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On-line capillary column immobilised metal affinity chromatography/electrospray ionisation mass spectrometry for the selective analysis of histidine-containing peptides

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Abstract

Capillary column immobilised metal affinity chromatography (IMAC) has been combined on-line with electrospray ionisation/quadrupole time-of-flight mass spectrometry for the fractionation of histidine-containing peptides. IMAC beads (Poros 20MC, 20 μm) containing imidodiacetate chelating groups on a cross-linked poly(styrene-divinylbenzene) support were packed into a fused silica column (250 μm i.d.), which was interfaced to the electrospray ion source of the spectrometer. A Cu(II) activated column was used to isolate histidine-containing peptides from tryptic and other peptide mixtures with an average breakthrough of 9.1%, to reduce the complexity of the mass spectral analysis. The analysis cycle time was reduced to less than 15 min, at an optimum flow rate of 7.5 μL/min, without sacrificing peptide selectivity. Direct coupling of capillary IMAC with MS allows on-line separation, using MS compatible loading and elution buffers, and detection in a high-throughput fashion when compared to off-line strategies.

Keywords: Capillary LC–MS; Immobilised metal affinity chromatography; Electrospray ionisation mass spectrometry; Copper(II); Histidine-containing peptides

1. Introduction

Immobilised metal affinity chromatography (IMAC) was first introduced in 1961 by Helferich, for the selective isolation of small molecules [1], although the first application of the technique to protein fractionation and purification was not reported until mid-1970s by Porath et al. [2]. IMAC fractionation of proteins is based on the interaction between an electron-donating group on a protein/peptide surface and a metal ion presenting one or more free coordination sites. The metal ion is immobilised on a multi-dentate chelating compound (such as imidodiacetate (IDA) or nitrilotriacetate (NTA)) [3], *via* coordination with electron pair donor atoms.

It has been calculated that 31% of tryptic peptides contain a histidine residue, providing 97% coverage of the human proteome [4], therefore selective isolation of histidine-containing peptides by IMAC has the potential to simplify complex tryptic and other peptide mixtures. The interaction of transition metal ions, such as Cu(II), Zn(II), Ni(II), classified as intermediate Lewis acids [5], with histidine-containing peptides can be achieved by exploiting the Lewis base properties of the imidazole nitrogen on the histidine side chain (see Fig. 1) [6]. The binding of histidine residues ($pK_a \sim 6.0$) to the Cu(II)-IMAC column occurs in the pH range 6–8, where the imidazole donor group is at least partially unprotonated [7]. The target proteins or peptides may be eluted from the IMAC column by lowering the pH, or facilitating a pH gradient using an eluting buffer, or by displacement using a competing ligand, such as imidazole. Yip et al. correlated the retention of peptides with their amino acid profile using immobilised Zn(II), Ni(II) and Cu(II) [8], which indicated that histidine has the highest retention ability,

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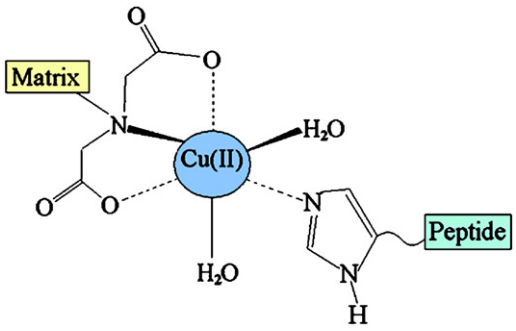

Fig. 1. Structure of imidodiacetate linked to a support matrix *via* a spacer arm, with an immobilised Cu(II) metal ion. The partial histidine residue is shown binding to the Cu(II) through its imidazole nitrogen.

and Cu(II)-IDA was the most selective adsorbent. The selection of histidine residues using Cu(II) activated IMAC columns has been widely reported for proteomic applications [9–11].

