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Capillary electrophoresis coupled to mass spectrometry to establish polypeptide patterns in dialysis fluids

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Abstract

Combination of capillary electrophoresis with mass spectrometry (CE–MS) allows generation of polypeptide patterns of body fluids. In a single CE–MS (45 min) run more than 600 polypeptides were analyzed in hemodialysis fluids obtained with different membranes (high-flux/low-flux). Larger polypeptides (M_r>10 000) were almost exclusively present in high-flux dialysates only, while in low-flux dialysates additional small polypeptides were detected. Comparison to the normal urine pattern yielded a surprisingly low consensus: a number of polypeptides present in urine were missing. We established a fast and sensitive technique, easily applicable to the monitoring of different modalities of dialyzers.

Keywords: Proteomics; Dialysis; Polypeptides; Peptides

1. Introduction

Analysis of the proteome is the main task in the post genomic era. A number of processes in the body may be reflected in body fluids such as serum, urine, etc. Unfortunately, protein assessment has thus far been hampered by the lack of a fast and reproducible technique which allows the simultaneous assessment of a large number of polypeptides. Western blotting and other immunological methods have been employed [1,2], but these techniques identify only a few polypeptides in one step. Proteomic analysis is now available for large-scale study of proteins in tissues and body fluids [3,4]. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is commonly used for protein separation and can be combined with mass spectrometry (MS) to yield identification of individual polypeptides. Over 1000 polypeptides spots can be discerned with 2D-PAGE [5]. However, since each single spot must analyzed separately by MS–MS for identification, these techniques are too cumbersome for routine use. Alternatively, HPLC in combination with MS, either by direct coupling or via off-line analysis of HPLC fractions, can be employed to analyze polypeptide patterns [6–8]. These methods unfortunately also reveal limitations. The typical reversed-phase HPLC appears not too well suited for the separation and analysis of larger polypeptides (M_r>4000) and, if separation of a complex mixture of polypeptides is required, is quite time-consuming. Recently, surface-

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enhanced laser desorption ionization (SELDI) has been employed to study polypeptides in body fluids [9,10]. While this method is certainly rapid and hence well suited to address clinical questions with respect to the speed of the analysis, it falls short of displaying the complete polypeptide pattern of body fluids due to its principle that only certain polypeptides are bound to the surface of the analyzer chip, depending on the conditions used.

The goal for clinical application must be to display a thorough pattern of a large number of polypeptides in a single, reproducible and time-limited step, which also enables comparison of different patterns. If this goal is met, proteomics could be widely used in clinical applications. As a step towards this goal, we established a technique based on capillary electrophoresis (CE) coupled to MS. This technique permits the analysis of several hundred polypeptides simultaneously in a short time in a small volume with high sensitivity. The combination of CE and mass spectrometry allows improved automation, speed and precision of proteome analysis [11]. Recently, several approaches to manage the limitations of capillary separation techniques, like the small sample amount, have been reported [12–14]. In addition, the technical problems provoked by the combination of these two systems have been solved by new and improved interfaces [15,16]. Hence it is possible to establish a robust tool for the routine detection of peptides in the low femtomole range [17].

Here we describe the use of this technique to study polypeptide patterns in dialysate obtained during treatment of patients with end stage renal disease (ESRD). Great efforts have been made to study the influence and behaviour of single proteins or peptides in dialysis [18–20], but only a few studies have been published that aim towards identification of the complete or even a part of the polypeptide spectrum involved in the process of renal replacement therapy [21,22]. On the other hand the characteristics as well as the assets and drawbacks of different membrane types have been thoroughly discussed [23,24]. In addition, the mechanisms for complete removal of uremic toxins are of great interest [25,26]. These deliberations clearly indicate that a fast and sensitive tool is needed to provide an exhaustive insight into the proteome of body fluids like serum and urine as well as related fluids like dialysate.

2. Material and methods

2.1. Samples

Dialysate obtained during treatment of patients undergoing dialysis with two different dialyzer membrane types (high-flux and low-flux) was collected at the Department of Nephrology, Medical School of Hannover, after informed consent was obtained. The total spent dialysis fluid was mixed thoroughly and small samples were stored frozen at −20 °C until use. Aliquots of 2 ml were adjusted to pH 10.0 using ammonia and cleared by centrifugation for 10 min at 13 000 *g*. The supernatant was applied to 0.2-ml bed volume of DEAE-Sepharose FF (Amersham Biosciences) in a 1-ml disposable column (Bio-Rad Labs., Hercules, CA, USA), equilibrated with 20 bed volumes of binding buffer (1%, v/v, ammonia). After washing with 10 bed volumes of binding buffer, proteins were eluted with 1% formic acid in water containing 30% methanol. The eluted fraction was frozen and lyophilized overnight in a Christ Speed-Vac RVC 2-18/Alpha 1-2 (Christ, Osterode am Harz, Germany). Shortly before use, the lyophilized samples were resuspended in 20 μl HPLC-grade water, sonicated for 1 min in an ultrasonic bath and centrifuged for 10 min at 13 000 *g* at 4 °C. HPLC-grade water, formic acid and ammonia were from Merck, Darmstadt, Germany. Methanol was from J.T. Baker, Deventer, The Netherlands.

2.2. CE–electrospray ionization (ESI) time-of-flight (TOF) MS

The samples were transferred to an appropriate vial and stored in the CE autosampler section. For capillary electrophoresis a P/ACE MDQ (Beckman Coulter, Fullerton, CA, USA) system equipped with a 90 cm×75 μm I.D. bare fused-silica capillary was used. The capillary was first rinsed with running buffer (30% MeOH, 0.5% formic acid, 69.5% water) for 3 min. The sample was injected for 20 s with 1 p.s.i. pressure, resulting in a sample plug of ~100-nl volume, equivalent to 0.25% of the capillary volume, as determined by initial experiments (1 p.s.i.= 6894.76 Pa). Separation was performed with +30 kV on the injection side and the capillary temperature

was set to 35 °C for the whole length up to the ESI interface. The spray tip potential was set to +3500 V, resulting in ~27 kV available for the whole capillary length. Between the runs the capillary was rinsed with 1 *M* NaOH for 5 min at a pressure of 30 p.s.i.

The CE–ESI-MS interface was accomplished using a CE–ESI-MS sprayer kit (Agilent Technologies). Sheath-flow contained 30% MeOH, 0.5% formic acid.

On-line TOF detection and data acquisition were performed on a Mariner Biospectrometry Workstation (Perseptive Biosystems, Farmington, USA). The data acquisition and the MS method were automatically controlled by the CE program via contact-close-relays. Spectra were accumulated for 3 s each over a mass-to-charge range from 400 to 2500.

MosaiquesVisu software [27] was used for peak detection, mass deconvolution, data three-dimensional visualization and generation of the polypeptide lists. Only molecular masses above 1000 were accepted. The program uses isotopic masses and conjugated masses for the determination of polypeptides. All detected polypeptides were deposited in a Microsoft Access database. Comparison between the samples and search for conformity were performed. Polypeptides were considered identical if the mass deviation was less than 0.05% and the CE time deviation was less than 20%.

3. Results

3.1. Sample preparation

While CE–MS is well or even better suited than HPLC–MS to examine complex biological samples (excellent separation efficiency and accurate mass determination), one problem of this technique is the sensitivity towards interfering matrix compounds such as salts or non-volatile buffers. Considering these requirements, samples from haemodialysis are easy to prepare for CE–MS analysis. We used anion-exchange chromatography (DEAE-Sepharose) and lyophilization to remove interfering salts, uncharged elements and to concentrate the final sample. This procedure has resulted in a reproducible and CE–ESI-MS-compatible sample matrix with only a minimum of polypeptide loss during the preparation, as shown in initial experiments utilizing internal standard polypeptides (data not shown).

