

Investigations of non-enzymatic glycation with new bioanalytical methods

Ph. D. Thesis

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Abbreviations

AC	Amadori compound	MALDI	matrix assisted laser
ACN	acetonitrile		desorption/ionization
AGE	advanced glycation endproduct	MALDI-TOF/MS	matrix assisted laser desorption/ionization
FL	fructosyl lysine		time of flight mass
FL-18	fructosyl lysine after loss of water	MS	spectrometry
HbA1c	hemoglobin A1c	PBS	mass spectrometry
HPLC	high performance liquid chromatography	SPE	phosphate buffers saline
HSA	human serum albumin	TFA	solid phase extraction
		TOF	trifluoroacetic acid
			time of flight

Introduction

Non-enzymatic glycation

Non-enzymatic glycation is one type of post-translational modification of the proteins structure that occurs after the synthesis by translation of the messenger RNA. This reaction is the covalent binding of single reducing sugars (glucose, fructose, ribose, etc.) to primary amino groups in proteins, such as the ϵ -amino group of the protein lysine residue. The initial product is a labile Schiff base intermediate. This type of molecules could be converted to isomeric products called the Amadori compounds (AC). The AC can undergo additional oxidation and rearrangement reactions and eventually dehydration, condensation, fragmentation, oxidation and cyclization reactions to form a series of biologically considerably more reactive constituents, termed advanced glycation end-products (AGEs). Formation of the Schiff base is relatively fast and highly reversible and the formation of the Amadori products from the Schiff base is slower, but much faster than the reverse reaction, so that the glycation product tends to accumulate on proteins.

As a result of the non-enzymatic glycation the AGEs can be formed because of the increased blood glucose level in hyperglycaemia, diabetes mellitus types I and II.

Clinical methods used for the detection of non-enzymatic glycation

Since the 1970s, the measurement of glycated hemoglobin A1c (HbA1c) has been used routinely as a clinical diagnostic marker for relatively long term (4-6 weeks) glucose control in diabetic patients.

The level of the glycated albumin in serum is thought to represent the condition of the blood glucose over the last 2 to 4 weeks, while HbA1c indicates glycaemia state over the last 1 or 2 months. Glycated human serum albumin (HSA) is an important midterm indicator of diabetes that is more sensitive to changes in blood glucose level than HbA1c.

Desalting of proteins and peptides by solid phase extraction (SPE)

Solid phase extraction (SPE) is an easily applicable method most frequently used for desalting, purification, preconcentration and fractionation of a complex mixture of peptides. Fractionation prior to analyses can have a significant effect on sequence coverage of proteins particularly implemented for those peptides obtained from the proteolysis of larger proteins. Further advantages of the SPE method include relatively high recovery rate, short extraction time, high enrichment factor and low consumption of organic solvents.

One common use of the SPE is desalting peptides or DNA oligonucleotides. While these required sample molecules are retained on the sorbents, non-volatile salts are washed off the sorbent with pure water.

Microextraction methods:

Miniaturization of solid phase extraction was a current trend in the last years. Miniaturized SPE tips are packed with a small amount (0.1mg) of reversed phase material or other sorbent, which allows eluting the desired components in small volume of mobile phase. Microscale SPE has been developed for MALDI-TOF/MS.

Boronate affinity chromatography

Boronate affinity columns were first used for the separation of sugars, polysaccharides and nucleic acid components by Weith et al in 1970. The application of the boronate affinity column in proteomics for the separation and quantification of glycohemoglobin was published by Mallia et al. in 1981.

Boric acid and boronic acid form stable esters with polyols and saccharides containing *cis*-diol groups. Up till now this method has been adopted for the separation of *cis*-diol compounds such as carbohydrates, enzymes, glycated proteins and glycated peptides. This method is described as a useful separation technique for the measurement of glycohemoglobin, the study of the glycation pattern of hemoglobin in diabetic patients, the

purification of arginine containing peptides and the separation of glycoproteins and glycopeptides.

Diabetes is an extensive disease, affecting 4% of the whole world population. For this reason, great efforts have been made to optimize the control of the acute complications of the disease (hypoglycaemic coma, ketoacidosis, and infections), but the long-term complications of the diabetes such as macroangiopathy, vasculopathy, immunodeficiency, nephropathy, retinopathy and neuropathy, which still remain widely spread.

Aims

Development in the application of Solid Phase Extraction:

- I. Determination of the binding capacity of C30 and C60(30) fullerene-silica with Leu-enkephalin*
- II. Identification of C18, C30, C60(10), C60(30) and C60(100) on the sequence coverage of the tryptic digests*
- III. Determination of glycosylated sites of Human Serum Albumin (HSA) and fibrinogen with C30 and C60(30)-silica*

Experiments of boronate affinity chromatography for the separation of glycosylated peptides:

- IV. Optimization of different approaches of elution in the case of boronate affinity tips*
- V. Desalting of glycosylated peptides using different sorbents*
- VI. Application of the new method for the detection of glycosylated peptides obtained from digested human serum albumin, collected from patients suffering from type 2 diabetes—compared to that of healthy volunteers*

Materials and methods

In-vitro glycation of HSA and fibrinogen

HSA (Sigma-Aldrich) (5 mg) was dissolved in D-glucose solution (0.167 M in phosphate buffered saline (PBS), pH 7.4). Fibrinogen (Sigma-Aldrich) does not dissolve in water; therefore 2 mg of protein was layered on top of warm (37°C) saline solution (0.9% m/v) consisting of D-glucose at a concentration of 0.167 M. Solutions of both proteins were incubated under aseptic conditions for 28 days at 37°C. Before further modifications and tryptic digestion the glycated HSA and glycated fibrinogen were purified by centrifugation through a membrane (Millipore, cut-off: 3000 Da).

