Synthesis and characterization of $^{13}$C labeled carnosine derivatives for isotope dilution mass spectrometry measurements in biological matrices

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**Abbreviations:** Boc, tert-butyloxycarbonyl; Boc$_2$O, Di-tert-butyldicarbonate; BSA, bovine serum albumin; CH$_3$CN, acetonitrile; CV%, variation coefficient percentage; DCM, dichloromethane; DIPEA, N,N-Diisopropylethylamine; HBTU, 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HCl, hydrochloric acid; HNE, 4-hydroxynonenal; K$_2$CO$_3$, potassium carbonate; HPLC-MS, high-performance liquid chromatography-mass spectrometry; LiOH, lithium hydroxide; MeI, methyl iodide; MeOH, methanol; MRM, multiple reaction monitoring; NaOH, sodium hydroxide; SOCl$_2$, thionyl chloride; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

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A B S T R A C T

Due to the physiological properties of L-carnosine (L-1), supplementation of this dipeptide has both a nutritional ergogenic application and a therapeutic potential for the treatment of numerous diseases in which ischemic or oxidative stress are involved. Quantitation of carnosine and its analogs in biological matrices results to be crucial for these applications and HPLC-MS procedures with isotope-labeled internal standards are the state-of-the-art approach for this analytical need. The use of these standards allows to account for variations during the sample preparation process, between-sample matrix effects, and variations in instrument performance over analysis time. Although literature reports a number of studies involving carnosine, isotope-labeled derivatives of the dipeptide are not commercially available. In this work we present a fast, flexible, and convenient strategy for the synthesis of the $^{13}$C-labeled carnosine analogs and their application as internal standards for the quantitation of carnosine and anserine in a biological matrix.
1. Introduction

L-carnosine (L-1) is an endogenous dipeptide synthesized by the enzyme carnosine synthase (EC 6.3.2.11) from β-alanine and L-histidine. The peptide is highly concentrated in skeletal muscle and other excitable tissues and has various physiological roles such as prevention of oxidation, inhibition of protein carbonylation and glycooxidation, and metal chelation [1]. Due to its properties, carnosine supplementation has been used for nutritional ergogenic application in the sport community [2], and in animal studies it is able to ameliorate several pathologies such as diabetes [3], atherosclerosis [4], and nephropathy [5]. However, L-carnosine presents limited activity in humans [6] due to the presence of the enzyme human carnosinase (CN1, EC 3.4.13.20), which rapidly hydrolyses the peptide upon absorption [7]. Besides L-carnosine, most of the animals (except *Homo sapiens*) have also the methylated analogs L-anserine (L-2) and/or L-balenine (L-3), in which the imidazole ring of L-histidine is methylated at the nitrogen atom closest to the side chain (N²) and away from the side chain (N⁵), respectively (Fig. 1). Although the relative expressions of the methylated forms differ from species to species, anserine is more frequently observed than balenine [1].

![Structure of L-carnosine (L-1) and its methylated analogs, L-anserine (L-2) and L-balenine (L-3).](image)

**Fig. 1.** Structure of L-carnosine (L-1) and its methylated analogs, L-anserine (L-2) and L-balenine (L-3).

In the past years scientific community has risen a great interest in carnosine studies, from its quantitation in food supplements to biological matrices. Therefore, several methods have been developed using high performance liquid chromatography coupled to either UV [8–10] or mass spectrometry detectors [11]. Moreover, methods relying on derivatization for improving either the retention time or the response (i.e. fluorometric detection) were developed [12,14]. Regarding the
detection of carnosine in complex matrices such as biospecimens, mass spectrometry detector is the most used [3,15–17]. Most of the methods for carnosine quantitation use no internal standard [18] or histidine containing dipeptides (i.e. L-tyrosyl-L-histidine or L-histidyl-L-leucine) [11,15,16] as internal standard, although isotope dilution (i.e. the use of an analyte isotopologue as internal standard) is the golden standard for mass spectrometry-based methods. The main explanation could be that stable isotopologues of carnosine are not available on the market or the synthesis might be expensive.

Isotope dilution mass spectrometry counterbalances spray instability, change of mobile phase solvent composition, background noise, sample interferences and competition from bulk ions [19–21]. This is critical, especially in biological matrices where a lot of compounds are present at different concentration in the samples and ion suppression phenomena can critically influence the output of a quantitative analysis [19,22,23].

However, reliable quantitation data can be obtained with isotope dilution techniques [24], where a stable isotope of the analyte is used as internal standard. The internal standard, which is characterized by identical physical and chemical properties to the analyte, will elute at the same retention time and undergo to the same phenomena in the ionization process [25,26]. On the contrary, the use of an isomer of the analyte is not always suitable since such an internal standard could have a different retention time and even if not so, it will undergo to a different ionization process due to different physical chemical properties of the molecule compared to the analyte [25].

The use of an internal standard for quantitative analysis has the function of correcting either random or systematic error [27]. To do so, internal standard has to be added as earlier as possible in the sample. Indeed, the longer the sample preparation is, the higher the chance to introduce error. Therefore, having a compound that behaves as the analyte during sample preparation procedure is desirable.

To date, in literature only a method reports an isotopic dilution quantitative analysis for the analysis of carnosine, although it is applied to quantified carnosine-HNE adduct and it has been used carnosine-HNEd11 adduct as internal standard [28]. In a previous work we have prepared a trideuterated analog of carnosine (L-carnosine-D3, L-1-[D3]) (Fig. 2a), which was successfully applied in an HPLC-MS method for the measurement of carnosinase activity in human serum [29]. Despite the result, deuterated standards present disadvantages that can limit the accuracy in quantitation by HPLC-MS. These limitations include loss of deuterium in solution due to deuterium-hydrogen exchange phenomena, loss of deuterium under mass spectrometry conditions, changes in fragmentation and changes in HPLC retention time relative to the unlabeled compound of interest
Furthermore, because of the metabolic instability of L-carnosine-D₃, the internal standard was not added during the sample preparation but following the blocking of the carnosinase hydrolytic activity.

**Fig. 2.** Synthetic strategies to obtain stable isotope-labeled carnosine and analogs: a) preparation of L-carnosine-D₃ (L-1-[D₃]) starting from L-histidine-D₃ (L-4-[D₃]) [29]; b) preparation of ¹³C labeled carnosine analogs starting from β-alanine-¹³C₃ (5-[¹³C₃]). H atoms connected to ¹³C atoms are omitted for clarity.

