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# Quantification of β-hydroxymethylbutyrate and leucine by ultrahigh performance liquid chromatography tandem mass spectrometry at different situations and stages of a rodent life

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ABSTRACT

The main objective of this work was to develop a method to measure Leucine (Leu) and β-hydroxymethylbutyrate (HMB) at basal levels in serum, urine, milk and brain microdialysates in rats. Ultrahigh performance liquid chromatography–electrospray-tandem mass spectrometry (UHPLC-ESI-MS/MS) was used as analytical technique. The sample treatment was simple and consisted of dilution with methanol and centrifugation for serum and urine, dilution with water and filtration with an Amicon filter for milk, and treatment with formic acid with no further dilution for microdialyzates. The procedures for sampling and the UHPLC-MS/MS parameters were accurately optimized to achieve the highest recoveries and to enhance the analytical characteristics of the method. For chromatographic separation, an Acquity UPLC BEH Amide column using acetonitrile-water gradient with formic acid as additive was used. The total run time was 4 min. The analytical characteristics (accuracy, selectivity and sensitivity) of the proposed method were evaluated. The limits of detection (LODs) obtained ranged from 0.4 to 7 ng $mL^{-1}$ and the limits of quantification (LOQs) from 1 to 22 ng $mL^{-1}$. Precision, expressed as relative standard deviation (% RSD), was lower than 15% in all cases, and the determination coefficient ($R^2$) was equal or higher than 99.0% with a residual deviation for each calibration point lower than ±25%. Mean recoveries were between 85 and 115%. The method was successfully applied to these matrices being able to detect significant differences between physiological situations, strains and stages of life.

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## 1. Introduction

β-hydroxy-β-methylbutyrate (HMB) is a metabolite of the amino acid leucine (Leu). The first step in HMB metabolism is the reversible transamination of Leu to α-ketoisocaproate (α-KIC) that occurs mainly extrahepatically. Following this enzymatic reaction, the majority of α-KIC is irreversibly oxidized to isovaleryl-CoA via the enzyme branched chain keto acid dehydrogenase (BCKAD). In the minority alternative pathway (approximately 5% of metabolized Leu), HMB is produced from α-KIC by the cytosolic enzyme KIC dioxygenase [1,2] (See Fig. 1). HMB functions are well known. It is an anti-catabolic agent associated with protein synthesis and attenuation of protein degradation [3,4], which makes it suitable for increasing strength and muscle mass in sport nutrition [5–9], as well as for clinical situations of increased protein degradation (cachexia), decreased rate of muscle protein synthesis (inactivity), or alteration of both (sarcopenia) [10–12]. In addition, it is a substrate for the synthesis of cholesterol needed to form and stabilize sarcolemmas in muscle [5], it has immune modulator properties [13,14], it downregulates apoptosis during immobilization and recovery [15] and also it produces improvement in oxidative metabolism [16].

Due to the catabolism of Leu people have a daily endogenous synthesis of HMB between 0.3 and 0.4 g, but plasma HMB levels can be increased by five- to ten-fold after feeding Leu (60 g) or HMB (3 g). Kinetic studies in animals have reported that the half-life of HMB in plasma is about 2 h and that about 34% of HMB is excreted in urine. In humans, the timecourse kinetics of HMB in plasma and urine showed that the half-life of HMB is also about 2 h while only 14–29% of the HMB is excreted in urine, depending on the dose. Those data suggest that 70–85% of the ingested oral HMB is retained in the body for further metabolism [1,2,17]. There may be a number

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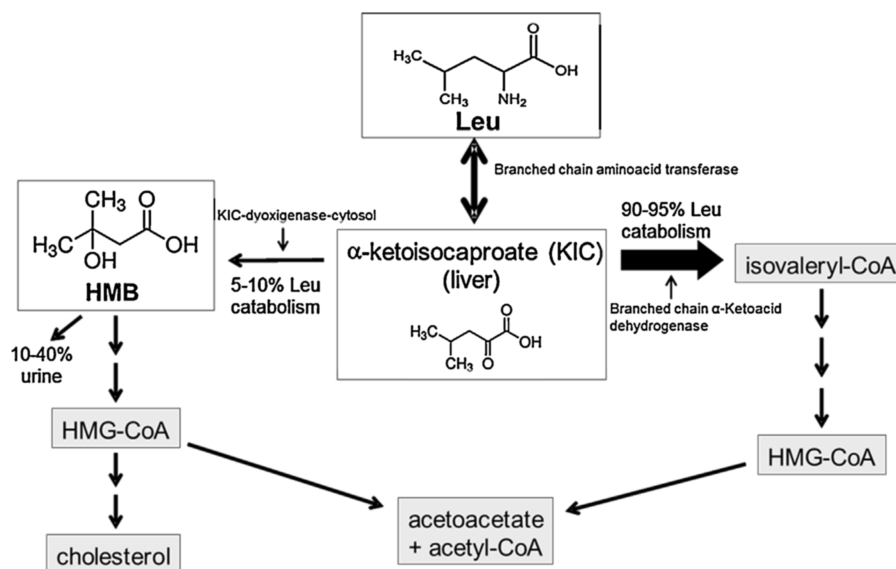



**Fig. 1.** Chemical structures of HMB and Leu and the metabolic route.

of factors such as gender, race, age or pathologies that could affect the basal levels and excretion of HMB and/or Leu. It is important to explore these levels of HMB and Leu in different biological fluids to have knowledge about how they vary naturally throughout the life of an animal under different conditions. This information could be relevant for the use of these compounds as dietary supplements or nutritional products with specific health outcomes.

Accurate and sensitive analytical methods are necessary for the analysis of low levels of HMB and Leu present in specific biological fluids. Classically, several methods have been published for determining amino acids such as Leu [18], with techniques such as HPLC [19–24] or gas chromatography [25–27]. So far, these methods had some shortcomings such as low specificity for HMB or the sample treatment complexity. Since HMB contains an α-hydroxy in place of the α-amino group, the techniques involving derivatization steps are not recommended. Nissen et al. [28] published a method to determine HMB in plasma by gas chromatography coupled with mass spectrometry (GC–MS) but this method requires high sample volumes and laborious protocols including derivatization steps. Recently, two analytical methods have been published to improve these inconveniences. First, in 2013 Deshpande et al. published a study of the bioavailability of HMB in plasma by LC–MS [29] and second, our research group has recently published a method to measure HMB and Leu by UHPLC-ESI-MS/MS in relatively clean biological fluids, namely cell cultures and brain microdialysates [30].

