Fatty Acid Composition, Enrichment of Polyunsaturated Fatty Acids and Bioactivities of Oils from Four Species of Animals Bone Marrow

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Abstract: In this study, four kinds of animal bone marrow powders were extracted with n-hexane using the Soxhlet extraction method. Polyunsaturated fatty acids were enriched by urea inclusion and low temperature crystallization method, then were further evaluated antioxidant and antibacterial activities. These results showed that the oil composition of the n-hexane extracts of four kinds of animal bone marrow primarily consisted of palmitic acid (18.57–31.01%), stearic acid (3.6–20.95%), and oleic acid (40.22–58.69%). The ratios of saturated fatty acids (SFA)/unsaturated fatty acids (UFA) were 1/1.417, 1/1.327, 1/2.140, and 1.285/1 for sheep, bovine, horse, and camel bone marrow oil, respectively. The SFA/UFA ratios determined by the urea inclusion method were 1/1.518, 1/1.390, 1/2.037, and 1.216/1, respectively. The SFA/UFA ratios according to the low temperature crystallization method with acetone were 1/1.920, 1/2.141, 1/2.360, and 1/1.157 for sheep, bovine, horse, and camel bone marrow oil, respectively. These enrichment methods effected the concentrations of UFAs from the camel bone marrow oil. Among the methods, the low temperature crystallization method effectively enriched the UFAs. All four bone marrow oils exhibited strong antioxidant and antimicrobial activities. The horse bone marrow oil showed the strongest antioxidant activity. Both antioxidant and antimicrobial activity improved after enrichment of the UFAs. These results lay a theoretical basis for application bone marrow oil resources in food and medicine.

Keywords: bone marrow oil; unsaturated fatty acid; urea inclusion; low temperature crystallization; GC/MS

1. Introduction

The nutritional value of polyunsaturated fatty acids (PUFAs) and their preventive effects on cardiovascular disease [1], neurological disorders [2], cancers [3, 4], rheumatoid arthritis [5], and other diseases have attracted widespread attention. Fatty acids possess numerous biological activities such as anti-inflammatory [6], antioxidant [7], antimicrobial [8], and neuroprotective [9] activities. At present, the technology for enrichment and separation of PUFAs has become a hot topic in oil and fat research. The oils in animal bone account for 5–15% of bone weight, and the oil content in bone marrow reaches 90–95% of bone weight, indicating that the available resources are quite abundant. Animal oils and fats have been widely used in the food processing industry because of their unique aroma. In addition, animal oils can also be directly used to produce stearic acid, oleic acid, soap, lubricants, household cosmetics, wax paper, and glycerin extracts [10, 11].

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The physiological activities of various PUFAs are significantly different, and separating them into a single high-purity product can enhance their activity. Therefore, it is necessary to carry out enrichment and analyses of unsaturated fatty acids (UFAs) as the main component of bone marrow oil to improve utilization of bone marrow.

Several studies have shown that bone marrow oils can be used to treat bone necrosis, enhance glucocorticoid therapy, and improve immunity with adiponectin [12]. Previous studies on sheep, bovine, horse, and camel bone marrow focused on the analysis of protein [13, 14], and few studies have investigated the chemical composition and biological activities of bone marrow oils or the oils that remain after extracting the protein. The relationship between mechanism of action and structure-function are unknown, which has led to a low utilization rate of bone marrow oil.

Several methods are available to enrich PUFAs, including enzymatic purification [15], supercritical-fluid extraction [16], urea inclusion [17], silica gel chromatography [18], and the low temperature crystallization method [19]. These methods used to concentrate PUFAs are based on differences in the polarity and spatial configuration of the fatty acids present in the extract. Thus, the degree of unsaturation plays an essential role in the separation [20]. The urea inclusion method is one of the most appropriate methods to concentrate PUFAs, as this process allows for large quantities of material with simple equipment, inexpensive solvents, and mild conditions [21]. The melting points of fatty acids change with the type and degree of unsaturation. At low temperatures, long chain saturated fatty acids (SFAs) have higher melting points and crystallize, leaving the PUFAs in liquid form [22]. Therefore, the proper solvent and temperature are necessary to achieve optimal yields [23].

In our study, bone marrow oil (that remaining after protein extraction) from bovine was extracted with different solvents (n-hexane, petroleum ether, ethanol, and methanol). n-Hexane was the ideal extraction solvent according to the contents of UFAs in the oil. Then, bone marrow oil from sheep, horses, and camels was extracted with n-hexane. After extracting the crude oils, the PUFAs were concentrated using the urea inclusion and low temperature crystallization methods. Antioxidant and antimicrobial activities were evaluated *in vitro*, and the nutritional value and bioactivities of the different types of animal bone marrow fatty acids were compared. This is the first study to investigate the relationship between animal bone marrow oil and bioactivity. Our results indicate that the different animal bone marrow fatty acids have different compositions, contents, and biological activities. These results will be useful to identify the nutrient contents of the oils. This study provides the technical and theoretical foundation for the development of health foods and drugs.

2. Materials and Methods

2.1. Materials

The fresh raw material was obtained from the Urumqi slaughter house (Xinjiang, China). All marrow was removed from the front and rear leg bones and was immediately frozen after rinsing with cold and warm water three times to remove crushed bone and blood. The samples were crushed into a powder in liquid nitrogen (1:6, w/v) and stored at −20°C until use.

