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Investigation of the use of immobilised metal affinity chromatography for the on-line sample clean up and pre-concentration of nucleotides prior to their determination by ion pair liquid chromatography-electrospray mass spectrometry: a pilot study

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Abstract

This study explored an alternative way to enrich and pre-purify biological samples containing nucleoside mono-, di- and triphosphates. These compounds were trapped by immobilised metal affinity chromatography (IMAC) on a Poros® 20 MC IMAC-column, which was conditioned with Fe^{3+}. The IMAC-column was implemented in a column switching set-up separating nucleoside mono-, di- and triphosphates on a Hypersil ODS 35 mm × 0.3 mm capillary column hyphenated to electrospray mass spectrometry resulting in the first miniaturised column switching liquid chromatography–mass spectrometry (LC–MS) system for nucleotides.

Keywords: Immobilised metal affinity chromatography; Nucleotides

1. Introduction

In order to understand the role nucleotides play in biochemical processes it was important to develop methods for their separation, quantitation and identification [1–9]. Additionally biochemical studies of the metabolism of anti-viral and cytostatic nucleoside-analogues justified the development of new liquid chromatography–mass spectrometry (LC–MS) methods. Their activity is often the result of intracellular phosphorylation [10]. As a consequence methods were needed to identify these nucleotides and to determine their intracellular levels. Several LC–MS(/MS) procedures identifying and quantitating nucleotide-analogues have been reported [11–20]. Most applications rely on the high selectivity of tandem mass spectrometric modes to identify or quantify the compounds of interest present in minimally processed samples. This approach is feasible once the analytes of interest are known and their mass spectrometric behaviour is described, yet it may be limited by the presence of matrix compounds which may compromise detection [21].

Our current research searched for more generally applicable methods. It focuses on the development of miniaturised column switching LC–MS(/MS) procedures for the simultaneous analysis of mixtures of nucleoside mono-, di- and triphosphates present in complex biological matrices. Multi-dimensional chromatography enables the pre-concentration of the compounds of interest and/or an on-line sample clean up. The latter permits the removal of matrix components which may interfere with mass spectrometric detection. Electrospray (tandem) mass spectrometry has a predominant position as an analytical tool as a result of its soft ionisation characteristics [22], the ability to generate both qualitative and quantitative information and its behaviour as a concentration sensitive detector [23]. The

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latter feature allows the reduction of the column diameters in chromatography (miniaturisation) leading to a gain in mass sensitivity [24].

The negative charges of the phosphate groups present in nucleotides were the most obvious targets to select for sample clean-up and pre-concentration. However charge is not a very selective criterion for trapping a group of compounds. Therefore another common feature of nucleotides is expedient. If one wants to include all common and uncommon endogenous nucleotides as well as a wide variety of phosphorylated nucleoside-analogues, the phosphate moiety is the only common feature. Literature shows that selective retention based on the phosphate moiety alone is feasible. Selective enrichment of phosphopeptides from a mixture containing non-phosporylated peptides has already been reported [25–33] and Buscher et al. noted the same for inositol phosphates [34]. A similar approach to enrich benzo[*a*]pyrene–DNA adducts was recently presented by Gennaro et al. [35]. This selective retention is achieved by using immobilized metal affinity chromatography, a technique based on the reversible complexation of electron-donating groups, such as phosphate, by an appropriate immobilised metal ion. In this paper, we present the first results of IMAC as an on line sample clean up and pre-concentration tool for nucleotides and its inclusion in a miniaturised column switching LC–MS.

2. Experimental

2.1. Chemicals and reagents

All nucleobases, nucleosides and nucleotides were purchased from Sigma–Aldrich (Bornem, Belgium) in the highest available grade. All 12 nucleotides were in the sodium salt form. Minimum Essential Medium Eagle (without sodium phosphate) was also from Sigma–Aldrich (Bornem, Belgium). *N*,*N*-dimethylhexylamine (*N*,*N*-DMHA), ammonium dihydrogen phosphate ($NH_4H_2PO_4$) acetic acid (HOAc; p.a.), methanol (MeOH; HPLC-grade), water (H_2O; HPLC-grade), ammonium hydroxide 28–30 wt.% (NH_4OH; p.a.), hydrochloric acid 37% min; (HCl; p.a.), ethylenediaminetetraacetic acid (EDTA; 99%), ammonium acetate (NH_4OAc; anhydrous, p.a.), iron(III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$; p.a.), copper(II) chloride dihydrate ($CuCl_2{\cdot}2H_2O$; p.a.) and sodium chloride (NaCl; p.a.) were from Acros (Geel, Belgium). Trichloroacetic acid (TCA) was purchased from Merck (Overijse, Belgium) and diethyl ether from Vel (Leuven, Belgium). 0.2 μm Nylon 4, 13 and 25 mm 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 between 10^{-2} and 10^{-3} M and stored at −21 °C until use. Fig. 1 shows the structures of the nucleotides used in this study. In addition Table 1 presents the relevant *m*/*z* values of each of the compounds under study. When required the stock solutions were diluted and properly mixed in the desired proportions in the appropriate solvent immediately before use. For general method development the nucleotides were diluted in 0.1% HOAc. In order to test the system's selectivity the stock solutions were spiked into phosphate-free cell culture medium. Before spiking, 8 mL of 10% TCA was added to 8 mL of medium. The mixture was incubated for 30 min on ice, followed by 15 min of centrifugation (457 × *g*; Mistral MSE, Loughborough, UK). The supernatant was three times extracted with diethyl ether (8 mL). The nucleotides were spiked in the resultant H_2O-phase. All samples were filtered through 0.2 μm 4 mm HPLC syringe filters prior to their analysis.

