Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 481–486

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Short communication

Plasma fatty acids metabolic profiling analysis of coronary heart disease based on GC–MS and pattern recognition

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ARTICLE INFO

Article history:
Received 13 June 2008
Received in revised form 16 October 2008
Accepted 18 October 2008
Available online 28 October 2008

Keywords:
Coronary heart disease
GC–MS
Metabolic profile
Patterns
Traditional Chinese Medicine

ABSTRACT

A simple approach for comprehensive profiling of fatty acids in human plasma based on gas chromatography–mass spectrometry (GC–MS) was described and validated. With this method, plasma fatty acid metabolic profiles of 23 coronary heart disease (CHD) patients and 25 healthy subjects were determined. Pattern recognition technology was used to establishing differences in the metabolic profiles of these two groups. Furthermore, CHD patients with two different patterns in Traditional Chinese Medicine clinical practices could be classified by the corresponding metabolic profiles, and several potential biomarkers were discussed.

1. Introduction

Coronary heart disease (CHD), as one of the most mortal diseases, is afflicting millions of people every year [1–3]. Till now, researchers have paid close attention to the correlation between blood lipid level and CHD. And plasma fatty acids were emphasized, for they not only provide an important energy source as nutrients, but also act as signaling molecules in various cellular processes in cardiovascular risks [4].

Nowadays, with the development of system biology, novel methods and techniques emerged to meet the needs of exploring the complex biological system. Metabolomic analysis has been shown to provide useful information in clinical diagnostics of some diseases, such as diabetes mellitus and liver failure [5–7]. Also, a ^{1}H-NMR-based metabonomics method has been established for rapid and noninvasive diagnosis of the presence and severity of coronary heart disease [8]. However, metabolic profile and especially lipid metabolic profile analysis were not involved. "Metabolic profile" is a spectrum of the composition and abundance of the metabolites, which can be used to monitor changes over time and in response to particular stimuli [9]. And lipids metabolic profile will contribute to appreciating how lipids react in a biological system and providing a powerful tool for elucidating the mechanism of lipid-based disease, screening novel biomarker, and monitoring clinical pharmacologic therapy [10].

Researchers have developed methodologies to monitor lipid composition in biological samples. Gas chromatography–mass spectrometry (GC–MS) has been a technique widely used for the identification of fatty acids in biological mixtures. Methyl esters are used almost universally for GC analysis of fatty acids [11,12]. Boron trifluoride (BF_3) in methanol is the most commonly used reagent for the derivatization step [13]. In addition to this method, there have been some reports on direct derivatization methods for the fatty acids in plasma extracts under selective conditions [7,14]. Recently, several benzylation, silylation and amide reagents were developed for robust and gentle derivatization of fatty acid for GC analysis, such as pentafluorobenzyl (PFB) [15], trimethylsilyl (TMS) [16], *tert*-butyldimethylsilyl (TBDMS) [17], deoxo-fluor [18] and so on.

The present paper utilize a simple method for comprehensive profiling of fatty acids in human plasma based on GC–MS, and apply to plasma samples of coronary heart disease patients. Metabolic profiles was used to reflect the perturbations of plasma fatty acids of CHD patients with different patterns, and pattern recognition methods were employed such as principal component analysis (PCA) to classify the CHD patients and healthy subjects, and partial least squares-discrimination analysis (PLS-DA) to discriminate two different patterns of CHD in Traditional Chinese Medicine (TCM) clinical practices. We attempted to find out whether differ-

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doi:10.1016/j.jpba.2008.10.018

ent patterns on which TCM always focused might have dissimilar mechanisms in the modern medicine research.