DPPH was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Petroleum ether, n-hexane, anhydrous methanol, anhydrous ethanol, anhydrous sodium sulfate, dimethyl sulfoxide, salicylic acid, H_2O_2, and methylbenzene were of analytical grade and purchased from local suppliers. GC/MS was performed using the 7890A-5975C instrument (Agilent Technologies, Palo

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Alto, CA, USA).

2.2. Methods

2.2.1. Bone marrow oil extraction

BBM powder (50 g) and 150 mL of n-hexane, petroleum ether, methanol, or ethanol were mixed. The extraction was performed on a Soxhlet extractor. The bone marrow oil was obtained by filtering the liquid mixture remaining after removing the n-hexane using a rotary evaporator (R210; Buchi Corp., New Castle, DE, USA). Then, one of the extraction solvents was chosen according to the UFA content in the oil to extract the bone marrow oil from the three other domestic animals (sheep, horse, and camel) as described above.

2.2.2. Preparation of mixed fatty acids

The four kinds of bone marrow oil were saponified by refluxing for 2 h at 72℃ using a mixture of NaOH-CH_3OH (4%, w/v). The saponified solution was cooled in a separatory funnel. An appropriate amount of distilled water was added to dissolve the saponified mixture, and the aqueous layer containing the saponified matter was acidified with 10% hydrochloric acid. n-Hexane was added to dissolve the oil layer. The oil layer was rinsed several times with water until neutral, then dehydrated with anhydrous sodium sulfate. The mixed fatty acids were obtained by removing the solvent using a rotary evaporator (Buchi R210) [24].

2.2.3 .Urea inclusion method

PUFAs were concentrated using the urea inclusion method according to a previous study with slight modifications [21]. The four kinds of bone marrow with mixed fatty acids were added to a urea-saturated methanol solution. The reaction was performed under reflux for 40 min at room temperature with a thermostatic heating magnetic stirrer for complete adduction. The solution was cooled to room temperature, stored at −10℃ for 24 h, and filtered under a vacuum. The filtrate was placed into a separatory funnel and an appropriate volume of water was added to remove the urea. The oil layer was rinsed several times with boiling water until the solution became colorless, and the mixed fatty acids were obtained after dehydration with anhydrous sodium sulfate. The solvent was evaporated with a rotary evaporator (Buchi R210).

2.2.4. Low temperature crystallization method

Low temperature crystallization of PUFAs was conducted according to a previous method with slight modifications [22]. The four kinds of bone marrow with mixed fatty acids were added to acetone and stored at −40℃ for 24 h. Thereafter, the samples were immediately centrifuged at −4℃ and 8,000 rpm for 10 min in a high speed refrigerated centrifuge (Hitachi Co., Tokyo, Japan). After centrifugation, the samples were stored again at −40℃ for 5 min, centrifuged again, and the crystallized fraction was separated from the liquid fraction. After separating the phases, the organic solvent was removed from the liquid fraction using a rotary vacuum evaporator (Buchi R210).

2.2.5. Methyl esterification analysis

Esterification was conducted according to a previous method [40] with slight modifications. The oil sample (0.1 g) was dissolved in a 2 mL petroleum ether-toluene mixture in a 10 mL

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volumetric flask, 3 mL of potassium hydroxide-methanol (0.4 M) was added, and the volume was bought up with distilled water. Then, the fatty acid methyl esters obtained were analyzed by GC/MS chromatography (7890A-5975C, Agilent Technologies) with a Superco elastic quartz capillary column (100 mm × 0.25 mm), with film thickness of 0.25 μm, FID detection, and a heat-up program. The column programmed heat-up temperature was as follows: initial injector temperature was 140℃ for 5 min, increased at a rate of 4℃/min to 200℃, held for 1 min, then increased at a rate of 3℃/min to 220℃, and held for 26 min.

The EI+ mode was used as the ionization source for the mass spectra, with ionization energy of 70 eV and ionization source temperature of 230℃. Full scan mode was utilized within the range of 40–500 u, and the transmission line temperature was 230℃. The relative proportions of each fatty acid were calculated using the area normalization method by comparing retention time and mass spectra from the GC/MS analysis.

2.2.6. Biological activity

1) DPPH radical scavenging activity

The DPPH radical scavenging activities of the oil samples were tested using a previous method [26]. The oil samples were dissolved in ethanol to obtain various test concentrations (0.25, 0.5, 0.75, 1, 1.25, 1.5, and 2 mg/mL). DPPH (0.2 mM) was freshly prepared in methanol. One mL of the sample was added to 1.0 mL of the DPPH-methanol solution and stored at 37℃ for 30 min in the dark. Absorbance was immediately determined at 517 nm against a blank. Vc was used as the positive control. The DPPH radical scavenging activity was calculated as follows:

$$\text{Scavenging ability (\%)} = 1 - (A_i - A_j)/A_0 \quad (1)$$

Where, A_0 is the absorbance of the DPPH solution in ethanol; A_i is absorbance of the sample mixed with the DPPH solution; and A_j is absorbance of the sample in ethanol.

2) Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was measured according to a method reported previously with slight modifications [42]. One mL of the sample in ethanol (0.25–2 mg/mL) was mixed with 1 mL of ferrous sulfate solution (6 mM) and salicylic acid in ethanol (6 mM). The mixture was shaken, 1 mL of 0.1% hydrogen peroxide was added, and the mixture was stored at 37℃ for 30 min in the dark. Absorbance was measured at 510 nm. V_C was used as the positive control. Hydroxyl radical scavenging activity was calculated using Eq. (1).

