Elution behaviour of low molecular weight compounds in size exclusion chromatography

Aki Sebastian Ruhl and Martin Jekel

ABSTRACT

Size exclusion chromatography (SEC) in combination with continuous organic carbon detection (OCD) is a powerful analytical technique that enables characterization of dissolved organic water constituents. Low molecular weight organic water constituents are ubiquitous but their behaviour in SEC analyses is not yet fully clarified. Therefore, we analysed a number of low molecular weight organic model compounds with various structural and functional characteristics by size exclusion chromatography combined with online OCD and ultraviolet light absorption measurement (UVD). The detection times of some low molecular weight organic compounds were much lower than expected. Elution behaviour of low molecular weight compounds (below 300 g/mol) was determined by functional groups rather than by molecular weight. Retention times of low molecular weight compounds varied between 51.9 min, close to that of humic substances, and 235 min of benzaldehyde, the double value of a standard chromatogram runtime of 120 min. Comparisons of both detectors' signals reveal that elution times and detection times are not identical. The elution sequence of various model compounds provided here facilitates identification of peaks in the low molecular weight range of SEC chromatograms.

Key words | humic substance, low molecular weight compound, NOM, organic carbon detection, size-exclusion chromatography

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INTRODUCTION

Dissolved organic compounds (DOC) are ubiquitous in both natural and technical systems. Advanced analytical systems that combine size exclusion chromatography with different detectors facilitate the characterization and quantification of different DOC-fractions and are used for fingerprint analysis of natural and treated waters (Huber & Frimmel 1996). According to the fingerprint analysis, the first eluting fraction is classified as biopolymers (mainly proteins and polysaccharides) followed by humic (and fulvic) substances and the building blocks. According to present assumptions, low molecular weight compounds elute following the sequence: (1) low molecular weight acids, (2) low molecular weight amphiphilic and (3) low molecular weight neutral compounds, with specific elution volume ranges (or elution times, respectively) for each fraction (Huber & Frimmel 1996; Huber *et al.* 2011). Hydrophobic compounds are assumed not to elute since they are completely retained in the SEC column (Huber *et al.* 2011).

Identification of large molecular weight compounds, such as biopolymers or humic and fulvic compounds, by this technique is applied in an increasing number of investigations (for example, Gruenheid *et al.* 2005; Laabs *et al.* 2006; Haberkamp *et al.* 2007; Genz *et al.* 2008; Nam & Amy 2008; Espinoza *et al.* 2009; Lankes *et al.* 2009; Zheng *et al.* 2010). Degradation of humic material to lower molecular weight compounds, namely formic, oxalic, succinic, and glutaric acid, was successfully monitored on the basis of SEC-OCD and SEC-UVD data in combination with ion chromatography (Espinoza *et al.* 2009). Haberkamp *et al.* (2007) estimated the removal of low molecular weight acids and low molecular weight neutrals according to elution time ranges. Acetate formed by homoacetogenic

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microorganisms under anoxic conditions could be distinguished from bulk natural organic matter (NOM) by size-exclusion chromatography (Ruhl *et al.* 2008).

The analytical technique itself has been subject to several detailed investigations (for example, Muller *et al.* 2000; Amy & Her 2004; Allpike *et al.* 2007). Specht & Frimmel (2000) investigated the influence of salt concentrations of the mobile phase on elution volumes of various organic compounds. Perminova *et al.* (1998) developed a model that covers structural properties of multiple model compounds. However, a systematic investigation of SEC data of different compounds with varying functional groups in comparison with typical humic substances has not yet been intensively discussed.

For the analyses of NOM constituents it is important to understand the influence of functional groups on the elution behaviour of organic compounds. Since certain sections in a SEC chromatogram are interpreted as low molecular weight acids and neutrals, model low molecular weight acids and low molecular weight neutrals are expected to elute within certain ranges the other way around. Here, various low molecular weight model organic compounds with carbonyl, hydroxyl, carboxylic, aldehyde, alkyl, alkene and ester functional groups were analysed by SEC-OCD and -UVD. Additionally, compounds containing cyclic and aromatic structures were analysed as well as heterocyclic model compounds.

