PEPTIDE MAPPING OF PROTEINS IN HUMAN BODY FLUIDS USING ELECTROSPRAY IONIZATION FOURIER TRANSFORM ION CYCLOTRON RESONANCE MASS SPECTROMETRY

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Human body fluids have been rediscovered in the post-genomic era as great sources of biological markers and perhaps particularly as sources of potential protein biomarkers of disease. Analytical tools that allow rapid screening, low sample consumption, and accurate protein identification are of great importance in studies of complex biological samples and clinical diagnosis. Mass spectrometry is today one of the most important analytical tools with applications in a wide variety of fields. One of the fastest growing applications is in proteomics, or the study of protein expression in an organism. Mass spectrometry

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has been used to find post-translational modifications and to identify key functions of proteins in the human body. In this study, we review the use of human body fluids as sources for clinical markers and present new data that show the ability of Fourier transform ion cyclotron resonance (FTICR) mass spectrometry (MS) to identify and characterize proteins in four human body fluids: plasma, cerebrospinal fluid (CSF), saliva, and urine. The body fluids were tryptically digested without any prior separation, purification, or selection, and the digest was introduced into a 9.4 T FTICR mass spectrometer by direct-infusion electrospray ionization (ESI). Even though these samples represent complex biological mixtures, the described method provides information that is comparable with traditional 2D-PAGE data. The sample consumption is extremely low, a few microliters, and the analysis time is only a few minutes. It is, however, evident that the separation of proteins and/or peptides must be included in the methodology in order to detect low-abundance proteins and other proteins of biological relevance. Mass Spec Rev 21:2–15, 2002; DOI 10.1002/mas.10016

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I. INTRODUCTION

In recent years, several investigators have emphasized the usefulness of the detection and identification of specific proteins in various body fluids as markers of metabolism, as well as their structural and/or regulatory roles in different disorders (for references, see below under each separate body fluid). It is of general interest to find a technique that allows for the simultaneous observation of many different proteins in body fluids in the search for new diagnostic markers of diseases and physiological correlates of behavior. The overall accepted approach to study the total protein expression of a cell, tissue, organ, or organism under particular physiological circumstances relies today only upon a coordinated use of 2D-PAGE (Klose, 1975; O'Farrell, 1975), image analysis, mass spectrometric protein identification, and database mining/bioinformatics (Mann, Hojrup, & Roepstorff, 1993). There is a need for proteomic approaches that can provide more information than traditional 2D-PAGE analysis. The ability of mass spectrometry (MS) to characterize complex biological samples without any complicated pre-purification is an attractive alternative to established time- and sample-consuming techniques. Current mass spectrometric techniques have developed into important tools in screening for clinical biomarkers, and should be investigated as a possible technique for total protein analysis.

Kelleher et al. (1998, 1999) have applied a 'top-down' approach in the analysis of proteins and mixture of proteins. In that approach, intact proteins are analyzed by electrospray ionization (ESI) MS, and are identified and characterized by MS/MS. The alternative, or 'bottom-up' approach, digests all proteins before analysis by MS, LC/MS, or CE/MS. This approach has been applied in the analysis of microbial and mammalian proteomes (Conrads et al., 2000, 2001; Smith et al., 2001, 2002).

The foci of this study were an inducement to review some of the more interesting findings of clinically relevant proteins in human body fluids, and to demonstrate the applicability of ESI Fourier transform ion cyclotron resonance (FTICR) MS in the screening of human body fluids for potential protein biomarkers of disease. To illustrate the concept behind this approach to protein identification, it is useful to compare with the traditional route, including 2D-PAGE, automated spot-picking, tryptic digestion, and protein identification by peptide mass fingerprinting and/or sequence tags generated by MS/MS. The alternative approach presented here "scrambles" the information by simultaneously digesting all proteins; separating these digests by direct infusion or a suitable liquid separation method coupled to the FTICR MS (Fig. 1), and identifying the proteins by highly accurate mass measurement. These two methods should be regarded as complementary—each has advantages and disadvantages over the other. This review describes that, for the first time such a thorough study has been made in human plasma, cerebrospinal fluid (CSF), saliva, and urine. The unsurpassed mass-resolving power and mass accuracy are the prominent features of FTICR-MS that are necessary to analyze such complex biological samples. The throughput and low sample consumption are also important features.

A. Human Body Fluids as Sources for Clinical Markers

A number of proteins are screened today by electrophoretic plasma protein analysis (agarose gel electrophoresis), and are used as clinical markers for, for example, CSF leakage (transthyretin), dehydration, inflammation, malignant tumors (albumin), inflammation, liver status, hemoglobin catabolism, spleen status, hemolytic processes (haptoglobin), and anemia (transferrin).

B. Plasma

It is well known that protein analysis of plasma and serum may provide valuable information on a large number of functions in the body. The analysis of plasma for diagnostic purposes has been described in numerous publications and book chapters (Putnam, 1987; Ganrot, Grubb, & Stenflo, 1997). Most of these proteins play their main role in the