IMAC allows single-step purification with minimal sample handling, making the technique a rapid, inexpensive, reproducible and reliable approach for the purification of peptides and proteins [11–13]. IMAC has also been applied successfully as an enrichment tool prior to MS analysis of phosphorylated peptides/proteins [14], using Fe(III), Ga(III), Al(III) and Zn(II) [15,16]. IMAC ligands have been used in Protein Chips for the specific enrichment of proteins, analysed by surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry (SELDI-TOF-MS) [17]. Other examples of the use of IMAC are the purification of DNA and oligonucleotide derivatives tagged with histidine residues, and the direct incorporation of phosphoramidies into PCR primers or oligonucleotides, utilised for the purification of PCR products or base-pairing oligonucleotide chains [18,19].

Capillary column liquid chromatography is used widely in mass spectrometry-based proteomic applications, employing fused silica columns usually of internal diameter (i.d.) less than 350 μm and mobile phase flow rates of less than 10 μL/min. Capillary columns have a number of advantages when compared with conventional columns (2.1 or 4.6 mm i.d.), such as a lower solvent usage and sample consumption, particularly when sample amounts are limited, and higher sensitivity as a result of the lower mobile phase flow rates when combined with concentration-sensitive electrospray (ESI) detection [20]. Despite these advantages the potential of interfacing capillary IMAC with mass-spectrometric detection has received little attention.

Direct coupling of capillary IMAC with mass spectrometry allows on-line separation and reproducible MS detection in a high-throughput fashion, minimising preparative steps, sample handling, sample loss, contamination and reducing analysis time when compared to off-line strategies [14]. Furthermore, the low flow rates of capillary IMAC are compatible with nanospray ESI ion sources and hyphenation with other capillary column techniques, such as on-line reversed phase capillary HPLC. To date, direct coupling of capillary column IMAC to ESI-MS has been reported only for the enrichment of phosphopeptides using Fe(III) activated columns. Watts et al. introduced another separation dimension, by combining capillary column IMAC with high-performance liquid chromatography (HPLC), prior to ESI mass-spectrometric analysis [21]. Other groups have combined capillary IMAC columns with nanoflow HPLC-μESI mass spectrometry and tandem mass spectrometry for the characterisation of phosphorylation sites on proteins and the determination of phosphorylated sequences [22–27]. However, no studies have been carried out for the enrichment of histidine-containing peptides from complex peptide mixtures, using a capillary IMAC column for direct on-line mass-spectrometric detection.

In this study, a Cu(II) activated capillary IMAC column has been combined on-line with electrospray ionisation/quadrupole time-of-flight mass spectrometer (ESI/Q-ToF-MS). The isolation of histidine-containing peptides from tryptic and other peptide mixtures is demonstrated and the selectivity, percentage breakthrough and ease of use of the capillary column have been evaluated.

2. Experimental

2.1. Materials

HPLC grade trifluoroacetic acid (TFA), methanol, acetonitrile and formic acid, ammonia, ammonium bicarbonate and ethylenediaminetetraacetic acid (EDTA) were purchased from Fisher Scientific (Loughborough, UK). Copper(II) acetate was purchased from Sigma–Aldrich (Poole, UK). Distilled and deionised water was obtained in-house using a Triple Red water purification system (Triple Red, Long Crendon, UK).

The following peptides were synthesised by Alta Bioscience (University of Birmingham, Birmingham, UK) at approximately 95% purity for Cu(II)-IMAC analysis; gp70, SPSYVYHQF; p53-193, HLIRVEGNL; p53-210, NTFRHSVVV; HAGE-551, LDVHDVTHV; HAGE-545, DLASRGLDVHDVTHV; LII, LIIPSLERL; SLE, SLERLVNAI; p53-217, VVPYEPPEV; HAGE-509, LGNISVESL.

Bovine serum albumin (BSA, 98% purity) was purchased from ICN Biomedicals Inc. (OH, USA) and trypsin (Trypsin Gold, Mass Spectrometry Grade) was supplied as lyophilised powder from Promega Ltd. (Southampton, UK).

2.2. Sample preparation

Synthetic peptides were reconstituted in DMSO (10 μg/μL) and then diluted to 60 pmol/μL in pH 7.2 loading buffer (10 mM formic acid/ammonia containing 10% (v/v) methanol) prior to Cu(II)-IMAC analysis.

