Bio-Medical Materials and Engineering 26 (2015) S2165–S2177
DOI 10.3233/BME-151522
IOS Press

Gas chromatography–mass spectrometry analysis of the free fatty acids in serum obtained from patients with Alzheimer's disease[1]

Yu Cui[a], Xiaobei Chen[b], Liyan Liu[b], Wei Xie[b], Yue Wu[b], Qunhong Wu[a,*] and Decai Wang[a,*]
[a]*Department of Social Medicine, College of Public Health, Harbin Medical University, Harbin, China*
[b]*Department of Nutrition and Food Hygiene, College of Public Health, Harbin Medical University, China*

Abstract. As the worldwide elderly population has grown, the incidence and prevalence of Alzheimer's disease (AD) has steadily increased. The differential lifelong exposure of populations to dietary fatty acids has raised concerns about the potential links between cognitive impairments and nutrition. However, few studies have addressed the levels of free fatty acids (FFAs) in AD patient serum. In this study, gas chromatography–mass spectrometry (GC–MS) was used to determine the levels of 15 serum FFAs in 31 AD patients and 33 healthy controls. The optimized methodology entailed the formation of methyl esters using 10% v/v H_2SO_4/CH3OH at 62°C for 2 hours. The linear range was 0.55–300 µg/mL, the range of recovery was 85.1–104.3%, and the detection limit was 0.03–0.08 µg/mL. Several FFAs in the AD patient significantly decreased when compared to the control, including three saturated fatty acids (C14:0, C16:0, and C18:0) and six unsaturated fatty acids (C16:1, C18:1, C18:2, γ-C18:3, C20:2, and C22:6). The serum level of C18:3 was significantly higher in the AD patients. The FFA profiles of the AD patients differed significantly from those of controls. The method effectively determined the FFA levels and could facilitate future studies regarding the relationship between AD and the metabolism of FFAs.

Keywords: Alzheimer's disease, fatty acid, GC–MS

1. Introduction

Alzheimer's disease (AD) is an insidiously, irreversible and progressive age related neurodegenerative disease. Although some AD cases are diagnosed as early onset, the vast majority of

[1] The authors would like to indicate that there are no actual or potential conflicts of interest pertaining to this paper.

[*] Address for correspondence: Qun-Hong Wu, Department of Social Medicine, College of Public Health, Harbin Medical University, 157 Baojian Road, Nangang District, Harbin 150086, P.R. China. Tel.: +86-451-8750-2836; Fax: +86-451-8750-2836; E-mail: wuqunhonghyd@163.com.
De-Cai Wang, Department of Nutrition and Food Hygiene, College of Public Health, Harbin Medical University, 157 Baojian Road, Nangang District, Harbin 150086, P.R. China. Tel.: +86-451-8750-2836; Fax: +86-451-8750-2836; E-mail: decaiwang@live.cn.

0959-2989/15/$35.00 © 2015 – IOS Press and the authors.

This article is published with Open Access and distributed under the terms of the Creative Commons Attribution and Non-Commercial License.

cases are diagnosed in patients over 65 years of age [1]. As the worldwide elderly population has increased, the prevalence of AD has also risen. Approximately 5.9% of all individuals aged 65 years or older in China will develop AD [2]. Furthermore, recent estimates have indicated that, by 2050, approximately 107 million people will be diagnosed with AD worldwide [3]. AD not only burdens caregivers, but also presents major adverse social, psychological, physical, and economic effects [4-6]. In 2010 alone, the worldwide costs associated with dementia were estimated to total 604 billion USD [7]. AD and other mental and neurological disorders cause significant morbidities and contribute to the global non-communicable disease burden [8]. Since the causes of AD are still unknown, more studies concerning factors that could predispose patients to the development of this disease should be conducted.

AD is characterized by the deposition of amyloid plaques in the brain. These amyloid plaques are comprised of the proteolytic fragments (AD) of neuronal amyloid precursor proteins (APP) and neurofibrillary tangles consisting of abnormal filamentous forms of the microtubule-associated protein tau. Although the biochemical events resulting in AD and tau deposition are not well understood, some studies have suggested that fatty acids could be contributory factors. FFAs have been shown to be capable of stimulating the formation of amyloid and tau filaments in vitro [9], similar to the neurodegenerative changes that occur during the early stages of AD [10, 11]. In addition, the oxidation products of two fatty acids, arachidonic acid and docosahexaenoic acid (DHA), are thought to play an important role in neurodegenerative processes [12]. Low omega-3 polyunsaturated fatty acid (PUFA) levels have been suggested to be associated with neurodegenerative disorders. Furthermore, the development of AD has been reported to be associated with low dietary consumption of fish, low n-3 PUFA intake, and suboptimal DHA status [13]. The cognitive benefits of high erythrocyte n-3 PUFA contents have also been investigated [14].

In previous studies, gas chromatography (GC) and flame ionization detectors (FID) have been used to determine serum FFA levels; however, the results obtianed using this method are sometimes inaccurate [15]. Another method involves the preparation of fatty acid derivatives, such as methyl esters, and silylation [16, 17]. Although silylation produces unstable derivatives, resulting in detector contamination, methyl ester preparation prevents this occurrence. Thus, in this study, a reliable method for the generation of methyl esters was developed. The proposed method was used to prepare the derivatives of serum FFAs obtained from AD patients and healthy controls. Then, GC–MS was used to compare the FFA profiles of the AD and control groups. Significant differences between the AD and control group FFA profiles were observed.

