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Method Development for Gas Chromatographic Analysis of Long-Chain Fatty Acids in Saliva and Other Human Matrices

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List of Abbreviations

Abbreviation	Definition
BF_3	Boron trifluoride
$CHCl_3$	Chloroform
FA(s)	Fatty acid(s)
FAME(s)	Fatty acid methyl ester(s)
FFA(s)	Free fatty acid(s)
GC	Gas chromatography
HCl	Hydrochloric acid
IS	Internal standard
MeOH	Methanol
MTBE	Methyl tert-butyl ether
NaOH	Sodium hydroxide
SD	Standard deviation
TGs	Triacylglycerols

1 Introduction

The COVID-19 pandemic has shown the importance of non-invasive, rapid, easy-to-use and reliable diagnostic tools. Saliva has been an essential diagnostic tool for the detection of SARS-CoV-2 and has sparked interest in further using it for the development of new diagnostic methods.[1]

Saliva is an ultrafiltrate of plasma and can thus be used as an alternative for the analysis of different biomarkers.[2] It plays an important role for the immune system, oral and general health.[3] Many diseases such as diabetes, certain cancers or autoimmune diseases like Sjogren´s syndrome significantly affect salivary flow and composition.[4,5,6] In recent years, the field of salivaomics has gained importance, focusing on identifying various salivary biomarkers for disease diagnosis. These biomarkers include viral RNA, antibodies,[7] and inflammatory cytokines, such as those linked to inflammatory bowel disease.[8] Lipids, in particular, have emerged as valuable biomarkers for different conditions. For instance, research has shown that patients with cystic fibrosis or diabetes exhibit distinct and elevated lipid profiles compared to healthy controls.[9]

In regards to nutritional sciences, saliva also has potential as a diagnostic tool for general eating habits, particularly fat intake. Traditional dietary assessment tools that rely on examining dietary habits like 24-hour recalls, food frequency questionnaires or dietary records are prone to reporting biases.[10] By analyzing various biomarkers such as carbohydrates, proteins, and lipids, saliva could offer a more precise alternative to traditional methods. However, as the use of saliva for diagnostic purposes is still relatively new, established methods for evaluating salivary lipid profiles remain limited.

For the analysis of lipids, a number of different types of chromatography have been used. However, gas chromatography (GC) has been proven to be one of the most reliable methods for fatty acid analyses.[11] A distinct characteristic of GC is that only volatile compounds can be analyzed. Since especially longer-chain fatty acids are not volatile, they have to be derivatized into their methyl esters before GC analysis is possible.[12] For this, various derivatization strategies can be used, typically categorized into base- and acid-catalyzed methods[13], with NaOH and HCl being most commonly used.

Before derivatization, lipids can optionally be extracted from the samples using one of several established methods.[13] The gold standard of lipid extraction is the method described by Folch et al.[14] It entails a number of disadvantages however, e.g. time inefficiency and a high usage of the toxic solvent chloroform.[15] Because of this, a number of alternatives, such as the Matyash method, have been developed. It is more precise and uses methyl tert-butyl ether (MTBE) as a less toxic alternative to chloroform.[16]
Additionally, samples can be concentrated using a vacuum concentrator to enhance detection sensitivity. However, this step is not always necessary and depends on the specific analytical aims. Both concentrated and non-concentrated samples were tested in this thesis to determine the optimal approach.

This thesis focuses on developing a reproducible and reliable method for analyzing fatty acids in saliva and cheek cells. A major challenge in saliva-based diagnostic tests is ensuring their diagnostic efficacy in terms of sensitivity, specificity, reproducibility, and correlation with established diagnostic criteria.[17]

For the initial experiments, the Salivette® sample collection tool was employed. However, despite multiple adjustments, fatty acid peaks did not increase as anticipated, and impurities remained prominent. To address these challenges, an alternative sampling approach was adopted. Instead of relying solely on saliva, cheek cells were collected using a toothbrush, based on a method described by Grindel et al. Their study investigated the impact of linseed oil supplementation on cheek cell fatty acid composition.[18]

Although the cheek cell sampling method showed promise, persistent impurities and limited improvement in fatty acid peak detection led to the exploration of a third biological matrix: capillary blood. While slightly more invasive than saliva, capillary blood remains a practical option due to its ease of collection, cost-efficiency, and time-saving nature. Preliminary results from experiments with capillary blood are presented in this thesis, offering valuable insights for future work.

The focus of the method development in this thesis lies on optimizing the sample preparation and methylation of fatty acids, as the chromatographic conditions have already been selected prior to this project. Method optimization was continued and completed, and the final steps will involve validating the method and conducting smaller studies to evaluate its application across various parameters such as gender, age, diet, and diseases. This approach ensures that while the chromatographic conditions were kept constant, the sample preparation process could be further refined to improve the overall quality of the analysis.

2 Literature Review

2.1 Characteristics and Functions of Saliva

2.1.1 Saliva Characteristics

Saliva is a complex biological fluid which components vary according to flow rate, diet and collection time.[13] Other factors that affect saliva compostion include age, sex, different levels of physical activity, diet and general health.[19]

Saliva is produced by three large (main) glands – the parotid (in front of and just underneath both ears), the submandibular (under the jaw) and the sublingual (below the tongue) gland – and hundreds of small (minor) glands.[3] After production, it is secreted into the oral cavity by salivary ducts.[20] Besides secretions of the salivary glands, whole-mouth saliva consists of gingival crevicular fluids and fluids exuded from mucosal membranes.[21]

Around the salivary glands, there is a dense network of capillaries, blood vessels, and acini. The capillaries in this region exhibit high permeability, allowing the exchange of molecules. As a result, biomarkers present in the bloodstream can permeate the acini and ultimately be released into saliva.[4] Different blood components migrate into the saliva by means of either active transport or passive diffusion across cell membranes.[22]

Saliva mainly consists of water (99%). The remaining 1% is made up of organic compounds like cholesterol, fatty acids, triglycerides, neutral lipids, glycolipids, glucose, amino acids, glycoprotein, steroid hormones, amylase, mucin, mucopolysaccharide, lysozymes, peroxidase and lactoferrin, as well as inorganic molecules (Na+, Cl–, Ca2+, K+, HCO3–, H2PO4–, F–, I–, Mg2+ and SCN–).[4,23] The protein and ion components are responsible for its viscoelastic properties.[23] Saliva also contains more than 700 microorganisms that correlate with different oral and systemic diseases.[4]

The average person produces around 600 mL of saliva daily.[4] Saliva secretion is influenced by nerve-mediated stimuli that lead to an activation of glandular fluid and protein secretory mechanisms. The frequency and intensity of these stimuli regulate the saliva volume produced by salivary glands and are influenced by both long-term and short-term factors. For instance, food intake leads to a sharp increase in saliva secretion in the short term, whereas long-term changes in saliva secretion can occur due to dietary shifts or aging. These physiological factors can modify the composition and functionality of saliva within the oral cavity.[24]

Salivary flow changes during the day and almost completely stops during sleep. Its buffering capacity helps maintain the pH within a relatively constant range between 6.6 and 7.1.[4] The neutral pH protects teeth and oropharyngeal mucosa from dietary and bacterial acids. Saliva is maintained in a state of supersaturation with hydroxyapatite, which serves as a protective measure against the demineralization of teeth.[25]

Saliva production can be restricted for a number of different reasons. For instance, certain medications can cause salivary hypofunction (= clinically objective decrease in

saliva production) and Xerostomia (= sensation of oral dryness). Additionally, HIV-AIDS and autoimmune diseases can affect saliva production.[20] In some rarer cases, infections or tumors in the salivary glands and stones (sialoliths) in the gland ducts can also impair salivary function. Individuals with these conditions are generally more susceptible to oral candidiasis, possibly due to a weakened antimicrobial defense mechanism in saliva.[26]

2.1.2 Functions of Saliva

Saliva has many important functions, the main ones being: 1) lubrication, 2) taste, 3) digestion, 4) buffering, 5) tooth integrity, 6) microbial homeostasis and protection and 7) wound healing.[27,22]

1) The compounds mainly responsible for lubrication are mucins (= complex protein molecules).[27] Lubrication of food particles facilitates their movement, improves food bolus cohesion and is essential for digestion.[19]

2) Food perception depends on both the composition of unstimulated saliva and the degree of salivation which changes according to the food consumed. Salivary components, including different proteins and low-molecular-weight compounds like ions and organic acids, modulate the sensitivity of taste receptors. This way, individual saliva composition changes each person´s sensation of taste. Different enzymes like α-amylase for carbohydrate-rich foods, lipolytic enzymes for the perception of high-fat foods and esterases for food aroma also change taste perception in individuals.[19]

3) In addition, these enzymes start the digestion process in the mouth with salivary amylase, for example, hydrolysing starch into maltose and occasionally glucose.[4]

4) In regards to buffering, there are three main components in whole saliva that allow a buffer capacity: bicarbonate, phosphate and protein buffer systems.[22] Buffer activity is affected by flow rate and is higher during stimulation (due to the higher levels of bicarbonate[28]) and lower in unstimulated saliva. It changes plaque and saliva pH, an important factor as pH needs to be above 5.5 for the remineralization process. For this, Ca^{2+} and phosphate ions repair the enamel.[27]

5) Different salivary proteins such as acidic proline-rich proteins, histatins, cystatins and especially statherins play a crucial role in tooth integrity as they impede the precipitation of calcium phosphate salts from saliva.[28]

6) Saliva also plays an important role in the innate immune system of humans. Components like lysozymes, hydrogen peroxide and thiocyanate ions have bactericidal properties.[4,3] At the same time, when a patient is infected with a virus such as HIV, rabies or polio, it is able to excrete and transmit the respective viruses. Similar effects are observed with substances such as potassium iodide (KI), lead (Pb), and mercury (Hg).[4] The balance of oral microbiota also depends on the salivary buffer capacity and its ability to maintain pH at a neutral level.[28]
Due to saliva's crucial role in the immune system, compromised saliva production leads to a higher incidence of oral issues like oral candidiasis, periodontal disease, and dental caries, as well as respiratory tract infections.[3]

7) Lastly, saliva aids the wound healing process in the mouth.[27] Different factors contribute to this process: the humid environment that fosters inflammatory cells essential for wound healing, tissue factors promoting blood clotting, and substances like epidermal growth factor necessary for epithelial cell proliferation.[29]

An additional characteristic of saliva is that it reflects hormone concentrations in blood and can therefore be used to assess endocrine function. It contains both nonsteroid hormones (e.g. catecholamines, somatostation, prolactin, thyroxine, gonadotrophins, etc.) and steroid hormones (e.g. aldosterone, sex hormones, etc.).[30]

2.2 Saliva as a Diagnostic Tool

2.2.1 Saliva as a Diagnostic Tool for Infectious Diseases

The COVID-19 pandemic has shown the importance of non-invasive, rapid, easy-to-use and reliable diagnostic tools.[21] Saliva represents a clinical alternative to blood plasma as it contains the same components, albeit in different concentrations.[31]
Saliva tests may provide a faster, cheaper and safer diagnostic alternative to blood sampling, especially in children and elderly patients.[32]
Another advantage is the simplicity of repeated saliva sampling, which makes it a valuable diagnostic tool for longitudinal studies.[24] Unlike other non-invasive methods such as urine, sweat, and tears, saliva presents fewer concerns regarding privacy intrusion and restricted access.[22]
Saliva tests are not expected to replace blood tests for all diagnostic applications, but they hold promise for detecting diseases where early diagnosis is critical.[33]

In regards to SARS-CoV-2 infections, the following advantages of salivary samples and the salivary diagnosis process have been described:
The procedure is minimally invasive, reliable, inexpensive, applicable for all ages and offers real-time diagnostic value. Saliva is also easy to handle and store.[7] Its high viral load, resulting from secretions originating from multiple sources, increases the likelihood of virus detection. Furthermore, since individuals can self-collect saliva samples, the risk of infection to healthcare personnel is reduced, making it a safer diagnostic tool.[34]
Many of these advantages are also applicable to the diagnosis of other infectious disesas like the ZIKA virus, Helicobacter pylori bacterial infection, fungal infections and others.[35]

2.2.2 Saliva as a Diagnostic Tool for Non-Communicable Diseases

In addition to COVID-19, saliva has been playing an increasingly important role in the diagnosis of other diseases. Using omics technologies, different disease biomarkers could be found through analyzing changes in saliva composition.[24] Biomarkers are molecular signatures and indicators of both normal biological processes and pathological conditions, as well as the body's response to treatment. Consequently, they can offer valuable insights for disease detection, diagnosis, and prognosis.[21]

The concept of salivaomics was introduced in 2008. Salivaomics defines a combination of genomics, transcriptomics, proteomics, metabolomics, microRNA (miRNA) and microbiome analysis.[4,36]

Today, saliva is recognized as a potential reservoir of biological indicators, encompassing alterations in biochemicals, DNA, RNA, proteins, and microbiota composition.[4] The increasing recognition of the microbiome's significance in human health has spurred researchers to examine the oral microbiome more closely. They utilize saliva as a sampling method to capture a glimpse of the microbial species present, aiming to identify microbial biomarkers associated with both oral and systemic diseases.[37] Saliva, being a readily collectible bodily fluid, can to some extent mirror the pathophysiological alterations within the human body.[5]

For instance, recent studies showed that immune cells**,** cytokine concentrations**,** and concentrations of other biomarkers were altered in the saliva of patients with Primary Sjogren´s Syndrome.[38,39,6] (In Sjogren´s Syndrome the salivary glands are, besides the tear glands, main targets of inflammation.[9])

Likewise, inflammatory cytokines were correlated with inflammatory bowel disease like Chron´s Disease or Ulcerative Colitis. However, in this case the state of the oral cavity, e.g. periodontitis and/or dental caries also affected the levels of oxidative biomarkers in saliva.[8] Moreover, correlations have been shown between salivary biomarkers, aging and Alzheimer´s Disease[40], as well as different cancers.[41,21,5] For Diabetes mellitus there was found a significant correlation between HbA1c levels and salivary glucose concentrations in individuals with type 2 diabetes. This suggests that saliva could serve as a viable means of monitoring blood glucose levels in patients with diabetes mellitus. In regards to CVD, affected patients showed lower salivary concentrations of α-2-HS-glycoprotein, while IL-1β, IL-6, TNF-α and prostaglandin E2 exhibited a significant increase in both atherosclerosis and periodontal disease. Acute myocardial infarction was mainly predicted by C-reactive protein.[4]

2.2.3 Salivary Lipids as Biomarkers

Patients with cystic fibrosis exhibit distinct salivary lipid profiles compared to healthy individuals. Their saliva contains significantly higher levels of certain lipids, including twice the amount of phospholipids, 54% more fatty acids, 35% more triglycerides, and 42% more cholesterol. Moreover, the glycerolipid pattern in cystic fibrosis patients differs from that in healthy individuals.[9]
In a rat model, induction of diabetes led to a rise in stearic acid (C18:0) and linoleic acid (C18:2 n-6) in all salivary glands, whereas oleic acid (C18:1 n-9) and arachidonic acid (C20:4 n-6) decreased significantly in certain glands. This decrease may be caused by an inhibition of desaturase enzymes which are activated by insulin. Insulin treatment has therefore been shown to reverse the effects diabetes poses on salivary lipids.[9]
Despite the numerous advantages of saliva as a diagnostic tool, every method also has limitations. In the case of saliva, both composition and volume vary between individuals, as does the concentration of specific biomarkers. Additionally, various medications can affect salivary composition and quality. Proteolytic enzymes in the oral cavity, such as amylase and proteases, may degrade salivary proteins, thereby reducing the reliability of diagnostic measurements.[7]

2.3 Saliva as an Indicator of Dietary Fat Intake

Apart from the diagnosis of different diseases, human saliva may also function as an indicator of dietary fat intake. Conventional methods of nutritional assessment are often susceptible to different reporting biases which leads to less reliable results. Therefore, saliva could potentially pose as a more precise alternative for the assessment of dietary fat intake. For instance, in one study salivary fatty acid content was determined by gas chromatography and compared between two different diets (mixed and vegetarian). Study participants with a vegetarian diet had higher salivary concentrations of alpha-linolenic acid (18:3 n-3) and lower concentrations of arachidonic acid (20:4 n-6) than those with mixed diets ($p = 0,045$). The salivary levels of arachidonic acid in vegetarian subjects also correlated with dietary levels obtained by Food Frequency Questionnaires.[10]

Moreover, since the quantity and quality of the consumed dietary fat is correlated with different diseases like cancer or cardiovascular diseases, the assessment of dietary fat through saliva could contribute to preventing fat-intake related diseases.[10] In a mouse model from 2021 excess uptake of n-3 and n-6 polyunsaturated fatty acids led to ferroptosis-mediated antitumor effects.[42] An intervention study from the same year demonstrated the positive effects of omega-3 fatty acids supplementation in pediatric cancer.[43] In contrast, cholesterol and saturated fatty acids might promote the malignant progression of prostate cancer.[44]

In a controlled study with monkeys, they fed one group hydrogenated coconut oil (HCO) and the other group corn oil. The HCO group demonstrated a decrease in linoleic acid (18:2 n-6) in submandibular and parotid saliva in comparison to the corn oil group.[45]

In a different study on the association of microbiome and metabolome composition with dietary protein intake and salivary pH, a positive correlation between overall dietary protein intake and salivary pH was found, with males exhibiting higher protein intake compared to females. This suggests that diet may play a significant role in altering salivary pH, which is one of the most influential environmental factors in the oral cavity.[46]

2.4 Salivary Lipids

Before 2005, lipids had simply been defined as hydrophobic compounds insoluble in water and mostly soluble in organic solvents.[47,9] In 2005, the International Lipid Classification and Nomenclature Committee, in collaboration with the LIPID MAPS Consortium, established a comprehensive worldwide classification system for lipids. This system divides lipids into the following 8 main categories: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides.[47] The classification system is based on well-defined chemical and biochemical principles. It has been accepted internationally and updated since 2005 to provide more detailed information.[48]
The first 5 of the lipid categories are found in saliva[47,48] and will be discussed below.

Salivary lipids were first mentioned by Doubleday in 1909.[49] Later analyses showed total lipid concentrations of 0.9 to 8 mg/100mL in submandibular saliva and concentrations ranging from 0.24 to 7.6 mg/100 mL in parotid saliva. The variations among studies might be due to differences in methodology like sample collection as well as analytical approaches.[10]

Lipids are a very important component of saliva, as different diseases and pathological states affect their composition in quality and quantity.[9] They play a role in intercellular transport and different signal transduction pathways between the salivary gland and other tissues and they influence the fluidity and permeability of cellular membranes.[9] Moreover, the free fatty acids released following lingual lipase activity could potentially exhibit antimicrobial properties, presenting an additional host mechanism in influencing the composition of the oral microbiota.[46]

2.4.1 Free Fatty Acids

Free fatty acids make up the biggest percentage of all salivary lipids. Free fatty acids are characterised by a hydrocarbon chain ending with a COOH group. The CH2- chain accounts for their hydrophobic properties, while the COOH group imparts hydrophilic properties. The combination of hydrophilic and hydrophobic groups in the molecule is responsible for the amphipathic nature of free fatty acids.
According to the number of carbon atoms, free fatty acids can be divided into short-chain (<6C), medium-chain (8-14C), and long-chain (>16C) free fatty acids, with the most prevalent molecules generally ranging from 4 to 30 carbon atoms.[9]

Fatty acids can also be differentiated by their number and position of double bonds. Fatty acids without any double bonds are known as saturated, whereas those with one or more double bonds are known as unsaturated. Regarding unsaturated fatty acids, only free fatty acids with a double bond at the n-9 position can be produced endogenously. Fatty acids with the double bond at the n-6 or the n-3 position on the other hand, linoleic acid and α-linolenic acid (ω-3) respectively, cannot be synthesized by the body, necessitating their acquisition through dietary sources. These are known as essential fatty acids.[9]

Fatty acids containing double bonds can occur as geometric isomers, namely cis and trans fatty acids. While in the trans configuration, the two hydrogen atoms adjacent to the double bond are positioned on opposite sides, in the cis configuration, these hydrogen atoms are located on the same side.[50]

In the human body, fatty acids are predominantly bound to other molecules, particularly proteins (e.g., cholesterol and phospholipids), and only rarely occur in their free fatty acid form.[13]

2.4.2 Glycerolipids

Glycerolipids are defined as all glycerol-containing lipids, the main group being acylglycerols. Acylglycerols are esters formed from fatty acids and glycerol and they contain three different groups: monoacylglycerols (MGs), diacylglycerols (DAGs) and triacylglycerols (TGs).[47,48] TGs are more common than MGs and DAGs and – unlike the other 2 – do not form dispersed micelles.[9]

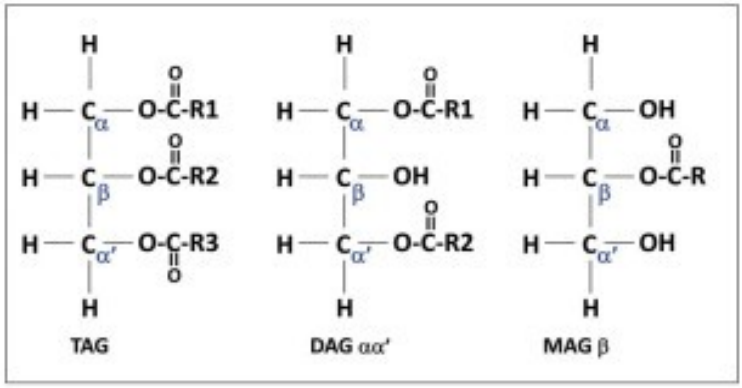


Figure 1: The three types of acylglycerols: triacylglycerol (TAG), diacylglycerol (DAG) and monoacylglycerol (MAG) [52]

Glycerophospholipids represent one of the most abundant subcategories within the glycerolipid group. These compounds share a common structural feature: phosphatidic acid (PA). The phosphate group of PA can bind, via an ester bond, to a molecule possessing a hydroxyl group. Examples of such molecules include choline, ethanolamine, serine, inositol, or another glycerol unit.[9,51]
Among linked fatty acids, acids attached at the C1 carbon atom are typically saturated, while those attached at the C2 atom are unsaturated with one or more double bonds.[9]
Related lipids include sphingolipids that contain aminoalcohol instead of glycerol, with sphingomyelin as its most well-known representative.[9,52]

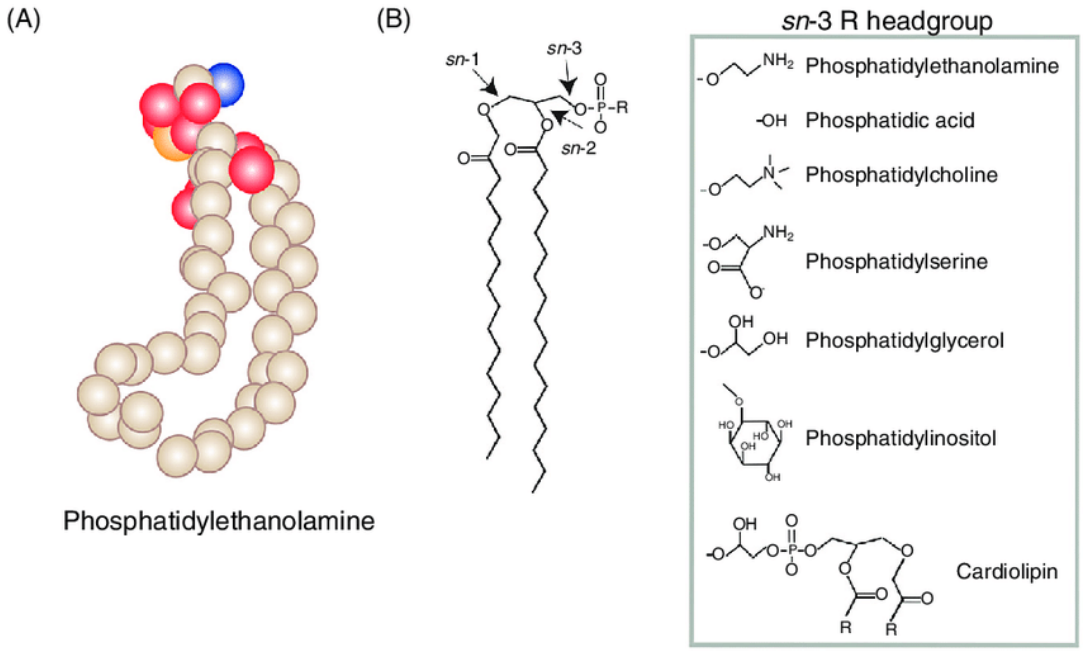


Figure 2: The glycerophospholipids

(A) Diagram showing the structure of phosphatidylethanolamine, with different spheres representing atoms: carbon (tan), oxygen (red), phosphate (orange), and nitrogen (blue). Hydrogen atoms are omitted.
(B) General structure of glycerophospholipids, where fatty acids attach to the glycerol backbone at the sn-1 and sn-2 positions, and the phosphate headgroup binds at the sn-3 position. Variations in headgroups are indicated. [55]

Cholesterol is a cyclic unsaturated alcohol with a single hydroxyl group. It can either be obtained from external sources or produced endogenously within the cytoplasm and endoplasmic reticulum of hepatocytes.[53,54] In bodily fluids, cholesterol is transported in conjunction with lipoproteins. It constitutes a significant portion of cell membranes, excluding the mitochondrial membrane, and within the cytoplasm, it exists in an esterified form, bound to fatty acids.[9]

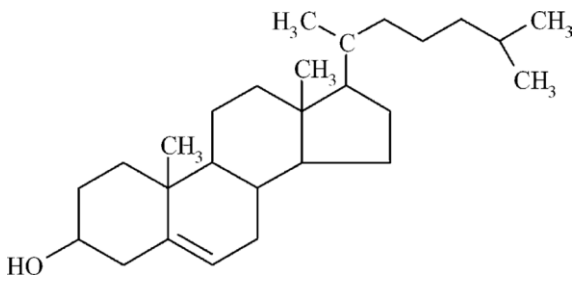


Figure 3: Chemical structure of cholesterol [57]

Phospholipids, cholesterol, and sphingolipids serve as essential structural and functional components for cell and intracytoplasmic membranes. Their presence significantly influences the flexibility, fluidity, and permeability of these membranes. Phospholipids such as inositol phospholipids can also play a crucial role as second messengers and in salivation processes.[9,55]

2.5 Analysis of Fatty Acids with Gas Chromatography

In general, gas chromatography for fatty acid identification is divided into three main steps: 1) extraction of the fatty acid from the sample matrix, 2) derivatization of the fatty acids and 3) GC analysis.[13]

The aim of lipid extraction is to separate as many lipids as possible from their non-lipid components. This process commonly involves the use of nonpolar organic solvents such as chloroform, methyl-tert-butyl ether, and heptane to effectively separate lipids from the aqueous phase.[31] There is a variety of well-established extraction methods and, in general, they can be used for distinct biological samples.[13] Most extraction protocols, however, have been developed for serum or plasma samples,[56] with fewer protocols specifically tailored for saliva and buccal cells. To receive optimal results for particular target analytes, method optimizaton is necessary.[13]

Instead of first applying an extraction method, lipids in the samples can also be directly derivatized.[13] Notably, derivatization is particularly crucial for fatty acids with more than 10 carbon atoms, as their volatility needs to be enhanced for gas chromatographic analysis. To achieve this, long-chain fatty acids are converted into their methyl esters, which allows for their identification.[12]

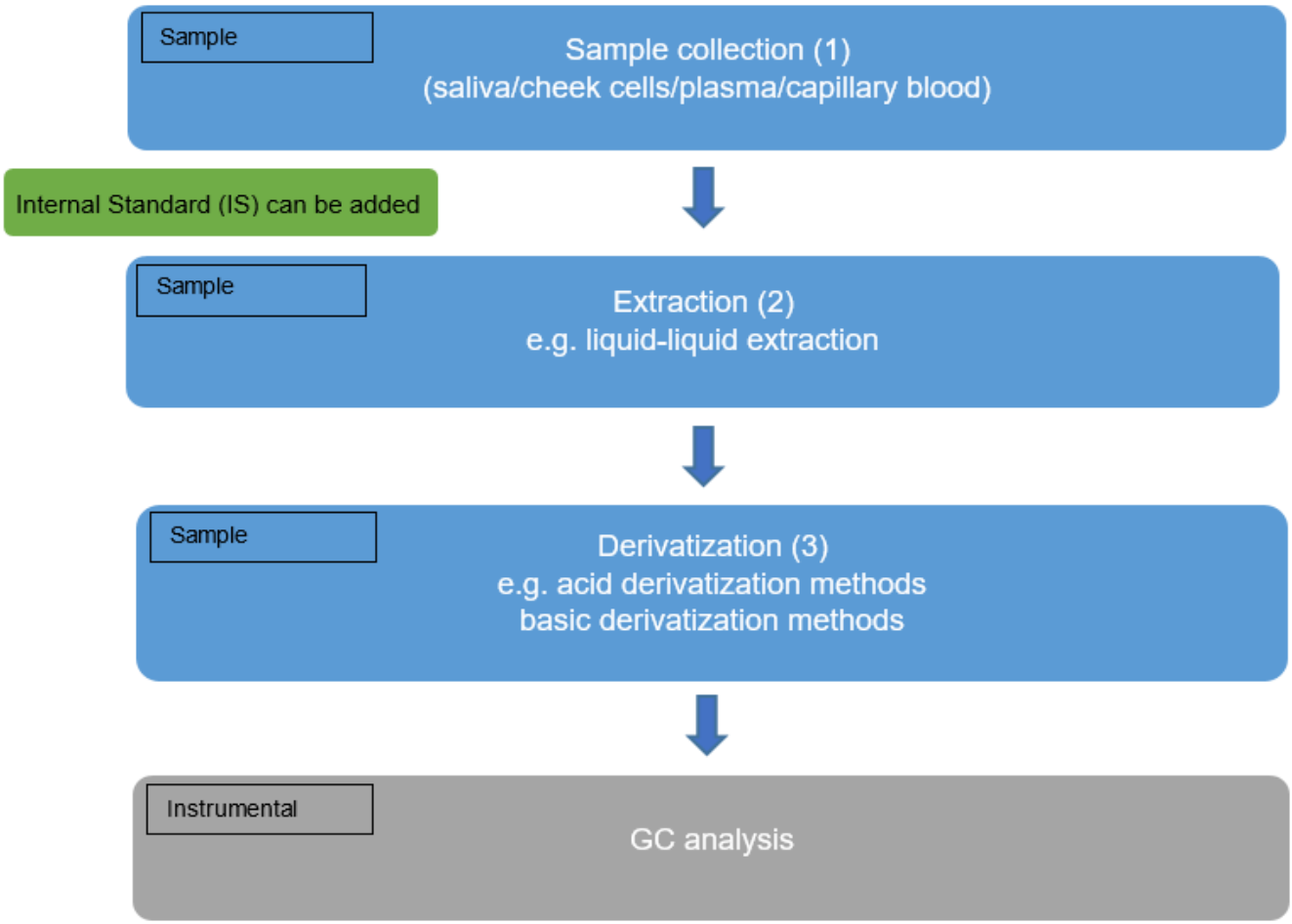


Figure 4: Required steps for sample preparation for GC analysis of fatty acids, adapted from reference [13]

2.5.1 Sample Preparation

2.5.1.1 Extraction Methods

Generally, there are two different extraction methods, liquid-liquid extraction and solid-phase microextraction. For this thesis, we worked exclusively with liquid-liquid extraction methods. Therefore, only this technique will be discussed further.

