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Research Article

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Determination of fatty acids in maternal serum by gas chromatography and mass spectrometry to evaluate the association with mental retardation in children

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ABSTRACT

Here a useful gas chromatography mass spectrometry method for determining levels of fatty acids in the maternal serum was developed and validated. Forty-five mixed standards of fatty acids methyl ester of more geometrical isomers and biological significance were separated on a capillary column with higher polarity and thinner film thickness. The total run time was approximate 36 min. Twenty serum fatty acids methylated by a H_2SO_4-CH_3OH-toluene mixture were subsequently identified. Measurements for each fatty acids methyl ester were linear over a wide range (0.05–100μg/mL, correlation coefficient > 0.99).The limits of detection and quantification for the targeted fatty acids were <9 and 22 ng/mL, respectively, satisfactory recoveries occurred in 75.07–98.09% of cases, and the relative standard deviation for each fatty acids was <12%. On a conditional logistic regression model, a high level of eicosapentaenoic acid was a protective factor against a low development quotient. This method was successfully applied to evaluate the association between maternal fatty acids level in early pregnancy and mental retardation in 2-year-old children.

Keywords: Gas Chromatography and Mass Spectrometry; fatty acid; early pregnancy; mental retardation

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INTRODUCTION

Since the brain undergoes a growth spurt in the third trimester of pregnancy and the neonatal period, many studies have investigated the effect of polyunsaturated fatty acid(PUFA) supplementation during these periods on the neurobehavioral development of children [1, 2].However, few studies have examined the association between maternal PUFA, positional, and *trans* fatty acids(FA) composition in the earlier weeks of gestation and the neurological conditions of children [3, 4].

To date, the simultaneous analysis of *cis/trans* FA has been extremely challenging and complex due to the wide range of positional monoene, diene, and triene isomers within biological fluids, while the analysis of FA in biofluids has yet to reach its full potential. Accordingly, a practical and reliable method for determining FA is becoming of increasing interest for basic research and human health. Various analytical approaches for FA have been discussed, such as gas chromatography (GC) (Wang et al., 2009, [5], GC and mass spectrometry (GC/MS) [6-8],high performance liquid chromatography (HPLC) [9, 10], and HPLC or ultra-performance liquid chromatography with

MS [11].GC with flame ionization detection (GC/FID)is the traditional strategy despite its important limitation of not providing FA structure information. Otherwise, GC/MS is still a relatively low-cost alternative that provides high separation efficiency for resolving complex biological mixtures [12]. The aims of this work were to optimize a reliable method for determining different FA isomers and prove its applicability for examining the association between maternal serum FA levels at 13 weeks' pregnancy and mental retardation in their children. This is the first prospective study of its kind in China.

EXPERIMENTAL SECTION

Reagents and Chemicals

A commercial standard mixture containing 37 fatty acids methyl ester (FAME,10 mg/mL; cat no. 47885-U) was purchased from Sigma (St. Louis, MO, USA). The eight FAME standards (100 mg each) – C9:0, C18:1 n7c, C18:2 n911t, C22:4 n6, C22:5 n3, C22:5 n6, C16:1 n7t, and C18:1 n7t– were obtained from Nu-Chek Prep, Inc. (Waterville, MN, USA). The 45 mixed standards consisted of the above FAME. The 20 FA standards (100 mg each) – including C12:0, C14:0, C16:0, C18:0, C16:1 n7c, C18:1 n7c, C18:1 n9c, C16:1 n7t, C18:1 n7t, C18:2 n6c, C22:2 n6, C18:3 n3 (ALA), C20:5 n3 (EPA), C22:5 n3 (DPA), C22:6 n3 (DHA), C18:3 n6 (GLA), C20:3 n6 (DGLA), C20:4 n6 (AA), C22:4 n6, and C22:5 n6 – were obtained from Nu-Chek Prep, Inc. All of the standards were prepared in 0.01% (w/v) butylated hydroxytoluene (BHT; Sigma, St. Louis, MO, USA). The 0.05% H_2SO_4 and H_2SO_4:CH_3OH:toluene(5:90:5,v/v)solutions were freshly prepared by the dilution of H_2SO_4 (purity > 98.0%) with methanol. The *n*-hexane, ethyl acetate, methanol, and toluene were of chromatographic grade, while the other chemicals were of analytical grade.