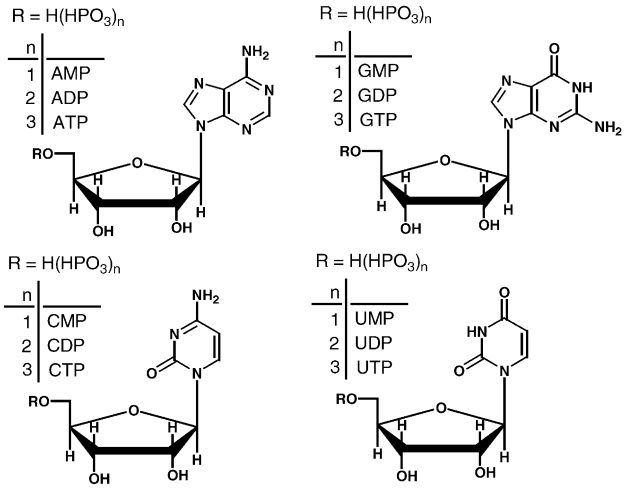

Fig. 1. The analysed nucleotides.

Table 1
Molecular masses and *m*/*z* values of the deprotonated nucleobases, nucleosides and nucleoside mono-, di-, and triphosphates

	Abbreviation	M_r	$[M-H]^-$
Adenine	Ade	135	134
Adenosine	Ado	267	266
Adenosine 5′-monophosphate	AMP	347	346
Adenosine 5′-diphosphate	ADP	427	426
Adenosine 5′-triphosphate	ATP	507	506
Guanosine 5′-monophosphate	GMP	363	362
Guanosine 5′-diphosphate	GDP	443	442
Guanosine 5′-triphosphate	GTP	523	522
Uridine 5′-monophosphate	UMP	324	323
Uridine 5′-diphosphate	UDP	404	403
Uridine 5′-triphosphate	UTP	484	483
Cytosine	Cyt	111	110
Cytidine	Cyd	243	242
Cytidine 5′-monophosphate	CMP	323	322
Cytidine 5′-diphosphate	CDP	403	402
Cytidine 5′-triphosphate	CTP	483	482

2.3. Solutions and mobile phases

All solvent compositions used for chromatography on a Poros® 20 MC IMAC phase were prepared in HPLC-grade H_2O. Solutions used in the IMAC-column conditioning procedure were 50 mM EDTA in 1 M NaCl, 0.5 M HCl, H_2O and 0.1 M NaCl. The metal ion solutions (Cu^{2+} and Fe^{3+}) were prepared in 0.1 M concentrations. In order to elute the IMAC-column several solvent compositions were investigated. The elution solutions can be found in Table 2 and were filtered just before use by means of the 0.2 μm 13 mm HPLC syringe filters.

The analytical separation of the nucleotides was achieved with a binary gradient program. The applied mobile phases were: (A) 5 mM *N*,*N*-DMHA/1 mM $NH_4H_2PO_4$ in water, pH adjusted to 7 with HOAc; and (B) 1 mM $NH_4H_2PO_4$ in 80:20 MeOH/H_2O mixture.

2.4. Capture of nucleoside mono-, di- and triphosphates by capillary IMAC chromatography

The Poros® 20 MC IMAC material was packed in 300 μm i.d. × 5 mm columns by LC Packings (Amsterdam, The Netherlands). In order to comply with the flow rates needed in capillary chromatography, a dedicated HPLC-system (CapLC; Micromass, Manchester, UK) equipped with three solvent lines (binary gradient + auxiliary) and an autosampler was used. Both the CapLC system and the mass spectrometer were controlled using the Masslynx software suite (Masslynx 3.5, Micromass, Manchester, UK). The IMAC-column conditioning sequence was automated. For that purpose a 20 μL sample loop was mounted on the 6-port injection valve of the autosampler. The injection loop was subsequently filled with each of the conditioning solutions (Fig. 6) and the content of the loop was delivered to the IMAC-column at 10 μL/min by the auxiliary solvent line. During the conditioning process, the solvent was sent to waste. This was accomplished by mounting the IMAC-column on a 10-port valve (VALCO, Houston, Texas) which was used as a stream select valve.

The IMAC-column was conditioned according to the instruction of the supplier of Poros® 20 MC IMAC material (The Nest Group, Massachusetts, USA) with subsequently: 50 mM EDTA in 1 M NaCl; H_2O; metal ion solution; H_2O; 0.1 M NaCl; H_2O. Because EDTA was not efficient to remove all metal ions from the IMAC-column, a 0.5 M HCl solution was used (The Poros® stationary phase is stable over the complete pH range 1–14). The injection of 0.1 M NaCl was omitted as no appreciable differences in chromatographic performance was observed. To ensure a maximal metal load two consecutive metal ion injections were programmed (loading more metal sometimes led to column clogging). The final injection sequence applied to condition the IMAC-column in the column switching LC–MS procedure was: 0.5 M HCl; H_2O; 0.1 M metal ion solution; 0.1 M metal ion solution; H_2O.

After the conditioning sequence 20 μL of a nucleotide sample was injected on the IMAC-column in the 0.1% HOAc carrier flow.

Subsequent nucleotide elution of the IMAC-column was carried out in different ways depending on the experiment. In the preliminary IMAC-experiments (cf. Section 3.1.1) elution was achieved by imposing a step gradient (0–100% in 1 s) between 0.1% HOAc (IMAC-column conditioning, sample loading) and aqueous NH_4OH (pH = 10). In the other experiments elution was realised by one or more 20 μL

Table 2
Summary of experiments in which the influence of the elution plug compositions on both (decoupled) dimensions of nucleotide separation, Fe^{3+}-IMAC and the ion pair LC, were tested