3.2. CE–ESI-MS analysis

The lyophilized sample was resuspended in HPLC-grade water shortly before use. For the separation the capillary was first flushed with buffer, followed by injection of the sample. At the injection-end the capillary was kept in buffer while a voltage of +30 kV was applied during the run. Temperature was held constant at 35 °C and no pressure was used during the separation. Using this set-up we obtained an optimized resolution with excellent peak characteristics for the subsequent data analysis. The CE–ESI junction was realized by a sheath-flow interface. Best results were obtained when sheath-flow and running buffer were identical (i.e. 30% methanol and 0.5% formic acid in water; pH 2.3–2.5) and the flow rate was kept below 10 μl/min.

Between the runs the capillary was rinsed with 1 *M* NaOH for cleaning and preconditioning. The CE–MS method was established to run fully automated without the need of any manual operation for at least 20 runs.

The CE separation was optimized with respect to good resolution for the polypeptides within an acceptable overall run time of less than 1 h. A set of standard polypeptides ranging from M_r 1700 to 14 500 was used to establish conditions suitable for both peptides and proteins. Utilizing the conditions described, 100 fmol of each of the standard compounds could be detected reproducibly. A typical example of a standard run is shown in Fig. 1.

The optimized method for both sample preparation and CE–MS analysis was subsequently utilized to analyze dialysis fluid. A total of eight samples from dialysis with high-flux membranes and 15 samples from dialysis with low-flux membranes were analyzed. Typical examples of raw data from high-flux and low-flux membranes displayed as three-dimensional contour plots (3D-plots) are shown in Fig. 2.

3.3. Subsequent data processing

As evident from the wealth of data, it is impossible to evaluate the raw data using commercially

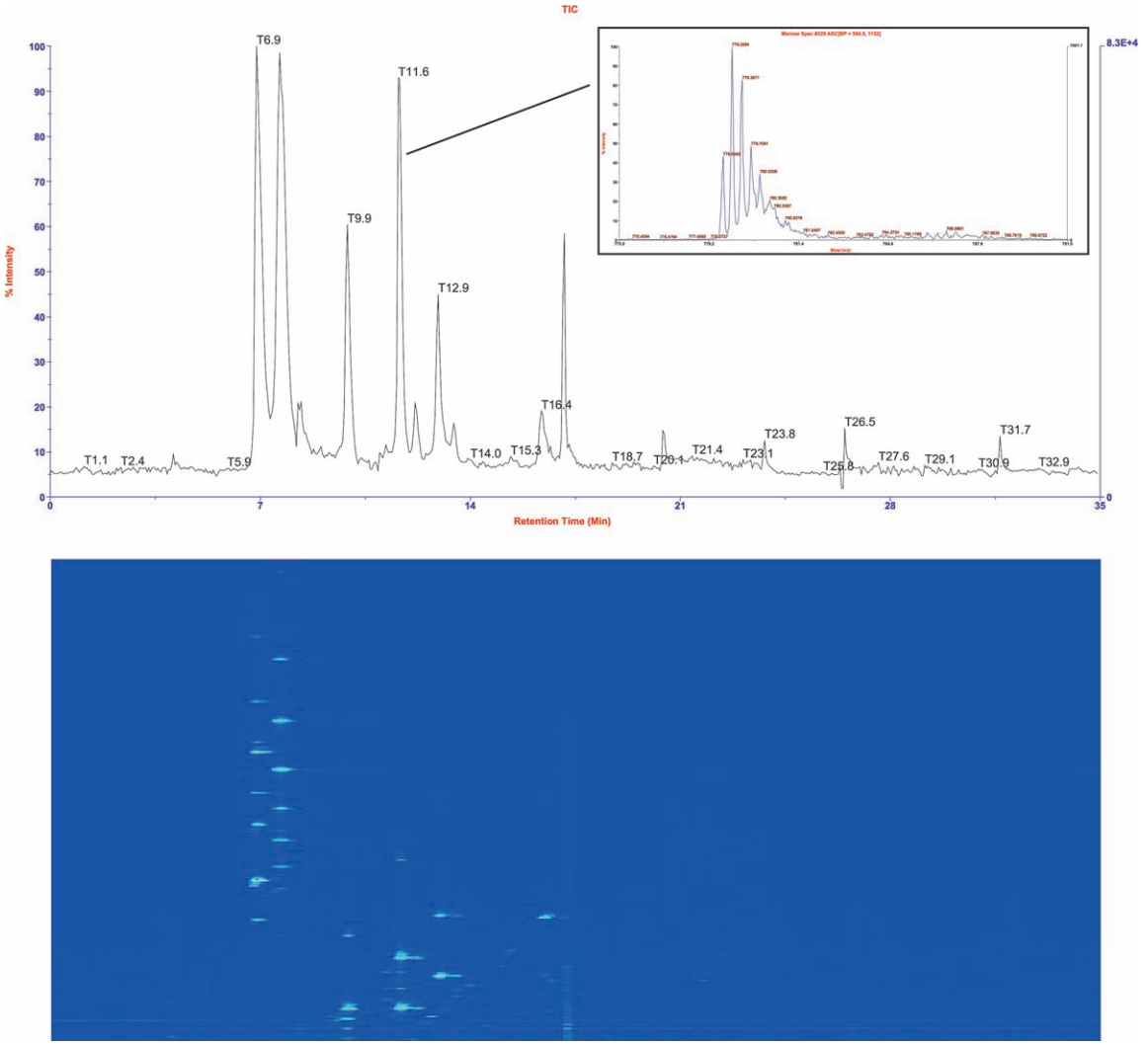
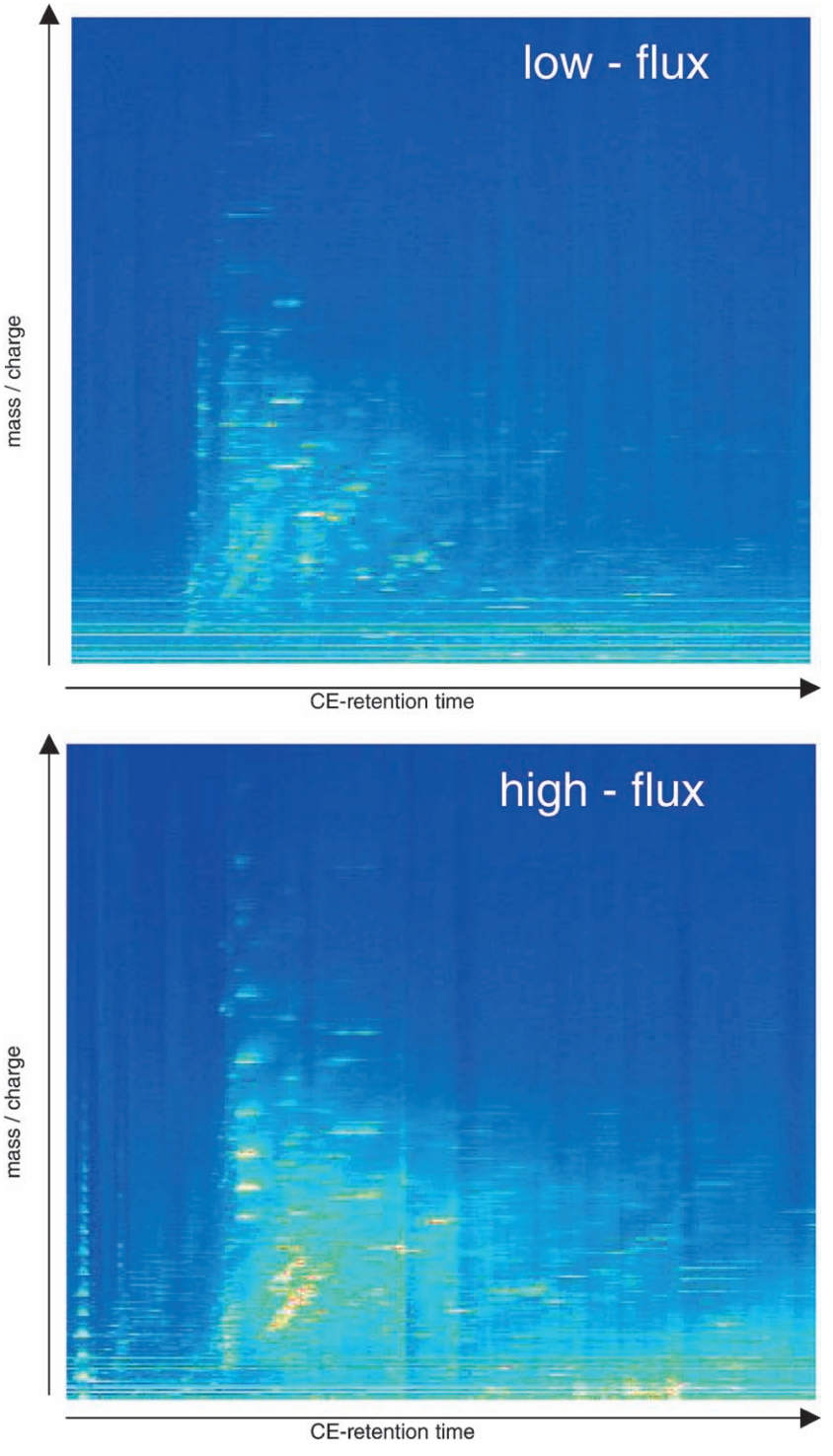


