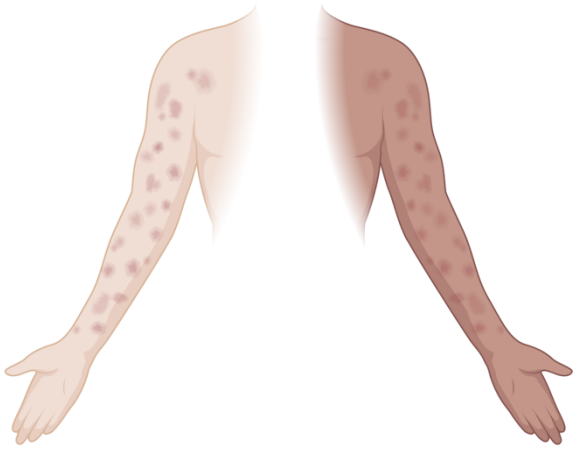
AALBORG UNIVERSITET

OPTIMIZING LIPID EXTRACTION PROTOCOLS FOR COMPREHENSIVE LIPIDOMIC ANALYSIS OF THE SKIN



Amaal Mohamed Hassan Guled
Medicine with Industrial Specialization
Biomedicine
2023

AALBORG UNIVERSITET

Title:

Optimizing Lipid Extraction Protocols for Comprehensive Lipidomic Analysis of the Skin

Semester:

10th semester

Project period:

September 1st, 2022 - October 16th, 2023

ECTS:

60 points

Project group:

9031

Supervisor

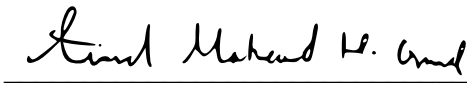
Christian Pablo Pennisi

Total pages: 35

Normal pages: 25

Appendix: 1

Participants:



Amaal Mohamed Hassan Guled, 20165960

Table of Content

Abbreviation

SC	Stratum corneum
SB	Stratum basale
TEWL	Transepidermal water loss
AD	Atopic dermatitis
CD	Contact dermatitis
ACD	Allergic contact dermatitis
ICD	Irritant contact dermatitis
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry
2,5-DHB	2,5-dihydroxybenzoic acid
Da	Dalton

Abstract

Aim: The objective of this study was to evaluate the efficiency of different solvents, including chloroform, methanol, a mixture of chloroform/methanol, and a buffer solution containing ammonium acetate in chloroform/methanol/propanol, in extracting lipids from the skin. The aim is to identify the optimal solvent for lipid extraction, contributing to advancements in lipidomic research.

Methods: In this study, skin samples were obtained from a healthy 24-year-old white female subject. Tape stripping was performed on the proximal forearm, while a control sample was taken from the alar crease of the nose using a scalpel. The lipid extraction process involved the optimization of solvents, including chloroform, methanol, and a 2:1 mixture of chloroform and methanol, and 2-propanol with the addition of ammonium acetate using MALDI-TOF MS analysis.

Results: The tape-stripping results indicate a non-zero signal on the mass spectrum, even in the absence of detected ions or compounds. The ceramide standard, encompassing four distinct ceramides with their respective Dalton (Da) values, revealed the presence of sodium adducts, as corroborated by corresponding mass spectrometry peaks. Subsequent solvent optimization confirmed a 2:1 combination of chloroform and methanol as the most effective extraction medium.

Conclusion: In conclusion, this study highlights the importance of solvent selection in lipid extraction. Methanol, due to its polar nature, was ineffective, while chloroform, especially in combination with methanol, proved more efficient. Caution is advised when applying solvents to tape discs. Integrating tape discs with MALDI-TOF MS presents a significant advancement in understanding dermatological disorders. With continued research and refinement, this method holds great potential for transforming the diagnosis and treatment of these prevalent dermatological conditions, ultimately leading to enhanced outcomes for affected individuals.

Resumé

Formål: Dette studie har til formål at evaluere effektiviteten af forskellige opløsningsmidler, herunder kloroform, metanol, en blanding af kloroform/metanol og en bufferopløsning indeholdende ammoniumacetat i kloroform/metanol/propanol, i udvindingen af lipider fra huden. Målet er at identificere det optimale opløsningsmiddel til lipidudvinding, hvilket bidrager til fremskridt inden for forskningen af lipider.

Metoder: I dette studie blev der indsamlet hudprøver fra en sund 24-årig, hvid kvindelig forsøgsperson. Der blev udført tape-stripping på den proksimale underarm, og en kontrolprøve blev taget fra næsen med en skalpel. Lipidudvindingsprocessen inkluderede optimering af opløsningsmidler, såsom kloroform, metanol og en blanding af kloroform og metanol i forholdet 2:1, samt 2-propanol med tilsætning af ammoniumacetat ved hjælp af MALDI-TOF MS-analyse.

Resultater: Resultaterne fra tape-stripping viser, at der er et påviseligt signal på masse-spektret, selvom der ikke er identificeret ioner eller forbindelser. Ceramidstandarden, som inkluderer fire forskellige ceramider med deres specifikke Dalton (Da) værdier, viste tilstedeværelsen af natriumaddukt. Dette blev bekræftet af tilsvarende topper i masse-spektrogrammet. Efterfølgende bekræftede optimeringen af opløsningsmidler, at en blanding af kloroform og metanol i forholdet 2:1 var den mest effektive metode til at udvinde lipider.

Konklusion: Afslutningsvis fremhæver dette studie vigtigheden af valg af opløsningsmiddel i lipidudvinding. Metanol, på grund af sin polare karakter, var ineffektiv, mens kloroform, især i kombination med metanol, viste sig mere effektiv. Der bør udvises forsigtighed ved anvendelse af opløsningsmidler på tape-skiver. Integration af tape-skiver med MALDI-TOF MS repræsenterer et betydeligt fremskridt inden for forståelse af dermatologiske lidelser. Med fortsat forskning og forbedring har denne metode stort potentiale til at transformere diagnosen og behandlingen af disse udbredte dermatologiske tilstande, hvilket i sidste ende fører til forbedrede resultater for berørte individer.

1. Introduction

1.1 The Integumentary System

The skin constitutes the body's largest organ, encompassing a surface area of approximately two square meters and a weight of around four kilograms in the adult human. The thickness of the skin is subject to variation based on the anatomical location. The skin covering the eyelids is characterized by minimal thickness, measuring approximately 0.5 millimeters. Conversely, the skin present on the palms and feet is notably thicker, measuring approximately four millimeters (Sobiepanek et al., 2019). The skin serves as the body's primary barrier, providing protection against harmful microorganisms causing infections, mechanical damage, chemical agents, heat, and preventing water loss (Baroni et al., 2012; Sobiepanek et al., 2019).

The skin comprises three primary layers: the epidermis, the dermis, and the subcutaneous tissue as seen in Figure 1 (Sobiepanek et al., 2019). The epidermis can be further subdivided into four layers, which will be elucidated in the subsequent section. The dermis is positioned between the epidermis and the subcutaneous layer, characterized by a rich composition of collagen and elastic fibers. This layer exhibits a high degree of vascularity and an extensive network of lymphatic vessels. Additionally, it encompasses sweat and sebaceous glands, along with hair follicles, providing essential structural support to the epidermis. Within the dermal layer, a variety of cells including fibroblasts and macrophages are found. Fibroblasts are primarily responsible for synthesizing and renewing the extracellular matrix, while macrophages play a vital role in eliminating foreign materials and damaged tissues resulting from various sources of injury or irritation. The deepest layer of the skin is the subcutaneous layer, also known as the hypodermis, composed primarily of fatty tissue. It plays a critical role in the body by providing mechanical shock absorption, insulating against external temperature changes, and regulating energy metabolism while also serving as a storage depot (Baroni et al., 2012; McLafferty et al., 2012).

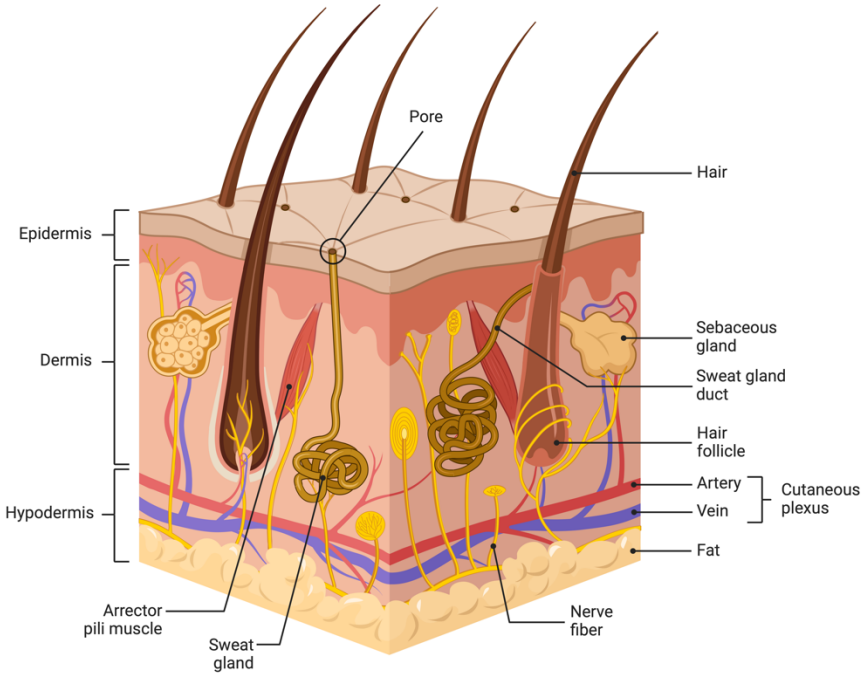


Figure 1: Anatomy of the Integumentary System: The integumentary system comprises three primary layers: the epidermis, dermis, and hypodermis (fat). It also includes specialized structures such as the arrector pili muscle, sweat gland, nerve fiber, cutaneous plexus, hair follicle, sweat gland duct, sebaceous gland, hair, and pore.

The integumentary system is a complex network comprising three to four million sweat glands, which play a crucial role in regulating body temperature by releasing sweat through hair follicles or directly onto the skin's surface via pores. Sebaceous glands contribute by producing sebum, an oily mixture of triglycerides, cholesterol, proteins, and organic salts. This sebum provides a protective shield for hair, preventing it from becoming dry and brittle while also controlling water evaporation from the skin, thereby maintaining its suppleness and hydration. Hair is primarily composed of keratinized epidermal cells bound by extracellular proteins, divided into the visible portion, or hair shaft, and the concealed portion within the skin, known as the hair root. The root is firmly anchored in the dermis of the subcutaneous layer and surrounded by a hair follicle. The arrector pili muscle, connected to the hair follicle, is responsible for the occurrence of goosebumps, often triggered by cold temperatures, excitement, or fear. The skin's vast network of nerve fibers enables it to react to external stimuli such as cold, heat, pain, touch, and pressure. With an estimated one million nerve fibers, the majority terminate in the face and extremities. Thermoregulation, a critical function of the integumentary system, involves insulation, sweating, and blood flow control. Subcutaneous adipose tissue beneath the dermis provides insulation, and at the junction of the dermis and subcutaneous fat layer, the deep cutaneous plexus ensures ample blood supply to these regions. Additionally, the superficial plexus

extends and delivers blood vessels to the boundary between the epidermis and dermis, contributing significantly to the process of thermoregulation (McLafferty et al., 2012).

