Mass Spectrometry
DOI: 10.5702/massspectrometry.A0151

Original Article

Determination of Choline-Containing Compounds in Rice Bran Fermented with *Aspergillus oryzae* Using Liquid Chromatography/Tandem Mass Spectrometry

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Choline-containing compounds are essential nutrients for human activity, as they are involved in many biological processes, including cell membrane organization, methyl group donation, neurotransmission, signal transduction, lipid transport, and metabolism. These compounds are normally obtained from food. Fermented brown rice and rice bran with *Aspergillus oryzae* (FBRA) is a fermented food product derived from rice and rice ingredients. FBRA exhibits a multitude of functional properties with respect to the health sciences. This study has a particular focus on choline-containing compounds. We first developed a simultaneous liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis method for seven choline-containing compounds. The method was subsequently applied to FBRA and its ingredients. Hydrophilic interaction chromatography (HILIC) and selected reaction monitoring were employed for the simultaneous analysis of seven choline-containing compounds. MS ion source conditions were optimized in positive ion mode, and the product ions derived from the choline group were obtained through MS/MS optimization. Under optimized HILIC conditions, the peaks exhibited good shape without peak tailing. Calibration curves demonstrated high linearity across a 300- to 10,000-fold concentration range. The application of the method to FBRA and other ingredients revealed significant differences between food with and without fermentation. In particular, betaine and α-glycerophosphocholine were found to be highest in FBRA and brown rice malt, respectively. The results indicated that the fermentation processing of rice ingredients results in alterations to the choline-containing compounds present in foods. The developed HILIC/MS/MS method proved to be a valuable tool for elucidating the composition of choline-containing compounds in foods.

Please cite this article as: Mass Spectrom (Tokyo) 2024; 13(1): A0151

Keywords: choline-containing compounds, simultaneous analysis, HILIC/MS/MS, fermented brown rice and rice bran with *Aspergillus oryzae*

(Received May 13, 2024; Accepted July 19, 2024; advance publication released online July 31, 2024)

INTRODUCTION

Choline is an essential nutrient that plays a multitude of vital roles within the human body. It is primarily obtained from the human diet.[1–3] First, choline is essential for the biosynthesis of various phospholipids that are integral to maintaining the structural integrity of cell membranes.[4–7] Second, choline functions as a cellular messenger.[8,9] Third, choline is associated with the balance between phospholipids and cholesterol and thus plays a pivotal role in the synthesis of a substance essential for the removal of cholesterol from the liver.[1,2] Consequently, inadequate choline supplementation results in the accumulation of fat in the liver.[10–13] Choline, in conjunction with other vitamins, including B12 and folate, plays a role in a process that is crucial for DNA synthesis.[14,15] Choline is utilized in the

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synthesis of acetylcholine, a vital neurotransmitter.[16,17] Acetylcholine is also implicated in memory and is intimately associated with cognitive functions.[18,19] Choline combines with phospholipids and is metabolized into various phospholipids.[1,17,20] In the initial stage of the synthesis of choline-containing lipids, choline is converted to phosphocholine by choline kinase.[21] Phosphocholine is converted to phosphatidylcholine (PC) *via* cytidine diphosphate-choline and diacylglycerol. PC is degraded to lysophosphatidylcholine (LPC) by lipoprotein-associated phospholipase A2 or lecithin-cholesterol acyltransferase.[22–24] In addition, α-glycerophosphocholine is a metabolite that is deacylated by two fatty acids.[25] Sphingomyelin is a sphingolipid that is formed by the conversion of choline from PC.[26,27] In a manner analogous to glycerolipids, sphingomyelin is deacylated to lysosphingomyelin (LSM).[28] These lyso-types of phospholipids function as lipid mediators within the human body.[29–32] Conversely, choline is oxidized to betaine *via* betaine aldehyde.[33,34]

Choline is derived from a variety of dietary sources, including beef liver, chicken liver, salmon, and eggs.[1] A deficiency in dietary choline has been associated with an increased risk of developing various disorders, including non-alcoholic fatty liver disease, cardiovascular disease, and chronic kidney disease. Furthermore, it has been linked to impaired cognitive function and the development of associated neuropsychiatric disorders.[35]

