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# Application of ESI-MS combined with $^1$H NMR in analyzing seed phospolipids of six species of *Michelia*

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**Abstract**: To date, there was no example to authenticate seed phospholipids based on their fingerprinting. By means of electrospray ionization mass spectrometry(ESI MS) and proton nuclear magnetic resonance($^1$H NMR) spectroscopy, phospholipids fraction extracted from seeds of six species from the genus *Michelia* were detected. It was firstly found that distinct difference in spectral fingerprinting region between $m/z$ 895-910 in ESI MS and 5.30—5.40 mg/L in $^1$H NMR among these six species. Thus, it suggested that the spectral differences shown by ESI MS and $^1$H NMR among seeds of these six species could be applied to identify them. And in a wider sense, for analyzing seed phospholipids of other plant species, a combination of ESI MS AND NMR was an effective tool.

**Key words**: Seed phospholipid; ESI MS; NMR; Fingerprinting; *Michelia*

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# 联合应用 ESI MS 和$^1$H NMR 分析含笑属植物种子磷脂

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**摘 要**: 首次尝试利用电喷雾电离质谱(ESI MS)和氢原子核磁共振($^1$H NMR)技术分析了含笑属六种植物种子的磷脂特性,发现在两个指纹图谱区发现明显的差异,即在质荷比($m/z$)895-910(ESI MS)和 5.30～5.40 mg/L($^1$H NMR)两个特异的区域存在显著差异。这些源于种子磷脂 ESI/MS 和$^1$H NMR 的谱带差异可以被用来分析不同植物的种子的磷脂特征。而且,相似地,在更广的层面上,特异性的谱带差异可用于分析其他植物种子的磷脂组成和特性,辅助鉴定种子。

**关键词**: 种子磷脂; 质谱; 核磁共振; 指纹; 含笑属

As for lipids, they provide both the structural basis of cell membranes and fuel for metabolism. Lipids consist of diverse types of hydrophobic biomolecules. One lipid classification system contrasts neutral lipids, such as sterols and acylglycerols, with polar lipids. Another scheme contrasts complex lipids, which were lipids that contain one or more fatty acyl species, with simple lipids that contain only a single building block, such as fatty acids or sterols(Fahy *et al*., 2005). Polar lipid classes, which include the major membrane lipids,

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were defined by a specific 'head group' or polar moiety (Fig. 1). Each polar lipid class was composed of various molecular species whose fatty acids or other hydrocarbon portions vary in chain length and degree of saturation (Brügger *et al.*, 1997). In plants, increasing evidence has demonstrated roles for lipids in cellular processes that include photosynthesis, signal transduction, vesicle trafficking, secretion and cytoskeletal rearrangement (Hallett and Bewley, 2002; Wang, 2002; Welti and Wang, 2004; Meijer and Munnik, 2003). Presumedly, there was certain relationship between lipid profiles and classification of species. To date, although various mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy were usually the methods of choice for the structure determination of natural (or synthesized) product and for both identifying and quantifying the metabolites in a cell or tissue type for being extremely rapid and without bias (Krishnan *et al.*, 2005; Ward *et al.*, 2007; Colquhoun, 2007). To our knowledge, NMR spectroscopy in combination with multivariate analysis has been applied to a number of classification problems including species and cultivar discrimination and quality assessment of foods and herbal medicines (Le Gall *et al.*, 2003; Wallis and Browse, 2002; Colquhoun, 2007), but few findings on seed lipids for *Michelia* plants by means of both MS and NMR have been reported. Mass spectrometry has established itself as the method of choice, but complementary information from other techniques, particularly NMR spectroscopy was potentially useful in extending

