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Review

A journey through PROTEOSONICS

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ABSTRACT

Ultrasonic energy is gaining momentum in Proteomics. It helps to shorten many proteomics workflows in an easy and efficient manner. Ultrasonic energy is nowadays used for protein extraction, solubilisation and cell disruption, to speed protein identification, protein quantification, peptide profiling, metal–protein complexes characterisation and imaging mass spectrometry. The present review gives a perspective of the latest achievements in ultrasonic-based sample treatment for proteomics as well as provides the basic concepts and the tools of the trade to efficiently implement this tool in proteomics labs.

Contents

Abbreviations: UE, ultrasonic energy; UF, ultrasound frequency; UI, ultrasound intensity; UA, ultrasound amplitude; TCA, trichloroacetic acid; ECM, extracellular matrix; MS, mass spectrometry; IMS, imaging mass spectrometry; DPD, decision peptide-driven; CH, cup horn; SR, sonoreactor; UB, ultrasonic bath; UP, ultrasonic probe

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1. Introduction

Ultrasonic energy (UE) as a tool in sample preparation is nowadays gaining momentum in proteomics. Some of the firsts attempts to use UE in proteomics were dedicated to protein extraction from complex matrices and to increase the kinetics of enzymatic reactions [1–5]. Since then UE has been reported as a tool to speed/improve several steps of sample handling in proteomics. At present, the use of UE in proteomics spans diverse topics, ranging from high throughput protein identification to quantification and biomarker discovery in biological fluids or tissue samples. UE finds its place to simplify and to shorten the daily work of proteomics researchers [3–5].

The present review is focused in two main aspects. Firstly, it is intended to make easier to the proteomics community to deal with UE. Therefore, the basic concepts about how to handle UE are explained in an easy and straightforward way. This is done on a step-by-step method, including the explanation of the differences among the devices for UE delivery at present available on the market. Secondly, the most important UE applications done to date in proteomics, to the best of our knowledge, are critically described in detail.

2. Ultrasonic energy: the tools of the trade

It is generally agreed that the word "ultrasound" refers to the sound with a frequency ranging from 20 KHz to 10 MHz, out of the 20 Hz–20 KHz, audible range of a healthy young person [6]. The ultrasonic frequency range is itself divided into two main zones, depending on the effects of the ultrasonic waves when passing a liquid medium (see Fig. 1A). High frequency ultrasound, comprised between 2 MHz and 10 MHz, also known as medical ultrasound, is widely used for medical purposes because the physical and chemical properties of the liquid media where the ultrasound is applied do not change. Low frequency ultrasound, is comprised between 20 KHz and 100 KHz, and causes many physical and chemical changes in the liquid media where they are used [7]. These noticeably changes are produced as consequence of a physical phenomena caused by low frequency ultrasound known as *cavitation* (Fig. 1B). Cavitation is the production of microbubles in a liquid, when a large negative pressure is applied [6]. Cavitation occurs when waves cross the liquid fast enough that the liquid molecules cannot follow the cycles of compression and decompression of the wavelength with the same speed. At certain point the forces that maintain liquid molecules together are broken and cavities are created. The formed cavities are known as *cavitation bubbles*. As more energy is delivered to the cavities in the form of ultrasound waves, the cavitation bubbles grow in size through the process called *rectified diffusion* [8]. There are two types of cavitation bubbles characterised by the different effects they promote. *Stable* cavitation is characterised by cycles of compression and decompression, as the wavelength passes through the liquid media but the cavitation bubble never implodes. In *transient cavitation*, the cavitation bubbles grow reaching an unstable size followed by a violent collapse. In these circumstances, cavitation bubbles acts as micro-reactors whereas, according to the hot-spot theory [9,10] temperatures and pressures near 5000 °C and 1000 atm, respectively, are reached. Additional effects are that the mass transfer processes in heterogeneous systems is increased and that the formation of micro-jets of liquid during the implosion at c. a. 400 Km h^{-1} causes erosion and disruption of solid surfaces [6,7]. Also, the described conditions facilitate the formation of highly reactive radical species (RRS) that can be used to enhance chemical reactions. The sonication of water results in the production of small quantities of $OH^{\bullet}$ and $H^{\bullet}$ radicals that undergo several subsequent reactions including the formation of H_2O_2, H_2,and O_2.

The correct application of the UE depends on different variables. In brief, these are ultrasound frequency, UF; ultrasound intensity, UI; ultrasound amplitude, UA; time of application; temperature, external pressure, type of liquid media, and type of gas present in the liquid media. These variables and their effects in the context of ultrasonic-based sample preparation have been discussed in previous publications [11–13] but will be shortly described below.

Common ultrasonic devices are sold delivering a wide range of electrical energy, which is referred as the "power" of the ultrasonicator. It is easy to find sellers classifying ultrasonic apparatus in function of the watts they deliver. The electrical energy is transformed into mechanical (vibration) energy. For instance, this can be visualised as a motion travelling through the ultrasonic tip, causing it to move up and down. The distance of the movement of vibration is called its amplitude. The amplitude of the vibration can be controlled up to a maximum depending on the power of the ultrasonicator. Ultrasonic amplitude and ultrasonic intensity have a direct relationship. The intensity of an ultrasonic wave is proportional to the square of the amplitude. Therefore, the highest is the amplitude the highest is the intensity.

For the same type of sample, if the output power is set to low values, low amplitude and low intensity are achieved. The lower amplitude and intensity the lower the effectiveness achieved with the ultrasonicator. The reverse is also true.

