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Title:	Development of an efficient extraction methodology to analyse potential inflammatory biomarkers from sebum
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Running title: Extraction methodology for analysis of potential inflammatory biomarkers from sebum

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Abstract

Introduction:

Proteins, such as cytokines and chemokines, are present in varying concentrations in a range of biofluids, with an important signalling role in maintaining homeostasis. Commercial tapes have been employed to non-invasively collect these potential biomarkers in sebum from the skin surface to examine their concentrations in conditions including acne, atopic dermatitis and pressure ulcers. However, the identification of robust biomarker candidates is limited by the low abundance of specific proteins extracted by current methodologies. Therefore, this study was designed to develop an optimized extraction method for potential inflammatory biomarkers in sebum collected with Sebutapes.

Methods:

Commercial tapes (Sebutapes) coated with synthetic sebum were used to systematically evaluate the effects of chemical and mechanical stimuli on extraction efficiency. Varying concentrations of high and low abundance biomarkers (IL-1α, IL-6, Il-8, INF-γ, TNF-α and IL-1RA) were used to spike the synthetic sebum samples. Methodological variables included different surfactants, mechanical stimuli and buffer volume. Extraction efficiency was estimated using immunoassay kits from the extracted buffer.

Results:

The results revealed that the use of a surfactant, i.e. β-dodecyl maltoside in addition to the mechanical stimuli, namely sonication and centrifugation resulted in an increased recovery of cytokines, ranging from 80% for high-abundant cytokines, such as IL-1α and IL-1RA, and up to 50% for low-abundance cytokines, including TNF-alpha, IL-6 and IL-8. Compared to previous methods, the new extraction protocol resulted in between an 1.5 - 2.0 fold increase in extraction efficiency.

Conclusion:

The study revealed that there was a high degree of variability in the extraction efficiency of different cytokines. However, improved efficiency was achieved across all cytokines with selective surfactants and mechanical stimuli. The optimised protocol will provide means to detect low levels of potential biomarkers from skin surface, enabling the evaluation of local changes in pro- and anti-inflammatory cytokines present in different skin conditions.

Keywords: Skin biomarkers, Protein Extraction, Inflammatory cytokines, sebum

Highlights:

- Extraction of inflammatory markers from skin surface using commercial tapes have been investigated using a synthetic sebum model
- A new protocol involving both surfactant and mechanical stimuli resulted in a 1.5 - 2.0-fold increase in extraction efficiency.
- There was a high degree of variability in extraction efficiency, with low abundance proteins observed to have up to 50% extraction efficiency.

1. INTRODUCTION

A biomarker has been defined as “a characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention” [1] . Biomarkers can be derived from molecular, histologic, radiographic, or physiologic characteristics. These are used in modern medicine to support care through diagnosis, monitoring, prediction and prognosis of a number of pathological processes [2]. Only a small number of studies have consistently reported the cutaneous biomarkers for skin diseases and chronic wounds [3]. This has presented a clinical challenge in both the early diagnosis and treatment of a range of skin conditions. This is despite the skin representing an accessible organ to sample biofluids e.g., sebum and sweat, through non-invasive collection at its surface.

There are many clinical situations where individuals with comorbidities and limited mobility are at risk of skin damage and chronic wounds. For example, pressure ulcers (PUs) are a localized phenomenon, where skin sites are damaged due to the exposure of pressure or pressure in combination with shear [4]. In the context of Pressure Ulcers, skin assessment is routinely practiced using visual skin inspection and risk assessment scales (RAS) that are based on clinical factors such as redness, erythema, blanchability, mobility and nutrition status of the individual [4] . Owing to the subjectivity, these scales often under or over-estimate the incidence of pressure ulcers [5, 6]. Therefore, there is a compelling need for an objective robust diagnostic test that could reliably determine skin status and inform clinical decision making prior to a loss in skin integrity. Moreover, individuals who suffer from PUs are associated with comorbidities including diabetes and malnutrition, that could lead to a change in the systemic inflammatory response [7-10]. To monitor local changes in skin integrity, there is a need to determine the inflammatory status of the skin at both localized sites and nearby control sites using methods that can distinguish spatial changes in skin health. These local changes in skin inflammation should be assessed in the light of systemic inflammation, which is often present in those at risk of skin damage. In recent years, sweat and sebum have been employed as biofluids in research studies evaluating early signs of skin damage, benefiting from the local, non-invasive sampling approach [11, 12].

To assess the integrity of loaded skin in a range of insult models as well as other skin conditions, protein markers including cytokines and chemokines, have been investigated as potential biomarkers [13-18]. When skin is subjected to external mechanical loads (pressure and/or shear), sustained cell deformation occurs leading to the disruption of the cellular membrane and local cytoskeleton network triggering a mechanotransduction response, including altered protein expression and proliferation responses [19, 11]. As an example, an *in-vitro* study involving reconstructed human epidermis has reported an upregulation in a set of cytokines, namely IL-1α, IL-1RA, TNF-α and IL-8 following prolonged mechanical loading [20]. Moreover, previous studies have highlighted the release of pro-inflammatory cytokines, namely INF-γ and IL-6, when human skin was subjected to prescribed mechanical and chemical insults [21, 22], as well as clinically relevant loads from prolonged lying on support surfaces [16] and the attachment of medical devices [21]. In addition, a study investigating cytokine release over the site of a category 1 pressure ulcer on a small number of participants, revealed a local upregulation over the sacral site compared to a control site 100 mm away, indicative of a localised area of tissue damage [23, 24].

Skin surface sampling could be achieved through various methods, namely, tape-stripping, cyanoacrylate skin surface stripping, skin blotting, abrasion methods, iontophoresis, transdermal analysis patch (TAP) and commercial adhesive tapes for sebum collection [25-30] .However, many of

these methods e.g. transdermal analysis patch requires specialised technologies and equipments. Other methods, namely, tape-stripping, cyanoacrylate surface stripping and skin abrasion causes damage to the skin surface to acquire cells and biofluids. By contrast, commercial tapes used to gently sample sebum, such as Sebutapes, offer a non-invasive sampling of local biofluid. Commercial tapes have been employed over several decades as a tool to non-invasively collect sebum from the skin surface to examine the concentrations of various potential biomarkers in conditions such as acne, atopic dermatitis and pressure ulcers [31, 15, 32] . Previous research in atopic and seborrheic dermatitis have employed several cytokines, namely, IL-13, IL-22, CCL17/TARC, in addition to IL-1alpha, IL-1RA and IL-8, to investigate the inflammatory status of the skin in patients with atopic dermatitis [31, 33, 34]. Amongst them, high-abundance cytokines typically IL-1alpha and IL-1RA, and low-abundance cytokines such as IL-8 have been previously extracted from Sebutapes [31]. Cytokines such as IL-1alpha, IL-6 and TNF-alpha collected from the skin surface, using Sebutape and skin blotting methods, have been investigated for their potential in diagnosing Pressure Ulcers in a patient cohort and have shown promising results for clinical utility, where a local upregulation was observed over the site of skin damage [35, 36]. The extraction of inflammatory markers from Sebutape have generally been performed using a standard protocol developed two decades ago [37]. However, the efficiency of the extraction method has not been fully reported. Moreover, previous studies have traditionally reported only IL-1α and IL-1RA in participant cohorts subjected to a range of external insults. By contrast, other low abundance cytokines, namely TNF-α, IL-6 and IL-8 are often cited as being below the limit of detection from commercial ELISA kits [22]. Therefore, the potential of these low-abundance cytokines as biomarkers have rarely been investigated.

