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Targeted quantification of lipid mediators in skeletal muscles using restricted access media-based trap-and-elute liquid chromatography-mass spectrometry

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Abstract

Lipid mediators (LMs) are a class of bioactive metabolites of the essential polyunsaturated fatty acids (PUFA), which are involved in many physiological processes. Their quantification in biological samples is critical for understanding their functions in lifestyle and chronic diseases, such as diabetes, as well allergies, cancers, and in aging processes. We developed a rapid, and sensitive LC-MS/MS method to quantify the concentrations of 14 lipid mediators of interest in mouse skeletal muscle tissue without time-consuming liquid-liquid or solid-phase extractions. A restricted-access media (RAM) based trap was used prior to LC-MS as cleanup process to prevent the analytical column from clogging and deterioration. The system enabled automatic removal of residual proteins and other biological interferences presented in the tissue extracts; the target analytes were retained in the trap and then eluted to an analytical column for separation. Matrix evaluation tests demonstrated that the use of the combined RAM trap and chromatographic separation efficiently eliminated the biological or chemical matrix interferences typically encountered in bioanalytical analysis. Using 14 LM standards and 12 corresponding deuterated compounds as internal standards, the five-point calibration curves, established over the concentration range of 0.031 to 320 ng mL⁻¹, demonstrated good linearity of $r^2 > 0.9903$ (0.9903 to 0.9983). The lower detection limits obtained were 0.016, 0.031, 0.062, and 0.31 ng mL⁻¹ (0.5, 1, 2, and 10 pg on column), respectively, depending on the specific compounds. Good accuracy (87.1–114.5%) and precision (<13.4%) of the method were observed for low, medium, and high concentration quality control samples. The method was applied to measure the amount of 14 target

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LMs in mouse skeletal muscle tissues. All 14 analytes in this study were successfully detected and quantified in the gastrocnemius muscle samples, which provided crucial information for both age and gender-related aspects of LMs signaling in skeletal muscles previously unknown. This method could be applied to advance the understanding of skeletal muscle pathophysiology to study the role of LMs in health and disease. Furthermore, we will expand the application of this methodology to humans and other tissues/matrices in the near future.

Graphical Abstract

Keywords

Lipid mediators; skeletal muscle; electrospray ionization (ESI); LC-MS/MS MRM; restricted access media (RAM); matrix effects

1. Introduction

Aging is associated with a progressive decline in structure and function of skeletal muscle. About 0.5–1% of muscle mass is lost per year in the individuals older than 30 years, and the rate of decline is dramatically accelerated in the elder persons over 65 years [1]. In addition, the loss of muscle mass is always combined with reduced muscle strength and endurance, thereby associated with adverse health outcomes such as physical disability, muscle injuries, poor life quality, and increased risk of death [1–3]. Furthermore, the reduction in muscle strength outpaces the loss the muscle mass, in that, there is a much larger decrease in force/strength compared to the amount of tissue lost, suggesting that the quality of muscle reduces with aging.

As a result of aging, dysregulation of a set of cellular processes, such as low-grade inflammation, increased oxidative stress, reduced capacity of muscle regeneration, and altered lipid metabolism contribute to this age-related muscle atrophy and muscle weakness, also called sarcopenia [4–6]. However, the primary molecular defects that lead to muscle dysfunction with aging are poorly understood. Even less understood is the mismatch between muscle mass and muscle strength during aging. Research designed to reveal the

biomarkers of sarcopenia and other age-related skeletal muscle disorders is essential for diagnosis of these diseases, thus promoting the development of effective treatments to improve health outcomes for older individuals.

Lipid mediators (LMs) are a class of bioactive metabolites of the essential polyunsaturated fatty acids (PUFA) which are involved in many physiological processes. They are generated locally through specific biosynthetic enzymes/receptors in response to extracellular stimuli, and play an important role through their signaling pathways on the regulation of pathophysiological states such as inflammation, metabolic syndrome, and cancer [7–9]. Thus, many LMs have been investigated as biomarkers and for drug development. LMs are structurally classified into three categories: 1) arachidonic acid (AA, ω -6 PUFA)-derived eicosanoids, including prostaglandins (PGs) and leukotrienes (LTs); 2) lysophospholipids and their derivatives; and 3) ω -3 PUFA derivatives, such as docosahexaenoic acid (DHA), α -linolenic acid (ALA), and eicosapentaenoic acid (EPA) [7].

To date, evidence from several studies suggests that lipid mediators may regulate skeletal muscle mass and function and potentially protect against muscle wasting in response to various pathological conditions. Prostaglandin E₂ (PGE₂), one of five major PGs produced from AA via the cyclooxygenase 1/2 (COX1/2) pathways, can accelerate skeletal muscle myogenic differentiation by promoting myoblast proliferation and blocking EP4 receptor results in increased production of intracellular reactive oxygen species (ROS) in myoblasts [8, 10]. Besides PGE₂, other major PGs also exhibited the regulatory roles on muscle cell growth and development. For example, an increased proliferation and reduced differentiation in L6 rat skeletal myoblasts can be observed by prostaglandin D₂ (PGD₂) [11], and prostaglandin F_{2 α} (PGF_{2 α}) can stimulate skeletal muscle growth by augmenting the size of myotube [12]. Additionally, LMs might also play a particular role in the mismatch between muscle mass and force/strength because of their potential to influence different aspects of muscle function, including the excitation-contraction coupling process. AA-derived lipid mediators eukotrienes and lipoxins have been reported for their regulating in whole-body cholesterol homeostasis and high-density lipoprotein (HDL) function in mammals [13], while cholesterol is enriched in the transverse-tubules (TT) of skeletal muscles and is essential for the formation and maintenance of these membrane structures [14–16] and maintenance of contractile force [17, 18]. Proper cholesterol content in the cell membrane is essential for optimal store-operated calcium entry (SOCE) in a variety of different cell types [19, 20], which we demonstrated to reduce with aging in skeletal muscles and contribute to sarcopenia and aging-muscle weakness [21, 22]. Moreover, numerous pre-clinical or clinical data have indicated the improved skeletal muscle health by ω -3 PUFA supplements (e.g. DHA or EPA), particularly in the elderly population [23–25]. However, the effects of ω -3 LMs on muscle growth have not yet been fully identified, even though recently these bioactive lipids (resolvins, protectins, etc.) attracted considerable attention due to their potent cell-protective and anti-inflammatory activity [26–28]. Therefore, quantitative analysis of LMs in skeletal muscle is necessary to elucidate the molecular mechanisms underlying the dissimilar lipid metabolism pathways in skeletal muscle from young and older individuals, and may provide a better understanding in the cause of and potential treatments for sarcopenia. Furthermore, this new technique has broader implications for the utilization of LMs in other fields.

