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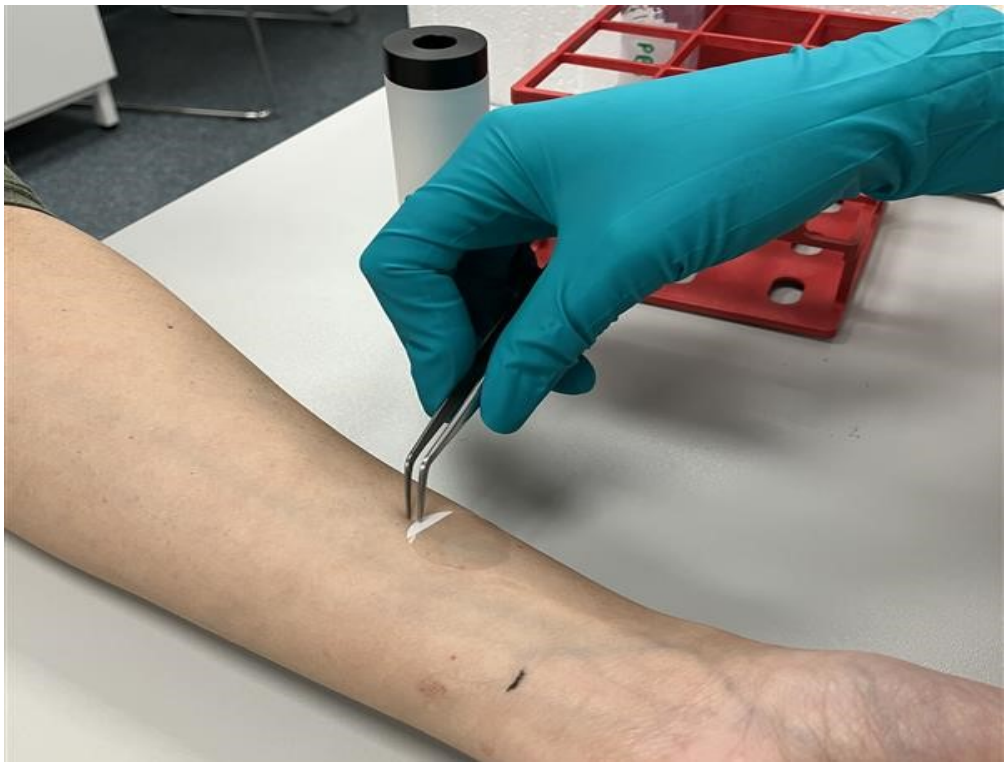

Non-invasive skin sampling: optimization of sample collection and extraction

Enhancing cytokine recovery by the modification of extraction solvents and the use of alternative sampling methods

Master's thesis within Biotechnology master's programme

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Cover: Application of a D-Squame disc on the volar forearm for skin surface sample collection.

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Abstract

Cytokines are a group of proteins that play a crucial role in cell communication within the immune system and are frequently studied as biomarkers for assessing skin condition. However, many inflammatory cytokines of interest are present at levels too low to be detected in skin surface samples. The aim of this master's thesis was to refine a non-invasive skin sampling technique, enhancing cytokine recovery by optimizing both the sample collection method and the cytokine extraction solvent. Improved cytokine recovery leads to more accurate analysis of the skin condition.

This clinical study involved two rounds of skin sample collection from test participants. In the first round, samples were collected using the tape stripping method, and cytokines were extracted from the tapes using combinations of two buffers and three detergents at three different concentrations. In the second round, samples were collected via tape stripping and skin swabbing. The samples were extracted using the most effective solvents identified in the first round, allowing for a comparison of the performance of the collection methods. Cytokine analysis was conducted using ELISA (enzyme-linked immunosorbent assay), complemented by the Micro BCA Protein Assay to determine the total soluble protein concentration of the samples. Statistical evaluations were carried out using ANOVA (analysis of variance).

The results indicate that Tris-HCl combined with 0.5% Triton X-100 and 0.5% β-dodecyl maltoside achieved the highest recovery of the cytokines IL-1α and IL-1RA, with swab being the most effective sampling method. For TNF-α, the optimal extraction solvent was Tris-HCl with 0.005% Tween 20, and the optimal collection method proved to be Leukofix tape, which was also true for IL-8. However, IL-8 and IL-6 levels were generally at or below the detection limit, which is why no definitive conclusions could be drawn from those data.

Keywords: non-invasive skin sampling, tape stripping, skin swabbing, inflammatory biomarkers, cytokines, protein extraction, detergents, ELISA

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To my beloved family, thank you for always being there for me, celebrating my successes and supporting me through any challenges. I am so grateful for the countless ways you have helped me along this journey.

LISA ARONSSON, Gothenburg, June 2025

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List of Acronyms

Below is a list of acronyms that have been used throughout this thesis:

AD	Atopic Dermatitis
AEs	Adverse Events
ACD	Allergic Contact Dermatitis
ANOVA	Analysis Of Variance
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CMC	Critical Micellization Concentration
CSFs	Colony-Stimulating Factors
CXCR1	Cysteine-X-Cysteine Receptor 1
CXCR2	Cysteine-X-Cysteine Receptor 2
DDM	n-Dodecyl β-D Maltoside
DEJ	Dermal-Epidermal Junction
ELISA	Enzyme-Linked Immunosorbent Assay
gp130	Transmembrane Glycoprotein 130
HRP	Horseradish Peroxidase
IFNs	Interferons
ILs	Interleukins
IL-1α	Interleukin-1 alpha
IL-1RA	Interleukin-1 Receptor Antagonist
IL-1RAcP	Interleukin-1 Receptor Accessory Protein
IL-6	Interleukin-6
IL-6R	Interleukin-6 Receptor
IL-8	Interleukin-8
ICD	Irritant Contact Dermatitis
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
NMFs	Natural Moisturizing Factors
TGFs	Transforming Growth Factors
TMB	Tetramethylbenzidine
TNFs	Tumor Necrosis Factors
TNF-α	Tumor Necrosis Factor alpha
TNFR1	Tumor Necrosis Factor Receptor 1
TNFR2	Tumor Necrosis Factor Receptor 2
Tris-HCl	Tris Hydrochloride
PBS	Phosphate-Buffered Saline

1

Introduction

The skin is the largest organ of the human body and functions as a protective barrier against harmful chemical, physical, and biological agents [1]. The skin surface offers a distinctive source of potential biomarkers (measurable indicators of a biological state), such as cytokines, which can provide valuable insights into the condition of the skin. Cytokines are small proteins that act as signaling molecules in the immune system, regulating inflammation and the body's response to infections [2]. Studies have shown that skin treated with an irritant, an allergen, or exposed to sunlight, has a different cytokine profile compared to uncompromised skin [3–5]. Similarly, research has demonstrated that inflammatory skin diseases exhibit unique cytokine expression patterns [6, 7]. Analyzing the skin cytokine profile therefore makes it possible to investigate the skin response to different external stimulants and to distinguish between different inflammatory skin diseases.

Typical cytokines to study for these purposes include interleukins (ILs) such as IL-1α, IL-1 receptor antagonist (IL-1RA), IL-6, and IL-8, as well as Tumor Necrosis Factor-α (TNF-α) [5, 8]. For non-invasive skin sample collection, tape stripping is a technique that is commonly applied. An adhesive tape is then applied to the skin for sampling of the outermost layer, known as the stratum corneum [9]. Currently, there is no standardized protocol for tape stripping or optimization for specific biomarkers [9]. This applies not only to the choice of collection method but also to the extraction procedure, which can further influence the recovery of cytokines. Extraction using several different buffers and detergents has been reported in the literature. Phosphate-buffered saline (PBS) and Tris-HCl are often used in combination with detergents like Tween 20, Triton X-100, and β-dodecyl maltoside [10–13].

In Essity's interest, employing a non-invasive skin sampling technique during clinical product evaluations allows for the inclusion of a broader range of participants, such as infants and the elderly. Infant and elderly skin is more sensitive due to thinner skin layers, lower levels of collagen and elastin, and a flatter dermal-epidermal junction (DEJ) [14]. These factors compromise the ability of the skin to function as a protective barrier and withstand mechanical stress. Therefore, it is crucial to implement non-invasive skin sampling procedures, such as tape stripping and swabbing, that cause no discomfort and do not disrupt the skin barrier. Expanding the list of detectable cytokines would further allow for more accurate evaluations of the effects of Essity's products on the skin. Ideally, this would lead to improved products on

the market, which is particularly relevant given the growing and aging population. The demand for products like diapers and incontinence pads is expected to rise. The world population is projected to grow from 8.2 billion in 2024 to 10.3 billion around 2085 [15]. By 2080, the number of people aged 65 and over will exceed those under 18 globally, reaching 2.2 billion. At that time, the elderly will make up approximately 22% of the world's population, compared to just 10% in 2024 [16].

As previously mentioned, skin surface sampling and cytokine analysis are not only used to assess the skin condition but also play a crucial role in diagnosing various inflammatory diseases and contributing to a deeper understanding of their underlying mechanisms. Psoriasis and atopic dermatitis (AD) are examples of chronic inflammatory skin diseases, affecting up to 4% [17] and 10% [18] of the population in Western countries, respectively. Psoriasis presents as red, scaly skin and is caused by the own immune system, while AD presents as itchy eczema resulting from a dysfunctional skin barrier [19]. Both conditions place a significant economic burden on society [20], underscoring the importance of accurate diagnosis and a thorough understanding of disease mechanisms to facilitate effective treatment.

1.1 Background

Essity Hygiene and Health AB is a global healthcare company that aspires to improve consumer well-being with their products and solutions [21]. The company has three main business areas: Health & Medical, Consumer Goods, and Professional Hygiene. Together, this comprises a broad range of products, including wound care products, sensor technology, incontinence products, baby care, feminine care, consumer tissue, and more. Examples of Essity-owned brands include Tork, TENA, Libresse, and Leukoplast.

As a producer of products that come into direct contact with the skin, it is crucial for Essity to consider the impact of their products on skin health. The use of absorbent hygiene products like diapers, incontinence pads, and feminine towels creates a warm and humid microclimate that can make the skin more prone to enzymatic degradation, microbial growth, chemical irritation, and mechanical damage caused by shear forces or pressure [22]. The occlusion also increases the pH of the skin. If these conditions continue to prevail, irritant contact dermatitis (ICD) and skin infections can develop. Symptoms of ICD include redness, swelling, itching, and pain [23]. The severity depends on the nature of the irritant, exposure time, frequency of use, and the individual's skin susceptibility. The prevalence of diaper dermatitis (DD) and incontinence-associated dermatitis (IAD) varies between 5-35% in infants and 5-42% among patients with incontinence, respectively [22].

For evaluating the effects of Essity's own products on skin, tape stripping and swabbing are two non-invasive skin sampling methods that have been implemented internally. However, there is no standard operative procedure in place. In case of tape stripping, sample collection and extraction have been performed using D-Squame tape and an extraction buffer reported in relevant publications. This methodology

has been successful for detection of natural moisturizing factors (NMFs), water-soluble compounds in the skin that help maintain skin hydration, using LC-MS/MS. Analysis of cytokines, on the other hand, has been limited to those present in high abundance. Since the majority of inflammatory cytokines of interest are present at very low concentrations in skin surface samples [24], the application of ELISA and possibility to investigate the inflammatory response become limited. When collecting tape stripping samples from normal stratum corneum, IL-1α and IL-1RA are typically the only cytokines present in sufficient amount to be quantified [25]. This highlights the need to optimize sample collection and extraction to enhance cytokine detection when using ELISA as the analytical technique.

The findings from this study will be applied in the ongoing skin barrier project at Essity and in future skin irritation investigations. Additionally, the study evaluated a tape from Essity's own assortment for skin sample collection. This, to determine if it performed comparably to D-Squame tape and if it could potentially become the new internal standard for tape stripping.

1.2 Aim

The aim of this master's thesis project was to optimize a non-invasive skin sampling method, with regards to sample collection and analyte extraction. It was a clinical study, thus involved taking skin samples from human participants. Sample collection was performed using two types of adhesive tapes (D-Squame and Leukofix tape) and a skin swab (FLOQSwabs). For analyte extraction, combinations of two types of buffers (PBS and Tris-HCl) with three detergents (Tween 20, Triton X-100, and β-dodecyl maltoside) at three different concentrations were evaluated. The analytes of interest were the following five cytokines: IL-1α, IL-1RA, IL-6, IL-8, and TNF-α, which served as inflammatory biomarkers. Cytokine analysis and protein quantification were performed using ELISA and the Micro BCA Protein Assay. The resulting data was tested for statistical significance using ANOVA.

1.3 Research Objectives

There were two main objectives with this research project:

- to determine which extraction solvent (combination of buffer and detergent) that resulted in the highest recovery of cytokines.
- to determine which sample collection method (tape stripping or swabbing) that resulted in the highest recovery of cytokines.

This applied to each specific analyte.

1.4 Limitations

This project was limited to investigating skin samples taken from the volar forearms of participants without active skin diseases, above the age of 18. It was further limited to only investigating non-invasive skin sample collection, with focus on tape stripping and swabbing, as well as restricted to evaluating two buffers, three detergents, and five cytokines. Analysis was conducted using ELISA together with a Micro BCA Protein Assay. Optical density was also measured for the collected D-Squame tapes. Besides that, no other analytical techniques were used.

1.5 Societal and Ethical Aspects

Prior to the execution of this study, an ethical application was sent in to and approved by the Swedish Ethical Review Authority (Etikprövningsmyndigheten) for sample collection from human subjects. The clinical investigation was further conducted in accordance with the Declaration of Helsinki, ICH-GCP guidelines, [26] and relevant national regulations. The test participants received oral and written information about the study and were required to sign an informed consent form. Participation was voluntary and could be discontinued at any time during the study. All personal information, including full name, gender, and age, was stored confidentially in line with the European General Data Protection Regulation (GDPR). The study data was pseudonymized so that a separately stored coding key, to which only qualified personnel had access, was required to link any of the data to the participants. The data will be stored for 10 years, while the samples will be stored at Essity's site in Mölndal for up to three months before being destroyed.

Having addressed the ethical considerations of the study's conduct, it is important to note that the sampling process itself was non-invasive, painless, and posed no risk to the participants. Tape stripping and swabbing are two well established skin sampling techniques. If a participant was not sensitive or allergic to tape adhesive or nylon, these methods were safe to use. Nevertheless, all participants were carefully monitored for any occurrence of Adverse Events (AEs).

For analysis of the samples, ELISA and a Micro BCA Protein Assay were performed. The current ELISA utilizes recombinant capture and detector antibodies, as well as recombinant cytokines for the standard curve. In contrast, the BCA assay uses bovine serum albumin (BSA) derived from bovine blood as standard, which may raise ethical concerns regarding animal welfare despite it being a byproduct of the cattle industry. Nevertheless, BSA is widely used for protein quantification.

Regarding societal impact, developing a standardized protocol for skin sample collection and extraction can enhance the accuracy and consistency of product evaluations. This will lead to the introduction of improved products on the market. Essity reaches one billion people in 150 countries every day with their hygiene and health solutions (data from 2023) [27], thereby having the potential for significant impact on the society. Additionally, the non-invasive sampling method will reduce

the need for invasive procedures, benefiting all test participants, particularly those with sensitive skin, such as babies and the elderly.

Worth mentioning is also the ecological aspect. While conducting this study did not significantly impact the environment or living organisms due to its low resource intensity, the possibility to develop better products because of this skin sampling method could ultimately reduce consumer waste. Improved product durability and functionality would lead to less frequent need for change of product, benefiting the environment. To address some negative ecological impacts, these include the energy consumption from operating the incubator and plate reader, as well as the plastic waste generated from laboratory consumables.

2

Theory

2.1 Anatomy and Physiology of the Skin

The human skin serves multiple functions, including maintaining the body's water and salt balance, regulating temperature and pH, enabling sensory perception, storing fat and vitamin D, and participating in endocrine and immunological signaling [1]. The skin's immunological function is attributed to the presence of immune cells, active in both the innate and adaptive immune system. As the largest organ of the body, the skin acts as a barrier to the external environment, providing protection against mechanical forces, UV-radiation, and harmful chemicals and pathogens.

It is particularly the outermost section of the skin, the epidermis, that regulates which substances can enter and exit the body [1]. The epidermal skin layer is typically 0.5 to 1.5 mm thick and consists primarily of keratinocytes responsible for the production of keratin filaments. Other cell types, including melanocytes (melanin-producing cells), Langerhans cells (antigen-presenting dendritic cells), and Merkel cells (mechano-receptor cells), are also present in the epidermis. The epidermis can be divided into four distinct layers: the basal layer, the stratum spinosum, the stratum granulosum, and the stratum corneum, each characterized by a different morphology of the residing keratinocytes. Keratinocytes originate from epidermal stem cells in the basal layer, differentiate and migrate upward through the epidermal layers, and finally reach the stratum corneum at the skin surface. There, they transform into corneocytes, which are dead cells lacking nuclei and organelles, and are integrated in a lipid matrix. These corneocytes eventually shed from the skin.

Located just below the epidermis lies the dermis, separated by a basement membrane called the dermal-epidermal junction (DEJ) [28]. The basal kerotinocytes anchor to the DEJ via hemidesmosome filaments. The main structural components of the dermis are collagen and elastin, providing tensile strength and elasticity to the skin. This connective tissue also contains blood- and lymphatic vessels, sweat- and sebaceous glands, hair follicles, nerves, immune cells, macrophages, and fibroblasts. The fibroblasts produce collagen and other components of the extracellular matrix. Beneath the dermis lies the innermost skin layer, the hypodermis, which consists of adipocytes (fat cells) and larger blood vessels, and functions as an energy storage.

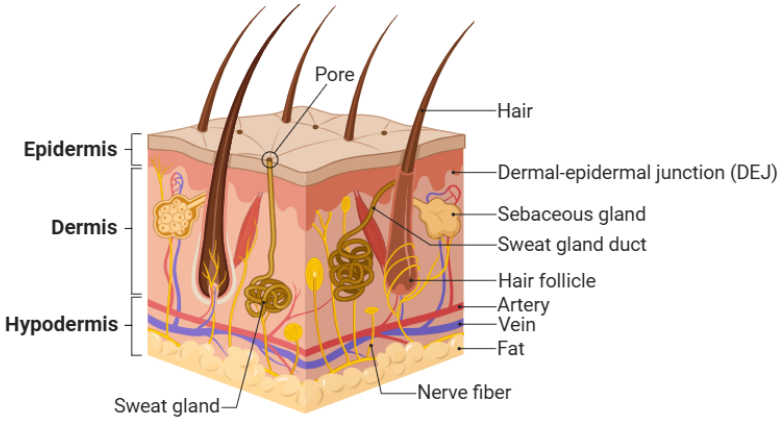

Figure 1: *Layers of the skin.*
(Image created in https://BioRender.com.)