Fig. 1. CE–MS spectrum of standard polypeptides. A mixture of seven polypeptides (1 pmol/μl each, injection volume ~100 nl) was used: REVQSKIGYGRQIIS (M_r 1732.96), GIVLYELMTGELPYSHIN (M_r 2048.03), TGSLPYSHIGSRDQIIFMVGR (M_r 2333.19), ELMTGELPYSHINNRDQIIFMVGR (M_r 2832.41), aprotinin (M_r 6517.5), ribonuclease (M_r 13 690.3) and lysozyme (M_r 14 313.1). The upper panel shows the total ion current, with the mass spectrum of the triply charged peptide TGSLPYSHIGSRDQIIFMVGR shown as an insert. Below, a contour plot of the same CE–MS run is shown.

available software. To obtain an effective tool for data evaluation, the MosaiquesVisu software was developed [27] combining the peak detection in each spectrum with mass deconvolution by well balanced isotopic and conjugated mass-to-charge determination. The software also includes a tool to visualize the raw and the processed data in a three-dimensional contour plot.

Fig. 2. Typical examples of raw data from high-flux and low-flux dialyzers displayed as three-dimensional contour plots. The background is displayed in blue while the signal is colour coded with increasing intensity from light blue to white to yellow to red. As is evident, several larger polypeptides, present as multiply charged peaks, are present in the high-flux dialysate, but absent in the low-flux dialysate.


low - flux
mass / charge
CE-retention time
high - flux
mass / charge
CE-retention time

Detected polypeptides were deposited in an MS-Access database and statistically analysed to identify common patterns. It was necessary to allow a wide deviation range of 20% for the CE time, due to differences between single runs, but the mass deviation could be set to 0.05%.

3.4. Low-flux membranes

A total of 15 samples from dialysis with low-flux membranes were analyzed and compared in the database. Overall 4096 data points (average of 273 per sample) could be found and were matched to 1639 polypeptides, characterized by their deconvoluted mass and CE time. About 90% of the detected polypeptides were in the M_r range <5000 (Fig. 4). A total of 47 polypeptides were found to be present in at least eight (>50%) of the 15 evaluated samples. The contour plot shown in Fig. 3A represents the processed data from a typical low-flux dialysate.

3.5. High-flux membranes

A total of eight samples from dialysis with high-flux membranes were available, and overall 4515 data points (average of 611 per sample) could be identified and were matched to 2515 different polypeptides. The mass distribution of these was slightly different from the low-flux samples. Here the 90% limit was in the M_r range <7000 and several larger polypeptides with M_r>20 000 were found (Fig. 4). Five polypeptides were found in all eight samples, while 179 proteins/peptides were detected in at least five samples, representing the >50% abundance group. A typical contour plot processed from a high-flux dialysis sample is shown in Fig. 3B.

3.6. Low-flux versus high-flux comparison

A third database containing all 23 record sets was constructed to compare both types of membranes and to evaluate if there are typical differences and potential marker polypeptides. This complete database consists of 8984 data points matched to 3827 different polypeptides, again characterized by their deconvoluted mass and CE time. As before, a mass deviation of 0.05% and a migration-time deviation of 20% was allowed.

For the determination of typical polypeptides, the 3827 polypeptides were classified into four categories, namely: (i) ''HF-typical'' with HF abundance >50% and LF abundance <50%; (ii) ''LF-typical'' with HF <50% and LF >50%; (iii) ''common'' with >50% in both types of membranes; and (iv) ''unspecific'' with an abundance lower than 50% in both types.

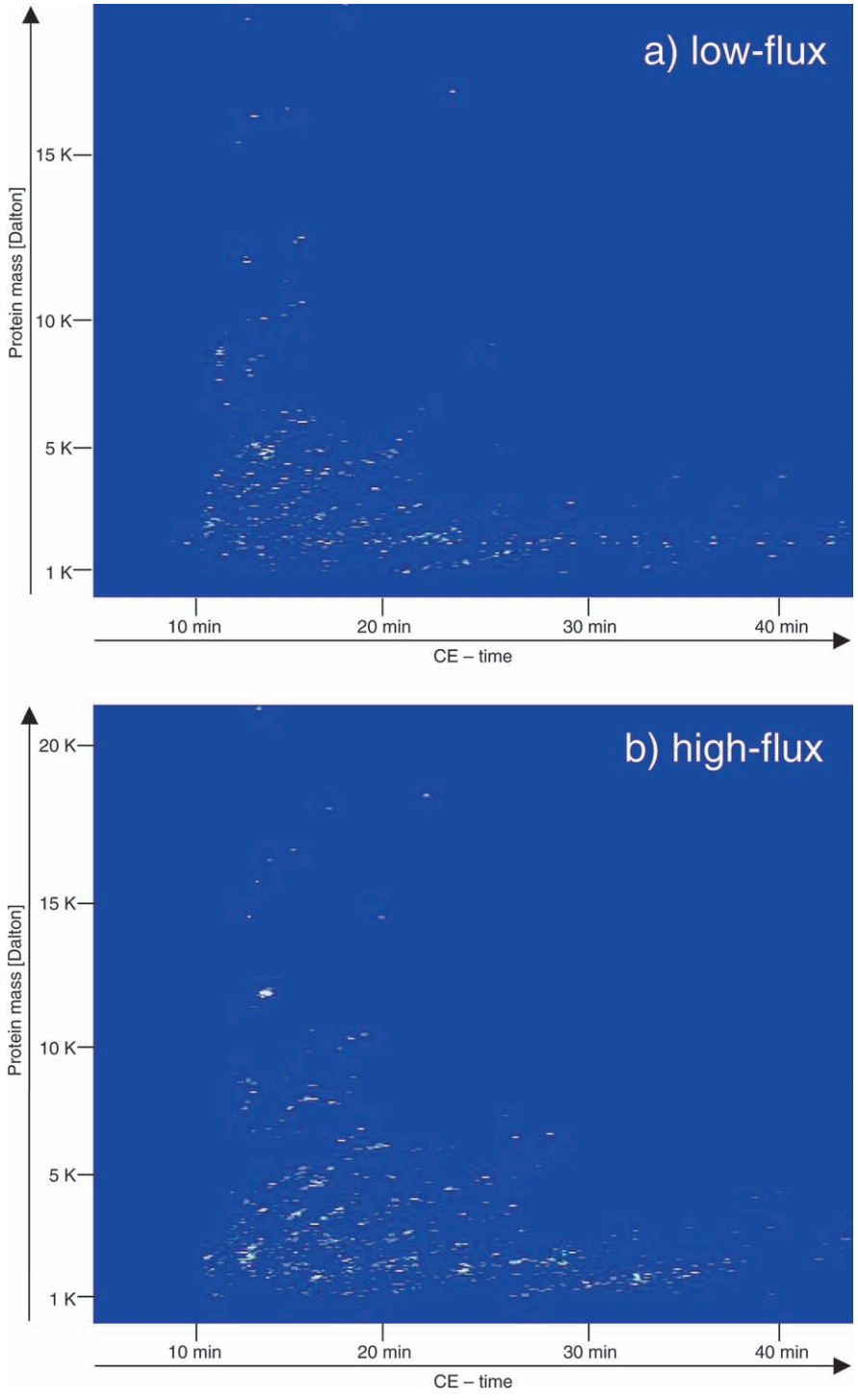
A total of 14 proteins/peptides were found to be typical for the low-flux membranes and 146 for the high-flux membranes, and another 33 of these were found in both high and low-flux dialysates (Table 1). The proteins/peptides detected only in LF membrane samples are in the M_r area up to 10 200, while the HF typical polypeptides show molecular masses up to 21 000, eight of these being larger than 10 000.

