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The Spectrophotometric Sulfo-Phospho-Vanillin Assessment of Total Lipids in Human Meibomian Gland Secretions

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Abstract

Human meibomian gland secretions (meibum) are the major lipid component of the human preocular tear film. The predominant lipid classes found in meibum include waxes (WE), cholesteryl esters (CE), and varying amounts of cholesterol (Chl). The classical sulfo-phospho-vanillin assay (SPVA), adapted for a microplate reader, was used to quantitate lipids in meibum. To account for varying reactivities of different lipids in SPVA, a model meibomian lipid mixture (MMx) that approximated the WE/CE/Chl composition of meibum was developed and used to quantitate meibomian lipids. The overall SPV responses of MMx and meibum were found to be close, with similar intermediate and final reaction products for both. Saturated WE that had not been expected to be reactive were found to be SPV-positive. A reaction mechanism for these compounds in SPVA which involves the formation of alkenyl ethers is proposed and discussed. Tested proteins were non-reactive in SPVA. Thus, by comparing the results of gravimetric analyses of meibum samples with the results of a properly calibrated SPVA, it was estimated that the SPV-reactive lipid content of dry meibum in tested samples was about 78 % (w/w). The SPV method can also be adopted for analyzing other types of complex lipids secretions, such as sebum, as well as whole lipid extracts from other lipid-enriched organs and tissues, if proper standards are chosen.

Keywords

Alkenyl ether; Carbocation; Meibum; Quantitation; Saturated wax esters

Introduction

The exposed outer surfaces of the eye (such as cornea and conjunctiva) are protected from desiccation and the external environment by a dynamic and continuously renewed multilayered tear film (TF) composed of water, carbohydrates, proteins, lipids and inorganic

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salts [1–3]. Lipids, which comprise the largest component of the thin outermost layer of the TF—the tear film lipid layer (TFLL)—are secreted onto the ocular surface predominantly from the meibomian glands (MG) located in both the upper and lower eyelids [4]. Alterations in the composition and/or function of the TFLL as a consequence of changes in the MG function have been implicate as having a pathological involvement in the development and/or progression of the most prevalent form of a common human condition referred to as dry eye (DE) [5, 6].

Toward understanding the pathological involvement of the MG in DE, extensive studies have been devoted to detailing changes in the anatomical presentation of the MG [7, 8], qualitative changes in the appearance and expressibility of meibum [9], as well as efforts to provide quantitative estimates of total meibum secretion and of the quantities of specific lipids present in expressed meibum [10–13]. Human meibum contains a complex mixture of hundreds to, potentially, thousands of individual lipid species derived from multiple chemically distinct classes of lipids. Qualitative analyses have characterized about 40 % of human meibum lipids as wax esters (WE), 30 % as cholesteryl esters (CE), with the remainder including (*O*-acyl)-omega-hydroxy fatty acids (OAHFA) and their cholesteryl esters, free fatty acids (FFA, less than 1 %) and cholesterol (Chl, between 0.1 and 0.5 %), triacylglycerols (TAG) and, possibly, diacylglycerols (DAG) [13–15]. Given the diverse chemical nature of the lipids in meibum, there is no analytical procedure analogous to e.g. protein assays, which can give simple accurate measurement of the total lipid pool. Most studies attempting quantitative analysis of meibum lipids have relied on gravimetric analysis of dried expressed meibum samples as an estimate of total sample lipids. In some studies authentic reference lipids, either commercially available or specifically synthesized, have allowed for accurate estimates to be made of selective lipid species. The availability of specific lipid reference standards is however limited [2], thus restricting much needed quantitative analysis of meibum lipids.

When it comes to evaluating (rather small) meibum samples, the current use of gravimetric analysis for estimation of total lipids suffers from two notable shortcomings: (1) collection of small samples increases the possibility of random errors in the estimation of sample weights, and (2) a (possibly) noticeable presence of non-lipid material in meibum, especially in samples collected from meibomian gland dysfunction patients [7], in which case the use of gravimetric analysis would constantly overestimate the starting weight of lipid material in such samples.

Any quantitative method for total meibum lipid analysis requires that it has sensitivity sufficient to allow for high throughput analyses of very small sample sizes composed of chemically very complex lipid mixtures. A thorough review of the literature identified the sulfo-phospho-vanillin (SPV) assay (SPVA) as a potentially useful method. The SPVA was initially reported as a method to estimate serum total lipids [16] and its chemical basis later investigated [17, 18]. The assay has recently been modified for use with small sized samples in a high throughput format using a microplate reader [19, 20]. The SPV reaction is performed in two steps, initial reaction of the lipids with concentrated (typically, ≥95 %) sulfuric acid at high temperature followed by a second reaction of the derived products with vanillin in the presence of phosphoric acid. Consensus understanding is that a positive SPV reaction requires the presence of double bonds or free hydroxyl groups within the lipid analytes [17, 18]. The chemical reactions are complex and are thought to involve formation of relatively stable (up to several hours [17]) carbonium ion (or carbocation) chromogen in the initial reaction followed by generation of a pink chromophore upon addition of vanillin to the reaction [17–19, 21].

The goal of this study was to evaluate the utility of the SPV reaction as a method to quantitate total meibum lipids in human clinical samples.

Materials and Methods

Reagents

Individual lipid standards were purchased from Avanti Polar Lipids Inc. (Alabaster, Al, USA), Nu-Chek Prep Inc. (Elysian, MN, USA), and from Sigma-Aldrich (St. Louis, MO, USA). The hydrocarbon heptadecane (HD), protein standards [fat free bovine serum albumin (BSA) and lysozyme], mucin isolated from bovine submaxillary gland (product M3895) and vanillin were all from Sigma-Aldrich.

Preparation of Lipid Standard Solutions

Individual standard lipid stocks were prepared at 1 mg/ml in chloroform and stored at −20 °C. All lipids were handled using only glass or stainless steel and were stored in Teflon-covered glass vials. Mixtures of lipids containing defined ratios of specific lipids were assembled from the stored stocks, keeping the final concentration of lipid in all at 1 mg/ml. The choice of lipids used to assemble a model meibum mixture (MMx) was based on current understanding of the major lipid classes present in human meibum and taking into consideration the saturated/ unsaturated lipid distributions within each class, (see Table 1). The chemical composition of MMx chosen for this study was as follows: behenyl oleate (BO):behenyl stearate (BS):cholesteryl stearate (Chl-S):cholesteryl oleate (Chl-O):Chl = 0.42:0.08:0.40:0.10:0.01 (by weight).

Meibum Collection

Samples of human meibum were collected by soft expression from healthy volunteers (5 female and 2 male samples, age range 30–56 years), as previously described [22]. All human sample collection procedures were approved by the UT Southwestern Institutional Review Board and were conducted in accordance with the Declaration of Helsinki. Dry meibum samples, analyzed gravimetrically, were used to prepare 1 mg/ml sample stock solutions in chloroform/ methanol (2:1, by vol).