In contrast to liquid-liquid extraction, solid-phase microextraction uses salting-out systems instead of solvents. Common salting-out reagents include sodium chloride (NaCl) and sodium sulfate (Na2SO4).[13]

2.5.1.1.1 Liquid-Liquid Extraction

Liquid-liquid extraction is commonly applied for the extraction of fatty acids in FA analysis.[13] Different combinations of organic solvents can be used for extraction, with the most well-known lipid extraction methods being those by Folch et al.[14] and Bligh and Dyer.[57]

The extraction method developed by Folch et al. has been established as the gold standard method for lipid extraction. With this method, a combination of chloroform and methanol (2:1 v/v) is used as the extraction solvent. After adding the solvent mixture to the sample, either water or a salt solution is applied to induce phase separation. Finally, the lower phase is collected for fatty acid analysis.[15]

For the method by Bligh and Dyer, a mixture of chloroform, methanol and water at a ratio of 2:2:1.8 is used mostly for water-rich biologial samples including tissue and blood. The Bligh and Dyer method combines the advantages of high recovery and low solvent consumption.[13]

The application of both the Bligh and Dyer method and the Folch method involves the use of highly toxic chloroform as one of the extraction solvents, which is a significant concern. The relatively high consumption of toxic solvents also makes these methods unsuitable for large-scale applications.[15]

For these reasons, there has been a search for alternatives to chloroform like water:ethanol:ethyl acetate or butanol:methanol (BUME) mixtures.[15,58] Another study advocates for the use of methyl-tert-butyl ether (MTBE) instead of chloroform, as their investigations showed that MTBE provided faster and cleaner lipid recovery. Furthermore, MTBE's lower density compared to chloroform causes the lipid-containing organic phase to form the upper layer during phase separation. This makes its collection substantially easier and reduces dripping losses. Matyash et al. reported overall recoveries of 90-98%, which is comparable to the Folch approach.[16]

In addition to these approaches, Hara and Radin developed a lipid extraction method applying the low toxicity solvents hexane and isopropanol.[59] This method was found to offer higher extraction recovery for total FAs than the Bligh and Dyer method and shorter sample preparation times**.** It has also been applied to both plasma and erythrocyte samples.[60,61]

2.5.1.2 Derivatization Methods

For GC analysis of fatty acids, particularly those with more than 10 carbon atoms, derivatization is typically required.[13] The derivatization increases the volatility of lipid components, enhances peak shape and therefore provides better separation. While numerous derivatization procedures are described in the literature, most of them convert fatty acids into their respective esters, typically methyl esters.[62] This section provides an overview of frequently used methods for fatty acid derivatization. Acid derivatization techniques can generally be applied to analyze total fatty acids (including both free and esterified forms), whereas basic derivatization methods are primarily suited for esterified fatty acids.[63]

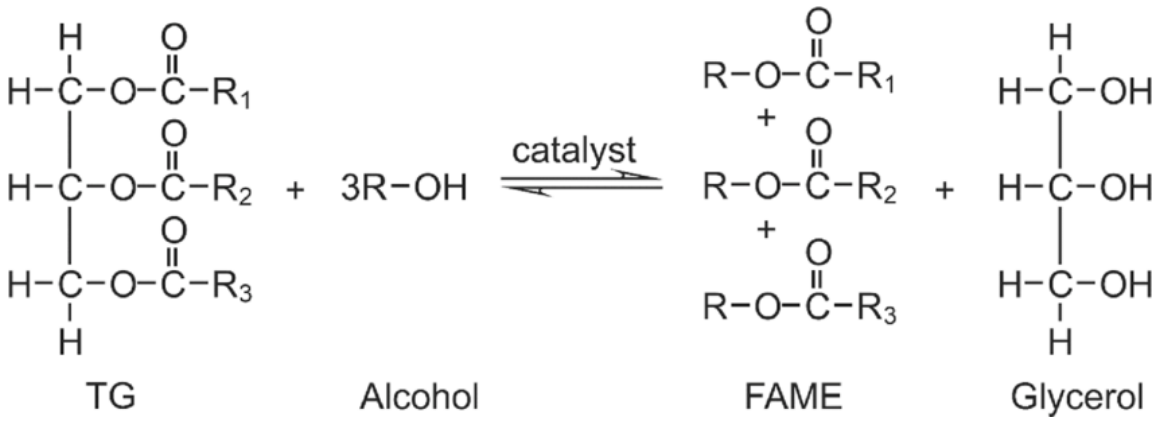


Figure 5: Transesterification of triglycerides [67]

2.5.1.2.1 Acid Derivatization Methods

The most frequently used acid derivatization reagents include hydrochloric acid (HCl), boron trifluoride (BF_3), acetyl chloride (CH3COCl) and sulfuric acid (H2SO4). The HCl derivatization method is widely favored for fatty acid analysis due to its straightforward operational procedure.[64,65] For the HCl derivatization, methanolic HCl is added to the sample and the mixture is subsequently heated for a specific time period. Limited solubility of certain lipids in methanolic HCl may necessitate the addition of a second solvent before derivatization.[64]

The BF_3 derivatization method is applicable to a variety of biological samples and has been used for fatty acid analysis for several decades.[66,67,68] While the BF_3 method offers effective derivatization, concerns have been raised in various studies regarding its stability and the potential formation of artifacts.[69,70,71]

In addition to HCl and BF_3 derivatization, other widely employed acid derivatization methods utilize acetyl chloride or H2SO4 as derivatizing agents.[72,73,74,75] However, these methods have limitations for PUFA analysis.[13]

In general, while acid-based derivatization methods are frequently applied in the analysis of biological samples due to their numerous advantages, it is important to

acknowledge their potential to influence the isomer distribution within the conjugated system as a notable concern.[70,76]

2.5.1.2.2 Basic Derivatization Methods

Basic derivatization methods offer several advantages, including short derivatization times, absence of double bond isomerization concerns, ease of operation and the use of less aggressive reagents. Nonetheless, they are unsuitable for the derivatization of free fatty acids (FFAs).[77]

Frequently used basic derivatization methods include the sodium methoxide (NaOCH3) and the potassium hydroxide (KOH) derivatization technique. The KOH method offers a straightforward protocol with a short reaction time. This involves adding methanolic KOH to the lipid extract, followed by incubation at room temperature or heating to 50°C for a few minutes for fatty acid derivatization. Subsequently, sodium bisulfate (NaHSO4) is introduced to achieve phase separation, and the supernatant is collected for GC analysis.[77]

Another common basic derivatization reagent includes NaOH.[62]
While derivatization methods using basic reagents can only be applied to esterified fatty acids, acid reagents can be used for total fatty acids (including free fatty acids and esterified fatty acids).[13] Therefore, basic and acidic derivatization reagents can be combined for a simplified methylation process and better results. A possible combination involves NaOH–BF_3–MeOH and has been applied by Quin et al. before.[78,82]

2.5.2 Fatty Acid Internal Standards

Internal standards are rountinely used in biomedical analysis to ensure correct and precise (quantification) results.[13]
A requirement for internal standards is that they possess an odd number of carbons because naturally only fatty acids with even numbers exist.[13] In the past, fatty acids like C13:0, C15:0, C17:0, C19:0, C19:1, C21:0 and C23:0 have been used as internal standards.[79,80,73,77]
They are added to the sample before or during preparation to correct for potential variations in the analysis.[13] The use of internal standards allows substances to be analyzed both qualitatively and quantitatively.[81]

2.5.3 Gas Chromatography Structure & Fundamentals

Chromatography is a widely used analytical technique for separating a mixture of substances into its individual components. There are many different types of chromatography, e.g. ion-exchange chromatography, high pressure liquid chromatography (HPLC), gas chromatography (GC), etc.[11]

Gas chromatography is one of the most reliable methods for fatty acids analyses.[11]
It is time-efficient, sensitive, offers a high peak capacity and can be combined with a number of selective detection methods, such as mass spectrometry (MS).[82,12]

However, it is only able to separate and identify volatile compounds, and derivatization of the samples may reduce reproducibility and be time-consuming.[12] Analyzed compounds must also be thermally stable,[82] and even then, the high temperatures required for sample vaporization inevitably cause some decomposition.[81]

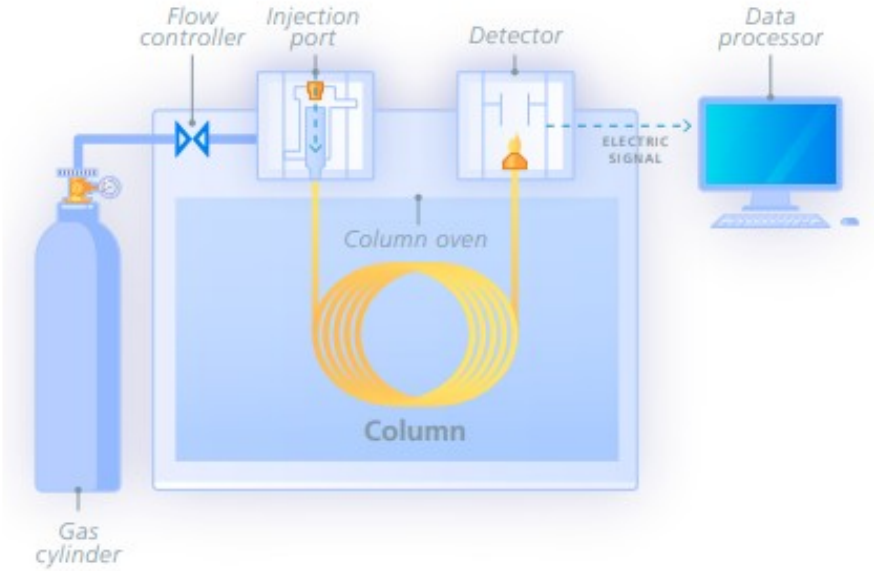


Figure 6: Typical GC configuration [86]

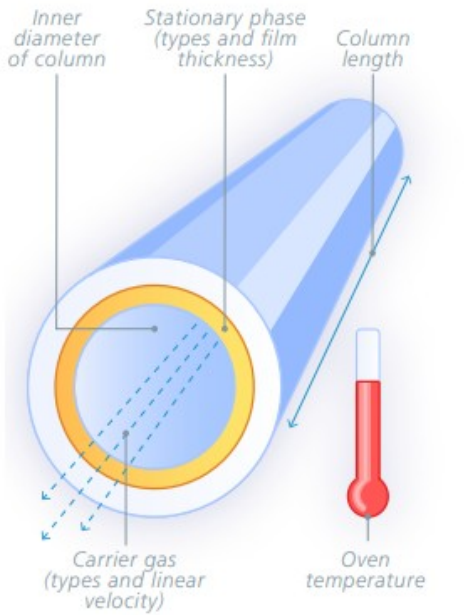


Figure 7: Factors that affect separation in a GC Column [85]

The gas chromatograph is made up of a stationary phase, which consists of a liquid film held by a solid support, and a moving phase. While the stationary phase comes in the form of a long column, the mobile phase consists of controlled gas.[81]

For the analysis, a sample gets injected into the gas chromatograph, is then converted into a gaseous state and transported by a carrier gas trough the column.[81]

The column is located in a temperature-regulated oven, as temperature affects the volatility of the analytes.[82] Volatile compounds with boiling points up to 350°C or 400°C can be analyzed with a GC column, depending on the substances undergoing the separation.[81]

The separation relies on the retardation of the individual compounds as they travel through the column with the carrier gas, typically helium or nitrogen. Sometimes also hydrogen or another inert gas is used.[82] The carrier gas has to be inert, as it should transport the sample, but not interact with it.[83]
The duration a compound spends traveling through the chromatographic column is referred to as its retention time. Retention times depend on characteristics such as the sample's boiling point and size, along with column properties like length, diameter, and temperature.[82] The column exit connects to a detector which registers when a compound leaves the column and sends this information to a computer that creates a chromatogram with it.[82]

Injectors:

A variety of GC injectors exist, including the following:

- Split
- Splitless
- On-column Injector[74]

For split/splitless injections, a sample is introduced into a small heated chamber via a syringe through a septum. The application of heat aids in the vaporization of both the sample and its matrix. For the split/splitless injection usually autosamplers are used that allow for a high throughput of samples.[83]
In split injection, the oldest and most widely used technique, only a portion of the vaporized sample enters the column, while the rest is diverted through a split or purge valve to avoid overloading. Split ratios usually range from 1:10 to 1:100. The split inlet is commonly used for the analysis of concentrated or pure samples.[84]

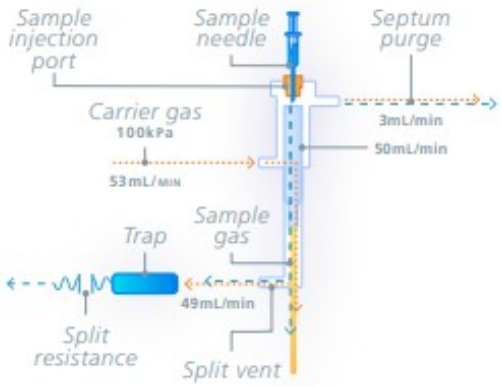


Figure 8: Schematics of a Split Injector Port [85]

With splitless injection on the other hand, the split valve is initially closed and most of the vaporized sample goes on to the column. The remaining vapors are purged out the split valve after 45 s. The splitless technique is utilized for trace analysis of pharmaceutical, biomedical and evironmental samples.[84]

On-column injection refers to direct injection of the sample into the head of the capillary column without applying heat. Other injector types include the solvent-free solid injector, the programmed temperature vaporization injector, and the Purge and Trap injector.[84]

Separation Columns:

There are two different main types of separation columns used in GC today: capillary and packed columns. A capillary column usually possesses an internal diameter ranging from 0.1 to 0.53 mm and a length of 10 to 100 meters, providing very high resolution. For instance, commonly used columns have an internal diameter of 0.25 mm and a length of 30 meters. The column, made of fused silica, features an inner

surface chemically bonded with the liquid phase. Its outer surface is coated with polyimide resin to enhance its robustness.
The choice of column depends on properties like the target components, the number of compounds and the instrument configuration. For a higher resolution, a smaller diameter and longer column should be used, while a larger diameter is acceptable when the resolution does not need to be as high.[83]
According to the type of analyte, an appropriate column, carrier gas and temperature program must be selected. Proper sample preparation and evaporation behaviour during injection must also be considered.[85]

Detectors:

According to the type of detector, properties regarding selectivity, detectability and dynamic range can vary. Examples of different detectors include flame ionization detectors (FID), thermal conductivity detectors, electron capture, flame photometric detector, etc.[82]
Gas chromatography is also often coupled with mass spectrometry, which provides detailed structural information on the analytes.[86]

3 Materials and Methods

3.1 Participants

The participants were exclusively members of the Wagner working group. Since this thesis focuses on pre-tests for method development, only a small number of participants was required. A smaller group also ensures quicker execution and evaluation of the experiments.

During the experimental work conducted for this thesis, participant composition fluctuated because not all individuals were consistently on site. For instance, one person was only present for a few months due to an internship, while others joined the working group at a later stage.

Regarding age and gender, most participants were in their twenties or early thirties, and the majority were female. No illnesses were reported among the participants.

Sample numbering within a participant

Each participant was assigned a unique ID (P1–P8) to anonymize the samples. Preliminary tests were conducted using samples from Participant 1 (P1), and subsequent participants were labeled consecutively (P2, P3, etc.).

During the initial phase, only saliva samples were collected. For Participant 1 (P1), different sample volumes were tested and labelled sequentially (e.g. P1_1mL, P1_500µL). For the subsequent participants, a standard saliva volume of 1 mL was used, so volume information was not included in the sample codes. With the introduction of additional sample matrices (e.g. oral mucosa cells) the sample codes were adapted to explicitly indicate the matrix (e.g., P1_saliva, P1_cells).

This system is applied consistently throughout the study in all text, tables, and figures, ensuring clear identification of each sample type, traceability of the data, and maintenance of participant anonymity.

3.2 Sample Collection

3.2.1 Types of Samples

While we initially focused on saliva as our primary sample matrix, limited success with saliva samples led us to explore two additional biological samples as non- and minimally invasive alternatives. In total, we worked with three different biological samples:

- Saliva
- Cheek cells
- Capillary blood

Additionally, we used plasma samples as a reference.

3.2.2 Sample Collection – Saliva, Cheek Cells and Capillary Blood

Samples were generally collected from participants in a fasted state, around 9:00 AM. Participants were asked to refrain from eating, but were allowed to drink water and brush their teeth in the morning. Some samples were also collected immediately after waking up, without prior water intake or tooth brushing.

Specific sampling procedures for each experiment, including any deviations from this standard protocol, are described in detail in the respective sections: cheek cell sampling (Chapter 4.6) and capillary blood sampling (Chapter 4.8).

3.2.2.1 Saliva Sampling Method using Salivette®

Saliva samples were collected using the Salivette® collection device by the company Sarstedt.
Salivette® offers a hygienic method to collect saliva without the need of medical staff. It collects total saliva through the use of a cotton roll.[87]

Figure 9: Application of the Salivette® [87]

1, 2 &3) The test subject takes out the suction toll and puts it into their mouth.
3) By gently moving the jaw for approximately 60 seconds, saliva flow is stimulated.
4) Subsequently, the saliva-moistened suction roll is placed back into the Salivette®.
5) The Salivette® is then sealed.
6) After centrifugation for 2 minutes at 1000 x g, the clear saliva is collected in the pointed tube.
7) Possible precipitates collect in the specially designated well of the Salivette® tube.

8) Then, the sealed hanging vessel, including the suction roll, is discarded. The obtained saliva can now be used for analysis.[87]

For the FAME methylation process, we generally used 1 mL of total saliva, unless otherwise specified in the respective experiment. Considerable variabilities in salivary flow and the amount of saliva produced were observed among test participants. Consequently, especially at the beginning of the experiments, collecting a full 1 mL of saliva was sometimes not possible, and volumes frequently ranged in the several hundred microliters.

Saliva samples were mostly prepared directly or put in the -20 °C freezer for storage.

3.2.2.2 Cheek Cell Sampling

Cheek cells were collected in the morning at the Department of Nutritional Sciences following a fasting period. Participants rinsed their mouths with ultrapure Milli-Q water before sampling. Using a children's toothbrush, both inner cheeks were brushed, and the toothbrush was immersed and rinsed in a Falcon tube containing 10 mL of Milli-Q water. Additional mouth rinses and pipette washes ensured thorough cell collection. The samples were centrifuged at 3700 x g for 10 minutes, after which the supernatant was removed, and the pellet was washed and resuspended in 1 mL of Milli-Q water. Finally, 10 µL of C19:0 IS were added, and the samples were dried in a SpeedVac for further processing.

3.2.2.3 Capillary Blood Sampling

Capillary blood was collected via fingertip puncture using a lancet, targeting fingers with good blood circulation. Blood was pipetted directly from the puncture site using sterile pipette tips and transferred into Falcon tubes containing MTBE, as this sample matrix was only used in combination with the Matyash extraction method. This step aimed to facilitate the extraction and analysis of lipids from the blood samples. Variations in blood flow and viscosity among participants led to differing blood volumes, with gentle squeezing of the puncture site aiding in blood collection.

3.2.3 Plasma Samples as Reference

Given the extensive research on plasma as a sample material and its higher concentration of fatty acids compared to saliva, we used plasma as a reference for specific tests. For this purpose, plasma samples from unidentified participants of a previous study conducted at the Department of Nutritional Sciences were utilized. These samples had been stored at -80°C.

3.3 Materials and Standards

3.3.1 Materials

Table 1: Reagents and suppliers

Reagents	Company name (product number)
Sodium hydroxide (NaOH)	Sigma Aldrich
Methanol (MeOH) ≥99.9% (GC)	Sigma-Aldrich (1060351000)
Boron-trifluoride methanol (14%)	Sigma Aldrich (B1252)
Hydrochloric acid (HCl)	Sigma Aldrich (1003171000)
Hexane (C6H14) ≥99% (GC)	Sigma Aldrich (2293)
Methyl tert-butyl ether (MTBE)	Sigma Aldrich (34875)
Chloroform ($CHCl_3$)	Sigma Aldrich
Nitrogen (N_2)	Sigma Aldrich
Internal Standards (C19:0 + C18:0)	Sigma Aldrich (C19: 72332-1G-F)
Fame Mix C4-C24	Carl Roth (8691.1)
Water, nuclease-free	Thermo Fisher (AM9937)

In all experiments, “water” refers to nuclease-free water unless otherwise specified.

Table 2: Laboratory equipment and suppliers

Devices	Company name (product number)
Water bath	Sigma-Aldrich
Pyrex® tubes with screw lids	Sigma-Aldrich
Ice-water bath	Styrofoam box, N/A
1000 µL pipette	Eppendorf
200 µL pipette	Eppendorf
20 µL pipette	Eppendorf
Repeat pipette (Multipette®)	Eppendorf
Glass tubes without lid	Sigma-Aldrich
GC vials with lids	Thermo Fisher
Vortex mixer	Sigma-Aldrich
Shaker	Sigma-Aldrich
Centrifuge	Eppendorf
Vacuum concentrator (SpeedVac™)	Thermo Fisher Scientific
Salivettes®	Sarstedt (511,534)
Tooth brushes (kids)	Today(dent)
Lancet	Sigma-Aldrich

3.3.2 Standards

C19:0 (nonadecanoic acid) was used as the primary internal standard (IS) for the majority of experiments. This odd-chain fatty acid was chosen because it is distinct from all naturally occurring fatty acids in saliva.[13] In some experiments, C18:0 was additionally used as an internal standard.

The internal standards were added to account for losses and under the assumption that they would behave similarly to the analytes themselves. Sample labels reflect the standards used: “IS” indicates C19:0 only, while “C18+IS” includes both C18:0 and C19:0. This labelling scheme was maintained to ensure consistency across the datasets.

For earlier experiments a 1,0 mg/mL C19:0 solution was used, which was adjusted to a 0,5 mg/mL standard solution later on (starting with chapter 4.4.2). This adjustment was necessary because the salivary fatty acid peaks were much smaller than the C19:0 peaks, making peak comparison and data analysis more difficult.

To achieve a concentration of 0,5 mg/mL C19:0/MeOH, 10 mg C19:0 nonadecanoic acid powder was diluted in 20 mL MeOH.

3.4 GC Method Development and Sample Preparation

3.4.1 Overview of Sample Preparation Steps

To prepare the samples for GC analysis, several preparatory steps were carried out to ensure reliable and reproducible results. Fatty acids were derivatized into fatty acid methyl esters (FAMEs) using two approaches:

- A combination of the acid reagent boron trifluoride (BF_3) and the base reagent sodium hydroxide (NaOH)
- An alternative derivatization method using hydrochloric acid, based on the protocol by Ichihara and Fukubayashi[64]

Lipid extraction methods were tested in selected experiments as additional preparatory steps to isolate lipids from specific sample matrices. These included the Folch method (using chloroform/methanol) and the Matyash method (using MTBE/methanol), both of which are well-established protocols for lipid extraction. A detailed description and discussion of these methods and their results can be found in the respective experiment sections.

For the GC analysis, all samples underwent the following workflow:

- After methylation, the samples were evaporated to dryness.
- The resulting residues were resuspended in 150 µL of hexane.
- Each sample was divided into two aliquots and transferred into separate GC vials for duplicate analysis.

3.4.2 Vacuum Concentration of Saliva Samples

To increase the detectability of fatty acids in saliva, some samples were concentrated using a vacuum centrifuge (SpeedVac). Depending on the initial volume and saliva composition, evaporation typically required 1–3 hours until the desired reduced volume was reached.

3.4.3 Derivatization methods

3.4.3.1 Derivatization via methanolic NaOH and BF_3

For most of the experiments we used the following protocol with methanolic NaOH and boron triflouride-methanol as derivatization reagents. It was developed by the team surrounding Professor Wagner as part of the preliminary tests and is based – amongst others – on a paper by Qin et al.[78]

FAME Protocol NaOH+BF_3:

- Prepare the methanolic NaOH: add 1 g NaOH pellets to a 50 mL flask and fill it with methanol → place the flask in an ultrasound bath for approx. 15-20 min.
- Bring the samples and 14% boron trifluoride-methanol to room temperature.
- Prepare a water bath at 100°C for the saponification and esterification steps.
- Prepare an ice water bath.
- Pipette 1 mL methanolic NaOH into a Pyrex test tube under the hood.
- Add X µl of the sample (and 10 µl of IS).
- Boil for 5 minutes in a water bath (100°C).
- Allow to cool thoroughly in an ice water bath.
- Add 1 mL of BF_3 and boil the water bath at 100°C for 5 minutes.
- Cool it thoroughly in the ice water bath.
- Add 1 mL hexane and shake for 5 min; repeat twice.
- Collect the hexane phase and transfer it to a glass test tube.
- Evaporate under N_2 in a water bath at 40°C for approximately 30 minutes.
- Mix with 150 µl of hexane.
- Transfer 70 µl (some losses due to evaporation are inevtiable) to a GC vial and store at -80°C or inject into the gas chromatograph.

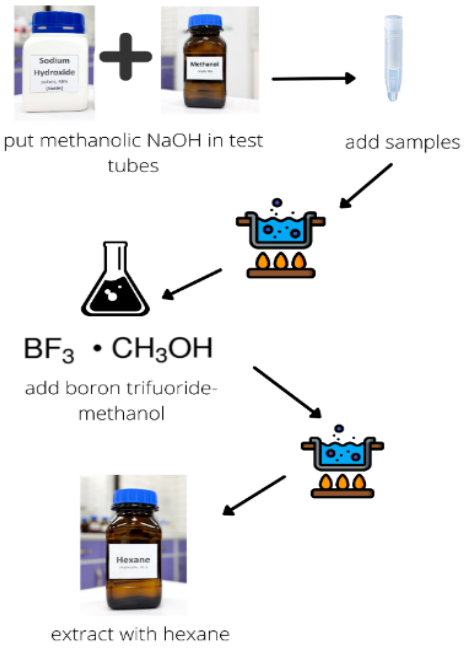


Figure 10: Schematic of the NaOH+BF3 derivatization process (created by the author)

3.4.3.2 Derivatization via methanolic HCl

For the second derivatization method we tested HCl as a derivatization reagent based on a method by Ichihara and Fukubayashi.[64]

FAME Protocol HCl:

To obtain a final concentration of 0.2% HCl in methanol per tube, the following procedure was carried out:

- Prepare a methanolic HCl solution by diluting 0.46 mL of 25% HCl with methanol to a final volume of 10 mL.
- Dispense 1.5 mL of MeOH into Pyrex tubes.
- Add 0.3 mL of the prepared methanolic HCl solution to each tube.
- Provide 1.5 mL of MeOH in Pyrex tubes.
- Add 0.3 mL of methanolic HCl to each tube.
- Add samples (and 10 µl of IS).
- Heat for 60 minutes at 45 °C in a heating block.
- Add 1 mL of Milli-Q H2O to each tube.
- Then add 1 mL of hexane.
- Shake for 5 minutes on the shaker at a speed of 750.
- Evaporate under N_2 for approximately 45 minutes.
- As with the other method, dissolve in 150 µL of hexane, divide each sample into 2 GC vials, and analyze in the gas chromatograph.

In the end, we chose to continue using the original method (using NaOH and BF_3 as derivatization agents) due to more consistent results and carried out most of the experiments with it.

3.4.4 Gas Chromatographic Conditions

GC analyses require several preparatory steps, including column selection (stationary phase and dimensions: column id, inner diameter, length, film thickness), carrier gas selection (helium/nitrogen and flow rate), temperature programming (initial temperature, initial hold, ramp rate, final temperature, final hold), injector temperature and detector temperature selection.[82]

Regarding the choice of carrier gas, helium is preferred in capillary columns because it is able to maintain separating resolution even at rapid linear flow rates.[83]

The gas chromatographic conditions had been developed prior to this thesis by my supervisors, who selected the column, carrier gas, and temperature program. These established conditions were adopted for all experiments conducted in this thesis and are summarized in Table 3.

Table 3: Gas chromatographic conditions

	Parameter	Condition
Hardware	GC	Shimadzu GC-2030 Nexis
	Autosampler	AOC-20s Plus
	Injection Unit	AOC-20i Plus

AOC-20i+s	Injection Volume	1 µL
	Rinse with Presolvent	2
	Rinse with Solvent (Post)	1
	Rinse with Sample	1
	Washing Volume	8 µL
	Washing Speed	High
SPL-1	Temperatur	250°C
	Injection Mode	Split
	Carrier Gas	He (Messner Helium 5.0)
	Flow Control Mode	Linear Velocity
	Pressure	165.9 kPa
	Total Flow	47.2 mL/min
	Column Flow	2.10 mL/min
	Linear Velocity	45.0 cm/s
	Purge Flow	3.0 mL/min
	Split	20.0
	Insert	Deactivated Insert with wool for split
	Syringe	10 µL Syringe 10F-S-0.63
	Septum	Long Life Septa LL-SP-20
Column	Column	Restek Rtx-2330
	Length	30 m
	Type	Capillary
	Inner Diameter	0.25 mm
	Film Thickness	0.2 µm
	Serial Number	1206687
	Lot Number	10723
	Activation Date	03.09.2020
	Temperature	90°C
	Equilibration Time	3.0 min
	Rate 1	13°C/min up to 155°C; Hold 0 min
	Rate 2	2.90°C/min up to 185°C; Hold 5 min
	Total Program Time	20.34 min
FID	Temperature	260°C
	Sampling Rate	40 ms
	Stop Time	20 min
	Makeup Gas	He
	Makeup Flow	24.0 mL/min
	H2 Flow	32.0 mL/min (Messner Wasserstoff 5.0)
	Air Flow	200.0 mL/min (Messner Synth. Luft 5.0 KW frei)
Software		LabSolutions by Shimadzu
		On Windows 10 Pro 22H2

3.4.5 Analysis and Identification of Fatty Acids

For the identification and analysis of individual FAMEs, standard retention times were applied to match and identify the fatty acid peaks observed in the chromatograms.

Each sample was analyzed in duplicate to ensure reproducibility and reliability of the results. The analysis was conducted in a qualitative and semi-quantitative manner, focusing on identifying the fatty acids present and assessing their relative abundances.

3.5 Statistical Analysis

Chromatograms were exported from the GC software “LabSolutions” (Shimadzu). As no direct export function was available, all fatty acid peak values were manually entered into an Excel spreadsheet. Both data processing and the generation of most figures were performed in Excel.

Peak areas were used as the primary parameter for comparing fatty acid levels. Reported as integrated values by the GC software, these areas are expressed in µV·min, but are treated here as counts. These values are suitable for relative comparison, but do not reflect absolute concentrations. Most values were also normalized to the internal standard. These normalized values are expressed as the ratio FA/IS and are therefore dimensionless. Accordingly, no units are used in either the text or the figures.

The mean area and the standard deviation of the fatty acid peaks, based on duplicate measurements, were calculated using Excel. The resulting mean values were then used for further calculations and visualizations.

4 Results

4.1 Initial Method Development and Pre-tests

4.1.1 Equipment Tests and Leakage Issues

Before I started my thesis, another master's student, together with two lab supervisors, worked on establishing a robust and reproducible protocol for sample preparation. This included conducting pre-tests of lab equipment, reagents, and internal standards.

Sealable Pyrex® glass test tubes in particular posed challenges, as some of them leaked. The derivatization step required boiling the samples in a water bath, which led to inconsistent evaporation of the sample material. The evaporation not only affected the measured results, but also posed a potential safety risk. Leaking tubes could release toxic methanol and BF_3 vapors. Furthermore, when the BF_3 reagent comes into contact with water, it forms the water adduct H_2O-BF_3, which subsequently loses HF to yield tetrafluoroboric acid and boric acid, both highly toxic compounds. The reaction proceeds as follows: $2\ BF_3 + 3\ H_2O \rightarrow HBF_4 + B(OH)_3 + 2\ HF$.[88]

Even after excluding visibly damaged tubes, individual tubes continued to leak unpredictably during repeated tests. First, the Pyrex® tubes without visible defects were tested with water, and any leaking tubes were discarded. They were then tested with methanolic NaOH, which revealed that additional tubes still leaked. After removing those, we proceeded to tests using actual samples containing methanolic NaOH, BF_3, and saliva. Nevertheless, some tubes continued to leak and lost a substantial volume already during the first boiling step with methanolic NaOH. The exact cause of these leaks remained unclear, although one possible explanation was variability in the age and quality of the lids. However, these differences could not be identified through visual inspection.