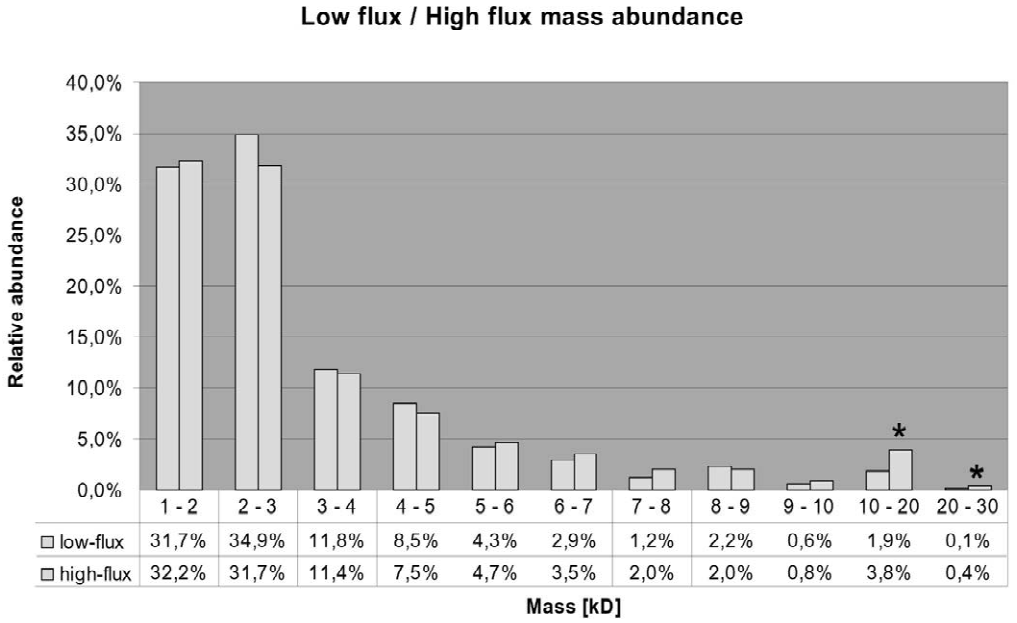
3.7. Dialysate versus urinary samples and serum

A number of urine samples was prepared according to the method for dialysis fluids [27] and matched to the databases. As evident from Table 1, we found surprisingly low conformity with the polypeptides found in dialysis fluids (Fig. 5). Of the 247 polypeptides representing the ''normal urinary polypeptide pattern'', only four (28%) LF-typical, 26 (18%) HF-typical and 13 (39%) common polypeptides were detected, as shown in the last column of Table 1. Of the polypeptides representing the ''normal dialysis pattern'' 70% were found in at least one urine sample. A substantial number of major urinary polypeptides were missing in the dialysate.

In initial experiments, we observed a 70% consensus between the peptides found in dialysate and in human serum.

Fig. 3. 3D-plots of CE–MS spectra from dialysate after data processing. (A) Low-flux dialysate; (B) high-flux dialysate. As is evident, the overall pattern observed is similar and comparable for both types of membranes, but the high-flux dialysate contains more polypeptides in the higher molecular mass range.


a) low-flux
15 K
10 K
5 K
1 K
Protein mass [Dalton]
10 min
20 min
30 min
40 min
CE – time
b) high-flux
20 K
15 K
10 K
5 K
1 K
Protein mass [Dalton]
10 min
20 min
30 min
40 min
CE – time



	1 - 2	2 - 3	3 - 4	4 - 5	5 - 6	6 - 7	7 - 8	8 - 9	9 - 10	10 - 20	20 - 30
low-flux	31,7%	34,9%	11,8%	8,5%	4,3%	2,9%	1,2%	2,2%	0,6%	1,9%	0,1%
high-flux	32,2%	31,7%	11,4%	7,5%	4,7%	3,5%	2,0%	2,0%	0,8%	3,8%	0,4%

Fig. 4. Comparison of the abundance of polypeptides detected in high-flux versus low-flux dialysates in dependence of their molecular mass. While the distribution, but not the absolute number, is quite similar in the low M_r (<10 000) range, a shift even in distribution can be observed in the high molecular range of the high-flux dialysates (marked with an asterisk).

4. Discussion

Peptides and proteins are major components in body fluids responsible for the initiation of signal transduction. Hence, their absence/presence and/or concentration can be used to establish a pattern displaying the health status of an organism. The aim of our work was to establish a fast, reproducible and robust technique that allows an exhaustive view of polypeptides in body fluids for diagnostic purposes and for the possible identification of therapeutic targets. To achieve this, capillary electrophoresis was coupled online to a mass spectrometer in combination with a sample preparation method that takes into account the special requirements for the polypeptides observed in body fluids.

To establish the technology and the software for data evaluation, samples that were expected to be of lower complexity (i.e. haemodialysis fluid) were analyzed. In the first set of experiments we aimed at visualizing the complex CE–MS data and comparing dialysis samples obtained with different dialyzer membranes. Moreover, the widespread application of renal replacement therapy indicates an important field of research, in terms of comparing different treatment modalities (such as haemodialysis and haemofiltration) and membrane types with the function of the native kidneys, aimed towards the identification and the monitoring of uremic toxins.

Utilizing our method, we are able to analyze the polypeptides present in dialysis samples in a fully automated manner. More than 600 polypeptides found in one individual sample can be characterized by their mass and retention time in less than 1 h. Combination of the highly efficient separation performance of capillary electrophoresis with the excellent resolution of ESI-TOF-MS and the Mosaiques-Visu software provides an excellent tool to analyze and compare the proteome of body fluids for diagnostic and scientific purposes.