Plug	IMAC	Ion pair LC			
		5 μL	3 μL	2 μL	1 μL
H_2O (1)		∼OK			OK
NH_4OH pH = 10 (2)	∼OK				
NH_4OH pH = 11 (3)	∼OK				
0.1% (w/v) NH_4OAc pH = 10 (4)	OK	Ø		Ø	Ø
0.1% (w/v) NH_4OAc pH = 6.7 (5)	Ø	∼OK/DP			
0.2% (w/v) NH_4OAc pH = 6.7 (6)	Ø	∼OK/DP			
0.5% (w/v) NH_4OAc pH = 6.7 (7)	Ø	Ø/DP			
0.1% (w/v) NH_4OAc pH = 8 (8)	Ø	∼OK/DP			
0.2% (w/v) NH_4OAc pH = 8 (9)	Ø	Ø/DP			
0.5% (w/v) NH_4OAc pH = 8 (10)	Ø	Ø/DP			
0.1% (w/v) $NH_4H_2PO_4$ pH = 4.7 (11)	Ø		OK		OK
0.2% (w/v) $NH_4H_2PO_4$ pH = 4.7 (12)	Ø		∼OK/DP		OK
0.5% (w/v) $NH_4H_2PO_4$ pH = 4.7 (13)	Ø		∼OK/DP		OK
0.1% (w/v) $NH_4H_2PO_4$ pH = 8 (14)	∼OK		∼OK/DP		∼OK
0.2% (w/v) $NH_4H_2PO_4$ pH = 8 (15)	∼OK		∼OK/DP		∼OK
0.5% (w/v) $NH_4H_2PO_4$ pH = 8 (16)	OK		∼OK/DP		∼OK

∼OK: moderate performance: IMAC, referring to peak width and recovery; ion pair LC, referring to the separation quality. Ø: no result: IMAC, no nucleotide elution; ion pair LC, no separation. DP: double peaks: ion pair LC, tendency of components to elute in two chromatographic peaks.

injections of elution plugs of different composition in the 0.1% HOAc flow.

After each analysis the IMAC-column was regenerated.

2.5. Ion pair chromatography

The employed ion pair chromatography was based on previous experiments [36]. Fast screening of the influence of the sample solvent composition (cf. Section 3.2.1) was done with the following gradient program: 5–15% B in 10 min (+ 10 min of equilibration). Full loop injections were used to introduce the nucleotide samples. For the ion pair chromatography in the final column switching LC–MS method (cf. Section 3.2.3) the following gradient elution steps were programmed: starting from 1 to 25% B in 24 min, from 25 to 100% B in 10 min and from 100 to 1% B in 1 min.

2.6. Column switching LC–MS for nucleotides

The IMAC-precolumn and the reversed phase (RP) analytical column were connected to a 10-port valve (VALCO, Houston, Texas), as shown in Fig. 2. In addition a 1 μL trapping loop was mounted on the 10-port valve. The auxiliary solvent line (0.1% HOAc 10 μL/min) was connected to the injection valve of the autosampler. The injector was coupled to the IMAC-precolumn. The gradient solvent lines were permanently delivering to the analytical column at a flow rate of 7 μL/min.

During both the IMAC-precolumn conditioning runs (3 min/each) and sample injection (7 min) the effluent of the precolumn was diverted to the waste via the trapping loop. Subsequent elution of the nucleotides from the IMAC-precolumn was achieved by a 20 μL injection of 0.5% (w/v) $NH_4H_2PO_4$ pH = 8 (Table 2: 16) in a 10 μL/min flow. At the same moment the analytical gradient was started. The elution plug released the compounds absorbed on the IMAC-precolumn. Once the bulk of the nucleotides was present in the 1 μL trapping loop the 10-port valve was switched. As such the content of the trapping loop could be

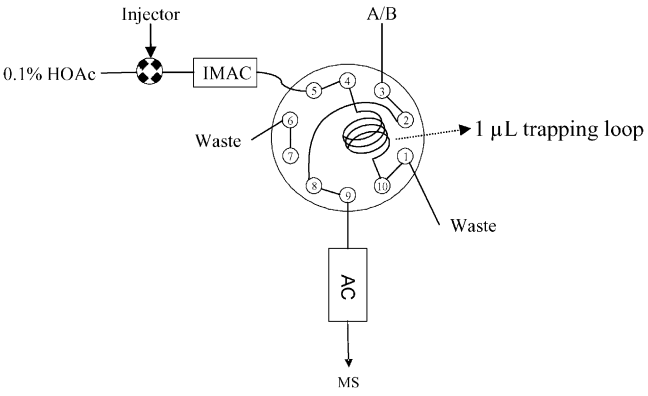

Fig. 2. The column switching set-up in trapping position. In LC–MS mode, connections are made between ports 1 and 2, 3 and 4, 5 and 6, 7 and 8, 9 and 10.

introduced in the gradient flow and as such "injected" on the analytical column. After completion of the gradient program a 0.1% (w/v) NH_4OAc pH = 10 (Table 2: 4) elution plug was injected on the IMAC-column, in order to remove residual nucleotides from the IMAC-column and diverted to the waste.

To determine the optimal switching time, the set-up described above was made but without analytical column connecting the 10-port valve directly to the mass spectrometer. A complete IMAC-conditioning sequence was executed and a 20 μL nucleotide test sample applied. Elution was realised by means of a 20 μL $NH_4H_2PO_4$ 0.5% (w/v) pH = 8 (Table 2: 16) elution plug. The start time of the elution peak was noted and was corrected for the dead volume between the 10-port valve and the mass spectrometer (silica tubing and the ES-capillary). At this moment the bulk of the nucleotides was still present in the 1 μL trapping loop.

2.7. Mass spectrometric conditions

Electrospray mass spectra were recorded in the negative ion mode (ES(−)) on a Q-TOF II mass spectrometer (Micromass, Manchester, UK) equipped with a pneumatically assisted Z-spray source (Micromass, Manchester, UK) using a standard electrospray probe. The ionisation voltage applied was 3.0 kV. In the experiments where the efflux of the IMAC-column was analysed, the cone voltage was set at 30 V and the source temperature at 80 °C. Full scan spectra with a mass range of *m/z* 100–600 were recorded. During the ion pair LC–MS experiments the experimental conditions were: ionisation voltage 3.0 kV, cone voltage 40 V, source temperature 80 °C and a mass range of *m/z* 50–800. For the column switching LC–MS the MS-conditions were different: ionisation voltage 3.0 kV, cone voltage 40 V, source temperature 150 °C and a mass range of *m/z* 100–800. Additionally a MeOH make-up flow of 3 μL/min was added through a T-piece in order to improve the ES spray characteristics and sensitivity of the column switching LC–MS. This flow was delivered by an additional HPLC pump (Kontron Instruments, Milan, Italy). For all experiments the flow rate of the nebulising gas (N_2) and drying gas (N_2) was ca. 20 and 300 L/h, respectively.

