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Original Article

Gas chromatography–mass spectrometry-based quantitative method using automated solid-phase analytical derivatization for plasma levels of methylmalonic acid, total homocysteine, and related metabolites in Japanese Black cattle

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Summary

High plasma levels of methylmalonic acid and total homocysteine are markers for vitamin B_{12} deficiency, whereas high plasma levels of total homocysteine indicate folate deficiency in animals, including cattle. Although a combination of solid-phase extraction and derivatization is effective for analyzing plasma methylmalonic acid and total homocysteine, these procedures can be time-consuming, complicating large-scale analyses. To address this, we focused on automated solid-phase analytical derivatization (SPAD), because both solid-phase extraction and derivatization can be conducted automatically in a short timeframe. In the present study, we evaluated a gas chromatography–mass spectrometry-based quantitative method utilizing automated SPAD to determine plasma levels of methylmalonic acid, total homocysteine, and related metabolites in Japanese Black cattle. Compared to manual derivatization without solid-phase extraction, the SPAD demonstrated superior analytical quality for the quantification of methylmalonic acid, total homocysteine, and some related metabolites, possibly because of solid-phase extraction. Additionally, the SPAD requires less time. These results suggest that the presented quantitative method has the potential to precisely and practically evaluate folate and vitamin B_{12} deficiencies in Japanese Black cattle.

Introduction

In animals, high plasma levels of methylmalonic acid and total homocysteine are markers of vitamin B_{12} deficiency, whereas high plasma levels of total homocysteine are indicative of folate deficiency. These markers are particularly useful in cattle, as both vitamins are important not only for cattle health but also for optimal productive performance[1,2]. Some studies have used these markers to evaluate the nutritional status of these vitamins in cattle[1,2]. However, to the best of our knowledge, no previous research has employed both markers in Japanese Black cattle.

For the analyses of methylmalonic acid and total homocysteine, some studies have used a combination of solid-phase extraction and derivatization to achieve precise quantification[3,4]. However, these pretreatment processes can be time-consuming, posing challenges for large-scale analyses. To address this issue, we focused on solid-phase analytical derivatization (SPAD), which allows both solid-phase extraction and derivatization to be conducted in a short timeframe[5]. In addition, a recent study has demonstrated that this process can be automated[6]. Therefore, in the present study, we evaluated a gas chromatography–mass spectrometry (GC/MS)-based quantitative method that utilizes automated SPAD to determine plasma levels of methylmalonic acid, total homocysteine, and related metabolites in Japanese Black cattle. Our goal was to compare the analytical quality of this SPAD method against manual derivatization without solid-phase extraction.

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Among the related metabolites, we selected tricarboxylic acid (TCA) cycle metabolites and total cysteine, as their levels could provide valuable insights into metabolism when interpreting the results for methylmalonic acid and total homocysteine. For derivatization, we chose *tert*-butyldimethylsilyl (TBDMS) derivatization because we have previously confirmed its usefulness in quantifying plasma metabolite levels (including some amino acids and related metabolites other than the metabolites investigated in the present study) in Japanese Black cattle[7]. For the solid-phase extraction, we employed two kinds of solid-phase extraction cartridges: an AX solid-phase, containing a polymer-based resin with quaternary alkylammonium functional groups, for the analysis of organic acids (methylmalonic acid and TCA cycle metabolites); and a CX solid-phase cartridge, containing a polymer-based resin with sulfonic acid functional groups, for the analysis of amino acids[6].

Materials and methods

Plasma of Japanese Black cattle

Plasma samples were collected from 18 Japanese Black cattle at 18, 20, 22, 24, 26, and 28 months of age. The plasma from each age group was pooled in equal proportions, dispensed into aliquots, and stored at −80℃ until analysis, serving as the quality control (QC) sample. All animal experiments were approved by the Animal Care Committee of the ZEN-NOH Central Research Institute for Feed and Livestock (Tsukuba, Ibaraki, Japan) under experimental number 2024-K6. Additionally, animal experiments were conducted in accordance with the ethical guidelines of the Institute of Livestock and Grassland Science.

External and internal standards

The external standards used in the present study included methylmalonic acid, homocysteine, and related metabolites (TCA cycle organic acids and cysteine). Methylmalonic acid, succinic acid, fumaric acid, L(-)-malic acid, L-cysteine hydrochloride monohydrate, and DL-homocysteine were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). α-Ketoglutaric acid and DL-isocitric acid trisodium salt hydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Citric acid monohydrate was purchased from NACALAI TESQUE, INC (Osaka, Japan).

