UV absorbance ratio index with size exclusion chromatography (URI-SEC) as an NOM property indicator

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ABSTRACT

An advanced approach is presented for identifying and characterizing biopolymers of natural organic matter (NOM) in water samples. It involves a simple method encompassing the ratio of peak heights of high performance size exclusion chromatography (HPSEC) chromatograms based on ultraviolet absorbance (UVA) detection at 210 nm and 254 nm. The HPSEC system eliminates inorganic interferences by separation and makes it possible to obtain ratio values associated with organic components as a function of molecular weight (MW). Certain biopolymers show a distinctive ultraviolet absorbance ratio index (URI, UVA_{210}/UVA_{254}) because they contain different compositional proportions of UV-absorbing functional groups and sp^2-hybridized carbon. URI values were found to be the lowest for humic acids (1.59 for a humic acid, highest aromaticity), intermediate for fulvic acids (1.88 for a fulvic acid, intermediate aromaticity), and highest for proteins (13.5 for BSA, lowest aromaticity). URI increases with the degree of eutrophication of natural waters by the increase of microbially derived components that have a high functional group proportion with a low sp^2-hybridized carbon, and increases with oxidation (e.g. ozonation) by the cleavage of unsaturated bonds (decrease in unsaturated bonds and increase in functional group proportions).

A particular functional group displays its characteristic chemical behaviour when it is present in a compound. The chemical behaviour of NOM is highly related to its functional groups. The concept of URI considers the main functional groups of NOM and can act as an important water quality index. It may be used as a surrogate for assessing the effects of oxidation of NOM. The method is simple and can be easily applied to characterization of bulk water samples.

Key words | biopolymers, high performance size exclusion chromatography (HPSEC), natural organic matter (NOM), UV absorbance ratio index (URI UVA_{210}/UVA_{254})

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INTRODUCTION

Natural organic matter (NOM) comprises a heterogeneous mixture of humic and fulvic acids, lignins, carbohydrates, and proteins of various molecular sizes and functional group compositions. The characterization of both physical and chemical properties of NOM is important because of its role in the fate, reactivity and transport of inorganic and organic pollutants, and its impacts on potable water treatment unit operations (Chin *et al.* 1994; Pelekani *et al.* 1999).

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Because the transport and reactivity of NOM are dependent on size, molecular weight (MW) distribution is an important physical property of NOM. The efficiency of water treatment processes (e.g. coagulation, adsorption and membrane separation) and the formation of disinfection by-products (DBPs) depend strongly on the MW of NOM (Reckhow *et al.* 1990). High performance size exclusion chromatography (HPSEC) has been widely used to estimate the MW distribution of NOM. This technique has various

advantages including small injection volumes, minimal sample pre-treatment, availability of equipment, and ease and speed of analysis (O'Loughlin & Chin 2000). Recently, a HPSEC system coupled with multiple detectors (UVA, fluorescence, and DOC [dissolved organic carbon] detectors) has been applied to characterize NOM as a function of MW (Her *et al.* 2002). This system effectively distinguishes between protein-like entities, polysaccharide-like entities and humic substances. However, it was more difficult to discern humic acids (HA) from fulvic acids (FA); moreover it requires an on-line DOC detector.

Even though HA and FA show similar chemical characteristics, their elemental compositions and the corresponding functional groups are different. The important functional groups present in NOM are carbonyls (including carboxylic acids, amides and esters), phenolic hydroxyls and alcoholic hydroxyls. Generally, FA has a greater oxygen content (greater concentration of functional groups) than HA and, as a result, FA tends to be more hydrophilic, more acidic and more effective in complexing metal ions (Pasto & Johnson 1979). Conversely, HA exhibits a greater aromaticity than FA. Therefore, it may be possible to differentiate them by these structural and functional group differences.

Wilson *et al.* (1999) studied the structure of MW fractions (after sample fractionation by dialysis) using numerous analytical approaches (NMR, pyrolysis-GC/MS, IR and thermogravimetric methods). However, these analyses are generally time-consuming, expensive, complex and require significant operator expertise. McKnight *et al.* (2001) developed a fluorescence index, the ratio of the emission intensity at a wavelength of 450 nm to that at 500 nm with an excitation of 370 nm, to distinguish microbially derived or terrestrially derived fulvic acids. However, the results of this method may depend on DOC concentration and inorganics owing to complexity associated with inner-filter light scattering and/or metal complexation.