MATERIAL AND METHODS

Analyses were carried out with a SEC-OCD system (DOC-Labor Dr. Huber, Karlsruhe, Germany) containing a typical SEC column (Toyopearl HW-50 (superfine), Tosoh Bioscience) that has been applied in a number of studies (for example, Muller *et al.* 2000; Dittmar & Kattner 2003; Haberkamp *et al.* 2007; Espinoza *et al.* 2009; Huber *et al.* 2011).

Roughly, an auto sampler injects a sample aliquot of 100 μL in an eluent flow (1 mL/min, phosphate buffer consisting of 8.4 mmol/L $Na_2HPO_4{\cdot}H_2O$ and 18.4 mmol/L KH_2PO_4, pH of 6.85) that leads into a UV detector (wave length of 254 nm) followed by a thin-film reactor (Graentzel type), where organic carbon is photochemically oxidized and the resulting carbon dioxide is carried by a nitrogen stream to an infrared detector. A sample aliquot of 1,000 μL is subsequently injected and flows through the SEC column prior to the UV detector and the thin-film reactor with continuous organic carbon detection. Phosphoric acid is injected to remove inorganic carbon. Detailed descriptions of the system are provided by Huber & Frimmel (1991, 1992, 1996) and Huber *et al.* (2011).

Chromatograms for environmental samples are typically recorded within 120 min (including the bypass peak). For certain organic compounds it was necessary to extend recording times up to two-fold, while other compounds were not detected within 600 min. Chromatographic data are digitally recorded and partly processed using the software Fiffikus (DOC-Labor Dr Huber, Karlsruhe, Germany).

All organic model compounds were dissolved in ultrapure water (ELGA, Germany) with concentrations of approximately 5 mg/L DOC. Initially, solutions were repeatedly analysed. Since both peak areas and detection times were highly reproducible, most solutions were analysed once.

RESULTS AND DISCUSSION

A chromatogram of a standard aquatic NOM model compound from Suwannee River (1R101N) is shown in Figure 1. The peak begins at approximately 45 min, reaches its maximum value at 51.9 min with a 'shoulder' and exhibits a small overlapping peak at 61.9 min. This shape is in good accordance with published chromatograms (for example, Huber & Frimmel 1992; Dittmar & Kattner 2003; Espinoza *et al.* 2009). To date, the shoulder is interpreted to consist of lower molecular weight compounds, so called 'building blocks' followed by a characteristic peak of organic acids (compare Huber *et al.* 2011).

Low molecular weight neutrals

Further chromatograms in Figure 1 show the characteristic behaviour of low molecular weight neutrals. Both chemical structure and molecular weights are indicated. Ethylene glycol with two hydroxyl functional groups is detected before alcohol with one hydroxyl function and one or two alkyl rests. Despite lower molecular weight, methanol is

