Analysis of glycopeptide biomarkers by on-line TiO_2 solid-phase extraction capillary electrophoresis-mass spectrometry

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Abstract

In this study is described an on-line titanium dioxide solid-phase extraction capillary electrophoresis-mass spectrometry (TiO_2-SPE-CE-MS) method for the analysis of the glycopeptide glycoforms obtained from the tryptic digests of recombinant human erythropoietin (rhEPO). The O_{126}-glycopeptide of rhEPO was used to optimize the methodology given its importance in quality control of biopharmaceuticals and doping analysis. Several aspects that affect the selective retention and elution, peak efficiency and electrophoretic separation of the O_{126} glycoforms were investigated to maximize detection sensitivity while minimizing non-specific retention of peptides. Under the optimized conditions, the microcartridge lifetime was around 10 analyses and repeatability was acceptable (%RSD values of 9-11% and 6-11% for migration times and peak areas, respectively). The method was linear between 0.5-50 $mg \cdot L^{-1}$ and 10-50 $mg \cdot L^{-1}$ for O_{126} glycoforms containing NeuAc and NeuGc, respectively, and limits of detection (LODs) were up to 100 times lower than by CE-MS. Although optimized for *O*-glycopeptides, the method proved also successful for preconcentration of N_{83}-glycopeptides, without compromising the separation between glycopeptide glycoforms with different number of sialic acids. Tryptic digests of other glycoproteins (i.e. human apolipoprotein CIII (APO-C3) and bovine alpha-1-acid glycoprotein (bAGP)) were also analyzed, demonstrating the applicability to glycopeptides with different glycan composition and nature.

Keywords: Capillary electrophoresis; glycopeptides; mass spectrometry; in-line solid-phase extraction; on-line solid-phase extraction; titanium dioxide

Introduction

In proteins, glycosylation is one of the most usual post-translational modifications, with more than half of the proteins in mammalian proteomes being glycosylated. The carbohydrate chains (glycans) are also known to play a crucial role in many processes such as recognition, signaling and adhesion on the cell surfaces [1,2]. Furthermore, *N*- and *O*-glycan structures are modified in recombinant biopharmaceuticals and altered in a wide range of disorders including cancer, inflammatory processes and alcoholism, among others [3–8]. Therefore, in recent years, characterization of aberrant glycosylation has aroused biomedical and biotechnological interest in order to detect and/or monitor several pathologies as well as to characterize recombinant biopharmaceuticals or discriminate between endogenous and recombinant variants of glycoproteins.

Human erythropoietin (hEPO) is a glycoprotein hormone produced mainly in the kidneys and responsible of maintaining red blood cell number and tissue oxygen supply at adequate levels. It shows four glycosylation sites, of which three are occupied by complex type N-glycans (at Asn24, Asn38 and Asn83) and one by an O-glycan (at Ser126). Recombinant variants of this glycoprotein (rhEPO) are widely used in the treatment of anemia in chronic kidney disease and cancer [9,10]. Nevertheless, rhEPO has become particularly popular in the last decades due to its misuse in endurance sport disciplines as a doping agent, hence forbidden by sport authorities since 1989 [11,12]. The presence of about 2% of non-human N-glycolylneuraminic acid (NeuGc) in the glycans of rhEPO O_{126}-glycosylation site enables distinction between recombinant and endogenous forms of hEPO in doping control. Furthermore, this content needs to be controlled to ensure the quality and safety of biopharmaceutical products due to adverse effects of NeuGc-contaminated pharmaceuticals [13]. Analysis of glycopeptide and

glycan biomarkers could be explored as an alternative to intact protein analysis, which presents certain limitations due to the high molecular mass and the detrimental effect of sugars in ionization [14,15]. In contrast to glycans, glycopeptide biomarkers offer information about the structure and composition of the carbohydrate moiety, as well as about glycosylation sites and their degree of occupation.

Capillary electrophoresis-mass spectrometry (CE-MS) has become a powerful hyphenated technique in glycoproteomics, tackling the analysis of intact glycoproteins, glycopeptides and glycans [7,16,17]. In addition to the CE-MS methods for the analysis of intact rhEPO [14,15], CE-MS methods for the analysis of rhEPO glycopeptides have previously been established for a more detailed protein characterization [18]. However, CE, as other microscale separation techniques, has low concentration sensitivity mainly due to the reduced sample volume injected to obtain optimum separations [19,20]. Over the years, different strategies have been described to decrease the limits of detection in CE [19–23]. Among these strategies, on-line solid-phase extraction capillary electrophoresis mass spectrometry (SPE-CE-MS) is one of the most versatile and successful alternatives [19–21]. In the typical SPE-CE configuration, a microcartridge, filled with a sorbent that shows affinity for the compounds of interest, is integrated in-line near the inlet of the separation capillary in order to clean-up and preconcentrate the target analytes from a large volume of sample (typically ~50-100 μL). Analysis of a broad variety of small molecules, peptides and proteins using different sorbents have been demonstrated by SPE-CE-MS [19–21], but not yet with titanium dioxide (TiO_2) sorbents or for the selective analysis of glycopeptides. In this regard, TiO_2 beads have been used for a long time for the off-line purification of phosphopeptides, or other phosphorylated analytes [24–26], and more recently for sialylated glycopeptides [27–29]. The mechanism of retention of both phosphopeptides and glycopeptides is mainly

attributed to interaction between the negatively charged phosphate or carboxyl groups, respectively, with the positively charged TiO_2 surface [26,28].

In this paper, for the first time to the best of our knowledge, TiO_2 beads were used as a sorbent in TiO_2-SPE-CE-MS to selectively analyze glycopeptides from the typical enzymatic digests of glycoproteins prepared in bottom-up proteomics approaches. The O_{126}-glycopeptide glycoforms of rhEPO were used to optimize the method, due to their relevance in quality control of biopharmaceuticals and doping analysis. Afterwards, the method was also evaluated for the analysis of rhEPO *N*-glycopeptides. Finally, it was applied to the tryptic digests of human apolipoprotein C-III (APO-C3), which contains only *O*-glycopeptides, and bovine alpha-1-acid glycoprotein (bAGP), which contains only *N*-glycopeptides.

2. Experimental Section

2.1. Chemicals

All chemicals used in the preparation of buffers and solutions were of analytical reagent grade. Acetic acid (HAc, glacial), formic acid (HFor 98-100%), sodium hydroxide and ammonia (25%) were supplied by Merck (Darmstadt, Germany). DL-Dithiothreitol (DTT, ≥99%), iodoacetamide (IAA, ≥98%), ammonium hydrogen carbonate (≥99.9%) and lactic acid (≥99.9%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Isopropanol was provided by Scharlab (Barcelona, Spain) while acetonitrile and water by Sigma-Aldrich (all of them of LC-MS quality grade). Trypsin (sequencing grade modified, 16000 $U \cdot mg^{-1}$) was purchased from Promega (Madison, WI, USA). ESI low concentration (ESI-L) tuning mix was supplied by Agilent Technologies (Waldbronn, Germany) for tuning and calibration of the mass spectrometer.

2.2. Glycoprotein samples

rhEPO produced in a chinese hamster ovary (CHO) cell line was provided by the European Pharmacopeia as a Biological Reference Product (BRP-lot4). Each sample vial contained 100 μg of rhEPO (a mixture of alpha and beta rhEPO), 24 mg of D-(+)-trehalose, 2.4 mg of arginine hydrochloride, 0.08 mg of Tween-20, 3.6 mg of NaCl and 2.5 mg of $NaH_2PO_4 \cdot 2H_2O$. The content of each vial was dissolved in water to obtain a 1000 $mg \cdot L^{-1}$ protein solution. Excipients of low-molecular mass were removed by ultracentrifugation using Microcon YM-10 centrifugal filters from Millipore (M_r cut-off 10,000, Bedford, MA, USA) as described elsewhere [18]. Centrifugations were carried out in a Mikro 20 centrifuge (Hettich, Tuttligen, Germany) at room temperature. bAGP (99%) and apoC-III (APO-C3) were obtained from Sigma-Aldrich. Stock solutions of 1000 $mg \cdot L^{-1}$ were prepared in water and aliquoted. Aliquots were evaporated to dryness

146 using a Savant SPD-111V SpeedVac concentrator (Thermo-Fisher Scientific, Waltham,
147 MA, USA) and stored at -20 ºC until enzymatic digestion.

