Cholesteryl esters as a depot for very long chain fatty acids in human meibum

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Abstract **Human meibomian gland secretions (also known as meibum) were analyzed for the presence of cholesteryl esters (Chl-E) using HPLC in combination with atmospheric pressure chemical ionization mass spectrometry. A special procedure based on detection of the in-source generated ion *m/z* 369 was developed to monitor all Chl-E simultaneously. The structures of the detected compounds were studied using in-source and postsource fragmentation of the precursor $(M+H)^+$ ions. In concordance with previous studies, Chl-E were found in all of the tested samples and comprised ~31% of the entire lipid pool (w/w, dry weight). There were at least 20 different saturated and unsaturated Chl-E species observed, whose fatty acid residues ranged from C18 to C34. Monounsaturated fatty acids were the most visible components of the Chl-E pool. The eleven most prominent compounds were: C20:0-, C22:1-, C22:0-, C24:1-, C24:0, C25:0-, C26:1-, C26:0-, C28:1-, C28:0-, and C30:1-Chl-E. Other Chl-containing compounds were detected but not identified at the time. Therefore, Chl-E are a depot for very long chain saturated and monounsaturated fatty acids in human meibum.**—Butovich, I. A. **Cholesteryl esters as a depot for very long chain fatty acids in human meibum.** ***J. Lipid Res.*** **2009.** 50: **501–513.**

Supplementary key words lipids • meibomian glands • HPLC • mass spectrometry

Meibomian glands that are located at the margins of human upper and lower eyelids produce secretions (also known as meibum) formed of a complex mixture of various nonpolar and polar lipids (1–31). The primary role of meibum is to form the bulk of a so-called preocular tear film lipid layer (TFLL), which covers the entire ocular surface and fulfills various roles including antievaporatory, antibacterial, lubricating, nutritional, and others. The lipid composition of TFLL is critical for maintaining the stability of the preocular tear film, deterioration of which has been associated with various ocular diseases and pathological conditions. Human meibum has been shown to have a large number of diverse lipid species, including wax esters (WE) (3, 5–7, 12, 16–20, 25, 26), cholesteryl esters (Chl-E) (3, 5, 7, 11, 12, 18, 20, 25, 26), mono-, di-, and triacyl glycerols (3, 5–7, 14, 16–18, 20, 21, 25, 26), hydrocarbons (6), free fatty acids (31), sterols (1, 3–7, 9, 11–13, 16–20), phospholipids (2, 6, 7, 15, 19, 21, 22, 25, 26), ceramides (6, 21), and many other poorly identified lipid compounds. Historically, the main methods used to characterize meibomian lipids were TLC (3–5, 15, 20–22), gas-liquid chromatography (6–10, 13), and gas chromatography-electron ionization GC-MS (11, 15, 20–22, 30, 31), HPLC with UV detection (15, 20–22), infrared spectrometry (27, 28), and, more recently, various types of modern mass spectrometry (MS) techniques with or without HPLC (16, 17, 19, 23, 25, 26). Recently, we developed and implemented normal phase (NP) HPLC-MS protocols that allowed us to evaluate the lipid composition of human meibum (25, 26). This approach has proven to be well-suited for analyses of very hydrophobic components of human meibum, which in our hands was found to be consisted primarily of WE, Chl-E, and triacyl glycerols, with all other compounds being minor constituents. For example, the overall content of free Chl in dry human meibum was estimated to be less than 1% (w/w). Our previous NP HPLC-MS and MS^n experiments allowed us to detect a large number of various $C_{18:1}$-based WE with saturated fatty alcohol components ranging from $C_{20:0}$ to $C_{30:0}$, and smaller amounts of linoleic acid ($C_{18:2}$) and stearic acid ($C_{18:0}$)-based WE with similar types of fatty alcohols in their structures (25, 26). However, the exact nature of Chl-E was not established at the time because: *1*) all tested Chl-E coeluted under the conditions of NP HPLC used in our earlier experiments, and *2*) low

The author acknowledges support from Research to Prevent Blindness (New York, NY) and from the National Institutes of Health in the form of unrestricted core grant EY-016664.

Manuscript received 11 August 2008 and in revised form 12 September 2008 and in re-revised form 24 September 2008.

Published, JLR Papers in Press, October 4, 2008.
DOI 10.1194/jlr.M800426-JLR200

Abbreviations: APCI, atmospheric pressure chemical ionization; Chl-E, cholesteryl ester; CID, collision-induced dissociation; LCFA, long-chain fatty acid; MS, mass spectrometry; NP, normal phase; RP, reversed-phase; RT, retention time; SIM, single ion monitoring; SRM, single reaction monitoring; TFLL, tear film lipid layer; TIC, total ion chromatogram; VLCFA, very long chain fatty acid; WE, wax ester.

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stability of Chl-E ions resulted in very low intensity signals of these compounds. Nevertheless, every tested sample of human meibum produced a strong signal *m/z* 369, more than 99% of which coeluted with authentic Chl-E standards, but not with standard Chl, and which consistently was one of the strongest MS peaks in the MS spectra. When fragmented in MS^n experiments, its product ions matched perfectly those of authentic Chl (26). Therefore, this signal was attributed to Chl-E. Our experiments with several authentic Chl-E showed that the signal *m/z* 369 originated from a Chl-E after it underwent a neutral loss of its fatty acid component, and/or from free Chl upon its dehydration, both happening spontaneously in the ion source of a mass spectrometer. This dual origin of *m/z* 369 signal could be advantageous when analyzing meibum samples as it can be used for easy and simultaneous monitoring of the elution profiles of various Chl-E and free Chl.

Earlier, human meibum was repeatedly reported to have large quantities of long chain and very long chain fatty acids (LCFA and VLCFA) of the C_{16} and above family esterified into various lipid species. Typically, the analyses were performed using GLC or GC-MS after transesterification of the entire lipid pool (7, 29, 30), which precluded researchers from identifying the intact lipid species present in meibum. As *1*) WE and Chl-E were estimated to comprise more than two-thirds of the entire lipid pool of human meibum (7), and *2*) WE detected in our experiments (25, 26) were mostly C_{18}-based, there was a distinctive possibility that it is the Chl-E that have VLCFA in their structures.

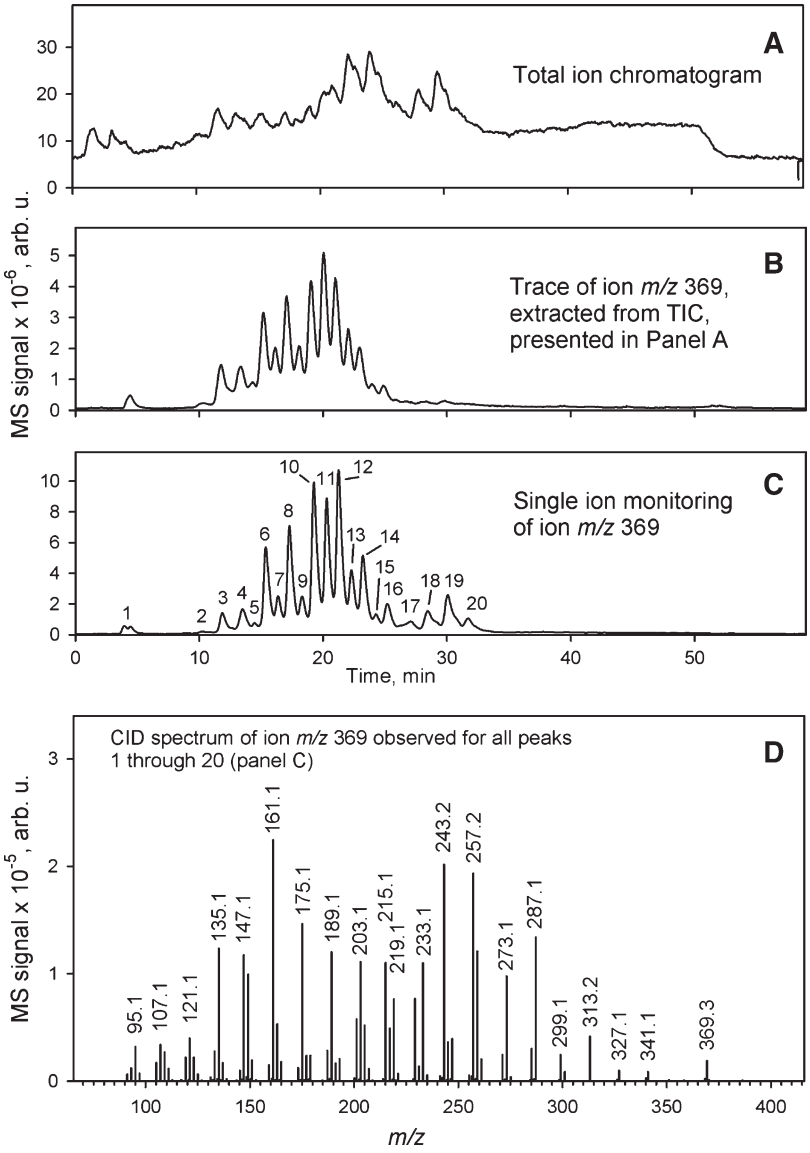


Fig. 1. Representative chromatograms of human meibum (A), its components producing characteristic analytical ion *m/z* 369 (B, C), and a collision-induced dissociation (CID) spectrum of ion *m/z* 369 (D). TIC, total ion chromatogram.

Therefore, in this paper we will discuss the results of HPLC-MS^n experiments conducted to test the hypotheses that Chl-E of human meibum is a depot for VLCFA, and that those compounds are the major components of human tear film.

