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Development of sample preparation methods for shotgun proteomic studies of white adipose tissue

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Master thesis in pharmacy
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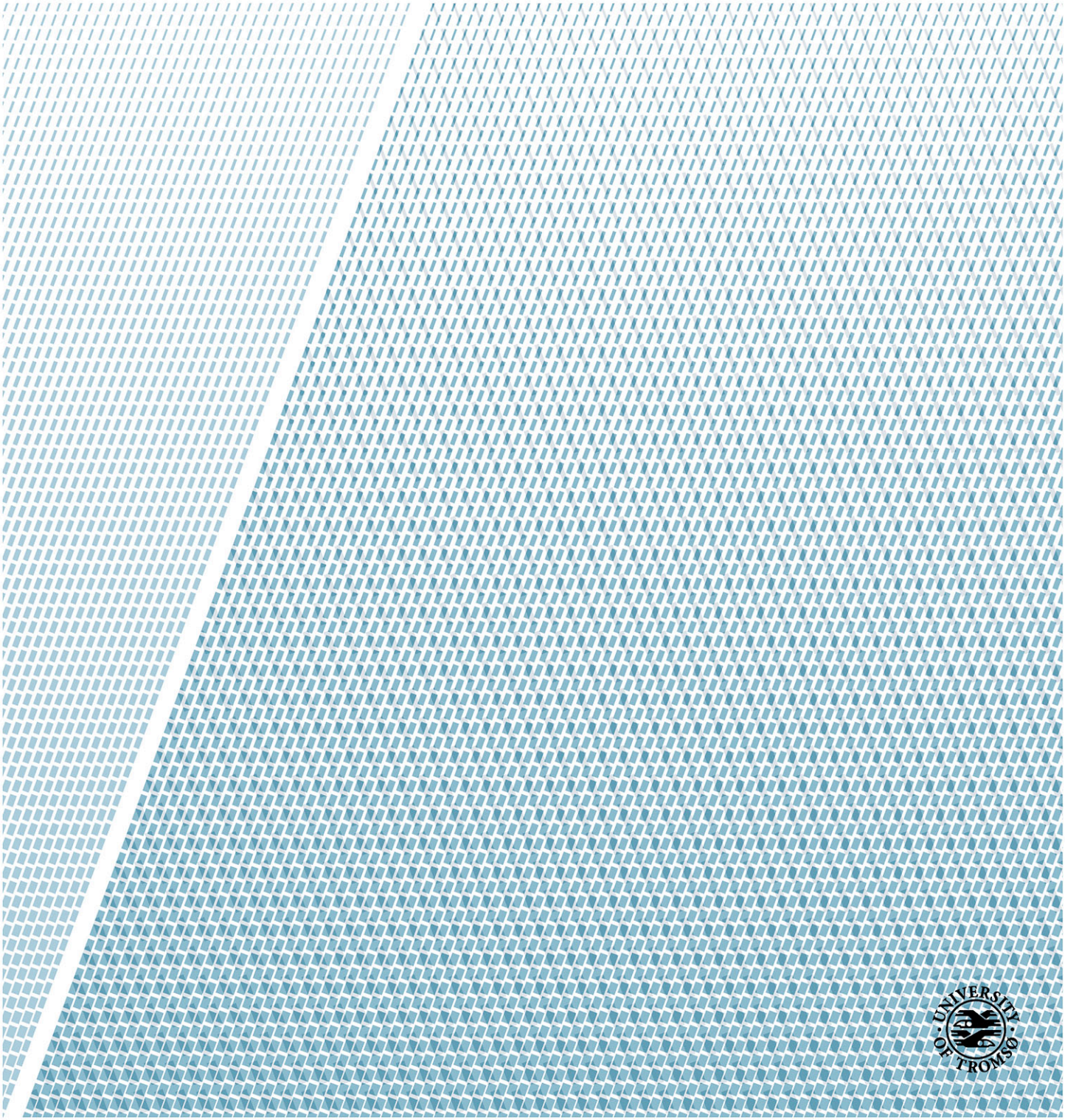

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I am fore mostly grateful to the almighty God for his divine protection and countenance bestowed upon me through my stormy academic journey.

I dedicate this thesis to my family, the late Rev. John D. Colnoe Sr, Mrs. Johnetta S. Colnoe, the late Elizabeth T. Talmon, John D. Colnoe Jr, Amos G. Colnoe, Hilary J. Colnoe and Blojay P. Colnoe for their love, support, prayers and guidance.

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ABSTRACT

Due to the lack of sunlight during the winter, people living in the arctic are unable to produce vitamin $D_{3;}$ hence deficiency of such vitamin is common in this part of the world. Given the geographic location of Norway, development of vitamin D deficiency is a common phenomenon. As vitamin D deficiency has become a public health issue, understanding the effects is vital for clinical implications. Studies have suggested that adipose tissues may be a direct target of vitamin D; especially the role of vitamin D in preventing the formation and development of adipocyte and of which has become of great interest to this study. Therefore, in order to investigate the potential role of vitamin D deficiency in relation to adipose tissue, Liquid chromatography-tandem mass spectrometry (LC-MS/MS) based shotgun analysis of adipose tissue proteome seems appropriate for this study.

This research seeks to develop a method that will quantify and identify white adipose tissue proteins. Hence, the appropriate method identify will be used in a vitamin D placebo controlled trial. To address this, the following seven different detergents efficiency methods to extract hydrophobic proteins were compared in triplicates for each sample as follows: PPS Silent (Sodium 3-(4-(1,1-bis (hexyloxy) ethyl) pyridinium-1-yl)- propane-1-sulfonate), ProteaseMAX (Sodium 3-((1-(furan-2-yl) undecyloxy) carbonylamino) propane-1-sulfonate), RapiGest (Sodium 3-[(2-methyl-2-undecyl- 1,3-dioxolan-4-yl) methoxy]- 1-propanesulfonate), Urea-Chaps (Urea-3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate), SDS (Sodium dodecyl sulfate), SDC (Sodium deoxycholate) and SL (Sodium laurate).

Two protein precipitation methods (acetone and ethanol) were compared using SDS and CHAPS-Urea samples as the basis for analysis after the acetone and ethanol precipitation (AP and EtOHP) procedures. Filter-aided sample preparation (FASP) was also carried out on the two samples mentioned above using a 30 kDa filter.

The results showed that PPS Silent, ProteaseMAX and RapiGest provided the total highest percentage yielded. Interestingly, SL and SDC yielded higher identification rates of proteins and peptides than the other methods. It is important to mention here that SL and SDC can be useful to anyone working on shotgun proteomics of adipose tissue.

This study is intended to be published, and is currently under revision by a publishing group.

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1.0 INTRODUCTION

In the last few years, several studies have focused on the role of vitamin D in curbing certain diseases. For example: obesity, diabetes, etc. Some studies have found a relationship between vitamin D status and adipose tissue, indicating that white adipose tissue formation and functions may be regulated by vitamin D (1). Vitamin D and the vitamin D receptor (VDR) are implicated in preadipocyte differentiation into adipocyte (2). Vitamin D_3 has shown to inhibit 3T3-L1 preadipocyte differentiations, by blocking transcription factors that regulate adipogenesis (3). In an in vitro study, a positive correlation between adiponectin and vitamin D could be shown (4).

1.1 Adipose tissue

Adipose tissue is an endocrine organ, which produces and secretes many bioactive proteins known as adipokines (adipose tissue hormones), in addition to its important role in fat storage; the adipokines are involved in several biological and physiological processes including energy metabolism and immune function (5-7).

There are two types of adipose tissues namely: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT stores triglycerides as energy and releases huge amount of adipocytokines, while BAT releases energy via the mitochondria to produce heat (6).

1.2 Vitamin D

Vitamin D is a steroid hormone and its active metabolite 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), binds to the VDR that can be found in several tissues. Vitamin D could regulate adipose tissue function and metabolism. The distribution of VDR in different tissues makes it interesting to study the functions of vitamin D (8).Vitamin D deficiency is usually defined as a serum concentration <20 ng/ml and over 30 ng/ml is referred to as sufficient (9).

The primary source of vitamin D_3 in humans is radiation from the sun, as production of vitamin D_3 relies on transformation of 7-dehydrocholesterol in the basal layers of the epidermis to pre-vitamin D_3 by ultraviolet B (UVB) light with a wavelength of 290-315 nm (10), shown in Figure 1.

The amount of sun ray that penetrates the skin determines the amount of vitamin D_3 synthesized (11). The skin synthesizes more than 80 % of the vitamin D_3 found in systematic circulation. The other 20% is derived from diet, animal cholecalciferol (vitamin D_3), or plant ergocalciferol (vitamin D_2), and through drugs supplementations (12). Pre-vitamin D_3 in the blood binds to the vitamin D-binding protein (DBP) and is transported to the liver by DBP. Hydroxylation takes place at position 25 to yield the precursor, 25-hydroxyvitamin D_3, which circulates in the body. The metabolite travels to all organs and hydrolysis occurs at position 1 to form the active1,25-$(OH)_2D_3$. The kidney is the most important organ for this process. The active endogenous hormone 1,25-$(OH)_2D_3$ carries out the physiological actions of vitamin D by binding to VDR (13).

Anything that hinders ultraviolet B radiation to the earth's surface or the penetration into the skin will affect the cutaneous synthesis of vitamin D_3 and leads to deficiency (14). Between October and March, people living in the arctic may not be able to produce vitamin D_3 via the skin due to decreased UVB radiation. Some factors that affect the sunlight at this time of the year are snow-covered ground, ozone, aerosol, altitude etc. (15). Öberg et, al, showed a high prevalence of vitamin D deficiency among youth in northern Norway, in particular among boys (16).

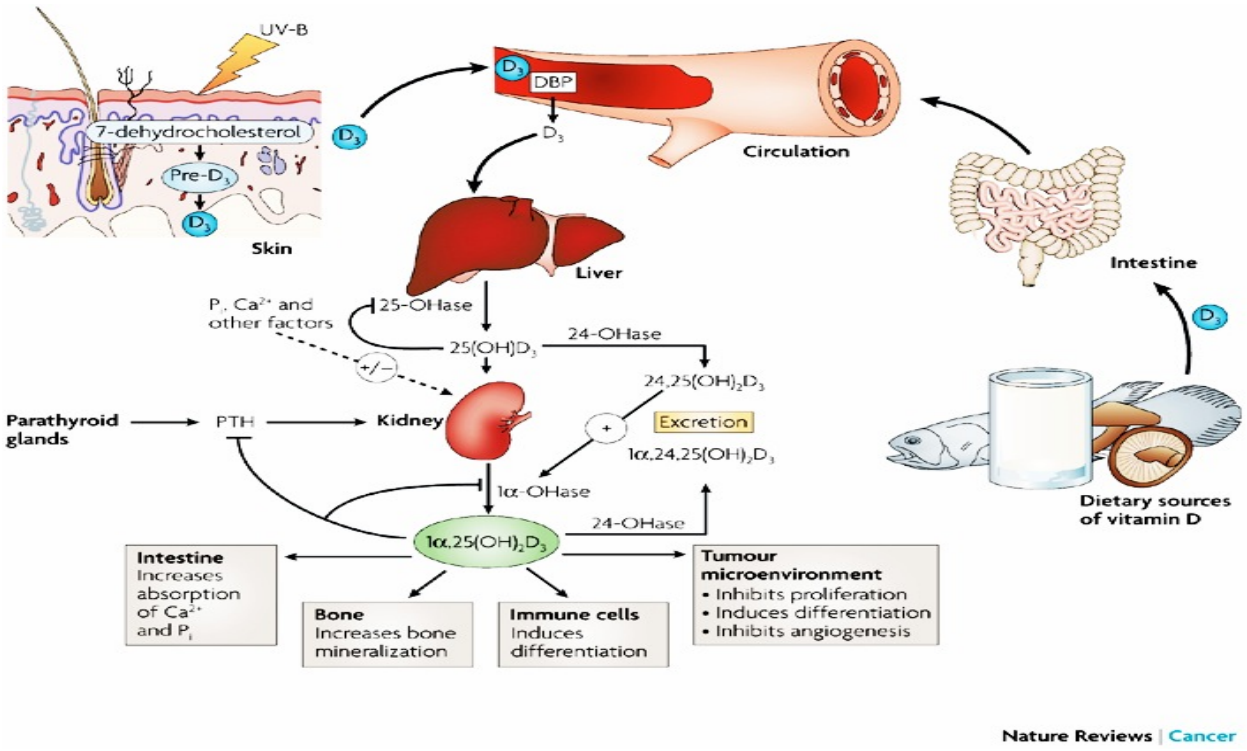


Figure 1: Synthesis of vitamin D (17)

1.3 Mass spectrometry (MS) based proteomics

Proteomics can be simply defined as the analysis of proteins on a large-scale or in detail; proteomics does not only involve identification and quantification of proteins, it also includes determination of protein localizations, modifications, interactions and eventually their functions (18).