148 rhEPO and bAGP, which contain cysteine residues that form disulfide bonds, were
149 firstly reduced and alkylated to facilitate digestion. Briefly, an aliquot of 50 μg of dried
150 glycoprotein was dissolved in 50 μL of 50 mM NH_4HCO_3 (pH 7.9) and 2.5 μL of 0.5M
151 DTT in the same buffer was added. The mixture was incubated in a thermoshaker at
152 56ºC for 30 min and then alkylated by adding 7 μL of 50 mM IAA in 50 mM NH_4HCO_3
153 (pH 7.9) and shaking for 30 min at room temperature in the dark. Excess of low-
154 molecular mass reagents was removed with Microcon YM-10 centrifugal filters as
155 described elsewhere [18]. The final protein residue was dissolved in 50 mM NH_4HCO_3
156 (pH 7.9) to obtain a final concentration of 1000 $mg \cdot L^{-1}$. *Trypsin digestion:* a 50 μL
157 aliquot of 1000 $mg \cdot L^{-1}$ protein solution in 50 mM NH_4HCO_3 (pH 7.9) was digested.
158 APO-C3 digest was directly prepared from aliquots of 10 μg of dried glycoprotein,
159 while rhEPO and bAGP digests were prepared from aliquots of 50 μg of reduced and
160 alkylated dried glycoprotein. Trypsin was added in an enzyme to protein ratio of 1:40
161 m/m. The mixture was vortexed and subsequently incubated at 37 ºC for 18h. Digestion
162 was stopped by heating at 100 ºC for 10 min, and the digest was stored at -20 ºC until
163 analysis [18]. All digestions were performed in triplicate. Incubations were performed
164 in a TS-100 thermoshaker (Biosan, Riga, Latvian Republic).

165 **2.3. CE-MS**

166 CE-MS experiments were performed in a $HP^{3D}CE$ system coupled to a 6220 oa-
167 TOF LC/MS mass spectrometer with an orthogonal sheathflow interface (Agilent
168 Technologies). The sheath liquid (50:50 (v/v) iPrOH/H_2O with 0.05% (v/v) of HFor)
169 was delivered at a flow rate of 3.3 $\mu L \cdot min^{-1}$ by a KD Scientific 100 series infusion
170 pump (Holliston, MS ,USA) and degassed for 10 min by sonication before use. CE

control and separation data acquisition (e.g. voltage, temperature and current) were performed using Chemstation software (Agilent Technologies) that was running in combination with the MassHunter workstation software (Agilent Technologies) for control, data acquisition and processing of the mass spectrometer. The mass spectrometer was tuned and calibrated following the manufacturer's instructions. A "check tune" of the instrument was performed every day in positive mode to ensure accurate mass assignments. Instrumental parameters were optimized for the analysis of rhEPO O_{126}- and N_{83}-glycopeptides in a previous study [18]. The optimized operational conditions in positive electrospray ionization (ESI) mode were: capillary voltage 4000 V, drying gas (N_2) temperature 200 ºC, drying gas flow rate 4 L min^{-1}, nebulizer gas (N_2) 10 psig, fragmentor voltage 190 V, skimmer voltage 60 V and OCT 1 RF Vpp voltage 300 V. Data were collected in profile (continuum) at 1 spectrum·s^{-1} (approx. 10,000 transients·$spectrum^{-1}$) between m/z 100 and 3200 working in the highest resolution mode (4 GHz).

A bare fused-silica capillary of 70 cm total length (L_T) x 75 µm internal diameter (I.D.) x 360 µm outer diameter (O.D.) (Polymicro Technologies, Phoenix, AZ, USA) was used in CE-MS. Activation and conditioning procedures were carried out off-line in order to avoid contamination with NaOH of the mass spectrometer. New capillaries were activated by flushing (930 mbar) sequentially for 30 min each with 1M NaOH, water and BGE (50 mM HAc and 50 mM HFor, pH 2.2). Capillaries were conditioned every day by flushing with NaOH (5 min), water (7 min) and BGE (10 min). Samples were reconstituted with BGE and injected for 15 s at 50 mbar. Electrophoretic separations were performed at 25 ºC and 25 kV under normal polarity (cathode in the outlet). Between runs, capillaries were flushed with water (1 min), 1 M HAc (3 min), water (1 min) and BGE (5 min). Capillaries were stored overnight filled with water.

196 Before CE-MS, all solutions were passed through a 0.22-µm nylon filter (MSI,
197 Westboro, MS, USA).

198 pH measurements were carried out with a Crison 2002 potentiometer and a Crison
199 electrode 52-03 (Crison instruments, Barcelona, Spain).

200 **2.4. SPE-CE-MS**

201 The TiO_2-coated magnetic beads (≤ 25 µm spherical) were obtained from the
202 Pierce Magnetic TiO_2 Phosphopeptide Enrichment Kit (Thermo Scientific,
203 Massachusetts, US). Bare fused silica capillaries (Polymicro Technologies) were used
204 for all the procedures.

205 Construction of the single-frit particle-packed microcartridge for TiO_2-SPE-CE-
206 MS was carried out as described elsewhere with little modifications [30]. The inlet end
207 was prepared by connecting the microcartridge (0.7 cm L_T × 250 µm i.d. × 365 µm o.d.
208 capillary) with a plastic sleeve to a previously conditioned inlet capillary (7.5 cm L_T ×
209 75 µm i.d. × 365 µm o.d.). The microcartridge was completely filled by vacuum with
210 TiO_2 sorbent beads. Another plastic sleeve was connected to the microcartridge, and a
211 small piece of cotton (approximately 1 mm) was placed in the plastic tube before
212 connecting the separation capillary (64.5 cm L_T × 75 µm i.d. × 365 µm o.d.). This
213 cotton frit in the microcartridge outlet end prevented the TiO_2 particles from leaking.
214 Particle leaking would promote current instability or breakdown and poor
215 reproducibility. Under the optimized conditions, the TiO_2 sorbent was first conditioned
216 by flushing (930 mbar) for 2 min with binding buffer (80% v/v acetonitrile, 10% v/v
217 HFor and 0.1 M lactic acid). Afterwards, protein digests were reconstituted in loading
218 buffer (80% v/v acetonitrile, 2% v/v HFor and 0.1 M lactic acid) to the desired
219 concentration and were loaded by flushing for 10 min (60 µL, estimated with the
220 Hagen-Poiseuille equation [31]). A final flush for 1 min with binding buffer and 1 min

with washing buffer (80% v/v acetonitrile, 2% v/v HFor) eliminated non-specifically retained molecules. All these steps were performed by switching off the nebulizer gas and the ESI capillary voltage to prevent the entrance of contaminants into the MS. Both were switched on and the capillary was filled by flushing for 2 min with BGE (the same as in CE-MS) to equilibrate the capillary before the electrophoretic separation. Then, a small volume of eluent (1 M NH_4OH) was injected at 50 mbar for 20 s (110 nL)[31] and, in order to prevent the eluent plug from traveling backwards due to the microcartridge backpressure, BGE was also injected at 25 mbar for 120 s. Separation was conducted at +20 kV for 35 min (cathode in the outlet) applying also a 50 mbar pressure to counteract microcartridge backpressure. Postconditioning to avoid carryover was performed by flushing for 1 min with water, followed by injection of eluent (50 mbar, 40 s) and application of a 100 mbar pressure during 10 min from the water vial.

2.5. Quality parameters

All quality parameters were calculated from data obtained by measuring peak area and migration time (t_m) from the extracted ion electropherogram (EIE) of rhEPO glycopeptide glycoforms (considering the m/z of the most abundant molecular ions, i.e. ions with charges +2, +3 and +4). Repeatability was evaluated as the percent relative standard deviation (%RSD) of peak areas and migration times obtained in consecutive analysis of digested rhEPO at 10 $mg \cdot L^{-1}$ (n=3). Linearity was studied by analyzing digested rhEPO at concentrations of protein between 25 and 1000 $mg \cdot L^{-1}$ for CE-MS and between 0.5 and 100 $mg \cdot L^{-1}$ for SPE-CE-MS. An estimation of the limits of detection (LODs) was obtained by analyzing digested rhEPO at low-concentrations (close to the LOD level, as determined from the approach based on S/N=3). The LODs of the different glycopeptide types (i.e. O_{126}-glycopeptide glycoforms containing NeuAc, O_{126}-glycopeptide glycoforms containing NeuGc and the most relevant N_{83}-

246 glycopeptide glycoforms) were established at the lowest concentration in which all the
247 expected glycoforms in each case were detected. The lifetime of the microcartridges
248 was evaluated by repeatedly analyzing digested rhEPO at a concentration of 10 $mg \cdot L^{-1}$.

