior to analysis does not produce artifactual changes in endogenous retinoid levels. More abundant endogenous retinoids, such as retinol and RE, are less susceptible to but not devoid of storage-induced degradation.

1. Keep samples frozen as tissue until immediately before homogenization.
2. Collect and flash freeze in liquid nitrogen as described in **Section 3.4** under yellow lights.
3. Shield from light exposure at all times during storage.

4. Tissues can remain in liquid nitrogen in an appropriate storage container until assay *or* remove samples from liquid nitrogen and place at -80°C until assay.
5. Analysis within several days is preferable, but is possible for up to 1–2 weeks without significant degradation. Same day or next day analysis of frozen samples produces the highest quality data.
6. Most samples stored over 1 month may show measurable loss, especially of RA.
7. Test stability of each sample/tissue type, if storage before analysis will take place (*see* Note 16).
8. Do not store homogenates (*see* Note 17).
9. Generally, do not freeze, thaw, and then refreeze tissues (unless stability has been verified).
10. Thaw all samples (regardless of type) on ice.

3.5. Tissue Homogenization and Preparation

Retinoids are susceptible to degradation during homogenization. Use the most gentle homogenization possible. Keep all samples on ice during homogenization. Homogenization methods, starting with the most gentle, include (1) hand homogenization with a ground glass homogenizer, (2) motorized homogenizer driving the pestle with a ground glass homogenizer, and (3) a Polytron motorized hand-held stainless steel homogenizer. Most tissues can be homogenized by hand. Very fibrous tissues may need motorized pestle or Polytron.

3.5.1. Tissue Homogenization

1. Perform all homogenization under yellow lights (*see* Note 3).
2. Clean homogenizers before use (*see* **Section 3.1**).
3. Homogenize on ice with ice-cold saline (0.9% NaCl) to make an approximately 10–25% homogenate (for approximate amounts of saline for different tissue types, *see* Table 1.3).
4. When using a hand homogenizer, use a slow motion and use as few strokes as possible to achieve a homogenate. When using a Heidolph homogenizer (or similar motor-driven homogenizer), use approximately five strokes at lowest possible speed that will achieve homogenization. When using a Polytron, use the lowest possible speed to achieve homogenization (~20–25% of maximum) pulsing the

¹⁶Different matrices/different tissue types have different susceptibility to degradation during storage.

¹⁷Homogenized samples frozen immediately and stored at -80°C for 1 day gave comparable results (within 10%) with those of freshly analyzed samples; however, homogenized samples stored for 1 month gave values that were 50% less than samples analyzed immediately. Therefore, it should be possible to store tissue homogenates overnight in the freezer, but long-term storage of homogenized samples should be avoided, even at -80°C (49).

homogenizer on and off in ~5–10 bursts of short duration (*see* Notes 18, ¹⁹, and ²⁰).

5. Aliquot homogenate with graduated 1 ml glass pipettes into 16 × 150 mm culture tubes.
6. Extract samples immediately after homogenization (*see* Notes 17 and ²¹).

3.5.2. Subcellular Fractionation—Retinoids can be quantified in subcellular fractions.

1. Perform all procedures under yellow lights (*see* Note 3).
2. Harvest tissue as described in **Section 3.4**.
3. Tissues should be homogenized (to make ~25% homogenate) in 10% sucrose, 10 mM Tris-HCl, 1 mM EDTA, 1.5 mM DTT, pH 7.4 at 1240 rpm in a Dounce homogenizing vial, Kontes, size 22.
4. Isolate subcellular fractions at 4°C by ultracentrifugation.
5. Freeze fractions at –80°C until assay (*see* Note 17).
6. Determine protein content as described in **Section 3.5.3**.

3.5.3. Protein Determination—If sample is too small to be weighed (e.g., localized area, embryo parts, cells, subcellular fraction), determine the total protein content of the sample as homogenate using the Bradford method or another method to assess total protein content. In this case, retinoids will be expressed as retinoid per gram protein instead of retinoid per gram tissue. Bradford assay is a dye-binding assay used to measure total protein content, where absorbance of the protein–dye solution is proportional to protein amount (*see* Notes 22, ²³, and ²⁴).

1. Dilute Bradford reagent with water immediately before use so that it is 20% concentrated Bradford reagent.
2. Aliquot 1 ml diluted Bradford reagent into disposable polystyrene cuvettes.
3. Add 20 µl protein to diluted Bradford reagent, mix well, and let react for 5–10 min.
4. Measure absorbance at 595 nm (*see* Note 25).
5. Calculate protein concentration from a standard curve. Use protein concentration to determine total gram of protein in homogenate aliquot.

¹⁸Alternate motion/position of pestle/homogenizer to avoid generating too much friction in a localized area. When homogenizing try not to pull any suction when moving the pestle up and down.

¹⁹Polytron homogenization should only be used if other homogenization methods are ineffective.

²⁰Homogenization of skin will have a non-homogenizable portion left over which should be subtracted from the tissue weight to give a net extracted tissue amount.

²¹Degradation and isomerization by the biological matrix will occur. Samples should be extracted within 30–60 min after homogenization. Homogenized samples will undergo significant degradation after 2 h at 4°C from matrix effects (48, 49).

²²For more information on Bradford dye-binding assay for total protein determination visit www.bio-rad.com.

²³Known amounts of BSA protein (or similar) should be used to generate a standard curve.

²⁴Protein should be diluted so absorbance readings from protein–dye are in the linear range (of the standard curve generated by known protein amounts).

²⁵Absorbance between 0.1 and 1.0 should fall in the linear range; however, this is dependent on standard curve (*see* Note 23).

3.6. Internal Standards

An internal standard improves accuracy by establishing extraction efficiency, handling loss, and revealing handling-induced isomerization/degradation. An internal standard should have similar chemical properties comparable to the analyte of interest, including structure, extraction efficiency, and chromatographic behavior. More than one internal standard may be added to follow multiple analytes.

1. Establish performance of chosen internal standard before use (*see* **Section 3.22.5**).
2. Internal standard solutions are prepared (*see* **Sections 3.1** and **3.2**) at a concentration that delivers an amount comparable to the analyte of interest.
3. Addition volume is usually 5–20 μ l, delivered in ethanol, acetonitrile, or DMSO.
4. Internal standard(s) are added to the homogenate with a calibrated glass syringe and the sample is vortex mixed (*see* Notes 26 and ²⁷).
5. Samples are then extracted as described in **Section 3.8**.

3.7. Conversion of Retinal to Retinal Oxime Derivatives

(*O*-ethyl)hydroxylamine is used to convert retinal into a stable oxime for accurate quantification (*see* Fig. 1.4). Retinal has a reactive aldehyde group susceptible to promiscuous reactions in the sample matrix, preventing efficient extraction. Reaction with hydroxylamine or (*O*-alkyl)hydroxylamines produces *syn*- and *anti*- isomers (93–95). It is more desirable to generate retinal oximes from (*O*-alkyl)hydroxylamines (e.g., (*O*-ethyl)hydroxylamine) instead of the nonalkylated hydroxylamine because the *anti*-retinaloxime isomer of the nonalkylated hydroxylamine tends to co-elute with retinol and/or elute as a broad asymmetrical peak in both reverse and normal-phase HPLC. Additionally, the variable contribution from the *syn*-retinaloxime prevents retinal quantification with only the *syn*-isomer.

1. Perform all procedures under yellow lights (*see* Note 3).
2. Collect and homogenize tissues as described in **Sections 3.4** and **3.5** and aliquot homogenate (*see* Table 1.3) into 16 $\times$ 150 mm disposable glass culture tubes.
3. To an appropriate amount of homogenate or serum (*see* Table 1.3), add 1–2 ml methanol and 0.5–2 ml of 0.1 M
O-ethylhydroxylamine in 0.1 M HEPES (pH 6.5).
4. Vortex samples well and let stand for 15 min at room temperature.
5. Extract retinal with hexane (*see* **Section 3.8.4**).

²⁶Dedicated syringes to each internal standard can prevent inadvertent contamination by retinoid residue in a syringe. Syringes should be cleaned before each use.

²⁷Take care not to touch the tip of the syringe to the sample to avoid cross-contamination.

3.8. Extraction

Most samples are extracted with a two-step acid–base extraction that recovers multiple retinoids: RA, retinol, RE, and RA polar metabolites. Retinal extraction requires pre-treatment to convert reactive retinal to a stable product (*see* **Section 3.7**). Extractions can be altered to target only one type of analyte, if desired.

3.8.1. Acid–Base Extraction: Retinol, RE, RA, and Polar Metabolites

1. Carry out all procedures under yellow lights (*see* Note 3).
2. Collect samples and homogenize as described (*see* **Sections 3.4** and **3.5**).
3. Aliquot homogenate into 16 × 150 mm disposable glass culture tubes (*see* Table 1.3).
4. Determine total protein content (if necessary) before extraction (*see* **Section 3.5.3**).
5. Add internal standard to homogenate/serum and vortex mix (*see* **Section 3.6**).
6. Add from 1 to 3 ml of 0.025 M KOH in ethanol to tissue homogenates/serum and vortex mix (at least 10 s).
7. If sample requires addition of acetonitrile to facilitate protein precipitation, add acetonitrile here (usually ~1 ml acetonitrile) and vortex mix (at least 10 s).
8. Add 10 ml of hexane to homogenate/serum and vortex mix (at least 10 s).
9. Centrifuge for 1–3 min at ~1,000 × *g* to facilitate phase separation.
10. Draw off the top (organic) phase containing nonpolar retinoids (retinol and retinyl ester(s)) to a new 16 × 150 mm disposable glass culture tube (*see* Note 28).
11. To the bottom (ethanolic aqueous) layer of homogenate/ serum, add 4 M HCl (60–240 µl) and vortex mix (at least 10 s).
12. Add 10 ml hexane and vortex mix (at least 10 s).
13. Centrifuge for 1–3 min at ~1,000 × *g* to facilitate phase separation.
14. Draw off top (organic) phase containing RA and polar retinoids to a new 16 × 150 mm disposable glass culture tube. *It is important not to disturb the bottom acid-containing layer! Any of the acid-aqueous layer drawn off with the hexane will destroy RA and/or cause major isomerization (see Fig. 1.5).*
15. Evaporate organic phases under nitrogen with gentle heating at ~25–300°C in a water bath (*see* Note 28).
16. Keep evaporated samples on ice until resuspension (*see* **Section 3.9**, resuspension).