MATERIALS AND METHODS

Reagents and equipment

Lipid standards were purchased from Nu-Chek Prep, Inc. (Elysian, MN) and Sigma Chemical Co. (St. Louis, MO). *epi*-Cholesteryl oleate was synthesized in-house using authentic *epi*-cholesterol (Steraloids, Inc., Newport, RI) and oleoyl anhydride. Solvents were of HPLC or spectroscopy grade. An Alliance 2695 HPLC Separations Module (Waters Corp., Milford, MA) operated under the Waters' Empower Pro (built 1154) software was used throughout. A reversed-phase (RP) Hypersil Gold HPLC column (150mm × 2.1 mm, 5 μm) from Thermo Electron (San Jose, CA) was used in HPLC experiments. An LCQ Deca XP Max ion trap mass spectrometer (Thermo Electron, San Jose, CA) was used as a detector in HPLC-style experiments and as a standalone unit in direct infusion experiments. All the experiments were carried out using an atmospheric pressure chemical ionization (APCI) ion source operating in the positive ion mode. An Xcalibur software (v. 1.4., Thermo Electron) was used to operate the mass spectrometer and collect and analyze the raw data. A SigmaPlot for Windows (v. 11.0; Systat Software, Inc., San Jose, CA) was used for curve-fitting purposes.

Sample collection

Meibum samples from five healthy volunteers were expressed from their meibomian glands, treated, and stored exactly as described earlier (25, 26). The procedures were approved by the Institutional Review Board and were performed in accordance with the principles of the Declaration of Helsinki. No conclusions about the effects of sex, age, diet, etc. on the meibum composition were to be made.

RP HPLC analyses of standard lipids and human meibum

Our current gradient RP HPLC procedure was partly based on a previously published isocratic HPLC routine (32). The analyses were performed at 35°C using a gradient of 5 mM aqueous ammonium formate (solvent A), acetonitrile (solvent B), and propan-2-ol (solvent C). The flow rate was maintained at 0.2 ml/min throughout the procedure. Before an injection, the column was pre-equilibrated with a solvent mixture A: B: C = 5: 45: 50 (v/v/v). The elution started with the same solvent mixture linearly changing to A: B: C = 5: 5: 90 (v/v/v) over the period of 35 min. Then, the solvent composition was maintained for 10 min, after which it was changed to the initial solvent mixture A: B: C = 5: 45: 50 (v/v/v) within the next 1 min. Finally, the column was reequilibrated for another 14 min with the same solvent. This completed the 1-h chromatographic routine.

The standard lipids were dissolved in either chloroform (to make 1 mg/ml stock solutions of each), or *n*-hexane: propan-2-ol = 50: 50 (v/v) (for diluted injection solutions in the concentration range between 20 and 100 μM). Between 0.5 and 1 mg of dry human meibum was dissolved in the *n*-hexane: propan-2-ol solvent mixture to give, typically, 0.1 mg/ml injection solution. Injection volumes for lipid standards and meibum were between 1 and 10 μl. It was found that larger sample volumes could degrade the quality of separation and shift (typically, reduce) the retention times (RT) of the analytes.

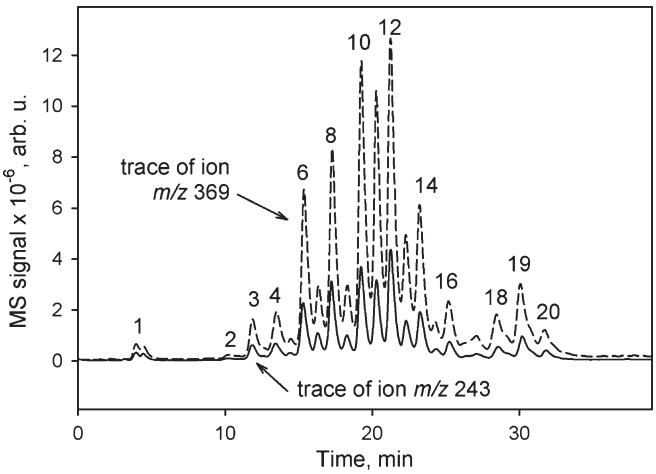


Fig. 2. Single ion monitoring (SIM) chromatogram of a precursor ion *m/z* 369 (MS1) and a selected reaction monitoring chromatogram of a product ion *m/z* 243 (MS2, transition 369 → 243).

MS detection of analytes

The entire flow of the effluent was directed to the APCI ion source operated in the positive ion mode. The following MS parameters were used: vaporization temperature (depending on the application), either 250°C or 350°C; sheath gas (nitrogen), 40 arbitrary units; source voltage, between 2.5 and 3 kV; source current, 2 to 5 μA; capillary temperature, either

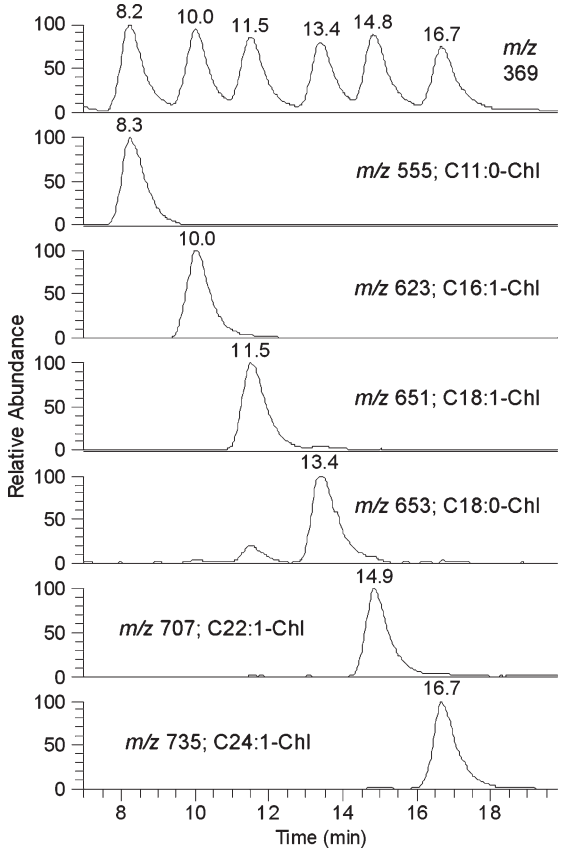


Fig. 3. Reversed-phase (RP) SIM chromatograms of an equimolar test mixture of six standard cholesteryl esters.

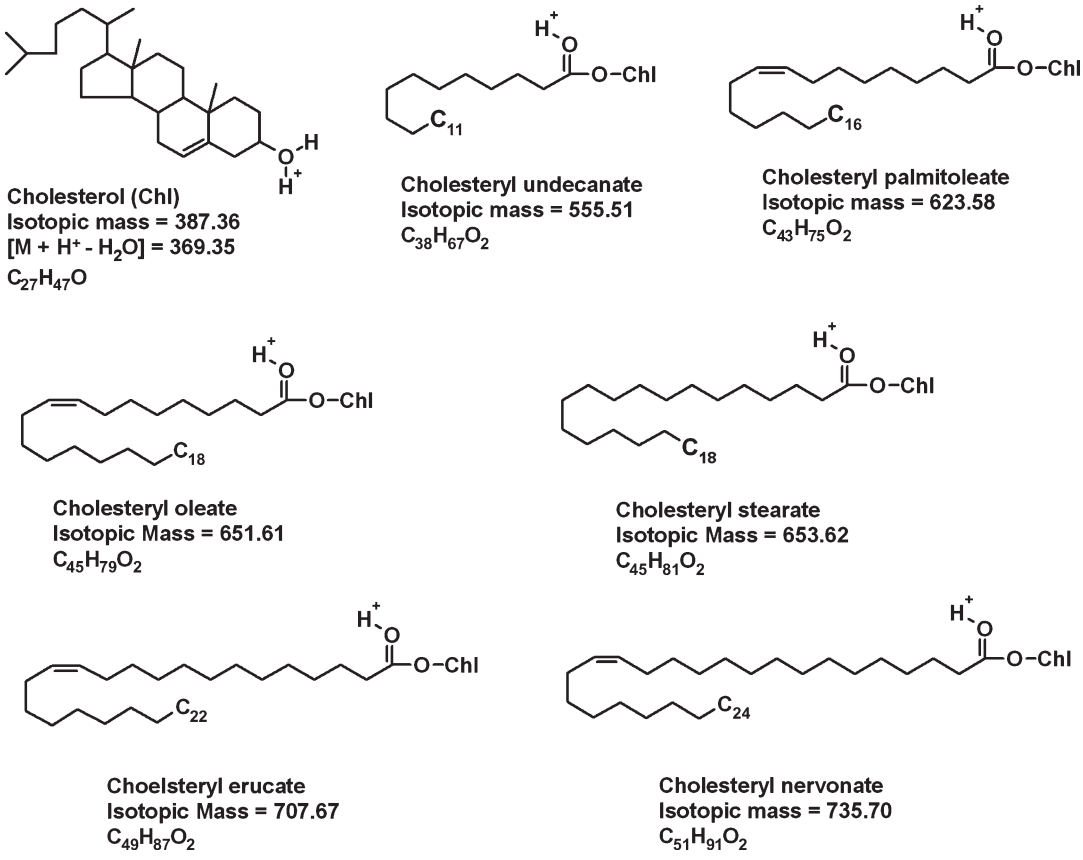
250°C or 350°C; capillary voltage, (+10V); tube lens offset, (−10 to −40 V).

Total ion chromatograms (TIC) were recorded in the *m/z* range of 205 to 2,000. For single ion monitoring (SIM) MS1 experiments, the range was modified accordingly to the analyte's expected *m/z* value. In MS2 (MS/MS) experiments, the normalized collision energy was optimized for each of the analytes, and typically fell in the range of 35% to 40%. The monoisotopic masses of the analytes were determined in zoom scan experiments in the range of *m/z* ±10 amu.

