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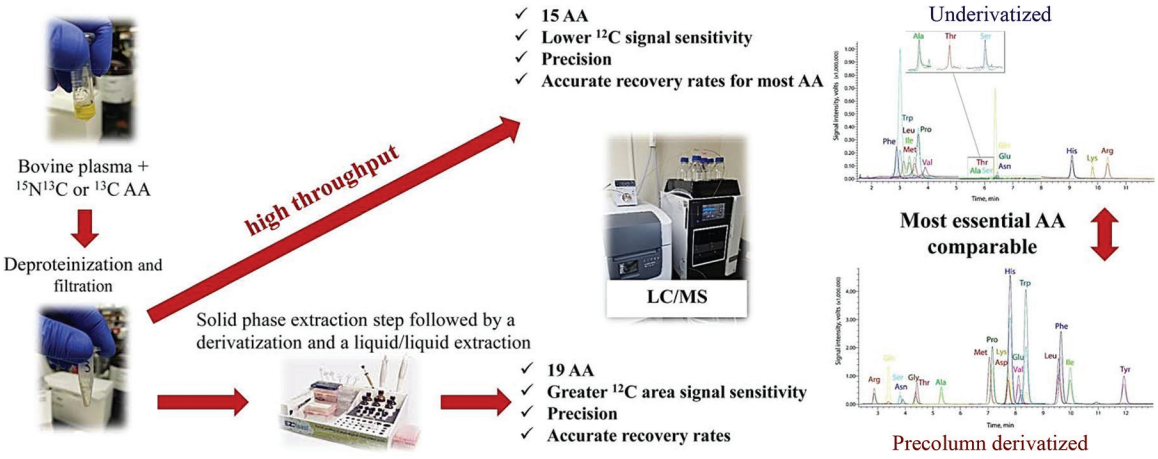
Quantification of bovine plasma amino acids via liquid chromatography–electrospray ionization-mass spectrometry: Comparison of underivatized and precolumn derivatized methods

Mateus Z. Toledo,[1] Caleb Nienow,[2] Daniel Luchini,[3] Sebastian I. Arriola Apelo,[1] and Milo C. Wiltbank[1]*

Graphical Abstract



Summary

We validated and compared 2 methods for quantification of bovine plasma amino acids (AA) via liquid chromatography (LC)-mass spectrometry. The underivatized method may be a cost-effective, high-throughput, and practical alternative for analysis of AA in dairy cattle, particularly if only essential AA values are required. The derivatized method has greater ^{12}C area signal sensitivity and linearity and recovery rates for all AA but requires more sample processing.

Highlights

- We validated and compared 2 methods for quantification of AA in bovine plasma.
- Our underivatized method may be a practical alternative for essential AA.
- The derivatized method has greater ^{12}C area signal sensitivity, linearity, and accuracy.

[1]Department of Animal and Dairy Sciences, University of Wisconsin, Madison 53706, [2]Shimadzu Scientific Instruments Inc., Columbia, MD 21046, [3]Adisseo, Alpharetta, GA 30022. *Corresponding author: wiltbank@wisc.edu. Received December 05, 2020. Accepted February 19, 2021.

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Quantification of bovine plasma amino acids via liquid chromatography–electrospray ionization-mass spectrometry: Comparison of underivatized and precolumn derivatized methods

Mateus Z. Toledo,[1] Caleb Nienow,[2] Daniel Luchini,[3] Sebastian I. Arriola Apelo,[1] and Milo C. Wiltbank[1]*

