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Online Comprehensive RPLC × RPLC with Mass Spectrometry Detection for the Analysis of Proteome Samples

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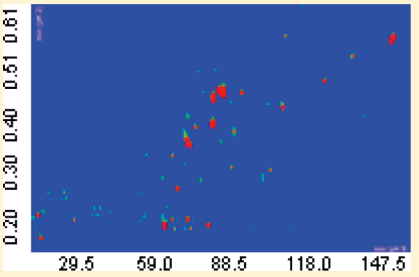
Supporting Information

ABSTRACT: LC-MS-based shotgun proteomics relies both on the power of the separation techniques and the sensitivity of detection methods. As a viable alternative to classical approaches in this field, we developed a fully automated, comprehensive 2D LC system, in which RPLC × RPLC was coupled to MS detection, for the first time, and applied for the analysis of tryptic digests obtained from α-casein and dephosphorylated α-casein. The use of a significantly different pH in the two dimensions allowed us to attain high peak capacity, despite the employment of novel identical stationary phases. Furthermore, such a combination addresses compatibility issues, thus allowing straightforward interfacing in online 2D LC configuration, as well as direct linkage to a mass spectrometer. A theoretical peak capacity of ca. 8500 was calculated for the setup, employing four serially coupled C18 columns in the first dimension (600 × 2.1 mm, 2.7 μm d.p.), operated under basic conditions, and 3 cm length of the same stationary phase (30 × 4.6 mm, 2.7 μm d.p. column), under acidic conditions, for fast second dimension analysis.



The analysis of proteome has always represented a challenging task for analytical chemistry, due to the enormous complexity and variability in dynamic range (up to 20 000 proteins may be present in a serum proteome, with a concentration range of 10^{10}). Additionally, proteins can undergo a variety of post-translational modifications (PTMs) which result in structural alterations, e.g., deamidation, oxidation, glycosilation, and phosphorylation.[1] In order to achieve a successful characterization, a great demand is therefore placed both on the power of separation technique and the sensitivity of detection methods.[2,3]

Since intact proteins are difficult to handle and may behave very differently due to their unique nature, proteome analysis is often performed at the peptide level, rather than at the protein level: hence, the term "peptidomics". On the one hand, proteolytic digestion of proteins prior to downstream processing yields a mixture of uniformly behaving peptides, thus overcoming many difficulties associated with the analysis; on the other hand, complexity of the sample is enormously increased, often overwhelming the peak capacity afforded by any single separation approach.

Hence, the need for more powerful and highly resolving separation methods has grown, along with the complexity of information to be gathered. To this regard, the combined use of orthogonal separation modes prior to MS or MS/MS analysis considerably alleviates this problem, by increasing the number of peptides that can be identified and quantified, including post-translationally modified peptides. High resolution front-end separation techniques is beneficial for several reasons: it better resolves peptides differing in charge and hydrophobicity and further simplifies the complexity of the ions entering the mass spectrometer, avoiding to overwhelm the limited dynamic range per spectrum of the mass detector.

As a result, the employment of multidimensional techniques has thrived over the last two decades, attempting to provide a more comprehensive knowledge of the biological networks in which expressed proteins are involved in, along with the assessment of their cellular localization and post-translational modifications.[4]

Traditionally, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), followed by enzymatic digestion of the separated protein spots, and MS identification has been the workhorse in analytical proteomics, its separation power being unrivaled. More recently, liquid-based, gel-free separation methods have drawn ever increasing interest, as they may address many of the limitations affecting 2D-PAGE, i.e., problematic detection of proteins characterized by large molecular weight, high p*I*, strong hydrophobicity, or low abundance and low accessibility of membrane-bound proteins. Difficulty of automation

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and scarce likelihood of straightforward hyphenation to mass spectrometry are also major drawbacks of these techniques. Besides, the rapid advances in both hardware and column technology, together with the development of dedicated software, have finally contributed to make liquid chromatography a very useful separation tool in modern proteomic research.[5,6]