Adsorption of peptides on C30 and C60 silica

After the activation and equilibration, 3 mg of both C30-silica and C60(30)-fullerene-silica were incubated overnight with a solution of Leu-enkephalin (0.1% TFA in water) at different concentrations ranging from 60 to 210 µg/mL under vigorous shaking at 20°C. Then the particles were centrifuged and the supernatants were analysed.

Solid phase extraction (SPE) of the digests

The tryptic digest (20 pmol) from HSA and fibrinogen in 200 µL 0.1% TFA/water were loaded onto the equilibrated particles, and the SPE tubes were washed three times with 1 mL 0.1% TFA/water to remove the non-bound peptides. The peptide pool was fractionated gradually with 5–70% v/v of ACN/water with 0.1% TFA, increasing the ACN concentration of the eluents with 5% in each step. From each eluent 300 µL was used. Fractions were collected, evaporated, redissolved in 5 µL water and analysed by MALDI-TOF/MS.

Optimization of composition of binding buffer and circumstances of elution using amperometric method

In the **first experiment**, an ammonium chloride solution at a concentration of 250 mM was prepared and the pH values of this solution were adjusted by adding ammonia to obtain binding buffers with pH values of 7.4, 7.8, 8.2, 8.6, 9.0, and 9.4. Each solution contained 50 mM magnesium sulphate. After rehydration boronate affinity tips were equilibrated with the binding buffers mentioned above, 0.01 mol D-glucose was dissolved in 10 ml of binding buffer and the solution was aspirated through the affinity gel. This was followed by a washing step using binding buffer to eliminate the

nonbound glucose. Finally, the elution of the bound D-glucose was carried out using 200 μ l of formic acid solution with a pH value of 2.0. In the **next experiment**, the pH was kept at a constant level and the concentration of the ammonium chloride was changed from 50 to 300 mM. Then 0.01 mol D-glucose was again dissolved in solution and aspirated through the gel slab of the tips. Elution of the bound glucose was implemented using 200 μ l of formic acid solution at pH 2.0.

From these experiments, the binding buffer in which the tip possessed the highest binding capacity toward glucose was chosen and the elution was investigated using formic acid from pH 2.0 to pH 5.0. Additional experiments for the optimization of the elution include the use of sorbitol at higher concentrations to remove D-glucose from the boronate affinity tips in the range from 0.1 to 1.6 M.

Isolation and purification of HSA from serum samples obtained from patients suffering from type 2 diabetes mellitus and from healthy volunteers.

Human sera taken from diabetic patients and healthy volunteers were diluted 20 times with double distilled water, and then 2 ml of this solution was centrifuged through a Centricon Ultracel YM-50 centrifugal filter tube (Millipore) at 5000g for 20 min (cutoff: 50,000 Da). Proteins remaining on the filter were further diluted with double distilled water 20 times, and the HSA was separated on a Kovasil MS-C18 non-porous column (Zeochem AG, Uetikon, Switzerland).

The HPLC instrument consists of a Dionex P680 gradient pump, Rheodyne 8125 injection valve, a Dionex UVD 340U UV–Vis detector (Dionex, Germany). Data acquisition was carried out using the Chromeleon software (version: 6.60 SP3 Build 1485).

Enrichment of glycosylated peptides using boronate affinity tips

Pipette tips containing 5 μ l of gel of immobilized m-aminophenylboronic acid with a total volume of 200 μ l (PhyTip 1000+ columns, PhyNexus, San Jose, CA, USA) were used for the enrichment of the glycosylated peptides. After rehydration, the tips were equilibrated with binding buffers. The digest of 100 μ g of protein (for each protein) was dissolved in the binding buffer, and the solution was aspirated through the affinity gel. Then the tips were washed with 1 ml of binding buffer to remove unselectively bound peptides. This was followed by an additional washing step with double distilled water to eliminate the trace of salts presented in the buffer if the elution was carried out using formic acid. Glycosylated peptides were eluted with 4x 50 μ l of formic acid solution at pH 2.0. Elution of the bound glycosylated peptides has also been accomplished using sorbitol at higher concentrations. In the case of elution with sorbitol, prior to MALDI measurement the glycosylated peptides needed to be desalted using different sorbents (C18, ZipTip, C60(30)).

Desalting of glycated peptides eluted from tips with sorbitol

For the desalting of glycated peptides eluted from boronate affinity tips, three different solid phase extraction (SPE) sorbents were compared. Here 5 mg from fullerene-derivatized silica particles made from a silica with a pore diameter of 30 nm (C60(30) silica) and 5 mg C18 silica was packed in SPE cartridges. Elution of the bound peptides was implemented with 300 µl of 80% ACN and 0.1% TFA in water. The eluate was evaporated to dryness, and the peptides were taken up in 5 µl of 0.1% TFA in double distilled water.

