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# Evaluation of nano-liquid chromatography–tandem mass spectrometry in a column switching setup for the absolute quantification of peptides in the picomolar range

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**Abstract**

A standard nanospray-liquid chromatography–tandem mass spectrometry system in a column switching setup for the absolute quantification of leucine-enkephalin was evaluated. Analytes were loaded on a C18 trapping column and back-flushed in the 75 μm analytical column. Quantification was performed with a triple quadrupole instrument. Validation results show that it is feasible, with a conventional nano-LC system in the column switching setup, to quantify peptides as low as 500 amol on column (50 pmol/L). Weighted linear regression analysis proves a good linearity in a dynamic range of almost three orders of magnitude. Nevertheless, robustness remains a key issue in nano-LC–MS/MS.


*Keywords:* Neuropeptides; Leu-enkephalin; Quantification

## 1. Introduction

Since LC–MS/MS is nowadays commonly used, miniaturisation is the key factor to gain sensitivity. This is especially true when one has only limited sample volumes of e.g. valuable proteins or peptides available. We are interested in the simultaneous analysis of neuropeptides in in vivo dialysis samples. To that end, leucine-enkephalin was chosen as the target of initial suitability oriented experimental work. Enkephalin is a small, five amino acid chain belonging to the neuropeptide family. Leucine-enkephalin is a naturally occurring opiate-like peptide that might be useful as an analgesic agent [1]. Several methods have been reported for the sensitive determination of leu-enkephalin and peptides in general, such as radio or enzyme immunoassay (RIA or ELISA), capillary electrophoresis (CE), capillary electrochromatography (CEC), liquid chromatography combined with UV, fluorescence, electrochemical or mass spectrometric detection [1–9]. Sensitive determination of neuropeptides is necessary, certainly in view of the low concentrated, low volume samples obtained by e.g. microdialysis. Although RIA provides high sensitivity, it has limited specificity [8,10]. Capillary zone electrophoresis is a simple and fast separation technique combining high separation efficiency with a low sample requirement and a high absolute sensitivity. One of the major disadvantages of CE and CEC compared to LC is, however, the limited loading capacity resulting in a low concentration sensitivity [2,6]. Gradient LC with UV detection is commonly used for the detection of enkephalins. However, low sensitivity due to low absorptivity of most peptides makes it inappropriate [7]. Fluorescence detection cannot detect all peptides and in general the sensitivity of the native fluorescence detection is slightly lower than UV, depending on the aromatic amino acid composition of the analyte. Pre-column or post-column derivatisation is required to solve this limitation [1,8,11]. Detection limits of electrochemical detection for leucine-enkaphalin are in the low nanomolar range [1]. Endogenous levels of opioid peptides in human CSF, as determined by radioimmunoassay lie in the fmol $mL^{-1}$ or picomolar range. Therefore, these methods are inadequate for the determination of endogenous levels of opioid peptides in human CSF [8].

Mass spectrometric detection has become almost indispensible in the field of bioanalysis. This is certainly the case for peptides and proteins. The development of the LC–MS

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interface, in combination with nanospray techniques dramatically improved the sensitivity limits to the picomolar, even femtomolar range [4,5,12,13]. Moreover, tandem MS is attractive because it offers the possibility of detecting peptides with sequence specificity and can be used, in principle, for any peptide. Although generally identified as an LC–MS interface, atmospheric pressure ionisation (API) interfaces accept liquid streams from either flow injection type systems or as an LC eluent. Both approaches are suitable for peptides. Flow type analytical systems, eventually with rudimentary separation on a small packed sprayer, have the advantage of simplicity and superior concentration type sensitivity, as is the case for API based mass selective detectors. However, despite earlier optimism regarding the selectivity of tandem MS, it has become clear that sound chromatographic separation is still a key factor in quantitative mass spectrometry.