A wide variety of analytical methods has been reported for LMs determination. The most commonly used detection methods are mass spectrometry (MS) coupled to either gas chromatography (GC) or liquid chromatography (LC) [29–31]. In comparison with other immunological methods such as ELISA, application of MS offers a fast, highly sensitive, and specific method for high throughput quantification. GC-MS was for a long time the preferred analytical technique for LM determination; however, in the past decade, tandem MS/MS instruments coupled with HPLC or UHPLC have become more popular [30, 32–34]. Combining the resolving power of LC and the detection specificity of MS/MS, LC-MS/MS provides a versatile and sensitive methodology for ultra-trace qualitative or quantitative bioanalysis [35]. Moreover, successful development of lipidomics profiling methods using LC-MS/MS has recently allowed for more comprehensive analysis of bioactive lipids formed by various metabolic pathways, and all can be determined simultaneously from a single sample [36–38]. However, for cell/tissue samples, though typically pre-extracted and protein precipitated with organic solvents, they often still contain significant amount of residual matrix components [39], including proteins and other large biomolecules which cause column clogging and deterioration, as well as matrix effects for targeted analysis [40]. It is therefore crucial to clean the samples prior to LC-MS. This is often accomplished with relatively laborious pretreatments, such as liquid-liquid extraction (LLE) and solid phase extraction (SPE) [41, 42]. These off-line techniques can also suffer from poor recovery and accuracy. Their advantages and disadvantages with regard to sample volume, recovery, analysis time, and automation have been comprehensively reviewed [42].

The use of on-line SPE, which minimizes sample manipulation and provides both high pre-concentration factors and recoveries, is increasingly used by many researchers to improve the sample throughput and overcome many of the limitations associated with manual LLE and SPE [43, 44]. For example, Ferreiro *et al.* developed a fast, efficient, and automatic method for trace analysis of eicosanoids in human serum and culture media using automated SPE LC-MS/MS [45].

In recent years, the use of restricted access media (RAM) based trap columns has received increasing attention as an alternative to traditional sample preparation techniques [23, 46–48]. Since the introduction of the concept of RAM through the pioneering works of Pinkerton, Boos, and Regnier [49–54], more than 20 years ago, RAM has been reasonably explored [40, 55–58] and a significant number of applications featuring the use of RAM have been reported [59–62]. The combination of size exclusion and conventional hydrophobic or hydrophilic retention in the inner pores of the particles packed in a trap column affords removal of macromolecules (excluded and unretained) from complex biological samples, whereas low molecular weight components are selectively retained and enriched. The analytes are then back- or forward-eluted and separated on an analytical column [42, 56, 63, 64]. Some researchers have comprehensively reviewed the development of analytical methods featuring RAM traps in the last decades [46, 64]. As an example, a reversed phase-based RAM LC-MS/MS system has been used for quantitative determination of dansylated bisphenol in human saliva [65]. A similar approach with RAM trap-and-elute system has been used for trace quantification of estrogens in cerebrospinal fluid and human serum samples [41, 46, 47]. Rao *et al.* developed and validated a highly sensitive and selective on-line two-dimensional LC-ESI/MS/MS method to determine sertraline

enantiomers in rat plasma. Their 2D-LC-MS/MS system consisted of a RAM column in first dimension for cleaning up the proteinaceous part of plasma and a chiral column as second dimension for separation of enantiomers and diastereomers without off-line pre-treatment [61].

The aim of this work was the development of an automatic RAM-based pretreatment and LC-MS/MS method for targeted LM quantification in skeletal muscle tissue samples. A RAM trap column interfaced with a two-position 6-port switching valve were used to streamline the automated removal of residual proteins, while trapping-and-eluting target analytes for direct on-line LC-MS/MS analysis. Fourteen standards of target compounds and twelve corresponding isotope-labelled LM internal standard compounds were used for calibration, quality control, matrix effect evaluation, and targeted quantification (Table 1). The method was validated and applied for simultaneous determination of fourteen bioactive lipids in skeletal muscles from male or female C56BL6 mice with different ages. Although most fatty acids were analyzed in negative ESI mode, this method also included positive ESI mode to obtain higher sensitivity of AEA and other LMs by using the high speed polarity switching functionality of the LC-MS instrument.

2. Experimental

2.1 Chemicals and Reagents

Fourteen lipid mediator standards (STD), including arachidonic acid (AA), docosahexaenoic acid (DHA), 6-keto prostaglandin $F_{1\alpha}$ (6-keto-PGF $_{1\alpha}$), prostaglandin $F_{2\alpha}$ (PGF $_{2\alpha}$), prostaglandin E_2 (PGE $_2$), prostaglandin A_2 (PGA $_2$), 5S,14R,15S-trihydroxy-6E,8Z,10E,12E-eicosatetraenoic acid (5S,14R-LXB4), 8-hydroxy docosahexaenoic acid (8-HDoHE), arachidonoyl ethanolamide (AEA), oleoyl ethanolamide (OEA), ($\pm$)17,18-dihydroxy-eicosa-5,8,11,14-tetraenoic acid (17,18-DiHETE), 9S-hydroxy-10E,12Z,15Z-octadecatrienoic acid (9-HOTrE), 13(S)-hydroxyoctadecadienoic acid (13-HODE), 9(S)-hydroxyoctadecadienoic acid (9-HODE), and twelve isotopically-labelled LM internal standards (IS) including AA-d $_8$, 6-keto-PGF $_{1\alpha}$ -d $_4$, PGF $_{2\alpha}$ -d $_4$, PGE $_2$ -d $_4$, prostaglandin D $_2$ -d $_4$ (PGD $_2$ -d $_4$), leukotriene B $_4$ -d $_4$ (LTB $_4$ -d $_4$), leukotriene C $_4$ -d $_5$ (LTC $_4$ -d $_5$), DHA-d $_5$, 5S-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic-5,6,8,9,11,12,14,15-d $_8$ acid (5-HETE-d $_8$), 15S-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic-5,6,8,9,11,12,14,15-d $_8$ acid (15-HETE-d $_8$), and 12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic-5,6,8,9,11,12,14,15-d $_8$ acid (12-HETE-d $_8$), and eicosapentaenoic acid- d $_5$ (EPA-d $_5$) were purchased from Cayman Chemical Co. (Ann Arbor, MI). Formic acid (reagent grade, 95%) was obtained from Sigma-Aldrich (St. Louis, MO). HPLC-MS grade acetonitrile, water, and methanol were purchased from J.T. Baker (Phillipsburg, NJ).

