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High sensitivity measurement of amino acid isotope enrichment using liquid chromatography–mass spectrometry

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ABSTRACT

Measurement of the incorporation or conversion of infused stable isotope enriched metabolites *in vivo* such as amino acids plays a key role in metabolic research. Specific routes are frequently probed in knockout mouse models limiting the available amount of sample. Although less precise as compared to combustion-isotope ratio mass spectrometry (C-IRMS), gas chromatography–mass spectrometry (GC–MS) or liquid chromatography–mass spectrometry (LC–MS) techniques are therefore often the method of choice to measure isotopic enrichment of target metabolites. However, under conditions of metabolic depletion, the precision of these systems becomes limiting. In this paper, studies were performed to enhance the sensitivity and precision of isotope enrichment measurements using LC–MS. Ion-statistics and resolution were identified as critical factors for this application when using a linear trap mass spectrometer. The combination with an automated pre-column derivatization and a carefully selected solvent mix allowed us to measure isotopic enrichments down to 0.005% at plasma concentrations as low as 5 μmol/l, an improvement by a factor of 100 compared to alternative methods. The resulting method now allowed measurement of the *in vivo* conversion of the amino acid arginine into citrulline as a marker for the production of nitric oxide in an *in vivo* murine endotoxemia model with depleted plasma levels of arginine and citrulline.

1. Introduction

The infusion of amino acids enriched with stable isotopes followed by measurement of their dilution in the body pool is considered a validated tool to study protein and amino acid metabolism in humans [1]. Arginine (ARG) and citrulline (CIT) enriched stable isotopes have been extensively used in both experimental and clinical studies to study the effects of interventions on arginine–nitric oxide metabolism [2]. To measure these stable isotope enrichments in blood and tissue, gas chromatography–mass spectroscopy (GC–MS) [2–4], isotope-ratio mass-spectroscopy (IRMS) [3,4] and liquid chromatography–mass spectrometry (LC–MS) [5–7] have been used.

Presently, IRMS systems still provide a superior precision compared to LC and GC–MS systems, but these systems are expensive, dedicated for this application only and require relatively large sample amounts, complicating the use of knockout mice models. Alternatively, GC–MS gained great popularity as a much more versatile approach [8], followed by LC–MS after the introduction of atmospheric pressure ionization (API) techniques. Even today, LC–MS systems with improved resolution, precision and sensitivity are introduced at increased frequency [4,7,9,10]. However, these enhanced system capabilities can only be put to their full deployment, provided current problems associated with the separation of important metabolites, like the amino acids CIT and ARG, can be solved. In the past, many strategies have been developed to determine amino acid concentrations in physiological samples [11–13]. Measurement of amino acid isotope enrichments as targeted in this study adds specific pre-requisites to the analysis. Important issues for this application are a fast and complete reaction at room-temperature enabling an automated pre-column derivatization, the formation of derivatives with a high ionization efficiency, which can be separated using MS-compatible solvents. In addition, minimal solvent and reagent based ion-suppression is important as well as robustness of the applied method.

Here, we describe a modified procedure [6] to chromatographically separate OPA-derivatized amino acids followed by mass spectrometric analysis of their isotopomeric envelop. In the present

