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Determination of very low stable isotope enrichments of $[^2H_5]$-phenylalanine in chicken liver using liquid chromatography–tandem mass spectrometry (LC–MS/MS)

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ABSTRACT

Stable isotope labeled amino acids are frequently used to examine nutritive effects on protein synthesis. This technique is characterized by tracing the incorporation of the label into newly synthesized proteins. In the present investigation, a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed for the determination of very low enrichment of protein-bound L-$[^2H_5]$-phenylalanine ($[^2H_5]$-phe) in chicken liver. The LC–MS/MS measurements were carried out in positive atmospheric pressure chemical ionization (APCI) mode. Two mass transitions each for $[^2H_5]$-phe (171.1/125.1 and 171.1/106.1) and L-phenylalanine (phe) (166.1/91.1 and 166.1/93.1) were chosen for quantification and qualification. Due to the high excesses of phe, less sensitive transitions were chosen in the case of phe. The separation was carried out on a phenyl-hexyl column using 0.1% formic acid as eluent A and methanol as eluent B. The method was calibrated with calibration standard solutions in the range of 0.01–0.5 mole percent excess (MPE). Linear regression analysis led to coefficients of determination (r^2) greater than 0.9995. The method was applied on liver samples from experiments investigating nutritive effects on tissue protein synthesis in broiler chickens. These samples were analyzed with a gas chromatography–mass spectrometry (GC–MS) method and reanalyzed with the developed LC–MS/MS method one year later. Compared to GC–MS, the main advantages of the LC–MS/MS method are its higher selectivity as well as the elimination of the need to convert and derivatize the samples prior to measuring.

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1. Introduction

Stable isotope labeled amino acids are frequently used as tracers to examine nutritive effects on protein synthesis and breakdown which are collectively referred as to protein turnover [1]. Among others, this technique can be used to trace the kinetics of incorporation of the labeled amino acid into newly synthesized proteins [2]. In this way, it becomes possible to investigate nutritive effects on the synthesis of target proteins on an individual protein, tissue protein or whole body protein level. Typical protocols involve the injection of a single bolus dose or a primed constant infusion of a stable isotope labeled amino acid [3] which then enters and mixes into the free amino acid precursor pool. By parallel measurement of the time dependent enrichment of the amino acid both in the free amino acid precursor pool and in the synthesized protein of interest it becomes possible to estimate the protein synthesis rate. If this protein synthesis is related to the absolute mass of that protein, a so-called fractional protein synthesis rate (FSR) can be estimated. Irrespective of the method used the enrichment of the free amino acid will be significantly higher than that of the same amino acid incorporated into the newly synthesized protein. These differences in the enrichment have consequences for the analytical methods which can be used and require more sensitive methods for measurement of the protein bound amino acids which are typically very low enriched. The stable isotope enrichment is usually reported as excess which can be expressed as molar percent excess (MPE) when the moles of the labeled amino acid are calculated as percentage of the sum of the moles of the unlabelled and labeled amino acid.

Expensive and technically sophisticated instruments like IRMS (isotope ratio mass spectrometer) are usually required for analyzing very low isotopic enrichments of the incorporated tracer amino acid. GC–MS methods were also tested for their suitability to detect such low enrichments because of the expense of IRMS methods. Calder et al. [4], Slater et al. [3] and Dänicke et al. [5]

Abbreviations: phe, L-phenylalanine; $[^2H_5]$-phe, L-$[^2H_5]$-phenylalanine; MPE, mole percent excess; ESI, electrospray ionization; APCI, atomic pressure chemical ionization.

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published GC–MS methods for the determination of low L-[$^2H_5$]-phenylalanine ([$^2H_5$]-phe) enrichment. All these methods included the enzymatic conversion and derivatization of L-phenylalanine (phe) to improve the chromatographic separation and discrimination of labeled ion fragments [4]. In contrast to Calder et al. [4], both Slater et al. [3] and Dänicke et al. [5] used the tertiary-butyldimethylsilyl (t-BDMS) derivates of phenylethylamine and not the heptafluorobutyryl anhydride, because the isotope ratio analysis of that derivative was unsatisfactory in both studies [3,5]. For determination of the enrichment of [$^2H_5$]-phe in proteins with the aforementioned GC–MS methods, the ratio of [$^2H_5$]-phe to phe was calculated based on the [M+5] and [M+2] peak of the t-BDMS derivate of phenylethylamine, because the mole peak generated an overloaded peak. Schweer et al. [6] reported a GC–MS/MS method for measuring very low levels of enrichment of L-[$^{13}C_6$]-phenylalanine and [5,5,5-$^2H_3$]-L-leucine in plasma and lipoprotein hydrolyzates. The amino acids were derivatized to their N-heptafluorobutyryl isobutyl ester derivatives and the isotope ratios were determined by GC–MS/MS in the negative ion chemical ionization mode, whereby the $[M-HF]^-$ ions were used as parent ions. The advantage of GC–MS/MS over GC–MS consists in its significantly higher selectivity.

