Stainless steel electrospray probe: A dead end for phosphorylated organic compounds?

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Abstract

A study of the interaction of phosphorylated organic compounds with the stainless components of a liquid chromatography–electrospray ionisation–mass spectrometry system (LC–ESI–MS) was carried out to disclose a (forgotten?) likely pitfall in the LC–ESI–MS analysis of phosphorylated compounds. The retention behaviour of some representative compounds of different important classes of phosphorylated biomolecules such as nucleotides, oligonucleotides, phosphopeptides, phospholipids and phosphorylated sugars was investigated during their passage through the injector and the stainless steel electrospray capillary. It became clear that the stainless steel components within the LC–ESI–MS setup were able to retain and trap phosphorylated compounds when these compounds were introduced under acidic conditions (0.1% acetic acid). Their release from these stainless steel parts was accomplished by applying an extreme basic mobile phase (25–50% ammonium hydroxide, ca. pH 12). From the data collected one could conclude that the availability of a primary phosphate group appeared imperative but was not always sufficient to realise adsorption on a stainless surface. Furthermore, the number of phosphate moieties seemed to enhance the adsorption properties of the molecules and hence roughly correlated with the analyte fraction lost. Corrosion of the inner surface caused by the mobile phase and the electrospray process was found to be an important factor in the course of these adsorption phenomena.

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1. Introduction

It was known that stainless steel parts mounted in liquid chromatography (LC) equipment can corrode under the influence of many of the conventionally used solvents [1–3]. Even passivated surfaces can be activated by reversed-phase solvents and the resulting corrosion can be extensive [1] leading to the malfunction of some parts of the instrument which may result in e.g. less accurate flow rates.

Yet more important was the observation of the irreversible adsorption of compounds to (corroded) stainless steel surfaces [4]. This phenomenon should be remembered as nowadays analysts are confronted with samples of increasing complexity. Moreover, the (partial) loss of specific compounds prior to detection is particularly important if limited amounts of sample are available. Within the context of high throughput methods producing massive amounts of data one might forget this problem and incorrect/incomplete data might be generated.

The culprit in the case of LC equipment is the availability of iron in the system. This issue was already addressed by some manufacturers of (nano-)LC instrumentation by designing iron-free chromatographic systems and injection/switching valves. Unfortunately, these designs are much more expensive and slightly the majority of analysts are indifferent towards the presence of iron.

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Although the phenomena of corrosion and adsorption are well documented their impact upon the acquisition of analytical data and more specific electrospray ionisation–mass spectrometry (ESI–MS) data are frequently overlooked. In general the physical situation in ESI capillaries has been described as a controlled current electrolytic (CCE) flow cell [5–8]. The occurrence of emitter corrosion was nicely exemplified by Van Berkel: the Fe(II) and Cu(II) ions formed by anodic corrosion of a nano stainless steel emitter produced a complex with phenanthroline the Fe(II) and Cu(II) ions formed by anodic corrosion of a nano stainless steel emitter produced a complex with phenanthroline and were detected as such by both MS and photo diode array oxidation/reduction processes in the ESI interfaces were exploited and even optimised for analytical benefits as recently outlined by Van Berkel et al.[11] and many references cited therein).

Yet the impact of the corrosion of the internal surface of the ESI capillary during ESI–MS analyses is – to the best of our knowledge – not yet explicitly accounted for [12]. One can deduce that in the course of time the surface of the emitter is altered by both solvents and redox processes resulting in an expanded contact surface. This would increase the prospect of compound losses by adsorption or even chemical binding.

In a study using Fe(III)-immobilised metal affinity chromatography (IMAC) as a tool for the on-line sample clean up and preconcentration of nucleoside mono-, di- and triphosphates, the loading and elution of the samples were monitored by ESI(+)–MS in order to probe the effectiveness of the trapping process [13]. The latter is the result of the selective chelation of terminal phosphate groups by Fe(III) under acidic conditions, a feature already put to use to enrich phosphopeptides [14–20]. In the course of these experiments analogous “IMAC effects” without an IMAC precolumn present were noticed. This intriguing observation prompted us to start additional experiments based on the assumption that the injector (needle, loop and valve), the tubing (either fused silica or poly(ether ether ketone) (PEEK)) and/or the stainless steel capillary of the ESI source were capable of mimicking the Fe(III)-IMAC behaviour and hence trap phosphorylated organic compounds.