1.2 Epidermis

The epidermis is a multilayered epithelium that undergoes continuous renewal. The several layers can be delineated starting with the basal layer, stratum basale (SB), located above the dermis, followed by the spinous (stratum spinosum) and granular (stratum granulosum). The outermost layer is known as the cornified layer, also known as stratum corneum (SC). These layers are illustrated in Figure 2. The epidermis consists of various cell types, with keratinocytes being the predominant type, constituting approximately 90% of its composition. Other significant cell types include Langerhans cells, Merkel cells, and melanocytes. Langerhans constitute the epidermal immune barrier while Merkel cells function as components of the nervous system and contribute to the sensory nerve aspect of the skin, whereas melanocytes protect the skin against ultraviolet radiation and produce melanin, the pigment responsible for skin coloration (Baroni et al., 2012; Sobiepanek et al., 2019).

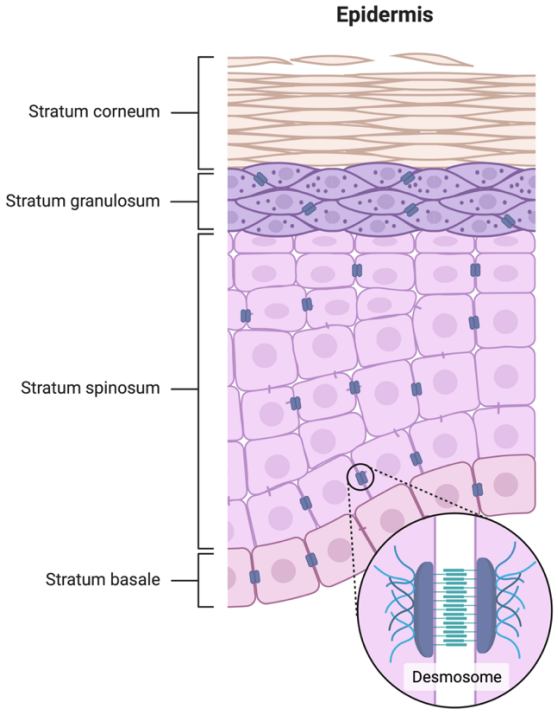


Figure 2: Layers of the Epidermis: The epidermis, comprising distinct layers, includes the stratum basale (active cell division), stratum spinosum (keratinocytes connected by desmosomes), stratum granulosum (lipid and keratohyalin granules), and stratum corneum (outermost layer of flat, dead corneocytes). Desmosomes serve as specialized cell junctions vital for structural integrity.

The SB is the deepest layer of the epidermis, adjacent to the dermis. It is composed of a single row of columnar keratinocytes, with additional cell types such as Merkel cells and melanocytes. This layer is rich in blood vessels, providing nourishment to the cells. The skin's color is influenced by both genetic factors and environmental exposure to UV light. Furthermore, Merkel cells in this layer establish contact with sensory neurons known as Merkel discs, collectively responsible for detecting the sensation of touch (McLafferty et al., 2012). The keratinocytes undergo proliferation and subsequently migrate towards the SC until they are eventually shed from the skin's surface in a process referred to as desquamation (Knox & O'Boyle, 2021). The stratum spinosum, located above the SB, is characterized by keratinocytes adopting a rounder and spikier shape. Comprising five to twelve cell layers, these cells are interconnected by intracellular bridges called desmosomes. These structures, akin to thorn-like projections, serve to enhance cohesion between adjacent cells. This arrangement contributes significantly to the skin's tensile strength and flexibility (McLafferty et al., 2012). Desmosomes play a crucial role in connecting neighboring keratinocytes, facilitating cell-cell cohesion within the nucleated layers. These structures are eventually shed as part of the desquamation process (Baroni et al., 2012). Additionally, Langerhans cells, vital for aiding the immune system in recognizing and combatting invading microorganisms, are found within the stratum spinosum. As cells progress toward the skin's surface, they become elongated and horizontally flattened, marking the stratum granulosum. This layer is comprised of three to five layers of flattened keratinocytes. In this stage, cells undergo a transformation where they lose their nuclei and transition into a keratinized state, primarily composed of the protein keratin. The presence of keratohyalin, a densely staining substance, is a notable feature of this layer. Keratohyalin converts tonofilaments, proteinaceous fibers commonly found in epithelial cells, into keratin. This process is fundamental for the formation of a durable outer layer in the epidermis. Lamellar bodies, also known as lamellar granules, may also be observed in this layer, producing lipids that extrude into intercellular spaces, contributing to cell cohesion. The outermost layer, the SC, is comprised of multiple layers of flattened, non-viable keratinocytes. These cells are arranged in a structured vertical alignment, giving the appearance of firmly interconnected cell membranes. The keratin within these cells is essential for the protection of both the skin and underlying tissues against external factors like heat, microorganisms, and chemicals. As the outermost protective barrier protecting the body from the external environment, the SC is susceptible to considerable mechanical stress and environmental factors, leading to wear and tear (McLafferty et al., 2012).

1.3 The Significance of Lipids in the Homeostasis of Skin Function

As keratinocytes progress from the basal layer to the granular layer they are considered viable because of their nucleated state, however, in the SC the keratinocytes have a flattened, anucleated morphology, leading to their classification as corneocytes. The corneocytes are arranged in layers, typically numbering between 15 and 25 layers (Baroni et al., 2012; Knox & O'Boyle, 2021). Keratinocytes play a pivotal role in lipid synthesis, deriving from either intermediate metabolic products or essential fatty acids. The synthesized lipids are then encapsulated within small organelles known as lamellar bodies. Upon release into the SC, they aggregate into intercellular layers and form a unique lipid matrix consisting of ceramides, cholesterol, and free fatty acids. These lipids are significant in the prevention of water loss from the skin, while simultaneously acting as a barrier against the penetration of water-soluble substances, thus essential for maintaining the skin's protective function (Baroni et al., 2012; Sobiepanek et al., 2019). The SC experiences a natural process of water movement into the atmosphere, termed transepidermal water loss (TEWL) (Baroni et al., 2012). When the skin barrier is disrupted, the nucleated epidermis initiates a homeostatic repair response, which leads to a rapid restoration of the barrier function. This restorative process involves an increased synthesis of all three crucial skin lipid classes mentioned. Notably, inhibiting the synthesis of cholesterol, fatty acids, or sphingolipids has been demonstrated to impede barrier repair (Berardesca et al., 2001). The breakdown of glycosphingolipids leads to the production of ceramides, while phospholipids are converted into free fatty acids. These changes in lipid composition and cellular structure result in the formation of a tightly packed structure within the SC. Ceramides, which make up a significant portion (30% to 40%) of the lipid content by weight, play a crucial role in the SC. It's important to note that this high concentration of ceramides is not found in the granular, spinous, and basal layers, emphasizing the importance of terminal differentiation in ceramide accumulation (Baroni et al., 2012). Consequently, the elevated lipid production in the nucleated layers of the epidermis supplies the essential lipids needed for effective barrier recovery (Berardesca et al., 2001). The corneocytes and lipids within the SC can be compared to a "brick-and-mortar" arrangement, where the lipids function as the mortar binding the corneocyte "bricks" together (Knox & O'Boyle, 2021). The SC's protective barrier relies on the interplay between sebaceous and epidermal lipids. Sebaceous lipids, primarily non-polar, encompass triglycerides, wax esters, and squalene. In contrast, epidermal lipids are a mixture of ceramides, free fatty acids, and cholesterol. This collaborative relationship is essential for maintaining the structural integrity and functionality of the SC's protective barrier (Pappas, 2009).

The balance between the processes of proliferation and desquamation is very important in the healthy epidermis, resulting in a complete renewal approximately every 28 days. This balance holds significant importance, as disruptions in these fundamental processes can give rise to certain skin disorders. Inflammatory skin conditions like psoriasis involve an elevated proliferation rate, which disturbs the normal differentiation process, ultimately leading to the formation of parakeratotic scales — a characteristic feature of hyperproliferative hyperkeratosis (Baroni et al., 2012). Simultaneously, the lipid composition within the SC serves a multifaceted role, exerting influence on various aspects of skin function. Changes in this lipid profile have been closely linked to skin disorders such as atopic dermatitis (AD) and psoriatic scales, which exhibit notable alterations in ceramide profiles. To grasp the underlying causes of impaired barrier function in diseased skin, it is crucial to comprehend the roles of distinct lipid classes and their influence on the phase behavior of SC lipids. Consequently, a profound understanding of lipid composition is crucial for identifying the cause of these diseases and devising treatments thereof (Bouwstra et al., 2000).

1.4 Contact Dermatitis

Contact dermatitis (CD) is categorized as an inflammatory skin disorder and is estimated to impact approximately 15% of the adult population during their lifetime based on five European countries (Johansen et al., 2022; Svensson et al., 2018). Annually, dermatologists in Denmark identify roughly 25,000 new cases of CD. Over five years, the all-encompassing healthcare cost for each case of occupational contact dermatitis (OCD) totaled €724. Moreover, productivity costs for cases of OCD during this period were estimated at €10,722. Additionally, productivity costs for cases of OCD during the same period were estimated at €10,722 (Sætterstrøm et al., 2014). This condition can manifest as acute or chronic inflammation of the skin, triggered by exposure to various chemical or physical agents.

CD can be classified into two main categories: allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD) (Bains et al., 2019). ACD results from repeated and direct skin exposure to contact allergens. In some cases, ACD can also be caused by exposure to airborne allergens, like volatile chemicals found in paints. This airborne pattern of ACD primarily affects areas of the skin not covered by clothing, notably the face. In individuals who are already sensitized, ACD can also be induced through systemic exposure, such as inhaling or ingesting a contact allergen. This can lead to widespread dermatitis and involvement of areas like the flexures and buttocks. Additionally, repeated skin

contact with proteins in food can initially present as urticaria, evolving into CD. This is particularly relevant for those in occupations involving food handling (Johansen et al., 2022). ICD is the result of skin exposure to irritants, predominantly caused by repeated exposure to mild irritants like soap and water (Bains et al., 2019). Though genetics may have a role, there are only a limited number of primary exposure types associated with ICD, including wet work, glove use, contact with detergents, handling food, and exposure to solvents or oils. It is crucial to emphasize that the integrity of the skin barrier significantly influences the development of ICD. This is why conditions like AD, characterized by a compromised skin barrier, frequently co-occur with cases of ICD (Johansen et al., 2022). Symptoms include discomfort, stinging, burning, and pain, which manifest within minutes to hours after exposure to an irritant. However, the rash is typically limited to the area of contact, unlike ACD where the rash can extend beyond the initial area of contact with the allergen (Bains et al., 2019). External factors, such as cleansing and sanitation agents, environmental pollutants, specific pharmaceutical components, and physical stressors, have the potential to impact the composition and structure of SC lipids. Additionally, age and mental state are recognized as internal factors that contribute to the composition and arrangement of lipids within the SC. Studies have consistently observed a decrease in the concentrations of essential SC lipids, particularly ceramides, with age. This often leads to dry skin in elderly patients due to reduced lipid content, subsequently impacting the skin's healing process and resulting in a compromised epithelial barrier (Sahle et al., 2015). Certain regions of the body exhibit differing susceptibility to irritation. Areas characterized by thinner skin, such as the face, dorsal hand, and finger webs, tend to be more prone to chemical irritation compared to regions with thicker skin, like the palms, soles, and back (Bains et al., 2019) The most observed clinical presentation is hand dermatitis, followed by occurrences on the face and foot (Johansen et al., 2022).

