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Profiling the triaclyglyceride contents in bat integumentary lipids by preparative thin layer chromatography and MALDI-TOF mass spectrometry

--Manuscript Draft--

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Abstract:	The mammalian integument includes sebaceous glands that secrete an oily material onto the skin surface. Sebum production is part of the innate immune system that is protective against pathogenic microbes. Abnormal sebum production and chemical composition is also a clinical symptom of specific skin diseases. Sebum contains a complex mixture of lipids, including triacylglycerides, that is species-specific. The broad chemical properties exhibited by diverse lipid classes hinder the specific determination of sebum composition. Analytical techniques for lipids typically require chemical derivatizations that are labor-intensive and increase sample preparation costs. This paper describes how to extract lipids from mammalian integument, separate broad lipid classes by thin-layer chromatography, and profile the triacylglyceride contents using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. This robust method enables a direct determination of the triacylglyceride profiles among species and individuals, and it can be readily applied to any taxonomic group of mammals.
Author Comments:	
Additional Information:	
Question	**Response**

May 20, 2013

Journal of Visualized Experiments
Editorial Office

Dear Michelle Kinahan:

Enclosed is a revised version of the manuscript "Profiling the triacylglyceride contents in bat integumentary lipids by preparative thin layer chromatography and MALDI-TOF mass spectrometry", which I co-authored with Thomas Risch and Brett Savary. The reviewers provided helpful comments that we used to strengthen the manuscript. All reviewers felt that this was a well written manuscript that thoroughly explained the techniques of TLC and MALDI-TOF MS in a fashion that novice readers could understand. There were only minor edits and formatting changes, which we were able to make. Listed below are responses to the reviewers comments and changes have been highlighted in the manuscript with the Track Changes under Review option in Microsoft Word.

Response to the reviewers:
Many of the revisions were minor and most were corrected accordingly. Referee 1 supplied a thorough review on MALDI procedures and data interpretation. Both Referees felt that it would be beneficial to include diseases in the introduction associated with altered sebaceous profiles. A list of diseases was included with a 2012 review paper cited. Some specific responses to Referee comments are included below.

EDITORIAL COMMENTS
The formatting issues have been addressed including the deletion of brand or company names.

REFEREE 1

4. Intro, p. 2: "free fatty acyls" should be changed to "free fatty acids"

In keeping with the terminology proposed by Fahy et al. 2005 and 2009, the usage of acyl was retained.

10. Protocol, 1 - It is unclear if the procedure is being performed on live animals or on dissected animal tissue. A clarification is needed, along with a few additional comments about handling live bats, if necessary (ie, how to hold them, are they anesthetized, etc.)

This was clarified in the procedures along with the IACUC approval and the citation for proper use of wild mammals in research, which was followed.

13. Protocol, 1, 1.3 - Is there a specific grade of filter paper required?

There is no particular grade that is required; however, we used qualitative P8 filter paper.

16. Protocol 3 - It might be helpful to make a new subheading called "Instrument calibration and optimization" or something like that, and have steps 3.1 to 3.5 under that subheading, since they are separate from preparing the samples for analysis.

While we feel that the Referee is correct in this suggestion, the protocol is at the length limit established by JoVE without incurring additional editorial processing.

18. Protocol 3, 3.11 and 3.12 - This could use a little more details...are peak lists exported from the instrument after smoothing and background subtraction? What are the typical settings for S/N or minimum intensity? Are there any restrictions on how the peaks are entered into the glycerolipids_batch tool? A screen shot of the tool would be helpful in the manuscript.

Peak lists are exported after smoothing and background subtraction, which is stated in 3.11. Restrictions on how peaks are processed in LIPID MAPS is dependent on the equipment operator, therefore operator proficiency is required for data acquisition and interpretation. The authors feel the level of detail given is sufficient for the manuscript.

17. Protocol 3, p. 6 - It is surprising that you are analyzing TG's in positive mode but you are adding NaOH...have you looked at any other sources of Na for complexation? Does the NaOH work best? If so, a comment to that effect should be added.

This topic has been addressed in detail in Gidden et al. 2007, which is cited in the manuscript. "Various concentrations (from 0.025 to 14 M) of NaOH, NH_3, $NaC_2H_3O_2$, and NH_4HCO_3 were prepared in water", added to samples of vegetable oil, and analyzed with a Bruker Ultraflex II. They concluded that the presence of base promoted Na^+ ion formation while suppressing H^+ ion formation. The H^+ ion is more unstable and prone to fragmentation, so the addition of the base promotes singly charged molecular ions that are more stable. A 1.0 M addition of NaOH was determined to give optimal results. A sentence was added to the results to explain the addition of NaOH.

20. Results, p. 6 - The discussion of the differences between prep and HP TLC plates is appreciated, but it needs clarification. It reads as though with prep plates there are 4 bands: sterols, FFA's, TAGs, and sterol esters/wax esters/squalene. But with HP plates there are only 3 bands, but it is stated that "the sterol esters, waxy esters and squalene will appear as only 3 separate bands" - are those 3 one band with prep plates and 3 bands with HP plates? Or are sterols, FFAs, TAGs, and sterol esters/wax esters/squalene 3 bands with HP plates, with sterols and waxy esters not separated? Please clarify.

We feel that the separation of broad lipid class was not clarified thoroughly and was confusing to read as the Referee commented. We added more specific terminology and explanation of broad lipid class separation should be less confusing.

23. Results - One problem with lipid analysis in positive mode is the spreading out of signal between protonated, sodiated, and potassiated species. Do you know how much protonated or potassiated signal you might be getting? Does the addition of NaOH allow only Na+ ions? A sentence or two addressing this topic is needed.

Please refer to the comment to number 17.

24. Discussion, p. 9 - add a source/vendor for Sebotape; also change "...Sebutape call also provide..." to "...Sebutape can also provide..."

Please refer to the editor's comment below. Sebutape was changed to "specialized tape products" and was included in the table of materials and reagents.

Editorial Comment: To comply with JoVE guidelines, please do not add any vendor information or company or brand names to the manuscript text but include this information in the table of materials and reagents.

REFEREE 2
Referee 2 had similar comments as Referee 1. All of the minor concerns have been addressed.

I would like to thank the referees for their time to improve the quality of the manuscript. Thank you for considering this manuscript for publication in *Journal of Visualized Experiments*.