In this paper we wish to demonstrate that this is a tangible concern for the analysis of a variety of phosphorylated organic compounds by liquid chromatography–electrospray ionisation–mass spectrometry system (LC–ESI–MS).

2. Experimental

2.1. Chemicals

All nucleosides and nucleotides were purchased from Sigma–Aldrich (Bornem, Belgium) in the highest available grade. The nucleotides were in the sodium salt form. Dimeric deoxyoligonucleotides were obtained from Sigma-Genosys (Pampisford Cambridge, UK). The phosphopeptide positive control set, the natural phospholipid 3-sn-phosphatidic acid sodium salt egg yolk lecithin, the glycerophosphate, the β-casein as well as the [Glu1]-fibrinopeptide B (human) were also from Sigma–Aldrich. The phosphopeptide control set consisted of a tetraphosphopeptide and a monophosphopeptide obtained by purification of a tryptic digest of β-casein. The phospholipid was a mixture containing combinations of different fatty acid residues (palmitic, stearic, oleic, linoleic and traces of other fatty acids). Raffinose was also obtained from Sigma–Aldrich. Trypsin was purchased from Promega (Madison, WI, USA).

Acetic acid (HOAc; analytical-reagent grade) and ammonium hydroxide 28–30 wt.% (NH4OH; analytical-reagent grade) were purchased from Merck (Overijse, Belgium). Ammonium bicarbonate (NH4HCO3; analytical-reagent grade) was from Fluka (Bornem, Belgium). Acetonitrile (ACN; HPLC supra gradient), methanol (MeOH; HPLC Absolute) and water (H2O; HPLC) were from Biosolve (Valkenswaard, The Netherlands). Twenty-five millimeters HPLC Syringe Filters were obtained from Alltech (Lokeren, Belgium).

2.2. Sample preparation

Prior to a series of measurements, stock solutions of the different compounds were prepared in HPLC grade water at concentrations of 10⁻³ and 10⁻⁴ M and stored at –21°C until use. Just before analysis they were diluted further according to the experimental needs.

For the experiments described under Section 3.1 an individual compound concentration of 2.5 × 10⁻⁴ M was maintained throughout all measurements. An exception was made for the sn-phosphatidic acid sodium salt: as the commercial product is a mixture itself it contains a concentration of ca. 5 × 10⁻⁴ M (based on the average mass of the mixture) was used in the samples. The samples were dissolved in 0.1% (v/v) HOAc (50:50 (v/v) MeOH/H2O) for the “injector + stainless steel capillary” (INJ + SSC), “injector” (INJ) and “stainless steel capillary” (SSC) effect experiments (see below). For the reference samples the compounds were made up in 0.1% (w/v) NH4OAc (25:25:50 NH4OH/H2O/MeOH). The nucleotide test set contained adenosine (Ado), adenosine mono-, di-, triphosphate (AMP/ADP/ATP), uridine (Urd), uridine mono-, di-, triphosphate (UMP/UDP/UTP) and raffinose (all 2.5 × 10⁻⁶ M). The phosphopentide test set was made of the tetraphosphopeptide RELEELNVPGEIVEpSpSpSpSEESITR (mppept), [Glu1]-fibrinopeptide B (EGVNDNEEGFFSAR) (glu-fib) and raffinose (all 2.5 × 10⁻⁶ M). The phospholipid test set contained the sn-phosphatidic acid sodium salt (5 × 10⁻³ M) and raffinose (2.5 × 10⁻⁴ M). The last test set was made up of glycerophosphate and raffinose (all 2.5 × 10⁻⁶ M).

The samples measured under Section 3.2 (Ado and ATP) were dissolved in 0.1% (v/v) HOAc (H2O) in a concentration of 10⁻⁵ M.

For the experiment described under Section 3.3 ca. 2 mg β-casein was dissolved in 1 mL 100 mM NH4HCO3 (90:10 H2O/ACN) pH 8.5 and digested for 3 h with 1:100 trypsin (1 µg/1 µL) at 37° C. Following digestion the sample was diluted to ca. 2.5 × 10⁻³ M in 0.1% (v/v) HOAc (50:50 (v/v) MeOH/H2O) and used as such.
2.3. LC conditions (Table 1)

A 0.1% (v/v) HOAc (50:50 MeOH/H2O) Sample loading, rinsing, acidic
B 100% ACN Rinsing
C 0.1% (w/v) NH4OAc (25:25:50 NH4OH/H2O/MeOH) Elution of adsorbed compounds, basic
D 0.1% (v/v) HOAc (H2O) Sample loading, rinsing, acidic
E 50% (v/v) NH4OH (H2O) Elution of adsorbed compounds, basic