Chromatography and Mass Spectrometry

The analysis was developed, validated, and performed on a GC/MS with an inert mass selective detector (GC 7890A, MS 5975C equipped with a 7693 auto sampler; Agilent Technologies, Shanghai, China). Mass spectra and retention times were acquired on a capillary column: DB-23 (60 m × 0.25 mm I.D., 0.15 μm film thickness; Agilent Technologies). The GC oven program started at 45°C (hold time, 2 min) and was increased 25°C/min to 105°C(hold time, 2 min), 15°C/min to 190°C (hold time, 12 min), and finally 1.5°C/min to 230°C (hold time, 2 min). The injector and detector temperatures were 250°C and 260°C, respectively. The ion source temperature was set to 150°C, while the mass spectrometer was operated in the electron impact mode at 70 eV in the scan range of 50–500 m/z. Helium (purity≥ 99.9996%) was used as the carrier gas with a flow rate of 1.0 mL/minute. Aliquots (1.0 μL each) were injected with a splitless ratio. Peaks were identified by comparing retention times and mass spectra of the FAME reference compounds. The qualitative ions detected using selected ion monitoring (SIM) are shown in Table 1, while the quantitative ions including *m*/*z* 74, *m*/*z* 55, *m*/*z* 67, and *m*/*z* 79 were determined after a solvent delay of 5.7 min throughout the run.

Preparation of Stock and Working Solutions

A standard stock mixture containing 45 of the FAME was prepared in *n*-hexane by dissolving of the appropriate amounts of the selected standards and stored at −40°Cuntil use. The working standard solutions (FAME) were prepared at concentrations of 0.05–100 μg/mL by dilution of the standard stock solution with hexane. The other standard mixture containing 20 FA was prepared in methanol as described above.

Sample Preparation

Non-fasting blood samples (5 mL) collected by antecubital venipuncture into evacuated serum tubes were taken from the mothers at 13 weeks'gestation enrolled in the China-Anhui Birth Cohort Study (C-ABCS). The serum samples (added to 0.01% BHT w/v) were stored at –80°C until analysis.

Extraction and Derivation of FA

The study aimed to detect the saturated, monounsaturated, diunsaturated, and PUFA (SFA, MUFA,DUFA,PUFA) levels within the maternal serum. Samples were prepared according to the reported method but with optimization [6, 13, 14].A 200-μL serum sample was added to 50 μL of 0.05% H_2SO_4, vortexed for at least 30 seconds, extracted with 2 mL of ethyl acetate using a vortex mixer for 60 seconds, and then centrifuged at 4000 rpm for 10 minutes at 4°C. The ethyl acetate phase was evaporated to dryness under nitrogen stream, followed by the addition of2 mL of the H_2SO_4-CH_3OH-toluene (5:90:5,v/v) mixture to the residue and incubated at 75°C for 1 hour with shaking every 20 minutes. Thereafter, the samples were cooled at room temperature and1 mL of saturated NaCl solution and 2 mL of hexane were sequentially added and mixed for 60 seconds by shaking using a vortex to obtain the targets. The organic phase was evaporated to dryness under nitrogen stream gas and the residue was re-dissolved in 200 μL of *n*-hexane, filtered using polytetrafluorethylene disc filters with 0.2μm pores, and stored at -20°C prior to the analysis.

Method Validation

External Calibration and Linearity

The calibration ranges for FAME were established using standard solutions. The following series of concentration gradients was created: 100, 50, 20, 10, 5, 4, 1 0.5 and 0.05μg/mL. Two calibration curves were generated for each FAME. One calibration curve was generated with 0.05–5μg/mL standard solutions for serum samples containing <5μg/mL and another with 5–100 μg/mL standard solutions for real samples where any of the species was present at concentrations >5μg/mL.

Recovery and Precision

The recoveries were calculated by comparison of the FA content of the spiked serum with the basal FA content of the untreated serum. The serum was spiked with known amounts of standard FA (0.05 and 0.5μg/mL) prior to the extraction. The intra- and inter-day assay (RSD%) was used to validate the method precision by determining the standard-added sample. Intra- and inter-day assay precision were determined five times on the same day and continuously for 5 days at the quality control concentrations (0.05and 0.5μg/mL) of the 20 FA standards in real serum.

Sensitivity

The limit of detection (LOD) and limit of quantification (LOQ) were defined as the concentrations providing signal-to-noise ratios of 3 and 10, respectively. In this method, the standard FAME mixture at the lowest concentration(0.05μg/mL) was selected to achieve a signal-to-noise ratio.