Different strategies have been used by researchers in designing multidimensional LC systems for proteomic studies, in order to maximize the separation of all components. A first distinction can be made between the directly coupled-column methods and the column switching methods. In the first approach, analysts use two columns with orthogonal separation selectivities, typically based on strong-cation-exchange (SCX) and reversed-phase (RP) materials, which are packed in tandem into a single capillary. Fractions are eluted from the first column by applying a series of pulsed steps of buffer with increasing ionic strength. At each pulse, peptides are eluted from the D2-RP column using a linear acetonitrile gradient into the mass spectrometer. A pulled tip placed at the outlet end serves as the electrospray ionization needle, with minimum delay volume. An example of the first method is the MudPIT (multidimensional protein identification technology) developed by Yates and co-workers.[7−9]

Regarding the choice of stationary phase, different separation modes have been exploited to deliver a wide range of selectivities in the first dimension (D1), while the use of reversed-phase LC turned out to be by far the most preferred choice for second dimension (D2) analysis, bringing in the fair advantages of robustness, easy handling, and high compatibility to linkage to MS detection and further affording superior resolution, peak capacity, and more homogeneous distribution of peptide elution in the separation window.[10−16]

Separated peptides are then used for database searching, through computer algorithms (such as MASCOT, Sonar, SEQUEST), and the matches are used to predict the identity and sequence of the protein. Peptide mass fingerprinting (PMF) uses data obtained from MALDI-MS analysis of intact peptides, while tandem MS data are obtained by further fragmentation of the peptides, by collision-induced dissociation (CID).

The first online comprehensive RPLC × RPLC separation of peptides with photo diode array (PDA) detection has been reported by Sandra and co-workers, who serially coupled four octadecylsilica (ODS) columns in the first dimension (D1), connected through two 2-position, ten-port switching valves to two parallel ODS columns in D2. Low selectivity correlation and, hence, very high peak capacity was achieved through multimode operation, i.e., employing acidic (pH 1.8) and basic (pH 10) mobile phase in D1 and D2, respectively.[17] A similar setup, developed for the separation of a high complex sample consisting of peptides from albumin digestion, was afterward investigated by Mondello and co-workers.[18] However, their system used only one short (3 cm length) column to obtain a very fast gradient in D2, which was operated under acidic (pH 2) conditions to be directly online coupled to MS. Both systems were based on the column switching method. In this contribution, we developed a comprehensive 2D LC system, in which the first online coupling of RPLC × RPLC to MS is investigated. The two dimensions were interfaced through an electronically activated 2 position, ten-port valve and consisted both of a novel shell-packed stationary phase specifically designed for peptide separation. The performance of the system was assessed by means of tryptic mapping (protein unfolding, trypsin digestion, and reversed-phase chromatography of the peptide samples), followed by electrospray ionization (ESI) MS characterization of α-casein and dephosphorylated α-casein.

EXPERIMENTAL SECTION

Chemicals. LC-MS grade water (H_2O) and acetonitrile (ACN) were purchased from Sigma-Aldrich (Milan, Italy). Eluent additives for LC-MS trifluoroacetic acid (TFA) and ammonium acetate (CH_3COONH_4) were purchased from Fluka (Buchs, Switzerland). Casein and casein dephosphorylated (α_{S2}, from bovine milk, min. 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypsin (from bovine pancreas) was purchased from Fluka (Buchs, Switzerland). HPLC peptide standard mixture was purchased from Sigma-Aldrich (Milan, Italy).

Samples and Sample Preparation. For the preparation of the tryptic digest, the procedure was based on the previous work of Bushey and Jorgeson,[19] one-tenth gram of α-casein or dephosphorylated α-casein was dissolved in 10 mL of 0.01 M $HCOONH_4$ buffer, and the pH was adjusted to 8.0 with NH_4OH; the solution was heated in a boiling water bath for 6 min. After the solution cooled, 2.0 mg of trypsin from bovine pancreas was added, and the mixture was allowed to react for 4 h at +37 °C; the reaction was quenched by adding 0.1% trifluoroacetic acid to pH 2. The digests were stored at +4 °C and filtered prior to injection through a 0.45 μm nylon membrane (Whatman). Aqueous solutions of the peptide standard mixture (500 ppm) were injected without any filtration.

