Chapter 5

Retinyl Ester Analysis by Orbitrap Mass Spectrometry

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Abstract

Retinoids are light-sensitive molecules that are normally detected by UV absorption techniques. Here we describe the identification and quantification of retinyl ester species by high-resolution mass spectrometry. Retinyl esters are extracted by the method of Bligh and Dyer and subsequently separated by HPLC in runs of 40 min. The retinyl esters are identified and quantified by mass spectrometry analysis. This procedure enables the highly sensitive detection and characterization of retinyl esters in biological samples such as hepatic stellate cells.

Key words Retinyl esters, Retinol, Vitamin A, Mass spectrometry, Orbitrap, Atmospheric pressure chemical ionization, High-performance liquid chromatography

1 Introduction

Vitamin A or retinol is an essential nutrient that must be acquired from the diet [1, 2]. Upon uptake of carotenoids like carotenes and β-cryptoxanthin, they can be converted to retinol via retinaldehyde [3]. Retinol and its derivatives are collectively called retinoids, with multiple isoforms of retinaldehyde and retinoic acid as the main biological active molecules. Retinoids are important for stem cell functions, reproduction, cell differentiation during embryogenesis and normal growth, cell metabolism in many different cell types, vision, and the immune system and play a role in the development of metabolic syndrome [4, 5].

Given the important roles of retinoids, it may not come as a surprise that unlike most other vitamins, their precursor molecule retinol can be stored within the body in relatively high levels to protect against the adverse effects of temporary insufficient dietary intake of vitamin A [6]. Retinol that is present in the body is stored as retinyl esters in specialized lipid droplets. The main storage places in the body are the liver and the lung, and low amounts of retinyl esters are also found in other tissues such as the eye, kidney, pancreas, adipose tissue, muscle, and brain [7–9].

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Two different enzymatic activities have been implicated in the esterification of retinol to retinyl esters. The enzyme responsible for most of the retinol esterification is lecithin/retinol acyltransferase (LRAT), which transfers the acyl-chain from the *sn*-1 position of phosphatidylcholine to retinol via a trans-esterification reaction [10]. The other enzymatic activity is an acyl-coenzyme A (Acyl-CoA)/retinol acyltransferase (ARAT) reaction. Acyl-CoA/diacylglycerol acyltransferase 1 (DGAT1) has been shown to contain ARAT activity as well [11].

As a result of these different reaction mechanisms, the retinyl ester species that are synthesized by these enzymes are different. In the liver, LRAT is the main enzyme responsible for storage of retinyl esters in a subset of liver cells named hepatic stellate cells (HSCs) [8, 9, 12]. Retinyl palmitate is the main retinyl ester synthesized in the liver and in HSCs, with lower amounts of retinyl oleate and retinyl stearate also present [9, 13]. In the absence of LRAT, the total retinyl ester content decreases, and retinyl palmitate is no longer the main retinyl ester. Other retinyl ester species such as retinyl oleate are relatively more abundant in the absence of LRAT [9, 13]. Thus, the retinyl species composition is indicative of the enzymatic activity involved in the storage of retinyl esters in lipid droplets.

For the analysis of retinyl ester species in cells and tissues, several methods have been developed. These methods are complicated by the light-sensitive nature of retinoids [14], so appropriate measures must be taken to avoid light exposure. Most methods are based on reverse-phase HPLC, due to the hydrophobic nature of retinyl esters [15–17]. The introduction of mass spectrometry allowed refinement of these methods by combining HPLC with mass spectrometry (LC-MS), facilitating the identification of specific retinyl ester species and improving the sensitivity. Further refinement was achieved by high-resolution mass spectrometry, allowing the specific monitoring of multiple predefined retinyl ester species [13].

Here we describe a highly sensitive LC-MS method that has been optimized to analyze the retinyl palmitate and retinyl oleate species. The ratio of these two retinyl ester species identifies the enzymatic activity involved in their synthesis, providing insight in the molecular mechanism of retinyl ester synthesis and storage. This method can easily be expanded to include other retinyl ester species as well.