UV (ultraviolet) spectroscopy has also been used to monitor the properties of NOM. Korshin *et al.* (1997) studied NOM composition and NOM alterations caused by coagulation and chlorination through the ratio of UV absorbance at 253 nm and at 203 nm (A_{253}/A_{203}). They used this ratio of absorbance as an indicator for the average degree of activation of the aromatic rings and for the prediction of the reactivity of the aromatic moiety in chlorination reactions. However, their absorbance ratio values may contain significant errors even with subtraction of a spectrum corresponding to the nitrate concentration from the raw spectrum because other inorganics such as sulfate and phosphate can also absorb UV light at less than 230 nm wavelengths. (Nitrate and other UV absorbance interferences can be eliminated by chromatographic separation of NO_3^- from larger organic nitrogen compounds such as proteins, the premise of this paper.) The E4/E6 ratio, the absorbance ratio at 460 nm and at 660 nm using an UV spectrophotometer, has also been applied to characterize NOM (Stevenson 1982; Rosell *et al.* 1989). A lower E4/E6 ratio value indicates a higher degree of condensation of the aromatic rings with higher MW. In general, humic acids have a lower E4/E6 ratio, representing more ageing or humification. However, this ratio does not account for the functional groups specifically associated with non-aromatic moieties in aquatic humic substances.

In spite of the intensive efforts by many researchers, some methods are too cumbersome for the frequent analyses of many bulk samples, and others are limited to identifying only certain NOM components. Therefore, the aim of this research was to develop an easy and rapid technique to identify NOM according to general biopolymers using the UV absorbance ratio index (URI, ratio of UV absorbance at 210 nm to that at 254 nm), based on UV detection following size exclusion chromatography (SEC). The measured URI values can provide further information such as the reactivity of NOM with disinfectants.

Without SEC, determinations of URI by a normal UV spectrophotometer would normally not be possible for natural samples owing to high interferences near 210 nm caused by inorganic ions (e.g. NO_3^-, SO_4^{2-} and PO_4^{3-}). The HPSEC-UVA system can portray URI values associated with organic components because SEC separates inorganics that elute later (at the salt boundary). Also, this system defines URI values for NOM as a function of molecular weight (MW). However, the URI values associated with smaller MW organic components are difficult to interpret because their peaks overlap with inorganic constituents. An inorganic interference range was estimated to be less than ~250 Daltons and, thus, effective URI values for organic

components can be obtained above this MW range. The URI values provide information on the relative proportions between UV-absorbing functional groups and unsaturated compounds in NOM. A higher density of these functional groups corresponds to a higher absorption at 210 nm, producing a higher URI (UVA_{210}/UVA_{254}).

MATERIALS AND METHODS

Surrogates for NOM

Suwannee River humic acid (SRHA) reference material (1R101H) and Suwannee River fulvic acid (SRFA) standard material (1S101F), obtained from the International Humic Substances Society, bovine serum albumin (BSA, MW: ~70,000 Da, Sigma), and asparagine (an amino acid, 132 Da, Sigma) were analysed by a UV/visible spectrophotometer (SPD-10A VP, Shimadzu) and an HPSEC with an UVA detector. Aliphatic compounds were not tested owing to their lack of UV absorptivity. All samples were used without further purification.

NOM sources

Various NOM source water samples were obtained: Silver Lake surface water (SL-SW, an allochthonous NOM drinking water source in Boulder, Colorado, during a spring snowmelt period), Barr Lake surface water (BL-SW, a eutrophic/autochthonous water/NOM source in Denver, Colorado, derived from a South Platte River diversion receiving wastewater effluent discharge, during the spring algal bloom period) and Oise River water (a source water for the Méry-sur-Oise nanofiltration (NF) membrane plant, France, during the spring algal bloom period). Oise River water samples corresponded to NF feed waters taken after pre-treatment, and consisted of sand filtered water (SFW) and sand filtered-ozonated water (SFOW, 3 mg l^{-1} of transferred ozone dose). The characteristics of these four water samples are tabulated in Table 1. Our method has been applied to identification and characterization of terrestrially derived (allochthonous) NOM (e.g. SL-SW) versus microbially derived (autochthonous) NOM (e.g. BL-SW), ozonation versus non-ozonated NOM.

Table 1 | Characteristics of natural waters

Source	pH	DOC (mg l^{-1})	UVA_{254} (cm^{-1})	SUVA (l mg – m^{-1})	Conductivity ($\mu S\ cm^{-1}$)
SL-SW	6.53	2.22	0.10	4.50	40.2
BL-SW	8.52	10.4	0.16	1.50	782
Oise River					
SFW	7.20	1.91	0.039	2.04	640
SFOW	7.11	1.74	0.018	1.03	640

Sample preparation

Solid samples were dissolved in pure water for UV scans and dissolved in eluent for HPSEC-UVA analysis. The ionic strength of the water samples was adjusted with a concentrated eluent solution (Her *et al.* 2002) to match the ionic strength of the HPSEC mobile phase.