Columns. The following columns were used: four Ascentis Express Peptide ES-C18, 150 × 2.1 mm, 2.7 μm d.p. (as D1 of the comprehensive 2D-LC system); one Ascentis Express Peptide ES-C18, 30 × 4.6 mm, 2.7 μm d.p. (as D2 of the comprehensive 2D-LC system). All columns were kindly donated by Supelco/Sigma-Aldrich (Bellefonte, PA, USA). Stainless steel tubing of 5 cm in length and a 0.07 in. I.D. (zero dead-volume) was used to serially connect the D1 columns.

Instrument. Comprehensive 2D-LC analyses were performed on a Shimadzu Prominence LC-20A (Shimadzu, Milan, Italy), consisting of a CBM-20A controller, two LC-20AD dual-plunger parallel-flow pumps (D1-LC), an LC-20 AB solvent delivery module, equipped with two dual-plunger tandem-flow pumps (D2-LC), a DGU-20 A5 degasser, an SPD-M20A photo diode array detector (8 μL detector flow cell volume), a CTO-20A column oven, and a SIL-20A autosampler. The two dimensions were connected using an electronically controlled 2-position ten-port high pressure switching valve (Rheodyne, supplied by Shimadzu, Kyoto, Japan), placed inside the column oven and equipped with two 0.254 mm I.D. PEEK loops of identical volume (100 μL). Both dimensions and the switching valve were controlled by the LCMSsolution software (Version 3.50.346, Shimadzu). The LC × LC data were visualized and elaborated into two and three dimensions using Chromsquare ver. 1.3 software (Chromaleont, Messina, Italy). The LC × LC system was coupled to an ion trap-time of flight (IT-TOF) mass spectrometer through an ESI source (Shimadzu, Kyoto, Japan). The column hold-up volume was 0.45 mL while the system extra column volume was estimated as 13.5 μL. A schematic of the system employed is depicted in Figure 1.

2D-LC-PDA-MS Conditions. D1 mobile phase: (A) 10 mM CH_3COONH_4 in H_2O v/v (pH 9); (B) 10 mM CH_3COONH_4 in H_2O/ACN 10:90, v/v (pH 9). Gradient: 0−40 min, 0−10% B, 40−60 min, to 20% B, 60−200 min, to 50%, 200−220 min, to 100% B (hold for 20 min). Flow rate: 100 μL/min. Column oven: 35 °C. Inj. vol.: 20 μL. Switching valve time (modula tion time): 60 s. D2 mobile phase: (A) 0.1% TFA in H_2O v/v

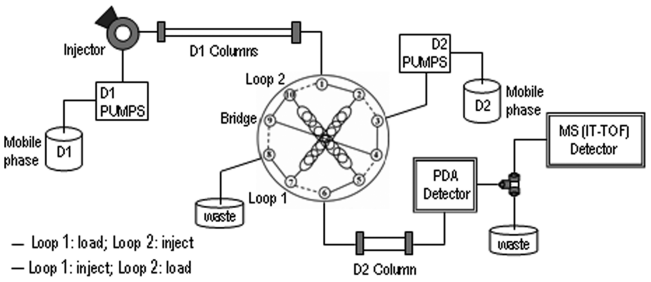

Figure 1. Schematic of the two-dimensional system employed in this study.

(pH 2); (B) 0.1% TFA in H_2O/ACN 10:90, v/v (pH 2). Gradient: 0−0.05 min, 0−20% B, 0.05−0.40 min, to 40% B, 0.40−0.50 min, to 50% B, 0.50−0.69 min, to 90% B, 0.69−0.70 min, to 0% B (hold for 0.3 min). Flow rate: 4 mL/min. Column oven: 35 °C.

PDA was used to monitor secondary column eluent during method development, allowing quick visual inspection of the separation obtained when optimizing the two chromatographic dimensions, independently. Only after a satisfactory and reproducible 2D separation was achieved, MS detection was connected to collect m/z data of the separated peptides, in such way limiting nebulizing gas (nitrogen) consumption. Being a nondestructive detector, PDA was maintained online before MS, to have a double check of experimental data. PDA detection: 215 nm (sampling rate 12.5 Hz, time constant 0.080 s). Data from PDA detector was background subtracted.