The desalting of glycated peptides was also carried out using the commercially available ZipTip (Millipore). In the case of ZipTip, the eluate was deposited directly onto a stainless steel target in 1 µl of 80% ACN and 0.1% TFA in water and then mixed with 1 µl of matrices and analyzed.

MALDI-TOF/MS analysis

Autoflex II MALDI instrument from Bruker Daltonics was used for the mass spectrometric measurements.

All mass spectra were acquired in positive mode with pulsed ionisation ($k = 337$ nm; nitrogen laser, maximum pulse rate: 50 Hz; maximal intensity 20–30% of the laser for peptides). Tryptic peptides were measured in reflectron mode using a delayed extraction of 120 ns and were monoisotopically resolved.

Proteins were measured in linear mode at a delayed extraction of 550 ns. The accelerating voltage was set to +19 kV, the reflectron voltage was set to +20 kV. Spectra of peptides and proteins were the sum of 1000 shots on the same sample spot, external calibration was used. Data processing was executed with Flex Analysis software packages (version: 2.4.). For the in-silico digestion Sequence Editor software (Bruker Daltonics) was used.

Results and discussion

Results of the solid phase (SPE) experiments

Comparison of the phases – recovery study (quantitative evaluation)

To identify the binding capacity of C30 and C60(30) the particles were incubated with a solution of Leu-enkephalin at different concentrations under vigorous shaking at 20°C. Then particles were centrifuged and the supernatants were analyzed. The concentrations of the supernatants represent equilibrium values with the adsorbed Leu-enkephalin. A calibration

curve was plotted by the measurement of standard solutions of Leu-enkephalin ($R^2 = 0.9899$) at 232 nm employing UV–Vis spectrophotometry. The adsorption isotherms can be depicted plotting the concentrations of supernatants (C_{equ} , expressed in $\mu\text{g} \times \text{mL}^{-1}$) against the adsorbed amount (m_{ads} , expressed in μg) (Figure 1/A). Data points fit the theoretical adsorption model of Langmuir with an excellent linear regression ($R^2 = 0.994$ for C60(30) and C30-silica, both) when plotting C_{equ} against $C_{\text{equ}}/m_{\text{ads}}$ (Figure 1/B). The binding capacities were calculated from the reciprocal slope of the lines.

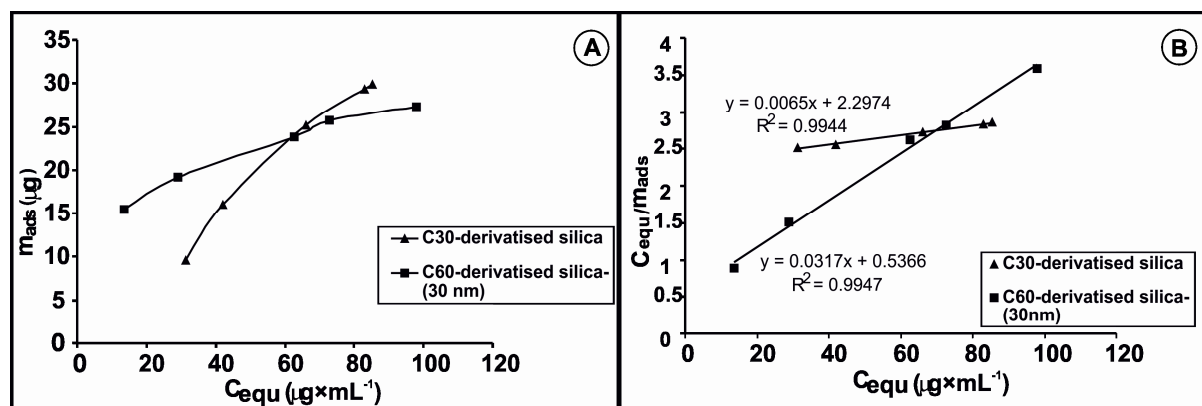


Figure 1. (A) Adsorption isotherms of Leu-enkephalin measured on C30-silica and C60(30) at 25°C. (B) The linearised forms of the isotherms. The reciprocal slope of the lines gives the binding capacity of the phases in saturation.

The binding capacity of C60(30) is $31.5 \text{ mg} \times \text{g}^{-1}$, which confirms a previously published result obtained for phosphopeptides ($33.6 \text{ mg} \times \text{g}^{-1}$). This value is $153.9 \text{ mg} \times \text{g}^{-1}$ for C30-silica showing a five times higher binding capacity.

Comparison of the phases based on the sequence coverage of HSA and fibrinogen digests received after stepwise SPE fractionation

Digests of HSA and fibrinogen were **fractionated on each SPE material**. In the case of fractionation of 20 pmol HSA digest, C18- and C30-silicas provide better coverages than the C60-silica materials in so far as 80.51% of the amino acids of HSA was identified after separating the peptides on C18-silica particles. This was found to be 80.68% on C30-silica, respectively. C60(30) enabled the identification of 70.77% of the amino acids of HSA.

