RAPID SOLID PHASE EXTRACTION OF DISSOLVED ORGANIC MATTER

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To my lovely wife, you are my strength. Thank You. A special thanks to Jesus, my friend, you walk with me all the way.

Dedication

To my mother, for her fire.

Abstract

Dissolved organic matter (DOM) is a complex mixture of organic molecules found ubiquitously in freshwater and saltwater environments. Contained within the heterogeneous mixture of DOM lies valuable information content on the source of molecules as well as the biotic mechanisms at work within an aquatic ecosystem. Recent advancements in high resolution mass spectrometry and liquid chromatography have made inroads into determinations of the molecular structures within DOM, which have been largely unknown until recently. Liquid chromatography-mass spectrometry (LC-MS) analysis, however, generally requires a prior step to concentrate/isolate DOM, and this step often limits the number of samples that can be analyzed. This study has developed a fast (<20 min) method to concentrate dissolved organic matter on commercially available online solid phase extraction (SPE) cartridges which can be directly eluted onto an LC-MS system. This method is generally faster and requires far less sample (10-100 mL) than previous SPE methods for DOM isolation. Additionally, this study tested a suite of very different SPE phases to find a combination of phases that could improve DOM recovery as compared with commonly used approaches. When a styrene divinylbenzene phase (RP1) was coupled with activated carbon, recoveries were found to be significantly higher than in previous SPE studies relying upon single phases (either C18 or styrene divinylbenzene-based). The SPE method proposed here was tested for a diverse set of salty and fresh water samples and percent recoveries ranged from 46-78% of the total dissolved organic carbon (DOC).

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Introduction:

Dissolved organic matter (DOM) found ubiquitously in aquatic environments comprises a massive reservoir of reduced carbon (Hedges et al. 1992). For example, the global ocean is estimated to contain a reservoir of 662 Pg of carbon as DOC (Hansell et al. 2012). As such, dissolved organic matter plays an important role in carbon and nutrient cycles from local to global scales (Mopper et al., 1991; Hedges, 1992; Jaio et al., 2010). While dissolved organic matter is ubiquitous in natural waters, the molecular level composition is heterogeneous and extracted DOM can be highly variable on spatial and temporal scales (Schwede-Thomas et al. 2005). Molecular composition in turn has effects on the rate of carbon remineralization and cycling in marine waters. Estimates of molecular life time range from 1.5 years for semi-labile DOM to 16,000 years for refractory DOM (Hansell et al. 2012). Additionally, DOM fluxes from rivers to oceans result in a globally significant transport of carbon (Hedges et al. 1997; Huang et al. 2012). Again, understanding molecular composition of DOM is important to understanding the rate, composition, and fate of riverine export as well as the processes of remineralization/burial (Hedges et al. 1997). For example, the molecular composition of soil DOM, described by C:N ratios, within a watershed is correlated to the quantity of riverine carbon exported to the ocean (Aitkenhead and McDowell, 2000). Hedges et al. (1992) stress the importance of DOM to carbon cycles which underlies the importance of DOM characterization and in turn DOM extraction techniques such that, “any improvement [in DOM extraction methods] will give a chemical glimpse of what is missing” (Hedges, 1992).

As an important part of the carbon cycle, dissolved organic matter production and consumption is often closely tied to planktonic communities (Jiao et al., 2010; Maranon et al., 2004; McCallister et al., 2005; Pace et al. 2007). In fact, most of the DOM in the surface ocean is produced by planktonic primary production (Hedges et al. 1997; Lihini et al. 1997) although riverine, stream and often small lake dissolved organic carbon (DOC) is usually terrestrially derived (Lapierre 2013; Cifuentes 1995). Using ^{13}C labeled carbonate, Pace et al. (2007) found that DOM is derived from production and grazing of various trophic levels in a lake planktonic community and was consumed by heterotrophic bacteria. Jiao et al. (2010) hypothesize that microbially mediated production and remineralization of DOM has significant effects on DOM and carbon cycling. This means that DOM is both an important component of heterotrophic production and is effective at exporting energy to other systems (Jiao et al., 2010). The relationship between DOM and marine microorganisms is described as the microbial loop. Essentially, DOM released by grazing zooplankton and pico-phytoplankton supports heterotrophic bacteria. Heterotrophic bacteria in turn support grazing zooplankton (Barber 2007). Of course, organic P and N cycles are closely tied with the carbon cycle as well because much of the dissolved P and N are in an organic form. As with the overall carbon cycles, the source, age, and exact structure of DOM could have an effect on microbial production and nutrient availability (McCallister et al. 2005). Implicitly, understanding these carbon and nutrient cycles requires better molecular level characterization of DOM.

In addition to its important role in carbon and nutrient cycles, DOM has other important ecological functions. For example, dissolved organic matter has an ecological function as a "sunscreen" absorbing light which both alters visible light availability for photosynthesis and the penetration of harmful UV radiation (Mopper and Kieber, 2002). Because phytoplankton tend to prefer certain wavelengths and irradiance levels, UV-visible light absorption is another way that DOM is tied to the planktonic community. Of course, this property can vary depending on the molecular structure of the DOM present. This is demonstrated by the fact that the rate of DOM photodegradation is highly dependent on the presence of specific functional groups (Thorn et al., 2010). In a review article on the bioavailability and photodegradation of DOM, Sulzberger and Durisch-Kaiser (2009) state, "chemical characterization of (C)DOM is key for rationalizing UV-induced transformations" (Sulzberger and Durisch-Kaiser, 2009). Indeed, improving our understanding of DOM molecular composition seems to be the next key step to more fully rationalize all important functions of DOM.

The composition of DOM is variable both spatially and temporally as a result of varied sources (Minor and Stephens, 2008), photobleaching (Dalzell et al., 2009; Minor et al., 2007; Mopper et al., 1991; Stephens and Minor, 2010), and changes caused by microbial degradation (Obernosterer and Benner, 2004). Because DOM composition is so variable and complex, the exact molecular structures present contain a high degree of information content. Molecular level resolution of

individual molecules within DOM (i.e., biomarkers) sheds light on both the source of DOM and the ecological interactions within the water column. However, to date such biomarker information, with a few exceptions, is generally limited to extractable, derivatizable, and GC-MS amenable molecules rather than the more polar compounds likely to be more representative of bulk DOM (Benner et al. 2002).

Extracting information content from DOM is as challenging as it is useful. The complex and heterogeneous nature of DOM is a formidable barrier to understanding the exact molecules within it. One of the useful tools to understanding DOM composition is liquid chromatography coupled with mass spectrometry (LC-MS) (Minor et al. 2002). The increasing resolution and sensitivity of high resolution mass spectrometry has allowed us to make inroads into this puzzle as well. Several recent studies have utilized the resolving power of Fourier-transform ion cyclotron resonance mass spectrometry (FTICR-MS) to identify the elemental compositions of individual molecules in DOM (Kim et al. 2003; Kujawinski et al. 2009; Longnecker and Kujwinski, 2011; Sleighter et al. 2012) and to determine the differences in DOM composition from different sources (Minor et al., 2012; Ohno et al. 2010; Bea et al. 2011). The number of studies in the past decade evidences the utility of such high resolution mass spectrometry approaches to this field.

One of the limiting factors in liquid chromatography/mass spectrometry (LC-MS) analysis is the low concentration and heterogeneous nature of DOM in large lake or ocean systems. The heterogeneous nature means that any given molecule is at a

very low level (Simpkins et al. 2010). A rapid, comprehensive method of concentrating/isolating DOM would greatly improve the efficiency of LC-MS or high resolution mass spectrometry analyses. Concentration/isolation of DOM from natural samples is generally done in one of four ways: 1. reverse osmosis coupled with electro dialysis (RO/ED), 2. ultrafiltration, 3. solid phase extraction (SPE), or 4. direct drying or freeze drying (lyophilization). The following section includes a review of these methods in the field of DOM analysis.

Review of DOM Extraction Techniques:

In order to place this work in the context of the larger field of dissolved organic matter extraction, a review of the topic is compiled here. As mentioned already concentration of DOM is generally done in one of four ways: 1. reverse osmosis coupled with electro dialysis (RO/ED), 2. ultrafiltration, 3. solid phase extraction, or 4. direct drying or freeze drying (lyophilization). This review will not focus on drying or freeze drying a sample because first, the process is self-evident and second, the process concentrates salts as well as organic matter. Directly drying a sample can only be done with samples that have exceptionally low ionic strength if the sample is intended for NMR, MS, or elemental analysis. However, drying and freeze drying are often coupled with the other three methods of DOM isolation after these methods have de-salted and concentrated the sample.

For this review, it is important to distinguish between the terms isolation, concentration, and extraction. Following the language of Koprivnjak (2006, PhD thesis), concentration refers to decreasing the total volume of the solution so that the DOC concentration increases; isolation refers to separating dissolved organic matter from inorganic compounds. Methods 1 through 3 above both concentrate and isolate dissolved organic matter; finally, extraction will be used here as a general term to refer to the combination of isolation and concentration. These methods will be compared by the recovery of dissolved organic carbon (DOC) within the DOM, which will be referred to as the efficiency of the method. Recovery is defined as in Eq. 1 or if blank data is available, Eq. 2.

$$RECOVERY = \frac{DOC_{ext} \times V_{ext}}{DOC_{sample} \times V_{sample}} \quad \text{Eq. 1}$$

$$RECOVERY = \frac{(DOC_{ext} - DOC_{blank}) \times V_{ext}}{DOC_{sample} \times V_{sample}} \quad \text{Eq. 2}$$

where V_{ext} is the volume of the extract; V_{sample} is the volume of the original sample; DOC_{ext} is the DOC concentration in the extract; DOC_{sample} is the DOC concentration of the original sample; and DOC_{blank} is the DOC concentration of a pure water or saltwater blank that has been run through the extraction process.

Reverse osmosis and ultrafiltration retain organic matter by a similar mechanism, namely, physical means by forcing water through a membrane which is

impermeable or semi-impermeable to most of the dissolved organic matter. The solution remaining, called the retentate, has increased DOC concentration. RO and ultrafiltration isolate DOM from inorganic salts by two different mechanisms. RO is coupled with electrodialysis, which uses electrical potential across a combination of cation and anion permeable membranes to desalt samples. In ultrafiltration, samples are desalted by diafiltration, which is essentially repeated dilution of the retentate with ultra-pure water and subsequent ultrafiltration, with more of the smaller molecular-weight salt moieties passing through the membrane with each dilution/filtration cycle.

Solid phase extraction retains organic matter by a very different mechanism than reverse osmosis or ultrafiltration. In solid phase extraction, molecules in a liquid sample are adsorbed onto a stationary phase and then eluted with a small volume of a solvent of suitable polarity. Formerly, XAD resins were the most commonly used stationary phases for DOM extraction. More recently other non-polar stationary phases based upon newer styrene divinylbenzene phases (e.g. PPL), C-18, or activated carbon are typically used. Non-polar molecules are retained on the stationary phase as sample is passed through a cartridge. Samples are desalted as needed by rinsing the cartridges with ultra-pure water and then eluted with organic mobile phase.

This review intends to compare RO/ED, ultrafiltration, and SPE for the extraction of bulk dissolved organic matter from natural water samples. We will outline the process for each method, and will consider the time and materials required. Also,

the advantages, disadvantages, and limitations of these extraction techniques will be discussed. Finally, recommendations for the field of dissolved organic matter characterization will be presented.

RO/ED:

Reverse osmosis (RO) as a method for concentrating natural dissolved organic matter was explored by Serkiz and Perdue (1990). While this method proved effective with DOM recoveries >90%, RO alone concentrates both dissolved organic matter and inorganic constituents. As salt concentration increases, carbonates and sulfates precipitate and can foul membranes (Gurtler et al. 2008). Like drying and freeze drying alone, this method was limited to waters with exceptionally low salinity until recently. In 2007, reverse osmosis was coupled with electrodialysis (ED) which is a technique to remove salts from the sample (Koprivnjak, PhD thesis). Since then, a number of papers have been published on this method demonstrating the effectiveness of RO/ED with freshwater (Koprivnjak 2007) and in sea water (Vetter et al. 2007; Gurtler et al. 2008; Koprivnjak et. al. 2009).

For marine samples, these studies (i.e., Vetter et. al. 2007; Gurtler et al. 2008; Koprivnjak et al. 2009) used generally the same method. RO/ED is done in three steps which are laid out clearly in Gurtler et al. (2008). First, ED is used alone to remove salts until the conductivity of the sample has decreased to 15 $\mu S\ cm^{-1}$. Next, RO and ED are used in conjunction. RO removes water while the retentate is

circulated through the ED stacks to keep the conductivity at 15 μS cm^{-1} which prevents salt precipitation on the membranes. Third, ED is again used alone to remove salts from the resulting concentrated sample. After the RO/ED process, the system is drained and rinsed with 0.01 M NaOH. Both the drained portion and the NaOH rinse are saved as the extracted sample. The entire RO/ED process is summarized in Figure 1 from Gurtler et al. (2008). This method is typically done with 20 L for fresh water samples (Koprivnjak 2007) and with 100-400 L for sea water samples (Koprivnjak et al. 2009); the sea water samples are reduced to a final volume of <10 L. The time required by the entire process per sample is not stated, but the RO portion of the process, when the majority of the water is removed, can be performed with a waste stream flow rate of 1.3 L/min to 2.7 L/min depending on the salinity.

Preliminary Handling

- Retrieve a seawater sample and filter the sample using a 0.45 µm filter – discard the first 20 L of filtered sample.
- Flush the RO/ED system with 50 L of filtered sample.
- Fill the sample processing tank with ~200 L of filtered sample.

Initial ED Phase

- While circulating the sample through both the RO and ED systems, engage the ED system to decrease the conductivity of the sample to around 15 mS cm^{-1}.

RO/ED Phase

- While circulating the sample through both the RO and ED systems, engage the RO system at a pressure of 1725 kPa to remove water until the targeted final volume is reached. Regulate the ED current to control the rate of desalting and maintain a conductivity of around 15 mS cm^{-1}.
- Turn off the RO system and drain it completely, transferring all recovered sample to the sample processing tank.

Final ED Phase

- Use pulsed ED, cycled on/off at 2 s intervals, to reduce conductivity to ~50 µS cm^{-1}.

Collect and Store DOM Samples

- Drain all sample from the sample processing tank, ED system, and all connecting tubing. Freeze immediately.
- Circulate 0.01 M NaOH through the RO system to recover adsorbed DOM, and drain completely.
- Circulate the same 0.01 M NaOH solution through the ED system to recover adsorbed DOM, drain completely, and freeze immediately.