The internal standards consisted of stable isotopes of selected metabolites. Methylmalonic acid (methyl-D_3, 98%) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). DL-Homocystine-3,3,3′,3′,4,4,4′,4′-d8 was purchased from C/D/N Isotopes, Inc (Pointe-Claire, Quebec, Canada). Stable isotope labeled organic acid mixture for mass spectrometry (including α-ketoglutaric acid-$^{13}C_5$, citric acid-$^{13}C_6$, fumaric acid-$^{13}C_4$, L-malic acid-$^{13}C_4$, and succinic acid-$^{13}C_4$) was purchased from Taiyo Nippon Sanso Corporation (Tokyo, Japan).

Pretreatment for automated SPAD

The pretreatment process for automated SPAD, used to analyze methylmalonic acid and TCA cycle metabolites, was carried out following previously established methods in the previous studies[8,9] with some modifications. A mixture consisting of 100 μL of plasma or standard solution (methylmalonic acid, succinic acid, fumaric acid, malic acid, α-ketoglutaric acid, and citric acid), 20 μL of internal standard mixture (comprising of 33.3 μM of methyl-d_3-malonic acid mixture and 8 μM of stable isotope - labeled internal standard mixture for TCA cycle metabolites), 80 μL of water, and 800 μL of acetonitrile was shaken at 1,200 rpm for 30 min at 37℃. The sample was centrifuged at 20,000 × g for 3 min at 4 ℃. Subsequently, 500 μL of the supernatant was collected, mixed with 500 μL of water, and adjusted to pH 7.0 using 0.1 N NaOH.

The pretreatment for automated SPAD to analyze total homocysteine and total cysteine was performed as follows: 100 μL of plasma sample or standard solution (homocysteine and cysteine), 20 μL of 50 μM internal standard (DL-homocystine-3,3,3',3',4,4,4',4'-d_8), 160 μL of water, 700 μL of acetonitrile, and 20 μL of 100 g/L Tris(2-carboxyethyl) phosphine hydrochloride (TCEP, Wako Pure Chemical Industries, Osaka, Japan) for disulfide bond reduction were shaken at 1,200 rpm for 30 min at 40℃. The sample was centrifuged at 20,000 × g for 3 min at 4℃, and 500 μL of supernatant was mixed with 500 μL of water and adjusted to pH 7.0 using 0.1 N NaOH.

GC/MS based quantitative analysis using automated SPAD

GC/MS analysis using automated SPAD was performed following a previously reported method[6] with some modifications. The sample preparation process, from extraction to injection, was automated using SGI-P100 and SPL-M100 (AiSTI Science, Wakayama, Japan). Solid-phase cartridges (Flash-SPE AXs and Flash-SPE CXs, containing 5 mg of adsorbent) were purchased from AiSTI SCIENCE. The AX solid phase, used for analysis of organic acids, contains a polymer-based resin with quaternary alkylammonium functional groups, while the CX solid phase, used for analysis of amino acids and amines, contains a polymer-based resin with sulfonic acid functional groups. In the present

