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Technical Note

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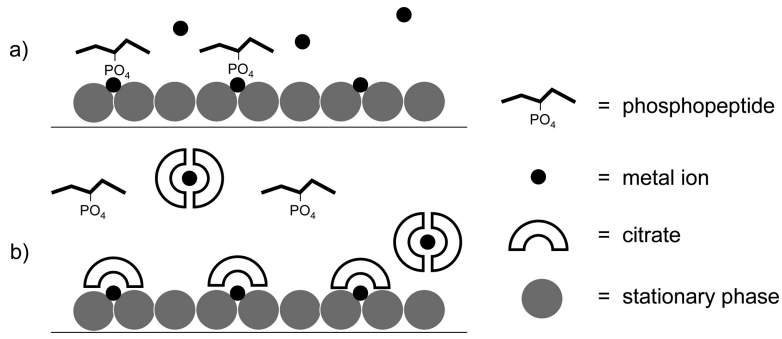


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# Citrate Boosts the Performance of Phosphopeptide Analysis by UPLC-ESI-MS/MS

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***Abstract:*** Incomplete recovery from the LC column is identified as a major cause for poor detection efficiency of phosphopeptides by LC-MS/MS. It is proposed that metal ions adsorbed on the stationary phase interact with the phosphate group of phosphopeptides via an ion-pairing mechanism related to IMAC (IMAC: immobilized metal ion affinity chromatography). This may result in their partial or even complete retention. Addition of phosphate, EDTA or citrate to the phosphopeptide sample was tested to overcome the detrimental phosphopeptide suppression during gradient LC-MS/MS analysis, while the standard solvent composition (water, acetonitrile, formic acid) of the LC system was left unchanged. With the use of UPLC, a citrate additive was found to be highly effective in increasing the phosphopeptide detection sensitivity. Addition of EDTA was found to be comparable with respect to sensitivity enhancement, but led to fast clogging and destruction of the spray needle and analytical columns due to precipitation. In contrast, a citrate additive is compatible with prolonged and stable routine operation. A 50 mM citrate additive was tested successfully for UPLC-MS analysis of a commercial four-component phosphopeptide mixture, a tryptic $\beta$-casein digest, and several digests of the 140 kDa protein SETDB1. In this protein, 27 phosphorylation sites could be identified by UPLC-MS/MS using addition of citrate, including the detection of several phosphopeptides carrying 3−5 pSer/pThr residues, compared to identification of only 10 sites without citrate addition. A 50 mM citrate additive particularly increases the recovery of multiply phosphorylated peptides, thus, extending the scope of phosphopeptide analysis by LC-MS/MS.

**Keywords:** highly phosphorylated phosphopeptides • reverse phase • LC-MS • additive • tandem mass spectrometry • IMAC

## Introduction

Because of its rich structural information, sensitivity and productivity, liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) has developed into the method of choice for analytical proteomics. For the analysis of a complete digest of a protein generated by a specific protease such as trypsin, one might expect similar signal intensities for each peptide by LC-ESI-MS, since all peptides should be formed in about equimolar amounts. However, experimental experience shows that for an individual protein some peptides are consistently observed with high abundance,[1] whereas others are observed with much lower intensity or not at all. This situation led to attempts to empirically predict proteotypic peptides.[1,2] Not surprisingly, detection of phosphopeptides by LC-MS shows a similar characteristic, that is, a highly variable response for individual phosphopeptides. Because of the outstanding biological significance of protein phosphorylation, phosphopeptide analysis by ESI-MS, LC-ESI-MS and MALDI-MS has attracted methodological interest. Efforts to understand their behavior in chromatography and mass spectrometric ionization have been undertaken with the aim to improve their detection.

In matrix-assisted laser desorption/ionization (MALDI)-MS matrix additives and matrix variations have been tested to improve phosphopeptide detection. Diammonium citrate has been demonstrated to improve phosphopeptide detection,[3] when added to the matrix 2,5-dihydroxybenzoic acid. Subsequently, 2,4,6-trihydroxyacetophenone with a diammonium citrate additive was demonstrated to be a very good matrix for phosphopeptide analysis.[4] Finally, addition of phosphoric acid to 2,5-dihydroxybenzoic acid was found to improve selectively the detection sensitivity of phosphopeptides compared to unmodified peptides.[5] The beneficial effects of the matrix additives were explained by their metal complexing properties (citrate) or their ability to 'salt out' phosphopeptides (phosphoric acid), which then are assumed to cocrystallize with the MALDI-matrix more efficiently.