2. Materials and methods

2.1. Solvents and standards

The ≥99%-purity myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1n-9), heptadecanoic acid (C17:0), stearic acid (C18:0), oleic acid (C18:1n-9), linoleic acid (C18:2n-6), linolenic acid (C18:3n-3), γ-linolenic acid (C18:3n-6), cis-11, 14-eicosadienoic acid (C20:2n-6), arachidonic acid (C20:4n-6), cis-5, 8, 11, 14, 17-eicosapentaenoic acid (C20:5n-3), cis-4, 7, 10, 13, 16-docosapentaenoic acid (C22:5n-6), cis-4, 7, 10, 13, 16, 19-docosahexaenoic acid (C22:6n-3), tetracosanoic acid (C24:0), and selacholeic acid (C24:1n-9) from Sigma (St. Louis, MO, USA). H_2SO_4 (purity: ≥98.0%), CH_3OH(GC), n-Hexane (GC), ethyl acetate (AR), NaCl, (AR) Reagent Co. (Tianjin, China).

2.2. Subjects

Elderly subjects (n = 64, aged 65–90 years) were recruited from the Centre of Harbin Elderly Care Service in Heilongjiang Province of northern China. Of these patients, 31 (18 males and 13 females) had been previously diagnosed with AD. The AD diagnoses were based on the criteria established by the National Institute of Neurological and Communicative Disorders and the Stroke/Alzheimer's Disease and Related Disorders Association (NINCDS/ADRDA). The cognitive functionality of the AD patients was assessed using the Mini Mental State Examination (MMSE), and the severity of dementia was assigned using the Clinical Dementia Rating (CDR) scale. The control group was comprised of 33 healthy, education- and age-consistent elderly subjects (16 males and 17 females) rehabilitating in the center.

Potential subjects were excluded if they had other neurological, psychiatric, or physiological diagnoses (e.g., affective disorder, Parkinson's disease, hypertension, diabetes, and dyslipidemia) or uncorrectable vision or hearing loss. Subjects exhibiting signs of alcohol abuse, risk factors for vascular disease, or a history of significant relevant head injury or stroke were also excluded. CT scans and laboratory tests were used to exclude other brain disorders. The characteristics of the AD and control groups (age, gender, and education) are presented in Table 1. No significant differences ($p > 0.05$) in age (t test), education (t test), or gender (chi-square test) were noted in the AD and control groups. The AD patients in this study were receiving AchE inhibitors. None of the subjects were institutionalized, following a special diet, or taking anticoagulants, trace elements, or vitamin supplements. A three-day food record was randomly obtained from a representative sample of the subjects. All subjects are familiar with the ethical agreement signed, voluntary participation and quit, voluntary associated with diseases such as questionnaire survey and laboratory examination.

2.3. Sample collection and preparation

Fasting blood samples (3 mL) were collected via venipuncture into tubes by a trained professional; each blood sample was immediately centrifuged (3000 g, 10 minutes, 4ºC) and stored at –80ºC for further analysis. The quantifications of the free fatty acids were analyzed using GC-MS, the methodology of which was determined in a previous study [18]. Serum aliquots (200 µL) were quickly spiked with internal standard (IS) working solution (200 µL heptadecanoic acid C17:0, 200 µg/mL). Then, add 1 ml0. 05% v/v H_2SO_4, 3 ml ethyl acetate, mix all, centrifugal: 4000 r/min. Concentrated dry. After 2 mL of 10% v/v Sulfuric acid/Methanol was added to the residual and incubated at 62ºC for 2 hour, 2 mL of saturated NaCl and 2 mL of n-Hexane were added sequentially and mixed for 1 minute in order to extract the $C_7H_{12}O_4$. The resulting organic phase was evaporated to dryness using N_2 gas, and the samples were drawn into 0.1ml of n-hexane prior to analysis.

Table 1

Characteristics of the AD and control subjects

Parameter	Healthy controls (n = 31)	AD patients (n = 33)	p value
Age (yr)	75.6 ± 8.4	76.8 ± 7.7	0.558
Gender (M/F)	15/16	15/18	0.814
Education (yr)	7.2 ± 3.4	6.5 ± 2.8	0.287

Note: The age and education data are expressed as means ± SD; gender denotes the number of male and female subjects as M/F. No significant differences in these parameters existed in the AD and control groups ($p > 0.05$).

2.4. Analysis conditions

The *Gas chromatography–mass spectrometry* analysis was performed using a gas chromatograph coupled to an ion-trap mass spectrometer (Thermo Finnigan, USA) [19]. DB-WAX capillary columns are 30 m × 0.25 mm i.d. and 0.25-μm film thickness. Helium was used as a carrier gas at a flow rate of 1.0 Ml/min. The temperature and split ratio of the injector were 230°C and 1:10, respectively. The FFA methyl esters were separated at a constant flow rate according to the following temperature program: (a) 50°C for 2 minutes; (b) increase to 200°C at 10°C/minute; (c) 200°C for 10 minutes; (d) increase to 220°C at 10°C/minute; and (e) 220°C for 15 minutes. The transfer line was maintained at 230°C. The ITMS was operated in the EI and full scan monitoring (m/z 30–350) modes. The source temperature was equal to 230°C, and the electron energy was equal to 70 Ev.

2.5. Sample analysis

Free fatty acid contents of the plasma were determined using GC-MS after conversion to Weinreb amides [20]. The mass spectrometer was calibrated using solutions containing reference standard methyl ester derivatives; then, 1.0 Ml aliquots were injected for GC–MS. The qualitative analysis was based on the characteristic ions of the fatty acids methyl esters and their relative retention times. The quantitative analysis was based on the peak areas of the fatty acids. Next, a regression curve equation was obtained using the peak area and quantity of each applied standard. The regression curve was then used to determine the contents of the free fatty acid methyl esters based on their total ion chromatograms.