A tryptic digest was prepared using BSA (26 μg/μL in 25 mM ammonium bicarbonate solution) and trypsin (0.5 μg/μL in 50 mM acetic acid). The trypsin solution was incubated at 30 °C in a water bath (30 min) and then chilled on ice. Digestion was performed by mixing BSA (250 μL, 26 μg/μL), ammonium bicarbonate (150 μL, 25 mM) and trypsin (100 μL, 0.5 μg/μL) in a plastic Eppendorf tube and incubated overnight at 37 °C. The reaction was quenched by the addition of 1% formic acid (150 μL). The digested BSA was diluted in the pH 7.2 starting buffer to give a final concentration of 50 pmol/μL.

2.3. Capillary IMAC column preparation

The capillary IMAC column was packed in-house using the following procedure. A deactivated fused silica tube (250 μm i.d. and 360 μm o.d.) was connected to a mini MicroFilter (Presearch, Herts, UK) fitted with a 1 μm titanium frit filter capsule. Approximately 1.0 mg of Poros 20MC beads (Applied Biosystems, Framingham, MA, USA) was weighed into a HPLC vial containing a small magnetic stirring flea. Methanol (1500 μL) was added to the HPLC vial and the capillary was inserted into the methanol solution. The column was packed to a column bed length of 75 mm (3.7 μL column bed volume), using a high-pressure bomb loader (Proxeon Biosystems, Denmark) connected to a nitrogen gas cylinder (∼150 psi). The pressure bomb was placed onto a magnetic stirrer during the packing. Finally, the packed capillary column was connected to a Waters 2790 HPLC pump (Waters, Manchester, UK) to be compressed at ∼1800 psi by a solvent flow of 50/50 (v/v) acetonitrile:water at 200–250 μL/min. A second mini MicroFilter was attached to the head of the column to minimise column blockage.

2.4. On-line capillary Cu(II)-IMAC/ESI/QToF-MS

A schematic diagram of the on-line capillary IMAC/ESI/QToF-MS set up is shown in Fig. 2. Loading and eluting buffers were delivered to the capillary IMAC column using a Waters CapLC capillary HPLC (Waters). The outlet of the column was connected to the electrospray ionisation source of a Q-ToF Ultima quadrupole time-of-flight mass spectrometer (Waters) controlled by MassLynx version 3.5 operating software (Waters). The loading buffer (10 mM formic acid/ammonia + 10% (v/v) methanol, pH 7.2) was

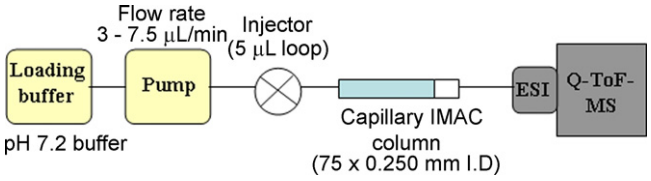

Fig. 2. Schematic diagram of the on-line capillary IMAC/ESI/Q-ToF-MS set-up. The mobile phase (loading buffer) is pumped continuously through the column to the ESI ion source. All other reagents and samples were loaded onto the column *via* the injector loop.

continuously pumped through the IMAC column at flow rates in the range 1.5–7.5 μL/min. Peptide samples, EDTA, Cu(II) acetate and the eluting buffer (0.15%, v/v, TFA) were injected onto the IMAC column using an injection valve and a 5 μL sample injection loop (Rheodyne, Model 7010, Alsbach a.d. Bergstrasse, Germany). The mass spectrometer was operated in positive ion mode with source and desolvation temperatures of 90–120 and 120–180 °C, respectively. A capillary voltage of 3.2–3.8 V was applied to the ESI probe tip and the nitrogen desolvation gas was set to a flow rate of 200–250 L/h. Full scan MS and MS/MS spectra were acquired in the *m/z* 400–1500 mass range (1.9 s acquisition time with a 0.1 s interscan delay).

