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Profiling of Endogenous Peptides by Multidimensional Liquid Chromatography

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1. Introduction

The state of the organism is reflected to the key process in the living body - protein metabolism. Proteomics is the large-scale study of gene expression at the protein level, which will ultimately provide direct measurement of protein expression levels and insight into the activity state of all relevant proteins (Pandey & Mann, 2000). The proteome analysis usually includes the following strategies: native protein pre-separation, then digestion followed by separation and identification, or alternatively straight digestion, separation and identification by mass spectrometry. Therefore, starting with one protein, after digestion we will end up with approximately 30 to 70 short peptide fragments. Identification of only very few of them will provide sufficient information which protein was present in the sample. The subproject of proteomics, namely the study of all peptides expressed by a certain cell, organ or organism, is termed peptidomics. The term was introduced in 2001 (Clynen et al., 2003). Peptides often have very specific functions as mediators and indicators of biological processes. They play important roles as messengers, *e.g.*, as hormones, growth factors, and cytokines, and thus have a high impact on health and disease. Peptidomics comprises not only peptides, originally synthesized by an organism to perform a certain task, but also degradation products of proteins (degradome). Therefore, proteolytic cleavage of proteins leads to peptides as indicators of protease activity, degradation, and degeneration therefore it is also reflects the organism state. The sensitivity of proteomics and peptidomics suffers from the lack of an amplification method, analogous to the polymerase chain reaction, to reveal and quantify the presence of low-abundance proteinaceous constituents therefore the display level is difficult. These challenges motivate the researches to develop reliable analytical platforms. Shortcomings in throughput are due to the absence of technologies that can deliver fast and parallel quantitative analysis of complex peptide distributions in an automated fashion. In the future, when peptidomics will be more analyzed and understood, and biomarkers identified straight capture step of biomarkers from complex bio-sample might be used. Peptidomics especially challenges the need for robust, automated, and sensitive high-throughput technologies. Most single-dimension separations lack sufficient resolution capability to resolve complex biological matrixes. For example, in human blood serum, 90 % of the protein content of serum is composed of 10 basic proteins. The remaining 10 % of serum consists of trace amounts of millions of different proteins. Thus, partial

purification of proteins is necessary so that proteins in trace amounts can be identified and their exact structural analysis can be performed. Chromatographic separation techniques are well suited for the analysis of complex multi-components samples. To overcome the limited peak capacity and concentration diversities of the analytes utilizing chromatographic separation systems, multidimensional chromatography (MD-LC) has been realized by analyte transfer between different separation modes through automated valve switching (Link, 2002). Another important prerequisite for the suitability of separation systems for proteomic analysis is the ability to handle very big and very small amounts of biological material (Machtejevas et al., 2006). However, the application of several orthogonal LC separation systems also bears the danger of severe sample losses due to adsorption on the separation and capture column and sample transfer. The mass loadability of LC columns is much higher than for 2D-gelelectrophoresis systems and can be tuned to the requirements of a MD system. LC modes can be implemented into the sample clean-up which in return becomes more selective, robust and reproducible, thus enhancing the quality of the final data. The most important feature is however, that MD-LC can be automated with a high degree of robustness and reproducibility.

Most sample-preparation procedures are performed manually and are thus time-consuming and laborious. On-line sample clean-up and on-column concentrations avoid this disadvantage. There are a number of important features that is gained by having the liquid phase separation system to be operated on-line. Direct injection techniques are generally preferable, since problems involved in off-line sample pretreatments, such as time consuming procedures, errors and risk for low recoveries can be readily avoided. Introduction of the Restricted Access Materials (RAM) offers a unique and intelligent solution. It designates a support family that allows direct injection of biological fluids by limiting the accessibility of interaction sites within the pores to small molecules only. The term restricted access material is a general term for a packing material having a hydrophobic interior covered by a hydrophilic barrier. The hydrophilic barrier allows passage of small molecules to the hydrophobic part of the stationary phase, while sterically preventing large molecules, such as proteins, from interacting with this part of the stationary phase. Macromolecules are excluded and may interact only with the outer surface of the particle support coated with hydrophilic groups, which minimizes the adsorption of matrix proteins.

In a search for new stationary-phase configurations the concept of monolithic silica stationary phases was explored and investigated in depth (Unger et al., 2011). A monolith consists of a continuous rod, of a rigid, porous polymer, that has no interstitial volume but only internal porosity consisting of micro-and macropores. All of the mobile phase is forced to flow through the channels of the porous separation medium, resulting in enhanced mass transport also improved chromatographic efficiency (Meyers & Liapis, 1999) and simultaneous extension of column life time.

2. Multidimensional LC/MS approaches in proteomics and peptidomics

Two-dimensional gel electrophoresis (2D-PAGE) and mass spectrometry are well-established and the most employed techniques in proteomics today. 2D-PAGE, however, provides limited information of the total amount of proteins. Low abundant proteins and

small peptides are not detected (Issaq, 2001). Additional methodologies and techniques in sample preparation, selective enrichment, high resolution separation, and detection need to be developed which would allow one to achieve even higher resolution than 2D-PAGE. Acceptable sensitivity to detect the low-abundant proteins is also still an issue. LC can address some of the above-mentioned problems. In comparison with gel-based separation methods, sample handling and preparation are simplified and automated. MD-LC has a number of advantages, such as a higher sensitivity, faster analysis time, variable sample size (preconcentration of the target substances is possible), possesses a large number of separating mechanisms, and, what is most important, and it is amenable to automation. However, because of the wide dynamic range, no single chromatographic or electrophoretic procedure is likely to resolve a complex mixture of cell or tissue proteins and peptides. Liquid chromatographic techniques are fast, quantitative, easy to automate, and can be coupled more readily to mass spectrometry than two-dimensional gel electrophoresis (Premstaller et al., 2001). The drawback of LC is the limited peak capacity of a single column. Thus, multidimensional LC is the choice, fractionating the eluent and transfer the fractions between different columns through automated valve switching (Cortes, 1990). Mass spectrometry has limitations with respect to sensitivity, therefore, a certain number of analyte molecules should be injected in order to be identified. Thus, higher amounts of the sample should be applied. Knowing the target analyte concentration in the sample provides the answer to the question: how much we should inject? In other words, the mass loadability of columns in the multidimensional column train plays a significant role, otherwise displacement phenomena and unwanted protein-protein-interactions will take place, which may change the down-stream composition of the individual fractions in an irreproducible way (Willemsen et al., 2004). Another important prerequisite for the suitability of a separation system for proteomic analysis is the ability to handle very small amounts of biological material (Premstaller et al. 2001). These methods allow one to detect low concentrations of peptides from complex mixtures with a high degree of automation.

Multidimensional (multistage, multicolumn) chromatography had been discovered early as a powerful tool to separate complex mixtures. Two of the protagonists were J.C. Giddings (Giddings, 1984, 1995) and J.F.K. Huber (Huber & Lamprecht, 1995). MD-LC is based on coupling columns in an on-line or off-line mode, which are operated in an orthogonal mode, i.e. separate the sample mixture by different separation mechanisms. The sample separated on the first column (first dimension) is separated into fractions which can then be further treated independently of each other. The practical consequence is an enormous gain in peak capacity (number of peaks resolved at a given resolution) and the potential of independent optimization of the separation conditions for each fraction. Simultaneously, there is the option of relative enrichment/depletion and peak compression by fractionation.

Multidimensional LC separation typically relies on utilizing two or more independent physical properties of the peptides to fractionate the mixture into individual components. Physical properties commonly exploited include are size, shape, charge, hydrophobicity and biomimetic or affinity interactions. These processes are the underlying phenomena for peptide/protein separations using different chromatographic modes, such as size exclusion, reversed phase, cation/anion exchange and hydrophobic interaction columns.

Biological, individual, and variations between individuals (such as gender, age and nutrition) affect peptidomes and require careful consideration in order to find valid biomarkers. A few, equally important factors for successful proteomic biomarker research are high sample quality, high sensitivity, and reproducibility which depend on proper selection of the high quality samples.

While MD-LC MS has found widespread use in the analysis of peptides from natural sources or generated by proteolytic digestion of larger proteins, the method is not suitable for analyzing proteins directly. First, proteins tend to denature under reversed-phase conditions either by stationary phase or mobile phase induced effects (strongly hydrophobic surfaces, low pH and high organic solvent concentrations) making their quantitative elution rather difficult. Observed recoveries are also often low and life time of the columns is compromised. Also, measuring the molecular mass of a protein by MS is not sufficient for its unambiguous identification. To circumvent these obstacles the proteins are digested and the separation is performed at the peptide level. One can distinguish two approaches (i) proteins are separated and then digested ("top-down" proteomics (Wolters et al., 2001)); (ii) in "shotgun" proteomics a complex protein mixture is first digested (see Figure 1, a) and peptides are then chromatographically resolved ("bottom-up" proteomics (see Figure 1, b) (Regnier et al., 2001)). In both cases, separation technologies play a critical role in protein identification and analysis.

