A simple and universal tool to remove on-line impurities in 1D- and 2D-LC/MS analyses

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INTRODUCTION

Liquid Chromatography coupled to Mass spectrometry (LC/MS) is routinely used for protein and peptide identification and sequencing. Several compounds are however known to interfere with peptide analyses in electrospray ionization mode (ESI) whether connected with mono- or bi-dimensional liquid chromatography systems (1D- or 2D-LC), or not. Among these are detergents such as triton and sodium dodecyl sulfate (SDS), polyethylene glycols (PEGs), plasticizers such as tris(2-butoxyethyl) phosphate and bis(2-ethylhexyl) phthalate or polydimethylcyclosiloxanes (PMC)[1]. Even if the greatest care is taken to select the chemicals and solvents of highest quality, it is well known in the proteomics community that some impurities may still be present and interfere with the analyses. Some of them are observed independently of any LC system and are due to volatile air contaminants like PMC, that are used as internal standard indeed. Others come from LC outlet with specific retention time and have to be addressed in priority.

In order to eliminate these contaminants, we modified our fluids pathways in both 1D- and 2D-LC configurations coupled to highly accurate, sensitive mass spectrometers with high mass resolution (LTQ-FT and LTQ Orbitrap, ThermoFisher Scientific, CA, USA).

We first attempted to identify the source of contamination, and then removed these contaminants using on-line self regenerating trapping setups. This robust and simple modification demonstrates that these impurities influence the quality of the analyses during 1D or 2D LC-MS with a strong suppressor effect. This effect is a major drawback because the retention time of these polymers is in the range of interest of tryptic peptides. After validation on standard samples, this setup has been implemented on every LC system of our laboratory. Here we present this simple, robust and self regenerating method is presented for on-line elimination of very common contaminants observed in RPLC-MS profiles.

METHODS

A tryptic digest consisting in 6 proteins from 11 kDa to 135 kDa (Cytochrome C, Lysozyme, Alcohol dehydrogenase, Bovine serum albumin, Serotransferrin and ß-Galactosidase, Dionex, Amsterdam,The Netherlands) was analyzed by nanoscale capillary LC-MS/MS (Ultimate 3000 Dionex) coupled on-line with FTMS instruments (LTQ-FT and LTQ-Orbitrap, ThermoFisher Scientific). RP separations were identical for 1D- or 2D-LC modes and conducted on a nano-column (C18 PepMap100, 3 µm, 100 Å, 75 µm id, 15 cm length, Dionex). A linear gradient from 0 to 50% solvent B in 35 min was used with a 220 nL/min flow (buffer A: H_20/ACN/FA, 98/2/0.1, v/v/v, buffer B: H_20/ACN/FA, 10/90/0.1, v/v/v). Similar series of experiments (data not shown) were also tested on the 1200 nanoLC system (Agilent Technologies, CA, USA).

To remove contaminants, a micro precolumn (C18 PepMap100, 5µm, 100 Å, 1 mm id, 15 mm length, Dionex) was placed between the loading pump outlet and the injection valve in the 1D-LC system (Figure 1). This precolumn was flushed for 25 min after sample injection at 40µL/min of solvent B.

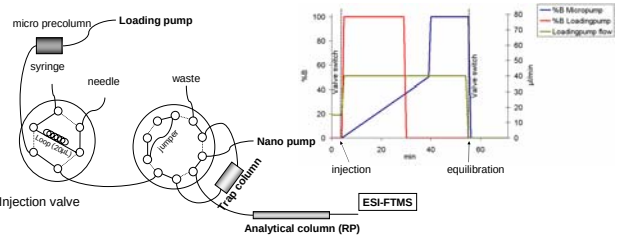

Figure 1 : Schematic representation of the 1D-LC system developed to remove polymers and RP gradient used

In 2D-LC mode, the peptides were first trapped on the capillary SCX column (BioBasic SCX 5µm, 300 Å, 0.32mm id, 15cm length, ThermoFisher Scientific). The gradient profile from 0 to 100 mM ammonium formate pH=3, ACN 5% (v/v) consists of 14 linear slopes with a flow rate of 2 µL/min. The eluted peptides were trapped alternatively on the trap 1 or on the trap 2 and desalted with buffer A. Valve V2 was then switched and the trapped peptides were flushed towards the analytical column for a RP-HPLC separation.