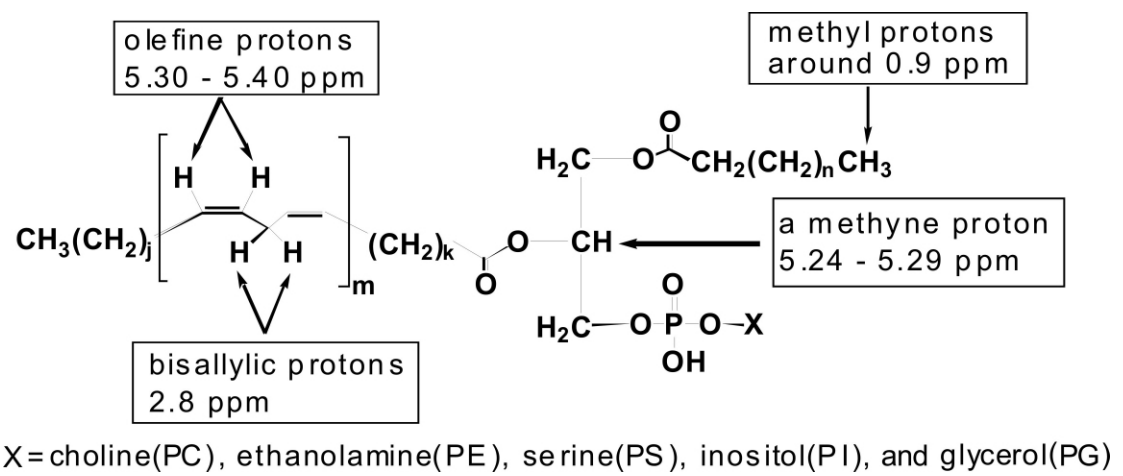


Fig. 1 A general structure of phospholipid with double bonds (olefinic structure) and chemical shift (δ, mg/L) in NMR spectrophotometry

the coverage of information.

To exactly test whether the new method was reliable, we used six species (which were well classified to certain systematic position) from the genus *Michelia*. In the investigation, both tandem electrospray ionization (ESI) MS and $^1$H NMR profiling were harnessed, which were already well-suited to generating the necessary data in this study. Seed phospholipid profiles of *M. hedyosperma*, *M. fulgens*, *M. yunnanensis*, *M. chapensis*, *M. foveolata* and *M. floribunda* were analyzed, which can be hardly differentiated by morphological features (Nooteboom, 2000; Liu *et al.*, 2004) by means of tandem ESI MS and $^1$H NMR, using spectral data on seed lipids of each species as chemical descriptors.

## 1 Materials and Methods

Mature seeds of *M. yunnanensis*, *M. chapensis*, *M. floribunda* and *M. hedyospernia* were collected at the Magnolia garden of the Kunming Institute of Botany, Chinese Academy of Sciences (25°07′24″ N, 102°44′37″ E, Alt. 1 982 m) on October 3, 2009. Seeds of *M. foveolata* and *M. fulgens* growing in east Yunnan province, China were collected (23°22. 28′ N, 103°47. 71′ E, Alt. 2 103 m) on 17 October, 2009.

Seeds (0. 5 g) were crashed in a china mortar with

pestle，and then total lipids were extracted in a mixture of $CHCl_3/CH_3OH$(2 ∶ 1，v/v) with addition of a trace of butylated hydroxytoluene(BHT) as an anti-oxidant to prevent further oxidation of non-conjugated olefinic structure in polyunsaturated fatty acyl group. After evaporation of the solvent under reduced pressure，the residue was rinsed with acetone to remove neutral lipid such as cholesterol and triglyceride to obtain polar phospholipids such as phosphatidylcholine(PC)，phosphatidylethanolamine(PE)，phosphatidylserine(PS)，phosphatidylinsitol(PI)，phosphatidylglycerol(PG) and sphingolipids(Bligh & Dyer，1959). The polar lipid fraction was dissolved in a mixture of methanol/acetonitrile/water(194 ∶ 215 ∶ 16) with 0.1% ammonium acetate to enhance protonation of phospholipids for ESI MS measurement. The tests of both ESI MS and following $^1$H NMR were repeated three times. ESI MS was conducted using a Perkin-Elmer SCIEX(Thornhill，ON，Canada) API-III tandem quadrapole mass spectrometer. The sample solution was introduced by direct infusion for spectral acquisition. Positive ion was scanned from $m/z$ 500-900 for most of the polar phospholipids. $^1$H NMR spectra were recorded on 600 MHz Varian INOVA UNITY 600 spectrophotometer and chemical shift(δ) were expressed in mg/L given relative to $CD_3OD$(3.30 mg/L).