Only recently, Meesters et al. [7] published an LC–MS/MS method for the analysis of stable isotope enrichments of phe and L-tyrosine in human plasma. A commercially available amino acid analysis kit was used for sample preparation, whereby the amino acids were derivatized. The ESI interface was used for LC–MS/MS analysis.

In the present paper, a sensitive and selective LC–MS/MS method is described for determination of very low stable isotope enrichments of protein-bound [$^2H_5$]-phe in chicken liver. The method was applied on liver samples from an earlier experiment where the FSRs of various tissues of broilers were measured by using [$^2H_5$]-phe as tracer amino acid [8]. The liver samples were analyzed with a gas chromatography–mass spectrometry (GC–MS) method and reanalyzed with the developed LC–MS/MS method approximately one year later. The results obtained by both methods were tentatively compared. An LC–MS/MS method for the determination of high enrichment of free [$^2H_5$]-phe is in preparation. It is intended to apply the developed methods on further physiological matrices.

2. Materials and methods

2.1. Chemicals

Stable isotope labeled [$^2H_5$]-phe was purchased from Chemotrade (98% purity, Leipzig, Germany), phe was purchased from Sigma–Aldrich Chemie (Seelze, Germany). Acetic acid and ammonium acetate (both 99% purity, LC/MS Optigrade) were obtained from LGC Promochem (Wesel, Germany), methanol and water (both LC–MS/MS Grade) were purchased from Carl Roth (Karlsruhe, Germany). PVDF (polyvinylidene fluoride) syringe filters (0.45 μm, 4 mm) were obtained from Amchro (Hattersheim, Germany).

2.2. Instrumentation

The LC–MS/MS system consisted of a 4000 Qtrap (Applied Biosystems, CA, USA) equipped with ESI (electrospray ionization) and APCI (atomic pressure chemical ionization) interface, coupled with a 1200 series HPLC equipped with a binary pump, high performance wellplate sampler, instant pilot and a column oven with switch valve (Agilent Technologies, Waldbronn, Germany).

2.3. Liver samples

Liver samples were obtained from a former experiment with broiler chickens for investigations of the inhibitory effects of the mycotoxin deoxynivalenol (DON) on the protein synthesis of various tissues as described in detail by Dänicke et al. [8]. Briefly, the animals were fed DON contaminated feed (15 mg/kg) ad libitum over 42 days. The tracer ([$^2H_5$]-phe) was administered via the *vena ulnaris* (10 mL/kg live weight, with a concentration of 150 mM phe in a NaCl-solution, in a mixture of phe and [$^2H_5$]-phe with an enrichment of 29 mole percent excess (MPE)). Ten minutes after injection, the broilers were stunned and then slaughtered by decapitation, and tissue and blood samples were collected. In total, 26 livers were available for measurements with GC–MS and LC–MS/MS.

Protein hydrolyzates of the liver samples were isolated for measurements by GC/MS one year ago as described in [9,10] according to the method of Dänicke et al. [5] (Fig. 1). This sample preparation method enables the determination of free and protein-bound [$^2H_5$]-phe and phe in addition to the determination of protein, RNA and DNA. The protein hydrolyzates for the determination of protein-bound [$^2H_5$]-phe and phe (low enrichment) were divided into two portions. One aliquot was analyzed with the GC–MS method of Dänicke et al. [5] directly after sample preparation and the remaining residue was kept frozen at −18 °C for one year before being reanalyzed with the developed LC–MS/MS method.

For GC–MS determination, phe was converted to phenylethylamine and derivatized to the t-BDMS phenylethylamine. Peak areas at *m*/*z* 180 (t-BDMS-phenylethylamine) and *m*/*z* 183 (t-BDMS-[$^2H_5$]-phenylethylamine) were recorded in the selected ion recording mode under electron ionization conditions and subsequently used for isotopic ratio analysis [5]. The reason why the peak at *m*/*z* 180 was used for t-BDMS-phenylethylamine and not the quasi molecular peak at *m*/*z* 178 was that the latter generated an overloaded peak.

For LC–MS/MS measurements, the stored aliquots of the hydrolyzates were thawed, resolved in 5 mL 0.1% formic acid, mixed in an ultrasonic bath, transferred to a tube and finally centrifuged at 10,000 rpm for 10 min. The supernatant was removed, filtered through a syringe filter and finally diluted in a ratio of 1:20 with 0.1% formic acid in order to match the phe concentration of the calibration range (see Section 2.4).