Sulfo-Phospho-Vanillin Assay

Defined aliquots of meibum or standard lipids were evaporated to dryness and incubated with 120 μl of 95 % sulfuric acid at 95 °C for 20 min in individual glass tubes. Then, the reaction mixtures were rapidly cooled by placing the tube rack on an ice pack, the tubes contents were vortexed and a 100-μl aliquot transferred to individual wells of a 96-well glass plate (Zinsser Analytic GmbH, Germany; purchased through Aldrich, Milwaukee, WI, USA; catalog number Z40644-9). Initial pre-vanillin absorbance was measured using a Beckman Coulter DTX880 Multimode Detector (Beckman Coulter, Indianapolis, IN, USA) and a 535-nm filter. This was followed by addition of 50 μl of 0.2 mg/ml vanillin in 17 % aqueous phosphoric acid, incubation at room temperature for 10 min in the dark and recording of a post-vanillin chromophore absorbance at the same wavelength (A_{535}). The final SPV response of samples was defined as the difference between the final post-vanillin and pre-vanillin 535 nm absorbance readings. In assays examining the effects of proteins or mucins, these components were added to the dry lipid samples before addition of sulfuric acid.

Spectrophotometric UV-Vis Scans

Experiments were performed using a Beckman Coulter DU800 scanning UV-Vis spectrophotometer. The SPVA reaction volumes were scaled up to 1 ml and 50 μg of lipid

and their UV-Vis absorption spectra were recorded from 200 to 700 nm. The formation of lipid chromophores generated after both the sulfuric acid (λ_{max} around 300 nm) and final SPV reaction (λ_{max} 530 ±10 nm) steps was observed and recorded. The differential spectra of pre- and post-vanillin mixtures revealed the actual UV-Vis chromophores generated from lipids in the SPVA.

Results

Spectrophotometric Evaluation of SPVA Products

The SPV reaction is performed in two steps and to derive an understanding of the different responses of individual lipid analytes in the assay, pre-vanillin lipid chromophore intermediates generated in the sulfuric acid incubation, as well as the final vanillin chromophores were analyzed by recording changes in their UV-Vis spectra. Generation of pre-vanillin intermediates requires heat but once cooled, such intermediates are stable in the absence of water [18] and can be characterized spectrophotometrically. The UV-Vis scans obtained for all WE treated with 95 % sulfuric acid showed a prominent absorption peak around 300 nm (Fig. 1a, data for only two WE are shown—BS and BO). This absorption maximum has previously been reported for other SPV responsive molecules [18].

A similar A_{300} absorption maximum was also present in the pre-vanillin spectra of all other SPV positive lipids examined in this study (Fig. 1), including a saturated aliphatic fatty alcohol 1-octadecanol (1-OD), Chl, and Chl-O. Where the spectra from different classes of lipids differed was in the presence of minor peaks with maxima above 300 nm and, sometimes, in the elevated baselines of the spectra between 400 and 600 nm (Chl, Chl-O, and MMx). In addition, when the latter were visually inspected after this step, they were observed to contain brown particulate material which readily dispersed with shaking, or in some cases showed the presence of a brown layer overlaying the solution, a layer which also readily dispersed. Such coloration was not observed with other types of standard lipids. Exclusion of oxygen from the reaction mixture by flushing it with nitrogen did not influence the generation of such material, nor did sonication of samples during the course of the incubation. It appeared that Chl-containing compounds easily charred during the incubation with 95 % sulfuric acid. All Chl-containing analytes showed evidence of such charring after incubation in sulfuric acid. In tubes containing only free Chl, the charring initially became evident even prior to initiation of the heating step. Also, the intensity of charring was related to total Chl +CE levels and thus all lipid mixtures containing Chl or CE showed some charring. This included the MMx reference mixture assembled for this study and all human meibum samples. Thus, to arrive at a true vanillin chromophore-mediated response for all individual lipids and lipid mixtures, the pre-vanillin A_{535} values had to be subtracted from the corresponding final post-vanillin A_{535} readings.

The added chemical complexity of the Chl reactions can also be seen in the UV-Vis absorption spectra of reaction intermediates which showed both a major maximum around λ 320 nm (Fig. 1b) and minor maxima at λ 385 and 420 nm, which were not observed in the spectra of non-Chl-containing lipids. While these additional maxima have been reported previously for Chl intermediates [18], no mention was made of an elevated baseline between 400 and 600 nm, though it certainly could affect quantitation of lipids.

Differential UV-Vis spectra of the final SPV-lipid products showed a broad common absorption maximum with λ_{max} 530 ± 10 nm for all classes of SPV positive lipids (Fig. 2), this despite their varied chemical nature. Notably, both saturated and unsaturated WE produced clear absorption maxima at 300 nm for intermediates (Fig. 1a) and at 530 ± 10 nm for the final products (Fig. 2a), and a concentration-dependent increase in their A_{535} values in the SPVA (see below). Intriguingly, such a maximum was virtually absent when the

reactions with related compounds—stearic acid (STA) and tristearin (TS)—were performed. In the case of TS, a large number of small insoluble particles formed. The latter, apparently, led to the observed increase in the baseline (Fig. 2a) likely due to the increased scattering of light on the particles. However, color development was minimal in these samples as reflected by a very weak absorption, or non-existent, maximum at 535 nm. Other SPV-positive classes of lipids also yielding A_{535} maxima in differential spectra were cholesteryl-containing lipids (Fig. 2b), saturated fatty alcohols [e.g. 1-OD (Fig. 2a)], and unsaturated TAG (e.g. TO, not shown)—the most abundant TAG reported in normal human meibum [23]. Saturated hydrocarbon HD (Fig. 2a) was un-reactive in the SPVA.

Evaluation of the SPVA for Lipid Standards Representative of Meibum Lipids

Meibum is a complex mixture of hundreds of individual lipids species which belong to many different lipid classes. To validate the SPVA for quantitation of meibum lipids, pure lipid standards that were representative of the major lipid classes found in human meibum and their mixtures were tested (Table 1).

The most prominent group of lipids in meibum is WE, which represent at least 40 % of the total lipids. The SPV response of WE was tested using the saturated BS and unsaturated BO. As expected, monounsaturated lipid BO was found to be highly reactive, showing a concentration-dependent increase in A_{535} (Fig. 3, upper panel). The observed increase was best fit with an empiric quadratic Eq. 1:

$$A_{535}=a+b\times[\text{Lipid amount},\mu\text{g}]+c\times[\text{Lipid amount},\mu\text{g}]^2. \quad (1)$$

Surprisingly, despite the absence of previously reported reactive groups in BS, the lipid was highly reactive in the assay. However, its response in the SPVA was somewhat lower than the response obtained for the matching levels of BO. A WE mixture of BO and BS (4:1, w/w), which mimicked the ratio of saturated to unsaturated WE in human meibum, gave an SPV response intermediate between BO and BS. In addition to BS, other saturated WE lipid standards examined were also SPV-positive [see, for example, stearyl stearate (SS)].

The second most abundant group of lipids in meibum is CE. Pure standards for CE containing both saturated and unsaturated FA residues, Chl-S and Chl-O respectively, were both responsive in the SPVA (Fig. 3, center panel). Both lipids showed a concentration-dependent increase in A_{535}. For a mixture of the two CE, Chl-O and Chl-S (1:4, w/w), reflecting the WE saturation/unsaturation ratio in human meibum, the SPV response was intermediate between that for the individual CE.

