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Ultrasonic Enhanced Applications in Proteomics Workflows: single probe versus multiprobe

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ABSTRACT

A 96-well plate-based platform in conjunction with an ultrasonic multiprobe of four tips was assessed to develop various fast proteomics workflows for gel-based proteomics. The use of such protocols reduce sample time and handling, allowing rapid processing whilst reducing the risk of contamination. The procedure reduces the time to indentify proteins separated by gel electrophoresis to just 8 min/each. In addition, the ultrasonic multiprobe was compared with the single probe as a tool to obtain high sample throughput in proteomics workflows entailing identification and/or quantification of proteins using mass-spectrometry based approaches. The 18-O labeling-based method was used to study the type of peptides extracted from the gels when the extraction was done with the aid of ultrasonic energy. The assessment was done in ten standard proteins separated by gel elecrophoresis. Two proteins obtained from *D. desulfuricans,* and from *Cyprinus carpio*, Split-Soret cytochrome c, and Vitellogenin respectively, were also indentified as a further proof-of-the concept.

Keywords: Ultrasonic, MALDI, Vitellogenin, ^{18}O, Inverse labeling.

1. Introduction

Ultrasonication has been recently appointed as a powerful tool in mass spectrometry-based proteomics workflows for protein identification [1-7]. Ultrasonic energy can be used to enhance from hours to minutes protein denaturation, protein reduction, protein alkylation and protein digestion, the four mais steps of any common procedure nowadays used for protein identification relaying on mass spectrometry. Furthermore, ultrasonic energy can also be used to speed protocols relaying on ^{18}O isotopic labeling, which is a widely used method to tracking changes in protein level expression as well as in sequencing of peptides by mass spectrometry-based techniques [6,7]. As a matter of fact ultrasonic energy has been recently integrated in rapid sample processing for ^{18}O-LC-MS-based quantitative proteomics [8].

Ultrasonic-based high throughput sample treatment for proteomics was recently reported for the treatment of liquid samples by joining a 96-well plate and an ultrasonic multiprobe. The present work shows a step forward of this protocol by applying it to proteins separated by gel-based approaches and also using it to study in an 18O labeling-based method the type of peptides extracted from the gels when the extraction is done with the aid of ultrasonic energy. The study was done through the identification of 10 standard proteins and two proteins obtained from *D. desulfuricans,* and from Cyprinus carpio, Split-Soret cytochrome c, and Vitellogenin respectively.

2. Material and methods

2.1 *Apparatus*

Protein digestion was done in a 96-well plate (Digilab-Genomic Solutions, USA). A vacuum concentrator centrifuge from UniEquip (Martinsried, Germany) model UNIVAPO 100H with a refrigerated aspirator vacuum pump model Uni-

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jet II was used for (i) sample drying and (ii) sample preconcentration. A minicentrifuge, model Spectrafuge-mini, from Labnet (Madrid, Spain), and a minicentrifuge-vortex, model Sky Line, from ELMI (Riga, Latvia) were used throughout the sample treatment, when necessary. A Simplicity™ 185 from Millipore (Milan, Italy) was used to obtain Milli-Q water throughout the experiments.

2.2 *Ultrasonic devices*

(i) Ultrasonic probe, model UP 100H (dr. Hielscher, Teltow, Switzerland, 200 W, 30 kHz ultrasonic frequency, 0.5 mm of diameter probe).

(ii) Ultrasonic multi-probe from Branson Ultrasonics Corporation (USA), model SLPe (150 W, 40 kHz ultrasonic frequency, 1 mm diameter probe). The ultrasonic generator SLPe is equipped with a multi-probe detachable horn (model 4c15), with four tips for simultaneous ultrasonication of four samples and it was used in conjunction with a 96-well plate, as it is depicted in video 1 of supporting information.

2.3 *Standards and reagents*

The following protein standards were used: α-lactalbumin from bovine milk (≥85%), BSA (>97%) and carbonic anhydrase (>93%) from Sigma (Steinheim, Germany), albumin from hen white (>95%) from Fluka (Buchs, Switzerland). Chymotrypsinogen A, catalase bovine and aldolase from rabbit were standards for gel filtration calibration kit high molecular weight from Amersham Biosciences (Piscataway, USA).

