Molecular Composition Study of Mumijo from Different Geographic Areas Using Size-Exclusion Chromatography, NMR Spectroscopy, and High-Resolution Mass Spectrometry

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Abstract Four mumijo samples originated from different mountain ranges were studied using size-exclusion chromatography (SEC), Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS), and ^{1}H and ^{13}C NMR spectroscopy. All the used analytical techniques demonstrated that the mumijo samples studied consist of a "high-molecular" fulvic-like and a low-molecular components. The second component is shown using FTICR MS and NMR methods to be predominantly a range of vegetative and animal metabolites.

Keywords Humics • Mumijo • Composition • NMR • SEC • FTICR MS

Introduction

Native mumijo commonly used in traditional medicine is a brown to blackish-brown exudation of variable consistencies that seeps from rock layers of different mountain regions. Mumijo is found in the Himalayas, Hindu Kush, Badakhshan,

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Pamir, Tian Shan, Altai, Ural, Caucasus (Eurasia), and also in northern Pollock Ranges (Australia) (Khanna et al. 2008). There are several regional synonyms for mumijo; the second most common one is shilajit. Being a complex mixture of humic substances, plant, and microbial metabolites occurring in rock rhizospheres, mumijo has been found to consist mostly of humic substances (80%) (Agarwal et al. 2007; Frolova and Kiseleva 1996). Fulvic acids extracted from shilajit recently were characterized using Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS) and nuclear magnetic resonance (NMR) techniques (Khanna et al. 2008); different analytical methods also were used to study a mumijo sample from Himalayas (Nepal) and its fulvic fractions (Schepetkin et al. 2009). The aim of the proposed work was a complex study of mumijo, without any additional fractionation, originated from different mountain ranges using modern physicochemical analytical techniques, such as FTICR MS and one- and two-dimensional NMR spectroscopy.

Materials and Methods

Four pharmaceutical mumijo preparations from Altai mountains (Russia), Himalayas (India), Tian Shan (*Kyrgyzstan), and Dzungarian Alatau (Kazakhstan) were studied*.

A liquid chromatographic system consisting of a solvent pump, a packed column, and a UV–vis detector with variable wavelength was used as described (Perminova et al. 2003). The UV absorbance was measured at 254 nm. The size-exclusion chromatography (SEC) column was 15 mm × 250 mm packed with Toyopearl HW-55S ("Toso-Haas," Japan). 0.03-M phosphate buffer with pH 6 and 8 was used as a mobile phase at a flow rate of 1 ml/min. The column was calibrated using sodium polystyrene sulfonates (PSS) (Da): 4,480, 14,000, 20,700, 45,100, and 80,840 (Polymer Standard Service, Mainz, Germany). Blue dextran (2,000 kDa) served as a void volume probe and acetone as a permeation volume probe. Molecular weights of the samples analyzed were calculated from the chromatograms using home-designed software *GelTreat*.

FTICR mass spectra were acquired on a 7-Tesla Finnigan LTQ FT mass spectrometer (Thermo Electron, Bremen, Germany) equipped with electrospray ion source (Finnigan Ion Max Source). The following conditions were used for electrospray: negative-ion mode, needle voltage 4.0 kV, syringe pump flow rate 2 μl/min, tube lens voltage 100 V, and heated capillary temperature 250°C. The spectra were acquired in SIM scan mode (selected ion monitoring) with resolution R = 400,000 at m/z 400 using multiple adjacent SIM windows of 100 Th apiece overlapped on 10 Th, and in full scan mode. For external mass calibration, LTQ FT tuning mix was used to give mass error at 2 ppm. Home-designed software *Transhumus* was used for automatic FTICR data processing which includes ion charge state and monoisotopic peak determination.

Quantitative ^{1}H and ^{13}C solution state NMR spectra were acquired using *Avance*-400 spectrometer (Bruker, Germany) operating at 400-MHz proton frequency. Proton spectra were acquired on the samples dissolved in entirely deuterated DMSO (99.9% isotope purity) at a concentration of about 10 mg/ml. Carbon-13 spectra were recorded on the samples dissolved in 99.9% D_2O at concentration of 80 mg/ml. The spectra were acquired using CPMG pulse program with 8-s relaxation delay and INVGATE procedure. The number of scans was about 5,000. All NMR spectra were acquired after centrifugation and with a 5-mm broadband probe.