1.4.1 Pathogenesis

ICD arises from the direct toxic impact of an irritant on epidermal keratinocytes, leading to a disruption of the skin barrier and initiation of the innate immune response. Different irritants act on keratinocytes in distinct ways; for instance, sodium lauryl sulfate, commonly found in detergents, directly harms epidermal keratinocytes. Conversely, organic solvents like acetone induce barrier disruption through lipid loss, further compromising the epithelial. This results in increased permeability, enabling irritants and potential allergens to penetrate. Prolonged epithelial damage, often due to repeated exposure to a mild irritant, activates the innate immune system. This results in the release of various proinflammatory cytokines, including IL-1α, IL-1β, TNF-α, GM-CSF, IL-6, and IL-8 from

keratinocytes. Subsequently, these cytokines activate a range of immune cell types, including Langerhans cells, dermal dendritic cells, and endothelial cells. As a result, these cells release chemokines, leading to the recruitment of neutrophils, lymphocytes, macrophages, and mast cells to the epidermis, thereby intensifying the inflammatory response.

The difference between ICD and ACD is based on their underlying mechanisms and immune responses. Both conditions originate from repeated exposure to low molecular weight haptens, which are alternatively referred to as contact allergens (Bains et al., 2019; Johansen et al., 2022). ICD can manifest without prior sensitization and may occur upon initial exposure to an irritant. With prolonged or significant exposure, ICD can develop in a wide range of individuals. Moreover, those affected by ICD demonstrate an increased susceptibility to developing sensitization to allergens. In contrast, ACD manifests as a delayed (type IV) hypersensitivity reaction to a hapten, involving an acquired immune response. It specifically engages antigen-specific T cells, and therefore, it exclusively occurs in individuals previously sensitized to the allergen. The presence of hapten-specific T cells in samples obtained from the skin, patch test sites, or through blood-ELISPOT assays, serves as a crucial diagnostic indicator for distinguishing between ACD and ICD. In summary, despite sharing a common trigger, ICD and ACD differ fundamentally in their immune responses and the prerequisite for prior sensitization. ACD is characterized by an acquired immune response involving antigen-specific T cells, whereas ICD can occur without prior sensitization. ICD, ACD, and AD often present with similar clinical features and have the potential to concurrently manifest within the same patient. These three conditions share a common hallmark: chronic inflammation (Bains et al., 2019).

1.4.2 Classification of Irritant Contact Dermatitis Subtypes

As previously mentioned, ICD can be categorized into acute and chronic stages. In acute ICD, dermatitis develops within minutes or hours of exposure to potent irritants such as acids and alkalis causing severe acute ICD, mainly reported in laboratory or occupational settings.

It may manifest with ulceration or trauma-like burns, resembling a chemical burn with a scalded appearance of the epidermis. Chronic ICD is the predominant type encountered in a physician's office and may arise from physical agents, repeated microtrauma, or prolonged exposure to weak irritants over the years. Its clinical manifestation presents as a dry, dull, red, scaly rash, and lichenified lesions. However, there is also an intermediate state known as sub-acute ICD or delayed acute ICD. Sub-acute ICD displays similarities with acute ICD, but it typically develops 8-24 hours after contact, commonly

triggered by typical irritants such as benzalkonium chloride. This surfactant is widely used in cosmetics, skin disinfectants, medicated shampoos, and other products. In summary, ICD can occur either after a single episode of exposure to strong irritants, or due to repeated exposure to weak irritants, without the requirement for prior sensitization.

The risk of developing ICD increases with certain occupations such as healthcare workers, hairdressers, metalworkers, etc. which may be due to repeated contact with water, detergents, organic compounds, and other chemicals (Bains et al., 2019).

Table 1: Categorization of ICD Subtypes and Associated Causes

Subtypes	Description	Clinical presentation
Acute ICD	Minutes to hours after exposure to a strong potent irritant (acids or alkalis)	Ulceration or trauma-like burns; scalded appearance of the epidermis
Sub-acute ICD	8-24 hours after initial exposure	Similar to acute ICD
Chronic ICD	Prolong exposure over to weak irritants over years Prevalent type of ICD	Erythema, scaling and lichenified lesions

1.6 Diagnosis and Conventional Treatment Approaches for Contact Dermatitis

ICD is typically a diagnosis of exclusion, it can be over-diagnosed, especially in occupations involving frequent wet work or wet-dry cycles. Accurate diagnosis of ICD can be challenging, as there are no universally accepted tests for it. It is important to first identify the irritant, which can include substances like cosmetics and fragrances, capable of causing both ACD and ICD. A positive patch test with current relevance makes ACD more likely, although both types can co-occur. Patch-testing is the gold standard for diagnosing ACD and requires experienced interpretation due to the clinical similarity between ACD and ICD. This process involves applying suspected contact allergens to the patient's upper back using specialized chambers affixed with tape, allowing for the identification of sensitization to specific allergens.

The management of ACD and ICD primarily centers around patient education regarding the avoidance of irritants, allergens, and other triggers. For individuals with ICD, it's crucial to guide how to

effectively steer clear of irritants both at home and in the workplace. Once the specific irritant is identified, implementing preventive measures, like using personal protective equipment at work, becomes essential to reduce the risk of future exposure. Additionally, restoring the skin barrier is crucial, achieved through the application of emollients, the use of anti-inflammatory topical and systemic agents, and occasionally phototherapy. In occupational settings, this might involve changes in the work environment, utilization of protective equipment, taking sick leave, and potentially considering a job change. Traditionally, treatments for ACD and ICD have encompassed the use of topical corticosteroids and, off-label, immunosuppressants typically employed in AD, such as methotrexate and azathioprine. In certain regions, alitretinoin is approved for treating chronic hand eczema. The ongoing development of novel treatments, including those for AD, holds promise for benefiting patients with ACD and ICD in the future (Bains et al., 2019; Johansen et al., 2022).

1.8 Methods to Investigate Skin Lipid Profile

Some conditions can be visually diagnosed, while others require sample collection, including blood, skin swabs, scraps, or biopsies (Sobiepanek et al., 2019). Analyzing skin lipids demands precise sampling, extraction, and analysis. Non-adhesive tape, specifically designed for this purpose, can capture surface lipids, providing a snapshot of the skin's outermost layer. These tapes are ingeniously engineered with variations, such as Sebutape for sebum lipids or D-Squame for SC removal. This allows researchers to target specific lipid profiles based on their research objectives. Moreover, tape-stripping is a non-invasive method, making it particularly advantageous for studies where invasive procedures like biopsies are less preferable. Tapes are primarily designed for sampling the non-viable layers of the SC. For a thorough examination of the deeper layers of the skin, an alternative method like a full-thickness skin biopsy is required (Kendall et al., 2018).

In lipid analysis, the crucial step of extraction relies on solvents to disrupt cellular membranes and solubilize lipids (Kendall et al., 2018). The selection of appropriate solvents is necessary for efficient lipid extraction. This involves a precise combination of polar solvents, which serve to disrupt protein-lipid complexes, and nonpolar solvents, which facilitate the dissolution of neutral lipids (Saini et al., 2021). Traditional lipid analysis techniques have predominantly relied on chromatographic methods, particularly liquid chromatography and thin-layer chromatography. While both approaches are widely employed, it's worth noting that liquid chromatography demands a significant level of experience, whereas thin-layer chromatography is somewhat constrained by its lower resolution capabilities. In lipid analysis and lipidomics, Matrix-Assisted Laser Desorption/Ionization Time of Flight

Mass Spectrometry (MALDI-TOF MS) is an invaluable tool and is known for its speed, simplicity as well and convenience. It can efficiently record mass spectra from diverse sources such as cells, tissues, and body fluid extracts within a few minutes. This technique relies on an ultraviolet-absorbing matrix, with 2,5-dihydroxybenzoic acid (2,5-DHB) commonly used for lipids. UV lasers and matrices that absorb in the UV spectral range are primarily employed. The matrix serves to absorb laser energy, preventing direct impacts on the analyte, as well as preventing aggregation of lipid molecules (Schiller et al., 2007). Furthermore, investigating skin lipid profiles offers valuable insights into the underlying mechanisms of skin disorders. Techniques like tape-stripping and MALDI-TOF MS are powerful tools in this regard. These methods not only allow for non-invasive sampling but also enable the comprehensive analysis of lipid composition, contributing to a deeper understanding of skin disorders' pathogenesis and potential therapeutic targets.

The extraction of lipids presents a significant challenge due to their wide-ranging chemical and structural diversity. Additionally, the intricate composition of biological samples, including body fluids, tissues, and cells, necessitates the careful refinement and optimization of extraction techniques (Saini et al., 2021). This challenge is particularly pronounced in the context of lipid extraction using tape for MALDI-TOF MS analysis to effectively capture the complexity of biological samples.

1.9 Aim

The objective of this study is to evaluate the efficiency of different solvents, including chloroform, methanol, a mixture of chloroform/methanol, and a mixture containing ammonium acetate in chloroform/methanol/2-propanol, in extracting lipids from the skin. The aim is to identify the optimal solvent for lipid extraction, contributing to advancements in lipidomic research.

2. Methods

2.1 Chemical Substances and Materials

Methanol (Sigma-Aldrich), Chloroform (Sigma-Aldrich), Dichloromethane (Sigma-Aldrich), Water with 0.1% formic acid (VWR Chemicals BDH), Milli-Q water (Merck Millipore), 2,5-Dihydroxybenzoic acid (Brucker Daltonics), Ammonium acetate (Sigma-Aldrich), 2-Propanol (Sigma-Aldrich), D-Squame D100 (Clinical and Derm LLC), D-Squame D101 (Clinical and Derm LLC.), D500 D-Squame pressure instrument (Clinical and Derm LLC), Scalpel (Swann-Morton)

2.2 Study Overview

This study was conducted as a collaborative effort between Aalborg University and the Department of Dermatology at Aalborg University Hospital as part of a Ph.D. project. Figure 3 illustrates the study's experimental overview.