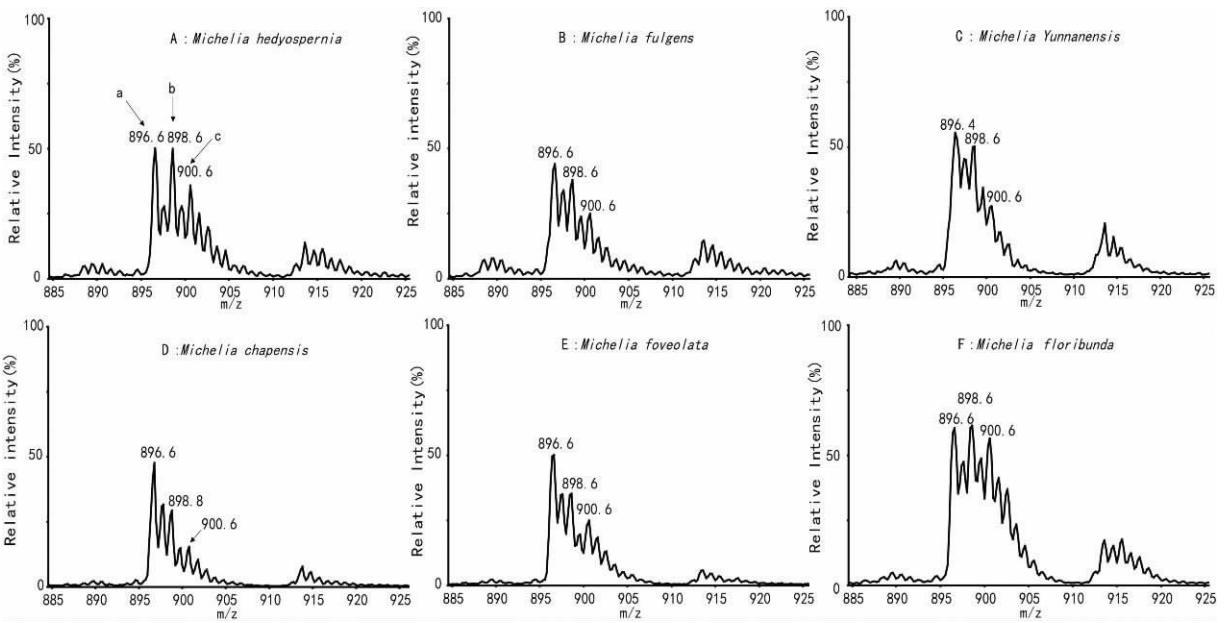


Fig. 2　ESI MS spectra of phospholipid fractions of six species in the genus *Michelia*

## 2　Results and Discussion

An ESI MS spectrum for the extract from *M. floridunda* was given in Fig. 2-A. Although a number of signals were observed，the complex spectrum was surprisingly similar to those of phospholipids from other seed species (data not shown). When a region between $m/z$ 895-910 was expanded to a full scale(Fig. 2)，distinct differences in signal patterns were observed among seed species investigated(spectra A-F). For example，between $m/z$ 895-910 in spectrum A，there were at least three kinds of signals at $m/z$ 896.6(peak a)，898.6(peak b) and 900.6(peak c). The signal at $m/z$ 896.6(peak c) overlaped a second isotopic peak of $m/z$ 896.6(peak a). Also，the signal at $m/z$ 900.6(peak c) overlaped a second isotopic peak of $m/z$ 898.6. The first important point was that the difference in $m/z$ between peak a and b，and peak b and c was equal to 2. This result suggested that chemical structures of three kinds of phospholipids (peak a，b and c) were very similar，and the differences in $m/z$ 2 between them indicated that the

difference was due to the number of double bond (Fig. 1) since the difference in mass number between $-CH_2-CH_2-$ and $-CH=CH-$ was equal to 2. A similar pattern can be seen in Fig. 2B-1F for the other five species. The second important feature was that, although three peaks at $m/z$ 896.6 (peak a), 898.6 (peak b) and 900.6 (peak c) were found for all the spectra, their relative intensities were significantly different among seed species. This indicated that all the seed lipids contained the same three kinds of phospholipids, their relative amounts were different species by species. Using this method (e.g., ESI MS), profiling of total phospholipid molecular species was considered better to analyze lipid

