Isolation and structural elucidation of vernix caseosa TG-estolides with TLC, HPLC-ESI/MS and GC-MS

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ISOLATION AND STRUCTURAL ELUCIDATION OF VERNIX CASEOSA TG-ESTOLIDES WITH TLC, HPLC-ESI/MS AND GC-MS

Diploma Thesis

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Hrvoje Dumić

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ABSTRACT

ISOLATION AND STRUCTURAL ELUCIDATION OF VERNIX CASEOSA TG-ESTOLIDES WITH TLC, HPLC-ESI/MS AND GC-MS

Hrvoje Dumić

A complete isolation of vernix caseosa triacylglycerol estolides with combination of thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC), and their partial structural elucidation with high-performance liquid chromatography – mass spectrometry (HPLC-MS) and gas chromatography – mass spectrometry (GC-MS) hyphenated systems was performed. New semi-preparative TLC method for isolation and semi-preparative HPLC method for additional purification of vernix caseosa triacylglycerol estolides were developed. Structural analysis of intact triacylglycerol estolides with LC-MS system revealed 126 structural isomers and after transesterification of triacylglycerol estolides, analysis of fatty acid methyl esters with GC-MS 71 different fatty acids that build vernix caseosa triacylglycerol estolides were observed.

(79 + XXII pages, 29 figures, 8 schemes,12 tables, 135 references, original in English)

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SAŽETAK

IZOLACIJA I STRUKTURNO ODREĐIVANJE TG- ESTOLIDA SIRASTOG MAZA (*VERNIX CASEOSA*) UPOTREBOM TANKOSLOJNE KROMATOGRAFIJE I VEZANIH SUSTAVA HPLC-ESI/MS I GC-MS

Hrvoje Dumić

Upotrebom tankoslojne kromatografije (TLC) i tekućinske kromatografije visoke djelotvornosti (HPLC) provedena je potpuna izolacija triacilglicerol estolida iz uzorka sirastog maza (vernix caseosa) te je djelomično određena njihova struktura upotrebom vezanih sustava tekućinska kromatografija visoke djelotvornosti – masena spektrometrija (HPLC-MS) i plinska kromatografija – masena spektrometrija (GC-MS). Razvijena je nova metoda semi-preparativne tankoslojne kromatografije za izolaciju i nova metoda semi-preparativne tekućinske kromatografije visoke djelotvornosti za dodatno pročišćavanje triacilglicerol estolida iz uzorka sirastog maza. Strukturna analiza triacilglicerol estolida, u njihovom intaktnom obliku, upotrebom vezanog sustava HPLC-MS otkrila je postojanje 126 strukturnih izomera. Također je, nakon transesterifikacije uzorka, strukturnom analizom metilnih estera masnih kiselina upotrebom vezanog sustava GC-MS otkriveno 71 različitih masnih kiselina koje grade triacilglicerol estolide u sirastom mazu.

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Ključne riječi: GC, HPLC, MS, sirasti maz, TLC, triacilglicerol estolidi

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PROŠIRENI SAŽETAK

I. SIRASTI MAZ (VERNIX CASEOSA)

Sirasti maz (vernix caseosa) je kremasti sloj, nalik na sir, koji prekriva površinu fetusa u periodu zadnjeg tromjesječja trudnoće.[I] Uglavnom se sastoji od vode (80 %) i složene smjese proteina (10 %) i lipida (10 %).[II] Do nedavno, kada je otkriven na površini kože novorođenih morskih lavova, za sirasti maz se oduvijek smatralo da nastaje samo na površini ljudskog fetusa.[III]

Sirasti maz nastaje na površini vanjskog sloja kože (stratum corneum) od glave prema nožnim prstima i od leđa prema prednjoj strain tijela,[IV] te se vjeruje da u zadnjem tromjesječju trudnoće, putem još nepoznatog mehanizma, potpomaže nastajanju tog vanjskog sloja kože.[V] Majčinski hormoni i hormoni posteljice imaju glavnu ulogu u kontroli nastajanja i u načinu prekrivanja površine kože fetusa sirastim mazom. Sirasti se maz nakon poroda uklanja pranjem novorođenčeta. Međutim, ukoliko se sirasti maz nebi uklonio na taj način, sam bi se nakon određenog vremena odvojio s porvšine kože procesom odvajanja, odnosno ljuštenja vanjske membrane tkiva.[VI] S obzirom da se već u sedamnaestom tjednu trudnoće sirasti maz može uočiti i na obrvama,[9] moguće je da stanice sirastog maza potječu od folikula kose.[VII]

Usporedbom sastava sirastog maza i stratum corneum-a uočene su mnoge sličnosti, ali također i neke razlike. Prijašnje studije pokazale su da sirasti maz grade hidrofilne rožnate stanice ili korneocite ugrađene u lipidni matriks, zbog čega se smatra da stanice sirastog maza zapravo potječu, odnosno nastaju iz odvojenih rožnatih stanica stratum corneum-a. Međutim,

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[I] S. B. Hoath, W. L. Pickens, M. O. Visscher, *Int. J. Cosmet. Sci.* **28** (2006) 319-333.
[II] A. Checa, T. Holm, M. O. D. Sjödin, S. N. Reinke, J. Alm, A. Scheynius, C. E. Wheelock, *Sci. Rep.* **5** (2015) 1–8.
[III] S. B. Hoath (ed.), W. L. Pickens, *Neonatal skin: Structure and Function*, Marcel Dekker, Inc., 2003, pgs. 193–210.
[IV] M. O. Visscher, V. Narendran, W. L. Pickensn, A. A. LaRuffa, J. Meinzen-Derr, K. Allen, S. B. Hoath, *J. Perinatol.* **25** (2005) 440–446.
[V] M. Visscher, V. Narendran, *Newborn Infant Nurs. Rev.* **14** (2014) 142–146.
[VI] E. A. Adejuyigbe, M. H. Bee, Y. Amare, B. A. Omotara, R. B. Iganus, F. Manzi, D. D. Shamba, J. Skordis-Worrall, A. Odebiyi, Z. E. Hill *BMC Pediatr.* **15** (2015) 1–7.
[VII] I. Kurokawa, F. W. Danby, Q. Ju, X. Wang, L. F. Xiang, L. Xia, W. Chen, I. Nagy,M. Picardo, D. H. Suh, R. Ganceviciene, S. Schagen, F. Tsatsou, C. C. Zouboulis, *Exp. Dermatol.* **18** (2009) 821–832.

suprotno od stanica stratum corneum-a, stanični dio sirastog maza ne posjeduje međustanične proteinske poveznice niti su lipidi lamelarno organizirani.[VIII] Što se tiče sličnosti lipidnog sastava sirastog maza i stratum corneum-a, svi glavni lipidi uočeni u stratum corneum-u su također uočeni i u sirastom mazu, što su Sumida i suradnici odredili analizom HPTLC.[IX] Međutim, glavninu lipida u stratum corneum-u čine slobodne masne kiseline, kolesterol i ceramidi, dok su u sirastom mazu glavne frakcije lipida sterolni i voštani esteri te triacilgliceroli.[VIII]

Kao što je rečeno ranije, sirasti je maz izgrađen od složene smjese proteina i lipida. Bez obzira na činjenicu da je daleke 1939. godine dr. Phil Rudolf Schmidt proveo prvu analizu sastava sirastog maza[X] i da je 1965. Käerkkärinen odredio detaljan lipidni sastav,[XI] znanstvenici i danas pokušavaju u potpunosti odrediti kemijske sastavnice proteina i lipida sirastog maza te odrediti njihove uloge.

Iako se uloge pojedinih kemijskih spojeva koji grade proteine i lipide sirastog maza ne znaju u potpunosti, uloge sirastog maza u cijelini su dobro poznate. Uz navedenu ulogu pomagača u stvaranju stratum corneum-a, otkrivene su mnoge druge funkcije. Zbog postojanja lipidnog hidrofobnog dijela, sirasti maz štiti fetus od mogućeg gubitka vode putem epidermisa. Istraživanja su pokazala da su svi uzorci sirastog maza sterilni. S obzirom da posjeduje lizozim (antimikrobni enzim), laktoferin (globularni glikoprotein koji je dio imunološkog sustava) i neke druge antimikrobne peptide, dokazana je njegova aktivnost protiv gljivičnih i bakterijskih patogena. Druge poznate uloge su: antioksidacijska, zaštita fetusa od plodne vode, regulacija temperature tijela fetusa te pomoć u njegovoj prilagodbi na vanjsku temperaturu nakon poroda, pomoć u zarastanju rana, te hidratacija i čišćenje kože, što se može upotrijebiti u farmaceutskoj industriji za proizvodnju preparata za kožu.[XII]

[VIII] R. Rissman, H. W. W. Groenink, A. M. Weerheim, S. B. Hoath, M. Ponec, J. A. Bouwstra, *J. Invest. Dermatol.* **126** (2006) 1823–1833.
[IX] Y. M. Y. Sumida, Y. Tokitsu, E. Al, S*tudies on the function of vernix caseosa: the secrecy of baby's skin*, 20th International Federation of Societies of Cosmetic Chemists Conference 1998 pg. 14–18.
[X] R. Schmid, *Arch. Gynakol.* **168** (1939) 445–450.
[XI] J. Kärkkäinen, T. Nikkari, S. Ruponen, E. Haahti, *J. Invest. Dermatol.* **44** (2010) 333–338.
[XII] H. Yoshio, M. Tolin, G. H. Gudmundsson, H. Lagercrantz, H. Jornvall, G. Marchini, B. Agerberth, *Pediatr. Res.* **53** (2003) 211–216.

II. ESTOLIDI

Estolidi su derivati masti i ulja. Njihova je struktura definirana sekundarnom esterskom vezom jedne molekule masne kiseline na alkilnom dijelu druge masne kiseline.[XIII] Ta esterska veza može nastati karboksilnom supstitucijom na hidroksilnoj skupini hidroksi masne kiseline ili adicijskom reakcijom na dvostruku vezu nezasićene masne kiseline. Nadogradnja masnih acilnih molekula može se na taj način odvijati dok god druga masna kiselina u svojoj strukturi na ugljikovodičnom lancu posjeduje hidroksilnu skupinu ili dvostruku vezu. Strukturno gledajući, estolidi mogu postojati u obliku tri različite klase: kao slobodne masne kiseline, kao esteri, i mogu biti uklopljeni u strukturu triacilglicerola.

Iako ih je daleke 1897. godine prvi otkrio Meyer u ricinusovom ulju,[XIV] struktura estolida, a pogotovo struktura triacilglicerol estolida i njihova točna uloga u prirodi još danas nisu poznate.[XV] To ne začuđuje toliko kada se uzme u obzir da su do 2011. triacilglicerol estolidi otkriveni u samo 11 biljnih vrsta,[XVI] a samo nekolicina znanstvenih istraživanja potvrđuje njihovo postojanje u određenim životinjskim tkivima.[XVII]

Iako triacilglicerol estolidi nisu u tolikoj mjeri rasprostranjeni u prirodi, estolidi jednostavnije strukture jesu, a pogotovo oni koji potječu od masnih kiselina. Dvije najrasprostranjenije klase estolida masnih kiselina su esteri masnih kiselina hidroksi masnih kiselina (FAHFA-a) i (O-acil)-ω-hidroksi masne kiseline (OAHFA-e). Iako pripadaju istoj klasi estolida; esteri masnih kiselina hidroksi masnih kiselina, između njih postoje neke strukturne razlike. FAHFA-e su strukturno jednostavnije, uobičajene dužine masnog lanca od 16 do 18 ugljikovih atoma i jednim stupnjem nezasićenosti odnosno jednom dvostrukom vezom. S druge strane, OAHFA-e posjeduju iznimno dugačke masne lance s 26 do 34 ugljikovih atoma te esterska veza nastaje isključivo na terminalnoj, ω-poziciji.[XVIII]

[XIII] S. Cermak, *Estolides: Synthesis and Applications.* Vol. 2, Elsevier Inc., 2011, pg. 433.
[XIV] H. Meyer, Chemische Notizen iiber Ricinusol. (1897).
[XV] M. A. Smith, H. Zhang, L. Forseille, R. W. Purves, *Lipids* **48** (2013) 75–85.
[XVI] T. A. Isbell, *Grasas y Aceites* **62** (2011) 8–20.
[XVII] S. McLean, N. W. Davies, D. S. Nichols, B. J. McLeod, *Lipids* **50** (2015) 591–604.
[XVIII] S. E. Hancock, R. Ailuri, D. L. Marshall, S. H. J. Brown, J. T. Saville, V. R. Narreddula, N. R. Boase, B. L. J. Poad, A. J. Trevitt, M. D. P. Willcox, M. J. Kelso, T. W. Mitchell, S. J. Blanksby, *J. Lipid Res.* **59** (2018) 1510–1518.

S obzirom da su estolidi derivati masnih kiselina, njihova se biosinteza, kao i biosinteza masnih kiselina odvija u citosolu stanice. I dok u prirodi uglavnom biljke, čija sintaza masnih kiselina proizvodi širok spektar hidroksi i nezasićenih masnih kiselina,[XIX] stvaraju estolide, velika je pažnja posvećena komercijalnoj sintezi estolida zbog njihovih izvanrednih svojstava. Estolidi se općenito mogu sintetizirati na dva načina: u kiselinski[XX] i enzimski[XXI] kataliziranoj reakciji. Iako je kiselinski katalizirana sinteza estolida puno raširenija, obje metode omogućuju pripremu velike količine estolida uz male troškove. Ipak, obje metode imaju određena ograničenja. Žestoki uvjeti u kiselinski kataliziranoj reakciji mogu, u reverznoj esterskoj reakciji uništiti ubačenu funkcijsku skupinu. To se može izbjeći upotrebom enzima. Međutim, ograničenje u enzimski kataliziranoj reakciji je kompatibilnost funkcijske skupine s aktivnim mjestom enzima. Također, obje metode su podložne kemijskoj ravnoteži zbog koje nastaju monomeri.[XXII] Zbog tih se ograničenja razvijaju nove metode i uvode poboljšanja u postojećim metodama sinteze te najveći doprinos u tom području imaju Cermak i Isbel. Oni su testiranjem različitih heterogenih i homogenih katalizatora sintetizirali širok spektar raznih estolida, poboljšali postojeće metode sinteze, a time i iskorištenja reakcija.[XXIII]

[XIX] R. C. Badami, K. B. Patil, *Prog. Lipid Res.* **19** (1980) 119–153. (1980).
[XX] S. C. Cermak, T. A. Isbell, *Ind. Crops Prod.* **29** (2009) 205–213.
[XXI] D. G. Hayes, R. Kleiman, *J. Am. Oil Chem. Soc.* **72** (1995) 1309–1316.
[XXII] J. A. Zerkowski, A. Nuñez, D. K. Y. Solaiman, *J. Am. Oil Chem. Soc.* **85**, (2008) 277–284.
[XXIII] T. A. Isbell, R. Kleiman, B. A. Plattner, *J. Am. Oil Chem. Soc.* **71** (1994) 169–174

III. IZOLACIJA TRIACILGLICEROL ESTOLIDA

Proces izolacije triacilglicerol estolida iz uzorka sirastog maza podijeljen je u četiri dijela: **1)** izolacija svih lipida sirastog maza, **2)** izolacija nepolarnih lipida, **3)** izolacija triaciglicerol estolida iz nepolarnih lipida, **4)** dodatno pročišćavanje triacilglicerol estolida upotrebom semi-preparativne metode HPLC. Svi lipidi sirastog maza izolirani su pomoću nekoliko uzastopnih koraka ekstrakcije, a za ekstrakciju je korištena smjesa metanola, kloroforma i ultra-čiste vode.

Ekstrakcija je proces koji se odvija na granici dviju faza, organske i vodene. Što je površina na kojoj se ekstrakcija odvija veća, to je veće njezino iskorištenje. Upravo je iz tog razloga prije same ekstrakcije provedena homogenizacija uzorka upotrebom vrtložne mješalice i sonikatora.

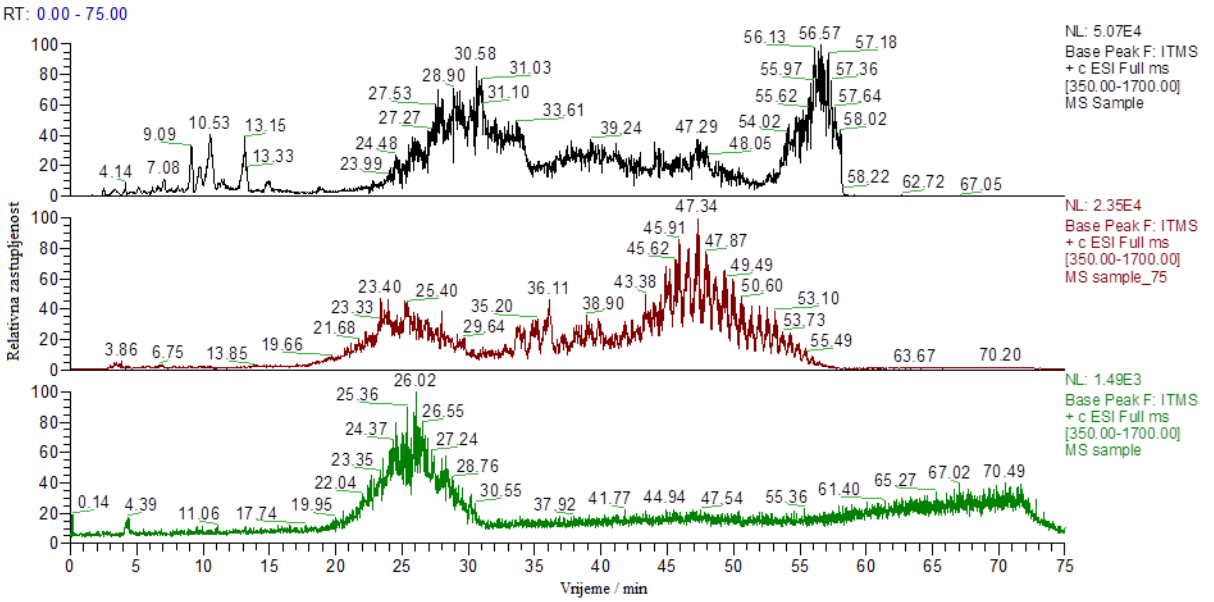
Kako se proces ekstrakcije odvijao, tako su nepolarni lipidi iz uzorka sirastog maza preneseni i otopljeni u organskoj fazi, dok su voda i proteini zaostali u vodenoj fazi. Nakon provedene ekstrakcije i nakon centrifugiranja uzorka, organska je faza odvojena od vodene upotrebom štrcaljkom. S obzirom da dva uzastopna ekstrakcijska koraka daju veće iskorištenje, odnosno veća je količina organskih nepolarnih spojeva prevedena u organsku fazu, provedena je druga ekstrakcija dobivene organske i vodene faze s istim otapalima.

Polarni membranski lipidi (pr. fosfolipidi, glikolipidi) se uobičajeno ekstrahiraju smjesom kloroforma, metanola i vode, dok se nepolarni lipidi (pr. triacilgliceroli, voštani i sterolni esteri) ekstrahiraju kloroform.[XXIV] U izoliranom lipidnom ekstraktu sirastog maza bile su prisutne sve klase lipida, stoga je dalje bilo potrebno zasebno izolirati samo nepolarne lipide. Kompleksna se smjesa lipida može frakcionirati, odnosno odvojiti kromatografijom u kojoj se odjeljivanje lipida bazira na različitoj polarnost svake pojedine klase lipida, stoga je za izolaciju nepolarnih lipida provedena semi-preparativna tankoslojna kromatografija. Nakon toga, dobiveni je uzorak sadržavao triaciglicerole, triacilglicerol estolide, kolesteril estere i voštane estere. Iako je treći korak u procesu izolacije triacilglicerol estolida sličan navedenom drugom koraku, jer je ponovno upotrebljena semi-preparativna tankoslojna kromatografija, treći je korak zbog iznimno male razlike u polarnosti između triacilglicerola i triacilglicerol estolida bio najzahtjevniji. Na kraju je četvrti korak uključivao upotrebu semi-preparativne metode

[XXIV] D. L. Nelson, M. M. Cox, *Lehninger Principles of Biochemistry*, W.H. Freeman and Company, 2008, pgs. 363, 364.

HPLC za za dodatno odvajanje i pročišćavanje estolida od male količine triacilglicerola i ostalih nečistoća prisutnih u uzorku.

Nakon svakog navedenog koraka izolacije čistoća uzorka je provjerena upotrebom metode HPLC te se proces izolacije i pročišćavanja može vidjeti na slici I. Prvi kromatogram prikazuje uzorak estolida nakon prve semi-preparativne TLC izolacije. Taj je uzorak sadržavao triacilglicerole (24 min – 33,6 min), triacilglicerol estolide (37 min – 47,3 min) i ostale nečistoće. Važno je naglasiti da je prvi kromatogram dobiven upotrebom "separacijske metode B" dok su ostala dva dobivene upotrebom "separacijske metode A", zbog čega je u usporedbi s prvim kromatogramom, svaki signal u druga dva kromatograma pomaknut u lijevo. Drugi kromatogram prikazuje uzorak estolida koji je, nakon ukoncentriravanja, pročišćen drugom semi-preparativnom tankoslojnom kromatografijom. Druga semi-preparativna metoda TLC, provedena nakon ukoncentriravanja uzorka, uklonila je triacilglicerole iz uzorka i ostavila triacilglicerol estolide (18 min – 30,5 min) i ostale nepoznate nečistoće. U zadnjem koraku izolacije, u potpunosti je pročišćen uzorak pomoću semi-preparativne metode HPLC te su ostali samo triacilglicerol estolidi, koji su dalje analizirani upotrebom metode HPLC-MS, te nakon transesterifikacije i trimetilsililacije upotrebom metode GC-MS.



Slika I Vizualna reprezentacija procesa izolacije i pročišćavanja triacilglicerol estolida iz ekstrakta nepolarnih lipida. 1) Kromatogram uzorka nakon prve semi-preparativne izolacije TLC, Separacijska metoda B 2) Kromatogram uzorka nakon druge semi-preparativne izolacije TLC, Separacijska metoda A, 3) Kromatogram potpuno pročišćenog uzorka triacilglicerol estolida dobiven nakon semi-preparativne izolacije HPLC, Separacijska metoda A.

IV. RAZVOJ METODE TLC ZA IZOLACIJU TRIACILGLICEROL ESTOLIDA IZ UZORKA NEPOLARNIH LIPIDA

Odvajanje triacilglicerol estolida od triacilglicerola i ostalih nepolarnih lipida temeljilo se na tankoslojnoj kromatografiji zbog mnogih prednosti ove tehnike. Tankoslojna kromatografija je jeftina, brza i jednostavna separacijska metoda koja ne zahtjeva upotrebu instrumenata, spojevi se mogu analizirati kvalitativno i kvantitativno te su kemijske interferencije prilikom procesiranja uzorka minimalne. Međutim, iznimno mala razlika u polarnosti između triacilglicerol estolida i triacilglicerola značajno je otežala njihovo razdvajanje. Stoga je bilo potrebno razviti novu metodu tankoslojne kromatografije za izolaciju triacilglicerol estolida od triacilglicerola i ostalih nepolarnih lipida te je prvi korak bila optimizacija mobilne faze. Ukupno je testirano 17 mobilnih faza na način da su jedan pored drugog na TLC pločici postavljeni ekstrakt nepolarnih lipida te standardi trioleina i triacilglicerol estolida.

Najbolje rezultate u smislu separacije, koncentracije spojeva na jednom mjestu i vidljivosti spojeva pod UV lampom dala je smjesa heksana, dietil etera i toluena u volumnom omjeru 92,5: 7,5: 7,5.

S obzirom da za izolaciju nisu korištene komercijalno dostupne silika gel pločice TLC, nego su korištene samostalno pripremljene pločice TLC, način njihove pripreme imao je veliku važnost u odvajanju i analizi uzorka tankoslojnom kromatografijom. Pločice TLC su pripremljene sa i bez upotrebe mehaničkog uređaja za raspodjelu silika gela na staklene pločice te je svaka pločica zbog prisutnosti ljudske greške bila neznatno drugačija, odnosno postojale su male razlike između njih. Razlike u pripremljenim pločicama TLC, koje mogu utjecati na odvajanje spojeva i konzistentnost rezultata, uočene su debljini sloja silika gela, gustoći silika gela, metodi pripreme pločica TLC i aduktima prisutnim u silika gelu. Nakon što je određena mobilna faza koja osigurava najbolje odvajanje spojeva, plan je bio dodatno poboljšati separaciju pa je od iznimne važnosti bilo osigurati konzistentnost rezultata. Zbog toga je razvijena metoda pripreme pločica TLC. Testirana su tri načina pripreme pločica TLC te su konzistentnost i značajnost rezultata provjereni statističkom analizom podataka.

Statistička analiza podataka temeljena je na dobivenim vrijednostima R_f standarda trioleina i triacilglicerol estolida. Iz dobivenih vrijednosti R_f određeni su srednja vrijednost R_f ($\overline{R_f}$), standardna devijacija (s), standardna devijacija svake točke izražena kao postotak

odstupanja od pojedine vrijednosti R_f (s[%]) i njezina srednja vrijednost ($\overline{s\,[\%]}$), relativna standardna devijacija (RSD), raspon vrijednosti R_f (w), apsolutna (E) i relativna pogreška (Er) i "Test Tn" te je na temelju tih podatak određena konzistentnost metode i značajnost dobivenih rezultata.

Tablica I prikazuje sumarne rezultate provedene statističke analize za sve tri metode pripreme silika gel pločica TLC. Pločice TLC s impregniranim metalom u silika gelu dale su rezultate najveće konzistentnosti s najmanjom greškom, zatim slijedi "standardna" metoda, i na kraju, najlošije rezultate dala je "brza" metoda pripreme pločica TLC.

Tablica I Usporedba rezultata statističke analize podataka dobivenih mjerenjem vrijednosti *R*f standarda trioleina i standarda triacilglicerol estolida na samostalno pripremljenim silika gel pločica TLC

Usporedba	N	$\overline{R_f(TO)}$	*s* (TO)	$\overline{s\,(TO)/[\%]}$	RSD	w	$\overline{R_f(Est)}$	*s* (Est)	$\overline{s\,(Est)/[\%]}$	RSD	w
„Standardna" priprema TLC pločica	21	0,23	0,0382	17,08	166	0,1285	0,11	0,021	18,8	181	0,0824
„Brza" priprema TLC pločica	20	0,27	0,0534	20,68	199	0,1934	0,14	0,0407	31,63	291	0,1604
Priprema TLC pločica s metalnim kationom	15	0,33	0,023	7,04	70	0,0661	0,19	0,0151	7,84	78	0,039

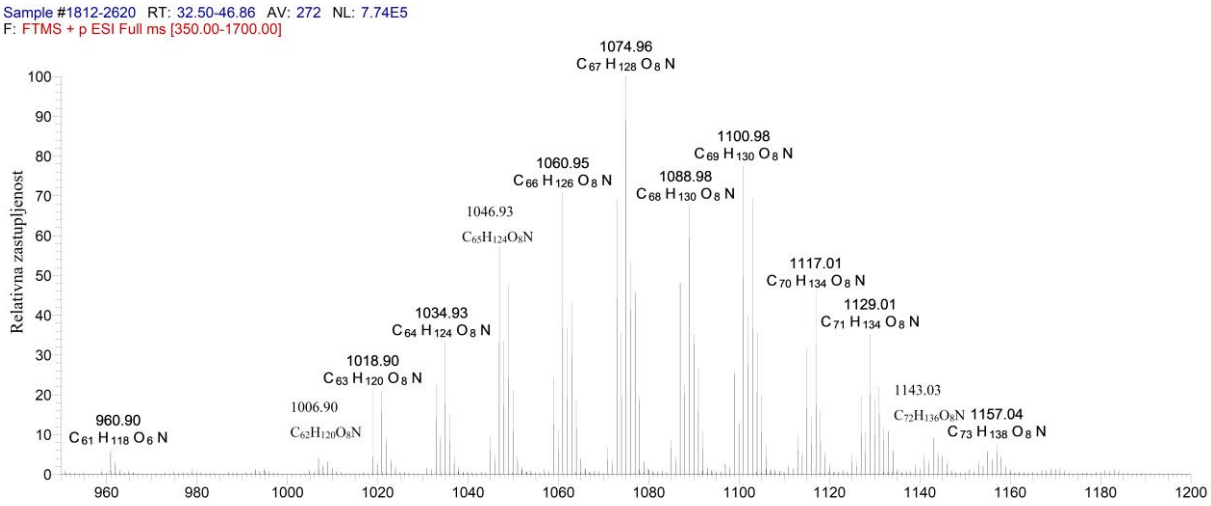
V. ANALIZA HPLC-ESI/MS

Dva različita instrumenta (LCQ Fleet i LTQ Orbitrap) i dvije različite separacijske metode ("Separacijska metoda A" i "Separacijska metoda B") upotrebljeni su za separaciju HPLC-MS, pročišćavanje i analizu triacilglicerol estolida sirastog maza. LCQ Fleet s ionskom stupicom 3D je nakon druge semi-preparativne izolacije TLC korišten za dodatno pročišćavanje uzorka triacilglicerol estolida, dok je LTQ Orbitrap zbog puno veće rezolucije i preciznosti određivanja mase korišten za analizu spektara masa.

"Separacijska metoda B" dala je čišće, jasnije i oštrije signale u usporedbi sa "Separacijskom metodom A". Iako elucija spojeva u "Separacijskoj metodi A" počinje ranije te je gradijentni program 10 minuta kraći, "Separacijskom metodom B" postignuto je bolje razdvajanje triacilglicerol estolida iz kompleksne smjese. Nadalje, signali u spektrima masa bili su većih intenziteta ($7{,}74 \cdot 10^5$ za "B" i $6{,}6 \cdot 10^5$ za "A"), što ne začuđuje s obzirom da je amonijev format, zbog kojeg se stvaraju molekulski adukti, dio mobilne faze, dok se on u "Separacijskoj metodi A" u izvor ESI uvodi zasebno. Stoga je za analizu spektara masa, zbog boljeg odvajanja triacilglicerol estolida iz kompleksne smjese i većeg intenziteta signala u masenim spektrima, odabrana "Separacijska metoda B".

Spektar masa svih triacilglicerol estolida može se vidjeti na slici II. Na njemu su naznačeni samo najzastupljeniji molekularni adukti $[M+NH_4]$ u određenom području masa. Međutim, analizom svakog pojedinog signala identificirano je 126 spojeva u rasponu *m/z* 934,85-1193,08.

Najzastupljeniji molekulski adukt ima *m/z* 1074,96. Djelomična struktura triacilglicerol estolida određena je analizom spektara MS^2 i MS^3 tog spoja. Ono što se nakon te analize moglo zaključiti je da triacilglicerol estolid s molekulskom formulom $C_{67}H_{124}O_8$ može biti izgrađen od jako velikog broja kombinacija masnih kiselina i OAHFA-a. U tablici II navedene su 42 kombinacije koje mogu izgraditi navedeni triacilglicerol estolid i to je samo ako se pozicija masnih kiselina i OAHFA-a ne uzme u obzir. U suprotnom, ako se pozicija masnih kiselina i OAHFA-a uzme u obzir, postoje 234 kombinacije koje mogu dati navedeni spoj. Broj mogućih kombinacija naglo raste ako se još uzme u obzir pozicija dvostruke veze i pozicija hidroksi skupine. Dakle, 126 identificiranih triacilglicerol estolida navedenih u TABLICI 10 zapravo čini smjesu strukturnih izomera te je stoga teoretski moguće postojanje tisuća različitih triacilglicerol estolida u uzorku sirastog maza.



Slika II Spektar masa FTMS p +ESI Full MS svih triacilglicerol estolida iz uzorka sirastog maza. Uzorak je snimljen upotrebom LTQ Orbitrapa u pozitivnom modu.. Retencijsko vrijeme u kojem su triacilglicerol estolidi uočeni; 32,5-46,86 min, raspon *m/z*: 350,00-1700,00

Tablica II Masne kiseline i (*O*-acil)-*ω*-hidroksi masne kiseline identificirane na temelju spektara MS^2 i MS^3 spoja $[M+NH_4]^+$ s *m/z* 1074.96 i njihove moguće kombinacije koje bi dale TG-estolid [M] *m/z* 1056,94 ukoliko redoslijed masnih kiselina na glicerolu nije uzet u

Masna kiselina	OAHFA	TG-Estolid s *m/z* 1056.94
14:0	31:0	14:0_16:0_37:2
14:1	32:0	14:0_16:1_37:1
15:0	33:1	14:1_16:0_37:1
15:1	33:2	14:1_16:1_37:0
16:0	34:0	14:0_17:0_36:2
16:1	34:1	14:0_17:1_36:1
17:0	34:2	14:1_17:0_36:1
17:1	35:0	14:1_17:1_36:0
18:0	35:1	14:0_18:0_35:2
18:1	35:2	14:0:18:1_35:1
	35:3	14:1_18:0_35:1
	36:0	14:1_18:1_35:0
	36:1	15:0_15:0_37:2
	36:2	15:1_15:0_37:1
	36:3	15:1_15:1_37:0
	37:0	15:0_16:0_36:2
	37:1	15:0_16:1_36:1
	37:2	15:1_16:0_36:1
	37:3	15:1_16:1_36:0
	37:4	15:0_17:0_35:2
		15:0_17:1_35:1
		15:1_17:0_35:1
		15:1_17:1_35:0
		15:0_18:0_34:2
		15:0_18:1_34:1
		15:1_18:0_34:1
		15:1_18:1_34:0
		16:0_16:0_35:2
		16:0_16:1_35:1
		16:1_16:1_35:0
		16:0_17:0_34:2
		16:0_17:1_34:1
		16:1_17:0_34:1
		16:1_17:1_34:0
		16:0_18:0_33:2
		16:0_18:1_33:1
		16:1_18:0_33:1
		16:1_18:1_33:1
		17:0_17:0_33:2
		17:1_17:0_33:1
		17:1:18:1_32:0
		18:1_18:1_31:0

VI. ANALIZA GC-MS

Nakon provedene analize intaktnih triacilglicerol estolida upotrebom tehnike HPLC-ESI/MS, provedena je i analiza masnih kiselina koje ih grade tehnikom GC-MS. S obzirom da triacilglicerol estolidi nisu hlapljivi, što ih čini neprikladnim za analizu GC-MS, najprije su prevedeni u metilne estere masnih kiselina. Metilni esteri masnih kiselina generirani su reakcijom transesterifikacije s acetil kloridom.[XXV] S obzirom da su nakon transesterifikacije uočene i slobodne masne kiseline, one su također esterificirane i to upotrebom trimetilsilil diazometana.[XXVI] U zadnjem su koraku hidroksilne skupine u metilnim esterima masnih kiselina sililirane s *N*,*O*-bis(trimetilsilil)acetamidom kako bi se odredila njihova pozicija u acilnom lancu.