In certain tubes, the lids appeared to generate elevated internal pressure, causing the rubber seal to be sucked into a vacuum-like state during cooling in ice water after the boiling steps. This indicated that the lids may not have sealed properly from the beginning. As no clear pattern emerged and no immediate solution was evident, we continued the experiments while focusing on adjusting other parameters.

After multiple tests with the Pyrex® tubes equipped with rubber-sealed lids and recurring leakage issues, we concluded that the leaks were likely caused by deterioration of the rubber seals. The tubes had been in use for several years, and the rubber may have become brittle over time, reducing the effectiveness of the seal and leading to sample evaporation.

Consequently, we decided to switch to tubes with PTFE-sealed lids (The relevant experiment is described in chapter 4.4.2). This change resulted in substantially fewer leaks and only a few instances in which the seal was drawn into a vacuum-like state during cooling.

4.1.2 Optimization of Experimental Conditions

In addition to testing the Pyrex® tubes, we also aimed to optimize the experimental conditions. A previous study reported that the rates of saponification and transesterification increase with higher reaction temperatures. However, when we tested different temperatures (80, 90, and 100 °C), we did not observe any meaningful differences in reaction performance.

Another unresolved issue during the pre-tests was the presence of impurities in the GC. To address this, we tested all reagents used for lipid derivatization and extraction, thoroughly washed the test tubes both manually and in the dishwasher and used only new GC vials. Despite checking all materials and solutions step by step and replacing the GC inlet liner, the impurities persisted. Therefore, we proceeded to test alternative derivatization approaches and other potential influencing factors to identify the source of the contamination.

4.1.3 Vacuum Concentration of Saliva Samples

Given the relatively low abundance of fatty acids in saliva, our objective was to concentrate 1 mL saliva samples to approximately 0.4 mL using a vacuum centrifuge. However, this proved challenging due to the high variability in saliva composition, which resulted in non-linear dehydration in the SpeedVac. In addition to day-to-day fluctuations within individuals, inter-individual variability likely caused further inconsistency. Preliminary GC runs indicated that reducing the saliva volume improved fatty acid detection, but additional testing was required.

4.2 Analysis of Saliva Samples

4.2.1 Experimental Framework

The initial tests were based on the protocol previously established by the team working with Professor Wagner at the Department of Nutritional Sciences. This protocol uses a double-derivatization approach, applying methanolic sodium hydroxide (NaOH) as the basic reagent and methanolic boron trifluoride (BF_3) as the acidic reagent. A previous study reported that BF_3 alone is insufficient for the transesterification of certain lipids, such as triacylglycerols (TGs) and cholesterol esters (CEs).[77] The combination of acidic and basic reagents therefore provides a more complete derivatization.

The NaOH + BF_3 method yielded good chromatographic performance, i.e. relatively high peaks, good separation, and low baseline noise, but a recurring issue was the high level of impurities. For this reason, we explored alternative derivatization procedures that might reduce impurities and/or produce higher peaks so that impurities would become negligible in comparison. Based on the method described by Ichihara and Fukubayashi,[64] we tested a derivatization approach using HCl instead.

HCl initially appeared promising, with seemingly lower impurity levels; however, after several runs, we observed that HCl reacted with the column material, causing baseline

bleeding and column degradation. Due to this issue, combined with the overall better peak performance obtained with the NaOH + BF_3 method, we returned to the original protocol. The corresponding experiments are described below.

4.2.2 Water Tests

Aim:

After detecting impurities in the preliminary test samples, the next step was to identify their source. To do this, we analyzed pure nuclease-free water to determine whether the peaks observed in the saliva samples originated from actual salivary fatty acids or from contaminants. The NaOH + BF_3 derivatization method was applied to two separate nuclease-free water samples, which were not expected to contain any lipids. As a reference, a sample containing only the C19:0 internal standard was also analyzed.

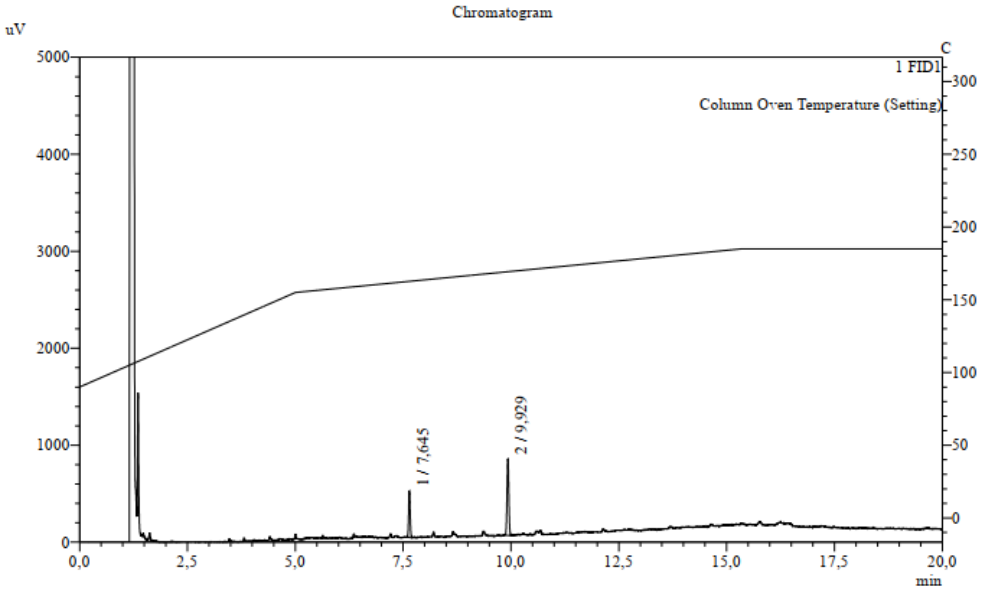
Procedure:

Samples:
- Water 1
- Water 2
- IS C19:0

After pipetting 1 mL of methanolic NaOH into each of three Pyrex® test tubes, 2 x 1 mL of nuclease-free water and 10 µL of the C19:0 internal standard (IS) were added. The FAME protocol was then followed as described and samples were analyzed by GC according to the established method.

Results and Discussion:

Below, gas chromatograms are shown as generated by the LabSolutions program by Shimadzu. The x-axis represents the retention times and the y-axis represents the signal intensity (peak height). The peak areas – calculated from the area under the curve (AUC) and expressed in counts – are shown in the accompanying peak table. The peak table lists all fatty acids measured in the sample, along with their respective retention times, peak areas, peak heights, and area percentages of the total peak sum. Peak areas were selected as the primary parameter for sample comparisons.

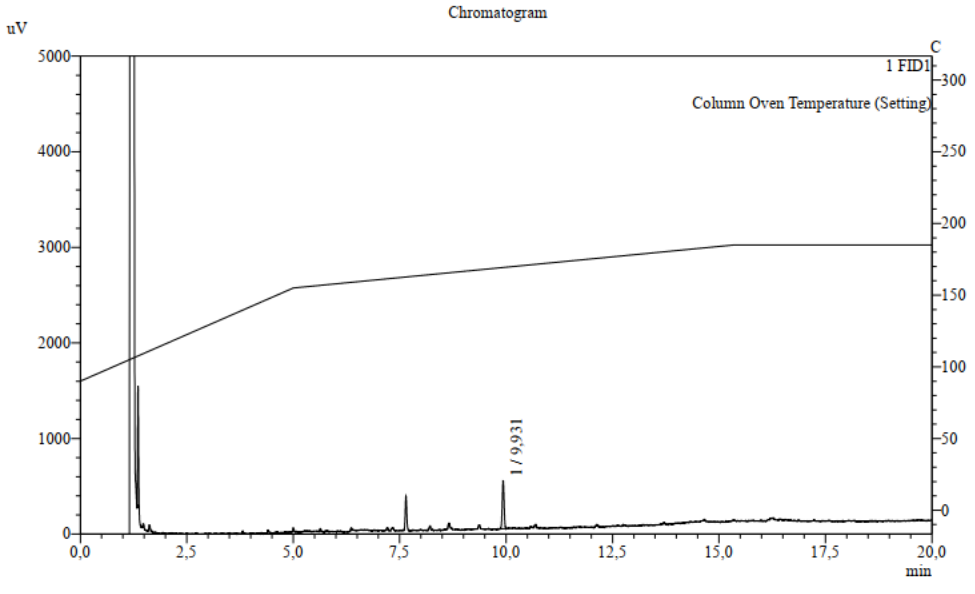


Peak Table

FID1

Peak#	Name	Ret. Time	Area	Height	Area%	Mark
1	C 16:0 Methyl palmitate	7,645	1151	475	33,439	
2	C 18:0 Methyl stearate	9,929	2292	786	66,561	
Total			3443	1262	100,000	

Figure 11: Chromatogram of nuclease-free water sample 1 (Water 1)

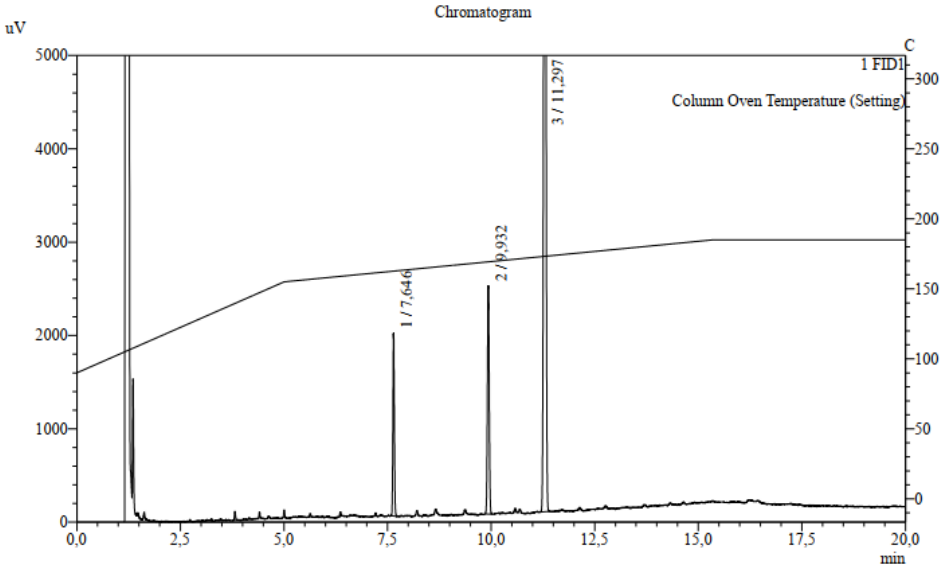


Peak Table

FID1

Peak#	Name	Ret. Time	Area	Height	Area%	Mark
1	C 18:0 Methyl stearate	9,931	1489	504	100,000	
Total			1489	504	100,000	

Figure 12: Chromatogram of nuclease-free water sample 2 (Water 2)



Peak Table

FID1

Peak#	Name	Ret. Time	Area	Height	Area%	Mark
1	C 16:0 Methyl palmitate	7,646	4638	1959	7,377	
2	C 18:0 Methyl stearate	9,932	7282	2440	11,582	
3	C 19:0 Methyl nonadecanoat C 19:0	11,297	50953	15655	81,041	
Total			62873	20054	100,000	

Figure 13: Chromatogram of IS C19:0 sample

The chromatograms of the water samples, which were not expected to contain any lipids, still showed fatty acid peaks. In the first water sample, peaks for C16:0 and C18:0 were detected, while in the second sample only a smaller peak at the retention time of C18:0 appeared.

The variation in peak areas suggests that the impurities did not (exclusively) originate from the water, but were introduced during the derivatization process. This is further supported by the fact that a freshly opened bottle of nuclease-free water was used. Similar results were observed in the sample containing the C19:0 IS. Despite the sample containing only C19:0, peaks for C16:0 and C18:0 were again detected. However, it is unlikely that the impurities in the samples stemmed (exclusively) from the IS, as the pure water samples contained impurities as well, albeit to a lesser extent.

Conclusion

The impurities did not (exclusively) originate from the water or the IS; therefore, they must have occured during the derivatization process.

4.2.3 Comparison of Sequential vs. Pooled Saliva Samples

4.2.3.1 Sequential Saliva Samples

Aim:

We tested how the fatty acid profile changes when saliva samples are collected successively from the same individual. The aim was to determine whether the salivary fatty acid concentration decreases with each consecutive sample and, if so, by how much. As usual, the C19:0 IS and pure water were also analyzed to assess whether the previously observed impurities persisted.

Procedure:

Samples:
- IS (C19:0)
- Water (200 µL)
- P1_1mL_1-3
- P1_500µL
- P1_300µL

- Saliva was collected from participant P1. Samples were numbered sequentially according to volume.
- After the first three 1 mL samples, insufficient saliva remained for a full Salivette®, so the next two samples contained only 500 µL and 300 µL, respectively.
- All saliva samples were centrifuged and transferred into Eppendorf tubes as described in Chapter 4.2.2.1.
- Samples were concentrated in the SpeedVac to approximately 400 µL.
- Evaporation times: ~2 h for the 500 µL sample and an additional 1 h for the 1 mL samples (P1_1–P1_3).
- 10 µL IS C19:0 was added to each sample, including the water control, at the beginning of the experiment.
- All samples were methylated using the standard NaOH + BF_3 method.
- Samples were extracted with hexane and analyzed via GC in a dual approach.

Results and Discussion:

For a more efficient representation of data, fatty acid peaks are hereafter summarized in graphs rather than showing individual chromatograms. For this, the peak areas of the measured fatty acids for all samples were entered into an Excel sheet. The samples were measured in duplicates, the average of which was calculated and used for the graphs. Moreover, standard deviations were generally calculated and represented in the graphs, except for the following experiments, where the duplicates had evaporated.

In addition, normalized values are often also presented. Normalization, based on the internal standard, compensates for variations in sample preparation or losses during analysis, allowing for a more accurate comparison of fatty acid levels across samples.

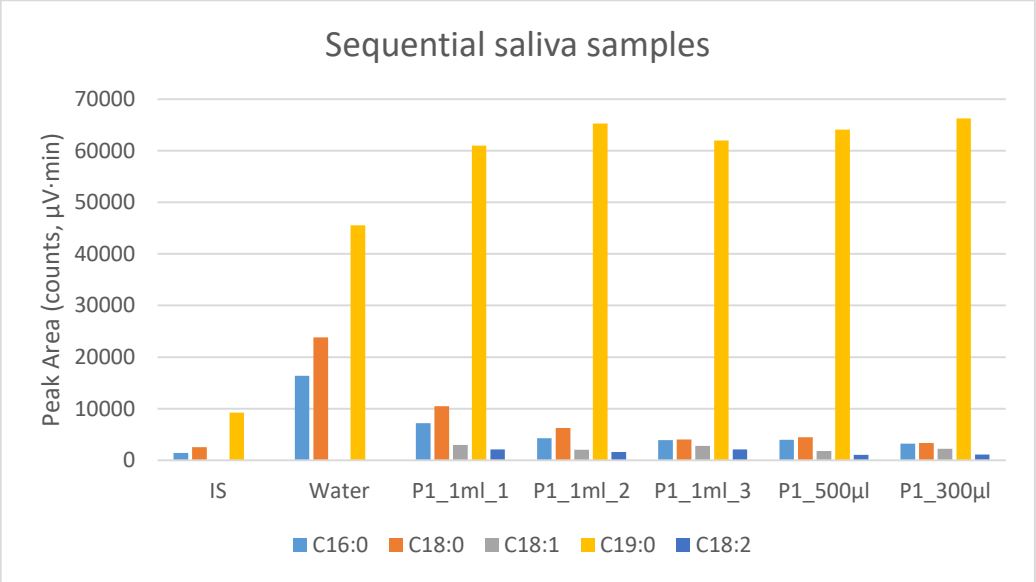


Figure 14: Peak areas of fatty acids in sequential saliva samples

Displayed are peak areas of various fatty acids and the IS C19:0 in sequential saliva samples. Only single measurements are shown, as half of the samples were lost due to evaporation and no SD could be calculated.

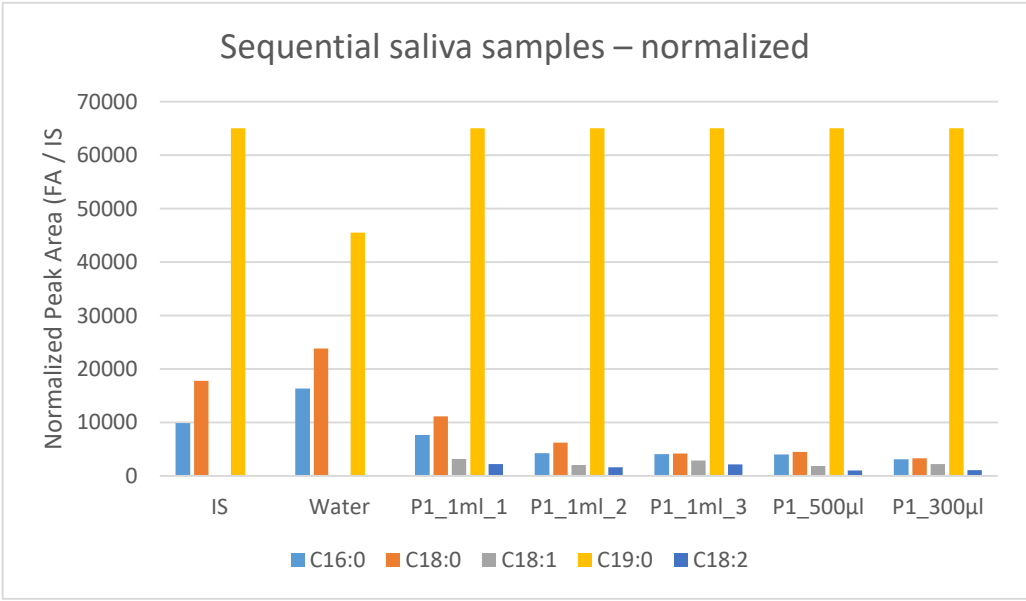


Figure 15: Normalized fatty acid values in sequential saliva samples

Displayed are normalized fatty acid values and the IS C19:0 in sequential saliva samples. Only single measurements are shown, as half of the samples were lost due to evaporation and no SD could be calculated. Values were scaled to an IS reference value of 65,000.

As shown in Figure 14, most of the IS C19:0 sample evaporated, as did the majority of the duplicates of the saliva samples. As a consequence, the fatty acid peak values in the diagrams are largely based on single measurements.

The metal caps of the GC vials were sealed using pliers to prevent evaporation of the FAMEs dissolved in hexane. Initially, some practice was required to achieve a tight seal and prevent sample loss. Over time, the technique was refined, ensuring consistent preservation of the samples.

Despite these precautions, the use of the internal standard allowed for accurate monitoring of sample evaporation. Accordingly, in the second graph, normalized fatty acid values are presented, scaled to a reference IS value of 65,000, which approximately represents the average peak area of the C19:0 IS.

As expected, the salivary fatty acid concentration gradually decreased with each saliva sample. However, the correlation was not linear, and samples with lower saliva volumes (500 µL and 300 µL) showed some higher fatty acid levels than those with higher saliva volumes. For example, for C18:0 a normalized value of 4504 was obtained in sample P1_500 µL, compared to 4188 in P1_1mL_3.

In addition, it is unclear to what degree impurities might have influenced fatty acid levels. This is supported by the observation that relatively high concentrations of impurities were detected in the water sample (with C16:0 and C18:0 peaks higher than in the saliva samples), despite the water being expected to be pure. The data suggest that the impurities were introduced during the derivatization process rather than in the vacuum concentrator, as the water samples were not processed in the SpeedVac, yet contained considerable impurities. Ideally, the internal standards should be added prior to sample preparation in the SpeedVac, as was done in this experiment.

Conclusion

Salivary fatty acids decreased with repeated sampling in the same test subject, albeit not linearly. Fatty acid peaks might also have been affected by impurities.

4.2.3.2 Comparison of Fatty Acid Profiles in Successively Collected vs. Pooled Saliva Samples

Aim:

The aim was to assess how the fatty acid content of saliva changes with successive sampling. To this end, five sequential saliva samples were collected and analyzed individually and compared with five samples that were pooled and analyzed together. The individually collected samples allowed assessment of intra-individual variability, while the pooled samples were used to evaluate method variability. Additionally, we compared the salivary fatty acid profile after breakfast versus in the fasted state.

Procedure:

Samples:
- P1_1mL_1-5 (sequential)
- P1_1mL_P_1-5 (pooled)

Day 1 (after breakfast):

- Breakfast consisted of oatmeal prepared with oat milk, some fruit (e.g., berries, banana slices) and a small handful of nuts.
- Saliva was collected 1 hour after finishing breakfast.
- Five individual saliva samples were collected via Salivettes® within ~8 min 30 sec.
- Five additional saliva samples were collected 5 minutes later within ~6 min.
- All 10 samples were centrifuged at 1,000 x g for 2 min at 20 °C.
- 1 mL from each of the first 5 Salivettes® was pipetted into 1.5 mL microtubes (P1_1mL_1-5).
- The remaining 5 Salivettes® were pooled in a 50 mL Falcon tube, mixed gently, and 1 mL pipetted into each of another 5 microtubes (P1_1mL_P_1-5).
- All 10 microtubes were placed in the SpeedVac for 2 h + 45 min (45 °C, 1 h, 14 Torr, Ramp 5, Auto Run) to concentrate the samples prior to derivatization.
- Samples were derivatized using the standard methanolic NaOH + BF_3 protocol.
- The derivatized samples were analyzed by GC the following day.

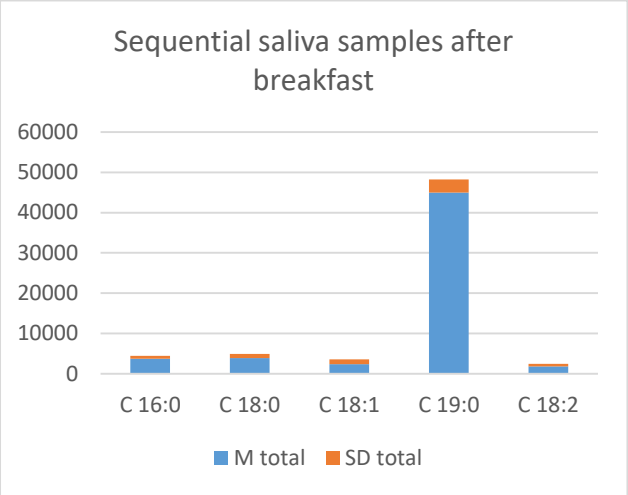
Day 2 (fasted, following day):

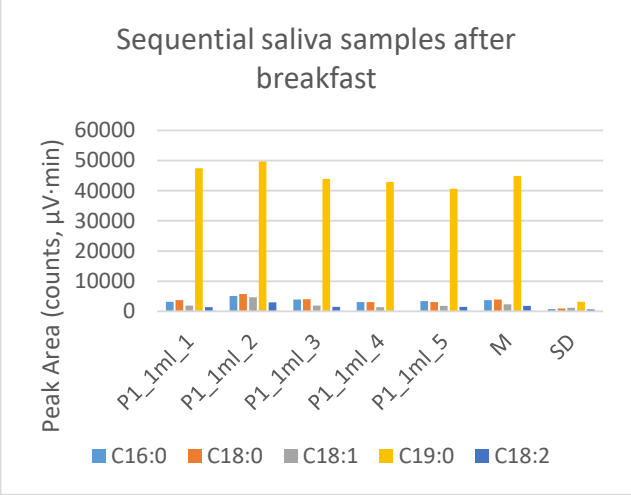
- Saliva was collected ~1 h after tooth brushing in a fasted state.
- Five individual saliva samples were collected within ~6 min 30 sec, followed by 5 additional samples within ~4 min 30 sec.
- Centrifugation and pipetting were performed as on Day 1, generating 10 microtubes (5 individual + 5 pooled).
- All 10 microtubes were concentrated in the SpeedVac for 2 h + 45 min.
- Samples were derivatized using the standard methanolic NaOH + BF_3 protocol.
- The derivatized samples were analyzed by GC the following day.

Results and Discussion:

All values reported in this experiment represent raw peak areas and are not normalized.

Results day 1 (after breakfast):

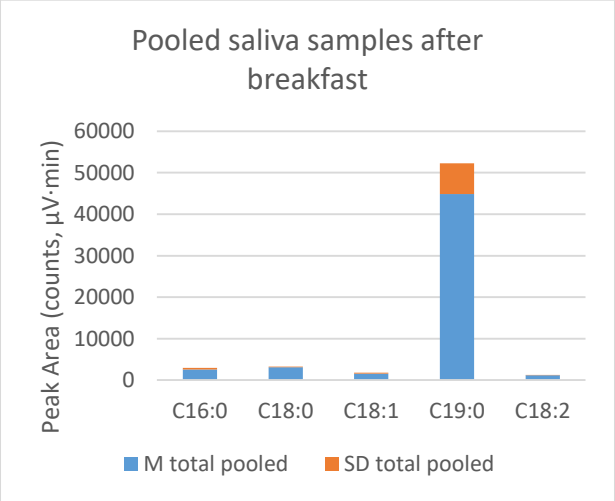


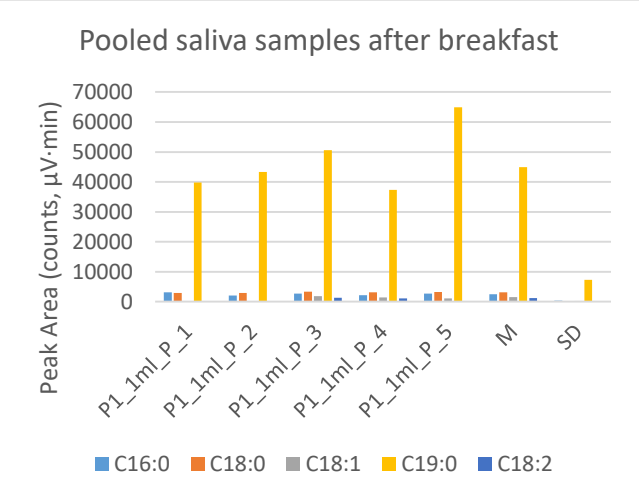


	C16:0	C18:0	C18:1	C19:0	C18:2
M total	**3740**	**3927**	**2361**	**44957**	**1836**
SD total	**735**	**961**	**1203**	**3256**	**647**

Figure 16: Peak areas of fatty acids in sequential saliva samples after breakfast

Displayed are peak areas of various fatty acids in sequential saliva samples collected after breakfast. Left: Mean values of all measured fatty acids. Right: Individual fatty acid values, including mean and SD of the 5 values.



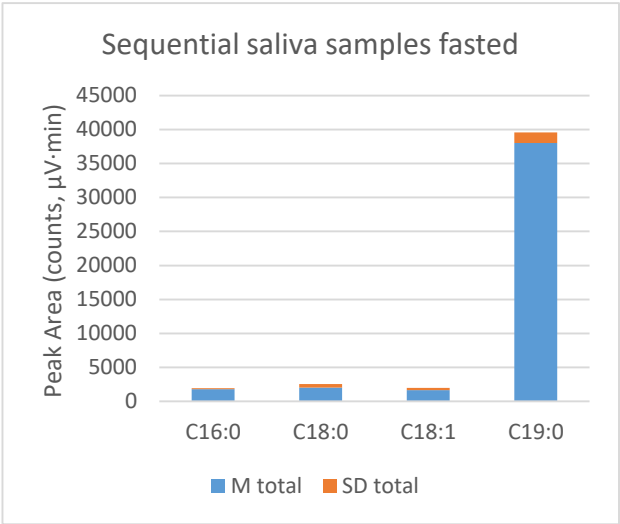


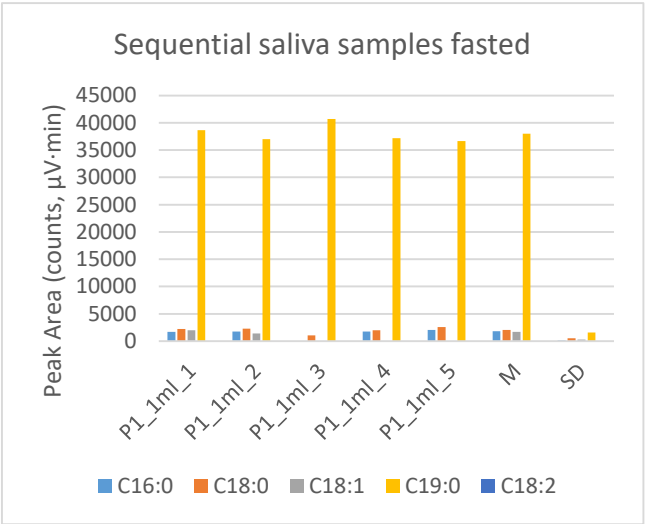
	C16:0	C18:0	C18:1	C19:0	C18:2
M total pooled	**2539**	**3113**	**1552**	**44900**	**1179**
SD total pooled	**396**	**175**	**262**	**7342**	**98**

Figure 17: Peak areas of fatty acids in pooled saliva samples after breakfast

Displayed are peak areas of various fatty acids in pooled saliva samples collected after breakfast. Left: Mean values of all measured fatty acids. Right: Individual fatty acid values, including mean and SD of the 5 values.

Results day 2 (fasted):

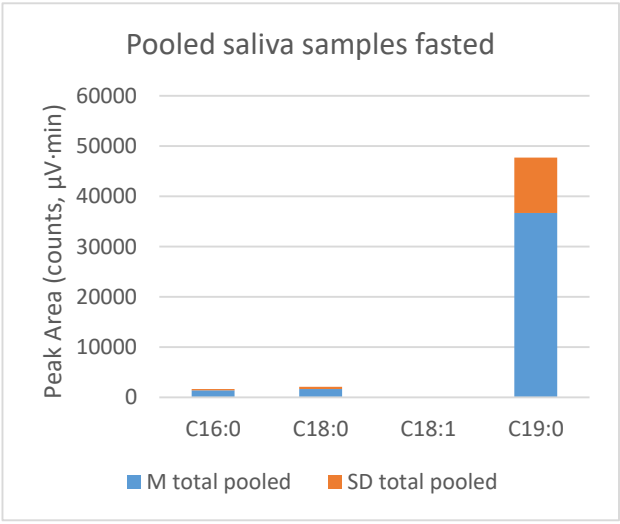


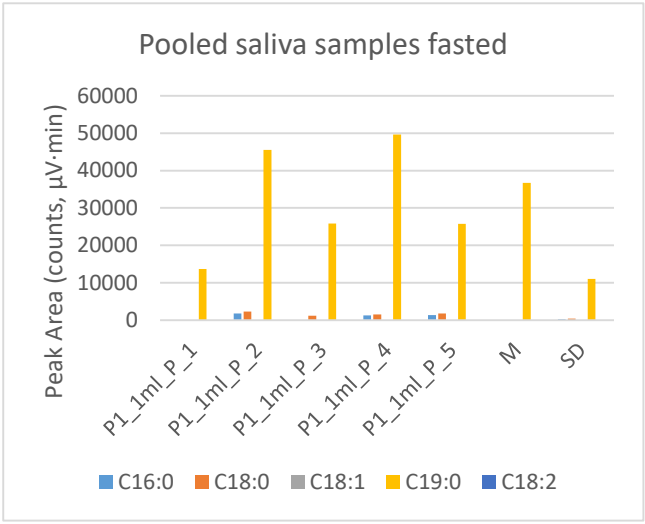


	C16:0	C18:0	C18:1	C19:0
M total	**1799**	**2021**	**1687**	**38028**
SD total	**133**	**531**	**296**	**1544**

Figure 18: Peak areas of fatty acids in <u>sequential</u> saliva samples fasted

Displayed are peak areas of various fatty acids in sequential saliva samples collected fasted. Left: Mean values of all measured fatty acids. Right: Individual fatty acid values, including mean and SD of the 5 values.