2. Experimental

2.1. Sample collection and study population

Fasting blood samples were collected from 25 healthy subjects, 23 CHD patients. CHD patients were characterized by the gold-standard angiographic technique and recruited to the coronary heart disease group who had significant coronary artery disease (defined as a reduction of more than 50% in the intralumenal diameter of major coronary arteries). In TCM clinical practices, CHD patients of 'heart deficiency of Qi' (HDQ) pattern customarily had repeat chest distress, palpitation, short breath and hypodynamia, while 'heart blood stasis' (HBS) patients often had chest pain on the fixed position and their sublingual vein had stagnant blood. The healthy subjects did not have chest pain, and electrocardiograms were normal. Consecutive subjects presenting at Traditional Chinese Medical Hospital of Zhejiang Province (Hangzhou, China) and the Sixth People's Hospital of Xiaoshan District (Hangzhou, China) who met the above criteria for either the CHD or healthy group were recruited to the study. All the CHD samples were collected before the medication regime been implemented. The informed consents were obtained. The clinical data for the two groups are shown in Table 1. Blood samples were drawn into Eppendorf tubes, and EDTA was used to prevent blood coagulation. Platelet-poor plasma was prepared by centrifugalization (3000 rpm for 10 min). Aliquots of plasma were stored at −80 °C until assayed.

2.2. Solvents and standards

Fatty acid methylester standards (FAME), myristic acid methyl ester (C14: 0 ME), pentadecanoic acid methyl ester (C15: 0 ME), palmitoleic acid methyl ester (C16: 1n-9 ME), palmitic acid methyl ester (C16: 0 ME), heptadecanoic acid methyl ester (C17: 0 ME), γ-linolenic acid methyl ester (C18: 3n-6 ME), linoleic acid methyl ester (C18: 2n-6c ME), oleic acid methyl ester (C18: 1n-9c ME), elaidic acid methyl ester (C18: 1n-9t ME), stearic acid methyl ester (C18: 0 ME), arachidonic acid methyl ester (C20: 4n-6 ME), *cis*-5,8,11,14,17-eicosapentaenoic acid methyl ester (C20: 5n-3 ME), *cis*-8,11,14-eicosatrienoic acid methyl ester (C20: 3n-6 ME), *cis*-11,14-eicosatrienoic acid methyl ester (C20: 2 ME), *cis*-11-eicosatrienoic acid methyl ester (C20: 1 ME), and 4,7,10,13,16,19-docos-ahexaenoic acid methyl ester (C22: 6n-3 ME) were purchased from Supelco (Bellefonte, PA) with more than 99% purity.

Chromatographic grade methyl nonadecanote as internal standard (I.S.) was purchased from Sigma (USA). The solution 5% H_2SO_4/CH_3OH was freshly prepared by diluting H_2SO_4 (98% purity) by analytical grade methanol, and 0.4 mol L^{-1} KOH/CH_3OH was freshly prepared in our laboratory by dissolving analytical grade KOH in methanol. 2-*tert*-Butyl-4-methyl-phenol (BHT, 99% purity) was obtained from Sigma (USA). Analytical grade *n*-hexane and anhydrous sodium sulfate were purchased from Sinopharm Chemical Reagent Co., Ltd. (China).

Table 1
Clinical data for the healthy group and the CHD group.

	Healthy subjects	CHD patients
Age	72.3 ± 7.8	72.6 ± 10.4
Sex		
Male, *n*	16	15
Female, *n*	9	8
Systolic BP (mmHg)	128 ± 15	129± 27
Diastolic BP (mmHg)	78± 11	73± 8
Current smokers, *n*	17	15
Creatinine (μM)	85.3 ± 9.1	83.0 ± 18.1
Glucose (mM)	4.2 ± 0.7	5.5 ± 1.4
Total cholesterol (mM)	4.0 ± 0.5	3.7 ± 1.0
Triglycerides (mM)	1.1 ± 0.5	1.2 ± 0.6
Platelets (10^9/L)	162.7 ± 38.0	180.9 ± 65.0

2.3. Sample preparation

Samples preparation procedures have been described previously in Ref. [7]. Briefly, aliquots (200 μL) of plasma were spiked with internal standard solution (100 μL C19:0 methyl ester) and antioxidant (20 μL BHT), lipid extraction was carried out using *n*-hexane after two-step methylation method. The methyl esters were extracted into hexane, and concentrated under nitrogen before GC–MS analysis.