study, we used Flash-SPE AXs for analysis of methylmalonic acid and TCA cycle metabolites, while and Flash-SPE CXs was used for the analysis of homocysteine and cysteine. The solid-phase cartridge was conditioned with 250 μL of 50% (v/v) acetonitrile in ultra-pure water (flow rate: 20 μL/s). Next, the AX cartridge was activated with 50 μL of 0.1 mol/L NaOH solution (flow rate: 5 μL/s), and the CX cartridge was activated with 50 μL of 0.1 mol/L HCl. Then, the following sequence was passed through the cartridge: 250 μL of ultra-pure water (flow rate: 10 μL/s), 250 μL of 50% (v/v) acetonitrile in ultra-pure water (flow rate: 20 μL/s), 50 μL of the sample (flow rate: 3 μL/s), 200 μL of 50% acetonitrile in ultra-pure water (flow rate: 10 μL/s), and 450 μL of acetonitrile (flow rate: 20 μL/s). After drying the resin in the cartridge using nitrogen gas, 3 μL of 5 g/L methoxyamine hydrochloride (MPBio, Tokyo, Japan) dissolved in pyridine was added, and the cartridge was left for 1 min for oximation. The resin was dried using nitrogen gas, followed by the addition of 10 μL of 75% (v/v) TBDMS (Thermo Scientific, Waltham, MA, USA) in toluene for TBDMS derivatization, and the cartridge was left for 1 min. Finally, the analytes were eluted using 25 μL of toluene (flow rate: 2 μL/s) and directly injected into the GC/MS system (GCMS-TQ8050, Shimadzu, Kyoto, Japan). A VF-5 MS column (30 m × 0.25 mm, i.d.; film thickness 0.25 μm; Agilent, Tokyo, Japan) was used for GC separation. The temperature program for the GC column was as follows: maintain an initial temperature of 60℃ for 2 min, then increase to 330℃ at a rate of 15℃/min, and finally hold at 330℃ for 3 min, resulting in a total GC run time of 23 min. The inlet temperature was maintained at 280℃, and helium was used as the carrier gas at a constant flow rate of 39.0 cm/s. Samples were injected in splitless mode, and the mass conditions were set as follows: ionization voltage, 70 eV; ion source temperature, 200℃; full scan mode in an m/z range 50–600, with a scan interval of 0.3 s. Chromatogram acquisition, mass spectral peak detection, and waveform processing were performed using GC/MS solution software (Shimadzu). Quantitative and qualitative ions for each metabolite were selected based on their specificity and analytical conditions for the specific ion monitoring (SIM) mode, which was used for quantification. In addition, the retention index was calculated based on the retention time of n-alkanes (hydrocarbon mixed solution; GL Sciences Inc., Tokyo, Japan) injected in the same run, aiding in the identification of metabolites. To confirm the detection of methylmalonic acid, homocysteine, and related metabolites in cattle plasma, the degree of chromatographic separation of the selected ions, the intensity ratio of quantitative and qualitative ions, and the linearity of the calibration curves for each metabolite was examined. For quantification, the peak areas of ions were calculated and normalized to that of the internal standard.

GC/MS based quantitative analysis using manual derivatization

To evaluate the analytical quality of the present method, a comparison was made with GC/MS-based quantitative analysis using manual derivatization. Manual derivatization was performed according to a previously reported method[6] with minor modifications.

For the analysis of methylmalonic acid and TCA cycle metabolites, 50 μL of plasma sample or standard solution mixture (methylmalonic acid, succinic acid, fumaric acid, malic acid, α-ketoglutaric acid, and citric acid) was combined with 10 μL of internal standard mixture (33.3 μM of methyl-d_3-malonic acid mixture and 8 μM of stable isotope - labeled internal standard mixture for TCA cycle metabolites), 40 μL of water, and 400 μL of acetonitrile.

For the analysis of total homocysteine and total cysteine, 50 μL of plasma sample or standard solution mixture (homocysteine and cysteine) was combined with 10 μL internal standard (DL-homocystine-3,3,3',3',4,4,4',4'-d_8), 10 μL of 100 g/L TCEP for disulfide bond reduction, 30 μL of water, and 400 μL acetonitrile. This mixture was shaken at 1,200 rpm for 30 min at 40℃.

Both types of mixtures were vortexed and centrifuged at 20,000 × *g* for 5 min at 4 ℃. Then, 400 μL of the solution was collected, centrifugally evaporated to dryness for 5 h after freezing at −80℃ for 1 h. For oximation, 50 μL of 40 mg/mL methoxyamine hydrochloride dissolved in pyridine was mixed with the dried sample, followed by shaking at 800 rpm for 60 min at 30℃. Next, 50 μL of N-*tert*-butyldimethylsilyl-N-methyltrifluoroacetamide (Thermo Scientific, Waltham, MA, USA) was added for TBDMS derivatization and incubated at 800 rpm for 60 min at 60℃. The resulting supernatant was subjected to GC/MS analysis (GCMS-QP2010 Ultra; Shimadzu, Kyoto, Japan). A DB-5 column (30 m × 0.25 mm, i.d.; film thickness 1.00 μm; Agilent, Tokyo, Japan) was employed for GC separation. The GC column temperature was programmed as follows: maintain an initial temperature of 100 ℃ for 4 min, then increase to 320℃ at a rate of 11℃/min, and finally maintain at 320℃ for 11 min, resulting in a total GC run time of 37 min. The inlet temperature was maintained at 280℃, with helium as the carrier gas at a constant flow rate of 39.0 cm per second. A sample volume of 1.0 μL was injected in splitless mode. The mass spectrometry conditions were as follows: ionization voltage, 70 eV; ion source

