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Cost-Effective Simultaneous Determination of τ- and π-Methylhistidine in Dairy Bovine Plasma from Large Cohort Studies Using Hydrophilic Interaction Ultra-High Performance Liquid Chromatography Coupled to Tandem Mass Spectrometry

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Abstract: The isomeric metabolites τ- and π-methylhistidine (formerly referred to as 3- and 1-methylhistidine) are known biomarkers for muscle protein breakdown and meat protein intake, frequently used in studies involving humans and animals. In the present study, we report the development and validation of a simple HILIC-MS/MS method for individual determination of τ-MH and π-MH in a large cohort of blood plasma samples from dairy cows. Their separate determination was achieved mainly through a mass spectrometry fragment ion study, which revealed that the two isomers exhibited distinct mass spectrometric behaviors at different collision energies. Chromatographic conditions were optimised to achieve better separation, minimizing inter-channel interference to less than 1% in both directions. A simple and effective sample clean-up method facilitated low laboratory manual workload. The analytical method was validated for the determination of τ-MH and π-MH in bovine plasma within a concentration range of 80 to 1600 μg/L and provided good linearity (>0.99 for both curves) and precision (<10%). Overall, the developed method enabled the determination of the two isomers in an efficient and economic-friendly manner suitable for large cohort bovine studies (involving hundreds to thousands of samples) mainly to provide data for statistical use.

Keywords: 1-methylhistidine; 3-methylhistidine; bovine plasma; muscle protein breakdown; lactating cows

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1. Introduction

The compound τ-Methylhistidine (tele-methylhistidine, τ-MH) is a known biomarker indicative of muscle protein breakdown [1]; increased levels of τ-MH in bodily fluids, such as blood or urine, indicate that the corresponding organism is in a catabolic state, effectively breaking down lean tissues to satisfy amino acid requirements. This biomarker is formed in the muscle by post-translational methylation of histidine residues in actin and myosin [2]. Its isomer, π-methylhistidine (pros-methylhistidine, π-MH) is also commonly found in

bodily fluids. However, π-MH is a product of anserine breakdown, thus, it is used as a meat protein intake marker in humans [3].

Dairy cows face significant metabolic adaptations when transitioning from late gestation to early lactation [4]. Substantial mobilization of fat and skeletal muscle tissue reserves can be caused by factors such as: a sharp increase in nutrient requirements and decreased dry matter intake along with a genetically driven hormonal tissue regulation [5,6]. Skeletal muscle tissue represents the most abundant pool of available amino acids during periods of negative protein balance. The rate of skeletal muscle mobilization can be determined by measuring plasma concentration of τ-MH. As τ-MH is not further metabolised in the body [6–8], it is quantitatively excreted in the urine [9].

Since its first isolation from human urine hydrolysates using ion exchange chromatography [10,11], τ-MH has been employed as a muscle protein turnover marker in a wide range of human and animal studies [12–15]. During intense muscle catabolism, τ-MH increases above basal levels that are indicative of normal muscle protein turnover. Recent studies have shown continuous research interest in this biomarker as demonstrated by applications of τ-MH in endurance training [16], in the study of human pathological conditions [17,18], and in veterinary research [19–21]. Although not a subject of extensive study, the isomer of τ-MH, π-MH, was a known breakdown product of anserine long before the discovery of the significance of τ-MH.

Since τ- and π-MH possess distinct biological roles, it is essential that the two isomers are individually determined [22]. However, in the literature, the nomenclatures of 1- and 3-MH are found interchanged, which may lead to confusion due to incorrect assignment of the methylated nitrogen atoms on the imidazole ring of anserine [23–26]. In this study, solely the recommendations of the International Union of Pure and Applied Chemistry (IUPAC) for histidine nomenclature were used, employing the prefixes τ- and π- instead of numbers [27]. Isomer structures along with other identifiers are provided in Figure S1 [28].

Early analytical methods for the determination of τ-MH in a variety of samples relied mainly on the use of ion exchange chromatography [10,11,29]. However, the rise in popularity of high-performance liquid chromatography (HPLC) during the 1980s and 1990s led to the development of several methods. Most methods used reverse phase HPLC (RP-HPLC), which relied on some form of derivatization to aid both the retention and detection of τ-MH. The reported methods are based on pre- or post-column derivatization using RP-HPLC [30–40] but may also include ion-pair chromatography [33]. However, derivatization has also been recently applied in the determination of τ- and π-MH in bovine cow plasma [22]. Despite their success, HPLC derivatization methods either require special reagents or equipment (mainly post-column methods), longer sample preparation times, or they may give relatively unstable derivatives.

Capillary electrophoresis has also been utilised for the analysis of both τ- and π-MH, focusing mainly on human urine [41–45]. The technique provides excellent separation of the τ- and π-MH isomers; however, the relatively high limits of detection (LOD) make the reported methods unattractive for the application on bovine blood plasma. Other methods for the analysis of τ-MH include utilization of conventional thin layer chromatography (TLC) [46], or specialised gas chromatography [47–49], or even pyrolysis-gas chromatography [50]. More recent methods include RP-HPLC coupled to tandem mass spectrometry with a separate determination of τ- and π-MH relying purely on spectral differences [51] and hydrophilic interaction liquid chromatography (HILIC) with specialised stationary phases [52], high salt concentrations [53], and isomer co-determination (profiling) [54].