Results and Discussion

The size-exclusion chromatograms recorded for the mumijo samples studied were characterized by two peaks (Fig. 1). The first one corresponded to the high-molecular part of the sample. This peak was the same that for the typical peat fulvic acids, PFA (Sakhtysh lake, Russia), also shown on Fig. 1. The second complicated chromatographic signal indicated the low-molecular part of mumijo.

Calculated molecular weights and column recovery values of the samples studied are comparable to typical peat fulvic molecular weight characteristics, except of the Himalayas mumijo that wascharacterized by less molecular mass (Table 1).

The ^{13}C and ^{1}H NMR spectra obtained were characterized with broad overlapping bands that are typical to NMR spectra of humics. Intensive sharp signals in the spectra obtained indicate non-humic low-molecular components of mumijo (Fig. 2). FTICR mass spectra acquired also look in the same manner: some intensive signals attended to non-humic mumijo components over a fulvic "background" (Fig. 3).

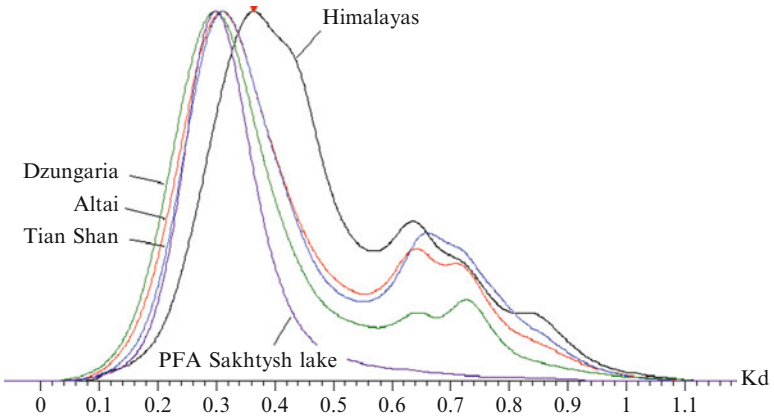

Fig. 1 Size-exclusion chromatograms of the mumijo samples compared with a typical PFA chromatogram

Table 1 Molecular weight characteristics and column recovery values of the samples studied

Sample	Mw (kDa)	Col. recovery (%)
Himalayas	5.1	62
Altai	8.0	73
Tian Shan	7.5	74
Dzungarian	9.0	84
Peat FA	9.1	76