MS detection: ESI^+ mode; 180 μL/min flow from the LC system was directed to the mass spectrometer (stainless steel microvolume connector, 1/16 in., 0.15 mm bore). Detector voltage, 1.60 kV; CDL (curved desolvation line) temperature, 200 °C; block heater temperature, 200 °C; nebulizing gas flow (N_2) 1.5 L/min; ion accumulation time, 40 ms; full scan, 200−2000 m/z; repeat, 3; ASC 70%. Resolution, sensitivity, and mass number calibration of the ion trap and the TOF analyzer were tuned using a standard sample solution of sodium trifluoroacetate. After the calibrant had flowed, cleaning operation of the tube and ESI probe was made by flowing acetonitrile (0.2 mL/min, 20 min). The acquired mass spectrometry data was manually processed, by averaging the number of scans within each chromatographic peak and performing deconvolution of charge envelope by the LCMS solution software (Version 3.50.346, Shimadzu). The experimental deconvoluted molecular mass of each peptide was then compared to the theoretical value obtained by in silico digestion of the proteins.

RESULTS AND DISCUSSION

The objective of this work was to develop a comprehensive LC platform in conjunction with ESI mass spectrometry for the analysis of α-casein and dephosphorylated α-casein.

Comprehensive 2D RPLC × RPLC of Tryptic Digests. The separation of both α-casein and dephosphorylated α-casein tryptic digests was first optimized in the two chromatographic dimensions, independently. The use of two RP dimensions operating at different pH values was meant to ensure orthogonality, easy handling, and optimum efficiency. The latter can be further enhanced by serially connected columns.[20,21] Hydrophobic interactions are responsible for peptide separation under gradient conditions, typically employing acetonitrile (ACN) as the organic modifier and trifluoroacetic acid (TFA) or formic acid (FA) as ion-pair reagents. Coupling online RP to RP fulfills the requirements for the mobile phase compatibility between the two dimensions; in general, the use of low percentages of organic solvent allows effective online desalting and concentration of peptides at the same time, providing excellent compatibility with ESI and MS detection. The most common RP stationary phases are octadecylsilica (ODS), which are commercially available for this type of application with different lengths, internal diameters, particle sizes, pore sizes, pH stability, and hydrophobicity. A number of researchers have demonstrated the high performance of a shell-packed (or fused-core) stationary phase recently introduced,[22,23] either to attain enhanced resolving power at moderate backpressure[24−26] or to perform fast repetitive analyses in D2, under gradient conditions.[27−30]

In this work, we report on the first application of newly introduced fused-core columns, specifically designed for ultrafast and ultrahigh resolution separation of peptides. The 160 Å pore size of these particles in fact allows efficient diffusion of peptide molecules in and out of the pores where they can fully interact with the bonded phase, while the use of extra stable bonding chemistry provides optimum separation of peptides up to 20 kDa. Furthermore, the use of sterically protected bonding technology avoids the loss of bonded phase (hydrolysis of siloxane bonds) when operated at high temperature or low pH mobile phase, an issue typically associated with peptide analysis.

On the basis of our previous investigations, we employed four serially coupled C18 columns (2.1 mm i.d. for a total length of 60 cm) of this type to obtain greater resolving power for D1 separation of the digests, under gradient conditions. A flow rate of 100 μL/min gave the best results, in terms of peak overlap and resolution, in accordance with the optimum value earlier reported for peptide elution.[17,18,31]

In order to evaluate the performance of the gradient separation, the peak capacity was calculated using the method defined by Neue (eq 1):[32,33]

$$n_c = 1 + \frac{t_g}{(1/n)\sum_1^n w} \quad (1)$$

in which t_g is the time of the gradient run time, n is the number of peaks selected for the calculation, and w the average peak width.

It is generally acknowledged that in peptide separations by reversed-phase LC the peak width is rather uniform throughout the gradient chromatogram; anyway, the high complexity of the sample hinders reliable calculation of the peak width over the spanning time scale. A standard mixture of five peptides was, therefore, analyzed under the same experimental conditions and used for estimation of the peak capacity in both dimensions of the 2D LC system.