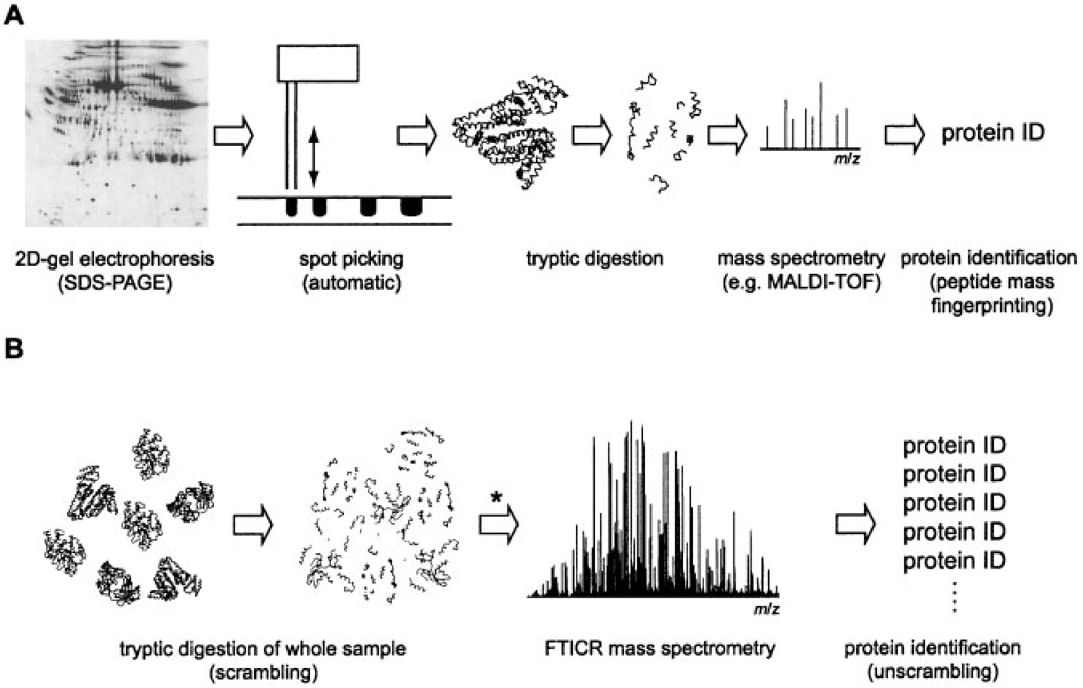

FIGURE 1. The scheme shows two different approaches to proteomics that involve mass spectrometry (MS). The established technique (**A**) is based on 2D-gel electrophoresis, spot picking, and tryptic digestion before MS (typically MALDI–TOF), and protein identification by peptide mass-fingerprinting, using one of several available search engines. The second approach (**B**), presented in detail in this study, involve digesting all components in the sample at once, separating the scrambled peptides by FTICR MS, and retrieving a list of identified proteins by unscrambling the available information. For complex mixtures, a liquid separation method such as LC or CE can be inserted at the *. A similar approach has also been used by Smith et al. (2001, 2002) in the analysis of bacterial and yeast proteomes.

extracellular space, and often have a molecular weight (Mw) above 45 kDa (e.g., human serum albumin 67 kDa). In the plasma, a large number of intracellular proteins are present due to the leakage from cells into the interstitial fluid. The leakage of these proteins, many of which are enzymes, is a result of normal cell catabolism. Plasma levels of specific proteins can, however, be used as diagnostic markers because, at various pathological conditions, the leakage—together with cellular death—dramatically increases the extracellular levels. The plasma proteins have many important functions in the circulation, for example, stabilizing the salt-water balance and transportation of low-Mw compounds (for instance, hydrophobic substances; e.g., fatty acids; or substances that are toxic in free form; e.g., bilirubin). There are also a number of smaller proteins bound to plasma proteins to avoid excretion by glomerular filtration in the kidney (e.g., retinol-binding protein bound to transthyretin (Lapin, Gabl, & Kopsa, 1989; Marshall & Williams, 1998), and globulin dimers bound to haptoglobin (Marshall & Williams, 1987)). Furthermore, many of these proteins act as protectors of various systems, such as extracellular protease inhibitors, immunoglobulins, complement factors, and coagulation factors. Most of the high-abundant plasma proteins are synthesized by the hepatocytes in the liver (except for the immunoglobulins, which are synthesized by the B-lymphocytes). Other cells synthesize less-abundant proteins (e.g., α_1-antitrypsin by the macrophages, factor VIII, and von Willebrand's factor by the endothelium (Ganrot et al., 1997)). The protein pattern is thus expected to vary greatly in time and space within the plasma samples.

Although the levels of different plasma proteins have a natural variation, the human serum albumin level is strictly regulated by the colloidosmotic pressure (salt–water balance) feedback mechanisms. This regulation results in a very low inter- as well as intra-individual relative standard deviation below 7% (note that the albumin level is reduced < 10% when lying down and during pregnancy (Ganrot et al., 1997)). Other protein levels are regulated by, for example, steroid hormones (such as ceruloplasmin and α_1-antitrypsin (Anderson & Anderson, 1977)) or by acute-phase reactions (e.g., C-reactive protein, α_1-antichymotrypsin, α_1-antitrypsin, orosomucoid, haptoglobin, complement factors C3 and C4, C4b-binding protein, fibrinogen, and factor VIII (Ganrot et al., 1997)), resulting in large variations during acute (e.g., pneumonia) as well as chronic processes (e.g., rheumatoid arthritis).

C. Cerebrospinal Fluid

The CSF is in continuum with the extracellular fluid of the central nervous system (CNS), and, therefore, reflects the biochemical environment of the brain. Diagnostic markers for patients with spinal muscular atrophy were found as early as 1976 by Kjellin & Stibler (1976), and Delmotte & Gonsette (1977) found CSF protein abnormalities in patients with multiple sclerosis. The number of disorders that have been screened for CSF proteins is very large. Especially different forms of cerebral inflammatory processes with intrathecally (in the brain) produced immunoglobulins (Blennow et al., 1993), different forms of amyloidosis (Stone, 1990; Bergquist, Andersen, & Westman, 2000), psychiatric disorders like schizophrenia (Blennow et al., 1996; Davidsson et al., 1996), dementia such as Alzheimer's disease (AD), and vascular dementia (Sjögren et al., 2001a,b) have developed much interest. However, the determination of brain-specific proteins present in low concentration in CSF is complicated because more than 80% of CSF proteins originate from the plasma (Thompson & Salinsky, 1988; Thompson & Keir, 1990). A number of strategies have been explored to circumvent this problem, using different forms of chromatography, electrophoresis, and combinations thereof (Wikkelsö et al., 1980; Davidsson et al., 1996; Davidsson et al., 2001). Several 2D-PAGE approaches have been taken for the analysis of proteins in CSF (Raymackers et al., 2000; Sickmann et al., 2000; Yuan et al., 2002; and references in the human body fluid database).

D. Saliva

Protein analysis of saliva in clinical diagnosis is attractive because saliva offers a simple, rapid, and non-invasive method for the short- and long-term monitoring of pathological disorders and drug therapy. However, the fluid collection must be clearly defined due to variations in saliva composition and flow-rate. A number of specific proteins in saliva are used as clinical markers. Among these proteins are phenotypic variants of α-amylase as indicators of inheritance of autosomal co-dominant alleles, disorders such as cystic fibrosis (Wolf et al., 1986), and diabetic ketoacidosis (Warshaw, Feller, & Lee, 1977). Beeley (1991) has reviewed clinical applications of saliva proteins.