Auxiliary substances have been added to phosphopeptide sample solutions to improve their analysis by positive LC-ESI-MS. The composition of the LC solvents has been left unchanged to avoid interference with chromatographic performance or with the stability of the LC-MS interface. Kim et al.[6] showed that addition of 0.1−1% of phosphoric acid to the sample improves the detection sensitivity for the quadruply phosphorylated tryptic fragment of $\beta$-casein by about 1 order

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of magnitude. However, even after phosphoric acid addition, the detection limit for the quadruply phosphorylated fragment of $\beta$-casein was still at 50 pmol compared to 50 fmol for the singly phosphorylated tryptic peptide. Use of the metal complexing agent EDTA as sample additive represents a different approach to improve the detection limits for phosphopeptides.[7] In that study, a set of highly acidic phosphopeptides was investigated, which contained 3−7 acidic residues in addition to 2−4 phosphoserine residues. The LC-ESI spectra of these highly acidic peptides were demonstrated to exhibit pronounced metal ion adduction (mostly of $Al^{3+}$ and $Fe^{3+}$) which was discussed as phosphopeptide specific metal ion adduction. Upon addition of EDTA to the sample solution, the absolute abundance of the protonated phosphopeptide signals increased, whereas the abundance of the phosphopeotide metal adducts decreased, and the LC elution behavior of the phosphopeptides improved. The combined use of phosphate and EDTA as sample additive has been shown to improve the performance of a four-component phosphopeptide mixture analysis[8] by ultra performance liquid chromatography[9] -tandem mass spectrometry (UPLC-MS/MS). In the study described below, we used addition of citrate as a metal-complexing additive to the sample solution to improve the detection sensitivity of UPLC-MS/MS for phosphopeptide analysis.

## Materials and Methods

**Chemicals and Standards.** Solvents and acids were from Biosolve (Valkenswaard, The Netherlands) in UPLC grade quality. Proteases were from Roche (Mannheim, Germany). EDTA powder was from Applichem (Darmstadt, Germany). Citric acid monohydrate was from Calbiochem (Darmstadt, Germany). The phosphopeptide standard mixture (MassPREP phosphopeptide standard) was from Waters (Milford, MA), composed of measured and equimolar amounts of the following synthetic tryptic enolase peptides: pT18 (= NVPLpYK), pT19 (= HLADLpSK), pT43 (= VNQIGpTLSESIK, and ppT43 (= VNQIGTLpSEpSIK). $\beta$-Casein and all other substances were from Sigma (Deisenhofen, Germany).

**UPLC-Mass Spectrometry.** UPLC-MS/MS analyses were performed using a nanoAcquity UPLC System (Waters, Milford, MA) in combination with a QTOF2 mass spectrometer (Waters Micromass, Manchester, U.K.). The column used was a 150 mm $\times$ 75 $\mu$m BEH $C_{18}$ column packed with 1.7 $\mu$m particles with a pore width of 130 Å. The LC was coupled with the mass spectrometer using a Pico Tip sprayer (Waters, Manchester, U.K.) operated using PicoTips (New Objective, Woburn, MA) with an inner diameter of 10 $\mu$m. UPLC-MS/MS analyses were carried out at a flow rate of 400 nL/min and a column temperature of 35 °C. Samples were loaded directly on the analytical column. After a washing period of 24 min with 1% B (acetonitrile with 0.1% formic acid), a linear gradient was from 99% A (water with 0.1% formic acid), 1%B to 70% A, 30% B in 30 or 60 min, respectively. This resulted in a total run time of 85 or 115 min, respectively.

The mass spectrometer was operated in positive ion mode with a capillary voltage of 2400 V and a cone voltage of 35 V. Data were acquired in DDA (data dependent acquisition) mode, one survey scan of 1 s was carried out followed by up to two MS/MS scans (of 1 s) of each of the three most intense precursor ions. Only multiply charged ions were allowed for fragmentation. MS/MS spectra were processed using MassLynx 4.1 to peak lists and searched against the protein database MSDB human using MASCOT 2.2.1. These searches were performed using full enzyme specificity (for elastase, the no enzyme option was selected) at a MS tolerance of 1 Da and a MS/MS tolerance of 0.5 Da including the following variable modifications: carbamidomethyl-Cys, oxidized Met, phosphorylation at Ser, Thr, Tyr.