The structure and functionality of the skin changes with age. Infant and elderly skin is particularly fragile [14]. Both skin types are defined by a thinner epidermis, dermis, and hypodermis, a collagen network with reduced supportive functionality and lower elastin levels leading to a lower skin elasticity, and a less pronounced DEJ leading to a decreased nutrient transfer between the layers. Overall, thinner skin and a flattened DEJ results in a decreased barrier function and protection against mechanical forces like pressure or friction. Skin pH is also elevated in premature and aging skin, which can inactivate enzymes responsible for pathogen destruction. Additionally, a reduction in water content within the stratum corneum, along with fewer small blood vessels, is characteristic for these skin types.

2.1.1 Skin Irritation

When it comes to the use of absorbent hygiene products, the primary associated skin condition is irritant contact dermatitis (ICD), with cases of allergic contact dermatitis (ACD) being relatively rare [22]. ICD and ACD are the two main classes of contact dermatitis, an inflammatory skin disorder [23]. ACD results from an allergic reaction to an allergen, mediated by the adaptive immune response. In contrast, ICD is caused by the irritant's harmful effects on the skin and involves the innate immune response, without the precise recognition of a foreign substance.

Numerous studies have confirmed that our skin is responsive to its environment. Compromised skin exhibits a different cytokine profile compared to control skin, often showing higher levels of pro-inflammatory cytokines. De Jongh et al. (2006) [3] performed a skin irritation study with the aim of better understanding the mechanism of ICD. The skin was exposed to a single or repeated application of sodium lauryl sulfate (SLS). The main finding was that IL-1RA and IL-8 levels significantly increased after exposure to the irritant. Morhenn et al. (1999) [4] investigated the cytokine mRNA levels of skin treated with SLS and dibutyl squarate (an allergen)

respectively. The skin reaction caused by the irritant generated a different expression of cytokines compared to the immunological skin reaction. Consequently, ICD could be differentiated from ACD. Furthermore, Perkins et al. (2001) [5] evaluated the cytokine profiles of rashed skin, skin treated with SLS, and skin exposed to sunlight. Their analysis focused on the expression levels of IL-1α, IL-1RA, and IL-8, all of which were elevated in compromised skin.

2.2 Immune System and Its Cytokines

Cytokines are small signaling proteins (5–20 kDa) involved in cell-to-cell communication [2]. Upon stimulation, they are secreted by one cell to bind to the cell surface receptors on a target cell. The ligand-receptor binding initiates the signaling process by inducing a conformational change in the receptor, thereby activating it [29]. This is followed by signal transduction, a cascade in which the signal is amplified and further transmitted intracellularly through the activation of phosphorylating protein kinases. Signal transduction pathways include for example the Jak-Stat pathway and Ras-mitogen-activated protein kinase (MAPK) pathway. Ultimately, this leads to the activation of transcription factors, which regulate specific gene expression, resulting in a cellular response or phenotypic effect. The signaling can be autocrine, paracrine, or endocrine, referring to communication with the same cell, nearby cells, or distant cells via the circulatory system [2].

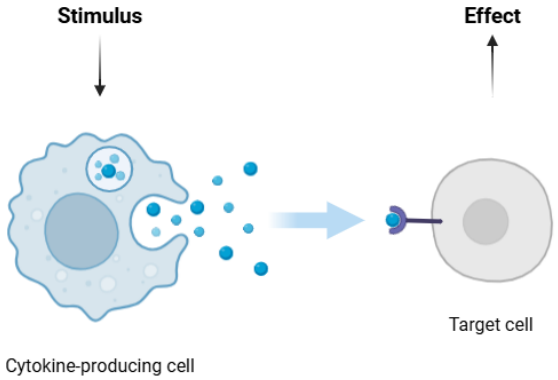

Figure 2: *Mechanism of paracrine signaling. (Image created in https://BioRender.com.)*

Cytokines play a crucial role in the immune system by regulating the inflammatory response during infection or injury. Inflammation aims to eliminate foreign objects, remove damaged cells and tissue, and promote tissue repair [30]. White blood cells, known as leukocytes, play a key role in this process. Among them, neutrophils are predominant in the acute phase of inflammation, while macrophages become more prevalent as the response progresses [31]. Both cell types are capable of phagocytosis, in which foreign objects and cellular debris are engulfed.

Our immune system includes both innate and adaptive immunity for protection against pathogens [2]. The innate immune response is rapid and non-specific, comprising physical barriers such as skin and mucous membranes, chemical barriers like the acidic and enzymatic environment in the stomach, as well as phagocytic cells, natural killer cells, and complement proteins. In contrast, the adaptive immune response is antigen-specific and involves lymphocytes, a type of white blood cell. It can be divided into the cell-mediated response, driven by T-lymphocytes (T-cells), and the humoral response, driven by antibody-producing B-lymphocytes (B-cells). Adaptive immunity is acquired first after the initial exposure to the pathogen, creating a memory that ensures that the subsequent encounters with the same pathogen results in a faster and more effective response. Cytokines are involved in both innate and adaptive immunity [30]. They are produced by activated phagocytes, primarily macrophages, to recruit more leukocytes to the site of inflammation, to activate T- and B-cells, and to attract new tissue cells. Cytokines are also secreted by activated T-helper cells to attract phagocytes and activate cytotoxic T- and B-cells.

Cytokines can be classified into six major families: interleukins (ILs), interferons (IFNs), chemokines, tumor necrosis factors (TNFs), transforming growth factors (TGFs), and colony-stimulating factors (CSFs) [32]. ILs enable communication primarily between leukocytes, IFNs are involved in anti-viral responses and regulation of cell growth, chemokines attract immune cells to the site of inflammation, TNFs can induce apoptosis (cell death) and act pro-inflammatory, TGFs control cell proliferation and differentiation as well as immune responses, and CSFs are hematopoetic cytokines responsible for the production of blood cells [33]. The cytokines of interest for this study are IL-1α, IL-1 receptor antagonist (IL-1RA), IL-6, IL-8, and Tumor Necrosis Factor-α (TNF-α). These were selected because they are frequently analyzed when evaluating skin conditions, as reported in the literature.

Cytokine production is carried out not only by macrophages, monocytes, neutrophils, and lymphocytes (T- and B-cells), but also by keratinocytes, fibroblasts, endothelial cells, epithelial cells, and several other cell types [34]. Besides exerting an immunological function in activating and attracting cells, cytokines can influence cell division and apoptosis [35]. Cytokines produced by skin cells can regulate keratinocyte proliferation and differentiation, thereby affecting the skin barrier [36]. A dysfunctional skin barrier is found in both atopic dermatitis (AD) and psoriasis patients, where cytokine expression is low. A common feature of all inflammatory skin diseases is precisely the clear dependence between skin barrier condition and cytokine expression [37]. Alterations in either skin barrier condition or cytokine expression influences the other, thereby affecting the disease progression.

The studied cytokines can be linked to inflammatory skin diseases [34]. For instance, all five cytokines are associated with psoriasis. IL-1α, IL-1RA, and TNF-α are also associated with AD. As such, these cytokines serve as key markers of inflammation in various skin conditions. Analyzing overall cytokine expression can help distinguish between different inflammatory conditions and thereby aid in disease diagnosis. Liu et al. (2022) [6] performed skin punch biopsies to examine the cytokine profiles associated with various inflammatory skin diseases. The study revealed that different

skin conditions exhibited distinct patterns of inflammatory biomarker expression. In another study, Portugal-Cohen et al. (2024) [7] extracted cytokines from the skin surface by placing a well containing PBS on the skin. The aim was to characterize the cytokine levels of different inflammatory skin diseases, with focus on IL-1α, IL-1β, IL-8, and IL-10. Indeed, the skin conditions could be correlated to their own signature cytokine expression, which further demonstrates that cytokine profiling facilitates disease prediction. For example, skin samples from psoriatic lesions had increased IL- 1β and IL-8 expression.

2.2.1 IL-1α and IL-1RA

IL-1α and IL-1RA belong to the IL-1 cytokine family [34]. IL-1α is initially translated into a pro-IL-1α molecule, which is then proteolytically processed and secreted in response to infection or injury. Although both IL-1α and IL-1RA act upon the same stimuli, IL-1α is pro-inflammatory and acts as an agonist to the IL-1 type I receptor (IL-1R1), while IL-1RA is anti-inflammatory and antagonistic to IL-1α. Binding of IL-1α to IL-1R1 induces a conformational change of the receptor, which attracts the IL-1 receptor accessory protein (IL-1RAcP) [38]. IL-1RAcP further enables the signal transduction where several phosphorylation and ubiquitination processes follow, activating various pathways. Collectively, this induces the expression of genes coding for IL-6 and IL-8, as well as IL-1α itself, hence amplifying the response. Conversely, when IL-1RA binds IL-1R1 it blocks its activation and thereby any downstream signaling.

2.2.2 IL-6

IL-6 is part of the IL-6-type cytokine family [34]. It has primarily a pro-inflammatory role in activating leukocytes, inducing production of acute phase inflammatory proteins, and promoting hematopoiesis. IL-6 binds to the IL-6 receptor (IL-6R) and then forms a complex with the transmembrane glycoprotein 130 (gp130) [39]. gp130 acts as a signal transducer.

2.2.3 IL-8

IL-8 is a chemoattractant belonging to the CXC chemokine family [34]. It recruits primarily neutrophils to promote inflammation and does so via chemotaxis, guiding neutrophil movement along the concentration gradient of the signal. IL-8 is produced in response to e.g., IL-1α and TNF-α signaling, and bind to the GTP-binding protein (G-protein) coupled receptors CXCR1 and CXCR2 [40].

2.2.4 TNF-α

TNF-α, part of the TNF family, has both pro- and anti-inflammatory effect [34]. It induces a potent inflammatory response, but also controls the extent and duration of that response. TNF-α further has a key role in the defense against pathogens and in promoting apoptosis. Elevated TNF-α levels are associated with the cardinal

symptoms of inflammation: heat, swelling, redness, pain, and loss of function. TNF-α is initially translated into transmembrane TNF-α and is subsequently cleaved into soluble TNF-α, hence the protein exists in both forms [41]. Secreted TNF-α then binds to the TNFR1 and TNFR2 receptors.

2.3 Skin Sampling

There exist several different skin sampling techniques, categorized as either invasive or non-invasive depending on whether the skin's surface is broken during the procedure or not. Skin punch biopsy is the most commonly used method, utilizing a sharp, circular blade to obtain a sample of all three layers of the skin [8]. Other invasive sampling techniques include collecting of blister fluid or scraping of the skin surface. Tape stripping has emerged as a non-invasive alternative to these methods, specifically sampling only the stratum corneum of the epidermis through the application and removal of adhesive tape on the skin [9]. It is a simple, efficient and minimally invasive technique, and is widely used in dermatology research to assess protein, RNA, lipid, and microbial expression. Furthermore, due to its minimal setup and training requirements, it has the potential to reach a larger population of patients unable or unwilling to travel for skin condition assessments [42].

The most commonly used tape for this application is D-Squame (Clinical and Derm, US), an adhesive disc that comes in various sizes, along with a pressure applicator and a device for measuring the tape transparency [43]. See Figure 3 for a demonstration of D-Squame application. Other non-invasive skin sampling methods include collecting of sebum from the skin surface using Sebutape (Clinical and Derm, US) [44], skin blotting with a hydrated nitrocellulose membrane [45], skin surface washing with PBS solution [7], and swabbing [11]. Swabbing involves gently rotating a swab with a cotton or nylon head, such as FLOQSwabs (Copan, Italy), back and forth along the skin surface.

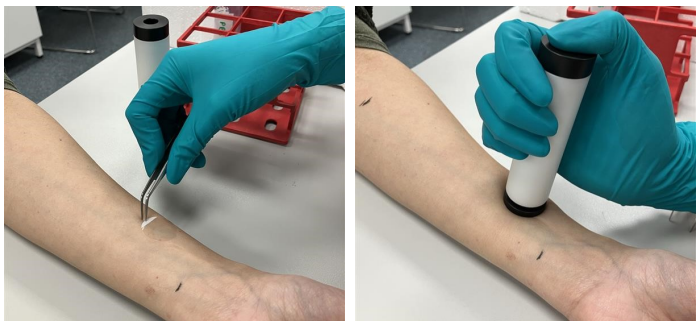

Figure 3: *D-Squame sampling with pressure application.*

This study included skin sample collection with D-Squame tape, FLOQSwabs, and a transparent medical tape from Essity's own assortment, Leukofix (Leukoplast, Germany). Images of the respective materials are presented in Figure 4. A visualization of Leukofix application and FLOQSwabs sampling is shown in Figure 5.

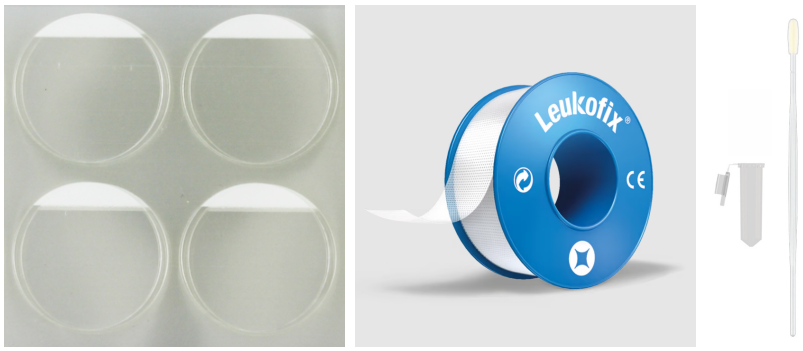

Figure 4: *From left to right; D-Square tape (Image adopted from: Clinical and Derm, URL: https://clinicalandderm.com/d100-d-square-standard-sampling-discs/, downloaded at 24-03-2025), Leukofix tape (Image adopted from: Leukoplast, URL: https://www.leukoplast.co.uk/products-professional-use/protecting-securing/fixing-securing/medical-tapes/leukofix, downloaded at 24-03-2025), and FLOQSwabs (Image adopted from: Copan, URL: https://www.copangroup.com/product-ranges/4n6flocswabs-genetics/, downloaded at 24-03-2025).*

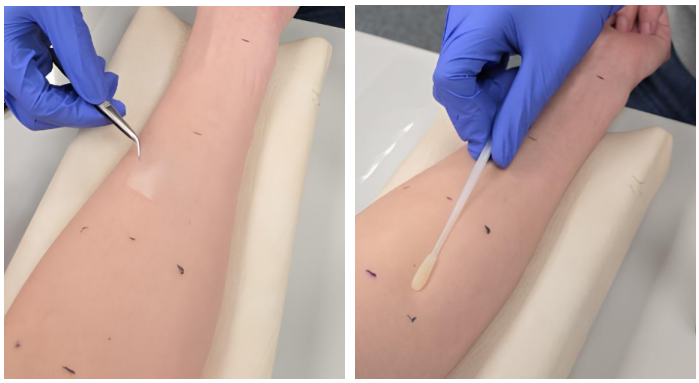

Figure 5: *Sampling with Leukofix tape (left) and FLOQSwabs (right).*

2.4 Cytokine Extraction and Analysis

To extract proteins from non-invasive skin sampling, an extraction buffer is employed. Several different buffer and detergent combinations for protein extraction have been reported in the literature. Morelli et al. (2021) [11] characterized the skin surface proteome in AD lesions, utilizing swab samples for their analysis. The authors report the use of an extraction solvent consisting of 50 mM Tris-HCl buffer with 4% (w/v) sodium dodecyl sulfate (SDS) and 6 M urea. In a different study, McAleer et al. (2019) [10] identified biomarkers of AD from tape stripping samples and used PBS with 0.005% (v/v) Tween 20 for cytokine extraction. Moreover, Kaleja et al. (2020) [12] performed a comparative analysis of different protein extraction solvents for tape stripping samples. They used 100 mM HEPES with protease inhibitor cocktail and (either): 0.1% RapiGest, 1%, 2% or 4% SDS, 6 M guanidine hydrochloride, or a combination of 1% SDS, 1% Triton X-100, 1% NP-40, 1% Tween 20, 1% deoxycholate, 50 mM NaCl, and 5 mM EDTA. The detergents that resulted in the highest protein detection were 1% SDS and the multidetergent mix. In another study, by

Jayabal et al. (2023) [13], the extraction of IL-1α, IL-6, IL-8, INF-γ, TNF-α, and IL-1RA from Sebutapes was optimized. The authors investigated the use of different detergents (Tween 20 and β-dodecyl maltoside) and detergent concentrations (0.025-0.1% and 0.075-0.15%, respectively), the extent of sample vortexing, sonication, and centrifugation, and the volume of PBS-buffer for enhancing the extraction efficiency. The optimized detergent concentrations were 0.05% (v/v) Tween 20 and 0.1% (w/v) β-dodecyl maltoside, where the use of β-dodecyl maltoside resulted in the highest overall extraction. However, PBS with 0.1% (w/v) β-dodecyl maltoside enhanced the extraction efficiency to a greater extent for the highly abundant cytokines IL-1α and IL-1RA, compared to the less abundant IL-6, IL-8, and TNF-α.

For then quantifying the extracted proteins, it is common to perform enzyme-linked immunosorbent assay (ELISA), mass spectrometry (MS), western blot, or immunostaining microscopy [9]. For this study, ELISA was employed as the analytical method. Therefore, the chosen detergents had to be non-denaturing of proteins. Although SDS is frequently mentioned in literature, it is primarily used for MS analysis, where it is not necessary for the protein to exist in its native form. SDS, being a anionic detergent, tends to denature proteins, making it less suitable for immunoassays [46]. Tween 20 is the most commonly used detergent for ELISA, typically paired with buffers of neutral or near-neutral pH, such as phosphate or Tris buffers [47]. In this project, PBS and Tris-HCl was used in combination with Tween 20, Triton X-100, and β-dodecyl maltoside, all of which are non-ionic detergents.

2.4.1 Buffers

Buffers are solutions composed of a conjugate acid-base pair, where either the acid or the base is of weak nature [48]. When a small amount of acid or base is added, a buffer can still maintain a stable pH.

2.4.1.1 PBS

Phosphate-buffered saline (PBS) is an isotonic buffer, meaning that it maintains the osmotic balance across cell membranes [49]. It is widely used as a diluent for biological samples. Dulbecco's PBS (DPBS) contains potassium chloride (KCl), potassium phosphate monobasic (KH_2PO_4), sodium chloride (NaCl), and sodium phosphate dibasic (Na_2HPO_4) [50]. pH ranges from 7.0 to 7.3 [51].

2.4.1.2 Tris-HCl

Tris hydrochloride (Tris-HCl) is a buffer created by dissolving tris(hydroxymethyl)-aminomethane ($C_4H_{11}NO_3$) in water and adding HCl to reach the desired pH level [52]. The chemical structure of Tris-HCl is presented in Figure 6. The buffer is commonly applied for controlling pH in various laboratory applications, including for electrophoresis and ELISA. In this project, Tris-HCl at pH 7.5 was used.