E. Urine

The analysis of human urine is one of the oldest diagnostic procedures known to medicine. The collection of urine samples is, in most cases, achieved by the natural, non-invasive method, and the amounts are often sufficient for any kind of analysis. By studying proteins in urine, it is possible to follow conditions that cause glomerular damage. The porous walls of the glomerular capillaries in the kidney normally allow only proteins with a MW < 65 kDa into the tubular system, whereas larger proteins stay in the blood stream. The tubular cells subsequently reabsorb the smaller proteins, and only low concentrations of proteins are normally present in the urine. Most of the renal diseases, as well as some extra-renal, may, however, be seen as proteinuria-defined by a loss of proteins into the urine. Marshall & Williams (1998) recently reviewed the clinical significance of analyzing urine, and listed a large number of clinical applications, including various renal diseases, diabetes, and Bence–Jones proteinuria.

II. REVIEW OF TYPICAL MATERIALS AND METHODS

A. General

Human body fluids of clinical interest—plasma, CSF, saliva, and urine—were digested by trypsin, and the digest was desalted on a ZipTip$_{C18}$® microcolumn prior to analysis; only 10-μL sample was consumed. Total protein content was analyzed, using the Bicinchoninic acid (BCA) Protein Assay (Pierce Chemical Co., Rockford, IL). The samples were introduced by continuous infusion, and the samples were ionized by sheathless ESI (Fenn et al., 1990). The analysis of the tryptic fragments was performed on a FTICR mass spectrometer, and the resulting mass spectra were used for peptide-mapping and database searches. All these steps are described in detail below.

B. Human Plasma Samples

Human plasma samples were collected from healthy blood donors. A volume of 10-mL EDTA-blood was centrifuged at 400*g* for 10 min (21°C), and the plasma was aliquoted into 1-mL fractions and kept at −80°C until further processing. Prior to the tryptic digestion, 4 μL (approximately 250 μg total protein) of the thawed sample was centrifuged to dryness in a small Eppendorf tube, using a Speedvac® system ISS110 (Savant Holbrook, NY).

C. Human CSF Samples

Human control CSF samples were obtained from the Department of Neurochemistry, Institute of Neuroscience, at Sahlgrenska University Hospital. This investigation was approved by the Human Ethics Committee at the Faculty of Medicine, Göteborg University, Sweden. Control samples were chosen from patients who did not have any symptoms or signs of major neurological or psychiatric disorders, and who were undergoing lumbar puncture for diagnostic purposes in order to exclude infectious disorders of the CNS. Routine CSF analyses gave normal values, without any sign of inflammation (normal white-cell count and no intrathecal IgG or IgM production) or damage to the blood–brain barrier function. Lumbar puncture was performed in the lateral decubitus position in the L4–L5 vertebral interspace. The first 12 mL of CSF was collected on ice in a chilled plastic tube, and was gently mixed to avoid any possible gradient effects. The CSF samples were centrifuged at 2,000*g* (4°C) for 10 min to eliminate any cells and other insoluble material; the supernatant was aliquoted into 1-mL fractions, and kept at −80°C until further processing. Prior to the tryptic digestion, 400 µL (approximately 140 µg total protein) of the thawed sample was centrifuged to dryness in a small Eppendorf tube, using an integrated Speedvac.

D. Human Saliva Samples

Human saliva samples were collected from a healthy male donor, who would spit into a small tube after rinsing the mouth with water. After a quick centrifugation to remove any particles, 400 µL (approximately 800 µg total protein (2 mg/mL)) of the saliva was centrifuged to dryness in a small Eppendorf tube, using a Speedvac.

E. Human Urine Samples

Human urine samples were collected from a healthy male donor in the morning, when the highest concentration of proteins is normally found. After a quick centrifugation to remove any particles, 400 µL (approximately 40 µg total protein) of the urine was centrifuged to dryness in a small Eppendorf tube, using a Speedvac.

F. Tryptic Digestion of Proteins

The dry protein pellet was dissolved in 250 µL of 8 M urea, 0.4 M NH_4HCO_3 followed by the addition of 25 µL of 45 mM dithiothreitol. The mixture was incubated at 50°C for 15 min, and cooled to ambient temperature. After incubation, 25 µL of 100 mM iodoacetamide was added, and the mixture was incubated for an additional 15 min in darkness. After the second incubation, 700 µL deionized water was added, together with 5% (w/w) trypsin. The digestion was performed at 37°C overnight in the dark.

G. Desalting

In order to remove salts and other contaminants, 10 µL of tryptic peptides (1/100 of the total volume) was acidified with acetic acid (HAc) to a final HAc concentration of 2.5%. The acidified mixture was desalted on a reversed-phase ZipTip$_{C18}$ pipette tip. The desalting procedure was conducted in the following way: the tip was first wetted in 50 µL 50% acetonitrile, and was equilibrated with 100 µL of 1% HAc. After equilibration, the sample was adsorbed to the reversed-phase media by 30 repeated cycles of sample loading. After sample loading, the tip was washed 5 × 100 µL with 1% HAc. Finally, the desalted tryptic peptides were eluted with 50 µL of 50% ACN in 1% HAc, followed by 50 µL of 50% MeOH in 1% HAc. The eluate was sampled in a microsediment tube (Sarstedt 72.702; Landskrona, Sweden), and was centrifuged to dryness in a small Eppendorf tube, using a Speedvac. The overall recovery from the desalting procedure was estimated to be ~75% (analysed with a BCA assay).

