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Application of Mass Spectrometry for Quantitative and Qualitative Analysis in Life Sciences

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Abstract: Due to its ability to analyze macromolecules, as well as polar and thermally labile low molecular weight compounds, mass spectrometry has become a very important tool in bioanalysis in recent years. The Life Sciences Mass Spectrometry Laboratory (LSMS) at the School of Pharmaceutical Sciences, University of Geneva, opened on April 1st, 2002. Its work is focused on the development and application of mass spectrometry for the qualitative and quantitative analysis of low molecular compounds and macromolecules present in biological fluids, tissues, organisms or cells. The instruments used for the research include: single quadrupole, triple quadrupole and hybrid MS instruments such as triple quadrupole linear ion traps; equipped with electrospray, nanoelectrospray, atmospheric pressure chemical ionization and matrix-assisted laser desorption.

Keywords: Chip-based infusion · MALDI · Mass spectrometry · Metabolite profiling · Non-covalent complex

1. Introduction

Mass spectrometry (MS) is increasingly playing a major role in academic and industrial pharmaceutical research for the identification and quantification of low molecular compounds or macromolecules, such as peptides or proteins involved in biological processes. The development in the mid 1980s, of matrix-assisted laser desorption (MALDI) and atmospheric pressure ionization (API) sample introduction techniques, highlighted the value of MS in life sciences. Impressive progress regarding robustness, sensitivity and performance, has also been made during the last decade with the mass analyzers. A large variety of MS instruments, including triple quadrupole, ion trap (IT), linear ion trap (LIT), time-of-flight (TOF) and Fourier transform ion cyclotron resonance (FTICR) systems are now commercially available. Subsequently, the use of MS is no longer restricted to highly specialized laboratories and it is quite common to find triple quadrupole or IT instruments in biological, pharmaceutical, chemical or medicinal laboratories. Despite the fact that MS is now widely used, the MS community remains very active in the development of new hardware, software and applications. Recent new combinations of hybrid mass spectrometers such as quadrupole-time of flight (QqTOF), triple quadrupole linear ion trap (QqLIT), or linear ion trap – Fourier transform ion cyclotron resonance (LIT-FTICR), open new doors for the application of mass spectrometry to solve life science challenges. Despite massive progress, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) still suffers from certain limitations regarding sample throughput in quantitative analysis and spectral information in qualitative analysis. The present paper focuses on investigations, by the LSMS group, highlighting alternative analytical approaches to overcome some of these limitations.

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2. Quantitative MS Analysis without Chromatography

LC-MS/MS with API plays a major role in the quantitative analysis of pharmaceutical compounds in biological fluids to support pharmacokinetic studies in drug discovery and development [1]. With LC-MS/MS several hundred samples can be analyzed on a daily basis. To achieve a limit of detection in the pg/ml range with good precision and accuracy, sample preparation remains essential to minimize matrix effects. Dilute and shoot approaches are widely used in discovery work but are often difficult to implement in drug development because the dilute and shoot assays are often not sufficiently robust or sensitive for routine analysis. Due to different chemical properties of pharmaceutical compounds and their metabolites, generic approaches are difficult to apply and the required method development is becoming a time-limiting step for multi-component analysis. LC remains important for the concentration of the analyte during the injection step, for separation of isobaric metabolites or to minimize matrix suppression. An LC-MS/MS assay with a run cycle time of ten seconds [2] has been successfully developed for the analysis of a

