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Indirect ultrasonication for protein quantification and peptide mass mapping through mass spectrometry-based techniques

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ABSTRACT

We report in this work a fast protocol for protein quantification and for peptide mass mapping that rely on ^{18}O isotopic labeling through the decoupling procedure. It is demonstrated that the purity and source of trypsin do not compromise the labeling degree and efficiency of the decoupled labeling reaction, and that the pH of the labeling reaction is a critical factor to obtain a significant ^{18}O double labeling. We also show that the same calibration curve can be used for MALDI protein quantification during several days maintaining a reasonable accuracy, thus simplifying the handling of the quantification process. In addition we demonstrate that ^{18}O isotopic labeling through the decoupling procedure can be successfully used to elaborate peptide mass maps. BSA was successfully quantified using the same calibration curve in different days and plasma from a freshwater fish, *Cyprinus carpio*, was used to elaborate the peptide mass maps.

1. Introduction

The quantification of proteins is a trade-off in numerous research studies, such as post-translational modifications, the searching of biomarkers for clinical diagnosis or prognosis or the development of new drugs [1–3]. Mass spectrometry is nowadays regularly used to quantify proteins in an absolute or relative manner through different approaches. Methods such as stable isotope-labeling with amino acids in cell culture (SILAC), isotope-coded affinity tagging (ICAT), isobaric tags for relative and absolute quantification (iTRAQ), and ^{18}O labeling have all been used in quantitative approaches. In addition, heavy isotope labeled peptides can be used to obtain absolute quantitative data. More recently, label-free methods for quantitative proteomics, which have the potential of replacing isotope-labeling strategies, are becoming popular [4].

Isotopic labeling of proteins with ^{18}O is a simple way to perform studies regarding differential protein expression levels, or protein quantification, among others. The isotope ratios of non-labeled to labeled peptides in the combined peptide mixtures are measured by mass spectrometry. Signal intensities of paired peptides provide relative quantification of their precursor proteins in the different pools; unpaired peptide signals indicate potential changes in amino-acid composition, caused by mutation or co/post-translational modifications [5].

^{18}O-labeling can be done in two manners [6]. On the one hand, the protein cleavage and the labeling process are completed at the same time; this procedure is commonly referred as direct ^{18}O-labeling. On the other hand, the protein cleavage and the labeling reactions are done in different steps; this procedure is named as decoupling ^{18}O-labeling. The reactions that take place during the labeling process are well known [5], the labeling is easy to be done and it only requires the use of ^{18}O-water and the presence of an enzyme, usually trypsin. Briefly, this is a two stage reaction comprised of a first step, where the enzyme forms an ester intermediate with the peptide and promotes the amide bond hydrolysis by incorporating one ^{18}O atom from $H_2{}^{18}O$ in the solvent; and a second step known as the carboxyl oxygen exchange reaction, where the enzyme forms another ester with the newly formed peptide and catalyzes the incorporation of the second ^{18}O atom. Although this type of protein quantification has recently received much attention in the research arena [6–10], there are still a number of shortcomings with this methodology. Thus, the direct labeling lacks in efficiency and accuracy, since the labeling and the degree of ^{18}O incorporation (1 ^{18}O or 2 ^{18}O) are peptide-dependant [6,11]. On the other side, the decoupled labeling allows better labeling efficiency

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and double ^{18}O incorporation, but it lacks in speed since the sample needs to be dried in a vacuum centrifuge several times [12]. Finally, immobilized trypsin can be also used for ^{18}O-labeling in a fast and efficient fashion, but in general time-consuming desalting/cleaning steps are required [13]. In addition, immobilized trypsin is an expensive reagent.

Recently, ultrasonic energy, UE, has emerged as a powerful tool in sample treatment for proteomics [14–19]. Previous studies have successfully used UE to reduce the sample treatment time for protein identification through mass spectrometry techniques and peptide mass fingerprint from 12/24 h to 8 min [20]. Notably, not only time was saved, but also the handling was simplified since there is no need to use high salt concentrations or chaotropic agents. Furthermore, UE has also been applied in the enhancement of the sample treatment for ^{18}O-labeling in both the decoupling and direct approaches [12,15].

In the present work we report for the first time on the study of several variables affecting the ^{18}O-labeling under the effects of an indirect ultrasonic field. The proposed methodology uses sonoreactor-based ultrasonic energy to simplify and to increment the speed and throughput of ^{18}O-labeling of proteins and complex proteomes. In addition we also demonstrate that the same calibration curve can be used in MALDI to quantify proteins in different days with a reasonable accuracy.