To assess the concentration of a panel of proteins from sebum, they must be liberated from the tapes and maintained in a soluble, native and functional form. The efficiency of the extraction process can be enhanced with both chemical and mechanical stimuli, and with the use of minimal extraction volumes during analysis. Chemical agents, particularly surfactants have been added to the extraction buffer to improve the extraction of proteins [38]. Depending on the ionic charge and the degree of hydrophobicity of the surfactant, the interaction with membrane proteins is influenced thereby affecting the extraction process. Non-ionic surfactants, in particular, Tween and Dodecyl maltoside (DDM), have been reported to extract proteins from biological membranes and prevent their denaturation when compared to their ionic counterparts [38-40]. Subsequently, Tween has been employed in the extraction of markers from Sebutape [37]. Standard laboratory practices, involving mechanical stimuli such as sonication, vortexing and shaking are typically employed to increase the liberation of proteins and ensure uniform suspension of proteins in solution. However, the effectiveness of the use of surfactants and mechanical stimuli on protein recovery from Sebutapes have not been systematically evaluated. Accordingly, the present study was designed to examine the influence of suitable surfactants and mechanical stimuli on the extraction efficiency of cytokines, namely, IL-1 α, IL-1RA, TNF- α, IL-6, IL-8 and INF- γ sampled from sebum using non-invasive tapes.

2. MATERIALS AND METHODS

2.1 Reagents and Equipment

The extraction efficiency of potential sebum biomarkers from the Sebutapes was assessed using a synthetic model, spiked with known concentrations of cytokines. Sebutapes are polypropylene-based lipophilic adhesive tapes purchased from Clinical and Derm LLC, USA. A combination of lipids were prepared using previously established constituents [41]. To review briefly, each selected compound (Table 1) was weighed (% w/w) in a glass container and heated at 60^{o}C using a hotplate magnetic stirrer (Cimarec, ThermoScientific, UK) with intermittent stirring until the solids became a

clear liquid for 10 minutes ensuring uniform mixing of the model lipids. The cooled mixture was stored and used for analysis within 1 week of preparation.

(Insert Table here – Table 1)

The lipids were handled using glass or stainless-steel tools and stored in scintillating glass vials with Teflon-coated caps. Two commonly used surfactants, namely, n-dodecyl β-D maltoside (DDM) and Tween-20, were purchased from Merck Millipore and milli-Q®-water was used for the preparation of reagent solutions. Mechanical stimulation was applied though sonication and centrifugation in an Ultrasonic bath (Grant Instruments XB3, UK) and refrigerated centrifuge (Fresco 17, ThermoScientific, UK), respectively. An array of selected cytokines, including IL-1α, IL-1RA, TNF- α, IL-6, IL-8 and INF-γ, were quantified using multiplex kits (MesoScale Diagnostics LLC, Rockville, US) and single cytokine kits (MesoScale Diagnostics LLC, Rockville, US). The electro chemiluminescent spectra were recorded with Meso QuickPlex SQ 120 (MesoScale Diagnostics LLC, Rockville, US).

2.2 Sample preparation

Sebutapes were coated with 0.5 µL of freshly prepared synthetic sebum and allowed to uniformly distribute over the surface. The sebum was applied using a sterile glass rod on the surface of the adhesive side of the Sebutape, ensuring complete transparency of the tape after 10 minutes. A sample volume of 100 µL for known concentrations of cytokine standards were dispensed with two 50 µL droplets. The cytokines involved those abundantly reported in sebum, namely IL-1α and IL-1RA, as well as low-abundant cytokines, including IL-6, IL-8, INF-γ and TNF- α. In the case of the multiplex kits, the calibrator standard solution constituted a mixture of each cytokine at different appropriate concentrations, as provided by the manufacturer. The tapes were further stored at 4^{o}C for a minimum of 14 hours to ensure that the Sebutapes were completely dry.

2.3 Extraction protocol

A series of experiments were conducted in a systematic stepwise design to identify optimum parameters for the extraction process. As shown schematically in Figure 1, the sequence initially involved the optimisation of chemical detergents in the form of two surfactants. Subsequent steps involved mechanical stimuli in the form of vortexing and sonication prior to investigation of buffer volume and centrifugation. The sonication and vortexing was carried out with tapes in-situ, whereas further concentration of the tape extracts with centrifugation was conducted following tape removal. In each case, the parameter of investigation was varied while the other extraction parameters were kept constant, as detailed in Table 2. The effect of surfactant, vortexing, sonication, centrifugation and buffer volume were studied using single cytokine kits, sensitive to concentrations of IL-1α, IL-1RA and IL-6. The influence of surfactant and centrifugation was assessed on a range of six selected cytokines, namely IL-1α, IL-1RA, IL-6, IL-8, TNF- α and INF- α, using multiplex ELISA kits (Table 2).

Surfactants

Two selected surfactants, namely Tween and DDM were investigated. The working concentrations of each surfactant ranged from 5-fold to 15-fold of the Critical micelle concentration (CMC), below which aggregates can form leading to protein denaturation [42]. The CMC of Tween and β-dodecyl maltoside are reported to be 0.06 mM and 0.15 mM, respectively. Sebutapes were extracted using the different extraction buffers and left unperturbed for 1 hour, while other extraction parameters were kept constant (Table 2).

Denaturation of cytokines with detergents was investigated by mixing known concentrations of cytokine solutions with the extraction buffers containing each detergent, namely Tween and

Dodecylmaltoside. The concentration range of cytokines investigated in the study are similar to the concentration range observed in samples derived from healthy volunteers [21, 11]. As an example, the concentration of IL-1α in these studies ranged between 20 - 120 pg/mL [21]. The maximum amount of cytokines coated on the Sebutapes ranged up to 142 pg, which is the product of the highest coating concentration (1420 pg/mL) investigated in the study and the coating buffer volume (0.1 mL). Changes in the cytokine concentrations were then quantified using immunoassay kits.

Sonication and vortexing

Following the extraction using an optimized buffer, Sebutapes were vortexed for different time periods, ranging from 0 to 3 minutes with no sonication. In parallel a further set was sonicated for different time periods, ranging from 0 to 15 minutes, in the absence of vortexing. An additional set of Sebutapes was sonicated for 10 minutes and vortexed for 1 minute in accordance with a previous protocol to estimate the combined effects of these mechanical stimuli [37]. A control sample was prepared in the absence of both sonication and vortexing.