H. Direct Infusion and Capillary Interface

The desalted tryptic peptides were dissolved in 10 µL of acetonitrile/deionized water 50:50 (v:v), 1% HAc (final protein concentration of 0.2 g/L in plasma, 0.1 g/L in CSF, 0.6 g/L in saliva, and 0.03 g/L in urine). An in-house designed capillary electrophoresis instrument that allowed pressurized injections was used for the direct infusion experiments. By applying a helium gas pressure of 0.3 bar, a flow-rate of 55 nL/min (0.9 nL/sec and scan, i.e., ~0.09 ng CSF protein/scan, i.e., ~0.6 fmol human serum albumin (HSA)/scan, assuming that HSA is approximately 45% of the total protein) was generated through a 33-cm bare fused-silica capillary (25 µm i.d. × 360 µm o.d.). The emitter end of the capillary was mechanically tapered, and was 'fairy dust' coated (Barnidge, Nilsson, & Markides, 1999). The emitter end was inserted in an in-house designed ESI interface that consisted of a 22-gauge stainless steel needle mounted into a Valco connector. The connector was fitted into a brass plate in the Analytica atmosphere-vacuum interface (Analytica, Branford, CT). The electrospray potential was set at 3 kV to provide a stable spray.

I. Mass Spectrometry

Experiments were performed on a Bruker Daltonics BioAPEX-94e FTICR mass spectrometer with a passively shielded 9.4 T superconducting magnet equipped with an Analytica ESI source (Palmblad et al., 2000). In the experiments described in this review, the Analytica atmosphere-vacuum interface was used, together with the home-built sprayer described above. The instrument was controlled by, and data were acquired on, a Silicon Graphics O2 workstation that operated with the Bruker Daltonics XMASS^{TM} software (version 5.0.6.). Broadband spectra, using 512-K data points and 2,048 scans, were acquired with a continuous infusion for all samples. Additional spectra with 1-M data points and 21,600 scans were acquired for plasma and CSF. Good mass calibration is vital in peptide mapping in complex mixtures. All spectra were initially externally mass-calibrated, and HSA could be identified in plasma, CSF, and urine. The plasma, CSF, and urine spectra were internally mass-calibrated, using up to 14 abundant tryptic peptides of HSA, HSA361-372 2+ (489.9526 Th), HSA35-44 2+ (613.8062), HSA361-372 2+ (734.4252), HSA438-452 2+ (820.4726), HSA169-183 2+ (950.5030), HSA599-609 1+ (1013.5990), HSA76-105 3+ (1138.5397), HSA45-75 3+ (1208.3012), HSA414-434 2+ (1300.6540), HSA250-286 3+ (1407.0053), HSA287-298 1+ (1443.6419), HSA299-310 1+ (1546.7967), HSA348-360 1+ (1623.7876), and HSA76-105 2+ (1707.3059) (the *m/z* values refer to the most abundant masses). Table 1 shows the result of the mass-calibration of the urine spectrum. The saliva spectrum was externally calibrated, using the calibration of the plasma spectrum.

J. Data Analysis

Primary data analysis was performed on a Silicon Graphics O2 workstation that operated with XMASS (version 5.0.6. or later). Peak lists were generated, using the built-in automatic functions for peak-picking and applying centroid-fitting to each peak. The automatic peak selection was inspected visually, and was adjusted when necessary. Peaks with a signal-to-background ratios < 2:1 were not included in the peak list. Peak lists were reduced to a list of isotopic clusters, using an algorithm implemented in Caml (www.inria.fr). To generate lists of possible tryptic fragments, the in-house written software DIG was used (www.angstrom.uu.se/ionphysics/software.html). In DIG, all cysteines were assumed to be carbamidomethylated, and one missed cleavage site was allowed. Only peptides of four amino acids or more were used. Peak lists were also submitted to Mascot (www.matrixscience.com) for matching tryptic fragments to the non-redundant OWL database 31.4 (312,942 sequences, 100,800,059 residues) or the subset of human sequences (26,988 sequences). Peak lists that contained more than 300 peaks were analyzed off-line. For peptide mapping and database searches, a program called DATACOMP was written in C and was run on a standard PC. The selection of sequences from the NCBI database to search was made according to what proteins were known to be present in the human body fluids (for references see Table 2) prior to these experiments. To calculate probabilities for random matches, a non-redundant set of 4,861 yeast proteins from SwissProt and TrEMBL was used as negative controls. This set was, in turn, compared to a non-redundant set of 24,679 SwissProt and TrEMBL human protein sequences to identify any systematic differences between yeast and human proteins.

III. RESULTS AND DISCUSSION

FTICR-MS analysis facilitates the identification and characterization of proteins in complex biological matrices such as easily sampled human body fluids. In ambitious proteomics projects, there is a search for clinically relevant disease markers that could be mutations or post-translational modifications in any (or several) locations in one (or more) proteins. The FTICR mass spectra for the continuous infusion of tryptically digested human body fluids generated several thousand peaks (~5,500 in the 21,600-scan plasma spectrum). These peaks could be

TABLE 1. Illustration of a good mass-calibration, showing the results from internal mass-calibration of the urine spectrum

Fragment peptide	Calculated *m/z*	Measured *m/z*	Mass measurement error (ppm)
HSA35–44 2+	613.8062	613.8062	0.0257
HSA361–372 2+	734.4252	734.4253	0.1299
HSA438–452 2+	820.4725	820.4724	0.1748
HSA169–183 2+	950.5030	950.5032	0.2038
HSA599–609 1+	1013.5990	1013.5988	0.1413
HSA287–298 1+	1443.6419	1443.6422	0.1632
HSA299–310 1+	1546.7967	1546.7958	0.5914
HSA348–360 1+	1623.7875	1623.7883	0.4419

Eight abundant tryptic peptides of human serum albumin (HSA) and a calibration formula, $m/z = a/f + b/f^2 + c$, where f is the frequency and a–c are the free parameters. A good calibration is paramount in order to differentiate between true and random matches.