Current proteomics workflows using ultrasonic energy as a tool in sample treatment relay in short times of exposure, generally less than 2 min, and in the use of high intensity devices with capabilities of delivering frequencies between 20 KHz and 40 KHz. Ultrasound amplitudes are generally set up to 50%. As will be seen in further sections, the ultrasonic probe (or multiprobe), the cup-horn and the sonoreactor, are the devices most commonly used nowadays to deliver ultrasonic energy in proteomics.

Temperature can be a problem as many proteomics protocols make use of chaotropes agents, which may covalently modify proteins. As an example, urea is routinely used as denaturing agent in proteomics. However, heat accelerates urea hydrolysis, which leads to the production of isocyanate. This chemical, in turn, promotes the carbamylation of proteins at the N-termini of lysine side chains [14]. However, for the majority of the proteomics applications the time of exposure, less than 2 min, and ultrasonic amplitude, below 50%, are not sufficient to promote an increase in the bulk temperature above the threshold to induce modifications on proteins. If temperature becomes a problem for the reason mentioned above, or some other reason, modern probes can be used in the "pulse" mode. In this working mode, the amplifier switches the power on and off repeatedly, avoiding excessive warming of the bulk sample. External cooling can also be applied.

The ultrasonic energy in proteomics has been always used with success, to the best of our knowledge, under atmospheric pressure. Therefore, external pressure is a variable not to be taken into account. Regarding the liquid media, ultrasound has been successfully applied

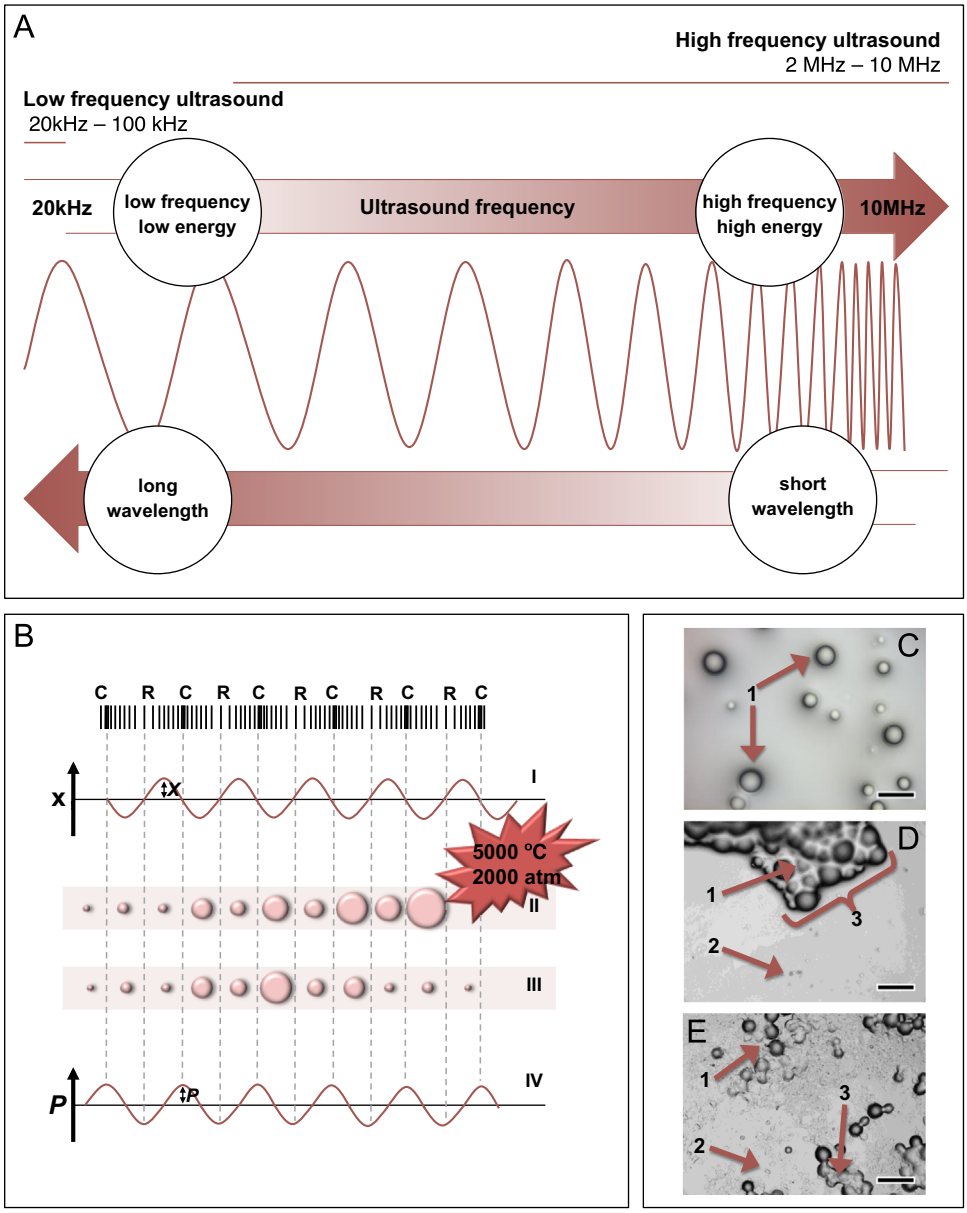

Fig. 1. Basics of ultrasonication. (A) Ultrasound frequency (20 kHz–10 MHz) is divided in two zones: low frequency ultrasound and high frequency ultrasound. High frequency ultrasound is widely used in medicine. Low frequency ultrasound, due to cavitation, can cause many physical and chemical changes in the liquid media where it is applied. (B) Cavitation phenomena: cavities are generated on the passing of the ultrasonic wave. The cavity can grow up and down (II) or just implode once has reached a maximum size (III). The implosion generates high pressures and temperatures. (C–E) Sepharose particles. (C) no ultrasonic energy was applied; (D) 20% ultrasonic amplitude was applied; (E) 80% ultrasonic amplitude was applied. (1: intact particles; 2: disrupted particles: 3: particles aggregated).
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to a wide variety of water or mixed organic-water solutions for protein cleavage in either in-solution or in-gel based approaches [15–18].