To quantify individual meibum lipids, standard curves were obtained for a number of lipid standards (see Results). The chromatograms were smoothed by using the Gaussian 3- to 9-point routine and then were integrated using the Xcalibur's built-in routine using the Avalon peak detection algorithm and assuming a flat baseline. This assumption was proven to be valid based on the analyses of individual lipid standards and their mixtures (see later discussion). Note that overly aggressive averaging of the mass spectra during an HPLC-MS experiment and/or excessive postprocessing smoothing somewhat degrades separation of the HPLC peaks. Thus, one needs to find a compromise between the peak resolution and noise. The best setting for spectra averaging for meibum samples appear to be between 2 and 5.

RESULTS

A representative TIC of a meibum sample is presented in **Fig. 1A**. The absence of clearly defined HPLC peaks is typical of HPLC-MS experiments as natural lipid extracts are usually complex and produce a large number of overlapping peaks, which create a diffused pattern similar to shown in Fig. 1A. However, extraction of a particular signal *m/z* 369 from the TIC led to a much better defined chromatogram (Fig. 1B). In a subsequent SIM MS1 experiment, an even better resolved elution pattern with more than 20 clearly identifiable HPLC peaks and shoulders producing a signal with *m/z* 369 was observed (Fig. 1C). Further HPLC-MS2 experiments revealed that upon collision-induced dissociation (CID), ion *m/z* 369 produced a family of product ions presented in Fig. 1D, which matched the fragmentation pattern of free cholesterol reported earlier (26). Importantly, every single HPLC peak depicted in Fig. 1B, C produced identical fragmentation patterns. This provided us with an additional valuable tool for determination of whether all the HPLC peaks visible in Fig. 1B, C were indeed Chl-E: when plotted together, HPLC traces of ions *m/z* 369 and 243 produced identical chromatograms (**Fig. 2**) in terms of both RT and relative peak intensities meaning that all the compounds detected as *m/z* 369 were derivatives of Chl (or its isomeric form *epi*Chl). Ion *m/z* 243 is, apparently, a product of neutral losses of the eight-carbon side chain of Chl and a methyl group. The higher resolution of chromatograms obtained in SIM experiments was, apparently, due to the higher (up to 30%) overall number of scans achieved in SIM experiments compared with the full-scan (TIC) experiments.



Scheme 1. Chemical structures of representative cholesteryl esters.

In an attempt to differentiate between Chl-E and *epi*Chl-E, two authentic model compounds, cholesteryl oleate ($C_{18:1}$-Chl) and *epi*-cholesteryl oleate ($C_{18:1}$-*epi*Chl), were analyzed side-by-side using HPLC-MS. Repeatedly, $C_{18:1}$-*epi*Chl eluted 0.5–1 min faster than $C_{18:1}$-Chl. As their CID spectra were found to be similar to each other (not shown), one needed to rely on the difference in their RT when assigning the structures. The RT of ~11.8 min of one of the components of human meibum (Figs. 1 and 2) was close to the RT of authentic $C_{18:1}$-Chl (~11.5 min). The meibum peak produced ions *m/z* 369 and 651 isobaric to $[M+H]^+$ ion of $C_{18:1}$-Chl (see later discussion). Therefore, the meibum component was tentatively identified as $C_{18:1}$-Chl.

To further elucidate the structures of meibomian Chl-E, an equimolar mixture of several authentic Chl-E and free Chl was tested to determine their RT (**Fig. 3**). The Chl-E ranged from a modestly long cholesteryl undecanate

TABLE 1. Characteristic ions and retention times (RT) of standard cholesteryl esters

Name of compound	Characteristic ions and their nature[a]	RT, min[b]	
		Saturated	Unsaturated
Chl	369 $(M-H_2O+H)^+$	4.4 (no fatty acid chain)	
	555 $(M+H)^+$		
$C_{11:0}$-Chl	572 $(M+NH_4)^+$	8.2	
	593 $(M+K)^+$		
	569 $(M+H)^+$		
$C_{12:0}$-Chl	586 $(M+NH_4)^+$	8.8	
	607 $(M+K)^+$		
	623 $(M+H)^+$		
$C_{16:1}$-Chl	640 $(M+NH_4)^+$		10.0
	661 $(M+K)^+$		
	625 $(M+H)^+$		
$C_{16:0}$-Chl	642 $(M+NH_4)^+$	11.6	
	663 $(M+K)^+$		
	651 $(M+H)^+$		
$C_{18:1}$-Chl	668 $(M+NH_4)^+$		11.5
	689 $(M+K)^+$		
	653 $(M+H)^+$		
$C_{18:0}$-Chl	670 $(M+NH_4)^+$	13.4	
	691 $(M+K)^+$		
	681 $(M+H)^+$		
$C_{20:0}$-Chl	698 $(M+NH_4)^+$	14.9	
	719 $(M+K)^+$		
	707 $(M+H)^+$		
$C_{22:1}$-Chl	724 $(M+NH_4)^+$		14.9
	745 $(M+K)^+$		
	709 $(M+H)^+$		
$C_{22:0}$-Chl	726 $(M+NH_4)^+$	16.8	
	747 $(M+K)^+$		
	735 $(M+H)^+$		
$C_{24:1}$-Chl	752 $(M+NH_4)^+$		16.7
	773 $(M+K)^+$		
	737 $(M+H)^+$		
$C_{24:0}$-Chl	754 $(M+NH_4)^+$	18.7	
	775 $(M+K)^+$		

[a] Potassium adducts of unsaturated Chl-E ($M+K^+$) were more visible than the adducts of saturated cholesteryl ester (Chl-E).

[b] RT of lipids may change within ±1 min depending on the uncontrollable fluctuations in the solvent composition and/or the equilibration state of HPLC columns. The change affects all compounds so that their retention time (RT) all shift in one and the same direction. The change affects slower moving compounds more than those that elute faster. Therefore, standard cholesteryl ester (Chl-E) should be used to verify the RT of unknowns.

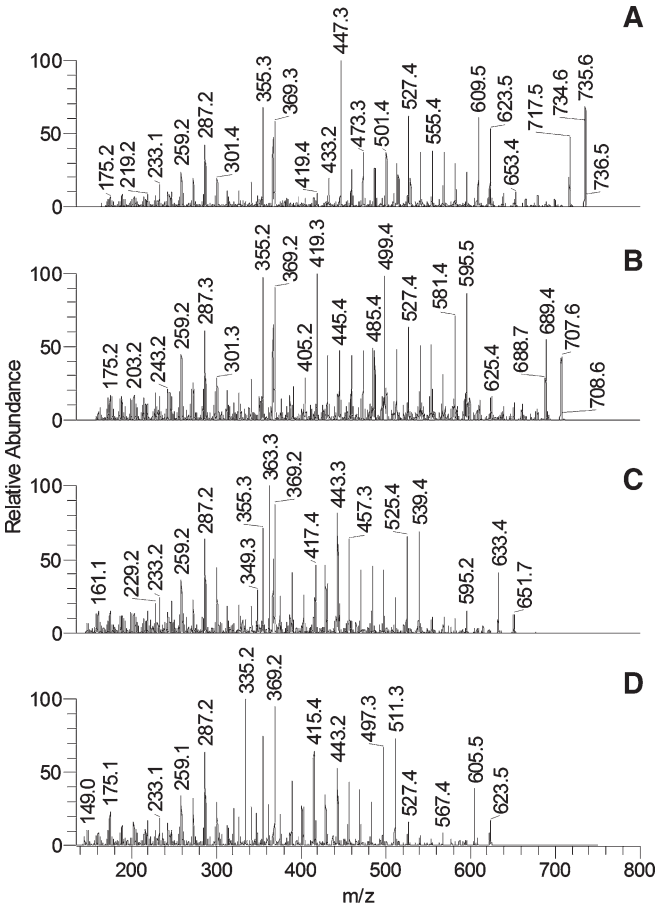
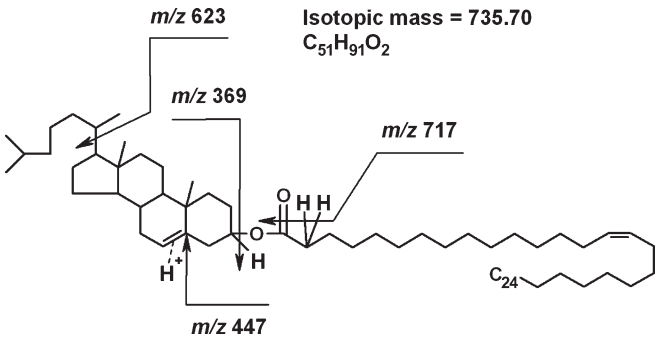


Fig. 4. Collision-induced dissociation atmospheric pressure chemical ionization (APCI) mass spectra of proton adducts of $C_{24:1}$-Chl (*m/z* 735, A), $C_{22:1}$-Chl (*m/z* 707, B), $C_{18:1}$-Chl (*m/z* 651, C), and $C_{16:1}$-Chl (*m/z* 623, D).

