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Serum Metabolomic Analysis of Cervical Cancer Patients by Gas Chromatography-Mass Spectrometry

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In this paper, gas chromatography-mass spectrometry (GC-MS) in combination with pattern recognition techniques were used to analyze serum metabolites in cervical cancer patients. The experimental parameters, including the extraction solvent, temperature and time of derivatization and chromatographic conditions, were investigated for serum metabolomic profiling. Forty-five endogenous metabolites included amino acids, fatty acids, carbohydrates and other intermediate metabolites were identified. Partial least squares discriminant analysis (PLS-DA), clustering analysis and principal component analysis based on these metabolites were applied to the discrimination of health group from cervical cancer group. The results showed that 18 metabolites were detected differently between the health group and cervical cancer group, but also demonstrated that the GC-MS technique was a valuable tool for the characterization of the serum metabolic profiling and the metabolomic study will be certainly benefit for monitoring the state of cervical patients, the prognosis and therapy evaluation of cervical cancer patients.

Keywords: Cervical cancer, Metabolomics, GC-MS, Statistical analysis.

INTRODUCTION

All over the world, cervical cancer is the second most common women cancer. It is the leading cause of the cancer death among women[1] and over 130000, 6600 and 12000 new cases of cervical cancer are diagnosed every year in China, Germany and US, respectively[2-4]. Moreover, this disease is more common in developing countries due to the lack of mass screening programs. However, there is an important problem to be faced, the diagnosis of cervical cancer is currently based on morphologic and histologic examinations and these clinical tests are neither sensitive nor peculiar. To improve the efficiency of disease screening, the current trend is the uses of the specified biomarkers in conjunction with available techniques. Current developments in molecular biology and proteomics are ushering a new era of the biomarker discovery and identification of new molecular targets related to carcinogenesis. Proteomics of cervical cancer[5,6] has yielded some novel potential biomarkers for cervical cancer, but the specific biomarkers of cervical cancer have not been well characterized due to proteomics is good at the identification of different proteins.

Metabolomics, as an omics science in systems biology, is the global quantitative assessment of endogenous metabolites within a biological system. There is potential for the metabolome to have a multitude of uses in oncology, including the early detection and diagnosis of cancer and as both a predictive and pharmacodynamic marker of drug effect[7,8]. It can distinguish between diseased and non-diseased status through comprehensive measurement of small molecules in easily accessible biofluids and diagnostic biomarker discovery.

With the development of metabolomics analysis, nuclear magnetic resonance spectroscopy and mass spectrometry have been successfully applied in separation, detection, characterization and quantification of the metabolome[9], but also have improved the sensitivity and spectral resolution of analytic assays on metabolomic samples in attempts to achieve a comprehensive biochemical assessment[10,11]. In recent years, these techniques are widely used in metabolomics studies, involving NMR spectroscopy[12], infrared and Roman spectroscopy[13], capillary electrophoresis (CE) and CE-MS[14-16], gas chromatography (GC) and GC-MS[17,18], liquid chromatography (LC) and LC-MS[19,20], matrix assisted laser desorption ionization-mass spectrometry (MALDI-MS)[21].

Currently, the metabolomics researches of cervical cancer are still in the exploratory stage. The metabolites of serum samples from cervical cancer patients were investigated by using a LC-MS based metabolomics approach[22]. Moreover, a GC-MS based metabolomics approach was applied to the discovery of urinary biomarker of women's cancers[23]. In this study, a proposed method based on GC-MS technique was used for the analysis of metabolites from the cervical cancer

patients, while healthy volunteers were used as controls. The objective of the paper are not only to compare the metabolite profiling from serum samples between cervical cancer and healthy subjects, but also to establish a diagnostic model from these metabolic biomarkers to make a distinction between cervical cancer and normal subjects using pattern recognition techniques (PLS-DA, clustering analysis, t-test and principal component analysis).

EXPERIMENTAL

Heptadecanoic acid and N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) were obtained from Sigma-Aldrich (St Louis, MO, USA). Acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany). *o*-Methylhydroxylamine (*o*-MHA) hydrochloride was supplied by J&K Company. Distilled water was produced by a Milli-Q Reagent Water System (Millipore, Bedford, MA, USA). All other common chemicals were analytical grade.