By the fractionation of the digest on C18-silica and C60(100) materials provided very similar results concerning the total coverage of fibrinogen (41.5% vs. 43.4%), however for B β -chain a slightly better result was achieved on C60(100).

While for both C30-silica and C60(30), the sequence coverage results were the same for A α - and B β -chains an outstanding value for c-chain (64.90%) was achieved on C60(30), which raised the total coverage up to 62.15%, and 55,59% on C30.

Identification of the possible glycation sites of HSA and fibrinogen after fractionation –comparison of the results provided by SPE experiments to boronate affinity chromatography

Glycated **HSA** is an excellent marker in diabetes and it can be used as a mid-term index of glycaemic control (approximately 20 days half-life). The role of glycated HSA in the identification of different stages of diabetes is clarified and the possible glycation sites of HSA have been extensively studied with a number of methods.

Fibrinogen may significantly contribute to an increased risk of cardiovascular disease in patients with diabetes both in that they generally have elevated fibrinogen levels and that fibrinogen undergoes non-enzymatic glycation in the presence of uncontrolled blood glucose levels in the diabetic subjects. This glycation can alter the structure and function of the fibrinogen; therefore it is important to gain information about the possible glycation sites of this protein.

In this work the identification of Amadori products (glucose molecules react with free amine groups forming glycosylamine residues through a reversible process, and later the glycosylamine can undergo an Amadori rearrangement to form a stable fructosamine residue) and fructosil-lysine were the object of the investigation.

The all possible single glycated sites of **HSA** obtained on C30-silica and C60(30) were compared with the unfractionated digest. Sixty-nine possible glycated sites were successfully identified. Fifty-nine of these have been described in the literature. Data obtained from SPE experiments made it possible to recognise glycation that could not be detected from the digest. Ten unknown modification sites have been identified: R81, K93, R98, K106, R114, R337, R348, R521, K560, K564

In the sequence of *A α -chain* of **fibrinogen** 72 modifications were found but from the digest only 41 of these sites were identified. Using C30-silica 67, by means of C60(30) 63 glycated residues could be identified in SPE experiments.

Of all the modified sites (59) on the *B β -chain*, 25 were found by analysing the digest, while this number increased with fractionations on C30-silica (53 sites) and C60(30) (47 sites). Forty-one possible glycosylated sites were found for the *c-chain* of fibrinogen. Of these, 24 were recognised from the digest. Results achieved using C30-silica (33) and C60(30) (34) were similar to each other.

Results of the experiments of the boronate affinity chromatography

Evaluation of binding conditions of boronate affinity tips

Electrochemical measurements proved to be a useful approach to optimize the **ionic strength and pH of the binding buffer** as well as the most appropriate circumstances of elutions. The results clearly demonstrate that the affinity tips bind the highest amount of glucose when the pH of the binding buffer varies between 7.7 and 8.2. Further enhancement of the pH of the binding buffer results in a decrease of the bound glucose. The maximal binding capacity of the tips was found to fall within the pH range of 7.8–8.2 in the curve; therefore, a value of approximately 8.2 was selected for this study.

When the binding capacity of the tips was plotted against the ionic strength of the binding buffer, 150 mM ammonium chloride provided the best results.

The effect of the pH of the eluent depicted in the function of the eluted glucose when using solutions of formic acid at different pH values for the elution. This relation is exponential; the lower the **pH of the eluent**, the higher the amount of glucose eluted. The elution of bound glucose on boronate-derivatised resin can also be accomplished by means of a highly concentrated sorbitol solution. The **effects of the concentrations of sorbitol** solution are plotted against the amount of released glucose. The curve reaches a plateau at 1.2 M; therefore, there is no need to employ sorbitol solutions for the elution at higher concentrations.

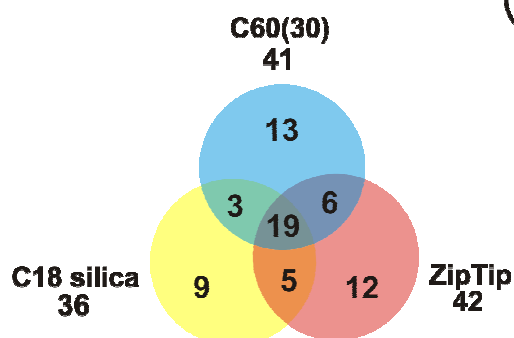
Evaluation of performance of boronate affinity tips toward glycosylated peptides enriched from glycosylated HSA tryptic digests

The application of the boronate affinity tips was introduced employing *in-vitro* glycosylated HSA as model proteins.

