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Surface enhanced laser desorptions ionization-time of flight-mass spectrometry analysis in complex food and biological systems

Harshadrai M. Rawel[1], Sascha Rohn[2], Jürgen Kroll[1] and Florian J. Schweigert[1]

[1]University of Potsdam, Institute of Nutritional Science, Department of Physiology and Pathophysiology, Potsdam, Germany
[2]Technical University of Berlin, Institute of Food Technology and Food Chemistry, Department of Food Analysis, TIB 4/3-1, Berlin, Germany

1 Introduction

Parallel to the expansion of gene technology, there has been an equally rapid development of MS. Two ionization techniques, MALDI and ESI have revolutionized the study of biology and biochemistry of biomolecules by permitting sensitive, rapid, and molecularly specific analyses of peptides and proteins [1–5]. Important information that can be determined by MALDI-MS includes the monomer mass, end group mass, and molecular weight distribution. Besides high mass accuracy, this technology also provides information on protein modifications such as phosphorylation and glycosylation, and allows the identification of proteins by peptide sequencing combined with protein database searches [3, 4, 6, 7]. MALDI-MS has also revealed itself as a powerful method for the characterization of synthetic and natural polymers [6] and was recently introduced for the analysis of condensed tannins or simple flavonols, mainly, in food science [8–15]. It is often used for the analysis of relatively pure proteins obtained from a complex food or biological sample (such as blood, serum, plasma, lymph, urine, cellular secretions *etc.*), by a rather lengthy set of isolation and purification procedures. Proper sample preparation is critical to this technique, and thus is an area of persistent interest [4, 6, 16–18]. In many cases, this means application of different protein purification techniques like chromatography (ion-exchange, size exclusion, affinity and RP), electrophoresis (SDS-PAGE, IEF, 2-D PAGE), centrifugation, membrane dialysis, immuno-thermal- and/or pH-dependant precipitation [16]. Some of these procedures not only require large amount of samples, but are also time and cost intensive being further associated with analyte loses, especially of minor components [16].

Correspondence: Dr. Harshadrai M. Rawel, University of Potsdam, Institute of Nutritional Science, Department of Physiology and Pathophysiology, D-14469 Potsdam, Germany
E-mail: rawel@rz.uni-potsdam.de
Fax: +49-33200-88-541

Abbreviations: ion exchange chips (anionic – SAX or cationic – WCX); **LDI,** laser desorption ionization; **SELDI,** surface enhanced laser desorption ionization; **TTR,** transthyretin

A general problem as exemplified by the protein composition of blood plasma is the great dynamic range of protein concentrations over at least nine orders of magnitude, the wide range of protein properties, including mass, p*I*, extent of hydrophobicity and post-translational modifications as well as the circumstance that the majority of plasma proteins are commonly present at very low abundances. The major protein constituents of serum include albumin, immunoglobulins, transferrin and haptoglobin. These proteins are accounting for 80% of the total plasma protein concentration of serum with 60–80 mg protein/mL [19–21]. Thus, a main challenge facing proteomics is reducing sample complexity prior to analysis. Among the many strategies used to achieve this goal is the use of different protein properties for preselection. This can be achieved by different means. Recent improvements to this end have been delivered by a promising new development termed surface-enhanced laser desorption/ionization -TOF-MS (SELDI-TOF-MS). The concept of SELDI was first introduced in 1993 by Hutchens and Yip [22]. This approach allows simplified sample extraction and facilitates effective on-probe investigations of proteins with on-chip binding and detection [16]. In SELDI, the sample support, typically referred to as probe, has been modified to accommodate extraction, modification and/or amplification of the sample. Principally two types of surface modifications can be distinguished, which are based on their unspecific or specific binding surface properties (Fig. 1): The first group includes the classic chromatographic separation moieties, such as RP, hydrophilic (normal phase, NP), ion exchange

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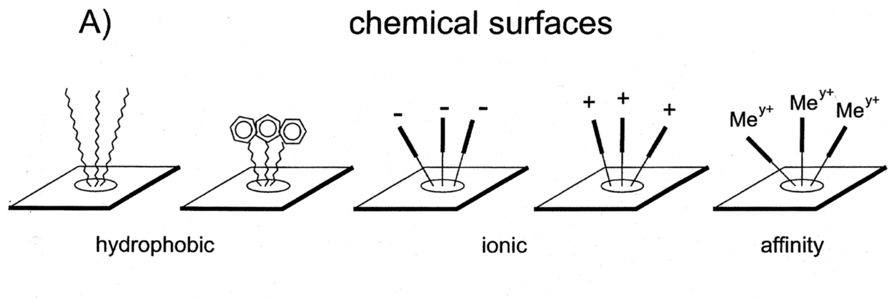

