Chapter 18
Sample Handling of Body Fluids for Proteomics

Joao A. Paulo, Ali R. Vaezzadeh, Darwin L. Conwell, Richard S. Lee, and Hanno Steen

Abstract This chapter provides an overview of different approaches that can be used for sample preparation of body fluids for proteomics. Sample collection, protein extraction, protease inhibitor supplementation, sample storage, and abundant protein depletion are presented here in the context of various human body fluids. We emphasize that the particular set of techniques chosen for such investigations tightly correlates with the fluid to be analyzed, as no consensus methods are appropriate for all body fluids. However, we stress the need for standardized methods for the individual body fluids which is paramount in obtaining reproducible and robust data when analyzing human body fluids. In addition, we provide examples of optimized sample handling techniques using a systemic (urine) and a proximal body fluid (pancreatic fluid).

Keywords Body fluids · Biomarkers · Sample handling · Urine · Pancreas · Pancreatitis · Kidney

18.1 Introduction

Standardization of the preanalytical phase, including sample collection, preparation, and storage must be established prior to sample collection and analysis so that proteomics can be extended to clinical use. Minimizing preanalytical disparities, analytical standardization and quality control measures are imperative for successful proteomic analysis of body fluids aimed at biomarker discovery in both a research setting and for translation into routine clinical testing. Human body fluids (such as

J.A. Paulo (✉)
Division of Gastroenterology, Hepatology and Endoscopy, Center for Pancreatic Disease, Brigham and Women's Hospital and Department of Medicine, Harvard Medical School, Boston, MA 02115, USA; Department of Pathology, Children's Hospital Boston, Harvard Medical School, Boston, MA 02115, USA
e-mail: Joao.Paulo@childrens.harvard.edu

Table 18.1 Common human body fluids

Fluid	Origin	Function
Amniotic fluid	Amniotic sac	Nourishing and protecting fetus
Aqueous humor	Eye	Maintains the intraocular pressure
Bile	Gall bladder/liver	Aids in digestion of lipids
Blood (plasma/serum)	Systemic	Maintenance of cells and tissues
Breast milk	Female breast	Nourishment
Bronchoalveolar lavage	Lungs	Exchange of gases from atmosphere into blood stream
Cerebrospinal fluid	Surrounds spine and brain	Cushion and maintenance
Gastroduodenal fluid	Stomach and duodenum	Digestion
Interstitial fluid	Surrounds extracellular space	Maintenance of cells and tissues
Lymph	Systemic/lymph node	Bathe cells with water and nutrients
Mucus/nasal lavage	Nasal cavity	Protection form infection/foreign agents
Pancreatic fluid	Pancreas	Digestion
Perilymph	Cochlea	Bathes spiral ganglion cell bodies of the auditory nerve
Pleural fluid	Pleural cavity, surrounds lungs	Aids in respiration
Saliva	Mouth	Digestion
Semen	Male gonads	Reproduction
Sweat	Skin	Thermoregulation
Synovial fluid	Synovial joints	Reduce friction between the articular cartilage
Tears	Eye	Clean and lubricate the eyes
Urine	Urinary system	Waste removal
Vaginal secretion	Vagina	Maintain vaginal moisture
Vitreous fluid	Eye	Maintains pressure between lens and retina

See references in text.

those listed in Table 18.1) may be a more attractive option than tissue biopsies for the diagnosis and prognosis of diseases as obtaining body fluid has the advantage of being less invasive, less costly, easier to collect and possibly easier to process. Body fluids are excellent sources of protein biomarkers because during their circulation, they come in contact with a variety of tissues and pick up proteins secreted or shed by tissues.

Despite the attractiveness of body fluids for biomarker discovery, the task of their quantitative analysis using proteomics technologies is challenging. Body fluids, such as plasma, are rich and complex reservoirs of proteins. Large differences in protein concentration (ranging from several milligrams to less than 1 pg/mL) and the high number of posttranslational modifications are among the challenges of body fluids proteomics. Other challenges are related to the development of standardized sample preparation and handling protocols. High variation in body fluids increases the necessity of standardized protocols and large sample cohorts to obtain statistically viable biological conclusions.

18.1.1 Proper Handling of Body Fluids
for Proteomics Is Essential

Establishing clear and consistent sample collection and processing methodologies
is an initial stage in the development of clinical proteomics assays. In translational
research, there often are insufficiently standardized protocols in regards to speci-
men collection, storage and processing. In urine proteomics, for example, several
articles have stressed the importance of standardized sample handling in reducing
experimental variability (Barratt and Topham, 2007; Decramer et al., 2008; Hortin
and Sviridov, 2007; Muller and Brenner, 2006; Munro et al., 2006; Thongboonkerd,
2007, 2008). Similarly, for blood and serum, there are ongoing efforts to optimize
sample handling protocols (Barelli et al., 2007; Hsieh et al., 2006; Luque-Garcia
and Neubert, 2007; Rai and Vitzthum, 2006). Significant changes in the proteomic
profile may be introduced during sample preparation if a consensus methodology
is not in place. In biomarker discovery, these procedural artifacts may significantly
impair the experimentation and analysis. All clinical and sample variables cannot be
entirely avoided; however, it should be kept to a minimum with standardized sample
handling methodologies.

18.1.2 Systemic and Proximal Body Fluids

In broad terms, there are two types of body fluids, proximal and systemic. Many
body fluids, such as urine, can be considered both. Both proximal and systemic
fluids have their advantages and disadvantages. A proximal fluid is the immediate
down-stream body fluid of a particular organ or disease. In contrast, systemic fluids,
such as blood or urine, can represent the entire body and may provide a snapshot
of the organism under a given set of conditions or disease. The majority of the
proteins detected in a systemic fluid may not necessarily be directly related to the
disease of interest (Issaq et al., 2007); and systemic fluid analysis may identify the
response of non-primary organs to the disease. For example, children with obstruc-
tive sleep apnea may have changes in renal function that can be detected in the
urinary proteome (Gozal et al., 2009).

The human blood/serum proteome originates from a variety of tissues as a result
of secretion or shedding, and can reflect human physiological states which can be
used for disease diagnosis and prognosis (Anderson et al., 2004). As blood comes
in contact with all organs of the body, it is a source of many potential biomarkers.
However, such biomarkers may be less specific than those obtained from proxi-
mal organ fluids, as the origin of suspected biomarkers may be difficult to attain.
Like other body fluids, blood contains proteases, cells, and lipids, and their removal
or deactivation may be necessary for protein analysis. The non-cellular fraction of
blood represents proteins essential for circulatory functions, organ secretions that
are released into circulation, and diseases such as myocardial infarctions (Omenn
et al., 2005). Blood (both serum and plasma) has one of the highest dynamic ranges

of any body fluid, with a protein concentration spanning over 10 orders of magnitude (Anderson and Anderson, 2002).

Urine can be considered both a systemic and proximal fluid. Specifically, urine represents the metabolic end product of blood, but it is also influenced by the status of the kidney and the lower urinary tract. It is estimated that up to 70% of the urinary proteome may originate from the urinary tract and kidney (Mischak et al., 2007). Urine is often considered the ideal body fluid source for diseases of the urogenital tract and kidney; however, at the same time, a large number of the proteins identified may be originating from other organ systems.

The potential advantage of analyzing a *proximal* body fluid is that it will increase the likelihood of biomarker discovery in the context of particular diseased organ (Rifai et al., 2006). For example, as cerebrospinal fluid (CSF) is in direct contact with the extracellular surface of the brain, its biochemical composition is altered by disorders related to the central nervous system. Synovial fluid, which is the fluid present in joints, has been used in the diagnosis of rheumatoid arthritis and similar inflammation. Synovial fluid reduces friction between cartilage and other tissues and may reflect that pathophysiological conditions of joints. Its investigation may result in effective markers of rheumatoid arthritis and osteoarthritis (De Ceuninck and Berenbaum, 2009; Wilson et al., 2009).