Abstract: The objectives of this experiment were to evaluate and compare underivatized (UND) and precolumn derivatized (DER) methods for quantification of bovine plasma AA by isotope dilution ratio via liquid chromatography-electrospray ionization (ESI)-single quadrupole mass spectrometry. Linearity of the mass-to-charge ratio signal and area signal sensitivity of ^{12}C were evaluated for each AA with 5-point standard curves (range: 1.1–500 μ*M*). Plasma from lactating dairy cows was isolated by centrifugation and deproteinized using 1 *N* perchloric acid with a final concentration of 0.5 *N*. Deproteinized plasma was filtered and injected into a 50 × 2-mm column (Imtakt) or extracted, derivatized, and injected into a 250 × 3-mm column (EZ:faast, Phenomenex) and analyzed via liquid chromatography-ESI-single quadrupole mass spectrometry. Coefficients of variation and recovery rates were evaluated using 4 replicates of pooled plasma samples spiked with each AA at concentrations of 10, 20, and 50 μ*M*. In addition, a subset of 24 plasma samples was used to directly compare methods using linear regression, correlation coefficient (r), concordance correlation coefficient (CCC), and Bland-Altman plot test. Both methods showed linearity within the dynamic range analyzed for all essential AA (coefficient of determination, $R^2 \geq 0.995$) and most other AA, although the UND samples had poor linearity ($R^2 \leq 0.990$) or peak resolution problems for Asp, Gly, Tyr, and Ser. Moreover, area signal sensitivity for ^{12}C AA was greater for DER samples than for UND samples [range: 2.2× (Pro) to 309.5× (Ala)]. Both methods had recovery rates ranging from 85.7 to 119.8.0%, and none differed from 100% except Gln [20 μ*M* (85.7%) and 50 μ*M* (87.6%)] and Val [50 μ*M* (119.8%)] using the UND method. The UND method had a coefficient of variation ranging from 0.9% (Val) to 7.8% (His), whereas for the DER method the range was 2.2% (Glu) to 8.8% (Asp). The highest correlation coefficient (>0.90) and CCC (>0.90) were observed for Arg, Ile, Leu, Met, Thr, Trp, Val, and Gln, with the Bland-Altman plot test showing minimal mean bias for these AA. Lowest values were observed for His (r = 0.46; CCC = 0.45), Lys (r = 0.76; CCC = 0.75), Ala (r = 0.83; CCC = 0.73), and Glu (r = 0.65; CCC = 0.42). The UND method showed linearity, precision, and accurate recovery rates for most AA, with most essential AA having comparable values between the 2 methods. However, the DER method had greater ^{12}C AA area signal sensitivity, linearity, and recovery rates.

Formulation of dairy cattle diets for AA has been an important focus of dairy nutritionists in recent years. Much of the research has been directed at improvements in milk fat and protein production, but research has also evaluated the potential for reductions in CP content in dairy diets to reduce dietary costs and excretion of nitrogen into the environment (Vyas and Erdman, 2009; Sinclair et al., 2014; Lean et al., 2018). Some AA are considered to be rate limiting for milk production and milk composition (NRC, 2001; Vyas and Erdman, 2009; Lee et al., 2012). The most studied rate-limiting AA are Met and Lys, although recent research has indicated that other AA, such as His and the branched-chain AA Val, Leu, and Ile, can also affect productive performance of lactating dairy cows (Lee et al., 2012; Haque et al., 2013; Zhao et al., 2019). In addition, AA have been associated with indicators of health and reproductive performance, with special emphasis on the time near parturition. For example, plasma concentration of AA during the transition period has been related to liver function and health disorders (Shibano and Kawamura, 2006; Zhou et al., 2016). Thus, the profile for circulating AA in cows can vary in herds or individual cows based on the characteristics of the diet or on the metabolic and health status of individual cows. Accurate and efficient quantification of plasma AA is important for validly evaluating the effectiveness of specific rumen-protected AA supplementation strategies, understanding AA metabolism, and determining AA profile status. Therefore, plasma AA analysis is an important tool for dairy researchers, and a simpler, accurate, low-cost methodology might make evaluation of AA status a practical approach for nutritionists and dairy farmers.

Different techniques are available for AA quantification. The AA analyzer, developed in the 1950s and based on ion-exchange chromatography with postcolumn ninhydrin detection, is the validated method of the Association of Official Analytical Chemists. This method, which is extremely reproducible and efficient for separation of each of the 20 AA used in mammalian proteins, is used with the greatest frequency (Walker and Mills, 1995; Rigas, 2013). However, this technique has limitations from a technical

[1]Department of Animal and Dairy Sciences, University of Wisconsin, Madison 53706, [2]Shimadzu Scientific Instruments Inc., Columbia, MD 21046, [3]Adisseo, Alpharetta, GA 30022. *Corresponding author: wiltbank@wisc.edu. Received December 05, 2020. Accepted February 19, 2021.