2.6. Statistical analysis

The statistical analysis was performed using SPSS 14.0 Trend (English Version). The results were presented as means ± the standard deviation (SD). The continuous variables were statistically evaluated using the t test. A p value of ≤0.05 was used to indicate statistical significance.

3. Results

3.1. Selection of the chromatographic conditions

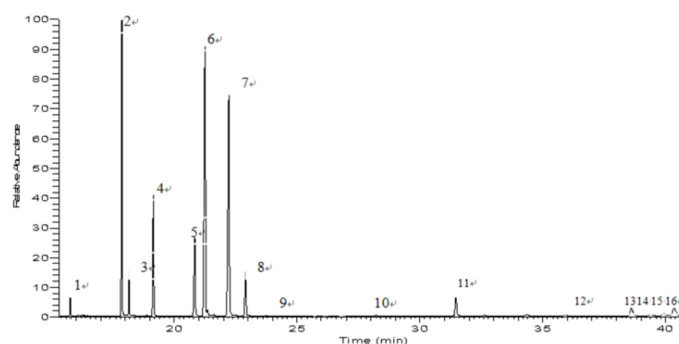

Fig. 1. Total ion chromatograms with the EI^+ scans of the FFA standards.
Note: 1. C14:0, 2. C16:0, 3. C16:1, 4. C17:0, 5. C18:0, 6. C18:1, 7. C18:2, 8. γ-C18:3, 9. C18:3, 10. C20:2, 11. C20:4, 12. C20:5, 13. C22:5, 14. C22:6, 15. C24:0, 16. C24:1.

The scans of the sixteen studied fatty acid methyl esters (including the C17:0 methyl ester) ranged from 30 to 350 m/z. In the preliminary experiments, the chromatographic peaks of the fatty acids (C18:0, C18:1, C22:5 and C22:6) were not entirely separate. The column temperature was optimized since it was a major factor governing chromatographic resolution. The three-stage temperature program ranging from 50°C to 230°C completely separated the sixteen fatty acid methyl esters. This technique was applied to all of the analyses. The total ion chromatograms of the standards and serum free fatty acid methyl esters are shown in Figures 1 and 2, respectively.

3.2. Selection of the characteristic ions

The FFA derivatives were identified by analyzing the characteristic ion (m/z) peaks obtained via hydrogen transfer, rearrangement, McLafferty rearrangement, i-judo fracture, double bond alpha-fracture, and carbonyl alpha-fracture. The characteristic molecular ion peaks for each species qualitatively analyzed. The peak intensities and characteristic ions of the sixteen fatty acid methyl esters (including the C17:0 methyl ester) are shown in Table 2; the mass chromatogram of each fatty acid methyl ester is presented in Figure 3.

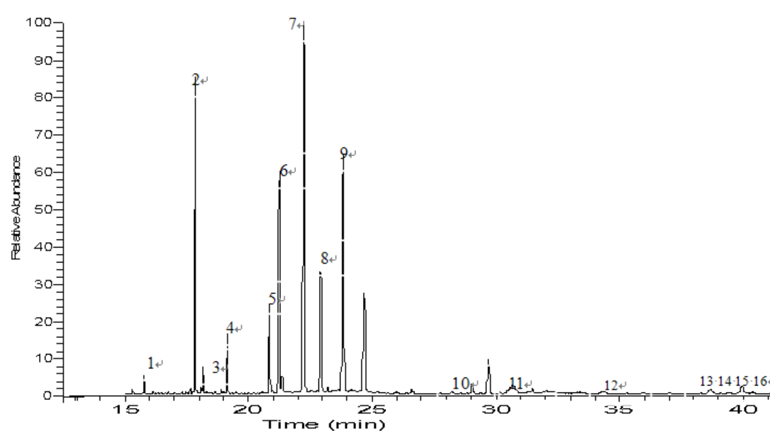

Fig. 2. Total ion chromatograms with the EI+ scans of the serum FFA methyl esters.
Note: 1. C14:0, 2. C16:0, 3. C16:1, 4. C17:0, 5. C18:0, 6. C18:1, 7. C18:2, 8. γ-C18:3, 9. C18:3, 10. C20:2, 11. C20:4, 12. C20:5, 13. C22:5, 14. C22:6, 15. C24:0, 16. C24:1

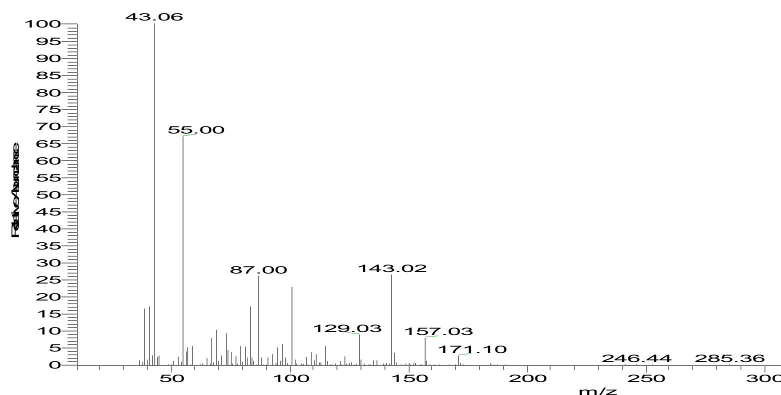

Fig. 3. Mass chromatograms with the EI^+ scan of the C14:0 methyl ester.