Although serum is typically considered to be a systemic fluid, in certain contexts it can be a proximal fluid. One particular example is of blood obtained directly from the coronary sinus of the heart, which is the collection of veins that collects blood from the myocardium of the heart. Blood obtained from this source may provide new insights into myocardial damage and ischemia (Gerszten and Wang, 2008). Other proximal fluids of interest include pleural fluid, which is the fluid between the layers of tissue surrounding the lungs. Abnormal accumulation of pleural fluid is a potential rich source of protein analysis for primary lung and pleural disease (Tyan et al., 2005a,b). Bronchoalveolar lavage (BAL) fluid is another potential source of proximal fluid for pulmonary disease, such as asthma. BAL fluid analysis demonstrates a great diversity of cellular origins and functions, and a comparative analysis of serum and bronchoalveolar lavage proteomes revealed that some proteins were more abundant in bronchoalveolar lavage than in plasma, suggesting that they are specifically produced in the airways (Noel-Georis et al., 2001; Wattiez and Falmagne, 2005). Nipple aspirates is another promising proximal fluid to identify biomarkers for breast cancer diagnosis and therapy. The analysis of tear fluid for specific eye disorders such as Sjogren's syndrome and dry eye syndrome is another example (Nguyen and Peck, 2009; Tomosugi et al., 2005).

Proteomic analysis of saliva may represent the proximal fluid of the upper airway and organs of the mouth and offers a rapid, simple and non-invasive method for short- and long-term monitoring of pathological disorders, such as oral squamous cell carcinoma (Wu et al., 2010) and periodontitis (Haigh et al., 2010). Pancreatic fluid is an excellent clinical specimen for identification of novel biomarkers of the upper gastrointestinal tract and pancreas, as its protein composition is of lower complexity compared to serum (Paulo et al., 2011). Pancreatic fluid primarily consists of fluid originating from the exocrine pancreas function, and may be an ideal fluid

source for the identification of novel biomarkers of pancreatitis and pancreatic ade-nocarcinoma (Grote and Logsdon, 2007; Lohr and Faissner, 2004; Wandschneider et al., 2001).

18.1.3 Sample Accessibility

While systemic fluids are often more easily accessible, proximal fluids may pro-vide a more immediate view of an organ's microenvironment at the expense of invasiveness. Although blood and urine can be accessed by relatively noninvasive means and allow for analysis of non-diseased patients for comparative proteomic studies, it may be difficult to obtain many other proximal body fluids from/for healthy controls. Fluid accessibility via noninvasive or minimally invasive meth-ods is imperative when performing comparative analyses that require sampling of a corresponding body fluid from a healthy individual. While some fluids are easy to access, such as urine, saliva, and blood, other fluids may be more difficult to acquire, necessitate well-trained individuals, and have inherent associated risks. Such less accessible proximal fluids, including cerebrospinal fluid, pleural fluid and synovial fluid must often be acquired only from individuals who are suspected of having the corresponding disease. Appropriate controls are sorely needed; however, controls usually consist of those with clinical suspicion of the disease, but whose diagnosis was negative. For example, in the study of pancreatic fluid and bile, fluid collection via ERCP (endoscopic retrograde cholangiopancreatography) is not recommended on a healthy individual due to the significant risk of complications which may arise and result in acute pancreatitis, and is usually only performed if underlying gas-trointestinal dysfunction is suspected (Crnogorac-Jurcevic et al., 2005; Lohr and Faissner, 2004). CSF collection involves a lumbar puncture, which may result in complications (Pendyala et al., 2009; Xiao et al., 2009). For such investigations, normal or healthy controls must be culled from patients who have a negative result for a particular test of that disease.

Proximal body fluids considered easily accessible include BAL fluid, urine (in specific diseases), nipple aspirates, tear fluid, and saliva. For example, BAL can be noninvasively obtained by the inhalation of a soluble hypotonic aerosol to induce expectoration (Beier et al., 2004). Collection of these body fluids is not associated with high risks to the patients, and normal controls may be more easily obtained. Overall, sample accessibility must be taken into consideration in the study design prior to undertaking any proteomic investigations.

18.1.4 Sample Variability

The composition of body fluids may be influenced by environmental factors, age, gender, sex, and confounding diseases, which may result in substantial subject-to-subject variability. Factors affecting composition of many body fluids, such as saliva, urine, pancreatic fluid, bile, and serum include circadian rhythm, health

status, exercise, medications, and food intake. For example, in a study of the cerebrospinal fluid proteomes from 10 individuals, 38% of the identified proteins were unique to individual subjects, whereas only 6% were common among all ten subjects (Wenner et al., 2004). Similarly, studies of human breast milk demonstrate a relationship between a mother's nutrition and milk proteome composition (Cavaletto et al., 2004). At the same time, many of the common milk proteins, such as immunoglobulins, casein, and serum albumin, are often modified by posttranslational modifications or proteolytic processes (D'Auria et al., 2005).

Urine is a prime example of how confounding factors can influence protein content. Urine is the metabolic end product of blood which can be affected by renal function. It is estimated that 70% of proteins from urine originate from the kidney, whereas the remaining proteins are derived from plasma (Mischak et al., 2007). The protein concentration of urine is also dependent on stability, binding, and clearance of small proteins that are usually reabsorbed into the blood via normal renal function. Typically, it is thought that the highest concentration of protein in urine is usually in the morning; however, this may be contaminated by bacterial protein contamination, protein degradation in the bladder, and longer storage times in the bladder. As such, the second urine of the day may be a more useful and less variable specimen for proteomic analysis. When investigating and designing discovery based proteomics studies of human body fluids, sample variability must be taken into consideration. Large individual and biological variability requires a larger sample size due and further highlights the need for standardized collection methodologies.

18.1.5 Fluid Mixing

Along with patient and environmental variability, certain body fluids are composed of mixtures from different glands, each of which if isolated separately may have its own unique proteome. For example, human saliva is secreted from multiple salivary glands including parotid, submandibular, sublingual, and other minor glands lying beneath the oral mucosa. Although most proteins isolated from saliva originate from the salivary glands, some blood (Huang, 2004), oral tissue (Kojima et al., 2000) and bacterial (Macarthur and Jacques, 2003) proteins have also been readily identified in this fluid. Seminal fluid is another complex mixture consisting of spermatozoa suspended within secretions of male sex glands including the seminal vesicles and prostate. Similarly, cervical–vaginal fluid, which has been analyzed to identify antimicrobial peptides, is composed of a mixture of different secretions (Di Quinzio et al., 2007; Shaw et al., 2007; Tang et al., 2007). In addition, the study of gastrointestinal diseases, such as pancreatitis, often involve the collection of fluid which is to some degree a mixture of gastric fluid, duodenal fluid, pancreatic fluid and bile (Paulo et al., 2010a). Amniotic fluid is another example of natural mixing of body fluids. Amniotic fluid surrounds the developing fetus and plays a crucial role in normal development. Amniotic fluid is a mixture of fetal and maternal proteins and corresponding degradation products. The composition of this body fluid includes proteins exchanged between mother and fetus, proteins excreted by the

fetus, and proteins from the maternal placenta. Along with the caveats of accessibility and variability, the innate mixing of different fluids may be unavoidable and must be considered throughout the body fluid analysis process.

18.2 Sample Preparation

No single sample preparation method is optimal for all body fluids. However, standardization of methods should be established at the clinical collection interface and the laboratory for each body fluid to avoid introducing potential confounders. Collection methods may vary depending on the location, or even the person who is acquiring the sample. Along with differences in collection methods, temporal and environmental (e.g. temperature of transport) differences in transporting the fluid from the clinic to the bench may result in the loss of reproducibility. Efforts should be made to standardize the protocols used to obtain the sample so as to increase the accuracy and reproducibility of the data.

18.2.1 Protein Extraction and Fractionation

The optimal method of protein extraction is highly dependent on the sample under investigation. Sample fractionation can be performed using a variety of methods including centrifugation, precipitation, liquid chromatography and electrophoresis. It is not uncommon, that for many body fluids, a large number of intracellular proteins are identified due to shedding of cells into the interstitial fluid, which is a common source of confounding proteins. At times, whole cells may be present, and can be removed via centrifugation prior to further sample processing. For body fluid proteomic analyses, proteins should be segregated from lipids, metabolites, and other non-proteinaceous substances. Most often, salt removal is accomplished via dialysis (spin, micro) (Manabe et al., 1999), ultrafiltration (Fountoulakis et al., 2004; Jiang et al., 2004), gel filtration/electrophoresis, precipitation with TCA or organic solvents, and/or solid-phase extraction.