2. Experimental

2.1. Materials and reagents

All protein standards, DL-Dithiothreitol (DTT), iodoacetamide (IAA), calcium chloride ($CaCl_2$) and acetonitrile (ACN, 99.9%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Trypsin from porcine pancreas (proteomics grade, Catalog No. T6567) and trypsin from bovine pancreas (Catalog No. T8802) were also from Sigma. Ammonium bicarbonate (NH_4HCO_3, ≥99.5%), formic acid (~98%) and α-cyano-4-hydroxycinnamic acid (α-CHCA, ≥99.0%) were obtained from Fluka (Buchs, Switzerland). Ammonium acetate (NH_4CH_3COO) was ordered from Pronalab (Lisbon, Portugal) and trifluoroacetic acid (TFA, 99%) from Riedel-de Haën (Seelze, Germany). $H_2{}^{18}O$ (97% ^{18}O abundance) was obtained form ISOTEC™ (Miamisburg, USA) and ZipTip® packed with C18 reversed-phase were from Millipore (Bedford, MA, USA). ProteoMass™ Peptide MALDI-MS Calibration Kit (MSCAL2) from Sigma was used as mass calibration standard for MALDI-TOF-MS.

2.2. Apparatus

Protein digestion and labeling was done in safe-lock tubes of 0.5 ml from Eppendorf (Hamburg, Germany). 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. Milli-Q natural abundance ($H_2{}^{16}O$) water was obtained from a Simplicity™ 185 from Millipore (Milan, Italy). A vacuum centrifuge from UniEquip (Martinsried, Germany) model UNIVAPO 100H with a refrigerated aspirator vacuum pump model Unijet II was used for sample drying and sample pre-concentration.

The Sonoreactor, model UTR200, from Hielsher Ultrasonics (Teltow, Germany) was used as source of ultrasonic energy. The sonoreactor provides indirect ultrasonication and can be considered a small high-intensity ultrasonic water bath. Up to six samples can be processed in sealed tubes or vials eliminating aerosols and cross-contamination. Like a traditional ultrasonic cleaning tank, the UTR200 (200 W, 24 kHz) sonicates from the bottom, but at a 50 times higher intensity [21].

2.3. Protein digestion

In-solution digestion was performed with a urea-free procedure as previously described with minor modifications [20]. Briefly, a stock solution of BSA, ovalbumin or α-lactalbumin was prepared in 25 mM ammonium bicarbonate buffer (pH 8.25) using natural abundance water and then mixed with ACN in a 1:1 ratio. To speed up protein denaturation in the organic solvent the sonoreactor was used at 50% amplitude to sonicate the solution for 1 min. Reduction was performed with DTT (10 mM) and alkylation was done with IAA (50 mM). In both cases the sonoreactor (50% amplitude) was used for 1 min to accelerate the respective reactions. Aliquots of 5 μl, corresponding to 15 μg of protein, were taken and diluted to a final volume of 20 μl with 12.5 mM ammonium bicarbonate to decrease the DTT and IAA concentrations. Trypsin was added (1:20 (w/w) enzyme-to-protein ratio) to a final volume of 24 μl and the protein solutions were sonicated with the sonoreactor during two intervals of 2.5 min at 50% of the maximum ultrasound amplitude. To stop the enzymatic reaction 2 μl of formic acid 50% was added. Finally, the samples were dried by vacuum centrifugation.

2.4. Post-digestion ^{18}O labeling

2.4.1. pH influence in the ultrasonic post-digestion labeling reaction

A stock solution of ovalbumin was prepared in ammonium bicarbonate 25 mM. After denaturation, reduction, alkylation and digestion with proteomics grade trypsin from porcine pancreas as described above, the 15 μg dried protein digests were dissolved in 10 μl of calcium chloride (50 mM), 10 μl of acetonitrile (20%) and 10 μl of ammonium acetate (100 mM, pH 6.75) with proteomics grade trypsin in a 1:40 (w/w) enzyme-to-protein ratio. A different set of samples was dissolved in 10 μl of natural abundance water/acetonitrile (4:1 ratio) with proteomics grade trypsin (1:40 (w/w) enzyme-to-protein ratio). The samples were dried again and finally resuspended in 10 μl of $H_2{}^{18}O$ (97% ^{18}O abundance) and sonicated with the sonoreactor in two intervals of 2.5 min. Trypsin (1:40 (w/w) enzyme-to-protein ratio) was added to the protein samples after the first ultrasonication interval. The labeling reaction was stopped by adding TFA till 1% (v/v).

2.4.2. Influence of the type of trypsin used in the ultrasonic labeling reaction

Aliquots of 15 μg of BSA or α-lactalbumin were in-solution digested, as explained above, with proteomics grade trypsin from porcine pancreas (TP1) or trypsin from bovine pancreas (TP2). The dried digests were then recomposed in 10 μl of 50 mM calcium chloride, 10 μl of 20% acetonitrile and 10 μl of 100 mM ammonium acetate (pH 6.75) with TP1 or TP2 in a 1:40 (w/w) enzyme-to-protein ratio. After drying again by centrifugal evaporation, the samples were resuspended in 10 μl of either $H_2{}^{16}O$ or $H_2{}^{18}O$ (97% ^{18}O abundance) and sonicated with the sonoreactor (50% amplitude) during 2.5 + 2.5 min. TP1 or TP2 (1:40 (w/w) enzyme-to-protein ratio) were added to each sample after the first ultrasonication interval. To stop the labeling reaction trifluoroacetic acid was added to 1% (v/v).