TABLE 2. Partial list of the most abundant proteins in the human body fluids studied

Protein	Reference literature			Levels in mg/L (range)		
	Plasma	CSF	Urine	Plasma	CSF	Urine
Actin beta	(Edwards, et al., 1979)	(Sanchez, et al., 1995)				
Actin gamma	(Edwards, et al., 1979)	(Sanchez, et al., 1995)				
Aldolase C4		(Thompson, 1988)			0.085	
Alpha globin	(Boggs and Fahey, 1960)					
Alpha-1-acid glycoprotein 1 precursor	(Anderson and Anderson, 1977;Ganrot, et al., 1997;Marshall and Williams, 1987;Marshall and Williams, 1991)	(Sanchez, et al., 1995;Thompson, 1988)	(Celis, et al., 1999;Ganrot, et al., 1997;Marshall and Williams, 1987;Marshall and Williams, 1998)	700 (550-1400)	3.6	<10
Alpha-1-acid glycoprotein 2	(Anderson and Anderson, 1977)		(Lapin, et al., 1989)	550-1400		
Alpha-1-actin precursor			(Celis, et al., 1999)			
Alpha-1-antichymotrypsin	(Anderson and Anderson, 1977;Ganrot, et al., 1997)	(Sanchez, et al., 1995)	(Celis, et al., 1999;Lapin, et al., 1989;Marshall and Williams, 1998)	400 (300-600)		
Alpha-1-antitrypsin (protease inhibitor 1 (anti-elastase))	(Ganrot, et al., 1997;Hughes, et al., 1992;Marshall and Williams, 1987;Marshall, et al., 1989;Marshall and Williams, 1991)	(Sanchez, et al., 1995;Thompson, 1988)	(Celis, et al., 1999;Ganrot, et al., 1997;Lapin, et al., 1989;Lapin and Feigl, 1991;Marshall and Williams, 1987;Marshall and Williams, 1998)	1400 (1900-3500)	8	<1.5
Alpha-1-beta glycoprotein	(Anderson and Anderson, 1977)	(Sanchez, et al., 1995)	(Celis, et al., 1999;Marshall and Williams, 1998)	150-300		
Alpha-1-microglobulin (protein HC)	(Anderson and Anderson, 1977;Ganrot, et al., 1997)	(Sanchez, et al., 1995;Thompson, 1988)	(Celis, et al., 1999;Ganrot, et al., 1997;Lapin, et al., 1989;Marshall and Williams, 1998)	34-90 (36-620)	0.035	<8
Alpha-2-actin			(Celis, et al., 1999)			
Alpha-2-antiplasmin	(Ganrot, et al., 1997; Hughes, et al., 1992)			60-70		
Alpha-2-glycoprotein 1, zinc	(Anderson and Anderson, 1977)	(Sanchez, et al., 1995;Thompson, 1988)	(Celis, et al., 1999;Lapin, et al., 1989;Lapin and Feigl, 1991;Marshall and Williams, 1998)	20-150	0.27	
Alpha-2-HS-glycoprotein	(Anderson and Anderson, 1977)	(Sanchez, et al., 1995;Thompson, 1988)	(Celis, et al., 1999;Marshall and Williams, 1998)	400-850	1.7	
Alpha-2-macroglobulin	(Anderson and Anderson, 1977; Ganrot, et al., 1997;Marshall and Williams, 1987;Marshall, et al., 1989)	(Thompson, 1988)	(Lapin, et al., 1989;Marshall and Williams, 1987;Marshall and Williams, 1998)	2000 (1500-4000)	2	
Alpha-2-microglobulin			(Lapin and Feigl, 1991)			
Alpha-fetoprotein			(Old and Chen, 1998)			
Alpha-lipoprotein	(Ganrot, et al., 1997)			3000		
Alpha-uterine protein			(Old and Chen, 1998)			
AMBP protein precursor	(Anderson and Anderson, 1977)	(Sanchez, et al., 1995)	(Lapin, et al., 1989)	34-90		
Amylase, alpha 1A			(Celis, et al., 1999)			
Anafylatoxin inactivator	(Ganrot, et al., 1997)			50		
Angiotensinogen	(Hughes, et al., 1992)			30-60		
Anti-chymotrypsin		(Thompson, 1988)			2.1	
Anti-thrombin III	(Ganrot, et al., 1997;Hughes, et al., 1992)			150 (120-390)		
Apolipoprotein AI	(Anderson and Anderson, 1977;Marshall and Williams, 1987;Marshall, et al., 1989;Marshall and Williams, 1991)	(Thompson, 1988; Sanchez, et al., 1995)	(Lapin, et al., 1989;Marshall and Williams, 1987;Marshall and Williams, 1998;Celis, et al., 1999)	900-2100	6	
Apolipoprotein AII	(Anderson and Anderson, 1977;Marshall and Williams, 1991)		(Marshall and Williams, 1998)	260-660		
Apolipoprotein AIII			(Marshall and Williams, 1998)			
Apolipoprotein AIV	(Hughes, et al., 1992)	(Sanchez, et al., 1995)		140		
Apolipoprotein CII	(Marshall and Williams, 1991;Zannis, et al., 1980)					
Apolipoprotein CIII	(Zannis, et al., 1980)					
Apolipoprotein D	(Anderson and Anderson, 1977;Marshall and Williams, 1991)	(Marshall and Williams, 1991; Sanchez, et al., 1995)		80-100		
Apolipoprotein E	(Anderson and Anderson, 1977)	(Wiederkehr, et al., 1987 ;Sanchez, et al., 1995)		30-50		
Apo-serum amyloid A	(Marshall and Williams, 1991)					
Asialotransferrin		(Davidsson, et al., 1997)				
Aspartate aminotransferase	(Lacher and Paolino, 1988)					

The BFPDB database is available at the department website (www.angstrom.uu.se/ionphysics/bfpdb.html).