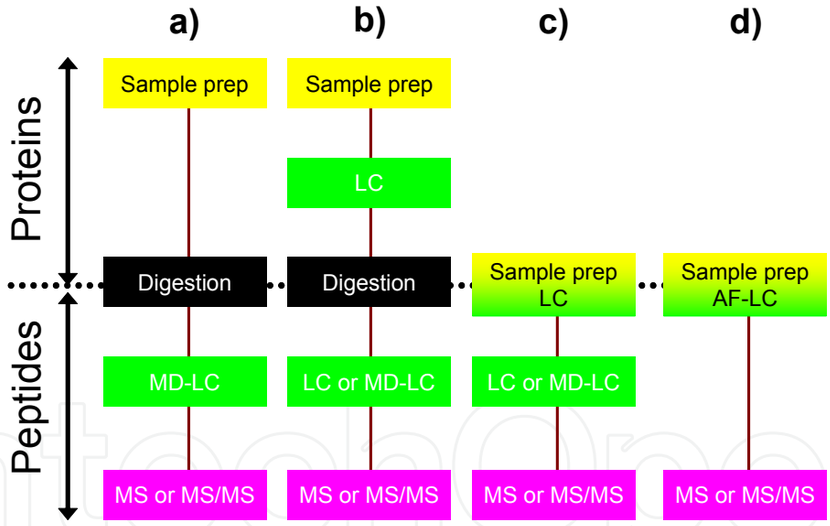


Fig. 1. Liquid chromatography workflow strategy options in proteomics. A – "bottom-up" approach, b – "top-down approach", c – selective sample clean-up directly combined with chromatographic separation ("digestion free" strategy), d – direct capture of target substances.

Even though in the "shotgun approach" sample complexity is vastly increased, there are an increasing number of reports on the comprehensive analysis of human proteomes using this strategy. Prior digestion gives access to the higher molecular weight proteins, however, at the expense of rendering the mixture much more complex. Assuming that a given biofluid contains 1,000 proteins and that each protein will generate approximately 50 proteolytic fragments, we are talking about 50,000 and more peptides to be resolved. This task can only

be approached by MD protein identification technologies (Gevaert et al., 2002, Griffin et al., 2002, Walters et al., 2001, Pang et al., 2002). The disadvantage of this approach is that one ends up with an extremely large number of peptides, which need to be resolved. However such an approach could be compared to the efforts of virtually to restore the forest look after it has been completely milled to the sawdust. No one would ague that this would require a lot of guessing and speculations, multiple details might be lost or misinterpreted.

Another attractive approach is to separate proteins first by ion exchange chromatography (see Figure 1 b) according to charge and charge distribution under "soft" (biocompatible) conditions and collecting fractions. The fractions are subjected to digestion and consecutive re-injection on to a RP column is performed, whereby the separation is based on the hydrophobicity. This is particularly favorable since the mobile phase in the second dimension (RP) is compatible with the solvent requirements of mass spectrometry. The restrictions associated with this method lie in the limited size of proteins that can be investigated (MW < 20,000 Daltons) and the insolubility or incomplete separation of very hydrophobic peptides. All peptide-containing fractions are then investigated by mass spectrometry to generate a peptide map (Schulz-Knappe et al., 2001). This approach has already been found to be sufficient to deal with smaller subsets of the proteome (i.e. several hundred proteins) (Hille et al., 2001). These studies also clearly demonstrate that this methodology is not yet suitable for the analysis of a whole proteome due to its enormous complexity. Therefore, pre-selection of the protein from a given tissue or a pre-separation seems mandatory. For example, for the analysis of human urine solid-phase extraction (C-18 packings) to trap peptides, followed by IEX chromatography in the first dimension collecting 30 fractions and analysis of the collected fractions by RP LC (C-18) in the second dimension (Heine et al., 1997) was successfully employed. A similar procedure was used for the separation of proteins and peptides in human plasma filtrate and plasma (Richter et al., 1999).

It is easy to be misguided by vast amount of publications usually dealing with standard protein digests. Separation of a few digested proteins peptides are shown in Figure 2a. Easy to recognize small differences in dynamic range, and even peak distribution, therefore the conclusion could be drawn, that all what we need for successful proteomics analysis is high peak capacity separation and one dimension then would be sufficient. However, using the same chromatographic conditions and column, also injecting eight times more of a real bio-sample (amniotic fluid) we are not observing nice and even separation any longer (Figure 2b). This is a common situation with all real bio-samples.

Direct analysis of biofluids (Figure 1c and d) without prior digestion is a definitive option in biomarker discovery peptidomics. Those routes could be accomplished by employing restricted access materials. RAM columns possess a dual function: firstly, they operate as size-exclusion columns to remove high molecular weight proteins and other undesired constituents. The size characteristics of proteins in pure SEC are known to be highly dependant on eluent composition such as pH, ionic strength (I) of the buffer (which includes salt type and concentration) and on the flow-rate (Quaglia et al., 2006). Ionic strength and pH, however, can vary significantly among biofluids such as plasma and urine. The consequence will be that the sample cleanup procedures have to be adjusted individually with respect to each type of biological sample and standardised protocols have to be worked out. Secondly, the RAM column serves as trap or capture column to selectively enrich target

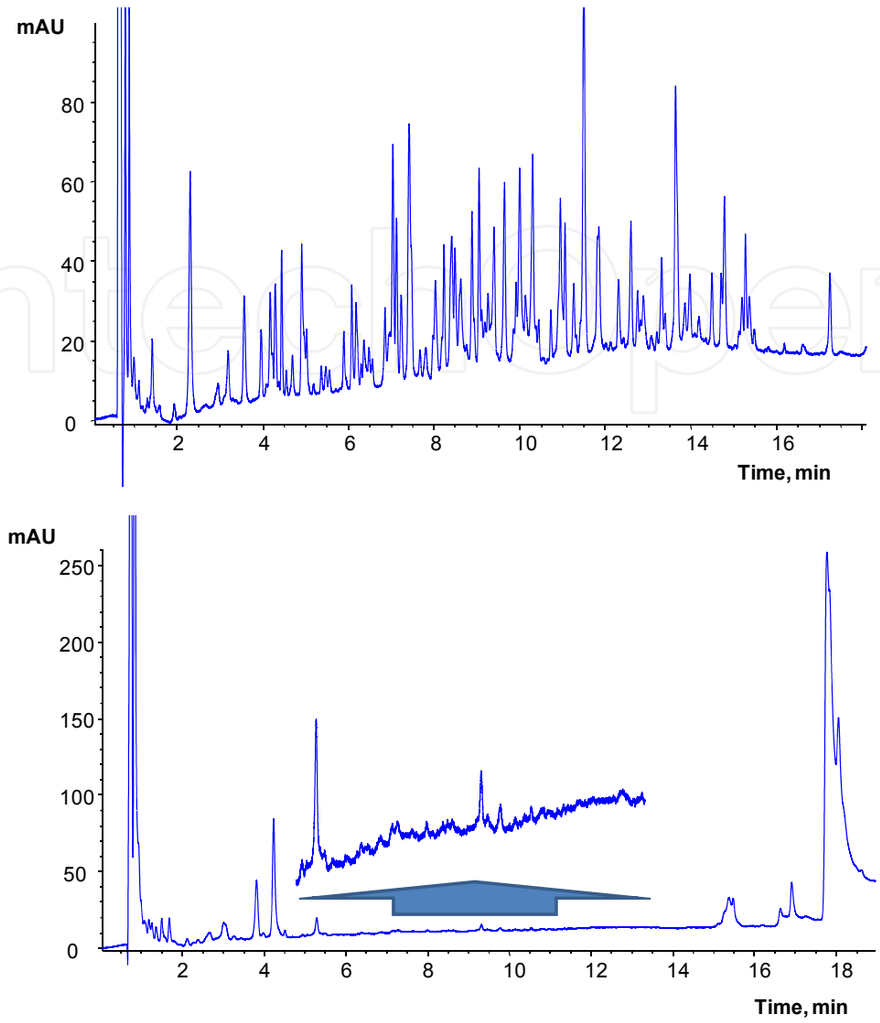


Fig. 2. Separation of standard protein digest and real biological sample analysis: upper chromatogram - 1 µl BSA digest (1mg/ml), lower chromatogram 8 µl of filtered amniotic fluid. Conditions: column - Chromolith Performance RP-18e 100 mm x 2 mm I.D.; eluents - A: 95% H_2O/5% ACN/0.1% TFA (v/v/v), B: 5% H_2O/95% ACN/0.085% TFA (v/v/v); gradient - from 5% B to 50% B in 20 min; flow rate - 0.3 ml/min, detection - UV 214 nm.

compounds in a reversed phase mode or in an ion-exchange mode. By regulating the pore size of the particles, the molecular weight exclusion can be varied as well as the molecular weight fractionation range, which allows certain analytes to be trapped at the internal surface. In this case, only proteins and peptides below a certain molecular shape and size have access to the inner pore surface of the RAM, are thus retained while the larger proteins encounter only the hydrophilic, non-adsorptive outer surface, and will be flushed out in the following washing step. Of the RAM, the strong cation exchanger with sulphonic ligands (RAM-SCX) was preferably employed in the sample clean up of proteins, which proved to show an acceptable capacity towards positively charged peptides and proteins. The features described above, when elegantly combined with column switching, become a powerful tool for direct analysis in the profiling of endogeneous peptides in a fully automated, multidimensional LC platform.