Analytical method

HPSEC was performed with a TSK-50S column (30 μm Toyopearl HW resin, nominal fractionation range of 100–20,000 g mol^{-1}) with a length of 25 cm and an inner diameter of 2 cm. The mobile phase was prepared with a phosphate buffer (0.0024 M NaH_2PO_4 + 0.0016 M Na_2HPO_4, pH 6.8) and 0.025 M Na_2SO_4, leading to an ionic strength of 0.1 M. The flow rate was 1 ml min^{-1} and the sample injection volume was 2 ml. A LC600 (Shimadzu) liquid chromatograph was used coupled with a SPD-6A (Shimadzu) variable wavelength UVA detector to track separated NOM components at 254 nm and 210 nm. Also, a DOC (Modified Sievers Total Organic Carbon Analyzer 800 Turbo) detector was employed for additional analyses according to methods described in Her *et al.* (2002). Even though polystyrene sulfonates (PSS) are regarded as alternative MW calibrants for the SEC-analysis of humic substances (hydrodynamic radii, viscosity, etc.) (Perminova *et al.* 1998), calibration was performed with polyethylene glycol (PEG) standards ranging from 200 to 10,000 Da because PSS displayed more interactions than PEG with the Toyopearl HW resin within the column. The calibration curve was semi-log linear ($r^2 > 0.99$) over the range

obtained by PEG standards, and was used to approximate MW of samples. The resultant calibration curve was used to represent NOM component separation as a function of molecular weight (Da) instead of retention time.

Why choose wavelengths of 210 nm and 254 nm?

The biopolymers in NOM that have different proportions of functional groups and aromatic rings show different molar absorptivities (ε) as a function of wavelength. Benzene displays several absorption bands (mostly *E*, *B* and *K* bands) in the UV region by $\pi \rightarrow \pi^*$ transitions. The wide absorption wavelengths are observed for aromatics at 180–210 nm for the *E* band (ethylenic band), at 250–295 nm for the *B* band (benzenoid band) and at 275–330 nm for the *K* band. The absorption wavelengths vary with the substituents on the benzene ring (Skoog & Leary 1992). However, the main NOM functional groups associated with non-aromatic groups display absorption maxima only at shorter wavelengths. In the case of the nonconjugated form, absorption maxima wavelengths are 206 nm (for carboxylic acids and esters) and 210 nm (for amides) in water by $n \rightarrow \pi^*$ transitions. When carboxylic acids or esters are conjugated, peak maxima absorption occurs by $\pi \rightarrow \pi^*$ transitions at an experimentally detectable spectral region (longer than 200 nm). The peak maxima absorption shifts to longer wavelengths with the substituents from the base absorption of 187 nm in water. The absorption band increment is dependent upon the substituent type and its position on the unsaturated carbonyl chromophore (Vance & David 1991).

RESULTS AND DISCUSSION

Analytical reproducibility and detection

When the same operating conditions were applied, the reproducibility of UVA and URI chromatograms showed indistinguishable differences in traces with multiple injections (Figure 1). The difference of URI values for four injections was less than 1% for this system, based on an injected DOC of 5 mg C l^{-1} of SRHA. Also, stable URI values were obtained at different concentrations of SRHA down to 0.5 mg C l^{-1} (Figure 2). This result is in contrast to the fluorescence index developed by McKnight *et al.* (2001) that showed significant variations depending on the sample concentration, based on our observations (data not shown).

pH effects

Figure 3 shows the variation of UV absorbance (SRHA, 10 mg l^{-1} as DOC) over a pH range of 2 to 10 (the ionic