Quite surprisingly, not only polypeptides, but also most probably synthetic organic compounds could be found in dialysis fluid. One example of this type of analyte is shown in Fig. 6. This compound does not

Table 1
Comparison of polypeptides found in more than 50% of either high-flux or low-flux dialysates

No.	Mass (M_r)	CE time (min)	Proteins found			Type	Low-flux sample #														High-flux sample #								Normal urine	
			Total	In LF	In HF		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23		
1	10 199.06±0.7	15.15±1.1	8	8	0	LF				+		+	+		+	+	+		+		+										
2	1423.41±0.1	16.89±1.5	11	11	0	LF	+	+	+		+	+	+	+	+		+		+		+									++	
3	2048.00±0.4	17.37±0.8	9	9	0	LF	+		+	+			+	+	+		+		+		+									++	
4	2064.57±0.4	18.54±1.0	11	11	0	LF	+	+	+	+	+	+	+	+			+		+		+									++	
5	2147.40±0.2	7.86±0.7	9	8	1	LF	+	+	+						+		+	+	+		+			+						+	
6	2170.03±0.5	23.86±1.4	8	8	0	LF	+		+	+			+	+	+		+				+									+	
7	2414.04±0.1	10.63±0.8	13	13	0	LF	+	+		+	+	+	+	+		+	+	+	+	+	+									+	
8	2658.02±0.3	10.42±0.8	14	13	1	LF		+	+	+	+	+		+	+	+	+	+	+	+	+				+					+	
9	2859.60±0.5	11.69±1.0	10	8	2	LF		+	+	+	+		+	+					+		+				+		+				
10	4322.02±0.8	20.53±1.7	15	13	2	LF	+	+	+	+	+		+	+	+	+	+	+	+		+	+							+		
11	5228.97±0.6	20.98±1.7	9	9	0	LF	+		+				+	+	+	+		+	+		+										
12	6799.19±0.8	15.21±0.7	9	8	1	LF	+		+				+	+	+	+	+				+							+			
13	8176.52±0.5	11.77±0.6	9	9	0	LF	+	+	+	+	+	+	+	+							+									+	
14	8289.72±0.9	11.49±0.8	9	9	0	LF	+	+	+		+	+	+			+			+		+									+	
15	1013.18±0.0	33.97±2.7	6	0	6	HF																+	+	+			+	+	+		
16	1044.60±0.0	12.09±0.3	5	0	5	HF																+	+	+		+			+		
17	1061.30±0.2	21.72±1.6	5	0	5	HF																+		+		+	+	+			
18	1062.60±0.0	12.04±0.4	6	0	6	HF																+	+	+		+	+		+		
19	1084.22±0.0	34.05±2.7	6	0	6	HF																+	+	+			+	+	+		
20	1085.22±0.1	25.38±1.9	5	0	5	HF																+	+				+	+	+	+	
21	1088.37±0.0	22.16±1.7	7	0	7	HF																+	+	+		+	+	+	+		
22	1091.40±0.0	22.13±1.6	7	0	7	HF																+	+	+		+	+	+	+		
23	1095.36±0.1	18.80±1.2	7	0	7	HF																+	+	+		+	+	+	+		
24	11 681.12±0.3	15.40±0.6	5	0	5	HF																+	+	+				+	+		
25	11 735.18±0.7	15.89±1.2	8	1	7	HF	+															+	+	+		+	+	+	+	+	
26	11 896.59±0.3	16.07±0.9	6	0	6	HF																+	+	+			+	+	+		
27	1209.34±0.1	35.29±3.2	5	0	5	HF																+		+			+	+	+	+	
28	1216.46±0.2	17.54±1.6	7	0	7	HF																+	+	+	+	+		+	+		
29	1244.50±0.0	23.93±1.9	7	0	7	HF																+	+	+		+	+	+	+	+	
30	1247.28±0.1	25.47±1.6	6	0	6	HF																+	+	+			+	+	+		
31	1287.43±0.1	39.99±3.6	6	0	6	HF																+	+	+			+	+	+	+	
32	1324.33±0.2	40.25±3.6	6	0	6	HF																+	+	+			+	+	+	+	
33	1347.48±0.2	39.20±2.8	5	0	5	HF																+	+	+			+	+		+	
34	1374.50±0.1	26.72±2.2	6	0	6	HF																+	+	+		+	+	+		+	
35	1386.40±0.1	41.29±3.6	5	0	5	HF																+	+	+				+	+	+	
36	14 556.73±0.3	14.13±0.5	6	0	6	HF																+	+	+		+	+		+		
37	14 682.82±0.4	14.00±0.6	5	0	5	HF																+	+			+	+	+			
38	1501.54±0.1	20.26±2.1	8	2	6	HF		+						+								+	+	+		+	+	+		+	
39	1511.29±0.1	20.06±1.5	7	2	5	HF											+		+			+		+		+		+	+	+	
40	1565.48±0.0	44.11±4.4	6	0	6	HF																+	+	+			+	+	+	+	
41	1568.65±0.1	19.78±1.1	6	0	6	HF																+	+	+		+	+	+		+	
42	15 816.48±0.9	14.61±0.9	8	2	6	HF	+						+									+	+	+			+	+	+		
43	1592.58±0.0	44.11±4.3	6	0	6	HF																+	+	+			+	+	+	+	
44	1638.58±0.1	17.92±0.9	6	0	6	HF																+	+	+		+	+	+		+	
45	1719.03±0.0	5.72±0.6	9	1	8	HF												+				+	+	+	+	+	+	+	+	+	*
46	1733.38±0.2	44.07±2.9	5	0	5	HF																+	+	+			+	+		+	
47	1750.70±0.0	12.66±0.5	6	0	6	HF																+	+	+			+	+	+	+	
48	1761.70±0.2	30.18±2.4	5	0	5	HF																+		+			+	+	+	++	
49	1764.55±0.1	36.29±2.8	5	0	5	HF																+	+	+				+	+	++	

Table 1. Continued

No.	Mass (M_r)	CE time (min)	Proteins found			Type	Low-flux sample #														High-flux sample #								Normal urine
			Total	In LF	In HF		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	
50	1775.56±0.1	17.35±1.0	7	0	7	HF																+	+	+		+	+	+	+	
51	1780.44±0.1	29.49±2.3	5	0	5	HF																+		+			+	+	+	+
52	1846.54±0.1	17.73±0.9	7	0	7	HF																+	+	+		+	+	+	+	+
53	1864.08±0.1	39.99±2.9	5	0	5	HF																+	+	+			+	+		+
54	1869.74±0.2	29.95±2.8	5	0	5	HF																+		+			+	+	+	
55	1871.16±0.1	5.80±0.4	5	0	5	HF																+	+	+			+	+		+
56	1876.56±0.1	27.67±1.9	6	0	6	HF																+	+	+			+	+	+	++
57	1878.79±0.0	11.78±0.5	7	0	7	HF																+	+	+		+	+	+	+	++
58	1932.42±0.4	18.34±0.8	5	0	5	HF																	+	+			+	+	+	++
59	1932.94±0.3	24.88±1.9	5	0	5	HF																+	+				+	+	+	++
60	1949.88±0.2	12.02±0.6	6	0	6	HF																+		+		+	+	+	+	++
61	1999.52±0.1	30.78±2.6	5	0	5	HF																+		+			+	+	+	+
62	2022.21±0.3	23.28±1.9	7	0	7	HF																+	+	+		+	+	+	+	
63	2055.64±0.1	34.15±2.1	5	0	5	HF																+	+	+			+	+		
64	2069.71±0.1	14.72±0.6	5	0	5	HF																+	+	+				+	+	++
65	2070.46±0.3	33.02±2.6	5	0	5	HF																+	+	+	+				+	++
66	2083.08±0.7	26.49±1.6	5	0	5	HF																+	+	+				+	+	+
67	2090.57±0.0	17.36±1.0	7	0	7	HF																+	+	+		+	+	+	+	
68	21 052.38±1.4	14.77±0.6	5	0	5	HF																	+	+		+	+		+	
69	2108.33±0.3	17.02±1.3	5	0	5	HF																+	+	+				+	+	
70	2112.31±0.3	31.66±2.6	6	0	6	HF																+	+	+			+	+	+	+
71	2127.70±0.2	32.32±2.7	5	0	5	HF																+		+			+	+	+	+
72	2138.70±0.2	28.59±2.5	6	0	6	HF																+	+	+			+	+	+	+
73	2149.32±0.2	16.75±1.4	7	2	5	HF								+					+					+	+		+	+	+	+
74	2149.47±0.1	38.06±3.3	6	0	6	HF																+	+	+			+	+	+	+
75	2153.52±0.4	34.09±2.6	8	2	6	HF											+	+				+	+	+			+	+	+	+
76	2161.85±0.2	12.49±0.7	6	0	6	HF																+	+	+		+	+	+		
77	2185.22±0.4	34.35±2.8	6	0	6	HF																+	+	+			+	+	+	+
78	2198.79±0.0	14.66±0.7	7	0	7	HF																+	+	+		+	+	+	+	+
79	2208.14±0.4	33.72±2.9	5	0	5	HF																+		+			+	+	+	+
80	2255.72±0.3	25.40±2.6	6	1	5	HF				+												+		+			+	+	+	++
81	2265.55±0.5	24.50±1.8	11	5	6	HF	+		+				+	+							+	+	+	+			+	+	+	+
82	2286.13±0.5	14.08±1.2	8	2	6	HF	+							+								+	+	+		+		+	+	
83	2291.56±0.2	36.77±3.0	6	0	6	HF																+	+	+			+	+	+	
84	2299.00±0.0	11.58±0.5	7	0	7	HF																+	+	+		+	+	+	+	
85	2307.90±0.1	16.64±1.0	5	0	5	HF																+		+		+	+	+		
86	2314.80±0.2	13.79±1.0	7	1	6	HF	+																+	+		+	+	+	+	++
87	2323.66±0.2	28.12±2.2	5	0	5	HF																+	+	+		+		+		
88	2324.42±0.5	14.56±0.8	6	1	5	HF						+										+	+	+	+				+	
89	2342.82±0.0	14.80±0.6	6	0	6	HF																+	+	+		+		+	+	+
90	2355.05±0.2	26.09±1.9	5	0	5	HF																+	+				+	+	+	+
91	2358.79±0.2	20.40±1.6	6	0	6	HF																+	+	+		+	+	+		+
92	2376.87±0.1	39.41±3.5	6	0	6	HF																+	+	+			+	+	+	+
93	2394.58±0.1	30.20±2.2	6	0	6	HF																+	+	+			+	+	+	++
94	2395.69±0.4	14.65±0.7	8	0	8	HF																+	+	+	+	+	+	+	+	++
95	2524.20±0.2	14.42±0.6	5	0	5	HF																	+	+		+		+	+	+
96	2542.87±0.4	13.68±0.9	7	1	6	HF	+																+	+		+	+	+	+	
97	2580.42±0.4	32.02±2.4	5	0	5	HF																+		+	+			+	+	
98	2622.73±0.3	14.62±0.7	7	0	7	HF																+	+	+		+	+	+	+	+
99	2635.94±0.4	29.91±2.3	7	2	5	HF	+														+	+	+				+	+	+	