2.2 LC-MS/MS conditions

The LC system (all components from Shimadzu Scientific Instruments, Inc.) was equipped with four-pumps (Pump A/B: LC-30AD, Pump C/D: LC-20AD XR), a SIL-30AC autosampler (AS), and a CTO-30A column oven containing a two-position six-port switching valve. On-line sample preparation was performed on a RAM trap column SUPELCOSIL LC-HISEP (5 cm $\times$ 4.6 mm, 5 μ m; Supelco, Bellefonte, PA) coupled to LC-

MS/MS. Fig. 1 is the schematic diagram of the streamlined injection, on-line pretreatment, and LC-MS analysis. Following sample injection onto the trap, the sample was washed for 2 minutes with mobile phase C (10 mM ammonium acetate) to remove proteins and other biological interferences. The valve was then switched to elute the analytes to the analytical column for separation. The LC separation was conducted on an Ultra C8 (150 × 2.1 mm, 3 μm d_p) (Restek, Bellefonte, PA) column, along with a Halo guard column (Optimize Technologies, Oregon City, OR). The oven temperature for the analytical column was set to 30 °C. Mobile phases A and B consisted of water and acetonitrile with 0.1% and 0.05% formic acid, respectively. The LC program consisted of three gradient steps over 0–100% of mobile phase B at a flow rate of 0.4 mL min⁻¹. In order to thoroughly clean the trap and analytical column, mobile phase D (1% formic acid in isopropanol/acetonitrile, 90/10 v/v) was used at the end of each analytical run for 5 minutes before returning to initial mobile phase conditions. The settings of flow rate and gradient program are summarized in Table 2.

The LC-MS/MS analysis was performed on a Shimadzu LCMS-8050 triple quadrupole mass spectrometer. The instrument was operated and optimized under both positive and negative electrospray ionization and multiple reaction monitoring modes (+/- ESI MRM). The optimized conditions were as follows: Interface voltage, 4.0 kV; interface temperature, 275°C; desolvation line (DL) temperature, 275°C; heating block temperature, 400°C; drying gas (N₂), 10 L min⁻¹; nebulizing gas (N₂), 3 L min⁻¹; heating gas (Air), 10 L min⁻¹; and CID gas (Ar), 230 kPa. The acquisition was divided into multiple segments. The *m/z* transitions (precursor to product ions) and their tuning voltages were selected based on the best MRM responses from instrumental method optimization software (Table 1). All analyses and data processing were completed on Shimadzu LabSolutions V5.65 software.

2.3 Tissue sample preparation

Gastrocnemius muscle was isolated after mice were sacrificed, then snap frozen in liquid nitrogen immediately and stored at -80°C. Before the experiment, the aliquoted frozen muscle tissue (50–100 mg) was defrosted on ice and in the dark, weighed carefully, and minced into small pieces on ice. The minced muscle was placed into a 2.0 mL round-bottom Low Retention microcentrifuge tube (Fisher Scientific, Waltham, MA) and 1.0 mL of ice-cold 80% methanol in water (v/v) was added. The mixture was homogenized using a TissueLyser II homogenizer (Qiagen, Germantown, MD) at the frequency of 30 s⁻¹, in 8 × 30-s bursts, waiting 20 s in between to avoid high temperature. The obtained homogenate was agitated on ice and in the dark for 1–2 h, followed by centrifugation at 6,000 × g at 4°C for 10 min, twice. The supernatant was transferred to another clean 1.5 mL Low Retention microcentrifuge tube, and introduced to an Eppendorf® 5301 concentrator centrifugal evaporator (Eppendorf, Hamburg, Germany) to remove all solvents. The dried extracts were stored at -80°C immediately. *Note. All procedures during preparation of extracted muscle samples should be performed on ice.* In the future LC-MS/MS analysis, dried extracts were reconstituted in 300 μL methanol/water (20/80 v/v), then 30 μL of the mixed solution was directly injected using the autosampler.

2.4 Sample preparation for matrix effect tests

Twelve deuterated LM standards were used as model compounds to evaluate the matrix effect in the procedure. These model analytes were spiked in 1) pure water/methanol (95/5 v/v) as control samples, and 2) evaporated tissue extracts (dissolved in 80/20 water/methanol) as matrix samples at 10 concentration levels: 0.0625, 0.125, 0.25, 0.5, 1.0, 2, 4, 8, 16, 32 ng mL⁻¹. The mixed samples were then vortexed for 4 min and shaken for another 10 min. Thirty microliter of each sample was directly injected for RAM LC-MS/MS analysis.

2.5 Preparation of standard calibration curve and other validation samples

Stock solutions of 14 lipid mediators and 12 deuterated standards (IS) were prepared in methanol at 1 mg mL⁻¹. Working standard solutions were prepared at 0.188, 0.375, 0.75, 3, 24, 96 pg μ L⁻¹ by diluting the stock solutions with methanol. The stock solutions of internal standards were prepared in methanol at 24 ng mL⁻¹. The calibration standard samples were prepared by adding 5 μ L of each working solution and 10 μ L of internal standard solution to 45 μ L methanol/water (5/95 v/v). The range of five compound-specific calibration solutions are from 0.031 to 320 ng mL⁻¹ (see compound-specific concentration values in Table 3). Quality control (QC) samples were similarly prepared in quintuplicate at 5, 10, and 40 ng mL⁻¹ for DHA and AA, and 0.5, 1, and 4 ng mL⁻¹ for other 12 analytes. Ten microliter of IS stock solution were added in all samples to reach the final concentration of 4 ng mL⁻¹. Finally 30 μ L of each sample was injected for RAM LC-MS/MS analysis.