Sincerely,
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Profiling the triacylglyceride contents in bat integumentary lipids by preparative thin layer chromatography and MALDI-TOF mass spectrometry

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Keywords:
Triacylglyceride, Plagiopatagium, Integument, Sebaceous gland, White-Nose Syndrome, Matrix-Assisted Laser-desorption/Ionization Time-of-Flight Mass Spectrometry, Thin-Layer Chromatography

Short Abstract: Mammalian integument contains solvent-extractable lipids that can provide chemical compositions characteristic of individual species. This paper presents a routine method for separating broad lipid classes isolated from integumentary tissues using thin layer chromatography and determining the triacylglyceride profile by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Long Abstract:
The mammalian integument includes sebaceous glands that secrete an oily material onto the skin surface. Sebum production is part of the innate immune system that is protective against pathogenic microbes. Abnormal sebum production and chemical composition is also a clinical symptom of specific skin diseases. Sebum contains a complex mixture of lipids, including

triacylglycerides, which is species-specific. The broad chemical properties exhibited by diverse lipid classes hinder the specific determination of sebum composition. Analytical techniques for lipids typically require chemical derivatizations that are labor-intensive and increase sample preparation costs. This paper describes how to extract lipids from mammalian integument, separate broad lipid classes by thin-layer chromatography, and profile the triacylglyceride contents using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. This robust method enables a direct determination of the triacylglyceride profiles among species and individuals, and it can be readily applied to any taxonomic group of mammals.

INTRODUCTION

Mammalian integumentary tissues include the epidermis, keratinous structures (e.g., hair and nails), and exocrine glands. Sebaceous-type exocrine glands are associated with hair follicles, which are collectively referred to as the pilosebaceous unit[1]. Sebaceous glands release an oily exudate onto the skin surface referred to as sebum. Sebum is composed largely of glycerolipids (e.g., triacylglycerides [TAGs]), free fatty acyls (FFAs), sterol/wax esters, and squalene. Sebum's chemical composition is species-specific[2]. In addition to being part of the innate immune system and providing antimicrobial function[3], sebaceous lipids affect important physiological processes including evaporative water loss through skin[4], cellular integrity and gene regulation[5], and drug absorption[6]. Sebaceous lipid compositions can also serve as disease markers. Altered ratios and amounts of sebaceous broad lipid classes are clinical signs of diseases such as acne vulgaris[7], dandruff[8], seborrheic dermatitis[8], asteatosis[9], among others[10]. Epidermal and hair tissues include variable profiles containing sterol and derivatives, TAGs, FFAs, ceramides, phospholipids, and other minor lipid components. Because integumentary lipids can function in disease processes, determining differences in chemical compositions of TAGs between healthy and diseased individuals may be useful for clinical diagnosis of disease.

Lipids are generally defined as being water-insoluble organic compounds with either nonpolar or nonpolar-polar substituents[11]. Lipid structures may be long hydrocarbon chains, oxygenated alkanes (including wax esters, FFAs, alcohols, ketones, and aldehydes), or complex ring structures such as cholesterol[12]. There are eight major classes of lipids based on structure (FFAs, glycerolipids [GL], glycerophospholipids [GP], sphingolipids [SP], sterol lipids [ST], prenol lipids [PR], saccharolipids [SL], and polyketides [PK]), which exhibit a wide range of chemical properties depending on class[13]. Due to the wide variation in the chemical properties of lipid classes, direct profiling without prior derivatization of lipid molecules is desired. One emergent method in lipid research is thin-layer chromatography (TLC) combined with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)[14].

MALDI-TOF MS is used extensively in proteomic research to identify proteins and associate them to specific amino acid sequences due to the highly accurate peptide ion mass "fingerprints" generated from trypsin-digested proteins[15]. MALDI-TOF MS can also be used to profile other biomolecule classes, including lipids such as TAGs[16-18]. MALDI requires the use of a matrix, typically an organic compound that contains aromatic and conjugated double bond

structures. The matrix molecules serve to gently transfer the energy of the laser to the analytes, promote proton transfer, and produce singly charged gas-phase ions[19-21]. Ions are subjected to a high voltage field under high vacuum and accelerated into a TOF mass analyzer where ions are subsequently separated by differences in velocities that are proportional to their mass-to-charge ratios. Even very large biomolecules can be ionized with little fragmentation, producing singly charged molecular ion species for simplified spectrum analysis. The ability to analyze lipid molecules directly without prior derivatization has promoted MALDI-TOF MS' ready adoption in lipidomic research[18].

This paper presents a routine method to isolate and analyze integumentary lipids from the hair, sebaceous secretions and plagiopatagium of the Eastern red bat (*Lasiurus borealis*). This is used to determine interspecific variations in bat integumentary lipids to elucidate the disease process of White-Nose Syndrome (WNS)[22]. WNS is a fungal disease of bats and is caused by the newly described psychrophilic species *Geomyces destructans*[23-25]. WNS has caused the death of over 5 million North American cave bats and threatens the extinction of vulnerable bat species, with potential economic impacts in the billions of dollars in damage to the agricultural industry[26,27]. To investigate the steps in *G. destructans* infection, lipids were extracted from the hair and wing tissues of Eastern red bats and separated into broad lipid classes by TLC to isolate the TAG fraction for subsequent analysis by MALDI-TOF MS. TAGs contain short acyl chains and are easily detected by MALDI-TOF MS with little matrix interference.

PROTOCOL

CAUTION: Obtain in advance all requisite state and federal permits for handling, transporting, and storing bats. Approvals must also be obtained from your institutional animal care and use committee, as well as from your institutional biosafety committee. If live bats (or nervous system tissue) are to be handled, animal handlers should be vaccinated for rabies. Bats used in the current study were collected from the Ozark St. Francis National Forest, AR during summer 2010 according to standard methods (Arkansas State University's Institutional Biosafety Committee approval # 135349-1)[28].

1.) Tissue treatment and lipid extraction

1.1) Clean all instruments with methanol before and between tissue collection from different individuals. Trim hair (about 1.0 g) from skin with a scissors and place in a 125 ml Erlenmeyer flask. Sample sebum from wing surface by scrubbing skin with 4 to 6 cotton balls wetted with chloroform:methanol solvent (C:M; 3:2 v/v), and place these in a separate flask.

1.2) Extract tissue with 10 ml of C:M (2:1 v/v) containing 0.5% butylated hydroxytoluene (BHT) to prevent oxidation[29]. Only use HPLC quality solvents.

1.3) After 2 hours, add about 0.5 g anhydrous sulfate to each flask, mix briefly, and collect the solvent by filtering through filter paper.

1.4) Repeat steps 1.2 and 1.3 twice. Once with 1:1 C:M and sequentially with 1:2 C:M. Pool filtrates together.

1.5) Evaporate the pooled filtrates under a stream of N_2, determine dry weight, and dissolve the lipid residue in 3:2 C:M (with 0.5% BHT) to a concentration of 10 mg/ml. Store sample in glass vials at -20°C. It is generally best to analyze samples within one month of sample collection and to minimize freeze thaw cycles.