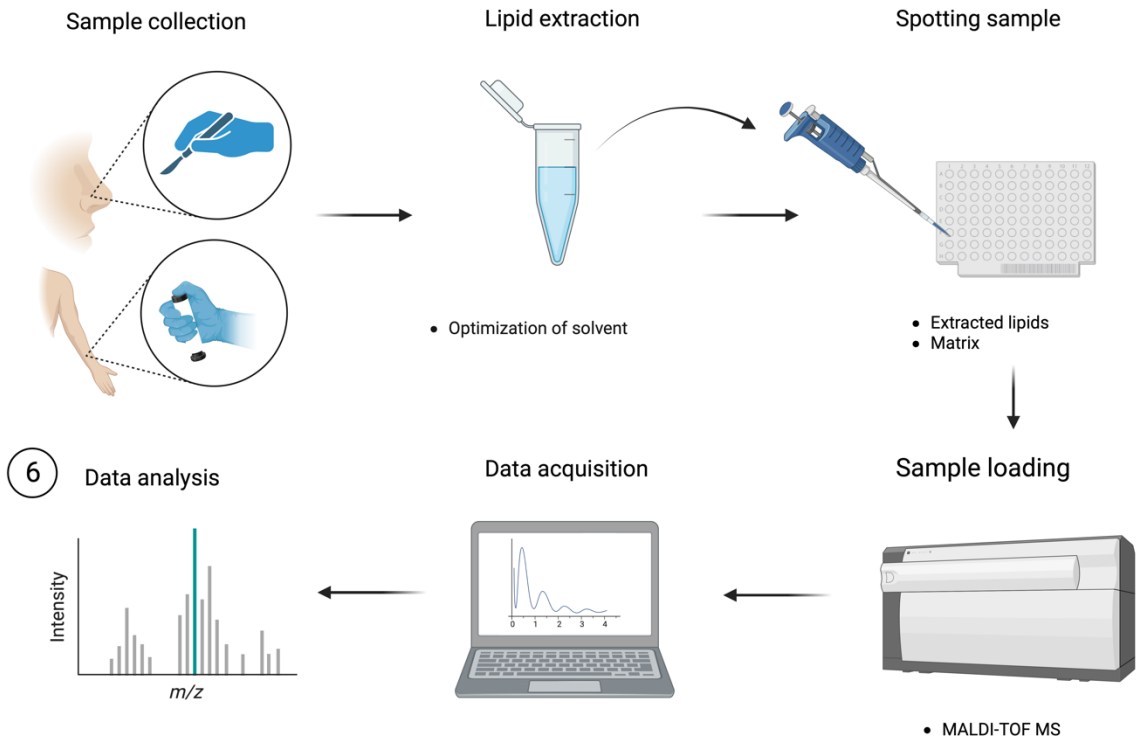


Figure 3: An illustration of our study design showing the different assays that have been performed.

2.3 Sample collection method

In this study, different collection methods were utilized. This included tape stripping of the proximal forearm alongside the acquisition of a skin sample from the alar crease of the nose, serving as a control in our tape stripping methodology. The study involved one test subject, a healthy white female who is 24 years of age; refer to Appendix 1 for study criteria.

The sampling site on the proximal forearm was prepared by using a D100 sampling disc to ensure the removal of any dirt and exogenous substances along with the top layer of the skin. This was done using the D-500 pressure instrument to provide a uniform pressure (225 g/cm²) for approximately 15

seconds. The disc was gently removed in a single, fluid motion using forceps. The prepared site was evident, leaving faint, quickly vanishing impressions on the skin where the edges of the discs had been. Following this, the smaller D101 sampling discs were employed for the tape-stripping process. This procedure was then repeated 5-7 times using the same tape disc - one tape strip is equivalent to one layer of the SC. Without touching the adhesive tape, the disc was placed in an Eppendorf tube with the adhesive tape facing the inside of the tube.

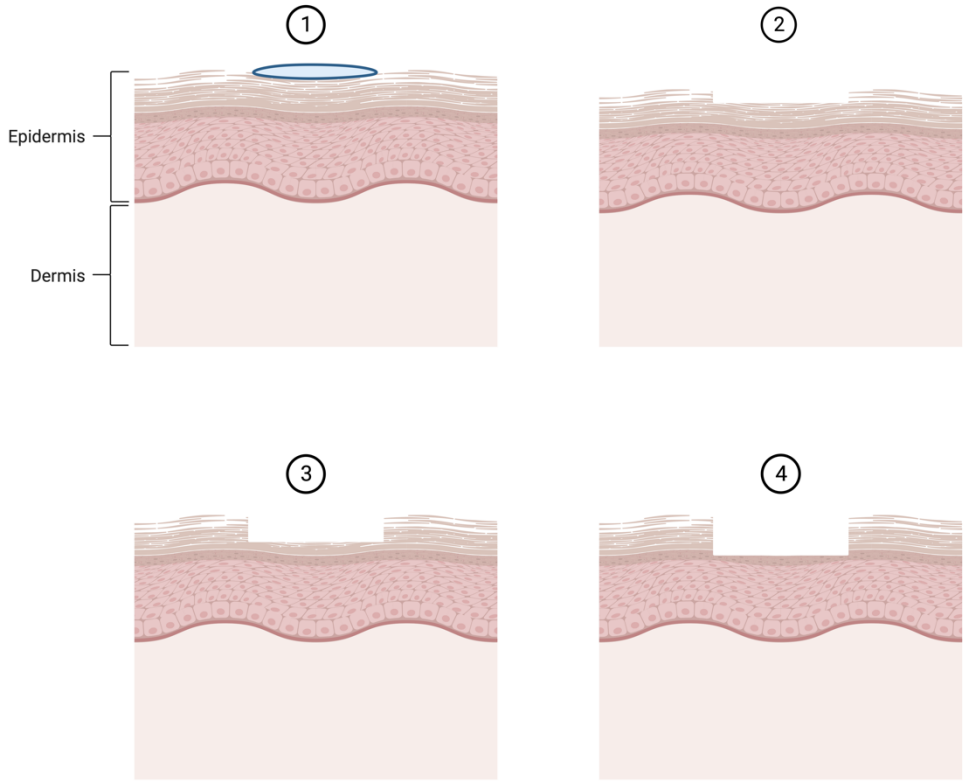


Figure 4: Tape-stripping*:* Illustration demonstrating the gradual removal of layers from the SC.

In addition to the tape stripping method, as seen in Figure 4, a control sample was collected from the alar crease of the nose. This region was chosen for its unique anatomical properties, providing a reliable baseline for comparison against the tape stripping method. For safety reasons, the participant personally performed the collection from the alar crease of the nose, using a disposable, sterile scalpel on the facial area. The scalpels were provided in pre-sterilized packaging.

2.4 Lipid Extraction

2.4.1 Lipid extraction from nose

Following the established procedure outlined by Sadowski et al., 2017, the extraction of lipids from the tape discs commenced with methanol. The samples were shaken at 4°C for one hour. The extracts were carefully transferred to new Eppendorf tubes and subjected to drying in a speed vacuum concentrator. Subsequently, the extraction process proceeded with the utilization of chloroform and methanol.

2.4.2 Solvent Optimization

In this study, the suitability of chloroform, methanol, and a combination of both as potential solvents for lipid extraction on skin samples collected from alar crease of nose was examined (Figure 5). To optimize the solvent for lipid extraction, three Eppendorf tubes were utilized. Each tube was designated for specific solvent testing. Tube 1 was allocated for chloroform, Tube 2 for a mixture of chloroform and methanol in a 2:1 ratio, and Tube 3 for methanol. After centrifugation at 14,000 x g for 5 minutes the supernatant was transferred to new Eppendorf tubes (Tube 4, Tube 5, and Tube 6) prepared for further adjustments. Tube 4 was designated for methanol, Tube 5 was left empty, and Tube 6 received chloroform. The supernatant from Tubes 1, 2, and 3 was meticulously transferred to Tubes 4, 5, and 6. This ensured a total volume of 600 µl, maintaining the chloroform/methanol ratio at 2:1.

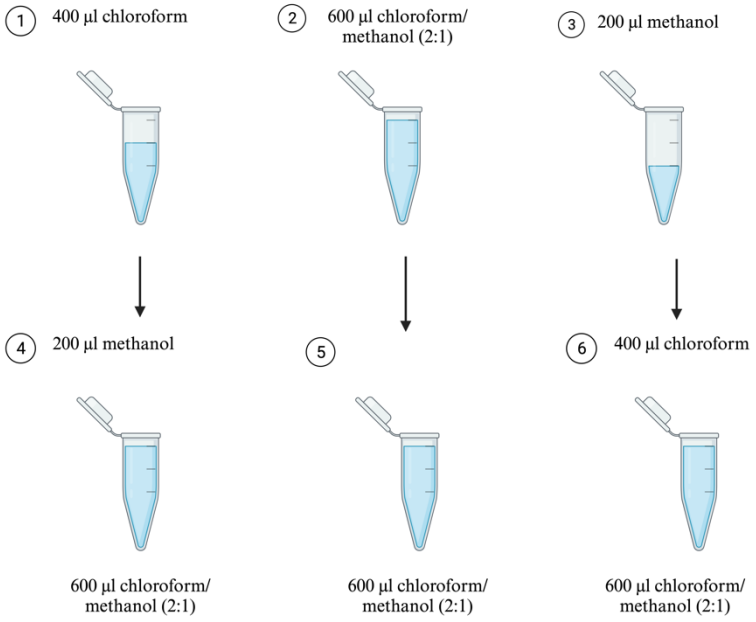


Figure 5: Solvent optimization: Evaluation of chloroform, methanol, and a 2:1 chloroform/methanol mixture as solvents for lipid extraction from nose alar crease skin samples. Tubes designated for each solvent were centrifuged, and supernatants were transferred for further adjustments, maintaining a consistent ratio.

Additionally, a mixture with ammonium acetate in chloroform/methanol/2-propanol, as outlined by Sadowski et al. in 2017, was incorporated into the extraction process.

