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Miniaturized Solid-Phase Extraction and Sample Preparation for MALDI MS Using a Microfabricated Integrated Selective Enrichment Target

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A microfabricated proteomic sample preparation and sample presentation device, Integrated Selective Enrichment Target, (ISET), comprising an array of 96 perforated nanovials is described. Each perforated nanovial can be filled with solid-phase extraction media for purification and concentration of peptides prior to matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS). The validity of the ISET sample preparation is shown by analysis of low nM-pM standard samples, as well as biological samples. The ISET solid-phase extraction sample preparation was compared to ZipTip and MassPREP PROtarget sample preparation, demonstrating a superior performance with respect to number of detected peptides and signal intensity of detected peptides.

Keywords: solid-phase microextraction • SPME • solid-phase extraction • SPE • mass spectrometry • proteomics • integrated selective enrichment target • ISET • MALDI MS

Introduction

Proteomics is highly dependent on biological mass spectrometry for scientific progress[1] and consecutively the success of mass spectrometry analysis relies on the quality of the preceding sample preparation steps. Solid-phase extraction (SPE) as a sample preparation step prior to biological mass spectrometry has gained huge importance, as these techniques (MALDI MS, ESI MS) require pure and concentrated samples in order to provide high sensitivity analysis. While there are many sample preparation methods that can be used to purify and concentrate samples prior to mass spectrometry, such as liquid chromatography, liquid−liquid extraction, dialysis or capillary electrophoresis, SPE remains one of the most used sample preparation techniques. The reason for this is probably the power of simultaneous sample cleanup and trace enrichment, together with the relatively high throughput that can be obtained by automation, short sample processing times and parallelization. An excellent review on the subject of SPE has been written by Gilar et al.[2]

In modern proteomics research one of the challenges is to effectively concentrate and purify microliter volumes of dilute samples, while ensuring minimal absorptive losses and maximum recovery in as small an elution volume as possible. To achieve this miniaturization has become necessary. By miniaturization, it is possible to handle limited sample volumes, minimize undesired adsorption with small surface areas and increase the apparent reaction rate of chemical reactions. Also, miniaturization allows for massive parallelization of sample processing which can lower the cost of analysis.

The two main ionization techniques used in mass spectrometry of biopolymers are MALDI MS and ESI MS. The two techniques are in many respects complementary,[3,4] but their differences makes ESI MS more suited to on-line SPE. There has been a lot of work published on miniaturization of on-line SPE coupled to ESI,[5−12] as well as off-line approaches.[13] Over the past decade MALDI MS has become an increasingly important technique for biopolymer analysis,[14] especially as MALDI since many years back has developed into a tandem mass spectrometry technique. The off-line nature of MALDI MS makes it ideal for the combining with off-line SPE. The advantage of off-line SPE is that it is easier to accomplish a disposable high throughput processing device with small dead volumes. A study of the literature will reveal that there are numerous different strategies that can be used to combine MALDI MS with off-line SPE. The main ones can be summarized as follows: (i) micropipet tips packed with SPE media; (ii) microtiter plates with integrated SPE; (iii) MALDI target plates for on-target (on-probe) purification of samples; and (iv) miniaturized/microfabricated chip based SPE technologies.

The micropipet tip based SPE began with tips developed in academic laboratories packed with chromatographic beads.[15,16] More recently, reports of SPE tips using monolith media[17] and pieces of SPE membranes[18] have been published. Today there

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are several commercial suppliers of different SPE tips, e.g., ZipTip (Millipore Corporation, Billerica, MA), SuproTip (The Nest Group, Inc, Southborough, MA), Omix pipet tips (Varian, Inc. Paulo Alto, CA), Eppendorf PerfectPure Tips (Eppendorf AG, Hamburg, Germany) and Agilent Cleanup C18 Pipet Tips (Agilent, Palo Alto, CA).

The microtiter plate based technologies such as the ZipPlate (Millipore Corporation, Billerica, MA) and the device presented by Nissum et al.,[19] where the SPE media is integrated in the bottom of each well offers a more an elegant solution to off-line SPE.

On-target purification (SPE) has been accomplished by modifying the surface of the MALDI target to promote binding of the analytes and allow contaminants to be washed away.[20−22] There are a large number of surfaces and strategies that can be used for on-target cleanup, as described in a review by Xu et al.[23] Examples of commercial on-target SPE technology include the protein chip used for SELDI analysis[24] (Ciphergen, Fremont, CA) and the recently presented MassPREP PROtarget (Waters, Milford, MA). The MassPREP PROtarget is a MALDI target that has a highly hydrophobic surface in order to enable the loading of volumes of 10 μL sample/position with a small binding area on each sample spot.

On-chip miniaturization of SPE has been demonstrated for many analytical applications.[25−29] The coupling of microfabricated SPE devices to MALDI MS has previously been presented by our group.[30,31] Our approaches along this line were initially based on pressure driven (flow injection systems) microfluidics,[30] which later were abandoned due to the difficulties of automation, multiplexing, concerns regarding carry-over effects, and the limited lifetime of such devices. The further developments in our group targeted a self-pumping capillary-flow driven system that circumvented the problems of pressurized pumping and yet provided a high analytical sensitivity.[32] An alternative and innovative way to solve the fluid transport problem in solid-phase extraction was commercialized by Gyros AB in the format of a microfluidic compact disk Gyro Lab MALDI SP1 (Gyros AB, Sweden), wherein centrifugal force was used to transport liquids for the purpose of SPE prior to MALDI MS.[33]

Other examples of SPE techniques that can be used in combination with MALDI MS are spin-columns, e.g., Vivapure C18 Micro spin columns (Vivascience AG, Hannover, Germany) or Agilent Peptide Cleanup C18 Spin Tubes. While effective for larger samples the spin-column approach is inherently not very well suited to small volumes and high throughput. Chromatographic beads can be used to concentrate and purify samples by incubating the beads directly with the sample solution, followed by a washing step, elution and spotting of the eluant onto a MALDI target.[34] A simplified approach for SPE where the beads with bound analytes were transferred onto the MALDI target followed by addition of matrix for elution on-spot has been suggested by Gevaert et al.[35,36] One example of a commercial approach to this methodology, that can be used for SPE, is the Bruker ClinProt system (Bruker Daltonics Inc., Bremen, Germany).

It is evident that any device used for high throughput SPE of biomolecules prior to mass spectrometry has to be able to rapidly and in parallel handle minute amounts of samples, while providing an efficient, economic and generic sample treatment process. The device also has to facilitate a high degree of automation, preferably using standard robotics, and a minimum of sample transfers. Finally, the device should be able to present the processed sample to the mass spectrometer in a way that avoids additional transfers and provides maximum sensitivity.