18.2.1.1 Centrifugation

One of the simplest methods of protein enrichment is ultracentrifugation using sequentially increasing centrifugal forces, or sucrose gradients, which permits separation at various subcellular levels. In addition to isolating proteins from membranes, mitochondria, or nuclei, differential centrifugation can also investigate vesicles such as exosomes, which may be an alternative source of biomarkers. Exosomes are small vesicles secreted by cells and may be a mechanism for selective removal of many plasma membrane proteins (Simpson et al., 2009). Recently, a nano-LC-MS/MS analysis identified 295 proteins in urinary exosomes, including multiple proteins known to be involved in renal and systemic diseases. Urinary

exosomes may be a rich source for disease biomarker discovery in urine (Gonzales et al., 2009; Pisitkun et al., 2004).

18.2.1.2 Precipitation

Several precipitation methods are available for protein isolation, these include acetone, trichloroacetic acid, ethanol, isopropanol, chloroform/methanol, and ammonium sulfate. The efficiency of protein precipitation varies among different organic solvents. For example, acetone appears to precipitate more acidic and hydrophilic proteins, whereas ultracentrifugation fractionates more basic, hydrophobic, and membrane proteins (Thongboonkerd et al., 2002). Alternatively, chloroform methanol extraction has been used to successfully extract hydrophobic proteins in human bile (Stark et al., 1999). For comparison, precipitation with 10% TCA/90% acetone/20 mM DTT is well-suited for salivary proteomics (Hu et al., 2005). Whereas, using 0.02% sodium bisulfate in ethanol and acetic acid (1:1, v/v) has been successful in protein extraction of sinonasal lavage, which has been used to investigate the effects of smoking on the nasal cavity proteins of smokers and non-smokers (Casado et al., 2005). Moreover, we have determined that trichloroacetic acid and acetone precipitation extracted the most proteins from pancreatic fluid and gastroduodenal fluid, respectively (Paulo et al., 2010b, c). Again, due to the intrinsic differences in the composition of the body fluid, such protein precipitation methodologies must be investigated for the particular fluid of interest and for the specific types of proteins that are to be isolated.

18.2.1.3 Depletion of Abundant Proteins

Due to sample complexity and the high dynamic range in some body fluids, depletion of abundant proteins may be necessary (Hortin et al., 2006). The depletion of proteins of relatively high abundance, such as those listed in Table 18.2, is advantageous for biomarker discovery. For example, albumin and immunoglobulins (main constituents of blood and urine) account for 80% of vitreous fluid proteins, which if

Table 18.2 Most abundant plasma proteins (Heide et al., 1997)

Protein	mg/mL
Albumin	50
IgG, IgA, IgM	12–15
Fibrinogen	3
α-, β-lipoproteins	2–6
α1-antitrypsin	2–5
α2-macroglobulin	2–4
Transferrin	2–3
α-1-acid glycoprotein	1
Hemopexin	1
α-lipoproteins	0.6–1.5
Haptoglobin	3
Ceruloplasmin	0.3

not depleted, may prevent the identification of lower abundant proteins (Koyama et al., 2003). Albumin is often depleted from serum or plasma using dye-based columns. Traditionally, Cibachrome Blue dye is used to deplete albumin (Thresher and Swaisgood, 1990). However, as this dye is nonspecific, monoclonal antibodies may be a better alternative (Bjorhall et al., 2005): anti-human serum albumin columns, for instance, have been shown to be more efficient and specific in the depletion of albumin from blood (Echan et al., 2005). Independently IgG is often removed with an immobilized protein A column.

In addition to albumin and IgG, there are other abundant proteins that are also often depleted to improve identification of low abundant proteins. For example, the most abundant proteins (human serum albumin, transferrin, IgG, IgA, IgM, fibrinogen, and hemoglobin) in CSF and blood are often immunodepleted to improve the identification of less abundant proteins (Hortin et al., 2006; Thouvenot et al., 2008). Multiple affinity removal columns that remove up to 7–14 of the most abundant proteins simultaneously are available (MARS – Multiple Affinity Removal System, Agilent) (Pieper et al., 2003; Bjorhall et al., 2005).

However, the disadvantage of removing albumin is that albumin functions as a carrier protein and its removal may result in the loss or co-depletion of other significant proteins (Zhou et al., 2004). Additionally, each additive step in the workflow may also lead to more sample loss and variability. In one study using blood, the depletion of albumin was associated with the additional reduction of 815 proteins possibly from co-depletion (Shen et al., 2005). Several other studies suggest that albumin binds to certain low-molecular weight proteins and peptides, which are lost while albumin is depleted (Decramer et al., 2008; Zolotarjova et al., 2005). To address the problem of co-depletion, addition of 5 to 20% acetonitrile may be useful in disrupting the binding of albumin to lower molecular weight proteins that may be otherwise lost during albumin depletion (Huang et al., 2005). Alternatively, albumin may be used to isolate small bound proteins, by specifically binding albumin under normal conditions to a solid matrix and releasing bound peptides for analysis via mass spectrometry (Lowenthal et al., 2005). Disrupting these protein–protein interactions by ultracentrifugation with detergents and/or chaotrophic agents can also help to avoid protein loss.

Currently, the vast majority of depletion methods have been developed specifically for serum. Adapting these methods to other body fluids, such as urine, can be challenging because of the inherent differences in each body fluid. The particular body fluid under investigation may contain a lower concentration of the high-abundant proteins of serum, or alternatively, other abundant proteins. The decision of whether to deplete proteins, and if so, with what modality, must be determined with the particular study and body fluid in mind.

18.2.2 Protease Inhibitors Supplementation

The use of protease inhibitors is assay-specific as their use may be detrimental to particular experiments. For example, certain protease inhibitors may interfere

with bottom-up proteomic experiments (particularly for in-solution digests) where specific enzymatic digestion (e.g., with trypsin) is necessary. Protease inhibitors should not be added to peptidomics studies aiming to investigate the temporal changes of enzyme activity (Ivanov and Yatskin, 2005; Schulz-Knappe et al., 2005). Although, the addition of specific protease inhibitors (e.g. phenylmethylsulfonyl fluoride (PMSF), aminoethyl benzylsulfonyl fluoride (AEBSF), ethylene diamine tetraacetic acid (EDTA), pepstatin, benzamidine, leupeptin, aprotinin) may be recommended in particular situations, it should be done so with caution, as inhibitors may modify proteins, introduce adducts, and interfere with further peptide studies. For example, certain peptide inhibitors, such as high-concentration aprotinin, may interfere with MS analysis and several small molecule inhibitors, such as PMSF and AEBSF have been shown to form covalent bonds with proteins (Finnie and Svensson, 2002), thereby changing pI and electrophoretic mobility (Rai et al., 2005). In addition, many protease inhibitor cocktails contain small molecule or peptide inhibitors, which can interfere with subsequent peptide ionization (Marshall et al., 2003).

Conversely, the addition of protease inhibitors might be advantageous for sample preservation during other applications, such as in-gel tryptic digests, in which low molecular weight protease inhibitors could be separated from the proteins and/or inactivated during SDS-PAGE. Body fluid that are shipped off site or are at risk for fragmentation because of temperature fluctuations during transport may require protease inhibitors during transport. The use of protease inhibitors should be tested to determine their need for each particular application or clinical sample situation.

18.2.3 Storage Conditions and Protein Stability

Both long- and short-term storage of body fluids may have an impact on sample quality. For example, blood is typically incubated at room temperature to allow for the initiation of the coagulation cascade. After clotting, the blood can be centrifuged to separate the serum and plasma components. However, significant variation of serum composition can occur secondary to variations of clotting time and temperature exposure. Plasma may be more stable by the nature of its collection process. To obtain plasma, various anticoagulants, such as heparin, citrate and EDTA (Lundblad, 2004) are added in defined amounts. Currently, HUPO (Human Proteome Organization) suggests the use of plasma with EDTA over serum because of the stability of plasma. Studies have shown improved resolution, sensitivity and reproducibility of protein identifications via tandem mass spectrometry in plasma verses serum (Omenn et al., 2005).