Na temelju retencijskih vremena standarda metilnih estera masnih kiselina i spektara masa te uz pomoć baze podataka NIST, određeni su metilni esteri masnih kiselina iz uzorka sirastog maza. Ukupno je određeno 71 metilni ester masnih kiselina, odnosno 71 masna kiselina koje izgrađuju triacilglicerol estolide sirastog maza te su one navedene u tablici III. Određen je širok spektar masnih kiselina koje grade triacilglicerol estolide: potpuno zasićene masne kiseline s acilnim lancima u rasponu od 9 do 28 ugljikovih atoma, širok spektar mono- i di-nezasićenih masnih kiselina, razne razgranate masne kiseline s grananjem obično na kraju lanca te zasićene hidroksi masne kiseline s hidroksi skupinom u α ili ω poziciji. Najzastupljenija masna kiselina koja gradi triacilglicerol estolide je palmitat (C16:0), što je bilo i za očekivati s obzirom da je jedini produkt djelovanja sintaze masnih kiselina 1, prisutne u kralježnjacima, upravo palmitat. Ostale su masne kiseline, koje grade triacilglicerol estolide, najvjerojatnije rezultat djelovanja širokog spektra enzima prilikom stvaranja sirastog maza u zadnjem tromješjećju trudnoće

[XXV] K. Stránsky, T. Jursik, *Lipid/Fett* **98** (2006) 71–77.
[XXVI] A. Presser, A. Hüfner, *Monatshefte fur Chemie* **135** (2004) 1015–1022.

Tablica III Masne kiseline koje grade triacilglicerol estolide sirastog maza određene analizom GC-MS

Retencijsko vrijeme / min	Struktura	Retencijsko vrijeme / min	Struktura
6,04	C9:0	21,172	C18:1n7c, C18:1n7t
6,922	C10:0	21,52	C18:2n9t; C?-grananje
8,863	C12:0; C10-grananje	21,627	C19:0; C17-grananje ili C19:0; C11-grananje
9,172	C13:0; C4-grananje	21,828	C18:2n6c ili C18:2n7tc ili C18:2n4t
9,339	C12:0	21,969	vjerojatno hidroksi masna kiselina ili oksidirana dvostruka veza
9,881	C13:0; C4-grananje	22,611	C19:0
10,366	C13:0; C10-grananje	22,71	C15:0; C2-OH
10,907	C13:0	22,968	C19:1n9c
11,5	C13:0; C11-grananje	23,615	C20:0; C18-grananje
11,802	C14:0; C12-grananje	23,735	C16:0; C2-OH
12,663	C14:0	24,459	C20:0
13,153	C14:1n3c	24,72	C16:0; C2-OH
13,298	C15:0; C12-grananje	24,974	C20:1n9c
13,388	C14:1n5c	25,144	C20:1n9t
13,639	C15:0; C13-grananje	25,875	C21:0; C18-grananje ili C21:0; C4-grananje
13,928	C15:0; C12-grananje	26,148	C18:0; C3-OH
14,095	C15:1n5t; C13-grananje	26,483	C21:0 ili C21:0; C4-grananje
14,568	C15:0	27,429	C22:0; C20-grananje
15,036	C15:1n5c	28,184	C16:0; C9-OH
15,229	C16:0; C4,8,12-grananje	28,333	C22:0
15,594	C16:0; C14-grananje	28,76	C22:1n9c
16,032	C16:1n6t; C14-grananje	28,923	C22:1n11c
16,566	C16:0	29,073	C16:0; C16-OH
17,007	C16:1n7c	29,488	C22:2 (Δ5,Δ13)c
17,106	C16:1n7t	29,579	C23:0; C20-grananje ili C23:0; C21-grananje
17,358	C16:1n5t	29,987	C20:2n7t
17,598	C17:0; C15-grananje	30,149	C23:0
17,695	C16:1n9t	31,026	C23:1n5c
17,917	C17:0; C14-grananje	31,335	C20:0; C2-OH
18,344	C17:1n7t; C14-grananje	31,896	C24:0
18,582	C17:0	33,062	C25:0
19,228	C17:1n7c	34,413	C26:0
19,627	C18:0; C16-grananje	34,8	C22:0; C2-OH
20,017	C18:1n12c	36,531	C28:0
20,612	C18:0	38,89	C24:0; C2-OH
21,02	C18:1n9t ili C18:1n10t		

§ 1. INTRODUCTION

Vernix caseosa (VC) is waxy white substance that covers the skin of the human fetus during the last trimester of pregnancy and it consists of water (80 %), proteins (10 %) and lipids (10 %).[1–5] VC forms on the surface of the developing stratum corneum (SC).[6] In fact, it is believed that VC cells derive from SC corneocytes,[7] which is why it is not surprising that a lot of similarities have been noticed in the lipid composition between VC and SC.[8] VC has a lot of different functions and roles: it facilitates the formation of SC,[9] it has a proven activity against fungal and bacterial pathogens,[10,11] protects the fetus from the amniotic fluid and helps regulating, that is maintaining its temperature immediately after birth,[1,6] and it has wound healing, skin hydrating and skin cleansing abilities.[9,12] First composition analysis of VC was conducted in 1939. by dr. Phil Rudolf Schmid[13] and first detailed free lipid composition was provided by Käerkkärinen in 1965.[14] So, to this day a lot of different lipid classes had been characterized in VC,[1,2,7,15,16] but now, a new class of lipids have been hypothesised; triacylglycerol estolides (TG-estolides).

In general, estolides are compounds derived from fats and oils which structure is identified by the secondary ester linkage of one fatty acyl molecule to the alkyl backbone of another fatty acid fragment.[17] They can occur in the form of free fatty acids, esters or triacylglycerols. Meyer was the first to mention estolides in 1897. when he discovered them in *Castor* oil.[18] Occurring in plants[19–24] and some mammals,[25–27] TG-estolides were, till this day, reported in handful of papers, so their structure and exact role is still quite unknown.

Since there are no papers on complete isolation and structural elucidation of VC TG-estolides, the goal of this work is to develop a complete semi-preparative TLC method for isolation and semi-preparative HPLC method for additional purification of VC TG-estolides. That will consist of testing different mobile phases for TLC and different TLC plate preparation techniques, and testing two different HPLC methods, which differ in gradient programs and mobile phase systems. After VC TG-estolides isolation will come their structural elucidation in their intact form with HPLC-ESI/MS and analysis of fatty acids that build them with GC-MS in form of fatty acid methyl esters (FAMEs).

§ 2. LITERATURE REVIEW

2.1. Vernix caseosa

Vernix caseosa (VC) is a creamy, cheese-like layer that covers the fetal skin during the last trimesters of the pregnancy (Figure 1).[1–3] It mainly consist of water (80 %), but a complex mixture of proteins (10 %) and lipids (10 %) is also a part of VC.[2,4,5] Until recently when it was discovered on the skin of a new-born sea lions,[28] VC has always been considered a substance formed uniquely on the skin of human fetus.[29,30]

Figure 1 Vernix caseosa covering the skin of a new-born baby[6]

The human epidermis, which is the outermost layer of the skin, is composed of four major layers; basal, spinous, granular and stratum corneum (SC) (Figure 2).[31] SC is the outermost and most important layer of epidermis, because it serves as a physical barrier from the environment.[5] That physical barrier is comprised of corneocytes, desmosomes (intracellular protein connections), nucleated epidermis cells and lipid enriched intracellular domains which

major components are cholesterol (CHOL), free fatty acids (FFA) and ceramides (Cers).[15,31,32] Comprising 50 % of all SC lipids,[33] Cers are the most abundant lipids in SC and have a crucial role in the characteristic organization of SC and in the prevention of trans-epidermal water loss (TEWL) both prenatally and postnatally,[7,15,34–36] which is why they are considered the key barrier lipids.[35] Even though the main role of SC is to prevent the loss of water,[5] skin of a new-born is susceptible to TEWL and skin trauma,[37] so additional protection is needed.

The formation of vernix takes place on the surface of SC proceeding from head to toe and from back to front,[6] and it is believed that it facilitates the formation of SC itself in the last three months of gestation, through yet unknown mechanism.[9,12] Maternal and placental hormones have the main role in controlling the formation and coverage of vernix on the entire skin surface of the baby. Vernix is removed from the skin after birth by washing the baby, but if not removed in this way, vernix would detach from the skin by itself with process similar to SC desquamation (shedding of the outermost membrane of a tissue).[12,38] Since vernix can already be noticed around and on the eyebrows during the seventeenth week of gestation,[9] it is plausible that vernix cells originate from hair follicles.[39]

A lot of similarities, but also some differences in the composition of VC and SC have been noticed. Previous studies showed that VC consist of hydrophilic corneocytes embedded in a lipid matrix, which is why is suggested that VC cells actually derive from detached SC corneocytes. But, unlike in SC, cellular part of vernix doesn't contain desmosomes and lacks lamellar lipid organization.[7,12,40,41] When it comes to similarities in the lipid composition of VC and SC, all major SC lipids have been noticed in VC. Those similarities were determined with HPTLC analysis conducted by Sumida et al.[8] However, the main lipids in SC are FFAs, CHOL and Cers, while major fractions of lipids in VC contain sterol esters (SE), wax esters (WE) and triacylglycerols (TGs).[7,14,32,42] Rissmann et al.[13] showed, using scanning electron microscopy in cryo-mode (Cryo-SEM) and freeze-fraction electron microscopy (FFEM), that in VC a large lipid pools in regions of clustered corneocytes are present. Those lipid pools haven´t been observed in SC. The absence of corneodesmosomes in VC allows the separation of corneocytes, which is not the case in SC. By analysing fatty-acid methyl esters (FAMEs) of VC lipid extract with GC they also determined that a majority of SC lipids have straight, unsaturated fatty-acid chains, while most of the fatty-acid chains in VC are branched. And finally, when considering the composition of Cers, which are, as mentioned before, the main barrier lipids with the role of preventing the TEWL, it has been determined that all ceramide classes present in SC are, as

well, present in VC, with the exception of Cer(NP), which are more abundant in the SC, than in VC.[7]

As said before, VC consists of a complex mixture of proteins and lipids. And even though the first composition analysis of the VC was conducted in 1939 by dr. phil. Rudolf Schmid[13] and the first detailed free lipid composition was provided by Käerkkärinen[14] in 1965., scientists are still trying to completely characterize the chemical constituents of VC proteins and lipids, and determine their roles.[1,2,7,15,16,30,40,43]

While roles of the individual constituents of VC lipids and proteins are still being determined, it is known that VC, as a whole, has a lot of diverse functions and roles. Besides

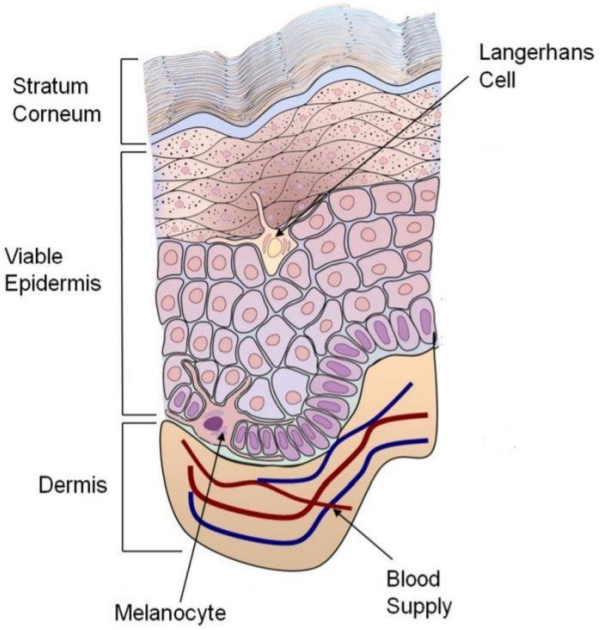


Figure 2 Composition of human epidermis[5]

aforementioned roles of VC as a facilitator of the SC formation and as protectant from the TEWL, a lot of other functions have been discovered. First of all, VC samples were found to be sterile.[44,45] Also, since it contains lysozyme (an antimicrobial enzyme produced by animals), lactoferrin (globular glycoprotein which is one of the component of immune system) and some other anti-microbial peptides, it has a proven activity against fungal and bacterial pathogens.[5,10,11] Other known functions are: anti-oxidant,[46] protection of the fetus from the amniotic fluid, temperature regulation and post-birth adaptation,[1,6] and finally, wound healing,[9] skin hydration[6,9] and skin cleansing,[9,12] which can be used in the pharmaceutical industry for production of skin preparatives .

2.2. Estolides

2.2.1. What are estolides?

Estolides are compounds derived from fats and oils. Their structure is identified by the secondary ester linkage of one fatty acyl molecule to the alkyl backbone of another fatty acid fragment.[17] The formation of that secondary ester linkage can occur either on the hydroxyl moiety of another fatty acid fragment by carboxylic substitution or on the double bond of unsaturated fatty acid by addition reaction. Addition of fatty acyl molecules can, in that way, continue as long as another fatty acid fragment have either hydroxyl moiety or double bond in its alkyl backbone, and such estolides, comprised of multiple hydroxy fatty acids, were found in the seed oils of *Trewia nudiflora* and *Mallotus phillipensis*.[19,27] The extend of oligomerization of estolide molecule is represented or defined with estolide number (EN), which is the average number of fatty acids added to a base fatty acid (Figure 3).[47]

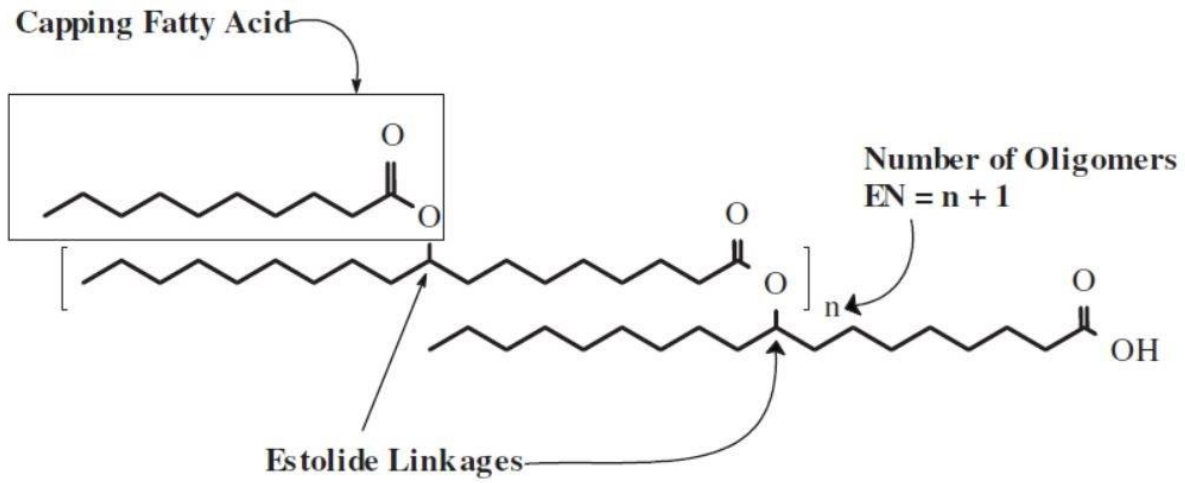


Figure 3 Free fatty acid estolide capped with C_{10} saturated fatty acid with an estolide number of 2 (EN=2)[39]

Structurally speaking, estolides can be in the form of three different classes: as free fatty acids (Figure 4), as esters (Figure 5) or formed within a triacylglycerols (Figure 6).

Even though first mention of estolides dates back to 1897. when they were discovered in *Castor* oil by Meyer[18], their structure, especially the structure of TG-estolides, and exact role in nature, where they occur mostly in plants, are still quite unknown.[19–24,48] Moreover, by 2011. TG-estolides have been reported in only 11 plants,[47] and only a handful of papers report TG-estolides in animal tissue.[25–27,49] It is known that TG-estolides are components of the storage oil

of certain plant and fungal species.[50] One such example is seed oil from *Lesquerella auriculate*, in which 96 % of oil was found to be in the form of estolides.[23,51]

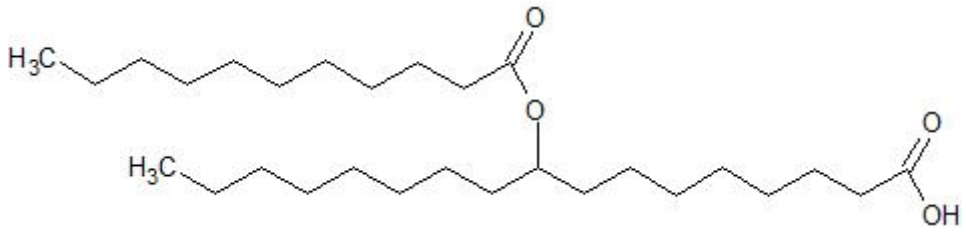


Figure 4 Free fatty acid estolide

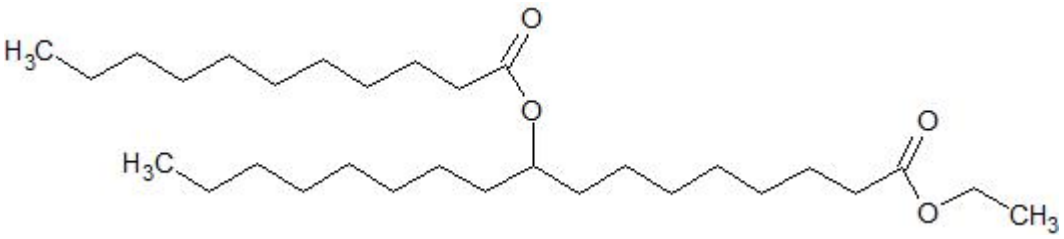


Figure 4 Ethyl ester estolide

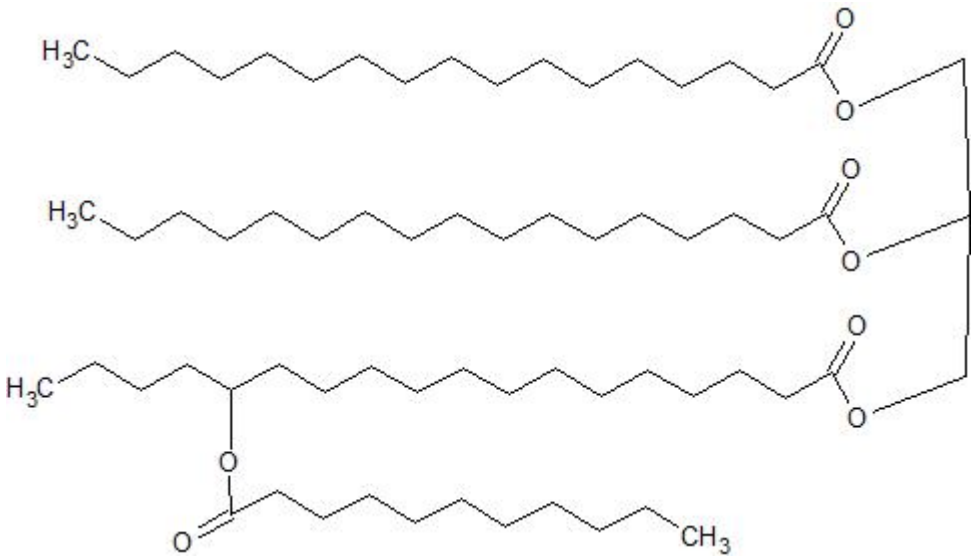


Figure 6 Triacylglycerol estolide

2.2.2. Physical and chemical properties of estolides

Physical properties of estolides have been investigated for quite some time, so they are known to have some extraordinary properties regarding their use as hydraulic fluids or lubricants. Since they derive from vegetable-based oils, which have been in use for years,[52–55] we can take a look at their properties from that point of view and see the improvements that they have over vegetable-based oils.

However, achieving those improvements is quite challenging, since the structure of estolide itself defines its properties. Some of the property defining features are chain length of base unit and capping material, and unsaturation of capping material. Cermak and Isbell had the greatest contribution to the investigation of physical properties of estolides, and they investigated pour points, cloud points, viscosities, oxidative stability, pKa value and gardner colour of estolides.[47,56–61]

The pour point is defined as the lowest temperature at which the sample is still in its liquid form and the cloud point as the lowest temperature at which the sample is opaque. This is really important for the engine or hydraulic pump to function properly at low temperatures, so the lower the pour point and cloud point, the better the properties of oil as lubricant. In the paper which compares physical properties of mono-capped with full-capped *Lesquerella* and *Castor* oil estolides[57], they reported two things; pour points improved, meaning that they decreased linearly when the shorter saturated fatty acid capping chain lengths were esterified with the hydroxy TGs and that the full-capped (meaning that all hydroxyl moieties within the TG are esterified) *Lesquerella* TG-estolides had slightly lower pour points for all cases than the mono-capped estolides. In another paper,[47] Isbell reported that estolides with mid-chain linkages will provide better low temperature properties and that molecular weight impacts low temperature properties, with higher molecular weight molecules yielding higher pour points. Generally speaking, varying the capping material on the estolide, which affects the crystal lattice at low temperatures, can lead to forming an estolide ester with low-temperature properties that are far more superior to other petroleum and vegetable-based oils.[17,62]

Besides low temperature properties, oxidative stability, pKa value and viscosity have great importance when it comes to lubricants. Due to the unsaturation of chains present in the structure of vegetable oils, they have poor oxidative stability. As said before, one way of forming an estolide is by adding a fatty acid to the double bond of another fatty acyl chain in its backbone. This, in turn, removes unsaturation of the fatty acyl chain and thus improves oxidative stability.[57] pKa value is dependent on the chain length. Fatty acids with shorter chains have lower pKa values, and therefore are more acidic. That can also affect the EN in a way where more acidic solution gives higher EN.[17] And finally, when considering viscosity, estolides again have far better properties compared to vegetable-based and mineral oils. Viscosity of lubricants is measured with so called VI value, which is a lubrication oil arbitrary quality indicator, or a measure of the change of kinematic viscosity with temperature. To put it

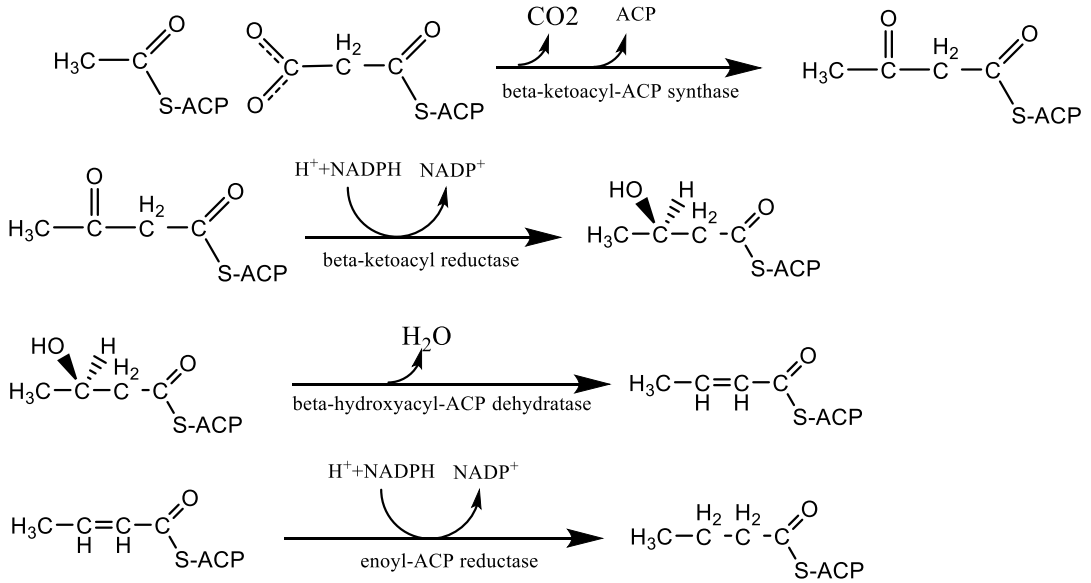
simply, VI shows how close the viscosities of a material are at 40 °C and 100 °C. The ideal material has the same viscosity at all temperatures. For comparison, most vegetable-based oils have VI value less than 100, while on the other hand, VI values of estolides can be higher than 200.[17]

2.2.3. Biosynthesis and biological significance of estolides

Even though TG-estolides are not so widespread in nature, that is in plants and animals, simpler estolides are, especially the ones derived from fatty acids. Two most abundant classes of fatty acid estolides are fatty acid esters of hydroxy fatty acids (FAHFAs) and (*O*-acyl)-*ω*-hydroxy fatty acids (OAHFAs).

Since estolides are derivatives of fatty acids, their biosynthesis, as well as synthesis of fatty acids occurs in the cell cytosol. In all organisms, the long carbon chains of fatty acids are assembled in a repeating four-step sequence represented in scheme 1: **1)** Claisen condensation of activated acetyl and malonyl groups forming acetoacetyl-ACP (acetoacetyl attached to Acyl carrier protein, which is the shuttle that holds the system together), **2)** Reduction of the carbonyl group at C-3 catalyzed by β-ketoacyl-ACP reductase forming D-β-hydroxybutyryl-ACP, **3)** Dehydration

catalyzed by β-hydroxyacyl-ACP dehydratase, which forms a double bond in the product, trans-Δ2-butenoyl-ACP, **4)** Reduction of double bond by enoyl-ACP reductase. A formed saturated acyl group becomes a substrate for subsequent condensation with an activated malonyl group.[63]



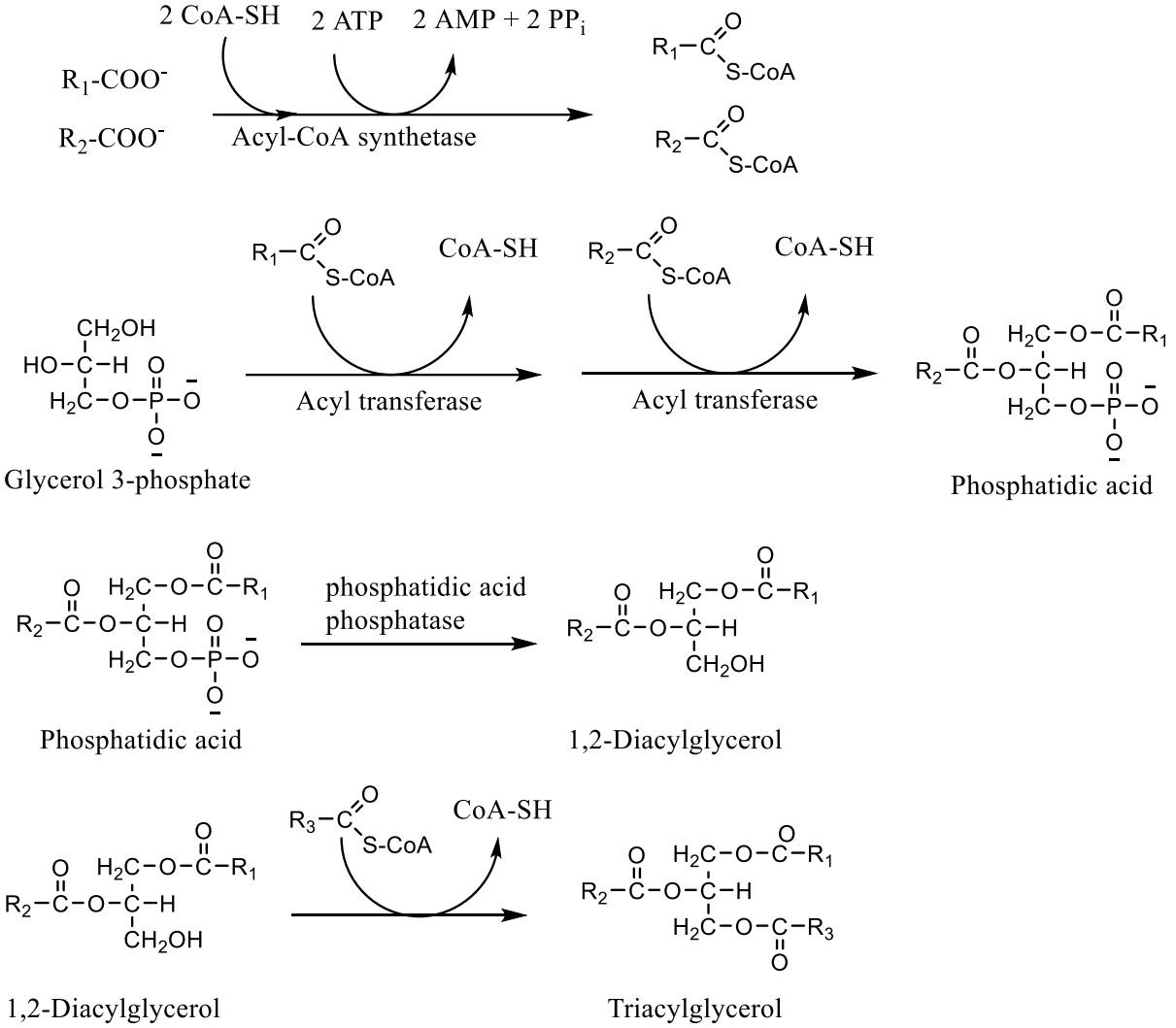
Scheme 1 Four basic repeating steps in the fatty acid biosynthesis

Fatty acids with even number of C-atoms are synthesized in that way, but fatty acids with odd number of C-atoms can be synthesized as well. Instead of acetyl-CoA, propionyl-CoA is used as the primer for the synthesis of long-chain fatty acids with an odd number of carbon atoms, which are found particularly in ruminant fat and milk.[63]

All enzymes involved in the biosynthesis of fatty acids are collectively referred to as fatty acid synthase (FAS). There are two major variants of FAS: FAS 1 found in vertebrates and fungi, and FAS 2 found in plants and bacteria. With FAS 1 systems, fatty acid synthesis leads to a single product (palmitate, 16:0) and no intermediates are released. Unlike FAS 1, FAS 2 generates a variety of products, including saturated fatty acids of several lengths, as well as unsaturated, branched, and hydroxy fatty acids. [64] There are two possible ways for vertebrates to obtain fatty acids with chains longer than C16; exogenously from food[65] and endogenously by synthesis with fatty acid elongation systems present in the smooth endoplasmic reticulum and in mitochondria.[66] The rate-limiting condensation step in this system is catalyzed by 1 of the 7 enzymes of the elongase of long chain fatty acid family (ELOVL1–ELOVL7), each member of which demonstrates its own substrate specificity. The only ELOVL that has been shown to have activity in in vitro studies toward substrates >C26 in length is ELOVL4.[67,68] ELOVL4 is required for synthesis of diverse lipid species containing very long chain FAs which are comprised of more than 28 carbons. Synthesis of such lipids occurs endogenously in the tissues where they are found; and loss of the unique lipid biosynthetic products of ELOVL4 can have significant biological consequences within the tissues where they are synthesized.

Most of the synthesized fatty acids have two fates: incorporation into triacylglycerols for storage of metabolic energy or incorporation into the phospholipid components of membranes. Biosynthesis of triacylglycerols (Scheme 2) occurs in several steps with two precursors: L-glycerol 3-phosphate derived from glycolytic intermediate dihydroxyacetone phosphate, and fatty acyl-CoAs, formed from fatty acids by acyl-CoA synthetases. The first step in the biosynthesis of triacylglycerols is the acylation of the two free hydroxyl groups of L-glycerol 3-phosphate by two molecules of fatty acyl-CoA to yield phosphatidic acid. Phosphatidic acid can then be converted either to triacylglycerol or to a glycerophospholipid. In the triacylglycerol pathway, phosphatidic acid is hydrolyzed by phosphatidic acid

phosphatase to form 1,2-diacylglycerol, which is then converted to triacylglycerols by transesterification with a third fatty acyl-CoA.[69]



Scheme 2 Biosynthesis of triacylglycerols

The term FAHFA was first used by Kahn et al.[25] in 2014. to describe a newly discovered endogenous mammalian lipids and Butovich[70] first confirmed OAHFAs in human meibum. Even though they belong to the same class of lipids; fatty acid esters of hydroxy fatty acids, there are distinct differences between them, which consequently means that they have different biochemistry. FAHFAs typically have acyl chains which are 16-18 carbons long and a single degree of unsaturation. On the other hand, OAHFAs have ultra-long fatty acid chains with 26-34 carbons and their hydroxy esterification is believed to be solely at the terminal, that is ω-position.[49] After the fatty acid biosynthesis, subsequent synthesis of FAHFAs include additional two steps, which are formation of hydroxy fatty acid (HOFA) catalyzed by hydrolase and

esterification of HOFA to FAHFA with FA acyltransferase.[25] The synthesis of OAHFAs requires additional three steps; **1)** Synthesis of very-long chain fatty acid (VLCFA), catalyzed by ELOVL4, **2)** oxidation and generation of ω-hydroxy fatty acid by microsomal ω-hydroxylase, **3)** Acyl transfer to the ω-hydroxy group of VLCFA by acyl-transferase.[70–72]

FAHFAs and OAHFAs are found in adipose tissue[25], meibum[73], amniotic fluid[71], equine sperm[74], plant food[75] etc. and they have extreme biological significance, and diverse and beneficial roles. Most significant biological characteristics of FAHFAs are anti-inflammatory and insulin-sensitizing potential.[25,75] Other known functions are: glucose uptake increase through the GLUT4 transporter, increase in de novo lipogenesis, decrease in free fatty acids production thus delaying diabetes onset[75] and they also have a potential in treating colon carcinoma.[76] On the other hand, OAHFAs are most abundant in meibum, so their main role is to spread and stabilize the meibomian lipid tear film by forming the interphase between its lipid and aqueous sublayer.[72,77] OAHFAs, as well as FAHFAs, have also anti-inflammatory and anti-diabetic effects through enhanced insulin production and glucose tolerance.[25,78] And finally, as amphiphiles, OAHFAs are suggested to have great surfactant properties, and as such may play a unique role in the functions of amniotic fluid in fetal development.[71,77]

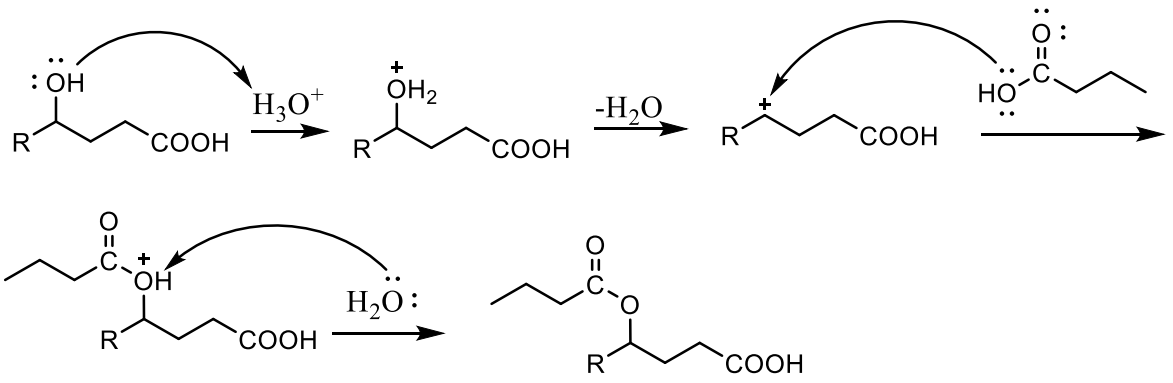
2.2.4. Synthesis of estolides

While estolides are mostly synthesized in nature by higher plants, which produce a wide variety of HOFAs and unsaturated FAs,[79] and some adipose tissues in animals synthesize them as well,[25–27] much attention has been payed to commercial synthesis of estolides, due to their extraordinary properties.