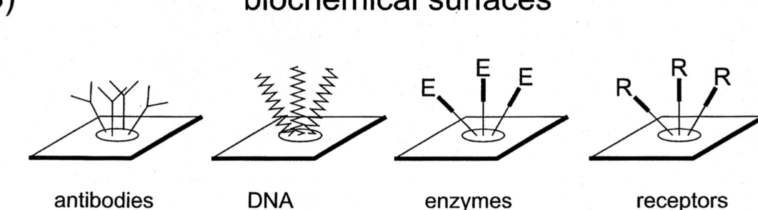

Figure 1. Principal types of surface modifications applied in SELDI-TOF-MS.

(anionic, SAX or cationic, WCX), IMAC and mixed-mode media. The second group contains arrays with biochemical surfaces, in which any molecule of interest may be linked covalently to the surface, like antibodies, receptors, enzymes, DNA, ligands, lectins *etc.*, allowing selective enrichment of captured analytes [16]. At commercial level, SELDI-TOF-MS is offered by Ciphergen ProteinChip® Array System (Ciphergen Biosystems, Palo Alto, CA, USA).

2 Materials and methods

2.1 Principle and instrument setup

The general principle (Fig. 2) of LDI-TOF-MS revolves around the rapid photo-volatilization of a sample embedded in an UV-absorbing matrix followed by time-of-flight mass spectra analysis [23]. The analyte material is carefully mixed with a suitable matrix, typically a low molecular weight organic acid, and the sample is irradiated with a laser beam (typically a 237-nm N_2 laser). The laser energy is absorbed by the matrix crystal, causing it to rapidly dissociate and release intact matrix molecules and small, volatile molecules, such as carbon dioxide and water [6]. These released gases carry intact analyte molecules into the gas phase. Any analytes cationized by available protons (H^+) or metals (for example, Na^+, K^+, or Ag^+) are subsequently mass analyzed [6]. Such ions are accelerated into the TOF mass analyzer, which consists of a field-free flight tube and travels in the linear operation mode to a detector at the end of the instrument [4]. Once the flight time is known, the *m/z* of the analyte ions can be calculated as reported in detail in [4, 16] for each instrument, which in turn needs to be calibrated with compounds of known mass (see also Fig. 2). The linear operation mode is limited in resolution, because an initial energy spread of the ions will cause a spread in the

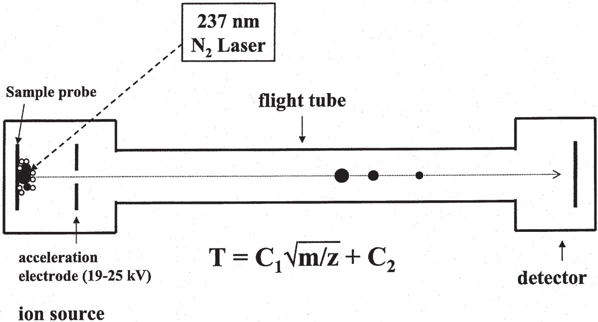

Figure 2. General principle of an LDI-TOF-MS instrument; where C1 and C2 are instrumental constants that can be determined with compounds of known mass [4].

TOF of the ions. This may be partially compensated by reflecting the ions in an electrostatic mirror and detecting the ions with a second detector (reflector mode) or by delayed ion extraction [4]. In this context, although SELDI-TOF-MS provides many other advantages described below, it still lacks in resolution power when compared to MALDI-TOF-MS.