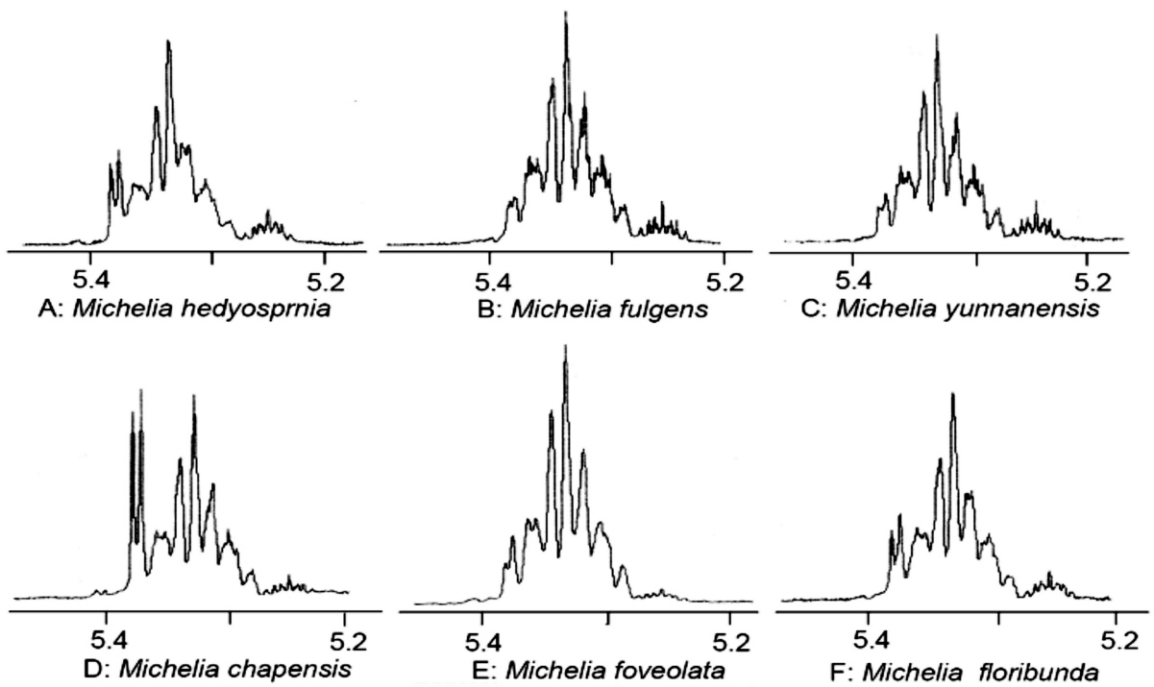


Fig. 3 $^1$H NMR spectra of phospholipid fractions of six species from *Michelia*
Solvent: deuteriomethanol; Abscissa: chemical shift (δ, mg/L)

molecular species in seeds.

As described above, the difference in ESI MS spectrum ($m/z$ 885-910) among 6 species was supposed to be originated from the difference in structure of PC and number of the olefinic bond (Fig. 1), similar spectral difference should be observed in $^1$H NMR since non-conjugated olefinic protons (Scheme 1) gave resonance signals specifically at δ 5.3—5.5 mg/L in general. $^1$H NMR was measured for the 6 phospholipid fractions extracted from each species in deuteriomethanol. The $^1$H NMR spectrum of the each species was also found to be highly similar to each other, and they were those of typical glycerophospholipids. Terminal methyl group of fatty acyl group at sn-1 and 2-posititon on glycerol backbone gave resonance signal at typically δ 0.9 mg/L for methyl protons, δ 2.0—2.1 mg/L (m, allylic protons; $-CH_2CH_2CH=CH$), δ 2.8 mg/L (m, bis-allylic protons; $-[CH=CH-CH_2-CH=CH]$) and δ 5.30—5.40 mg/L (m, olefin protons; CH=CH) and δ 5.24—5.29 pm for a methyne proton (Baba *et al.*, 2001; Yamomoto *et al.*, 2007) (Fig. 1). Unfortunately, analyses of multiplet signals at δ 5.3—5.5 mg/L for olefine protons was not possible since the multiplicity was due to proton-proton coupling, magnetic non-equvalence of each olefine proton and overlap of olefinic signals from different phospholipids. Although significant differences seen between spectra, they were not as clear as ESI MS spectra in Fig. 2. The difference between spectrum B and F in Fig. 3 was not clear. Another feature seen from Fig. 3 was the relative intensity of the olefinic proton signals between δ 5.29—5.40 mg/L and those of multiplet signals of the hydrogen

(5.25 mg/L) on sn-2 carbon of glycerol moiety in phospholipids. For example, the spectrum C(*M. yunnanensis*), the ratio was 9 ∶ 1 and in spectrum E(*M. foveolata*), the ratio was 27 ∶ 1. This indicated that the total number of double bonds in the phospholipid fractions of seed lipids from *M. yunnanensis* was less than that in the seed lipid fractions from *M. foveolata*. This outcome was consistent with the results from comparison between the spectra C and E in Fig. 2. In spectrum E, the height of a peak at $m/z$ 896.6 was apparently higher than those at $m/z$ 898.6 and 900.6 comparing from the relative intensity of the peak at $m/z$ 896.4 in the spectrum C, indicating that the number of double bonds in phospholipid fractions from M. foveolata appears to be more than those in phospholipid fractions from *M. yunnanensis*. Similarly, NMR fingerprintings were applied to discrimination of three Ephedra species (Kim *et al.*, 2005) and Strychnos species (Frédérich *et al.*, 2004). Based on our results and other studies published, a successful model could be established for every species, better in combination with MS spectral data.

As observed from the ESI MS and $^1$H NMR spectra of 6 species, the phospholipids patterns were similar, but their fingerprint regions were significantly different from one species to another. Therefore, it was possible that spectral difference may be utilized to analyze seed phospholipids species and to a certain extent, potentially used to classify plant species. It should be a valuable approach to detect lipid based on spectral analytical methodology in seeds in the future.

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