2.1 Designing a MD-LC system

The primary criteria for the choice of a separation phase system are selectivity and orthogonality, mass loadability, and biocompatibility (in case of quantitation). As a rule of thumb, the first dimension should possess a high mass loadability combined with sufficient selectivity and maintenance of bioactivity. Ion exchange chromatography (IEC) therefore is the method of choice offering charge selectivity. In principle, there are two options in IEC, either to employ a cation or anion exchanger, which in return influences the pH working range. Note that either cationic or anionic species are resolved, i.e. only a limited number of species from the whole spectrum. The IEC columns are operated via salt gradients with increasing ionic strength. Consequently, the salt load must be removed before the fractions are transferred to the second dimension column.

It is most common to use reversed phase chromatography as the second dimension. The term RP stands for a number of columns with different degrees of hydrophobicity. The most commonly applied phases are n-octadecyl bonded silicas (RP-18 columns). An intrinsic feature of RP columns is their desalting property. Salts are eluted at the front of the chromatogram, when running a gradient elution with an acidic buffer/acetonitrile mobile phase with increasing acetonitrile content. The hydrophobic surface of the RP packing and the hydrophobic eluent are not favorable with respect to providing a biocompatible environment for proteins: they may change their conformation or denature which may be seen by the appearance of broad peaks, splitting of peaks etc. RP columns possess a much lower mass loadability than IEC columns (10 mg of protein per gram of packing as compared to 100 mg in IEC). An advantage of RP is the fact that the eluents are compatible to MS, provided volatile buffers such as ammonium acetate are employed.

In case of an on-line MD-LC system, the speed of analysis in the second dimension should be as high as possible (Wagner et al., 2002). This, however, conflicts with the requirement of high resolution or high peak capacity. The highest peak capacity in gradient elution RPC is obtained with a shallow gradient at relatively low flow-rate. Thus, a compromise between the desired peak capacity and the gradient time is inevitable. Often gradient times of several hours are applied for the analysis of peptides from protein digests.

A question often arising is: How many dimensions do we need in MD-LC? It becomes obvious that as the number of dimensions increases, the peak capacity will increase. In an ideal case the total peak capacity of the MD-LC system is equal to the product of the individual orthogonal dimensions. At the same time, above two dimensions an on-line MD-LC system becomes very sophisticated in its instrumental setup and may be difficult to control. The major goal in proteomics for the common user is to design a highly efficient, error minimizing and easy-to-handle system. Reduction of the system complexity is the major demand. It is essential to select a minimum number of dimensions to handle complex separations which also should preferably include on-line sample clean-up steps.

2.2 Advantages of on-line sample clean-up approaches

Most sample-preparation procedures are performed manually and are thus time-consuming and laborious. On-line sample clean-up and on-column concentrations avoid this disadvantage. There are a number of important features that is gained by having the liquid phase separation system to be operated on-line. The overall yield in most cases is improved

compared to off-line approaches and methodologies. Exposed surfaces are kept to a minimum that usually is the main cause of sample losses. Overall precision can also be controlled by having yields above 50 %. It is possible to handle yields that are lower; however, it generally is a real analytical challenge to obtain operational stability within such analytical processes. Direct injection of samples onto HPLC columns is substantially advantageous in the clinical laboratories in terms of its time- and labor-saving capabilities, in addition to other advantages given below. General direct injection methods have been devised which deal with the problem of many different proteins being present in the sample. The methods include the pre-column technique, restricted access materials, and chromatography in mobile phases containing surfactant. High performance affinity chromatography is also a direct injection technique will demonstrate its power in near feature (Figure 1 d). The characteristic and performance of each direct injection technique are comprehensively discussed below for the analysis of biological samples.

The pre-column technique is the direct injection technique that is mostly reported. The pre-column technique utilizes two columns in series (pre-column and analytical) connected by a switching valve. The most common pre-column technique employs a reversed phase pre-column and a reversed-phase analytical column: the sample is injected into an aqueous mobile phase flowing through a pre-column (1-4 cm in length, 3 - 4.6 mm I.D.) which retains lipophilic compounds, passing non-retained hydrophilic compounds to waste. The switch in valve is then changed and components retained on the pre-column are eluted onto the analytical column by increasing the solvent strength of the mobile phase. This technique serves the dual function of concentration of analyte and removal of hydrophilic substances. There are many advantages of the pre-column injection technique in comparison to traditional sample preparation techniques: time saving in comparison to the labor-intensive liquid-liquid extraction and precipitation techniques, high reproducibility and high, also a superior detection limit capabilities due to its allowance for injection of large sample volumes.

Several types of column-switching designs have been applied. The back flush design is most often used because it reduces band broadening (Yamashita et al., 1992). However some prefer the forward-flush mode to protect the analytical column from possible impurities at the head of the column. Use of an on-line ~0.5μm filter is recommended, which needs periodic replacement. A design that incorporates a second pre-column parallel to the first has been employed which increases sample throughput, by alternating injection on one pre-column and back flushing of retained compounds on the other pre-column.

Most biofluids contain large amounts of well-known proteins such as albumin and IgGs, which overwhelm the separation system and make the detection of the low abundant proteins and peptides very difficult. It is thus advantageous to remove these proteins prior to digestion or direct separation. There are alternative ways of reducing the overall protein load by specific adsorption of albumin and IgG to affinity matrices (Nakamura et al., 2002, Wang et al., 2003, Govorukhina et al., 2003). While usually an affinity matrix is generally highly specific, in high content samples the affinity ligand is limited to exhibit its specificity. There are degrees of specificity between highly selective immunoaffinity matrices and less selective but more robust affinity supports using synthetic ligands. In an effort to reduce the amount of albumin from human serum, a number of affinity matrices has been evaluated based on antibodies or dye ligands. Antibody-mediated albumin removal was efficient and

selective. Dye ligand chromatography, a technique that is extensively used in protein chromatography was surprisingly effective (Andrecht et al., 2004) in particular with regard to high binding capacities and a long column lifetime, however, at the expense of selectivity.

2.3 Restricted access material columns

The direct injection of biological samples onto the chromatographic column without any sample preparation is in most cases highly problematic and may lead to an irreversible contamination of the separation columns, which deteriorate selectivity and column performance. A powerful asset to circumvent all the named problems is the implementation of restricted access material for sample preparation. Special Solid Phase Extraction (SPE) supports possessing restricted access properties have been developed (Hagestam and Pinkerton, 1985, Yu et al., 1997, Boos and Rudolphi, 1997). In 1991, Desilets et al. (Desilets, et al., 1991) introduced the restricted access term. Silica based Restricted Access Materials have been developed for the clean-up in bioanalysis; first for low molecular weight compounds in biofluids (Rbeida, et al. 2005) and subsequently for biopolymers such as peptides (Wagner et al. 2001). Those supports were able to withstand several hundred plasma or serum injections (total volume of 5–7 ml) without losing performance. The concept and the methodology were successfully used for the sample clean-up of peptides and proteins out of biofluids by extending the range of available materials employing cation and anion-exchanger RAM (Machtejevas et al., 2004)). Specific non-silica based RAM were also developed for the investigation of the food (Bovanova & Brandsteterova 2000) and environmental matrices (Hogendoorn et al., 1999). Vijayalakshmi and co-workers (Pitiot et al., 2004) have presented a new RAM called a bi-dimensional chromatographic support operating on a size exclusion mode and an affinity or pseudoaffinty mode. A survey on the current state-of-art of RAM-columns in sample pre-treatment is given Souverain et. al. (Souverain et al., 2004). A RAM support developed by Boos and Grimm (Boos & Grimm, 1999) is based on SCX-diol modification to improve performances in terms of efficiency, retention and reproducibility. Račaitytė et al. (Račaitytė et al. 2000) have shown that this type of RAMs is highly suitable for the on-line extraction and analysis of neuropeptides in plasma. Machtejevas et al. (Machtejevas et al. 2006) analyzed the pore structural parameters and size exclusion properties of LiChrospher strong cation-exchange and reverse phase restricted access materials. For peptide analysis out of the biofluids, the strong cation-exchange functionality seems to be particularly suitable mainly because of the high loadability of the strong cation-exchange restricted access material (SCX-RAM) and the fact that one can work under non-denaturing conditions to perform effective chromatographic separations. The proper column operating conditions leads to the total effective working time of the RAM column to be equal to approximately 500 injections (depending on the type of sample).

