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Determination of free methionine in human blood plasma by species-specific isotope dilution HPLC-ICP-MS using ^{34}S-labelled methionine.

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Abstract

A species-specific Isotope Dilution (ID) method is described for the determination of free methionine in human blood plasma by High Performance Liquid Chromatography - Inductively Coupled Plasma Mass Spectrometry (HPLC-ICP-MS) using ^{34}S-labelled methionine as species-specific spike. The ^{34}S-labelled methionine was obtained from yeasts grown in a medium enriched with ^{34}S (>91%) in the form of sulphate. Methionine was extracted from yeast using an enzymatic digestion with protease XIV followed by isolation using preparative reverse phase HPLC. Two separate batches of the labelled methionine standard were obtained. The ^{34}S-labelled methionine standard solutions obtained were characterised both in terms of ^{34}S isotope enrichment (82.7±0.6%) and total sulfur concentration (396 ± 6 µg/g) by reverse Isotope Dilution HPLC-ICP-MS using a natural abundance methionine standard solution and a multicollector instrument working in the pseudo-high resolution mode to avoid spectral interferences. Additionally, the identity of the ^{34}S-labelled methionine isolated by preparative HPLC and its isotope enrichment was confirmed by Gas Chromatography-Mass Spectrometry (GC-MS).

Human blood plasma samples were spiked with the ^{34}S-labelled methionine spike. Then, plasma proteins were precipitated with trifluoroacetic acid and separated by centrifugation. Methionine was separated from the rest of sulfur containing compounds by reversed phase chromatography in isocratic mode using a mobile phase of 75 mM ammonium acetate (pH 7.4) containing 2% of methanol. The retention time of methionine (8.3 minutes) was confirmed by fortifying the samples with natural abundance methionine and also by collecting the methionine peak and further analysis by GC-MS. Finally, methionine in human blood plasma samples was determined by measuring the signals for ^{32}S and ^{34}S in a double focusing ICP-MS instrument working at medium resolution (R=4000). Concentrations were calculated by integrating the methionine peak for both masses and applying the isotope dilution equation after mass bias correction. The recoveries for samples fortified at different concentration levels ranged between 98.4 and 100.5 %. Additionally, good agreement was obtained between the results found with this method and those reported by the clinical laboratory using a validated routine method.

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Keywords: species-specific isotope dilution, ^{34}S-labelled yeast, ^{34}S-labelled methionine, blood plasma samples, ICP-MS.

Introduction

The measurement of free methionine in blood is currently performed in clinical studies to detect aminoacidopathies in paediatric population[1,2], an inherited enzyme catabolic alteration affecting the metabolism of amino acids. The principal aminoacidopathy where methionine is involved is the hypermethioninemia (MET)[3], a disorder characterized by elevated concentrations of methionine in the body. MET patients usually do not show signs of the illness. However, if MET is untreated, it can cause learning delays, muscle weakness, and other health problems. On the other hand, methionine is also monitored in blood to evaluate the nutritional status of certain patients such as those with liver, kidney or neoplastic diseases[4], burned[5], with endocrine disorders[6], etc. Furthermore, in the last decades researchers have been studying the methionine dependency of tumour cells[7]. They found a link between the methionine concentration in blood and tumour growth. Different antitumor approaches have been applied drawing on this unique characteristic of tumour cells[8,9,10].

Quantification of methionine and other amino acids in biological, food and beverage samples has been carried out using a chromatographic separation, usually liquid chromatography with fluorescence[11], UV-VIS[12] or mass spectrometry[13] detection. One of the most popular methods for amino acid analysis, including methionine, is based on a liquid chromatography separation and post-column derivatization with ninhydrin. The coloured product formed can be detected by UV-VIS absorption[14].

As it is well known, the validation of these routine methodologies requires the use of standard reference materials, the comparison with other analytical methodologies, usually reference methods, or the participation in intercomparison exercises. In this sense, Isotope Dilution Mass Spectrometry (IDMS) is internationally regarded as a reference or highly qualified primary ratio method which has been used for the validation of clinical routine procedures[15]. However, if the advantages of IDMS are to be fully exploited, the use of a spike solution containing the compounds to be analysed in an isotopically labelled form (species-specific spiking) is required. To do that, the composition and structure of the species of interest must be exactly known in order to either synthesize the corresponding enriched species or acquire the proper spike solution if it is commercially available[16].

In our laboratory, a ^{77}Se-labelled selenomethionine standard was synthesised by growing yeast on a ^{77}Se-rich culture medium[17]. After harvesting, the yeast cells were disrupted and the cytosol content hydrolysed using Protease XIV. Finally, the ^{77}Se-labelled selenomethionine was isolated by anion exchange liquid chromatography from the protein digest and its concentration determined by reverse IDMS using a natural selenomethionine standard. The ^{77}Se-labelled selenomethionine standard was applied to the determination of selenomethionine in a selenized

yeast candidate reference material, and the results obtained were in good agreement with those reported by other laboratories[17].

Thereafter, yeast labelled with ^{34}S was also prepared in our laboratory by yeast growth on a ^{34}S-enriched specially prepared, culture medium in the absence of natural abundance sulfur[18]. The final product was characterised both with reference to isotope enrichment and total sulfur concentration by IDMS and isotope pattern deconvolution using a MC-ICP-MS instrument. The ^{34}S-labelled yeasts have been used directly in metabolic studies with laboratory animals such as rats[19] and mice[20]. The extraction of ^{34}S-labelled methionine from the yeast and its use as standard for species-specific IDMS has not been, as far as we known, previously employed for analytical purposes[21].

Consequently, the main objective of this research was the isolation and characterisation of ^{34}S-labelled methionine from yeasts in order to prepare a standard spike solution that could be used for species-specific IDMS. The identity of the species isolated, its isotope enrichment and concentration were evaluated by both ICP-MS and GC-MS. Once analytically characterised, the spike standard solution was applied to determine free methionine in human blood plasma and the results compared with the methodology currently used in the Clinical Biochemistry service of the Central Hospital of Asturias (HUCA, Oviedo, Spain) for the analysis of free amino acids in human blood plasma (Biochrom AAA, amino acid analyser).

Experimental

Reagents

A stock solution of 1000 mg/L of natural abundance S (as sulphuric acid in water) was purchased from Merck (Darmstadt, Germany). Further dilutions of this stock solution were made using ultra-pure water obtained from a Milli-Q system (Millipore Co., Bedford, MA, USA) to prepare the different working aqueous standard solutions as required. Enriched ^{33}S and ^{34}S were supplied from Cambridge Isotope Laboratories (Andover, MA, USA) as elemental powder. Stock solutions of about 1000 mg/L were prepared dissolving the elemental powder with nitric acid obtained by sub-boiling distillation and diluting with ultra-pure water. The standard spike solutions in the form of sulphate were characterized in terms of concentrations and isotopic abundances as described previously[18]. The natural abundance S and enriched ^{33}S and ^{34}S solutions were kept refrigerated at 4°C. A mixture of the different sulfur isotopes, containing ca. 30 mg/L of natural S, enriched ^{33}S and enriched ^{34}S was made by diluting the stock solutions using ultra-pure water. This mixture standard solution was employed for the configuration of the cups and the optimisation of conditions in the multicollector instrument as described previously[20]. On the other hand, a neutral pH enriched $^{34}SO_4^{2-}$ solution was prepared by neutralization of the stock solution with ammonia (Fluka Analytical, Buchs, Switzerland). This neutral solution was employed in the yeast growth procedure.

Natural abundance DL-methionine (>99%) was purchased from Sigma–Aldrich (St. Louis, MO, USA).Ammonium acetate and HPLC-grade methanol were purchased from Fluka Analytical and Merck, respectively. Both reagents were used to prepare the chromatographic mobile phases.

To carry out the enzymatic hydrolysis of the ^{34}S-labelled yeast, protease type XIV was supplied by Sigma–Aldrich.

Yeast cells were grown using high purity nutrients obtained from Sigma–Aldrich: D-(+)-Glucose, ammonium chloride, amino acids (arginine, glutamic acid, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, tryptophan and, valine), adenine, uracile, vitamins (thiamine hydrochloride, riboflavin 5´-monophosphate sodium salt, nicotinic acid, D-pantothenic acid hemicalcium salt, pyridoxine hydrochloride, (+)-biotin N-hydroxysuccinimide ester, myo-inositol, folic acid and 4-aminobenzoic acid), boric acid, potassium iodide, iron(III) chloride, sodium molybdate dehydrate, manganese(II) chloride tetrahydrate, zinc chloride, copper(II) nitrate hemi(pentahydrate), potassium phosphate monobasic, magnesium chloride hexahydrate, sodium chloride and calcium chloride dihydrate.

N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) with 1% tert-Butyldimethylchlorosilane (TBDMSCl) was purchased from Sigma-Aldrich. MTBSTFA and TBDMSCl were used as derivatizing and catalyst reagents in the GC-MS experiments, respectively.

Trifluoroacetic acid (ReagentPlus®, 99%) was purchased also from Sigma-Aldrich. This reagent was used to precipitate plasma proteins in the blood plasma samples.

Instrumentation

The multicollector inductively coupled plasma mass spectrometer (MC-ICP-MS) used was a Neptune Plus from ThermoFisher Scientific (Bremen, Germany) and was operated in the pseudo medium resolution mode. The instrument was equipped with 9 Faraday cups. The sample introduction system was a PFA-100 microconcentric nebulizer and a cyclonic spray chamber. The nebulizer gas flow rate, torch position and ions lens settings were optimized for higher sensitivity and the acquisition parameters were optimized for better precision of the measurements of the sulfur isotope ratios. Experimental conditions were similar to those described previously[20]. The double focusing inductively coupled plasma mass spectrometer (DF-ICP-MS) used was an Element II from ThermoFisher Scientific (Bremen, Germany), and was operated at the medium resolution mode (R=4000). All measurements were made with the standard sample introduction configuration of the instrument, that is, a Scott-type spray chamber working at room temperature, a Meinhard concentric nebulizer and a Fassel torch. The optimum instrumental settings for the measurement of sulfur isotope ratios in both instruments are summarized in Table 1.

The preparative isolation of the ^{34}S-labelled methionine was performed on an HPLC system (Agilent 1260 Infinity, Agilent Technologies, Santa Clara, CA) with an Agilent Zorbax Eclipse

XDB column (9.4x250 mm, 5µm) equipped with an analytical-scale fraction collector and a variable wavelength detector. Liquid chromatography separations for methionine characterisation and blood plasma samples were performed on a Surveyor LC Pump Plus (ThermoFisher Scientific, Bremen, Germany) using a Discovery BIO Wide Pore C18 reverse phase column (15 cm X 2.1 mm, 5 µm particle size, Supelco, Bellefonte, Pennsylvania, USA).

Methionine characterization by GC-MS was carried out using a gas chromatograph, Agilent 7890, coupled to a triple quadrupole mass spectrometer; Agilent 7000 Series Triple Quad GC/MS (Agilent Technologies, Wilmington, DE, USA) operating at 70 eV. The GC was fitted with a split/splitless injector and a DB-5 MS capillary column (cross-linked 5% phenyl-methyl siloxane, 30 m x 0.25 mm i.d., 0.25 µm coating).

A Spill Mill Plus from Analitik Jena AG (Jena, Germany) was used for the disruption of the yeast cells. A thermomixer compact from Eppendorf (Hamburg, Germany) was used to carry out the enzymatic hydrolysis and the derivatization for GC-MS analysis. A centrifuge 5810R D from Eppendorf were employed for the centrifugation of the samples. All samples were dried using a centrifugal vacuum concentrator (Genevac, Suffolk, UK) to remove water and organic solvents. All standard solutions were prepared gravimetrically using an analytical balance model AB204-S (Mettler-Toledo GmbH, Greifensee, Switzerland).

A pH-meter model Basic 20 from Crisson (Barcelona, Spain) was used for pH adjustment. The yeast growth was followed by measuring the optical density spectrophotometrically at 600 nm in a Spectronic 20 (Milton Roy).

The Biochrom AA analyser (Biochrom Ltd, Cambridge, UK), used in the clinical laboratory of HUCA, is a PC controlled automatic cation exchange liquid chromatograph with a post-column detection system based on the oxidative deamination reaction of the α-amino group with ninhydrin, liberating ammonia, carbon dioxide, an aldehyde with one less carbon atom and a reduced form of ninhydrin, hydrindantin. The produced ammonia reacts with the hydrindantin and another molecule of ninhydrin to yield a purple substance (Ruhemann's purple) that absorbs around 570 nm.

Procedures

Synthesis and isolation of ^{34}S-labelled methionine.