Added gas in a reaction mixture will act as nucleation sites for the cavitation spots and thus will enhance the cavitation [13]. As for the case of pressure, we hypothesise that this variable is not critical, as the applications developed up to now have been successfully done without the need to add gas into the solutions.

3. Current ultrasonic tools to shorten and simplify proteomics workflows

A detailed revision about the characteristics and performance of ultrasonic devices has been previously published [12]. UE is regularly applied in proteomics in two different forms, as follows. *Direct ultrasonication* refers to the use of ultrasonic probes where a titanium probe (sonotrode) is dipped into the sample. *Indirect ultrasonication* refers to any system in which the ultrasound reaches the sample through the walls of the sample container.

3.1. Direct ultrasonication

Sonotrodes can deliver UE through one, four, or up-to 96 sonotrodes. This means that nowadays UE can be used almost without restrictions concerning to the number of samples. Recently, it has been shown that 96 samples can be reduced, alkylated and digested in 3 h using a four sonotrode-based multiprobe [19]. In addition this time could be reduced to tens of seconds using a 96 sonotrode-based probe [19]. Recently, the company Digilab has introduced in its line of robotic platforms for proteomics applications, ProprepII (Disclaimer: specific company, product and equipment names are given to provide useful information; their mention does

not imply recommendation or endorsement by the authors) a multiprobe of four sonotrodes to speed the classic proteomics workflows comprising the steps of protein solubilisation/denaturation, protein reduction, protein alkylation and protein digestion.

Direct sonication must be used with low amplitudes (up to 50%), and when applied to both in solution or in gel workflows the following concerns are advised. In the case of in-solution preparation, high amplitudes of ultrasonication will lead to aerosol formation and eventually to cross-contamination of the samples. In the case of in gel-separated proteins, the main concern refers to disruption of the gel and eventually to the clogging of the LC column, if no preventive measures are adopted such as centrifugation or sample cleaning using, for example, ZipTips. In a similar manner, silica and sepharose particles are disrupted by direct sonication [20]. For this reason, other supports for enzyme immobilisation, such as glass or magnetic beads, should be preferably selected. It is advised to clean the sonotrode between samples to avoid contamination.

Further to the comments given above, an important issue that normally is forgotten by most users concerns to the sonotrode diameter. Each sonotrode is designed to work in a certain range volume. The minimum volume that can be treated with an ultrasonic probe is as low as 10 μl, which match the requirements of low sample volume demanded by most common proteomics applications.

Another point to remark deals with the shape of the reaction vessel, which should be conical-type and with the diameter as small as possible. This shape aids to rise up the liquid level allowing a deeper insertion of the sonotrode into the processed sample [21]. A dip insertion of the sonotrode avoids aerosol and foaming formation, which are a problem that, ultimately, leads to a lack in cavitation efficiency. The sonotrode must not touch the walls of the sample container; otherwise fracture of the sonotrode can occurs.

3.2. Indirect ultrasonication

Indirect ultrasonication can be performed with many different devices. It is noteworthy that ultrasonic baths, perhaps the most frequent ultrasonic tool found in research laboratories are not recommended to speed proteomics workflows as they deliver UE with low intensity, being not able to speed the protein digestion process conveniently [22]. Therefore, to apply indirect sonication the cup-horn or the sonoreactor are the recommended tools [12]. With these devices, it is possible to handle a minimum of six samples at a time, and many models have the possibility to attach a closed cooling water circuit to avoid heating of the sample. In addition, other advantages beyond high sample throughput are that (i) samples can be treated in a sealed container, thus avoiding to jeopardise important samples and allowing hazardous samples to be treated without external contact, and (ii) that an inert environment can be used, as oxygen can be purged off the sample before the ultrasonication takes place. In addition, there is no metal contamination risk as in the case of the use of the sonotrode, where the metal becomes in touch with the solution. It must be remarked that indirect sonication provides intensity 50 times lower than the direct sonication [15,23].

4. Ultrasonic energy in proteomics: where, when and how?

The range of application of UE in the field of proteomics is large: (i) enhancement of protein extraction from cells/biological tissues; (ii) reducing the use of interfering detergents; (iii) enhancement of protein extraction/solubilisation and (iv) enhancement of sample treatment for protein identification and protein quantification. In this section some general roles are provided about how to address correctly UE in a classic proteomics workflow.