2 Materials

2.1 Laboratory Equipment

1. Workspace with yellow/red light (*see* **Notes 1** and **2**).
2. Chemical fume hood.

3. Vortex mixer.
4. Centrifuge with relative centrifugal force of 2000 RCF.
5. Nitrogen evaporator unit.
6. (U)HPLC system fitted with column oven and cooled autosampler (e.g., Vanquish, Thermo Fisher Scientific).
7. High-end Orbitrap mass spectrometer interfaced with APCI (e.g., Q-Exactive HF (Thermo Fisher Scientific).
8. HALO 90 Å C8 Column 2.7 μm, 3.0 × 150 mm (Advanced Technologies).
9. Kinetex SecurityGuard Ultra C8, 3.0 mm with holder.
10. Gas: high-purity N_2 (for drying samples and atmospheric pressure ionization).

2.2 Basic Consumables and Chemicals

1. Borosilicate amber glass tubes (16 × 150 mm, 15 mL) with Teflon-lined caps (*see* **Notes 3** and **4**).
2. Borosilicate glass Pasteur pipettes (long).
3. Amber autosampler vials and caps with Teflon-liner.
4. Pipette tips.
5. Vial racks.
6. Vial trays.
7. Milli-Q-grade deionized water (18 MΩ) or bottled HPLC-grade water.
8. Formic acid (100%) (LC-MS Grade) (*see* **Note 5**).
9. Chloroform (LC-MS Grade).
10. Methanol (LC-MS Grade).
11. Ethanol (LC-MS Grade).
12. Acetonitrile (LC-MS Grade).
13. Acetone (LC-MS Grade).
14. Butylated-hydroxytoluene.

3 Methods

To avoid photodecomposition [14], the extraction of retinoids from serum, cells, and tissues must be carried out rapidly "in the dark" (*see* **Note 1**). Laboratory windows should be covered with appropriate materials such as aluminum foil. A room with no windows is the ideal setting to perform this procedure. Artificial lighting should be provided by preferably a red or alternatively yellow light bulb. During the extraction procedures, butylated-hydroxytoluene is added to avoid free radical-mediated oxidation

and photoisomerization. After extraction, retinoids must be stored in amber glass tubes.

In the liver, retinyl esters are stored in hepatic stellate cells. Procedures for the isolation of primary hepatic stellate cells from mouse liver have been described elsewhere [18]. In short, after liver perfusion, primary hepatic stellate cells were isolated from livers of mice by pronase/collagenase digestion followed by Nycodenz density gradient centrifugation. Due to the abundant presence of retinyl ester-containing lipid droplets, primary hepatic stellate cells have a lower buoyant density than other liver cells and float on the Nycodenz layer. The isolated hepatic stellate cells were cultured for 1 day on plastic culture dishes as described [19], before retinyl ester analysis was performed.

3.1 Preparation of Solutions (see Note 6)

1. Chloroform/methanol (1:2, v/v): In a clean 0.5-liter borosilicate bottle with Teflon-lined cap, add 100 mL chloroform to 200 mL methanol, and mix.
2. Methanol/acetonitrile/chloroform/water (46:20:17:17, v/v/v/v): In a clean 0.5-liter borosilicate bottle with Teflon-lined cap, add 230 mL methanol to 100 mL acetonitrile, 85 mL chloroform, and 85 mL Milli-Q water, and mix.
3. Solvent A (95% acetonitrile, 5% Milli-Q water, 0.1% formic Acid): To 950 mL of acetonitrile, add 50 mL Milli-Q water and 1 mL formic acid (100%). De-gas by sonication in a sonifier bath for 5 min.
4. Solvent B (85% acetone, 15% chloroform, 0.1% formic acid): To 850 mL of acetone, add 150 mL chloroform and 1 mL formic acid (100%). De-gas by sonication in a sonifier bath for 5 min.

