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Mapping of peptides and protein fragments in human urine using liquid chromatography–mass spectrometry

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Abstract

A method for the mapping of peptide mixtures, heterogeneous with respect to the concentration and the size of individual peptides, was established with the aim of obtaining a comprehensive analysis of human urine peptides. Peptide extraction and fractionation were optimized to achieve a two-step analysis, using reversed-phase and ion-exchange chromatography. Highly sensitive detection of peptides was performed by coupling microbore HPLC with electrospray mass spectrometry (ESI-MS). Peptides such as urodilatin, angiotensin and fragments of psoriasin, granulin and uromodulin were isolated and sequenced. The procedure presented here is a tool for the analysis of complex peptide mixtures from human urine. © 1997 Elsevier Science B.V.

Keywords: Peptide hormones; Peptide sequencing

1. Introduction

Biological samples are usually complex mixtures which contain a large variety of different components over a wide range of molecular masses. Up to several thousand different peptides and protein fragments in varying concentrations may be present. In addition to blood or serum, human urine is a relevant source for detecting peptides, proteins and their metabolized fragments [1–5], and it can be obtained and processed in sufficient quantities. Up to now, little information has been available about the composition of peptides in human urine. As elaborated for human hemofiltrate [6], urine is an additional source for the isolation of yet unknown peptides, which may play a role in medical diagnostics and in basic research of physiological processes. The characterization of these mixtures with respect to peptides may contribute to a better understanding of the physiological and pathophysiological processes in the organism, such as the metabolism of proteins and the processing of peptide hormones.

Malfunctions of the organism may be caused by the over- or underproduction of a regulatory peptide or result in a changed metabolism of larger proteins. The regulatory peptides and protein fragments ranging in molecular mass (M_r) from 300 to 15 000 may be excreted into the urine. Usually, peptides of diagnostic interest are determined by immunological methods such as radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) or by two-dimensional (2D) separation techniques. Immunological detection is limited to peptides or proteins against which specific antibodies have been generated. Furthermore, only substances bearing certain immunoepitopes can be detected by these methods. A useful characterization technique currently avail-

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able to specifically identify proteins in mixtures is 2D sodium dodecyl sulfate–polyacryamide gel electrophoresis (SDS-PAGE) [7–9]. With this method a complex mixture of proteins is separated into over a thousand different spots on a polyacryamide gel. The individual spots can be visualized and studied using a variety of techniques. A characteristic of SDS-PAGE is that the position of a spot in a gel can be correlated to the molecular mass of either a protein or a protein subunit. However, the accuracy of the molecular mass estimation can vary by at least 5–10%. In addition, this method is usually restricted to proteins with a M_r over 5000. In contrast, electrospray mass spectrometry (ESI-MS) gives a mass accuracy better than 0.1% and is extremely well suited for the detection of peptides of low and high molecular mass.

LC–MS coupling was introduced at the beginning of this decade when its importance was recognized for the discovery of biomolecules [10–13]. The combination of HPLC and MS in a tandem setting is an innovative device for the rapid characterization of peptides and proteins in complex mixtures. In contrast to the immunological and 2D techniques, it is possible to detect several substances within a wide M_r range in one measurement with LC–MS.

LC–MS analysis of peptide mixtures has been described for a number of applications. Most of these analyses have been carried out with either relatively simple mixtures, containing only a few peptides, or with mixtures containing peptides in equal amounts, like enzymatic digests [14], or with very complex mixtures, but with defined peptides in a narrow molecular mass range, like the MHC-peptides [15]. These publications describe the identification of proteins by the fragment mass fingerprint, the identification of post-translational modifications based on the known amino acid sequence, the identification of genetic variants in known proteins, such as serum albumin [16], or the characterization of the small MHC-peptides by LC–MS–MS. The MHC-peptides are short peptides with 15 to 25 amino acids, and thus well suited for MS–MS analysis.

In order to perform LC–MS analysis, peptide prepurification is necessary in order to remove non-peptidergic compounds such as lipids, cellular debris etc. Due to the wide range of concentrations and distribution of different peptides, a comprehensive analysis of the peptide extract is not possible in one single chromatographic separation step.

