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Separation of Amadori peptides from their unmodified analogs by ion-pairing RP-HPLC with heptafluorobutyric acid as ion-pair reagent

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Abstract Glycation is a common class of nonenzymatic posttranslational modifications relevant for several diseases and cell aging in general, such as D-glucose-derived modifications at the ε-amino groups of lysine residues in blood proteins, especially albumin, immunoglobulin, and hemoglobin, for diabetic patients. These Amadori compounds are identified on the peptide level after enzymatic digestion and chromatographic separation by mass spectrometry. Their syntheses usually rely on a global glycation approach. Both areas require the reliable separation of glycated peptides from their unmodified congeners present in different ratios, which is typically not achieved by standard eluent systems in ion-pairing RP-HPLC (IP-RPLC). Here, we compare aqueous acetonitrile and methanol gradients containing either trifluoroacetic acid (TFA) or heptafluorobutyric acid (HFBA) as ion-pairing agents to separate such peptide pairs. TFA-containing eluents resulted in rather low resolutions, and the glycated and unglycated peptides often coeluted. HFBA increased the retention times of the unmodified peptide more than for the glycated peptide thereby improving the separation of all eight studied peptide pairs, even achieving baseline separations for some sequences. Thus the use of HFBA as ion-pair reagent provides a universally applicable eluent system in IP-RPLC to separate glycated peptides from their unmodified counterparts, even at the preparative scale required for synthetic peptides.

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Introduction

Many proteins are enzymatically or nonenzymatically modified in living organisms. Important and well-studied examples of the former type are N- and O-glycosylation, phosphorylation, methylation, and acetylation [1]. The term nonenzymatic modification refers to chemical reactions in living cells directed towards specific functional groups accessible for small molecules at, or close to, the surface of proteins, such as oxidation, desamidation, and glycation [2–4]. In glycation (also called nonenzymatic glycosylation), which is commonly known as the Maillard reaction or browning, carbonyl compounds react with amino groups present in amino acids, amines, phospholipids, peptides, or proteins [5]. D-Glucose, for example, can interact with the free N-terminus [6], ε-amino groups of Lys residues [7], or guanidinium groups of Arg residues [8] to reversibly yield a Schiff base, which undergoes a rapid rearrangement to form more stable *N*-(1-deoxy-D-fructose-1-yl)amino acid derivatives. The so-called Amadori products gradually undergo a chain of mostly irreversible chemical reactions, such as oxidation, dehydration, fragmentation, and cross-linking, forming many diverse chemical structures referred to as advanced glycation end products (AGEs) [9]. A remarkable increase of such endogenous and often colored or fluorescent compounds is characteristic of ageing, diabetes mellitus complications, renal failure, and Alzheimer's disease [10, 11].

The analysis of such compounds relies mostly on different high-performance liquid chromatography (HPLC) techniques

to separate modified amino acids or peptides obtained by hydrolysis or enzymatic digestion of proteins. At the amino acid level, N_α-glycated amino acids were successfully analyzed by anion-exchange HPLC [12, 13]. Ion-pairing reversed-phase HPLC (IP-RPLC) is the separation technique of choice for peptides because of its general applicability and high separation efficiency. Moreover, it can be favorably coupled online to ESI-MS [14–16] or offline to MALDI-MS [17, 18] even at the nanoscale required for proteome studies. Typically acidic aqueous acetonitrile gradients and C_8 or C_{18} stationary phases are used [18, 19]. Medium-sized Amadori peptides, however, are only partially separated from their unmodified analogs and often coelute in IP-RPLC, as the polar side chain does not influence the retention times significantly [20]. For short polar peptides this problem can be partially overcome by using methanol-based eluents [21, 22], but these conditions are not generally applicable to complex peptide mixtures and have to be optimized for each sequence. This seriously limits the purity of Amadori peptides synthesized by a global glycation approach [18, 19, 22, 23].

Here we study the influence of different mobile phases on the separation of glycated peptides from their unmodified homologs in IP-RPLC on C_{18} phases to accomplish a better purification of synthetic Amadori peptides as well as to allow easier analyses of accordingly modified peptides. Thus methanol and acetonitrile were tested as organic solvents, and trifluoroacetic acid (TFA) and heptafluorobutyric acid (HFBA) were tested as ion-pair reagents. Whereas TFA is typically used for peptide separations the more hydrophobic ion-pair reagent HFBA has been rarely applied but specifically increases the retention of basic peptides. HFBA was used recently to improve the separation of glutathione-derived Amadori products [24]. To the best of our knowledge, the use of HFBA has not been studied in IP-RPLC to separate Amadori peptides and the corresponding unmodified congeners.