2.4. Determination of the phe concentration in the liver sample hydrolyzates

In the diluted liver sample hydrolyzates, the phe concentration was determined with LC–MS/MS using the same instrument parameters for phe as described in Section 2.6. A calibration curve was generated by means of standard solutions in the range of 0.1–20.0 μg/mL phe, by which the exact phe concentration of the 26 diluted liver sample hydrolyzates was determined. The calculation was performed with Analyst-Software (Version 1.4.2, Applied Biosystems).

2.5. LC parameters

The analytical column was a BETASIL phenyl-hexyl column (100 mm × 2.1 mm, 3 μm) (Thermo Scientific, Karlsruhe, Germany) preceded by a phenyl-hexyl guard column (4 mm × 2 mm) (Phenomenex Ltd., Aschaffenburg, Germany). The flow rate of the mobile phase was set to 450 μL/min. Eluent A consisted of 0.1% formic acid in water and eluent B of 100% methanol. A binary gradient was applied as follows: the initial composition of the mobile phase, 2% eluent B, was kept constant for 0.5 min, then eluent B was increased to 90% within 0.5 min and kept constant for 2 min. Finally,

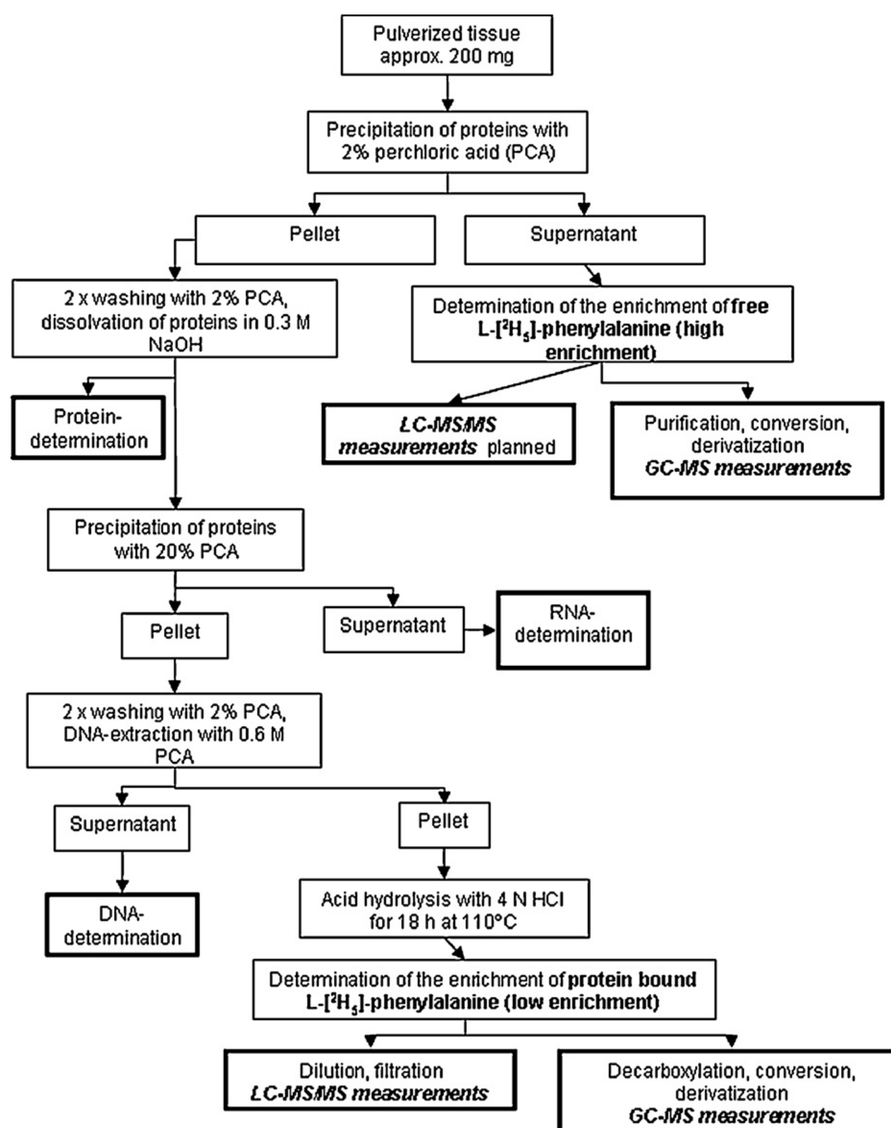

Fig. 1. Scheme of sample preparation for determination of the enrichment of protein-bound L-[$^2H_5$]-phenylalanine and of free L-[$^2H_5$]-phenylalanine in chicken liver – in addition to the determination of protein, RNA and DNA – as used for determination with GC–MS including modifications for LC–MS/MS; PCA – perchloric acid.

in order to re-equilibrate the column the eluent B was decreased to 2% within 0.5 min and kept constant for 6.5 min. In total, one analysis lasted 10 min. The injection volume was 10 μL and the column temperature was set to 50 °C. The retention time of [$^2H_5$]-phe amounted to 2.66 min and that of phe to 2.74 min.