In this study, extraction, fractionation and concentration of peptides were carried out in two consecutive steps based on different separation principles including solid-phase extraction (SPE) and ion-exchange chromatography. The fractions obtained were analysed by highly sensitive LC–MS coupling. Additionally, peptides obtained as pure fractions were identified by N-terminal sequencing. Identification of peptides was supported by database comparison.

In the present study we demonstrate the usefulness of LC–MS coupling for the analysis of complex peptide extracts from human urine. Together with the identification of the peptides in human urine by chemical sequencing or MS–MS analysis, the work described here will lead to a complete map of the peptides, secreted from the organism.

2. Experimental

2.1. Human urine

Human urine samples were collected from healthy male and female volunteers, acidified immediately with hydrochloric acid to pH 2.7 and cooled to 4°C to prevent bacterial growth and proteolysis.

2.2. Peptide preparation

Peptide extraction was performed using SPE (C_{18}) followed by ion-exchange chromatography. To remove cellular debris and low-molecular-mass substances, tangential flow filtration (M_r 1000, Prep/scale TFF cartridge, Millipore, Eschborn, Germany) was performed. The filtrate (600.0 ml; conductivity 15.2 mS/cm) was applied to C_{18} octadecyl cartridges (Mega Bond Elute, 20 CC/5 GRM, Varian, Darmstadt, Germany). The cartridges were preconditioned with 100% methanol and washed with 0.1% TFA in water. After application of the sample the cartridges were then rinsed with 2% NaCl solution in 0.1% TFA to remove unbound material. The peptides were eluted from the cartridges with methanol–water–TFA (80:19.9:0.1, v/v/v). After dilution with deionized water to a conductivity of 0.7 mS/cm at pH 3.2,

the eluate was loaded onto a strong cation exchanger (PolySulfoethyl A, 100×4.6 mm I.D., 5 μm, 300 Å, ICT, Frankfurt, Germany). The elution was carried out with a linear gradient starting from 90% buffer A: 20 m*M* CH_3COOH, 10% CH_3CN, pH 3.2 to 80% buffer B: 20 m*M* CH_3COOH, 10% CH_3CN, 1 *M* NH_4Ac, pH 6.6 in 40 min with a flow-rate of 0.4 ml/min. Optical density (OD) was measured at 280 nm. Fractions were taken every 2 min, freeze-dried and stored at −20°C until further analysis by LC–MS. Analysis of the obtained fractions was performed using LC–MS coupling.

2.3. LC–MS coupling

2.3.1. Liquid chromatography system

Microbore RP-HPLC was performed on an ABI Model 140 B, dual syringe solvent delivery system and an ABI Model 785, programmable absorbance detector (Applied Biosystems, Weiterstadt, Germany), equipped with a capillary Z-cell (LC-Packings, Amsterdam, Netherlands). A Rheodyne Model 8125 injection valve with a 5 μl loop was used. Microbore separations were carried out with a C_{18} column (100×1 mm I.D., ODS-AQ, 3 μm, 120 Å, YMC, Schermbeck, Germany). The column outlet was directly connected to the electrospray interface of the mass spectrometer using a fused-silica capillary with an I.D. of 50 μm. The separation was performed at a flow-rate of 20 μl/min using a linear gradient from 0.06% TFA in water to 0.05% TFA in 80% acetonitrile with an increase of either 1%/min or 0.5%/min. The gradient conditions are depicted in the corresponding figure legends (Fig. 1). A 5-μl aliquot of each sample was loaded on the column. The UV detector was set at a wavelength of 220 nm at an absorbance range of 0.2 au.