The Integrated Selective Enrichment Target (ISET) was conceived with the above requirements in mind. The main purpose of the ISET device is to maintain the benefits of miniaturization, while keeping the complexity of the analytical platform low. The ISET was realized as a silicon microfabricated MALDI target comprising 96 perforated nanovials that can be filled with extraction media (e.g., beads) for enrichment of biomolecules by SPE. The ISET device has previously been used for investigation of protein biomarkers in prostate disease.[37] In this paper, a thorough study of the basic sample preparation steps and the analytical performance of the ISET technology is reported, including a performance comparison with some of the commercially available SPE techniques. The inherent principle of ISET ensures a focusing effect of the eluting sample spots that will result in an amplification of the MS read-out signal.

Experimental Section

Chemicals. Unless otherwise specified all chemicals were purchased from Sigma-Aldrich. Co. (St. Louis, MO) and used without any further purification. Trypsin type IX, porcine pancreas was from Promega (Madison, WI), Poros R2 50, 20 and 10 μm beads were from Applied Biosystems (Foster City, CA).

In-Solution Digestion. Alcohol dehydrogenase (ADH) and casein (α-CAS) 100 μM in 50 mM NH_4HCO_3, were digested with trypsin, (1:40 enzyme:substrate) at 37 °C for 4 h. Dilutions to applied concentrations were made from stock solutions containing 2 μM digested protein.

In-Gel Digestion. Approximately 50−100 μg of cell lysate was separated by 2-DE. The isoelectric focusing (IEF) step was performed in an IPGphor (Amersham Pharmacia Biotech) and the second-dimension was carried out on 14% homogeneous Duracryl slabgels. The proteins were visualized with silver staining, excised and subjected to in-gel digestion in 50 mM NH_4HCO_3.[38] After in-gel digestion of a protein spot the resulting peptide solution was acidified through addition of TFA prior to the extraction/sample cleanup. For the comparison, experiments using real samples the same spots were picked from 3 identical gels and pooled.

ISET Manufacturing. ISET targets were manufactured by standard chemical wet etching of 360 μm thick ⟨100⟩-silicon wafers. Briefly, the wafers were oxidized for 8 h to provide a 1 μm thick oxide layer. A pattern defining an array of 96 466 × 466 μm squares was transferred to the wafer by UV photolithography and subsequent oxide layer etching in buffered HF. The wafer was subsequently etched in KOH, resulting in pyramidal pits (54.74° angle), that stopped when the pyramid was fully developed. After reoxidizing the wafer to full oxide thickness, 1 μm, the oxide on the backside of the wafer was removed, whereafter the anisotropic etching was resumed until the pyramids penetrated the wafer. This last etch step was stopped when the through-holes were 15 μm ± 2 μm, resulting in a 310 μm thick wafer. Each nanovial defined a volume of 48 nL. The ISET described herein had 96 perforated nanovials with the same pitch as spot positions on a standard Waters MALDI target and was diced from the silicon wafer in a format that allowed fitting into a stainless steel holder, made by milling down an ordinary target 320 μm, for insertion into a MALDI LR instrument, Figure 1.

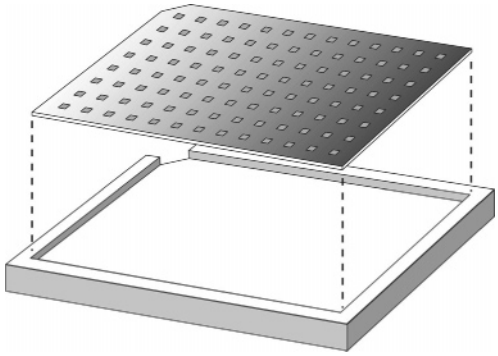


Figure 1. Stainless steel MALDI MS adaptor cassette for the microfabricated 96 position ISET.

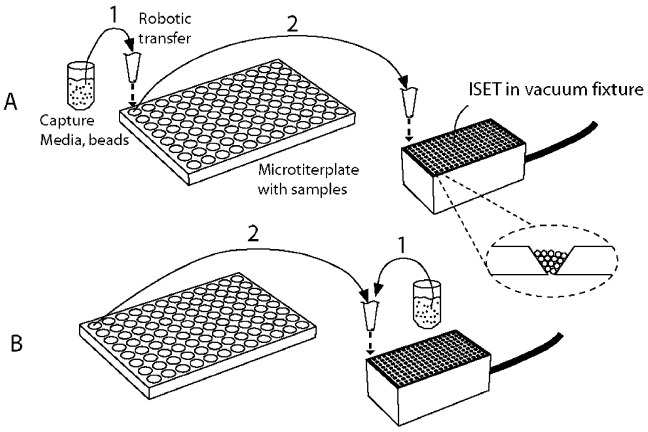


Figure 2. Beads and sample can be loaded into the ISET by applying a vacuum to the backside of the plate, either by (A) transferring the sample while bound to the beads, or (B) by first adding the beads to the perforated nanovial and subsequently aspirate the sample through the bead in the nanovial.

ISET Sample Preparation for Solid-Phase Extraction of Peptides. The core of the ISET is that the fluid handling in each analysis position is performed straight through the plate. The beads, in this case POROS R2 beads, are trapped in the perforated nanovial in each position and fluid transport is realized by applying a uniform vacuum across the plate. This was accomplished by placing the ISET in an in-house made Plexiglass fixture. The vacuum was supplied by a vacuum pump (Vacuubrand GMBH, Wertheim, Germany), controlled and gauged with a valve (Qiagen vacuum regulator, product no. 19530) inserted between the vacuum pump and the fixture. Sample preparation for peptide mapping with the ISET was performed by two different sample protocols A or B, shown in Figure 2.

Protocol A, (Figure 2A). (1) A 1-μL portion of a suspension of Poros R2 beads in 50% ACN/0.1%TFA was added to the sample (acidified with TFA) while stored in an external container, e.g., a microtiter plate. The analytes (peptides) were allowed to bind for at least 30 min.

(2) The beads, with the captured analytes, were transferred to the ISET by aspiration of 5 μL sample/beads from the bottom of the sample container and loaded into the perforated nanovials under maximum vacuum, −600 to −800 mbar as measured at the gauge.

(3) Wash with 2 × 3 μL, 0.1% TFA, under maximum vacuum, followed by removing the ISET from the vacuum fixture and rinsing the entire backside with MQ.

(4) Elution of the analytes onto the backside of the ISET with 2 × 0.3 μL, 50% ACN/0.1% TFA containing 1 mg/mL of cyano-4-hydroxy-cinnamic acid and ACTH 18−39/μL (internal calibrant). The elution was done at a lower vacuum, approximately −100 mbar.

Protocol B, (Figure 2B). (1) Each perforated nanovial was filled with approximately 40 nL Poros R2 beads in 50% ACN/0.1%TFA, at maximum vacuum.

(2) A 5-μL portion of acidified sample was applied to each position and drawn through the bead volume by applying maximum vacuum.

(3) Wash with 2 × 3 μL, 0.1% TFA, under maximum vacuum, followed by removing the ISET from the vacuum fixture and rinsing the entire backside with MQ.