3) Antimicrobial activity

A 20 μL aliquot of sample solution was taken from the concentrated 50 mg/mL sample and placed in a 37℃ incubator for 30–60 min. Vernier calipers were used to measure and record the diameter of the bacteriostatic rings after 16–18 h. The samples were considered ineffective against the microbe when the inhibition zone diameter was ≤ 7 mm. Antimicrobial activity was determined using *C. albicans* (ATCC10231) and *E. coli* (ATCC11229) [43].

3. Results

3.1. Fatty acid content of four kinds of solvents from bovine bone marrow oil

The fatty acid contents of the bovine bone marrow extracted with the four kinds of solvents

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were determined by gas chromatography/mass spectroscopy (GC/MS) analysis. Table 1 shows that the content of UFAs was higher than that of SFAs for the four kinds of oils. The ratios of SFA/UFA were in the order from low to high as: n-hexane > petroleum ether > ethanol > methanol extract, data were 1.41>1.40>1.25>1.24. The oil extracted with n-hexane had the highest UFA content. The main fatty acids are oleic acid (45.32% - 50.42%), palmitic acid (22.20% - 24.10%), stearic acid (13.41% - 16.49%), linoleic acid (1.91% - 3.75%) for four kinds of animal bone marrow oils. Moreover, UFAs content were higher than SFAs, but the extent is not high. Bone marrow oil is a kind of multicomponent fatty acid with different physicochemical properties. The physiological activities of UFAs are quite different, in order to make them better works their own function, it need to separate the mixture into a single and high purity products or reduce the content of UFAs. Consequently, the aim of this study was to enrich the UFAs. Thus, we selected n-hexane as the ideal extraction solvent.

Table 1. Fatty acid composition and contents of the four kinds of oils from bovine bone marrow

Fatty acid	petroleum ether	n-hexane	methanol	ethanol
	Content (%)			
Myristic acid (C_{14}:0)	2.33	2.54	2.89	2.23
Pentadecanoic acid (C_{15}:0)	1.25	-	1.34	0.31
Palmitic acid (C_{16}:0)	24.1	23.84	22.20	23.21
Heptadecylic acid (C_{17}:0)	0.62	1.48	3.44	2.01
Stearic acid (C_{18}:0)	13.41	13.62	14.9	16.49
Nonadecanoic acid (C_{19}:0)	-	-	0.58	-
eicosanoic acid (C_{20}:0)	-	-	0.21	-
Myristoleic acid (C_{14}:1)	0.47	0.31	0.68	0.3
pentadecenic acid (C_{15}:1)	-	-	0.05	-
Hexadecenoic acid (C_{16}:1)	1.49	2.03	0.05	1.92
9,12-Octadecadienoic acid (C_{18}:2)	1.49	0.74	-	-
Linoleic Acid (C_{18}:2)	2.41	3.03	1.91	3.75
Linolenic Acid (C_{18}:3)	-	-	0.06	-
Eicosapentaenoic acid (C_{20}:5)	-	-	0.03	-
SFA	41.71	41.48	45.56	44.45
MUFA	54.39	54.64	51.49	52
PUFA	3.9	3.77	2.11	3.75
UFA	58.29	58.42	53.60	55.75
SFA/UFA	1/1.4	1/1.41	1/1.24	1/1.25

Note: -, the fatty acid content is less than 0.01% or not detected under the selected experimental condition; SFA was the sum of C_{14}:0, C $_{15}$:0, C_{16}:0, C_{17}:0, and C_{18}:0; MUFA was the sum of C_{16}:1, C_{17}:1, C_{18}:1, C $_{19}$:1, C_{20}:1, C_{21}:1, C_{26}:1; PUFA was the sum of C_{18}:2, C_{18}:3, C_{20}:2, C_{20}:3, C_{20}:4, C_{20}:5; UFA was the sum of MUFA and PUFA.

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As shown in Table 2, the ratios of SFA/UFA obtained from the four kinds of bone marrow with mixed fatty acids were in the order of horse bone marrow (HBM) > sheep bone marrow (SBM) > bovine bone marrow (BBM) > camel bone marrow (CBM), date were 1/1.38, 1/1.264, 1/1.912, 1.285/1. The main fatty acids were palmitic acid and stearic acid, and their contents were 18.57–31.01% and 3.50–20.95%, respectively. The CBM had the highest palmitic acid content (31.01%), while the SBM had the highest stearic acid content (20.95%). (Z)-9-Octadecenoic acid (40.96–58.69%) was the main UFA, and its content was higher in HBM (58.69%) than the others.

Table 2. Composition and content of the main fatty acids from the four kinds of bone marrow oil