Low molecular weight standard protein mixture of glycogen phosphorylase b, bovine serum albumin, BSA, ovalbumin, carbonic anhydrase, trypsin inhibitor and α-lactalbumin were purchased from Amersham Biosciences (Piscataway, USA). Thyroglobulin and Lactate dehydrogenase were purchased from Amersham Biosciences (Piscataway, USA)

Carp vitellogenin standard was purchased from Biosense Laboratories (Bergen, Norway).

Trypsin enzyme, sequencing grade was purchased from Sigma. All materials were used without further purification. α-cyano-4-hydroxycinnamic acid (α-CHCA) puriss for MALDI–MS from Fluka was used as MALDI matrix. ProteoMass™ Peptide MALDI-MS Calibration Kit (MSCAL2) from Sigma was used as mass calibration standard for MALDI-TOF-MS.

The following reagents were used for protein depletion: sodium chloride puriss. p.a. and magnesium chloride hexahydrate puriss. p.a. were purchased from Fluka; ethylenediaminetetraacetic acid disodium salt dehydrate puriss. p.a. was from Riedle-de Haën (Seelze, Germany).

The following reagents were used for protein digestion: acetonitrile, iodoacetamide (IAA) and DL-dithiothreitol (DTT) (99%) were purchased from Sigma; formic acid and ammonium bicarbonate (>99.5%) were from Fluka; trifluoroacetic acid (TFA, 99%) were from Riedel-de-Haën (Seelze, Germany); and urea (99%) was from Panreac (Barcelona, Spain).

2.4 *Sample treatment*

2.4.1. Protein separation by 1D-SDS-PAGE

Amounts of protein ranging from 0.5 to 3.7 μg were dissolved in 5 μL of water plus 5 μL of sample buffer (5 mL of 0.5 M Tris-Base + 8 mL of 10 % SDS + 1 mL of β-mercaptoethanol + 2 mL of glycerol + 4 mg of bromophenol blue in a final volume of 20 mL in water) for analysis by sodium dodecyl sulphate polyacrilamide gel electrophoresis (SDS-PAGE) (10% 0.5 mm thickness). After gel electrophoresis (65 min, 120 V, 400 mA), the gel was stained with Coomassie blue R-250 and destained in order to visualize the proteins bands.

2.4.2. In-gel sample treatments

(i) Overnight method. For in-gel digestion optimization, 2.1 μg of BSA and 2.9 μg of α-lactalbumin were loaded onto 10% SDS-PAGE gels. Coomassie Blue-stained protein bands were excised from the gels, cut into pieces and subjected to digestion. Excised gel bands were then washed with water (3 times with agitation/centrifugation, 10min each), and dehydrated with acetonitrile (2 times, 3 min each + 1 time, 20 min with agitation/centrifugation) and dried in a vacuum centrifuge. Gel pieces were further rehydrated with 10 mM of DTT in 25 mM ammonium bicarbonate buffer and incubated 10 min at 60 ºC for protein reduction. Then DTT solution was replaced by IAA 55 mM in 25 mM ammonium bicarbonate buffer and incubated in the dark and room temperature by 35 min. After protein reduction and alkylation gel pieces were dried and rehydrated in ice bath in a 0.025 μg/μL solution of trypsin in 12.5 mM ammonium bicarbonate buffer, to a final volume of 25 μL, during 1 h.

After the rehydration step, samples were digested overnight at 37 °C. Next, trypsin activity was stopped by the addition of 20 μL of 5% formic acid. The supernatant was withdrawn and retained, and the peptides were extracted from the gel pieces by adding 50-100 μL of a mixture of acetonitrile/TFA (500 μL H_2O+500 μL AC+1 μL TFA) and incubating them for 15 min at 37 ºC in a shaker. This extraction was done twice. Then, all supernatants were combined and evaporated to dryness in a vacuum concentrator centrifuge and finally the dried peptides obtained were reconstituted with 10 μL of 0.3% v/v formic acid.