The present work aims to extend the use of this method to other biological fluids such as serum, urine and milk. Brain microdialysates were also revisited to improve sensibility. Sample treatments were optimized for the fluids mentioned above and the method was validated according to the US Food and Drugs Administration (FDA) guideline for bioanalytical assays [31]. Subsequently, basal levels HMB in these sample matrices and on different biological conditions and stages of life were measured.

## 2. Materials and methods

### 2.1. Chemicals and reagents

All reagents were analytical grade unless specified otherwise. Water (18.2 MΩ cm) was purified and filtered by a specific LC–MS filter using a Milli-Q system from Millipore (Bedford, MA, USA). β-Hydroxymethylbutyrate (HMB) and Leu were supplied by Sigma-Aldrich (Madrid, Spain). LC-MS grade methanol (MeOH), acetonitrile (MeCN), ethanol (EtOH) and formic acid (FA) were purchased from Scharlab (Barcelona, Spain). Artificial cerebrospinal fluid (aCSF) was purchased from Harvard Apparatus (Holliston, Massachusetts, USA).

A stock solution of compounds was prepared by weighing 0.04 g HMB and 0.01 g Leu into a 10 mL flask and dilution with water. The solution remained stable for at least one month at 4 °C. Five work standard solutions for calibration purposes were prepared specifically depending on the studied matrix. For serum and urine, an intermediate solution (No. 1) was obtained by dilution of 50 μL of the stock solution to a final volume of 10 mL with MeOH. A second intermediate solution (No. 2) was prepared by diluting 200 μL of solution No. 1 to 1 mL with MeOH. Work standards for calibration purposes, named WS1, WS2, WS3 and WS4 were prepared by diluting 10, 50, 200 and 500 μL of the intermediate solution N° 2 to a final volume of 1 mL with MeOH in a maximum recovery vial. The standard WS5 was the solution No. 2. The same process was followed for other matrices, but in that case the solvent used for dilutions was different. Specifically, to measure HMB and Leu in milk the solvent was LC–MS water.

For microdialysates from rat brain a modification of the previously published article was done. The use of an improved chromatographic column by the manufacturer caused higher sensitivity than in the previous work due to narrower and higher peaks were obtained compared with those obtained in previous experiments by HILIC column. So with this improvement basal levels could be detected. Furthermore, as these levels were close to the limit of quantification, it was decided avoid dilution to improve the signal and work within the linear dynamic range of the method. For both calibration and samples processing, and in order to concentrate the basal level to the optimal value, no dilution of samples was made.

The calibration standards were injected at the beginning and end of each sample series. A quality control standard (WS3) was injected after every twenty injections. Calibration standards were freshly prepared from the original stock solution in each experiment.

### 2.2. Apparatus and software

Detection and quantification of the analytes were performed using an UPLC® Acquity system from Waters (Milford, MA, USA) equipped with a binary pump, vacuum membrane degasser, a thermostated column compartment, an autosampler, and an automatic injector was used. The chromatograph was connected on-line to a triple quadrupole mass spectrometer detector (TQD) with electrospray ionization (ESI) interface. The following chromatographic columns were tested: Acquity UPLC BEH C18 (2.1 mm × 100 mm i.d., 1.7 μm particle size), Acquity UPLC BEH HILIC (2.1 mm × 150 mm i.d., 1.7 μm particle size), Acquity UPLC BEH HILIC (2.1 mm × 100 mm i.d., 1.7 μm particle size), and Acquity UPLC BEH Amide (2.1 mm × 100 mm i.d., 1.7 μm particle size) from Waters. MassLynx software version 4.1 from Waters (Milford, MA, USA) was used for instrument control and for data acquisition and analysis.

An analytical balance with a precision of 0.1 mg, a vortex-mixer, a speed vac evaporator from Heraeus Instrument Thermo Scientific (Madrid, Spain), maximum recovery LC vials and screw caps from Waters, 0.2 μm nylon filter, 0.2 μm polytetrafluoroethylene (PTFE) filter and 0.5 mL 10K Amicon filter from Millipore (Bedford, MA, USA) and an eVOL automated analytical syringe from SGE Analytical Science (SGE Europe, United Kingdom) and were also used.

### 2.3. Animal work

Sprague-Dawley (SD) rats were provided by Janvier and Charles River Laboratories (France). Zucker Lean Rats (ZLR) were obtained from Charles River Laboratories. Adult animals were fed with a purified rodent diet according to the AIN-93 recommendations [32]. Pups were nursed by their own mothers. Animals were housed at constant room temperature (22 ± 2 °C) and 45–55% humidity under a regular 12-h light/dark schedule.

Serum and urine samples were measured in: a) pup rats (male and female) at postnatal day (PND) 1–3, 12–14 and 19–20, b) 13 week-old rats (male and female), c) gestating rats at gestational day (GD) 0, 5, 12 and 18 (13–16 weeks-old), d) mothers rats after delivery at PND 1–3, 12–14 and 19–20 (16–18 weeks-old), e) adult female rats which had been ovariectomized (OVX) at 24 weeks of age simulating menopause and rats of the same age which suffered the surgery but the ovaries were not removed (SHAM), and g) Zucker lean rats (ZLR), a strain that is the natural non-diabetic control for Zucker diabetic rats at several time points (10, 15 and 20 weeks of age). In addition, serum of 70 week-old males was measured, milk samples were also obtained from SD dams at PND 1–3, 12–14 and 19–20, and microdialysate samples were determined in adult male SD rats.

The following techniques were used to obtain blood samples depending on the experimental design: terminal bleeding in dead endpoint experiments and in rat pups experiments, and tail punction in longitudinal studies. Urine was obtained after involuntary urination or full day collection in metabolic cages in longitudinal studies or by punction of the bladder after dead. Milk samples were taken by manual expression in anesthetized animals after stimulation of milk secretion by intraperitoneal administration of oxytocin. The samples were taken either after 3–4 h fasting or overnight fasting (12–18 h) depending on the experimental design. All the samples were stored at −80 °C until analysis. Microdialysate samples were taken in the same way that in our previous published article mentioned above.

Protocols for all experimental procedures were carried out according to ethical guidelines for animal experimentation at the Spanish National Research Council (RD 53/2013).