2.6. MS/MS parameters

For optimization of the MS/MS parameters, the ESI interface was used in both negative and positive ion mode and standard solutions of phe as well as [$^2H_5$]-phe (1 μg/mL) dissolved in water/methanol (80/20, v/v) were directly infused via a syringe pump (Harvard Apparatus; Model 11; South Natick, MA, USA) at a flow of 10 μL/min. Additionally, these standard solutions were tested with and without adding ammonium acetate (1 M) as well as with and without the addition of formic acid (0.1%).

The ion source parameters and gases were optimized by means of flow injection analysis (FIA) for both ESI interface and APCI interface in positive mode. For this purpose, a solution of phe and [$^2H_5$]-phe in 0.1% formic acid in water was injected into a flow of 0.1% formic acid and 5 mM ammonium acetate in water (eluent A, 98%) and methanol (eluent B, 2%). Finally, the APCI interface in positive mode was chosen with the following settings: temperature (TEM): 500 °C, curtain gas (CUR): nitrogen, 20 psi; nebulizer gas (GS1): 25 psi; needle current (NC): 3 μA. The operating mode of the mass spectrometer was the MRM (multiple reaction monitoring) mode.

2.7. Standards and calibration

The isotopic excess was expressed as mole percent excess (MPE) and calculated as the ratio between [$^2H_5$]-phe and the sum of [$^2H_5$]-phe plus phe multiplied by 100. To determine the enrichment of the protein-bound [$^2H_5$]-phe, series of 12 standard solutions containing phe and [$^2H_5$]-phe in the range from 0.01 to 0.5 MPE with different concentrations (5, 10, 15 and 20 μg/mL) of phe were calculated and prepared out of stock solutions with concentrations of 10.3 mg/mL [$^2H_5$]-phe and 10 mg/mL phe, respectively. The calibration standard solutions were measured with the developed LC–MS/MS method and calibration curves were constructed by plotting MPE versus the calculated peak area ratios of mass transitions 171.1/125.1 ([$^2H_5$]-phe) to 166.1/91.1 (phe). The peak areas

Table 1
MS/MS parameters for the determination of L-[$^2H_5$] phenylalanine and L-phenylalanine in positive APCI mode.

Analyte	Precursor-ion [m/z]		Product-ion [m/z]	DP [V]	CE [eV]	CXP [V]
L-Phenylalanine	[M+H]+	166.1	91.1[a]	36.00	49.00	14.00
			93.1	36.00	33.00	6.00
L-[$^2H_5$]-Phenylalanine	[M+H]+	171.1	125.1[a]	41.00	17.00	10.00
			106.1	41.00	39.00	6.00

DP, declustering potential; CE, collision energy; CXP, cell exit potential.
[a] Mass transition used for quantification.

were determined with Analyst-Software (Version 1.4.2, Applied Biosystems).

2.8. Preliminary matrix effect studies

To investigate possible matrix effects of the liver hydrolyzates in LC–MS/MS, two liver samples with pre-analyzed phe concentrations of 7.4 μg/mL and 4.8 μg/mL for samples A and B, respectively, were spiked with different amounts of phe (0.5, 1, 1.5, 2 μg/mL). After spiking the samples, the phe concentrations of these samples were determined again as described above.

2.9. Calculations and statistics

The repeatability of the MPE measurements with the developed LC–MS/MS method was investigated by intra- and inter-day validation of 13 calibration standard solutions (range 0.01–0.5 MPE) with a phe concentration of 5 μg/mL in three replicates on three different days (Days 1, 4 and 7). Out of the measured peak areas of [$^2H_5$]-phe and phe, peak area ratios were calculated (interpolated concentration) and evaluated using the calibration equation $y = 0.1508x - 0.0022$ (x: MPE, y: peak area ratio). The accuracy of the LC–MS/MS measurements was calculated applying Eq. (1) described by Braggio et al. [11].

$$\text{Accuracy}\quad(\%) = \frac{\left|\text{theoretical concentration} - \text{interpolated concentration}\right| \times 100}{\text{theoretical concentration}} \tag{1}$$

The relative standard deviation (RSD %) was calculated from multiple replicated measurements of the 13 calibration standard solutions. The intra- and inter-day consistency of the calibration data was calculated by analysis of variance (ANOVA) from results obtained in the intra- and inter-day validation with calibration standard solutions. To enable calibration throughout several orders of magnitude, a logarithmic transformation of the calibration data was performed to ensure homoscedasticity. Afterwards an ANOVA including the factors “days”, “replicates” and “theoretical MPE” was performed ($p < 0.05$) [12].