standpoint, such as instability of ninhydrin, coeluting compounds, and slow throughput time, with approximately 40 min for sample preparation (protein precipitation, removal of interfering compounds, and derivatizations) and 2- to 4-h runs (Walker and Mills, 1995; Rigas, 2013). Separation techniques developed in recent decades using gas or liquid chromatography coupled with detection by MS and quantification by isotope ratio are more robust approaches with the potential to overcome these limitations (Badawy et al., 2008; Dietzen et al., 2008; Kaspar et al., 2008). These techniques are now widely used in dairy research, and several commercial kits are available. Mass spectrometry allows the analysis of AA using isotopically labeled forms of the same AA as internal standards, which improves quantification by limiting the effect of coeluting compounds (Calder et al., 1999). In addition, these methods require less sample preparation time (~8 min) and run time (8–20 min; Krummen et al., 2004; Dietzen et al., 2008; Krumpochova et al., 2015). More recently, underivatized (**UND**) methods have become available; these methods use an ion pair, hydrophilic mode, or normal phase plus ion exchange, which allow shorter analysis times and less labor for sample preparation (Nemkov et al., 2015; Takano et al., 2015; Prinsen et al., 2016). However, UND methods have not yet been rigorously evaluated and compared with the more traditional precolumn derivatized (**DER**) method using bovine plasma.

Therefore, this study evaluated and compared UND (mixed mode with normal phase plus ion exchange) and precolumn DER (ion exchange and reversed phase mode) methods using bovine plasma samples analyzed via single-quadrupole liquid chromatography-electrospray ionization (**ESI**)-MS. We predicted that both methods would have linearity and accuracy for all AA evaluated and that the UND method would have acceptable precision and accuracy and would be comparable with the DER method for all AA evaluated.

Standards of all 20 NEAA and EAA were purchased from Sigma-Aldrich (AAS18) at a concentration of 2,500 μ*M* in 0.1 *N* HCL. This standard contained 17 AA, and Asn, Gln, and Trp were added separately to achieve similar concentrations for all AA. Labeled AA-^{13}C-^{15}N or labeled AA-^{13}C (Metabolomics Amino Acid Mix Standard-MSK, 1.2 mL, modified and added labeled AA-^{13}C of Asn, Gln, and Trp; Cambridge Isotopes Laboratory) were used as internal standards (Calder et al., 1999). Plasma from lactating dairy cows was isolated by centrifugation and deproteinized using 1 *N* perchloric acid at a final concentration of 0.5 *N*. Deproteinized plasma was filtered, and extraction and derivatization were performed according to the manufacturer's directions. Briefly, the procedure consists of solid-phase extraction using a sorbent tip that binds AA while allowing interfering compounds to flow through. Subsequently, AA are eluted into the sample vial and derivatized in aqueous solution. Derivatized AA migrate to the organic layer for additional separation from interfering compounds, and the organic layer is then removed and dried under nitrogen. The AA were redissolved in aqueous mobile phase, and either 1 μL (0.5 μL for the highest curve points, 250 and 500 μ*M*) was injected into a 250 × 3-mm column (DER method; ion exchange and reverse phase: EZ: faast, Phenomenex) or 5 μL was injected into a 50 × 2-mm column (UND method; mixed mode with normal phase plus ion exchange; Intrada Amino Acid, Imtakt Inc.) for analysis.