The principle of the restricted access support is based upon the presence of two chemically different surface properties of porous silica particles (Figure 3). The outer surface of the particles (25 – 40 mm O.D.) is highly biocompatible: it possesses diol modification and hence is hydrophilic, while the pore surface chemistry is tailored as a hydrophobic dispersion phase with C-18 functionality or as a strong cation exchanger with SO3 functionality. An advantage of these adsorbents relies on the simultaneous occurrence of two chromatographic separation mechanisms: selective reversed-phase interaction or ion exchange chromatography of lower molecular mass analytes and size exclusion

chromatography for the macromolecular sample constituents. By regulating the pore size of the particles, a physical restriction barrier is adjusted to regulate the interval of molecules that may penetrate and, in the case of penetration, may be trapped in the functionalized pore structure. A pore size of 6 nm allows access to the pores only for analytes with a molecular mass below 15 kDa. The proteins (>15 kDa) can thus be eluted with the void volume directly into the waste. Smaller analytes, however, such as drugs and metabolites from body fluids, pesticides or hormone residues from milk or animal tissue samples, may enter the pores and interact with the n-alkyl chains or ion-exchange groups bound to the inside of the pores. When dealing with complex samples i.e. human bio-fluids, the sample clean-up and fractionation of the sample into matrix and target analytes can be achieved. Depending on the ligands present in the pores, small molecules with hydrophobic or ionic properties are selectively enriched.

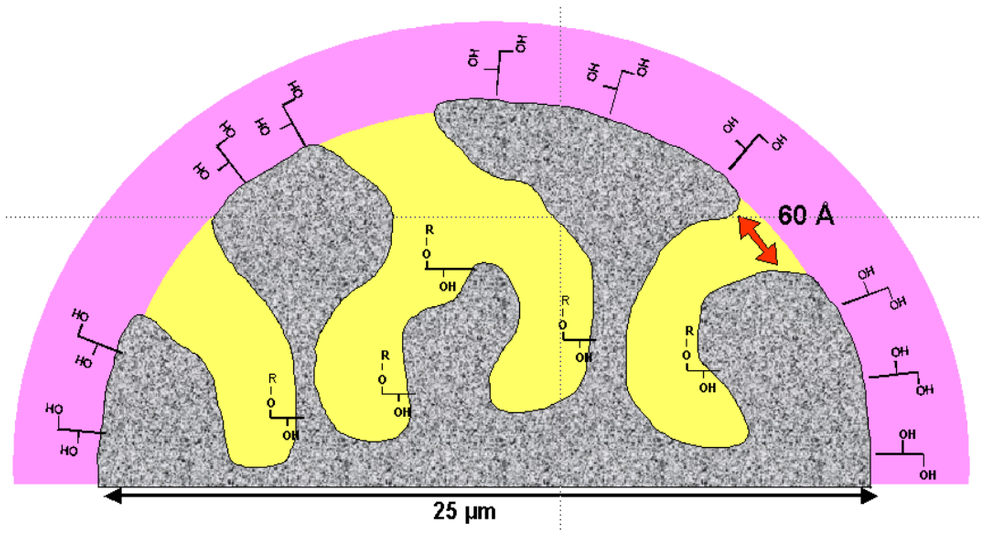


Fig. 3. Artistic representation of a SCX-RAM silica particle (LiChrospher 60 XDS (SO_3/Diol), Merck KGaA, Germany). The external surface is coated with hydrophilic, electroneutral diol-groups for the exclusion of high molecular weight components (>15 kDa); the internal surface is functionalized with ion-exchange groups accessible for low molecular weight components which may be trapped by electrostatic interaction.

The diffusion barrier can be accomplished in two ways: (i) the porous adsorbent particles have a topochemically different surface functionalization between the outer particle surface and the internal surface. The diffusion barrier is then determined by an entropy controlled size exclusion mechanism of the particle depending on the pore size of adsorbent (Pinkerton 1991); (ii) the diffusion barrier is accomplished by a dense hydrophilic polymer layer with a given network size over the essentially functionalized surface. In other words, the diffusion barrier is moved as a layer to the interfacial layer inside the adsorbent particles, the exclusion properties are controlled by the size of the polymeric network protecting the internal surface and is no longer dependant on the average pore diameter of the adsorbent (Mazsaroff & Regnier, 1988).

The SEC process is entropically driven; i.e proteins with decreasing shape and size penetrate an increasing volume of the porous particles. The SEC of proteins is commonly carried out

with buffer solution containing a high salt concentration, e.g. 0.1 M, at pH 5 to 7. The high salt concentration is needed to suppress electrostatic interactions between the solute and the charged surface. In the sample clean up of the RAM-SCX column the concentration of salt is much lower e.g smaller than 20 mM and the pH is kept at approximately 3. Under these conditions, electrostatic attraction forces are dominant between the positively charged peptides and proteins whereas the negatively charged species are excluded from the pores of the RAM-SCX column through electrostatic repulsion forces (Figure 4 a).

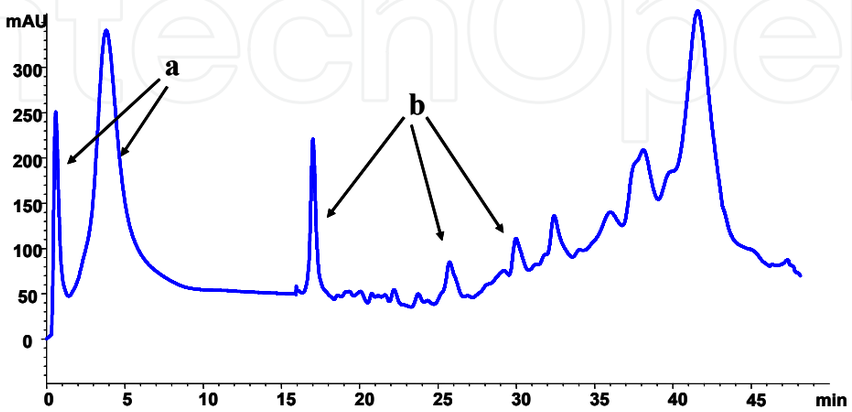


Fig. 4. Typical SCX-RAM column separation profile: peaks (a) represent physical exclusion by pore size. Trapped retained bio-molecules are separated by a gradient in the second step (b). Conditions: column - LiChrospher 60 XDS (SO_3/Diol), 25 x 4 mm I.D., flow rate - 0.5 ml/min, gradient from 0 to 1 M NaCl in 20 mM KH_2PO_4 pH 2.5, containing 5 % ACN in 30 min. Sample: 100 µl Human Hemofiltrate (3.7 mg/ml), UV detection at 214 nm.

After loading the RAM-SCX column the washing step elutes at isocratic conditions all the excluded compounds between the start and 15 minutes. After 15 minutes switching occurs and the trapped analytes are eluted from the RAM-SCX column with a strong eluent under gradient condition in the period between 15 and 45 minutes (Figure 4 b). Thus it is a charge and charge distribution selective process combined with SEC. Use of RAM-SCX allows one the direct application of biofluids onto the column. Small peptides are selectively trapped in the pores by cationic functional groups while large molecular weight biopolymers are directed to waste. This strategy performs the sample clean up and selective peptide enrichment in one simultaneous step (Figure 1 c).

Mass loadability of SPE and RAM columns play a key role in executing the sample clean-up. It is advisable to work below the overload regime of the column. Otherwise, displacement effects and other phenomena such as secondary interaction by adsorbed species might take place, which will lead to non-reproducible results (Wilemsen et al., 2004). Last statement is particularly important when the task is to monitor medium to low abundant proteins, therefore, usually large sample volumes in the millilitre range are applied. As the column lifetime is known to be limited a control measure has to be applied to check the condition of the RAM-SCX column and, if necessary, replace it by a new one. In our experience the column endured about 200 injections of urine.

Although compatibility of these stationary phases with direct biological sample injection is high, one still has to keep in mind that samples have to be filtrated or centrifuged prior to

injection to remove the solid contaminants and precipitations. Even so, some components tend to agglomerate/precipitate with the time while samples queue up in autosampler. Therefore, an additional in-line filter is highly recommended. One should keep in mind that the operational flow rate has enormous impact on the molecular size distribution when employing a RAM column. Higher flow rates can shift the molecular range of the trapped molecules to lower values as smaller molecules need less time to penetrate the pores. Also higher flow rates could alter the hydrodynamic volume of the biomolecules. Higher molecular mass molecules will be enriched operating at lower flow rates. Column temperature affects the viscosity of the mobile phase and, consequently, the diffusion ratio and influences the speed of mass transfer. Carefully performed optimizations of the chromatographic parameters ensure the success of the analysis.

2.4 Monolithic silica columns

Special features of monolithic silica columns circumstanced the successful application in proteomics. In contrast to conventional particle-packed columns, monolithic silica columns are made of a continuous piece of porous silica, utilizing a sol-gel process leading to rod columns, which possess a defined bimodal pore structure with macro and meso pores in the micro- and nanometer range (see Figure 5).