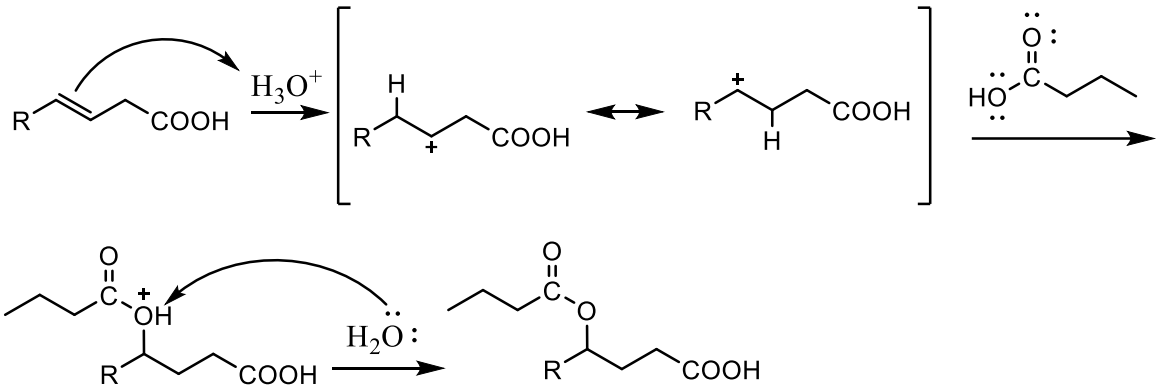
Penoyer was a pioneer in the field of estolide synthesis. He has been able to synthesize the simplest estolides by homopolymerization of ricin oleic acid which had hydroxyl moiety attached to the 12th carbon atom under high temperature (250 °C) with CO_2 sparge.[80] After that point, a lot of other methods for estolide synthesis have been developed, which in turn allowed the production of estolides with much more complex structure.[58–62] Today, the most synthesized estolides are the ones derived from triacylglycerols of *Castor* and *Lesquerella* oil, because of their aforementioned properties. The high production started with Lawate´s patent which describes the synthesis of TG-estolides from *Castor* and *Lesquerella* with heptanoic, iso-stearic, adipic and fumaric acids as the capping fatty acid using p-toluene-sulfonic acid as the catalyst.[17,47,81]

There are many different estolides that can be produced from any number of fatty acids, as long as they have hydroxyl moiety or double bond in their acyl backbone. Generally, there are two basic ways of making them; acid-catalyzed[17,58–62] and enzyme-catalyzed.[82–84] While acid-catalyzed estolide synthesis is much more widely used, both methods are capable of inexpensively preparing large amounts of product. Nevertheless, they do have some limitations. Harsh conditions in acid-catalyzed synthesis can easily destroy inserted functional groups in reverse esterification reaction. This can be avoided by the use of enzymes, but a limitation here is the compatibility of those functional groups with the active site of an enzyme. Additionally, both reaction routes are subject to equilibrium which produces monomers.[85] Considering that, there are more and more methods and improvements being reported and the highest contribution in that field again had Cermak and Isbel. They synthesized to this date, many different estolides from different seed oils and improved synthesizing methods and yields by testing various heterogenous and homogenous catalysts.[17,47,56–59,61,62,86]

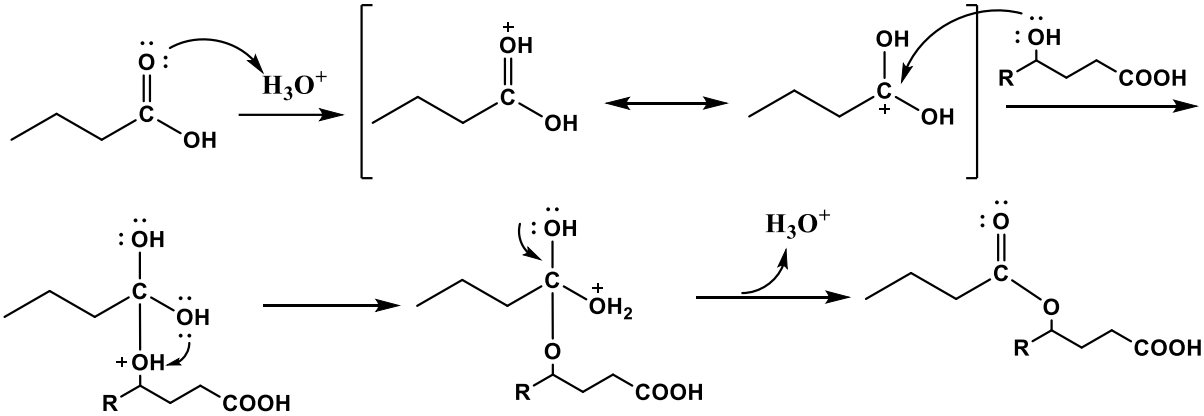
As said before, estolides can be formed from unsaturated and hydroxy fatty acids. When considering the mechanism of that formation, there are three possible routes of estolide synthesis, which are depicted in scheme 3, scheme 4 and scheme 5. Carbocation formation is included in two out of three mechanistic routes. The difference between the two involving carbocation formation is that in hydroxy fatty acids, carbocation is formed by the elimination of water, while in unsaturated fatty acids carbocation is formed by protonation of double bond. That carbocation then undergoes nucleophilic addition by another fatty acid, with or without carbocation migration along the length of the chain, to form an ester linkage. The third possible mechanism is nucleophilic carboxylic substitution, where hydroxyl moiety attacks carboxylic carbon, after which the elimination of water as leaving group occurs.



Scheme 3 Mechanism of acid catalyzed estolide synthesis from hydroxy fatty acid involving formation of carbocation and nucleophilic attack of carboxylic group to that carbocation



Scheme 4 Mechanism of acid catalyzed estolide synthesis from unsaturated fatty acid



Scheme 5 Mechanism of acid catalyzed carboxylic substitution for estolide synthesis from hydroxy fatty acid

2.2.5. Uses, applications and roles of estolides

Besides aforementioned application of estolides as lubricants in hydraulic pumps and engines, they have a lot of other uses in the industry and they are also believed to have certain roles in some animals.

To start with the simplest estolides, the ones derived from hydroxy fatty acids. Isbel, Kleiman and Erhan produced mono-estolides from hydroxy fatty acids (HOFAs) with little dimer or trimer formations, which have potential use in lubricant, cosmetic and ink formulation and in plasticizers.[87] Further on, estolides synthesized from unsaturated fatty acids have shown good use in cosmetics, since a wide range of new structures can be introduces, depending on the position of double bond in acyl backbone of starting fatty acid.[47,88] As said before, a wide variety of estolides can be synthesized by inserting fatty acids of various chain lengths and branching, and molecular weight can be controlled by continuous homo-polymerization which can serve in the production industrial materials of wanted physical properties.[47] The widest application of the most complex estolides, TG-estolides, is in the lubrication of hydraulic pumps

and engines due to the better hydrolytic stability of the secondary ester linkage between the fatty acids, which gives them excellent cold temperature properties and higher thermal oxidative stability compared to vegetable based oils.[47,89] Also, TG-estolides serve in the formation of films,[56] in controlling the viscosity of chocolates, and as pigment dispersants in ink, paint and cosmetics.[60,90,91]

Since they are only recently discovered in adipose tissue of some animals, precise roles of TG-estolides are still quite unknown, but some assumptions and hypothesis have been made. McLean et al.[27] were the first ones who discovered TG-estolides in mammalian tissue. To be more precise, estolides were found in the paracloacal gland of the brushtail possum. It is known that lipids, steroids, steroid esters and alcohols have a role of semi chemicals which are in charge of controlling the release of volatile compounds which then act as chemical messengers, and can also have a roll in conspecific recognition.[92,93] McLean et al. discovered hundreds of different molecular species in the paracloacal gland of brushtail possum. Those compounds made a complex mixture of lipids which can be divided into three classes; diacyl glycerol esters, TGs and estolides. And even though the biological significance of them was not determined, one can assume, given the previously mentioned role of lipids in animals, that they also have some role as chemical messengers.[27]

And in the end, it is worth mentioning, that recently fatty acid esters of hydroxy fatty acids (FAHFA) were discovered in the adipose tissue of transgenic mice overexpressing glucose transporter 4 ($GLUT_4$) and it is known that FAHFAs are a class of bioactive lipids which show great promise for treating diabetes and inflammatory diseases.[25,26,94]

2.3. Chromatography

Chromatography is a physical method for separation of compounds, in which they are divided between two phases, one of which is stationary and the other moves in a certain direction. It was first introduced by Mikhail Semyonovich Tsvet in 1906. when he separated herbal pigments on a glass column packed with finely divided $CaCO_3$ as the stationary phase.[95] Today, there are a lot of different chromatographic techniques, which can be used for separating mixtures of fatty acids, amino acids, lipids, proteins, sugars, enantiomers of organic compounds etc.[96] There are five chromatographic techniques, based on the separation mechanism; adsorption, partition, ion-exchange, affinity chromatography and chromatography by exclusion. And, based on the physical state of the mobile phase, there are gas (GC) and liquid chromatography (LC), and fluid chromatography under super-critical conditions (SFC). We will take a closer look at adsorption in thin-layer chromatography (TLC) and partition in high-performance liquid chromatography (HPLC), and at GC and LC.

Results of a chromatographic analysis, whether is qualitative or quantitative, are represented with chromatogram. Chromatogram is a graphical representation of the detector´s response versus the volume of mobile phase or time that is needed for certain compound to be eluted from the column. The quality of compounds separation depends on the frequency of establishing equilibrium between stationary and mobile phase while mobile phase caries the compound through the column or up the plate. The efficiency of the chromatographic separation is defined with several values. Number of theoretical plates represents the number of established equilibriums between the mobile and stationary phase, and it is calculated with equation 2.1

$$N = 16 \times \left(\frac{V_R}{w_b}\right)^2 = 16 \times \left(\frac{t_R}{w_b}\right)^2 \tag{2.1}$$

where V_R is total retention volume, t_R is total retention time of certain compound and w_b is the width of the peak at the base. Column efficiency can also be expressed with height equivalent to the theoretical plate (Equation 2.2).

$$H = \frac{L}{N} \tag{2.2}$$

where L is the length of the column. Two other values worth mentioning are selectivity of the chromatographic column (α) and resolution (R_S). Selectivity is expressed with so called separation factor (α) (Equation 2.3)

$$\alpha = \frac{t_{R2} - t_D}{t_{R1} - t_D} \tag{2.3}$$

where t_{R2} and t_{R1} are total retention times of two compounds that are being separated, and t_D is dead time (time needed for compound that does not retain to pass through the column). The greater the difference between total retention times of compounds, the more selective the column. Resolution (R_S) is a measure of separating two chromatographic peaks which includes selectivity and efficiency of chromatographic column (Equation 2.4).[97]

$$R_S = \frac{t_{R2} - t_{R1}}{(w_{b1} - w_{b2})/2} \tag{2.4}$$

2.3.1. Thin-layer chromatography (TLC)

Thin-layer chromatography is, alongside paper chromatography and electrochromatography, one of the planar chromatographic methods. It was first used in 1962. for the sterol, cholesteryl esters and triacylglycerol analysis.[98,99] TLC is a form of liquid-solid chromatography in which stationary phase is a thin layer of some adsorbent (e.g. SiO_2) which, in most cases, coats glass or aluminium plate, and the mobile phase caries the compounds over the plate with capillary forces.

TLC plates can easily be prepared in the lab by spreading an aqueous slurry of the finely ground solid onto the clean surface of a glass plate. When the plate is completely dry and the layer has set and adheres tightly to the surface, sample can be applied. Sample application is a critical part of TLC, because one must be careful not to pierce the stationary phase all the way to the glass (or aluminium) with capillary tube, and sample must be uniformly applied each time onto each spot to assure the consistency of analysis. Mechanical dispensers, which are commercially available, assure the uniformity of sampling. After sampling, comes plate development, where TLC plate is positioned in a closed container containing developing solvent or mixture of solvents. Once again, one must be careful to place the plate straight and not to submerge the line where samples are in the solvent itself. The last step of TLC analysis is detection of compounds, which can be accomplished in many different ways using instruments such as mass spectrometer (MS) or infrared (IR) spectrometer, but there are two simplest and most commonly used techniques that require no instruments. One of them is spraying the plate with sulfuric acid or some other reagent that will react with organic compounds, and after heating the plate burn them, yielding dark products. Disadvantage of such detection is that compounds are being destroyed, so they cannot be used for further analysis or

for semi-preparative TLC. The other, non-destructive, way of detection is accomplished by spraying some fluorescent material onto the plate, such as rhodamine 6G or primuline, after which the compounds are located and distinguished under the UV-light.[100]

In normal-phase TLC, where stationary phase is polar (e.g. silica gel) and mobile phase non-polar, adsorption of compounds onto the stationary phase takes place. As the non-polar solvent carries compounds up the plate equilibrium is being established, because compounds interact with the stationary phase. So, more polar compound bind to stationary phase stronger than non-polar, thus travel shorter distance up the plate, and have lower R_f value (ratio of the distance that specific compound travelled and the distance that mobile phase travelled).

Today, TLC is used a lot and is often used prior to the application of more selective and sensitive methods or prior to conducting the separation on a larger scale (column liquid chromatography) because of its many advantages. It is a cheap, fast and simple separation method that requires no instrumentation, the compounds can be quantitatively and qualitatively analysed and there are little chemical interferences during the sample processing. TLC can be successfully applied to the separation of mono-, di-, and triacylglycerols and their derivatives. Typical mobile phases for the separation of simple lipids contain hexane, diethyl ether and formic acid in various proportions. Complex lipids such as phospholipids and glycosphingolipids do not migrate under this condition, so they can only be detected as if they were a single lipid class.[101,102]

2.3.2. High-performance liquid chromatography (HPLC)

Out of all liquid chromatographic methods, high-performance liquid chromatography (HPLC) is the most versatile and widely used. Its development started in the late 1960s when packings with particle diameters of 3 to 10 μm were being produced. Characteristic of HPLC are pumps which produce extremely high pressures (15 to 100 MPa). All HPLC systems are composed of mobile phase reservoirs which are connected to high-pressure-producing pumps, sample injection system, HPLC column and detector.

Modern HPLC systems usually have four mobile phase reservoirs for different mobile phases. Solvents must be extremely pure and must not contain dissolved gases or solid particles. If present, those impurities can be eliminated by filtration through small pore filters under vacuum (for solid particles) and with degasser (for gases). HPLC pumps produce extreme pressures which pumps mobile phases into the column and through the rest of the system.

Elution can be isocratic where single solvent or solvent mixture of constant composition is used for analysis, or it can be gradient where two solvent systems that differ in polarity are used and varied in composition during the separation. Every HPLC pump must provide high pressures, continuous flow and reproducibility.

Given that injection with syringe through septum is not reproducible, sample injection system in HPLC is based on a sampling loop. Also, most HPLC instruments are equipped with an autosampler which has an automatic injector. While sample is being injected into the loop, mobile phase does not go through it, but is directly introduced into the column. Sample is injected into the column by directing the mobile phase through filled sample loop. Introducing the sample into the column in that way ensures high pressure of sample injection and constant flow.

HPLC columns can be made from stainless steel tubing, which are most widely used, and glass and polymer tubing. They are usually 5 to 25 cm long, have an inside diameter of 3 to 5 mm and particle size of packing is 3 to 5 μm. Precolumns are placed, as the name itself says, in front of the main column, and their role is to protect the main column which increases its lifetime. Two types of precolumns exist; scavenger which is placed between the mobile phase reservoir and the injector, and guard column, positioned between the injector and the analytical column.

In liquid chromatography, there are two types of detection; indirect, in which a change of specific property of mobile phase is being detected and direct, in which a change of specific property of compounds that are being separated is measured. The most widely used detectors for LC are based on absorption of ultraviolet or visible radiation. Spectrophotometric detectors are considerably more versatile than photometers and are also used in HPLC instruments. Great advantage of HPLC systems is that it can be connected to mass spectrometer as a detector which makes a powerful analytical tool.

In HPLC, partition is mostly the mechanism with which compounds are being separated and it is based on a difference in the adsorption of compounds in stationary phase and solubility of compounds in mobile phase. Based on the relative polarities of two phases, two types of partition chromatography are distinguishable; normal-phase and reversed-phase. It has been estimated that more than three-quarters of all HPLC separations are performed with reversed-phase packings.[103] In contrast to normal-phase chromatography, mobile phase in reversed-

phase is polar, and aqueous solutions and mixtures of methanol, iso-propanol (IPA), acetonitrile (ACN) or tetrahydrofuran (THF) are usually being used.

Applications of HPLC are practically endless. It is used in pharmaceutical and food processing industry, biochemical and clinical chemistry and in forensics, for analysis of antibiotics, sedatives, amino-acids, proteins, lipids, pesticides, herbicides, drugs, narcotics, poisons etc. When it comes to analysis of lipids, HPLC has a great advantage over classical TLC analysis due to better separation efficiency and the availability of different methods offering greater versatility and ease of quantification. Lipids can be detected and quantified with normal-phase, silver-ion and nonaqueous reversed-phase liquid chromatography. Because of the easiness of hyphenation to a variety of detectors, reversed-phase liquid chromatography (RPLC) techniques have a wide application, especially when connected to mass spectrometer (RPLC-MS). Additionally, mobile phases used in RPLC are compatible with biological and lipophilic molecules, such as lipids. The possibility of lipid identification and analysis with RPLC-MS was demonstrated by Holčapek when he and his group unambiguously identified 133 TGs in 9 plant oils.[103–105]

2.3.3. Gas chromatography (GC)

Gas chromatography is an instrumental analytical method in which components of vaporized sample are separated by being distributed between mobile gaseous phase and a liquid (GLC) or a solid (GSC) stationary phase. The elution of compounds is performed with the flow of inert gas through the column. Unlike in other types of chromatography, mobile phase in gas chromatography does not interact with molecules of the analyte. The only function of the mobile phase is to transport the analyte through the column. However, molecules of the analyte still interact with stationary phase, which is why retention time of compounds also depends on the length and type of column.

Major components of the GC instrument are gas tank, flow control valve, thermostat, sample injection system, GC column and detector.

Carrier gases, which are the mobile phases in GC must be chemically inert, and most commonly used are helium, argon, nitrogen and hydrogen. Flow rate of carrier gas is controlled with pressure regulators, gauges and flow meters. Flow rates differ depending on used columns; it can range from 25 to 150 mL/min for packed columns and from 1 to 25 mL/min for open tubular capillary columns, but typical flow rates used in the GC analysis ranges from 1 to 2 mL/min.

Liquid sample can be injected manually or with autosampler with calibrated micro syringes through a rubber or silicone septum, into a heated sample port located at the head of the column, where it evaporates. Temperature of the sample port is usually kept at 50 °C higher than the boiling point of the least volatile component of the sample. Volume of the sample used in GC analysis can vary from few tenths of a microliter to 20 μL.

There are two types of GC columns; packed and capillary. They can be made from stainless steel, glass, fused silica or Teflon. Thermostat is in charge of controlling the temperature of the column and the optimal temperature depends on the sample being analysed. Temperature can be constant during the entire analysis or, for samples with broad boiling range, it can increase continuously or in steps as the separation proceeds. For the fastest, most efficient and sensitive analysis capillary columns with length from 2 to 60 meters and radius of 0,1 – 0,5 mm are being used.

Different detectors can be used in GC and they are based on flame ionization, thermal and electrical conductivity, photoionization, electron capture and mass, IR and UV spectrometry. GC is used for qualitative and quantitative separation and analysis of volatile, non-polar and thermally stable compounds. And even compound that do not have those characteristics can be analysed after their derivatization, which can be performed by simple chemical reactions: esterification, acylation, alkylation and silylation. All in all, GC has been widely applied to the separation and determination of the components in a variety of sample types.[106]

2.4. Mass spectrometry

Mass spectrometry is an instrumental analytical method in which gaseous (e.g. in GC-MS) or liquid (e.g. in HPLC-ESI/MS) molecules of analyte are being ionized, after which comes the separation and detection of formed ions based on their mass to charge ratio (*m/z*). It has qualitative and quantitative application and has been used for analytical purposes since the early 1940s when it was first applied in the petrochemical industry to quantitatively analyse the hydrocarbon mixture. Mass spectrometry had a great breakthrough in the industry, as well as in academic purposes when the advancement of technology enabled the production of linked systems, especially chromatographic-mass spectrometry systems.

The beginnings of mass spectrometry dates back to 1897. when J. J. Thomson discovered an electron[107,108] and determined its *m/z* ratio, for which he was awarded with the Nobel prize in physics in 1906. He also constructed the first mass spectrometer in 1912. In 1918. A. J. Dempster constructed the first electron ionization ion source.[109] The first commercial mass spectrometer, designed for analysis of organic compound, was constructed in 1942., and in 1958. McLafferty and Gohlke were the first to connect gas chromatograph with mass spectrometer.[110]

Today, mass spectrometry is used for determination of the isotope ratio of atoms in a sample, the elemental composition of compounds in a sample, the structure of the inorganic, organic and biological molecules, the qualitative and quantitative composition of the mixture, and the structure and composition of the solid surface, all of which has a widespread application in medicine, pharmaceutical and petrochemical industry, forensic tests, food, drugs and pesticide analysis and more.[111] The reason for such a large application is many advantages over other analytical, instrumental techniques. Depending on the analyte, extremely high sensitivity (LOD = 10^{-15} mol -10^{-18} mol), excellent accuracy in determination of atomic mass (10^{-9} μ) and extremely high resolution ($8 \cdot 10^{6}$), which is the ability to separate two ion signals with a small difference in masses, can be achieved.

The mass spectrometer consists of a sample injection system, ion source, mass analyser, detector, and data processing system. Components before data processing system can be placed in an evacuated region (10^{-5} – 10^{-8} Torr), which ensures a relatively low collision frequency between various species in the mass spectrometer, which is vital for the production and maintenance of free ions. The sampling system serves to bring the sample into the ionization

chamber (ion source) where molecules are ionized, and generated ions are directed to the mass analyser.

The spectrum layout strongly depends on the way that molecules are being ionized, that is on the ion source that is being used, and on the type of mass analyser. There are a lot of ionization techniques which can be divided based on the physical-chemical properties of an analyte and on the amount of internal energy that is being transferred during the ionization process (Table 1). We will take a closer look at electron ionization (EI) and electrospray ionization (ESI). Function of mass analysers it to separate ions formed in the ion source based on their *m/z* ratio. Available mass analysers are: magnetic sector, quadrupole (Q), ion trap (IT), time-of-flight (TOF), Fourier-transform ion cyclotron resonance (FT-ICR) and orbital trap. Since instruments used in this thesis have ion trap and orbital trap as mass analysers, we are going to take a closer look at those two.[112]

Table 1 Mass spectrometry ion sources

Aggregate state of sample	Ionization technique
Gas	Electron ionization (EI)
	Chemical ionization (CI)
	Desorption chemical ionization (DCI)
Liquid or solid	Fast atom bombardment (FAB)
	Secondary ion mass spectrometry (SIMS)
	Field desorption (FD)
	Plasma desorption (PD)
	Laser desorption (LD)
	Matrix-assisted laser desorption/ionization (MALDI)
Solution	Thermospray ionization (TSI)
	Electrospray ionization (ESI)
	Atmosperic pressure chemical ionization (APCI)

2.4.1. Electron ionization (EI)

At the beginnings of mass spectrometry, EI was the most widely used type of ionization technique, and it is still used today. In it, molecules interact with a high-energy beam of electrons. This produces positive and negative ions, and neutral species. The positive ions are directed toward the analyser by electrostatic repulsion.

Electron impact ionization is applicable to volatile and thermally stable compounds with masses up to roughly $1 \cdot 10^3$ Da, and mass spectra obtained by this technique are highly repeatable and can be stored in spectral databases. It works on the principle of heating the

tungsten or rhenium wire, which then emits electrons that accelerate at a potential of 70 eV and collide with the gaseous molecules of the analyte. The collision of accelerated electrons with analyte molecules causes an energy transfer which expels valence electrons from the molecule, resulting in a molecule ion in the form of a positively charged radical, after which fragmentation occurs. Such a way of ionization causes a strong molecular fragmentation and the signal of the molecular ion is often small or even missing. However, fragments allow insight into the structure of the analysed compound, which may facilitate its identification. After ionization and fragmentation, formed ions are being separated by mass analyser based on their *m/*z ratio.[113]

2.4.2. Electrospray ionization (ESI)

ESI is a soft ionization technique which is applicable to analysis of compounds in a solution. It can be used for determination of small and big molecules, ionic and non-charged compounds, non-covalent complexes, and it allows direct connection between liquid chromatography and mass spectrometry.

The solution containing the analyte is introduced into a metal capillary to which a high electric field is applied under atmospheric pressure. That causes separation of positive and negative charges in the solution. When the capillary is connected to a positive end of the voltage source, positive ions travel to the cathode and accumulate on the surface of the liquid. At the critical field strength, the so-called Taylor cone is produced in which the droplets enriched by the positively charged ions are continuously produced, which under the influence of potential and pressure gradient travel to the mass analyser. By altering the polarity, it is possible to produce negatively charged particles.

Today ESI-MS is used for analysis of a wide variety of compounds; peptides, proteins, lipids, sugars, nucleic acids etc. By reducing the flow of the analysed solution, the sensitivity of the method is increased, and the detection limits decreased to the order of attomole. One advantage of electrospray ionization is that it produces multiply charged ions, which enables the analysis of large biomolecules even with mass analysers that have smaller measuring range of *m/z* values.[114]

2.4.3. 3D-Ion trap

There are two types of ion traps; linear, that is 2D which is composed of four cylindrical electrodes and 3D ion trap.

3D ion trap is comprised of upper and lower endcaps, and ring electrode, to which a combination of alternating and direct potential is applied, creating a "three-dimensional quadrupole". Ions of all *m/z* ratios are trapped in the centre of that 3D quadrupole. Trapped ions oscillate under the influence of electric field. Those oscillations form a complicated trajectory. To avoid loss of ions, which would be caused by spread of ions into too much space, helium is introduced into the ion trap which eliminates the excess of energy by colliding with them. By changing the alternating potentials, the trajectory of an ion with particular *m/z* ratio becomes unstable and exits through the lower or upper opening on the endcap electrode. Because of that, only half of ions are directed towards the detector. Thus, potential and pressure gradients direct the ions formed in the ionic source, and the separation takes place in time.

Great advantage of 3D ion trap is the possibility to conduct MS/MS and MS^n experiments. In the contrast to the system with three quadrupoles where separation takes place in space, separation and fragmentation in MS/MS experiments in ion trap takes place in time. Wanted ion, that is ion on which the MS/MS experiment is to be carried out, stays in the ion trap while all other ions are eliminated. Helium present in the ion trap ensures the energy needed for fragmentation. Formed fragments can then be analysed or another fragmentation step can occur.[115,116]

2.4.4. Orbital trap

Orbital trap is mass analyser which consists of a central, spindle-shaped electrode and an external, barrel-like electrode. When positive ions are being analysed a negative potential of several kilovolts is applied to the central electrode while the outer electrode is grounded, that is it has no potential. Ions of a certain kinetic energy are captured in the electrostatic field between the electrodes. Stable trajectories of the ions include rotation around the central electrode and harmonic oscillations along that electrode. The frequency of harmonic oscillations is independent of the kinetic energy of the ion and depends only on the *m/z* ratio. The current induced by the oscillations is, using Fourier transformations, translated into the frequencies and intensities, that is to mass spectrum. Orbital trap can be directly linked to pulse ionization sources. If continuous sources are used, the ions are introduced into Orbitrap through an ion trap, called C-trap. This mass analyser is characterized by high resolution and accuracy of 1 ppm with internal calibration.[117]

2.5. Linked systems or hyphenated techniques

Linked systems or hyphenated techniques represent the combination or coupling of different instrumental analytical techniques. Its development started in 1958. when McLafferty and Gohlke created the first linked system; GC-MS.[110] Hirschfeld first used the term "hyphenation" in 1980. to describe a possible combination of two or more instrumental analytical methods in a single run.[118] Development of technology enabled the combination of many different instrumental analytical techniques, which significantly broadened their applications in analysis of a complex samples, such as natural products.[119]

When talking about hyphenated techniques, usually combination of chromatography with spectrometric methods is meant. Complex mixture that is being analysed is divided into pure fractions with chromatography, and then spectroscopy provides selective information for identification of individual compounds in those fractions. Today, they can be divided into double and triple hyphenated techniques, depending on the number of connected instruments, and they range from the combination of separation-separation (SPE-LC), separation-identification (GC-MS, HPLC-MS) and identification-identification (MS-MS) .[120]

There are many advantages of hyphenation, compared to single instrument analysis, but the main idea is to increase signal-to-noise ratio, and thus improve detection limit.[121] Yost explained how signal-to-noise ratio is increased by hyphenation; "While in hyphenation the response or signal achieved decreases with the increasing number of coupling or dimensions, the chemical noise decreases even faster due to the increased selectivity, thus resulting in an improved signal-to-noise ratio."[122] Other advantages are; fast and accurate analysis, higher degree of automation, higher sample throughput, better reproducibility, reduction of contamination due to its closed system, and separation and quantification achieved at the same time.[120] Two of the most used hyphenated techniques today are LC-MS and GC-MS.

2.5.1. HPLC-MS

HPLC-MS is a separation-identification double hyphenated technique, most widely used in pharmaceutical industry for qualitative and quantitative analysis of various compounds.[123–126] It combines the power of LC to chemically separate individual compounds or classes of compounds into fractions, and ability of MS to unambiguously confirm their molecular identity.

The ionization techniques used in HPLC-MS, or generally LC-MS are mild ionization techniques, such as aforementioned ESI. Because of mild ionization which mostly produces

only molecular adducts, information of the compounds structure obtained in one run is quite poor. That is why in most HPLC-MS analysis tandem mass spectrometry (MS-MS) experiments are conducted, which provide fragments through collision-induced dissociation (CID) of the produced molecular adducts and thus give another dimension to structural analysis of compounds.

Usually, a direct connection of HPLC and MS is not possible, because of relatively high flows of mobile phases, which would disturb the vacuum present in the MS. To avoid this and to enable the HPLC-MS connection, different types of interfaces are used. They are designed in such a way that they offer adequate nebulization and vaporization of the liquid, ionization of the sample, removal of the excess solvent vapor, and extraction of the ions into the mass analyser. ESI and APCI are the two most widely used interfaces, especially for analysis of natural products.[119] ESI is more suitable for ionization of polar and ionic compounds and is capable of ionizing both small and large biomolecule, while APCI can ionize less polar and neutral compounds.[127]

2.5.2. GC-MS

GC-MS is another widely used hyphenated technique, which combines gas chromatography with mass spectrometry. GC-MS instruments have been used for the identification of thousands of components that are present in natural and biological systems.

Unlike in HPLC-MS, the flow rate from capillary columns in GC is usually low enough to allow direct connection with MS. Electron ionization (EI) and chemical ionization (CI) are the two most common ionization techniques used for GC-MS, and most common analysers are quadrupoles.

The result of the GC-MS experiment is a set of vectors in three-dimensional space which is triangulated to three axes: retention time, mass-to-charge ratio and intensity. Each retention time has a chromatogram containing a set of *m/*z ratios with corresponding intensities. That means that the chromatogram is a two-dimensional representation of the detector's response over time and the mass spectrum is just a set of all points scanned at the same time. Data in GC-MS can be represented as total-ion chromatogram or as mass chromatogram. In total ion chromatogram, all ions formed by ionization are represented as the function of retention time, and in mass chromatogram, which is used to improve detection sensitivity and selectivity, *m/z* values of selected ions are monitored.

§ 3. EXPERIMENTAL SECTION

3.1. Materials

Vernix caseosa (1 – 2 g) was collected from healthy new-born subjects delivered at full term (gestation weeks 39 – 42) immediately after the delivery. The samples were stored in amber glass vials at –80 °C. The study was approved by the Ethics Committee of the General University Hospital, Prague (910/09 S-IV) and the samples were collected with written informed parental consent.