For the DER method, optimal chromatographic separation was obtained with a flow rate of 0.5 mL/min using a gradient with solvent A (10 m*M* ammonium formate in water) and solvent B (10 m*M* ammonium formate in methanol). Initial conditions were 32% of A and 68% of B. Concentration of solvent B was linearly increased to 83% over 13 min and then returned to 68%. The column was re-equilibrated for 4 min, with a total run time of 17 min. For the UND method, optimal chromatographic separation was obtained with a flow rate of 0.3 mL/min using a gradient with solvent A (acetonitrile containing 0.1% of formic acid) and solvent B (100 m*M* ammonium formate). Initial conditions were 86% of A and 14% of B. Concentrations of solvent B were linearly increased to 100% from 3 to 10 min, followed by a decrease from 100% to 14% of solvent B over 10 to 12 min, and the column was re-equilibrated (14% solvent B) for 3 min, for a total run time of 15 min. For both methods, the eluent was ionized using ESI and analyzed using a positive selected ion monitoring mode. The ^{12}C ions (mass-to-charge ratio; *m*/*z*) monitored were as follows. For the DER method, EAA: 303.0 (Arg), 370.0 (His), 260.0 (Ile and Leu), 361.0 (Lys), 278.0 (Met), 294.0 (Phe), 248.0 (Thr), 333.0 (Trp), and 246.0 (Val); NEAA: 218.0 (Ala), 243.0 (Asn), 304.0 (Asp), 275.0 (Gln), 318.0 (Glu), 204.0 (Gly), 244.0 (Pro), 234.0 (Ser), and 396.0 (Tyr). For the UND method, EAA: 175.2 (Arg), 156.2 (His), 132.2 (Ile and Leu), 147.2 (Lys), 150.2 (Met), 166.2 (Phe), 120.1 (Thr), 205.0 (Trp), and 118.2 (Val); NEAA: 90.2 (Ala), 133.0 (Asn), 134.1 (Asp), 147.0 (Gln), 148.1 (Glu), 76.3 (Gly), 116.1 (Pro), 106.1 (Ser), and 182.2 (Tyr). In addition, internal standards (^{13}C-^{15}N or ^{13}C) for each AA were monitored. Analyses were performed via liquid chromatography (Nexera-i LC-2040C; Shimadzu) coupled with a mass spectrometer (LCMS-2020; Shimadzu). Analytical protocol conditions included the following: nebulizing gas, 1.5 L/min; drying gas, 20 L/min; desolvation line, 250°C; heat block, 400°C; interface bias, +4.5 kV, interface current, 19.9 μA; detector, −1.35 kV; and oven, 35°C. Quantification of AA was based on area under the curve and ratio between ^{12}C-AA and labeled AA-^{13}C-^{15}N or AA-^{13}C. Data processing was performed using the LabSolutions software (Shimadzu).

Method evaluations were performed with area signal sensitivity for ^{12}C, linearity of *m*/*z*, precision (percent coefficient of variation; **CV**), and recovery rates. Signal sensitivity was defined as the area under the peak for the lowest point on the standard curve for each AA, measured by the signal intensity (V) and time (min). Recovery rates were evaluated with a total of 4 replicates using pooled plasma samples spiked with each AA at concentrations of 10, 20, and 50 μ*M*. Selection of the 2 spiking concentrations to be evaluated was based on the physiologic range for each AA in cattle. The percent CV was also determined using 4 pooled plasma samples with no spiking. Recovery rates were analyzed using the TTEST procedure of SAS 9.4 (SAS Institute Inc.) for testing if rates were different from 100%. Difference was considered significant at $P \leq 0.05$, whereas differences between $P > 0.05$ and $P \leq 0.10$ were considered a tendency.

In addition, a subset of 24 plasma samples from multiparous Holstein cows (80 ± 3 DIM and 49.7 ± 7.9 kg of milk/d; mean ± SD) were analyzed using both methods and directly compared between the methods. Method comparison was performed using linear regression, correlation coefficient (r), and concordance correlation

Table 1. Linear range, ^{12}C area signal sensitivity, and linearity to mass-to-charge ratio for quantification of AA using an underivatized (UND) or a precolumn derivatized (DER) method via liquid chromatography-MS

		UND		DER		
Item	Linear range (μ*M*)	Signal sensitivity (V/min)	R^2	Signal sensitivity (V/min)	R^2	Fold increase in signal sensitivity[1]
EAA						
Arg	7.8–125	96,630–1,449,740	0.999	304,612–4,458,940	0.999	3.2
His	7.8–125	65,033–1,230,166	0.999	3,947,154–42,417,158	0.999	60.7
Ile	31.3–500	416,518–6,745,694	0.999	3,892,695–22,208,884	0.999	9.3
Leu	31.3–500	320,796–4,536,577	0.999	5,013,883–25,338,084	0.999	15.6
Lys	15.6–250	92,202–2,071,644	0.999	1,522,443–12,500,338	0.999	16.5
Met	3.9–62.5	118,273–784,719	0.999	734,916–5,771,980	0.999	6.2
Phe	7.8–125	131,062–2,057,001	0.999	1,545,947–23,681,392	0.999	11.8
Thr	15.6–250	5,247–121,651	0.999	534,786–6,579,017	0.999	101.9
Trp	7.8–125	144,456–2,463,199	0.999	1,196,296–19,119,764	0.999	8.3
Val	31.3–500	392,681–3,811,317	0.999	3,135,716–16,125,131	0.999	8.0
NEAA						
Ala	31.3–500	5,543–141,431	0.996	1,715,763–8,864,006	0.999	309.5
Asn	3.9–62.5	8,282–74,135	0.996	57,781–476,904	0.999	7.0
Asp[2]	1.1–62.5	—	—	92,027–5,162,366	0.999	—
Gln	31.3–500	102,056–3,095,812	0.999	1,389,088–7,886,8242	0.999	13.6
Glu	15.6–250	21,040–349,258	0.998	658,437–5,601,688	0.999	31.3
Gly[2]	31.3–500	—	—	481,726–7,184,010	0.999	—
Pro	7.8–125	401,394–3,634,331	0.999	897,674–16,039,115	0.999	2.2
Ser	15.6–250	6,654–26,583	0.988	422,637–3,391,547	0.999	63.5
Tyr[2]	7.8–125	—	—	667,446–10,036,002	0.999	—