### 2.4. Sample preparation

Serum samples were diluted in a specific volume of methanol depending on the concentration of the analyte to be within the linear working range. The addition of methanol precipitates proteins and cleans the sample before injection into the LC system. Dilutions were done always in methanol LC–MS grade and were as follows:

- Normal rats including pups, young and adult animals: 50 μL of serum + 50 μL of MeOH.
- Zucker rats (ZLR): 40 μL of serum + 260 μL of MeOH.

The dilutions were vigorously shaken for 1 min, centrifuged at 14,000 × *g* for 10 min at 8 °C, collected into maximum recovery vials and injected into the UPLC®.

Urine samples were also diluted in a specific volume of MeOH depending on the concentration of the analyte to be within the linear working range:

- Normal rats: 10 μL + 1.5 mL of MeOH LC–MS grade.
- ZLR: 50 μL + 8 mL MeOH.

The dilutions ware shaken for 1 min and the liquid layer was transferred to maximum recovery vials and injected directly into the UPLC®.

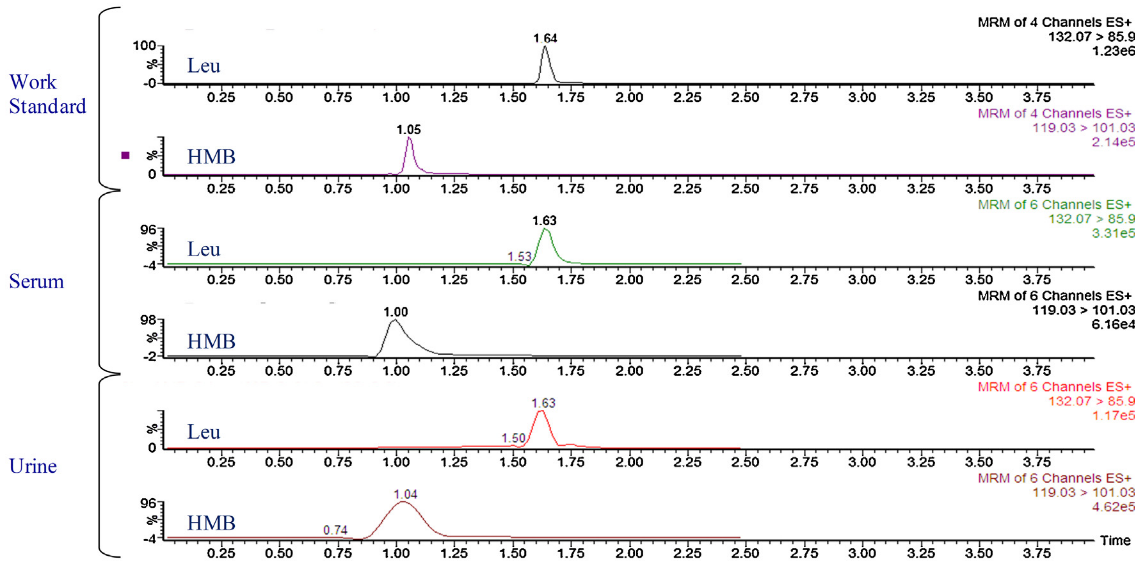
For rat milk samples, 50 μL of milk were diluted with 100 μL of purified LC–MS water. They were shaken vigorously for 1 min and centrifuged in a 10K Amicon Filter at 14,600 × *g* for 30 min at 10 °C. The extract was collected in maximum recovery vials to inject into the UPLC®. A second dilution was made to determine Leu within the linear dynamic range (LDR) of work. In that case, 20 μL of the extract were taken in a new vial and 480 μL of LC–MS quality water were added. The mixture was shaken and then injected into the chromatographic system.

Finally, for microdialysates from rat brain, a modification of the work previously published by our research group [30] was done for the determination of the basal levels of HMB and Leu in microdialysate samples. Microdialysate samples (typically 40 μL) were automatically collected every 20 min directly into vials and frozen at −80 °C until analysis. They were placed in maximum recovery vials. Two microliters of FA were added and then mixed for 10 sec on a vortex-mixer; it was ready to direct injection into the UPLC®.

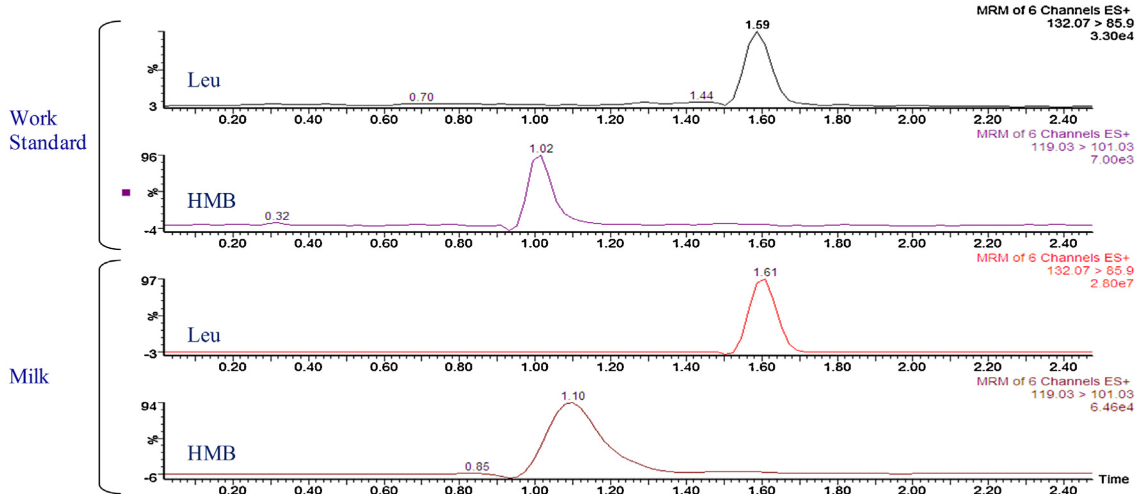
### 2.5. Liquid chromatography-mass spectrometry conditions

The development of the liquid chromatographic method is based in the previously reported in a published article [30]. Two LC columns were checked again: an Acquity UPLC BEH HILIC (2.1 mm × 100 mm i.d., 1.7 μm particle size) and an Acquity UPLC BEH Amide (2.1 mm × 100 mm i.d., 1.7 μm particle size) from Waters, and in this case, since the manufacturer has recently improved the stability of the amide column, better separations, showing a higher resolution in the shortest times were obtained. Accordingly, the sensitivity was increased by 10 times. The best separation was obtained in the same way using a mobile phase composed of water as solvent A and 0.1% (v/v) of FA in acetonitrile as solvent B. A linear gradient was established as follow: 0.0–1.0 min, 90 to 20% B; 1.0–3.0 min, 20 to 10% B; 3.0–3.1 min, back to 90% B; and maintaining 90% B until 4 min. Flow rate was 0.3 mL min$^{-1}$, injection volume 2 μL, the column temperature was maintained at 30 °C, the sample temperature at 20 °C and total run time was 4 min. Injection or washing weak and strong solvents were a mixture of 450 mL of acetonitrile and 50 mL of water, and pure water respectively. These solutions were stable for at least one week at room temperature.

