Development of ultrahigh resolution FTICR mass spectrometry methods for clinical proteomics

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Chapter 1

Standardized and automated solid-phase extraction procedures for high-throughput proteomics of body fluids

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ABSTRACT

In order to balance the speed of analytical sample preparation procedures with mass spectrometry (MS)-based clinical proteomics the application of high-throughput robotic systems for body fluid workup is essential. In this paper we describe the implementation of various solid-phase extraction (SPE) sample preparation protocols on two different platforms, namely: 1) Magnetic bead-based SPE of peptides and proteins from body fluids on a Hamilton liquid handling workstation; 2) Cartridge-based SPE on a SPARK Symbiosis system. All SPE protocols were optimized for MS-based proteomics and compared with respect to obtained peptide- and protein profiles. Throughput numbers that were achieved in a 24 hour time frame for the sample workup procedures were more than 700 samples for the magnetic bead-based method and over 1000 samples for the cartridge-based method.

INTRODUCTION

Proteomics has matured into a standard technology and with that interest has broadened from studying fundamental biological processes to screening large patient cohorts in clinical research [1]. Improved and more detailed genetic knowledge of various biological processes or organisms is now widely used in proteomics research [2;3]. The last decade sample throughput in MS-based proteomics has increased enormously due to the development of highly automated and robust mass spectrometers and improved speed in data handling and -processing. As a consequence, the analytical methods that involve peptide- and protein profiling of body fluids require high-throughput approaches. It should be stressed that sample workup is an essential part in all proteomics workflows because of the large complexity of any biological material [4-6]. In order to handle sample complexity and obtain full coverage multidimensional separations have been reported [7;8]. However, such approaches are not feasible for large screening studies taking into account time, costs and robustness. Most biomarker discovery studies that have reported on candidate peptides and proteins that were found to correlate with the presence and/or a stage of a certain disease were based on relatively small numbers of samples. Not surprisingly, a large majority of these discoveries could not be validated and did not make it into a diagnostic clinical assay [9;10]. Thus a high-capacity pipeline is needed to perform screening studies of clinical cohorts containing at least 1,000 different individuals [11]. Such a high-throughput workflow consists of robust and relatively fast sample processing and preparation (resulting in a reduced complexity) as well as automated MS [1;12]. Moreover, high-end MS in combination with specific sample workup allows mapping of modifications such as glycosylation and phosphorylation [13;14].

In this paper we present two automation platforms for SPE-based sample preparation and subsequent MS-measurements. Automation of sample preparation not only increases sample throughput but also improves robustness by eliminating human errors. Here, two different instrumental setups are described: The first one involves magnetic bead-based SPE, whereas the second SPE-setup is based on column-like cartridges. Both setups aim for peptide- and protein profiling of body fluids. For this purpose a subset of proteins and peptides is captured from body fluids such as serum or urine using an SPE approach. The captured components are measured on a mass spectrometer to obtain a "profile" (mass spectrum) of the body fluid [15]. Pattern analysis

of these profiles allows the classification of individuals into groups (*e.g.* healthy versus diseased). In general, these methods consist of incubation-, washing- and elution steps. In addition, a protocol is described that involves spotting of samples onto a MALDI-target plate for analysis on for instance a MALDI time-of-flight (TOF) or MALDI Fourier Transform Ion Cyclotron Resonance (FTICR) system. We have chosen to implement the first application on a Hamilton liquid-handling workstation. Advantages of this robotic system are the flexibility in deck layout, user-friendly programming software and the possibility to include in-house developed and third party consumables or equipment. The second application has been implemented on a SPARK Symbiosis SPE system, specifically adapted to increase sample throughput. The advantages of this system are the availability of a wide range of different cartridges and the ability to keep the samples at 4°C during sample storage and after elution.

The general availability of functionalized magnetic beads for peptide- or protein profiling is decreasing, or has even been discontinued in the case of (polymeric) reversed-phase (RP) C18-beads. Other players have entered the market by supplying magnetic beads based on silica material that are currently under investigation in our group. Furthermore, the magnetic bead protocol results in peptide- or protein eluates that are "optimized" for MALDI-MS measurements while in some instances direct infusion ESI-MS is the preferred MS method, *e.g.* to obtain multiply charged species that are more suitable for MS/MS-fragmentation. For these reasons an alternative SPE system was considered. This paper describes the implementation and comparison of both systems.

MATERIALS AND METHODS

Consumables and reagents. All microtitration plates (PCR-plates) were obtained from Greiner (Alphen a/d Rijn, The Netherlands). Functionalized magnetic beads were either purchased from Bruker Daltonics (weak cation exchange (WCX); Bremen, Germany) or from Invitrogen (reversed-phase (RP) C18; Breda, The Netherlands). The elution buffer for WCX magnetic beads was a 130 mM ammoniumhydroxide solution (J.T. Baker, Deventer, The Netherlands) and the stabilization buffer consisted of 3% trifluoroacetic acid (TFA; Sigma, St. Louis, USA) in water. MALDI matrix α-cyano-4-hydroxycinnamic acid (CHCA) was obtained from Bruker Daltonics and used as a 3 mg/ml solution in

acetone:ethanol 1:2. Magnets were purchased from Webcraft GmbH (http://www.supermagnete.de/; Gottmadingen, Germany).