[1]Ratio of signal sensitivity for the lowest concentration on the standard curve for each AA (DER/UND).
[2]Not obtained using the UND method due to resolution problems.

coefficient (**CCC**) using the REG, CORR, and IML procedures of SAS 9.4, respectively. The CCC index contains measurements of both accuracy and precision and ranges from −1 to 1, with 1 being perfect agreement, −1 being perfectly reversed agreement, and 0 being no agreement (Lin, 1989). Performance of regressions was assessed using root mean squared error of prediction (RMSEP). The mean squared error of prediction (**MSEP**) was decomposed into mean bias (**MB**), slope bias (**SB**), and random errors (Theil,

Table 2. Comparison of underivatized (UND) and precolumn derivatized (DER) methods to quantify bovine plasma AA (mean ± SD; n = 24) via liquid chromatography-MS[1]

	Bovine plasma AA, μ*M*		Mean bias			RMSEP	MSEP decomposition (%)		
Item	UND	DER	(μ*M*)	r	CCC	(μ*M*)	Mean bias	Slope bias	Random error
EAA									
Arg	60.5 ± 19.4	61.1 ± 19.4	−0.06	0.99	0.99	3.12	4.2	0.5	95.2
His	34.6 ± 8.7	36.7 ± 9.4	−2.11	0.46	0.45	9.39	5.1	30.9	64.0
Ile	89.4 ± 25.8	93.9 ± 26.1	−4.50	0.94	0.93	9.94	20.5	3.2	76.3
Leu	118.5 ± 33.6	118.6 ± 36.5	−0.07	0.98	0.98	7.30	0.0	21.9	78.1
Lys	67.5 ± 21.0	70.4 ± 23.6	−2.91	0.76	0.75	15.56	3.5	22.7	73.8
Met	20.9 ± 10.2	23.0 ± 11.9	−2.06	0.99	0.96	3.04	45.2	33.1	21.0
Phe	36.9 ± 6.5	40.4 ± 7.0	−3.49	0.95	0.84	4.04	74.8	3.1	22.2
Thr	98.1 ± 24.2	99.4 ± 26.3	−1.27	0.97	0.96	6.67	3.6	18.6	77.7
Trp	52.0 ± 12.0	48.8 ± 11.8	3.20	0.97	0.93	4.37	53.5	0.1	46.4
Val	270.0 ± 67.2	254.2 ± 73.8	15.84	0.96	0.94	25.31	39.2	12.2	48.6
NEAA									
Ala	206.5 ± 36.2	226.1 ± 38.2	−19.56	0.83	0.73	29.09	45.2	7.8	47.0
Asn	27.7 ± 14.7	33.0 ± 18.5	−5.21	0.98	0.91	7.25	51.6	30.4	17.9
Asp[2]	—	—	—	—	—	—	—	—	—
Gln	161.4 ± 54.8	148.7 ± 50.0	12.53	0.98	0.95	17.28	53.6	4.1	42.3
Glu	95.5 ± 15.3	78.2 ± 28.5	17.31	0.65	0.42	27.58	39.4	43.6	17.0
Gly[2]	—	—	—	—	—	—	—	—	—
Pro	64.9 ± 13.4	61.3 ± 12.7	3.65	0.90	0.86	6.88	28.2	0.9	70.8
Ser[2]	—	—	—	—	—	—	—	—	—
Tyr[2]	—	—	—	—	—	—	—	—	—

[1]Mean bias was determined with Bland-Altman plot test. CCC = concordance correlation coefficient; MSEP = mean squared error of prediction; RMSEP = root mean squared error of prediction.
[2]Not compared due to poor chromatogram or linearity using the underivatized method.

