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Ultrasonic multiprobe as a new tool to overcome the bottleneck of throughput in workflows for protein identification relaying on ultrasonic energy

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ABSTRACT

We studied in this work the performance of the new ultrasonic multiprobe in terms of throughput, handling and robustness. The study was conducted using the multiprobe to speed two different proteomics workflows. The "classic" method relaying on overnight protein digestion (12 h), was used as the standard procedure. This work clearly shows the importance of testing variables such as ultrasonic amplitude and ultrasonic time when adapting an ultrasonic-based treatment to a new ultrasonic device. The results here presented also shown and confirm the advantage of speed up sample treatment workflows with the aid of ultrasonic energy in combination with a 96-well plate. The methods compared were similar in terms of robustness, but the desalting free method was the fastest, requiring only 2 min/sample for completion. In addition it was also the simplest in terms of handling, since no desalting step was needed. The following standard proteins were successfully identified using the methods studied: bovine serum albumin, α-lactalbumin, ovalbumin, carbonic anhydrase, fructose-bisphosphate aldolase A, catalase, chymotrypsinogen A. As case study, the identification of the protein Split-Soret cytochrome *c* from *D. desulfuricans* ATCC 27774 was carried out.

1. Introduction

The enzymatic cleavage of proteins is regularly used in proteomics for protein identification through peptide mass fingerprint and mass spectrometry-based techniques [1]. Nowadays high throughput in sample treatment is generally recognized for the scientific community as a priority demand in proteomics approaches. Over the last years we have witnessed the reporting of different strategies to (i) reduce the time needed to perform protein digestion and to (ii) simplify the handling for protein identification [2]. Thus, warming [3], ultrasonic energy [4–6], infrared radiation [7,8], high pressure [9] or spinning [10] are recent strategies that allow performing sample treatment for protein identification of complex mixtures in a fast, efficient and reproducible manner.

From the strategies mentioned above, the utilization of ultrasonic energy as a way to speed the enzymatic kinetics of protein cleavage from overnight (hours) to minutes was first reported in 2005 [4] and it was soon validated by different research groups [11,12]. Later, the use of ultrasonic energy was successfully extended to the different steps of the sample handling for protein identification, namely protein solubilization/denaturation, protein reduction and protein alkylation [13]. The most recent sample treatment reported in literature making use of ultrasonic energy to accelerate sample handling for protein identification claims a time to complete the process of 8 min in a clean method that avoids desalting procedures [14].

The present work shows a step forward in the application of ultrasonic energy in proteomics workflows, since high sample throughput is obtained by jointing for the first time an ultrasonic multiprobe, allowing the treatment of four samples at once, with a 96-well plate. The comparison study was conducted through the identification of seven target proteins by mass spectrometry and peptide mass fingerprint using three different sample treatment workflows. In addition, as a case study, the identification of Split-Soret cytochrome *c* from *D. desulfuricans* ATCC 27774 was carried out using the three methods compared in this work.

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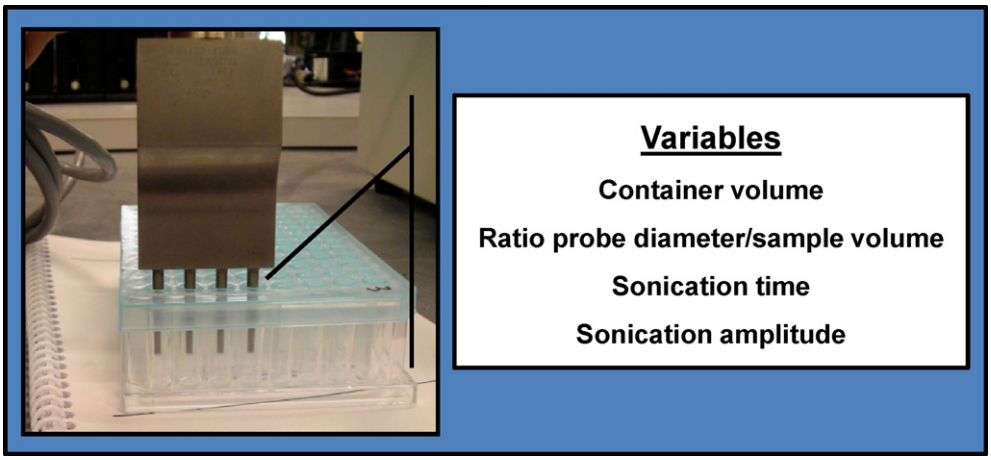