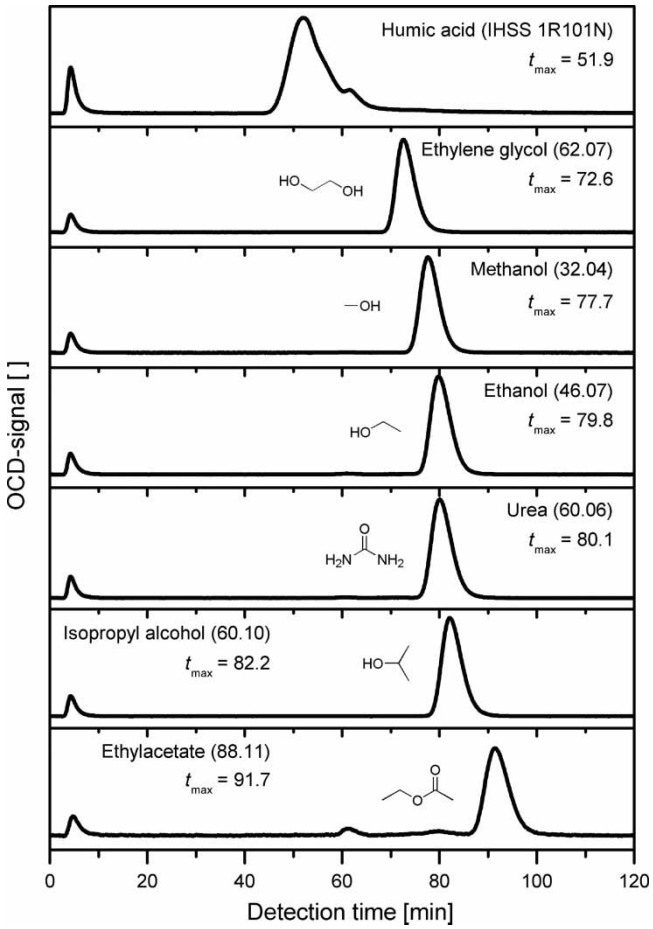

Figure 1 | SEC-OCD bypass peaks and chromatograms of a large molecular weight model substance (Suwannee River aquatic NOM 1R101N) and hydrophilic non-carboxylate low molecular weight compounds with different functional groups (molecular weight in brackets and detection time of peak maxima t_{max}).

detected before ethanol. We conclude by comparing detection times of the four alcohols that hydroxyl functions accelerate while methyl groups decelerate elution. An influence of molecular weight is not significant, while the hydrodynamic volume of a compound provokes an influence. The hydrodynamic volume is the size of a dissolved molecule including its hydrate shell. The structure and functional groups of a molecule determine the number of associated hydrate water molecules.

Urea, consisting of two amines and a carbonyl functional group between, can also be considered as a neutral compound under environmental conditions. Urea follows ethanol but precedes isopropyl alcohol and ethyl acetate, an ester of ethanol and acetic acid. Ethyl acetate is another neutral compound, and it is detected last among the neutrals in spite of its highest molecular weight. The sequence of methanol, ethanol and isopropyl alcohol is in accordance with previously reported data (Specht & Frimmel 2000; Dittmar & Kattner 2003).

Low molecular weight acids

Another important group of low molecular weight compounds that is often discussed in conjunction with SEC-OCD chromatograms are organic acids. Historically, that class of constituents is discussed as acids although under environmental conditions their carboxylates are expected. Chromatograms of various low molecular weight model compounds with carboxylic functional groups are shown in Figure 2. Considering retention times of carboxylate anions of different low molecular weight acids, no direct correlation with molecular weight was observed. Bypass peaks are included in order to allow comparison of relative peak heights and areas. The areas below the chromatographic peaks are approximately ten times larger than the bypass peak, corresponding to a ten times larger injection volume. Some chromatograms consist of a narrow and high peak while other compounds exhibit a broader chromatogram with even two peaks. However, double peaks were not observed in the bypass peak.

In particular, di- and trivalent anions, corresponding to citric (triprotic), oxalic and succinic (both diprotic) acids, exhibit a retention time approaching that of large molecular weight humic substances. Citrate, the carboxylate of citric acid is an alpha hydroxy acid consisting of three carboxylic acid functional groups and one hydroxy functional group. It can therefore be considered as a highly polar organic compound with a large hydrate shell and therefore a large hydrodynamic volume. The peak maximum is detected at 54.2 min, less than 3 min after the maximum of the humic model compound (Figure 1). Citric acid is often used as cleaning agent for various membrane types (for example, Teodosiu *et al.* 1999; Plottu-Pecheux *et al.* 2002) and diluted residuals might interfere with the peak that is interpreted as humic acid.