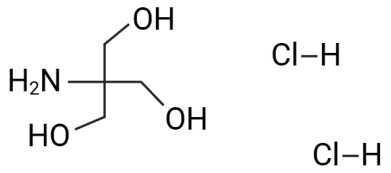

Figure 6: *Chemical structure of Tris-HCl.*

2.4.2 Detergents

To extract cellular protein content, cells are typically lysed through the addition of a detergent solution and treatment with either heat or sonication, which is then followed by centrifugation [53]. Detergents, also known as surfactants, are surface active agents that lower the surface tension of liquids [54]. This property makes them highly effective in cleaning products as well as in cell lysis buffers and in other laboratory applications. Detergents consist of a hydrophobic hydrocarbon tail and a hydrophilic head. They can be classified into four groups based on the nature of the head group: non-ionic, anionic, cationic, or zwitterionic (containing an equal amount of positively and negatively charged functional groups). Due to their amphiphilic structure, detergents can aggregate into spherical micelles in an aqueous solution, with the hydrophobic tails pointing inward and the hydrophilic heads facing outward towards the water.

The critical micellization concentration (CMC) is the lowest detergent concentration at which micelles form, specified at a certain temperature [54]. The formation of micelles facilitates the solubilization of membrane proteins by disrupting the lipid bilayer of cell membranes. Detergents insert themselves into the membrane to form mixed micelles with the membrane lipids. This leads to the breakdown of the membrane structure, resulting in cell lysis and releasing of cell contents, including of cytokines. The process ensures a complete cytokine recovery, which remain in a soluble form due to the presence of the detergent. For protein extraction where it is necessary to maintain the structure and function of the protein, including immunoassays, non-ionic and zwitterionic detergents are preferred as they are non-denaturing.

2.4.2.1 Tween 20

Tween 20, also under the name of polyoxyethylene sorbitan monolaurate ($C_{58}H_{114}O_{26}$), is a non-ionic detergent [55]. It consists of hydrophilic ethylene oxide subunits binding to a sorbitol ring and hydrophobic hydrocarbon chains, see Figure 7. Tween 20 is commonly applied for protein extraction prior to ELISA and western blot [54]. CMC at 25°C is 0.059 mM. With a density of 1.1 g/ml and a molar weight of 1228 g/mol, the lowest tested concentration of 0.005% (v/v) Tween 20 in this project is just below the CMC at 0.045 mM, whereas 0.05% corresponds to 0.45 mM (8 fold CMC) and 1% to 9 mM (153 fold CMC).

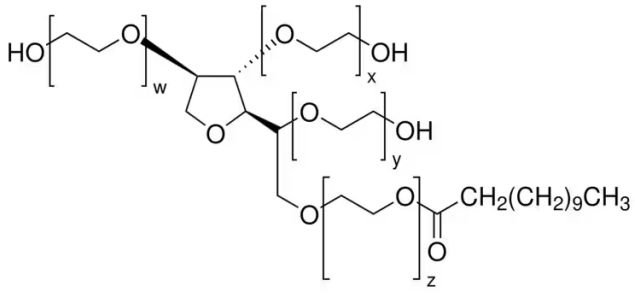

Figure 7: *Chemical structure of Tween 20. (Image adopted from: Merck, URL: https://www.sigmaaldrich.com/NL/en/product/sigma/p9416, downloaded at 12-03-2025)*

2.4.2.2 Triton X-100

Triton X-100, or polyethylene glycol *tert*-octylphenyl ether ($(C_2H_{40})_nC_{14}H_{220}$), is another non-ionic detergent commonly used in cell lysis buffers for performing various proteomic analyses [56]. The chemical structure is displayed in Figure 8. CMC at 25°C is between 0.17-0.3 mM [54]. With a density of 1.07 g/ml and a molar weight of 625 g/mol, the lowest tested concentration of 0.5% (v/v) Triton X-100 is above CMC at 8.6 mM, and 1% corresponds to 17 mM and 2% to 34 mM.

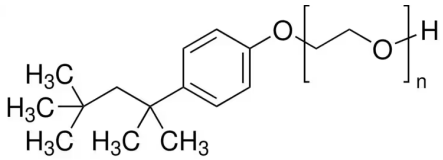

Figure 8: *Chemical structure of Triton X-100.*
(Image adopted from: Merck, URL: https://www.sigmaaldrich.com/NL/en/product/sial/x100?msockid=3db9ab53efd86aeb38f1be29eede6b1f, downloaded at 12-03-2025)

2.4.2.3 β-dodecyl maltoside

β-dodecyl maltoside, or n-dodecyl β-D maltoside (DDM), is a non-ionic detergent commonly applied for protein solubilization [57]. The chemical formula is $C_{24}H_{46}O_{11}$, and the structure is presented in Figure 9. CMC at 25°C is 0.15 mM [54]. With a molar weight of 511 g/mol, the lowest tested concentration of 0.1% w/v β-dodecyl maltoside is above CMC at 2 mM (13 fold CMC), and 0.5% corresponds to 10 mM (67 fold CMC) and 1% to 20 mM (133 fold CMC).

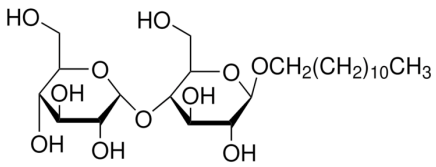

Figure 9: *Chemical structure of β-dodecyl maltoside.*
(Image adopted from: Merck, URL: https://www.sigmaaldrich.com/NL/en/product/sigma/d4641?msockid=3db9ab53efd86aeb38f1be29eede6b1f, downloaded at 21-03-2025)

2.4.3 ELISA for Cytokine Analysis

Proteins are inherently unstable and prone to degradation over time [53]. Factors such as improper temperature, pH, and choice of buffer can cause proteins to denature or aggregate. Despite the use of protease inhibitors to prevent protein breakdown, it is challenging to inhibit all proteolytic activity. Protein levels may even decrease throughout an experiment due to way of handling, necessitating sensitive analysis methods. Additionally, since the amount of protein in a sample cannot be amplified, it is crucial to recover as much protein as possible during extraction.

ELISA is a sensitive and highly selective technique that utilizes antibodies to detect specific proteins in various samples. Antibodies exhibit high specificity for their antigens, minimizing the risk of unwanted interactions [58]. The method offers significant advantages, including minimal sample preparation, rapid analysis, and cost-effectiveness, factors that are particularly important for diagnostic and research laboratories. ELISA is described as the gold standard for quantitative analysis of cytokines and was anticipated to provide reliable results in this study. However, cytokines that are normally present at very low concentrations, like TNF-α, IL-6, and IL-8, can be difficult to detect using ELISA [13], highlighting the need for this study. The working mechanism of ELISA is described in the Methods section.

3

Methods

During this degree project, a complete clinical study was conducted with two rounds of skin sampling from test participants. The aim of the first round of sample collection was to compare extraction solvents with varying chemical compositions for their ability to enhance cytokine detection and quantification. With the same basis of comparison, the aim of the second round of sample collection was to compare the performance of different collection methods. In this section, the methodology of sample collection, extraction, and analysis is explained further, and the extent of the literature research is defined. Figure 10 presents an overview of the project.

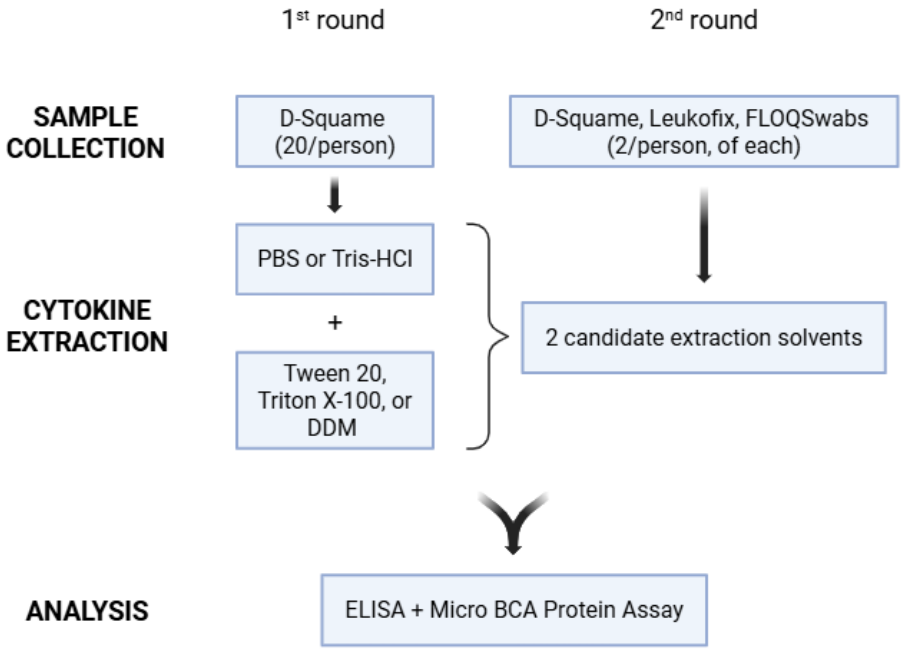

Figure 10: *Flowchart over the master's thesis project. (Image created in https://BioRender.com.)*

3.1 Literature Research

Literature research was conducted throughout the entire master's thesis project, especially in the initial phase of the project for writing of the introduction. Tape stripping (as well as other non-invasive sampling techniques) and skin biomarker studies were of primary focus, in order to gather background information on the topic and to determine which buffers, detergents, detergent concentrations, and cytokines that were of interest to study.

3.2 Sample Collection

During this clinical investigation, skin samples were taken from test participants by different types of adhesive tapes and swab. The study population consisted of healthy women and men, above the age of 18. The study however excluded individuals with active skin diseases or damage to the skin barrier, individuals with a known sensitivity to adhesives or nylon, and individuals with hairy forearms. Before any procedure was initiated, the participants were informed in writing and orally about the course of the study and signed an informed consent form.

Nineteen participants (n = 19; 17 females, 2 males; median age at 54, ranging between 18-79 years) were recruited for two rounds of sample collection, set approximately one month apart. In the first round, sampling was performed using solely D-Squame tape. For each participant, 10 samples were taken per volar forearm. In the second round, sampling was performed using D-Squame tape, Leukofix tape, and FLOQSwabs. For each participant, 3 samples were taken from different spots on each volar forearm, that is one sample for each collection method. After D-Squame tape sampling, the tape transparency was measured using a densitometer. All samples were then stored in a freezer until extraction and analysis.

The areas for sample collection are further defined by Figure 11. Sampling was performed 2-3 cm from the wrist and arm fold. Randomization of sample analysis could be ensured due to the fact that a certain position on the arm had a specific numbering. In the second round of sample collection, the sampling area for D-Squame, Leukofix, and FLOQSwabs - each covering one-third of the total area - was continuously rotated among participants, as demonstrated in Table 1. This approach further contributed to sample randomization.

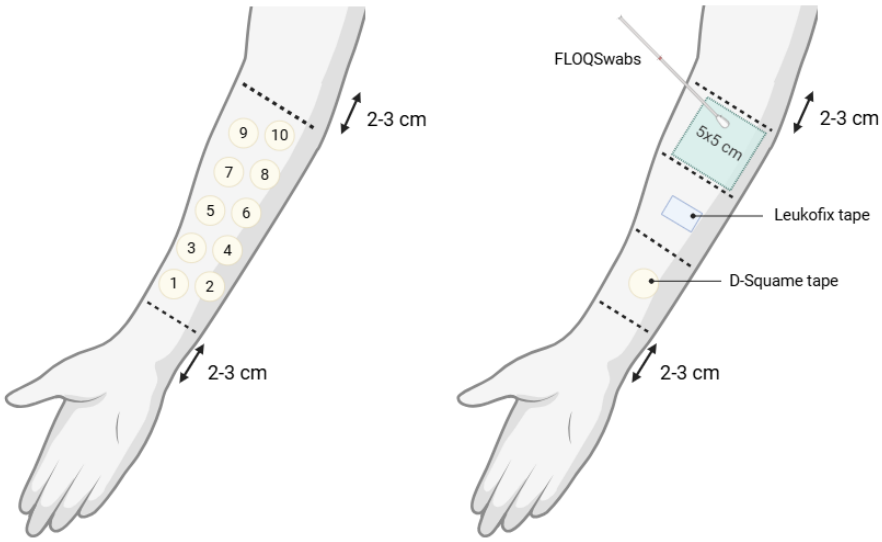

Figure 11: *Placement of D-Squame tapes in the first round of sample collection (left), and areas for sampling with D-Squame tape, Leukofix tape, and FLOQSwabs in the second round of sample collection (right). (Image created in https://BioRender.com.)*

Table 1: *Rotation of D-Squame, Leukofix, and FLOQSwabs sampling area on the volar forearm*

Test participant	$\frac{1}{3}^{rd}$ **closest to wrist**	$\frac{1}{3}^{rd}$ **in the middle**	$\frac{1}{3}^{rd}$ **closest to elbow**
1	D-Squame	Leukofix	FLOQSwabs
2	Leukofix	FLOQSwabs	D-Squame
3	FLOQSwabs	D-Squame	Leukofix
4	D-Squame	Leukofix	FLOQSwabs
Continued	...	...	...

3.2.1 Sampling with D-Squame Tape

D-Squame tapes were used in the first and second round of sample collection. The sample collection protocol is presented in Appendix A.1. The tapes were applied to the volar forearms using a pressure device and rotated twice in the same spot. A densitometer (SquameScan; Clinical and Derm, US) was used to measure the optical absorption of the tapes at 850 nm (infrared light), in order to estimate the amount of skin material collected. The output was a proportion of light absorbed by the collected tape relative to a reference tape strip, and this value can be converted into protein amount using the equation y=a·x-b [59]. The tapes were then placed in low protein binding tubes and stored in a freezer until extraction and analysis.

3.2.2 Sampling with Leukofix Tape

Leukofix tapes were used in the second round of sample collection. The sample collection protocol is presented in Appendix A.2. The 2.5×3 cm sized tape strips were applied to the volar forearms for one minute. The tapes were then removed and placed in low protein binding tubes to be stored in a freezer until extraction and analysis.

3.2.3 Sampling with FLOQSwabs

FLOQSwabs was used in the second round of sample collection. The sample collection protocol is presented in Appendix A.3. A 5×5 cm area was marked on each volar forearm. The swabs were immersed briefly in PBS buffer before swabbing back and forth over the defined area. The swab tips were cut off and placed in low protein binding tubes to be stored in a freezer until extraction and analysis.

3.3 Sample Extraction and Analysis

The collected samples were submerged in extraction solvent, sonicated, and centrifuged. See Appendix B for details of the respective extraction protocols. For cytokine extraction of the first round of samples, two types of buffers (PBS and Tris-HCl) and three detergents (Tween 20, Triton X-100, and β-dodecyl maltoside) at three different concentrations were evaluated and compared to the current standard extraction solvent at Essity. Tween 20 was tested at 0.005, 0.05 and 1% v/v, Triton X-100 at 0.5, 1 and 2% v/v, and β-dodecyl maltoside at 0.1, 0.5, and 1% w/v, as decided by literature research. Essity's current extraction solvent consists of PBS with 0.005% Tween 20. The top two candidate solvents, that allowed for cytokine detection at higher sensitivity and consistency, were then used for analyte extraction of the second round of samples.

Analysis of the samples, i.e. detection and quantification of cytokines, was conducted using enzyme-linked immunosorbent assay (ELISA) and a protein assay for measuring the total soluble protein concentration. Given the constraints on time and resources, technical replicates were not performed. For sample collection with D-Squame tape, the correlation between the total soluble protein concentration and the value from the densitometer was calculated. The statistical significance of the different extraction solvents and sample collection methods for analyte detection was determined using ANOVA. Thereafter, a recommendation of extraction solvent and collection method for each specific analyte could be made.

3.3.1 ELISA

Enzyme-linked immunosorbent assay, or ELISA, is an enzymatic immunoassay that utilizes enzymes to detect antibody-antigen binding [58]. This study implemented a variant of sandwich ELISA for the quantification of the following cytokines: IL-1α, IL-1RA, IL-6, IL-8, and TNF-α. In sandwich ELISA, the antigen (cytokine)

is captured between two antibodies, a capture antibody and a detector antibody, allowing for very high sensitivity.

Abcam's SimpleStep ELISA Kits [60] were used, see protocol in Appendix C.1. Each kit includes a 96-well microplate precoated with an anti-tag antibody, with affinity for the capture antibody. Samples and an antibody cocktail, containing both the capture and detector antibodies, are added to the plate. The detector antibody is enzyme-conjugated to horseradish peroxidase (HRP). After incubation, unbound antibodies are washed away, and the substrate 3,3',5,5' tetramethylbenzidine (TMB) is added. The enzyme converts this colorless substrate into a blue product, which can be measured at 600 nm using a microplate reader. Thereafter, sulfuric acid (H_2SO_4) is added as a stop solution, changing the solution from blue to yellow. The color signal can then be measured at 450 nm with a microplate reader. The color intensity is proportional to the amount of cytokine in the sample.

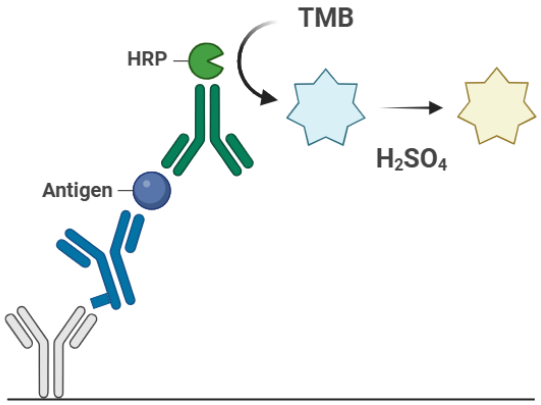

Figure 12: *Mechanism of Abcam's SimpleStep ELISA Kits, displaying the anti-tag antibody in grey, capture antibody in blue, and detector antibody in green. (Image created in https://BioRender.com.)*

3.3.2 Micro BCA Protein Assay

Thermo Fisher's Micro BCA Protein Assay Kit [61] was used to measure the total soluble protein concentration of the samples, see protocol in Appendix C.2. The assay is based on the reduction of Cu^{2+} to Cu^{+} by the presence of protein in an alkaline solution. Two molecules of bicinchoninic acid (BCA) then forms a purple-colored chelation complex with Cu^{+}, which exhibits absorbance at 562 nm. The absorbance is directly proportional to the protein amount.

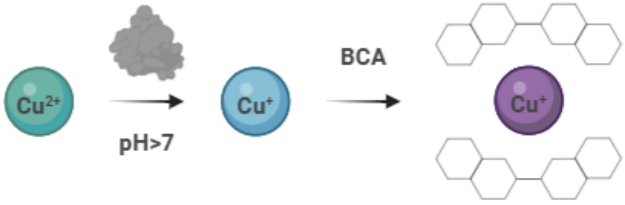

Figure 13: *Mechanism of Thermo Fisher's Micro BCA Protein Assay. (Image created in https://BioRender.com.)*

3.3.3 Statistics

In this study, ANOVA was applied to the obtained cytokine recoveries to determine the effect of using extraction solvents with varying chemical composition as well as using the different collection methods. The analysis was executed in the statistical software GraphPad Prism 10.