To outdo these limitations, we have developed a carbon-13 [¹³C] labeling-based strategy for the synthesis of stable isotope carnosine and analogs. Moreover, we decided to prepare the ¹³C labeled isotopologues as D-enantiomers to overcome the L-enantiomers instability towards tissue and serum carnosinase degradation. Interestingly, D-carnosine having the D-histidine instead of the L-histidine, displayed a carbonyl quenching activity comparable to that of L-carnosine but resulted to be stable to the enzymatic hydrolysis [31,32]. The new ¹³C labeled standards suffer from none of the previously mentioned deficiencies and, despite ¹³C is usually a more expensive labeling technique, they were prepared with a fast and efficient synthetic route which has roughly the same cost as the deuterium-based strategy. This novel approach offers four main advantages over the previous methods. First, ¹³C labeling has higher isotopic purity (up to 99%) compared to deuterated compounds, as it is not prone to exchange/loss of label. Second, it is prevented the chromatographic interference due to deuterium atoms, in fact it is reported that deuterium could change retention time of the internal standard [33]. Third, the flexible synthetic pathway developed here allows the synthesis of a ¹³C labeled carnosine derivative along with a variety of analogs, such as labeled derivatives of stereoisomers and methylated carnosine analogs (Fig. 2b). Finally, the use of a non-physiological D-
stereoisomer determines an increased metabolic stability of the labeled compound which therefore can be applied from the beginning of the sample preparation process, resulting to be a more reliable and robust internal standard.

On this basis, in the present study we report the synthesis and characterization of the two novel $^{13}$C labeled analogs of L-carnosine and L-anserine, D-1-$[^{13}$C$_3]$ and D-2-$[^{13}$C$_3]$ respectively, to be used as internal standards, along with their application for the quantitation of histidine dipeptides in a biological matrix.

2. Experimental

2.1. Materials and reagents

All chemicals were purchased from Sigma-Aldrich Srl (Milan, Italy) and Cambridge Isotope Laboratories Inc (Andover, USA) and used without further purification. Anhydrous solvents were purchased in sure seal bottles and used without further drying.

2.2. Equipments

The reactions were monitored by thin-layer chromatography (TLC) on commercial aluminum plates precoated with silica gel 60 (F-254, Merck). Visualization was performed with UV light at 254 nm. Spots were further evidenced by staining with a dilute alkaline potassium permanganate solution or an ethanol solution of ninhydrin and acetic acid. The synthesized compounds were purified on glass chromatography columns packed with silica gel (230-400 mesh particle size, pore size 60 Å, Merck) or DOWEX 50WX2 H$^+$ form resin. $^1$H NMR and $^{13}$C NMR spectra (Supplementary data S3-S11) have been registered with a Varian Mercury 300 instrument (300 MHz and 75 MHz, respectively). Chemical shifts (δ) are expressed in parts-per-million (ppm) and coupling constants (J) in hertz (Hz). Abbreviations used for peak multiplicities are given as follows: s (singlet), bs (broad singlet), d (doublet), dd (doublet of doublets), ddd (doublet of doublet of doublets), qd (quadruplet of doublets), dt (doublet of triplets), m (multiplet). Optical rotations were determined with a JASCO P-1010 polarimeter. HPLC-MS analysis was carried out on an ExionLC-100 coupled to an API4000 via Turbo-V ESI source (AB-Sciex, Milan, Italy). Instrument control and data analysis were carried out with Analyst (version 1.6.3; AB-Sciex, Milan, Italy) and Prism (GraphPad, California, USA).

2.3. Synthesis of Isotopically Labeled Compounds
2.3.1. *Methyl D-histidinate dihydrochloride* (6). D-histidine (2.00 g, 12.89 mmol) was suspended in methanol (50 mL) and cooled in an ice bath. Thionyl chloride (7.67 g, 64.45 mmol) was added dropwise, and the reaction was stirred at room temperature for 12 h. The solvent was removed under reduced pressure, resulting in compound 6 as a white powder (3.07 g, 98%): \(R_f = 0.3\) (dichloromethane/methanol/ammonia solution 8:2:0.1). \([\alpha]_D^{20} = -10.1\) (c 0.01 g/100 mL, H₂O). \(^1\)H NMR (300 MHz, CD₃OD) \(\delta\) 8.93 (s, 1H; H₂ imidazole), 7.54 (s, 1H; H₅ imidazole), 4.46 (t, \(J = 6.1\) Hz, 1H; CH₂CH), 3.86 (s, 3H; OMe), 3.52 – 3.33 (m, 2H; CH₂CH). \(^1\)C NMR (75 MHz, CD₃OD) \(\delta\) 169.38 (C OMe), 136.05 (C₂ imidazole), 128.09 (C₄ imidazole), 119.82 (C₅ imidazole), 53.85 (CH₂CH), 52.99 (OMe), 26.52 (CH₂CH).

2.3.2. *tert-butyl (R)-4-(2-((tert-butoxycarbonyl)amino)-3-methoxy-3-oxopropyl)-1H-imidazole-1-carboxylate* (8). Di-tert-butyl dicarbonate (4.00 g, 19.00 mmol) was added to a stirring suspension of 6 (2.00 g, 8.26 mmol) in methanol (20 mL). The suspension was cooled in an ice bath, then triethylamine (2.29 mL, 16.52 mmol) was added dropwise and the reaction was stirred at room temperature for 3 h. After completion, the mixture was concentrated under reduced pressure. Ethyl acetate (50 mL) was added, and the formed solution was washed with water (2 x 25 mL). The organic phase was dried over Na₂SO₄ and the solvent was removed under reduced pressure. The crude was purified via column chromatography (cyclohexane/ethyl acetate 6:4), providing compound 8 as a white powder (1.80 g, 59%): \(R_f = 0.5\) (dichloromethane/methanol 98:2). \([\alpha]_D^{20} = -4.01\) (c 1 g/100 mL, MeOH). \(^1\)H NMR (300 MHz, CDCl₃) \(\delta\) 8.11 (s, 1H; H₂ imidazole), 7.20 (s, 1H; H₅ imidazole), 5.70 (d, \(J = 7.3\) Hz, 1H; NH), 4.58 (dd, \(J = 5.4\) Hz, 1.2 Hz, 1H; CH₂CH), 3.74 (s, 3H; OCH₃), 3.11 (d, \(J = 4.8\) Hz, 2H; CH₂CH), 1.62 (s, 9H; Boc imidazole), 1.43 (s, 9H; BocNH). \(^1\)C NMR (75 MHz, CDCl₃) \(\delta\) 172.41 (C OMe), 155.58 (NHCO), 146.96 (N°CO), 138.71 (C₂ imidazole), 137.05 (C₅ imidazole), 114.70 (C₄ imidazole), 85.75 (N°COOC(CH₃)₃), 79.90 (NHCOOC(CH₃)₃), 53.30 (CH₂CH), 52.43 (COOMe), 30.39 (CH₂CH), 28.43 (N°COOC(CH₃)₃), 28.00 (NHCOOC(CH₃)₃).