	C16:0	C18:0	C18:1	C19:0
M total pooled	**1460**	**1697**	**/**	**36701**
SD total pooled	**229**	**411**	**/**	**11000**

Figure 19: Peak areas of fatty acids in <u>pooled</u> saliva samples fasted

Displayed are peak areas of various fatty acids in pooled saliva samples collected fasted. Left: Mean values of all measured fatty acids. Right: Individual fatty acid values, including mean and SD of the 5 values.

Regarding the samples collected after breakfast, the individually analyzed samples exhibited greater variability in fatty acid values, whereas the standard deviation was lower in the pooled samples. The only exception was the IS C19:0, which showed higher variation in the pooled samples, likely due to pipetting differences. C19:0 was the only synthetic fatty acid and was added by hand. It was added after the saliva samples were pooled and divided among the microtubes, leading to some variability. Total salivary fatty acid values were lower in the pooled samples, which were collected immediately after the five individual samples, indicating that fatty acid concentrations in saliva decreased with sequential sampling.

Comparing the fasted samples with those collected after breakfast, it is evident that eating prior to saliva collection led to higher fatty acid levels. Although the meal was not particularly high in fat, it still had a noticeable effect on salivary fatty acid concentrations. Further experiments will be required to assess the impact of meals with higher fat contents.

In the fasted samples, salivary fatty acid levels decreased with successive collections, with C18:1 dropping below the detection limit in the pooled samples. Linoleic acid (C18:2) was only detectable in the post-breakfast samples. Standard deviations of the fatty acids in the pooled samples were generally comparable to those of the individually collected samples. The only exception was C19:0, which exhibited higher variability in the pooled samples, most likely due to pipetting inaccuracies or sample evaporation during derivatization.

Conclusion

Salivary fatty acids were higher in samples collected after breakfast than in fasted samples. Pooling the post-breakfast samples reduced the standard deviations, reflecting the decrease in fatty acid concentrations with successive sampling. In the fasted samples, no such clear trend was observed.

4.3 Comparison of Derivatization Methods

4.3.1 Testing the HCl Derivatization Method

Aim:

Due to recurring impurities in the samples derivatized using the methanolic NaOH + BF_3 method, we tested an alternative derivatization approach using HCl as the methylation catalyst. The aim was to achieve higher peak intensities and lower levels of impurities. The protocol employed was based on the method described by Ichihara and Fukubayashi[64] and is detailed in the Materials and Methods chapter.

Testing of standards and water samples

Procedure:

Samples:

- C18_1-2 (10 µL)
- C18+IS_1-2 (10 µL + 10 µL)
- Water_1-2 (200 µL)
- Water+IS_1-2 (200 µL + 10 µL)

The samples were methylated following the HCl protocol.

Results and Discussion:

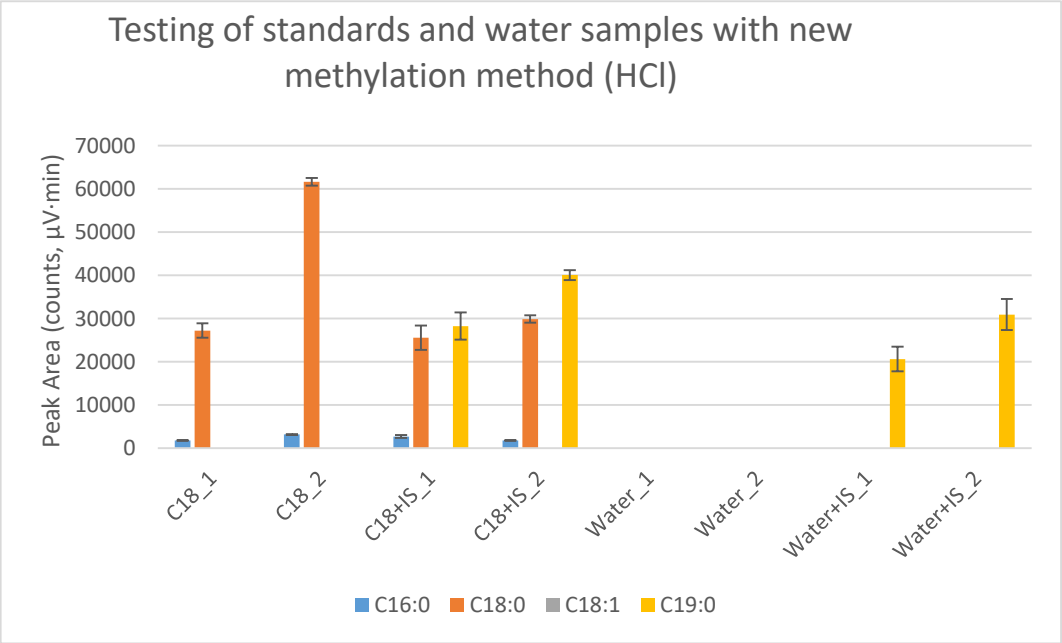


Figure 20: Peak areas of different IS and water samples derivatized with HCl

Displayed are peak areas of pure C18:0 internal standards, C18:0 + C19:0 IS combinations, pure water samples and water + C19:0 IS. Values are shown as the mean of two measurements ± SD.

The fatty acid peaks appeared satisfactory, although some impurities were still present. For instance, small C16:0 peaks were observed in the C18:0 IS samples. No impurities were detected in the pure water samples. In the Water+IS (C19:0) samples, only C19:0 peaks were observed, with no unexpected fatty acid peaks. However, both C18:0 and C19:0 IS exhibited relatively high variability across samples.
These results suggest that the impurities might have originated from the C18:0 standard, indicating the need to prepare a fresh C18:0 standard solution for subsequent experiments.
At this point, the HCl derivatization method appeared promising, producing fewer impurities than the NaOH + BF_3 method.

Testing of saliva samples vs. blood plasma

Procedure:

Samples:
- Blank
- P1_saliva_100µl
- P1_saliva_200µl
- P1_saliva_500µl_1-2
- P1_saliva_1mL
- Plasma (10 µL)

Sample Preparation / Evaporation:

- Saliva samples were collected via Salivettes®, centrifuged, and transferred into Eppendorf tubes.
- 10 µL C19:0 IS was added to each sample.
- Samples were concentrated in the SpeedVac. Evaporation was uneven: after 2 hours, P1_saliva_500µL_1 and P1_saliva_500µL_2 were fully evaporated; the remaining samples were frozen at −20 °C.
- Four days later, P1_saliva_1mL, P1_saliva_200µL, and P1_saliva_100µL were returned to the SpeedVac for an additional hour until fully evaporated.

Methylation with Methanolic HCl:

- 1.94 mL 37% HCl was diluted with MeOH to 10 mL in a volumetric flask.
- 1.125 mL MeOH and 0.225 mL methanolic HCl were added directly to the samples, as well as to two additional microtubes: one empty (blank) and one containing 10 µL plasma + 10 µL IS.
- Microtubes were vortexed for several minutes and transferred to Pyrex tubes.
- Samples were heated at 45 °C for 60 min.
- 1 mL Milli-Q water and 1 mL hexane were added; tubes were shaken for 5 min.
- **Note:** Emulsification of the hexane phase occurred in P1_saliva_100µL, P1_saliva_500µL_1, and P1_saliva_500µL_2.
- Supernatants were transferred into glass tubes without lids.
- The hexane extraction was repeated twice.

Final Steps / Analysis:

- Attempts to evaporate the solvent under N_2 failed due to an empty N_2 bottle.
- Samples were left overnight in the fridge (tubes covered with parafilm) and were evaporated and analyzed the next day by GC.

Results and Discussion:

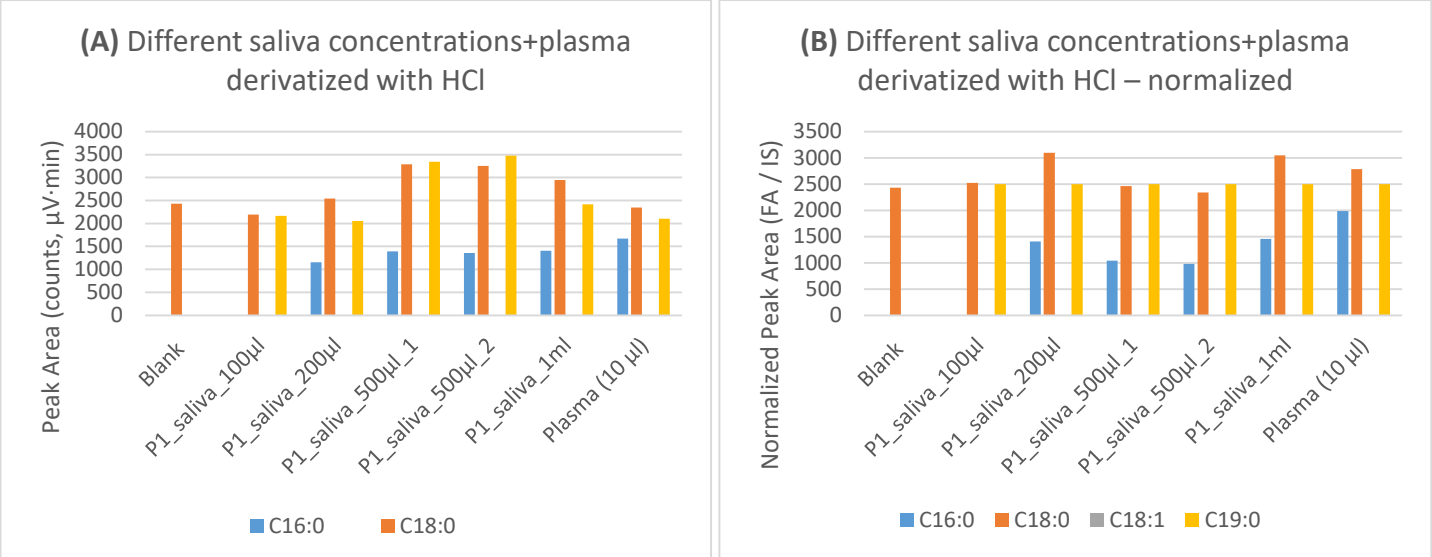


Figure 21: Fatty acid values in saliva samples of varying volume (participant P1) derivatized with HCl

Displayed are fatty acid values in saliva samples of different volumes, along with a blank and a plasma sample, collected from participant P1.
(A) Raw peak areas.
(B) Normalized fatty acid values (scaled to an IS reference value of 2,500).
No SD is shown due to single measurements.

The fatty acid concentrations in these samples were lower overall because the samples were left in the fridge (in tubes covered with parafilm) overnight.

No clear linear relationship was observed between fatty acid peak areas and saliva volume. For example, both C16:0 and C18:0 levels in P1_saliva_200 µL were larger than in P1_saliva_500 µL_1 and P1_saliva_500 µL_2 and roughly the same as in P1_saliva_1 mL. However, sampling order must be considered. With successive collection, salivary fatty acid concentrations decrease over time. Therefore, higher fatty acid concentrations may have been present in the smaller saliva volumes collected earlier.

Figure 21 also shows a relatively high C18:0 peak area in the blank sample, which must have been introduced during the derivatization process, as the blank microtube was not processed in the SpeedVac.

To minimize contamination, we increased our focus on maintaining a clean working environment and avoiding any contact between skin and pipette tips or Eppendorf tubes, as this could potentially introduce fat contamination into the samples.

Conclusion

Although the first experiment using the HCl method showed minimal impurities, they reappeared in the second experiment. Further tests are required to evaluate the HCl method and to identify the source of the impurities.

4.3.2 Comparison of NaOH/BF_3 and HCl Derivatization Methods

Aim:

In this experiment, the same samples were prepared using the two different methylation methods to directly compare their performance. It should be noted, however, that the samples were not entirely comparable, as successive saliva sampling leads to a decrease in fatty acid content with each subsequent sample.

Procedure:

Derivatization with methanolic HCl:

Samples:

- C18:0+IS(C19:0)_1-2
- IS(C19:0)_1-2
- P1_saliva_200µl_1-2
- P1_saliva_500µl_1-2
- P1_saliva_1mL_1-2
- Plasma_1-2 (10 µL)

- Saliva samples (P1_saliva_200µL_1, P1_saliva_200µL_2, P1_saliva_500µL_1, P1_saliva_500µL_2, P1_saliva_1mL_1, P1_saliva_1mL_2) were collected with Salivettes® and concentrated in the SpeedVac for ~2 hours until fully evaporated.
- Plasma_1 and Plasma_2 vials: 10 µL plasma added.
- C18+IS_1 and C18+IS_2 vials: 10 µL C18:0 added.
- 10 µL IS (C19:0) was added to all samples.

Derivatization with methanolic NaOH + BF_3:

Samples:

- C18:0+IS(C19:0)_1-2
- IS(C19:0)_1-2
- P1_saliva_200µl_3-4
- P1_saliva_500µl_3-4
- P1_saliva_1mL_3-4
- Plasma_1-2 (10 µL)

- Saliva samples (P1_saliva_200µL_3, P1__saliva_200µL_4, P1_saliva_500µL_3, P1_saliva_500µL_4, P1_saliva_1mL_3, P1_saliva_1mL_4) were collected and concentrated in the SpeedVac for ~2 hours until fully evaporated.
- Evaporated samples were dissolved in 50 µL MeOH in Eppendorf tubes, then transferred to glass vials.
- Plasma_1 and Plasma_2 vials: 10 µL plasma added.
- C18+IS_1 and C18+IS_2 vials: 10 µL C18:0 added.
- 10 µL IS was added to each of the 12 vials.

Methylation:

- Methylation was performed using the HCl method or NaOH + BF_3 protocol, as indicated for each subset of samples.

Analysis:

- All GC vials were frozen at −80 °C and analyzed a few days later.
- **Note:** P1_saliva_200 µL_1 and P1_saliva_500 µL_2 (HCl samples) were excluded from the diagram due to an extraction error.

Results and Discussion:

Figures 22 and 23 are shown on the same y-axis scale (0-90,000, 10,000 increments) to allow direct comparison of peak intensities.

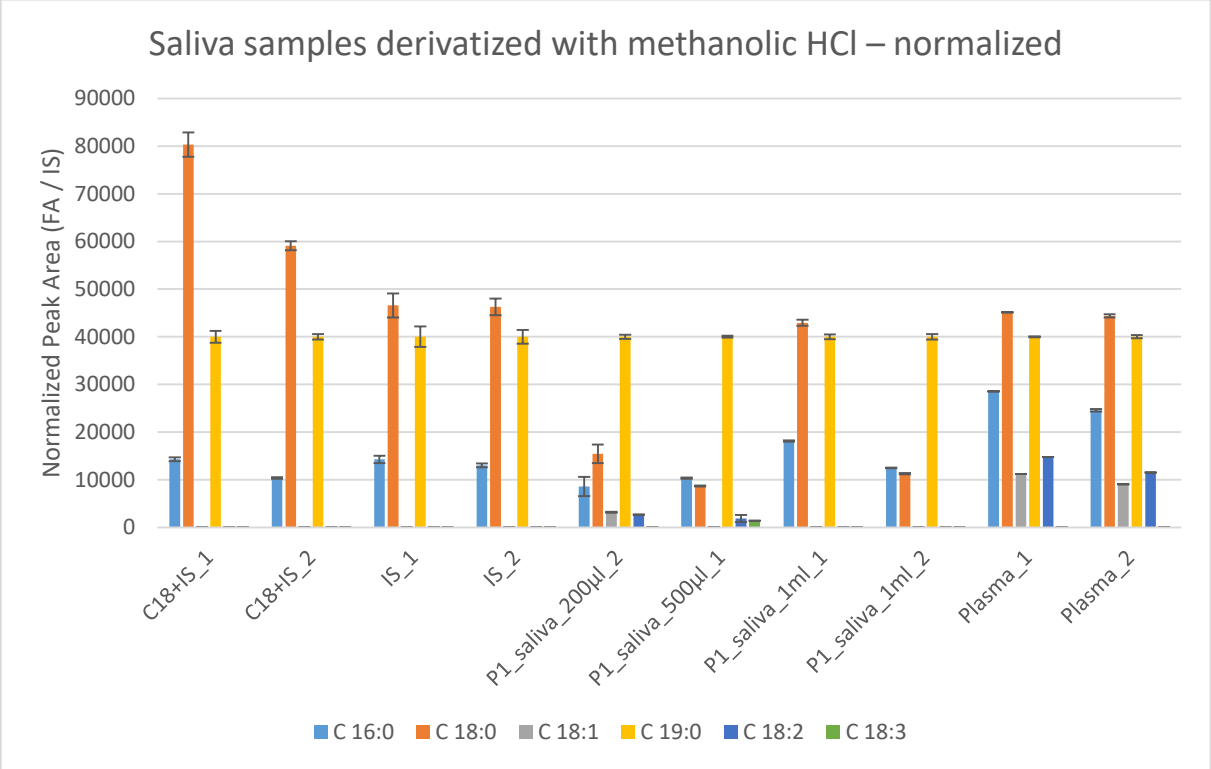


Figure 22: Normalized fatty acid values in internal standards, saliva, and plasma samples after derivatization with methanolic HCl

Displayed are normalized fatty acid values of different internal standard solutions (C18:0 + C19:0 IS; C19:0 IS), saliva samples of varying volumes, and plasma samples after derivatization using methanolic HCl. Values were scaled to an IS reference value of 40,000 and represent the mean of two measurements ± SD.

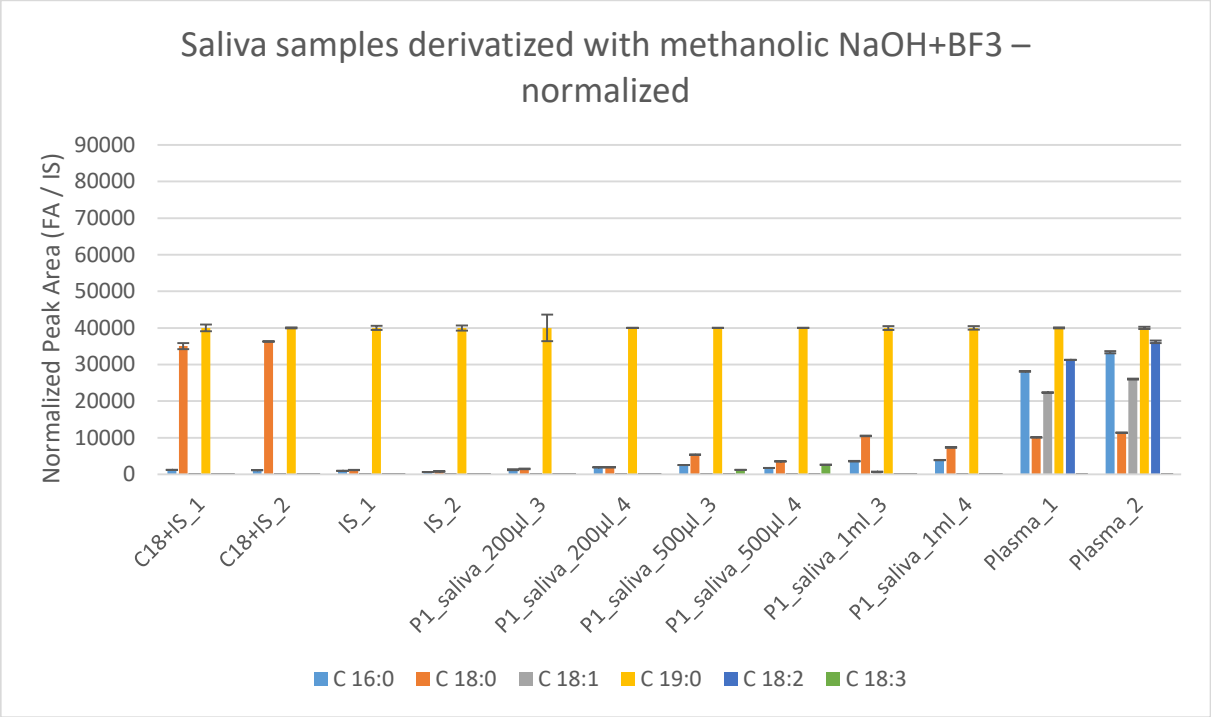


Figure 23: Normalized fatty acid values in internal standards, saliva, and plasma samples after derivatization with methanolic NaOH + BF_3

Displayed are normalized fatty acid values of different internal standard solutions (C18:0 + C19:0 IS; C19:0 IS), saliva samples of varying volumes, and plasma samples after derivatization using methanolic NaOH + BF_3. Values were scaled to an IS reference value of 40,000 and represent the mean of two measurements ± SD.

When derivatized with methanolic HCl, the normalized C18:0 levels in the C18:0+IS standards reached up to 80,000, whereas they only reached approximately 35,000 when derivatized with methanolic NaOH + BF_3. This difference was particularly notable because –unlike C18:0 – the C19:0 peaks showed comparable average areas across both derivatization methods, with mean values around 40,000. However, the individual C19:0 measurements exhibited substantial variability, ranging from approximately 20,000 to nearly 70,000. The higher C18:0 peaks in the HCl-derivatized samples could potentially be attributed to impurities. This interpretation is supported by the substantially higher C16:0 impurities detected in C18:0+IS_1+2, as well as C16:0 and C18:0 impurities in IS_1+2, in the samples prepared with methanolic HCl.

Even though the same plasma samples were used for derivatization with both methods, the resulting chromatograms revealed notable differences in fatty acid composition. In the plasma samples, C18:1, and C18:2 values were substantially higher in the NaOH + BF_3 chromatograms compared to the HCl ones, whereas C18:0 levels were consistently higher in the HCl-derivatized plasma samples. These differences could, however, also be influenced by impurities.

In the saliva samples, C16:0 and C18:0 values were higher in the methanolic HCl-derivatized samples compared to the NaOH + BF_3 samples. In contrast, C18:3 was detected in more NaOH + BF_3-derivatized saliva samples. C18:2 was only measured

at low concentrations in two HCl-derivatized saliva samples, while no C18:2 peaks were observed in the NaOH + BF_3-derivatized saliva samples.

Conclusion

The peak areas rose with increasing saliva concentrations when using the NaOH + BF_3 derivatization method, whereas HCl derivatization produced higher peaks for certain fatty acids in some samples.

Overall, the NaOH + BF_3 method was more promising due to its higher peak intensities and lower potential for impurities, and was therefore selected for subsequent experiments.

Column corrosion due to the use of HCl

For a more comprehensive evaluation of the differences between the two derivatization methods, additional experiments would be needed to better understand potential variations in peak formation and reproducibility. However, this was ultimately unnecessary, as the use of HCl was found to corrode the GC column and was therefore no longer an option. As shown in the chromatogram below, it also caused a baseline drift that got worse with each analyzed sample and made it more challenging to accurately interpret the chromatogram and identify peaks. Therefore, we decided not to use HCl further for FAME methylations.

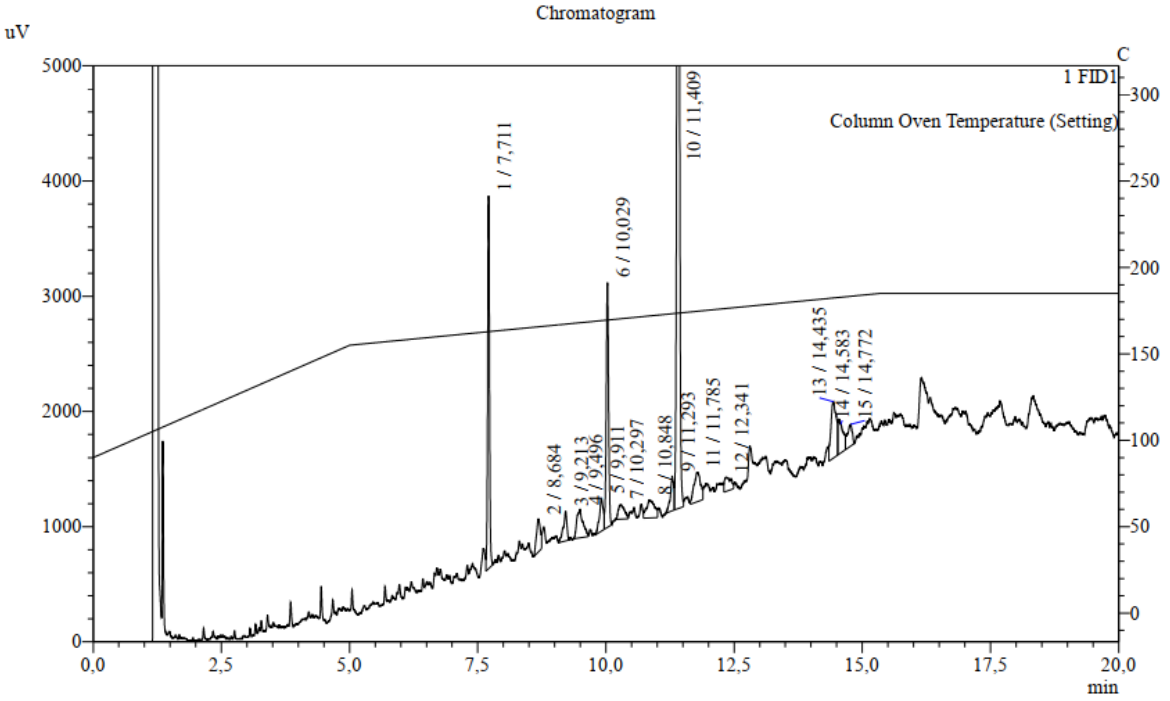


Figure 24: Chromatogram about the impact of HCl on the GC column

4.4 Plasma Dilution Series with vs. without the use of a Vacuum Concentrator

4.4.1 Plasma Dilution Series with Water

Aim:

To test the linearity and sensitivity of the analytical method, we prepared a plasma dilution series. The aim was not only to assess how well plasma concentrations correlated with the fatty acid peak areas in the chromatogram, but also to evaluate the suitability of the SpeedVac as a sample preparation tool. For this purpose, the plasma dilution series was analyzed twice, first by direct derivatization of the samples and a second time after sample preparation using the SpeedVac.

Procedure:

Without the use of the SpeedVac:

Samples:

- Water (10 µL)
- Water+IS (10 µL + 10 µL)
- Plasma (10 µL, undiluted)
- Plasma dilution series (6 samples)

- A serial dilution series was prepared using 7 microtubes:
    1. 10 µL pure plasma (1×)
    2. 10 µL plasma + 90 µL nuclease-free water (1:10)
    3. 10 µL of previous dilution + 90 µL water (1:100)
    4. 1:1,000
    5. 1:10,000
    6. 1:100,000
    7. 1:1,000,000
- 10 µL of each sample (Water, Water+IS, Plasma, and all 6 plasma dilutions) were added to glass vials.
- IS was added to all samples except pure water.
- Samples were methylated with NaOH and BF_3 as per the standard methylation protocol and analyzed by GC.

With the use of the SpeedVac:

Samples:

- Water (200 µL)
- Water + IS (1 mL + 10 µL)
- Plasma (10 µL, undiluted)
- Plasma dilution series (6 samples)

- The same dilution series was prepared, but each dilution was supplemented with 1 mL nuclease-free water.

- The dilution series and Water+IS were placed in the SpeedVac.
- Evaporation was incomplete due to empty cooling liquid and delivery issues, but samples were processed nonetheless.
- The contents of all microtubes were fully transferred into glass eprouvettes.
- An additional glass tube with 200 µL Water was prepared.
- 10 µL IS was added to all samples except the (200 µL) Water sample.
- Samples were methylated according to the standard protocol and analyzed by GC.

Results and Discussion:

To improve comparability, the fatty acid values in Figures 25 and 26 were normalized.

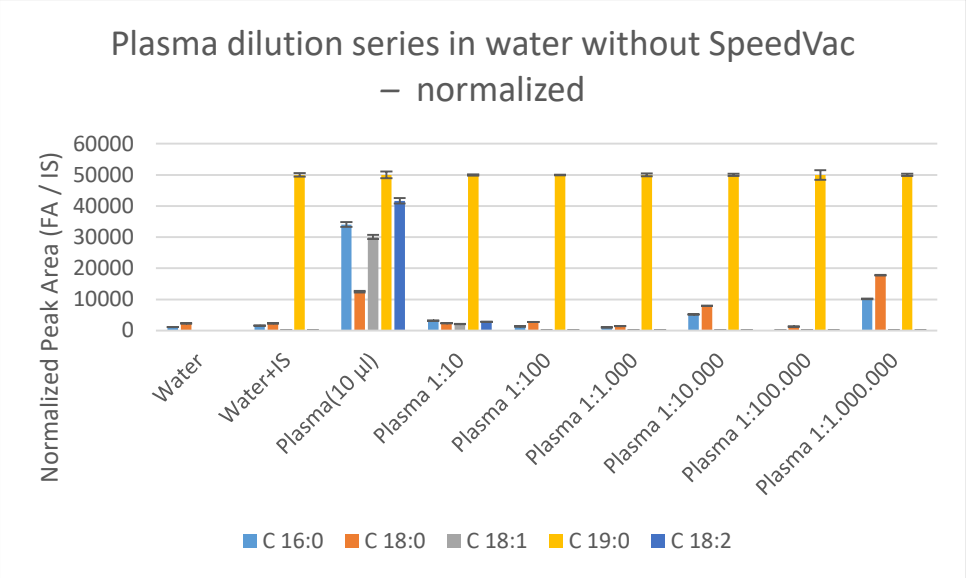


Figure 25: Normalized fatty acid values in a plasma dilution series in water without SpeedVac preparation

Displayed are normalized fatty acid values of a plasma dilution series prepared in water without using the SpeedVac for sample concentration. Values were scaled to an IS reference value of 50,000 and represent the mean of two measurements ± SD.

While the decrease in fatty acids from pure plasma (10 µL) to the 1:10 dilution was within the expected range in Figure 25, the fatty acid levels in the subsequent dilutions were not. Notable C16:0 and C18:0 peaks appeared in the highly diluted plasma samples, particularly in the 1:10,000 and 1:1,000,000 dilutions. At these dilution levels, all fatty acids (except the IS) should have been below the detection limit. Therefore, the detected C16:0 and C18:0 peaks were likely caused by impurities. This interpretation is further supported by the presence of C16:0 and C18:0 impurities in the pure water and water+IS samples. Additional testing was deemed necessary to identify the source of these impurities and minimize their impact.

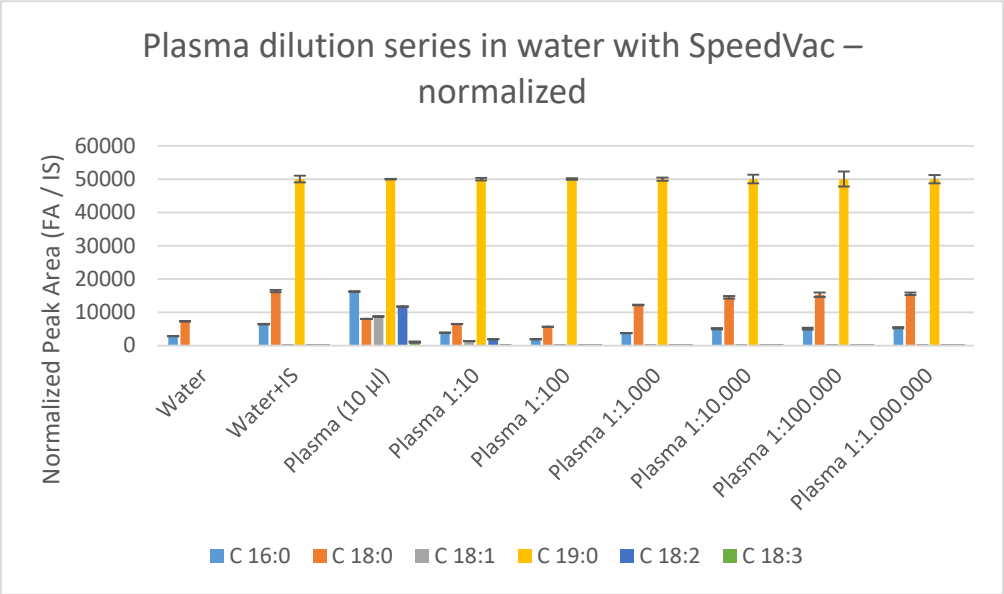


Figure 26: Normalized fatty acid values in a plasma dilution series in water with SpeedVac preparation

Displayed are normalized fatty acid values of a plasma dilution series prepared in water using the SpeedVac for sample concentration. Values were scaled to an IS reference value of 50,000 and represent the mean of two measurements ± SD.