Oxalic acid is the dicarboxylic (diprotic) acid with the lowest molecular weight, succinic acid having a slightly larger one. Oxalate is a particularly small compound, consisting of two carbon atoms, each involved in a carboxylic

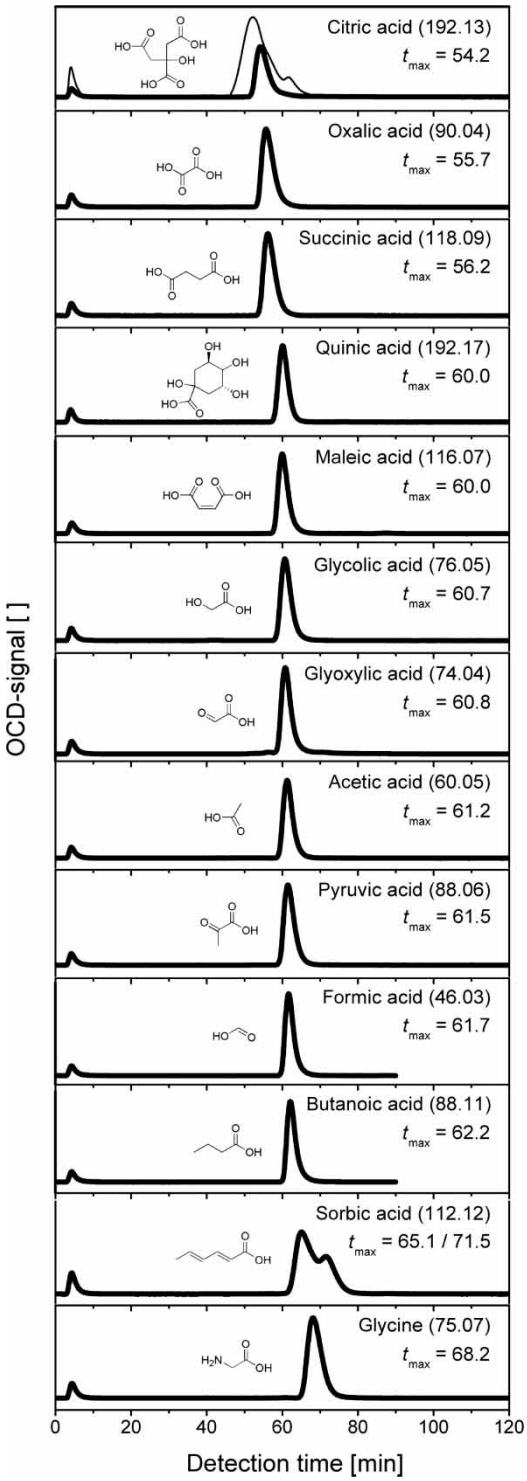

Figure 2 | SEC-OCD bypass peaks, chromatograms and chemical structures of various low molecular weight aliphatic and cyclic mono-, di- and tricarboxylic acids without and with further functional groups and molecular weights in brackets and t_{max} in min (humic acid indicated as week line in the chromatogram of citric acid). Acids are indicated since names of their carboxylates are used less.

functional group. Oxalate occurs as ozonation product of NOM and should be carefully distinguished from the humic acid peak. Succinic acid involves two more aliphatic carbon atoms linking two carboxylic functional groups. Maleic acid with a double bond between two carboxylic groups is similar to succinic acid with a slightly lower molecular weight. The intermolecular hydrogen bonding leads to a smaller hydrate shell and therefore hydrodynamic volume. The carboxylates of these low molecular weight acids eluted much earlier than expected according to the elution sequence (Huber & Frimmel 1996; Huber *et al.* 2011).

Low molecular weight compounds with one carboxylic functional group are retained for slightly longer. Quinic acid contains a cyclic aliphatic structure with four hydroxyl groups besides one carboxylic function. Though quinate is much larger due to a higher carbon and oxygen content, it elutes after oxalate and succinate but at almost the same time as maleate. Glycolic acid, an alpha hydroxy acid, consists of a carboxylic acid functional group and a hydroxy functional group. Glyoxylic acid differs only in an aldehyde group instead of the hydroxyl group. Glycolate, glyoxylate and pyruvate are somehow similar to oxalate with a hydroxyl, an aldehyde and a ketone functional group, respectively, instead of a second carboxylic group. Comparing those compounds, the following sequence of additional functional groups can be deduced: hydroxyl function followed by aldehyde function followed by ketone function. Considering the structural similarity, carboxylic functional groups have a slightly accelerating effect on elution behaviour.