Table 1

Abbreviations, compositions and purpose of mobile phases used

Mobile phase Composition Purpose
A 0.1% (v/v) HOAc (50:50 MeOH/H2O) Sample loading, rinsing, acidic
B 100% ACN Rinsing
C 0.1% (w/v) NH4OAc (25:25:50 NH4OH/H2O/MeOH) Elution of adsorbed compounds, basic
D 0.1% (v/v) HOAc (H2O) Sample loading, rinsing, acidic
E 50% (v/v) NH4OH (H2O) Elution of adsorbed compounds, basic

2.3. LC conditions (Table 1)

For the experiments described in Section 3.1 a ternary gradient pump (CapLC, Waters, Manchester, UK) with integrated injector was used. The mobile phases were all delivered at 10 μL/min and were 0.1% (v/v) HOAc (50:50 MeOH/H2O) (A), 100% ACN (B) and 0.1% (w/v) NH4OAc (25:25:50 NH4OH/H2O/MeOH) (C). They were, respectively used as the loading, rinsing and elution solvent. Prior to use they were filtered through a 0.2 μm nylon 25 mm syringe filter (Alltech). Samples were injected via a 10 μL PEEK loop mounted on a 6 port valve type stainless steel (SS) Nitronic 60 injector (Valco Instruments, Houston, TX, USA). The injector was connected to the electrospray probe via PEEK tubing (Upchurch Scientific, Oak Harbor, WA, USA). The total of all the components associated with the injection of the sample and its deliverance to the mass spectrometer (actual injector and tubings) was called injector (INJ) throughout the text. An amount of 10 μL of the analyte was injected (2.5 × 10−8 M (25 pmol/injection)). Samples were electrosprayed by means of the standard stainless steel ESI capillary (SSC) (184 mm × 100 μm I.D.).

In all experiments described under Section 3.1 sample injections and LC eluents were monitored by ESI-MS in the negative ion mode (ESI−) unless specified.

Studies of the INJ + SSC effects (Sections 3.1 and 3.3): experimental conditions

Samples were injected in mobile phase A and the whole system was flushed for 8 min followed by 8 min of additional rinsing with mobile phase B. Finally mobile phase C was applied. The 8 min intervals were arbitrarily chosen and represented ca. 55 times the internal volume of the SSC.

Study of the INJ effect (Section 3.1): experimental conditions

Prior to sample injection the INJ was disconnected from the ESI-MS setup. After sample injection the INJ was rinsed with both mobile phases A and B, respectively for 8 and 10 min. Just before switching to mobile phase C the INJ was reconnected to the MS. Coupling and decoupling of the injector to the MS was always done manually as the use of an extra switching valve might involve extra adsorption sites.

Study of the SSC effect (Section 3.1): experimental conditions

During injection and subsequent flushing with mobile phase A the INJ was attached to the MS (8 min). Subsequent the INJ was manually disconnected and rinsed with mobile phases C (8 min) and B (10 min). After that the MS was again hooked up with the INJ. An additional wash of the INJ + SSC with mobile phase B preceded the definitive application of mobile phase C.

Reference (Section 3.1): experimental conditions

Samples were dissolved in 0.1% (w/v) NH4OAc (25:25:50 NH4OH/H2O/MeOH) (C) and flow injected in mobile phase C.

The applied solvent sequences for the different experiments described under Section 3.1 were summarized in Fig. 1.

A Waters CapLC (Waters) system equipped with a 996 diode array detection (DAD) system (Waters) with capillary flow cell was used for the experiments described under Section 3.2. The total flow path consisted of a SS injector, a 20 μL PEEK loop, 75 μm I.D. silica tubing and the SSC of the ESI interface. Samples were dissolved in 0.1% HOAc (H2O) (10−4 M) and an amount of 20 μL was injected (ca. 200 pmol). The carrier solvent was 0.1% (v/v) HOAc (H2O) (D) and was delivered at a flow rate of 10 μL/min. After injection of the sample, the complete setup was flushed for at least 20 min with mobile phase D at the same flow rate. Elution of adsorbed compounds was achieved by multiple injections of 20 μL of 50% (v/v) NH4OH (H2O) (E). Eluting compounds were monitored by UV (DAD) or by ESI−-MS on a triple quadrupole mass spectrometer (Quattro II, Waters) or both.