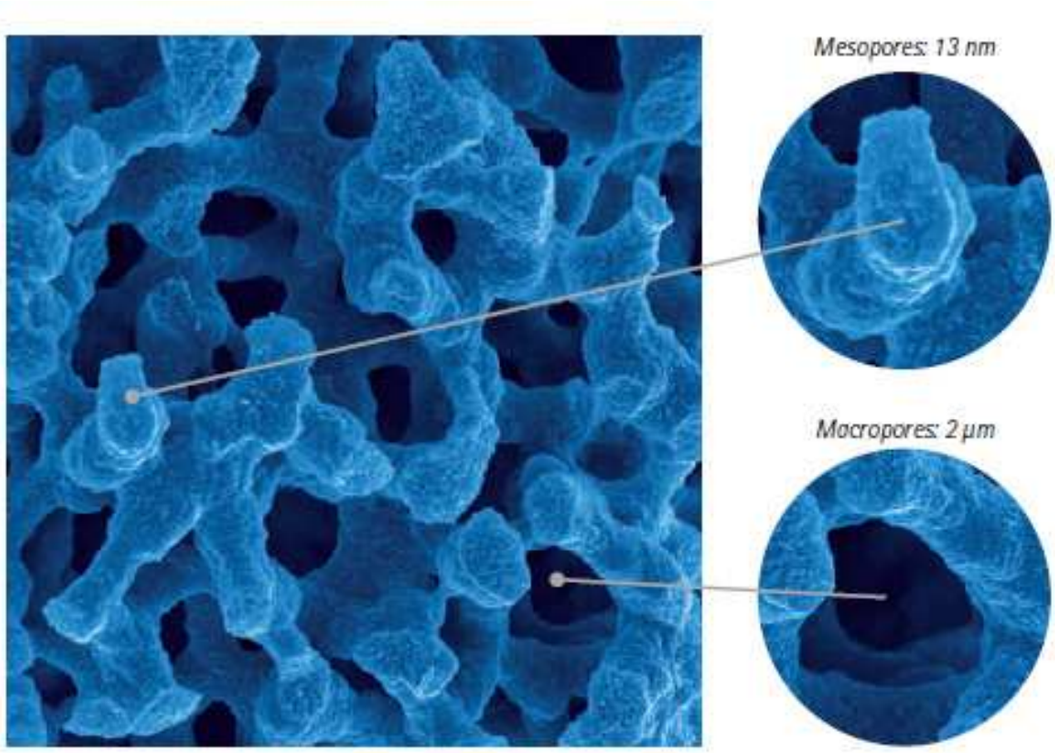


Fig. 5. SEM picture of a cross section from a silica monolith. Total porosity > 80%. The mesopores form the fine porous structure (average pore size 13 nm) and create the large uniform surface area on which adsorption takes place, thereby enabling high performance chromatographic separation. The macropores allow rapid flow of the mobile phase at low pressure. Their average size is 2 µm.

One of the major of those special features is low column backpressure. Low backpressure is not only nice to have, but a must, as setting up multidimensional separation platform for proteomics it allows one to select a desired flow-rate from a broader range (see Figure 6).

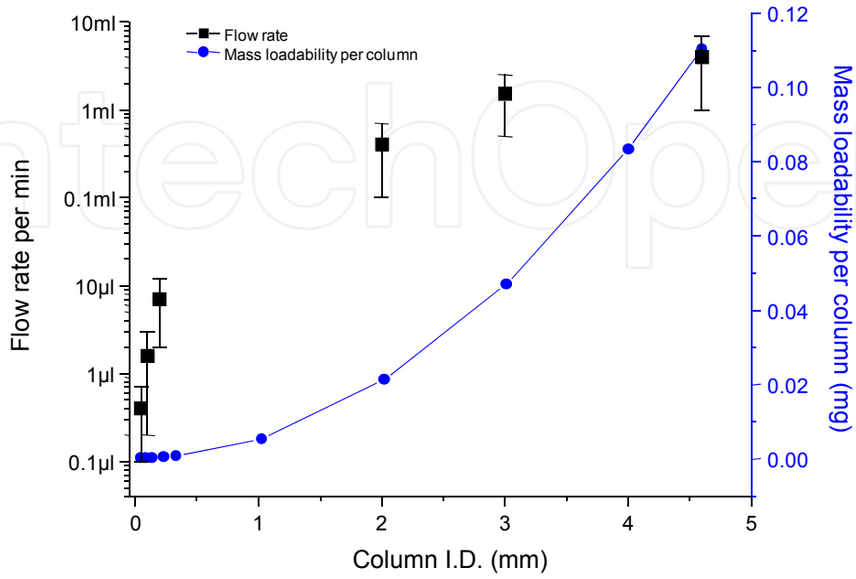


Fig. 6. Estimation of monolithic silica columns flow rates and mass loadability per column. Columns 2, 3 and 4.6 mm I.D. are 10 cm long; columns 50 µm, 100 µm and 200 µm are 15 cm long.

Prof. Regnier group, demonstrated the advantage of flow rate variation possibility for 4.6 mm I.D. Chromolith Performance column. It was concluded that silica monolith reversed-phase chromatography columns show little loss in the resolution of peptides ranging up to several thousand in molecular weight as mobile phase velocity is elevated from the conventional 2.5–25 mm/s (Xiong et al., 2004). Moreover, at 25 mm/s with a 100 mm length column, operating pressure did not exceed 150 bar. This is well within the pressure limit of most commercial LC instruments. The separation of a tryptic digest of cytochrome *C* in 6 and 60 min seemed almost identical. Resolution at 25 mm/s linear velocity was 77% of that at 2.5 mm/s. It was conluded that the fact that peptide separations could be achieved 10 times faster than with a conventional packed column with moderate loss in resolution could have a major impact on analytical throughput in proteomics.

Combination of different sizes fulfils the injection volume requirement for various samples. The possibility of being able to vary the flow-rate over a large area up to very high linear flow velocities combined with the robustness of the monoliths also reduce considerably the "down times" during washing and re-equilibration of the column (Rieuxet al., 2005).

Important to notice, that comparing a particulate and a silica monolithic guard column showed that the particulate column was clogging much faster than the monolithic column (Machtejevas et al., 2007). 120 injections of plasma (50 µl each injection) led to an increase of approximately 6 bar at the particulate column back pressure, while at the monolithic column the back pressure rise was only approximately 1 bar. The life time of the short silica monolithic columns used as a trap column or as a guard column heavily depends on a type

and a volume of the bio-fluid injected. After injecting plasma column performance dropped drastically when the volume of half column volume was injected. For urine the column stability was at least 20 times higher. This is definitely related to the sample complexity. Comparing the life time of the same dimension of monolithic silica columns and particle packed columns under same conditions; monolithic silica column life time was at least double compared with particulate packed column of the similar dimension. This is not a surprise, as any particle packed column contains particles and frits to maintain particles in the column. The flow through between particles is much smaller that the particle size itself, for example, if the column packed with 2 µm particles the space between particles is about 0.5 µm. Monolithic silica column made as one single spongy rod, does not contains frits, and flow through pores (macropores) are about 2 µm diameter.

Capillary separations, although delivering much improved sensitivity, especially when combined with mass spectrometry, often have the drawback of reduced robustness. This is partially due to the limited stability of packed capillary columns and the risk of clogging (same aspect as discussed above). Monolithic capillary columns made of polymeric (Svec et al., 2003) or silica-based materials promise to overcome some of the limitations mentioned above, namely that of packing stability. An interesting study was performed by the Guryca et. al. to provide a side-by-side comparison of monolithic nano-LC columns used in reversed-phase chromatography of proteins tryptic digests (Guryča et al., 2008). They compared PepMap (LC Packings, Amsterdam, The Netherlands, 3 µm 100Å, ID 75 µm, 15 cm), Chromolith CapRod (Merck KGaA, Darmstadt, Germany; silica monolith-C18, ID 100 µm, 15 cm) and PS-DVB (LC Packings; polystyrene monolith, ID 100 µm, 5 cm) columns (all C18 modification), in terms of the number of peptides identified and also with respect to their chromatographic characteristics. In terms of performance the peak shapes obtained on Chromolith CapRod and PepMap columns appeared to be very similar, and the peak widths for both columns were in the range 0.3–0.4 min. The PS-DVB column exhibited somewhat disappointing performance which could be attributed, to the mobile phase composition used. However, it was concluded, that generally the performance of both silica based columns was superior to that of monolithic PS-DVB (Guryča et al., 2008). Also a similar finding was observed comparing peptide identification power. Comparing column throughput Chromolith CapRod column was superior with 5.0 µl/min in contrast to flow rates of up to 0.8 µl/min for PS-DVB column and to 0.5 µl/min for the particulate (PepMap) column. Moreover, it was found that, for short gradients, the number of identifications is not affected by the flow rate (3–10 mm/s). The results shown demonstrate the greater potential of monolithic compared to particle-based columns, as higher flows can be utilized, enabling the number of identifications per unit of time to be significantly increased. Furthermore, due to their higher porosity they have fewer tendencies to get clogged. Usually, micro columns for LC are fabricated by packing beads with a controlled range of diameters and pore sizes. To obtain a better efficiency, columns have been packed with particles of ever smaller diameters (Szabolcs et al., 2009) bringing about another practical limitation: the increase of the back pressure. To circumvent this problem, alternative chromatographic modalities such as ultrahigh-pressure liquid chromatography, open tubular chromatography, and capillary electro-chromatography have been investigated. All this has led to the use of particle sizes in the range of 3 to 5 μm as a good compromise between column efficiency and pressure drop. Moreover, it was demonstrated that the recently developed monolithic-type HPLC columns could be operated at high flow rates while maintaining a high efficiency. In this context,