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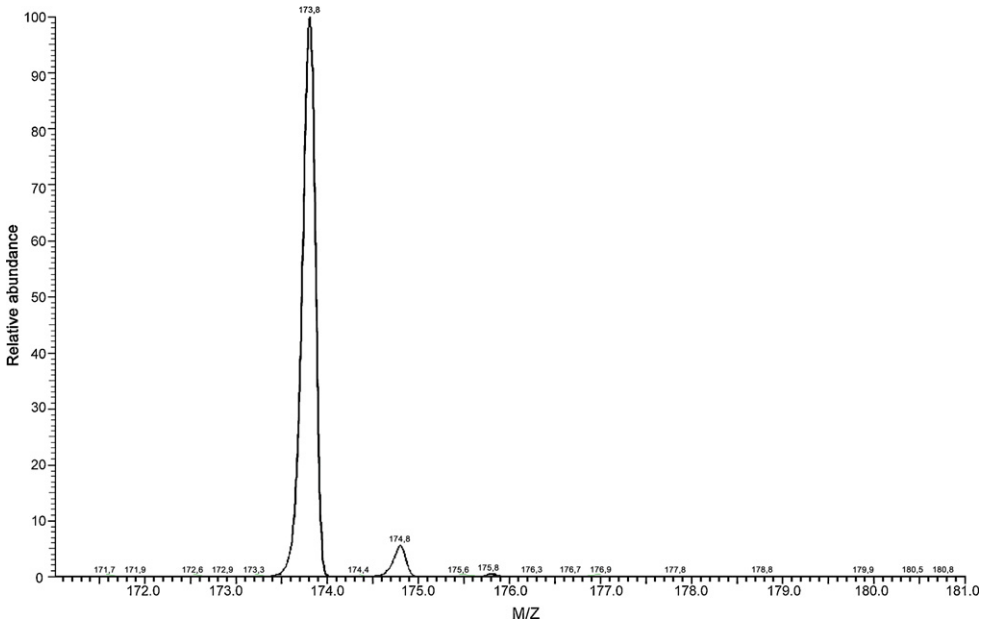

Fig. 1. Isotopomeric envelop of naturally enriched arginine.

procedure, we adapted previously described procedures [2,5,6] to eliminate unwanted reagent side reactions causing rapid deterioration of the separation yielding a robust chromatographic method. In addition, we investigated which factors are critical to obtain maximal precision when using a modern linear trap high sensitivity LC–MS system for the measurement of the lowly concentrated amino acid isotopomeric peaks.

To validate this approach, calibration curves were constructed to determine the linear ranges of both concentration and isotopic enrichment of an LTQ-XL mass spectrometer. In addition, the suitability of the method was evaluated by measuring the isotopic enrichment of plasma samples obtained from an animal experiment.

2. Materials and methods

2.1. Instrumentation

The HPLC system consisted of a Gilson Model 233 XL sample processor, equipped with cooled sample and reagent trays (10 °C) and a Rheodyne 6 way high-pressure valve (Meyvis, Bergen op Zoom, The Netherlands) equipped with a 10 μl sample loop. A high pressure gradient at a flow of 0.35 ml/min was generated using two Model PU1580 pumps connected through a T-piece (Jasco Benelux, Maarssen, The Netherlands). The separation was performed on a 150 × 3 mm (i.d.) Allsphere C18, 3 μm column (Grace/Alltech, Breda, The Netherlands), mounted in a Spark Mistral column oven (Separations, Hendrik Ido Ambacht, The Netherlands) set to 22 °C. The bench-top mass spectrometer was an LTQ XL (ThermoElectron Veenendaal, The Netherlands), equipped with an ion-max electrospray (ESI) probe. The system was operated in negative mode. Baseline separation was achieved of the isotopomeric peaks using the enhanced full scan mode. Maximal sensitivity was obtained with the heated capillary set to 220 °C, sheath and auxiliary gas set to 99 and 35 units respectively. The tube lens offset was 20 V and the spray-voltage was 4.5 kV.

2.2. Reagents and solvents

2.2.1. OPA reagent

OPA reagent consisted of 15 mg o-phthaldialdehyde (Fluoraldehyde, Pierce/Omnilabo, Breda, The Netherlands) dissolved in 0.5 ml methanol, buffered with 3.5 ml of 1 M, pH 10.4 potassium borate buffer and with the addition of 15 μl 3-mercaptopropionic acid (3-MPA).

2.2.2. Solvents and gradient

Solvent A was 10 mmol/l acetic acid set to pH 6 with triethylamine (TEA) and solvent B was a mixture of acetonitrile and milliQ water (40/60, v/v). Prior to use, helium sparking degassed solvents. At $T = 0$ min, 95% solvent A was pumped at 0.35 ml/min. Within 40 min the percentage of solvent B was increased to 54%, followed by an increase to 100% B in 5 min. During 5 min 100% solvent B was maintained to regenerate the column, after which initial conditions were restored within 1 min. After 9 min equilibration, the system was ready for the next run.