To remove contaminants, two micro precolumns, same as described above, were inserted after loading pump 2 and after micropump 1 (Figure 2). These precolumns were regularly flushed at 40µL/min of solvent B.

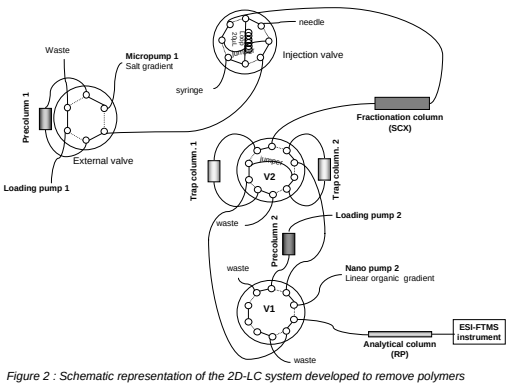

Figure 2 : Schematic representation of the 2D-LC system developed to remove polymers

With LTQ-FT, data were acquired in automatic high dynamic mode consisting of alternate acquisitions in FTMS full scan survey mode (m/z range 500-2000), 3 FTMS SIM scan mode for exact mass and charge state determination of peptide and 3 LIT MS/MS mode for sequencing as previously described[2].

With the LTQ-Orbitrap, data acquisition was realized in automatic mode with alternate acquisitions in FTMS full scan survey mode (m/z range 500-2000) and 3 to 7 LIT MS/MS.

Data were processed using Bioworks 3.2 software (ThermoFisher Scientific) and searched against Swissprot from UniProtKB release 9.6 (257,964 entries) indexed for tryptic peptides with up to 2 miscleavages, carboxyamidomethylation of cysteins (+57.022 uma) and methionin oxidation (+15.995 uma). Protein identification was validated according to the published standards[3] : at least 2 different sequences identified as first candidates for the protein with a minimal Xcorr and delta CN of 2.5 and 0.1 respectively. Mass accuracy tolerance was set to 10 ppm in MS mode, and 1 Da in MS/MS mode.

RESULTS

In our experiments interfering peaks (m/z of 520.333, 564.359, 608.385, 652.411, 696.438) were observed in every RP chromatogram with reproducible retention times corresponding to a percentage of AcN between 34 and 37 % and with a signal to noise ratio of up to 2000 (Figure 3A). This well-known PEG pattern (with m/z values 44.026 units apart) was detected over approximately 3 min of retention time in every LC run. Contaminants were not directly detected into any solvent, chemical or LC systems if analyzed without trap column. Indeed we tested different LC systems (Ultimate 3000, Dionex and 1200 LC Agilent Technologies, CA, USA), different acids (type, batch and supplier) without any significant change (data not shown). According to recent studies[4], we observed that polymers disappeared when we removed the trap column (data not shown) and concluded that these contaminants are enriched on stationary phase of the trap column. That is why we inserted an extra micro precolumn between the loading pump outlet and the injection valve to continuously eliminate polymeric contaminants. To avoid contaminant saturation, the micro precolumn was efficiently flushed by a 40µL/min flow of solvent B in parallel of the analytical gradient. All contaminants were then removed (Figure 3B).

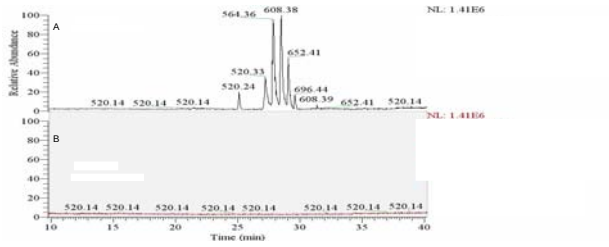

Figure 3 : Extracted ion chromatograms (EICs) of ions m/z 520.333, 564.359, 608.385, 652.411, 696.438 obtained from blank run injection without (A), and with (B) a micro precolumn in 1D-LC system.