(Kele & Guiochon, 2002) investigated the reproducibility of the preparation of the first columns getting reproducibilities higher than with particle based columns. Because of their capacity to perform fast separations they can be used for fast screening methods and applications in multidimensional chromatography systems. Conditioning and regeneration of these monolithic columns can be done in a short time when compared with the corresponding capillary packed columns, thus making more effective use of costly LC-MS equipment. They can be easily integrated in fully automated systems to perform unattended runs. These columns are flexible and they show a good performance at both low (1.5 µl/min) and high (4.5 µl/min) flow rates. Such flow rate range is highly compatible with MALDI plate spotting strategy. Fraction could be spotted directly, then the flow of 4 µl/min allows to spot up to 8 fractions. If the flow rate is set to 3 µl/min, and an equal flow of MALDI matrix solution is added post-column (7 mg/ml re-crystallized α-cyanohydroxycinnamic acid, 2 mg/ml ammonium phosphate, 0.1% trifluoroacetic acid, 80% acetonitrile) and the combined eluant is automatically spotted onto a stainless steel MALDI target plate every 6 s (0.6 µl/spot), a total of 370 spots obtained per original SCX fraction (Fort et al., 2009). Haffey demonstrated similar approach and obtained 3828 MALDI-TOF spots from the 12 SCX fractions (Haffey et al., 2009). Such a separation strategy offers enormous discrimination power and imposing peak capacity.

3. Application example: The case study

The analysis concept is based on an on-line sample preparation and a two-dimensional LC (see Figure 7) system: pre-separating the majority of the matrix components from the analytes which are retained on a RAM-SCX (LiChrospher 60 XDS (SO_3/Diol), two 25 x 4mm

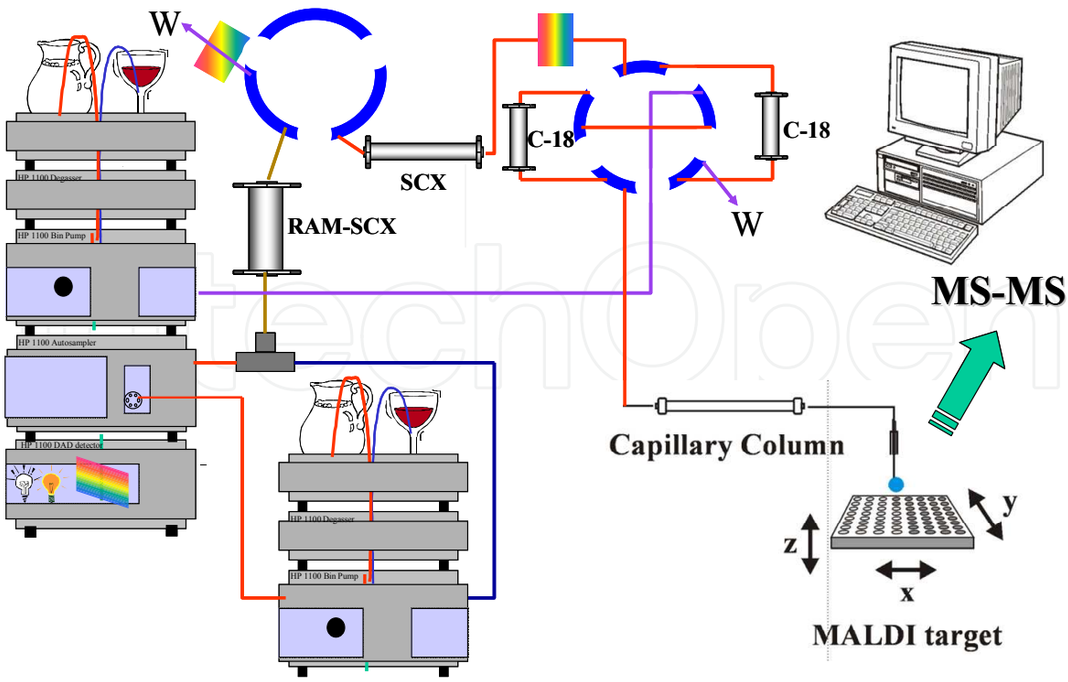


Fig. 7. Multidimensional chromatographic separation platform with integrated on-line sample clean-up.

id) column followed by a solvent switch and transfer of the trapped peptides using five salt steps by mixing 20 mM phosphate buffer (pH 2.5) (eluent A1) and 20 mM phosphate buffer with 1.5 M sodium chloride (eluent B1) at following proportions: 85/15; 70/30; 65/45; 45/55; 0/100 at the constant 0.1 ml/min flow rate was performed after the switching for the second dimensional strong cation exchange analytical separation and trapped onto the RP column by means of column switching in a way to perform two-dimensional orthogonal separations.

Desorption of the adsorbed species from the RAM-SCX column could be accomplished by employing an eluent with a higher solvent strength or pH than the eluent at the loading. We preferred the salt steps as the pH needed double time for re-equilibration. The desorption step was repeated several times to eliminate memory effects. In order to avoid sample to sample cross contamination, two blank gradients were typically applied (with specific analytes or higher loadings it could reach up to five blank gradients). The further steps of the analysis e.g. the transfer from the RAM-SCX column to the next (analytical cation exchange column) is heavily dependant on the way this transfer is performed. Three different modes could be chosen to elute the trapped sample from the RAM-SCX column: isocratic, one step elution with a strong solvent, elution with a linear gradient and elution with pulsed gradient.

A desalting and preconcentration of the fractions containing proteinaceous components were performed on two identical trap columns Zorbax 300 SB-C18, 5 µm particles, 5 x 0.3 mm I.D. obtained from Agilent (Agilent, Waldbronn, Germany). As a final column a monolithic fused silica RP-18 endcapped capillary column of dimensions 150 x 0.1 mm I.D. (Chromolith CapRod, Merck KGaA, Darmstadt, Germany) was used. We preferred the monolithic type of column over particulate capillary column for the following reasons: (a) monolithic silica columns offers high variability of flow rates adjustments, which is particularly useful in the set up of multidimensional LC MS system to adjust for different column sizes; (b) the monolithic silica columns implemented in the multidimensional LC MS system meets the requirement of high reproducibility as with particulate columns; (c) in terms of column robustness and usage flexibility monolithic silica columns are superior that packed particulate columns eg.: one could cut the top end column when damaged, furthermore, there is no change in the permeability as a result of pressure fluctuation; the end of the capillary directly connected to the MS; no frits are required etc. Standard acetonitrile gradient with 0.1 % formic acid at constant 2 µl/min flow rate separated trapped peptides in 40 min. The end of reverse phase capillary column directly inserted in an in house made robotic spotting apparatus so that the droplets are accumulated above MALDI plate and directed consequently from spot to spot with 2 minute intervals filling 100 spot MALDI plate per sample (5 fractions from the RAM-SCX column (salt steps), 20 fractions from the monolithic capillary RP 18e column, 5x20=100). After all plate positions were filled and dried out properly matrix material, consisting of α-cyano-4-hydroxycinamonic acid in 50 % acetonitrile / 4 % formic acid / water (v/v/v) of a volume of 0.5 µl was spotted on the top. The MALDI plate was kept in dark place and analysed within the 12 hours.

As already mentioned the system performs an on-line directly injected human plasma, cerebrospinal fluid and urine sample separation in a fully automated way, by a scale down strategy, gaining in sensitivity. In all peptide displays (Figure 8), between 1,000 and 4,000 mass spectrometric signals appeared, which correspond to 500 – 2,000 individual peptides.

This usually reflects redundancy (peptides that elute in more than one fraction), peptide species with and without oxidative states, and a small number of mass spectrometric derivatives, such as fragment ions.