Figure 1. General protocol for shipboard isolation of marine DOM using RO/ED method (directly from Gurtler et al. 2008)

RO/ED has been shown to effectively remove salts from sea water. This was first demonstrated in Vetter et al. (2007) where the final conductivity for isolated seawater was reduced to 9.5 mS cm^{-1}, thus improving the mass ratio of DOM to sea salts from roughly 1:17,500 to 1:180. Gurtler et al. (2008) further improved the ED method by using pulsed electrical current in the final step of ED phase. The method in Gurtler et al. (2008) was able to reduce the final salt concentrations to < 0.1 mS cm^{-1}, a level that allowed samples to be analyzed by NMR and FTICR-MS in Koprivinjak et al. (2009).

The equipment necessary for a shipboard RO/ED system for seawater is described in detail in Vetter et al. (2007). For the RO process the system uses the following: commercial RO module (Dow FilmTec TW30-4021, The Dow Chemical Company, Midland, MI); Standex Procon CMP-7500 SS pump (Procon, Murfreesboro, TN); stainless steel pressure vessel, and stainless steel tubing and fittings. For the ED process the system requires cationic and anionic exchange membranes (Neosepta AMX (strongly basic, 2.0–3.5cm2 at 25 ◦C) and CMX (strongly acidic, 1.8–3.8cm^2 at 25 ◦C; Ameridia, Somerset, NJ, manufactured by Astom Corp., Tokyo, Japan) as well as paired 100 cell and 50 cell electrodialysis stacks (Type 100, DeukumGmbH,Frickenhausen, Germany). A schematic for the system is shown in Figure 2.

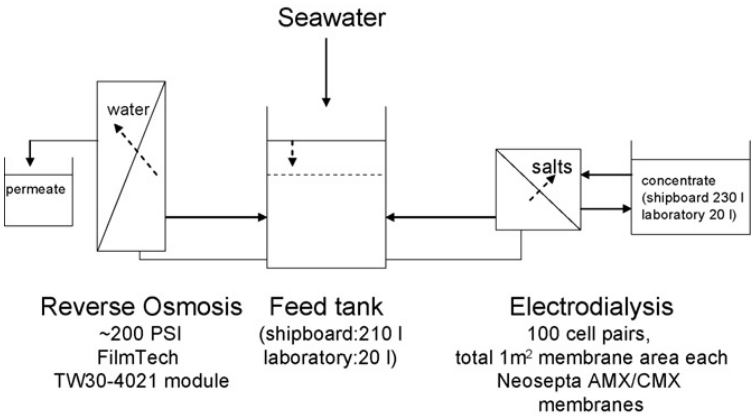


Figure 2. Ship board RO/ED system schematic (directly from Vetter et al. 2007)

While the RO/ED system described above is much more expensive than either an SPE or ultrafiltration set-up, the proponents argue that it is able to yield much

higher recoveries than other DOM extraction methods. RO/ED is able to recover an average of 75% of the DOC from a set of 16 different seawater samples; for some samples, DOC recovery greater than 90% is reported (Koprivnjak et al. 2009). In addition to retaining a higher amount of the dissolved organic carbon, Koprivnjak et al. (2009) argue that the dissolved organic matter in RO/ED extracts are more representative of the DOM found in the initial sea water samples. This conclusion is based on the fact that UV-Vis spectra and C/N ratios of extracted DOM resemble those of the initial sea water samples. Also, RO/ED is able to retain additional portions of the DOM pool compared to SPE or ultrafiltration which is evidenced by additional peaks in the ^{13}C-NMR and ^{1}H-NMR spectra as well as more alkyl carbon peaks found in the FTICR-MS spectra (Koprivnjak et al. 2009). One of the caveats with RO/ED is that it requires a chemically harsh 0.01 M NaOH (pH 12) rinse to remove organic matter from the RO membrane. This rinse may degrade molecules present in the DOM pool.

One of the limitations of RO/ED for DOM extraction is the time and cost required to run this system. As a result, blanks and loss of material have not been well characterized. Koprivnjak et al. (2009) assumed that loss of material during the RO/ED process was by adsorption to the membrane because losses across the RO membrane could not account for it. However, they did not quantify the extent to which carry-over occurred from one sample to the next. A single blank sample was run through the ship board RO/ED system in Koprivnjak et al. (2009). The amount of DOC (μmol/L) recovered in the procedural blank was on average 14.5% (range:

2.2 to 23%) of the amount recovered in the sea water samples. The amount of DOC material found in the blank samples was not subtracted to calculate recovery. If blanks are subtracted from samples, than the average recovery is 64% rather than 75% (calculated from Koprivnjak et al. 2009 data). Prior to extraction, the RO/ED system was rinsed with each sample and the blank sample had a lower NaCl concentration which could mean that the system was not flushed as effectively for the blank. This could have possibly inflated the amount of DOC found in the blank relative to the other sea water samples (Koprivnjak et al. 2009). If this method is to be useful to the field of DOM analysis, carry-over from sample to sample, loss of material and blanks need to be better characterized.

Ultrafiltration:

Ultrafiltration has been widely used since it was first proposed as a DOM extraction technique by Benner (1991). The fundamental difference between RO and ultrafiltration is the membrane permeability. RO membranes theoretically retain all sizes of organic matter as well as inorganic salts while ultrafiltration theoretically retains only the high molecular weight fraction (typically >1000 Da) of the DOM; small molecules and inorganic salts pass through the membrane. DOM is isolated with diafiltration where the membrane is rinsed with ultra-pure water as opposed to the ED method used with RO because ultrafiltration membranes are permeable to inorganic salts. The primary advantage of this method over SPE is that it can generally recover a higher portion of the dissolved organic matter (Kruger et al. 2011; Simjouw et al. 2005) although few head-to-head comparisons have been done. Also, ultrafiltration does not require "harsh" chemical manipulations i.e. acidification to pH 2 for SPE (Benner et al. 1992). However, this method is limited to extracting only the high molecular weight (HMW) portion of the dissolved organic matter and results are highly dependent on experimental procedures and equipment used (Buesseler et al 1996; Guo and Santchi 1996; Gustaffson et al. 1996).

The method for DOM extraction by cross-flow ultrafiltration is outlined in Benner et al. (1991) and essentially the same procedure, with minor changes, remains in use. Sample is first filtered through a 0.2 μm filter to remove particulate matter.

Typically 100-200 L of sample is isolated at a time. The ultrafiltration system must be thoroughly rinsed (>50 L) with ultra-pure water immediately before use (Gustafffson et al. 1996). Then, the system is conditioned with 5 L of sea water to reduce the loss of DOC by sorption to the membrane. As differences in pressure can change the amount of material that is recovered, the pressure should be held constant (Buesseler et al. 1996). For example pressures were held at 50-55 psi at the inlet and 42-48 psi at the outlet in Benner et al. (1997) which resulted in a flow rate of 13-18 L/hr for a single cartridge. Filtrate is removed until a desired concentration factor is achieved (typically concentration factors of 10-30 are used for natural samples). Then, diafiltration with 6-9 volumes of ultra-pure water is used to remove salts (Benner 1991).

A number of different commercial ultrafiltration set-ups are available. However, these different systems have varied responses and recoveries (Buessseler et al. 1996). To reduce the amount of membrane sorption and fouling, either cross-flow filtration (CFF) (also called tangential flow filtration) or stirred cell systems are used. The most commonly used CFF ultrafiltration systems are an Amicon DC-1OL or DC30 system (Benner 1991; Benner et al. 1997; Guo and Santschi 1996) and Amicon 8400 for stirred cell ultrafiltration (Kruger et al. 2011; Sinjouw et al. 2005). The major difference between CFF ultrafiltration and stirred cell ultrafiltration is the volumes that the system can handle with stirred cell ultrafiltration usually used for volumes < 1 L and CFF used for volumes of 100s to 1000s of liters per sample. Initially, polysulfone filters (Amicon SlONl) with a pore size of 1 nm and a molecular

weight cutoff of 1,000 Daltons were used. More recently, regenerated cellulose polymer membranes have been shown to be less subject to DOC sorption than polysulfone membranes (Hoffmann et al. 2000).

The limitations of ultrafiltration have been extensively characterized by a scientific collaboration organized by Buesseler in 1996 (Buesseler et al. 1996). A series of studies done as a part of this collaboration found significant differences in organic matter extraction when different ultrafiltration systems or operating conditions were used (Buesseler et al. 1996). Additionally, studies were done to quantify the blanks, loss of material by sorption to the membrane, and the effective molecular weight cutoff of a 1,000 Da membrane (Guo and Santschi 1996; Gustaffson et al. 1996).

These studies found that good blanks can be achieved with large-volume ultrafiltration but only after extensive flushing of the CFF system, i.e. >50 L (Guo and Santschi 1996; Gustaffson et al. 1996). Also, Gustaffson et al. (1996) stresses the importance that the system is flushed immediately before use.

Loss of material by sorption to the membrane is a significant problem. When various molecular weight standards were tested, initial loss of 80-100% occurred, presumably from sorption to the membrane. However, once the membrane was saturated with material an equilibrium condition arose such that molecules were both adsorbed and desorbed from the membrane and no further loss of material

occurred (Gustaffson et al. 1996). For natural samples, loss to the membrane was prevented by conditioning the system with 5 L of sample before ultrafiltration. Still, DOC material was lost during the first few liters of extraction until equilibrium was established (Guo and Santschi 1996). Both of these studies strongly recommend that the system is checked by mass balance where DOC is quantified in both the filtrate and the rententate every time that ultrafiltration is done (Guo and Santschi 1996; Gustaffson et al. 1996). Additionally, Gustaffson et al. (1996) point out that even if 100% of the material is accounted for by mass balance, the system could still be functioning improperly because there are the competing processes of blank issues and loss of material by sorption to the membrane. While still subject to the same issues, regenerated cellulose polymers were later shown to be less subject to molecular sorption (Hoffmann et al. 2000).

For DOM extraction, the membranes typically used have nominal molecular weight cutoff of 1,000 Da which corresponds to 1 nm. The effective molecular weight cutoff varies depending on experimental conditions. Effective molecular weight cutoff is defined as the molecular weight for which 90% of the molecules are actually retained by the membrane under experimental conditions. According to the manufacturers, 1,000 Da is the effective molecular weight cutoff for a 1 kDa membrane. However, ultrafiltration systems are generally designed for industrial processes with highly concentrated solutions rather than dilute natural samples, for which, the effective molecular weight cutoff is highly dependent on the experimental conditions (Buesseler et al. 1996; Gustaffson et al. 1996) and the

concentration factor (Benner et al. 1997; Guo and Santschi 1996). Also, the effective molecular weight cutoff is higher for dilute natural samples. For example, using artificial seawater samples, the effective molecular weight cutoff for a 1 kDa membrane was reported to be as high as 50 kDa (Gustaffson et al. 1996). Though, other studies report 5-6 kDa as the effective molecular weight cutoff (Benner et al 1997; Guo and Santschi 1996).

Concentration factor (Volume of initial sample/Volume of rententate) is an important factor determining effective molecular weight cutoff. Molecules of intermediate size are occasionally rejected by the membrane such that at a given concentration factor molecules of different sizes will be retained at different proportions (Figure 3) (Guo and Santschi 1996). As the concentration

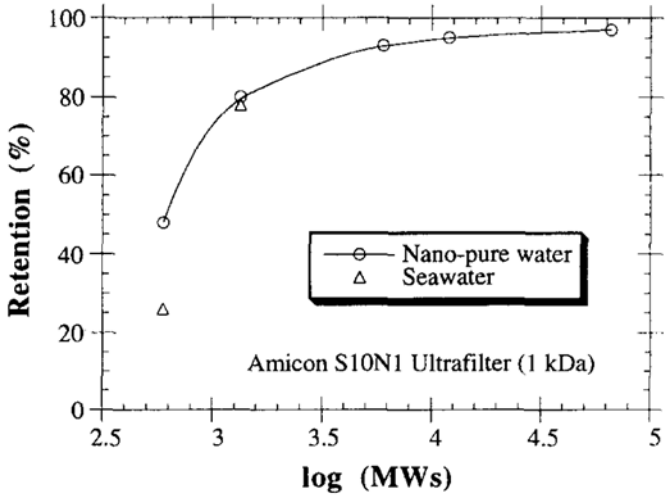


Figure 3. From Guo and Santschi (1996). Retention coefficient (%) of different model macromolecular organic compounds by a 1-kDa ultrafilter (Amicon SlONl). Macromolecules used include raffinose, vitamin B-12, insulin, cytochrome-C, and albumin for a concentration factor of 12-13.

factor increases, the retained material shifts toward higher molecular weight size fractions which is modeled in figure 4A. This means that the total percentage recovery of DOM decreases as the concentration factor increased which is modeled in Figure 4B (Benner et al. 1997).

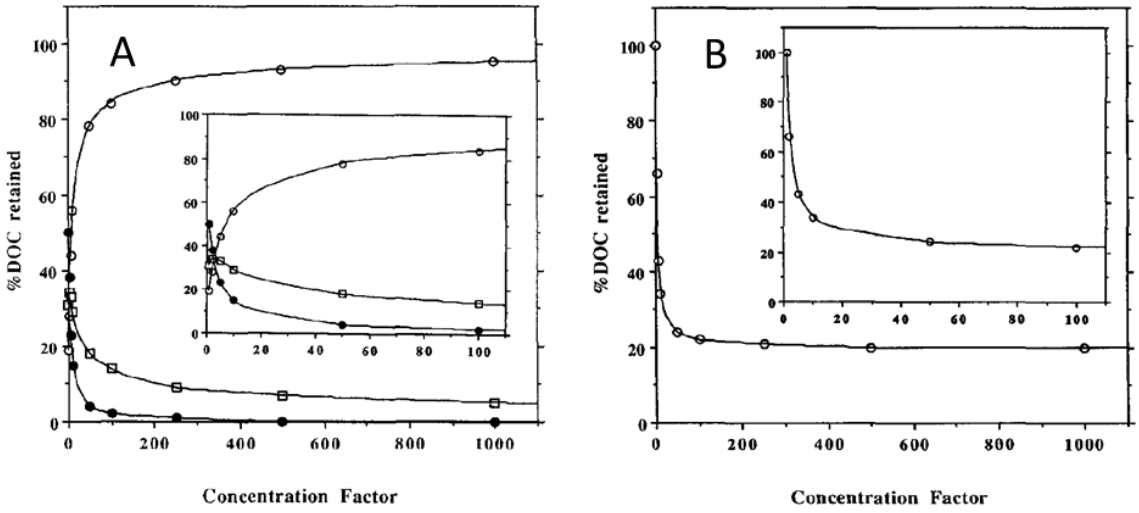


Figure 4. Directly From Benner et al. (1997) **A**. Modeled retention at varying concentration factors of a mixture of three components of DOC with different membrane rejection coefficients during tangential-flow ultrafiltration. The high-molecular-weight component (open circles) with a rejection coefficient of 1.0 accounted for 20% of the initial DOC. The intermediate-molecular-weight component (open squares) with a rejection coefficient of 0.5 accounted for 30% of the initial DOC, and the low-molecular-weight component (solid circles) with a rejection coefficient of 0.0 accounted for 50% of the initial DOC. **B.** Modeled retention of total dissolved organic carbon (DOC) at varying concentration factors during tangential-flow ultrafiltration of the mixture of three components shown in part A.