An HPLC procedure identical to that described

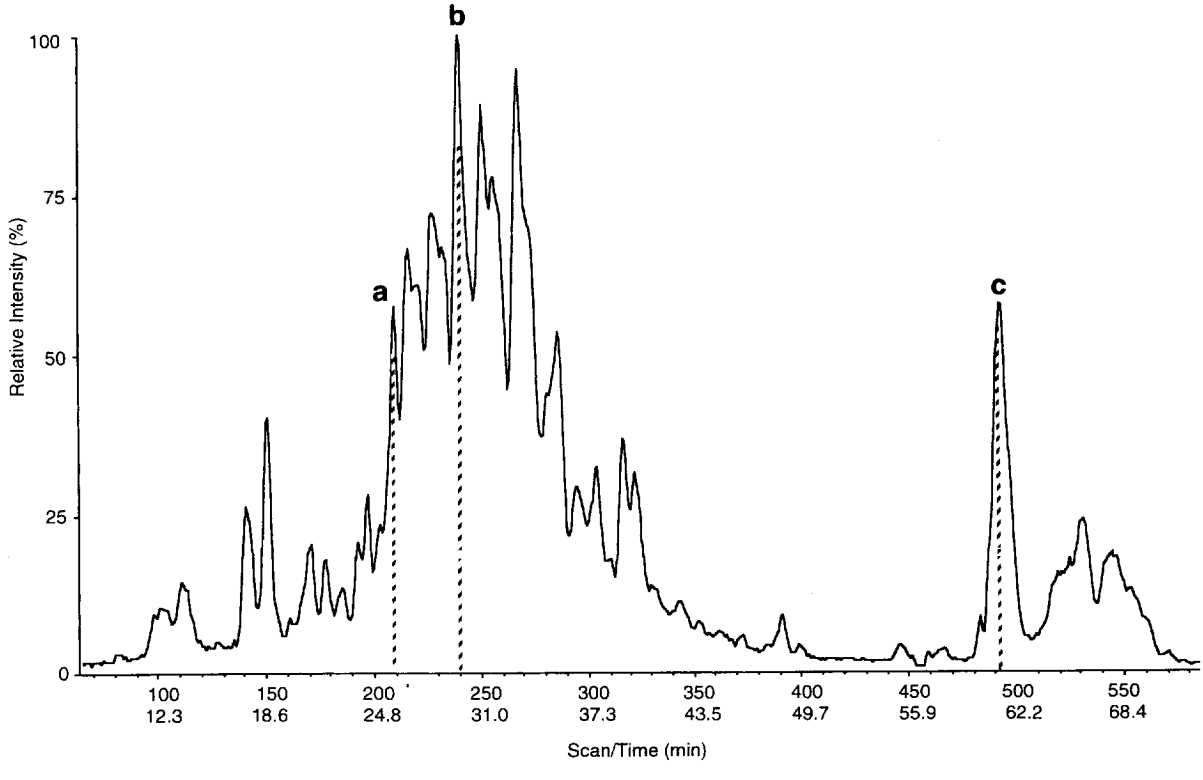

Fig. 1. Full-scan total ion current (TIC) profile of a subfraction from human urine. 160 ml equivalent of human urine separated in thirty fractions after SPE and cation-exchange were measured on-line in positive ion mode. a, b and c depict regions of the chromatogram that are analysed in Fig. 2.

above was used to collect the fractions for consecutive protein sequence analysis. Fractions were manually collected or also automatically sampled by a fraction collector.

2.3.2. Electrospray mass spectrometry

On-line mass analysis was performed on an API III mass analyser (SCIEX, Perkin-Elmer, Langen, Germany) equipped with an articulated ion spray source. Settings were balanced between sensitivity and mass resolution during the calibration. Mass analysis was performed at 5000 V interface voltage and 70 to 100 V orifice voltage, scans ranged from 300 to 2400 u with 0.3 u steps and 0.1 ms measurement dwell time for each step. All analyses were performed under single MS conditions, using the first quadrupole as a mass filter. The chromatograms obtained were analysed manually using the programs recommended by the instrument manufacturers.

2.4. Protein sequence analysis

Several peptides identified in single LC–MS runs were subsequently purified from aliquots of 40 ml to 160 ml urine using routine reversed-phase and cation-exchange chromatography in single fractions. For automatic Edman degradation of pure peptides, a pulse-liquid sequencer (Applied Biosystems 473A or 494A) was used with a fast cycle program and a micro cartridge. Six to ten amino acid residues were sequenced to characterize a primary structure and confirm the peptide by a data base search in EMBL, Swiss-prot and GenBank using the fasta and tfasta program package [17].

3. Results and discussion

Fast and comprehensive mapping of urinary peptides may result in the detection of novel peptides and diagnostic markers. Up to now, there has been no suitable procedure available for a multidimensional analysis of this type. Since microbore RP-HPLC and highly sensitive/high resolution ESI-MS are both techniques for complex mixture analysis, we attempted to use a combination of both, the LC–MS technique, to analyse peptide fractions from human urine.