Table 1. Continued

No.	Mass (M_r)	CE time (min)	Proteins found			Type	Low-flux sample #														High-flux sample #								Normal urine
			Total	In LF	In HF		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	
100	2636.82±0.1	41.39±2.7	5	0	5	HF																+	+	+			+	+		
101	2639.80±0.1	15.30±1.0	8	1	7	HF	+															+	+	+		+	+	+	+	+
102	2695.49±0.5	13.93±1.2	7	2	5	HF					+		+											+		+	+	+	+	++
103	2728.30±0.4	14.70±0.8	7	0	7	HF																+	+	+		+	+	+	+	
104	2774.55±0.4	19.48±1.5	8	2	6	HF							+	+								+	+	+		+	+	+		
105	2807.08±0.2	29.97±2.2	6	0	6	HF																+	+	+			+	+	+	+
106	2819.82±0.3	14.91±1.2	7	1	6	HF	+																+	+		+	+	+	+	
107	2923.43±0.5	21.25±0.8	5	0	5	HF																+		+			+	+	+	
108	2939.16±0.2	20.99±1.5	5	0	5	HF																	+	+		+		+	+	
109	2942.05±0.5	25.31±2.0	8	0	8	HF																+	+	+	+	+	+	+	+	
110	2955.97±0.1	20.72±1.1	5	0	5	HF																+	+	+		+		+		
111	2987.58±0.6	13.79±1.1	5	0	5	HF																			+	+	+	+	+	+
112	3016.30±0.2	13.34±0.8	7	1	6	HF										+						+	+	+			+	+	+	+
113	3022.41±0.3	17.38±0.9	6	1	5	HF															+	+	+	+			+	+		+
114	3068.92±0.2	20.89±1.4	6	0	6	HF																+	+	+		+	+	+		
115	3197.13±0.2	18.05±0.8	6	1	5	HF	+															+	+	+			+	+		
116	3208.89±0.4	27.98±2.5	5	0	5	HF																+	+	+		+		+		+
117	3237.93±0.2	14.42±0.7	7	0	7	HF																+	+	+		+	+	+	+	+
118	3265.69±0.4	25.53±1.8	7	0	7	HF																+	+	+	+		+	+	+	++
119	3300.16±0.2	19.47±0.9	5	0	5	HF																+	+	+				+	+	+
120	3328.88±0.1	43.20±4.5	5	0	5	HF																+		+			+	+	+	+
121	3366.10±0.1	13.40±0.4	6	0	6	HF																+	+	+		+		+	+	
122	3453.11±0.3	13.43±0.5	7	0	7	HF																+	+	+		+	+	+	+	+
123	3630.52±0.9	13.65±0.8	5	0	5	HF																	+		+		+	+	+	
124	3729.19±0.1	14.11±0.6	7	0	7	HF																+	+	+		+	+	+	+	+
125	3923.32±0.4	20.57±1.5	5	0	5	HF																+	+	+		+			+	
126	3944.58±0.0	29.92±2.4	5	0	5	HF																+		+			+	+	+	+
127	4032.24±0.1	15.48±0.8	7	0	7	HF																+	+	+		+	+	+	+	
128	4044.42±0.1	17.52±1.0	6	0	6	HF																+	+	+		+	+	+		++
129	4070.30±0.1	21.26±1.5	5	0	5	HF																	+	+		+		+	+	
130	4088.62±0.8	15.68±1.0	5	0	5	HF																+	+	+				+	+	
131	4233.36±0.1	24.66±1.7	5	0	5	HF																+		+			+	+	+	
132	4244.38±0.2	14.17±0.6	5	0	5	HF																+	+	+			+	+		
133	4347.97±0.3	28.20±2.5	7	0	7	HF																+	+	+		+	+	+	+	++
134	4359.00±0.2	14.56±0.8	6	0	6	HF																+	+	+		+	+		+	+
135	4378.91±0.6	27.08±1.9	7	1	6	HF										+						+	+	+			+	+	+	
136	4414.56±0.2	25.29±2.4	5	0	5	HF																	+			+	+	+	+	
137	4440.84±0.1	27.02±2.4	7	0	7	HF																+	+	+		+	+	+	+	
138	4456.89±0.3	26.23±1.7	6	0	6	HF																+	+	+			+	+	+	
139	4780.52±1.0	30.07±3.0	5	0	5	HF																+		+		+	+	+		+
140	4800.23±0.4	30.11±2.5	6	0	6	HF																+	+	+			+	+	+	+
141	4817.32±0.6	28.87±2.2	5	0	5	HF																+		+			+	+	+	+
142	4833.05±0.1	29.02±2.1	6	0	6	HF																+	+	+			+	+	+	+
143	4849.27±0.4	29.89±3.5	5	0	5	HF																+	+			+	+		+	+
144	5243.72±0.3	18.52±1.3	7	0	7	HF																+	+	+		+	+	+	+	
145	5452.08±0.5	17.31±0.9	6	0	6	HF																+	+	+		+	+	+		
146	5839.13±1.1	17.35±1.5	8	3	5	HF							+			+			+			+	+	+	+		+			
147	6366.22±0.3	19.65±0.6	5	0	5	HF																+	+	+			+	+		
148	6387.63±0.6	22.17±1.4	6	0	6	HF																+	+	+		+	+	+		
149	6651.60±0.5	20.25±1.4	6	1	5	HF													+			+		+		+	+	+		