Analysis of variance (ANOVA) is a statistical method used for determining the differences in mean variance between and within three or more data groups [62]. Ordinary ANOVA assumes that the data is parametric, i.e. normally distributed within each group and of equal variance across groups. ANOVA implements the 'F statistic' which is calculated as the ratio of the variance between groups and the variance within groups, a signal-to-noise ratio. If the F statistic is high, there is a significant difference between the groups and the null hypothesis (being there is no difference in the group means) can be rejected. However, ANOVA does not tell which groups (if any) that are different from each other, for this a multiple comparison test or "post hoc test" is employed. The resulting p-values are then adjusted to reduce the occurrence of Type I errors (false positives), using multiple testing correction. As the number of comparisons increases, so does the probability of incorrectly rejecting the null hypothesis, known as the family-wise error rate (FWER).

The Kruskal–Wallis H-Test for one-way ANOVA is a non-parametric test that serves as an alternative to the ordinary one-way ANOVA, comparing group ranks instead of group means [63]. It was employed in this study to determine significances based on a single factor, using a threshold of $p<0.05$. Following the Kruskal-Wallis test, a Dunn's multiple comparison test was conducted. Dunn's test, also non-parametric, ranks all data and performs pairwise comparisons of the mean ranks [64]. The p-values were then adjusted to control the FWER at α=0.05.

A two-way ANOVA is conducted to assess the significances between groups based on two factors and to examine the interaction between these factors. This parametric test was implemented together with Tukey's multiple comparison test. Tukey's test identifies which groups are significantly different through pairwise comparisons of the means [65], controlling the FWER at α=0.05.

To determine if there was a significant difference between two groups, the Mann-Whitney U test was used. This non-parametric test, also known as the Wilcoxon rank sum test, was conducted two-tailed with a threshold for statistical significance set at $p<0.05$. Unlike the Student's t-test, the Mann-Whitney U test does not compare the means of the groups but rather compares their ranks to determine if one group has higher or lower values than the other [66].

4

Results

All samples were analyzed using ELISA and the Micro BCA Protein Assay in a microplate reader. For each assay, a standard curve (signal versus concentration) was plotted, allowing sample concentrations to be calculated from the linear equation. Cytokine concentrations (pg/ml) were then normalized to total soluble protein concentrations from the BCA assay (μg/ml) in order to adjust for the variable amount of stratum corneum removed during collection. This yielded an estimate of cytokine amount in pg/μg soluble protein.

Since the majority of the data did not follow a Gaussian distribution, non-parametric statistical testing was applied. The Kruskal-Wallis one-way ANOVA with Dunn's multiple comparisons test was used on data from the first round to compare the performance between every extraction solvent and between the detergents. For the second round, it was used to compare between each combination of collection method and extraction solvent, as well as between the collection methods alone. For then assessing the significance of the two buffers and the two extraction solvents, used in the first and second round respectively, the Mann-Whitney U-test was applied. Additionally, two-way ANOVAs with Tukey's multiple comparisons test (parametric testing) was conducted to further evaluate the significance of the buffers versus detergents, and the collection methods versus extraction solvents, and to identify any interaction between them.

The notations used for significance in the following graphs are according to GraphPad software and represent these thresholds: ns >0.1234, * <0.0332, ** <0.0021, *** <0.0002, and **** <0.0001.

4.1 Optimization of Extraction Solvent

The samples from the initial collection were extracted using the 18 different combinations of buffers and detergents. For IL-6 and IL-8, all signals were below the detection limit, so no conclusions could be drawn from those data. TNF-α also had very low signal output, but some general trends were still observable. The selection of the best extraction solvents to continue with for the next round of samples was therefore based primarily on data from IL-1α and IL-1RA, and to some extent TNFa. The obtained data is presented in the following sections.

4.1.1 IL-1α Recovery

Figure 14 displays IL-1α recovery by use of the different extraction solvents. Individual participant data points are shown as dots. The bars represent the mean values, with error bars indicating the standard deviation. The PBS buffer is represented in orange, while Tris-HCl is shown in blue. The concentrations of the different detergents are indicated by varying intensities of color. This format will be consistent across the following graphs. The value corresponding to Essity's current extraction solvent (0.005% Tween 20 in PBS) is indicated by a gridline and labeled as 'REF'.

The figure illustrates a general trend where Tris-HCl outperforms PBS, with Triton X-100 and β-dodecyl maltoside yielding a higher IL-1α recovery than Tween 20. Interestingly, for Tween 20, the highest IL-1α recovery is observed at 0.005% concentration, followed by 0.05%, and then 1%. This pattern is consistent with the trends seen for IL-1RA and TNF-α. In contrast, for the other two detergents, varying concentrations do not show differences in cytokine recovery.

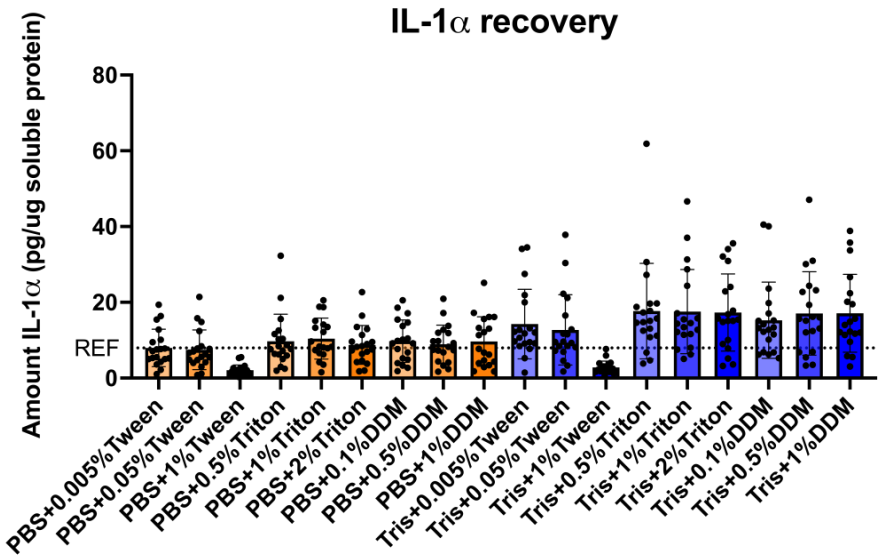

Figure 14: *IL-1α recovery over extraction solvent.*

Subsequently, the normalized cytokine amounts were divided with the reference value for each participant, resulting in a ratio. This approach simplifies the comparison of different extraction solvents against the reference. It reduces data variability by accounting for individual variability in protein expression. The obtained values are presented in Figure 15. Values above 1 indicate that the extraction solvent performed better than the current standard.

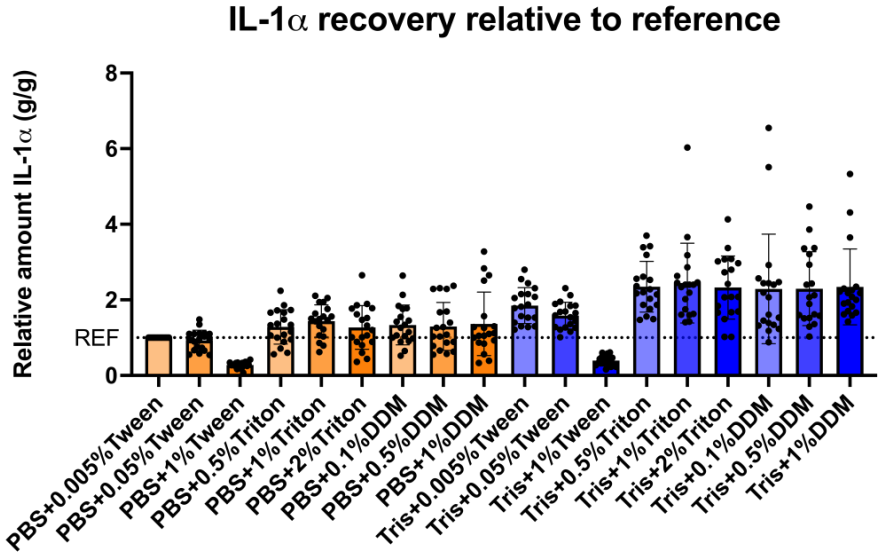

Figure 15: *Relative IL-1α recovery over extraction solvent.*

Significant comparisons between extraction solvents for both real (pg/μg) and relative (g/g) amounts of IL-1α are presented in Appendix, Table D.1. To highlight some findings, PBS in combination with 1% Tween 20 resulted in significantly lower IL-1α recovery than all combinations of Tris-HCl, except for 1% Tween 20. Additionally, PBS with 1% Tween 20 was significantly less effective than all combinations of PBS with Triton X-100 and β-dodecyl maltoside. Tris-HCl in combination with 1% Tween 20 was significantly lower than all combinations of Tris-HCl with Triton X-100 and β-dodecyl maltoside. When comparing the same detergent for the two different buffers, only 2 out of 9 combinations were significantly different. The reference extraction solvent (PBS with 0.005% Tween 20) was significantly less effective compared to Tris-HCl with all Triton X-100 and β-dodecyl maltoside concentrations, as well as to Tris-HCl with 0.005% Tween 20 (considering relative values).

To determine the two extraction solvents that performed best across all participants, the solvents were ranked based on the ratio relative to the reference. The ranks for each solvent were summed to create an aggregated rank. A lower aggregated rank indicates a higher overall performance, as the solvent then consistently ranked among the best for many participants. These results were compared with the frequency with which the respective extraction solvents performed best, second best, or third best for all participants combined, see Table 2.

Table 2: *Extraction solvents ranked and counted based on number of times performed best, second, and third place, for IL-1α*

Extraction solvent	Aggregated rank	Overall rank	Best	2^{nd} best	3^{rd} best
PBS+0.005%Tween	259	15	0	0	0
PBS+0.05%Tween	266	16	0	0	0
PBS+1%Tween	338	18	0	0	0
PBS+0.5%Triton	214	14	0	0	0
PBS+1%Triton	188	9	0	0	1
PBS+2%Triton	213	12	0	0	0
PBS+0.1%DDM	205	10	0	0	0
PBS+0.5%DDM	213	12	0	0	0
PBS+1%DDM	210	11	0	0	2
Tris+0.005%Tween	126	7	0	2	2
Tris+0.05%Tween	168	8	0	0	0
Tris+1%Tween	324	17	0	0	0
Tris+0.5%Triton	73	1	3	2	5
Tris+1%Triton	84	4	4	3	3
Tris+2%Triton	83	2	3	2	2
Tris+0.1%DDM	114	6	5	0	0
Tris+0.5%DDM	83	2	3	5	1
Tris+1%DDM	88	5	1	5	3

As can be seen in the table, the use of Tris-HCl over PBS generally results in a better overall ranking. Tris-HCl with 0.5% Triton X-100 was ranked as best, and Tris-HCl with 2% Triton X-100 and 0.5% β-dodecyl maltoside was ranked as second best. This trend is consistent when considering the number of times the respective extraction solvents performed the best, second best, or third best.

Figure 16 presents the data divided according to the use of different buffers and detergents. Significances are included in both graphs, based on adjusted p-value.

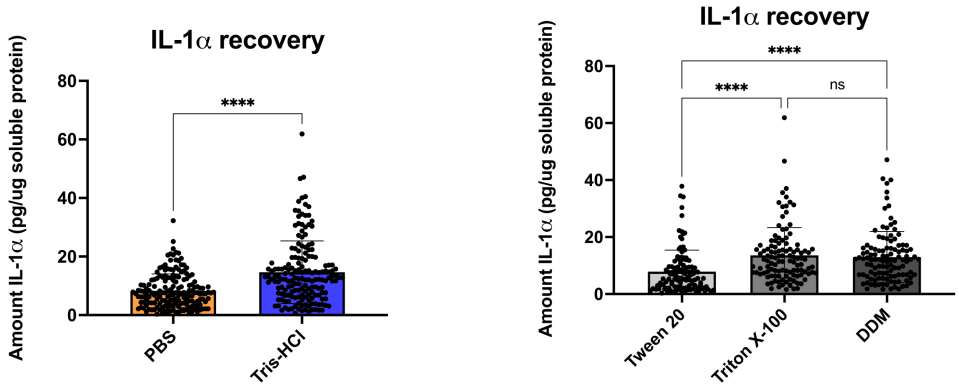

Figure 16: *IL-1α recovery over different (a) buffers (t-test) and (b) detergents (one-way ANOVA).*

Tris-HCl significantly enhanced the recovery of IL-1α compared to PBS. There is also a significantly improved effect for both Triton X-100 and β-dodecyl maltoside compared to Tween 20, but no significant difference between Triton X-100 and β-dodecyl maltoside.

In Figure 17, the effects of buffers and detergents are further investigated using a two-way ANOVA, instead of by two non-parametric tests. This approach reveals that the previously described relationship between Tween 20, Triton X-100, and β-dodecyl maltoside is still valid for Tris-HCl, but not for PBS, where no significant differences are detected. Additionally, there is a significant improvement for the use of Tris-HCl with Triton X-100 and β-dodecyl maltoside compared to all PBS and detergent combinations.

The two-way ANOVA assumes normally distributed data, which is not the case for this dataset, potentially affecting the accuracy of the significance levels. However, the main purpose of using two-way ANOVA is to identify interactions between the two factors; buffers and detergents. The analysis concludes that there is no significant interaction between the effects of buffers and detergents, consistent with the subsequent findings for IL-1RA and TNF-α.

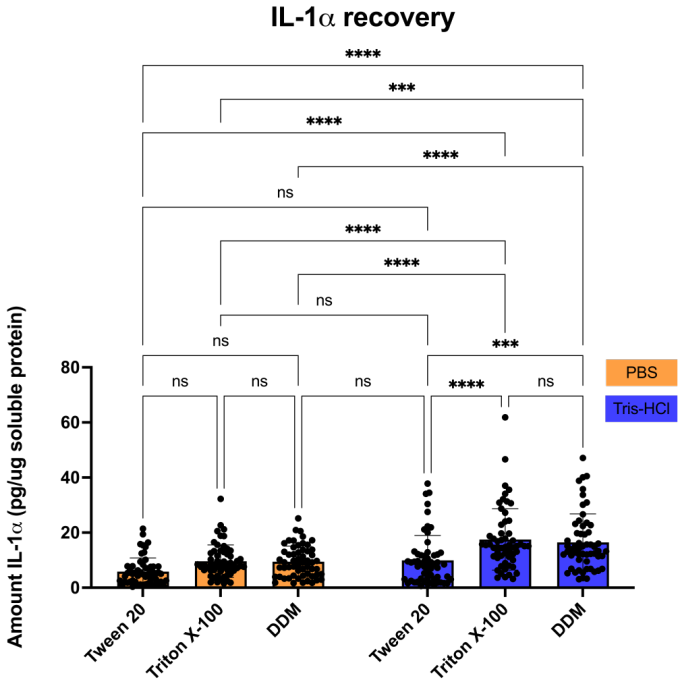

Figure 17: *IL-1α recovery over buffer and detergent (two-way ANOVA).*

4.1.2 IL-1RA Recovery

In Figure 18 and 19 the recovery of IL-RA is presented. Similar to the results for IL-1α, Tris-HCl proves to be the most effective buffer, with Triton X-100 and β-dodecyl maltoside generally outperforming Tween 20. The significant comparisons between extraction solvents are presented in Appendix, Table D.2. As was the case for IL-1α, PBS with 1% Tween 20 performed significantly worse compared to all combinations of Tris-HCl, except for 1% Tween 20, and Tris-HCl with 1% Tween 20 was significantly less effective than Tris-HCl with all concentrations of Triton X-100 and β-dodecyl maltoside. This time, 4 out of 9 comparisons involving the same detergent but different use of buffer were significant. The reference extraction solvent had a significantly reduced performance compared to Tris-HCl with all concentrations of Triton X-100 and β-dodecyl maltoside, as well as to Tris-HCl with 0.005% Tween 20 (considering relative values), same as for IL-1α.

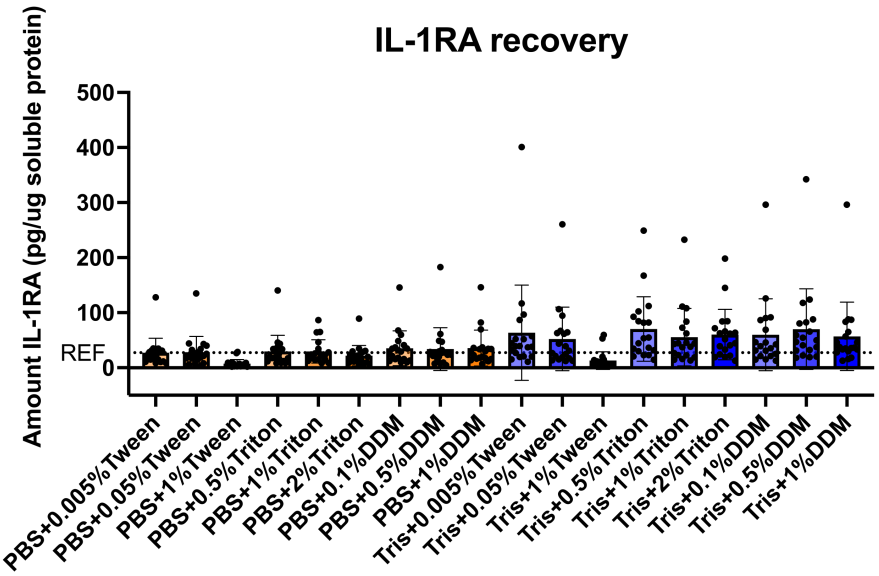

Figure 18: *IL-1RA recovery over extraction solvent.*

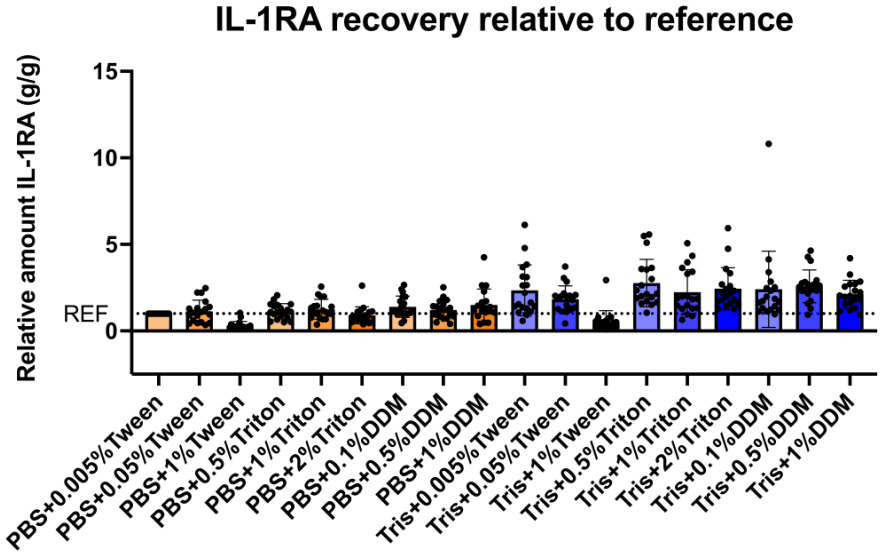

Figure 19: *Relative IL-1RA recovery over extraction solvent.*

The ranks of the extraction solvents based on their relative IL-1RA recovery is displayed in Table 3. The two best extraction solvents were Tris-HCl with 0.5% Triton X-100 and 0.5% β-dodecyl maltoside.

