CHINESE JOURNAL OF ANALYTICAL CHEMISTRY
Volume 44, Issue 3, March 2016
Online English edition of the Chinese language journal

Available online at www.sciencedirect.com
ScienceDirect

Cite this article as: Chin J Anal Chem, 2016, 44(3), 329–334.

RESEARCH PAPER

Direct Analysis of Phospholipids in Biological Tissues Using Internal Extractive Electrospray Ionization Mass Spectrometry

LU Hai-Yan[1], ZHANG Jian-Yong[2], ZHOU Wei[1], WEI Yi-Ping[2], CHEN Huan-Wen[1,*]

[1] East China University of Technology, Jiangxi Key Laboratory for Mass Spectrometry and Instrumentation, Nanchang 330013, China
[2] Second Affiliated Hospital of Nanchang University, Nanchang 330006, China

Abstract: Phospholipids and their metabolites play an important role in a variety of cellular processes including cell-cell adhesion, cell growth and differentiation, apoptosis, phagocytosis as well as storage of energy. In this study, the phospholipid composition of cancer tissue and adjacent normal tissue from humans and animals were analyzed by internal extractive electrospray ionization mass spectrometry (iEESI-MS). Extractive solvent at high voltage (+5.5 kV) was injected into tissue samples using a fused silica capillary at a flow rate of 0.5–1.0 μL min^{-1}, producing fine charged droplets containing analytes of tissue samples at the tip of the sample. Charged droplets were directly sampled to the atmospheric inlet of a mass spectrometer. Out of 21 different ratios of CH_3OH-H_2O solvent mixture, the ratio CH_3OH-H_2O (30:70, *V/V*) showed the optimal phospholipids extraction and visibility in MS. A large number of phospholipids from different tissue samples (such as cancer tissue and adjacent normal tissue of lung cancer, esophageal tissue, pork, beef, porcine heart and porcine lung) were obtained simultaneously by iEESI-MS analysis. The experimental results demonstrated that iEESI-MS was characterized by minimal sample pretreatment, low sample consumption, and rapid analysis (the analysis time per sample was less than 1 min), and the selectivity and sensitivity of iEESI-MS could be improved by choosing proper solvent. Importantly, the experimental results provided new information for further studies of phospholipids in biological tissues.

Key Words: Biological tissue; Phospholipids; Extractive solvent; Internal extractive electrospray ionization mass spectrometry

1 Introduction

Phospholipids and their metabolites play an important role in a variety of cellular processes including cell growth and differentiation, apoptosis and phagocytosis, storage of energy, and cell signaling[1,2]. Phospholipids are among the most popular targets in single-cell study, because of their high concentrations in cell membrane[3,4]. Accordingly, the development of various diseases is related to the changes in the levels of phospholipids and metabolism disorders in organism[5], which suggests that phospholipids would be used as biomarkers of diseases. Therefore, phospholipids profiling within different biological tissue samples would contribute to revealing the mechanism of organisms, seeking biomarkers of diseases and the development of pharmaceutical drugs.

Unfortunately, cumbersome sample pretreatments such as sample grinding, centrifugation, and chemical extraction are commonly needed for phospholipids analysis in complex samples (such as plasma, tissue sample). These procedures are time-consuming[6] and might bring uncertainty to the detection results. Recently, ambient ionization mass spectrometry methods have emerged as a promising strategy for rapid analysis of raw biological tissue samples. These methods such as desorption electrospray ionization (DESI)[7,8], laser ablation electrospray ionization (LAESI)[9], feature minimal sample pretreatment, low sample consumption and high sensitivity,

Received 14 January 2016; accepted 20 January 2016
*Corresponding author. Email: chw8868@gmail.com
This work was supported by the National Natural Science Foundation of China (Nos. 21565004, 21225522), and the Program for Changjiang Scholars and Innovation Research Team in Universities (PCSIRT) of China (No. IRT13054).

DOI: 10.1016/S1872-2040(16)60910-6

and were successfully applied to the simultaneous and direct analysis of a broad range of phospholipids from biological tissue samples. However, these methods mainly analyzed phospholipids on the surface of tissue sample. More phospholipids could be recovered by sampling bulk biological tissue rather than the surface of the tissue sample.