The text sample contained the following peptides, eluted according to their hydrophobicity: Gly-Tyr (M_W 238); Val-Tyr-Val (M_W 370); Tyr-Gly-Gly-Phe-Met (methionine enkephalin acetate, free base, M_W 573); Tyr-Gly-Gly-Phe-Leu (leucine enkephalin, free base, M_W 555); Asp-Arg-Val-Tyr-Ile-His-Pro-Phe (angiotensin II acetate, free base, M_W 1045). A theoretical peak capacity (n_C) of 244 was calculated for the separation attained in D1, using the average base peak width given by the software (4σ standard deviation).

Subsequently, applications were run on a fully automated LC × LC system, configured around an electronically activated

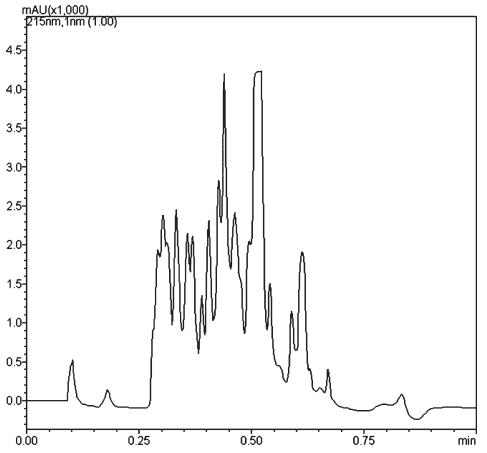

Figure 2. One min-D2 separation of tryptic digested α-casein on a 30 × 4.6 mm, 2.7 μm dp Ascentis Express Peptide ES-C18 column.

two-position, ten-port valve for within-loop automated fraction collection/reinjection, equipped with two identical 100 μL storage loops. In the comprehensive setup, the whole effluent from the D1 narrow-bore C18 columns was transferred online to the secondary column, consisting of a 3 cm length, 4.6 mm i.d. of the same stationary phase. The first dimension effluent was fractionated every 60 s into aliquots of 100 μL, corresponding to the volume of the first dimension mobile phase quantity eluting per sampling time.

The choice of switching valve modulation time and gradient profile was made to meet the following stringent requirements: each fraction injected onto the secondary column must be completely eluted before the following transfer occurs; the second dimension analysis time had to be kept as short as possible, not to impair the separation achieved in the first dimension (in this respect, the transfer of a large number of cuts is highly beneficial). For all these reasons, the second dimension separation must be fast and on-column focusing must be achieved.[34] To this regard, for D2 analyses, we employed a very high flow rate (4 mL/min) and a gradient program starting with 100% concentration of the weaker solvent (water), to ensure peak focusing at the head of the column; then, a slow gradient ramp was set, to elute peptides with very similar behavior. As it can be appreciated from the chromatogram in Figure 2, in the final part of the gradient program, a fast ramp up to 90% of the stronger solvent (ACN) was run, to ensure the elution of all the peptides within the fast analysis time of 1 min, including reconditioning time of 18 s. A peak capacity of 35 was estimated, under these conditions, in the same way as for the first dimension separation.

The overall peak capacity of the comprehensive separation was calculated as 8540, being multiplicative of the individual values obtained for the two dimensions ($n_{C1} \times n_{C2}$). These values are merely theoretical, however, and highly inflated, not taking into account any effect of the first dimension undersampling (about 1 fraction per peak capacity), nor the selectivity correlation (orthogonality), or the retention window in both dimensions, which does not cover the whole gradient duration. Therefore, some adjustments were made, in the calculation, which are fundamental for realistic peak capacity calculations.