2.3. Animal experimentation

2.3.1. Surgical and experimental procedure

Male C57Bl6/j mice were obtained from the breeding facilities at the Central Animal Facilities of Maastricht University. The Animal Ethics Committee of Maastricht University approved all experiments (DEC nr 2008-042). Mice were either treated with saline ($N = 4$) or lipopolysaccharide (LPS) ($N = 9$) as described previously [14]. In brief, prior to surgery, mice were pre-medicated with 0.01 mg/kg buprenorfine subcutaneously and anesthesia was induced with 4% isoflurane. During surgery, anesthesia was maintained with 2% isoflurane. To gain continuous venous access, mice received a right jugular vein cannulation. Four days after cannulation a continuous infusion of LPS (*Escherichia coli* O55:B5, Sigma–Aldrich, St. Louis, MO) was started to induce endotoxemia. In total 200 μg LPS was infused during an 18 h period, with a continuous rate of 83 μl/h. At the end of the LPS infusion period, a primed-constant infusion of L-[guanidino-$^{15}N_2$]-arginine and L-[ureido-^{13}C–$^{2}H_2$]-citrulline was infused in the jugular vein. The priming dose was 146 nmol/10 g bodyweight [$^{15}N_2$]-arginine and 44 nmol/10 g [^{13}C–$^{2}H_2$]-citrulline and the infusion dosage was 960 nmol/10 g bodyweight [$^{15}N_2$]-arginine and 90 nmol/10 g [^{13}C–$^{2}H_2$]-citrulline. Previous experiments [14] indicated isotopic steady state between 20 and 60 min. At the end of the experiments