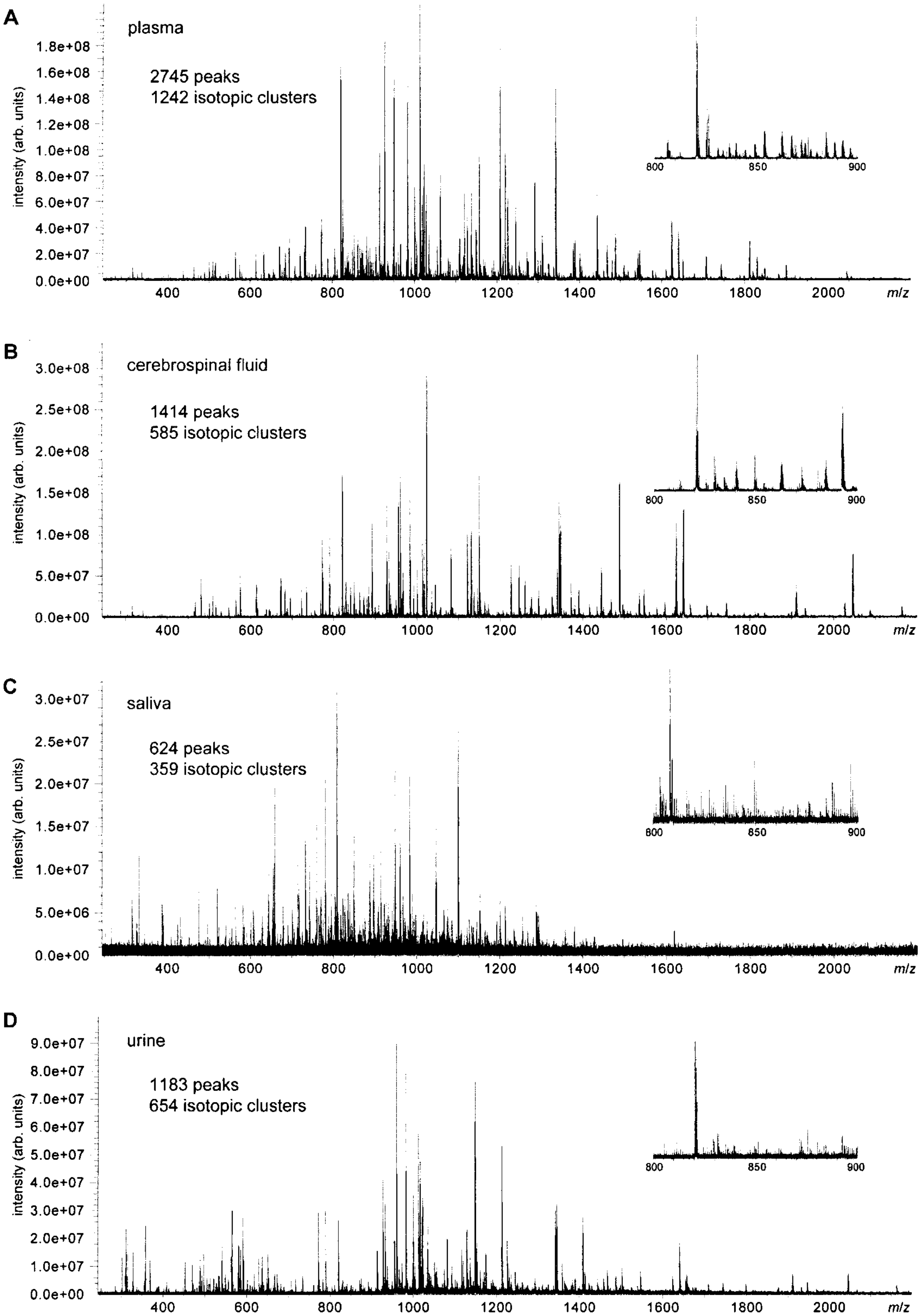

FIGURE 2. Examples of FTICR mass spectra of plasma (**A**), CSF (**B**), saliva (**C**), and urine (**D**) tryptic digests. The insets illustrate the density of isotopic clusters in the region *m/z* 800–900. Information on the number of peaks and isotopic clusters is also provided in the spectra. Approximately 50% of all peaks could be assigned to a specific protein in the body fluid database.

reduced to 350–2,000 isotopic clusters, originating from up to 1,000 unique masses (or 1,300 in the 21,600-scan plasma spectrum). Approximately 50% of all masses could be assigned to a specific protein. In Figure 2A,D, continuous infusion spectra of 512-K data points and 2,048 scans are shown for tryptically digested human plasma, CSF, saliva, and urine, respectively. It is not an obvious advantage to collect 21,600 scans compared to 2,048. What is gained in signal-to-noise ratio could be lost in accuracy due to instrument drift. Also, ~18 μL of sample was consumed when collecting 21,600 scans compared to ~1.7 μL when collecting 2,048 scans.

Figure 3 shows a region of a plasma tryptic digest spectrum with the possible identity of some of the peaks indicated.

Table 2 lists some of the proteins and protein precursors identified as possible analytes in various human body fluids. This information has been collected from the scientific literature and other databases (see references in Table 2), and will be made available on the department web site (www.angstrom.uu.se/ionphysics/bfpdb.html).

Protein identification based on peptide mapping is always probabilistic; i.e., a match is never certain, only more or less probable. The accuracy needed to assign peptides depends on the nature of the sample and on the mass of the peptides. The 150 proteins in our database generate > 16,000 theoretical tryptic peptides, assuming a maximum of one missed cleavage site and no variable modifications. On average, 0.53 peptides and at most nine peptides were found within 5 ppm from another peptide. These numbers increase with the number of proteins in the database. For 1,000 randomly chosen yeast proteins, the average number of peptides within 5 ppm was 1.78, and the maximum was 54 peptides. The standard deviation of the mass measurement error in the 9.4 T FTICR-MS is ~2 ppm.

To get an idea of the statistical significance for a certain identification, it is appropriate to compare with random peptide mass matches from sequences derived from an unrelated organism. The random matches from the *Saccharomyces cerevisiae* proteome can be used to calculate the probability (or risk) for a random match (false positive) when identifying a human protein, and thus to calculate the significance. The biologist may object that *Homo sapiens* and *S. cerevisiae* are indeed related organisms, but the evolutionary relationship must be much closer and protein sequence homologies near 100% to generate any significant cross-talk in peptide mass fingerprinting (i.e., common tryptic peptides in distantly related human and yeast proteins). There are other means to discriminate between true and random matches. For