(4) Elution of the analytes onto the backside of the ISET with 2 × 0.3 μL, 50% ACN/0.1%TFA containing 1 mg/mL of cyano-4-hydroxy-cinnamic acid and ACTH 18−39/μL (internal calibrant). The elution was done at a lower vacuum (approximately −100 mbar).

The ISET was reused after each analysis by washing away beads with a paint brush under running water, followed by three washes in 50% ACN/0.1% TFA and a final wash in the vacuum setup with 100% ACN.

The POROS R2 bead suspension used was prepared by adding beads to a wetting solution of 50% ACN/0.1% TFA. To get a homogeneous size of the beads, the suspension was shaken and allowed to settle until the supernatant was lightly cloudy. The supernatant was then discarded and new wetting solution was added. This process was repeated three times. Prior to use the bead suspension was diluted in such a manner that 1 μL of the suspension contained enough beads to fill an entire nanovial.

Comparison Studies. Samples of α-CAS or ADH were prepared by dilutions to specified concentrations with 50 mM NH_4CO_3/0.5% TFA immediately prior to use and aliquots of 25 μL were placed in microtiter plates. The samples to be compared were then chosen randomly and all sample handling was performed by careful manual pipetting. For the ISET, the sample preparation scheme described in Figure 2A was used. ZipTip$_{\mu-C18}$ and MassPREP PROtarget (Waters, US) sample preparation was performed according to the manufacturers specifications.

Briefly, for the ZipTip$_\mu$-C18 wetting was done by aspirating and dispensing 2 × 10 μL of 100% ACN, followed by equilibration with 2 × 10 μL 0.1% TFA. A minute volume (1 μL) of equilibration liquid was left in the ZipTip to avoid dewetting of the capture media. Binding of analytes was done by 10 aspirating and dispensing cycles of the sample, followed by washing with 2 × 10 μL 0.1% TFA. Elution of bound analytes from ZipTips was done directly onto a standard stainless steel MALDI target by slowly dispensing 1 μL of 50% ACN/0,1%TFA containing 5 mg/mL of α-cyano-4-hydroxy-cinnamic acid. Extra care was taken to avoid air bubbles in the elution. For the MassPREP PROtarget the protocol suggested for high sensitivity was used, i.e., the target spots were conditioned by first applying 1 μL ACN, followed by addition of 2 μL 0.1% TFA to avoid dewetting before applying the sample solution. The samples used for the MassPREP PROtarget were prepared as to contain 30% ACN. Washing was performed by adding and removing 4 × 5 μL 0.1% TFA to each spot. After drying 1 μL of 90% ACN/0.1% TFA containing 0.1 mg/mL of α-cyano-4-hydroxy-cinnamic acid was applied. All solutions used in the comparative studies were prepared daily as common stock

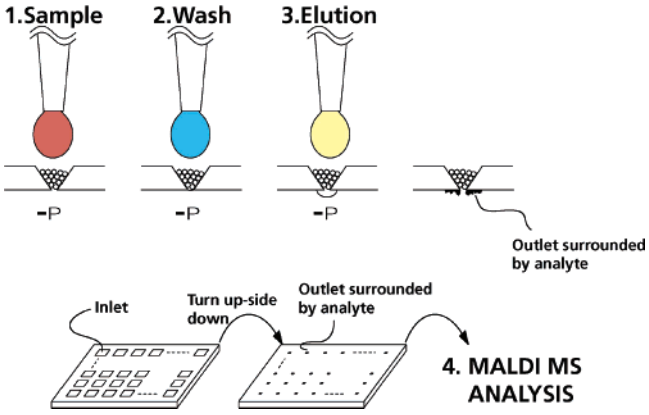


Figure 3. Workflow when using the ISET, sample (1), wash (2) and elution fluid (3) is drawn through the media by applying vacuum to the backside of the ISET. After elution, the analytes form a spot (0.5−1 mm) in an analysis zone surrounding the outlet. In the readout step the ISET is turned up side down making the analyte positions accessible for laser desorption/ionization in the MALDI MS instrument (4).

solutions. Mass spectra was acquired the same day by the same operator using identical instrumental settings, only the laser was allowed to be fine-tuned.

MALDI MS Analysis. The MALDI MS analysis was performed on an MALDI LR (Waters) running Masslynx 3.5/proteinlynx 1.1. All spectra were acquired manually, as an average of approximately 100 shots. The peptide mass fingerprinting[39−43] (800−3000 Da) was made in reflector mode. In the peptide mapping experiments internal calibration was performed by the software on ACTH 18−39 (2465.199 Da) or the peptide fragment (2211.104 Da) originating from autodigestion of trypsin. The protein identification was performed using the ProteinLynx 1.1 software and a local copy of Swiss-Prot. The database searches allowed for 50 ppm mass accuracy, carboxamidomethyl cysteine, oxidation of methionine residues, one missed cleavage site and species was set to human.

In the evaluation and comparison experiments with standard samples, the monoisotopic masses and corresponding intensity from the acquired spectra were exported as mass-intensity text files. These text files were then analyzed with an in-house developed Labview program that quickly provided the number of occurrences, mean intensity, and standard deviation of the intensity for each observed peptide peak throughout the collected mass spectra.

Results and Discussion

Principle of ISET Sample Preparation. The ISET technology offers an all-in-one-solution to biological/proteomic sample preparation by enrichment, cleanup and presentation to MALDI MS. The fundamental part of the ISET platform is the perforated nanovial (consisting of a pyramidal through-hole) with a 15 μm outlet hole. Functionalized beads are loaded into the nanovial and trapped, forming a packed bed, by means of conventional pipetting or in an industrial setting by means of high-throughput robotics. The principal of the ISET technology is briefly illustrated in Figure 3, where the beads are packed in an ISET nanovial, (1) sample is drawn through the SPE bed, or transferred to the nanovial while bound to the beads, and after washing (2) the sample is eluted (3) with matrix solution onto the ISET backside via the applied under pressure across the plate. The ISET is subsequently loaded into the MALDI MS instrument facing the backside up and each MALDI spot is analyzed (4).

Microfluidic Characterization. In a first experiment, the elution properties of the ISET sample preparation were investigated by applying 50 fmol ADH digest to 48 positions (4 rows of perforated nanovials) in the ISET. Elution was performed by stepwise applying 0.3 μL elution liquid (50% ACN/0.1%TFA), i.e., the first row (12 samples) of ADH digest was eluted with 0.3 μL, the second row with 0.6 μL (2 × 0.3 μL), the third row with 0.9 μL (3 × 0.3 μL) and the fourth with 1.2 μL (4 × 0.3 μL). To keep the spot size and amount of matrix as equal as possible, only the first 0.3 μL of elution liquid contained matrix and the elution liquid was allowed to dry before each new addition.

The acquired spectra were subsequently analyzed with regard to the number of observed peptides and their signal intensity. It was found that most of the analyte is eluted in the first 0.3 μL and that a second elution, while not providing any additional peptide peaks, leads to an overall increase in intensity of the observed peaks.

