# Metabolic Fingerprinting of *Ephedra* Species Using $^1$H-NMR Spectroscopy and Principal Component Analysis

Hye Kyong KIM,[a] Young Hae CHOI,[a] Cornelis ERKELENS,[b] Alfons W. M. LEFEBER,[b] and Robert VERPOORTE*,[a]

[a] *Division of Pharmacognosy, Section Metabolomics, Institute of Biology, Leiden University; and* [b] *Division of NMR, Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University; P. O. Box 9502, 2300 RA Leiden, The Netherlands.* Received August 2, 2004; accepted September 27, 2004

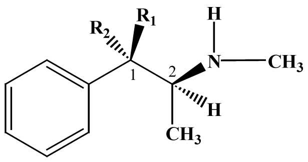
**The metabolomic analysis of *Ephedra* species was performed using $^1$H-NMR spectroscopy and multivariate data analysis. A broad range of metabolites could be detected by $^1$H-NMR spectroscopy without any chromatographic separation. The principal component analysis used to reduce the huge data set obtained from the $^1$H-NMR spectra of the plant extracts clearly discriminated three different *Ephedra* species. The major differences in *Ephedra sinica*, *Ephedra intermedia* and *Ephedra distachya* var. *distachya* were found to be due to benzoic acid analogues in the aqueous fraction and ephedrine-type alkaloids in the organic fraction. Based on this metabolomic recognition, one of nine commercial *Ephedra* materials evaluated was shown to be a mixture of *Ephedra* species. This method will be a useful tool for chemotaxonomic analysis and authentification of *Ephedra* species including quality control of plant materials.**

**Key words** metabolic fingerprinting; *Ephedra*; ephedrine; $^1$H-NMR; principal component analysis

*Ephedra*, known as Ma huang, is one of the oldest medicinal herbs known to mankind. The genus *Ephedra* (Ephedraceae) contains more than 50 species.1) In both traditional and more recent popular uses, the pharmacological effects are mainly attributable to ephedrine-type alkaloids (Fig. 1) such as ephedrine, pseudoephedrine, methylephedrine and norephedrine.2) Although *Ephedra sinica* has been the primary source for the ephedrine alkaloids or dietary supplements containing *Ephedra* plant material, other species such as *Ephedra equistina*, *Ephedra intermedia*, *Ephedra geradiana*, *Ephedra alata*, *Ephedra distachya*, *Ephedra botschantzevii*, *Ephedra fragilis*, *Ephedra major*, *Ephedra minuta*, *Ephedra monosperma*, *Ephedra pachyclada*, *Ephedra likiagensis*, *Ephedra saatilis*, *Ephedra lomatolepis*, *Ephedra lepidosperma*, *Ephedra przewalskii*, and *Ephedra regelianan* contain a certain amount of the ephedrine alkaloids.3) The concentration of the alkaloids varies from 0.02 to 3.4%.4) In spite of the variation in the alkaloid content, previous studies related to quality control or chemotaxomic work on *Ephedra* species were performed focusing on the ephedrine alkaloids.4—6) To confine the analysis to a small range of metabolites (ephedrine-type alkaloids in the case of *Ephedra* species) may be misleading in a chemotaxonomic study. For distinguishing the metabolic features of the plant, a broad analysis is demanded. Therefore chemical fingerprinting covering a whole range of metabolites is necessary in order to confirm or deny the plant material being used for the manufacturing of a product. Recently, Schaneberg *et al.*7) reported two carboxylic acid derivatives as possible chemical markers in *E. sinica* detected by HPLC. However, to use the whole "metabolome" of *Ephedra* and obtain the profile of all metabolites might be more suitable to exclude adulterants or wrong identification.