2.3.3. *methyl Nα-(tert-butoxycarbonyl)-Nπ-methyl-D-histidinate* (9). In a flame-dried reaction tube flushed with argon, 8 (500 mg, 1.35 mmol) was dissolved in dry CH₃CN (6 mL). Methyl iodide (230 mg, 1.62 mmol) was added dropwise and then the reaction was stirred at 90 °C for 2 h. After completion, the solvent was evaporated under reduced pressure. The resulting yellow solid was dissolved in methanol (5 mL) and K₂CO₃ (112 mg, 0.81 mmol) was added. After stirring the mixture at 67 °C for 4 h, the solvent was removed under reduced pressure. The crude was purified via column chromatography (dichloromethane/methanol 100:0 → 95:5), providing compound 9 as a white solid.
(1.80 g, 59%): \( R_f = 0.5 \) (dichloromethane/methanol 98:2). \([\alpha]_D^{20} = +11.69 \) (c 1 g/100 mL, MeOH). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \( \delta \) 7.53 (s, 1H; H5 imidazole), 6.81 (s, 1H; H2 imidazole), 5.19 (d, \( J = 6.5 \) Hz, 1H; NH), 4.54 dd, \( J = 11.6, 5.4 \) Hz, 1H; CH\(_2\)CH), 3.74 (s, 3H; OMe), 3.60 (s, 3H; NMe), 3.10 (qd, \( J = 15.3, 5.8 \) Hz, 2H; C\(_2\)H), 1.42 (s, 9H; Boc).

\(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \( \delta \) 171.71 (COOCH), 155.18 (COO(CH\(_3\))\(_3\)), 138.27 (C2 imidazole), 127.66 (C4 imidazole), 126.89 (C5 imidazole), 80.49 (C(CH\(_3\))\(_3\)), 53.13 (CH\(_2\)C\(_H\)), 52.76 (OMe), 31.67 (NMe), 28.41 (C(CH\(_3\))\(_3\)), 27.02 (C\(_H\)\(_2\)CH).

2.3.4. (R)-1-methoxy-3-[1-methyl-1H-imidazol-5-yl]-1-oxopropan-2-aminium \(^2,2,2\)-trifluoroacetate \(^{10}\). Trifluoroacetic acid (0.245 mL, 3.18 mmol) was added dropwise to a stirring solution of 9 (60 mg, 0.21 mmol) in dichloromethane (5 mL). The reaction was stirred at room temperature for 2 h and then the solvent was removed under reduced pressure to afford compound \(^{10}\) as a yellow solid (60 mg, 95%). \([\alpha]_D^{20} = –11.6 \) (1 g/100 mL, H\(_2\)O).

\(^1\)H NMR (300 MHz, CDCl\(_3\)) \( \delta \) 8.79 (s, 1H; H5 imidazole), 7.42 (s, 1H; H2 imidazole), 4.40 (t, \( J = 7.1 \) Hz, 1H; CH\(_2\)CH), 3.83 (s, 3H; NMe), 3.78 (s, 3H; OMe), 3.34 (ddd, \( J = 24.2, 16.4, 6.6 \) Hz 1H; C\(_2\)H).

\(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \( \delta \) 169.37 (COOCH), 137.73 (C2 imidazole), 130.15 (C4 imidazole), 120.42 (C5 imidazole), 54.14 (CH\(_2\)C\(_H\)), 52.24 (OMe), 34.04 (NMe), 28.79 (C\(_H\)\(_2\)CH).

2.3.5. 3-((tert-butoxycarbonyl)amino)propanoic acid \(^{13}\)C\(_3\) \(^{11}\). 1N NaOH aq. (1 mL) was added dropwise to a stirring solution of 5 (100 mg, 1.12 mmol) in a mixture of dioxane (2 mL) and water (1 mL). The mixture was cooled with an ice bath and di-\(\text{tert-}\)butyl dicarbonate (282 mg, 1.29 mmol) was added. After stirring the reaction at room temperature for 1 h, the solvent was concentrated under reduced pressure. The resulting residue was diluted with ethyl acetate (3.5 mL) and acidified with NaHSO\(_4\) aq. (10%) to pH 2-3. The aqueous phase was washed with ethyl acetate (3 x 1.5 mL). The pooled organic phases were washed with water (2 x 2 mL), dried over Na\(_2\)SO\(_4\), filtered and concentrated under reduced pressure to afford compound \(^{11}\) as a white powder (200 mg, 94%); \( R_f = 0.7 \) (cyclohexane/ethyl acetate 1:1). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \( \delta \) 10.87 (s, 1H; \(^{13}\)COOH), 5.20 (s, 1H; NH), 3.33 (d, \( J = 137.8 \) Hz, 2H; \(^{13}\)CH\(_2\)NH), 2.51 (d, \( J = 124.7 \) Hz, 2H; \(^{13}\)CO\(_{\text{Boc}}\)), 1.39 (s, 9H; Boc).

\(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \( \delta \) 176.79 (dd, \( J = 67.2, 55.2 \) Hz; \(^{13}\)COOH), 156.16 (CONH), 79.76 (C(CH\(_3\))\(_3\)), 36.63 (dd, \( J = 93.1, 37.0 \) Hz; \(^{13}\)CH\(_2\)NH), 34.44 (dd, \( J = 53.7, 37.2 \) Hz; \(^{13}\)CO\(^{13}\)CH\(^{13}\)CH2), 28.40 (C(CH\(_3\))\(_3\)).