Herein, without sample pretreatment, a novel strategy termed as internal extractive electrospray ionization mass spectrometry (iEESI-MS)[10–12] was applied to probe 21 kinds of different proportion of CH_3OH-H_2O extraction solvent. The experimental results showed that CH_3OH-H_2O (30:70, *V/V*) was the optimal solvent system favorable for phospholipids extraction. Furthermore, a new mass spectrometry method was established for the analysis of phospholipids in biological tissue samples.

2 Experimental

2.1 Instruments and reagents

The iEESI-MS experiments were carried out by a homemade internal extractive electrospray ion source coupled with a linear trap quadruple (LTQ) mass spectrometer (Thermo Scientific, San Jose, U.S.A.). The temperature of MS capillary inlet was typically set at 150 °C. The tube lens voltage was set at 100 V and the capillary voltage kept at 10 V. The voltage used for spray ionization was 5.5 kV under the positive ion detection mode. Tandem mass spectrometry for structural confirmation was carried out using collision-induced dissociation (CID) experiments. An isolation window of 1.5 Da and normalized collision energy of 10%–30% was chosen. Besides, other parameters were set as the default values of instrument, and no further optimization was performed. The fused silica capillary (0.10 mm i.d., 0.15 mm o.d., Agilent Technologies Co., Ltd., U.S.A.) was used to inject extractive solution into tissue samples. Methanol (HPLC grade) was purchased from ROE Scientific Inc. (Newark, U.S.A). The deionized water used for the experiments was provided by ECIT chemistry facility at Laboratory.

2.2 iEESI-MS analysis

The principle schematic diagram of direct tissue analysis using iEESI-MS is shown in Fig.1. The fused silica capillary was parallelly inserted into the tissue sample with certain volume, allowing the distance between the fused silica capillary tip inserted the tissue and the apex of tissue was 2 mm. The apex of tissue was pointed to the ion entrance of the mass spectrometer at a distance of 5–6 mm. Extraction solution (mixture of CH_3OH and H_2O at certain proportion) biased with a high voltage (+5.5 kV) was straightforwardly infused into the analyzed samples through the inserted fused

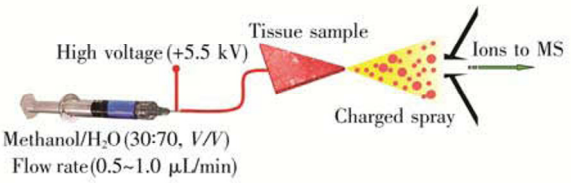

Fig.1 Schematic diagram of the internal extractive electrospray ionization mass spectrometry (iEESI-MS)

silica capillary, with a flow rate of 0.5–1.0 μL min^{-1}. The analytes were extracted by the injected extractive solution and carried along the electric field gradient inside the bulk volume of the sample. Fine charged droplets containing analytes were generated on the apex of analyzed tissue sample. After desolvation, the ions of analytes were brought into mass spectrometer ion inlet for interrogation.

The tested animal tissue samples such as pork, beef, porcine heart and porcine lung were purchased from local markets. Adjacent normal tissue and cancer tissue of lung cancer, esophageal cancer tissue from human were provided by Second Affiliated Hospital of Nanchang University. All samples were stored around −80 °C in ultra-low refrigerator and thawed to room temperature (25 °C) before performing iEESI-MS analysis. Because of the repeatability of experimental results was vital for establishment of reliable method, hence when performing optimization experiment, each tissue sample was measured more than 3 times as CH_3OH and H_2O at different proportion.

3 Results and discussion

3.1 Optimization of extraction solvent composition

To obtain abundant information of phospholipids with higher signal intensity, choosing appropriate extraction solvent is of great importance for the performance improvement of the analysis method. The chloroform-methanol system (Folch or bligh and dyer recipes) is commonly used for lipid extraction[4]. This method used $CHCl_3$-CH_3OH (2:1, *V/V*) mixed solvent, but chloroform is carcinogenic[13], and its degradation easily generated phosgene, hydrogen chloride gas. Some unstable phospholipids might occur chemical reaction[14] and affect the accuracy of experimental results. Though dichloromethane-methanol ($CHCl_2$-CH_3OH)[15], methyl tert-butyl ether methanol (MTBE-CH_3OH)[1] for lipids extraction were reported in previous literatures, these systems were not used in direct mass spectrometry analysis. Generally, both extractive system and the property of solvent have great influence on the extractive efficiency of analytes.