temperature, 200℃; full scan mode within an m/z range 50–600, with a scan interval of 0.3 s. Chromatogram acquisition, mass spectral peak detection, and waveform processing were performed using Shimadzu GC/MS solution software (Shimadzu). In addition, the retention index was calculated based on the retention time of n-alkanes (hydrocarbon mixed solution; GL Sciences Inc., Tokyo, Japan) injected in the same run. For SIM mode, the same quantitative and qualitative ions used in the SPAD method were employed. This mode was used for quantification. To confirm the detection of methylmalonic acid, homocysteine, and related metabolites in cattle plasma, we examined the degree of chromatographic separation of the selected ions, the intensity ratio of quantitative and qualitative ions, and the linearity of the calibration curves for each metabolite. For quantification, the peak area of each ion was calculated and normalized to that of the internal standard.

Intra-day repeatability, inter-day reproducibility of assay, and spike recovery rate

The intra-day repeatability and inter-day reproducibility of the assay were investigated for metabolites detected in plasma. QC samples were analyzed three times a day over three separate days. The same analytical conditions, as described in the previous section for the SPAD, were applied. The relative standard deviation was deemed acceptable at less than 20%, following guidelines from a previous untargeted metabolomics study on Japanese Black cattle[7].

To assess the spike recovery rates of the SPAD method, spiked metabolites were divided into three groups: low, medium, and high, based on their quantified levels. For the analysis of methylmalonic acid and related metabolites using the SPAD, the following were used: 100 μL of plasma sample or standard solution mixture (methylmalonic acid, succinic acid, fumaric acid, malic acid, α-ketoglutaric acid, and citric acid), 100 μL of the spiked mixture of standards, 20 μL of internal standard mixture (33.3 μM of methyl-d_3-malonic acid mixture and 8 μM of stable isotope - labeled internal standard mixture for TCA metabolites), and 780 μL of acetonitrile. For the analysis of homocysteine and cysteine using the SPAD, 100 μL of plasma sample or standard solution mixture (homocysteine and cysteine), 100 μL of the spiked mixture of standards, 20 μL of 50 μM internal standard (DL-homocystine-3,3,3',3',4,4,4',4'-d_8), 60 μL of water, 700 μL of acetonitrile, and 20 μL of 100 g/L TCEP were used for disulfide bond reduction. The subsequent pretreatment and analytical conditions were the same as those described in the previous section. The acceptable recovery rates in this study ranged from 80 to 120%.

Similarly, the spike recovery rates for the manual derivatization method were assessed using the SPAD quantifications. Spiked metabolites were also divided into low, medium, and high. For the analysis of TCA metabolites and methylmalonic acid, 50 μL of plasma sample or standard solution mixture (methylmalonic acid, succinic acid, fumaric acid, malic acid, α-ketoglutaric acid, and citric acid), 50 μL of the spiked mixture of standard, 10 μL of internal standard mixture (33.3 μM of methyl-d_3-malonic acid and 8 μM of stable isotope - labeled internal standard mixture for TCA cycle metabolites), and 390 μL of acetonitrile were used. For the analysis of homocysteine and cysteine, 50 μL of plasma sample or standard solution mixture (homocysteine and cysteine), 50 μL of the spiked mixture of standard, 10 μL internal standard (DL-homocystine-3,3,3',3',4,4,4',4'-d_8), 10 μL of 100 g/L TCEP for disulfide bond reduction, 30 μL of water, and 400 μL acetonitrile were used. The subsequent pretreatment and GC/MS analytical conditions were the same as those described in the previous sections. The acceptable recovery rates in the present study ranged from 80 to 120%.

SPAD to manual derivatization ratio

We calculated the SPAD to manual derivatization ratio using the mean values obtained by both methods. The acceptable ratio in the present study ranged from 80 to 120%.

Statistical analysis

All statistical analyses were performed using Microsoft MS-Excel (ver. 2306).

Results

Confirmation of analytical conditions of methylmalonic acid, homocysteine, and related metabolites

Methylmalonic acid, homocysteine, and related metabolites were detected using both SPAD and manual derivatization methods. The analytical conditions used are detailed in Table 1. For both derivatization methods, the retention times and mass spectra of eight external and seven internal standards were confirmed. The derivatization types, as well as quantitative and qualitative ions, were also determined. Additionally, the retention index for each metabolite was calculated based on the retention time of the n-alkanes analyzed during the same run.