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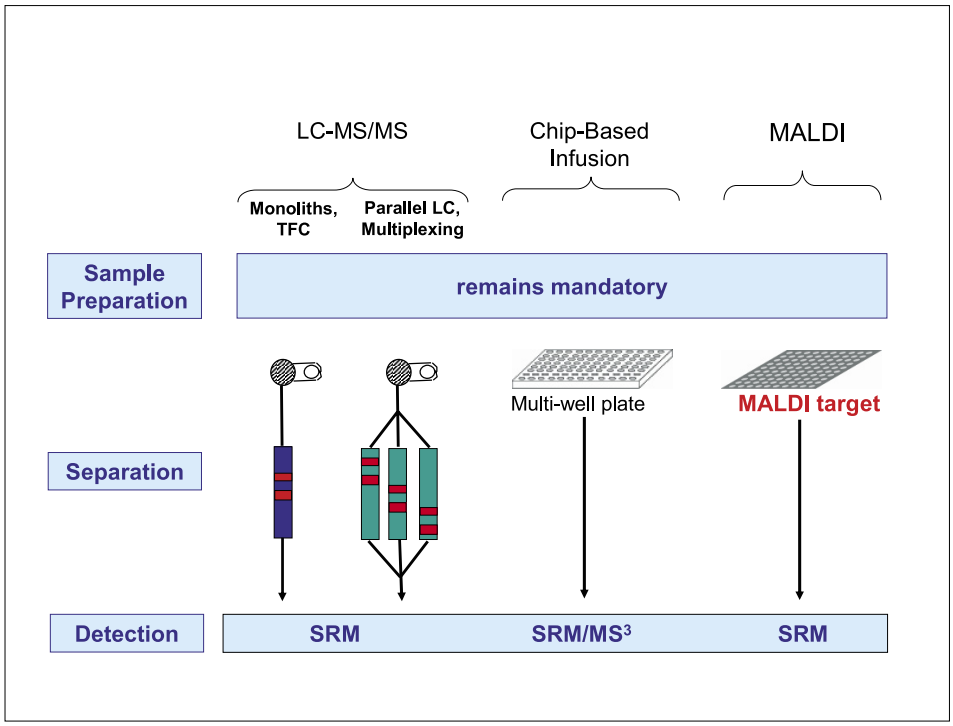

Fig.1. Various strategies for quantitative analysis in biological fluids

pharmaceutical compound in plasma, however the system configuration is quite challenging for routine application.

Sensitivity is often obtained at the cost of analysis time or throughput. However, many studies do not require ultimate sensitivity. With the high selectivity of tandem mass spectrometry, baseline separation of the analytes is no longer required and the question arises as to the real need for LC. However, as illustrated in Fig. 1, sample preparation remains a key step in bioanalysis. To achieve a run cycle time of well below 1 min, it becomes almost mandatory to by-pass liquid chromatography, and two approaches using chip-based infusion or MALDI, have been successfully investigated.

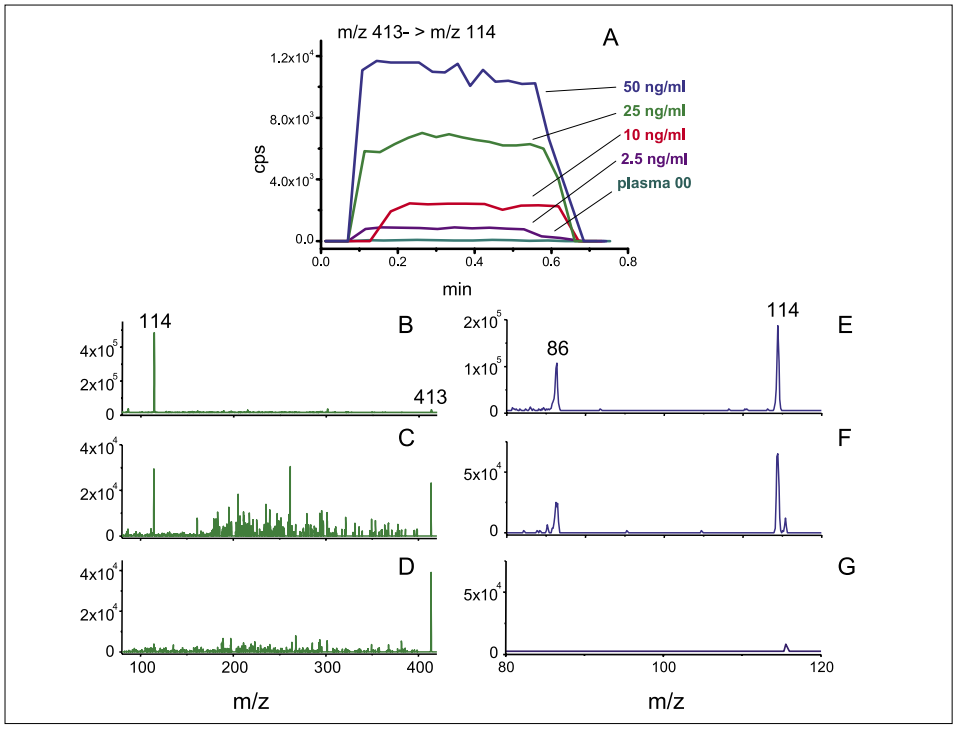

Fig. 2. A) SRM infusiogram of the parent drug plasma extract at various concentrations; B) Enhanced product ion (EPI) spectrum of a reference solution of parent drug; C) EPI spectrum of calibration sample 2.5 ng/ml of parent drug; D) EPI spectrum of blank plasma; E) MS3 spectrum of a reference solution of parent drug; F) MS3 spectrum of calibration sample 2.5 ng/ml of parent drug; G) MS3 spectrum of blank plasma.