**A:** HMB and Leu: Work standard, Serum extract and Urine extract (MeOH)




**B:** HMB and Leu: Work Standard and Milk extract ($H_2O$)




**C:** HMB and Leu: Work Standard and Microdialysates from rat brain (aCSF solution)

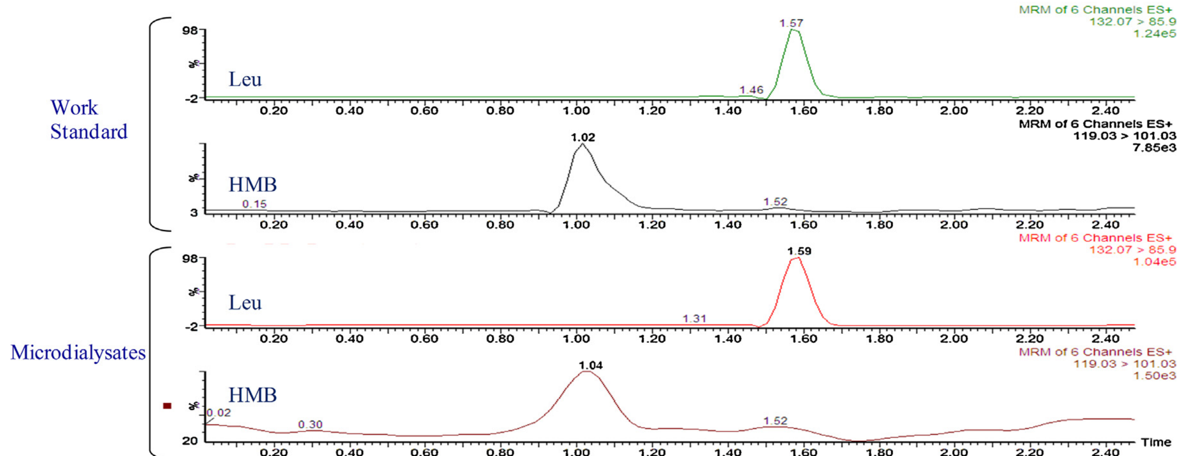



**Fig. 2.** Chromatograms of each analyte in calibration solution and in sample extracts. Quantification transition is shown. (A) HMB and Leu in work standard, serum extract and urine extract (MeOH); (B) HMB and Leu in work standard and milk extract ($H_2O$); (C) HMB and Leu in work standard and microdialysates from rat brain (aCSF solution).

**Table 1**
Analytical and statistical parameters.

| Sample Type | Solvent | | $b$ (mL µg$^{-1}$) | $R^2$ (%) | LOD (ng mL$^{-1}$) | LOQ (ng mL$^{-1}$) | LDR (ng mL$^{-1}$) |
|---|---|---|---|---|---|---|---|
| Serum, Urine | Methanol | HMB | 1767 | 99.1 | 6 | 19 | 19–4500 |
| | | Leu | 44842 | 99.1 | 0.9 | 3 | 3–1600 |
| Milk | Water | HMB | 2091 | 99.6 | 7.0 | 22 | 22–4500 |
| | | Leu | 45939 | 99.0 | 0.4 | 1 | 1–1600 |
| Brain Microdialysates | Ringer Solution | HMB | 862 | 99.0 | 4 | 15 | 15–4500 |
| | | Leu | 44992 | 99.9 | 0.8 | 3 | 10–1600 |

$b$ – slope; $R^2$, coefficient of determination; LOD – limit of detection; LOQ – limit of quantification; LDR – linear dynamic range; $P$-value for lack-of-fit test >5% in all cases.

For MS/MS detection, ESI was performed in positive ion mode. The tandem mass spectrometer was operated in multiple reaction monitoring (MRM) mode and Q1 and Q3 quadrupoles were set at unit mass resolution. Electrospray ionization spray voltage was 4500 V. Nitrogen was used as desolvation gas at 850 L min$^{-1}$ and as auxiliary gas in the cone at 50 L min$^{-1}$. The temperature of the source was 120 °C and the desolvation temperature was 400 °C. Argon (99.999 % purity) was used as collision gas at an approximate rate of 0.1 mL min$^{-1}$. Precursor ion selected for HMB was 119 *m/z* while product ions were 101 and 59 m/z for quantification and confirmation respectively. For Leu the precursor ion was 132 and the product ions were 86 *m/z* (quantification) and 44 *m/z* (confirmation). Regarding cone voltage, 17 V was optimized for HMB and 23 V for Leu. The collision energies were 12/5 eV for HMB and 20/10 eV for Leu. Other common parameters for both compounds were dwell time, 100 ms; Delay time, 5 ms; Inter Channel Delay, 5 ms and InterScan time, 5 ms. Fig. 2 shows the chromatogram of a standard mixture of compounds in MeOH, water and aCSF solution used to test the matrix effect in different solvents.

### 2.6. Data presentation and statistical calculation

The results were presented as mean ± SD in Tables and Figures. Significant differences are stated in figures. One-way ANOVA and post-hoc comparison by Bonferroni's correction or Student's *t*-test were applied when appropriate.

## 3. Results and discussion

### 3.1. Sample treatment for the different matrices

The optimal sample processing method for each matrix was studied. Since HMB is a molecule with amphipathic chemical character, it was reasonable to avoid protocols that include liquid-liquid partition steps, where polar and non polar solvents are used, and then HMB could be partitioned between both mediums. In fact, Deshpande et al. [29] used the liquid-liquid extraction for plasma samples, obtaining very low recovery rates. In the present work, a simple treatment of sample by dilution with a solvent was evaluated to reduce the interferences and to degrade proteins.