2.5. Calibration curve

A stock solution of BSA was prepared in 25 mM ammonium bicarbonate and diluted to 7.2, 6.0, 4.8, 3.8, 3.4, 3.1, 2.4, 1.9, 1.7, 1.4, 1.0 and 0.5 μg/μl. Following the sample treatment described above, the samples were in-solution digested and ^{16}O-labeled during (2.5 + 2.5) min with the sonoreactor (50% amplitude) in natural abundance water media. At the same time a 5 μg BSA digest was ^{18}O-labeled in $H_2{}^{18}O$ (97% atom abundance) and spiked into the

^{16}O samples before the MALDI-TOF-MS analysis. A calibration curve with at least 8 data points based on the $^{16}O/^{18}O$ ratio was made for each one of the following tryptic peptides: YLYEIAR ([M+H]$^+$ 927.49 m/z); RHPEYAVSVLLR ([M+H]$^+$ 1439.81 m/z); and LGEYGFQNALIVR ([M+H]$^+$ 1479.80 m/z). Four replicates were made for each point of the calibration curve, and the ratios $^{16}O/^{18}O$ were calculated as described below in the isotopic deconvolution section.

2.6. Ultrasonic ^{18}O-labeling: a case study

Cyprinus carpio blood was collected from the caudal vein and centrifuged at 4000 × *g* for 15 min (4 °C) to obtain the plasma. Then an 80 μl aliquot of plasma was diluted to 100 μl with phosphate-buffered saline (PBS, pH 7.4) and 5 vols. of cold acetone were added. After one day at −20 °C, the samples were centrifuged at 10,000 × *g*, for 30 min (4 °C), the supernatant was removed and the pellet was air dried. For in-solution digestion, the pellet was suspended in 80 μl of ammonium bicarbonate 25 mM and divided into two aliquots of 40 μl. After addition of 40 μl of acetonitrile the samples were sonicated in the sonoreactor (50% amplitude) during 2 min. Reduction and alkylation were performed like described above but with the double ultrasonication time for each reaction (2 min). Protein digestion was performed during 5 min (2.5 + 2.5 min) in the sonoreactor (50% amplitude) with proteomics grade trypsin (1:20 (w/w) enzyme-to-protein ratio). Formic acid was added to 1% to stop the enzymatic digestion and the samples were concentrated to 10 μl in the vacuum centrifuge. Sample cleaning was performed before the labeling reaction with C18 reversed-phase ZipTip® as follows:

(i) *activation*—aspirate and dispense, A&D, 10 μL of acetonitrile (2×), then A&D 10 μL of 50% acetonitrile/0.1% TFA (2×), and then A&D 10 μL of 0.1% TFA (2×);
(ii) *peptide binding*—10 μL of sample (A&D the sample 20 cycles);
(iii) *washing*—A&D 10 μL of 0.1% TFA (3×).
(iv) *peptide elution*—10 μL of 90% acetonitrile/0.1% TFA (A&D the sample 20 cycles).

After cleaning the samples, the reagents used in the labeling reaction were added as described above. The digests were dried again in a vacuum centrifuge and finally dissolved in $H_2{}^{18}O$ (97% atom abundance) or in $H_2{}^{16}O$. Three labeling methods were tested: (a) overnight – 12 h (6 + 6 h) at 37 °C; (b) ultrasonication with sonoreactor – 10 min (5 + 5 min) at 50% amplitude; and (c) without ultrasonication – 10 min (5 + 5 min) at RT. The labeling reaction was stopped by adding TFA to 1% and the labeled peptides were analyzed by MALDI-TOF-MS.

2.7. MALDI-TOF-MS analysis

MALDI-TOF-MS spectra were obtained with a Voyager DE-PRO Biospectrometry Workstation model from Applied Biosystems (Foster City, USA), equipped with a nitrogen laser radiating at 337 nm. The analysis was performed after mixing the samples in a 1:1 ratio with the matrix solution of α-CHCA (10 μg/μl) prepared in 50% acetonitrile/0.1% TFA and hand-spotting 1 μl of each sample onto a MALDI-TOF-MS stainless steel well plate. Measurements were done in the reflector positive ion mode, with a 20 kV of accelerating voltage, 75.1% of grid voltage, 0.002% of 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 [M+H]$^+$: 757.3997, 1046.5423, 1533.8582 and 2465.1989, respectively). 300 laser shots were summed per spectrum. Spectra were processed using Data Explorer™ software (version 4.0) from Applied Biosystems.