In addition to the Chl residues present in CE, meibum also contains low levels of free Chl [14, 15, 23]. Chl—a molecule with a double bond and a free hydroxyl group—also showed a concentration-dependent SPV response (not shown). However, the response of Chl differed significantly from that observed for CE, where its hydroxyl group was esterified to fatty acids.

Normal meibum also contains small amounts of TAG [23, 24], with oleic acid being the most abundant fatty acyl residue. A molecule of TO has three oleic acid residues. However, the observed increase in A_{535} did not differ much from that observed for other oleic acid-containing lipids with just one oleic acid residue in their structures.

Typical skin lipids—ceramides—were also tested. Surprisingly, they showed only minimal reactivity (see results for ceramide *N*-lignoceroyl-D-erythro-sphingosine; Fig. 3, lower panel, curve 10).

Validation of a Model Meibum Mixture for Analysis of Human Meibum

Given the complex nature of the SPV response of individual lipid species, accurate estimation of meibum lipids requires a reference standard with a composition mimicking that of lipids present in normal human meibum (Table 1). In the SPVA, the MMx showed increasing A_{535} with increasing lipid input and, as found for individual pure lipids, this increase was best fit with Eq. 1. The SPV-lipid chromophore was detectable as early as a few minutes post addition of vanillin (Fig. 4a), but continued to steadily increase with longer incubation times. The standard reaction time was chosen to be 10 min, which was a compromise between the duration of the experiment and the sensitivity of the analysis.

Addition of a low level of Chl to the MMx (1 %, w/w, or less) slightly reduced the final SPV response when compared to the response of the MMx without Chl (Fig. 4b, c). Using an unpaired *t* test analysis of the two responses, the observed difference reached a significance of $p = 0.032$. A 1 % concentration of Chl in the MMx is on the higher end of what has been reported for normal human meibum, but is on the lower end of what was found for aqueous tears (Butovich et al., unpublished). Regardless of the concentration of free Chl in the MMx, the resulting calibration curves were very close (Fig. 4b).

Finally, by varying the saturation/unsaturation ratios of the WE and CE standards in the MMx, we showed that the assay tolerated the changes without significantly impacting the observed response of the model meibum lipid mixture in the assay (Fig. 4c).

To provide a clearer overview of the relative reactivity of individual lipids, it was beneficial to re-plot the data shown in Fig. 3 using the actual molecular masses and molar amounts of the analytes (expressed in nmol) instead of the physical weights (expressed in μg) of their tested aliquots. The latter approach is more suitable for complex lipid mixtures with fairly unknown, or changing, composition (like meibum and the MMx), while the former is more informative when comparing the individual reactivity of pure lipid standards or simple mixtures. Also, the analytes were grouped and analyzed in many different ways according to their chemical structures (e.g. groups of Chl-containing compounds, saturated lipids, unsaturated lipids etc.). Some of the recalculated plots are shown in Fig. 5a, b.

Finally, to visualize the molecular reactivity of individual lipids of various types, the SPV responses of a range of lipids (100 nmol each) were compared in a side-by-side experiment (Fig. 5c). One can see that the individual responses of the lipids did vary with their degree of unsaturation, but were close for groups of lipids of similar types and could be placed in the following order: Chl-O ≅ BO ≅ SO ≅ TO, and STA ≪ Chl <Chl-S ≤BS ≅ SS. However, the inhibitory effect of the cholesteryl rings on the A_{535} values for Chl and CE is obvious.

Spectrophotometric Comparison of the SPV Response of the MMx and Meibum

Meibum and the MMx are both complex lipid mixtures, with meibum in addition containing many lipid species not represented in the MMx, e.g. OAHFA and Chl-OAHFA. However, UV-Vis spectra showed that the sulfuric acid incubation products of both mixtures generated similar chromophores (Fig. 6a). In addition, the shape of the baseline and minor absorption peaks above 400 nm for both were similar and showed a pattern intermediate between the patterns obtained for pure WE and Chl-containing standards (compare Figs. 6a, 2). Differential UV-Vis spectra for the pre- and post-vanillin reaction products of the MMx and human meibum revealed final chromophores with an absorption maximum at 535 nm in both, consistent with their measured positive response in the SPV assay (Fig. 6b).

Effects of Non-Lipid Ocular Components on the SPVA Results

Clinical meibum samples, while predominantly composed of lipids, can potentially contain non-lipid products such as salts, carbohydrates, and proteins derived from meibomian glands, the ocular surface, and the aqueous tears. Human aqueous tears have been shown to be enriched with lysozyme (LYZ), albumins, and mucins (MUC) as major proteins [3, 25, 26]. To model potential influences of such compounds on the SPV estimate of meibum lipids, we performed analyses of 20 μg of MMx alone or with addition of authentic LYZ in the amounts of 10 and 20 μg, MUC from bovine submaxillary gland (25 and 50 μg), and BSA (10 and 20 μg).

MUC (a mixture of glycosylated proteins), when added alone to the assay, was found to be SPV-negative. In addition, the SPV analysis of the MMx premixed with MUC was not impacted. Similarly to MUC, neither BSA nor LYZ gave any significant SPV response. When pre-mixed with the MMx, the presence of either of these proteins did not altered the outcome of the lipid estimate. The exact range of lipid-to-protein weight ratios in human meibum is, to the best of our knowledge, not known, but is expected to be less than the levels tested in our experiments (which were between 1:2.5 and 2:1). Paired *t* test analysis showed no effects of non-lipid additives on the results of the SPV lipid analysis. Thus, this data suggests that the presence of high levels of protein in meibum samples will not impact the use of the SPVA to obtain an estimate/index of the lipid content of different clinical samples of meibum.

Comparison of SPV and Gravimetric Analyses of Human Meibum

Human meibum samples were collected and analyzed gravimetrically and their recorded dry weights were used to prepare stock solutions at 1 mg total meibum/ml. In the SPVA, addition of increasing amounts, 0 to 40 μl, of meibum collected from 2 normal patients (1 male, 1 female) both showed a quasi-linear increase in A_{535}. For the female sample calculated parameters of Eq. 1 were $a = -0.0042 \pm 0.0017$; $b = 0.0077 \pm 0.0007$; $c = 0$ ($r^2 = 0.982$), while for the male sample—$a = 0.0206 \pm 0.0164$, $b = 0.0080 \pm 0.0007$; $c = 0$ ($r^2 = 0.984$). Data are shown as means ± SD (n=3). To compare gravimetric estimates to the lipid levels in meibum estimated by the SPVA, replicates of 20 μl were withdrawn from 7 different clinical meibum collections (1 mg/ml gravimetric stocks) and their lipid contents assayed. Evaluation of the individual meibum gravimetric stocks using the SPVA showed that the mean SPV-reactive lipid content was about 15.6 ± 4.6 μg (mean ± SD), or 78 % (w/w) of the whole meibum sample (Table 2). Finally, it must be emphasized that, as is true for protein assays, to compensate for any experiment-to-experiment variability, one needs to run fresh calibration curves together with meibum samples each time the experiment is performed. Fortunately, this is not a problem when using a microplate reader with a 96-cell plate.