Herein, we have developed a simple, cost-effective and time-efficient method for the separate determination of both τ- and π-MH in bovine blood. The study is based on a thorough but straightforward sample clean-up combined with chromatographic separation. It uses the unique spectral characteristics of τ- and π-MH, as revealed by a thorough ion breakdown study of the two isomers. Contrary to methods described in the available literature, our method does not require sample derivatization, specialised stationary phases,

ion-pairing, or high buffer/salt concentrations. It is intended for analyzing large numbers of samples with the lowest possible cost and downtime, while providing data of τ- and π-MH, which can be used in statistical analysis of large cow cohort studies (which may involve hundreds or thousands of animals). The developed method was applied in >600 samples to study protein metabolism and skeletal muscle tissue mobilization in transition dairy cows in practice. It provided results for various types of statistical analyses, such as group differentiations between animals based on their experimental characteristics.

2. Materials and Methods

2.1. Chemicals

All the reagents used in this study were of analytical grade or better. Methanol (MeOH), acetonitrile (MeCN), and formic acid were purchased from VWR. Chloroform ($CHCl_3$) was purchased from Alfa Aesar. Isomers τ- and π-methylhistidine (τ-MH and π-MH, respectively) were from Sigma Aldrich (Sigma catalog numbers 67520 and M9005, respectively).

2.2. Samples

Blood samples originated from a field study involving Holstein dairy cows raised in different farms from September 2016 to October 2019. The study was approved by the Research Committee of the Aristotle University of Thessaloniki, Greece (approval protocol number 62/15 December 2015), and details of the experimental design are outlined in recent publications by Siachos et al. [55,56].

In particular, bovine blood samples were collected from cows after the morning milking and at 5 different time-points relative to the day of calving (at 21 and 7 days prepartum and at 7, 21, and 28 days postpartum). The samples were collected using coccygeal venipuncture directly into vacuum evacuated 6 mL sterile glass tubes that contained Lithium-Heparin as an anticoagulant (BD Vacutainer®; Plymouth, UK). The samples were placed in a portable cooler immediately after collection. Heparinized plasma was collected from the blood samples by centrifugation (3000× *g* 15 min) within 1–2 h of sampling and stored in polyethylene tubes of 1.5 mL and frozen at −40 °C pending analysis.

2.3. Solutions and Control Samples

A series of solutions were prepared for use in method development: a global pooled heparinized bovine plasma sample representing the average endogenous concentration of τ- and π-MH in bovine plasma; initial stock solutions of τ- and π-MH; standard solutions to be used for recovery and parallelism assessment (parallelism includes the method's linearity and calibration range assessment); standard solutions for evaluating the instrument's linearity range for τ- and π-MH in the pure mobile phase (with a larger range than the calibration range of the actual method); and, finally, a series of quality control samples. The prepared solutions are described in detail below.

A pooled heparinized plasma sample was prepared by mixing equal volumes (200 μL) from a random selection of 60 heparinized plasma samples for a sample bank that included samples from multiple farms sampled across several years.

Stock solutions of τ- and π-MH were prepared at a concentration of 1000 μg/mL in MeOH:H_2O, 1:1 (*v*/*v*).

For the recovery study, aqueous standard solutions were prepared from stock solutions at concentrations of 1500, 3000, and 6000 μg/L.

For parallelism assessment (which includes the method's linearity assessment), aqueous standard solutions were prepared from stock solutions at concentrations of 560, 1120, 2240, 3920, 5600, 8400, and 11,200 μg/L.

For quality control (QC) purposes, four (4) samples were prepared: the pooled sample constituted the medium QC (MQC) and its corresponding concentration was 850.4 μg/L for τ-MH and 708.9 μg/L for π-MH (as determined during parallelism assessment); a low concentration QC (LQC) was generated by diluting the pooled sample to half its concentration with pure water; a higher medium QC (HMQC) and a high QC (HQC) by

spiking the pooled sample with additional concentrations of 320 and 560 μg/L, respectively, using aqueous solution containing τ- and π-MH. The final concentrations of the QCs are presented in Table S1. When running batches, within-batch injection precision was assessed by preparing a pooled extract sample from the first 30 sample extracts about to be run within the batch.

2.4. Sample Preparation

The sample preparation procedure included two basic steps: a step for lipid (and partial protein) removal using liquid-liquid extraction (LLE) with chloroform, and a step for complete protein removal by precipitation with a methanol:acetonitrile mixture (an adaptation of the approach described in Deda et al. [57]). The exact procedure differs slightly between non-spiked and spiked samples.