2.5. Cu(II)-IMAC/ESI-MS analysis

The Cu(II)-IMAC column was activated by stripping the Poros MC support with 8 × 5 μL aliquots of 50 mM EDTA, which were loaded onto the column *via* the 5 μL loop fitted to the injection valve at a flow rate of 5 μL/min. The column was then washed and equilibrated for 20 min with the pH 7.2 starting buffer flow. Cu(II) acetate solution (36 nmol/μL, 5 μL

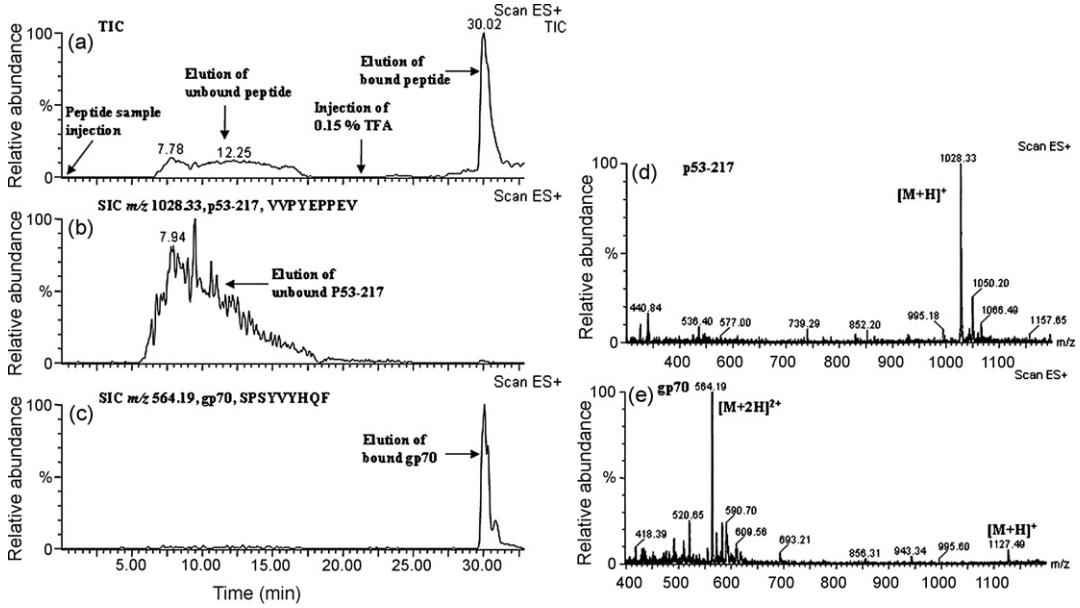

Fig. 3. Ion chromatograms for a two peptide mixture loaded onto the on-line capillary IMAC column and then eluted in 0.15% aqueous TFA, (a) total ion chromatogram (TIC), (b) selected ion chromatogram (SIC) for the non-histidine-containing peptide p53-217 (*m/z* 1028.33), (c) SIC for the histidine-containing peptide gp70 (*m/z* 564.19), (d) the mass spectrum of the unbound peptide during the loading stages (7.0–19.0 min) and (e) the mass spectrum of the bound peptide during elution at 29.5–32.5 min.

volume) was injected onto the column at a reduced flow rate of 1.5 μL/min, to allow copper to interact with the imidodiacetate chelating groups on the surface of the Poros media. The amount of Cu(II) loaded onto the column was calculated based on the capacity of the Poros media, so that two-thirds of the column was activated to minimise metal ion leakage. Following activation, the Cu(II)-IMAC column was washed with the starting buffer for 10 min (flow rate of 5 μL/min) to remove any unbound Cu(II) ions. The Cu(II)-IMAC column was connected to the ESI ion source (Fig. 2) of the Q-ToF mass spectrometer and synthetic peptides or BSA tryptic digest samples were loaded at a flow rate in the range 3–7.5 μL/min. Bound peptides were eluted by an injection of aqueous TFA (5 μL, 0.15%, v/v).

3. Results and discussion

A fused silica capillary column (250 μm i.d.) was packed in-house with Poros 20MC beads and combined successfully on-line with electrospray mass spectrometry for the selective isolation of histidine-containing peptides using immobilised Cu(II) ions. Poros 20MC presented the highest level of non-specific binding, but retained the least number of histidine-containing peptides in a study by Ren et al. [27], who examined various support matrices for the isolation of histidine-containing peptides with off-line mass-spectrometric detection. However, Poros MC exhibits high physical stability and rigidity, allowing high flow rates and back-pressures to be utilised during packing into the capillary and for direct on-line MS analysis.