First, the practical peak capacity of the separation, which accounts for orthogonality between the two dimensions (retention

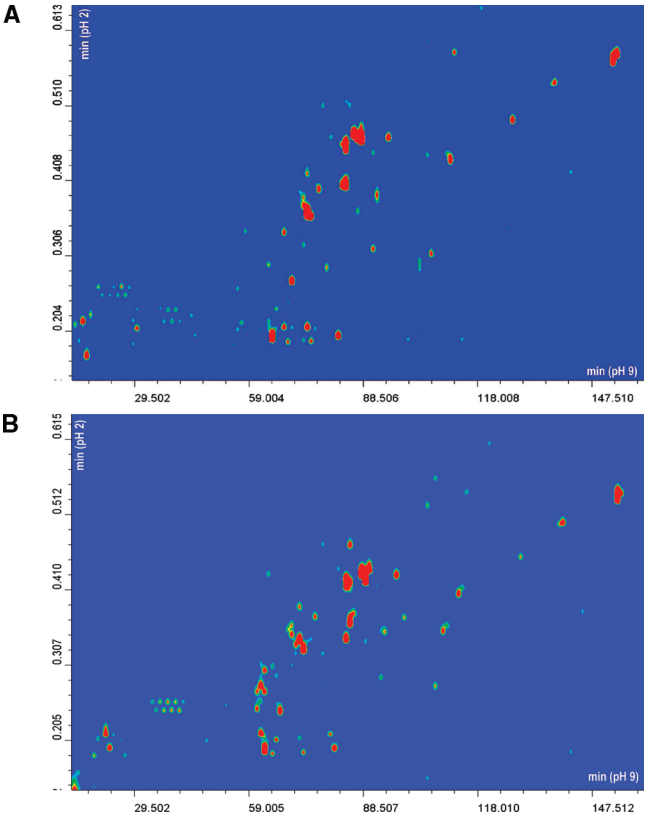

Figure 3. 2D RPLC × RPLC plot of α-casein (A) and dephosphorylated α-casein (B) tryptic digest separation. Colors are used to represent the value of absorbance at a given point. The relation between colors and absorbance is ruled by a color map, that can be configured by the user.

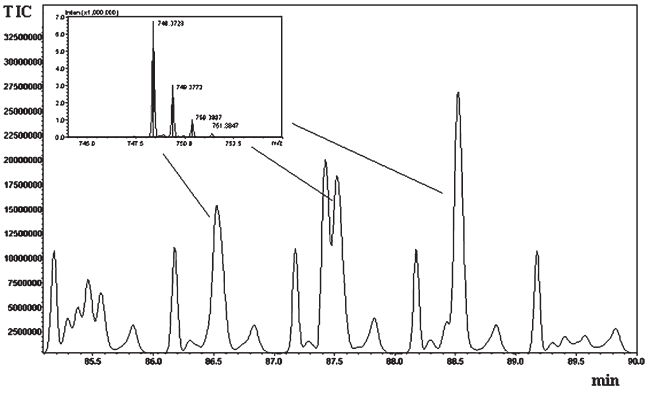

Figure 4. Five minute enlargement of the raw chromatogram obtained for the 2D RPLC × RPLC separation of α-casein tryptic digest (plot in Figure 3A). The inset shows m/z values corresponding to three consecutive cuts from the first dimension.

correlation derived from solute retention vectors), was calculated using the equation developed by Liu et al.[35] This calculation is based on solute retention parameters and, therefore, is more accurate in describing resolving power than those calculated by the multiplicative rule. A value of 3982 was obtained.

For the quantitative estimation of the undersampling effect, a very recent approach developed by Carr's research group was employed,[36] which also accounts for the effective retention time window and the second dimension gradient time. When such a

Table 1. Mass Accuracy for MS-IT-TOF Determination of a Standard Peptide Mixture

AA sequence	monoisotopic mass	theoretical $[M + H]^+$	measured $[M + H]^+$	error (amu)	error (ppm)
Gly-Tyr	238.09540	239.10333	239.1023	−0.00103	4.30
Val-Tyr-Val	370.21079	380.21860	380.2164	−0.00229	6.02
Tyr-Gly-Gly-Phe-Met	573.22579	574.23369	574.2334	+0.00031	0.53
Tyr-Gly-Gly-Phe-Leu	555.26936	556.27726	556.2767	−0.00560	1.00
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe	1045.53457	1046.54247	1046.5457	+0.00323	3.08