The term "metabolome" has been used to describe the observable chemical profile or fingerprint of the metabolites in whole tissues.8) To obtain the most complete metabolomic profile, it is necessary to use a wide spectrum of analytical techniques which are rapid, reproducible, and stable in time and require only a very simple sample preparation. NMR is one of the techniques that meet those requirements. Although NMR method development has mainly been driven toward the enhancement of qualitative information for general structure elucidation, the quantitative aspect has also been recognized since the early days of NMR.9) Moreover, in the last decade a number of techniques have been devised to develop NMR spectroscopy as a fingerprinting tool for the quality assessment of crude plant materials. Multivariate or pattern recognition techniques such as the well-described principal component analysis (PCA) are important tools for the analysis of data obtained by NMR. Recently, NMR in combination with PCA has been applied to the metabolomic profiling of several types of plants10,11) and phytomedicines.12,13)

In this study, we report a $^1$H-NMR spectroscopy method coupled with PCA for the metabolic fingerprinting of *Ephedra* species. Based on these data, discrimination is performed for three different *Ephedra* species, and nine commercial *Ephedra* herbs were evaluated using this metabolomic approach. The method might be a useful tool for quality control of *Ephedra* pharmaceuticals and authentification of phytomedicines.



$R_1$ = OH, $R_2$ = H, Ephedrine

$R_1$ = H, $R_2$ = OH, Pseudoephedrine

Fig. 1. Structures of Ephedrine and Pseudoephedrine

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* To whom correspondence should be addressed. e-mail: verpoort@chem.leidenuniv.nl

## Results and Discussion

A mixture of $CHCl_3$–MeOH–$H_2O$–$NH_4OH$ was used for the extraction of the metabolites of *Ephedra* plants. When this basic extraction solvent was used, most of alkaloids were transferred to the organic $CHCl_3$ phase, with a recovery of more than 94%. When a neutral solvent system without $NH_4OH$ was used, ephedrine-type alkaloids were present both in the $CHCl_3$ fraction and the water fraction, which makes it difficult to interpret the metabolites in the water fraction. After extraction with the basic solvent, two different fractions from the plant material, the $CHCl_3$ and aqueous fractions, were measured with $^1H$-NMR. Organic fractions in which most ephedrine-type alkaloids were present showed quite distinct spectra in different *Ephedra* species (Fig. 2). Characteristic signals of (−)-ephedrine such as N–$CH_3$ at $\delta$ 2.59 (s), H-1 at $\delta$ 4.7—4.9 (d, $J$=3.8 Hz), and methyl protons at $\delta$ 0.8—0.9 (d, $J$=6.7) were found in the spectrum of $CHCl_3$ fractions of *E. sinica*. In addition to these signals, another singlet at $\delta$ 2.56 (s) was detected and identified as N–$CH_3$ of (+)-pseudoephedrine.[14] When the intensity of the signals at $\delta$ 2.56 and $\delta$ 2.59 was compared, the relative intensity of the signal at $\delta$ 2.56 (s) to $\delta$ 2.59 (s) was high in *E. intermedia*, which confirmed that (+)-pseudoephedrine is the major alkaloid in *E. intermedia*.[14] In the case of *E. distachya* var. *distachya*, no signals of ephedrine-type alkaloids were detected.

The $^1H$-NMR spectra of the aqueous fractions of *Ephedra* species are shown in Fig. 3. The patterns in the aromatic region ($\delta$ 6.0—9.0) are markedly different from each other. In *E. intermedia*, the proton signals which belong to benzoic acid analogues such as $\delta$ 8.08 (d, $J$=7.2 Hz), $\delta$ 7.70 (t, $J$=7.5 Hz), and $\delta$ 7.56 (t, $J$=7.8 Hz) were found to be higher than in *E. sinica* (Figs. 3A, B). In *E. distachya* var. *distachya*, the characteristic signals due to two *trans* olefinic protons ($J$=15—16 Hz) in the region of $\delta$ 6.1—6.5 and $\delta$ 7.6—7.7 were observed (Fig. 2C). These signals were identified as olefinic protons of phenylpropanoids, H-8 and H-7, respectively.[15]