4.1. Where should it be applied?

4.1.1. Protein extraction and solubilisation from complex samples

Many proteomics approaches are based on cell culture and cell lysate preparations. UE can be used for both cell lysis and protein resuspension after protein precipitation. In the first case, cells are washed with an appropriate buffer to remove any left culture media and then, mixed with the appropriate lysis buffer containing a protease inhibitor cocktail. The resulting cell suspension is then sonicated using an ultrasonic sonotrode, typically, three cycles of 15 s at 50–80% ultrasonic amplitude. Sonication helps to break the cell walls and thus freeing the cell content into the solution. However, for each cell type and buffer used, careful optimisation must be done to achieve optimal cell lyses. A Bradford or BCA assays, depending upon the lysis buffer composition, would be an effective way to measure protein concentration of the supernatant. Chan et al. [24] have shown that cell disruption and homogenisation with glass beads under the effects of an ultrasonic field results in higher protein recovery yields from cells. They applied ultrasonication on approximately 150 mg (dry weight) of *Prorocentrum triestinum*, a model causative agent of harmful algal blooms.

Xavier et al. [25] showed that lysis of cells in urea/thiourea solution followed by subsequent sonication to disrupt the nucleic acids, and protein concentration using ultrafiltration led to enrichment of proteins and minimal nucleic acid contamination. The 2D-GE obtained with UE extraction shows better resolution of spots in comparison with other methods were no UE was used.

Many sample preparation protocols include a step of protein precipitation prior downstream analysis. It is common to use organic solvents or strong acids to induce protein precipitation, leading to a protein pellet free of contaminants. However, complete resuspension of such protein precipitates are somehow difficult to achieve. In those cases, the application of 3–4 cycles of 15 s at 50% ultrasonic amplitude are in general enough to maximise protein solubility. Manadas et al. have shown that TCA-precipitated proteins can be solubilized with higher ratios of total protein recovery and reproducibility just using ultrasonication after TCA precipitation followed by elution in sample buffer. In addition, this approach improves reproducibility and matching ratios between gels when analysed by specialized software [26].

Epithelial cell behaviour is coordinated by the composition of the surrounding extracellular matrix, ECM. The study of the protein composition of ECM is of critical importance for understanding normal and disease states. Insights into the ECM proteome have been hampered by its low solubility. In addition, enzymatic cleavage of this proteome is difficult. Hansen et al. proposed a sample treatment that combines ultrasonication and surfactant assisted digestion [27]. When this method was compared with a traditional overnight digestion it was found that ultrasonication improves the sequence coverage for many proteins. In addition, hundreds of previously unidentified proteins were found.

In some applications UE can be used as a detergent helping to separate undesired compounds of the targeted analytes. As an example sugars and storage proteins are interfering compounds in the study of the starch granule proteome, but they can be easily removed from the surface of starch granules using a washing buffer solution in conjunction with ultrasonication [28].

4.1.2. Protein identification workflows

There are a number of different steps that can be undertaken during sample preparation, before a protein is analysed by MS. Depending on the sample origin and biological question to be

answered, these steps might include subcellular fractionation, protein extraction/solubilisation, protein fractionation, protein digestion, peptide fractionation and desalting. Both, direct and indirect ultrasonication can be used to accelerate and to enhance the protein(s) solubilisation, denaturation and digestion steps.

After gel electrophoresis, the protein spot/band of interest is excised and rinsed several times. Depending on the reagents used for gel staining the washing steps can be laborious and time consuming. The steps required to remove staining reagents from the protein spots/bands can be accelerated by the aid of ultrasonication. Using an ultrasonic bath (indirect ultrasonication) at 60–100% ultrasonic amplitude, 35 kHz, and 2–5 min of ultrasonication time, is in general, enough to remove the excess of dye from the gel [29]. After elimination of the interfering staining products, protein reduction and alkylation are common steps used to first reduce disulphide bonds of proteins and then to promote the alkylation of the formed –HS groups. Both steps are needed to prevent intramolecular and intermolecular disulphide bonds from forming between cysteine residues of proteins, thus allowing a better enzymatic cleavage of proteins. Reduction of the disulphide bonds is routinely done with up to 1-h incubation with dithiothreitol (DTT) and the alkylation of the cystines is conducted by incubating the sample with iodoacetamide (IAA) for up to 45 min in the dark. Direct and indirect ultrasonication, can be used to speed those steps to just a couple of minutes, with the same efficiency for both in terms of peptides matched and protein sequence coverage [22,23].

Protein digestion is by far the most time consuming step in most proteomics applications. It can be afforded in two different ways, between homogeneous or between heterogeneous phases.

Protein digestion in homogeneous (liquid-liquid) phases can be speed from the traditional overnight (up to 18 h) method to just some minutes with UE [16,17]. It must be noted that UE have a negative effect on the enzyme, inactivating it in a couple of minutes [18,23]. Therefore to achieve a complete and effective digestion of complex samples, such as cell lysates, addition of trypsin may be required during the digestion step.

Solid-liquid digestion of proteins (heterogeneous phases) can be done in two different manners. The protein is immobilised inside a gel and the enzyme is in solution, or the protein is in solution and the enzyme is immobilised in a solid support. In the first case, UE acts as a micro-syringe, helping the enzyme to penetrate into the small gel pores and getting into contact with the protein inside the gel [30,31]. It is important to choose the right amplitude for the ultrasound. For this application, by choosing an amplitude too low (10%) the enzyme might not be delivered into the gel and by choosing it too high (80%), UE will degrade the solid surface, gel or enzyme's solid support [19,20]. Solid, microscopic particles will interfere the downstream analysis, particularly when HPLC is connected in the pipeline by blocking the micro-capillary systems (auto sampler, column switching unit, pre-analytical column) [19]. When the proteins are in solution whilst the enzyme is immobilised in a solid support, the solid support must be carefully chosen, as some materials are easily degraded by UE (Fig. 1C) [20]. Recent findings also suggest that UE enhancement of protein digestion between heterogeneous phases is produced by mass transfer caused by cavitation, more likely [20].