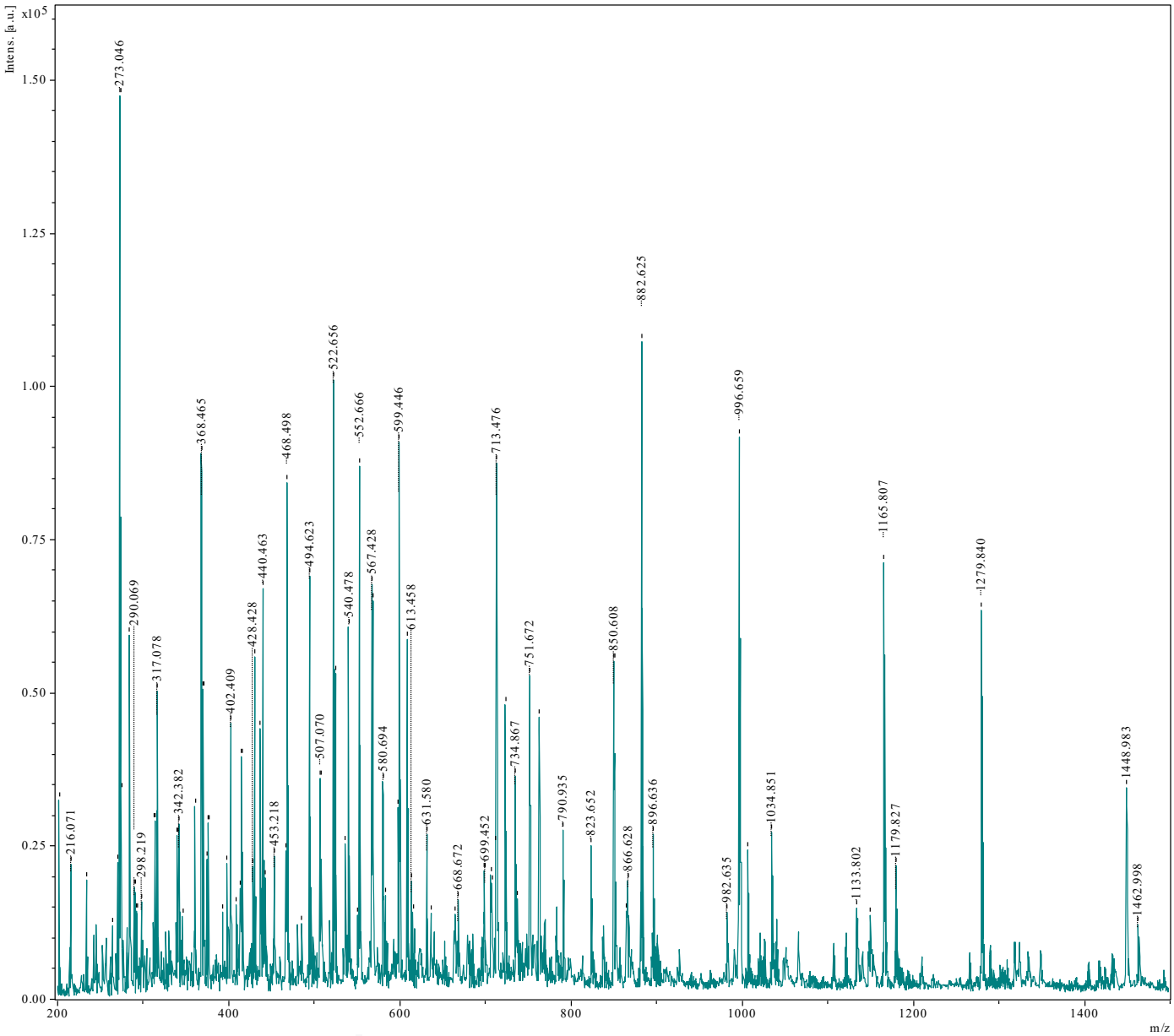
2.5 Matrix-Assisted Laser Desorption and Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS)

The MALDI-TOF MS analysis was performed using a Bruker Ultraflextreme mass spectrometer with a 2,5-DHB matrix (Bruker Daltonics). The matrix was prepared by combining methanol and water in equal parts, 0.1% formic acid, and 2,5-DHB. The samples were applied onto a ground steel MTP 384 target plate with a volume of matrix twice that of the sample. In each case, approximately 0.5 µL of material was carefully deposited onto the MALDI target. The mass spectrometry (MS) analysis was conducted in positive reflector mode, focusing on molecular weights ranging from 200 to 1500 Da to avoid matrix noise. Peak identification and subsequent analysis were performed using FlexControl and FlexAnalysis (version 3.4, Bruker GmBH). The study utilized a ceramide standard sourced from Avanti Polar Lipids, Inc. (Avanti Polar Lipids 330712, solution (1:1 dichloromethane/methanol)). This standard was a mixture of four distinct ceramides, each characterized by specific fatty acid chain compositions: C16 Ceramide (d18:1/16:0), C18 Ceramide (d18:1/18:0), C24 Ceramide (d18:1/24:0), C24:1 Ceramide (d18:1/24:1(15Z)). These ceramides served as reference compounds, aiding in the calibration and validation of the mass spectrometric analysis conducted in the study. The ceramide standard sample was calibrated and followed by a “lift” process.

3. Results

In this study, we systematically investigated the performance of different solvent systems, including chloroform, methanol, a chloroform/methanol mixture, and a buffer solution containing ammonium acetate. The mass spectrum depicts the distribution of ions based on their mass-to-charge ratios (m/z). The x-axis represents the m/z values, while the y-axis indicates the intensity or abundance of ions. Peaks in the spectrum correspond to distinct molecular species, with higher peaks representing more abundant ions. The relative heights and positions of the peaks provide valuable information about the composition and structure of the analyzed sample.

3.1 Tape stripping results

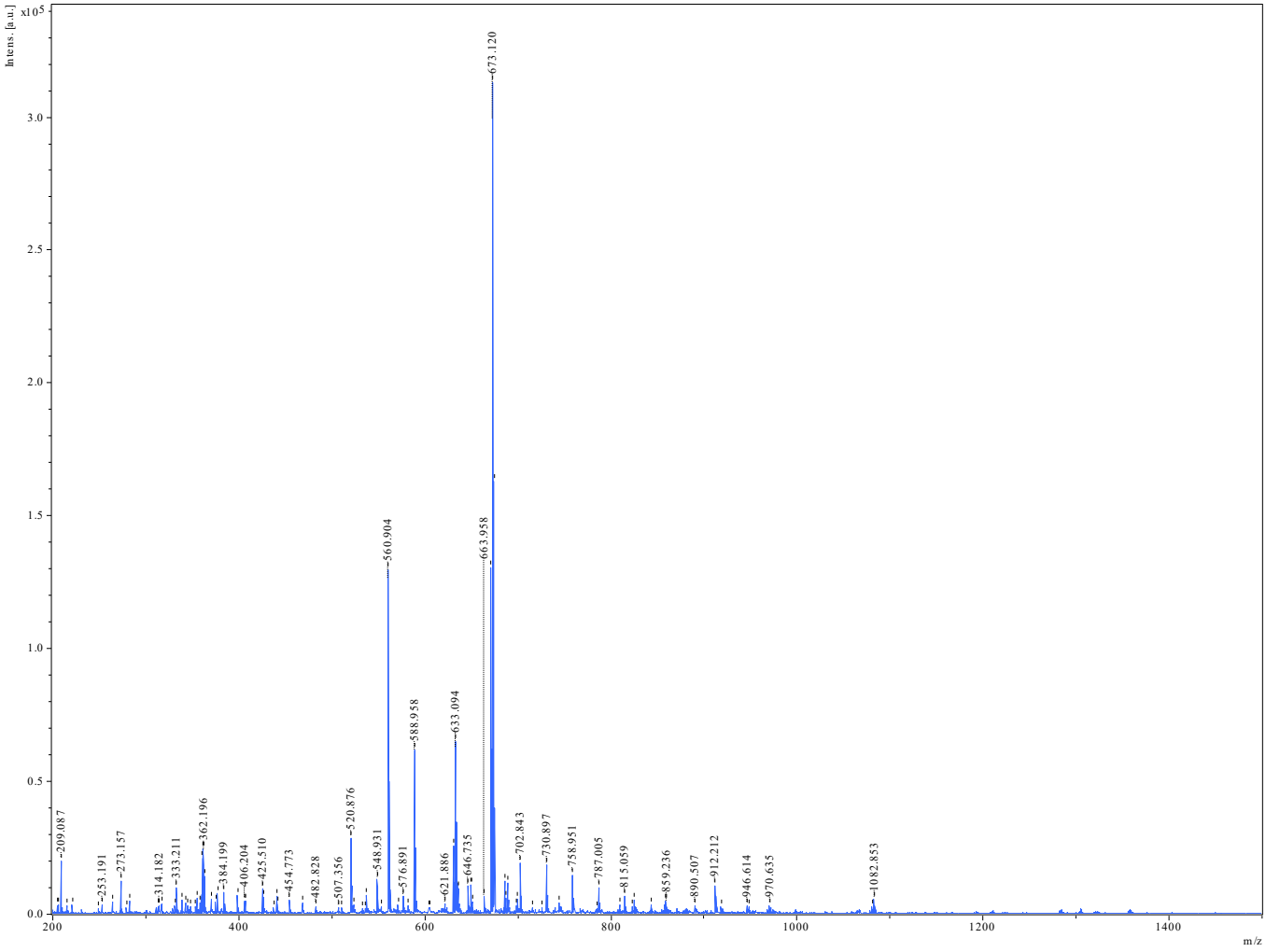


Graph 1**: **Tape-stripping results: This figure displays the outcomes of the tape-stripping procedure.

In Graph 1 depicting the tape-stripping results, it is noticeable that the baseline signal does not originate from the x-axis. When the baseline signal does not start from the x-axis in a mass spectrum, it indicates the presence of a non-zero signal even in the absence of detected ions or compounds. In other words, a measurable intensity or signal is being recorded by the instrument, suggesting the existence of background noise or interference present in the measurement.