Extraction efficiency of analytes is also strongly related to the composition of solvent and solubility of analytes in the solvent. Solubility is still subjected to similarity-

intermiscibility theory[16]. In this aspect, extractive solvent of CH_3OH and H_2O at different proportion (0−100%) were applied for iEESI-MS analysis of phospholipids. The signal intensity changes of dipalmitoyl phosphatidylcholine (*m/z* 757, $[DPPC + Na]^+$) (A), phosphatidylcholine (*m/z* 783, $[POPC + Na]^+$) (B), oleoyl phosphatidylcholine (*m/z* 809, $[DOPC + Na]^+$) (C) and arachidonic acid stearoyl phosphatidylcholine (*m/z* 833, $[SAPC + Na]^+$) (D) from cancer tissue (2a) and adjacent normal tissue (2b) of lung cancer with concentration of methanol in solvent were shown in Fig.2.

As shown for cancer tissue (Fig.2a), the signal intensity of above four kinds of phospholipids significantly increased with an increase in CH_3OH proportion from 0 to 5%, from 20% to 30%, and from 75% to 80%. Overall, the signal intensities were maximized at 30% CH_3OH concentration. Similarly, it could be seen from adjacent normal tissue (Fig.2b), higher signal intensity of above four kinds of phospholipids was obtained with the CH_3OH proportion at 30%. The results indicated that CH_3OH-H_2O (30:70, *V/V*) was the optimal proportion for phospholipids extraction from cancer tissue and adjacent normal tissue of lung cancer. Thus, CH_3OH-H_2O (30:70, *V/V*) was selected as optimized solvent in the following experiments.

3.2 Analysis of cancer patients samples

Mass spectrum of cancer tissue (Fig.3a), adjacent normal tissue (Fig.3b) of lung cancer and esophageal cancer tissue (Fig.3c) obtained using CH_3OH-H_2O (30:70, *V/V*) was shown in Fig.3. Isolated precursor ions conducted collision-induced dissociation (CID) experiment, through comparison of their characteristic fragments with literatures validated that the majority of dominant peaks in the mass range *m/z* 700−900 were identified as phospholipids. Identified compounds included *m/z* 757 $[PC(32:0) + Na]^+$, *m/z* 773 $[PC(32:0) + K]^+$, *m/z* 783 $[PC(36:4) + Na]^+$, *m/z* 799 $[PC(36:4) + K]^+$, *m/z* 809 $[PC(36:2) + Na]^+$, *m/z* 825 $[PC(36:2) + K]^+$, *m/z* 833 $[PC(38:4) + Na]^+$ etc.

The relative abundance of *m/z* 783 $[PC(36:4) + Na]^+$, *m/z* 799 $[PC(36:4) + K]^+$, *m/z* 809 $[PC(36:2) + Na]^+$, *m/z* 825 $[PC(36:2) + K]^+$, *m/z* 833 $[PC(38:4) + Na]^+$ in the cancer tissue (Fig.3a) were significantly higher than the relative abundance of the same species in adjacent tissue (Fig.3b), whereas the relative abundance of *m/z* 757 $[PC(32:0) + Na]^+$ in adjacent normal tissue was higher than that in cancer tissue. Previous literatures reported that predominant signal peak of *m/z* 757, 783, 809 and 833 were the main components of pulmonary surfactant[17]. Pulmonary surfactant is secreted by alveolar type 2 (AT2) cells (the constitution of Alveolar wall). The main components of pulmonary surfactant include phospholipids (DPPC 80%) and a small amount of pulmonary surfactant binding protein. It was proved that canceration of AT2 cells were caused by gene mutations[18], and familial pulmonary fibrosis and lung cancer are correlated with the surfactant protein D (SP-D) mutations[19]. Some studies have even suggested that the pulmonary surfactant binding protein B (pro-SP-B) in serum is a potential biomarker of lung cancer[20]. However, the relationship between the abnormal metabolism of pulmonary surfactant and convertion of AT2 cells to cancer cells remained to be unclear.