3. Results and discussion

3.1 Optimization of RAM trapping and elution conditions

The RAM trap and elution conditions were selected in the prepared tissue extracts spiked with 12 deuterated model compounds. The dried extracts were reconstituted in methanol/water (20/80 v/v), the solvent showing the minimal matrix effect and satisfied trapping efficiency for the LMs studied in this paper, before direct injection into LC-MS. Prior to analytical separation, a RAM-based trap column SUPELCOSIL LC-HISEP was placed as an automated cleanup treatment to remove proteins and other large or ionic interferences. The RAM trap used is characterized by hydrophilic outer- and hydrophobic inner-pore phases which exclude large biomolecules in the void volume, while retaining selectively low molecular weight analytes in the bonding phase through a reversed phase mode [56, 61]. It is therefore critical to use proper buffer composition to obtain best trapping efficiency when small target molecules penetrate into the porous support and interact with the stationary phases bound to the inner surface [40, 56, 66]. Loading/washing buffers of varying compositions were tested to optimize trapping efficiency using different methanol-water and acetonitrile-water mixtures containing different concentrations of ammonium acetate (0 to 20 mM). Buffer C (10 mM ammonium acetate in water), exhibited the best trapping efficiency and was selected and used as loading/washing mobile phase for all tests in this study. Similarly, the trapping efficiency was also related to the loading/washing flow rate and time, and the optimized settings, 0.2 to 2 mL min⁻¹ in 2 min, respectively, were chosen as they demonstrated optimum trapping efficiency for the compounds studied [67]. The detailed LC values and programs are presented in Table 2.

3.1.1 Evaluation of trapping efficiency—Ideally, the trapping efficiency should be evaluated based on the comparison between the MRM LC-MS responses from analytes in tissue extracts with and without the trap column being used. However, the analytical column would be damaged immediately if protein- and other large molecule-containing samples were directly injected. Therefore, in this study, the trapping efficiency was assessed by comparing the MRM LC-MS/MS responses of LMs standards dissolved in methanol/water (20/80 v/v) with and without trap column being used. The trapping efficiencies were calculated by dividing the relative peak area (STD/IS) of analytes obtained with trap by those without the trap. Data of quintuplicate analyses (2 ng mL^{-1}) from each condition are summarized in Table 4. With the average recoveries from 86 to 119%, these results indicated that the RAM trap used in this method has high trapping efficiency for the target analytes.

3.2 Method validation

The method was validated for determining a suitable calibration model, limit of detection, accuracy, precision, and possible presence of matrix effects using pure standard solutions, as well as tissue extracts from gastrocnemius muscles from young and aged male and female C57BL6 mice. The validation followed the Food and Drug Administration (FDA) Bioanalytical Method Development Guidance for Industry [68].

3.2.1 Evaluation of matrix effects—Although a powerful analytical technique for quantitative bioanalysis due to its high sensitivity and selectivity, LC-MS/MS is susceptible to matrix effects in biological samples. Matrix components can have significant influence on the accuracy, precision, and robustness of bioanalytical methods [39, 63, 69, 70]. Therefore, it is crucial to first evaluate the matrix effects in method validation.

A matrix effect, in terms of electrospray ionization – mass spectrometry, is defined as the effect of co-eluting residual matrix components on the ionization of the target analyte. It has been commonly assessed as the difference between the mass spectrometric response for an analyte in pure standard solution and the response for the same analyte in biological matrix [71]. Due to the fact that lipid mediators are ubiquitous and are potentially found endogenously in most of tissues and other biological samples, we selected twelve deuterated LM standards as model compounds (listed in Table 1 as compounds 1, 3, 5, 8, 9, 12, 16, 17, 18, 22, 23, and 25) to evaluate the matrix effects of the procedure. The matrix effects were assessed by the comparison between the MRM responses of these deuterated compounds spiked in pure water/methanol (95/5 v/v) versus in extracted tissue samples (dissolved in methanol/water, 20/80 v/v). The samples prepared in these two sets of solutions were analyzed using the developed RAM-LC-MS/MS method. The LC-MS MRM peak areas of these compounds obtained were plotted on x and y axis, and their linear coefficient values were calculated and are presented in Table 5. In an ideal situation, a slope with a value of one indicates the absence of matrix effects. The deviations of the slope to a value less than or greater than unity indicates ion suppression or enhancement, respectively, in the presence of matrix [69, 71, 72]. The slopes of the linear equations from the 10 concentration-level samples ($0.0625, 0.125, 0.25, 0.5, 1.0, 2, 4, 8, 16, 32 \text{ ng mL}^{-1}$) from the two solutions were close to unity (0.86 to 1.18), indicating essentially no or very minimal matrix effects. The minimal matrix effect in this approach can be attributed to efficient removal of proteins and

other biological interferences by the RAM trap in the first dimension, good chromatographic separations in the second-dimension analytical column, and the high ionization efficiency of the fatty acid analytes.

3.2.2 Calibration curve and linearity—As the above tests demonstrated that matrix effects were found to be absent or minimal using the developed method, pure standard solutions prepared in methanol/water (5/95 v/v) were used to establish the standard curves, validate the method, and quantify the analytes in our study. This is fortuitous because target-free biological matrix is often not available; many of the target lipids are present in all relevant blood and tissue sample types.

The prepared standard samples were analyzed in our RAM-based trap-and-elute LC-MS method. Figure 2 shows a representative MS/MS chromatogram of 14 target LMs and 12 deuterated compounds (Table 1) used as standards and internal standards, respectively. The calibration curves were generated at 5 compound-specific concentration levels: 0.031, 0.062, 0.25, 2, and 8 ng mL⁻¹ for AEA and OEA; 0.062, 0.25, 2, 8, and 16 ng mL⁻¹ for 6-keto-PGF_{1α}, PGF_{2α}, PGE₂, 5S,14R-LXB₄, 13-HODE, 9-HODE, 8-HDoHE and PGA₂; 0.25, 2, 8, 18, and 64 ng mL⁻¹ for 17,18-DiHETE and 9-HOTrE; 0.62, 2.5, 20, 80, and 320 ng mL⁻¹ for DHA and AA. The ranges varied depending on the sensitivity of the individual analytes. The regression coefficients (R²) were calculated using the linear regression analysis of relative area responses [(peak area of analyte/peak area of Internal Standard) served as y-axis] vs concentration ratios [(concentration of analyte/concentration of Internal Standard) served as x-axis]. The calibration curves exhibited good linearity (R² = 0.9903 to 0.9983) in the linear ranges established (Table 3).