2.2 Sample and matrix application

The requirements of the matrix molecules are that they must have UV absorption at the wavelength of the laser (typically a 237-N_2 laser), low volatility and ability to transfer protons to the analyte molecules [4]. The analyte and the matrix are first embedded in a crystal or thin film, whereby the analyte is an integral part of the matrix crystal or film. Laser energy is applied to the sample, creating gas phase neutrals and ions [16]. During LDI, thermal energy is

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Table 1. Common matrices used in LDI MS [11, 15, 23–26]

Matrix	Structure	Analyte molecules
CHCA α-Cyano-4-hydroxycinnamic acid	N≡C–C=CH·COOH, OH	Peptides, (polymers), theaflavins
SA Sinapinic acid 3,5-Dimethoxyl-4-hydroxycinnamic acid	HC=CH·COOH, H_3CO, OCH_3, OH	Proteins, polymers
DHB 2,5-Dihydroxybenzoic acid	COOH, OH, HO	Peptides, proteins, lipids, nucleic acids, condensed tannins, isoflavones
HPA 3-Hydroxy-2-pyridinecarboxylic acid	COOH, OH, N	Nucleic acids
Dithranol 1,8,9-Trihydroxyanthracen	OH OH OH	Polymers
IAA 3-β-Indole acrylic acid	COOH, N, H	Polymers, condensed tannins
Retinoic acid	CH_3, CH_3, CH_3, COOH, CH_3, CH_3	Polymers
THAP 2′,4′,6′-Trihydroxyacetophenone	O=C–CH_3, HO, OH, OH	Anthocyanins, flavonol glycosides, isoflavones, theaflavins

absorbed by the matrix molecules from the laser causing them to be heated via direct or secondary thermal changes. This thermal energy is transferred to the analyte molecules, thus producing desorbed ions [16].

Typical matrices suitable for LDI-MS of different analyte molecules are given in Table 1 [11, 15, 23–26]. A number of MALDI-MS sample preparation techniques in terms of their suitability have been developed and classified [7, 27, 28]. The most commonly applied techniques include dried-droplet method, thin-layer methods and sandwich method, the details of which are given in [27]. The importance of matrix selection, matrix and analyte concentration, pH adjustment, crystallization conditions and the use of additives, the tolerance of different sample preparations towards salts, buffers, synthetic polymers, detergents, denaturants and other contaminants, as well as the influence of the preparation methods on undesired amino acid side-chain oxidation have been evaluated for MALDI-MS in [27]. For example, dramatic mass discrimination effects in the MALDI-MS response of peptides, proteins, and their mixtures using CHCA as matrix have been demonstrated underlining the importance of sample preparation [28]. These effects were found to be strongly dependent on the sample-matrix solution composition, pH, and the rates at which the sample-matrix co-crystals are grown [28]. During sample preparation, high concentrations of salts (NaCl, $CaCl_2$, KH_2PO_4), conventional detergents (SDS, *n*-octylglucoside, Tween, Triton), denaturing reagents (urea, guanidine salts) or solvent additives (DMSO, glycerol) interfere with MALDI-MS and need to be eliminated or reduced prior to analysis. In this context, ammonium dodecyl sulfate has been suggested as an alternative to SDS for protein sample preparation with improved performance in MALDI-MS [29]. A new methodology employs cleavable detergents to enhance the solubility of intracellular and membrane proteins to be analyzed by MALDI-MS without degrading spectral quality [30]. Cleavable detergents are surfactants that have an easily cleavable bond connecting the hydrophilic head to the hydrophobic tail of the surfactant. Introduction of this chemical group into the surfactant provides a mechanism by which unwanted surfactant properties, *e.g.* foaming and aggregation, can be eliminated in a controlled way following protein solubilization [30]. On-target DTT application allows the reduction of disulfide bonds. This is usually done by placing a droplet (1–5 μL) of DTT solution (*e.g.* in ammonium hydrogen carbonate, pH 7.8) on top of the matrix-sample crystals as reported in [27, 31, 32].

SELDI-TOF-MS on the other hand relies on the selective retention of proteins on a functionalized surface. Selective retention allows analysis of samples incompatible with MALDI samples containing above-mentioned constituents such as residual salts, buffers, detergents, and organics. The samples can be applied directly to the ProteinChip Array and the constituents can then be washed free from the selectively bound proteins. In addition, the retained proteins are homogeneously presented to the matrix molecules and laser energy stream with SELDI technology, enhancing signal sensitivity, reproducibility, and quantifiability [33].

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3 Results and discussion

3.1 General remarks

With regard to proteome analysis, SELDI-TOF-MS can be applied to a great variety of applications in the analysis of peptides and proteomes from complex biological matrices (see Table 2 for some typical examples). It can be used for comparative protein profiling, for protein purification and protein identification, to monitor peptide and protein modifications. In the following, selective aspects related to food and biological matrixes such as blood plasma or saliva will be described.