3.8.2. RA-Only Extraction from Serum—If only RA quantification from serum is desired, a one-step acetonitrile extraction is possible.

1. Follow **Section 3.8.1**, acid–base extraction: steps 1, 2 and 3.

²⁸Hexane extracts are evaporated under nitrogen gas immediately or kept on ice until evaporation (as soon as possible).

2. Add 1 ml of acetonitrile and 60 μ l of 4 M HCl and vortex mix (at least 10 s).
3. Add 10 ml of hexane and vortex mix.
4. Centrifuge for 1–3 min at $\sim 1,000 \times g$ to facilitate phase separation.
5. Draw off top (organic) phase containing RA and polar retinoids to a new 16 $\times$ 150 mm disposable glass culture tube. *It is important not to disturb the bottom acid-containing layer! Any of the acid-aqueous layer drawn off with the hexane will destroy RA and/or cause major isomerization (see Fig. 1.5).*
6. Evaporate organic phases under nitrogen with gentle heating at ~ 25 – 30°C in a water bath (*see* Note 28).
7. Keep evaporated samples on ice until resuspension (*see* **Section 3.9**).

3.8.3. Retinol and RE Extraction—If only retinol and RE are desired for quantification, use **Section 3.8.1**, acid–base extraction: omitting steps 8, 9, 10, and 11.

3.8.4. Retinal (Oxime) Extraction—Retinal oxime derivatives are extracted efficiently with hexane (>95% recovery in most cases).

1. Follow **Section 3.7** to prepare retinal (*O*-ethyl) oxime derivatives.
2. Add 10 ml hexane to homogenate and vortex mix (at least 10 s).
3. Centrifuge for 1–3 min at $\sim 1,000 \times g$ to facilitate phase separation (*see* Note 29).
4. Draw off top (organic) layer to a new 16 $\times$ 150 mm disposable glass culture tube.
5. Evaporate organic phases under nitrogen with gentle heating at ~ 25 – 30°C in a water bath (*see* Note 28).
6. Keep evaporated samples on ice until resuspension (*see* **Section 3.9**).

3.9. Resuspension

Several factors are important to consider when selecting a resuspension solvent for analysis of retinoids. These include (1) solubility of retinoid in resuspension solvent, (2) compatibility of resuspension solvent with HPLC mobile phase, (3) volume of resuspension solvent necessary to make amount of analyte in injection volume appropriate to linear range of the analysis method, and (4) stability of sample in resuspension solvent.

1. Perform all procedures under yellow lights (*see* Note 3).
2. Extract and evaporate samples (*see* **Section 3.8**).
3. Add appropriate volume of solvent for the desired analyte and separation method (*see* Tables 1.4 and 1.5 and Notes 30, ³¹, ³², and ³³).

²⁹There should be a crisp layer between phases. If a center layer is observed there may be inefficient extraction of the retinal *O*-ethyl oxime derivatives. Adjust reagents to eliminate.

³⁰Samples resuspended in acetonitrile were more stable than suspensions in other solvents, including mixtures of methanol/ acetonitrile/water, methanol/acetonitrile, and methanol.

³¹Samples should not be resuspended or stored in mobile phases that contain acid.

³²Resuspension volume may depend on vitamin A status. For example, liver resuspension volumes can range from 120 to 1000 μ l.

4. Vortex mix for 10–20 s.
5. Transfer sample with a 9" Pasteur pipette to a low-volume glass insert for analysis.
6. Analyze according to analyte (*see Sections 3.12–3.21*).

3.10. Extracted Sample Storage

1. Place samples in amber vials and keep shielded from light.
2. Samples in acetonitrile are stable at room temperature for 2 days; at 4 days a 20% loss occurs (at room temperature) (49). Cooling of the autosampler helps preserve sample quality.
3. If samples are not analyzed immediately, store at –20°C (preferable) or 4°C.
4. Resuspended samples (in acetonitrile) stored at –20°C remain unchanged for ~5–7 days (49–51).
5. Test stability of each sample type if samples are to be stored for any length of time before analysis (*see Note 16*).

3.11. Sample Preparation Optimization Strategies

Attention to sample preparation provides dividends in sensitivity and accuracy, especially in the low-femtomole range. The sample preparation described here, although not extensive, purifies the matrix sufficiently to enhance accuracy and the lives of guard and analytical columns. The simpler sample preparations available are adequate only for samples with less complicated matrixes, such as serum from normal subjects, or certain cell culture extracts (61, 71). Most tissues (e.g., liver, kidney, testis) and/or serum from metabolically altered subjects present a more complex matrix, which requires sufficient matrix cleanup to prevent deterioration of assay performance (*see Note 34*). The following experimental variable should be optimized:

1. Amount of tissue extracted. Small tissues/limited tissue amounts may require pooling of multiple tissue samples for analysis. Large tissue samples/tissues abundant in retinoids require only a fraction of the entire tissue for analysis.
2. Amount of homogenate extracted. Too little extracted will yield low signal, whereas too much sample extracted will yield poor extraction efficiency and/or high background.

³³For adipose tissue (and other high lipid content tissues), there is often a sizable amount of lipid present after evaporation in the retinol/RE extract. Resuspend in a slightly larger volume (200 vs. 120 µl), vortex mix, and then transfer the sample avoiding the lipid drop. Lipid collection in the bottom of the low volume insert is problematic for injection and analysis. An internal standard can account for sample loss to the lipid drop (*see Note 56*).

³⁴The acid–base extraction described here (*see Section 3.8*) is preferable to analyses that use saponification. Saponification, an alkaline digestion that frees retinoids from the stabilizing matrix and lipids while hydrolyzing RE to retinol to yield a total retinol measurement, can be problematic. The elevated temperature and exposure to alkali often causes retinoid degradation and isomerization of 4–40% (2). This loss is illustrated by 30–65% lower total ROL values obtained after saponification compared with the sum of ROL and RE values obtained separately (16, 22).

3. % homogenate. Dilution of homogenate can improve extraction efficiency. For very small samples it is often easier to handle a more dilute sample to minimize transfer losses (addition of 0.5–1.0 ml saline for homogenization).
4. Ratio of extraction reagents. The amount of 0.025 M KOH in ethanol, 4 M HCl, and/or hexane effect extraction efficiency. Tissue homogenate typically needs more extraction reagent(s) than cells or dilute/small samples.
5. Precipitation of protein. 0.025 M KOH in ethanol precipitates some protein. Acetonitrile can be added during the KOH step to assist in precipitating additional protein. Tissues that are protein rich can display higher background and/or interfering peaks in LC/MS/MS chromatograms.
6. Resuspension volume. Resuspension volume can be adjusted according to the abundance of the analyte, the size of the sample, and the type of analysis. *See* Table 1.4 for starting guidelines.

3.12. Separation Methods and Sample Preparation

The separation methods here are optimized according to analyte. Some separation methods allow quantification of multiple species and/or isomeric forms. Additionally, the sample preparation protocol reported here allows analysis of greater than 5,000–10,000 samples (~6–12 months) before requiring column replacement. Methods that shorten sample preparation modestly report changing guard columns daily and replacing analytical columns frequently, even with the relatively simple matrix of normal serum, which could be quite costly (61).

3.13. RA Isomer Separations

Two reverse-phase separation protocols were developed to resolve RA and its isomers: one predominantly for cultured cells or sub-cellular fractions (gradient 1) and one predominantly for tissue samples (gradient 2), which have higher background. An alternate normal-phase separation is also provided.

3.13.1. Cell/Subcellular Fraction RA (Gradient 1)

1. Use a high performance liquid chromatograph (HPLC) consisting of a vacuum degasser, binary pump, temperature-controlled column compartment, and a temperature-controlled autosampler.
2. Maintain the column compartment at ~20–25°C and the autosampler at 10°C.
3. Inject 20–30 µL. Inject 30 µl for small/dilute/low abundance samples (*see* Note 35).
4. Use a Supelcosil ABZ+PLUS Supelguard cartridge column (Supelco, 2.1 × 20 mm, 5 µm) before the analytical column.
5. Use a Supelcosil ABZ+PLUS column (Supelco, 2.1 × 100 mm, 3 µm).

³⁵Small samples include those ~10–20 mg or less of tissue. Small samples can also include samples of low retinoid abundance such as VAD diet, genetically manipulated animals, or exogenous compound-treated animals.

6. Use the following solvents: A, H₂O with 0.1% formic acid; B, acetonitrile with 0.1% formic acid (*see* Note 36).
7. Gradient 1 separation is effected at 400 µl/min with the following linear gradient: 0–5 min, 60% B to 95% B; 5–8 min, hold at 95% B; 8–9 min, 95% B to 60% B; 9–12 min, re-equilibrate with 60% B.
8. Use MS/MS detection (*see* **Section 3.18**).
9. Retention times of RA isomers are as follows: 6.8 min (13cRA), 7.5 min (9cRA), 8.0 min (atRA), and 8.8 (4,4-dimethyl-retinoic acid) (*see* Fig. 1.6, Note 37).
10. Quantify each RA isomer from a calibration curve generated from standard amounts of that isomer using the gradient 1 separation.