Glycosylated HSA underwent tryptic digestion. The optimized conditions, including the ionic strength and pH of the binding buffer and the ways of the different types of elutions, were applied for HSA glycosylated for 28 days. This is why the highest number of glycosylated peptides is expected in this case; therefore, the differences among the applied approaches taken are obviously better expressed. Results received from the experiments carried out with a sorbitol solution at a concentration of 1.2 M were evaluated first. Under optimized conditions, the binding of the glycosylated peptides was carried out using ammonium chloride/ammonia buffer and with taurine buffer using both at the same pH value (8.2) and the same concentration (150 mmol). After the elution of glycosylated peptides of HSA with sorbitol prior to MALDI-TOF analysis, the eluate consequently needed to be **desalted**. This was implemented using three different types of sorbents: the commercially available ZipTip, fullerene(C60)-derivatised silica material (C60(30)) and the one made on the basis of silica with a 30-nm pore radius), and a homemade densely coated C18 silica. The experimental circumstances, including the composition of the binding buffer, the comparison of the **elution using sorbitol and formic acid**, and the **different types of sorbents** employed for desalting the eluates after using a sorbitol solution at a concentration of 1.2 M, were evaluated on the basis of the identified single glycosylated peptides and the number of possible glycosylated sites on HSA glycosylated for 28 days. These can be plotted by means of Venn diagrams. Figure 2/A and B below depict the numbers of *single glycosylated peptides* captured by the boronate affinity tips employing ammonium chloride/ ammonia buffer and taurine buffer, respectively, for the enrichment of glycosylated constituents. In both cases, the bound glycosylated peptides were eluted with a highly concentrated sorbitol solution (1.2 M) and desalted on the three materials mentioned above. As shown in the number of glycosylated peptides bound and eluted from each sorbent, ammonium chloride/ammonia buffer proved to be more efficient than taurine buffer in that 67 single glycosylated peptides could be selectively captured by means of using ammonium chloride/ammonia buffer, whereas 55 peptides modified with only 1 sugar unit were possible to be monitored after the elution from all of the employed phases.

Ammonium chloride/ammonia buffer

Number of single glycosylated peptides



Taurine buffer

Number of single glycosylated peptides

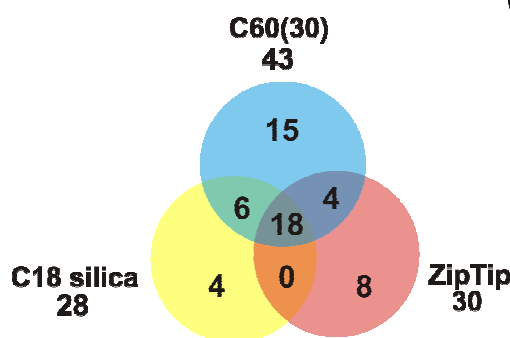


Figure 2: Comparison of different sorbents used for desalting when elution is carried out with sorbitol. (A) Venn diagram showing the numbers of detected single glycosylated peptides when the loading buffer was ammonium chloride/ammonia at optimized circumstances. (B) Venn diagram depicting the numbers of detected single glycosylated peptides in the case of using taurine buffer.

After the desalting step, 13 unique peptides of the total single glycosylated peptide pool adsorbed by the C60(30) particles (from 41 peptides) were possible to acquire using ammonium chloride/ammonia as binding buffer (Figure 2/A). Figure 2/B above shows unique single glycosylated peptides also bound exclusively by C60(30) particles when the enrichment of the glycosylated peptides was carried out using taurine buffer. In total, 43 of the eluted peptides could be identified when desalting them by means of employing C60(30) silica material. Of 43 single glycosylated peptides, 15 were identified only from the eluate of C60(30) and the remaining 28 peptides of the other sorbents could also be detected. It may be important to emphasize that these 15 unique glycosylated peptides must be interpreted only in the context of evaluating the three different sorbents with respect to the number of bound single glycosylated peptides. If the unique peptides detected in the eluates of C60(30) SPE material after the enrichment with the two above-mentioned buffer systems are compared, it can be concluded that their numbers were very similar to each other so long as 13 of them were found when enriching them with an ammonium chloride/ammonia buffer and 15 glycosylated peptides were found when the binding buffer was composed of taurine. Only 5 single glycosylated peptides with the same sequence and modifications could be analyzed from the unique peptides enriched with the two buffers and desorbed from C60(30). The majority of the unique peptides were found to be different despite the fact that they had been eluted from the same sorbent. This

observation reveals that the use of different buffer systems alters the selectivity of the boronate phase insofar as the captured peptide profile and the identified potential glycosylated sites show great variety.

Using ammonium chloride/ammonia buffer for the enrichment of glycosylated constituents with boronate affinity tips, as described earlier in this study, 41 single glycosylated peptides were bound by C60(30) silica particles. In addition, 13 single glycosylated unique peptides were explored in the eluted peptide pool. Single glycosylated peptides involve 8 possible glycosylated sites, namely K106, R114, R257, R348, K372, K436, K439, and K466.

These results were compared with those received after the enrichment of glycosylated peptides using taurine buffer. Among those peptides released from the affinity tips and desalted from the sorbitol solution on C60(30) particles, 15 single glycosylated unique peptides were analyzed. Single glycosylated peptides made possible the identification of 9 unique glycosylated sites. In single glycosylated unique peptides K190, K372, K413, K444, R445, K466, K536, K538, and K541, unique glycosylated sites were localized.

ZipTip is a commercially available product, probably the most frequently and efficiently used tool for desalting the solutions of proteins and peptides prior to mass spectrometric measurements. When binding peptides to affinity tips with ammonium chloride/ammonia buffer, the desalting procedure carried out with *ZipTip* allowed the identification of 12 single glycosylated unique peptides modified at the residues of K93, R98, K402, and K524. Thus, in terms of the number of unique glycosylated sites localized in single unique glycosylated peptides, *ZipTip* does not seem to be as efficient as C60(30); however, it must be noted that 42 single glycosylated peptides were desalted by means of *ZipTip*, and 58 possible glycosylated sites were monitored.