Materials and methods

Reagents

TFA (UV-spectroscopy grade) and HFBA (UV-spectroscopy grade) were purchased from Fluka (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). Methanol and acetonitrile (both HPLC-S gradient grade) were obtained from Biosolve V.B. (Valkenswaard, Netherlands). Water was purified on an ELGA system (LabWater Supplier, Bucks, Great Britain) in house (resistance > 18 MΩ cm).

Peptides

A total of eight site-specifically glycated peptides, i.e., H-AK_{Am}ASASFL-NH_2, H-ASK_{Am}ASKFL-NH_2, H-AK_{Am}ASADFL-NH_2, H-AK_{Am}DSASFL-NH_2, H-AGGK_{Am}AFLMP-NH_2, H-AGGK_{Am}AFRNCEA-NH_2, H-AGGK_{Am}AAFL-NH_2, and H-AGGK_{Am}AAFL-OH (K_{Am} = glycated lysine residue) referred to as peptides A to H, as well as the corresponding unmodified peptides were synthesized on solid phase with 9-fluorenylmethoxycarbonyl/*tert*-butyl (Fmoc/^{t}Bu) chemistry [25]. Parts of the eight resin-bound peptides were cleaved directly with TFA to obtain the unmodified peptides. The remaining parts were selectively deprotected at the lysine residue to be glycated [26], incubated with D-glucose, and finally cleaved with TFA [22]. The crude peptides were purified on a Jupiter C_{18} column (internal diameter 10 mm, length 150 mm, particle size 10 μm, pore size 30 nm, Phenomenex, Aschaffenburg, Germany) with a flow rate of 5 mL/min using a linear aqueous acetonitrile gradient in the presence of 0.1% TFA. Absorption was monitored at 220 nm. Purified peptides were lyophilized and stored at −18 °C.

IP-RPLC

Peptide separations were optimized on a Jupiter C_{18} column (internal diameter 4.6 mm or 10 mm, length 150 mm, particle size 5 μm, pore size 30 nm, Phenomenex) at room temperature using an Äkta™ purifier HPLC system (GE Healthcare Amersham Biosciences Europe GmbH, Freiburg, Germany) equipped with a P903 gradient pump, UV-900 detector, INV-907 injection valve (25-μL injection volume), A-905 autosampler, and Frac-950 fraction collector controlled by the Unicorn 5.1 software package for different eluent compositions and ion-pair reagents (Table 1). The column was equilibrated with 5% eluent B (or eluent C) for at least 15 min. After injection the initial eluent composition was held for 5 min before the content of eluent B (eluent C) was increased linearly to 20% within 10 min. The peptides were then eluted by a linear gradient using a slope of 0.25% eluent B (eluent C) per min. Flow rates were 1 mL/min (5 mL/min) for analytical (semipreparative) separations. Absorption was monitored at 220 nm.

Mass spectrometry

Peptides were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

Table 1 Eluent systems studied for separation of Amadori peptides from their unmodified analogs by IP-RPLC

Ion-pair reagent	Water	60% aqueous acetonitrile	80% aqueous methanol
0.1% (v/v) TFA	Eluent A1	Eluent B1	Eluent C1
0.1% (v/v) HFBA	Eluent A2	Eluent B2	Eluent C2

using a 4700 proteomic analyzer (Applied Biosystems GmbH, Darmstadt, Germany) operated in positive ion reflector TOF mode and α-cyano-4-hydroxycinnamic acid (Bruker Daltonics GmbH, Bremen, Germany) as matrix.