To summarize the concluding sentiment on ultrafiltration after the scientific collaboration in 1996, ultrafiltration is an effective method of DOC isolation, but the methods have to be carefully constrained to reduce the amount of material lost and to achieve high quality blanks. In the words of Guo and Santschi (1996), "Ultrafiltration is a time-consuming procedure and involves the handling of multiple

fractions [to achieve a satisfactory mass balance] which can contribute to large errors in the final result [if not done carefully]."

Finally, this review will discuss the effectiveness of ultrafiltration in comparison to the SPE and RO/ED in terms of the type and quantity of DOM recovered. DOC recoveries by ultrafiltration are generally higher for samples with terrestrially derived DOC and decrease for ocean samples (Simjouw et al. 2005; Guo and Santschi 1996). The average molecular weight of DOC decreases as a result of photodegradation (Opsahl and Benner, 1998) and microbial degradation, so that open ocean systems have a smaller average molecular weight than coastal or terrestrial systems. Also, the apparent molecular weight of a sample decreases as salinity increases which is presumably due to coiling of large molecules (Kruger et al. 2011). Further, ultrafiltration has a lower DOC recovery for deep ocean systems than for surface waters (Benner et al. 1992; Benner et al. 1997). This suggests a possible mechanism of a "diagenetic sequence from macromolecular material to small refractory molecules" with depth where macromolecules are produced by phytoplankton primary production (Benner et al. 1997). In summary, the typical DOC recoveries for open ocean samples range from 20-40%, and 50-70% or higher for coastal and freshwater samples (Table 1).

Ultrafiltration is highly effective at retaining large polysaccharides (Benner et al. 1992) and their degradation products as well as amino sugars (Simjouw et al. 2005). Beyond that generality, the type of DOM recovered by ultrafiltration is highly

dependent on the sample. For example, Kruger et al. (2011) found elevated $SUVA_{254}$ values relative to initial sterile-filtered water in ultrafiltration extracts from fresh water (river and lake) samples which suggest a high portion of aromatics in the HMW fraction. Conversely, Simjouw et al. (2005) found that chromophoric material was preferentially recovered with SPE rather than ultrafiltration for Chesapeake Bay estuarine samples.

Table 1. This table summarizes the typical DOC recovery by ultrafiltration from a range of samples.

Study	% DOC Recovery	Sample type
Benner et al. 1992	33	North Pacific Surface
Benner et al. 1992	22	North Pacific 4000 m
Benner et al. 1997	23-35	Surface Atlantic and Pacific (n=9)
Benner et al. 1997	20-24	Depth sample (2400-4000 m) Atlantic and Pacific (n=4)
Guo and Santschi 1996	35	Surface Pacific
Guo and Santschi 1996	55	Coastal North Atlantic
Simjouw et al. 2005	50.8	Chesapeake Bay mouth
Simjouw et al. 2005	50.7	Elizabeth River, Chesapeake Bay Estuary, VA, USA
Kruger et al. 2011	64	Lake Superior, MN
Kruger et al. 2011	59	Lester River, Duluth, MN
Kruger et al. 2011	78	Brule River, Brule, WI

It is debatable if the DOM recovered by ultrafiltration is representative of the DOM in the initial samples. Benner et al. (1992) found that the C/N ratios for ultrafiltration extracts were similar to the C/N ratios of the initial sea water samples. Similarly, the E2/E3 ratio of the ultrafiltration extracts is very close to the E2/E3 ratios in the initial fresh water samples (Kruger et al. 2011). However, when

mass balance was quantified by both DOC concentrations and UV-VIS absorbance, recoveries were very different which suggests a bias in the material retained by ultrafiltration (Kruger et al. 2011). Regardless of how well ultrafiltration recovers a representative DOM extract, the DOM extracted by ultrafiltration is different from that extracted by C-18 SPE as shown by FTIR and DT-MS (Simjouw et al. 2005).

One of the major advantages of ultrafiltration compared to SPE or RO/ED is that ultrafiltration does not require chemical manipulations which may alter or degrade dissolved organic matter composition. (SPE attains the highest recoveries when the sample is acidified to pH 2, and RO/ED extract is rinsed from the membrane by NaOH solution at pH 12.) Granted, all extraction methods could cause chemical changes to the dissolved organic matter; removing salts and concentrating the DOM alone would certainly change the chemical environment (Kruger et al. 2011), and adsorbing/desorbing from the ultrafiltration membrane could cause some chemical changes as well. Still, ultrafiltration is chemically the gentlest of these extraction methods.

SPE:

Solid Phase Extraction (SPE) has been, and still is, the most widely used DOM extraction method. This is probably because SPE is the "easier and quicker technique" (Simjouw et al. 2005) compared to either RO/ED or ultrafiltration. Also, SPE is by far the cheapest of these three methods (Green et al. 2014). The general

principle of SPE is that molecules are retained on a solid phase as sample is pulled or pushed through a cartridge; molecules are then eluted by appropriate solvent. Typically, nonpolar materials are used as solid phases to retain hydrophobic molecules from aqueous solution. Hydrophobic molecules are then eluted by organic solvent such as methanol or acetonitrile. Before the mid-1990's, XAD resins were the SPE sorbent of choice for DOM extraction. Currently, in the field of DOM extraction, XAD resins have been largely replaced by C18 sorbents and more recently additional modified styrene divinylbenzene phases (i.e. PPL by 3M or RP1 by Phenomenex, ect.) because these sorbents have been found to be more effective. As such, this review will focus primarily on the SPE methods using C-18 or the newer styrene divinylbenzene phases such as 3M PPL, Bond Elute. Unlike the other methods of DOM extraction, the DOM material recovered by SPE is inherently biased in the types of molecules that are recovered because the method selects for non-polar fractions.

The method for DOM extraction by SPE is quite simple compared to ultrafiltration or RO/ED. SPE can be done with either disks or cartridges as discussed below. For SPE cartridges the method is described in Dittmar et al. (2008). SPE cartridges are prepared by rinsing with organic mobile phase (often 1 cartridge volume methanol). After filtered samples are acidified to pH 2 -3 with 6 N HCl, samples are generally loaded at no more than 40 mL/min (Dittmar et al. 2008). Some studies, though, state that loading efficiency is independent of flow rate up to 150 mL/min (Louchouarn et al 2000). Dittmar et al. (2008) recommends that for every gram of sorbent no more

than 2 mmol DOC or 10 L of sample be loaded. Cartridges are rinsed with 0.01 M HCl in ultra-pure water to remove salts. Then, DOM is eluted with organic mobile phase (1 cartridge volume of methanol) (Dittmar et al. 2008). For disks, the method is the same except for a few small changes. Disks are rinsed and eluted with 3 times 10 mL of 90:10 methanol/water as described in Kim et al. (2003) and Simjouw et al. (2005). If methanol is an appropriate solvent for the particular analysis technique, the extract can be used as is. If not (i.e. TOC analysis, RP-LC-MS, NMR), the extract can be dried down and made up in ultra-pure water or appropriate solvent. A general SPE method for DOM extraction is illustrated in figure 5.

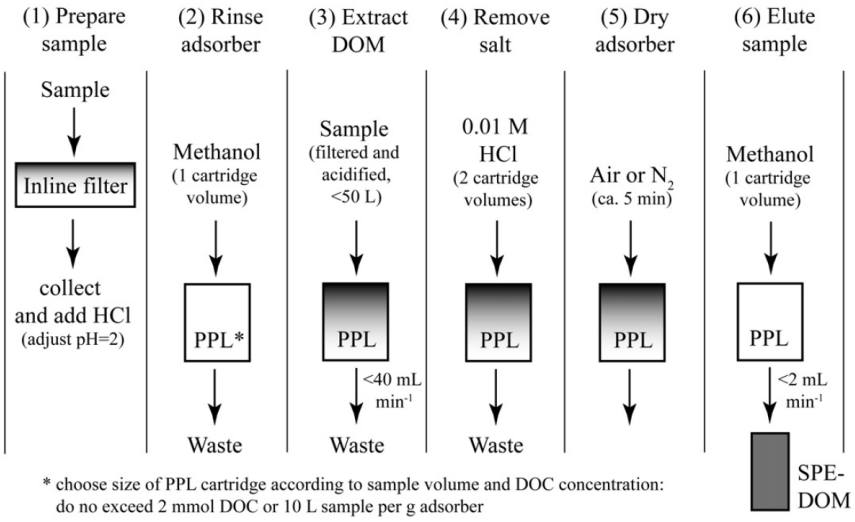


Figure 5. DOM extraction from sea water method from Dittmar et al. (2008)

If sample storage space or preservation is an issue, samples can be loaded on cartridges in the field and cartridges can be preserved by freezing or refrigeration and eluted later without detrimental effects on the recoveries (Louchouarn et. al 2000).

There is a small debate in the literature about the use of disks or cartridges for SPE extractions. Kim et al. (2003) argue that the disks can be used with higher flow rates than cartridges and report a high recovery of ~60% of the DOC although in this case recovery is determined only by UV-Visible absorbance and not by DOC concentrations. However, the highest reported DOM recoveries for SPE extractions have been with modified styrene divinylbenzene PPL cartridges (Varian, now Agilent, Bond Elut) (Dittmar et al. 2008). Both cartridges and disks are commercially available and both methods seem to be robust. Increased recoveries using styrene-divinylbenzene based phases (PPL, Varian/Agilent Bond Elut and SDB-XC, 3M) relative to C-18 based phases have been seen in both cartridge studies (Dittmar et al. 2008) and disk studies (Minor et al. 2014).

The following materials are required for SPE: SPE cartridges (such as Varian Bond Elute, PPL) or disks (such as 3M Empore, C_{18}) as well as a generic vacuum pump or hand pump, filter flask, and rubber stopper to connect the SPE cartridge with the filter flask.

DOC recoveries by SPE are summarized in Table 2. A few patterns in DOC recoveries are evident. First, XAD resins had lower recoveries than C-18 or newer, modified styrene divinylbenzene phases. XAD resins were able to recover only 5-15% of the DOC in ocean water (Thurston, 1985). Second, when the same method is used, intra-study variability between samples is greater than inter-study variability. This

suggests that sample to sample variability in DOM composition is the biggest factor in the amount of material that is recovered. Third, samples from similar locations have similar DOC recoveries even when they are analyzed by different studies. This suggests that the method itself is fairly robust. Forth, as with ultrafiltration, recoveries are lower for ocean samples than for more terrestrially derived samples.

SPE is typically described as recovering lower amounts of material than ultrafiltration or RO/ED. Certainly, XAD resins had lower recoveries than the other methods. However, to the authors' knowledge, only two studies have done a direct comparison quantifying recoveries in SPE and ultrafiltration using the same samples. Simjouw et al. (2005) found that C-18 SPE had 10-15% lower recoveries than ultrafiltration, but drew these conclusions based on only 2 samples (Simjouw et al. 2005). Kruger et al. (2011) tested three fresh water samples and found higher recoveries by ultrafiltration for two of them and lower recovery for the third. Also, since this study was done, SPE methods have improved by using styrene divinylbenzene based sorbents which have higher recoveries than C-18 sorbents (Dittmar et al. 2008). Like ultrafiltration but contrasting with RO/ED, SPE tends to have higher recoveries with fresh water and coastal samples than open ocean samples. Apparently, both overall molecular weight and hydrophobicity of the DOM is lower for open ocean samples. Finally, a combination of SPE and ultrafiltration can be applied to achieve higher recoveries then either method alone, up to 70% recoveries for estuary samples (Simjouw et al. 2005) because apparently SPE and ultrafiltration retain different portions of the overall DOM (Simjouw et al. 2005).

Also, one recent study has reported that SPE and RO/ED can be used together to achieve ~100% recoveries for open ocean samples based upon single extractions of two samples from the Pacific: a surface water and a deep water (Green et al. 2014).

Solid phase extraction has an inherent bias in the type of material recovered toward the more hydrophobic material. C-18 SPE enriches alkane/alkene structures as well as aromatic proteins and phenolic (lignin like) compounds (Simjouw et al. 2005). Reviewing several early studies using SPE, Benner et al. (1992) concludes that the material recovered by SPE is invariant with depth and oceanic environment based on ^{1}H- and ^{13}C-NMR. For Benner et al. (1992), this suggests that SPE recovers primarily older, more refractory components of marine DOM. In comparing deep-water humic extracts and ultrafiltered material, Benner et al. (1992) pointed out that the humic extract had more unsubstituted alkyl carbon and less carbohydrate character than the ultrafiltered DOM. These observations are consistent with the current hypothesis that a large portion of the refractory dissolved organic matter in the oceans consists of carboxyl-rich alicyclic molecules (CRAM) with a structure of primarily fused alicyclic rings with carboxylic acid groups (Hertkorn et al. 2006). Such material, upon sample acidification, should be retained well by hydrophobic phases. Consistent with Benner's observations of carbohydrate enrichment in ultrafiltered DOM relative to XAD-extracted DOM, we have observed that large polysaccharides are not well retained by C-18 or PPL phases (Swenson, unpublished data), and that ultrafiltered DOM is enriched in polysaccharide and aminosugar moieties relative to C-18 extracts (Simjouw et al. 2005).

Table 2. This table reviews the percent of DOC that can be recovered by SPE extraction from various studies. Clearly, composition of the dissolved carbon, which varies spatially and temporally, is the biggest factor in terms of the percent of material that can be recovered. This is seen by the fact that variability from different sites within the same studies is greater than variability across studies. In this case, PPL phase (Bond Elut PPL, Varian/Agilent) stands for a modified styrene divinylbenzene polymer.