3. Results and discussion

3.1. Investigation of the retention behaviour of nucleoside mono-, di- and triphosphates on the IMAC-precolumn

3.1.1. Nucleotide retention on IMAC resins

In the literature a dominant affinity of copper (Cu^{2+}) loaded iminodiacetate gels for the heterocyclic bases of the common nucleo(s/t)ides [37–39] is described. This is in contrast to iron (Fe^{3+}) loaded iminodiacetate gels which have been reported to exhibit a strong affinity towards the phosphate groups present in nucleotides [40] but no affinity to

nucleosides or nucleobases. Several models are used to explain the differential retaining behaviour of metal ions in IMAC. The primary action of binding is based on the formation of co-ordination bonds between the immobilised metal ion and electron donating groups delivered by the compounds of interest [41–43]. The different selectivity exerted by various metal ions towards different kinds of nucleophiles is generally explained by the Hard and Soft Acids and Bases (HSAB) principle of Pearson [44]. Hard Lewis acids like Fe^{3+} tend to bind with hard Lewis bases, like oxygen. In Pearson's classification Cu^{2+} is considered to be an acid of intermediate strength, which is predicted to prefer binding to borderline bases as the nitrogen in aminoaryl groups and azaheterocycles [44].

For the reasons explained earlier our primary interest laid in creating an environment allowing retention using the presence of phosphate moieties of nucleoside mono-, di- and triphosphates. Nevertheless both metal ions (Cu^{2+} and Fe^{3+}) were tested for their capability to retain/trap nucleotides. The first aspect explored was the study of the behaviour of nucleotides on a Poros® 20 MC IMAC resin which was originally developed for trapping phospho-peptides.

Column conditioning was achieved by means of a series of subsequent 20 μL injections in a HOAc flow. The applied test mixtures were made of cytosine, cytidine, CMP, CDP, CTP, adenine, adenosine, AMP, ADP and ATP dissolved in 0.1% HOAc and each at a concentration of 1×10^{-5} M. Elution, based on competing hydroxyl binding [42], was realised by changing the mobile phase to aqueous NH_4OH (pH = 10), 2 min after injection. The effluent of the column both during the sample loading- and eluting step was monitored using full scan ES(−)MS. An evaluation of the extracted ion chromatograms and the corresponding MS-spectra of the compounds under investigation showed that the nucleobases and nucleosides were not retained at all and that they eluted with the void volume of the column. All nucleotides were trapped on the column until aqueous NH_4OH was applied (Fig. 3). Surprisingly, identical results were obtained using Cu^{2+}-loaded columns (results not shown) which is in disagreement with literature data mentioned above.

The apparently inconsistent behaviour of Cu^{2+} could tentatively be explained. One could consider its intermediate position in Pearson's HSAB classification which implies that phosphate co-ordination can not be ruled out. Furthermore the affinity Cu^{2+} exerts towards nucleobases was reported only to hold true in a very limited pH range (about pH = 7) [38]. Therefore we could conclude that under our experimental conditions, both Cu^{2+} and Fe^{3+} enforced retention of nucleotides based on phosphate co-ordination. It is worthwhile to emphasize that all nucleotides eluted at the same moment, suggesting that only one phosphomonoester was responsible for binding. This agrees well with a previous report [40]. The simultaneous elution of all nucleotides also opened the possibility to use IMAC in a column-switching set-up. Since both metal ions gave similar results Fe^{3+} was arbitrarily chosen for further experiments.

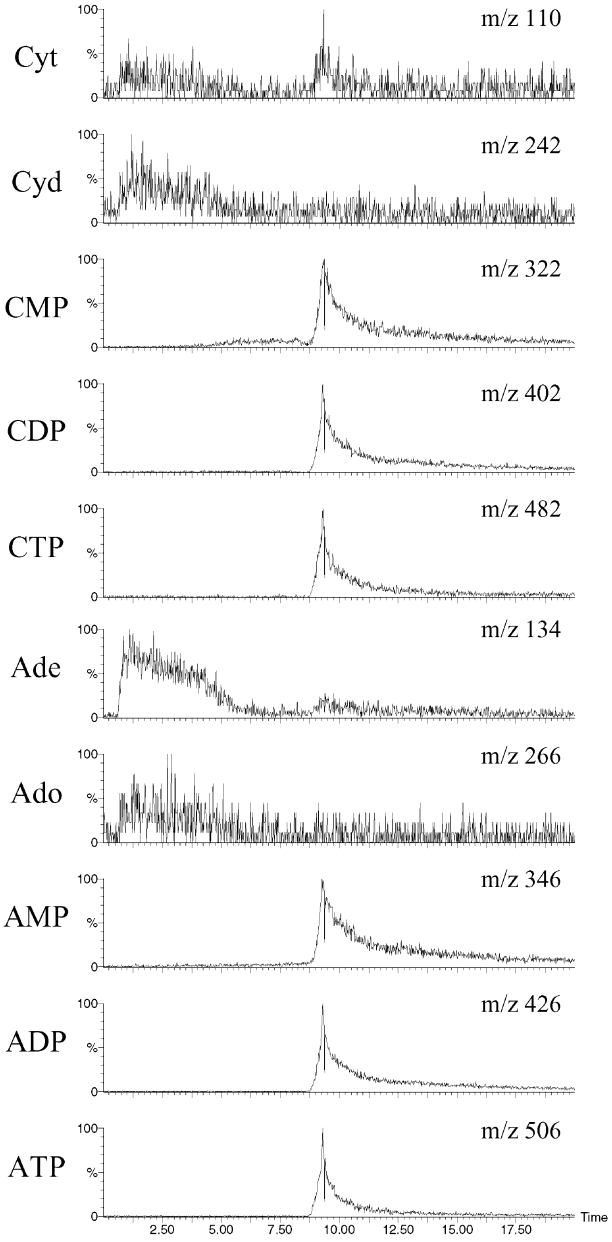

Fig. 3. Extracted ion chromatograms for $[M-H]^-$ ions showing selective retention of the nucleotides on a 300 μm i.d. × 5 mm Fe^{3+}-IMAC-column.

