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# Combined utilization of ion mobility and ultra-high-resolution mass spectrometry to identify multiply charged constituents in natural organic matter

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**Natural organic matter as complex biogeochemical non-repetitive material was investigated with ion mobility mass spectrometry (IMS) and ultra-high-resolution Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) approaches in order to unravel the existence of multiply charged state constituents. Hereby we describe and assign the potential molecular formulae of these doubly charged species, derived from FTICR-MS, and the existence of these species was confirmed via IMS. The parallel application of these powerful techniques enabled the boundaries of the understanding of natural organic matter to be pushed further. Copyright © 2009 John Wiley & Sons, Ltd.**

Natural organic matters are complex biogeochemical non-repetitive materials existing in such vast amounts in various environmental compartments that their quantity easily exceeds the amount of functional biomolecules.[1,2] During diagenesis, they continuously undergo reformation, degradation, governed by the fundamental restraints of thermodynamics and kinetics, resulting in extreme intricacy. The natural diversity of these complex organic materials denotes high variability and density of binding sites, which enable them to behave as a natural buffer against environmental and chemical extremes. Furthermore, natural organic matter defines the bioavailability and cycling of organic and inorganic nutrients and pollutants,[3–5] making the molecular level understanding of such supermixtures essential. The proper characterization of such systems on a molecular level is feasible only if minimally invasive sampling techniques[6,7] and several high-end analytical techniques are combined, since generally applied methods, such as elemental composition analysis, UV-Vis and IR spectroscopy, describe them only with averaged characteristics.[8,9]

Intensive mass spectrometric information is presented here, using 12 Tesla Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) combined with integrated mathematical data analysis and quadrupole time-of-flight ion mobility mass spectrometry (Q-TOF-IMS). FTICR-MS measurements give sufficient mass accuracy and resolution even in broad-band mode,[10] enabling exact molecular formulae assignment with corresponding charge states. The identification of a singly (or doubly) charged constituent is based on the general fact that the corresponding $^{13}C^{12}C_{n-1}$ ion of a $^{12}C_n$ nuclide will be presented higher in *m*/*z* by 1.00335 (or 0.50168) Da.

Since the occurrences of poly-hydroxyl, -carboxylic acids had been shown in these types of mixtures,[11–13] the formation of multiply charged negative ions in the electrospray ionization (ESI) experiments would have been expected. However, it should be noted that the exhibited properties of large molecules might change during their transformation from the polyelectrolyte state in solution to the detected ions in the gas phase. On the other hand, until now, the presence of probable multiply charged constituents was only assumed; moreover, sometimes depicted as 'bumps', but without further details.[14,15] Furthermore, fragmentation studies on model compounds and Suwannee River Fulvic Acid (SRFA) were also carried out, presenting potential fragmentation pathways, often leading to singly charged ions.[16,17] However, these lower resolution MS analyses did not provide information about individual species. Hereby, due to the ultra-high resolution, the obtained exact masses enabled assignment of individual molecular formulae also for doubly charged constituents. In addition, IMS was also applied for the first time for this purpose, with its unique IMS dimension, that allows ion separation on the basis of their size/charge ratios.

