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## Current trends and advances in analytical techniques for the characterization and quantification of biologically recalcitrant organic species in sludge and wastewater: A review

Sylvain Faixo, Nicolas Gehin, Stéphane Balayssac, Véronique Gilard, Sofiane Mazeghrane, Mathieu Haddad, Gilberte Gaval, Etienne Paul, Jean-Christophe Garrigues

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1 **Current trends and advances in analytical techniques for the characterization and**  
2 **quantification of biologically recalcitrant organic species in sludge and wastewater: A**  
3 **review**

4  
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21

22

23 **Abstract**

24 The study of organic matter in wastewater is a major regulatory and environmental issue and  
25 requires new developments to identify non-biodegradable refractory compounds, produced

26 mainly by thermal treatments. Recent advances linking physicochemical properties to  
27 spectroscopic analyzes (UV, Fluorescence, IR) have shown that the refractory property is  
28 favored by several physicochemical parameters: weight, hydrophobicity, aromaticity and  
29 chemical functions. Currently, the most effective developments for the quantification of  
30 refractory compounds are obtained with hyphenated methods, based on steric separation of  
31 the macromolecular species by steric exclusion chromatography (SEC)/PDA/Fluorescence  
32 systems. Hyphenated techniques using High Resolution Mass Spectrometry (HRMS), ultra-  
33 high-resolution mass spectrometry with Fourier-transform ion cyclotron resonance mass  
34 spectrometry (FT-ICR MS) and NMR have been developed to analyze macromolecules in  
35 wastewater with minor sample preparation procedures. A particular class has been identified,  
36 the melanoidins, generated by Maillard reactions between sugars, amino acids, peptides and  
37 proteins present in wastewater and sludge, but low molecular weight compounds formed as  
38 intermediates, such as ketones, aldehydes, pyrazines, pyridines or furans, are also recalcitrant  
39 and are complex to identify in the complex matrices. The lack of available standards for the  
40 study of these compounds requires the use of specific techniques and data processing.  
41 Advances in chemometrics are obtained in the development of molecular or physicochemical  
42 indices resulting from the data generated by the analytical detectors, such as aromaticity  
43 calculated by  $SUVA_{254}$  and determined by UV, fluorescence, molar mass, H/C ratio or  
44 structural studies (measuring the amount of unsaturated carbon) given by hyphenated  
45 techniques with SEC. It is clear that nitrogen compounds are widely involved in  
46 refractoriness. New trends in nitrogen containing compounds characterization follow two  
47 axes: through SEC/PDA/Fluorescence and HRMS/NMR techniques with or without  
48 separation. Other techniques widely used in food or marine science are also being imported to  
49 this study, as it can be seen in the use of "omics" methods, high-performance thin layer  
50 chromatography (HPTLC) and chromatography at the critical condition, rounding out the

51 important developments around SEC. While improving the performance of stationary phases  
52 is one of the challenges, it results in a fundamental understanding of the retention mechanisms  
53 that today provide us with more information on the structures identified. The main objective  
54 of this review is to present the spectroscopic and physicochemical techniques used to qualify  
55 and characterize refractoriness with a specific focus on chemometric approaches.

56

## 57 **Keywords**

58 Melanoidins, recalcitrant organic compounds, wastewater, hydrothermal carbonization, size  
59 exclusion chromatography, hyphenated techniques