MS-based proteomics approaches for protein analysis are also referred to as “top-down”, “middle-down” and “bottom-up”. The “top-down” approach involves direct mass spectrometric (MS) analysis of intact proteins. The “bottom-up” approach includes digestion of proteins into peptides prior to MS analysis. When a mixture of proteins is applied in a “bottom-up” approach, this is referred to as “shotgun proteomics”. In “middle-down” approach larger peptides fragments are analyzed than in the “bottom-up” approach (19), see figure 2.

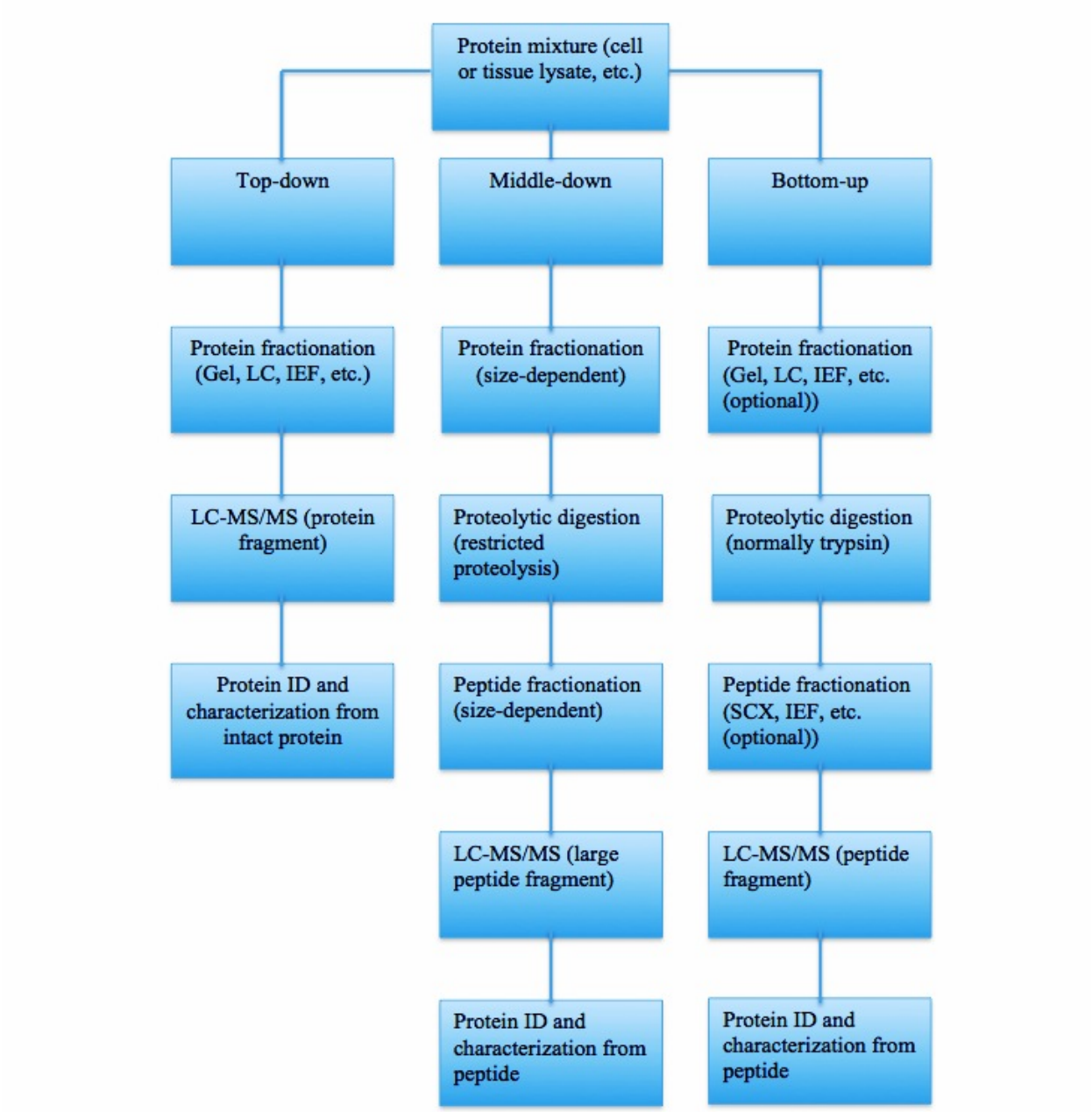


Figure 2: Strategies for MS-based protein identification and characterization

In a "top-down" approach intact proteins are analyzed instead of peptides as in the "bottom-up" approach, this diminishing sample complexity in contrast to the "bottom-up" approach where complexity is a problem due to digestion of proteins to generate peptides. Since proteins are analyzed intact, all information connecting to the proteins e.g. post-translational modifications (PTMs), mutation causing sequence variation, etc, are maintained. Nevertheless, the "top-down" approach has its own limitations, which includes ionization and fractionation of proteins and fragmentation in the gas phase. The use of peptides rather than

intact proteins in a shotgun “bottom-up” approach makes it easy to fractionate, ionize and fragment proteins. This approach has gained popularity in the field of proteomics and is also referred to as “workhorse” of proteins analysis. The “middle-down” approach has the same advantages as the “top-down” approach in addition to reducing peptides repetition between proteins (19).

1.4 Shotgun Proteomics

Shotgun proteomics is a powerful way to investigate the proteome. It is a “bottom-up” LC-MS/MS proteomic technique used to identify high coverage of proteins in an untargeted approach, shown in figure 3. The workflow consists of the extraction of proteins from serum, plasma, tissues, cells, etc. Proteins obtained are hydrolyzed into peptides by protease enzymes like trypsin. Trypsin is often preferred in an MS analysis, because it specifically cleaves peptide bonds at the carboxyl group of lysine (Lys) and arginine (Arg), resulting in a positive charged amino acid at the c-terminal end of the peptide. Peptides mixtures after digestion are usually desalted and concentrated before they are separated by ultra-high performance liquid chromatography (UHPLC) coupled to an online high-resolution mass spectrometer and ionized by electrospray ionization (ESI) techniques using aqueous to organic solvents together with a hydrophobic (C18) stationary phase. Longer column length and small bead sizes yield an effective separation. Fragment masses generated in a mass spectrometer are used for identification of proteins while intensities are used for quantification. The currently most used technologies of mass spectrometers for identification and quantification in proteomics are, Orbitraps, Time-of- flight (TOF) and ion traps (20).

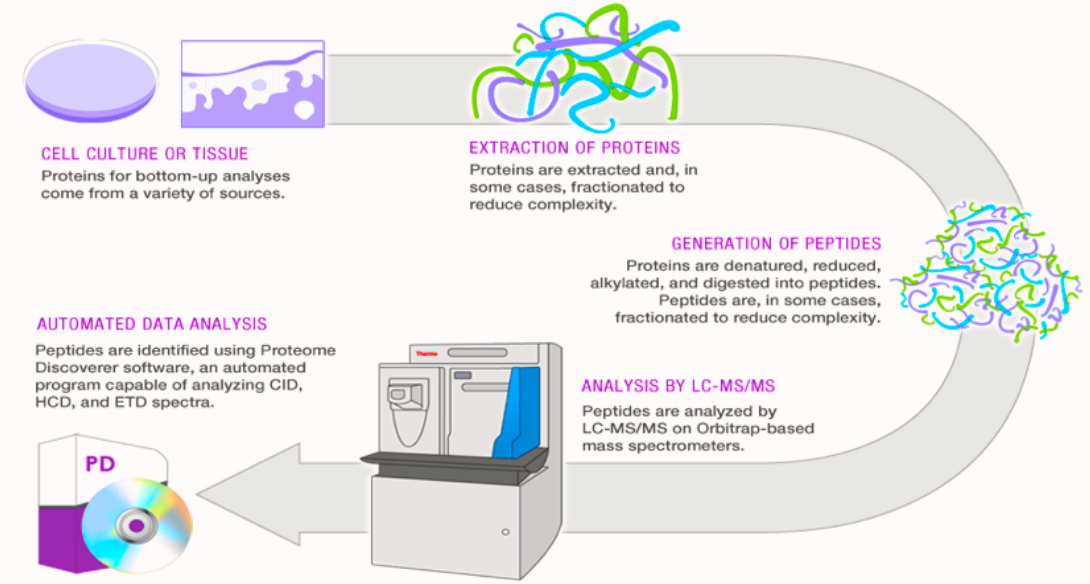


Figure 3: Shotgun proteomics workflow (http://planetorbitrap.com/bottom-up-proteomics#.VJDNr1ourG4) (51)

Protein digestion is normally the most import step in shotgun proteomics. It facilitates the depletion of intact proteins to peptides of appropriate size for MS analysis. One of the main challenges with this approach is the ability to detect as many as possible proteins in the complex mixtures (20, 21).

1.5 Gel electrophoresis-based and gel-free proteomics approaches

The two traditional sample preparation methods are the gel electrophoresis based and the gel-free or in-solution approaches, shown in figure 4.

The gel electrophoresis involves the separation of proteins by one or two-dimensional gel (Isoelectric focusing [IEF]), according to their sizes and charges. In other words, electrophoresis involves the grouping of molecules based on size. Molecules travel through a gel made of agar or polyacrylamide by the help of an electric field. The gel consists of a negative charge at one end and positive charge at another end. When electric current is applied, smaller molecules travel faster while lager molecules travel slowly through the gel, leaving different sizes of molecules forming clear bands of gel. The proteins are then visualized using different visualization methods (radio labeling, colloidal comassie blue, silver staining). The image generated can be analyzed, proteins bands are cut out of gel and the proteins are afterward digested inside the gel. After digestion, the peptides generated can

be analyzed by peptide mass fingerprint (PMF) usually by matrix-assisted laser desorption/ionization TOF-MS (MALDI-TOF MS) or by UPLC-MS similar to the shotgun approach. The peptides masses (and possible fragments) are matched against different protein databases for identification (22, 23).

The 2D gel electrophoresis (2DE) method has several limitations, with perhaps most serious being issues related to the reproducibility of the method. In addition, hydrophobic proteins are often not displayed on the 2DE, proteins with high molecular weight or highly basic/acidic proteins are difficult to resolve, low abundant proteins are difficult to detect and automation of the gel-based method can be difficult (24-27).

In gel-free approaches, proteins are digested in-solution. Proteins, peptides or both are separated using a liquid chromatography (LC) system. The gel-free approach is easier with regards to sample handling and speed, but requires an advanced LC-MS system (28).