Specificity

A fine separation of *cis*/*trans* and positional isomers FAME, such as C16:1, C18:1, C18:2, and C18:3 in the standard mixture and serum samples, was used as a typical scheme during the method optimization.

A *nested case-control study*

The C-ABC Srecruited pregnant women from Ma'anshan city of Anhui province in China. A detailed description of the cohort study protocol was published elsewhere [15].A total of811 pregnant women from the C-ABCS (n = 4,669) during the first trimester were selected in this study from April to October 2011. When their infants reached 2 years of age, their mental and psychomotor development levels were assessed using the Bayley Scales of Infant Development of China Revision (BSID-CR). A total of 43 children were assigned to the low development quotient group (case) and 129 children were in the normal intelligence group (control) in accordance with the purpose of the study. The FA concentrations in population data analysis were expressed as μmol/L. Statistical analysis was performed using statistical software SPSS Statistics version 16.0 (SPSS Inc., Chicago, IL, USA). Written informed consent was obtained from the parents. Ethical approval for the study was obtained from the Ethics Committee of Anhui Medical University.

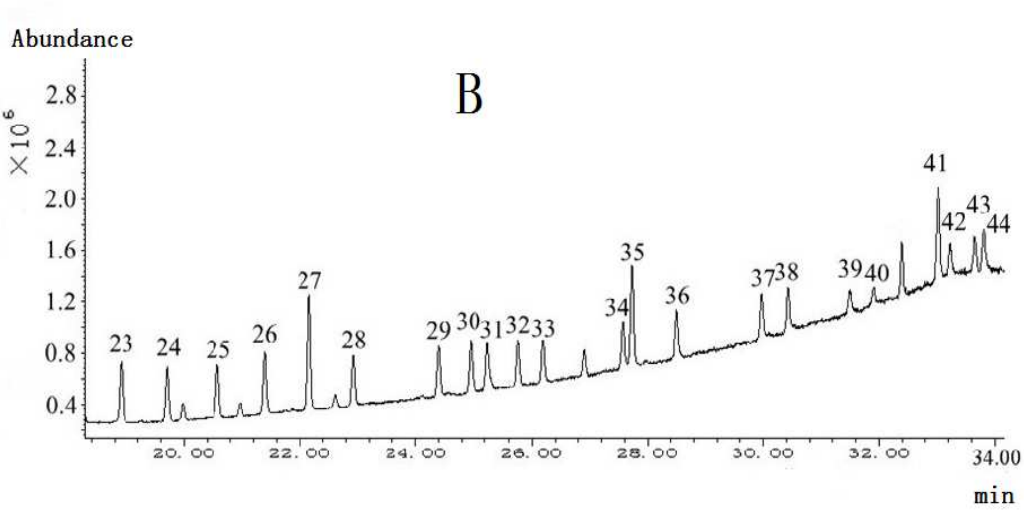
RESULTS AND DISCUSSION

Method Optimization

The proper choice of the stationary phase plays a pivotal role in improving a GC method. According to other studies [16, 17],to obtain satisfactory resolution of these FAME, different stationary phases such as wax,HP-5MS, DB7MS, and SP-2560 types should be used. We found that even with optimized chromatography conditions, overlapping elution of some isomers still occurred, so we ultimately chose the cyanopropyl stationary phase on a DB-23 column with 50% cyanopropyl-methylpolysiloxane. In terms of separation of the geometric or *cis/trans* FAME isomers such as C18:1 and C18:2,using columns with different lengths and film thicknesses, the DB-23 column with a 60-meter length and 0.15-μm film thickness could provide interesting possibilities in the elution behavior. Although the mixture contained 45 FAME standards, it is a pity that the butyric acid (C4:0) methyl ester was eluted with the solvent and not included in data set (Table 1, Fig. 1), so lighter alkanes that eluted with the solvent were similarly omitted from the study [18]. After optimization of the temperature gradient program, full resolution over a wide range of FAME can be achieved(Figs. 1, 2). No overlap in FAME was found using SIM. Regarding the total run time, the value was approximately 40 min in the reported method, which was longer than the running time in the present study [19, 20].