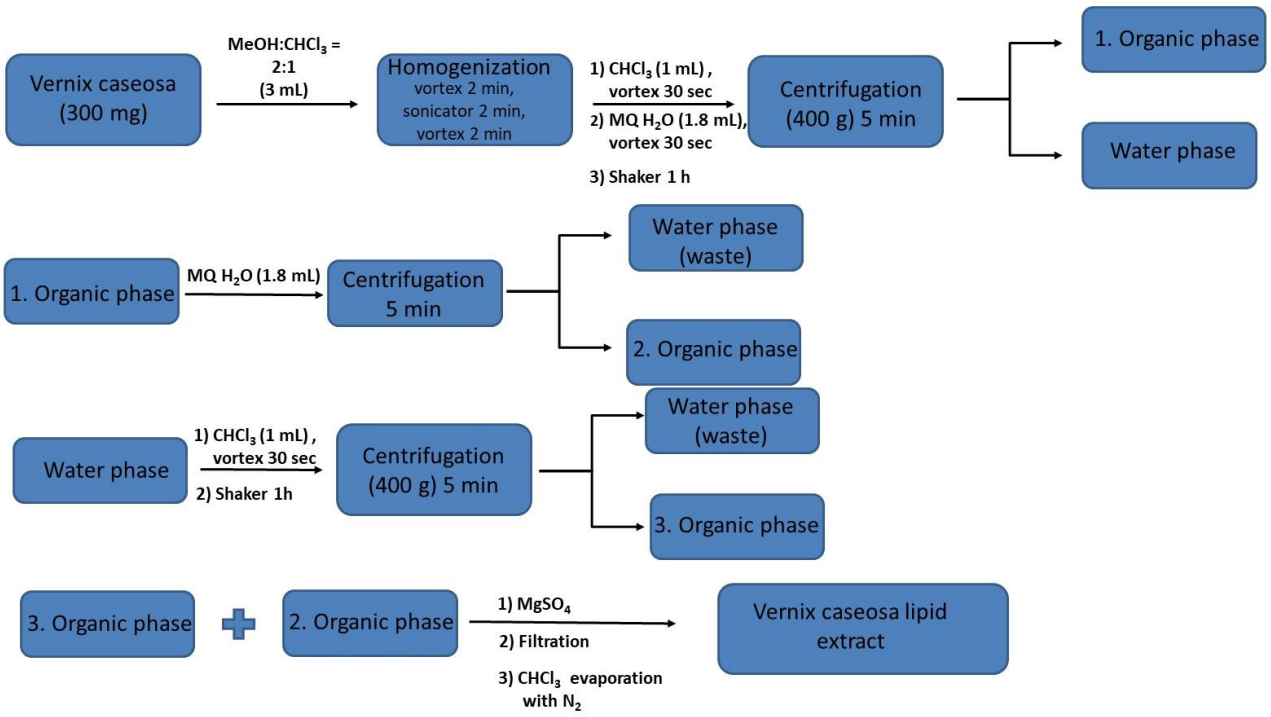
3.2. Chemicals

LC-MS grade methanol (Merck KGaA, Germany), hexane, 2-propanol (both from Honeywell, Germany), ethyl acetate (Sigma – Aldrich, St. Louis, MO) and HPLC grade toluene, acetone, cyclohexane (all from Sigma – Aldrich) and acetonitrile (VWR chemicals, USA) were used as received. Chloroform and diethyl ether (both from Penta, Czech Republic) were distilled from analytical-grade solvents. For everything, except TLC plate making, ultra-pure water was used instead of distilled water. It was obtained by purifying water on the spot with Milli-Q purifier from Merck & Co. Reagent grade primuline, rhodamine 6G, trimethylsilyl diazomethane (all from Sigma – Aldrich) and silica gel 60G (Merck KGaA, Germany) were used as received as well as analytical-grade concentrated acetic acid, sodium chloride, formic acid (all from Lach-Ner, Czech Republic), , *N*,*O*-bis(trimethylsilyl)acetamide, acetyl chloride, ammonium formate, lithium(I) carbonate (all from Fluka Analytical, Germany), magnesium(II) sulphate, magnesium(II) chloride, copper(II) chloride, manganese(II) chloride, palladium(II) nitrate, nickel(II) nitrate, iron(III) nitrate, aluminium(III) nitrate nonahydrate, rubidium(I) iodide, cesium(I) iodide, silver(I) carbonate (all from Sigma-Aldrich), potassium(I) chloride (Penta, Czech Republic), calcium(II) chloride, anhydrous tin(IV) chloride (both from LaChema N.P. Brno) and cobalt(II) chloride (Janssen Chimica, Belgium). The standard of triolein (Nu-Chek-Prep, Inc., Waterville, MN) was used as received and estolide standards AMR-1921-3-1 and AMR-1919-2 were synthesized at the IOCB´s division of Medicinal Chemistry by Aleš Machara, Ph.D. The structures of estolide standards are given in the appendix (Figure D1, Figure D2).

3.3. Isolation of Lipids

3.3.1. Isolation of all vernix caseosa lipids

Vernix caseosa lipids were extracted from 4 samples equally representing two genders of new-borns. All the other lipid extracts and non-polar lipids were received from previous, related research. Scheme 6 shows the isolation procedure of all vernix caseosa lipids. Each sample was processed separately as follows: The sample of vernix caseosa (approximately 300 mg) was suspended in a mixture of methanol : chloroform (2:1 by volume, 3 mL) in a conical-bottom glass centrifuge tube and homogenized using vortex shaker (2 min) followed by treatment in the ultrasonic bath (2 min) and then again on vortex shaker (2 min). After that, chloroform (1 mL) and ultra-pure water (1.8 mL) were added to the suspension which was then put on a shaker



Scheme 6 Schematic procedure for isolation of all vernix caseosa lipids

(1 hour). After shaking the suspension for 1 hour, the sample was centrifuged for 5 minutes at 400 g. The organic phase was separated from the water phase and was put to a new glass tube where it was again extracted with ultra-pure water (1.8 mL) and collected after centrifugation. Re-extraction was also performed with the water phase by adding chloroform (1 mL) and shaking the solution for 1 hour. The given solution was centrifuged as before, and the organic phase was separated from the water phase. Obtained organic phases were mixed together and two tablespoons of anhydrous magnesium(II) sulfate was added for the removal of excess of

water from the solution. The mixture was filtered through pre-cleaned cotton wool and chloroform was evaporated using nitrogen gas. After weighting obtained lipids, they were suspended in a chloroform: methanol mixture (19:1 by volume) and stored in a freezer at –30 °C. The concentration of prepared lipid solution was 30 mg mL^{-1}.

3.3.2. Isolation of non-polar lipids

To get non-polar lipids from total vernix caseosa lipid extract, semi-preparative TLC was performed. It was done on silica gel TLC plates using hexane: diethyl ether: formic acid as the mobile phase (80:20:2 by volume). After plate development, compounds were visualized with rhodamine 6G under the UV-light. The zone which contained triacylglycerols, as well as the rest of silica gel plate above that zone, was scraped off into a glass column, which had purified cotton wool at the bottom and a small amount of silica gel powder on top of that cotton wool. Non-polar lipids were extracted into a heart-shaped flask using freshly distilled diethyl ether. Diethyl ether was evaporated with nitrogen gas, non-polar lipids were weighted and chloroform solution (γ = 30 mg/mL) was made. Until the next use, the chloroform solution of non-polar lipids was stored in a freezer at the temperature of –33 °C.

3.3.3. Isolation of TG-estolides from non-polar lipids

To isolate TG-estolides from non-polar lipids, semi-preparative TLC was again used. It was performed on silica gel TLC plates impregnated with sodium chloride, with hexane: diethyl ether: toluene (92.5:7.5:7.5 by volume) as the mobile phase. To achieve good resolution, each plate was developed three times. After plate development, compounds were visualized with primuline under the UV-light. To know where on a plate wanted estolides are, that is to know which part of the plate should be scraped into the glass column, analytical TLC with triolein and TG-estolide standards (AMR-1921-3-1 and AMR-1919-2) was performed beforehand. Part of the plates which contained TG-estolides, between the starting point and triacylglycerols, was scrapped into the glass column, which had purified cotton wool at the bottom and a small amount of silica gel on top of that cotton wool. The procedure for extracting estolides is the same as the procedure for extracting non-polar lipids, except the concentration of the final solution, which is now much lower. The obtained estolide sample was purified with yet another semi-preparative TLC using the same procedure, but this time the solution was pre-concentrated by evaporating most of chloroform, and the whole sample was put onto one wider plate. Even after all that, there were still some TGs present, so additional purification with semi-preparative HPLC was later conducted.

3.4. Development of the TLC method for isolating estolides from non-polar lipids

3.4.1. Mobile phase

To optimize the mobile phase for separation of estolides from other non-polar lipids, mainly triacylglycerols, 17 mobile phases were tested in total (Table 2). Most of them were three-component mobile phases, so they were basically prepared in the same way. Mobile phases (100 mL) were prepared by mixing solvents in the desired ratio. It is important to point out, that before any mobile phase was prepared and before any TLC analysis was performed, all glassware, as well as all silica gel TLC plates were purified with chloroform: methanol mixture (1:1 by volume). Different mobile phases were tested by using them in the analytical TLC of triolein and TG-estolide standards. Which mobile phase gave the best separation of estolides from triacylglycerols was decided visually.

3.4.2. Adsorption TLC, "standard" TLC plate making method

Silica gel TLC plates were made by stacking glass slides on a base plate and covering the slides by silica gel slurry using a TLC spreader. Silica gel (38.2 g) was weighted in a glass beaker and a 100 mL of distilled water was poured into the Erlenmeyer flask with a glass stopper. Silica gel was slowly added with a spoon to the water and the flask was constantly shaken to avoid the formation of clumps and lumps. When the whole amount of silica gel was used, obtained silica gel slurry was poured into the TLC spreader, the leaver on it was turned for 180 ° and the spreader was pulled across the base plate, thus distributing the silica gel slurry onto the slightly wet glass plates. Silica gel TLC plates were left at room temperature to dry a little (30 min) and then each of them was lifted, that is separated from a base plate with a spatula and left on a filter paper for 3 days to dry completely. Before analysis, each plate was purified with chloroform: methanol mixture (1:1 by volume). The consistency and significance of the results obtained on plates which were prepared in this way, which is the separation of TG-estolides from TGs, that is the R_f values of TG-estolides and TGs, were tested with the statistical analysis, which was conducted with Microsoft Excel. Those results were later compared with two other TLC plate preparation methods.

3.4.3. Adsorption TLC, "fast" TLC plate making method

Here, silica gel TLC plates were made without the TLC spreader, nor the big glass plate. Silica gel slurry was made by simply pouring water into the beaker with weighted silica gel and by mixing it all up with a spoon. Silica gel to water ratio was 2.5:1 by mass. So, for example, on 40 g of silica gel, 100 mL of water was used. A small amount of silica gel slurry was poured to the bottom of one plate, and that plate was then being moved in all directions, so the silica gel gets distributed evenly on the entire surface of the plate. Silica gel covered plates were put onto the filter paper and left to dry for 3 days. Again, the consistency and the significance of the results obtained on plates which were prepared in this way were tested with statistical analysis, which was conducted with Microsoft Excel. Those results were later compared with two other TLC plate preparation methods.

3.4.4. Metal ion TLC, "standard" TLC plate making method

In this method, silica gel TLC plates were made again with the TLC spreader. The difference between this and the first one is, that here metal salt is added to the silica gel to make use of interactions between metal ions and ester group oxygens. To determine which metal cation would bind the best to the compounds that are being separated, estolides and TGs, metal cation-ester complex formation was monitored. For that, equimolar solutions ($c = 1 \cdot 10^{-7}$ mol dm^{-3}) of metal nitrates, chlorides, iodides and carbonate were prepared in water and 5 % of ethyl acetate was added into each of them. Formation of the metal cation-ethyl acetate complex was observed with ESI/MS analysis. Silica gel, water and metal salt were mixed in a mass ratio of 14:48:1. The procedure for making the plates is the same as in the first method. And, as well as with the other two methods, the consistency and the significance of the results obtained on plates which were prepared in this way were tested with statistical analysis, which was conducted with Microsoft Excel. Those results were later compared with two other TLC plate preparation methods.

3.5. HPLC-ESI/MS

Analysis of purified TG-estolides was performed on two different HPLC-MS systems and with two methods differing in gradient program and used mobile phases. On both instruments and in both methods purified mixture of TG-estolides was separated on reversed-phase HPLC using Kinetex stainless-steel C18 column (150 × 3 mm, particle size 2.6 μm) at a column oven temperature of 40 ºC.

3.5.1. LCQ Fleet

The first analysis was performed on LCQ Fleet 3-D Ion trap mass spectrometer (Thermo Fisher Scientific) with ESI probe installed. The mass spectrometer was coupled to an HPLC system consisting of Rheos Allegro 210 quaternary gradient pump (Flux Instruments) and Accela autosampler (Thermo Fisher Scientific).

The capillary temperature was set to 275 ºC. Sheath and auxiliary gas (nitrogen) were set at a flow rate of 35 and 5 arbitrary units respectively. Discharge current was 5 μA and capillary and tube lens voltages were 5 V and 120 V respectively. Positively charged ion spectra were recorded with ammonium formate as the source of ammonium ions, which was delivered differently into the MS, depending on the separation method.

The MS method consisted of 3 scan events: 1) a full MS in the *m/z* 350 – 1700 range; 2) collision-induced dissociation (CID) MS^2 scan event of the most intense ion in the *m/z* 1000 – 1200 range with normalized collision energy of 22 arbitrary units; and 3) collision-induced dissociation MS^3 scan event of the most intense ion in the *m/z* 1000 – 1200 range with normalized collision energy of 26 arbitrary units.

In order to additionally purify TG-estolide sample from any remaining triacylglycerols, LCQ Fleet was used for semi-preparative HPLC as well. Using separation method A, all compounds which were determined to be estolides (t_r = 18 – 30.5 min) were separated into a glass heart-shaped beaker. Ten consecutive analysis, that is separations of an estolide sample (γ = 10 mg/mL) with injection volume of 10 μL were performed. Completely purified estolides were, until transesterification, stored in chloroform solution in the freezer at -33 ºC.

3.5.2. LTQ Orbitrap XL

The second analysis was performed on LTQ Orbitrap XL hybrid FT mass spectrometer (Thermo Fisher Scientific), which also had an ESI probe installed. The mass spectrometer was coupled to an HPLC system consisting of Rheos Allegro 2200 quaternary gradient pump (Flux

Instruments) and PAL HTS autosampler (CTC Analytics). The capillary temperature was set to 275 °C. Sheath and auxiliary gas (nitrogen) were set at a flow rate of 35 and 5 arbitrary units respectively. Discharge current was 5 μA and capillary and tube lens voltages were 9 V and 150 V respectively. Positively charged ion spectra were recorded with ammonium formate as the source of ammonium ions, which was again delivered differently into the MS, depending on the separation method.

The MS method consisted of the same 3 scan events as on LCQ Fleet.

3.5.3. Separation method A

Mobile phases used in this method were acetonitrile (**A**) and 2-propanol (**B**). The gradient program lasted 75 minutes and was set as follows: 30 – 100 % **B** from 0 – 60 min at 150 μL min^{-1}; 100 % **B** from 60 – 65 min at 120 μL min^{-1}; 100 – 30 % **B** from 65 – 70 min at 120 μL min^{-1}; 30 % **B** from 70 – 75 min at 150 μL min^{-1}. Since ammonium formate was not a part of the mobile phases, the aqueous solution of ammonium formate (c = 0.5 mol dm^{-3}) was separately delivered, after the chromatographic column, with UltiMate 3000 HPLC pump (Dionex) at a flow rate of 5 μL min^{-1}.

3.5.4. Separation method B[26]

Mobile phases used in this method were mixture of methanol and water (64:40 by volume) with 0.1 % of formic acid and 5 % of ammonium formate (**A**) and mixture of 2-propanol and methanol (90:10 by volume) with 0.1 % of formic acid and 5 % of ammonium formate (**B**). The gradient program lasted 68 minutes and was set as follows: 0 – 70 % **B** from 0 – 5 min; 70 – 85 % **B** from 5 – 50 min; 85 – 100 % **B** from 50.0 – 50.1 min; 100 % **B** from 50.1 – 55 min; 100 – 0 % **B** from 55 – 55.1 min; 0 % **B** from 55.1 – 68 min. Since ammonium formate was a part of the mobile phases there was no need for additional, separated delivery of it into MS. The flow was constant during the whole gradient program (250 μL min^{-1}).

3.6. Transesterification

Chloroform solution of purified TG-estolides was first evaporated with nitrogen gas. Then, methanol – chloroform mixture (3:2 by volume) was added to make a solution with concentration of 1 mg/mL. This solution of estolides was transferred to an ampule and acetyl chloride was added. Acetyl chloride was added in high surplus based on the paper published by Karel Stránský and Tomáš Jursík[128] to ensure complete transesterification. Immediately after the addition of acetyl chloride, the ampule was set onto the dry ice for 5 minutes. The ampule was then sealed with a burner and another piece of glass and shaken in a thermomixer (1 h, 70 °C, 600 rpm). After completion of the reaction, excess of hydrochloric acid, formed during the reaction, was neutralized with silver carbonate. Transesterified sample was at the end decanted of solid silver chloride and stored in a freezer.

After the GC-MS analysis, FFAs were detected in the sample so additional esterification step was performed with trimethylsilyl diazomethane ($TMSCHN_2$). Solvent from previous analysis was evaporated under stream of nitrogen. Remained sample was first dissolved in toluene – methanol mixture (3:2 by volume). An etheric solution of $TMSCHN_2$ was added dropwise until the yellow colour persisted. The reaction mixture was placed on an orbital shaker for 30 minutes. After 30 minutes the reaction was quenched with three drops of concentrated acetic acid and the excess of solvent was evaporated under stream of nitrogen, thus concentrating the FAMEs solution.

3.7. Trimethylsilylation

For the GC-MS analysis of hydroxyl groups in fatty acid methyl esters, trimethylsilylation of the sample was also performed. FAMEs sample was dissolved in dried acetonitrile and excess of *N*,*O*-bis(trimethylsilyl)acetamide was added. The reaction mixture was placed in a dry bath for 10 minutes at 40 °C. The excess of solvent was evaporated under a stream of nitrogen and the sample was injected onto the GC column

3.8. GC-MS

Transesterified TG-estolides, that is fatty acid methyl esters (FAMEs) were analysed using Agilent 7890A GC system coupled to Agilent 5975C quadrupole mass spectrometer equipped with DB-WAX column (30 m x 250 μm, 0.25 μL; Agilent). Helium was used as the carrier gas at 1 mL/min. 1 μL of sample was injected and injector was held at 260 °C and operated in splitless mode. The temperature program was: 50 °C (1 min), then 25 °C/min to 140 °C (0 min)

and then 4 °C/min to 260 °C. The 70 eV EI mass spectra were recorded in the range of *m/z* 25-400. Source and quadrupole temperatures were set to 230 °C and 150 °C respectively.

Silylated hydroxy fatty acid methyl esters were analysed using Agilent 6890N GC system coupled to Agilent 5975B quadrupole mass spectrometer equipped with HP-5ms column (30 m x 250 μm, 0.25 μL). Again, helium was used as the carrier gas at 1 mL/min. 1 μL of sample was injected and injector was held at 280 °C and operated in splitless mode. The temperature program was: 60 °C (2 min) and then 7 °C/min to 320 °C. The 70 eV EI mass spectra were recorded in the range of *m/z* 25-750. Source and quadrupole temperatures were set to 230 °C and 150 °C respectively.

§ 4. RESULTS AND DISCUSSION

4.1. Isolation of Lipids

Vernix caseosa consists of water, proteins and lipids. Since, in this thesis, hypothesised lipids in VC; TG-estolides are the only compounds of interest, their isolation was first out of three experimental parts in estolide analysis. Isolation of VC TG-estolides can also be divided into four parts: **1)** isolation of all VC lipids, **2)** isolation of non-polar lipids, **3)** isolation of TG-estolides from non-polar lipids, **4)** additional purification of TG-estolides with semi-preparative HPLC.

Isolation of all VC lipids consisted of several consecutive extraction steps. As said in the chapter 3.3.1. mixture of methanol, chloroform and ultra-pure water was used for extraction. Given that VC mostly consists of water (80 %), it is in a solid state when stored in a freezer at – 33 °C, but at a room temperature it has a creamy consistency, because of its lipid composition and ice melting, which is why weighting of the sample had to be quick. Extraction is a process which happens at the border of two phases, organic and aqueous, and the larger the surface area on which extraction takes place, the higher the yield of extraction. Because of that, sample homogenization was performed beforehand. Vortex mixer and sonicator were used for homogenization. First, vortex mixer ensured proper suspension and mixing of VC sample with methanol – chloroform solution, which also broke apart molecules. Since VC cells are mostly ruptured, proteins and lipids were released into the solution. Then sonicator, which uses sound waves to agitate particles in a solution, caused several other things. It accelerated the dissolution of VC sample, facilitated further mixing of it with MeOH – $CHCl_3$ solution and removed dissolved gases from liquid. After sonication, additional vortexing step was applied to ensure proper mixing of released proteins and lipids. Next step was the extraction itself. It is based on Nernst distribution law which says: "At constant temperature, a solute distributes itself between two immiscible solvents only in a particular ratio", meaning that, at equilibrium, the ratio of the concentrations of a component A in two liquid phases is constant, and can be expressed with the equation 2.5

$$k = \frac{c\,(A)_{org}}{c\,(A)_{H_2O}} \tag{2.5}$$

where *k* is the distribution coefficient, which is temperature and pH dependent, and $c\,(A)_{org}$ and $c\,(A)_{H2O}$ are the molar equilibrium concentrations of the component A in the organic and aqueous phase. In this case two immiscible solvents were chloroform and water. After their addition to the sample, obtained suspension was mixed on the shaker for 1 hour. As the extraction proceeded, non-polar lipids from VC were transferred and dissolved into the organic phase, while water and proteins remained in the aqueous phase. After the extraction was done, and after centrifugation, organic phase was removed from the aqueous phase with syringe. Given that two consecutive extraction steps provide higher yield compared to the one extraction with larger amount of solvent, that is higher amount of organic non-polar compounds is transferred to the organic phase, second extraction of obtained organic and water phases was performed. Two obtained organic phases were put together, and since after extraction process there is always a small amount of water present in the organic phase, anhydrous $MgSO_4$, which is a drying agent, was added. By adding anhydrous inorganic salt to the organic phase, dissolved water binds to the salt and thus converts to crystalline water, which is then removed by filtering through a cotton wool. Mass of the used VC sample was 300 mg and the mass of isolated lipids was 29,1 mg, which is almost exactly theoretically mentioned 10 %.

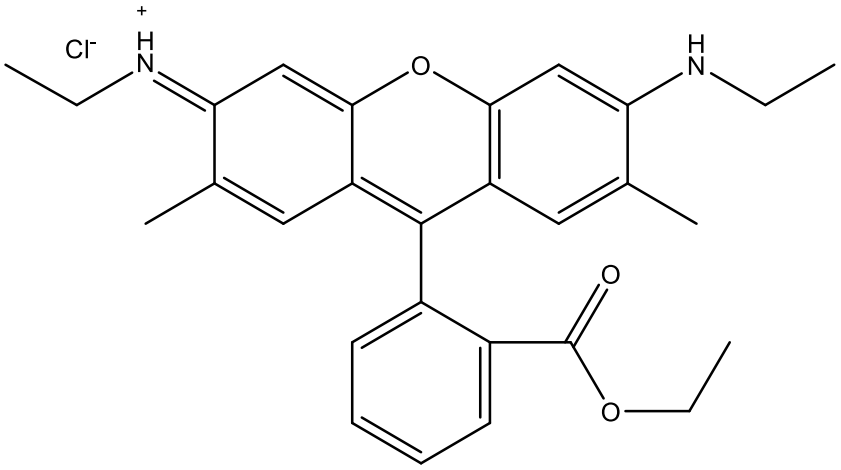


Figure 8 Rhodamine 6G structural formula

Since commonly used extractant for polar lipids (e.g. phospholipids, glycolipids) is a mixture of chloroform, methanol and water, and non-polar lipids (e.g. TGs, WEs, SEs) are extracted with chloroform,[129] in isolated VC lipid extract all of them are present, so the next step was to isolate only non-polar lipids. Complex mixtures of lipids can be fractionated by chromatographic procedures based on the different polarities of each class of lipid, so semi-preparative TLC was performed for the isolation. Many solvent systems can be

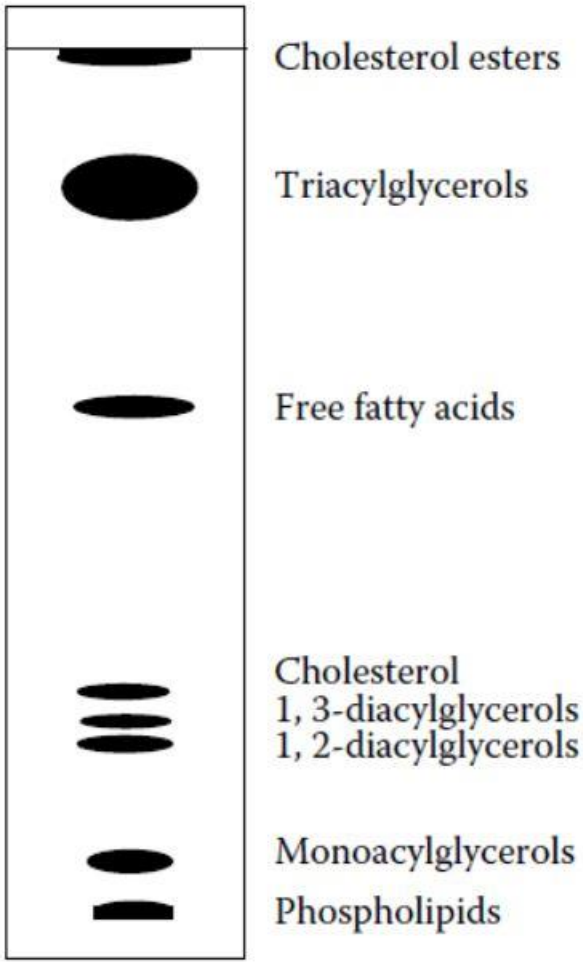


Figure 7 Schematic TLC separation of simple lipids on silica gel G as stationary phase, Mobile phase; hexane: diethyl ether: formic acid (80:20:2 by volume)[128]

used for separation of simple lipid classes, but one that is used most frequently is hexane : diethyl ether : formic acid in different ratios (Figure 7).[130] Polarity of compounds decreases from starting point towards solvent front. To be able to detect compounds under UV-light, it is necessary that they fluoresce, and only molecules that absorb radiation of certain wavelength and then emit radiation of greater wavelength do. Will the molecule fluoresce or not is determined by its structure. Molecules that have covalently bound unsaturated groups which

absorb UV-light (chromophores) will fluoresce. Since lipids do not fluoresce, TLC plates were sprayed with rhodamine 6G (Figure 8). Spots which contained lipids quenched rhodamines fluorescence, thus allowing their detection under UV-light. As said in the experimental section 3.3.2., zone on TLC plate which contained TGs, as well as every other zone less polar than TGs was scrapped into the glass column. Since TG-estolides are slightly more polar than TGs, one might argue that by scrapping the zone containing TGs and other zones above it will cause the loss of TG-estolides, but, as it can be seen from figure 11, used mobile phase (hexane: diethyl ether: formic acid in 80:20:2 ratio by volume) cannot separate TG-estolides from TGs. Diethyl ether is known as an excellent solvent for non-polar lipids,[129] while rhodamine 6G is insoluble in it. Non-polar lipids, which were dissolved in ether were collected into the heart-shaped flask, while rhodamine 6G and silica gel stayed in the column.

Now, the sample contained TGs, TG-estolides, cholesteryl esters, wax esters and most likely other minor lipids with low polarity. Even though the procedure itself is pretty similar to

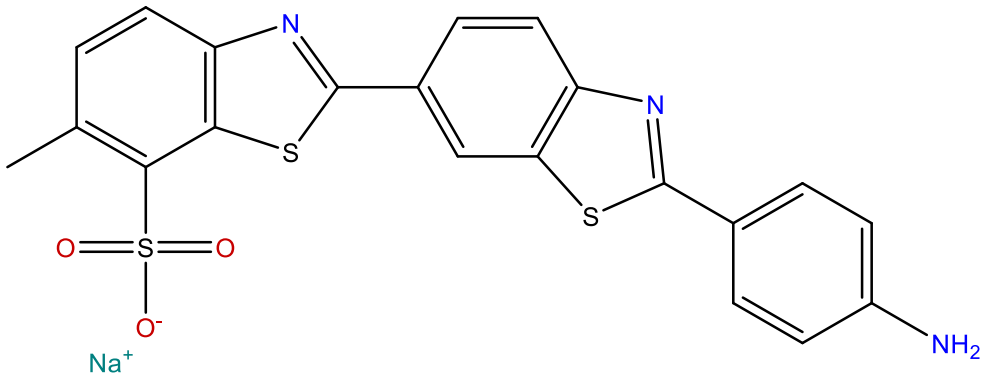


Figure 9 Primuline structural formula

the second step, third step of the estolide isolation was the most challenging one, because of the extremely small difference in the polarity between TGs and TG-estolides. Performing an analytical TLC of triolein standard and TG-estolide standards (AMR-1919-2 and AMR-1921-3-1) slightly greater polarity of TG-estolides was observed compared to TGs. Fatty acyl chain decreases the polarity of a compound, but on the other hand additional carboxylic group increases it even more. Small difference in polarity required a complete development of the TLC method for separation of TG-estolides from TGs and other non-polar lipids, which will be explained in the chapter 4.2. In short, some differences compared to the second step of isolation are; different mobile phase, different type of TLC plate, development of the TLC plate, that is the number of times TLC plate was developed, and visualization reagent used. Mobile phase

and different TLC plate will be explained later, but for now, each TLC plate was developed three times, because in that way compounds were more concentrated onto the one spot on a plate, meaning that the resolution was drastically improved compared to the single development. Additionally, primuline (Figure 9) was used instead of rhodamine 6G, because with it, the compounds were more visible under UV-light. After they were isolated, the chloroform solution of obtained estolides was concentrated by evaporating most of chloroform, and the third step was performed again, but this time on only one TLC plate, which was, compared to previously used TLC plates, twice as wide.

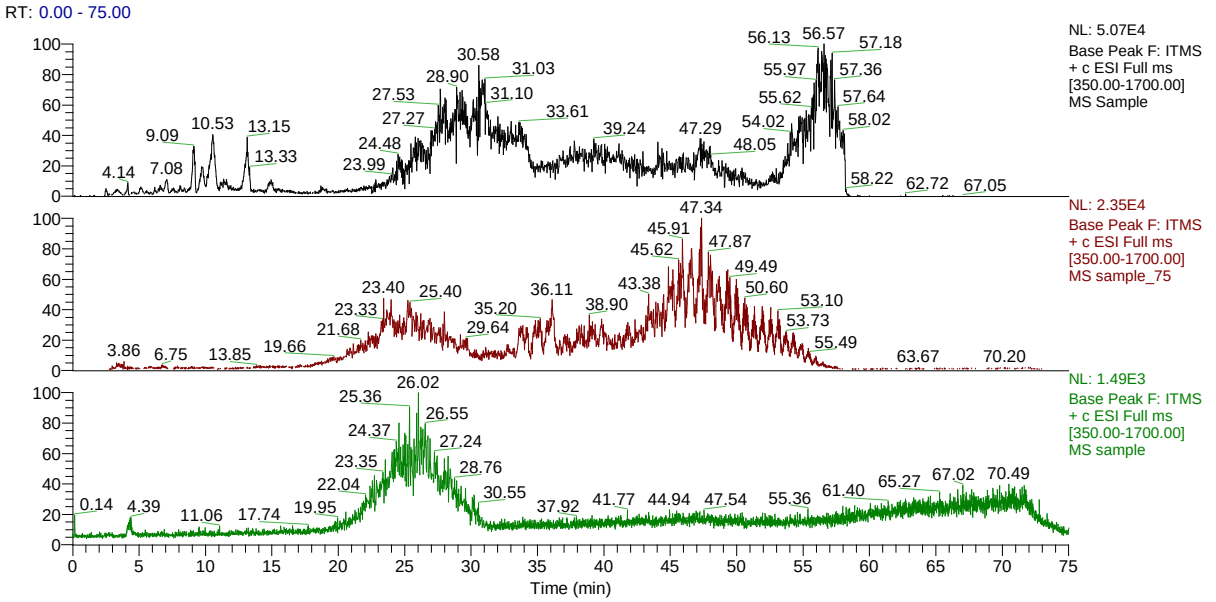


Figure 10 Visual representation of estolide isolation and purification from non-polar lipids. 1) Chromatogram of "unpurified" estolide sample, after first semi-preparative TLC isolation, Separation method B, 2) chromatogram of second semi-preparative TLC purified estolide sample, Separation method A, 3) chromatogram of semi-preparative HPLC purified estolide sample, Separation method A

And finally, fourth step of isolation included use of HPLC for additional separation and purification of estolides from small amount of TGs and other impurities that were present in the sample. Difference here regarding the stationary phase, compared to the TLC isolation is that it was performed in the reversed-phase mode, that is the stationary phase was non-polar and mobile phases polar.

After every isolation step of estolides, the sample was checked with HPLC, and process of isolation and purification can be seen in figure 10. First chromatogram represents "unpurified" estolide sample, measured after first semi-preparative TLC isolation, which

contained TGs (24 min – 33.6 min), TG-estolides (37 min – 47.3 min) and other impurities. Not to get confused, it is important to mention that first chromatogram was obtained using "Separation method B", while two others were obtained using "Separation method A". Mobile phases in "Separation method B" are more polar than in "Separation method A" which is why, compared to the first one, every peak in two other chromatograms is shifted to the left. Second chromatogram shows estolide sample which was, after concentrating it, purified with second semi-preparative TLC. That second semi-preparative TLC expelled TGs from the sample and left TG-estolides (18 min – 30.5 min) and other unknown impurities. Final semi-preparative HPLC purification step ("Separation method A") left nothing but TG-estolides, which were then used for further analysis on HPLC-MS and, after transesterification and trimethylsilylation, on GC-MS.