3.13.2. Tissue RA (Gradient 2)

1. Use a high performance liquid chromatograph (HPLC) consisting of a vacuum degasser, binary pump, temperature-controlled column compartment, and a temperature-controlled autosampler.
2. Maintain the column compartment at 25°C and the autosampler at 10°C.
3. Inject 20–30 µl. Inject 30 µl for small/dilute/low abundance samples (*see* Note 35).
4. Use a Supelcosil ABZ+PLUS Supelguard cartridge column (Supelco, 2.1 × 20 mm, 5 µm) before the analytical column.
5. Use an Ascentis RP-Amide column (Supelco, 2.1 × 150 mm, 3 µm).
6. Use the following solvents: A, H₂O with 0.1% formic acid; B, acetonitrile with 0.1% formic acid (*see* Note 36).
7. Gradient 2 separation is effected at 400 µl/min with the following linear gradient: 0–3 min, hold at 70% B; 3–15 min, 70% B to 95% B; 15–20 min, hold at 95% B; 20–21 min, 95% B to 70%B; 21–25 min, re-equilibrate at 70% B.
8. Use with MS/MS detection (*see* **Section 3.18**).
9. Retention times of RA isomers are as follows: 12.8 min (13cRA), 13.8 min (9cRA), 14.3 min (atRA), and 17.1 (4,4-dimethyl-retinoic acid) (*see* Fig. 1.6, Note 37).
10. Quantify each RA isomer from a calibration curve generated from standard amounts of that isomer using the gradient 2 separation.

3.13.3. Alternate Normal-Phase Separation

1. Use a high performance liquid chromatograph (HPLC) consisting of a vacuum degasser, binary pump, temperature-controlled column compartment, and a temperature-controlled autosampler.
2. Maintain the column compartment at 25°C and the autosampler at 10°C.

³⁶Some stationary phases are susceptible to pH-dependant degradation. Manufacturer's recommendations should be followed.

³⁷Confirm retention times and peak identity with authentic standards frequently.

3. Inject 100 μ l (for UV detection or less if using MS/MS detection).
4. Use a Zorbax SIL, 4.6 $\times$ 250 mm, 5 μ m column.
5. Use isocratic 0.4% 2-propanol/hexane at 2 ml/min (*see* Note 38).
6. A representative chromatogram with HPLC/UV detection at 340 nm is shown; however, separation could be adapted to MS/MS detection (*see* Fig. 1.6).
7. Retention times of RA isomers are as follows: 10.9 min (13cRA), 12.1 min (9cRA), and 13.1 min (atRA) (*see* Note 37).
8. Quantify each RA isomer from a calibration curve generated from standard amounts of that isomer using the normal-phase separation (*see* Note 39).

3.14. Retinal

Retinal quantification requires summing the *syn*- and the *anti*-(*O*-ethyl)retinaloxime forms in chromatograms (of samples prepared and extracted as described in **Sections 3.7** and **3.8**). A reverse-phase separation quantifies total retinal, whereas a normal-phase separation can separate *cis*- and *trans*-isomeric forms of retinal (*O*-ethyl) oximes.

3.14.1. Total Retinal

1. Use a high performance liquid chromatograph (HPLC) consisting of a vacuum degasser, binary pump, temperature-controlled column compartment, and a temperature-controlled autosampler.
2. Maintain the column compartment at 25°C and the autosampler at 10°C.
3. Inject 100 μ l.
4. Use a Zorbax SB-C18, 4.6 $\times$ 100 mm, 3.5 μ m column.
5. Analytes are separated at 1 ml/min with a linear gradient from 40% H₂O/60% acetonitrile/0.1% formic acid to 5% H₂O/95% acetonitrile/0.1% formic acid over 5 min. Final conditions were held for 9 min (*see* Notes 37, ⁴⁰, and ⁴¹).
6. Use UV detection at 368 nm. Retinol can be monitored simultaneously at 325 nm.
7. Retinal (*O*-ethyl) oximes elute at 6.6 min (*anti*-) and 10.9 min (*syn*-), and retinol elutes at 7.2 min (*see* Fig. 1.7, Note 37).
8. The sum of the *syn*- and the *anti*- oximes is used to quantify retinal (*O*-ethyl) oxime from a calibration curve generated from standard amounts of retinal (*O*-ethyl) oxime.

³⁸Prepare mobile phase fresh as small changes in (normal phase) mobile phase composition (due to evaporation, etc.) will result in significant changes in retention time(s).

³⁹Although levels of RA in vivo were not detectable above background and/or were the same magnitude as random/interfering peaks, this method is useful for applications in which RA is high such as enzyme assays. Quantification of in vivo levels of RA is best accomplished with more sensitive detection methods (49, 50).

⁴⁰Note that the column needs to be flushed periodically when quantifying ester-rich tissue (e.g., liver) to reduce RE accumulation. A flush with the total retinol and RE separation described in **Section 3.15.1** followed by equilibration with a blank total retinal separation as described in **Section 3.14.1** is effective.

⁴¹The amount of RE present can be significantly higher than retinol and require re-injection of a smaller volume to give an RE peak area within the linear range.

3.14.2. Retinal *cis*- and *trans*-Isomers

1. Use a high performance liquid chromatograph (HPLC) consisting of a vacuum degasser, binary pump, temperature-controlled column compartment, and a temperature-controlled autosampler.
2. Maintain the column compartment at 25°C and the autosampler at 10°C.
3. Inject 100 µl.
4. Use *two* (2) Zorbax SIL, 4.6 × 250 mm, 5 µm columns, connected in series.
5. Use 11.2% ethyl acetate, 2% dioxane, 1.4% 1-octanol in hexane at 1 ml/min flow rate (*see* Note 38).
6. Use UV detection at 325 nm.
7. Representative chromatograms can be found in Furr (81) and in Landers and Olson (92) (*see* Note 37).
8. The sum of the *syn*- and *anti*- oximes from each isomer is used to quantify retinal (*O*-ethyl) oxime from a calibration curve generated from standard amounts of retinal (*O*-ethyl) oxime.

3.15. Retinol and RE

The total retinol and RE method is a reverse-phase separation that is effective with tissue quantification, as well as cell systems and subcellular fractions. The retinol isomer method is an isocratic normal-phase separation that can quantify the isomeric distribution of retinol, which may be of interest when investigating precursors to RA isomers.

3.15.1. Total Retinol and RE

1. Use a high performance liquid chromatograph (HPLC) consisting of a vacuum degasser, binary pump, temperature-controlled column compartment, and a temperature-controlled autosampler.
2. Maintain the column compartment at 25°C and the autosampler at 10°C.
3. Inject 100 µl. Perform a separate 10 µl injection for liver RE quantification to ensure that the RE signal occurred within the linear detection range (*see* Note 41).
4. Use a guard column or inline filter.
5. Use a Zorbax SB-C18, 4.6 × 100 mm, 3.5 µm column (Agilent).
6. Separate analytes at 1 ml/min with 11% H₂O/89% acetonitrile/0.1% formic acid for 9 min, followed by a linear gradient over 2 min to 100% acetonitrile. Then maintain 100% acetonitrile for 2 min, followed by a linear gradient over 2 min to 5% acetonitrile/1,2-dichloroethane. Hold final conditions for 2 min before returning to initial conditions (*see* Notes 42 and ⁴³).

⁴²For Sections 3.14.1 and 3.15.1 reservoirs can be set up as follows: A: H₂O, B: H₂O with 10% formic acid, C: acetonitrile, D: 1,2-dichloroethane. For example, to achieve 40% H₂O/60% acetonitrile/0.1% formic acid HPLC flow can be set to 39% H₂O, 1% H₂O with 1% formic acid, and 60% acetonitrile.

7. Use with UV detection at 325 nm.
8. Retention times for analytes: retinol at 4.8 min, retinyl acetate (IS) at 8.9 min, and RE (shown as retinyl palmitate) at 16.5 min (*see* Fig. 1.8, Notes 37 and ⁴⁴).
9. Quantify retinol and RE from calibration curves generated from standard amounts of retinol and RE separated using the total retinol and RE method (*see* Note 45).

3.15.2. Retinol Isomers

1. Use a high performance liquid chromatograph (HPLC) consisting of a vacuum degasser, binary pump, temperature-controlled column compartment, and a temperature-controlled autosampler.
2. Maintain the column compartment at 25°C and the autosampler at 10°C.
3. Inject 100 µl. Perform a separate 10 µl injection for liver RE quantification to ensure that the RE signal occurred within the linear detection range (*see* Note 41).
4. Use a Zorbax SIL, 4.6 × 250 mm, 5 µm column.
5. Resolve analytes using 0.4% 2-propanol/hexane at 2 ml/min (*see* Note 38).
6. Use UV detection at 325 nm.
7. Retention times for retinol isomers: 20.9 min (13c-retinol), 27.0 min (9c-retinol), and 28.9 min (at-retinol). Other retinoids eluted at 2.0 min (retinyl palmitate and other RE) and 3.6 min (retinyl acetate) (*see* Fig. 1.8, Notes 37 and ⁴⁶).
8. Quantify retinol isomers from calibration curves generated from standard amounts of each isomer separated using the retinol isomer method.

3.16. Polar Metabolites

Polar metabolites can be separated using the same mobile phase solvents as in **Sections 3.14** and **3.15** using a gradient of water, acetonitrile, and formic acid. Blumberg et al., White et al., and Taimi et al. provide other separation methods (96–98). The separation method described by Taimi et al. using a 2.1 mm ID column and a flow rate of 0.2 ml/min is compatible with MS/MS detection (98).