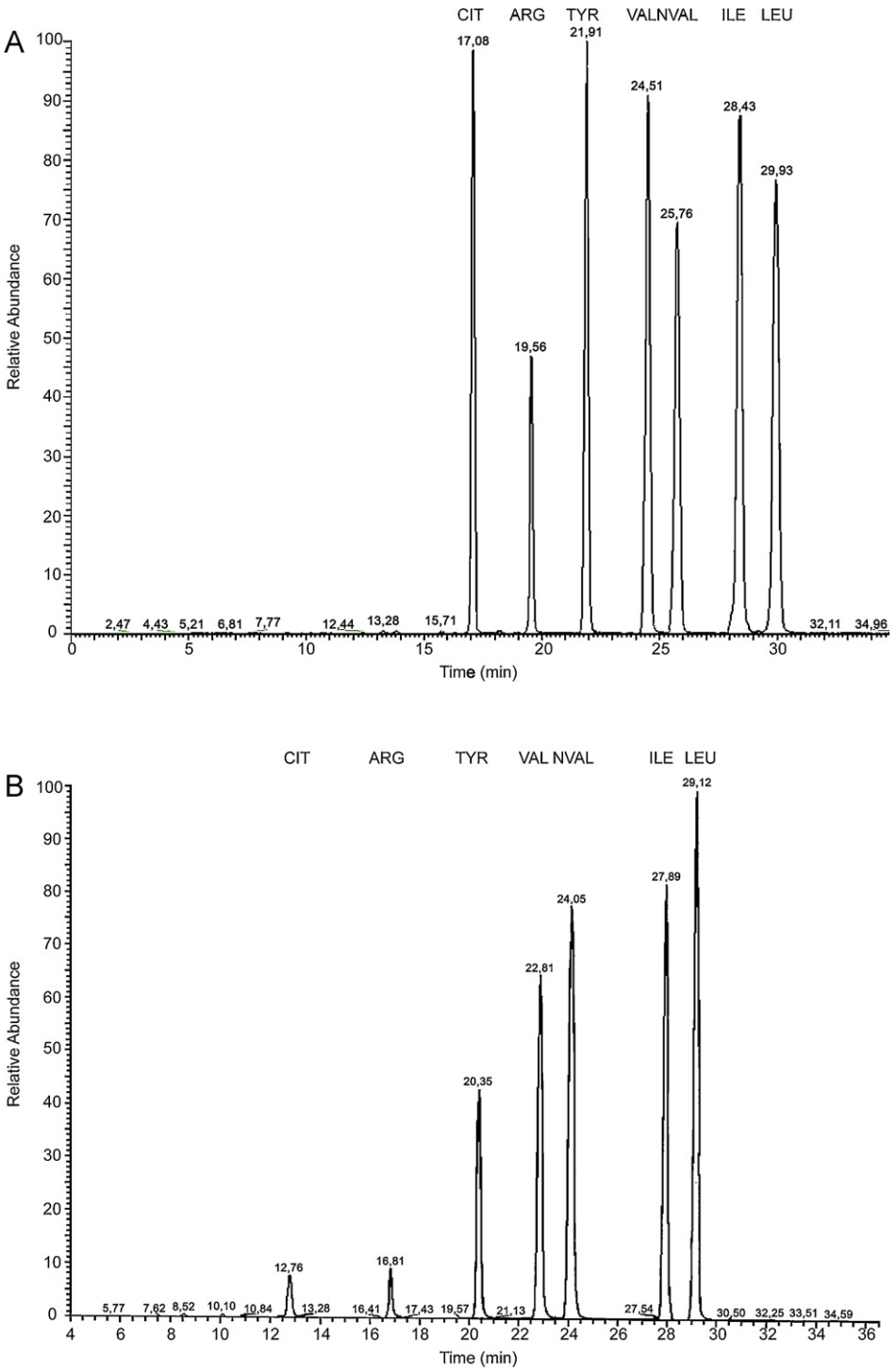

Fig. 2. Relative response of selected OPA amino acid derivatives. (A) New enhanced response of early eluting amino acid derivatives and (B) old situation [6].

mice were euthanized via a cardiac puncture for arterial blood sampling.

2.3.2. Blood sampling and sample processing

A total of 600 μl arterial blood was sampled in pre-chilled, heparinized cups (Sarstedt, Nȳmbrecht, Germany) on ice and centrifuged (4 °C for 15 min at 8500 × *g*) to obtain plasma. For determination of amino acid concentrations, acetonitrile (200 μl) was added to 100 μl plasma for deproteinization, vortex-mixed immediately and stored at −80 °C until further analysis by LC–MS.

2.4. Principle of tracer technology

Many elements in nature have isotopes and some of these are stable. In biochemistry, the most abundant stable isotope is ^{13}C, with a natural abundance of about 1.1%. Consequently, a biological molecule containing for instance 5 carbons has a 5 × 1.1% chance of incorporating an ^{13}C isotope, which using a mass spectrometer can be observed as peak 1 Da (M1) higher than the expected molecular mass (M) with an abundance of 5.5% compared to the peak of the expected mass. The molecule also has a (much lower) chance to incorporate 2 (M2) or more isotopes (M3 or higher), and thus the

given molecule appears in a mass spectrum not as a single peak, but as an isotopomeric envelop (Fig. 1). The ratio between the heavier isotopomeric peaks (the tracer) and the non-heavy isotope containing base peak (the tracee) can be expressed as the tracer–tracee ratio (TTR):

$$\mathrm{TTR}\,(\%) = \frac{[\mathrm{tracer}]}{[\mathrm{tracee}]} \times 100\%$$

If test subjects are provided with a metabolite enriched with one or more stable isotopes, these isotopes will mix with the bodie's own metabolite and increase the concentration of the supplied isotope. Consequently, the TTR for this isotope will increase according to:

$$\text{Increase in TTR} = \mathrm{TTR}\,(\text{measured}) - \mathrm{TTR}\,(\text{natural})$$

Considering the amount of supplied tracer is known, one can calculate the amount of dilution and thus the original pool size and by monitoring the change in TTR in time, the activity of metabolic pathways.