Study	SPE conditions	% Recovery	Sample
Thurman, 1985	XAD, pH 2	5-15	General review of XAD recoveries in ocean waters
Hedges et al. 1992	XAD-2	~10	North Pacific
Hedges et al 1992	XAD-8	~30	Amazon River
Mills et al. 1982	C-18 pH 2	38	Estuarine DOC
Lara and Thomas, 1994	XAD, pH 2	50	Cultures grown in Antarctic seawater, of the 50% retained only 30% could be eluted by standard means.
Simjouw et al. 2005	C-18 pH 2	44.9, 27.4	Two samples from the same location on the Elizabeth River collected on different dates.
Simjouw et al. 2006	C-18 pH 2	38.8, 36.4	Two samples from the same location in the Chesapeake bay Mouth, This study had higher recoveries by ultrafiltration at 50.8% and 50.7% for Chesapeake Bay mouth and Elizabeth River respectively.
Dittmar et al. 2008	PPL pH 2	62 +/-6	North Brazil shelf and coastal zone
Dittmar et al. 2008	PPL pH 2	62 +/-6	Apalachicola River and tributaries
Dittmar et al. 2008	PPL pH 2	65 +/- 6	Apalachicola, salt marshes
Dittmar et al. 2008	PPL pH 2	43 +/-2	Gulf of Mexico, deep sea
Dittmar et al. 2008	PPL pH 2	43 +/-5	Weddell Sea (surface to bottom)
Kruger et al. 2011	C-18 pH 2	63 +/-6	Lester River, Duluth, MN
Kruger et al. 2011	C-18 pH 2	38+/- 11	Lake Superior, open lake
Kruger et al. 2011	C-18 pH 2	25 +/- 3	Brule River, MN
Kim et al. 2003	C-18 pH 2	60%	Stream water from New Jersey black water stream and a pristine mountain stream in Costa Rica. Poor blanks
Dalzell et al. 2009	C-18 pH 2	44	Great Bridge, Elizabeth River, Upper River
Dalzell et al. 2009	C-18 pH 2	54	Town Point, Elizabeth River (mid river)
Dalzell et al. 2009	C-18 pH 2	24	Chesapeake Bay mouth

One of the major criticisms of SPE is that SPE is chemically fairly harsh (Benner 1992, Koprivenjak 2006, etc.). SPE with C-18 or newer styrene divinylbenzene-

based sorbents is not as harsh as former SPE methods using XAD resins because XAD resins generally require several steps to elute molecules including treatments with strong base. Still, C-18 and the newer styrene divinylbeneze phases retain the highest amount of DOM when the sample is first acidified to pH 2 with HCl (Mills et al. 1982; Lara and Thomas, 1994; Dittmar, et al. 2008). This step could potentially alter the dissolved organic matter in the sample by breaking ester and peptide bonds as well as altering the higher order structure of large molecules (Koprivnjak, PhD thesis, 2007). While acidification does improve the recovery of DOM by SPE, it does not necessarily have to be part of the SPE process. Additionally, all DOM extraction methods will alter the DOM molecular structure to some extent.

Finally, based on experience in our lab, SPE tends to be fairly robust and have low blanks. However, we highly recommend replicate extractions of the same sample and using blanks each time SPE is preformed because occasionally, we have found significant variability or contamination.

Overview

In summary, RO/ED, ultrafiltration, and SPE can all be used effectively for DOM extraction in both marine and freshwater systems. RO/ED is able to achieve the highest recoveries and arguably representative samples of DOM, but needs to be better characterized in terms of blanks and loss of material. Also, RO/ED has limited availability. (Thus far, most of the RO/ED work to extract DOM had been done by a

single lab group.) Ultrafiltration has had much more wide spread use and is better characterized than RO/ED. Ultrafiltration does not require any chemical manipulations such as pH changes that could alter the DOM structure and, like RO/ED, recovers arguably representative samples of DOM. However, the effectiveness of ultrafiltration can be highly variable depending on the methods and equipment used and must be done with a high degree of care to reduce contamination or loss of material. Solid phase extraction is the cheapest and fastest of these three methods and seems to be "the method of choice" (Simjouw et al. 2005). Historically, SPE has been thought to have lower recoveries than the other two methods, but recent advancements in the sorbents used, particularly styrene divinylbenzene has improved SPE recoveries. As a chemical separation, this method selectively biases the material recovered for the more hydrophobic fraction of DOM.

In the words of Simjouw et al. (2005) "Which of these techniques is more appropriate for the isolation of DOM depends on the focus of the research." Ultrafiltration seems to have the highest recoveries for polysaccharides and large molecules while SPE extract contains a high portion of lipids and aromatic compounds. DOM recovery for both of these methods is highly sample dependent.

Project Background/Goals:

As described above the main methods for DOM extraction are RO/ED, ultrafiltration, and SPE. All three of these methods have major limitations. None of these methods

is able to recover all of the DOM present in a sample. Reverse osmosis has limited availability and has not been well characterized; ultrafiltration needs to be done with a high degree of care to ensure good blanks and reduce the loss of material; SPE retains a biased subset of DOM because it extracts molecules by chemical means. All of these methods are time consuming (several hours to days) on a per sample basis with SPE being generally the fastest and easiest method of DOM extraction (Simjouw et al., 2005).

As such, SPE is the most commonly used method for DOM extraction when mass spectrometric (MS) and LC-MS analysis are done; indeed, this extraction method is used almost exclusively in the LC-MS and FTICR-MS studies listed above (i.e. Minor et al. 2002; Kim et al. 2003; Kujawinski et al. 2009; Longnecker and Kujwinski, 2011; Sleighter et al. 2012; Minor et al., 2011; Ohno et al. 2010; Bea et al. 2011). Again, as stated above, SPE works by running sample (typically 1-5 L) over a resin (typically of C-18 or modified styrene divinylbenzene). Non-polar compounds are retained and later eluted using organic mobile phase. This mobile phase is then dried off and the samples are made up in solvent appropriate for analysis.

The percentage of the total DOM that can be recovered by SPE is highly variable from sample to sample. For ocean water, a lower percentage of the total DOM is typically retained than for more terrestrial based carbon sources (Dalzell et al. 2009; Dittmar et al., 2008; Kim et al., 2003). Generally, SPE has been thought to have lower recoveries than ultrafiltration. However, these recoveries may now be

comparable since recent improvements over C-18 SPE using modified styrene divinylbenzene sorbents (Dittmar et al. 2008); no direct comparisons have done between SPE with these newer styrene divinylbenzene phases and ultrafiltration. The percentage of the DOC material that can be recovered is compiled in Table 2 from a range of studies and natural samples. The highest recoveries reported by SPE are 65% using PPL, a modified styrene divinylbenzene sorbent (Dittmar et al. 2008). Clearly, a large portion of the DOC is not recovered in SPE extraction and conclusions drawn from (at best) 65% of the total organic matter may have significant bias. Further improvements to DOM recoveries would be valuable to the field of DOM analysis. Additionally, most of the studies above that use SPE to concentrate DOM for MS analysis are based on 1-2 sample replicates. The reason for this is probably the time and cost of collecting and storing large volumes of sample as well as the time required to concentrate samples via standard SPE. Improving the speed of the extraction step would allow more samples and sample replicates to be done.

The first objective of this study was to develop an SPE method for DOM analysis that requires less time and sample than traditional SPE methods. It should be noted that Morales et al. (2009) proposed a micro SPE method for DOM that was fully automated and attached to a FTICR-MS instrument. The method proposed here takes approximately the same amount of time as that method. However, the method proposed here is more generalized and requires less equipment. Also, this study

quantifies the DOC recoveries to compare with standard SPE methods, and this study improves on the DOC recoveries by experimenting with different SPE phases. Here rapid SPE was accomplished by concentrating a small volume (10-100 mL, depending on DOM concentration) of sample for a single analysis and by using cartridges that can be directly eluted into an LC-MS system, if desired. This method was tested against the standard SPE method outlined in Dittmar et al. (2008) to demonstrate that it works comparably well in terms of the percentage of material that can be recovered. The second objective of this study was to improve on the amount of DOM that can be recovered by SPE. To do this, a suite of stationary phases were tested to find a combination of phases that could improve on the DOM recovery. The third objective of this study was to characterize blanks and ensure that this method could be applied to a wide range of sample types.

Finally, this study proposes to make suggestions to generally improve solid phase extraction techniques depending on DOM source and concentration.

Materials and Methods

Previously, the highest recoveries of bulk DOM with SPE were reported in Dittmar et al. (2008). The method proposed here is similar in many respects to the solid phase extraction (SPE) method proposed in Dittmar et al. (2008) except that small volumes of sample are loaded on small-scale SPE cartridges, and the cartridges can be directly eluted into a liquid chromatography (LC) system. This means that the

proposed method can be done much more rapidly than the SPE method in Dittmar et al. (2008). This method requires two types of commercially available cartridges: RP-1, Guard Column, 3x4 mm (PN AJ0-5808 Phenonenex, Inc) (See fig. 6.B.c-d) and 3x10 mm, Hypercarb, Thermo Scientific Javelin Direct-Connection Columns (PN 60310-502, Thermo Fisher Scientific Inc) (See figure 6.B.b). The RP-1 guard columns have a polymeric styrene divinylbenzene stationary phase which is similar to the PPL cartridges that were found to have the highest recoveries in Dittmar et al. (2008). The Hypercarb cartridges from Thermo Scientific have an activated carbon stationary phase which is known to be useful in retaining relatively polar organic molecules (Manufacturer, Thermo Scientific). For the rest of this report, Hypercarb cartridges will be denoted as CAR. The proposed method can use either RP-1 cartridges on their own (in which case the recoveries are similar to the method proposed in Dittmar et al. 2008), or use stacked RP-1/CAR cartridges together to achieve higher DOM recovery.

Prior to concentration by SPE, the samples must first be prepared as in Dittmar et al. (2008) by filtering through either a 0.2 μm pore size Nucleopore polycarbonate filter cartridge which should be thoroughly rinsed with DI water prior to use or a Whatman G/FF (glass fiber filter, nominally 0.7 μm pore size, precombusted at 450°C for 5 h). Filter size and composition should be chosen depending on the goals of the study. (The size of the filters (0.8 or 0.2 μm pore size) is not particularly important to the SPE method so long as particulate matter which could gum up the cartridges is removed.) Then, samples are acidified to pH 2 with 6 N HCl.

Cartridges are thoroughly flushed with 2 cycles of 20-30 mL of mobile phase B (5:95 ultra-pure water/HPLC grade acetonitrile with HPLC grade 0.10 M formic acid) followed by >10 mL pH 2 ultra-pure water acidified with 6 N HCl. The loading pump is also flushed with mobile phase B followed by pH 2 ultra-pure water followed by sample. Cartridges are loaded with 5-100 mL of sample at 4 mL/min. The amount loaded depends on sample concentration and analysis type. (For our purposes, loading 60 mL of even the most dilute samples was at the upper limit of the electrospray mass spectrometer (ESI-MS) detector.) The cartridges are then rinsed with 0.3 mL of pH 2 ultra-pure water at 1 mL/min to remove salt. Finally, the above mentioned cartridges can be directly eluted into an LC-MS system with either 100% mobile phase B (1 mL/min for 1.5 min) or a gradient elution of increasing mobile phase B from 0-100-100-0% in 0-10-12-0 min at 1 mL/min. These cartridges can be attached directly to the inlet of an HPLC column (as shown in Fig. 6 C-D). For all liquid chromatography experiments presented in this study an Agilent 1100 system was used with a flow rate of 1 mL/min and at a column temp of 25^{o} C, and for mass spectrometry a Thermo Finnigan LCQ-Advantage Max Mass Spectrometer was used experimental parameters used for specific experiments are described below.

As mentioned above, the cartridges require a fairly thorough flushing to achieve good blanks. However, once the cartridges have been initially flushed, less flushing is required between samples (i.e., 1-2 mL of flushing with mobile phase B followed by 0.5 mL of ultra-pure water at pH 2). Each cartridge can be reused tens to hundreds of times but cartridges have a shorter lifespan for salty samples.

Cartridges should be replaced when either high blanks or erratic recovery of DOC is observed.

The total time to concentrate one sample is typically <10-25 minutes total compared to up to 4.5 hours ("up to 10 L of sample are loaded at no more than 40 mL/min") in the method proposed by Dittmar et al. (2008). There are two reasons that the proposed method of rapid SPE is faster than the methods in Dittmar et al. (2008) or Kim et al. (2003):

1. Smaller volume of sample is required for rapid SPE. This is not because rapid SPE is drastically more efficient than standard SPE but because standard SPE is inherently wasteful. Typically several liters of sample are concentrated into an extract that is 1 to 10 mL. From this extract, generally less than 100 µL is used per LC-MS analysis. In rapid SPE the concentration factor is equivalent, but the amount of sample concentrated is exactly what is required for a single analysis.

2. Rapid SPE can be directly eluted into an LC-MS system. In standard SPE methods, samples are typically eluted from the cartridges or disks with methanol or with 90:10 methanol/water (Dittmar et al. 2008; Kim et al. 2003; Simjouw et al. 2005). If methanol is not an ideal solvent for the analysis technique being used, the extracts have to be dried down and reconstituted in appropriate solvent which is another time consuming step. In contrast, the cartridges used here are designed to be eluted

directly into the inlet of an analytical HPLC column or into a mass spectrometer (Fig. 6C).

If the above method is used with only the RP-1 (polystyrene divinylbenzene) cartridge, the efficiency in terms of the percent of DOM recovered is similar to the Dittmar et al. (2008) method. This is expected as they both use similar solid phases. However, the efficiency of the rapid SPE method can be improved by loading sample on stacked RP-1 / CAR cartridges. In this case, the RP-1 cartridge should be the first one loaded in series because this order gives the highest recoveries (see recommendation section below). The method above can be followed as written; however, it may be preferable to elute the two cartridges separately because they contain different portions of the dissolved organic matter.

One of the biggest criticisms of SPE is that it requires acidifying the sample to pH 2 which could potentially cause degradation of the dissolved organic matter by catalyzing the hydrolysis of ester and peptide bonds. This concern is raised by a number of different DOM extraction studies (Benner et al. 1992; Koprivnjak 2007; ect.). The degradations caused by acidification can be greatly reduced simply by acidifying the sample immediately before loading it on the cartridges.