Quantitative analysis in the selected reaction monitoring (SRM) using an automated chip-based infusion nanoelectrospray device has been recently demonstrated [3] for the analysis of verapamil in plasma. The chip-based infusion system (NanoMate, Advion BioSciences) is made of an array of 100 or 400 nozzles and enables the rapid infusion of the sample at a flow rate of approximately 200 nl/min. For each analysis a new pipette tip and a new nozzle are used to eliminate the carry-over effect encountered with LC-MS/MS analysis. Detection is performed on a triple quadrupole mass spectrometer operated in SRM mode. With chip-based infusion the software is no longer integrating chromatographic peaks but rather an 'infusiogram' or a square-shaped 'peak' is created by the extracted ion chromatogram from the infusion process (Fig. 2A). Construction of the calibration curve is done in a similar way to LC-MS/MS by plotting the area ratio of the analyte over the internal standard. Using a plasma aliquot of 100 µl an assay could be developed for an oxadiazole drug and its major metabolite in human plasma from 2.5 to 1000 ng/ml [4]. Infusion time of approximately one minute was long enough to collect sufficient data. Using chip-based infusion, sample carry-over, evident during LC-MS, was completely eliminated and sample throughput was also improved. A batch of 96 samples could be analyzed in less than 90 min. Method development is also simplified because: i) no chromatographic separation means that no optimization is necessary, ii) the analytes and the internal standard always co-elute and non-labeled internal standards can be used. Another advantage is that deterioration of chromatographic separation during analyses of very large batches is no longer an issue. However, with chip-based infusion good sample preparation such as liquid–liquid extraction was found to be very important to minimize matrix suppression and to develop rugged analytical methods.

Method development and assay validation of pharmaceutical compounds in biological matrices are typically performed with spiked samples. However study samples may behave differently and the screening of potential interferences of metabolites or co-administrated drugs should be performed. Triple quadrupole linear ion trap mass spectrometers and precise SRM quantization can be performed in the same analysis, resulting in the detection of full scan MS/MS or MS3 spectra [5][6]. This feature becomes particularly interesting for method validation using *in vivo* samples and in drug metabolism. This concept was extended to chip-based infusion by performing simultaneous SRM and MS3 quantitation (Fig. 2B-G) in the same run,

thus increasing analytical specificity [4]. A concept for monitoring quantitative assay performance using SRM/MS3 ratio was proposed. One limitation of the approach is that phase II metabolites can, under certain circumstances, fragment into the aglycon form by up-front CID in the interface of the instrument and interfere with quantitation.

MALDI is mainly used for the analysis of peptides and proteins. It is a promising approach when combined with a triple quadrupole instrument for the quantitative analysis in SRM mode of small molecules and peptides, because it allows real, accurate and precise high-throughput analysis. The application of MALDI-SRM has been demonstrated for the analysis of talinolol in human plasma without LC separation, using a prototype orthogonal MALDI triple quadrupole mass spectrometer. The assay was found to be linear from 1 to 1000 ng/ml using a 200 µl plasma aliquot [7]. Even by using a non-deuterated internal standard (propranolol), precision and accuracy were found to be sufficient to perform quantitative analysis. Liquid–liquid extraction was selected as sample preparation method to minimize matrix suppression. Direct protein precipitation with perchloric acid failed due to severe matrix suppression. However, an *in situ* wash of the spot with an aqueous solution permitted restoration of the signal, allowing quantitative analysis with a simplified sample preparation procedure. With a high repetition laser (1400 Hz) the analysis of one spot takes less than 2 s, allowing the analysis of 96 samples in approximately 4 min.

In bioanalysis, the decoupling of sample preparation and chromatographic separation from MS analysis should offer a very attractive alternative compared to on-line LC-MS/MS: multiplex sample analysis is easier and the time required to analyze a batch of sample from a preclinical or clinical study is dramatically reduced.