The calibration curves for quantification are presented in Table 2. In the SPAD method, the calibration curves for all metabolites showed acceptable linearity ($R^2 > 0.99$). However, in manual derivatization, the calibration curve

for succinic acid showed poor linearity (R^2 = 0.981).

Confirmation of GC/MS chromatogram peak of each metabolite in bovine plasma

GC/MS chromatogram peak of each metabolite in bovine plasma was confirmed based on analytical conditions shown in the previous paragraph. Among all peaks detected, based on retention time and index, only the peak of methylmalonic acid in bovine plasma was not the same as that of its own standard in manual derivatization (Fig. 1 A and 1B). On the other hand, retention time and index of bovine methylmalonic acid in SPAD is identical to those of

Table 1. Mass spectrometry analytical conditions, retention time, and retention index for methylmalonic acid, total homocysteine, and related metabolites

Metabolite Name	Derivatization type	Quantitative Ion (*m/z*)	Qualitative Ion (*m/z*)	Internal Standard	SPAD		Manual derivatization	
					Retention time	Retention index	Retention time	Retention index
Organic acids								
Methylmalonic acid	2TBDMS	289	189	Methyl-d_3-malonic acid	11.123	1646	17.533	1668
Methyl-d_3-malonic acid	2TBDMS	292	192		11.123	1646	17.533	1668
Succinic acid	2TBDMS	289	189	Succinic acid-$^{13}C_4$	11.915	1753	18.078	1766
Succinic acid-$^{13}C_4$	2TBDMS	293	191		11.915	1753	18.078	1766
Fumaric acid	2TBDMS	287	329	Fumaric acid-$^{13}C_4$	12.138	1784	18.326	1788
Fumaric acid-$^{13}C_4$	2TBDMS	291	333		12.138	1784	18.326	1788
α -Ketoglutaric acid	MO-TBDMS	346	258	*α*-Ketoglutaric acid-$^{13}C_5$	13.565	1996	20.663	2011
α -Ketoglutaric acid-$^{13}C_5$	MO-TBDMS	351	263		13.565	1996	20.663	2011
Malic acid	3TBDMS	419	187	Malic acid-$^{13}C_4$	14.137	2088	21.751	2125
Malic acid-$^{13}C_4$	3TBDMS	423	191		14.137	2088	21.751	2125
Citric acid	4TBDMS	459	431	Citric acid-$^{13}C_6$	16.861	2582	26.118	2642
Citric acid-$^{13}C_6$	4TBDMS	465	437		16.861	2582	26.118	2642
Isocitric acid	4TBDMS	405	363	(not quantified)	16.920	2594	26.318	2655
Amino acids								
Cysteine	3TBDMS	302	378	Homocystine-3,3,3′,3′,4,4,4′,4′-d_8	14.719	2179	22.732	2232
Homocysteine	3TBDMS	392	420	Homocystine-3,3,3′,3′,4,4,4′,4′-d_8	15.449	2308	23.844	2360
Homocystine-3,3,3′,3′,4,4,4′,4′-d_8	3TBDMS	396	424		15.449	2317	23.844	2360

SPAD: solid-phase analytical derivatization

Table 2. Comparison of calibration curve between two derivatization methods

Metabolite Name	SPAD		Manual derivatization	
	Regression equation	Coefficient of determination (R^2)	Regression equation	Coefficient of determination (R^2)
Organic acids				
Methylmalonic acid	y = 0.144 x + 0.142	0.9998	y = 0.1495 x + 0.0529	0.9993
Succinic acid	y = 0.473 x + 5.4931	0.9993	y = 0.395 x + 27.3654	0.9819
Fumaric acid	y = 0.514 x + 0.7469	0.9985	y = 0.454 x + 2.4807	0.9940
α-Ketoglutaric acid	y = 0.484 x + 0.1386	0.9998	y = 0.57 x + 0.5733	0.9991
Malic acid	y = 0.505 x + 0.7707	0.9995	y = 0.394 x + 1.4994	0.9947
Citric acid	y = 0.869 x + 2.0242	0.9999	y = 0.657 x + 2.1549	0.9990
Amino acids				
Cysteine	y = 0.106 x + 5.5657	0.9921	y = 0.09 x + 1.3913	0.9943
Homocysteine	y = 0.057 x + 0.2954	0.9992	y = 0.037 x + 0.0086	0.9999