Table 2. Some typical examples of SELDI-TOF-MS applications

Applications
– Serum proteomics/protein profiling; serum protein expression
– cancer related differentiation of serum protein and peptide profiles
– ProteinChip array-based assays, *e.g.* for beta amyloid
– new proteomic tests, *e.g.* urinary test for patients with urolithiasis.
– characterization of potential biomarkers in biological fluids *e.g.* cerebrospinal fluid biomarkers for the diagnosis of Alzheimer's disease
– protein identification, *e.g.* thymosin beta-4 from lymphoblastoid cell lines
– protein digestion and structural characterization
– dentification of posttranslational protein modifications in food systems and biological fluids
– protein-protein interactions; protein/non-protein interactions

3.2 Characterization of complex analyte mixtures for comparative protein profiling

One of the most important steps in proteome analysis involves the separation and subsequent identification of the proteins. 2D-PAGE is the most frequently applied technique for separation of majority of the proteins from a given cell type [4]. Recent advances based on retentate chromatography-MS (RC-MS) or shortly termed SELDI-MS can contribute to improvements in many aspects of protein identification, including fermentation/cell-culture optimization, purification development, process monitoring, and product analysis [17, 33, 34]. This basic system applying a biochemical platform is one of the key features of SELDI-TOF-MS in its ability to provide a rapid protein expression profile from a variety of biological and clinical samples. It has been used for biomarker identification as well as the study of protein-protein, and protein-DNA interaction. The versatility of SELDI-TOF-MS has allowed its use in projects ranging from the identification of potential diagnostic markers for a variety of diseases such as cancer and neurological impairments or to describe age dependant changes or kidney function [35, 36] as well as to the study of biomolecular interactions and the characterization of post-translational modifications [37, 38].

In following, the advantage of SELDI-TOF-MS over more commonly applied MALDI-TOF-MS can be illustrated by applying this technique to the characterization of saliva. Proteins are known to play an important role in identifying genetic differences. ProteinChip Arrays that are applied for SELDI-MS have been described above and characteristically possess multiple "spots" with all spots on a given array carrying the same chromatographical separation media. After applying, *e.g.* saliva samples to selected spots, non-bound components can be rinsed from the array. Bound components can then be selectively desorbed by washing individual spots with appropriate buffers, as would be done during step-elution chromatography. In our case, four different classical chromatographic separation moieties, such as RP, hydrophilic (normal phase, NP) and ion exchange (anionic – SAX or cationic – WCX) were tested with human saliva. The obtained results are illustrated in Fig. 3. All four tested chips are suited for peptide analysis in range of 1000–7000 *m/z* and document different retentate behavior. For a reproducible high-throughput testing/screening purpose, the SAX chip was found to be most valuable for characterization of whole saliva peptides and proteins in the range 2–20 000 *m/z* and the WCX Chip in the range of 20–80 000 *m/z* under the applied conditions as illustrated in Fig. 4. Further, there is still a large potential in optimizing the retention behavior by using more appropriate buffer systems. These findings can also then be applied to matching preparative chromatographic sorbents to attain the comparable behavior under elution conditions. In a similar way, the complexity of the protein mixtures from cereals and milk was also investigated and is reported in detail in [39]. In case of wheat, the WCX chip was found to be well suited for characterization of the gliadin faction (soluble in 70% ethanol) in the range of 15–45 kDa [39]. This methodical approach would allow characterizing the art of specific differences in protein composition of cereals and helping in screening for celiac immunoreactive proteins in complex formula food samples [40, 41]. The procedure would also permit the study of the alteration of gliadins in food during the baking process [42]. Further, LDI-MS may be preliminarily established as a unique system with the ability to discriminate the specific type of gluten toxic fractions present in food samples [43].

