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Study on the loss of nucleoside mono-, di- and triphosphates and phosphorylated peptides to a metal-free LC–MS hardware

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ABSTRACT

In our earlier LC–MS experiments on the analysis of phosphocompounds like nucleotides (mono-, di- and triphosphate) and phosphopeptides and in literature, low sensitivity and severe losses of analyte to the instrumental setup were observed. Since we noticed that the stainless steel parts of the setup (e.g., the electrospray needle) adsorbed important quantities of the phosphorylated analytes, we made a LC–ESI setup without metallic surfaces. The first results were disappointing since also the fused silica surface of the LC-ESI coupler adsorbed part of the nucleotides and phosphopeptides injected in flow analysis experiments. We present experiments documenting the contribution of the different components of the setup. A number of potential solutions to the adsorption problem are proposed and tested. Only dimethyldichlorosilane deactivation of fused silica capillaries gave satisfactory results as adsorption of nucleotides and phosphopeptide was minimised.

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1. Introduction

1.1. Importance of phosphorylated compounds

Phosphorylated organic compounds such as (oligo-) nucleotides and phosphoproteins represent an important class of biologically active compounds. Nucleotides are the building blocks of our genome and nucleoside triphosphates (e.g., ATP) are the main universal energy carriers of cells. Several nucleoside and nucleotide analogues are often used as pro-drugs in the treatment of various viral infections and cancer [1–3]. In most cases, their mode of action includes metabolic phosphorylation [4]. (Cyclic-)nucleotides are chemical carriers of signals within the cell, signals that are transduced by (de-)phosphorylation of target proteins. Phosphorylation of proteins is a ubiquitous posttranslational modification that dramatically influences structure, activity, subcellular location and degradation of the protein [5]. The research area connected to this – phosphoproteomics – is an increasingly important research domain [6].

These phosphorylated biomolecules (nucleotides, nucleotide analogues and phosphorylated peptides) are intensively studied by means of different chromatographic methods such as reversed phase [7], ion-pairing [8–10], ion exchange [11,12] and affinity chromatography [13–16] for both concentration and separation. These analytical chromatographic techniques are often coupled to tandem mass spectrometry for identification and/or structural elucidation of the compounds.

1.2. Analytical challenge

Although comprehensive studies have been published recently [17,18] the chromatographic and mass spectrometric analysis of phosphorylated organic compounds has never been straightforward (e.g., Piggee [19] and references therein). Due to their high polarity, multiply phosphorylated peptides and nucleotides show only limited retention in reversed phase LC–MS analysis [20,21]. Moreover, because of their lower isoelectric point resulting from the low pK_a's of the phosphate moiety they are inherently more difficult to detect in positive ion mode mass spectrometry. This is especially unfavourable for phosphopeptide analysis since positive ion mode tandem MS is the method of choice for peptide sequencing and subsequent protein identification.

A major technical challenge that needs to be tackled when dealing with (multiply) phosphorylated compounds, is the fact that they interact strongly with specific parts of an LC–MS setup [22]. This phenomenon is of critical impact when dealing with low con-

Abbreviations: AMP, 5′-adenosine monophosphate; ADP, 5′-adenosine diphosphate; ATP, 5′-adenosine triphosphate; LC, liquid chromatography; MS, mass spectrometry; ICP, inductively coupled plasma; PEEK, polyether ether ketone; PAEK, olyaryl ether ketone; EDTA, 2,2′,2″,2‴-(ethane-1,2-diyldinitrilo)tetraacetic acid; PA, phosphoric acid; ID, internal diameter; LOD, limit of detection; DMDCS, dimethyldichlorosilane.

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centrations of phosphorylated analyte. Especially stainless steel parts show high affinity for phosphorylated molecules. This problem becomes even more apparent when these metal surfaces get corroded over time, a process that is known to happen in stainless steel emitters [23].

1.3. New approach and new problem

To avoid undesirable adsorption of phosphorylated compounds and consequent sample loss, a new capillary LC–MS setup was designed, free of metal surfaces that are in contact with the analyzed samples. The standard electrospray probe was replaced by electrospray chip technology [24].

Yet the problem of adsorption of multiply phosphorylated organic compounds at low pH could not be overcome by this approach and the main site of adsorption in this case could be traced back to the fused silica tubing used in the setup. Silanol moieties on silica surfaces are notorious in liquid chromatography for their capacity to adsorb and cause tailing of organic bases [25]. In order to minimise this problem, different strategies have been developed which included modifying the free silanol groups of the silica by endcapping. Though even then some silanol moieties remain available [26]. Castillo and colleagues also reported unexpected secondary interactions between hydroxyl containing analytes and fused silica capillaries in an LC-ICP–MS approach [27]. Little information is found in the literature about the specificity and pH dependent interaction of silanols with phosphorylated organic compounds [28,29].

With the current tendency towards analysis of minute amounts of highly complex samples, these adsorption phenomena become a tangible concern. In high throughput experiments that produce vast amounts of data, processes like the adsorption of phosphorylated compounds could easily be overlooked, resulting in the underrepresentation of entire classes of these important biomolecules. Moreover, when accurate quantification of compounds is desired, loss and carryover of any compounds on parts of the analytical setup becomes an issue.