($C_{11:0}$-Chl) to the longest Chl-E available commercially, cholesteryl nervonate ($C_{24:1}$-Chl) (**Scheme 1**). It became clear that various Chl-E eluted according to their overall hydrophobicity: extension of their chains by two methylene groups increased their RT by ~1.5–2.0 min, while introduction of a double bond while maintaining the carbon chain length reduced RT by approximately the same time (**Table 1**). In the studied range of standard Chl-E concen-



Scheme 2. APCI MS collision-induced dissociation pattern of cholesteryl nervonate.

trations, a simple polynomial equation adequately represented the experimental data:

$$y = k_1 + k_2 \times (N - 2M) + k_3 \times (N - 2M)^2 \quad (Eq.\ 1)$$

where y is RT; N, a number of carbon atoms in the fatty acid chain of a Chl-E (between 11 and 24); M, a number of double bonds in the fatty acid chain (0 or 1); while k_1, k_2, and k_3 were regression coefficients that were found to be 4.4, 0.142, and 0.019, respectively. The r^2 (the coefficient of determination) value was 0.999. It is clear that the regression coefficient k_1 has a physical meaning of RT of free Chl. Note that the regression coefficients will slightly change if the RT change due to inadvertent fluctuations in the solvent composition and temperature, and/or equilibration state of the column (see a footnote to Table 1).

Importantly, in an RP HPLC-MS1 experiment the test Chl-E were visible as a mixture of proton $(M+H)^+$, ammonium $(M+NH_4)^+$, and potassium $(M+K)^+$ adduct ions, although their intensities were very low compared with the major product ion *m/z* 369. Chl-E with unsaturated fatty acids were easily detectable, while signals of those with saturated fatty acid residues were weaker.

In RP HPLC-MS2 experiments, proton adducts of standard monounsaturated Chl-E produced characteristic product ions, which were useful in determining of the nature of their sterol moieties and of the overall lengths of the fatty acid chains in Chl-E. For example, an $[M+H]^+$ ion of $C_{24:1}$-Chl (*m/z* 735) dissociated releasing a series of characteristic ions depicted in **Fig. 4A**, **Scheme 2**. Ion *m/z* 623 apparently originated from a neutral loss of a C_8H_{16} (112 amu.) side chain of Chl. The same loss of the C_8H_{16} fragment was observed for every tested Chl-E: $C_{22:1}$-Chl (*m/z* 707, $[M+H]^+$), $C_{18:1}$-Chl (*m/z* 651, $[M+H]^+$), and $C_{16:1}$-Chl (*m/z* 623, $[M+H]^+$), and produced ions with *m/z* values of, respectively, 595, 539, and 511 (Fig. 4B–D). An important ion in the $C_{24:1}$-Chl CID spectrum is ion *m/z* 447. It apparently originated upon fragmentation of the Chl moiety of the compound without affecting its fatty acid chain (Scheme 2). Indicatively, similar ions were detected for every tested monounsaturated Chl-E. Thus, $C_{22:1}$-Chl, $C_{18:1}$-Chl, and $C_{16:1}$-Chl produced related product ions *m/z* 419, 363, and 335.

Another group of characteristic product ions were a ubiquitous ion *m/z* 369 and its fragments 287, 273, 259, 233, and others formed spontaneously from the precursor $[M+H]^+$ ion. These were used to identify Chl-E in human samples. All of the standard Chl-E tested in MS2 and MS3 experiments produced ion *m/z* 369 and its product ions, which were identical to those reported for authentic Chl (26).

Unexpected was product ion *m/z* 367 detected in human samples. This ion, which appeared among the product ions in the CID spectra of the $[M+H]^+$ of the Chl-E-like compounds (e.g., $C_{24:1}$-Chl and $C_{26:1}$-Chl) in MS2 experiments, was tentatively identified as a cholestatriene (a product similar to cholestadiene with an additional double bond $[Chl-H_2O-2H+H]^+$) (**Fig. 5**). Note that in an MS3 experiment, most of its prominent product ions had *m/z* values 2 amu lower than those originated from ion *m/z* 369. Both of the spectra were obtained in one experiment while analyzing the same sample.

Spontaneous fragmentation of Chl-E with saturated fatty acids in MS1 experiments, on the other hand, did not produce any clearly identifiable fragments except for ion *m/z* 369, primarily because of the very low intensity of their $(M+H)^+$ ions (not shown). Therefore, the only practical way of monitoring elution of the saturated Chl-E from an HPLC column was to monitor ion *m/z* 369.

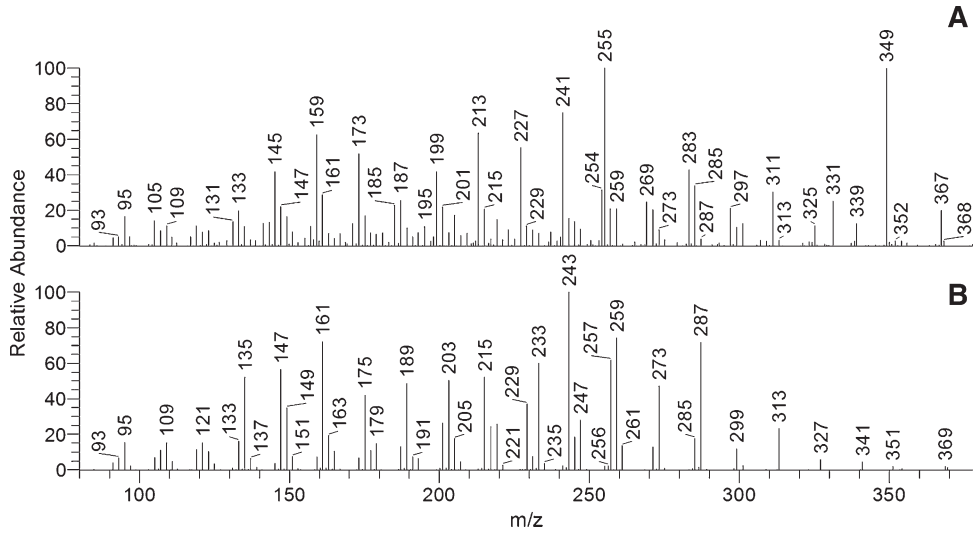


Fig. 5. Collision-induced dissociation mass spectra of ions *m/z* 367 (spectrum A) and 369 (spectrum B) generated from a meibum ion *m/z* 763 (a proton adduct of $C_{26:1}$-Chl) in MS3 experiments [*m/z* 763 (MS1) → *m/z* 369 (MS2) → products (MS3) and *m/z* 763 (MS1) → *m/z* 367 (MS2) → products (MS3)]. Similar ions were observed in CID spectra of other monounsaturated cholesteryl ester (Chl-E) from meibum.

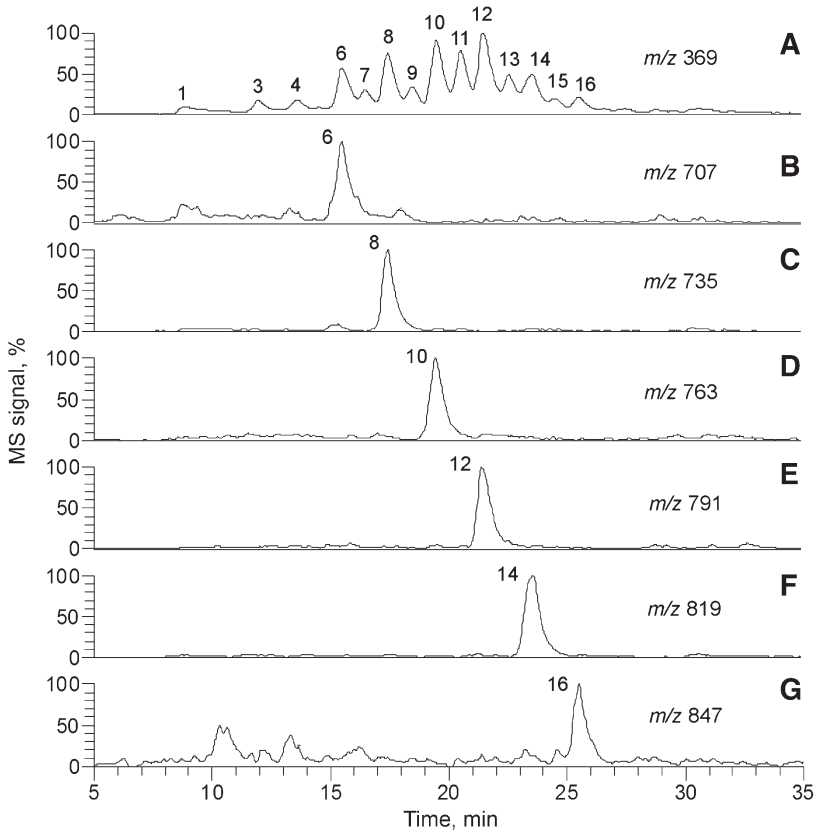


Fig. 6. Reconstructed chromatograms of Chl-E found in human meibum. Trace A: all Chl-E detected by monitoring ion *m/z* 369. Individual Chl-E (traces B–G) were monitored as $[M+H]^+$ ions. Trace B: $C_{22:1}$-Chl; trace C: $C_{24:1}$-Chl; trace D: $C_{26:1}$-Chl; trace E: $C_{28:1}$-Chl; trace F: $C_{30:1}$-Chl; trace G: $C_{32:1}$-Chl.

A systematic drift of the baseline due to changes in solvent compositions, which is often observed in gradient HPLC-MS experiments, was not an issue in our experiments, as our solvent mixture did not produce such interfering ions. After extraction of chromatograms of ion *m/z* 369 from TIC, or during SIM experiments, the baseline remained conveniently flat, which facilitated determination of correct RT and peak areas.