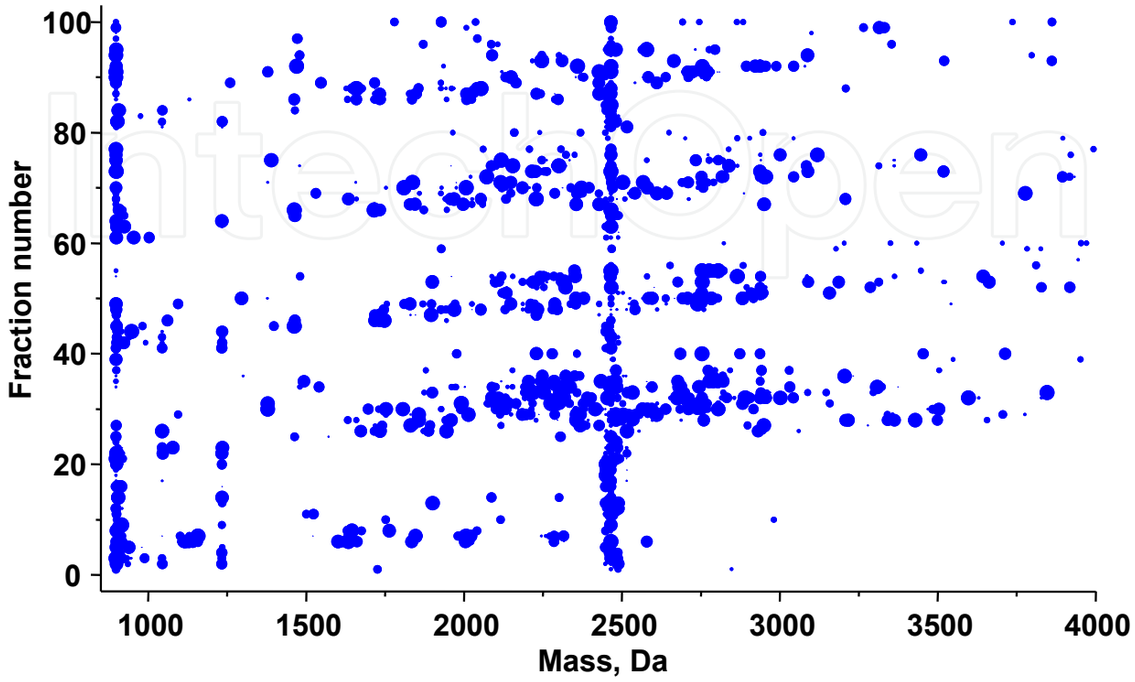


Fig. 8. Example of a human plasma peptide map. Injection volume is 48µl.

The fully automated 2D-LC system performing an effective fractionation combined with off-line MALDI TOF MS offers an enormous potential for human peptidomics screening on a daily basis. The system offers a high flexibility to be optimized for effective analysis of other biofluids such as amniotic fluid, sputum, urine, etc. The systems are completely automated and perform a high number of analysis cycles with low cost per analysis. Fast and comprehensive mapping of bio peptides and protein fragments will open possibilities to recognize novel and specific biomarkers that will help to diagnose disease and possibly provide valuable information for new drug development.

4. Conclusion

Novel restricted access materials have shown high efficiency in sample clean-up after direct on-line biofluid injections. Benefits of monolithic silica columns such as: super eminent low backpressure compared to particulate packed columns, therefore high variability of flow rates adjustments is possible; superior long term stability and data reproducibility analyzing various proteinaceous samples; much higher flow rates allows speeding up the overall analysis: fast separation, washing and re-equilibration. When those two novel developments are combined in a elegant multidimensional and fully automated way proteomic analysis could be accentuated.

In the future, proteomics will play the major role in drug discovery, accelerating the various steps involved – target identification, target validation, drug discovery (efficacy, selectivity

and mode of action). Operated on a routine basis, MD-LC may provide with the desired data, and after interconnection with the biology outcome could be found.

5. References

Andrecht, S., Anders, J., Hendriks, R., Machtejevas, E., Unger, K. (2004). Effiziente Probenvorbereitung fűr die Proteomanalyse von Kőrperflűssigkeiten. *Laborwelt*, 5/2, 4–7

Boos, K.S. and Rudolphi, A. (1997). The use of restricted-access media in HPLC, Part I – Classification and review. *LC-GC Int*., 15, 602-611

Boos, K.S. and Grimm, C.H. (1999). High-performance liquid chromatography integrated solid-phase exctraction in bioanalysis using restricted access precolumn packings. *Trends Anal. Chem*., 18, 175-180

Bovanova, L., Brandsteterova, E., (2000). Direct analysis of food samples by high-performance liquid chromatography. *J. Chromatogr. A*, 880 149-168

Clynen, E., De Loof, A. and Schoofs, L. (2003). The use of peptidomics in endocrine research. *Gen. Comp. Endocr*., 132, 1-9

Cortes, H.J. (1990). *Multidimensional Chromatography. Techniques and Applications*, Marcel Dekker, New York

Desilets, C.P., Rounds, M.A., Regnier, F.E. (1991). Semipermeable-surface reversed-phase media for high-performance liquid chromatography. *J. Chromatogr*., 544, 25-39.

Fort, P.E., Freeman, W.M., Losiewicz, M.K., Sigh, R.S.J., Gardner, T.W. (2009). The Retinal Proteome in Experimental Diabetic Retinopathy: Up-regulation of Crystallins and Reversal by Systemic and Periocular Insulin. *Molecular & Cellular Proteomics*, 8, 767-779

Giddings, J.C. (1984). Two-dimensional separations: concept and promise. *Anal. Chem*., 56, 1258–1270

Giddings, J. C. (1995). Sample dimensionality: a predictor of order-disorder in component peak distribution in multidimensional separation. *J. Chromatogr. A*, 703, 3–15

Gevaert, K., Van Damme, J., Goethals, M., Thomas, G.R., Hoorelbeke, B., Demol, H., Martens, L., Puype, M., Staes, A. and Vandekerckhove, J. (2002). Chromatographic isolation of methionine-containing peptides for gel-free proteome analysis. *Mol. Cell. Proteomics*, 1, 896-903

Govorukhina, N.I., Keizer-Gunnink, A., van der Zee, A.G.J., de Jong, S., de Bruijn, H.W.A., Bischoff, R. (2003). Sample preparation of human serum for the analysis of tumor markers. Comparison of different approaches for albumin and γ-globulin depletion. *J. Chromatogr. A*, 1009, 171-178

Guryča, V., Kieffer-Jaquinod, S., Garin, J., Masselon, C.D. (2008). Prospects for monolithic nano-LC columns in shotgun proteomics. *Anal. Bioanal. Chem*., 392, 1291-1297.

Griffin, T.J., Gygi, S.P., Ideker, T., Rist, B., Eng, J., Hood, L. and Aebersold, R. (2002). Complementary profiling of gene expression at the transcriptome and proteome levels in Saccharomyces cerevisiae. *Mol. Cell. Proteomics*, 1, 323-333

Hagestam, I.H., Pinkerton, T.C. (1985). Internal surface reversed-phase silica supports for liquid chromatography. *Anal. Chem*., 57 1757-1763

Haffey, W.D., Mikhaylova, O., Meller, J., Yi, Y., Greis, K.D., and Czyzyk-Krzeska M.F. (2009). iTRAQ Proteomic identification of pVHL-dependent and -independent

targets of Egln1 prolyl hydroxylase knockdown in renal carcinoma cells. *Adv Enzyme Regul*., 49(1), 121–132

Heine, G., Raida, M. and Forssmann, W.-G. (1997). Mapping of peptides and protein fragments in human urine using liquid chromatography – mass spectrometry. *J. Chromatogr. A*, 776, 117–124

Hille, J.M., Freed, A.L. and Wätzig, H. (2001). Possibilities to improve automation, speed and precision of proteome analysis: A comparison of two-dimensional electrophoresis and alternatives. *Electrophoresis*, 22, 4035–4052

Hogendoorn, E.A., Dijkman, E., Baumann, B., Hidalgo, C., Sancho, J.-V., Hernandez, F. (1999). Strategies in using analytical restricted access media columns for the removal of humic acid interferences in the trace analysis of acidic herbicides in water samples by coupled column liquid chromatography with UV detection. *Anal. Chem*., 71 1111-1118

Huber, J.F.K. and Lamprecht, G. (1995). Assay of neopterin in serum by means of two-dimensional high-performance liquid chromatography with automated column switching using three retention mechanisms. *J. Chromatogr. B*, 666, 223–232

Issaq, H.J. (2001). The role of separation science in proteomics research. *Electrophoresis*, 22, 3629-3638

Issaq, H.J., Chan, K.C., Janini, G.M., Conrads, T.P. and Veenstra, T.D. (2005). Multidimensional separation of peptides for effective proteomic analysis. *J. Chromatogr. B,* 817, 35–47

Kele, M., Guiochon, G. (2002). Repeatability and reproducibility of retention data and band profiles on six batches of monolithic columns. *J. Chromatogr. A*, 960, 19-49.