Fig. 1. This figure shows the new ultrasonic multiprobe coupled to the 96-well plate.

2. Experimental

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 Unijet II was used for (i) sample drying and (ii) sample pre-concentration. 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

An ultrasonic multiprobe 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 multiprobe 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 Fig. 1.

2.3. Standards and reagents

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

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 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 treatments

The sample treatments summarized below are based in works previously developed in our laboratory. However, they are applied for the first time with an ultrasonic multiprobe in a 96-well plate.

2.4.1. Classic method

The overnight digestion was performed after protein denaturation with 6.5 M urea and reduction with 10 mM DTT in 12.5 mM ammonium bicarbonate at 37 °C for 1 h. Iodoacetamide was then added to a final concentration of 50 mM. The resulting mixture was incubated at room temperature in darkness for 45 min. The mixture was then diluted 4-fold to reduce urea concentration and an aliquot of 50 μL was taken to perform the enzymatic digestion. After trypsin addition (1:20, w/w trypsin-to-protein ratio), all samples were incubated at 37 °C overnight (12 h). Then, 1 μl of formic acid 50% (v/v) was added to stop the trypsin activity (final formic acid concentration in sample 5%). Finally, to avoid high saline concentration in the MALDI, desalting using ZipTips® was done as follows:

(a) *Activation*: aspirate and dispense, A&D, 10 μl of acetonitrile (×1), then A&D 10 μl of [50% acetonitrile/0.1% TFA] (×1), and then A&D 10 μl of 0.1% TFA (×2),
(b) *Peptide binding*: 10 μl of sample (A&D the sample 20 cycles),
(c) *Washing*: A&D 10 μl of 0.1% TFA (×3),
(d) *Peptide elution*: 10 μl of [90% acetonitrile + 0.1% TFA] (A&D the sample 20 cycles).

2.4.2. Accelerated urea method

In brief, the method described above and referred as "classic method" was followed but protein alkylation, protein reduction, and protein digestion were done in 10, 10, and 4 min respectively, under the effects of an ultrasonic field [15]. ZipTip® were used thorough the sample treatment to avoid high saline concentration in the MALDI as described above.

2.4.3. Accelerated clean method

This method was recently reported by our group [14]. In brief, the proteins were dissolved in mixed acetonitrile/aqueous solutions, and (i) denaturation, (ii) reduction and (iii) alkylation

of proteins were done in steps of 1 min whilst protein digestion was done during 4 min. Ultrasonic energy was used in all steps.

2.5. A Case study

2.5.1. D. desulfuricans ATCC 27774

D. desulfuricans ATCC 27774 cells were cultured in sulfate–lactate medium. Cells were collected by centrifugation (8000 × *g* during 15 min at 4 °C), resuspended in 10 mM Tris–HCl buffer and ruptured in a French press at 9000 psi. After centrifugation (10,000 × *g*, 45 min) and ultracentrifugation (180,000 × *g*, 60 min) the supernatant was dialyzed against 10 mM Tris–HCl buffer. The soluble extract was loaded in a DEAE-Cellulose™ and then in a Q-Sepharose™ column both equilibrated with 10 mM Tris–HCl and eluted with a linear gradient to 250 mM Tris–HCl. The third purification step included a hydroxyapatite column equilibrated with 100 mM Tris–HCl and eluted with a potassium phosphate linear gradient from 1 to 200 mM. Finally, the fraction containing the haemic-protein was concentrated in a diaflow system (membrane YM 10) and loaded in a Superdex 200 column (Pharmacia) equilibrated with 300 mM Tris–HCl. The purity of the proteins was evaluated by SDS-PAGE and UV–vis spectroscopy. All purification procedures were performed under aerobic conditions at 4 °C and pH 7.6.