TABLE 2. Theoretical and experimental masses of Chl-E found in human meibum[a]

HPLC peak #	Compound	Retention time (min)	Molecular formula $(M+H)^+$	Theoretical *m/z* of $(M+H)^+$	Experimental *m/z*
3	$C_{18:1}$-Chl	11.8	$C_{45}H_{79}O_2$	651.6	651.3
4	$C_{20:1}$-Chl	13.4	$C_{47}H_{83}O_2$	679.6	679.3
6	$C_{20:0}$-Chl	15.3	$C_{47}H_{85}O_2$	681.6	681.3
6	$C_{22:1}$-Chl	15.2	$C_{49}H_{87}O_2$	707.7	707.3
7	$C_{21:0}$-Chl	16.2	$C_{48}H_{87}O_2$	695.7	695.3
8	$C_{22:0}$-Chl	17.2	$C_{49}H_{89}O_2$	709.7	709.3
8	$C_{24:1}$-Chl	17.1	$C_{51}H_{91}O_2$	735.7	735.3
9	$C_{23:0}$-Chl	18.2	$C_{50}H_{91}O_2$	723.7	723.4
10	$C_{26:1}$-Chl	19.0	$C_{53}H_{95}O_2$	763.7	763.4
10	$C_{24:0}$-Chl	19.1	$C_{51}H_{93}O_2$	737.7	737.3
11	$C_{25:0}$-Chl	20.3	$C_{52}H_{95}O_2$	751.7	751.4
12	$C_{28:1}$-Chl	21.1	$C_{55}H_{99}O_2$	791.8	791.4
12	$C_{26:0}$-Chl	21.1	$C_{53}H_{97}O_2$	765.7	765.4
13	$C_{27:0}$-Chl	22.1	$C_{54}H_{99}O_2$	779.8	779.5
14	$C_{30:1}$-Chl	23.0	$C_{57}H_{103}O_2$	819.8	819.5
14	$C_{28:0}$-Chl	23.5	$C_{55}H_{101}O_2$	793.8	793.4
15	$C_{29:0}$-Chl	24.1	$C_{56}H_{103}O_2$	807.8	807.5
16	$C_{32:1}$-Chl	25.0	$C_{59}H_{107}O_2$	847.8	847.6
17	$C_{30:0}$-Chl	25.0	$C_{57}H_{111}O_2$	821.8	821.5
18	$C_{34:1}$-Chl (minor)	26.9	$C_{61}H_{111}O_2$	875.9	875.5
	$C_{36:1}$-Chl and above	Not detected	$C_{63}H_{115}O_2$	903.9	Not detected

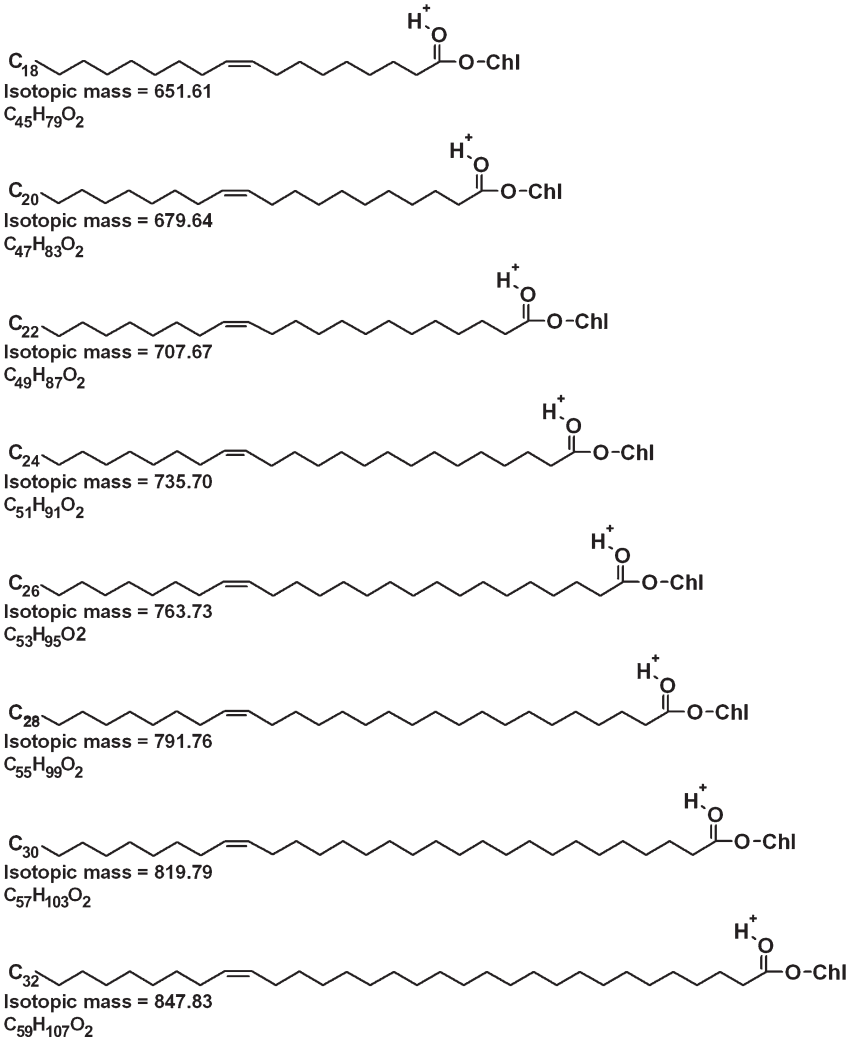
[a] All reported RT and *m/z* were determined from the same chromatogram.

With this information in hand, a more accurate structural evaluation of Chl-E present in human meibum became possible.

A side-by-side comparison of SIM chromatograms of human meibum samples (**Fig. 6**) with those of Chl-E standards (Fig. 3) showed that meibum contained noticeable amounts of compounds, which coeluted with very long chain $C_{24:1}$-Chl and $C_{22:1}$-Chl, while the shortest Chl-E detected in appreciable quantities in meibum was tentatively identified as $C_{18:1}$-Chl. To determine whether the natural Chl-E in meibum were in fact $C_{18:1}$-, $C_{22:1}$-, and $C_{24:1}$-Chl, the meibum was analyzed for the presence of the corresponding proton adduct ions *m/z* 651, 707, and 735. To stabilize $(M+H)^+$ ions, the vaporization and capillary temperatures were maintained at 250°C. Those ions were indeed detected in all of the tested samples. To find if meibum had other Chl-E, we computed theoretical *m/z* values for a range of Chl-E with C_{16} to C_{36} LCFA and VLCFA as acyl components (**Table 2**). The RT for those hypothetical compounds were estimated using Equation 1. Many of such saturated and monounsaturated compounds were found to be present in meibum (Figs. 1 and 6) (Table 2) (**Scheme 3**). The identification of compounds was performed on the basis of their *m/z* values, fragmentation patterns, and RTs. For instance, ion *m/z* 763 with RT ~19 min when fragmented at a normalized collision energy of 38%, produced fragments *m/z* 369 and 651, which were consistent with a $C_{26:1}$-Chl. Other parent ions were analyzed in a similar fashion. The longest Chl-E detected in the meibum samples was a $C_{34:1}$-Chl.

When plotted using equation 1, the data from Table 2 produced regression coefficients $k_1 = 4.26$, $k_2 = 0.273$, and $k_3 = 0.014$, respectively ($r^2 = 0.995$). Note that the RT for each of the compounds in Table 2 can be, and were, determined from one and the same chromatogram. The elution pattern of the meibum compounds followed the same rule as the authentic Chl-E. Thus, it provided an additional evidence that all of the compounds from Table 2 are indeed saturated and monounsaturated Chl-E.

Further analysis of the meibum (**Fig. 7**) revealed a group of even slower moving (thus, more hydrophobic) Chl-E-like compounds than the VLC-Chl-E discussed above.



Scheme 3. Proposed structures of major unsaturated cholesteryl esters in human meibum.

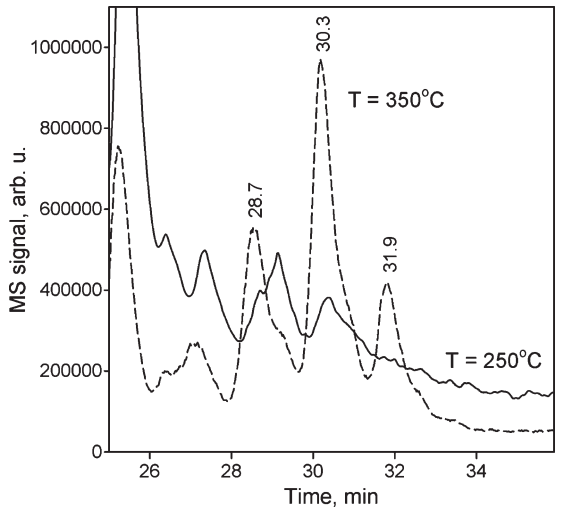


Fig. 7. Partial chromatogram of meibum recorded SIM experiment (*m/z* 369) at vaporization and capillary temperatures of 250°C (solid line) and 350°C (broken line).

Their RT were approximately 29, 30, and 32 min. Note that these compounds became visible only after the vaporization temperature of the APCI ion source was increased to 350°C vs. 250°C (compare Fig. 7 with Figs. 1 and 6). All those unidentified Chl-E-like compounds spontaneously produced ion *m/z* 369, which fragmented exactly as free Chl and standard Chl-E in MS2 experiments did. Their long RT would require Chl to be esterified to VLCFA with more than 34 carbon atoms. However, after computing hypothetical Chl-E structures and their corresponding *m/z* values, no such $(M+H)^+$ ions above the noise level were detected in human meibum.