2.8. Isotopic peak deconvolution

Isotopic peak deconvolution was done using the deisotope function of the Data Explorer™ software (version 4.0) from Applied Biosystems. This function is an advanced peak filtering method that uses a deisotoping algorithm to determine the relative abundance of multiple components with overlapping isotope distributions [22]. Thus, the deisotope function allows reducing a spectrum to a centroided plot by deconvoluting the monoisotopic peaks from the peak list. For each peak in a spectrum, the software inspects the peak list for the higher theoretical masses and areas associated with additional expected peaks in a theoretical isotopic cluster, relative to the peak in question. Moreover, for comparative purposes, in order to test the correct applicability of this function, the mathematical algorithm for deconvolution described by Yao et al. (Eq. (1)) was also used in the first steps of this work and in the calculation of the $^{16}O/^{18}O$ ratios used in the calibration curve [23]:

$$\left(\frac{^{16}O}{^{18}O}\right) = \frac{I_0}{I_4 - \frac{M_4}{M_0}I_0 - \frac{M_2}{M_0}\left(I_2 - \frac{M_2}{M_0}I_0\right) + \left(I_2 - \frac{M_2}{M_0}I_0\right)} \quad (1)$$

where M_0, M_2 and M_4 correspond to the theoretical relative intensities of the monoisotopic peak and the monoisotopic peaks with masses 2 Da and 4 Da higher, respectively; and I_0, I_2 and I_4 are the measured relative intensities of the first, the third and the fifth peaks in the isotopic cluster.

3. Results and discussion

The decoupled ^{18}O-labeling reaction is a two step process: (i) in the first part of the procedure the enzymatic cleavage is done in natural abundance water, which is then removed by drying the sample in a vacuum centrifuge to avoid the interferences of any remaining ^{16}O in the following labeling step with ^{18}O; (ii) in the second part of the procedure, the dried peptides are dissolved in $H_2{}^{18}O$ enriched medium and ^{18}O-labeled in the presence of an enzyme, generally trypsin. In a previous work we have studied the effect of time and ultrasonic energy in the labeling reaction, and the results showed that the labeling reaction, i.e. the second step of the decoupled procedure, could not be improved with ultrasonic energy [24]. Briefly, it was proved that BSA peptides could be double labeled with ^{18}O with the same yield as the overnight (6 + 6 h) labeling in just 5 min without ultrasonic energy. It was also concluded that better results could be obtained if the amount of enzyme used to catalyze the labeling reaction was divided in two. Therefore, one part of trypsin is added to the dried peptides before the labeling reaction, and the second part is added at the reaction's half time to maintain trypsin's activity. However, the influence of important variables, such as the pH effect and the type of trypsin used in the labeling reaction were not assessed when the reaction was performed with ultrasonication. Another important variable to take into account when performing ultrasonication is the temperature effect. It is known that when a liquid media is under the effect of an ultrasonic field the temperature rises. Therefore, in order to control the temperature and avoid sample overheating, all the experiments were performed in the sonoreactor with a water recirculation system.

3.1. The influence of the pH

It has been claimed in the literature [23,25] that the pH at which the isotopic labeling is done in the decoupling procedure is a critical factor to obtain highly (>90%) and reproducible double labeling. To evaluate the influence of pH in the labeling efficiency under the effects of an ultrasonic field, two different set of experiments were carried out. In one set, after the proteolysis of ovalbumin, the sample was resuspended in natural abundance

Table 1
Influence of buffer pH in the ^{18}O-labeling reaction of ovalbumin (15 μg) under the effects of an ultrasonic field. (a) ^{18}O-Labeling in ammonium acetate 100 mM buffer solution (pH 6.75); (b) ^{18}O-labeling in ammonium bicarbonate 25 mM buffer solution (pH 8.5). The labeling reaction was performed in the sonoreactor (50% ultrasonic amplitude) in two intervals of 2.5 min. Two shots of trypsin (1:40 (w/w) enzyme-to-protein ratio) were added: the first before ultrasonication and the second after the first 2.5 min period of ultrasonication ($n = 3$).

Peptide	$^{18}O_2$ (%)[1]	$^{18}O_{total}$ (%)[2]	
VYLPR $[M+H]^+$ = 647.39 m/z	64.94 ± 4.32	100	a (pH 6.75)
	*	*	b (pH 8.5)
HIATNAVLFFGR $[M+H]^+$ = 1345.74 m/z	90.81 ± 1.35	100	a (pH 6.75)
	76.93 ± 5.01	97.91 ± 1.76	b (pH 8.5)
GGLEPINFQTAADQAR $[M+H]^+$ = 1687.84 m/z	90.86 ± 0.73	99.17 ± 1.18	a (pH 6.75)
	90.96 ± 2.30	99.99 ± 0.01	b (pH 8.5)
ELINSWVESQTNGIIR $[M+H]^+$ = 1858.97 m/z	88.49 ± 0.19	99.23 ± 1.09	a (pH 6.75)
	32.59 ± 1.85	74.64 ± 2.12	b (pH 8.5)
LYAEERYPILPEYLQCVK $[M+H]^+$ = 2284.17 m/z	88.16 ± 1.53	100	a (pH 6.75)
	15.52 ± 5.45	32.52 ± 17.73	b (pH 8.5)

[1] % of double labeled peptides.
[2] % of single or double labeled peptides.
[*] Peptide not present in the spectra.

water/acetonitrile (4:1) (pH ca. 8). In the other set, the sample was resuspended in a solution of ammonium acetate 100 mM/calcium chloride 50 mM/acetonitrile 20% (pH c.a. 6.75). The samples were dried again, and dissolved in ^{16}O or ^{18}O water. The samples were recomposed in a mixture of acetonitrile/water, to facilitate the dissolution of hydrophobic peptides; since it has been claimed that solubilization of peptides is the rate-limiting step in the labeling reaction [5]. It can be seen in Table 1 that the percentage of peptides labeled with one or two ^{18}O (% $^{18}O_{total}$), was the same in both set of experiments, except for the two larger peptides, meaning that almost all peptides were at least single labeled with both protocols. However, the percentage of double labeled peptides (% $^{18}O_2$), was higher when the labeling was performed at pH of ca. 6.75. Under such conditions most peptides of ovalbumin were labeled with two ^{18}O atoms in a percentage higher than 90%, hence reflecting the pH as a critical parameter to obtain efficient and reproducible double labeling, even under the effects of an ultrasonic field. For further experiences the labeling was done at a pH of ca 6.75.