For serum and urine, four treatments were compared: deproteinization with EtOH; MeOH; a mixture MeOH:FA (1:1, v/v) and pure FA. The best results were obtained using MeOH, which provided recovery rates and other validation parameters within acceptance levels (recovery rates between 85 and 115%, relative standard deviation <15%). For rat milk, similar experiments were done. Different solvents (MeOH, EtOH, water), with and without additives (FA) and filtration devices (Amicon filter with centrifugation, nylon filter, PTFE filter) were tested, obtaining the best results with a simple dilution with water (without additives) and filtering with a 10K Amicon filter.Finally, for microdialysate samples a protocol similar to the one previously published [30] was applied, but in this case the new Amide column increased 10 times the sensitivity since were obtained narrower and higher peaks than in the previous Hilic column and higher ratio signal/noise (S/N) were achieved. Therefore basal levels were detected, although the values were close to quantification limits. A slight modification in the procedure involved an acidification of the medium with 2 µL FA with no further dilution. No significant matrix effects were observed after studying with different dilutions of samples where each analyte was quantified to confirm that the final concentrations, after applying the appropriate dilution factor, were the same in all cases.

### 3.2. Calibration curves

A calibration curve was obtained for each compound by injecting 2 µL of different standard solutions in different solvents depending on the sample, at concentration levels ranging from 0.03 to 4.00 µg mL$^{-1}$ for HMB and 0.01 to 1.00 µg mL$^{-1}$ for Leu. Standards for quantification of analytes in serum and urine were prepared in MeOH; water was used for milk and aCSF solution was used in the case of microdialysates. The lack-of-fit test was applied to two replicates and three injections of each standard (five concentration levels). The results for every type of sample are summarized in Table 1.

Quality controls were injected after every 20 injections to assure the validity of the calibration curve. The predicted value was expected to not exceed ±15% of the theoretical value.

### 3.3. Method validation

The analytical method was validated in terms of linearity, selectivity, sensitivity and accuracy (trueness and precision), according to the protocols described in the US Food and Drugs Administration (FDA) guideline for Bioanalytical Method Validation [31].

Linearity of the calibration curves. It was evaluated using coefficients of determination (%$R^2$) and $P$-values for the lack-of-fit test (%$P_{lof}$). The values obtained for $R^2$ ranged from 99.0 to 99.9% for Leu and from 99.0 to 99.6% for HMB, and $P_{lof}$ values were higher than 5% in all cases. This indicated a good linearity within the stated ranges.

Selectivity. The specificity of the method was determined by comparing the chromatograms of blank solvents with those corresponding to the samples. The two compounds of interest were found in all the cases at basal levels. No interferences from endogenous substances were observed at the retention times of each respective analyte (Fig. 2), which eluted at 1.0 min and 1.6 min for HMB and Leu respectively. This finding suggested that the LC–MS/MS conditions provided sufficient selectivity.

Accuracy: precision and trueness. Due to the lack of certified reference materials, a spike/recovery assay was performed to validate the method in terms of trueness, which was evaluated by determining the recovery of known amounts of the compounds of interest spiked into blank samples. The samples were analyzed using the proposed method and the concentration of each compound was determined by interpolation in the standard calibration

**Table 2**
Recovery and precision of target compounds in samples.

| | | Spiked ($\mu g\,mL^{-1}$) | Found[a] ($\mu g\,mL^{-1}$) | Recovery (%) | RSD (%) | *n* |
|---|---|---|---|---|---|---|
| Serum | HMB | 0.79 | 0.78 | 98.7 | 14.5 | 15 |
| | | 1.58 | 1.58 | 100.0 | 11.7 | 15 |
| | | 3.17 | 3.03 | 95.6 | 13.4 | 15 |
| | Leu | 0.09 | 0.09 | 101.1 | 9.9 | 15 |
| | | 0.18 | 0.17 | 94.7 | 8.4 | 15 |
| | | 0.37 | 0.37 | 101.9 | 13.7 | 15 |
| Urine | HMB | 0.24 | 0.24 | 101.6 | 10.0 | 15 |
| | | 0.64 | 0.64 | 100.8 | 8.9 | 15 |
| | | 1.61 | 1.61 | 101.2 | 7.1 | 15 |
| | Leu | 0.24 | 0.22 | 103.7 | 13.9 | 15 |
| | | 0.63 | 0.59 | 97.5 | 10.8 | 15 |
| | | 1.57 | 1.33 | 86.5 | 5.7 | 15 |
| Milk | HMB | 0.32 | 0.29 | 89.5 | 14.6 | 15 |
| | | 0.59 | 0.53 | 90.2 | 13.6 | 15 |
| | | 4.04 | 4.58 | 113.4 | 12.7 | 15 |
| | Leu | 0.05 | 0.05 | 99.7 | 12.2 | 15 |
| | | 0.50 | 0.45 | 89.8 | 11.9 | 15 |
| | | 1.00 | 0.97 | 96.5 | 13.9 | 15 |

[a] Mean value; RSD – relative standard deviation; *n* – number of determinations.

curve. Recoveries were calculated by comparing the found amounts with the theoretical amounts (spiked amounts). As is shown in Table 2, the recoveries were between 85% and 115% in all cases for serum, urine and milk; plasma samples were also tested but the results obtained were not acceptable and this matrix was discharged for further experiments. Based on this fact, serum was selected to continue the experiments.

The use of a filter is generally recommended to obtain clean samples and to avoid obstruction of the UPLC® Acquity system. However, we found that if the sample was centrifuged and the supernatant collected unfiltered and directly injected, there were no obstruction issues and recovery rates were maintained. Filtration was only used for milk samples.

The precision of the method (as relative standard deviation, RSD) was assessed at three concentration levels for each compound. Three replicates at each level were analyzed on the same day in order to evaluate intra-day variability and the procedure was repeated for five days to determine inter-day variability in order to obtain a total of 15 measurements ($n = 15$). The results of within-laboratory reproducibility are summarized in Table 2. RSD values were between 5.7% and 14.8%. Therefore, all compounds were within the acceptable limits for bioanalytical method validation, which are considered ≤15% of the actual value.