4.1.3. Protein quantification workflows

Mass spectrometry-based strategies for protein quantification can be done in many different ways [32]. One of those strategies is based on the isotopic labelling of peptides with ^{18}O [32], which has recently drawn the attention of the proteomics community, because it can be used in an easy and straightforward way for protein differential expression studies, i.e., biomarker discovery, and for relative protein quantification [32].

The ^{18}O labelling of peptides can be done using two different labelling approaches named direct labelling (proteolytic labelling) and decoupled labelling (post-digestion labelling). In the direct approach, protein digestion and labelling are coupled and can be accelerated from overnight (12 h) to a couple of minutes with the aid of ultrasonic energy [33,34]. The labelling time can be substantially decreased but the disadvantage of this approach is that yields of labelling are generally below 80%. The labelling also lacks in reproducibility, having higher RSDs than the classic overnight labelling protocol. Different algorithms have been developed to overcome this problem [35–37]. In the decoupled procedure or post-digestion labelling, proteins are first digested, and then the sample is dried and finally reconstituted in ^{18}O water [38]. Even though the UE does not accelerate the labelling process, the use of ultrasonic energy in the prior steps (destaining of the gel piece, reduction, alkylation and digestion) accelerates the sample treatment pipeline significantly. The main advantage of decoupling the protein digestion and the labelling over the direct labelling is that a higher degree of peptides are efficiently double labelled, which is extremely important for the accuracy of the quantification. One interesting application of UE in heterogeneous phases recently reported entails the separation of proteins through gel electrophoresis and its quantification by ^{18}O labelling. The ultrasonic treatment of the gel piece is done for the reduction, alkylation and digestion steps. Then, the sample is dried and peptide reconstitution in ^{18}O water can be done with the aid of UE in an ultrasonic bath or using centrifugation and shaking. This methodology was successfully compared to the ELISA method for protein quantification [39,40]. The above described methodologies for ultrasonic ^{18}O based labelling are addressed for work on homogeneous phases; this is, digestion and labelling, decoupled or not, are done with the proteins and the enzyme dissolved in a liquid media. However, immobilised enzyme can be also used to label peptides. As it was explained in previous sections, UE must be carefully used because not all solid supports can be used to immobilise trypsin if ultrasonication is going to be used.

The absolute quantification of proteins separated by gel electrophoresis lacks accuracy and reproducibility [41]. To overcome those difficulties Wang et al. described a method called inverse labelling [42]. In this method two different states of the sample (disease and control) are labelled and then mixed with their unlabelled counterparts. Samples are analysed in parallel and by comparing the results of the two experiments, the differentially expressed proteins can be recognised through quick pattern recognition. The work combining the inverse labelling workflow and ultrasonic energy has been published [39,40]. The method has four main steps: (1) gel electrophoresis separation of the protein(s); (2) fast ultrasonic in-gel digestion of the protein(s); (3) ^{18}O-labelling through the decoupled method, and (4) quantification through selected peptides previously chosen using the ^{18}O inverse labelling approach and software specifically developed to select the peptides that will drive the quantification of the protein in an automated mode [40]. In brief, Inverse labelling is a designation or a workflow were the sample (S) and the internal standard (IS) are divided in two aliquots as follows: (i) S1 and S2; (ii) IS1 and IS2. Then samples are digested and incubated with $H_2{}^{16}O$ (S1, IS1) and $H_2{}^{18}O$ (S2, IS2). The sample S1 ($16O$) is mixed with IS2 ($18O$) and analysed. The sample S2 ($18O$) is mixed with IS1 ($16O$) and analysed. For these experiments it is expected to obtain an $18O/16O$ ratio for the mixture IS2/S1 and another ratio for the S2/IS1. Since the amount of protein in S1 and S2 is the same and IS1 has the same amount of proteins as IS2 the ratio IS2/S1 should be the same as $(S2/IS1)^{-1}$. Deviation on such ratio highlights those peptides that cannot be used for quantification.

4.1.4. Peptide mapping

Mass spectrometry peptide-based mapping of complex proteomes, also termed as fingerprinting or profiling, has gained momentum in proteomics. Carreira et al. have recently demonstrated that the acceleration of the classic proteomics workflow (solubilisation/denaturation, reduction alkylation and digestion)

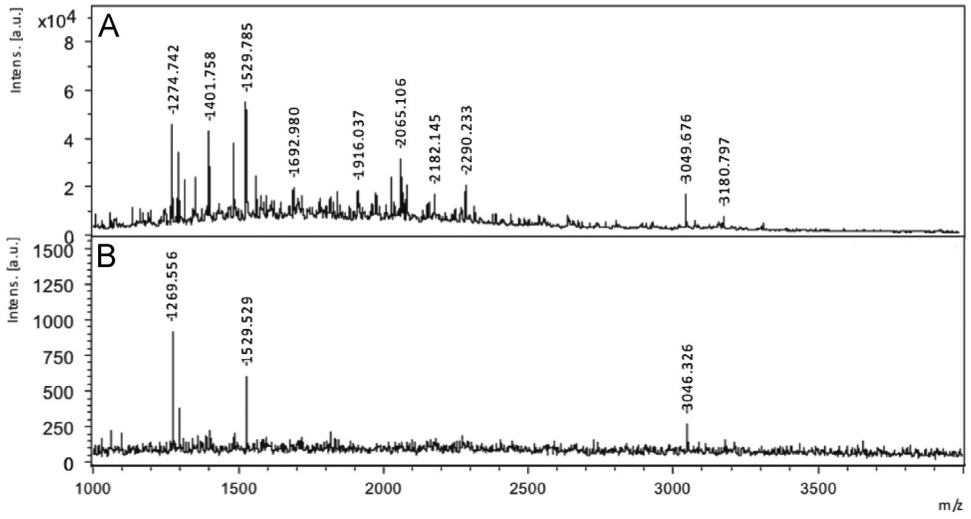

Fig. 2. MALDI spectra of liver tissue from *Mus musculus* digested with 40 ng/mm^2 of trypsin. (A) Digestion aided with UE, 50% UA, and 30 s UT. (B) Digestion done in 30 s without ultrasonication. Intensity scales are not proportional.