60

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86

87 **Abbreviations**

88 ACN: acetonitrile

89 AIC: akaike information criterion

90 ANOVA: analysis of variance

91 BBI: broad band inverse

92 CA: cluster analysis

93 HCA: hierarchical CA

94 CE: capillary electrophoresis

95 CMP: comprehensive multiphase

96 COD: chemical oxygen demand

97 COSY: correlation spectroscopy

98 CP/TOSS cross polarization/total sideband suppression

99 CP-MAS: cross polarization magic-angle spinning

100 DBE: double bound equivalent

101 DEPT: distortionless enhancement by polarization transfer

102 DOC: dissolved organic carbon

103 DOM: dissolved organic matter

104 rDOM: refractory DOM

105 DON: dissolved organic nitrogen

106 rDON: refractory DON

107 DOSY: diffusion-ordered spectroscopy

108 DP/MASS: direct polarization/magic angle spinning

109 DP/TOSS: direct polarization/ total sideband suppression

110 EEM: excitation emission matrices

111 FA: fulvic acid

112 FIFFF: flow field-flow fractionation

113 FT-ICR MS: fourier-transform ion cyclotron resonance mass spectrometry

114 HA: humic acid

115 HiA: hydrophilic acids

116 HiB: hydrophilic bases

117 HiN: hydrophilic neutrals

118 HIX: humification index

119 HMBC: heteronuclear multiple-bond correlation

120 HoA: hydrophobic acid

121 HoB: hydrophobic bases

122 HoN: hydrophobic neutrals

123 HPAEC-PAD: high-performance anion-exchange chromatography with pulsed amperometric  
124 detection

- 125 HRMS: high-resolution mass spectrometry
- 126 HS: humic substances
- 127 HSQC: heteronuclear single quantum correlation
- 128 HTC: hydrothermal carbonization
- 129 IC: inorganic carbon
- 130 LCCC: liquid chromatography at the critical condition
- 131 LLE: liquid-liquid extraction
- 132 MRP: Maillard reaction products
- 133 MW: molecular weight
- 134       HMW: high MW
- 135       LMW: low MW
- 136       MWD: MW distribution
- 137 NOM: natural organic matter
- 138 OCD: organic carbon detection
- 139 OND: organic nitrogen detection
- 140 PCA: principal component analysis
- 141 PDA: photo diode array detector
- 142 PLS-DA: partial least squares discriminant analysis
- 143       OPLS-DA: orthogonal PLS-DA
- 144 POM: particulate organic matter
- 145 PRAM: polarity rapid assessment method
- 146 RAMP: ramped amplitude
- 147 RP: reversed phase
- 148 SFS: synchronous fluorescence spectroscopy

149 SPE: solid-phase extraction  
150 SUVA<sub>254</sub>: specific ultraviolet absorbance at 254nm  
151 TC: total carbon  
152 TCI: proton-optimized triple resonance NMR inverse probe  
153 THP: thermal hydrolysis process  
154 TKN: total Kjeldahl nitrogen  
155 TN: total nitrogen  
156 TOC: total organic carbon  
157 TOCSY: total correlation spectroscopy  
158 WWTP: wastewater treatment plant

159

## 160 **1. Introduction**

161 The development of new analytical methods for wastewater treatment to identify the  
162 wide variety of often toxic compounds present and those specifically generated during  
163 treatment processes, is a major present and future challenge. Wastewater treatment plants  
164 (WWTP) are a necessary step in depollution because they prevent the discharge of  
165 anthropogenic pollutants in natural waters. Prevention of some environmental issues as  
166 eutrophication or chemical contamination is secured by the increasingly stringent discharge  
167 concentration standards (depending on geographical area) for global parameters such as  
168 chemical oxygen demand, COD (125 mg O<sub>2</sub>.L<sup>-1</sup> in Europe), or total nitrogen, TN (10-15 mg  
169 N.L<sup>-1</sup> in Europe) [1–4]. Most urban WWTPs use biological processes for water treatment to  
170 reduce the concentration of dissolved compounds and suspended solids and the main waste  
171 produced is composed of biomass called sludge, and this waste to be processed [5].

172 The increasing amount of sewage sludge produced from WWTPs represents a  
173 significant challenge for wastewater facilities operations and their environmental footprint



174 [6]. Anaerobic digestion (AD) is thus implemented within various WWTPS with the purpose  
175 of energy recovery, sludge volume reduction and biomass stabilization, as it can reduce the  
176 organics, odor and pathogens, as well as generating biogas for the production of electricity  
177 and heat. Pretreatment, and in particular the hydrolysis step of AD, will change the  
178 composition of the sludge flocs by introducing organic substances from the cellular organelles  
179 of the bacteria used in the processes. Thus, various pretreatment methods before AD have  
180 been widely developed to promote hydrolysis efficiency and improve the AD performance,  
181 including mechanical, thermal, chemical and biological treatments [7].

182 Thermal treatment is a technology that has recently been implemented in WWTPs to  
183 improve sludge processing. Sludge is heated to temperatures of 140-200 °C to improve biogas  
184 production through anaerobic digestion [8] and/or sludge dewaterability [9], thus reducing  
185 overall costs [8–12]. The consequences of these thermal processes are the formation of  
186 significant amounts of soluble molecules, which are not degraded by biological processes and  
187 are defined as refractory dissolved organic matter (rDOM) [13]. Refractory compounds also  
188 named recalcitrant or persistent, have been defined as molecules not degraded by the  
189 biological treatment of wastewater. However, if the definition of refractory compounds is  
190 quite relative and depends on the method of degradation used by the WWTP, refractoriness  
191 has been well studied and standardized in the literature [14,15]. There is therefore an analytical  
192 challenge linked to regulatory constraints on the concentration of these refractory compounds  
193 in wastewater and to the constant development of sludge thermal treatment technologies  
194 [2,16–18]. Refractory compounds in WWTPs may schematically originate from 3 sources:  
195 initially present in the raw wastewater, formed during the main biological processes [19] and  
196 formed during other processes like anaerobic digestion [20] or thermal processes [21–26].  
197 The resulting refractory organic matter of various origins, then, represents a challenge when  
198 following the WWTPs discharge standards. It appears that thermal treatments generate high