4.2. Development of the TLC method for isolating TG-estolides from non-polar lipids

4.2.1. Mobile Phase

Separation of estolides from TGs and other non-polar lipids was based on TLC, because of its advantages mentioned in chapter 2.3.1. However, small difference in polarity between TGs and TG-estolides made their separation really challenging. That is why TLC method for separation of estolides from primarily TGs, and then from other non-polar lipids had to be developed, and first was the optimization of mobile phase. In total, 17 different mobile phases (Table 2) were tested by separating non-polar lipid extract, and triolein and TG-estolide standards, which were placed side by side on TLC plates.

Table 2 Mobile phases tested in the optimization of estolide separation from triacylglycerols

N	Mobile phase	Ratio of solvents (v:v)
1	Hexane: diethyl ether: formic acid	70 : 30 : 2
2	Hexane: diethyl ether: formic acid	80 : 20 : 2
3	Hexane: diethyl ether: formic acid	90 : 20 : 2
4	Hexane: diethyl ether: formic acid	92.5 : 7.5 : 2
5	Hexane: diethyl ether: formic acid	95 : 5 : 2
6	Hexane: diethyl ether	92 : 5 : 7.5
7	Toluene: diethyl ether: formic acid	95 : 5 : 2
8	Hexane: formic acid	100 : 2
9	Hexane: ethyl acetate: formic acid	92.5 : 7.5 : 2
10	Hexane: diethyl ether: ethyl acetate	92.5 : 7.5 : 7.5
11	Hexane: diethyl ether: ethyl acetate: formic acid	92.5 : 7.5 : 7.5 : 2
12	Hexane: diethyl ether: toluene	92.5 : 7.5 : 7.5
13	Hexane: diethyl ether: toluene	90 : 10 : 7.5
14	Hexane: diethyl ether: toluene	90 : 10 : 10
15	Hexane: diethyl ether: chloroform	92.5 : 7.5 : 7.5
16	Cyclohexane: diethy ether	92.5 : 7.5
17	Cyclohexane: diethyl ether: toluene	92.5 : 7.5 : 7.5

As mentioned before, solvent system hexane: diethyl ether: formic acid in different ratios is most commonly used for chromatographic separation of lipids. So, it was natural to first try isolating TG-estolides from other non-polar lipids using that solvent system. First, it was tested in the 80:20:2 ratio by volume, but, as it can be seen from figure 11, there was practically no separation at all. By decreasing the polarity of developing solvent, more polar TG-estolides should bind stronger to polar stationary phase, while non-polar mobile phase

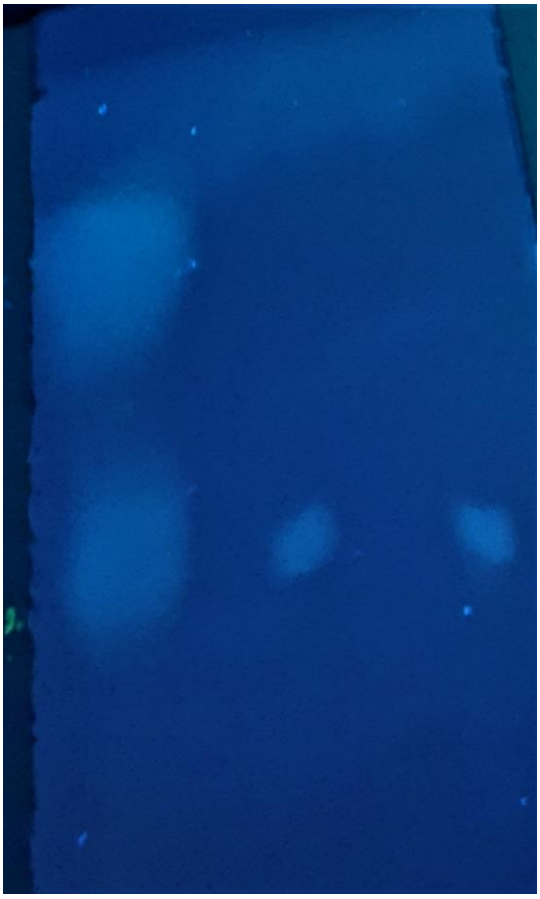


Figure 11 Analytical TLC of (from left to right) non-polar lipid extract, triolein standard and TG-estolide standard, solvent system → Hexane: Diethyl ether: formic acid = 80:20:2

carries less polar TGs up the plate. That way of thinking was, from that point, the base for further modification of mobile phase, but too much decrease in polarity had an opposite effect. For example, when hexane: formic acid in ratio 100:2 by volume was used, compounds remained at the starting point (Figure 12). That happened because mobile phase didn't contain polar solvent at all, which would otherwise limit the binding of applied compounds to stationary phase and carry them up the plate. But applied compounds bound stronger to the stationary phase and were not carried up the plate. Getting back to hexane: diethyl ether: formic acid mobile phase, there were, indeed some indications of separation with that developing solvent in the 95:5:2 ratio (Figure 13), but that was still quite far from satisfactory result. Next, the polarity of mobile phase was slightly increased by using mentioned solvents in 92.5:7.5:2 ratio, and also the same developing solvent, but without formic acid (92.5:7.5) was tested (Figure 14). From it, the role of formic acid can clearly be seen; formic acid concentrated the compounds onto one spot, but the separation was affected, meaning that it was slightly worse. Since, the separation of compounds was better without formic acid, it was expelled from further testing.

Figure 12 Analytical TLC of (from left to right) non-polar lipid extract, triolein standard and TG-estolide standard, solvent system → Hexane: formic acid = 100:2

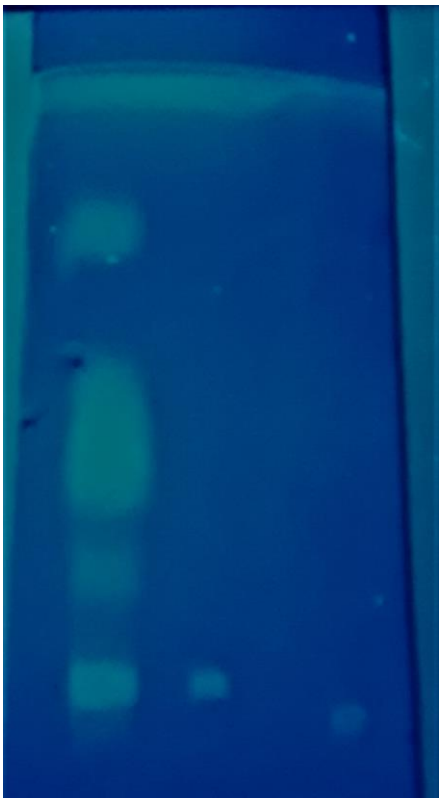


Figure 13 Analytical TLC of (from left to right) non-polar lipid extract, triolein standard and TG-estolide standard, solvent system → Hexane: Diethyl ether: formic acid = 95:5:2

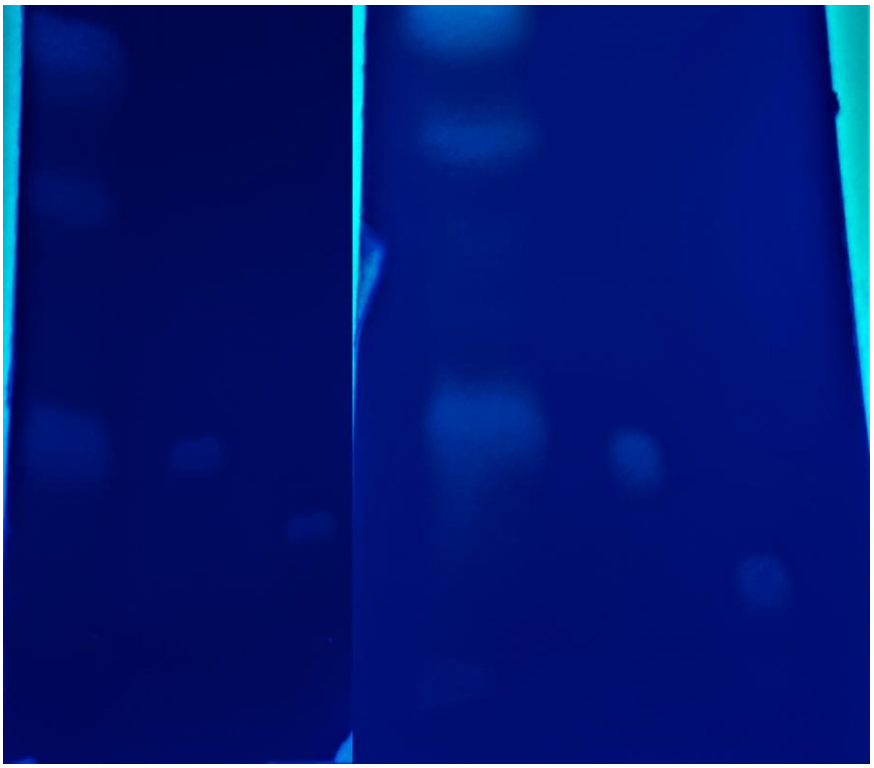


Figure 14 Analytical TLC of (from left to right) non-polar lipid extract, triolein standard and TG-estolide standard, a) Left TLC plate developed with Hexane: Diethyl ether: formic acid = 92.5:7.5:2, b) Right TLC plate developed with Hexane: Diethyl ether = 92.5:7.5

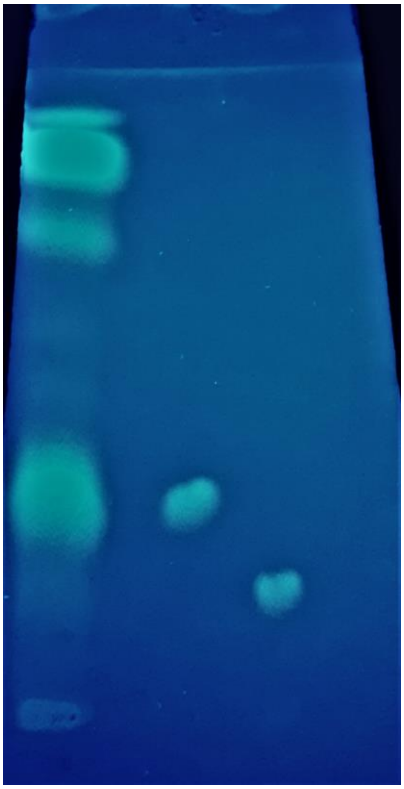


Figure 15 Analytical TLC of (from left to right) non-polar lipid extract, triolein standard and TG-estolide standard, solvent system → Hexane: Diethyl ether: toluene = 92.5:7.5:7.5

After many trial and error runs with other developing solvents, the best results, in the means of separation, concentration of compounds onto one spot and visibility of compounds under the UV-light, were obtained with hexane: diethyl ether: toluene in 92.5:7.5:7.5 ratio by volume (Figure 15). Other developing solvents from table 2 didn't gave satisfactory results.

4.2.2. Adsorption TLC, "standard" TLC plate preparation method

Given that I did not use commercially available silica gel TLC plates, but I used the ones that I prepared myself, plate preparation had a great importance when it came to TLC sample separation and analysis. Plates were prepared with or without TLC spreader, and because of plane human error, which was always present and unavoidable, every TLC plate was slightly different. Density of silica gel slurry, thickness of applied silica gel layer, method of plate preparation and modifiers present in silica gel slurry affect separation of compounds and consistency of the results. After finding the best developing solvent the plan was to get even better separation of compounds and it was of utmost importance to obtain consistent results, hence the development of TLC plate preparation method.

First TLC plate preparation method was standard one which requires TLC spreader for spreading silica gel slurry over the glass plates. Thickness of silica gel layer in this method depends on the speed with which the spreader is dragged across the plates and on the density of silica gel slurry. If it was dragged slowly, the layer would be thicker and vice versa. Also, slurry shouldn´t be too dense or too transparent, and after a lot of trial and error runs, density of silica gel slurry was found to be best with 38.2 g of silica gel on 100 ml of water (1:2.6 by mass). Plates were prepared as described in chapter 3.4.2., and consistency and significance of results checked with statistical analysis.

Statistical analysis was done based on the R_f values of triolein and TG-estolide standards. Data derived from R_f values that was used to describe consistency and significance of the results comprised of average R_f value ($\overline{R_f}$), standard deviation (s), standard deviation of each data point expressed as percentage value of R_f (s [%]) and its average value ($\overline{s\ [\%]}$), relative standard deviation (RSD), range of R_f values (w), absolute (E) and relative (E_r) error and "Test Tn". Data obtained for each plate with this plate preparation method can be seen in table D1, and data which was used for determination of result´s significance in table 3.

Table 3 Significant data obtained from statistical analysis of "standard" TLC plate preparation method

"Standard"	Triolein standard	Estolide standard
N	21	21
$\overline{R_f}$	0.23	0.11
s	0.038	0.021
$\overline{s\ [\%]}$	17.08	18.77
RSD	166	181
W	0.13	0.08
E, E_r	→ 0	→ 0
Test Tn	Positive	Positive

Until they are compared with statistical results of another two plate preparation methods, it is hard to tell whether these results are good or bad, but they will serve as a standard. One thing that can tell whether the results, that is data points are, if anything, consistent with one another is the "Test Tn", which is a statistical test that determines whether each and every data point belongs to the measured population. Tn of each data point is calculated with equation 4.1.

$$T_n = \frac{\left|R_f - \overline{R_f}\right|}{s} \tag{4.1}$$

If the Tn value is lower that T_n critical, which is empirically determined and tabled, data point is good and can stay as a part of the population. If opposite happens, that means that data point differs too much from the population and has to be thrown out. Every data point from this statistical analysis had T_n value lower than T_n critical, meaning that the results are consistent.

The advantage of this plate preparation method is, if done properly, consistency of layer thickness, but it has some shortcomings as well. One of them is definitely stripes of silica gel, which are present if the spreader is not dragged across the plates with constant speed. The biggest shortcoming is that it is time consuming, hence the second, "fast" TLC plate preparation method.

4.2.3. Adsorption TLC, "fast" TLC plate preparation method

In this TLC plate preparation method described in chapter 3.4.3. TLC spreader wasn't used. Not using TLC spreader had two major advantages: it saved an extraordinary amount of time, because aforementioned 24 silica gel TLC plates can be prepared in just 20 minutes, and there were no stripes present on the plates. One shortcoming of this method is the possibility of an unequal distribution of silica gel slurry on the TLC plates.

Even though this method has more advantages than shortcomings, the results of statistical analysis, however, show different picture (Table D2, Table 4). Standard deviation (s), average value of standard deviation of each data point ($\overline{s\,[\%]}$), relative standard deviation (RSD) and range of R_f values (w) were used in the comparison with the first plate preparation method for determination of result´s significance. And even though absolute and relative error strive towards zero, and all data points "passed" Tn test, it is clearly visible that this plate

Table 4 Significant data obtained from statistical analysis of "fast" TLC plate preparation method

"Fast"	Triolein standard	Estolide standard
N	20	20
$\overline{R_f}$	0.27	0.14
s	0.053	0.041
$\overline{s\,[\%]}$	20.68	31.63
RSD	199 ppt	291 ppt
W	0.19	0.16
E, E_r	→ 0	→ 0
Test Tn	Positive	Positive

preparation method has all four values higher than the first one, meaning that the consistency of separation is worse. Also, results can be compared using Student´s T-test, which tells if there is a significant difference between two sets of data, or simply, if two sets of data belong to the same population. If T-test is, for example positive, that would, in this particular case, mean that there is no difference between data obtained with "standard" and data obtained with "fast" TLC

plate preparation method, and either one could be used for plate preparation, because the results would statistically be the same. But, table 5 shows that t_{stat} value is higher than $t_{critical}$ for both triolein and TG-estolide standard, which is why the four aforementioned values (s, $\overline{s}$ [%], RSD and w) determine which results are better, and obviously the "standard" TLC plate preparation method has lower error, that is results obtained on TLC plates prepared in that way have higher consistency and significance.

Table 5 Student´s T-test comparing two sets of data, assuming unequal variances, obtained with "fast" and "standard" TLC plate preparation method for triolein standard and TG-estolide standard

Based of R_f (TO) **T-test: Two-Sample Assuming Unequal Variances**			**Based of R_f (Est)** **T-test: Two-Sample Assuming Unequal Variances**		
	„Fast"	„Standard"		„Fast"	„Standard"
Mean	0.268129	0.230458	**Mean**	0.139714	0.114033
Variance	0.002854	0.001462	**Variance**	0.001657	0.0004267
Observations	20	21	**Observations**	20	21
Hypothesized Mean Difference	0		**Hypothesized Mean Difference**	0	
df	34		**df**	28	
t Stat	2.585303		**t Stat**	2.528019	
P(T<=t) one-tail	0.007095		**P(T<=t) one-tail**	0.008696	
t Critical one-tail	1.690924		**t Critical one-tail**	1.701131	
P(T<=t) two-tail	0.014191		**P(T<=t) two-tail**	0.017392	
t Critical two-tail	2.032245		**t Critical two-tail**	2.048407	

4.2.4. Metal ion TLC, "standard" TLC plate preparation method

Since "fast" TLC plate preparation method did not give good result, I had to return to "standard" one which uses TLC spreader, but this time metal cation was incorporated into silica gel. It is known that metal cation can bind to oxygen in carbonyl, carboxyl, or in this case to oxygen in ester groups, thus modifying adsorption onto the silica gel plate.[131] Since TGs and TG-estolides differ in the number of ester linkages, the separation on the TLC silica gel plate incorporated with metal cation should be different. One other well-known example of separating different fatty acids on modified silica gel TLC plates is separation of fatty acids that differ in the number of double bonds on silver impregnated silica gel TLC plates.[132]

Table 6 Inorganic salts used in the complexation with ethyl acetate to determine which metal cation binds the best to the ester group

Class	Inorganic salt	Metal cation
Nitrates	$AgNO_3$	Ag^+
	$Mg(NO_3)_2$	Mg^{2+}
	$Ni(NO_3)_2*6H_2O$	Ni^{2+}
	$Pd(NO_3)_2$	Pd^{2+}
	$Al(NO_3)_3*9H_2O$	Al^{3+}
	$Fe(NO_3)_3$	Fe^{3+}
Chlorides	KCl	K^+
	$CaCl_2$	Ca^{2+}
	NaCl	Na^+
	$MgCl_2$	Mg^{2+}
	$CuCl_2$	Cu^{2+}
	$MnCl_2$	Mn^{2+}
	$SnCl_4*5H_2O$	Sn^{4+}
	$CoCl_2$	Co^{2+}
Iodides	RbI	Rb^+
	CsI	Cs^+
Carbonate	Li_2CO_3	Li^+

But before any TLC plate preparation, metal cation that binds the best to the ester group had to be determined. Different metal cation – ethyl acetate complexes were formed and formation of that complexes was monitored with ESI/MS as described in chapter 3.4.4. All compounds, that is metal cations that were tested are listed in table 6. Predicting the binding strength of metal cation with carboxylic or ester group is challenging, because many different factors affect it. First thing that anybody would consider is the difference in the metal cation-oxygen ionic radii, and the trend would be like this; the smaller the difference in the ionic radius between metal cation and oxygen the higher the binding strength. But Bala et al.[131] found, using molecular modeling and charge density plots, that ionic radius based explanations fail to describe ion binding strength trends of metal cation with carboxylic group, and proposed that geometry, that is coordination of individual cations have a crucial role in determination of binding strengths of different cations with carboxylic acid group.

ESI/MS analysis showed that only six metal cations formed a complex with ethyl acetate (Figure 16). Since some metal cations, especially alkali and earth alkali are always present in the surroundings, intensities were obtained by subtracting the intensity of metal cation-ethyl acetate from intensity of ethyl acetate as a blank. If anything, it was expected of alkali and earth alkali metals to form a complex. It is clear that sodium has the highest binding strength with

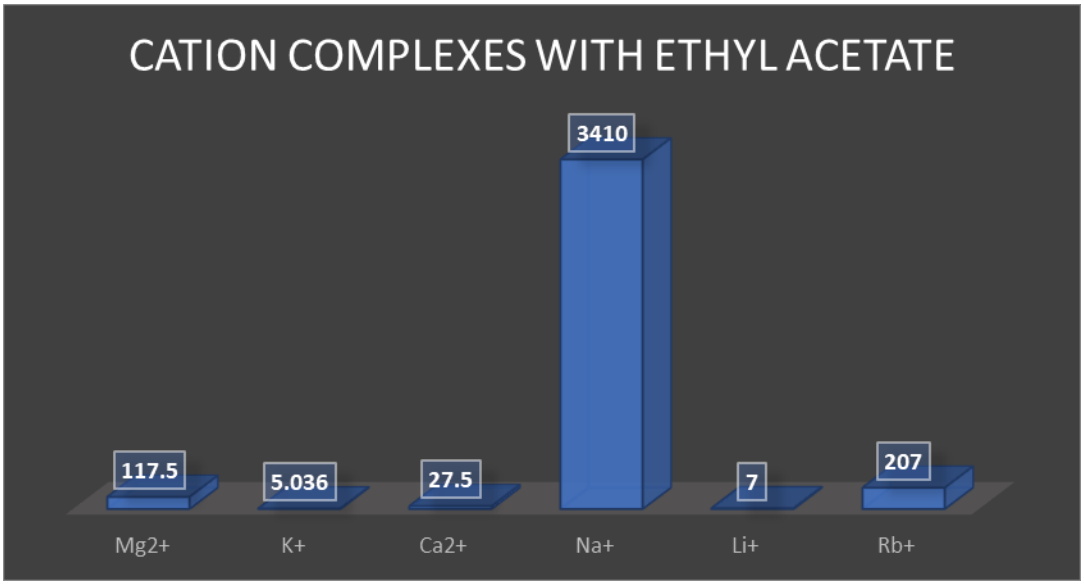


Figure 16 Intensities of metal cation-ethyl acetate formed complexes measured with ESI/MS and expressed in arbitrary units

ethyl acetate, so it was used as a modifier in silica gel. Most of tested metal cations would probably also form complex with ethyl acetate, but their concentration must have been too low.

Now when it was found that sodium cation binds the best to the oxygen in the ester group, TLC plates impregnated with it could be prepared, and another statistical analysis conducted. Table D3 and table 7 show “metal ion” TLC plate preparation results of statistical analysis.

Table 7 Significant data obtained from statistical anaylsis of "metal ion" TLC plate preparation method

“Metal ion”	Triolein standard	Estolide standard
N	15	15
$\overline{R_f}$	0.33	0.19
s	0.023	0.015
$\overline{s\ /[\%]}$	7.04	7.84
RSD	70	78
w	0.066	0.039
E, E_r	→ 0	→ 0
Test Tn	Positive	Positive

Table 8 Student´s T-test comparing two sets of data, assuming unequal variances, obtained with "metal ion" and "standard" TLC plate preparation method for triolein standard and TG-estolide standard

Based of R_f (TO) T-test: Two-Sample Assuming Unequal Variances			**Based of R_f (Est) T-test: Two-Sample Assuming Unequal Variances**		
	„Metal Ion“	„Standard“		„Metal Ion“	„Standard“
Mean	0.329734	0.230458	**Mean**	0.194038	0.114033
Variance	0.000534	0.001462	**Variance**	0.000229	0.0004267
Observations	15	21	**Observations**	15	21
Hypothesized Mean Difference	0		**Hypothesized Mean Difference**	0	
df	33		**df**	34	
t Stat	9.678472		**t Stat**	13.41142	
P(T<=t) one-tail	1.82E-11		**P(T<=t) one-tail**	1.95E-15	
t Critical one-tail	1.69236		**t Critical one-tail**	1.690924	
P(T<=t) two-tail	3.65E-11		**P(T<=t) two-tail**	3.9E-15	
t Critical two-tail	2.034515		**t Critical two-tail**	2.032245	

Student´s T-test was also conducted to compare results obtained with “metal ion” and “standard” TLC plate preparation methods (Table 8), and by looking at t_{stat} values it shows that this two sets of data differ even more compared to the “fast” – “standard” TLC plate preparation methods.

And now, when we have all data for all three TLC plate preparation methods, and when we have the confirmation that sets of data obtained from “standard”, “fast” and “metal ion” TLC plate preparation methods do not belong to the same population, we can put significant data for TO standard and TG-estolide standard side by side, just to get a clearer view (Table 9). “Metal ion” TLC plate preparation method gives the highest consistency of results with the lowest error, then follows “standard” one, and the worst results were obtained with “fast” method. One thing that can also be seen is that the separation of TO standard from TG-estolide standard is the best on silica gel TLC plates impregnated with metal cation, with the difference in R_f value of 0.14, while difference in the R_f values obtained on plates prepared with “standard” and “fast” preparation method is 0.12 and 0.13 respectively.

Table 9 Comparison of significant data obtained with "standard", "fast" and "metal ion" TLC plate preparation methods for TO and TG-estolide standards

Comparison	N	$\overline{R_f(TO)}$	s (TO)	$\overline{s\,(TO)}/[\%]$	RSD	w	$\overline{R_f(Est)}$	s (Est)	$\overline{s\,(TO)}/[\%]$	RSD	w
Standard TLC preparation	21	0.23	0.0382	17.08	166	0.1285	0.11	0.021	18.8	181	0.0824
Fast TLC preparation	20	0.27	0.0534	20.68	199	0.1934	0.14	0.0407	31.63	291	0.1604
Metal ion TLC preparation	15	0.33	0.023	7.04	70	0.0661	0.19	0.0151	7.84	78	0.039

4.3. HPLC-ESI/MS analysis

Two different instruments (LCQ Fleet and LTQ Orbitrap) and two different separation methods ("Separation method A" and "Separation method B") were used for HPLC-MS separation, purification and analysis of VC TG-estolides. LCQ Fleet equipped with 3D ion trap as mass analyser was, after the second semi-preparative TLC, used for additional purification of estolides, while LTQ Orbitrap was, because of its higher resolution and better mass accuracy compared to 3D ion trap, used for mass spectra analysis. Purification of estolide sample with semi-preparative HPLC was achieved on LCQ Fleet with "Separation method A" and was explained in chapter 4.1., so in this chapter we are going to focus on mass spectra analysis and fragmentation patterns.

First thing first, which separation method would be best for the analysis of mass spectra has to be determined. Figure 17 shows two reconstructed full MS chromatograms of estolide mixture with mass range *m/z* 1000-1200, in time range within which estolides are observed. Those chromatographs were obtained with "Separation method A", which was developed on spot, and "Separation method B", which was used in separation of FAHFA-containing TGs.[26] As explained in experimental section (chapters 3.5.3. and 3.5.4.) they differ in used mobile phases and gradient programs. "Separation method B" uses more polar mobile phases compared to "Separation method A" which is why compounds start eluting later on the reversed-phase

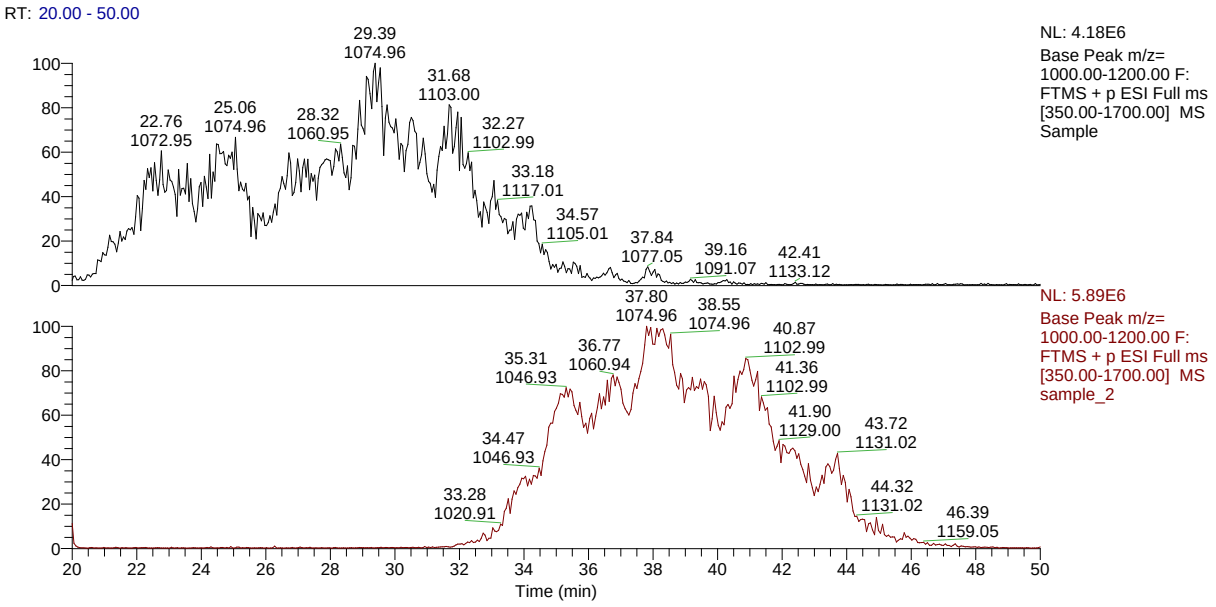


Figure 17 Reconstructed chromatograms of TG-estolide mixture (γ = 0.625 mg/mL) represented in time range in which TG-estolides were identified and obtained with "Separation method A" (upper one) and "Separation method B" (lower one). LTQ Orbitrap in full MS, ESI positive mode and mass range *m/z* 1000-1200

column. In fact, they start exiting the column 12,5 minutes later than in "Separation method A", and the retention time of most abundant compound (*m/z* 1074.96) is almost 9 minutes higher in "Separation method B" (t_R = 38.05 min) than it is in "Separation method A" (t_R = 29.39 min). Since, in both separation methods, all estolides exited the column much sooner than the program ended, it is clear that gradient program in both of them could be shortened; "A" for 25 minutes (from 75 to 50) and "B" for 8 minutes (from 68 to 60), because 10 minutes of isocratic run of mobile phases is enough to expel possible impurities and purge the column for subsequent run. In reversed-phase column, compounds of lower molecular mass are eluted earlier, as well as are more unsaturated compounds, because they are more polar, so they do not bind to the non-polar stationary phase as saturated ones do.

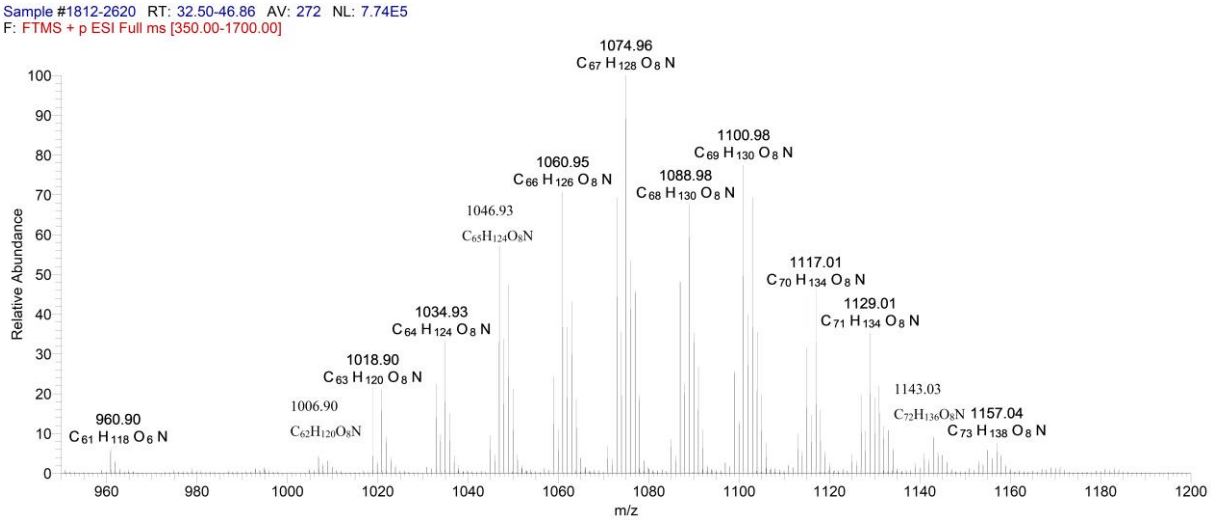


Figure 18 FTMS p +ESI Full MS mass spectrum of all identified TG-estolides in vernix caseosa sample, measured on LTQ Orbitrap in positive mode. Retention time; 32.5-46.86 min, measured *m/z* 350.00-1700-00

A lot of peak overlapping is present in both chromatograms, but by analysing *m/z* values of compounds exiting the column, slightly better separation was observed with "Separation method B." Even though compounds start eluting earlier in "Separation method A" than they do in "Separation method B", and even though chromatographic run is 10 minutes shorter in "Separation method A", better separation of TG-estolides in a complex mixture was achieved with "Separation method B". Moreover, signals in mass spectra had slightly higher intensities ($7.74 \cdot 10^5$ for "B" and $6.6 \cdot 10^5$ for "A"). So, because of better separation of estolide mixture and higher intensities in the mass spectra, I have decided to analyse mass spectra obtained from HPLC-ESI/MS run conducted with "Separation method B".