2.) Lipid separation by preparative thin-layer chromatography

2.1) Prepare in advance solvent-washed 1.5 ml microcentrifuge tubes by filling with 3:2 C:M, rinsing with acetone, and air-drying. This is done to remove plasticizers that can interfere with later mass spectrometry analysis. Store sample tubes in a dust-free container and handle these tubes only with gloves to prevent contamination from skin oils.

2.2) Activate the TLC plate by first pre-developing it with 3:2 C:M. Add enough solvent to the TLC chamber to a depth of 1 cm, then place plate in the chamber (close with glass lid) and allow the solvent to run completely to the top of the plate. This takes about 45 min.

2.3) Remove the plate and dry in a fume hood until solvent evaporates (about 15 min), then place in an oven for at least 10 minutes at 120°C. Place a pencil mark at the top of the plate to maintain orientation when samples are applied. Place a straight pencil line, 1.5 cm from the bottom edge of the plate, to mark the baseline where the sample and standards will be placed.

2.4) Prepare TLC chamber by cutting a piece of filter paper large enough to line the two short walls and one long wall. Place the filter paper liner into the chamber. It will fully wet when solvent is added to the chamber.

2.5) Prepare 100 ml of the mobile phase solvent, which is hexane:diethyl ether:acetic acid (H:E:A; 80:20:2, v/v/v). Pour solvent into the chamber to give a depth of about 1 cm. Cover with the glass lid, using a silicone grease seal along the top edge of the chamber. Allow the chamber to equilibrate overnight before use.

2.6) Apply the sample manually to the prepared plate with a capillary tube or pipette as a continuous streak from about 1.5 cm from one edge to 1.5 cm to the other edge. An automated sample applicator is preferred since it will load the sample in a more homogenous streak. Use the outer lanes of TLC plate to spot about 20 µl containing a mixture of sterol, FFAs, TAGs, and sterol ester standards (use at 10 mg/ml; premade mixtures can be obtained).

2.7) Place the loaded TLC plate into the equilibrated chamber, close with the lid, and develop the plate until the solvent runs to the top edge. This takes about 45 min with the mobile phase

described here. Remove the plate from the chamber and allow the excess solvent to evaporate from the plate in a fume hood for about 1 min.

2.8) Spray with 0.05% rhodamine 6G in 95% ethanol. Visualize lipid bands under a long wavelength ultraviolet lamp. Mark with a pencil the R*f* position for the fluorescent bands resolved in the sample and standards. A photographic record can be taken at this point.

2.9) Identify the band corresponding with the TAG standard and remove it from the plate by scraping the silica off with a spatula onto a large piece of glassine weigh paper. Transfer silica to a 1.5 ml solvent-washed microcentrifuge tube.

2.10) Add 1.0 ml of 3:2 C:M solvent to the sample tube, sonicate for 1 minute, pellet the silica by centrifugation, and then transfer the solvent into a new pre-weighed tube. Repeat the previous step, pool the filtrates, and evaporate the solvent under a stream of N_2.

2.11) Store the dried residue containing TAGs under N_2 in the dark at 4 °C while additional tissue samples are separated by TLC. Rhodamine 6G is present in samples, but it is insoluble in hexane and is removed during sample dissolution immediately before MS analysis.

3.) TAG analysis by MALDI-TOF MS

3.1) Prepare a fresh α-cyano-4-hydroxy-cinnamic acid (CHCA) matrix solution by dissolving 10 mg in 1 ml CHCA solvent (49.5% ethanol, 49.5% acetonitrile, and 1% aqueous 0.1% TFA).

3.2) Prepare adrenocartocotropic hormone (ACTH; 18-39 clip, 2465.1989 Da) for instrument resolution and sensitivity testing by mixing 1 µl of ACTH (1 mg/ml) with 39.5 µl of 0.1% trifluoroacetic acid (TFA) to obtain a 10 pmol/µl stock solution. This can be stored at -20°C for future use.

3.3) Prepare a fresh working ACTH solution (1 pmol/µl) by taking 1 µl of the 10 pmol/µl stock solution, mixing with 9 µl 0.1% TFA, and then mixing (1:1) with 10 µl of the CHCA matrix solution to obtain a 500 fmol/µl concentration.

3.4) Prepare TAG standards at 10 mg/ml in 3:2 C:M for MALDI instrument calibration (e.g., tricaprin [470.361 Da], tricaprylin [554.455 Da], trilaurin [638.549 Da], tripalmitin [722.642 Da], tripalmitolein [800.689 Da], trimyristin [806.736 Da], triolein [884.783 Da], tri-11-eicosenoin [968.877 Da], and trierucin [1052.971 Da]).

3.5) Prepare triolein at 10 mg/ml in 3:2 C:M for an external lock-mass calibration TAG standard.

3.6) Dissolve the stored TAG samples in hexane to a 10 mg/ml solution.

3.7) Prepare a 0.5 M (77.06 mg/1.0 ml) stock solution of 2,5-dihydroxybenzoic acid (DHB) with 90% methanol for use as sample and standard matrix. Also prepare a 1.0 M (2.0 g/50.0 ml) solution of NaOH. Cover the tube with DHB solution using aluminum foil to protect from light.

3.8) Mix (in pre-washed tubes) 10.0 µl DHB matrix, 10.0 µl sample or standard, and 5.0 µl 1.0 M NaOH. Mix and briefly centrifuge the tube to bring mixture to the bottom.

3.9) Spot 1.0 µl of standard, sample, or ACTH onto a MALDI stainless-steel target plate and place in a desiccator until dry. Place the target plate into the instrument for data acquisition.

3.10) Perform MALDI-TOF MS analyses in positive reflectron mode. Tune and calibrate the instrument as described by the instrument's manufacturer using the ACTH and TAG standard solutions.

3.11) Acquire spectra for each sample spotted on the target plate (consistent with instrument specifications; parameters for this work were 5 Hz laser firing rate, ~100 shots per spot to obtain an average spectrum). After smoothing and subtracting the background from MALDI spectra, process ion peaks manually with the online search engine LIPID MAPS (www.lipidmaps.org/tools/ms/glycerolipids_batch).

3.12) Copy and paste the list spectra into the list of precursor ions and intensity box. Limit the search to acyl composition desired. Identify TAGs by the mass/charge (m/z) ratios from ions present in the spectrum for each sample. If fatty acid methyl ester (FAME) percentages are available from separate GC/MS analysis, add these data to obtain probabilities of TAGs present.

INSTRUMENT: The mass spectrometer used in this study is a Waters MALDI Micro MX (equipped with a 337 nm 20 Hz N_2 laser). Any manufacturer's MALDI instrument can be used having positive reflectron mode capability. General settings used are pulse voltage, 2000 V; reflectron, 5200 V; source, 15,000 V, with data acquisition using MassLynx software (ver. 4.0). These operating conditions provide mass resolution greater than 12,000.