3.2 Ceramide Standard Analysis

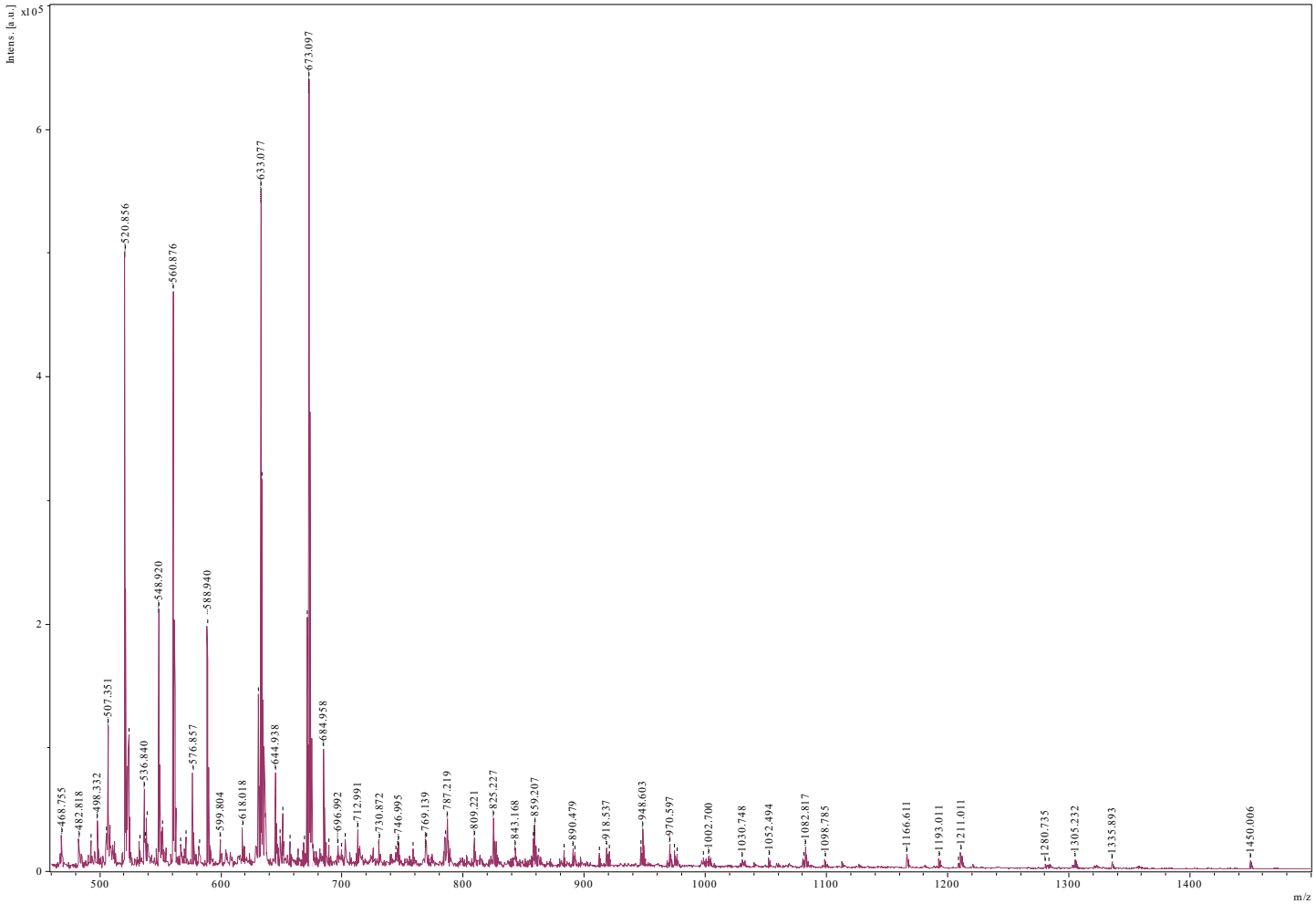
3.2.1 Standard Calibration



Graph 2: Calibration: This mass spectrum depicts the results of the standard calibration process.

A calibration procedure was conducted prior to the analysis of the standard sample to ensure accurate measurements.

3.2.2 Characterization and Identification of Ceramide Standards



Graph 3: Ceramide Standards with Sodium Adducts: The ceramide standards exhibit the following Dalton (Da): C16 Ceramide: 537.90 Da**,** C18 Ceramide: 565.95 Da**,** C24 Ceramide: 650.11 Da and C24:1 Ceramide: 648.10 Da.

The specified ceramide standard includes four distinct ceramides, each associated with their respective Dalton (Da):

- C16 Ceramide (d18:1/16:0) - 537.90 Da
- C18 Ceramide (d18:1/18:0) - 565.95 Da
- C24 Ceramide (d18:1/24:0) - 650.11 Da
- C24:1 Ceramide (d18:1/24:1(15Z)) - 648.10 Da

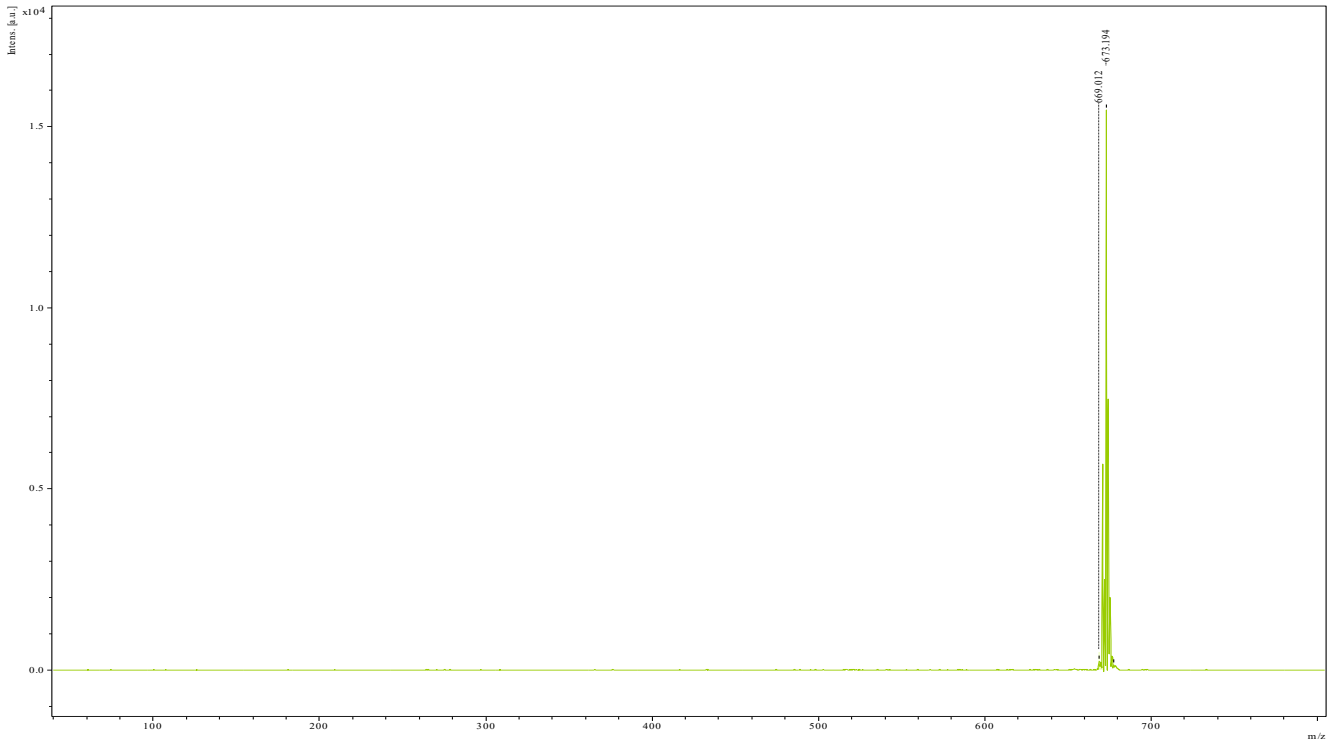
However, it's important to note that these values do not perfectly align with the peaks observed in the MS graph. This discrepancy is primarily attributed to the presence of proton adducts. In MALDI MS, adducts refer to charged complexes formed when ions, such as protons (H+) or sodium ions (Na+), interact with the analyte molecules in the presence of the matrix. In this case, Na+ was attached to

the analyte molecule, resulting in a positively charged sodium adduct. The atomic weight of sodium (Na) is approximately 22.99 atomic mass units (amu), thus giving the following Da:

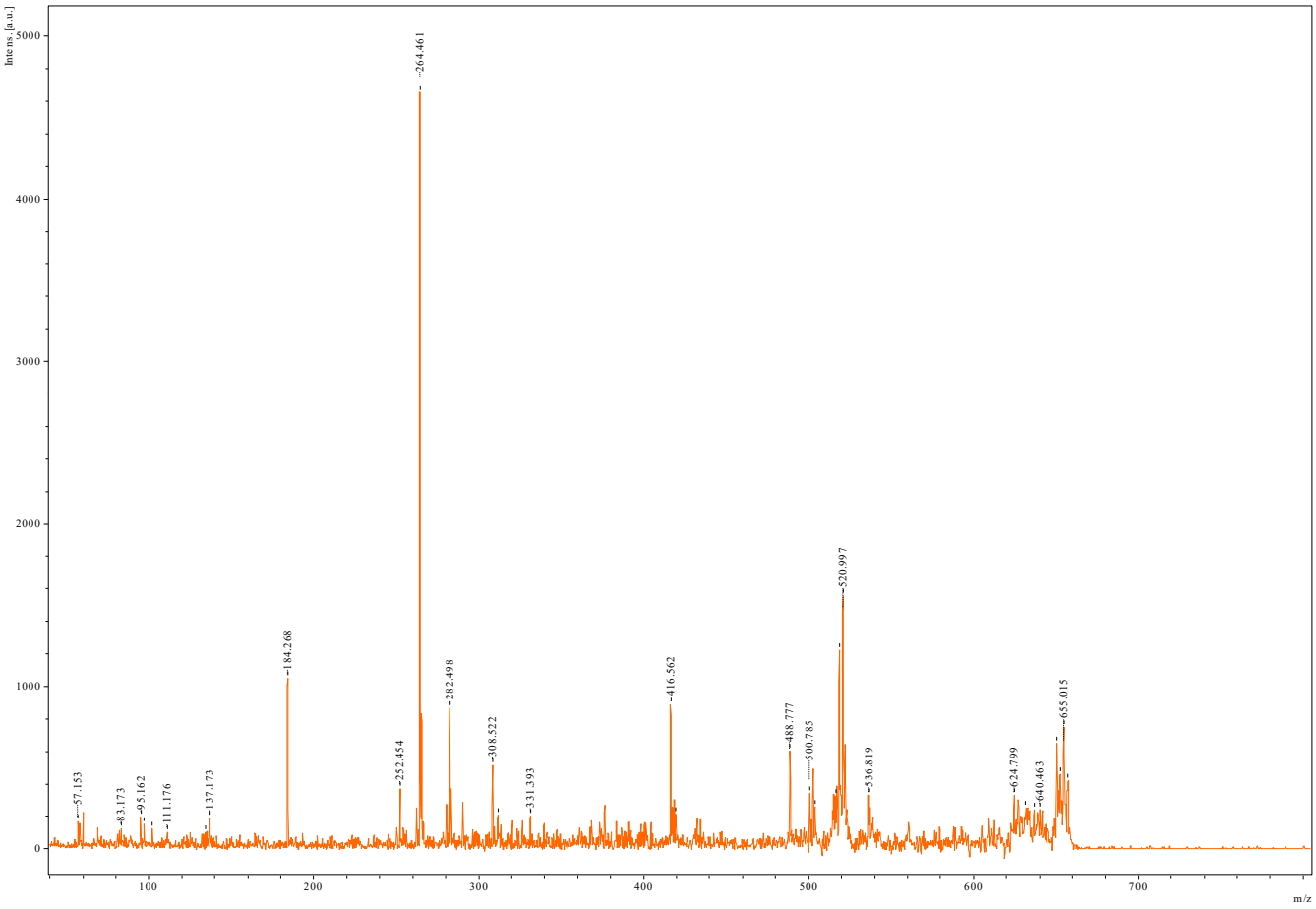
- C16 Ceramide (d18:1/16:0) with sodium adduct - 559.90 Da
- C18 Ceramide (d18:1/18:0) with sodium adduct - 587.95 Da
- C24 Ceramide (d18:1/24:0) with sodium adduct - 672.11 Da
- C24:1 Ceramide (d18:1/24:1(15Z)) with sodium adduct - 670.10 Da

These values also correspond to the peaks observed in the mass spectrometry Graph 3, confirming the presence of the sodium adducts. However, the presence of structural similarity of C24 Ceramide (d18:1/24:0) and C24:1 Ceramide (d18:1/24:1(15Z)) leads to nearly identical mass-to-charge ratio (m/z) values in the mass spectrum. When these ceramides are ionized and detected in the mass spectrometer, their m/z peaks are so close that they can overlap or "shadow" each other. This means that the peaks representing these two ceramides may appear as a single, broader peak on the mass spectrum, which is applicable in this case. The peak for C24:1 Ceramide (d18:1/24:1(15Z)) with a sodium adduct at 670.10 is obscured by the more prominent peak of C24 Ceramide (d18:1/24:0) with a sodium adduct at 672.11.