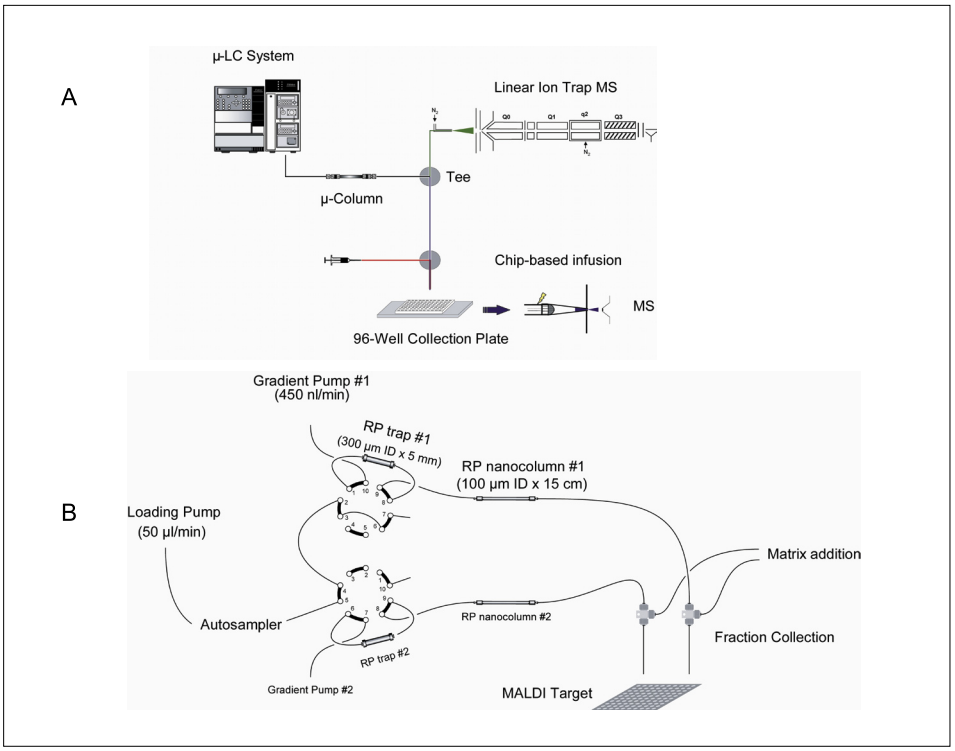

Fig. 3. A) Micro-LC combined with fraction collection into 96-well plate; B) Parallel nano-LC combined with dual collection onto MALDI target.

3. The Application of Mass Spectrometry in Drug Metabolism

Drug metabolism is playing an increasingly important role in drug discovery and early drug development. Metabolite stability, identification, and structural characterization are crucial for lead optimization. For this purpose, efficient strategies based on non-radiolabeled parent drugs are needed. Due to the need for high sensitivity, LC-MS/MS is currently the method of choice for early drug metabolism studies [8]. However, LC-MS/MS has an intrinsic problem for qualitative analysis because most LC peaks elute too quickly to perform all of the necessary MS experiments in one run. Therefore, structural characterization of metabolites often requires re-analysis to obtain high quality multiple stage MS or accurate mass data on various types of mass spectrometers. Fig. 3A illustrates a new strategy which combines micro-LC-MS fraction collection *via* a post-column split into a 96-well plate, with simultaneous on-line MS monitoring, followed by automated nano-electrospray chip-based infusion for rapid re-analysis of the fractions of interest [9]. The information-dependent LC-MS/MS analysis enables the characterization of the major metabolites, while the chip-based infusion is used to obtain good product ion spectra for lower level metabolites. This generates complementary MS information on potential metabolites identified in the LC-MS trace and enables screening for unexpected metabolites. Fractions from the chromatographic analysis are collected in 20 second steps into a 96-well plate. The fractions of interest can be re-analyzed with chip-based infusion on a variety of mass spectrometers including QqLIT (Q TRAP, AB/MDS Sciex) and QqTOF systems (QSTAR XL, AB/MDS Sciex), at any time. Acquiring data for several minutes using multi-channel acquisition (MCA), or signal averaging while infusing the fractions at approximately 200 nl/min, permits about a 50 times gain in sensitivity (signal-to-noise) in MS/MS mode [9]. A 5–10 µl sample fraction can be infused for more than 30 min, which allows sufficient time to perform various MS experiments. As the 96-well plate containing the sample fractions may be transferred to different instruments, MSn, precursor or neutral loss, accurate mass measurement (either in positive or negative mode), can be performed. The time saving is significant because the original sample needs neither to be re-analyzed by re-injection nor to be pre-concentrated. As previously mentioned MALDI, which is a typical off-line MS technique, is gaining attention for the analysis of low molecular weight compounds. Fig. 3B presents a strategy where metabolite sample analysis can be multiplexed, which is more appropriate for screening purposes [10]. In this configuration the fractions are spotted after the post-column addition of matrix onto a target plate. As the analytes are embedded in the matrix crystal, conservation of the sample and re-analysis are greatly simplified. One limitation of this setup is that the chromatographic information is lost. In our laboratory these types of sample are typically analyzed onto a quadrupole–time of flight instrument (QSTAR XL) equipped with an o-MALDI source.