The selectivity of the capillary Cu(II)-IMAC column for histidine-containing peptides was evaluated initially using a simple test mixture consisting of one histidine-containing peptide (gp70, SPSYVYHQF) and a non-histidine-containing peptide (p53-217, VVPYEPPEV). The total ion chromatogram (TIC) of the peptide mixture is shown in Fig. 3a, and the selected ion chromatograms of p53-217 at *m/z* 1028.33 (singly charged) and gp70 at *m/z* 564.19 (doubly charged) are shown in Fig. 3b and c, along with the corresponding mass spectra (Fig. 3d and e, respectively). Preliminary results demonstrated that gp70 had a strong affinity for Cu(II) and was retained on the capillary IMAC column with high efficiency due to copper/peptide complexation at the pH of the loading buffer (pH 7.2, 10 mM formic acid/ammonia), which is approximately one unit above the pK_a of the histidine imidazole side chain (∼6.0). Elution of gp70 was achieved by injection of an aliquot (5 μL) of the elution buffer (0.15% aqueous TFA), resulting in a decrease in pH and protonation of the imidazole nitrogen, which disrupted the histidine–metal bond. The non-histidine-containing p53 peptide was not retained and was eluted through the column in the loading buffer with no non-specific binding to the column, indicating that the on-line capillary Cu(II)-IMAC procedure has good selectivity for histidine-containing peptides.

3.1. Analysis of a nine-peptide mixture

The IMAC procedure was evaluated using a mixture containing nine synthetic peptides (listed in Section 2), five of which contained at least one histidine residue. Three of the histidine-containing peptides (gp70, p53-193 and p53-210) were retained by the immobilised Cu(II) ions on the column, but two were not retained using the original formic acid/ammonia loading buffer (data not shown). However, addition of methanol to the buffer resulted in the specific enrichment of all five histidine-containing peptides on the Cu(II)-IMAC column. The addition of an organic modifier (methanol) can increase surface contact of peptides with the Poros media, by minimising secondary structure formation of peptides, which can often hinder the accessibility of histidine residues [28]. Various methanol concentrations (0–50%, v/v) were evaluated and the highest specificity was obtained when 10% (v/v) methanol was added to the loading buffer (data not shown). Addition of methanol therefore allows a wider peptide coverage then that possible without the methanol modifier, but at the expense of greater spectral complexity.