2.6. 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 (1 ml + 1 ml + 2 μl). 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.

2.7. Statistical analysis

The *t*-test was used to determine differences between the ultrasonic-based methods and the classic overnight methods. Statistical analysis was performed with the significance level of 5%, using the software Statistica 6.0 (StatSoft, Inc. 1984–2001, Tulsa, OK, USA).

3. Results and discussion

3.1. Classic method

Table 1 shows that all the target proteins were identified when the classic method was carried out. The target proteins scored always first and out of the random region in all replicates done in this study ($n = 4$). The number of peptides matched and the sequence coverage obtained for each protein were used to compare performance with the other two methods studied in this work.

3.2. Accelerated urea method

Next, we carried out a set of experiments to compare the performance of the ultrasonic multiprobe in the acceleration of the classic method. Previous research developed in our group has shown that the classic method can be accelerated using ultrasonic energy in the different steps of its workflow [5,6,13–15]. A pitfall of this procedure when the treatment in done with single probe is that samples must be handled one by one. In other words, sample throughput is still a bottleneck in the application of ultrasonic-based approaches for the acceleration of methods commonly used for protein identification. Nevertheless, the ultrasonic-probe-based devices have evolved in such a way that multiprobes for the simultaneous treatment of samples ranging from 4 to 12 are nowadays available [16]. Their performance for proteomics applications has not been tested yet, to the best of our knowledge. It must be stressed that it is necessary to adequately address the challenges of high sample throughput while maintaining data quality. Therefore, the most important variables affecting ultrasonic-based sample treatments, namely amplitude and time of ultrasonication [16] were studied. This set of experiments was carried out in a 96-well plate which is the regular plate used in robotic platforms. It must be noteworthy that when a single probe is used to speed proteomics workflows, the diameter of the tip regularly used is 0.5 mm, whilst the new multiprobes are manufactured with a diameter of 1 mm [16]. This difference is important since the lower is the sample container and the ratio sample volume/probe diameter, the higher is the risk of to lose sample by aerosol formation and subsequent sample spreading out of the sample container. In addition, aerosol formation can led to cross-contamination among the wells of the 96-well plate. For those reasons, testing the changes in sample treatment performance any time that a new ultrasonic device is tested is very important. Fig. 2 shows the results obtained for the identification of BSA and α-lactalbumin under different conditions of ultrasonication. For both proteins studied, the results suggest that jointing long times of ultrasonication (i.e. 240 s) with low ultrasonication amplitudes, the number of peptides matched and the protein coverage obtained allow protein identification at the same confidence level that with the classic protocol, as it is also showed in Table 1. This conclusion may be also observed for higher amplitudes. This result suggests that amplitude of ultrasonication is not an important variable in order to accelerate the in-solution digestion of proteins, at least for the ultrasonic multiprobe here assessed. This finding is important since high ultrasonic amplitudes can degrade the protein with the result of failing in protein identification. More important, to use amplitudes as low as possible when working with a 96-well plate, is also advantageous because it avoids the spreading out of the sample through drops or aerosol formation, overcoming cross-contamination. As an example, it is noteworthy that when we assayed an ultrasonic amplitude of 75%, sample

Table 1
Protein sequence coverage and number of peptides matched for the in-solution protein digestion: overnight method, accelerated method with urea and ZipTip® and clean method in H_2O/acetonitrile ($n = 4$, $p_t = 0.05$[a]).