3.3 Protein modification

The nutritional value of food proteins is governed by amino acid composition, ratios of essential amino acids, susceptibility to hydrolysis during digestion, source, and the effects of processing. Many protein modifications occurring due to

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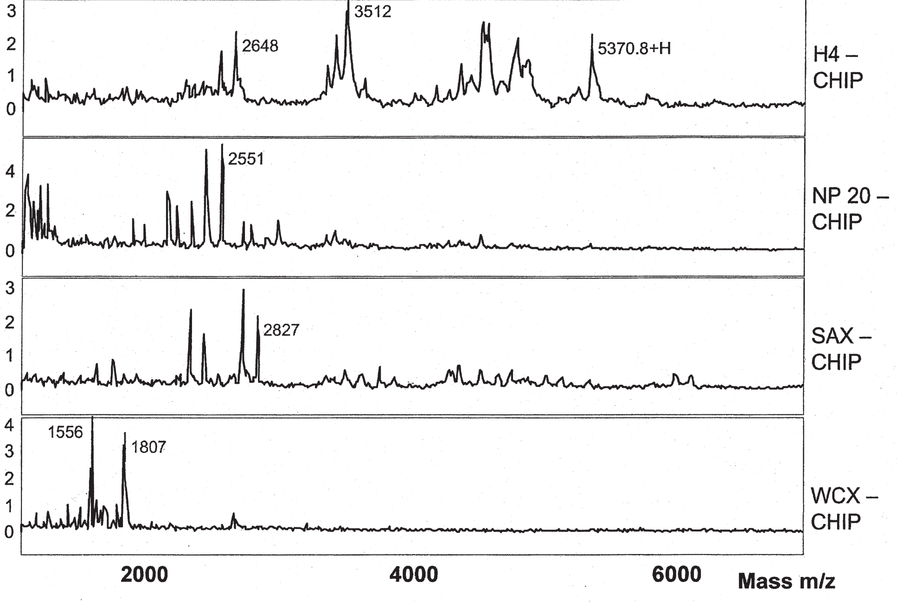

Figure 3. SELDI-TOF-MS of whole saliva peptides on ProteinChip1 Arrays. Code: reverse phase (RP), hydrophilic (normal phase - NP) and ion exchange (anionic - SAX or cationic - WCX); matrix applied – α-cyano-4-hydroxycinnamic acid.

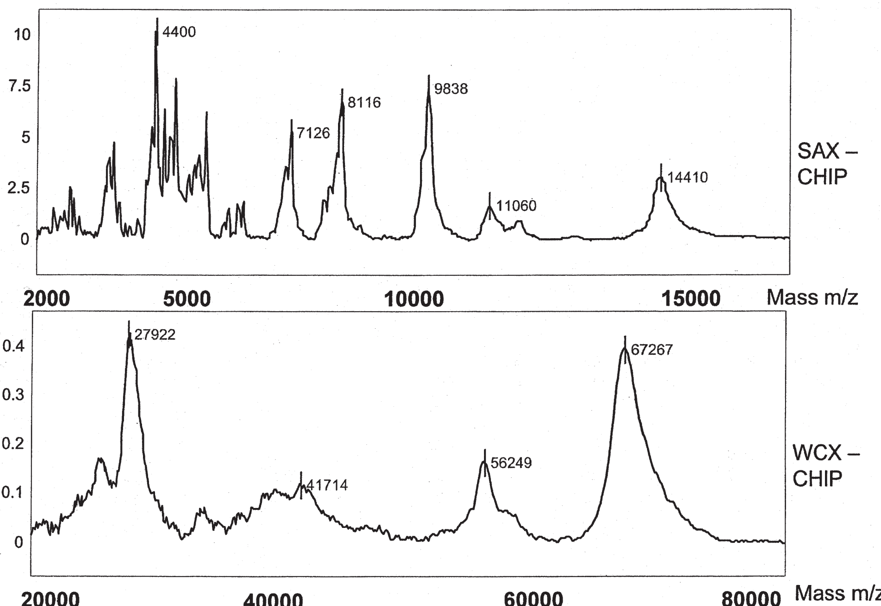

Figure 4. SELDI-TOF-MS of whole saliva peptides (2–20 000 *m/z*) and proteins (20–80 000 *m/z*) on ion exchange (anionic - SAX or cationic - WCX) ProteinChip1 Arrays; matrix applied – sinapinic acid.

reactions with other food constituents (*e.g.* oxidized plant phenols) in course of food storage and processing may lead to a loss in nutritional quality, which is especially serious in underprivileged countries [44, 45]. One of the classical ways to characterize such protein modifications is the application of a soft ionization technique using mass spectrometry. One of our main research fields includes the interactions of food proteins with secondary plant metabolites. The latter are native food components, which are becoming more and more interesting due to their physiological effects on human beings. In context of these studies, it was possible to characterize the modifications of selected proteins in model systems in presence of plant phenols [46–50] and in presence of breakdown products of glucosinolates [51] by using both MALDI- and SELDI-TOF-MS. Figure 5 shows exemplary the covalent binding of quercetin and rutin to whey proteins.