**Standard Mixture Preparation.** The phosphopeptide standard mixture was dissolved in water or 50 mM citrate solution before UPLC-MS/MS analysis. $\beta$-Casein was dissolved in 0.1 M $NH_4HCO_3$, denatured using 6 M urea, reduced and alkylated using DTT and iodoacetamide and digested overnight by trypsin in solution at 37 °C. The resulting mixture was diluted using water or citrate, respectively, to $\beta$-casein concentrations between 5 fmol/$\mu$L and 2.5 pmol/$\mu$L and a citrate concentration of 50 mM.

**SETDB1 Preparation.** FLAG-tagged SETDB1 was expressed in HEK293 cells; 24 h after transfection, the cells were treated with the radiomimetic drug neocarzinostatin (NCS) for 1 h. SETDB1 was subsequently immunoprecipitated from protein extracts using FLAG antibody-coated M2 beads (Sigma). Aliquots representing about 2.5 $\mu$g per lane of the immune complexes were separated in 10% SDS-PAGE and the gel was stained with 0.25% Coomassie Brilliant Blue R 250 dissolved in 45% methanol/10% acetic acid. Bands were excised from the gel; destaining and reduction/alkylation were performed as described elsewhere.[10] Then, the gel pieces were dried in a SpeedVac (Savant, Thermo Fischer Scientific, Bremen, Germany) and the protease in its recommended buffer was added to the gel pieces (0.1 M Tris/HCl, pH 8, for AspN; 0.1 M Tris/HCl, pH 8.5, for elastase; 0.1 M Tris/HCl, pH 7.8, 10 mM $CaCl_2$ for chymotrypsin and 0.1 M $NH_4HCO_3$, pH 7.8, for trypsin). The buffer was added in an amount sufficient to cover the gel pieces. The samples were incubated at 37 °C overnight. Extracted peptide fractions were phosphopeptide enriched using $Ga^{3+}$-IMAC (Phosphopeptide Isolation Kit, Pierce, Rockford, IL) following the manufacturer's protocol, but using $NH_4OH$ as elution buffer. Phosphopeptides were eluted using 20 $\mu$L of 400 mM $NH_4OH$ solution, then the eluate fraction was acidified using 3 $\mu$L of 15% FA. The volume of the resulting fractions was reduced using a SpeedVac to approximately 6 $\mu$L. The fraction was split into 2 identical aliquots and 1 $\mu$L of water or 1 $\mu$L of 200 mM citrate solution was added.

## Results

**General Consideration.** The LC-MS/MS analyses in this study were consistently performed without trap column by direct sample injection on the analytical column to avoid sample losses due to incomplete trapping. Addition of a metal-complexing agent to the sample was selected to retain the normal LC solvent system. The idea was that the bolus of the metal-complexing agent introduced with the sample travels through the analytical column directly before the analytes and, thus, generates a modified (cleaned or capped) surface of the stationary phase in each analytical run.

**Selection of Citrate as Additive.** Addition of phosphoric acid was tried but showed only a weak enhancement of phosphopeptide signals. Also, phosphate showed a memory effect on the column with enhancement of the background level as already described.[6] Therefore, phosphate was not studied further. Addition of EDTA showed a significant improvement of phosphopeptide detection, in particular for higher phosphorylated peptides. However, EDTA also shows retention on the LC column, and following a few injections, massive precipitations were observed at the ESI spray needle resulting