Patient recruitment and sample collection: The study protocol was approved by the local ethics committee. All serum samples were obtained through Haidian Maternal and Child Health Hospital, Beijing, China. Informed consent was supplied by all participants. Serum samples of cancer group were collected from patients (n = 5, No. 7-11) with gynecological pathologically confirmed cervical cancer from March 2010 to January 2011. Serum samples of healthy group were collected from healthy female volunteers (n = 6, No. 1-6). The age of cervical cancer patients was in the range of 35-43, while the age of normal controls was from 25 to 28.

Sample preparation: The collected blood was allowed to clot at room temperature for at least 1 h and centrifuged at 3,500 rpm for 20 min at 4 °C. The supernatant was aliquoted and frozen stored at -80 °C until use.

Serum samples were derivatized and analyzed by GC-MS. To extract low molecular weight metabolites, the frozen serum samples were defrosted on ice and centrifuged at 3,500 rpm for 5 min at 4 °C. 600 μL of acetonitrile was added into 200 μL serum sample spiked with 10 μL of internal standard (heptadecanoic acid, 1 mg/mL in chloroform) and vortexed for 1 min. Then, the mixture was centrifuged at 12,000 rpm for 15 min at 4 °C and 650 μL of the supernatant was collected and concentrated to complete dryness by nitrogen with ice bath. Finally, the residue was derivatized as followed: *o*-MHA solution (100 μL, 20 mg/mL in pyridine) was added and vortexed for 1 min. The resolved solution was incubated for 120 min at 40 °C water bath followed by 100 μL MSTFA incubated for another 120 min.

GC-MS analysis: 1 μL aliquot of the derivatized solution was injected split (1:30) into a GC/MS-QP2010 (Shimadzu Co., Kyoto, Japan) coupled with a RTX-50 column (30 m × 0.25 mm i.d., 0.25 μm film thickness; Thames Restek U.K LTD) as the separation column in GC system. The inlet temperature was kept at 270 °C and helium was used as carrier gas at a constant flow rate of 0.8 mL/min. The GC column temperature was initially at 70 °C for 4.5 min and then increased to 190 °C at a rate of 10 °C/min, to 200 °C at 5 °C/min, to 220 °C at 2 °C/min, to 290 °C at 10 °C/min in sequence and hold at 290 °C for 4.5 min. The MS conditions were as follows: ionization voltage: 70 eV; transfer line temperature: 250 °C; ion source temperature: 230 °C; Full scan mode in the m/z range 50-800 with a 0.2 second per scan velocity.

Data processing: After GC-MS analysis, the serum profiles were represented by total ion current (TIC). Chromatograms of all serum samples were subjected to noise reduction prior to peak area integration and then the peak areas of serum metabolites were integrated using Shimadzu GC-MS solution software Version 2.53 (Shimadzu Co.). The peak of low molecular weight metabolites in the chromatogram was identified by comparing the mass spectrum with a standard mass spectrum in the National Institute of Standards and Technology (NIST) Mass spectra Library. Peaks with a similarity index more than 80 % were the assigned compound names. All known artificial peaks were excluded from the data set.

Statistics: Relative peak area of each compound to heptadecanoic acid as the internal standard was calculated as the response. The resulting two-dimensional matrix was established including sample information and the relative peak areas. Two sample t-test statistics was used to compare the differences of metabolite levels between cervical cancer and health group. P values of < 0.05 was considered as statistically significant. Principal component analysis and cluster analysis were used to differentiate the samples and performed in the MATLAB software (version 7.5, MathWorks, USA). Besides, partial least squares discriminant analysis was performed using SIMCA-P$^+$ Software Version 11.5 (Umetrics, Umeå, Sweden).

RESULTS AND DISCUSSION

Development of GC-MS method: To obtain more information of metabolomic profiles, the experimental parameters including the extraction solvent, the temperature and time of the derivatization and chromatographic conditions were optimized, respectively. In the process of optimization, five metabolites were selected as study objects, whose retention time were in different time periods and intensities of peaks were moderate. First, investigating extraction solvents of metabolites including acetonitrile, acetone, methanol, ethanol and chloroform. The results showed that acetonitrile had a higher extraction efficiency and better reproducibility. In addition, the time and temperature of the derivatization were optimized, the experimental results indicated that the intensities of peaks for these five metabolites were optimal at 40 °C for 120 min. Chromatographic temperature-programmed and the flow of carrier gas were optimized and selected 0.8 mL/min of flow rate.