3.2. The influence of the type of trypsin

To test the importance of the type of trypsin in our methodology, two enzymes with different origins and purity were used: (i) proteomics grade trypsin from porcine pancreas (TP1); and (ii) trypsin from bovine pancreas (TP2). As can be seen in Table 2, results clearly show that the purest trypsin (TP1) does not have better performance when compared with the less pure trypsin (TP2). For instance, peptide fragment (YLYEIAR)H^+ −927.49 m/z from BSA presented a double labeling yield of 94% and 98% when labeled, respectively, with TP1 and TP2 under an ultrasonic field. Yet, peptide fragment (KVPQVSTPTLVEVSR)H^+ −1639.94 m/z, also from BSA, presented a double labeling yield around 84% when TP1 was used and 71% when TP2 was used instead. Regarding α-lactalbumin, the labeling degree obtained for the different peptides was, in general, higher when TP1 was used. In addition, no differences were found concerning the signal to noise ratio in the MALDI spectra, as it is showed in Figs. S-1 and S-2 of Supplementary Data. This result is important, if we consider the differences in the prices between both types of trypsin: the purest enzyme used here, trypsin from porcine pancreas, is nearly 1000 times more expensive than the trypsin from bovine pancreas.

For comparative purposes, results regarding the labeling degree obtained for samples labeled without ultrasonic energy are also presented in Table 2. In a general manner, similar double labeling yields were obtained with and without ultrasound. Peptide (ALKAWSVAR)H^+ −1001.59 m/z from BSA presents a double labeling yield of 92% with TP1 and 98% with TP2, regardless of the method used. The same similarity can be found for the majority of the peptides from BSA or α-lactalbumin. This is in agreement with already published results [24], where is stated that the labeling reaction in the decoupled procedure cannot be accelerated with ultrasound, even when a different type of trypsin is used, as described above.

3.3. Analytical applications

3.3.1. Protein quantification using a calibration curve

Different amounts of BSA, comprised between 1 and 15 μg were digested and then labeled in natural abundance water. At the same time a 5 μg BSA aliquot was labeled in 97% $H_2{}^{18}O$ with the same reaction conditions, to produce the internal standard that was spiked into each one of the ^{16}O samples before MALDI-TOF-MS analysis. Three tryptic peptides were monitored (YLYEIAR; RHPEYAVSVLLR; and LGEYGFQNALIVR) and a calibration curve was

Table 2
The influence of the purity and type of trypsin in the labeling degree ($^{18}O_2$%) of BSA (15 μg) and α-lactalbumin (15 μg). Results were obtained after labeling the dried digests of each protein with the sonoreactor at 50% amplitude during 5 min (2.5 + 2.5 min). Two types of trypsin were used: (i) proteomics grade trypsin from porcine pancreas (TP1); and (ii) trypsin from bovine pancreas (TP2). See Section 2 for detailed conditions ($n = 3$).

	Mass (m/z)	$^{18}O_2$ (%)			
		Ultrasound		No Ultrasound	
		TP1	TP2	TP1	TP2
BSA	927.49	93.82 ± 0.59	98.59 ± 1.99	96.24 ± 5.31	96.69 ± 4.68
	1001.59	92.14 ± 5.18	97.67 ± 3.29	92.67 ± 6.36	98.09 ± 2.70
	1439.81	85.89 ± 3.37	95.37 ± 1.91	91.63 ± 4.58	91.32 ± 0.93
	1479.80	90.17 ± 1.32	90.50 ± 0.21	90.75 ± 0.36	89.52 ± 2.68
	1639.94	83.27 ± 7.40	71.18 ± 3.10	86.01 ± 3.24	72.02 ± 1.35
α-Lactalbumin	710.33	69.89 ± 0.45	57.07 ± 3.61	71.92 ± 2.26	64.50 ± 2.68
	1200.65	22.11 ± 1.26	15.42 ± 3.31	22.57 ± 1.90	11.30 ± 0.54
	1669.94	9.48 ± 0.29	19.56 ± 12.58	9.57 ± 4.18	5.04 ± 3.56

Table 3
Calibration curves for protein quantification. Ten samples of BSA with different amounts of protein (comprised between 1 and 15 μg) were digested in natural abundance water and spiked with 5 μg of BSA labeled in $H_2{}^{18}O$ (97% atom abundance). After MALDI-TOF-MS analysis the $^{16}O/^{18}O$ ratios were calculated from the spectra with Eq. (1). Two calibration curves—C1 and C2 were derived for each one of the tryptic peptides: YLYEIAR; RHPEYAVSVLLR; and LGEYGFQNALIVR. Experimental values of two samples of BSA, 7.0 and 3.5 μg, reported on the table were calculated from the equations of the calibration curves for each one of the 3 tryptic peptides. The average value for the BSA concentration present in these two samples was calculated from the values obtained for each peptide monitored. Each data point in the calibration curve corresponds to 4 replicates.