In addition to natural inherent variables of samples, multiple freeze–thaw cycles can have a detrimental effect on proteome stability. Freeze–thaw cycles can induce precipitation, as has been shown in urine and saliva (Nurkka et al., 2003). Even though proteins from a particular fluid may be resistant to degradation or precipitation resulting from freeze–thaw cycles, it may be of additional benefit to store

samples for extended periods in smaller aliquots to minimize, or essentially eliminate, the need for multiple sample thaws. Several studies have been performed to investigate the stability of urine, which has a lower complexity and relatively high thermostability compared to serum, under different storage conditions. In urine proteomics, for example, several recent reviews have stressed the importance of standardized sample storage conditions in reducing experimental variability (Barratt and Topham, 2007; Decramer et al., 2008; Hortin and Sviridov, 2007; Muller and Brenner, 2006; Munro et al., 2006; Thongboonkerd, 2007, 2008). For pancreatic fluid, such variations are especially pronounced due to its inherent high concentration of active proteolytic enzymes, and for which proteolysis is evident after 30 min at room temperature on SDS-PAGE gels. In addition, proteins in particular are subject to modifications, degradation, precipitation, adductions (e.g. acetylation, oxidation), which again can confound studies aimed to identify biomarkers. Storage conditions may vary with the body fluid under investigation, but a cautious and conservative approach should be considered until all variables are suitably investigated. Currently, we recommend minimal transport time, transport at low temperatures, separating clinical samples into experiment specific single use aliquots, and long term storage at –80°C.

18.2.4 Sample Analysis

There are numerous different methodologies that can be used to perform sample analysis. Each method has its advantages and disadvantages in the context of the particular body fluid analysis. Typically the down-stream needs of the workflow and complexity of the sample will determine the optimal method of sample analysis. We provide examples of different methodologies.

18.2.4.1 Protein Fractionation

The substantial complexity of body fluid proteomes requires multiple dimensions of separation in order to be resolved and comprehensively studied (as briefly mentioned in Section 2.1). Separation is usually based on different physicochemical properties of the species and can be performed at the protein level (prior to proteolytic digestion), at the peptide level (post-proteolytic digest), or both. A diverse range of separation methods, which can be used in tandem are available to reduce the complexity of a given body fluid sample.

To reduce sample complexity at the protein level, proteins can be fractionated by differential centrifugations, size exclusion chromatography or centrifugation, reversed-phase liquid chromatography, or gel electrophoresis. A combination of these methods can be utilized if further fractionation is necessary due to sample complexity.

GeLC-MS/MS is one of the most common methods of separation. In GeLC-MS/MS, one-dimensional SDS-PAGE gel electrophoresis is initially performed to fractionate proteins by electrophoretic mobility/molecular weight. The entire gel

lane is cut into numerous gel slices and in-gel proteolytic digestion results in numerous peptide fractions, which are then submitted to secondary separation by standard reverse phase liquid chromatography prior to MS/MS analysis. GeLC-MS/MS can be advantageous in body fluid proteomics, because proteins are immobilized to the gel matrix which allows for cleaning of salts, lipids, and other substances (which are common in body fluids) that can interfere with mass spectrometry (Shevchenko et al., 1996, 2006). However, large scale studies involving multiple samples can be difficult.

18.2.4.2 2D-GE

Another commonly used protein separation method is two-dimensional gel electrophoresis (2D-GE). Proteins are first separated according to their isoelectric point followed by separation based on their electrophoretic mobility/molecular weight. Gel spots are excised and proteins can be proteolytically digested prior to mass spectrometry analysis. Although progress has been made to increase reproducibility, there remains still significant run-to-run variability, which is a deterrent for comparative proteomics experiments designed to detect differences between two samples. Additionally, interfering compounds and salts in many of the body fluids can make 2D-GE difficult. Furthermore, a large number of clinical samples may prohibit the efficiency of 2D-GE.

Many of the difficulties of 2D-GE have been overcome by the development of difference imaging gel electrophoresis (DiGE) (Seike et al., 2004; Tian et al., 2008; Walsh et al., 2009). In DIGE, two or three samples are labeled with spectrally distant isobaric fluorescent dyes (Cy2, Cy3, or Cy5) and run simultaneously on a single gel. Images for each wavelength are then merged for quantitative and qualitative assessment of differences without need of cross gel comparisons, thus eliminating gel-to-gel variation (Mujumdar et al., 1989; Unlu et al., 1997). Furthermore, gel spots corresponding to proteins that differ between images, or proteins of interest that overlap in both samples can be excised and analyzed by mass spectrometry.

18.2.4.3 Peptide Fractionation

Protocols typically would begin with in-solution digestion of the protein sample resulting in peptides which are fractionated in a single dimension (revered-phase, strong cation exchange, isoelectric focusing) (Manadas et al., 2009) or via MUDPIT (multidimensional protein identification technology) (Peng et al., 2003; Washburn et al., 2001).

As opposed to separating at the protein level, initial separation can occur at the peptide level to reduce sample complexity. Orthogonal methods of separation include high pH reversed-phase chromatography, strong cation exchange chromatography, and/or isoelectric focusing prior to mass spectrometric analysis. Several recent studies have compared the various fractionation methods mentioned above (Delmotte et al., 2007; Dwivedi et al., 2008; Elschenbroich et al., 2009; McDonald et al., 2002; Motoyama and Yates, 2008). However, such studies are

performed to answer specific questions, and their advantages may not be applicable to all study designs.

18.2.4.4 SELDI

Surface-enhanced laser desorption/ionization (SELDI) (Hauskrecht et al., 2005; Koopmann et al., 2004; Ortsater et al., 2007; Rosty and Goggins, 2002; Scarlett et al., 2007; Verma et al., 2001) is commonly used to investigate body fluids prepared by the methods outlined above. A subfraction of proteins are isolated by adsorptive surfaces on a chip and the proteins or peptides that bind are analyzed by matrix-assisted laser desorption/ionization (MALDI). The SELDI chip contains a chromatographic coating on which sample components of a certain type (i.e., hydrophobic, or metal binding) are captured. Using this technique, the mass-to-charge peak pattern is identified; however, the corresponding proteins or peptides identifications are not. More recent advances have allowed for some protein identification when the ProteinChip (BioRAD) technology is coupled to tandem mass spectrometers.

18.2.4.5 Quantitative Proteomics

Although DiGE does overcome some of the limitations of 2D-GE, it still carries over biases against very small, very large and hydrophobic proteins, many of which are of great importance when attempting to discover biomarkers of human body fluids. For such analyses, other methods of quantitative proteomics may provide an unbiased approach to comprehensive proteome analysis. SILAC (stable isotope labeling in cell culture) is one of the original methods of labeling for quantitative proteomics. Unfortunately it is only applicable in cell culture or animal models. For cell culture, stable isotope labels may be introduced during cell growth to attain up to 100% labeling efficiency. Typically, two cell states are prepared, with one set of cells being grown in media with heavy isotope labeled arginine and/or lysine, combined, and chromatographically separated to assess relative differences and similarities in protein content. Currently, SILAC cannot be used with human body fluids. Human body fluids can only be labeled after they are collected.

Currently, all labeling methods occur at the peptide level. Among the simplest method of labeling is the incorporation of ^{18}O into the C-terminus of the peptides during proteolysis, e.g. with trypsin (Stewart et al., 2001). Similarly, peptides can be labeled with chemical tags. Chemical labeling can be performed in a multiplexed manner using isobaric tags (e.g., TMT (Thompson et al., 2003) or iTRAQ (Ross et al., 2004)). From two to eight disease states, time points, or samples can be chemically labeled and compared in a single experiment. After samples are differentially labeled, they are pooled, undergo separation as described above, and then further fractionation by liquid chromatography prior to tandem mass spectrometry analysis. The fragmentation of the attached tag generates low molecular mass reporter ions that can be used to relatively quantify the peptides and the proteins from which they originate. These quantitative methods allow slight changes in the proteome to

be detected, which would otherwise not be if only qualitative (presence vs. absence) of proteins were investigated. Once, the protein of interest has been defined, directed quantitation methods such as isotope dilution (also known as AQUA for Absolute Quantitation) can be applied. Using this method, a synthetic heavy-isotope-labeled standard peptide is introduced into cell lysates at a known concentration and selected reaction monitoring is used to detect and quantitate the peptide of interest (Gerber et al., 2003, 2007; Kirkpatrick et al., 2005).