According to the above optimal conditions, injection of serum sample was continuous for six times to calculate relative standard deviations (RSDs) of retention time and peak areas of these five metabolites and the results demonstrated that relative standard deviations of the retention time and peak areas these five metabolites were less than 0.05 and 13 %, respectively.

Metabolomic profiling of cervical cancer: Representative GC-MS TIC of serum samples from the health group and cancer group were showed in Fig. 1. The majority of the peaks in the chromatograms were identified as endogenous metabolites by NIST mass spectra library, including amino acids, organic acids, carbohydrates and fatty acids[24].

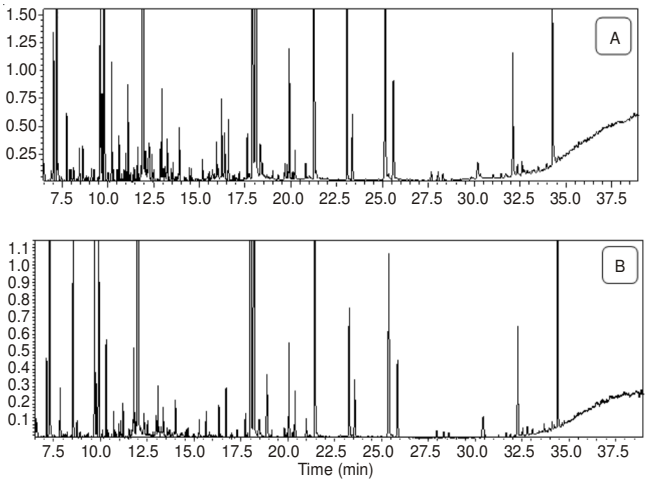

Fig. 1. Representative GC-MS total ion current of the samples from health control (A) and cervical cancer patient (B) after chemical derivatization

Combined with search results based on NIST database, 45 metabolites were selected as the study objects and the relative peak areas of these metabolites were calculated (Table-1). Then the relative peak area values were expanded 100 times as the relative content values.

Independence-sample *t*-test statistics (SPSS 17.0 software) was used for the comparison of the metabolite levels to determine their significant differences between the cancer group and the health group. As shown in Table-1, the levels of 14 of 45 metabolites were found to be significantly different between cervical cancer patients and the normal controls ($p < 0.05$). The differentially expressed metabolites ($p < 0.05$) were considered to be statistically significant. Among these differentially expressed metabolites, the levels of ten metabolites were up-regulated and other four metabolites were down-regulated.

Pattern recognition: The relative content data of 45 metabolites were imported into Matlab and SIMCA-P, respectively. principal component analysis, the most generally used algorithm in metabolomics studies[25], as a basic form of multiple classification analysis (MCA), was employed in processing the GC-MS data[26]. The principal component analysis scores plots showed that the two groups (healthy group and cancer group) were completely scattered into two different regions (Fig. 2). As shown in Fig. 2, sample points of the health group gathered well, while sample points of the cancer group showed discrete state, which may be caused by the individual differences.

Clustering analysis was used to assign a set of observations into subsets (called clusters) so that observations in the same cluster are similar in some senses. In this study, the cluster trees were achieved according to the distances matrices by using the ward algorithm. As shown in Fig. 3, a clear discrimination between the health group and the cancer group was obtained by clustering analysis, numbered 1 to 6 for the health group and numbered 7 to 11 for cervical cancer patients.

Finally, the metabolomic data using PLS-DA for all subjects were analyzed and displayed with scores plots of PLS-DA to investigate metabolite patterns of the health group and cancer group. Consistently, cervical cancer patients were distinct from health group and the sample points were completely separated from the two groups (Fig. 4).

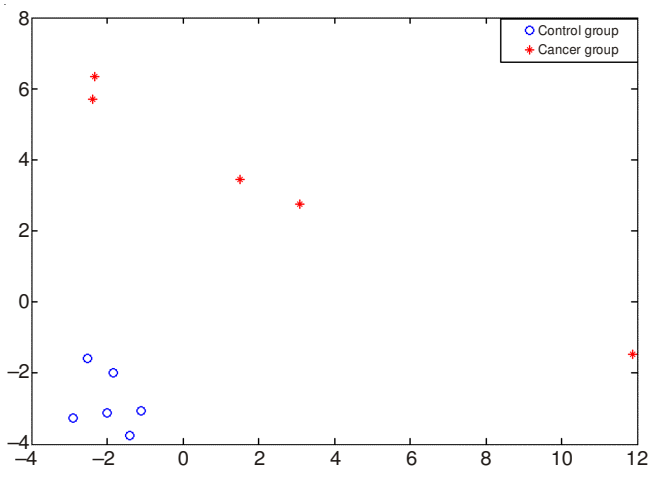

Fig. 2. Principal component analysis (PCA) scores plot discriminating cervical cancer group from health group based on GC-MS metabolites