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## EXPERIMENTAL

The samples, SRFA (Suwannee River Fulvic Acid) and SRNOM (Suwannee River Natural Organic Matter) standards (obtained from IHSS), were dissolved in methanol directly before the analysis and analyzed at different concentrations (1000, 100, 10, 1, 0.1 μg/mL) and at two different values of pH (3–4 and 9–9.2). Dissolved samples, in methanol, without pH adjustment denoted pH 3 to 4. In the case of pH 9, ammonium hydroxide was added to the samples, dissolved in methanol. Ultra-high-resolution mass spectra of these samples were acquired at the Helmholtz Zentrum München with a Bruker APEX Qe Fourier transform ion cyclotron resonance (FTICR) mass spectrometer equipped with a 12 Tesla superconducting magnet and an Apollo II ESI source. The samples were injected by direct infusion at a flow rate of 2 μL/min. The spectra were obtained in negative ion mode with the number of data points per measurements of 4 MW (time domain size) with a typical resolution of $5 \times 10^5$. The number of scans (512 for sample concentrations 1000 and 100 μg/mL, 1000 for 10 μg/mL, 11000 for 1 μg/mL and 45000 scans for 0.1 μg/mL sample concentration) were increased in order to compensate the dilution effect and to obtain comparable datasets. The spectra were externally calibrated on arginin clusters and then internally recalibrated with fatty acids. Once the exact masses of the molecules had been determined, their molecular formulae were batch-calculated by a software tool, written in-house (E.V. Kunenkov). The following chemical constraints were applied: the maximum number of elements C(70)H(140)O(50)N(1)S(2), tolerance of mass error by $^{13}$C isotope search ≤0.3 ppm, and by direct formulae assignment ≤0.6 ppm. The composition of elements was selected based on the generally observed elemental composition of such organic matter.[18] Predominant C,H,O numbers, relative to other elements like N or S, were allowed in the formulae calculation since these types of materials and their potential sources (i.e. lignin) denote also similar distribution in their elemental composition.

For the online Supporting Information, elemental compositions were computed with the Data Analysis software, version 3.4 (Bruker) using the following restrictions: C,H,O unlimited; N, S 0–2; H/C ratio <3, mass error ≤0.5 ppm; observance of the N-rule; double-bond equivalent (DBE) must be non-negative and radicals must be disallowed. Charge states were considered during the formulae calculations; therefore, the presented masses were automatically multiplied by the correspondent charge values. The molecular formula of given mass was considered only if the corresponding $^{12}C_{n-1}{}^{13}C_1$ peak was found. Generally, the observed error was ≤90 ppb and, in both charge states, the number of calculated formulae occurred in the order CHO >> CHOS, CHON, CHONS.

High efficiency IMS-based measurements were performed using a Synapt$^{TM}$ High Definition MS$^{TM}$ system (Waters Corporation).[19] This hybrid quadrupole orthogonal acceleration time-of-flight (oa-ToF) mass spectrometer combines high-resolution tandem mass spectrometry with high efficiency IMS-based gas-phase separations, capable of distinguishing isobaric molecules based on their mobility (combination of size, shape and charge). Measurements were made using a Synapt High Definition MS system in the Waters European Application Laboratory (Manchester, UK). The instrument was operated using argon as a collision gas for collision-induced dissociation (CID) fragmentation experiments and with nitrogen for the IMS-based measurements. The nitrogen pressure was 0.5 mbar. For measurements up to *m/z* 1000 the pusher frequency was approximately 20 000 pushes s$^{-1}$. Each IMS-based measurement required 200 pushes; therefore, each measurement took approximately 10 ms. The duty cycle across the IMS region approaches 100% due to ions being trapped prior to and released in packets into the IMS region. Furthermore, radiofrequency (rf) confinement of ions within the trap, IMS, and transfer T-wave devices ensure that there are no diffusive losses of ions.

## RESULTS AND DISCUSSION

Herein, the obtained high-resolution mass spectra of SRNOM (Suwannee River dissolved organic matter) and SRFA (Suwannee River fulvic acid) standards revealed, besides the usually characterized singly charged constituents, further multiply charged species, that were described for the first time. A parallel series of measurements was conducted at a different concentration (Table 1) in order to confirm (i) the discussed multiply charged compounds are not only due to the measurements settings, but might originate from the sample itself; and (ii) the necessity for

**Table 1.** Observed numbers of assigned molecular formulae of SRNOM and SRFA at different settings

| | | Assigned molecular formulae (1–) | | Assigned molecular formulae (2–) | | |
|---|---|---|---|---|---|---|
| Number of scans | Applied conc. (ppm) | SRNOM | SRFA | SRNOM | SRFA | Applied pH |
| 45000 | 0.1 | — | 1868 | — | 180 | 3–4 |
| 11000 | 1 | 3142 | 3162 | 3213 | 1839 | |
| 1024 | 10 | 2199 | 1539 | 2174 | 1862 | |
| 512 | 100 | 2794 | 2375 | 1629 | 1201 | |
| 512 | 1000 | 2900 | — | 1825 | — | |
| 11000 | 1 | 2462 | 2379 | 87 | 176 | 9–9.2 |
| 1024 | 10 | 1632 | 1496 | 77 | 17 | |
| 512 | 100 | 1497 | 1722 | 182 | 2444 | |
| 512 | 1000 | 1732 | 2523 | 356 | 2073 | |