Buffer volume

To investigate the influence of buffer volume, sets of coated Sebutapes were placed with the adhesive side upwards in square polypropylene boxes (MB26P009, MOCAP Limited, UK). The effect of buffer volume was assessed with a representative high abundant cytokine, IL-1alpha and a low-abundant cytokine, IL-6. With respect to IL-6, two sets of Sebutapes were extracted with 1.7 and 1.0 mL of the extraction buffer. In addition to the investigated buffer volumes, IL-1alpha was further investigated for a reduced buffer volume of 0.7 mL. These samples were sonicated for 5 minutes and the extraction efficiencies were examined.

Centrifugation

A further set of Sebutapes were extracted with the optimised extraction buffer and mechanical stimulation. One set of tapes were removed using clean forceps and the resultant solution was centrifuged in refrigerated conditions (4^{o}C) for a period of 10 minutes to prevent protein degradation. A 200 µL sample of the extracted solution was left in the vials in pellet form, while the remainder of the volume was removed from the vial as supernatant without disturbing the pellet. The extracted solution and the pellets were subsequently analysed using immunoassays. The influence of centrifugation speed, ranging from 1000 g to 15000 g, was investigated and optimized with the use of IL-1RA and the optimized speed was employed to investigate the extraction efficiency on the six selected cytokines.

(Insert Figure here : Figure 1)

2.4 Data analysis

Raw data were imported into Excel (Microsoft Office,2019, USA) for analysis. The normality of the data was assessed using Shapiro-Wilk test. Each condition was performed using 4 replicates (n=4) and the mean $\pm$ SD was calculated. The recovery percentage was calculated using the following formulae:

$$\text{Recovery (\%)} = \frac{\text{Amount of cytokine recovered} \times 100}{\text{Amount of cytokine coated}}$$

$$= \frac{\text{Concentration obtained from the calibration curve} \times \text{Volume of extraction buffer} \times 100}{\text{Coating concentration} \times \text{Volume coated}}$$

The data followed a normal distribution. Accordingly, pairwise t-test was used to compare the test conditions. Differences were considered to be statistically significant at the 5% level ($p < 0.05$).

(Insert Table here : Table 2)

3. RESULTS

3.1 Chemical stimuli

The recovery of IL-1α for each of the extraction buffers is illustrated in Figure 2. It is evident that compared with PBS alone, both Tween and DDM increased the recovery of cytokines from 50% to approximately 70%. In particular, 0.05% Tween and 0.1% DDM yielded the highest values for IL-1α recovery, ranging between 70-80%. In addition, it is also evident that with Tween the recovery is dependent on the protein concentration, whereas with DDM the recovery was similar for the two protein concentrations. It should be noted that there was variability within some of the replicates. As an example, the recovery of replicates for a cytokine concentration of 500 pg/mL extracted with 0.075% Tween ranged from 48% to 70%.

Selecting the optimal concentration for each surfactant i.e., 0.05% Tween and 0.1% DDM, the recovery efficiency of the panel of six cytokines is presented in Figure 3. The maximum recovery of each cytokine was variable and highly dependent on the cytokine. As an example, the maximum recovery for IL-1α was 65%, whereas the corresponding value for TNF-α was 27%. PBS with 0.1% DDM consistently produced the highest recovery for each of the cytokines across the range of concentrations. In addition, for all cytokines, except IL-1RA, the maximum recovery corresponded to the highest cytokine concentration. Moreover, the denaturation study revealed that there was minimal denaturation of the target proteins. As an example, IL-8 had a maximum recovery loss of 15% following exposure to the DDM detergent (data not shown).

(Insert Figure here : Figure 2)

(Insert Figure here : Figure 3)

3.2 Mechanical stimuli - Sonication and Vortexing

Table 3 summarises the effect of two mechanical stimuli. With respect to sonication over the period of 5 to 15 minutes, an increased recovery of cytokines was observed at each of the 3 durations. By contrast, there was a minimal effect of vortexing in isolation on the extraction efficiency. An increase in recovery of IL-1α, equivalent to ~10%, was observed with the use of sonication and vortexing from the coated Sebutapes compared to no mechanical stimulation. It should also be noted that the recovery remained at 75% for 10 minutes of sonication irrespective of vortexing (0 and 1 minute)

(Insert Table here : Table 3)

3.3 Buffer volume

Figure 4 indicates the percentage recovery of both a high-abundant cytokine i.e., IL-1α and a low-abundant cytokine i.e., IL-6 in different volumes of optimised extraction buffer (DDM, 0.1%). It is evident that there was an increased recovery in lower extraction volumes for varying concentrations

of both cytokines with the exception of IL-6 at 80 pg/mL. Moreover, there was limited variability associated within the replicates, as an example, the replicates for IL-1alpha with a coating concentration of 560 pg/mL extracted with a buffer volume of 0.7 mL displayed recovery values ranging between 83-92%.

(Insert Figure here : Figure 4)

3.4 Centrifugation

The recovery of cytokines from coated Sebutape for the supernatant, pellet and the uncentrifuged samples at three different centrifugation speeds is illustrated in Figure 5. It is evident that at higher speeds of centrifugation i.e., at 15000 g, a 30 % increase in recovery was observed with IL-1RA in the pellet when compared to that of the uncentrifuged samples (Figure 5a). Subsequent experiments employing a centrifugation at 15000 g, revealed that the percentage recovery of the six cytokines in the pellets are consistently higher than that of the uncentrifuged samples. Close examination revealed that the recovery of cytokines in the pellet, supernatant and the uncentrifuged samples increased with respect to the individual molecular weights. As an example, low-molecular weight cytokines namely, IL-8, INF-gamma and IL-6 demonstrated recovery values in the pellet of less than 40 %, whereas the recovery of high molecular weight cytokines, namely IL-1α and IL-1RA demonstrated corresponding values in excess of 70% (Figure 5b). However, the relative increase in pellet recovery when compared to uncentrifuged samples was comparable for each of the cytokines. These values ranged from ~5% to 15% with the exception of IL-1RA, wherein the recovery increased from 45% in the uncentrifuged samples to 75% in the pellet.

(Insert Figure here : Figure 5)

4. DISCUSSION

The extraction and quantification of both high-abundance and low-abundance cytokines from the skin surface using non-invasive methods represents an important challenge in the assessment of skin conditions, such as pressure ulcers. This motivated the study to examine the influence of mechanical and chemical stimuli on the extraction of protein markers from Sebutapes coated with synthetic sebum. A stepwise optimization process was performed to assess the recovery efficiency for a range of cytokines using a synthetic sebum model. The study revealed that specific modifications from an established methodology [31] achieved an improved extraction efficiency.