2.3.6. General procedure for the synthesis of intermediates \(^{12}\) and \(^{13}\). In a two-necked flask flushed with argon, \(^{11}\) (200 mg, 1.06 mmol) was dissolved in dry CH\(_3\)CN (4 mL). HBTU (400 mg, 1.06 mmol) was added and the stirring mixture was cooled with an ice bath. DIPEA (273 mg, 2.12 mmol)
was added dropwise, and the reaction was stirred for 10 min at 0 °C. D-His-OMe dihydrochloride or \(N^\alpha\)-methyl-D-His-OMe dihydrochloride were suspended in dry CH\(_3\)CN (10 mL) and added dropwise to the stirring mixture at 0 °C. The reaction was allowed to warm to room temperature and stirred for 12 h. After removal of the solvent under reduced pressure, the residue was diluted with CH\(_2\)Cl\(_2\) (10 mL) and washed with saturated aqueous solutions of NaHCO\(_3\) (4 mL) and NH\(_4\)Cl (4 mL). The organic phase was dried over Na\(_2\)SO\(_4\), filtered and concentrated under reduced pressure. The crude was purified via column chromatography (ethyl acetate/methanol 75:25), providing intermediates 12 and 13 as pure compounds.

2.3.6.1. methyl (3-((tert-butoxycarbonyl)amino)propanoyl)-D-histidinate-\(^{13}\)C\(_3\) (12). The title compound was obtained by reacting D-His-OMe dihydrochloride (256 mg, 1.06 mmol) following the general procedure. 12 was obtained as a white foam (250 mg, 70%): \([\alpha]_D^{26} = -19.08\) (c 0.45 g/100 mL, CH\(_2\)Cl\(_2\)). \(^1\)H NMR (300 MHz, CdCl\(_3\)) \(\delta\) 7.58 (s, 1H; H2 imidazole), 7.10 (s, 1H; CH\(_2\)CHNH), 6.80 (s, 1H; H5 imidazole), 5.53 (s, 1H; \(^{13}\)CH\(_2\)NH/Boc), 4.82 (d, \(J = 4.14\), Hz 1H; CH\(_2\)CH), 2.69 – 2.60 (m, 1H; \(^{13}\)CH\(_2\)CH), 2.69 – 2.60 (m, 1H; \(^{13}\)CO\(^{13}\)CHH), 2.25 – 2.19 (m, 1H; \(^{13}\)CO\(^{13}\)CHH), 1.43 (s, 9H; Boc). \(^{13}\)C NMR (75 MHz, CdCl\(_3\)) \(\delta\) 171.80 (d; \(J = 44.9\) Hz, CO), 156.28 (CONH), 135.22 (C\(_2\) imidazole), 127.87 (C4 imidazole), 116.07 (C5 imidazole), 79.38 (C(CH\(_3\))\(_3\)), 52.78 (CH\(_2\)CH), 52.41 (OCH\(_3\)), 38.04 – 35.73 (m; \(^{13}\)CH\(_2\)NH, \(^{13}\)CO\(^{13}\)CH\(_2\)CH), 29.15 (CH\(_2\)CH), 28.44 (C(CH\(_3\))\(_3\)).

2.3.6.2. methyl \(N^\alpha\)-((tert-butoxycarbonyl)amino)propanoyl)-\(N^\alpha\)-methyl-D-histidinate-\(^{13}\)C\(_3\) (13). The title compound was obtained by reacting \(N^\alpha\)-methyl-D-His-OMe ditrifluoroacetate (217 mg, 0.528 mmol) following the general procedure. 13 was obtained as a white foam (180 mg, 96%): \([\alpha]_D^{26} = +7.3\) (c 1 g/100 mL, MeOH). \(^1\)H NMR (300 MHz, CdCl\(_3\)) \(\delta\) 7.58 (s, 1H; H2 imidazole), 7.06 (bs, 1H; CHNH), 6.71 (s, 1H; H5 imidazole), 5.20 (s, 1H; \(^{13}\)CH\(_2\)NH/Boc), 4.79 (qd, \(J = 6.4, 2.9\) Hz, 1H; CH\(_2\)CH), 3.72 (s, 3H; NMe), 3.55 (s, 4H; OMe + \(^{13}\)CH\(_2\)CH), 3.08 – 3.04 (m, 3H; CH\(_2\)CH, \(^{13}\)CH\(_2\)CHNH), 2.59 (d, \(J = 5.9\) Hz, 1H; \(^{13}\)CO\(^{13}\)CHH), 2.17 (d, \(J = 11.3\) Hz, 1H; \(^{13}\)CO\(^{13}\)CHH), 1.40 (s, 9H; Boc). \(^{13}\)C NMR (75 MHz, CdCl\(_3\)) 171.65 (d; \(J = 44.9\) Hz, COOMe), 170.87 (COOMe) 156.12 (CONH), 138.48 (C2 imidazole), 127.87 (C4 imidazole), 126.65 (C5 imidazole), 79.38 (C(CH\(_3\))\(_3\)), 52.72 (CH\(_2\)CH), 51.67 (OMe), 37.56 – 35.39 (m; \(^{13}\)CO\(^{13}\)CH\(_2\)CH, \(^{13}\)CH\(_2\)NH), 31.51 (NMe), 28.46 (C(CH\(_3\))\(_3\)), 26.52 (CH\(_2\)CH).