Separation parameters

Retention (capacity) factor k' was calculated as the quotient of the net retention time ($t_R'=t_R-t_0$) and the dead time (t_0):

$$k' = (t_R - t_0)/t_0 \quad (1)$$

Selectivity α was calculated as ratio of the retention factor of the unmodified peptide (k'_{Pept}) and the corresponding Amadori peptide (k'_{Am}):

$$\alpha = k'_{Pept} \Big/ k'_{Am} \quad (2)$$

Resolution R_s was calculated from the retention times of the unmodified ($t_{R_{Pept}}$) and corresponding glycated peptides ($t_{R_{Am}}$) as well as peak widths at the peak basis (w_b) of the corresponding peaks:

$$R_s = 2 \times \frac{t_{R_{Pept}} - t_{R_{Am}}}{w_{b_{Pept}} + w_{b_{Am}}} \quad (3)$$

As w_b is difficult to determine in a chromatogram, especially for partially separated peaks, we determined the peak widths at half height (w_h) and calculated w_b for a Gaussian peak:

$$w_b = \sqrt{\frac{2}{\ln 2}} \times w_h \approx 1.699 \times w_h \quad (4)$$

Results and discussion

In recent years both the analytical characterization of Amadori-modified proteins and the chemical synthesis of Amadori peptides have greatly advanced. Glycation sites can be routinely identified in proteins after enzymatic digestion by using mass spectrometry directly or after selective enrichment. On the solid phase glycated peptides can be synthesized basically at any length using a global glycation approach [18]. However, in both cases the separation of glycated peptides from the homologous unglycated peptides is only achieved for short sequences by RPC. Typically medium-sized peptides will coelute with their glycated versions. Although boronic acid affinity chromatography [27] and cation-exchange chromatography should be able to solve this problem, the high salt concentrations usually applied are incompatible with the MS techniques, contaminate synthetic peptides, and thus require an additional desalting step. Moreover, IEC is not routinely used in most laboratories working on MS analysis or peptide synthesis. Thus we looked for alternative mobile phase compositions on C_{18} phases to achieve a good separation between glycated and unglycated peptides independent of the peptide sequence.

Seven different peptide sequences (C-terminal amides) were selected ranging in length from eight to eleven amino acid residues and containing the glycation sites at the ε-amino group of lysine in positions two to four of the peptide chain (Table 2, peptides A to G). Peptides were synthesized on solid phase and their identity confirmed by MALDI-TOF-MS. Retention times and peak widths at half height were determined by injecting all peptides individually for the different eluent systems to calculate the retention factor k'. The selectivity α and resolution R_s were determined by analyzing a mixture of the glycated and corresponding unmodified peptide pairs. The most commonly used eluent system with an aqueous acetonitrile gradient in the presence of 0.1% TFA as ion-pair reagent did not provide a good separation at an increase of 0.6% acetonitrile per min. The separation was improved with shallower gradients for some peptides using slopes of 0.3% and 0.15% acetonitrile per min. A further decrease of the gradients did not improve the separation anymore but resulted in broader peaks limiting the resolution. Thus the optimal separation was achieved at an increase of 0.15% acetonitrile per min. Despite the shallow gradient most peptides were only partially separated as indicated by α values between 1.01 and 1.05 for peptide pairs A to F (Table 2), which is in agreement with our earlier observations. The best separation was achieved for peptide pair G with a selectivity of 1.08 (Table 2, Fig. 1a). But even this would not allow semipreparative purification of a synthetic Amadori peptide contaminated with its unmodified analog, which is common for a global glycation approach. The glycated peptides eluted slightly earlier than the unmodified peptides.

As Horvat's research group successfully used aqueous methanol gradients in the presence of TFA to separate N-terminally glycated peptides from the corresponding unmodified peptides [21], we applied this eluent system to our peptide pairs. Methanol is more hydrophilic than acetonitrile and therefore peptides have to be eluted with higher organic contents. More important is the presence of the hydroxyl group, however, which will undergo similar interactions as the sugar side chain, and thereby elute the glycated peptides in a different way than the cyano group of acetonitrile. For five of the seven tested sequences, the resolution was worse. The modified and unmodified peptides of peptide pairs D and F even coeluted (Table 2). Only for sequences A and C was the separation improved, indicating that sometimes a methanol gradient can be advantageous. The elution order of the separated peptide pairs was identical to the acetonitrile eluent, i.e., the Amadori peptide eluted first.