Table.1 Positional and geometrical FA isomers, retention times (RT), fragment ions used for quantification and Characterization of the FAME. The number refers to the chromatogram shown in Fig.1

Number	FAME	Retention time(min)	Quantitative ion(m/z)	Characteristic ions(m/z)		
1	C6:0	6.059	74	116	74	87
2	C8:0	7.209	74	144	74	87
3	C9:0	7.823	74	101	115	87
4	C10:0	8.466	74	172	74	87
5	C11:0	9.142	74	186	74	87
6	C12:0	9.868	74	143	74	87
7	C13:0	10.663	74	199	74	87
8	C14:0	11.558	74	199	74	87
9	C14:1n9c	11.995	55	123	55	69
10	C15:0	12.581	74	143	74	87
11	C15:1n10c	13.103	55	222	55	69
12	C16:0	13.796	74	270	74	87
13	C16:1n7t	14.076	55	236	55	111
14	C16:1n7c	14.266	55	268	55	97
15	C17:0	15.267	74	270	74	87
16	C17:1n10c	15.861	55	268	55	69
17	C18:0	17.115	74	298	74	87
18	C18:1n7t	17.456	55	296	55	97
19	C18:1n9c	17.576	55	264	55	69
20	C18:1n9t	17.704	55	296	55	83
21	C18:1n7c	17.877	55	222	55	69
22	C18:2n9t	18.248	67	263	67	109
23	C18:2n6c	18.915	67	294	67	81
24	C18:3n6	19.711	79	292	79	95
25	C18:3n3	20.577	79	261	67	79
26	C18:2n9c11t	21.393	67	280	67	55
27	C20:0	22.163	74	312	74	87
28	C20:1n9	22.922	55	264	55	69
29	C20:2n6	24.393	67	308	67	81
30	C21:0	24.946	74	326	74	87
31	C20:3n6	25.238	79	320	67	79
32	C20:4n6(AA)	25.762	79	304	67	79
33	C20:3n3	26.191	79	320	55	95
34	C20:5n3 (EPA)	27.572	79	316	91	79
35	C22:0	27.724	74	340	74	87
36	C22:1n9	28.503	55	338	55	69
37	C22:2n6	29.971	67	336	67	81
38	C23:0	30.426	74	354	74	87
39	C22:4n6	31.491	79	119	105	91
40	C22:5n6	31.904	79	105	91	105
41	C24:0	33.021	74	368	74	87
42	C22:5n3 (DPA)	33.223	79	119	91	105
43	C22:6n3 (DHA)	33.639	79	313	79	91
44	C24:1n9c	33.845	55	348	55	97



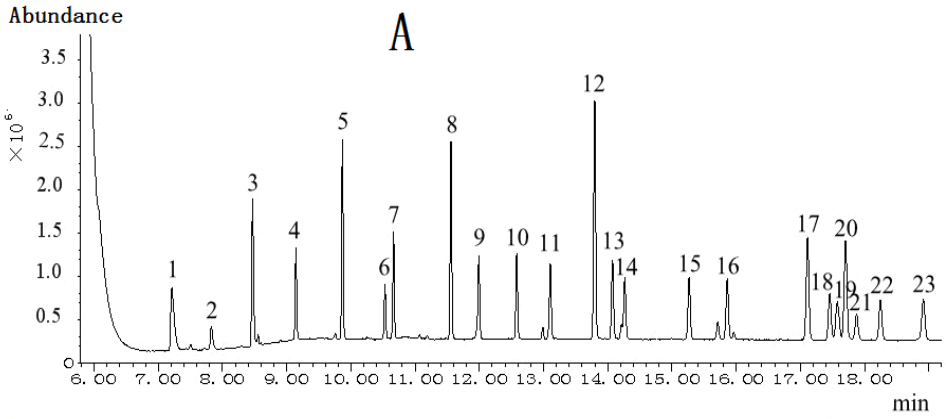


Fig.1.（A and B）The total ion chromatogram of 45 mixed standards of FAME at 40μg/mL（except for C4:0 FAME, showing a satisfactory separation of positional and geometric isomers of the mono-, di- and tri-unsaturated ones .Peak numbers in chromatograms are labelled in Table 1