Table 3: *Extraction solvents ranked and counted based on number of times performed best, second, and third place, for IL-1RA*

Extraction solvent	**Aggregated rank**	**Overall rank**	**Best**	**2^{nd} best**	**3^{rd} best**
PBS+0.005%Tween	242	15	0	0	0
PBS+0.05%Tween	240	14	0	0	1
PBS+1%Tween	333	18	0	0	0
PBS+0.5%Triton	221	12	0	0	0
PBS+1%Triton	207	11	0	0	0
PBS+2%Triton	270	16	0	0	0
PBS+0.1%DDM	187	10	0	0	1
PBS+0.5%DDM	222	13	0	0	0
PBS+1%DDM	184	9	0	0	0
Tris+0.005%Tween	111	5	4	2	0
Tris+0.05%Tween	137	8	1	2	0
Tris+1%Tween	311	17	0	1	0
Tris+0.5%Triton	72	1	5	2	4
Tris+1%Triton	128	7	0	3	2
Tris+2%Triton	86	3	1	5	3
Tris+0.1%DDM	116	6	2	0	5
Tris+0.5%DDM	74	2	3	3	2
Tris+1%DDM	108	4	3	1	1

As presented in Figure 20, there is a significant difference between the two buffers for IL-1RA recovery, and Triton X-100 and β-dodecyl maltoside enhance the recovery compared to Tween 20. Specifically, Tris-HCl combined with Triton X-100 or β-dodecyl maltoside is significantly better than all PBS solutions, which is demonstrated by the two-way ANOVA in Figure 21. However, the two-way ANOVA reveals no significant differences between the detergents when using the same buffer, contrary to the findings of the one-way ANOVA in Figure 20b.

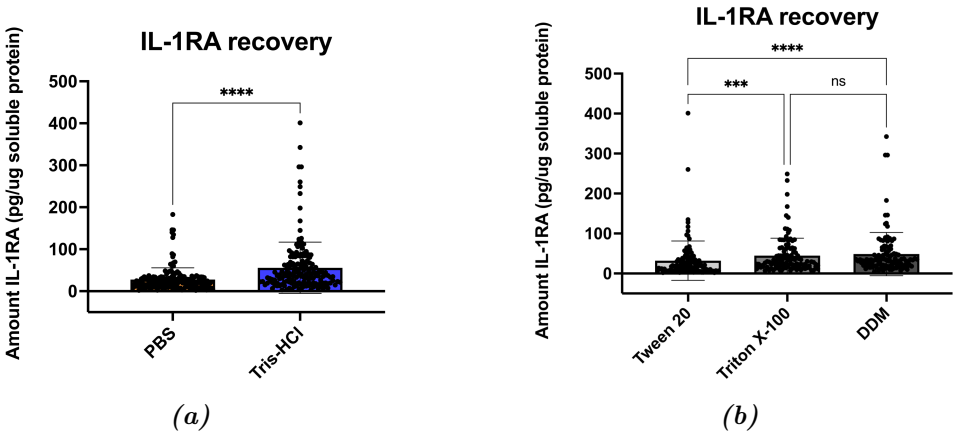

Figure 20: *IL-1RA recovery over different (a) buffers (t-test) and (b) detergents (one-way ANOVA).*

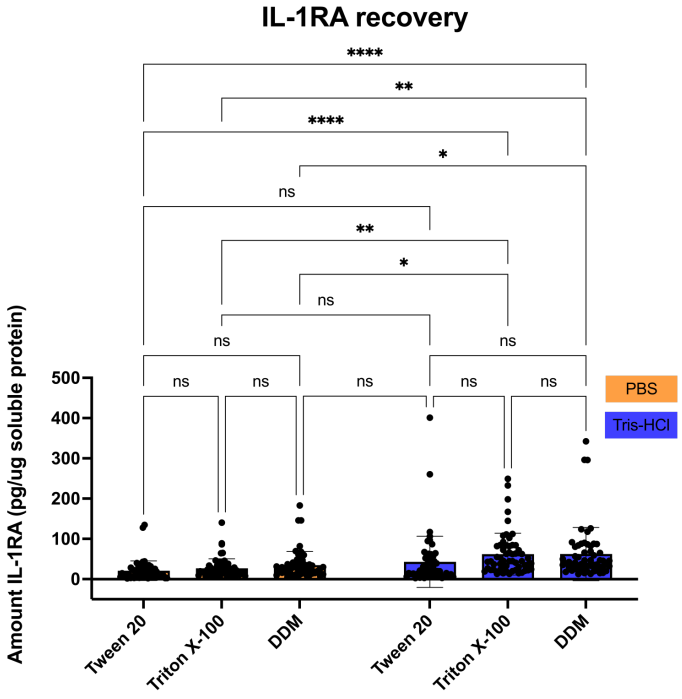

Figure 21: *IL-1RA recovery over buffer and detergent (two-way ANOVA).*

4.1.3 TNF-α Recovery

TNF-α levels were detectable in 14 out of 19 participants (73.4 %), and the data is presented in Figure 22 and 23. Consistent with the results for IL-1α and IL-1RA, there is a clear trend indicating that Tris-HCl facilitates higher TNF-α recovery compared to PBS. However, unlike for IL-1α and IL-1RA, the two lowest concentrations of Tween 20 appear superior to Triton X-100 and β-dodecyl maltoside. The multiple comparisons are found in Appendix, Table D.3. PBS with 1% Tween 20 yielded significantly lower TNF-α recovery compared to all combinations of Tris-HCl, except for 1% Tween 20. However, Tris-HCl with 0.005% and 0.05% Tween 20 were significantly better than all combinations of PBS with Triton X-100 and β-dodecyl maltoside, as well as than Tris-HCl with 1% Tween 20. For TNF-α, 6 out of 9 extraction solvents were significantly different when comparing the same detergent for the two different buffers (considering relative values).

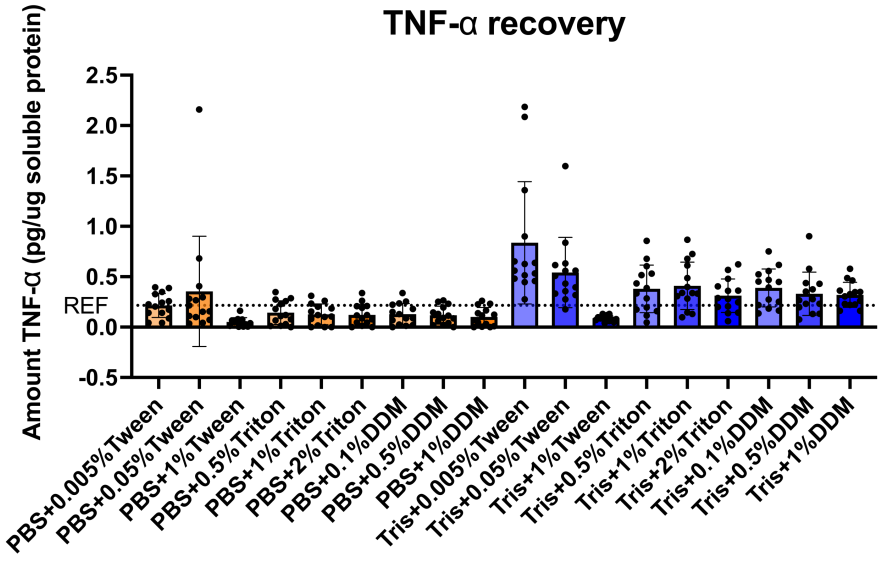

Figure 22: *TNF-α recovery over extraction solvent.*

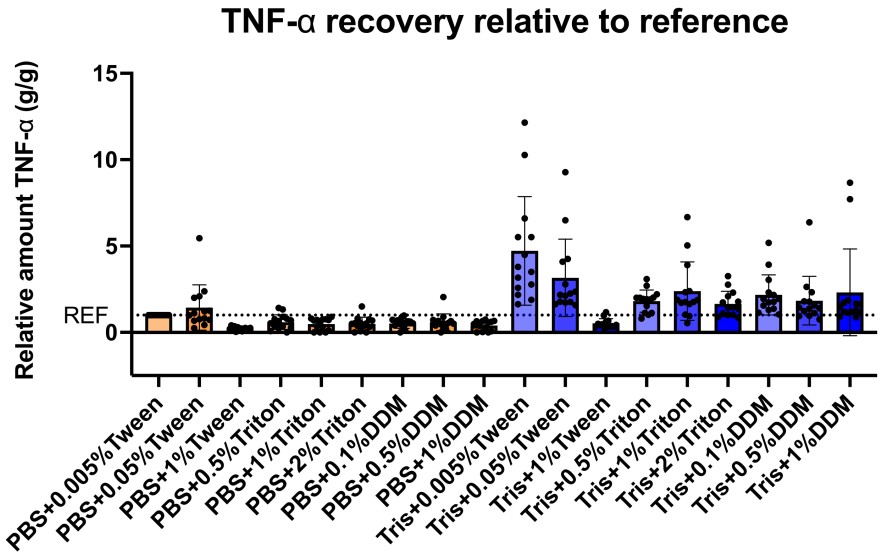

Figure 23: *Relative TNF-α recovery over extraction solvent.*

The ranking of the extraction solvents is presented in Table 4. The results support the previous remark that Tris-HCl is the superior buffer choice. Additionally, Tris-HCl with 0.005% and 0.05% Tween 20 are identified as the best and second-best extraction solvents, respectively.

Table 4: *Extraction solvents ranked and counted based on number of times performed best, second, and third place, for TNF-α*

Extraction solvent	**Aggregated rank**	**Overall rank**	**Best**	**2^{nd} best**	**3^{rd} best**
PBS+0.005%Tween	128	10	0	0	0
PBS+0.05%Tween	122	9	1	1	2
PBS+1%Tween	237	18	0	0	0
PBS+0.5%Triton	178	11	0	0	0
PBS+1%Triton	191	14	0	0	0
PBS+2%Triton	192	15	0	0	0
PBS+0.1%DDM	184	12	0	0	0
PBS+0.5%DDM	189	13	0	0	0
PBS+1%DDM	212	17	0	0	0
Tris+0.005%Tween	20	1	11	1	1
Tris+0.05%Tween	46	2	1	5	3
Tris+1%Tween	202	16	0	0	0
Tris+0.5%Triton	73	4	0	1	1
Tris+1%Triton	74	5	0	1	3
Tris+2%Triton	92	8	0	1	0
Tris+0.1%DDM	67	3	0	1	3
Tris+0.5%DDM	90	7	0	2	1
Tris+1%DDM	88	6	1	1	0

Figures 24 and 25 once again demonstrate an improved TNF-α recovery for Tris-HCl compared to PBS, at least when comparing the same detergent across different buffers. Although, neither data analysis can definitively identify differences between the detergents. The large standard deviation for using Tween 20 may contribute to this outcome.

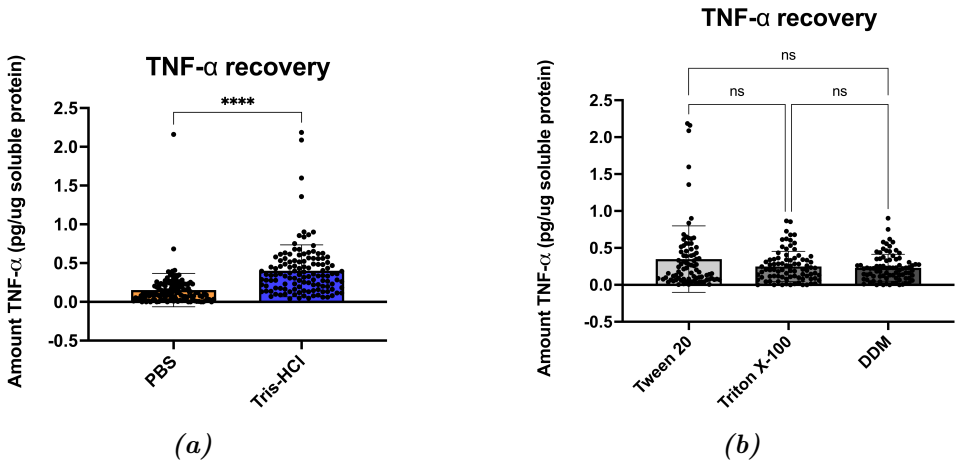

Figure 24: *TNF-α recovery over different (a) buffers (t-test) and (b) detergents (one-way ANOVA).*

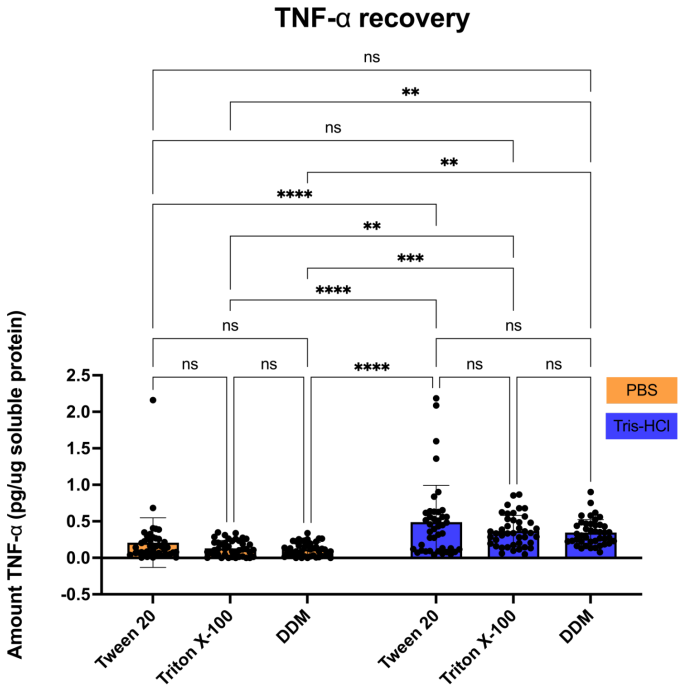

Figure 25: *TNF-α recovery over buffer and detergent (two-way ANOVA).*

4.2 Optimization of Collection Method

For the second round of sample extractions, Tris-HCl was used with 0.5% Triton X-100 and 0.5% β -dodecyl maltoside, as these two extraction solvents ranked the overall highest for IL-1α and IL-1RA recovery in the first round. IL-6 remained undetectable for all participants, but IL-8 could be detected for some, and TNF-α for more participants than the previous round.

4.2.1 IL-1α Recovery

The IL-1α recovery is presented in Figure 26, as well as in Figure 27 with the data separated according to collection method and extraction solvent, and in Figure 28 analyzing the two factors simultaneously. In these figures, D-Squame is represented in green, Leukofix in red, and FLOQSwabs in purple, with lower color intensity for Triton X-100 and higher intensity for β-dodecyl maltoside. Leukofix performed significantly worse than both D-Squame and FLOQSwabs. However, no significant differences were found between the extraction solvents (0.5% Triton X-100 and 0.5% β-dodecyl maltoside) when using the same collection method. Additionally, the two-way ANOVA indicates no significant interaction between collection method and extraction solvent, which remains true also for IL-1RA, TNF-α, and IL-8.

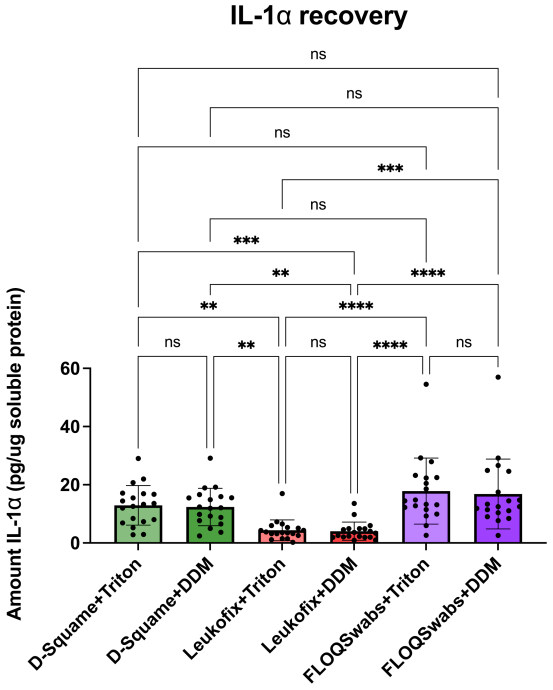

Figure 26: *IL-1α recovery over collection method and extraction solvent (one-way ANOVA).*

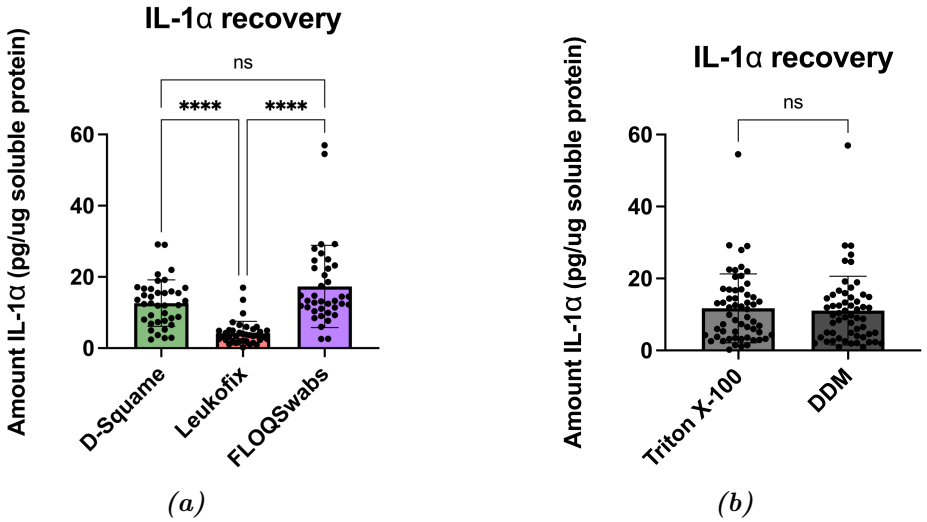

Figure 27: *IL-1α recovery over different (a) collection methods (one-way ANOVA) and (b) extraction solvents (t-test).*

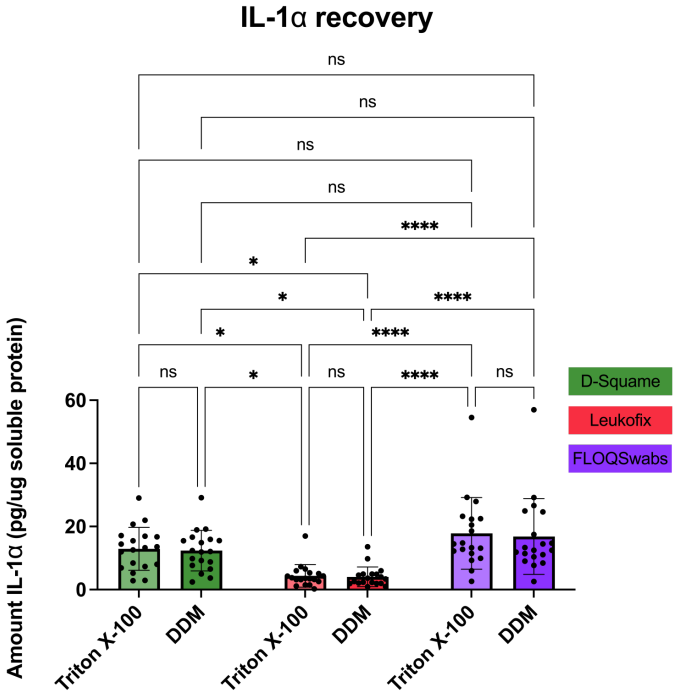

Figure 28: *IL-1α recovery over collection method and extraction solvent (two-way ANOVA).*

4.2.2 IL-1RA Recovery

The recovery of IL-1RA is presented in Figure 29, 30, and 31. The results from the non-parametric tests (t-test and one-way ANOVAs) indicate primarily that FLOQSwabs significantly improve IL-1RA recovery compared to Leukofix, while the choice of extraction solvents does not have a significant impact. However, when analyzing the data with a two-way ANOVA, no significant effects of either the collection method or extraction solvent were observed.