Table 2

Peak intensity and characteristic ion data of the serum FFAs

FFA	Characteristic ion	Relative peak intensity	FFA	Characteristic ion	Relative peak intensity
C14:0	43	100	C18:3	79	100
	55	68.06		91	50.75
	143	24.6		292	4.26
C16:0	43	100	C20:2	67	100
	87	27.08		81	46.95
	270	6.81		322	1.67
C16:1	55	100	C20:4	79	100
	67	98.62		91	94.82
	83	69.68		316	5.81
C17:0 [a]	43	100	C20:5	91	100
	87	24.89		79	72.57
	284	0		316	0.12
C18:0	43	100	C22:5	79	95.29
	74	6.05		91	100
	298	1.23		340	0.21
C18:1	55	100	C22:6	79	85.54
	67	94.47		91	100
	297	1.41		342	2.61
C18:2	67	100	C24:0	74	43.67
	79	74.37		143	100
	294	0.85		382	3.47
γ-C18:3	79	100	C24:1	55	100
	91	63.75		96	83.39
	292	0.21		380	2.48

Note: [a]C17:0, international reference standard.

Table 3

Calibration equations, correlation coefficients, and detection limits of the FFAs

Fatty acid	Retention time	Calibration equation[a]	Regression coefficient	Range (μg)	Limit of detection (μg/mL)	Limit of quantification (μg/mL)
C14:0	15.75	Y = 0.4414X – 0.2206	0.9964	2.48–12.50	0.05	0.10
C16:0	17.86	Y = 0.0289X + 0.418	0.9957	24.89–225.25	0.03	0.12
C16:1	18.15	Y = 0.0425X – 0.0822	0.9996	2.55–25.50	0.08	0.15
C18:0	20.82	Y = 0.0248X – 0.0479	0.9992	30.50–101.50	0.03	0.10
C18:1	21.22	Y = 0.0319X – 0.4984	0.9991	25.00–300.00	0.04	0.15
C18:2	22.19	Y = 0.0331X – 0.2653	0.9999	25.00–250.00	0.05	0.13
γ-C18:3	22.89	Y = 0.2107X – 0.4323	0.9995	2.65–12.80	0.08	0.16
C18:3	23.77	Y = 0.0689X – 0.0033	0.9991	0.55–25.00	0.08	0.20
C20:2	27.25	Y = 0.0483X – 0.0267	0.9988	0.65–15.50	0.08	0.15
C20:4	29.67	Y = 0.0269X –	0.9997	10.00–75.00	0.05	0.24

		0.034				
C20:5	31.45	Y = 0.0242X + 0.0328	0.9953	2.45–25.00	0.05	0.14
C22:5	38.61	Y = 0.0069X + 0.0051	0.9975	1.25–28.00	0.06	0.18
C22:6	39.91	Y = 0.0241X – 0.1348	0.9993	10.00–100.00	0.07	0.20
C24:0	39.37	Y = 0.0201X + 0.0483	0.9976	1.20–15.50	0.08	0.25
C24:1	40.35	Y = 0.0117X + 0.013	0.9967	2.45–22.80	0.05	0.27

Note: [a]X: quality of the fatty acids; Y: relative peak area = peak area of fatty acid/ peak area of internal standard C17:0; the retention time of the international standard was equal to 19.14 minutes.

3.3. Selection of the FFA methylation conditions

Since the fatty acid methyl ester yields were affected by the catalyst concentration, reaction time, and temperature, all of these parameters were investigated. The use of 10% (v/v) H_2SO_4/CH_3OH at 62°C for 2 hours was found to reliably generate methyl esters.

3.4. Linear range and detection limits

A series of mixed reference standard solutions were used to generate the regression curve for each standard based on the concentration applied to the peak area of each FFA (see Table 3). The detection limits were calculated based on the concentration of each FFA using the equation S/N (signal-to-noise ratio) = 10. The correlation coefficients ranged from 0.9953 to 0.9999 and the linear range was 0.55–300 μg/mL. The detection limit of each fatty acid ranged from 0.03 to 0.08 μg/mL (see Table 3) and the limit of quantification (LOQ) is from 0.10 to 0.27 μg/mL.

3.5. Accuracy and precision

In order to evaluate the accuracy and precision of the methodology, the FFA contents of three serum samples were determined. Then, different concentrations of mixed reference standards were added (ranging from 5 to 500 μg/mL), and extraction was performed. This allowed for the calculation of the recovery values, which ranged from 85.1% to –104.3% (see Table 4). Next, the low-, medium-, and high-concentration FFA standard solutions were added to the serum samples, and each was subjected to six determinations under optimal experimental conditions. The coefficients of variation in all cases ranged from 0.66% to 5.07% (Table 5).

3.6. Analysis of the AD serum samples

The serum FFA levels of the 31 AD patients and 33 control subjects were determined using the optimized methodology. As shown in Figure 4, the mean levels of the three saturated fatty acids (C14:0, C16:0, and C18:0 with p values of $p < 0.05$, $p < 0.01$, and $p < 0.05$, respectively) and six unsaturated fatty acids (C16:1, C18:1, C18:2, γ-C18:3, C20:2, and C22:6 with p values of $p < 0.01$, $p < 0.01$, $p < 0.05$, $p < 0.05$, $p < 0.01$, and $p < 0.01$, respectively) were significantly lower in the AD serum than in the control serum. In contrast, the C18:3 levels were significantly higher in the AD serum than in the control serum ($p < 0.05$).