3.2.3 MS "Lift" and Fragmentation



Graph 4: Lift: This graph depicts the ”lift” procedure performed on C24:1 Ceramide (d18:1/24:1(15Z))



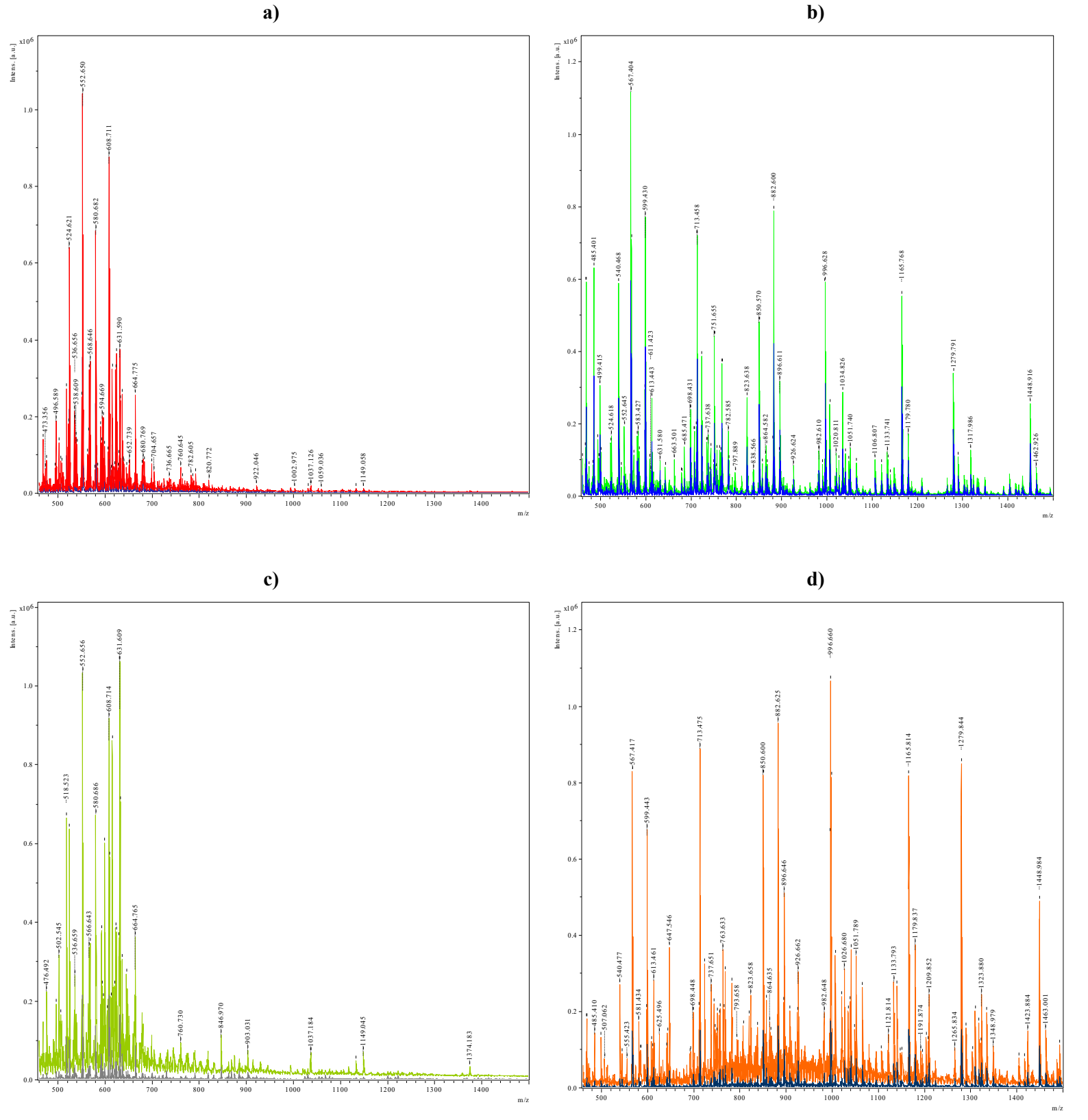
Graph 5: Fragmentation: This graph depicts the distinct 'fingerprint' specific to the original molecule.

A "lift" procedure was performed, involving the precise selection and isolation of specific ions (peaks) from the sample mixture for further analysis or fragmentation. By applying the 'lift' procedure, we achieved the essential isolation of specific ions from the sample mixture, leading to a notably enhanced and informative MS spectrum. In the calibrated sample, the most evident peak corresponds to C24 Ceramide (d18:1/24:0) at 673.120 Da as seen in Graph 4. Additionally, in this MS graph, C24:1 Ceramide (d18:1/24:1(15Z)) is now distinctly discernible, appearing at 669.012 Da, no longer obscured. Subsequently, the isolated peak underwent an additional round of fragmentation, enhancing the resolution of the fragment ion spectrum. The resulting fragmentation pattern displayed a unique 'fingerprint' characteristic of the original molecule, which is vital for precise identification. When a peak of 673 Da is found in a clinical sample, “lift” can give a fragmentation pattern, a 'fingerprint,' to identify the molecule.

3.3 Solvent Optimization Results

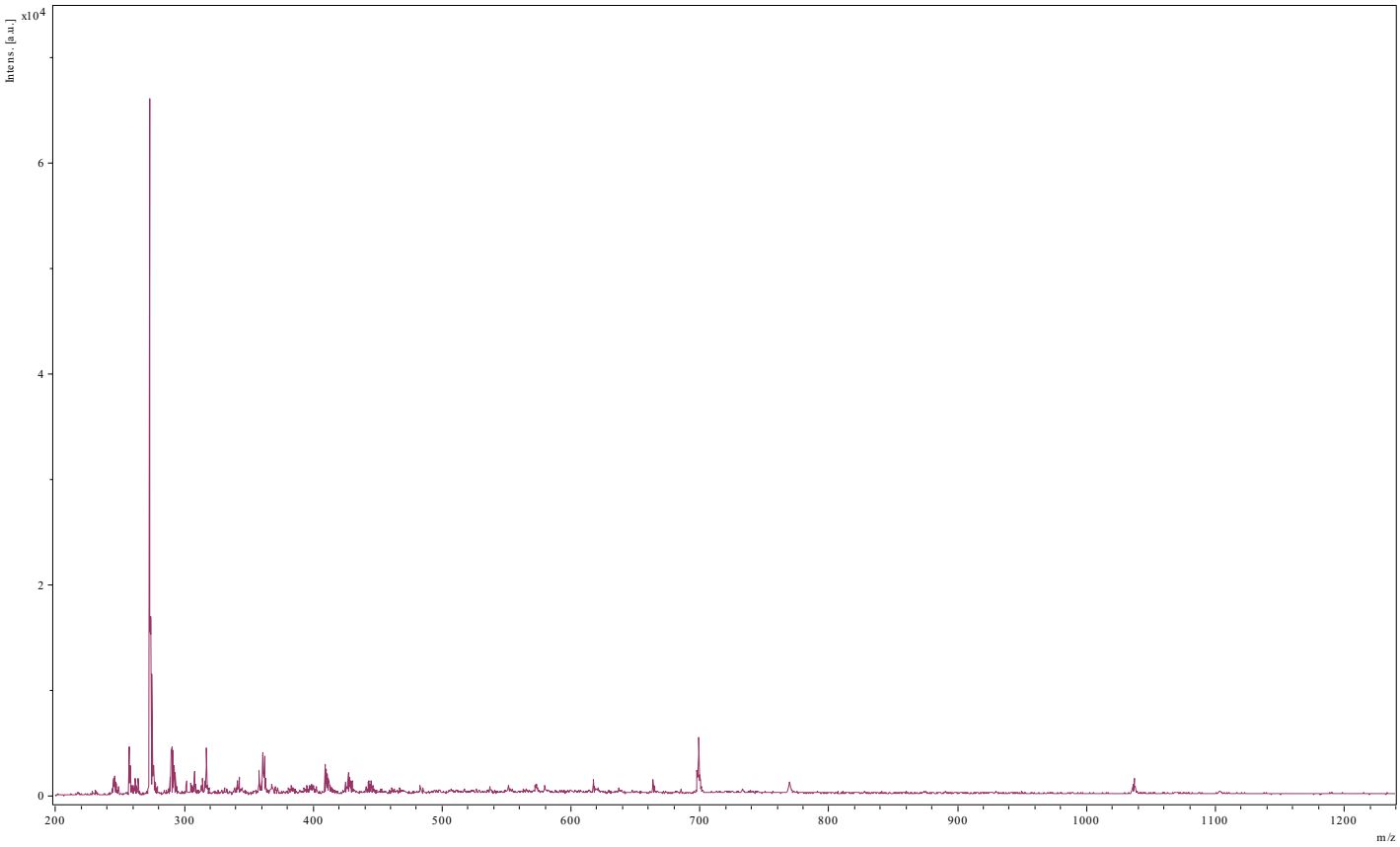
The figures presented in this section exhibit two distinct colors, illustrating the overlay of two separate mass spectra obtained from the same sample. This was performed to assess the reproducibility of the results, providing valuable insights into the consistency and reliability of the analytical process (Graph 6).

In Graph 6a, the mass spectrum obtained using chloroform as the primary extraction solvent is illustrated. Background noise is noticeable, indicating random variations in signal intensity that do not correspond to actual analyte peaks and appear as irregularities in the baseline of the mass spectrum. This noise can arise from various sources, including electronic interference, impurities in the sample or solvent, fluctuations in instrument sensitivity, and other factors. Utilizing a combination of chloroform and methanol for lipid extraction (Graph 6b) displays distinct and well-separated ion peaks with sharply defined intensity. This suggests an efficient extraction and ionization of lipids, with minimal interference from contaminants. The balanced solvent system, combining the non-polar efficiency of chloroform and the polar disruption ability of methanol, maximizes lipid recovery, providing a comprehensive lipid profile. Mass shifts of 14 Da (two H and one C) corresponding to the length difference of two lipid chains were observed. The 2 Da change also observed corresponds to the difference between a double- and single bond between carbon atoms, in agreement with the peaks being lipids. The signal representing extracted lipids is prominent in comparison to background noise, enhancing data accuracy and reliability. Graph 6c, utilizing methanol as the primary extraction solvent, presents peaks resembling those in Graph 6a. The presence of significant background noise around the detected peaks suggests the possibility of impurities or an incomplete extraction process. Moreover, the baseline exhibits a noticeable deviation from the x-axis, indicating a non-zero signal even in the absence of detected ions or compounds. This also applies to extraction results using the mixture of ammonium acetate in chloroform/methanol/2-propanol shown in Graph 6d. Additionally, there is notable background noise despite somewhat well-separated ion peaks.