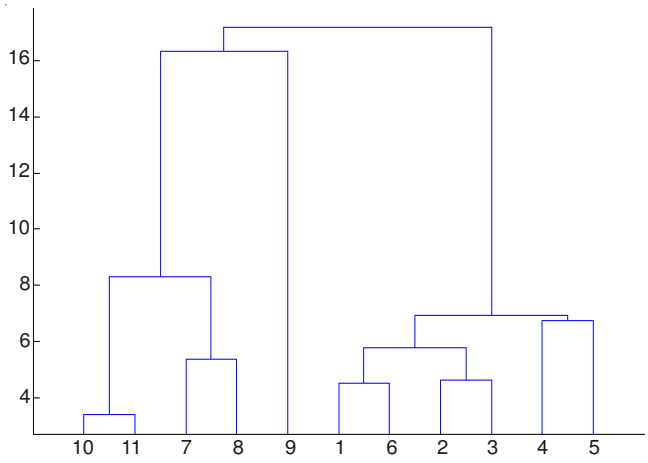

Fig. 3. Result of clustering analysis (CA) analysis on the cervical cancer patients and healthy samples

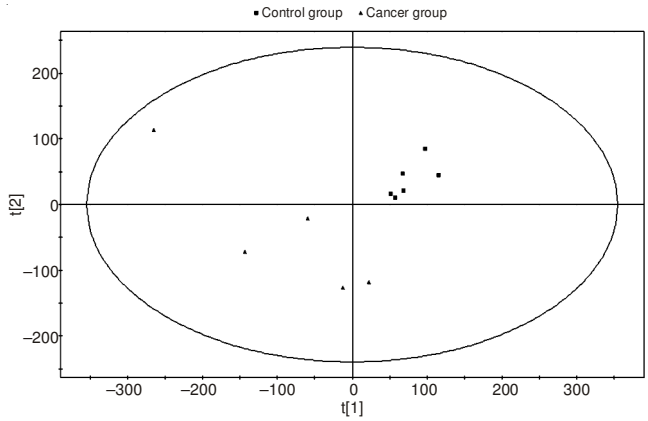

Fig. 4. PLS-DA scores plots discriminating between the cervical cancer patients and healthy samples using the metabolomic data

Combined with PLS-DA loadings plots and *t*-test results, we found that some of selected markers ($p < 0.05$) were worth to further investigate. Hence, we would intend to debate their roles during the process of tumor metabolism associated with cervical cancer. Compared with the healthy group, the level of ethanedioic acid, phosphate and gulonic acid was significant higher, while the level of eleven compounds (ethoxypropionic