PCA is an unsupervised method performed without using knowledge of sample class, which reduces the dimensionality of the data input while in a 2- or 3D map.[16] By producing new linear combinations of the original variates, in this case the integrated NMR spectral regions, it is possible to plot such data in order to indicate relationships between samples in a multidimensional space. It enables the easy comparison of plant metabolic profiles. To analyze the data set obtained from *Ephedra* species, the covariance method was applied. In applying PCA to the organic fractions, the *Ephedra* species evaluated are well separated from each other (Fig. 4A). The main principal component (PC) to differentiate the plants is PC1. The *E. sinica*, *E. intermedia*, and *E. distachya* var. *distachya* were clearly distinguished by PC1. In the loading plot of PC1, it was found that PC1 was mainly affected by ephedrine signals such as from the $CH_3$ at $\delta$ 0.9—0.8, N–$CH_3$ at $\delta$ 2.60, and aromatic signal at $\delta$ 7.3 (Fig. 4B). Among those species, *E. sinica* showed higher amounts of ephedrine than other species. *E. distachya* var. *distachya* showed an unusual location in the PCA score plot where it was outside the 95% Hotelling T2 confidence eclipse because ephedrine was not detected in the plant. The commercial *Ephedra* herbs evaluated in this study were not clearly separated because the PC values from the $CHCl_3$ fraction are dominantly affected by ephedrine and pseudoephedrine. This narrow range of metabolites is not sufficient to differentiate. For further metabolic analysis to obtain clear differentiation, the $^1H$-NMR spectra of the aqueous fraction was assessed for PCA.

PCA of the aqueous fraction is shown in Fig. 5. In the

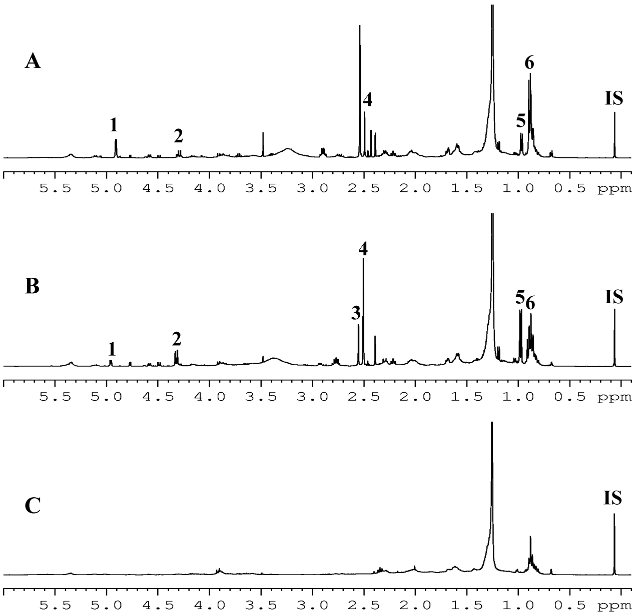

Fig. 2. $^1H$-NMR Spectra of Organic Fractions of *Ephedra*: (A) *E. sinica*, (B) *E. intermedia*, (C) *E. distachya* var. *distachya*

IS, internal standard of HMDSO. 1, Signals of H-1 proton of (−)-ephedrine; 2, signals of H-1 proton of (+)-pseudoephedrine; 3, signals of N–$CH_3$ protons of (−)-ephedrine; 4, signals of N–$CH_3$ protons of (+)-pseudoephedrine; 5, signals of $CH_3$ protons of (−)-ephedrine; 6: signals of $CH_3$ protons of (+)-pseudoephedrine.