REPRESENTATIVE RESULTS

The extraction method for isolating total lipids from tissue described by Folch[30] is a straightforward procedure, which is adapted here. After tissue extraction and solvent evaporation, the lipids often appear as a yellowish film. The yellow color most likely is from protein contaminants, which can be removed by performing a liquid-liquid extraction. This additional sample processing is not needed in this procedure because preparative TLC separates the TAG fraction from such contaminants. The addition of fresh anhydrous sodium sulfate at all filtering stages helps reduce water contamination that affects accurate lipid weight determinations.

Mammalian integumentary lipid analyses by preparative TLC with H:E:A as the mobile phase will usually resolve four distinct bands corresponding to (starting from origin) sterols, FFAs, TAGs, and sterol esters/wax esters/squalene (Figure 1). On occasion when using analytical high performance (HP) TLC with the H:E:A mobile phase, the sterol esters, waxy esters, and squalene will separate and appear as three separate bands. Under the conditions used in the present study, the sterol/waxy esters are not separated. If these bands are of interest, the mobile phase can be switched to isooctane:ethyl ether (95:5 v/v), and the HPTLC plate can be analyzed by scanning densitometry. Other factors can cause poor separation. These are usually eliminated by placing the filter paper in the chamber, applying grease for a tight seal on the lid, equilibrating the chamber overnight, and keeping clean TLC chambers, to obtain consistent quality TLC separations and data.

Representative MALDI-TOF mass spectra obtained for TAGs isolated from the Eastern red bat are shown in Figures 2 and 3. These spectra contain TAG ion peaks in the mass range between *m/z* 850-910, which is typical for TAGs isolated from non-aquatic mammals. The addition of 1.0 M NaOH promotes singly charged Na^+ ions that are more stable than H^+ ions. In addition to stability the absence of H^+ and K^+ ions increases ease of spectrum analysis. The *m/z* 850-910 ion peaks correspond to 16:0, 18:0, 18:1, and 18:2 FA moieties being the dominant acyl constituents in TAGs (Table 1). Possible FA moieties of TAGs can be initially determined by total ion *m/z* present in MALDI-TOF MS spectra, and differences among species and individuals deduced. However, if the specific ratios of acyl content are required, then MS/MS or gas chromatography (GC/MS) must be used. Further information on acyl ratios can be deduced by observing the peaks in the diacylglycerides region of the spectrum (Figure 4). Diacylglycerides are produced from TAG fragmentation in the MALDI source and can be found in the *m/z* 590-650 region. TAG fragmentation can be increased by omitting the addition of 1.0 M NaOH[16]. Eastern red bat wing tissue is characterized by a dominant peak at *m/z* 879.7 and wing tissue with a dominant peak at *m/z* 881.8 (Figure 2 and 3 respectively). Peaks at *m/z* 907.8, 879.7, and 855.7 (POP, PPoS) are approximately even in intensity (~50%) in hair tissue with the peak at 853.7 being ~40%.

Table 1: Fatty acid composition, elemental composition, and isotopic mass of sodiated adducts of triacylglycerides and diacylglycerides.

Composition	**Elemental Composition**	**Observed Mass**
Na^+TAGs		*Na^+TAGs*
SSO	$C_{57}H_{108}O_6$	911.8
OOS, LSS	$C_{57}H_{106}O_6$	909.8
OOO, LnSS, LSO	$C_{57}H_{104}O_6$	907.8
LOO, LLS	$C_{57}H_{102}O_6$	905.8
LLO, OOLn	$C_{57}H_{100}O_6$	903.7

LLL	$C_{57}H_{98}O_6$	901.7
LLLn	$C_{57}H_{96}O_6$	899.7
LLnLn	$C_{57}H_{94}O_6$	897.7
LnLnLn	$C_{57}H_{92}O_6$	895.7
OSP	$C_{55}H_{104}O_6$	883.8
LSP, OOP, SOPo	$C_{55}H_{102}O_6$	881.8
LOP, LnSP, LSPo	$C_{55}H_{100}O_6$	879.7
LLP, LnOP, LOPo	$C_{55}H_{98}O_6$	877.7
LnLP, LLPo, LnOPo	$C_{55}H_{96}O_6$	875.7
LnLnP, LnLPo	$C_{55}H_{94}O_6$	873.7
LnLnPo	$C_{55}H_{92}O_6$	871.7
PPS	$C_{53}H_{102}O_6$	857.8
POP, PPoS	$C_{53}H_{100}O_6$	855.7
OOM, PPL, PoPoS, POPo	$C_{53}H_{98}O_6$	853.7
PPLn, PPoL, PoPoO, MyOO	$C_{53}H_{96}O_6$	851.7
LLM, LnOM	$C_{53}H_{94}O_6$	849.7
PPP, SSLa	$C_{51}H_{98}O_6$	829.7
PPPo, OSLa	$C_{51}H_{96}O_6$	827.7
PPoPo, PMyO	$C_{51}H_{94}O_6$	825.7
LnLnLa	$C_{51}H_{86}O_6$	817.6
MMS, SLaP, PPM	$C_{49}H_{94}O_6$	801.7
SLaPo, PPoM, PPMy	$C_{49}H_{92}O_6$	799.7
PoPoM, OOCa	$C_{49}H_{90}O_6$	797.7
MMP	$C_{47}H_{90}O_6$	773.7
MMPo, OCaP	$C_{47}H_{88}O_6$	771.7
OCaPo	$C_{47}H_{86}O_6$	769.6
MMM, PPCa, PMLa	$C_{45}H_{86}O_6$	745.6
PoPCa, PoMLa	$C_{45}H_{84}O_6$	743.6
PoPoCa	$C_{45}H_{82}O_6$	741.6
LaLaP, MMLa, MCaP	$C_{43}H_{82}O_6$	717.6
LaLaPo	$C_{43}H_{80}O_6$	715.6
OO	$C_{39}H_{72}O_5$	643.5
OL	$C_{39}H_{70}O_5$	641.5
LL	$C_{39}H_{68}O_5$	639.5
SP	$C_{37}H_{72}O_5$	619.5
OP	$C_{37}H_{70}O_5$	617.5
LP	$C_{37}H_{68}O_5$	615.5

Ln=linolenic acid (18:3), L=linoleic acid (18:2), O=oleic acid (18:1), S=stearic acid (18:0), P=palmitic acid (16:0), Po=palmitoleic acid (16:1), M=myristic acid (14:0), My=myristoleic acid (14:1) La=lauric acid (12:0), Ca=Capric acid (10:0).