In specific, microcentrifuge tubes containing approximately 1200 μL heparinized plasma were rigorously vortexed for 30 s and centrifuged at 10,600× *g* for 15 min at 4 °C to precipitate any suspended material (mainly cells). In the case of non-spiked samples, a volume of 280 μL of the centrifuged plasma was transferred to a clean microcentrifuge tube, and 20 μL of a 1.5% aqueous formic acid solution was added to the tube followed by rigorous vortexing for 30 s. Regarding spiked samples, the sample volume differs (210 μL of pooled sample and 70 μL of the spike solution) and the procedure is reported in detail in the corresponding sections.

After vortexing, 300 μL of $CHCl_3$ was added to the tube followed by rigorous vortexing for a further 30 s. Then, the tube (containing a cloudy solution of both phases) was centrifuged at 10,600× *g* for 15 min at 4 °C for complete phase separation. Following centrifugation, 200 μL of the upper (aqueous) layer were transferred into a clean microcentrifuge tube, and 600 μL of a MeOH-MeCN (1:1, *v*/*v*) mixture were added to the tube. The tube was then rigorously vortexed for 30 s and centrifuged at 10,600× *g* for 15 min so that all remaining proteins would precipitate in the sedimented phase. The liquid phase was then collected, transferred to a new microcentrifuge tube, and stored at −20 °C pending analysis.

2.5. UHPLC-MS/MS Method Development and Validation

All samples were analysed on a Thermo Scientific™ TSQ Quantum™ Access MAX Triple Quadrupole Mass Spectrometer coupled to an Accela™ 1250 UHPLC pump and an Accela™ autosampler. Chromatographic separation was performed using a Waters™ ACQUITY UPLC BEH Amide Column (1.7 μm, 2.1 mm × 150 mm). Data were processed using Thermo Scientific™ Qual Browser in Thermo Xcalibur version 3.063 and Microsoft Excel. The performed experiments and validation criteria are based on guidelines published by EMA [58].

2.5.1. Specificity

An MS/MS fragmentation study was performed for each isomer to acquire ion fragment intensity data so that ion breakdown curves could be constructed, similar to those described by Mörlein et al. [59]. The main purpose of this procedure was to find which fragment ions and collision energies offered the highest selectivity and sensitivity. Individual, 1000 μg/L solutions of τ- and π-MH in H_2O:MeOH 1:1 (*v*/*v*) were infused in the mass spectrometer at a rate of 10 μL/min using a syringe pump and the following experimental conditions: the mass spectrometer was set in product scan mode and 50 scans were taken in 5 V collision energy (CE) increments (5–50 V) in profile mode. Scans were averaged and further process to centroid to acquire absolute intensity data. Then, ion breakdown curves of the most selective and abundant ion fragments were prepared. Source parameters for infusion were as follows: spray voltage 3500 V, vaporiser temperature 50 °C, sheath and auxiliary sheath gas pressures 10 and 0 arbitrary units, respectively, and capillary temperature 240 °C. Ion breakdown curves were prepared by plotting absolute intensity with collision energy. It is noted that extensive clean-up of the glass syringe and transfer

capillaries was required to remove all traces of the previously analysed isomer between fragmentation experiments.

Chromatographic conditions were determined by trial and error. In short, optimum elution conditions, mobile phase, and column temperature were determined for the fastest and best possible separation and peak shape of both isomers. After the initial isocratic conditions and elution of the peaks of interest, a gradient elution step was applied to flush any remaining polar constituents from the column. Mobile phase A was MeCN with 0.1% formic acid, and mobile phase B was H2O with 0.1% formic acid. The elution program used was as follows: A–B, 65:35 (v/v), hold for 3 min, then to A-B, 40:60 (v/v) over two minutes, hold for 2 min, then to A–B, 65:35 (v/v) hold for 10 min (total analysis time 17 min). The required equilibration step was applied as per column manufacturer's instructions to prevent retention time shifts. The flow rate was set at 350 µL/min, the column temperature at 60 °C, and the injection volume was 5 µL. Source conditions for sample analysis were as follows: spray voltage 3500 V, vaporiser temperature 250 °C, sheath and auxiliary gas pressures 40 and 10 arbitrary units, respectively, and capillary temperature was set at 320 °C. Selected transitions for quantitation were m/z 170 -> 124 (15 V) for τ-MH and m/z 170 -> 95 (30 V) for π-MH.

Inter-channel interference between the selected transitions for the two isomers was assessed by injecting high concentrations (4× the upper limit of quantitation (ULOQ)) of individual τ- and π-MH and determining the percentage of peak area present in the transition of the other isomer. This was calculated using the ratio of the peak area observed in the channel of the other isomer to the peak area of the injected isomer.

2.5.2. Recovery

Recovery and precision were determined at three different concentration levels. Aqueous spiking standard solutions were prepared from stock solutions at the following concentrations: 1500, 3000, and 6000 µg/L. Recovery was assessed by spiking 210 µL aliquots of the pooled sample with 70 µL of the spiking standard solution before performing the extraction and further treated as a non-spiked sample, as described in the sample preparation section, resulting in concentrations of 375 µg/L (low), 750 µg/L (medium), and 1500 µg/L (high). Concentration values were selected to be representative of the higher and lower part of the calibration range. Pooled sample extracts (after the 2nd extraction step and before injection) were also spiked accordingly (considering all extraction steps) to reach the same resulting concentrations pre- and post-extraction since the extraction process includes no evaporation steps. Extractions were performed in triplicates to assess precision at each concentration level. Pooled samples were also extracted as-is in triplicate to determine pooled sample peak area. Recoveries were calculated as follows: (peak area pre-extraction − peak area pooled)/(peak area post-extraction − peak area pooled) × 100. Precision was expressed as %RSD.