3.1.2. The concept of plug elution

The exploration of the feasibility to use IMAC as a selective retention tool for nucleotides fell within our objective to develop a column switching LC–MS system. The approach we adopted was a column switching set-up in which the precolumn was never put on-line with the analytical column [33]. As such the contamination of the LC–MS system by possible metal leaking would be kept minimal. In order to be able to apply a binary gradient elution and to deliver the sample to and from the IMAC-column with only three solvent lines available, we introduced the concept of the elution plug. In this approach elution of sample from the IMAC-column to the analytical column was performed

by a 20 μL injection of a suitable solvent plug using the autosampler.

Three elution-solutions (Table 2: 2–4) were tested: NH_4OH pH = 10; NH_4OH pH = 11 and 0.1% (w/v) NH_4OAc pH = 10. With these compositions competing hydroxyl binding was exploited as the main elution mechanism.

After conditioning the IMAC-column, 20 μL of the sample mix (see Section 3.2.1) was injected at a flow rate of 10 μL/min, followed by a 5 min washing cycle. Introduction of the wash step was done in anticipation of future experiments with real life samples. This would allow to discard impurities to the waste, while capturing the compounds of interest. After this step 20 μL of the elution plug was introduced, again followed by 5 min of rinsing. The nucleotides liberated by the elution plug were expected to elute during the 2 min injection time of the plug since the void volume of the column was far less than 20 μL. Subsequently a second 20 μL injection of the same elution plug composition was applied in order to check any memory effects. Sample loading and elution were monitored using full scan ES(−)MS.

As expected the nucleobases and the nucleosides were not retained on the column. In addition it was noted that during the washing step some of the nucleoside monophosphates and to a lesser extend nucleoside diphosphates were already eluting (Section 3.1.3).

The three different elution plugs described above were able to liberate the bulk of the retained nucleotides in one shot, as concluded by comparison of the elution profiles (cf. the extracted ion chromatograms) and spectra after applying a second elution plug. The composition consisting of 0.1% (w/v) NH_4OAc pH = 10 (Table 2: 4) gave the best results in terms of peak widths and shapes. The elution volume in which the nucleotides were present was approximately 3 μL. This corresponded with a pre-concentration factor of about 7.

3.1.3. Selectivity of Fe^{3+}

As stated above a slightly different behaviour of the nucleoside mono- and diphosphates versus the nucleoside triphosphates was observed during the washing step. In order to get some more insight in this phenomenon additional experiments were conducted. A test mixture containing AMP, ADP, ATP, GMP, GDP, GTP, CMP, CDP, CTP, UMP, UDP and UTP was prepared each at a concentration of 5×10^{-6} M. An aliquot of 20 μL of the sample was introduced in 0.1% HOAc at a flow rate of 5 μL/min for 15 min. This step allowed capture of the nucleotides.

As can be seen a break through of the nucleotides was observed (Fig. 4). This was most pronounced in the case of the nucleoside monophosphates and to a lesser extend for the nucleoside diphosphates. Also it was noted that the