(ii) Accelerated method. In this method, the protocol described above and referred as "overnight method" was followed but (i) washing steps (ii) alkylation, (iii) reduction, (iv) digestion of gel bands and (v) extraction peptides were done in 8 min (2 min each washing step, 30% ultrasonic amplitude, total of 3), 5 min (30% ultrasonic amplitude), 5 min (30% ultrasonic amplitude), 4 min (25% ultrasonic amplitude) and 8 min (two extraction steps, 2 min each) respectively, using ultrasonication at 30 kHz, with a single probe or 40 kHz with the four tip multiprobe.

2.5 *Case studies*

2.5.1. Desulfovibrio desulfuricans ATCC27774

Desulfovibrio desulfuricans ATCC27774 cells were cultured in sulfate-lactate medium. Cells were collected by centrifugation (8000 × g during 15 min at 4 ºC), resuspended in 10 mM Tris-HCl buffer and ruptured in a French press at 9000 psi. After centrifugation (10000 × g, 45 min) and ultracentrifugation (180000 × g, 60 min) the supernatant was dialyzed against 10 mM Tris-HCl buffer. Both proteins were isolated from the soluble extract using chromatographic columns (anionic exchange, Hydroxyapatite column and molecular exclusion chromatography). The purity of the proteins was evaluated by SDS-PAGE and UV-visible spectroscopy. All purification procedures were performed under aerobic conditions at 4 ºC and pH 7.6. Split-soret cytochrome c from *Desulfovibrio desulfuricans* ATCC27774 was in gel digested according to the accelerated method described in 2.5.2. Protein identification was done using the PMF procedure by MALDI-TOF-MS.

2.5.2 Plasmatic vitellogenin from Cyprinus carpio

80 µL of plasma from *Cyprinus carpio* was diluted to 100 µL with cold PBS (Phosphate Buffer Solution). 300 µL of -20 ºC cold acetone were added into the diluted plasma solution and kept overnight on ice. The sample was centrifuged at 10000 g, 4 ºC for 30 min. The supernatant was removed and the pellet was suspended in 100 µL of buffer (10 mM Tris-HCl pH 7.4; 2% of SDS; 1% of β-mercaptoethanol) [21]. Amounts of delipidated plasma (5 µL) were mixed with 5 µL of sample buffer (5 mL of 0.5 M Tris-Base + 8 mL of 10 % SDS + 1 mL of β-mercaptoethanol + 2 mL of glycerol + 4 mg of bromophenol blue in a final volume of 20 mL in water) for analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% 0.5 mm thickness). After gel electrophoresis (65 min, 120 V, 400 mA), the gel was stained with Coomassie blue R-250 and destained in order to visualize the proteins bands. Vitellogenin was in gel digested according to the accelerated method described in 2.5.2. Protein identification was done using the PMF procedure by MALDI-TOF-MS.

2.6 *Inverse ^{18}O labeling of peptides.*

Protein BSA was used to study the effect of ultrasonication in the release of peptides from gels. BSA was separated by 1D-PAGE, and then submitted to the protocol described in section 2.5.2 (ii). Once the protein was digested, gel pieces were removed and the solutions containing the peptides were dried in a speed vacuum. Then, peptides obtained were recomposed in (i) normal water or (ii) ^{18}O water and then both methods were compared following the inverse ^{18}O labeling protocol as described by Wang et al [10].

2.7 *MALDI-TOF-MS analysis*

A MALDI-TOF-MS model Voyager DE-PRO Biospectrometry Workstation equipped with a nitrogen laser radiating at 337 nm from Applied Biosystems (Foster City, USA), was used to obtain the PMF. MALDI mass spectra were acquired as recommended by the manufacturer and treated with the Data Explorer™ software version 4 series. Prior to MALDI-TOF-MS analysis, the sample was mixed with the matrix solution. α-CHCA matrix was used throughout this work and was prepared as follows: 10 mg of α-CHCA was dissolved in 1 mL of Milli-Q water/acetonitrile/TFA (1mL+1mL+2µL). Then, 2 µL of the aforementioned matrix solution was mixed with 2 µL of sample and the mixture was shaken in a vortex for 30 s. Finally, 1 µl of the sample/matrix mixture was spotted on a well of a MALDI-TOF-MS sample plate and was allowed to dry.