Precision and trueness data indicated that the methodology to determine the target compounds in serum, urine and milk samples was highly accurate and that the presence of co-extracted matrix

**Table 3**
Basal levels of HMB and Leu in serum at different conditions and stages of life.

| Rat strain | Gender/condition | Age in weeks (stage in days) | Fasting | Sample concentration ($\mu g\,mL^{-1}$) HMB | Leu |
|---|---|---|---|---|---|
| SD | Female/lactating | 16 (PND1–3) | 3–4 h | 0.55 ± 0.33 | 9.22 ± 2.94 |
| | | 17 (PND12–14) | | 1.08 ± 0.69 | 7.82 ± 5.85 |
| | | 18 (PND19–20) | | 0.37 ± 0.12 | 10.8 ± 2.8 |
| SD | Female/gestating | 13 (GD-0) | 3–4 h | 0.90 ± 0.30 | 15.7 ± 5.1 |
| | | 14 (GD-5) | | 0.77 ± 0.26 | 12.0 ± 3.1 |
| | | 15 (GD-12) | | 0.67 ± 0.12 | 9.95 ± 3.64 |
| | | 16 (GD-18) | | 0.68 ± 0.11 | 6.13 ± 2.22 |
| SD | Male/pup | 1 (PND1–3) | 3–4 h | 1.64 ± 0.40 | 14.8 ± 9.8 |
| | | 2 (PND12–14) | | 0.42 ± 0.30 | 14.9 ± 3.5 |
| | | 3 (PND19–20) | | 0.41 ± 0.02 | 9.91 ± 2.20 |
| SD | Female/pup | 1 (PND1–3) | 3–4 h | 1.29 ± 0.36 | 7.78 ± 2.24 |
| | | 2 (PND12–14) | | 0.52 ± 0.07 | 13.5 ± 5.9 |
| | | 3 (PND19–20) | | 0.42 ± 0.03 | 7.59 ± 1.71 |
| SD | Male/virgin | 13 | Overnight | 0.53 ± 0.03 | 7.49 ± 0.37 |
| SD | Female/virgin | 13 | 3–4 h | 0.94 ± 0.33 | 18.7 ± 7.9 |
| | | 13 | Overnight | 0.51 ± 0.10 | 9.43 ± 4.34 |
| SD | Female/OVX | 30 | Overnight | 0.39 ± 0.06 | 16.6 ± 7.6 |
| | Female/SHAM | 30 | | 0.44 ± 0.15 | 15.9 ± 3.6 |
| SD | Male | 72 | 3–4 h | 0.27 ± 0.06 | 12.8 ± 3.6 |
| ZLR | Male | 10 | 3–4 h | 0.17 ± 0.12 | 25.8 ± 3.4 |
| ZLR | | 15 | | 0.14 ± 0.17 | 18.0 ± 6.2 |
| ZLR | | 20 | | 0.28 ± 0.34 | 23.3 ± 6.0 |

SD – Sprague–Dawle rats; PND – Postnatal day; GD – gestational day; OVX – Ovariectomized rats; SHAM – rats wich suffer the surgery but the ovaries are not removed; ZLR – Zucker Lean Rats. Significant differences are displayed in the figures.

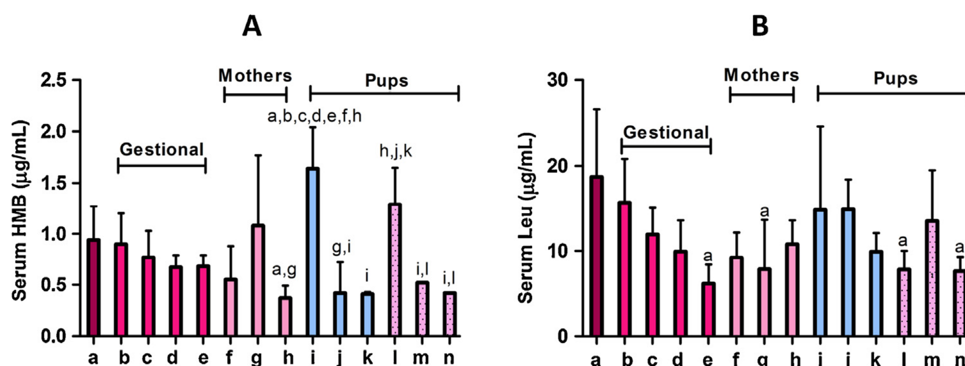



**Fig. 3.** (A) Concentration of HMB in serum during perinatal status; (B) concentration of Leu in serum during perinatal status. Letters express one-way ANOVA test significant differences with refereed groups. Letters are defined as a: Virgin; b: GD0; c: GD5; d: GD12; e: GD18; f: Mum-PND1–3; g: Mum-PND12–14; h: Mum-PND19–20; i: Male PND1–3; j: Male PND12–14; k: Male PND19–20; l: Female PND1–3; m: Female PND 12–14 and n: PND 19–20.

**Table 4**
Basal levels of HMB and Leu in urine at different conditions and stages of life.

| | | | | Sample concentration ($\mu g\,mL^{-1}$) | |
|---|---|---|---|---|---|
| Rat Strain | Gender/condition | Age (weeks) | Fasting | HMB | Leu |
| SD | Male/pup | 1 (PND1–3) | 3–4 h | 7.86 ± 2.97 | 6.54 ± 3.27 |
| | | 2 (PND12–14) | | 6.10 ± 2.84 | 6.43 ± 1.63 |
| | | 3 (PND19–20) | | 16.8 ± 7.4 | 12.9 ± 4.3 |
| SD | Female/pup | 1 (PND1–3) | 3–4 h | 11.6 ± 6.8 | 7.48 ± 3.92 |
| | | 2 (PND12–14) | | 6.46 ± 1.73 | 6.80 ± 2.07 |
| | | 3 (PND19–20) | | 17.8 ± 11.4 | 13.1 ± 7.3 |
| SD | Female | 13 | 3–4 h | 5.44 ± 2.58 | 5.70 ± 4.91 |
| | | 13 | Overnight | 11.8 ± 4.7 | 5.84 ± 1.45 |
| SD | Female/Gestating | 13 (GD-0) | 3–4 h | 7.35 ± 2.48 | 2.06 ± 0.28 |
| | | 14 (GD-5) | | 9.81 ± 1.73 | 2.28 ± 0.30 |
| | | 15 (GD-12) | | 11.0 ± 3.6 | 1.79 ± 0.41 |
| | | 16 (GD-18) | | 10.3 ± 0.9 | 2.14 ± 0.45 |
| SD | Female | 18 (PND19–20) | 3–4 h | 14.6 ± 6.4 | 6.72 ± 2.63 |
| SD | Female/ OVX | 24 | Overnight | 4.85 ± 2.94 | 0.49 ± 0.14 |
| | Female/ SHAM | 24 | | 6.16 ± 3.29 | 0.48 ± 0.06 |
| ZLR | Male | 10 | 3–4 h | 37.2 ± 11.0 | 10.4 ± 6.2 |
| | | 15 | | 34.4 ± 12.6 | 11.9 ± 1.9 |
| | | 20 | | 28.6 ± 19.5 | 10.9 ± 10.5 |

SD: Sprague–Dawle rats; PND – Postnatal day; GD – gestational day; OVX – Ovariectomized rats that were mothers at 13 weeks; SHAM – rats wich suffer the surgery but the ovaries are not removed and that were also mothers at 13 weeks; ZLR – Zucker Lean Rats.

components, which typically suppress the analyte signal in mass spectrometry, did not affect the performance of the method. Microdialysate samples were not revaluated in terms of accuracy due to the recent publication about the validation in this matrix [30]. Only different dilutions were quantified to confirm that there was no matrix effect in microdialysates.