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carefully chosen experimental settings (e.g. the applied concentration of analytes, internal standards and additional ions (salt) might suppress the ion yield of the sample). In the absence of ammonium hydroxide, the number of assigned molecular formulae, in both cases, could be increased with an increased number of scans. In parallel, an increased sample concentration did not necessarily mean an elevation in the number of formulae obtained. Especially in the case of the doubly charged constituents, lower sample concentration and longer acquisition time turned out to be more beneficial. However, an SRFA concentration of 0.1 μg/L appeared too low even with an extremely long acquisition time (45000 scans, approx. 60 h). In the case of the buffered samples (pH 9–9.2), similar trends could not be observed particularly within the doubly charged assignments. Rather similar results were obtained if the applied concentration was high (1 mg/L) and the number of scans were low and if a low concentration (1 μg/L) with a high number of scans were applied. A sizable number of doubly charged constituents could be assigned only if higher sample concentration was applied. This phenomenon is due to ion suppression that takes place during the ionization process if ammonium hydroxide is added. Despite using sample concentrations of 0.1–1 mg/L, only SRFA showed a notable increase in the number of doubly charged formulae, while, in SRDOM, doubly charged species and a sizable part of the singly charged ones remained suppressed.

The general outcome of these optimized measurements indicated the benefit of the use of lower concentration (1–10 μg/mL) with longer acquisition time than that generally suggested and applied (up to 5 mg/mL).[20,21] Another important outcome of the systematic measurement of dilution rows indicates that the multiply charged species that occur are not aggregates,[22] since these constituents exist even at low concentrations in diluted samples.

An FTICR mass spectrum of a 1 μg/mL SRNOM sample (experimental settings are detailed in the Experimental section) is shown in Fig. 1. Beside the regular pattern of singly charged constituents, where the most abundant isotopes ($^{12}C$) and their relevant $^{13}C^{12}C_{n-1}$ isotopes are located 1 Da mass width from each other, a second series of masses with 0.5 Da mass distances is revealed. Due to the low intensities this is not readily visible among the singly charged species along the complete mass range. It was only seen when a selected region was enlarged (e.g. 330–342 *m/z* in Fig. 1). However, the general observations denoted doubly charged species in the 250–560 *m/z* mass range. The observed signals, with significantly lower intensities, denote the $^{13}C^{12}C_{n-1}$ isotopes of doubly charged species. Similar results were obtained from an IMS spectrum of SRNOM, where two groups, with a shorter and a longer drift time, were observed (respectively also 1 Da and 0.5 Da mass distances in their simulated mass spectra, Fig. 2). FTICR mass spectra of SRNOM were converted into molecular formulae with a