Moreover, by comparison of the mass spectra data of lung cancer tissue (Fig.3a) with esophageal cancer tissue (Fig.3c), the signal peak of phospholipids including *m/z* 726, 757, 773, 783, 799, 809, 825 and 849 were mainly obtained both in lung cancer tissue and esophageal cancer tissue, but the information of phospholipids obtained from lung cancer tissue were much more than esophageal cancer tissue in the mass range of *m/z* 700–900, which was attributed to the differences between tissue samples.

Phospholipids are the main components of cell membrane and play a significant role in a variety of cellular processes such as storage of energy[1] and cell signaling[2]. Emerging evidences showed that cancer and diverse human diseases are strongly correlated with changes of phospholipids, which

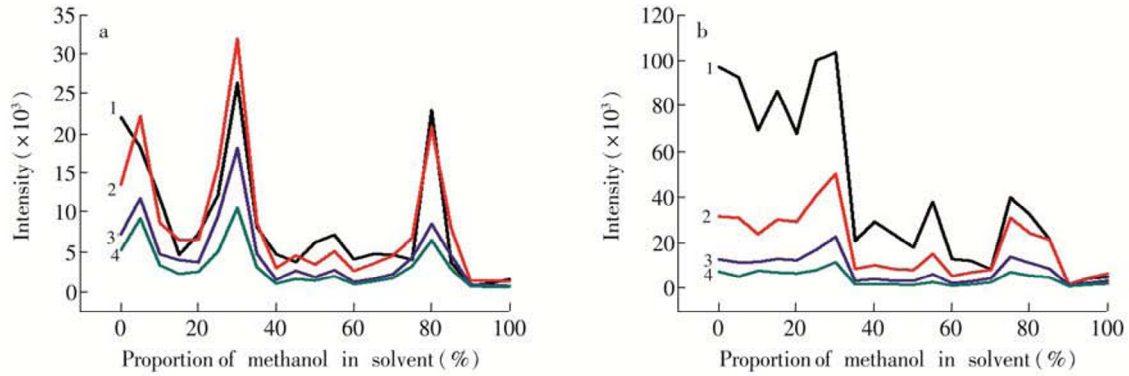

Fig.2 Effect of solvent constitution of iEESI. The change of signal intensity of dipalmitoyl phosphatidylcholine (*m/z* 757, $[DPPC + Na]^+$) (A), phosphatidylcholine (*m/z* 783, $[POPC + Na]^+$) (B), oleoyl phosphatidylcholine (*m/z* 809, $[DOPC + Na]^+$) (C) and arachidonic acid stearoyl phosphatidylcholine (*m/z* 833, $[SAPC + Na]^+$) (D) from cancer tissue (a) and adjacent normal tissue (b) of lung cancer with different proportion of methanol.