TABLE-1
RELATIVE INTENSITY OF 45 METABOLITES IN SERUM SAMPLES OF CERVICAL CANCER PATIENTS AND HEALTHY GROUP

No.	Metabolites	Retention time (min)	Healthy group (n = 6)	Cancer group (n = 5)	P value
1	Ethoxypropionic acid*	7.22	5.90 ± 0.54	1.75 ± 0.61	0.000
2	Pyruvic acid*	7.31	6.21 ± 2.06	0.72 ± 0.57	0.000
3	*l*-Alanine	7.77	22.90 ± 7.90	41.17 ± 21.36	0.082
4	Pyridine-3-carboxylic acid	8.02	8.02 ± 8.43	2.45 ± 1.10	0.180
5	Ethanedioic acid*	8.46	19.31 ± 20.52	115.67 ± 44.83	0.001
6	*l*-Valine*	8.66	22.53 ± 1.22	10.32 ± 1.92	0.000
7	Butanoic acid	9.11	12.32 ± 7.96	4.95 ± 3.14	0.085
8	Propanoic acid*	9.25	5.51 ± 0.43	3.48 ± 1.20	0.004
9	Ethanedioic acid	9.60	99.87 ± 27.41	144.42 ± 39.32	0.054
10	Glycine	9.82	279.59 ± 26.50	327.62 ± 110.42	0.391
11	Glycerol	10.22	55.11 ± 12.28	51.17 ± 29.51	0.771
12	*l*-Isoleucine	10.31	5.20 ± 0.74	4.00 ± 0.81	0.31
13	Butanoic acid*	10.52	5.59 ± 0.40	2.81 ± 0.83	0.000
14	Glycine	11.11	29.76 ± 5.40	27.05 ± 11.30	0.614
15	2-Ketoisocaproic acid*	11.24	4.16 ± 0.26	2.60 ± 0.76	0.001
16	Phosphate*	11.69	16.99 ± 7.55	48.43 ± 19.61	0.020
17	Urea	11.93	844.10 ± 178.64	1106.38 ± 458.40	0.282
18	Isotridecyl alcohol*	12.52	7.56 ± 2.16	3.39 ± 3.40	0.001
19	Hexadecanoic acid	12.89	15.25 ± 0.76	18.11 ± 4.79	0.254
20	α-Hydroxycaproic acid	12.97	28.72 ± 1.99	26.56 ± 7.87	0.529
21	*l*-Methionine	15.17	11.15 ± 4.07	10.57 ± 3.99	0.816
22	Adenosine*	15.94	16.68 ± 1.45	4.45 ± 3.67	0.000
23	*l*-Oroline*	16.22	30.87 ± 2.25	17.81 ± 7.13	0.002
24	Dimethylpyruvic acid*	16.39	20.82 ± 1.19	2.016 ± 0.67	0.000
25	Phenylalanine	16.59	35.71 ± 36.30	18.62 ± 6.73	0.535
26	Proline	17.18	4.04 ± 0.63	4.88 ± 1.80	0.310
27	Glucose	17.91	1198.83 ± 129.36	1827.72 ± 631.59	0.089
28	Glucose	18.09	568.74 ± 64.88	670.04 ± 201.40	0.333
29	Gulonic acid*	19.80	2.13 ± 0.56	53.87 ± 25.48	0.010
30	Glycoside	19.91	8.36 ± 5.23	4.19 ± 2.35	0.136
31	Tyrosine	20.22	60.75 ± 5.27	59.45 ± 22.31	0.904
32	2-Deoxy-galactopyranose	20.81	18.67 ± 3.27	21.20 ± 7.08	0.492
33	Heptadecanoic acid (is)	23.06	-	-	
34	Aspartic acid	23.36	43.37 ± 10.39	46.60 ± 15.08	0.684
35	Octadecanoic acid	25.15	199.86 ± 24.47	191.32 ± 79.11	0.806
36	9,12-Octadecadienoic acid	25.60	90.65 ± 31.16	69.48 ± 35.36	0.318
37	Aspartic acid	27.67	8.25 ± 2.57	16.40 ± 23.37	0.481
38	Tryptophan	28.04	7.53 ± 2.89	8.58 ± 6.06	0.715
39	Carbamic acid	28.30	6.85 ± 2.03	5.00 ± 1.59	0.135
40	Arachidonic acid	30.20	21.68 ± 3.64	26.46 ± 9.94	0.298
41	Tridecanol*	31.08	2.87 ± 0.74	1.74 ± 0.75	0.034
42	2-Hydroxy caprylic acid	31.64	3.12 ± 0.94	2.99 ± 1.07	0.830
43	Glycerol monopalmitate	32.11	80.91 ± 43.48	70.25 ± 26.09	0.664
44	Glyceryl trilaurate	34.05	3.49 ± 0.32	6.84 ± 2.97	0.065
45	Glycerol monopalmitate	34.27	100.49 ± 11.97	164.17 ± 73.33	0.063

* Indicate lower p values than 0.05

acid, pyruvic acid, *l*-valine, propanoic acid, butanoic acid, 2-ketoiso-caproic acid, isotridecyl alcohol, adenosine, *l*-proline, dimeth-ylpyruvic acid and tridecanol) was significant lower in cervical cancer group.