Table 2. Results for the 2D LC-MS Method Evaluation for a Tryptic Digest from α-Casein and Dephosphorylated α-Casein[a]

				molecular mass (monoisotopic)			
				casein α_{-S2}		casein α_{-S2} dephosph.	
residue #	peptide sequence	MC #	phosph. site	theoretical	experimental	theoretical	experimental
1-21	-KNTMEHVSSSEESIISQETYK(Q)	1	8, 9, 10, 16	2745.9997	2745.9964	2426.1344	2426.1148
1-24	-KNTMEHVSSSEESIISQETYKQEK(N)	2	8, 9, 10, 16	3131.1958	3131.1961	2811.3305	2811.3268
2-21	(K)NTMEHVSSSEESIISQETYK(Q)	0	8, 9, 10, 16	2617.1642	2617.9018	2298.0394	2298.0270
25-32	(K)NMAINPSK(E)	0	46	953.4114	n.d.	873.4451	873.4420
25-65	(K)NMAINPSKENLCSTFCKEVVR(N)	2	46	2462.1230	n.d.	2382.1567	2382.0052
42-45	(K)EVVR(N)	0		501.2983	n.d.	501.2983	501.2882
46-70	(R)NANEEEYSIGSSSEESAEVATEEVK(I)	0	56, 57, 58, 61	3007.0295	3007.0228	2687.1642	2687.1394
77-80	(K)HYQK(A)	0		574.2936	574.3344	574.2936	n.d.
81-91	(K)ALNEINQFYQK(F)	0		1366.6954	1366.6944	1366.6954	1366.6876
92-114	(K)FPQYLQYLYQGPIVLNPWDQVKR(N)	1		2864.5086	2864.3628	2864.5086	2864.3725
115-125	(R)NAVPITPTLNR(E)	0		1194.6793	1194.6786	1194.6793	1194.6754
126-136	(R)EQLSTSEENSK(K)	0	129, 131	1250.5699	n.d.	1250.5699	1250.5546
126-137	(R)EQLSTSEENSKK(T)	1	129, 131	1538.5975	1538.6032	1378.6649	1378.6564
137-152	(K)KTVDMESTEVFTKKTK(L)	3	143	1950.9446	1950.9534	1870.9783	1870.9828
138-149	(K)TVDMESTEVFTK(K)	0	143	1465.6120	1465.6274	1385.6457	1385.6358
138-150	(K)TVDMESTEVFTKK(T)	1	143	1593.7070	1593.7212	1513.7407	1513.7324
150-152	(K)KTK(L)	1		375.2554	375.1509	375.2554	n.d.
150-160	(K)KTKLTEEEKNR(L)	3		1374.7539	n.d.	1374.7539	1374.6226
151-158	(K)TKLTEEEK(N)	0		976.5149	976.5104	976.5149	n.d.
153-158	(K)LTEEEK(N)	0		747.3650	747.3650	747.3650	747.3643
159-160	(K)NR(L)	0		288.1619	288.1595	288.1619	n.d.
161-165	(R)LNFLK(K)	0		633.3922	633.3862	633.3922	633.3556
161-166	(R)LNFLKK(I)	1		761.4872	761.4928	761.4872	n.d.
167-170	(K)ISQR(Y)	0		502.2936	502.3006	502.2936	502.3008
174-181	(K)FALPQYLK(T)	0		978.5470	978.5470	978.5470	n.d.
182-188	(K)TVYQHQK(A)	0		902.4683	902.4660	902.4683	n.d.
182-205	(K)TVYQHQKAMKPWIQPKTKVIPYVR(Y)	3		2938.6440	2938.2763	2938.6440	n.d.
200-205	(K)VIPYVR(Y)	0		745.4559	745.4472	745.4559	n.d.

[a] MC = missed cleavage.

calculation was applied, the peak capacity calculated for the 2D-LC system was further halved, yielding a value of 1802.