Mass spectrum representing all TG-estolides eluting between 32.5 and 46.86 minutes can be seen in figure 18. Only molecular adducts $[M+NH_4]^+$ of highest abundance in certain mass range are represented here, but by analysing every single peak in it, 126 compounds ranging from *m/z* 934.85 to 1193.08 have been identified and they are listed in table 10. Since ammonium formate was added, molecular adducts $[M+NH_4]^+$ are observed in the spectra. By subtracting molecular mass of ammonium cation, molecular masses and molecular formulae of TG-estolides, as well as the ring-double bond (RDB) value in each TG-estolide was obtained. It is assumed that only double bonds are present in fatty acyl chains, rather than triple bonds and ring structures, which was later proved with GC-MS analysis. Most of determined TG-

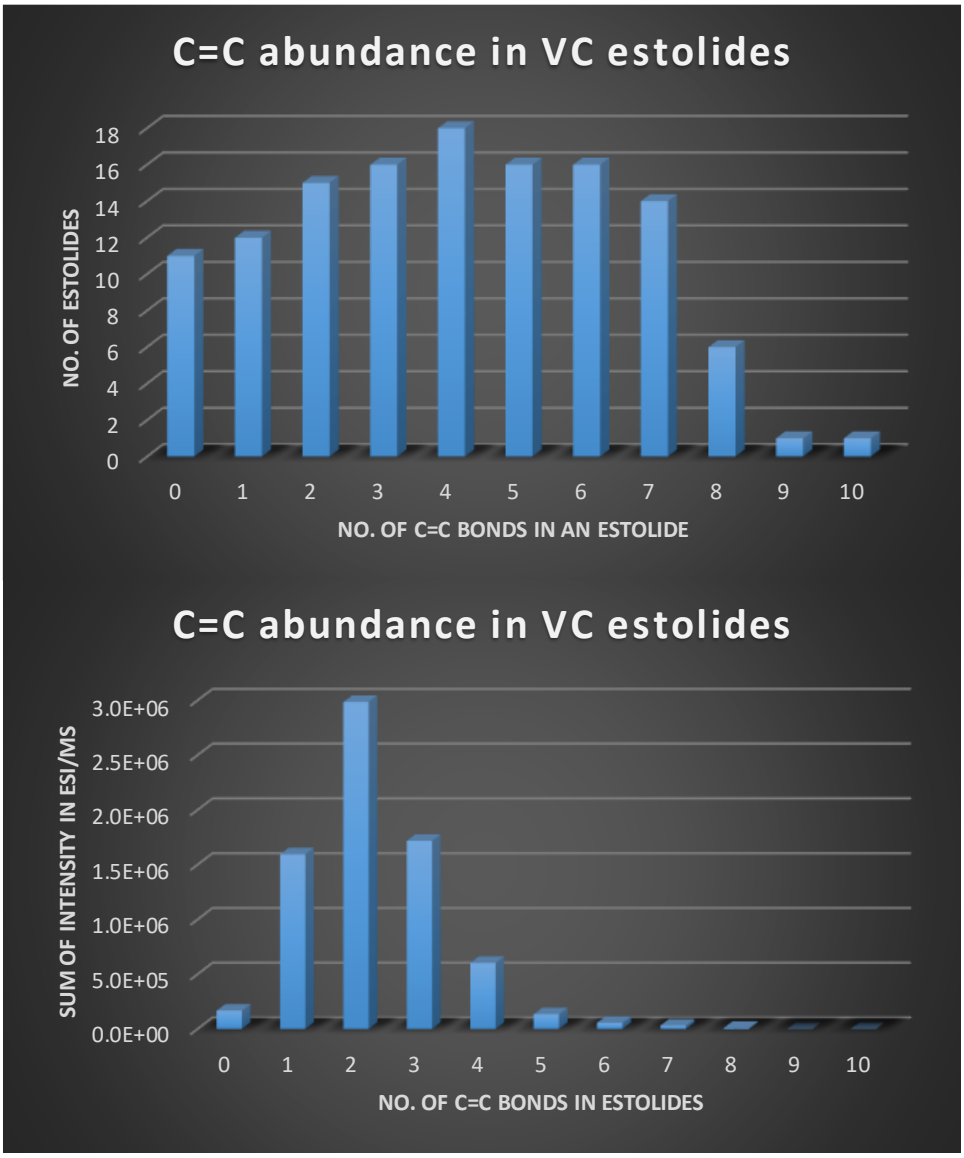


Figure 19 Abundance of C=C bonds in identified vernix caseosa TG-estolides by a) total number of TG-estolides in VC sample that contain them, b) sum of obtained ESI/MS intensities of TG-estolides with certain number of C=C bonds

estolides are unsaturated, and number of double bonds in structure of identified compounds varies from 1 to 10 (Figure 19a). Such distribution of double bonds is not surprising, because that means that most fatty acyl chains have 1 or two double bonds, which is also confirmed when looking at the sum of intensities versus the number of double bonds (Figure 19b). It is also possible that in some TG-estolides one fatty acyl chain has more than two double bonds, while others have none, but that cannot be observed or determined with HPLC-ESI/MS, so GC-MS analysis was conducted as well. Most abundant compound observed with ESI/MS has *m/z* 1074.95968, which after subtracting molecular mass of ammonium cation gives TG-estolide

Table 10 List of all vernix caseosa TG-estolides identified with HPLC-ESI/MS using LTQ Orbitrap in positive mode and "Separation method B"

$[M+NH_4^+]$	Molecular formula	RDB $[M+NH_4^+]$	INTENSITY	[M]	Formula	RDB [M]	No. C=C	$[M+NH_4^+]$	Molecular formula	RDB $[M+NH_4^+]$	INTENSITY	[M]	Formula	RDB [M]	No. C=C
952.85	$C_{58}H_{114}O_8N$	2.5	1.63E+03	934.85	$C_{58}H_{110}O_8$	4	0	1080.92	$C_{68}H_{122}O_8N$	8.5	5.53E+03	1062.92	$C_{68}H_{118}O_8$	10	6
960.82	$C_{59}H_{110}O_8N$	5.5	5.24E+04	942.82	$C_{59}H_{96}O_8$	7	3	1082.93	$C_{68}H_{124}O_8N$	7.5	6.64E+03	1064.93	$C_{68}H_{120}O_8$	9	5
962.83	$C_{59}H_{112}O_8N$	4.5	1.62E+02	944.83	$C_{59}H_{108}O_8$	6	2	1084.94	$C_{68}H_{126}O_8N$	6.5	5.05E+04	1066.94	$C_{68}H_{122}O_8$	8	4
964.85	$C_{59}H_{114}O_8N$	3.5	2.22E+03	946.85	$C_{59}H_{110}O_8$	5	1	1086.96	$C_{68}H_{128}O_8N$	5.5	3.12E+05	1068.96	$C_{68}H_{124}O_8$	7	3
966.87	$C_{59}H_{116}O_8N$	2.5	2.57E+03	948.87	$C_{59}H_{112}O_8$	4	0	1088.98	$C_{68}H_{130}O_8N$	4.5	4.04E+05	1070.98	$C_{68}H_{126}O_8$	6	2
972.82	$C_{60}H_{110}O_8N$	6.5	1.09E+02	954.82	$C_{60}H_{106}O_8$	8	4	1090.91	$C_{69}H_{120}O_8N$	10.5	1.40E+03	1072.91	$C_{69}H_{120}O_8$	12	8
976.85	$C_{60}H_{114}O_8N$	4.5	1.11E+03	958.85	$C_{60}H_{110}O_8$	6	2	1090.99	$C_{68}H_{132}O_8N$	3.5	1.90E+05	1072.99	$C_{68}H_{128}O_8$	5	1
978.87	$C_{60}H_{116}O_8N$	3.5	4.57E+03	960.87	$C_{60}H_{112}O_8$	5	1	1092.92	$C_{69}H_{122}O_8N$	9.5	3.38E+03	1074.92	$C_{69}H_{118}O_8$	11	7
980.88	$C_{60}H_{118}O_8N$	2.5	5.46E+03	962.88	$C_{60}H_{114}O_8$	4	0	1094.93	$C_{69}H_{124}O_8N$	8.5	4.65E+03	1076.93	$C_{69}H_{120}O_8$	10	6
986.84	$C_{61}H_{112}O_8N$	6.5	1.66E+02	968.84	$C_{61}H_{108}O_8$	8	4	1096.94	$C_{69}H_{126}O_8N$	7.5	1.68E+04	1078.94	$C_{69}H_{120}O_8$	9	5
988.85	$C_{61}H_{114}O_8N$	5.5	1.74E+02	970.85	$C_{61}H_{110}O_8$	7	3	1098.96	$C_{69}H_{128}O_8N$	6.5	1.79E+05	1080.96	$C_{69}H_{124}O_8$	8	4
990.87	$C_{61}H_{116}O_8N$	4.5	3.85E+03	972.87	$C_{61}H_{112}O_8$	6	2	1100.98	$C_{69}H_{130}O_8N$	5.5	4.85E+05	1082.98	$C_{69}H_{126}O_8$	7	3
992.88	$C_{61}H_{118}O_8N$	3.5	1.23E+04	974.88	$C_{61}H_{114}O_8$	5	1	1102.99	$C_{69}H_{132}O_8N$	4.5	4.39E+05	1084.99	$C_{69}H_{128}O_8$	6	2
994.89	$C_{61}H_{120}O_8N$	2.5	1.18E+04	976.89	$C_{61}H_{116}O_8$	4	0	1104.92	$C_{70}H_{122}O_8N$	10.5	2.19E+03	1086.92	$C_{70}H_{118}O_8$	12	8
996.83	$C_{62}H_{110}O_8N$	8.5	1.00E+02	978.83	$C_{62}H_{106}O_8$	10	6	1105.00	$C_{68}H_{134}O_8N$	3.5	1.27E+05	1087.00	$C_{69}H_{130}O_8$	5	1
998.84	$C_{62}H_{112}O_8N$	7.5	2.45E+02	980.84	$C_{62}H_{108}O_8$	9	5	1106.93	$C_{70}H_{124}O_8N$	9.5	4.00E+03	1088.93	$C_{70}H_{120}O_8$	11	7
1000.85	$C_{62}H_{114}O_8N$	6.5	2.77E+02	982.85	$C_{62}H_{110}O_8$	8	4	1108.95	$C_{70}H_{126}O_8N$	8.5	4.86E+03	1090.95	$C_{70}H_{122}O_8$	10	6
1002.87	$C_{62}H_{116}O_8N$	5.5	7.86E+02	984.87	$C_{62}H_{112}O_8$	7	3	1110.96	$C_{70}H_{128}O_8N$	7.5	1.22E+04	1092.96	$C_{70}H_{124}O_8$	9	5
1004.88	$C_{62}H_{118}O_8N$	4.5	8.05E+03	986.88	$C_{62}H_{114}O_8$	6	2	1112.98	$C_{70}H_{130}O_8N$	6.5	6.07E+04	1094.98	$C_{70}H_{126}O_8$	8	4
1006.89	$C_{62}H_{120}O_8N$	3.5	3.56E+04	988.89	$C_{62}H_{116}O_8$	5	1	1114.99	$C_{70}H_{132}O_8N$	5.5	1.97E+05	1096.99	$C_{70}H_{128}O_8$	7	3
1008.91	$C_{62}H_{122}O_8N$	2.5	1.99E+04	990.91	$C_{62}H_{118}O_8$	4	0	1117.01	$C_{70}H_{134}O_8N$	4.5	2.15E+05	1099.01	$C_{70}H_{130}O_8$	6	2
1008.82	$C_{63}H_{110}O_8N$	9.5	1.15E+02	990.82	$C_{63}H_{106}O_8$	11	7	1118.93	$C_{71}H_{124}O_8N$	10.5	1.38E+03	1100.93	$C_{71}H_{120}O_8$	12	8
1010.84	$C_{63}H_{112}O_8N$	8.5	2.43E+02	992.84	$C_{63}H_{108}O_8$	10	6	1120.95	$C_{71}H_{126}O_8N$	9.5	3.66E+03	1102.95	$C_{71}H_{122}O_8$	11	7
1012.86	$C_{63}H_{114}O_8N$	7.5	9.03E+02	994.86	$C_{63}H_{110}O_8$	9	5	1122.96	$C_{71}H_{128}O_8N$	8.5	6.05E+03	1104.96	$C_{71}H_{124}O_8$	10	6
1014.87	$C_{63}H_{116}O_8N$	6.5	7.46E+02	996.87	$C_{63}H_{114}O_8$	8	4	1124.98	$C_{71}H_{130}O_8N$	7.5	2.88E+04	1106.98	$C_{71}H_{126}O_8$	9	5
1016.88	$C_{63}H_{118}O_8N$	5.5	4.55E+03	998.88	$C_{63}H_{114}O_8$	7	3	1126.99	$C_{71}H_{132}O_8N$	6.5	1.36E+05	1108.99	$C_{71}H_{128}O_8$	8	4
1018.90	$C_{63}H_{120}O_8N$	4.5	3.32E+04	1000.90	$C_{63}H_{116}O_8$	6	2	1129.01	$C_{71}H_{134}O_8N$	5.5	2.45E+05	1111.01	$C_{71}H_{130}O_8$	7	3
1020.91	$C_{63}H_{122}O_8N$	3.5	1.45E+05	1002.91	$C_{63}H_{118}O_8$	5	1	1130.94	$C_{72}H_{124}O_8N$	11.5	6.98E+02	1112.94	$C_{72}H_{120}O_8$	13	9
1022.84	$C_{64}H_{112}O_8N$	9.5	3.35E+01	1004.84	$C_{64}H_{108}O_8$	11	7	1131.02	$C_{72}H_{136}O_8N$	4.5	1.58E+05	1113.02	$C_{71}H_{132}O_8$	6	2
1022.93	$C_{63}H_{124}O_8N$	2.5	2.54E+04	1004.93	$C_{63}H_{120}O_8$	4	0	1132.95	$C_{72}H_{126}O_8N$	10.5	2.74E+03	1114.95	$C_{72}H_{122}O_8$	12	8
1024.86	$C_{64}H_{114}O_8N$	8.5	1.02E+03	1006.86	$C_{64}H_{110}O_8$	10	6	1134.96	$C_{72}H_{128}O_8N$	9.5	4.23E+03	1116.96	$C_{72}H_{124}O_8$	11	7
1026.87	$C_{64}H_{116}O_8N$	7.5	1.97E+03	1008.87	$C_{64}H_{112}O_8$	9	5	1136.98	$C_{72}H_{130}O_8N$	8.5	5.71E+03	1118.98	$C_{72}H_{126}O_8$	10	6
1028.88	$C_{64}H_{118}O_8N$	6.5	1.49E+03	1010.88	$C_{64}H_{114}O_8$	8	4	1138.99	$C_{72}H_{132}O_8N$	7.5	1.42E+04	1120.99	$C_{72}H_{128}O_8$	9	5
1030.90	$C_{64}H_{120}O_8N$	5.5	1.10E+04	1012.90	$C_{64}H_{116}O_8$	7	3	1141.01	$C_{72}H_{134}O_8N$	6.5	3.53E+04	1123.01	$C_{72}H_{130}O_8$	8	4
1032.91	$C_{64}H_{122}O_8N$	4.5	1.55E+05	1014.91	$C_{64}H_{118}O_8$	6	2	1143.02	$C_{72}H_{136}O_8N$	5.5	6.78E+04	1125.02	$C_{72}H_{132}O_8$	7	3
1034.93	$C_{64}H_{124}O_8N$	3.5	2.18E+05	1016.93	$C_{64}H_{120}O_8$	5	1	1145.04	$C_{72}H_{138}O_8N$	4.5	3.34E+04	1127.04	$C_{72}H_{134}O_8$	6	2
1036.85	$C_{65}H_{114}O_8N$	9.5	1.85E+02	1018.85	$C_{65}H_{110}O_8$	11	7	1146.96	$C_{73}H_{128}O_8N$	10.5	1.10E+03	1128.96	$C_{73}H_{124}O_8$	12	8
1036.94	$C_{64}H_{126}O_8N$	2.5	2.75E+04	1018.94	$C_{64}H_{122}O_8$	4	0	1148.98	$C_{73}H_{130}O_8N$	9.5	2.97E+03	1130.98	$C_{73}H_{126}O_8$	11	7
1038.87	$C_{65}H_{116}O_8N$	8.5	2.07E+03	1020.87	$C_{65}H_{112}O_8$	10	6	1150.99	$C_{73}H_{132}O_8N$	8.5	7.32E+03	1132.99	$C_{73}H_{128}O_8$	10	6
1040.88	$C_{65}H_{118}O_8N$	7.5	3.26E+03	1022.88	$C_{65}H_{114}O_8$	9	5	1153.01	$C_{73}H_{134}O_8N$	7.5	2.03E+04	1135.01	$C_{73}H_{130}O_8$	9	5
1042.90	$C_{65}H_{120}O_8N$	6.5	5.19E+03	1024.90	$C_{65}H_{116}O_8$	8	4	1155.02	$C_{73}H_{136}O_8N$	6.5	4.12E+04	1137.02	$C_{73}H_{132}O_8$	8	4
1044.91	$C_{65}H_{122}O_8N$	5.5	6.36E+04	1026.91	$C_{65}H_{118}O_8$	7	3	1157.04	$C_{73}H_{138}O_8N$	5.5	5.18E+04	1139.04	$C_{73}H_{134}O_8$	7	3
1046.93	$C_{65}H_{124}O_8N$	4.5	4.29E+05	1028.93	$C_{65}H_{120}O_8$	6	2	1159.05	$C_{73}H_{140}O_8N$	4.5	1.62E+04	1141.05	$C_{73}H_{136}O_8$	6	2
1048.94	$C_{65}H_{126}O_8N$	3.5	3.06E+05	1030.94	$C_{65}H_{122}O_8$	5	1	1165.01	$C_{74}H_{134}O_8N$	8.5	4.02E+03	1147.01	$C_{74}H_{130}O_8$	10	6
1050.87	$C_{66}H_{116}O_8N$	9.5	1.39E+03	1032.87	$C_{66}H_{112}O_8$	11	7	1167.02	$C_{74}H_{136}O_8N$	7.5	7.39E+03	1149.02	$C_{74}H_{132}O_8$	9	5
1050.96	$C_{65}H_{128}O_8N$	2.5	2.95E+04	1032.96	$C_{65}H_{124}O_8$	4	0	1169.04	$C_{74}H_{138}O_8N$	6.5	1.21E+04	1151.04	$C_{74}H_{134}O_8$	8	4
1052.89	$C_{66}H_{118}O_8N$	8.5	4.03E+03	1034.89	$C_{66}H_{114}O_8$	10	6	1170.97	$C_{75}H_{128}O_8N$	12.5	1.55E+02	1152.97	$C_{75}H_{124}O_8$	14	10
1054.90	$C_{66}H_{120}O_8N$	7.5	3.34E+03	1036.90	$C_{66}H_{116}O_8$	9	5	1171.05	$C_{74}H_{140}O_8N$	5.5	1.26E+04	1153.05	$C_{74}H_{136}O_8$	7	3
1056.91	$C_{66}H_{122}O_8N$	6.5	8.97E+03	1038.91	$C_{66}H_{118}O_8$	8	4	1177.01	$C_{75}H_{134}O_8N$	9.5	2.22E+03	1159.01	$C_{75}H_{130}O_8$	11	7
1058.93	$C_{66}H_{124}O_8N$	5.5	1.62E+05	1040.93	$C_{66}H_{120}O_8$	7	3	1179.02	$C_{75}H_{136}O_8N$	8.5	4.61E+03	1161.02	$C_{75}H_{132}O_8$	10	6
1060.94	$C_{66}H_{126}O_8N$	4.5	4.54E+05	1042.94	$C_{66}H_{122}O_8$	6	2	1181.04	$C_{75}H_{138}O_8N$	7.5	8.87E+03	1163.04	$C_{75}H_{134}O_8$	9	5
1062.87	$C_{67}H_{116}O_8N$	10.5	2.35E+03	1044.87	$C_{67}H_{112}O_8$	12	8	1183.05	$C_{75}H_{140}O_8N$	6.5	1.07E+04	1165.05	$C_{75}H_{136}O_8$	8	4
1062.96	$C_{66}H_{128}O_8N$	3.5	2.71E+05	1044.96	$C_{66}H_{124}O_8$	5	1	1185.07	$C_{75}H_{142}O_8N$	5.5	6.55E+03	1167.07	$C_{75}H_{138}O_8$	7	3
1064.89	$C_{67}H_{118}O_8N$	9.5	2.27E+03	1046.89	$C_{67}H_{114}O_8$	11	7	1189.10	$C_{75}H_{146}O_8N$	3.5	3.33E+02	1171.10	$C_{75}H_{142}O_8$	5	1
1064.98	$C_{66}H_{130}O_8N$	2.5	2.57E+04	1046.98	$C_{66}H_{126}O_8$	4	0	1191.03	$C_{76}H_{136}O_8N$	9.5	1.28E+03	1173.03	$C_{76}H_{132}O_8$	11	7
1066.90	$C_{67}H_{120}O_8N$	8.5	2.66E+03	1048.90	$C_{67}H_{116}O_8$	10	6	1191.12	$C_{75}H_{148}O_8N$	2.5	8.47E+02	1173.12	$C_{75}H_{144}O_8$	4	0
1068.91	$C_{67}H_{122}O_8N$	7.5	4.51E+03	1050.91	$C_{67}H_{118}O_8$	9	5	1193.04	$C_{76}H_{138}O_8N$	8.5	1.66E+03	1175.04	$C_{76}H_{134}O_8$	10	6
1070.93	$C_{67}H_{124}O_8N$	6.5	5.47E+04	1052.93	$C_{67}H_{120}O_8$	8	4	1195.05	$C_{76}H_{140}O8N$	7.5	2.97E+03	1177.05	$C_{76}H_{136}O8$	9	5
1072.94	$C_{67}H_{126}O_8N$	5.5	4.55E+04	1054.94	$C_{67}H_{122}O_8$	7	3	1197.07	$C_{76}H_{142}O_8N$	6.5	3.45E+03	1179.07	$C_{76}H_{138}O_8$	8	4
1074.96	$C_{67}H_{128}O_8N$	4.5	6.36E+05	1056.96	$C_{67}H_{124}O_8$	6	2	1205.04	$C_{77}H_{138}O_8N$	9.5	9.82E+02	1187.04	$C_{77}H_{134}O_8$	11	7
1076.98	$C_{67}H_{130}O_8N$	3.5	2.83E+05	1058.98	$C_{67}H_{126}O_8$	5	1	1207.05	$C_{77}H_{140}O_8N$	8.5	2.08E+03	1189.05	$C_{77}H_{136}O_8$	10	6
1078.90	$C_{68}H_{120}O_8N$	9.5	4.84E+03	1060.90	$C_{68}H_{116}O_8$	11	7	1209.07	$C_{77}H_{142}O_8N$	7.5	2.34E+03	1191.07	$C_{77}H_{138}O_8$	9	5
1078.99	$C_{67}H_{132}O_8N$	2.5	1.77E+04	1060.99	$C_{67}H_{128}O_8$	4	0	1211.08	$C_{77}H_{144}O_8N$	6.5	2.56E+03	1193.08	$C_{77}H_{140}O_8$	8	4

with molecular formula $C_{67}H_{124}O_8$. RDB value tells us that this compound has two double bonds present in fatty acyl chains, but position of those double bonds, position of hydroxyl branching and exact structure of that TG-estolide, as well as structure of all other identified TG-estolides cannot be determined with HPLC-MS analysis. Nevertheless, mass spectra, that is MS/MS and MS^3 fragmentation can give us a good insight into its structure. Figure 20 shows total ion

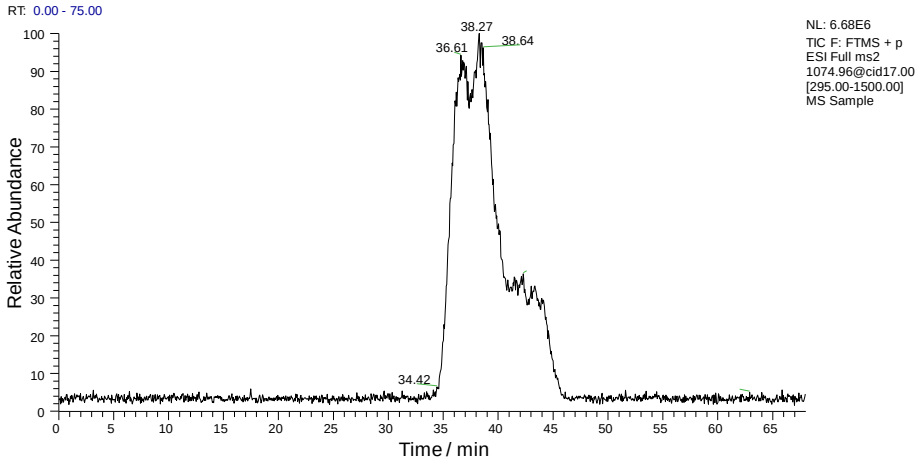


Figure 20 Reconstructed total ion current (TIC) FTMS + p ESI Full MS^2 1074.96@CID 17.00 [295.00-1500.00] chromatogram of *m/z* 1074.95968, obtained on LTQ Orbitrap in ESI positive mode with "Separation method B"

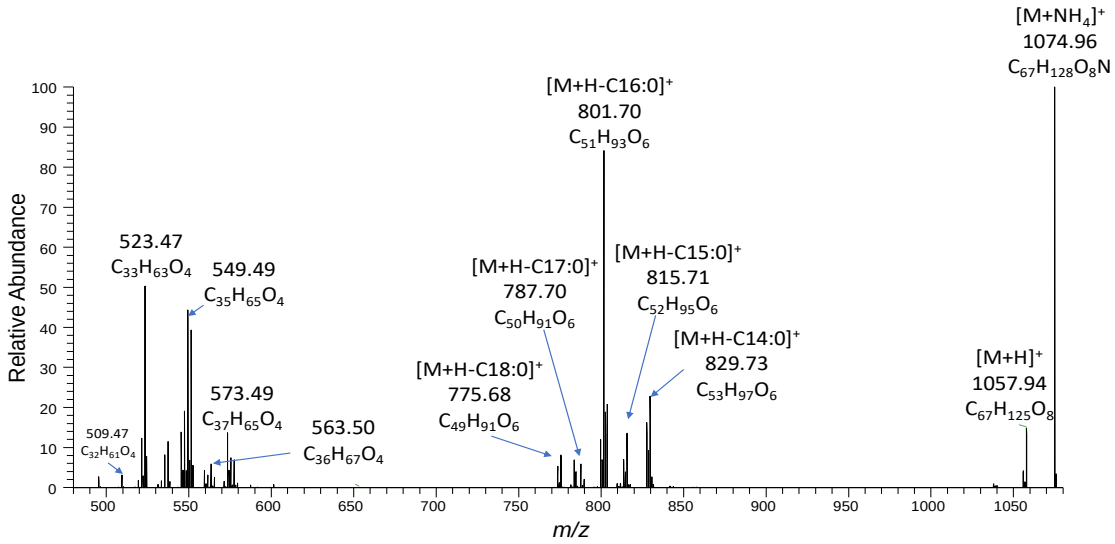


Figure 21 FTMS + p ESI full MS^2 mass spectrum of compound *m/z* 1074.95968 obtained from highest peak (37.42-38.91 min) in reconstructed TIC chromatogram of *m/z* 1074.95968. 1074.96@CID 17.00 [*m/z* 295.00-1500.00]

current (TIC) reconstructed chromatogram for most abundant compound (*m/z* 1074.95968) which is present in the RT range 34.42 – 44.57 min and figure 21. MS/MS spectrum of said compound. Peak with the highest abundance *m/z* 1074.95968 corresponds to molecular adduct $[M+NH_4]^+$ and the one next to it; *m/z* 1057.94, with the loss of *m/z* 17 [NH_3], corresponds to $[M+H]^+$. From that peak, specific losses can be observed and molecular formulae of compounds in the *m/z* 773-830 and *m/z* 505-580 ranges identified. In *m/z* 773-830 range ion losses with the highest abundance corresponds to the loss of one fatty acid (C14:0-C18:0) and formed compounds are highlighted in the spectrum, but by analysing the spectrum further, unsaturated fatty acid losses (C14:1-C18:1) were observed as well. Mass spectrum analysis of TG-estolide standard (Figures D3-D5) showed that dissociated fatty acid was bound to glycerol rather than to hydroxyl group of another fatty acid, so same is applied to analysed compound. That tells us that higher energy is required for cleaving ester group on hydroxyl fatty acid than it is on glycerol. 1,2-diacylglycerol-estolide (1,2-DAG-estolide) is formed after loss of one fatty acid which can further be fragmented. The interpretation of all fragments from that mass spectrum can be seen in table D4. Also, OAHFA neutral losses were deduced from fragments in *m/z* 505-580 range. To get further insight into the structure of analysed compound, MS^3 spectra of the highest peak from MS^2, in the *m/z* 210.00-815.00 range (*m/z* 801.70) was obtained. Figure 22 shows reconstructed TIC chromatogram for said compound with the highest abundance at 36.87 min and figure 23 MS^3 spectrum. Again, a mixture of fatty acid losses can be observed (C14:0,

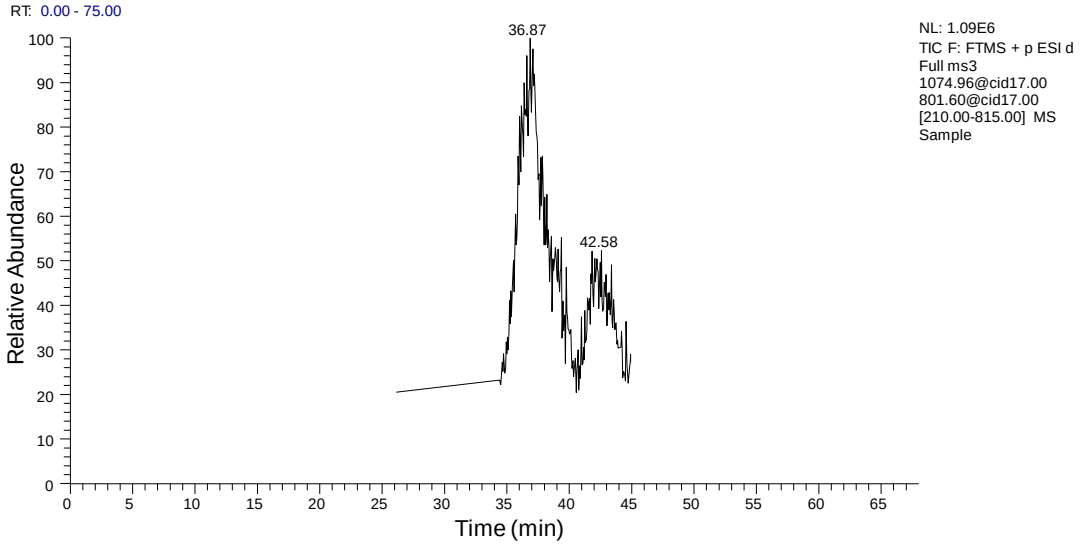


Figure 22 Reconstructed total ion current (TIC) FTMS + p ESI d Full MS^3 1074.96@CID 17.00, 801.60@CID 17.00 [*m/z* 210.00-815.00] chromatogram of *m/z* 801.7, obtained on LTQ Orbitrap in ESI positive mode with "Separation method B"

C14:1, C15:0, C16:0, C16:1, C16:2, C17:2, C18:2) in the spectrum. Based on the MS^3 mass spectrum analysis of AMR-1919-2, where second dissociation of fatty acyl chain can occur from hydroxy fatty acid, as well as from glycerol, it is difficult to say from which part of compound that second dissociation occurred. MS^4 experiment would give additional information regarding that second dissociation, but even then, the exact position from which the dissociation occurred would be difficult to determine, because of the complexity of the sample. Also, one other problem with MS^4 experiments is the sensitivity of the instrument, which is not high enough to detect formed fragments.

Considering fatty acid losses observed in MS^2 spectra of *m/z* 1074.95968 the one thing that can be said for sure is that TG-estolide with molecular formula $C_{67}H_{124}O_8$ can be formed from a high variety of fatty acid and OAHFA combinations. There are 42 combinations of FAs and OAHFAs listed in table 11 that can form TG-estolide of molecular formula $C_{67}H_{124}O_8$, and that is if the positions of FAs and OAHFAs on the glycerol (sn-1, sn-2 and sn-3) are not taken into consideration. Otherwise, there are 234 possible combinations that can give said compound. That number rapidly rises if positions of double bonds in fatty acyl chains and position of hydroxyl moiety in hydroxy fatty acids are considered as well. So, 126 compounds identified with HPLC-ESI/MS listed in table 10 contain different structural isomers, and theoretically thousands of different TG-estolides could be found in VC sample.

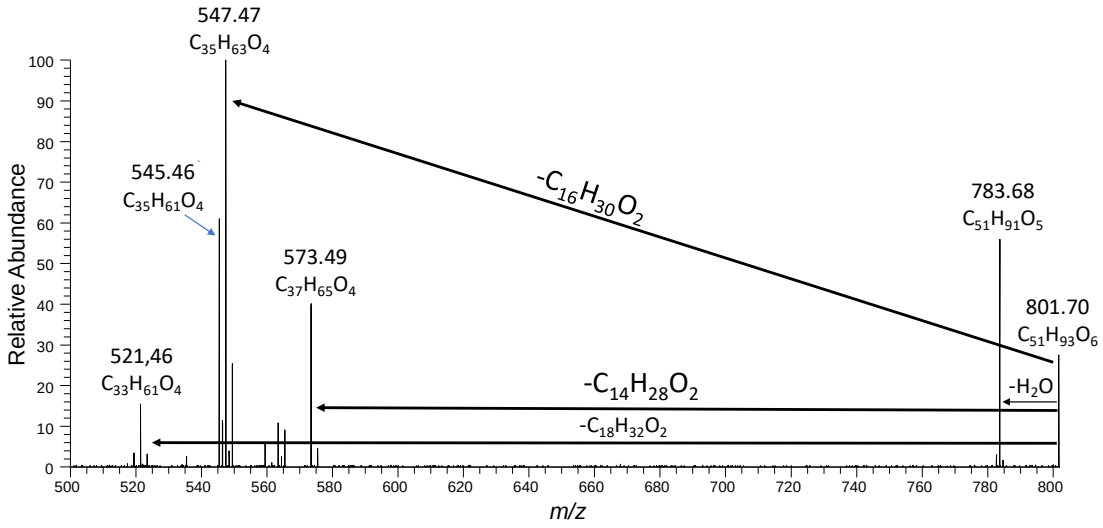


Figure 23 FTMS + p ESI full MS^3 mass spectrum of compound *m/z* 801.70 obtained from highest peak in reconstructed TIC chromatogram of *m/z* 801.70. 1074.96@CID 17.00, 801.70@CID 17.00 [*m/z* 210.00-815.00]. LTQ Orbitrap in ESI positive mode

Table 11 Identified fatty acids and (*O*-acyl)-*ω*-hydroxy fatty acids bound to glycerol from MS^2 spectra of $[M+NH_4]^+$ *m/z* 1074.95968, and possible combinations of FAs and OAHFAs that can give TG-estolide [M] *m/z* 1056.94 if the order of FAs and OAHFAs is not considered

FA	OAHFA	TG-Estolide m/z 1056.94
14:0	31:0	14:0_16:0_37:2
14:1	32:0	14:0_16:1_37:1
15:0	33:1	14:1_16:0_37:1
15:1	33:2	14:1_16:1_37:0
16:0	34:0	14:0_17:0_36:2
16:1	34:1	14:0_17:1_36:1
17:0	34:2	14:1_17:0_36:1
17:1	35:0	14:1_17:1_36:0
18:0	35:1	14:0_18:0_35:2
18:1	35:2	14:0:18:1_35:1
	35:3	14:1_18:0_35:1
	36:0	14:1_18:1_35:0
	36:1	15:0_15:0_37:2
	36:2	15:1_15:0_37:1
	36:3	15:1_15:1_37:0
	37:0	15:0_16:0_36:2
	37:1	15:0_16:1_36:1
	37:2	15:1_16:0_36:1
	37:3	15:1_16:1_36:0
	37:4	15:0_17:0_35:2
		15:0_17:1_35:1
		15:1_17:0_35:1
		15:1_17:1_35:0
		15:0_18:0_34:2
		15:0_18:1_34:1
		15:1_18:0_34:1
		15:1_18:1_34:0
		16:0_16:0_35:2
		16:0_16:1_35:1
		16:1_16:1_35:0
		16:0_17:0_34:2
		16:0_17:1_34:1
		16:1_17:0_34:1
		16:1_17:1_34:0
		16:0_18:0_33:2
		16:0_18:1_33:1
		16:1_18:0_33:1
		16:1_18:1_33:1
		17:0_17:0_33:2
		17:1_17:0_33:1
		17:1:18:1_32:0
		18:1_18:1_31:0

4.4. GC-MS analysis

After the analysis of intact vernix caseosa TG-estolides with HPLC-ESI/MS, analysis of fatty acids that build them was performed with GC-MS. Since intact TG-estolides are not volatile, which makes them unsuitable for GC analysis, they were first transferred to volatile compounds; fatty acid methyl esters (FAMEs). FAMEs were generated in transesterification reaction with acetyl chloride based on the paper published by Karel Stránský and Tomáš Jursík[128]. Since, after transesterification reaction, free fatty acids were also generated, they were esterified in the next step using trimethylsilyl diazomethane.[133] In the final step, hydroxyl moieties in FAMEs were silylated with *N*,*O*-bis(trimethylsilyl)acetamide in order to try determining their position in fatty acid chains. First, we will take a quick look at the aforementioned derivatization reactions, and then chromatograms and some mass spectra from which FAMEs, that is fatty acids that build vernix caseosa TG-estolides, were determined.