199 concentrations of refractory dissolved organic nitrogen (rDON), measured by total Kjeldahl  
 200 nitrogen (TKN) (260 to 470 mg N.L<sup>-1</sup>), and rDOM, measured by COD (5.25-12.5 g O<sub>2</sub>.L<sup>-1</sup>),  
 201 depending on the temperature used in the process (120 – 208 °C). Table 1 gives values for the  
 202 amount of refractory organic matter found by different authors and its potential impact on  
 203 WWTP discharge quality.

204

205

206

207 Table 1. Quantification of rDOM and rDON produced by thermal treatments of sewage  
 208 sludge.

Sludge treatment	Thermal treatment	rDOM in COD (g O <sub>2</sub> .L <sup>-1</sup> )	rDON in TKN (mg N.L <sup>-1</sup> )	WWTP outlet	Reference
140-165 °C, 6bar	THP	N/A	N/A	Increase of TKN from 1.8 to 2.8 mg N.L <sup>-1</sup>	[21]
Thermal treatment, anaerobic and aerobic	THP	5.25	260	-	[22]
170 °C, anaerobic and aerobic	THP	Increase of rDOM from 2.6 to 8.4 % in the recycled stream.		Increase of COD from 52 to 63 mg O <sub>2</sub> .L <sup>-1</sup>	[24]

170 °C, 7.9 bar,	THP		260	-	[27]
anaerobic					
120-170 °C, 25	THP	9 - 12.5	380 - 470	-	[28]
psi, anaerobic					

---

209

210 In addition to these refractory compounds formed during thermal processes, natural  
 211 organic matter (NOM) is present in surface water or soil and is also found in wastewater. This  
 212 organic matter can be separated between natural and anthropogenic species, and then in each  
 213 class between soluble and solid aggregates. From the total organic matter present in  
 214 wastewater samples, natural organic matter (NOM), DOM, rDOM and biodegradable DOM  
 215 are identified by analytical methods before and after the different treatment processes as  
 216 shown in Fig. 1. There are wide chemical families of rDOM (surfactants, pharmaceuticals,  
 217 chemicals, Amadori compounds, melanoidins, etc.) and the chemical structures range from  
 218 small molecules to polymers, as shown in Fig. 2, with the identified structures of humic  
 219 substances (HS), polyphenols, Amadori compounds and melanoidins.

220 In Fig. 2, HS and phenols can come from both natural and anthropogenic sources.  
 221 Maillard compounds and melanoidins are anthropogenic compounds. Structural studies of  
 222 rDOM show that the majority of the refractory compounds are nitrogen compounds, forming  
 223 the dissolved organic nitrogen (DON) and refractory dissolved organic nitrogen (rDON)  
 224 families [21,23,24,33], as presented in Table 1.

225 Determinations of COD and total nitrogen (TN) are global measurements commonly  
 226 used to evaluate the efficiency of wastewater treatment. However, they give global and  
 227 nonspecific information about molecules. Therefore, COD and TN measurements are not  
 228 relevant for understanding the processes of refractory compound formation, which limits the  
 229 optimization strategies of the technologies involved such as thermal treatment or anaerobic

230 digestion or the evaluation of technologies able to degrade (chemical oxidation) or remove  
231 (adsorption) these troublesome compounds. As the main goal up until the last decade was the  
232 optimization of biogas production, very few authors worked on the characterization of the  
233 structure of the rDOM formed. More stringent discharge standards and stronger human health  
234 concerns have, however, led to an increasing interest in the characterization of rDOM  
235 [22,23,34–36]. The rDOM found after thermal treatment has been studied in other industrial  
236 fields that are developing identification methods that can be dedicated to wastewater, such as  
237 food [37,38], animal waste [39], and surface water [40]. The analytical development  
238 performed for the characterization of these rDOM will therefore be considered in this review.