Table 4

Recovery dates of the serum FFAs (n = 6 in each case)

Fatty acid	Background value (μg/mL)	Added (μg/mL)	Measured (μg/mL)	RSD (%)	Bias (%)
C14:0	6.72	5	11.51	5.8	95.7
	6.72	10	16.15	3.2	94.3
	6.72	15	21.36	4.1	97.6
C16:0	305.33	200	492.93	2.4	93.8
	305.33	300	600.53	3.2	98.4
	305.33	500	790.83	1.8	97.1
C16:1	18.52	10	28.37	3.4	98.5
	18.52	20	38.84	5.9	101.6
	18.52	30	49.54	1.1	103.4
C18:0	168.45	100	268.15	4.0	99.7
	168.45	150	321.30	3.6	101.9
	168.45	200	377.05	1.5	104.3
C18:1	250.50	100	347.90	2.7	97.4
	250.50	200	432.90	2.9	91.2
	250.50	300	545.40	1.3	98.3
C18:2	372.15	250	610.65	3.3	95.4
	372.15	350	709.90	2.8	96.5
	372.15	450	792.45	2.2	93.4
γ-C18:3	15.04	10	23.96	5.4	89.2
	15.04	20	34.00	6.3	94.8
	15.04	30	42.97	4.8	93.1
C18:3	6.15	5	10.48	5.9	86.5
	6.15	10	15.27	4.1	91.2
	6.15	15	19.55	3.7	89.3
C20:2	14.87	10	23.78	5.1	89.1
	14.87	20	32.57	6.5	88.5
	14.87	30	42.92	4.7	93.5
C20:4	135.26	50	184.06	2.3	97.6
	135.26	100	230.06	1.9	94.8
	135.26	150	282.86	3.5	98.4
C20:5	19.42	10	28.51	4.89	90.9
	19.42	20	39.19	2.02	98.9
	19.42	30	47.10	1.47	92.3
C22:5	54.10	20	72.23	1.20	90.7
	54.10	50	104.21	1.26	100.2
	54.10	100	141.59	0.89	87.5
C22:6	67.34	20	85.26	3.4	89.6
	67.34	50	113.09	4.1	91.5
	67.34	100	155.94	5.8	88.6
C24:0	110.61	100	195.71	0.46	85.1
	110.61	150	245.38	1.00	89.8
	110.61	200	278.27	1.70	87.3
C24:1	10.03	10	19.21	4.53	91.8
	10.03	20	28.62	5.43	93.0
	10.03	30	38.56	2.11	95.1

4. Discussion

GC coupled with FID has previously been used to determine serum FFA levels [14, 21, 22]. Although this method is highly sensitive, its dependability is questionable. Furthermore, qualitatively

Table 5

Precisional data of the serum FFAs (n = 6 in each case)

Fatty acid	Concentration (μg/mL)						Mean	RSD%	CV%
	1	2	3	4	5	6			
C14:0	8.11	8.16	8.30	8.21	8.29	8.07	8.19	1.00	2.89
	4.79	4.56	4.89	4.69	4.42	4.68	4.67	3.56	
	2.23	2.99	2.65	2.76	3.09	3.12	2.81	4.11	
C16:0	345.21	339.04	351.24	331.89	340.22	350.37	343.00	2.16	1.44
	764.40	747.56	745.23	752.23	736.32	749.98	749.29	1.23	
	969.08	959.22	977.11	952.30	966.00	973.05	966.13	0.94	
C16:1	50.92	51.02	51.47	51.38	51.19	51.77	51.29	0.61	1.26
	115.68	114.57	113.98	116.12	110.65	115.69	114.45	1.77	
	142.20	144.00	147.12	147.56	145.38	146.19	145.08	1.40	
C18:0	216.15	217.68	217.71	216.92	217.77	218.00	217.31	0.31	0.66
	444.93	435.86	440.36	440.05	430.12	437.25	438.10	1.14	
	630.10	625.72	624.51	626.79	631.56	632.33	628.50	0.52	
C18:1	459.22	460.00	469.70	453.82	455.50	465.11	460.56	1.30	1.16
	761.86	755.23	760.23	762.31	750.46	740.64	755.12	1.12	
	950.26	961.72	970.01	977.21	973.55	975.90	968.11	1.07	
C18:2	501.70	509.00	547.88	512.30	498.26	515.45	514.10	3.45	2.57
	805.33	797.22	802.36	804.02	785.23	793.78	797.99	0.96	
	209.03	198.76	190.12	202.37	197.36	205.20	200.47	3.30	
γ-C18:3	2.11	2.17	2.32	2.30	2.26	2.01	2.20	5.50	4.87
	6.23	5.91	6.12	6.01	5.32	5.67	5.88	5.67	
	9.75	9.38	9.09	9.00	9.77	9.31	9.38	3.44	
C18:3	6.29	6.01	6.57	5.93	6.26	5.47	6.09	6.21	5.07
	13.77	12.95	13.08	13.14	12.05	12.86	12.98	4.28	
	18.50	18.79	17.01	17.32	18.99	17.45	18.01	4.71	
C20:2	10.36	10.00	10.71	11.09	11.28	9.76	10.53	5.71	4.13
	16.16	15.78	16.14	15.96	14.96	15.14	15.69	3.30	
	26.33	26.71	26.32	26.69	26.97	26.01	26.51	3.37	
C20:4	100.33	104.70	112.31	114.20	109.71	111.16	108.74	4.80	2.82
	184.30	183.30	184.03	185.01	179.42	182.36	183.07	1.09	
	272.20	279.81	278.31	285.20	266.90	268.71	275.19	2.57	
C20:5	20.32	20.98	21.39	19.85	20.01	21.12	20.61	3.09	1.74
	56.17	58.30	57.12	58.19	58.18	56.73	57.45	1.57	
	126.09	127.38	127.75	126.47	125.99	127.00	126.78	0.56	
C22:5	34.85	34.91	33.26	34.01	33.57	33.89	34.08	1.97	0.93
	101.32	102.56	101.93	102.28	101.01	101.95	101.84	0.57	
	173.16	174.28	174.50	173.91	173.88	174.02	173.96	0.26	
C22:6	10.32	10.97	11.28	9.86	10.01	11.33	10.63	6.10	3.28
	33.01	32.08	32.45	32.67	31.25	30.94	32.07	2.55	
	74.96	76.87	74.32	75.67	75.06	75.99	75.48	1.19	
C24:0	15.71	15.03	14.87	15.21	15.56	14.62	15.17	2.73	1.44
	58.26	58.93	57.20	57.79	58.31	58.50	58.17	1.03	
	86.21	85.19	85.37	85.92	86.30	86.28	85.88	0.57	
C24:1	2.56	2.77	2.62	2.61	2.59	2.68	2.64	2.87	4.98
	4.89	4.14	4.92	4.99	4.61	4.43	4.66	7.15	
	7.76	7.68	7.01	6.98	7.00	7.17	7.27	4.93	