2.5.3. Parallelism

A surrogate matrix validation approach (by adapting some of the tests suggested by Houghton et al. and Jones et al. [60,61] for other endogenous metabolites) was undertaken to ensure that precision and accuracy were properly assessed, since a stable isotope labelled (SIL) or other suitable internal standards (IS) were unavailable in our lab for the metabolites under investigation. This approach was performed on a "fit-for-purpose" basis and is further explained in the results and discussion section.

As the surrogate matrix approach was adapted for quantification, a parallelism study was required. Proof of parallelism of the surrogate matrix calibration to the standard addition curves within the limits of quantitation, ensures that matrix effects are accounted for and that the quantification is conducted properly. This approach aims to determine if the selected matrix (in this case water) is a suitable matrix for the quantitation of τ-MH and π-MH in heparinized plasma. For this purpose, 7 spiked standards were prepared in water at the following concentrations: 560, 1120, 2240, 3920, 5600, 8400, and 11,200 µg/L.

For the standard addition curve, 240 μL of pooled sample were transferred in seven 1.5 mL microcentrifuge tubes. Finally, 20 μL of 1.5% aqueous formic acid solution and 40 μL of every aqueous standard were added to each tube. For the surrogate matrix curve, the same procedure was applied but using water instead of pooled sample. The tubes were vortexed and extracted following the procedure described in the sample preparation method section and injected directly to the LC-MS/MS for analysis. Standard addition and surrogate matrix curves were constructed for both τ- and π-MH, from spiked pooled sample and spiked water, and their slopes were assessed for parallelism as a percentage by dividing the pooled curve slope with the water curve slope. The final resulting calibration range was: 80, 160, 320, 560, 800, 1200, and 1600 μg/L. (It is noted that this calibration range refers to the initial bovine plasma concentration in the sample. Since all samples are diluted with a ratio of 1:4 during the sample preparation step for protein precipitation and no preconcentration step is applied, the on-column concentration range is 20–400 μg/L.)

2.5.4. Validation and Quality Control

For within-day and between-days precision (calculated as percent relative standard deviation (%RSD)) and accuracy (calculated as percent recovery (%R) or measured concentration to original QC concentration) assessment, but also for within-batch validation, 4 quality control (QC) samples were used as described above. QC samples were analysed 4 times for within-day and 12 (3 × 4) times for 3 consecutive days for between-days precision and accuracy.

The developed method was applied in the analysis of a large number of bovine plasma samples (>600). Results of a representative batch of 100 samples are provided. Two calibration curve replicates were run at the beginning and at the end of the batch. Quality control samples (ranging at all 4 QC levels) and a blank were run every 10 samples. Batch precision was assessed by running a pooled extract (prepared by mixing equal volumes from the first 30 samples of the batch) at equally spaced intervals within the batch.

3. Results and Discussion

3.1. Method Development and Validation

3.1.1. Specificity

Direct infusion of τ- and π-MH at increasing CE values demonstrated that the two isomers have distinct ion-abundances in their fragmentation patterns with increasing CE. At CE 15 V, the transition 170 -> 124 is selective for τ-MH since it is the predominant ion, while it is insignificant in the spectrum of π-MH. Similarly, the transition 170 -> 95 is selective for π-MH at a CE of 30–35 V (Figure 1).

The mass spectra (profile) of each isomer at the selected CE are provided in Figure 2. The acquired mass spectra were compared and confirmed with mass spectra library sources [62,63]. Indicative mass spectra at CE of 10, 20, and 30 V for τ- and π-MH are provided in Figures S2 and S3, respectively.

Regarding the transitions selected above, the inter-channel interference from τ-MH to π-MH and vice versa was calculated at 0.98 ± 0.03% for τ- to π-MH and 0.42 ± 0.12% for π- to τ-MH. Regardless of such a limited amount of inter-channel interference, the difference in retention time between the two isomers following chromatography assists in enhancing the method's specificity for these two isomers.

Optimization of chromatographic conditions through trial and error showed that long equilibration of the initial conditions and column temperature is required to achieve both isomer separation and acceptable peak shape in the shortest possible time. Separation of the isomers' peaks was achieved by applying isocratic conditions. A gradient step to 60% aqueous conditions was also necessary to flush off the column polar constituents of the samples. A chromatogram indicative of the separation between τ- and π-MH is provided in Figure 3. Small structural differences allow for a slightly different degree of interaction in the HILIC mode with the amide stationary phase.