Discussion

Mechanistic Considerations

Since its introduction almost 75 years ago by Chabrol and Charonnat [16], the SPV reaction has been used, with varying degrees of success, for measuring the total lipid content of samples of biological origin. The strength of the approach is in its low specificity, so that diverse lipid classes can be quantitated if proper standards are chosen. However, it has been noted time and again that different lipid classes and, often, different lipids within one class, provided different responses in the SPVA. Apparently, the disparate reactivity of different types of lipids with the reagent, and variations in the methodology were responsible for this variability. The molecular mechanisms of the SPV reaction are very complex. Knight et al. [17] advocated for the role of the (–CH=CH–) olefinic group (found in unsaturated lipids) in

the mechanism which involves the formation of a carbonium ion, also known as alkyl carbocation (–CH_2–C^+H–), upon treatment of unsaturated lipids with concentrated sulfuric acid. Johnson et al. [18], on the other hand, proposed the formation of an alkenyl carbocation R_1–C^+H–CH=CH–R_2 as an intermediate product. Two considerations are critical for understanding the role of hydroxyl groups in the SPV reaction: (1) the classical sulfuric acid-catalyzed elimination reaction in which an alcohol loses a molecule of water to produce an alkene with a C=C double bond, and (2) the Komarowski reaction [27] (as described by Duke in 1947 [28]), in which newly generated aliphatic aldehydes (see below) in the presence of strong acids react with aromatic aldehydes to produce aldol condensation products.

Regardless of the mechanism, a general consensus is that the SPV reaction requires either a C=C double bond or a hydroxyl group to be present in the lipid molecule for it to be SPV-positive. Therefore, we were surprised to see that, in our hands, compounds that satisfy neither of these two requirements—such as saturated WE and, to a certain extent, saturated free FA and saturated TAG—were SPV-positive. Earlier, free FA and saturated TAG (such as tripalmitin) were reported to be either SPV-negative [18], or provided a minimal SPV response [17]. Unlike data presented in those reports, the reactivity of STA in our experiments was noticeable, while saturated WE (SS and BS) reacted almost as efficiently as their unsaturated counterparts (Fig. 3). These observations necessitated a more detailed investigation and discussion of the mechanisms of these reactions.

It appeared that all tested compounds, with the exception of fully saturated HD, did produce the first intermediate product of the SPV reaction—a chromophore with λ_{max} around 300 nm and a symmetrical UV spectrum—which was formed from the lipids upon treating them with hot concentrated sulfuric acid. As no phospho-vanillin was present in the reaction mixture at this stage, this first intermediate product should be a carbocation, almost certainly one of those discussed above. Earlier observations by Deno et al. [29], who reported on the formation of aliphatic alkenyl carbocations in acidic conditions and described their UV spectra, corroborate our conclusions. The authors noted that such carbocations had nearly symmetrical UV absorption spectra with λ_{max} about 300 nm, minima at around 250 nm, and a monotonic increase in the UV absorption from 240 to 200 nm. This essentially describes the UV spectra presented in our Figs. 1 and 6a for all tested SPV-positive compounds, including fully saturated wax esters, the MMx, and human meibum. Note that the two related compounds—saturated BS and monounsaturated BO—produced almost identical UV-Vis chromophores (Fig. 1a). The alikeness of the two chromophores implied similarities in their structures and in the mechanisms of their formation. However, while BO has an apparently mandatory olefinic group, BS has none. Therefore, the mechanism of its reaction should be somewhat different (and more complex) than those listed above.

For the readers' convenience, a generalized scheme of the lipid transformations in the SPV reaction is presented in Fig. 7. The two traditionally assumed pathways in the reaction involve a pre-existing olefin, or an aliphatic alcohol. In concentrated sulfuric acid solutions, either mechanism proceeds through a crucial step of formation of carbocations, which, once formed, engage in a reaction with phospho-vanillin. However, only carbocations generated from unsaturated precursors were expected to produce a chromophore with λ_{max} ~300 nm. The saturated WE, which have neither a free hydroxyl group, nor a pre-existing C=C double bond, must generate a reactive group before they can produce a carbocation.

Two plausible mechanisms for this step are envisioned. The first mechanism is based on a simple (reversible) reaction of hydrolysis of wax esters in acidic solutions to produce a free alcohol and a free fatty acid, as shown in Fig. 7. From there, the reaction could proceed through a traditional reaction of elimination of water from the alcohol (a dehydration step)

to produce a terminal diene (or alkyl vinyl) as shown in Fig. 7 for free fatty alcohols. However, this explanation is somewhat self-contradictory as the hydrolytic step requires water, and is usually performed in diluted aqueous solutions of acids, while the dehydration step, and the further transformation of the newly generated olefins in carbocations, are better carried out in concentrated, almost anhydrous sulfuric acid. Thus, it is rather unlikely that the hydrolytic reaction is indeed the first step in these transformations.

The second mechanism (also depicted in Fig. 7) involves, as the first step, formation of alkenyl (or vinyl) ethers through the reaction of elimination of a molecule of water from an aliphatic ester (such as SS and BS). Alkenyl and vinyl ethers have been shown to be hydrolytically unstable in aqueous acids producing a free aldehyde and a free alcohol [30–32]. These two products could then enter the SPV and/or the Komarowski reactions as described above. Alternatively, the newly generated C=C double bonds in the presence of concentrated sulfuric acid could directly produce the SPV-positive carbocations. Either of these two mechanisms explains the reactivity of saturated WE in the SPV reaction, and the formation of two critical chromophores with λ_{max} 300 and 500 nm. As alkyl carbocations do not possess a conjugated double bond, they seem to be unlikely to produce chromophore λ_{max} 300 nm. Therefore, the reaction of formation of allylic or alkenyl carbocations seems to be the more plausible pathway. Experiments are in progress to elucidate the mechanism of this reaction in further detail.

Three unsaturated oleic-acid containing lipids (BO, Chl-O, and TO) demonstrated almost identical molecular reactivity in the SPVA, and so did another group of lipids which was based on saturated stearic acid—SS, BS, and Chl-S. Notably, the overall molecular reactivity of oleic acid-containing lipids was about 50 % or so higher than that of stearic acid-based lipids, which can be reflective of the presence of double bonds in the former group of lipids. A direct comparison of pairs of saturated and unsaturated lipids (BO/BS, and Chl-O/Chl-S) was especially revealing—they all produced similar spectra of the carbocation intermediate (λ_{max} 300 nm) and of the final SPV chromophore (λ_{max} 500 nm). Importantly, nonesterified, free STA (Fig. 5c) was found to be somewhat reactive as well, but much less so than its esterified products. It seems that its direct reactivity in the SPVA is limited, and the reaction might involve an extra step, for example a classical reaction of formation of STA anhydride or a terminal vinyl (both being plausible scenarios of dehydration in concentrated sulfuric acid). Free Chl was also found to be less reactive than CE, which further accentuated the role of the ester bond in the reactions. On the other hand, saturated Cer, which do not have ester bonds, double bonds, or free hydroxyl groups, barely reacted in the SPVA (not shown). Interestingly, a direct comparison of TO and TS demonstrated that TO was highly reactive, while TS produce much smaller amounts of the SPV chromophore. It seems that the glycerol moiety in saturated TAG inhibits formation of intermediate products of carbocation nature, which are necessary for the SPV reaction to proceed, while in unsaturated TAG this was less of a factor. Thus, the presence of an aliphatic ester bond similar to that found in WE and CE (but not TAG) could be added to the list of factors that make a compound highly SPV-positive.