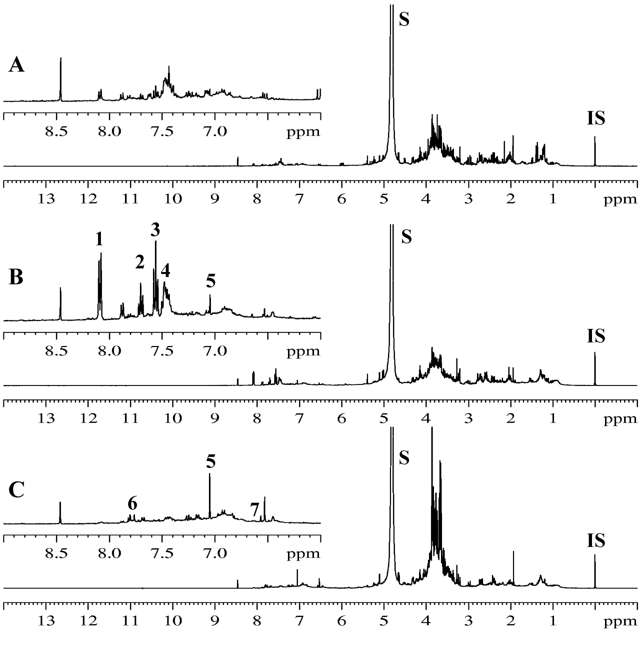

Fig. 3. $^1H$-NMR Spectra of Aqueous Fractions of *Ephedra*: (A) *E. sinica*, (B) *E. intermedia*, (C) *E. distachya* var. *distachya*

S, residual solvent signal of $H_2O$; IS, internal standard of TSP. 1—3, Signals of benzoic acid analogue; 4, signals of phenylalanine; 5, proton signal of polyphenol; 6, H-8 region of phenylpropanoid; 7, H-7 region of phenylpropanoid.