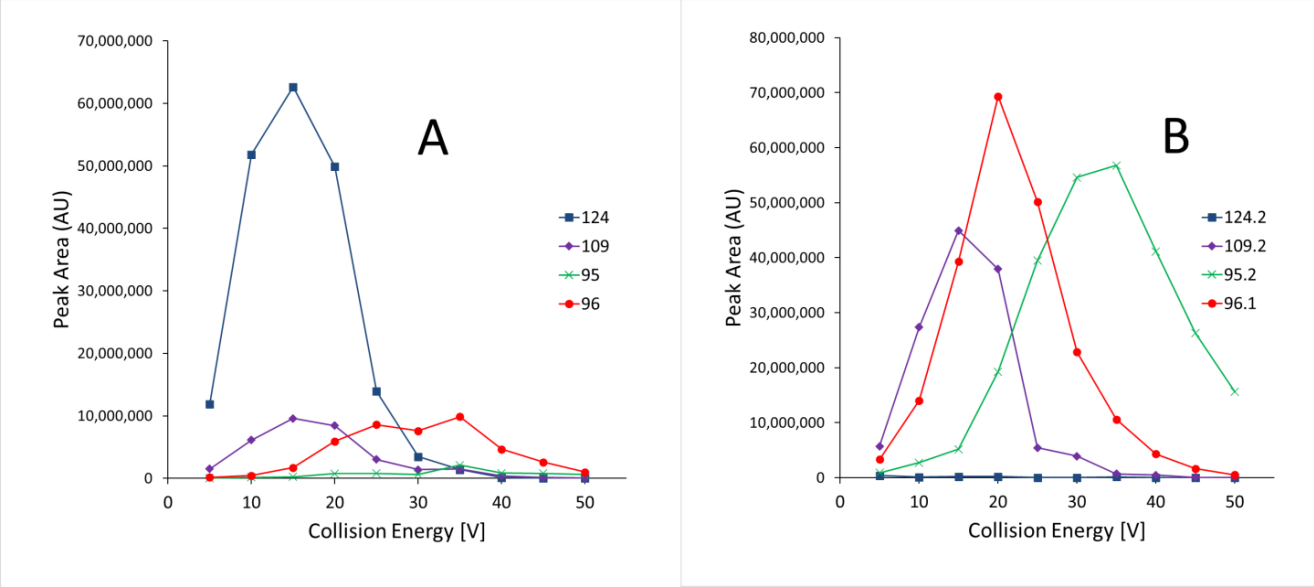

Figure 1. Ion breakdown curves in absolute intensities of the most selective and intense fragment ions for (**A**) τ-MH and (**B**) π-MH. Each value corresponds to an average of 50 scans. It is demonstrated that the most intense ion fragments are 124 in the case of τ-MH and 96 and 95 in the case of π-MH. The intensity of ion 124 is significantly higher in the mass spectrum of τ-MH, while that of ion 95 is significantly higher in the mass spectrum of π-MH. Fragment ions 109 and 96 are present in significant intensities in both isomer breakdown curves.

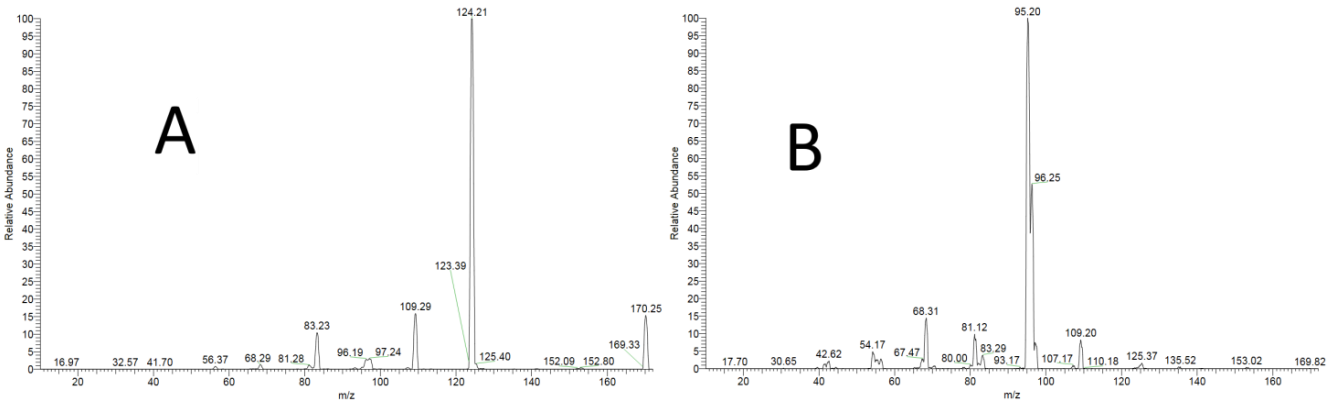

Figure 2. (**A**): Mass spectrum at a CE of 15 V for τ-MH. The spectrum is an average of 50 scans, from 10 to 172 m/z for the precursor 170 m/z. Ion 124 is the most intense peak in the spectrum, (**B**): Mass spectrum at a CE of 30 V for π-MH. The spectrum corresponds to the average of 50 scans, from 10 to 172 m/z for the precursor 170 m/z. Ion 95 is the most intense peak in the spectrum. Readers should refer to Figure S4 for more details.