⁴³The total ROL/RE reverse-phase method was modified from previous methods to use an acetonitrile/water/formic acid mobile phase that transitions to an acetonitrile/dichloroethane mobile phase (31, 99). The acetonitrile/water/formic acid mobile phase gave sharper ROL peaks than did the previous methanol/water-based ROL separations

⁴⁴Not all endogenous REs were resolved using this method; it was intended to quantify only total RE. Figure 1.3a shows retinyl palmitate only, which is up to 90% of the endogenous ester. Retinyl oleate co-eluted with retinyl palmitate, whereas other esters, such as retinyl linoleate, retinyl myristate, and retinyl stearate, eluted just before or after retinyl palmitate.

⁴⁵The sum of all ester peaks was used to calculate total RE. Retinyl palmitate was used as the calibrant to calculate total ester because retinyl palmitate and other RE have similar absorbance maxima (44).

⁴⁶Retinyl palmitate can be quantified using the retinol isomer method, but because of its minimal retention and the possible background contribution from minimally retained matrix components, the total ROL/RE method described here is preferable for RE quantification.

3.16.1. Polar Metabolites with 4.6 mm ID column

1. Use a high performance liquid chromatograph (HPLC) consisting of a vacuum degasser, binary pump, temperature-controlled column compartment, and a temperature-controlled autosampler.
2. Maintain the column compartment at 25°C and the autosampler at 10°C.
3. Inject 100 µl.
4. Use a Zorbax SB-C18, 4.6 × 100 mm, 3.5 µm column (Agilent).
5. Separate analytes at 1 ml/min with a linear gradient from 75% H₂O/25% acetonitrile/0.1% formic acid to 1% H₂O/99% acetonitrile/0.1% formic acid over 30 min; hold at 1% H₂O/99% acetonitrile/0.1% formic acid for 7 min; return to initial conditions over 4 min; and hold for four additional minutes to equilibrate.
6. Use UV detection at 355 nm or MS/MS detection (*see Section 3.18*).
7. RA elutes at 30 min and polar metabolites elute between 18 and 23 min (4-OH-RA, 19.5 min; 4-oxo-RA, 20.1 min) (*see Note 37*).
8. Calibration curves are generated from standard amounts of each retinoid with the gradient solvent system used.

3.16.2. Polar Metabolites with 2.1 mm ID Column (from Taimi et al. (98))

1. Use a high performance liquid chromatograph (HPLC) consisting of a vacuum degasser, binary pump, temperature-controlled column compartment, and a temperature-controlled autosampler.
2. Use a Zorbax C18 Eclipse XDB 150 × 2.1, 5 µm column (Agilent).
3. Use water (solvent A), acetonitrile (solvent B), and 10% acetic acid (solvent C).
4. Separate polar metabolites at a flow rate of 0.2 ml/min starting with mixture of solvents A:B:C in the ratio 64:35:1 for 2 min, a linear gradient for 28 min up to 95% of solvent B with a constant flow rate of 1% solvent C, an isocratic hold at 95% B for 10 min, a linear gradient to initial conditions over 5 min, and an equilibration at initial conditions for 5 min.
5. Use MS/MS detection (*see Section 3.18*) or UV detection at 355 nm.
6. RA elutes at 33.2 min and polar metabolites elute between 15 and 20 min (4-OH-RA, 15.8 min; 4-oxo-RA, 16.9 min; 18-OH-RA, 19.0 min) (*see Note 37*).

3.17. Separation Optimization Strategies

Proper and sufficient chromatographic separation of analytes is essential to accurate quantification. Species of interest should be baseline resolved (peaks not overlapping) and have good peak shape (sharp peaks) (*see Figs. 1.6, 1.7, and 1.8*). Use standard solutions of the retinoids of interest to evaluate the separation before unknown analysis. If insufficient resolution is observed, several factors can be optimized to effect an adequate separation:

1. Column stationary phase. Surface chemistry differences lead to different selectivity for analyte separation. Choose column based on the selectivity of the stationary phase for the analytes to be separated. For example, C-16 alkylamide column stationary phases have greater resolving power for reverse-phase separations of RA isomers compared to C-18 stationary phases. C-18 columns work well for reverse-phase retinol, retinal, and retinyl ester separations. Silica columns are effective for normal-phase retinoid separations.
2. Column dimensions. Optimize column diameter and length to effect a separation. For analytical separations, columns with ID of 4.6 mm or smaller are typically used. MS-based detection commonly uses columns with ID of 2.1 mm or smaller. Smaller inner diameter columns not only increase resolution but also increase pressure. Capillary columns require a high pressure pump. Increasing column length increases resolution, but doubling column length results in double the elution time and solvent consumption with a 1.4-fold increase in resolution (100).
3. Stationary phase particle size. Smaller diameter stationary phase particles not only increase resolution but also increase pressure. However, greater resolution resulting from smaller stationary phase particles will reduce the column length needed for a given separation.
4. Mobile phase composition. Mobile phase composition must be compatible with column stationary phase and with the detection method. Mobile phase composition can affect MS ionization efficiency. Mobile phase choice can also influence peak shape, for example, acetonitrile-based retinol separations give sharper peak shapes as compared to methanol-based separations for retinol on a C-18 column (*see* Fig. 1.9).
5. Mobile phase gradient. Altering the gradient of a separation either in slope or in composition can assist in adjusting the retention time of analytes.
6. pH. The pH of some mobile phases is important and aids in the separation of charged species through ion pairing (*see* Note 36).
7. Flow rate. Optimum flow rate will give optimum resolution through maximizing plate height (*see* Note 47).
8. Temperature. Controlling temperature greatly assists in reproducibility of retention times. Elevating or reducing temperature can also be useful in optimizing a separation.
9. Separation mode/type. Switching from reverse phase to normal phase (or vice versa) may be necessary to effect sufficient resolution between species of interest. This modification requires switching column type (stationary phase).

⁴⁷Optimum plate height and flow rate occur when contributions from eddy diffusion, longitudinal diffusion, and resistance to mass transfer are minimized (100).

3.18. LC/MS/MS Detection

Low abundance of endogenous RA requires sensitive detection. MS/MS is currently the most sensitive method of RA detection and is readily coupled to LC separations capable of resolving RA isomers that have varied biological activity in vivo. Typically, quantitative MS/MS uses a triple-quadrupole MS instrument where the parent ion mass is selected in Q1, the parent ion is collisionally fragmented by N₂ in Q2, and a product ion mass is selected in Q3 for detection. MS/MS offers appropriate sensitivity for RA detection through background reductions of 100–1000-fold over MS (*see* Fig. 1.10). MS/MS also imparts specificity by requiring analytes to meet both parent ion and product ion m/z conditions for detection. The sensitivity and background reduction advantage of MS/MS allows for analysis of smaller tissue samples and produces superior chromatograms for quantification (*see* Fig. 1.10). Information obtained from MS/MS fragmentation is also useful in the identification of unknown molecules.

Several reports have shown that positive atmospheric pressure chemical ionization (APCI) has numerous advantages for RA analysis (as well as other retinoids), including favorable ionization efficiency based on the conjugated structure and carboxylic acid group (*see* Fig. 1.6), greater sensitivity and lower background than negative APCI, and greater signal intensity and linear dynamic range than electrospray ionization (49, 60, 77). APCI is also less susceptible than ESI to matrix suppression that can interfere with accurate quantification. Negative ESI–MS/MS, however, has been used effectively to identify polar metabolites produced from RA catabolism (96–98).

3.18.1. MS/MS Detection of RA Isomers

1. Use the separation described in **Section 3.13.1** or **3.13.2**.
2. Use an Applied Biosystems API-4000 triple-quadrupole mass spectrometer (or comparable instrument) equipped with APCI operated in positive ion mode.
3. Operate in multiple reaction monitoring (MRM) mode: monitor RA using an m/z 301.1 [M + H]⁺ to m/z 205.0 transition; monitor 4,4-dimethyl-RA using an m/z 329.4 [M + H]⁺ to m/z 151.3 transition, and use a dwell time of 150 ms for both RA and 4,4-dimethyl-RA (*see* Note 48).
4. The optimum positive APCI conditions on an API-4000 (Applied Biosystems) included the following: collision gas, 7; curtain gas, 10; gas1, 70; nebulizer current, 3; source temperature, 350; declustering potential, 55; entrance potential, 10; collision energy, 17; collision exit potential, 5. Source position was vertical 790, horizontal 750 (*see* Note 49) (50).
5. Data from previous work were acquired with an Applied Biosystems API-3000 triple-quadrupole mass spectrometer equipped with APCI using the conditions described (49).

⁴⁸Optimize parent ion/fragment ion transition for your conditions to maximize background and minimize background and interferences from biomatrix species.

⁴⁹Source conditions listed are guidelines. Optimize conditions for your instrument/conditions. Tuning of instrumental conditions is essential to obtaining sufficient sensitivity to collect in vivo sample data.

6. Assess detector capabilities and assay performance prior to quantification; then quantify each analyte from a calibration curve generated from standard amounts of that compound (*see* **Section 3.22**).
7. Peak identity/instrument performance for each method should be verified daily by injecting a mixture of authentic retinoid standards (*see* Note 37).

3.18.2. MS/MS Identification of RA Polar Metabolites (Taimi et al. (98))

1. Use with separation of polar metabolites described in **Section 3.16**.
2. Use a Micromass Quattro Ultima triple-stage quadrupole mass spectrometer (Manchester, UK) in negative ESI mode.
3. Operate in either full mass scan mode (m/z 200–500) or product ion scan mode (m/z 50–400). Metabolites were characterized using MS/MS in the product ion scan mode (*see* Note 50).
4. Negative ESI conditions were capillary voltage, -3.15 KV; cone voltage, -37 V; desolvation gas (nitrogen) flow, 871 l/h; collision gas pressure, 2.3×10^{-3} torr; collision energy, 25 V.