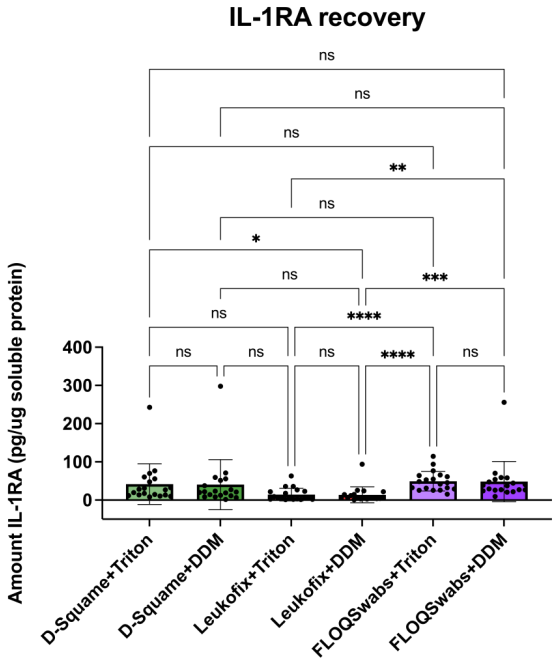

Figure 29: *IL-1RA recovery over collection method and extraction solvent (one-way ANOVA).*

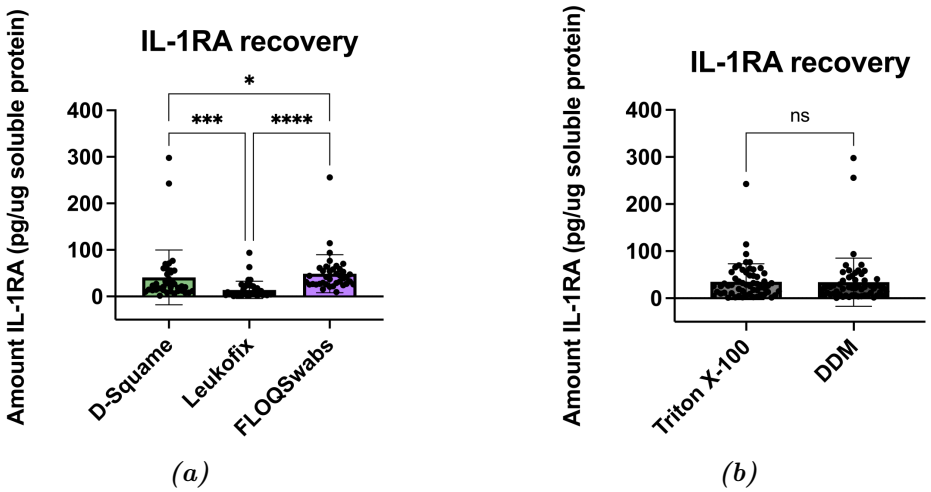

Figure 30: *IL-1RA recovery over different (a) collection methods (one-way ANOVA) and (b) extraction solvents (t-test).*

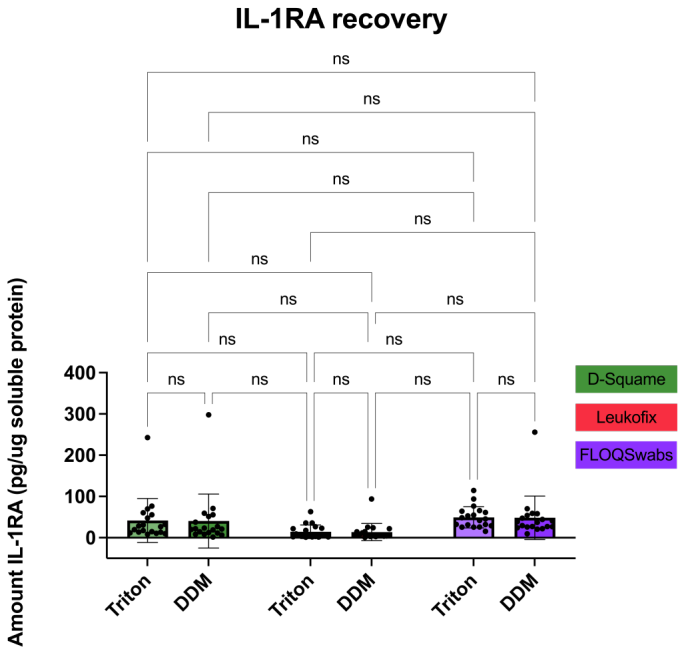

Figure 31: *IL-1RA recovery over collection method and extraction solvent (two-way ANOVA).*

4.2.3 TNF-α Recovery

For the second round of sample collection, TNF-α was detectable in 18 out of 19 participants (94.7%). The recovery of TNF-α is presented in Figure 32, 33, and 34. The one-way ANOVA results generally conclude that both Leukofix and D-Squame outperform FLOQSwabs, which contrasts the findings for IL-α and IL-1RA, where FLOQSwabs performed best. Nevertheless, the extraction solvents showed no significant differences, consistent with the results for IL-α and IL-1RA. However, the two-way ANOVA reveals that all but two comparisons are non-significant (Leukofix with Triton X-100 compared to FLOQSwabs with Triton X-100 and to FLOQSwabs with β-dodecyl maltoside, respectively)

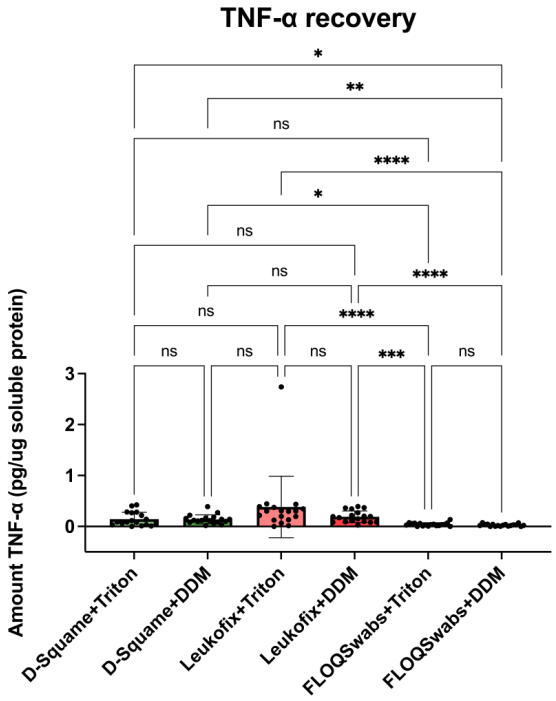

Figure 32: *TNF-α recovery over collection method and extraction solvent (one-way ANOVA).*

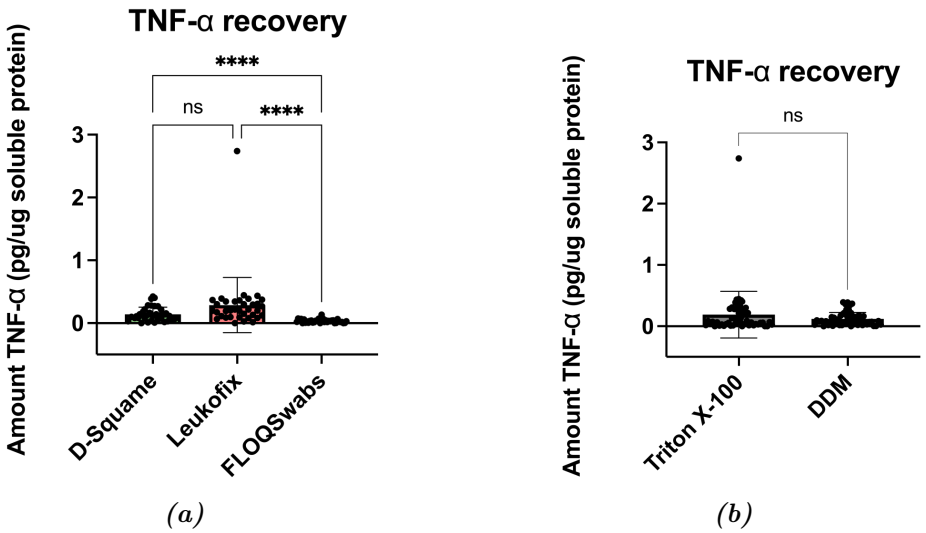

Figure 33: *TNF-α recovery over different (a) collection methods (one-way ANOVA) and (b) extraction solvents (t-test).*

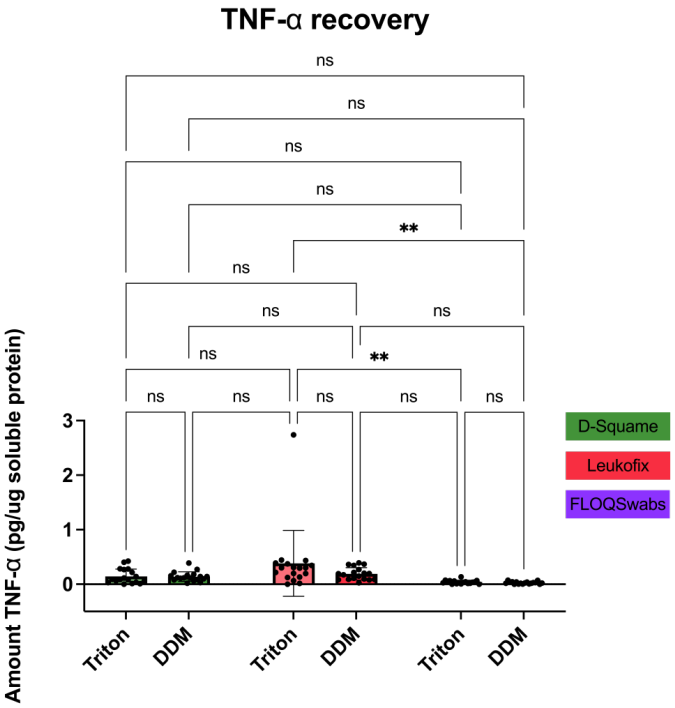

Figure 34: *TNF-α recovery over collection method and extraction solvent (two-way ANOVA).*

4.2.4 IL-8 Recovery

IL-8 was detectable for 13 out of 19 participants (68.4%) with the data presented in Figure 35, 36, and 37. In general, neither the collection method nor the extraction solvent had an impact on IL-8 recovery, at least not that could be determined statistically. However, visual inspection of the graphs suggests that Leukofix is the superior collection method, as for TNF-α.

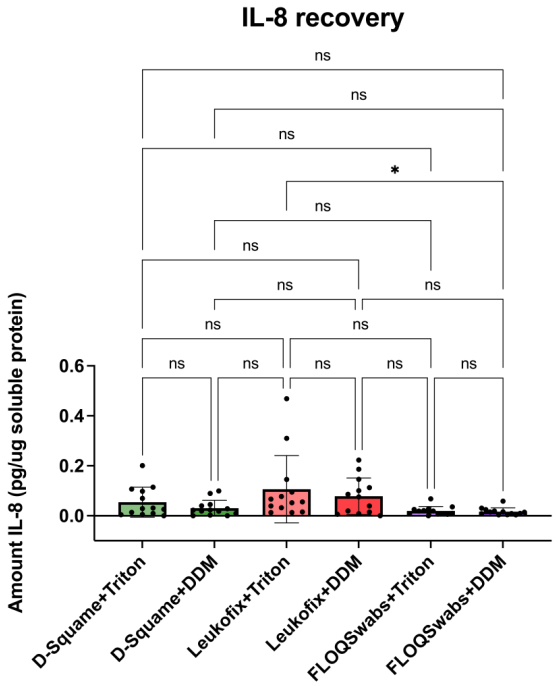

Figure 35: *IL-8 recovery over collection method and extraction solvent (one-way ANOVA).*

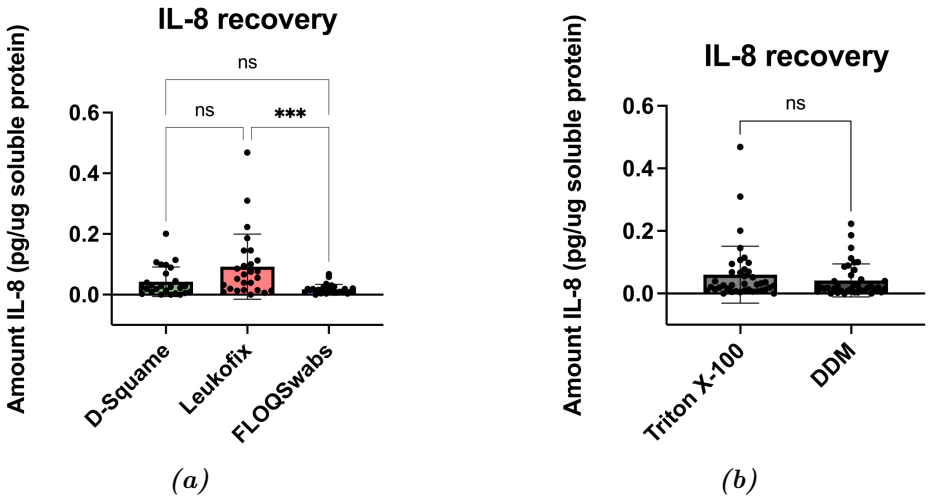

Figure 36: *IL-8 recovery over different (a) collection methods (one-way ANOVA) and (b) extraction solvents (t-test).*

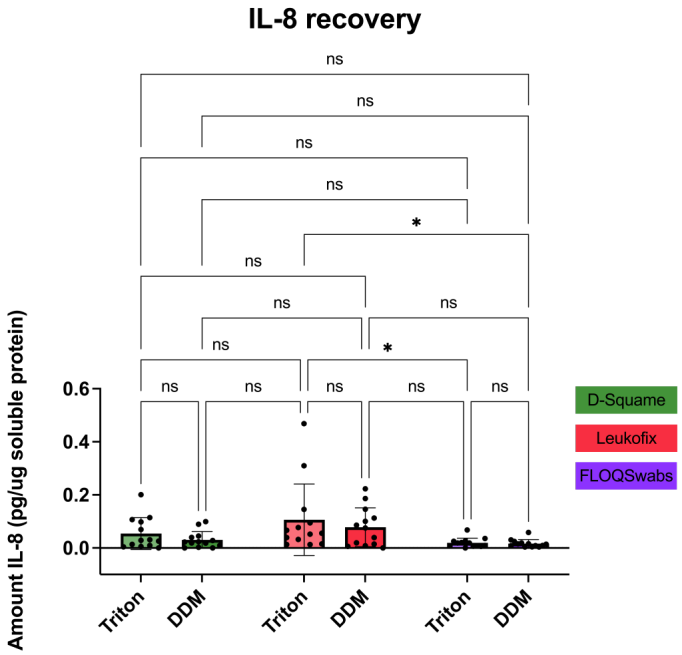

Figure 37: *IL-8 recovery over collection method and extraction solvent (two-way ANOVA).*

4.3 Summary Over Cytokine Recovery

Table 5 presents the cytokine recoveries for the respective buffers and detergents from the first sample collection, and Table 6 for the different collection methods and the use of the two best extraction solvents from the second round of collection. The data is shown as a mean with standard deviation, and also as a range from the minimum to the maximum recovery in pg cytokine/μg soluble protein. In Table 6, 'Triton' and 'DDM' refer to Tris-HCl with 0.5% Triton X-100 and β-dodecyl maltoside, respectively.

In round 1, Tris-HCl resulted in higher cytokine recovery compared to PBS. For IL-1α and IL-1RA, Triton X-100 and β-dodecyl maltoside outperformed Tween 20, while TNF-α showed the opposite trend. In round 2, for IL-1α and IL-1RA, sampling with FLOQSwabs was superior to D-Squame, which in turn was better than Leukofix. However, TNF-α and IL-8 showed the opposite relationship.

Table 5: *Cytokine recovery in the first round for use of different buffers and detergents*

Cytokine	**Recovery (mean ± SD (range)) (pg/μg)**				
	PBS	**Tris-HCl**	**Tween**	**Triton**	**DDM**
IL-1α	8.3±5.8 (0.3-32.3)	14.6±10.7 (0.6-61.9)	7.9±7.6 (0.3-37.8)	13.6±9.7 (1.6-61.9)	13.0±9.0 (1.7-47.1)
IL-1RA	27.7±28.1 (2.0-182.8)	55.8±61.1 (2.6-400.9)	32.0±49.1 (2.0-400.9)	44.5±43.8 (7.0-249.0)	48.7±54.1 (4.7-342.4)
IL-6	N/A	N/A	N/A	N/A	N/A
IL-8	N/A	N/A	N/A	N/A	N/A
TNF-α	0.15±0.21 (0.00-2.16)	0.40±0.34 (0.04-2.18)	0.35±0.45 (0.00-2.18)	0.25±0.21 (0.00-0.87)	0.23±0.18 (0.00-0.90)

Table 6: *Cytokine recovery in the second round for use of different collection methods and extraction solvents*

Cytokine	**Recovery (mean ± SD (range)) (pg/μg)**				
	D-Squame	**Leukofix**	**FLOQSwabs**	**Triton**	**DDM**
IL-1α	12.64±6.53 (2.4-29.1)	4.2±3.3 (0.2-17.0)	17.3±11.5 (2.6-57.0)	11.7±9.6 (0.2-54.5)	11.1±9.6 (0.9-57.0)
IL-1RA	41.0±58.8 (1.7-297.7)	14.0±18.5 (1.0-94.0)	48.8±40.8 (9.3-255.8)	35.1±37.9 (1.3-242.7)	34.1±51.3 (1.0-297.7)
IL-6	N/A	N/A	N/A	N/A	N/A
IL-8	0.04±0.05 (0.00-0.20)	0.09±0.11 (0.00-0.47)	0.02±0.02 (0.00-0.07)	0.06±0.09 (0.00-0.47)	0.04±0.05 (0.00-0.22)
TNF-α	0.14±0.11 (0.00-0.42)	0.29±0.44 (0.00-2.74)	0.03±0.03 (0.00-0.14)	0.19±0.38 (0.00-2.74)	0.12±0.11 (0.00-0.39)

Table 7 displays the cytokine concentrations (in pg cytokine/ml) obtained from performing of ELISA, combining all data in the first round and all data in the second round. The highest cytokine concentrations were observed for IL-1RA, followed by IL-1α, TNF-α, and IL-8. IL-6 had the lowest concentration and was undetectable.