3.2.3 Limits of detection and quantification—The limits of detection and quantification (LODs and LOQs) were determined as the lowest concentration of each analyte detected with a signal-to-noise ratio >3 and 10, respectively, and <15% of relative standard deviation (RSD) in 5 replicates. The analyses were carried out on samples prepared with the lowest concentration adjusted to be close to the LOD of each analyte. The limits of detection were found to be: 0.016 ng mL⁻¹ for AEA, and OEA; 0.031 ng mL⁻¹ for 6-keto-PGF_{1α}, PGF_{2α}, PGE₂, 8-HDoHE, 5S,14R-LXB₄, PGA₂, 13-HODE, and 9-HODE; 0.062 ng mL⁻¹ for 17,18-DiHETE and 9-HOTrE; and 0.31 ng mL⁻¹ for DHA and AA (detailed values presented in Table 3). These LOD values correspond to the detection limits of 0.5, 1, 2, 10 pg, respectively, on column for the four group of compounds. As shown at the lowest concentration levels of each calibration range in Table 3, the LOQs were 0.031, 0.062, 0.25, 0.62 ng mL⁻¹, respectively, for these groups of the analytes.

3.2.4 Accuracy and precision—The accuracy and precision of the method were determined by analyzing the QC samples in quintuplicate at three concentration levels: 5, 10, and 40 ng mL⁻¹ for AA and DHA; and 0.5, 1, and 4 ng mL⁻¹ for the other 12 LMs. The accuracy of the method was expressed by [(mean observed concentration)/(spiked concentration)] × 100. The precision of the method was presented as [(the calculated standard deviation)/(the calculated mean)] × 100. As shown in Table 3, the accuracies for all three levels of QC samples are in the range of 87.1% to 114.2% for all 14 LMs analyzed. All results met assay acceptance criteria for accuracy as required in FDA guidance (±15%).

Precisions for all three levels of QC samples were in the range of 2.0% to 13.4% and were also in accordance to FDA assay acceptance criteria.

3.2.5 Targeted LM quantification in mice tissue extract—Direct or indirect interference of lipid signaling systems, primarily the eicosanoid and the endocannabinoid lipid families, can significantly disrupt cellular signaling processes; thus, alterations in the levels of these lipids have been associated with the development of various pathophysiological conditions [36]. Both eicosanoids and endocannabinoids are synthesized and distributed widely in different tissues in the body; however, very limited results have been reported as for the generation and metabolism of these bioactive lipids in skeletal muscles. The developed LC-MS/MS method was applied in this study to quantitatively analyze the levels of endogenous LMs in gastrocnemius muscles from mice with different age or gender. Among 14 LMs determined in this study, 12 LMs belong to the eicosanoid system, including prostaglandins (PGE₂, PGA₂, PGF_{2α}, 6-keto-PGF_{1α}), leukotrienes (5S, 14R-LXB₄), and HODE (13-HODE and 9-HODE) derived from ω-6 PUFAs AA and LA, as well as other minor eicosanoids (9-HOTrE, 17,18-DiHETE, and 8-HDoHE) derived from ω-3 PUFAs ALA, EPA, and DHA, respectively. Additionally, OEA and AEA from endocannabinoid lipid signaling pathway system were investigated in this study. One-way analysis of variance (ANOVA) was used to compare multiple groups of measurements. All statistical analyses were performed using IBM SPSS Statistics version 23, and $p < 0.05$ was considered significant.

Five-point calibration curves were used to quantify 14 lipid mediators of interest. For the samples with higher LM concentration, they were diluted 10 times in the buffer, and then analyzed again on LC-MS/MS. The target analytes were identified and quantified based on their characteristic fragment ions and retention times. As summarized in Table 6, all 14 interested LMs were successfully detected and quantified. The RSDs of the replicate measurements are mostly less than 15%, suggesting that the precision of the present method for all determined LMs is in the FDA acceptable range.

To date, most simultaneous lipidomic assays developed by other researchers focused mainly on LC-MS/MS analysis of the samples from brain, liver, pancreas, kidney, spleen, heart, solid tumor, plasma, and spinal cord [36, 73–75], but little information on LM levels in skeletal muscles was reported. While caution should be exercised when comparing directly the lipidomic data obtained from different tissues, because profiles of PUFA-derived LMs are tissue-dependent, the importance of our new methodology and its immediate application to skeletal muscles should not understated. Skeletal muscles are the largest organ-system in the body, comprising nearly 50% of its mass and largely responsible for the regulation and modulation of overall metabolism.

Our LC-MS results indicate both gender- and age-associated differences in the levels of the determined LMs in mice gastrocnemius muscles (Table 6). In the young age groups, the levels of 11 out 14 LMs were remarkably high in females compared to males. Moreover, significant changes were observed for most tested LMs during aging, and an age-related reduction is more prominent in females than in males. Similar gender- and age-related changes in LMs levels were previously reported in human blood samples [76, 77]. Such

differences might derive from the complex sex- or age- specific biological process in the body. For example, as compared to males, the higher DHA concentrations in muscles from females (Table 6) could be contributed by the combined effects of higher estrogen expression levels (upregulation to DHA levels) and lower testosterone concentrations (downregulation to DHA levels) in the female's body [78, 79]. This regulation effect from sex hormones reduces significantly during aging, leading to the reduced DHA levels in the aged female mice and elevated levels in the aged male mice. Moreover, among the AA metabolites, the expression level of 5S,14R-LXB₄, derived from the lipoxygenase (LOX) pathway, is much lower than other metabolites derived from the cyclooxygenase (COX) pathway. This result is consistent with the previous findings that AA metabolites by the LOX pathway are highly expressed in blood and immune cells, whereas the metabolites via COX pathway are highly expressed in tissues [36, 74].

Furthermore, our new methodology, led for the first time to the quantification of LMs with key roles on tissue and organism homeostasis processes such as: a) wound-healing, b) inflammation; c) angiogenesis; d) insulin sensitivity; e) insulin resistance; and f) PPARs stimulation [27, 80–83]. We therefore envision both future prognostic and diagnostic potential for this new approach as we plan to establish it in skeletal muscle and other tissues across species, health and disease conditions. As aforementioned, lipids play a key role on membrane composition and EC coupling and even on skeletal muscle force generation. Our new technique offer a new venue to quickly look into the role of specific LMs in muscle physiology and beyond.