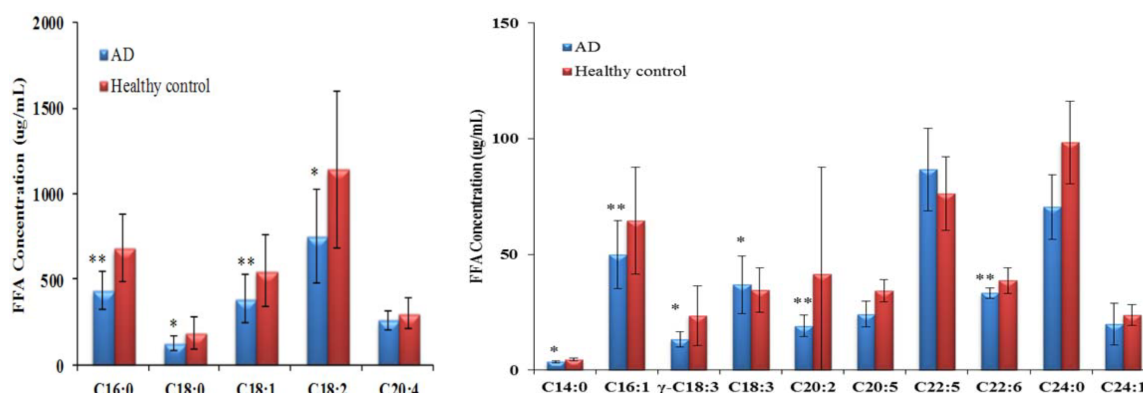

Fig. 4. Serum FFA concentrations of the AD and healthy control groups.

analyzing the target compounds in samples is difficult with this method. High-performance liquid chromatography (HPLC) can also be applied to serum FFA detection, but is less sensitive than GC coupled with FID [23]. The recent development of metabolomics has led to the more frequent application of GC–MS to human body fluid analyses [24, 25]. Metabolomics has been used to analyze the metabolites in patients with different diseases in order to understand their underlying mechanisms and the development of their biological markers.

In this study, an optimized method for the detection of serum FFA derivatives using GC–MS was established. Due to the high thermal stability and long separation times of fatty acids as well as the temperature limits of chromatographic separation columns, all of the serum FFAs were derivatized. Some catalysts commonly used in derivatization include BF_3/CH_3OH and H_2SO_4/CH_3OH. After exploring the types, concentrations, reaction times, and temperatures, an optimized method was established using H_2SO_4/CH_3OH. The optimum reaction conditions in this study included 10% v/v H_2SO_4/CH_3OH at 62°C for 2 hours.

The GC–MS analysis was also optimized in order to ensure the complete separation of all of the FFA derivatives, including the *trans* and *cis*-fatty acids. Under these conditions, the retention times of the *cis* molecules were significantly longer than those of the *trans* molecules. The MS identification of the characteristic ions generated from the saturated fatty acids via rearrangement, McLafferty rearrangement, alpha-fracture, gamma-hydrogen transfer, and i-judo fracture permitted the unambiguous identification of each unsaturated fatty acid. The double- and polyunsaturated fatty acids generated different ions via carbonyl and double-bond alpha-fracture, permitting the individual identification of these FFA derivatives.

In this study, the linear range, detection limit, accuracy, and precision of the proposed method were also investigated. The linear ranges of eleven fatty acids (C14:0, C16:1, γ-C18:3, C18:3, C20:2, C20:4, C20:5, C22:5, C22:6, C24:0, and C24:1) were 0.55–100 μg/mL; the other linear ranges (C16:0, C18:0, C18:1 and C18:2) were 24.89–300 μg/mL. The correlation coefficients ranged from 0.9953 to 0.9999, and the recovery ranged from 85.1 to 104.3%; the coefficients of variation (CV%) of the intra-day precision values of the high, medium, and low concentrations ranged from 0.66 to 5.07%, and the detection limits ranged from 0.03 to 0.08 μg/mL. These results indicated that the optimized experimental method suitably detected and analyzed the serum FFA contents.