The reactivity of Chl-containing compounds was further influenced by the presence of the cholesteryl rings, which complicated the reaction mechanism even more, and produced a series of additional, albeit less intense, chromophores with much longer λ_{max} 350 to 600 nm (Fig. 1b). The discussion of the nature of these intermediates goes beyond the scope of the current paper. However, it is important to note that all tested Chl-containing lipids still produced a carbocation with λ_{max} of about 320 nm as a major intermediate product. It is not clear at the moment whether additional chromophores impact generation of the main chromophore λ_{max} 320 nm, or are formed independently from it. There are indications (such as excessive charring) that in Chl-containing lipids reactions other than the desired reaction

of the aliphatic carbocation formation become pronounced, which potentially could lead to various types of cyclic by-products formed from the Chl moiety.

Quantitation of Complex Lipid Mixtures and Model Meibum Mixtures

In studies directed towards evaluation of complex lipid extracts of biological origin, including human meibum, gravimetric analysis of dry samples has been the starting point of most experiments. However, where pure lipid standards have been available, quantitative studies of specific meibum lipids have been possible. Our study with the SPVA was undertaken to facilitate further quantitative studies and to address a real need for identification of procedures that would allow accurate, routine estimation of the total lipid present in small clinical samples of meibum. Considering that MG produce and secrete meibum through a holocrinic mechanism, dry meibum samples, in addition to lipids, also contain a number of other non-lipid components which may include proteins, carbohydrates, and salts. Thus, gravimetric methods may tend to overestimate the total lipid content in study samples, a problem which in addition has the potential to be more pronounced in clinical samples collected from patients with MG pathology, e.g. in the non-aqueous form of DE, where histological studies have shown the accumulation of non-lipid material in the central meibomian ducts [7]. Thus, in addition to accommodating the diversity of different lipid chemical groups that are found in meibum, another requirement would be that other non-lipid meibum components not significantly affect the outcome of the lipid estimate.

Initial searches of the literature for total lipid assay procedures for biological samples identified the SPVA as the most promising, if not the only, method for such analysis. This colorimetric method has been used to derive estimates of serum lipids [17, 18] and more recently for analysis of lipids in smaller sample sizes obtained from insects [33] (and references therein). In addition, a microplate reader-adapted format of the assay was recently presented [20], further suggesting its utility for high through-put analysis of small lipid-containing samples.

The variability in the calibration curves obtained for individual lipids (Figs. 3, 5) necessitated the use of a lipid mixture that approximated the human meibomian lipidome as the most appropriate reference standard for the SPVA (Table 1). In this mixture, the molar ratios of the most abundant lipid classes identified in meibum (CE, WE, and Chl), and their degrees of saturation/unsaturation were chosen to make the mixture resemble human meibum thus bringing its SPV response as close to that of meibomian lipids as currently possible. This information was used to derive the MMx reference standard (BO:BS:Chl-S:Chl-O:Chl = 0.42:0.08:0.40:0.10:0.01, by weight) used for our human meibum studies. Reactivity of all the lipids in the MMx contribute to the final SPV response and, by analogy, all of the most abundant lipid classes present in meibum were expected to generate a similar SPV response. While the ratios of WE to CE, and saturated to unsaturated lipid species could possibly vary between meibum samples, our data suggest that the interdonor- and intersample variability is relatively small for normal, non-DE donors [10, 13–15], and for a population of dry eye patients that participated in a recent clinical trial [34]. Note, however, that in the latter paper only gross MS signals of samples were reported and analyzed. Importantly, our new data prove that the assay can tolerate some variability in the lipid ratios without significantly impacting its outcomes—the A_{535} values (Fig. 4).

This study did show that addition of Chl to mixtures of WE and CE would only minimally affect the results of the SPVA (Fig. 4). Values for Chl in normal meibum have been reported to be 0.2–1 % (w/w) of total lipid content [14, 22, 23]. The value of 0.2 % was derived in a study where estimates were based on quantitative calibration of the detection method using a pure Chl standard. In the current study, Chl was added to the MMx in the amounts of 0.5–1 % (w/w), thus its levels were on the high end of their reported normal values in meibum.

However, when present even at these relatively high levels, Chl did not significantly alter the total lipid estimates as measured in the SVP assay (Fig. 4).

Comparison of the SPV responses of the MMx lipid mixture and human normal meibum showed that both responded in a similar manner in the assay. For both lipid mixtures, UV-Vis analyses showed generation of similar chromophores in both the intermediate and final vanillin steps of the assay (Fig. 6). In addition, assay sensitivity was such that as little as 10 μl of a 1 mg/ml gravimetric stock (or 10 μg) was sufficient to obtain a measurable response for most meibum samples, thus allowing for using assay replicates for samples. The SPV response of meibum was linear from 0 to 40 μg gravimetric (not shown). Comparison of the SPV lipid quantitation versus gravimetric analysis of normal meibum revealed in general a lower SPV lipid estimate when compared to the gravimetric estimates (Table 2): meibum samples estimated to have 20 μg (gravimetric) of lipid were measured to contain on average only 15.6 ± 4.6 μg, or 78 %, of the lipid as measured in the SVP assay.

There are a number of possibilities to be considered to explain why the SPV estimates are almost universally lower than those obtained gravimetrically, including: (1) lipids in meibum which are not responsive in the SPVA, (2) meibum components which negatively impact the assay results, or (3) meibum components other than lipids which contribute to the gravimetric measurements, but are not SPV-positive. As (1) no major presence of saturated hydrocarbons has been detected in meibum in a recent GC/ MS study [13], (2) saturated TAG and Cer are relatively minor components of normal meibum, and (3) other identified meibomian lipids (such as oleic acid, OAHFA, Chl-esters of OAHFA, diacylated diols, TO and other unsaturated TAG [24]) are supposed to be SPV-positive as they are essentially similar to the WE, CE, and TO tested in this paper, it is reasonable to assume that about 22 % (w/w) of meibum are SPV-negative compounds of non-lipid nature. Human meibum and tear film samples, while predominantly of lipid nature, may also contain proteins [35] and possibly other non-lipid components (such as salts and polysaccharides). Mucin may be one such component, originating either from the tear film or, possibly, from the ocular surface given that goblet cells were recently documented in the lid wiper region in human eyelids, placing them in close proximity to the opening of the meibomian ducts [36]. The current analysis of the SPV method shows that protein components did not impact estimation of lipid levels with this assay, but the presence of such components will, however, contribute to gravimetric estimates for meibum samples.