Graph 6: Solvent optimization result: a) 400 µl chloroform, b) 600 µl chloroform/methanol (2:1), c) 200 µl methanol, and d) 7.5 mM ammonium acetate in chloroform/methanol/2-propanol.

3.4 Matrix control



Graph 7: Matrix of 2,5-DHB

The use of 2,5-DHB as a control resulted in minimal to no signal. It is imperative, that the matrix control does not introduce any unwanted interferences or contaminants that could potentially disrupt the analysis or lead to skewed results.

4. Discussion

The objective of this study was to assess the efficiency of various solvents for extracting lipids from the skin. This investigation aimed to determine the optimal solvent for lipid extraction, thereby advancing lipidomic research. Due to the inefficient extraction of lipids from tape, the study's solvent optimization was carried out using sample controls from the alar crease of the nose. Four solvents were examined, namely chloroform, methanol, a combination of chloroform and methanol, and a buffer solution containing ammonium acetate in chloroform, methanol, and 2-propanol.

After conducting the experiments, it became evident that employing methanol for lipid extraction did not produce the anticipated results. This outcome highlights a significant issue in the methodology, which warrants discussion and consideration. Methanol is a polar solvent with the ability to interact with a wide range of compounds due to its hydroxyl group (Saini et al., 2021). However, in the context of lipid extraction, methanol's polar nature poses a problem. Lipids are primarily composed of hydrophobic fatty acid chains, which are inherently repelled by polar solvents like methanol. This leads to reduced affinity between the solvent and the lipids, resulting in a less efficient extraction process. The inefficiency of methanol in this context could be attributed to its inability to sufficiently disrupt the intermolecular interactions within the lipid structure. Unlike nonpolar solvents, which can readily immerse themselves in the lipid matrix, methanol struggles to penetrate and effectively solubilize the lipids. As a result, the yield of extracted lipids is substantially lower compared to solvents better suited for this purpose. Therefore, the choice of solvent plays a critical role in determining the selectivity of lipid extraction. Chloroform, for instance, is well-known for its high affinity towards lipids due to its nonpolar nature, making it an effective choice for lipid extraction. The mixture of chloroform and methanol also proved to be more efficient than methanol alone, likely due to the presence of chloroform which compensates for the shortcomings of methanol. However, adding an organic solvent directly to the tape discs may be a problem because it could potentially lead to structural damage or dissolving of the tape material, introducing an unwanted source of contamination in the lipid sample. Given these results, it is crucial to reassess the selection of solvent for extracting lipids from tape discs when utilizing MALDI-TOF MS.

Common dermatological disorders such as ACD, ICD, AD, and psoriasis are characterized by skin inflammation. The role of lipids in the development and progression of these disorders has gained significant attention. In these disorders, the interplay between skin lipids and external factors holds substantial importance. Any disturbances in lipid composition and distribution have the potential to

compromise the skin barrier, resulting in increased vulnerability to allergens, irritants, or immune dysregulation. Understanding the role of lipids and employing advanced techniques like MALDI-TOF MS along with innovative sample collection methods like tape discs, can significantly enhance our understanding and management of these disorders. When combined with MALDI-TOF MS, tape disc collection offers a unique opportunity for precise lipidomic analysis. The collected lipids can be subsequently analyzed using MALDI-TOF MS, providing a comprehensive profile of the lipid composition associated with each dermatological disorder. This enables the identification and quantification of specific lipid species, offering valuable insights into the pathogenesis of these conditions. Tape discs offer a range of distinct advantages in dermatology research. One of the primary benefits of employing tape discs is their non-invasive nature. This method provides a gentle and painless means of collecting skin lipids. For patients, this represents a significant advantage as it eliminates the need for more invasive procedures, reducing discomfort and potential complications. The use of tape discs proves to be an efficient and practical approach to sample collection. It allows for a quick and straightforward process, enabling rapid acquisition of samples. This efficiency minimizes both the time and resources required for sample collection, which is particularly advantageous for large-scale studies and facilitates the ease of longitudinal monitoring. Tape disc collection is designed to primarily target the SC, which is the outermost layer of the epidermis. This targeted approach ensures that the deeper layers of the skin, where the barrier functions are located, remain largely undisturbed. As a result, the lipids collected through this method are highly representative of the skin's surface. This is crucial as many of the abnormalities in barrier function, particularly in dermatological conditions, are concentrated in this outer layer. While tape discs offer a practical collection method, standardization of the technique, appropriate storage of collected samples, and validation against other collection methods are important considerations for reliable research outcomes.

The integration of tape discs as a sample collection method, in combination with MALDI-TOF MS, represents a significant advancement in our ability to understand and manage dermatological disorders. By efficiently collecting and extracting surface lipids, researchers can gain valuable insights into the underlying mechanisms of ACD, ICD, AD, and psoriasis. With further research and refinement, this innovative approach holds great potential to revolutionize the diagnosis and treatment of these prevalent dermatological conditions, ultimately improving outcomes for affected individuals.

5. Conclusion

In conclusion, this study emphasizes the significance of solvent selection in lipid extraction. While versatile in its interactions, methanol proved ineffective due to its polar nature. Chloroform, particularly in combination with methanol, demonstrated greater efficiency. Nevertheless, caution is warranted when applying solvents directly to tape discs. These findings underscore the importance of reevaluating solvent choices, especially in the context of MALDI-TOF MS for lipid extraction from tape discs. Furthermore, the integration of tape discs as a non-invasive sample collection method, combined with advanced techniques like MALDI-TOF MS, represents a significant leap forward in comprehending and addressing dermatological disorders. This innovative approach relies on the effective extraction of lipids from tape discs, enabling the efficient retrieval of surface lipids and offering crucial insights into the underlying mechanisms of conditions such as ACD, ICD, AD, and psoriasis. With continued research and refinement, this method holds great potential for transforming the diagnosis and treatment of these prevalent dermatological conditions, ultimately leading to enhanced outcomes for affected individuals.

6. References

Bains, S. N., Nash, P., & Fonacier, L. (2019). Irritant Contact Dermatitis. In *Clinical Reviews in Allergy and Immunology* (Vol. 56, Issue 1, pp. 99–109). Humana Press Inc. https://doi.org/10.1007/s12016-018-8713-0

Baroni, A., Buommino, E., De Gregorio, V., Ruocco, E., Ruocco, V., & Wolf, R. (2012). Structure and function of the epidermis related to barrier properties. In *Clinics in Dermatology* (Vol. 30, Issue 3, pp. 257–262). https://doi.org/10.1016/j.clindermatol.2011.08.007

Berardesca, E., Barbareschi, M., Veraldi, S., & Pimpinelli, N. (2001). Evaluation of efficacy of a skin lipid mixture in patients with irritant contact dermatitis, allergic contact dermatitis or atopic dermatitis: A multicenter study. *Contact Dermatitis*, *45*(5), 280–285. https://doi.org/10.1034/j.1600-0536.2001.450505.x

Bouwstra, J. A., Dubbelaar, F. E. R., Gooris, G. S., & Ponec, M. (2000). *The Lipid Organisation in the Skin Barrier*. https://doi.org/https://doi.org/10.1080/000155500750042826

Johansen, J. D., Bonefeld, C. M., Schwensen, J. F. B., Thyssen, J. P., & Uter, W. (2022). Novel insights into contact dermatitis. *Journal of Allergy and Clinical Immunology*, *149*(4), 1162–1171. https://doi.org/10.1016/j.jaci.2022.02.002

Kendall, A. C., Koszyczarek, M. M., Jones, E. A., Hart, P. J., Towers, M., Griffiths, C. E. M., Morris, M., & Nicolaou, A. (2018). Lipidomics for translational skin research: A primer for the uninitiated. In *Experimental Dermatology* (Vol. 27, Issue 7, pp. 721–728). Blackwell Publishing Ltd. https://doi.org/10.1111/exd.13558

Knox, S., & O'Boyle, N. M. (2021). Skin lipids in health and disease: A review. In *Chemistry and Physics of Lipids* (Vol. 236). Elsevier Ireland Ltd. https://doi.org/10.1016/j.chemphyslip.2021.105055

McLafferty, E., Hendry, C., & Alistair, F. (2012). *The integumentary system: anatomy, physiology and function of skin*. https://doi.org/10.7748/ns2012.10.27.7.35.c9358

Pappas, A. (2009). Epidermal surface lipids. *Dermato-Endocrinology 1:2, 72-76*, *1*(2).

Sadowski, T., Klose, C., Gerl, M. J., Wójcik-Maciejewicz, A., Herzog, R., Simons, K., Reich, A., & Surma, M. A. (2017). Large-scale human skin lipidomics by quantitative, high-throughput shotgun mass spectrometry. *Scientific Reports*, *7*. https://doi.org/10.1038/srep43761

Sætterstrøm, B., Olsen, J., & Johansen, J. D. (2014). Cost-of-illness of patients with contact dermatitis in Denmark. *Contact Dermatitis*, *71*(3), 154–161. https://doi.org/10.1111/cod.12231

Sahle, F. F., Gebre-Mariam, T., Dobner, B., Wohlrab, J., & Neubert, R. H. H. (2015). Skin diseases associated with the depletion of stratum corneum lipids and stratum corneum lipid substitution therapy. *Skin Pharmacology and Physiology*, *28*(1), 42–55. https://doi.org/10.1159/000360009

Saini, R. K., Prasad, P., Shang, X., & Keum, Y. S. (2021). Advances in lipid extraction methods—a review. In *International Journal of Molecular Sciences* (Vol. 22, Issue 24). MDPI. https://doi.org/10.3390/ijms222413643

Schiller, J., Suss, R., Fuchs, B., Muller, M., Zschornig, O., & Arnold, K. (2007). MALDI-TOF MS in lipidomics. In *Frontiers in Bioscience* (Vol. 12).

Sobiepanek, A., Kobiela, T., & Galus, R. (2019). Application of the tape stripping method in the research on the skin condition and its diseases. *Review and Research on Cancer*, *5*(1).

Svensson, A., Ofenloch, R. F., Bruze, M., Naldi, L., Cazzaniga, S., Elsner, P., Goncalo, M., Schuttelaar, M. L. A., & Diepgen, T. L. (2018). Prevalence of skin disease in a population-based sample of adults from five European countries. *British Journal of Dermatology*, *178*(5), 1111–1118. https://doi.org/10.1111/bjd.16248

Appendix

Appendix 1: The Study Design

Initially, the plan was to include around 50 participants. Unfortunately, due to logistical constraints, achieving this target sample size proved unfeasible. As a result, the study involves one test subject, specifically a healthy white female who is 24 years of age. Eligibility criteria for participation were limited to females aged 20 to 30 years with Fitzpatrick skin types I to III Exclusion criteria were applied to individuals with specific medical histories, including a record of AD, psoriasis, hay fever, asthma, and urticaria. In preparation for the test, the participant was asked to refrain from bathing or applying topical moisturizer for 24 hours.