Similarly, MALDI-TOF-MS was also applied in the investigation of glycation processes [31, 32, 52–54]. Glycation summarizes non-enzymatic reactions between amino groups of proteins and sugars or sugar degradation products, leading to early glycation products (intact sugar attached) and advanced glycation end products (AGEs). Protein glycation is involved in the progression of several diseases, such as diabetes, uremia, and atherosclerosis [31,

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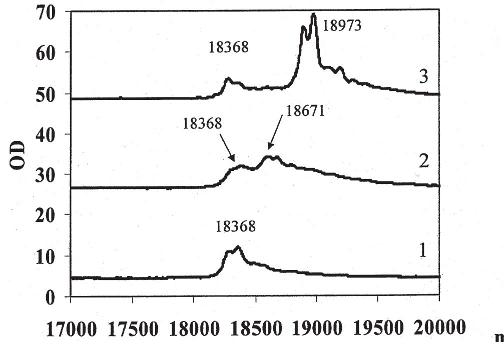
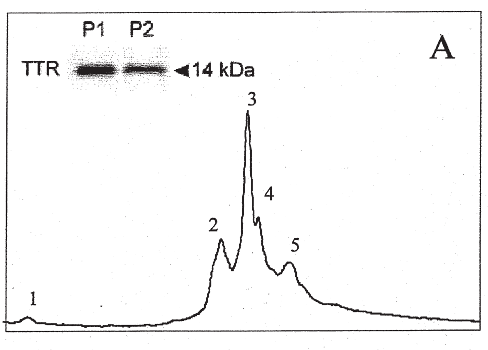


Figure 5. SELDI-TOF-MS of β-lactoglobulin derivatives. Code: 1 = control β-lactoglobulin; 2 = quercetin β-lactoglobulin derivative; 3 = rutin β-lactoglobulin derivative.

52]. Therefore, it was possible to qualitatively study the formation of early Maillard products of lysozyme, produced upon incubation with different sugars in model experiments [31] as well as to determine the average number of sugar residues attached to serum albumin or immunoglobulins of diabetic or uremic patients [52] by applying MALDI-TOF-MS.

3.4 Analytical approaches based on biochemical properties

Among the selective binding surfaces, a most promising analytical approach for the characterization of both post-translational modifications and protein complexes is the use of high-affinity antibodies to capture proteins of interest and their interacting proteins. Antibodies are very appropriate receptor elements in a protein detection array, as they posses the specificity required to identify a target epitope [37, 38, 55]. The SELDI platform can be readily adapted for developing such an immunoassay format. The immuno SELDI-TOF-MS functions by attaching the antibody directly or via protein A to the spots of a pre-activated ProteinChip1 Array (PS20; Ciphergen Biosystems). The PS20 array consists of a surface with epoxy groups that dock proteins by covalently reacting with their amine groups. After incubation under appropriate conditions, blocking of residual active sites and washing steps, the antibody can be added. The unbound antibodies are than removed by washing again followed by the addition of the biological samples to be investigated like plasma, urine *etc.* [56]. This assay has been successfully applied in our laboratory to identify transthyretin (TTR, formerly called prealbumin) [55–57]. Transthyretin belongs to a group of proteins including thyroxin-binding globulin and albumin, which bind and transport thyroid hormones in the blood [56]. Since TTR is found not only on its own but also with post-



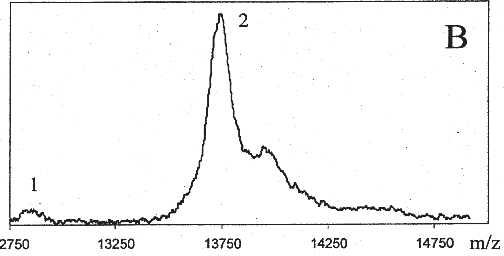


Figure 6. Representative mass spectrum of TTR of a healthy human after on-chip immunological affinity enrichment using a polyclonal TTR antibody (A) and after incubation of the TTR variants with DTT (B); Inlay: P1 and P2 represent two different plasma samples; Western blot of TTR monomer of ~14 Da after SDS-PAGE and Western blotting using a polyclonal TTR antibody in plasma of healthy individuals. Peak labels: 1 = truncated TTR; 2 = native TTR; 3 = S-cysteinylated TTR; 4 = cysteinglycinylated; 5 = S-glutathionylated TTR [55].