3. Results and discussion

3.1. LC–MS/MS optimization

First, full scan experiments (Q1 scan mode, scan range 100–210 amu, scan time 3 ms) of [$^2H_5$]-phe and phe were performed using the ESI interface in both positive and negative modes to select the most suitable precursor ions. The ionization in positive ESI mode provided two to ten times higher intensities for both [$^2H_5$]-phe and phe compared with ionization in negative mode. The M+1 peak showed the most intensive signal and was chosen as precursor ion for phe as well as for [$^2H_5$]-phe. By adding of 1 M ammonium acetate the less intensive sodium adduct peak was suppressed. The addition of 0.1% formic acid had a positive effect on the protonation of the molecules in the positive ionization mode. Then, suitable mass transitions for [$^2H_5$]-phe and phe were chosen and subsequently the parameters declustering potential (DP), collision energy (CE) and cell exit potential (CXP) were optimized for each selected mass transition. The optimized parameters are presented in Table 1. For [$^2H_5$]-phe two product ions were selected, showing the most intensive signals. In contrast, for phe two less intensive product ions were chosen, since the phe concentration at an MPE of 0.01 (lowest calibration standard solution) is 10,000 times higher than the [$^2H_5$]-phe concentration and the most intensive signal (166.1/146.8) of phe generated an overloaded signal. Further optimization was carried out using only positive ionization mode. Using flow injection analysis (FIA) the interface parameters for both ESI and APCI interface were optimized. Using the APCI interface, twice the signal intensities could be observed when compared to the results of the ESI interface. Therefore, the APCI interface was used for further method development.

After preliminary studies with acetonitrile and water as eluents A and B, in the final method 0.1% formic acid in water was used as eluent A and methanol as eluent B due to lower background noise and better peak properties. After elution of [$^2H_5$]-phe and phe, the proportion of eluent B was increased up to 90% to remove impurities in the HPLC column, because high impurities were expectable when working with physiological samples. To enable the relatively high flow rate of the mobile phase of 450 μL/min, the column oven temperature was set up to 50 °C in order to reduce the back-pressure in the system. Increasing oven temperature had no negative impact on the peak intensities observed and hence the detection limits were not influenced negatively. When testing different injection volumes (10 and 20 μL), no improvement could be observed with the higher injection volume. Therefore, an injection volume of 10 μL was used to avoid LC–MS/MS impurities from matrix compounds.

3.2. Standards and calibration

Calibration standard solutions in the range of 0.01–0.5 MPE with different phe concentrations (5, 10, 15 and 20 μg/mL) were analyzed. The objective was to find out the lowest possible phe concentration in order to minimize LC–MS/MS contamination from matrix compounds. Calibration curves were generated by plotting the MPE versus the calculated peak area ratios of mass transitions 171.1/125.1 ([$^2H_5$]-phe) to 166.1/91.1 (phe). Linear regression analysis led to the equations presented in Table 2. Comparing the slopes of the calibration curves, it can be observed that increasing phe concentrations of the calibration standard solutions led to increased slopes of the regression lines. In this context systematically lower MPE would be determined out of the same peak area ratios, when calibration curves with higher phe concentrations than in the investigated sample solution were used for evaluation. Downwards the calibration range is restricted by the lowest measurable [$^2H_5$]-phe signal with the lowest enrichment of 0.01 MPE at a phe concentration of 5 μg/mL and upwards by the signal with the highest phe concentration where no overloading of the phe signal occurred. With the phe concentrations in liver

Table 2
Calibration data for the MPE determination (0.01–0.5) at different L-phenylalanine concentrations.

L-Phenylalanine-concentration [μg/mL]	Equation	Coefficient of determination (r^2)
5	$y = 0.1508x - 0.0022$	0.9999
10	$y = 0.1638x - 0.0024$	0.9999
15	$y = 0.1788x - 0.0013$	0.9995
20	$y = 0.1842x - 0.0031$	0.9998

y = peak area ratio of L-[$^2H_5$]-phenylalanine (171.1/125.1)/L-phenylalanine (166.1/91.1); x = calculated MPE.

varying depending on individual animal factors, it is not possible to set the concentration of phe to a certain value. Therefore, the phe concentration in the liver sample hydrolyzates has been adjusted to the range of 5–10 μg/mL phe. As can be seen in Fig. 2A, the phe signal of a liver hydrolyzate with 8.5 μg/mL phe is not overloaded. For calculation of MPE's of the liver samples, means of the results with calibration curves at 5 μg/mL and 10 μg/mL phe were used. Alternatively, a calibration curve with 7.5 μg/mL phe can be used.