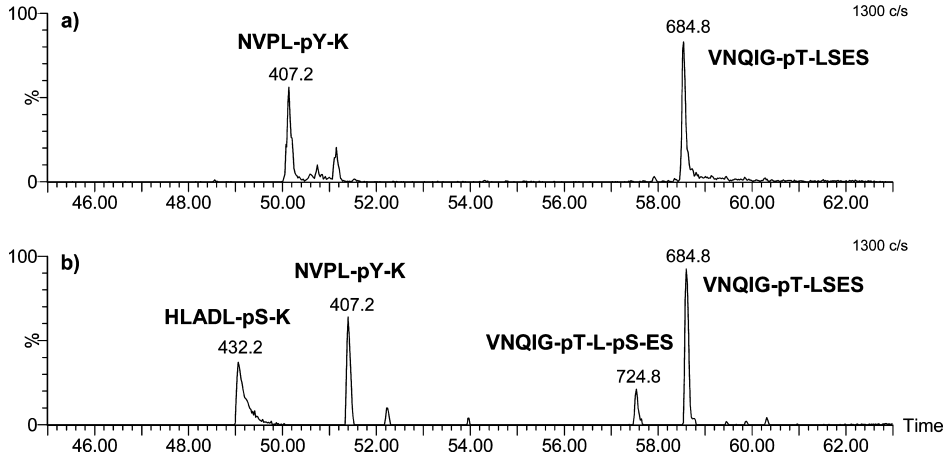


**Figure 1.** UPLC-ESI-MS analysis of the MassPREP phosphopeptide standard containing equimolar amounts of the four phosphopeptides as annotated in (b). The sample amount injected was 200 fmol of each peptide; (a) sample injected in water; (b) sample injected in 50 mM citrate.

in poor performance of the electrospray. Additionally, repetitive injection of EDTA led to the destruction of the analytical column which was probably due to precipitation of EDTA at the column outlet. In our opinion, this effect is caused by the chromatographic separation of the EDTA solubilizing agent ($NH_4^+$ or $Na^+$) and EDTA itself. Because of the low solubility of EDTA at the acidic pH of the LC solvent system (0.1% FA), it precipitates and thus blocks the analytical column. In view of these results, a different metal complexing agent with better solubility at acidic pH was searched. For this selection, the stability constants of $Fe^{3+}$ complexes were considered in particular, since $Fe^{3+}$ is a common trace metal contamination in LC systems, and since complexes with $Fe^{3+}$ and other trivalent metals are used for selective enrichment of phosphopeptides. The stability constant for $Fe^{3+}$−citrate complexes ($K_s$ = 13.5)[11] is lower compared to that of the $Fe^{3+}$−EDTA complex ($K_s$ = 16.1)[11] but still indicates a high stability. In addition, citrate shows a better solubility at acidic pH and less retention on a reversed phase LC column compared to EDTA. Thus, citrate was selected as sample additive for the experiments presented in this study.

**Analysis of a Phosphopeptide Standard Mixture.** As first sample, a mixture of 4 synthetic phosphopeptides (MassPREP phosphopeptide standard, see Materials and Methods) was analyzed by UPLC-ESI-MS with and without addition of citrate. The citrate concentration, the sample volume, the pH of the sample, and the column temperature were investigated with respect to maximum phosphopeptide signal intensities. A 50 mM citrate and an injection volume of 4 $\mu$L delivered the best results; the sample pH value and the column temperature did not show a significant effect. With the use of the optimized conditions and standard conditions without citrate additive, the commercial four-component phosphopeptide mixture was analyzed (see Figure 1).

As shown in Figure 1, the performance of phosphopeptide detection is significantly improved using citrate as sample additive. After addition of citrate, all four species were clearly observed, whereas the histidine-containing monophosphopeptide and the doubly phosphorylated peptide were not observed at all without citrate additive.

**Analysis of a Tryptic $\beta$-Casein Digest.** Tryptic digests of $\beta$-casein are often used as test mixtures to demonstrate the performance of methodological innovations in phosphopeptide analysis work flows, since this sample contains two phosphopeptides of very different structure, a singly phosphorylated (FQ-pS-EEQQQTEDELQDK) and a quadruply phosphorylated peptide (RELEELNVPGEIVE-pS-L-pS-pS-pS-EESITR). In numerous published assays, the $(pSer)_4$-peptide is missing and the widely accepted interpretation for this finding is adsorption on the LC column. We observed that, for injections without citrate addition, elution of the $(pSer)_4$-peptide could only be observed for sample amounts >10 pmol. Following addition of 50 mM citrate, the $(pSer)_4$-peptide could be detected at sample amounts as low as 20 fmol injected, which indicates a reduction of the detection limit for this phosphopeptide by a factor of 500. Figure 2 demonstrates this finding.

In summary, the data in Figure 2 demonstrate the efficiency of citrate to establish roughly uniform detection sensitivity for the two main tryptic phosphopeptides of $\beta$-casein.