2. Experimental

2.1. Chemicals

Raffinose was obtained from Sigma-Aldrich (Bornem, Belgium). Nucleotides (AMP, ADP and ATP) were purchased as sodium salts from Sigma-Aldrich as well and were of the highest grade available. The phosphopeptide control set consisted of a monophosphopeptide and a tetraphosphopeptide from β-casein. This phosphopeptide control set and the non-phosphorylated (Glu[1])fibrinopeptide B were also obtained from Sigma-Aldrich, as was the EDTA (Na^+-salt). Water (H_2O) and acetonitrile (ACN) were from HPLC grade quality and obtained from Acros (Geel, Belgium). n-Hexane 95% was obtained from Acros as well. Ammonium acetate (NH_4OAc, p.a.) was from Janssen Chimica (Geel, Belgium). Acetic acid (HOAc, p.a.) and ammonium hydroxide (NH_4OH, 25%, p.a.) were purchased from Merck (Overijse, Belgium). Methanol (MeOH; LC–MS grade) came from Biosolve (Valkenswaard, The Netherlands). Phosphoric acid (H_3PO_4; 85%) was obtained from Chem-lab (Zedelgem, Belgium). The dimethyldichlorosilane (DMDCS) was purchased from Fluka (Bornem, Belgium).

Stock solutions of all samples were prepared in HPLC grade water at stock concentrations of 10^{-3} or 10^{-4} M and stored at −20 °C until use. Just before analysis, samples were diluted to working concentrations of 10^{-5} M for each compound. EDTA was dissolved in 50% MeOH at a concentration of 50 mM and stored at −20 °C until use.

Two sets of test mixtures were prepared. The nucleotide test mixture consisted of equal concentrations of raffinose, 5′-AMP, 5′-ADP and 5′-ATP. The peptide test mixture consisted of fibrinopeptide B (EGVNDNEEGFFSAR) as an unphosphorylated reference peptide, a monophosphorylated (FQpSEEQQQTEDELQDK) and tetraphosphorylated (RELEELNVPGEIVEpSLpSpSpSEESITR) peptide obtained from bovine β-casein.

The mobile phases used throughout this study were A: 0.1% (v/v) HOAc (50:50 H_2O/MeOH), B: 100% ACN and C: 25:25:50 NH_4OH/H_2O/MeOH.

2.2. Instruments

For all experiments, a capillary ternary gradient pump with integrated injector was used (CapLC, Waters, Manchester, UK). The mobile phases were delivered at a flow rate of 8 μL/min unless stated otherwise. Samples were injected trough a 10 μL PEEK loop (Upchurch scientific, Oak Harbor, WA, USA) mounted on the 6 port valve type injector (Valco Instruments, Houston, TX, USA), modified with a PAEK rotor (C2-13R6) and PEEK stator (C-1C46) to omit contact of the sample with stainless steel surfaces (standard stator).

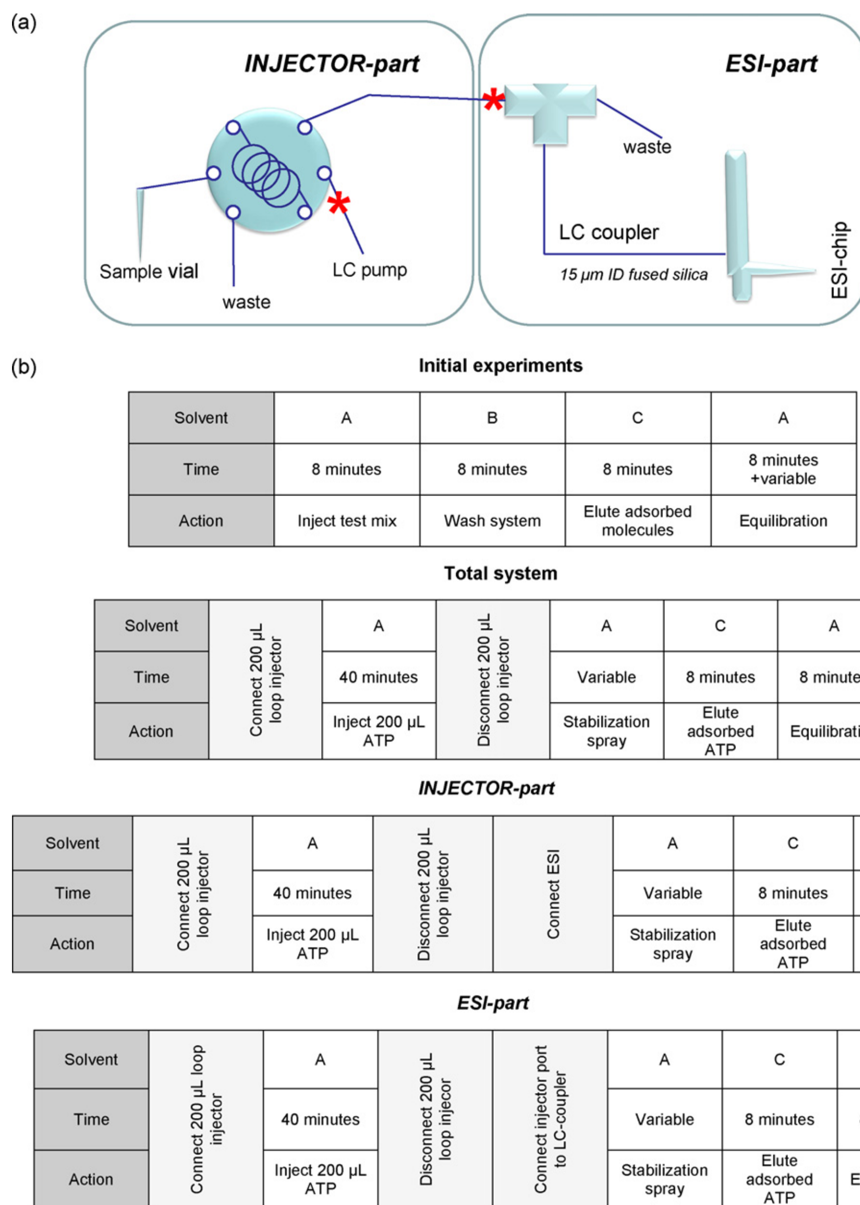
The injector was connected to the modified PEEK splitting tee (Upchurch scientific) of the Nanomate via PEEK tubing (360 μm OD, 50 μm ID, Upchurch scientific). The mobile phase was split in order to obtain an analytical flow of approximately 500 nL/min through the 30 cm long, 15 μm ID fused silica LC-coupler of the Nanomate ESI-source. As needed, an auxiliary injector with a 200 μL PEEK loop was used. A scheme of the LC setup is outlined in Fig. 1a.