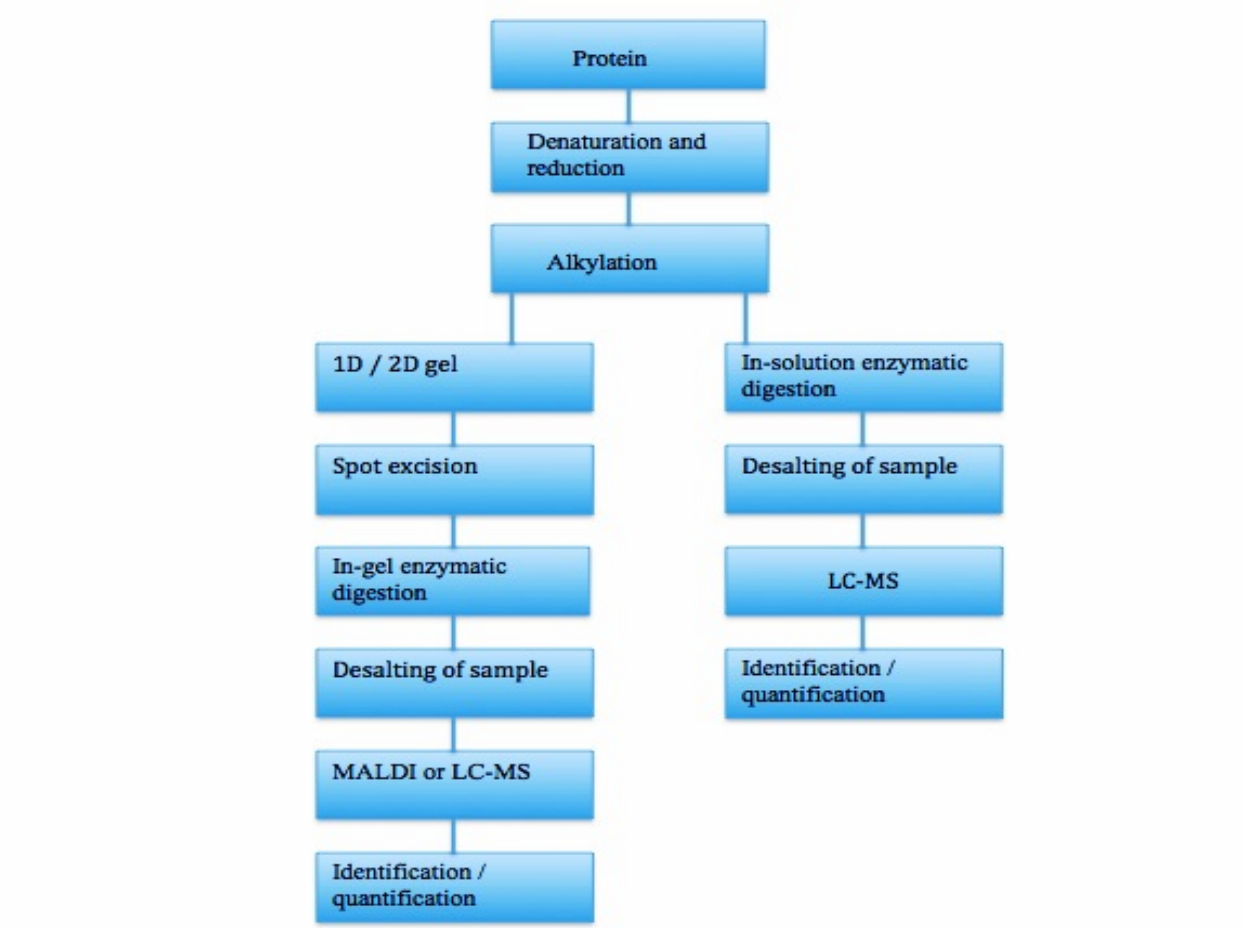


Figure 4: Workflows of in-gel (left) and in-solution (right) digestion, followed by LC-MS analysis of a protein sample

1.6 Mass spectrometry-compatible surfactants

Membrane proteins are difficult to solubilize and analyze because they are highly hydrophobic and low in abundance. Extraction and solubilization of proteins are vital for a successful enzymatic digestion. Many detergents, chaotropes, aqueous-organic solvents, organic acids, etc., have been added to different buffers to improve extraction and solubilization of membrane/hydrophobic proteins (29, 30).

Among all the additives used, some detergents have shown to be highly effective when it comes to denaturation and solubilization of all proteins. One detergent with a high capacity to extract membrane proteins is sodium dodecyl sulfate (SDS). The main disadvantages of SDS are its incompatibility with MS and its ability to reduce protease activity. MS compatible

detergents were developed to improve protein digestion for MS analysis. An acid-labile surfactant (ALS), RapiGest was found to promote intact protein analysis by MS and is referred to as an SDS analogue. RapiGest does not lower trypsin or other protease enzymes activities and is compatible with MS because it hydrolyzed at low pH to generate surfactants that do not interfere with MS analysis. Moreover, ALS has shown to solubilize and improve in-solution digestion of hydrophobic proteins. RapiGest has been used in both in-gel and in-solution digestions, prior to LC-MS/MS analysis and has shown no trypsin inhibition, contrary to SDS (31-35). MS compatible detergents (PPS Silent, RapiGest, Invitrosol (IVS)) improve solubility), enhance proteins digestion and increase the number of identify proteins and peptides (21).

Two other MS compatible and enzyme enhancing detergents are sodium laurate (SL) and sodium deoxycholate (SDC). SDC and SL can precipitate at low pH; which enable removal of SDC and SL from digested sample by acid precipitation strategy prior to MS analysis, which improves protein identification especially of integral membrane proteins. If the SDC or SL concentration in the sample is too high, it affects trypsin activity, leading to decrease protein identification. SDC extracts more proteins compared to common additive like Urea (36-38).

SL is similar to SDS in structure (figure 5 and 6). SL has the same ability to extract and solubilize hydrophobic proteins as SDS and more efficient than RapiGest and SDC. SL at a low concentration increases enzymatic activity, but at high level it can impair digestion. SL is removed from the sample before MS analysis in a similar way as SDC (37).

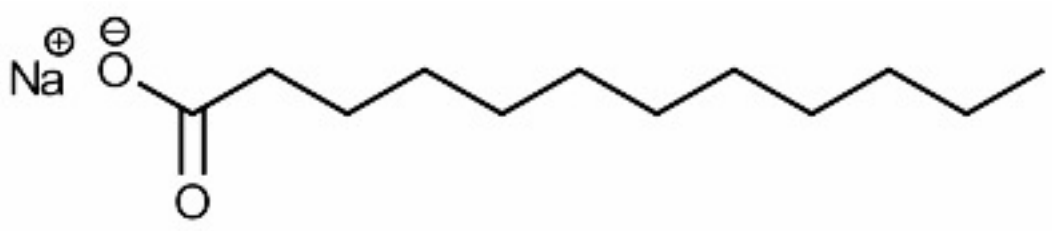


Figure 5: Sodium laurate (SL)

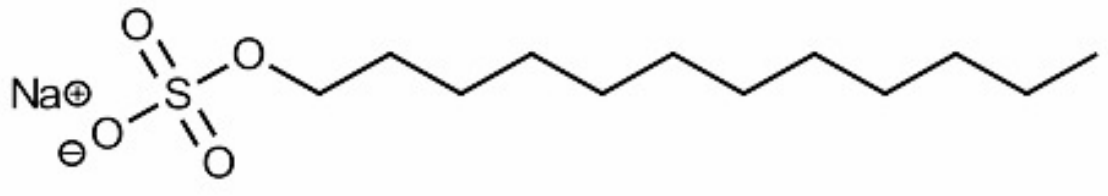


Figure 6: Sodium dodecyl sulfate (SDS)

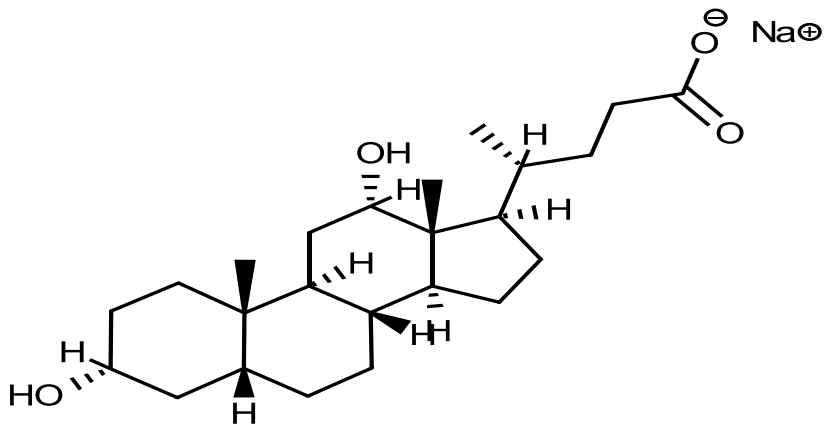


Figure 7: Sodium deoxycholate (SDC)

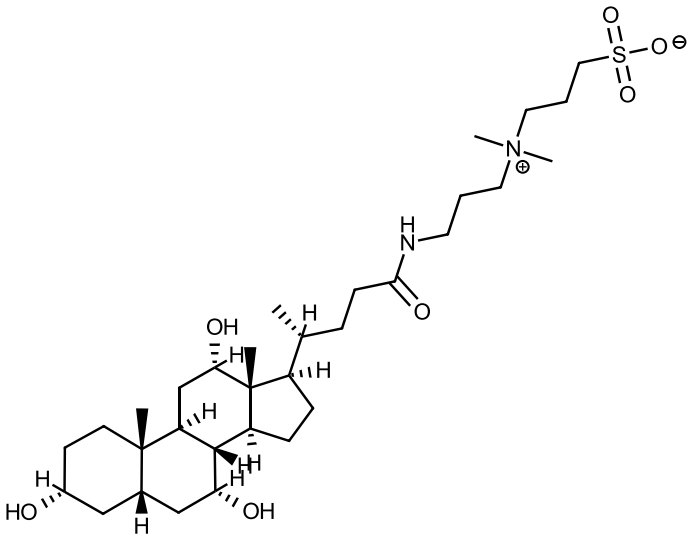


Figure 8: 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)

1.7 Protein purification

The quality of the results obtained from a proteomic study depend on the quality of the start material, but proper sample preparation purification of proteins is crucial in order to obtain a reliable and reproducible data in the study (39).

Precipitation is a method used to concentrate and fractionate target molecules from different contaminants. The type of protein precipitation method chosen is vital, because it should be effective in order to remove contaminants and concentrate samples. Trichloro acetic acid (TCA), acetone (Ac), methanol and other compounds are used for protein precipitation. Some of these methods can lead to low recovery of proteins after precipitation, depending on the solubility of the pellet. If the pellet is not dissolved properly, high loss of proteins can be observed (40, 41).

1.8 Filter-aided sample preparation (FASP)

FASP is a sample preparation method for MS- based proteomic analysis where proteins are purified by ultrafiltration before digest; hence generate peptides that are free of interference after digestion. Reduction, alkylation and digestion of samples can be done on a filter and generated peptides are collected. Samples are repeatedly washed and centrifuged with buffers, which usually contain urea. In repeated washing steps any remaining detergents, reduction agent (dithioerythritol (DTT)), alkylation agent (iodacetamide (IAA)) and low

molecular weight components are removed (42). Washing of sample with buffer solution that does not contain urea is less effective and does not enable complete depletion of SDS (43).

FASP was developed using filters with small pores with nominal molecular weight (MW) cut-offs of 3 000 and 10 000, but larger pores filters facilitate the sample preparation process. Filters with larger nominal molecular weight cut-offs of 30 and 50 kDa have shown advantages in sample preparation time and peptides yield. There is no difference in the number of peptides and proteins identified using filters of different brands (44, 45).

In this method, a variety of digestion conditions can be used. This is an important advantage compared with the in-gel and in-solution methods (42).

1.9 Proteomics in biomarkers discovery

Identification of biomarkers can help to speed up the development of drugs by identifying novel drugs targets of pathways involved in a certain disease and can be an important factor in diagnosis and evaluation of disease progression (46).

Since proteins are expressed at a given time under a given condition, a particular set of proteins are expected to be seen at a certain disease state, 2DE approach and protein chips techniques have also been utilized in proteomics for identification of biomarkers (46-48). High-performance LC-MS/MS provides a faster and less time consuming analysis of samples. This technique has been employed in the discovery of biomarkers in order to achieve a better understanding of diseases (46).

