A comprehensive method for determination of fatty acids in the initial oral biofilm (pellicle)

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Abbreviated title

Determination of fatty acids in the oral biofilm

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Abbreviations: BAME, bacterial acid methyl ester; CV, coefficient of variation; EDTA, ethylenediaminetetraacetic acid; FA, fatty acid; FAME, fatty acid methyl ester; GC, gas chromatography; GTFCh, Gesellschaft für Toxikologische und Forensische Chemie (Society of Toxicological and Forensic Chemistry, Germany); IS, internal standard; LOD, limit of detection; LOQ, limit of quantification; QC, quality control; RSD, relative standard deviation; RT, retention time; SIM, selected ion monitoring; S/N ratio, signal-to-noise ratio; TEM, transmission electron microscopy
Abstract

The acquired pellicle is a tenacious organic layer covering the surface of teeth, protecting the underlying dental hard tissues. Lipids account for about one quarter of the pellicle’s dry weight, and are assumed to be of considerable importance for the protective properties. Nevertheless, only preliminary information is available about the nature of lipids in the pellicle. Gas chromatography coupled with electron impact ionization mass spectrometry (GC/EI-MS) was used to establish a convenient analytical protocol in order to obtain a qualitative and quantitative characterization of a wide range of fatty acids (FAs) (C\textsubscript{12} - C\textsubscript{22}). \textit{In situ} biofilm formation was performed on bovine enamel slabs mounted on individual splints carried by 10 subjects. A modified Folch extraction procedure was adopted to extract the lipids from the detached pellicle, followed by transesterification to fatty acid methyl esters (FAMEs) using methanol and conc. HCl. Tridecanoic and nonadecanoic acid were used as internal standards (IS) suitable and reliable for robust, precise and accurate measurements. The present study demonstrates, for the first time, a procedure based on a combination of innovative specimen generation and convenient sample preparation with sensitive GC-MS analysis for the determination of the fatty acid profile of the initial oral biofilm.

Supplementary key words: GC-MS, derivatization, saliva, oral biofilm, \textit{in situ}
Introduction

Biofilm formation on dental hard tissues is fundamental for caries and periodontitis, two diseases with extremely high prevalence and considerable economic relevance (1–6). Dental hard tissues are the only non-shedding surfaces in the human organism. Accordingly, the process of bioadhesion at tooth surfaces is of particular significance for oral diseases (7). The first step is the formation of the pellicle layer, which is mainly composed of adsorbed proteins and other macromolecules from the oral environment (saliva, crevicular fluids), and is clearly distinguished from the microbial biofilm (plaque) (7, 8). The selective process of pellicle formation is driven by physicochemical interactions such as van der Waals forces as well as electrostatic and hydrophobic interactions (7, 9). Serving as a protective lubricant, diffusion barrier and buffer, the pellicle layer participates in all interfacial events taking place in the oral cavity (8). Furthermore, several antibacterial proteins and enzymes are present in this proteinaceous layer of high tenacity (10, 11). Nevertheless, several bacteria have adapted to this protective structure, as certain pellicle components provide specific receptors for bacterial adhesion to the tooth surface, making the pellicle a conditioning film for bacterial biofilm formation (12, 13). All in all, the pellicle is a key structure, mediating the process of bioadhesion at the tooth surface and the interaction between bacteria, saliva and teeth. Some properties of the pellicle, such as ultrastructure, amino acid composition, or enzyme activity, have been investigated in detail (7, 8, 13). Thereby three types of studies have to be differentiated: *In vitro* studies (pellicle formed *in vitro* from collected saliva on different materials), *in vivo* studies (pellicle harvested by scrapping with a curette from the tooth surface) and *in situ* approaches (samples exposed to the oral cavity with splints) (7, 14). *In vitro* studies do not adequately mimic the situation in the oral cavity due to lacking
maturation processes, thus the *in vitro* pellicle differs considerably from the *in vivo* situation (15). Harvesting the *in vivo* pellicle yields to only very small amounts of sample material and the basal structures of the pellicle are not removed sufficiently (14). Accordingly, *in situ* setups with enamel slabs are preferable and allow evaluation of the pellicle with many elaborate methods. However, there is only limited information on the nature, function and composition of lipids in the pellicle. Data is predominantly derived from studies carried out in the 1980s and refers exclusively to the workgroup around Slomiany (16, 17). Therefore, further research is required to get a wider understanding of their biological effect in the oral cavity. Lipids in the pellicle are assumed to hamper bacterial adhesion and to protect the tooth surface against erosive noxae. Methods such as GC-MS offer the opportunity to analyze the lipid composition of the pellicle layer more precisely than in previous studies. The aim of the present study was to establish and to validate a precise method for the evaluation of the fatty acid pattern of the *in situ* formed pellicle. Harvesting of the pellicle and the small amount of sample material represents considerable challenges (14).