Fermented brown rice and rice bran with *Aspergillus oryzae* (FBRA) is a multifunctional food that is thought to treat various diseases and is considered to be rich in various bioactive metabolites as well as fiber and carbohydrates.[36] Evidence has been presented that FBRA has the potential to confer health benefits, including anti-cancer and anti-inflammatory effects.[37–40] The fermentation process increases the concentration of various antioxidant phytochemicals in FBRA. Consequently, fermentation enhances bioactive compounds in rice bran, which may contribute to its antioxidant potential.[41] The bioactive components obtained from FBRA have been demonstrated to exert a range of biological activities.[38,42,43] In summary, FBRA is regarded as a functional food with the potential to confer health benefits, particularly in the context of cancer prevention and enhancement of antioxidant capacity.

From the background information available on FBRA, we postulated that choline-containing compounds in FBRA, which are produced by the fermentation of rice ingredients, might increase during the fermentation process. The objective of this preliminary study was to quantify the choline-containing compounds in rice and ingredients derived from rice, including FBRA. Choline is a quaternary ammonium ion that is continuously and positively charged. Choline-containing compounds are all highly polar compounds with molecular weights below 1,000. Therefore, the elution of these compounds in liquid chromatography presents significant challenges. To address this challenge, various columns have been employed in previous studies.[44–46] Hydrophilic interaction liquid chromatography (HILIC) is a more effective method for retaining and separating polar compounds than other chromatographic techniques.[47–49] The use of a HILIC column can facilitate the attainment of more optimal peak shapes and enhanced reproducibility for choline-containing compounds.[50–52] In this study, we developed a simultaneous analysis method for seven choline-containing compounds (Fig. 1) using HILIC-mass spectrometry/mass spectrometry (HILIC/MS/MS). The method was subsequently applied to FBRA and other ingredients to analyze the contents of choline-containing compounds.

MATERIALS AND METHODS

Chemicals

The following compounds were procured from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan): ammonium formate, betaine-$^2H_{11}$, choline-$^2H_9$, formic acid, and α-glycerophosphocholine. The choline was procured from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). The following compounds were procured from Merck KGaA (Darmstadt, Germany): acetylcholine chloride, betaine, phosphocholine chloride, and phosphocholine-$^2H_9$. Acetonitrile was procured from Kanto Kagaku (Tokyo, Japan). The following compounds were purchased from Avanti Polar Lipids, Co. Ltd. (Alabaster, AL, USA): α-glycerophosphocholine-$^2H_9$, LPC (sn-1/16:0), LSM, and LSM (d17:1). Ultrapure water was prepared using Puric-α (Organo Co. Ltd., Tokyo, Japan) and utilized in all experiments.

Liquid chromatography/tandem mass spectrometry (LC/MS/MS) equipment

The LC/MS/MS instrumentation was assembled using a QTRAP6500 (SCIEX, Framingham, MA, USA) quadrupole linear ion trap hybrid tandem mass spectrometer with an electrospray ionization (ESI) probe attached to the ion source and connected to an ultra-high-performance liquid chromatograph system (Nexera, Shimadzu, Kyoto, Japan). The analysis was conducted using LC/MS/MS in positive ion mode. The analytical software Analyst 1.6.2 (SCIEX) and the peak area integration software MultiQuant version 2.1.1 (SCIEX) were employed for the analysis and integration of peak areas, respectively. Statistical analysis was conducted using JMP Pro version 17.1 software (SAS Institute Inc., Cary, NC, USA).

Optimization of MS/MS conditions

The selected reaction monitoring (SRM) transitions were investigated using 1 μg/mL of each standard solution at a flow rate of 10 μL/min. The SRM parameters, namely the precursor ion (*m/z*) and product ion (*m/z*), declustering potential (DP), collision energy (CE), and cell exit potential (CXP), were optimized under SRM conditions. The ion source parameters in MS/MS were optimized through the use of a flow injection analysis of a standard mixture. The ion source parameters, including collision gas (CAD), curtain gas (CUR), turbo gas (GS1), nebulizer gas (GS2), turbo gas temperature (TEM), and ion spray voltage (ISV), were also optimized.