2.0 AIMS OF THE STUDY

The primary objective of this study was to develop a sample preparation method for identification of proteins expressed in the proteome of adipose tissue by LC-MS/MS. The appropriate method developed will be used to perform a proteomics analysis of adipose tissue in a randomized placebo controlled trial at samples taken before and after two months of vitamin D treatment during the arctic winter.

- Comparison of the efficiency of different buffers used to extract hydrophobic proteins from adipose tissue will be evaluated by:
  - Total number of proteins, peptides and yield (%)
  - Evaluation of methods for purification of proteins
  - Number of identified proteins and peptides
  - Average sequence coverage
  - Transmembrane domains
  - Subcellular locations
  - Molecular weight of identified proteins
  - GRAVY index of identified proteins
  - Number of unique peptides

3.0 MATERIALS AND METHODS

3.1 Materials and Chemicals

The following materials and chemicals were identified and used in this study. 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), acetone (Ac), acetonitrile CROMASOLV® (Reidel-de Haen), ammonium bicarbonate (ABC), bovine serum albumin (BSA), Dithioerythritol (DTT), ethanol (EtOH), guanidine hydrochloride (GuHCl), hydrochloride acid (HCl), acetonitrile (ACN), iodacetamide (IAA), sodium deoxycholate (SDC), sodium dodecyl sulfate (SDS), sodium laurate (SL), formic acid (FA), thiourea, trifluoroacetic acid (TFA), Trizma® hydrochloride (Tris-HCl), 30 000 kDa MWCO Nanospin filter, trypsin and urea, were purchased from Sigma-Aldrich. Sodium 3-(4-(1,1-bis (hexyloxy) ethyl) pyridinium-1-yl) propane-1-sulfonate (PPS Silent), sodium 3-((1-(furan-2-yl) undecyloxy) carbonylamino) propane-1-sulfonate (ProteaseMSX), sodium 3-[(2-methyl-2-undecyl- 1,3-dioxolan-4-yl) methoxy]-1-propanesulfonate (RapiGest), MagNA Lyser Green Beads and Agilent Bond Elut OMIX C18 tip 100 µL were purchased from Agilent Technologies, Promega, Waters and Roche. Ultra-pure 18.2- MΩ water was obtained from Millipore Milli-Q system. All other reagents were domestic products of highest grade available.

3.2 Tissues

Adipose tissue from hip surgery was acquired through the University Hospital of Northern Norway (UNN). The adipose tissue obtained was immediately washed three times with phosphate-buffered saline, frozen in liquid nitrogen and stored at -20 ^{0}C.

3.3 Instruments

Table 1: Instruments

Instruments	Supplier and address
Biofuge fresco micro-centrifuge	Heraeus instruments, Osterode, Germany
Heidolph™ Reax Top Vortex Mixer	Heidolph instruments, Schwabach, Germany
EASY-nLC 1000 Liquid chromatography	Thermo Fisher Scientific, Bremen, Germany
IKA® Magnetic stirrers with heating, IKA®-Werke GmbH & Co. KG	Staufen, Germany
IKA® MS 1 shaker	IKA laboratory technology, North Carolina, USA
MagNA Lyser	Roche
Methrohm 744-pH meter, Metrohm AG	Metrohm AG, Herisau, Switzerland
Millipore water purification system, 0,22 µm filter	Molsheim, France
Nanodrop® ND-1000 Spectrophotometer	Thermo Fisher Scientific, Bremen, Germany
Q-Exactive hybrid quadrupole -Orbitrap mass spectrometer	Thermo Fisher Scientific, Bremen, Germany
Spectra-max 190 micro-plate Spectrophotometer	Molecular devices, Sunnyvale, California, USA
Termaks TS 8056 laboratory incubator	Bergen, Norway
Bransonic® ultrasonic cleaner	BRANSON, USA

3.4 Methods

3.4.1 Samples names and buffers compositions

Table 2: Buffers compositions

Buffers names	Compositions
ABC-buffer	50 mM ABC and Milli-Q water (pH 8.0)
Urea-buffer	8 M Urea in 100 mM Tris-HCl (pH 8.5)
PPS Silent lysis buffer	0.2% PPS Silent surfactant in ABC buffer
ProteaseMAX lysis buffer	0.2% ProteaseMAX surfactant in ABC buffer
RapiGest lysis buffer	0.2% RapiGest surfactant in ABC buffer
CHAPS-Urea lysis buffer	7 M Urea, 2 M Thiourea, 4% CHAPS, 50 mM Tris-HCl and 10 mM DTT
SDS lysis buffer	1% SDS, 10 mM DTT in 50 mM Tris-HCl
SDC lysis buffer	1% SDC in ABC buffer
SL lysis buffer	1% SL in ABC buffer

3.4.2 Proteins extraction from adipose tissue

For cell lysis 50 mg adipose tissue were placed into MagNA Lyser Green Beads and 500 µl of the different lysis buffer were added (see Table 2). Cell lysis was performed in a MagNA lyser instrument with two cycles of 60 seconds and at a speed of 6500 Hz. After allowing tubes to stand for half an hour, they were centrifuged at 16 000 x g at 5 ^{0}C for 5 minutes. Approximately 200 µl cell lysate was extracted from underneath the lipid bilayer with a syringe and needle. The whole procedure was carried out under cooled conditions.

3.4.3 Protein quantification

After cell lysis protein concentrations of the cell lysates were quantified with the, "Pierce™ BCA Protein Assay Kit" according to manufacturer's protocol. Protein concentration could not be measured for the CHAPS-Urea Sample due to incompatibility of urea with the assay.

3.4.4 Acetone (Ac) and ethanol (EtOH) precipitation

Ac and EtOH precipitation strategies for protein purification were employed and compared. The whole procedure was carried out under cooled conditions.

Ice-cold Ac (100 µl) was added to extracted SDS (25 µl) and CHAPS-Urea (25 µl) samples, vortexed and incubated -20 ^{0}C for 1 hour to precipitate the proteins. After centrifugation at 16 000 x g for 15 minutes at 4 ^{0}C, supernatants were discarded. The pellets were washed once with ice-cold Ac (100 µl) and centrifuged for 10 minutes at 16 000 x g at 4 ^{0}C. Remaining residues of Ac in the vials were evaporated under the fume hood. The pellets were re-suspended in 5 µl 2 M GuHCl by pipetting samples slowly up and down.

Ethanol precipitation (EtOHP) was performed in the same manner as the acetone precipitation (AP), but with 180 µl ethanol and centrifugation at 16 000 x g for 30 minutes and 4^0 C in a pre-cooled centrifuge.

3.4.5 In-solution digestion

For enzymatic digestion of samples containing GuHCl, the samples were diluted to a concentration of 0.1 M GuHCl, which is tolerated by trypsin. All samples were placed on a shaker at low speed, reduced with 10 mM DTT at 56 ^{0}C for 30 minutes and alkylated at room temperature with 20 mM IAA for 30 minutes in the dark.

To increase the efficiency of the digest 1 mM $CaCl_2$ was added. Trypsin was added in an enzyme: protein ratio of 1:20 and mixed into the sample carefully by pipetting slowly up and down. The vials were placed on a shaker at low speed and incubated overnight at 37 ^{0}C for 14 hours.

3.4.6 Filter-aided sample preparation (FASP)

ABC buffer (25 µl) was added to lysates containing SDS (25 µl) and CHAPS-Urea (25 µl). Samples placed on a shaker at low speed were reduced with 10 mM DTT in an incubator at 56 ^{0}C for 30 minutes and alkylated at room temperature with 20 mM IAA for 30 minutes in the dark. The samples were filled up to 200 µl with Urea buffer (Table 3). The spin filter devices were equilibrated once with ABC/Urea buffer (50 µl: 50 µl) before the samples were loaded on the filter. The samples were washed twice with Urea buffer (200 µl and 100 µl respectively), and four times with ABC buffer (100 µl). Afterwards proteins were digested in ABC buffer (100 µl) with trypsin (enzyme: protein ratio 1:20). The samples were mixed carefully on the filter, placed on a shaker at low speed and incubated at 37 ^{0}C for 14 hours. Peptides were eluted by centrifugation; afterwards the filters were washed with ABC buffer (50 µl) and milli-Q water (50 µl) and the flow troughs were combined. All centrifugation steps were performed at 16 000 x g for 20 minutes.

3.4.7 Sample preparation for PPS Silent samples

The cell lysate containing PPS Silent (25 µl) was diluted with ABC buffer to obtain a concentration of 0.1% PPS Silent for digestion. After in-solution digest (see 3.4.5), PPS Silent was hydrolyzed according to the manufacturer's protocol. HCl was added to a final concentration of 250 mM, the cleavage reaction was allowed to proceed for one hour at room temperature and the samples were centrifuged at 16 000 x g for 10 minutes. Supernatants were collected after centrifugation.

3.4.8 Sample preparation for ProteaseMAX samples

The cell lysate containing ProteaseMAX (25 µl) was diluted with ABC buffer to obtain a concentration of 0.05% ProteaseMAX for digestion. After digestion, the samples were centrifuged at 16 000 x g for 10 seconds. TFA was added to a final concentration of 0.5% and

incubated at room temperature for 5 minutes. The mixtures were centrifuged again for 10 minutes at 16 000 x g and the supernatants collected for further analysis.

3.4.9 Sample preparation for RapiGest samples

The cell lysate containing RapiGest (25 µl) was diluted with ABC buffer to obtain a concentration of 0.1% RapiGest for digestion. After digestion, the mixtures were acidified to a final concentration of approximately 0.5% TFA (pH < 2) and incubated at 37 ^{0}C for 45 minutes. Slight cloudiness was observed. The samples were centrifuged at 16 000 x g for 10 minutes, but no precipitation was observed. The solution was transferred to new vial.

3.4.10 Sample preparation for SDC and SL samples

The cell lysate containing SDC and SL (each 25 µl) were diluted with ABC buffer to a concentration of 0.5% for digestion. After digest (according to 3.4.5), SDC and SL were removed by acid precipitation. The procedure was carried out as described before (38) with some modifications. Digested samples were acidified briefly with 1% TFA v/v to about pH 2. The samples were vortexed, allowed to rest for 5 minutes and centrifuged at 16 000 x g for 15 minutes. The supernatants were collected for further analysis.

3.4.11 Sample clean up

Table 3: Overview of buffers for C18 desalting

Washing solution	0.1% TFA
Conditioning solution	50:50 Acetonitrile: Milli-Q water
Elution solution	75:25 Acetonitrile: 0.1% TFA

The samples were adjusted to a concentration of 0.1% TFA (pH about 2). The tips were conditioned twice with conditioning solution (100 µl) and equilibrated twice with washing solution (100 µl). After conditioning, contact of air with the tips was avoided. Pretreated samples were bound by aspirating and dispensing of the samples in 15 cycles in order to gain maximum efficiency. The samples were purified with washing solution (100 µl) once and eluted with 50 µl elution solution.

3.4.12 Peptides quantification

Nanodrop-1000 spectrophotometer was used to determine the absorbance of peptides after digest in all samples. The samples (2 µl) were placed in the spectrophotometer and the absorbance was measured at a wavelength of 205 nm.

The concentration (µg/µl) of peptides was calculated using the following formula:

$$Concentration\left(\frac{\mu g}{\mu l}\right) = \frac{A205}{31 * 0.1}$$

3.4.13 LC-MS/MS analysis

Nano-LC-MS/MS analysis was conducted on a Q-Exactive mass spectrometer coupled to an EASY-nLC 1000. For each sample 1.5 µg peptides were injected, according to concentration measurement with a Nanodrop 1000 spectrophotometer at a wavelength of 205 nm. Peptides were pre-concentrated on a reverse phase trapping column (Acclaim PepMAP® 100, ID 75 µm, length 2 cm, nanoViper, pore size 100 Å, Thermo Fisher Scientific) with 0.1% formic acid (FA) on a flow rate of 20 µl/min, followed by separation on a reverse phase main column (EASY-Spray, PepMAP® RSLC, ID 75 µm, length 50 cm, C18 particle size 2µm, pore size 100 Å, Thermo Fisher Scientific) using a binary gradient (solvent A: 0.1% FA, solvent B: acetonitrile (ACN), 0.1% FA) at a flow rate of 200 nl/min and at 60 ^{0}C. Peptides were eluted with a gradient of 0% ACN to 5% ACN at 19 minute, further to 30% at 180 min and to 100% at 200 min. Subsequently the column was regenerated with 100% ACN for additional 10 minute. Wash gradients were used between the samples for minimizing memory effects. For MS analysis the Q-Exactive mass spectrometer was run in positive more, the global settings were a chromatographic peak width of 15 s and a default charge state of 2. Full MS survey scans from 400 to 2000 m/z were acquired at a resolution of 70.000. AGC values set to 3e^{6} and maximum injection time to 100 minutes for MS scans. The 15 most intense peaks were subjected to MS/MS with a resolution of 17.500. The following settings were chosen: dynamic exclusion 10 second, under fill ratio 1%, charge

states +2, +3, and +4, exclude isotopes, normalized collision energy 28, isolation width 2 m/z, AGC target $5e^4$ and maximum injection time 50 minutes.