Fatty acid	Formula	SBM	BBM	HBM	CBM
		Content (%)			
Dodecanoic acid (C_{12}:0)	$C_{12}H_{24}O_2$	-	-	0.12	0.24
Myristoleic acid (C_{14}:1)	$C_{14}H_{26}O_2$	0.07	0.15	0.20	0.17
Myristic acid (C_{14}:0)	$C_{14}H_{28}O_2$	1.25	0.96	2.61	5.86
Pentadecanoic acid (C_{15}:0)	$C_{15}H_{30}O_2$	0.49	0.19	0.12	1.46
(Z)-9-Hexadecenoic acid (C_{16}:1)	$C_{16}H_{30}O_2$	1.41	1.64	5.12	3.37
(Z,Z)-7,10-Hexadecadienal (C_{16}:2)	$C_{16}H_{28}O$	-	0.19	-	-
Palmitic acid (C_{16}:0)	$C_{16}H_{32}O_2$	18.57	26.39	27.75	31.01
Heptadecylic acid (C_{17}:0)	$C_{17}H_{34}O_2$	-	0.70	0.16	2.21
(Z)-10- Heptadecenoic acid (C_{17}:1)	$C_{17}H_{32}O_2$	1.23	-	0.44	-
(Z)-9- Octadecenoic acid (C_{18}:1)	$C_{18}H_{34}O_2$	54.28	53.7	58.69	40.22
Stearic acid (C_{18}:0)	$C_{18}H_{36}O_2$	20.95	16.08	3.60	15.45
(E)-9- Octadecenoic acid (C_{18}:1)	$C_{18}H_{34}O_2$	0.51	1.48	0.23	-
(Z)-10-Nonadecyenoic acid (C_{19}:1)	$C_{19}H_{36}O_2$	0.34	-	0.44	-
Nonadecanoic acid (C_{19}:0)	$C_{19}H_{38}O_2$	0.10	-	-	-
(Z,Z)-2-Methyl- 3,13-Octadecadienol (C_{19}:2)	$C_{19}H_{36}O$	0.64	-	0.31	-
(Z)-11- Eicosenoic acid (C_{20}:1)	$C_{20}H_{38}O_2$	-	0.16	0.71	-
SFA		42	44.16	34.36	56.23
MUFA		58	55.54	65.39	43.76
PUFA			0.19	0.31	0
UFA		58	55.84	65.7	43.76
SFA/UFA		1/1.38	1/1.264	1/1.912	1.285/1

Note: same as Table 1.

3.2 Enrichment of unsaturated fatty acids

3.2.1 Urea inclusion

The urea inclusion method was used to enrich the UFAs, and the results are shown in Table 3. The contents of palmitic acid and stearic acid decreased to 16.81–29.94% and 3.01–19.1%, respectively, compared with the fatty acid composition of the crude oil, after treatment by the urea inclusion method, the contents of (Z)-9-octadecenoic acid increased to 41.18–61.02% at a 2% rate of increase. PUFAs, such as (Z,E)-9,11-octadecadienoic acid and (Z,Z,Z)-11,14,17-tricosenoic acid, also appeared in the extracts. (Z,E)-9,11-Octadecadienoic acid is a characteristic component

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after urea inclusion that was detected in the CBM. Moreover, the quantity of UFAs in the HBM was more than twice that of SFAs.

Table 3. Composition and content of the main fatty acids from the four kinds of bone marrow oils enriched by urea inclusion

Fatty acid	Formula	SBM	BBM	HBM	CBM
		Content (%)			
Dodecanoic acid (C_{12}:0)	$C_{12}H_{24}O_2$	-	-	0.14	0.22
Myristic acid (C_{14}:0)	$C_{14}H_{28}O_2$	1.28	1.02	2.29	5.78
Myristoleic acid (C_{14}:1)	$C_{14}H_{26}O_2$	-	0.18	0.18	0.12
Pentadecanoic acid (C_{15}:0)	$C_{15}H_{30}O_2$	0.52	0.41	0.11	1.32
(Z)-9-Hexadecenoic acid (C_{16}:1)	$C_{16}H_{30}O_2$	1.47	1.78	4.80	3.21
Palmitic acid (C_{16}:0)	$C_{16}H_{32}O_2$	16.81	25.08	27.15	29.94
Heptadecylic acid (C_{17}:0)	$C_{17}H_{34}O_2$	2	1.74	0.15	2.01
(Z,Z)-7,10-Hexadecadienal (C_{16}:2)	$C_{16}H_{28}O_2$	-	0.45	-	-
(Z)-9-Octadecenoic acid (C_{18}:1)	$C_{16}H_{30}O_2$	58.82	55.75	61.2	41.18
(Z,E)-9,11-Octadecadienoic acid (C_{18}:2)	$C_{18}H_{32}O_2$	-	-	-	0.62
Stearic acid (C_{18}:0)	$C_{18}H_{36}O_2$	19.1	13.58	3.09	15.61
(Z,Z,Z)-11,14,17-Tricosenoic acid (C_{20}:3)	$C_{20}H_{34}O_2$	-	-	0.51	-
SFA		39.71	41.83	32.93	54.88
MUFA		60.29	57.71	66.56	44.51
PUFA		-	0.45	0.51	0.62
UFA		60.29	58.16	67.07	45.13
SFA/UFA		1/1.518	1/1.390	1/2.037	1.216/1

Note: same as Table 1.

3.2.2. Low temperature crystallization

The UFAs were enriched using the low temperature crystallization method, and the results are shown in Table 4. The contents of palmitic acid and stearic acid decreased to 14.44–26.22% and 2.80-13.35%, respectively, compared with the fatty acid composition of the crude oil after treatment by the low temperature crystallization method, the rate of decrease for stearic acid was 13%. However, the content of UFAs increased to 52.34–68.92%, with a rate of increase of 7–10%. The (Z)-9-octadecenoic acid content increased to 47.12–66.01%. The SFA/UFA ratio of the CBM decreased significantly after low temperature crystallization compared with the results after urea inclusion, as the amount of UFAs was remarkeble higher than that of SFAs. These results indicate that the low temperature crystallization method effectively enriched the UFAs.