3.1. Extraction of peptides from urine

The varying composition of human urine with respect to peptide and protein content, electrolytes, pH, lipids, cellular debris, urea and pigments makes it a heterogeneous source for peptide extraction. Different SPE methods as well as precipitations were performed, including reversed-phase extraction, cation- and anion-exchange extraction, batch extraction using alginic acid and ethanol precipitation, were investigated in our laboratory for their suitability and reproducibility. The most practical approach proved to be the SPE, using crude reversed-phase material in single-use cartridges as supplied by many different companies. These materials were of equal suitability for extraction. Initial experiments using the obtained crude extracts showed a high number of different peptides, indicating that the resolution of the LC–MS method was not sufficient to characterize individual peptides at this stage of purification. For this reason, a fractionation using cation-exchange HPLC had to be performed to obtain a 2D separation including the reversed-phase LC–MS column. Separation of peptides was adequate when a strong cation exchanger with peptide binding at low pH values was used. Elution was then carried out with a pH-like gradient (pH 3.2 to 6.6), in combination with a volatile buffer since the urine samples contain both acidic and basic peptides. From the resulting chromatographic separation, thirty fractions were directly applied for the LC–MS measurements.

3.2. LC–MS measurements

Several reports have been published on LC–MS characterization of large biomolecules and known peptides [14–16,18,19], but there are limited reports on successful on-line electrospray LC–MS analysis of complex mixtures like urine or blood plasma. We intended to establish a versatile technique for the multidimensional analysis of such samples. The combination of the full capabilities of high resolution microbore reversed-phase HPLC and ESI-MS in a tandem format is adequate for the characterization of peptides or proteins. The coupling of microbore HPLC with the ESI-MS improves the method and provides the additional molecular mass information

that is difficult or impossible to obtain by other methods.

In combination with a suitable prepurification method, it is possible to analyse the peptides and protein fragments from urine extracts and obtain a peptide map on the basis of the following three parameters: charge, hydrophobicity and mass. Very little urine is needed for the analysis, and most experiments are carried out with 30 to 50 ml equivalents, an amount which can be easily obtained from a single person. From this amount of urine, an analysis of peptides in a concentration range from approx. 100 pmol/l to nmol/l becomes possible. This amount is also enough for consecutive purification of peptides for sequence determination. To analyse the less abundant peptides, more urine can be processed by this technique.

Highly-sensitive microbore LC–MS with a mobile phase flow of 20 μl/min is routinely performed using the articulated ion-spray interface and on-line MS detection without post-column split.

A typical full-scan total ion current (TIC) profile from microbore LC–MS analysis of a subfraction is shown in Fig. 1. A 5-μl aliquot of the sample, representing the equivalent of 160 ml human urine is applied to the LC–MS using the 100×1.0 mm I.D. microbore column. Separation was accomplished by applying a linear gradient from 10% B to 60% B over 90 min. The mass spectrometer was set to scan from 300 to 2400 m/z every 7 s, varying the orifice voltage from 70–100 V. The gradient was suitable to achieve the necessary separation required for this complex mixture. As seen in the TIC already, this spectrum shows more than forty different components. Since peptides are present in electrospray ionization as multiple charged ions, series of ions with distinct m/z ratios are characteristic for a given peptide. As an illustration, the ESI mass spectra corresponding to the three chromatographic components in Fig. 1 (denoted as peaks a–c) are shown (Fig. 2a–c and Table 1). The mass spectrum of peak a shows six peaks representing the charged ions of one peptide listed below with the calculated molecular mass of 4389±1.02 u. For peak b, a molecular mass of 4753±1.0 u is calculated from five charged ion species and for c, eleven charged ion species allow the calculation of the mass of 18 162±3.3 u for this peptide. In addition, many more peptides are present in these scans at a lower concentration. Subsequent purification and sequence analysis of the peaks reveal partial sequences of fragments of pancreatic ribonuclease, α-1-microglobulin and albumin. An extensive analysis of this LC–MS run is shown in Fig. 3, where the calculated peptide masses are shown at the corresponding peaks of the TIC from Fig. 1. Calculating only peptide masses with a