Acid catalysed lipid transesterification with acetyl chloride is a simple and reproducible method utilisable for sample quantities from several hundreds of μg to several hundreds of

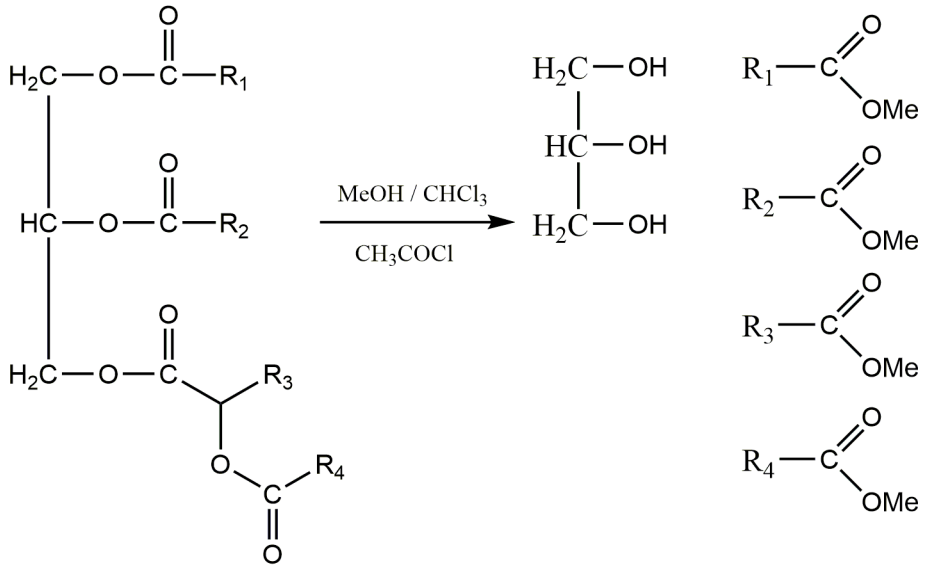


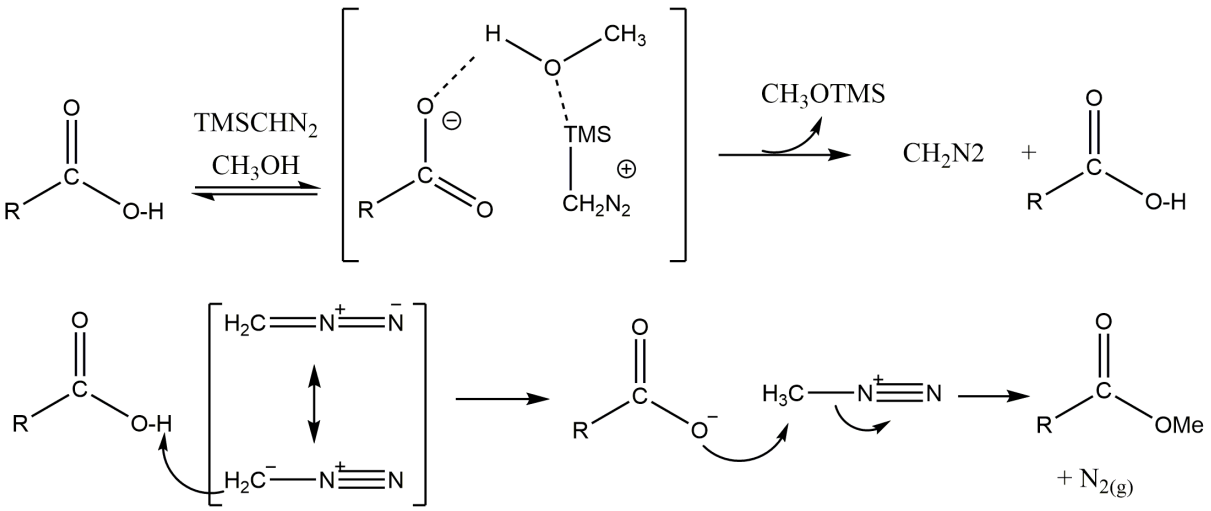
Figure 24 Transesterification of TG-estolide with acetyl chloride in methanol-chloroform solution

mg.[128] Transesterification reaction with acetyl chloride is displayed in figure 24. First, methanol reacts with acetyl chloride in carboxylic substitution, thus generating hydrochloric acid needed for transesterification reaction. Then, in another reaction, acid catalysed carboxylic substitution, methanol reacts with each ester group of TG-estolide, cleaving fatty acids from glycerol and forming fatty acid methyl esters. Excess of formed hydrochloric acid was neutralized with silver

carbonate. Silver carbonate was added until the formation of CO_2 stopped. Small amount of water, which can react with FAMEs in reverse reaction and form free fatty acids, was formed in neutralization reaction (Equation 4.2) and that is why some free fatty acids were also observed in the first GC-MS analysis. Silver chloride precipitate was simply removed by decanting it, and sample dissolved in methanol – chloroform mixture was injected into the GC-MS.

$$2\ HCl + Ag_2CO_3 \rightarrow 2\ AgCl_{(s)} + H_2O_{(l)} + CO_{2(g)} \qquad (4.2)$$

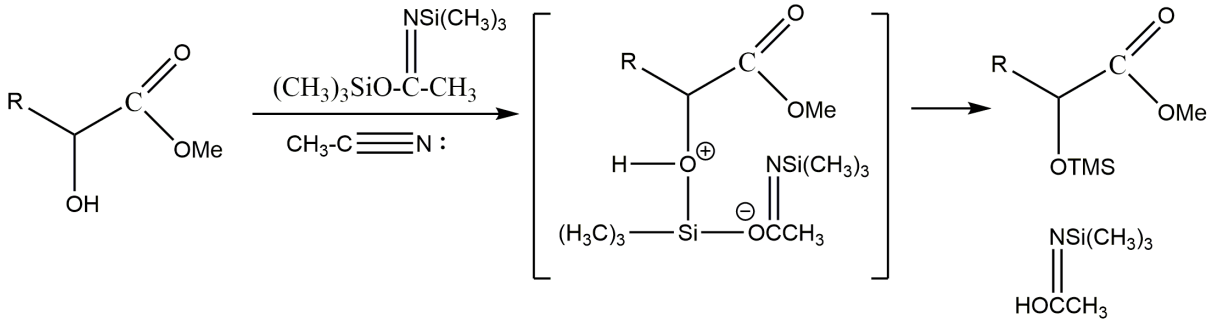
Free fatty acids also needed to be transferred into FAMEs, and even though methyl esters are usually prepared in acid catalysed reaction with methanol, another approach was applied. Based on the paper published by Hashimoto et al.[134] the reaction of carboxylic acids with $TMSCHN_2$ in the presence of methanol quickly gives methyl esters in excellent yields at



Scheme 7 Mechanism of free fatty acid esterification with trimethylsilyl diazomethane ($TMSCHN_2$)

room temperature, reaction can easily be monitored and the method can be efficiently applied for determination of fatty acids with gas chromatography. Reaction mechanism can be seen in scheme 7. $TMSCHN_2$ was used instead of diazomethane (CH_2N_2), because CH_2N_2 is highly toxic, thermally labile and explosive, while $TMSCHN_2$ is not.

And final reaction that was performed for analysis of fatty acids that build vernix caseosa TG-estolides was silylation of hydroxyl groups in order to determine their position in fatty acid chains. Silylation of hydroxyl groups was carried out with *N*,*O*-



Scheme 8 Mechanism of hydroxy fatty acid derivatization with *N,O*-bis(trimethylsilyl)acetamide (BSA)

bis(trimethylsilyl)acetamide (BSA) in dried acetonitrile. BSA is usually used as a protecting group for hydroxyl and carboxyl moieties during organic synthesis, but it is also widely used for derivatization of wide range of functional groups prior to GC-MS characterization. Additionally, silyl derivatives are generally more volatile, less polar and more thermally stable. Since reaction is performed on FAMEs, and BSA does not react with ester groups, only hydroxyl groups are silylated. Mechanism of the reaction can be seen in scheme 8. The reaction is viewed as a nucleophilic attack upon silicon atom of the silyl donor, producing a bimolecular transition state. The silyl compound leaving group possesses low basicity, meaning that it has the ability to stabilize a negative charge in transition state. Unfortunately, in this case reaction was not highly successful, so only a handful of silylated products were observed with GC-MS

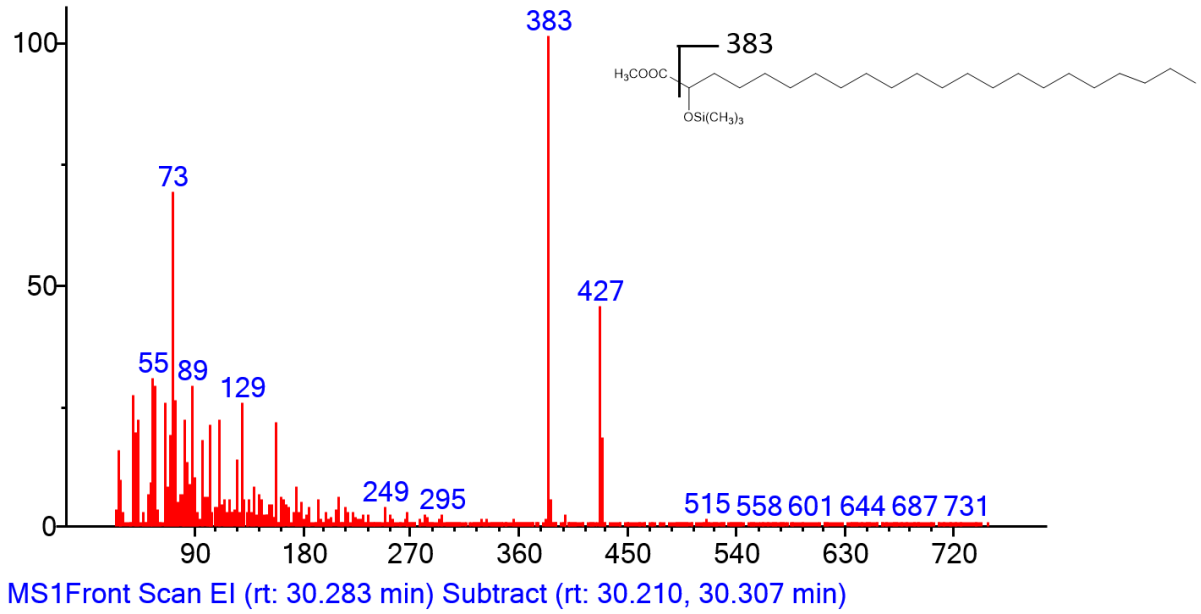


Figure 25 Mass spectrum of trimethyl silylated methyl 2-hydroxy docosanoate obtained using Agilent 5975C quadrupole mass spectrometer, 70 eV EI mass spectrum, *m/z* 25-400

analysis which were near the noise area, because the highest signal in chromatogram was the one from trimethylsilyl acetamide (Figure D7). One of the silylated products that was observed has molecular formula $C_{26}H_{54}O_3Si$ and its mass spectrum can be seen in figure 25. Molecular ion $[M]^+$ *m/z* 442 is not visible, because of low signal-to-noise ratio. Signal at *m/z* 427 represents the loss of a methyl group from the trimethylsilyl moiety. The base at *m/z* 383 is the result of cleavage between carbons 1 and 2 and the position of hydroxyl moiety on the fatty acyl chain can be determined from it. Mass spectrum of that compound showed that hydroxyl group is positioned on α-carbon atom. So, that compound corresponds to fatty acid C22:0; C2-OH, which was determined in the previous GC-MS analysis. Having that in mind, it is possible to determine hydroxy fatty acids and positions of hydroxyl groups from mass spectra and spectral database without derivatization with BSA. Chromatogram of a standard mixture of FAMEs can

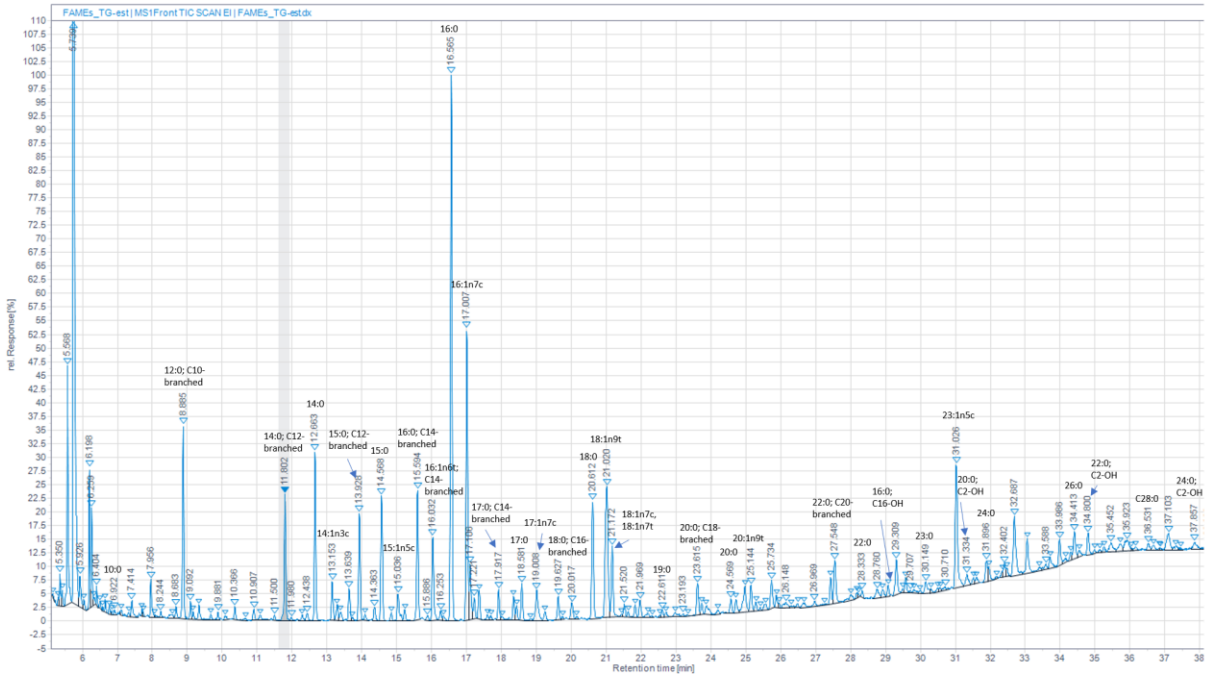


Figure 26 Gas chromatogram of a mixture of fatty acid methyl ester from vernix caseosa sample obtained on Agilent 7890A GC system, DB-WAX column (30 m x 250 μm, 0.25 μm; Agilent), He at 1 mL/min, 1 μL of sample

be seen in figure D6 and chromatogram of FAMEs sample from vernix caseosa in figure 26. Based on retention times of FAMEs in chromatogram of standard solution and based on mass spectra with the help of NIST spectral database, FAMEs in the sample of vernix caseosa were determined. In total, 71 FAMEs, that is fatty acids that build vernix caseosa TG-estolides were determined, and they are listed in table 12. Wide variety of fatty acids that build vernix caseosa

TG-estolides have been observed; completely saturated fatty acids ranging from C9 to C28, wide range of mono- and di- unsaturated fatty acids, branched fatty acids with branching usually at the end of the chain and saturated hydroxyl fatty acids with hydroxyl group in α or ω position. The most abundant fatty acid that builds vernix caseosa TG-estolide is palmitate (C16:0), which was expected given that FAS1 synthesises fatty acids in vertebrates, and its only product is palmitate.[64] Other present fatty acids that build vernix caseosa TG-estolides are result of action of a wide variety of enzymes during formation of vernix caseosa.[41]

Figure 27 shows mass spectrum of most abundant FAME observed in vernix caseosa sample; methyl palmitate. Signal of molecular ion *m/z* 270 is relatively low but can still be seen. Base signal *m/z* 74 is the result of McLafferty rearrangement and that ion is central to the identification of most ester derivatives of fatty acids. A site-specific rearrangement is involved in which a hydrogen atom at position 4 of the aliphatic chain migrates to the carbo-methoxy

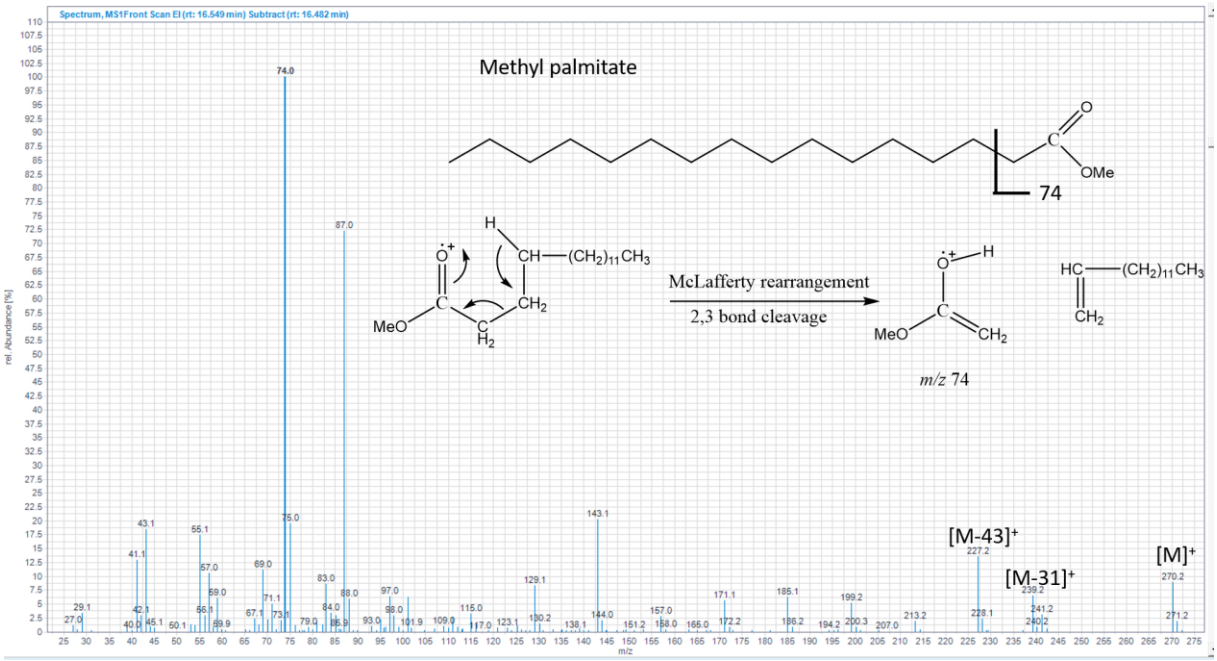


Figure 27 Mass spectrum of methyl palmitate obtained using Agilent 5975C quadrupole mass spectrometer, 70 eV EI mass spectrum, *m/z* 25-400

group through six-membered transition state, which is sterically favoured. Also, ion at *m/z* 239 $[M-31]^+$ is the result of loss of methoxy group which confirms that the compound is indeed methyl ester. The long homologous series of related ions whose difference is 14 (*m/z* 87, 101,

Table 12 Fatty acids that build vernix caseosa TG-estolides determined with GC-MS analysis

Retention time / min	Structure	Retention time / min	Structure
6.04	C9:0	21.172	C18:1n7c, C18:1n7t
6.922	C10:0	21.52	C18:2n9t; C?-branched
8.863	C12:0; C10-brached	21.627	C19:0; C17-branched OR C19:0; C11-branched
9.172	C13:0; C4-branched	21.828	C18:2n6c OR C18:2n7tc OR C18:2n4t
9.339	C12:0	21.969	probably hydroxy fatty acid or partially oxidised double bond?
9.881	C13:0; C4-branched	22.611	C19:0
10.366	C13:0; C10-branched	22.71	C15:0; C2-OH
10.907	C13:0	22.968	C19:1n9c
11.5	C13:0; C11-branched	23.615	C20:0; C18-branched
11.802	C14:0; C12-branched	23.735	C16:0; C2-OH
12.663	C14:0	24.459	C20:0
13.153	C14:1n3c	24.72	C16:0; C2-OH
13.298	C15:0; C12-branched	24.974	C20:1n9c
13.388	C14:1n5c	25.144	C20:1n9t
13.639	C15:0; C13-branched	25.875	C21:0; C18-branched OR C21:0; C4-branched
13.928	C15:0; C12-branched	26.148	C18:0; C3-OH
14.095	C15:1n5t; C13-branched	26.483	C21:0 OR C21:0; C4-branched
14.568	C15:0	27.429	C22:0; C20-branched
15.036	C15:1n5c	28.184	C16:0; C9-OH
15.229	C16:0; C4,8,12-branched	28.333	C22:0
15.594	C16:0; C14-branched	28.76	C22:1n9c
16.032	C16:1n6t; C14-branched	28.923	C22:1n11c
16.566	C16:0	29.073	C16:0; C16-OH
17.007	C16:1n7c	29.488	C22:2 (Δ5,Δ13)c
17.106	C16:1n7t	29.579	C23:0; C20-branched OR C23:0; C21-branched
17.358	C16:1n5t	29.987	C20:2n7t
17.598	C17:0; C15-branched	30.149	C23:0
17.695	C16:1n9t	31.026	C23:1n5c
17.917	C17:0; C14-branched	31.335	C20:0; C2-OH
18.344	C17:1n7t; C14-branched	31.896	C24:0
18.582	C17:0	33.062	C25:0
19.008	C17:1n7c	34.413	C26:0
19.627	C18:0; C16-branched	34.8	C22:0; C2-OH
20.017	C18:1n12c	36.531	C28:0
20.612	C18:0	38.89	C24:0; C2-OH
21.02	C18:1n9t OR C18:1n10t		

115, 129, 143, 157, 171, 185, 199, 213) of general formula $[CH_3OCO(CH_2)_n]^+$ is evidence that there are no other functional groups in the chain and they are formed by losses of neutral aliphatic radicals from the terminal part of the molecule. The ion at *m/z* 227 $[M-43]^+$ is formed via rearrangement of the chain and one hydrogen atom, followed by expulsion of a propyl radical, again via six-membered transition state. Mass spectra of other identified saturated, non-branched FAMEs can be analysed in similar way.

Mass spectrum of methyl 2-hydroxyhexadecanoate is shown in figure 28. Signal of molecular ion *m/z* 258 $[M]^+$ is relatively small but can still be seen. The ion at *m/z* 90 is the result of McLafferty rearrangement, which is mechanistically the same as with previously analysed

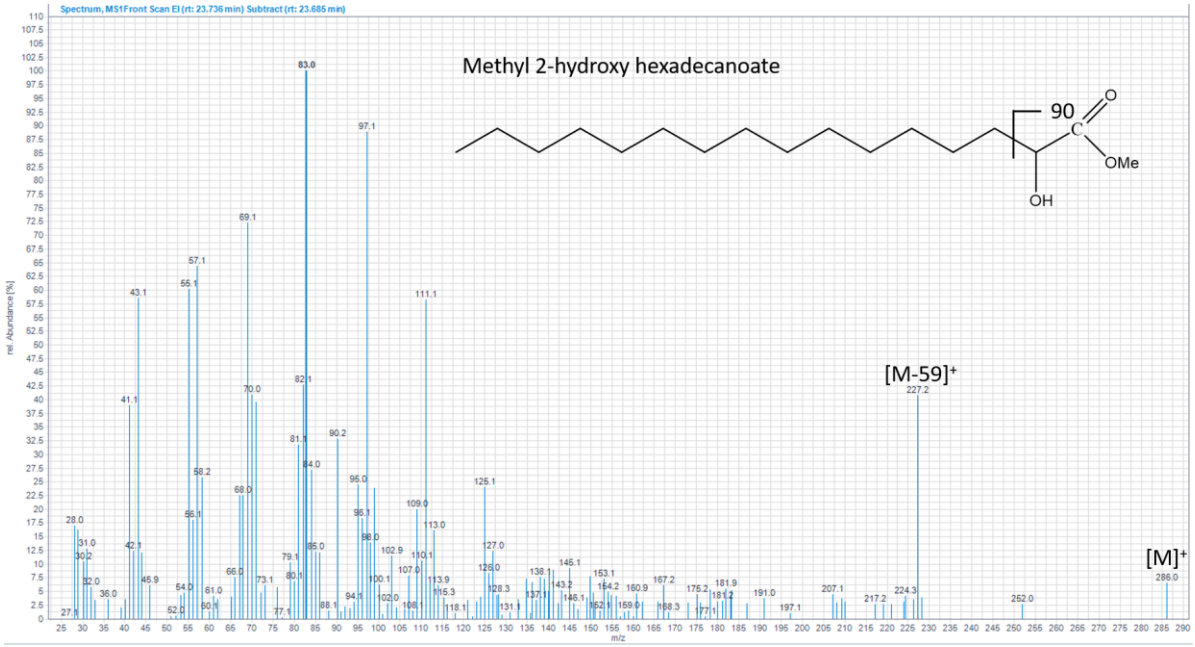


Figure 28 Mass spectrum of methyl 2-hydroxyhexadecanoate obtained using Agilent 5975C quadrupole mass spectrometer, 70 eV EI mass spectrum, *m/z* 25-400

methyl palmitate. Ion at *m/z* 227 $[M-59]^+$ corresponds to fragmentation of methyl ester group. The long homologous series of related ions whose difference is 14 (*m/z* 69, 83, 97, 111, 125, 139, 153, 167, 181) can also be seen which is again evidence that there are no other functional groups in the chain and they are formed by losses of neutral aliphatic radicals from the terminal part of the molecule. Even though the loss of water which produces molecular ions $[M-17]^+$ and $[M-18]^+$ is common in mass spectra of hydroxy fatty acids, they are very small or non-detectable with saturated homologues. Mass spectra of other identified saturated, non-branched hydroxy FAMEs can be analysed in similar way.

Finally, representative of branched fatty acids that we will take a look at is 14-methyl-methylhexadecanoate, and its mass spectrum is shown in figure 29. First thing that can be noticed is that mass spectrum appears to be noisier in higher *m/z* values compared to analogous straight-chain ester, which is often the case.[135] Signal of molecular ion *m/z* 284 $[M]^+$ is probably not visible because of that. Signal that distinguishes *anteiso*-isomer represented with this spectrum from *iso*-isomer and branched isomer from straight-chain analogue is that of an ion $[M-29]^+$ (*m/z* 255), because it is more abundant than that equivalent to $[M-31]^+$ and it corresponds to the loss of C_2H_5 from the end of fatty acid chain. Base signal *m/z* 74, which is the result of McLafferty rearrangement is again clearly visible, as is the long homologous series of signals with the *m/z* difference of 14 (*m/z* 87, 101, 115, 129, 143, 157, 171, 185, 199, 213,

227, 241). Mass spectra of other identified saturated, branched FAMEs can be analysed in similar way.

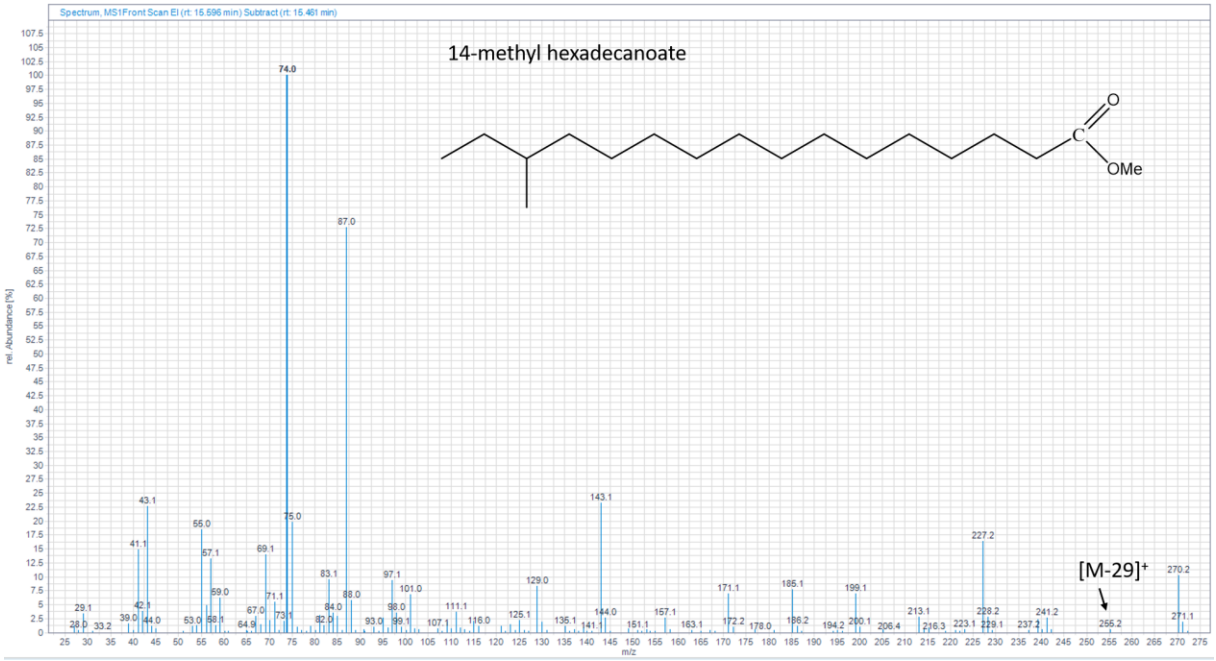


Figure 29 Mass spectrum of 14-methyl hexadecanoate obtained using Agilent 5975C quadrupole mass spectrometer, 70 eV EI mass spectrum, *m/z* 25-400

§ 5. CONCLUSION

A complete isolation of vernix caseosa TG-estolides with combination of semi-preparative TLC and HPLC, and their partial structural elucidation with HPLC-ESI/MS and GC-MS was performed.

Isolation of vernix caseosa TG-estolides included four separated steps: **1)** isolation of all VC caseosa lipids, **2)** isolation of non-polar lipids, **3)** isolation of TG-estolides from non-polar lipids, **4)** additional purification of TG-estolides with semi-preparative HPLC. Known methods for isolation from previous researches were used in first two steps, while new methods for isolation and purification were developed in third and fourth step. Given that difference in polarity between TGs and TG-estolides is really small, completely new method for their separation was developed. That included new mobile phase which in TLC gave the best possible separation of TG-estolides from TGs; hexane: diethyl ether: toluene in 92.5: 7.5: 7.5 ratio by volume, and new TLC plate preparation method. Out of three tested TLC plate preparation methods, the "standard" one, which uses TLC spreader, with sodium cations incorporated into silica gel slurry proved to be the best with the highest consistency of results and lowest error. New HPLC method which served for additional isolation and purification of TG-estolides was developed. "Separation method A" which uses 2-propanol and acetonitrile as mobile phases with gradient program of 75 minutes completely purified the sample, leaving only a complex mixture of vernix caseosa TG-estolides.

For structural analysis of vernix caseosa TG-estolides "Separation method B", which uses mixture of methanol and water (64:40 by volume) with 0.1 % formic acid and 5 % of ammonium formate (**A**) and mixture of 2-propanol and methanol (90:10 by volume) with 0.1 % of formic acid and 5 % of ammonium formate (**B**) with 68 minute gradient program gave higher intensities and sharper, distinct chromatographic peaks. HPLC-ESI/MS analysis revealed 126 compounds ranging from *m/z* 934.85 to 1193.08. Number of double bonds in those compounds range from 0 to 10, but compounds that have two double bonds in total were most abundant. Next highest abundant were compounds with three, then one and four double bonds. Those compounds were, after analysis of mass spectra in MS/MS and MS^3 experiments, concluded to be structural isomers.

Finally, after derivatization reactions, GC-MS analysis revealed 71 FAMEs, that is fatty acids that build vernix caseosa TG-estolides; completely saturated fatty acids ranging from C9 to C28, wide range of mono- and di- unsaturated fatty acids, branched fatty acids with branching usually at the end of the chain and saturated hydroxy fatty acids with hydroxyl group in α or ω position.