Serum samples. Serum samples were obtained from healthy volunteers at the Leiden University Medical Center (LUMC). Samples were collected, anonymized and processed according to a standardized protocol: all blood samples were drawn by antecubital venapuncture while the individuals were seated and had not been fasting. The samples were drawn in an 8.5 cc Serum Separator Vacutainer Tube (BD Diagnostics, Plymouth, UK) and within maximally 4 hours centrifuged at room temperature at 1000 g for 10 minutes. The samples were kept in sterile 500 µl barcode labeled polypropylene tubes (TrakMate, Matrix TechCorp.) at -80ºC until further use.

SPE using magnetic beads. For each (biological) sample fresh magnetic beads were used. The RPC18-beads were first activated by a three-step washing with a 0.1% TFA solution. Then, the samples were added to the activated beads and incubated for 5 minutes at room temperature. The beads were washed again three times with 0.1% TFA and peptides were eluted with a 1:1 mixture of water and acetonitrile.
For the application of WCX-beads, the samples were added directly to the beads together with binding buffer since no activation was needed. The beads were washed three times with washing buffer and eluted with a high pH elution buffer, which was prepared as described in 'Consumables and reagents'. Thus obtained eluates were mixed with stabilization buffer to acidify the sample.
Two microliters of each (stabilized) eluate were mixed with 15 microliters of a MALDI matrix solution in a 384-well PCR plate. Then, 1 microliter of this mixture was spotted in quadruplicate onto a MALDI-target plate (Bruker Daltonics).

SPE using cartridges. For each (biological) sample a new cartridge was used. Cartridges were pre-washed with 1 ml 100% acetonitrile and equilibrated with 1 ml 1% acetic acid / 2% acetonitrile (wash solvent) prior to sample application. Serum samples were diluted 4 times with 0.1% acetic acid and 100 µL of the diluted serum was applied to the cartridge with 1 ml wash solvent. The cartridges were washed with 2 ml wash solvent and eluted

with 100 µL 50% acetonitrile / 0.1% acetic acid. After elution the tubing was rinsed with 500 µL wash solvent before proceeding to the next sample.

Target plate spotting and mass spectrometry. The protocol for spotting onto a MALDI-target plate is part of the other two procedures described above. The implementation will be described in the Results and Discussion section. MALDI-TOF experiments were performed on an UltraFlex II (Bruker Daltonics) either operating in positive reflectron mode in the *m/z*-range of 600–4,000 or in positive linear mode in the *m/z*-range of 1000-11000. The spectra were acquired using FlexControl software ver. 3.0 (Bruker Daltonics). A Smartbeam 200 Hz solidstate laser, set at a frequency of 100 Hz, was used for ionization. A profile, or summed spectrum, was obtained for each MALDI-spot by adding 20 spectra of 60 laser shots each at different rasters. To this end, FlexControl software decided on-the-fly whether or not a scan was used for the summed spectrum. For reflectron mode acquisitions, a resolution higher than 2,000 was required. Peaks were detected using the SNAP centroid peak detection algorithm with signal-to-noise threshold of 1 and a "TopHat" baseline subtraction. All mass scans not fitting these criteria were excluded. For linear mode acquisitions, a resolution higher than 100 was required and peaks were detected using the centroid peak detection algorithm with signal-to-noise threshold of 2 and a "TopHat" baseline subtraction. The measurement of a MALDI spot was finished when 1200 laser shots had been summed in one profile. FlexAnalysis Software 3.0 (Bruker Daltonics) was used for visualisation and data processing.

MALDI-FTICR experiments were performed on a Bruker 15 tesla solariX™ FTICR mass spectrometer equipped with a novel CombiSource [16]. The MALDI-FTICR system was controlled by Compass solariXcontrol software and equipped with a Bruker Smartbeam-II™ Laser System that operated at a frequency of 500 Hz. The "medium" predefined shot pattern was used for the irradiation. Each mass spectrum was obtained from a single scan of 600 laser shots using 512 K data points. Typically, the target plate offset was 100 V with the deflector plate set at 180 V. The ion funnels operated at 100 V and 6.0 V, respectively, with the skimmers at 15 V and 5 V. The trapping potentials were set at 0.60 V and 0.55 V, the analyzer entrance was maintained at -7 V, and side kick technology was used to further optimize peak shape and signal intensity. The required

excitation power was 28% with a pulse time of 20.0 µs. MALDI-FTICR profiles were obtained from the same target plate that had been used for the MALDI-TOF acquisitions.