Protein[b]	Theor. Mr (kDa)	Classic method, $x \pm$ S.D.			Accelerated urea method, $x \pm$ S.D. (p_{ex}[c])			Accelerated clean method, $x \pm$ S.D. (p_{ex}[c])		
		Mascot score	Sequence coverage (%)	Peptides matched	Mascot score	Sequence coverage (%)	Peptides matched	Mascot score	Sequence coverage (%)	Peptides matched
α-Lactalbumin *B. taurus*	16.7	137 ± 7	47 ± 2	11 ± 1	74 ± 2	49 ± 7 (0.65)	9 ± 1 (0.10)	89 ± 8	46 ± 4 (0.71)	9 ± 1 (0.07)
Chymotrypsinogen A *B. taurus*	26.2	139 ± 8	61 ± 8	10 ± 2	76 ± 2	51 ± 7 (0.10)	8 ± 2 (0.26)	118 ± 6	51 ± 7 (0.08)	9 ± 2 (0.39)
Carbonic anhydrase II *B. taurus*	29.1	104 ± 8	53 ± 5	13 ± 3	107 ± 7	52 ± 4 (0.66)	11 ± 2 (0.41)	178 ± 4	59 ± 4 (0.12)	16 ± 4 (0.16)
Fructose-bisphosphate aldolase A *O. cuniculus*	39.8	164 ± 9	48 ± 4	13 ± 2	104 ± 15	47 ± 2 (0.92)	12 ± 1 (0.51)	90 ± 7	44 ± 6 (0.41)	11 ± 2 (0.19)
Ovalbumin *G. gallus*	43.2	121 ± 3	50 ± 9	15 ± 2	134 ± 6	43 ± 5 (0.25)	15 ± 2 (0.77)	147 ± 7	46 ± 9 (0.56)	15 ± 4 (1.00)
Catalase *B. taurus*	60.1	159 ± 6	43 ± 4	16 ± 1	129 ± 7	39 ± 7 (0.32)	14 ± 4 (0.44)	254 ± 5	43 ± 2 (0.14)	17 ± 4 (0.18)
Serum Albumin *B. taurus*	71.2	288 ± 10	61 ± 5	39 ± 4	307 ± 10	62 ± 5 (0.39)	37 ± 4 (0.47)	207 ± 9	56 ± 12 (0.22)	34 ± 7 (0.24)
Split-Soret cytochrome *c* *D. desulfuricans* ATCC 27774	27.8	128 ± 8	62 ± 8	18 ± 3	119 ± 9	53 ± 2 (0.09)	15 ± 2 (0.10)	107 ± 8	65 ± 12 (0.70)	15 ± 2 (0.10)

[a] p_t = theoretical significance level.

[b] Initial protein concentration: 0.3 μg/μl. Accelerated method with urea: protein reduction and protein alkylation were done with 10 min ultrasonication time and 25% ultrasonication amplitude each one, whilst protein digestion was done with 4 min ultrasonication time and 10% ultrasonication amplitude. Accelerated clean method with acetonitrile: protein reduction and protein alkylation were done with 1 min ultrasonication time and 25% ultrasonication amplitude each one, whilst protein digestion was done with 5 min ultrasonication time and 10% ultrasonication amplitude.

[c] Experimental significance level.