Tryptic Mapping and MS Characterization. To the best of our knowledge, the only comprehensive column-switching LC system in combination with MS detection for tryptic mapping of protein isoforms has been reported by Cortes and co-workers,[37] who tuned a 2D-LC system composed of a cation-exchange column in the first dimension and two parallel C18 columns in the second dimension. The primary differences in our experimental setup consisted of the use of RPLC in both dimensions (both consisting of shell-packed particles), thus avoiding the use of salt concentrations in the first dimension, and the use of a single column switching valve as an interface in place of a trap and a secondary column. The contour plots obtained for α-casein and dephosphorylated α-casein tryptic digest separations on our 2D LC-PDA-MS system are shown in Figure 3A,B respectively, where the "*x*" axis represents the retention times of the peptides eluting in D1 RPLC, whereas the "*y*" axis represents the different retention times by D2 RPLC of the resolved peptides. Replicate analyses ($n = 3$) of the digests were run on the comprehensive system and reproducibility of retention times was calculated for five selected peaks spread throughout the elution window, yielding an average CV% of 0.555 (averaged CV% in the two chromatographic dimensions).

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Figure 4 shows a 5 min enlargement of the raw 2D RPLC × RPLC chromatogram corresponding to the plot in Figure 3A obtained for the separation of α-casein tryptic digest; the average mass spectrum obtained for three consecutive peaks (1 min interval, corresponding to the modulation time set) is depicted in the inset. This demonstrates that at least three fractions of the peptide with m/z 747.3650 have been transferred from the first to the second dimension of the comprehensive system.

Mass spectrometric data obtained by IT-TOF detection were used to identify peptides separated by means of the 2D RPLC × RPLC system. Instrument parameters had been previously optimized, in terms of resolution, sensitivity, and ionization efficiency, by analysis of the standard peptide mixture; the results obtained are reported in Table 1.

The acquired mass spectrometry data were manually processed, by averaging the number of scans within each chromatographic peak, and deconvolution of charge envelope was afterward performed for $[M + H]^+$ and $[M + nH]^{n+}$ ions. The experimental deconvoluted molecular masses of the peptides were then compared to the theoretical values obtained by in silico digestion of the proteins, as reported in Table 2.

In silico digestion of α-casein and dephosphorylated α-casein was performed using PeptideMass software available at Expasy site (www.expasy.ch/tools/peptide-mass.html), selecting up to three missed cleavages (MC) for the generation of peptides. For each identified peptide, monoisotopic molecular masses of the phosphorylated and the corresponding dephosphorylated forms are reported, together with the position of the modified aminoacidic residues (phosph. site). Many of the differences depicted in the plots in Figure 3 are obviously related to the presence or absence of phosphorylated peptides; in more detail, peptide residues 1-21, 1-24, 2-21, 46-70, 126-136, 137-152, 138-149, and 138-150 are detected in the phosphorylated form in α-casein, whereas they lack one or multiple phosphate groups in dephosphorylated α-casein. Since phosphorylation occurs on the serine residue and a difference of (roughly) 80 Da was observed for removal of each phosphate group, it must be nailed down that only HPO^{3-} is removed by dephosphorylation, leaving the serine residue intact.[38] When the results obtained from the two RPLC dimensions were combined, sequence coverage of 90.3% and 76.3% for α-casein and dephosphorylated α-casein, respectively, were obtained. Comparison with the corresponding values of 68.2% and 56.4% obtained for the monodimensional system (D1, four coupled columns) clearly demonstrates the usefulness of the 2D-LC system.

CONCLUSIONS

In this contribution, we developed the first fully automated, comprehensive RPLC × RPLC system, coupled to PDA and MS detection. Due to the ionic nature of peptides, the use of different pH values ensured enough separation selectivity between the two dimensions, although both consist of the same stationary phase. Furthermore, such a combination addresses compatibility issues, thus allowing straightforward interfacing in online 2D LC configuration, as well as direct linkage to a mass spectrometer.

High efficiency at moderate backpressure was achieved through the use of fused-core stationary phases in the two dimensions; being specifically designed for peptide separations, these columns are feasible to be operated at extreme pH values or under high temperature, thus addressing the stability issues typically associated to this type of analysis.

The results achieved so far hold promise for further optimization of the technique, both in terms of chromatography and detection; current efforts are devoted to obtain reliable tandem MS data, which can be directly used to identify peptides, and subsequently infer the nature of native and recombinant proteins of clinical relevance.

ASSOCIATED CONTENT

Supporting Information. Table of mass accuracy for MS-IT-TOF determination of a standard peptide mixture. This material is available free of charge via the Internet at http://pubs.acs.org.

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