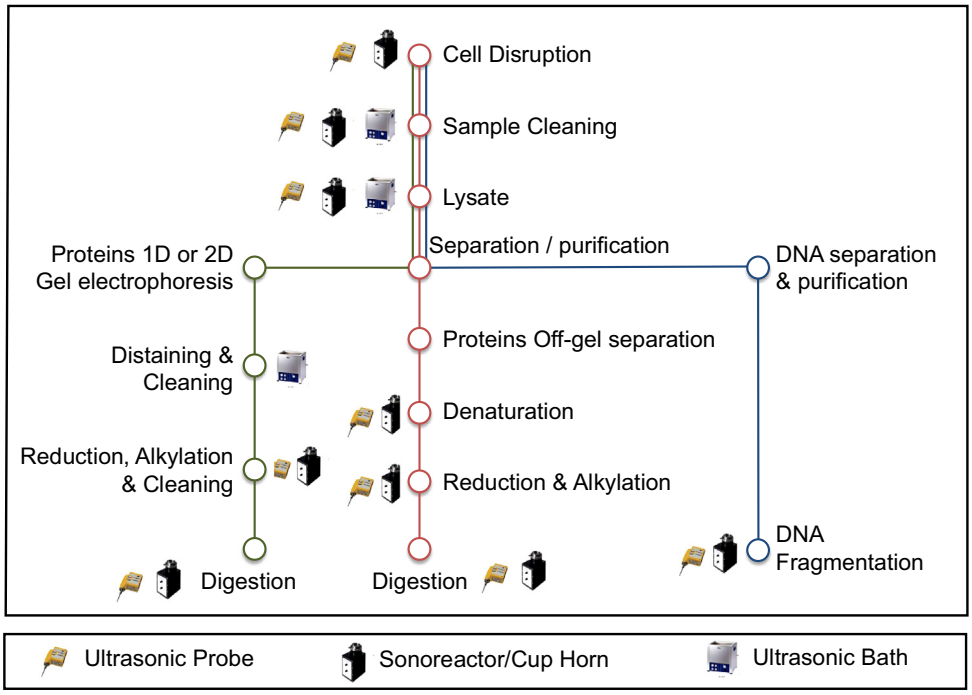

Fig. 3. Comprehensive scheme showing the ultrasonic devices that should be used in the different steps of most common proteomics workflows.

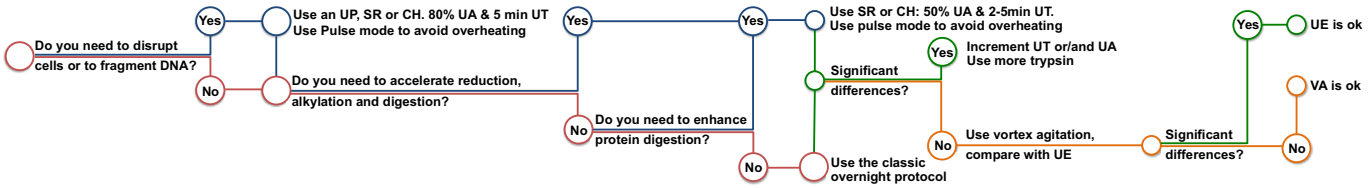

Fig. 4. Comprehensive scheme showing how to use ultrasonic energy in most common proteomics workflows. UA: ultrasonic amplitude; CH: cup horn; UT: ultrasonic time; IT: immobilised trypsin; IHIUE: indirect high intensity ultrasonic energy; SR: sonoreactor; VA: vortex agitation.

Table 1
Brief description of literature dealing with ultrasonication and proteomics.