2.3.7. 3-aminopropanoyl)-D-histidine-\(^{13}\)C\(_3\) (D-1-\(^{13}\)C\(_3\)). Trifluoroacetic acid (0.3 mL, 3.97 mmol) was added dropwise at 0 °C to a stirring solution of 12 (90 mg, 0.26 mmol) in CH\(_2\)Cl\(_2\) (5 mL). The
reaction was allowed to warm to room temperature and stirred for 1 h. After removal of the solvent under reduced pressure, the residue was diluted with THF (5 mL) and water (0.5 mL) and cooled to 0°C. 2 M LiOH aq. solution (2 mL) was added dropwise to the mixture and stirring was continued at room temperature for 4 h. Then the solvent was evaporated under reduced pressure and the crude product was purified via DOWEX 50WX2 resin, providing target compound D-1-[13C3] as a yellow solid (57 mg, 95%). [α]26 = −20.40 (c 0.5 g/100 mL, H2O). 1H NMR (300 MHz, d2O) δ 7.68 (s, 1H; H2 imidazole), 6.94 (s, 1H; H5 imidazole), 4.46 (dd, J = 8.7, 4.7, 2.8 Hz, 1H; CH2CH), 3.41 (dt, J = 11.2, 5.7 Hz, 1H; 13CH2CH, 13CO13CHH), 3.13 (dd, J = 15.0, 4.6 Hz, 1H; CHHCH), 3.00 – 2.80 (m, 3H; 13CH2H2, CHHCH, 13CO13CHH), 2.45 – 2.39 (m, 1H; 13CO13CHH2, 13CHH2, 13CO13CHH), 177.91 (COOH), 171.74 (d, J = 49.0 Hz; 13CONH), 135.72 (C2 imidazole), 128.85 (C4 imidazole), 126.02 (C5 imidazole), 53.66 (CH2CH), 36.02 (d, J = 15.5, 4.8 Hz, 1H; CHHCH), 2.94 – 2.74 (m, 3H; 13CH2H2, CHHCH, 13CO13CHH), 2.38 – 2.29 (m, 1H; 13CO13CHH). 13C NMR (75 MHz, d2O) δ 177.29 (COOH), 172.00 (d, J = 48.5 Hz; 13CONH), 138.66 (C2 imidazole), 135.72 (C4 imidazole), 135.72 (C5 imidazole), 36.02 (d, J = 15.5, 4.8 Hz, 1H; CHHCH), 2.94 – 2.74 (m, 3H; 13CH2H2, CHHCH, 13CO13CHH), 2.38 – 2.29 (m, 1H; 13CO13CHH). HRMS: theoretical m/z = 230.12393, experimental m/z = 230.12397, accuracy = 0.17 ppm (Fig. S1).

2.3.8. Nα-(3-aminopropanoyl)-Nπ-methyl-D-histidine-[13C3] (D-2-[13C3]). A 1M aqueous solution of NaOH was added dropwise up to pH 13 (1 mL) to a solution of 13 (180 mg, 0.508 mmol) in MeOH (5 mL), and the reaction was stirred at room temperature for 4h. After completion, the solvent was evaporated, and the residue suspended in water (5 mL). 1M HCl was added dropwise up to pH 1 (2 mL) and the mixture was stirred overnight. The solvent was evaporated under reduced pressure and the crude product was purified via DOWEX 50WX2 resin, providing the final compound (D)-2-[13C3] as a yellow solid (90 mg, 95%). [α]26 = −9.0 (1 g/100ml, H2O). 1H NMR (300 MHz, d2O) 7.52 (s, 1H; H2 imidazole), 6.75 (s, 1H; H5 imidazole), 4.42 (ddd, J = 9.0, 4.8, 2.9 Hz, 1H; CH2CH), 3.58 (s, 3H; NMe), 3.31 – 3.28 (m, 1H; 13CH2NH2), 3.12 (dd, J = 15.5, 4.8 Hz, 1H; CHHCH), 2.94 – 2.74 (m, 3H; 13CH2H2, CHHCH, 13CO13CHH), 2.38 – 2.29 (m, 1H; 13CO13CHH). 13C NMR (75 MHz, d2O) 177.29 (COOH), 172.00 (d, J = 48.5 Hz; 13CONH), 138.66 (C2 imidazole), 126.02 (C5 imidazole), 53.66 (CH2CH), 36.02 (d, J = 36.6 Hz; 13CH2NH), 33.61 (dd, J = 48.8, 36.4 Hz; 13CO13CH213CH2), 31.01 (NMe), 26.14 (CH2CH). HRMS: theoretical m/z = 244.13997, experimental m/z = 244.14014, accuracy = 0.69 ppm (Fig. S2).

2.4. Enantiopurity evaluation

Enantioselective chromatography was performed using a teicoplanin-based column (Chirobiotic-T, 250 × 4.6 mm, 5 µm particle size, Sigma Aldrich, Milan, Italy) as previously described [34]. Briefly, sample were injected in the HPLC system and the chromatographic separation
was carried out at 1 mL/min flow rate with a mobile phase consisting of 70 % water with 0.1 % of formic acid and 30 % methanol under isocratic conditions. The column was thermostated at 30 °C. Eluents were ionized in positive ion mode by using +3 kV ionization potential, 25 units of curtain gas, 50 units of gas 1, and 60 units of gas 2 heated at 550 °C. The detector was working in MRM mode following the transition reported in Table 1. Collision gas was set at 4 units, declustering potential at 45 V, entrance potential at 10 V, collision cell exit potential at 10 V, and scan time at 100 ms.

**Table 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor ion (m/z)</th>
<th>Product ion (m/z)</th>
<th>Collision Energy (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carnosine L-1</td>
<td>227.20</td>
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<tr>
<td></td>
<td>227.20</td>
<td>156.20</td>
<td>22</td>
</tr>
<tr>
<td>D-1-[13C3]</td>
<td>230.20</td>
<td>110.20</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>230.20</td>
<td>156.20</td>
<td>22</td>
</tr>
<tr>
<td>Anserine L-2</td>
<td>241.20</td>
<td>109.20</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>241.20</td>
<td>170.20</td>
<td>20</td>
</tr>
<tr>
<td>D-2-[13C3]</td>
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<td>109.20</td>
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</tr>
<tr>
<td></td>
<td>244.20</td>
<td>170.20</td>
<td>20</td>
</tr>
</tbody>
</table>

2.5.  **Tissue sample isolation**

Skeletal muscle was isolated from the Tibialis Anterior of a C57BL/6 mouse model. Mice were kept in pathogen-free conditions and sacrificed at 8 weeks of age. Then, muscles were collected and analyzed. In particular, the fascia of Tibialis Anterior muscles was removed and frozen in liquid nitrogen.

All the procedures on animals were conformed to Italian law (D. Lgs n 2014/26, implementation of the 2010/63/UE) and approved by the University of Milan Animal Welfare Body and by the Italian Ministry of Health.

2.6.  **Biospecimens Handling**

Tissues were homogenized with Bead Bug tissue homogenizer (Benchmark Scientific, USA) to produce a final concentration of 100 mg/mL in phosphate buffer saline (PBS). The homogenization process was done at 4 °C. Protein concentration expressed as equivalent of BSA in mg/mL was quantified with the Bradford method [35].
Urine were centrifugated at 14000 g at 4 °C for 10 minutes. Supernatant was stored at –20 °C until use.