A recent study showed that 44 different FAME could be separated in a short amount of time (17.2 min) on a highly polar BPX70 column, but fewer c*is/tran*-C18:1 and C18:2 FAisomers of greater significance were included than in our study [21].During transesterification, a boron trifluoride (BF_3)–methanol reagent was added to the mixture to achieve a higher transesterification ratio because BF_3-methanol is harmful if inhaled or absorbed through skin. We ultimately preferred the H_2SO_4-catalyzed FA transesterification method. We compared several transesterification reactions in our experiments, and all tested derivation methods were acid-catalyzed methylations using 5% H_2SO_4,10% H_2SO_4, or 5% HCl in methanol (containing 5% toluene) at 75°C, 5% H_2SO_4 resulted in a higher methanolysis yield. Thereafter, the reaction yield at 75°C was evaluated at several time points (30, 45, 60, and 90 min). The maximum derivation yield was obtained at 60 min. Overall, we believe our procedure is more practical for most researchers since a standard GC/MS instrument can be used without the need for an additional apparatus or instrument modification.

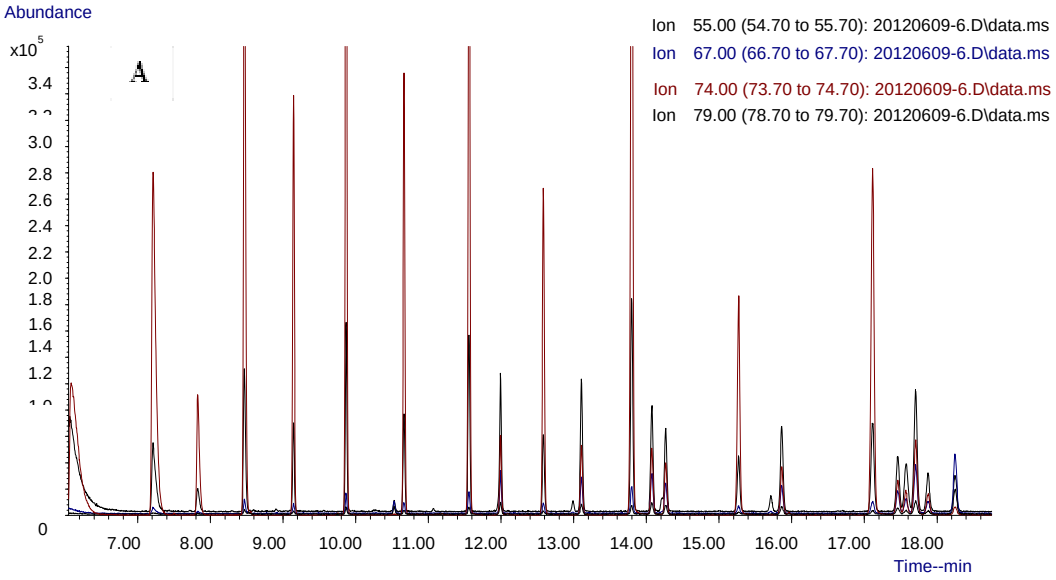
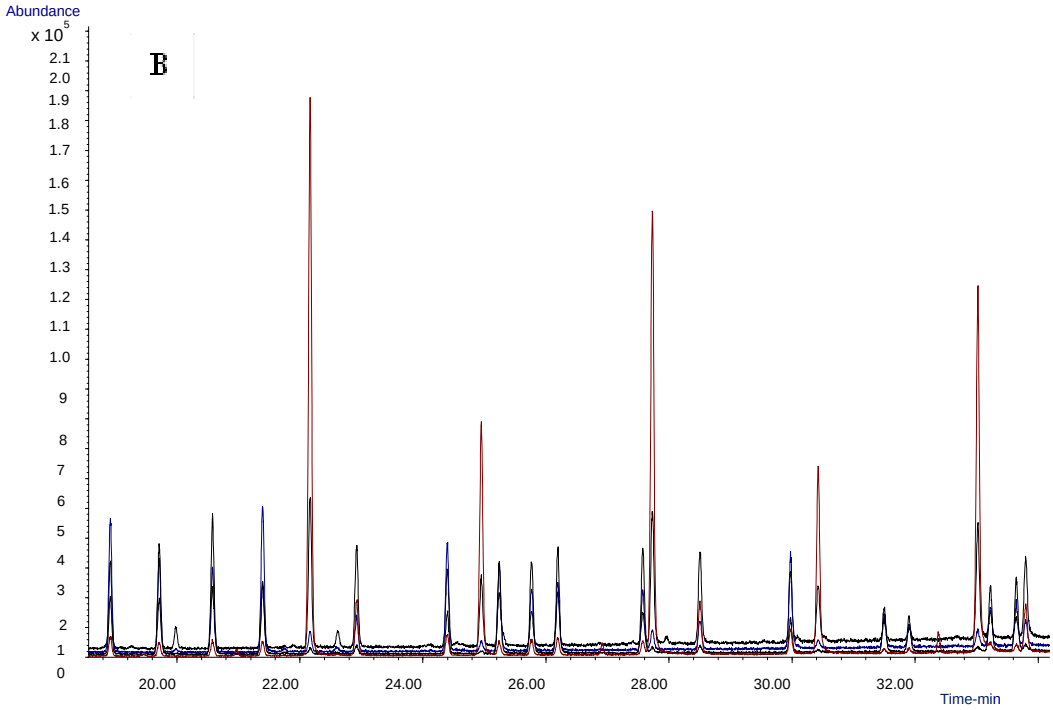