Mass spectra were recorded on a Q-TOF 2 mass spectrometer (Waters) used in negative (−) or positive (+) ion mode. The Waters ESI-source was replaced by a Nanomate ESI-chip system (Advion biosystems, Ithaca, NY, USA). The ESI-chip consists of an integrated 20 × 20 array of nanoelectrospray nozzles that are etched from a planar silicon wafer. The electrospray was initiated by applying −1.75 to −1.9 kV (negative ion mode) or 1.7 kV (positive ion mode) to the polymeric coupler on the backside of the chip. Both ion modes gave the same adsorption results. Operation of the nanospray proved to be more stable in positive ion mode whereas negative ion mode yields better sensitivity for the nucleotides. Voltage and chip position were optimised using the Chipsoft 7.1.1 software (Advion Biosystems). For all nucleotide experiments, a mass range of m/z 50–750 was chosen and a cone voltage of 30 V was used. In case of the (phosphorylated) peptides, the mass range was m/z 300–2000 with a 40 V cone voltage. A scan time of 1 s was always used.

2.3. LC conditions

The adsorption of nucleotides and phosphopeptides to the LC-ESI setup was examined using the same sequence as used earlier [20]. The 10 μL sample was injected in solvent A at a flow rate of 8 μL/min, the setup washed with solvent B and any adsorbed compound eluted with solvent C (see Fig. 1b). Finally the system was reset to initial conditions (solvent A) for the next sample injection.

To assess which components of the LC hardware adsorbed the injected phosphorylated compounds, the LC setup was divided into two parts: the *INJECTOR (INJ)-part* on one hand, which included the injector, sample loop, and PEEK tubing. On the other hand the *ESI-part*, which comprised the PEEK splitting tee, the LC-coupler and the ESI-chip (Fig. 1a). To test the different parts separately, they were disconnected and a PEEK loop of 200 μL (750 μm ID) was placed upstream of the part to be tested (connection point is marked with * in Fig. 1a). 200 μL 10^{-5} M of the nucleotide mixture was injected at a flow rate of 8 μL/min and the part was rinsed in solvent A afterwards. Then both parts were reconnected and rinsed for 10 more minutes before switching to alkaline solvent C for elution. During separate experiments to test the capacity of the complete setup,



Solvent	A	B	C	A
Time	8 minutes	8 minutes	8 minutes	8 minutes +variable
Action	Inject test mix	Wash system	Elute adsorbed molecules	Equilibration

Solvent	Connect 200 µL loop injector	A	Disconnect 200 µL loop injector	A	C	A
Time		40 minutes		Variable	8 minutes	8 minutes
Action		Inject 200 µL ATP		Stabilization spray	Elute adsorbed ATP	Equilibration

Solvent	Connect 200 µL loop injector	A	Disconnect 200 µL loop injector	Connect ESI	A	C	A
Time		40 minutes			Variable	8 minutes	8 minutes
Action		Inject 200 µL ATP			Stabilization spray	Elute adsorbed ATP	Equilibration

Solvent	Connect 200 µL loop injector	A	Disconnect 200 µL loop injecor	Connect injector port to LC-coupler	A	C	A
Time		40 minutes			Variable	8 minutes	8 minutes
Action		Inject 200 µL ATP			Stabilization spray	Elute adsorbed ATP	Equilibration

Fig. 1. (a) A schematic drawing of the LC-ESI setup. To investigate where the site of adsorption was located, the setup was split into two parts: the *INJECTOR-part* and the *ESI-part* and these parts were assessed separately by means of a 200 µL injection of the nucleotide mixture from an auxiliary injector. The * marks the connection points for the auxiliary 200 µL injection loop. (b) A timing scheme of the different experiments. Timing of the experiments investigating the adsorption of phosphocompounds on the LC–MS setup. From top to bottom: initial experiments (cfr. Tuytten et al. [22]), total system, *INJECTOR-part* and *ESI-part*.

both parts were connected and the 200 µL loop was placed before the injector. After injection of the 10^{-5} M nucleotide mixture, the setup was rinsed in solvent A and nucleotides could be eluted by switching to solvent C. The exact timing of these experiments is depicted in Fig. 1b.

For the EDTA experiments, a 10 µL plug of 50 mM EDTA was injected on the LC setup. After rinsing for 30 min in solvent A, the nucleotide mixture was injected and analyzed as described above. These experiments were repeated with 0.085% and 0.425% phosphoric acid as the initial plug.