4. Simultaneous Analysis of Pharmaceutical Compounds and Endogenous Metabolites

In general, bioanalysis focuses on the qualitative and quantitative analysis of pharmaceutical compounds and their metabolites circulating in biological fluids to support pharmacokinetic, pharmacodynamic or drug metabolism studies. Metabolomics, metabonomics, and metabolite profiling analyze changes in endogenous metabolites

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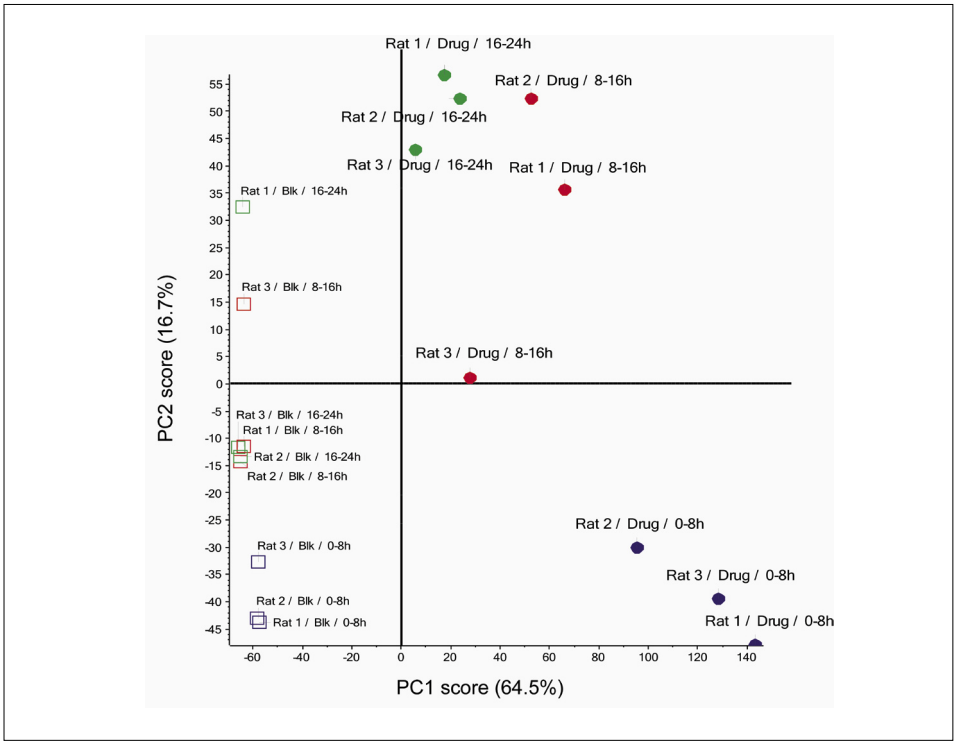

Fig. 4. Multi-component analysis of treated (drug) and non-treated rats (blk)

generally containing low molecular weight compounds. The effects of genes on metabolic pathways and changes in efficacy, toxicity and disease state after drug treatment can be monitored. Biomarker analysis determines compounds, generally proteins and peptides, which are indicative of a disease. In all cases, mass spectrometry and in particular LC-MS and MALDI-MS, play an essential role as an analytical technique to generate the data. With low molecular weight compounds, it becomes particularly interesting to correlate variation of endogenous metabolites with drug concentration. This may be particularly attractive for toxicology studies where the onset of toxicological effects could be monitored in a non-invasive fashion. Fig. 4 shows the principal component analysis of the LC-MS analysis of three rats before and after administration of a pharmaceutical compound. For this purpose various urine fractions were collected and analyzed without any sample preparation by LC-MS on a QqTOF (QSTAR XL) instrument in gradient mode. The plots are constructed based on the m/z ratio in positive ion mode of the compounds present in urine and their respective LC retention time. The plot clearly shows which of the rats received the drug and the rate of metabolism. This particular example illustrates the potential of mass spectrometry to perform multi-component analysis but also shows the need to develop new analytical strategies for sample analysis and data treatment. By combining various MS and MS/MS experiments performed simultaneously in the same LC analysis, metabolites of the parent drug can be characterized based on accurate mass; possible changes in endogenous metabolite profiles can also then be explored.