The optimized protocol included the introduction of centrifugation and the use of a new detergent (DDM). In comparison to the previously established methodology [37], efficiency in analytical approach has been achieved by removing the vortexing stage and reducing buffer volume (Figure 1). These changes in extraction protocol resulted in 1.5-2.5-fold increase in efficiency from the original methodology. The optimised extraction protocol can be summarised by the following steps:
1) Sebutapes are extracted in 700 µL of the extraction buffer (i.e., PBS+0.1% DDM) and the samples should be shaken for 1 hour ensuring complete immersion of the tape in the buffer.
2) The samples are then sonicated for a period of 5 minutes
3) The Sebutapes are removed and the contents transferred into Eppendorf tubes for centrifugation at 15000 g in refrigerated conditions for 10 minutes
4) 500 µL of supernatant are removed and the pellet then stored for analysis using ELISA kits.

The present findings revealed that high recovery could not be consistently achieved for all the selected protein markers. As an example, the recovery of high abundance markers, such as IL-1α and

IL-1RA, ranged between 70-80 %. By contrast, the recovery of low-abundance, low molecular weight protein markers ranged between 20-30%. This reduced recovery in samples containing low protein concentrations could be attributed to their inherent thermal instability, in addition to their inability to maintain their functional form at low concentrations [43, 44]. It should also be noted that in five of the six cytokines, the maximum recovery was observed for highest concentrations (Figure 3). By contrast, this was not observed with IL-1RA, which presents with a high molecular weight (60 kDa), where the protein aggregates would tend to form at lower concentrations in comparison to the other low molecular weight cytokines [45]. Further studies are required to confirm the dependence of molecular weight on cytokine stability and recovery. Increased extraction could be achieved with ionic detergents, although this might be associated with the denaturation of proteins [46].

Previous studies investigating the influence of surfactants on proteins suggests that DDM offers a combination of high extraction efficiency with enhanced protein stability when compared with Tween [40]. This supports the present findings where a PBS solution with 0.1% DDM offered improved extraction efficiency for a panel of cytokines (Figure 3). In addition, our evaluation of denaturation also indicated minimal (<15%) changes in the stabilization of the cytokines (data not shown). Nonetheless, the recovery varied between proteins, which could be attributed to the protein structure and their tendency to maintain their functional form when exposed to surfactants [47]. Furthermore, the present study suggests that certain mechanical stimuli, such as centrifugation and sonication, offer an increase in the recovery of cytokines when compared to others such as vortexing. The process of shaking also ensured a more uniform contact of the buffer volume with the tapes. In addition, a reduction in buffer volume prevented dilution of the cytokines in solution, thereby further facilitating the quantification of low-abundance proteins such as IL-6 (Figure 4).

The original evaluation of sebum extraction from Sebutapes used sonication alone [37] and reported a 100% recovery of IL-1α. However, in the present study, a maximum recovery of up to 80% was observed (Figure 4a). This difference could be a direct result of the use of synthetic sebum in the present study to simulate real-life conditions in addition to the extended drying period of Sebutapes (>14 hours). Recent studies employing the established extraction protocol have revealed that the detection of low-abundance proteins, namely, IL-6, IL-8, INF-gamma and TNF-α, was challenging owing to their low concentrations, which were often below the detection limit of commercial immunoassay kits [48, 49, 22]. Low abundance of certain markers on the skin surface could be attributed to the transport properties of the protein molecules as well as the barrier function of skin [50-52]. To assess whether the extraction process removed all proteins from the Sebutape surface, the present study also conducted a two-stage extraction process and evaluation of the subsequent recovery. The results yielded only minimal additional protein recovery from the second extraction, indicating almost complete protein recovery from the first extraction (data not shown).

There are certain limitations in the current study. For example, it only used a selected panel of six cytokines which have been previously reported in the inflammatory processes preceding skin damage [21, 48, 32, 11] and two ionic surfactants. Furthermore, it is to be noted that the use of surfactants in the extraction process requires extensive protocols to concurrently analyse metabolites, using sensitive analytical techniques such as chromatography and spectrometry [53]. In addition, the effect of selected parameters, namely, buffer volume and sonication were evaluated using selected cytokines IL-1alpha and IL-6, which precludes generalising the finding to all potential biomarkers. Moreover, a further limitation of the study is that the effect of buffer volume was not tested at the lowest volume i.e. 0.7 mL as there were marginal changes in the recovery between 1 and 1.7 mL (Figure 4). It is to be noted the recovery of cytokines from each of the studies was also influenced by the incubation time required to dry the coating solution. However, each phase of the

present study was conducted with separate controls to establish the relative change in recovery. In addition, although sebum could be collected locally and non-invasively, it is only available in relatively small volumes, particularly at skin sites with limited density of sebaceous glands. For example, sites such as the plantar foot has low gland density (1-34 μg/cm^2) in comparison to the nasal bridge of the face (146-231 μg/cm^2) [54, 55].

The combination of commercial non-invasive tapes for sebum sampling and this optimised extraction protocol provides the potential to develop novel predictive point of care diagnostic tools for skin conditions, similar to biosensors developed for sweat analysis [56] . This would further aid in designing preventive strategies and treatments to prevent the incidence of progressive skin damage. Indeed, in a recent study the influence of pH on skin health demonstrated that the present extraction protocol yielded a 10-fold increase in the low abundance cytokines [57], when compared to the previous studies employing similar insult models [49]. Recent clinical studies at the host investigation investigating the response on sites presenting with a Category I PU and employing the optimised extraction method indicated promising results in the analysis of low abundance biomarkers, namely, IL-6,IL-8, TNF-α and INF-γ [58].

In conclusion, the improved extraction methodology from this study has enabled the detection of low abundance protein markers, with 1.5 – 2.5-fold increases in extraction from the previously established protocol. The identification of such robust predictive biomarkers of skin integrity requires further evaluation of the spatial and temporal profiles to establish robust thresholds by which changes in skin status can be established. This could provide the basis for more objective monitoring of skin health and provide a complimentary assessment to support clinical decision making.

STATEMENT OF ETHICS

Ethical approval is not required for this study in accordance with local or national guidelines as the study was an experimental work with proteins.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare

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AUTHOR CONTRIBUTIONS

All authors contributed to the conception of the study. The study was performed by Hemalatha Jayabal. The first draft was written by Hemalatha Jayabal. All authors were involved in data interpretation. The manuscript was reviewed and edited by Peter Worsley and Dan Bader.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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Figure legends