3.3. Performance of the LC–MS/MS method

On average the accuracies were about 2.4 ± 1.3% for intra-day measurements and 3.2 ± 2.2% for inter-day measurements. The relative standard deviations (RSD %) ranged from 0.24 to 2.2% for intra-day measurements and from 2.0 to 9.0% for inter-day measurements. All obtained intra- and inter-day accuracy and RSD values were acceptable for our study and showed that the developed method leads to reproducible analytical results. The ANOVA calculated non-significant effects at all tested interactions (Table 3). The terms of interest in the ANOVA are "replicate" and "replicate x theoretical MPE" interaction. Thus the intra- and inter-day consistency of the developed method for the determination of very low stable isotope enrichments of [$^2H_5$]-phe in proteins is proved.

The lowest calibration standard solution (0.01 MPE, 5 μg/mL phe) generated a signal-to-noise ratio (S/N) of 9.6 for the peak of the mass transition of [$^2H_5$]-phe (171.1/125.1) (Fig. 2D). Therefore, a MPE of 0.01 is defined as the limit of the MPE quantification (LOQ).

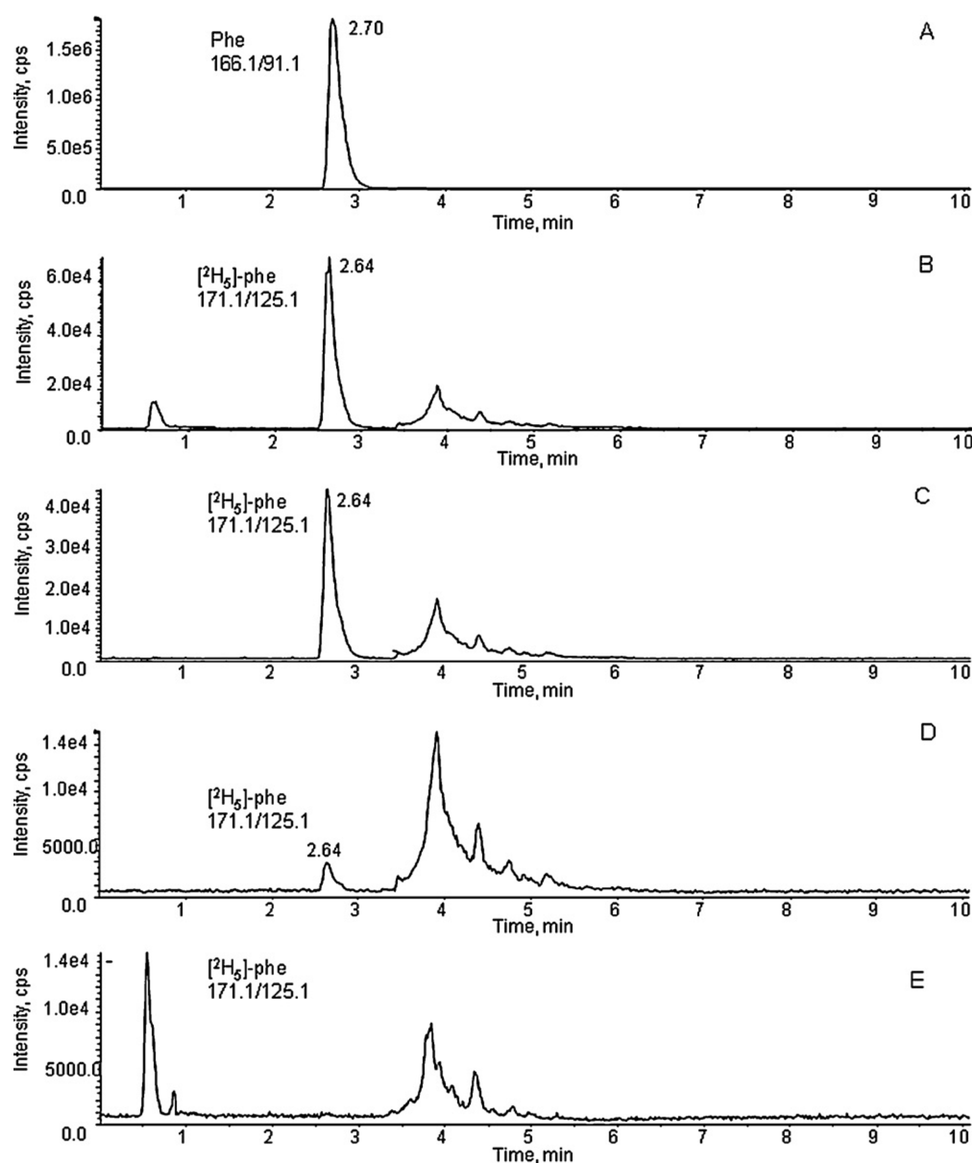

Fig. 2. Extracted ion chromatograms (XIC) of: L-phenylalanine (phe) of a liver sample hydrolyzate with 8.5 μg/mL phe and 0.20 MPE (mole percent excess) (A), L-[$^2H_5$]-phenylalanine ([$^2H_5$]-phe) of a liver sample hydrolyzate with 8.5 μg/mL phe and 0.20 MPE (B), [$^2H_5$]-phe of a standard solution with 5 μg/mL phe and 0.20 MPE (C), [$^2H_5$]-phe of a standard solution with 5 μg/mL phe and 0.01 MPE (LOQ) (D) and of [$^2H_5$]-phe of a blank liver sample hydrolyzate with 8.8 μg/mL phe (E).

Table 3
Validation results of the ANOVA of the LC–MS/MS measurements of calibration standard solutions ($n = 9$; MPE 0.01–0.5; 5 μg/mL L-phenylalanine) and p-values at a significance level of 0.05.

Source	d.f.	SQ	MQ	F-Value	p-Value	Significant at $\alpha = 0.05$
Day	2	0.0161	0.0080	0.0368	0.9639	n.s.
Replicate	2	0.0142	0.0071	0.0326	0.9679	n.s.
Replicate × theoretical MPE	24	0.1602	0.0067	1.0200	0.4530	n.s.

d.f., degrees of freedom; SQ, sum of squares; MQ, expected mean squares; n.s., not significant; α, significance level.

3.4. Liver sample preparation for LC–MS/MS measurements

The liver sample preparation for LC–MS/MS measurements was done according to a method for GC–MS measurements (see Section 2.3). In contrast to GC–MS, it was not necessary to convert and derivatize the liver hydrolyzates obtained at the end of the sample preparation. The liver hydrolyzates were only resolved in 0.1% formic acid, filtered and diluted by a factor of 20 in order to adjust the phe concentration to the range of 5–10 μg/mL (see Section 