Saccharomyces cerevisiae (common baker's yeast), AMW13C strain, was used for the synthesis of ^{34}S-labelled yeast using a protocol developed in our laboratory[18] but with some changes that will be discussed later. Briefly, a synthetic culture medium was prepared avoiding the use of sulphates in order to achieve a culture medium low in natural abundance sulfur. Thus, manganese, zinc, copper and magnesium sulphates, usually used in this kind of culture media, were substituted by the corresponding chloride salts. The sulfur source was ammonium sulphate enriched in ^{34}S which was prepared in our laboratory as described above. The cells

grew up in a controlled temperature room at 28°C with agitation. The stationary phase of yeast growth was reached at 24 hours. Finally, cells were collected by centrifugation and rinsed with ultra-pure water to remove the excess of inorganic sulfur. The ^{34}S-labelled yeast was stored at -20°C until use.

The stored yeast cells were mechanically disrupted with beads in a mill for 90 seconds. Then, 0.15 g of yeast was hydrolysed with 10 mg of protease XIV in a Tris-HCl 0.1M pH 7.5 medium. Samples were incubated at 37°C during 24 hours. Hydrolyzed samples were further centrifuged (4000 g, 15 min) and the supernatant collected and stored at -20°C until use.

Finally, methionine was isolated by injecting the supernatant in a reverse phase HPLC column coupled on-line with a UV-VIS detector (set at 210 nm) followed by a fraction collector. The chromatographic conditions used are shown in Table 2. Fractions containing methionine from several injections were collected between 9-9.8 minutes. The collected fractions were mixed, dried, reconstructed in pure water, aliquoted in vials of 100 µL and stored at -80 °C until use. Using this strategy, the spike material demonstrated to be stable for at least two years. About 300 µg of methionine were obtained for each gram of yeast (dry weight) prepared.

Characterization of the purified ^{34}S-labelled methionine

The characterization of the purified ^{34}S-labelled methionine was carried out both in terms of purity, concentration and isotope composition. Purity was tested by injecting the isolated ^{34}S-labelled methionine standard in the reverse phase chromatograph with ICP-MS detection The concentration of sulfur in the ^{34}S-labelled standard was measured both by conventional IDMS and species-specific isotope dilution HPLC-MC-ICP-MS. To do that, the ^{34}S labelled methionine solution was diluted 1:100 with ultra-pure water. Then, a 1:1 mixture with a ^{33}S enriched (conventional IDMS) or natural methionine (species-specific isotope dilution HPL-MC-ICP-MS) standard solutions of 1 µg/g (as sulfur) were prepared. The ^{34}S/^{33}S isotope ratio of the mixture was measured in the multicollector ICP-MS instrument using the conditions given in Table 1. A natural abundance sulfur standard solution was measured prior to the mixtures for mass bias correction. Finally, the concentration of methionine was calculated using the isotope dilution equation described elsewhere[16]. The isotope composition of sulfur in the isolated methionine standard was also measured by multicollector ICP-MS.

On the other hand, confirmation of the presence of ^{34}S-labelled methionine and an alternative measurement of its isotope enrichment was carried out by molecular mass spectrometry using GC-MS. To do that, 200 µL of the stock solution were taken to dryness. The residue was dissolved in 150 µL of MTBSTFA in 1% TBDMSCl and incubated for 10 min at 60°C on a thermomixer. Finally, samples were centrifuged for 2 min at 14000g and the clear liquid was transferred into vials for GC-MS analysis using the conditions summarized in Table 2. The ^{34}S enrichment was calculated from the molecular isotope envelope using a procedure previously developed in our laboratory[22].

Methionine analysis in human blood plasma samples

Human blood plasma samples were supplied by the Clinical Biochemistry service of the HUCA. For methionine quantification, a weighed amount of the ^{34}S-labelled methionine solution was added and then plasma proteins were precipitated using an excess of 10 volumes of trifluoroacetic acid (TFA) for 1 volume of plasma. The mixture was brought to an ice bath for 15 min to favour protein precipitation. Then, the samples were centrifuged at 15000 g for 5 minutes and the supernatants were collected and stored at -20°C until use.

The analysis of methionine in the supernatant solutions was performed by reverse phase HPLC column coupled on-line with a double focusing ICP-MS. The chromatographic conditions used for the separation of methionine are shown in Table 2. Finally, human blood plasma methionine concentration was calculated using the isotope dilution equation[16].

An aliquot of the same blood plasma samples had been previously analysed on a Biochrom Amino Acid Analyser at HUCA, after a brief pretreatment consisting on fully deproteinisation with sulphosalicylic acid, addition of n-Leu as internal standard, pH adjustment to 2.2 with lithium hydroxide and filtration through a 0.2 µm membrane filter to remove any remaining particulate material. The obtained sample was injected from a temperature controlled autosampler onto a column of cation exchange resin. Lithium citrate buffers of varying ionic strength and pH from 2.8 to 3.55 are then pumped through the column to separate the various amino acids. The column temperature is accurately controlled and adjusted automatically as necessary to produce the separation. The column eluent is mixed with a ninhydrin reagent and then passed through the reaction coil, maintained at 138 ºC. From the reaction coil, the eluent/ninhydrin mixture is pumped to the photometer unit, which measures the amount of light absorbed at 570 nm and 440 nm. After each sample analysis, the column is regenerated by pumping a strong base (0.3 M lithium hydroxide) through de column followed by equilibrating buffer (pH 2.8), which adjusts the analytical column resin to its correct pH prior to the next analysis.

Results and discussion

Synthesis, isolation and confirmation of ^{34}S-labelled methionine

We have previously studied the optimal reagent quantities for the medium where yeast could growth[18] for the preparation of ^{34}S-labelled yeast. However, such studies were carried out on a small scale (50 mL). For the isolation of enough ^{34}S-labelled methionine from yeast cells it was required to scale-up the procedure. It was observed that, as the volume of the culture medium increased the isotopic enrichment of sulfur in the yeast decreased. To overcome this problem, several modifications in the culture medium were performed in order to increase de volume of the culture medium to 2 L with minimal reduction in the sulfur isotope enrichment. Thus, glutamic acid, phenylalanine, proline and serine were added to the mixture of amino acid and the amount of each amino acid was increased from 20 to 75 mg per litre. The nitrogenous bases uracil and adenine were also added (75 mg per litre). With this modified culture medium

an isotopic enrichment between 91% and 94% in ^{34}S was achieved in the twelve separated batches of yeast prepared. About 8 g of ^{34}S-labelled yeast were obtained from each batch of 2 litres of culture medium. The yeast obtained was mixed, homogenised and stored frozen at -20ºC until use. Enzymatic hydrolysis was done with protease XIV for the release of methionine from the yeast proteins. The amount of protease XIV was optimized by measuring the amount of methionine extracted. The range assayed was between 5 and 50 mg of protease XIV. The optimum value was found at 10 mg of protease XIV (for 150 mg of yeast). For example, Figure 1 shows one of the reverse phase HPLC-ICP-MS chromatograms obtained after the hydrolysis of yeast. The second peak in the chromatogram corresponds to ^{34}S-labelled methionine while the first peak could be cysteine and/or gluthatione as both compounds elute close to the dead volume under the experimental conditions used. The isotope enrichment for ^{34}S in methionine is higher than that of the first peak in the chromatogramwhile the isotopic enrichment of both sulfur-containing compounds is clearly lower than the original isotopic enrichment of ^{34}S in the yeast (91 to 94%) probably due to contamination with natural sulfur amino acids impurities from protease XIV. of a methionine standard

The final isolation of the ^{34}S-labelled methionine was performed by injecting the hydrolysed samples in a HPLC preparative system. Figure 2 shows the chromatograms obtained for a pure standard of natural abundance methionine (Fig. 2A) and the hydrolysed sample (Fig. 2B) detected by UV absorption at 210 nm. As can be observed, methionine eluted between 9-9.8 min (Figure 2A). Consequently, the system was set to collect fractions between 9-9.8 min. As it can be seen in Figure 2B, when the hydrolysed samples were injected, a chromatographic peak was also detected at the same retention time.

Final purity of the ^{34}S-labelled methionine spike was tested by injecting it in the reverse phase chromatograph with ICP-MS detection. As it can be seen in Figure 3, a single peak was obtained with retention time corresponding to that of a natural abundance methionine standard indicating that no other sulfur-containing compounds were present.

The confirmation of the identity of ^{34}S-labelled methionine isolated in the previous step was performed by GC-MS. The isolated sample and a natural abundance methionine standard were derivatised as described in the procedures. The total ion chromatograms showed that, methionine eluted at 27.9 minutes. Other peaks observed corresponded to side products of the derivatising reagent. The fragmentation patterns of the two methionine solutions at 27.9 minutes retention time are shown in Figure 4. Peaks at nominal masses 320, 292 and 218 in Figure 4A (standard of natural abundance methionine) correspond to fragments which contain sulfur because in Figure 4B (isolated ^{34}S-enriched methionine from yeast) they appear at nominal masses 322, 294 and 220 (2 units higher due to the presence of ^{34}S instead of ^{32}S).

Peaks at nominal masses 320 and 218 (322 and 220 in Figure 4B) correspond to the loss of C_4H_9 (-57 units) and $C_7H_{15}O_2Si$ (-159 units), respectively, probably following an α-homolitic cleavage. Peak at nominal mass 292 (294 in Figure 4B) could correspond to the loss of C_6H_{13} or C_5H_9O (-85 units in both cases) but the cleavage mechanism is unclear[23]. Other main peaks at

nominal masses 147 and 73 do not contain sulfur as they appear at the same m/z in both spectra. The high peak at nominal mass 73 could correspond to C_2H_7NSi formed from the neutral loss of $C_{11}H_{23}O_2SSi$ (-247 and -249 units for ^{32}S and ^{34}S, respectively) in the fragment of nominal mass 320 (322 in Figure 4B).

Characterization of the ^{34}S-labelled methionine

The isotopic enrichment of the isolated ^{34}S-labelled methionine standard solution was determined by multicollector ICP-MS and the values obtained confirmed by alternative GC-MS measurements. The ^{34}S isotopic enrichment obtained by direct nebulisation of the standard solution in the multicollector instrument and by injecting such solution in the HPLC-MC-ICP-MS system was 79.5 ± 0.5% and 82.7±0.6%, respectively. The small difference observed could be due to a small contamination of natural abundance sulfur when analysing the spike solution by direct nebulisation.

For GC-MS, a natural sulfur methionine standard solution (5 µg/g as sulfur) was derivatised and injected in the GC-MS system in SIM mode measuring nominal masses 319 to 327 to study cluster purity. The experimental isotope distributions were calculated as the ratio of each peak area obtained for each mass divided by the sum of all peak areas measured. The experimental isotope distribution observed was compared with the theoretical isotope distribution calculated using the method of Kubinyi[24] and a Visual Basic macro for Excel developed in our laboratory[25,26]. It was observed that the contribution of the loss of hydrogen or the tailing of the peaks at the low mass side in the experimental spectrum was only 0.2% of the measured spectrum. Then, the isotope distribution of the labelled methionine spike was measured in the same way and the isotope enrichment determined as described previously[22].

In brief, a Visual Basic macro for Excel was prepared to calculate 100 different theoretical isotope distributions for the derivatised fragment of ^{34}S-labelled methionine using tentative ^{34}S isotope enrichments between 80% and 100%. Then, the theoretical spectra were compared with the experimental one by linear regression analysis. The right isotope enrichment was selected as to that giving the minimum in the square sum of residuals between the calculated and observed distributions.

The ^{34}S enrichment obtained by GC-MS was 82.0 ± 0.2%. This value is close to that calculated by HPLC-MC-ICP-MS. In conclusion, the ^{34}S enrichment in methionine decreased around 10% in comparison with the ^{34}S enrichment in the original yeast. These differences could be attributed to natural sulfur methionine impurities in the proteases employed to hydrolyse the yeast proteins. Finally, the enrichment value obtained by HPLC-MC-ICP-MS was employed in further IDMS calculations.

The concentration of sulfur in the purified ^{34}S-methionine spike was determined by two alternative IDMS procedures. First, by direct nebulisation of the solution after spiking it with a ^{33}S enriched spike and, second, by HPLC-MC-ICP-MS after spiking it with a standard solution of

natural abundance methionine (species specific reverse IDMS). The final methionine concentration (as sulfur) found in the purified amino acid solution was 401 ± 5 µg/g by direct nebulisation and 396 ± 6 µg/g by species specific reverse IDMS, respectively. Both procedures produced the same results as no other sulfur containing compounds were detected in the ^{34}S spike.

It was calculated that a total amount of 296 µg of ^{34}S-enriched methionine was obtained per gram of (dry weight) yeast following the proposed procedure.

Analysis of human blood plasma samples

Figure 5 shows the HPLC-ICP-MS chromatogram of a human blood plasma sample spiked with the ^{34}S-labelled methionine spike. As it can be observed, free methionine can be separated from other sulfur-containing compounds in human blood plasma samples following the reverse phase chromatographic method described in the procedures. The $^{34}S/^{32}S$ isotope ratio for the unknown sulfur compounds present in the blood plasma samples was 0.045±0.001. This value indicates that the addition of the ^{34}S labelled methionine spike did not change the natural sulfur isotope ratio of those compounds.