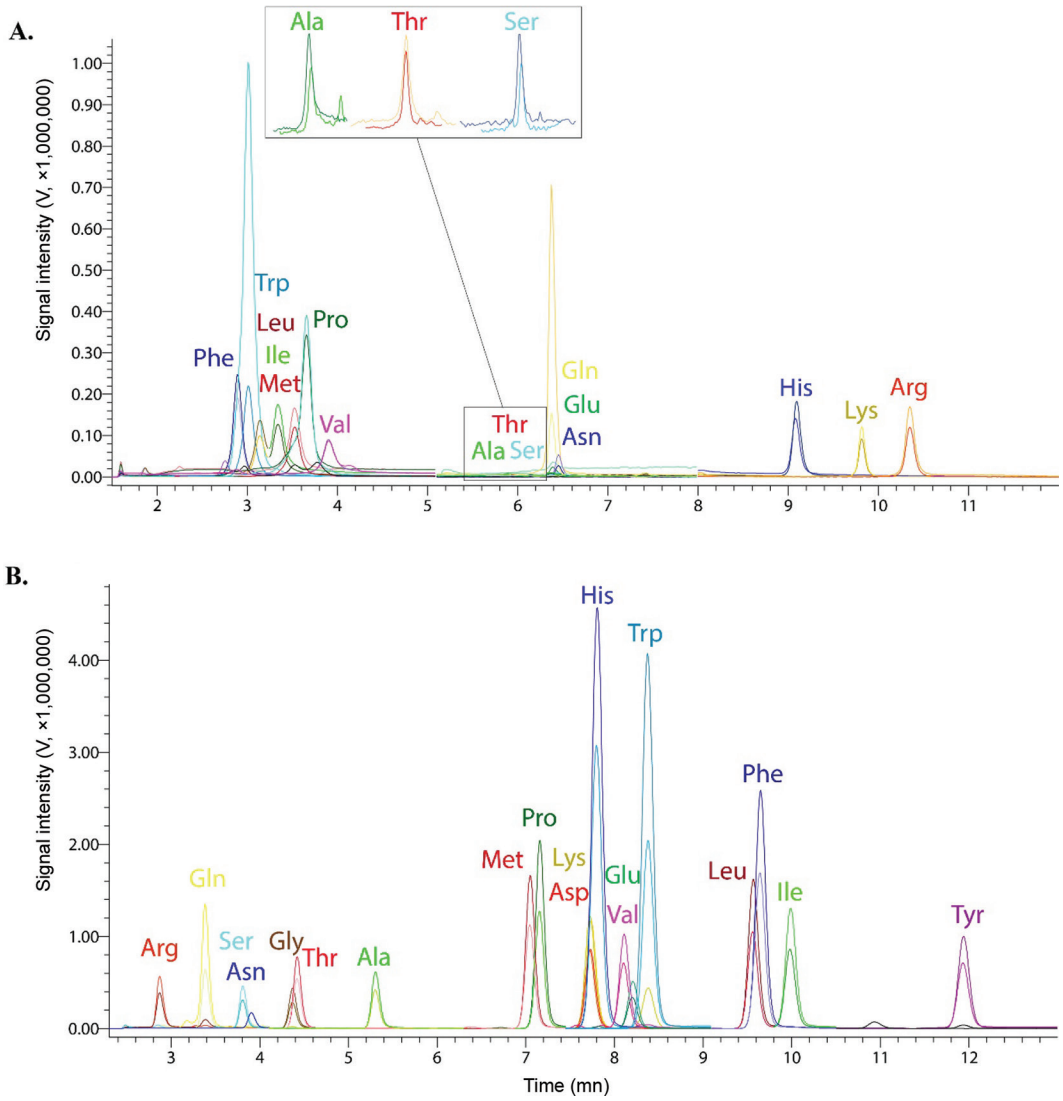

Figure 1. Selected ion monitoring chromatograms of EAA and NEAA standards (125 μ*M*) obtained using (A) underivatized (UND) and (B) precolumn derivatized (DER) methods via liquid chromatography-mass spectrometry. The ^{12}C ions (mass-to-charge ratio) monitored were as follows. UND method: EAA: 175.2 (Arg), 156.2 (His), 132.2 (Ile and Leu), 147.2 (Lys), 150.2 (Met), 166.2 (Phe), 120.1 (Thr), 205.0 (Trp), and 118.2 (Val); NEAA: 90.2 (Ala), 133.0 (Asn), 134.1 (Asp), 147.0 (Gln), 148.1 (Glu), 76.3 (Gly), 116.1 (Pro), 106.1 (Ser), and 182.2 (Tyr). DER method: EAA: 303.0 (Arg), 370.0 (His), 260.0 (Ile and Leu), 361.0 (Lys), 278.0 (Met), 294.0 (Phe), 248.0 (Thr), 333.0 (Trp), and 246.0 (Val); NEAA: 218.0 (Ala), 243.0 (Asn), 304.0 (Asp), 275.0 (Gln), 318.0 (Glu), 204.0 (Gly), 244.0 (Pro), 234.0 (Ser), and 396.0 (Tyr); In addition, isotopes (AA-^{13}C-^{15}N or ^{13}C) for each AA were monitored as the internal standards. The UND method provides 16 AA. The AA Ala, Thr, and Ser had ^{12}C AA area signal sensitivity lower than 15,000 V/min; peaks are magnified in the upper part of the figure. Poor linearity ($R^2 \leq 0.990$) or peak resolution problems were found for Asp, Gly, Tyr, and Ser using the UND method. Peaks with a slightly different color eluting at a similar time are the labeled internal standards of the same AA.

1961; Bibby and Toutenburg, 1977) using the software Model Evaluation System (http://nutritionmodels.tamu.edu/mes.html) as described by Tedeschi (2006). In this study, the MSEP is the sum squared difference between UND observed values and model-predicted values divided by the number of samples analyzed in the linear regression.

Linearity of AA to *m*/*z* and ^{12}C signal sensitivity (Table 1) were evaluated for each AA with duplicate samples of 5-point standard curves with a range of 1.1 to 500 μ*M*, and the points were selected for each AA based on physiological ranges observed in dairy cows (Patton et al., 2015). Both methods showed linearity within the dynamic range analyzed for all EAA ($R^2 \geq 0.995$). For