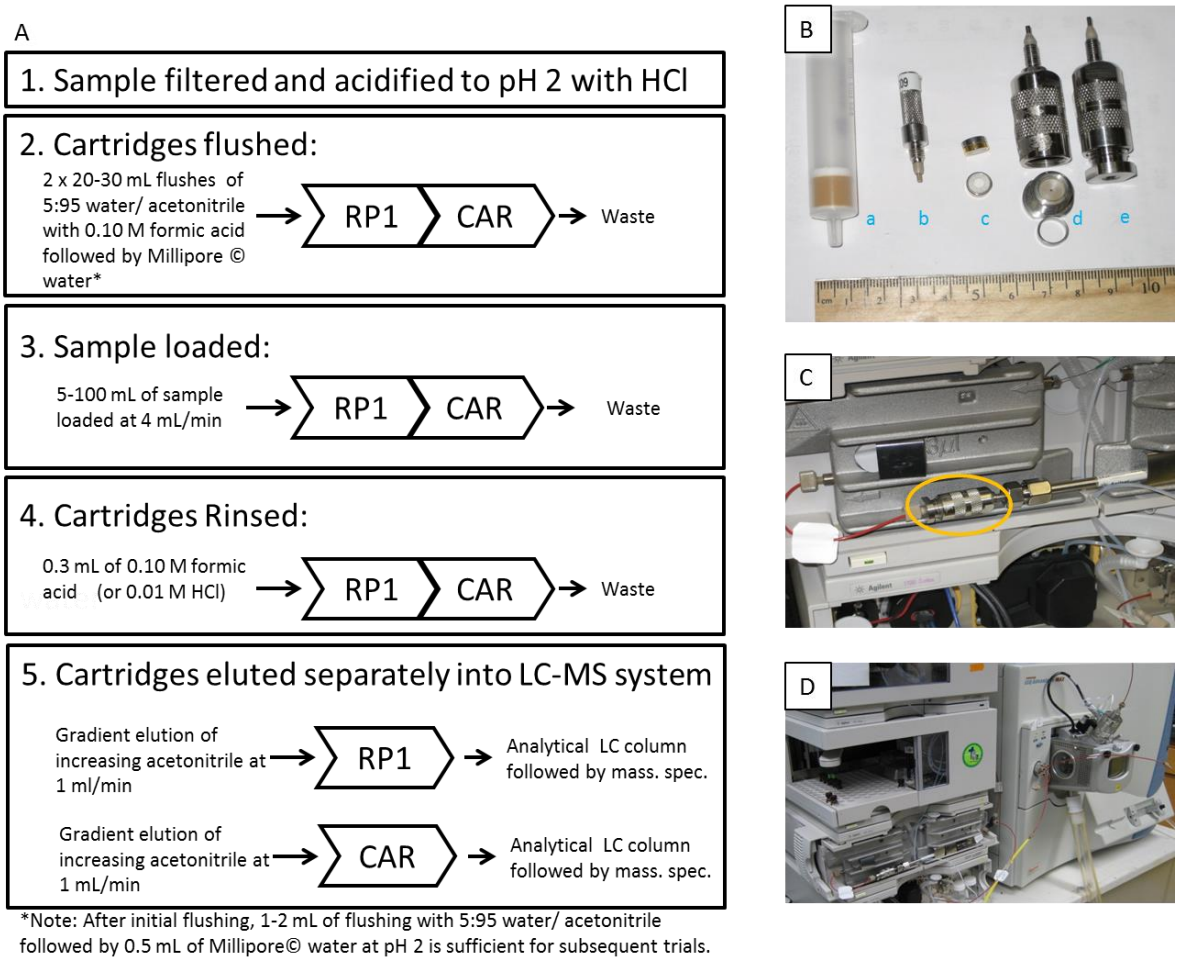


Figure 6. Scheme of rapid SPE method and images of SPE cartridges. A) Scheme of the rapid SPE method proposed here; B) Images of cartridges: B.a 3M empore PPL SPE cartridge, B.b 3x10 mm, Hypercarb, Thermo Scientific Javelin Direct-Connection Column, B.c RP-1, Guard Column cartridges, 3x4 mm, Phenonenex, B.d Phenomenex guard column cartridge holder, and B.e two RP-1 guard cartridges inside of a cartridge holder; C) Image showing the SPE guard cartridge directly connected to an analytical LC column; D) Image showing an SPE guard cartridge being directly eluted into an analytical LC column followed by a UV detector and a mass spectrometer.

Assessment

1. **Does this work as well as standard SPE in less time?**

The proposed method of rapid SPE was compared to the standard SPE method for DOM concentration (as in Dittmar et al. 2008). This head to head comparison was done with two samples and was analyzed based on the percentage of the DOM that could be recovered. Our hypothesis was that these two methods would work comparably well because the sorbent in both cases was polymeric styrene divinylbenzene and samples were loaded under essentially identical conditions. The two samples used for this comparison were water collected on 11/28/2012 at the mouth of Lester River, Duluth, MN ("MLR") and near shore water from Lake Superior collected at Leif Erikson Park, Duluth, MN ("LEP1"). Both samples were filtered through 0.2 μm cartridge filters and acidified to pH 2 with 6 N HCl. They were stored under refrigeration and analyzed within one week of collection. Both samples were collected and analyzed in duplicate.

Standard method: Following the Ditmar et al. (2008) protocol, sample was loaded on a Bond Elute PPL (3M Empore) cartridge and eluted with one cartridge volume of methanol. For the LEP1 sample, 4 L was loaded. For the MLR sample 1 L was loaded. The eluent was then dried down to remove methanol and dissolved in 40 mL (LEP1 sample) or 10 mL (MLR sample) in ultra-pure water. Aliquots of concentrated sample were injected onto an LC-MS system or placed into a combusted glass vials and acidified to pH 2 for subsequent total carbon analysis. The chromatographic analysis was done with the following method: Column: Agilent ZORBAX Eclipse XDB C-8 column 4.6 x 150 mm, 5μm particle size, PN 993967-906: LN USRK020038. Mobile phase gradient: 0-100-100-0 Mobile phase B in 0-10-12-0 min. where Mobile

Phase A is 0.01 M formic acid in ultra-pure water and mobile phase B is 5/95 ultra-pure water: HPLC grade acetonitrile with 0.01 M formic acid. UV detection was done using the inline Agilent 1100 UV-vis detector. Mass spec detection was done with a Thermo Finnigan LCQ-Advantage Max Mass Spectrometer with an ESI ion source in positive ion mode (4.5 kV). The amount of material recovered was quantified by the total UV-210 and UV-254 peak areas as well as the total ion chromatogram (TIC) peak area for ESI-MS (Table 4). TIC and UV 210 nm peak areas were first found to be linearly correlated with quantity of sample by injecting various volumes of concentrated sample.

Rapid SPE method: To test recoveries in the rapid SPE method, 25 to 50 mL of sample were loaded onto 4 stacked RP-1 HPLC guard cartridges from (Phemomenex, 3x4 mm) at 4 mL/min. DOC was directly eluted with 15:85 Ultra-pure water/ HPLC grade acetonitrile with 0.05 M formic acid into a combusted TOC vial. The vial was then dried down, made up to 40 mL with Ultra-pure water, acidified, and analyzed on a Shimadzu TOC_{VSH} (as in Zigah et al, 2011)

Table 3. This table shows the amount of organic carbon recovered with a standard SPE method (as in Dittmar et. al. 2008) compared to the rapid SPE method where results are measured by TOC analysis. The amount recovered in mg/L is normalized for the concentration factor. Errors are the average deviation from duplicate extractions of the same sample.

Sample	TOC concentration, Filtered and acidified sample (mg/L)	Standard SPE (mg recovered/L original sample)	Standard SPE % recovered	Rapid SPE (mg recovered/L original sample)	Rapid SPE % recovery
Lake Superior shore sample collected at Leif Erickson park on 11/28/2012 (LEP)	1.80 +/- 0.05	0.55 +/- 0.06	31% +/- 3%	0.49 +/- 0.08	27% +/- 5%
Sample Collected from the mouth of Lester River 11/28/2012 (MLR)	4.16 +/- 0.16	2.38 +/- 0.12	57% +/- 6%	2.86 +/- 0.36	69% +/- 13%

As expected, rapid SPE worked comparably well to the standard method for both samples. The amount of material recovered and the percent recovery for both methods is shown in Table 3. In the case of the Lake Superior near shore sample (LEP1) 31% of the DOC could be retained by standard SPE and 27% of the DOC could be retained by rapid SPE. For the Lester River sample (MLR) 57% and 69% of the DOC could be recovered for the standard SPE method and the rapid SPE method respectively. For both samples, the percentage of DOC recovered was not significantly different for the standard and rapid techniques. Table 4 compares the chromatographic response for the concentrated extracts from both methods. The 210 nm peak area for the rapid SPE extract was not significantly different from the peak area for the standard SPE extract. The chromatographic response confirms the pattern seen by TOC analysis, namely that both methods preform equally well in terms of the amount of DOM that can be retained.

Table 4. This table compares the chromatographic response in terms of UV-210 nm and ESI-TIC peak area for extracts from rapid SPE and standard SPE. In all cases, the reported values are the average of two replicates. LC method used: column: Agilent ZORBAX Eclipse XDB C-8 column 4.6 x 150 mm, 5µm particle size, PN 993967-906: LN USRK020038. Mobile phase gradient: 0-100-100-0 Mobile phase B in 0-10-12-0 min. where Mobile Phase A is 0.01 M formic acid in ultra-pure water and mobile phase B is 5/95 ultra-pure water: HPLC grade acetonitrile with 0.01 M formic acid. UV detection was done using the inline Agilent 1100 UV-vis detector. Mass spec detection was done with a Thermo Finnigan LCQ-Advantage Max Mass Spectrometer with an ESI ion source in positive ion mode (4.5 kV). Before analysis, both ESI mass spec. TIC and UV 210 nm peak areas were found to be linearly correlated with quantity of sample. UV 210 nm confidence interval represent 95% confidence. Rapid SPE cartridges were eluted directly into the HPLC column.

Standard SPE	ESI TIC Area	UV 210 nm
MLR sample	3.99E+09	3.75E+07 +/- 2E+6
LEP sample	2.64E+09	1.41E+07 +/- 8E+05
Rapid SPE	ESI TIC Area	UV 210 nm
MLR sample	4.11E+09	3.78E+07 +/- 6E+6
LEP sample	1.67E+09	1.38E+07 +/- 6E+5

2. Can we improve on standard SPE recoveries with additional phases?

In Dittmar et al. (2008), a styrene divinylbenzene sorbent (PPL) was found to have the highest DOM recoveries among the tested stationary phases. This study looked at a number of stationary phases not tested in Dittmar et al. (2008) with the goal of finding a combination of stationary phases that could recover a higher percent of the DOM than a modified styrene divinylbenzene phase alone. The stationary phases tested here were 1. Polymeric styrene divinylbenzene (RP-1 as described above), 2. C-18 bound to silica (3x4 mm C-18 HPLC Guard Column, Phenomenex), 3. Activated carbon (CAR, as described above), 4. Amine embedded styrene divinylbenzene polymers (3x10 mm, Polar embedded, Thermo Scientific Javelin Direct-Connection

Columns), 5. Strong anion exchange (SAX, 3x4mm HPLC Guard Column, Phenomenex), and 6. Strong cation exchange (SCX, 3x4mm HPLC Guard Column, Phenomenex). Because the goal was to find a combination of phases that could retain the highest amount of DOC, phases were tested by loading a combination of cartridges in sequence and eluting them separately into an LC system. The LC elution was done with no column to reduce extra sample fractionation and to analyze solely the material that could be retained on the SPE cartridges. LC elution was done with an Agilent 1100 and detection was done with an in line Agilent 1100 UV-Vis detector. SPE cartridges were eluted in two stages to look at material that was easily eluted from the cartridge (1.5 min at 1 mL/min of 0.05 M ammonium formate, in 5% HPLC grade acetonitrile in ultra-pure water) and material that was more strongly bound to the cartridges (1.5 min at 1 mL/min of 0.05 M formic acid in 25:75 ultra-pure water/acetonitrile). Amount of MLR sample that could be recovered was analyzed by the total UV-210 nm and UV-254 nm peak area. Various combinations of stationary phases were tested in this way using sample at both pH 7 and pH 2. Results (not shown) confirmed that recoveries were highest when the sample was acidified to pH 2 (as in most previous SPE studies) and that the styrene divinylbenzene phase (RP-1) was the best single solid phase over a range of pH conditions (i.e pH2 pH 7 pH 8.5). Further, these results showed that additional material could be recovered on the activated carbon phase when it was loaded together with the RP-1 phase. The combination of RP-1 and CAR had the highest recoveries when the RP-1 phase was loaded in front of the CAR phase. Apparently, if the carbon phase is used exclusively, material is irreversibly bound. Loading the RP-

1 phase in front of the carbon phase reduces the amount of potentially irreversibly-retained material that reaches the carbon phase. Finally, a very small amount of additional material could be retained on the strong anion exchange phase over a range of pH conditions. More material could be retained on the anion exchange phase at pH 7 than at pH 2 as expected, but this amount was far less than the amount retained on RP-1.

3. Do our results with C-18 vs. RP-1 support previous study findings?

Dittmar et al. (2008) showed that a modified styrene divinylbenzene phase (PPL) was the best phase for solid phase extraction for a variety of natural samples. Still, many studies continue to use C-18 phases or stacked C-18 /styrene divinylbenzene for solid phase extraction of DOM. For comparison with Dittmar et al. (2008), RP-1 and C-18 cartridges were compared. MLR sample (25 mL) was loaded on either 4 stacked RP-1 cartridges or 4 stacked C-18 cartridges, eluted into an LC system and quantified by UV-254 nm or UV-210 nm peak area. The LC method used here was the same method that is described in Table 4. These wavelengths had a linear response which was confirmed by injecting various amounts of MLR concentrate onto the LC-MS system. The results in Table 5 show that these two phases recover equal amounts of UV-absorbent material from this sample when loaded at pH 2. Also, the four RP1 cartridges were loaded behind the four C-18 cartridges and vice versa. Then, the phases were eluted separately into the LC system as above. No additional UV-254 absorbent material was found on the phase loaded second. The

means that, at least for UV-254 absorbent material, these two phases retain a similar portion of the DOM pool and no advantage is gained by stacking the two phases.

Table 5. This table shows the total area from the UV chromatograms at 254 and 210 nm when 25 mL of sample is loaded and eluted from 4 RP1 cartridges or 4 C-18 cartridges. The sample used for these tests was collected from the mouth of Lester River (MLR).

	Peak Area 210 nm	Peak Area 254 nm
RP1 cartridges	1.16E+08 +/- 0.08E+08	4.9E+07 +/- 0.5E+07
C18 cartridges	1.2E+08 +/- 0.1E+08	4.8E+07 +/- 0.8E+07

4. Can rapid SPE with RP-1/CAR be applied to a wide range of samples and retain more material than RP-1 alone?

Having found that stacked polystyrene divinylbenzene (RP-1) and activated carbon (CAR) and possibly strong anion exchange (SAX) could retain more colored dissolved organic material than RP-1 alone, the amount of material that could be recovered by the combination of these phases was quantified by TOC analysis for a range of samples. Because the recoveries by SPE are highly dependent on the composition of dissolved organic matter, the goal was to test this method with representative samples containing very different compositions of DOM.

The samples used in these experiments are listed below:

1) Suwannee River Fulvic Acid Standard II (2S101F, International Humic Substances Society, http://www.humicsubstances.org/source.html). Dried

sample was dissolved (at 1.0 mg/L) in ultra-pure water and acidified to pH 2.6 with 6 N HCl. This sample was made up at least 24 hours in advance of analysis to allow the sample to fully hydrate and equilibrate. Sample was analyzed within two weeks of creation. Sample name: SWF.

2) Fresh water samples: Near shore water collected from Lake Superior at Leif Erikson Park, Duluth, MN, and from Chester Creek, Duluth, MN on 4/17/2013 at 7:00 AM and 7:10 AM respectively. Also, water collected from Oregon Creek on 9/20/2013 at 11:00 AM. All three samples were filtered through pre-combusted 0.7 μm G/FF filters and acidified to pH 2.6 with 6 N HCl. These samples were stored in the dark at room temperature and analyzed within two weeks of collection. Sample names: LEP2, CHC, and ORC respectively.