2.7. Sample Preparation of biospecimens

Aliquots of urine samples (20 µL) were spiked (1 µL) with increasing amounts of carnosine (0.25, 1, 2.5, 5, 7.5, 10 µM) and fixed amount (5 µM) of two internal standards (i.e. tyrosyl-L-histidine and L-carnosine-D3). Samples were then deproteinized at 4 °C with 10 volumes of acetonitrile and centrifugated at 14000 rpm for 10 minutes at 4 °C. Supernatants were directly analyzed with the HPLC-MS system.

20 µL of muscle homogenates were deproteinized at 4 °C with 10 volume of acetonitrile for 10 minutes and then centrifugated at 14000 rpm for 10 minutes at 4 °C. The supernatant was spiked (1 µL) with increasing amounts of L-carnosine or L-anserine (2, 4, 6, and 8 µM) and with a fixed amount (4 µM) of the corresponding internal standards.

2.8. HPLC-ESI-MRM analysis

The samples obtained were analyzed on the HPLC-MS system. Chromatographic separation was obtained with a Hypersil Gold HILIC column (150 × 2.1 mm, 3 µM, 175 Å, Thermo-Fisher, Milan, Italy) thermostated at 40 °C. The elution was performed at 250 µL/min with a gradient method reported in Table 2.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Ammonium formate 100 mM pH 3 (%)</th>
<th>Acetonitrile (%)</th>
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</thead>
<tbody>
<tr>
<td>0.00</td>
<td>5.00</td>
<td>95.00</td>
</tr>
<tr>
<td>0.20</td>
<td>5.00</td>
<td>95.00</td>
</tr>
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<td>6.70</td>
<td>70.00</td>
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</tr>
<tr>
<td>15.00</td>
<td>5.00</td>
<td>95.00</td>
</tr>
</tbody>
</table>
Eluents were ionized in positive ion mode by using +3.5 kV ionization potential, 25 units of curtain gas, 40 units of gas 1, and 60 units of gas 2 heated at 550 °C. The detector was working in MRM mode following the transition reported in Table 1 and Table 3. Collision gas was set at 4 units, declustering potential at 45 V, entrance potential at 10 V, collision cell exit potential at 10 V, and scan time at 100 ms.

Table 3
MRM transitions of tyrosyl-L-histidine and L-carnosine-D3.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor ion (m/z)</th>
<th>Product ion (m/z)</th>
<th>Collision Energy (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosyl-L-histidine</td>
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<td>110.30</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>319.30</td>
<td>156.10</td>
<td>25</td>
</tr>
<tr>
<td>L-carnosine-D3</td>
<td>231.20</td>
<td>159.10</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>231.20</td>
<td>167.20</td>
<td>25</td>
</tr>
</tbody>
</table>

The method was qualified for carnosine and anserine quantitation in mouse muscle (Tibialis Anterior) following the validation procedure reported by Matuszewski et al. [36].

3. Results and discussion

3.1. Design and synthesis of $^{13}$C labeled carnosine analogs

With this study we present a fast and convenient strategy for the synthesis of $^{13}$C-labeled carnosine analogs. To demonstrate the flexibility of this synthetic method we have prepared the $^{13}$C-carbon isotope derivatives of both the D-isomer of carnosine (D-1-$[^{13}$C$_3$]) and its N$^\pi$ methylated analog D-anserine (D-2-$[^{13}$C$_3$]).

The chemical synthesis of L-carnosine (L-1) and its methylated analogs, L-anserine (L-2) and L-balenine (L-3), has been widely reported [37–39]. Most of the methods are based on the acylation of the amino acid histidine with β-alanine. The activation of the carboxylic group of β-alanine is required and the amino group of β-alanine has to be protected to avoid the formation of unwanted by-products because of the poor specificity of the coupling reaction [40]. By following this method, in a previous work we reported the synthesis of L-carnosine-D$_3$ (L-1-$[^{13}$C$_3$]), which was obtained from a trideuterated analog of L-histidine (L-4-$[^{13}$C$_3$]) (Fig. 2a) [29]. However, although the successful result, by applying such histidine-D$_3$-based approach the preparation of labeled D-stereoisomers of the dipeptide is not feasible and the synthesis of methylated analogs appears to be inefficient and expensive. Conversely, the novel strategy we report here is based on the application of commercially available β-alanine-$^{13}$C$_3$ isotope ($5-[^{13}$C$_3$]) and allows to synthesize the desired carnosine methylated analogs by simply
modifying the histidine moiety before the coupling reaction, saving on the number of reaction steps where the labeled compound is involved. Moreover, since the synthesis ensure the retention of the configuration, it is possible to prepare stereoisomer of the dipeptides by using D-histidine as starting material.

We initially synthesized the histidine moiety D-6 and its N\textsuperscript{\pi} methylated analog D-10 starting from the commercial D-histidine (D-4) by following the synthetic route depicted in Scheme 1. The amino acid D-4 was treated with thionyl chloride in methanol to give the corresponding methyl ester D-6. While different methods were developed to methylate the less reactive N\textsuperscript{\pi} on the imidazole ring \[39,41\], we decided to Boc(\textit{tert}-butyloxy carbonyl)-protect the methyl ester intermediate D-6 yielding the two N\textsuperscript{\pi} and N\textsuperscript{\tau} Boc imidazole regioisomers, D-7 and D-8. Boc protected also on the primary amine. D-7 and D-8 were then separated by silica gel column chromatography and obtained in a 2:8 ratio. Intermediate D-8 was submitted to N\textsuperscript{\pi} imidazole methylation, which was performed in a two-step reaction, first by treatment of D-8 with methyl iodide in acetonitrile under reflux condition, and subsequent Boc removal from the imidazole N\textsuperscript{\tau} with potassium carbonate in methanol. This mild deprotection [42] that preserves the primary amine protected allows a convenient chromatographic purification of the methylated compound D-9. Subsequent Boc group cleavage from the primary amine of intermediate D-9 upon treatment with trifluoroacetic acid (TFA) afforded compound D-10.