Peptide	7.0	3.5	Expected (μg)	Calibration curve
YLYEIAR	6.8[a]	2.4[a]	Experimental (μg)	$y = 0.2174x - 0.5059$ ($R^2 = 0.9848$)[a]
([M+H]$^+$ 927.49 m/z)	6.5[b]	2.9[b]		$y = 0.2844x - 0.8066$ ($R^2 = 0.9836$)[b]
RHPEYAVSVLLR	6.6[a]	2.5[a]		$y = 0.3209x - 0.4915$ ($R^2 = 0.9888$)[a]
([M+H]$^+$ 1439.81 m/z)	7.0[b]	2.3[b]		$y = 0.3588x - 0.528$ ($R^2 = 0.9906$)[b]
LGEYGFQNALIVR	6.9[a]	2.5[a]		$y = 0.3047x - 0.405$ ($R^2 = 0.9756$)[a]
([M+H]$^+$ 1479.80 m/z)	7.3[b]	2.1[b]		$y = 0.2748x - 0.4584$ ($R^2 = 0.9728$)[b]
	6.8 ± 0.2[a]	2.5 ± 0.1[a]	Average (μg)[c]	
	6.9 ± 0.4[b]	2.5 ± 0.4[b]		

[a] Values calculated from the 1st calibration curve (C1).
[b] Values calculated from the 2nd calibration curve (C2).
[c] Average of the results obtained for all peptides.

made based on the $^{16}O/^{18}O$ ratios obtained for each one of the peptides. These peptides were chosen because they corresponded to the most intense peaks in the BSA spectra, thus having less interference of spectrum noise, and because the efficiency in double ^{18}O incorporation ($^{18}O_1/^{18}O_2$) and total ^{18}O incorporation ($^{16}O/^{18}O$) was higher than 90% in all of them. In the first part of this experiment two calibration curves were done in two different days. These curves were then used to calculate the amount of BSA of two target samples spiked with the same internal standard used for each calibration curve. No differences between the results obtained with the calibration curve 1 (C1) and the calibration curve 2 (C2) were observed in the calculated values for each target sample as it may be seen in Table 3. The average quantities of BSA calculated from C1 were 6.8 μg and 2.5 μg, and from C2 were 6.9 μg and 2.5 μg, which are very close to the expected 7.0 μg and 3.5 μg.

In the second part of this experiment we tried to demonstrate that a calibration curve done in one day could be used for further quantification during the same week, if the experimental conditions are carefully reproduced in the lab. This fact would simplify enormously protein quantification since it would not be necessary to do a daily calibration curve. Therefore, four different amounts of BSA (3.5; 4.0; 5.0 and 7.0 μg) were digested, labeled in natural abundance water and spiked with a 5 μg BSA ^{18}O internal standard. The results presented in Fig. 1 show a great concordance between the values calculated from C1 and C2. Regarding the accuracy of the quantification method, calculated values showed an error ranging from 15% to 25% of the expected value in all the BSA quantities tested. These findings suggest that it is possible to quantify proteins in MALDI using a very simple calibration curve methodology, and that the same curve can be used during different days. This makes possible protein quantification in different days, without having to repeat a new daily calibration curve, thus saving time in the analysis. In addition there is no need to use an old ^{18}O internal standard which might become degraded with storage time.

3.3.2. Peptide mass mapping

In order to test if our methodology for protein labeling could be applied to complex crude mixtures of proteins, the labeling of a plasma sample from *Cyprinus carpio* was attempted. It is important to stress here that we were not interested in protein identification at this point, but only in the study of the labeling efficiency of our procedure when applied to complex biological samples. With this in mind, proteins of a 40 μl fish plasma sample were recomposed in ammonium bicarbonate 25 mM after acetone precipitation. The digestion of each sample was done with 2 μg of trypsin in 5 min using the sonoreactor technology. After enzymatic digestion, all samples were concentrated to 10 μl and cleaned with

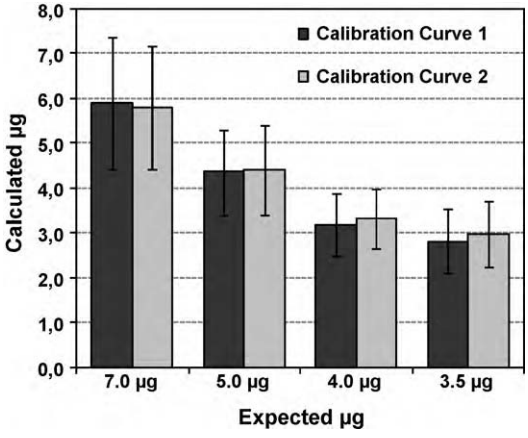

Fig. 1. Calibration curve robustness. Four samples of BSA, comprised between 3.5 and 7.0 μg, were digested and labeled in natural abundance water and then spiked with 5.0 μg of BSA labeled in ^{18}O (97% abundance) water. After MALDI-TOF-MS analysis, the $^{16}O/^{18}O$ ratios were calculated from the spectra with the Eq. (1). The expected values in μg are the average of the values obtained for the three tryptic peptides YLYEIAR; RHPEYAVSVLLR; and LGEYGFQNALIVR; based on the two calibration curves derived previously (see Table 3). RSDs were below 25% ($n = 4$).