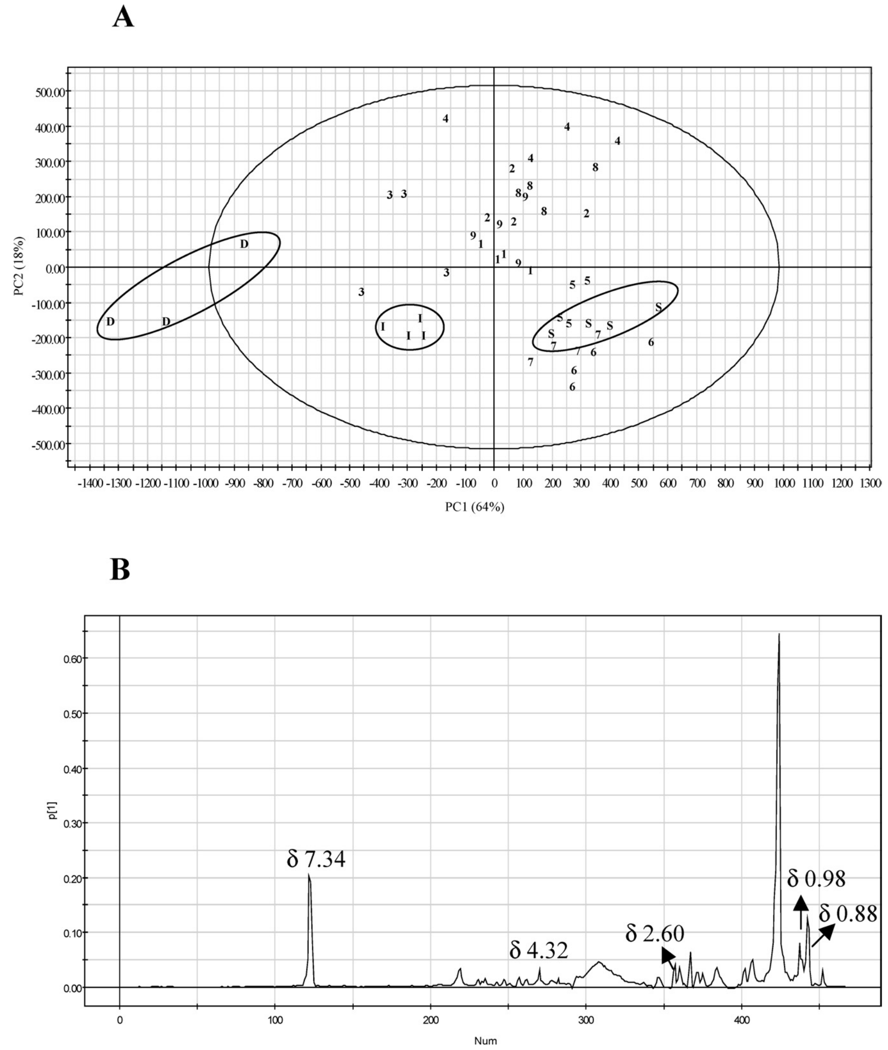

Fig. 4. (A) Score Plot of PC1 and PC2 Scores of Organic Fraction of *Ephedra* Species Following PCA Analysis and (B) Loading Plots of PC1
S, *E. sinica*; I, *E. intermedia*; D, *E. distachya* var. *distachya*. 1—9, *Ephedra* herbs 1—9 purchased from a Taiwanese market.

aromatic region, clear differences could be seen. In the sugar region ($\delta$ 3.0—5.0), it was difficult to identify each peak because a number of signals were overlapping. For this reason, only the aromatic region ($\delta$ 6.0—10.0) was used for further PCA analysis. With PC1 and PC2, all *Ephedra* species were well separated and nine *Ephedra* herbs obtained from a Taiwanese commercial market clustered near *E. intermedia* except for herb no. 6. The relatively higher PC1 of *E. intermidia* showed that it contained more benzoic acid analogues at 8.08 (d, $J$=7.2 Hz), 7.70 (t, $J$=7.5 Hz), and 7.56 (t, $J$=7.8 Hz) and monosubstituted phenolic compounds like phenylalanine at $\delta$ 7.46 (m). In the case of commercial herbs, *Ephedra* herb no. 6 was located between *E. sinica* and *E. intermedia* in the score plot, which might mean that it is a mixture of these two species. To confirm herb no. 6, the $^1$H-NMR spectra obtained from mixtures of authentic *E. sinica* and *E. intermedia* (1 : 1, 1 : 2, 2 : 1, w/w) were analyzed using PCA, together with that of no. 6 commercial *Ephedra* herb. The PC cluster of *Ephedra* no. 6 is very close to that of the mixture (Fig. 6). It was presumed that this plant material comes from two different species. In ANOVA of PC values of the mixture and *Ephedra* no. 6, there was no significant difference between them.

If synthetic ephedrine alkaloids are added to the plant material, it is not possible to differentiate plant materials using methods focusing on ephedrine analogues alone, but the NMR method combined with multivariate analysis can differentiate materials because it is based on total metabolic fingerprinting.

The method using $^1$H-NMR and multivariate analysis may allow consistent discrimination of *Ephedra* species based on metabolomic profiles and is of interest as a tool for chemotaxonomic studies. This approach also can be applied for quality control and authentification of pharmaceuticals.

## Experimental

**Plant Material** *E. sinica* and *E. intermedia* were obtained from Taiwan Pharmaceutical Company (Sun Ten Phytotech Co., Taipei, Taiwan, ROC) and authentified by Prof. Y. S. Chang (Institute of Chinese Pharmaceutical Sciences, Chinese Medical University, Taichung, Taiwan, ROC). *E. distachya* var. *distachya* was generously donated by the National Herbarium Netherland, Leiden, The Netherlands. Nine *Ephedra* plant materials were purchased from a local Taiwan market. All voucher specimens are deposited in the Division of Pharmacognosy, Leiden University, The Netherlands.

**Solvents and Chemicals** Analytical grade chloroform and methanol were purchased from Merck Biosolve Ltd. (Valkenswaard, The Netherlands). $CDCl_3$ (99.96%) and $D_2O$ (99.00%) were obtained from Cambridge Isotope Laboratories Inc. (Miami, FL, U.S.A.), and NaOD was from Cortec (Paris, France).