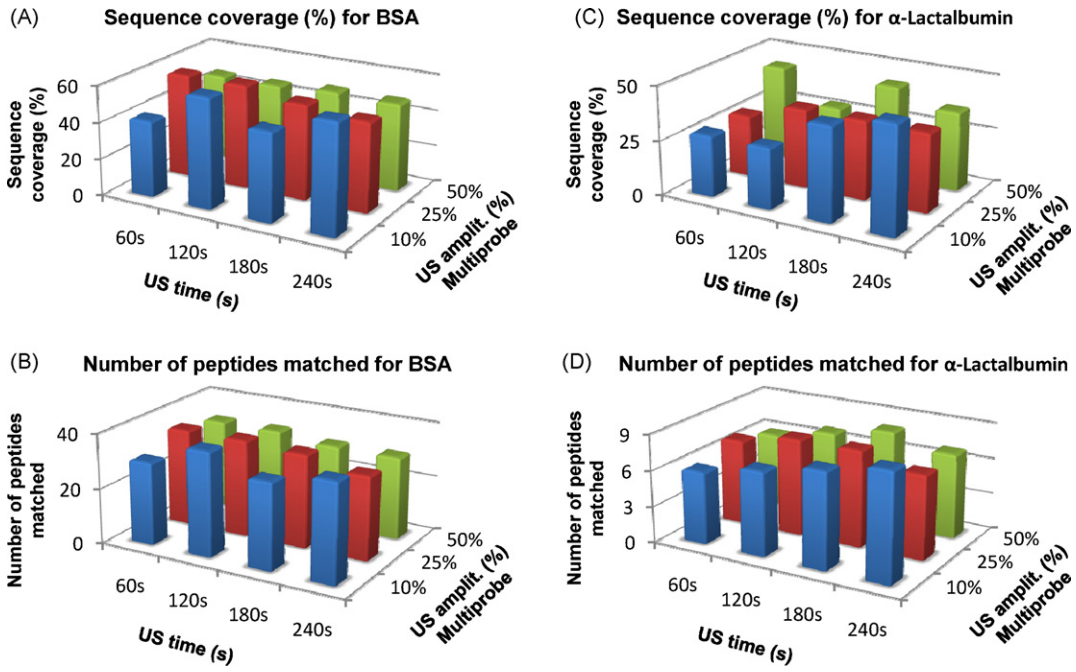

Fig. 2. Number of peptides matched and sequence coverage for bovine serum albumin and α-lactalbumin as a function of time, and amplitude of ultrasonication. Sequence coverage and peptides matched for the overnight method was 61 ± 5 and 39 ± 4 for bovine serum albumin and 47 ± 2 and 11 ± 1 for α-lactalbumin, respectively.

was lost, being withdrawing from the well as an aerosol due to the effects of the high ultrasonic amplitude used. As consequence, cross-contamination caused by the aerosol formation was also verified, being detected peptides of BSA protein in well plates that in theory only contained protein α-lactalbumin and vice versa. Consequently the highest amplitude used in further experiments was 10%. Moreover, the maximum volume of sample recommended to work with is 50 μl, higher volumes can lead to random cross-contamination.

Regarding ultrasonication time, as may be seen in Fig. 1, this variable was found to affect the results; since the number of peptides matched and their respective sequence coverage for either BSA or α-lactalbumin were slightly improved when this variable was increased in the digestion step from 60 to 240 s. These findings are consistent with data previously reported in the literature [14,15]. We hypothesize that a complex relation between the type of enzyme, the type of substrate, the ultrasonic amplitude and the ultrasonic time, influences the efficiency of the enzymatic process when it is carried out under the effects of an ultrasonic field. Thus, Sakakibara et al. have shown an enhancement in the reaction's kinetic for the hydrolysis of sucrose, when it was used the enzyme invertase in conjunction with ultrasonication [17]. Nevertheless, other authors have pointed out, that ultrasonic energy can inactivate enzymes. Thus, Bracey et al. have reported an inhibitory effect in the activity of the enzyme subtilisin when the subtilisin-catalyzed interesterification reaction in an organic solvent was studied under the effects of ultrasonication [18]. As further example, although the enzyme protease XIV was inactivated towards casein substrate after 2 min of ultrasonication with probe, the same enzyme in the same conditions was active towards mussel tissue substrate after 4 min of ultrasonication [19].

Next, with the best conditions found in the set of experiments above described, we proceed to identify other proteins, as showed in Table 1, The number of peptides matched and the protein coverage were statistically compared with those obtained with the classic method and no differences were found at a significance level of $p > 0.05$ (test t, $n_1 = 4$, $n_2 = 4$). This result indicates that with the right conditions chosen the multiprobe can be used in conjunction with a 96-well plate to obtain fast and high throughput sample treatment for protein identification.