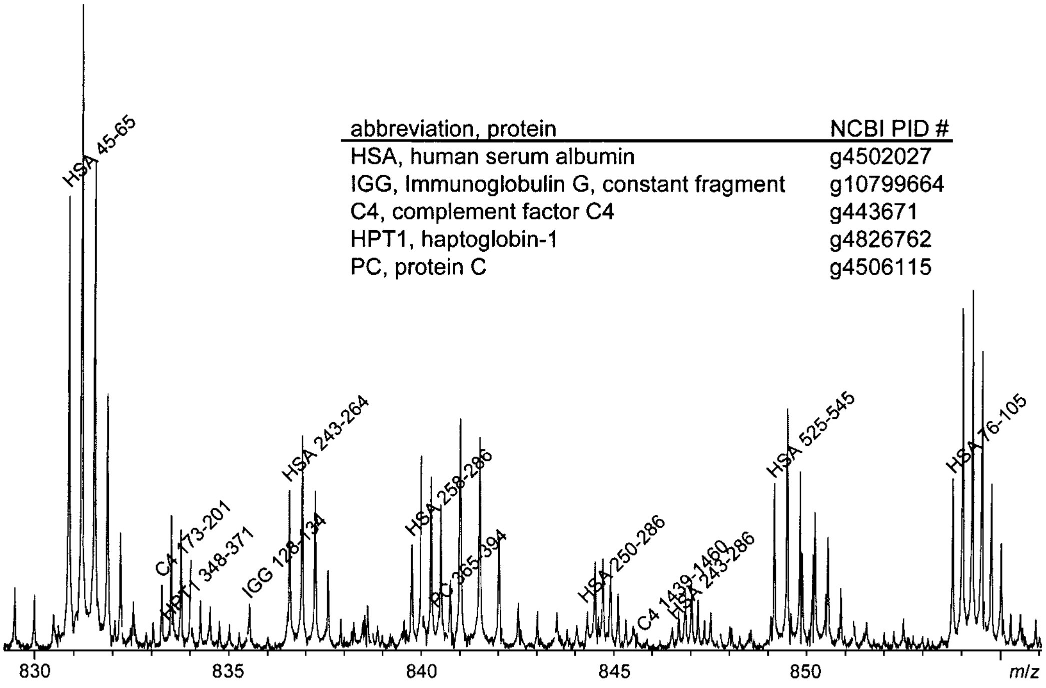

FIGURE 3. Detailed view of a region of a plasma tryptic digest spectrum with some of the peaks putatively identified from the small subset of the NCBI protein database. As can be seen, there is much overlap between isotopic clusters in the densest regions of the spectra, sometimes with as many as four to five overlapping clusters of isotopic peaks.

instance, the patterns of matching peptides in the sequence differ between true and random matches. In general, true matches have non-random distributions of peptides in the protein sequence. One way of quantifying this one-dimensional pattern is to look at the distributions of peptides with zero, one, or two close neighbors in the sequence—here simply called the 'connectivity' in a peptide map. The choice of a spatial pattern descriptor of peptide maps such as this aspect is supported by the empirical notion that true peptide matches are often contiguous. This pattern also appears as a factor in the Bayesian model used in the ProFound algorithm (Zhang & Chait, 2000).

A number of proteins could be identified in the body fluids by the direct infusion-FTICR MS of tryptic digests with a significance of 99%; i.e., 1% probability that the match is random. The number of peptides required for a protein match depends on the size of the protein, or rather, on the number of theoretical tryptic fragments of the protein.

The most significant matches were HSA (in plasma, CSF, and urine) and amylase (saliva). The most abundant proteins in the body fluids were also those identified with the highest significance (Table 3). At most, 1–2 of the 99% and 4–8 of the 95–99% significant can be expected to be false (random) matches, given the number of hypotheses tested (150). The matches are ranked according to the product of the mass measurement error likelihood ratio and the peptide connectivity.

The significance was calculated by taking the product of likelihood ratios (LR) for the measured mass measurement error distribution, and comparing the distribution of random matches (from yeast) with the expected distribution of true matches (in the presence of the background of random matches) according to the Neyman–Pearson lemma. The random and true mass measurement error distributions are both approximately normal, but the true matches have a much smaller variance. A small negative skewness of the random distribution could be observed. This actual, measured distribution for the random matches (from yeast) and a Gaussian fit to measured errors of HSA in plasma (discarding outliers) were used.

Information on the non-randomness in the sequence coverage was also included to improve significance of true matches. In this case, the 'connectivity,' i.e., the distribution of peptides having 0, 1, and 2 neighbors in the sequence for all matching peptides (within 5 ppm), was compared with that derived from the yeast proteins (Fig. 4). Protein identification (via peptide mass fingerprinting), using, for example, MASCOT or ProFound, also returns the most abundant proteins; serum albumin (in plasma, CSF, and urine) and amylase (saliva) as the top match. However, these programs assume a fixed number (1–4) of proteins in the mixture.

The abundance of a specific protein, the cleavage efficacy, the likelihood that a number of peptides from that protein are detected, and the total number of peptides in the sample determines whether this protein will be identified. In human body fluids, the number of proteins varies, and there is probably no good estimate of this number. If all proteins are included, from the most abundant (e.g., HSA 45 g/L) to the least abundant (a few copies per liter), then there are thousands of possible proteins in these fluids. Looking at the proteins detected in the various body fluids here, the dynamic range ranges from 45 g/L (HSA in plasma) to > 0.27 mg/L (Alpha-2-glycoprotein 1 in CSF). However, the specific levels of all proteins in the database are not known.

As we have shown in this review, it is possible to successfully analyze protein mixture tryptic digests by direct infusion. The high-sequence coverage typically

TABLE 3. Matches between BFPDB (150 sequences) and direct infusion ESI–FTICR mass spectra of the four body fluids

Plasma	CSF	Saliva	Urine
Serum albumin	**Serum albumin**	**Amylase alpha 1A**	**Serum albumin**
Immunoglobulin G	**Transferrin**	**Apolipoprotein A-IV**	**Uromodulin**
Immunoglobulin A	**Cystatin C**	Fibrinogen alpha	**Haptoglobin**
Fibrinogen alpha	**Immunoglobulin G**	Complement factor B	**Cytokeratin**
Transferrin	**Perlecan**	Complement factor 9	**AMBP protein**
Plasma kallikrein	**Alpha-1-antitrypsin**	Immunoglobulin G	**Alpha-1-microglobulin**
Complement factor C4	Complement factor C4	Complement C3	**Epidermal growth factor**
Haptoglobin	Prostaglandin D synthase	Vitronectin	**Apolipoprotein D**
Cystatin C	Alpha-2-glycoprotein 1		Complement component C4
	Haptoglobin		Alpha-1-acid glycoprotein 1
	Fibulin 1 isoform D		Protein Z
	Actin gamma		Prostaglandin D2 synthase
	Alpha-1-antichymotrypsin		Alpha-2-HS glycoprotein

Proteins in boldface are 99% significant, others are 95–99% significant when tested individually. The abundancies (levels in milligram per liter) of different proteins in each fluid can, in most cases, be found in BFPDB or in Table 2.