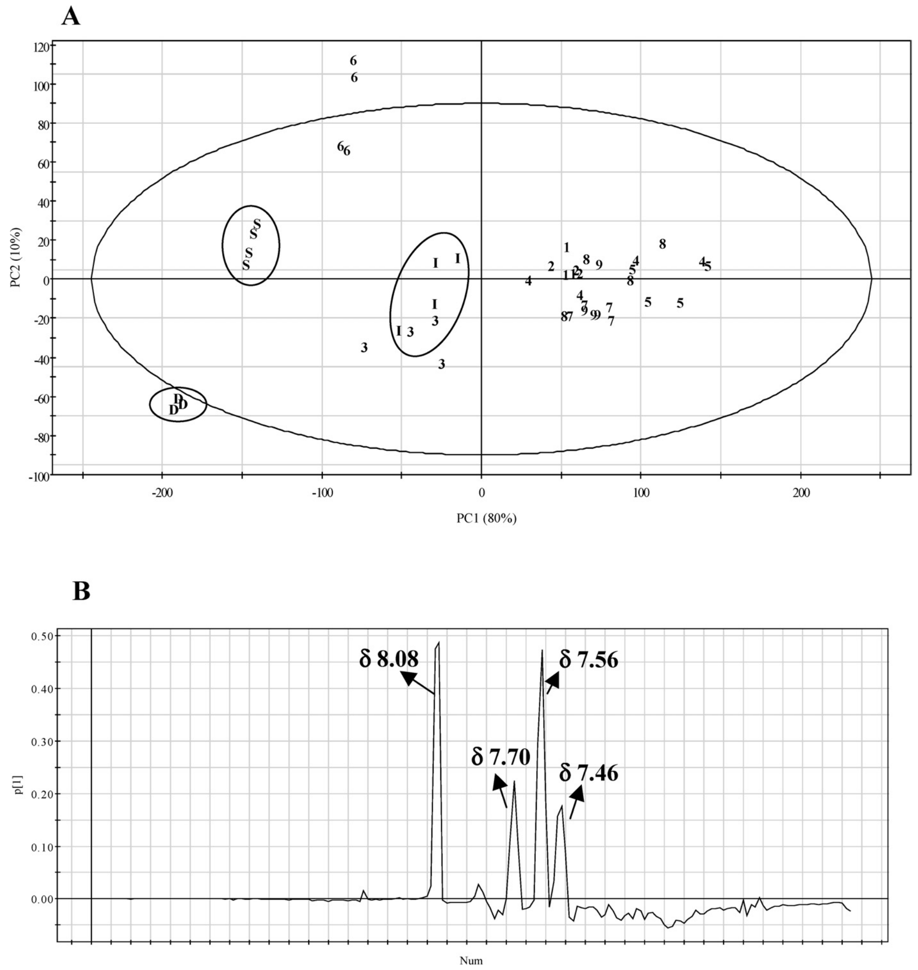

Fig. 5. (A) Score Plot of PC1 and PC2 Scores of Aqueous Fraction of *Ephedra* Species Following PCA Analysis and (B) Loading Plot for PC1

S, *E. sinica*; I, *E. intermedia*; D, *E. distachya* var. *distachya*; 1—9, *Ephedra* herbs 1—9 purchased from a Taiwanese market.