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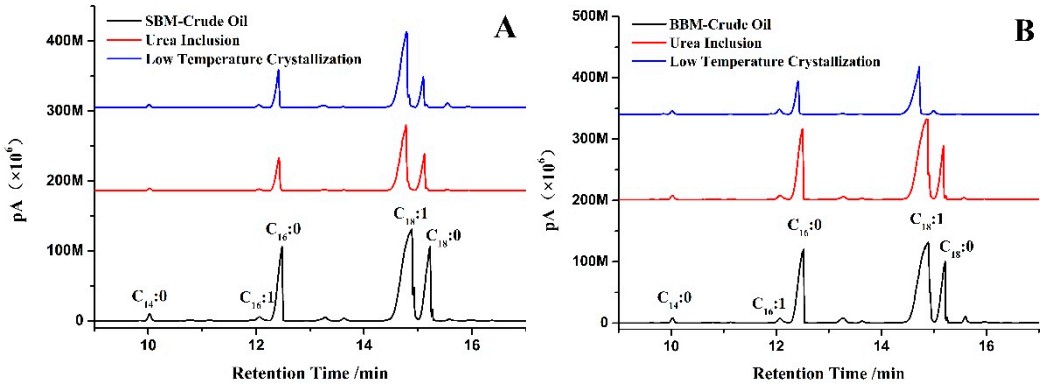
Table 4. Composition and content of the main fatty acids in the four kinds of bone marrow oil enriched by low temperature crystallization

Fatty acid	Formula	SBM	BBM	HBM	CBM
		Content (%)			
Dodecanoic acid (C_{12}:0)	$C_{12}H_{24}O_2$	-	-	0.13	0.26
Tridecanoic acid (C_{13}:0)	$C_{13}H_{26}O_2$	-	0.21	-	
Myristic acid (C_{14}:0)	$C_{14}H_{28}O_2$	1.23	2.42	2.07	5.89
Myristoleic acid (C_{14}:1)	$C_{14}H_{26}O_2$	-	0.24	0.18	0.21
Pentadecanoic acid (C_{15}:0)	$C_{15}H_{30}O_2$	0.52	-	0.11	1.66
(Z)-9-Hexadecenoic acid (C_{16}:1)	$C_{16}H_{30}O_2$	10.7	4.84	4.67	4.42
Palmitic acid (C_{16}:0)	$C_{16}H_{32}O_2$	14.44	26.22	25.29	25.13
Heptadecylic acid (C_{17}:0)	$C_{17}H_{34}O_2$	0.51	-	0.16	2.96
(Z)-10- Heptadecenoic acid (C_{17}:1)	$C_{17}H_{32}O_2$	-	0.43	-	-
(Z)-9-Octadecenoic acid (C_{18}:1)	$C_{18}H_{34}O_2$	66.01	62.26	62.81	47.24
Stearic acid (C_{18}:0)	$C_{18}H_{36}O_2$	13.35	2.80	3.01	10.46
(Z,Z)-9,12-Octadecadien-1-ol (C_{18}:2)	$C_{18}H_{34}O_2$	0.51	-	0.30	0.76
(Z,Z,Z)-6,9,12-Octadecatrienoic acid (C_{18}:3)	$C_{18}H_{30}O_2$	-	-	-	0.55
Glycidyl palmitate (C_{19}:1)	$C_{19}H_{36}O_3$	-	-	-	0.29
(Z)-13- Eicosenoic acid (C_{20}:1)	$C_{20}H_{38}O_2$		0.59	0.76	0.18
SFA		31.79	31.65	30.77	46.36
MUFA		67.71	68.36	68.92	52.34
PUFA		0.5	0	0.3	1.31
UFA		68.21	68.36	69.22	53.65
SFA/UFA		1/2.146	1/2.160	1/2.250	1/1.157

Note: same as Table 1.

3.3 Comparison of the enrichment effect of unsaturated fatty acids



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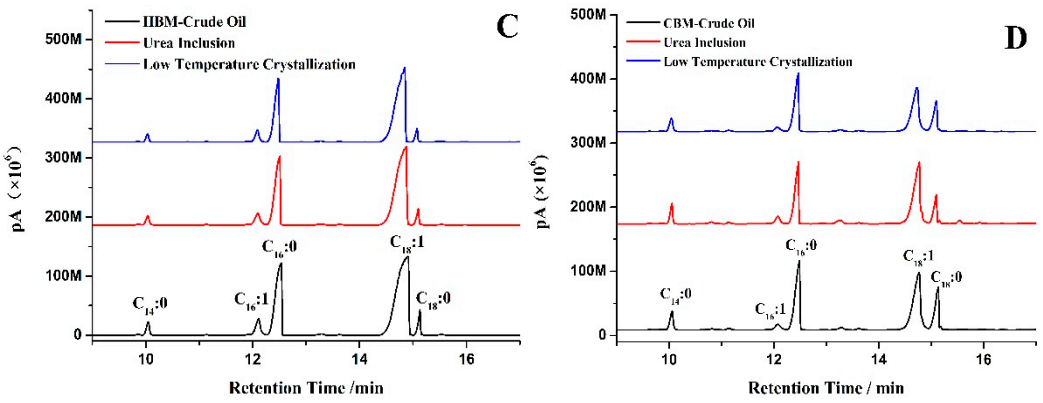

Fig. 1. Total ion chromatograms of the fatty acids [A. sheep bone marrow (SBM); B. bovine bone marrow (BBM); C. horse bone marrow (HBM); and D. camel bone marrow (CBM)]

The total ion chromatograms of the fatty acids from crude oil treated by urea inclusion and low temperature crystallization are shown in Fig. 1. saturated fatty acids and unsaturated fatty acids peak in proper sequence.enrichment method mainly effect myristic acid (C_{14}:0), (Z)-9-hexadecenoic acid (C_{16}:1),palmitic acid (C_{16}:0), (Z)-9-0ctadecenoic acid (C_{18}:1), stearic acid (C_{18}:0) peak level and content.