3.2 Preparation of Standards

Prepare stock solutions of 1 mg/mL by dissolving 1 mg of the desired retinyl ester species in 1 mL of ethanol or chloroform.

3.3 Sample Extraction

Hepatic stellate cell samples are extracted "in the dark" using red light by Bligh and Dyer extraction [20].

1. Defrost the samples on ice (*see* **Note 7**).
2. Transfer 800 µL cell suspension in phosphate-buffered saline (PBS) to an amber pointed glass tube with Teflon-lined cap.
3. Add internal standards and butylated hydroxytoluene (*see* **Note 8** and **9**).
4. Add 3 mL of chloroform/methanol (1:2) (v/v) (*see* **Notes 8** and **9**).
5. Shake or vortex vigorously for 30 s.

6. Incubate for 20 min on room temperature in a rotational shaker (*see* **Note 10**).
7. Centrifuge for 5 min at 2000 RCF.
8. Transfer supernatant to clean amber pointed glass tube with Teflon lined cap (*see* **Note 11**).
9. Add 2 mL Milli-Q water to the supernatant.
10. Add 2 mL $CHCl_3$ to the supernatant.
11. Shake or vortex vigorously for 30 s.
12. Centrifuge for 5 min at 2000 RCF.
13. Transfer lower (organic) phase to a clean amber pointed glass tube with Teflon-lined cap (*see* **Note 12**).
14. To the remaining upper (water) phase, add 2 mL $CHCl_3$.
15. Shake or vortex vigorously for 30 s.
16. Centrifuge for 5 min at 2000 RCF.
17. Combine the lower (organic) phase with the previous lower phase.
18. Discard the remaining upper (water) phase.
19. Evaporate combined organic phase under a stream of nitrogen gas (*see* **Note 13**).

3.4 LC-MS Analysis

1. Set up the LC-MS system with APCI source, cooled autosampler, column oven, and reversed phase C8 column fitted with Ultra Guard column (*see* **Note 14**).
2. Set column oven temperature to 30 °C.
3. Purge LC solvent lines A and B for 5 min with solvent A and B, respectively.
4. Equilibrate the system for 10 min at 10% solvent B at a flow of 300 μL/min.
5. Set up the following method in your LCMS software: Flow of the LC, 300 μL/min; injection =10 μL sample on column.
6. The following gradient is applied with a total run time of 40 min:

Time (min)	Solvent A (%)	Solvent B (%)
0	90	10
15	90	10
35	0	100
35.1	90	10
40	90	10

7. Operate the mass spectrometer in atmospheric pressure chemical ionization (APCI) mode with spray voltage set to 3000 V (*see* **Notes 15** and **16**).
8. Capillary temperature is set to 325 °C; Probe Heater Temperature is set to 350 °C. (*see* **Note 17**).
9. Auxiliary gas flow rate is set to 20 AU and sheath gas flow rate to 60 AU.
10. Set the MS mode to full scan mode starting from 250 to 650 m/z with a scan resolution of 120 K.
11. Prepare samples by dissolving in methanol/acetonitrile/chloroform/water (46:20:17:17, v/v/v/v), and transfer to amber autosampler vials (*see* **Notes 18** and **19**).
12. Start the run with a blank (methanol/acetonitrile/chloroform/water (46:20:17:17, v/v/v/v) to equilibrate the system, followed by the samples in randomized order. End with a blank.
13. Shut down system according to factory manual.

3.5 Data Analysis

1. Extract and analyze data with processing software, for example, TraceFinder (Thermo Fisher Scientific).
2. The m/z values of relevant retinyl ester species by MS analysis are shown in Table 1.
3. LC-MS analysis of retinoids with APCI source results in source fragmentation of retinoids, generating a characteristic retinol fragment [M-H2O]+ (m/z 269.2264). The extracted peak areas of these retinol fragments are used for quantification of the retinyl ester species (Figs. 1 and 2).
4. The different retinyl esters are separated by retention time and are identified based on the [M+H]+ ions (Fig. 1).
5. LC-MS analysis of hepatic stellate cells identifies the presence of retinyl palmitate, retinyl oleate, and retinyl stearate (Fig. 3).