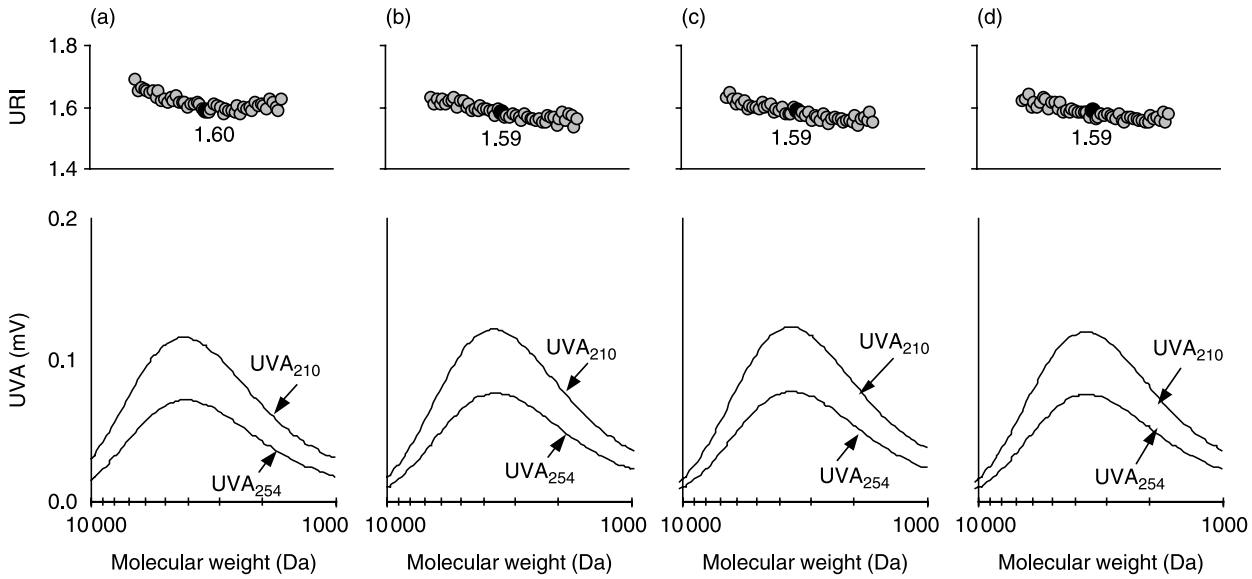

Figure 1 | Reproducibility of both UVA and URI chromatograms (four injections of SRHA of 5 mg C l^{-1}) (numerical value corresponds to peak maximum).

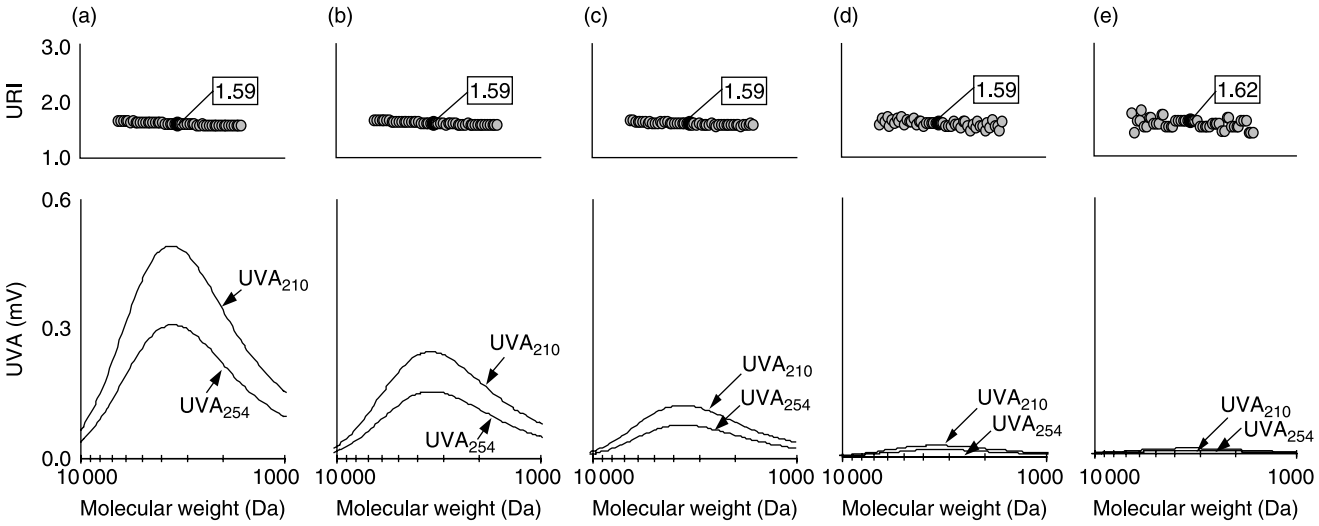

Figure 2 | Comparison of URI values at different concentrations of SRHA: (a) 20 mg C l^{-1}, (b) 10 mg C l^{-1}, (c) 5 mg C l^{-1}, (d) 1 mg C l^{-1} and (e) 0.5 mg C l^{-1}.

strength was constant for all samples). UV absorbance exhibited the most significant differences at pH 3 to 5 and above a pH of 9. The de-protonation of functional groups of SRHA induced the increase of UV absorbance, and the variations of UV absorbance were greatest at lower wavelengths (Figure 3). As a result, 210 nm was chosen for the detection of NOM functional groups. In the case of sp^2 – hybridized carbons, even though they show absorbance over a wide UV wavelength range, 254 nm was chosen because of their recognizable absorption (benzenoid band) in this wavelength. Moreover, 254 nm is widely used for aromatic detection. NOM contains many chromophores that are mainly unsaturated groups responsible for electronic absorption such as C = C and C = O. Therefore, the observed UV absorption spectra of NOM show a broad appearance produced by the sum of the absorption bands of each chromophore. However, the peak intensity (molar absorbance) is different for the diverse composition of aromatic and non-aromatic compounds with various functional groups at different wavelengths.