2.4. Preparation of standard solution

The standard stock solution was prepared with *n*-hexane of C16: 1n-9 ME (0.746 mmol L^{-1}), C16: 0 ME (2.22 mmol L^{-1}), C18: 2n-6c ME (0.680 mmol L^{-1}), C18: 1n-9c ME (1.35 mmol L^{-1}), C18: 1n-9t ME (0.676 mmol L^{-1}), C18: 0 ME (1.34 mmol L^{-1}), C20: 4n-6 ME (0.629 mmol L^{-1}), C20: 3n-6 ME (0.625 mmol L^{-1}), and C22: 6n-3 ME (0.585 mmol L^{-1}). The stock solution was further diluted with *n*-hexane, adding I.S. (1.12 mmol L^{-1}) in each diluted solution. The I.S. solution was dissolved at a concentration of 0.641 mmol L^{-1} in *n*-hexane, in order to obtain relative concentrations of FAs in plasma samples.

2.5. GC–MS analysis

GC–MS analyses were performed on an Agilent Technologies 6890N Network gas chromatograph coupled to an Agilent Technologies 5973 Network quadrupole mass selective spectrometer. Separation was performed on a ZB-5MS (Phenomenex) capillary columns (30 m × 0.25 mm I.D., 0.25 μm film thickness). Helium was used as carrier gas with a flow rate of 1.0 mL min^{-1}. The temperature of the injector was 270 °C, and the sample (0.2 μL) was injected in the splitless mode. The column temperature was set at 160 °C, and ramped at 1 °C min^{-1} to 220 °C, where after the temperature was ramped again at 7 °C min^{-1} to 260 °C. The tandem quadrupole mass spectrometer was operated in electron impact (EI) mode and full scan monitoring mode (*m*/*z* 80–800). The quadrupole temperature was set at 150 °C, the source temperature at 230 °C, electron energy at 70 eV.

Mass spectrometry operating in SIM mode was used for quantitative analysis of fatty acids. Those characteristic fragment ions which gave a satisfactory intensity of response and at the same time were specific only for plasma fatty acids. This choice had permitted to obtain the best compromise between sensitivity and specificity of analysis, resulting in the highest value of signal-to-noise (S/N) ratio. Selected ion monitoring was carried out by monitoring *m*/*z* 83 for C16: 1n-9, *m*/*z* 87 for C16: 0, *m*/*z* 81 for C18: 2n-6c, *m*/*z* 83 for C18: 1n-9c, *m*/*z* 83 for C18: 1n-9t, *m*/*z* 87 for C18: 0, *m*/*z* 91 for C20: 4n-6, *m*/*z* 93 for C20: 3n-6, *m*/*z* 91 for C22: 6n-3, and *m*/*z* 87 for I.S. A dwell time of 100 ms was used for all SIM ions and EM voltage 2400 V.

2.6. Method validation

2.6.1. Linearity and range

Linearity and range of the analytical procedure were performed by serial dilution of standard stock solution. 7-Point

multi-component internal standard calibration curves were performed by calculating the ratios between the peak area of each substance and the peak area of the relative internal standard.

2.6.2. Precision and accuracy

Precision was evaluated both intra- and inter-day by analysis of plasma samples from CHD patients and the data compared after six consecutive runs (intra-day) and from data obtained over a 6-day period (inter-day). To validate the accuracy of the method, the recoveries experiments were performed by adding accurate amounts of the standards to plasma samples of CHD patients, and then samples were treated and analyzed.

2.6.3. Limit of quantification and method reproducibility

Quantification limits were determined using a signal-to-noise approach, which corresponded to the concentration that gave a signal-to-noise ratio of 10:1. The method reproducibility was examined by six duplicate plasma samples from the same CHD patient, treated according to the same preparation procedure and analyzed with the optimized GC-SIM-MS method.