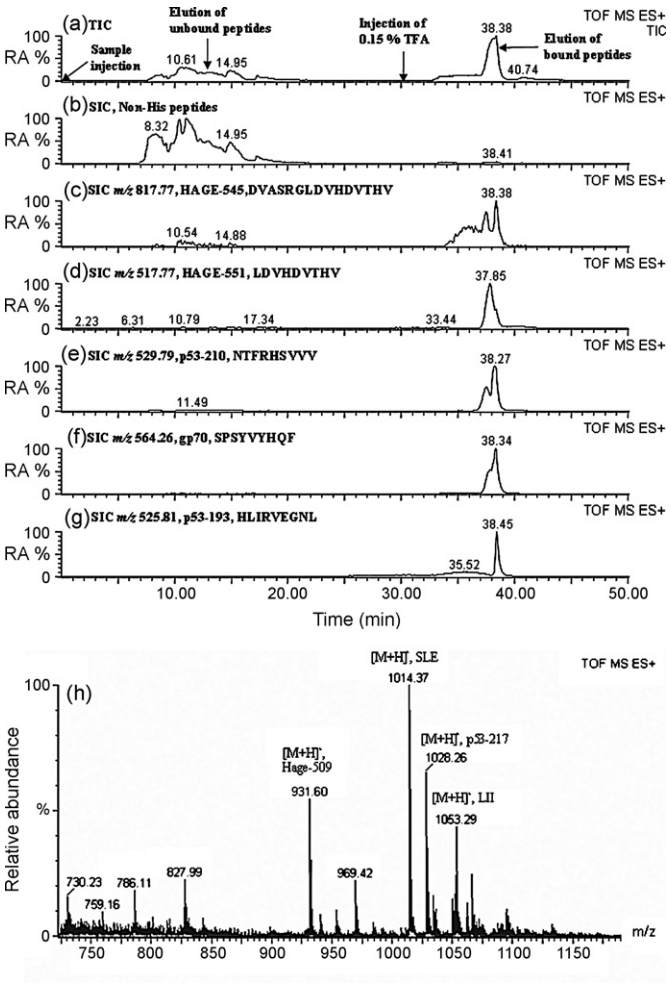

Fig. 4. Ion chromatograms of a nine peptide mixture loaded onto the on-line capillary IMAC column (a) total ion chromatogram, (b) combined selected ion chromatogram for the non-histidine-containing peptides (HAGE-509, *m/z* 931.60; LII, *m/z* 1053.29; SLE, *m/z* 1014.37; p53-217, *m/z* 1028.26; LII, *m/z* 1053.29) and selected ion chromatograms for individual histidine-containing peptides, (c) HAGE-545, *m/z* 817.77, (d) HAGE-551, *m/z* 517.77, (e) p53-210, *m/z* 529.79, (f) gp70, *m/z* 564.26, (g) p53-193, *m/z* 525.81, and (h) mass spectrum of unbound peptides (non-histidine-containing) eluted during the loading stage (7.0–20.0 min).

The mass-spectrometric responses of the bound and unbound peptides are shown in Fig. 4. The total ion chromatogram (TIC) of the peptide mixture is shown in Fig. 4a, and the combined selected ion chromatogram of the non-histidine-containing peptides is shown in Fig. 4b. The corresponding mass spectrum is presented in Fig. 4h. Non-histidine-containing peptides did not interact with the Cu(II)-IMAC column and were eluted from the column in the loading buffer, presenting a broad chromatographic peak (between 7 and 20 min). The selected ion chromatograms (SIC) of $[M+2H]^{2+}$ for histidine-containing peptides HAGE-545 (*m/z* 817.77), HAGE-551 (*m/z* 517.77), p53-210 (*m/z* 529.79), gp70 (*m/z* 564.26) and p53-193 (*m/z* 525.81) are shown in Fig. 4c–g, respectively. All histidine-containing peptides in the peptide mixture were retained on the capillary IMAC column. These targeted peptides were successfully eluted at a flow rate of 3 μL/min, as a sharp chromatographic peak at ∼36.5–38.0 min on injection of an aliquot of eluting buffer (0.15% aqueous TFA, 5 μL), shown in Fig. 4c–g. All the histidine-containing peptides retained on the column in the loading buffer were eluted in a single 5 μL injection of aqueous TFA. Injection of a second of TFA yielded no further peptide responses. The retention efficiency of the capillary IMAC column was determined by comparing the breakthrough responses (SIC peak areas) for the individual bound peptides during the loading stages (7–20 min) with their elution responses (37–39 min). The capillary Cu(II)-IMAC column isolated histidine-containing peptides an average breakthrough 9.1% (range 4–17%).

3.2. Analysis of a BSA tryptic digest

The ability of the capillary Cu(II)-IMAC column to retain histidine-containing peptides selectively in a more complex mixture of peptides was studied by using a BSA tryptic digest. None of the peptides, including histidine-containing peptides, were retained on an unactivated column (i.e. prior to immobilisation of copper onto the Poros 20MC beads). The resulting mass spectrum of the unfractionated BSA digest (shown in Fig. 5b) is very complex, with a high degree of spectral noise, and shows the presence of both histidine-containing (indicated by H) and non-histidine-containing BSA peptide peaks. Data analysis of such spectra can often be problematic due to isobaric mass-spectrometric responses and peak overlapping.