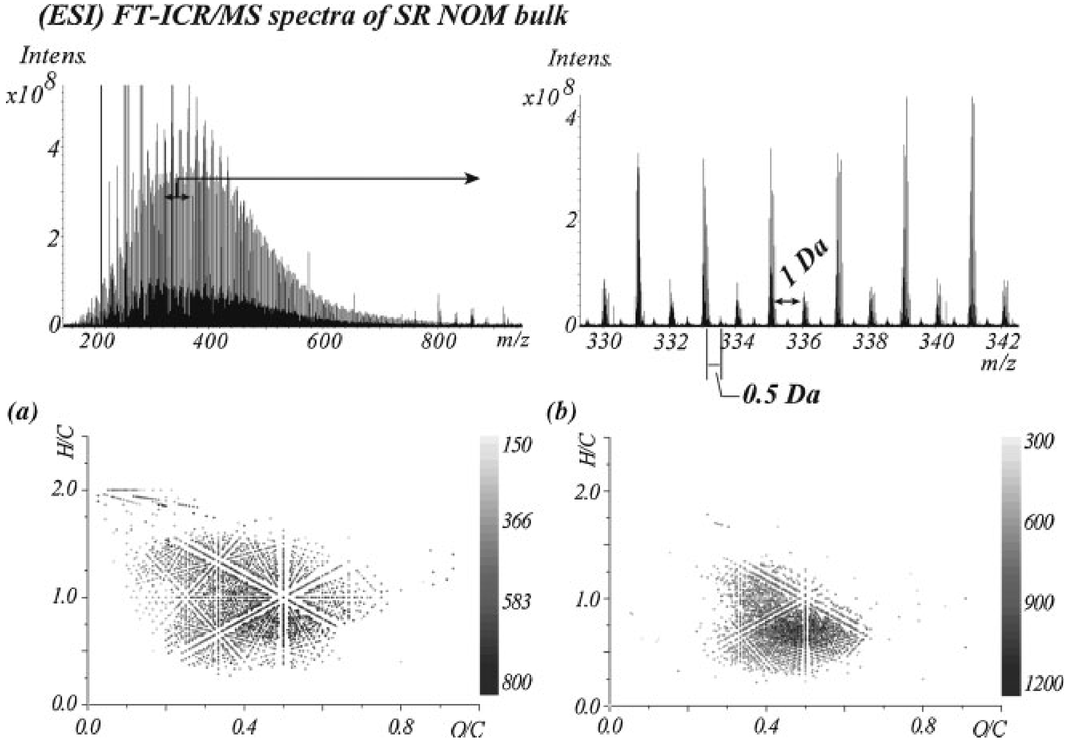

**Figure 1.** ESI negative ion 12 Tesla FTICR mass spectra of SRNOM (1 μg/mL) (uppermost left). A selected expanded section (uppermost right) is given to demonstrate (i) the very high resolution in this spectra (120 000 resolved peaks, with an average resolution of $\geq$300 000 (at *m/z* 400)) and (ii) the most significant mass differences between $C_cH_hO_oN_nS_s$ and $^{13}CC_{c-1}H_hO_oN_nS_s$ ($\Delta m = 1$ Da (singly charged), 0.5 Da (doubly charged)). Beneath the FTICR mass spectra, charge sorted ((a) only single charged, (b) only doubly charged constituents) van Krevelen diagrams denote over 6000 assigned molecular formulae, plotted based on their H/C and O/C elemental ratios and color-coded according to detected masses. This figure is available in color online at www.interscience.wiley.com/journal/rcm.

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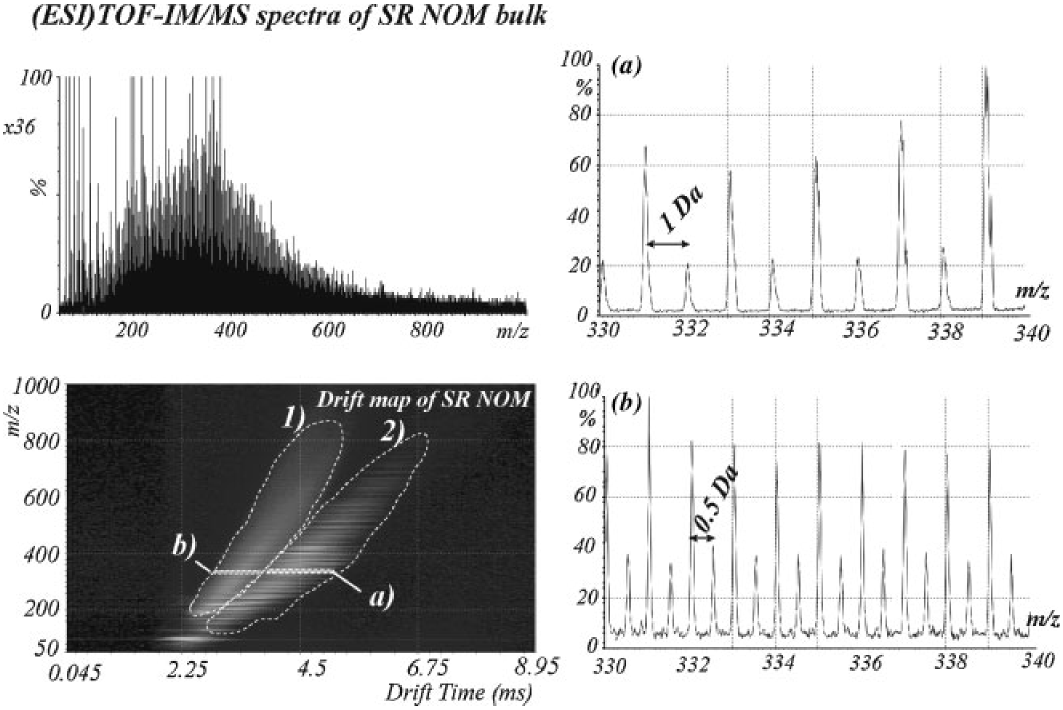