Table 1. Continued

No.	Mass (M_r)	CE time (min)	Proteins found			Type	Low-flux sample #															High-flux sample #								Normal urine	
			Total	In LF	In HF		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23		
150	6651.68±0.3	33.92±3.0	5	0	5	HF																+	+	+			+		+		
151	6688.28±0.5	21.95±1.7	6	0	6	HF																	+	+		+	+	+	+		
152	6813.60±0.5	20.20±1.3	5	0	5	HF																+	+				+	+	+		
153	7659.99±0.3	14.21±0.6	7	0	7	HF																+	+	+		+	+	+	+		
154	7870.27±0.4	20.85±1.7	6	0	6	HF																+		+		+	+	+	+		
155	8000.72±0.2	19.18±0.8	5	0	5	HF																+	+	+		+		+			
156	8015.71±1.0	19.10±1.1	7	0	7	HF																+	+	+		+	+	+	+	+	
157	8512.43±1.4	19.15±1.4	6	0	6	HF																+	+	+			+	+	+		
158	8667.44±0.1	14.11±0.5	7	0	7	HF																+	+	+		+	+	+	+		
159	8837.30±0.3	21.89±1.3	6	0	6	HF																+	+	+			+	+	+	+	
160	9866.23±0.2	21.08±1.2	6	0	6	HF																+	+	+			+	+	+	+	
161	10 329.33±1.0	16.23±1.3	17	10	7	CO	+					+	+	+	+	+	+	+	+		+	+	+	+		+	+	+	+	+	*
162	11 721.46±1.2	12.22±1.0	16	9	7	CO	+						+		+	+	+	+	+	+	+	+	+	+		+	+	+	+		*
163	1250.47±0.2	21.73±2.0	16	9	7	CO	+	+	+	+	+			+		+		+	+			+	+	+	+		+	+	+	++	*
164	1409.48±0.1	16.96±1.5	20	13	7	CO	+	+	+		+	+	+	+	+	+	+	+		+	+	+	+	+		+	+	+	+		*
165	1723.15±0.1	36.85±2.9	19	13	6	CO	+		+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+		+	+		+	*
166	1787.30±0.2	13.75±1.2	18	13	5	CO	+	+	+	+	+	+		+	+	+	+		+	+	+	+	+			+	+	+		+	*
167	1882.62±0.1	12.67±1.1	19	11	8	CO		+	+	+	+	+	+	+		+		+	+	+		+	+	+	+	+	+	+	+	+	*
168	2015.19±0.2	27.39±2.5	20	13	7	CO	+	+	+	+	+	+	+	+		+		+	+	+	+	+	+	+		+	+	+	+	+	*
169	2079.22±0.3	36.88±3.3	18	11	7	CO	+	+	+	+	+	+	+	+		+	+		+			+	+		+	+	+	+	+	+	*
170	2133.74±0.3	21.66±2.1	18	10	7	CO		+	+	+	+	+		+	+	+	+		+			+	+	+	+		+	+	+	+	*
171	2169.68±0.4	18.95±1.3	15	8	7	CO				+	+	+	+			+		+	+	+		+	+	+	+		+	+	+	+	*
172	2204.75±0.2	22.69±2.2	17	11	6	CO	+		+		+	+	+		+	+	+	+	+		+	+	+	+			+	+	+	+	*
173	2220.87±0.2	22.57±2.3	17	11	6	CO	+		+		+	+	+	+	+	+	+		+		+	+	+	+			+	+	+	+	*
174	2226.84±0.3	22.24±1.8	17	11	6	CO	+		+		+		+	+	+	+	+	+	+		+	+	+	+			+	+	+	+	*
175	2249.39±0.4	20.36±2.0	18	11	7	CO	+			+	+	+		+		+	+	+	+	+	+	+	+	+	+		+	+	+	++	
176	2559.07±0.2	10.62±0.8	19	12	7	CO	+	+	+	+	+	+	+	+	+		+		+		+	+	+	+		+	+	+	+	+	*
177	2565.04±0.5	18.12±2.0	16	8	8	CO	+	+	+	+	+	+	+	+								+	+	+	+	+	+	+	+	+	*
178	2824.70±0.4	18.94±1.5	17	10	7	CO		+	+	+	+	+		+	+		+		+		+	+	+	+		+	+	+	+	++	*
179	2999.02±0.4	16.82±1.8	19	12	7	CO	+	+		+	+	+	+	+	+	+	+		+		+	+	+	+		+	+	+	+	++	*
180	3013.04±0.6	16.73±1.7	14	8	6	CO	+	+		+			+	+		+	+				+	+	+	+			+	+	+	++	*
181	3314.29±0.2	13.34±1.1	15	9	6	CO	+	+	+	+		+	+	+			+				+	+	+	+		+	+	+			*
182	3891.49±0.2	19.81±1.8	14	9	5	CO	+		+		+	+	+	+		+	+				+	+		+			+	+	+		*
183	4418.51±0.5	16.38±1.4	19	13	6	CO	+	+	+		+	+	+	+	+	+	+	+	+		+	+	+			+	+	+	+		*
184	4933.20±0.5	13.67±1.1	21	13	8	CO	+		+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		*
185	4960.16±0.9	13.59±1.1	21	14	7	CO	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+		+	+	+	+		*
186	4976.04±0.9	13.84±1.1	18	11	7	CO	+	+	+	+	+	+	+	+	+				+		+	+	+	+		+	+	+	+	+	*
187	4999.86±0.7	20.40±1.9	14	9	5	CO	+		+		+	+	+	+	+		+		+			+	+				+	+	+	+	*
188	5121.84±0.5	14.42±1.0	17	10	7	CO	+		+		+	+	+	+	+		+		+		+	+	+	+		+	+	+	+		*
189	5258.24±0.7	15.74±1.4	19	12	7	CO		+	+	+	+	+	+	+		+	+	+	+	+		+	+	+	+		+	+	+		*
190	6237.65±0.5	14.84±1.4	19	12	7	CO		+	+	+	+	+	+	+	+	+		+	+	+		+	+	+	+		+	+	+	+	*
191	6542.27±0.8	14.90±1.1	20	13	7	CO	+	+	+	+		+	+	+	+	+	+	+	+		+	+	+	+		+	+	+	+	+	*
192	8499.69±0.8	13.42±1.1	15	10	5	CO	+	+	+	+	+	+	+	+				+		+		+		+		+		+	+		*
193	8559.00±0.7	11.85±0.9	19	12	7	CO	+	+	+		+		+	+	+	+	+	+		+	+	+	+	+		+	+	+	+	+	*

The presence of each polypeptide in an individual sample is indicated by a "+". The data were also compared to data from urine [27]. "+" indicates presence in at least one sample, "++" indicates presence in >50% of the analyzed samples.

resemble a peptide, due to its charge state of exclusively 1, which is never observed when analyzing peptides. The isotopic distribution pattern observed is unlike any seen when analyzing peptides, but it resembles a single, chlorinated compound, which is not found in natural products like body