3. Results and discussion

3.1. Chromatography

To measure an accurate amino acid isotope enrichment, it is essential that amino acids with an overlapping isotopomeric envelop are baseline separated from each other (e.g. consider isoleucine, leucine and arginine, citrulline). Even though the separation of underivatized amino acids is currently feasible [4,15], derivatization can still be useful to enable the use of MS-compatible solvents [16] and to enhance the ionization response [17]. Based on these considerations, we previously reported 2 different strategies, using either derivatization of amino acids with o-phthaldialdehyde (OPA) [6] and later 9-fluorenylmethylchloroformate (FMOC) [5], both allowing an automated on-line derivatization procedure. Still, adequate measurement of isotopic enrichments of low concentrated amino acids like citrulline remained difficult to achieve even at normal physiological levels of 60 μmol/l [16]. Under pathological conditions, when citrulline concentrations can drop below 15 μmol/l [18], accurate measurements of low isotopomeric enrichments were impossible. Furthermore, both methods required a technically vulnerable column-switching step to exclude reagent surplus [5]. In the case of the o-phthaldialdehyde (OPA)-method [6] this would otherwise cause a rapid deterioration of the separation due to accumulation of the purple colored reaction product of solvent ammonia and OPA reagent excess. Because OPA reacts only with primary amines, we hypothesized that if solvent ammonia could be replaced by an alternative volatile base like the secondary amine triethylamine (TEA) the column switching step could be omitted. Indeed, even after more than 200 injections of the amino acid–OPA reagent mix, no decrease of column performance was observed, making the method much more robust.

In addition, we observed that TEA–acetic acid based solvents cause a significant change in selectivity, compared to ammonia–acetic acid based solvents applied before. Critical separations like isoleucine (ILE)-leucine (LEU) and ARG-CIT, which otherwise required the addition of tetrahydrofuran (THF) were now possible without THF addition. This is important as THF caused a severe ion-suppression of especially the early eluting OPA–amino acid derivatives. Omission of THF resulted in an enhancement of the ionization response of CIT and ARG derivatives by a factor of 10–20 (Fig. 2A and B) [5].

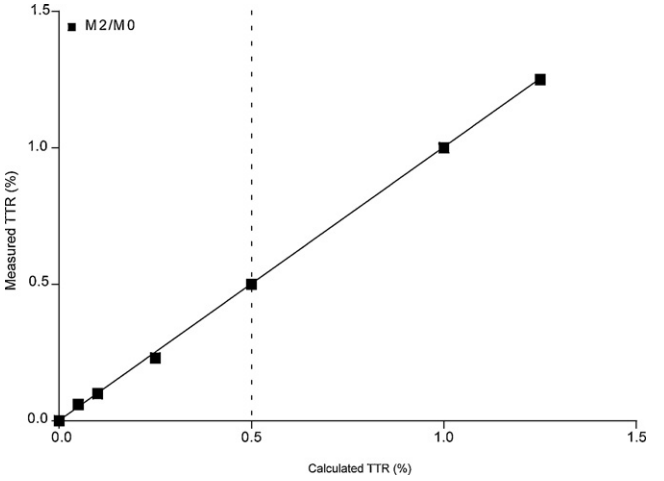

Fig. 3. Enrichment calibration curve of the M+2 isotope of guanidino-$^{15}N_2$-arginine obtained in enhanced full scan mode: $Y = (1.000 \pm 0.008129)X - (0.001429 \pm 0.005221)$, $R^2 = 0.9997$. The dotted line represents the lowest concentration at which the TTR could be measured using the previous method [6].