2.6.4. Freeze–thaw stability and room temperature stability

All stability experiments were performed on plasma samples from CHD patient. Stability experiments included evaluation of the stability of fatty acids during three freeze–thaw cycles and stability of derivatized fatty acids at room temperature for 16 h.

2.7. Processing and pattern recognition of GC–MS data

The plasma samples from the healthy subjects group and patients group were analyzed in a random order. Enhanced Chemstation software (Agilent Technology) was used for automatic peak detection and calculating peak areas of internal standard and specific compounds. Identification of the interested peaks was supported by NIST v1.0.0.12 mass spectra library and FAME mix standards. Automatic peak detection was performed with peak width set to 0.2 s, and initial threshold set to 15.6. Peaks whose signal-to-noise ratios were lower than 3 had been rejected. The common peaks in all GC–MS profiles were matched and extracted, and relative area values of these common peaks were used as the variables to construct a multidimensional vector to characterize the biochemical pattern of each plasma sample. The further study depended on the pattern recognition of the multidimensional data, principal component analysis (PCA) and partial least squares-discrimination analysis (PLS-DA) were employed. All of the multivariate analysis was performed with SIMCA-P 11.0 software (Umetrics, Sweden).

3. Results and discussion

3.1. Derivatization

Some derivatization methods for fatty acids have been reported till now. Two common methods: silylation with BSTFA and TMCS and methylation methods (direct esterification (DE) and saponification–esterification (SE)) were tested. The results showed that firstly saponification–esterification and then direct esterification could get more kinds of fatty acids than the other methods in this experimental condition. Considering the purpose of metabolic profiling, this rapid, convenient, and less expensive methylation method was chosen.

3.2. Method validation

The mean correlation coefficients (R^2) of the calibration curves, which is higher than 0.996 showed good linearity of the method in the range of 0.025–14.780 mmol L^{-1}. The precisions were evaluated as ranging from 4.77% to 14.63% for intra-day assay and from 5.65% to 14.96% for inter-day assay, except C22: 6n-3 confirming that the method has good precision. Due to its polyunsaturation, C22: 6n-3 had a larger value of 16.08% and 20.99% for intra-day and inter-day, respectively. The results of accuracy test showed that the recovery of fatty acids were in the range of 85.07–104.49%, within $100 \pm 15\%$ to validate this method. The LOQs of the fatty acids varied from 0.4 ng L^{-1} to 13.8 ng L^{-1}, which meet the demand of sensitivity of metabolic profiling analysis. The R.S.D.s of reproducibility were all below 11.51%, which indicating a good repeatability of this method. Biological samples were found to be stable for at least 16 h at room temperature, allowing for overnight autosampler loading. And during three freeze–thaw cycles, fatty acids were found to be stable.

3.3. Application of analyzing plasma samples of healthy subjects and CHD patients

In this method, plasma fatty acids metabolic profiles of 23 CHD patients and 25 healthy subjects were obtained by GC–MS with this method. Typical GC–MS total ion current (TIC) chromatogram is shown in Fig. 1. In this profile, there are more than 40 peaks, in which 34 peaks are common peaks in the TIC chromatograms of the 48 samples, and among the 34 peaks, 21 fatty acids are unequivocally determined by the corresponding standards in the FAME mix standards according to their retention times and mass spectra characteristics. The other metabolites were tentatively determined based on their electron ionization (EI) MS data according to the NIST mass spectra library.

3.4. Principal component analysis of GC–MS data

A total of 21representative fatty acids peaks with their relative peak areas were used to construct a vector to describe the biochemical characteristics of each sample. Principal component analysis summarizes a data set with many variables by creating a few new variables (called principal components, PCs) containing most of the information [19]. The score plot of PCA represented the distribution of all samples. It is shown in Fig. 2, in which data of one patient and two healthy people were discarded for they were out of the 95% confidence interval (the ellipse). The clustering of the two groups was within the 93.5% correct rate. It might be concluded from the PCA results that plasma fatty acids metabolic profiling could reflect part of the perturbations of the CHD patients.