Fig.2. （A and B）Characteristic or quantitative ions chromatography of corresponding 44 standard FAME in merged format from the Fig.1



Method Validation

Due to the fact that the concentration range of FA in human serum could be highly variable, quantitation of the identified FA was performed using two calibration curves with a mixture of representative FAME standards. It was previously difficult to simultaneously measure a wide spectrum of FA using earlier reported methods [22]. Generally speaking, in this study, the calibration lines were linear under our conditions and showed regression line coefficients (SFA $R^2 \geq 0.9970$, MUFA $R^2 \geq 0.9921$, DUFA $R2 \geq 0.9903$, PUFA$R^2 \geq 0.9912$).

The Assay recoveries and precisions are shown in Table 2. The recoveries were in the range of 75.07–98.09% and the RSD% for each FA derivative was <12%. The FA standards with concentrations of 0.5 μg/mL had RSD%< 10%, whereas those at 0.05μg/mL had RSD% > 10%, similar to the previously reported method [21, 23].

Table. 2Recoveries , Precisions and Sensitivities of20 fatty acids derivatives after transmethylation for 1 h at 75°C , in serum sample spiked with the fatty acids at 0.5 and 0.05 μg/mL (Mean ± SD, n=3)

Fatty acid	Inter-day Recovery				Intra-day Recovery				Sensitivity (ng/mL)	
	0.5 μg/ mL(spkiked))		0.05 μg/ mL		0.5 μg/mL		0.05μg/ mL			
	Recovery	RSD%	Recovery	RSD%	Recovery	RSD%	Recovery	RSD%	LOD	LOQ
C12:0	98.09±1.20	1.23	95.56±1.92	1.89	96.34±1.07	1.11	90.10±4.58	5.09	4	9
C14:0	95.23±3.48	3.49	91.23±2.75	3.13	94.67±2.20	2.34	89.21±3.37	3.79	4	10
C16:0	97.56±0.94	0.97	93.09±2.79	3.19	91.23±3.83	4.21	91.02±6.39	7.03	3	8
C18:0	94.12±0.92	1.09	90.21±2.02	2.31	97.27±0.94	0.99	89.07±1.09	1.23	3	9
C16:1n7c	91.23±3.67	4.13	93.07±7.54	8.32	90.41±3.79	4.21	87.23±5.21	5.99	4	19
C18:1n7c	93.78±5.38	5.78	89.19±7.05	7.92	96.20±7.80	8.13	90.42±3.28	3.65	9	17
C18:1n9c	90.23±4.48	4.98	88.67±6.09	6.56	93.30±5.57	5.99	91.64±7.63	8.33	6	20
C16:1n7t	92.19±2.71	3.33	91.08±5.16	5.67	95.23±6.43	6.77	89.12±7.02	7.89	6	15
C18:1n7t	89.09±7.23	8.31	90.78±3.76	4.19	90.78±4.83	5.33	87.09±7.03	9.02	4	17
C18:2n6c	87.05±6.98	7.89	86.71±6.21	6.98	91.78±6.71	7.32	85.01±7.04	3.78	6	16
C22:2n6	79.14±7.14	9.08	77.67±8.57	10.21	80.01±7.73	9.66	79.09±7.05	11.06	6	21
C18:3n3	85.32±5.17	6.32	86.71±7.83	9.11	81.08±7.23	8.93	76.01±7.37	9.71	7	19
C20:5n3	78.17±4.34	5.38	76.09±5.38	7.09	79.09±3.69	4.67	75.10±2.46	3.29	9	20
C22:5n3	83.32±6.68	8.21	81.62±5.31	6.56	85.20±4.43	5.21	79.20±3.86	4.89	4	13
C22:6n3	81.01±2.86	3.44	80.68±1.85	2.33	83.05±5.79	6.98	86.12±2.00	2.32	5	22
C18:3n6	85.67±5.90	6.89	82.98±4.87	5.89	81.50±5.92	7.32	88.25±6.23	7.09	6	14
C20:3n6	82.21±5.79	7.33	84.52±7.61	9.33	83.10±7.93	9.56	76.01±4.99	6.57	5	17
C20:4n6	79.04±7.19	9.09	87.27±9.27	10.66	77.09±8.99	11.67	75.07±6.01	8.02	4	13
C22:4n6	80.01±8.08	9.81	82.61±9.28	11.32	81.32±7.12	8.79	89.18±8.05	9.05	3	11
C22:5n6	77.56±5.51	7.11	80.54±6.65	8.32	79.20±4.81	6.09	84.24±4.47	5.32	4	15