Measurements were done in the reflector positive ion mode, with a 20 kV accelerating voltage, 75.1 % grid voltage, 0.002 % guide wire and a delay time of 100 ns. Two close external calibrations were performed with the monoisotopic peaks of the Bradykinin, Angiotensin II, P14R and ACTH peptide fragments (m/z: 757.3997, 1046.5423, 1533.8582 and 2465.1989, respectively). Monoisotopic peaks were manually selected from each of the spectra obtained. Mass spectral analysis for each sample was based on the average of 500 laser shots. Peptide mass fingerprints were searched with the MASCOT [http://www.matrixscience.com/search_form_select.htmL] search engine with the following parameters: (i) SwissProt. 2006 Database; (ii) molecular weight (MW) of protein: all; (iii) one missed cleavage; (iv) fixed modifications: carbamidomethylation (C); (v) variable modifications: oxidation (M); (vi) peptide tolerance up to 150 ppm. A match was considered successful when the protein identification score is located out of the random region and the protein analyzed scores first.

3. Results and Discussion

3.1. *96 well plate method for proteins separated by gel electrophoresis*

The protein concentration loaded onto the gel was 2,1 µg/µL for BSA and 2.9 µg/µL for α-lactalbumin. Results, including the values setting for each variable, are presented in Fig. 1. As may be seen, ultrasonic amplitude was found a critical parameter. For BSA protein, when the lower amplitude was used in the digestion step (10%), longer treatment times were required to obtain good results, whilst the highest amplitude used (50%) degrades the gel in such a way that protein identification by MALDI was not possible. In the case of the α-lactalbumin this problem was even worst since using the lower amplitude in the digestion step no identification was possible at all in the range time assessed (60s-240s), whilst for the highest amplitude the identification was only possible in times comprised between 120 and 240 s. These results could be explained on the basis of recent research in ultrasonic applications in medicine and drug delivery that has estimated the pressure at the tip of the jet generated by bubble collapse (cavitation phenomena) around 60 MPa [11]. This is high enough to penetrate small pores, such as the ones