2.4. *Treatment of fused silica*

2.4.1. *Etching*

Fused silica capillaries (50 µm ID) were subjected to 10% (w/v) HF that was pulled through the capillary by a vacuum pump operating at 80 Torr for 10 min, followed by rinsing with 0.1 M HCl for 2 min and then another round of etching for 10 min. Finally, the treated capillaries were washed in 0.1 M HCl and water for 5 min each.

2.4.2. *DMDCS deactivation*

Before derivatization fused silica capillaries (15 µm ID) were conditioned in methanol for 30 min followed by hexane for 30 min. All solutions were "pushed" through the capillary using a pressure bomb at 50 bar containing a vial with the desired solvent. The capillary is mounted through the pressurised vessel lid by a Swagelock connection. The capillaries were then derivatized by flushing with DMDCS solution for 60 min. Afterwards, they were flushed for 60 min, first in hexane and then in methanol and dried overnight at 65 °C in a GC oven (Hewlett-Packard, series II 5890, Palo Alto, CA, USA).

Table 1
Constituents and relevant m/z values for the test mixtures.

Name	Calculated mass	Negative ion mode $[M-H]^-$	Positive ion mode $[M+H]^+$
Raffinose	504.17	503.16	505.18
Nucleotide mixture			
AMP	347.06	346.05	348.07
ADP	427.03	426.02	428.04
ATP	507.00	505.99	508.01
Phosphopeptide mixture		$[M-2H]^{2-}$	$[M+2H]^{2+}$
Glu-fib	1569.70	783.85	785.86
Mppept	2060.82	1029.40	1031.42
		$[M-3H]^{3-}$	$[M+3H]^{3+}$
Tppept	3121.26	1039.41	1041.43

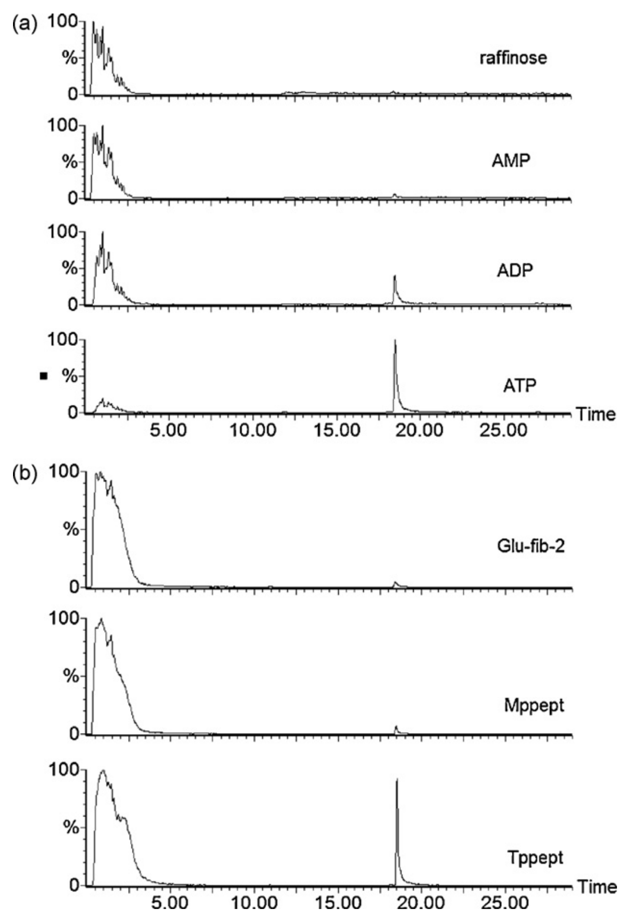


Fig. 2. (a) Nucleotide adsorption on a metal-free LC–MS setup. Selected ion profiles (ES-) for raffinose m/z 503, AMP m/z 346, ADP m/z 426 and ATP m/z 506 (top to bottom). Adsorbed nucleotides elute at ca. 18.5 min together with the mobile phase C elution front. (b) Phosphopeptide adsorption on a metal-free LC–MS setup. Selected ion profiles (ES-) for Glu-Fib-2 m/z 783.8, Casein-β monophosphopeptide m/z 1029.4 and casein-β tetraphosphopeptide m/z 1039.4 (top to bottom). Adsorbed phosphopeptides elute at ca. 18.5 min together with the mobile phase C elution front.

3. Results and discussion

3.1. Metal-free LC–MS setup

In order to analyze minute amounts of phosphorylated organic compounds such as nucleotides or phosphopeptides without the adsorption effects and concomitant sample loss and carry over as described earlier by our group and others [22,30], all metal parts needed to be excluded from the chromatographic setup. The construction of a metal-free LC setup was accomplished by replacing the stainless steel stator from the 6-port injection valve of the autosampler by a PEEK stator (VALCO part nr. C-1C46). Instead of using the standard stainless steel electrospray probe, the flow was splitted and approximately 500 nL/min was diverted to a chip based nanoelectrospray interface (Advion Biosystems) through fused silica tubing. The only metal parts in the LC system are the pumps and tubing upstream of the injector and will never be in direct contact with the analytes.