**Scheme 1.** Synthesis of the histidine moieties D-6 and D-10. \textit{Reagents and conditions:} i) MeOH, SOCl\textsubscript{2}, 0 °C \textelah to rt, 12 h; ii) Boc\textsubscript{2}O, TEA, MeOH, rt, 2 h; iii) MeI, CH\textsubscript{3}CN, 90 °C, 1.5 h; iv) K\textsubscript{2}CO\textsubscript{3}, MeOH, 67 °C, 4 h; v) TFA, DCM, rt, 2 h.
The desired final $^{13}$C labeled compounds D-1-$^{13}$C$_3$ and D-2-$^{13}$C$_3$ were obtained as shown in Scheme 2. The amino group of β-alanine-$^{13}$C isotope 5-$^{13}$C$_3$ was protected with tert-butylxycarbonyl resulting in 11-$^{13}$C$_3$. The carboxylic group of 11-$^{13}$C$_3$ was activated using the coupling agent hexafluorophosphate benzotriazole tetramethyl uronium (HBTU) and was then reacted with the previously prepared histidine moieties D-6 and D-10, to afford the protected dipeptides, D-12-$^{13}$C$_3$ and D-13-$^{13}$C$_3$, respectively. The desired labeled compounds D-1-$^{13}$C$_3$ and D-2-$^{13}$C$_3$ were finally obtained as pure and neutral derivatives after a two-step deprotection followed by a purification via cation exchange resin. Because of the different solubilities, D-12-$^{13}$C$_3$ was deprotected in two steps with TFA in dichloromethane and lithium hydroxide in a tetrahydrofuran/water mixture, whereas D-13-$^{13}$C$_3$ was treated with sodium hydroxide in methanol followed by hydrochloric acid in water.

Scheme 2. Synthesis of D-carnosine-$^{13}$C$_3$ (D-1-$^{13}$C$_3$) and D-anserine-$^{13}$C$_3$ (D-2-$^{13}$C$_3$) isotopes. Reagents and conditions: (i) Boc$_2$O, NaOH, dioxane/water, $0^\circ$C → rt, 1 h; (ii) HBTU, DIPEA, CH$_3$CN, $0^\circ$C → rt, 3 h; (iii) TFA, DCM, rt, 2 h; (iv) LiOH, THF/water, $0^\circ$C → rt, 1.5 h; (v) DOWEX 50WX2; (vi) NaOH, MeOH, rt, 4 h; (vii) HCl, water, $0^\circ$C → rt, 12 h.
3.2. *Enantiopurity evaluation*

Configuration purity of the final product was evaluated by means of polarimetric analysis and enantioselective chromatography. Chromatographic analysis of compounds D-1-[13C3] detected only one peak at a retention time corresponding to the D-enantiomer of carnosine (Fig. S3). Whereas chromatographic analysis of compound D-2-[13C3] detected two peaks. The most abundant was the D-enantiomer of anserine, while the L-enantiomer was less abundant. The data of the corresponding peak area were extracted, and the chromatographic enantiomeric excess (ee) was calculated and turned out to be 92 % (Fig. S4).

Presumably, this loss in enantiomeric purity results from the basicity of the imidazole ring of histidine which is highly prone to racemization in mild basic condition. Without the Boc protection the imidazole Nπ can extract the proton from the chiral center of histidine causing its racemization through the temporary formation of the enolate form [43]. Therefore, the decrease of enantiomeric purity of D-2-[13C3] could occur during the Boc-protection of intermediate D-6 which is subjected to reaction in the presence of triethylamine for 3 hours. A similar result was reported by Torikai et al. [44] while Boc-protecting the L-histidine stereoisomer. The cause of racemization was identified in the prolonged exposure of L-histidine methyl ester to basic conditions, and it was possible to increase the enantiomeric excess by reducing the reaction time.

However, the enantiomeric impurity of compound D-2-[13C3] does not have any impact on the precision and accuracy of the method based on HILIC column, since it coelutes with the D enantiomer, and it is detected at the same m/z ratio.

3.3. *Carnosine and anserine quantitation in muscle tissue*

The use of a stable isotope of carnosine as internal standard for HPLC-MS analysis of carnosine in biospecimens has been recently reported by our group [29]. Although it still retains drawbacks such as deuterium exchange in the synthesis process and metabolic instability, L-carnosine-D3 (L-1-[D3]) resulted to be more effective than tyrosyl-L-histidine in correcting error in quantitative data.

Tyrosyl-L-histidine has been used as internal standard for carnosine quantitation in many papers in the past [15,16]. Nevertheless, it has numerous drawbacks such as different retention time than carnosine (depending on the chromatographic gradient developed), and a different intrinsic ionization. Such differences, altogether prevent tyrosyl-L-histidine to be as effective as carnosine isotopologues as internal standard for MS applications.
Fig. 3 shows the correction efficacy of tyrosyl-L-histidine and L-carnosine-D₃ (L-1-[D₃]) on a calibration curve of carnosine in urine. Each concentration point was done in a single urine sample coming from a different animal, to increase matrix effect differences as much as possible. Carnosine alone does not show a good response linearity, as measured by the goodness of fit (R²) to a linear model (Fig. 3a). The use of tyrosyl-L-histidine as internal standard slightly improved the linearity, although not completely (Fig. 3b). Only the correction with L-carnosine-D₃ (L-1-[D₃]) can fully compensate the matrix effect at each concentration point, resulting in a good correlation between sample concentration and peak area (Fig. 3c). Such results are expected since matrix effect can be different from sample to sample and affect analyte response in many different ways (e.g. by affection ionization or by affection analyte separation and elution). However, the retention time of the analyte and tyrosyl-L-histidine are different and the matrix impact on their ionization and elution efficiency are not necessarily the same. For this reason, correcting the analyte response by the response of tyrosyl-L-histidine can neither re-establish a linear response to analyte concentration, nor make acceptable the accuracy and precision of the method (Supplementary data Table S1).

Fig. 3. Calibration curve in urine of: a) carnosine; b) carnosine corrected by tyrosyl-L-histidine; c) carnosine corrected by L-carnosine-D₃ (L-1-[D₃]).
As mentioned before, to increase the versatility of the internal standard by increasing its metabolic stability we decided to synthetize the D enantiomer of carnosine. The labeled amino acid β-alanine-\(^{13}\)C\(_3\) (5-[\(^{13}\)C3]) was chosen as isotope moiety, preventing deuterium exchange processes, and allowing the synthesis of carnosine analogs with small adjustment of the synthetic route.