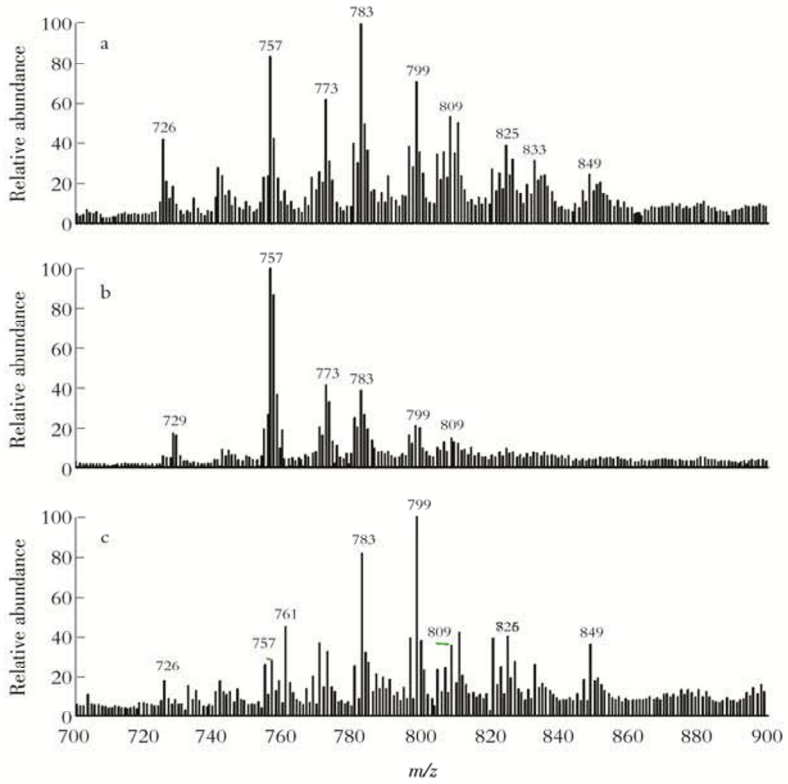

Fig.3 iEESI-MS analysis of different cancer tissues using CH_3OH-H_2O (30:70, *V/V*) as solvent, (a) lung cancer tissue, (b) lung cancer adjacent tissue, (c) esophageal tissue.

suggested that phospholipids could be used as potential biomarkers of diseases[5]. Recent research proved the significant role of phospholipids in the cardiovascular diseases[21,22]. Therefore, the establishment of a fast and accurate method to analyze phospholipids in the complex biological tissue sample would exert great influence on seeking the biomarkers of diseases, probing the relationship between the mechanism of diseases and variations in phospholipids metabolism.

3.3 Analysis of other biological tissue samples

As shown in Fig.4, the mass spectra of pork (Fig.4a), beef (Fig.4b), porcine heart (Fig.4c), porcine lung (Fig.4d) were obtained by iEESI-MS using CH_3OH-H_2O (30:70, *V/V*) as extractive solvent. In the mass range of *m/z* 700–900, it could be observed from Fig.4 that phospholipids such as *m/z* 757, 773, 783, 799, 809, 825, 833 and 849 were mainly obtained in these biological tissue samples. Similarly, because of heterogeneity among different tissue samples, the great variability of mass signals was observed. These results further demonstrated that this solvent system (CH_3OH-H_2O, 30:70,

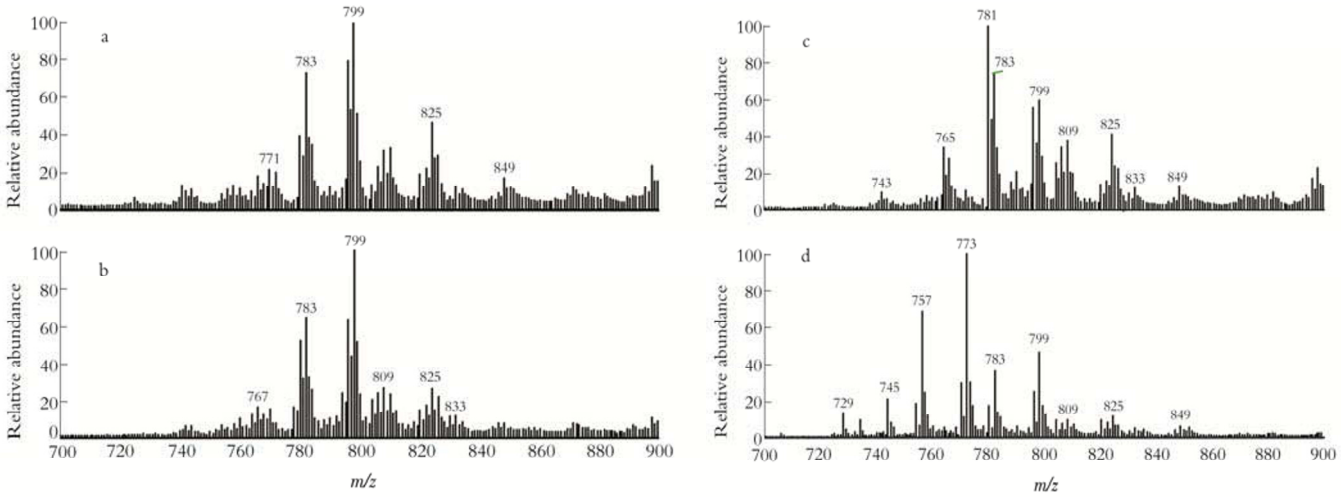

Fig.4 iEESI-MS analysis of different animal tissues using CH_3OH-H_2O (30:70, *V/V*) as solvent, (a) pork, (b) beef, (c) porcine heart, (d) porcine lung

V/*V*) was favorable for the phospholipids extraction in both human tissue samples (including cancer tissue and adjacent normal tissue) and different animal tissue samples (such as pork, beef, porcine heart, porcine lung).