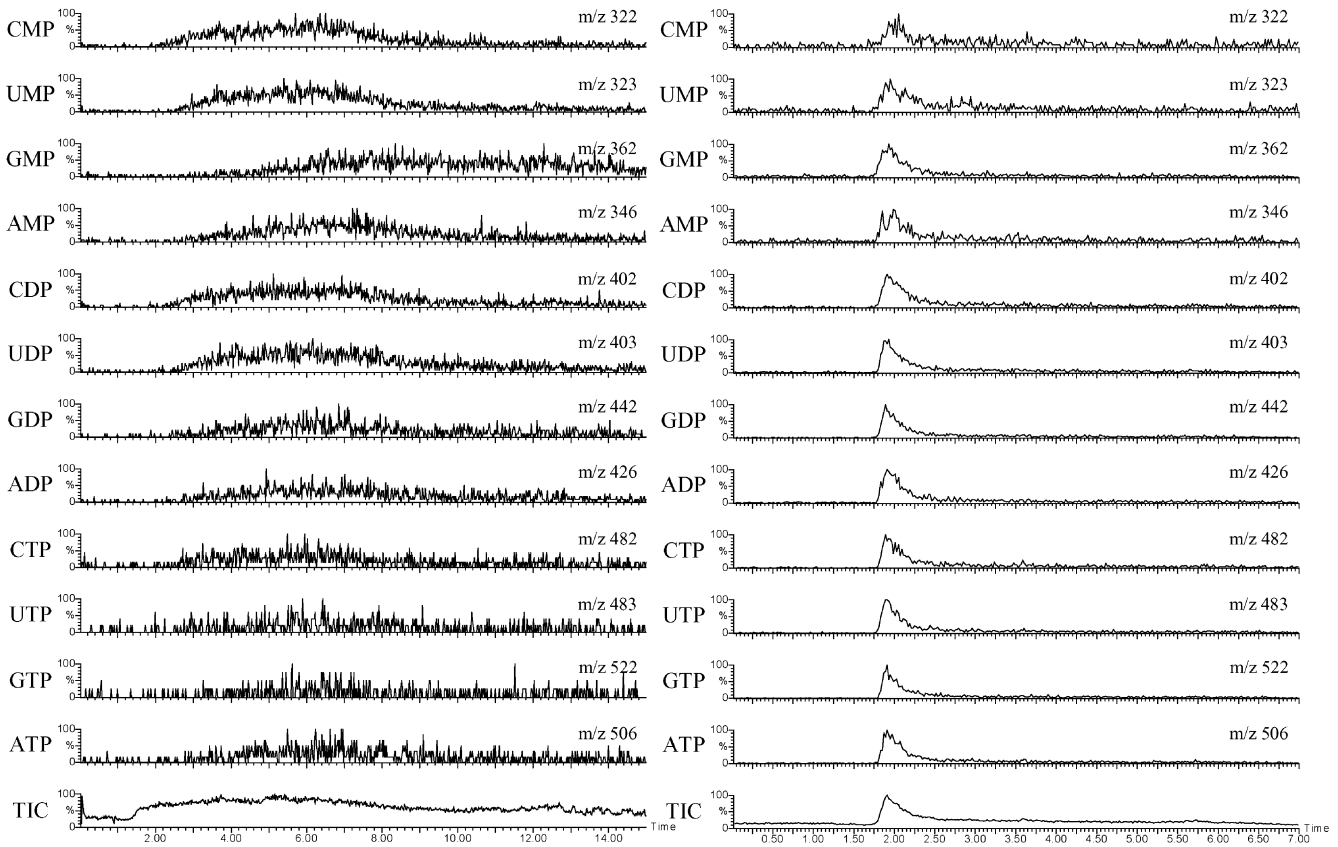

Fig. 4. Retention behaviour of 12 nucleotides on a Fe^{3+}-IMAC-column. Left trace: reconstructed ion chromatograms for the $[M-H]^-$ during the loading step. Right trace: reconstructed ion chromatograms for $[M-H]^-$ during the elution step using a 0.1% (w/v) NH_4OAc pH = 10 plug.

pyrimidine nucleotides had less retention compared to their purine analogues. This was confirmed by examination of the elution profiles after introducing 0.1% NH_4OAc pH = 10 (Table 2: 4), which released the compounds of the IMAC-column: less intense signals of the nucleoside monophospates were observed, especially for CMP and UMP.

These experiments gave rise to a preliminary classification in terms of affinity towards immobilised iron(III): nucleoside triphosphates > nucleoside diphosphates > nucleoside monophosphates. These data suggested that trapping on the column could not solely be explained by [Fe^{3+}-phosphate] complexation alone but that other effects were involved. Dobrowolska et al. already noted a possible influence of the nucleobase when studying the retention behaviour of 2′-deoxy-CMP, -AMP and -GMP but this was considered to play a minor role in the interaction with immobilised trivalent iron [40].

In order to check whether nucleoside monophosphates were really trapped by Fe^{3+}-IMAC and to proof that the $[M - H]^-$ signals in the ES(−) run were not the result of in source collision activated dissociation (CAD) of the di- and triphosphates, a mixture of CMP, UMP, GMP and AMP (2×10^{-5} M) was injected. From these results their trapping on the Fe^{3+}-IMAC-column was confirmed. Furthermore here also it was found that purine nucleotides (GMP > AMP) were favourably bound compared to the pyrimidine analogues (UMP > CMP).

3.1.4. Selective retention of nucleotides present in a matrix

If IMAC is intended to be used as an alternative for ion-exchange chromatography to enrich nucleotides, it has to prove its merits under more challenging conditions. Therefore, in order to test its selectivity towards nucleotides available in a complex biological matrix, a phosphate-free cell culture medium was spiked with a mixture of 12 nucleotides to a concentration of 10^{-5} M each. Proteins, more particularly phospho-proteins, were removed by a TCA–ether extraction. The injection sequence described above for standard mixtures was applied and both sample loading/washing (7 min) and plug elution were monitored in full scan ES(−)MS mode. Soon after 20 μL of the sample was introduced, the total ion current (TIC) increased and subsequently decreased slowly as the matrix compounds were washed off. Upon injection of 20 μL of 0.1% (w/v) NH_4OAc pH = 10 (Table 2: 4), an elution peak in the TIC was observed. When the MS-spectrum of the elution peak was examined, the most abundant ions corresponded to the $[M - H]^-$ of the different nucleotides spiked in the cell culture medium and some phosphate clusters. Also in these experiments it was noted that the nucleoside monophosphates were less prominent in the spectra (Fig. 5).