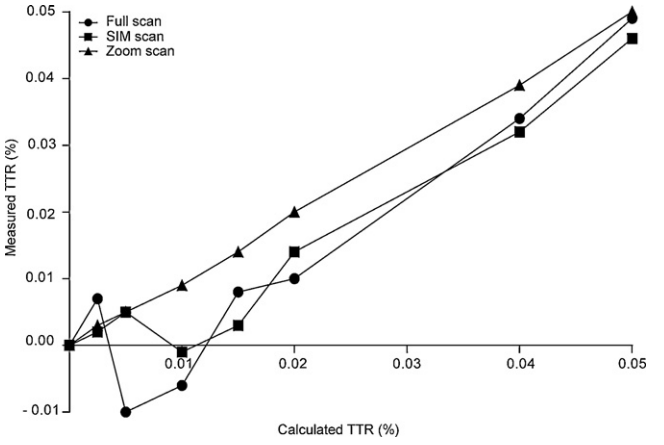

Fig. 4. Enrichment curves of the M+2 isotope of guanidino $^{15}N_2$-aginine constructed using different scan modes: full scan: $Y = (1.035 \pm 0.1621)X - (0.006933 \pm 0.003995)$, $R^2 = 0.8716$; selected ion monitoring (SIM) scan: $Y = (0.9172 \pm 0.1039)X - (0.003712 \pm 0.002559)$, $R^2 = 0.9285$; and zoom scan: $Y = (0.9917 \pm 0.01289)X - (0.0001648 \pm 0.0003175)$, $R^2 = 0.9917$.

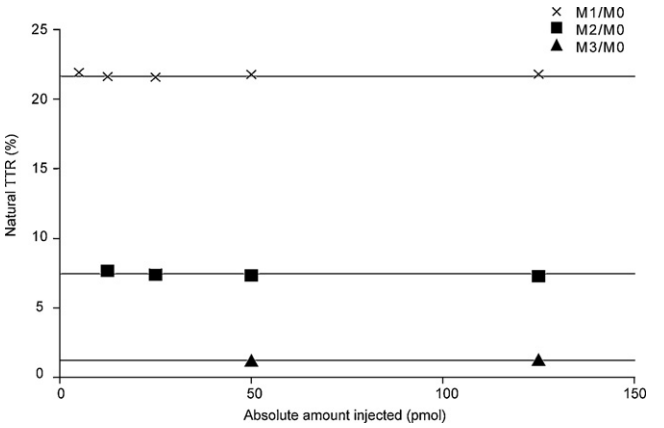

Fig. 5. Relation between injected amount and stability of the TTR ratio of the first 3 isotopes of naturally enriched citrulline. The drawn lines represent the theoretical TTR's of the measured isotopes.