249 **3. Results and Discussion**

250 **3.1. CE-MS**

251 With the aim of establishing a robust and reliable method to analyze sialylated
252 glycopeptides by TiO_2-SPE-CE-MS, glycopeptides from the tryptic digest of rhEPO
253 were first analyzed by CE-MS. rhEPO was chosen as a model glycoprotein for its
254 importance in quality control of biopharmaceutical and doping analysis, but also
255 because of the broad microheterogenity, as it presents both *N*- and *O*- glycosylation
256 with varying degrees of glycan branching and sialylation. The coverage of peptides and
257 glycopeptides in the tryptic digest of rhEPO was mapped by CE-MS using a method
258 previously developed in our group with a minor modification [18,32]. That
259 methodology used a bare fused capillary of 50 μm i.d. for the electrophoretic separation
260 of peptides and glycopeptides with an acidic BGE (50 mM HAc:50 mM HFor, pH 2.2)
261 and a sheath liquid of iPrOH:H_2O (60:40, v/v; 0.05% HFor). By contrast, in this study, a
262 75 μm i.d. capillary was preferred, as a wider capillary provided better performance
263 later by TiO_2-SPE-CE-MS. In the typical unidirectional SPE-CE configuration, the
264 microcartridge is mounted in series to the separation capillary, and TiO_2 microcartridges
265 were introducing a certain backpressure that resulted in poor performance with 50 μm
266 i.d. capillaries [20].

267 Samples of rhEPO at 1000 $mg \cdot L^{-1}$ were digested with trypsin and analyzed by
268 CE-MS in positive mode. Table 1 shows the average peak area and migration time of all
269 the detected peptides and glycoforms of O_{126}- and N_{83}-glycopeptides and the mass error
270 calculated from three replicate measurements. Full peptide sequence coverage and the

detected glycoforms of O_{126}- and N_{83}-glycopeptides agreed with our previous studies [18,32]. It is worth mentioning, that digestion with trypsin also results in a 2N-glycopeptide (N_{24}-N_{38}) that is poorly detected by CE-MS because its size and total charge promote very poor ionization efficiency. In general, the peak area values obtained for peptides were significantly large, which could therefore hinder the ionization yield of both O_{126}- and N_{83}-glycopeptides. Furthermore, as expected, the peak area values of the O_{126}-glycopeptide glycoforms containing N-acetylneuraminic acid (NeuAc) were substantially higher than those containing N-glycolylneuraminic acid (NeuGc), which were found at very low concentrations. Regarding the N_{83}-glycopeptide, some of the peak area values of the glycoforms were very small due to the low abundance and the high sialylated carbohydrate content, which promotes lower ionization yields. Figure 1 shows the extracted ion electropherograms (EIEs) of the most abundant O_{126} (A-i) and N_{83} (B-i) glycopeptide glycoforms. As can be observed, baseline separation of O_{126} and N_{83}-glycopeptide glycoforms containing different number of sialic acids were achieved with a 75 μm i.d. capillary, as in our previous studies with a 50 μm i.d. capillary [18,32]. Linearity of the method was investigated at concentrations of digested rhEPO varying from 25 to 1000 $mg \cdot L^{-1}$ for the most abundant O_{126} glycopeptide glycoforms, which contained NeuAc, and from 100 to 1000 $mg \cdot L^{-1}$ for the most relevant N_{83}-glycopeptide glycoforms, which presented 4Ant, 2, 3 or 4 NeuAc and 1 Fuc (see Figure 1A-ii and 1B-ii, respectively), with coefficients of determination of $R^2 > 0.99$. With respect to the O_{126}-glycopeptide, the LOD was established at 25 $mg \cdot L^{-1}$ of digested rhEPO to detect all glycoforms containing NeuAc and at 100 $mg \cdot L^{-1}$ of digested rhEPO for the less abundant glycoforms with NeuGc. In the case of N_{83} glycoforms, they were not detected at concentrations lower than 100 $mg \cdot L^{-1}$ of digested rhEPO.

3.2. TiO_2-SPE-CE-MS optimization

The TiO_2 beads obtained from the commercial kit designed for off-line purification of phosphopeptides and sialylated glycopeptides were used to prepare the microcartridges for TiO_2-SPE-CE-MS. The composition of the different buffers supplied in the kit was unknown and non-compatible with mass spectrometry, as we confirmed with some preliminary experiments. Typically, phosphopeptides and glycopeptides have been selectively enriched off-line with TiO_2 using hydroorganic mixtures with high contents of acetonitrile and HFor or trifluoroacetic acid for conditioning, loading and washing, and NH_4OH solutions for the elution [27–29,33,34]. In this study, the TiO_2 microcartridges were conditioned using a binding buffer containing 80% v/v acetonitrile and 10% v/v HFor. In the preliminary experiments, 50 $mg \cdot L^{-1}$ rhEPO tryptic digests were loaded at 930 mbar for 5 min in a loading buffer with 80% v/v of acetonitrile and 2% v/v of HFor. After washing with binding buffer (1 min) and loading buffer (1 min) and filling with BGE (50 mM HAc and 50 mM HFor, pH 2.2) (2 min), the retained glycopeptides were eluted with 1 M NH_4OH. Some experiments were also performed washing only with loading buffer (2 min), but the short washing step with binding buffer was necessary to reduce non-specific retention of peptides, which were in part eluted during this washing step (see Supplementary Figure 1). The graph bar of Figure 2A shows the total peak area of peptides and the different O_{126} glycoforms detected by TiO_2-SPE-CE-MS under different conditions. As can be observed (see the bars labelled as “preliminary conditions”), a small amount of peptides were still retained in the TiO_2 sorbent, but non-specific retention was lower compared to selective retention of the O_{126} glycoforms. Anyway, these preliminary results needed to be improved.

Most authors using TiO_2 for off-line purification of phosphopeptides and glycopeptides use lactic or glycolic acid in the loading and binding buffers to improve the selectivity by reducing unspecific binding of peptides [24,27]. According to them, 0.1M of lactic acid was added to the binding and loading buffers and a 50 $mg \cdot L^{-1}$ rhEPO digest was analyzed by TiO_2-SPE-CE-MS. Under these conditions, retention of peptides decreased while the intensity of O_{126} glycoforms significantly increased, as can be seen in Figure 2A. Higher concentrations than 0.1M of lactic acid produced higher noise and current instabilities during separation. Using 0.1 M of lactic acid in the loading and binding buffer, it was also tested the effect of adding an extra washing step with a lower percentage of acetonitrile (20% v/v of acetonitrile with 2% v/v of HFor, 1 min) in order to remove the most hydrophilic peptides non-specifically retained in the sorbent, as proposed by other authors in off-line protocols [27,29]. However, while the total peptide area was scarcely reduced (Figure 2A), the area of O_{126} glycoforms drastically decreased. This demonstrated that glycopeptides were partially lost when the TiO_2 sorbent was washed with high water contents. Therefore, this extra washing step was discarded, and binding and loading buffers with 80% v/v of acetonitrile, 0.1 M lactic acid and 10% or 2% v/v of HFor, respectively, were used for the rest of experiments. At this point, it is also important to note that, despite the TiO_2 sorbent has typically been described to retain sialylated glycopeptides, the O_{126}/0NeuAc glycoform was also detected using these conditions (see Figure 2), as also reported for other non-sialylated glycopeptides [28]. This was probably due to non-specific interactions with the peptide moiety and hydrophilic interactions with the attached glycans. Another remarkable fact is that the separation between glycoforms with different number of sialic acids was maintained, despite 50 mbar of pressure were necessary to be applied during separation to counteract the microcartridge backpressure (see in Figure 2Bi the

346 separation for the O_{126} NeuAc glycoforms). Even applying this positive pressure,
347 migration times increased around 10 minutes compared to CE-MS (Figure 1Ai).