**MATERIALS AND METHODS**

**Chemicals and standards**

A Supelco 37-component FAME mix, a Supelco 23-component Bacterial Acid Methyl Ester (BAME) mix, as well as additional standards of single FA target compounds (12:0, 14:0, a15:0, 15:0, 16:0, 16:1n-9, 18:0, 18:1n9c, 18:2n-6, 20:0, 22:1n-9) and the two IS (13:0, 19:0) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Hydrochloric acid, chloroform, methanol and n-hexane were purchased from Carl Roth (Karlsruhe, Germany) in GC ultra grade and LC-MS grade. Water utilized for
preparation of standard and extraction solutions was deionized with a Milli-Q purification system (Millipore, Schwalbach/Ts, Germany).

**Instrumental conditions**

GC/EI-MS analyses were performed with a Fisons GC 8065 gaschromatograph interfaced with a single-quadrupole Fisons 800 MSD. The samples (1 µl) were injected via a CTC A200S autosampler (splitless, split open after 90 sec). The injector and transfer line temperatures were kept at 260 °C. A Select FAME fused silica capillary column (50 m x 0.25 mm ID, 0.25 µm film thickness; Agilent Technologies, Waldbronn, Germany) was used for separation of the target compounds. Helium (purity 5.0) was used as carrier gas with a constant pressure of 100 kPa. The GC temperature program started at 50 °C (hold time 5 min) and was increased to 260 °C (hold time 8 min) at a ramp rate of 6.5 °C/min. A solvent delay of 8 min was applied. The electron energy was 70 eV and the temperature of the ion source was set to 250 °C. In the GC/EI-MS full scan mode, \( m/z \) 60-400 was recorded. For GC/EI-MS in the SIM mode, fragment ions including \( m/z \) 74, \( m/z \) 87, \( m/z \) 81, and \( m/z \) 79 for FAME were recorded throughout the run (18).

**Calibration standards and quality control samples**

The Supelco FAME mix and the Supelco BAME mix, including 49 different FAMEs, were used as reference standards to identify the FAs of the pellicle samples. After screening the pellicle samples for the most abundant FAs, a stock solution containing 11 FAs of two levels of concentration (1 mg/ml each of 12:0, 14:0, a15:0, 15:0, 16:1n-9, 18:2n-6, 20:0; 5 mg/l each of 16:0, 18:0, 18:1n-9, 22:1n-9; in methanol) was prepared from the individual FA standards for quantitative analysis. Calibration standards were made up of seven different concentrations depending on the particular FA, ranging from 12.5 ng/ml to 250 ng/ml and 62.5 ng/ml to 1250 ng/ml.
respectively. The final concentrations were yielded by diluting the stock solution with methanol. Quality control (QC) samples were prepared at four different concentrations (30, 175 ng/ml and 150, 875 ng/ml; in 0.4% EDTA solution). The FA stock solution and the QC samples were aliquoted and stored at -20 °C under nitrogen.