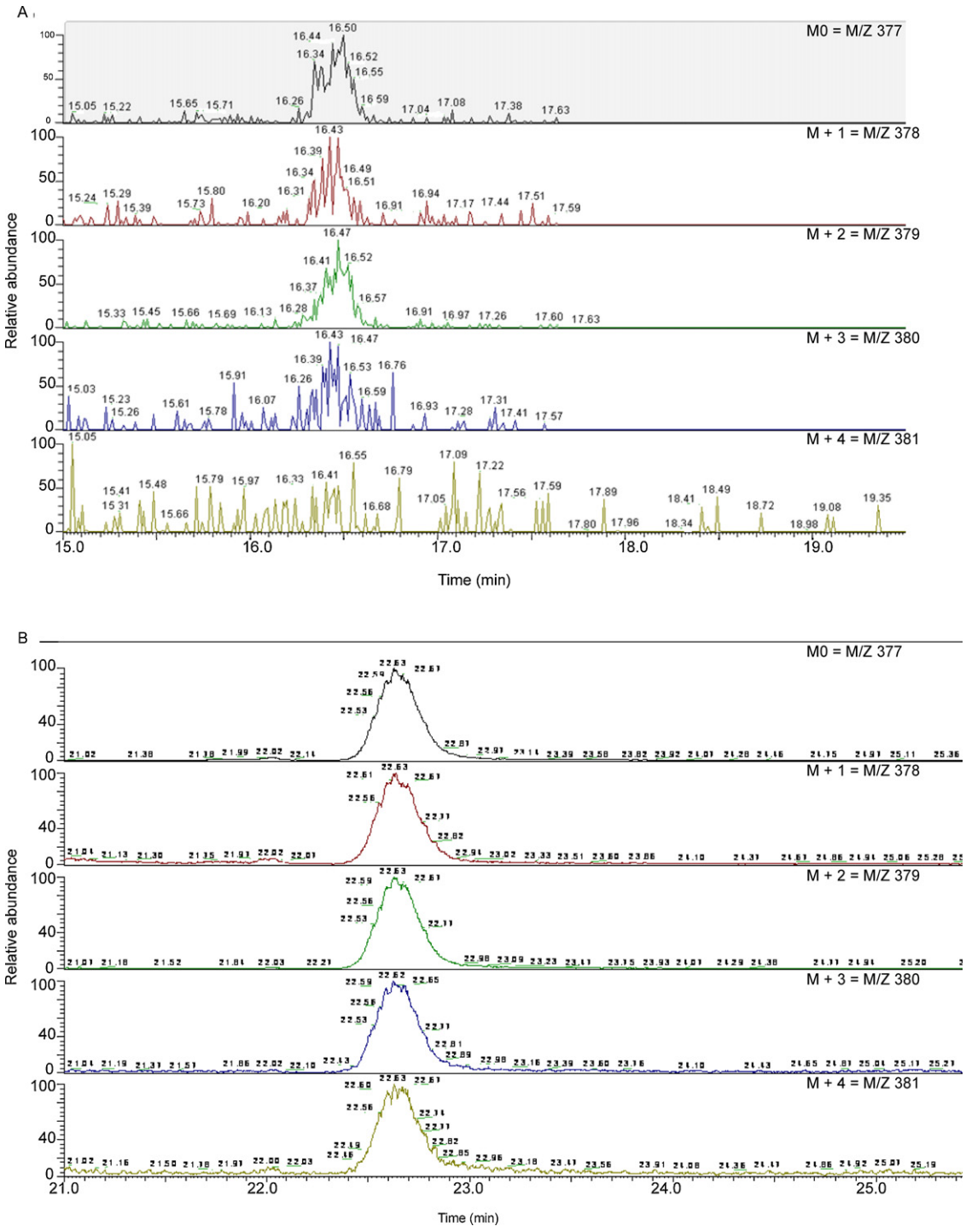

Fig. 6. Response of the isotopes of naturally enriched plasma arginine–OPA derivatives obtained using the "old" OPA method (A) [6] and the present method (B).

3.2. Mass spectrometry

To maximize the signal of the mass spectrometer for OPA-derived amino acids, their infusion into the system is required. However, OPA amino acids are unstable in the reaction mixture and their degeneration starts within minutes after formation [16]. The reaction mixture itself contains a concentrated (non-volatile) borate buffer required for the generation of the OPA derivatives, prohibiting direct infusion into the electrospray probe which otherwise would quickly be blocked. Thus, tuning by infusion of the reaction mix is no option.

Instead, we injected a high concentration of freshly derivatized OPA–amino acids and collected the solvent fraction containing the amino acid of interest and infused this fraction into the MS system at a flow rate of 10 μl/min. To mimic chromatographic conditions, the infused sample flow was mixed with the HPLC effluent pumped at 0.35 ml/min through a PEEK T-piece. Optimal gas flows found were 99 units for sheath gas and 35 units for auxiliary gas.