Due to issues with the SpeedVac, specifically the absence of cooling liquid, which prevented proper evaporation, the results shown in Figure 26 are not entirely reliable.

Although the IS was added after SpeedVac processing, the C19:0 peaks still showed substantial variability. Figure 26 displays only the normalized C19:0 values, although the interpretation also considers the variability observed in the raw peak areas. The irregular C19:0 intensities may have resulted from inaccurate pipetting as well as partial sample loss during derivatization due to faulty tube seals. As a result, the use of alternative test tubes was considered.

Another notable observation in the SpeedVac samples was the presence of relatively constant C16:0 and C18:0 impurities across samples. In the last three plasma dilutions, the C16:0 and C18:0 peaks were all approximately the same size, suggesting that the plasma fatty acids were no longer detectable at these dilutions and that the measured peaks likely originated from impurities. Similar C16:0 and C18:0 impurities were also found in the water and water+IS samples.

When comparing samples processed without the SpeedVac to those concentrated using the SpeedVac, the directly derivatized samples showed considerably higher fatty acid peak intensities in pure plasma, with maximum normalized fatty acid values reaching up to over 40,000 compared to below 20,000 in the SpeedVac-processed samples. In contrast, C18:0 peaks, likely impurity-related, were slightly higher across SpeedVac samples. Consistently, impurity levels in the water samples were also higher in the SpeedVac-prepared set.

Conclusion

The results of this experiment favor direct derivatization over pre-derivatization sample concentration using the SpeedVac. However, these findings should be interpreted with caution, as the SpeedVac preparation did not function properly under the conditions of this experiment.

4.4.2 Plasma Dilution Series with MeOH

Aim:

To confirm the results of the previous experiment, the plasma dilution series was repeated, this time diluting the plasma samples in methanol instead of water. Organic solvents such as methanol generally provide better solubility for fatty acid derivatives than water. Moreover, methanol is already used in the FAME methylation procedure.

For the following experiments, a new C19:0 standard solution with a lower concentration was prepared. In the previous experiments, a 1.0 mg/mL IS solution had been used; however, the IS peaks in the chromatograms were much higher than most of the salivary fatty acid peaks. To facilitate analysis and comparison, a lower-concentration C19:0 solution was prepared. For this purpose, 10.03 mg of C19:0 powder was dissolved in 20 mL MeOH to obtain a 0.5 mg/mL standard solution.

Additionally, for all subsequent experiments, test tubes with rubber-lined caps were replaced by test tubes with PTFE-lined caps due to repeated leakages observed in earlier experiments.

Procedure:

Without the use of the SpeedVac:

Samples:

- Water (10 µL)
- Water+IS (10 µl + 10 µL)
- Plasma (10 µL, undiluted)
- Plasma dilution series with MeOH (6 samples)

- The same plasma dilution series as in the previous experiment was prepared, using MeOH instead of water.
- 10 µL of each sample (Water, Water + IS, Plasma, and all 6 plasma dilutions) were transferred into glass vials.
- IS was added to all samples except the Water sample.
- Samples were directly methylated with NaOH and BF_3 as per the standard methylation protocol and analyzed by GC.

With the use of the SpeedVac:

Samples:

- Water (1 mL)
- Water + IS (1 mL + 10 µL)
- Plasma (10 µL, undiluted)
- Plasma dilution series with MeOH (6 samples)

- Another plasma dilution series was prepared, again using MeOH.
- All plasma dilutions and the Plasma sample were supplemented with 1 mL nuclease-free water.
- Two additional tubes containing 1 mL Water and 1 mL Water+IS were prepared.
- 10 µL IS was added to all plasma samples before SpeedVac evaporation, except for the pure Water sample.
- All samples were concentrated in the SpeedVac of the pharmacy department. (**Note:** Uneven evaporation!)
- The contents of all tubes were fully transferred into glass eprouvettes.
- Samples were methylated according to the standard protocol and analyzed by GC.

Results and Discussion:

In Figure 27 and 28, raw peak areas are presented because the newly introduced lower-concentration IS and the technical issues with the SpeedVac in the second series prevent meaningful normalization across all samples.

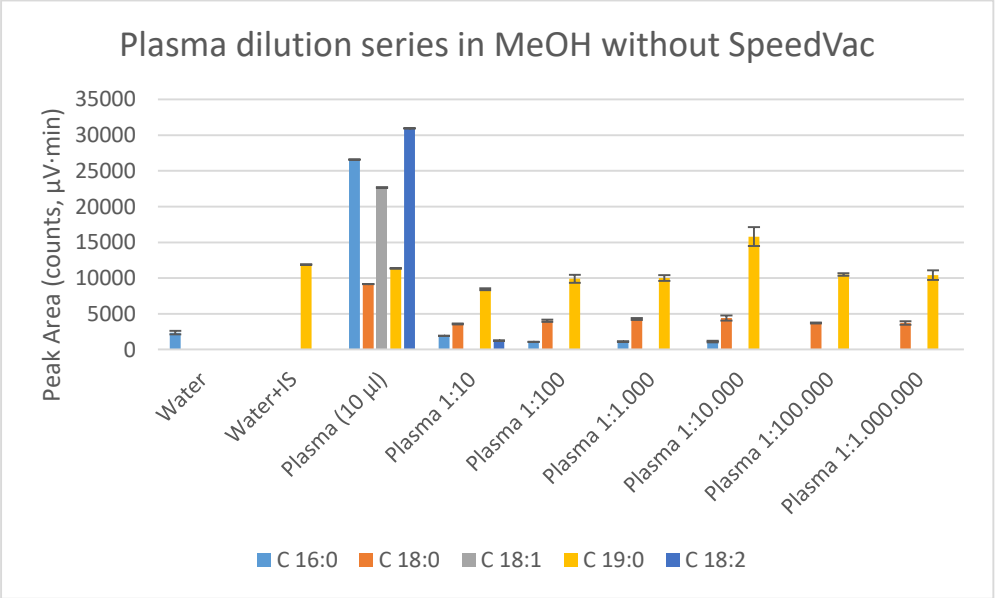


Figure 27: Peak areas of fatty acids in a plasma dilution series in MeOH without SpeedVac preparation

Displayed are peak areas of fatty acids in a plasma dilution series prepared in MeOH, without using the SpeedVac as a sample preparation tool. Values represent the mean of two measurements ± SD.

As shown in Figure 27, the lower-concentration C19:0 IS resulted in correspondingly lower peaks in the chromatograms. While the water+IS sample was free of fatty acid impurities, some C16:0 contamination was detected in the pure water sample. The 10 µL pure plasma sample exhibited expectedly high fatty acid concentrations; however, C16:0 and C18:0 did not decrease in a linear fashion across the subsequent dilutions. From the 1:100 plasma dilution onwards, no fatty acids other than C16:0, C18:0 and the IS (C19:0) were detectable. Notably, the C18:0 peaks remained relatively constant across all dilutions in the raw data, indicating the presence of impurities.

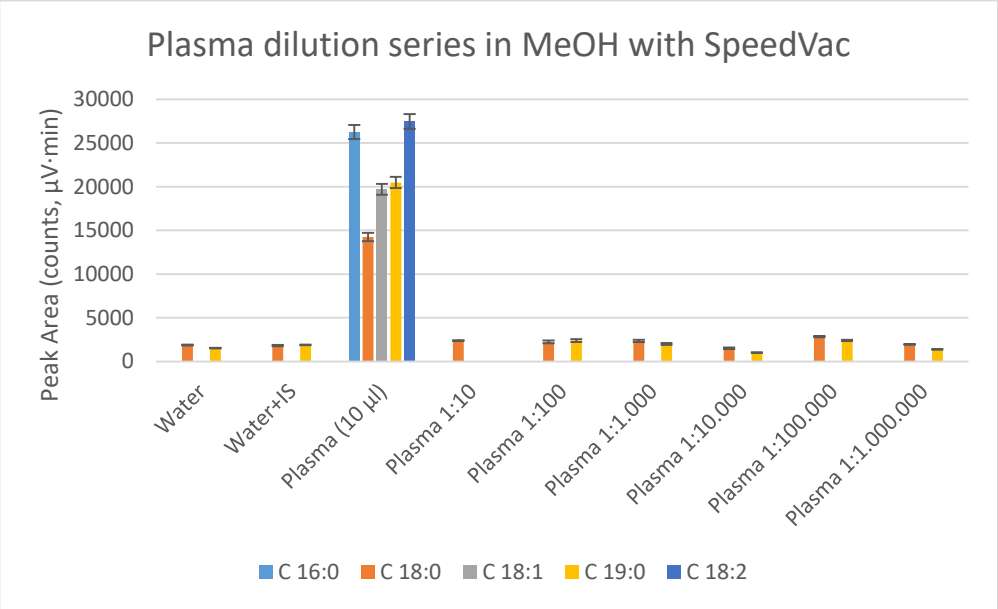


Figure 28: Peak areas of fatty acids in a plasma dilution series in MeOH with SpeedVac preparation

Displayed are peak areas of fatty acids in a plasma dilution series prepared in MeOH, with the use of the SpeedVac as a sample preparation tool. Values represent the mean of two measurements ± SD.

The plasma series prepared using the SpeedVac did not provide reliable results. For these samples, the SpeedVac at the pharmacy department was used. This may explain why considerable portions of the samples evaporated. The C18:0 and C19:0 peaks in the water and water+IS samples were approximately the same as in the other samples, suggesting that some plasma fatty acids might have transferred to the water samples during concentration in the SpeedVac.

The 10 µL pure plasma sample showed reasonably high fatty acid peaks, even though these were lower than in the samples prepared without the SpeedVac. Considering that a greater proportion of the IS in the pure plasma sample prepared without the SpeedVac had evaporated, the naturally occurring fatty acid peaks in the SpeedVac-prepared plasma were even lower when normalized.

Conclusion

Fatty acid levels in the plasma dilution series were higher in samples prepared without the SpeedVac compared to those prepared with the SpeedVac. However, due to the issues encountered with the SpeedVac and the resulting unreliable data, no definitive conclusions can yet be drawn regarding its general suitability as a sample preparation tool for saliva.

4.5 Time-Based Variations and Dietary Influence

4.5.1 Saliva Analysis over 3 Consecutive Days

Aim:

This experiment aimed to investigate how salivary fatty acid profiles vary among different individuals over three consecutive days

Procedure:

Samples

- 3 x Water (1 mL)
- 3 x Water+IS (1 mL + 10 µL)
- 3 x P1_1, P1_2
- 3 x P2_1, P2_2
- 3 x P3_1, P3_2
- 3 x P4_1, P4_2
- 3 x P5_1, P5_2

Participants and Sample Collection:

- Five members of the working group participated (P1, P2, P3, P4, P5).
- Saliva samples were collected using Salivettes®: first in the morning immediately after waking up (P1-P5_1), and again 1.5–2 hours later at the department in a fasted state, after brushing teeth and drinking water (P1-P5_2).
- Each participant was responsible for collecting their own samples.
- Sampling was repeated over three consecutive days, though not all participants collected their samples on the same days. On day 3, samples from P1 were not collected.

Sample Preparation / Evaporation:

- **Day 1:** 1 mL of each participant's saliva was transferred into 1.5 mL microtubes, 10 µL IS C19:0 added, then evaporated in the SpeedVac for 2 h 45 min + 30 min. Water and water+IS samples were reduced to ~0.5 mL; saliva samples to ~0.1 mL. After two additional 1-hour cycles with minimal further evaporation, samples were left as they were.
- **Day 2:** Samples were treated as on day 1. Evaporation was more even, except water and water+IS (water: 1 → 0.9 mL; water+IS: 1 → 0.7 mL).

- **Day 3:** Same procedure as Days 1–2. Saliva samples evaporated evenly; water and water+IS volumes changed little.
- **Note:** Leakage was observed on all three days, though less severe with PTFE-lined tubes compared to glass tubes with rubber-lined lids.

Methylation:

- All samples were methylated using methanolic NaOH and BF_3 according to the standard protocol.

Analysis:

- Samples were analyzed by GC according to the established method.

Results and Discussion:

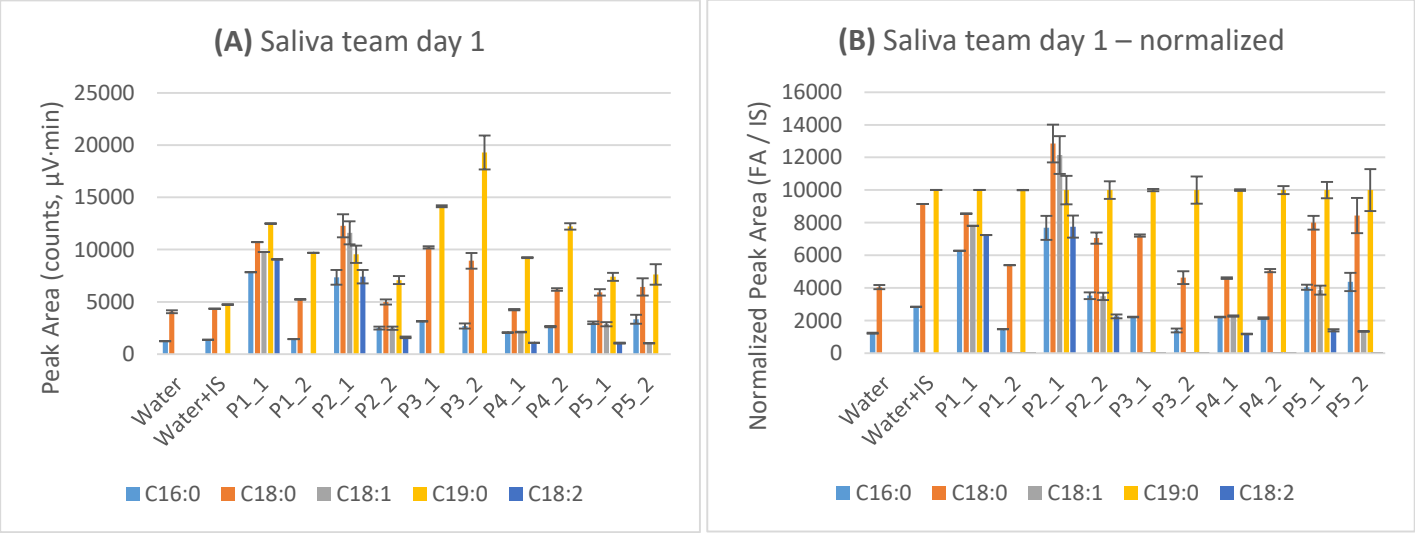


Figure 29: Fatty acid values from five participants and reference samples (day 1)

Displayed are fatty acid values from five participants and reference samples on day 1 of sample collection; P1-P5_1: morning saliva, P1-P5_2: taken 1.5-2 h later.
(A) Raw peak areas.
(B) Normalized fatty acid values (scaled to an IS reference value of 10,000).
Values are presented as the mean of two measurements ± SD.
Note: Panels (A) and (B) use different y-axis scales due to the normalization.

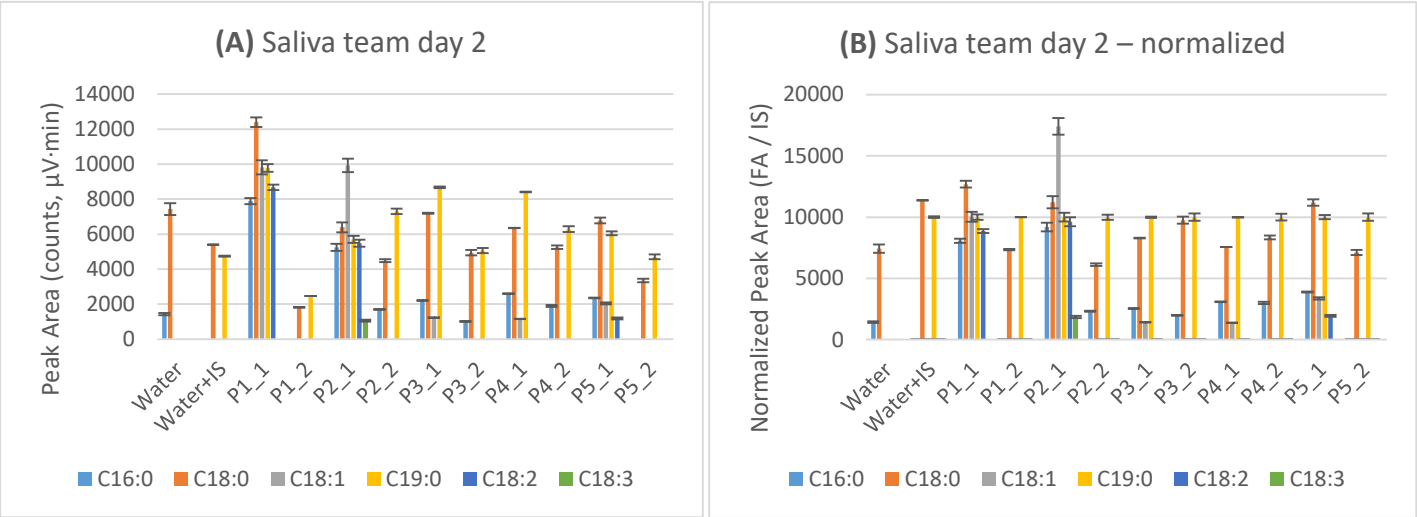


Figure 30: Fatty acid values from five participants and reference samples (day 2)

Displayed are fatty acid values from five participants and reference samples on day 2 of sample collection; P1-P5_1: morning saliva, P1-P5_2: taken 1.5-2 h later.
(**A)** Raw peak areas.
(B) Normalized fatty acid values (scaled to an IS reference value of 10,000).
Values are presented as the mean of two measurements ± SD.
Note: Panels (A) and (B) use different y-axis scales due to the normalization.

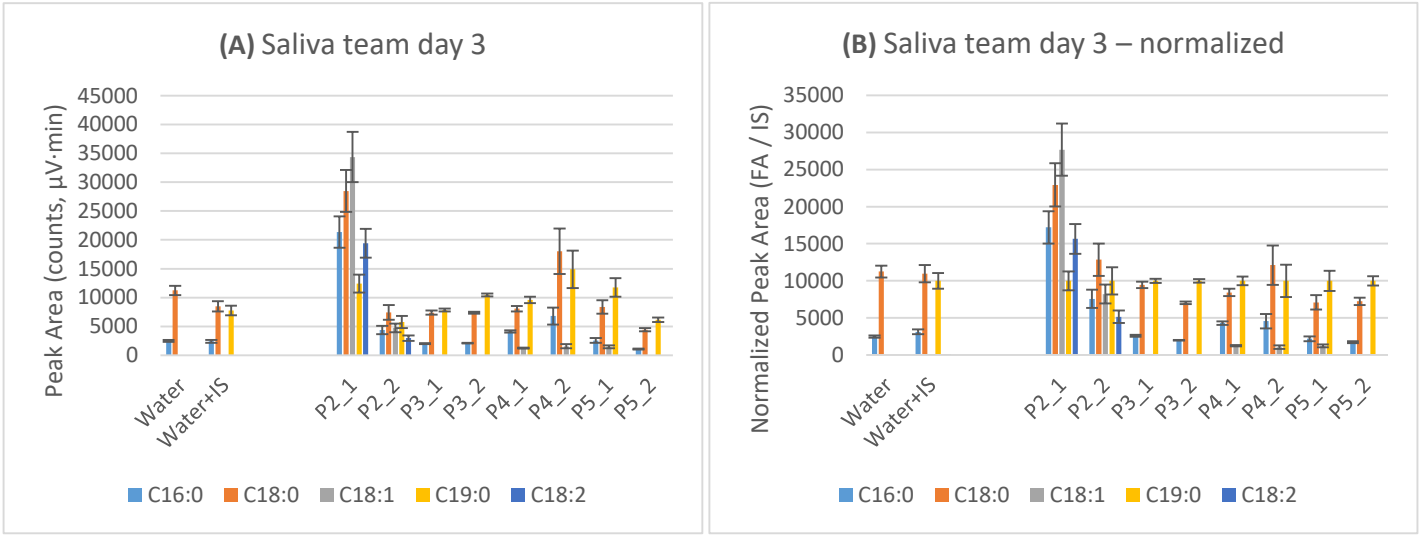


Figure 31: Fatty acid values from five participants and reference samples (day 3)

Displayed are fatty acid values from five participants and reference samples on day 3 of sample collection; P1-P5_1: morning saliva, P1-P5_2: taken 1.5-2 h later.
(A) Raw peak areas.
(B) Normalized fatty acid values (scaled to an IS reference value of 10,000).
Values are presented as the mean of two measurements ± SD.
Note: Panels (A) and (B) use different y-axis scales due to the normalization.

Although the water samples ideally should not have contained any fatty acids, C16:0 and C18:0 impurities were observed on all three days. Both the C16:0 and C18:0 values in the water samples increased over the three test days. It remains unclear whether this increasing contamination resulted from cumulative impurities in the

nuclease-free water bottles or from random introduction of impurities during the FAME methylation process. Previous tests had also shown a variable degree of impurities, further complicating the identification of the underlying cause.

In the water+IS samples, C18:0 impurities were detected on all three days, while C16:0 impurities were observed on two days. The sample peaks on days one and three were of similar magnitude, with day two being the exception, containing only C18:0 impurities. As no consistent pattern was observed in the C16:0 and C18:0 peaks across the water+IS samples, it can be assumed that the impurities occurred randomly or were introduced during the methylation process rather than originating from the nuclease-free water. However, the variation in peak areas could also be partially attributed to uneven evaporation of the samples in the SpeedVac.

As expected, the salivary fatty acid profile changed when comparing morning saliva with samples collected 1.5–2 hours later. In most cases, fatty acid levels decreased for each participant, with a few exceptions, such as C18:0 in P4 samples, which showed a slight increase at the second time point on all three days. However, this may have been due to variations in the sample preparation procedure.

It was also noticeable that some participants exhibited substantially higher fatty acid peaks than others. For instance, P1 and P2 showed higher concentrations of nearly all measured fatty acids compared to P3, P4, and P5. These differences may have been influenced by dietary factors or other variables affecting salivary fatty acid profiles, as discussed in the literature review. Notably, C18:1 and C18:2 peaks were considerably higher in P1 and P2 samples than in the samples from the other participants. However, these peaks decreased markedly during the second sampling and in some cases disappeared entirely, such as in P1_2 on the first test day, as well as in P1_2 and P2_2 on the second test day.

Comparing the development of the fatty acid profiles of the same participants over the three test days, larger changes were observed in some participants than in others. These changes were particularly pronounced in participants who initially exhibited higher and more diverse fatty acid peaks, especially in the P1 and P2 samples. For example, normalized C18:1 levels in P2_1 increased from approximately 12,000 on the first test day to over 17,000 on the second day, and slightly above 27,500 on the third day. Most other fatty acids in P2's saliva also increased across the test days (except for C18:3), resulting in a steady rise in total salivary fatty acid content over the testing period. This pattern was likely influenced by dietary intake on the days preceding each collection, possibly reflecting consumption of higher-fat foods prior to the second and third test days. However, dietary factors are only one potential explanation; other contributors may include individual metabolic differences, salivary gland activity, hormonal fluctuations, or variations in the oral microbiota.

For P1, no saliva samples were collected on the third day, limiting the assessment of fatty acid changes, although higher overall levels appeared on the second day. Among the remaining participants, notable variability in fatty acid peaks was observed, with C16:0 and C18:0 consistently dominating. Peaks of other fatty acids were frequently much smaller or even absent. It should also be noted that the extent to which C16:0 and C18:0 peaks were affected by impurities remains unclear.

Conclusion

This experiment demonstrated that salivary fatty acid profiles can vary considerably between individuals on a given day and may also fluctuate to varying degrees over the course of several days. Therefore, it would be valuable to analyze saliva samples from a larger cohort in future experiments. However, it is important to note that the current method may not consistently produce high fatty acid peaks in all participants.

4.5.2 Impact of Dietary Oils on Salivary Fatty Acids

Aim:

The aim of this experiment was to examine how the consumption of different oils affects the fatty acid profile in saliva. Three culinary oils with varying levels of saturated and unsaturated fatty acids, linseed oil, olive oil, and palm oil, were selected. The choice was based on their frequency of consumption (particularly olive oil and palm oil, with palm oil mainly used in processed foods) and, most importantly, their distinct fatty acid compositions. While linseed oil is particularly rich in linolenic acid,[90] olive oil contains the highest oleic acid content[91] and palm oil is known for its high saturated fatty acid content.[92]

Figures 32–34 provide an approximate overview of the fatty acid composition of these oils. However, depending on the type of oil, processing and analytical method, the proportions of the different fatty acids may naturally vary.

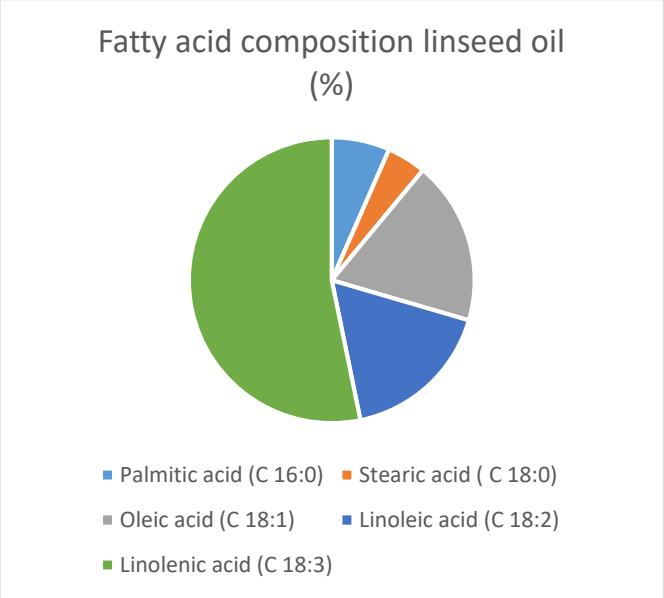


Figure 32: Fatty acid composition of linseed oil in percentages, created based on [93]

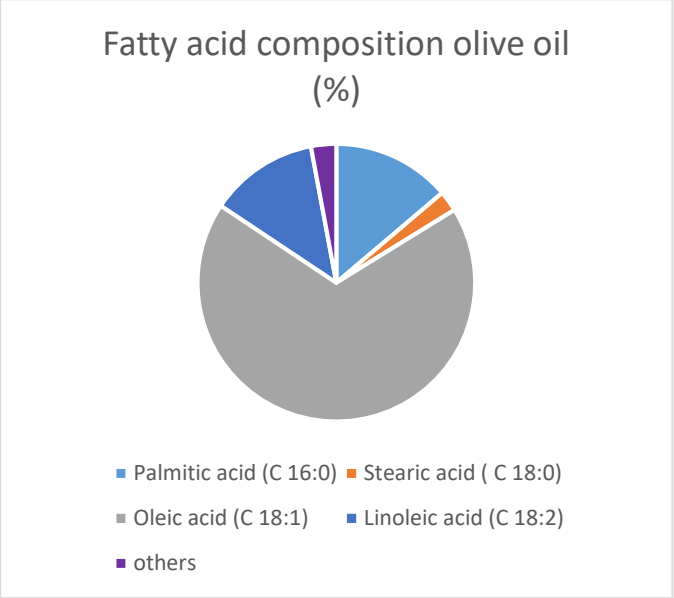


Figure 33: Fatty acid composition of olive oil in percentages, created based on [95]

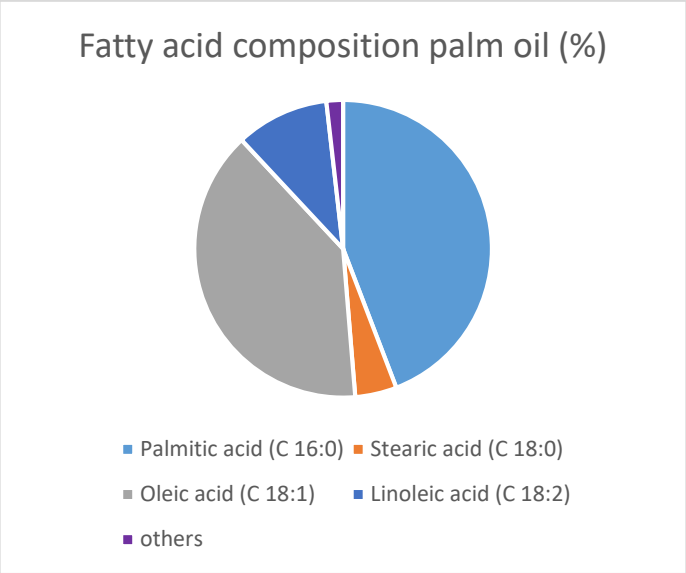


Figure 34: Fatty acid composition of palm oil in percentages, created based on [96]

Procedure:

Samples:

- Linseed Oil:
  - Water (1 mL)
  - Water + IS (1 mL + 10 µL)
  - P1_1, P1_2, P1_3, P1_4
  - P2_1, P2_2, P2_3, P2_4
  - P3_1, P3_2, P3_3, P3_4
- Olive Oil:
  - Water (1 mL)
  - Water + IS (1 mL + 10 µL)
  - P1_1, P1_2, P1_3, P1_4
  - P2_1, P2_2, P2_3, P2_4
  - P3_1, P3_2, P3_3, P3_4
  - P6_1, P6_2, P6_3, P6_4
- Palm Oil:
  - Water (1 mL)
  - Water + IS (1 mL + 10 µL)
  - IS (10 µL C19:0)
  - P1_1, P1_2, P1_3, P1_4
  - P2_1, P2_2, P2_3, P2_4
  - P3_1, P3_2, P3_3, P3_4
  - P6_1, P6_2, P6_3, P6_4

Sample Collection:

- Four participants were included; three of them (P1, P2, P3) took part in all oil experiments.
- Participant 6 (P6) participated in the olive and palm oil experiments only and collected saliva samples at home.
- Saliva samples were collected at four time points:
    1. Morning saliva (immediately after waking)
    2. 30 seconds after oil consumption
    3. 1 hour after oil consumption
    4. 3 hours after oil consumption
- Salivettes® were used as usual. Participants brushed their teeth and drank water only after the first sample. Each oil was rinsed in the mouth for 30 seconds, swallowed, and a Salivette® was taken immediately. Participants were instructed not to consume food or water between samplings.

Evaporation:

- All samples were evaporated in the SpeedVac: initial 2 hours, followed by an additional 1 hour.
- **Notes:**
    - Water and water+IS samples did not evaporate properly.
    - For palm oil samples, after 2 hours, tubes remained in the SpeedVac without active vacuum for ~10 minutes (unplanned step, may have influenced results).

Methylation and Analysis:

- All fully evaporated samples were subjected to standard methylation and GC analysis according to the established protocol.

Results and Discussion:

Linseed oil:

For the three oil experiments, the raw GC data were first plotted using the default y-axis scaling, which represented the full detector signal range. In these initial graphs, most fatty acid peaks were barely visible due to the large differences in peak areas. To improve visibility, separate graphs with adjusted y-axes were created for each oil. These adjusted plots make the peaks easier to distinguish while still preserving the relative proportions of the fatty acids.