NEAA, UND samples had poor linearity ($R^2 < 0.995$) or resolution problems (signal-to-noise ratio <3 or poor peak resolution) for Asp, Gly, Tyr, and Ser; however, all NEAA showed linearity using the DER method. In addition, the UND method had some AA with an intercept that was significantly different from zero ($P \leq 0.05$; Met, Val, Pro, Asn, Pro, and Ser). Moreover, signal sensitivity of ^{12}C AA was much greater for DER samples than for UND samples. It is worth noting that injection volume was 5 μL using the UND

method, whereas only 1 µL was adequate for the DER method, as indicated by the manufacturer's instructions. Regardless of injection volume, the DER method had greater area signal sensitivity than the UND method, ranging from 305.9× (Ala) to 2.2× (Pro). (Fold increase in signal sensitivity, DER divided by UND, was calculated for the lowest concentration on the standard curve for each AA.) Of particular interest, Met, Lys, and His had 6.2×, 16.5×, and 60.7× greater area signal sensitivity, respectively. However, most of these AA with lower signal sensitivity showed linearity for *m*/*z* using the UND method ($R^2 \geq 0.995$).

The precision and accuracy of both methods were evaluated by calculating the CV for the pooled plasma samples (standard deviation divided by mean) and the recovery rate for each AA by spiking 2 different concentrations of AA (low and high), selected to be near the physiological range for each AA. The UND method had a CV ranging from 0.9% (Val) to 7.8% (His), whereas for the DER method the range was 2.2% (Glu) to 8.8% (Asp). The UND method showed recovery rates ranging from 85.7% to 119.8%. The majority of the EAA and NEAA had recovery rates that did not differ from 100%, except for Val ($P = 0.02$), with the spiking of 50 µ*M* (119.8%), and Gln, which showed lower recovery rates (85.7% for 20 µ*M*, $P < 0.01$; and 87.6% for 50 µ*M*, $P < 0.01$). On the other hand, the DER method showed recovery rates ranging from 90.4% (Ser) to 113.7% (Leu), and none of the EAA or NEAA had recovery rates that differed from 100% ($P > 0.05$).