In summary, peaks of acetate and pyruvate (61.2 and 61.5 min, respectively) correspond to the small peak that is currently assumed to originate from the so defined low molecular weight acids (Huber *et al.* 2011) while other compounds show a different elution behaviour.

Aromatic carboxylic acids

Aromatic, besides aliphatic, structures are typical constituents of NOM. Size exclusion chromatographic results of low molecular weight mono-aromatic compounds with various functional groups are shown in Figure 3.

Detection time of the peak maximum of benzenetetracarbonic acid (52.2 min) is very close to that of the aquatic

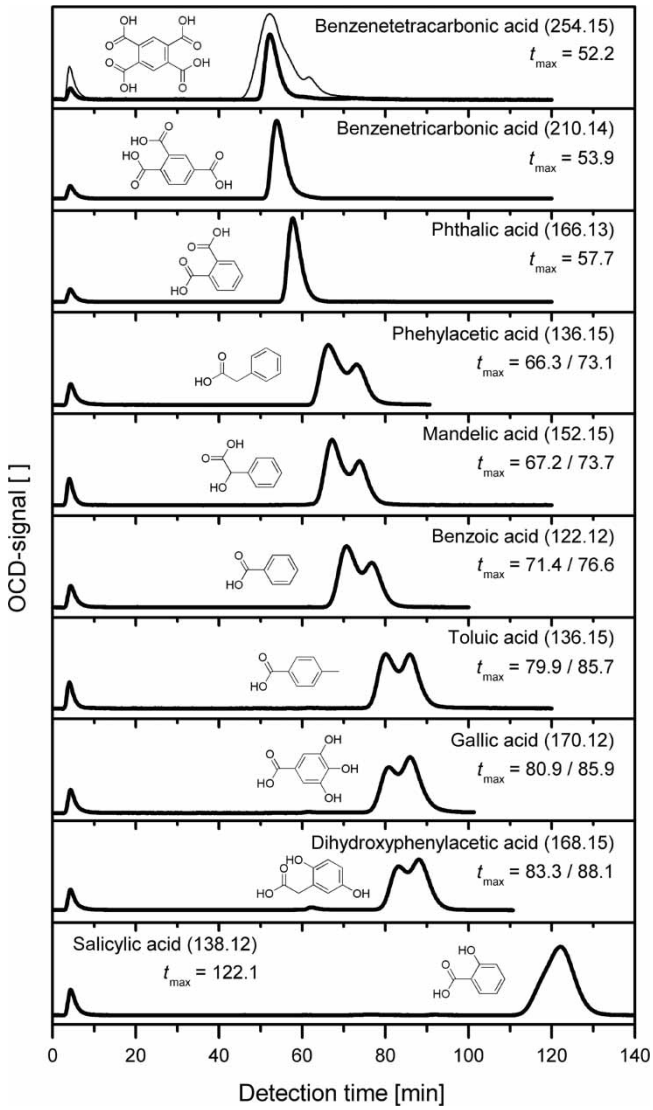

Figure 3 | SEC-OCD bypass peaks and chromatograms and chemical structures of different low molecular weight aromatic carboxylates (molecular weight of acids in brackets and indicated peak detection time t_{max} in min) with up to four carboxylic functional groups (humic acid included as week line in the chromatogram of benzenetetracarbonic acid). Names of acids instead of carboxylates are indicated for simplification.

NOM compound. Benzenetricarbonic acid and phthalic acid are detected quite closely. Although the aromatic di-, tri- and tetravalent ions can be considered as low molecular weight compounds, they are detected in the time range of large molecular weight humic substances and therefore contribute to the humic substance peak.