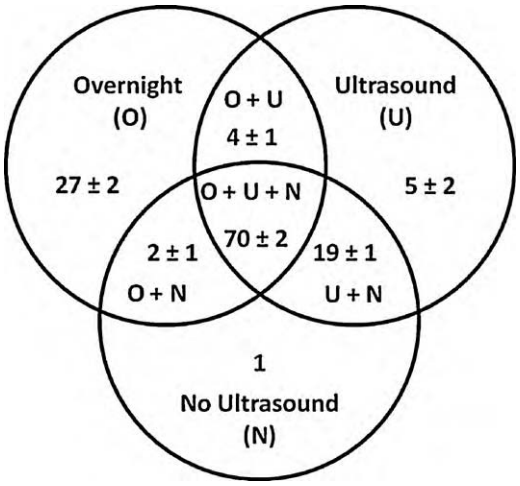

Fig. 2. Overlap of the *Cyprinus carpio* plasma peptides ^{18}O-labeled with the following different methods: (a) Overnight (O) at 37 °C (6 + 6 h); (b) sonoreactor (U) at 50% amplitude (5 + 5 min) and (c) no-ultrasound (N) at RT (5 + 5 min). See Section 2 for detailed conditions ($n = 3$).

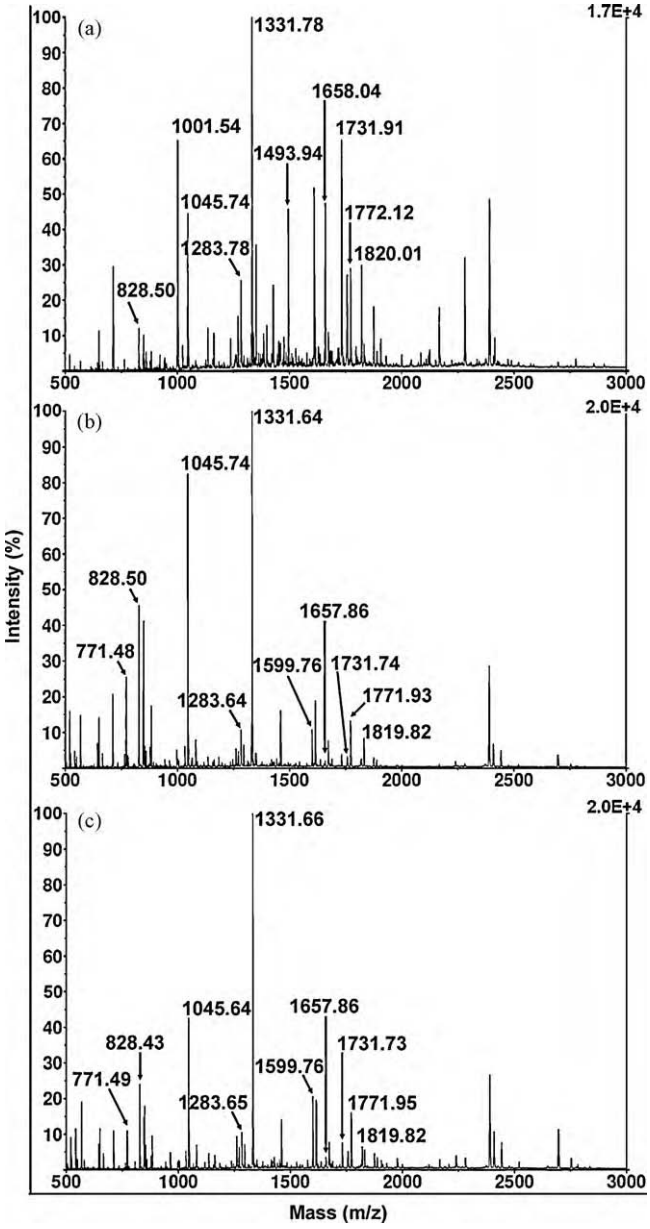

Fig. 3. MALDI mass spectra of *Cyprinus carpio* (~40 μg) plasma samples labeled using the following conditions: (a) overnight ^{18}O-labeling at 37 °C (6 + 6 h); (b) ^{18}O-labeling with the sonoreactor (50% amplitude; 5 + 5 min); (c) ^{18}O-labeling without ultrasound at room temperature (5 + 5 min).