As the characteristic MS peak of any Chl-E (*m/z* 369) was constantly one of the major ions in any of the tested meibum samples (25, 26), its quantitation was necessary to expand our views on the actual composition of human meibum. The most aggravating factors in such an undertaking were: *1*) absence of commercial VLC-Chl-E standards with fatty acids longer than C_{24}; and *2*) relatively low stability of Chl-E $(M+H)^+$ and $(M+NH_4)^+$ ions under the conditions of APCI MS analysis. Thus, an alternative approach to the sample quantitation had to be developed. Considering that all tested Chl-E were easily releasing their characteristic ion *m/z* 369, this was used in an attempt to quantify its precursors.

An equimolar mixture of several authentic Chl-E and free Chl (100 μM each) was prepared and analyzed by RP HPLC-MS as described above. Conveniently, all tested Chl-E produced HPLC peaks of the same size: after integration, their relative areas were astonishingly close to each other at 15.1 ± 1.4% (mean ± SD) with free Chl being approximately 7% of the TIC (all peaks being 100%) (Fig. 3).

These numbers were similar for four different detection techniques implemented in the experiment: *1*) recording TIC with subsequent extraction and plotting of ion *m/z* 369 spontaneously generated in-source in an MS1 experiment [$(Chl\text{-}E+H)^+ \rightarrow$ *m/z* 369)]; *2*) recording a chromatogram of ion *m/z* 369 in a SIM MS1 experiment; *3*) recording a TIC of all product ions of ion *m/z* 369 in a single reaction monitoring (SRM) MS2 experiment (*m/z* 369@38% → Σ product ions); and *4*) extracting and plotting a signal of a product ion (e.g., *m/z* 243.2) in a SRM MS2 experiment (*m/z* 369@38% → 243) (all shown in **Fig. 8**). Therefore, neither the length, nor the degree of saturation of a fatty acid chain of a Chl-E had a noticeable effect on the intensity of the generated characteristic ion *m/z* 369 or its products. As standard Chl-E of different structures taken at equimolar concentrations produced MS and HPLC peaks of very similar (if not identical) intensities, this allowed us to determine the ratio of Chl-E of human meibum by comparing relative intensities of their *m/z* 369 signals.

The next step was to generate a standard curve for Chl-E and free Chl. As an example, three types of standard curves for $C_{24:1}$-Chl are shown in **Fig. 9**. Curve 1 was obtained using $(M+H)^+$ adduct to monitor the compound. Curve

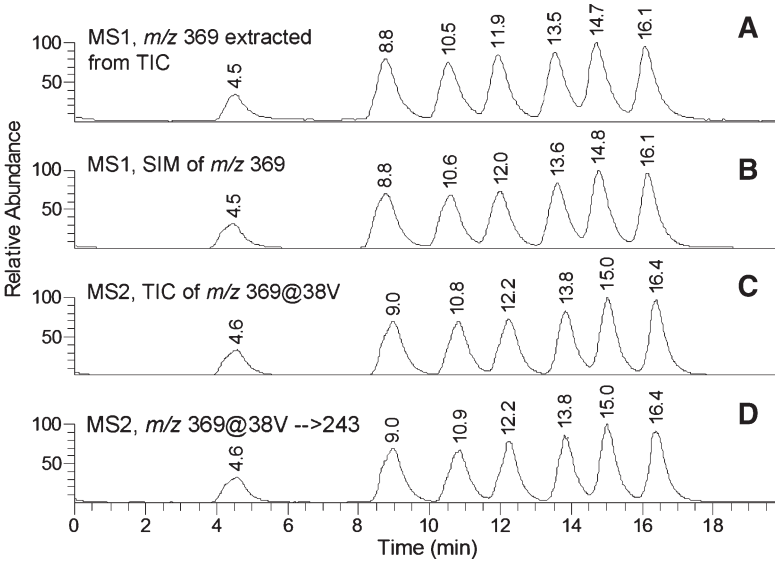


Fig. 8. Comparison of four analytical techniques proposed for analysis of cholesteryl esters by HPLC APCI MS in the positive ion mode. A: Chromatogram of ion *m/z* 369 extracted from a TIC in an MS1 experiment; B: SIM of ion *m/z* 369 in an MS1 experiment; C: monitoring of (*m/z* 369@38% → Σ all product ions) in a single reaction monitoring (SRM) MS2 experiment; D: SRM of transition *m/z* 369@38% → 243.

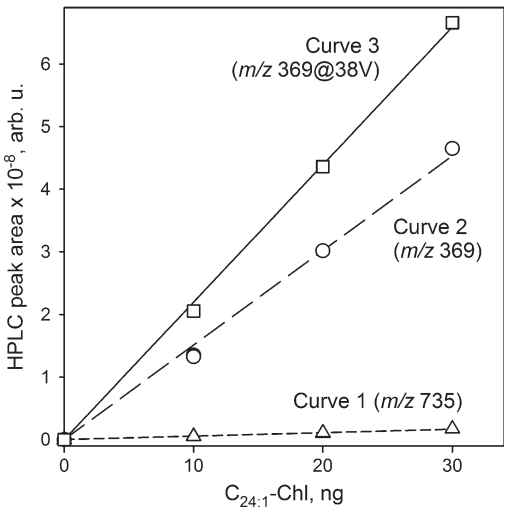


Fig. 9. Standard curves for determination of cholesteryl nervonate using HPLC APCI MS. The measured intensities of the following analytical ions were used to quantify the compound: proton adduct of the compound $(M + H)^+$, *m/z* 735 (triangles, Curve 1); fragment ion *m/z* 369, spontaneously generated in-source and extracted from TIC (circles, Curve 2); fragment ion *m/z* 369, spontaneously generated in-source and monitored in a SIM experiment (squares, Curve 3).

2 was built using the spontaneously in-source-generated characteristic fragment ion *m/z* 369, while curve 3 was obtained by monitoring ion *m/z* 369 in an MS1 experiment and integrating the resulting HPLC peaks. Among the three, the last chart showed the highest magnitude and the highest degree of linearity with $r^2 > 0.999$. Thus, this was used to quantify $C_{24:1}$-Chl in human meibum. The apparent $C_{24:1}$-Chl to dry meibum weight ratio was found to be approximately 3.1%, which puts this particular compound on the list of major meibum constituents. Similar experiments were performed to quantify some other Chl-E and free Chl of human meibum. Based on these data, to estimate the weight ratio of other Chl-E, it seemed to be sufficient to integrate HPLC traces obtained by monitoring ion *m/z* 369 in an MS1 SIM experiment (**Fig. 10A, B**). Moreover, because of the high degree of symmetricity of the HPLC peaks of standard Chl-E, their heights (H) have been found to strongly correlate with the peaks' areas (A) (Fig. 10C). Thus, either parameter (H or A) could be used to estimate the molar ratio of Chl-E in meibum.

As *1*) saturated Chl-E gave very weak signals of $(M+H)^+$, $(M+NH_4)^+$, and/or $(M+K)^+$; *2*) all tested standard Chl-E in the tested range of standard Chl-E from $C_{11:0}$ to $C_{24:1}$ showed virtually identical efficacy in spontaneous production of ion *m/z* 369 in MS1 experiments; and *3*) many of the necessary Chl-E standards are currently unavailable from any commercial source, monitoring of ion *m/z* 369 in MS1 experiments or its products in MS2 experiments looked like a reasonable, if not the only, choice. The noted absence of Chl-E standards with VLCFA above C_{24} makes it impossible to check the efficiency of ionization of longer-chain Chl-E. However, it looks reasonable to assume that if the efficiency did not change for the tested standards $C_{11:0}$–$C_{24:1}$, it will remain relatively unchanged for compounds with VLCFA in the range of C_{24}–C_{32}. This should be verified in the future when longer-chain Chl-E standards become available.

The apparent composition of human Meibomian Chl-E and free Chl estimated as shown in Figs. 9–**11** is presented in **Table 3**. Routinely, there were more than 20 individual Chl-E species detected. Their molar ratios were calculated from SIM chromatograms (Figs. 1B, 2, 11) assuming that each individual Chl-E species produced ion *m/z* 369 with similar (if not identical) efficiency (Figs. 8 and 10). Then, the actual mass ratio of $C_{24:1}$-Chl in human meibum was calculated using meibum preparations and the calibration curve presented in Fig. 10B. This gave us a reference point to calculate the mass ratios of other Chl-E found in meibum by using their molar ratios to $C_{24:1}$-Chl-E (Table 3). The same procedure was repeated with other authentic $C_{18:1}$-Chl, $C_{18:0}$-Chl, and $C_{22:1}$-Chl-E with a similar outcome. However, considering that $C_{24:1}$-Chl-E was

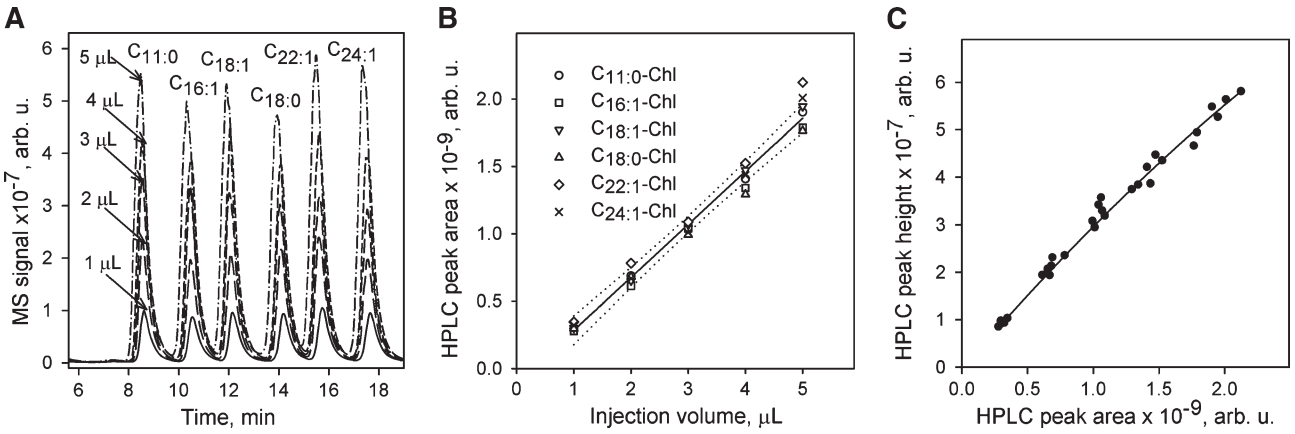


Fig. 10. Quantitative analysis of standard cholesteryl esters using HPLC APCI MS. A: HPLC analyses of a mixture of six authentic cholesteryl esters (100 µM each; detection of ion *m/z* 369. B: A single standard curve for all six model cholesteryl esters. Confidence interval 95% is shown (dotted line). All 30 data points (six esters, five concentrations) were used to compute the standard curve and the confidence interval. C: A correlation between peak areas and peak heights for six standard cholesteryl esters.