239         The rDOM is made up of thousands of different compounds, each in small  
240 concentrations leading to a large overall concentration, particularly in thermally-treated  
241 sewage sludge [23]. Therefore, the use of advanced analytic methods is necessary, first to  
242 fractionate the compounds and then to get accurate chemical information about the diverse  
243 molecules that compose the rDOM. The rDOM is found in an extremely wide range of  
244 molecular masses, physicochemical properties and concentrations. In particular, a large  
245 variance is observed in aromaticity, hydrophobicity, and molar mass distribution for organic  
246 molecules in the samples. Inorganic compounds are also present, some compounds are  
247 complexed with anions, cations or small molecules such as phenols, adding still more  
248 complexity, and thus making the matrix effect responsible for the analytical variance very  
249 important. This justifies the use of complex, time consuming and expensive, hyphenated and  
250 analytical techniques. This complexity in the matrix makes sample preparation a crucial step  
251 for each analytical objective, in order to simplify matrices and split species before analysis. A  
252 broad spectrum of spectroscopic techniques (IR, UV, fluorescence, mass spectrometry,  
253 NMR.) is used to advance structural studies. Hyphenated techniques using the high-resolution  
254 analyzers also provide an orthogonal experimental space for improving analytical resolution.

255 Table 2 summarizes the methods, mainly used for the analyses of rDOM, depending on the  
256 structural information sought. The orthogonality will be a key element in the analytical chain,  
257 notably for identifying certain classes of nitrogen compounds, aromatic compounds,  
258 macromolecular compounds or intermediates of certain chemical reactions encountered in  
259 thermal treatment processes. The choice of the components in the analytical chain of Table 2,  
260 from sample preparation to data processing, will be guided by two main objectives:  
261 quantifying and identifying structures. In both cases, the absence of the quantification  
262 standards and isotopic standards needed for the mass spectrometry analysis of rDOM will be a  
263 key point to be resolved. Chemometrics are increasingly used to overcome the lack of  
264 standards, allowing a relative quantification and classification of isolated compounds in these  
265 complex matrices, and they can add value to the standardization of the interpretation of  
266 results. All the data must be linked to isolate the refractory species and identify the key  
267 factors for a more detailed study. The hyphenated method's final objective is to correlate  
268 COD with resolutive monitoring and speciation specifically for compounds generated during  
269 the treatment processes [41].

270 In this review, the state of the art of all analytical methods dedicated to the study of  
271 DOM will be considered, with a specific focus on rDOM. Due to the structural complexity of  
272 rDOM and wastewater matrices, the main objective of this review is to present the  
273 spectroscopic and physicochemical techniques used to qualify and characterize rDOM. A  
274 large part of the review is devoted to new chemometrics developments, allowing a  
275 classification of the isolated species based on key analytical parameters linked to rDOM  
276 structure and properties. Advances in NMR and mass spectrometry will also be discussed,  
277 including the identification in other matrices of methods that can be applied to the study of  
278 wastewater rDOM.

279 Table 2: Analytical methods used for the analysis and characterization of recalcitrant compounds.

Methods	Advantages	Drawbacks	Structural information	Recalcitrant species
Dissolved organic carbon (DOC) and nitrogen (DON)	Fast Simple Inexpensive Normalized for quantification	No resolution Narrow monitoring scale	Soluble C and N DOC/DON ratio	No specificity
UV/Vis spectrophotometry	Fast Simple Inexpensive	Matrix effect UV-quenching Possible interferences No split Low resolution	Aromaticity (UV 254 nm) SUVA <sub>254</sub> nm	Hydrophilic and hydrophobic rDOM
Fluorescence spectroscopy	Rather specific Good sensitivity	Possible interferences No split	Protein-like or humic-like peaks with specific Ex/Em wavelength couples	Serine and Tyrosine like rDOM Melanoidins
Fourier transform infrared spectroscopy	Inexpensive	Not specific	Functional groups	Melanoidins HA, FA

Nuclear magnetic resonance spectroscopy (liquid and solid state)	Holistic Structural information, Quantitative information without specific standard. Easy sample preparation for CP-MAS	Expensive Low sensitivity	Relative proportions of functional groups. Full structural characterization after isolation. Presence of specific nuclei (ex: $^{31}\text{P}$ ).	Product from human activities (surfactants, phthalates) HA Protein-like
Reversed-phase high performance liquid chromatography	Fast Simple fractionation for 2 to 5 species	No discrimination between macromolecules, proteins, polysaccharides and micro-pollutants.	Macromolecular DOM and rDOM fractionation based on structural properties: C/O ratio, hydrophobicity	Pharmaceuticals HA Phenolic compounds Amadori compounds
Steric exclusion chromatography (SEC)	Fractionation controlled by molecular weight and other interactions. Wide variety of SEC columns for linear or branched polymers. Split of	High influence of the composition and pH of the buffer eluent. Low resolution of HPLC SEC columns.	Size distribution. Correlation with carbon and molecular nitrogen with the use of simple detectors	HA, FA Melanoidins Proteins Polysaccharides