3.18.3. Optimization Strategies for MS/MS Detection—Optimization of MS/MS conditions for maximum signal are essential to effective quantification efforts. Infusing a standard solution (~ 1 nM– 1 μ M) via a syringe pump (~ 1 – 100 μ l/min) is necessary to tune the instrument properly before analysis (*see* Note 51). Optimize conditions by infusion and confirm with the chromatographic conditions used during quantification.

1. Instrument type. The model, vendor, and instrument type all affect the potential sensitivity. Different instruments will have different “base” sensitivities.
2. Molecular ion. Infuse each analyte to confirm the molecular ion obtained (*see* Note 52).
3. Ionization method. Whereas positive APCI has been reported to be most sensitive for retinoids, different ionization modes should be investigated for their sensitivity on a particular instrument.
4. Source position. Optimize the physical position of the source in relation to the orifice. This is essential when analytes are of low abundance.
5. Ionization conditions. Optimize all ionization conditions including gas flows, various voltages, and collision/fragmentation conditions.
6. Solvent composition. Optimize solvent composition (including mobile phase solvents and modifiers) which can affect sensitivity.

⁵⁰ESI is more susceptible to ion suppression effects than APCI and is less desirable for quantification.

⁵¹Always start with a more dilute solution (rather than a concentrated solution) when beginning tuning.

⁵²Some retinoids lose part of the parent molecule during ionization resulting in a molecular ion m/z of (parent-lost group). For example, retinol $[MH - H_2O]^+$ and retinyl acetate $[MH - AcOH]^+$ have been reported (60).

7. Product ion. Examine product ions produced by collisionally activated precursor ion fragmentations with N₂ for intensity and background levels.

3.19. HPLC/UV

UV detection after HPLC separation of retinoids offers analysis specificity because very few compounds absorb at wavelengths characteristic of retinoids. The intrinsic absorption of most compounds in the sample milieu is significantly more blue (maxima at shorter wavelength) than that for retinoids. Additionally, UV detection of retinoids has specificity through structure-dependent absorbance maxima (*see* Table 1.2). Benefits of UV detection also include simplicity and cost-effectiveness (compared to MS-based detection methods). Whereas single wavelength and diode array detection (DAD) are effective for *in vitro* assay retinoid quantification and quantitation of abundant endogenous retinoids (retinal, retinol, RE) *in vivo*, they lack the necessary sensitivity for endogenous RA detection. Endogenous RA concentrations in the assay tissue amounts described here (*see* Table 1.3) are up to several orders of magnitude below the limit of detection and/or limit of quantification for both DAD and single-wavelength detection (50).

3.19.1. Typical UV Detection Conditions

1. Use separations described or other (*see* Sections 3.13, 3.14, 3.15, 3.16, and 3.17).
2. Acquire absorbance spectra for analytes of interest in the mobile phase solvent to determine appropriate detection wavelength (*see* Table 1.2).
3. Use either a single-wavelength UV detector set at/near the absorbance maxima or use a DAD to collect an appropriate wavelength range to cover the entire absorbance spectra (*see* Note 53).
4. Assess detector capabilities and assay performance prior to quantification; then quantify each analyte from a calibration curve generated from standard amounts of that compound (*see* Section 3.22).
5. Peak identity/instrument performance for each method should be verified daily by injecting a mixture of authentic retinoid standards (*see* Note 37).

3.19.2. Optimization Strategies for UV Detection

1. To maximize signal. Choose a wavelength close to the absorbance maximum in the mobile phase solvent to maximize signal.
2. To maximize specificity. Choose a wavelength (not necessarily the maximum) that does not overlap or minimally overlaps with other retinoid species.
3. DAD vs. single wavelength. Single wavelength is often more sensitive than DAD; however, because DAD acquires the entire absorbance spectra, compound identity can be confirmed by its spectral signature.

⁵³If multiple analytes are being detected using a single-wavelength detector, use a detection wavelength appropriate for all analytes.

3.20. GC/MS

For GC/MS detection, consult work by Napoli (45).

3.21. ECD

For detection by electrochemical detection consult work by Hagen et al., Sakhi et al., and Ulven et al. (70–72).

3.22. Essential Assay Characterization and Application

Several experiments must be performed to verify assay performance in order to obtain reliable, reproducible retinoid quantification data. Assay characterization includes determination of limits of detection (LOD), limits of quantification (LOQ), linear range, reproducibility, accuracy and precision, recovery, and handling-induced degradation.

3.22.1. LOD and LOQ—The LOD is defined by a signal/noise ratio of 3:1 and the LOQ is defined by a signal/noise ratio of 10:1. LOD and LOQ provide sensitivity measures. Determine for each analyte, with each method, and on each instrument.

1. Prepare a series of standard solutions on the day of use from a stock solution with a spectrophotometrically verified concentration (*see Section 3.2*).
2. Collect replicate data (at least triplicate) for each concentration.
3. Assess concentration of analyte that results in $S/N=3$ for LOD and $S/N=10$ for LOQ (*see Fig. 1.11*).

3.22.2. Linear Range—The concentration range of the standard solutions should span several orders of magnitude and encompass the amount of analyte that will be encountered in a physiological sample.

1. Prepare a series of standard solutions on the day of use with spectrophotometrically verified concentrations (*see Section 3.2*).
2. Collect replicate data (at least triplicate) for each concentration.
3. Plot average peak area as a function of concentration (*see Fig. 1.11*).
4. Exclude data that deviate from linearity at the high and low ends of the concentration range, if necessary. The remaining linear data define the working linear range.
5. Use linear regression to obtain the best fit line to the data and assess goodness of fit according to r^2 (*see Note 54*).
6. Use the slope of the line to determine unknown concentrations of analyte according to peak area.

3.22.3. Accuracy/Precision—Accuracy is the agreement between applied and measured amounts and should be assessed at different areas of the linear range (low, mid, and high

⁵⁴ r^2 of 1 is a perfect correlation between peak area and concentration. r^2 of 0.99 or greater is desirable.

concentration). Precision is measured by the instrumental coefficient of variance (CV) which is obtained by repeat measurements of standard samples on the same day (*see* Note 55).

1. Prepare a series of standard solutions on the day of use with spectrophotometrically verified concentrations similar in concentration to that which will be encountered in a physiological sample (*see* **Section 3.2**).
2. Collect replicate data (at least triplicate) for each concentration.
3. Assess agreement between applied (prepared concentration) and measured (via calibration curve) amounts for accuracy.
4. Assess instrumental CV from repeat injections of the same sample concentration (*see* Note 56).

3.22.4. Recovery/Handling During Preparation—Percentage recovery reflects extraction efficiency and handling losses. The ability of an internal standard to reflect the loss and extraction efficiency of an endogenous retinoid must be evaluated before use in a quantitative assay. An internal standard should have a structure similar to the analyte, similar chromatographic behavior, and similar extraction efficiency. For example, 4,4-dimethyl-RA has a structure similar to atRA (*see* Fig. 1.6), has similar chromatographic behavior (*see* Fig. 1.6), and has similar extraction efficiency. Internal standard should be used at a level similar to the level of analyte and evaluated for each matrix (*see* Note 57).

3.22.5. Evaluation of Internal Standard Performance

1. Obtain sample (tissue, serum, cells, etc.) in sufficient quantity to perform multiple measurements (*see* **Section 3.4**).
2. Evaluate 3–10 samples per matrix type; allocate several samples as for background/blank determination.
3. Add internal standard to homogenized samples (*see* **Section 3.6**).
4. Add a known amount of exogenous retinoid to the same homogenate samples (comparable to endogenous level of analyte).
5. Extract and resuspend samples (*see* **Sections 3.8** and **3.9**).
6. Prepare several “100%” standard samples with the same amount of internal standard added to homogenate in the appropriate resuspension volume. Also prepare “100%” samples for exogenous retinoid.

⁵⁵The coefficient of variation (CV), also known as “relative variability,” equals the standard deviation divided by the mean. It can be expressed either as a fraction or a percent.

⁵⁶% CV of 5–10% or less is desirable.

⁵⁷Internal standard performance can vary by analyte and biological matrix. For example, retinyl acetate reflected RE recovery accurately for all tissues investigated using the acid–base extraction in **Section 3.8**. For adipose and other lipid-rich tissue tissues, retinyl acetate also accurately reflected the recovery of retinol. However, retinyl acetate did not always accurately reflect retinol recovery from liver and, thus, was not used to adjust liver retinol values (51). Retinyl acetate used as an internal standard represents the recovery of RE and retinol in adipose with reasonable accuracy.

7. Analyze samples with desired separation and detection method (*see Sections 3.12–3.21*).
8. Calculate % of internal standard recovered and % of exogenous retinoid recovered. Amount of endogenous “background” should be accounted for in % exogenous retinoid recovery. Interferences from the biological matrix should also be evaluated in the “blank” samples (*see Note 58*).
9. Compare the % recovery of internal standard and the % recovery of retinoid of interest. Values need to be similar for use as an internal standard in quantitation.

3.22.6. Quantitation with an Internal Standard

1. Evaluate internal standard for ability to reflect endogenous retinoid of interest before use (*see Section 3.22.5*).
2. Add internal standard(s) to each homogenized sample and vortex well (5–10 s) (*see Section 3.6*).
3. Multiple internal standards can be used to reflect multiple analytes.
4. Extract and resuspend samples (*see Sections 3.8 and 3.9*).
5. Prepare several “100%” standard samples with the same amount of internal standard added to homogenate in the appropriate resuspension volume.
6. Analyze samples with desired separation and detection method (*see Sections 3.12–3.21*).
7. Calculate % of internal standard recovered in each sample.
8. Use % recovery value to obtain absolute amount of retinoid in sample.