Link, A.J. (2002) Multidimensional peptide separations in proteomics. *Trends Biotechnol.* 20(12S), 8-13

Machtejevas, E., John, H., Wagner, K., Standker, L., Marko-Varga, G., Forssmann, W-G., Bischoff, R., Unger, K.K. (2004). Automated multidimensional HPLC: sample preparation and identification of peptides from human blood filtrate. *J. Chromatography B*, 803 121-130

Machtejevas, E., Unger, K.K., Ditz, R. (2006). Multidimensional Column Liquid Chromatography (LC) in Proteomics – Where Are We Now? in *Proteomics in Drug Research*, ed. M. Hamacher, K. Marcus, K. Stühler, A. van Hall, B. Warscheid, H.E. Meyer, 89-111, Wiley-VCH, Weinheim, Germany

Machtejevas, E., Denoyel, R., Meneses, J.M., Kudirkaitė, V., Grimes, B.A., Lubda, D., Unger, K.K. (2006). Sulphonic acid strong cation-exchange restricted access columns in sample cleanup for profiling of endogeneous peptides in multidimensional liquid chromatography Structure and function of strong cation-exchange restricted access materials. *J. Chromatogr. A*, 1123, 38–46

Machtejevas, E., Andrecht, S., Lubda, D., Unger, K.K. (2007). Monolithic silica columns of various formats in automated sample clean-up/multidimensional liquid chromatography/mass spectrometry for peptidomics. *J. Chromatogr. A*, 1144 97-101

Machtejevas, E., Unger K.K. (2008). Proteomics Sample Preparation, in: Jörg von Hagen (Editor), *Sample preparation for HPLC – based proteome analysis*. 245-264, Wiley-VCH, Weinheim, Germany

Machtejevas, E., Unger, K.K. (2008). Multidimensional Liquid Chromatography: Theory and Applications in Industrial Chemistry and the Life Sciences. Steven A. Cohen and Mark R. Schure (Editors), *Peptidomics*. 207-220. John Wiley & Sons Inc., Hoboken, New Jersey, USA

Machtejevas, E., Marko-Varga. G., Lindberg, C., Lubda, D., Hendriks, R., Unger, K.K. (2009). Profiling of Endogenous Peptides by Multidimensional Liquid Chromatography: On-line Automated Sample Cleanup Procedures for Biomarker Discovery in Human Urine. *J. Sep. Sci*. 32, 2223 – 2232

Mazsaroff, I., Regnier, F.E. (1988). Phase ratio determination in an ion-exchange column having pores partially accessible to proteins. *J. Chromatogr.*, 442: 15-28

Meyers, J.J., Liapis, A.I. (1999). Network modeling of the convective flow and diffusion of molecules adsorbing in monoliths and in porous particles packed in a chromatographic column. *J. Chromatogr. A*, 852, 3-23

Nakamura, K., Suzuki, T., Kamichika, T., Hasegawa, M., Kato, Y., Sasaki, H., Inouye, K. (2002). Evaluation and applications of a new dye affinity adsorbent. *J. Chromatogr. A*, 972, 21-25

Pandey, A. and Mann, M. (2000). Proteomics to study genes and genomes. *Nature*, 405, 837-846

Pang, J.X., Ginnanni, N., Donge, A.R., Hefta, S.A. and Opiteck, G.J. (2002). Biomarker discovery in urine by proteomics. *J. Proteome Res*., 1, 161-169

Pinkerton, T.C. (1991). High-performance liquid chromatography packing materials for the analysis of small molecules in biological matrices by direct injection. *J. Chromatogr.*, 544: 13-23

Pitiot, O., Poraht, J., Guzmann, R., Vijayalakshmi, M.A. (2004). 9th International Symposium on Biochromatography, 5–7 May, Bordeaux, France, Programme & Abstracts, p. 34

Premstaller, A., Oberacher, H., Walcher, W., Timperio, A.M., Zolla, L., Chervet, J-P., Cavusoglu, N., van Dorsselaer, A., Huber, Ch.G. (2001). High-Performance Liquid Chromatography-Electrospray Ionization Mass Spectrometry Using Monolithic Capillary Columns for Proteomic Studies. *Anal. Chem*., 73: 2390-2396

Quaglia, M., Machtejevas, E., Hennessy, T. and Unger, K.K. (2006). Size Exclusion Chromatography (SEC) of biopolymers) Optimization strategies and trouble shouting. S. Kromidas (Editor), *HPLC Made to Measure – A Practical Handbook for Optimization*,- Wiley-VCH, Weinheim, Germany, 383-403

Račaitytė, K., Lutz, E.S.M., Unger, K.K., Lubda, D., Boos, K.S. (2000). Analysis of neuropeptide Y and its metabolites by high-performance liquid chromatography–electrospray ionization mass spectrometry and integrated sample clean-up with a novel restricted-access sulphonic acid cation exchanger. *J. Chromatogr. A*, 890: 135-144

Rbeida, O., Christiaens, B., Hubert, Ph., Lubda, D., Boos, K.-S., Crommen, J. and Chiap, P. (2005). Integrated on-line sample clean-up using cation exchange restricted access sorbent for the LC determination of atropine in human plasma coupled to UV detection. *J. Pharm. and Biomed. Anal*., 36/5: 947-954

Regnier, F., Amini, A., Chakraborty, A., Geng, M., Ji, J., Riggs, L., Sioma, C., Wang, S. and Zhang, X. (2001). Multidimensional chromatography and the signature peptide approach to proteomics. *LC-GC*, 19, 200–213

Richter, R., Schulz-Knappe, P., Schrader, M., Standker, L., Jurgens, M., Tammen, H. and Forssmann, W.-G. (1999). Composition of the peptide fraction in human blood plasma: database of circulating human peptides. *J. Chromatogr. B*, 726, 25–35

Rieux, L., Niederlander, H., Verpoorte, E., Bischoff, R. (2005), Silica monolithic columns: Synthesis, characterisation and applications to the analysis of biological molecules. *J. Sep. Sci*., 28, 1628–1641

Schulz-Knappe, P., Zucht, H-D., Heine, G., Jürgens, M., Hess, R. and Schrader, M. (2001). Peptidomics: the comprehensive analysis of peptides in complex biological mixtures. *Combinatorial Chemistry & High Throughput Screening*, 4, 207–217

Souverain, S., Rudaz, S., Veuthey, J.-L., (2004), Restricted access materials and large particle supports for on-line sample preparation: an attractive approach for biological fluids analysis. *J. Chromatogr. B*, 801, 141-156

Svec, F., Tennikova, T., Deyl, Z. (editors) (2003). *Monolithic materials: preparation, properties and applications*. Elsevier Amsterdam, The Netherlands. P. 800.

Szabolcs, F., Jeno, F., Katalin, G. (2009). Characterisation of new types of stationary phases for fast liquid chromatographic applications. *J. Pharm. Biomed. Anal*., 50(5), 703-712

Unger, K.K., Machtejevas, E. (2007). Mehr als nur eine Säule – die Möglichkeiten der mehrdimensionalen LC. *Nachrichten aus der Chemie*, 6 661-664

Unger, K.K., Machtejevas, E. (2009). Peptid-Profilanalyse aus Körperflüssigkeiten. *ChromChat*, 4 44 -47

Unger K.K., Tanaka, N. and Machtejevas, E. (2011). *Monolithic silicas in separation science: Concepts, syntheses, characterization, modeling and applications*. Wiley-VCH, Weinheim, Germany, P. 374

Wagner, K., Miliotis, T., Marko-Varga, G., Bischoff, R. and Unger, K.K. (2002). An automated on-line multidimensional HPLC system for protein and peptide mapping with integrated sample preparation. *Anal. Chem*., 74, 809–820

Wang Y.Y., Cheng, P., Chan, D.W. (2003). A simple affinity spin tube filter method for removing high-abundant common proteins or enriching low-adundant biomarkers for serum proteomic analysis. *Proteomics,* 3, 243-248

Westermeier, R., Naven, T., and Hēpker, H.-R. (2008). *Proteomics in Practice. A Guide to Successful Experimental Design* 2nd Ed. WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, P. 482

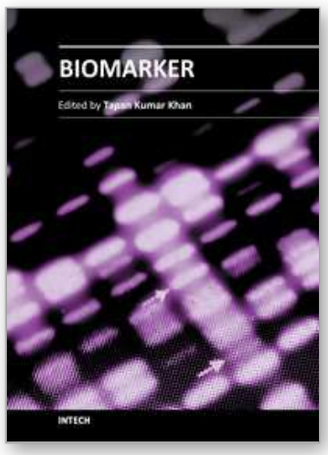
Willemsen, O., Machtejevas, E., Unger, K.K. (2004). Enrichment of proteinaceous materials on a strong cation-exchange diol silica restricted access material (RAM): protein-protein displacement and interaction effects. *J. Chromatography A*, 1025, 209-216.

Wolters, D.A., Washburn, M.P. and Yates III, J.R. (2001). An automated multidimensional protein identification technology for shotgun proteomics. *Anal. Chem*., 73, 5683-5690

Xiong, L., Zhang, R., Regnier F.E. (2004). Potential of silica monolithic columns in peptide separations. *J. Chromatogr. A*, 1030, 187–194

Yamashita, K,, Motohashi, M., Yashiki, T. (1992). Automated high-perfomance liquid chromatographic method for the simultaneous determination of cefotiam and delta 3-cefotiam in human plasma using column switching. *J. Chromatogr*., 577, 174-179

Yu, Z., Westerlund, D. and Boos, K.S. (1997). Determination of methotrexate and its metabolite 7-hydroxymethotrexate by direct injection of human plasma into a column-switching liquid chromatographic system using post-column photochemical reaction with fluorimetric detection. *J. Chromatogr. B*, 689, 379-386



Biomarker

Edited by Prof. Tapan Khan

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Clinicians, scientists, and health care professionals use biomarkers or biological markers as a measure of a person's present health condition or response to interventions. An ideal -biomarker should have the following criteria: (I) ability to detect fundamental features of the disease, (II) ability to differentiate from other closely related diseases, (III) ability to detect early stages and stages of progression, (IV) the method should be highly reliable, easy to perform and inexpensive, and (V) sample sources should be easily accessible from body. Most of the chapters in this book follow the basic principle of biomarkers.

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