3.2. Retention of phosphocompounds

In the first experiments, two mixtures representing the most important classes of terminally phosphorylated organic compounds (nucleotides and phosphorylated peptides) were flow injected to test their behaviour on the newly build metal-free chromatography setup. As raffinose is a widely used calibration compound in negative ion mode mass spectrometry, it was chosen as a non-phosphorylated reference and added to all samples in an equimolar amount. The nucleotide test mixture further consisted of equal concentrations of adenosine 5′-mono-, di- and triphosphate. The peptide test mixture consisted of fibrinopeptide B as an unphosphorylated reference peptide, a monophosphorylated and a tetraphosphorylated peptide obtained from bovine β-casein. The constituents, molecular masses and m/z values of these compounds in the two test mixtures are listed in Table 1. In analogy to our earlier experiments [22] the initial flow injection experiment was composed of injecting (mobile phase A), then rinsing (mobile phase B) and subsequently elution of any adsorbed analyte in mobile phase C (see Fig. 1b).

Fig. 2 depicts the extracted ion elution profiles of the selected test mixtures. These profiles show a clear difference between non-phosphorylated and phosphorylated compounds. Non- or monophosphorylated biomolecules eluted in the void volume. The multiply phosphorylated compounds showed a weaker signal at the void volume. When the setup was flushed with the alkaline solvent (C), a strong signal for these compounds was visible around 18 min, the moment the alkaline solution breaks through in the ESI-source, while no signal was detected for the non-phosphorylated compounds. This phenomenon was apparent for both nucleotides and phosphorylated peptides. Contrary to our expectations (multiply) phosphorylated organic compounds were still heavily retained on the metal-free LC–MS setup and could only be eluted under strong alkaline conditions. This adsorption could have an enormous effect on LOD of these analytes when not dealt with appropriately.

In these experiments, each injection contained 100 pmol of the β-casein tetraphosphopeptide. As can be seen in Fig. 2b, a large part of it was trapped on the LC–MS setup. This adsorption could account for the very high LOD values (160, 60 and 50 pmol) previously reported for this tetraphosphopeptide [31–33].

No signals were detected when the solvent was switched to 100% acetonitrile (solvent B) in the rinsing step. This indicates that there was no considerable adsorption caused by hydrophobic interaction of analyte with parts of the LC setup, which was to be expected as the injection solvent already contained 50% MeOH. The 100% organic conditions were detrimental for the stability of the electrospray ionisation. Therefore the rinsing step was omitted in our further analyses.

3.3. pH dependancy

The pH of the solvents has an important effect on the both the charge of the analyte molecules and the surface charge of the LC-components. This could have consequences for ionisation but also for the observed adsorption phenomena. Therefore the acetic acid in the injection solvent was replaced by 0.5 mM ammonium acetate to change its pH from acidic) to neutral (pH 3.3 and pH

7.0, both prior to adding the organic solvent). When the nucleotide test mixture was injected under these conditions, no adsorption to the system was detected, i.e., no signal for the test analytes was observed upon flushing with alkaline solvent C.

It was anticipated that an increased pH would reduce the interaction with the hardware since a strong alkaline solution is used to release the adsorbed nucleotides or phosphopeptides from the setup. However, acidic conditions are often preferred, especially for peptide applications, since they guarantee full protonation in ESI-MS and ameliorate peak shapes of basic analytes [34].

3.4. Time dependency

When conducting the first experiments, a large unexplained variation between measurements was observed in the amount of nucleotide that was adsorbed. This variation somehow correlated with the time between successive experiments. In this time the chromatographic setup was flushed with solvent A. Therefore we conducted a series of experiments where the equilibration time between consecutive injections of the nucleotide test mixture was variable but strictly timed (Fig. 3a). The equilibration times were 0, 15, 30 and 60 min between consecutive injection cycles and were executed in a random order. The areas of the nucleotides eluted in the alkaline solvent were plotted against the equilibration time preceding the analysis as shown in Fig. 3b. These data indicate that longer equilibration between measurements enhances adsorption of phosphorylated organic compounds to the chromatographic setup.

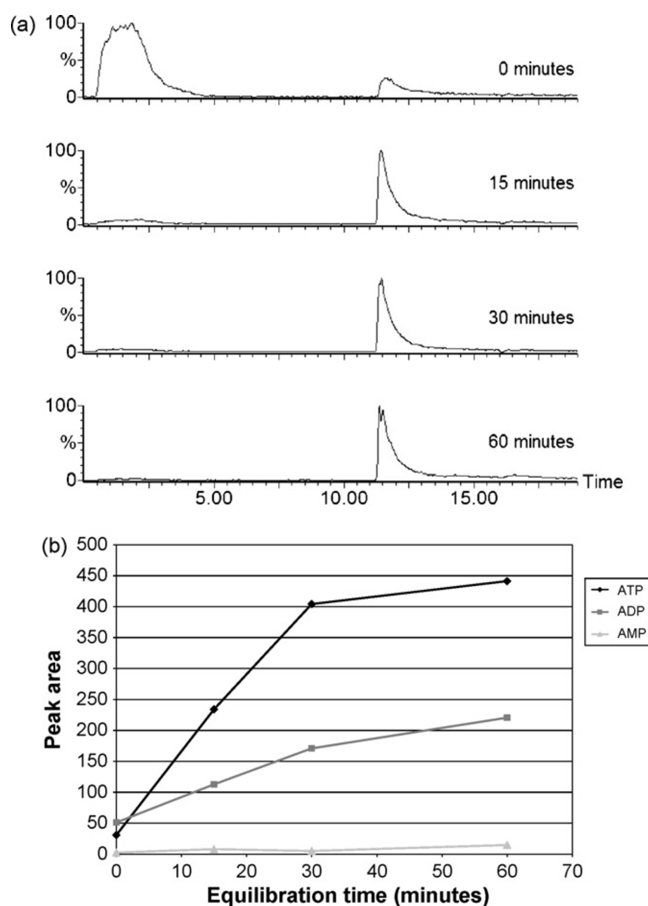


Fig. 3. (a) Effect of system equilibration time. Selected ion profiles (ES-) for ATP using different equilibration times prior to injection of the nucleotide sample. The signal at ca. 11 min (release of adsorbed nucleotide together with the mobile phase C elution front) increases with increasing equilibration time. Equilibration time 0, 15, 30 and 60 min (top to bottom). (b) Effect of system equilibration time. Integration of AMP, ADP and ATP signals released upon alkaline wash showing increased nucleotide adsorption with increasing equilibration time (system flushed with solvent A) between experiments. The data was obtained from successive experiments.