348 The eluent composition was also investigated trying to improve the results
349 obtained with the 1M NH_4OH solution. Some authors have suggested the use of higher
350 NH_4OH concentrations than 1M to elute highly sialylated glycopeptides in off-line
351 procedures [29,35]. To test this hypothesis, a 10 $mg \cdot L^{-1}$ rhEPO digest was analyzed
352 using as eluents 1M, 3M and 6M NH_4OH solutions. As can be observed comparing
353 Figures 2B-i and 2B-ii, the peak area of O_{126} glycoforms decreased when 3M NH_4OH
354 was used, especially the one with higher sialic acid content (O_{126}/2NeuAc). Results
355 were even worse when using 6M NH_4OH, obtaining data with increased noise and
356 observing current instabilities. The use of ammonium phosphate (pH 9) as eluent was
357 also evaluated. Due to the high affinity of the phosphate groups for the TiO_2 sorbent, it
358 was expected to improve glycopeptide recoveries. Nonetheless, the peak area of O_{126}
359 glycoforms decreased, being unable to detect O_{126}/0NeuAc glycoform (see Figure 2B-
360 iii). Finally, an acidic eluent (0.1% v/v HFor) was also tested [28], but the obtained
361 results were very poor. Therefore, the 1M NH_4OH solution was confirmed as the eluent
362 for the analysis of glycopeptides by TiO_2-SPE-CE-MS. Under these conditions, sample
363 loading time was investigated loading a 10 $mg \cdot L^{-1}$ rhEPO digest for 5, 10, 20 and 30
364 min at 930 mbar. The peak area of O_{126}-glycopeptide glycoforms increased
365 progressively from 5 to 20 min and then started decreasing due to analyte breakthrough
366 (see Supplementary Figure 2). Despite the increased sensitivity, loading the sample for
367 20 minutes caused current instabilities that affected the separation repeatability.
368 Therefore, a loading time of 10 minutes was selected for the optimized method.

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3.3. Analysis of rhEPO glycopeptides by TiO_2-SPE-CE-MS

The optimized method was repeatable in terms of migration times and peak areas. The %RSDs (n=3) were between 9-11% and between 6-11% for O_{126} glycoforms, respectively, in consecutive analysis of a 10 $mg \cdot L^{-1}$ rhEPO digest. With respect to the microcartridge lifetime, it worked properly for around 10 analyses. The method was linear (R^2 >0.99) between 0.5 and 50 $mg \cdot L^{-1}$ for O_{126} glycoforms containing NeuAc and between 10 and 50 $mg \cdot L^{-1}$ for O_{126} glycoforms with NeuGc (see Figures 3A-i and 3A-ii, respectively). When loading higher concentrations, the sorbent was saturated, and the expected increase in the peak areas was not observed. The LOD was established at 0.25 $mg \cdot L^{-1}$ to detect all the O_{126} glycoforms with NeuAc and at 10 $mg \cdot L^{-1}$ for those with NeuGc, which corresponds to an improvement of up to 100 times with respect to CE-MS method. The developed method lowers significantly the working concentrations for O_{126} glycoforms from rhEPO containing both NeuAc and NeuGc. This is an attractive trait, since it enables detection of glycoforms with NeuGc at low concentrations, which is particularly important in quality control of biopharmaceuticals and doping analysis. Figure 3B shows the EIEs of the O_{126} glycoforms with NeuGc at 100 $mg \cdot L^{-1}$ of digested rhEPO.

The average peak areas of all detected O_{126}-glycopeptide glycoforms by TiO_2-SPE-CE-MS are presented in Table 1, as well as the values for the peptides and the N_{83} glycopeptide glycoforms. The TiO_2-SPE-CE-MS method, although developed and optimized for the O_{126} glycopeptide glycoforms of rhEPO enabled also detection of all N_{83} glycopeptide glycoforms reported by CE-MS (see Table 1). It was found that allowed a sensitivity enhancement of 10 times, detecting at 100 $mg \cdot L^{-1}$ of digested rhEPO all the N_{83} glycoforms identified at 1000 $mg \cdot L^{-1}$ by CE-MS, without any reoptimization for *N*-glycopeptides. The LOD for the N_{83} glycoforms was higher than

396 for the O_{126} glycoforms, as expected because these larger and more negatively-charged
397 glycoforms are harder to ionize in positive ionization mode. By way of an example,
398 Figure 3C shows the EIEs of the most relevant N_{83} glycoforms obtained by TiO_2-SPE-
399 CE-MS and previously presented for CE-MS in Figure 1B-i. As in CE-MS, N_{83}
400 glycoforms migrated close and after the O_{126} glycoforms (compare Figure 2B-i and 3C).
401 Moreover, separation between glycoforms containing different number of sialic acids
402 was not compromised with or without on-line preconcentration.

403 Another important concern was to determine if the optimized method was
404 providing an accurate and reliable glycopeptide glycoform fingerprint of rhEPO. In
405 order to study if the TiO_2 sorbent preferentially retained the glycoforms containing
406 more sialic acids, rhEPO digests were analyzed at different concentrations by CE-MS
407 and TiO_2-SPE-CE-MS. Figure 4 shows the bar graphs for the peak areas of each O_{126}
408 glycoform (i.e. O_{126}/ONeuAc, O_{126}/1NeuAc or O_{126}/2NeuAc) relative to the sum of all
409 O_{126} glycoforms. As can be observed, in all the studied concentrations, the relative peak
410 area of O_{126}/2NeuAc using TiO_2-SPE-CE-MS was slightly higher (around 36%) than by
411 CE-MS (around 25%). Consequently, the relative peak areas of the glycoforms
412 containing less sialic acids slightly decreased compared to CE-MS. This proved that the
413 TiO_2 sorbent shows certain preference for more sialylated glycoforms. In the case of N_{83}
414 glycopeptide, using as a case study the N_{83} tetraantennary glycoforms with two, three
415 and four sialic acids, no significant differences were found between CE-MS and TiO_2-
416 SPE-CE-MS (relative peak areas were around 5%, 30% and 65% for N_{83}/4Ant2NeuAc,
417 N_{83}/4Ant3NeuAc and N_{83}/4Ant4NeuAc, respectively, in both cases). Therefore, it can
418 be tentatively assumed that the slightly preference of the TiO_2 sorbent for the more
419 sialylated structures in rhEPO was observed because O_{126} glycoforms have an small
420 carbohydrate moiety, which is basically composed by sialic acid residues.

3.4. Analysis of other model glycoproteins

In order to test the potential of the TiO_2-SPE-CE-MS method developed for rhEPO with substantially different glycopeptides, the tryptic digests of APO-C3 and bAGP were also analyzed by CE-MS and TiO_2-SPE-CE-MS. APO-C3 is a small glycoprotein containing 70 amino acids with an O-glycosite at position 74, while bAGP is a 42 kDa glycoprotein contaninig 5 N-glycosylation sites at positions 16, 39, 76, 86 and 118.

Regarding APO-C3, the tryptic digests were analyzed at 600 $mg \cdot L^{-1}$ and 10 $mg \cdot L^{-1}$ of digested glycoprotein by CE-MS and TiO_2-SPE-CE-MS, respectively. Results of detected peptides and O_{74} glycoforms in both cases, including the mass error and peak areas, are listed in Table 2. At these concentrations, the same peptides and glycopeptides were detected using both methods. Even though the analyzed concentration by TiO_2-SPE-CE-MS was 60 times lower than by CE-MS, the peak areas obtained for O_{74} glycoforms were higher compared to those obtained by CE-MS. At the same time, the opposite was in general observed for peptides (Table 2), proving that the TiO_2 sorbent selectively retained the glycopeptides. As happened with rhEPO digests, the glycoform without sialic acids (O_{74}/0NeuAc) was also detected by TiO_2-SPE-CE-MS. Similarly, comparison between the relative peak areas obtained for O_{74} glycoforms by CE-MS and SPE-CE-MS (see Table 2) revealed again certain preference of the TiO_2 sorbent for glycoforms containing more sialic acids. Thus, the relative peak area of the O_{74}/2NeuAc glycoform by TiO_2-SPE-CE-MS was higher (around 88%) than by CE-MS (around 75%), whereas the relative peak areas of the glycoforms with less sialic acids were similar (O_{74}/0NeuAc) or lower (O_{74}/1NeuAc).

With regard to bAGP, the tryptic digests were analyzed at 1000 $mg \cdot L^{-1}$ and 100 $mg \cdot L^{-1}$ of digested glycoprotein by CE-MS and TiO_2-SPE-CE-MS, respectively. Table 3 lists all the detected peptides and glycopeptide glycoforms with both techniques, including the mass error and peak areas. Although at ten times lower concentration of digested bAGP by TiO_2-SPE-CE-MS, the same peptides and glycopeptides were detected as by CE-MS. In addition, selective retention of glycopeptides was obtained by TiO_2-SPE-CE-MS. No glycoforms from the N_{16}-glycopeptide could be detected probably because the peptide size of this glycopeptide is about two to three times larger than for the rest of glycopeptides, making it harder to ionize. Figure 5 shows as an example the EIEs of some N_{39} and N_{86} biantennary glycoforms obtained by CE-MS and TiO_2-SPE-CE-MS. Regarding migration time, no separation between N_{39}, N_{76}, N_{86} and N_{118} glycopeptide glycoforms with two sialic acids was obtained (see Figure 5 for some N_{39} and N_{86} glycoforms). This is to be expected since all these glycoforms contain two negative charges and share very similar molecular masses. On the other hand, separation between N_{39} glycoforms with two and three sialic acids was obtained using both CE-MS and TiO_2-SPE-CE-MS methods, as shown in Figure 5. Furthermore, a higher affinity of the TiO_2 sorbent for glycans with more sialic acids was also revealed in this case. As can be observed in Figure 5 and Table 3, the relative peak area of the N_{39}/2Ant3NeuGc and N_{118}/2Ant3NeuGc was higher by TiO_2-SPE-CE-MS than by CE-MS. These results indicate that, as with the *O*-glycopeptides, the TiO_2 sorbent presents certain preference for more sialylated structures in *N*-glycopeptides probably because the carbohydrate moiety is smaller (biantennary *N*-glycopeptide glycoforms) than in rhEPO (tri and tetraantennary *N*-glycopeptide glycoforms).