URI values of reference samples determined by UV scans

Figure 4 shows absorptivities (ε, l/mol-C cm) based on 1 mol l^{-1} of organic carbon for SRHA, SRFA, BSA and asparagine dissolved in pure water (adjusted to pH 7 with

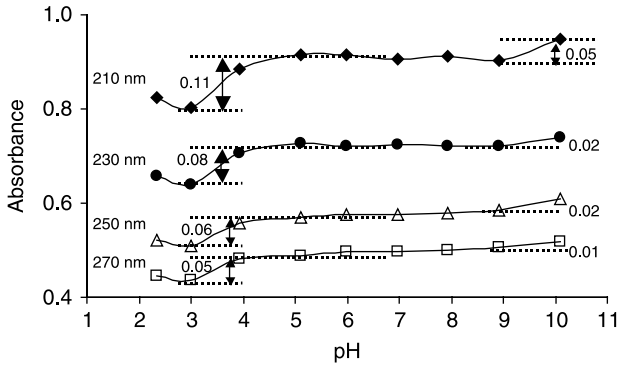

Figure 3 | UV absorbance of SRHA (10 mg l^{-1} as DOC) at different pH conditions (in MQ) for 210 nm, 230 nm, 250 nm and 270 nm.

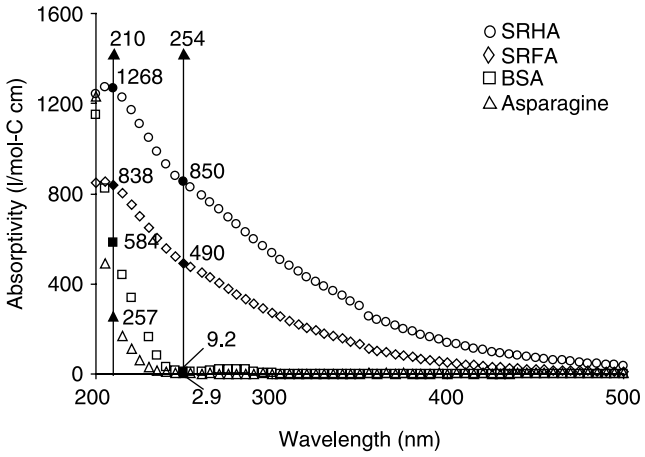

Figure 4 | Absorptivities based on 1 mol l^{-1} of organic carbon for SRHA, SRFA, BSA and asparagine in pure water (pH 7) as a function of wavelength.

NaOH) from 200 to 500 nm. As expected, absorptivities are not the same for all samples across all wavelengths. At 254 nm, SRHA and SRFA exhibit a higher ε, whereas BSA and asparagine show low or negligible ε. At 210 nm, ε becomes much more significant for BSA and asparagine compared with SRHA and SRFA. The increments of changes in ε as a function of wavelength are also not the same for SRHA and SRFA.

Table 2 summarizes the URI values derived from UV scans. The obtained URI values were 1.49 for SRHA, 1.71 for SRFA, 63.5 for BSA and 88.6 for asparagine. These differences in URI values were caused by differences in the density of functional groups and unsaturated groups in the NOM structures. Even though the chemical structures are similar for both SRHA and SRFA, SRFA contains a higher density of functional groups (i.e. OH and COOH), whereas SRHA has a higher density of aromatic rings.

BSA is composed of various amino acids. Even though a few amino acids contain aromatic rings (e.g. phenylalanine, tyrosine and tryptophan) and double bonds (e.g. histidine), the aromaticity of proteins is generally low (SUVA, Table 2). This is due to a high density of functional groups (peptide bonds including carbonyl groups) associated with aliphatic components. Therefore, BSA shows a low UV absorbance at 254 nm and a high UV absorbance at 210 nm, resulting in a high URI of 63.5; asparagine shows a similar trend.

URI values of reference samples determined by HPSEC-UVA

Figure 5 shows the URI values obtained by HPSEC-UVA for SRHA, SRFA, BSA and asparagine. The URI values for peaks on SEC chromatograms correspond to 1.59 for SRHA, 1.88 for SRFA, 13.5 for BSA and 41.0 for asparagine (Table 3). While the URIs for BSA and asparagine by HPSEC-UVA show lower values than by UV scan, they are still higher values than the URIs for other biopolymers. The discrepancy of URI values obtained by UV scan and by HPSEC-UVA may be attributed to the higher baseline due to the high ionic strength of eluent or interference by sulfate and phosphate ions that are used for ionic strength control in the eluent. Nevertheless, the URI values consistently increased in the order: SRHA, SRFA, BSA and asparagine, and thus can be used as an NOM type/property indicator. It is noteworthy that URI is relatively constant for SRHA and SRFA, suggesting chemical similarity among humic acid and fulvic acid components; in the case of BSA and asparagine, the peak values of URI correspond to the most sensitive analytical recognition of these single component chromatograms. As formulated, the URI is an index for dominance of non-humic/autochthonous over humic/allochthonous NOM components and, as such, behaves in an inverse manner to SUVA.