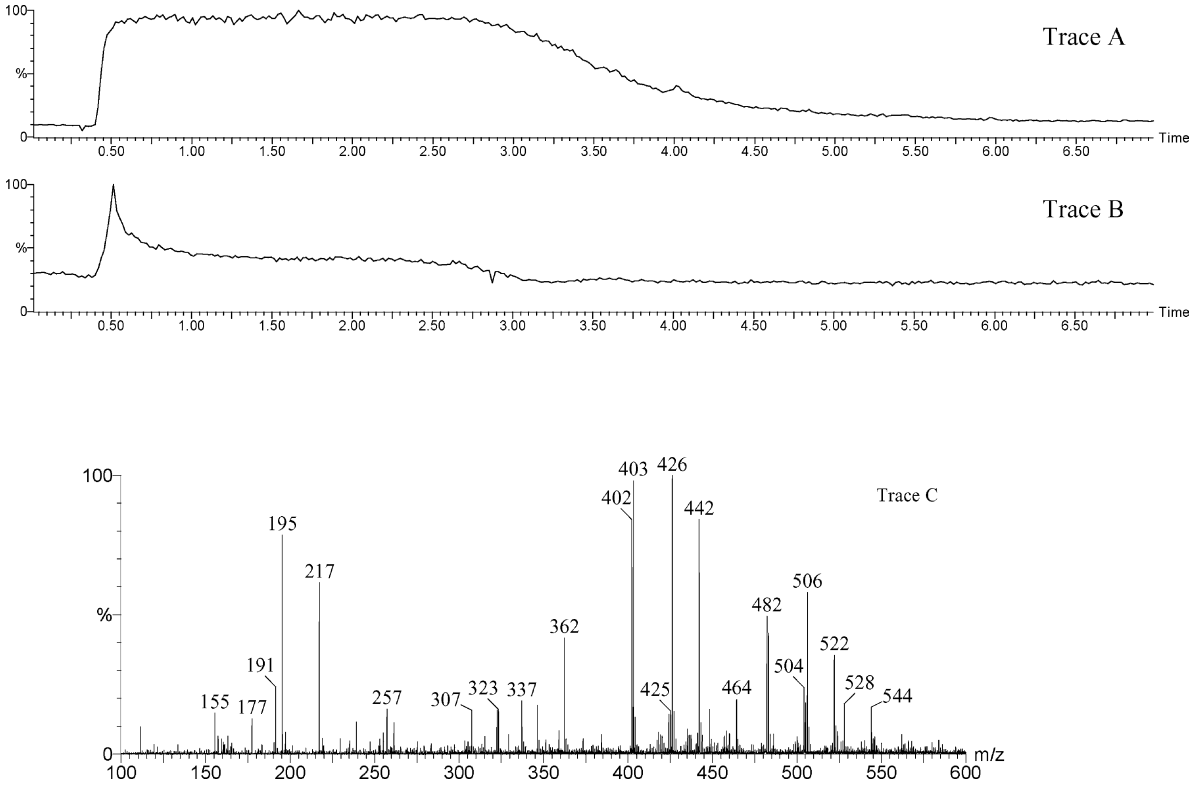

Fig. 5. Injection of phosphate free growth medium spiked with 12 nucleotides (10^{-5} M each). (Trace A) Total ion current during the loading step. (Trace B) Total ion current dring chaotropic elution with 0.1% NH_4OAc pH = 10. (Trace C) $[M - H]^-$ and $[M - 2H + Na^+]^-$ signals of the eluting nucleoside mono-, di- and triphosphates. In the low *m/z* region phosphate clusters can be recognized: the *m/z* values 195, corresponding with $[H_5P_2O_8]^-$, and 217 as a result of addition of $[H_3PO_4]$ to *m/z* 195.

3.2. The development of a column switching LC–MS method for nucleoside mono-, di- and triphosphates: Fe^{3+}-IMAC ion pair LC–MS

The experiments described above demonstrated that Fe^{3+}-IMAC could be used as a purification and pre-concentration tool for nucleotides and as such could be incorporated in a column switching LC–MS.

Since separation of nucleoside mono-, di- and triphosphates by anion-exchange is incompatible with ESMS, ion pair chromatography was the method of choice.

It was predictable that the difficult step in the column switching set-up would be the composition of the plug containing the nucleotides on the analytical column. On one hand this composition should provide sufficient elution power to release the nucleotides trapped on the Fe^{3+}-IMAC-column and on the other hand the composition of the plug should not completely deteriorate the analytical separation. Furthermore the direct implementation of the total column switching set-up would require a rigorous fine tuning of the switching time. Therefore we decoupled both separation dimensions and evaluated the influence of different plug compositions on both columns separately. In Table 2, a summary of all the implemented elution plugs and their impact on both chromatographic dimensions was summarised.

3.2.1. Composition of the elution plug

Compliant with the results described above (cf. Section 3.1.2) the first composition examined, with respect to its influence on the analytical separation, was 0.1% (w/v) NH_4OAc pH = 10 (Table 2: 4). Injection of the nucleotides in this composition, turned out to ruin the ion pair chromatography completely. An explanation for this observation was the effect of the alkaline plug on *N*,*N*-DMHA which being an amine, needed to be protonated in order to act as an ion pair reagent for nucleotides. Therefore an extra constraint was imposed on the pH setting it to a maximum value of 8.

Since this lower pH-value has a decreased elution in the IMAC-column different concentrations of NH_4OAc were investigated, with and without adjusting the pH to 8 (Table 2: 5–10). However little or no nucleotide elution was seen on the IMAC-column. Furthermore these plugs, if introduced on the analytical column disturbed chromatography causing the nucleotides to elute as double peaks. Thus a 0.1% (w/v) NH4OAc pH = 10 (Table 2: 4) plug was the best choice from the IMAC point of view (cf. Section 3.1.2).

In order to find a solution free phosphate was introduced and tested on its ability to elute nucleotides trapped on a Fe^{3+}-loaded IMAC-column. Unlike acetate ions, inorganic phosphate has two possible binding sites and therefore it is believed to be inherently more competitive than acetate [42,45]. One could expect the free phosphate to have a similar affinity towards immobilised iron(III) as the phosphate groups present in nucleotides. Therefore it was assumed that a displacement mechanism could be exploited. Different concentrations of $NH_4H_2PO_4$ were investigated at different pH settings (Table 2: 11–16). The data revealed that the influence of free phosphate alone (pH = 4.7) was not sufficient to provoke an instant elution of the nucleotides. Yet a similar elution to that produced with the 0.1% (w/v) NH_4Ac pH =10 plug was achieved with $NH_4H_2PO_4$ 0.5% (w/v) adjusted to pH = 8 (Table 2: 16).

The key test for this new approach was the study of its influence on the nucleotide-separation via ion pair chromatography. The effect of the $NH_4H_2PO_4$ (0.1% (w/v) to 0.5% (w/v), pH 4.7–8) was tested by injecting nucleotides dissolved in these different compositions onto the ion pair LC system. Although pH = 4.7 (Table 2: 11–13) yielded better results from a chromatographic point of view the 0.5% (w/v) $NH_4H_2PO_4$ pH = 8 elution plug (Table 2: 16) was preferred, since only the latter composition yielded satisfactory elution from the IMAC-column.

3.2.2. Injection volume on the ion pair LC–MS

As described above the nucleotides eluted from the IMAC-system in a volume of approximately 3 μL. In the experiments designed to investigate the influence of the elution plugs onto the ion pair LC–MS (second dimension) we initially injected 5 μL of the premixed samples (nucleotide + elution plug composition) onto the LC–MS system, in order to maximise the nucleotide recovery present in the IMAC-eluate. However in this study we had to decrease the injection volume in order not to disturb the analytical separation. It turned out that 1 μL injection yielded the best results.