SPAD: solid-phase analytical derivatization

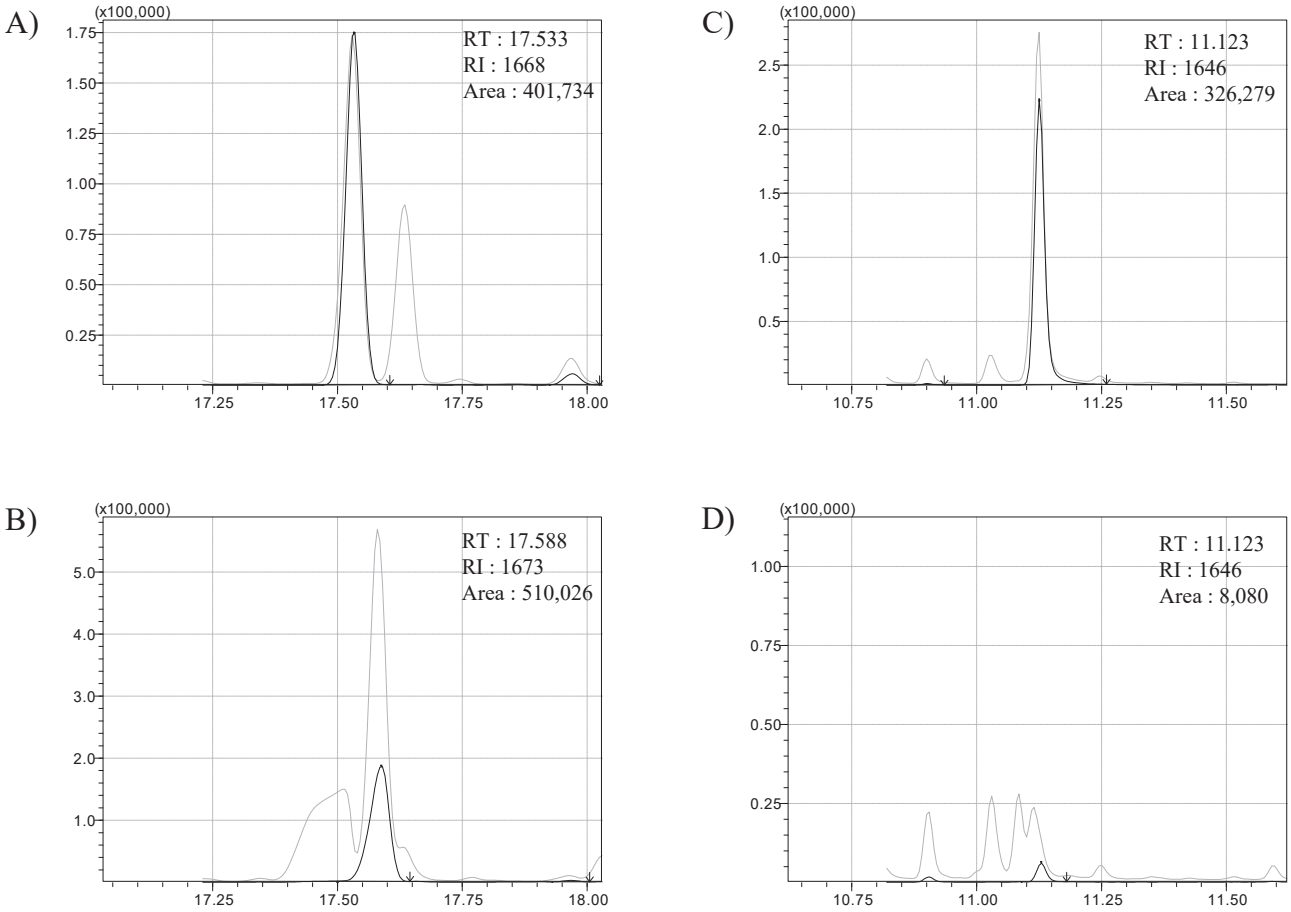

Fig. 1. Comparison of GC/MS Chromatogram peak of methylmalonic acid between two methods
Black: Quantitative Ion (m/z: 289), Gray: Qualitative Ion (m/z: 189).
A: Chromatogram peak of methylmalonic acid standard (50 μM) in manual derivatization. B: Chromatogram peak of methylmalonic acid in bovine plasma in manual derivatization. C: Chromatogram peak of methylmalonic acid standard (50 μM) in SPAD. D: Chromatogram peak of methylmalonic acid in bovine plasma in SPAD. RT: Retention time, RI: Retention index.

the standard (Fig. 1 C and 1D). Therefore, we judged that methylmalonic acid in the manual derivatization was not enough for the quantification and should be excluded for further evaluation.