In conclusion, though not a “silver bullet”, the SPVA seems to be a useful tool for measuring total lipid content in samples predominantly formed from lipids of the CE, WE, and unsaturated TAG classes, e.g. meibum. Surprisingly, the method is capable of detecting even saturated WE, which is critically important for meibum analysis as human meibum has a high percentage of these kind of lipids. If a proper reference lipid mixture is chosen (such as the MMx mixture that mimics the composition of human meibum) and used alongside the biological samples, the SPV reaction can provide meaningful estimates of the overall lipid content of the samples, using tools that are available in most biochemical settings and clinical laboratories. The nature of the chemical transformations involved in the SPV reaction is of high interest and complexity and is to be reported separately.

Acknowledgments

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Abbreviations

BO	Behenyl oleate
BS	Behenyl stearate
BSA	Bovine serum albumin
Chl	Cholesterol
CE	Cholesteryl ester
Chl-O	Cholesteryl oleate
Chl-S	Cholesteryl stearate
FA	Fatty acid
HD	Heptadecane
LYZ	Lysozyme
MMx	Model meibum lipid mixture
MUC	Mucin
OAHFA	(*O*-Acyl)-omega-hydroxy fatty acid
1-OD	1-Octadecanol
SPVA	Sulfo-phospho-vanillin assay
STA	Stearic acid
SO	Stearyl oleate
TAG	Triacylglycerol
TO	Triolein
TS	Tristearin
UV-Vis	Ultraviolet-visible
WE	Wax ester

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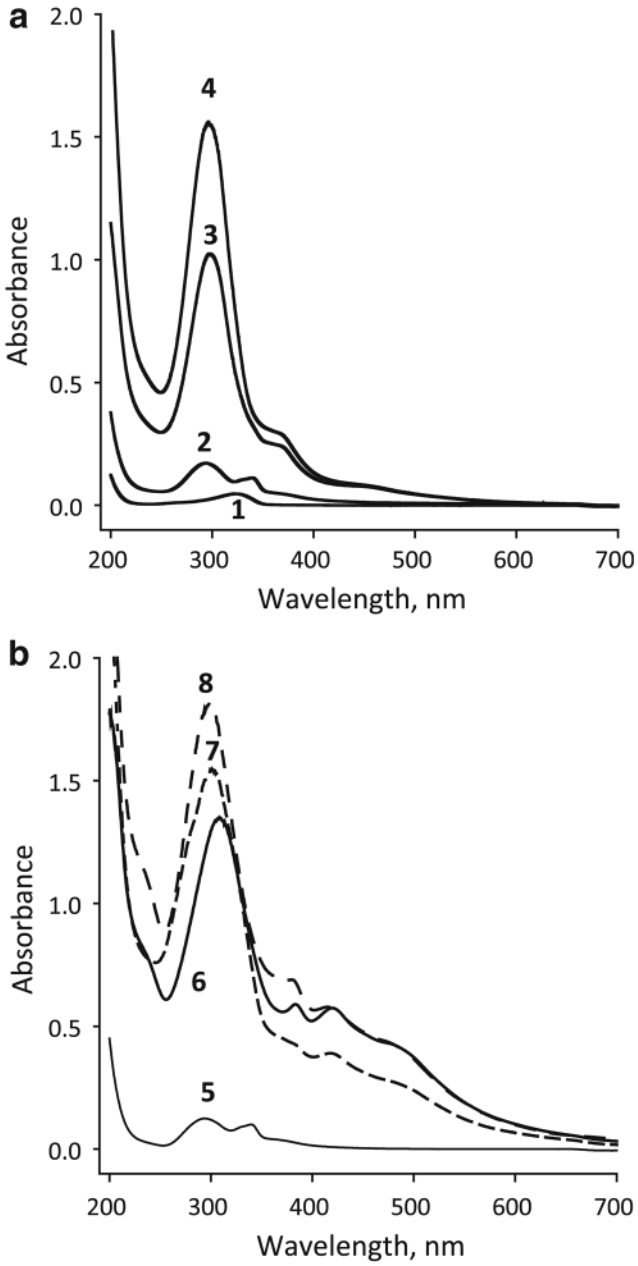

Fig. 1.

UV-Vis absorption spectra of standard lipids in concentrated sulfuric acid. The following lipids were tested: heptadecane (*1*), stearic acid (*2*), behenyl stearate (*3*), behenyl oleate (*4*), tristearin (*5*), cholesterol (*6*), model meibum lipid mixture (*7*), cholesteryl oleate (*8*)

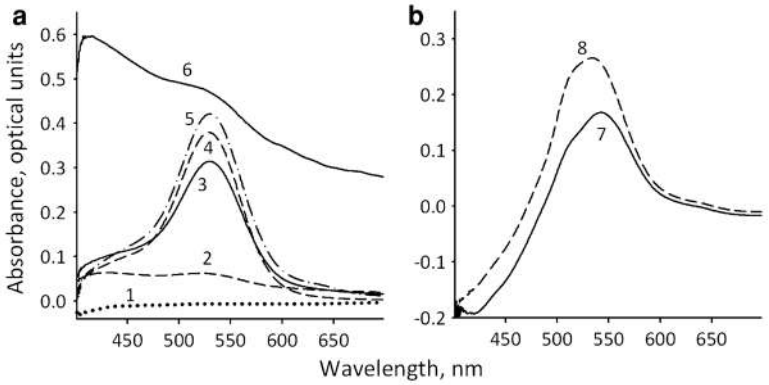

Fig. 2.

Differential UV-Vis absorption spectra of the sulfo-phospho-vanillin products of standard lipids. The spectra of the following compounds are shown: heptadecane (*1*), stearic acid (*2*), stearyl stearate (*3*), stearyl oleate (*4*), 1-octadecanol (*5*), tristearin (*6*), cholesterol (*7*), and cholesteryl oleate (*8*)

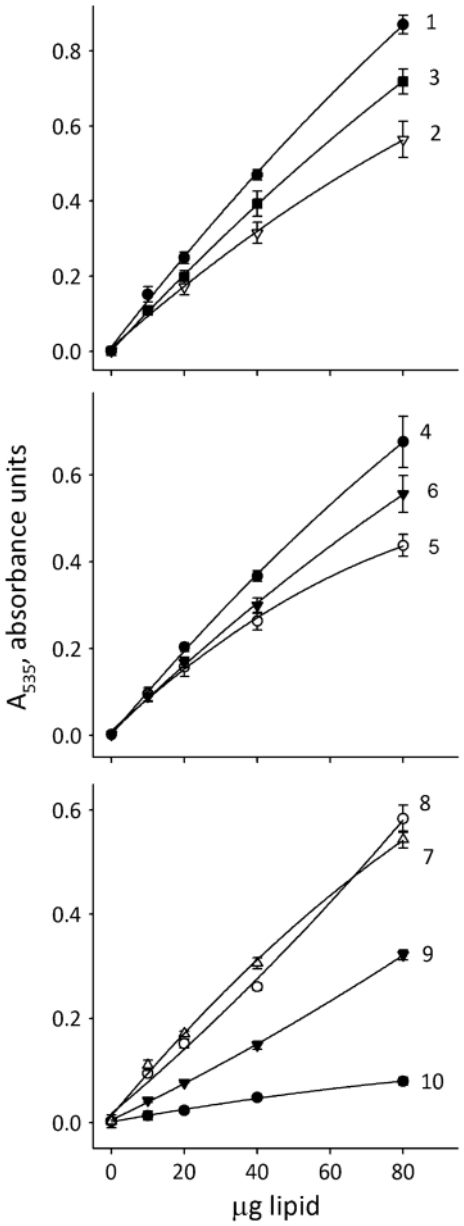

Fig. 3.