**Figure 2.** ESI negative ion TOF-IMS spectra and drift time vs. *m/z* distribution (bottommost left) of SRNOM was obtained on the Synapt HDMS system (Waters, Manchester, UK). In the drift map, at least two distributions of ions were detected: ***1)*** doubly charged constituents with shorter drift time and ***2)*** singly charged molecules with longer drift time. Insets ***a)*** and ***b)*** (right panel) show an expanded view of the *m/z* region 330–340. Inset ***a)*** denotes only singly, while inset ***b)*** only doubly charged ions, as species containing one or more $^{13}C$ will appear 1/*z* above the *m/z* value of the corresponding monoisotopic ions. This figure is available in color online at www.interscience.wiley.com/journal/rcm.

software tool, written in-house. The elemental ratios (H/C, O/C) of each constituent were plotted in a van Krevelen diagram separately, based on their denoted charge state. Such a presentation of assigned molecular formulae enables individual structural properties (oxygenation, aromaticity, aliphaticity) to be described of the observed constituents within this supermixture.[23] Although we have to note that, in several cases, differentiation between charge states is not possible in the van Krevelen representation, since constituents with $C_xH_yO_z^-$ and $C_{2x}H_{2y}O_{2z}^{2-}$ elemental composition denote the same position. However, one of the most remarkable differences between charge states is that molecules assigned as doubly charged are densely situated in higher O/C and lower H/C fields than the singly charged ones (Fig. 1), thus related to structures functionalized with potential functional groups with oxygen content, for instance hydroxyl (condensed polyphenols, tannins, lignin derivatives) or carboxyl groups (polycarboxylated aromatics).[24–27]

Since formulae assignment within a complex spectrum obtained from organic matter is hardly possible without an automated approach, a validation of the assignment tool is needed. A revision of the reliability of these elemental compositions is evitable; therefore, within the mass range of 348–350 *m*/*z*, the molecular formulae, obtained from the manual (see Supporting Information), and the automated assignments were compared, resulting in good accordance. This result points to the fact that the application of this software tool describes possible constituents realistically (see Supporting Information). Enlargement of the same mass range in the mass spectra obtained by IMS indicates similar intensity patterns as in the spectrum obtained from FTICR-MS (Fig. 3). However, the provided resolution was lower in the case of IMS; the spectrum exhibited ‘humps’ around masses 349, 349.5, 350 and 350.5 *m*/*z*. Meanwhile FTICR-MS with its ultra-high resolution and accuracy was able to assign individual constituents for the similar nominal mass range. Since the results originated from the same population of constituents, in other words, from the same sample, general charge states (drift times) and elemental compositions (molecular formulae), derived from the two different methods, could be confirmed. The distribution of these parameters in drift time is in good correlation with the expectations. Disregarding the differences, due to the conformation/ structural properties, doubly charged species had lower drift times than the singly charged species. The observed distribution in drift time is typical for the overall mass range, where doubly charged species were observed, although the interval of the measured drift time increased as a function of the masses (Fig. 2).