Notes : RSD relative standard deviation, SD standard deviation, LOD limit of detection, LOQ limit of quantitation.

The LOD and LOQ for the targeted FA were <9 and 22 ng/mL, respectively. As expected, the use of the SIM mode for quantification greatly increased sensitivity compared to the use of extracted ions. In addition, compared with the other published method, the proposed assay had higher MUFA and PUFA sensitivities[24,25].

The chromatographic procedure provides baseline separation between saturated and unsaturated FAME standards of different chain lengths as well as between most positional isomers, which might provide a valuable hint. A representative chromatographic separation of long-chain saturated and unsaturated FA (>12 carbons) included in maternal serum is shown in Figure 3. The data showed that the temperature gradient and column selection achieved high peak resolution.

The identified *cis/trans* FAME were separated completely in the 45 standards mixture or real serum. These peaks could be more completely separated from adjacent peaks than the ones published [26]. Compared with our approach, the performance of liquid chromatography–electrospray ionization–MS (ESI-MS) for FA separation was poorer; therefore, ESI-MS was less ideal for determining FA content despite its higher sensitivity since it could not better separate the geometric and positional isomers [12].

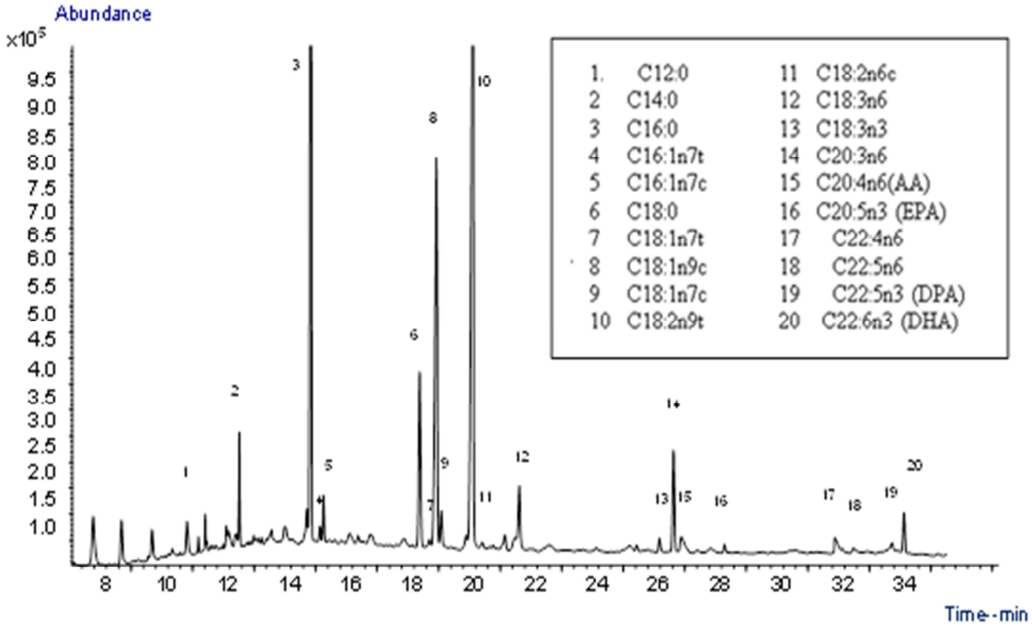


Fig.3. The total ion chromatogram of 20 kinds of transesterificated FFA in maternal serum, including geometrical (cis/trans) and positional isomers of biological importance