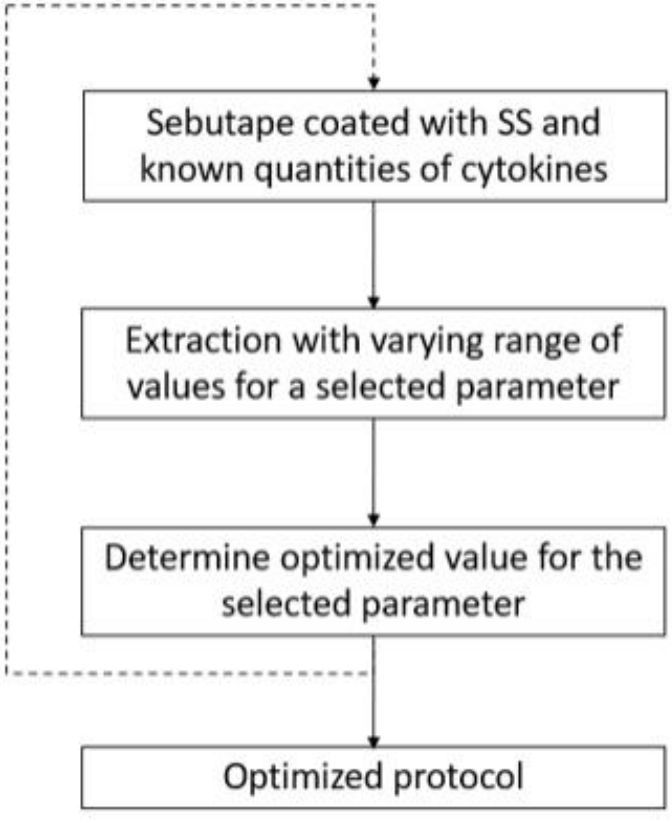
Figure 1: (a) Schematic of the experimental protocol illustrating the stepwise process implemented to develop and optimize the protocol and (b) the sequence in which the parameters were investigated

Figure 2:Plot illustrating the recovery (%) of IL-1α for different extraction buffers employed for two different coating concentrations of the cytokine. Each test condition was carried out in quadruplicates

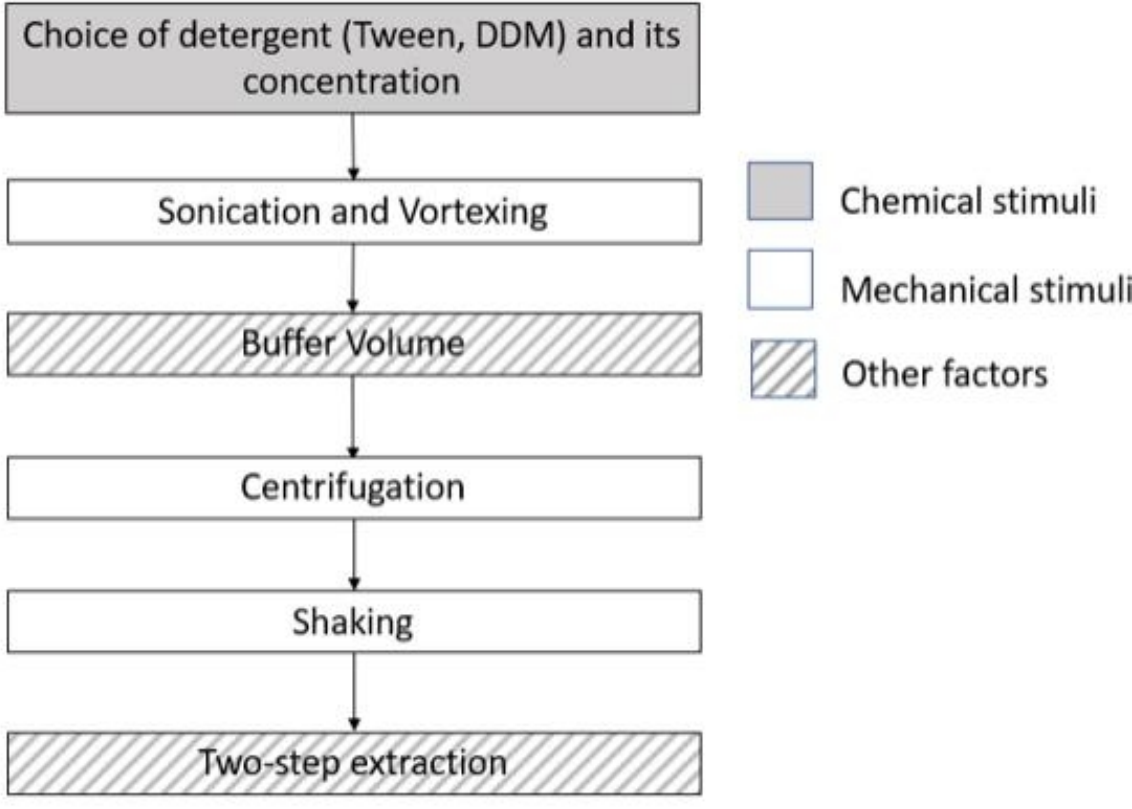
Figure 3: The percentage recovery of different concentrations of cytokines, namely (a) IL-1α (b) IL-1RA (c) TNF-α (d) IL-6 (e) INF-gamma (f) IL-8 for three different buffers, namely, PBS+0.1 % DDM, PBS+0.05% Tween, PBS

Figure 4: The estimated percentage recovery of (a) IL-1α and (b) IL-6 for different extraction buffer volumes. Significance was assessed using a t-test with *, ** and *** indicating $p<0.05$, $p<0.005$, $p<0.001$ respectively.

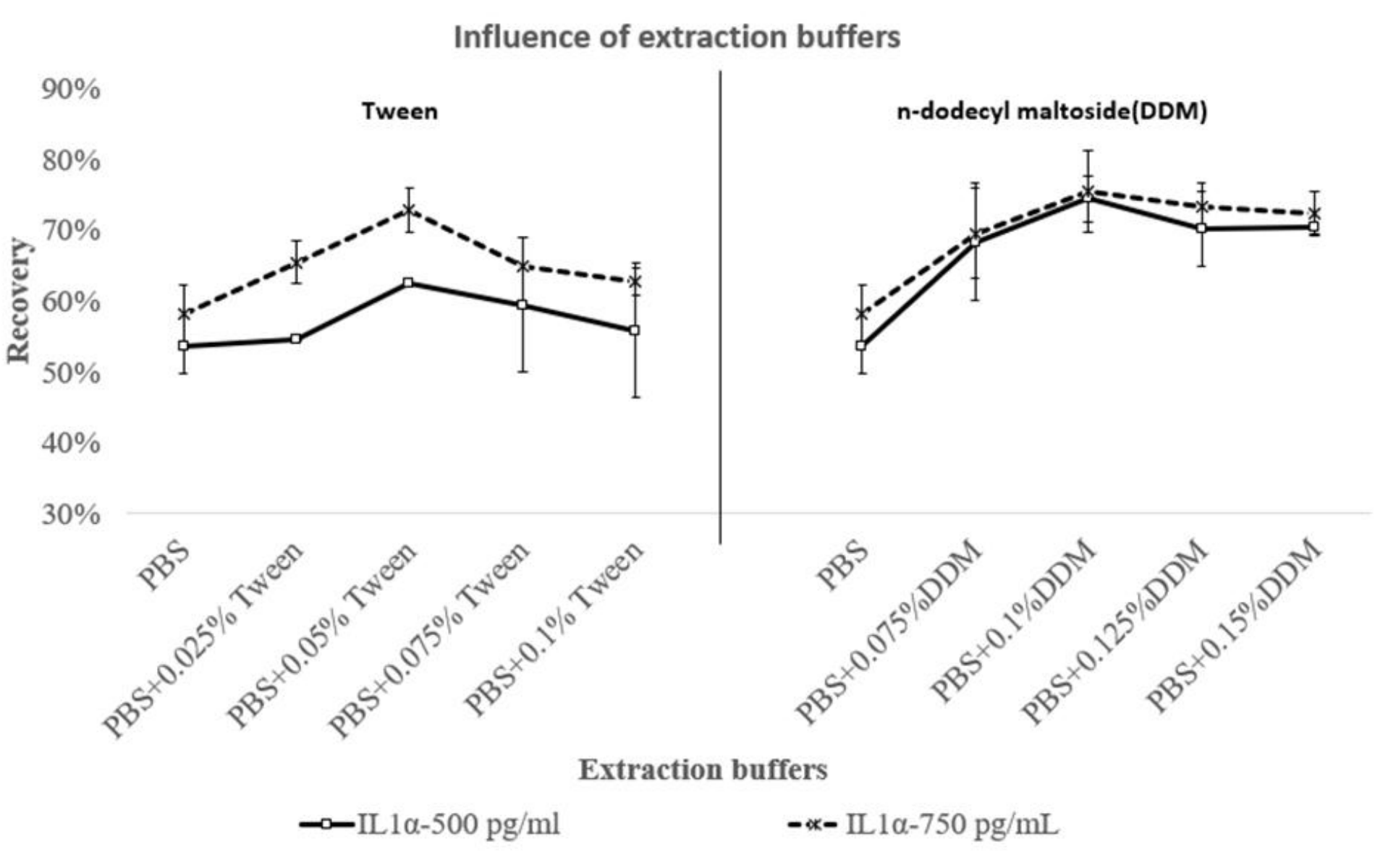
Figure 5: Plots illustrating (a) the influence of centrifugation speeds on recovery (%) of IL-1RA and (b) the influence of centrifugation on recovery (%) of different cytokines with their molecular weights listed (Molecular weights retrieved from phosphosite.org). Significance was assessed using a t-test with *, ** and *** indicating $p<0.05$, $p<0.005$, $p<0.001$ respectively.