**Subjects and sample collection**

Bovine incisors were acquired from two year old cattle (BSE-negative). After extraction, the teeth were stored in thymol solution. For sample generation, round enamel slabs (5 mm diameter) were gained from the labial surface of the teeth with a trepan bur. The surface of the enamel slabs was wet ground up to 4000-grit with abrasive paper. Afterwards, the samples were disinfected in a sequential procedure in an ultrasonic bath. After 3 min in sodium hypochlorite (2%), the slabs were washed twice in deionized water for 5 min each, followed by ultrasonication in ethanol (70%) for 10 min and final cleaning in deionized water for another 10 min. Before exposure to the oral fluids, the slabs were stored in deionized water for 24 h to form a hydration layer (19, 20).

For pellicle formation, the slabs were fixed into small cavities on individual upper jaw splints with silicon impression material (Aquasil, Dentsply De Tray, Konstanz, Germany), so that only the surface was exposed to the oral fluids. 12 slabs per splint were fixed on buccal and palatal sites of the premolars and the 1\textsuperscript{st} molar (14, 19). After oral exposure for 30 min, the slabs were rinsed for 10 s with saline solution to remove loosely attached salivary fractions. Then the slabs were removed from the splints with a dental probe and transferred to 15 ml Falcon tubes. For the purpose of desorption, the samples were incubated in an ultrasonic bath with 1 ml 0.4% EDTA (pH 7.4) for 60 min (14). The pellicle is a biofilm of high tenacity,
therefore direct and complete extraction of pellicle components is difficult. A previous study indicates that the adopted desorption procedure allows complete and quantitative detachment of the \textit{in situ} formed pellicle as validated by TEM (14). The desorbed pellicle was pipetted into 1.5 ml amber screw vials and stored at -20 °C until analysis.

The age of the subjects participating in this study ranged between 26 and 57 (4 male, 6 female). The subjects showed no signs of caries and periodontitis, the plaque indices were close to zero. The study protocol was approved by the ethics committee of the medical faculty, University of Freiburg (# 222/08).

\textbf{Sample preparation}

The pellicle sample, dissolved in 1 ml 0.4 % EDTA solution, was spiked with 30 µl of tridecanoic and nonadecanoic acid (25 µM each, in methanol) as internal standards prior to extraction. A modified Folch extraction procedure (21) was applied, in which 3.9 ml of a CHCl$_3$/MeOH (2:1, v/v) solution were added to the desorbed pellicle sample. After vortexing, the mixture was centrifuged at 900 g for five minutes. The lower phase, containing virtually all the lipids, was isolated in a screw-capped glass test tube (16.5 x 105 mm), and the solvent was evaporated under a gentle stream of nitrogen. Transesterification was carried out based on the method of Ichihara and Fukubayashi (22) and adapted to the pellicle matrix. The sample was dissolved in 0.2 ml of chloroform. 2 ml of methanol and 0.1 ml of conc. HCl (35 %, w/w) were added in this order to the lipid solution. The final HCl concentration was 1.5% (w/v) in a total volume of 2.3 ml. The solution was overlaid with nitrogen and the tube was tightly closed. After vortexing, the tube was heated at 100 °C for 1 h. Once cooled to room temperature, 2 ml of hexane and 2 ml of water were added for extraction of FAMEs. The tube was vortexed, and after phase separation, the hexane phase was
isolated and evaporated under a gentle stream of nitrogen. The residue was re-
dissolved in 0.1 ml of hexane and 1 µl of this solution was injected for GC-MS
analysis.

Data evaluation

Retention times (RT) of the separated FAs as well as the respective mass spectra,
gained from full scan measurement, were used for qualitative analysis. Although
EI-Ionization was applied, the molecular ion (M+) of each FA was visible in the mass
spectrum. Quantification of data obtained from SIM mode measurements was
performed using the peak area ratios relative to that of the IS. Least squares
regression analysis was implemented, using the peak area ratios against increasing
standard concentrations to obtain calibration linearity. Peak area ratios of the
unknown samples were referred to this calibration curve. Prior to the sample run, a
blank sample and the seven calibration standards were measured. Measurements of
the pellicle samples were bracketed by injections of QC samples to validate the
results.