4. Conclusions

A rapid and sensitive analytical method was developed and validated for targeted quantification of 14 bioactive lipid mediators. It was used to quantify the amounts of LMs in extracted mice skeletal muscles. In contrast to many previous methods which involve time-consuming off line liquid-liquid or solid phase extraction, this method effectively streamlined the direct injection, on-line cleanup, and LC-MS/MS quantification in a single analysis of <35 min. By using RAM as an automated pretreatment process, the method enabled efficient and unattended removal of residual proteins and other biological interferences present in complex tissue extracts, and allowed pre-concentration of the target compounds and LC-MS analysis with minimal matrix effects. The method showed good sensitivity, linearity, precision, and accuracy, and was successfully applied to obtain the concentrations of 14 target LMs in mice skeletal muscle tissue. The concentration profiling of targeted lipid mediators obtained provided crucial information related to both gender and age aspects of skeletal muscle physiology/metabolism with insights on hormonal influences as well as for the first time, the absolute quantification of LMs involved with essential homeostatic functions ranging from tissue repair, inflammation, to insulin resistance and PPARs activation. While the targeted MS-based approaches described herein are valuable for targeted LM quantification, it may be also suitable for untargeted applications and biomarker discovery in tissues, cells or other biological samples.

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Abbreviations

AA	arachidonic acid
AEA	arachidonoyl ethanolamide
ALA	α -linoleic acid
COX	cyclooxygenase
DHA	docosahexaenoic acid
17,18-DiHETE	($\pm$)17,18-dihydroxy-eicosa-5,8,11,14-tetraenoic acid
DL temperature	desolvation line temperature
EA	ethanolamide
EPA	eicosapentaenoic acid
ESI	electrospray Ionization
IS	Internal standard
GC-MS	gas chromatography–mass spectrometry
GC-MS/MS	gas chromatography–tandem mass spectrometry
HDL	high-density lipoprotein
8-HDoHE	8-hydroxy docosahexaenoic acid
5-HETE-d₈	5S-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic-5,6,8,9,11,12,14,15-d ₈ acid
12-HETE-d₈	12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic-5,6,8,9,11,12,14,15-d ₈ acid
15-HETE-d₈	15S-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic-5,6,8,9,11,12,14,15-d ₈ acid
9-HODE	9(S)-hydroxyoctadecadienoic acid
13-HODE	13(S)-hydroxyoctadecadienoic acid
9-HOTrE	9S-hydroxy-10E,12Z,15Z-octadecatrienoic acid
HPLC	high-performance liquid chromatography
6-keto-PGF_{1α}	6-keto prostaglandin F _{1α}

LA	linoleic acid
LC	liquid chromatography
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LLE	liquid–liquid extraction
LM	lipid mediators
LOD	limit of detection
LOQ	limit of quantification
LOX	lipoxygenase
LTB₄-d₄	leukotriene B ₄ -d ₄
LTC₄-d₅	leukotriene C ₄ -d ₅
LTs	leukotrienes
5S	14R-LXB ₄ , 5S,14R,15S-trihydroxy-6E,8Z,10E,12E-eicosatetraenoic acid
MRM	multiple reaction monitoring
MS	mass spectrometry
m/z	mass-to-charge ratio
OEA	oleoyl ethanolamide
PGA₂	prostaglandin A ₂
PGD₂-d₄	prostaglandin D ₂ -d ₄
PGE₂	prostaglandin E ₂
PGF_{2α}	prostaglandin F _{2α}
PGs	prostaglandins
PUFA	polyunsaturated fatty acid
QC	quality control
RAM	restricted-access media
RSD	relative standard deviation
SPE	solid-phase extraction
STD	standard
TT	transverse-tubules

UPLC

ultra-performance liquid chromatography

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Highlights

- Streamlined RAM-trap pretreatment and LC-MS/MS quantification simultaneously.
- Time savings and higher throughput.
- Rapid and sensitive quantification of lipid mediators in skeletal muscles.
- Biological or chemical matrix interferences are eliminated efficiently.
- Advancing the knowledge of lipid signaling in musculoskeletal health and disease.

Fig. 1.
Schematic diagram of the on-line trap-elute pretreatment and the LC-MS/MS analyses.

Fig. 2. Representative MS/MS chromatogram of target LMs and deuterated standards. (Peaks # labelled correspond to the compounds 1–26 in Table 1).

Table 1

Target and deuterated lipid mediator compounds and MRM transitions

#	Compounds	Retention (min.)	MRM (+/-)	MRM Transition (M/Z)	MS parameters			Compound type
					Q1(V)	CE(V)	Q3(V)	
1	6-keto-PGF _{1α} -d ₄	10.7	MRM(-)	373.20>249.10	22	26	25	Deuterated
2	6-keto-PGF _{1α}	10.7	MRM(-)	369.20>245.20	22	25	24	Target(1 [*])
3	PGF _{2α} -d ₄	12.6	MRM(-)	357.20>197.20	12	26	19	Deuterated
4	PGF _{2α}	12.6	MRM(-)	353.20>193.10	15	24	18	Target(3 [*])
5	PGE ₂ -d ₄	13.1	MRM(-)	355.20>275.20	21	17	29	Deuterated
6	PGE ₂	13.1	MRM(-)	351.20>271.20	21	16	28	Target(5 [*])
7	5S,14R-LXB ₄	13.4	MRM(-)	351.30>221.20	12	15	22	Target(5 [*])
8	PGD ₂ -d ₄	13.5	MRM(-)	355.20>275.20	21	17	29	Deuterated
9	LTC ₄ -d ₅	14.2	MRM(+)	631.40>308.20	-28	-14	-22	Deuterated
10	PGA ₂	15.3	MRM(-)	333.20>271.20	20	15	17	Target(9 [*])
11	17,18-DiHETE	16.2	MRM(-)	335.20>247.20	20	16	25	Target(12 [*])
12	LTB ₄ -d ₄	16.4	MRM(-)	339.20>197.10	11	15	11	Deuterated
13	9-HOTe	18.3	MRM(-)	293.20>171.10	17	16	30	Target(16 [*])
14	13-HODE	19.5	MRM(-)	295.20>195.10	24	18	18	Target(16 [*])
15	9-HODE	19.5	MRM(-)	295.20>171.10	13	19	29	Target(16 [*])
16	15-HETE-d ₈	19.8	MRM(-)	327.20>226.20	26	14	22	Deuterated
17	12-HETE-d ₈	20.2	MRM(-)	327.20>184.30	13	15	18	Deuterated
18	5-HETE-d ₈	20.4	MRM(-)	327.20>116.10	14	15	19	Deuterated
19	8-HDoHE	20.4	MRM(-)	343.20>109.10	16	14	18	Target(18 [*])
20	AEA	21.5	MRM(+)	348.20>62.10	-15	-15	-25	Target(18 [*])
21	OEA	22.6	MRM(+)	326.20>62.10	-28	-36	-35	Target(18 [*])
22	EPA-d ₅	22.6	MRM(-)	306.20>262.20	10	11	26	Deuterated
23	DHA-d ₅	23.0	MRM(-)	332.20>288.20	11	11	20	Deuterated
24	DHA	23.0	MRM(-)	327.20>283.20	11	12	30	Target(23 [*])