3.22.7. Evaluation of Extraction Efficiency Without an Internal Standard—

Quantitative analysis using a very efficient extraction in the sample preparation can be effective without an internal standard. Evaluate extraction efficiency with physiological levels of added exogenous retinoid.

1. Obtain sample (tissue, serum, cells, etc.) in sufficient quantity to perform multiple measurements (*see Section 3.4*).
2. Evaluate 3–10 samples per matrix type; allocate several samples as for background/blank determination.
3. Add a known amount of exogenous retinoid to homogenized sample (comparable to endogenous level of analyte).
4. Extract and resuspend samples (*see Sections 3.8 and 3.9*).
5. Prepare several “100%” standard samples with the same amount of exogenous retinoid added to homogenate in the appropriate resuspension volume.

⁵⁸If significant interference is observed with either the analyte of interest or the internal standard, analysis conditions must be altered.

6. Analyze samples with desired separation and detection method (*see Sections 3.12–3.21*).
7. Calculate % of exogenous retinoid recovered. Amount of endogenous “background” should be accounted for in % exogenous retinoid recovery.
8. % recovery of retinoid of interest should consistently be >90–95% for extractions without an internal standard (*see Notes 59 and 60*).

3.22.8. Quantitation Without an Internal Standard

1. Evaluate extraction efficiency for retinoids of interest prior to sample analysis (*see Section 3.22.5*).
2. Extract and resuspend samples (*see Sections 3.8 and 3.9*).
3. Analyze samples with desired separation and detection method (*see Sections 3.12–3.21*).
4. Assume extraction losses to be negligible to obtain absolute amount of retinoid in sample (*see Notes 59 and 60*).

3.22.9. Evaluation of Handling-Induced Degradation by an Internal Standard—

An internal standard may also act as an indicator of handling-induced degradation, such as isomerization of RA (*see Note 61*).

1. Obtain sample (tissue, serum, cells, etc.) in sufficient quantity to perform multiple measurements (*see Section 3.4*).
2. Evaluate 3–10 samples per group; include a control group.
3. Add internal standard(s) to each homogenized sample and vortex well (5–10 s) (*see Section 3.6*).
4. Expose to mild and severe degradation stress (e.g., light, acid).
5. Extract and resuspend samples (*see Sections 3.8 and 3.9*).
6. Prepare several “100%” standard samples with the same amount of exogenous retinoid added to homogenate in the appropriate resuspension volume.

⁵⁹For extraction described in **Section 3.3.8.1**, exogenous retinol spiked into liver homogenate before extraction was recovered $94 \pm 4\%$ ($n = 3$) and extraction losses were assumed to be negligible (No IS was used to adjust this value.) (51).

⁶⁰For extraction described in **Section 3.3.8.4**, recovery of the retinal *O*-ethylloximes routinely exceeded 95% and extraction losses were assumed to be negligible (No IS was used.) (51).

⁶¹An example of an internal standard indicating analyte degradation is illustrated by 4,4-dimethyl-RA (*see Figs. 1.2 and 1.5*). In these panels the internal standard isomerization closely mirrors that of endogenous atRA under both mild and severe conditions. The ability of an RA internal standard to indicate handling-induced isomerization is a valuable characteristic, because it helps distinguish endogenous RA isomers from those formed during handling. Samples that have been handled properly have no isomers in the 4,4-dimethyl-RA chromatogram, indicating that observed isomers in the RA chromatogram are endogenous (*see Fig. 1.5a*). Samples with artifactual isomers also show isomers in the 4,4-dimethyl-RA chromatogram (*see Fig. 1.5b*). Note the difference in the decrease in atRA and increase in *cis*-RA isomers is concurrent with isomers occurring in the internal standard chromatogram. Samples with significant isomerization of the internal standard (>10–15%) should be discarded. Previous reports have concluded that the biological matrix can cause ~7% isomerization of atRA into *cis*-isomers (48, 49). *cis*-Isomers exceeding this proportion should be endogenous, provided that the internal standard shows no isomerization.

7. Analyze samples with desired separation and detection method (*see Sections 3.12–3.21*).
8. Evaluate degradation of internal standard compared to degradation of retinoid (*see Note 61, Figs. 1.2 and 1.5*).

3.22.10. Reproducibility—Reproducibility of the assay is evaluated in terms of the intra-assay (same day) and inter-assay (different day) CV (*see Note 55*). Samples should be prepared using all procedures of the assay to reflect assay variability, including sample preparation and chromatographic analysis.

3.22.11. Intra-assay CV

1. Obtain sample (tissue, serum, cells, etc.) in sufficient quantity to perform multiple measurements (*see Section 3.4*).
2. Prepare multiple samples on the same day. For example, 3–10 samples prepared separately from a single minced mouse or rat liver.
3. Homogenize, extract, and resuspend samples individually (*see Sections 3.5, 3.6, 3.7, 3.8, and 3.9*).
4. Collect data according to desired method on the same day (*see Sections 3.12–3.21*).
5. Quantify the amount of endogenous retinoid (*see Sections 3.22.2 and 3.22.4*).
6. Calculate % CV (*see Note 56*).

3.22.12. Inter-assay CV

1. Obtain sample (tissue, serum, cells, etc.) in sufficient quantity to perform multiple measurements (*see Section 3.4*).
2. Prepare multiple samples on several different days. For example, 3–10 samples each day on 3–5 days over the course of a week. Ideally samples will be identical, for example, prepared separately from a single minced mouse or rat liver.
3. Samples should be stored at -80°C until assay (*see Section 3.4*).
4. Homogenize, extract, and resuspend samples individually (*see Sections 3.5, 3.6, 3.7, 3.8, and 3.9*).
5. Collect data according to desired method (*see Sections 3.12–3.21*).
6. Quantify the amount of endogenous retinoid (*see Sections 3.22.2 and 3.22.4*).
7. Calculate % CV (*see Note 56*).

3.23. Identity Confirmation Strategies

The identity of an analyte should be confirmed by multiple methods. This is especially important when new compounds are being identified. Identity confirmation is also important for new and existing assays to confirm that there are not any additional interfering species co-eluting during chromatography or during detection that could interfere with accurate

identification and quantification. Interferences can be due to components of the biological matrix remaining in the prepared sample or other endogenous retinoids that are not sufficiently chromatographically resolved.

3.23.1. Mass Signature

1. Use MS to determine characteristic mass of analyte.
2. Preferably, for more specificity, use MS/MS to determine characteristic precursor to product ion mass transition.
3. If analyte is unknown species, MS/MS can assist in providing structural information

3.23.2. Co-elution with Authentic Standards

1. Compare the retention time of analytes in a sample with the retention time of authentic standards.
2. Data for pure standards and sample analytes should be collected under identical conditions (separation method, detection, etc.).

3.23.3. Addition of Authentic Standards During Sample Preparation—It is important to confirm that species have not been artifactually produced or converted into another analyte during sample preparation (e.g., by hydrolysis, isomerization). Stability of compounds during preparation can be evaluated by spiking in physiologically relevant levels of exogenous retinoid and comparing to a non-spiked control (*see* Notes 62 and ⁶³).

1. Spike-in before homogenization. Exogenous retinoid is spiked into homogenization solution (e.g., saline) (*see* **Section 3.2**) and then samples are homogenized, extracted, and resuspended similar to control. The desired result is an increase only in the peak of analyte postulated to be of the same identity as the spiked-in retinoid (and observed augmentation is consistent with the concentration of added exogenous retinoid) (*see* Note 64, Fig. 1.12).
2. Spike-in after homogenization but before extraction. Spike-in exogenous retinoid (as in step 1) but after homogenization to tissue.
3. Spike-in after extraction to resuspended sample. Spike-in exogenous retinoid (as in step 1) but after extracted sample is resuspended. Here, sample can be injected and analyzed followed by the addition of exogenous retinoid, re-injection, and analysis.
4. Evaluate all three spike-in scenarios (steps 1, 2, and 3).

⁶²It is important to not overwhelm endogenous levels or saturate analysis conditions to the point that augmentation of the retinoid of interest or changes to other retinoids cannot be observed.

⁶³Stability of analyte(s) during preparation should be tested for each analyte and each matrix type as various tissues have different potential for analyte hydrolysis, isomerization, etc.

⁶⁴Control and spike-in samples should be sufficiently similar to distinguish that changes in peak area are from exogenously added retinoid and not sample variation.

3.23.4. Use of Chromatography of Varied Selectivity—Altering the selectivity of the chromatographic separation should be done to confirm that no additional species are co-eluting with the analyte of interest.

1. Reverse phase, normal phase. Switching from reverse phase to normal phase or vice versa will significantly change the separation selectivity and analyte retention characteristics. Similar results should be obtained with both separation types.
2. Stationary phase. Switching column stationary phase chemistry can be effective if the stationary phases have sufficiently different selectivity.
3. Mobile phase. Similar to step 2.
4. 2D chromatography. The analyte of interest is separated with one column and separated again on a chromatographic column of different selectivity. Can be performed in tandem or a fraction containing the analyte of interest can be collected from the first separation and then re-chromatographed on the alternate selectivity system.

3.24. Application

3.24.1. Tissue—The assays described here have been applied to various tissues. A summary of retinoid values is provided (*see* Table 1.6). Retinoid levels vary according to some or all of the following: strain, age, sex, diet, genotype, and/or exogenous treatment. Ideally each experimental comparison has a cohort of control animals and a cohort of experimental animals side by side.