Method validation

Statistical analysis was done, referring to the guidelines for method validation of the
Society of Toxicological and Forensic Chemistry (GTFCh) (25). The main
performance characteristics evaluated were selectivity over the analyte, linearity of
the response, closeness to the true value, precision of the obtained results and
detection and quantification limits. The limit of detection (LOD) and limit of
quantification (LOQ) were defined to be the lowest concentration with a
signal-to-noise (S/N) ratio > 3 for LOD and 10 for LOQ. The precision expressed as
the coefficient of variation (% CV) and the accuracy as the percentage relative error
(% bias) were determined from the QC samples at two different concentrations based
on the calibration range of each FA. For intraday repeatability, five replicates were analyzed, whereas the interday reproducibility was measured from samples run over five non-consecutive days.

RESULTS AND DISCUSSION

Method validation

The characterization of lipids and their fatty acid profiles via GC-MS is a widely accepted practice (23, 24). Nevertheless, analysis of fatty acids can be complicated due to cross-contamination, because lipids are omnipresent in nature and are constituents of commercial plastics, surfactants, and lubricants (23). As with any analytical procedure, the validity of the results depends on proper sampling and preservation of the sample prior to analysis. The importance of sample preparation is often underestimated and therefore carried out hurriedly and incorrectly. It must be kept in mind that, in case of errors occurring during the extraction procedure, even the best analytics is worthless. The described analytical method is reliable when plastic products are avoided whenever possible, and all the glassware used is cleaned (e.g. rinsed with methanol) prior to use.

For method validation, parameters such as accuracy, precision, selectivity, and the analytical limits (LOD, LOQ) were evaluated (Table 1). The GC/EI-MS analysis in the SIM mode provided LOQs ranging from 7.6 to 91.8 ng/ml, whereas those of most FAs ranged from 7.6 to 28.8 ng/ml, except for 18:0 (83.9 ng/ml) and 22:1n9c (91.8 ng/ml).

The calibration curve, obtained from a blank sample and seven calibration standards, was linear over a 20-fold concentration range, with coefficients of determination $r^2 > 0.995$ for all analyzed FAs.
Precision and accuracy were determined by analyzing the QC samples acquired for the intra- and interday assays. The intraday (n=5) precision ranged from 1.1 to 12.0 % (% CV), and accuracies ranged from 90.0 to 106.9 % (% bias). Interday (n=5) precision and accuracy were between 1.2 to 13.4 % and 84.4 to 106.3 % (Table 1). Bias values within an interval of ±15% of the nominal value are accepted as a tolerance limit, except for compounds with concentrations close to the LOQ, where 20% is acceptable (25). With respect to the nature and available sample volume of the matrix, these results demonstrate the applicability of the method.

**Fatty acid profile of the initial oral biofilm (pellicle)**

The lipid content of the pellicle has not been investigated thoroughly, even though lipids seem to be a significant constituent of the pellicle formed *in vivo* (16). Regarding the nature, function and composition of lipids in the acquired pellicle, the current state of research provides only preliminary information. Studies on pellicle composition are hampered by the fact that only limited amounts of pellicle material can be harvested and recovered from human teeth *in vivo* for analytical investigation. The thickness of the pellicle layer is variable and depends on the oral exposure time as well as the localization in the oral cavity. It ranges between 10 to 20 nm after 3 min and up to 500 nm on buccal sites after 2 h (8, 20, 26). Despite these limitations, precise analysis of FAs in the pellicle is possible with the presented procedure. The chromatographic separation of the 13 FAs as their methyl ester derivatives was achieved with excellent peak shapes and high responses (Fig. 1).