Conclusions

An on-line TiO_2-SPE-CE-MS method was optimized to selectively retain and enrich glycopeptides obtained from glycoprotein digests. Conditions for the TiO_2-SPE-CE-MS analysis of rhEPO O_{126}-glycoeptide glycoforms were carefully fine-tuned to maximize detection sensitivity and reduce non-specific retention of peptides, without compromising separation between glycoforms. Under the optimized conditions, repeatability was good (9-11 and 6-11% RSD for migration times and peak areas) and the microcartridge lifetime was around 10 analyses. The method was linear and the LODs were up to 100 times lower than by CE-MS. It was also demonstrated that the optimized method allowed preconcentrating the rhEPO N_{83}-glycopeptide glycoforms, as well as APO-C3 *O*-glycopeptides and bAGP *N*-glycopeptides. Therefore, the established TiO_2-SPE-CE-MS method can be used to analyze glycopeptides with different compositions and regardless of the protein derived from. It has to be taken into account the higher affinity of the TiO_2 sorbent for more sialylated glycoforms, which would decrease with the size of the carbohydrate moiety. Results are promising with immediate application in the analysis of diluted protein digests of biopharmaceuticals or in comparative glycoproteomic studies.

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635

636 **Figure legends**

637

638 **Figure 1:** CE-MS analysis of rhEPO tryptic digest. A) Extracted ion electropherograms
639 (EIEs) of the most abundant O_{126}-glycopeptide glycoforms (1000 mg·L^{-1} rhEPO) (i) and
640 linearity (25-1000 mg·L^{-1} rhEPO) (ii). B) EIEs of the most relevant N_{83}-glycopeptide
641 glycoforms (1000 mg·L^{-1} rhEPO) (i) and linearity (100-1000 mg·L^{-1} rhEPO) (ii).

642 **Figure 2:** A) Bar graph showing the effect on the peak areas of rhEPO O_{126}-
643 glycopeptide glycoforms and the total peptides detected by TiO_2-SPE-CE-MS of the
644 presence in the binding and loading buffers of 0.1M of lactic acid and of an extra
645 washing step B) EIEs of the most abundant O_{126}-glycopeptide glycoforms using the
646 established binding and loading buffers with 0.1M of lactic acid and different eluents:
647 (i) 1M NH_4OH, (ii) 3M NH_4OH and (iii) 50 mM $(NH_4)_3PO_4$ (pH 9) (10 mg·L^{-1} rhEPO).

648 **Figure 3:** A) Linearity of the optimized TiO_2-SPE-CE-MS method for the rhEPO O_{126}-
649 glycopeptide glycoforms. B) EIEs of rhEPO O_{126} glycoforms with NeuGc and C) the
650 most relevant N_{83} glycoforms by TiO_2-SPE-CE-MS under the optimized conditions
651 (100 mg·L^{-1} rhEPO).

652 **Figure 4:** Bar graph showing the relative peak areas of the most abundant O_{126}-
653 glycopeptide glycoforms at different concentrations of digested glycoprotein by CE-MS
654 and TiO_2-SPE-CE-MS. (Relative peak area was calculated as the peak area of each
655 glycoform divided by the sum of the peak areas of all glycoforms).

656 **Figure 5:** EIEs of bAGP N_{39}/2Ant2NeuGc, N_{39}/2Ant3NeuGc and N_{86}/2Ant2NeuGc
657 glycopeptide glycoforms by (A) CE-MS (1000 mg·L^{-1} bAGP) and (B) TiO_2-SPE-CE-
658 MS (100 mg·L^{-1} bAGP).

Table 1.- Summary of peptides, O_{126}- and N_{83}-glycopeptide glycoforms detected by CE-MS and TiO_2-SPE-CE-MS in the tryptic digest of rhEPO at a concentration of 1000 mg·L^{-1} and 100 mg·L^{-1} of digested glycoprotein, respectively (n=3).

			CE-MS					SPE-CE-MS
	Sequence	**M_{theo}**	**Error[a] (ppm)**	**Area (x10^6 a.u.)**	**%RSD**	**t_M (min)**	**%RSD**	**Area (x10^6 a.u.)**
Peptides	APPR	439.2543	3.2	1.0	4.3	5.0	1.4	0.01
	LICDSR-(Cys-IAA[b])	762.3694	1.1	2.8	2.7	5.6	1.3	0.3
	VLER	515.3067	3.4	8.9	4.9	5.2	2.7	0.05
	YLLEAK	735.4167	4.2	8.3	5.6	5.5	2.6	0.07
	VNFYAWK	926.4650	1.2	4.1	1.5	5.6	1.3	0.009
	MEVGQQAVEVWQGLALLSEAVLR	2525.3312	5.3	0.3	3.1	6.8	3.1	0.02
	AVSGLR	601.3548	1.9	4.6	0.002	5.4	1.3	0.01
	SLTTLLR	802.4913	7.3	19.2	7.7	5.6	1.3	0.01
	ALGAQK	586.3439	1.7	3.6	0.3	5.3	2.7	0.1
	TITADTFR	923.4712	2.4	5.4	4.0	5.8	2.4	0.03
	VYSNFLR	897.4709	1.4	3.8	0.7	5.6	2.5	0.2
	LYTGEACR-(Cys-IAA[b])	968.4386	1.1	3.7	4.9	5.8	2.4	0.2
	TGDR	447.2077	5.8	0.05	2.5	5.2	2.7	0.05
O_{126} glycoforms[c]	/0NeuAc	1829.8895	5.4	0.5	0.9	6.6	1.1	0.5
	/1NeuAc	2120.9849	4.5	13.7	2.3	7.5	2.8	10.7
	/2NeuAc	2412.0803	4.9	4.8	2.6	8.4	2.5	6.2
	/1NeuGc	2136.9798	5.7	0.2	1.8	7.5	2.8	0.1
	/1NeuGc1NeuAc	2428.0752	3.0	0.1	8.6	8.4	2.5	0.1
N_{83} glycoforms	/3Ant2NeuAc1Fuc	5074.1962	9.1	0.06	11.6	7.9	1.8	0.1
	/3Ant3NeuAc1Fuc	5365.2919	5.7	0.2	4.9	8.5	3.3	0.3
	/4Ant2NeuAc1Fuc	5439.3279	5.1	0.08	12.3	7.9	2.7	0.09
	/4Ant3NeuAc1Fuc	5730.4234	3.7	0.6	4.2	8.5	2.5	0.5

	/4Ant4NeuAc1Fuc	6021.5188	1.9	1.5	6.6	9.1	3.9	1.1
	/4Ant1LacNAc2NeuAc1Fuc	5804.4602	1.1	0.09	9.7	8.0	2.7	0.09
	/4Ant1LacNAc3NeuAc1Fuc	6095.5556	2.2	0.8	0.2	8.5	2.5	0.5
	/4Ant1LacNAc4NeuAc1Fuc	6386.6510	3.5	1.9	1.5	9.1	4.7	1.4
	/4Ant2LacNAc3NeuAc1Fuc	6460.6878	6.5	0.3	8.5	8.5	3.3	0.3
	/4Ant2LacNAc4NeuAc1Fuc	6751.7832	4.6	0.7	12.1	9.1	3.1	0.5

Detected peptides with less than 4 amino acids were not condidered (ie. R, K, LFR, GK,LK).

[a] Error was calculated in ppm as: $(|M_{exp} - M_{theo}|/M_{theo}) \times 10^6$ (exp = experimental and theo = theoretical).

[b] IAA stands for iodoacetamide.

[c] O-glycopeptide glycoforms are composed by one N-acetylglucosamine and one galactose monosaccharides plus the indicated number of sialic acids in each case.