To confirm this elution profile, another strategy was employed where the first elution/elutions were washed away from the analysis zone surrounding the outlet with 100% ACN. In this experiment, all elutions contained matrix in the elution liquid. This way, the first row of samples consisted of the first 0.3 μL, the second row had the first 0.3 μL washed away and contained the analytes from the second elution (0.3−0.6 μL), and the third row contained the third elution (0.6−0.9 μL). Again, the first elution contained most of the analyte, while the second elution lacked some peptide peaks which were observable in the first elution (i.e., those of low intensity in the first elution) and the observed overall intensities were ca. 20−30% of those observed in the first elution. In the third elution, 4 of 12 spots provided low intensity peaks from one or two peptides, which had been observed at very high intensity in the previous elutions.

At higher analyte loading (200 fmol), peptides were observed in the third and fourth elution, but in these cases, the intensity of the peptide peaks in the first two elutions were so high (some at detector saturation) that this loss of sample was of no great consequence for the analysis result.

Due to the "semiquantitative" nature of MALDI MS these experiments do not provide sufficient information to specify the recovery of the ISET sample preparation. To quantitative measure of the recovery additional experiments with isotopically labeled peptides need to be conducted.[44] However, the results indicate that any losses are relatively negligible in respect to the analysis results, and thus an elution protocol with two elutions of 0.3 μL, both containing matrix at a lower concentration, was adopted.

Sample losses due to unspecific absorption and incomplete elution are of course unavoidable during solid-phase extraction and sample handling. While the design of the ISET sample preparation is such that sample transfer steps are minimized and the short path length (and total surface exposure) of the device minimizes the unspecific absorption, there are still room for improvements. There are two areas that we are currently addressing to improve the observed losses. One is the pyramidal shape of the nanovials, as this geometry allows for a less restricted fluid flow at the edges of the pyramid. The other is the fact that when the first 0.3 μL of elution liquid is applied to the inlet of a perforated nanovial analytes are immediately desorbed from the capture beads, and even though the elution

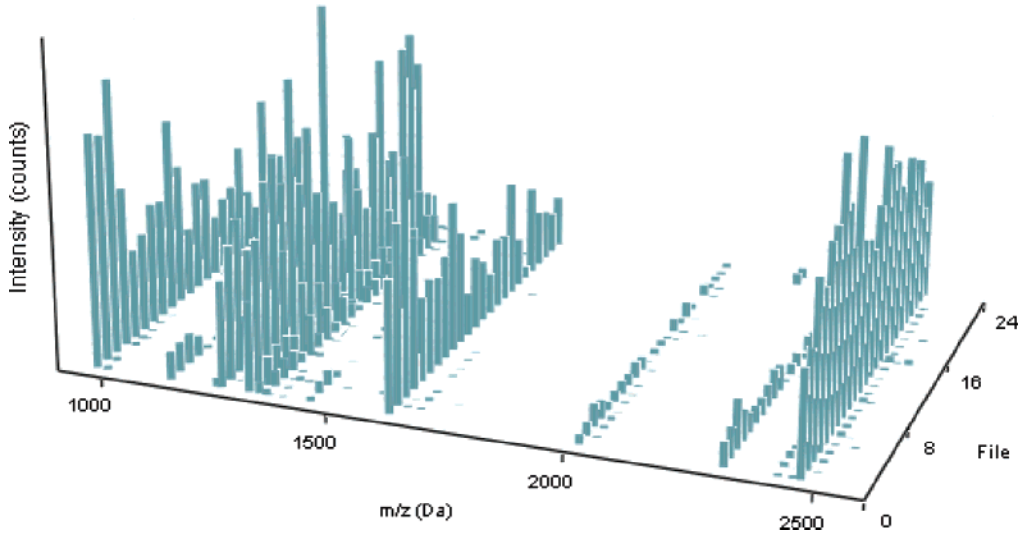


Figure 4. 3D representation of 24 MALDI MS spectra obtained after ISET solid-phase extractions of samples containing 25 fmol ADH digest.

liquid is instantly drawn through the nanovial some crystallization takes place around the edges and in the nanovials.

Influence of Sample Loading Strategy. Two different sample loading strategies for the ISET sample preparation were investigated, as described in Figure 2. One strategy where the analytes were transferred to the perforated nanovials while bound to the beads (Figure 2A) and another where the perforated nanovials were pre-filled with beads and the analytes were captured on the beads by aspirating the sample through the bead volume (Figure 2B). To conclude which strategy provided the best analysis results, 48 samples of 25 fmol (5 μL of 5 nM) ADH digest were analyzed with each strategy using POROS R2 50 beads. Analysis of the resulting spectra revealed that the sensitivity was significantly higher, when the analytes were transferred to the perforated nanovials while bound to the beads, i.e., path A in Figure 2. With an average of 11 peptide peaks originating from ADH compared to 8 peptides in average for the strategy where sample is drawn through the bead volume for analyte capture (path B, Figure 2). The signal intensity variations for the peptides when the beads were incubated with the sample for analyte capture prior to loading into the ISET (path A, Figure 2) were also lower. Figure 4 shows a 3D representation of the resulting spectra obtained from 24 different positions, using the strategy where sample was transferred while bound to beads.

One reason for the difference between the two strategies is probably that the sample flow through the pre-filled perforated nanovials varied. In some nanovial positions the samples passed straight through the bead volume in less than 10 s and for a few others it took minutes. This stresses the importance of a perfectly packed column with homogeneous flow resistance for reproducible results, when analyte binding is done in flow-through fashion. By using smaller beads (POROS R2, 20 and 10 μm) the difference in flow resistance was reduced and reproducibility slightly improved, but for approximately 5−10% of the nanovial positions the maximum obtainable vacuum, with the used setup, was not sufficient to draw the aqueous (high surface tension) sample solution through the nanovials. Another reason for the lower performance of pre-filled perforated nanovials was that the beads are dewetted between loading of the beads and application of the sample, leading to decreased binding of the analyte. Neither of these two phenomena will affect the strategy where the analyte is transferred while bound to beads.

Transferring the analytes to the perforated nanovial while bound to beads has a number of advantages;

1. With pre-filled perforated nanovials no more than 5 μL sample solution can be added at once. Using transfer of the analytes while bound to beads is not as restricted by the initial sample solution volume, as "all" of the analytes are delivered to the perforated nanovial by aspirating 5 μL liquid from the bottom the incubation volume holding the bead sediment. Naturally, some residual beads are left in the sample solution container, but this strategy still provided the highest sensitivity.

2. The analytes are absorbed to the beads in their original compartment and additional analyte losses by unspecific absorption are avoided. This aspect becomes more important with decreased analyte concentration.

3. When performing solid-phase extraction of in-gel digested 2-DE spots the beads can be added directly to the sample solution after digestion (still containing the gel plug). Unconfirmed results indicate that this provides better recovery of analytes than transferring the digest supernatant and following extractions of the gel plug to a second microtiter plate.