3) Salt water samples: Samples collected from 2 sites in the Elizabeth River subestuary in the Chesapeake Bay, Great Bridge, VA, sampled on 9/15/2013 at 5:15 AM, salinity 18 +/-1 ppt (sample name: GBV) and Norfolk, VA near Old Dominion University campus sampled at 6:00 AM on 9/15/2013, salinity 23 +/- 1 ppt (sample name: ERM). Both samples were filtered through 0.2 μm filters to prevent microbial degradation and shipped to the Large Lakes Observatory, Duluth, MN where they were acidified to pH 2.5 with 6 N HCl.

Samples were stored under refrigeration and analyzed within 2 weeks of collection. For comparison with these naturally brackish to salt water samples, some of the freshwater Oregon Creek sample described above was salted to 30 ppt salt with combusted 9:1 NaCl/$MgSO_4$ to look at the effects increased ionic strength on DOM recovery. Sample name: SOC.

All cartridges and the pump were thoroughly flushed with RP-mobile phase B (5:95 ultra-pure water/HPLC grade acetonitrile with 0.10 M formic acid) followed by pH 2, ultra-pure water. Samples were run through an inline 2.6 μm stainless steel sieve just prior to the cartridges. For earlier tests (samples LEP2, CHC, and SWF samples), samples were loaded on stacked RP1 (4 x)/C-18 (2 x) cartridges followed by CAR (2x) and finally SAX (2x). For later experiments (samples GBV, ERM, ORC, and SOC), samples were loaded on RP1 (2x) cartridges followed by CAR (1x) cartridges. The C-18 and SAX phases were removed in later experiments because no additional material could be retained on stacked RP1/C-18 relative to RP1 alone and no additional material could be significantly retained on the SAX phase.

Cartridges were loaded together in a train with between 15 and 60 mL of sample at 4 mL per min. For salty samples, cartridges were flushed with 0.3 mL of ultra-pure water acidified to pH 2 with formic acid to remove salt prior to elution. Each SPE phase was eluted separately (with the exception of RP-1/C-18) into a combusted TOC vial. Reversed phase SPE cartridges (i.e., RP1, C-18 and CAR) were eluted with

1-2 mL of RP-mobile phase B at 1 mL/min. SAX cartridges were eluted with 0.05 M phosphate buffer (pH 3) in Ultra-pure water. Samples were lightly covered with aluminum foil and dried down to remove acetonitrile in an oven at 50^0 C. Dry samples were reconstituted with 15 mL of ultra-pure water and acidified to pH 2 with 6 N HCl. Extracts were then analyzed for total organic carbon on a Shimadzu TOC_{VSH}. The total organic carbon in the extract was compared to the initial sample to determine the percent recovery. Each sample was extracted and analyzed either in duplicate or quadruplicate.

For fresh water method blanks, 15 mL aliquots of pH 2 ultra-pure water were loaded onto the cartridges and eluted, dried, and analyzed as above (n=6). For salt water blanks, pH 2, ultra-pure water was salted to 10 ppt (n=3) and 30 ppt (n=3) using combusted 9:1 NaCl / $MgSO_4$. Salted blanks were loaded and eluted as above. Blank data is shown in Table 6. For the fresh water blanks, the TOC concentration is not significantly different from zero and the standard deviation is close to the instrumental variability. The salt water blanks are slightly higher with TOC concentrations of 0.29 mg/L for RP1 and 0.38 mg/L for CAR but still not significantly different from zero. The higher salt water blanks may have been due to the fact that the cartridges used on that day were new and not sufficiently flushed. In general, to get good salt water blanks required much more flushing than fresh water blanks because additional material can be removed from the cartridges when they are exposed to mobile phases with high ionic strength.

Table 6. This table shows the TOC results in mg/L from loading and eluting cartridges with ultra-pure water and salted ultra-pure water at pH 2. Blanks for the SAX cartridges were much higher and one of the reasons that the SAX cartridges were not used in subsequent experiments.

	Fresh water blanks		Salted blanks	
	RP1	CAR	RP1	CAR
Average	0.1	0.1	0.3	0.4
SD	0.2	0.3	0.3	0.4

For each sample, the average blank value was subtracted from the TOC concentration for a conservative calculation of DOM recovery even though blanks were not significantly different from zero. Then, the TOC concentration was averaged from multiple replicates of extractions plus analysis and a 95% confidence interval was calculated with a propagation of error based on the instrumental error (n=5-7, based on instrumental blanks and check standards and standard deviation (from replicate extractions, n=2-4) of the results. Figure 7 shows the DOC concentration of the extracts for all samples. Figure 7a shows that a significant amount of material can be retained on the RP1 phase for all samples. Figure 7b shows that a significant amount of additional material can be retained on the CAR phase for all samples except for the Suwannee River fulvic acid standard. For all samples, the CAR cartridge was loaded behind the RP1 cartridge and eluted separately. This means that the material retained on the carbon cartridge is additional material which, in turn, means that this method improves on the bulk DOM recovery. The variations seen in Fig. 7 are representative of, more than anything else, the various DOC concentrations in the initial samples (as reported in Table 7). This figure portrays the data in this way to show that a significant amount of additional material can be recovered by using stacked RP1 and CAR phases. The

SAX cartridges were not able to retain a significant amount of additional material and also had much larger blank issues than either the RP1 or the CAR phase. After initial tests, the SAX phase was not used.

To compare across samples and to previous studies, the amounts of material recovered on the RP1 phase and the CAR phase were added together and normalized as a percentage of the DOC material in the initial sample (Figure 8). The percentage of material retained by rapid SPE with RP1 and CAR (Table 7) is relatively high compared to previous extractions of DOM by SPE found in the literature (Table 1 and 2). For example, in this study two stream samples from the north shore of Lake Superior, Chester Creek and Oregon Creek, had recoveries of 79.6% and 75.6%; the north shore stream sample (Lester River) from Kruger et al. (2011) had 63% recovery by SPE. The Lake Superior sample in this study had 78% recovery while the Lake Superior sample in Kruger et al. (2011) had 38% recovery by SPE. For samples on the Elizabeth River this study recovered 48% and 52% of the DOM while Simjouw et al. (2005) recovered 44.9% and 27.4% for two different samples on the Elizabeth River. Further, SPE recoveries reported here are higher than those reported for ultrafiltration in Kruger et al. (2011) and comparable to the recoveries by ultrafiltration in Simjouw et al. (2005) when similar samples are compared.

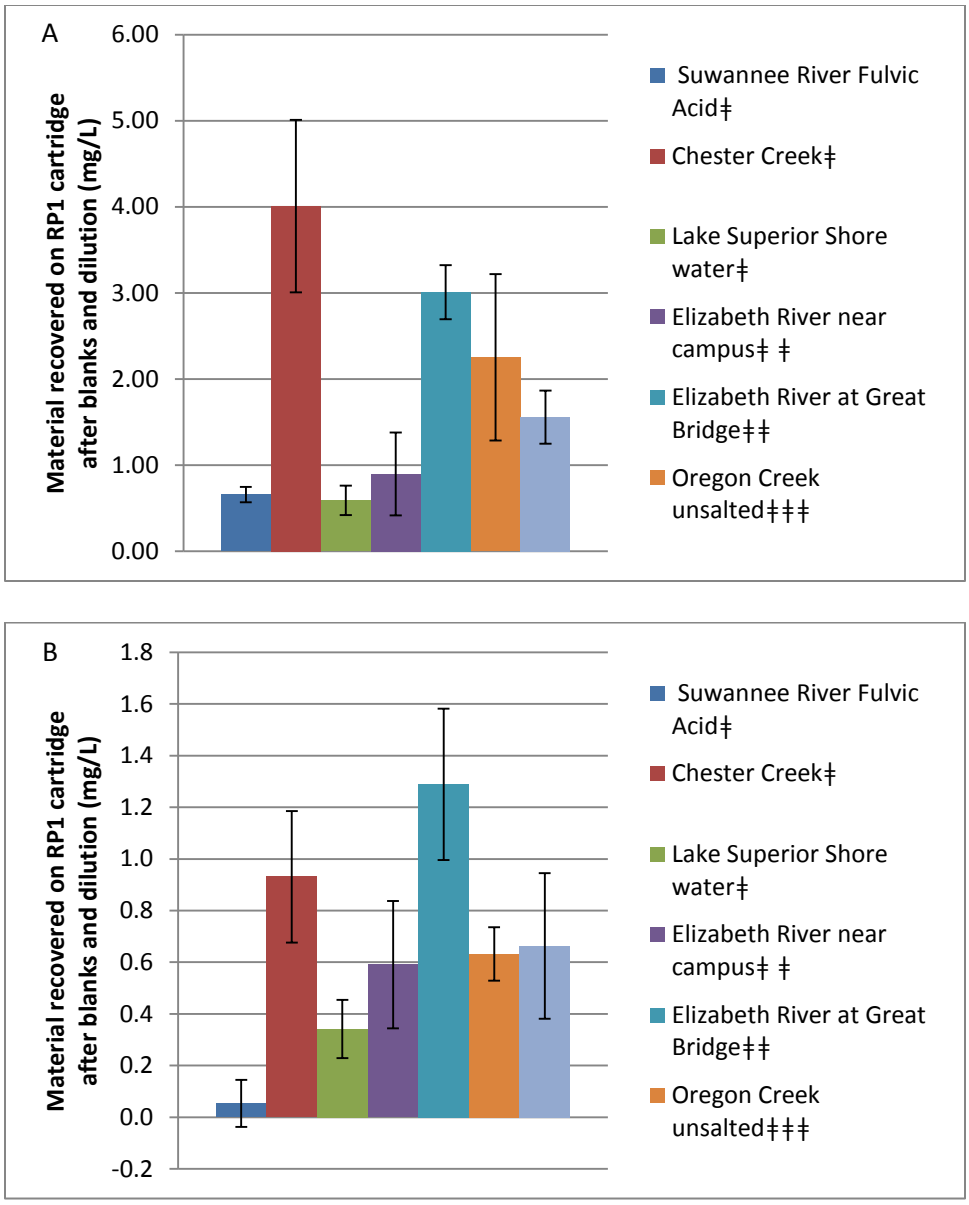


Figure 7. This figure shows the amount of material that can be retained on A) RP1 and B) CAR SPE phases for various samples where CAR phases were loaded behind RP1 cartridges and eluted separately. The data here shown here is after blank data is subtracted and the values have been scaled for dilution. The initial [DOC] for each sample is shown in Table 7.

To look at the direct effects of salinity on DOM recovery, the Oregon Creek sample was salted to 30 ppt with combusted 9:1 NaCl:$MgSO_4$. The salted sample had slightly lower recovery than the fresh water sample.

The errors seen in Figure 7 and 7 represent the 95% confidence interval. These errors are largely reflective of the instrumental error which was +/- 0.2 mg/L DOC based on our blanks and check standards.

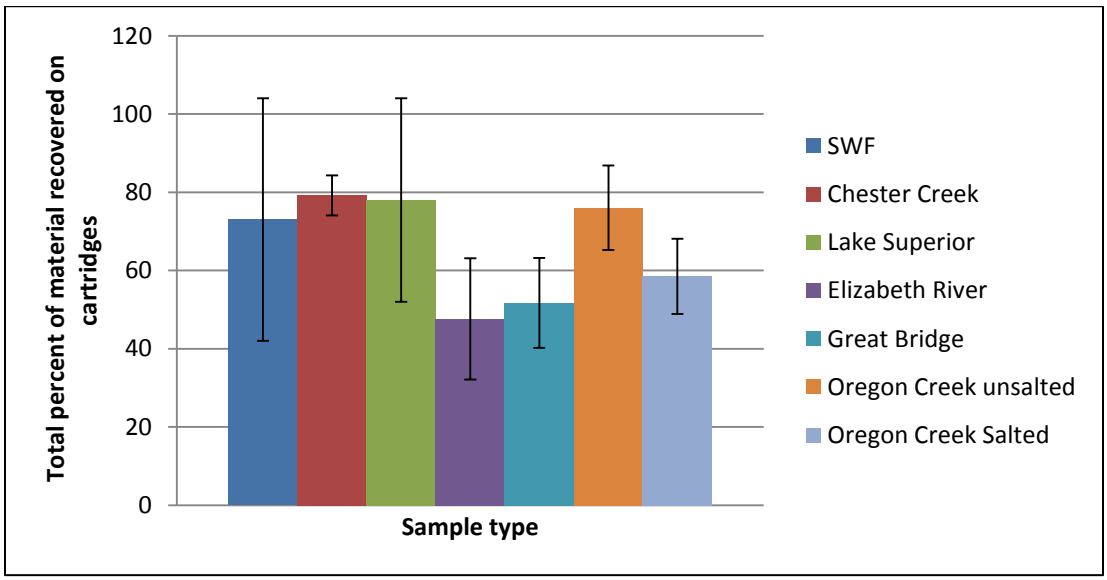


Figure 8. This figure shows the total percentage of DOC material that can be retained on a combination of RP1 and CAR SPE phases from various samples.

Table 7. This table shows the % of material retained for different samples on the RP1, carbon (CAR), and anion exchange phases (SAX). The values reported here are after blanks (from Table 6) are subtracted. Note: The three cartridges were loaded together with the RP1 first followed by the CAR and the SAX cartridge was last. This means that material retained on the carbon cartridge is additional material.

Sample	DOC conc. (mg/L)	Total % of material retained	% retained on RP1 cartridge	Additional % Retained on CAR cartridge	Additional % Retained on SAX cartridge
Suwannee River Fulvic Acid‡	0.9 +/- 0.1	73% +/- 31%	73% +/- 31%	5% +/- 27%*	-7% +/- 27%*
Chester Creek‡	6.6 +/- 0.3	79% +/- 5%	61% +/- 4%	14% +/- 3%	1% +/- 3%*
Lake Superior Shore water‡	1.3 +/- 0.2	78% +/- 26%	47% +/- 20%	27% +/- 17%	5% +/- 15%*
Elizabeth River near campus‡‡	3.1 +/- 0.3	48% +/- 15%	29% +/- 11%	19% +/- 11%	n/a
Elizabeth River at Great Bridge‼	8.3 +/- 0.2	52% +/- 12%	36% +/- 6%	16% +/- 10%	n/a
Oregon Creek unsalted‼‼	3.8 +/- 0.2	75% +/- 10%	59% +/- 6%	17% +/- 9%	n/a
Oregon Creek salted‼‼	3.7 +/- 0.2	58% +/- 10%	41% +/- 4%	17% +/- 9%	n/a

‡n=4, ‼n=3, ‼‼n=2

* Not included in the total percent because the % retained is not significant.