2.4. MS conditions

Electrospray mass spectra were recorded in ESI− either on a Q-TOF II mass spectrometer (Waters) or a Quattro II triple quadrupole mass spectrometer (Waters) both equipped with a pneumatically assisted Z-spray source (Waters) and standard SS electrospray probes. The type of SS used for the electrospray probes was not released by the manufacturer.

In the experiments described under Sections 3.1 and 3.3 the Q-TOF was used. For each test set the instrument was tuned separately to maximise response for the particular m/z range under study. The ionisation voltage applied was −3 kV throughout Sections 3.1 and 3.3. The source and desolvation temperature were both set at 100 °C. A cone gas flow rate of ca. 60 L/h and a desolvation gas flow rate of ca. 150 L/h were applied. Spectra were recorded in full scan mode. In case of the nucleotide test set ESI− mass spectra were recorded in the range of 200–750 Da at a cone voltage of 30 V. The oligonucleotide set was recorded between 200 and 800 Da at a cone voltage of 30 V. For the phosphopeptide test set a wide range of 300–2000 Da was covered and a cone voltage of 40 V was applied. For the phospholipid mixture the mass range covered and cone voltage applied were, respectively, 200–1000 Da and 30 V. The glycophosphatase was monitored between 100 and 600 Da and a cone voltage of 30 V was used.

For the experiments under Section 3.2 the Quattro II mass spectrometer was used for ESI− spectra recording; the covered
mass range was 350–650 Da at a cone voltage of 40 V. Flow rates for the cone and desolvation gases were similar to those of the Q-TOF experiments.

3. Results

3.1. Hardware contributions to retention/losses of phosphorylated analytes

In a first series of experiments we studied the behaviour of a limited set of terminally phosphorylated and non-terminally phosphorylated representatives of important biochemical compound classes, i.e. nucleotides, oligonucleotides, phospholipids, phosphosugars and phosphopeptides. An equimolar amount of a non-phosphorylated compound, i.e. raffinose was added to all samples as reference. In Table 2 the molecular mass and relevant m/z values of all compounds used were summarised, together with some structures.

We subdivided the sample/solvent path of the analytical system in two parts: the SSC and the INJ, the latter covering all the hardware components associated with the injection of the sample and its delivery to the mass spectrometer. The idea was to probe the retention behaviour of the phosphorylated compounds in both SSC and INJ. Any retention was investigated by plotting the extracted ion chromatograms for [M-H]⁻ of the analytes ([M-2H]²⁻ or [M-3H]³⁻ in the case of the (phospho)peptides), by comparing the areas under the chromatographic peaks and by interpretation of the according mass spectra.

Initially the cumulative INJ + SSC effects on the compounds under test were investigated: after injection of a particular sample the complete instrumental setup was subjected to a cycle involving: (1) mobile phase A; (2) mobile phase B and (3) mobile phase C, as outlined in Fig. 1. Preliminary experiments (data not shown) learned that only mobile phase C (high NH₄OH concentration) was able to release compounds adsorbed.

Next the contribution of each individual component (INJ and SSC effect) to the retention of phosphorylated compounds was examined. To probe only the INJ effect the LC and MS were decoupled during sample injection and rinsing of the PEEK tube. Just before applying mobile phase C the LC system was coupled to the MS. Any signal then observed by ESI(−)-MS must originate from compounds previously adsorbed in the INJ under the acidic loading conditions.

The contribution of the SSC (SSC effect) to the adsorption of phosphorylated compounds was investigated in a similar way. During injection and subsequent washing with the mobile phase A the INJ was attached to the MS. In this way eventual elution of compounds could be detected. Subsequently the INJ was manually disconnected from the MS system and rinsed with mobile phase B. By doing so all compounds adsorbed in the INJ were removed. After that the INJ was reinstalled and coupled to the electrospray SSC. An additional rinsing step of the INJ and SSC with 100% ACN (mobile phase B) preceded the definitive delivery of mobile phase C. If any compounds were adsorbed on the SSC ions should now be detected.

In order to validate all the data gathered in the experiments described above and make a relative quantification possible a reference experiment was needed. It was assumed that no retention on the LC–ESI–MS hardware would occur if the compounds were dissolved and in-flow injected in mobile phase C. The elu-
Table 2

Molecular masses (monoisotopic) and relevant m/z values of the deprotonated compounds used, together with some structures

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<tr>
<th>Name</th>
<th>Exact mass [M-H]−</th>
<th>*[M-2H]2−</th>
<th>**[M-3H]3−</th>
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3.1.1. Nucleotides

The nucleotide test mixture containing uridine, adenosine, uridine 5′-mono-, di- and triphosphate, adenosine 5′-mono-, di- and triphosphate together with raffinose was subjected to the experiments described above.