3.3. Accelerated clean method

A drawback of the classic method is that, prior to MS analysis, ZipTip® tips or other kind of home-made mini-columns containing C_{18} beads are often used as peptide microextraction and purification columns. It has been demonstrated; however, that sample loss can be as high as 90%, when ZipTips® columns are used [20]. This loss depends on the absolute concentration of the initial peptide digest loaded into the ZipTips® and is peptide type-dependant [20]. In addition, using ZipTips® sample handling becomes time consuming, labour intensive and expensive. Therefore we also tested the performance of the multiprobe in a clean method relaying in the use of a mixture of water/acetonitrile to solve the sample. The initial trials were done applying ultrasonic energy in the reduction, alkylation and digestion steps of our proteomic workflow (25% ultrasonic amplitude and 5 min ultrasonic time in each step). Results, however, were unexpectedly low in terms of protein sequence coverage and peptides matched, especially for protein α-lactalbumin. This can be explained because amino acid residues valine and isoleucine has the potential to sterically hinder trypsin binding when an incomplete protein denaturation has been done. Therefore, a step was added in which protein denaturation was done by applying ultrasound to the sample before proceed with the subsequent protein reduction. Remarkably, after this change, the results obtained in terms of peptides matched and protein coverage for BSA, α-lactalbumin and for the other proteins used in this study, as showed in Table 1, were as good as for the classic or the accelerated urea methods. The number of peptides matched and the protein coverage obtained were statistically compared with those of the classic method and differences were not found (test t, $p > 0.05$, $n_1 = 4$, $n_2 = 4$).