Table 7: *Summary over cytokine concentrations in the first and second round*

Cytokine	**Concentration (mean ± SD (range)) (pg/ml)**	
	First round	**Second round**
IL-1α	585±386 (51-2129)	897±1133 (6-5705)
IL-1RA	2228±2460 (250-19567)	2593±3340 (28-16966)
IL-6	N/A	N/A
IL-8	N/A	2.0±2.0 (0.0-10.0)
TNF-α	12.9±12.6 (0.0-138.7)	5.4±7.2 (0.0-71.6)

4.4 D-Squame Densitometer Measurements

The total soluble protein concentrations determined through the Micro BCA Protein Assay showed only a weak positive linear relationship to the D-Squame densitometer measurements (the total protein amount, both soluble and insoluble) from both rounds of sample collection, with a correlation of 0.154. The proportion of light absorbed by the D-Squame tape strips varied between 7.1 and 38.2%, with a mean and standard deviation of 25.1±5.1%. The soluble protein concentrations measured by the BCA assay for the D-Squame ranged from 20.3 to 264.8 μg/ml, with a mean and standard deviation of 67.7±52.1 μg/ml.

In theory, the optical density of the tapes could be used to normalize the cytokine concentrations obtained from ELISA, rather than using the total soluble protein concentration. However, that method does not appear to be suitable in this context, making densitometer measurements unnecessary to perform for future investigations of this nature. However, densitometer measurements can still provide valuable insights when comparing treated or compromised skin with control skin.

5

Discussion

From the first round of samples, data on cytokine recovery was obtained for IL-1α, IL-1RA, and TNF-α. For all three cytokines, the use of 50 mM Tris-HCl outperformed PBS. For IL-1α and IL-1RA, Tris-HCl combined with Triton X-100 and β-dodecyl maltoside was superior to Tween 20. However, for TNF-α, the two lowest concentrations of Tween 20 (0.005% and 0.05%) with Tris-HCl were most effective.

For the extraction of the second round of samples, Tris-HCl with 0.5% Triton X-100 and 0.5% β-dodecyl maltoside was used, as these solvents ranked best for IL-1α and IL-1RA. IL-1α, IL-1RA, TNF-α, and IL-8 were present at detectable levels. For IL-1α, the use of FLOQSwabs and D-Squame resulted in significantly higher cytokine recovery than Leukofix. For IL-1RA, FLOQSwabs was significantly better than Leukofix. Conversely, for TNF-α, Leukofix and D-Squame were significantly better than FLOQSwabs. These findings are primarily based on the one-way ANOVA results. Two-way ANOVA analysis for IL-1RA and TNF-α showed non-significant effects of sample collection. The two extraction solvents were not significantly different from each other, which agrees with the results from the first round. For IL-8, almost none of the multiple comparisons were significant.

Overall, across both rounds, IL-1RA had the highest recovery, followed by IL-1α, TNF-α, IL-8, and IL-6, which was undetectable. However, it is important to note that the cytokine concentrations could vary heavily between individuals, which likely contributed to the relatively high standard deviations observed in Tables 5, 6, and 7. Moreover, no single extraction solvent or collection method proved to be optimal for all participants.

5.1 Optimization of Extraction Solvent

The choice of Tris-HCl with 0.5% Triton X-100 and 0.5% β-dodecyl maltoside for the second extraction of samples was not entirely straightforward, as there was no statistically significant difference between all combinations of Tris-HCl with Triton X-100 and β-dodecyl maltoside; all performed similarly. The decision was based on their rankings and the number of times they performed best for IL-1α and IL-1RA, which is not a perfect decision-making system.

This choice meant that the extraction solvents were not optimal for TNF-α, for which Tris-HCl in combination with Tween 20 performed better than the other detergents. Nevertheless, it was necessary to select the extraction solvent that worked best for most cytokines. Otherwise, additional samples would have needed to be collected in the second round, or the tapes would have had to be cut in slices to allow treatment with different extraction solvents.

This study highlights that there is no universal extraction solvent suitable for all cytokines, and that it is challenging to test for both highly abundant and low abundant cytokines simultaneously. The structure of the cytokines likely influences their interaction with and affinity for the different extraction solvents. IL-1α and IL-1RA have similar structures and bind to the same receptor, which can explain why the same extraction solvent works optimally for both. The two cytokines have a molecular weight around 17 kDa and a structure consisting of α-helices with a barrel-shaped hydrophobic core of β-strands [67]. In contrast, TNF-α has a different structure and receptor. It is also approximately 17 kDa, but forms a cone-shaped homotrimer of β-sheets [68].

Additionally, cytokines are inherently present at the skin surface at different levels, so extraction is not the only factor influencing cytokine detection. Keratinocytes, located in the epidermis, continuously express IL-1α and maintain a reservoir of the cytokine which is released in response to infection or injury [69, 70]. This promotes the expression of IL-6, IL-8, and TNF-α, among other cytokines [70]. Since this study did not compromise the condition of the skin that was sampled, it is not surprising that IL-6, IL-8, and TNF-α are not expressed at levels as high as IL-1α. The cytokines are also produced by other cell types, including macrophages, fibroblasts, and lymphocytes [34]. However, under normal conditions, these cells reside in the dermis and are only recruited in response to injury or infection. Therefore, collecting skin samples using tape stripping and swabbing, which only samples the stratum corneum, was expected to yield lower recovery of these cytokines.

Furthermore, the buffers used in this project, PBS and Tris-HCl, are both water-based, which theoretically make them both effective for extracting hydrophilic cytokines. However, significant differences in their performance were observed. Tris-HCl is less ionic than PBS and slightly basic (pH 7.5), while PBS has a neutral pH, factors that may contribute to these differences. The addition of detergents also influences cytokine compatibility with the extraction solvent. All three detergents used are non-ionic and non-denaturing of proteins, but differ in polarity. Tween 20 is the most hydrophilic, with multiple ethylene oxide chains. β-dodecyl maltoside is less hydrophilic but still contains a hydrophilic maltoside group. Triton X-100 is the least hydrophilic, with a hydrophobic phenyl group as its core and a polyethoxy chain that is less hydrophilic than maltoside. These variations in polarity, together with the effect of concentration, may impact the extent of cytokine extraction. However, for Triton X-100 and β-dodecyl maltoside, changes in concentration did not significantly affect cytokine recovery. This may be because all tested concentrations were above the critical micellization concentration (CMC). Increasing the concentration beyond CMC leads to the formation of micelles composed solely of the detergent,

rather than promoting detergent-protein binding, thereby not further enhancing protein solubilization [71]. Nevertheless, a concentration effect was observed for Tween 20, where a lower concentration resulted in higher recovery. Although, the two lowest concentration were tested at or near its CMC, whereas the highest concentration was 153 times above CMC, representing a significant concentration gap.

Several studies have compared the effectiveness of different extraction solvents for cytokine recovery from skin samples, as previously exemplified by Kaleja et al. (2020) [12] and Jayabal et al. (2023) [13]. Jayabal et al. (2023) concluded that β-dodecyl maltoside worked better for IL-1α and IL-1RA than for TNF-α, consistent with the results in this study. However, β-dodecyl maltoside still performed better than Tween 20 for TNF-α. In a different study by Hendrix et al. (2007) [72], the extraction of skin biomarkers from D-Squame tape was optimized. Different SDS concentrations were tested, two buffers (citrate-PBS and PBS), buffer volumes, extraction temperature, and other parameters were tested. The highest SDS concentration in PBS yielded the most effective extraction. However, higher detergent concentrations do not always guarantee improved recovery, as they also increase the risk of protein denaturation. Other studies have investigated the performance when combining non-ionic detergents with anionic, cationic, and zwitterionic detergents to promote protein solubilization while also limiting denaturation [73, 74].

5.2 Optimization of Collection Method

The extraction of cytokines using Tris-HCl with 0.5% Triton X-100 and 0.5% β-dodecyl maltoside improved the detection of TNF-α, from being detectable in 14 to 18 participants. In addition, IL-8 was detectable in 13 participants. However, despite the improved recovery of IL-8 in the second round, it was often present near the limit of detection and had relatively large variation between participants. IL-6 levels remained below the detection limit across all the participants. The baseline expression of these two cytokines may be too low for this type of analysis.

Nevertheless, IL-6 and IL-8 could be studied using a non-invasive skin sampling method with ELISA as the analytical technique *if* skin irritation is induced. This would allow for studying the cytokine expression in irritated skin or studying the effects of different treatments on irritated skin. In such cases, the control cytokine levels would be higher, and treatment would lead to a decrease in these elevated levels. Alternatively, using a concentrating column to concentrate the cytokines after extraction could make their analysis more feasible.

Regarding the collection methods, FLOQSwabs enhanced IL-1α and IL-1RA recovery, while Leukofix was more effective for TNF-α and IL-8 recovery. The swab was first submerged in PBS before being used on the forearm, which may have facilitated the uptake of certain cytokines. Leukofix, a much stickier tape compared to D-Squame, may yield higher cytokine recovery due to its strong adhesive properties. However, this increased adhesion can also make it more challenging to extract the cytokines from the tape. In a recent study by Chen et al. (2025) [75], three tapes

with varying adhesive properties were tested for skin lipid sampling, with D-Squame tape yielding the highest lipid recovery. As a matter of fact, there is a limited number of research articles comparing different skin sample collection methods, and none specifically comparing tape stripping with skin swabbing for cytokine analysis. However, numerous studies confirm the effectiveness of various non-invasive skin sampling methods for cytokine profiling.

The potential implementation of Leukofix requires additional consideration, as participants may be more resistant to using it due to it being more uncomfortable to remove. Leukofix might still be suitable for sampling of uncompromised skin, but not for sampling of irritated skin without an intact skin barrier. Additionally, Leukofix has not yet been optimized for this type of sampling. For instance, the tapes had to be cut to the right measurements by hand, and it was challenging to place them in their sampling tubes, unlike D-Squame, which is very user-friendly.

5.3 Comment on Statistical Analysis

In the second round, except for IL-1α, the two-way ANOVAs did not conclude any significant effects of either the collection method or extraction solvent. This could be due to the large variation in cytokine recovery within the groups, resulting in standard deviations larger than the respective means. The reason for using the two-way ANOVA was to mitigate the risk of false positives associated with performing two separate tests (one-way ANOVA and t-test) instead of one. The two-way ANOVA helps avoid this issue by analyzing the individual effects of each factor as well as the interaction between the two factors. However, a two-way ANOVA assumes the data follow normality, which was not true for this data set. Therefore, one-way ANOVA and t-test were used for the non-parametric testing. Sampling from 19 different participants is still a considerably small number, so it is not surprising that the data does not follow normality.

5.4 Sources of Error

For the analysis of samples using ELISA and the Micro BCA Protein Assay, no technical replicates were performed due to time constraints. This limitation increases the susceptibility of the data to variability, making it more challenging to identify outliers. However, this study did include biological replicates, with the same treatments (extraction solvents and collection methods) tested for all 19 participants. The addition of extraction solvents to the samples was randomized, ensuring that they were not applied to samples taken from the same position on the arm, and that they did not consistently occupy the same positions on the plate. This is crucial to avoid plate effects. Plate effects are a source of error that arise from inconsistencies in the surface of the wells, which can affect protein binding and reaction rates, as well from variations in temperature and evaporation based on the well position.

It would further have been beneficial to include a positive control for each participant, such as using sodium lauryl sulfate (SLS) to induce local irritation and then sample from the affected area. This would result in higher levels of inflammatory cytokines, providing a way to confirm that the ELISA assay is functioning correctly and that the participant has an active immune response. However, this approach would require a more extensive ethical review process. Additionally, the study's exclusion criteria already specified that participants should not have any ongoing skin diseases or a compromised immune defense.

Another source of error is the inherent variability in cytokine concentrations between individuals, which can make it difficult to detect significant effects of different extraction solvents and collection methods. To address this, in the first round, cytokine concentrations were normalized against the value of the reference extraction solvent for each participant, yielding a ratio. This normalization proved to make comparisons between extraction solvents more significant, presumably by accounting for the individual variability of skin.

5.5 Future Research

The data from this project can be further analyzed to explore differences in baseline concentrations of the five cytokines considering demographic factors, such as sex and age. This information would be valuable for future studies at Essity when investigating the effects of a product on the skin.

Additional investigations could focus on the use of Tris-HCl with a broader concentration range of Triton X-100 and β-dodecyl maltoside for the extraction of IL-1α and IL-1RA. The current concentrations were selected based on the lowest and highest reported literature values, along with an intermediate concentration. For TNF-α, it would be beneficial to examine the use of Tris-HCl with a wider range of Tween 20 concentrations. Additionally, for IL-6 and IL-8, exploring other buffers, detergents, and even alternative non-invasive collection methods is necessary.

Moreover, the effects of varying vortex and sonication durations, as well as centrifugation speed and time, should be investigated, similar to in the study conducted by Jayabal et al. (2023) [13].

6

Conclusion

This master's thesis project aimed to optimize a non-invasive skin sample collection and extraction method for the enhanced recovery of cytokines IL-1α, IL-1RA, IL-6, IL-8, and TNF-α, which are signaling molecules in the immune system and important inflammatory biomarkers. The project focused on optimizing extraction solvents (including buffer type, detergent, and detergent concentration) and the choice of sample collection method (tape stripping or swabbing). The gold standard for cytokine analysis, ELISA, was used as the analytical technique, although it has traditionally been limited to analyzing highly abundant cytokines while cytokines are present at low concentrations at the skin surface.

The results showed that Tris-HCl with Triton X-100 and β-dodecyl maltoside yielded the highest recovery of IL-1α and IL-1RA. Based on overall performance, at all tested concentrations, 0.5% Triton X-100 and 0.5% β-dodecyl maltoside were identified as the optimal concentrations. For TNF-α, the best extraction solvents were Tris-HCl combined with the two lowest concentrations of Tween 20 (0.005 and 0.05%), with 0.005% being the most effective, although not significantly better. Regarding collection methods, FLOQSwabs performed best and Leukofix worst for IL-1α and IL-1RA, whereas for TNF-α and IL-8, Leukofix was most effective and FLOQSwabs least effective. Further optimization of the extraction solvent for TNF-α is recommended, starting with Tris-HCl and Tween 20, as well as for IL-6 and IL-8, which were generally at or below the limit of detection.

The results from this project will have implications for future skin irritation studies and product safety evaluations at Essity, as the current protocol for non-invasive skin sample collection and cytokine extraction has not been optimized. Improved cytokine detection is important for evaluating skin condition and enhances the ability to diagnose various inflammatory skin diseases characterized by different cytokine expression patterns. The proposed buffers, detergents, detergent concentrations, and collection methods for the studied cytokines represent a significant step towards enhancing cytokine detection of both highly abundant and low abundant cytokines using non-invasive skin sampling and applying ELISA as the analytical technique.

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A

Protocols for Sample Collection

A.1 Sampling with D-Squame Tape

Material and Equipment

- Monaderm GO-DS22 D-Squame Discs, 22 mm diameter (LOT: 240150)
- Clinical and Derm D500 D-Squame Pressure Instrument
- Clinical and Derm D501 SquameScan® 850A Instrument (SN: 0417110142)
- Thermo Fisher Scientific Low Protein Binding Microcentrifuge Tubes, 1.5 ml (LOT: AA402053)
- Tweezer
- Marker pen
- Ruler
- Ice box

Methodology

Listed below is the procedure for application of each tape:

1. Apply the tape on the volar forearm.
2. Apply pressure (225 g/cm^2) on the tape for 10 seconds with the pressure device.
3. Rotate the tape 90 degrees on the same spot and apply the pressure again.
4. Repeat the turning and pressuring one more time.
5. Remove the tape from the skin.
6. Measure the transparency of the tape using the SquameScan densitometer.
7. Place the tape in a 1.5 ml tube.
8. Store the tube in a -20°C freezer until extraction and analysis.

A.2 Sampling with Leukofix Tape

Material and Equipment

- Essity Leukoplast Leukofix®, 2.5 cm×5 m (LOT: 44250251)
- Eppendorf Protein LoBind® Tubes, 5 ml (LOT: N217180N)
- Tweezer
- Marker pen
- Ruler
- Scissor
- Timer
- Ice box

Methodology

Listed below is the procedure for application of each tape:

1. Cut the tape to a 2.5×3 cm size.
2. Apply the tape on the volar forearm.
3. Let the tape stay on the skin for 1 minute.
4. Remove the tape from the skin.
5. Place the tape in a 5 ml tube.
6. Store the tube in a -20°C freezer until extraction and analysis.

A.3 Sampling with FLOQSwabs

Material and Equipment

- Copan 4N6FLOQSwabs®Genetics 4520CA; including 2 ml tube (LOT: 2308465)
- Corning Science Falcon Polypropylene Conical Tube, 50 ml (LOT: 24724015)
- Thermo Fisher Scientific Gibco™ DPBS (1X), 500 ml (LOT: 2572191)
- Marker pen
- Ruler
- Scissor
- Ice box

Methodology

Listed below is the procedure for application of each swab:

1. Take the swab out of its plastic pouch and place in a 50 ml tube with PBS.
2. Define a 5×5 cm area for swab sampling on the volar forearm.
3. Take the swab out of the PBS, hold it so that the shaft is parallel to the skin surface, and rub back and forth 20 times in each direction (up and down and side to side) while rotating the swab head continuously.
4. Cut off the tip of the swab and place in a 2 ml tube.
5. Store the tube in a -20°C freezer until extraction and analysis.

B

Protocols for Sample Extraction

Material and Equipment

- Thermo Fisher Scientific Gibco™ DPBS (1X), 500 ml (LOT: 2572191)
- Thermo Fisher Scientific Fisher BioReagents™ Tris-HCl buffer, 1 M (pH 7.5), 500 ml (LOT: 243466)
- Merck Sigma-Aldrich Tween®20, 50 ml (LOT: SLBR6201V)
- Merck Triton® X-100, 1 L (LOT: K34979303 611)
- Merck Sigma-Aldrich n-Dodecyl β-D-maltoside, 1 g (LOT: 0000365742)
- Thermo Fisher Scientific Pierce™ Protease Inhibitor Tablets (LOT: YJ4033516)
- Thermo Fisher Scientific Low Protein Binding Microcentrifuge Tubes, 1.5 ml (LOT: AA402053)
- Elma Transsonic® Sonicator 660/H (SN: 001759050)
- VWR Micro Star 17R® (SN: 42103940)
- Scale
- Vortex
- Ice

Methodology

1. Prepare the extraction solvent (buffer with 1X Protease Inhibitor and detergent at certain concentration). The Tris-HCl buffer is first diluted with MilliQ water to reach a concentration of 50 mM. One tablet of Protease Inhibitor is added to 50 ml buffer, followed by the addition of the respective detergent.