Sensitivity. The limits of detection and quantification were calculated from the signal-to-noise ratio. The calculated LODs (signal-to-noise ratio = 3) were in the range from 4–7 ng $mL^{-1}$ for HMB and 0.4–0.9 ng $mL^{-1}$ for Leu; and the corresponding LOQs (signal-to-noise ratio = 10) ranged from 15–22 and 1–3 ng $mL^{-1}$, respectively. The values obtained are shown in Table 1.

### 3.4. Method application. Determination of HMB and Leu contents

*Serum.* Table 3 gathers all the results in serum from normal animals at different ages and situations of life under overnight fasting (12–18 h) and mild fasting (2–4 h). The biological variability was relatively high being the RSD > 15% in most of the cases. Despite this, it is worth pointing out the following biological results that are depicted also in figures (see the reference to the figures where appropriate):

(a) There were no differences on HMB serum levels during gestation being the levels similar to those found before gestation (V).

**Table 5**
Basal levels of HMB and Leu in milk from Sprague–Dawley rats in different points of the lactation period.

| | | | Sample concentration ($\mu g\,mL^{-1}$) | |
|---|---|---|---|---|
| Rat strain | Age (weeks) | Feeding | HMB | Leu |
| Sprague–Dawley | 15 (PND1–3) | Unrestricted | 1.35 ± 0.50 | 9.23 ± 2.05 |
| Sprague–Dawley | 17 (PND12–14) | Unrestricted | 1.37 ± 0.42 | 7.61 ± 4.39 |
| Sprague–Dawley | 18 (PND19–20) | Unrestricted | 1.84 ± 0.34 | 11.1 ± 5.7 |

PND – Postnatal day.

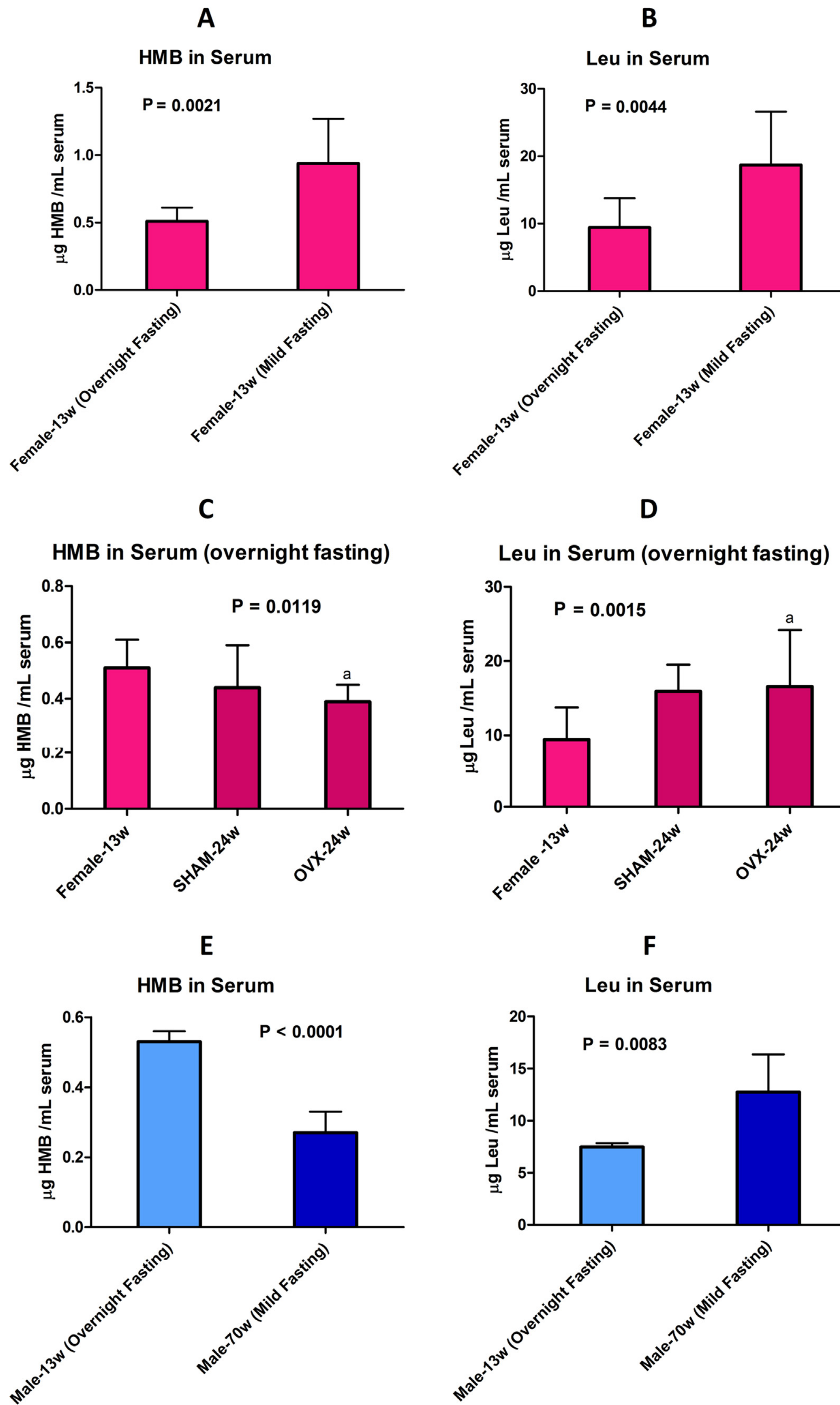



**Fig. 4.** HMB and Leu in serum at different stages; Student's *t*-test were applied and significant differences were found. Comparison between overnight and mild fasted female rats at 13 weeks of age (A for HMB and B for Leu); comparison between young female rats versus SHAM and OVX (a) rats (C for HMB and D for Leu); comparison between young male rats of 13 weeks old and male rats of 70 weeks old (E for HMB and F for Leu).