RESULTS AND DISCUSSIONS

Implementation of SPE using magnetic beads on the robotic liquid handling system. The robotic system for automated liquid handling is built up from three units (Figure 1A). Currently, this system is used for (1) in-gel and in-solution digestions, (2) desalting of samples using ziptips, and (3) magnetic bead based SPE for peptide- and protein profiling. In this study data is reported from the third application, which involves the unit depicted on the right-hand side of Figure 1A. This unit consists of a two-arm Hamilton STARplus robotic workstation that is equipped with eight independently controlled 1 ml pipetting channels, a 300 µL 96 channel pipetting head, an 8+1 channel nanospotter, a 96-well magnet, a vacuum system, a heated/cooled shaker, a temperature controlled carrier and a plate CORE-gripper. Description of the second and third unit (left-hand side and middle of Figure 1A) is outside the scope of this work.

For the application of magnetic bead based SPE it was necessary to develop specific labware that was not readily available from the supplier. For example, to prevent evaporation of volatile solvents from the supplied open reagent containers, special containers have been implemented with automated sliding lids to open and close on demand. In addition, a wireless and non-fixed mini-camera that can be picked-up by one of the eight 1 ml pipetting channels has been implemented for quality control purposes. Finally, a specific magnet plate incorporating 96 individual magnets to accommodate 96-well plates was in-house developed. With this magnet different sample workup steps can be performed as will be described in the following paragraph.

In MS-based clinical profiling studies proteins and peptides are isolated from a biological fluid (blood, urine, CSF) using SPE material, which is normally used in a cartridge or column-like format or as paramagnetic beads. In the here described automated system paramagnetic beads coated with a variety of functionalities, are applied to extract various subsets of peptides and proteins. The platform allows multiplexing, *i.e.* a sample plate can be processed with different types of magnetic beads, either simultaneously or sequentially. The deck layout for this protocol is shown in Figure 1B.

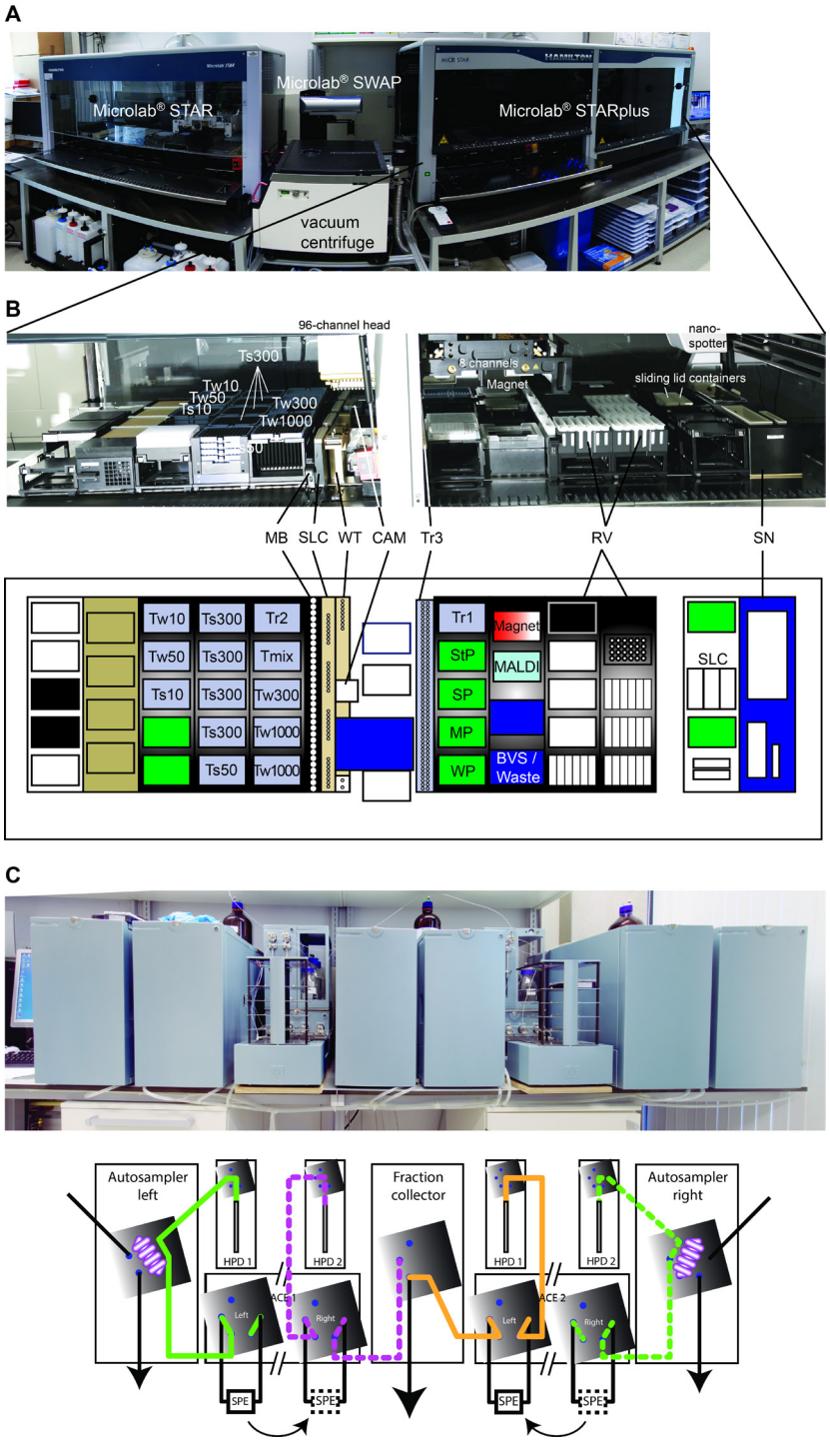
The various protocols for the different bead types have an identical workflow in terms of "order of events", however slightly differ with regard to the number of activation cycles, wash cycles and incubation times and the specific buffers used. All protocols for the extraction procedures provided by the manufacturer for manual processing were adapted and further optimized to allow implementation on the robotic system. Initially, these optimizations have been described on a previous 8-channel liquid handling platform (http://www.hamiltonrobotics.com/fileadmin/user_upload/prodb/app_notes/Proteomics/BR-0504-01_PeptideExtraction_for_ProteinProfiling_01.pdf). However, for the current platform these protocols were further adjusted to obtain an even higher throughput. In short, each protocol consists of a binding step, several washing steps and an elution step. Optionally there might be an activation and/or equilibration step, and a stabilization step of the eluate. All eluates are collected in a fresh PCR-plate and then spotted in quadruplicate onto a target plate for further analysis by MALDI-TOF-MS and/or MALDI-FTICR-MS. In order to reduce loss of magnetic beads to a minimum PCR-plates were used throughout the protocol. These plates allow for maximum aspiration of solvents with a minimal residual volume in the wells and minimal disturbance of the magnetic bead pellet through a smaller surface to height ratio in comparison with other types of microtitration plates.