Sample type	Conclusions/comments	[Ref.]
I. Solubilizing difficult samples with the aid of UE		
Prorocentrum triestinum, a model causative agent of harmful algal blooms	Ultrasonication applied on approximately 150 mg (dry weight) of *P. triestinum*. Cell disruption and homogenisation with glass beads under the effects of an ultrasonic field results in higher protein recovery yields from cells	[24]
TCA-precipitated proteins	TCA-precipitated proteins can be solubilized with higher ratios of total protein recovery and reproducibility just using ultrasonication after TCA precipitation followed by elution in a sample buffer. This approach improves reproducibility and matching ratios between gels when analysed by dedicated software	[26]
Protein composition of extracellular matrix, EMC	Sample treatment that combines ultrasonication and surfactant assisted digestion. Insights into the ECM proteome have been hampered by its low solubility. In addition enzymatic cleavage of this proteome is difficult. The use of UE helps to overcome those drawbacks. When this method was compared with a traditional overnight digestion it was found that ultrasonication improves the sequence coverage for many proteins. In addition, hundreds of previously unidentified proteins were found	[27]
Soybean proteins	Using UE to enhance the tryptic digestion of soybean proteins, it was successfully obtained the profiling of soybean peptides from transgenic and non-transgenic soybeans and from different pigmented beans commercialised as soybeans	[44]
Platinum drugs with proteins	Proteomics studies dealing with the characterisation of platinum drugs with proteins can be done performing the digestion process under the effects of an ultrasonic field without the destruction of the metal–protein complex. This finding opens new lines of work in metallomics as the time required for sample treatment in no more a bottleneck in this type of studies, allowing for the first time high sample throughput	[45]
II. In gel-based workflows		
Adenylylsulfate reductase alpha subunit from a cell culture of *desulfovibrio desulfuricans* ATCC27774	Remarkably, whole sample treatment time was reduced by shortening the digestion time from 12 h to only 2 min	[50]
Characterisation of a new allergen from the *Cupressus arizonica*	Ultrasonic energy was utilised for speeding up gel-based workflows	[51]
Protein containing a new heterometallic Mo–Fe cluster in *Desulfovibrio alaskensis*	Ultrasonic energy was applied for speeding up the enzymatic cleavage After the UE aided digestion the Mo–Fe cluster was still intact While the UE can be used to lyse cells, it can be also used in a more settled way with less destructive power	[52]
Digestion of proteins extracted from plants, namely *Hordeum vulgare* and *Arabidopsis thaliana*	The effectiveness of protein digestion using infrared energy and ultrasonic energy versus the traditional 16 h at 37 °C sample treatment is compared in the digestion of proteins extracted from plants. It was concluded that ultrasonic energy is a method of comparable performance in terms of protein identification to the traditional 16 h-digestion methods	[53]
III. Off gel-based workflows		
Macrophages RAW 264	Proteomic analysis for protein identification was done following a shotgun approach. Sample treatment was based on the use of direct high intensity ultrasonic energy Cellular extracts were denatured in 8 M urea, reduced (3 min), alkylated (3 min) and digested with trypsin (1 min) under the effects of ultrasonic energy	[54]
Split soret cytochrome c from D. desulfuricans ATCC27774	A clean method for protein identification that avoids the use of detergent and/or urea in the solubilisation of proteins, and that does not need desalting with zip-tips was proposed through the identification of the protein split soret cytochrome *c* from *D. desulfuricans* ATCC27774 and five model proteins with masses comprised between 14.4 kDa and 97 kDa. The purified proteins were solubilized in a mixture of water/acetonitrile and sonicated for 1 min at 50% ultrasonic amplitude. Sample cleanup before MS is not needed	[17]
Whole cell lysate	The development and validation of a spectral library searching method for peptide identification from MS/MS data is described, This library was done using a whole cell lysate that was first reduced, then alkylated and finally digested with trypsin using UE provided by an ultrasonic probe	[55]
Seafood species	Systematise the use of ultrasonic energy with probe to shorten sample treatment time and to simplify sample handling in their approaches to food chemistry. In this case for seafood identification. Selected tandem mass spectrometry ion monitoring, SMIM, was used in combination with an ion-trap mass spectrometer in a shotgun proteomics approach for the fast identification of seafood species. Using this methodology the authors were capable to classify seven commercial, closely related, species of Decapoda shrimps and also all commercial fish species belonging to the *Merlucciidae* family. The proposed methodology makes use of high intensity focused ultrasound-assisted trypsin digestion for ultra fast sample preparation. Peptide separation and identification by reverse phase capillary LC coupled to an ion-trap working in the SMIM scanning mode was then done	[56,57]
Detection of parvalbumins beta allergens in fish	The rapid and direct detection of parvalbumins beta allergens in fish The proposed methodology is based on the purification of β-PRVBs by treatment with heat, the use of accelerated in-solution trypsin digestion under an ultrasonic field provided by High-Intensity Focused Ultrasound and the monitoring of only 19 β-PRVB peptide biomarkers by Selected MS/MS Ion Monitoring (SMIM) in a linear ion trap (LIT) mass spectrometer	[58]
IV. Shortening ^{18}O labelling protocols with the application of UE (protein quantification)		
Shortening ^{18}O based labelling protocols	Different ultrasonic devices have been tested to speed the direct ^{18}O labelling of proteins. The UB, the UP and the SR were tested and it was found that SR yielded the best results yet with a worse performance than the classical overnight protocol, specially for peptides with masses over 1500 Da. In order to avoid misinterpretations of the data different algorithms have been developed to aid the data interpretation	[34,43]
Immobilised trypsin used to digest and to label peptides	It has been suggested and later confirmed that UE could help to enhance digestion and labelling through immobilised trypsin. There are few drawbacks when using immobilised trypsin. First, the	[20,59]

Table 1 (*continued*)

Sample type	Conclusions/comments	[Ref.]
	common solid supports to immobilise trypsin will not tolerate the ultrasonic energy but will disrupt. Second, ultrasonic energy will inactivate the immobilised trypsin, thus making of the immobilised trypsin a single use item	
Protein quantification in proteins separated by mass spectrometry. Use of the ^{18}O inverse labelling method	The absolute quantification of proteins separated by gel electrophoresis lacks accuracy and reproducibility. The inverse labelling method overcomes these difficulties The method has four main steps: (1) gel electrophoresis separation of the protein(s); (2) fast ultrasonic in-gel digestion of the protein(s); (3) ^{18}O-labelling through the decoupled method, and (4) quantification through selected peptides previously chosen using the ^{18}O inverse labelling approach and software specifically developed to select the peptides that will drive the quantification of the protein in an automated mode	[39,40]

can be also used for the fast mapping of complex samples, such as plasma [43]. This remarkable finding has been also reported by Domínguez-Vega et al. in the chromatography-based mapping of peptides from soybean proteins. Using UE to enhance the tryptic digestion of soybean proteins, they successfully obtained the profiling of soybean peptides from transgenic and non-transgenic soybeans and from different pigmented beans commercialised as soybeans [44].