4 Conclusions

In conclusion, the experimental results indicated that the selectivity and sensitivity of iEESI-MS could be significantly improved by selecting appropriate solvent. 21 kinds of different ratios of CH_3OH-H_2O solvent were systematically tested for the efficient phospholipid analysis by iEESI-MS and the CH_3OH-H_2O (30:70, *V*/*V*) ratio displayed the highest extraction efficiency for different biological tissue samples, including animal and human tissues. Overall, direct iEESI-MS analysis without chemical degradation is a promising approach for the study of phospholipids in biological tissue samples.

References

[1] Wang H, Manicke N E, Yang Q, Zheng L, Shi R, Cooks R G, Ouyang Z. *Anal. Chem*., **2011**, 83(4): 1197–1201

[2] Santos C R, Schulze A. *FEBS J.*, **2012**, 279(15): 2610–2623

[3] Ellis S R, Ferris C J, Gilmore K J, Mitchell T W, Blanksby S J, In Het Panhuis M. *Anal. Chem*., **2012**, 84(22): 9679–9683

[4] Xie W, Gao D, Jin F, Jiang Y, Liu H. *Anal. Chem*., **2015**, 87(14): 7052–7059

[5] Guo S, Wang Y, Zhou D, Li Z. *Sci. Rep*., **2014**, 4: 5959

[6] Paglia G, Ifa D R, Wu C, Corso G, Cooks R G. *Anal. Chem*., **2010**, 82(5): 1744–1750

[7] Girod M, Shi Y, Cheng J X, Cooks R G. *Anal. Chem*., **2011**, 83(1): 207–215

[8] Lostun D, Perez C J, Licence P, Barrett D A, Ifa D R. *Anal. Chem*., **2015**, 87(6): 3286–3293

[9] Nemes P, Woods A S, Vertes A. *Anal. Chem.*, **2010**, 82(3): 982–988

[10] Zhang H, Zhu L, Luo L, Wang N, Chingin K, Guo X, Chen H. *J. Agr. Food Chem.*, **2013**, 61(45): 10691–10698

[11] Zhang H, Chingin K, Zhu L, Chen H. *Anal. Chem*., **2015**, 87(5): 2878–2883

[12] Zhang H, Gu H, Yan F, Wang N, Wei Y, Xu J, Chen H. *Sci. Rep.*, **2013**, 3: 2495

[13] Nagano K, Kano H, Arito H, Yamamoto S, Matsushima T. *J. Toxicol. Env. Heal. A*, **2006**, 69(20): 1827–1842

[14] Xi C, Chen Y H, Yang W, Zhang R P, He J M, Zhang H B, Xiao X H, Zeper A. *Chinese J. Anal. Chem*., **2013**, 41(9): 1308–1314

[15] Hu C, van Dommelen J, van der Heijden R, Spijksma G, Reijmers T H, Wang M, Slee E, Lu X, Xu G, van der Greef J. *J. Proteome Res.*, **2008**, 7(11): 4982–4991

[16] Jia B, Zhang X L, Ding J H, Yang, S P, Chen H W. *Chinese Science Bulletin* (Chinese Version), **2012**, 57(20): 1918

[17] Wei Y, Chen L, Zhou W, Chingin K, Ouyang Y, Zhu T, Wen H, Ding J, Xu J, Chen H. *Sci. Rep*., **2015**, 5: 10077

[18] Sutherland K D, Berns A. *Mol. Oncol.*, **2010**, 4(5): 397–403

[19] Ishii T, Hagiwara K, Ikeda S, Arai T, Mieno M N, Kumasaka T, Muramatsu M, Sawabe M, Gemma A, Kida K. *COPD*, **2012**, 9(4): 409–416

[20] Sin D D, Tammemagi C M, Lam S, Barnett M J, Duan X, Tam A, Auman H, Feng Z, Goodman G E, Hanash S. *J. Clin. Oncol*., **2013**, 31(36): 4536–4543

[21] Zwaal R F A, Comfurius P, Bevers E. *Cell Mol. Life Sci.*, **2005**, 62(9): 971–988

[22] Aboagye E O, Bhujwalla Z M. *Cancer Res*., **1999**, 59(1): 80–84