Leu concentrations tended to decrease in gestation, being significantly different at the end of gestation with regard to the levels in non-pregnant rats (Fig. 3).

(b) Pup rats (both males and females) had higher levels of HMB in serum at PND1-3 (Fig. 3).

(c) No differences were found by sex both in pups (Fig. 3) or adult animals (overnight fasted 13 weeks-old male vs females in Table 3).

- There was an effect of the fasting type: overnight fasting lowered the levels of HMB and Leu in serum (comparison between overnight fasted and mild fasted female rats at 13 weeks of age in Fig. 4: A for HMB and B for Leu).
- Ovariectomization by itself did not affect basal levels of HMB or Leu as the comparison with Sham operated animals did not yield significant differences. However, the levels of HMB tended to decrease and those of Leu to increase in ovariectomized adult rats to the point of being significantly different from young animals (Fig. 4: C for HMB and D for Leu).
- HMB levels decreased and Leu levels increase with age. In this regards, there were significant differences between males at 13 weeks and 70 weeks of age (data in Table 3, Fig. 4: E for HMB and F for Leu). Although both groups were not in the same fasting conditions, young animals were overnight and old animals were 3–4 h fasted, this would favour even higher differences between both groups, as mild fasting was associated with higher levels particularly of HMB. This result is also in line with the finding in overiectomized rats, where the combination of ovariectomization and age reduced the content of HMB. A reasonable explanation for this effect would be that the catabolism of Leu (which is about 5% in normal conditions) could be reduced with aging.
- Fig. 4 With regard to differences between strains, ZLR rats had less content of HMB and more of Leu in serum than SD rats (Fig. 5). As in explained in point (f), the difference in fasting conditions do not invalidate this finding as it also predicts higher difference in serum levels.

*Urine.* Regarding urine the results are shown in Table 4. It was not possible to obtain all urine samples due to the difficulty of the sampling process itself. There were no samples for 13 and 70 weeks-old males, and only one time point in the mother during lactation (PND 19–20) could be sampled. For the rest of the groups, between 4 and 10 samples were analyzed. The following results were found:

- HMB and Leu concentrations were higher at PND 19–20, just the opposite to plasma in which they increased at the beginning of life. No differences were found in gestation and lactation and there were no difference with the level before gestation.
- HMB excreted in ovariectomized animals was lower than in virgin animals with 13 weeks of life, being also coherent with the low HMB level found in serum.
- The excretion of both compounds was higher in the ZLR than in SD rats.

*Milk.* Table 5 shows the HMB and Leu concentrations found in this biological fluid. No significant differences were found during the lactation period. It is interesting to remark that HMB levels in milk was in a concentration around 1.5 μg mL-1 milk and close to HMB concentration in serum from pups at PND1-3.

Microdialysates from rat brain. Finally the analytical method was applied to microdialysates from rat brain in a study in vivo. The microdialysis technique was previously described in two recent publications by our group [30,33], and in the first of them HMB and Leu were also measured. The modifications of the method accomplished in this work allowed us to detect basal levels of HMB and Leu

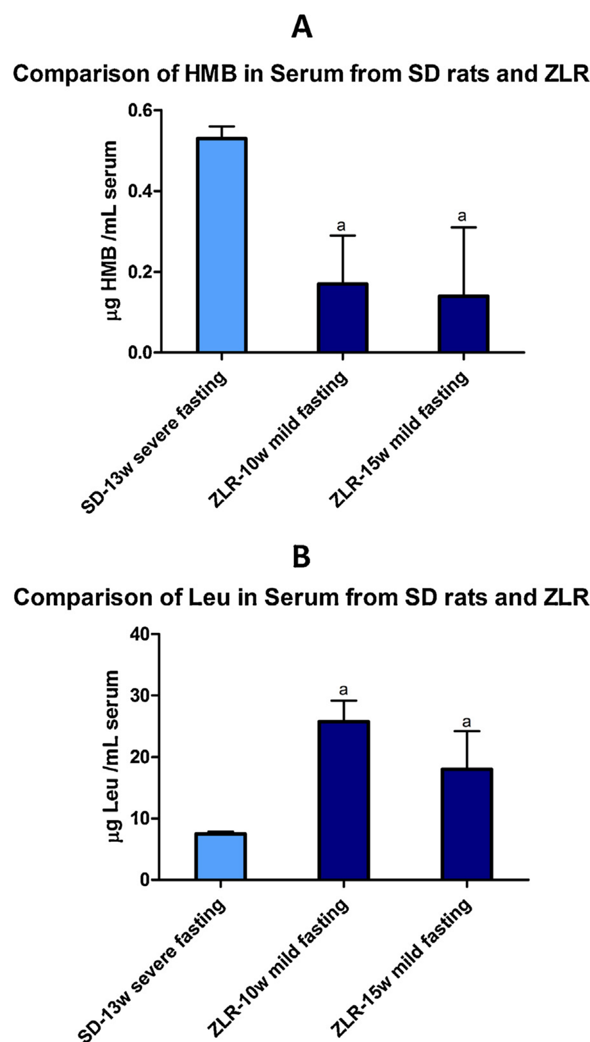



**Fig. 5.** Comparison of HMB (A) and Leu (B) in Serum from SD rats and ZLR (a). After one-way ANOVA test strong significant differences were found between male SD and male ZLR with similar ages in both cases, with an inverse behaviour between HMB and Leu.

in brain fluids, where we could not detect them before. The experiments were done in females SD rats with 18 weeks of life and under mild fasting, and the basal concentrations of HMB and Leu were $0.24 \pm 0.09\ \mu g\ mL^{-1}$ and $0.15 \pm 0.19\ \mu g\ mL^{-1}$, respectively. Unlike other tissues, the concentrations of both compounds were of the same order of magnitude.

## 4. Conclusions

The proposed method is a powerful tool for the simultaneous determination of HMB and Leu in different biological fluids. The determination and quantification of these compounds using LC–MS/MS was successfully performed on an Acquity UPLC BEH Amide column, using pure water and 0.1% (v/v) formic acid in acetonitrile as mobile phases and triple quadrupole detection in positive electrospray ionization mode. The sample treatment was adapted to each matrix, namely serum, urine, milk, and brain microdialysates, and was kept as simple as possible. The analytical performance of the method was validated providing a reliable tool for the simultaneous determination of these two compounds in several matrices, which in turn, would offer important information regarding the metabolic pathway of Leu and HMB at different stages of the life or in a variety of physiological situations.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2015.05.013

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