   Tween 20 was prepared at 0.005, 0.05, and 1% v/v concentration, Triton X-100 at 0.5, 1, and 2% v/v concentration, and β-dodecyl maltoside at 0.1, 0.5, and 1% w/v concentration. For the preparation of Triton X-100 and β-dodecyl maltoside extraction solvents, the solutions only fully mixed after about 20 minutes during which the flasks had to be occasionally swirled by hand. Note

also that Triton X-100 is highly viscous, so when pipetting it into the buffers, the pipette tip was held above the liquid to prevent crystallization on the tip.

2. Add extraction solvent to the sample tubes and vortex:
   - For D-Squame extraction, add 600 μl extraction solvent to the 1.5 ml tube containing the tape and vortex.
   - For Leukofix extraction, add 2.5 ml extraction solvent to the 5 ml tube containing the tape and vortex.
   - For FLOQSwabs extraction, add 1 ml extraction solvent to the 2 ml tube containing the swab and vortex.
3. Sonicate the tubes in an ice bath for 15 minutes (at 35 kHz).
4. Centrifuge the tubes at 15 000 g for 1 minute.
5. Transfer the supernatant to a 1.5 ml tube.
6. Store in a -20°C freezer or continue with analysis.

C

Protocols for Sample Analysis

C.1 ELISA

Material and Equipment

- Abcam ab178008 Human IL-1a ELISA Kit (LOT: 2101062897), ab211650 Human IL-1ra ELISA Kit (LOT: 2101054803), ab178013 Human IL-6 ELISA Kit (LOT: 2101063224), ab214030 Human IL-8 ELISA Kit (LOT: 2101056348), ab181421 Human TNF alpha Elisa Kit (LOT: 2101061973); including:
  - Capture Antibody (10X)
  - Detector Antibody (10X)
  - Lyophilized Recombinant Protein
  - Antibody Diluent 5BI
  - Wash Buffer PT (10X)
  - TMB Development Solution
  - Stop Solution
  - Sample Diluent NS
  - SimpleStep Pre-Coated 96 Well Microplate
  - Plate Seal
- Tecan Spark Microplate Reader 10M (SN: 1610006080)
- Tubes, 10 ml
- Thermo Fisher Scientific Low Protein Binding Microcentrifuge Tubes, 1.5 ml (LOT: AA402053)
- Ice box
- Vortex
- MilliQ water
- Automatic pipette
- Multi- and single-channel pipettes
- Plate shaker

Methodology

The following protocol is adjusted from the protocol of Abcam ab17800 Human IL-1 alpha SimpleStep ELISA Kit [60]:

1. Prepare the Antibody Cocktail in a 10 ml tube. For a volume sufficient for 96 wells, combine 600 μl 10X Capture Antibody, 600 μl 10X Detector Antibody, and 4.8 ml Antibody Diluent.
2. Prepare 8 standard solutions in 1.5 ml tubes, with the concentrations indicated in the protocol. First, for preparation of the stock standard, add Sample Diluent directly to the tube containing the Lyophilized Recombinant Protein (add the volume indicated on the label). Let the solution stand for 10 minutes until fully dissolved. Add the appropriate volume of Sample Diluent to the respective tubes, then perform the dilution series.
3. For performing of IL-1α or IL-1RA ELISA, dilute the samples 1:2 by combining 50 μl sample with 100 μl Sample Diluent. For analysis of the other cytokines, undiluted samples are used.
4. To the microplate, add 50 μl standard or sample in the corresponding wells, then add 50 μl Antibody Cocktail to all wells.
5. Seal the plate with a plastic film and place in a plate shaker at 300 rpm for 1 hour.
6. Prepare 1X Wash Buffer by diluting the 10X Wash Buffer with MilliQ water. For a volume sufficient for 96 wells, mix 10 ml 10X Wash Buffer with 90 ml MilliQ water.
7. When the incubation is finished, wash the wells three times using 350 μl 1X Wash Buffer. Ensure that all excess liquid is removed after each wash, particularly after the final wash.
8. Add 100 μl Development Solution to each well and place the plate in the plate reader to measure the development of substrate kinetically at 600 nm.
9. After the kinetic reading, add 100 μl Stop Solution to each well and put the plate back in the plate reader to measure the endpoint at 450 nm.
10. Analyze the data: Plot a standard curve, calculate the sample concentrations from the standard equation (remember to multiply with the 3X dilution factor for IL-1α and IL-1RA), normalize the concentrations against the total soluble protein concentration of each sample (from the Micro BCA Protein Assay), and visualize the normalized values.

C.2 Micro BCA Protein Assay

Material and Equipment

- Thermo Fisher Scientific Micro BCA™ Protein Assay Kit (LOT: XH348515)
  - Micro BCA Reagent A (MA), 240 ml
  - Micro BCA Reagent B (MB), 240 ml
  - Micro BCA Reagent C (MC), 12 ml
  - Bovine Serum Albumin Standard Ampules, 2 mg/ml
- Thermo Fisher Scientific Corning™ Costar® 96-Well Flat-Bottom Microplate; including low evaporation lid (LOT: 08219002)
- Thermo Fisher Scientific Low Protein Binding Microcentrifuge Tubes, 1.5 ml (LOT: AA402053)
- Incubator
- Tecan Spark Microplate Reader 10M (SN: 1610006080)
- Ice box
- Vortex
- MilliQ water
- Automatic pipette
- Single-channel pipettes

Methodology

The following protocol is adjusted from the protocol of Thermo Fisher Scientific Micro BCA Protein Assay Kit [61]:

1. Prepare the albumin standard solutions in 1.5 ml tubes according to Table C.1, starting from the 2000 μg/ml albumin stock.

Table C.1: *Albumin standard dilution series*

	Volume of MilliQ water (μl)	**Volume of previous solution (μl)**	**Final concentration (μg/ml)**
Std 1	900	100	200
Std 2	800	200	40
Std 3	500	500	20
Std 4	500	500	10
Std 5	500	500	5
Std 6	500	500	2.5
Std 7	600	400	1
Std 8	1000	0	0

2. Dilute all samples 1:4 by combining 50 μl sample with 200 μl MilliQ water.
3. Add 150 μl diluted sample or albumin standard in the corresponding wells of the microplate.
4. Prepare the Working Reagent by adding 25 parts Reagent A, 24 parts Reagent B, and 1 part Reagent C. For a volume sufficient for one plate, combine 12.5 ml of Reagent A, 12 ml Reagent B, and 0.5 ml of Reagent C.
5. Add 150 μl Working Reagent to each well of the plate.
6. Put on a lid and incubate the plate in 37°C for 2 hours.
7. When the incubation is finished, let the plate stay in room temperature until it reaches 25°C before performing any measurement.
8. Place the plate in the plate reader and measure the absorbance at 562 nm.
9. Analyze the data: Plot a standard curve and calculate the sample concentrations from the standard equation (remember to multiply with the 5X dilution factor).

D

Multiple Comparison Tests

Table D.1, D.2, and D.3, present the significant effects of different extraction solvents for IL-1α, IL-1RA, and TNF-α, both when the input data is cytokine amount and amount relative to the reference. Only significant comparisons are included in the tables. Adjusted p-values are marked accordingly: ns >0.1234, * <0.0332, ** <0.0021, *** <0.0002, **** <0.0001.

Table D.1: *Multiple comparisons of extraction solvents for initial IL-1α recovery*

Extraction solvent	**IL-1α**	**IL-1α_{rel}**
PBS+0.005%Tween vs. Tris+0.005%Tween	ns	**
PBS+0.005%Tween vs. Tris+0.5%Triton	ns	****
PBS+0.005%Tween vs. Tris+1%Triton	ns	****
PBS+0.005%Tween vs. Tris+2%Triton	ns	****
PBS+0.005%Tween vs. Tris+0.1%DDM	ns	**
PBS+0.005%Tween vs. Tris+0.5%DDM	ns	***
PBS+0.005%Tween vs. Tris+1%DDM	ns	****
PBS+0.05%Tween vs. Tris+0.005%Tween	ns	**
PBS+0.05%Tween vs. Tris+0.5%Triton	ns	****
PBS+0.05%Tween vs. Tris+1%Triton	ns	****
PBS+0.05%Tween vs. Tris+2%Triton	ns	****
PBS+0.05%Tween vs. Tris+0.1%DDM	ns	**
PBS+0.05%Tween vs. Tris+0.5%DDM	ns	***
PBS+0.05%Tween vs. Tris+1%DDM	ns	****
PBS+1%Tween vs. PBS+0.5%Triton	*	**
PBS+1%Tween vs. PBS+1%Triton	***	***
PBS+1%Tween vs. PBS+2%Triton	*	**
PBS+1%Tween vs. PBS+0.1%DDM	**	**
PBS+1%Tween vs. PBS+0.5%DDM	*	**
PBS+1%Tween vs. PBS+1%DDM	**	**
PBS+1%Tween vs. Tris+0.005%Tween	****	****
PBS+1%Tween vs. Tris+0.05%Tween	***	****
PBS+1%Tween vs. Tris+0.5%Triton	****	****
PBS+1%Tween vs. Tris+1%Triton	****	****
PBS+1%Tween vs. Tris+2%Triton	****	****
PBS+1%Tween vs. Tris+0.1%DDM	****	****
PBS+1%Tween vs. Tris+0.5%DDM	****	****
PBS+1%Tween vs. Tris+1%DDM	****	****
PBS+0.5%Triton vs. Tris+1%Tween	ns	*
PBS+0.5%Triton vs. Tris+0.5%Triton	ns	*
PBS+0.5%Triton vs. Tris+1%Triton	ns	*
PBS+1%Triton vs. Tris+1%Tween	**	**
PBS+2%Triton vs. Tris+1%Tween	ns	*
PBS+2%Triton vs. Tris+0.5%Triton	ns	*
PBS+2%Triton vs. Tris+1%Triton	ns	*
PBS+0.1%DDM vs. Tris+1%Tween	*	*
PBS+0.1%DDM vs. Tris+0.5%Triton	ns	*
PBS+0.1%DDM vs. Tris+1%Triton	ns	*
PBS+0.5%DDM vs. Tris+1%Tween	ns	*
PBS+0.5%DDM vs. Tris+0.5%Triton	ns	*
PBS+0.5%DDM vs. Tris+1%Triton	ns	*
PBS+1%DDM vs. Tris+1%Tween	ns	*
PBS+1%DDM vs. Tris+0.5%Triton	ns	*
PBS+1%DDM vs. Tris+1%Triton	ns	*
Tris+0.005%Tween vs. Tris+1%Tween	****	****
Tris+0.05%Tween vs. Tris+1%Tween	**	****
Tris+1%Tween vs. Tris+0.5%Triton	****	****
Tris+1%Tween vs. Tris+1%Triton	****	****
Tris+1%Tween vs. Tris+2%Triton	****	****
Tris+1%Tween vs. Tris+0.1%DDM	****	****
Tris+1%Tween vs. Tris+0.5%DDM	****	****
Tris+1%Tween vs. Tris+1%DDM	****	****

Table D.2: *Multiple comparisons of extraction solvents for initial IL-1RA recovery*

Extraction solvent	**IL-1RA**	**IL-1RA$_{rel}$**
PBS+0.005%Tween vs. Tris+0.005%Tween	ns	*
PBS+0.005%Tween vs. Tris+0.5%Triton	ns	***
PBS+0.005%Tween vs. Tris+1%Triton	ns	*
PBS+0.005%Tween vs. Tris+2%Triton	ns	***
PBS+0.005%Tween vs. Tris+0.1%DDM	ns	*
PBS+0.005%Tween vs. Tris+0.5%DDM	ns	****
PBS+0.005%Tween vs. Tris+1%DDM	ns	**
PBS+0.05%Tween vs. Tris+0.5%Triton	ns	**
PBS+0.05%Tween vs. Tris+2%Triton	ns	*
PBS+0.05%Tween vs. Tris+0.5%DDM	ns	**
PBS+1%Tween vs. PBS+1%Triton	*	*
PBS+1%Tween vs. PBS+0.1%DDM	**	**
PBS+1%Tween vs. PBS+0.5%DDM	*	*
PBS+1%Tween vs. PBS+1%DDM	**	***
PBS+1%Tween vs. Tris+0.005%Tween	****	****
PBS+1%Tween vs. Tris+0.05%Tween	****	****
PBS+1%Tween vs. Tris+0.5%Triton	****	****
PBS+1%Tween vs. Tris+1%Triton	****	****
PBS+1%Tween vs. Tris+2%Triton	****	****
PBS+1%Tween vs. Tris+0.1%DDM	****	****
PBS+1%Tween vs. Tris+0.5%DDM	****	****
PBS+1%Tween vs. Tris+1%DDM	****	****
PBS+0.5%Triton vs. Tris+0.5%Triton	ns	**
PBS+0.5%Triton vs. Tris+2%Triton	ns	*
PBS+0.5%Triton vs. Tris+0.5%DDM	ns	**
PBS+1%Triton vs. Tris+0.5%Triton	ns	*
PBS+1%Triton vs. Tris+0.5%DDM	ns	*
PBS+2%Triton vs. Tris+0.005%Tween	ns	**
PBS+2%Triton vs. Tris+0.05%Tween	ns	*
PBS+2%Triton vs. Tris+0.5%Triton	*	****
PBS+2%Triton vs. Tris+1%Triton	ns	**
PBS+2%Triton vs. Tris+2%Triton	*	***
PBS+2%Triton vs. Tris+0.1%DDM	ns	**
PBS+2%Triton vs. Tris+0.5%DDM	*	****
PBS+2%Triton vs. Tris+1%DDM	ns	***
PBS+0.1%DDM vs. Tris+1%Tween	ns	*
PBS+0.5%DDM vs. Tris+0.5%Triton	ns	*
PBS+0.5%DDM vs. Tris+0.5%DDM	ns	**
PBS+1%DDM vs. Tris+1%Tween	ns	*
Tris+0.005%Tween vs. Tris+1%Tween	***	****
Tris+0.05%Tween vs. Tris+1%Tween	**	****
Tris+1%Tween vs. Tris+0.5%Triton	****	****
Tris+1%Tween vs. Tris+1%Triton	***	****
Tris+1%Tween vs. Tris+2%Triton	****	****
Tris+1%Tween vs. Tris+0.1%DDM	***	****
Tris+1%Tween vs. Tris+0.5%DDM	****	****
Tris+1%Tween vs. Tris+1%DDM	****	****

Table D.3: *Multiple comparisons of extraction solvents for initial TNF-α recovery*

Extraction solvent	**TNF-α**	**TNF-α_{rel}**
PBS+0.005%Tween vs. PBS+1%Tween	ns	*
PBS+0.005%Tween vs. Tris+0.005%Tween	*	ns
PBS+0.05%Tween vs. PBS+1%Tween	ns	**
PBS+1%Tween vs. Tris+0.005%Tween	****	****
PBS+1%Tween vs. Tris+0.05%Tween	****	****
PBS+1%Tween vs. Tris+0.5%Triton	***	****
PBS+1%Tween vs. Tris+1%Triton	****	****
PBS+1%Tween vs. Tris+2%Triton	**	****
PBS+1%Tween vs. Tris+0.1%DDM	****	****
PBS+1%Tween vs. Tris+0.5%DDM	**	****
PBS+1%Tween vs. Tris+1%DDM	***	****
PBS+0.5%Triton vs. Tris+0.005%Tween	***	****
PBS+0.5%Triton vs. Tris+0.05%Tween	**	***
PBS+0.5%Triton vs. Tris+0.5%Triton	ns	*
PBS+0.5%Triton vs. Tris+1%Triton	ns	**
PBS+0.5%Triton vs. Tris+0.1%DDM	ns	**
PBS+1%Triton vs. Tris+0.005%Tween	****	****
PBS+1%Triton vs. Tris+0.05%Tween	**	****
PBS+1%Triton vs. Tris+0.5%Triton	ns	**
PBS+1%Triton vs. Tris+1%Triton	ns	**
PBS+1%Triton vs. Tris+2%Triton	ns	*
PBS+1%Triton vs. Tris+0.1%DDM	ns	***
PBS+1%Triton vs. Tris+0.5%DDM	ns	*
PBS+1%Triton vs. Tris+1%DDM	ns	*
PBS+2%Triton vs. Tris+0.005%Tween	****	****
PBS+2%Triton vs. Tris+0.05%Tween	***	****
PBS+2%Triton vs. Tris+0.5%Triton	ns	**
PBS+2%Triton vs. Tris+1%Triton	*	**
PBS+2%Triton vs. Tris+2%Triton	ns	*
PBS+2%Triton vs. Tris+0.1%DDM	*	***
PBS+2%Triton vs. Tris+0.5%DDM	ns	*
PBS+2%Triton vs. Tris+1%DDM	ns	*
PBS+0.1%DDM vs. Tris+0.005%Tween	****	****
PBS+0.1%DDM vs. Tris+0.05%Tween	**	****
PBS+0.1%DDM vs. Tris+0.5%Triton	ns	**
PBS+0.1%DDM vs. Tris+1%Triton	ns	**
PBS+0.1%DDM vs. Tris+2%Triton	ns	*
PBS+0.1%DDM vs. Tris+0.1%DDM	ns	***
PBS+0.1%DDM vs. Tris+0.5%DDM	ns	*
PBS+0.1%DDM vs. Tris+1%DDM	ns	*
PBS+0.5%DDM vs. Tris+0.005%Tween	****	****
PBS+0.5%DDM vs. Tris+0.05%Tween	***	****
PBS+0.5%DDM vs. Tris+0.5%Triton	ns	**
PBS+0.5%DDM vs. Tris+1%Triton	*	**
PBS+0.5%DDM vs. Tris+0.1%DDM	*	**
PBS+1%DDM vs. Tris+0.005%Tween	****	****
PBS+1%DDM vs. Tris+0.05%Tween	***	****
PBS+1%DDM vs. Tris+0.5%Triton	*	***
PBS+1%DDM vs. Tris+1%Triton	**	***
PBS+1%DDM vs. Tris+2%Triton	ns	**
PBS+1%DDM vs. Tris+0.1%DDM	*	****
PBS+1%DDM vs. Tris+0.5%DDM	ns	**
PBS+1%DDM vs. Tris+1%DDM	ns	**
Tris+0.005%Tween vs. Tris+1%Tween	****	****
Tris+0.05%Tween vs. Tris+1%Tween	****	****
Tris+1%Tween vs. Tris+0.5%Triton	*	**
Tris+1%Tween vs. Tris+1%Triton	**	**
Tris+1%Tween vs. Tris+2%Triton	ns	*
Tris+1%Tween vs. Tris+0.1%DDM	**	***
Tris+1%Tween vs. Tris+0.5%DDM	ns	*
Tris+1%Tween vs. Tris+1%DDM	*	*

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