SPV reactivity of standard lipids. The following pure lipid standards and their mixtures representative of the lipids in human meibum were tested: (*1*) behenyl oleate, (*2*) behenyl stearate, (*3*) behenyl oleate/behenyl stearate=4/1 (by weight), (*4*) cholesteryl oleate, (*5*) cholesteryl stearate, (*6*) cholesteryl oleate/cholesteryl stearate=1/4 (by weight), (*7*) stearyl stearate, (*8*) triolein, (*9*) stearic acid, (*10*) ceramide. All analyses were performed in quadruplicates and data shown as means ± SD. *Curves* were fit using Eq. 1

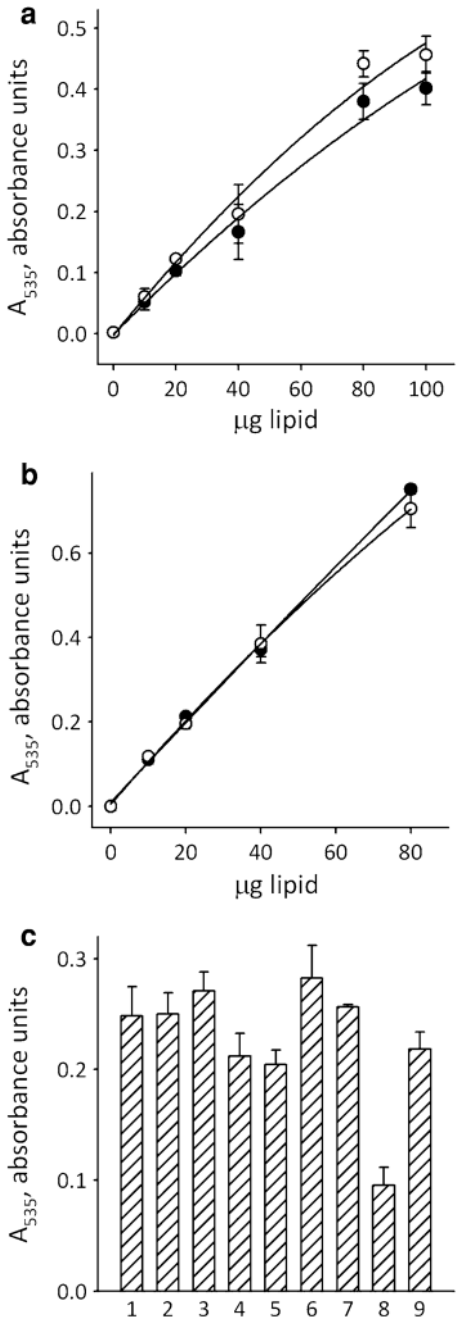

Fig. 4.

Derivation of a reference mixture of pure lipids. **a** The SPV response of the MMx (BO:BS:Chl-S:Chl-O:Chl = 0.42:0.08:0.40:0. 10:0.01, by weight) for two different incubation times—5 min (*closed circles*) and 10 min (*open circles*)—after addition of the phospho-vanillin reagent. **b** Comparison of the SPV response of the MMx containing either 0.5 or 1 % Chl (*closed* and *open circles*, respectively). c SPV responses of 40 µg of standard lipid mixtures illustrating the influence of the chemical nature of WE and CE and of the presence of Chl on the assay. The following mixtures were tested (all ratios by weight): BO:BS = 0.5:0.5 (*1*), BO:BS = 0.83:0.17 (*2*), BO:BS = 0.95:0.5 (*3*), Chl-S:Chl-O = 0.5:0.5 (*4*), Chl-S:Chl-O = 0.8:0.2 (*5*), BO:BS:Chl-S:Chl-O = 0.42:0.08:0.40: 0.10 (*6*), BO:BS:Chl-

S:Chl-O = 0.48:0.02:0.40:0.10 (*7*), Chl alone (*8*), and BO:BS:Chl-S:Chl-O:Chl = 0.42:0.08:0.40:0.10:0.01 (*9*). Analyses were performed in triplicates for **a** and **b** and in quadruplicate for **c**. The data are expressed as means ± SD

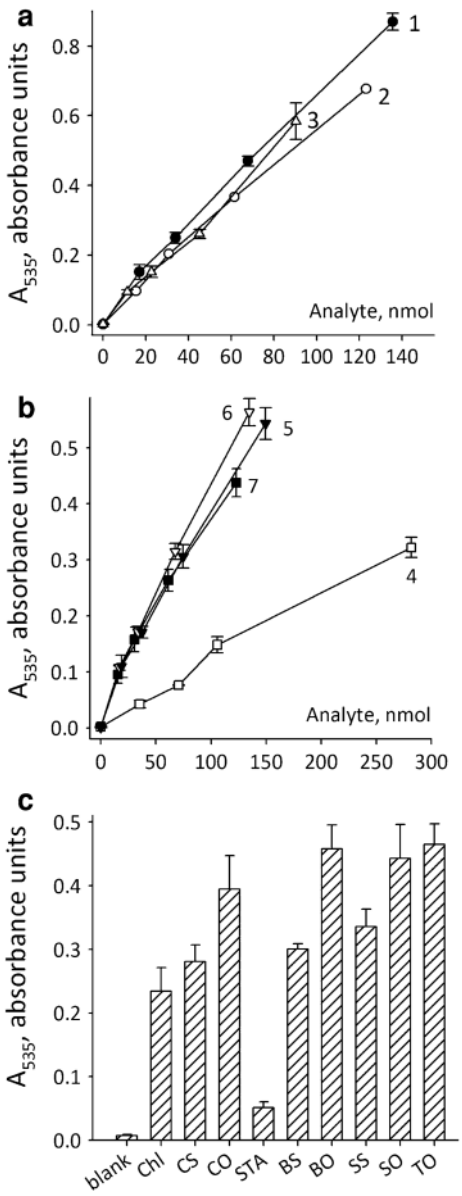

Fig. 5.