All of the individuals in this study underwent evaluations, including medical history assessments, physical and neurologic examinations, laboratory tests, and neuropsychological assessments. The control group subjects exhibited no signs or symptoms of cognitive decline or neurologic disease,

including epilepsy. Subjects with hypertension, diabetes, and dyslipidemia were also excluded before the study. On this basis, the serum FFAs of 31 AD patients and 33 control subjects were analyzed using GC–MS. The mean levels of the individual free fatty acids in the two groups were subjected to pairwise comparisons. The results indicated significant differences in the FFA levels (including C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, γ-C18:3, C18:3, C20:2, and C22:6) of the AD and control groups. This suggested that the serum free fatty acids levels in the AD patients, notably the reduced levels of n-3 polyunsaturated fatty acids, could be associated with the onset and development of AD. The n-3 fatty acids, including C18:3, C20:5, and C22:6 (DHA), accounted for ~10% of the brain lipids. Some studies have reported that the n-3 PUFAs are closely associated with the AD. Morris and Wu found that consumption of fish with high n-3 fatty acids may reduce the risk of incident Alzheimer disease [26, 27]. DHA, as the important n-3 fatty acids, plays an important role in the functionality of cell membranes in the brain [28-30]. DHA has also been reported to be capable of modulating the neuronal production of AD, releasing and reuptaking neurotransmitters, and ameliorating neurodegenerative disease [29, 31, 32]. Several studies emphasize the neuroprotective properties of DHA-derived neuroprotectin D1 (NPD1) and its potential beneficial effects on AD [33, 34]. All of these studies demonstrated that the DHA is correlated to the AD.

DHA levels have also been found to be significantly reduced in AD patients in this study, which suggest that Fatty acid metabolic disorders in the body is the AD of a potential risk factors. Further studies combining metabonomic techniques must be conducted in order to screen for potential FFA biomarkers.

The fatty acids of human erythrocyte membranes are primarily comprised of docosahexaenoic acid (C22:6), arachidonic acid (C20:4, linoleic acid (C18:2), palmitic acid (C16:0), oleic acid (C18:1), and stearic acid (C18:0). In normal human plasma, FFA compositions are similar to erythrocyte membrane compositions, except FFA compositions include myristic acid instead of docosahexaenoic acid (C14:0). The fatty acid profiles of erythrocyte membranes can be affected by plasma FFAs [35]. Higher erythrocyte n-3 PUFA contents have been suggested to be associated with higher cognitive scores; however, this difference was only significant in the absence of the *APOE* ε4 allele [14]. Further studies addressing the possible relationship between AD and the fatty acids of human erythrocyte membranes should be conducted.

In this study, a method of serum fatty acid determination was optimized and used to conduct a preliminary analysis of the serum FFAs in AD patients. Nine FFA levels were significantly lower in the AD serum, and one FFA level was higher. The serum FFA levels of the AD and control groups differed. Thus, the differences in the FFA levels could have influenced the development of AD. The detection and quantification method developed in this paper could allow for the investigation of the relationship between FFA levels and AD. The results of this study could be used to perform a large-scale study on the potential relationship between serum FFA levels and the development of AD.

Acknowledgments

We would like to thank the Harbin Center of Elderly Care Services who provided invaluable assistance in collecting the serum samples.

Our project was supported by the National Natural Science Foundation of China under grant number 81273193.