**Phosphorylation Analysis of SETDB1.** To further substantiate the positive effect of citrate addition for phosphopeptide analysis by UPLC-ESI-MS, we analyzed the 140 kDa phosphoprotein SETDB1. SETDB1 is a SET domain histone methyltransferase that specifically trimethylates Lys-9 of histone H3. H3 Lys-9 trimethylation represents a specific tag for epigenetic transcriptional repression by recruiting heterochromatin protein 1 (HP1;CBX1;CBX3) to methylated histones. SETDB1 functions mainly in euchromatic regions, where H3 Lys-9 trimethylation is coordinated with DNA methylation. SETDB1 is targeted to histone H3 by TRIM28/TIF1$\beta$, a general transcriptional corepressor recruited by KRAB zinc-finger proteins.[12−14]

Four identical gel bands of immunoprecipitated SETDB1 were in-gel digested using either trypsin, chymotrypsin, endoproteinase AspN or elastase. Peptides were extracted from the gel and phosphopeptides were enriched using $Ga^{3+}$-IMAC. The phosphopeptide eluate fractions were divided into two identical aliquots, which were analyzed by UPLC-ESI-MS/MS in identical manner, except for the addition of citrate in one aliquot. The MS/MS data files obtained were both processed using an identical MASCOT search against the MSDB human protein database. The MASCOT identity/homology threshold scores (≥95% confidence level) for these searches were 53 for the analysis of data generated by specific enzymes (trypsin, chymotrypsin, AspN). For the analysis of data generated using a nonspecific enzyme (elastase), the threshold was 63. The number of phosphopeptides assigned with scores above these threshold values were 2 without addition of citrate, and 8 after

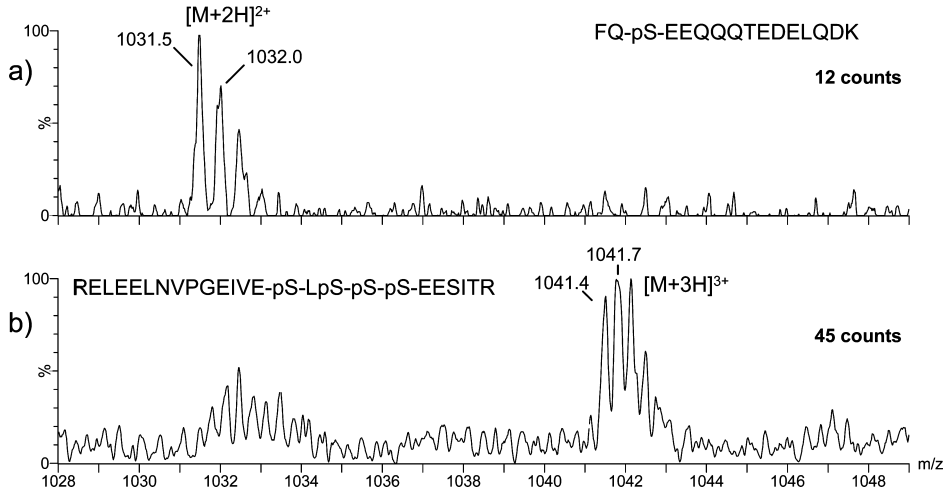


**Figure 2.** Analysis of 20 fmol of a tryptic $\beta$-casein digest using UPLC-MS/MS with citrate additive; (a) molecular ion signal for the T6 phosphopeptide, retention time 56.2 min; (b) molecular ion signal for the T1−2 phosphopeptide, retention time 68.2 min. Both phosphopeptide signals exhibit signal-to-noise ratios sufficient for their detection.

**Table 1.** Summary of the Phosphorylation Analysis by UPLC-MS/MS of SETDB1[a]

| number of phosphorylation sites/peptide | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| **Peptides assigned with citrate** | **16** | **10** | **4** | **2** | **1** |
| peptides assigned without citrate | 7 | 1 | 1 | - | - |
| *ratio [with citrate]/ [without citrate]* | *2.7* | *10* | *4* | - | - |
| **summed peak areas with citrate** | **11362** | **2942** | **2046** | **921** | **511** |
| summed peak areas without citrate | 5304 | 183 | 73 | -- | -- |
| *ratio [with citrate]/ [without citrate]* | *2.1* | *16* | *28* | -- | -- |

[a] For details, see Table S1 in Supporting Information). Trypsin, AspN, chymotrypsin, and elastase were employed for in-gel digestion of SETDB1 and were evaluated with respect to the phosphopeptides assigned by MASCOT. With the use of the four proteases, the following numbers of phosphopeptides were assigned, trypsin (13), elastase (7), AspN (7), and chymotrypsin (6). For data evaluation, the signal intensities (peak areas of the extracted ion chromatograms of each phosphopeptide signal), and the number of identified phosphopeptides were considered. The data are ordered according to the number of phosphate groups per peptide.

addition of 50 mM citrate. The results are summarized in Table 1 and displayed in more detail in Table S1 in Supporting Information. All phosphopeptide annotations shown in Table S1 including those with subthreshold scores were ranked on position one within the individual MASCOT peptide hit lists, meaning that they represent the best hits among the other possibilities. The annotations were checked manually with respect to peptide sequence and phosphorylated residue(s), and are considered to be correct including those with subthreshold scores.