For confirmation purposes, the fraction where the plasma blood methionine eluted under these conditions (8-8.5 minutes) was collected and brought to the GC-MS instrument after preconcentration and derivatisation. The total ion chromatogram obtained was similar to those obtained for natural and ^{34}S-labelled methionine solutions, with the methionine peak eluting at 27.9 minutes. The mass spectrum for this peak eluting at 27.9 minutes from a human blood plasma sample is shown in Figure 6. The pattern of this spectrum is identical to that obtained for the natural sulfur methionine standard (Figure 4A) confirming the presence of methionine in the fraction collected between 8-8.5 minutes in the HPLC system.

Recoveries studies were carried out by adding a known amount of natural methionine to the human blood plasma samples. The natural methionine was added before the precipitation of plasma proteins. Three concentration levels were assayed by fortifying a human blood plasma sample with 2, 4 and 8 µg/g of natural methionine (as sulfur). Fortified samples were analysed by ID-HPLC-ICP-MS following the above described method. The recoveries found were 98.4±0.6%, 100.5±0.9% and 99.2±0.7%, respectively.

Limits of detection and quantification were calculated as three and ten times the standard deviation of the blank concentration[27], respectively. To do that, the blank solution was analysed several times following the same procedure as the samples. The values obtained for the limits of detection and quantification were 0.07 and 0.2 µg/g (0.5 and 1,6 µmol/L) of methionine, respectively.

Finally, 13 human blood plasma samples were analysed three times by the method above described and the results compared with those given by the Clinical Biochemistry Service of the HUCA using the routine method described previously. Results obtained by both analytical

methodologies, expressed in µmol/L, appear in Table 6. Please note that plasma methionine concentrations as well as their related reference values for clinical applications usually are expressed with only two significant figures. A statistical least squares fitting test was used to compare the results found by both analytical methodologies. There were no evidences that both methods give different results. On the other hand, the results found are within the reference range for healthy adults.

Conclusions

The procedure to synthesize high volumes of ^{34}S-labelled yeast has been slightly improved by changing the culture medium and avoiding contamination with natural abundance sulfur as far as possible. The ^{34}S enrichment in the yeast was in all cases greater than 91% which means an improvement of 25% with regard to previous works[19]. Unfortunately, it could be difficult to improve such enrichment in future experiments due to the inevitable presence of several sources of natural sulfur in the culture medium.

A method to extract and isolate ^{34}S-labelled methionine from ^{34}S-labelled yeast has been developed. In order to produce standard solutions of ^{34}S-labelled methionine, characterization, in terms of concentration and isotope abundance, has been performed by multicollector ICP-MS and the results compared with an alternative method based on GC-MS. Additionally, the identity of the methionine standard was confirmed by GC-MS. Results show a small decrease in the ^{34}S enrichment of the methionine (about 80%) with regard to the ^{34}S enrichment of the yeast (more than 90%) due probably to contamination with natural sulfur methionine in the solutions obtained.

Nevertheless, once characterized, these solutions can be used as standard solutions of ^{34}S-labelled methionine for species-specific isotope dilution of methionine in real samples. The analysis of methionine in human blood plasma and the comparison of the results with the routine, validated, method employed in the Clinical Biochemistry Service of the HUCA demonstrate the validity of this procedure.

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Table 1.- Instrumental operating conditions and acquisition parameters

Parameters	Element II	Neptune Plus	
Rf power	1350 W	1200W	
Cool gas flow	14 L min^{-1} Ar	14 L min^{-1} Ar	
Auxiliary gas flow	0.95 L min^{-1} Ar	0.9 L min^{-1} Ar	
Sample gas flow	0.91L min^{-1} Ar	0.89 L min^{-1} Ar	
Acquisition method	5 runs, 200 passes, 0.01 s sample time, 10 samples per peak, 3 s setting time	5 blocks, 10 cycles, 4.194 s integration time, 3 s idle time	
Detector	SEM	Faraday cups	
Cup Configuration		L4	^{32}S
		C	^{33}S
		H4	^{34}S

Table 2.- Instrumental parameters used in the chromatographic separations.

HPLC conditions for methionine quantification in plasma blood samples	
Column	Discovery BIO Wide Pore C18
Injection Loop	20 µL
Flow rate	80 µL/min
Mobile phase	Ammonium acetate 75 mM, pH 7.4 , 2% methanol
Gradient	Isocratic mode
HPLC conditions for methionine isolation	
Column	Agilent Zorbax Eclipse XDB
Injection Loop	100 µL
Flow rate	1.5 mL/min
Mobile phase	Ammonium acetate 75 mM, pH 7.4 , 2% methanol
Gradient	Isocratic mode
GC conditions for methionine characterization	
Column	DB-5 MS capillary column
Injection volume	2 µL
Injection mode	Splitless mode with 1 min of purge time
Flow rate	2 mL/min
Carrier gas	Helium
Injector temperature	250°C
Interface temperature	280°C
Ion source temperature	230°C
Gradient	The column temperature was initially held at 60°C for 1 min, and then a temperature ramp of 5°C/min was applied until 320°C for 10 min. The total run time was 68 min

Table 3.- Results obtained for 13 human blood serum samples using the ID-HPLC-ICP-MS method (proposed method) and the routine method (HUCA).

Samples	Biochrom (µmol/L)	Proposed method (mean ± standard deviation in µmol/L, n=3)
1	18	18.3 ± 1.2
2	25	24.9 ± 2.0
3	31	31.4 ± 1.8
4	21	21.9 ± 1.0
5	14	14.4 ± 0.6
6	27	26.6 ± 1.4
7	29	30.1 ± 2.5
8	18	18.4 ± 1.3
9	25	26.2 ± 1.5
10	16	16.7 ± 0.4
11	32	32.5 ± 1.6
12	24	25.1 ± 0.9
13	35	35.3 ± 0.8

LEGEND OF FIGURES

Figure 1.- Chromatograms obtained after the enzymatic hydrolysis of yeast with protease XIV but before methionine isolation. Dashed and solid lines correspond to ^{32}S and ^{34}S, respectively

Figure 2.- Chromatograms obtained in the preparative HPLC system with UV-VIS detection at 210 nm injecting: A) a natural standard of 10 µg/g methionine and B) sample from the enzymatic hydrolysis of yeast.

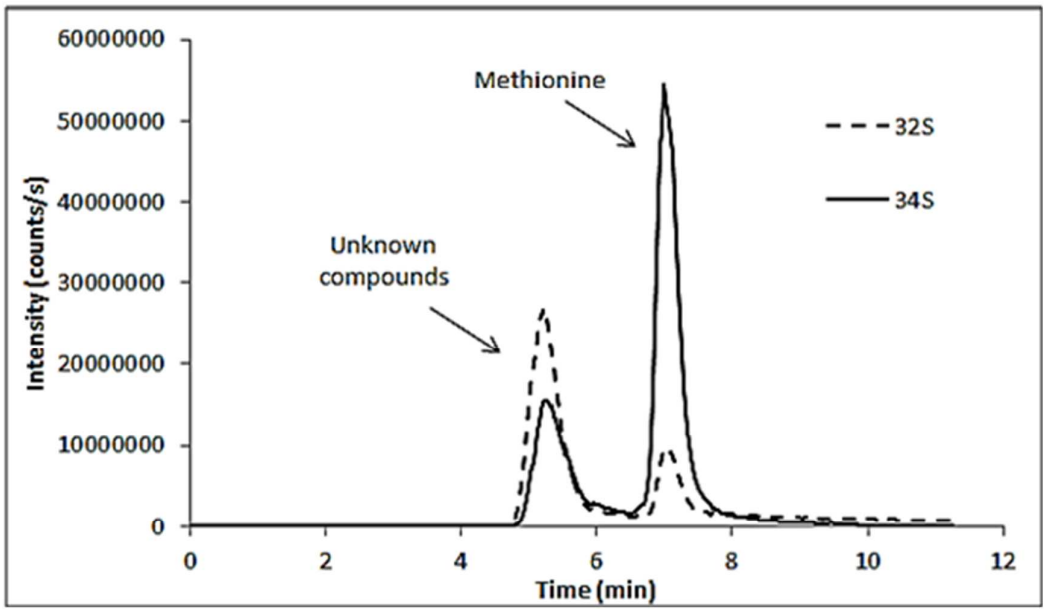
Figure 3.- HPLC-ICP-MS chromatogram of the isotopically enriched methionine standard solution obtained after isolation in the preparative HPLC system.

Figure 4.- GC-MS mass spectra obtained for: A) 5 µg/g natural methionine solution and B) diluted ^{34}S enriched methionine solution

Figure 5.- HPLC chromatogram obtained for a human blood plasma sample after spiking with ^{34}S enriched methionine. Dashed and solid lines correspond to ^{32}S and ^{34}S, respectively.

Figure 6.- GC-MS mass spectrum for the fraction collected between 8-8.5 minutes using the HPLC separation

Figure 1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Intensity (counts/s)
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4
6
8
10
12
Time (min)
Methionine
Unknown compounds
32S
34S