Figures:

Figure 1: Thin-layer chromatogram of broad lipid class separation by hexane:diethyl ether:acetic acid (80:20:2 v/v/v) as the mobile phase. The band between sterol and FFA was not identified by a standard but may be a fatty alcohol or wax diester.

Figure 2: Expanded TAG region of MALDI-TOF mass spectrum of sodiated TAGs (*m/z* 700-950) from Eastern red bat (*L. borealis*) wing tissue. Peaks identified at *m/z* 853.7 (OOM, PPL, PoPoS, POPo) and *m/z* 879.7 (LOP, LnSP, LSPo).

Figure 3: Expanded TAG region of MALDI-TOF mass spectrum of sodiated TAGs (*m/z* 700-950) from Eastern red bat (*L. borealis*) hair tissue. Peaks identified at *m/z* 905.8 (LOO, LLS) and *m/z* 907.8 (OOO, LnSS, LSO).

Figure 4: DAG region of MALDI-TOF mass spectrum of sodiated DAG fragments (*m/z* 530-730) from Eastern red bat (*L. borealis*) wing tissue. Peaks identified at *m/z* 643.5 (OO) and *m/z* 615.5 (LP).

DISCUSSION

This paper presents a simple and robust method for separating broad lipid classes isolated from mammalian integument by preparative TLC and determining TAG profiles by MALDI-TOF MS, without time-consuming derivatization of the lipid molecules. The critical steps in producing quality spectra of TAGs with MALDI-TOF MS include: 1) Successful extraction of the compound with minimal contamination or oxidation; 2) Sufficient separation and isolation by chromatography; and 3) High resolution and mass accuracy by MALDI-TOF MS.

This paper demonstrates the method by extracting and separating the neutral lipid fraction from the plagiopatagium of the Eastern red bat to obtain TAG MS profiles. While the present study used a bat species (Mammalia: Chiroptera) these methods can be extended to study integumentary lipids of any mammalian species. Bat integument is characterized by being predominantly cholesterol, with lower amounts of TAGs, FFAs, squalene, and sterol/wax esters. Sebum lipid ratios in bats differ from humans in that squalene is present in low amounts (as opposed to up to 16% in humans), whilst cholesterol occurs in larger ratios (1-7% in humans but 26-62% in bats)[22,31]. Human hairs contain about 3% TAGs, while ratios up to 28% are found in Eastern red bat hair. The extraction of lipid samples from bats is similar for other species. While in this study cotton balls soaked in solvent are used to remove sebum, one can also invert a vial or sample tube containing solvent onto the surface of the integument multiple times. Specialized tape products also provide alternative means to extract surface lipids[32]. A critical part of a proper lipid extraction is to minimize contamination from skin oils. This is easily accomplished by keeping a squeeze bottle with methanol and spraying all glassware and utensils, wiping utensils with a tissue between all samples and wearing examination gloves.

Oxidation of polyunsaturated acyls is prevented through the addition of BHT, and it should be used regardless of the temperature samples are stored at.

Biomolecule analysis usually requires a chromatographic step to separate molecules of interest from contaminants. TLC is used in this method, which avoids instrumentation requirements for gas or liquid chromatography and requires less technical experience to achieve robust and repeatable results. Depending on the lipid class of interest, many different mobile phases can be incorporated. Furthermore, the use of HPTLC plates and scanning densitometry can be used to achieve quantitative results. While the variations of TLC methods are too numerous to list here, some common mobile phases used in lipid TLC include chloroform:methanol:water for separation of phospholipids and glycolipids or isooctane:ethyl ether for separation of non-polar lipids[28]. In terms of separating TAGs from other lipid classes, the H:E:A solvent system works consistently and provides comparable results.

Another advantage for using TLC is that bands of interest can be rapidly profiled using MALDI-TOF MS without prior derivatization of the analytes. In this study the silica is removed from the TLC plate, and the analyte is eluted from it by sonication in a solvent and subsequent centrifugation to separate the adsorbent and evaporation of the eluting solvent. Alternatively, matrix may be applied directly onto analyte bands separated on TLC plates and then directly analyzed by MALDI-TOF MS[33]. Successful profiling by MALDI-TOF MS does rely on sufficient sample preparation and the operator's proficiency with tuning and calibrating the instrument. The instrument should be calibrated daily with standards covering molecular weight range appropriate for the molecules of interest. The suitable sensitivity and resolution (e.g., $\geq$10,000) of the equipment should also be confirmed daily with ACTH.

The sebaceous lipid constituents found on the surface of mammalian integument may play a role in colonization by bacterial/fungal pathogens. Therefore knowledge of the chemical composition between species and individuals may provide clues about processes of human and wildlife diseases. Intraspecific differences among diseased and healthy individuals may represent clinical signs that aid in disease detection and diagnosis. Furthermore, if specific compounds are present that inhibit microbial growth, these may be identified for use in disease treatment and prevention.

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DISCLOSURES

The authors declare no competing financial interests.