Obviously, the manipulation of magnetic beads requires a magnet for capturing. Ideally, in a 96-well PCR plate all magnetic beads are captured at the bottom of each well upon using a magnet. Different types of commercially available magnets to accommodate such 96-well plates were tested on the Hamilton system, namely magnets in ring-, bar- and pin form. Unfortunately, in our hands none of these magnets allowed suitable implementation in the robotic system due to "off-center" or "well-above-the-bottom" capturing of magnetic beads in a PCR plate. As a result of non-optimal capture proper resuspension of magnetic beads was not possible and the SPE-cleanup of serum samples failed. Therefore, a magnet block was in-house developed with a single magnet at the bottom of each well, resulting in optimal capture of beads. Proper resuspension of the bead pellet needs to be performed without the magnet. This implicates that two deck positions are required for processing one sample plate. Therefore, an electronically controlled height-adjustable magnet was designed to allow collection and resuspension of beads at the same position in the robot deck.

The here described SPE-protocols based on magnetic bead fractionation were implemented in the year 2008. Since then, more than 15,000 different serum samples have been processed in biomarker screening studies [17-23]. These studies included statistical validation tests. From the results of the 96-channel system it was concluded that the reproducibility of the peptide- and protein profiles obtained from MALDI-TOF-MS was better than in the case of manual sample processing.

Typically, the coefficient of variation (CV) of the peak areas in the profiles was on average 15% when processing biological serum sample replicates, and for some specific peptides even below 5%. Note that this value results from a combination of variation in the SPE-procedure itself, the MALDI-spotting and MALDI-TOF measurement. This aspect is further discussed in the last paragraph of this section (comparison of beads and cartridges).

Implementation of online SPE using cartridges on the Symbiosis. Basically, a Symbiosis system consists of one autosampler (Reliance), one High Pressure Dispenser (HPD) and one Automated Cartridge Exchange (ACE) unit. In Figure 1C, the setup of our customized Symbiosis system is shown. In this setup three Reliance systems are combined with two HPD units, each equipped with two syringes, and two ACE units. Two Reliance systems are used as an autosampler (left- and right-hand side of Figure 1C) and the third Reliance acts as a fraction collector (middle of Figure 1C). The fraction collector is reached by both systems in an alternating way: when one system is in the "load position", *i.e.* equilibrating and washing a cartridge (left-hand side in Figure 1C), the other one is in the "elute position" (right-hand side in Figure 1C). Note that the cartridge is swapped from the left clamp to the right clamp in the ACE unit on the left-hand side upon elution, and vice-versa for the cartridge in the second ACE. Moreover, for each sample a new cartridge is applied, and the used cartridge is placed back into the storage container. In this approach a throughput of about 45 samples per hour can be achieved.