4.1.5. Characterisation of metal–protein complexes

Moreno-Gordaliza et al. [45] have demonstrated that proteomics studies dealing with the characterisation of proteins, which react with platinum drugs, can be done performing the digestion process under the effects of an ultrasonic field without the destruction of the metal–protein complex. This finding opens new lines of work in metallomics, allowing for the first time high sample throughput.

4.1.6. Imaging mass spectrometry—IMS

Nowadays tissue analysis by mass spectrometry is a technique that has been gaining importance among the scientific community [46,47]. Imaging mass spectrometry, IMS, is able to generate images from chemical information from a tissue sample. Literally proteins, peptides lipids and other molecules are turned into pictures [48]. UE has been applied in our lab to speed tissue digestion for MS profiling from 3 h to just 30 s opening this way sample treatment of tissues to high throughput (see Fig. 2). As can be seen in Fig. 2, excellent results in terms of digestion were obtained in just 30 s whilst when no ultrasonication was used enzymatic cleavage was almost negligible [49].

4.2. When should it be applied?

Fig. 3 depicts most common proteomics workflows and it includes recommendations about the steps in which UE should be applied. The UE can be used from the very beginning of a sample workflow. Indeed, when cell well disruption is required, the simplest way to do it is using high intensity UE. Generally this is preferentially done with sonotrodes at high intensities, normally 80–100%. Although less known to this application, high intensity indirect UE provided by a cup-horn or by the sonoreactor can be also used to disrupt cells. In this case the use of 100% UA is recommended. Once the content of the cells has been released into solution different approaches are used to separate and isolate the part of the proteome wanted. In these steps, sample drying and subsequent resuspension is frequently required. In such cases, resuspension can be done with the aid of UE, which facilitates the process.

Reduction, alkylation and digestion of single proteins or whole proteomes are done using the cup-horn or the sonoreactor. When possible, cup-horn or sonoreactor should be the tools chosen as they allow for high sample throughput whilst helping to avoid cross contamination (sealed treatment). Protein cleavage is not properly accelerated with the ultrasonic bath [22].

4.3. How should it be applied?

As a general role, of all variables affecting ultrasonic energy application, UA should be carefully controlled. The high amplitudes (above 60%) are only recommended for cell disruption, and for this application the ultrasonic bath should not be used. For any other applications, indirect high intensity UE provided by the cup horn or the sonoreactor, is recommended. The sonotrode, the sonoreactor or the cup-horn are the devices recommended for proteomics under the following general conditions: (1) ultrasonic amplitude: 50%; (2) ultrasonication time: up to 5 min, for the cup-horn and for the sonoreactor, 1–2 min for the sonotrode: (3) ultrasonic frequency 20–40 kHz. It is important to avoid overheating of the sample during sonication, especially for longer sonication times (5 min). In such cases, it is advised to apply US in two cycles of 2.5 min. A comprehensive diagram depicting how to apply UE is presented in Fig. 4. In addition, Videos 1–3 given in supplementary material are also provided to help in the implementation of proteomics workflows in the proteomics lab.

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.qbiomech.2009.12.001.

5. Applications

Table 1 shows the main applications currently available in literature dealing with ultrasonic energy and its application in proteomics. This includes shortening protein identification-based workflows, either in-gel-based or off gel-based; the shortening of ^{18}O based labelling protocols for protein quantification; peptide mapping; the solubilisation of difficult samples for the characterisation of metal–protein complexes and the simplification of sample treatment for imaging mass spectrometry—IMS

6. Final remarks and future prospects

The use of UE in proteomics is gaining momentum in Proteomics. This method was initially addressed to speed the enzymatic digestion of proteins and whole proteomes. Since then, new applications have been reported regularly and constantly,

including fast protein identification, fast protein quantification, fast mapping of proteins and fast in-situ digestion of tissues. Different research teams have addressed the uses of UE here reported, such as protein identification and protein quantification, being achieved similar results. This confirms the robustness of ultrasonic-based sample treatments. However, other approaches described are relatively new. Therefore, new papers validating those ones are anticipated. Some potential applications of ultrasonic energy in proteomics are still to be reported, such as studies dealing with protein phosphorylation, protein glycosylation, and protein quantification with other labelling reagents than ^{18}O water, high throughput peptide mapping and bacterial identification.

7. Conclusions

Today in day ultrasonic energy can be considered as a powerful tool to speed most of the worldwide daily used proteomics workflows. Steps such as cell disruption and protein extraction, protein solubilisation/denaturation, protein reduction, protein alkylation, protein digestion, isotopic labelling of peptides as well as tissue digestion can be done in an easy way if they are done with the aid of ultrasonic energy.

The ultrasonic bath, the sonotrode, the cup-horn as well as the sonoreactor, can be used to provide ultrasonic energy. However, on base on the expertise acquired during the last years working with ultrasonic tools, the use of the cup-horn or the sonoreactor are indeed recommended as it brings a number of advantages. Thus, high indirect sonication intensity and the possibility to work with the sample containers closed make of cup-horns and sonoreactor the ultrasonic tool to be chosen for proteomics.

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