3.2). This simplified sample preparation proved to be appropriate as no endogenous interference was detected at the retention time of [$^2H_5$]-phe in the LC–MS/MS chromatogram of a blank liver sample in the corresponding mass transit channel (Fig. 2E). In addition to it, hardly any matrix peaks occurred in the corresponding chromatogram of a liver sample with 0.20 MPE compared to a standard solution with the same MPE (Fig. 2B and C).

3.5. Determination of the phe concentration in the liver hydrolyzates

The phe concentration in broiler liver was estimated based on literature data in order to adjust the phe concentration of the hydrolyzate solutions to the phe concentration of the selected calibration standard solutions (5–10 μg/mL). According to previous report [13], phe concentrations of 12.3 mg/g liver were found in laying hens. Corresponding to this value and considering the sample preparation steps, a 1:20 dilution of the liver sample hydrolyzates was used. The exact phe concentration of the diluted liver hydrolyzates ($n = 26$) of the broiler chicks (see Section 2.3) was measured with an adapted LC–MS/MS method. Calibration standard solutions were compounded in the range of 0.1–20 μg/mL for calculation of the phe concentrations in the hydrolyzates. Because of the relatively broad calibration range a quadratic regression was chosen and the data were evaluated using the generated equation. The mean phe concentration of the diluted hydrolyzates amounted to 7.61 ± 1.09 μg/mL corresponding to 8.12 mg/g liver. Compared to the data reported in relevant literature, the liver hydrolyzates of the broiler chicks contained approximately 34% less phe. This difference could potentially be explained by individual differences among animals such as the genetic background, the nutritional status, the broiler's age, and the fat content of the livers. Moreover, the liver samples used in this study were obtained from an animal experiment with broiler chicks, whereas the data from relevant literature were generated with hens.

3.6. Analysis of [$^2H_5$]-phe enriched liver samples

Based on the phe concentrations analyzed in the liver hydrolyzates (mean concentration 7.61 ± 1.09 μg/mL), the calibration equations with 5 μg/mL and 10 μg/mL phe (Table 2) were used for calculation of the mean MPE of the liver samples. By using the calibration curve with 5 μg/mL phe, a mean value ($n = 26$) of 0.16 ± 0.04 MPE was calculated, while the use of the 10 μg/mL phe calibration curve provided a mean value ($n = 26$) of 0.15 ± 0.04 MPE. Hence, for every sample a mean MPE value was calculated from the evaluation using both (5 and 10 μg/mL phe) calibrations.