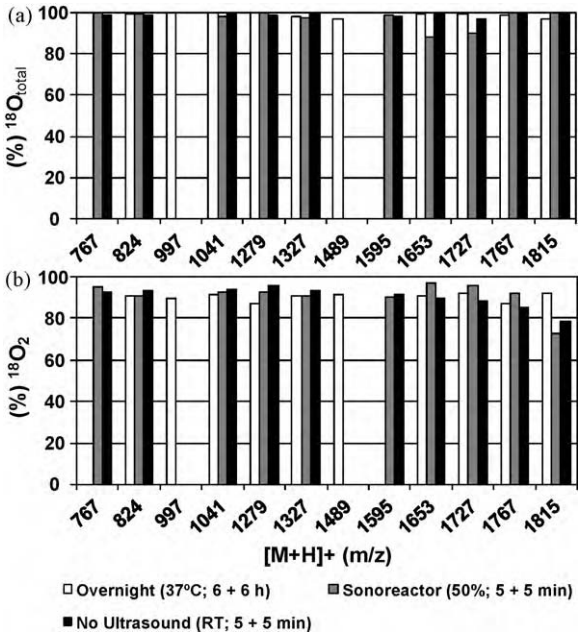

Fig. 4. Influence of the ultrasonic energy and time in the labeling of proteins from a *Cyprinus carpio* plasma sample. (a) Labeling efficiency – percentage of peptides with one or two ^{18}O atoms incorporated in the C-terminal carboxyl group (% $^{18}O_{total}$). (b) Labeling degree – percentage of double labeled peptides with ^{18}O in the C-terminal carboxyl group (% $^{18}O_2$). Three different methods were used for ^{18}O-labeling: (i) overnight labeling at 37 °C (6 + 6 h); (ii) sonoreactor labeling at 50% amplitude for (5 + 5 min.); (iii) labeling with no ultrasound at RT for (5 + 5) min (n = 2).

zip-tip to eliminate salts from biological origin. It is never too much to remember that no other protein purification procedures rather than acetone precipitation and zip-tip cleaning were performed, and no kind of protein separation by liquid chromatography or electrophoresis was done. In the second step of the decoupling procedure three labeling experiments were tested: (i) ^{18}O-labeling with no ultrasound at 37 °C for 12 h (overnight); (ii) ^{18}O-labeling with no ultrasound at room temperature for 10 min; and (iii) ^{18}O-labeling with ultrasonic energy provided by the sonoreactor for 10 min. At the same time and with the same experimental conditions plasma samples were labeled in natural abundance water. After a carefully inspection and comparison between MALDI-TOF-MS spectra of labeled and unlabeled samples (Figs. 2 and 3), we were able to find 103 labeled peptides for the overnight procedure (O), and 98 and 92 labeled peptides when ultrasound (U) and no-ultrasound (N) were used respectively. The searching and comparison of the peptides was done using dedicated software and all of them were manually verified. Of the 103 labeled peptides found during the overnight reaction, 27 were found exclusively in this treatment and 70 were common to the three methods, whilst 19 peptides were only found when the digestion was done during 10 min.

Concerning the labeling efficiency ($^{18}O_{total}$ %), we found that all peptides were single or doubly ^{18}O-labeled in a percentage higher than 98% (Fig. 4a) with no significant differences between the various methodologies. The results obtained for the labeling degree ($^{18}O_2$%) showed no major differences between the three methods, with a percentage of double ^{18}O ($^{18}O_2$%) incorporation higher than 90% in all peptides, even the peptides with higher m/z ratios (Fig. 4b). These very promising results show that it is possible to perform ^{18}O labeling in real and complex biological samples in only 10 min with a high labeling efficiency, making possible a faster protein relative differentiation in protein expression studies. In addition, it can be also concluded that ultrasonication is ineffective to improve the double ^{18}O incorporation, which is in agreement with the previous report results by our group [24].

4. Conclusions

This work clearly demonstrates that there is no need in performing the ^{18}O labeling reaction with the highest quality trypsin. The results obtained with proteomics grade trypsin from porcine and with trypsin from bovine pancreas, which is ca. 1000 times less expensive than the proteomics grade trypsin, were similar. In addition, even when the labeling reaction was performed with the bovine trypsin and with no ultrasonic energy the results presented no differences to the ones obtained with ultrasound.

Regarding the pH of the labeling reaction, it was observed that the percentage of double ^{18}O incorporation at the peptide's carboxyl group could not be improved by using ultrasonic energy when the pH of the reaction was c.a. 8, the same pH that is used in the

protein digestion step. Thus, the pH of the labeling reaction should be carefully controlled and a pH around 6.75 is recommended.

The results obtained after labeling a complex plasma sample from *Cyprinus carpio* showed that the ultrasonic energy is only useful to accelerate sample treatment in the first part of the decoupling procedure, where protein denaturation, reduction, alkylation and digestion take place. Doing this, the workflow for the decoupling ^{18}O labeling of proteins is reduced in 12–24 h, depending on the complexity of the sample.

Finally, concerning protein quantification, we have shown the robustness of our approach, where protein quantification is done trough a simple calibration curve. We also demonstrated that the same calibration curve can be used in several different days allowing protein quantification with an acceptable error.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2010.05.011.

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