Phenylacetic, mandelic, benzoic, toluic, gallic, dihydroxyphenylacetic and salicylic acid consist of an aromatic ring with only one carboxylic functional group. Although gallic acid contains three hydroxyl functional groups attached to the aromatic ring, it is detected beyond benzoic and toluic acid. In the case of mandelic acid, a hydroxylic function is incorporated between the aromatic ring and the carboxylic function, while the carboxylic and the hydroxyl functions are directly attached to the aromatic ring in the case of salicylic acid. The reason for the difference of 64.3 min between the peak maxima is therefore surprising. Even toluic acid that contains an alkyl besides the carboxylic function is detected 42.2 min prior to salicylic acid. Normally an alkyl group decreases hydrophilic properties and is therefore assumed to increase elution time due to hydrophobic interactions.

Double peaks were observed for the carboxylates of sorbic acid (Figure 2) and phenylacetic, mandelic, benzoic, toluic, gallic and dihydroxyphenylacetic acid (Figure 3). Occurrence of a double peak for a sole compound was explained for acetone by a bridging model in that two acetone molecules are bound with a sulphate ion (present in the eluent) leading to a higher molecular weight species (Her *et al.* 2002). In the case of anionic carboxylates, bridging through anions such as phosphate (or sulfate) is not likely. The eluent only contains monovalent cations of sodium and potassium and no divalent cations. Therefore, bridging is not expected to occur. Interestingly only one clear peak was observed for the bypass, leading to the conclusion that double peaks originate from the passage of the column. Despite containing a carboxylic functional group, salicylate elutes beyond the conventionally recorded elution time windows of 120 min (at 122.1 min). Further investigations are needed to resolve these phenomena.

Heterocyclic and hydrophobic compounds

The elution behaviour of heterocyclic compounds is not primarily determined by molecular weight either. Caffeine, with the highest molecular weight, reveals the strongest retention (see Figure 4). Odd peak shapes were observed for piperazine, barbituric acid and ascorbate. Barbituric acid and especially piperazine, both heterocyclic compounds incorporating nitrogen, revealed a significant tailing both in bypass and chromatographic peaks (Figure 4). This observation indicates that oxidation of nitrogen

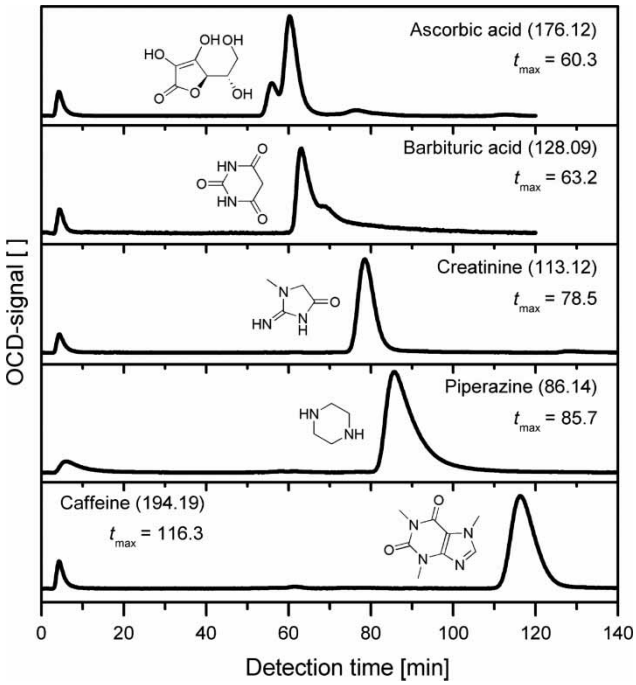

Figure 4 | SEC-OCD bypass peaks, chromatograms and chemical structures of heterocyclic (non-carboxylic) organic compounds (molecular weights in brackets).

heterocyclic compounds by UV radiation might be kinetically restricted. Oxidation of C-N bonds appears to be slower compared to C-O or C-H bonds. However, no similar effects were observed for nitrogen containing creatinine, a hetero monocyclic compound, or caffeine, a hetero polycyclic compound. Peak shapes of most other compounds are symmetric, too. Nevertheless, humic substances include up to 5% by weight nitrogen. If nitrogen is included in heterocyclic structures, a retarded oxidation comparable to piperazine with a similar tailing might occur. The shape of the NOM peak with tailing was explained by the partly overlap of building blocks, low molecular weight acids, amphiphiles and neutrals (for example, Huber *et al.* 2011).