#	Compounds	Retention (min.)	MRM (+/-)	MRM Transition (M/Z)	MS parameters			Compound type
					Q1(V)	CE(V)	Q3(V)	
25	AA-d ₈	23.1	MRM(-)	311.20>267.2	11	13	17	Deuterated
26	AA	23.1	MRM(-)	303.20>259.2	24	16	25	Target(25 [*])

^{*} the IS used for this target compound.

Table 2

Mobile phase and LC program

Time (min)	A,B flow (mL min ⁻¹)	%B	C flow (mL min ⁻¹)	D flow (mL min ⁻¹)	Switching valve position	Loading/Washing
0–2	0.4	0	0.2–2	0	1	
2–5	0.4	25	0	0	0	
5–10	0.4	40	0	0	0	
10–20	0.4	75	0	0	0	Elution of retained analytes
20–25	0.4	100	0	0	0	
25–30	0	100	0	0.35	0	
30–35	0.4	0	5	0	1	Equilibration of trap and column

Compositions of mobile phases A–D are 0.1% formic acid in water, 0.05% formic acid in acetonitrile, 10 mM ammonium acetate in water, and 1% formic acid in isopropanol/acetonitrile (90/10 v/v), respectively.

Table 3
Limit of detection (LOD), calibration range, linearity, precision and accuracy of the RAM LC-MS/MS assay

LOD (ng mL ⁻¹)	Linearity Range (ng mL ⁻¹)	Linearity (R ²)	Prepared concentration (ng mL ⁻¹)	Measured concentration (ng mL ⁻¹)	Precision (CV %)	Accuracy (%)
6-keto-PGF_{1α}						
0.031	0.062–32 <i>I</i>	0.9995	0.5	0.44 ± 0.01	3.0	88.3
			1	1.07 ± 0.07	6.5	106.6
			4	3.87 ± 0.13	3.4	96.6
PGF_{2α}						
0.031	0.062–32 <i>I</i>	0.9992	0.5	0.45 ± 0.05	11.3	90.5
			1	0.98 ± 0.09	9.3	98.4
			4	3.73 ± 0.16	4.2	93.2
PGE₂						
0.031	0.062–32 <i>I</i>	0.9995	0.5	0.45 ± 0.02	4.7	90.0
			1	1.00 ± 0.04	4.1	99.8
			4	3.86 ± 0.09	2.4	96.4
5S,14R-LXB₄						
0.031	0.062–32 <i>I</i>	0.9994	0.5	0.46 ± 0.01	3.2	92.9
			1	1.01 ± 0.07	7.1	100.6
			4	3.78 ± 0.19	5.0	94.6
PGA₂						
0.031	0.062–32 <i>I</i>	0.9995	0.5	0.56 ± 0.05	8.8	111.6
			1	1.06 ± 0.04	4.0	106.4
			4	3.97 ± 0.23	5.8	99.2
17,18-DHETE						
0.062	0.25–64 <i>I</i>	0.9980	0.5	0.51 ± 0.05	9.1	101.6
			1	0.99 ± 0.07	6.6	99.3
			4	4.56 ± 0.35	7.6	114.0
9-HOTrE						
0.062	0.25–64 <i>I</i>	0.9999	0.5	0.51 ± 0.02	3.5	101.7
			1	1.03 ± 0.07	6.3	102.9
			4	4.00 ± 0.22	5.6	100.0
13-HODE						
0.031	0.062–32 <i>I</i>	0.9999	0.5	0.53 ± 0.04	7.2	106.9
			1	1.08 ± 0.05	4.6	108.3

LOD (ng mL ⁻¹)	Linearity Range (ng mL ⁻¹)	Linearity (R ²)	Prepared concentration (ng mL ⁻¹)	Measured concentration (ng mL ⁻¹)	Precision (CV %)	Accuracy (%)
9-HODE						
0.031	0.062–32 ¹	0.9998	4	3.93 ± 0.29	5.0	100.4
			0.5	0.52 ± 0.03	6.8	103.1
			1	1.04 ± 0.07	6.5	104.1
			4	3.95 ± 0.12	2.9	98.8
8-HDoHE						
0.031	0.062–32 ¹	0.9997	4	0.44 ± 0.03	6.2	87.1
			0.5	0.95 ± 0.03	3.1	95.3
			4	3.98 ± 0.15	3.8	99.5
AEA						
0.016	0.031–8 ³	0.9976	4	0.47 ± 0.05	10.5	93.1
			0.5	0.94 ± 0.06	6.9	94.3
			4	3.82 ± 0.08	2.0	95.6
OEA						
0.016	0.031–8 ³	0.9903	4	0.56 ± 0.04	7.0	112.2
			0.5	1.14 ± 0.06	5.6	114.2
			4	4.21 ± 0.31	7.3	105.3
DHA						
0.31	0.62–320 ⁴	0.9998	40	4.9 ± 0.7	13.4	97.4
			5	10.1 ± 0.4	3.6	101.4
			40	38.5 ± 3.3	8.5	96.3
AA						
0.31	0.62–320 ⁴	0.9968	40	4.6 ± 0.2	4.1	92.3
			5	10.0 ± 0.8	8.0	100.3
			40	41.2 ± 3.1	7.6	103.1

¹0.062 – 32 ng mL⁻¹ from 5 concentration levels: 0.062, 0.25, 2, 8, 32 ng mL⁻¹;²0.25 – 64 ng mL⁻¹ from 5 concentration levels: 0.25, 2, 8, 32, 64 ng mL⁻¹;³0.031 – 8 ng mL⁻¹ from 5 concentration levels: 0.031, 0.062, 0.25, 2, 8 ng mL⁻¹;⁴0.62 – 320 ng mL⁻¹ from 5 concentration levels: 0.62, 2.5, 20, 80, 320 ng mL⁻¹.