5. Proteomic Analysis

The *Caenorhabditis elegans* is a small free-living soil nematode found in temperate regions that feeds on microbes such as bacteria. It has become an important model organism for the investigation of fundamental problems in the life cycle, in particular behavior, development, and aging. The overall goal of our research was to develop and apply analytical methods based on the combination of various sample preparation approaches, liquid chromatography and mass spectrometry (1D & 2D LC-MS) for the analysis, identification and quantification of proteins expressed in wild-type and knock-out *C. elegans* strains.

Fig. 5 illustrates a two-dimensional dual nanoscale liquid chromatography arrangement, combined with a triple quadrupole linear ion trap mass spectrometer, which was applied to the analysis of a tryptic digest of a total proteins extract of the nematode *Caenorhabditis elegans*. Peptides were separated with a strong cation exchange microcolumn in the first dimension and two reversed phase C18 nanocolumns were used for the second dimension. MS experiments were performed using an information-dependent data acquisition (IDA) strategy where two precursor ions per cycle were selected from enhanced MS (EMS) or from enhanced multi-charged ions (EMC) survey scans. Then two enhanced resolution scans were performed to determine precursor ion charge states. When the IDA criteria were met, two enhanced product ion scans were performed as dependent scans. The advantages of a 2D dual nanoscale LC-MS/MS system are reflected in the significant increase, almost doubling the number of peptides and proteins that were successfully identified. Indeed, the use of twice the number of salt fractions in the first dimension allowed a better sample fractionation