3.5. Partial least squares-discrimination analysis of discrimination of different patterns of CHD patients

PLS-DA is a multivariate classification method based on PLS, the regression of PCA. PLS-DA explains maximum separation between defined class samples in the data set [20]. After the crude screening by PCA, a PLS-DA model was constructed in order to discriminate two different patterns of CHD in Traditional Chinese Medicine clinical practices.

In TCM, observed signs and symptoms of patients (e.g. pulse tracing, coating on the tongue, etc.) are analyzed to identify the type of internal maladjustments. All these information together reflect a special functional state which is called a 'pattern' [21]. In practices, TCM practitioners would give individualized therapy based on 'pattern differentiation' even when treating the same patho-

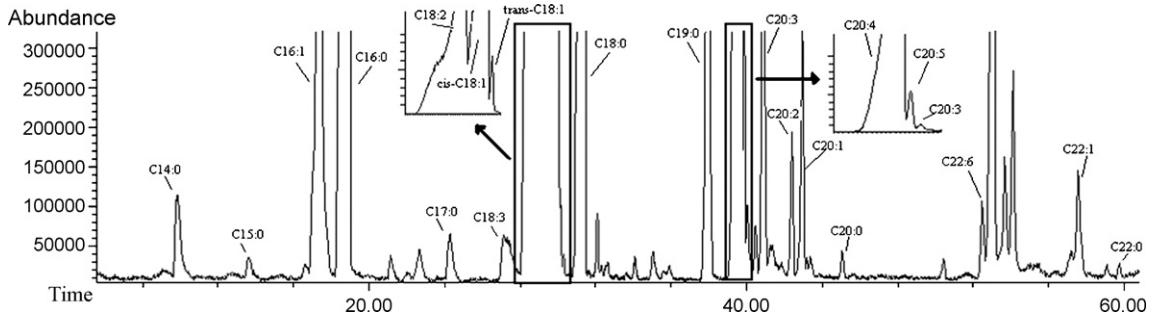

Fig. 1. A typical total ions current chromatogram with two parts showed in suitable size. 21 fatty acids are identified and labeled.

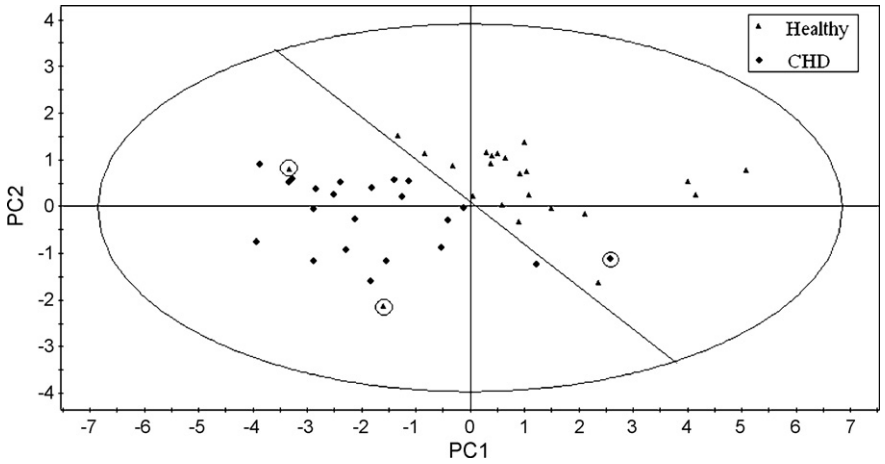

Fig. 2. PCA score plot of 25 healthy subjects and 23 CHD patients with the two-dimension visualization of the first two PCs in which data of one patient and two healthy people were discarded for they were out of the 95% confidence interval (the ellipse).

logical change. For instance, CHD patients are identified mainly as two patterns: 'heart blood stasis' and 'heart deficiency of Qi', to which different herbal formulae are administered [22], and produce good clinical curative effect. Considering the inter-relationship of therapeutic targets in these complicated disease, the pattern classification of TCM is worthy of exploring by modern medicine science.