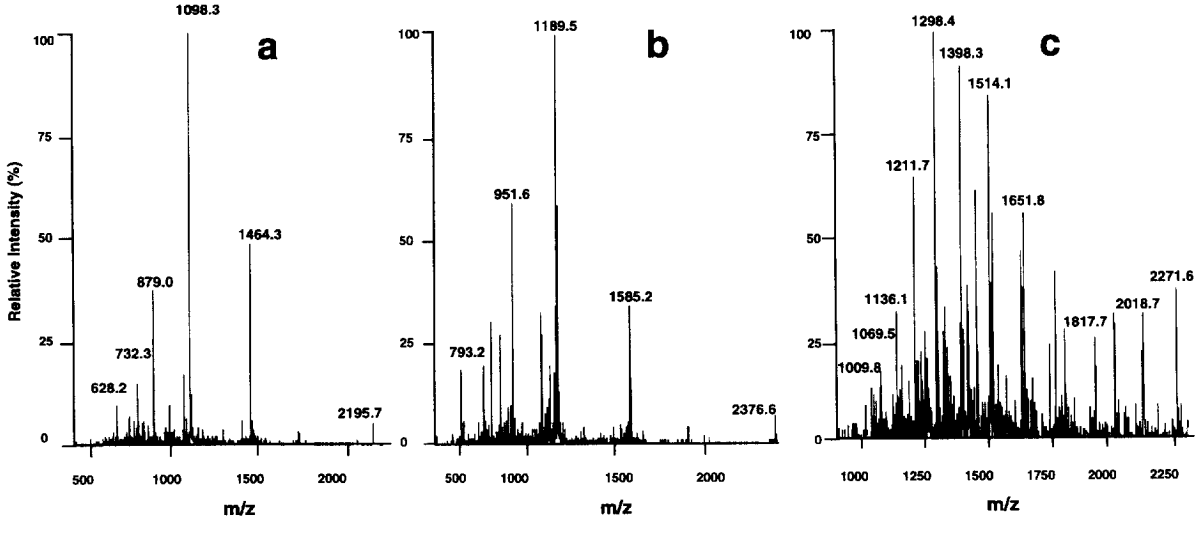

Fig. 2. Electrospray mass spectra of the regions a–c from Fig. 1.

Table 1
Peptide fragments of plasma proteins identified by chemical sequencing from the regions a–c from Fig. 1

Mass spectrum	Calculated mass (u)	Charges (z)	N-terminal fragment sequence	Identified protein
a	4389.41	+2 to +6	ESRAKKFQRQHMD	Pancreatic ribonuclease glycosylated form
b	4752.77	+2 to +6	FLTKKFSRHHGP	α-1-Microglobulin
c	18 161.77	+8 to +18	VRPEVDVMXTAFHDNEETFLKKYLEIARR (X=C, n.d. by chemical sequencing)	Serum albumin

standard deviation of less than 0.1%, over twenty peptides are unambiguously identified and characterized in this peptide fraction. As expected from the elution behaviour of peptides with respect to their M_r from RP-HPLC, larger peptides are found in fractions which elute at higher organic solvent concentrations.

All thirty fractions from cation-exchange were investigated according to the scheme given in Section 2. Thus, a comprehensive mapping of the peptides from human urine may be performed in the future. Single peptides were collected in separate runs and sequenced. Using a database comparison we identified different peptides and protein fragments as shown in Table 2. The substances identified were mainly fragments from larger proteins like serum albumin, uromodulin and collagen. In addition, several peptides like granulin, urodilatin and angiotensin-I were detected.