In contrast, histidine-containing BSA peptides were selectively isolated from the BSA tryptic digest when the capillary IMAC column was activated with copper. The total ion chromatogram (Fig. 5a) demonstrates that on loading of BSA tryptic digest, unbound peptides are observed to elute through the column, and on injection of the eluting buffer (at ∼11 min) a sharp chromatographic peak is observed as the peptides are released from the column. Various flow rates in the range 3.0–10.0 μL/min were examined for the BSA digest and the optimum flow rate was found to be 7.5 μL/min. This reduced the total analysis time, from injection to detection of analyte, from 40 min (Fig. 4a) to less than 15 min (Fig. 5a) without sacrificing selectivity. Mass spectra corresponding to the unbound (2.5–10.5 min) and bound (13.7–14.7 min) fractions are presented in Fig. 5c and d, respectively. None of the histidine-containing BSA pep-

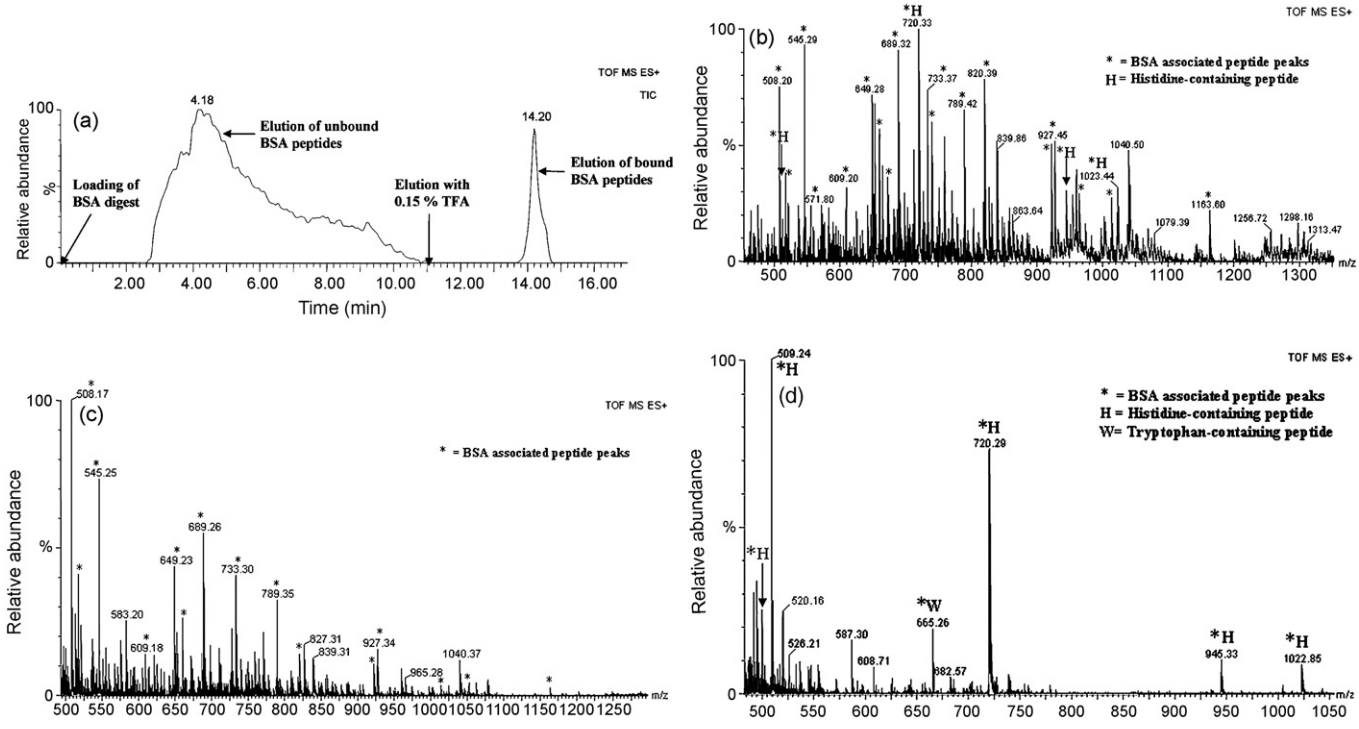

Fig. 5. (a) Total ion chromatogram for a BSA tryptic digest loaded onto the on-line capillary Cu(II)-IMAC column and eluted with 0.15% aqueous TFA, (b) the mass spectrum of BSA tryptic digest on an unactivated column, (c) the mass spectrum of unbound BSA digest peptides injected onto a Cu(II) activated IMAC column during the loading stage (3.0–10.0 min) and (d) the mass spectrum of bound BSA digest peptides on a Cu(II) activated IMAC column during elution with 0.15% aqueous TFA (13.7–14.7 min).