Late eluting compounds are inserted in Figure 5 (left) in comparison with other model compounds and chemical structures are provided. Benzyl alcohol was detected with a peak maximum at 168.8 min, despite having a hydroxyl functional group. Aniline eluted at 193.5 min, 73.5 min after the typically recorded elution time window. Before extending the recording time, aniline occurred at 73.5 min in the subsequent measurement (as 'ghost peak'). Benzaldehyde was detected at 235.0 min. While the difference in detection times of glycolic acid and glyoxylic acid was as small as 0.1 min, the aromatic aldehyde elutes more than 50 min after the corresponding alcohol. Both dinitrobenzene and methyl benzoate were detected bypassing the SEC column but were not found in the effluent of the SEC column within 6 h, the five-fold of conventional data acquisition time. According to conventional interpretation, both compounds are therefore classified as hydrophobic compounds that do not elute from the SEC column. Very long retention times might explain occasionally occurring unexpected peaks ('ghost peaks') in one of the subsequent SEC

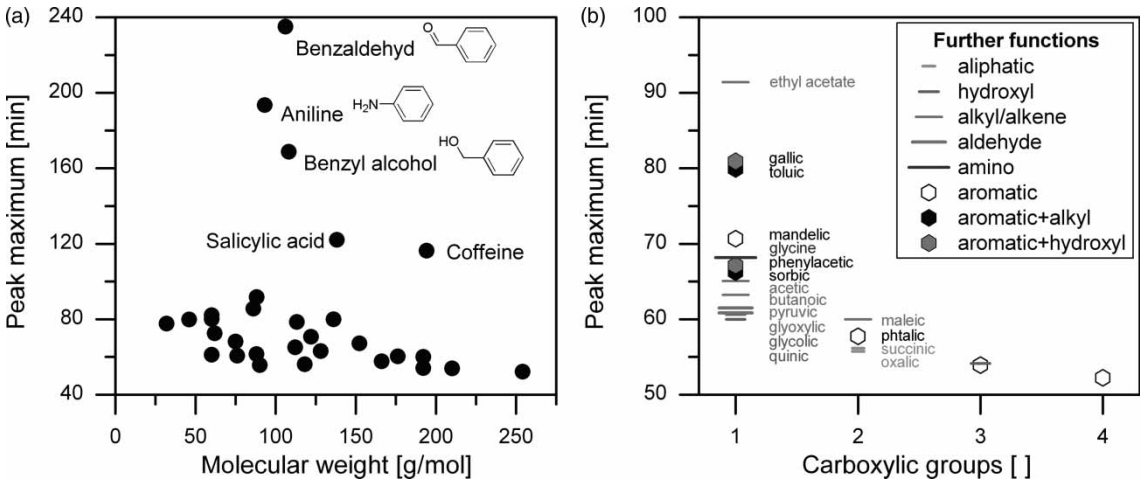

Figure 5 | Correlation between molecular weight and detection time of peak maxima (left) and number of carboxylic with further functional groups and peak maxima (right). Names of acids instead of carboxylates are indicated for simplification.

runs when no cleaning is carried out between the measurements.

Detection times of peak maxima are referred to the molecular weight of the organic compounds in Figure 5 (left). Even compounds with a molecular weight below 100 g/mol are detected very early. Molecular weight therefore does not exhibit the major influence on the retention. A good correlation was observed in dependence of carboxylic functional groups (Figure 5, right). The greatest variation is shown for monocarboxylates according to the greatest variation in functions. An interaction of the SEC solid phase and carboxylic groups overcompensate the size effect.