The results given in Table 1 clearly demonstrate the positive influence of a citrate additive. When citrate is used, the number of phosphopeptides detected is 33, whereas only 9 phosphopeptides were identified by conventional analysis. This leads to the identification of 27 nonredundant phosphorylation sites with and of only 10 sites without citrate addition (for details see Figure S1 and Table S1 in Supporting Information). The summary of the results in Table 1 shows the general superiority of the UPLC-MS/MS data generated with citrate additive. Already for the singly phosphorylated peptides, the sum of the peak areas is increased by a factor of 2 by citrate addition. This factor increases to a value of about 15 for the doubly phosphorylated peptides and to nearly 30 for triply phosphorylated peptides. Finally, this factor cannot be calculated for peptides which contain four or five sites of phosphorylation, since these are not detected at all without citrate additive. When the number of assigned phosphopeptides is compared in the same way as the peak areas, the ratios in favor of the citrate addition are somewhat smaller, but still consistently in favor of the citrate addition (enhancements between 2.7 and 10). Thus, all data evaluation methods prove the strongly positive influence of citrate addition on phosphopeptide detection and identification. To visualize the necessity of citrate addition for identification of highly phosphorylated peptides, Figure 3 shows survey scans accumulated over the elution period of the 1−5 times phosphorylated forms of the tryptic T131-T132 fragment of SETDB1.

It can be clearly seen that in both analyses the singly and doubly phosphorylated forms exhibit comparable signal intensities. In contrast, the triply phosphorylated species exhibit significantly lower signal intensities using injection without additive (Figure 3a), and the forms with four or five sites of phosphorylation are not detected at all under these conditions. Following the addition of citrate to the sample buffer (Figure 3b), a clear identification of all phosphorylated peptides up to the 5 times modified form is possible. The signal intensities and the quality of the corresponding MS/MS spectra are of excellent quality and allow a straightforward pinpointing of the phosphorylated residues. As an example, Figure 4 shows the MS/MS spectrum of the pentaphosphorylated peptide at $m/z$ 1268. Although no neutral losses of $H_3PO_4$ from the molecular ion are observed, the neutral losses from the corresponding sequence ions are present. Extensive sequence information and identification of both peptide ends confirm the presence of the assigned peptide including the positions of phosphorylation.

## Discussion

The data in Figure 1a show that in standard LC-MS/MS analysis the LC recovery of diphosphorylated peptides and of monophosphorylated peptides with a histidine residue