Table 5.Comparison of fatty acids from the four bone marrow oils

	Crude Oil				Urea inclusion				Low temperature Crystallization			
	SBM	BBM	HBM	CBM	SBM	BBM	HBM	CBM	SBM	BBM	HBM	CBM
	Content (%)											
C_{16}:0	18.57	24.23	27.75	31.01	16.81	24.86	27.15	29.94	14.44	26.22	25.29	25.13
C_{18}:0	20.95	15.59	3.60	15.45	18.51	13.23	3.09	15.61	12.57	2.80	3.01	9.78
C_{18}:1	54.28	52.08	58.69	40.96	57.79	54.93	61.05	41.18	63.56	62.26	62.42	45.30
SFA	42	44.16	34.36	56.23	39.71	41.83	32.93	54.88	31.79	31.65	30.77	46.36
MUFA	58	55.54	65.39	43.76	60.29	57.71	66.56	44.51	67.71	68.36	68.92	52.34
PUFA	0	0.19	0.31	0	0	0.45	0.51	0.62	0.5	0	0.3	1.31
UFA	58	55.84	65.7	43.76	60.29	58.16	67.07	45.13	68.21	68.36	69.22	53.65
SFA/UFA	1/1.38	1/1.26	1/1.91	1.26/1	1/1.52	1/1.39	1/2.04	1.21/1	1/2.15	1/2.16	1/2.25	1/1.16

As shown in Fig. 1 and Table 5, the content of UFAs was in the order of HBM > SBM > BBM > CBM. The contribution of urea inclusion method to the increase of UFAs content in crude oil was 1-2%, the increase rate were 7.76%, 12.32%, 3.52%, 9.89% for SBM, BBM, HBM and CBM, respectively. The decrease of stearic acid content was 1-2%. After urea inclusion, the order of SFA/UFA ratio was same with crude oil, and the content of SFA in HBM was twice as much as that of UFA. After low temperature crystallization, the increase rate of UFAs was about 3-12%, and the content ordered as HBM > BBM > SBM > CBM. The effect of low temperature crystallization on stearic acid is more obvious, and it can be reduced by 7 times (BBM). The increase in the rate of UFAs content and the decrease in the rate of SFA content were clear after the low temperature crystallization treatment compared with urea inclusion. The variations in the SFA and UFA contents were larger in the BBM, SBM, and CBM, particularly in the CBM, as the

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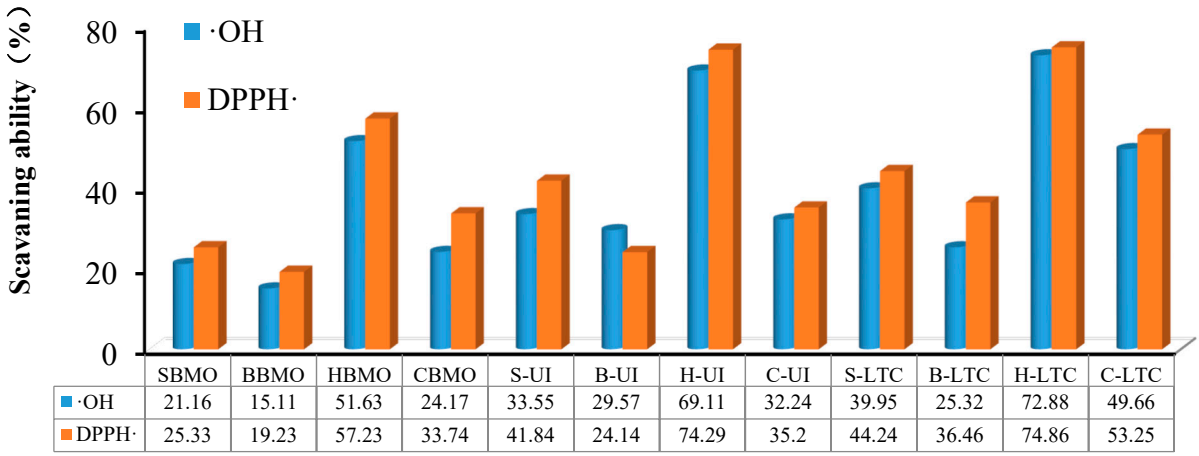
UFA content in the CBM was higher than that of SFAs. Therefore, low temperature crystallization using acetone was the most effective method to enrich the UFAs.

3.4 Biological activities

3.4.1 Antioxidant activity

As shown in Fig. 2, all of the tested bone marrow oils demonstrated scavenging ability against DPPH and hydroxyl free radicals at a concentration of 1 mg/mL. Scavenging ability was 19.23–57.23% for the DPPH free radical and 15.12–51.63% for the hydroxyl free radical from crude oil. The scavenging ability against DPPH and hydroxyl free radicals was strengthened after treatment by urea inclusion, and the rates of increase were 14.91–17.06% and 14.45–17.61% for DPPH and hydroxyl free radicals, respectively. Scavenging ability was also enhanced after the low temperature crystallization treatment. The rates of increase were 17.23–17.58% for the DPPH free radical and 9.99–21.25%% for the hydroxyl free radical, respectively. The HBM had the highest antioxidant ability. All of the fatty acids obtained by n-hexane extraction and treated with the urea inclusion and low temperature crystallization methods exhibited some antioxidant activity, scavenging activity against DPPH was stronger than that against the hydroxyl free radical. Antioxidant activity increased after enrichment of the UFAs, and the increase in the rate of scavenging ability about 8-25%. Moreover, the low temperature crystallization method had a greater improving effect on antioxidant activity.