These stable isotope labeling remain the 'gold standard', but they have not been widely used for large-scale, multiplexed analyses owing to their relatively high cost, the limited availability of different mass-coded labels, and the frequent under-sampling associated with tandem mass spectrometry. Label-free methods for LC-MS represent attractive alternatives (Lundgren et al., 2010). They are based on the principle that the relative abundance of the same peptide in different samples can be estimated by the precursor ion signal intensity across consecutive LC-MS runs, given that the measurements are performed under identical conditions. In contrast to differential labeling, every biological specimen needs to be measured separately in a label-free experiment. Typically, peptide signals are detected at the MS1 level and distinguished from chemical noise through their characteristic isotopic pattern. These patterns are then tracked across the retention time dimension and used to reconstruct a chromatographic elution profile of the monoisotopic peptide mass. The total ion current of the peptide signal is then integrated, or the counts of spectra corresponding to a particular protein are recorded, and used as a quantitative measurement of the original peptide concentration. Label-free methods are very replicate dependent. To be statistically significant, chromatographic separation reproducibility must be very high. The exact alignment of the chromatograms and a highly reproducible ion count are mandatory. Not every chromatographic system is prepared for this performance, and the competition for the ionization may be problematic when very complex samples are separated. Advances in dedicated software are still needed to study the comparative behavior of both samples during the analysis in a trustable manner.

18.3 Examples

18.3.1 Urine

Urine is one of the ideal biological sources for prognosis and diagnosis of human diseases. Before the proteomics era, urine was analyzed by physicians for hundreds of years. Urine is produced by the kidney and was typically thought to be the filtered waste product of blood; however, it is now known that urine may contain many potential markers of systemic disease and the genitourinary tract. In a healthy normal human approximately 150–180 mg of proteins are daily excreted into human urine (Bramham et al., 2009). The majority of these proteins are derived from the kidney and the urogenital tract (70%) with the rest originating from plasma

(30%) (Gonzalez-Buitrago et al., 2007). A low amount of exosomes and apical membranes of renal tubular epithelial cells are also shed into urine. During normal kidney function, glomerular filtration results in removal of proteins with molecular weights greater than 40,000 Da. Smaller proteins (less than 15,000 Da) pass freely through the glomerular barriers, but are almost completely reabsorbed in the proximal tubules (99%). About two thirds of the filtered proteins is comprised of albumin, transferrin, some immunoglobulins and low molecular weight proteins (Barratt and Topham, 2007). Alteration of normal kidney function may lead to disruption of the normal transport mechanisms and subsequent changes in the urinary proteome.

Urine has several characteristics that make it a preferred clinical specimen as compared to other body fluids. First and foremost is the fact that urine is readily available, noninvasively obtained, and usually present in large volumes. This availability simplifies repeated sampling from the same individual and permits critical longitudinal studies. Urine has the specific advantage of being the proximal fluid (primary effluent) to the majority of urogenital organs. In contrast to samples such as blood, urine is exceptionally stable (Lee et al., 2008). It can sit at room temperature for hours or be stored at –20°C for long periods without any significant quantitative change to the general proteome. However, the proteins stability should not be interpreted in a generalized manner, since several studies have shown alteration in the peptidome (Fiedler et al., 2007) and the exosomes (Zhou et al., 2006) after long exposures to room temperature. In addition, it is unclear how different subproteomes would change under similar conditions.

Another advantage of urine is that it appears to be less complex than other most widely used body fluids. Although the urine proteome's dynamic range still spans over several orders of magnitude, its simplicity over serum makes it a rather attractive alternative (Fliser et al., 2007). Furthermore, urine contains a high amount of natural peptides and low-molecular weight proteins, which are soluble and can be directly analyzed. Capillary electrophoresis mass spectrometry (CE-MS) has been extensively used to study these compounds (Fliser et al., 2007). Finally, urine is a rich source of glycoproteins, where half of all proteins are thought to contain a glycan group (Wang et al., 2006). Glycosylation is a widespread and important post-translational modification and is involved in many biological processes such as immune response, signal transduction, inflammatory reaction and cell adhesion (Haltiwanger and Lowe, 2004).

Despite attractiveness of urine for biomarker discovery, some confounding effects have made its use in routine clinical applications challenging (Vaezzadeh et al., 2009). The composition of urine is highly variable. These changes are due to diet, hydration, circadian rhythm, metabolic and catabolic processes, exercise, diseases and other environmental factors. This variability has rendered the task of defining "normal" human urine proteome extremely intricate. The diurnal variation of the urinary proteome is also important to take into account (Bottini et al., 2002). Another major obstacle is the presence of high concentrations of interfering compounds such as urea, salts, metabolites and other charged compounds. Another significant drawback is the presence of a few proteins such as uromodulin, immunoglobulins and albumin in high abundance. These proteins can mask

the detection of low-abundant proteins. Finally, the inconsistency of urine pH may result in greater variability in the composition of particular peptide fragments.

The advantages and challenges of urine as a source for biomarker discovery demonstrate the necessity of developing standard sample collection and preparation procedures. The initiation of Human Kidney and Urine Proteome Project (HKUPP) by Human Proteome Organization (HUPO) is an indication of the international effort of the scientific community towards this goal (Yamamoto et al., 2008). In this section, we provide a step-by-step practical review of current urinary proteomics sample handling challenges and offer recommendations based on our own experience and the literature.

18.3.1.1 Sample Collection and Storage

Urine can be collected at different time points during the day. However, Bottini et al. showed that the 24 h and first morning urine samples are at risk of contamination from bacteria or bladder epithelial cells (Bottini et al., 2002). Twenty-four hour urine samples should not be used for discovery based proteomics analysis because of their inherent nature of contamination in collection. The second-morning sample is recommended for urinary proteomics. Another aspect of the sample collection is when to collect during urination. Ideally a catheterized sample would be obtained. However, typically a voided sample is what is usually collected. It is suggested to use a midstream urine sample to decrease the risk of contamination, particularly for females (Schaub et al., 2004). Recommendations on methods to clean the genital area, to avoid contamination in the urine sample, can be found in the literature (Lifshitz and Kramer, 2000; Schaub et al., 2004; Vaillancourt et al., 2007).

In the past it was suggested to add the protease inhibitors for urine collection (Zhou et al., 2006). However, since urine is stagnated in the bladder for a long term, the proteases may not be as active as in other body fluids. Moreover, their addition might result in interference with protein digestion and analysis and down-stream mass spectrometry steps (Rai et al., 2005). Previous reports described increased bacterial activity in samples exposed to room temperature for >8 h or stored at 4°C for a >12 h (Thongboonkerd and Saetun, 2007). They suggest the addition of preservatives such as sodium azide (0.1–1 mM) or boric acid (200 mM) to retard bacterial growth. However, in our hands a sample, which was exposed to room temperature for 24 h did not show any noteworthy bacterial growth (Lee et al., 2008). Our search of the entire Swiss-Prot database using this sample resulted in identification of only three single-peptide protein identifications from *Escherichia coli*, where two of these three proteins had a high degree of homology to their human counterparts. In addition, our comparison of urine samples exposed at room temperature for 0, 4, 8 and 24 h showed that 97.5% of the top 50th percentile of the identified proteins were seen at all times (Lee et al., 2008). By performing label-free quantitation, we also showed that peptide spectral counts levels were stable at room temperature up to 24 h (Lee et al., 2008). Earlier reports of contamination may have been more a function of the voided collection methodology.