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	the salts, low small molecules and polymers.	Time consuming separations.		
Hyphenated techniques:	Combination of SEC		Amino-acids composition	Specific tyrosine- and serine-
- SEC/PDA/Fluorescence	fractionation and specificity of the fluorescence emission of known species		Aromaticity	like proteins.
	Relative quantification		Size distribution	HA, FA
			SUVA <sub>254</sub> , UV 280 nm and UV 210nm of each peak	Melanoidins
- SEC/HRMS	Structural information	Matrix effect	Elemental analysis	HA, FA
	Macromolecule elemental analysis for FT-ICR MS	Multiple adducts in ESI	O/C and N/C ratio	Melanoidins
			Aromaticity	Proteins
			Double bond equivalents	Polysaccharides
			CH <sub>2</sub> and C <sub>2</sub> H <sub>4</sub> O homolog series	Amadori compounds

---



280 **2. Trends in molecular characterization: dissolved organic matter and recalcitrant organic**  
281 **compounds**

282 2.1 Recalcitrant species

283 First, part of DOM is natural organic matter that can contain recalcitrant compounds  
284 such as HS, for example. Consistent review from [42] describes NOM, the analytical method  
285 used and treatment options. In addition to natural recalcitrant compounds, many recalcitrant  
286 organic species are produced by human activities such as landfill leachates [43] or food  
287 industry [44]. Specifically, in WWTPs, low quantities of refractory compounds have always  
288 been reported throughout the process, but recently-implemented thermal treatments form  
289 additional, new recalcitrant compounds that can show biological activity and effect on human  
290 health: Genotoxicity, cytotoxicity, antioxidative, antimicrobial activity [32]. Such effluents  
291 can contain up to 100 times more refractory compounds than treated or natural waters and are  
292 problematic for compliance with WWTP discharge standards. Furthermore, recalcitrant  
293 compounds formed during thermal processes can contain nitrogen, which is the most  
294 problematic element as its discharge standards are already low [23,45]. Lastly, while many  
295 natural recalcitrant compounds are inert, some anthropogenic compounds may be hazardous  
296 for the environment and/or human health [46,47].

297 Refractory compounds are remarkable in that they cannot be attributed to a simple  
298 family or chemical class. The literature shows the development of a wide variety of analytical  
299 methods that can be improved to optimize the detection limit, or resolution [48]. Depending  
300 on the desired objectives, which may be regulatory, qualitative, quantitative or related to  
301 structural analysis, the methods developed will involve a wide variety of processes and  
302 detection systems.

303 Refractoriness is generally correlated by several physicochemical parameters such as  
304 high molecular weight (HMW), hydrophobicity, aromaticity or chemical functions, especially

305 certain substitutions such as chlorinated aromatics. These characteristics can be found in the  
306 humic substances (HS), humic acids (HA) and fulvic acids (FA) that constitute the majority of  
307 rDOM species isolated in raw water, but also in many refractory compounds of anthropogenic  
308 origin. For example, HA and FA are acidic organic polymers recalcitrant to biodegradation  
309 [49,50]. Their complex molecular structures show a link between aromaticity, which can be  
310 measured by UV absorption at 254 nm or the H/C ratio, and molar mass calculated for each  
311 chromatographic peak identified in water samples, as shown on Fig. 3. It is, therefore,  
312 essential to be able to access resolute analytical methods for the fractionation of refractory  
313 compounds and to be able to measure other related properties such as aromaticity for access to  
314 sample monitoring. Fig. 3 shows the classification possibilities for various HAs and FAs  
315 isolated from surface water samples according to these 2 parameters. This classification  
316 methodology will be used with other rDOM species for analytical developments allowing  
317 rapid comparison of recalcitrant compounds in wastewater samples.