Table 2.- Summary of peptides and O_{74}-glycopeptide glycoforms detected by CE-MS and TiO_2-SPE-CE-MS in the tryptic digest of APO-C3 at a concentration of 600 mg·L^{-1} and 10 mg·L^{-1} of digested glycoprotein, respectively (n=3).

				CE-MS	SPE-CE-MS
	Sequence	**M_{theo}**	**Error[a] (ppm)**	**Area(x10^7 a.u.)**	**Area(x10^7 a.u.)**
Peptides	SEAEDASLLSFMQGYMK	1905.8488	9.3	0.03	0.004
	HATK	455.2492	7.4	1	0.5
	DALSSVQESQVAQQAR	1715.8438	2.9	0.8	1
	GWVTDGFSSLK	1195.5873	2.1	4	0.01
	DYWSTVK	897.4232	2.4	2	0.05
O_{74} glycoforms[c]	/0NeuAc	2501.1486	3.7	0.003 (0.2%[b])	0.005 (0.1%[b])
	/1NeuAc	2792.2440	0.4	0.3 (24.9%[b])	0.7 (12.3%[b])
	/2NeuAc	3083.3394	1.7	0.9 (74.8%[b])	5 (87.6%[b])

Detected peptides with less than 4 amino acids were not condidered (ie. TAK, DK).

[a] Error was calculated in ppm as: $(|M_{exp} - M_{theo}|/M_{theo}) \times 10^6$ (exp = experimental and theo = theoretical).

[b] Relative peak area was calculated as the peak area of each glycoform divided by the sum of the peak areas of all glycoforms.

[c] O-glycopeptide glycoforms are composed by one N-acetylglucosamine and one galactose monosaccharides plus the indicated number of sialic acids in each case.

Table 3.- Summary of peptides and N-glycopeptide glycoforms detected by CE-MS and TiO_2-SPE-CE-MS in the tryptic digest of bAGP at a concentration of 1000 mg·L^{-1} and 100 mg·L^{-1} of digested glycoprotein, respectively (n=3).

				CE-MS	SPE-CE-MS
	Sequence	**M_{theo}**	**Error[a] (ppm)**	**Area(x10^6 a.u.)**	**Area(x10^6 a.u.)**
Peptides	WFYIGSAFR	1145.5658	2.9	3	0.05
	AIQAAFFYLEPR	1424.7452	1.7	3	0.01
	HAEDK	598.2711	3.4	2	3
	LITR	501.3275	2.6	4	0.2
	EYQTIEDK	1024.4713	4.1	2	0.2
	VESDR	604.2816	2.8	3	0.1
	EHFVDLLLSK	1199.655	5.3	6	0.4
	NVGVSFYADKPEVTQEQK	2038.0007	3.3	5	1
	EFLDVIK	862.48	1.7	11	0.02
	CIGIQESEIIYTDEK-(Cys-IAA[b])	1796.8502	6.2	1	0.04
	DACGPLEK-(Cys-IAA[b])	888.4011	4.6	5	0.3
	QHEEER	826.3569	6.5	0.9	0.8
	ETEAS	535.2126	7.2	0.8	0.03
N_{39} glycoforms	/2Ant2NeuGc	3000.1123	2.6	0.28 (70%[c])	0.27 (53%[c])
	/2Ant3NeuGc	3307.2026	4.3	0.12 (30%[c])	0.24 (47%[c])
N_{76} glycoforms	/2Ant2NeuAc	3394.2984	8.4	0.14 (28%[c])	0.12 (31%[c])
	/2Ant2NeuGc	3426.2882	4.0	0.15 (30%[c])	0.14 (36%[c])
	/2Ant1NeuAc1NeuGc	3410.2933	1.1	0.21 (42%[c])	0.13 (33%[c])
N_{86} glycoforms	/2Ant2NeuAc	2951.1647	8.8	0.14 (21%[c])	0.20 (27%[c])
	/2Ant2NeuGc	2983.1545	4.7	0.25 (37%[c])	0.26 (36%[c])
	/2Ant1NeuAc1NeuGc	2967.1596	4.6	0.28 (42%[c])	0.27 (37%[c])

N_{118} glycoforms	/2Ant2NeuAc	3530.4162	2.8	0.32 (25%[c])	0.10 (15%[c])
	/2Ant2NeuGc	3562.4060	1.1	0.39 (31%[c])	0.16 (24%[c])
	/2Ant1NeuAc1NeuGc	3546.4111	1.8	0.46 (36%[c])	0.18 (27%[c])
	/2Ant3NeuGc	3869.4963	8.2	0.10 (8%[c])	0.23 (34%[c])

Detected peptides with less than 4 amino acids were not condidered (ie. SAR, IYR, HFR, K).

[a] Error was calculated in ppm as: $(|M_{exp} - M_{theo}|/M_{theo}) \times 10^6$ (exp = experimental and theo = theoretical).

[b] IAA stands for iodoacetamide.

[c] Relative peak area was calculated as the peak area of each glycoform divided by the sum of the peak areas of all the glycoforms of a certain glycosylation site.