On the basis of this rationale, the sample preparation strategy of transferring the analytes to the perforated nanovial while bound to beads should be used, when maximal sensitivity is of importance. Figure 5 shows the spectra resulting from (A) ISET solid-phase extraction of 5 μL from an in-gel digest of Actin using pre-filled perforated nanovials and (B) ISET solid-phase extraction by adding beads to the remaining digest solution of 25−30 μL containing the gel plug.

Influence of Spot Size. As desorption/ionization in MALDI MS is performed with a laser having a spot size of 100−200 μm, it is desirable to have as high analyte concentration as possible in the area that is irradiated. The fact that a small spot

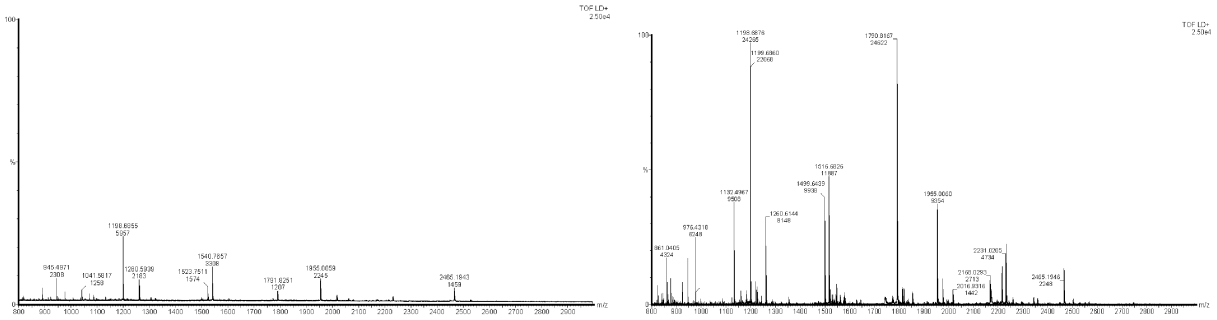


Figure 5. Spectra resulting from (A) ISET solid-phase extraction of 5 μL from an in-gel digest of actin using pre-filled perforated nanovials and (B) ISET solid-phase extraction by adding beads to the remaining digest solution of 25−30 μL containing the gel plug. Note that the scaling of the *y*-axis is normalized so that 100% corresponds to 25 000 counts in both spectra.

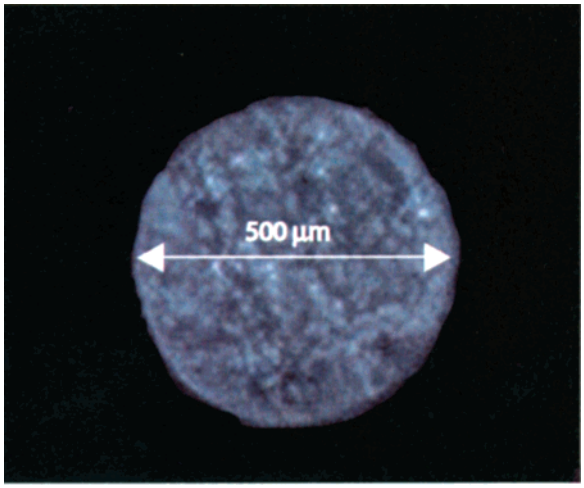
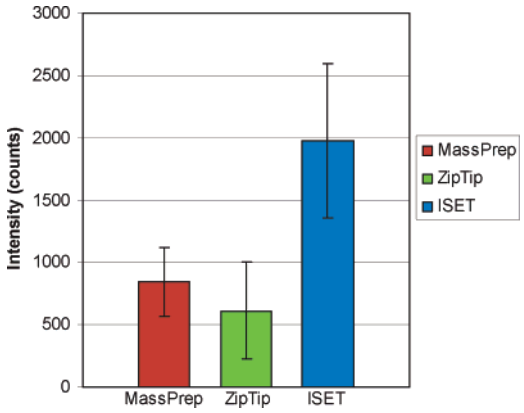


Figure 6. Matrix spot generated by ISET sample preparation using α-cyano-4-hydroxy-cinnamic acid matrix.

size is beneficial to MALDI MS analysis is well-known.[45,46] Several methods can be applied to ensure a small spot, e.g., drop on demand dispensing,[47−50] hydrophilic anchor point on a hydrophobic target[51] deposition on a hydrophobic target,[52] capillary deposition[53−56] or electrospray deposition.[57,58]

During ISET sample preparation the spot size is determined by the amount of matrix/elution liquid that is added, the composition of the elution liquid and the speed (vacuum level) which is used to draw the elution liquid through the nanovials. By controlling the vacuum and using 50% ACN/0.1%TFA as elution liquid, a spot size of 0.5−1 mm was obtained, Figure 6. An ordinary dried-droplet sample preparation gives a spot size having a diameter of 2.5 mm, thus the analytes are distributed on an area of 4.9 mm^2. The spot size of the samples eluted from ZipTips was approximately 2 mm in diameter, i.e., 3.1 mm^2. The binding motif (Activewell) on the MassPREP PROtarget is specified to be approximately 1 mm^2, but the spots resulting from adding 1 μL of 90% ACN/0.1% TFA to the MassPREP PROtarget were not confined to this well and the estimated average spot size was closer to 1.5 mm in diameter, corresponding to an area of 1.8 mm^2. The ISET sample spots were between 0.5 and 1 mm in diameter, assuming an average spot size of 0.75 mm gives a sample area of 0.4 mm^2. Thus, the analytes are approximately 4 times more concentrated in the ISET spot, as compared to a MassPREP PROtarget spot. Assuming that a spot contains 50 fm of analyte, a MassPREP PROtarget would have an analyte density of 28 fmol/mm^2 and an ISET spot 113 fmol/mm^2.

Chart 1. Mean Intensity and Standard Deviation (n = 12) of the Internal Calibrant as Observed in an Experiment Comparing MassPREP PRO, ZipTip, and ISET Solid-Phase Extraction.



In experiments comparing the ISET to ZipTip$_\mu$-C18 and the MassPREP PROtarget, the same amount of internal calibrant was added to the elution/matrix solution, e.g., 25 fmol ACTH 18−39/μL. It was found that the intensity of the internal calibrant was higher in the ISET sample preparation than the ZipTip and MassPREP PROtarget. In the MassPREP PROtarget and ZipTip sample preparation, 1 μL was used for elution, i.e., 25 fmol of ACTH 18−39. With the ISET approximately 0.6 μL of elution solution was applied, corresponding to 15 fmol ACTH 18−39.

In Chart 1, the mean intensity and standard deviation (n = 12) of the internal calibrant, while analyzed with the three different SPE techniques is presented, as can be seen the ISET provides the highest intensity, despite having 40% less internal calibrant in each spot. This phenomenon is believed to mainly be a function of spot size, as the internal calibrant is included in the elution liquid and not subject to solid-phase extraction. Also, the signal of other peptides present in the sample was more intense using the ISET sample preparation and thus the higher intensity of the internal calibrant is not due to ion suppression phenomena.