The pH of urine may vary largely (4.4–8.0) based on the dietary acid-base load, fruit and vegetables and meat intakes and also for pathological reasons (Welch et al., 2008). Despite the fact that it has been demonstrated that urine's pH has no effect on proteome analysis using 2D-GE (Thongboonkerd et al., 2009), sample pH can have a significant influence on the partitioning of proteins between sediments and the soluble portion of urine (Saetun et al., 2009). Further work has to be done to assess pH effects on posttranslational modifications and protease activity in samples with proteinuria. We found that while depleting albumin from urine using Hitrap Blue columns (GE Healthcare), the pH can have a significant effect on the efficiency of the depletion as well as the recovery (manuscript in preparation). Interestingly at low pH, the depletion was very effective; however, the recovery was not optimal. Since the reverse was true for basic pH, we systematically adjust the sample's pH to neutral in order to obtain high recovery and efficient depletion.

Samples are usually centrifuged at low speed or filtrated to remove cells and debris. Immediately after this step, urine samples are stored at –20°C or lower (we recommend –80°C). Repetitive freeze–thaw cycles have been reported to alter the proteome (Schaub et al., 2004). We experimented five freeze–thaw cycles and found that 98.3% of the top 50th percentile of the identified proteins remained unchanged (Lee et al., 2008). Using spectral count quantitation on the top 200 abundant peptides, we also found that freeze–thaw cycles did not have any significant effect on the peptide quantification. These results are confirmed by Fiedler et al. using MALDI to compare the peptidome of once frozen sample to three freeze–thaw cycles (Fiedler et al., 2007).

18.3.1.2 Sample Preparation

To date, there is no consensus on methods for concentrating, purifying and depleting urine samples for proteomics analysis. It is well known that using different methods will yield different proteins. In addition, variation in sample handling may significantly alter the urinary proteome and create artificial differences between comparable specimens.

Protein Concentration and Purification

Isolating and concentrating urinary proteins is an essential and important step as urine specimens usually have a very low protein concentration in non-nephrotic diseases, and in comparison to blood and tissue samples. Numerous methods have been employed including lyophilization, ultrafiltration, centrifugal filtration, reversed-phase extraction, and precipitation with organic solvents. Lyophilization was shown in several studies to afford the best quantitative yield since it has the least protein loss (Lee et al., 2008; Thongboonkerd et al., 2006). However, the most important drawback of this method is that it concentrates the proteins and also salts and other contaminations. Precipitation with organic solvents such as ethanol (Lee et al., 2008), acetone (Thongboonkerd et al., 2006) and acetic acid (Thongboonkerd et al.,

2006) favors isolation of mostly hydrophilic proteins. Proteins isolated with precipitation methods may still contain salt and lipids. Centrifugal filtration has a superior consistency compared with other methods but results in some protein loss (Lee et al., 2008). Reversed-phase trapping columns made of C4 or C18 resins are efficient tools to concentrate urinary proteins and peptides, respectively (Lee et al., 2008). In addition, the trapping columns can be used to for desalting of electrolytes and urea, but other interfering compounds, such as lipids, might remain in the sample.

We compared four different protein extraction methods to determine the most effective method: ethanol precipitation, lyophilization, microconcentrators and C4 trapping column. We found that out of the top 100 proteins identified, 89% were found in all four methods. It has been shown that a combination of different methods can result in obtaining a better coverage of the proteome. By combining 10 different protocols, Thongboonkerd and colleagues were able to increase the number of 2D-GE detected protein spots from around 100 in each method to 700 (Thongboonkerd et al., 2006). We believe that the choice of the method should be based on the technical ease, throughput, cost and compatibility with the down-stream protocols. While methods such as ethanol or dual-phase methanol/chloroform precipitation are efficient in enriching the urinary protein, the samples remain salty and are not suitable for protein digestion. In our hands, C4 purification was efficient to remove the salts but some other compounds remained in the samples which resulted in high current issues during isoelectric focusing, usually a sign of interference from charged molecules.

High-Abundant Protein Depletion

The dynamic range of protein concentrations in urine spans several orders of magnitude. Albumin, uromodulin (Tamm–Horsfall protein), transferrin, immunoglobulins and α1-antitrypsin constitute the majority of the high abundant proteins in the urinary proteome. Depletion of high abundant proteins may provide access to previously unreachable protein, particularly high potential biomarker proteins with low-abundance (Pieper et al., 2004; Zolotarjova et al., 2005). However, some of these high-abundant proteins may provide invaluable information in some diseases. A repetitive pattern of albumin fragments and some of its specific fragments have been associated with nephrotic diseases (Candiano et al., 2006) and type 2 diabetics (Mischak et al., 2004). Albumin oxidation has also been reported in patients with active focal segmental glomerulosclerosis (Musante et al., 2007). Further discussion concerning arguments for and against the depletion of abundant proteins is discussed in Section 18.2.1.

Recently, a different approach entitled "Proteominer" has been introduced (Boschetti et al., 2007). This method is based on combinatorial library of hexameric peptide ligands bound to polyacrylate beads. Any protein in the complex body fluid mixture can interact with one of the millions of the peptide ligands. This allows the equilibration of proteins concentrations in contrast to removing specific high-abundant species. This technology has been applied to the analysis of urine (Castagna et al., 2005). Castagna et al. reported that the use of the Proteominer in

conjunction with nano-LC-MS/MS increased the number of identified proteins from 134 for the non-treated urine to 300 proteins. However, since the natural abundance of proteins is altered with this method, performing quantitative analysis might prove to be challenging.

18.3.2 Pancreatic Fluid

The search for evidence of early chronic pancreatitis eventually leads to an exhaustive lists of invasive (endoscopic) and non-invasive (radiologic) pancreatic imaging studies. If endoscopic and radiologic findings are absent or equivocal, patients are generally referred to a tertiary care center for further testing. Pancreas function testing is considered the non-histological gold standard to diagnose mid-to-late stage chronic pancreatitis (Chowdhury et al., 2005). Routine pancreatic biopsy is not recommended due to potential complications of bleeding and fistulae formation. Chronic inflammation in the pancreas will decrease acinar and duct cell secretory function. This degree of dysfunction can be determined by measurement of specific concentrations of cellular secretory components in pancreatic fluid after hormone stimulation. Thus, pancreatic dysfunction is a "surrogate" marker of chronic pancreatitis (DiMagno et al., 1973). Function testing is advantageous as it may detect abnormalities in secretion before the development of steatorrhea or radiologic abnormalities. Earlier chronic pancreatic disease detection is needed to assess the benefit of treatments that may retard or modify disease progression, and decrease the number of diagnostic tests which will ultimately lower the healthcare costs.

18.3.2.1 Sample Processing To Date

Several mass spectrometry-based proteomic analyses of pancreatic fluid have been reported to date (Chen et al., 2007a,b; Gronborg et al., 2004, 2006). The studies used different approaches in all aspects including: sample collection, method of collection, protein extraction, the use of protease inhibitors, and data analysis. Several of these proteomic investigations of pancreatic fluid have been performed using specimens collected surgically or via ERCP (Chen et al., 2005, 2007a,b; Cui et al., 2008; Gronborg et al., 2004, 2006; Ke et al., 2009; Li et al., 2008; Zhao et al., 2009). Although these specimens were not directly collected for proteomics, as they were considered excess specimens, both methods are highly invasive and associated with significant risks for the patients. In contrast, the ePFT (endoscopic pancreatic function test) collection method, which is much less invasive compared to ERCP and surgery, and permits the safe collection of 10-fold larger volumes of pancreatic fluid, making it a well-suited method for comprehensive proteome analysis (Conwell et al., 2002, 2003a,b; Stevens et al., 2004a,b; Wu and Conwell, 2009).