This indicates a slow equilibration effect after the alkaline plug has passed the fused silica tubing. The nature of this process is unknown. However, the electric current profile as measured on the Nanomate interface shows a similar profile (data not shown). Upon change from solvent A to solvent C, a prompt increase in the electric current is recorded. When switching back from the ammonia solution to the acidic mobile phase A, the current decreases much slower and requires up to an hour to restore the initial value. A similar slow equilibration process was already described in reversed phase columns upon switching from neutral or alkaline to acidic conditions [35].

These experiments were repeated with the same controlled equilibration times, programmed in a random order. Although the same trend was visible, the area for ATP eluting in the alkaline solvent was still not fully reproducible. When these consecutive measurements with the same equilibration time were plotted against the injection number a declining trend became apparent on a time scale of several days. Since all these experiments were done using the same LC-coupler, this seems to indicate that the number of active phosphocompound trapping sites on the LC setup diminishes with the time the LC-coupler was in use. The origin of this phenomenon was not investigated further. However, replacing the LC-coupler with a new LC-coupler restored the capacity of the setup to trap nucleotides that are released upon wash with solvent C, rendering the LC-coupler the most suspicious part of the setup.

3.5. Contribution of different parts

3.5.1. Total setup, INJECTOR-part, ESI-part

To investigate which part of the chromatographic setup was the source of phosphocompound adsorption, the sample path was divided into two parts: the *INJECTOR-part* on one hand, covering the injection valve and the 50 μm ID PEEK tubing from the injector to the splitting tee. On the other hand, the *ESI-part*, consisting of the splitting tee, the 15 μm ID fused silica LC-coupler from the Nanomate Triversa and the ESI-chip itself. The setup is pictured in Fig. 1a. For these experiments, the nucleotide test mixture was used and the mass spectrometer was operated in positive ion mode.

Simple experiments allowed us to assay the different parts of the chromatographic system separately in order to locate the site of adsorption of the nucleotides. The contribution of the different parts was probed by disengaging the tubing between injector and chip based electrospray source at the PEEK splitting tee and exposing the part (*INJECTOR* or *ESI*) under study to 200 μL of the nucleotide mixture. This was done by using a second injector mounted with a 200 μL PEEK loop. After injection of this volume with nucleotide solution and extensive rinsing with solvent A, both parts were connected again and any adsorbed nucleotide was eluted with the alkaline solvent. The effect of the entire system was analyzed using the same 200 μL sample injection by the second injector but without disconnecting the PEEK splitting tee. Any nucleotide signal now detected in ESI-MS, must come from adsorption to the studied part under the loading conditions used, since this is the only part of the setup that comes in contact with the sample during injection.

Saturation of the adsorption sites of the different parts and analysis of the eluting nucleotides from the separate parts allows us to give a relative estimate of the contribution of the different parts to the total adsorption. Therefore the amount of ATP loaded on the system needed to be large enough. 200 μL was an arbitrarily chosen volume that the CapLC could inject in a reasonable amount of time. A single injection of 200 μL of the test mixture was able to saturate the chromatographic setup completely, as multiple con-

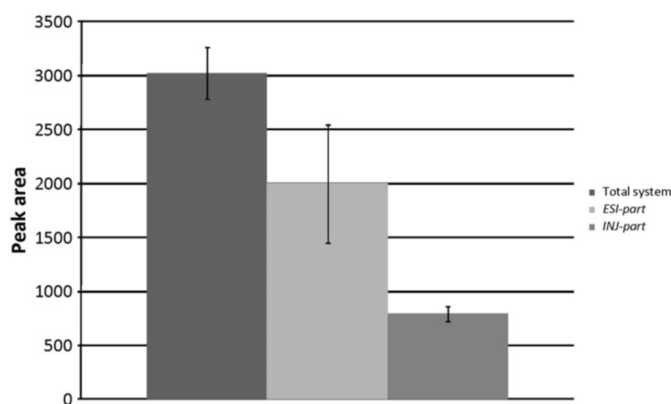


Fig. 4. The contribution of the different parts of the LC-ESI setup to ATP adsorption.

secutive loop injections of 200 μL did not increase the signal of the eluted nucleotide upon washing with the alkaline solvent (data not shown).

The signals obtained for the nucleotides after desorption from the setup in the ammonia containing solvent were integrated and plotted in Fig. 4. It is clear that the *ESI-part* is the main source of adsorption. This part is responsible for adsorbing about 2/3 of the total amount, compared to almost 1/3 of the total signal originating from the *INJECTOR-part*.