Having in mind a wide variety of fatty acids determined with GC-MS analysis, every possible combination of those fatty acids which can give TG-estolide and 126 compounds determined to be structural isomers, it is clear that vernix caseosa can have thousands of different TG-estolides in its lipid structure. This is a relatively new class of lipids discovered in vertebrates and completely new in vernix caseosa so, to elucidate vernix caseosa TG-estolides completely, additional structural analysis, in which MS^4 experiments would give a great contribution, needs to be performed.

§ 6. LIST OF ABBREVIATIONS AND SYMBOLS

ACN – acetonitrile

APCI – atmospheric pressure chemical ionization

BSA – *N*,*O*-bis(trimethylsilyl)acetamide

CER – ceramide

CHOL – cholesterol

CID – collision induced dissociation

Cryo-SEM – cryo scanning electron microscopy

DAG – diacylglycerol

EI – electron ionization

ELOVL – elongation of very long chain fatty acids protein

EN – estolide number

ESI – electrospray ionization

FAHFA – fatty acid ester of hydroxy fatty acid

FAME – fatty acid methyl ester

FAS – fatty acid synthase

FFA – free fatty acid

FFEM – freeze-frame electron microscopy

FR-ICR – Fourier transform ion cyclotron resonance

GC – gas chromatography

HOFA – hydroxy fatty acid

HPLC – high-performance liquid chromatography

HPTLC – high-performance thin-layer chromatography

IHD – index of hydrogen deficiency

IPA – isopropyl alcohol

IR – infrared

IT – ion trap

LC – liquid chromatography

LOD – limit of detection

MAG – monoacylglycerol

MS – mass spectrometer

OAHFA – (*O*-acyl)-*ω*-hydroxy fatty acid

Q – quadrupole

RDB – ring-double bond

RPLC – reversed-phase liquid chromatography

RT – retention time

SC – stratum corneum

SE – sterol ester

SFC – supercritical fluid chromatography

TEWL – trans-epidermal water loss

TG – triacylglycerol

THF – tetrahydrofuran

TIC – total ion current

TLC – thin-layer chromatography

TOF – time-of-flight

VC – vernix caseosa

VLCFA – very long chain fatty acid

WE – wax ester

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§ 8. APPENDIX

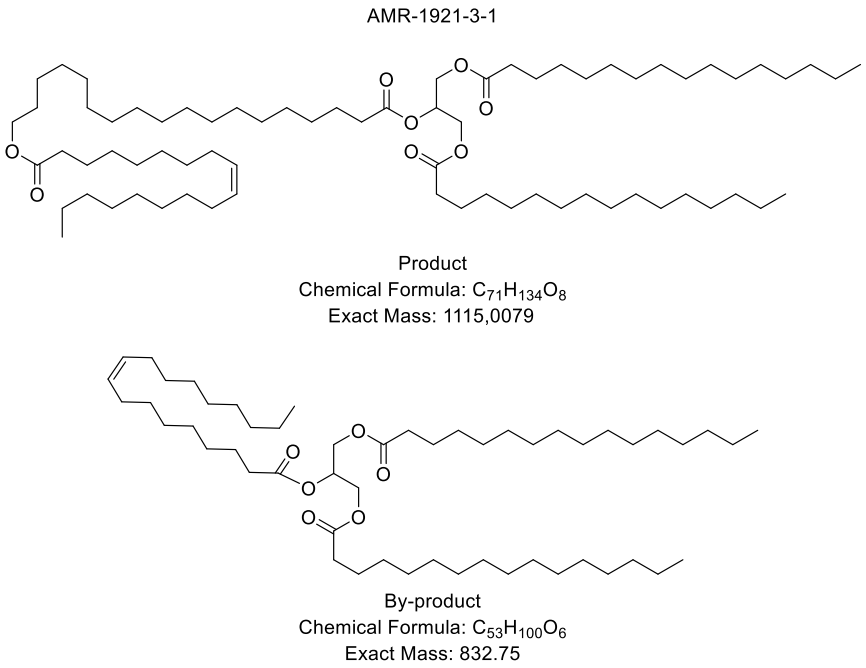

Figure D1 Estolide standard AMR-1921-3-1 and the by-product of its synthesis; 2,3-Dipalmito-1-Olein

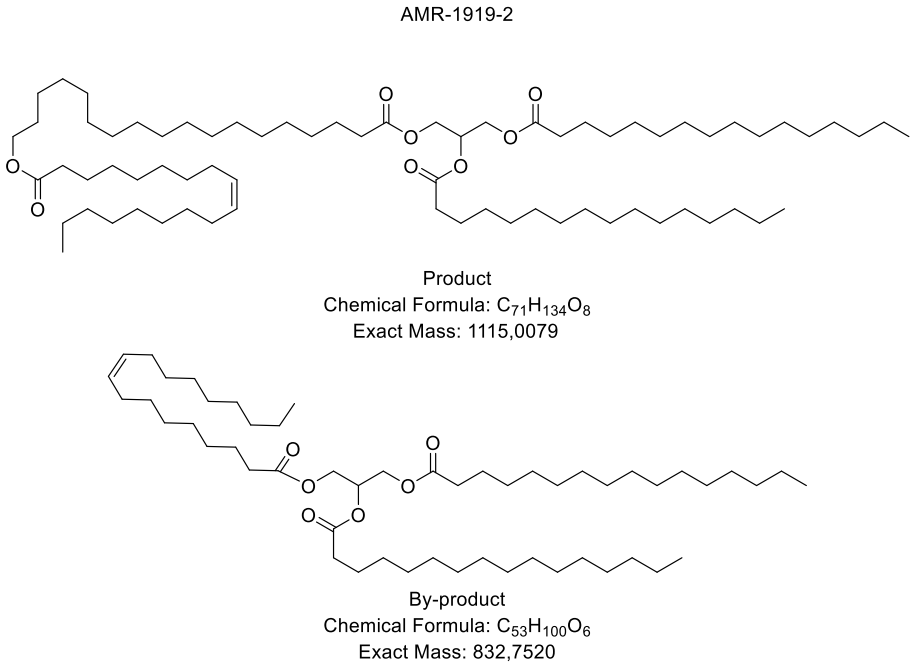

Figure D2 Estolide standard AMR-1919-2 and the by-product of its synthesis

Table D1 Data obtained from statistical analysis of "standard" TLC plate preparation method

Standard TLC preparation	y	x (TO)	Rf (TO)	$\overline{R_f(TO)}$	s (TO)	s (TO) / [%]	$\overline{s(TO)}/[\%]$	RSD (TO)	w	E (TO)	Er (TO)	Test Tn	x (Est)	Rf (Est)	$\overline{R_f(Est)}$	s (Est)	s (Est)/[%]	$\overline{s(Est)}/[\%]$	RSD (Est)	w	E (Est)	Er (Est)	Test Tn
1	9.2	2.3	0.2500	0.2305	0.0382	15.2935	17.0816	166	0.1285	0.0195	8.4796	0.5111	1.1	0.1196	0.1140	0.0207	17.2806	18.7679	181	0.0824	0.0055	4.8514	0.2678
2	9.2	2.3	0.2500			15.2935				0.0195	8.4796	0.5111	1.2	0.1304			15.8405				0.0164	14.3834	0.7938
3	9.1	2.2	0.2418			15.8149				0.0113	4.9033	0.2956	1.1	0.1209			17.0927				0.0068	6.0036	0.3313
4	9.2	2.1	0.2283			16.7500				-0.0022	-0.9534	0.0575	1.1	0.1196			17.2806				0.0055	4.8514	0.2678
5	9	2.5	0.2778			13.7642				0.0473	20.5329	1.2376	1.2	0.1333			15.4962				0.0193	16.9252	0.9341
6	9	2.2	0.2444			15.6411				0.0140	6.0689	0.3658	1.2	0.1333			15.4962				0.0193	16.9252	0.9341
7	8.8	2.5	0.2841			13.4583				0.0536	23.2723	1.4028	1.2	0.1364			15.1518				0.0223	19.5826	1.0808
8	9	2.5	0.2778			13.7642				0.0473	20.5329	1.2376	1.4	0.1556			13.2824				0.0415	36.4127	2.0097
9	9.5	2.3	0.2421			15.7922				0.0116	5.0539	0.3046	1	0.1053			19.6285				-0.0088	-7.6906	0.4245
10	9.5	2.2	0.2316			16.5101				0.0011	0.4864	0.0293	1	0.1053			19.6285				-0.0088	-7.6906	0.4245
11	9.7	2	0.2062			18.5434				-0.0243	-10.5323	0.6348	1.2	0.1237			16.7014				0.0097	8.4873	0.4684
12	9.7	2.1	0.2165			17.6604				-0.0140	-6.0589	0.3652	1.1	0.1134			18.2197				-0.0006	-0.5533	0.0305
13	9.3	2.4	0.2581			14.8156				0.0276	11.9789	0.7220	1.1	0.1183			17.4684				0.0042	3.7240	0.2055
14	9.6	2.4	0.2500			15.2935				0.0195	8.4796	0.5111	1.2	0.1250			16.5292				0.0110	9.6174	0.5308
15	9.2	2.6	0.2826			13.5289				0.0522	22.6291	1.3640	1.1	0.1196			17.2806				0.0055	4.8514	0.2678
16	9.4	2.1	0.2234			17.1142				-0.0071	-3.0608	0.1845	1.1	0.1170			17.6562				0.0030	2.6205	0.1446
17	8.2	1.5	0.1829			20.9011				-0.0475	-20.6247	1.2432	0.7	0.0854			24.2035				-0.0287	-25.1393	1.3875
18	8.2	1.5	0.1829			20.9011				-0.0475	-20.6247	1.2432	0.7	0.0854			24.2035				-0.0287	-25.1393	1.3875
19	8.2	1.5	0.1829			20.9011				-0.0475	-20.6247	1.2432	0.7	0.0854			24.2035				-0.0287	-25.1393	1.3875
20	8.2	1.4	0.1707			22.3941				-0.0597	-25.9164	1.5621	0.6	0.0732			28.2374				-0.0409	-35.8337	1.9777
21	9	1.4	0.1556			24.5789				-0.0749	-32.5016	1.9591	0.8	0.0889			23.2442				-0.0251	-22.0499	1.2170

Table D2 Data obtained from statistical analysis of "fast" TLC plate preparation method

Fast TLC preparation	y	x (TO)	Rf (TO)	$\overline{R_f(TO)}$	s (TO)	s (TO) / [%]	$\overline{s(TO)}/[\%]$	RSD (TO)	w	E (TO)	Er (TO)	Test Tn	x (Est)	Rf (Est)	$\overline{R_f(Est)}$	s (Est)	s (Est) / [%]	$\overline{s(Est)}/[\%]$	RSD (Est)	w	E (Est)	Er (Est)	Test Tn
1	8.75	2.9	0.3314	0.2681	0.0534	16.1193	20.6824	199	0.1934	0.0633	23.6080	1.1849	1.5	0.1714	0.1397	0.0407	23.7482	31.6285	291	0.1604	0.0317	22.6994	0.7790
2	9	2.9	0.3222			16.5799				0.0541	20.1744	1.0125	1.5	0.1667			24.4267				0.0270	19.2911	0.6620
3	9.1	2.3	0.2527			21.1374				-0.0154	-5.7366	0.2879	1.1	0.1209			33.6792				-0.0188	-13.4812	0.4627
4	9.5	1.7	0.1789			29.8546				-0.0892	-33.2607	1.6693	0.7	0.0737			55.2509				-0.0660	-47.2608	1.6219
5	9.4	3.5	0.3723			14.3482				0.1042	38.8662	1.9506	2.2	0.2340			17.3948				0.0943	67.5151	2.3170
6	9.5	3.1	0.3263			16.3719				0.0582	21.7011	1.0892	1.8	0.1895			21.4864				0.0498	35.6151	1.2223
7	9.4	2.8	0.2979			17.9352				0.0297	11.0930	0.5567	1.5	0.1596			25.5123				0.0199	14.2148	0.4878
8	9.3	3.1	0.3333			16.0272				0.0652	24.3184	1.2205	1.8	0.1935			21.0341				0.0538	38.5315	1.3223
9	10.3	3.3	0.3204			16.6748				0.0523	19.4905	0.9782	1.9	0.1845			22.0697				0.0448	32.0309	1.0992
10	9.3	2.1	0.2258			23.6592				-0.0423	-15.7843	0.7922	1.2	0.1290			31.5511				-0.0107	-7.6456	0.2624
11	9.3	2.2	0.2366			22.5838				-0.0316	-11.7741	0.5909	1.3	0.1398			29.1241				0.0001	0.0506	0.0017
12	9.3	2.2	0.2366			22.5838				-0.0316	-11.7741	0.5909	1.3	0.1398			29.1241				0.0001	0.0506	0.0017
13	9.3	2	0.2151			24.8422				-0.0531	-19.7946	0.9935	1.1	0.1183			34.4194				-0.0214	-15.3418	0.5265
14	9	2	0.2222			24.0408				-0.0459	-17.1211	0.8593	0.95	0.1056			38.5685				-0.0342	-24.4490	0.8391
15	9	2	0.2222			24.0408				-0.0459	-17.1211	0.8593	0.95	0.1056			38.5685				-0.0342	-24.4490	0.8391
16	9	2.8	0.3111			17.1720				0.0430	16.0305	0.8045	0.85	0.0944			43.1059				-0.0453	-32.4017	1.1120
17	9.4	2.1	0.2234			23.9136				-0.0447	-16.6803	0.8372	1.2	0.1277			31.8904				-0.0121	-8.6281	0.2961
18	9.4	2.4	0.2553			20.9244				-0.0128	-4.7774	0.2398	1.2	0.1277			31.8904				-0.0121	-8.6281	0.2961
19	9.4	2.4	0.2553			20.9244				-0.0128	-4.7774	0.2398	1.2	0.1277			31.8904				-0.0121	-8.6281	0.2961
20	9.4	2.1	0.2234			23.9136				-0.0447	-16.6803	0.8372	0.8	0.0851			47.8356				-0.0546	-39.0854	1.3414

Table D3 Data obtained from statistical analysis of "metal ion" TLC plate preparation method

Metal ion TLC preparation	y	x (TO)	Rf (TO)	$\overline{R_f(TO)}$	s (TO)	s (TO) /[%]	$\overline{s(TO)}/[\%]$	RSD (TO)	w	E (TO)	Er (TO)	Test Tn	x (Est)	Rf (Est)	$\overline{R_f(Est)}$	s (Est)	s (Est) /[%]	$\overline{s(Est)}/[\%]$	RSD (Est)	w	E (Est)	Er (Est)	Test Tn
1	9.6	3.2	0.3333	0.3297	0.0231	6.9329	7.0408	70	0.0661	0.0036	1.0915	0.1557	2	0.2083	0.1940	0.0151	7.2616	7.8433	78	0.0399	0.0143	7.3674	0.9450
2	9.6	3.2	0.3333			6.9329				0.0036	1.0915	0.1557	2	0.2083			7.2616				0.0143	7.3674	0.9450
3	8.7	3	0.3448			6.7018				0.0151	4.5774	0.6531	1.7	0.1954			7.7421				0.0014	0.7032	0.0902
4	8.7	3.1	0.3563			6.4856				0.0266	8.0633	1.1505	1.8	0.2069			7.3120				0.0129	6.6269	0.8500
5	8.7	3.1	0.3563			6.4856				0.0266	8.0633	1.1505	1.8	0.2069			7.3120				0.0129	6.6269	0.8500
6	8.7	3	0.3448			6.7018				0.0151	4.5774	0.6531	1.8	0.2069			7.3120				0.0129	6.6269	0.8500
7	9.7	3.5	0.3608			6.4047				0.0311	9.4290	1.3453	2	0.2062			7.3372				0.0121	6.2605	0.8030
8	9.7	3.5	0.3608			6.4047				0.0311	9.4290	1.3453	2	0.2062			7.3372				0.0121	6.2605	0.8030
9	9.7	3	0.3093			7.4722				-0.0205	-6.2038	0.8852	1.9	0.1959			7.7234				0.0018	0.9475	0.1215
10	9.7	3	0.3093			7.4722				-0.0205	-6.2038	0.8852	1.9	0.1959			7.7234				0.0018	0.9475	0.1215
11	9.5	3	0.3158			7.3181				-0.0139	-4.2291	0.6034	1.7	0.1789			8.4541				-0.0151	-7.7770	0.9975
12	9.5	2.9	0.3053			7.5704				-0.0245	-7.4215	1.0589	1.7	0.1789			8.4541				-0.0151	-7.7770	0.9975
13	9.5	3	0.3158			7.3181				-0.0139	-4.2291	0.6034	1.7	0.1789			8.4541				-0.0151	-7.7770	0.9975
14	9.5	2.9	0.3053			7.5704				-0.0245	-7.4215	1.0589	1.6	0.1684			8.9824				-0.0256	-13.2019	1.6933
15	9.5	2.8	0.2947			7.8408				-0.0350	-10.6138	1.5144	1.6	0.1684			8.9824				-0.0256	-13.2019	1.6933

Table D4 List of identified compounds from MS/MS spectra of *m/z* 1074.95968

m/z	*m/z* loss	*Molecular loss*	Molecular formula	Molecular adduct	*C=C*	*Structure*
1074.96	0	-	$C_{67}H_{128}O_8N$	$[M+NH_4]^+$	2	N/A
1057.94	17	NH_3	$C_{67}H_{124}O_8$	$[M+H]^+$	2	N/A
831.74	226.19	$C_{14}H_{26}O_2$	$C_{53}H_{98}O_6$	$[M+H-C14:1]^+$	1	N/A
829.73	228.21	$C_{14}H_{28}O_2$	$C_{53}H_{96}O_6$	$[M+H-C14:0]^+$	2	N/A
817.73	240.21	$C_{15}H_{28}O_2$	$C_{52}H_{96}O_6$	$[M+H-C15:1]^+$	1	N/A
815.71	242.22	$C_{15}H_{30}O_2$	$C_{52}H_{94}O_6$	$[M+H-C15:0]^+$	2	N/A
803.71	254.22	$C_{16}H_{30}O_2$	$C_{51}H_{94}O_6$	$[M+H-C16:1]^+$	1	N/A
801.7	256.24	$C_{16}H_{32}O_2$	$C_{51}H_{92}O_6$	$[M+H-C16:0]^+$	2	N/A
789.68	268.24	$C_{17}H_{32}O_2$	$C_{50}H_{92}O_6$	$[M+H-C17:1]^+$	1	N/A
787.68	270.26	$C_{17}H_{34}O_2$	$C_{50}H_{90}O_6$	$[M+H-C17:0]^+$	2	N/A
775.68	282.26	$C_{18}H_{34}O_2$	$C_{49}H_{90}O_6$	$[M+H-C18:1]^+$	1	N/A
773.66	284.27	$C_{18}H_{36}O_2$	$C_{49}H_{88}O_6$	$[M+H-C18:0]^+$	2	N/A
579.47	478.4	$C_{30}H_{54}O_4$	$C_{37}H_{70}O_4$	N/A	0	37:0
577.46	480.42	$C_{30}H_{56}O_4$	$C_{37}H_{68}O_4$	N/A	1	37:1
575.47	482.43	$C_{30}H_{58}O_4$	$C_{37}H_{66}O_4$	N/A	2	37:2
573.46	484.45	$C_{30}H_{60}O_4$	$C_{37}H_{64}O_4$	N/A	3	37:3
571.47	486.46	$C_{30}H_{62}O_4$	$C_{37}H_{62}O_4$	N/A	4	37:4
565.48	492.42	$C_{31}H_{56}O_4$	$C_{36}H_{68}O_4$	N/A	0	36:0
563.5	494.43	$C_{31}H_{58}O_4$	$C_{36}H_{66}O4$	N/A	1	36:1
561.48	496.45	$C_{31}H_{60}O_4$	$C_{36}H_{64}O_4$	N/A	2	36:2
559.47	498.46	$C_{31}H_{62}O_4$	$C_{36}H_{62}O_4$	N/A	3	36:3
551.46	506.43	$C_{32}H_{56}O_4$	$C_{35}H_{66}O_4$	N/A	0	35:0
549.45	508.45	$C_{32}H_{60}O_4$	$C_{35}H_{64}O_4$	N/A	1	35:1
547.46	510.46	$C_{32}H_{62}O_4$	$C_{35}H_{62}O_4$	N/A	2	35:2
545.46	512.48	$C_{32}H_{64}O_4$	$C_{35}H_{60}O_4$	N/A	3	35:3
537.46	520.45	$C_{33}H_{60}O_4$	$C_{34}H_{64}O_4$	N/A	0	34:0
535.46	522.46	$C_{33}H_{62}O_4$	$C_{34}H_{62}O_4$	N/A	1	34:1
533.46	524.48	$C_{33}H_{64}O_4$	$C_{34}H_{60}O_4$	N/A	2	34:2
523.47	534.46	$C_{34}H_{62}O_4$	$C_{33}H_{62}O_4$	N/A	0	33:0
521.46	536.48	$C_{34}H_{64}O_4$	$C_{33}H_{60}O_4$	N/A	1	33:1
519.44	538.5	$C_{34}H_{66}O_4$	$C_{33}H_{58}O_4$	N/A	2	33:2
509.46	548.48	$C_{35}H_{64}O_4$	$C_{32}H_{60}O_4$	N/A	0	32:0
495.44	562.5	$C_{36}H_{66}O_4$	$C_{31}H_{58}O_4$	N/A	0	31:0

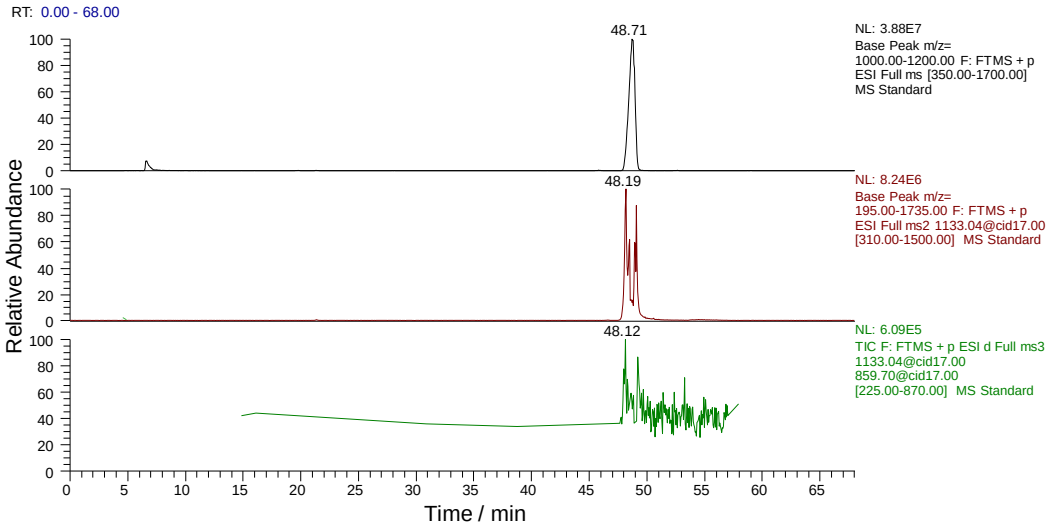

Figure D3 Reconstructed full MS, full MS^2 of *m/z* 1133.04@CID 17.00 [*m/z* 310.00-1500.00] and full MS^3 of *m/z* 1133.04@CID17.00, 859.70@CID 17.00 [*m/z* 225.00-870.00] chromatograms of AMR-1919-2 standard obtained on LTQ Orbitrap, ESI; positive mode

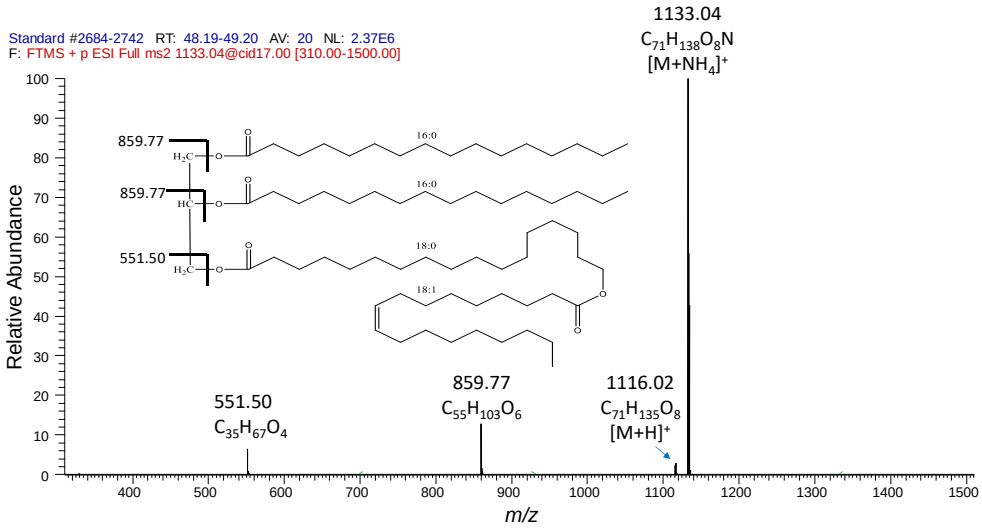

Figure D4 Full MS^2 mass spectrum of AMR-1919-2 standard. FTMS + p ESI full MS2 of *m/z* 1133.04@CID 17.00 [*m/z* 310.00 - 1500.00]

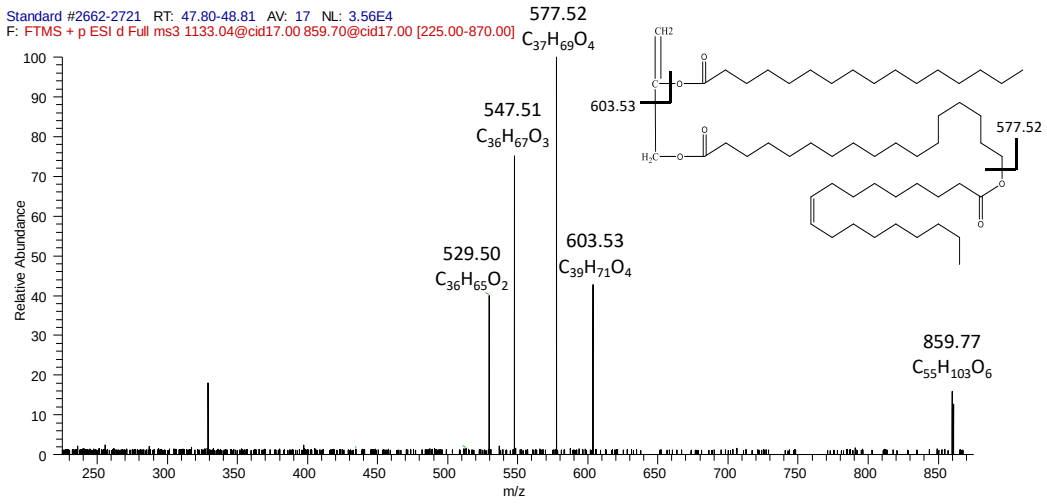

Figure D5 Full MS^3 mass spectrum of AMR-1919-2 standard. FTMS + p ESI full MS^3 of *m/z* 1133.04@CID 17.00, *m/z* 859.70@CID 17.00 [*m/z* 225.00 – 870.00]

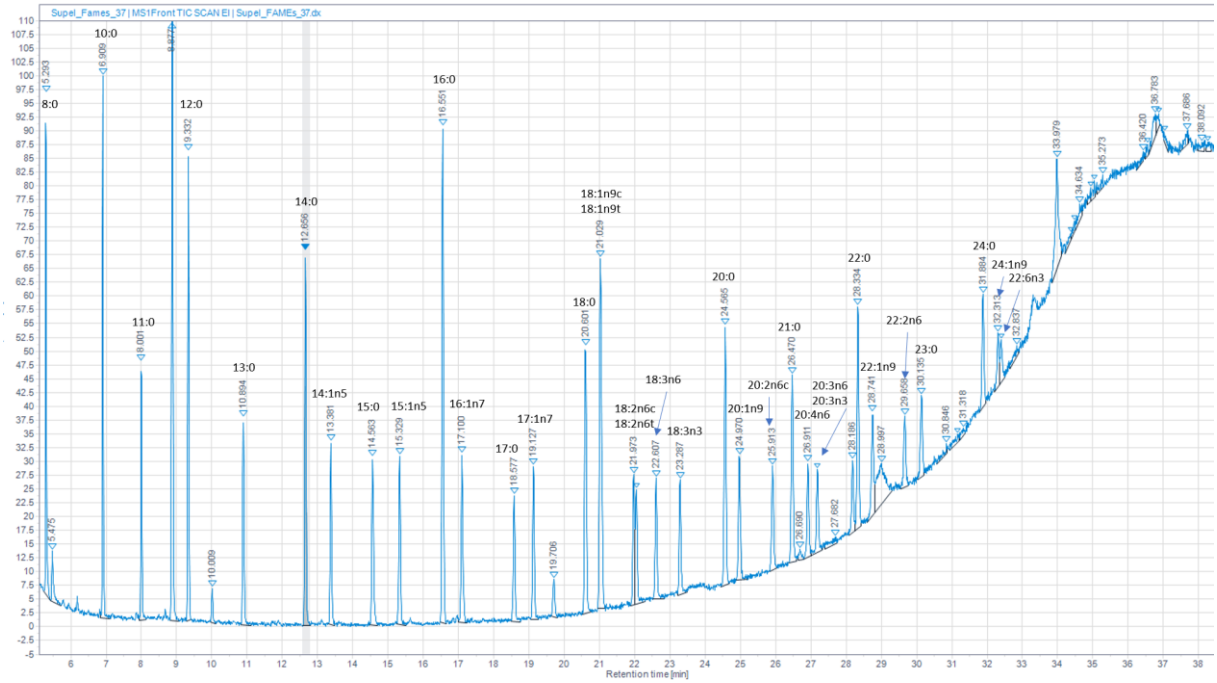

Figure D6 Chromatogram of a mixture of fatty acid methyl ester standards obtained on Agilent 7890A GC system, DB-WAX column (30 m x 250 µm, 0.25 µm; Agilent), He at 1 mL/min, 1µL of sample

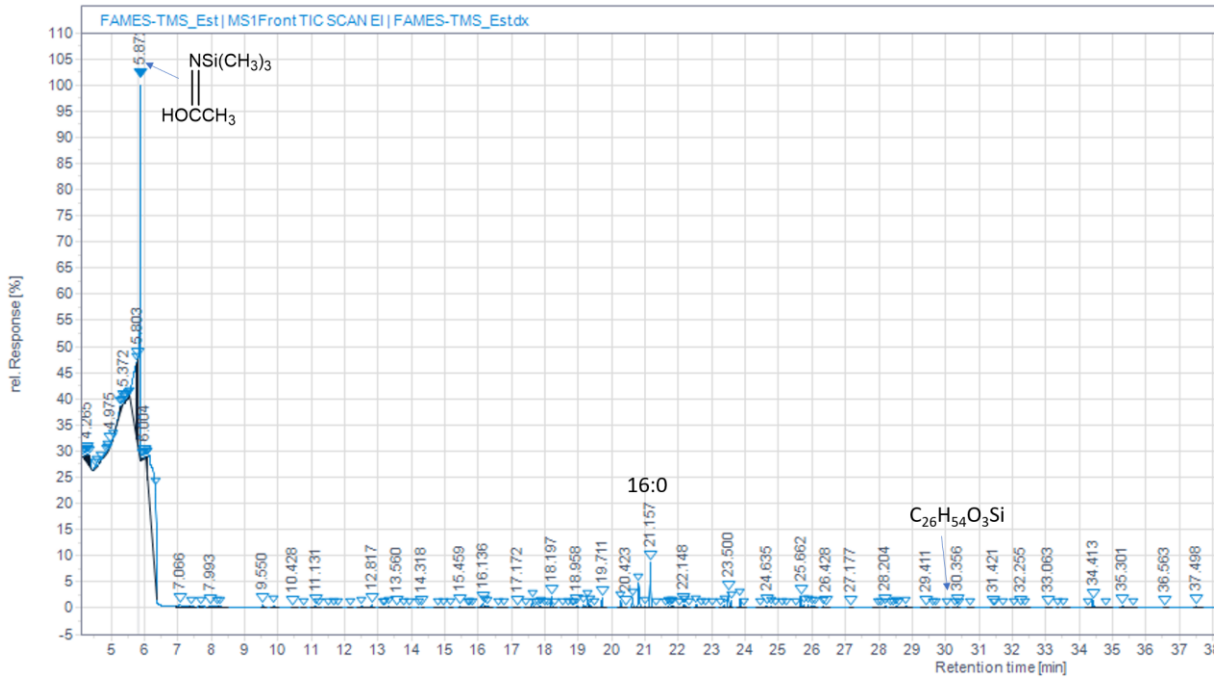

Figure D7 Chromatogram of a mixture of O-TMS-FAMEs from vernix caseosa sample obtained on Agilent 6890N GC system, HP-5ms column (30 m x 250 µm, 0.25 µm), He at 1 mL/min, 1µL of sample

§ 9. CURRICULUM VITAE

Personal Information

Name and surname: Hrvoje Dumić

Date of birth: 26. September 1994.

Place of birth: Zagreb

Education

2001–2009	Osnovna škola [Osnovna škola Stenjevec, Zagreb]
2009–2013	Srednja škola [Gimnazija Lucijana Vranjanina, Zagreb]
2013–2017	Preddiplomski studij [Undergraduate study of Chemistry, Faculty of Science, University of Zagreb, Zagreb]
2019	Međunarodna studentska razmjena [Erasmus+ Internship, The Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, Prague, Czech Republic]

Honours and Awards

2018	Rector´s award for individual scientific work
2018	1st place at regional competition for students; STEM Games 2018, knowledge, Science category

Activities in Popularization of Science

2015	“Dani otvorenih vrata Prirodoslovno-matematičkog fakulteta”

Participation at Scientific Meetings and Conferences

1. Lecturer at 5th Symposium of Chemistry students; “Kinetic study of acid catalysed reduction of nitrobenzene”, Faculty of Science (University of Zagreb), 2018.