Figure 2A

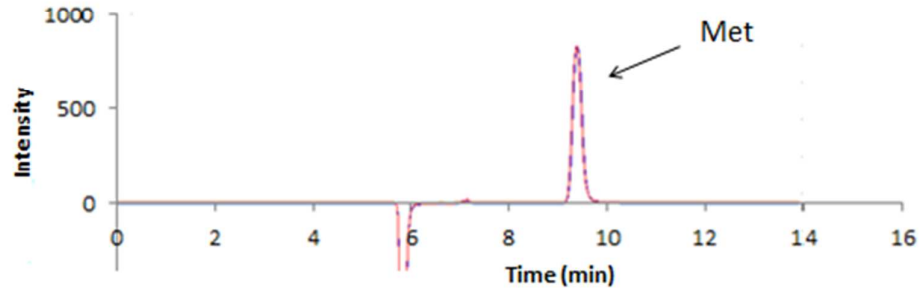


Figure 2B

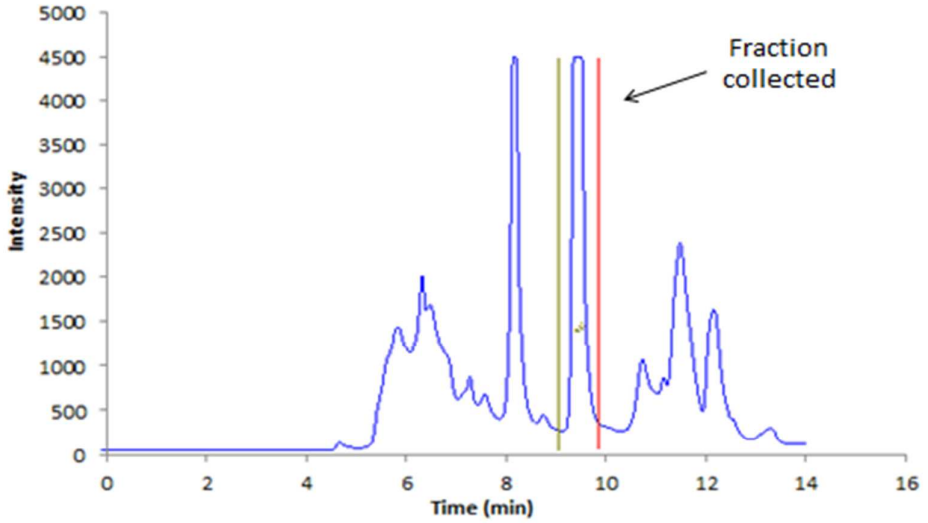


Figure 3

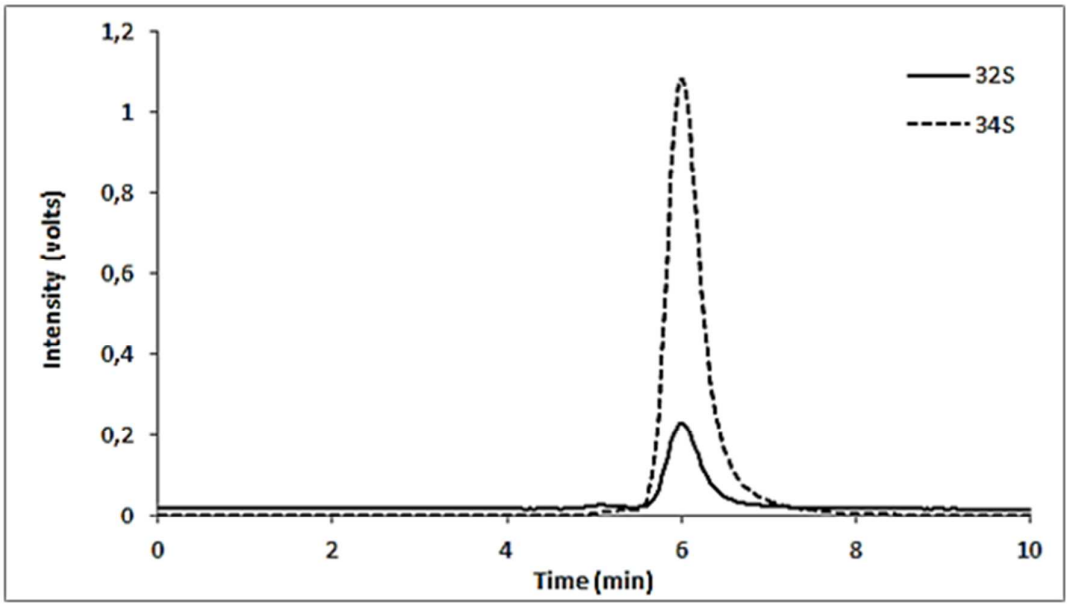


Figure 4A

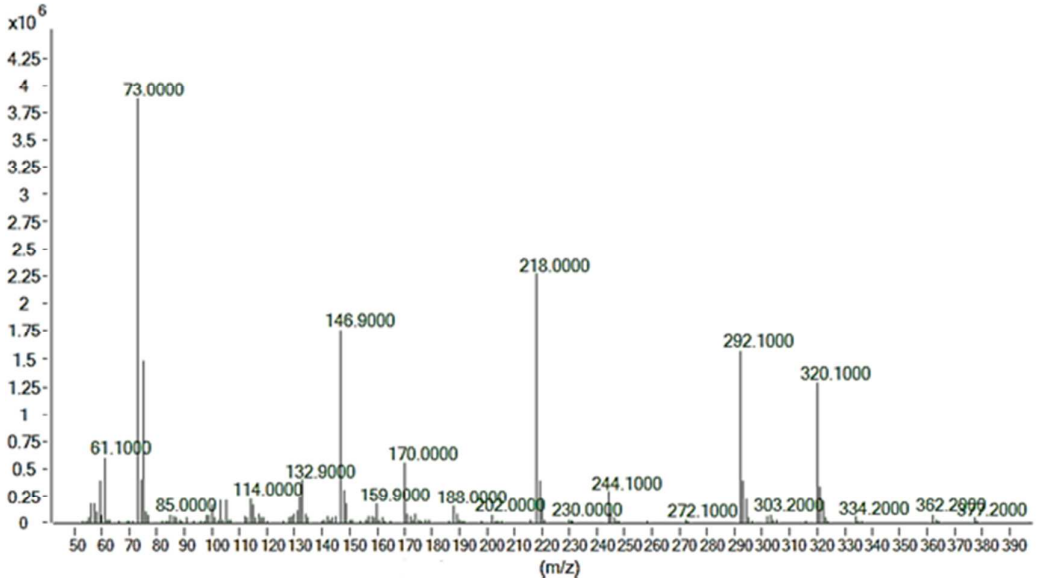


Figure 4B

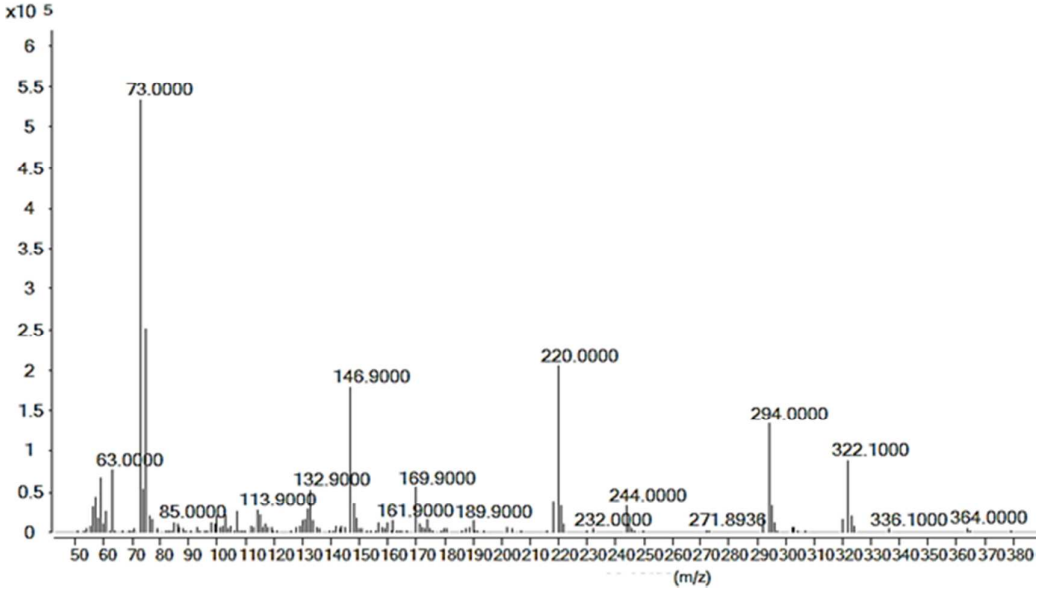
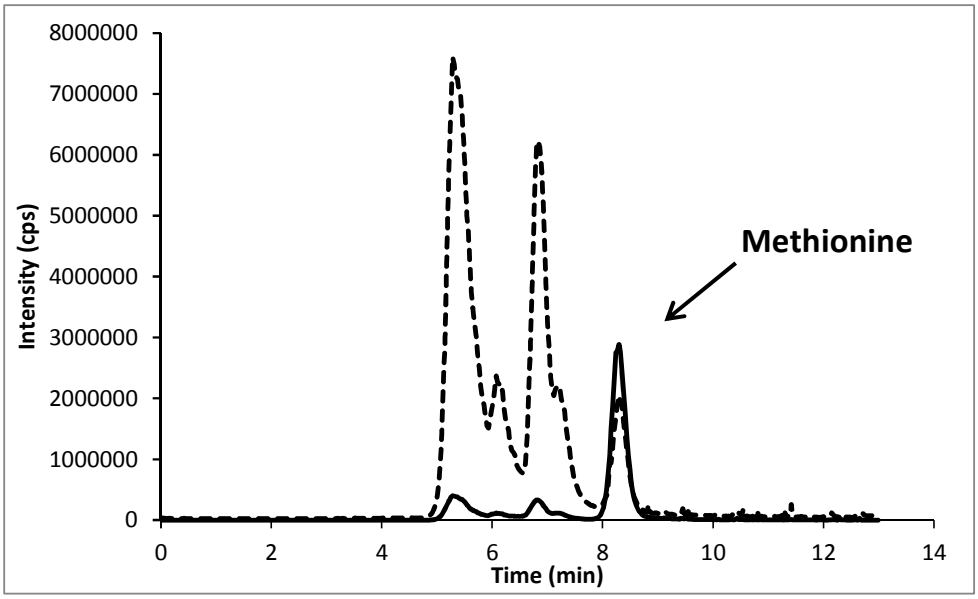


Figure 5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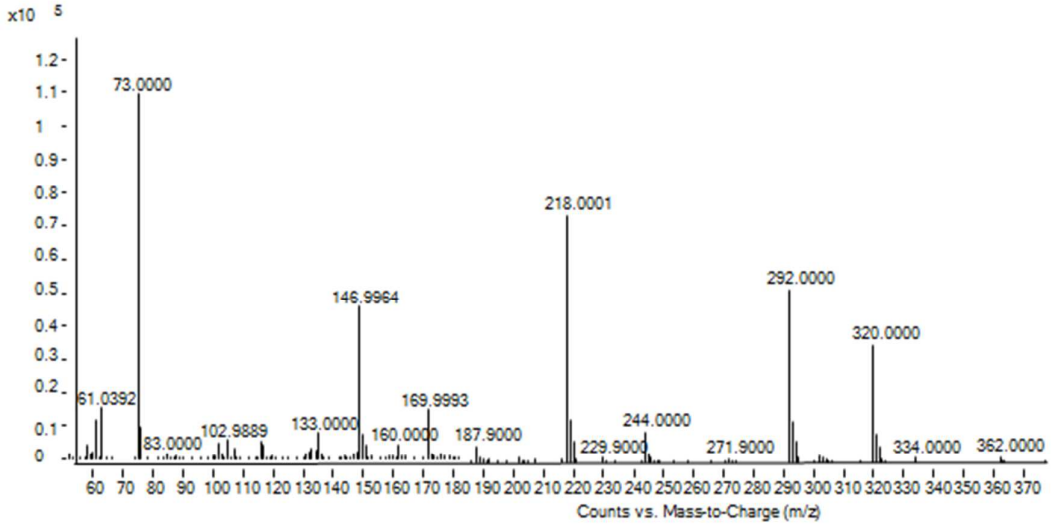
14
Time (min)
Methionine

Figure 6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73.0000
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320.0000
61.0392
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160.0000
187.9000
229.9000
271.9000
334.0000
362.0000
60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320 330 340 350 360 370
Counts vs. Mass-to-Charge (m/z)

ANSWERS TO THE REVIEWERS

Comments to reviewer 1

Question 1. In this work, the authors present an idea to determine free methionine in human blood plasma based on IDMS strategy. To some extent, the proposed strategy could make sense. However, there is still many important experimental data not included in this manuscript.

Answer 1. We appreciate your careful revision of the manuscript. Many changes have been introduced in order to take into account your comments and suggestions including new experiments to better characterize the spike solution.

Q2. In the confirmation of 34S-labelled methionine, there are two peaks in figure 1. It should be noticed that the content of the first peak is also higher and could not be ignored. Therefore, the author should give evidence to prove that the unknown sulfur containing component did not affect the quantification of methionine in IDMS procedure.

A2. The chromatogram shown in Figure 1 was obtained after the enzymatic hydrolysis of yeast with protease XIV but before the isolation of the isotopically enriched methionine by preparative LC. In order to check the elimination of the unknown sulfur compounds present in the hydrolysed sample, the solution obtained following the isolation procedure was injected again in the HPLC-ICP-MS system. This chromatogram is the new Figure 3. As it can be seen in the new figure, only one peak, which appears at the retention time of methionine, was observed confirming the absence of other sulfur compounds in the purified solution of ^{34}S enriched methionine. This fact has been clarified in the new text. Also, this chromatogram was employed to measure again the isotope enrichment of methionine.

Q3. As we know, the prerequisite of species specific IDMS is that the spike can not have influence on any other sulfur-contained proteins in blood plasma. In figure 4, it was observed that the 32/34S ratio of methionine was significantly changed. However, the author should give data to demonstrate the 32/34S ratio of other sulfur proteins was not changed. Unfortunately, this aspect of data was missed.

A3. The $^{34}S/^{32}S$ isotope ratio for the unknown sulfur compounds present in the blood plasma samples was 0.0451±0.001. This value indicates that the addition of the ^{34}S labelled methionine spike did not change the natural sulfur isotope ratio of those compounds. This information has been now included in the text.

Q4. The information of Table 4 is far from enough. A new methodology should have a convincing method validation process. No any uncertainty information is in Table 4, and compared to traditional methods, the advantage of IDMS was not emphasized. In table 4, I cannot get the conclusion that the IDMS approach is better than the traditional one.

A4. The reproducibility of the values found by the proposed methodology has been included in the new table 4. Unfortunately, the data from the traditional method were given without uncertainty for medical reasons.

Q5. Overall, this work is far from a professional analytical article. Too much experiment information was not included.

A5. Sorry to give this impression. We have tried to improve the manuscript in the revised version.

Comments to reviewer 2

Q6. The paper deals with the interesting topic of producing species specific spike material for amino acids relying on biosynthesis via yeast and subsequent purification. Moreover, the paper covers the proof of principle application, which is the quantitative determination of methionine in human plasma. Overall I support publication in JAAS, because I think it is an interesting work. However, prior to publication I would like to see additional data especially regarding the quantification of methionine in plasma samples.

A6. We appreciate your careful revision of the manuscript. Many changes have been introduced in order to take into account your comments and suggestions.

Q7. Since the authors claim to introduce a method which is of higher metrological order than the routine methods applied in the clinics, it would be required to address basics of method validations such as the uncertainty of the quantification method (or at least give repeatability values for the investigated plasma samples). Moreover, limits of detection and quantification should be added. Since there is a certified reference material for methionine (SRM 1950, NIST) in human plasma, it would have been very elegant to use this material in the study.

A7. The reproducibility of the values found by the proposed methodology has been included in the new table 4. Additionally, the values of the limits of detection and quantification have been included in the new text. Regarding the use of a Reference Material, you are completely right. Unfortunately, such CRM for amino acids in plasma was not available in our laboratory or in the Hospital at the time when the experiments were performed. The method used in the Hospital was validated by intercomparison exercises so it could be considered as a reference method.

Specific comments

Q8. Culture medium: Just out of curiosity, do you really produce isotopically enriched sulfate by oxidizing elemental sulfur via dissolution in nitric acid. I was wondering about this, since in fact this oxidation would result in sulfite and not sulfate?