Once fluid is collected, it must be processed to efficiently extract proteins for analysis. Following a short centrifugation spin to remove any particulates, protein extraction is generally performed on the pancreatic fluid sample. Care must be taken to prevent proteolysis as it is prevalent due to the elevated amounts of pancreatic

proteolytic enzymes. Although some publications investigating fluids of pancreatic origin do not specifically mention the protein precipitation method used (Chen et al., 2007a,b; Gronborg et al., 2004, 2006; Wandschneider et al., 2001), other publications have cited acetone precipitation for protein extraction (Ke et al., 2009; Zhou et al., 2007). In addition, there is data reported for and against the use of protease inhibitors in pancreatic fluid studies (Gronborg et al., 2006; Lohr and Faissner, 2004; Thongboonkerd, 2007; Wandschneider et al., 2001). Finally, mass spectrometry-based studies of pancreatic fluids investigating either pancreatic cancer (Chen et al., 2007b; Cui et al., 2008; Ke et al., 2009; Li et al., 2008; Zhao et al., 2009) or chronic pancreatitis have utilized GeLC-MS/MS (Gronborg et al., 2004; Paulo et al., 2010a), SELDI (Rosty and Goggins, 2002), and ICAT (Chen et al., 2007a). Proteins identified varied in number from 22 to over 170 based on the MS methodology utilized.

18.3.2.2 Sample Optimization

In efforts to standardize sample processing methodologies, we initiated a study using SDS-PAGE protein profiling to establish a robust protocol (Fig. 18.1) for pancreatic fluid analysis for future studies (Paulo et al., 2010b). These experiments were designed to maximize the integrity of our pancreatic fluid samples, as protein degradation was expected in this endogenously protease-rich body fluid. We have shown that a sample of pancreatic fluid that is incubated at 37°C shows progressive degradation overtime, most noticeably for time points beyond 1 h (Fig. 18.2). Similarly, we have shown that protein degradation was prevalent even if the sample was maintained at room temperature for longer than 1 h. However, no degradation was evident after 8 h if the samples were maintained on ice. Thus, to limit sample
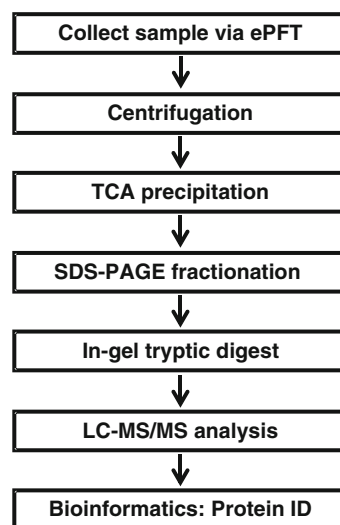
Fig. 18.1 General workflow of an ePFT-mass spectrometry-based proteomics experiment

**Fig. 18.2** Autodigestion of pancreatic fluid at 36°C over 16 h
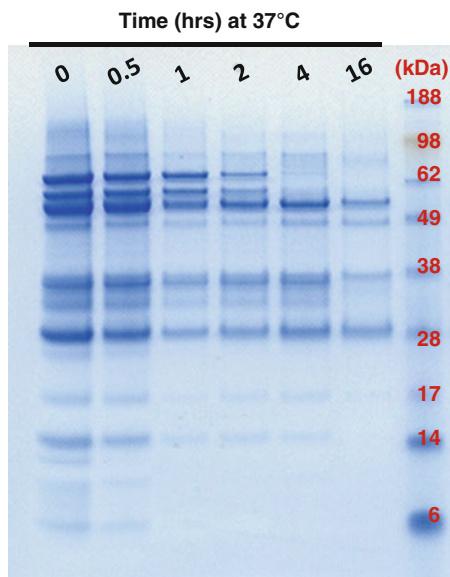
degradation, we maintain our pancreatic fluid samples at 4°C and minimize its handling prior to precipitation or storage at –80°C. Such results support the importance of maintaining samples, which are rich in proteolytic enzymes, such as pancreatic fluid, at cold temperatures during preparation for subsequent proteomic analysis.

In addition, we examined the effects of multiple freeze–thaw cycles on the degree of protein degradation. As samples often require repeated analysis, their integrity following freeze–thaw cycles should be examined. To test this for pancreatic fluid, we subjected samples to repeated cycles of freezing at –80°C and thawing on ice for approximately 1 h with intermittent agitation. Following five freeze/thaw cycles, SDS-PAGE protein pattern analysis revealed only very slight differences in the protein bands (Fig. 18.3). Even though we show that pancreatic fluid proteins are resistant to degradation resulting from freeze–thaw cycles, it may be of additional benefit to store samples for extended periods in smaller aliquots to minimize, or essentially eliminate, the need for multiple sample thaws.
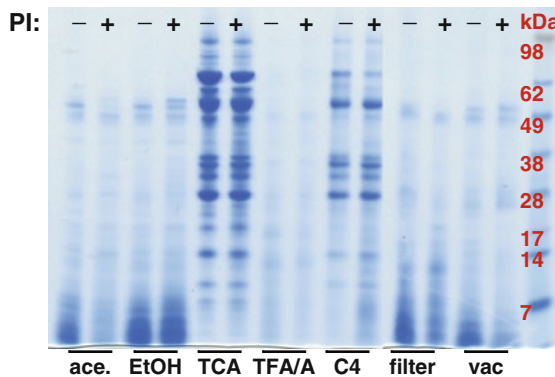
Protein extraction from pancreatic fluid was optimized. Both chemical and non-chemical protein extraction techniques were tested for maximal protein extraction with minimal degradation including vacuum centrifugation, 5 kDa molecular weight cut-off ultrafiltration, C4 trapping column, trifluoroacetic acid/acetonitrile precipitation, trichloroacetic acid (TCA) precipitation, ethanol precipitation (Fig. 18.4). Of the methods investigated, TCA precipitation maximized protein extraction. While the cold temperatures slow enzymatic reactions, TCA precipitation most efficiently and rapidly inactivated pancreatic enzymes as a result of the decrease in pH, and thus results in less overall protein degradation. However, this method did not apply to all fluids, including those of the upper gastrointestinal tract. We have shown by mass

Fig. 18.3 Evaluation of
freeze–thaw (FT) cycles on
pancreatic fluid
auto-digestion. SDS-PAGE
separation of pancreatic fluid
samples undergoing a total of
five freeze–thaw cycles. FT,
freeze–thaw cycle
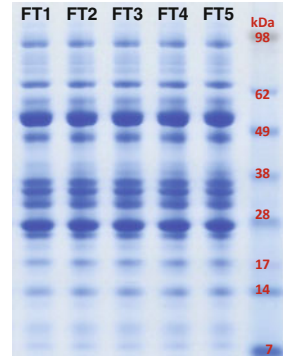
spectrometry-based proteomic analysis that protein degradation in gastroduodenal
fluid is exacerbated by TCA, potentially due to the presence of gastric enzymes
which are activated under acidic conditions (Paulo et al., 2010c). We suggest sim-
ilar screening of precipitation protocols for other body fluids to optimize protein
extraction for proteomic analysis.

Furthermore, we have shown that the addition of protease inhibitors may be
ineffective in the case of pancreatic fluid (at the manufacturer's recommended
concentration), as there was no difference in the protein profile with or with-
out their addition, as was depicted in Fig. 18.4. This is possibly the result of
the unusually high protease concentration encountered in pancreatic fluid. There
are conflicting reports in the literature, both for and against the use of protease
inhibitors in pancreatic fluid studies (Gronborg et al., 2006; Lohr and Faissner, 2004;

Fig. 18.4 Pancreatic fluid protein extraction methods. SDS-PAGE gel image of pancreatic fluid
proteins extracted using the following techniques: *vac*, vacuum centrifugation; *filter*, 5 kDa molec-
ular weight cut-off Centricon filtration; *C4*, C4 trapping column; *TFA/A*, trifluoroacetic acid/
acetonitrile precipitation; *TCA*, trichloroacetic acid precipitation; *EtOH*, ethanol precipitation; *ace*,
acetone precipitation; *PI*, protease inhibitors

Fig. 18.5 Processing of pancreatic fluid from 11 patients using our optimized methodology. Samples were collected by secretin-stimulated ePFT and processed using our optimized methodology
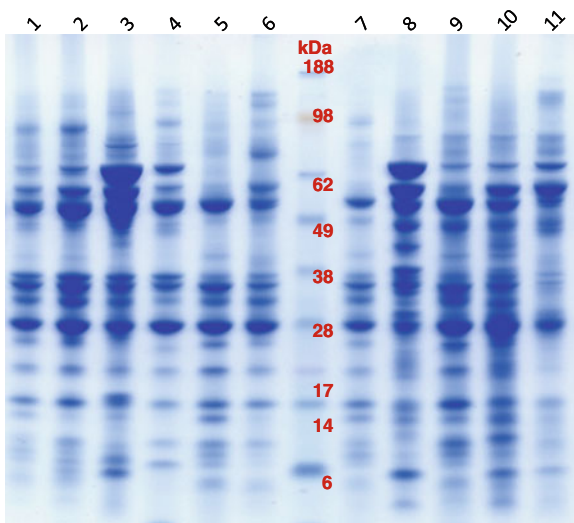
Thongboonkerd, 2007; Wandschneider et al., 2001). Our results have shown that auto-digestion is minimized whether or not the sample is supplemented with the protease inhibitors, as long as samples are handled on ice and/or are TCA precipitated (Paulo et al., 2010b).