3.24.2. Cell Systems—Retinoids and retinoid assays can be quantified from cell systems, including isolated cells, primary culture cell systems, and established cell lines. If endogenous retinoids are to be quantified, handling precautions must be observed during isolation and culture. For enzyme assays, precautions must be observed during assay (*see* Sections 3.1, 3.2, 3.3, and 3.4).

1. Cells should be switched to serum-free media before assay. Media should always also be measured for presence of retinoids (*see* Note 65).
2. Cells and/or media can be quantified for retinoid content either endogenous or as a result of treatment.
3. Typical amount of cells and/or media analyzed depends on situation (*see* Note 66).
4. If retinoid production is to be monitored, retinoid precursors should be added to cells/samples via a calibrated glass syringe via a concentrated solution (freshly prepared), so that addition volume does not exceed 1–2% of sample volume (usually ~5–10 μ l).
5. Retinol as substrate should be purified before use (*see* Section 3.3).

⁶⁵All serum-containing media contains retinol (and sometimes RE and RA) and many media formulations have supplements that include retinoids.

⁶⁶0.5–1 ml media and/or a confluent amount of cells in a 6-or 12-well plate has been successfully used in a variety of experiments in the Napoli Lab.

3.25. Bio-analysis Limitations and Potential Pitfalls

Whereas some assays offer benefits over others, no method is devoid of limitations. Listed are some general potential pitfalls when assaying biological samples.

1. LC/MS/MS. LC/MS/MS can have interfering background contributions from the biomatrix (*see* Note 67). Contribution of biomatrix interference to overall retinoid signal should be assessed and chromatography and/or MS/MS conditions adjusted to eliminate or minimize background contributions (*see* **Sections 3.17** and **3.18.3**).
2. LC/MS. LC/MS has less specificity and higher background than MS/MS.
3. LC/UV. UV detection is more effective with abundant retinoids (RE, retinol, retinal) than RA. Both single wavelength and DAD are less sensitive than MS/MS for RA detection and lack the positive mass identification provided by mass spectrometry. Chromatography and UV detection can also be optimized to eliminate or minimize background contributions and co-elution (*see* **Sections 3.17** and **3.18.3**).
4. LC/ECD. Retinoid quantification with ECD is potentially susceptible to interference from other species in the biomatrix and also lacks positive mass identification.
5. GC/MS. GC/MS presents challenges for isomer separation and derivatization is often necessary.
6. Isomer separations. Insufficient chromatographic resolution of isomers can potentially skew quantification due to co-elution. This is especially problematic for RA detection (*see* **Section 1**).
7. ESI. ESI (electrospray ionization) is susceptible to matrix suppression effects that hinder reproducibility needed for accurate quantification. Retinoid signal can be suppressed to varying degrees by components of the biomatrix during ionization. These matrix suppression effects can fluctuate according to matrix components (sample type, preparation method, etc.) and also across a chromatographic gradient.

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⁶⁷Some reports show significant background contributions that could be problematic during detection of in vivo levels of RA in a biomatrix (79).

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Fig. 1.1.

Structures of analytes in the central pathway of retinoid metabolism. Typical in vivo levels of each analyte are listed. Ranges reflect variation among tissues and dietary conditions.

Fig. 1.2.

Similar isomerization rates of atRA (**a** and **b**) and 4,4-dimethyl-RA (**c** and **d**). (**a**) and (**c**) show standard solutions (~50 nM) exhibiting mild isomerization after 10 min of exposure to standard fluorescent room lights. (**b**) and (**d**) show standard solutions with severe isomerization after 30 min exposure to sunlight. Note the similar rate of production of isomers from the all-*trans* forms for both retinoids ($t_R \approx 16.2$ min for atRA; $t_R \approx 19.8$ for 4,4-dimethyl-RA) to *cis*-isomers ($t_R = 10\text{--}16$ min for RA and $12\text{--}18$ min for 4,4-dimethyl-RA). Adapted from Ref. (49).

Fig. 1.3.
Absorbance spectra of select retinoids in ethanol.

Fig. 1.4.
Conversion of retinal into retinal-(*O*-ethyl)oxime.

Fig. 1.5.

Application of internal standard with mouse kidney illustrating acid-induced isomerization. (a) Chromatogram for atRA with correct sample handling. *Inset:* 4,4-dimethyl-RA (internal standard) shows only all-*trans* form. (b) Chromatogram for atRA illustrating handling-induced isomerization by acid contamination. *Inset:* *cis*-isomers (*arrows*) of 4,4-dimethyl-RA. Note the decrease in atRA and increase in *cis*-isomers concurrent with *cis*-isomers in the internal standard. Peak identities: (1) 4,4-dimethyl-RA, (2) atRA, (3) 9cRA, and (4) 13cRA. Peaks 5 and 6 most likely indicate 9,13-di-*cis*-RA and 11-*cis*-RA, respectively. Adapted from Ref. (49).

Fig. 1.6. RA isomer separations and structures. **(a, b)** SRM chromatograms of standard solutions: **(a)** gradient 1, cultured cell/subcellular fraction protocol; **(b)** gradient 2, tissue protocol. The *solid line* corresponds to m/z Q1:301/Q3:205 for RA, and the *broken line* corresponds to m/z Q1:329/Q3:151 for 4,4-dimethyl-RA. 9,13-dcRA elutes between 9cRA and 13cRA (data not shown). **(c)** HPLC/UV chromatograms of standard solutions monitored at 340 nm. **(d)** Structures of atRA, its isomers, and the internal standard 4,4-dimethyl-RA. Reprinted with permission from Ref. (50). Copyright © 2008, American Chemical Society; see **Section 3.13**.

Fig. 1.7.

Retinal separation. HPLC/UV chromatograms of standard solutions using the retinal oxime method showing the *anti*-retinal-(*O*-ethyl)oxime (6.6 min), retinol (7.2 min), and the *syn*-retinal-(*O*-ethyl)oxime (10.9 min). *Solid line*, left axis; *broken line*, right axis. Reprinted from Ref. (51). Copyright © 2008, with permission from Elsevier; see **Section 3.14**.

Fig. 1.8.

Retinol and retinol isomer separations. HPLC/UV chromatograms of standard solutions using (a) the total retinol/total RE method and (b) the retinol isomer method. The RE standard shown is retinyl palmitate and REA is retinyl acetate (internal standard). Reprinted from Ref. (51). Copyright © 2008, with permission from Elsevier, *see* **Section 3.15**.

Fig. 1.9.

Comparison of methanol-based and acetonitrile-based mobile phases for total retinol and RE separation. **(a)** Methanol based and **(b)** acetonitrile based. *Panels a and b* show identical mouse kidney samples separated with the same gradient of methanol or acetonitrile/water/1,2-dichloroethane. Reprinted from Ref. (51). Copyright © 2008, with permission from Elsevier; see **Section 3.15**.

Fig. 1.10.

Background reduction by MS/MS detection. (a, b) RA mass spectra showing (a) Q1 scan with $[M + H]^+$ (m/z : 301.1); (b) Q3 scan with $[M + H]^+$ and product ions obtained after fragmentation. Both scans were obtained by infusing 200 nM RA at 10 μ l/min. Note the reduction in background in (b) compared to (a). (c, d) Comparison of UV detection and MS/MS detection showing (c) UV detection at 350 nm (using a separation similar to alternate normal-phase separation) and (d) MS/MS detection using m/z 301.1 205.0 transition (and separation similar to tissue separation (gradient 2)). Note the reduction in background in (d) compared to (c). Also note the 50-fold lower tissue requirement for MS/MS detection in (d). Reprinted in part with permission from Ref. (50). Copyright © 2008, American Chemical Society; see **Section 3.13**.

Fig. 1.11.

Assay characterization. (a) LOD and LOQ for atRA using gradient 2, tissue protocol (*see Section 3.22.1*). LOD is 0.75 fmol and LOQ is 0.125 fmol. (b–d) Representative calibration curve for atRA obtained using the cultured cell protocol (r^2 , 0.999) (*see Section 3.22.2*). (b) Full linear range. (b, inset) Zoom in on data less than 50 fmol to show the functionality and linearity of the assay with low fmol. Reprinted in part with permission from Ref. (50). Copyright © 2008, American Chemical Society *see Sections 3.13 and 3.22*.

Fig. 1.12.

Identity verification by spike in of exogenous retinoids. (a–d) Addition of exogenous retinoids to mouse liver before homogenization to show that 9c-retinol is not formed artifactually during sample preparation. The *left panel* of each pair is a magnified view of the *right panel* to show 9c-retinol more clearly. (a) The addition of 9c-retinol increases 9c-retinol only. (b) The addition of at-retinol increases at-retinol only. (c, d) The addition of either 9c-retinol or at-retinol does not increase either 9c-retinol or at-retinol. Reprinted from Ref. (51). Copyright © 2008, with permission from Elsevier.