Table 2 | URI values obtained by UV scans

		Molar absorptivity (l/mol-C·cm)		
	SUVA (l mg-m^{-1})	UVA_{210}	UVA_{254}	URI
SRHA	7.35	1,268	850	1.49
SRFA	4.21	838	490	1.71
BSA	0.09	584	9.2	63.5
Asparagine	0.02	257	2.9	88.6

Interpretation of URI values for natural water samples

Figure 6 shows the URI values and HPSEC-UVA chromatograms obtained at 210 nm and 254 nm for SL-SW and BL-SW. The inorganic interference range highly depends on the inorganic constituent concentrations; clearly, the much higher conductivity of BL-SW compared with SL-SW (Table 1) is manifested by a greater inorganic interference at less than 250 Da. The SL-SW (allochthonous NOM, high SUVA in Table 1), collected under spring runoff (snowmelt) conditions, generally showed lower URI values than BL-SW (autochthonous NOM, low SUVA in Table 1) over the MW range. The low URI value of 1.52 for SL-SW at 25,000 Da may be humic-like substances, even though the NOM components at this MW range are generally thought to be polysaccharide-like (Hesse *et al.* 1999) and/or protein-like substances. The SL-SW components at 2,800 Da show a URI of 1.65 in a range between HA and FA; and the URI increases with decreasing MW up to 2.26, indicating an increase in functional group proportion.

The URI was much higher for the BL-SW sample, which can be explained by the eutrophic state of this source

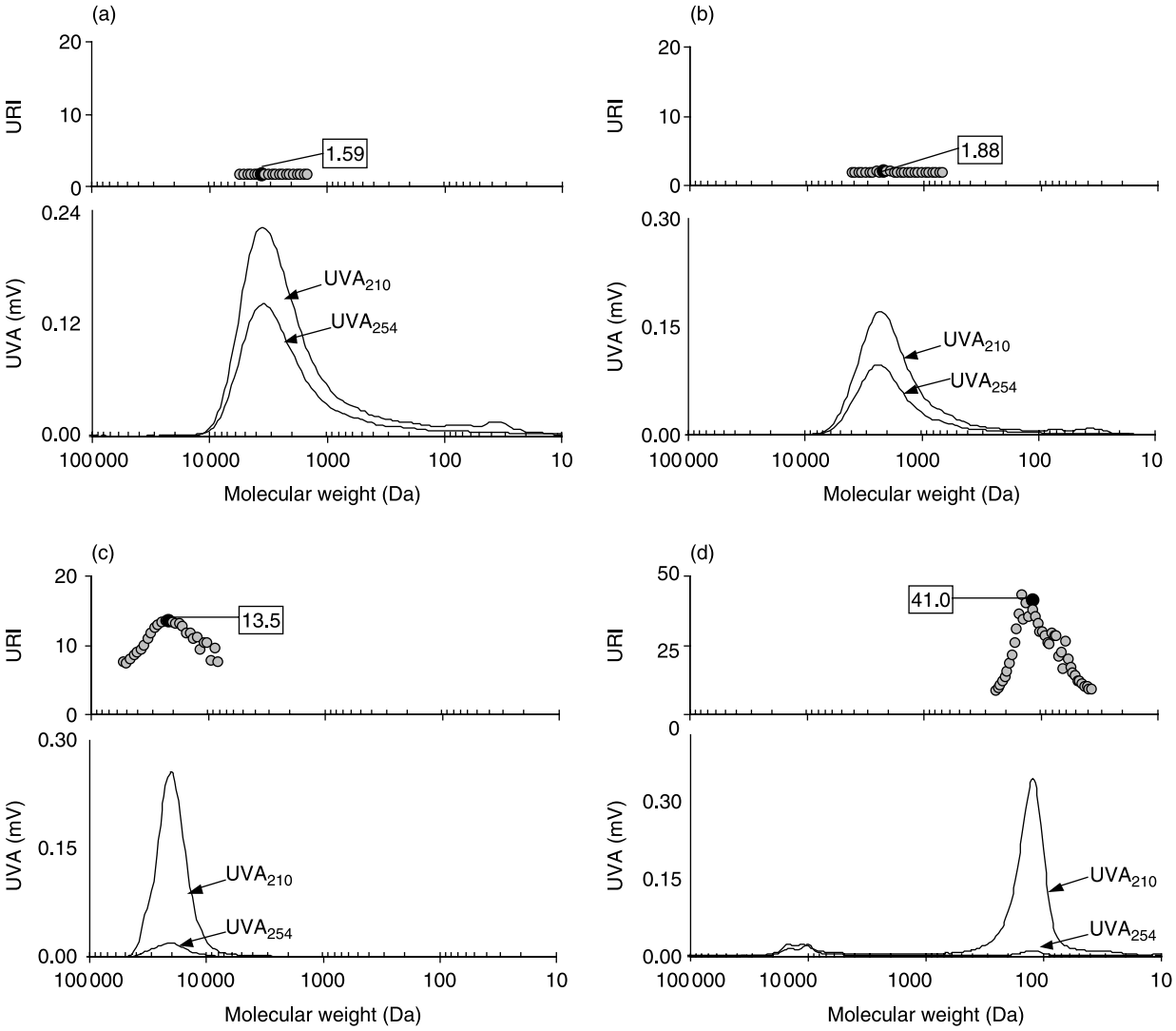

Figure 5 | Comparison of URI values and HPSEC-UVA chromatograms obtained at 210 nm and 254 nm: (a) SRHA, (b) SRFA, (c) BSA and (d) asparagine.