Table 2 Effect of four different eluent systems on separation parameters of unmodified and glycated peptide pairs by IP-RPLC

Eluent system	Parameters	Peptide A	Peptide B	Peptide C	Peptide D	Peptide E	Peptide F	Peptide G	Peptide H
CH_3CN,TFA	$t_{R_{Pept}}$	66.40	55.63	61.69	65.92	81.47	37.08	58.87	68.91
	$t_{R_{Am}}$	65.59	52.96	58.92	64.09	78.12	36.33	56.05	71.09
	$w_{h_{Pept}}$	1.83	2.46	2.32	2.06	1.77	1.91	1.56	1.19
	$w_{h_{Am}}$	0.57	2.59	2.06	2.01	2.00	0.70	1.73	1.25
	α	1.01	1.05	1.05	1.03	1.04	1.02	1.08	1.03
	R_s	0.40	0.62	0.74	0.53	1.05	0.34	0.86	1.05
CH_3OH, TFA	$t_{R_{Pept}}$	53.75	80.53	88.05	93.83	117.20	75.45	43.29	60.29
	$t_{R_{Am}}$	51.55	78.31	83.95	93.83	114.32	75.45	42.49	57.72
	$w_{h_{Pept}}$	1.22	3.68	1.84	3.94	1.83	2.16	4.34	3.02
	$w_{h_{Am}}$	1.29	2.39	3.35	3.94	3.88	2.16	4.34	3.25
	α	1.05	1.03	1.05	1.00	1.03	1.00	1.02	1.05
	R_s	1.03	0.43	0.93	0.00	0.59	0.00	0.11	0.48
CH_3CN, HFBA	$t_{R_{Pept}}$	65.53	76.09	64.24	67.09	78.96	56.57	63.48	80.35
	$t_{R_{Am}}$	62.60	72.01	60.41	63.36	74.71	54.04	58.77	73.96
	$w_{h_{Pept}}$	1.56	2.18	1.57	1.64	1.34	1.27	2.55	2.72
	$w_{h_{Am}}$	1.78	2.63	2.12	2.10	1.88	1.29	3.26	4.15
	α	1.05	1.06	1.07	1.06	1.06	1.05	1.08	1.09
	R_s	1.03	1.00	1.22	1.17	1.55	1.16	0.95	1.10
CH_3OH, HFBA	$t_{R_{Pept}}$	107.12	152.68	66.68	72.27	87.58	95.81	64.99	79.40
	$t_{R_{Am}}$	103.17	146.93	62.16	68.00	82.92	92.75	59.53	73.12
	$w_{h_{Pept}}$	2.24	2.45	2.05	1.98	1.91	1.97	1.85	2.59
	$w_{h_{Am}}$	2.30	3.39	2.86	2.54	2.52	2.03	2.93	3.47
	α	1.04	1.04	1.08	1.07	1.06	1.06	1.10	1.09
	R_s	1.02	1.16	1.08	1.11	1.24	0.90	1.34	1.22

Peak widths at half height (w_h) and retention times (t_R) and the resulting selectivity coefficients (α) and resolutions (R_s) are listed for unmodified and glycated peptide pairs A–H (for sequences see Materials and methods). Eluent compositions are provided in Table 1. A linear gradient from 20% eluent B to 45% eluent B in 100 min (A1–B1 and A2–B2) or from 30% eluent C to 65% eluent C in 140 min (A1–C1 and A2–C2) was applied at a flow rate of 1 mL/min