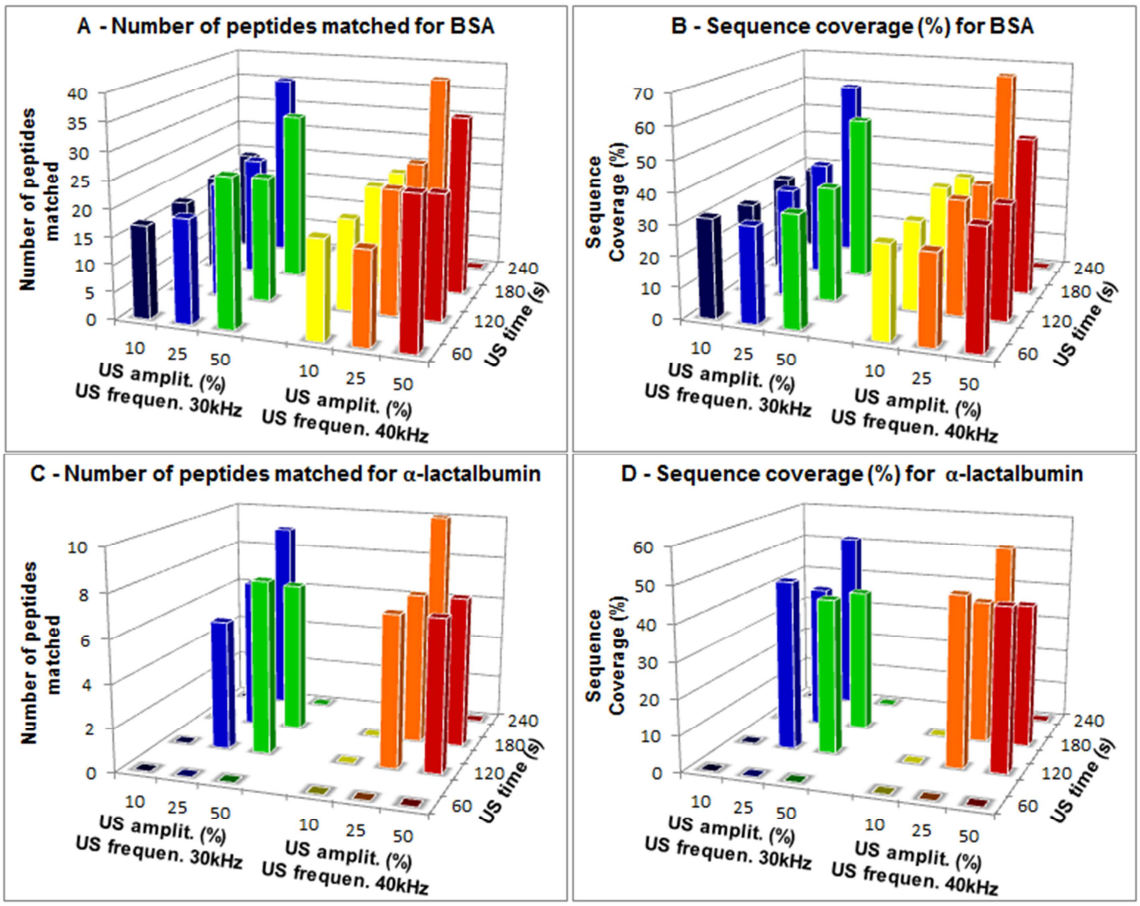

Figure 1. Number of peptides matched and sequence coverage for BSA and α-lactalbumin as a function of time, amplitude and frequency of sonication. Proteins were separated by Gel electrophoresis. Peptides matched and sequence coverage for the overnight method was 42±6 and 70±4 respectively for BSA and 11±2 and 51±1 for α-lactalbumin.

present in the gels in which proteins are separated. Hence, liquid jets may act as microsyringes, delivering the enzyme to a region of interest. However, the ultrasonic energy needed to do such delivering must be reached, and we hypothesized that this is the reason why the lower amplitude studied did not perform well, because it is not powerful enough to deliver the enzyme inside the gel, as it was the case for α-lactalbumin. As the amplitude and time is increased, the enzymatic cleavage performs better for both proteins, until a maximum is reached. Then, the gel is degraded and the protein identification becomes difficult or impossible, as it was the case for both proteins for the highest amplitude and time studied. Gel degradation can be explained on basis on the fact that when a cavitation bubble collapses near the surface of a solid sample particle, micro-jets of solvent propagate toward the surface at velocities greater than 100 m s^{-1}, causing pitting and mechanical erosion of the solid surface, thus leading to particle rupture (i.e., disruption) [12]. Although this process could be favorably used to enhance peptide release from the gel, at some point it becomes a problem interfering the subsequent analysis by mass spectrometry or even blocking HPLC columns. However, it is noteworthy that the multi-probe and the 96-well plate performed well for gel-based protein separations, once the process has been optimized. In addition, it was not found differences between the two frequencies studied. Furthermore, the best results obtained with the multi-probe were not different from the ones obtained using the overnight protocol.

To complete the study a set of 8 further proteins was mixed and separated using gel electrophoresis to proceed to protein identification through the ultrasonic method. Results showed in Table 1 demonstrate the successful accomplishment between the 96-well plate and the multi-probe since all proteins were identified with similar protein coverage and number of peptides matched than using the overnight protocol.

A comparison of the total time and number of steps involved in the handling of the five sample treatments reported in this manuscript is presented in Table 2. As may be seen, the ultrasonic method with multiprobe allows for the treatment of 4 samples in 30 minutes. This number can be exponentially increased using the new generation of multiprobes that allow treating 96 samples at once [11]. Hence, this method has a great potential for clinical purposes, where a high number of samples are generally handled daily.

3.2 *Effect of ultrasonication in the release of peptides from gels.*

To investigate the role of ultrasonication in the release of

Table 1. Number of peptides matched and protein sequence coverage for in gel-protein digestion by the overnight method and accelerated method.