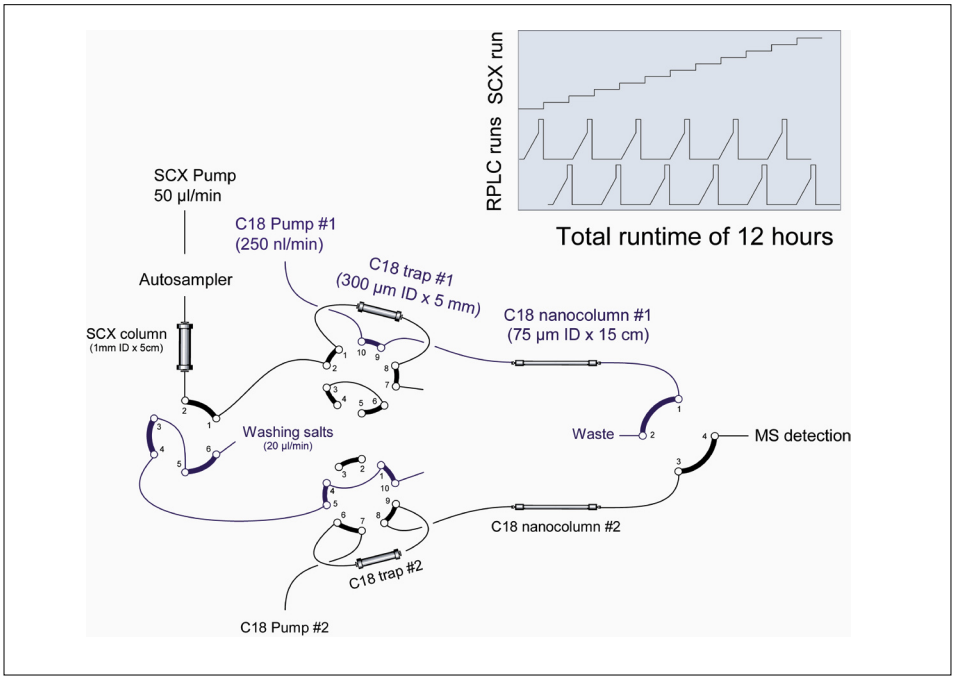

Fig. 5. 2D dual nanoscale LC-MS/MS configuration for peptide analysis

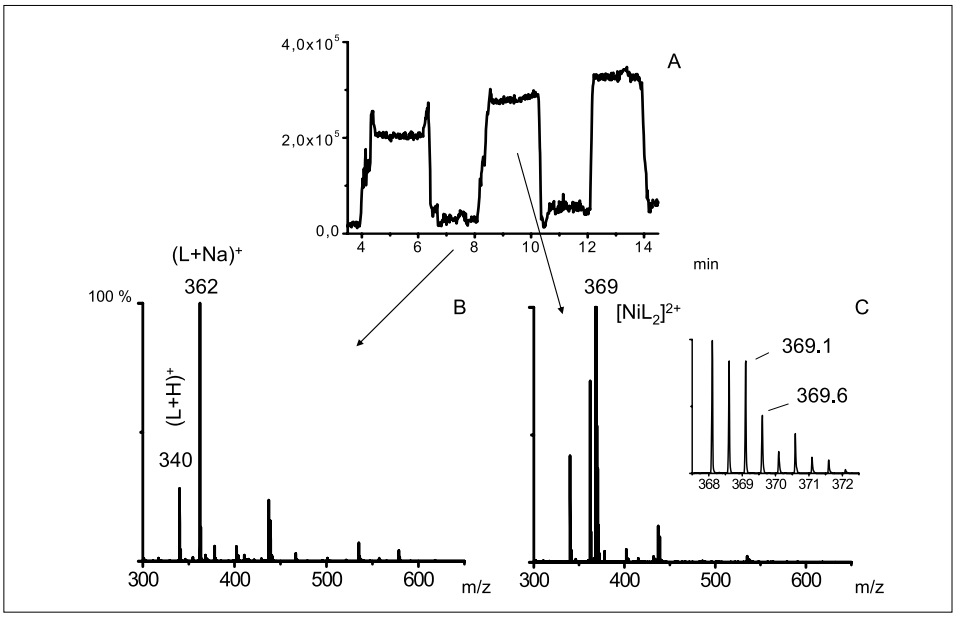

Fig. 6. Dual-channel microchips: ligand and metal, complex formation in the spray A) V1 = 3.3 kV ON, V2 = 2.8 kV OFF B) V1 = 3.3 kV ON, V2 = 3.5 kV ON

reducing the number of peptides transferred to the second chromatographic dimension per salt fraction. The dual C18 nanocolumns configuration improved MS detection duty time by a factor of two, which resulted in sampling more peptides eluting from the second dimension within the same total analysis time. Finally, the use of EMC instead of EMS as the survey scan allowed selective removal of singly charged precursor ions with improved results, in terms of MS/MS spectra quality and the number of identified peptides.

6. Dual Plastic Chip-based Infusion

Miniaturization of analytical instrumentation is of great interest because it allows the design of unique analytical devices. Plastic polymers are particularly attractive since they are cheap and relatively simple to work with. diagnoSwiss (Monthey, Switzerland) has recently developed single and dual disposable plastic chips that allow infusion of various analytes at nanoflow rate ranges in front of an MS. In our laboratory the application of dual-channel microchips was investigated i) as lock mass devices for accurate mass measurements and ii) to infuse separately a metal ion and a ligand solution to obtain formation of the non-covalent complex in the condensed phase. Both channels can be switched on or off by the application of an electric potential. Fig. 6 illustrates the dual-channel microchips allowing separate infusion of a solution containing the ligand and metal ion solutions. Depending on the voltage applied to the metal ion solution, the non-covalent complex $[NiL_2]^{2+}$ was formed spontaneously in the condensed phase on the top of the spray. This configuration is particularly interesting for investigating the affinity of various metal ions towards a specific ligand or the affinity of various ligands to a specific metal ion, by minimizing the amount of analyte necessary to perform these kinds of experiments.

7. Conclusions

In qualitative and quantitative analysis on-line LC-MS/MS is the most common way of analyzing samples. Fraction collection and off-line analysis offer real benefits over the on-line approach regarding analysis speed, multiplexing, and data quality. It could be predicted that liquid chromatography, in particular with chip-based approaches, could become part of the sample preparation and analysis procedure.

On-line approaches such as LC-MS are restricted to API in combination with liquid chromatography, while off-line approaches can be performed either with API or MALDI. The application of MALDI is no longer restricted to peptides and proteins, but in combination with a triple quadrupole instrument in the SRM mode, it will offer the possibility to perform real, high speed analysis of small molecules such as metabolites.

Finally, expected improvements in mass spectrometer sensitivity and resolution, or new ionization techniques, will strongly affect the way current bioanalysis is performed.

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