Table 1
m/z values of specific retinoids

Analyte	Molecular formula	Exact mass	[M+H]+	[M-H_2O]+
Retinyl palmitate	$C_{36}H_{60}O_2$	524.45933	525.4666	507.4560
Retinyl oleate	$C_{38}H_{62}O_2$	550.47498	551.4823	533.4717
Retinyl stearate	$C_{38}H_{64}O_2$	552.49063	553.4979	535.4873
Retinol fragment	$C_{20}H_{30}O$	286.22967	287.2369	269.2264
Retinol acetate	$C_{22}H_{32}O_2$	328.24023	329.2475	311.2369

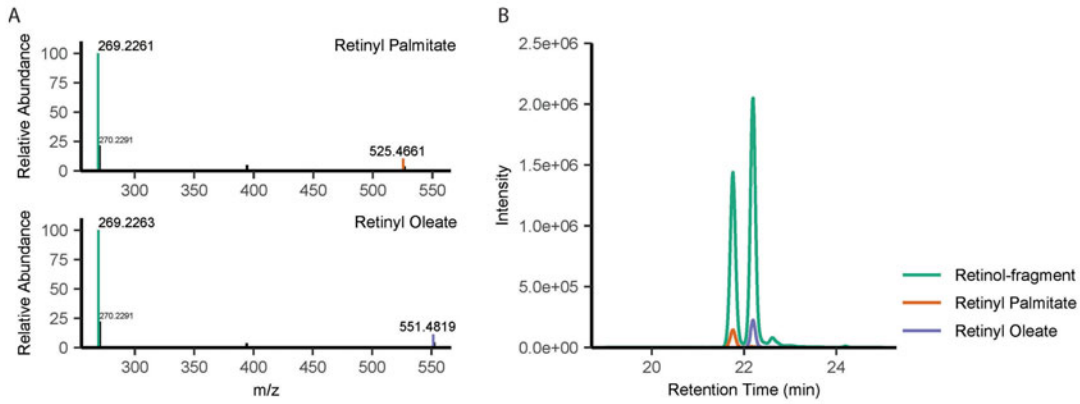

Fig. 1 Lipidomic spectra of retinyl ester standards. (**a**) Mass spectrum of retinyl palmitate (*top*) and retinyl oleate (*bottom*). (**b**) Extracted ion chromatograms of 269.2264, 525.4661, and 551.4823 (within 5 ppm), respectively (*see* also **Note 20**)

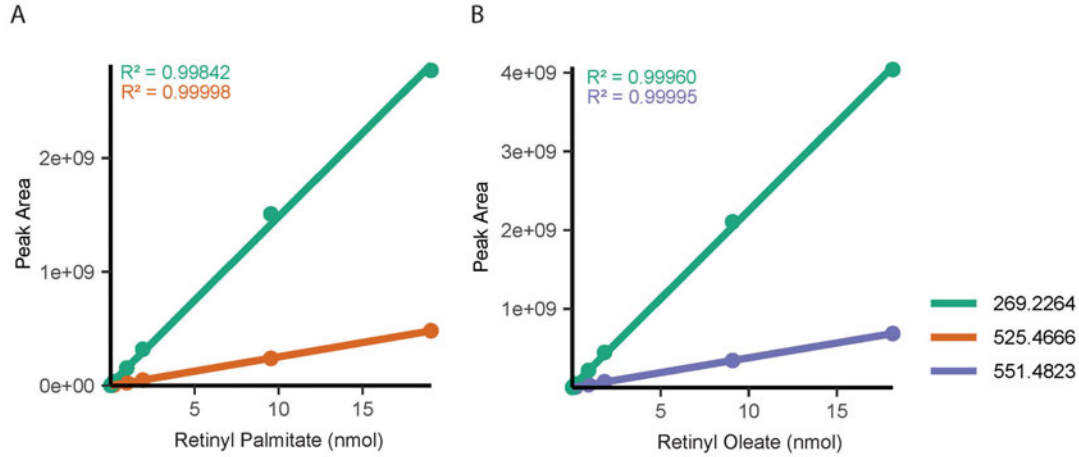

Fig. 2 Calibration curves of retinyl palmitate and retinyl oleate (for details, *see* **Notes 21** and **22**)