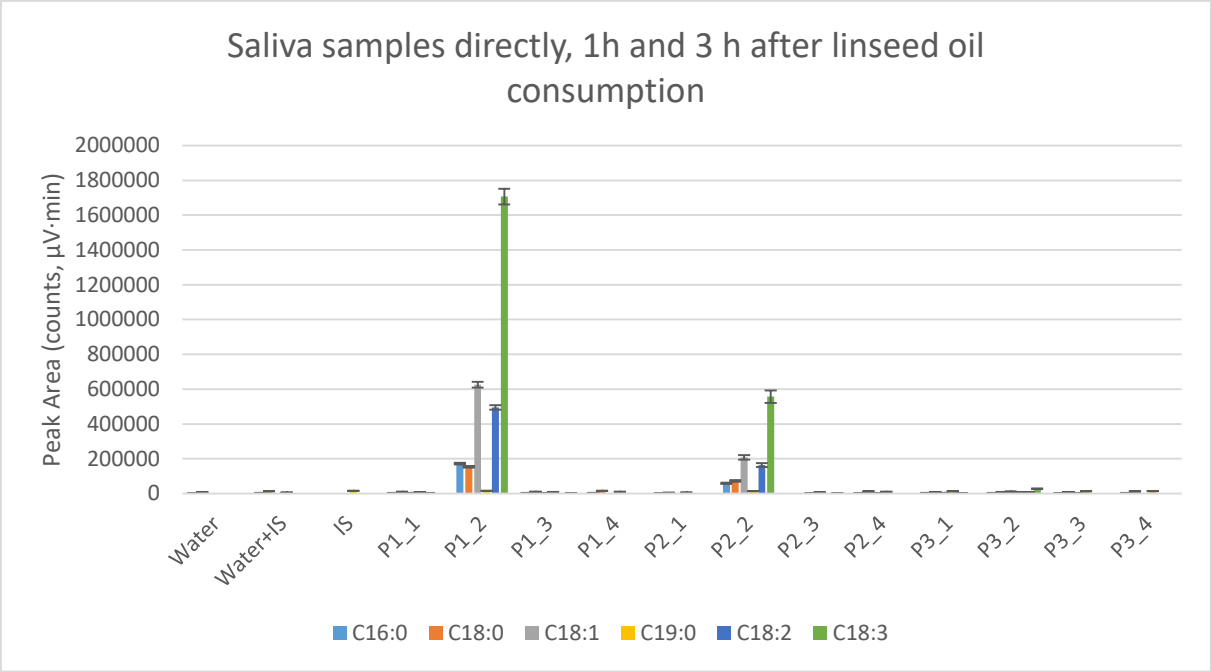


Figure 35: Peak areas of fatty acids in saliva before and after linseed oil consumption

Displayed are peak areas of various fatty acids in saliva samples from three participants (P1, P2, P3) and reference samples, collected at four time points relative to linseed oil consumption:
P1/P2/P3_1 – fasted morning saliva;
P1/P2/P3_2 – directly after oil consumption;
P1/P2/P3_3 – 1 hour after oil consumption;
P1/P2/P3_4 – 3 hours after oil consumption.
Values are shown as the mean of two measurements ± SD.

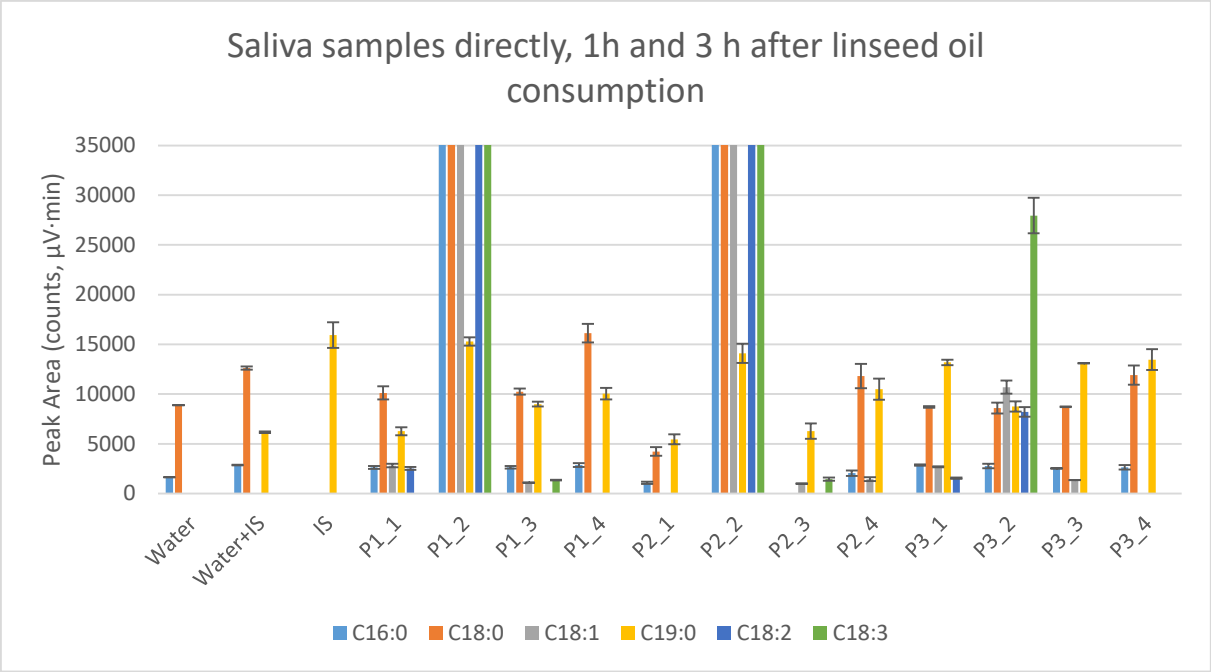


Figure 36: Peak areas of fatty acids in saliva before and after linseed oil consumption with an adjusted y-axis

Displayed are peak areas of various fatty acids in saliva samples from three participants (P1, P2, P3) and reference samples, collected at four time points relative to linseed oil consumption:
P1/P2/P3_1 – fasted morning saliva;
P1/P2/P3_2 – directly after oil consumption;
P1/P2/P3_3 – 1 hour after oil consumption;
P1/P2/P3_4 – 3 hours after oil consumption.
Values are shown as the mean of two measurements ± SD, with an adjusted y-axis for improved visibility.

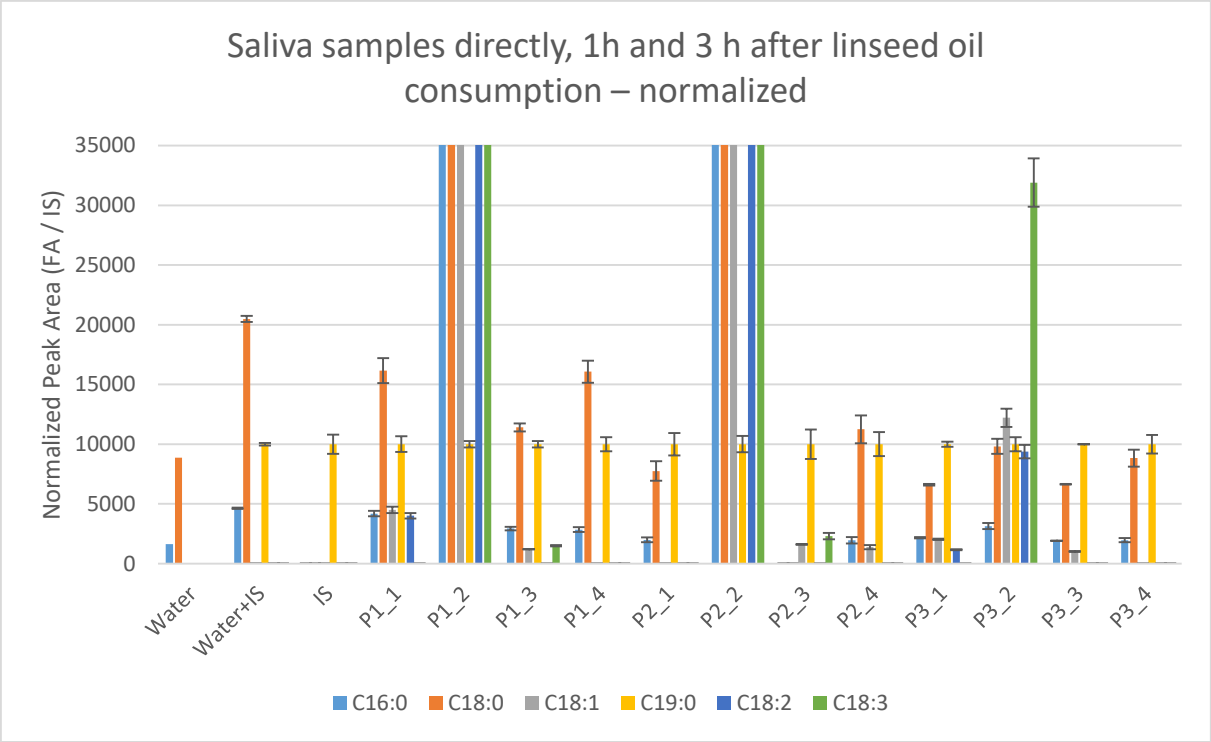


Figure 37: Normalized fatty acid values in saliva before and after linseed oil consumption with an adjusted y-axis

Displayed are normalized fatty acid values in saliva samples from three participants (P1, P2, P3) and reference samples, collected at four time points relative to linseed oil consumption:
P1/P2/P3_1 – fasted morning saliva;
P1/P2/P3_2 – directly after oil consumption;
P1/P2/P3_3 – 1 hour after oil consumption;
P1/P2/P3_4 – 3 hours after oil consumption.
Values are scaled to an IS reference value of 10,000 and are shown as the mean of two measurements ± SD, with an adjusted y-axis for improved visibility.

Figure 35 clearly shows that the fatty acid peaks in the P1_2 and P2_2 samples (i.e., the samples collected immediately after oil consumption) were considerably higher than in the other samples. This is not surprising, as these samples were taken only 30 seconds after the oil was swallowed, meaning that some oil residues were likely still present in the mouth. The differences in fatty acid peak areas between participants may be due to variations in the amount of oil swallowed or slight differences in timing, with one person possibly waiting a little longer than the intended 30 seconds, allowing more of the oil to be washed away by saliva.

As expected for linseed oil, which typically contains over 50% linolenic acid, the C18:3 peak was the highest among the fatty acid peaks at the second time point (immediately after oil consumption). However, the fatty acid content rapidly decreased by the third sampling, with only traces – or none at all, as in the case of P3_3 – of C18:3 remaining. In fact, by the third sampling, no C18:3 was detected in any of the participants

Figure 36 shows little variation between the third and fourth sampling, with some fatty acid peaks even increasing slightly at the fourth time point. It remains unclear whether these fluctuations in salivary fatty acid levels occurred naturally, despite no food intake

between samplings, or resulted from variations in sample processing (such as partial evaporation of the samples).

Olive oil:

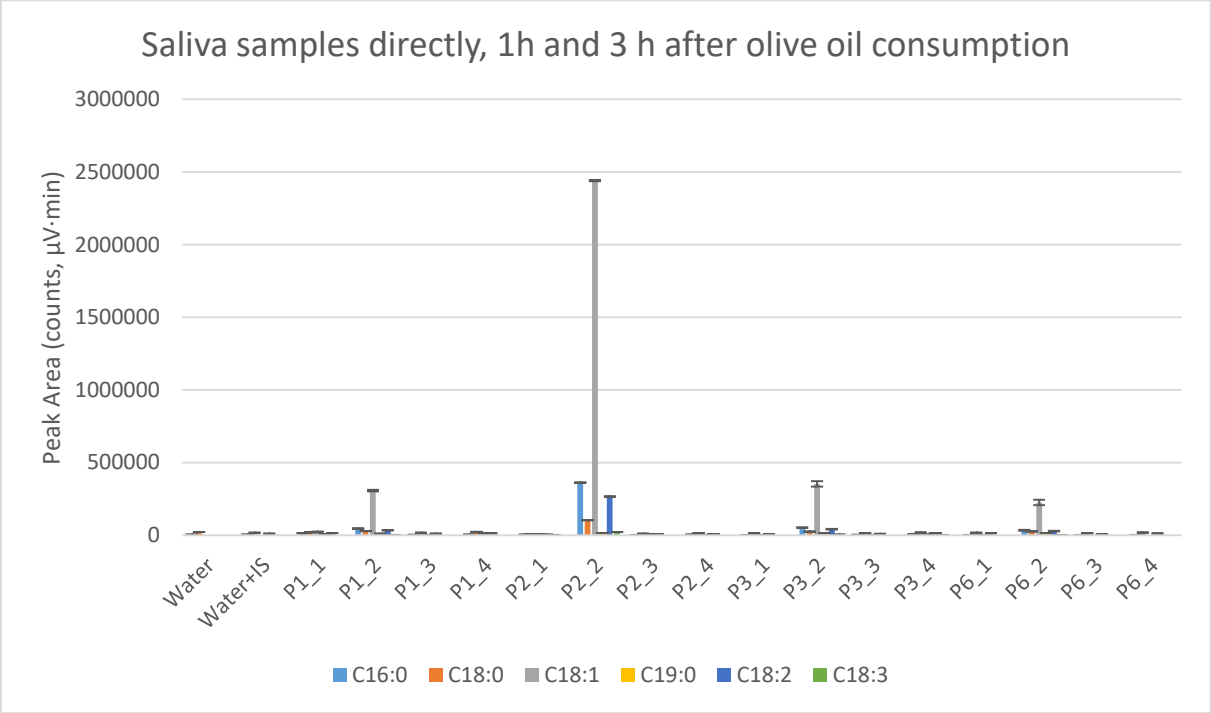


Figure 38: Peak areas of fatty acids in saliva before and after olive oil consumption

Displayed are peak areas of various fatty acids in saliva samples from four participants (P1, P2, P3, P6) and reference samples, collected at four time points relative to olive oil consumption:
P1/P2/P3/P6_1 – fasted morning saliva;
P1/P2/P3/P6_2 – directly after oil consumption;
P1/P2/P3/P6_3 – 1 hour after oil consumption;
P1/P2/P3/P6_4 – 3 hours after oil consumption.
Values are shown as the mean of two measurements ± SD.

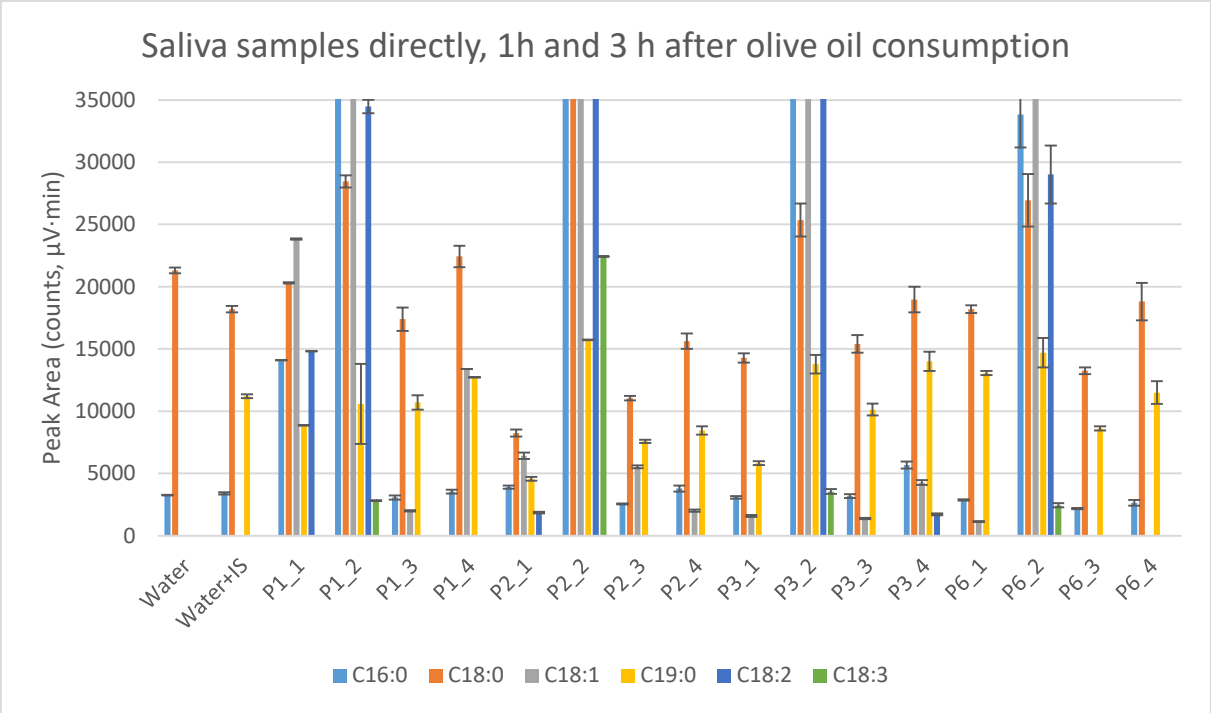


Figure 39: Peak areas of fatty acids in saliva before and after olive oil consumption with an adjusted y-axis

Displayed are peak areas of various fatty acids in saliva samples from four participants (P1, P2, P3, P6) and reference samples, collected at four time points relative to olive oil consumption:
P1/P2/P3/P6_1 – fasted morning saliva;
P1/P2/P3/P6_2 – directly after oil consumption;
P1/P2/P3/P6_3 – 1 hour after oil consumption;
P1/P2/P3/P6_4 – 3 hours after oil consumption.
Values are shown as the mean of two measurements ± SD, with an adjusted y-axis for improved visibility.

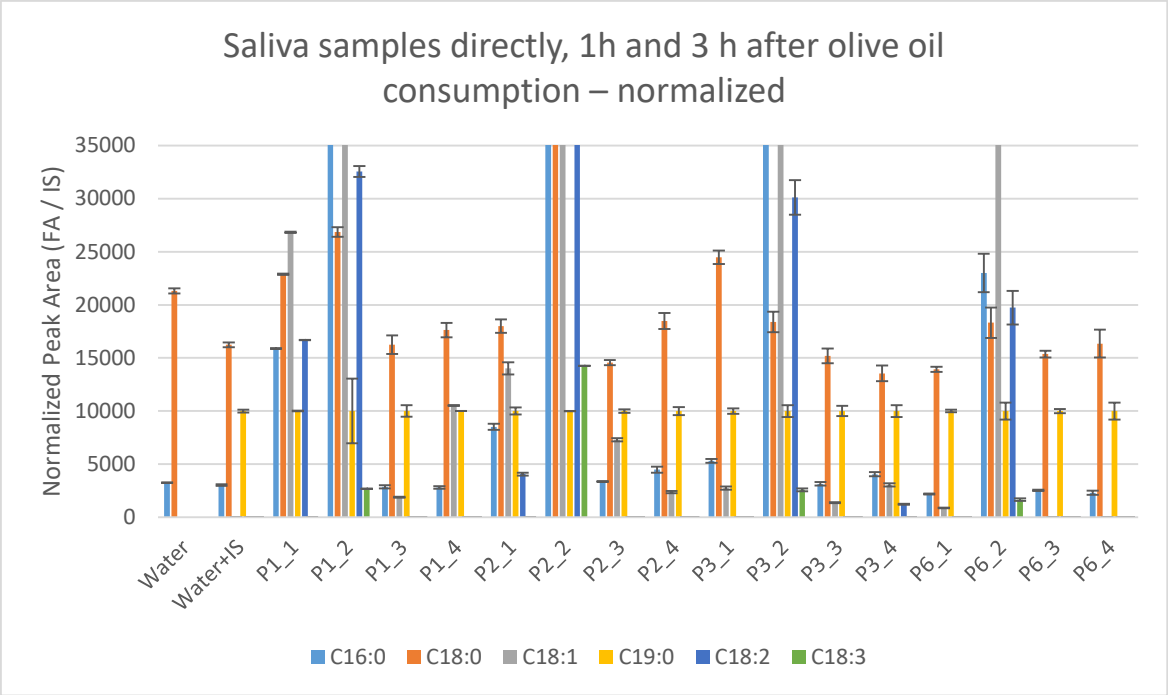


Figure 40: Normalized fatty acid values in saliva before and after olive oil consumption with an adjusted y-axis

Displayed are normalized fatty acid values in saliva samples from four participants (P1, P2, P3, P6) and reference samples, collected at four time points relative to olive oil consumption:
P1/P2/P3/P6_1 – fasted morning saliva;
P1/P2/P3/P6_2 – directly after oil consumption;
P1/P2/P3/P6_3 – 1 hour after oil consumption;
P1/P2/P3/P6_4 – 3 hours after oil consumption.
Values are scaled to an IS reference value of 10,000 and are shown as the mean of two measurements ± SD, with an adjusted y-axis for improved visibility.

Figure 38 shows the unadjusted data for the olive oil test. As oleic acid (C18:1) is the predominant fatty acid in olive oil, it was expected that C18:1 would show the highest peak after oil consumption. Similar to the test with linseed oil, the peak areas at the second sampling varied substantially between participants. This is likely due to P2 having more oil residues in the mouth when collecting the saliva sample after oil consumption compared to the others. C16:0 and C18:2 fatty acid peaks were also notably high at the second sampling. However, by the third sampling, C18:1 peaks (raw values) had dropped below 6,000 counts and were undetectable in P6_3. The extent of the fatty acid decrease varied between participants. For instance, even though P3 had higher C18:1 levels at the second sampling, the levels fell below those of P1 by the third sampling. Some inconsistencies were also present, such as the C18:1 (and C18:2) values in P3_4 being higher than those in P3_3.

Palm oil:

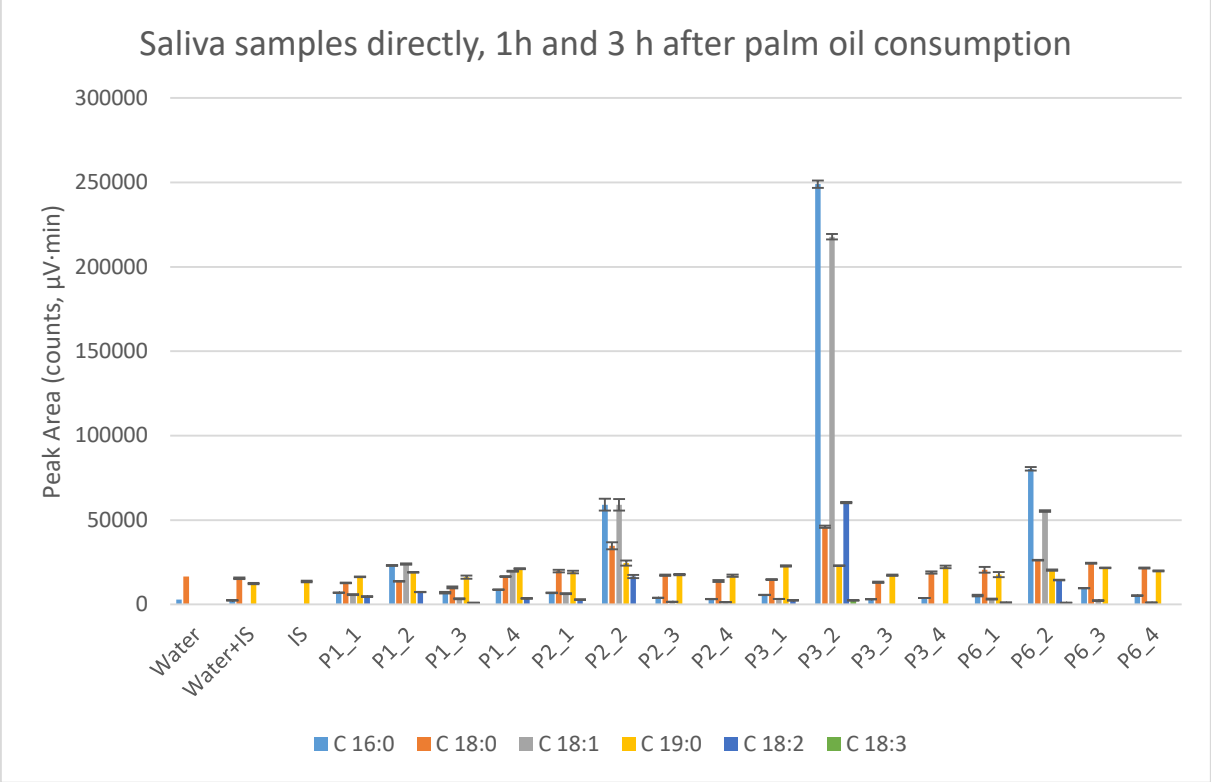


Figure 41: Peak areas of fatty acids in saliva before and after palm oil consumption

Displayed are peak areas of various fatty acids in saliva samples from four participants (P1, P2, P3, P6) and reference samples, collected at four time points relative to palm oil consumption:
P1/P2/P3/P6_1 – fasted morning saliva;
P1/P2/P3/P6_2 – directly after oil consumption;
P1/P2/P3/P6_3 – 1 hour after oil consumption;
P1/P2/P3/P6_4 – 3 hours after oil consumption.
Values are shown as the mean of two measurements ± SD.

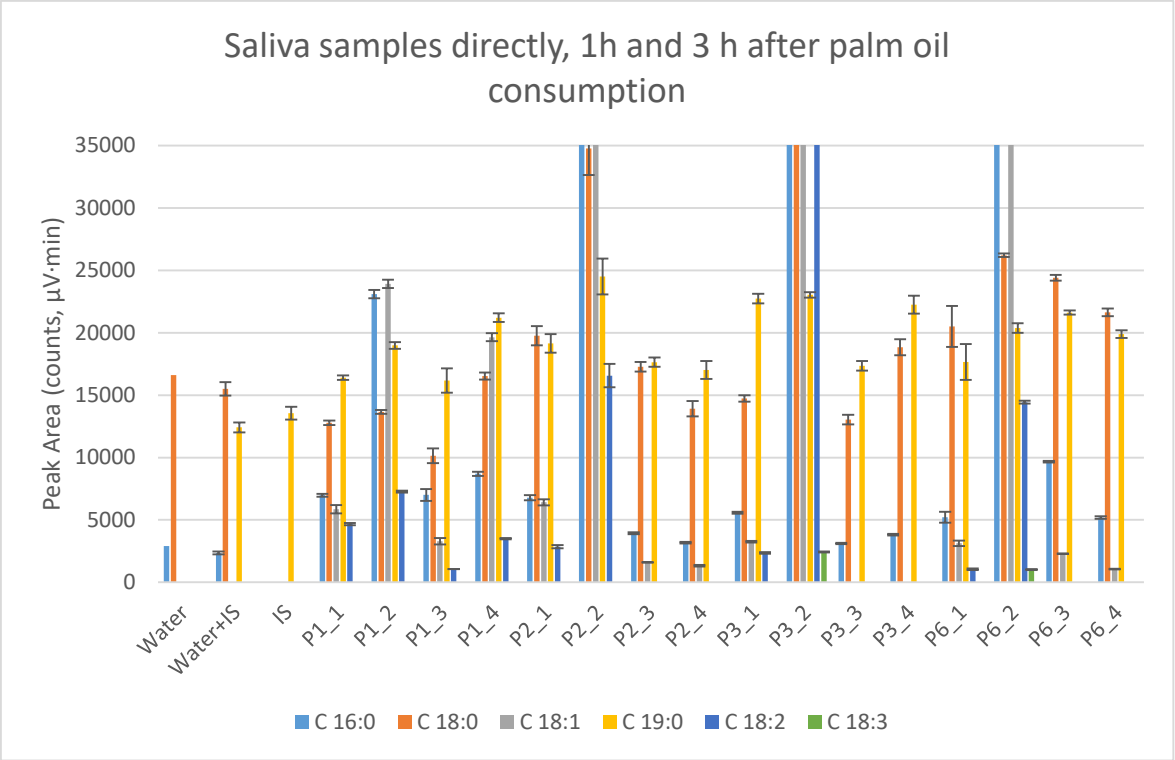


Figure 42: Peak areas of fatty acids in saliva before and after palm oil consumption with an adjusted y-achsis

Displayed are peak areas of various fatty acids in saliva samples from four participants (P1, P2, P3, P6) and reference samples, collected at four time points relative to palm oil consumption:
P1/P2/P3/P6_1 – fasted morning saliva;
P1/P2/P3/P6_2 – directly after oil consumption;
P1/P2/P3/P6_3 – 1 hour after oil consumption;
P1/P2/P3/P6_4 – 3 hours after oil consumption.
Values are shown as the mean of two measurements ± SD, with an adjusted y-axis for improved visibility.

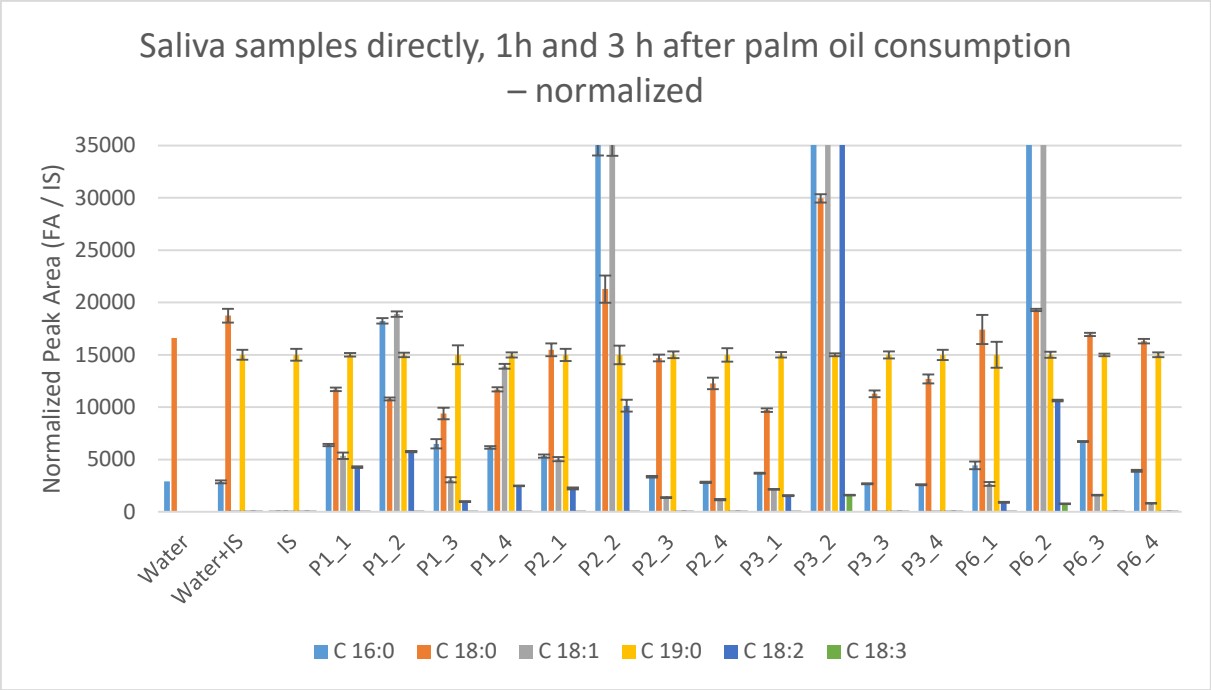


Figure 43: Normalized fatty acid values in saliva before and after palm oil consumption with an adjusted y-axis

Displayed are fatty acid values in saliva samples from four participants (P1, P2, P3, P6) and reference samples, collected at four time points relative to palm oil consumption:
P1/P2/P3/P6_1 – fasted morning saliva;
P1/P2/P3/P6_2 – directly after oil consumption;
P1/P2/P3/P6_3 – 1 hour after oil consumption;
P1/P2/P3/P6_4 – 3 hours after oil consumption.
Values are scaled to an IS reference value of 15,000 and are shown as the mean of two measurements ± SD, with an adjusted y-axis for improved visibility.

According to the fatty acid composition reported in the literature, palmitic acid levels were highest after palm oil consumption, followed by oleic acid. Smaller amounts of linoleic acid were also detected in some saliva samples. However, as observed in previous experiments, fatty acid peaks rapidly decreased by the third sampling. The difference between the third and fourth samplings, however, was minimal, with the exception of P1_4, where levels seemed to rise again. The possibility that samples 1_3 and P1_4 were accidentally switched cannot be excluded.

Additionally, a considerable amount of impurities was still detected in the water and water+IS samples, with the C18:0 contamination even exceeding the C18:0 peaks found in the saliva samples

When comparing the results of the palm oil experiment to those of the other oil experiments, substantial variation in fatty acid peak areas was observed among participants. It was difficult to predict which participants would exhibit the highest fatty acid peaks after oil consumption. To determine whether these variations occured randomly, future experiments should place greater emphasis on strictly controlling the interval between oil consumption and sample collection.