3.5.2. ESI- part: investigating the fused silica capillary

To rule out the possibility that the polymer contact surface of the LC-coupler, albeit very small, was the main source of adsorption in the *ESI*-part, the nucleotide mixture was infused in acidic solution (solvent A) using the polymer tips as delivered by Advion Biosciences. This was followed by infusing an alkaline solution (solvent C) through the same tip. These infusion experiments showed no detectable signal for the nucleotides in the alkaline solvent that could indicate adsorption on the polymer. As the polymer used for the tips is the same material as the polymer contact surface of the LC-coupler, the only remaining potential material that could account for adsorption of the nucleotides in the *ESI*-part is the fused silica tubing of the LC-coupler.

In order to further document the adsorption phenomenon, we used a separate piece of fused silica to investigate the ability of the silica surface to adsorb nucleotides. An equal length of nominally identical fused silica (50 μm ID, 50 cm long) purchased from two different suppliers was mounted between the injector and the PEEK splitting tee. This resulted in significantly different signals for the amount of nucleotide adsorbed for the capillaries of both suppliers (see Fig. 5). Although identical in dimensions, the fused silica capillaries of both suppliers behave differently in terms of adsorption of the ATP.

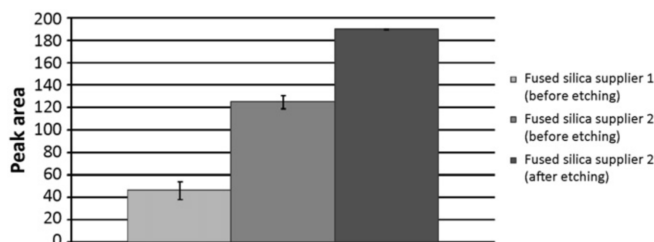


Fig. 5. Effect of etching and different suppliers on ATP adsorption to fused silica. Peak areas for the signals of adsorbed ATP eluting from different fused silica lines upon switching to solvent C. Fused silica obtained from two suppliers (left and middle) and after HF treatment (right).

In order to demonstrate that the surface characteristics of the fused silica were important for the adsorption, we etched one of the capillaries by exposure to HF as described (Section 2.4.1). The rationale behind this was that the etching would create a larger contact surface in the capillary and more free and reactive silanol groups. As we expected, an increase in the signal for the adsorbed nucleotide was observed after etching of the capillary.

The surface of fused silica, and more specifically its silanol groups, are notorious for its capacity to interact with and cause tailing of basic analytes in liquid chromatography [25]. Few reports mention the possibility of silanol groups capable of specifically trapping (multiply) phosphorylated analytes. Liu and colleagues noticed the formation of complexes consisting of phosphopeptides and metals originating from solvents, stainless steel, glassware and C18 material. This caused a dramatic increase in LOD and they solved his problem by adding EDTA to their sample as a chelator [28]. Kim *et al.* succeeded in improving the detection of multiphosphorylated peptides in reversed phase LC by the addition of phosphoric acid to the sample. Their rationale was that the phosphate group from phosphoric acid would block residual silanol groups on the C18 material [29]. In CE, background electrolyte containing ammonium phosphate was also used to mask silanols and prevent them from binding nucleotides [36]. Nevertheless, this is to our knowledge the first time that the adsorption of multiply phosphorylated compounds to silanol groups on an unmodified fused silica surface was documented to such an extent.

3.6. Potential solutions

Rather than conducting “in depth” experiments to describe the adsorption isotherms, we decided to focus in this initial study on finding practical solutions to overcome the observed phenomena and enable qualitative and quantitative analysis of nucleotides, phosphopeptides and other multiply phosphorylated organic molecules.

3.6.1. EDTA

It is known that phosphorylated compounds show strong affinity for metal ions such as Fe(III) and Al(III). This feature is already put to use to selectively isolate phosphorylated peptides and nucleotides in the IMAC procedure (Immobilised Metal Affinity Chromatography) [13,14]. Furthermore, we demonstrated earlier that phosphorylated compounds are adsorbed by (corroded) metal surfaces in an LC setup [22] in a process that is likely to be analogous to IMAC. As Liu and colleagues described the formation of phosphopeptide-metal ion complexes in liquid chromatography [28], we tested whether these metal complexes were present and responsible for the observed adsorption in our setup.

Closer analysis of the spectra indeed showed the presence of Al(III) and Fe(III) complexes with ATP in the alkaline elution plug. 10 μL of the 50 mM EDTA solution was injected to remove all metal ions from the LC system. Afterwards, the system was flushed in solvent A for 30 min in order to prevent ion suppression caused by the presence of EDTA. The nucleotide mixture was then injected as described (Section 3.2). The mass spectra recorded at the EDTA injection show the presence of EDTA-Al(III) (m/z 315) and EDTA-Fe(III) complexes (m/z 344). The formation of ATP-Fe(III) (m/z 559) and ATP-Al(III) (m/z 530) complexes is clearly reduced by the injection of the EDTA plug as the signal at their corresponding m/z values disappeared in the subsequent nucleotide injections. Nevertheless, no noticeable difference could be observed in the adsorption behaviour of ATP and ADP. This indicates that the formation of ATP-iron or ATP-aluminium complexes does occur but that it does not play a significant role in the adsorption processes we observed.