Such experiments (involving precipitation optimization, autodigestion, freeze–thaws, use of protease inhibitors) are vital, not only for pancreatic fluid, but for proteomics of any body fluids which may susceptible to degradation. We can apply our optimized methodology to pancreatic fluid from various individuals and produce robust and relatively reproducible SDS-PAGE protein banding patterns (Fig. 18.5). Additionally, using our sample preparation methodology coupled with GeLC-MS/MS, we have been able to consistently identify over 250 proteins from a single sample of ePFT-collected pancreatic fluid (manuscript in preparation). Our optimized sample preparation methods can be utilized in future studies investigating the proteome of pancreatic fluids, but can also be extended to studies of other body fluids.

18.4 Conclusions

Sample preparation is an indispensible step in the study of body fluid proteomics, as it has a tremendous impact on subsequent analyses. It is crucial to decide on a particular body fluid preparation strategy with the final goal in mind. No single method can be applied to all samples, and it is imperative to optimize procedures for a particular sample type. The depletion of highly abundant proteins is

often desired, but caution must be taken as not to deplete proteins which may be of interest. Similarly, protein degradation must be minimized. Care must be taken to ensure that bedside-to-bench transport is rapid and efficient, samples are processed cold temperatures, freeze–thaw cycles are limited and proteases are inactivated, by denaturation, or if suitable for subsequent studies, by the addition of protease inhibitors. The combination of high-resolution separation techniques and powerful mass spectrometric analysis allows previously unattainable information to be acquired. The comprehensive analysis of protein mixtures and the identification of hundreds or thousands of proteins is possible for clinical applications with recent developments in high-throughput mass spectrometry (Han et al., 2008; Kentsis et al., 2009; Latterich et al., 2008; Tsangaris, 2009; Yates et al., 2009). Proteomics can facilitate the elucidation of proteins which regulate the pathogenesis of disease and facilitate the discovery of clinically-relevant biomarkers. However, the quality of results of such proteomic studies depends heavily on the methodology by which samples are prepared. Variations in methods may introduce discrepancies that can impede the progress of body fluid proteomics. Standardized methods, such as those that we investigate herein, can maximize protein extraction and minimize the heterogeneity of samples by reducing protein degradation.

The establishment and optimization of clear and consistent sample collection, handling, and processing methodologies is paramount to the development of clinical proteomics to create a platform upon which such assays can be further developed. Quantitative proteome profiling may be key, as a protein may be present, albeit at varying levels, both in the normal and in the diseased state, and without quantitative information, the value of that protein as a biomarker may be overlooked. In comparative proteomics, sample preparation is of utmost importance as minor differences in experimental and control samples are under investigation. Whereas the genome is relatively stable and identical in all cells, the proteome varies by organ, cell, subcellular location, temporally, and due to stimuli, such as changes in health, diet, and environment. Differences exist not solely in tissue/cell-specific protein content, but also in protein processing, such as posttranslational modifications and splice variants. Biological fluids are attractive for biomarker discovery, owing to several factors including ease of accessibility, avoidance of risky, costly and invasive biopsies, monitoring of the disease state by multiple samplings, and resulting in the development of relatively easily implemented prognostic/diagnostic tests (Good et al., 2007). Consideration must be taken that the samples remain stable during collection, transportation from site of collection to site of analysis, storage, and preanalytical preparation. To this end, one should meticulously document the conditions for sample preparation and handling and, thereby carefully track all preanalytical variables. Thus, although comprehensive proteomic analysis is a valuable tool for biomarker discovery and understanding the pathophysiology of disease, fluid-specific standardized methodology must be established for successful comprehensive proteomic analyses.

Protocol Box

Below are protocols, culled from a variety of sources including Ke et al. (2009), Lee et al. (2008), Thongboonkerd et al. (2006), Zhou et al. (2007), which have been mentioned throughout the chapter. Volumes are appropriate for pancreatic and gastroduodenal fluids (0.5–1.5 mg/mL), but may be adjusted for less protein rich body fluids, such as urine.

   Acetone precipitation. Add four sample volumes (800 µL) of ice-cold 100% acetone to 200 µL of sample, vortex briefly, and incubate at –20°C for 3 h. Subsequently, centrifuge the samples at $20,000 \times g$ at 4°C for 30 min. Carefully aspirate the supernatant and air dry the pellets at 23°C.

   Chloroform-methanol precipitation. Solubilize or reduce sample volume to 100 or 200 µL in water (or aqueous buffer). Add 1 mL of a chloroform/methanol (2:1, vol:vol) solvent. Vortex thoroughly and place on ice. Centrifuge at 4°C for 20 min at 14,000 rpm. Carefully extract the lower phase containing the chloroform with glass syringe. Add 500–1000 µL of MeOH to the supernatant and vortex thoroughly. Centrifuge at 4°C for 20 min at 14,000 rpm. Discard the supernatant and dry the pellet in speed-vac.

   C4 column. A final concentration of 0.1% trifluoroacetic acid (TFA) and 2% acetonitrile (ACN) was added to 200 µL of pancreatic fluid. Loaded the samples onto a Michrom C4 trapping column which has been pre-equilibrated with 0.1% TFA/2% ACN (Buffer A). Wash the samples twice with 500 µL of Buffer A and elute with 0.1% TFA/90% ACN. Dry the eluted samples at 23°C in a vacuum centrifuge.

   Ethanol precipitation. Add a total of 800 µL of 100% ethanol to a 200 µL aliquot of sample. Vortex thoroughly and place on ice for 30 min. Centrifuge at $14,000 \times g$ at 4°C for 30 min. Carefully aspirate the supernatant and air dry the pellets at 23°C.

   Microfiltration. Load 200 µL of sample onto a Millipore 5 kDa Microcon centrifugal filter concentrators and centrifuge at $14,000 \times g$ at 4°C until the volume is at least one-tenth of the original sample volume. Wash the retained samples twice with 200 µL of water in the centrifugal filter concentrator. Dry the final 20 µL sample at 23°C in a vacuum centrifuge.

   TFA/A precipitation. Add two sample volumes (400 µL) of ice-cold 0.1% trifluoroacetic acid (TFA) in 99.9% acetonitrile (ACN) to 200 µL of sample. Vortex thoroughly and place on ice for 30 min. Centrifuge at $14,000 \times g$ at 4°C for 30 min. Carefully aspirate the supernatant and air dry the pellets at 23°C.

   TCA precipitation. Add 25 µL of ice-cold 100% trichloroacetic acid to 200 µL of sample. Vortex thoroughly and incubate at 4°C for 2 h. Centrifuge the sample at $20,000 \times g$ at 4°C for 30 min and carefully aspirate the supernatant. Add 1 mL of 100% ice-cold acetone to the pellets. Vortex briefly and incubate at –20°C for 1 h. Centrifuge the sample at $20,000 \times g$ at 4°C for 30 min. Gently

wash the pellet twice with 100% ice-cold acetone. Allow the final pellets air dry at 23°C.

   Vacuum centrifugation. Dry 200 µL aliquots of sample at 23°C in a vacuum centrifuge (SPD1010 Thermo Savant, Waltham, MA) for approximately 3–4 h.

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