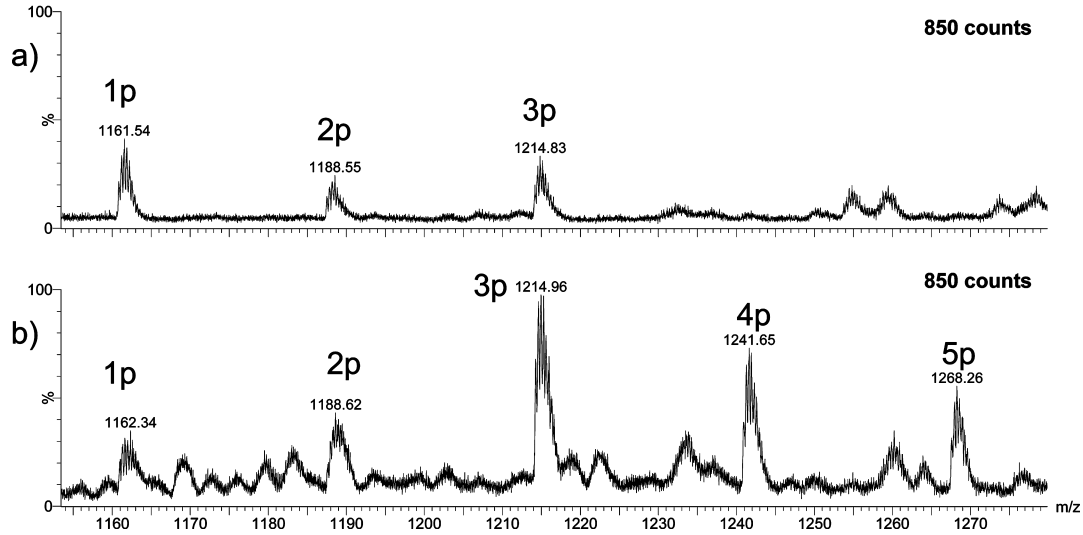


**Figure 3.** Summarized survey scans of an UPLC-MS/MS run of a tryptic digest of SETDB1 over the elution period of the 1−5 times phosphorylated form of the tryptic 33mer peptide KPTAGQTSATAVDSDDIQTISSGSEGDDFEDKK (T131−132); (a) injection without citrate additive; (b) injection with citrate at a concentration of 50 mM. For one analysis, a total amount of about 1 $\mu$g of the immunoglobulin/protein complex of SETDB1 was loaded onto a gel and analyzed after in-gel digestion and phosphopeptide enrichment.

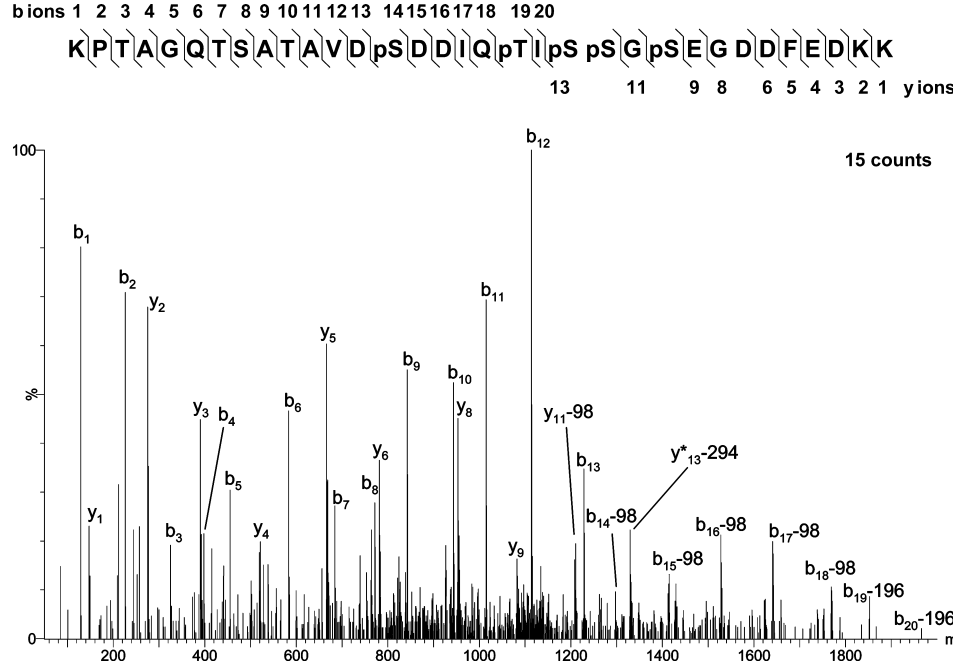


**Figure 4.** LC-MS/MS spectrum of the pentaphosphorylated tryptic 33mer peptide T131−132 derived from SETDB1. A tryptic digest of this protein was injected using a 50 mM citrate additive. The $[M + 3H]^{3+}$ ion at *m/z* 1268 was selected as precursor ion. The fragment ions from the b and y ion series deliver extensive sequence information and confirm the positions of the phosphorylated residues. The error graphs showing the deviations of the experimental and calculated fragment ion *m/z* values are shown in Figure S3 in Supporting Information.