In LC, as in the MS interfaces, miniaturization is mandatory, certainly in our case with neuropeptides as sensitivity was the most important requisite. To that end, a nano-LC system was chosen. Theoretically, diminishing the column diameter in LC–MS, gives a quadratic improvement in sensitivity [14]. With routine analytical application in mind, we opted for the commercially available 75 μm type of nano columns. On such a system, sample injection volumes are limited to more or less 10 nL, which is not practical for the analysis of biological extracts containing low levels of analytes. In order to be able to inject large sample volumes on a nano column, on-column analyte focusing or a column switching setup is necessary [15,16]. Since on-column focusing needs a prohibitively long time at a flow in the nanoliter-per minute level, the column switching setup [17–19] was evaluated. Sample injection is in this case made off-line, at a flow rate of 10 μL/min and the trapping column is then moved in-line to the analytical column by switching a valve. An extra advantage of such an approach is desalting of the sample and a certain level of sample clean-up. In the case of neuropeptides, analysis is often in support of in vivo microdialysis experiments. Samples are thus relatively clean but desalting is mandatory. For optimum sensitivity and selectivity, the mass spectrometric analysis was performed in multiple reaction monitoring (MRM) on a triple quadrupole instrument. That MS/MS method increases significantly the molecular specificity of the quantitative measurements [20] and is necessary for the quantification of peptides in the lower concentration range, i.e. the picomolar range.

Nevertheless, a relatively complex column switching LC–MS/MS system of this kind should not only be sensitive. It should also be linear, robust and reproducible in order to routinely and absolutely quantitate peptides. This was emphasised in recent work of Wan et al. who used isotopically labelled internal standardisation to obtain a sensitive and robust quantitative analysis method on a packed emitter tip for the quantification of a synthetic opioid peptide analog [Dmt$^1$] DALDA [21].

In this paper we describe the experimental evaluation of the potential of a standard nano-LC–MS/MS system in a column switching setup, as generally used in proteomics, to absolutely quantify peptides. The neuropeptide leu-enkephalin is thereby used as a model. Special attention has been paid to the overall robustness of the nanospray system.

## 2. Experimental

### 2.1. Chemicals

Standards [glu$^1$]-fibrinopeptide and leu-enkephalin were purchased from Sigma-Aldrich (Bornem, Belgium). HPLC grade acetonitrile and formic acid were supplied by Merck (Darmstadt, Germany). A Synergy 185 system (Millipore Corporation, Bedford, MA, USA) was used to generate high-purity water for the preparation of all aqueous solutions.

### 2.2. Sample preparation

Stock solutions of leu-enkephalin and [glu$^1$]-fibrinopeptide were prepared in water (1.0 nmol/mL). Working standards of leu-enkephalin were prepared in the concentration range of 10 fmol/mL–10 pmol/mL by dilution with 0.1% (v/v) formic acid in water, with the internal standard [glu$^1$]-fibrinopeptide present in a final concentration of 1 pmol/mL.

### 2.3. Mobile phases

LC solvents A and B consisted respectively of 0.1% (v/v) formic acid in water and 0.1% (v/v) formic acid in a 80/20 (v/v) acetonitrile/water mixture. Both solvents (A) and (B) were filtered through a 0.45 μm membrane filter.

### 2.4. Liquid chromatography

Chromatography was performed in a standard column switching setup, as commonly used in proteomics. Our system consisted of a Famos™, Fully Automated Micro Autosampler equipped with a 10 μL loop (LC-Packings, Amsterdam, The Netherlands), for injection of samples. The capillary liquid chromatography system consisted of a low pressure gradient micropump (Ultimate™, LC-Packings, Amsterdam, The Netherlands). Injection parameters were optimised in the microliter pick up mode, meaning that exactly 10 μL of the samples could be injected, without any sample loss. This method is particularly suited for limited sample volumes. A Switchos™ column switching device (LC-Packings, Amsterdam, The Netherlands) was operated at a loading flow of 10 μL/min. Samples were loaded for 3 min in 100% solvent A (i.e. water + 0.1% (v/v) formic acid) on a Pepmap® C18 trapping column (5 μm, 100Å, 300 μm i.d. × 1 mm; LC-Packings, Amsterdam, The Netherlands). After 3 min, the valve was switched and the analytes were back-