LC conditions

The mobile phases A and B were composed of 20 mM ammonium formate/formic acid (100:0.1, v/v) and acetonitrile, respectively, and were delivered in a gradient flow mode. The column utilized was an InertSustain Amide PEEK (2.1 mm i.d. × 150 mm, 1.9 μm, GL Sciences, Tokyo, Japan). The column temperature was set to 40°C. The gradient profile was configured as follows: B (%) 95→70→65→10→10, time (min); 0→4→8→8.1→15 min, with an equilibration period of 5 min at B 95%.

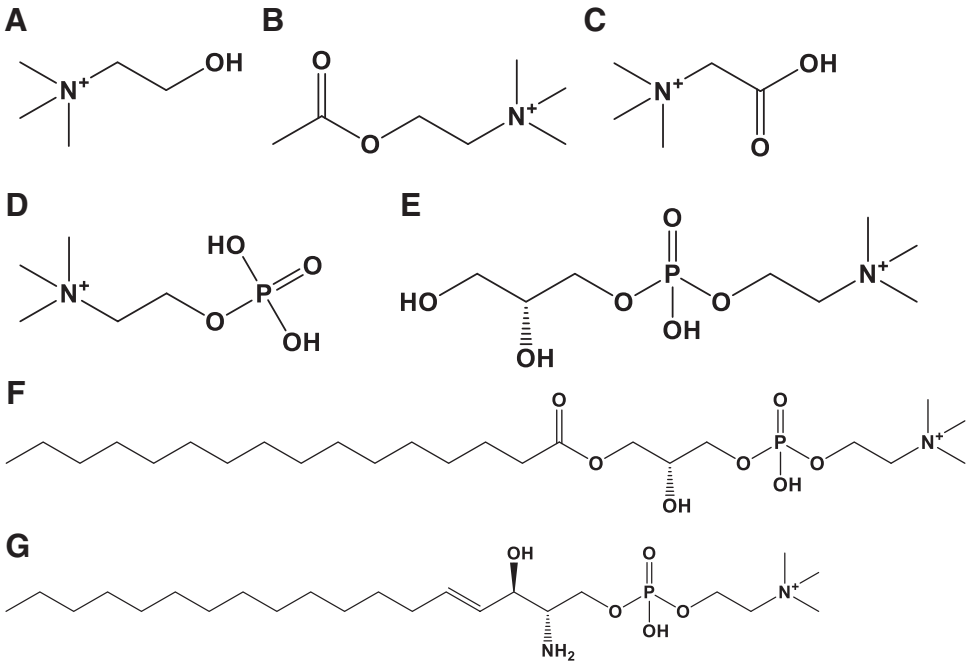

Fig. 1. Chemical structure of analytes. (A) choline, (B) acetylcholine, (C) betaine, (D) phosphocholine, (E) glycerophosphocholine, (F) lysophosphatidylcholine (*sn*-1, 16:0), and (G) lysosphingomyelin (d18:1).

Preparation of stock and working solutions

All the analyte and internal standard (IS) solutions were prepared in a water/methanol (1:1, v/v) mixture. All the analyte solutions were combined and diluted to concentrations of 10,000, 3000, 1000, 300, 100, 30, 10, 5, 3, 2, and 1 ng/mL, respectively. Subsequently, each IS solution was combined and diluted to a concentration of 100 ng/mL, thereby preparing an IS mix solution. Calibration curves were generated by plotting the peak area of the diluted working standards against that of the IS.

Food samples and method for analysis of choline-containing compounds

A total of five distinct sample types were employed in this study: white rice, brown rice, rice bran, brown rice malt, and FBRA. White rice is produced by the removal of the germ and bran (the outer layer of the rice grain) from brown rice. Brown rice is rice that has undergone a process of removing the hull from the rice seed, leaving the germ and bran intact. Rice bran is a byproduct of milling brown rice and contains the seed coat and germ. Brown rice malt is produced by fermenting brown rice with malt. FBRA is a product derived from brown rice and rice bran by fermentation with *Aspergillus oryzae*. Fermentation of rice bran using *Aspergillus oryzae* was carried out according to standard procedures at Koken Co., Ltd. (Tobetsu-cho, Hokkaido, Japan).[53] Rice bran was sprayed with water and mixed with steamed brown rice (1/10 the amount of rice bran) as a fermentation accelerant. The mixture was then processed in steam (10°C) for 40 min (first steaming) and then steamed in a drum-type fermenter at 100°C for 70 min (second steaming). The raw material was collected as follows. The steamed raw material was cooled to approximately 35°C using cold air and then allowed to undergo static fermentation in the fermentation liquid. The *Aspergillus oryzae* inoculation was carried out until the temperature of the intermediate product reached 40°C (about 12 hours after *Aspergillus oryzae* inoculation). The drum fermenter was then rotated. The intermediate product was further fermented while being stirred at a temperature of 37–43°C for about 20 hours. The fermentation product was then dried with hot air (approximately 50°C) for approximately 8 hours to obtain the FBRA (final product). As fresh FBRA has a high moisture content (approximately 30%), this sample (approximately 1 g) was dried at approximately 50°C for 1 hour. All samples were made into powder and stored at −20°C until use.