The extracted ion chromatograms of the cumulative INJ + SSC effect experiment showed a clear difference between the behaviour of the non-phosphorylated and phosphorylated compounds. This is illustrated in Fig. 2 showing the reconstructed ion chromatograms for the [M-H]− of raffinose (m/z 503), Urd (m/z 243), UMP (m/z 323), UDP (m/z 403) and UTP (m/z 483). In general the non-phosphorylated compounds (raffinose and Urd) emerged immediately after injection. During the following steps, i.e. rinsing with mobile phases A and B and subsequent change to mobile phase C no raffinose or Urd eluted. It is clear that within 5 min after injection of the sample plug also signals of the phosphorylated compounds were observed. However, the question remained whether a fraction of these compounds was retained by the INJ and/or the SSC, since this could have a tremendous effect on the LODs of phosphorylated compounds.

When the mobile phase was changed to C no increment of the signals of the raffinose and the nucleosides were observed. However, [M-H]− signals were recorded pointing to the elution of the nucleoside 5′-mono-, di- and triphosphates.

As can be seen from Fig. 2 the UMP signal at ca. 18.5 min is quite low in intensity. Therefore, we injected a solution of solely
Fig. 2. The extracted ion chromatograms of uridine, UMP, UDP and UTP and raffinose when monitoring the INJ + SSC effect. The increments in the traces of UDP and UTP at ca. 18.5 min matches the mobile phase C elution front.

UMP. This experiment showed that UMP was not adsorbed by the stainless steel surfaces. The signal observed around 18.5 min could therefore be explained by in source CAD of UDP or UTP. Analogous results were obtained for AMP. The SSC and INJ effect experiments yielded similar elution profiles. In either case the extracted ion chromatograms and the according spectra unambiguously indicated considerable amounts of the nucleoside di- and triphosphates were recovered when applying the basic C mobile phase. In the case of the SSC effect experiment only the electrospray SSC itself could account for these losses. For the INJ effect two potential contributors could be responsible: the stainless steel injection valve and the PEEK tubing linking the valve with the ESI probe. Since there is no reason/literature suggesting interactions of nucleoside di- and triphosphates with PEEK tubing the injection valve remained the only suspect.

In order to get an insight in the relative amount of compound adsorbed on the stainless steel surface the intensity of the different \([M-H]^-\) signals observed in the experiments described above was compared to the intensity of the corresponding \([M-H]^-\) signal obtained in the reference experiment. These areas were plotted in a normalised bar graph (Fig. 3). From these data it can be seen that the non-phosphorylated compounds (nucleosides + raffinose) experienced no adsorption whatsoever. Yet the results for the di- and triphosphates were striking: recoveries from the different hardware components –compared to the reference experiment– varied between 12 and 31%. When the cumulative effect of INJ + SSC was considered recoveries up to 64% were recorded. These values did not take into account ion suppression phenomena and should as such be interpreted with caution; nevertheless the adsorption effect cannot be neglected. Furthermore, there seemed to be a certain correlation between the aptitude to adsorption and the number of phosphate moieties present in the molecule.

To exclude any possible electrical effects in the SSC similar experiments were conducted but under ESI(+) (data not shown). The same effects and tendencies were observed.

3.1.2. Oligonucleotides

Two dimeric \(2'\)-deoxyoligonucleotides of guanosine and cytidine together with raffinose made up the oligonucleotide test set. The selected dimers were GpC \([(M-H)^- = 555]\) and \(5'\)-pGpC \([(M-H)^- = 635]\), the former only having a 3'–5' phosphodiester bond, the latter having an terminal 5'–phosphate group.

In search for the INJ + SSC effect it was found that, by examining the extracted ion chromatograms for \([M-H]^-= 555\), \(5'\)-pGpC experienced already some retention in the liquid pathway and exhibited a delayed return to the background values whilst flushing the solvent path with the acidic mobile phase A. During the ACN wash (B) none of the traces showed a signal increment. The application of the mobile phase C led again to important increment at \(m/z\) 635 for \(5'\)-pGpC, yet also a signal at \(m/z\) 555 was seen as is illustrated in Fig. 4. The observed pattern for the traces of raffinose and GpC showed a square wave form as expected for flow injection. As GpC showed no retention following injection its presence in the trace at 18.35 min was explained again by in source CAD of \(5'\)-pGpC. In order to be sure the experiment was repeated with GpC as the sole compound injected, which proved the previous assumption.