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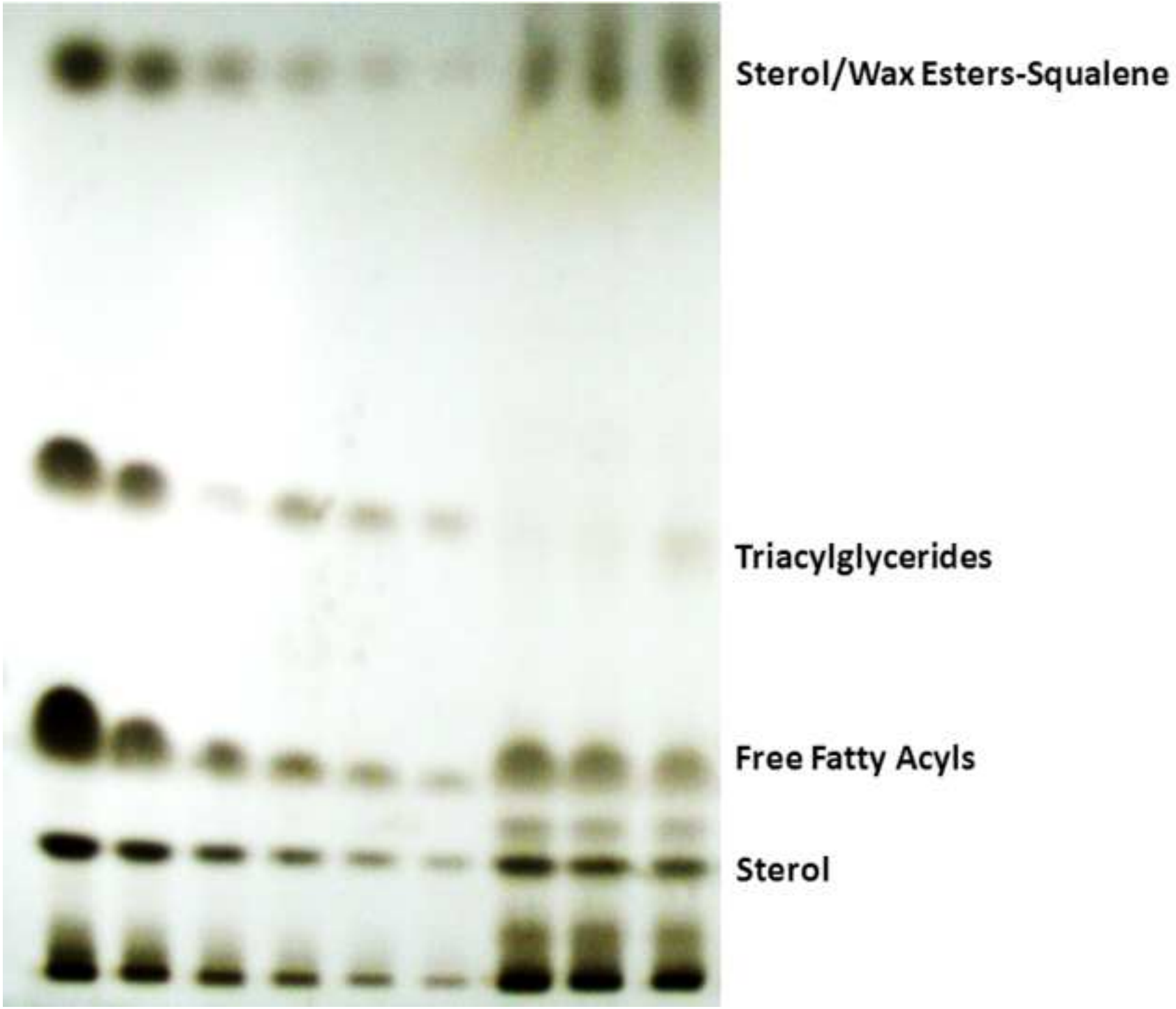

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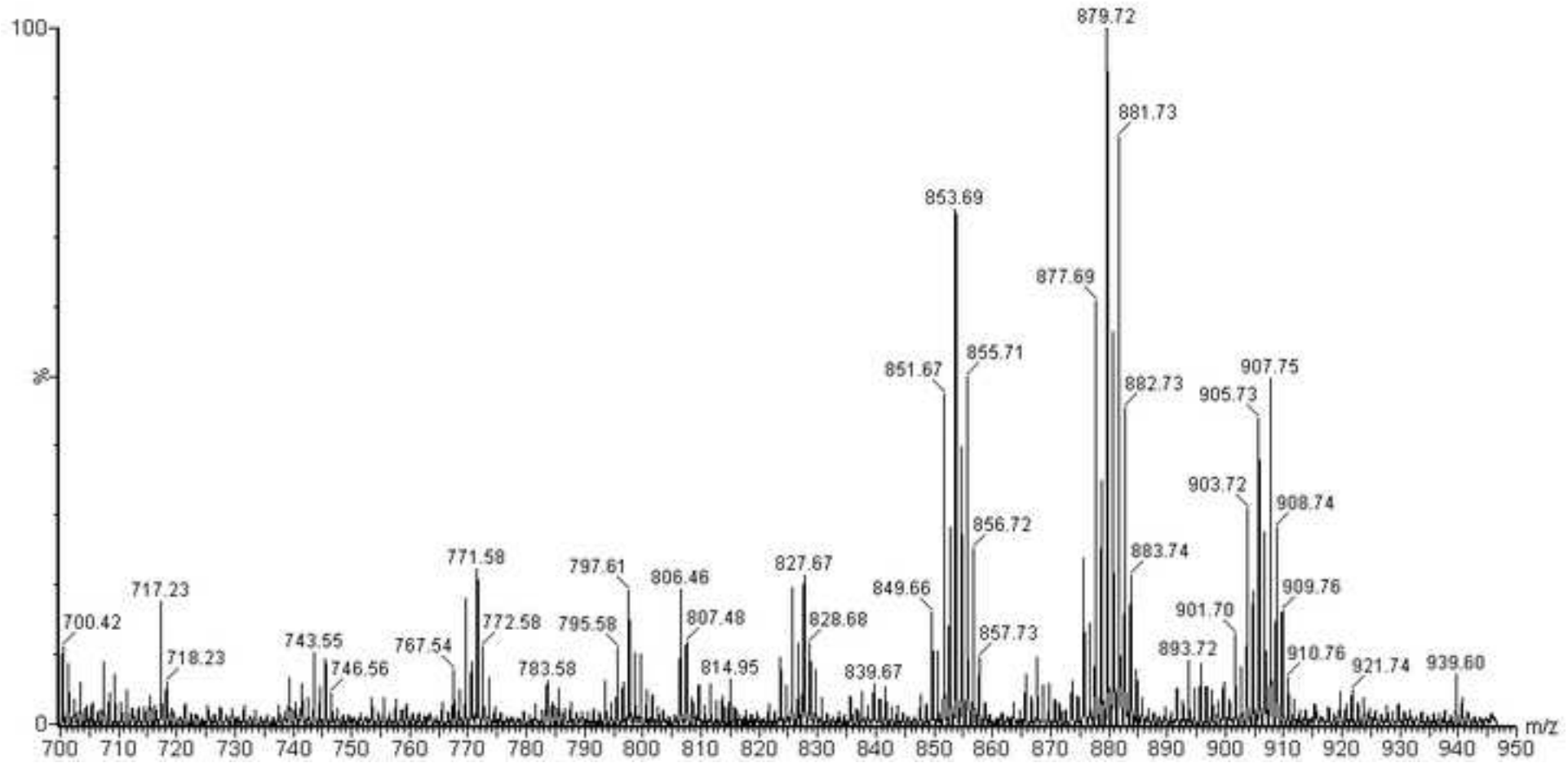