Matrix Crystallization. Another factor that contributes to the sensitivity of the ISET sample preparation might be the matrix crystallization conditions. As the matrix is included in the elution liquid and drawn through the perforated nanovial by means of vacuum, the crystallization is done in a low vacuum environment that affects the crystallization process.

In an experiment, spots generated from 0.3 μL of elution liquid (50 fmol ACTH 18−39/μL in 50% ACN/0.1% TFA con-

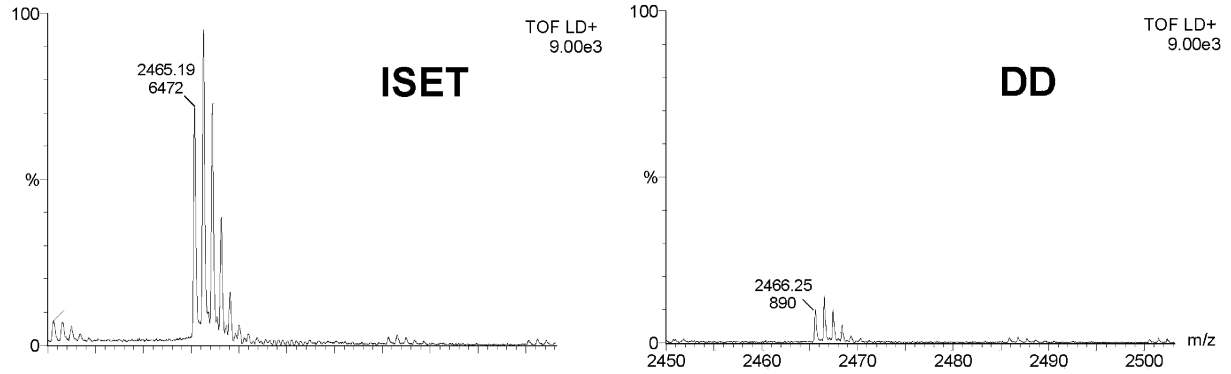


Figure 7. Spectra generated from analysis of 0.3 μL 50 fmol/μL ACTH 18−39, prepared by ISET sample preparation and dried-droplet sample preparation. Spot size was similar approx 0.75 mm in diameter. Note that scaling of the *y*-axis is normalized to 9000 counts = 100%.

taining matrix) having passed through empty perforated nanovials and allowed to crystallize in a vacuum conditions, were compared to spots generated by depositing 0.3 μL of elution liquid directly on the backside of the ISET as a dried-droplet. The resulting MALDI spots were of similar size, but the observed intensity for the ISET sample preparation was 3−6 times higher compared to the dried-droplet spots, see Figure 7.

The matrix crystals resulting from ISET sample preparation were more homogeneous than those resulting from dried-droplet sample preparation. It has previously been shown that vacuum deposition provides favorable crystallization conditions[59] and in this perspective, it was concluded that the vacuum conditions for the crystallization on the ISET were equally favorable.

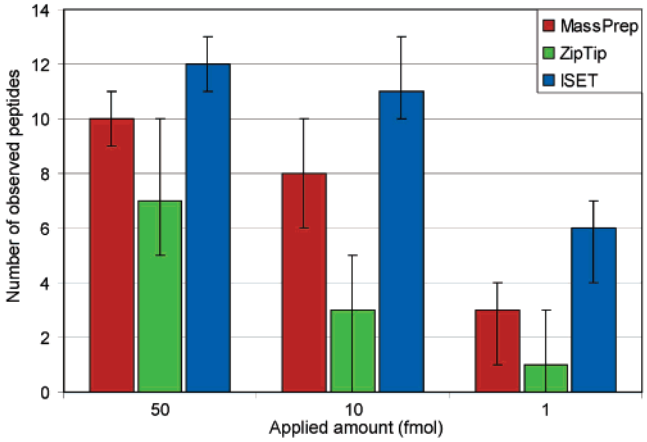
Comparasion with ZipTip$_{\mu-C18}$ and MassPREP PROtarget. Evaluation of SPE performance from MALDI MS result is difficult due to phenomena such as ion suppression[60] and large signal variations (hot-spots). Therefore, the presented experiment should be viewed from the relative outcome of the comparison, rather than the exact applied concentrations and amounts.

Using the ISET sample preparation with incubation of beads in the sample solution, the ISET sample preparation was compared with ZipTip$_{\mu}$-C18 and MassPREP PROtarget sample preparation. In one experiment, a α-Casein digest, 25 μL/sample in 50 mM NH_4HCO_3 was analyzed at three different concentrations, 2 nM, 0.4 nM and 0.04 nM. As 25 μL was used this corresponded to total loads of 50, 10 and 1 fmol analyte applied on the beads. The spectra were then analyzed with respect to the average number (n = 12) of observed peptides originating from α-Casein, Chart 2. For an analyte load of 50 fmol (2 nM) an average of 10 peptides originating from α-Casein was observed for the MassPREP PROtarget, ZipTip$_{\mu}$-C18 gave an average number of 7 peptides and ISET provided an average of 12 peptides. The 2 nM sample solution was the only one in which peptides could be observed using dried-droplet sample preparation (average of 2).

At a load of 10 fmol (0.4 nM) the MassPREP PROtarget provided an average of 8 peptides, ZipTip$_{\mu}$-C18 gave an average number of 3 peptides and ISET provided an average of 11 peptides.

Using the lowest concentration 0.04 nM (1 fmol total load) the MassPREP PROtarget sample preparation provided an average of 3 peptides, ZipTip$_{\mu}$-C18 gave an average number of 1 peptide and ISET provided an average of 6 peptides. The ISET solid-phase extraction provided the highest average number of peptides for the three applied amounts of α-Casein, Chart 2.

Chart 2. Mean (n = 12) Number of α-Casein Peptides Observed after MassPREP PRO, ZipTip, and ISET Solid-Phase Extraction Performed at Three Different Concentrations, Giving Total Loads of 50, 10 and 1 fmol. Note that Error Bars Show the Maximum and Minimum of Observed Peptides with Each Technique, and Not Standard Deviation.



The ISET also provided the highest (overall) signal intensity for the observed peptides, Figure 8 shows 5 spectra each from ZipTip$_{\mu}$-C18, MassPREP PROtarget, and ISET and gives an impression of the difference and variation of the signal intensity obtained by the different sample preparation techniques.

Comparison was also made using (previously identified) in-gel digested 2-DE separated proteins. In this case, 10 μL of digest supernatant from each sample was processed with the three sample preparation techniques. In the case of the dried-droplet sample preparation, 1 μL of digest supernatant was used. The ISET provided the highest number of peptides from the respective protein, Table 1, and the highest (overall) signal intensity. Figures 9 and 10 show resulting spectra from Annexin and Heat shock 27. Note that the scaling of the mass spectra is normalized to show the difference in intensity obtained by the ISET sample preparation, which makes it difficult to see all of the observed peptides for the other SPE sample preparation techniques.