Although fatty acid analysis of the lipid classes in pellicle samples was reported here, saliva samples can also be analyzed using this protocol as well as other biofilms relevant for the pathogenesis of certain diseases. Examples are contact lenses or bypass.
Using the devised method, 11 FAs (12:0, 14:0, a15:0, 15:0, 16:0, 16:1n9c, 18:0, 18:1n9c, 18:2n6c, 20:0, 22:1n9c) were detected and quantified by GC-MS analysis of the pellicle samples. Among these, palmitic- (16:0) (32 %), stearic- (18:0) (21 %), oleic- (18:1n9c) (14 %), erucic- (22:1n9c) (10 %) and linoleic acid (18:2n6c) (5 %) account for the majority of FAs in the pellicle. The fatty acid profile of the pellicle seems to be characteristic for this biological structure (Fig.2). The composition is very stable. However, the total amount of investigated fatty acids shows distinctive interindividual differences among the ten study subjects (Fig.3). Values vary from 680 to 1600 ng per cm² pellicle formation surface. As compared to other pellicle parameters, the natural variability is rather low (11). Further research based on the presented method is necessary to evaluate the influence of saliva, oral localization and pellicle formation time on the fatty acid composition of the pellicle layer. Thereafter, epidemiological studies on the lipid composition of the pellicle in patients suffering from diseases such as xerostomia, periodontitis, dental erosions or caries are possible. This offers further insight into the respective pathological mechanisms and new approaches in dental prophylaxis are conceivable. This is of considerable relevance as hydrophobic interactions are essential for the process of pellicle formation and bacterial adhesion as well as for the protective properties of the pellicle layer (7). Furthermore, the method allows for investigation of potential effects of rinses with mouthwashes or edible oils on the composition and on the functional properties of the pellicle layer.

In conclusion, a comprehensive GC-MS method was developed as a practical and feasible assay, which allows the quantification of pellicle FAs and helps to understand the initial process of bioadhesion in the oral cavity, which is also governed by hydrophobic interactions. The presented study demonstrates, for the first time, a procedure based on a combination of innovative specimen generation
and convenient sample preparation with sensitive GC-MS analysis for the
determination of the fatty acid profile of the initial oral biofilm.
Notice of grant support

This study was supported by a scientific grant from the German Research Foundation (DFG; HA 5192/2-1; KU 1271/6-1).
REFERENCES


**Fig. 1.** GC/MS chromatogram of a pellicle sample (formation time 30 min) acquired in SIM mode. The sample was separated through a thermally stable Select FAME capillary column (50 m x 0.25 mm ID, 0.25 µm film thickness). The GC oven program started at 50 °C (hold time 5 min) and was increased to 260 °C (hold time 8 min) at a ramp rate of 6.5 °C/min. Characteristic fragment ions ($m/z$ 74, $m/z$ 87, $m/z$ 81, and $m/z$ 79) were monitored throughout the run.
Fig. 2. Fatty acid composition of pellicle sample (formation time 30 min). Values represent the means ± SD of 10 subjects, expressed as % of the investigated FAs. This profile seems to be characteristic for the biological structure of the pellicle.
Fig. 3. Amount of total investigated fatty acids of the 30 min pellicle of the ten study subjects (A-J). Values vary from 680 to 1600 ng per cm$^2$ pellicle formation surface, illustrating interindividual differences (dashed line marks the mean amount of total fatty acids of the ten subjects).
TABLE 1. Validation results of the overall method in the intra- and interday assays.

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<th>FA</th>
<th>RT</th>
<th>Linearity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>LOD&lt;sup&gt;b&lt;/sup&gt;</th>
<th>LOQ&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Intraday QC&lt;sub&gt;low&lt;/sub&gt; (n=5)</th>
<th>Intraday QC&lt;sub&gt;high&lt;/sub&gt; (n=5)</th>
<th>Interday QC&lt;sub&gt;low&lt;/sub&gt; (n=5)</th>
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<td>ng/ml</td>
<td>CV&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Accuracy&lt;sup&gt;d&lt;/sup&gt;</td>
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<sup>a</sup> Calibration range from 12.5 ng/ml to 250 ng/ml and 62.5 ng/ml to 1250 ng/ml.

<sup>b</sup> The limit of detection was measured at S/N ratio > 3.

<sup>c</sup> The limit of quantification was measured at S/N ratio > 10.

<sup>d</sup> Precision and accuracy were expressed as the mean values of data obtained from QC samples (QC<sub>low</sub>: 30, 150 ng/ml and QC<sub>high</sub>: 175, 875 ng/ml, depending on the particular FA) through intra- and interday assays.