Concerning **taurine binding buffer**, for single glycosylated peptides, *ZipTip* provided a worse result than C60(30) so long as only 30 peptides were bound; of these, 8 were found to be unique. Taking into account the glycosylated residues detected in peptides bound on *ZipTip*, 54 possible glycosylated sites were found, in contrast to C60(30), where this number was slightly higher (61). Six unique glycosylated sites at the positions of K402, K432, K313, K317, K500, and R485 could be observed.

Finally, the desalting of the glycosylated peptides was also tried with **octadecyl silica** with high surface coverage. In the case of ammonium chloride/ammonia buffer 36 single glycosylated peptides were successfully bound, 9 of which were unique peptides, modified at K199, K317, K276, K564, and K573 as well. The poor performance of this material toward hydrophilic glycosylated peptides is also expressed in the number of unique peptides found, that is, only 6

with 4 possible unique glycosylated sites at R98, K413, K414 and R428. When using taurine buffer, the results of the desalting on C18 particles were even worse. In total, 28 single glycosylated peptides of the 4 unique peptides analyzed were bound; none of them comprised any unique glycosylated sites.

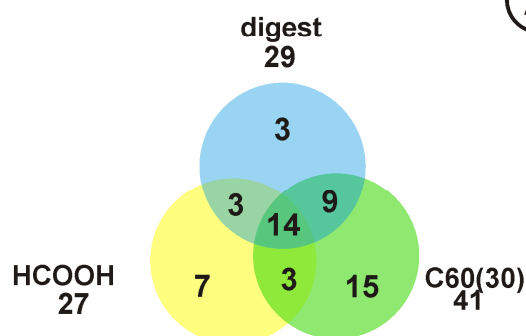
Elution of the bound constituents is frequently implemented in acidic media. A **formic acid** solution of pH 2.0 proved to be the best in terms of the amount of eluted D-glucose evidenced by amperometric measurements. Elution with this solution was compared to elution implemented with sorbitol (desalted by C60(30)) regarding the number of eluted single glycosylated peptides and the possible identified glycosylated sites as well. These two ways of elution were compared with the digest of HSA incubated with D-glucose for 28 days to assess the efficiency of the enrichment. Surprisingly, the elution with formic acid yielded the worst results in terms of the number of identified glycosylated peptides and the possible sites of glycosylations.

Figure 3/A below shows that in 100 pmol of the digest of overglycosylated HSA *without enrichment*, 29 single glycosylated peptides were recognized and 3 were found to be unique peptides with 5 unique glycosylated sites. When binding glycosylated peptides with *ammonium chloride/ ammonia buffer*, the elution of single glycosylated peptides with *formic acid* resulted in the release of 27 single glycosylated peptides from the boronate affinity tips; of these, 7 were unique peptides with 4 possible sites of modification. As mentioned earlier, the *elution with sorbitol* yielded the detection of 41 single glycosylated peptides; nevertheless, the eluate was subjected to SPE on C60(30) particles prior to MALDI-TOF analysis. However, in this context of the comparison, 15 unique peptides comprising 12 unique residues were explored. As also demonstrated in Figure 3/B above, formic acid made possible the release of only 11 peptides. Elution with sorbitol again showed the best results after desalting. This was expressed by 24 identified peptides; of these, 14 were observed to be unique constituents containing 16 unique glycosylated sites.

Ammonium chloride/ammonia buffer

Number of single glyated peptides

(A)



Taurine buffer

Number of single glyated peptides

(B)

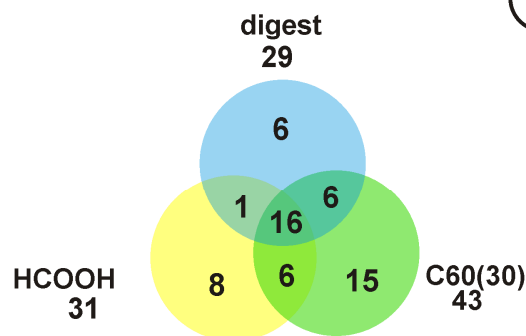


Figure 3: Comparison of elutions of glyated peptides from affinity tips with digest of HSA glyated for 28 days with the numbers of detected glyated peptides indicated. (A) Venn diagram showing the numbers of single glyated peptides when using ammonium chloride/ammonia binding buffer. (B) Venn diagram of the single glyated peptides released from the tips in the case of employing taurine binding buffer

Figure 3/B above show those cases where glyated peptides were bound to the affinity tips in the presence of **taurine buffer**. Briefly, from the digest, 29 single glyated peptides were detected; of these, 6 were unique and each had 3 unique residues. Eluting the peptides with *formic acid* solution allowed the recognition of 31 single glyated peptides; of these, 8 were unique with 4 possible unique sites of glycation. When eluting the peptides with *sorbitol*, 43 single glyated peptides could be detected; of these, 15 were unique and incorporated 8 unique glyated sites. As demonstrated above, with the elution of the bound glyated peptides with formic acid solution, it seems that no enrichment of the modified constituents occurred in spite of the fact that the highest amount of glucose could be measured at pH 2.0. Therefore, the acidic elution is not recommended by Takátsy et al..