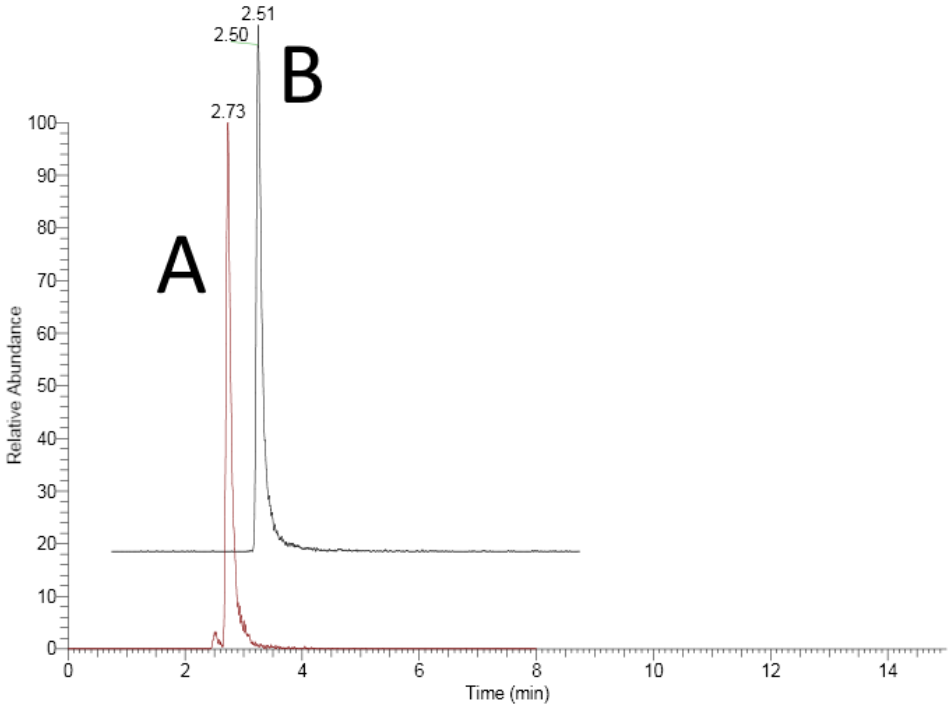

Figure 3. Chromatogram depicting the separation of π-MH (**A**) and τ-MH (**B**).

3.1.2. Recovery

Recovery was assessed at three spiked concentrations, low (375 µg/L), medium (750 µg/L), and high (1500 µg/L). Each extraction was performed in triplicate, along with the extraction of the non-spiked pooled sample, to access precision at the pooled level. Recoveries for both isomers were within an acceptable range (78.54–91.84%), and precision (expressed as %RSD) was below 10% in all cases (Table 1).

Table 1. Recovery values and extraction repeatability values (n = 3) for τ- and π-MH. Recoveries are within the 70–120% range, which is considered by the authors acceptable for the purpose of this study. Precision in all cases is below 10%.

Solution Type	Recovery [%R]		Precision [%RSD]	
	τ-MH	**π-MH**	**τ-MH**	**π-MH[1]**
Low spike	78.54	84.90	1.85	9.33
Medium spike	84.02	79.33	4.22	5.53
High spike	91.84	87.29	5.00	9.10
Pooled sample	-	-	4.33	7.78

3.1.3. Parallelism

Parallelism was assessed using a standard addition curve by spiking and extracting, as above, a pooled plasma sample with τ- and π-MH at the following resulting concentrations: 80, 160, 320, 560, 800, 1200, and 1600 µg/L. The surrogate matrix was spiked in the same quantities to the same concentrations, resulting in the sample calibration curve. The surrogate matrix used in this case was ultrapure water. Both curves were analysed in triplicate, and the injection means for each concentration were plotted to assess parallelism by calculating the ratio of the slopes of the standard addition over the sample calibration curve. For the purpose of this study, the authors considered a slope ratio in the range 110 to 90% as satisfactory. The calculated ratios for parallelism were 90.40% for τ-MH and 90.37% for π-MH (Figure 4), indicating that the matrix effect was insignificant, thus quantification can be applied without bias using the surrogate matrix calibration curve.