Again the contributions of both the INJ and the SSC were investigated and compared with the reference experiment (Fig. 5). The observed adsorption effects were less pronounced compared to the nucleoside-di- and triphosphates, though the recoveries of \(5'\)-pGpC were about 9 and 26% for the SSC and INJ, respectively. From these results one could conclude that in the case of dimeric oligonucleotides, a primary phosphate was imperative for adsorption to the SS surfaces available in LC–ESI–MS hardware. However, more experiments would be necessary to determine whether this is a general rule or not.
3.1.3. Phosphopeptides

Within the context of the current worldwide focus on proteomics it was important to investigate whether phosphopeptides were also prone to (ir)reversible interactions with LC–ESI–MS hardware. For that reason a monophosphorylated peptide (Mppept), a tetraphosphorylated peptide (Tppept), [Glu]-fibrinopeptide B (Glu-fib) and raffinose were mixed to make up another test set. The commercially available phosphopeptides originated from a tryptic digest of β-casein (cf. Section 2). The sample was injected and subjected to the INJ + SSC solvent sequence. The different reconstructed ion chromatograms of the negatively charged deprotonated molecules were generated and plotted (Fig. 6). For the raffinose the [M-H]− at m/z 503 was used and for the peptides the summed isotopic cluster was used (Mppept: Σ m/z 1029.5–1031; Tppept: Σ m/z 1039.5–1040.5; Glu-fib: Σ 783.8–785.3). For evaluation of the phosphopeptides, respectively [M-2H]2− and [M-3H]3− were selected and for the Glu-fib: [M-2H]2−.

The effect seen for the Tppept was impressive: the signal trace exhibited a huge increment when mobile phase C reached the ESI interface. Furthermore, the Tppept exhibited retention compared to raffinose once injected. The effects for Mppept were less pronounced: a slight retention was observed together with a limited recovery by mobile phase C. The Glu-fib trace agreed well with the behaviour expected for a non-retained compound, yet some Glu-fib was also recovered when the mobile phase was switched to mobile phase C. This was contradicting our rationale that correlated the SS interaction with the presence of (multiple) phosphorylations.
Fig. 7. Part of the spectra (m/z 800–1600) obtained from recovery of peptides from the different hardware components after injection of the peptide test set and injection of the test set made up in solvent C. The spectra are dominated by the double and triple charged isotopic envelopes of the peptides, sodium adduction is also observed. Intensities of the different spectra are mutually compared and normalised to 100%. The complete spectrum of the reference is also given, which makes the dominance of Glu-fib in the spectrum clear. Ion suppression of the phosphopeptide signals by Glu-fib in the reference results in the overestimation of the recoveries in the other experiments.

On the other hand it was observed that Glu-fib is far more prominent in the spectra compared to the other compounds even though it was present in an equimolar quantity. This preferential detection might overestimate the actual (absolute) presence of Glu-fib. Nevertheless, it did not alter the fact that Glu-fib was recovered to some extent and thus previously adsorbed.

Additionally the individual INJ and SSC effects were then investigated for the peptide test set, followed by the reference experiment (Fig. 7). The latter confirmed that Glu-fib was the most abundant ion when recorded under the eluent conditions (mobile phase C) and that at the same time the signals of the other compounds were suppressed. More interesting was the observation that again from both the SSC and INJ a significant amount of the phosphorylated compounds, especially Tppept, was recovered. At the same time an impurity in the commercially phosphopeptides sample showed: the signal at m/z 987.7 agrees with another triply charged tetraphosphorylated peptide originating from β-casein, with mass 2965.15 [21]. The Glu-fib contribution from the INJ if compared to the SSC contribution was more pronounced. This might ensue from a memory on the PEEK tubing rather than from interaction with the stainless steel surfaces as Glu-fib is rather lipophilic. The bar graphs derived from the areas under the elution peaks of the different extracted ion chromatograms were not as informative as before because of the Glu-fib ion suppression effect. This gave rise to an apparent recovery for Tppept in the INJ + SSC effect of >100% compared to the reference data (Fig. 8). The quenching was even more pronounced when a similar experiment was conducted in the positive ion mode, though the same tendencies were there: the loss of phosphorylated compounds in acidic conditions to SS hardware components was significant and correlated with the number of phosphate groups present.