flushed onto a PepMap® C18 (3 μm, 100 Å, 75 μm i.d. × 15 cm) analytical column. A linear gradient from 6% to 75% solvent B over 43 min (i.e. 80/20 acetonitrile/water + 0.1% (v/v) formic acid) was used for the analytical separation at a column flow rate of 150 nL/min. The trapping column and analytical column were maintained at 75% solvent B for 9 min at 150 nL/min, followed by a second switch and 15 min of re-equilibration of, respectively, the trapping and analytical column at 100% solvent A (10 μL/min) and 6% solvent B (150 nL/min). The autosampler, switching device and HPLC pumps were controlled by Chromeleon® software (Dionex, Amsterdam, The Netherlands).

### 2.5. Mass spectrometry

Detection was performed with a Quattro Ultima triple quadrupole instrument (Waters, Manchester, UK) equipped with an orthogonal nano-electrospray source (Z-spray®) in the electrospray positive ion mode (ESI+). In the nano-electrospray source (Waters, Manchester, UK) an on-line PicoTip™ emitter (distal coated SilicaTip™, 360 μm o.d., 20 μm i.d., 10 μm i.d. at the tip; New Objective, Woburn, MA, USA) was used as a spray capillary. Cone and capillary voltage were optimised at 65 V and 2500 V, respectively. Source temperature was controlled at 80 °C and the $N_2$ cone gas was maintained at 55 L/h. Collision energy was set at 25 eV for [glu$^1$]-fibrinopeptide and 20 eV for leu-enkephalin. The mass spectrometer was operated in the MRM mode using argon as collision gas. The MRM method selected both protonated molecules, *m/z* 786.21 for [glu$^1$]-fibrinopeptide and *m/z* 556.41 for leu-enkephalin. Transitions of the doubly charged 786.21 > 480.49 and 786.21 > 684.49 for [glu$^1$]-fibrinopeptide and the singly charged 556.41 > 278.2 and 556.41 > 397.31 for leu-enkephalin were recorded. Data were collected and processed using the MassLynx® software (Waters, Manchester, UK). Alternatively, all stainless steel spray capillaries were used. These nano-bore emitters (Proxeon Biosystems, Odense, Denmark) with an inner diameter of 25 μm (untapered) were fitted in a home made adaptor to fit the Waters nano-electrospray source. Alternative gold coated emitters (75 μm o.d., 5.2 μm i.d. at the tip) were a kind gift of Nanoseparations (Nieuwkoop, The Netherlands).

## 3. Results and discussion

### 3.1. Chromatographic performance

In this study we evaluated a nano column switching setup for the absolute quantification of leucine-enkephalin. Injection volumes in nanoflow LC systems are limited to nanoliter volumes only [22,23]. Column switching overcomes this limitation by giving the possibility of injecting microliter volumes. The microliter pick-up mode enables the injection of exactly 10 μL without any sample loss. This is particularly suited for limited sample volumes, as in the case of the restricted sample quantity of e.g. microdialysis. Future adaptations to even greater sample volumes could be easily made, e.g. to obtain lower detection limits.