Table 4Trapping efficiency^a of lipid mediators with and without the RAM trap^b

Lipid mediators	Normalized Area (STD/IS)		Efficiency (%)
	Without RAM trap	With RAM trap	
6-keto-PGF_{1α}	7.11 ± 0.10	7.68 ± 0.22	108
PGF_{2α}	5.90 ± 0.22	5.69 ± 0.23	96
PGE₂	2.89 ± 0.14	2.71 ± 0.09	93
5S,14R-LXB₄	0.73 ± 0.05	0.68 ± 0.03	93
PGA₂	10.34 ± 1.04	10.24 ± 0.78	99
17,18-DiHETE	1.06 ± 0.02	1.14 ± 0.05	107
9-HOTrE	2.84 ± 0.21	3.22 ± 0.25	114
13-HODE	5.80 ± 0.25	6.07 ± 0.16	105
9-HODE	2.09 ± 0.14	2.33 ± 0.07	111
8-HDoHE	2.61 ± 0.30	2.25 ± 0.08	86
AEA	18.73 ± 3.14	22.30 ± 3.40	119
OEA	3.60 ± 0.50	3.46 ± 0.27	96
DHA	11.99 ± 1.34	12.19 ± 1.30	102
AA	4.18 ± 0.30	4.37 ± 0.26	105

^a Mean ± SD, n=5.^b Concentration of standard solution is 2 ng mL⁻¹.

Table 5

Slopes (k) and coefficients (R^2) of linear regression analysis for 12 deuterated lipid mediator compounds spiked in pure methanol/water versus in tissue extracts to assess matrix effects.

Deuterated compounds	Slope (k)	R^2
6-keto-PGF _{1α} -d ₄	1.06	0.9811
PGF _{2α} -d ₄	0.97	0.9981
PGE ₂ -d ₄	0.89	0.9958
PGD ₂ -d ₄	0.93	0.9974
LTC ₄ -d ₅	1.11	0.9997
LTB ₄ -d ₄	1.09	0.9947
15-HETE-d ₈	1.02	0.9920
12-HETE-d ₈	1.00	0.9899
5-HETE-d ₈	0.98	0.9906
EPA-d ₅	0.86	0.9990
DHA-d ₅	1.18	0.9933
AA-d ₈	1.05	0.9966

Table 6
Lipid mediators (LMs) quantification (pg mg⁻¹ muscle) and associated metabolic pathways

Metabolic pathways	Lipid mediators	Male		Female	
		4-month old	16-month old	4-month old	18-month old
Linoleic acid (LA)	13-HODE	31.1 ± 2.3 (7.5)	57.3 ± 3.8*** (6.7)	96.0 ± 5.2 (5.5)	38.9 ± 2.0*** (5.2)
	9-HODE	31.7 ± 1.6 (5.0)	60.5 ± 3.2*** (5.3)	66.3 ± 2.9 (4.4)	44.1 ± 2.3*** (5.1)
Arachidonic Acid (AA)	AA	2016.7 ± 196.3 (9.7)	1602.6 ± 123.6 (7.7)	4404.0 ± 263.5 (6.0)	3860.3 ± 348.7* (9.0)
	6-keto-PGF _{1α}	1.41 ± 0.14 (10.2)	0.90 ± 0.12 (13.1)	4.43 ± 0.30 (6.8)	4.50 ± 0.62 (13.7)
	PGF _{2α}	2.41 ± 0.07 (3.0)	1.37 ± 0.08*** (6.1)	5.34 ± 0.12 (2.3)	3.71 ± 0.24*** (6.6)
	PGE ₂	2.92 ± 0.22 (7.5)	1.93 ± 0.09*** (4.5)	2.21 ± 0.11 (5.1)	0.92 ± 0.09*** (9.5)
	PGA ₂	0.10 ± 0.01 (14.7)	0.13 ± 0.03 (25.4)	0.55 ± 0.07 (12.4)	0.35 ± 0.04*** (11.9)
	5S,14R-LXB ₄	0.08 ± 0.02 (20.2)	N.D.	0.13 ± 0.01 (5.1)	N.D.
α-Linoleic acid (ALA)	9-HOTRE	0.85 ± 0.04 (4.9)	1.77 ± 0.12*** (6.6)	3.02 ± 0.24 (8.0)	1.22 ± 0.07*** (5.5)
Eicosapentaenoic acid (EPA)	17,18-DIHETE	5.06 ± 0.33 (6.5)	9.44 ± 0.48*** (5.1)	4.79 ± 0.41 (8.5)	2.43 ± 0.22*** (8.9)
Docosahexaenoic acid (DHA)	DHA	1927.9 ± 149.1 (7.7)	2322.7 ± 125.6 (5.4)	5781.0 ± 472.3 (8.2)	5068.7 ± 342.2** (6.8)
	8-HDoHE	5.66 ± 0.26 (4.5)	3.73 ± 0.08*** (2.1)	17.3 ± 0.7 (4.2)	3.36 ± 0.22*** (6.5)
Ethanolamide (EA)	AEA	2.32 ± 0.14 (6.0)	1.96 ± 0.46 (2.1)	1.99 ± 0.22 (10.9)	2.30 ± 0.26 (11.4)
	OEA	23.0 ± 2.1 (8.9)	15.3 ± 1.4*** (9.1)	31.6 ± 2.4 (7.5)	19.6 ± 2.7*** (13.7)

Mean ± SD (RSD%), n=5.

One-way ANOVA with Tukey post hoc test (**p* 0.05; ***p* 0.01; ****p* 0.001) was used to compare LMs levels in gastrocnemius muscles from young and aged male and female C57BL/6 mice.

N.D., not detected.