References

[1] R. Brookmeyer, S. Gray and C. Kawas, Projections of Alzheimer's disease in the United States and the public health impact of delaying disease onset, American Journal of Public Health **88** (1998), 1337-1342.
[2] Z.X. Zhang, G.E. Zahner and G.C. Román, Dementia subtypes in China: Prevalence in Beijing, Xian, Shanghai, and Chengdu, Arch Neuro **62** (2005), 447-453.
[3] R. Brookmeyer, E. Johnson and K. Ziegler-Graham, Forecasting the global burden of Alzheimer’s disease, Alzheimer’s Dement **3** (2007), 186-191.
[4] J. Murray, J. Schneider and S. Banerjee, Eurocare: A cross-national study of co-resident spouse carers for people with Alzheimer's disease, II--A qualitative analysis of the experience of caregiving, International Journal of Geriatric Psychiatry **14** (1999), 662-667.
[5] J. Schneider, J. Murray and S. Banerjee, Eurocare: A cross-national study of co-resident spouse carers for people with Alzheimer's disease, I--Factors associated with carer burden, International Journal of Geriatric Psychiatry **14** (1999), 651-661.
[6] C.A. Thompson, K. Spilsbury and J. Hall, Systematic review of information and support interventions for caregivers of people with dementia, BMC Geriatrics **7** (2007), 18.
[7] World Alzheimer’s Report 2010: The global economic impact of dementia, London, Alzheimer’s Disease International, 2010.
[8] Political Declaration of the High-level Meeting of the General Assembly on the Prevention and Control of Non-communicable Diseases, 19-20 September 2011, New York, United Nations, 2011.
[9] D.M. Wilson and L.I. Binder, Free fatty acids stimulate the polymerization of tau and amyloid beta peptides, American Journal of Pathology **150** (1997), 2181-2195.
[10] N. Schuff, N. Woerner and L. Boreta, MRI of hippocampal volume loss in early Alzheimer’s disease in relation to Apo Egenotype and biomarkers, Brain **132** (2009), 1067-1077.
[11] P.A. Thomann, E. Kaiser and P. Schönknecht, Association of total tau and phosphorylated tau 181 protein levels in cerebrospinal fluid with cerebral atrophy in mild cognitive impairment and Alzheimer’s disease, Journal of Psychiatry Neuroscience **34** (2009), 136-142.
[12] M.R. Prasad, M.A. Lovell, M. Yatin, et al., Regional membrane phospholipid alterations in Alzheimer's disease, Neurochemical Research **23** (1998), 81-88.
[13] A.M. Tully, H.M. Roche and R. Doyle, Low serum cholesteryl ester-docosahexaenoic acid levels in Alzheimer's disease: A case-control study, British Journal of Nutrition **89** (2003), 483-489.
[14] L.J. Whalley, I.J. Deary and J.M. Starr, n–3 Fatty acid erythrocyte membrane content, APOE ε4, and cognitive variation: An observational follow-up study in late adulthood, American Journal of Clinical Nutrition **87** (2008), 449-454.
[15] Z.H. Chen, J. Miao and J.Y. Liu, Analysis of free fatty acids compositions in human serum by gas chromatography, Journal of Tianjin of Medical University **6** (2000), 142-144.
[16] L.S. Basconcillo and B.E. McCarry, Comparison of three GC/MS methodologies for the analysis of fatty acids in Sinorhizobium meliloti: Development of a micro-scale, one-vial method, Journal of Chromatography B-analytical Technologies in The Biomedical and Lift Sciences **871** (2008), 22-31.
[17] P.A. Golay, F. Giuffrida and F. Dionisi, Streamlined methods for the resolution and quantification of fatty acids including trans fatty acid isomers in food products by gas chromatography, Journal of Aoac International **92** (2009), 1301-1309.
[18] D.C. Wang, C.H. Sun and L.Y. Liu, Serum fatty acid profiles using GC-MS and multivariate statistical analysis: Potential biomarkers of Alzheimer’s disease, Neurobiology of Aging **33** (2013), 1057-1066.
[19] S. Michalski, R. Shaler and F.L. Dorman, The evaluation of fatty acid ratios in latent fingermarks by gas chromatography/mass spectrometry (GC/MS) analysis, Journal of Forensic Sciences **58** (2013), S215-220.
[20] S.J. Ubhayasekera, J. Staaf, A. Forslund, P. Bergsten and J. Bergquist, Free fatty acid determination in plasma by GC-MS after conversion to Weinreb amides, Analytical and Bioanalytical Chemistry **405** (2013), 1929-1935.
[21] C.O. Kangani, D.E. Kelley and J.P. Delany, New method for GC/FID and GC-C-IRMS analysis of plasma free fatty acid concentration and isotopic enrichment, Journal of Chromatography B-analytical Technologies in The Biomedical and Lift Sciences **873** (2008), 95-101.
[22] H.L. Lau, C.W. Puah and Y.M. Choo, Simultaneous quantification of free fatty acids, free sterols, squalene, and acylglycerol molecular species in palm oil by high temperature gas chromatography flame ionization detection, Lipids **40** (2005), 523-528.
[23] A. Kotani, Y. Miyaguchi and E. Tomita, Determination of organic acids by high-performance liquid chromatography with electrochemical detection during wine brewing, Journal of Agricultural and Food Chemistry **52** (2004), 1440-1444.
[24] M.Z. Cai, Y.C. Jin and H.Q. Wu, Simultaneous analysis of eight mental drugs with amide structure by GC-MS, Journal of Clinical Laboratory Analysis **26** (2007), 46-50.

[25] C. Boudrault, R.P. Bazinet and D.W. Ma, Experiment models and mechanisms underlying the protective effects of n-3 polyunsaturated fatty acids in Alzheimer's disease, Journal of Nutritional Biochemistry **20** (2009), 1-10.
[26] M.C. Morris, D.A. Evans and J.L. Bienias, Consumption of fish and n-3 fatty acids and risk of incident Alzheimer disease, Archives of Neurology **60** (2003), 940-946.
[27] S. Wu, Y. Ding and F. Wu, Omega-3 fatty acids intake and risks of dementia and Alzheimer's disease: A meta-analysis, Neuroscience and Biobehavioral Reviews **48** (2015), 1-9.
[28] M.R. Miller, J. Seifert and N.J. Szabo, Erythrocyte membrane fatty acid content in infants consuming formulas supplemented with docosahexaenoic acid (DHA) and arachidonic acid (ARA): An observational study, Maternal and Child Nutrition **6** (2010), 338-346.
[29] G.M. Cole and S.A. Frautschy, Docosahexaenoic acid protects from amyloid and dendrtic pathology in an Alzheimer's disease mouse model, Public Health Nutrition **18** (2006), 249-259.
[30] F. Panza, V. Frisardi and C. Capurso, Possible role of S-Adenosylmethionine, S-Adenosylhomocysteine, and polyunsaturated fatty acids in predementia syndromes and Alzheimer's disease, Journal of Alzheimers Disease **16** (2009), 467-470.
[31] M.C. Irizarry, Biomarkers of Alzheimer's disease in plasma, Neuroreport **1** (2004), 226-232.
[32] L.S. Libow, Alzheimer's disease: Overview of research and clinical advances, Managed Care Interface **20** (2007), 20-23.
[33] S. Lorente-Cebrián, A.G. Costa and S. Navas-Carretero, An update on the role of omega-3 fatty acids on inflammatory and degenerative diseases, Journal of Physiology and Biochemistry **71** (2015), 341-349.
[34] Y. Zhao, F. Calon and C. Julien, Docosahexaenoic acid-derived neuroprotectin D1 induces neuronal survival via secretase and PPARgamma-mediated mechanisms in Alzheimer's disease models, Plos One **6** (2011), e15816.
[35] G.Y. Yang, L. Li and S.Q. Zhan, Composition analysis of free fatty acid in erythrocyte membrane and plasma and their correlative study with membrane microviscosity, Chin Journal of Histochem Cytochem **8** (1999), 87-90.