Comparison of elution and detection time

UV active compounds are first detected in the UV detector followed by its oxidation to and detection of carbon dioxide (Figure 6). In this study, differences in detection times of UVD peak maxima and OCD peak maxima (Δt) were compared to examine the oxidation behaviour in the thin-film reactor.

If a compound reveals a refractory behaviour in the photochemical oxidation step, an increased time lag between the two types of detection can be expected. The volume flow is assumed to have been constant during the presented analyses, although in the long term a decrease of permeability in the SEC column due to ageing (contamination, cake formation) might reduce the volume flow and therefore increase the time lag between UV and OC detection. UV and OC detection times of chromatographic peak maxima are listed in Table 1.

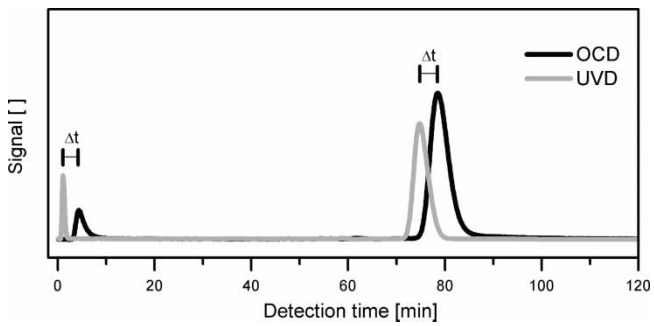

Figure 6 | SEC-OCD and SEC-OCD bypass and chromatograms of a UV active compound with indicated time lag (Δt) between UVD and OCD maximum response for creatinine.

Table 1 | OC and UV detection times of chromatographic peak maxima of UV active model compounds and calculated time lags (only the greater peak maximum is considered for double peaks)

Model compound	OCD [min]	UVD [min]	Δt [min]
Oxalic acid	55.7	52.2	3.5
Pyruvic acid	61.5	58.0	3.5
Benzentetracarbonic acid	52.2	48.7	3.6
Benzenetricarbonic acid	53.9	50.2	3.7
Phthalic acid	57.7	54.1	3.7
Benzoic acid	70.7	67.7	3.8
Salicylic acid	122.1	120.0	3.8
Caffeine	116.3	112.4	3.9
Benzyl alcohol	168.8	164.7	4.1
Aniline	193.5	189.4	4.1
Benzaldehyd	235.0	230.9	4.1
Humic acid (1R101N)	51.9	47.5	4.4

Among the model compounds, significantly increased time differences are observed for compounds that are well retained by the SEC column. Increased dispersion due to longer residence time might have an influence. Interestingly, the greatest time lag was discovered for the aquatic NOM model compound. Shorter retention times of a more UV active subgroup might increase the time lag. However, it is more likely that complete oxidation to carbon dioxide is more time consuming. NOM is oxidized more slowly (by almost 1 min) than the UV absorbing model compounds.

These findings imply that the elution/retention time differs from the detection time for OCD analysis with a thin film reactor. Assuming that UV absorption and carbon dioxide yield are caused by the same compounds, comparison of both detection times might provide further information about stability toward oxidation.

CONCLUSIONS

In size exclusion chromatography the molecular structure and functional groups mainly determine the elution and detection time of low molecular weight organic compounds, not the molecular weight. Retention and detection times of large molecular weight compounds are probably similarly influenced by functional groups. Correlations between

carboxylic functional groups and elution times were observed for low molecular weight compounds. It is likely that the quantity of carboxylic functional groups, besides molecular weight, might also influence retention times of large molecular weight compounds. However, elution behaviour of low molecular weight carboxylates is more heterogeneous and complex than previously assumed. On the other hand, it is possible to differentiate between most low molecular weight compounds on the basis of elution times in SEC chromatograms. Relative sequences of model compounds provided here can be used to identify substances behind peaks in the low molecular weight range for comparable SEC columns and operating conditions.

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