3.4.14 Data analysis

The data was analyzed using Thermo Scientific Proteome Discoverer™. Two search engines were used for protein identification (SEQUEST® and Mascot™). The search parameters are shown in Table 4.

GRAVY scores were determined using the Gravy calculator (www.gravy-calculator.de), whereas the predications for trans-membrane domains (TMDs) were carried out using the transmembrane hidden markov model (TMHMM) algorithm. It is accessible at http://www.cbs.dtu.dk/services/TMHMM-2.0/.

Table 4: Search conditions for all search engines, classical FDR and percolator

Proteome Discoverer™	Version 1.4.0.288
Discover Demon	Version 1.4
Mascot™	Version 2.3.0
SEQUEST®	Version 1.4
FASTA	Human (Uniprot / Swissprot)
Peptide tolerance:	10 ppm
Fragment tolerance	0.02 Da
Enzyme	Trypsin, max. 2 missed cleavages
Dynamic modifications: SEQUEST®: Mascot™:	 Oxidation/ +15.995 Da (M) Deamidated / +0.984 Da (N, Q) Oxidation (M) Deamidated (NQ)
Static modifications: SEQUEST®: Mascot™:	 Carbamidomethyl / +57.021 Da (C) Carbamidomethyl (C)
FDR	0.01

4.0 RESULTS AND DISCUSSION

In this study, the protein extraction efficiency and LC-MS/MS performance with different extraction buffers for adipose tissue are evaluated, according to the experimental design summarized in Figure 9. As shown in Figure 13, the experiment was carried out in triplicates.

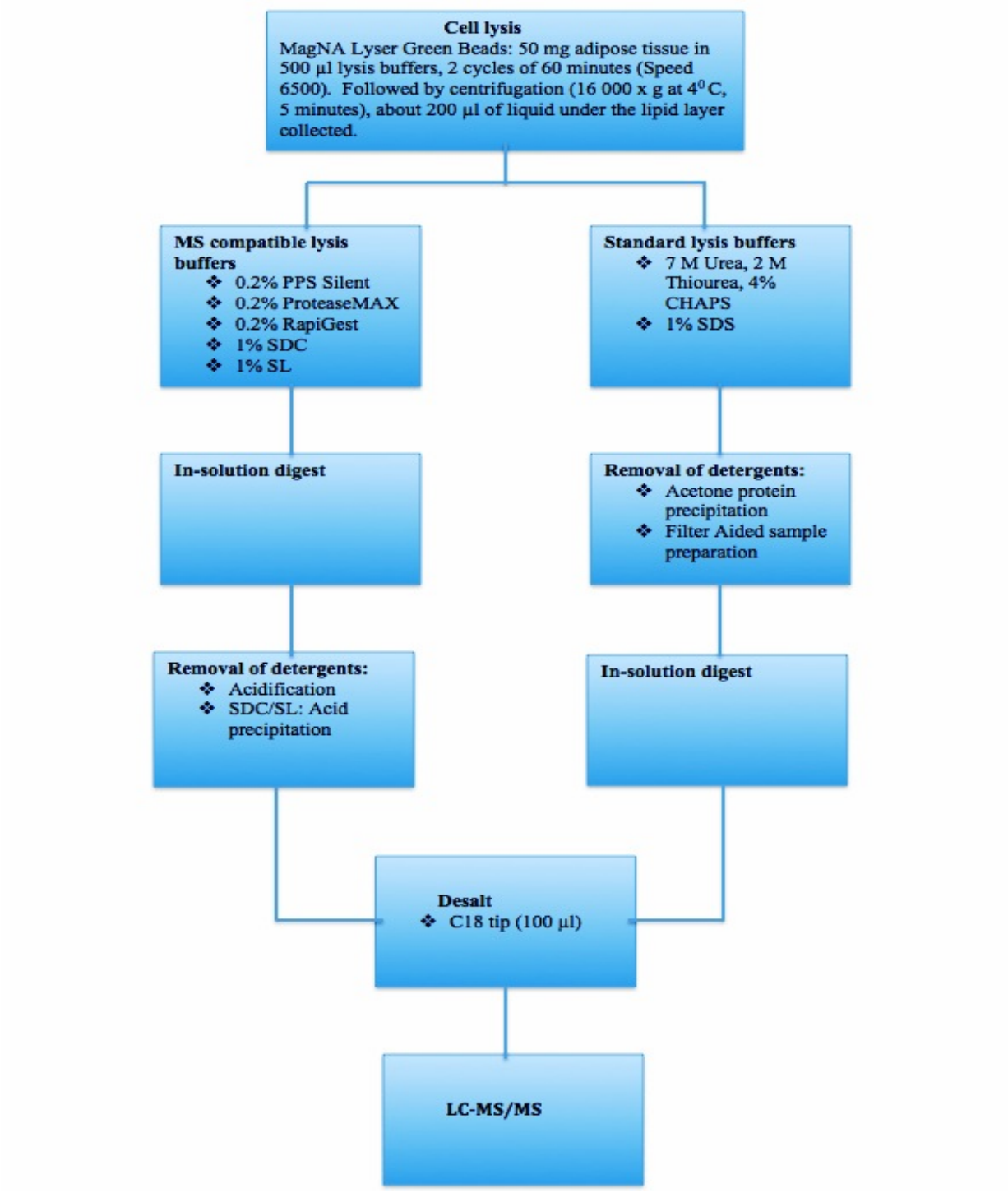


Figure 9: Schematic presentation of the workflow used to compare extraction buffers

4.1 Evaluation of the efficiencies of extraction protein and digestion based on different buffers

Protein extraction and digestion are two of the most important steps in a shotgun based LC-MS/MS proteomics approach. Detergents and additives that have been used for protein extraction and digestion should have a strong ability to disrupt membrane and extract proteins, in particular membrane proteins that are difficult to dissolve. In addition, the detergents should not reduce enzyme activity. Protein identification depends on the ability of a detergent to extract and facilitate digested proteins. For this reason different chaotropes, aqueous-organic solvents, detergents and organic acids have been used in sample preparation (29).

Based on this, the ability of different buffers to extract and digest proteins was compared in this study. The results are shown in Table 5.

Table 5: Comparison of different detergents/additives capacity to extract and digest proteins, standard deviation and total protein yield

Samples	**Protein after cell lysis in 25 µl (µg)**	**Protein after Acetone precipitation (µg)**	**Peptides (µg) after digest**	**Peptides (Standard deviation)**	**Total yield (%)**
PPS Silent	31.61		11.08	0.21	35%
ProteaseMAX	30.92		9.20	0.67	30%
RapiGest	36.41		10.02	0.16	28%
CHAPS-Urea FASP			4.76	1.09	
CHAPS-Urea AP		13.40	10.90	1.21	
SDS FASP	35.36		6.42	1.29	18%
SDS AP		7.07	6.76	2.84	19%
SDC	46.90		11.26	0.45	24%
SL	59.96		11.79	0.13	20%

SDS is one of the most efficient detergents for extraction, solubilizing and denaturing of proteins, especial membrane proteins (31). The limitations with SDS as mention previously, include the incompatibility with MS and the reduction of enzyme activity (32, 33).

In this study, it is demonstrated that SL has a strong ability to extract proteins as well as facilitate the digestion of proteins. Its capacity to extract proteins is even higher than that of SDS as seen in Table 5. Although SL and SDC are similar in structure, SL has higher protein

extraction efficiency than SDC (Table 5). Both detergents consist of a hydrophilic head (carboxyl group), but SL has a long hydrophobic chain similar to SDS, and this might be the reason for the differences in effect, Figure 5 and 6. SL has also previously been shown to have the same ability to extract and solubilize proteins than SDS and a higher ability than of SDC or RapiGest. (37).

In this study, SL and SDC have shown higher abilities to lyse membranes and extract proteins compared to the other detergents (RapiGest, ProteaseMAX, PPS Silent and SDS). As previously mentioned the protein concentration for the Chaps-Urea sample was not measured due to incompatibility with the method (BCA).

The protein amount after acetone precipitation of the SDS was found to be extremely low (Table 5). The low amount was probably due to high protein loss during the precipitation. Because of this, the experiment was done twice and combined prior to MS analysis. Removal of the solvent from the pellet is one of the main factors that affect the amount of protein yield after precipitation. The desire to remove all of the solvents from the pellet might explain the loss of proteins observed, as the pellet was very small and hard to see. Washing the pellet more than once has shown to reduce the concentration of SDS in the sample, which is positive for MS analysis (33). However, in order to conserve as much protein as possible the pellet was washed once prior to digestion. There is a possibility that the amount of SDS in the samples might have interfered with the MS-analysis, although no evidence of this was evident in the chromatography of the sample. As seen in figure 13, SDS AP sample yielded low numbers of proteins and peptides identifications.

Table 5 shows that more mass of peptides (µg) was generated after digestion from the SL, SDC and PPS Silent samples compared to the other samples. This can possibly be explained by that enzyme activity was better in these samples, leaving undigested proteins in the other samples that were then removed upon desalting. The percentages of missed cleavages are low in PPS Silent and ProteaseMAX, but slightly higher in SDS FASP, CHAPS-Urea and SL samples compared with the other samples, Figure 14. Regardless of the high percentages of missed cleavages; SL gives a high peptides yield.

The samples that in general showed a low peptide mass were the CHAPS-Urea FASP, SDS FASP and SDS AP samples. This observation can be explained by several factors, but it is noteworthy that both SDS samples (AP and FASP) showed low yields while for the CHAPS-Urea sample only the FASP sample showed low peptide mass, while the CHAPS-Urea AP showed an close to average peptide mass yield. The most probable explanation for the high losses in the FASP samples is the losses of proteins going through the filter (MW less then filter cut off) and peptides attaching to the filter and hence not being properly removed from the filter after digestion. For the SDS AP sample the strong detergent effect of the SDS can explain the bad yields in the precipitation by interactions between the proteins and the SDS, making the proteins more prone to stay in solution during high acetone concentrations.

Even though, SL, SDC and PPS Silent samples have high peptides yield, PPS Silent (35 %), ProteaseMax (30 %) and RapiGest (28%) have the highest percentage yield. This means that protein loss in these samples from extraction to digestion is less compare with the other samples.

4.2 Comparison between acetone and ethanol precipitation methods

The LC-MS/MS performance of Ac and EtOH based protein precipitation was compared for adipose tissue as written in 3.4.4. The number of proteins identified in each run is shown in Figure 10. The number of protein identifications by the two methods is comparable, but AP strategy identified slightly more proteins than EtOH, both for CHAPS-Urea samples and SDS samples.