	SBMO	BBMO	HBMO	CBMO	S-UI	B-UI	H-UI	C-UI	S-LTC	B-LTC	H-LTC	C-LTC
·OH	21.16	15.11	51.63	24.17	33.55	29.57	69.11	32.24	39.95	25.32	72.88	49.66
DPPH·	25.33	19.23	57.23	33.74	41.84	24.14	74.29	35.2	44.24	36.46	74.86	53.25

Fig. 2. Comparison of scavenging ability against ·OH and DPPH· of the bone marrow oils (1 mg/mL) (UI: urea inclusion; LTC: low temperature crystallization)

3.4.2 Antimicrobial activity

As shown in Table 6, all of the tested bone marrow oils exhibited some antimicrobial activity; the activity against *Escherichia coli* was stronger than that against *Candida albicans*. Antimicrobial activity was enhanced in fatty acids with a second double bond (linoleic acid). cis-fatty acids had stronger antimicrobial activity than the trans-isomers. After enrichment of the UFAs, antimicrobial activity was also enhanced with an increase in cis-fatty acid content. In particular, the increase in the rate of antimicrobial activity of the CBM was stronger than that of SBM and BBM, which may have been caused by the emergence of PUFAs, such as

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(Z,Z,Z)-6,9,12-octadecatrienoic acid. All four animal bone marrow oils exhibited strong antimicrobial activities. Further studies are in progress to gain more insight into the biological activities of these animal bone marrow oils to improve their biological utility.

Table 6. Antimicrobial activities of the four bone marrow oils

Sample	CA (mm)	EC (mm)	Sample	CA (mm)	EC (mm)	Control
SBMO	+	-	H-UI	++	++	
BBMO	+	+	C-UI	++	+	CA (mm) +++
HBMO	+	+	S-LTC	++	+	EC (mm) ++++
CBMO	+	+	B-LTC	+	+	
S-UI	+	+	H-LTC	++	++	
B-UI	++	++	C-LTC	++	++	

Note, CA: *Candida albicans*; EC: *Escherichia coli;* UI: urea inclusion; LTC: low temperature crystallization

4. Discussion

Animal bone has widespread applications in the food processing industry. Animal bone oil is in a solid state at room temperature, and they can be used directly as a pharmaceutical raw materials, whereas their use as a food ingredients bring certain difficulties. After enrichment of the UFAs, efficient embedding can avoid deterioration by oxidation and expand the application range. The UFAs in bone marrow oil are beneficial for cholesterol metabolism and distribution, and they reduce arteriosclerosis and prevent cardiovascular disease [29]. Bone marrow oil does not accumulate after it is consumed, so it can prevent obesity as a new type of healthcare fat. Fat and fatty acids play an important role in the development of noncommunicable diseases, such as overweight, obesity, metabolic syndrome, and nonalcoholic fatty liver disease [30].

4.1. Fatty acid content

Previous studies have shown that the SFAs (palmitic acid and stearic acid) and the UFAs (oleic acid and linoleic acid) are the main components in animal oils, and the ratio of the two is close to 1:1. Liu et al. [31] reported that crude oil from sheep bone is composed of seven kinds of fatty acids (three SFAs and four UFAs). Sheep bone mainly includes oleic acid (458.90 mg/g), palmitic acid (155.59 mg/g), and stearic acid (128.25 mg/g). Lin [32] compared the effects of different methods on bovine bone degreasing. Hexyl hydride, ethanol, and ordinary pressure cooking were used to treat bovine bone and determine the effect on the basal components and color of the bone. The experimental results showed that ordinary pressure cooking was the best for degreasing, and the fat content of the bone was 4.05%. Qin [33] reported extracting horse bone oil with n-hexane. Chicken bone oil [34] and yak bone oil [35] have also been studied.

Our research indicates that the four kinds of bone marrow oil were comprised of palmitic acid (18.57–31.01%), stearic acid (3.6–20.95%), and oleic acid (40.22–58.69%). Total SFA contents were 34.36–56.23%, whereas that of UFAs was 43.76–65.7%. For example, the content of SFAs among the fatty acids in bone marrow oil was 53–58%, and UFA content was 41–45%, among which palmitic acid was 22–24% and stearic acid was 13–16% of total content. The content of fatty acids in bovine bone marrow oil is similar to that in human milk and milk from other animals [36]. Previous studies on animal bone have directly used it to manufacture related

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products, but few studies have separated the bone from the bone marrow. Bone oil has a lower extraction yield than bone marrow oil (90–95%), consequently resulting in reduced nutritional value of the products on a large scale. Microcapsules have been widely used in medicine, food, pesticides, cosmetics, additives, and other fields because of the various functions of encapsulation, including protecting substances from the environment, effectively isolating active ingredients, reducing volatility and toxicity, and controlling sustained release. Some progress has been made in the refining technology of pig bone oil and oil microencapsulation [37]. A few studies have investigated encapsulation of bovine and sheep bone oil. Microencapsulation of bone marrow oil is an effective way to improve its comprehensive utilization value.