The extracted peak areas of the retinol fragments are directly proportional to the relative abundance of the individual retinyl ester species in biological samples (Fig. 2). For quantification, *see* **Notes 23–25**.

4 Notes

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

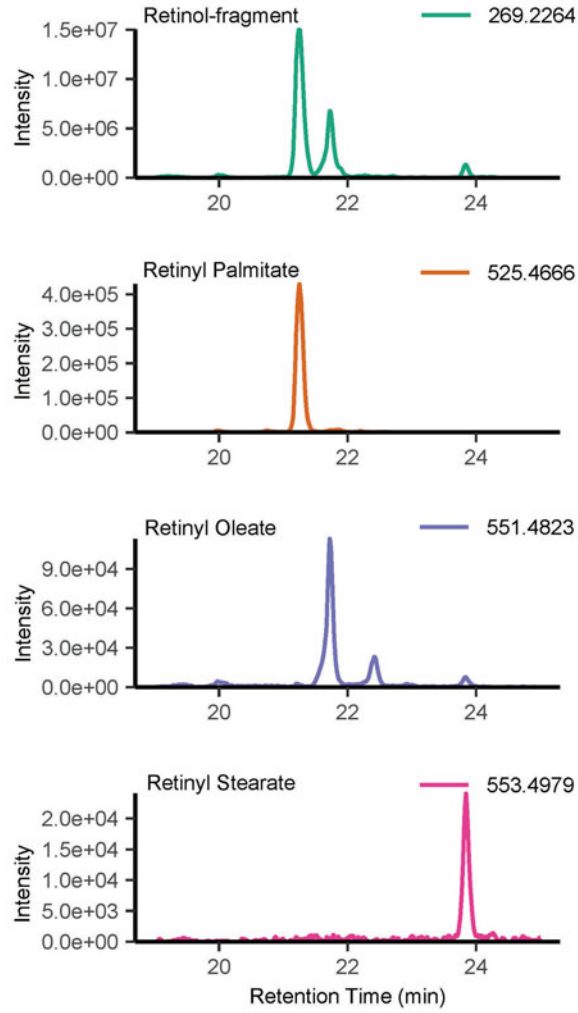

Fig. 3 LC-MS analysis of retinyl ester species in hepatic stellate cells. Example of extracted ion chromatograms of the retinol fragment (m/z 269.2264) in hepatic stellate cells (*top*) that were used for quantification of different retinyl ester species. Identification of retinyl ester species is based on the parent ions. Full scan monitoring allows monitoring of additional retinol species, i.e., retinol stearate (m/z 553.4979, *bottom*)

2. If 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.
3. The use of amber glassware minimizes exposure to full-spectrum light.
4. To prevent adhesion of retinoids to glassware or plastics, it is recommended to use silanized glassware.