may be impaired, since IMAC has been developed for enrichment of His-containing proteins.[15] The $\beta$-casein digest analysis in Figure 2 shows that after citrate addition the tetraphosphorylated $\beta$-casein tryptic fragment can be detected at a level of 20 fmol injected. Finally, the results obtained for SETDB1 clearly show that under standard conditions the detection efficiency for phosphopeptides is negatively correlated to the number of their phosphate groups (Table 1). A characteristic of standard LC conditions is the signal suppression of highly phosphorylated peptides, as visible in Figure 3a. This Figure displays five peptides with identical amino acid sequence, which differ only in their phosphorylation status. The observed nonuniform detection sensitivities can be directly ascribed to the number of phosphate groups present, leading to the general conclusion that an increase in the number of phosphorylation sites causes a decrease in detection sensitivity. This feature can also be recognized indirectly from sets of phosphopeptides identified in phosphoproteomic studies, for example, in the analysis of the yeast phosphoproteome.[16] In these studies, preferentially singly and doubly phosphorylated peptides are found. It appears that, in this property of phosphopeptide

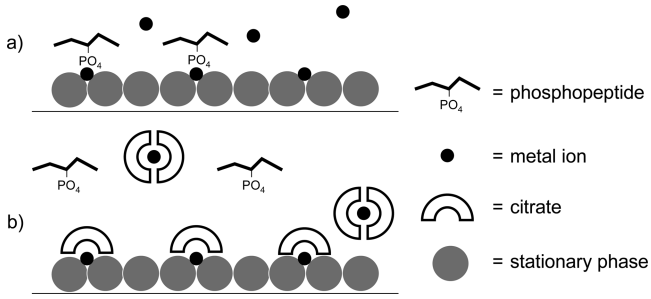


**Figure 5.** Model proposed for the ion-pair interaction of phosphopeptides with metal ions in solution and metal ions immobilized at the stationary phase of a reversed phase LC column: (a) at standard acidic conditions used for LC-MS of peptides, phosphopeptides are coordinated to metal ions, which are present as contaminants on the surface of the reversed phase material; (b) in the presence of citrate, dissolved metal ions are complexed and the free coordination sites of adsorbed metal ions are capped, so that phosphopeptides are not bound to metal ions in solution or at the stationary phase.

retention, the reversed phase column performs like an IMAC column, for which preferential retention of highly phosphorylated, highly acidic, and of histidine-containing peptides is characteristic. Thus, an IMAC-related ion-pairing mechanism is proposed for interpretation of the suppression effects of reversed phase columns in phosphopeptide analysis. The model proposed is shown schematically in Figure 5:

Because of the production process[17] and due to the continuous contact with the trace elements released from LC system parts or contained already in the LC solvents, the column material may contain adsorbed/partially complexed metal ions, such as $Fe^{3+}$, $Al^{3+}$, $Ni^{2+}$, and $Ca^{2+}$. These immobilized cations still have free coordination sites, which can capture phosphopeptides via their phosphate group (see Figure 5a). The positive effect of a citrate additive is caused by complexation of the free coordination sites of the metal ions immobilized on the reversed phase column. A small percentage of adsorbed metal ions may be washed out by complexing agents like citrate, but we assume that a substantial portion of the adsorbed metals is 'capped' by citrate and not removed (see Figure 5b). This capping of the immobilized metal ions blocks the detrimental interaction with phosphopeptides, in particular with highly phosphorylated and/or histidine-containing phosphopeptides so that a normal recovery of these species in the LC-eluent is observed. Because the probability for identification of a peptide is directly related to its intensity, a higher molecular ion intensity is generally beneficial for identification of phosphopeptides. A reduced ionization efficiency of phosphopeptides in positive ion mode electrospray ionization may reduce the detection efficiency for these species. However, these effects are of minor importance, and sometimes the ionization efficiency of phosphopeptides may even exceed that of their unmodified counterparts, in particular for highly basic peptides.[18]

## Conclusions

It can be concluded that the major cause for a low detection efficiency of phosphopeptides in LC-ESI-MS/MS analysis is adsorption to the reversed phase column material, for which an IMAC-related mechanism is proposed. This study shows that a citrate additive to the sample solution is highly effective to overcome this adsorption. Compared to other additives like EDTA or phosphate, citrate is an easy-to-use sample additive and shows excellent compatibility with continuous operation of an LC-ESI-MS system.

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**Supporting Information Available:** Sequence of SETDB1_human; extracted ion chromatograms of the differentially phosphorylated forms of the tryptic peptide KPTAGQTSATAVDSDDIQTISSGSEGDDFEDKK (T131-T132); mass errors within the MS/MS spectrum of the phosphopeptide KPTAGQTSATAVD-pS-DDIQ-pT-I-pS-pS-G-pS-EGDDFEDKK This material is available free of charge via the Internet at http://pubs.acs.org.

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