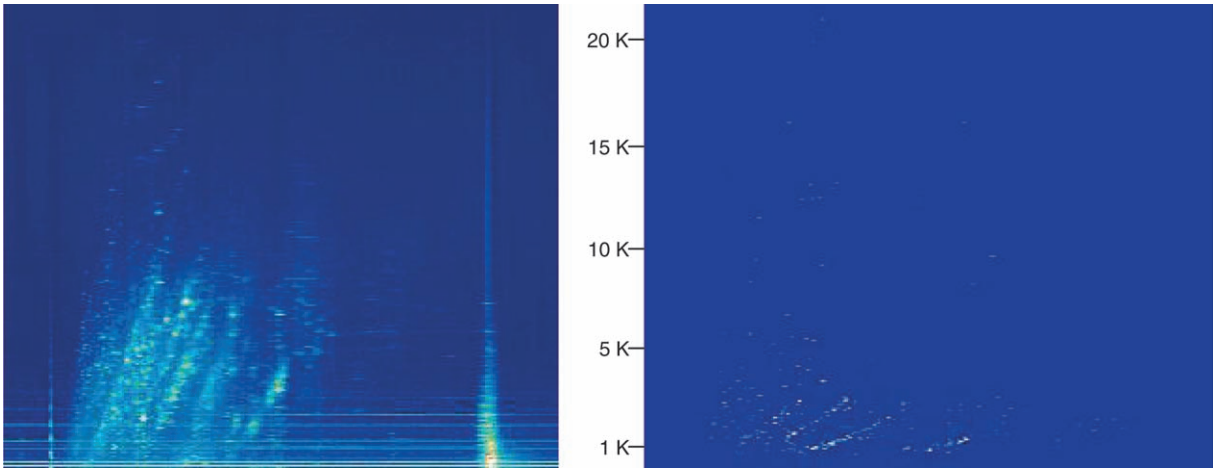


Fig. 5. Typical CE–MS contour plot of raw and processed data from a urine sample obtained from a healthy volunteer. As is evident, no obvious similarity can be observed when comparing urine to dialysate.

fluids. Most likely, this and similar compounds which were found in other samples, are introduced into the system (and hence into the patient) via dialysis tubing, membranes, etc. These compounds are not due to artefacts stemming from sample preparation, since they could reproducibly be detected in the individual samples only but not in any other. These results emphasize the sensitivity of the described method and show that this technology allows more thorough dialysis examination in order to minimize unwanted side-effects like the contamination with synthetic organic compounds and to maximize the removal of the ''uremic substances''.

Our data reveal that a number of larger polypeptides can only be found in high-flux dialysates. While no discrete cut-off molecular mass can be established, evidently starting at ~8000 the number of polypeptides identified in the high-flux membranes exceeds by far that of the low-flux membranes; essentially no polypeptide above 12 000 could be detected in the dialysates from the low-flux membranes. While this was to be expected, we did not anticipate that also a large number of smaller peptides that were present with high incidence in the high-flux dialysates were either completely absent or could be detected with significantly lower incidence in the low-flux dialysates. This might indicate that the high-flux membranes are in general better suited to removing polypeptides during haemodialysis.

Our results further indicate that a typical ''dialysis polypeptide pattern'' can be established using this technology. This is encouraging and might open new ways towards the assessment and improvement of dialysis membranes and even technologies.

Urine and dialysis fluid are not highly comparable with respect to the protein/polypeptide pattern. Since dialysis does not substitute all aspects of renal function concerning protein metabolism and/or elimination, such differences in protein/polypeptide patterns are not unexpected. This assumption is substantiated by our data and further analysis might allow investigation of how uremic toxins could efficiently be removed from the serum of dialysis patients. During the last few years renal replacement techniques have been improved considerably, allowing removal of larger molecules due to the application of high-flux membranes combined with filtration pressure. Still, removal of uremic toxins and proteins, such as β_2-microglobulin could be improved, since even treatment with haemofiltration using high-flux membranes does not lead to complete removal [28]. The method described here allows direct comparison of dialysis fluid and serum of individual patients and patient groups. Monitoring the removal of proteins under different conditions may further enhance the quality of renal replacement therapy and thus lead to even better care for patients with ESRD.

Taken together, the results presented here show

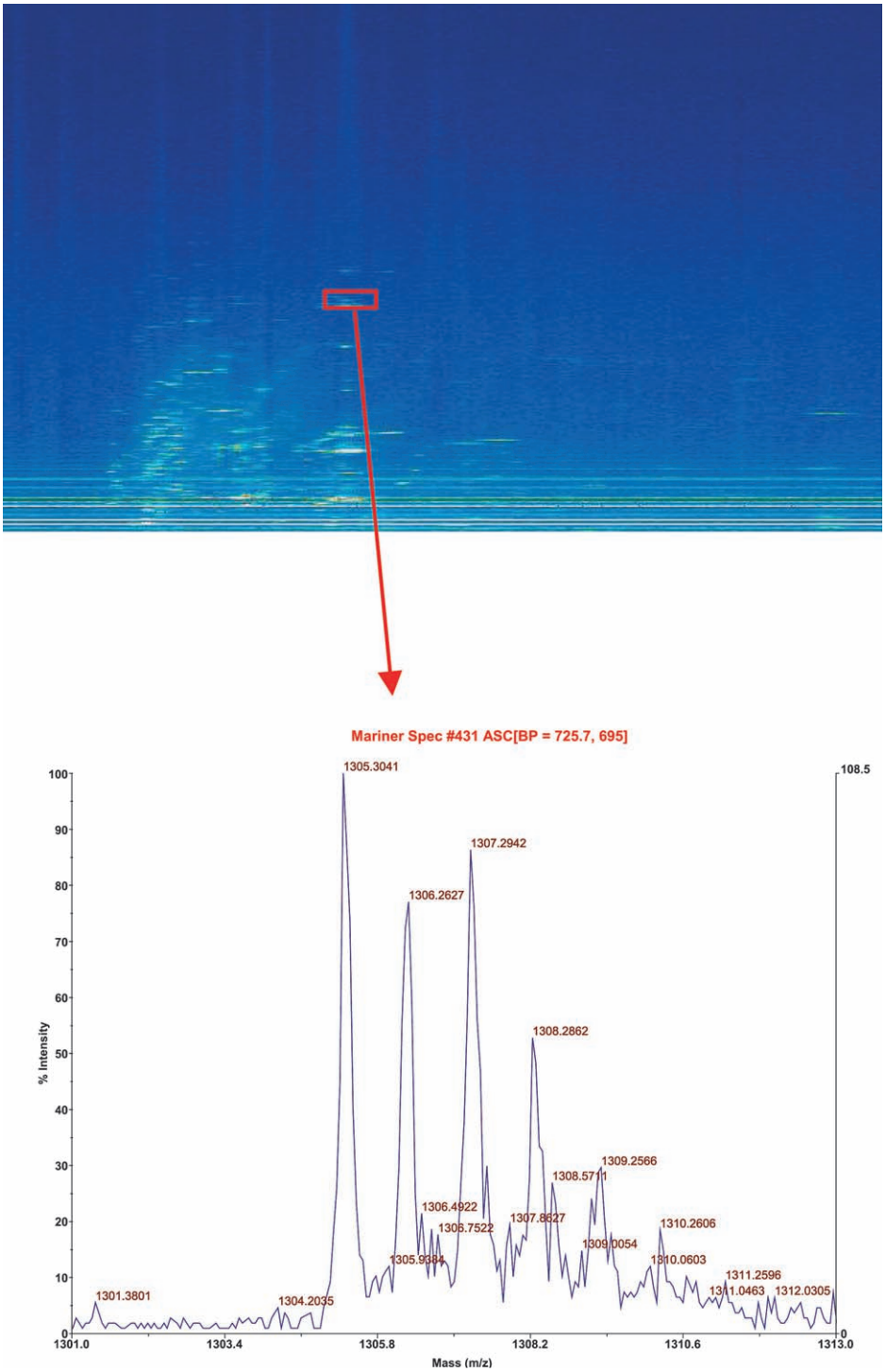


Fig. 6. Example of non-biological compound in dialysate. The upper panel shows a contour plot of raw data of a CE–MS run. In the lower panel, the isotopic distribution of a substance, which appears to be a chlorinated organic compound, is shown.

that CE–MS is excellently suited to establish polypeptide patterns of complex biological samples. The data provide hope that a number of ''uremic toxins'', still largely unknown, can be identified by applying the technique for screening patient samples.

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