The most efficient way to gain information about the modified glyated sites of a protein by MALDI-MS is to bind glyated peptides from the digest of the protein using, for instance, ammonium chloride/ammonia binding buffer under optimized circumstances, and then the selectively bound constituents are eluted with a concentrated solution of a proper sugar such as sorbitol. Furthermore, prior to analysis, the presence of undesirable sugar can be eliminated using an effective sorbent for desalting. As was demonstrated, C60(30) proved to be as efficient as ZipTip; no significant differences could be observed in the binding efficacy of either phase toward glyated peptides.

Application of boronate affinity tips for selective enrichment of Amadori products from digest of HSA collected from sera of patients suffering from type 2 diabetes mellitus and healthy volunteers

Four healthy volunteers and four patients suffering from type 2 diabetes mellitus were chosen to test the method described above for the mapping of the level of glycation of HSA. Patients were selected on the basis of the level of HbA1c and fasting plasma glucose. For healthy volunteers, besides some important clinical parameters, the fasting plasma glucose was always less than $6.1 \text{ mmol} \times \text{L}^{-1}$. These values were by far the highest in patients 1 and 2 (12.17 and $13.02 \text{ mmol} \times \text{L}^{-1}$, respectively). For patients 3 and 4, slightly lower values were obtained (8.53 and $7.50 \text{ mmol} \times \text{L}^{-1}$, respectively).

For all of the investigated patients, HbA1c was considered to be rather high (10.30 , 11.50 , 11.82 , and 11.30% as measured for patients 1, 2, 3, and 4, respectively)

These two series of data served for the estimation of the glycaemic state of patients. HSA reflects the amount of blood glucose more rapidly than HbA1c; moreover, accounting for its relatively short (17 days) biological half-life, the glycation level of HSA is relevant to the condition of blood glucose over 2 weeks preceding the test.

Approximately $300 \mu\text{g}$ of the digest of HSA collected from each patient and healthy volunteer was enriched under optimized conditions using boronate affinity tips. The bound constituents were released from the tips by means of a 1.2 M sorbitol solution. The eluate was then desalted on C60(30) and preconcentrated. Peptides remaining after this procedure were measured by MALDI–TOF/MS.

Besides the privileged glycation sites of K12, K233, and K525 being present in each individual, many other glycated K and R residues described previously were recognized. Regarding the numbers of glycated peptides and the possible identified glycated residues with the exception of patient 4 (whose fasting plasma glucose was the lowest), the clinical parameters can be associated with the glycation pattern obtained from mass spectrometric data.

Glycated peptides with a mass shift of 144.042 were explored. They can be either FL (Amadori product) after a loss of water or tetrahydropyrimidine bound directly to arginine residues. Results also indicate that peptides appearing at m/z 1077.519 and 1783.938 are worth considering. The latter site was observed in all of the investigated digests belonging to the patients but was utterly absent from samples taken from healthy individuals. The presence

of a modification at m/z 144.042 was further corroborated by the corresponding PSD spectrum, where the presence of a neutral loss was detected at m/z 1639.896, showing that the location of the modification can be at either K414 or R428. Similarly, the peptide at m/z 1077.519 could be detected in only three diabetic patients. This modification located at R81 is considered to be a tetrahydropyrimidine attached, as expected, to arginine.

More patients and control individuals need to be involved to make a decision as to whether these 2 peptides with the possible modifications can serve as potential biomarkers in diabetes mellitus. The method elaborated and reported here seems to be a powerful tool for the investigation of non-enzymatic glycation.

New results reported in the dissertation

I. Determination of the binding capacity of C30 and C60(30) fullerene-silica with Leu-enkephalin

After the activation and equilibration, C30-silica and C60(30) were incubated overnight with a solution of Leu-enkephalin at different concentrations. The peptide concentrations of the supernatants represent equilibrium values with the adsorbed Leu-enkephalin. The adsorption isotherms can be depicted plotting the concentrations of supernatants (C_{equ}) against the adsorbed amount. Data points fit the theoretical adsorption model of Langmuir with an excellent linear regression when plotting C_{equ} against $C_{\text{equ}}/m_{\text{ads}}$. The **binding capacity of C60(30)** is 31.5 mg/g, which is confirmed by a previously published result. In the case of **C30-silica** the value is 153.9 mg/g showing a five times higher binding capacity.

II. Identification of solid phases (C18, C30, C60(10), C60(30), C60(100)) on the sequence coverage of tryptic digest of HSA and fibrinogen

Twenty pmol HSA digest was fractionated on each stationary phase: C18, C30, C60(10), C60(30), C60(100). The C18- and C30-silicas provided better sequence coverages (approximately 80%) than the C60-silicas.

In the case of fractionation of 20 pmol fibrinogen the **C30 and C60(30)** materials yielded nearly the same results for A α - and B β -chains of fibrinogen, but there was a remarkable increment for c-chain (64.9%) on C60(30) that means the total sequence coverage reached the best result 62.15%. The hydrophobicity and the length of the peptide do play an important part since those peptides have better access to the alkyl moieties and therefore generated interactions stronger than to the spherical C60 molecules. Due to the highest percentage of the aromatic amino acids in the c-chain of fibrinogen the π - π interactions between the peptides and the fullerene play more important role than in the case of A α - and B β -chains.