Retention time (RT) stability is a crucial factor in (nano)-LC, especially when time-dependent mass spectrometric functions are used. Moreover, specificity considerations in MRM analysis require retention time as an extra identity confirmation criterion. Therefore, particular attention was devoted to loading and analytical flow and thus retention time reproducibility. In that respect, it proved absolutely vital that all mobile phases were continuously sparged with helium to prevent gas bubble formation in the Switchos™ and Ultimate™ micropumps which seem to be rather prone to pump cavitation, thereby dramatically influencing nanoflow reproducibility. For optimum separation, trifluoroacetic acid (TFA) is commonly added in reversed-phase liquid chromatography of peptides. Since it is well known that the combination of TFA and ESI-MS results in signal reduction and spray instability [24–27], formic acid (0.1%, v/v) was used as additive in the mobile phases. 0.1%, v/v of TFA results in an immense drop in sensitivity and even a small amount of TFA (0.01%, v/v), even when added to the formic acid containing mobile phases, resulted in a significant signal suppression at infusion experiments. Due to the volatility of formic acid, one would suspect a loss of formic acid due to helium degassing, thus gradually affecting retention behaviour. This effect is, however, negligible: pH is the key to retention behaviour rather than modifier concentration and the pH of solvent A (water + 0.1% (v/v) formic acid) remained constant (pH 2.68 ± 0.02), even after one week of continuous sparging with helium. Reproducibility of retention times was checked by repeated injections of standard mixtures of [glu$^1$]-fibrinopeptide and leu-enkephalin. Resulting retention times showed maximally 5.75% relative standard deviation ($n$ > 50). Retention times usually never shifted more than 0.75 min, which is reasonable at such low flow rates, generated by flow splitting. However, larger shifts have occasionally been observed, probably due to gas bubble formation in the pumps, going by unnoticed, but having a major influence in the nanoflow region of the system. A recorded pressure profile proves a good help in such cases. Relative retention times (RT leu-enkephalin/RT [glu$^1$]-fibrinopeptide) at the other hand resulted in 0.38% relative standard deviation ($n$ > 50), which presents a good value for qualitative and quantitative analysis. It is clear that rather than absolute retention times, relative retention times are preferably used as they are very reproducible, allowing unambiguous identification or confirmation of, in this case, leu-enkephalin.

Chromatographic separation was performed using a linear gradient up to 75% of solvent B. Particular consideration should be paid to the reequilibrating time, which clearly tends to the unacceptable. We evaluated that an equilibrating time of 15 min at the end of each run is an absolute minimum to prevent influences on the retention time of the next chromatographic run.

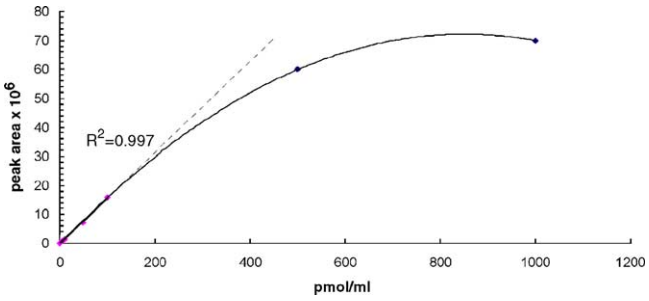


Fig. 1. Saturation of the LC–MS system at higher loadings of [glu$^1$]-fibrinopeptide.

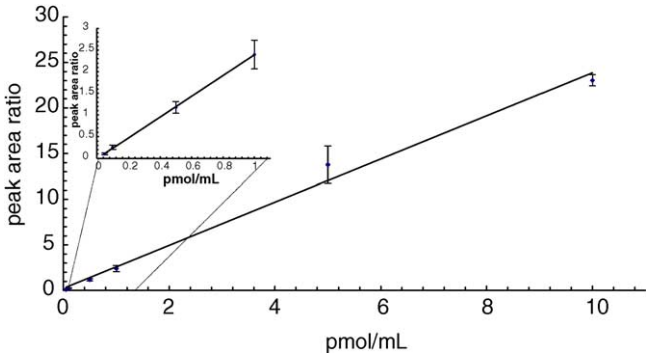


Fig. 2. Linear regression of leucine enkephalin, using [glu$^1$]-fibrinopeptide as internal standard ($n$ = 6). Error bars clearly indicate the influence of spray variability on the between day reproducibility.

### 3.2. Quantitative characteristics

The linear dynamic range of the column switching setup was initially evaluated by injection of increasing concentrations of standards of leu-enkephalin, respectively, [glu$^1$]-fibrinopeptide. Care should be taken when bringing more than 1 pmol on column, as saturation of the system is seen at higher loading, resulting in a quadratic fit (Fig. 1). Although linearity is good up to 100 pmol/mL (1 pmol oc, 10 μL injections), these relatively high concentrations resulted in sticking of the peptides in the LC system. This seriously affects the possibility of sensitive determinations, as carry-over is seen in several runs thereafter. Sticking will also occur at low concentrations, but the effects will not be witnessed because the stuck amounts are below the limit of detection. To prevent overloading the LC-system, we never injected more than 100 fmol on column. MRM detection offers a good sensitivity with a great selectivity, which are both very important in high sensitivity analytical methods. Good linearity ($R^2$ > 99.9%) could be achieved over a concentration range of three orders of magnitude (100 amol oc–100 fmol oc, 7 data points) for series of standards of both leu-enkephalin and [glu$^1$]-fibrinopeptide, based on absolute peak areas. However, this could only be achieved with a new sprayer tip and on the condition it remained in good condition for the duration of the whole batch of measurements. As will be discussed later, this kind of robustness is not evident.