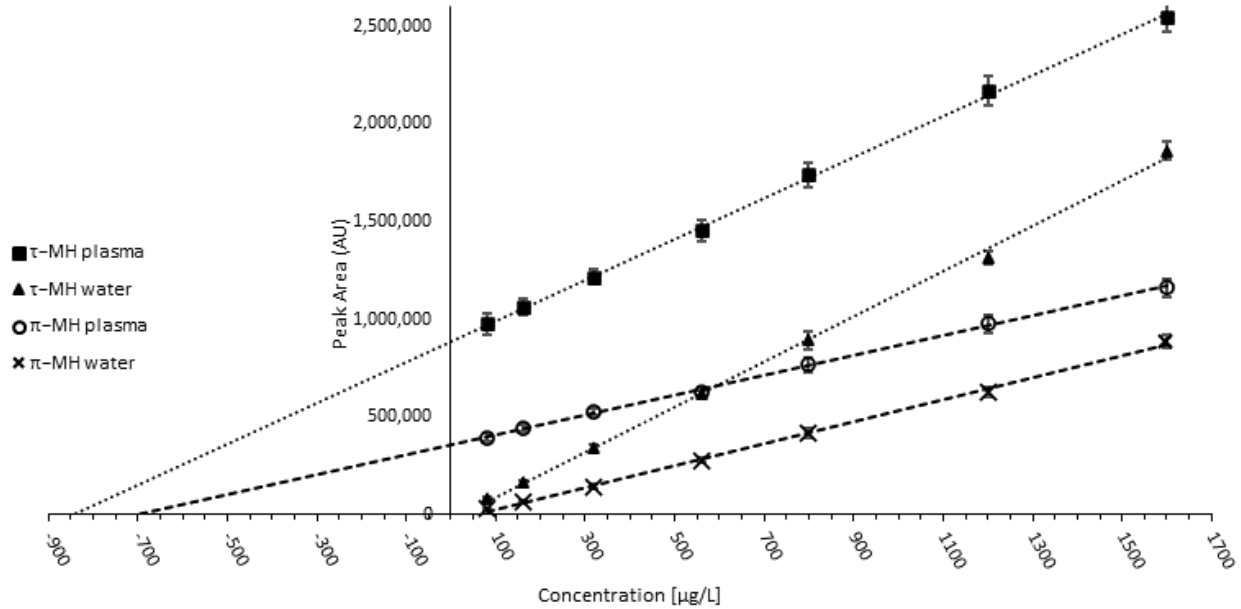

Figure 4. Standard addition and surrogate matrix calibration curves for τ- and π-methylhistidine at the selected concentrations. Extrapolation of the standard addition curve indicates the endogenous value of τ- and π-MH in the pooled sample.

The endogenous concentration of the pooled cow plasma sample can be determined by extrapolating the standard addition curve to the point where the spike concentration equals zero (Figure 4) or simply by dividing the slope to the intercept of the calibration equation. For τ- and π-MH, the endogenous concentrations were determined as 850.4 µg/L and 708.9 µg/L (or 5.03 µM and 4.19 µM), respectively, which is in the range of expected literature values. The lower and upper limits of quantitation (LLOQ and ULOQ) were set at 80 and 1600 µg/L (0.5 and 9.93 µM), respectively, since for concentrations above 1600 µg/L parallelism would drop below 90%. Any sample with a concentration exceeding the method's calibration range should be diluted appropriately with H_2O and re-analysed.

3.1.4. Validation and Quality Control

As already described in Section 2.3, QC samples were prepared at four concentration levels. Each QC concentration was determined experimentally using the surrogate matrix calibration curve and compared to the QC concentration that was determined using the endogenous extrapolated concentration values. QCs were run at four replicates each day in Day 1, Day 2, and Day 3. Precision and accuracy results are given in Table 2. The limit of quantitation for the method is set at 80µg/L (0.5 µM), the same as the LLOQ.

Table 2. Results for within-day and between-days trueness (%R) and precision (%RSD). All values appear to be within the acceptable validation range.

Figure of Merit	Within-Day (Day 1) *n* = 4		Between-Days (Day 1, Day 2, Day 3) *n* = 12	
	τ-MH	π-MH	τ-MH	π-MH[1]
Accuracy LQC [%]	103.4	112.9	99.7	113.2
Accuracy MQC [%]	100.6	102.7	96.0	104.0
Accuracy HMQC [%]	97.9	102.5	95.6	105.2
Accuracy HQC [%]	99.8	100.3	97.1	106.2
Precision LQC [RSD%]	3.5	4.2	2.3	4.8
Precision MQC [RSD%]	6.5	6.2	3.7	4.2
Precision HMQC [RSD%]	3.9	4.7	3.4	3.7
Precision HQC [RSD%]	2.0	2.6	1.5	1.8

As shown in Table 1, recovery and precision of the developed method can be considered acceptable, taking into account the specific purpose of the method, which is to provide concentration values of τ- and π-MH for use as ad-hoc variables in statistical analyses (e.g., confounding effects) in very large cohort cow studies.

3.2. Sample Analysis

As mentioned above, the developed method was applied for the analysis of a large number of samples (>600) in a series of batches derived from a cow metabolism study with the aim of providing data for use in statistical analysis. Here the results of a representative batch of 100 heparinized plasma samples are provided: within batch results of QC samples gave an average trueness of 102.7% for τ-MH and 105.16% for π-MH, ranging from 93.7% to 111.2% for τ-MH and 91.3% to 115.9% for π-MH. Within-batch injection precision was at 5.0% and 5.6% (%RSD) for τ- and π-MH, respectively. Fresh surrogate matrix calibration curve and QC samples were prepared for each batch.

The average concentration in the representative batch for τ-MH was 1201 μg/L (7.1 μM), ranging from 152 to 2859 μg/L (0.9–16.9 μM), and for π-MH 947 μg/L (5.6 μM), ranging from 321 to 2369 μg/L (1.9–14 μM), both of which are in line with the literature [13].