In this study, carnosine and anserine were quantified in mouse muscle (\textit{Tibialis Anterior}). Their determination was performed by using HPLC-MS analysis and applying the isotope dilution concept to a standard addition method. Each sample was analyzed in experimental triplicates. In Fig. 4 is shown the standard addition curve obtained for carnosine (Fig. 4a) and anserine (Fig. 4b) by employing as internal standard \(^{13}\)C-carnosine (D-1-[\(^{13}\)C3]) and \(^{13}\)C-anserine (D-2-[\(^{13}\)C3]), respectively. From the linear regression analysis, we determined a final concentration of 6.02±0.52 nmol/100 mg of wet tissue or 1.10±0.09 nmol/mg of proteins for carnosine and 14.82±0.69 nmol/100 mg of wet tissue or 2.71±0.13 nmol/mg of proteins for anserine. As reported in literature, anserine and carnosine are both present in mice with anserine in a 3-fold excess compared to carnosine, which is consistent with our finding [45].

![Graph](https://via.placeholder.com/150)

\textbf{Fig. 4.} Standard addition curves for a) carnosine (R\(^2\) = 0.98) and b) anserine (R\(^2\) = 0.98) in tibial anterior homogenate.

The use of D-1-[\(^{13}\)C3] and D-2-[\(^{13}\)C3] as internal standard allowed to quantify the corresponding analytes, carnosine and anserine, with more precision and accuracy. This effect is detectable by a decrease in the coefficient of variation for each point after correcting the analyte peak area by the internal standard (Table 4).
Table 4
Concentration in mouse muscle (Tibialis Anterior) of carnosine and anserine as nmol/100 mg wet tissue.

<table>
<thead>
<tr>
<th>CARNOSINE</th>
<th>Spiked standard</th>
<th>Nominal concentration&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Average concentration&lt;sup&gt;b&lt;/sup&gt;</th>
<th>SD&lt;sup&gt;c&lt;/sup&gt;</th>
<th>CV%&lt;sup&gt;d&lt;/sup&gt;</th>
<th>ER%&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Data not corrected by the internal standard (D-1-[13C₃])</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>4.85</td>
<td><strong>6.00</strong></td>
<td><strong>0.99</strong></td>
<td>16.42</td>
<td>23.65</td>
<td></td>
</tr>
<tr>
<td>2</td>
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<td>1.13</td>
<td>15.20</td>
<td>8.73</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>8.85</td>
<td>9.26</td>
<td>1.66</td>
<td>17.94</td>
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<tr>
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<tr>
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<td>13.78</td>
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<td>23.41</td>
<td>7.24</td>
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<table>
<thead>
<tr>
<th>ANSERINE</th>
<th>Spiked standard</th>
<th>Nominal concentration&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Average concentration&lt;sup&gt;b&lt;/sup&gt;</th>
<th>SD&lt;sup&gt;c&lt;/sup&gt;</th>
<th>CV%&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Bias%&lt;sup&gt;e&lt;/sup&gt;</th>
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<tbody>
<tr>
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<td>Data not corrected by the internal standard (D-2-[13C₃])</td>
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</tr>
<tr>
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<td>33.68</td>
<td>4.29</td>
<td>12.75</td>
<td>-2.20</td>
<td></td>
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</tbody>
</table>

<sup>a</sup>Nominal concentration is the spiked standard concentration +X=0 intercept.
<sup>b</sup>Average concentration is the concentration calculated from experimental data using regression line.
<sup>c</sup>Standard deviation.
<sup>d</sup>Coefficient of variation percentage is the intermediate precision of experimental measures.
<sup>e</sup>Bias percentage is the accuracy of experimental measures.

Looking at the value reported at each calibration curve point, the coefficient of variation percentage (CV%) is too high to consider the analysis acceptable from a precision point of view. This
is because we are analyzing a complex matrix such as a tissue homogenate. As reported in Fig. 5, in such a matrix we observed an intense matrix effect reducing the signal intensity down to 30% of the signal observed in blank matrix (buffer used for homogenation).

However, the coefficient of variation (CV%) of internal standard peak area across all samples was 15.33% for D-1-[\(^{13}\text{C}_3\)] and 13.45% for D-2-[\(^{13}\text{C}_3\)]. This demonstrates an acceptable fluctuation of signal intensity across samples due to matrix effect.

![Signal intensity over time](image)

**Fig. 5.** Comparison of relative intensities of the signal of a carnosine standard (5 pmol/µL) spiked in water (blue line), in mouse muscle homogenate supernatant (orange line) or in homogenized mouse muscle before protein precipitation (gray line).

The recovery from sample preparation procedure (protein precipitation) was calculated from the ratio of the area of the analyte spiked into tissue homogenate before protein precipitation, compared with the area of the analyte spiked into tissue homogenate after protein precipitation. A recovery of about 85% of the analyte was calculated.

The implementation of the stable isotope as internal standard allowed to correct the error of each HPLC-MS course. Both intermediate precision and accuracy improved after the internal standard correction.

Mass spectrometry ionization is highly influenced by several uncontrolled factors \([19–21]\). Our obtained data for carnosine and anserine quantitation show that the use of a stable isotope as internal standard allows to reduce the impact of these variations, making the analysis more reliable.
4. Conclusion

In the present work, a new, fast, and convenient synthesis of carnosine $^{13}$C stable isotopologues was described for the first time. Compared to the deuterated derivatives [29], $^{13}$C-based isotopologues have shown more stability during the synthetic process and analysis since $^{13}$C-carbon do not undergo exchange processes such as the proton/deuteron exchange. Moreover, the use of β-alanine-$^{13}$C$_3$ isotope 5 as labeled moiety enables the synthesis of several stable isotopologues of carnosine methylated derivatives and enantiomers, by working on the less expensive unlabeled building block for almost the entire synthetic process while employing the expensive stable isotope precursor only in the final two steps of the synthesis. The stable isotopologues $^{13}$C-carnosine d-1-$^{13}$C$_3$ and $^{13}$C-anserine d-2-$^{13}$C$_3$ were prepared and successfully employed as internal standard in HPLC-MS analysis for the determination of carnosine and anserine in mouse muscle tissue. Analyte isotopologues are indeed the best internal standards for quantitative analysis in mass spectrometry experiments. However, high costs and low availability of them on the market, make their use less common. Taken together, we developed a convenient synthetic way to produce multiple carnosine stable isotopes starting from the same isotope precursor. The stable isotopologues were used as internal standards and gave optimal results in quantitative isotope dilution mass spectrometry determinations.

Credit author statement


Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
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Appendix A. Supplementary data

Supplementary data to this article can be found online.

References