Conclusion

In summary, the fatty acid profiles across the different oil experiments varied considerably between participants, with peak areas after oil consumption remaining largely unpredictable. While the fatty acid composition of the oils was generally reflected in the saliva samples and salivary fatty acid concentrations decreased over time, inconsistencies in timing, possible sample mislabeling, and recurring impurities may have affected the results. Future experiments should therefore prioritize stricter control of the time intervals between oil consumption and sample collection to reduce variability.

4.6 Development and Testing of Cheek Cell Sampling

Inspired by the work of Annemarie Grindel, a former researcher at the University of Vienna, we implemented a new cell sampling method. In 2013, Grindel co-published a study on the effect of linseed oil supplementation on fatty acid levels in cheek cells. [18] Given the challenges we faced in obtaining higher fatty acid concentrations from saliva, and the limited success of adjusting our sample preparation methods, we decided to test cheek cells as an alternative sample matrix.

Following Grindel et al.'s protocol, samples were collected in the morning at the Department of Nutritional Sciences, after teeth brushing but while the participants were still fasted. No liquids, including water, were consumed before sampling.
To begin, the mouth was rinsed with 20 mL of Milli-Q water. For each sample, 10 mL of Milli-Q water were placed in a 50 mL Falcon tube. Both inner cheeks were brushed 20 times using a children's toothbrush (todaydent, Bipa), and the toothbrush was then inserted into the Falcon tube, where it was moved back and forth to dislodge the collected cells.
The mouth was then rinsed again with 20 mL of Milli-Q water, which was added to the Falcon tube containing the toothbrush. The toothbrush was rinsed using a 1000 µL pipette with two 1 mL washes and one 0.5 mL wash of Milli-Q water to ensure any remaining cells were collected. The Falcon tubes were centrifuged at 3700 x g for 10 minutes at room temperature. After centrifugation, the supernatant was carefully decanted, and the pellet was washed with 1 mL of Milli-Q water (1 mL was added and gently pipetted out again). The pellet was then resuspended in 1 mL of Milli-Q water, transferred to Eppendorf tubes, and 10 µL of C19:0 IS were added. Finally, the tubes were placed in the SpeedVac for further processing.

4.6.1 Evaluation of the Cheek Cell Sampling Method

Aim:

This experiment aimed to identify potential sources of contamination and determine whether the C16:0 and C18:0 fatty acid impurities originated from the nuclease-free water, the IS, the SpeedVac, or were introduced at some other point during sample handling.

Procedure:

Samples:
- IS (10 µL C19:0)
- IS+MeOH (10 µL + 1 mL)
- IS+Water (10 µL + 1 mL)
- Empty
- P1_cells
- P2_cells

Sample Collection:

- Cheek cell samples were collected using the method described above.

Evaporation:

- The IS C19:0 sample was placed in the SpeedVac for 5 minutes until fully evaporated.
- The IS+MeOH sample was evaporated for 2 hours until fully dry.
- The IS+Water sample, empty tube, and cheek cell samples (P1_cells, P2_cells) were left in the SpeedVac for an additional hour, reducing their volume to about half.

Reconstitution and Transfer:

- 1 mL MeOH was added to the empty tube and the fully evaporated samples (IS, IS+MeOH). The solvent was aspirated and dispensed multiple times with a pipette to ensure thorough mixing.
- These solutions were transferred into test tubes containing NaOH/MeOH.
- The remaining samples (IS+water, P1_cells, P2_cells) were directly transferred into test tubes with NaOH/MeOH.
- All microtubes were rinsed with 20 µL and subsequently 80 µL NaOH/MeOH, and the rinses were added to the respective test tubes.

Methylation and Analysis:

- All samples were methylated and analyzed by GC according to the standard protocol.
- **Note:** The IS+water sample could not be evaporated under N_2 prior to dissolving in hexane and SpeedVac treatment; therefore, it was not analyzed.

Results and Discussion:

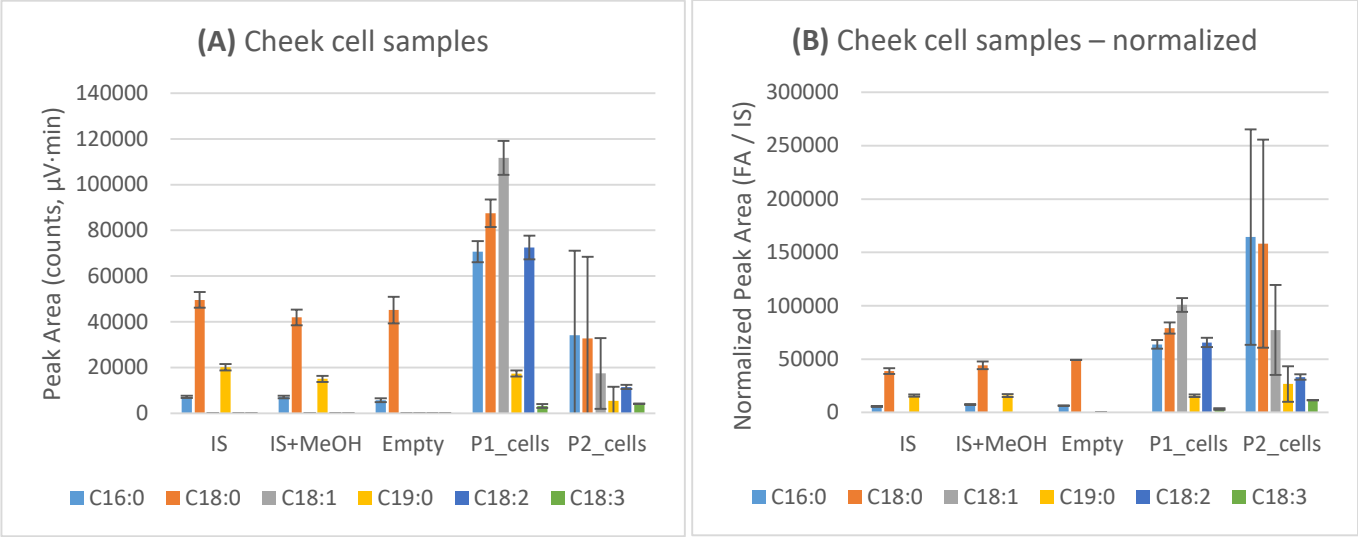


Figure 44: Fatty acid values in reference, control and cheek cell samples

Displayed are fatty acid values of reference samples (IS and IS+MeOH), the empty control, and cheek cell samples from two participants.
(A) Raw peak areas.
(B) Normalized fatty acid values (scaled to an IS reference value of 15,000).
Values are shown as the mean of two measurements ± SD.
Note: Panels (A) and (B) use different y-axis scales due to the normalization.

In the IS sample, both C16:0 and C18:0 fatty acids were detected, even though only C19:0 had been intentionally added. Notably, the amount of C18:0 was roughly twice that of C19:0, and the empty control showed contamination levels comparable to those in the IS and IS+MeOH samples. Since contamination was similar in the empty control and in samples containing only the IS, the C16:0 and C18:0 contaminants cannot be attributed to the IS itself. Rather, they likely originated during evaporation in the SpeedVac or at another stage of sample preparation.

The peaks in the P1_cells and P2_cells samples were quite pronounced, with noramlized C18:1 values in P1_cells approaching 100,000 and C16:0 reaching 60,000 in Figure 44 (panel B), far exceeding the levels of C16:0 contamination observed in the empty control and IS samples. In P2_cells, normalized fatty acid levels were even higher than in P1_cells, however, standard deviations were notably high.
The fatty acid peak areas from the cheek cell samples were promising, though not necessarily higher than those found in salivary samples. Nevertheless, this new sampling method will be used in the subsequent experiments of this thesis.

Conclusion

The new sampling method using cheek cells seems promising but will require further testing to assess its effectiveness. However, contamination issues persisted, with considerable levels of C16:0 and C18:0 fatty acids detected in the IS, IS+MeOH and empty control samples, likely introduced during sample processing.

4.6.2 Repetition to Identify Contamination Sources

Aim:

The experiment was repeated with additional samples to clarify potential contamination sources and the effects of the SpeedVac, as the water sample from the previous test could not be analyzed.

Procedure:

Samples:
- IS (10 µL C19:0)
- IS+MeOH (10 µL + 1 mL)
- IS+Water (10 µL + 1 mL)
- Empty 1-2
- P1_cells_1-2
- P2_cells
- P3_cells

Sample Collection:

- Cheek cell samples were collected according to the method described by Grindel et al.

Evaporation:

- The IS (C19:0) sample was placed in the SpeedVac for 5 minutes until fully evaporated.
- When the SpeedVac was opened after 5 minutes, a drop of liquid was observed in empty microtube 1 (Empty 1). As a result, an additional empty tube (Empty 2) was added to the experiment.
- The IS+MeOH sample was evaporated for 2 hours until completely dry.
- The remaining samples (IS+Water, Empty 2, P1_cells_1, P1_cells_2, P2_cells, P3_cells) were kept in the SpeedVac for an additional hour, reducing their volume to about half.

Methylation and Analysis:

- All samples were subsequently processed according to the standard protocol and analyzed by GC.

Results and Discussion:

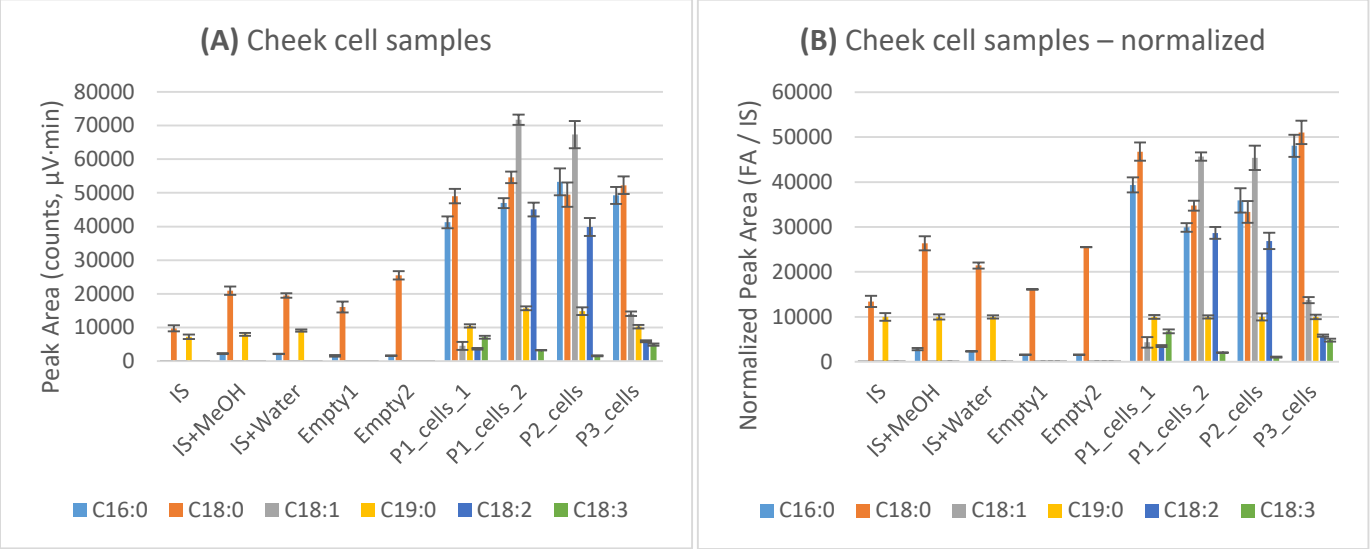


Figure 45: Fatty acid values in reference, control and cheek cell samples (repeat experiment)

Displayed are fatty acid values of reference samples (IS, IS+MeOH, IS+W), empty controls, and cheek cell samples.
(A) Raw peak areas.
(B) Normalized fatty acid values (scaled to an IS reference value of 10,000).
Values are shown as the mean of two measurements ± SD.

This time, there was no C16:0 peak in the IS sample, but small peaks were present in the IS+MeOH, IS+water, Empty 1, and Empty 2 samples. There was also considerable C18:0 contamination in these samples, with values reaching ~10,000–25,000 counts. Empty 2 showed more impurities than Empty 1, in which a drop of liquid had entered during the SpeedVac process. This suggests that the contamination could have partly been caused by the SpeedVac, with some liquid possibly entering Empty 2 as well, or that a higher amount of contaminants than those contained in the drop of liquid was introduced into the Empty 2 sample during the methylation process.

Normalized fatty acid levels were lower than in the previous experiment, with the highest values ranging from ~40,000 to just over 50,000. This variation may also reflect differences in dietary intake on the day prior to sampling.

Conclusion

The impurities were lower compared to the previous experiment, but so were the fatty acid peaks. While the SpeedVac may have contributed to sample contamination, further tests are needed to confirm this.

4.7 Testing Different Lipid Extraction Methods

4.7.1 Folch Lipid Extraction Method

Aim:

Initially, we avoided using the lipid extraction method by Folch et al.[14] due to the relatively high amounts of toxic solvents, chloroform and methanol, required. Additionally, considering the small sample sizes, the volumes of chloroform and methanol used are quite large. Frequent liquid transfers further increase the risk of sample loss.

However, since it was difficult to reduce impurities and enhance fatty acid peaks with the previously used method, we decided to explore alternative lipid extraction techniques. Professor Wagner suggested the Folch et al. method, as it is the gold standard for lipid extraction and one he had successfully used in the past. A department-specific protocol for the Folch et al. method was followed.

Procedure:

Samples:
- IS
- P1_saliva
- P1_cells
- P2_saliva
- P2_cells

Sample Preparation/Day 1:

- Four 50 mL sealable screw-cap jars were rinsed with chloroform under the chemical hood.
- 2 mL of each saliva sample (P1_saliva, P2_saliva) and 2 mL of each cell sample P1_cells, P2_cells) were added to the jars. (For the cell samples, the pellets dissolved in 1 mL of Milli-Q water were further diluted with an additional 1 mL of Milli-Q water.)
- 10 mL of chloroform and 5 mL of MeOH were added to each sample.
- 10 µL of IS C19:0 was added to each jar.
- The jars were briefly purged with nitrogen (N_2) and sealed.
- The samples were shaken overnight in a cold room at low speed using a shaker.

Sample Preparation/Day 2:

- Each sample was poured into six 100 mL separation funnels, using glass funnels with folded filters.
- The Pyrex glass jars were rinsed with 3 sets of 2 mL chloroform, and the liquid was transferred to the separating funnels. The filters were rinsed with approximately 2x1 mL chloroform, and then left to rest for 10 minutes.
- Once the entire extract had passed into the separation funnels, the filters were discarded.

- 3 mL of calcium chloride (CaCl2) solution was added to the separation funnels, which were then sealed with glass stoppers and shaken vigorously for 1 minute with intermittent venting.
- The phases were allowed to separate (~5 minutes).
- The lipid phase was drained into a 50 mL measuring cylinder using a folded filter with 3 spatula portions of sodium sulfate.
- 5 mL of chloroform was added to the aqueous phase, and the funnel was shaken with intermittent venting.
- The lipid phase was drained once more.
- The folded filter was rinsed with two sets of 1 mL chloroform and then discarded.
- The lipid extract was transferred into opaque glass containers and degassed under nitrogen (N_2).

Methylation and Analysis:

- The glass tubes were stored in the freezer at -20°C, methylated and analyzed with GC the following day.

Results and Discussion:

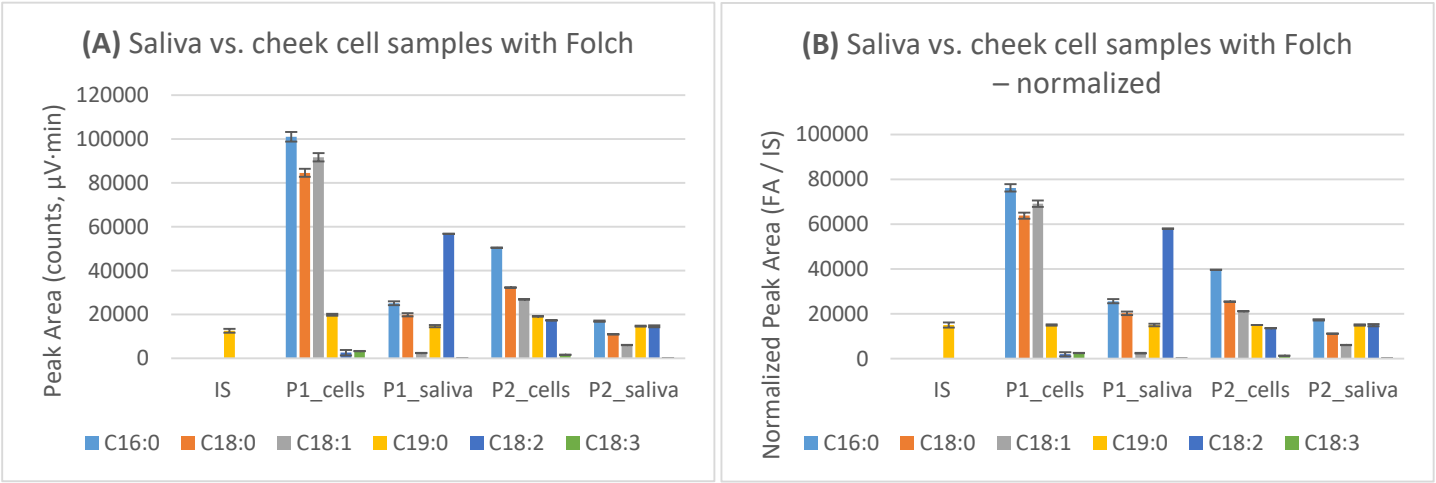


Figure 46: Fatty acid values in saliva and cheek cell samples after Folch extraction

Displayed are fatty acid values of the IS and cheek cell samples (of the two participants P1 and P2), after lipid extraction according to Folch et al.[14]
(A) Raw peak areas.
(B) Normalized fatty acid values (scaled to an IS reference value of 15,000).
Values are shown as the mean of two measurements ± SD.

The IS sample (10 µL IS C19:0 alone) was mistakenly not prepared using the Folch et al. method like the other samples. Instead, it was added only during the FAME methylation step, which limits its utility as an internal standard. Without the IS sample undergoing the lipid extraction process as a control, it is impossible to accurately assess potential fatty acid losses during the extraction procedure.

The peaks, particularly those from the cheek cell samples, appeared promising. The normalized values of P1's cheek cell fatty acids reached nearly 80,000, while those of P2 reached up to 40,000. Figure 46 also effectively illustrates the difference in peak

area between cheek cell and saliva samples. Most of the fatty acids in the cheek cell samples were considerably higher than those in the saliva samples.

Nevertheless, the Folch et al. lipid extraction method presents practical challenges, including extended processing time and difficulties in maintaining procedural cleanliness and precision. These limitations raise concerns about its efficiency for routine application, despite potential favorable analytical outcomes.

Conclusion

The cheek cell samples showed promising fatty acid peaks, with values considerably higher than those from saliva samples. However, practical constraints of the Folch et al. extraction method may limit its suitability for routine use.

4.7.2 Repetiton of Folch Extraction Tests

Aim:

To test the reproducibility of the lipid extraction method according to Folch et al. and to include a comparative control with C19:0 IS only, the extraction was repeated once more.

Procedure:

Samples:
- IS
- Empty
- P1_saliva
- P3_saliva
- P4_saliva
- P1_cells
- P3_cells
- P4_cells

Sample Preparation / Day 1:

- Six sealable 50 mL screw-cap jars were rinsed with chloroform under the chemical hood.
- 2 mL of saliva samples (P1_saliva & P4_saliva; P3_saliva sample was only around 1.8 mL) and 1.5 mL each of cell samples (P1_cells, P3_cells, P4_cells) were added to the jars. (For this, cell pellets were dissolved in 1.5 mL of Milli-Q water.)
- 10 mL of $CHCl_3$ and 5 mL MeOH were added to each jar.
- 10 µL of IS C19:0 was added to each sample.
- Jars were briefly purged with N_2 and the lids closed.
- Samples were shaken for 60 min in a cold room on a shaker at low speed and left in the cold room overnight.

Sample Preparation / Day 2:

- Two additional 50 mL Pyrex jars were rinsed with $CHCl_3$.

- 10 mL of $CHCl_3$ and 5 mL of MeOH were added to each jar.
- In one jar, an additional 10 µL of IS was added (samples Empty and IS).
- Lipid extraction was performed on all 8 samples following the same procedure as in the previous experiment.

Methylation and Analysis:

- FAME methylation and GC analysis were carried out as usual.

Results and Discussion:

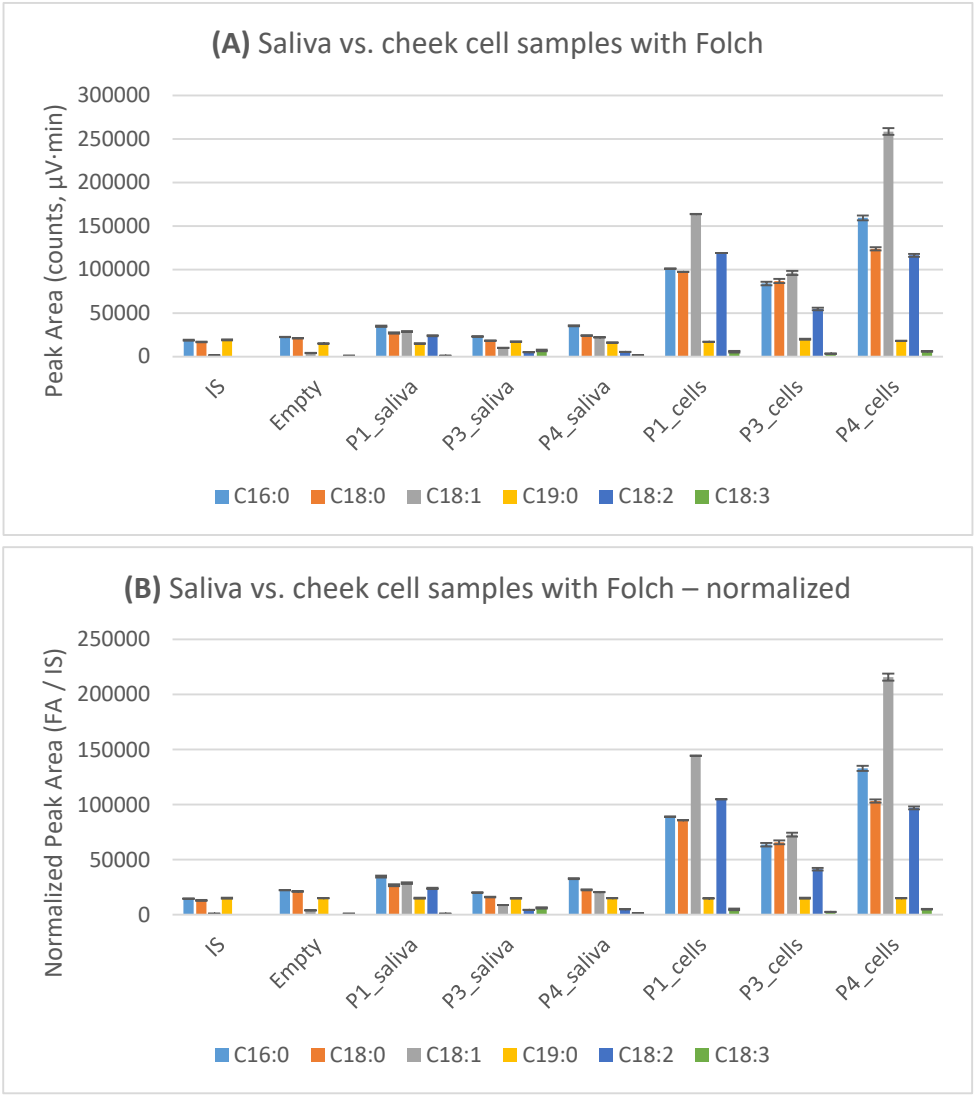


Figure 47: Fatty acid values in control, saliva and cheek cell samples after Folch extraction

Displayed are fatty acid values of the IS, empty control, saliva samples, and cheek cell samples (of the three participants P1, P3 and P4), all prepared using the Folch method.
(A) Raw peak areas.
(B) Normalized fatty acid values (scaled to an IS reference value of 15,000).
Values are shown as the mean of two measurements ± SD.

As with the previous experiment, the fatty acid peaks in the cheek cell samples were considerably higher than those in the pure saliva samples. Compared to FAME tests conducted without prior lipid extraction using the Folch et al. method, the total peak areas were notably high, with many fatty acids surpassing 100,000 counts and a single fatty acid (C18:1 in P4_cells) even exceeding 250,000 counts in Figure 47 (graph A).

The IS and Empty samples also contained considerable amounts of contaminants. While these levels were comparable to the peaks observed in the saliva samples, they were relatively negligible compared to the much higher peaks seen in the cheek cell samples.

Conclusion

These results indicate that the Folch et al. method provides high peak areas for fatty acids, especially in cheek cell samples. However, given the method's extensive use of toxic solvents, increased processing time, and procedural complexity, newer, more effective, and environmentally friendly lipid extraction methods may offer better alternatives for our needs.

4.7.3 Matyash Lipid Extraction Method

Following the suggestion of my lab supervisor, Patrick Zöhrer, we explored the use of methyl-tert-butyl ether (MTBE) as an alternative to the traditional Folch method for lipid extraction. The Folch method has been refined by various researchers,[16,93] and the method developed by Matyash et al. offers several advantages over Folch, including a faster and cleaner extraction process. One notable benefit is the ease of collection and reduced risk of dripping losses, as the lipid-containing organic phase separates on top of the aqueous phase.[16] In contrast, the Folch method positions the organic phase below the aqueous phase.[14] However, the MTBE-based method may present challenges, such as decreased reproducibility due to the high volatility of MTBE.[94]

Comparison of N_2 treatment with SpeedVac as sample preparation tools

Aim:

The aim was to evaluate whether N_2 gas treatment was a more effective sample preparation method compared to the SpeedVac. The results obtained with the SpeedVac were not entirely convincing, as it remained unclear whether it effectively increased peak areas. Additionally, the persistent, untraceable impurities could not be ruled out as originating from the SpeedVac. To address this, the SpeedVac´s performance was compared to that of N_2 gas evaporation prior to fatty acid saponification and methylation.

Procedure:

Samples:
- P1_cells_N_2 (nitrogen)
- P3_cells_N_2 (nitrogen)
- P7_cells_N_2 (nitrogen)
- P1_cells_SV (SpeedVac)

- P3_cells_SV (SpeedVac)
- P7_cells_SV (SpeedVac)

Sample Collection:

- For this experiment, the cheek cell extraction method described by Grindel et al. was used again, with a slight modification: the cell pellet was not diluted in Milli-Q water after centrifugation.
- Instead, the supernatant was carefully removed using a Pasteur pipette, and any remaining liquid was collected with a 100 µL pipette.

Matyash Extraction / Modifications in Sample Preparation:

- The cell pellets were directly diluted with MTBE in the Falcon tube, filling each tube up to 5 mL. (Note: MTBE drips heavily, so a 5 mL pipette could not be used.)
- 10 µL of IS C19:0 was added to each sample.
- Falcon tubes were placed on a shaker at medium-high speed (750 RPM) for 1 hour at room temperature.

Matyash Extraction / Phase Separation:

- After shaking, 1.25 mL of nuclease-free water was added to each sample using a multipipette to induce phase separation.
- The samples were incubated on a shaker at low speed (300 RPM) for 10 minutes, followed by centrifugation at 1,000 x g for 10 minutes.
- The upper organic phase was carefully removed and divided into two treatment groups:
    - **N_2 treatment group**: Organic phase transferred into glass tubes with screw caps (P1_N_2, P3_N_2, P7_N_2) for evaporation under nitrogen.
    - **SpeedVac group**: Organic phase transferred into 15 mL Falcon tubes (P1_SV, P3_SV, P7_SV) for vacuum centrifuge evaporation.

Matyash Extraction / Additional Washing Step:

- In a black, 50 mL glass container (rinsed with MTBE), 10 mL MTBE, 3 mL MeOH, and 2.5 mL nuclease-free water were mixed.
- 2 mL of this mixture was added to each Falcon tube, followed by shaking at low speed (300 RPM) for 5 minutes and centrifuging at 1,000 x g for 10 minutes.
- The supernatant was then added back to the glass tubes (N_2 treatment) or Falcon tubes (SpeedVac group).

Evaporation:

- **N_2 treatment group**: Glass tubes were treated with N_2 in a heating block for 30 minutes until fully evaporated.
- **SpeedVac group**: Falcon tubes were placed in the vacuum centrifuge for 1 hour at standard settings (no temperature, ramp 5) until nearly fully evaporated.

- The following day, the SpeedVac samples (P1_SV, P3_SV, P7_SV) were placed in the vacuum centrifuge for an additional 1 hour and 20 minutes, with periodic checks, until fully evaporated.

Methylation and Analysis:

- All samples were then methylated as per the usual protocol and analyzed using GC.

Results and Discussion:

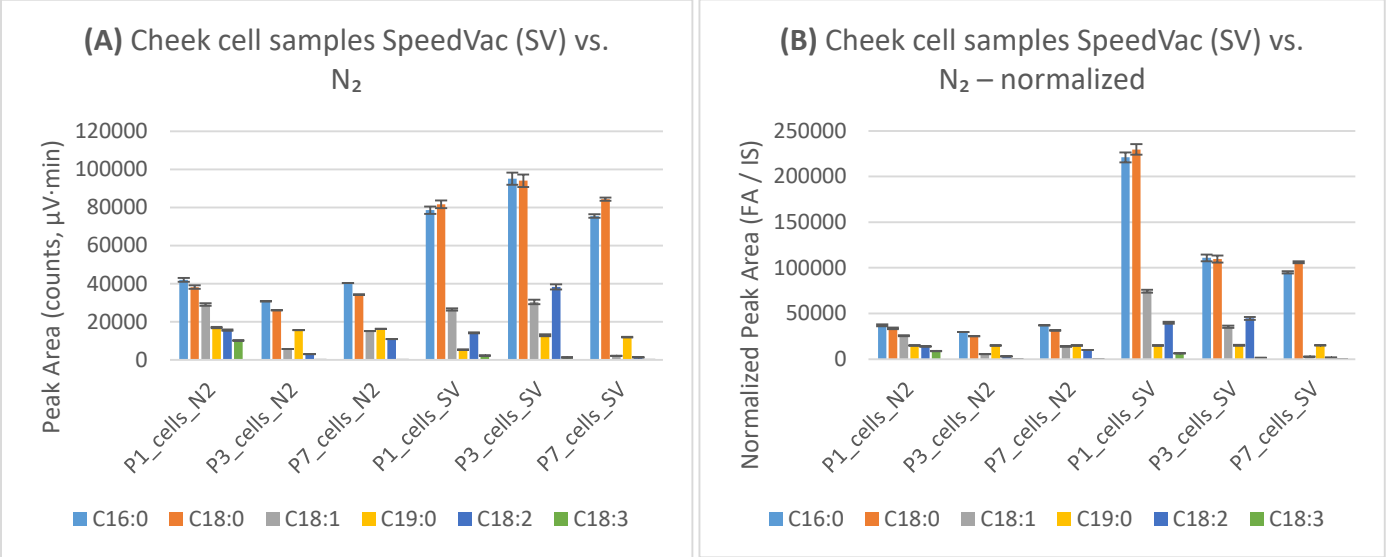


Figure 48: Fatty acid values in cheek cell samples after Matyash extraction with SpeedVac (SV) or N_2 evaporation

Displayed are fatty acid values of cheek cell samples from three participants (P1, P3, P7), prepared using either SpeedVac (SV) concentration or nitrogen (N_2) evaporation.
(A) Raw values.
(B) Normalized fatty acid values (scaled to an IS reference value of 15,000).
Values are presented as the mean of two measurements ± SD.
Note: Panels (A) and (B) use different y-axis scales due to the normalization.