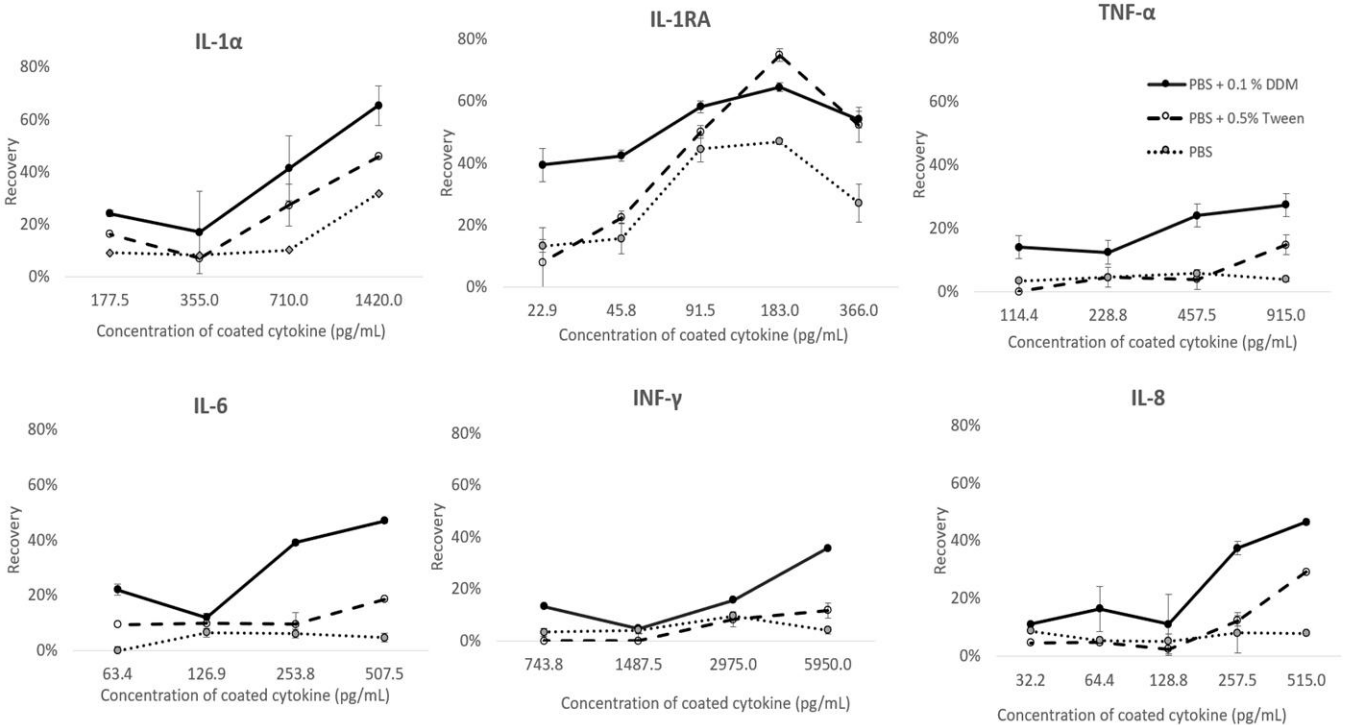

Sebutape coated with SS and known quantities of cytokines
Extraction with varying range of values for a selected parameter
Determine optimized value for the selected parameter
Optimized protocol

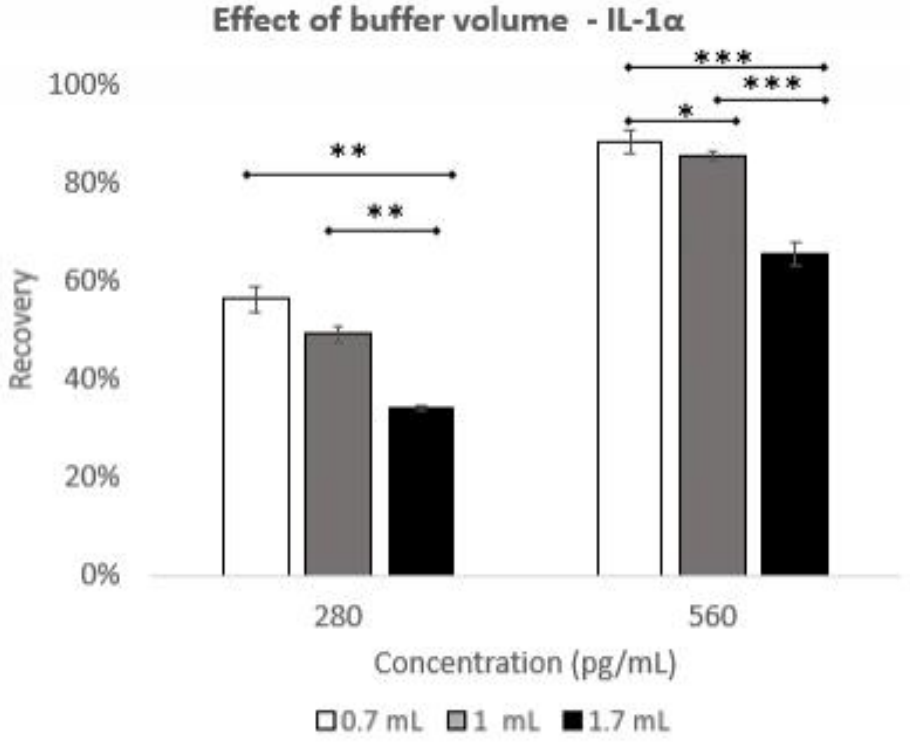
(a)