Figure

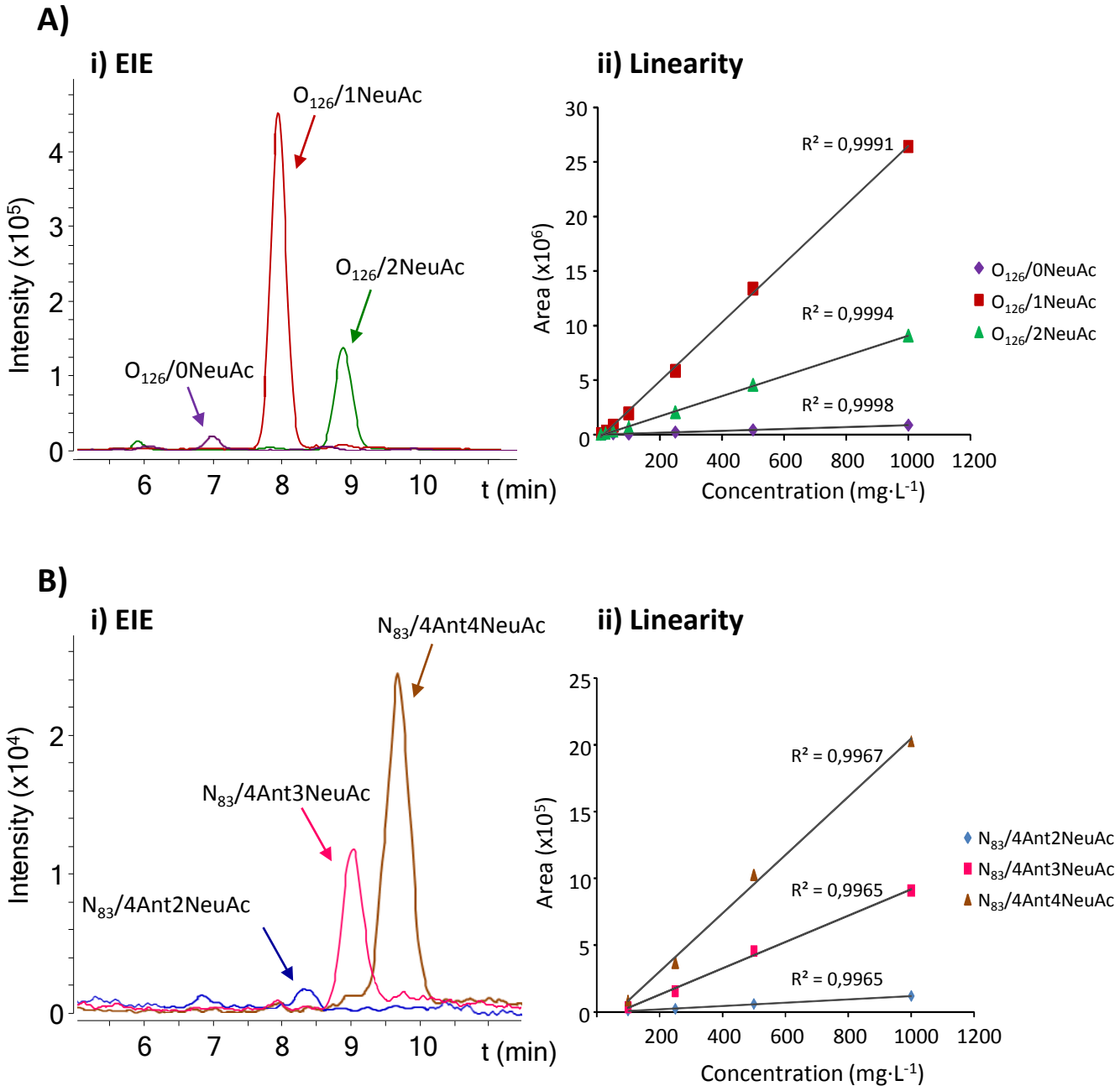


Figure 1

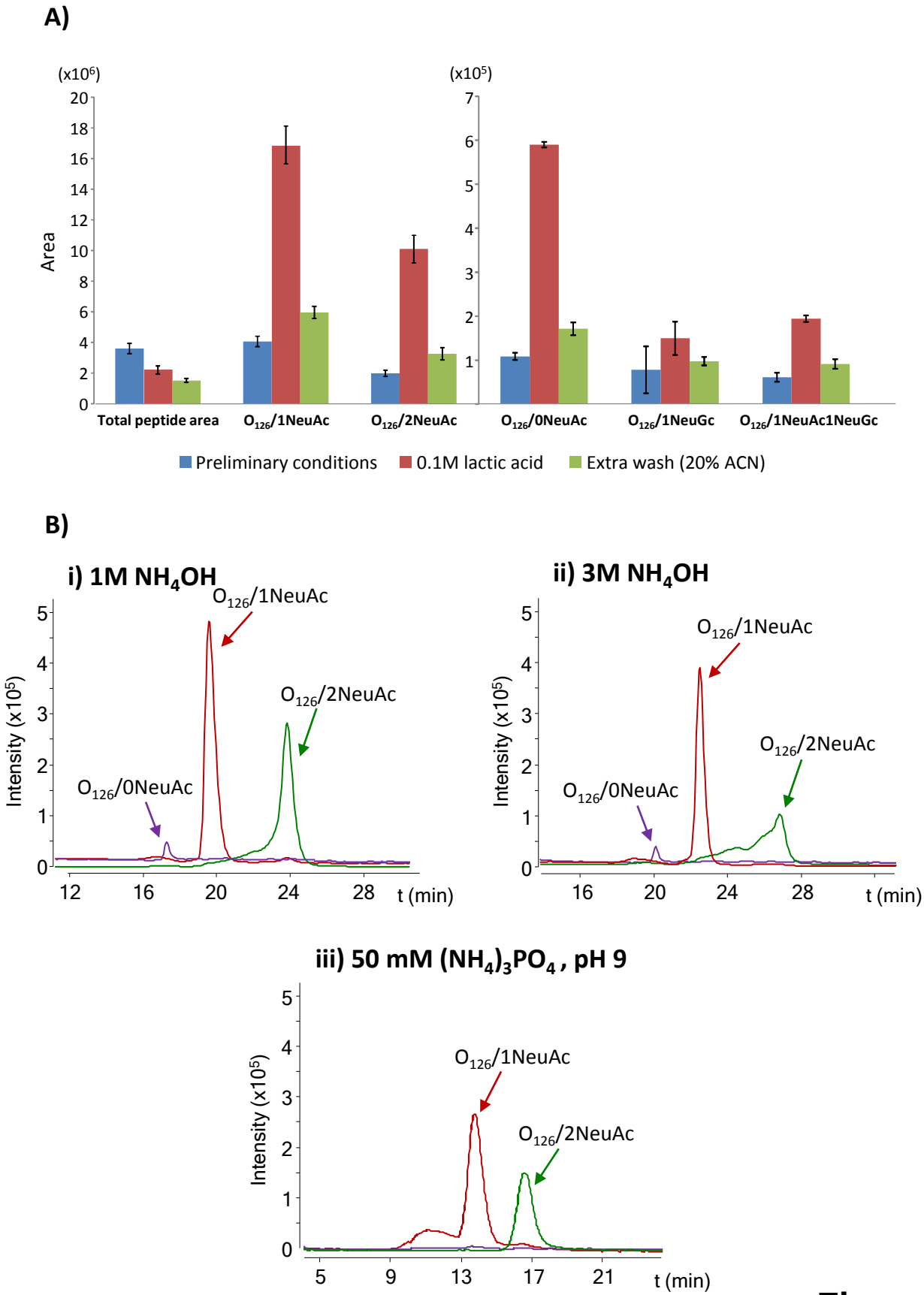
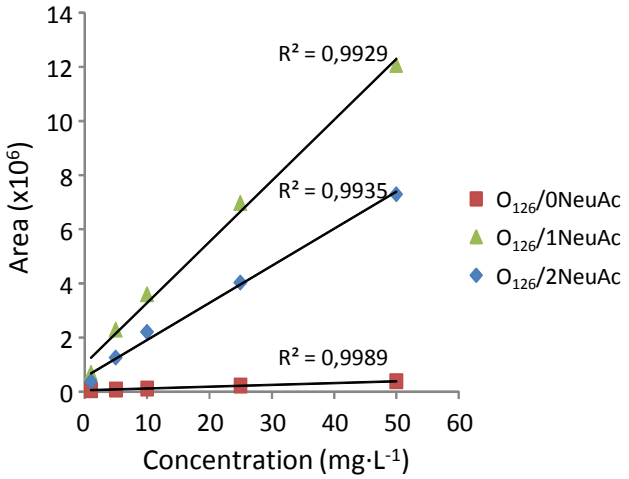
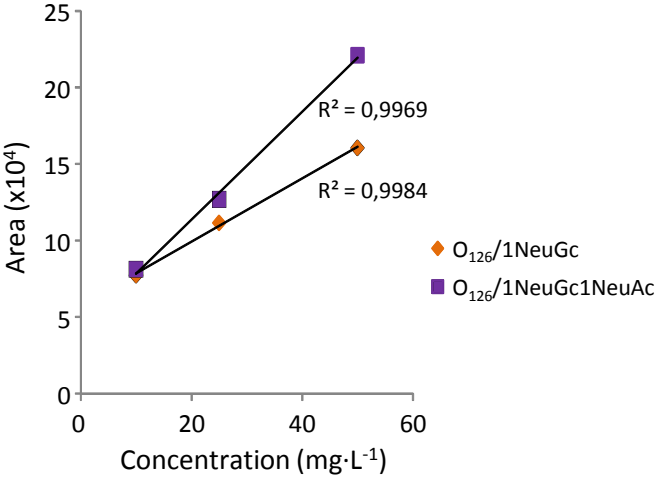

A)
(x10⁶)
(x10⁵)
Area
Total peptide area
O126/1NeuAc
O126/2NeuAc
O126/0NeuAc
O126/1NeuGc
O126/1NeuAc1NeuGc
Preliminary conditions
0.1M lactic acid
Extra wash (20% ACN)
B)
i) 1M NH4OH
ii) 3M NH4OH
iii) 50 mM (NH4)3PO4 , pH 9
Intensity (x10⁵)
t (min)
O126/1NeuAc
O126/2NeuAc
O126/0NeuAc

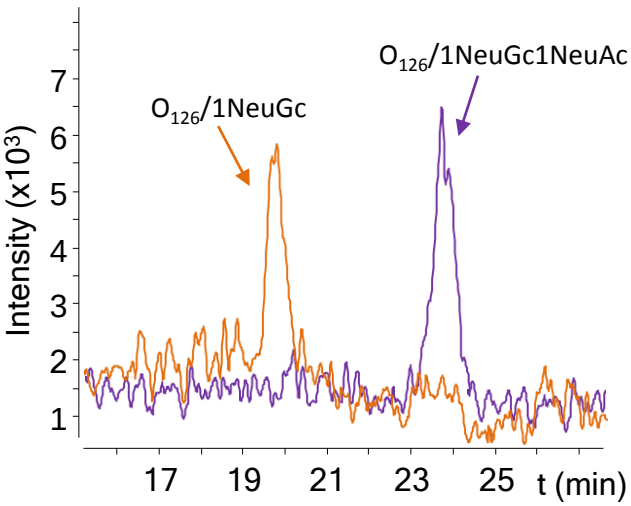
Figure 2

A)


R² = 0,9929
R² = 0,9935
R² = 0,9989
Area (x10⁶)
Concentration (mg·L⁻¹)
O126/0NeuAc
O126/1NeuAc
O126/2NeuAc


R² = 0,9969
R² = 0,9984
Area (x10⁴)
Concentration (mg·L⁻¹)
O126/1NeuGc
O126/1NeuGc1NeuAc

B)


O126/1NeuGc
O126/1NeuGc1NeuAc
Intensity (x10³)
t (min)

C)