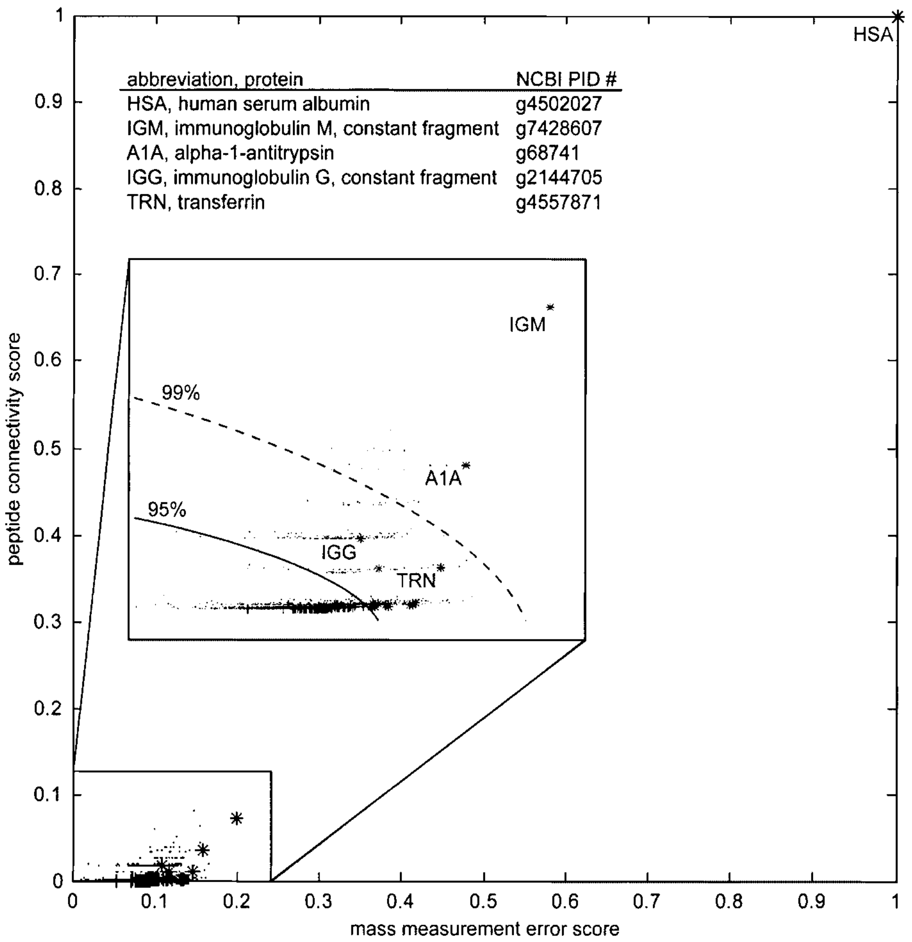

FIGURE 4. The probability-based mass measurement error and ‘peptide connectivity’ scores for 4,861 yeast sequences and 150 sequences of human proteins suggested to be present in the body fluids for a plasma tryptic digest spectrum, illustrating that spectra contain information on more proteins than the most abundant protein (HSA). The scores were logarithmically scaled and were normalized. Two areas that contain 95% (solid) and 99% (dashed) of the yeast distribution of 4,861 sequences (dots) are indicated. Nineteen sequences of the 150 in the database were outside this 95% region. In the best spectra, about 10 proteins could be identified with 99% significance or better (using the statistical method described in this study), demonstrating that even complex protein mixtures can successfully be analyzed by direct infusion.

achieved for abundant proteins allows the localization (with some significance) of almost any post-translational modification at different positions in the protein. In this way, the most abundant proteins in the body fluids can be identified already in direct infusion experiments, and with additional two-dimensional approaches it is highly likely that this number will increase significantly. It is known that separation methods are particularly useful in the analysis of real biological samples, where sensitivity and a wide dynamic range are more critical. Liquid separations are instrumental in reducing ion-suppression effects in the electrospray process and space-charge effects in the FTICR analyzer cell; both effects limit the dynamic range and sensitivity in mass spectrometric analyses. Also, new means to fragment peptides and proteins to achieve structural information, such as electron capture dissociation (ECD) (Zubarev, Kelleher, & McLafferty, 1998), have been shown to further extend the capabilities of FTICR MS. ECD can successfully generate short sequence tags of abundant components in a tryptic digest on the time-scale of a chromatographic separation (Palmblad et al., 2002).

IV. CONCLUSIONS

The resolving power and accuracy of the FTICR-MS technique facilitated the screening of complex biological samples without any extensive pre-purification. The tremendous amount of information (more than 5,500 peaks in a single spectrum) that this procedure reveals may well be used for fingerprint analysis and for screening of clinically relevant markers. The megabyte amounts of raw data generated in the described analyses require efficient computer algorithms for processing spectra, and statistically robust methods for searching protein sequence databases to identify and characterize the peptides and proteins in complex biological samples. In mass spectra of the intact body fluids, no (or very few) proteins could be identified, whereas in the digested samples several of the abundant proteins were characterized with a high-sequence coverage (between 50–95% with mass measurement errors below 5 ppm). Thus, this approach may become an important complement to ordinary tools not only in clinical diagnosis, but also in proteomics and functional genomics. Present work includes the optimization of capillary electrophoresis-FTICR MS, capillary electrochromatography-FTICR MS, liquid chromatography-FTICR MS, and liquid chromatography-ECD-FTICR MS. These hyphenated techniques typically generate 10,000–100,000 peaks in each analysis. The separation methods will be applied in the characterization of peptides and proteins in human body fluids from healthy controls as well as clinically sampled material, whereas direct infusion can provide a rapid first screening of the samples.

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