3.3. Discussion

Older methods for amino acid determination (or other polar endogenous metabolites) that relied on pre- or post-column derivatization are slowly being replaced by modern methods employing tandem mass spectrometry and hydrophilic interaction liquid chromatography [54,64–67]. In the authors' opinion, wide availability of modern tandem mass spectrometry instruments means that the arduous sample preparation accompanying derivatization procedures of amino acids is, in most cases, becoming a thing of the past.

The purpose of the developed method is to be applied for the economic, simple, and fast quantitation of τ- and π-MH in bovine plasma in samples deriving from large cohort Holstein cow studies (involving hundreds or thousands of samples) with the aim to correlate the levels of mainly τ-MH to the nutritional status of the animals by means of statistical analysis (e.g., sample classification, multivariate statistical analysis, investigation of confounding effects, etc.). The method reported herein includes simple and straightforward sample preparation with a bovine plasma specific quantitation range for τ- and π-MH of 80 and 1600 μg/L (0.5 and 9.93 μM).

The main advantages of the presented method compared to those published previously are the simplicity of procedures and availability of materials used for both sample preparation and analysis. Additionally, the method offers lower cost, less instrument downtime, and a specific focus on bovine plasma. A similar method reported by Wang et al. for the determination of τ- and π-MH in human urine [51] requires minimal sample preparation, no derivatization, and relies solely on mass spectroscopic differences without any chromatographic separation between isomers. Since there is little analyte retention, the isomers are expected to co-elute with a significant amount of matrix components; hence to minimise ion suppression and matrix effects, the urine samples were diluted (approximately 82 times according to the authors' calculations using the reported data) before injection. The reported LLOQ for the method is at 5 μM, which is too high for bovine plasma (which has a reported average of approx. 5 μM [22]). The method presented herein accounts for matrix effects and focuses on bovine plasma offering an LLOQ of 0.5 μM (80 μg/L).

Kochlik et al. reported a method for the individual determination of τ- and π-MH in human plasma [52] with excellent LLOQ (0.015 μM), which could potentially be applied for bovine plasma. However, the method requires lengthy sample preparation (3 h and 20 mins), and the separation is achieved using a specialised amino acid stationary phase. The extended sample preparation length makes this method unattractive for large cohort studies; herein sample preparation takes approx. 40 mins. Additionally, using a generic

and widely employed/available column (such as the one employed herein) makes for a more feasible alternative for the average lab.

Roggenshack et al. published a method where τ- and π-MH were determined separately in biofluids by utilizing a steep gradient and very high concentration of buffer salt [53] (the authors reported LLOQ at approx. 10µM for both τ- and π-MH). Such high salt concentrations may benefit retention and peak shape in some stationary phases in the HILIC mode [68]. However, the use of high salt concentrations may impact analyte ionization and electrospray ionization (ESI) performance [69,70]. It may also lead, in the authors' experience, to blockage of ESI components (e.g., spray needle), especially in the heated ESI mode. This may pose a severe hindrance when the analysis of hundreds or thousands of samples is required, both regarding cost and time (ESI needles may need replacement after blockage), leading to additional instrument downtime. Additionally, the high cost of obtaining and using high purity mobile phase additives must also be taken into consideration.

As mentioned above, the presented method was developed specifically for the quantitation of τ- and π-MH in bovine plasma and large cohort studies to provide results for statistical analysis. If the method was to be adapted to provide study-specific results of, e.g., diagnostic nature (in either a veterinary or other medical setting), a stricter method validation approach would probably be more appropriate (e.g., employing carbon-13 isotope labelled standards). Although the employed parallelism assessment is representative of many animals and spanning several years, due to possible variations in sample parameters (anticoagulation method, geographical location, health status of animals, breed, etc.) and lab-specific settings (e.g., different source design and mass spectrometry detector), parallelism should be reconfirmed before starting a new study.

The presented method is developed to minimise costs, sample preparation, and analysis time, including instrument downtime, with the aim of analyzing hundreds to thousands of samples. Sample preparation time is approximately 40 min (sample number bottlenecked by the available centrifuge positions) while sample and data analyses are approximately 20 min per sample. Based on these figures, a batch of 100 samples (complete with calibration and QC) would require approximately two working days for full processing. As for cost-effectiveness, apart from the capital equipment requirements, all materials are affordable and common in analytical labs.

4. Conclusions

A novel, economically friendly, simple, and fast method was developed for the determination of τ- and π-methylhistidine in Holstein cow heparinized plasma. The method is based on both chromatographic separation and spectral ion abundance, utilising hydrophilic interaction liquid chromatography (HILIC) coupled to tandem mass spectrometry (LC-MS/MS). The method includes simple and straightforward, clean-up driven, sample preparation and does not require specialised extraction, derivatization, or other labour-consuming techniques. Hence, the analysis of τ- and π-MH in large numbers of samples that are associated with lactating Holstein cow studies is made possible in an efficient and economically friendly manner.

Supplementary Materials: The following Supporting Information can be downloaded at: https://www.mdpi.com/article/10.3390/separations10020144/s1.

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