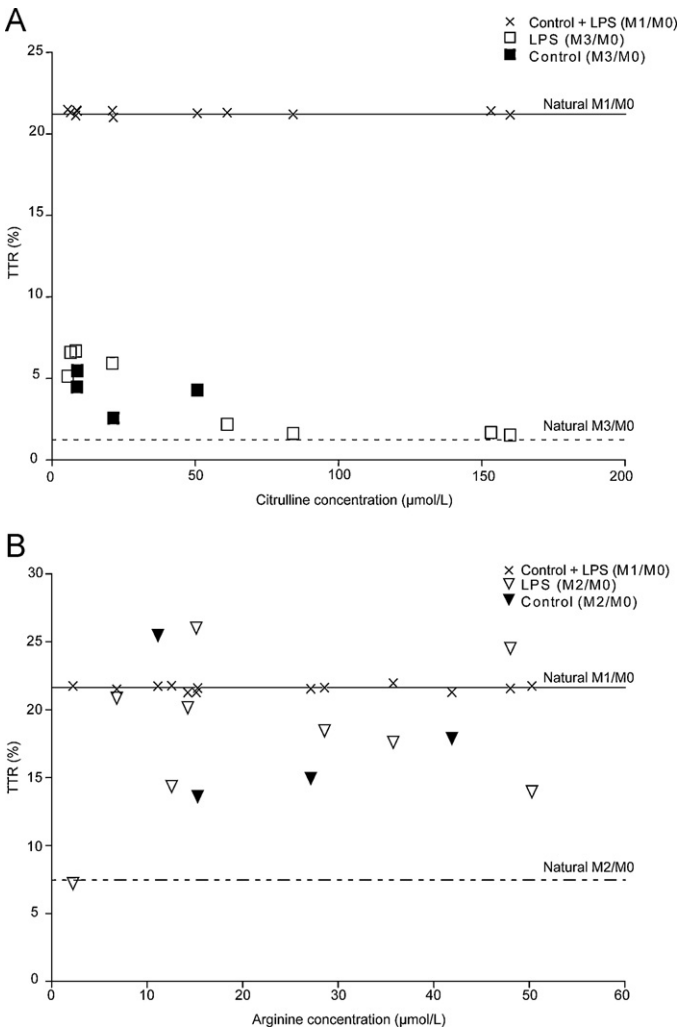

Fig. 7. *In vivo* ^{13}C–$^{2}H_2$-citrulline and guanidino $^{15}N_2$-arginine enrichment during pathological depleted conditions using the present optimized LC–MS technique. (A) Isotopic enrichment of citrulline and (B) isotopic enrichment of arginine.

Optimal heated capillary temperature was 220 °C and tube lens was 20 V.

3.3. Enrichment curves

To investigate the influence of the system resolution on the sensitivity of the newly developed method, enrichment curves were constructed at the level of 50 μM. In Fig. 3 the measured enrichment of the $^{15}N_2$-arginine tracer was plotted against the theoretical value. Measurements were first performed in the enhanced full scan mode. In this mode, compatible with the resolution of a triple-quadrupole system, we demonstrate enrichments can be measured reliably down to 0.05% above natural. To compare, the previous limit at 0.5%, indicated as a doted line, was included [5,6]. Below 0.05%, the background noise level of the isotopomeric peaks interfered too much. By increasing the resolution from full-scan to SIM-scan and zoom-scan, target isotopomeric peaks could better be resolved from the background enabling more reliable measurements at lower enrichments (Fig. 4). Using the zoom-scan mode, an accurate enrichment could be realized down to 0.005% above natural at the 5 μmol/l level, and additional increase by a factor of 10.

Sensitivity in relation to the concentration was also investigated (Fig. 5). Increasing amounts of ARG were injected and the natural (theoretical) enrichment of all the isotopomeric peaks up to the M5 peak were plotted against the calculated (measured) TTR. It can be observed that for the M2/M0 and M1/M0 ratio's correct TTR's can be measured down to 5 pmol injections (comparable to a 5 μmol/l concentration).

To visualize the gain in precision in the above mentioned conditions, we compared measurement of the ARG enrichment in plasma samples using the previously described OPA method [6] and the current method (Fig. 6).

3.4. In vivo enrichment

Administration of LPS resulted in depletion of the CIT and ARG pool. Concentrations of CIT and ARG dropped to minimal values of 5 μmol/l. Despite this depletion of citrulline and arginine during the endotoxemia, both for CIT and ARG the theoretical TTR for the M1 isotope could be measured adequately even at these low concentrations. Enrichment of the supplied tracer, ARG M2 and CIT M3 was clearly picked up, ranging from 7 to 19% for ARG M2 and 0 to 5% for CIT M3 (Fig. 7). These results indicate that also at low concentrations, reliable enrichments can be measured in plasma, demonstrating that this method is a valuable tool to study ARG and CIT metabolism.

4. Conclusion

In view of the results presented above, here described approach can be used to determine isotopic ratios on-line of several physiologically important amino acids in one run. Especially during conditions of low baseline concentrations of important amino acids like citrulline and arginine, this method now enables estimation of isotopic enrichments down to 0.005% at sample concentrations down to 5 μmol/l. The same methodology can be applied to also measure several other amino acid enrichments in the same analytical run (not shown). Considering these results, the present technique now bridges the gap between dedicated IRMS and LC–MS.

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