Comparison of the 2 methods for the 24 plasma samples using linear regression analysis resulted in 11 AA (15 AA were compared) with $r \geq 0.90$. Similarly, 9 AA had CCC ≥ 0.90. Most of the EAA showed minimal mean bias as well as agreement between methods (Table 2). Among these, Arg, Ile, Leu, Met, Thr, Trp, and Val were comparable between methods ($r > 0.90$; CCC > 0.90). However, the least comparable EAA were His (r = 0.46; CCC = 0.45) and Lys (r = 0.76; CCC = 0.75). The regression analysis of His and Lys showed that a greater percentage of MSEP was due to SB (30.9 and 22.7%, respectively) than to MB (5.1 and 3.5%, respectively). Among NEAA, only 2 AA (only 5 were available for comparison due to no linearity or peak resolution of the UND method) showed $r \geq 0.90$ and CCC ≥ 0.90 (Asn and Gln; Table 2), whereas Ala (r = 0.83; CCC = 0.73), Glu (r = 0.65; CCC = 0.42), and Pro (r = 0.90; CCC = 0.86) were less comparable between methods. The MSEP decomposition showed a greater percentage of MB than SB for these AA, except for Glu. The regression analysis and coefficients indicated lack of precision and accuracy between the 2 methods for these AA.

The discrepancy between methods for some AA may be explained by multiple factors. Besides the derivatization step, the DER method involves a step of sample precipitation and 2 sample extraction phases (solid and liquid-liquid; Phenomenex, 2005; Fonteh et al., 2007; Badawy et al., 2008; Dziagwa-Becker et al., 2015). This results in the removal of most compounds such as phospholipids and small peptides or other molecules that can interfere with ionization, detection, or both. In addition, it is well known that derivatization improves selectivity and signal sensitivity (Zhu et al., 2015). The UND method involves only a precipitation and filtration step, which may not remove all interfering compounds from the plasma matrix. This results in a much higher noise level and elevated baseline, which was also evidenced by the signal sensitivity differences between the methods (see chromatogram in Figure 1). For the UND method, we observed differences in peak shape using bovine plasma, and there were compounds that eluted with similar *m*/*z* in the same chromatogram segment, or coeluting compounds with a different *m*/*z* but eluting near or at a similar time, indicating interference. Nevertheless, linearity to *m*/*z* and recovery rates were accurate for most AA even though there was lower signal sensitivity for UND samples compared with DER samples. Several studies have reported the effect of matrix on ion suppression (Cappiello et al., 2008; Furey et al., 2013). It is possible that some AA are more affected by ion suppression than others. In addition, it is worth noting that a single-quadrupole mass analyzer was used for this study rather than triple-quadrupole tandem MS; the triple-quadrupole tandem MS has a greater ability to differentiate compounds that are being measured from interfering compounds in the sample matrix, resulting in improved signal sensitivity (Dietzen et al., 2008; Pitt, 2009; Grebe and Singh, 2011). Future investigation, potentially using more intense sample preparation techniques or more sensitive mass analyzers such as triple-quadrupole tandem MS, is warranted to optimize UND methods.

In conclusion, we evaluated and compared 2 methods for quantification of AA in bovine plasma. Both had linearity to *m*/*z*, precision, and accurate recovery rates for most EAA despite the lower signal sensitivity observed with the UND method. Nevertheless, some NEAA had poor peak resolution or linearity for *m*/*z*. Most EAA had high correlation coefficients and CCC and minimal mean bias, and, in general, the two methods were in agreement and were comparable. In contrast, NEAA evaluated using the UND method were generally less comparable with the values obtained with the DER method. The DER method required more sample processing including derivatization to achieve greater signal sensitivity, resulting in greater costs than using the UND method. Thus, the UND method may be a cost-effective, high-throughput, and practical alternative for analysis of AA in dairy cattle, particularly if only EAA values are required.

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Notes

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