Protein	theor. M_r (kDa)	overnight method (n = 2)			accelerated method (n = 2)					
		Mascot score	Sequence coverage (%)	No. of peptides matched	Mascot score		Sequence coverage (%)		No. of peptides matched	
					30 kHz	40 kHz	30 kHz	40 kHz	30 kHz	40 kHz
α-Lactalbumin	16.7	130±5	51±1	11±2	110±2	104±5	50±2	51±0	9±1	10±0
Trypsin inhibitor	24.3	87±3	51±1	18±0	69±3	64±5	28±2	27±1	7±1	7±0
Carbonic anhydrase	29.1	115±3	69±0	20±0	240±2	238±3	71±2	64±0	19±0	18±1
Ovalbumin	43.2	100±2	49±6	20±1	82±2	84±5	50±2	44±4	16±2	15±0
BSA	71.2	152±4	70±4	42±6	220±1	222±3	64±2	66±5	40±3	37±1
Aldolase rabit	39.8	152±6	72±6	21±2	109±3	103±4	68±1	68±0	20±1	19±0
Catalase bovine	60.1	98±4	43±2	26±2	218±2	220±0	37±4	43±0	22±1	24±0
Phosphorylase b	97.7	92±5	66±1	64±1	298±1	305±3	48±3	52±2	40±2	38±1
Thyroglobulin subunit*	310.0	194±8	28±1	76±6	188±2	194±6	28±1	25±7	80±2	81±1
Lactate dehydrogenase subunit*	36.9	79±2	46±4	21±1	83±1	182±4	46±2	49±0	20±1	21±0
Split-Soret cytochrome c *D. desulfuricans*	27.8	128±4	51±4	11±1	100±4	96±6	40±5	37±0	10±2	10±1
Vitellogenin *Cyprinus carpio*	148.8	176±6	42±4	49±1	150±3	141±6	42±1	41±1	50±2	51±1

* HMW-Native standard under denaturant conditions.

peptides from gels, a set of experiments using the ^{18}O isotopic labeling of the protein BSA was done to compare, the type of peptides released using the overnight, ON, or the ultrasonic, US, treatments [6,7]. The same amount of BSA was loaded onto a gel, and the protein was in-gel digested, using the overnight or the ultrasonic protocol. The supernatants were withdrawn from the tubes and dried down in a vacuum centrifuge. Then samples were recomposed in ^{16}O or in ^{18}O water. Since under conventional conditions, the yield of peptides extracted from a gel is protein-dependant and it varies for different peptides that originate from the same protein, we decide to use the inverse labeling as described by Wang et al [10] to ensure a correct identification of peptides extracted and unambiguous assessment of differential peptide extraction. To avoid any biased yields own to the sample treatment, all steps were exactly the same for the overnight or ultrasonic protocols, with the exception of the protein digestion step. Fig. 2 shows the inverse labeling method for the unambiguous identification of peptides used. The result of this set of experiments are shown in Table 3, where may be seen that a total of 40 peptides were identified as BSA peptides. Interestingly, from those 40 peptides, 15 peptides were labeled either in the direct or in the reverse process, whilst 21 peptides from the total, were also identified but they were not labeled. This put forwards that after sample recomposition not all peptides are labeled. This result is in agreement with data published by other authors [13]. From this 21 non labeled peptides, 6 were observed in the direct and reverse method, suggesting that they are produced regardless of the digestion method used; 9 were found in the mixture US^{16}O / ON^{18}O and six were found in the mixture US^{18}O / ON^{16}O. Therefore it can be concluded that some peptides are preferentially formed as a function of the digestion method. Thus, peptides (GACLLPK)H^+; (LCVLHEKTPVSEK)H^+; (MPCTEDYLSLILNR)H^+ are formed in the overnight digestion, whilst peptides (VLASSAR)H^+; (ALKAWSVAR)H^+; (HLVDEPQNLIK)H^+ are formed in the ultrasonic method. On the overall, both methods produced the same number of total peptides, c.a. 40, confirming that both methodologies work well for protein digestion. Another interesting finding is that protein modifications as consequence of the heating/cavitation caused by the ultrasonic energy were not

Table 2. Comparison of handling and time consumed for the five methods studied with the 96 well plate ultrasonic method.