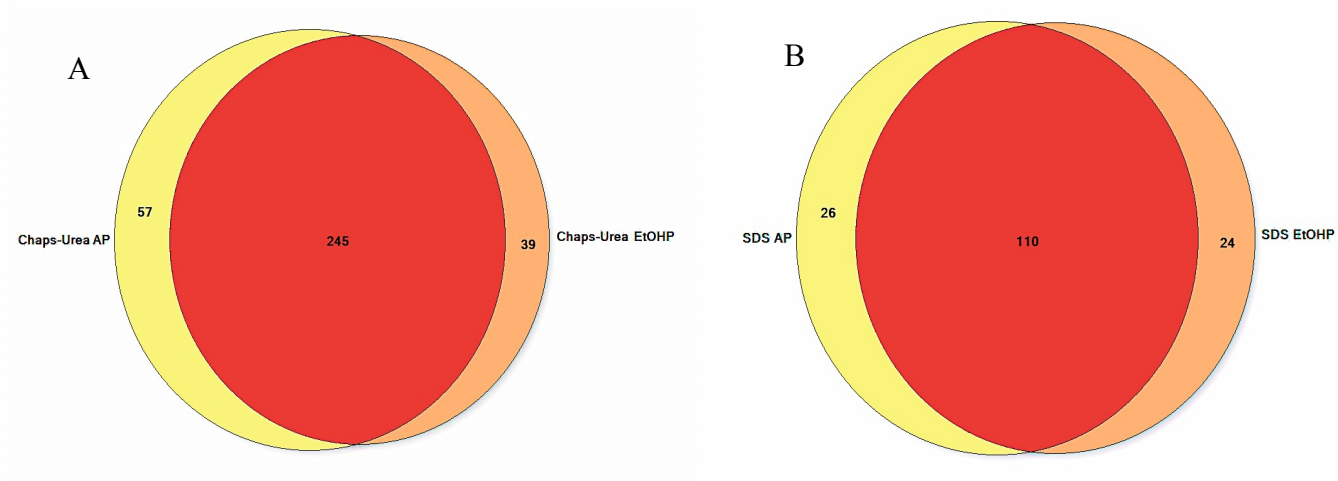


Figure 10: Shows a Venn diagram comparison of protein groups identified with different precipitation methods in A and B

The total number of proteins identified in the CHAPS-Urea AP sample was 302 compare to 284 in the CHAPS-Urea EtOHP, and 136 against 134 in the SDS AP and SDS EtOHP sample.

The Acetone precipitation method was selected for further evaluation based on the results shown in Figure 10.

4.3 Comparative analysis of protein groups and peptides identified by LC-MS/MS based on the sample preparation methods

In this study, we compared different search engines and conditions to reveal the most efficient settings to identify proteins using the Thermo Scientific Proteome Discoverer 1.4. The number of protein groups identified can be seen in Figure 11.

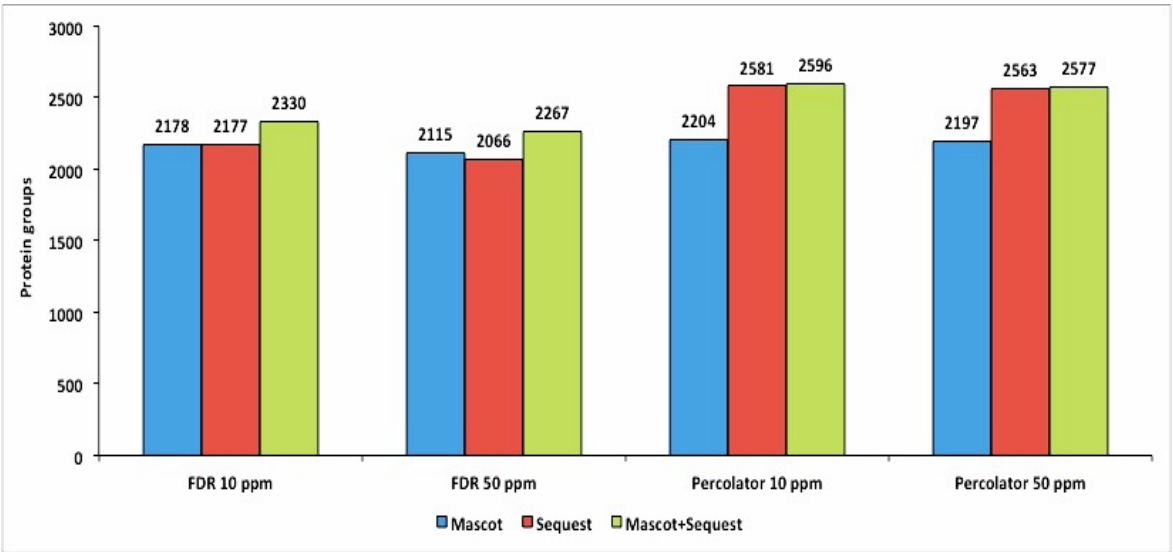


Figure 11: Comparison of the number of protein groups identified by the different analysis strategies and conditions

The following search algorithms were compared. SEQUEST® versus Mascot™ versus SEQUEST® + Mascot™ combined, mass tolerance 10 versus 50 ppm and percolator versus FDR. A 10 ppm mass deviation filter afterwards filtered the results for the 50 ppm mass deviation filter.

SEQUEST® + Mascot™ combined, Percolator and mass tolerance 10 ppm yielded in higher protein group identifications than just one search algorithm (SEQUEST® or Mascot™), FDR and mass tolerance 50 ppm followed as before with a 10 ppm mass deviation filter.

Based on the results, the following workflow was used for processing all raw files: SEQUEST® + Mascot™, mass tolerance 10 ppm and percolator. The workflow used is shown in figure 12.

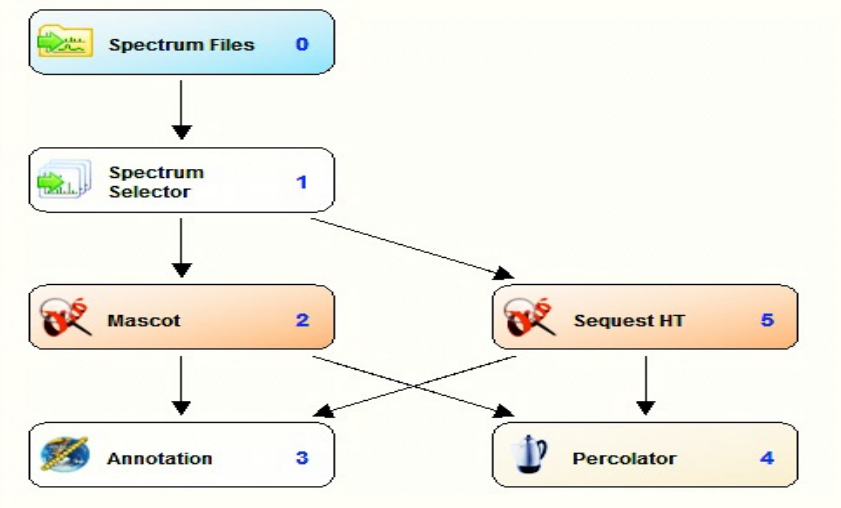
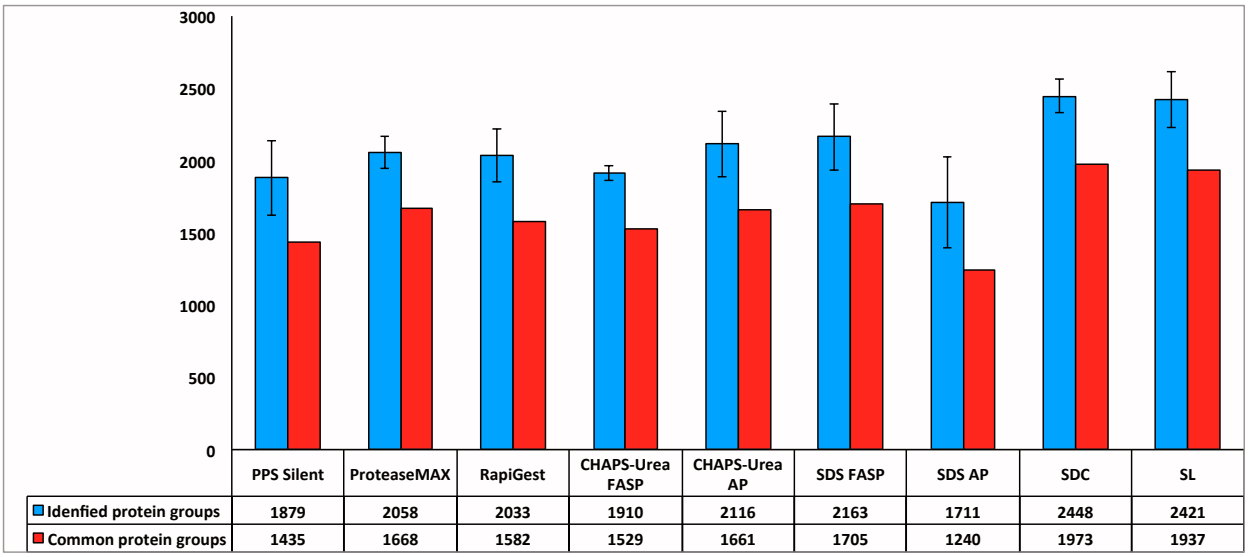


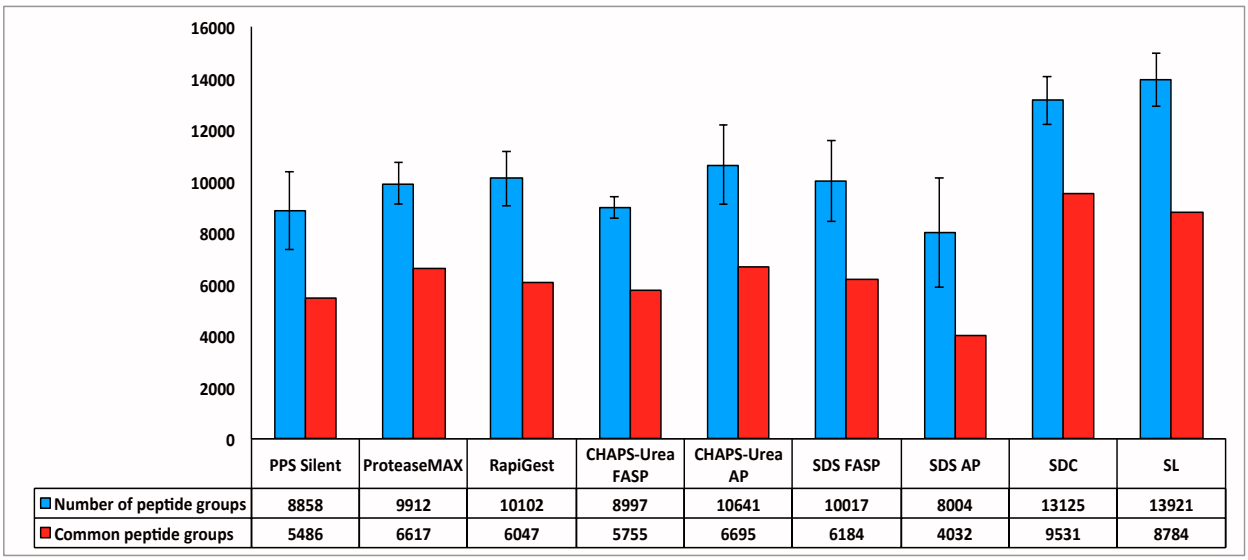
Figure 12: Overview of the Protein Discoverer workflow

LC-MS/MS runs were carried out in triplicates for each sample and the number of proteins groups, peptides and common proteins groups and peptides for all samples can be seen in Figure 13 and 14 respectively.