The reason for the better analysis result obtained by ISET sample preparation as compared to MassPREP PROtarget and ZipTip is a combination of the smaller spot size, more effective

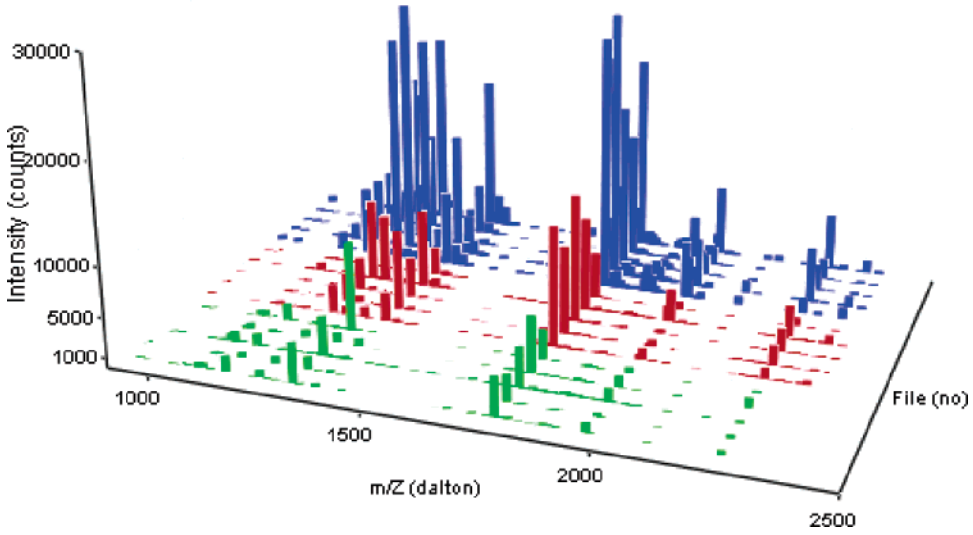


Figure 8. 3D representation of 15 mass spectra, 5 each as obtained by solid-phase extraction of 50 fmol α-Casein samples with ZipTip (Green), MassPREP PROtarget (Red) and ISET (Blue).

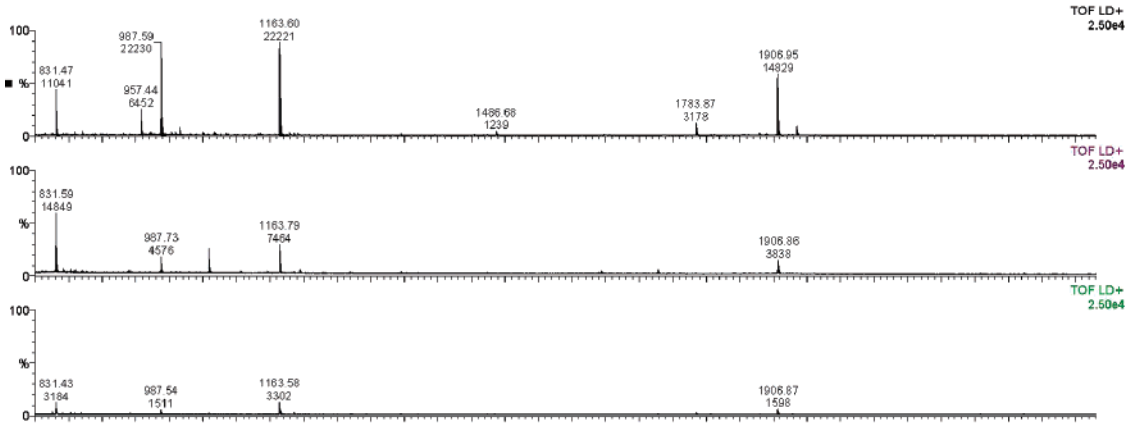


Figure 9. Resulting mass spectra from 10 μL of in-gel digested Heat shock protein as obtained after ISET (top), MassPREP (middle) and ZipTip (bottom), sample preparation. Note γ-axis scaling normalized so that 100% corresponds to 25 000 counts.

Table 1. Number of Peptides Identified for 10 In-Gel Digested Proteins with the Different Sample Preparations

accession	ISET	MassPREP	ZipTip	DD	description
ALBU_HHUMAN	27	18	17	17	Serum albumin precursor
TPM4_HUMAN	23	15	7	5	Tropomyosin alpha 4 chain
ANX5_HUMAN	22	15	9	5	Annexin A5
ACTG_HUMAN	21	14	8	8	Actin, cytoplasmic 2 (γ-actin)
LAMC_HUMAN	20	12	9	10	Lamin C
1433E_HUMAN	15	13	5	2	14-3-3 Protein epsilon
PDX2_HUMAN	12	9	4	4	Peroxiredoxin 2
ENOA_HUMAN	12	7	3	0	Alpha enolase
LEGI_HUMAN	11	8	4	3	Galectin 1 Beta galactos
HSPB1_HUMAN	9	6	4	0	Heat-shock protein beta-1

analyte capture by incubation and fewer sample transfers. Like the ZipTip and MassPREP PROtarget, the ISET sample preparation allows for SPE of sample solutions containing MALDI incompatible contaminants.

There are some fundamental differences (advantages as well as disadvantages) between the available SPE technologies. The advantage of the micropipet tip based SPE is that it is easy to automate using ordinary pipet robotics and relatively inexpensive, with a cost per sample of $2−3. The drawbacks are that there are many sample transfers, where sample can be lost and that the requirement of a low flow resistance (large pores) in the tips leads to a slow mass transfer of analyte to the SPE media and tip-to-tip variations. Thus, several aspiration dispensing cycles are needed and recovery (i.e. sensitivity) has been questioned for the ZipTips when small elution volumes are used.[44,61]

In the microtiterplate SPE sample preparation methodologies sample losses due to unnecessary sample transfers are minimized and this is claimed to result in higher sensitivities. Standard pipetting robotics can be used to perform most of