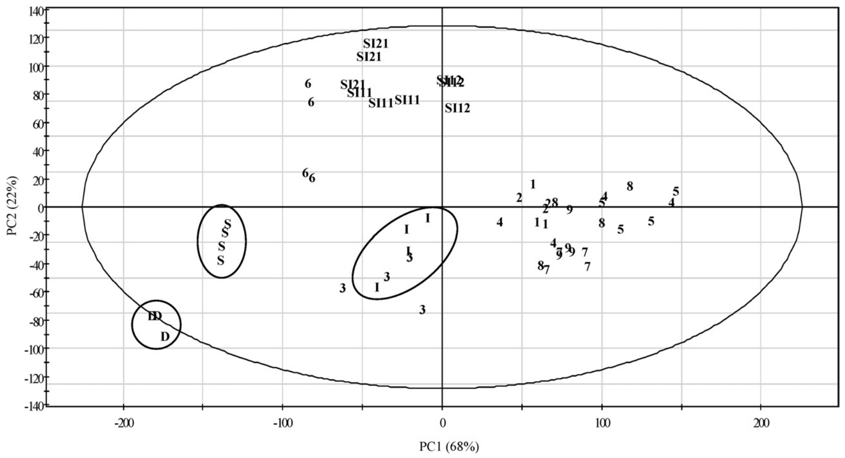

Fig. 6. Score Plot of PC1 and PC2 Scores of Aqueous Fraction for *Ephedra* Including Mixture of *E. sinica* and *E. intermedia* Following PCA Analysis

SI12, mixture of *E. sinica* and *E. intermedia* in the ratio of 1 : 2 (w/w); SI11, mixture of *E. sinica* and *E. intermedia* in the ratio of 1 : 1 (w/w); SI21, mixture of *E. sinica* and *E. intermedia* in the ratio of 2 : 1 (w/w), S, *E. sinica*; I, *E. intermedia*; D, *E. distachya* var. *distachya*; 1—9, *Ephedra* herbs 1—9 purchased from a Taiwanese market.

**Extraction** Ground material (300 mg) was transferred into a 15-ml centrifuge tube. Five milliliters of 50% water–methanol mixture, 5 ml of chloroform, and 100 $\mu$l of 28% (v/v) $NH_4OH$ were added to the tube, followed by vortexing for 30 s and sonication for 1 min. The material was then centrifuged at 3000 rpm for 20 min. The extraction was performed twice. The organic and aqueous fractions were transferred separately into a 25-ml round-bottomed flask and dried with a rotary vacuum evaporator. The dried sample was dissolved in 1 ml of NMR solvent and used for $^1$H-NMR measurement.

**$^1$H-NMR Apparatus and Parameters** The organic fractions were dissolved in $CDCl_3$. For aqueous fractions, $KH_2PO_4$ was added to $D_2O$ as a buffering agent. The pH of the $D_2O$ was adjusted to 6.0 using a NaOD (1 M) solution. All spectra were recorded on a Bruker AV-400 NMR spectrometer operating at a proton NMR frequency of 400.13 MHz. For each sample, 128 scans were recorded with the following parameters: 0.126 Hz/point; pulse width (PW)$=$4.0 $\mu$s (30°); and relaxation delay$=$2.0 s. FIDs were Fourier transformed with LB$=$0.3 Hz. The spectra were referenced to residual solvent $CHCl_3$ at 7.26 ppm for organic fractions and trimethyl silane propionic acid sodium salt (TSP) at 0.00 ppm for aqueous fractions. For scaling of all NMR signals, hexamethyl disiloxane (HMDSO, 0.01%, v/v) and TSP (0.01%, w/v) were used as internal standards.

**Data Reduction of the $^1$H-NMR Spectra and Multivariate Analysis** The $^1$H-NMR spectra were automatically reduced to ASCII files using AMIX (v. 3.8, Brucker Biospin). Spectral intensities were scaled to HMDSO and TSP for the $CHCl_3$ and aqueous fraction, respectively, and reduced to integrated regions, referred to as buckets, of equal width (0.02 ppm) corresponding to the region of $\delta$ 10.0 to $-0.1$. The regions between $\delta$ 5.1 and $\delta$ 4.70 were removed prior to statistical analyses for the aqueous fraction. The residual proton signals corresponding to $CHCl_3$ (7.26), TSP, and HMDSO were also removed at this stage. The generated ASCII file was imported into Microsoft Excel for the addition of labels and then imported into SIMCA-P 10.0 (Umetrics, Umeå, Sweden) for PCA analysis.

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