A standard protein mixture (20 fmol) was analyzed in our 1D-LC system before and after introduction of the micro precolumn. Without the trapping of contaminants, serotransferrin was not identified and lysozyme was identified only with one peptide (Table 1). Moreover only 10 different peptides were validated for all identified proteins. After introduction of a micro precolumn, all proteins were identified with at least two different peptides and 34 different peptides were validated for all proteins identification. This test was sequentially repeated to check the efficiency and the robustness of our setting for several months.

Protein Name	Accession number	Number of peptides		Score	
		Without Micro Precolumn	With Micro Precolumn	Without Micro Precolumn	With Micro Precolumn
Beta-galactosidase	P00722	2	7	20	60
Serum albumin precursor	P02769	3	8	30	80
Serrotransferrin	Q29443	0	11	0	110
Alcohol dehydrogenase 1	P00330	2	4	20	20
Lysozyme C precursor	P00698	1	2	10	20
Cytochrome c	P00015	2	2	20	20

Table 1 : Identification of 20fmol protein mixture with and without precolumn.

In 2D-LC experiments, interfering peaks were also observed in RP chromatograms of every SCX step (Figure 5A) with a S/N of up to 1300. A similar approach as in 1D-LC was implemented (Figure 2). Interfering peaks were still observed but with a S/N of up to 300 (Figure 5B).

It was necessary to remove also these impurities on the first dimension of our 2D-LC system. A second micro precolumn was inserted after micropump 1. To regenerate the C18 phase, this precolumn is flushed with a flow of 40µl/min of solvent B after last SCX fraction. PEG's peaks were still observed but with a S/N of up to 50 (Figure 5C), which was far below the background signal coming from the PMC ion ($(Si(CH_3)_2O)_7$; m/z 536.170 with S/N of 200).

Recent studies suggested that ammonium formate is more contaminated than formic acid[4]. Accordingly, we prepared our buffer with formic acid 99-100%, ammonia solution 25% and TFA. The contamination was then not detected anymore (Figure 5D).

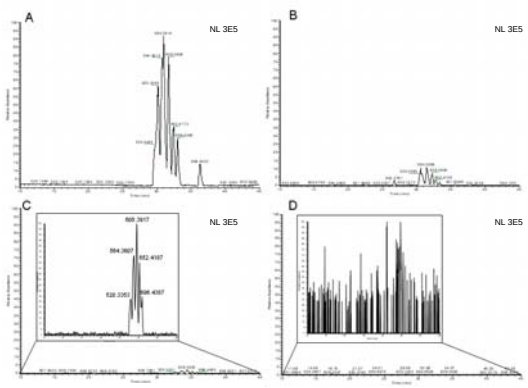

Figure 4 : Extracted ion chromatograms (EICs) of ions m/z 520.333, 564.359, 608.385, 652.411, 696.43 obtained from standard injection in 2D-LC system without any micro precolumn (A), with one micro precolumn (B), with two micro precolumns (C) and with a new ammonium formate solution (D). A relative intensity scale was also performed (C and D).

CONCLUSIONS

We have demonstrated that different common series of impurities can be observed whatever MS or LC instruments, batches or suppliers for solvents and chemicals used. These impurities may be harmful to the quality of the analyses during 1D- and 2D- LC-MS. This work presents robust and simple solutions to reduce dramatically and continuously the amount of impurities by using an on-line purification of the mobile phase by micro precolumns so that you improve your analyses.

REFERENCES

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Acknowledgments : We thank L'Oréal for Anne-Marie Hesse PhD fellowship ; RNG and FRRT for fundings