Choice of detergent (Tween, DDM) and its concentration
Sonication and Vortexing
Buffer Volume
Centrifugation
Shaking
Two-step extraction
Chemical stimuli
Mechanical stimuli
Other factors

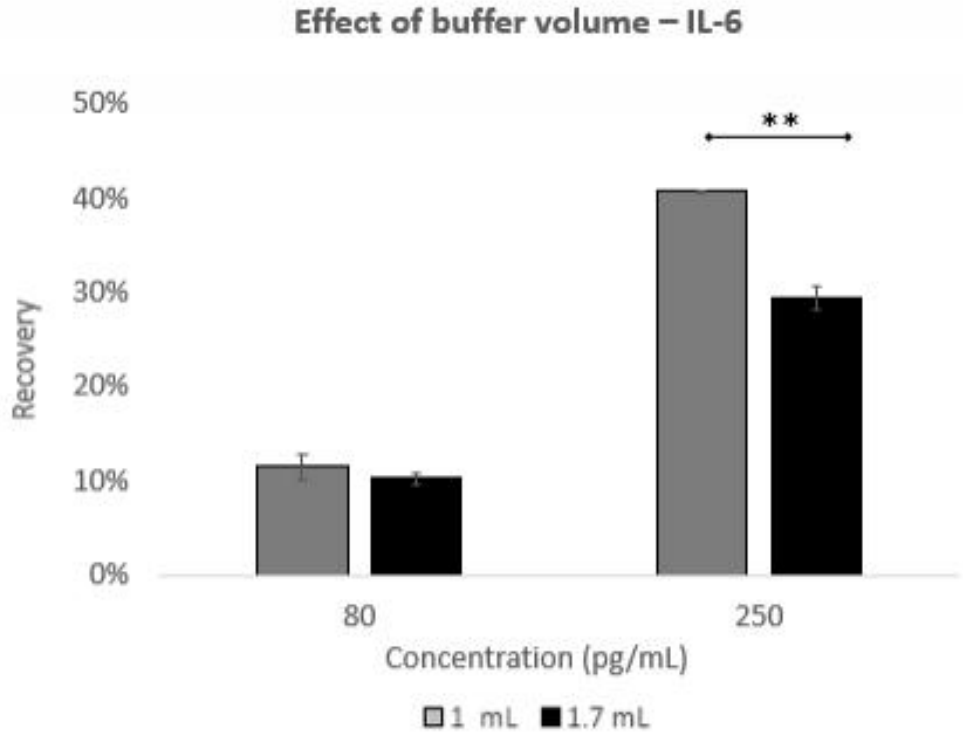
(b)


Influence of extraction buffers
Tween
n-dodecyl maltoside(DDM)
Recovery
90%
80%
70%
60%
50%
40%
30%
PBS
PBS+0.025% Tween
PBS+0.05% Tween
PBS+0.075% Tween
PBS+0.1% Tween
PBS
PBS+0.075%DDM
PBS+0.1%DDM
PBS+0.125%DDM
PBS+0.15%DDM
Extraction buffers
IL1α-500 pg/ml
IL1α-750 pg/mL

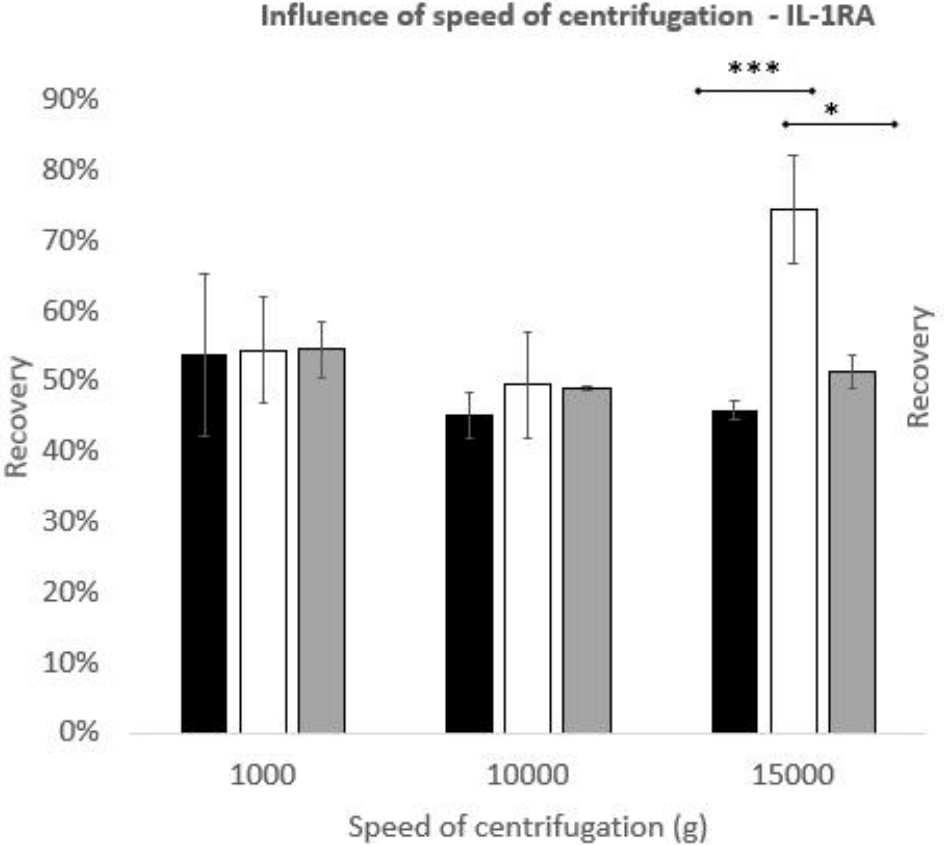

IL-1α
80%
60%
40%
20%
0%
Recovery
177.5
355.0
710.0
1420.0
Concentration of coated cytokine (pg/mL)
IL-1RA
80%
60%
40%
20%
0%
Recovery
22.9
45.8
91.5
183.0
366.0
Concentration of coated cytokine (pg/mL)
TNF-α
80%
60%
40%
20%
0%
Recovery
PBS + 0.1 % DDM
PBS + 0.5% Tween
PBS
114.4
228.8
457.5
915.0
Concentration of coated cytokine (pg/mL)
IL-6
80%
60%
40%
20%
0%
Recovery
63.4
126.9
253.8
507.5
Concentration of coated cytokine (pg/mL)
INF-γ
80%
60%
40%
20%
0%
Recovery
743.8
1487.5
2975.0
5950.0
Concentration of coated cytokine (pg/mL)
IL-8
80%
60%
40%
20%
0%
Recovery
32.2
64.4
128.8
257.5
515.0
Concentration of coated cytokine (pg/mL)