A8. As far as we know, hot concentrated nitric acid oxidizes sulfur to sulfate according to:

$$S_{(s)} + 6HNO_{3\,(aq.\,conc.)} \rightarrow H_2SO_{4\,(aq.)} + 6NO_{2\,(g)} + 2H_2O_{(l)}$$

Ion chromatography separations showed that the oxidized sulfur compound eluted at the retention time of sulfate (experiments performed a few years ago).

Q9. Did the authors address the stability of the spike material, since methionine is known to rapidly degrade? Are there any investigations in this regard?

A9. After purification by preparative LC, the spike material was aliquoted in vials of 100 µL and stored at -80ºC. The different vials employed during the last two years did not show any degradation. This fact has been now mentioned in the procedures.

Q10. Figure 1: Was this chromatogram obtained with or without implementing protein precipitation? What would be the retention time of cysteine or glutathione in this separation? Please flag the peaks within the figure, it should not only be described in the text that peak 1 corresponds to unknown and peak 2 to methionine. The explanation that there are sulfur amino acid impurities in the protease would not result in a less enriched unknown peak, but would in fact reduce the relative 34S content of the methionine as well?

A10. The chromatogram of Figure 1 was obtained directly after enzymatic hydrolysis of the ^{34}S-enriched yeast with protease. Please note that no protein precipitation was performed in this procedure. All sulfur peaks observed should correspond to sulfur-containing amino acids or glutathione. The first peak eluted close to the dead volume of the column and could be either cysteine or glutathione (both eluted at the dead volume under these conditions, so they could be present in the first peak). This is clarified in the text. Peaks have been flagged in the new figure. Finally, the sulfur isotopic enrichment in the first unknown peak is much lower than in the methionine peak. This fact could be attributed to the presence of higher amounts of impurities of cysteine or other sulfur peptides (as glutathione) from the protease XIV and the lower amounts of such compounds in the hydrolyzed sample. The isotope enrichment of methionine was also reduced to 82% from the original 92% in the yeast.

Q11. The amount of spike material produced by the described procedure is not clear, the authors give just a concentration of 400 µg /g and it is not clear whether that is just the concentration obtained by preparative HPLC in the purified amino acid solution? It would be good to know how much it was per gram dry weight yeast or at least what amount of 34S was the result of the fermentation. Moreover, what would be the ideal conditions to store the spike? In solution? Speedvac treatment? What is the overall stability of the spike?

A11. The total amount of spike material produced is now indicated in the text. As it was described in the procedures, the collected fractions were mixed, dried and reconstructed in pure water. The concentration of 400 µg/g is the concentration in the solution obtained after reconstruction in pure water. As it has been indicated above, the spike material was stored at -80ºC in vials of 100 µL. Using this strategy, the spike material demonstrated to be stable at least two years. This information is now available in the manuscript.

Q12. It would be desirable to give recoveries with uncertainty for the fortified plasma samples.

A12. These uncertainties have been indicated in the revised text.

Q13. I would like to express once more the need for giving uncertainties along with quantitative values (or at least precision values) in a paper on isotope dilution. (Table 3). Moreover, it would be required to address limit of detection and quantification.

A13. We have included all these data in the revised version of the manuscript.

Determination of free methionine in human blood plasma by species-specific isotope dilution HPLC-ICP-MS using ^{34}S-labelled methionine.

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Abstract

A species-specific Isotope Dilution (ID) method is described for the determination of free methionine in human blood plasma by High Performance Liquid Chromatography - Inductively Coupled Plasma Mass Spectrometry (HPLC-ICP-MS) using ^{34}S-labelled methionine as species-specific spike. The ^{34}S-labelled methionine was obtained from yeasts grown in a medium enriched with ^{34}S (>91%) in the form of sulphate. Methionine was extracted from yeast using an enzymatic digestion with protease XIV followed by isolation using preparative reverse phase HPLC. Two separate batches of the labelled methionine standard were obtained. The ^{34}S-labelled methionine standard solutions obtained were characterised both in terms of ^{34}S isotope enrichment (82.7±0.679.5%) and total sulfur concentration (396401 ± 65 µg/g) by reverse Isotope Dilution HPLC-ICP-MS using a ^{33}S enriched inorganic standardnatural abundance methionine standard solution and a multicollector instrument working in the pseudo-high resolution mode to avoid spectral interferences. Additionally, the identity of the ^{34}S-labelled methionine isolated by preparative HPLC and its isotope enrichment was confirmed by Gas Chromatography-Mass Spectrometry (GC-MS).

Human blood plasma samples were spiked with the ^{34}S-labelled methionine spike. Then, plasma proteins were precipitated with trifluoroacetic acid and separated by centrifugation. Methionine was separated from the rest of sulfur containing compounds by reversed phase chromatography in isocratic mode using a mobile phase of 75 mM ammonium acetate (pH 7.4) containing 2% of methanol. The retention time of methionine (8.3 minutes) was confirmed by fortifying the samples with natural abundance methionine and also by collecting the methionine peak and further analysis by GC-MS. Finally, methionine in human blood plasma samples was determined by measuring the signals for ^{32}S and ^{34}S in a double focusing ICP-MS instrument working at medium resolution (R=4000). Concentrations were calculated by integrating the methionine peak for both masses and applying the isotope dilution equation after mass bias correction. The recoveries for samples fortified at different concentration levels ranged between

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* To whom correspondence should be addressed.

98.4 and 100.5 %. Additionally, good agreement was obtained between the results found with this method and those reported by the clinical laboratory using a validated routine method.

Keywords: species-specific isotope dilution, ^{34}S-labelled yeast, ^{34}S-labelled methionine, blood plasma samples, ICP-MS.

Introduction

The measurement of free methionine in blood is currently performed in clinical studies to detect aminoacidopathies in paediatric population[1,2], an inherited enzyme catabolic alteration affecting the metabolism of amino acids. The principal aminoacidopathy where methionine is involved is the hypermethioninemia (MET)[3], a disorder characterized by elevated concentrations of methionine in the body. MET patients usually do not show signs of the illness. However, if MET is untreated, it can cause learning delays, muscle weakness, and other health problems. On the other hand, methionine is also monitored in blood to evaluate the nutritional status of certain patients such as those with liver, kidney or neoplastic diseases[4], burned[5], with endocrine disorders[6], etc. Furthermore, in the last decades researchers have been studying the methionine dependency of tumour cells[7]. They found a link between the methionine concentration in blood and tumour growth. Different antitumor approaches have been applied drawing on this unique characteristic of tumour cells[8,9,10].

Quantification of methionine and other amino acids in biological, food and beverage samples has been carried out using a chromatographic separation, usually liquid chromatography with fluorescence[11], UV-VIS[12] or mass spectrometry[13] detection. One of the most popular methods for amino acid analysis, including methionine, is based on a liquid chromatography separation and post-column derivatization with ninhydrin. The coloured product formed can be detected by UV-VIS absorption[14].

As it is well known, the validation of these routine methodologies requires the use of standard reference materials, the comparison with other analytical methodologies, usually reference methods, or the participation in intercomparison exercises. In this sense, Isotope Dilution Mass Spectrometry (IDMS) is internationally regarded as a reference or highly qualified primary ratio method which has been used for the validation of clinical routine procedures[15]. However, if the advantages of IDMS are to be fully exploited, the use of a spike solution containing the compounds to be analysed in an isotopically labelled form (species-specific spiking) is required. To do that, the composition and structure of the species of interest must be exactly known in order to either synthesize the corresponding enriched species or acquire the proper spike solution if it is commercially available[16].

In our laboratory, a ^{77}Se-labelled selenomethionine standard was synthesised by growing yeast on a ^{77}Se-rich culture medium[17]. After harvesting, the yeast cells were disrupted and the cytosol content hydrolysed using Protease XIV. Finally, the ^{77}Se-labelled selenomethionine was isolated by anion exchange liquid chromatography from the protein digest and its concentration

determined by reverse IDMS using a natural selenomethionine standard. The ^{77}Se-labelled selenomethionine standard was applied to the determination of selenomethionine in a selenized yeast candidate reference material, and the results obtained were in good agreement with those reported by other laboratories[17,17,17].

Thereafter, yeast labelled with ^{34}S was also prepared in our laboratory by yeast growth on a ^{34}S-enriched specially prepared, culture medium in the absence of natural abundance sulfur[18]. The final product was characterised both with reference to isotope enrichment and total sulfur concentration by IDMS and isotope pattern deconvolution using a MC-ICP-MS instrument. The ^{34}S-labelled yeasts have been used directly in metabolic studies with laboratory animals such as rats[19] and mice[20]. The extraction of ^{34}S-labelled methionine from the yeast and its use as standard for species-specific IDMS has not been, as far as we known, previously employed for analytical purposes[21].

Consequently, the main objective of this research was the isolation and characterisation of ^{34}S-labelled methionine from yeasts in order to prepare a standard spike solution that could be used for species-specific IDMS. The identity of the species isolated, its isotope enrichment and concentration were evaluated by both ICP-MS and GC-MS. Once analytically characterised, the spike standard solution was applied to determine free methionine in human blood plasma and the results compared with the methodology currently used in the Clinical Biochemistry service of the Central Hospital of Asturias (HUCA, Oviedo, Spain) for the analysis of free amino acids in human blood plasma (Biochrom AAA, amino acid analyser).

Experimental

Reagents

A stock solution of 1000 mg/L of natural abundance S (as sulphuric acid in water) was purchased from Merck (Darmstadt, Germany). Further dilutions of this stock solution were made using ultra-pure water obtained from a Milli-Q system (Millipore Co., Bedford, MA, USA) to prepare the different working aqueous standard solutions as required. Enriched ^{33}S and ^{34}S were supplied from Cambridge Isotope Laboratories (Andover, MA, USA) as elemental powder. Stock solutions of about 1000 mg/L were prepared dissolving the elemental powder with nitric acid obtained by sub-boiling distillation and diluting with ultra-pure water. The standard spike solutions in the form of sulphate were characterized in terms of concentrations and isotopic abundances as described previously[18,18,18]. The natural abundance S and enriched ^{33}S and ^{34}S solutions were kept refrigerated at 4°C. A mixture of the different sulfur isotopes, containing ca. 30 mg/L of natural S, enriched ^{33}S and enriched ^{34}S was made by diluting the stock solutions using ultra-pure water. This mixture standard solution was employed for the configuration of the cups and the optimisation of conditions in the multicollector instrument as described previously[20,20,20]. On the other hand, a neutral pH enriched $^{34}SO_4^{2-}$ solution was prepared by

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neutralization of the stock solution with ammonia (Fluka Analytical, Buchs, Switzerland). This neutral solution was employed in the yeast growth procedure.

Natural abundance DL-methionine (>99%) was purchased from Ssigma–Aldrich (St. Louis, MO, USA).

Ammonium acetate and HPLC-grade methanol were purchased from Fluka Analytical and Merck, respectively. Both reagents were used to prepare the chromatographic mobile phases.

To carry out the enzymatic hydrolysis of the ^{34}S-labelled yeast, protease type XIV was supplied by Sigma–Aldrich ~~(St. Louis, MO, USA)~~.

Yeast cells were grown using high purity nutrients obtained from Sigma–Aldrich: D-(+)-Glucose, ammonium chloride, amino acids (arginine, glutamic acid, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, tryptophan and, valine), adenine, uracile, vitamins (thiamine hydrochloride, riboflavin 5´-monophosphate sodium salt, nicotinic acid, D-pantothenic acid hemicalcium salt, pyridoxine hydrochloride, (+)-biotin N-hydroxysuccinimide ester, myo-inositol, folic acid and 4-aminobenzoic acid), boric acid, potassium iodide, iron(III) chloride, sodium molybdate dehydrate, manganese(II) chloride tetrahydrate, zinc chloride, copper(II) nitrate hemi(pentahydrate), potassium phosphate monobasic, magnesium chloride hexahydrate, sodium chloride and calcium chloride dihydrate.

N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) with 1% tert-Butyldimethylchlorosilane (TBDMSCl) was purchased from Sigma-Aldrich. MTBSTFA and TBDMSCl were used as derivatizing and catalyst reagents in the GC-MS experiments, respectively.

Trifluoroacetic acid (ReagentPlus®, 99%) was purchased also from Sigma-Aldrich. This reagent was used to precipitate plasma proteins in the blood plasma samples.

Instrumentation

The multicollector inductively coupled plasma mass spectrometer (MC-ICP-MS) used was a Neptune Plus from ThermoFisher Scientific (Bremen, Germany) and was operated in the pseudo medium resolution mode. The instrument was equipped with 9 Faraday cups. The sample introduction system was a PFA-100 microconcentric nebulizer and a cyclonic spray chamber. The nebulizer gas flow rate, torch position and ions lens settings were optimized for higher sensitivity and the acquisition parameters were optimized for better precision of the measurements of the sulfur isotope ratios. Experimental conditions were similar to those described previously[20~~2020~~]. The double focusing inductively coupled plasma mass spectrometer (DF-ICP-MS) used was an Element II from ThermoFisher Scientific (Bremen, Germany), and was operated at the medium resolution mode (R=4000). All measurements were made with the standard sample introduction configuration of the instrument, that is, a Scott-type spray chamber working at room temperature, a Meinhard concentric nebulizer and a Fassel torch. The

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optimum instrumental settings for the measurement of sulfur isotope ratios in both instruments are summarized in Table 1.