Table 3 | URI values obtained by HPSEC-UVA chromatograms

	Responses at peak maxima (mV)		
	UVA_{210}	UVA_{254}	URI
SRHA	0.223	0.140	1.59
SRFA	0.180	0.096	1.88
BSA	0.256	0.019	13.50
Asparagine	0.245	0.006	41.0

water. The components at 25,000 Da (URI 8.6) and at 280 Da (URI 15.1) may be protein-like substances and simple amino acids associated with algal organic matter. The URI values of 2.1 to 2.3 between 2,200 and 1,050 Da indicate humic substances. The relatively higher URI values compared with SRHA and SRFA in this region indicate that this NOM source contains a higher functional group proportion and is not likely to be terrestrially derived (i.e. allochthonous).

URI values and HPSEC-UVA chromatograms are shown in Figure 7 (a) and (b) for two different nanofiltration (NF) feed waters, sand filtered water (SFW) and sand

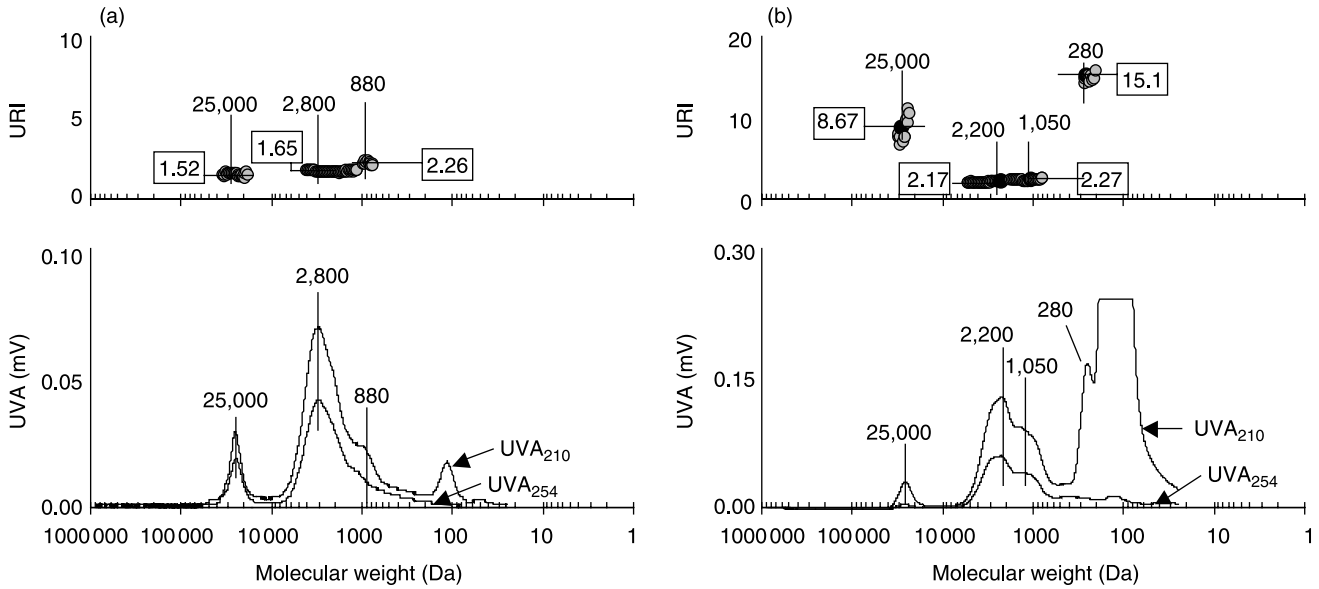

Figure 6 | URI and HPSEC-UVA chromatograms: (a) SL-SW (Silver Lake) and (b) BL-SW (Barr Lake).

filtered/ozonated water (SFOW), derived from the Oise River source. The SFW water was preceded by coagulation/flocculation and clarification; the SFOW water followed SFW. An algal bloom occurred during the sampling period and the URI (1.97) of SFW at 2,050 Da was higher than that of SRFA (1.88). While ozonation significantly decreases the UV absorbance (significant decrease of SUVA, Table 1), a greater decrease was observed at 254 nm compared with 210 nm, yielding a higher URI. The reason for the decrease of UV absorbance at 210 nm, even with the increasing functional group density by ozonation, is because UV absorption at 204 nm (E_2-band, ε_{max} 7900) associated with sp^2-hybridized carbon also decreases. The URI (2.70) of SFOW at 2,050 Da is much higher compared with that (1.97) of SFW, probably because of the decrease (cleavage) of double bonds and activated aromatic moieties, and increase (formation) of