When comparing the raw values with the normalized ones, it seemed that more fatty acids were lost when using the SpeedVac than with N_2 gas treatment. This was indicated by lower levels of the C19:0 IS in the SpeedVac-treated samples (P1_SV, P3_SV, P7_SV) compared to those treated with N_2 (P1_N_2, P3_N_2, P7_N_2) and a more pronounced change in fatty acid peak areas.

However, the total fatty acid peaks in the SpeedVac samples were notably higher than those in the N_2-treated samples. This suggested that while some losses occurred with the SpeedVac, it still yielded higher fatty acid concentrations in terms of peak area.

Regarding the lipid extraction method based on Matyash et al., the peak areas, especially from the samples prepared using the SpeedVac, were high and promising.

Conclusion

The SpeedVac method yielded higher total fatty acid peak areas compared to N_2 treatment, but further tests are needed to confirm reproducibility. Additionally, the lipid extraction method based on Matyash et al. showed promising results, particularly in the SpeedVac-prepared samples.
To improve future analyses and detect potential impurities, a pure C19:0 IS should be included and processed in parallel with the cheek cell samples.

4.7.4 Testing the Matyash Method for Saliva and Cheek Cells

Aim:

To evaluate whether the Matyash extraction method yields better results for cheek cell samples compared to saliva samples, we collected samples from three subjects using both sampling methods. Additionally, an empty Falcon tube without samples or IS was included as a reference during the Matyash extraction and methylation process to assess potential leaching of substances from the tube itself. Furthermore, to compare the effects of N_2 treatment versus vacuum concentration, three saliva samples were prepared using the SpeedVac, while the remaining samples were prepared with N_2.

Procedure:

Samples:
- IS (no extraction before methylation)
- Empty (N_2)
- IS_SV
- IS_N_2
- P1_saliva_SV
- P7_saliva_SV
- P1_saliva_N_2
- P3_saliva_N_2
- P7_saliva_N_2
- P1_cells_N_2
- P3_cells_N_2
- P7_cells_N_2

Sample Collection:

- Cheek cell samples were collected according to the previously described protocol.
- Saliva samples were collected using Salivettes® immediately upon awakening, followed by centrifugation for 2 minutes at 1,000 x g and 20 °C.

Matyash Extraction / Sample Preparation:

- 5 mL MTBE was added directly to cheek cell samples in Falcon tubes.
- For saliva samples (P1_saliva_SV, P7_saliva_SV, P1_saliva_N_2, P3_saliva_N_2, P7_saliva_N_2), 1 mL of sample was transferred to 50 mL Falcon tubes and mixed with 5 mL MTBE.

- For the empty sample, a 50 mL Falcon tube was filled with 30 mL Milli-Q water, centrifuged with the cheek cell samples, emptied, and refilled with 5 mL MTBE.
- IS_SV and IS_N_2 each received 5 mL MTBE in empty 50 mL Falcon tubes.
- 10 µL C19:0 IS was added to all samples except the empty control.
- All samples were shaken for 1 hour at room temperature.

Matyash Extraction / Phase Separation:

- 1.25 mL nuclease-free water was added to each tube, followed by shaking for 10 minutes at room temperature.
- Samples were centrifuged at 1,000 x g for 10 minutes.
- The upper phase was transferred to glass vials for N_2 evaporation or to 15 mL Falcon tubes for SpeedVac treatment (IS_SV, P1_saliva_SV, P7_saliva_SV).

Matyash Extraction / Additional Washing Step:

- A mixture of 30 mL MTBE, 9 mL MeOH, and 7.5 mL nuclease-free water was prepared.
- 2 mL of this mixture was added to each Falcon tube, shaken at low speed for 5 minutes, and centrifuged at 1,000 x g for 10 minutes.
- **Observation:** emulsion formation occurred in the upper phase of P1_saliva_SV, and the P3_cells_N_2 sample showed a cloudy upper phase with white flakes.

Evaporation:

- Glass vials (N_2 group) were evaporated under nitrogen at 40 °C in a heating block.
- Falcon tubes (SpeedVac group) were evaporated for 1 hour (no temperature, ramp 5), followed by an additional 30 minutes.

Methylation and Analysis:

- Fully evaporated SpeedVac samples (IS_SV, P1_saliva_SV, P7_saliva_SV) were dissolved in 200 µL MeOH and transferred to glass vials. Falcon tubes were rinsed with 50 µL MeOH, which was also transferred.
- 1 mL NaOH/MeOH solution was added to all samples, as well as to an empty IS reference vial.
- 10 µL C19:0 IS was added specifically to the IS reference.
- All samples underwent standard methylation and were analyzed by GC.

Results and Discussion:

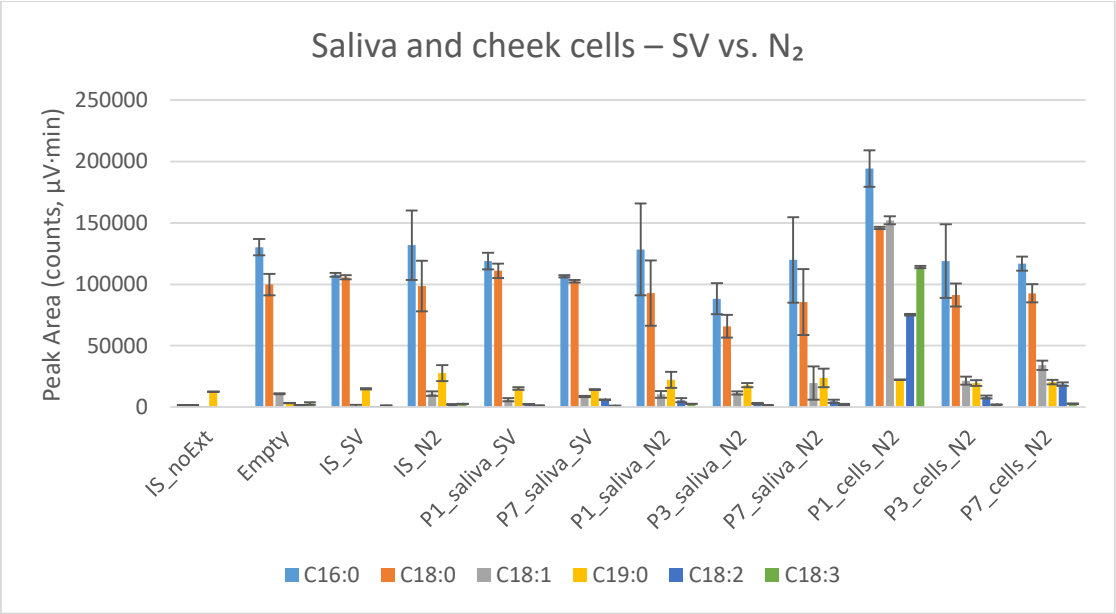


Figure 49: Peak areas of fatty acids in saliva and cheek cell samples after Matyash extraction with SpeedVac (SV) or N_2 evaporation

Displayed are peak areas of fatty acids from samples of three participants (P1, P3, P7) and reference samples, prepared using either SpeedVac (SV) concentration or N_2 evaporation. Values are shown as the mean of two measurements ± SD.

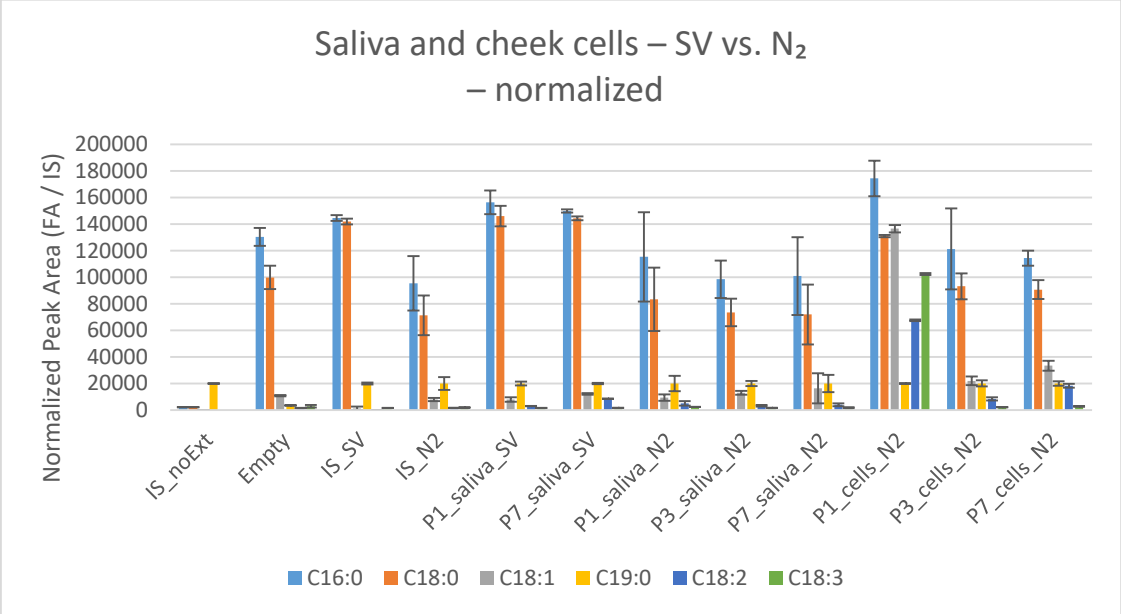


Figure 50: Normalized fatty acid values in saliva and cheek cell samples after Matyash extraction with SpeedVac (SV) or N_2 evaporation

Displayed are normalized fatty acid values from samples of three participants (P1, P3, P7) and reference samples, prepared using either SpeedVac (SV) concentration or N_2 evaporation. Values are scaled to an IS reference value of 20,000 and are shown as the mean of two measurements ± SD.
Note: Figures 49 and 50 use different y-axis scales because Figure 50 shows normalized values.

This experiment suggests that impurities were introduced into the samples prior to the methylation procedure. Evidence for this comes from the IS sample, which contained the fewest impurities. This sample was prepared solely for the FAME methylation process and did not undergo the Matyash extraction, in contrast to the empty control, which underwent the entire extraction process and contained a notable amount of impurities, particularly C16:0 and C18:0. Figure 49 and 50 show that the empty control displayed even higher levels of C16:0 and C18:0 fatty acids than many of the actual samples.

This indicates that the impurities were likely introduced during the Matyash extraction or evaporation with SpeedVac/N_2. However, it is unlikely that the impurities were caused exclusively by the SpeedVac, as both the Empty (N_2) and IS_N_2 samples also contained impurities. Contamination could have occurred at multiple stages, including during the FAME methylation.

Consequently, the fatty acid profiles of the other saliva and cheek cell samples cannot be considered reliable, as it is unclear whether the detected fatty acids originated from the samples themselves or from contaminants. Both reference samples, IS_SV and IS_N_2, also contained similar levels of impurities, with the IS_SV sample (prepared using the SpeedVac) showing higher C16:0 and C18:0 levels. Figure 49 further confirms that more of the SpeedVac samples evaporated compared to those prepared with N_2, consistent with previous observations. As expected, fatty acid content in the cheek cell samples was consistently higher than in the saliva samples from the same subjects.

Conclusion

The results indicate that impurities were introduced at multiple stages of the extraction and methylation process, rendering the fatty acid profiles of the saliva and cheek cell samples unreliable. Despite this, the cheek cell samples consistently showed higher fatty acid content compared to saliva samples, in line with expectations.

4.8 Testing Capillary Blood as an Alternative Matrix

4.8.1 Comparison of Capillary Blood Samples with Cheek Cell Samples

Aim:

To explore minimally invasive, easy and quick methods for fatty acid analysis, we compared capillary blood samples with cheek cell samples from three participants. The goal was to assess whether capillary blood could serve as an alternative matrix while providing comparable fatty acid profiles.

Procedure:

Samples:
- IS
- P1_blood
- P3_blood

- P8_blood
- P1_cells
- P3_cells
- P8_cells

Sample Collection:

- 5 mL MTBE and 1.25 mL nuclease-free water were pre-added to each 50 mL Falcon tube.
- **Note:** The addition of water at this stage deviates from the standard method, where water is typically added only after the first incubation.
- Capillary blood was collected by puncturing the ring finger and little finger using a lancet.
- Blood was pipetted directly from the finger immediately after puncture using a sterile pipette tip and transferred to the Falcon tubes. Skin around the puncture site was gently squeezed to increase droplet size.
    - Approximate volumes: P1: 9 µL, P3: 15 µL, P8: 13 µL.
- To ensure consistent treatment across sample types, for each cheek cell sample, 5 mL MTBE and 1.25 mL nuclease-free water were also added**,** even though in the standard protocol water would normally be added later.
- For the IS sample, 5 mL MTBE and 1.25 mL nuclease-free water were added to an empty Falcon tube.

Matyash Extraction / Sample Preparation:

- 10 µL C19:0 IS was added to all seven tubes.
- Tubes were placed on a shaker at low speed (100 RPM) for 1 hour.
- Since the water had already been added in a previous step, no additional water was introduced.

Matyash Extraction / Phase Separation:

- Samples were centrifuged at 1,000 x g for 10 minutes.
- The supernatant was transferred to glass vials.

Matyash Extraction / Additional Washing Step:

- To extract any remaining fatty acids, 2 mL of the MTBE:MeOH:H_2O mixture (20:6:5) was added to the original Falcon tubes.
- Tubes were shaken at 100 RPM for 10 minutes and centrifuged again at 1,000 x g for 10 minutes.
- Supernatants were combined with the previously collected fractions in the glass vials.

Evaporation:

- Samples were evaporated under N_2 at 30 °C for ~45 minutes.
- Evaporated samples were frozen at -20 °C until further analysis.
- **Note:** The samples were analyzed on a different day after freezing.

Methylation and Analysis:

- All samples underwent standard methylation and were analyzed by GC.

Results and Discussion:

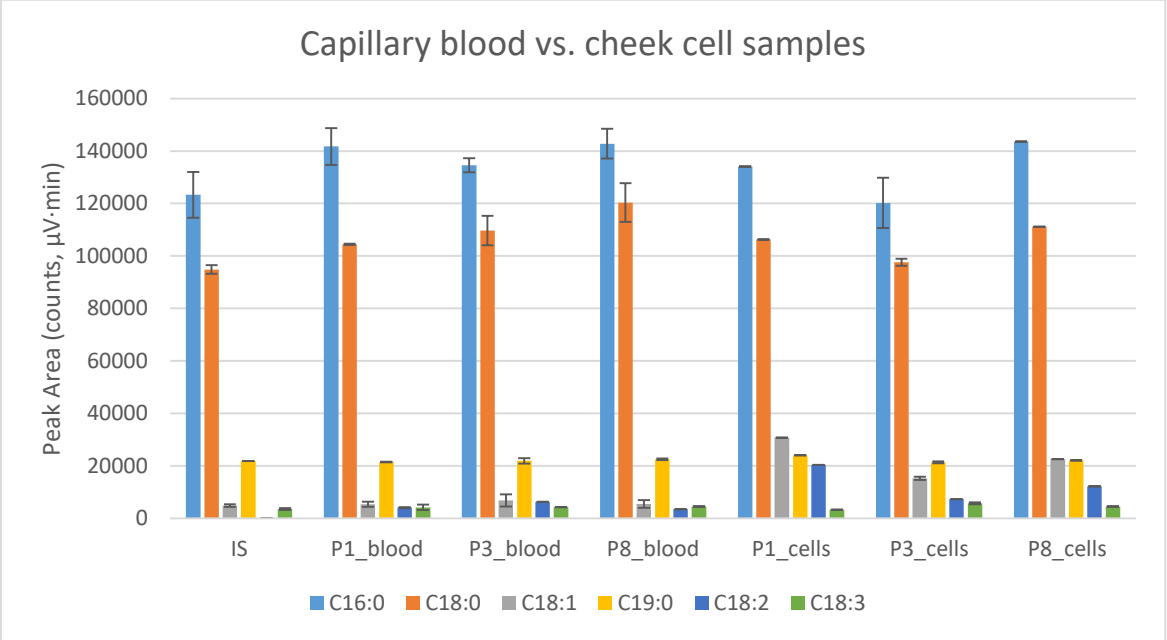


Figure 51: Peak areas of fatty acids in capillary blood and cheek cell samples after Matyash extraction

Displayed are peak areas of fatty acids in capillary blood vs. cheek cell samples from three participants (P1, P3, P8), along with the reference sample. Values are not normalized and are shown as the mean of two measurements ± SD with the exception of samples P1_cells and P8_cells, as the second sample of each evaporated.

As shown in Figure 51, the IS sample still exhibited notable impurities, despite the absence of the SpeedVac, which had been suspected as a major contributor to recurring contamination. The levels of fatty acids C16:0 and C18:0 were roughly comparable across all samples; however, it remains uncertain or even likely, that these peaks originated from contaminants. Additionally, cheek cell samples displayed higher concentrations of fatty acids C18:1 and C18:2 compared to capillary blood samples.

It is also noticeable that the IS values were quite stable and remained at similar levels across the different samples, particularly compared to some previous experiments.

Conclusion

In summary, while the cheek cell samples showed elevated levels of C18:1 and C18:2 fatty acids compared to capillary blood samples, the persistent impurities in the IS sample highlight the need for improved extraction protocols to ensure reliable results.

4.8.2 Replication of Capillary Blood Sample Testing

Aim:

The previous experiment was repeated to assess the reproducibility of the results and to determine if the impurities could be reduced.

Procedure:

The same blood and cheek cell samples (P1, P3, P8), along with the internal standard, were used as in the previous experiment.

Sample Collection:

- Cheek cell sampling was conducted following the standard protocol.
- For capillary blood samples, 5 mL MTBE was pre-added to each of four 50 mL Falcon tubes.
- Capillary blood was collected by puncturing the ring finger with a lancet. Blood was immediately pipetted into the Falcon tubes using a sterile pipette tip.
    - Approximate volumes: P1: 13 µL, P3: 20 µL, P8: 12 µL.
- For cheek cell samples, 5 mL MTBE was added to each pellet.

Matyash Extraction / Sample Preparation:

- All samples were placed on a shaker for 1 h at 150 RPM.
    - **Observation:** Blood clotting and incomplete dissolution of cheek cells.
- 10 µL of C19:0 IS was then added to each sample.
- An additional IS control was prepared by adding 5 mL MTBE and 10 µL IS to an empty Falcon tube. (**Note:** These steps are typically performed at the beginning of the procedure.)
- 1.25 mL nuclease-free water was added to each sample and shaken for 10 min at 150 RPM.
    - **Observation:** blood clotting persisted, and cheek cells did not fully dissolve.
- Samples were shaken for another 10 min at 150 RPM until fully dissolved.

Matyash Extraction / Phase Separation:

- Samples were centrifuged at 1,000 x g for 10 min.
- Supernatants were carefully transferred to glass vials that had been rinsed with MTBE to minimize impurities.

Matyash Extraction / Additional Washing Step:

- 2 mL of MTBE:MeOH:H_2O (20:6:5) was added to the Falcon tubes.
- Tubes were shaken for 5 min at 150 RPM and centrifuged at 1,000 x g for 10 min.
- Resulting supernatants were combined with those from the previous step.

Evaporation:

- Samples were evaporated under N_2 at 30 °C for ~1 h.

- Evaporated samples were stored at −20 °C.

Methylation and Final Steps:

- On the following day, samples underwent standard methylation and were analyzed by GC.

Results and Discussion:

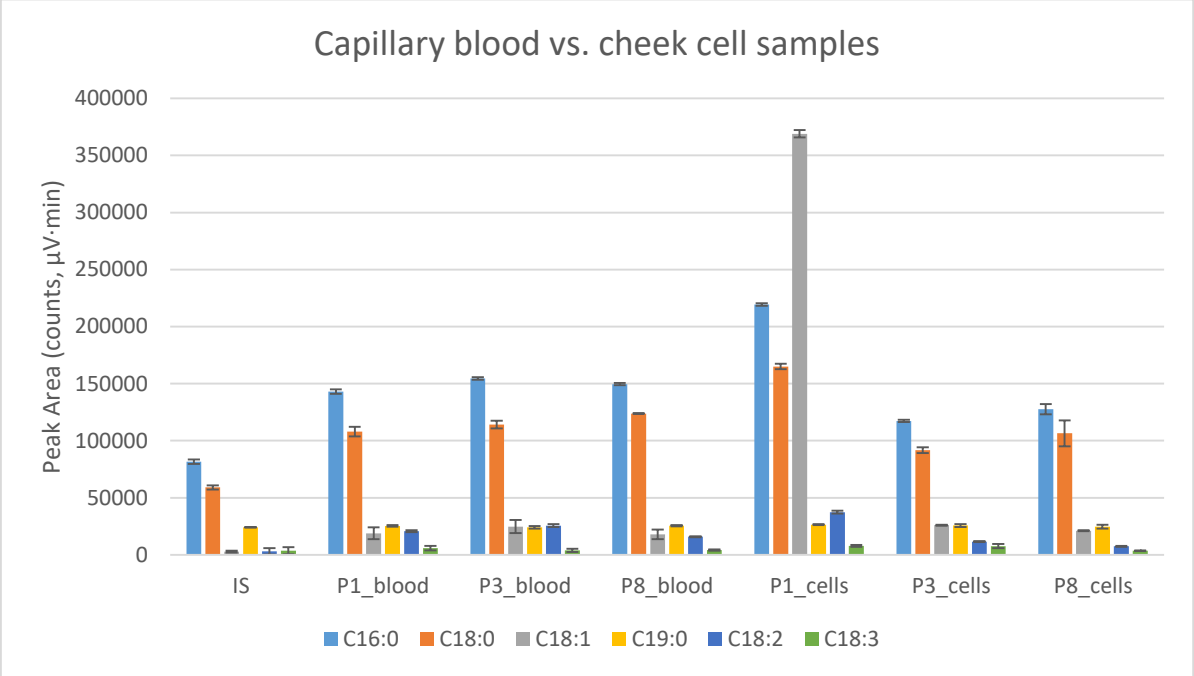


Figure 52: Peak areas of fatty acids in capillary blood and cheek cell samples after Matyash extraction (repeat experiment)

Displayed are peak areas of fatty acids in capillary blood vs. cheek cell samples from three participants (P1, P3, P8), along with the reference sample. Values are not normalized and are shown as the mean of two measurements ± SD.

The reference sample IS still exhibited contamination with various fatty acids, although at lower concentrations than in previous experiments. In the test samples, C16:0 and C18:0 levels exceeded those in the IS, suggesting that at least a portion of these fatty acids originated from the cheek cells and capillary blood. However, the potential contribution of impurities to these peaks cannot be ruled out.
C16:0 and C18:0 levels were consistent across blood samples. In comparison, the P1_cells sample showed higher concentrations, while the P3_cells and P8_cells samples exhibited lower levels relative to their corresponding blood samples. Unlike previous tests, C18:1 was only elevated in the cheek cell sample P1_cells compared to P1_blood, with no notable differences observed among the other test subjects. Although C18:2 was slightly higher in P1_cells than in P1_blood, it was lower in the cheek cell samples P3_cells and P8_cells compared to the respective capillary blood samples.

Moreover, the IS values once again remained relatively stable across the different samples, particularly when compared with some of the previous experiments.

Conclusion

The capillary blood samples did not exhibit a clear correlation with their corresponding cheek cell samples, suggesting variability in the fatty acid profiles between the two sample types.

5 Discussion

The objective of this master's thesis was to develop and refine a method for the non-invasive, cost-effective extraction and derivatization of fatty acids from saliva and cheek cell samples. Initially, our efforts were focused solely on saliva samples; however, due to limited success in increasing fatty acid peak areas to reliable levels, we introduced cheek cell sampling as an alternative. Toward the end of the research period, we also experimented briefly with capillary blood as an additional sample matrix.

Method development:

Methylation Methods

After conducting several pre-tests to establish a robust and reproducible protocol, various methylation techniques were evaluated, including both acid- and base-catalyzed methods. The initial approach involved a combination of basic NaOH and acidic BF_3 reagents. Persistent issues with impurities prompted us to test an alternative methylation technique using HCl as a catalyst. However, despite attempts to improve clarity, the HCl method led to column degradation and did not yield substantial improvements. As a result, we reverted to the original NaOH/BF_3 methylation method and continued with it for the rest of the experiments.

Extraction Methods

In response to persisting issues with contamination and unreliable results, different extraction methods were explored. We began with the gold standard in lipid extraction, the Folch method, but it did not deliver satisfactory results. The method not only failed to sufficiently increase fatty acid peak areas or reduce impurities, but also required a lengthy processing time and large quantities of toxic solvents, such as chloroform.

To address these drawbacks, we transitioned to the more modern Matyash extraction method, which uses MTBE, a safer, less toxic solvent. This approach was also faster and more efficient. However, despite these advantages, the Matyash method did not lead to considerable improvements in fatty acid peak areas following the FAME methylation step.

Vacuum concentrator

We also tested the effect of using a vacuum concentrator during sample preparation. However, the results were inconclusive, with no relevant improvements in peak intensity or clarity. Moreover, the SpeedVAc even appeared to introduce or increase impurities in several experiments.

Testing the Method:

To assess the method's potential as a diagnostic tool, we compared fatty acid profiles under several conditions:

- Sequential vs. pooled saliva samples,
- The effect of eating breakfast vs. fasting before sample collection,

- Variations in the fatty acid profile over three consecutive days from the same individuals.

Additionally, we examined the impact of different dietary fats, specifically linseed, olive and palm oil, on the fatty acid composition in saliva samples from participants. While these experiments provided valuable data, they also highlighted the ongoing difficulties with obtaining clear and reproducible results due to contamination and inadequate peak intensities of the salivary fatty acids.

Challenges with Different Sample Matrices:

One of the major challenges encountered during method development was the variability between sample matrices – saliva, cheek cells, and capillary blood. Each matrix presented unique difficulties:

- **Saliva** contained lower fatty acid concentrations, making it harder to detect distinct peaks and differentiate them from impurities.
- **Cheek cells** provided higher fatty acid content, but were more prone to contamination.
- **Capillary blood**, while promising, displayed inconsistencies in fatty acid profiles when compared to cheek cell samples from the same participants.

Of these, cheek cells showed the most promise in terms of fatty acid content, but contamination remained a key challenge. Capillary blood samples, though inconsistent, might also warrant further exploration. The differing properties of these matrices likely affected the efficiency of lipid extraction and subsequent methylation, complicating the development of a reliable method.

Conclusion:

While progress was made in reducing impurities and exploring various extraction and methylation techniques, a fully reliable method for analyzing fatty acids in saliva or cheek cell samples has not yet been achieved. Impurities remained a persistent challenge, and fatty acid peak areas did not increase sufficiently to offset their impact.

Among the matrices tested, cheek cells showed greater promise than saliva, with capillary blood as a potential alternative for future studies. Despite these limitations, this thesis provides valuable insights into the effects of various extraction and methylation methods and sample preparation techniques. It also highlights factors influencing fatty acid profiles in biological samples, such as dietary fat consumption, matrix-specific challenges, and impurity handling.

Although further refinement is needed, this work provides a foundation for optimizing salivary and cheek cell lipid analysis and validating its potential diagnostic and nutritional applications.

6 Outlook

My colleague and master's student Sabrina Seewald will continue the method development, with a particular focus on capillary blood analysis alongside cheek cell analysis. In addition to testing capillary blood from the finger, she will also explore blood sampling from the earlobe.

The primary objective will be to reduce impurities and improve both the clarity and intensity of fatty acid peaks, thereby enhancing the overall accuracy of the method. A deeper investigation into contamination sources, along with the implementation of stricter control measures, will be crucial to minimizing impurities.

Once the method is validated and the results are reproducible, future research will center on small internal trials and dietary studies involving oils or other fat-rich foods. This will provide further insights into the influence of fat consumption on the fatty acids in saliva.

While a fully reliable diagnostic method for salivary or cheek cell lipids has not yet been achieved, this project has laid important groundwork for further research and method development. Ultimately, the work conducted as part of this master's thesis has brought us one step closer to developing easy-to-use, affordable, non-invasive diagnostic methods.

Abstract

While plasma samples have traditionally been used for long-chain fatty acid analysis, saliva diagnostics have gained importance as a non-invasive, cost-effective and user-friendly alternative in recent years. Given the need for robust analytical techniques in both matrices, gas chromatography (GC) is commonly employed for fatty acid profiling. However, as GC can only analyze volatile compounds, long-chain fatty acids must first be derivatized into their corresponding methyl esters.

This thesis focuses on optimizing sample preparation by exploring and comparing different derivatization and extraction methods, as well as evaluating the impact of tools like a vacuum concentrator. The method's reliability was further assessed by examining the effects of different conditions on fatty acid profiles, including sampling (pooled vs. sequential saliva samples), dietary (fasting vs. breakfast before collection) and temporal factors (variations over three consecutive days).

A key challenge was achieving sufficiently high GC peaks for reliable data while minimizing impurities. Due to persistent difficulties with saliva samples, cheek cells and capillary blood were tested as alternative matrices. Cheek cells showed the highest fatty acid peaks, while capillary blood, despite inconsistencies, may warrant further investigation. The original methylation method using NaOH and BF_3 was the most successful, while none of the evaluated extraction methods led to a notable increase in fatty acid peak areas. The results from the vacuum concentrator were inconclusive.

Although a fully reliable diagnostic method for salivary or cheek cell lipids is still to be established, this thesis provides a solid foundation for advancing non- or minimally invasive, affordable diagnostic solutions.

Zusammenfassung

Während Plasmaproben traditionell für die Analyse langkettiger Fettsäuren verwendet werden, haben Diagnostikmethoden auf Basis von Speichel in den letzten Jahren als nicht-invasive, kostengünstige und benutzerfreundliche Alternative zunehmend an Bedeutung gewonnen. Beide Probenarten erfordern zuverlässige analytische Verfahren, weshalb die Gaschromatographie besonders häufig eingesetzt wird. Da sie jedoch nur flüchtige Verbindungen nachweisen kann, müssen langkettige Fettsäuren vor der Analyse zunächst in ihre entsprechenden Methylester derivatisiert werden.

Diese Masterarbeit konzentriert sich auf die Optimierung der Probenvorbereitung durch die Testung verschiedener Derivatisierungs- und Extraktionsmethoden sowie die Bewertung des Einflusses von Probenvorbereitungstools wie einem Vakuumkonzentrator. Die Zuverlässigkeit der Methode wurde außerdem durch die Untersuchung verschiedener Einflussfaktoren auf die Fettsäureprofile überprüft, darunter methodische Aspekte (gepoolte vs. sequenzielle Speichproben), diätetische Bedingungen (Frühstück vs. nüchtern vor der Probenentnahme) sowie zeitliche Faktoren (Veränderungen über drei aufeinanderfolgende Tage).

Eine zentrale Herausforderung bestand darin, ausreichend hohe Peaks zu erzielen, um zuverlässige Ergebnisse zu erhalten und gleichzeitig Verunreinigungen zu minimieren. Aufgrund anhaltender Schwierigkeiten mit den Speichelproben wurden Wangenepithelproben und Kapillarblut als alternative Probenarten getestet. Wangenepithelproben zeigten das größte Potenzial für die Fettsäureanalyse, während Kapillarblut trotz seiner Schwankungen potenziell für weitere Analysen in Betracht gezogen werden kann. Die ursprünglich verwendete Derivatisierungsmethode mit NaOH und BF_3 erwies sich als die erfolgreichste, während keine der getesteten Extraktionsmethoden die Peak-Flächen wesentlich verbessern konnte. Die Ergebnisse zur Verwendung des Vakuumkonzentrators waren nicht eindeutig.

Obwohl eine vollständig zuverlässige diagnostische Methode für Speichel- oder Wangenzelllipide erst etabliert werden muss, bietet diese Masterarbeit eine solide Grundlage, um die Entwicklung nicht- oder minimalinvasiver, kostengünstiger Diagnostiklösungen voranzutreiben.

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