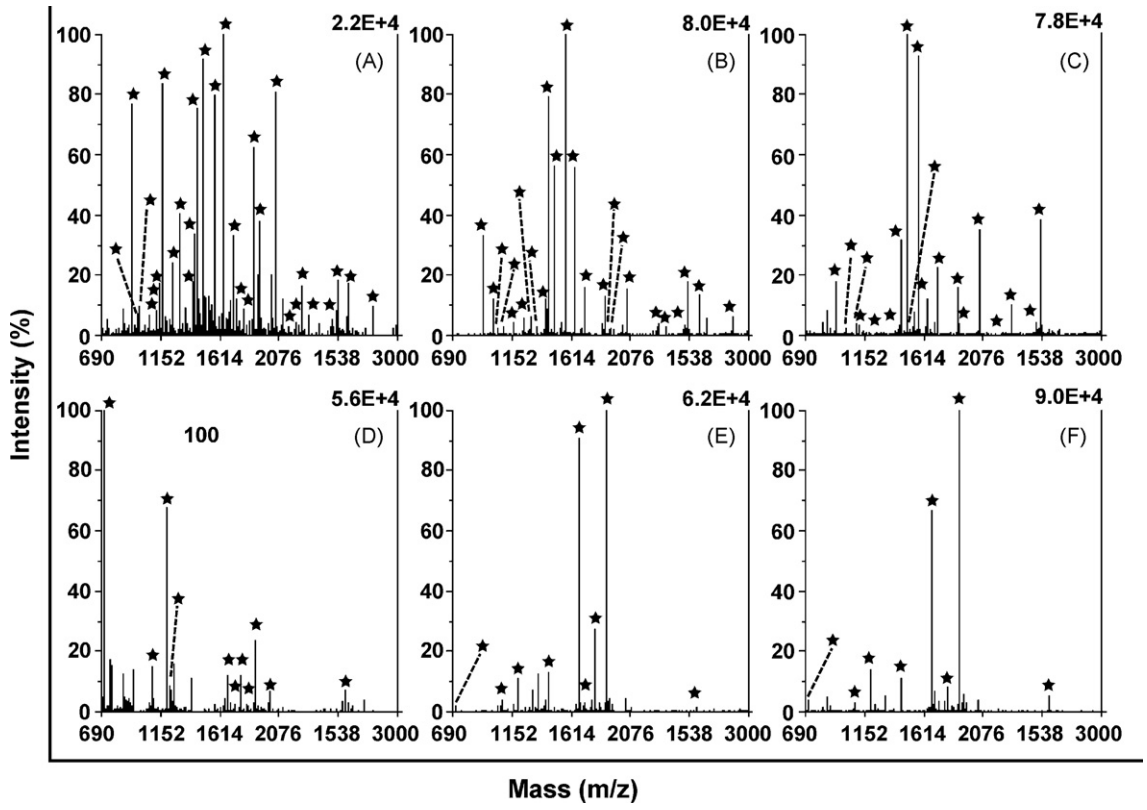

Fig. 3. MALDI spectra obtained of in-solution digestion of 3 μg/μl of bovine serum albumin and α-lactalbumin. Panel A, B and C, spectrum of digested bovine serum albumin with classic method (A), accelerated urea method (B) and (C) accelerated clean method. Panel D, E and F, spectrum of digested α-lactalbumin with classic method (D), accelerated method (E) and (F) accelerated clean method.

3.4. MALDI spectra

MALDI spectra of the sample treatments here compared are presented in Fig. 3 for BSA and α-lactalbumin. For both proteins, the spectrum belonging to the classic method shows a different pattern of peak intensities, when compared to the ultrasonic-based ones. This could suggest that under the effects of an ultrasonic field some peptides are preferentially formed. Furthermore, when the

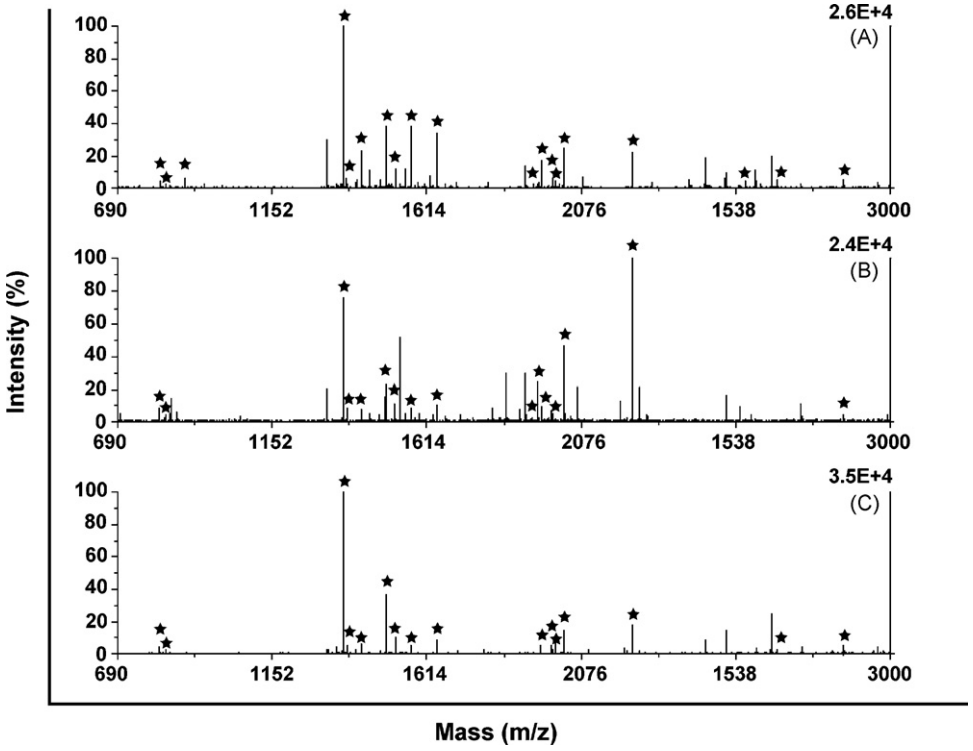

Fig. 4. MALDI spectra of Split-Soret cytochrome *c* from *D. desulfuricans*, (A) classic method, (B) accelerated urea method and (C) accelerated clean method.

Table 2
Comparison of handling and time consumed for the three methods studied in this work.