		Denaturation	Reduction	Alkylation	Digestion	Desalting	Total steps	Total time
Proteins in gel	Overnight	5 min heating before electrophoresis	10 min US	35 min US	12 hours US	no	25*	24 h
	Ultrasonic	5 min heating before electrophoresis	5 min	5 min	4 min	no	20**	30 min

* Including: electrophoresis, band excision, 12 steps for gel washing, 10×3 min each and 2×20 min each, trypsin incubation on ice before digestion, peptides extraction (2 extractions) and evaporated to dryness (3 times).

** Including: electrophoresis, band excision, 8 steps for gel washing, 2×2 min each, digestion and peptides extraction and evaporated to dryness (3 times). With the in gel ultrasonic digestion no incubation of trypsin on ice is needed.

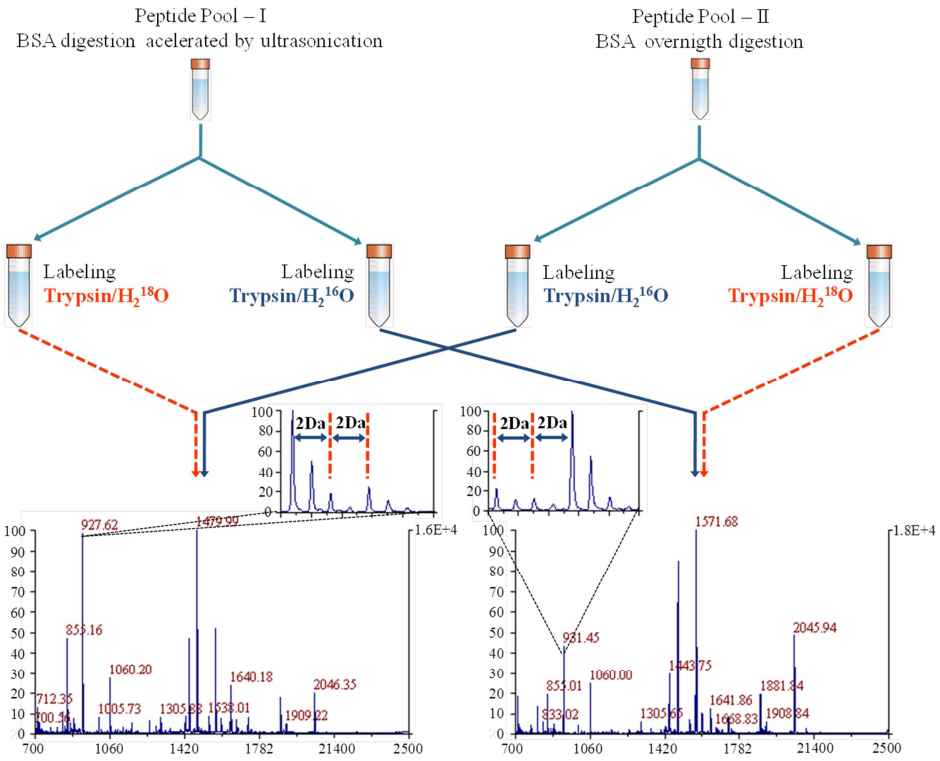

Figure 2. The inverse labeling method for the unambiguous identification of peptides released from gels using the overnight or the ultrasonic digestion protocol.