All rice samples were subjected to drying and milling processes, resulting in the formation of a powder. Subsequently, weighed sample powders were treated with a solution of methanol and water (1:1, v/v) in a 10-fold volume ratio (v/w). This mixture was then mixed for 1 hour, then subjected to sequential sonication for 1 hour, and centrifugation at 15,000 × *g* and 4°C for 10 min. Subsequently, an IS mix solution (50 μL) and methanol (50 μL) were added to the supernatant and thoroughly mixed. One microliter of the resulting solution was injected for LC/MS/MS analysis. The quantities of choline-containing compounds were determined using calibration curves. A Wilcoxon's test was employed to investigate significant differences between each compound.

RESULTS AND DISCUSSION

Optimization of LC/MS/MS conditions for choline-related metabolites

We developed simultaneous analytical conditions for all the analytes and the ISs. The parameters of the SRM and ion source are presented in Table 1. In the product ion spectra, the most intense product ion at *m/z* 58 was observed for choline and betaine. In previous reports, *m/z* 60 was observed as the product ion,[50] but in our results, *m/z* 58

Table 1. MS/MS conditions for choline-containing compounds

A. SRM parameters and the retention time

Compound	Category	Q1 (*m/z*)	Q3 (*m/z*)	DP (V)	EP (V)	CE (V)	CXP (V)	Retention time (min)
Choline	Analyte	104	58	40	10	39	8	4.65
Acetylcholine	Analyte	146	87	20	10	19	4	4.23
Betaine	Analyte	118	58	20	10	37	12	4.89
Phosphocholine	Analyte	184	125	200	10	25	14	6.11
Glycerophosphocholine	Analyte	258	104	20	10	23	12	5.84
LPC (*sn*-1, 16:0)	Analyte	496	184	160	6	31	10	3.77
LSM (d18:1/18:0)	Analyte	465	184	140	4	29	8	4.69
Choline-$^2H_9$	IS	113	66	30	6	35	12	4.65
Betaine-$^2H_{11}$	IS	129	68	36	8	23	14	4.89
Phosphocholine-$^2H_9$	IS	193	95	20	10	23	6	6.10
Glycerophosphocholine-$^2H_9$	IS	267	113	20	8	23	26	5.84
LSM (d18:1/17:0)	IS	451	184	120	6	27	12	4.66

CE, collision energy; CXP, cell exit potential; DP, declustering potential; IS, internal standard; LPC, lysophosphatidylcholine; LSM, lysosphingomyelin; MS/MS, tandem mass spectrometry; SRM, selected reaction monitoring.

B. Ion source parameters

Items	Optimized values
CUR	30 psi
GS1	80 psi
GS2	90 psi
ISV	5500 V
TEM	600°C
CAD	11 unit

CAD, collision gas; CUR, curtain gas; GS1, turbo gas; GS2, nebulizer gas; ISV, ion spray voltage; TEM, turbo gas temperature.

was observed with the most intense, so we adopted the *m/z* 58 as the monitoring ion for choline. It is postulated that $[NC_3H_8]^+$ is derived from the choline group. The product ions of acetylcholine (*m/z* 87) and phosphocholine (*m/z* 125) were considered to result from the neutral loss of 59 Da derived from the choline group $[N(CH_3)_3]$. The most intense product ion observed in the α-glycerophosphocholine experiment was choline (*m/z* 104). The LPC and LSM produced a phosphocholine group at *m/z* 184.[54,55] The stable isotope-labeled compounds provided common CID patterns for the analytes, as detailed in Table 1A. The ion source parameters were optimized to maximize the intensity of all analytes (Table 1B).