5. Does the amount of sample processed affect the percent recovered and the quality of the isolated DOM?

One of the key advantages of this method is that it can be used to rapidly concentrate DOM. This makes rapid SPE flexible; if an analysis technique, for example, requires larger or smaller amounts of the analytes to be within the calibration range, the concentration step can quickly be repeated with a different volume of sample. To demonstrate this, different volumes of MLR sample were loaded on 4 stacked RP1 cartridges at 4 ml/min and eluted into an LC system. In this case, cartridges were eluted through an analytical LC column (Agilent ZORBAX Eclipse XBD C-18 column, 4.6 x 100 mm, 5 μm particle size, PN 946975-902 SN USBE001190) into an Agilent 1100 UV-VIS Detector with 0.10 M formic acid in 15:85 ultra-pure water/HPLC grade acetonitrile at 1 mL/min. For each volume of sample that was loaded and eluted, the UV-254 chromatographic peak area was measured. As the volume of sample increased the UV-254 peak area increased with a linear response (Fig. 9). At least for UV-absorbent material, the amount of material recovered with this method is proportional to the volume of sample loaded from 5 to 80 mL sample MLR or a maximum loading of 0.23 mg of DOC. The high degree of linearity in Fig. 9 ($r^2 > 0.99$) and the low y-intercept suggests that, at least for the UV-absorbent portion of DOM, material is neither lost nor gained in the concentration process. While chromatographic peaks do broaden as more sample is loaded onto cartridges, this effect is reasonable. When 80 mL sample was loaded, the peak width was <150% of the peak width when 25 mL of sample was loaded.

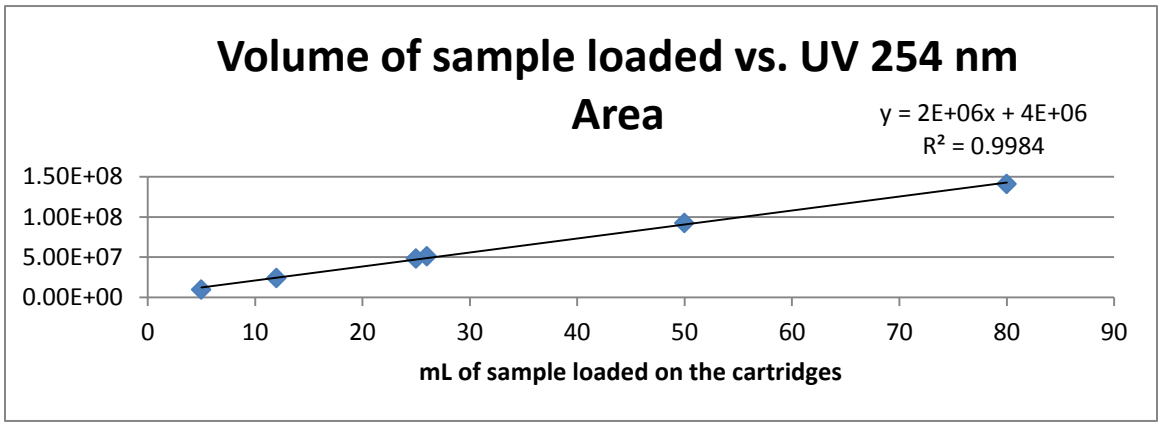
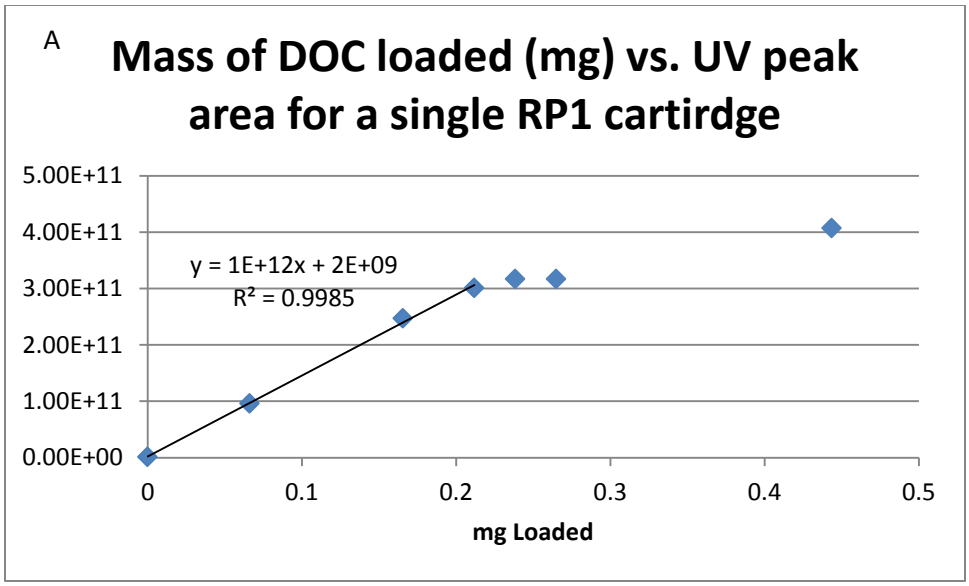


Figure 9. This figure shows the linear relationship between the amount of river sample loaded and the total UV 254 nm peak area in the subsequent chromatogram. Various amounts of sample (MLR) were loaded on 4 RP1 cartridges at 4 mL/min and eluted into an analytical LC column followed by a UV detector. Similar data was found in at 210 nm however the upper limit of the detector was reached.

A similar experiment was used to determine the limits of material that can be retained on a single RP1 or CAR cartridge. Sample CHC was loaded on a single RP1 cartridge or a single CAR cartridge at 4 mL/min and eluted into an Agilent UV-Vis Detector at 1 mL/min following the same LC method as above. The response was again measured by UV 254 nm peak area. These results are shown in Figure 10. Up to 0.20 mg carbon can be loaded on a single RP1 cartridge (Figure 10a) while greater than 0.5 mg carbon can be loaded on a single CAR cartridge. While the maximum amount that can be retained may be more dependent on moles than on the mg of carbon, it is reasonable to assume that the DOC in the sample used is heterogeneous enough that the limit of 0.2 mg C per cartridge is applicable for most natural samples.



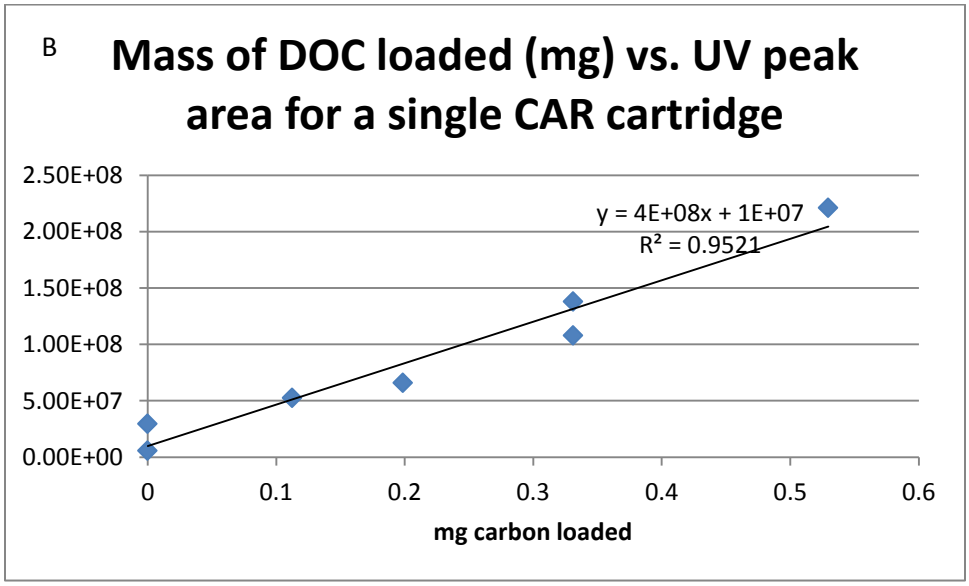


Figure 10. This figure shows the UV peak areas as a function of the amount of DOC material loaded. For A) A single RP1 cartridge response measured at 210nm, and B) A single CAR cartridge, response measured at 254 nm. Various amounts of sample (CHC) were loaded on 1 RP1 cartridge or 1 CAR cartridge at 4 mL/min and eluted into an analytical LC column followed by a UV detector.

6. Can we confirm what we see above with model compounds?

To better characterize the way that online SPE cartridges recover organic matter, four model compounds were loaded and recovered on SPE cartridges under various conditions. The four model compounds used were an organic base, (caffeine, Sigma-Aldrich, C-0750, Lot# 104K0823), organic acid (vanillic acid, Sigma Aldrich, V-2250, Lot# 71K2539), a small peptide (met-arg-phe-ala), and a large polysaccharide, blue dextran (Average mol. weight 2,000,000 Da, Sigma Aldrich D5751, Lot# 014K0036).

Individual stock solutions of caffeine, vanillic acid, and the peptide were prepared and injected onto the LC system. After an LC method was developed for each of these three molecules, a calibration curve of mg injected vs. UV-254 nm peak area was created for each of these molecules by injecting various amounts of the stock solution in order to create a quantitative relationship between material recovered and UV-254 peak area. For all three molecules liquid chromatography was done on an Agilent 1100 system at a flow rate of 1 mL/min and a column temp of 25^{o} C with a Agilent ZORBAX Eclipse XBD C-18 column, 4.6 x 50 mm, 5 μm particle size, PN 946975-902 SN USBE001190. Mobile Phase A was 95:5 ultra-pure water/HPLC grade acetonitrile with 0.10 M formic acid; Mobile Phase B was 5:95 ultra-pure water/ HPLC grade acetonitrile with 0.10 M formic acid. A gradient elution was used, for caffeine 5-40-40-5-5% Mobile Phase B in 0-5-6-6.01-7 minutes; for the peptide, 0-40-40-0-0% Mobile Phase B in 0-5-6-6.01-7 minutes; and for Vanillic Acid with the RP1 cartridge, 0-40-40-0-0% Mobile Phase B in 0-5-6-6.01-7 min. and for

the CAR cartridge, Vanillic Acid was eluted with a gradient 0-100-0-0% Mobile phase B in 0-7-7.01-9 min.

The stock solution was then diluted with ultra-pure water and loaded on the RP1 (styrene divinylbenzene) SPE cartridges. The cartridges were eluted into the LC system by the same LC method used in the calibration curve for each molecule. The percent recovery was determined by the UV 254 nm peak area. The various conditions used for loading the samples were circumneutral pH and pH 2 with or without 30 ppt salt. These four conditions are described in more detail below:

1. Circumneutral pH (For caffeine, pH 7.5; for the peptide, pH 6.3; for vanillic acid both pH 7.6 and pH 5.7 were tested with little difference in the result)
2. Neutral pH with 30 ppt salt (pH as above for the three solutions and with 30 ppt of combusted 9:1 $NaCl/MgSO_4$)
3. pH 2 (Samples were acidified to pH 2.2-2.6 with 1 ml/L of 6 N HCl)
4. pH 2 with 30 ppt salt (as above).

Table 8. Percentage of material recovered on an RP1 cartridge for an organic base (Caffeine), acid (Vanillic Acid) and a peptide (met-arg-phe-ala) loaded under various conditions and eluted onto an LC column. Quantification was done with UV-254 nm. Bold font corresponds to significantly different from 100% recovery (n=2 for all samples; error represents the 95% confidence interval).

	Neutral	Neutral with 30 ppt salt	pH 2	pH 2 with 30 ppt salt
Caffeine	106 +/- 15%	115 +/- 19%	101 +/- 21%	101 +/- 22%
Vanillic Acid	**21 +/- 28% (pH 5.7)** **13 +/- 35% (pH 7.3)**	**0 +/- 12% (pH 5.7)**	94 +/- 11%	92 +/- 11%
Peptide	94 +/-15%	**93% +/- 5%**	**88 +/- 5%**	**89 +/- 8%**

Caffeine was recovered on the RP1 cartridge at ~100% under all conditions (Table 7). This is true even though caffeine is below its pKa value and thus is a positively charged species. The peptide sample was recovered at around 90% under all tested conditions (Table 7). Because this is a small polar peptide, I would expect that most peptides and peptide-like DOM would be well retained on the styrene divinylbenzene phase which supports results found previously (Simjouw et al. 2005).

Vanillic Acid is retained at low pH with 100% recovery. However, at a pH above the pKa (tested at both pH 5.7 and 7.3) vanillic acid is not highly retained on an RP1 (styrene divinylbenzene) phase. Interesting to note is that the amount of vanillic acid recovered at pH 5.7 decreased from ~20% to 0% when the solution was salted to 30 ppt. Apparently, ionic strength can affect the partitioning between the aqueous phase and the stationary phase for molecules that are weakly retained (Table 7). Although this is not a surprising result, it is a third reason that recoveries by SPE could be lower for salt water samples. Three possible reasons that DOM recovery is lower for salt water samples compared to fresh water samples are: 1) differences in the DOM composition, 2) ionic–strength-dependent changes in the tertiary structure of large molecules (as in Kruger et al. 2011), and 3) ionic strength effects on the partitioning of weakly retained molecules.

The recovery of vanillic acid from salt and freshwater was also tested on the carbon cartridge at pH 5.7. In both cases, the recovery of vanillic acid on the CAR cartridge was not significantly differently from 100%. While this is only one example, it demonstrates that the carbon cartridge is able to retain molecules that cannot be retained on the RP1 phase. This includes organic acids near their pKa.

Some molecules are retained on SPE cartridges at 100% under a wide range of conditions while some are not. This means that concentrating a sample under different conditions (i.e. salt vs. fresh water samples) could select for certain types of molecules.

Blue Dextran was used to model the recovery of large polysaccharide on our SPE cartridges. The amount of Blue Dextran that could be retained on the cartridges was measured via colorimetry because blue dextran did not have any retention on the C-18 LC column used for the other molecules. To measure this, 0.5 mL of 900 mg/L blue dextran in DI water was run through two stacked RP1 cartridges. The absorbance of the eluent was compared to the absorbance of the initial solution at 635 nm. If blue dextran was retained on the cartridges, the absorbance in the eluent would be lower than the absorbance in initial solution. In fact this did not happen; the absorbance remained constant after the solution was run through the cartridges. This means that no blue dextran could be retained or recovered by RP1 cartridges.

To see if any of the SPE phases available could successfully retain the model large polysaccharide, blue dextran, this test was repeated for all six of the SPE stationary phases that were available: black carbon, styrene divinylbenzene, styrene divinylbenzene with polar embedded amine groups, C-18, strong cation exchange, and strong anion exchange phases (as described previously). Also, a solution of blue dextran was loaded at neutral pH and at pH 2 for all six phases. Finally, blue dextran was loaded on a strong anion exchange cartridge at pH 10.5. None of the SPE phases or conditions tested could retain blue dextran. While the literature has several examples of concentrating saccharides by SPE (many using similar phases and conditions as this study) (i.e. Fu et. al. 2010, de Villers et. al. 2004, Smits et. al. 1998), most of them are monosaccharides and small oligosaccharides. Dextran, of course, is large polysaccharide which probably behaves in a similar fashion to large semi-labile polysaccharides found in DOM. This supports what has been found in other studies; SPE is not effective at retaining large polysaccharides compared to ultrafiltration (Simjouw et al. 2005). For example, open Lake Superior DOM is proportionally higher in carbohydrate material than its tributaries (Stephens and Minor 2010), which corresponds to a lower percent recovery of DOC by SPE for Lake Superior water (Kruger et al. 2011). We hypothesize that much of the DOM that we cannot retain by SPE is composed of large saccharides.