In this study, a PLS-DA model was established for the classification of these two patterns and the detection of potential biomarkers. The PLS-DA score plot is shown in Fig. 3, with one outlier been discarded. The absolute coefficients plot shown in Fig. 4 interpreted the influence of each variable on the classification of the two groups, and the corresponding compounds which had the stronger influence would be the candidates of the potential biomarkers.

3.6. Potential biomarkers and possible regulate mechanism

PCA was used to analyze the multidimensional data, and different plasma fatty acids metabolic profiles were observed between the samples of CHD patients and healthy people. It suggests that

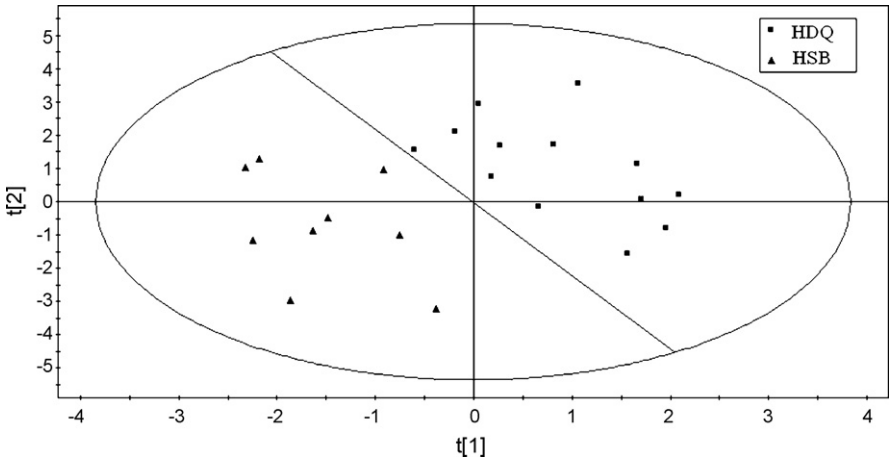

Fig. 3. Two-dimension plots of PLS-DA of the 23 CHD samples. The discrimination between two different patterns in Traditional Chinese Medicine of CHD was obtained (HSB for heart blood stasis, and HDQ for heart deficiency of Qi).

Table 2
The potential biomarkers and their possible regulate mechanism on CHD.

Type of FA	Members	Possible regulate mechanism on CHD	Ref.
SFA	C14:0 C16:0 C18:0	High capacity to raise LDL cholesterol levels	[23]
trans-FA	*trans*-C18:1	(1) Led to changes in the phospholipid fatty acid composition in the aorta, the target tissue of atherogenesis (2) This inhibition of EFA to PUFA by the isomeric fatty acids in hydrogenated fat is a risk factor in the development of CHD	[24]
n-3 PUFA	C22:6 C20:5	(1) Has beneficial impact on mitochondrial biogenesis, intrinsic antioxidant enzyme expression and efficiency in oxygen consumption (2) May affect the adjustment of myocardiac metabolism to tolerate hypoxia and cardiac output	[26] [28–31]
n-6 PUFA	C20:4	(1) Alter the artery endothelial cell function, and enhance the inflammatory response (2) Vary hemorheological indices and lead to coronary artery plaque and artery stenosis ultimately	[25–27]

SFA for saturated fatty acid, PUFA for polyunsaturated fatty acid, and EFA for essential fatty acid.