The preparative isolation of the ^{34}S-labelled methionine was performed on an HPLC system (Agilent 1260 Infinity, Agilent Technologies, Santa Clara, CA) with an Agilent Zorbax Eclipse XDB column (9.4x250 mm, 5µm) equipped with an analytical-scale fraction collector and a variable wavelength detector. Liquid chromatography separations for methionine characterisation and blood plasma samples were performed on a Surveyor LC Pump Plus (ThermoFisher Scientific, Bremen, Germany) using a Discovery BIO Wide Pore C18 reverse phase column (15 cm X 2.1 mm, 5 µm particle size, Supelco, Bellefonte, Pennsylvania, USA).

Methionine characterization by GC-MS was carried out using a gas chromatograph, Agilent 7890, coupled to a triple quadrupole mass spectrometer; Agilent 7000 Series Triple Quad GC/MS (Agilent Technologies, Wilmington, DE, USA) operating at 70 eV. The GC was fitted with a split/splitless injector and a DB-5 MS capillary column (cross-linked 5% phenyl-methyl siloxane, 30 m x 0.25 mm i.d., 0.25 µm coating).

A Spill Mill Plus from Analitik Jena AG (Jena, Germany) was used for the disruption of the yeast cells. A thermomixer compact from Eppendorf (Hamburg, Germany) was used to carry out the enzymatic hydrolysis and the derivatization for GC-MS analysis. A centrifuge 5810R D from Eppendorf were employed for the centrifugation of the samples. All samples were dried using a centrifugal vacuum concentrator (Genevac, Suffolk, UK) to remove water and organic solvents. All standard solutions were prepared gravimetrically using an analytical balance model AB204-S (Mettler-Toledo GmbH, Greifensee, Switzerland).

A pH-meter model Basic 20 from Crisson (Barcelona, Spain) was used for pH adjustment. The yeast growth was followed by measuring the optical density spectrophotometrically at 600 nm in a Spectronic 20 (Milton Roy).

The Biochrom AA analyser (Biochrom Ltd, Cambridge, UK), used in the clinical laboratory of HUCA, is a PC controlled automatic cation exchange liquid chromatograph with a post-column detection system based on the oxidative deamination reaction of the α-amino group with ninhydrin, liberating ammonia, carbon dioxide, an aldehyde with one less carbon atom and a reduced form of ninhydrin, hydrindantin. The produced ammonia reacts with the hydrindantin and another molecule of ninhydrin to yield a purple substance (Ruhemann's purple) that absorbs around 570 nm.

Procedures

Synthesis and isolation of ^{34}S-labelled methionine.

Saccharomyces cerevisiae (common baker's yeast), AMW13C strain, was used for the synthesis of ^{34}S-labelled yeast using a protocol developed in our laboratory[18,18] but with some changes that will be discussed later. Briefly, a synthetic culture medium was prepared avoiding

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the use of sulphates in order to achieve a culture medium low in natural abundance sulfur. Thus, manganese, zinc, copper and magnesium sulphates, usually used in this kind of culture media, were substituted by the corresponding chloride salts. The sulfur source was ammonium sulphate enriched in ^{34}S which was prepared in our laboratory as described above. The cells grew up in a controlled temperature room at 28°C with agitation. The stationary phase of yeast growth was reached at 24 hours. Finally, cells were collected by centrifugation and rinsed with ultra-pure water to remove the excess of inorganic sulfur. The ^{34}S-labelled yeast was stored at -20°C until use.

The stored yeast cells were mechanically disrupted with beads in a mill for 90 seconds. Then, 0.15 g of yeast was hydrolysed with 10 mg of protease XIV in a Tris-HCl 0.1M pH 7.5 medium. Samples were incubated at 37°C during 24 hours. Hydrolyzed samples were further centrifuged (4000 g, 15 min) and the supernatant collected and stored at -20°C until use.

Finally, methionine was isolated by injecting the supernatant in a reverse phase HPLC column coupled on-line with a UV-VIS detector (set at 210 nm) followed by a fraction collector. The chromatographic conditions used are shown in Table 2. Fractions containing methionine from several injections were collected between 9-9.8 minutes. The collected fractions were mixed, dried, reconstructed in pure water, aliquoted in vials of 100 µL and stored at -80 °C until use. ~~in vials of 100 µL, so only one vial remains unfrozen until it is exhausted~~. Using this strategy, the spike material demonstrated to be stable for at least two years. About 300 µg of methionine were obtained for each gram of yeast (dry weight) prepared.

Characterization of the purified ^{34}S-labelled methionine

The characterization of the purified ^{34}S-labelled methionine was carried out both in terms of purity, concentration and isotope composition. Purity was tested by injecting the isolated ^{34}S-labelled methionine standard in the reverse phase chromatograph with ICP-MS detection. ~~A single peak was obtained with retention time corresponding to that of a natural abundance methionine standard.~~ The concentration of sulfur in the ^{34}S-labelled standard was measured both by conventional IDMS and species-specific isotope dilution HPLC-MC-ICP-MS. To do that, the ^{34}S labelled methionine solution was diluted 1:100 with ultra-pure water. Then, a 1:1 mixture with a ^{33}S enriched (conventional IDMS) or natural methionine (species-specific isotope dilution HPL-MC-ICP-MS) standard solutions of 1 µg/g (as sulfur) ~~was~~ were prepared. The $^{34}S/^{33}S$ isotope ratio of the mixture was measured in the multicollector ICP-MS instrument using the conditions given in Table 1. A natural abundance sulfur standard solution was measured prior to the mixtures for mass bias correction. Finally, the concentration of methionine was calculated using the isotope dilution equation described elsewhere[16~~16~~]. The isotope composition of sulfur in the isolated methionine standard was also measured by multicollector ICP-MS.

On the other hand, confirmation of the presence of ^{34}S-labelled methionine and an alternative measurement of its isotope enrichment was carried out by molecular mass spectrometry using GC-MS. To do that, 200 µL of the stock solution were taken to dryness. The residue was dissolved in 150 µL of MTBSTFA in 1% TBDMSCl and incubated for 10 min at 60°C on a

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thermomixer. Finally, samples were centrifuged for 2 min at 14000g and the clear liquid was transferred into vials for GC-MS analysis using the conditions summarized in Table 2. The ^{34}S enrichment was calculated from the molecular isotope envelope using a procedure previously developed in our laboratory[22].

Methionine analysis in human blood plasma samples

Human blood plasma samples were supplied by the Clinical Biochemistry service of the HUCA. For methionine quantification, a weighed amount of the ^{34}S-labelled methionine solution was added and then plasma proteins were precipitated using an excess of 10 volumes of trifluoroacetic acid (TFA) for 1 volume of plasma. The mixture was brought to an ice bath for 15 min to favour protein precipitation. Then, the samples were centrifuged at 15000 g for 5 minutes and the supernatants were collected and stored at -20°C until use.

The analysis of methionine in the supernatant solutions was performed by reverse phase HPLC column coupled on-line with a double focusing ICP-MS. The chromatographic conditions used for the separation of methionine are shown in Table 2. Finally, human blood plasma methionine concentration was calculated using the isotope dilution equation[16~~16~~].

An aliquot of the same blood plasma samples had been previously analysed on a Biochrom Amino Acid Analyser at HUCA, after a brief pretreatment consisting on fully deproteinisation with sulphosalicylic acid, addition of n-Leu as internal standard, pH adjustment to 2.2 with lithium hydroxide and filtration through a 0.2 µm membrane filter to remove any remaining particulate material. The obtained sample was injected from a temperature controlled autosampler onto a column of cation exchange resin. Lithium citrate buffers of varying ionic strength and pH from 2.8 to 3.55 are then pumped through the column to separate the various amino acids. The column temperature is accurately controlled and adjusted automatically as necessary to produce the separation. The column eluent is mixed with a ninhydrin reagent and then passed through the reaction coil, maintained at 138 ºC. From the reaction coil, the eluent/ninhydrin mixture is pumped to the photometer unit, which measures the amount of light absorbed at 570 nm and 440 nm. After each sample analysis, the column is regenerated by pumping a strong base (0.3 M lithium hydroxide) through de column followed by equilibrating buffer (pH 2.8), which adjusts the analytical column resin to its correct pH prior to the next analysis.

Results and discussion

Synthesis, isolation and confirmation of ^{34}S-labelled methionine

We have previously studied the optimal reagent quantities for the medium where yeast could growth[18~~18~~] for the preparation of ^{34}S-labelled yeast. However, such studies were carried out on a small scale (50 mL). For the isolation of enough ^{34}S-labelled methionine from yeast cells it was required to scale-up the procedure. It was observed that, as the volume of the culture medium increased the isotopic enrichment of sulfur in the yeast decreased. To overcome this problem, several modifications in the culture medium were performed in order to increase de

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volume of the culture medium to 2 L with minimal reduction in the sulfur isotope enrichment. Thus, glutamic acid, phenylalanine, proline and serine were added to the mixture of amino acid and the amount of each amino acid was increased from 20 to 75 mg per litre. The nitrogenous bases uracil and adenine were also added (75 mg per litre). With this modified culture medium an isotopic enrichment between 91% and 94% in ^{34}S was achieved in the twelve separated batches of yeast prepared. About 8 g of ^{34}S-labelled yeast were obtained from each batch of 2 litres of culture medium. The yeast obtained was mixed, homogenised and stored frozen at -20ºC until use. Enzymatic hydrolysis was done with protease XIV for the release of methionine from the yeast proteins. The amount of protease XIV was optimized by measuring the amount of methionine extracted. The range assayed was between 5 and 50 mg of protease XIV. The optimum value was found at 10 mg of protease XIV (for 150 mg of yeast). For example, Figure 1 shows one of the reverse phase HPLC-ICP-MS chromatograms obtained after the hydrolysis of yeast. The second peak in the chromatogram corresponds to ^{34}S-labelled methionine while the first peak could be cysteine and/or gluthatione as both compounds elute close to the dead volume under the experimental conditions used. The isotope enrichment for ^{34}S in methionine is~~with isotope enrichment~~ higher than that of the first peak in the chromatogram ~~(unknown sulfur-containing component). The~~while the isotopic enrichment of both sulfur-containing compounds is clearly lower than the original isotopic enrichment of ^{34}S in the yeast (91 to 94%) probably due to contamination with natural sulfur amino acids impurities from~~in~~ protease XIV. ~~The isotopic enrichment in the first unknown peak is much lower than in the methionine peak of a methionine standard. This fact could be attributed to the presence of higher amounts of impurities of natural cysteine or other sulfur peptides as glutathione (their retention times match the unknown peak) from the protease XIV and the lower amounts of such enriched compounds in the hidrolysed sample.~~

The final isolation of the ^{34}S-labelled methionine was performed by injecting the hydrolysed samples in a HPLC preparative system. Figure 2 shows the chromatograms obtained for a pure standard of natural abundance methionine (Fig. 2A) and the hydrolysed sample (Fig. 2B) detected by UV absorption at 210 nm. As can be observed, methionine eluted between 9-9.8 min (Figure 2A). Consequently, the system was set to collect fractions between 9-9.8 min. As it can be seen in Figure 2B, when the hydrolysed samples were injected, a chromatographic peak was also detected at the same retention time.

Final purity of the ^{34}S-labelled methionine spike was tested by injecting it in the reverse phase chromatograph with ICP-MS detection. As it can be seen in Figure 3, a~~A~~ single peak was obtained with retention time corresponding to that of a natural abundance methionine standard indicating that no other sulfur-containing compounds were present.

~~In order to check the elimination of the unknown sulfur compounds present in the hydrolysed sample (see Figure 1), the solution obtained following the isolation procedure was injected in the HPLC-ICP-MS system. As it can be seen in Figure 3, only one peak, which appears at the~~

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~~retention time of methionine, was observed confirming the absence of other sulfur compounds in the purified spike solution.~~

The confirmation of the identity of ^{34}S-labelled methionine isolated in the previous step was performed by GC-MS. The isolated sample and a natural abundance methionine standard were derivatised as described in the procedures. The total ion chromatograms showed that, methionine eluted at 27.9 minutes. Other peaks observed corresponded to side products of the derivatising reagent. The fragmentation patterns of the two methionine solutions at 27.9 minutes retention time are shown in Figure ~~3~~4. Peaks at nominal masses 320, 292 and 218 in Figure ~~3A~~ 4A (standard of natural abundance methionine) correspond to fragments which contain sulfur because in Figure ~~3B~~ 4B (isolated ^{34}S-enriched methionine from yeast) they appear at nominal masses 322, 294 and 220 (2 units higher due to the presence of ^{34}S instead of ^{32}S).