7. Can the cartridges be directly eluted into an LC-MS system without detrimental peak broadening?

If high resolution mass spectrometry is used to analyze DOC, there may not be as much of a need for high-powered chromatographic separation. In this case, it is unimportant if analytes elute from the cartridge in broad bands or a narrow bands so long as they do elute. However, if a high quality separation is desired as part of LC-MS analysis, peak broadening can be detrimental. To confirm that analytes can be eluted from the cartridges in narrow peaks, a mixture of vanillin, vanillic acid, tannic acid, and caffeine (all from Sigma-Aldrich) were injected (100 µL) into an LC-MS system. The LC method used was 0-100-100% mobile phase B in 0-10-12 minutes where mobile phase A was 95:5 DI water/acetonitrile with 0.05 M formic acid and mobile phase B was 5:95 DI water/acetonitrile with 0.05 M formic acid. The LC column was a Zorbax Extend, 4.6 x 150 mm, 3.6 mm i.d. (P# 763953-902, USK C001762). The same solution was diluted (100X), acidified to pH 2, and 10 mL was loaded onto 4 x RP1 cartridges. The cartridges were eluted into the LC-MS system according to the same method and the chromatograms were compared (Figure 11). Vanillin, vanillic acid and caffeine all eluted from the cartridges (Figure 11b) in a narrow band similar to when the molecules were directly injected onto the system (Figure 11a). Tannic acid is a diverse mixture of molecules and did not have a narrow peak width in either case. Slight differences in retention time are caused by differences in dead volume due to the injection loop.

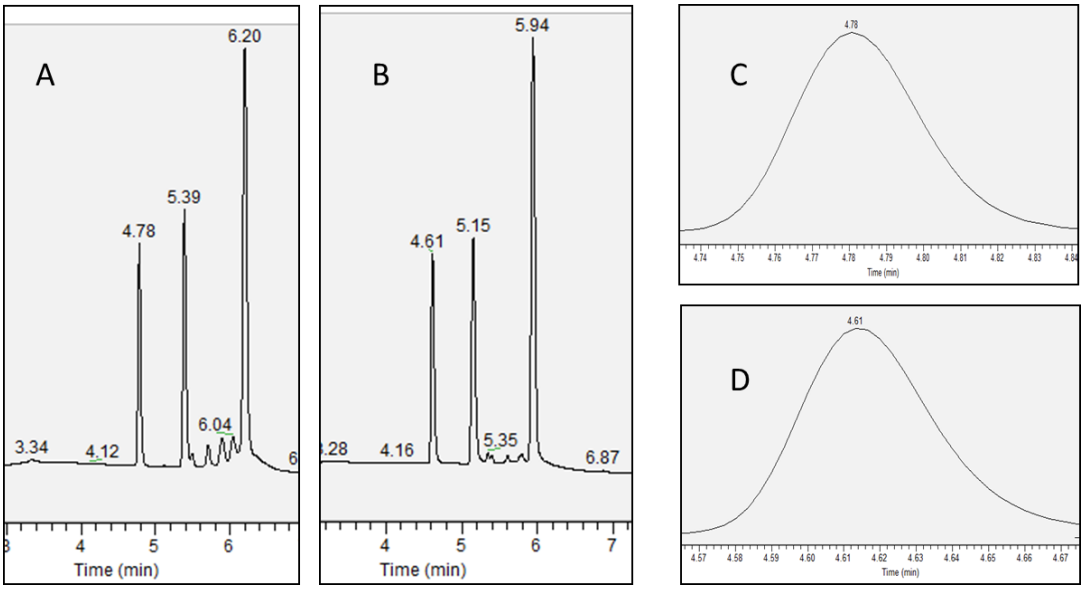


Figure 11. This figure shows the chromatograms of vanillic acid, caffeine, and vanillin loaded on RP1 cartridges and eluted into an LC system (B and D) or directly injected into an LC system (A and C). C and D show a zoomed in profile of the vanillic acid peak to demonstrate that the peak width is similar.

Finally we demonstrated that rapid SPE could be directly coupled with LC-MS to analyze DOM in a natural water sample. The sample was collected through the ice in the St. Louis River Estuary (sample collected on 3/4/2014 at the mouth of Woodstock Bay, coordinates: 46 deg 42.653 min N X 92 deg 8.998 min W). The sample was filtered through a combusted 0.7 µM G/FF filter, and then acidified to pH 2.6 with 6 N HCl. The sample was stored in the dark and analyzed within two days of collection: Sample name: SLE.

After thoroughly flushing the cartridges (as described above), 10 mL of SLE sample was loaded on 2xRP1 followed by 1xCAR cartridges at 4 mL/min. The cartridges were then directly connected to the inlet of an analytical LC column (Zorbax Eclipse Plus C8, 4.6 x 150 mm, 3.5 µm, PN 959963-906, SN USUTC01019). Organic matter

that was retained on the SPE cartridges was then eluted into the LC column according to the following method: flow rate, initially 0mL/min to prevent loss of material before the analysis began than 1.0 mL/min, temp. 25^0 C, gradient elution 10-100-10-10% mobile phase B in 0-8-8.01-10.50 min where mobile phase A is 95:5 ultra-pure water/HPLC grade acetonitrile with 0.10 M formic acid and mobile phase B is 5:95 ultra-pure water/HPLC grade acetonitrile with HPLC grade 0.10 M formic acid. UV absorbance was detected at 210 nm and 254 nm. RP-1 and CAR cartridges were loaded together and eluted separately as described above to determine if the material recovered by the two phases was different.

The LC system was connected to a Thermo Finnigan LCQ-Advantage Max Mass Spectrometer and organic matter was detected with both atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) ionization sources in positive and negative ion modes. Separate trials were done for detection by the different ionization sources. For all mass spectrometric data the detection window was set from 50 m/z to 2000 m/z. APCI mass spectrometric parameters: source voltage, 4.0 kV; source temp, 400^0 C, capillary temp, 250^0 C; capillary voltage, 4.10 V. ESI mass spectrometric parameters: source voltage, 4.55 kV; capillary voltage, 36.5 V; capillary temp, 350^0 C.

Representative LC-MS chromatograms are shown in Figure 5 for the RP-1 cartridge (Fig. 5a) and the CAR cartridge (Fig. 5b). These demonstrate that the SPE method can be directly coupled with LC-MS analysis. Comparison with the blanks (when

ultra-pure water was loaded on the cartridges rather than SLE sample) clearly supports the fact that dissolved organic matter could be recovered with the RP-1 cartridge and additional material could be recovered on the CAR cartridge.

The material recovered with the two different SPE phases was compared using weight- (MWw) and number-averaged (MWn) molecular weight calculations as in Dalzell et al. (2009). . This is possible because APCI produces only singly charged ions, and ESI of natural organic matter also produces predominately singly charged ions (Koch et al., 2005). As seen in Table 6 and exemplified in Fig. 5c-d, there is a shift toward lower molecular weight species recovered by the carbon cartridge. This is not because the carbon cartridge has a higher affinity to lower molecular weight material *per se* but because the CAR cartridge recovers material which is not retained on the RP-1 phase. The additional material recovered on the CAR phase is generally lower molecular weight. This leads us to hypothesize that much of the organic matter which has not been recovered by SPE to date is low molecular weight material.

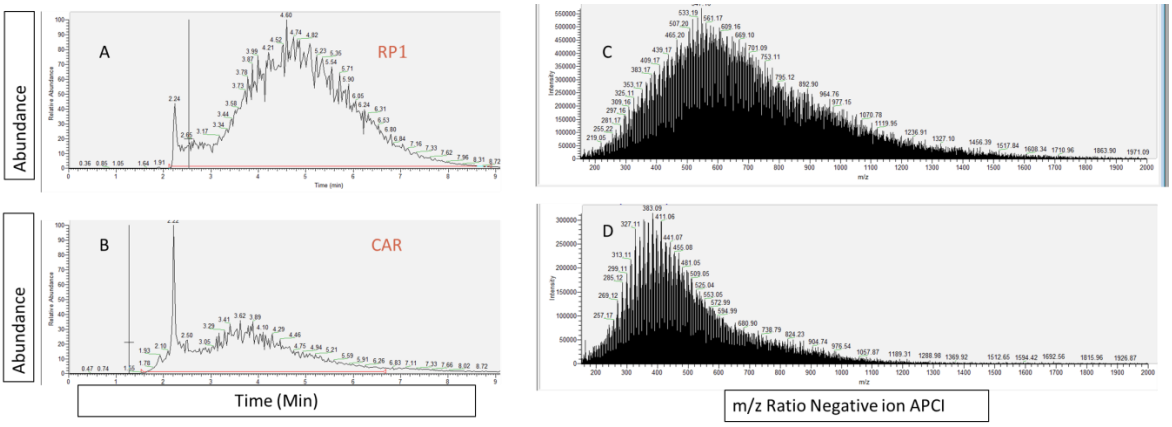


Figure 5. This figure shows the total ion chromatogram from negative ion APCI analysis for the SLE sample loaded and eluted off of the RP-1 cartridge (A) and additional material eluted from the CAR (B). The integrated mass spectra corresponding with the chromatogram for the RP-1 cartridge (C) and the CAR cartridge (D)

Table 6. This table shows the number averaged molecular weight (MW_n) and the weight averaged molecular weight (MW_w), which are calculated as in Dalzell et al. (2009). These numbers represent an "average" molecular weight for a complex mixture of molecules eluted from the RP-1 and CAR cartridges. Different ionization sources detect overlapping but different sets of molecules. Averages are based on nominal molecular weight (Nearest integer m/z ratio) due to the limitations of the instrument.

Number averaged molecular weight (MWn)

	APCI (-)	APCI (+)	ESI (-)	ESI (+)
RP1	620	563	885	753
CAR	448	456	801	734

Weight averaged molecular weight (MW_w)

	APCI (-)	APCI (+)	ESI (-)	ESI (+)
RP1	727	674	1139	1000
CAR	525	551	1081	987

Discussion

Here a method is presented for rapidly concentrating DOM by SPE extraction; this method can be directly coupled to various analyses, including LC-MS analysis. Comparing the efficiency of a DOM extraction method to previous studies is difficult because the efficiency is highly dependent on the DOM composition of the starting sample. The DOM composition in turn is variable across sites and even at the same site at different sampling times. However, this study completed a head-to-head comparison between a previous SPE method (as in Dittmar et al. 2008) and the present method. Both extraction methods were used on the same two samples. The

rapid solid phase extraction method preformed equally as well in less time and with less solvent used. The efficiency of DOM recovery for this rapid method was then improved by combining styrene divinylbenzene cartridges with activated carbon cartridges. Additional DOM was recovered using stacked modified styrene divinylbenzene phase/ activated carbon compared to modified styrene divinylbenzene alone. This was demonstrated for a diverse set of samples. Again, admitting that DOM recovery is highly sample dependent, the present method performed very well in comparison to previous studies that quantified DOM recovery by SPE. This study tested samples from similar locations to many of the previous studies, i.e. small rivers on the north shore of Lake Superior and Lake Superior (as in Kruger et. al. 2009), and samples from the Elizabeth River/ Chesapeake Bay (as in Simjouw et. al. 2003). DOM recovery was found to be higher than in previous studies that used SPE for similar sample locations and rapid-SPE recoveries were either comparable to or higher than recoveries reported for ultrafiltration; they approach levels obtained by RO/ED.

In addition to less time required to concentrate samples and higher DOM recovery than previous SPE methods, there are a few distinct advantages to rapid SPE. First, less sample is required. To the extent that sample volume and storage is a limiting factor, this method would allow more samples to be collected. Because the pool of molecules in dissolved organic matter is so complex and highly variable, the importance of collecting and analyzing more samples across spatial and temporal scales cannot be overstressed. Second, while SPE is fairly robust and reproducible,

there is some variability introduced by sample processing and, to date, this has not been well addressed in limnological and oceanographic studies. In order to do true replicates, the concentration step has to be repeated as well as the instrumental analysis. This is much more feasible with a fairly rapid concentration method. Third, in a similar way, there is the statistical importance of many blanks. Blank injections, or even several methods blanks, can be performed directly on the instrument that will be used for sample analysis. This can be done in a fairly short time and is valuable because different analysis techniques may have varying sensitivity to different contaminants.

This method uses commercially available, relatively inexpensive, and reusable cartridges. The method is also fast and highly efficient, and the extract can be directly eluted onto an LC-MS system. Considering all of these factors, the rapid SPE method reported here is easily available to most labs. Because dissolved organic matter is so variable and ubiquitous, we see an advantage in streamlining DOM analysis as much as possible to allow for comparisons across different studies and labs. The value of streamlining DOM isolation re-iterates the sentiment of Buesseler et al. (1996). Perhaps, this method could be a basis for a universal DOM extraction method for LC-MS analysis.

Comments and Recommendations

The authors of this study cannot stress enough the importance of using blanks for any technique to concentrate dissolved organic matter. In the case of this method, the blanks were generally not significantly different from zero. However, the cartridges require sufficient flushing prior to sample processing. The high ionic strength of salt water samples tends to enhance the release of organic carbon from the cartridges. To get a clean salt water blank, cartridges should be flushed with salted pH 2 ultra-pure water in addition to the flushing procedure recommended in the methods section. Also, blanks should be done separately for the two cartridge phases because carbon cartridges generally require more flushing than modified styrene divinylbenzene cartridges. That being said, once cartridges are sufficiently flushed, minimal flushing needs to be done between samples. The amount of flushing required to achieve a good blank in this method is probably not very different from the amount of flushing required for standard SPE techniques. And, several blanks, like samples, can be run quickly with rapid SPE.

We recommend that RP1 cartridges be loaded in front of CAR cartridges. If a sample is loaded directly into carbon cartridges, some of the molecules are irreversibly bound to the stationary phase. With the RP1 cartridge in front of the CAR cartridge, the most well retained molecules are removed from the sample by the RP1 phase before reaching the carbon cartridge.

Also, we recommend eluting these two phases separately into an analysis system because different portions of the DOM pool can be retained on each. As such, the molecules are already resolved into at least two classes of molecules.

Finally, no more than 0.2 mg of DOM should be loaded per cartridge per analysis. However, if more material is required for a given analysis, more cartridges can simply be stacked together and loaded.

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