A
Microlab® STAR
Microlab® SWAP
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vacuum centrifuge
B
96-channel head
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HPD 1
HPD 2
ACE 1
Left
Right
SPE
SPE
Fraction collector
HPD 1
HPD 2
ACE 2
Left
Right
SPE
SPE
Autosampler right

Figure 1. Systems setup. Picture of both sample preparation systems, consisting of a Hamilton Microlab® STAR, a Hamilton SWAP, an automated CHRIST vacuum centrifuge and a Hamilton Microlab® STARplus (A), a photo and schematic representation (B) of the Microlab® STARplus deck layout used for magnetic bead based protein profiling and a photo with flow-scheme (C) of the Symbiosis system. To carry out a protocol on the Microlab® STARplus with optimal use of deck space and without user-intervention stacks of pipette tips are needed. This storage of tips on the deck is further indicated as "Tip-stacking" (Ts) positions. To allow for tip pickup within a certain protocol one rack of tips is initially transferred to a so-called "Tip-working" (Tw) position. TCC: Temperature Controlled Carrier; Tw10: tip work sequence 10 microliter tips; Tw50: tip work sequence 50 microliter tips; Tw300: tip work sequence 300 microliter tips; Tw1000: tip work sequence 1000 microliter tips; Ts10: tip stack 10 microliter tips; Ts50: tip stack 50 microliter tips; Ts300: tip stack 300 microliter Tips; Tmix: spare position for all tip types to be used by 96-channel head; Tr1, Tr2, Tr3: empty racks for tips to be re-used; MB: Magnetic beads; WP: working plate; MP: mixing plate for mixing sample and MALDI-matrix; SP: sample plate; StP: eluent storage plate; SLC: Sliding Lid Containers for volatile solvents; MALDI: MALDI-target plate; RV: reagent vessels; WT: waste and tool carrier. Tools include CORE-gripper and CORE-camera (CAM); SN: service carrier nano-head for washing and waste.

Both paramagnetic beads and the cartridges are available with a wide variety of functionalities. However, cartridges can also be specifically packed with any solid phase material of choice, making the availability of such material the only limiting factor. In this study six out of eight functionalities from the SPARK method development kit were tested with respect to peptide- and protein isolation from serum samples, as well as three other functionalities, namely: CN-SE, C2-SE, C8 EC-SE (End Capped), C18 HD (High Density), Resin GP (General Phase), Resin SH (Strong Hydrophobic), PLRP, C18-OH and C18-EC. A general protocol was used for all cartridges. In short, the cartridges were activated with acetonitrile and equilibrated with wash solvent. Serum samples were diluted 4 times with 0.1% acetic acid and applied to the cartridges using wash solvent. The cartridges were washed and eluted with elution solvent. The eluates were collected as 100 µL fractions. The resulting eluates were spotted in quadruplicate using the Hamilton STARplus as described below.

Figure 2 (inset) shows a typical FTICR-MS profile of SPE eluates, such as obtained with samples processed with the nine cartridges mentioned above. Based on the number of features (peaks) in the spectrum and the total signal intensity in both the low- and the high-mass range, the C8 EC-SE, C18-HD and C18-EC cartridges performed best and to a lesser extent also the C2-SE in the high-mass range. Preliminary results also indicated that the eluates from the C18-EC cartridges performed best in direct infusion FTICR-MS experiments, making these cartridges the type of choice for future experiments.

Using these C18-EC cartridges, four samples were processed in eightfold and spotted in quadruplicate onto a MALDI-target plate. For each processed sample two spots were used for MALDI-FTICR-MS measurements in the low mass range (1000-3700 Da) and two spots were used for MALDI-FTICR-MS measurements in the high mass range (3.5-10kDa), resulting in 16 spectra for each sample in both mass ranges. For 23 peaks of known composition and present in all spectra [16;24], the intensities expressed as area under the curve were averaged over the 16 spectra. The intensities were normalized against the total intensity of the 17 (low-mass range) and 6 (high-mass range) peaks, respectively. The results are depicted in Figure 2. It can be seen that the intra-sample reproducibility (standard deviation) is dependent on the peptide itself (*i.e.* the *m/z*-value), and in some cases this error is even larger than the inter-sample variation (*e.g.* at *m/z*-value 1465). However, in general the standard deviations are within acceptable limits and allow accurate comparison between peptide intensities in different serum samples. For example, the peptide intensity at $m/z = 1778.023$ is two-fold higher in sample 4 compared to sample 1.