3.7. Preliminary matrix effect studies

The LC–MS/MS technique often exhibits matrix effects, especially in the case of complex matrices like food, urine and tissues [14]. These matrix effects can be compensated by the use of internal standards, at best by the use of stable isotopic labeled analytes. Because peak area ratios of [$^2H_5$]-phe and phe are used in the present method possible matrix effects should be compensated. However, given the high excess of phe in case of the very low enrichment of [$^2H_5$]-phe, a full compensation for possible matrix effects cannot be ensured.

Matrix effects can be evaluated by comparing standard and matrix matched calibration curves. In an LC–MS/MS method for the analysis of staple isotope enrichments of L-phe and L-thyrosine in human plasma [7], a matrix matched calibration could be used since the phe concentration in ovalbumin is very constant. In contrast, in the present method, a matrix matched calibration was not feasible as liver naturally contains phe in varying concentrations. For preliminary investigations on matrix effects, a method of standard addition was applied on the measurement of the phe concentration of two liver hydrolyzates. In the case of matrix effects a difference in the expected phe concentration (phe concentration of the sample + spiked level) and measured concentration of the spiked samples would be detectable.

Results of the measurements for the unspiked liver hydrolyzates A and B showed a phe concentration of 7.4 μg/mL and 4.8 μg/mL, respectively. In the liver hydrolyzates A and B which were spiked with 0.5, 1, 1.5 and 2 μg/mL phe, the expected phe concentration was measured within ±0.1 μg/mL. These preliminary results do not indicate a matrix influence on the phe determination and correspondingly [$^2H_5$]-phe determination with LC–MS/MS.

3.8. Preliminary method comparison (LC–MS/MS method versus GC/MS method)

The mean values of MPE of the 26 liver hydrolyzates which were determined with the developed LC–MS/MS method, were plotted against the MPE values determined with GC–MS one year ago (Fig. 3). The compared results show a linear correlation ($r = 0.8681$). Based on the linear regression coefficient, the LC–MS/MS determination provides approximately 36% higher MPE values for the same liver hydrolyzates. Because the samples were analyzed with the two methods at a distance of approximately one year, a proper comparison between both methods is not possible. However, possible reasons for the variant results can be discussed. One reason for these deviations might be alterations due to the storage time of the hydrolyzates at −18 °C for one year. Another source of variation might be the use of two different measuring principles – LC–MS/MS in the MRM (multiple reaction monitoring) mode and GC–MS in the SIM (selected ion monitoring) mode. Furthermore, another reason could be the enzymatic conversion and derivatization with t-BDMS associated biases inherent in the GC–MS methods, which can be excluded for LC–MS/MS measurements. Frequently, this sample preparation step has the potential to contain errors in GC–MS measurements, since an exchange of isotopic label can occur especially at hydrogen/deuterium during sample processing [15].

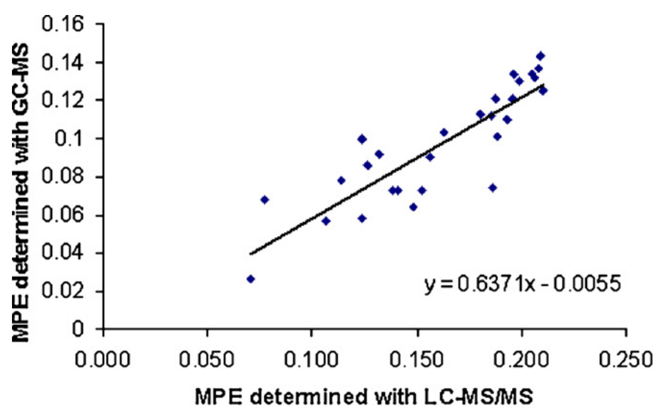

Fig. 3. Results for MPE (mole percent excess) in 26 liver samples measured with LC–MS/MS in comparison to GC/MS measurements one year ago.

The enzymatic conversion and derivatization of labeled and unlabeled phenylalanine are necessary to enhance the gas-chromatographic separation and sensitivity. Derivatization was also necessary in case of the GC–MS/MS method presented by Schweer et al. [6]. With the use of LC–MS/MS, improvements like higher selectivity and no need for derivatization were expected. However, an LC–MS/MS method for the analysis of staple isotope enrichments of L-phe and L-thyrosine in human plasma of Meesters et al. [7] still required a derivatization step in the sample preparation.

4. Conclusions

The developed LC–MS/MS method enables the routine measurement of very low stable isotope enrichment of protein-bound [$^2H_5$]-phe down to 0.01 MPE in chicken liver. Thus, this method is well suited for investigations of nutritive effects on tissue protein synthesis. Compared to GC–MS methods, which have been the methods of choice up until now, the main advantages of the LC–MS/MS method are the higher selectivity and no need to convert and derivatize the samples prior to measuring, thus avoiding confounding effects. A method for the determination of high enrichment of free [$^2H_5$]-phe is in preparation. It is intended to apply the methods on further physiological matrices.

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