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Peaks at nominal masses 320 and 218 (322 and 220 in Figure ~~3B~~4B) correspond to the loss of C_4H_9 (-57 units) and $C_7H_{15}O_2Si$ (-159 units), respectively, probably following an α-homolitic cleavage. Peak at nominal mass 292 (294 in Figure ~~3B~~4B) could correspond to the loss of C_6H_{13} or C_5H_9O (-85 units in both cases) but the cleavage mechanism is unclear[23]. Other main peaks at nominal masses 147 and 73 do not contain sulfur as they appear at the same m/z in both spectra. The high peak at nominal mass 73 could correspond to C_2H_7NSi formed from the neutral loss of $C_{11}H_{23}O_2SSi$ (-247 and -249 units for ^{32}S and ^{34}S, respectively) in the fragment of nominal mass 320 (322 in Figure ~~3B~~4B).

~~Final purity of the ^{34}S-labelled methionine spike was tested by injecting it in the reverse-phase chromatograph with ICP-MS detection. A single peak was obtained with retention time corresponding to that of a natural abundance methionine standard indicating that no other sulfur-containing compounds were present.~~

Characterization of the ^{34}S-labelled methionine

The isotopic enrichment of the isolated ^{34}S-labelled methionine standard solution was determined by multicollector ICP-MS and the values obtained confirmed by alternative GC-MS measurements. The ^{34}S isotopic enrichment obtained by direct nebulisation of the standard solution in the multicollector instrument and by injecting such solution in the HPLC-MC-ICP-MS system was 79.5 ± 0.5% and 82.7±0.6%, respectively. The small difference observed could be due to a small contamination of natural abundance sulfur when analysing the spike solution by direct nebulisation.

For GC-MS, a natural sulfur methionine standard solution (5 µg/g as sulfur) was derivatised and injected in the GC-MS system in SIM mode measuring nominal masses 319 to 327 to study cluster purity. The experimental isotope distributions were calculated as the ratio of each peak area obtained for each mass divided by the sum of all peak areas measured. The experimental isotope distribution observed was compared with the theoretical isotope distribution calculated using the method of Kubinyi[24] and a Visual Basic macro for Excel developed in our

laboratory[25,26]. It was observed that the contribution of the loss of hydrogen or the tailing of the peaks at the low mass side in the experimental spectrum was only 0.2% of the measured spectrum. Then, the isotope distribution of the labelled methionine spike was measured in the same way and the isotope enrichment determined as described previously[222222].

In brief, a Visual Basic macro for Excel was prepared to calculate 100 different theoretical isotope distributions for the derivatised fragment of ^{34}S-labelled methionine using tentative ^{34}S isotope enrichments between 80% and 100%. Then, the theoretical spectra were compared with the experimental one by linear regression analysis. The right isotope enrichment was selected as to that giving the minimum in the square sum of residuals between the calculated and observed distributions.

The ^{34}S enrichment obtained by GC-MS was 82.0 ± 0.2%. This value is close ~~but slightly higher than~~to that calculated by ~~multicollector~~ HPLC-MC-ICP-MS. In conclusion, the ^{34}S enrichment in methionine decreased around 10% in comparison with the ^{34}S enrichment in the original yeast. These differences could be attributed to natural sulfur methionine impurities in the proteases employed to hydrolyse the yeast proteins. Finally, the enrichment value obtained by ~~multicollector~~ HPLC-MC-ICP-MS was employed in further IDMS calculations.

The concentration of sulfur in the purifiede ^{34}S-methionine spike was determined by two alternative IDMS procedures. First, by direct nebulisation of the solution after spiking it with a ^{33}S enriched spike and, second, by HPLC-MC-ICP-MS after spiking it with a standard solution of natural abundance methionine (species specific reverse IDMS) ~~as it has been explained above~~. ~~This procedure was possible due to the fact that no other sulfur containing compounds were detected in the ^{34}S-labelled methionine spike.~~ The final methionine concentration (as sulfur) found in the purified amino acid solution ~~obtained by preparative HPLC~~ was 401 ± 5 µg/g by direct nebulisation and 396 ± 6 µg/g by species specific reverse IDMS, respectively. Both procedures produced the same results as no other sulf~~ph~~ur containing compounds were detected in the ^{34}S spike.

It ~~can be~~was calculated that a total amount of 296 µg of ^{34}S-enriched methionine was obtained per gram of (dry weight) yeast ~~could be obtained~~ following the proposed procedure.

Analysis of human blood plasma samples

Figure ~~4~~ 5 shows the HPLC-ICP-MS chromatogram of a human blood plasma sample spiked with the ^{34}S-labelled methionine spike. As it can be observed, free methionine can be separated from other sulfur-containing compounds in human blood plasma samples following the reverse phase chromatographic method described in the procedures. The ^{34}S/^{32}S isotope ratio for the unknown sulfur compounds present in the blood plasma samples was 0.045±0.001. This value indicates that the addition of the ^{34}S labelled methionine spike did not change the natural sulfur isotope ratio of those compounds.

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For confirmation purposes, the fraction where the plasma blood methionine eluted under these conditions (8-8.5 minutes) was collected and brought to the GC-MS instrument after preconcentration and derivatisation. The total ion chromatogram obtained was similar to those obtained for natural and ^{34}S-labelled methionine solutions, with the methionine peak eluting at 27.9 minutes. The mass spectrum for this peak eluting at 27.9 minutes from a human blood plasma sample is shown in Figure 56. The pattern of this spectrum is identical to that obtained for the natural sulfur methionine standard (Figure 3A4A) confirming the presence of methionine in the fraction collected between 8-8.5 minutes in the HPLC system.

Recoveries studies were carried out by adding a known amount of natural methionine to the human blood plasma samples. The natural methionine was added before the precipitation of plasma proteins. Three concentration levels were assayed by fortifying a human blood plasma sample with 2, 4 and 8 µg/g of natural methionine (as sulfur). Fortified samples were analysed by ID-HPLC-ICP-MS following the above described method. The recoveries found were 98.4±0.6%, 100.5±0.9% and 99.2±0.7%, respectively.

Limits of detection and quantification were calculated as three and ten times the standard deviation of the blank concentration[27], respectively. To do that, the blank solution was analysed several times following the same procedure as the samples. The values obtained for the limits of detection and quantification were 0.07 and 0.2 µg/g (0.5 and 1,6 µmol/L) of methionine, respectively.

Finally, 13 human blood plasma samples were analysed three times by the method above described and the results compared with those given by the Clinical Biochemistry Service of the HUCA using the routine method described previously. Results obtained by both analytical methodologies, expressed in µmol/L, appear in Table 6. Please note that plasma methionine concentrations as well as their related reference values for clinical applications usually are expressed with only two significant figures. ~~A statistical two tailed paired t-test was used to compare the results found by both analytical methodologies. For 12 degrees of freedom the value of t (P=0.05) is 2.18 while the experimental t was 1.69 which confirms the null hypothesis, that is,~~A statistical least squares fitting test was used to compare the results found by both analytical methodologies. ~~t~~There ~~are~~ were no evidences that both methods give different results. On the other hand, t~~T~~he results found are within the reference range for healthy adults.

Conclusions

The procedure to synthesize high volumes of ^{34}S-labelled yeast has been slightly improved by changing the culture medium and avoiding contamination with natural abundance sulfur as far as possible. The ^{34}S enrichment in the yeast was in all cases greater than 91% which means an improvement of 25% with regard to previous works[19,19,19]. Unfortunately, it could be difficult to improve such enrichment in future experiments due to the inevitable presence of several sources of natural sulfur in the culture medium.

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A method to extract and isolate ^{34}S-labelled methionine from ^{34}S-labelled yeast has been developed. In order to produce standard solutions of ^{34}S-labelled methionine, characterization, in terms of concentration and isotope abundance, has been performed by multicollector ICP-MS and the results compared with an alternative method based on GC-MS. Additionally, the identity of the methionine standard was confirmed by GC-MS. Results show a small decrease in the ^{34}S enrichment of the methionine (about 80%) with regard to the ^{34}S enrichment of the yeast (more than 90%) due probably to contamination with natural sulfur methionine in the solutions obtained.

Nevertheless, once characterized, these solutions can be used as standard solutions of ^{34}S-labelled methionine for species-specific isotope dilution of methionine in real samples. The analysis of methionine in human blood plasma and the comparison of the results with the routine, validated, method employed in the Clinical Biochemistry Service of the HUCA demonstrate the validity of this procedure.

Acknowledgements

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Table 1.- Instrumental operating conditions and acquisition parameters

Parameters	Element II	Neptune Plus
Rf power	1350 W	1200W
Cool gas flow	14 L min^{-1} Ar	14 L min^{-1} Ar
Auxiliary gas flow	0.95 L min^{-1} Ar	0.9 L min^{-1} Ar
Sample gas flow	0.91L min^{-1} Ar	0.89 L min^{-1} Ar
Acquisition method	5 runs, 200 passes, 0.01 s sample time, 10 samples per peak, 3 s setting time	5 blocks, 10 cycles, 4.194 s integration time, 3 s idle time
Detector	SEM	Faraday cups
Cup Configuration		L4 ^{32}S
		C ^{33}S
		H4 ^{34}S

Table 2.- Instrumental parameters used in the chromatographic separations.

HPLC conditions for methionine quantification in plasma blood samples	
Column	Discovery BIO Wide Pore C18
Injection Loop	20 µL
Flow rate	80 µL/min
Mobile phase	Ammonium acetate 75 mM, pH 7.4 , 2% methanol
Gradient	Isocratic mode
HPLC conditions for methionine isolation	
Column	Agilent Zorbax Eclipse XDB
Injection Loop	100 µL
Flow rate	1.5 mL/min
Mobile phase	Ammonium acetate 75 mM, pH 7.4 , 2% methanol
Gradient	Isocratic mode
GC conditions for methionine characterization	
Column	DB-5 MS capillary column
Injection volume	2 µL
Injection mode	Splitless mode with 1 min of purge time
Flow rate	2 mL/min
Carrier gas	Helium
Injector temperature	250°C
Interface temperature	280°C
Ion source temperature	230°C
Gradient	The column temperature was initially held at 60°C for 1 min, and then a temperature ramp of 5°C/min was applied until 320°C for 10 min. The total run time was 68 min

Table 3.- Results obtained for 13 human blood serum samples using the ID-HPLC-ICP-MS method (proposed method) and the routine method (HUCA).

Samples	Biochrom (µmols/L)	Proposed method (mean ± standard deviation in µmols/L, n=3)
1	18	18.3 ± 1.218
2	25	24.9 ± 2.025
3	31	31.4 ± 1.831
4	21	21.9 ± 1.022
5	14	14.4 ± 0.614
6	27	26.6 ± 1.426
7	29	30.1 ± 2.530
8	18	18.4 ± 1.318
9	25	26.2 ± 1.526
10	16	16.7 ± 0.417
11	32	32.5 ± 1.632
12	24	25.1 ± 0.925
13	35	35.3 ± 0.835

LEGEND OF FIGURES

Figure 1.- Chromatograms obtained after the enzymatic hydrolysis of yeast with protease XIV but before methionine isolation. Dashed and solid lines correspond to ^{32}S and ^{34}S, respectively.

Figure 2.- Chromatograms obtained in the preparative HPLC system with UV-VIS detection at 210 nm injecting: A) a natural standard of 10 µg/g methionine and B) sample from the enzymatic hydrolysis of yeast.

Figure 3.- HPLC-ICP-MS chromatogram of the isotopically enriched methionine standard solution obtained after isolation in the preparative HPLC system.

Figure ~~3~~4.- GC-MS mass spectra obtained for: A) 5 µg/g natural methionine solution and B) diluted ^{34}S enriched methionine solution

Figure ~~4~~5.- HPLC chromatogram obtained for a human blood plasma sample after spiking with ^{34}S enriched methionine. Dashed and solid lines correspond to ^{32}S and ^{34}S, respectively.