Table 1.1

Comparison of atRA limits of detection (LOD) for validated assays

References	atRA LOD ^a	Detection	Assay application/demonstrated matrices
Napoli (45)	120 fmol	GC/MS	Serum, plasma, cells
Wang et al. (60)	(~211 fmol) ^b	LC/MS	Prostate
Schmidt et al. (48)	186 fmol	LC/UV	Tissues, serum
Sakhi et al. (71)	26.6 fmol	LC/ECD	Tissues (embryo)
Ruhl (79)	23.3 fmol	LC/MS/MS	Serum, cell pellets (no tissues)
Kane et al. 2005 (49)	10 fmol	LC/MS/MS	Tissues, serum, cells
Gundersen et al. (61)	6.6 fmol	LC/MS/MS	Plasma only
Kane et al. 2008 (50)	0.5 fmol	LC/MS/MS	Tissues, serum, cells

^a atRA LOD expressed as mol on column and defined as $S/N=3$

^b Estimated based on a listed LOQ of 702 fmol (30)

Table 1.2

Optical properties of select retinoids^{a,b,d}

Compound	Solvent	λ_{max}	ϵ	$E_{1\text{ cm}}^{1\%}$	References
All- <i>trans</i> -retinol	Ethanol	325	[52,770]	1845	(84)
	Hexane	325	[51,770]	1810	(84)
9- <i>cis</i> -retinol	Ethanol	323	42,300	[1477]	(85)
9,13-di- <i>cis</i> -retinol	Ethanol	324	39,500	[1379]	(85)
13- <i>cis</i> -retinol	Ethanol	328	48,305	1689	(85, 86)
11- <i>cis</i> -retinol	Ethanol	319	[34,890]	1220	(84)
	Hexane	318	[34,320]	1200	(84)
All- <i>trans</i> -retinyl acetate	Ethanol	325	51,180	1560	(87)
	Hexane	325	[52,150]	1590	(88)
All- <i>trans</i> -retinyl palmitate ^c	Ethanol	325	[49,260]	940	(88)
All- <i>trans</i> -retinal	Ethanol	383	[42,880]	1510	(84)
	Hexane	368	[48,000]	1690	(84)
9- <i>cis</i> -retinal	Ethanol	373	36,100	[1270]	(86)
9,13-di- <i>cis</i> -retinal	Ethanol	368	[32,380]	1140	(89)
13- <i>cis</i> -retinal	Ethanol	375	[35,500]	1250	(84)
	Hexane	363	[38,770]	1365	(84)
11- <i>cis</i> -retinal	Ethanol	380	[24,935]	878	(84)
	Hexane	365	[26,360]	928	(84)
All- <i>trans</i> -retinoic acid	Ethanol	350	45,300	[1510]	(85)
9- <i>cis</i> -retinoic acid	Ethanol	345	36,900	[1230]	(85)
9,13-di- <i>cis</i> -retinoic acid	Ethanol	346	34,500	[1150]	(85)
13- <i>cis</i> -retinoic acid	Ethanol	354	39,750	[1325]	(85)
11- <i>cis</i> -retinoic acid	Ethanol	342	[27,780]	926	(89)
4-oxo-retinoic acid	Ethanol	360	[58,220]	1854	(90)
	Hexane	350	[54,010]	1720	(90)

Compound	Solvent	λ_{max}	ϵ	$E_{1\text{ cm}}^{1\%}$	References
4-oxo-13- <i>cis</i> -retinoic acid	Ethanol	361	[39,000]	1242	(91)

λ_{max} , maximum wavelength (nm)

ϵ , molar absorptivity ($\text{M}^{-1}\text{ cm}^{-1}$)

E , mass attenuation coefficient ($\text{ml g}^{-1}\text{ cm}^{-1}$)

^a Adapted from Furr (83)

^b Values in brackets are calculated from corresponding ϵ or $E_{1\text{ cm}}^{1\%}$ values

^c Medium- and long-chain fatty-acyl esters of retinol have identical molar extinction coefficients (ϵ) (92)

^d ϵ and $E_{1\text{ cm}}^{1\%}$ values are given for the maximum wavelength

Table 1.3Typical tissue harvest amounts from adult mouse^{**}

Tissue	Tissue harvest amount	Volume of saline for homogenization	Extract amount for RA/retinol/RE	Extract amount for retinal
Blood (isolated serum)	200–600 µl (100–300 µl)	N/A None	N/A 100–300 µl	N/A 100–300 µl
Liver	0.2 g	2 ml	0.5–0.75 ml	0.5–1 ml
Kidney	1–2 kidney	1–2 ml	0.75–1 ml	0.5–1 ml
Testis	1–2 testis	1–2 ml	1 ml	0.25–1 ml
White adipose	0.2 g	1–1.5 ml	0.75–1 ml	0.75–1 ml
Brown adipose	0.1–0.2 g	1 ml	1 ml	–
Muscle	0.1–0.2 g	1 ml	1 ml	–
Skin	0.3–0.4 g	1–1.5 ml	1–1.5 ml	–
Spleen	Whole spleen	1 ml	1 ml	–
Pancreas	Whole pancreas	1 ml	0.3–1 ml	0.3–1.0 ml
Mammary gland	0.1–0.2 g	1–1.5 ml	1 ml	–
Retinae	Both retinae, pool ~4 retinae	1 ml	1 ml	–
Brain (whole)	Entire brain	2 ml	0.5–0.75 ml	–
Hippocampus	2 hippocampi	1 ml	1 ml	–
Cortex	0.1–0.2 g	1–1.5 ml	0.75–1 ml	–
Cerebellum	Whole region	1 ml	1 ml	–
Striatum	Whole region	1 ml	1 ml	–
Thalamus	Whole region	1 ml	1 ml	–
Olfactory bulb	Whole region	1 ml	1 ml	–
Hypothalamus	Whole region, pool 2–3 mice	1 ml	1 ml	–

–, not determined

N/A, not applicable

^{**} Amounts listed are guidelines. Extract conditions should be optimized for each tissue. See **Section 3.11** for optimization strategies

Table 1.4

Typical resuspension and injection volumes**

Tissue type	RA resuspend (μl)	RA inject (μl)	Retinol RE resuspend (μl)	Retinol RE inject (μl)	Retinal resuspend (μl)	Retinal inject (μl)
Serum/plasma	60	20-30	120	100	120	100
Liver ^a	60	20-30	500	100 and 10-20 ^d	120	100
Adipose and high lipid content tissues ^b	60	20-30	200	100	150-200	100
All other tissues	60	20-30	120	100	120	100
Small samples ^c	40-60	20-30	110-120	100	110-120	100

^a See Note 32
^b See Note 33
^c See Note 35
^d See Note 41

** Volumes listed are guidelines. Extract conditions should be optimized for each tissue

Table 1.5

Typical resuspension solvents**

Separation method	Sections	Resuspension solvent
RA isomers (gradient 1)	3.13.1	Acetonitrile
RA isomers (gradient 2)	3.13.2	Acetonitrile
RA isomers (normal phase)	3.13.3	Hexane with 0.4% isopropyl alcohol
Total retinal	3.14.1	Acetonitrile
Retinal isomers	3.14.2	Retinal isomer mobile phase (11.2% ethyl acetate, 2% dioxane, 1.4% 1-octanol, 85.4% hexane)
Total retinol and total RE	3.15.1	Acetonitrile
Retinol isomers	3.15.2	Hexane with 0.4% isopropyl alcohol
Polar metabolites	3.16.1, 3.16.2	Acetonitrile or acetonitrile/water mixture up to 50% water

**
Solvents listed are guidelines. Extract conditions should be optimized for each tissue

Table 1.6Select retinoid levels in adult mouse^a

Tissue	atRA (pmol/g)	Total retinal (pmol/g)	Total retinol (nmol/g)	Total RE (nmol/g)
Serum ^b	2.7 ± 0.3 (21)	32.2 ± 6.2 (6)	0.81 ± 0.04 (70)	0.22 ± 0.02 (69)
Liver ^b	38.1 ± 3.4 (18)	160.9 ± 14.3 (26)	9.6 ± 0.9 (60)	562.6 ± 75.9 (55)
Kidney ^b	15.2 ± 2.2 (30)	187.3 ± 31.2 (12)	0.60 ± 0.04 (37)	1.8 ± 0.2 (37)
Adipose ^b (epididymal)	14.2 ± 2.4 (18)	63.5 ± 5.2 (12)	0.63 ± 0.03 (37)	0.59 ± 0.09 (29)
Muscle ^b	1.5 ± 0.2 (15)	–	0.15 ± 0.02 (38)	0.25 ± 0.03 (31)
Spleen ^b	7.3 ± 0.6 (14)	–	0.60 ± 0.06 (26)	1.2 ± 0.1 (26)
Testis ^b	8.9 ± 1.0 (14)	90.7 ± 10.1 (12)	0.08 ± 0.01 (27)	0.31 ± 0.02 (27)
Brain ^b	17.1 ± 3.7 (19)	–	0.32 ± 0.01 (5)	0.22 ± 0.02 (5)
Brain ^c	33.9 ± 3.9 (8)	–	0.68 ± 0.23 (19)	0.84 ± 0.16 (19)
Hippocampus ^c	45.3 ± 5.2 (8)	–	0.30 ± 0.03 (27)	0.70 ± 0.05 (27)
Cortex ^c	16.0 ± 1.3 (7)	–	0.08 ± 0.01 (18)	0.35 ± 0.04 (18)
Olfactory bulb ^c	76.5 ± 21.3 (4)	–	0.20 ± 0.02 (4)	0.63 ± 0.03 (4)
Thalamus ^c	80.9 ± 6.0 (4)	–	0.20 ± 0.04 (4)	0.47 ± 0.06 (4)
Cerebellum ^c	54.8 ± 3.6 (8)	–	0.42 ± 0.08 (8)	0.73 ± 0.10 (8)
Striatum ^c	78.0 ± 33.2 (3)	–	0.21 ± 0.05 (4)	0.41 ± 0.08 (4)

^a Only a partial list of recovered in vivo retinoid levels. For a full description see Kane et al. (49–51)^b Data were obtained from 2- to 4-month-old male SV129 mice fed and bred from dams fed an AIN93G diet with 4 IU vitamin A/g^c Data were obtained from 4-month-old male C57BL/6 mice fed an AIN93M with 4 IU vitamin A/g from weaning and bred from dams fed a stock diet (>30 IU vitamin A/g). Values are means ± SEM (*n*). Serum values are either pmol/ml or nmol/ml.

–, not measured