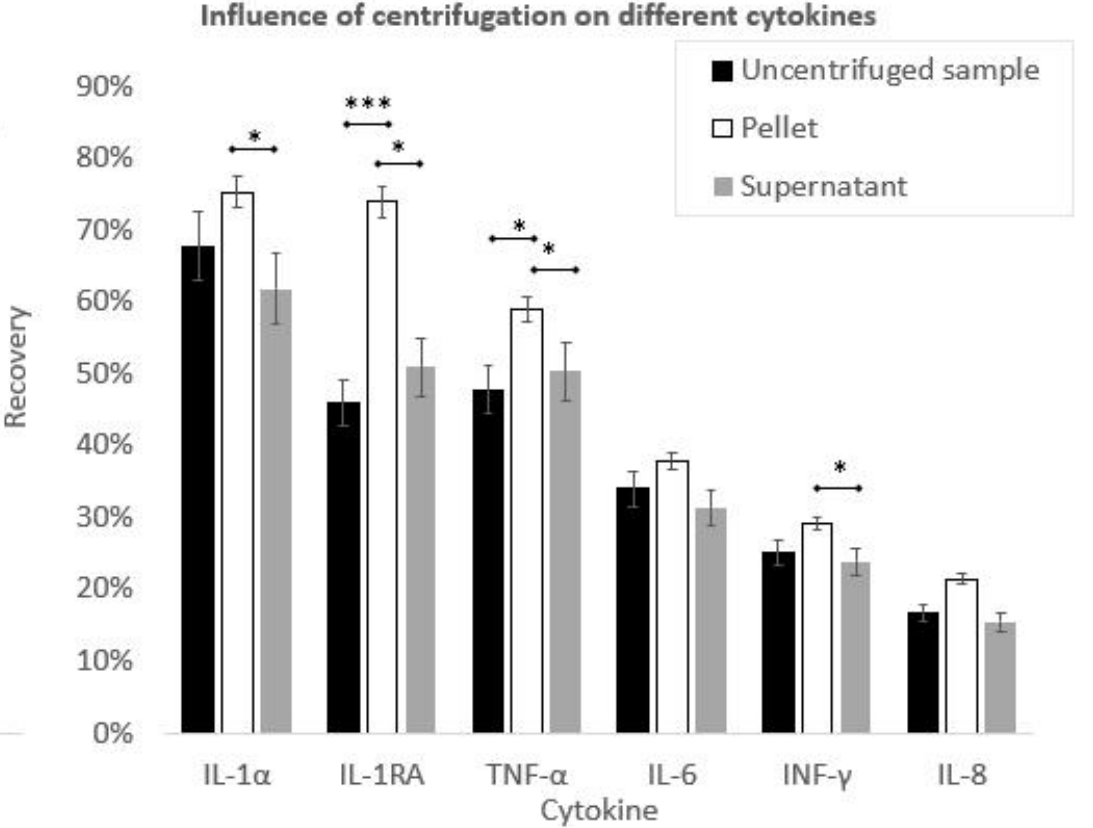

Effect of buffer volume - IL-1α
100%
80%
60%
40%
20%
0%
Recovery
**
**
*
***
***
280
560
Concentration (pg/mL)
0.7 mL
1 mL
1.7 mL

(a)


Effect of buffer volume – IL-6
50%
40%
30%
20%
10%
0%
Recovery
**
80
250
Concentration (pg/mL)
1 mL
1.7 mL

(b)





Cytokine	IL-1α	IL-1RA	TNF-α	IL-6	INF-γ	IL-8
Molecular weight (Da)	30607	65402	25644	23718	19348	11098

Table 1: Constituents of synthetic sebum

Compound	Weight (%)	Manufacturer
Squalene	15	MP Biomedicals Inc.,
Paraffin Wax	10	Aldrich
Jojoba Oil	15	Sargent-Welch
Olive Oil	10	Merck
Coconut oil	10	Aldon Corporation
Cottonseed oil	25	MP Biomedicals
Oleic acid	1.4	Aldrich
Palmitoleic acid	5	MP Biomedicals
Palmitic acid	5	EMD Chemicals
Cholesterol	1.2	Sigma Aldrich
Cholesterol oleate	2.4	Tokyo Kasei Kogyo Co.,

Table 2: Summary of the separate studies and the associated parameters involved in optimizing the protein extraction from Sebutapes

Parameter assessed	Parameter values	Cytokine	Concentration of coating solution (pg/mL)	Other extraction parameters				
				t_{son} (minutes)	t_{vort} (minutes)	Sur, C_{sur}	V_{buffer} (mL)	t_{cent} (minutes)
	Tween - 0.025 – 0.01% DDM – 0.075-0.175%	IL-1α	500,750	10	2	-	1.7	0
Surfactant (Sur, C_{sur})	0.05 % Tween, 0.1 % DDM	IL-1α IL-6 IL-8 TNF-α INF-γ IL-1RA	177.5 - 1420 63.4 - 507.5 32.2 - 515 114.4 - 915 743.8 - 5950 22.9 - 366	10	2	-	1.7	0
Sonication time (t_{son})	0,5,10,15 minutes	IL-1α	750	-	0	DDM, 0.1%	1.7	0
Vortex time (t_{vort})	0,0.5,1,2,3 minutes	IL-1α	750	0	-	DDM, 0.1%	1.7	0
Buffer volume (V_{buffer})	0.7mL, 1.0mL, 1.7mL 1.mL, 1.7mL	IL-1α IL-6	560,280 250,80	5	0	DDM, 0.1%	-	0
Centrifugation (S_{cent})	1000g, 10000g, 15000g	IL-1α IL-6 IL-8 TNF-α INF-γ IL-1RA	177.5 - 1420 63.4 - 507.5 32.2 - 515 114.4 - 915 743.8 - 5950 22.9 - 366	5	0	DDM, 0.1%	0.7	10

Sur – Surfactant, C_{sur}- Concentration of surfactant, t_{son} – Time of sonication, t_{vort} – Time of vortexing, V_{buffer} - Volume of extraction buffer, t_{cent} - Time of centrifugation, S_{cent} – Speed of centrifugation

Table 3: Recovery (%) of IL-1α from coated Sebutapes for different time periods of mechanical stimuli

Sonication time –t_{son} (minutes)	**Vortex time – t_{vort} (minutes)**	**Recovery – mean ± SD (%)**
0	0	66.5 ± 2.0
5	0	77.2 ± 0.8
10	0	75.1 ± 2.1
15	0	77.6 ± 0.2
0	0.5	67.2 ± 0.5
0	1	67.0 ± 1.8
0	2	67.2 ± 3.0
0	3	66.5 ± 2.0
10	1	75.0 ± 4.0

t_{son} – Time of sonication, t_{vort} – Time of vortexing