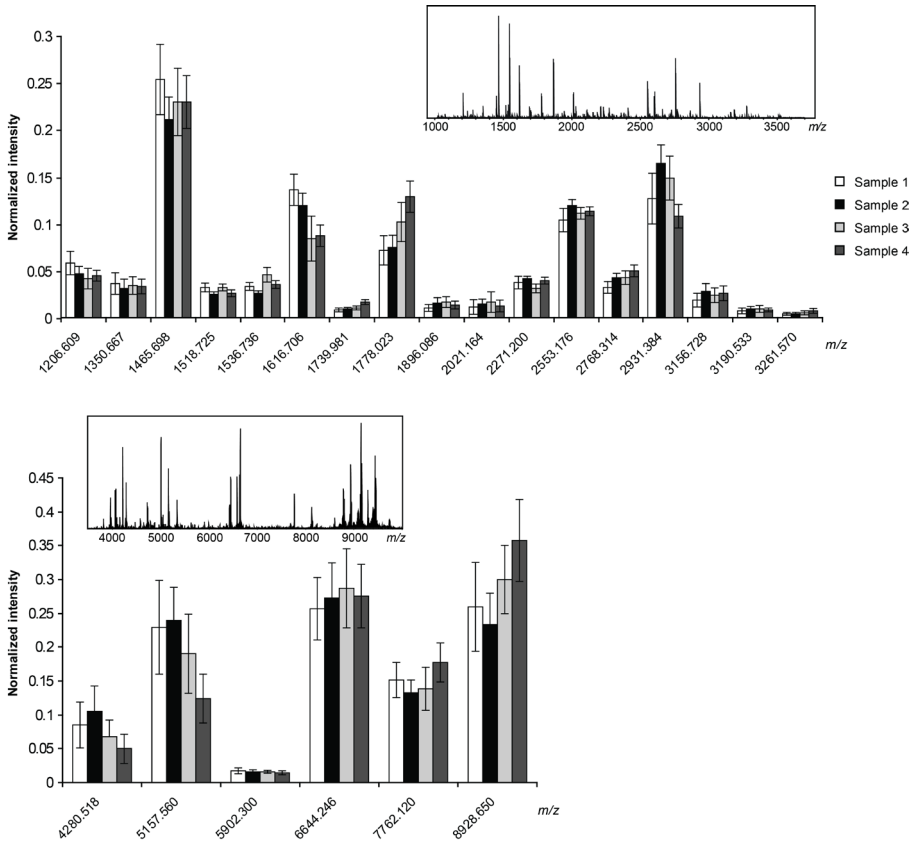

Figure 2. Repeatability and Reprocucibility of the cartridge-based SPE method. Peptides used for this analysis are observed as $[M+H]^+$ at monoisotopic *m/z* values: 1206.6 = EGDFLAEGGGVR; 1350.6 = SGEGDFLAEGGGVR; 1465.7 = DSGEGDFLAEGGGVR; 1518.7 = ADS(-H2O)GEGDFLAEGGGVR; 1536.7 = ADSGEGDFLAEGGGVR; 1616.7 = ADSpGEGDFLAEGGG VR; 1739.9 = NGFKSHALQLNNRQI; 1778.0 = SKITHRIHWESASLL; 1896.0 = RNGFKSHALQLN NRQI; 2021.1 = SSKITHRIHWESASLLR; 2271.1 = SRQLGLPGPPDVPDHAAYHPF; 2553.1 = SSSYSKQ FTSSTSYNRGDSTFES; 2768.2 = SSSYSKQFTSSTSYNRGDSTFESKS; 2931.3 = SSSYSKQFTSSTSYNR GDSTFESKSY; 3156.6 = NVHSGSTFFKYYLQGAKIPKPEASFSPR; 3190.4 = SSSYSKQFTSSTSYNRGDSTFESKSYKM; 3261.5 = SSSYSKQFTSSTSYNRGDSTFESKSY KMA; 4280.5= NVHSAGAAGSRMNFRPGVLSSRQLGLPGPPDVPDHAAYHPF; 5902.4 = SSSYSKQFTSSTSYNRGDSTFESKSYKMADEAGSEADHEGTHSTKRGHAKSRPV; 6644.3 = PSPTFLTQVKESLSSYWESAKTAAQNLYEKTYLPAVDEKLRDLYSKSTAAMoxSTYTGIFT; 8928.8 = TQQPQQDEMoxPSPTFLTQVKESLSSYWESAKTAAQNLYEKTYLPAVDEKL-RDLYSKSTAAMSTYTGIFTDQVLSVLKGEE

Implementation of a MALDI-target plate spotting protocol on the robotic liquid handling system. A spotting protocol for MALDI plates has been developed previously (http://www.hamiltonrobotics.com/fileadmin/user_upload/prodb/app_notes/Proteomics/BR-0302-01_MALDITargetSpot.pdf); however this protocol included a washing step on the MALDI target. Since our procedures result in purified samples such a washing step was not needed anymore. To this end, we compared two methods of matrix and sample deposition on the MALDI-target: 1) Premixing sample with matrix in a 384-well plate and 2) deposition of sample on a MALDI target plate followed by application of matrix with the nanospotter. The two methods differ in sample to matrix ratio and the final concentration of organic solvent. For the first method the sample to matrix ratio was 2:15 and the organic solvent concentration was 94% while for the second method the sample to matrix ratio was 10:4 and the organic solvent concentration was 64%, when calculated for a sample composition containing 50% acetonitrile as is the case for most of the applications described in this paper. This ratio is of influence on the drying speed and crystal formation. The premixing method was found to perform consistently better than the nanospotter method. In principle it would be possible to reverse the second method (apply sample instead of matrix with the nanospotter). This would change the sample to matrix ratio and the final organic solvent concentration, but processing times would increase dramatically. Moreover, to prevent sample carry-over disposable tips are to be preferred over washable tips. Depending on the method applied before spotting, using only a single spot for each sample will not always result in a good spectrum.