Application

After the data processing by analysis of covariance, there was no significant difference in total *trans* FA between the cases and controls, but the serum levels of total PUFA (F = 7.309, P = 0.008), n-3 PUFA (F = 7.971, P = 0.006), n-6 PUFA (F = 4.100, P = 0.045), DHA (F = 6.377, P = 0.013), EPA (F = 11.803, P = 0.001), AA (F = 4.747, P = 0.031), DPA (F = 6.115, P = 0.015), EPA + DHA (F = 7.933, P = 0.006), and DHA + AA (F = 6.618, P = 0.011) showed a statistically significant difference (P < 0.05) (Fig. 4). A conditional logistic regression model was fitted, a high level of EPA was a protective factor against a low development quotient (β =30, SE = 0.43, Wald value = 9.04, P = 0.003, odds ratio = 0.27, 95% confidence interval, 0.12–0.64) after the adjustment for confounding factors including the mother's education and bodyweight, child's age, and relative psychiatric diseases.

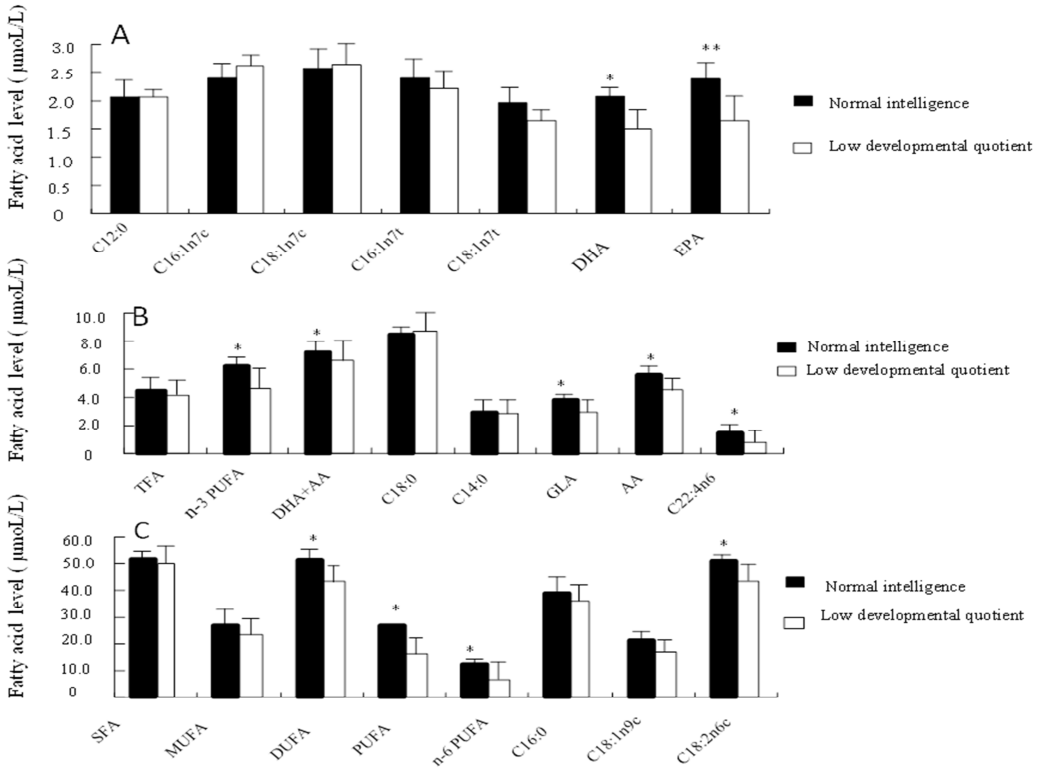


Fig.4.There was no significant difference in *trans* FA ,SFA levels between the cases and controls, but the total PUFA, n-3 PUFA, n-6 PUFA, and DHA, EPA, AA,C22:4n6 (DPA), EPA + DHA, DHA + AA showed significant difference. *:*P*< 0.05, **: *P* < 0.05,as compared with the controls

CONCLUSION

Here we presented a reliable and sensitive method for FA profiling of positional and geometrical isomers. Our findings indicated that the GC/MS approach can offer a technical platform for the comprehensive quantification of FA in serum. A low EPA level might be a predictor of mental retardation, especially when no other plausible factors can be identified.

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