For quantification of leu-enkephalin, peak area of the analyte divided by the peak area of [glu$^1$]-fibrinopeptide, used as internal standard, was plotted against the amount of analyte injected on the column. Weighted linear regression was applied to calculate the calibration curve. In doing so, a higher weight is allocated to the lower concentrations in the calibration curve, leading to an improved quantification of very low-level concentrations. A weighing factor 1/$X$ was used. The MRM method permitted the construction of linear response curves between 50 fmol/mL or 500 amol on column and 10 pmol/mL, respectively, 100 fmol on column (Fig. 2). Correlation coefficients of this weighted linear regression were between 0.9928 and 0.9997 ($n$ = 6). An average correlation coefficient of 0.9962 was obtained for the relationship between the peak area ratio (leu-enkephalin/[glu$^1$]-fibrinopeptide) and the corresponding calibration concentrations. The 95% confidence interval of the $y$-intercept of the linear response curves always included zero, corresponding with significant $P$-values. Mean residuals of the weighted linear regression were between 95 and 109.6% (maximum R.S.D. 15.6%) (Table 1). These data indicate quantitative analysis with the proposed set-up is feasible. Nevertheless, robustness proved to be a key issue.

The limit of detection (LOD) and limit of quantitation (LOQ) were established at 16 fmol/mL and 54 fmol/mL, as determined by the formula LOD = $3S_{\text{dev. intercept}}$/m, respectively LOQ = $10S_{\text{dev. intercept}}$/m. Additionally, signal-to-noise ratio ($S/N$) was minimally 3 for the LOD and minimally 10 for the LOQ. Proportionally lower concentration limits could be reached by increasing the injection volume. Unfortunately, the major application targeted by a quantitative neuropeptide analysis, i.e. in vivo dialysis experiments, often precludes the use of larger sample volumes.

Between day precision of the various standards (50 fmol/mL–10 pmol/mL) was evaluated and included in Table 1. Injection of 50 fmol/mL (500 amol on column, LOQ) revealed a precision of 23.3% R.S.D.

### 3.3. Nanospray performance

The SilicaTip™ emitters have a multi-layered conductive coating. Durability of these emitters appeared crucial for routine quantitative measurements. To our experience, average life time of these tips is about one week. Optimal spray usually lasts for only approximately 2 days. We consider this an almost insurmountable problem for routine quantitative measurements. Not only does this negatively influence reproducibility within a single, e.g. overnight, sample batch, but spray and thus ionisation characteristics of different tips, in conjunction with cumbersome, poor repeatability of the critical tip positioning in the nanosource configuration result in large batch-to-batch variations in detection and quantification limits. The main reason for spray instability is droplet formation at the tip orifice, resulting in a sputtering spray. This can be temporarily adjusted by manually increasing the capillary voltage but this is unrealistic in batch operation. Ap-

Table 1
Linearity and total reproducibility data

| Linearity | Slope | Intercept | Standard error | $R^2$ | | |
|---|---|---|---|---|---|---|
| Average ($n$ = 6) | 2.53 | −0.0214 | 0.0415 | 0.9962 | | |
| S.D. | 0.2573 | 0.0130 | 0.0204 | 0.2371 | | |
| Precision | | | | | | |
| Concentration (pmol/mL) | 0.05 | 0.1 | 0.5 | 1 | 5 | 10 |
| R.S.D. ($n$ = 6) | 23.30% | 19.85% | 11.45% | 13.73% | 11.13% | 2.67% |
| Mean residuals | | | | | | |
| $n$ = 6 | 97.81% | 109.65% | 94.97% | 95.80% | 109.58% | 98.81% |
| R.S.D. | 11.40% | 13.85% | 7.09% | 11.45% | 15.59% | 3.79% |