Intra-day repeatability, inter-day reproducibility, and spiked recovery rate

The intra-day repeatability and inter-day reproducibility for methylmalonic acid, total homocysteine, and related

metabolites quantified in bovine plasma are shown in Table 3. Using the SPAD method, all eight metabolites, including methylmalonic acid and total homocysteine, in bovine plasma demonstrated acceptable results. In manual derivatization, the levels of three metabolites (total homocysteine, total cysteine, and citric acid) met acceptable criteria.

The spiked recovery rates for methylmalonic acid, total

Table 3. Comparison of intra-day repeatability and inter-day reproducibility between two derivatization methods

Metabolite Name	Intra-day repeatability								Inter-day reproductivity		SPAD to manual derivatization ratio
	Day 1		Day 2		Day 3						
	Measured concentration (μmol/L)	RSD (%)	Measured concentration (μmol/L)	RSD (%)	Measured concentration (μmol/L)	RSD (%)	Measured concentration (μmol/L)	RSD (%)			
SPAD											
Methylmalonic acid	1.25 ± 0.05	7.45	1.27 ± 0.05	7.47	1.41 ± 0.02	2.77	1.31 ± 0.04	5.24	-		
Succinic acid	7.29 ± 0.35	8.38	11.91 ± 1.34	19.45	9.76 ± 0.90	16.03	9.65 ± 1.09	19.57	0.45		
Fumaric acid	0.91 ± 0.04	8.04	1.13 ± 0.01	1.01	1.01 ± 0.02	2.71	1.02 ± 0.05	8.81	0.12		
α-Ketoglutaric acid	20.76 ± 0.49	4.07	21.91 ± 1.29	10.16	17.51 ± 0.48	4.75	20.06 ± 1.08	9.29	0.91		
Malic acid	3.89 ± 0.15	6.74	3.59 ± 0.09	4.33	4.34 ± 0.05	1.83	3.94 ± 0.18	7.82	2.05		
Citric acid	133.22 ± 1.06	1.38	149.30 ± 1.18	1.37	144.17 ± 1.96	2.35	142.23 ± 3.87	4.72	0.83		
Total cysteine	115.92 ± 4.74	7.09	140.53 ± 3.19	3.94	101.44 ± 3.59	6.13	119.30 ± 9.32	13.53	1.03		
Total homocysteine	6.21 ± 0.12	3.24	6.54 ± 0.10	2.62	6.45 ± 0.09	2.41	6.40 ± 0.08	2.21	0.65		
Manual derivatization											
Methylmalonic acid	-		-		-		-		-		
Succinic acid	20.38 ± 2.47	21.00	25.56 ± 2.99	20.23	17.83 ± 1.20	11.64	21.26 ± 1.86	15.13	-		
Fumaric acid	7.59 ± 0.35	8.06	12.49 ± 1.07	14.84	4.73 ± 0.64	23.49	8.27 ± 1.85	38.77	-		
α-Ketoglutaric acid	19.99 ± 0.39	3.37	15.31 ± 0.16	1.80	30.81 ± 0.53	2.96	22.04 ± 3.75	29.45	-		
Malic acid	3.30 ± 0.10	5.43	1.80 ± 0.27	26.07	0.66 ± 0.04	10.67	1.92 ± 0.62	56.27	-		
Citric acid	168.69 ± 4.34	4.46	185.41 ± 0.81	0.76	159.49 ± 5.54	6.01	171.20 ± 6.19	6.27	-		
Total cysteine	114.78 ± 2.98	4.50	117.35 ± 3.33	4.92	115.23 ± 8.07	12.13	115.79 ± 0.65	0.97	-		
Total homocysteine	9.37 ± 0.19	3.60	9.22 ± 0.21	3.86	10.99 ± 0.88	13.82	9.86 ± 0.46	8.12	-		