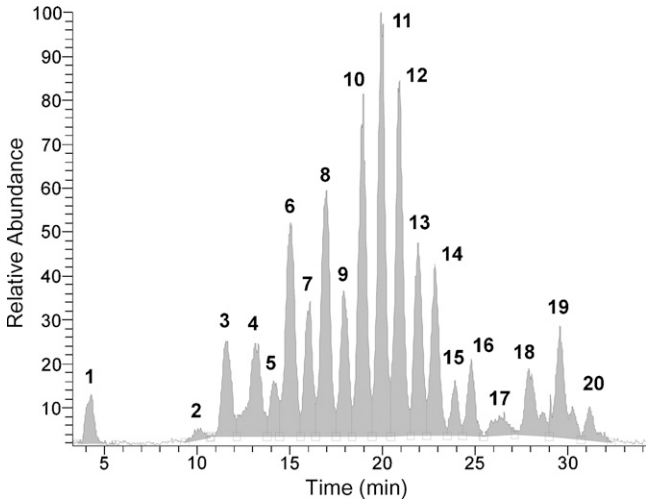


Fig. 11. Determination of molar percentage of individual Chl-E components of human meibum in a SIM experiment. Analytical ion: *m/z* 369. Individual HPLC peaks 1 to 20 were integrated using Xcalibur's built-in Avalon routine.

the closest of the standard Chl-E to being an "average" meibomian Chl-E, the data presented in Table 3 are based on its absolute quantitation in meibum. Samples collected from five healthy volunteers were analyzed 3 to 9 times each. The detected unsaturated Chl-E had predominantly C22:1 to C32:1 chains. An interesting compound was $C_{25:0}$-Chl, which produced a large peak with RT ~20 min (Table 3). Note that some of the HPLC peaks in Figs. 1, 2, and 11 are formed by two (or more) coeluting compounds. The overall content of Chl-E in dry meibum was found to be very close to the earlier reported ~31% (w/w) (7).

DISCUSSION

Complete lipidomic analysis of human meibomian gland secretions is a challenging task because of the small size of samples and the great diversity of the meibomian lipid species. Unlike other "omics" where researchers can count on a few fairly universal and standardized techniques (e.g., protein and gene sequencing), those working in lipidomics cannot. The lipids are too diverse chemically to be analyzed by using just one routine, and a cautious multi-step, approach to the lipid analyses needs to be taken. Currently, HPLC-MS is the most powerful tool in lipidomic analysis. It combines high sensitivity, high selectivity, and the ability to perform structural analysis of the compounds in MS/MS and MS^n-capable instruments. Compared with plain MS, HPLC-MS offers an additional analytical tool, determination of the RT of analytes, which greatly increases the chances of correct identification of unknowns by helping to separate isobaric compounds that are abundantly present in biological samples. Therefore, we utilized HPLC-MS^n to analyze human meibum for the presence of Chl-E and to elucidate their structures.

Chl-E were found in every single sample analyzed so far regardless whether it was a normal donor or a dry eye patient. This contradicts an earlier report on the complete absence of Chl-E in meibum of certain normal donors (13). Our observation is also different from an earlier paper where no traces of Chl-E could be found in the presented data (31). However, our current observations are in line with other publications where Chl-E were reported to be the major components of human meibum (3, 5, 7, 11, 12, 16–20). This has also been predicted in our recent publications on the topic (25, 26). Yet, structural characterization of meibomian Chl-E had not been

TABLE 3. Estimated qualitative and quantitative composition of Chl-E found in normal human meibum

Compound	Molar ratio in meibum of 5 normal volunteers[a] Median ± SD	Mass ratio (mg/mg Chl-E fraction)[b]	Mass ratio (mg/mg dry meibum)[b]
Chl	1.2 ± 0.3	0.005	0.002
Unknown	0.4 ± 0.1	0.003	0.001
$C_{18:1}$-Chl	4.8 ± 1.4	0.026	0.008
$C_{20:1}$-Chl	3.1 ± 0.7	0.025	0.009
Unknown	1.9 ± 0.6	0.009	0.003
$C_{22:1}$-Chl + $C_{20:0}$-Chl	7.7 ± 1.8	0.073	0.023
$C_{21:0}$-Chl	4.0 ± 1.0	0.036	0.011
$C_{24:1}$-Chl + $C_{22:0}$-Chl	10.0 ± 0.8	0.11	0.034
$C_{23:0}$-Chl	3.7 ± 0.6	0.04	0.012
$C_{26:1}$-Chl + $C_{24:0}$-Chl	13.1 ± 0.7	0.136	0.043
$C_{25:0}$-Chl	12.9 ± 1.4	0.127	0.039
$C_{28:1}$-Chl + $C_{26:0}$-Chl	15.1 ± 1.3	0.164	0.051
$C_{27:0}$-Chl	7.0 ± 0.9	0.068	0.021
$C_{30:1}$-Chl + $C_{28:0}$-Chl	7.1 ± 1.0	0.083	0.026
$C_{29:0}$-Chl	1.4 ± 0.5	0.025	0.008
$C_{32:1}$-Chl + $C_{30:0}$-Chl	2.4 ± 1.2	0.054	0.017
$C_{34:1}$-Chl	0.7 ± 0.4	0.016	0.005
Unknown	1.6 ± 0.7	n/a	n/a
Unknown	1.7 ± 0.3	n/a	n/a
Total	~100	1	0.313

[a] Determined from individual chromatograms by using HPLC-MS at vaporization/capillary temperature of 250°C. Each sample was analyzed 3 to 9 times.

[b] Determined by using standard curves for $C_{18:1}$-Chl, $C_{18:0}$-Chl, $C_{22:1}$-Chl, and $C_{24:1}$-Chl and the molar ratios from this table.

performed at the time. In this paper, for the first time we present results of structural elucidation and quantitation of major Chl-E species found in human meibum.

There were more than 20 distinctively different Chl-E species detected in human meibum (Figs. 1, 2, and 6) (Tables 2 and 3). The shortest positively identified Chl-E was $C_{18:1}$-Chl, while the longest was $C_{34:1}$-Chl. The most abundant species were $C_{22:1}$-Chl to $C_{30:1}$-Chl, with $C_{26:1}$-Chl, $C_{25:0}$-Chl and $C_{28:1}$-Chl being on the top of the list. We assume that most (if not all) of the intact unsaturated Chl-E posses *cis*-double bonds as the *trans*-isomers are typically associated with oxidation or temperature-induced isomerization of natural *cis*-isomers. The plots have virtually identical slopes for the Chl-E with 11 to 24 carbons in their fatty acid residues ($k_1 = 4.4$; $k_2 = 0.2695$; $k_3 = 0.0143$) for saturated compounds and ($k_1 = 4.4$; $k_2 = 0.2587$; $k_3 = 0.0144$) for monounsaturated ones. Therefore, equation 1 is useful in determination of approximate RT of unknown or expected Chl-E and to reject false isobaric positives, which are unlikely to fit the equations.

Interestingly, at least half of the Chl-containing compounds detected in human meibum eluted later than the longest Chl-E standards, C_{22} and C_{24} Chl. However, for some of them (e.g., compounds eluting as HPLC peaks 19 and 20), no matching *m/z* signals that would be expected of Chl-E were found. This may be caused either by (expected) low stability of their $(M + H)^+$ and/or $(M + NH_4)^+$ ions, or by the different chemical nature of these compounds. Experiments are in progress to address this issue.

The chemical nature of identified Chl-E partly answers a question of which particular lipid class (or classes) the LCFA and VLCFA reported in a recent study by Joffre et al. (29, 30) were esterified into. In their study, the source of C_{20} to C_{26} fatty acids in human meibum remained unknown as the entire meibum samples were transmethylated immediately after collection in a reaction with methanolic boron trifluoride and then analyzed by GC-MS. This allowed the researchers to detect various methyl esters of LCFA and VLCFA, but precluded them from making a definite conclusion about their sources. According to our earlier data, WE could not be such a source as the vast majority of them were based on a $C_{18:1}$ (oleic) acid, followed by $C_{18:0}$ and $C_{18:2}$-based WE (25, 26). Neither such source could be di- or triacyl glycerols, which in the same studies were found to be far less prominent components of human meibum than WE and Chl-E. Phospholipids, ceramides, monoacyl glycerols, and alike were detected in even lesser concentrations (if any). Therefore, they could not be a major source of the LCFA and VLCFA either. Our current findings show that such a source is Chl-E. Interestingly, our data show that the longest fatty acid detected in Chl-E of human meibum has up to 34 carbons, far longer than 26 carbons reported by Joffre et al. (29, 30) and, possibly, even longer than that if the Chl-containing HPLC peaks 19 and 20 are indeed Chl-E. Also, a large portion of the LCFA and VLCFA were monounsaturated. This discrepancy has most likely to do with the well-known fact that long chain fatty acids are unstable under the conditions of GC-MS analysis and quickly decompose at high temperatures at which the GC columns typically operate. This especially concerns unsaturated LCFA and VLCFA, which are even less stable than the saturated ones. The VLCFA also suffer from low volatility, which is inversely proportional to their length and molecular mass. Therefore, the notable absence of VLCFA beyond C_{26} for saturated compounds, and anything beyond $C_{18:1}$ for unsaturated VLCFA in the data by Joffre et al. (29, 30) can be related to these limitations of the GC-MS technique. The exact location and geometry of the double bonds in the structures of monounsaturated Chl-E from meibum remain to be investigated. However, we were able to match some of the meibomian Chl-E with authentic Chl-E standards (see previous discussion) and expect other compounds to follow the trend and be based on *n-9* monounsaturated fatty acids.