plying desolvation gas did not inhibit any droplet formation and effectively reduced MS signal at infusion, thus negatively influencing sensitivity. Increasing the source temperature to 120–140 °C also does not offer any solution. Clogging of the emitters has also been observed. Besides filtering of the solvents, it seems nothing can be done to avoid this. For all of these reasons, gold-coated fused silica tapered tips and non-tapered nano-bore stainless steel emitters were evaluated as alternatives. The average life time of the gold coated tips is considered to be about one week [23]. Non-tapered stainless steel tips are normally less susceptible to clogging and the durability of plain stainless steel versus coated silica would appear to be better. Continuous infusion with a Hamilton syringe at 400 nL/min of leu-enkephalin and [glu$^1$]-fibrinopeptide resulted in comparable responses in view of sensitivity, for both alternative emitters. When coupled to an LC-system, similar droplet formation and variable spray, however, appeared. Even the non tapered nano-bore stainless steel emitters steadily suffered from the increasing formation of droplets at the tip orifice after only 24 h at 2500 V. Fig. 3 indicates variations in signal intensity, seen at infusion experiments with the Picotip and gold coated emitters. Each time point represents the highest possible signal attainable after optimal tuning with that emitter. High variability is seen, sometimes over very short time intervals, for example, a drop in sensitivity of 50% over 20 min at infusion of [glu$^1$]-fibrinopeptide with a gold coated emitter.

Drawbacks of nanospray emitters, such as fragility, causing a high propensity to fracture the sharp end of the tip, corrosion of the conductive coating, clogging and manufacturing difficulties (the orifice of the nanotip is less uniform than in

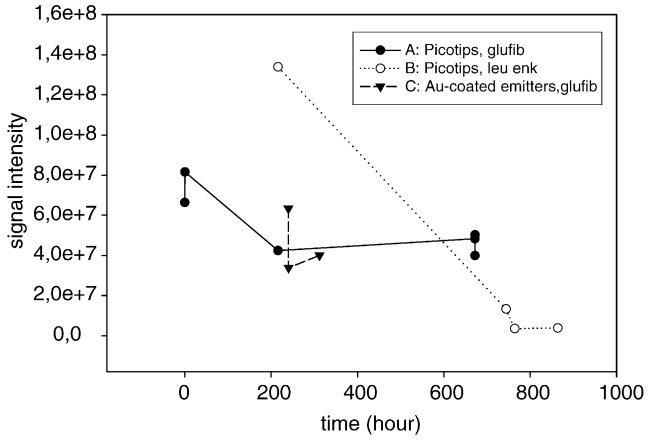


Fig. 3. Tuning experiments at infusion of [glu$^1$]-fibrinopeptide (A and C) or leucine enkephalin (B). MS parameters were always optimised at each point to obtain the highest possible signal intensity. As such, variability of absolute signal intensity, due to spray instability, is clearly stressed.