3.1.4. Phospholipids and phosphosugars

Some other classes of monophosphorylated biomolecules were briefly investigated, namely terminally phosphorylated phospholipids and a phosphorylated sugar. The phospholipid sample, 3-sn-phosphatidylcholine contained a typical fatty acid ester content of mainly palmitates and oleates and considerable amounts of stearates, linoleates, etc. When subjected to the initial INJ + SSC experiment the bulk only eluted if mobile phase B was applied. This agreed well with their hydrophobic properties and as such their anticipated interaction with the PEEK tubing. Subsequent application of the mobile phase C released some more of the phospholipids, which would agree with our observations for the other studied compounds. However, partial
hydrolysis of the available fatty acid esters gave rise to phospholipids in the sample with only one instead of two fatty acid residues. The shape of the extracted ion chromatograms of these compounds resembled a square wave form as anticipated for a non-retained compound and minimal recoveries were found after elution with mobile phase C. Further testing (cf. INJ and SSC effects, reference) made clear that the hydrophobic interactions were predominant and as such these concise experiments made deductions whether adsorption to SS surfaces based on the presence of a phosphate group occurred or not, impossible. It is not inconceivable that the different effects (hydrophobicity and interactions with SS) reinforced each other. In view of this all no further experiments were performed.

As target compound for a phosphorylated sugar glycerophosphate was chosen. The associated [M-H]− trace at m/z 171 mimicked perfectly the unretained compound trace (cf. rafinose). This agreed well with the observations made for the nucleoside monophosphates, which also had no retention.

3.2. Trapping capacity of the SSC

The above experiments showed interactions with SS surfaces of the LC–ESI–MS hardware to be responsible for the (partial) loss of (multi) phosphorylated compounds. In this process the electrospray SS capillary was an important contributor. It was assumed that the physical state of the inner wall of the capillary would be an important factor in this phenomenon. Furthermore, it felt expedient to establish additional proof for the (possible) actual adsorption properties of the SSC without using a mass spectrometer to bypass any unforeseen instrumental effect and at the same time excluding all electrical effects. To do so an SSC of an ESI probe which was, intensively (and trouble free) used in different LC–ESI–MS studies in our laboratory was evaluated. The analytical system used for this purpose consisted of an injector, silica tubing and the aforementioned SSC. MS and/or DAD were used to monitor the compounds.

To study the trapping capacity solely associated with the SSC of the ESI probe ATP, adenosine and blank solutions were used as test samples. In all experiments injections of ATP or adenosine were always preceded by a number of 200 pmol injections of 50% NH₄OH (H₂O) (E) and a blank injection, the latter being 0.1% HOAc (H₂O), mobile phase D. If necessary additional injections of mobile phase E were made to reduce the signal of the adsorbed compounds to back ground level.

In a first experimental setup ca. 200 pmol ATP (in 0.1% HOAc) was injected twice via a SS valve directly connected to the DAD system via a fused silica line. The elution of ATP was monitored at 258 nm and identified by its UV–absorption profile (Fig. 9, upper half). A different result was obtained when the same ATP sample was injected on the system in which the SSC was mounted between injector and DAD system: none of the ATP reached the detector (Fig. 9, lower half). As both systems only differed in the presence/absence of the SSC, it was concluded that the SSC had a considerable trapping capacity for ATP.

In a third experiment the solvent path consisted of the SS injection valve, silica tubing, the DAD system and the SSC. Identical injections were made and monitored via both the DAD and the ESI–MS systems. Following its injection in mobile phase D ATP was detected by DAD, yet no signal was observed by ESI(−)-MS at [M-H]− (m/z 506) unless the solvent composition was changed to mobile phase E. If adenosine was injected under analogous conditions no adsorption was observed.
To gain a deeper appreciation of the possible implications of these findings for "real" samples we injected a crude tryptic digest of β-casein. This tryptic digest would contain Mpppept and Tppept. Prior to ESI-MS analysis the sample was diluted to ca. 2.5 pmol/μL in 0.1% (v/v) HOAc (50:50 MeOH/H₂O). The above described INJ + SSC solvent stream has not yet, to the best of our knowledge, been reported. This is especially embarrassing since electrospray mass spectrometry is a widespread technique in (phospho)peptide studies.