3.6.2. Phosphate

Since the phosphate group is the functional group responsible for the adsorption to the system, we tried to saturate the surfaces with phosphate by injecting a phosphoric acid solution (0.425% and 0.085%) prior to the injection of the nucleotides. This approach was successfully used earlier by Kim et al. [29], who observed a remarkably lower detection limit for phosphopeptides and nucleotides when injected in a solvent containing phosphoric acid. However, in our current experiments, no reduction of the adsorption to the LC–MS setup was found. The reason for this is unclear. The main difference between our experiments and those conducted by Kim et al., was that we injected phosphoric acid prior to injecting the phosphorylated compounds, while they dissolved the sample in a solvent containing phosphoric acid. We avoided injecting phosphoric acid together with the analytes in order to prevent massive ion suppression of the nucleotides caused by the presence of phosphate clusters. Since Kim et al. described a carryover effect (increased sensitivity) of the phosphoric acid injection in the subsequent analyses of phosphopeptides, we expected that an initial injection with phosphoric acid could avoid the adsorption observed in our experiments. The detrimental effect of phosphoric acid cluster formation in nanoelectrospray of phosphorylated peptides was also observed by others [37].

3.6.3. Deactivation by dimethyldichlorosilane

In accordance with the suggestion by Kim et al., we expect hydrogen-bonding to the available silanol groups to be the cause for the adsorption of the phosphocompounds. Therefore, deactivation and derivatisation of the fused silica tubing should reduce the adsorption. Dimethyldichlorosilane is a silanizing agent that is used to passivate glass surfaces for various purposes. It reacts with available silanol groups at the fused silica surface, resulting in the formation of dimethylsiloxane bridges. Unlike silanol groups, these siloxanes are unable to form hydrogen bridges and, based on our hypothesis, will not adsorb phosphorylated compounds.

Initial experiments demonstrated that derivatisation of the fused silica capillary of the LC-coupler with dimethyldichlorosilane significantly reduced the adsorption. The signal upon injection of ATP increased and the signal after switching to alkaline conditions, i.e., setting free any adsorbed phosphorylated compound, disappeared (see Fig. 6). However, the capacity to selectively trap the phosphates is gradually restored after some analyses, most likely due to hydrolysis of the dimethylsiloxane derivatization under the alkaline conditions (pH 12) used in the elution step of the experiments.

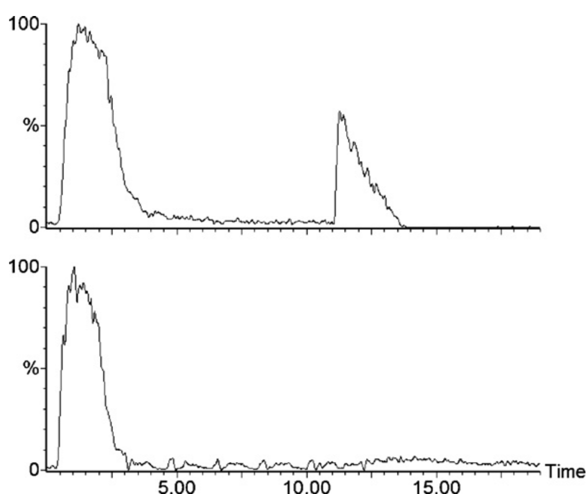


Fig. 6. Effect of fused silica deactivation with DMDCS. Selected elution profiles (ES−) for ATP m/z 506 immediately before (upper trace) and after (lower trace) derivatization of the fused silica of the LC-coupler.

4. Conclusions

Although we removed all metal surfaces from our LC-ESI setup and replaced them by fused silica and PEEK components, the adsorption of multiply phosphorlylated analytes to the setup was still apparent. Non-phosphorylated compounds (raffinose and (Glu^1)fibrinopeptide B) showed no adsorption to the LC-ESI components. Retention on the LC setup was observed for compounds that contain multiple phosphate groups, despite the clear difference between trinucleotides, which are triphosphates containing two phosphoanhydride bonds and peptides containing multiple phosphate groups in the peptide sequence. This loss of analytes to the instrument is detrimental to all quantitative analyses of nucleotides and phosphopeptides.

Both the fused silica LC-coupler of the nanoelectrospray interface and the injector part (from injector up to splitter of the Nanomate ESI-source) showed to some extend adsorption of the nucleotides and phosphopeptides. Although being the smaller part of the integral setup, the larger fraction of the adsorption could be attributed to the fused silica LC-coupler.

By etching the fused silica with HF and by using fused silica tubing from different suppliers we could demonstrate that the fused silica surface is important in the interaction with the nucleotides and phosphopeptides. Apparently the surface of nominally identical fused silica tubing behaves different towards phosphocompounds. The exact nature of the interaction with the fused silica is not studied, however pH is an important factor. This is in line with results of Kim et al. [29] who pointed at hydrogen bridging as responsible for the interactions. Increasing the pH could improve the results for nucleotides, but is no solution for the phosphopeptides. Interaction with metal ions does exist [26] and could be seen in our data by the presence of Fe(III) and Al(III) adduct-ions with nucleotides in our MS spectra. Removal of these ions could be accomplished by flushing the setup with EDTA, but this did not avoid adsorption of the analytes to the system.

Flushing the setup with a phosphate, an approach similar to our earlier experiments with stainless steel electrospray needles [38], and used by other authors [29] did not solve the adsorption problem.

The adsorption was minimised by deactivation of the fused silica by treatment with dimethyldichlorosilane. As long as the experimental conditions preserve the dimethyldichlorosilane derivatization, this deactivation prevents the massive adsorption of multiply phosphorylated compounds to the LC-ESI setup and opens options for an important improvement in sensitivity for the analysis of important phosphorylated biomolecules as nucleotides and (multiply) phophorylated peptides.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2010.05.022.

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