Figure ~~5~~6.- GC-MS mass spectrum for the fraction collected between 8-8.5 minutes using the HPLC separation

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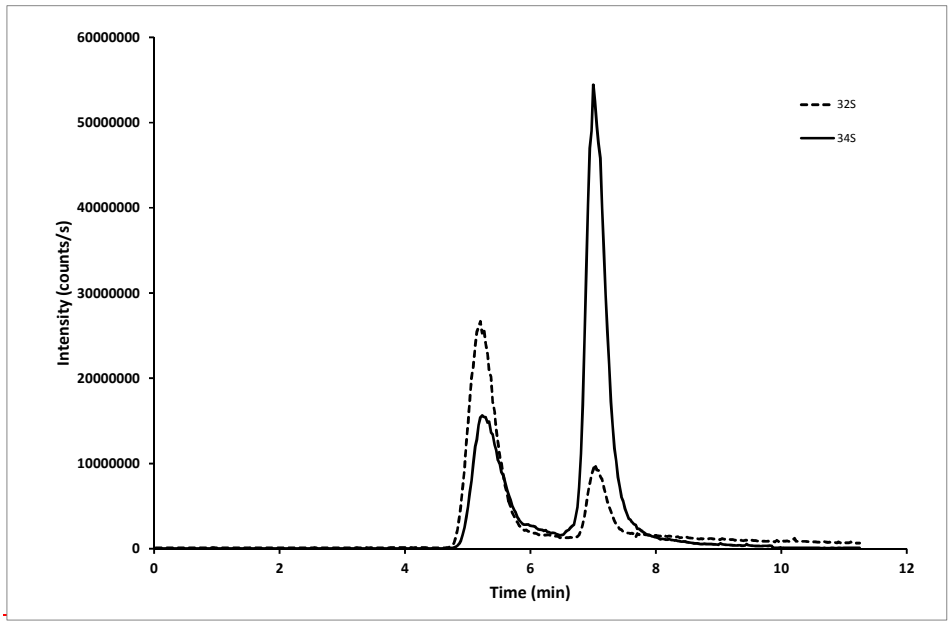
Figure 1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8
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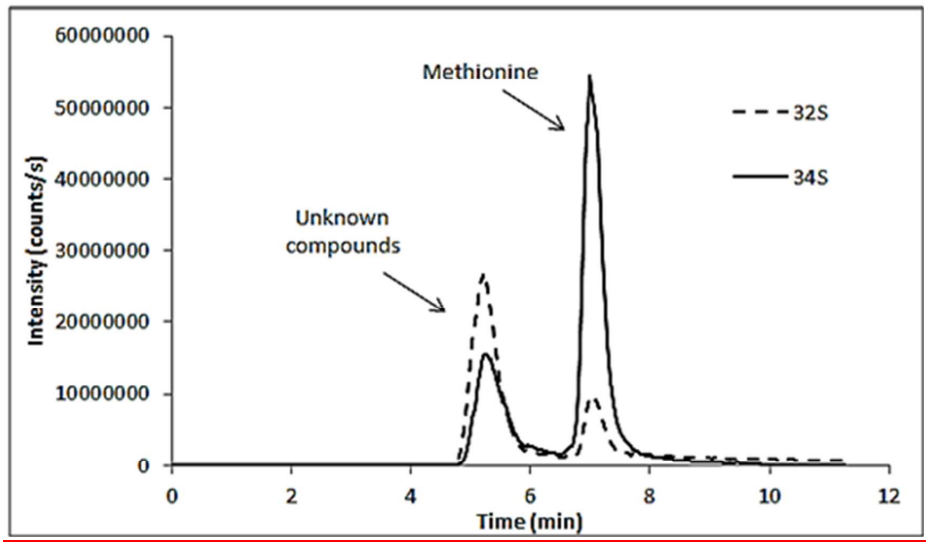
12
Time (min)
32S
34S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34S
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4
6
8
10
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Time (min)

Figure 2A

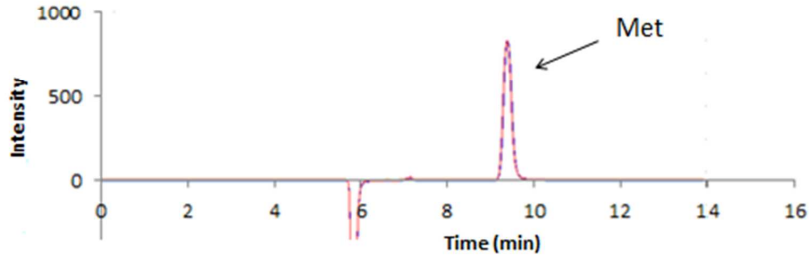


Figure 2B

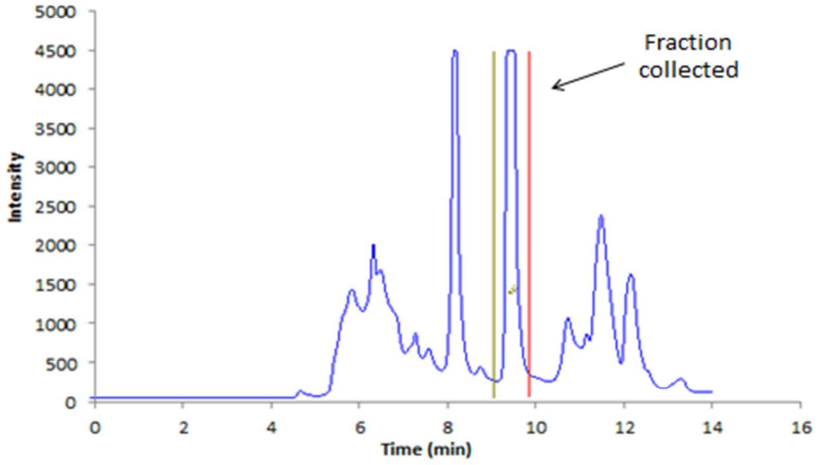


Figure 3

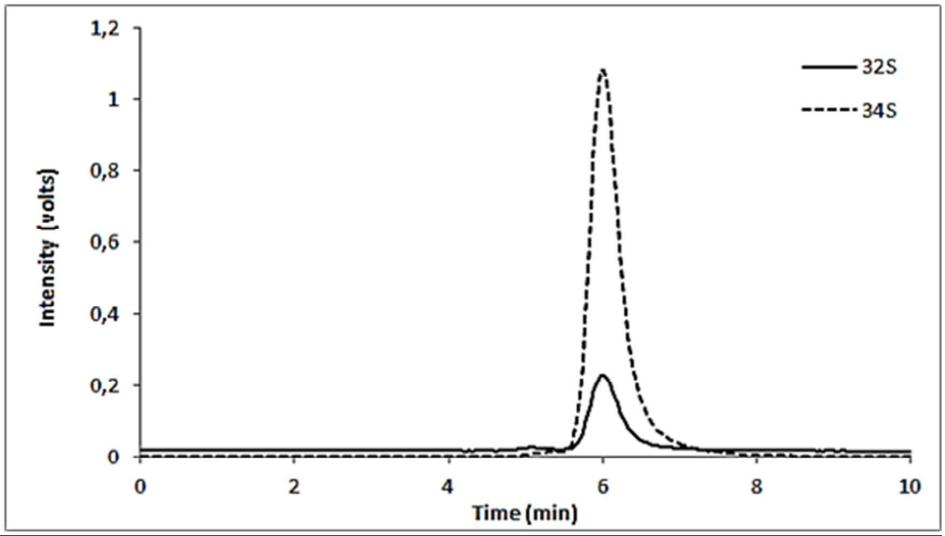


Figure ~~3A~~4A

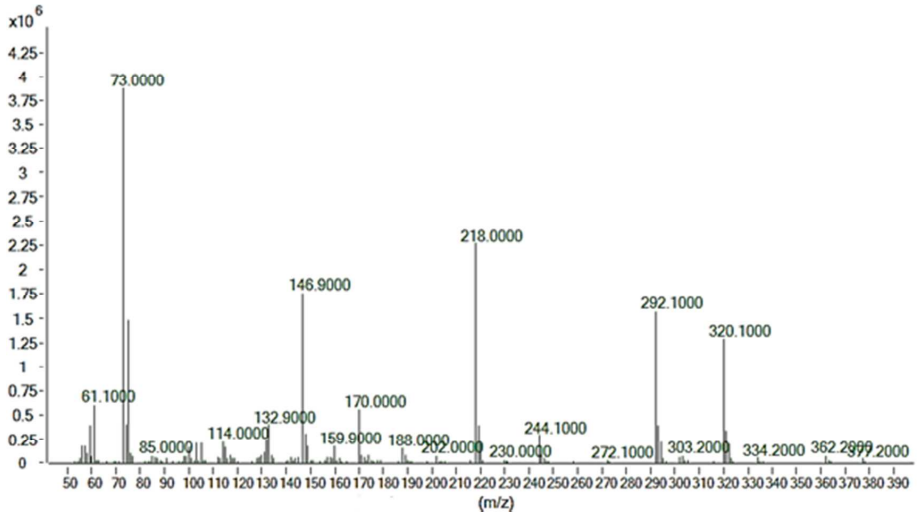


Figure ~~3B~~4B

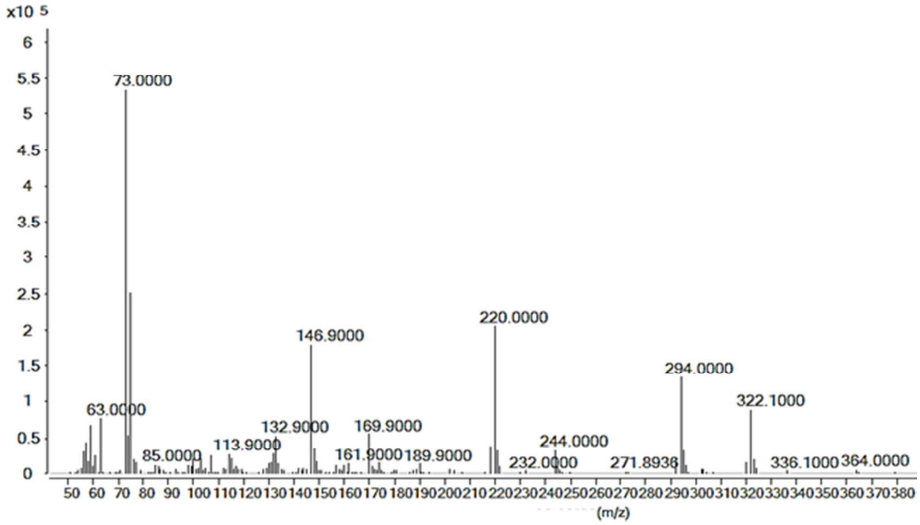
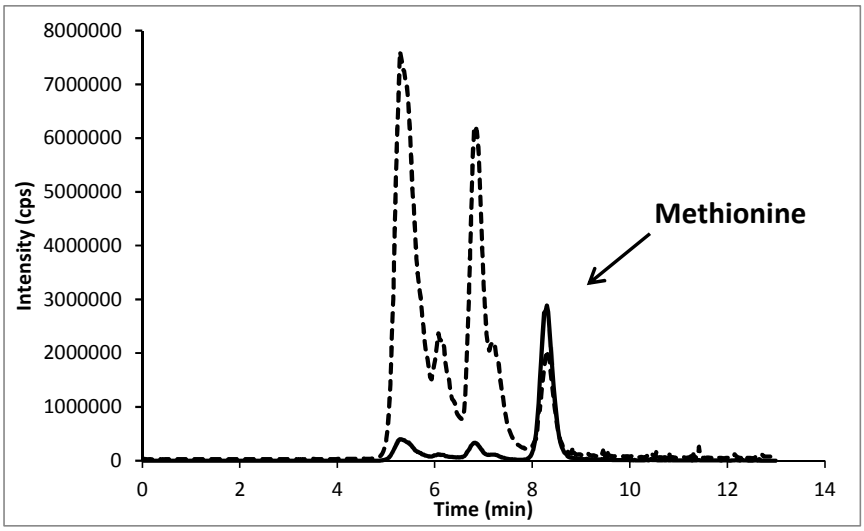


Figure 45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Intensity (cps)
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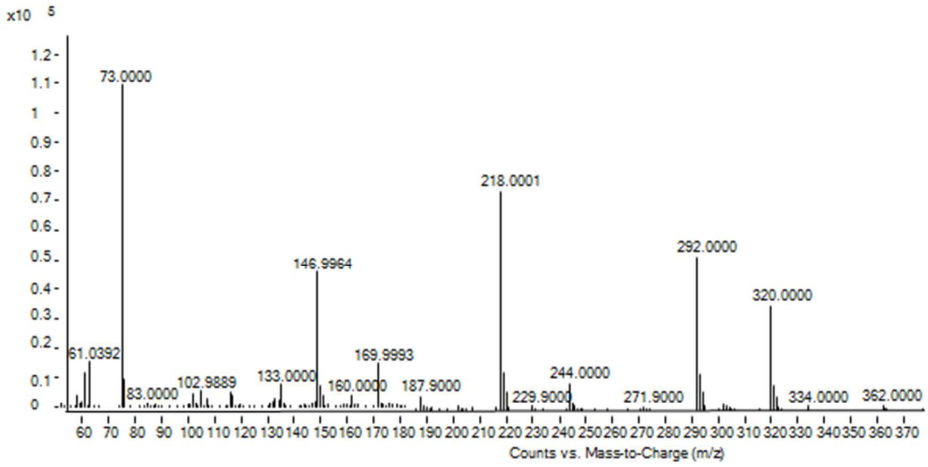
2
4
6
8
10
12
14
Time (min)
Methionine

Figure ~~5~~6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1.1
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0
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218.0001
292.0000
146.9964
320.0000
61.0392
169.9993
244.0000
83.0000
102.9889
133.0000
160.0000
187.9000
229.9000
271.9000
334.0000
362.0000
60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320 330 340 350 360 370
Counts vs. Mass-to-Charge (m/z)