	PPS Silent	ProteaseMAX	RapiGest	CHAPS-Urea FASP	CHAPS-Urea AP	SDS FASP	SDS AP	SDC	SL
Idenfied protein groups	1879	2058	2033	1910	2116	2163	1711	2448	2421
Common protein groups	1435	1668	1582	1529	1661	1705	1240	1973	1937

Figure 13: Average number of protein groups identified from the triplicates with error bars showing standard deviation (SD) and total number of common proteins groups found in each sample

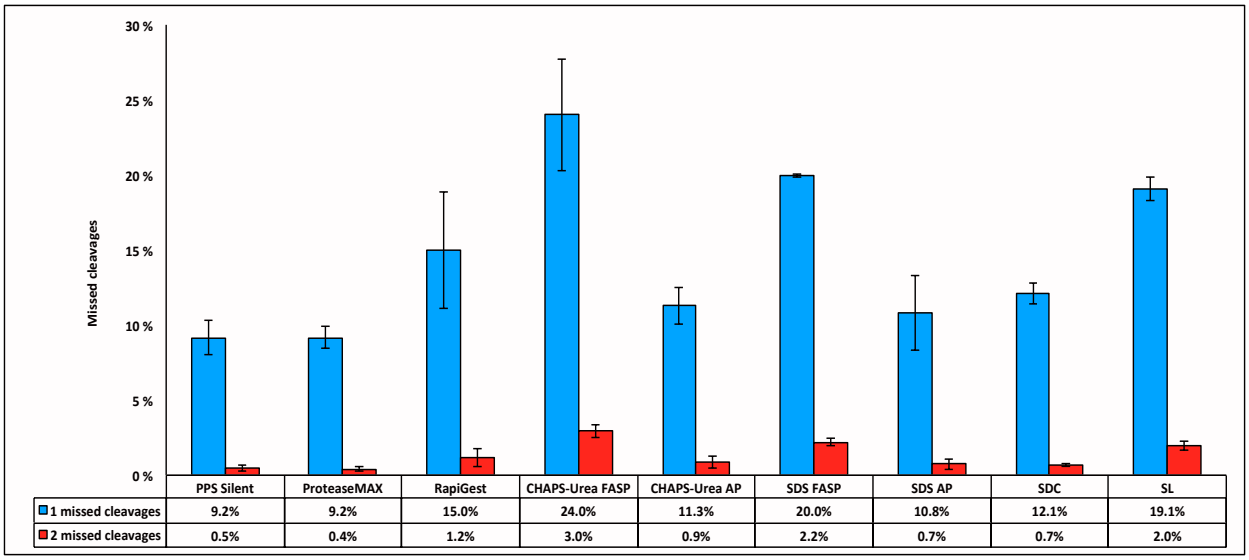


	PPS Silent	ProteaseMAX	RapiGest	CHAPS-Urea FASP	CHAPS-Urea AP	SDS FASP	SDS AP	SDC	SL
Number of peptide groups	8858	9912	10102	8997	10641	10017	8004	13125	13921
Common peptide groups	5486	6617	6047	5755	6695	6184	4032	9531	8784

Figure 14: Average number of peptides identified from the triplicate and total number of common peptides from the SEQUEST® search.

These comparative results demonstrate that SL and SDC are more efficient in the identification of proteins as shown in Figure 13, but the number of peptides identified is slightly higher in SL compared with SDC. PPS Silent and SDS AP have the lowest number of proteins and peptides identified. The number of common proteins and peptides corresponds

with the number of proteins and peptides identified (figure 13 and 14). Higher number of protein and peptides identification gives high common proteins and peptides.



	PPS Silent	ProteaseMAX	RapiGest	CHAPS-Urea FASP	CHAPS-Urea AP	SDS FASP	SDS AP	SDC	SL
1 missed cleavages	9.2%	9.2%	15.0%	24.0%	11.3%	20.0%	10.8%	12.1%	19.1%
2 missed cleavages	0.5%	0.4%	1.2%	3.0%	0.9%	2.2%	0.7%	0.7%	2.0%

Figure 15: Percentages of missed cleavages with error bars showing standard deviation.

The number of missed cleavages in each protein group identified is shown in Figure 15. CHAPS-Urea has the higher percentage of missed cleavages, followed by SDS FASP, SL, RapiGest, SDC, SDS AP, Chaps-Urea AP, PPS Silent and ProteaseMaX. The number of missed cleavages gives an idea of the efficiency of the digest. Samples with high numbers of missed cleavages are often partially digested, resulting in peptides with intact cleavage site.

CHAPS-Urea FASP has the higher percentage of missed cleavages, but as seen in figure 14, it does not yield the lowest number of identified peptides, hence it seems like the number of missed cleavages is not of high importance when it comes to identification of proteins. However, missed cleavages can be a serious problem with regard to quantitative studies and therefore a detergent with a low number of missed cleavages should be chosen when quantitative studies are conducted.

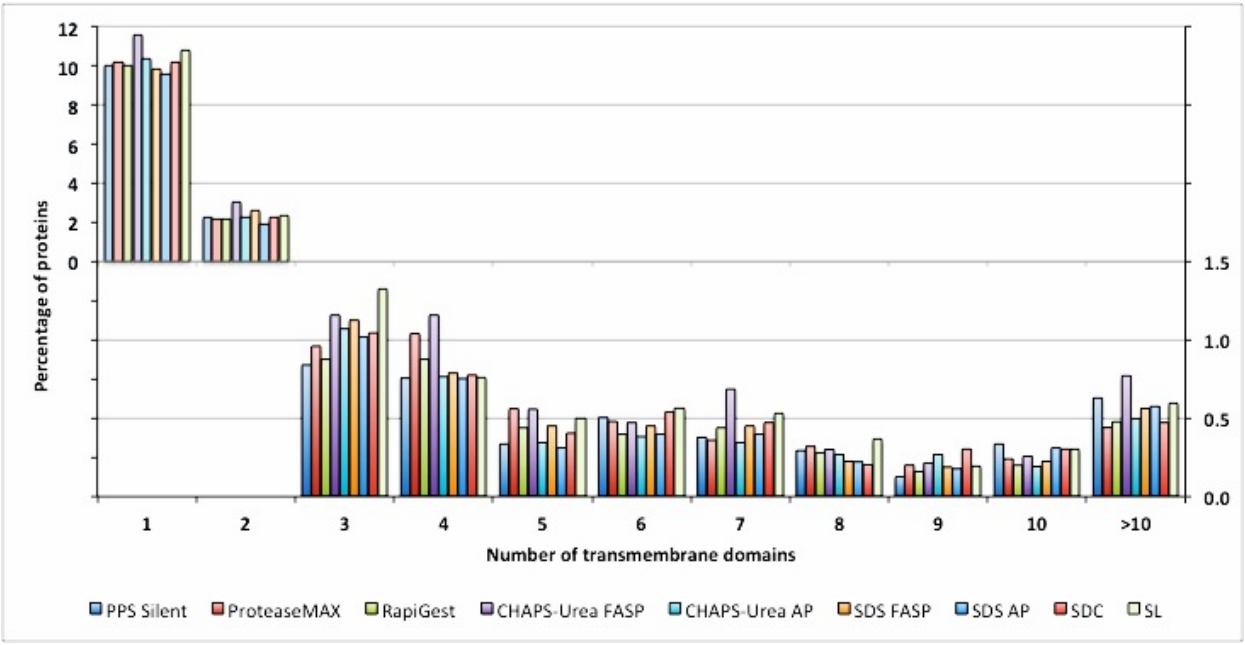


Figure 16: Distribution of proteins identified with predicted transmembrane domains (TMDs)

Transmembrane proteins are difficult to analyze because they contain one or more hydrophobic TMDs. We studied the ability of various buffers to identify proteins with TMDs. The distribution profile of the identified TMDs was compared in Figure 16. The general distribution of TMDs identified in transmembrane proteins is similar in all samples. Most of the transmembrane proteins identified had 1-2 TMDs. Based on the data, CHAPS-Urea FASP and SL were more effective for digestion and identification of proteins containing TMDs compared with the other methods.

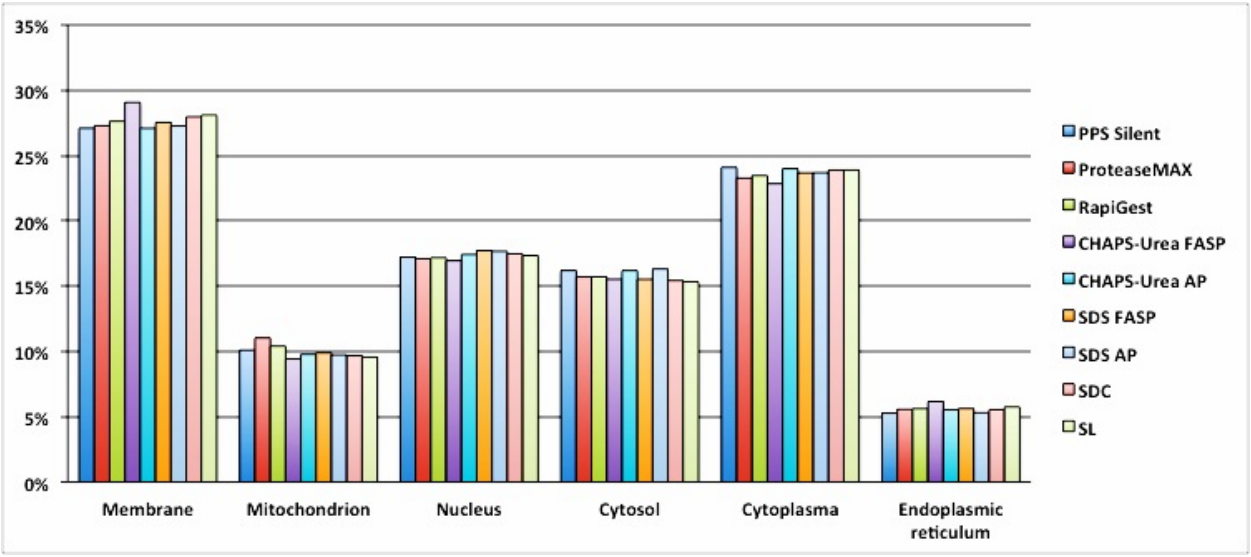


Figure 17: Distribution of the subcellular locations of proteins identified.

The subcellular location of protein changes during cell cycle and under internal (e.g. stress) and external conditions (e.g drugs). Many approaches have been use to study the proteome of cells under different conditions, which is of great interest, because it reveals the functions of the cell and how it responds under different circumstances (49, 50).

In this study, we determine the subcellular distribution of proteins identified in adipose tissue by the different samples, in order to evaluate if the different lysis buffers or strategies facilitated a shift in information about proteins in different subcellular localizations. The percentages of the cellular location of proteins are shown in Figure 17.

There is no marked difference between the samples. Membrane and cytoplasmic proteins were more abundantly detected. CHAPS-Urea FASP samples had a fairly higher percentage of membrane and endoplasmic reticulum proteins followed by SL and SDC. The proportion of cytoplasmic proteins was slightly higher in the PPS Silent and CHAPS-Urea AP samples.

The percentage of mitochondrial proteins identified is scarcely higher in ProteaseMax, followed by RapiGest. Both SDS FASP and SDS AP, followed by SDC, SL and Urea-Chaps AP yielded a high percentage of nucleus proteins. The samples that identified slightly higher cytosolic proteins are PPS Silent and Chaps-Urea FASP.