Subsequently, we investigated the LC/MS/MS conditions. The optimal conditions were those that led to the optimal peak shapes shown in Fig. 2. The analytes were successfully detected within 7 min without peak tailing in HILIC/MS/MS.

Calibration curves for analytes

Calibration curves were prepared for seven choline-containing compounds. The results are summarized in Table 2. The five compounds for which stable isotope-labeled compounds were available were used as IS. For the two compounds (LPC and LSM) for which it was not possible to procure a stable isotope label, a calibration curve was created by combining them with other molecules, and the combination with the highest linearity was selected. Six compounds were subjected to $1/x^2$ weighting, while betaine was subjected to 1/x weighting. A choline calibration curve with a 10,000-fold range was prepared. Similarly, analytes with stable isotope compounds were analyzed over a wide linear range. The linear range of LPC was the narrowest because it was corrected not only by the stable isotope but also by α-glycerophosphocholine-$^2H_9$.

Application of the method to rice-related foods

The LC/MS/MS method was employed to analyze several rice-based ingredients (Fig. 2). The complete results are presented in Table 3, and the individual contents of each analyte are illustrated in Fig. S1. The choline concentration is highest in rice bran (Fig. S1A). The analysis revealed that brown rice exhibits a higher concentration of choline than white rice. Brown rice malt and FBRA exhibit relatively low levels of choline content (Fig. S1A). Acetylcholine has its highest concentration in rice bran, followed by FBRA (Fig. S1B). Betaine has its highest concentration in FBRA, representing the highest amount observed in all analytes (Fig. S1C) and samples (Table 3). Phosphocholine was detected only in white rice and brown rice (Fig. S1D). The result suggested that phosphocholine is metabolized into other compounds by fermentation processing. By contrast, the α-glycerophosphocholine concentration is higher in fermented rice foods, rice bran, brown rice malt, and FBRA than in white rice and brown rice (Fig. S1E). LPC and LSM concentrations are higher in white rice and brown rice than in fermented rice foods (Fig. S1F and G). In these LC/MS/MS analyses, it was found that fermentation processing affects the levels of choline-containing compounds in foods. Figure 3 illustrates the changes that occur. Betaine in FBRA was the choline-related compound with the highest concentration of all the samples in this study. Betaine has several health benefits. First, betaine is beneficial for non-alcoholic hepatitis.[56] By converting homocysteine to methionine, betaine may reduce the risk of heart disease.[57,58]