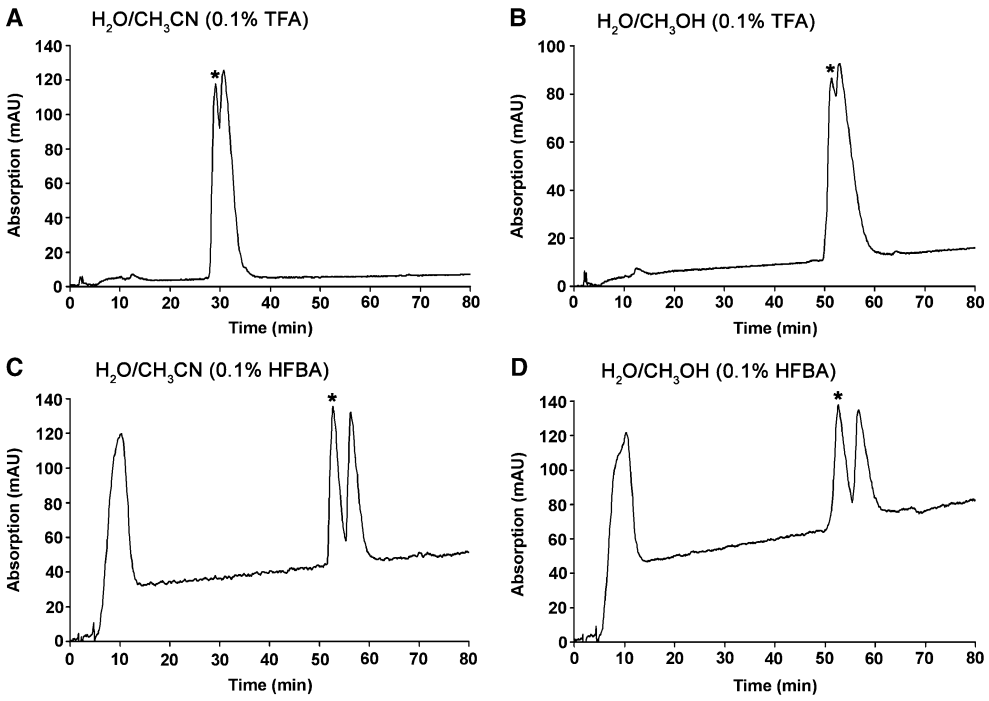

Fig. 1 IP-RPLC chromatograms of unmodified and glycated H-AGGKAAFL-NH_2 (peptide G). Eluent systems were A1–B1 (**A**), A1–C1 (**B**), A2–B2 (**C**), and A2–C2 (**D**) as provided in Table 1. A Jupiter C_{18} column and a flow rate of 1 mL/min were used. Linear gradients with an increase of 0.15% acetonitrile per min started at 9% (TFA, **A**) or 12% (HFBA, **C**) aqueous acetonitrile. For methanol gradients an increase of 0.20% per min was used starting at 12% (TFA, **B**) or 28% (HFBA, **D**) aqueous methanol. The unmodified and glycated peptides (marked with an *asterisk*) eluted at 30.8 and 29.1 min (**A**), 53.0 and 51.5 min (**B**), 56.3 and 52.8 min (**C**), and 56.9 and 52.8 min (**D**), respectively

As both TFA-containing eluent systems did not fulfill our requirement to generally separate glycated and unglycated peptides, we replaced TFA by HFBA. The rationale was to increase the retention of the unmodified peptide by interaction of the more hydrophobic ion-pair reagent with its free ε-amino group. The glycated lysine residue instead should not bind to HFBA and therefore elute earlier than the unmodified peptide. The separation was indeed enhanced by the increased retention of the unmodified peptide compared with the glycated peptide for both acetonitrile- and methanol-containing eluents. The selectivity improved slightly to a range from 1.05 to 1.08 (Table 2). The resolution increased to 0.95–1.55 for the aqueous acetonitrile gradient and 0.90 to 1.34 for the methanol system. Thus a partial separation for all peptides with similar R_s values was obtained (Fig. 1c,d). For peptide E, a baseline separation was obtained, i.e., an R_s value above 1.5. The aqueous methanol gradient was slightly better than the acetonitrile gradient for most sequences. The only drawback of the HFBA system was the peak broadening for all modified and unmodified peptides, which will limit the analysis of complex peptide samples. Perhaps the peak widths could be reduced with higher temperatures or lower flow rates but this was not further studied.

As only peptide amides had been studied so far, we also tested the separation efficiency of all four eluent systems for a peptide with a C-terminal free acid function (peptide H). Expectedly the replacement of a single functional group in such a large molecule altered the selectivity α and resolution R_s only slightly (cf. peptide G, Table 2).

As the separation problem studied here is often encountered during the synthesis of Amadori peptides by the global glycation approach, we finally studied the optimal eluent system, i.e., aqueous acetonitrile containing 0.1% HFBA, for the semipreparative purification of crude peptide G (Fig. 2a). The unmodified peptide eluted about 11 min later than the Amadori peptide corresponding to 1.6% acetonitrile. Despite the relatively broad peaks the collected peptide fraction appeared homogenous in IP-RPLC (data not shown) and MALDI-MS (Fig. 2b).