Pyruvic acid is the product of glycolysis process. In our study, the level of pyruvic acid in cervical cancer patients was lower than healthy group and it may be caused by the decrease of glycolysis. Glucose plays another important role in glycolysis. The level of glucose was higher in the serum of cervical cancer patients, it maybe demonstrate that the less processes of glycolysis would be taken place in cervical cancer patients and it led to the decrease level of pyruvic acid. It is worth noting that decreased pyruvic acid level was also found in reference[27] and it was employed as the candidate metabolite biomarker for gastric cancer.

Butanoic acid exists in animal fat in the form of butyrate and it not only can inhibit deacetylase activity, but also regulate the gene expression. The decreased level of butanoic acid was also found in gastric cancer, but the reason why butanoic acid decreased is unclear[28].

According to the literature[29], the expression of ethanedioic acid in the breast cancer patients also showed significant changes, but the trends of change were opposite compared with cervical cancer patients. Moreover, there were some similar results *e.g.*, the level of ethanedioic acid was down-regulated in the urine samples of hepatocellular carcinoma patients[30] and the serum of hepatocarcinoma in rats with lung metastasis[31].

l-Valine not only is an essential amino acid in human body, but also is the precursor of succinate, which is a part of Krebs

cycle. *l*-Proline is one of the parts of plant proteins and it is the precursor of α-ketoglutarate which is also a part of Krebs cycle. The decreased levels of these two amino acids were detected in the serum of cervical cancer patients due to a decreased glycolysis, on the contrary, the increased level of *l*-valine was found in oesophageal cancer[26] and gastric cancer[32].

Adenosine, which is an inhibitory neurotransmitter, it is an intermediate of adenosine triphosphate and phosphate. The increased level of phosphate may lead to the decreased level of adenosine in the serum of cervical cancer patients.

These compounds in the clinical diagnosis of cervical cancer and related applications should be remained for further confirmations. But it should be noted that in this study, number of sample cases is too few and we will further analyze more clinical samples to verify the trends of the characterization of the metabolites. Moreover, a comparative analysis of results is questionable based on different technology platforms of metabolomics. In addition, the development and progression of cervical cancer is through cervicitis, cervical intraepithelial neoplasia and cervical cancer at different stages. Adopting established analytical method based on GC-MS in this paper, the analysis of metabolomics was conducted for different pathological processes of cervical cancer and pathological mechanism of cervical cancer need to further study.

Conclusion

In our present work, we established an analytical method based on metabolomic profiling of serum in cervical cancer patients by chemical derivatization and gas chromatography-mass spectrometry. GC-MS was proved to be a viable and complementary analytical platform for serum metabolic profiling in terms of its high sensitivity and reproducibility. In addition, the results suggested that metabolomics is capable of representing the differences between cervical cancer patients and normal controls. GC-MS based qualitative and quantitative profiling analysis contributed to deduce the cancer biomarkers combined with statistical analysis. The changes in the serum levels of metabolites may also provide promising biomarkers not only for the early diagnosis of cervical cancer, but also for its prognosis.