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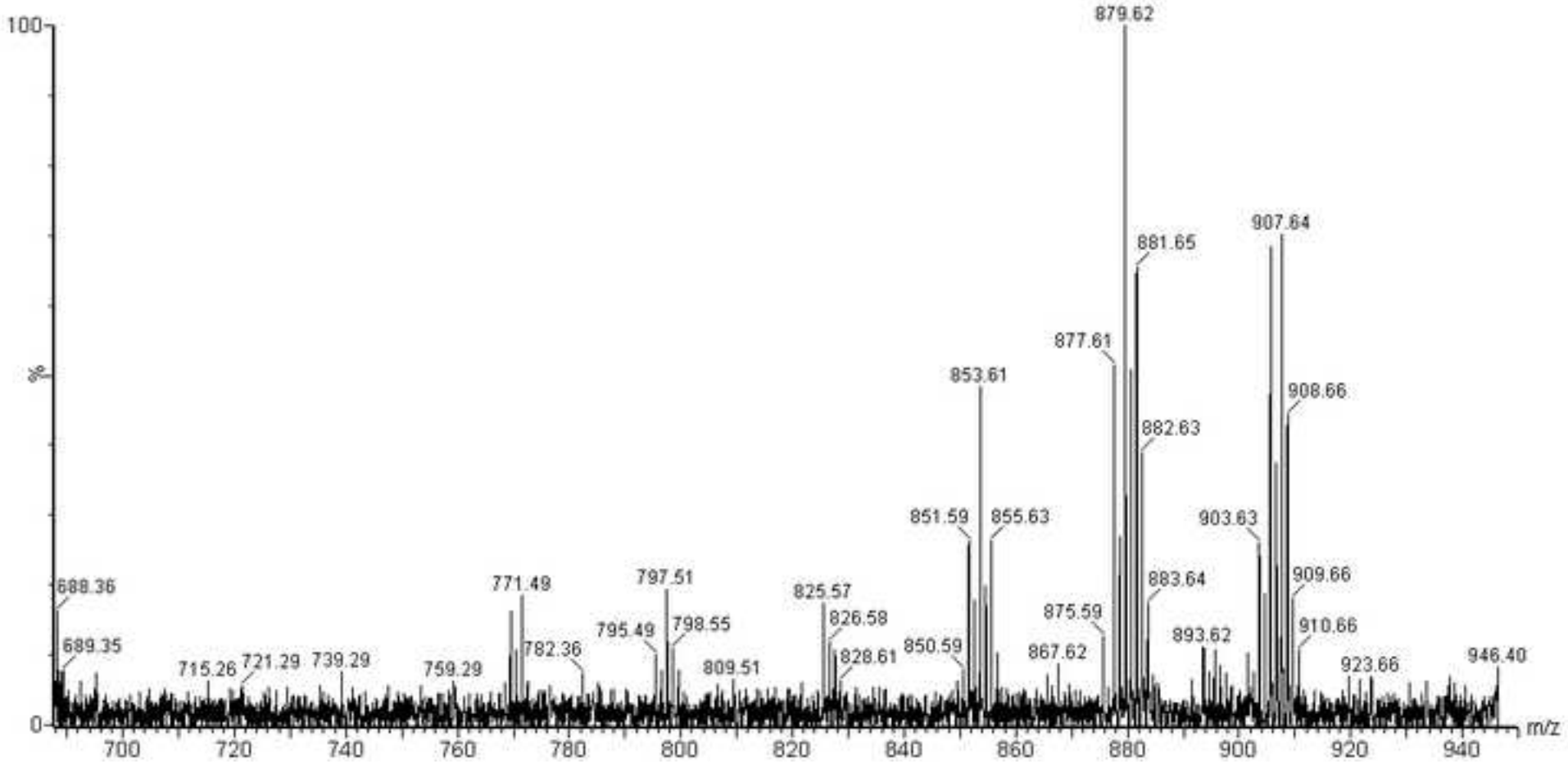

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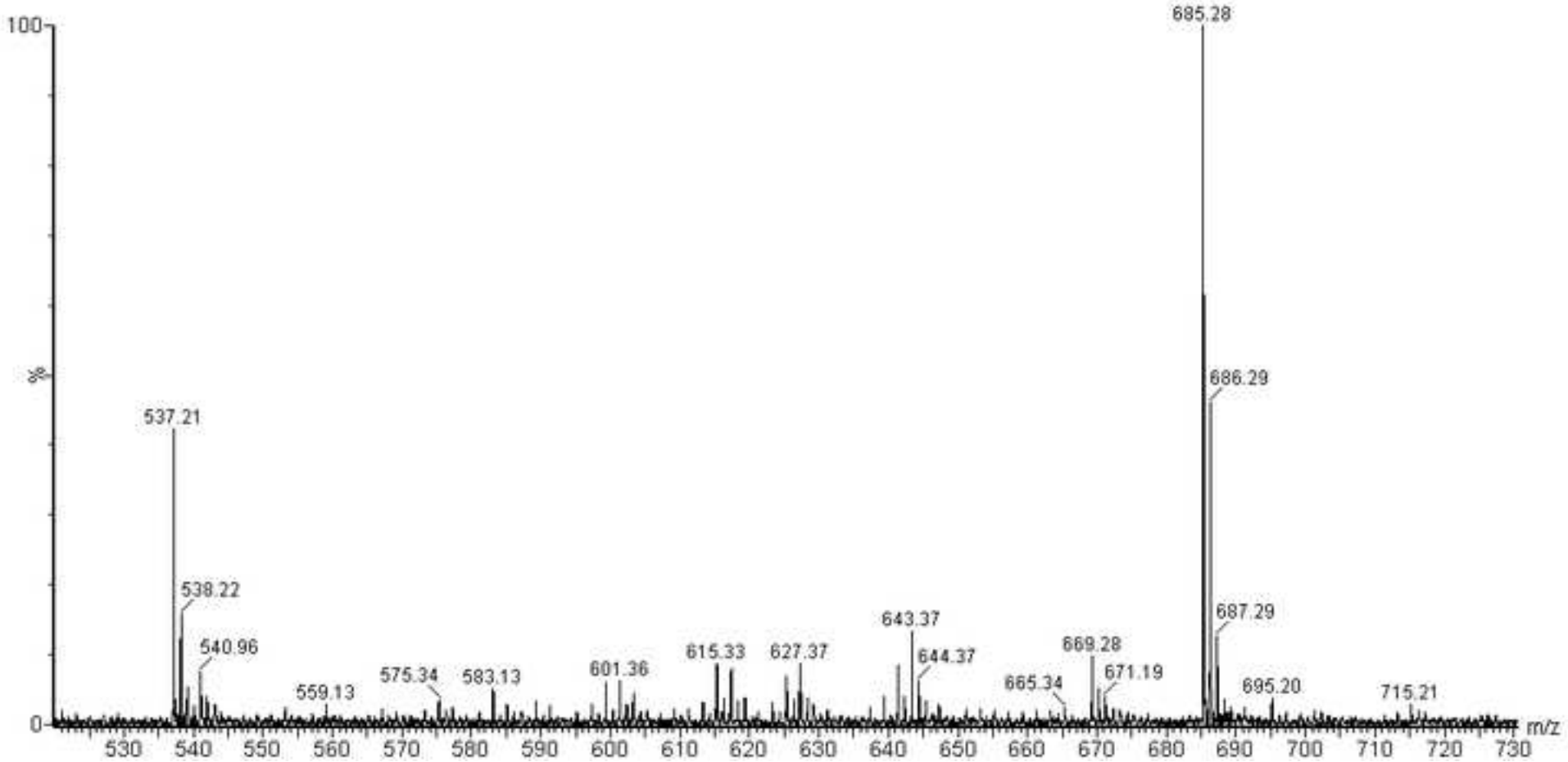