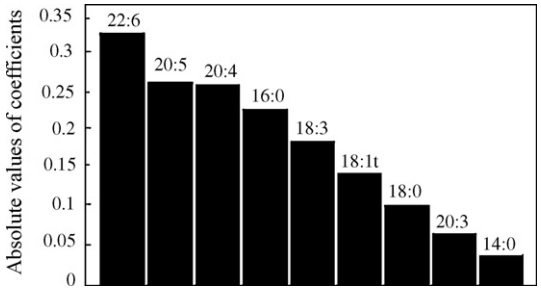

Fig. 4. Absolute values of coefficients of the nine fatty acids had the most strongly influence of the PLS-DA model. The absolute values of coefficients can render the influence of corresponding variables on separation between these two patterns.

modification of fatty acids may contribute diversely in the development of coronary heart disease.

In our study, several potential biomarkers were found to be related with the cause of CHD. Their possible regulate mechanisms are summarized in Table 2.

It was conjectured that the modification of *n*-6 and *n*-3 polyunsaturated fatty acids (PUFAs) might have the main influence on the 'heart blood stasis' and 'heart deficiency of Qi' patterns. High levels of *n*-6 fatty acids and low levels of *n*-3 might lead to an atherogenic state, which may at last lead to the heart blood stasis pattern. Also they may affect the adjustment of myocardiac metabolism to tolerate hypoxia and cardiac output, which finally cause the repeat chest distress, palpitation, short breath and hypodynamia of 'heart deficiency of Qi' patterns of patients.

4. Conclusions

In this paper, an optimized assay was established and validated for comprehensive determination of fatty acids in human plasma. The results suggested that the GC–MS-based method developed herein was useful and reliable for discriminating CHD patients and healthy persons and for identifying other metabolic syndromes related to fatty acids. The employment of multivariate analysis of GC–MS data made it possible to classify CHD patients and health controls, and a PLS-DA model was constructed to represent the alterations of two patterns of CHD in Traditional Chinese Medicine. Furthermore, several potential biomarkers which were related to the development of CHD were discussed. This method could be an alternative for disease diagnosis, pathogenesis research, and the modern research of Traditional Chinese Medicine theories. Limited to the time and cost, we studied only a small number of subjects. However, the aim of the present study is to investigate the science that underpins this novel field. Further studies will be required to compare different methodological approaches in various clinical applications.

Acknowledgements

We appreciate the work of the teams of Traditional Chinese Medical Hospital of Zhejiang Province and the Sixth People's Hospital of Xiaoshan District in the blood sample collection. We thank the advice of Professor Xiaoping Zhao on the writing of this manuscript. This work is financially supported by the Key Program of Zhejiang Province (No.: 2005C13026), National Natural Science Foundation (No.: 30772615 and No.: 30701068) and Program for New Century Excellent Talents in University (NCET-06-0515).