Using α-cyanohydroxycinnamic acid (CHCA) as the MALDI matrix and a Bruker Anchorchip^{TM} an optimal spot is a very thin uniform layer which is slightly larger than the anchor (Figure 3A). Causes for non-ideal spots could *e.g* be the absence of analytes in the sample or extract, resulting in very dense spots with the size of the anchor (Figure 3B) or, in case of magnetic bead purification, the presence of magnetic beads on the MALDI target caused by incomplete separation of beads from the eluate, resulting in brown spots (Figure 3C). Furthermore, aberrant spot volumes, caused for instance by droplets at the outside of the tips, may result in different crystal formation and subsequently worse spectra (Figure 3D). Therefore, each sample is generally spotted multiple times.

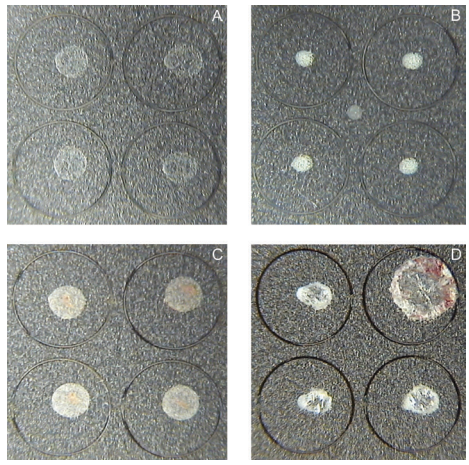

Figure 3. Examples of MALDI spots. (A) Optimal spot: thin homogeneous matrix layer, which is larger than anchor. (B) Small spots almost the size of the anchor, due to absence or (very) low concentration of analytes. (C) Brown spots: presence of magnetic beads due to incomplete separation of magnetic beads from eluate. (D) One very large spot due to the presence of a droplet on the tip during spotting. Also visible are magnetic beads (brown color) and an inhomogeneous matrix layer due to changed evaporation conditions (lower organic solvent concentration).

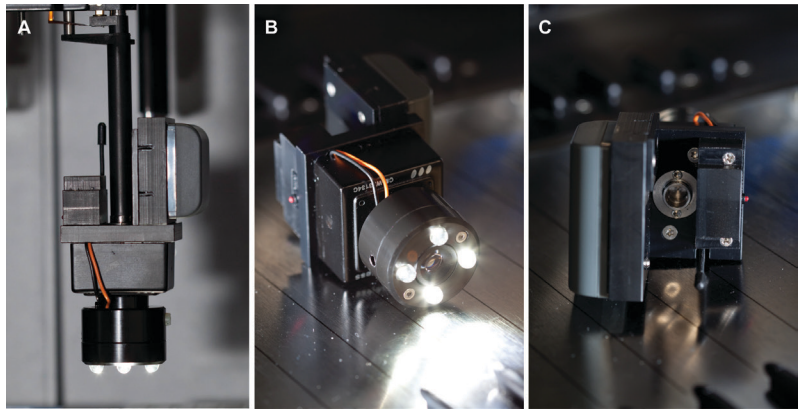

Figure 4. Wireless camera mounted on a pipetting channel (A) top-view showing the CORE-head, battery and switch (B) and bottom view showing the LEDs (C).

Theoretically it would be possible to check the quality of the spots and the envisaged quality of the spectra by imaging and automated evaluation of the images. For this purpose it would be possible to use a camera mounted on the Hamilton STARplus, as described in an application note on the Hamilton website (http://www.hamiltonrobotics.com/fileadmin/user_upload/prodb/app_notes/Genomics/BR-0506-01-EasyPick_ColonyPicking.pdf). However, this is a serious investment. Therefore, an affordable system was developed which, in addition, is not fixed to the robot, but is placed on the deck as separate labware. To implement this, a commercially available wireless mini camera was mounted in a frame, together with a battery and LEDs for illumination. To grab the camera the Hamilton's CORE technology was used. For this purpose a CORE head, similar to a tip-head, was also included in the frame. Furthermore, a switch was integrated which activates the camera when this is picked up and stops it when placed back on the deck (Figure 4). So far, no software is available which allows automated image evaluation, but we foresee that general software for this purpose will become available in the future.

Comparison of MALDI-FTICR profiles obtained from magnetic beads-based SPE or cartridge-based SPE. The same samples from the reproducibility test described above were also processed with C18 magnetic beads. Here, the comparison of the bead-processed samples with the cartridge-processed samples is based on MALDI-FTICR profiles, although similar profiles were obtained using MALDI-TOF-MS (data not shown). Recently, we have reported on the beneficial effects of ultrahigh resolution MS in terms of determined mass measurement errors and alignment of data [16]. Typical examples of these precision profiles are depicted in Figure 5a, in which a so-called low mass and high mass spectra are shown. The comparison of eluates from C18 magnetic beads to those of C18-EC cartridges is illustrated in Figure 5b, where peak area CVs (four samples, eight independent workups each) are given for a set of known peptides (same as in Figure 2). At a first glance, especially the low mass region seems different between the two SPE protocols. However, upon careful analysis it was found that on average the same peptides are captured by both the cartridges and the magnetic beads, although for some peptides in a different ratio. For instance, *m/z* 1465.7 and 1616.7 were captured to a higher