Table 3. Results from the BSA ^{18}O-inverse labeling experiments. All peptides were manually verified. (n=2)

	Nº. of peptides	Peptide fragment
Direct & reverse Labeled peptides US^{16}O / ON^{18}O and US^{18}O / ON^{16}O	15±2	(SEIAHR)H$^+$; (YLYEIAR)H$^+$; (LVNELTEFAK)H$^+$; (HPEYAVSVLLR)H$^+$; (SLHTLFGDELCK)H$^+$; (RHPEYAVSVLLR)H$^+$; (YICDNQDTISSK)H$^+$; (TCVADESHAGCEK)H$^+$; (LGEYGFQNALIVR) H$^+$; (HPEYAVSVLLRLAK)H$^+$; (KVPQVSTPTLVEVSR) H$^+$; (MPCTEDYLSLILNR)H$^+$; (RPCFSALTPDETYVPK) H$^+$; (LFTFHADICTLPDTEK)H$^+$; (RHPYFYAPELLYYANK)H$^+$
Direct & reverse non-labeled peptides US^{16}O / ON^{18}O and US^{18}O / ON^{16}O	6±1	(CASIQK)H$^+$; (TPVSEKVTK)H$^+$; (CCTESLVNR)H$^+$; (DTHKSEIAHR)H$^+$; (FKDLGEEHFK)H$^+$; (TVMENFVAFVDK)H$^+$
Labeled peptides from mixture US^{16}O / ON^{18}O	3±1	(GACLLPK)H$^+$; (LCVLHEKTPVSEK)H$^+$; (MPCTEDYLSLILNR)H$^+$ Oxidation (M)
Non-labeled peptides from mixture US^{16}O / ON^{18}O	9±1	(ALKAWSVAR)H$^+$; (HLVDEPQNLIK)H$^+$; (TVMENFVAFVDK)H$^+$ Oxidation (M); (VTKCCTESLVNR)H$^+$; (ETYGDMADCCEK)H$^+$; (LKHLVDEPQNLIK)H$^+$; (DDPHACYSTVFDK)H$^+$; (AEFVEVTKLVTDLTK)H$^+$; (YNGVFQECCQAEDK)H$^+$; (HPYFYAPELLYYANK)H$^+$;
Labeled peptides from mixture US^{18}O / ON^{16}O	3±0	(VLASSAR)H$^+$; (ALKAWSVAR)H$^+$; (HLVDEPQNLIK)H$^+$
Non-labeled peptides from mixture US^{18}O / ON^{16}O	6±1	(LVTDLTK)H$^+$; (LSQKFPK)H$^+$; (IETMREK)H$^+$; (SEIAHRFK)H$^+$; (NECFLSHK)H$^+$; (CCTKPESER)H$^+$; (LKECCDKPLLEK)H$^+$

found. For instance, no extra oxidations or carbamydomethcarbamydomethylations were detected.

These results confirm the utility of ^{18}O labeling in relative proteomics discovering and confirms the usefulness of the combination of ultrasonication and a 96-well plate for proteomics applications.

4. Future prospects

The speed, simplicity, high throughput and number of potential proteomics applications that can be developed jointing 96-well plate and ultrasonic multiprobes, it makes of this combination an ideal tool for robotic platforms. As an example, if a multiprobe of 96 tips was acomplisehd with a 96

well plate, the time need to identified a protein separated by gel electrophoresis could be reduced to just 20 s. Therefore developments in this area of research are anticipated.

5. Conclusions and perspectives

It has been proven that the combination of a 96-well plate and an ultrasonic multi-probe is a powerful tool in sample treatment for proteomics, allowing high sample throughput and a potentially enormous number of different proteomics applications. The huge variety of protocols that can be used with this accomplishment has it been demonstrated through different proteomics sample treatments for protein identification and 18-O based labelling. Sample preparation steps including destaining, washing, reduction & alkylation, digestion, spotting on MALDI targets or transfer to LC/MS input plates can be combined on a single automated platform making use of ultrasonication. This allows for rapid processing, minimizes the risk of contamination and therefore reduces the chance of application errors and improves the quality of data. The results showed that for protein digestion low or high ultrasonic amplitudes must be avoided when using a 96 well plate and an ultrasonic multiprobe. We have not found differences in performance for protein identification when using ultrasonic amplitudes of 30 (single probe) or 40 kHz (multiprove).

We have demonstrated that using the direct and reverse ^{18}O labeling the effectiveness of different procedures for in-gel protein digestion can be compared in terms of number and type of peptides produced. In fact our findings showed a similar number of peptides obtained by either the overnight or the ultrasonic method. However, some peptides were preferentially formed for each digestion protocol.

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