Soon after injection a huge increment in the TIC trace was observed at the moment the injection plug reached the detector (Fig. 10). When the mobile phase was changed to mobile phase C eluent the TIC trace became more alike to the surface seen in a new SSC. This showed irreversible compound losses due to SS hardware is not new: already in the 1980s Sadek et al. demonstrated irreversible protein losses due to the presence of stainless steel surfaces especially in the high linear flow rates could become "active" and corroded under influence of many reverse phase blends especially in the high linear flow regions (valves, connectors, capillary tubing) [1]. As a result pitting and the formation of metal oxide layers occurred. Had dad and Foley reported that under acidic conditions detectable quantities of Fe(III) were observed following the corrosion of stainless steel surfaces and especially to stainless steel column frits [4].

Furthermore, our preliminary data indicated that an essential part of the detector can bias the results: The observation that the electrospray capillary contributes to the removal of phosphorylated compounds from the solvent stream has not yet, to the best of our knowledge, been reported. This is especially embarrassing since electrospray mass spectrometry is a widespread technique in (phospho)peptide studies.

In this process the degree of corrosion of the inner surface of the ESI probe would not be susceptible to this phenomenon of mobile phase corrosion, which will add to the corrosion inherent to the electrospray process itself [10]. It has to be noted that "differences" in appearance for SS nanospray emitters were already observed after 48 h of use [25].

To illustrate the detrimental impact of corrosion the inner surfaces of a used and a brand new SSC were examined by electron microscopy. As can be seen in Fig. 11 the tip of the used capillary was heavily corroded whereas the new capillary was undamaged. The same observations were made for the inner surface close to the tip. Away from the spraying tip the surface became more alike to the surface seen in a new SSC. This showed
Fig. 11. Electron microscopy images of a used SSC (left) and a new SSC (right). Images (a) and (b) show the outlook of the tips of the respective SSCs. It is clear the used one (a) is heavily corroded even at the outside. Images (c) and (d) show lengthwise cross-sections (of parts) of both capillaries near the tip. The new one is obviously not entirely smooth but already displays small lobes (d). The inner surface near the tip showed an heavily damaged inner layer (c). When a cross-section of the used SSC further away from the spraying tip was evaluated its appearance was more alike the new SSC inner surface (not shown). Image (e) displays the inner surface at the very end of the used SSC: it is obviously heavily corroded and an increase in inner surface is apparent.
In soil science, it was well established that inorganic phosphates adsorb well to iron (hydr)oxides [26–29]. A maximum sorption of phosphates was observed at low pH and the sorption decreased at higher pH [29]. In the same paper it was stated that sorption was rapid at pH < 8.5; above this value the sorption became kinetically slow. Furthermore, ligand exchange between the (inorganic) phosphate and the surface hydroxyl groups of the oxide surfaces will – according to these studies – lead to the (pH dependent) bidentate binding of the phosphate by the metal ion (chemisorption). Not by coincidence this bidentate formation is believed to be the underlying mechanism in the purification of phosphorylated analytes by Fe(III)-IMAC [30–32]. This information side by side with the here presented data and the electron microscopy images suggested that SS surfaces and especially activated/corroded SS surfaces can chemisorb phosphorylated analytes, most probably via mechanisms also governing Fe(III)-IMAC. However, our observations concerning the monophosphorylated analytes do not fit this model completely. So additional effects as the possibility of multiple interactions, pH conditions, interaction times, etc. might be involved. It is worthwhile mentioning that Fe(III)-complexation concerning the monophosphorylated analytes do not fit this model completely. So additional effects as the possibility of multiple interactions, pH conditions, interaction times, etc. might be involved. It is worthwhile mentioning that Fe(III)-complexation concerning the monophosphorylated analytes do not fit this model completely. So additional effects as the possibility of multiple interactions, pH conditions, interaction times, etc. might be involved. It is worthwhile mentioning that Fe(III)-complexation concerning the monophosphorylated analytes do not fit this model completely. So additional effects as the possibility of multiple interactions, pH conditions, interaction times, etc. might be involved. It is worthwhile mentioning that Fe(III)-complexation concerning the monophosphorylated analytes do not fit this model completely. So additional effects as the possibility of multiple interactions, pH conditions, interaction times, etc. might be involved. It is worthwhile mentioning that Fe(III)-complexation concerning the monophosphorylated analytes do not fit this model completely. So additional effects as the possibility of multiple interactions, pH conditions, interaction times, etc. might be involved.
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