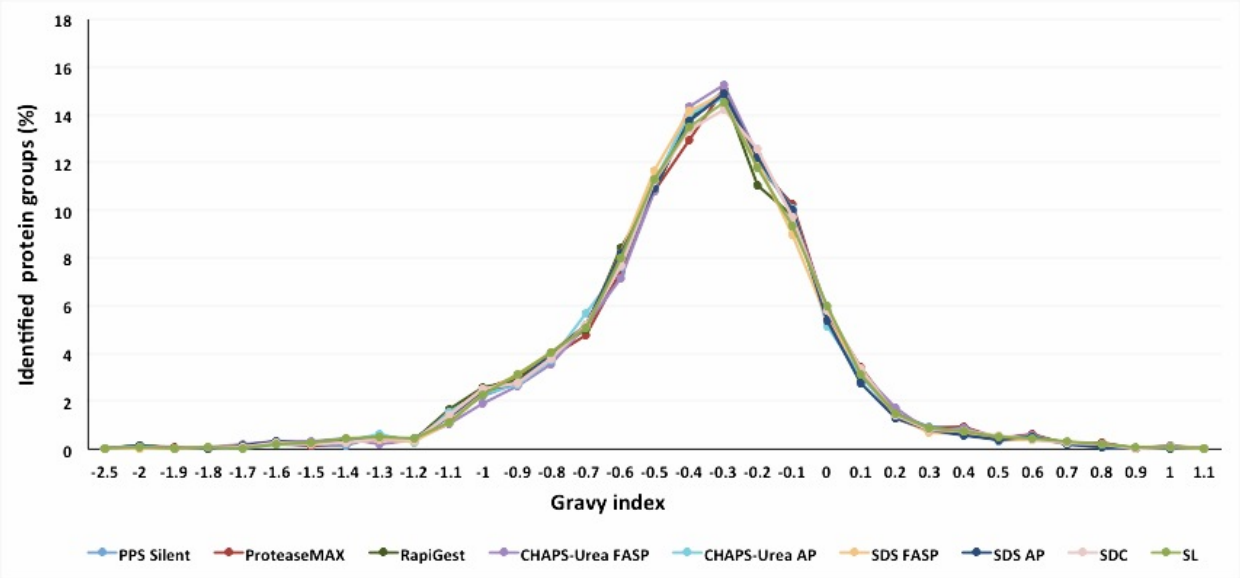


Figure 18: Hydrophobicity distribution of protein groups identified from all samples

The identified protein groups were analyzed using the gravy calculator, and the calculated values are shown in Figure 18. The GRAVY distribution of the proteins seems to be very similar in all the samples. The GRAVY distribution illustrates that the efficiency of all the detergents to solubilize lipophilic proteins is comparable. Proteins groups with negative GRAVY value are referred to as hydrophilic and positive values as hydrophobic. Most of the proteins groups identified have a negative GRAVY value, indicating that a lower number of hydrophobic proteins were detected in all samples. Most of the proteins groups identified have a GRAVY index between -0.5 and 0.

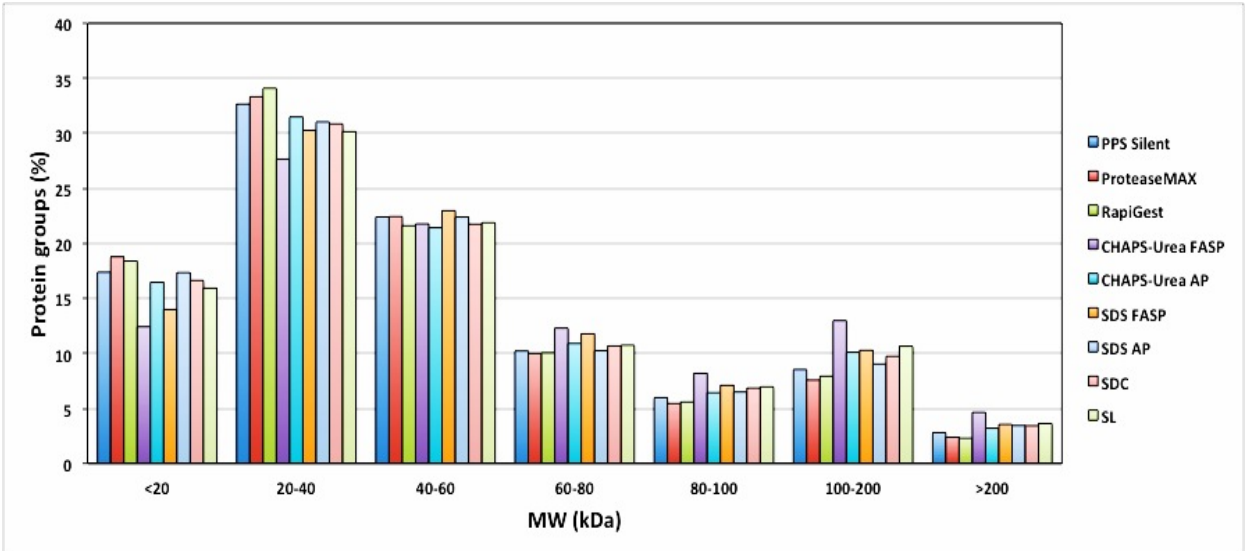


Figure 19: Molecular Weight distribution of identified proteins

Figure 19 shows the comparison of molecular weight distribution in all samples. The majority of the identified protein groups are distributed in the range of 20-40 kDa.

The FASP samples were processed using a 30 kDa molecular weight cut off filter. In theory, smaller proteins than 30 kDa should be washed through the filter and hence lost, but as figure 19 shows some small proteins are also retained. This can be explained by the unfolded nature of proteins so that they can appears to be bigger, or an interaction between the filter and the proteins. The FASP samples yielded to higher percentages of the high MW groups, especially for the CHAPS-Urea FASP sample. This means that the FASP samples identified more high MW proteins compared with the other samples.

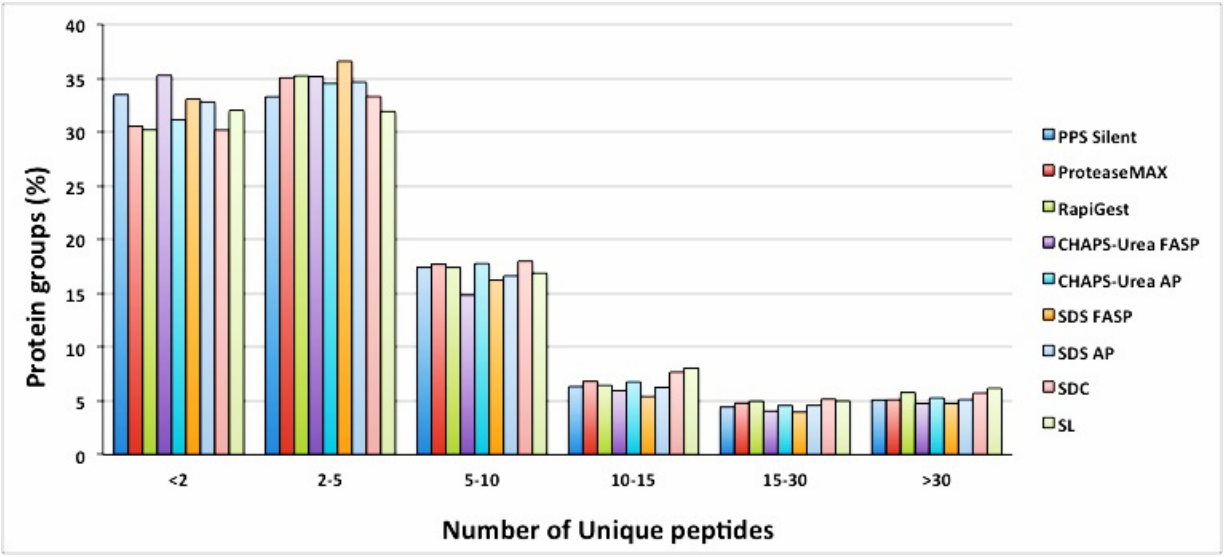


Figure 20: Protein group distribution based on the number of unique peptides identified

To identify proteins based on their peptides implicate some uncertainty, because a peptide can be found in more than one protein. Peptides that are unique to a protein/protein groups increases the confidence of proteins identified. For this reason the distribution of number of unique peptides was analyzed and the result can be seen in Figure 20.

The desired number of unique proteins varies between different types of proteomic experiments; in quantitative studies more than one peptide is usually used to quantify a protein in order to reduce errors. However, in deep proteome sequencing there is a desire to have just one unique peptide per protein in order to reach as deep as possible in the proteome in one run.

The distribution profile or number of unique proteins is similar in all samples. Most of the protein groups identified have 2-5 unique peptides.

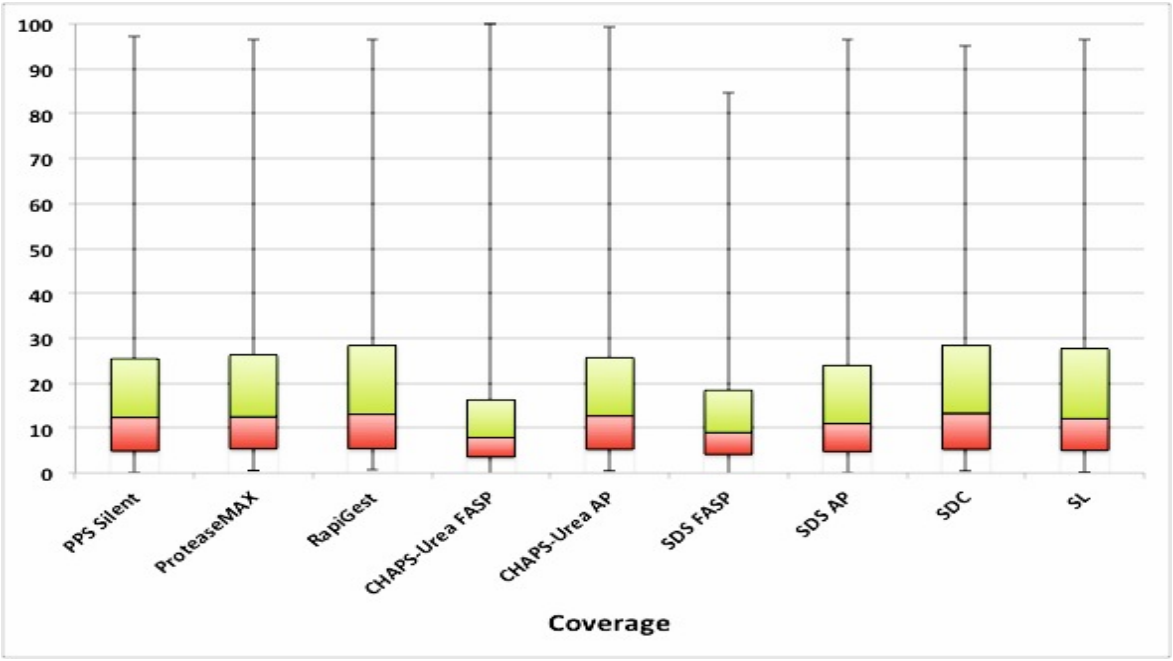


Figure 21: Box and whiskers plots of the average sequence coverage range of the data. There are no outliers in the data

To further characterize the differences between the different methods, box and whisker plots were used to provide a statistical comparison of the coverage of the proteins identified (figure 21). The spread in the data seems to be from almost 0% to over 95%, except for the SDS FASP sample which never returned more than 85% coverage.

The background for this is unknown, but also the average coverage of this sample is lower than the other, together with the CHAPS-Urea FASP sample. Since equal masses of peptides were injected on the LC it seems like the FASP method resulted in fewer proteins and peptides, but higher relative concentration of those proteins or peptides.

5.0 CONCLUSION

This study conducted, first looked at the efficiency of different detergents to extract hydrophobic proteins from adipose tissue. SL has shown to have a strong ability to lyse membrane and solubilize proteins compared with the other methods. Even though SL has a high protein yield, the amount of peptides generated is comparable to that of SDC and PPS Silent. Whiles, PPS Silent, ProteaseMax and RapiGest have the highest amount of Yield (%).

Comparison of AP and EtOHP shows no major difference between the different methods in the SDS samples, but in the CHAPS-Urea samples. Based on the result the acetone precipitation method was thus chosen as the method for further proteins purification. The FASP method using a 30 kDa retained proteins with molecular weight less than 30 kDa in contrary to theory. However, higher molecular weight proteins were identified in the FASP samples compared to the other samples.

From the different search engines and conditions tested, SEQUEST® and Mascot™ combined, with Percolator and a mass tolerance of 10 ppm search gave the best results in identifying proteins compared to the others strategies and conditions examined in this study.

Evaluation of the number of identified proteins, peptides, missed cleavages, TMDs, cellular locations, unique peptides, average coverage, MW and GRAVY index was performed. This comparative study demonstrated that the SL and SDC were superior to that of the other methods for the identification of proteins and peptides, regardless there was no marked difference for the identification of hydrophobic proteins with multiple TMDs.

In summary we have established a method for identification of proteins expressed in the proteome of adipose tissue by LC-MS/MS that can be further developed into a quantitative method for use in the randomized placebo controlled trial of vitamin D. However, further development is needed prior to identification of biomarkers for vitamin D treatment.

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