5. Formic acid is caustic and irritant. Wear personal protective equipment (gloves and eye protection), and handle carefully.
6. Handling and preparation of organic solvents should be performed in a fume cabinet wearing chemical resistant gloves. Chloroform is a possible carcinogen in humans, so take preventive measures.
7. Frozen samples should be completely thawed (on ice) for efficient retinoid extraction.
8. Add 1 nmoL retinyl acetate and 5 nmoL of a non-biological cholesterol-d7 to monitor extraction efficiency and to determine photodegradation (*see* **Notes 23–25**). The amounts of these standards can be adjusted in subsequent experiments to be preferably in the same range as the amounts of retinyl ester and cholesterol expected in the samples.
9. Add 10 nmoL butylated hydroxytoluene to prevent oxidation and photoisomerization of retinyl esters to be quantified.
10. Make sure the caps tightly seal the tubes to prevent leakage of fluids.
11. Carefully decant supernatant into clean tube, or transfer with use of a Pasteur pipette.
12. Be careful not to disturb the protein interface between the organic (lower) phase and water (upper) phase. To transfer the lower phase more accurately, a Plastipak (3 mL) syringe adapted on a glass Pasteur pipette can be used. Apply a slight positive pressure when inserting the pipette through the upper phase into the lower phase. Replace the Pasteur pipette for every new sample to prevent cross contamination.
13. To speed up the process, you can also make use of a water bath (37 °C).
14. To prolong the lifetime of the analytical column, a guard column is advised.
15. (H)ESI (heated electro-spray ionization) is more susceptible to ion suppression effects than APCI and therefore less desirable for quantification.
16. Linear range of APCI (<10 pmoL–20 nmoL) is greater than (H)ESI (Fig. 4).
17. The listed source conditions are guidelines. Optimize conditions for your instrument/conditions. Tuning of instrumental conditions is essential to obtain sufficient sensitivity for the analysis of biological samples.
18. For better peak separation, methanol/acetonitrile/chloroform/Milli-Q (46:20:17:17) is used instead of the commonly used chloroform/methanol (1:1) to dissolve LC-MS standards/samples.

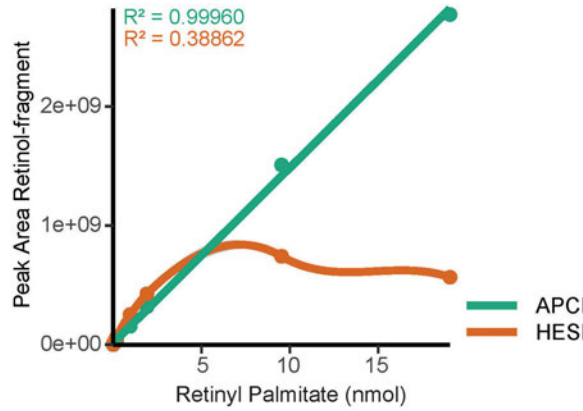

Fig. 4 Linear range of APCI vs HESI

19. For low volumes (<100 μL), use glass inserts in autosampler vials.
20. Due to in-source fragmentation, the fragment ion m/z 269.2264 is much more abundant than the precursor ions m/z 525.4661 (retinol palmitate) and m/z 551.4819 (retinol oleate). Therefore, the fragment ion m/z 269.2264 is more suitable for quantification, and the precursor ions m/z 525.4661 and m/z 551.4819 are used for identification (Fig. 1).
21. Confirm retention times and peak identity with authentic standards frequently.
22. Limitation of maximum amount is the solvation factor of retinoids in organic solvents.
23. For absolute quantitation, a standard curve is made with a stock solution containing retinyl palmitate or retinyl oleate solution (Fig. 2).
24. The extraction recovery can be determined by addition of the internal standard retinyl acetate to the samples prior to extraction. Retinyl acetate is a non-biological retinyl ester that also generates the characteristic retinol fragment $[M\text{-}H_2O]+$ (m/z 269.2264).
25. Potential problems with photodegradation of retinyl esters can be identified by addition of the internal standards retinyl acetate and cholesterol-d7 retinyl acetate to the samples prior to extraction. Retinyl acetate is sensitive to photodegradation, whereas cholesterol-d7 is not sensitive to photodegradation. The ratio of the retinyl acetate-derived retinol fragment $[M\text{-}H_2O]+$ (m/z 269.2264) and a characteristic cholesterol-d7 fragment (protonated cholestadiene) of *m/z* 376.3955 must remain constant in all samples.

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