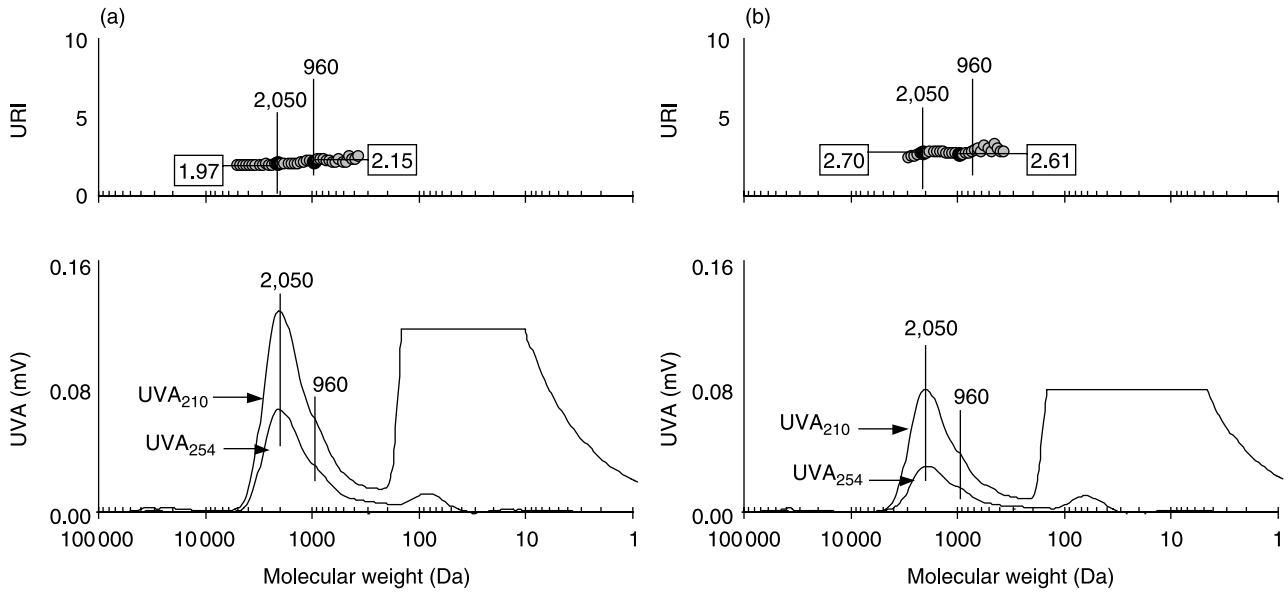

Figure 7 | URI and HPSEC-UVA chromatograms: (a) sand filtered and (b) sand filtered/ozonated Oise River water.

Table 4 | URI summary

	Carbonyl groups*	Unsaturated bonds	URI
SRHA	Low	High	1.59
SRFA	Intermediate	Intermediate	1.88
BSA	High	Low	13.50
Asparagine	High	Low	41.0
Eutrophic NOM	High	Low	> 1.90
Oxidation (ozonation) effects	Increase	Decrease	Increase

*Carbonyl groups include carboxylic acids, amides, esters, etc.

carbonyl-containing compounds including carboxylic acids upon ozonation (Von Gunten 2003). As an aside, the high UVA_{210} readings at less than ~250 Da are caused by high nitrate levels in Oise River water samples (Figure 7).

CONCLUSIONS

This research demonstrates that URI can be used as an important water quality index to estimate the functional group proportions of NOM, and to provide general insight into NOM type (i.e. allochthonous versus autochthonous). As described previously, the URI values exhibit an opposite trend to that of SUVA values: high URI generally shows low SUVA. Therefore, further study is required to verify these relationships. However, URI could provide specific information as a function of molecular sizes and more stable results compared with corresponding SUVA values when non-UV absorbing components such as polysaccharides are mixed with certain UV-absorbing components. Generally, a particular functional group displays its characteristic chemical behaviour when it is present in a compound, and the role and reactivity of NOM is highly related to functional groups. Table 4 summarizes general URI values. URI may be used as a surrogate index for assessing the effects of (ozone) oxidation on NOM. One of the drawbacks of URI is an inability to 'recognize' non-UV absorbing NOM components such as polysaccharides. However, this limitation can be overcome by coupling HPSEC with a DOC detector (Her *et al.* 2002). However, the measurements of URI are easier, and more precise detection is possible, especially when employing a dual wavelength UV detector.

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