The presence of large quantities of VLC Chl-E in meibum may have important implications in the physiology and biophysics of the ocular surface and tear film as these compounds are extremely hydrophobic and create self-assembling liquid-crystal-like structures whose properties can be modulated in a broad range by mixing with each other and with other lipid molecules and ions (33). Therefore, qualitative and quantitative characterization of Chl-E in meibum seems to be a critical step in determining how tear film is organized and functions.

It is noteworthy to mention that neither individual WE, described in our recent reports on the topic (25, 26), nor Chl-E studied in this paper, had been identified and reported in earlier publications on the topic. This emphasizes a necessity of choosing a proper technique in separating, detecting, and analyzing lipids that are tremendously diverse in their chemical and physical properties. As WE and Chl-E combined represent ~60% of meibum (7), this vast pool of lipids must be taken into consideration while designing a model of tear film and TFLL.

The analyses described are not intended to cover all species of Chl-E present in meibum in all the details, much less so other steroid-based compounds, which may apparently be there. However, this paper presents compelling evidence that among various lipid classes tested so far, Chl-E are the most likely and prominent depot for monounsaturated LCFA and VLCFA. This needs to be taken into account when studying biochemistry and biophysics of tear film and TFLL.

REFERENCES

1. Pes, O. 1897. Ricerche microchimiche sulla secrezione delle ghiandole sebacee palpebrali. *Arch. Ottal.* **5:** 82–91.
2. Linton, R. G., D. H. Curnow, and W. J. Riley. 1961. The meibomian glands: an investigation into the secretion and some aspects of the physiology. *Br. J. Ophthalmol.* **45:** 718–723.
3. Nicolaides, N. 1965. Skin lipids. II. Lipid class composition of samples from various species and anatomical sites. *J. Am. Oil Chem. Soc.* **42:** 691–702.
4. Andrews, J. S. 1970. Human tear film lipids. I. Composition of the principal non-polar component. *Exp. Eye Res.* **10:** 223–227.
5. Cory, C. C., W. Hinks, J. L. Burton, and S. Shuster. 1973. Meibomian gland secretion in the red eyes of rosacea. *Br. J. Dermatol.* **89:** 25–27.

6. Tiffany, J. M. 1978. Individual variations in human meibomian lipid composition. *Exp. Eye Res.* **27:** 289–300.
7. Nicolaides, N., J. K. Kaitaranta, T. N. Rawdah, J. I. Macy, F. M. Boswell 3rd, and R. E. Smith. 1981. Meibomian gland studies: comparison of steer and human lipids. *Invest. Ophthalmol. Vis. Sci.* **20:** 522–536.
8. Nicolaides, N., and E. C. Ruth. 1982–1983. Unusual fatty acids in the lipids of steer and human meibomian gland excreta. *Curr. Eye Res.* **2:** 93–98.
9. McCulley, J. P., J. M. Dougherty, and D. G. Deneau. 1982. Classification of chronic blepharitis. *Ophthalmology.* **89:** 1173–1180.
10. Dougherty, J. M., and J. P. McCulley. 1986. Analysis of the free fatty acid component of meibomian secretions in chronic blepharitis. *Invest. Ophthalmol. Vis. Sci.* **27:** 52–56.
11. Harvey, D. J., J. M. Tiffany, J. M. Duerden, K. S. Pandher, and L. S. Mengher. 1987. Identification by combined gas chromatography-mass spectrometry of constituent long-chain fatty acids and alcohols from the meibomian glands of the rat and a comparison with human meibomian lipids. *J. Chromatogr.* **414:** 253–263.
12. Osgood, J. K., J. M. Dougherty, and J. P. McCulley. 1989. The role of wax and sterol esters of meibomian secretions in chronic blepharitis. *Invest. Ophthalmol. Vis. Sci.* **30:** 1958–1961.
13. Shine, W. E., and J. P. McCulley. 1991. The role of cholesterol in chronic blepharitis. *Invest. Ophthalmol. Vis. Sci.* **32:** 2272–2280.
14. Shine, W. E., and J. P. McCulley. 1996. Meibomian gland triglyceride fatty acid differences in chronic blepharitis patients. *Cornea.* **15:** 340–346.
15. Shine, W. E., and J. P. McCulley. 1998. Keratoconjunctivitis sicca associated with meibomian secretion polar lipid abnormality. *Arch. Ophthalmol.* **116:** 849–852.
16. Krenzer, K. L., M. R. Dana, M. D. Ullman, J. M. Cermak, D. B. Tolls, J. E. Evans, and D. A. Sullivan. 2000. Effect of androgen deficiency on the human meibomian gland and ocular surface. *J. Clin. Endocrinol. Metab.* **85:** 4874–4882.
17. Sullivan, B. D., J. E. Evans, K. L. Krenzer, M. Reza Dana, and D. A. Sullivan. 2000. Impact of antiandrogen treatment on the fatty acid profile of neutral lipids in human meibomian gland secretions. *J. Clin. Endocrinol. Metab.* **85:** 4866–4873.
18. Shine, W. E., and J. P. McCulley. 2000. Association of meibum oleic acid with meibomian seborrhea. *Cornea.* **19:** 72–74.
19. Sullivan, B. D., J. E. Evans, J. M. Cermak, K. L. Krenzer, M. R. Dana, and D. A. Sullivan. 2002. Complete androgen insensitivity syndrome: effect on human meibomian gland secretions. *Arch. Ophthalmol.* **120:** 1689–1699.
20. Shine, W. E., J. P. McCulley, and A. G. Pandya. 2003. Minocycline effect on meibomian gland lipids in meibomianitis patients. *Exp. Eye Res.* **76:** 417–420.
21. Shine, W. E., and J. P. McCulley. 2003. Polar lipids in human meibomian gland secretions. *Curr. Eye Res.* **26:** 89–94.
22. Shine, W. E., and J. P. McCulley. 2004. Meibomianitis: polar lipid abnormalities. *Cornea.* **23:** 781–783.
23. Nichols, K. K., B. M. Ham, J. J. Nichols, C. Ziegler, and K. B. Green-Church. 2007. Identification of fatty acids and fatty acid amides in human meibomian gland secretions. *Invest. Ophthalmol. Vis. Sci.* **48:** 34–39.
24. Butovich, I. A., T. J. Millar, and B. M. Ham. 2008. Understanding and analyzing meibomian lipids – a review. *Curr. Eye Res.* **33:** 405–420.
25. Butovich, I. A., E. Uchiyama, M. A. Di Pascuale, and J. P. McCulley. 2007. Liquid chromatography-mass spectrometric analysis of lipids present in human meibomian gland secretions. *Lipids.* **242:** 765–776.
26. Butovich, I. A., E. Uchiyama, and J. P. McCulley. 2007. Lipids of human meibum: mass-spectrometric analysis and structural elucidation. *J. Lipid Res.* **48:** 2220–2235.
27. Borchman, D., G. N. Foulks, M. C. Yappert, and D. V. Ho. 2007. Temperature-induced conformational changes in human tearlipids hydrocarbon chains. *Biopolymers.* **87:** 124–133.
28. Borchman, D., G. N. Foulks, M. C. Yappert, D. Tang, and D. V. Ho. 2007. Spectroscopic evaluation of human tear lipids. *Chem. Phys. Lipids.* **147:** 87–102.
29. Joffre, C., M. Souchier, S. Grégoire, S. Viau, L. Bretillon, N. Acar, A. M. Bron, and C. Creuzot-Garcher. 2008. Differences in meibomian fatty acid composition in patients with meibomian gland dysfunction and aqueous-deficient dry eye. *Br. J. Ophthalmol.* **92:** 116–119.
30. Souchier, M., C. Joffre, S. Grégoire, L. Bretillon, A. Muselier, N. Acar, J. Beynat, A. Bron, P. D'Athis, and C. Creuzot-Garcher. 2008. Changes in meibomian fatty acids and clinical signs in patients with meibomian gland dysfunction after minocycline treatment. *Br. J. Ophthalmol.* **92:** 819–822.
31. Ham, B. M., J. T. Jacob, M. M. Keese, and R. B. Cole. 2004. Identification, quantification and comparison of major non-polar lipids in normal and dry eye tear lipidomes by electrospray tandem mass spectrometry. *J. Mass Spectrom.* **39:** 1321–1336.
32. Snow, J. W., J. M. Glick, and M. C. Phillips. 1992. The phase behavior of cholesteryl esters in intracellular inclusions. *J. Biol. Chem.* **267:** 18564–18572.
33. Ginsburg, G. S., D. Atkinson, and D. M. Small. 1984. Physical properties of cholesteryl esters. *Prog. Lipid Res.* **23:** 135–167.