Beyond charge-state differentiation, IMS data, in combination with FTICR-MS results, can be used for structural assignments (Fig. 4). The drift section was compared with its corresponding nominal mass range, derived from high-resolution mass spectrometric (HRMS) data. Individually assigned formulae along the selected nominal mass (349 *m*/*z*),

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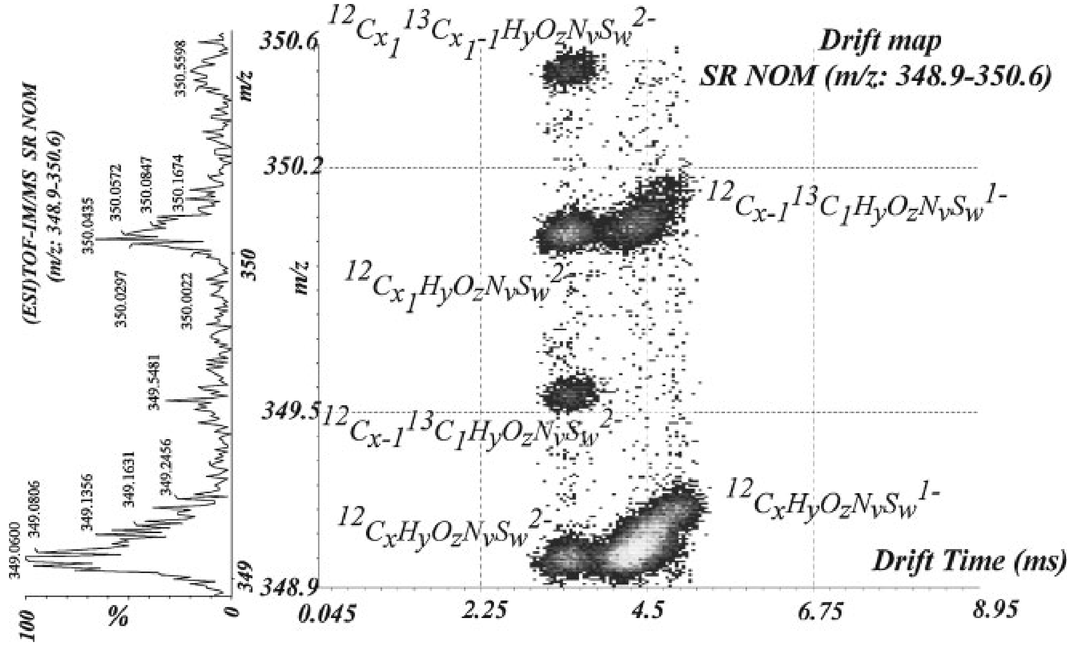

**Figure 3.** Expanded mass spectrum (left) and the corresponding drift map selection (right) of *m/z* region 348.8–350.6, obtained by (ESI)TOF-IMS. The observed drift time distributions are labeled by their matching general elemental composition (obtained by FTICR-MS) and their charge state (in agreement with Fig. 1). This figure is available in color online at www.interscience.wiley.com/journal/rcm.

obtained by FTICR-MS analysis, exhibited increased H/C and decreased O/C ratios with an increased mass. In parallel, aromaticity indices (AIs) were calculated[28] from the obtained molecular formulae and the calculated values were compared with the observed drift times (Fig. 4). The AI is known as an effective descriptor for potential aromatic or condensed aromatic structures. The AI, due to the isotopic mass defect phenomenon, was found to be inversely proportional within the particular nominal mass (Fig. 4). Comparing this fact, derived from the specific elemental composition, with the observed drift time distribution along the highlighted nominal mass resulted in a potential correlation between drift time shifts and structures with different aromaticity.

Among compounds with similar charge states, differences in drift times occurred, generally due to the variation in masses or structural properties.[29] Compared with unfolded molecules compact structures have a shorter drift time; however, this can