III. Determination of glycosylated sites of HSA and fibrinogen with C30 and C60(30)-silica

Digest of HSA glycosylated for 28 days were fractionated on C60(30) and C30 solid phase particles, the results were compared with unfractionated glycosylated HSA digest and boronate affinity. **Sixty-nine possible glycosylated sites** of HSA could be identified altogether, 10 of them have **never been described** in the literature before. Data provided from SPE experiments made it possible to recognise glycosylations that could not be detected from the unfractionated digest.

In the case of fractionation of glycosylated fibrinogen digest, the result cannot be compared with other data in the literature, since it has not been reported before. In the sequence of A α -chain 72 modifications were found, using C30-silica 67, by means of C60(30) 63 glycosylated residues could be identified in SPE experiments. Of all the modified sites in B β -chain there were **59**, with fractionations on C30-silica nearly the same number **53 sites**, and on C60(30) **47 sites**. Forty-one possible glycosylated sites were found for the c-chain of fibrinogen. Results achieved using C30-silica (33 sites) and C60(30) (34 sites) were similar to each other. For instance, in c-chain glycosylation on R8, R35, R123, R246 and R421 residues were only possible to be explored employing C60(30). The **possible glycosylated sites of fibrinogen were described first** by Böddi et. al.

In the SPE experiments of glycosylated proteins C30-silica is a competitive candidate with regard to C60(30) concerning both the number of glycosylated sites and the number of bound peptides. The selectivity of these materials differs; C30-silica adsorbs larger peptides, while C60(30) possesses excellent binding capacity towards hydrophilic, arginine-rich small peptides. The

findings may lie in the basis of a feasible SPE-off-line-MALDI method to be used for the investigation of polar constituents of complex biological samples.

IV. Optimization of different approaches of elutions in the case of boronate affinity tips

Miniaturized boronate affinity chromatography was found to be the optimal method for the selective enrichment of non-enzimatically modified peptides. The proper binding condition is known (**150 mM ammonium chloride/ammonia buffer, pH 8.2**) but the appropriate circumstances of elution have never been optimized before. Data obtained from electrochemical measurements allowed us to find the optimal elution procedure of the bound glucose. It could be carried out employing either acidic conditions or a highly concentrated solution of sorbitol. Elution carried out using a **sorbitol solution** at a concentration of **1.2 M** was found to be more efficient than elution with formic acid at pH 2.0, although the more acidic solution was used; the more efficient the acidic media for the elution was. The solution of formic acid allowed us to identify 27 single glycosylated peptides, as long as elution with sorbitol has given 41 single glycosylated peptides.

V. Desalting of glycosylated peptides using different sorbents

After the elution of glycosylated peptides with sorbitol prior to MALDI-TOF analysis, the eluate consequently needed to be desalted. This was implemented using three different types of sorbents: the commercially available ZipTip, C60(30) and a homemade C18 silica. The eluates after using a sorbitol solution at a concentration of 1.2 M, were evaluated on the basis of the identified single glycosylated peptides and the number of possible glycosylated sites on HSA glycosylated for 28 days.

Ammonium chloride/ammonia buffer has proven to be the best binding buffer for selective enrichment of glycosylated peptides by boronate affinity chromatography. As the Venn diagrams show, 41 single glycosylated peptides were adsorbed with ammonium chloride/ammonia buffer by C60(30), of these 13 were unique with 8 possible glycosylated sites. While using ZipTip, 42 single glycosylated peptides can be found, 12 were unique with 4 unique glycosylation sites.

Regarding the numbers of single glycated peptides and the possible modified residues, **C60(30)** was considered as a promising sorbent with respect to its performance toward glycated constituents. Moreover, its efficacy is competitive with commercial that of **ZipTip**. The application of C18 did not meet the requirements concerning the number of glycated peptides bound during the desalting procedure.

VI. Application of the new method for the detection of glycated peptides achieved from digested human serum albumin, collected from patients suffering from types 2 diabetes – compared to healthy volunteers

The applicability of the method was demonstrated for the digest of HSA isolated from healthy volunteers and diabetic patients. However, both the control and the diabetic groups included only four individuals, **more glycated peptides**, and consequently **more glycated sites** were recognized for patients suffering from type 2 diabetes mellitus having been under insufficient diabetic control when being monitored FL (Amadori product).

The privileged glycation sites of K12, K233, and K525 were present in each individual. Results also indicate that peptides appearing at m/z 1077.519 and 1783.938 are worth considering, although peptide at m/z 1077.519 could be detected in only three diabetic patients. This modification, which located at R81, is considered to be an attached tetrahydropyrimidine.

When detecting FL after a loss of water (FL-18), a distinctive peptide appearing at **m/z 1783.9** was observed in each diabetic patient but was not detected in healthy control individuals. This peptide is located in the region of [414–428] of HSA and incorporates K414 and R428. This peptide and its glycated sites may serve as a **potential biomarker for diabetes**; however, further research is required to confirm this observation.

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