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REFERENCES

1. X.D. Ma, G.Q. Cai, W. Zou, Y.H. Huang, J.R. Zhang, D.T. Wang and B.L. Chen, *Gene*, **524**, 208 (2013).
2. U. Siebert, G. Sroczynski, P. Hillemanns, J. Engel, R. Stabenow, C. Stegmaier, K. Voigt, B. Gibis, D. Hölzel and S.J. Goldie, *Eur. J. Public Health*, **16**, 185 (2005).
3. A. Hasim, M. Ali, B. Mamtimin, J.Q. Ma, Q.Z. Li and A. Abudula, *Exp. Ther. Med*., **3**, 945 (2012).
4. E.S. Amirian, K. Adler-Storthz and M.E. Scheurer, *Cancer Lett*., **336**, 18 (2013).
5. E.K. Yim and J.S. Park, *Expert Rev. Proteomics*, **3**, 21 (2006).
6. N.S. Ye and J. Li, *Anal. Lett*., **46**, 250 (2013).
7. G.A.N. Gowda, S. Zhang, H. Gu, V. Asiago, N. Shanaiah and D. Raftery, *Expert Rev. Mol. Diagn*., **8**, 617 (2008).
8. Y.S. Kim, P. Maruvada and J.A. Milner, *Future Oncol*., **4**, 93 (2008).
9. M. Yoshida, N. Hatano, S. Nishiumi, Y. Irino, Y. Izumi, T. Takenawa and T. Azuma, *J. Gastroenterol*., **47**, 9 (2012).
10. J.L. Spratlin, N.J. Serkova and S.G. Eckhardt, *Clin. Cancer Res*., **15**, 431 (2009).
11. G. Theodoridis, H.G. Gika and I.D. Wilson, *Mass Spectrom. Rev*., **30**, 884 (2011).
12. B. Sitter, T.F. Bathen, M.B. Tessem and I.S. Gribbestad, *Prog. Nucl. Mag. Res. Sp*., **54**, 239 (2009).
13. D.I. Ellis and R. Goodacre, *Analyst*, **131**, 875 (2006).
14. R. Ramautar, O.A. Mayboroda, G.W. Somsen and G.J. de Jong, *Electrophoresis*, **32**, 52 (2011).
15. R. Ramautar, G.W. Somsen and G.J. de Jong, *Electrophoresis*, **30**, 276 (2009).
16. R. Ramautar, G.W. Somsen and G.J. de Jong, *Electrophoresis*, **34**, 86 (2013).
17. Z.M. Wu, Z.Q. Huang, R. Lehmann, C.X. Zhao and G.W. Xu, *Chromatographia*, **69**, S23 (2009).
18. M.M. Koek, R.H. Jellema, J. Greef, A.C. Tas and T. Hankemeier, *Metabolomics*, **7**, 307 (2011).
19. P.A. Guy, I. Tavazzi, S.J. Bruce, Z. Ramadan and S. Kochhar, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci*., **871**, 253 (2008).
20. R. Llorach-Asunción, O. Jauregui, M. Urpi-Sarda and C. Andres-Lacueva, *J. Pharm. Biomed. Anal.*, **51**, 373 (2010).
21. D.A. Pirman, E. Efuet, X.P. Ding, Y. Pan, L. Tan, S.M. Fischer, R.N. DuBois and P. Yang, *PLoS ONE*, **8**, e61379 (2013).
22. J. Chen, Y.H. Shan, Q. Yan, S.M. Zhao, P.Y. Yin, X.J. Zhao, X. Lu, X.W. Xi, X.P. Wan and G.W. Xu, *Sci. China Chem*., **39**, 1268 (2009).
23. H.M. Woo, K.M. Kim, M.H. Choi, B.H. Jung, J. Lee, G. Kong, S.J. Nam, S. Kim, S.W. Bai and B.C. Chung, *Clin. Chim. Acta*, **400**, 63 (2009).
24. H. Wu, T.T. Liu, C.G. Ma, R.Y. Xue, C.H. Deng, H.Z. Zeng and X.Z. Shen, *Anal. Bioanal. Chem*., **401**, 635 (2011).
25. O. Beckonert, M. E. Bollard, T.M.D. Ebbels, H.C. Keun, H. Antti, E. Holmes, J.C. Lindon and J.K. Nicholson, *Anal. Chim. Acta*, **490**, 3 (2003).
26. H. Wu, R.Y. Xue, C.L. Lu, C.H. Deng, T.T. Liu, H.Z. Zeng, Q. Wang and X. Shen, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci*., **877**, 3111 (2009).
27. A. Ikeda, S. Nishiumi, M. Shinohara, T. Yoshie, N. Hatano, T. Okuno, T. Bamba, E. Fukusaki, T. Takenawa, T. Azuma and M. Yoshida, *Biomed. Chromatogr*., **26**, 548 (2012).
28. J.D. Hu, H.Q. Tang, Q. Zhang, J. Fan, J. Hong, J.Z. Gu and J.L. Chen, *World J. Gastroenterol*., **17**, 727 (2011).
29. J. Wang, L.F. Yu, P. Shen and S.F. Wang, *J. Zhejiang Univ. Med. Sci*., **38**, 478 (2009).
30. H. Wu, R.Y. Xue, L. Dong, T.T. Liu, C.H. Deng, H.Z. Zeng and X.Z. Shen, *Anal. Chim. Acta*, **648**, 98 (2009).
31. Z.F. Li, J. Wang, C. Huang, S. Zhang, J. Yang, A. Jiang, R. Zhou and D. Pan, *Rapid Commun. Mass Spectrom*., **24**, 2765 (2010).
32. H. Wu, R.Y. Xue, Z.Q. Tang, C.H. Deng, T.T. Liu, H.Z. Zeng, Y.H. Sun and X.Z. Shen, *Anal. Bioanal. Chem*., **396**, 1385 (2010).