3.2.3. Column switching LC–MS system for nucleotides

Since a 0.5% (w/v) $NH_4H_2PO_4$ pH = 8 (Table 2: 16) plug gave the best IMAC-results and in order to maintain chromatographic integrity only a 1 μL heart cut of the approximately 3 μL total volume of the plug was injected on the analytical column. The procedure leading to the optimal switching time maximising the nucleotide recovery is described in Section 2.6. In order to compensate for the effect of the introduction of the elution plug on the separation, the gradient program previously described [36] was slightly adapted. Better on-column focusing was achieved by changing the starting conditions of the mobile phase composition to 1% B instead of 5% B. The total analysis sequence comprised the conditioning of the IMAC-column (stripping, Fe^{3+}-loading, washing), the sample introduction, the sample elution by means of a plug injection, the heart cut of the IMAC effluent in the 1 μL loop and ultimately the chromatographic separation and mass spectrometric detection of the nucleoside mono-, di- and triphosphates. This methodology was then used for the analysis of 20 μL of a cell culture medium spiked with a mixture of 12 nucleotides (5×10^{-6} M each). The analysis of this cell culture medium sample showed selective trapping of the nucleotides on the IMAC-column followed by good chromatography and mass spectrometric detection of the compounds of interest. In agreement with the

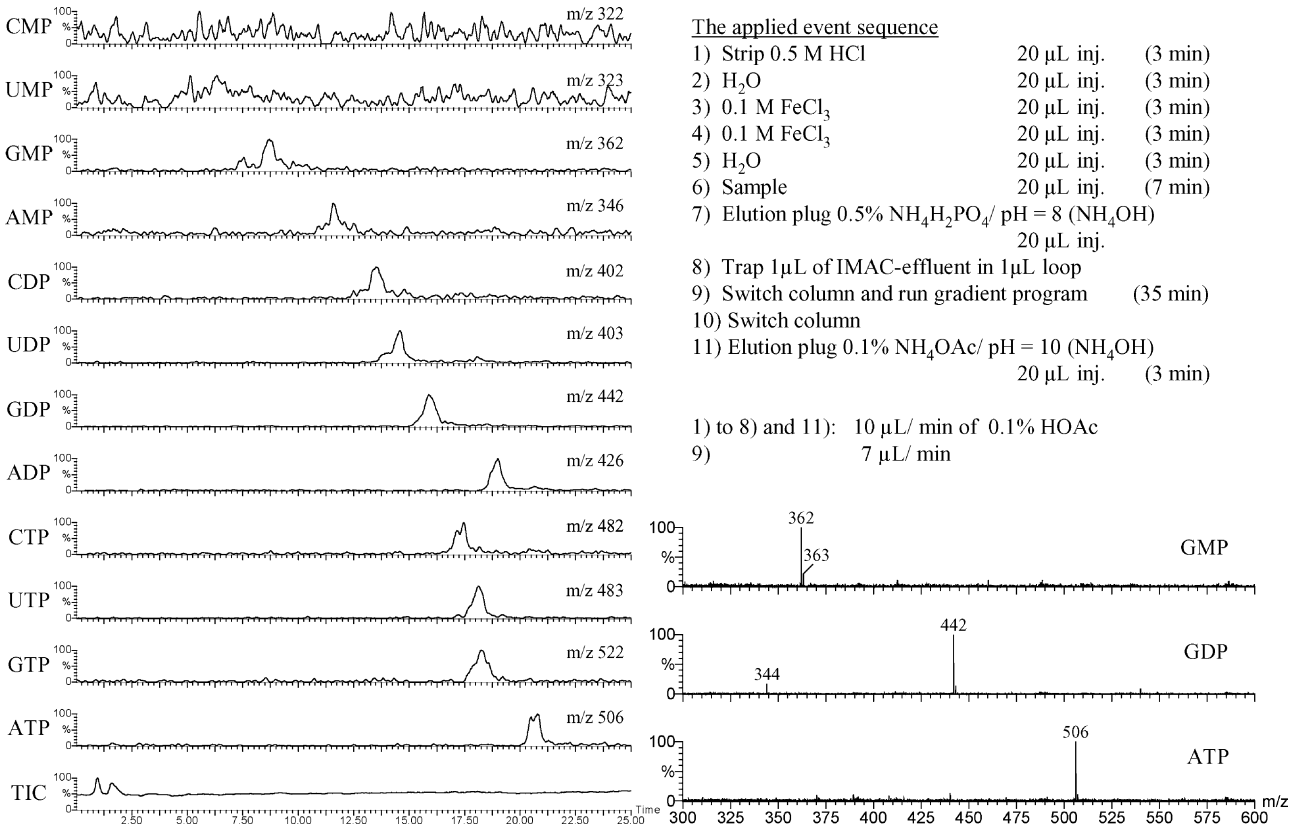

Fig. 6. Capillary column switching LC–MS analysis of a phosphate free growth medium spiked with 12 nucleotides using Fe^{3+}-IMAC ion pair LC–MS. The complete event sequent to produce these data is summarised. Extracted ion chromatograms and TIC are shown, together with three examples of mass spectra corresponding with the chromatographic peaks of GMP, GDP and ATP (after background subtraction).

above results the capture of the nucleoside monophosphates CMP and UMP on the Fe^{3+}-IMAC-column was insufficient to allow their detection (Fig. 6).

From this experiment it may be concluded that the overall sensitivity of the column switching LC–MS system was still unsatisfactory. The main reason is the restricted sensitivity of the ion pair LC–MS system used, partly due to the use of some $NH_4H_2PO_4$ in the mobile phases which gave rise to important background increments and possible ion suppression. It was also due to the poorer detection limits achieved in the ES negative ion mode [21]. In addition it was observed that the recoveries of nucleotides from the Fe^{3+}-IMAC-column was not complete by applying the current elution plug as still some nucleotides elute in a second, control elution plug. Furthermore the current heart cut approach puts restraints to the method.

4. Conclusion

IMAC was proven to be a valuable alternative for anion exchange chromatography for the selective retention of nucleoside mono-, di- and triphosphates. For the chaotropic elution of the latter compounds using a LC–MS compatible mobile phase a 0.1% (w/v) NH4OAc pH = 10 solution should be preferred. However if the IMAC-precolumn was introduced in an ES/MS column switching system a 0.5% (w/v) $NH_4H_2PO_4$ pH = 8 solution should be used in order to have a minimal disturbance of the ion pair chromatography used in the separation of the nucleotides under electrospray LC–MS conditions. However one should realise that this was a pilot study and that further experiments using different IMAC-supports (bidentate, tridentate, ...), different immobilised metal ions and different conditions (pH, competing agents, ...) could lead to an improved methodology.

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