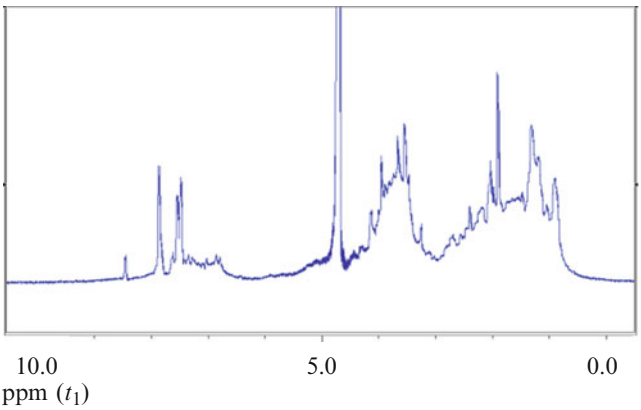

Fig. 2 ^{1}H NMR spectrum of Altai mumijo

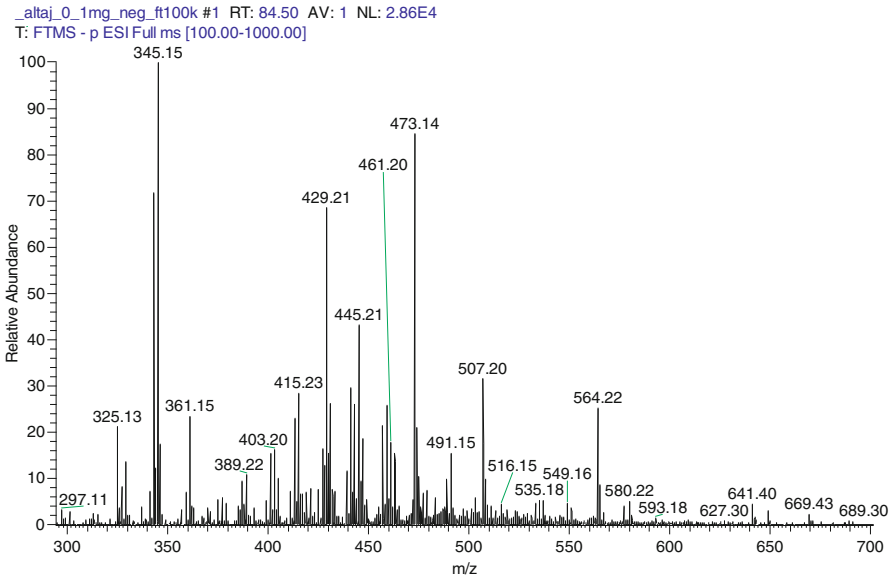

Fig. 3 FTICR mass spectrum of Altai mumijo

Table 2 Molecular peaks from FTICR MS and their structural ascriptions using NMR data

Molecular mass	Intensity, % of the highest peak				Chemical formula (without of a proton)	Ascriptions (confirmed by NMR)
	Altai	Himalayas	Tian Shan	Dzungarian		
127.12576	3.06	4.84	7.01	2.09	$C_7H_{15}N_2$	Methyldiaminocyclohexane
173.04558	n/a	0.61	n/a	n/a	$C_7H_9O_5$	Shikimic acid
178.05087	7.60	1.34	14.95	n/a	$C_9H_8NO_3$	Hippuric acid
191.05598	6.13	11.97	9.22	n/a	$C_7H_{11}O_6$	Quinic acid
194.04575	3.58	1.53	9.24	n/a	$C_9H_8NO_4$	Hydroxyhippuric acid
255.23259	15.83	45.28	19.00	11.44	$C_{14}H_{29}N_4$	Methyldiaminocyclohexane, dimmer
283.26367	12.71	35.77	14.45	9.12		
325.12833	21.55	3.61	22.70	0.82		

Some of the molecular peaks from FTICR MS data and their structural ascriptions confirmed by NMR are shown in Table 2.

Thus, SEC, NMR, and FTICR MS analytical methods demonstrated that the mumijo samples studied consist of two principal components. The first one is of fulvic nature, and the second represents a range of vegetative and animal metabolites.

References

Agarwal, S.P., R. Khanna, R. Karmarkar, M.K. Anwer, and R.K. Khar. 2007. Shilajit: A review. *Phytotherapy Research* 21: 401–405.

Frolova, L.N., and T.L. Kiseleva. 1996. Chemical composition of mumijo and methods for determining its authenticity and quality (a review). *Pharmaceutical Chemistry Journal* 30: 543–547 DOI: 10.1007/BF02334644.

Khanna, R., M. Witt, M.K. Anwer, S.P. Agarwal, and B.P. Koch. 2008. Spectroscopic characterization of fulvic acids extracted from the rock exudate Shilajit. *Organic Geochemistry* 39: 1719–1724.

Perminova, I.V., F.H. Frimmel, A.V. Kudryavtsev, N.A. Kulikova, G. Abbt-Braun, S. Hesse, and V.S. Petrosyan. 2003. Molecular weight characteristics of humic substances from different environments as determined by size exclusion chromatography and their statistical evaluation. *Environmental Science and Technology* 37: 2477–2485.

Schepetkin, I.A., G. Xie, M.A. Jutila, and M.T. Quinn. 2009. Complement-fixing activity of fulvic acid from shilajit and other natural sources. *Phytotherapy Research* 23(3): 373–384.