translational modifications as well as in a protein-protein-complex with retinol-binding protein (RBP) it is ideal for use in illustrating these analytical approach [55–57]. Figure 6A shows a representative spectrum of TTR variants normally occurring in plasma such as the native, the cysteinylated and the gluthationylated form. A classical Western blot would only result in a single band at ~14 kDa as depicted in the inlay of Fig. 6. Using the example of TTR it can be shown that the analytical system can be as well used to perform on-chip modification of proteins. This includes not only the limited proteolysis for protein fragmentation but also the treatment of proteins with DTT. In the case of TTR this treatment result in a complete loss of all variants of TTR in favor of the native form (Fig. 6B), indicating that all modifications are due to changes at the position of Cys 10.

Considering that this on-chip procedure using the SELDI-TOF technology is quite easy to use, is much less time consuming and is much more sensitive at least in the low molecular weight range than Western blotting, it therefore may provide a very promising approach for the screening of

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autoantibodies in autoimmune diseases [58]. Due to its versatility, this on-chip technology could allow the large-scale screening for complex autoantibody distributions for diagnostic purposes and early detection of autoimmune diseases might be possible [58].

3.5 Protein identification and structural characterization

Structural information can be easily generated by mass spectrometric peptide mapping. Such an application is suggested for characterization of saliva proteins in Fig. 7. Peptide mass fingerprinting is a powerful technique in which experimentally measured *m/z* values of peptides that result from a protein digest with specific endoproteases form the basis for a characteristic fingerprint of the intact protein [59]. The specific endoproteases frequently applied include trypsin (arg, lys), LysC, GlucC, Chymotrypsin (tyr, trp, phe) and AspN. The generated lists of peptide masses are used to identify the target protein using different protein data banks such as Swiss-Prot database (http://us.expasy.org/), Protein Identification Resource (PIR; http://pir.georgetown.edu/home.shtml) or National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). A digestion protocol producing peptide maps with broad amino acid sequence coverage of a protein will result in a greater number of peptide fragment masses belonging to a particular protein that can be entered into a search program, and therefore the confidence obtained in a match to a database will also be higher. This strategy relies on a good relative or absolute mass accuracy as the criterion that discriminates false positive results. Mass accuracy depends to a large extent on good resolution, which in turn is one of the key features of MALDI-TOF-MS. In addition, all ions undergo a certain degree of decomposition after acceleration, termed PSD. Sequence information on peptides can be generated in MALDI-TOF-MS by taking advantage of this PSD as described in [4]. Recent instrumental developments provide for levels of sensitivity and accuracy that make these techniques major analytical tools for proteome analysis [60, 61].

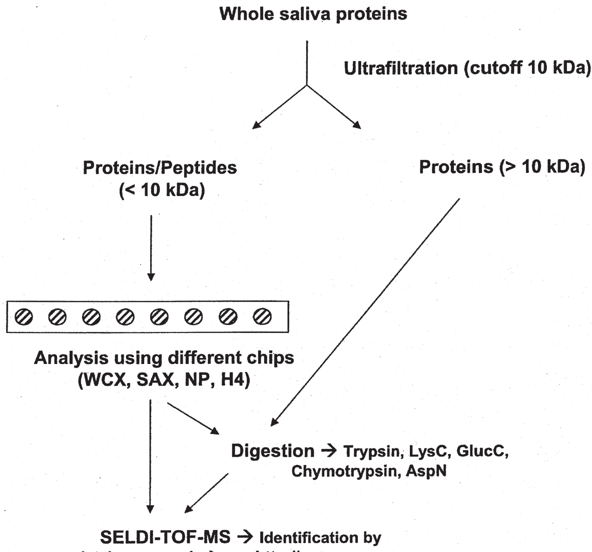

Figure 7. Suggestion to proteomics of saliva – protein separation, digestion and identification.

4 Concluding remarks

In conclusion, SELDI-TOF-MS offers a miniaturized, high-throughput approach that allows the on-chip pre-fractionation of complex samples. This will not only be of importance in biomarker discovery on specific diseases, but will also open new areas in the analysis of peptides, proteins and other ingredients in food samples. This will greatly improve our understanding of biological processes and will help in identification of positive and detrimental food constituents.

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