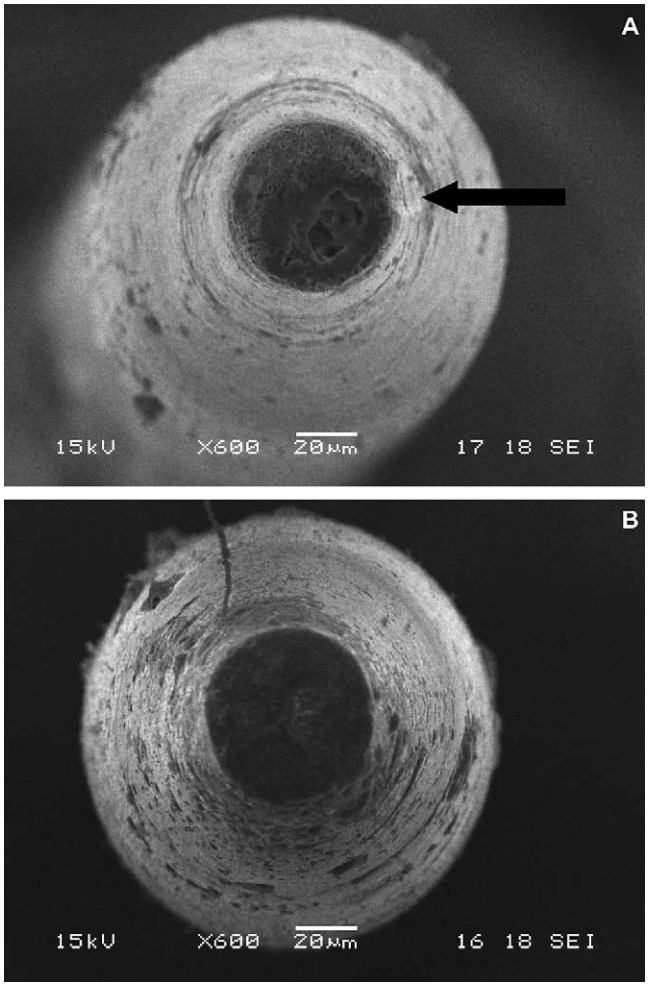


Fig. 4. Scanning electron microscopy images of employed nanospray emitters: (A) stainless steel emitter after 48 h of use, the arrow indicates a damage/difference with the non-used tip, probably due to manufacturing difficulties or electric degradation; and (B) non-used stainless steel emitter.

conventional ES) make their use very troublesome in quantitative analysis [28,29]. Electrochemical reactions, occurring at the emitter during the ESI process are, apart from clogging, the major limiting factors for the lifetime of the emitters [30]. The processes that cause the failure of sheathless electrospray ionisation emitters, based on different kinds of gold coatings on fused-silica capillaries, are described and explained by Nilsson et al. [31]. Besides, several papers have been published, devoted to the development of enhanced, more robust, better nanoelectrospray needles [32–36]. The lack of durability and chemical stability of the commercially available emitters used in this research, seriously affects the possibility of unattended LC–MS operation [23,34–36]. Comparison of scanning electron microscopy images of the stainless steel emitters show the difference between two of the investigated emitters (Fig. 4). Another issue is the ability to obtain reproducible charge state distributions, which can have an enormous impact on the accuracy of studies that rely on a single charge state for quantification. The orifice diameter is in that case very important to control charge state distributions. Success of tandem MS can hinge on the ability to control the charge state of specified ions [28]. The poor quality and thus short life-time of the metal coatings has also been reported in CZE-nano-ESI with sheathless interfaces. The lifetime of metal coatings are often short varying from minutes to days, due to electrochemical/electrical degradation under CE/ESI-MS [2,37,38]. We can conclude that droplet formation, durability, clogging and spray instability of the ESI emitters effectively reduce the feasibility of routine nanospray in absolute quantitative measurements.

## 4. Conclusions

It is possible, with a conventional nano-LC system in the column switching setup, to determine peptides as low as 100 amol on column in MS/MS. Multiple reaction monitoring (MRM), in combination with perfectly reproducible relative retention times, allow the unambiguous confirmation of peptides. Linearity is good in a dynamic range of almost three orders of magnitude.

However, between day precision variability, mean residuals and the average linearity data already reveal the in essence non-robustness of the nano-LC–MS/MS system. Tuning and continuous infusion of standard peptides also revealed signal instability of the Picotip® nanospray system. Gold coated and stainless steel emitters seem to offer no real solution for the spray instability. Internal standardisation with another peptide, in this case [glu$^1$]-fibrinopeptide, is hardly sufficient to solve this. The nanosource and electrospray ionisation process, are obviously far from stable, when used in absolute quantitative measurements.

Nano-electrospray is a technique with great advantages concerning sensitivity. To our experience, this advantage is substantially negated by its instability and the lack of robustness. Stability of the nano-ionisation process should clearly be improved in the future to effectively enlarge its analytical possibilities and to make nano-LC–MS/MS a “workable” instrument to routinely measure peptides in the picomolar range. Isotopically labeled internal standards might also be essential to obtain this goal as was clearly illustrated in a recent example [21].

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