Thus, the described LC–MS method is sensitive,

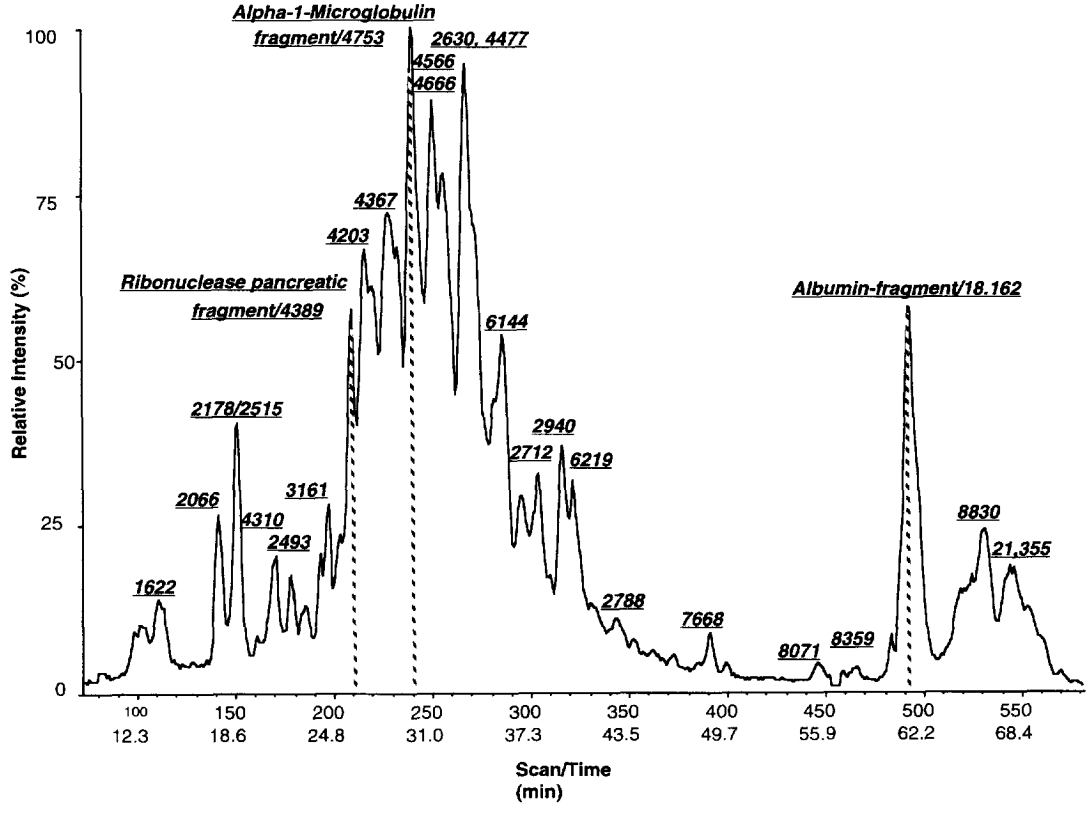

Fig. 3. TIC with peptide masses analysed as described in Section 2.3.

Table 2
Peptides and protein fragments isolated from human urine and characterized by chemical sequencing

Peptide hormones	Fragments of plasmaproteins	Fragments of enzymes, inhibitors	Fragments of extracellular matrix
Angiotensin 1	Albumin	α-1-Antitrypsin	Collagen-α-1 (X) (CA1A)
Granulin[a]	Apolipoprotein D	Argininosuccinate lyase	Collagen-α-1 (VIII) (CA18)
Urodilatin	Apolipoprotein C-III	Kininogen, HMW/LMW	Collagen-α-1 (IX) (CA19)
	α-2-HS Glycoprotein (Fetuin)	Pancreatic ribonuclease	Procollagen-α-1 (I) (CA11)
	α-1-Microglobulin	Pepsinogen A	Procollagen-α-1 (I) (CA12)
	Fibrinogen A	Prostaglandin-H2 D-isomerase	Procollagen-α-1 (III) (CA13)
	IG γ-2-chain C-region	RAS-related protein RAP-1B	Procollagen-α-1 (IV) (CA14)
	IG γ-3-chain C-region	Stromelysin-3	Procollagen-α-1 (V) (CA15)
	IG κ-chain C-region	Uroporphyrinogen-III synthase	Psoriasin[a]
	IG λ-chain C-region		
	Keratin, type I		
	Parathymosin		
	Uromodulin		

[a] Proteins with unknown functions.

with respect to the amount of urine used for the analysis, precise and stable. The further analysis and identification of a great number of peptides in human urine will contribute to establish a peptide map of this body fluid.

4. Conclusions

The combination of different chromatographic procedures and the on-line mass determination by LC–MS permits the analysis of complex mixtures of peptides or protein fragments. The advantage of MS detection is the extra dimension of information available regarding the molecular mass. Consequently, highly sensitive peptide mapping from human urine may be achieved by LC–MS coupling. The complementary information obtained by ion-exchange, RP-HPLC and LC–MS allows the physicochemical characterization of peptides based on three parameters: charge, hydrophobicity and molecular mass. The application of LC–MS for the analysis of thirty standardized subfractions obtained from cation-exchange is shown to detect numerous peptides and protein fragments in prepurified crude extracts from human urine.

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