Method	Denaturation	Reduction	Alkylation	Digestion	Desalting	Total steps	Total time (h)[a]
Overnight (urea)	Urea	60 min	45 min	12 h	Yes	4	14
Ultrasonic (urea)	Urea	10 min US	10 min US	4 min US	Yes	4	6.5
Ultrasonic (H_2O/acetonitrile)	1 min US	1 min US	1 min US	5 min US	No	4	3

[a] Total time needed to complete the analysis for 96 samples (96-well plate).

spectra of the accelerated method are compared with the ones of the clean method, some differences in peak intensities are also observed that can be attributed, in this case, to the differences between both sample treatments: the use of urea/ziptips or organic solvents respectively. It is also possible that the different reagents used in the sample treatments might influence the peptide distribution within the matrix spot. It must be pointed out that, despite of the differences in peak intensities, when the optimum conditions find out for each method were used, protein identification was always possible.

3.5. Application to a case study

To compare the sample treatments studied in this work, we test the identification of a cytochrome produced by *D. desulfuricans* ATCC 27774. This organism, which is a facultative nitrate/sulfate bacterium, considerably expresses a protein named Split-Soret [21] in the presence of nitrate. This fact suggests that this protein can be involved in the nitrate metabolism [22].

The production and purification of this protein was explained in Section 2.5.1. A sample containing 0.3 μg/l of Split-Soret cytochrome *c* was used in this set of experiments. The samples containing the protein were then submitted to the three methods studied in this work and the results presented in Table 1 clearly demonstrate that the classic method and the accelerated classic method provided protein coverage and peptides matched that were not found statistically different (test *t*, $p > 0.05$, $n_1 = 4$, $n_2 = 4$).

Fig. 4 shows the spectra of the Split-Soret cytochrome *c* for the three sample methods used.

3.6. Final remarks

Table 2 shows the differences in time and handling for the three sample treatments compared in this work. As may be seen, if we consider the workflow as composed of five main steps as follows: (i) denaturation, (ii) reduction, (iii) alkylation, (iv) digestion and (v) desalting, handling is not the same. The clean method avoids the use of ZipTips or any other kind of desalting processes. This means that it becomes also economically cheaper, since ZipTips are an expensive reagent. In terms of time consumed, the best is again the clean method, being necessary only 2 min/sample to complete the workflow.

The new ultrasonic multiprobe-device can work as efficiently as the single probe. In terms of throughput, however, it allows to work 4–12 times faster, depending on the multiprobe chosen to work with. It must be stressed that whilst an analysis runned using all the wells of a 96-well plate takes 12 h with the classic protocol, it last only for 3 h with the ultrasonic clean method. Due to simplicity of use and high throughput, it may be advanced that the ultrasonic multiprobe-device will be implemented in robotic platforms.

4. Conclusions

The new ultrasonic multiprobe-device has been studied in conjunction with a 96-well plate in the acceleration of two different proteomic workflows in terms of speed, throughput, handling and robustness. We have demonstrated that to avoid cross-contamination between samples in this approach, low amplitudes must be used. For the six standard proteins studied, the two workflows accelerated with ultrasound give results that were found similar in terms of robustness, as their utilization provide results comparable with a classic non-ultrasonic method. The clean fast method has the best performance in terms of speed and handling since only 2 min/sample are necessary to complete it, being desalting not necessary, thus diminishing the total number of steps.

Regarding throughput, it has been proven that the combination of a 96-well plate and an ultrasonic multiprobe is a potential powerful tool in sample treatment for proteomics, allowing high sample throughput. The methods proposed allow for rapid processing, minimizing the risk of contamination and reducing the chance of application errors. In addition, a potentially enormous number of different proteomics applications are advanced, such as fast and high throughput protein quantification using isotopic labeling [23]. Sample preparation steps, including reduction and alkylation, digestion, spotting on MALDI targets or transfer to LC/MS input plates can potentially be combined on a single automated platform making use of ultrasonic energy provided by ultrasonic multiprobes.

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