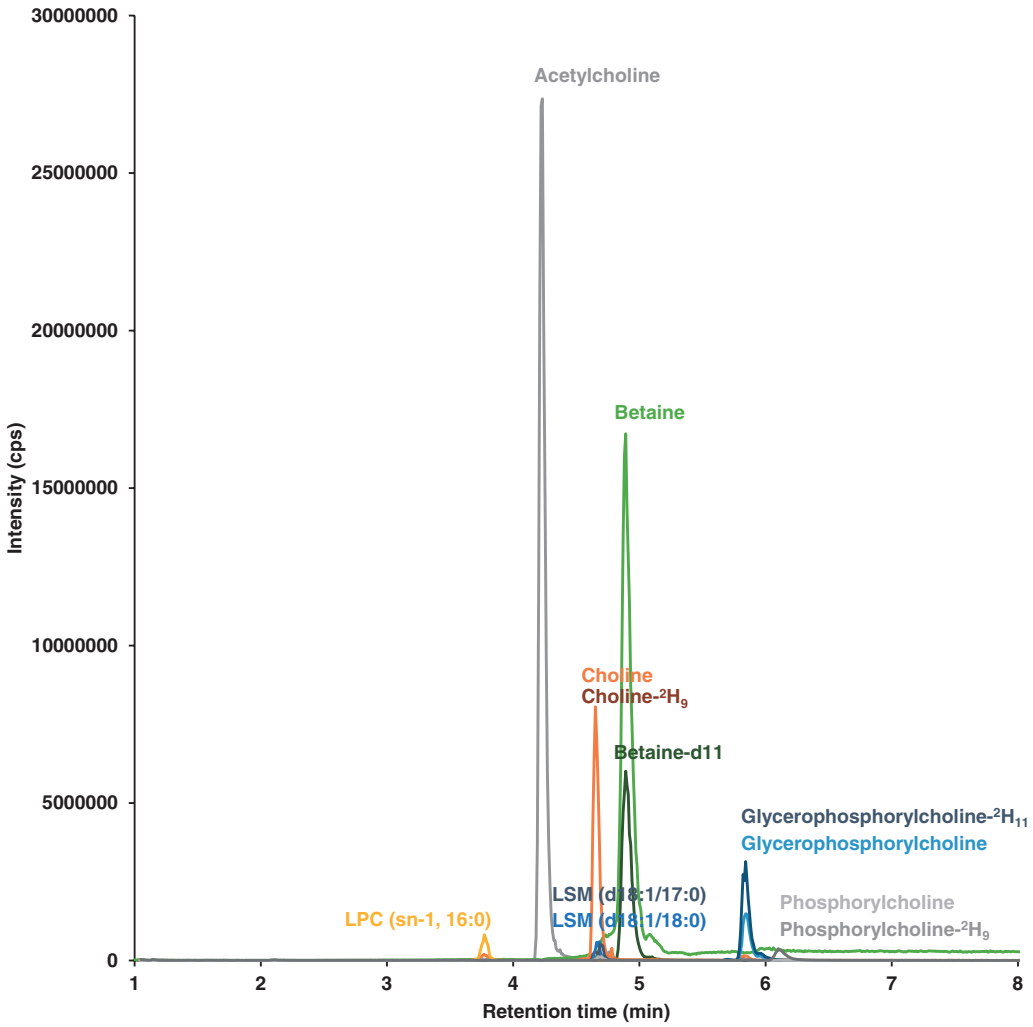

Fig. 2. Optimized SRM chromatograms of all compounds. SRM, selected reaction monitoring.

Table 2. Calibration curves for choline-related compounds.

Analyte	IS	Slope	Intercept	Correlation coefficient	Weighting	LLOQ (ng/mL)	ULOQ (ng/mL)
Choline	Choline-$^2H_9$	0.27984	2.46808	0.99575	$1/x^2$	1	10,000
Acetylcholine	Choline-$^2H_9$	0.99342	−0.04749	0.99779	$1/x^2$	3	10,000
Betaine	Betaine-$^2H_{11}$	0.0262	0.61983	0.99681	1/x	5	10,000
Phosphocholine	Phosphocholine-$^2H_9$	0.00095	0.11448	0.99541	$1/x^2$	10	10,000
Glycerophosphocholine	Glycerophosphocholine-$^2H_9$	0.00535	0.00073	0.99708	$1/x^2$	2	10,000
LPC (*sn*-1, 16:0)	Glycerophosphocholine-$^2H_9$	0.00172	0.03764	0.98973	$1/x^2$	30	10,000
LSM (d18:1/18:0)	LSM (d18:1/17:0)	0.0087	−0.01043	0.99487	$1/x^2$	2	5,000

IS, internal standard; LLOQ, lower limit of quantification; LPC, lysophosphatidylcholine; LSM, lysosphingomyelin; ULOQ, upper limit of quantification.

Table 3. The amounts of choline and the related metabolites in rice-related foods.

Analyte	White rice (ng/mg)	Brown rice (ng/mg)	Rice bran (ng/mg)	Brown rice malt (ng/mg)	FBRA (ng/mg)
Choline	34.5 ± 10.6	102 ± 20.9	450 ± 55.5	17.2 ± 4.12	41.7 ± 11.3
Acetylcholine	0.191 ± 0.0825	0.802 ± 0.140	10.6 ± 1.99	1.79 ± 0.332	7.06 ± 2.03
Betaine	4.85 ± 9.13	5.11 ± 1.24	32.6 ± 5.33	51.8 ± 27.7	677 ± 111
Phosphocholine	46.5 ± 53.3	33.4 ± 41.2	N.D.	N.D.	N.D.
Glycerophosphocholine	33.1 ± 8.33	83.4 ± 31.1	553 ± 262	961 ± 170	555 ± 124
LPC (*sn*-1, 16:0)	56.3 ± 20.5	54.9 ± 24.4	13.5 ± 24.0	22.4 ± 8.11	11.9 ± 10.4
LSM (d18:1/18:0)	0.258 ± 0.324	0.13 ± 0.00707	0.126 ± 0.00506	N.D.	0.122 ± 0.00439

FBRA, fermented brown rice and rice bran with *Aspergillus oryzae*; LPC, lysophosphatidylcholine; LSM, lysosphingomyelin.