SPAD: solid-phase analytical derivatization

Table 4. Comparison of spiked recovery rate between two derivatization methods

Metabolite Name	Measured concentration (μmol/L)	Added concentration			Recovery rate		
		Low concentration (μmol/L)	Middle concentration (μmol/L)	High concentration (μmol/L)	Low Recovery rate (%)	Middle Recovery rate (%)	High Recovery rate (%)
SPAD							
Methylmalonic acid	1.31	1	2	4	81.57	83.68	100.27
Succinic acid	9.65	3	5	10	53.01	72.63	102.24
Fumaric acid	1.02	0.5	1	2	84.73	102.74	112.61
α-Ketoglutaric acid	20.06	15	30	60	94.84	94.56	115.07
Malic acid	3.94	2	4	8	107.87	111.75	103.04
Citric acid	142.23	75	150	300	102.28	106.96	104.41
Total cysteine	119.30	62.5	125	250	123.19	110.04	97.35
Total homocysteine	6.40	3	6	12	108.64	116.59	112.06
Manual derivatization							
Methylmalonic acid	-		-			-	
Succinic acid	21.26	3	5	10	< 0	8.95	102.24
Fumaric acid	8.27	0.5	1	2	392.01	550.06	515.13
α-Ketoglutaric acid	22.04	15	30	60	108.21	111.90	101.24
Malic acid	1.92	2	4	8	57.85	73.21	79.31
Citric acid	171.20	75	150	300	108.97	111.36	92.19
Total cysteine	115.79	62.5	125	250	95.34	105.90	116.58
Total homocysteine	9.86	3	6	12	67.47	85.29	94.34

SPAD: solid-phase analytical derivatization

homocysteine, and related metabolites quantified in bovine plasma are shown in Table 4. In the SPAD, six among eight metabolites (methylmalonic acid, total homocysteine, fumaric acid, α-ketoglutaric acid, malic acid, and citric acid) were acceptable across all spiked levels. For manual derivatization, three metabolites (α-ketoglutaric acid, citric acid, and total cysteine) were acceptable at all spiked levels.

SPD to manual derivatization ratio

The SPD to manual derivatization ratios for methylmalonic acid, total homocysteine, and related metabolites in

bovine plasma are shown in Table 3. Only three metabolites (α-ketoglutaric acid, citric acid, and total cysteine) had an acceptable ratio, the others being either not calculable (methylmalonic acid) or out of range (succinic acid, fumaric acid, malic acid, and total homocysteine).

Discussion

The present study could quantify plasma levels of methylmalonic acid and total homocysteine in Japanese Black cattle using the SPAD. Compared to manual derivatization without solid-phase extraction, the analytical quality for both methylmalonic acid and total homocysteine in bovine plasma was clearly improved and more acceptable. Given that the cattle in this study had adequate dietary cobalt (substrate of vitamin B_{12}) (data not shown), it is crucial to highlight that plasma levels of methylmalonic acid and total homocysteine obtained via SPAD align with those previously reported for cobalt sufficient beef cattle (plasma methylmalonic acid level < 2 μM and plasma total homocysteine level < 10 μM)[1]. Therefore, we speculated that quantification by the SPAD would be more reliable for detecting plasma levels of methylmalonic acid and total homocysteine in Japanese Black cattle.

Furthermore, for the eight related metabolites analyzed alongside methylmalonic acid and/or homocysteine, seven demonstrated superior analytical quality when using the SPAD. The remaining one, total cysteine, showed little difference in overall analytical quality, although it was partially inferior compared to manual derivatization. On the other hand, only three metabolites (α-ketoglutaric acid, citric acid, and total cysteine) had an acceptable SPD to manual derivatization ratio. SPD is partly different from manual derivatization in terms of quantitative value. Therefore, it can be concluded that the SPAD is basically better for quantifying the related metabolites of methylmalonic acid and homocysteine than manual derivatization.

The total analytical time is an important factor for practical applications. For example, to analyze 12 samples and 4 standards, the time required for pretreatment before GC/MS analysis in the SPAD (1 h) was shorter than the time for manual derivatization (9 h in total: 1 h for early-stage pretreatment, 1 h for freezing, 5 h for evaporation, and 2 h for manual derivatization). This time-saving advantage makes the SPAD highly beneficial for large-scale analyses.

In conclusion, the present quantitative method has the potential to precisely and practically evaluate nutritional deficiencies of folate and vitamin B_{12} in Japanese Black cattle.

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