Molecular reactivity of standard lipids in the SPVA. The following compounds were tested: **a** Behenyl oleate (*1*), cholesteryl oleate (*2*), triolein (*3*); **b** stearic acid (*4*), stearyl stearate (*5*), behenyl stearate (*6*), and cholesteryl stearate (*7*); **c** SPV responses of standard lipids (100 nmol each) compared in a side-by-side experiment. The samples were analyzed in quintuplicates. The *data* are shown as means ± SD. Panels **a** and **b** show recalculated data from Fig. 3

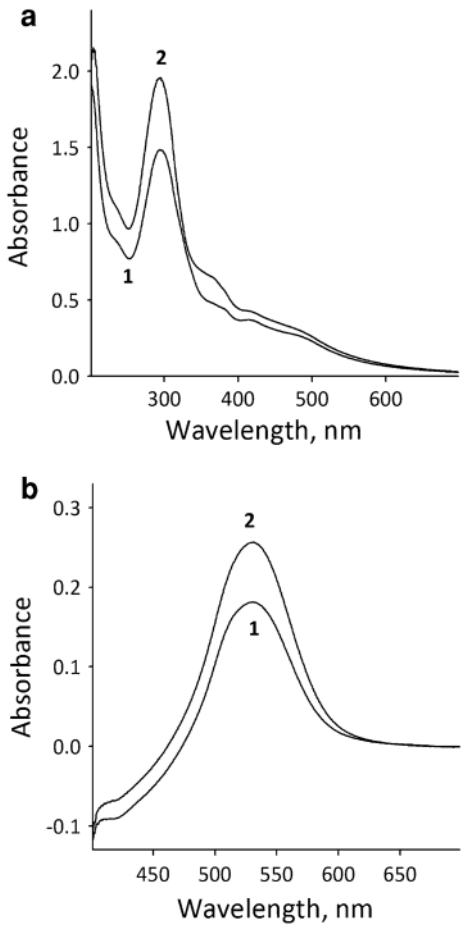

Fig. 6.

UV-Vis spectra of the intermediate and the final SPV reaction products of human meibum and the model meibum lipid mixture. **a** Differential UV-Vis spectra showing similar pre-vanillin reaction intermediates that are formed upon incubation of: (*1*) the MMx standard lipid mixture (BO:BS:Chl-S:Chl-O:Chl = 0.42:0.08:0.40: 0.10:0.01, by weight), and (*2*) human meibum with sulfuric acid. **b** Differential UV-Vis absorption spectra show that the same final vanillin-lipid chromophores, with absorption maxima at 535 nm, are generated by both the MMx (*1*) and human meibum (*2*) in the SPVA

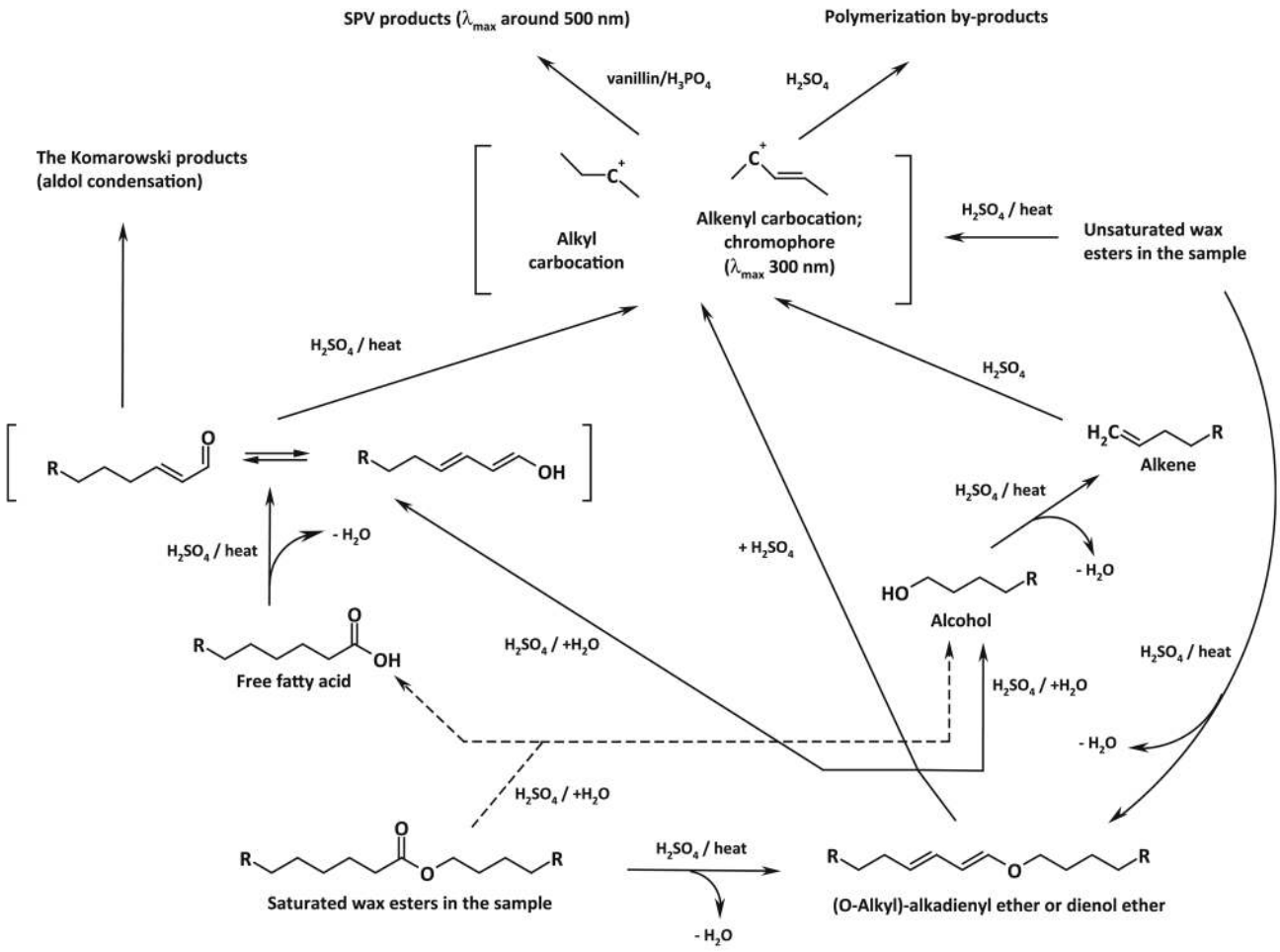

Fig. 7.
Proposed reactions of typical unsaturated and saturated aliphatic compounds in the sulfo-phospho-vanillin assay

Table 1

Major lipid classes in human meibum

Lipid class	% of total meibum lipid (w/w)	Ratio of saturated to unsaturated lipids within each class	References
Wax esters	41 ± 8	1:4.6	[14]
Cholesteryl esters	~30	4:1	[15, 16]
Cholesterol	<2	n/a	

Table 2

Comparison of gravimetric and SPV lipid estimates for human meibum

Meibum collections	Total sample weight (μg)[a]	Gravimetric aliquot (μg)[b]	SPV estimate (μg)[a, b]
S1	740	20	13.6
S2	490	20	16.5
S3	800	20	12.4
S4	1,030	20	9.9
S5	90	20	24.2
S6	1,337	20	17.7
S7	1,103	20	14.9

[a]The results shown in this table have passed the Shapiro–Wilk normality test, which indicated the Gaussian distribution of the values and the apparent absence of outliers, which would have been needed to be removed from calculations

[b]There was a statistically significant difference between the gravimetric weights and the SPV estimates ($p = 0.026$). Random sample variations would have resulted in the SPV estimates being randomly higher and lower than the gravimetric weights, which was not the case