References

[1] F. Barzi, A. Patel, D. Gu, P. Sritara, T.H. Lam, A. Rodgers, M. Woodward, A.P.C.S. Collab, J. Epidemiol. Community Health 61 (2007) 115–121.
[2] J. Fang, G.A. Mensah, M.H. Alderman, J.B. Croft, Am. Heart J. 152 (2006) 1035–1041.
[3] J.T. Parissis, K. Fountoulaki, G. Filippatos, S. Adamopoulos, I. Paraskevaidis, D. Kremastinos, Int. J. Cardiol. 116 (2007) 153–160.
[4] M.W. Giuseppe Marazzi, C. Vitale, R. Patrizi, F. Pelliccia, H.P. Otavio Gebara, J.A.F. Ramires, M. Volterrani, G.M.C.R. Massimo Fini, Int. J. Cardiol. 120 (2007) 79–84.
[5] K.L. Yuan, H.W. Kong, Y.F. Guan, J. Yang, G.X. Xu, J. Chromatogr, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 850 (2007) 236–240.
[6] L.Z. Yi, J. He, Y.Z. Liang, D.L. Yuan, F.T. Chau, FEBS Lett. 580 (2006) 6837–6845.
[7] G.S. Ke Yu, J. Sheng, Y. Chen, W. Xu, X. Liu, H. Cao, H. Qu, Y. Cheng, L. Li, J. Proteome Res. 6 (2007) 2413–2419.
[8] J.T. Brindle, H. Antti, E. Holmes, G. Tranter, J.K. Nicholson, Nat. Med. 8 (2002) 1439–1444.
[9] G. Schlotterbeck, A. Ross, F. Dieterle, H. Senn, Pharmacogenomics 7 (2006) 1055–1075.
[10] X. Han, R.W. Gross, Mass Spectrom. Rev. 24 (2005) 367–412.
[11] A. Darbre, in: K. Blau, G. King (Eds.), Handbook of Derivatives for Chromatography, Heyden and Son, London, 1977, pp. 39–103.
[12] N.C. Shantha, G. Napolitano, J. Chromatogr. 624 (1992) 37–51.
[13] R.R. Wolfe, Principles and Practice of Kinetic Analysis, Wiley-Liss, New York, 1992.
[14] C. Lin, E.W. Blank, R.L. Ceriani, N. Baker, Lipids 26 (1991) 548–552.
[15] R.J. Pawlosky, J.R. Hibbeln, N. Salem Jr., J. Lipid Res. 48 (2007) 935–943.
[16] U. Caruso, B. Fowler, M. Erceg, C. Romano, J. Chromatogr. 562 (1991) 147–152.
[17] T. Ohie, X. Fu, M. Iga, M. Kimura, S. Yamaguchi, J. Chromatogr. B 746 (2000) 63–73.
[18] C.O. Kangani, D.E. Kelley, J.P. DeLany, J. Chromatogr. B 873 (2008) 95–101.
[19] E. Pohjanen, E. Thysell, J. Lindberg, I. Schuppe-Koistinen, T. Moritz, P. Jonsson, H. Antti, Metabolomics 2 (2006) 257–268.
[20] L. Eriksson, E. Johansson, N. Kettanah-Wold, S. Wold, Introduction to Multi and Megavariate Data Analysis Using Projection Methods (PCA and PLS-DA), Umetrics AB, Malmo, Sweden, 1999.
[21] W.Y. Jiang, Trends Pharmacol. Sci. 26 (2005) 558–563.
[22] Z. Xie (Ed.), Practical Traditional Chinese Medicine, Foreign Language Press, 2000.
[23] T.K.Q. Seo, K. Qi, C. Chang, Y. Liu, T.S. Worgall, R. Ramakrishnan, R.J. Deckelbaum, J. Clin. Invest. 115 (2005) 2214–2222.

[24] F.A. Kummerow, Q. Zhou, M.M. Mahfouz, M.R. Smiricky, C.M. Grieshop, D.J. Schaeffer, Life Sci. 74 (2004) 2707–2723.
[25] J. Goodfellow, M.F. Bellamy, M.W. Ramsey, C.J.H. Jones, M.J. Lewis, J. Am. Coll. Cardiol. 35 (2000) 265–270.
[26] A. Leaf, J.X. Kang, Y.F. Xiao, G.E. Billman, Circulation 107 (2006) 2646–2652.
[27] A.P. Simopoulos, L.G. Cleland, Omega-6/Omega-3 Essential Fatty Acid Ration, Karger, Farmington, CT, 2003.
[28] E.M. Balk, A.H. Lichtenstein, M. Chung, B. Kupelnick, P. Chew, J. Lau, Atherosclerosis 184 (2006) 237–246.
[29] P.L.M.S. Pepe, Circulation 105 (2002) 2303–2308.
[30] R.J. Deckelbaum, T.S. Worgall, T. Seo, Am. J. Clin. Nutr. 83 (2006) 1520s–1525s.
[31] S. Pepe, N. Tsuchiya, E.G. Lakatta, R.G. Hansford, Am. J. Physiol. 276 (1999) H149–H158.