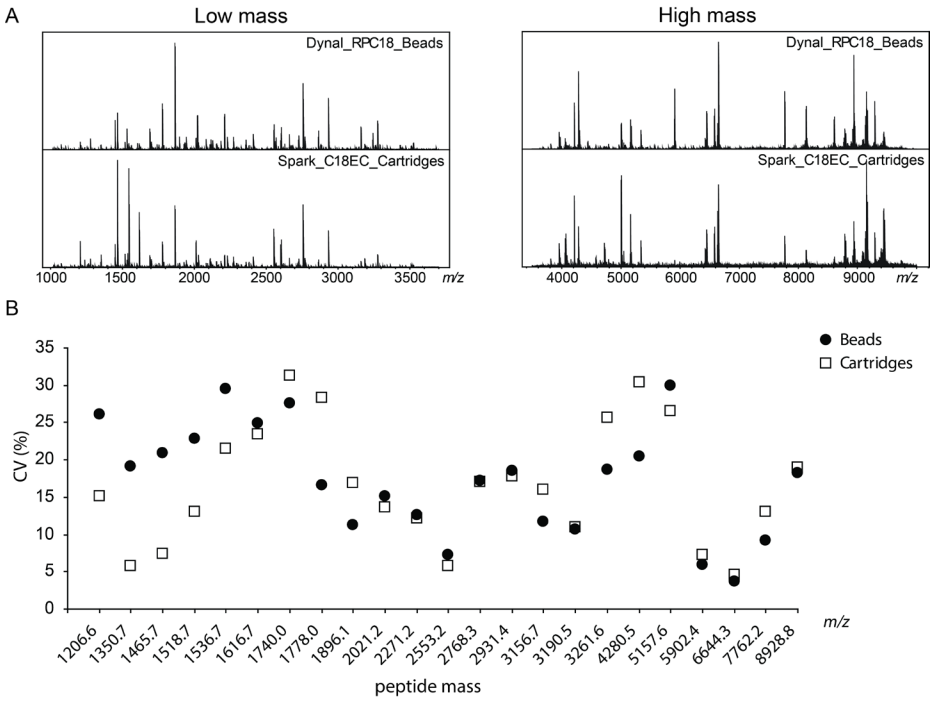

Figure 5. Comparison of magnetic bead-based SPE with cartridge-based SPE. Spectra (A) and average normalized intensities (B). Peptides used for this analysis are the same as in Figure 2.

extent by the cartridges, while *m/z* 2021.2 and 3156.7 were isolated in higher amounts by the magnetic beads. Similarly, in the high mass region differences were observed for the signal at *m/z* 5905.4. From Figure 5b it becomes clear that the peak area CVs are often similar for both the magnetic beads and the cartridges. Note that for some of the peptides the two workup protocols yield results with different efficiency and reproducibility. For these peptides the less efficient method usually exhibits the largest variation. It is obvious that biologically relevant differences should be considered with regard to the determined CV of each specific peptide.

In summary, precision profiles obtained from the same sample after work-up using the here described two SPE-protocols show a large amount of overlap in a qualitative analysis, *i.e.* the same peptides are captured by both procedures. From these results we conclude that the C18 cartridges provide a suitable alternative for C18

magnetic beads. Nevertheless, for some species large differences in the absolute amounts (ratios) are observed. Whether or not these intensity differences have an effect on classification of samples of specific patient cohorts is currently under investigation.

CONCLUSIONS

The implementation of the sample preparation processes on automated liquid handling platforms, as described in this paper, allows to both speed up the analysis of a large number of samples but also to eliminate errors. Apart from the throughput, the latter aspect is essential for application in clinical diagnosis where standardization and quality control (QC) are pivotal in meeting good laboratory practice (GLP) requirements [25]. In combination with the previously described automated MALDI-target loading and the high-throughput MS-based methods developed in our group this workflow is a next step towards implementation of proteomics in a clinical setting [26;27].

In this paper, two sample preparation techniques based on SPE on a highly automated platform are described. It was found that the cartridge-based Symbiosis system is a suitable alternative for the magnetic bead-based sample preparation and cleanup. In view of the fact that the production of specific types of beads has been discontinued this is an important aspect. Furthermore, the use of cartridges increases flexibility in sample preparation, since the cartridges can be packed with virtually any desired solid phase material. The peptides captured with C18-SPE material by both techniques are similar, although the capture efficiency may differ for each peptide. Furthermore, it was shown that the standard deviations for both the magnetic beads and the cartridges were very similar in inter- as well as intra-sample comparisons. As discussed in this paper, the CV of the intensity of a certain peak in magnetic bead profiles was often well below 10% when processing biological serum sample replicates, and on average about 15%. Based on the results presented here we expect this to be the same for the cartridges, which requires further investigation with larger sample cohorts.

SUPPLEMENTAL INFORMATION

A movie of the CORE-Camera in action can be found on http://www.lumc.nl/con/1040, then navigate to 'biomolecular mass spectrometry/Mass spectrometry-based clinical proteomics/methods'.

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