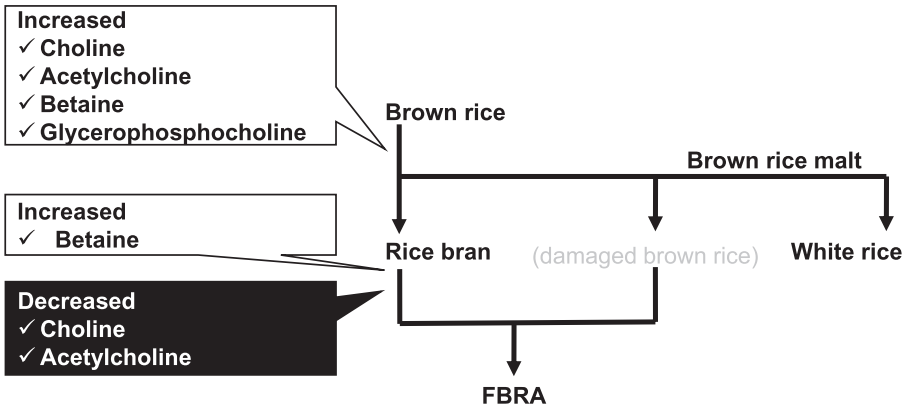

Fig. 3. Summary of fermentation effect on the content of choline-containing compounds in rice ingredients. FBRA, fermented brown rice and rice bran with *Aspergillus oryzae*.

The association of choline intake with betaine and the inflammation process in free-eating is suggested.[59] In addition, betaine has a neuroprotective role, potentially preserving cognitive functions and preventing neurological disorders.[60] Betaine supplements may help improve muscle strength and body composition and reduce muscle loss.[61,62] In addition, betaine has a protective effect against Alzheimer's disease.[63] Figure S1E illustrates that α-glycerophosphocholine is present in greater quantities in fermented rice foods. α-Glycerophosphocholine is currently under investigation for its potential to improve cognitive function.[64–66] In addition, it is postulated that it functions as a precursor to acetylcholine, a neurotransmitter implicated in memory, cognitive function, and attention. Consequently, it may enhance the effectiveness of acetylcholinesterase inhibitors, which are employed for the treatment of Alzheimer's disease, potentially improving behavioral symptoms, functional outcomes, and cognitive symptoms.[67] The fermentation process of rice affects the composition of choline-containing compounds, and it is hypothesized that FBRA supplements may have beneficial effects on human health. However, it should be noted that an analytical method validation was not performed, which may have implications for the reliability of the results obtained.

CONCLUSION

In this study, we developed a simultaneous analysis method for seven choline-containing compounds using HILIC/MS/MS and applied the method to quantify them in FBRA and its ingredients. Concentrations of betaine and α-glycerophosphocholine, which have been linked to numerous beneficial health effects, significantly increased through fermentation processing in FBRA and brown rice malt. This suggests that the production of rice ingredients by fermentation may be a promising avenue for food-based health development. Consequently, further investigation will be necessary in the future.

ABBREVIATIONS

CAD, collision gas; CE, collision energy; CXP, cell exit potential; CUR, curtain gas; DP, declustering potential; FBRA, fermented brown rice and rice bran with Aspergillus oryzae; GS1, turbo gas; GS2, nebulizer gas; HILIC, hydrophilic interaction chromatography; ISV, ion spray voltage; LC/MS/MS, liquid chromatography/tandem mass spectrometry; LPC, lysophosphatidylcholine; LSM, lysosphingomyelin; SRM, selected reaction monitoring; TEM, turbo gas temperature.

ACKNOWLEDGMENT

We express our gratitude to Genmaikoso Co., Ltd. (Sapporo, Japan) for its generous grant, which has enabled us to defray a portion of our budget. Moreover, they provided us with food samples that have proven to be invaluable in our research endeavors.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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