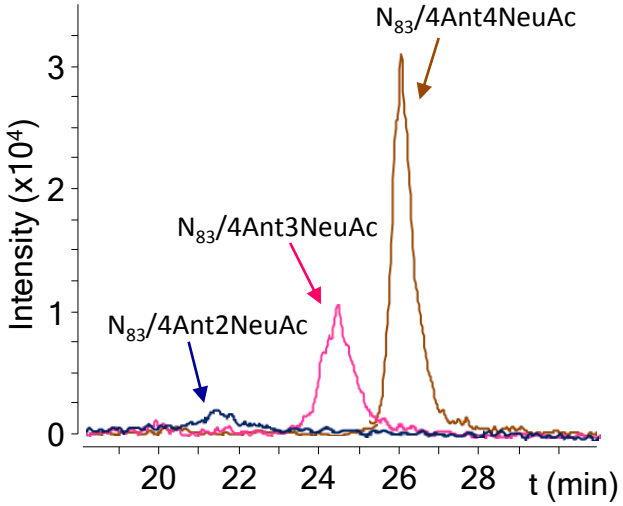

N83/4Ant4NeuAc
N83/4Ant3NeuAc
N83/4Ant2NeuAc
Intensity (x10⁴)
t (min)

Figure 3

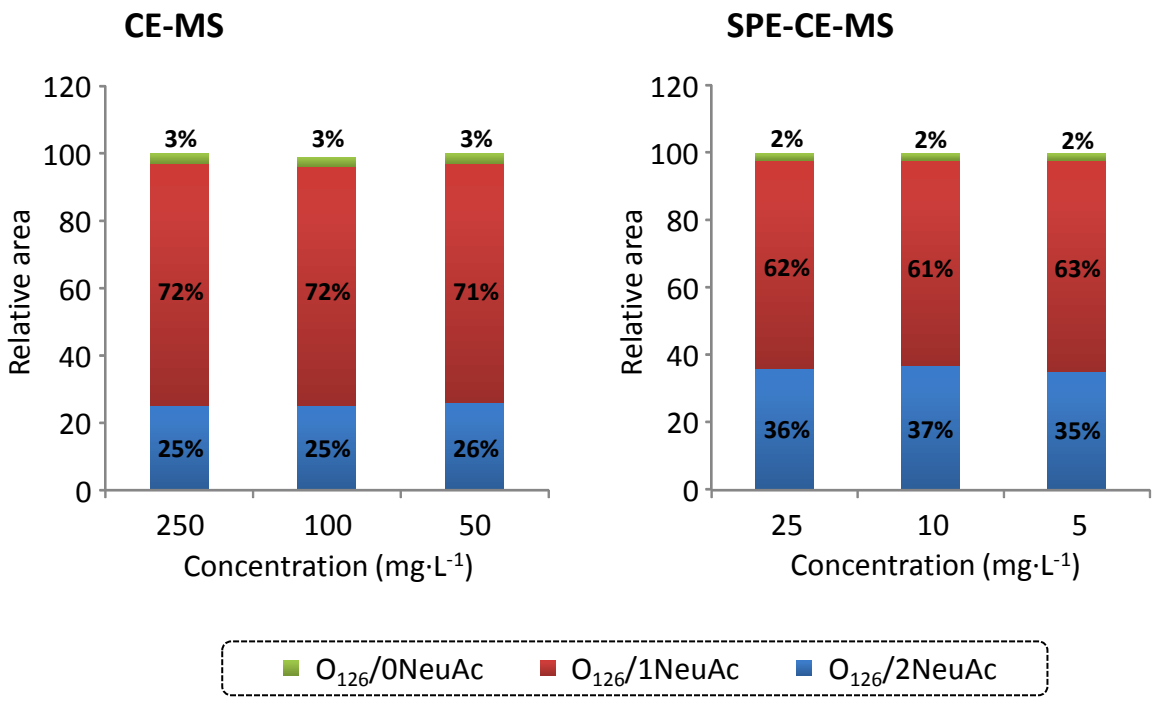
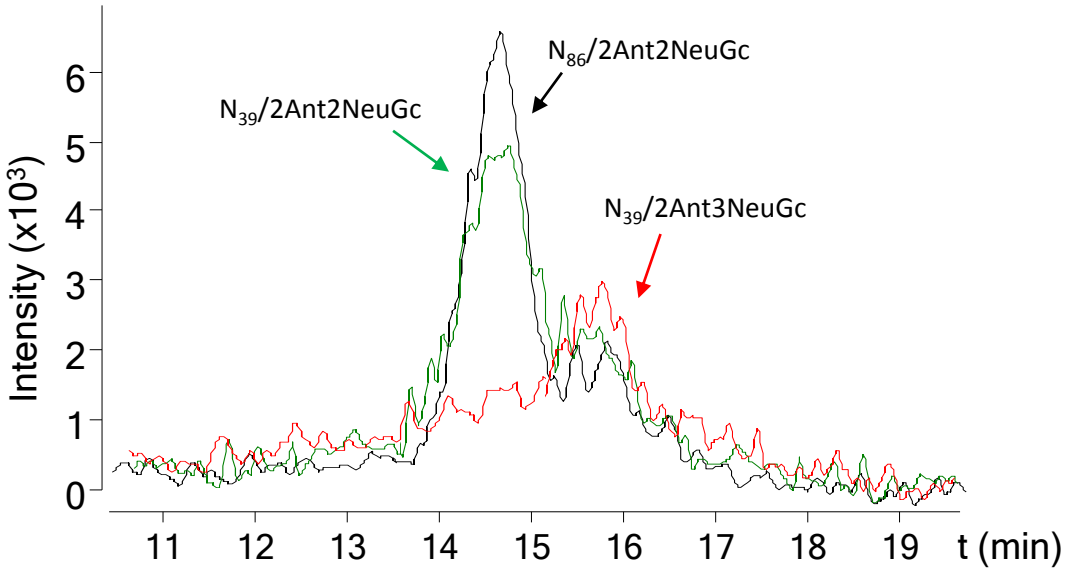

CE-MS
SPE-CE-MS
Relative area
120
100
80
60
40
20
0
3%
72%
25%
3%
72%
25%
3%
71%
26%
250
100
50
Concentration (mg·L-1)
2%
62%
36%
2%
61%
37%
2%
63%
35%
25
10
5
Concentration (mg·L-1)
O126/0NeuAc
O126/1NeuAc
O126/2NeuAc

Figure 4

A) CE-MS (1000 mg·L^{-1})



B) SPE-CE-MS (100 mg·L^{-1})

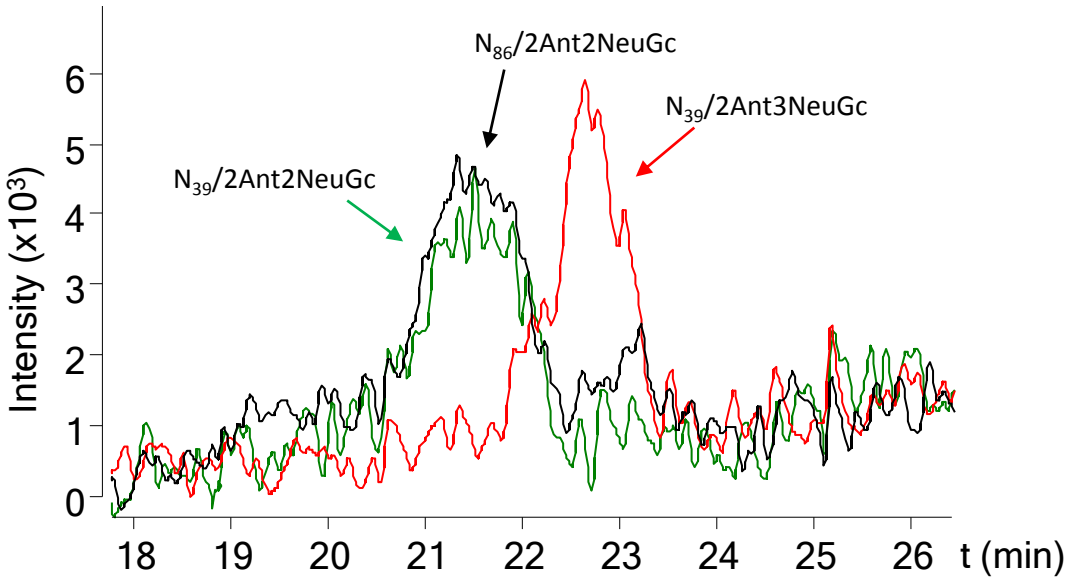


Figure 5