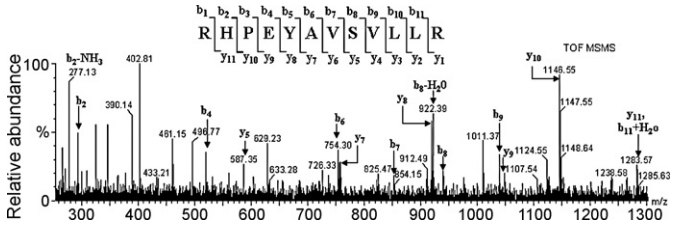

Fig. 6. Tandem mass spectrum of *m/z* 720.3 precursor ion, derived from a histidine-containing BSA tryptic peptide (RHPEYAVSVLLR).

tides (indicated by an asterisk) was in the unbound fraction. BSA peptide peaks eluted with 0.15% aqueous TFA all contained histidine residues (Fig. 5d), except for the peak at *m/z* 665.3. This peptide does not contain a histidine residue, but the sequence includes tryptophan. It has previously been shown that the tryptophan indole side chain shows some affinity for Cu(II) ions [29], but to a lesser extent than histidine. In comparison to the mass spectrum obtained without IMAC separation (Fig. 5b), Fig. 5c and d demonstrates a marked reduction in sample complexity by using the capillary Cu(II)-IMAC method. This suggests that IMAC separation has the potential to greatly simplify the mass spectrum and concomitantly increase confidence in protein identification. BSA tryptic peptide peaks (indicated by an asterisk) in Fig. 5b–d were identified using the Mascot protein search engine (http://www.matrixscience.com), mass spectrometry (peptide mass fingerprint) peak data were entered into the database and BSA was identified with high confidence. Average percentage breakthrough of histidine-containing BSA peptides was determined to be 9.2% (range 5–15%), demonstrating the high retention efficiency of the Cu(II) activated capillary IMAC column for the isolation of histidine-containing peptides from a tryptic digest.

The capillary Cu(II)-IMAC column was capable of performing multiple IMAC separations without the need for regeneration (by EDTA) or reactivation (by Cu(II) ions). There was also negligible leaching of Cu(II) ions, indicated by the absence of Cu(II)–peptide adducts, which are detectable by ESI [29]. In addition, Cu(II) ion leakage was minimised by underloading the metal ion on the Poros media.

Tandem mass-spectrometric analysis (MS/MS) was carried out on the isolated histidine-containing peptide at *m/z* 720.3 ($[M+2H]^{2+}$), to demonstrate the potential of this developed on-line system to provide tandem MS data and obtain peptide sequencing information. The product ion spectrum is shown in Fig. 6, these MS/MS fragmentation ions were also searched using Mascot, and confirmed that the mass-spectrometric peak at 720.3 is derived from a histidine-containing BSA tryptic peptide RHPEYAVSVLLR. Capillary Cu(II)-IMAC coupled with mass spectrometry and tandem mass spectrometry is therefore able to fractionate a tryptic peptide mixture with minimal non-specific binding, and provide peptide sequence information.

4. Conclusions

A robust, on-line capillary Cu(II)-IMAC/ESI/Q-ToF-MS system has been developed for the isolation of histidine-containing peptides with high efficiency. This approach demonstrates the selectivity of capillary column IMAC activated with copper(II) to isolate histidine-containing peptides from complex mixtures, allowing enhanced detection of biomolecules using both MS and MS/MS routines. Total analysis time from injection of sample to detection of histidine-containing peptides was reduced to less than 15 min, without sacrificing selectivity. The low flow rate of capillary IMAC is compatible with nanoflow ESI and has the potential for integration within multidimensional systems, such as capillary SCX, SAX, and reversed phase columns to further reduce the complexity of samples and enhance detection by mass spectrometry.

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