It appears that the ion-pair reagent HFBA is superior for separation of peptides glycated at ε-amino groups of lysine residues from their unmodified analogs by forming an ion pair with the free amino groups and thereby increasing the retention of the unmodified peptides relative to the glycated peptides. Moreover, the separation depends mostly on the modified site and, therefore, can be applied to all peptides without optimizing the eluent system for each new sequence. Though HFBA is less volatile than TFA it can be mostly removed by lyophilization and thus should not interfere with most peptide assays compatible with trifluoroacetate peptide salts. For other applications HFBA had to be removed by ultrafiltration, dialysis, or size-exclusion chromatography, which all work well at the typical peptide synthesis scales between 1 and 200 μmol. Removal of HFBA is also advantageous for both MALDI-MS and ESI-MS, as it can suppress signal intensities similarly to TFA due to formation of very strong ion pairs. These clusters are only partially broken apart in ESI sources and can seriously reduce the sensitivity of IP-RPLC-ESI-MS analyses [28]. Such limitations, however, can be overcome by addition of weak volatile acids, which compete with perfluorated acids to pair with the analyte or organic carrier solvents [29, 30]. Postcolumn addition of propionic acid-2-propanol, for example, improved the signal-to-noise ratio up to 100-fold for TFA-containing eluents. The lower volatility and relatively high molecular mass of HFBA, however, are disadvantageous for online coupling to ESI-MS as the instruments will be contaminated. This limits its general use in protein analytics and proteome-wide studies. Thus peptide digests should be fractionated in two dimensions by RP-HPLC using first the methanol/HFBA eluent system and afterwards an aqueous acetonitrile gradient in the presence of formic acid that also allows online coupling to ESI-MS. The two eluent

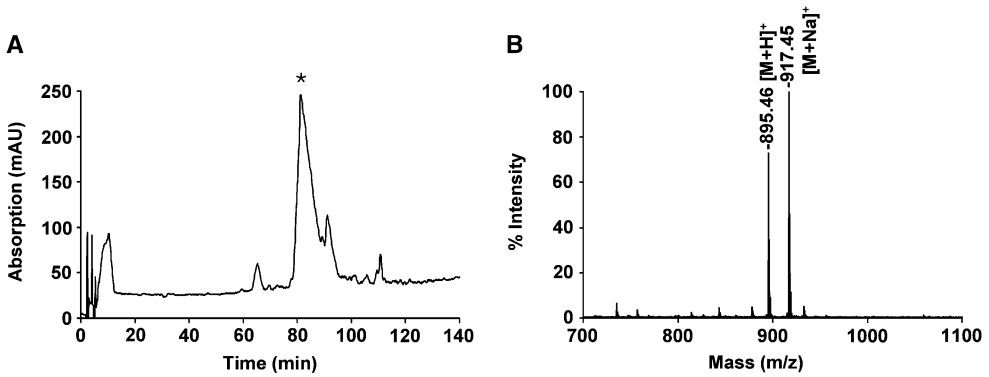

Fig. 2 Semipreparative purification of crude H-AGGK$_{Am}$AAFL-NH$_2$ (**A**) and MALDI-MS of the purified peptide (**B**). A linear gradient from 12 to 27% aqueous acetonitrile in the presence of 0.1% HFBA within 100 min at a flow rate of 10 mL/min was applied (Jupiter C_{18} column). The mass spectrum of the purified fraction (marked with an *asterisk*) containing the glycated peptide was recorded in positive ion mode on the 4700 proteomic analyzer using α-cyano-4-hydroxycinnamic acid as matrix

systems are even partially orthogonal, especially if different stationary phases are applied.

Conclusion

Amadori peptides derived from D-glucose by modifying the ε-amino groups of lysine residues are not or only partially resolved by aqueous acetonitrile gradients in the presence of 0.1% TFA on C_{18} phases. This unfavorable separation efficiency is mostly independent of the peptide sequence and obvious for all medium-sized peptides longer than approximately five amino acid residues. These limitations were overcome by replacing TFA by HFBA thus providing a fairly good separation of all studied peptide pairs, i.e., unmodified versus glycated, up to eleven residues with a typical resolution of 1.1; moreover, a baseline separation was achieved for some sequences. Importantly, this resolution was mostly independent of the sequence and amino acid composition, indicating a universal eluent system that can be used to generally separate at least medium-sized Amadori peptides from the corresponding unmodified sequences. The organic solvent influenced the separation only slightly with methanol providing, in most cases, a better separation than acetonitrile.

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