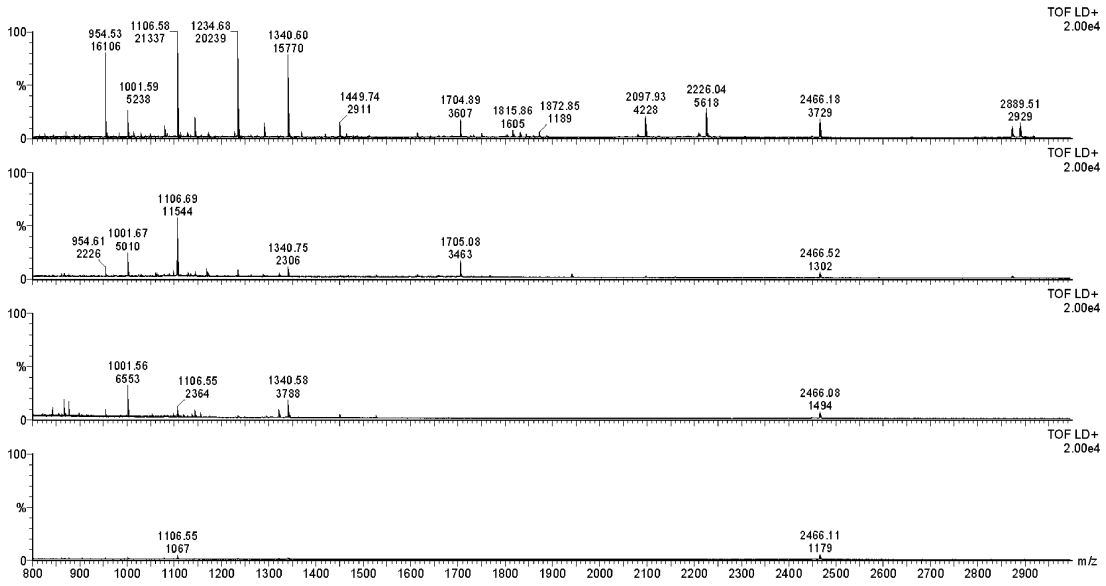


Figure 10. Resulting mass spectra from 10 μL of in-gel digested Annexin 5 as obtained after ISET (top), MassPREP (second down), ZipTip (third down) and dried-droplet (bottom), sample preparation. Note *y*-axis scaling normalized so that 100% corresponds to 20 000 counts.

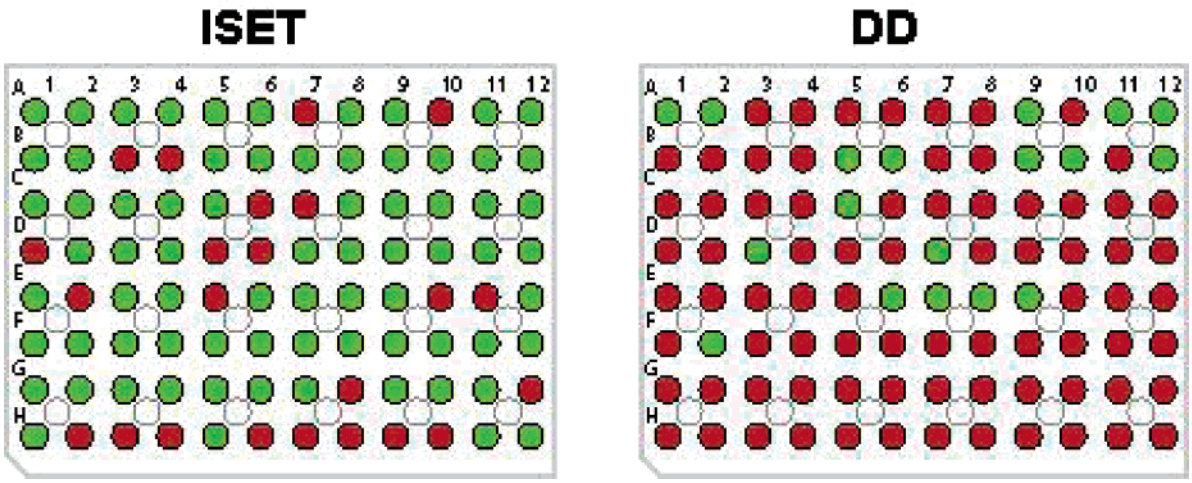


Figure 11. Analysis read-out after PMF of 96 in-gel digested proteins using ISET or Dried-Droplet sample preparation, green spot denotes significant hits and red no hit after the database interrogation.

the sample transfers and the sample can be eluted directly onto a MALDI MS target, of 96 well microtiter plate format, placed under the device. Although, special adaptations have to be made in order to facilitate the final elution onto the MALDI target. These devices are relatively new and there is not a lot of data published on their performance, but the methodology is sound and as long as the cost per analysis is kept low, the use of this type of devices can become as common as the tip based SPE approach.

For on-probe SPE the final elution step is avoided as elution is done by addition of the matrix solution to the spot, i.e., the recovery should be 100% unless analytes are lost during washing. A potential drawback of this approach to SPE is that it still requires many sample transfers to be performed leading to sample losses. It will also become important to avoid spreading of the sample spot when the matrix is added to the analyte spot as sensitivity will suffer with increasing spot size. Another concern which might be of less importance when the goal is purification/concentration of peptides from, e.g., in-gel digest is the limited capacity of a "planar" surface. This capacity deficient becomes more of a concern when the goal is biomarker discovery, where the sample may contain many different species of a broad concentration range.

While miniaturization has the prerequisites needed for optimizing the SPE process and provides the desired parallelization and sensitivity, it can often lead to quite complicated design solutions, which are expensive to produce, and require specialized robotics for sample delivery/transfers, as there often are problems of interfacing the microworld to the macroworld in the laboratory. The fluid transport mechanism is a fundamental issue in the design of a microsystem for solid-phase extraction and if array formats are desired parallel fluid handling systems are needed, which eventually makes, e.g., syringe driven systems obsolete. As a potential alternative solution, massively parallel chip integrated peristaltic pumping and valving has been demonstrated by Quake et al.[62] The ISET technology provides the ability to perform massively parallel

analysis in an array format and includes the advantageous aspects of both the microtiterplate and on-probe methods.

96 Samples Run on ISET vs Dried Droplet. The benefits of solid-phase extraction as a sample preparation step for in-gel digested proteins are well-known and can clearly increase the number of significant protein hits. In an experiment, 96 in-gel digested proteins were analyzed by dried-droplet sample preparation and after ISET solid-phase extraction sample preparation. In the case of dried-droplet sample preparation, 1 μL of the digest supernatant was deposited on a stainless steel MALDI target immediately followed by deposition of 1 μL matrix. For the ISET sample preparation, 10 μL of digest supernatant and transfer of sample while bound to beads was used. The difference in analysis result, using a 95% confidence limit, gave that ISET sample preparation provided significant hits for 73 of 96 samples, while the dried-droplet only provided significant hits for 18 of 96 samples, Figure 11.

Conclusions

The ISET sample preparation achieves a signal amplification that is superior to the compared commercial systems, e.g., MassPREP PROtarget MALDI targets or ZipTip solid-phase microextraction tips. Almost of as great importance as the higher sensitivity achieved by ISET sample preparation, is the ease of use. Even a first time user can manually prepare 96 samples, one ISET target, in less than 1 h.

The uncomplicated design of the ISET device provides several beneficial aspects, such as ease of fabrication with a high-density of array positions and interfacing to standard laboratory robotics. Another important aspect is the short path length in the ISET, which ensures minimal unspecific adsorption of the analytes on surfaces in the device.

The ISET device can be used in a number of generic applications, where the user chooses a suitable capture medium or several different (complementary) capture medium types, depending on the application. The ISET methodology was proven successful in the performance evaluation as a generic integrated microscale proteomic sample enrichment, cleanup, and MALDI MS analysis platform. Its ease of use and the qualitative results are currently driving our efforts to develop a low cost disposable version of the ISET for evaluation at large scale in proteomics laboratories.

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