4.2. Bioactivities of bone oils

A considerable number of studies have reported that bone has antioxidant, antibacterial activities, and promotes human osteoblasts, hematopoiesis, immune regulation, biomaterial production, treatment of various bone diseases, subcutaneous cell metabolism, and delayed aging [38]. We have concluded that these biological activities of bone are mainly due to bone proteins and bone charcoal, as few studies have investigated the bioactivities of bone marrow oil. Bone marrow oils are believed to have better antioxidant (DPPH radical scavenging activity was 30.45–50.56%) and antimicrobial [8] activities than bone marrow protein. In particular, biological activity is enhanced after enrichment of the UFAs. Yang [39] reported that ostrich oil demonstrates excellent antimicrobial ability against *Pseudomonas aeruginosa*, *Candida albicans*, and *Penicillium glaucum,* and the optimum concentration for antimicrobial activity was 20 mg/mL. The horse oil studied by Jing [8] exhibited significant antimicrobial activities against *Candida albicans*, and the minimum inhibitory concentration of horse oil against *Candida albicans* was 11 mg/mL. The oils extracted from sheep, bovine, horse, and camel in our study showed distinct inhibitory effects on *Candida albicans* and *Escherichia coli*, which may have been the result of differences in the composition and content of SFAs and UFAs in the different species of animal oils.

Further research is progressing to gain more insight into applying bone oils in food, medicine, and healthcare products. Extensive studies are required to identify the potential biological activities of bone marrow oil to improve product quality. This approach will provide a new way to improve the nutritional function and value of animal bone.

4.3. Comparison of different extraction solvents and enrichment methods

Oil was extracted from BBM with n-hexane, petroleum ether, methanol, and ethanol, and the SFA/UFA ratios were 1/1.41, 1/1.40, 1/1.25, and 1/1.24, respectively. The UFA content of the oils extracted with n-hexane was higher than the others. Moreover, it had the highest extraction rate. In addition, preliminary bioactivity tests showed that the antimicrobial activity of the BBM extracted with the different solvents was in the order: methanol > n-hexane > petroleum ether > ethanol. Methanol is not suitable for large-scale extraction or use in health foods because of its toxicity. Therefore, n-hexane was selected as the ideal extraction solvent.

UFAs have numerous biological activities, so enriching the UFAs from animal bone marrow not only enhances the bioactivities of the oil, but also improves utilization. The most commonly used enrichment methods are urea inclusion and low temperature crystallization, and both methods are simple, quick, and feasible for mass production. UFA contents generally increase

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after treatment with the urea inclusion and low temperature crystallization methods, such as that observed for salmon [40] and seal oils [41], which increased 14.20% and 52.20%, respectively after treatment by urea inclusion and for silkworm pupa [42] and horse fat [43], which were 47.0% and 24.39%, respectively, after treatment by low temperature crystallization. The recovery yield of UFAs was 10–40%. The rate of recovery increased 3–12% after low temperature crystallization in our study. The SFA/UFA ratios of the four kinds of bone marrow oil were 1/2.15, 1/2.16, 1/2.25, and 1/1.16 for SBM, BBM, HBM and CBM, respectively, and the content of UFAs was close to 70%. The increase in the rate of UFA content in the bone marrow oil was lower, but recovery yield was 50–70%. In conclusion, multiple operations will be required to further increase of UFA contents.

5. Conclusion

The four kinds of animal bone marrow oil were extracted with n-hexane as the extraction solvent. The SFA/UFA ratios were 1/1.38, 1/1.26, 1/1.91, and 1.285/1 for SBM, BBM, HBM and CBM, respectively for the crude oil. The SFA/UFA ratios obtained from the urea inclusion and low temperature crystallization treatments were 1/1.52, 1/1.39, 1/2.03, 1.22/1 and 1/2.15, 1/2.16, 1/2.25, 1/1.16, respectively for the four kinds of bone marrow oils. The low temperature crystallization method was more suitable for enriching UFAs from the bone marrow oil. The scavenging ability against the DPPH and hydroxyl free radicals of the crude oils were in the order: HBM > SBM > CBM > BBM; the urea inclusion results were the same as those of the crude oil. After low temperature crystallization, the antioxidant activities were in the order: HBM > BBM > CBM > SBM. Among them, the HBM crude oil and that treated with the enrichment methods always had the strongest antioxidant activity. All of the bone marrow crude oils and extracted oils exhibited strong antimicrobial activities after enrichment of the UFAs. In conclusion, the low temperature crystallization method effectively enriched UFAs and improved the biological activities of the animal bone marrow oils.

All of the fatty acids obtained by n-hexane extraction and treated with enrichment methods of UFAs have demonstrated strong antimicrobial and antioxidant activity. Moreover, thier activity enhanced after enrichment of UFAs. In the further research, increasing the type of test microbial or introducing other biological activities could develop more bioactivities of bone marrow. Bone marrow oil is a kind of multicomponent fatty acid with different physicochemical properties. The physiological activities of UFAs are quite different, in order to make them better works their own function, it need to separate the mixture into a single and high purity products or reduce the content of UFAs. Therefore, four kinds of bone marrow powdered oils were prepared firstly, and the ratio of saturated to unsaturated fatty acids was checked. Then unsaturated fatty acids were prepared by urea saturation and freezing crystallization method, its need carry out several times, It provides basis for later microencapsulation or other uses. Therefore, in our study, four kinds of bone marrow powder oils were prepared firstly, and the ratio of saturated to unsaturated fatty acids was determined. Then UFAs were prepared by urea inclusion and low temperature crystallization method. It provides basis for later microencapsulation or other uses of animal bone marrow oil.

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