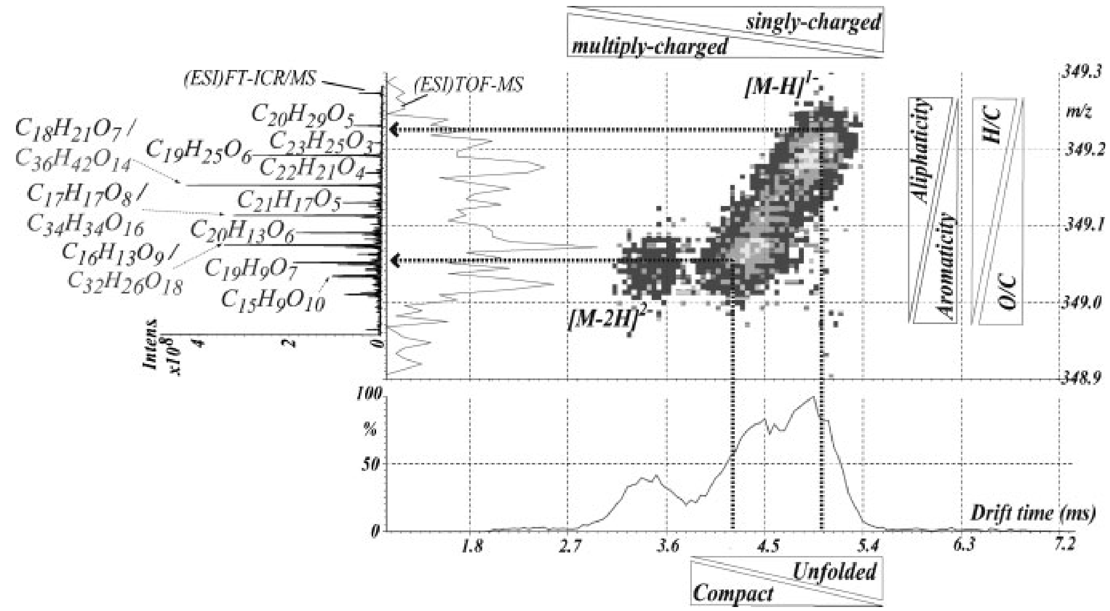

**Figure 4.** Correlation between the potential structural properties, obtained from IMS and FTICR-MS measurements. Drift map selection was compared with the corresponding TOF-IMS and FTICR-MS spectra of mass range 348.9–349.3 *m/z*. This figure is available in color online at www.interscience.wiley.com/journal/rcm.

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be also affected by the individual molecular weight. This was clearly not the case when singly charged constituents within one nominal mass, with a deviation of ± 0.2 Da, were investigated (Fig. 4). Therefore, differences in drift times might only be affected by the structural properties (compactness) of the different constituents. Comparing exact masses, assigned molecular formulae with the corresponding drift section of singly charged molecules exhibited correlation between aromaticity, O/C ratio and compactness. Shorter drift times were observed in parallel with higher aromaticity and higher O/C ratio, while, with decreasing aromaticity, the drift time was increased. This result, considering similar charge states, might lead to a direct correlation between structural properties, drift time, and assigned molecular formulae. In cases of increased ($>0.5$) and extremely high values of AI ($>0.67$), condensed aromatic structures with fewer side chains exhibit compact structures, resulting in decreased drift times. Based on these observations, besides charge-state assignments, IMS can be also used to gain further insights into structure. However, it was not possible to obtain a similar correlation between drift time and possible structural properties of doubly charged species, since these constituents were less abundant. In order to gain such information, enrichment of these species would be necessary. This can be obtained with electrophoretic methods (free flow electrophoresis), where separation takes place based on size and charge distribution and the doubly charged species are expected to be enriched in the mobile fractions.

## CONCLUSIONS

Overall, it can be stated that the combined utilization of methods with different principles has the major advantage that each method complements the weak points of the other. The FTICR mass spectrometer equipped with a 12 Tesla magnet was able to provide sufficient resolving power and mass accuracy; however, it could not differentiate between the isomers of the constituents. Parallel IMS did not have the necessary resolution to assign individual molecular formulae but, with its unique feature, it is capable of distinguishing isobaric molecules based on their mobility, which depends on the size, shape and charge of the specific compound. Consequently, the authors are convinced of the inherent potentials of a possible hyphenation of these advanced mass spectrometric tools. Therefore, we eagerly look forward to an ultimate combination of these approaches in a single instrument that will be able to push further the boundaries of humic substance chemistry.

## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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