*Excel Spreadsheet- Table of Materials/Equipment

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Name of the reagent	Company	Catalogue number	Comments (optional)
Butylated Hydroxytoluene	MP Biomedicals, LLC www.mpbio.com	101162	
TLC flexible plates	Whatman www.whatman.com	4410-222	
ACTH	Sigma-Aldrich Chem. Co. www.sigmaaldrich.com	A8346-5X1VL	
Triolein	Sigma-Aldrich Chem. Co.	44895-U	
TLC Lipid Standard	www.nu-chekprep.com	TLC 18-1	
MALDI TAG Standard	Nu-Check Prep., Inc.	NIH Code 53B	
MALDI TAG Standard	Sigma-Aldrich Chem. Co.	17810-1AMP-S	
Glass Vials w/ Teflon cap	U.S. National Scientific Co. www.nationalscientific.com/	B7800-2	
DHB	Sigma-Aldrich Chem. Co.	50862-1G-F	
CHCA	Sigma-Aldrich Chem. Co.	C8982-10X10mg	
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Dear Michelle Kinahan:

Enclosed is a revised version of the manuscript "Profiling the triacylglyceride contents in bat integumentary lipids by preparative thin layer chromatography and MALDI-TOF mass spectrometry", which I co-authored with Thomas Risch and Brett Savary. The reviewers provided helpful comments that we used to strengthen the manuscript. All reviewers felt that this was a well written manuscript that thoroughly explained the techniques of TLC and MALDI-TOF MS in a fashion that novice readers could understand. There were only minor edits and formatting changes, which we were able to make. Listed below are responses to the reviewers comments and changes have been highlighted in the manuscript with the Track Changes under Review option in Microsoft Word.

Response to the reviewers:
Many of the revisions were minor and most were corrected accordingly. Referee 1 supplied a thorough review on MALDI procedures and data interpretation. Both Referees felt that it would be beneficial to include diseases in the introduction associated with altered sebaceous profiles. A list of diseases was included with a 2012 review paper cited. Some specific responses to Referee comments are included below.

EDITORIAL COMMENTS
The formatting issues have been addressed including the deletion of brand or company names.

REFEREE 1

4. Intro, p. 2: "free fatty acyls" should be changed to "free fatty acids"

In keeping with the terminology proposed by Fahy et al. 2005 and 2009, the usage of acyl was retained.

10. Protocol, 1 - It is unclear if the procedure is being performed on live animals or on dissected animal tissue. A clarification is needed, along with a few additional comments about handling live bats, if necessary (ie, how to hold them, are they anesthetized, etc.)

This was clarified in the procedures along with the IACUC approval and the citation for proper use of wild mammals in research, which was followed.

13. Protocol, 1, 1.3 - Is there a specific grade of filter paper required?

There is no particular grade that is required; however, we used qualitative P8 filter paper.

16. Protocol 3 - It might be helpful to make a new subheading called "Instrument calibration and optimization" or something like that, and have steps 3.1 to 3.5 under that subheading, since they are separate from preparing the samples for analysis.

While we feel that the Referee is correct in this suggestion, the protocol is at the length limit established by JoVE without incurring additional editorial processing.

18. Protocol 3, 3.11 and 3.12 - This could use a little more details...are peak lists exported from the instrument after smoothing and background subtraction? What are the typical settings for S/N or minimum intensity? Are there any restrictions on how the peaks are entered into the glycerolipids_batch tool? A screen shot of the tool would be helpful in the manuscript.

Peak lists are exported after smoothing and background subtraction, which is stated in 3.11. Restrictions on how peaks are processed in LIPID MAPS is dependent on the equipment operator, therefore operator proficiency is required for data acquisition and interpretation. The authors feel the level of detail given is sufficient for the manuscript.

17. Protocol 3, p. 6 - It is surprising that you are analyzing TG's in positive mode but you are adding NaOH...have you looked at any other sources of Na for complexation? Does the NaOH work best? If so, a comment to that effect should be added.

This topic has been addressed in detail in Gidden et al. 2007, which is cited in the manuscript. "Various concentrations (from 0.025 to 14 M) of NaOH, NH_3, $NaC_2H_3O_2$, and NH_4HCO_3 were prepared in water", added to samples of vegetable oil, and analyzed with a Bruker Ultraflex II. They concluded that the presence of base promoted Na^+ ion formation while suppressing H^+ ion formation. The H^+ ion is more unstable and prone to fragmentation, so the addition of the base promotes singly charged molecular ions that are more stable. A 1.0 M addition of NaOH was determined to give optimal results. A sentence was added to the results to explain the addition of NaOH.

20. Results, p. 6 - The discussion of the differences between prep and HP TLC plates is appreciated, but it needs clarification. It reads as though with prep plates there are 4 bands: sterols, FFA's, TAGs, and sterol esters/wax esters/squalene. But with HP plates there are only 3 bands, but it is stated that "the sterol esters, waxy esters and squalene will appear as only 3 separate bands" - are those 3 one band with prep plates and 3 bands with HP plates? Or are sterols, FFAs, TAGs, and sterol esters/wax esters/squalene 3 bands with HP plates, with sterols and waxy esters not separated? Please clarify.

We feel that the separation of broad lipid class was not clarified thoroughly and was confusing to read as the Referee commented. We added more specific terminology and explanation of broad lipid class separation should be less confusing.

23. Results - One problem with lipid analysis in positive mode is the spreading out of signal between protonated, sodiated, and potassiated species. Do you know how much protonated or potassiated signal you might be getting? Does the addition of NaOH allow only Na+ ions? A sentence or two addressing this topic is needed.

Please refer to the comment to number 17.

24. Discussion, p. 9 - add a source/vendor for Sebotape; also change "...Sebutape call also provide..." to "...Sebutape can also provide..."

Please refer to the editor's comment below. Sebutape was changed to "specialized tape products" and was included in the table of materials and reagents.

Editorial Comment: To comply with JoVE guidelines, please do not add any vendor information or company or brand names to the manuscript text but include this information in the table of materials and reagents.

REFEREE 2
Referee 2 had similar comments as Referee 1. All of the minor concerns have been addressed.

I would like to thank the referees for their time to improve the quality of the manuscript. Thank you for considering this manuscript for publication in *Journal of Visualized Experiments*.

Sincerely,
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