318 With the large variation of reactive compounds in raw wastewater and of their  
319 physicochemical properties, the analytical challenge is present at all levels: sample  
320 preparation, fractionation and structural analysis. For HS, structural heterogeneity is  
321 demonstrated by the analytical approach following fractionation of specific physicochemical  
322 data: molecular mass and hydrophobicity. Modelling of polymeric structures generated in a  
323 glycine/glucose model showed the formation of three groups of macromolecules between 10  
324 kDa and 20 kDa. The composition of the polymers will depend on the ratio, the type of  
325 reactants and the reaction parameters, and can be determined using fractionation techniques.  
326 In this model, the origin of nitrogen-containing polymers is attributed to specific Amadori  
327 intermediate and the non-nitrogen-containing polymers to glucosone and deoxyglucosone  
328 [52]. All these intermediates can be identified by GC/MS analysis of reaction product dialysis.  
329 New developments using direct analysis or derivatization aim to gain better knowledge of the

330 reaction mechanisms involved through the structural study and quantification of the  
 331 recalcitrant compounds. The optimization of treatment processes can also be managed on an  
 332 analytical basis. Table 3 summarizes the analytical techniques developed for the study of the  
 333 Maillard products that this review focuses on (see part 2.2).

334

335 Table 3. Analytical techniques used for the analysis of Maillard reaction products. Reprinted  
 336 from [48].

Maillard reaction products (MRPs)	Analytical techniques
Amadori compounds (Direct analysis)	Column chromatography HPLC differential refractometry detection HPLC involving derivatization HPAEC coupled electrochemical and/or DAD FAB-MS ESI coupled HPLC and EC, EC coupled MS MALDI-TOF NMR LC-MS
Indirect analysis (2-FM-AA)	Ion-pair RP-HPLC CEC UV-detection HPLC-MS
Advanced Maillard products	HPLC-DAD
Carboxymethyl-lysine	RP-HPLC RP-HPLC o-phthalaldehyde pre- column derivatization GC-MS
Pyrraline	RP-HPLC
Cross linking products Lysine	LC-MS with ESI
Dimmers Arginine-lysine	LC-MS with ESI

	Ion-exchange chromatography FAB–MS
Other amino acid derivatives,	HPLC-coupled GC–MS
Argyrimidine	
Pyrazinones	HPLC with UV and fluorescence detection
Lysine aminoreductone	HPLC-DAD
Final stage MRPs	HPLC, NMR, MS, UV, IR spectrometry
General melanoidins	MALDI-TOF mass spectrometry

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337

338

## 339 2.2 Maillard reactions

340 Thermal treatment with temperatures greater than 120 °C applied to sewage sludge  
341 solubilizes organic matter and breaks bacteria cell walls [53,54] with the production of low  
342 and HMW molecules. Some of them, such as the carbonyl and amino functional groups,  
343 include reducing carbohydrates, amino acids, peptides and proteins which may react with  
344 each other in special conditions. An analytical survey of sewage sludge shows that samples  
345 composed of 15-30 % carbohydrates and 20-50 % proteins generate ideal conditions for  
346 Maillard reactions [55–57]. Maillard reactions are non-enzymatic condensation, controlled by  
347 temperature, that occurs between reducing sugars and proteins [58]. Refractory compounds in  
348 thermally treated sewage sludge were modelled with a Maillard reaction scheme by  
349 identifying specific Amadori intermediates [26,33]. Maillard reactions have been widely  
350 studied in the food industry due the physicochemistry and especially the brown color of the  
351 synthesized compounds at high temperature. It can be responsible for flavor adjustments in  
352 many types of food [59,60]. Depending on the conditions, these reactions can also be the  
353 source of a waste that requires specific treatment, like brewery stillage [44]. Maillard  
354 reactions are a complex system of interconnected chemical reactions and are not yet well

355 understood. Generated compounds depend on many parameters such as the nature of the  
356 reactants, pH and temperature [48,61]. Low molecular weight (LMW) compounds are formed  
357 as intermediates through the earliest stages of Maillard reactions, including ketones,  
358 aldehydes, pyrazines, pyridines and furans [48,62,63]. All these intermediates rapidly react in  
359 an aldol-type condensation. The degradation of monosaccharides such as aldose sugars leads  
360 to the formation of furaldehydes and furanones, such as furan-2-carboxaldehyde and furan-2-  
361 aldehyde [64]. The subsequent steps involve dehydration, cyclization, retro-aldolization,  
362 isomerization, rearrangement and condensation. In the last steps, all the intermediates react  
363 with amino acids and peptides to form LMW monomers leading to the production of HMW  
364 molecules, called melanoidins, by polymerization [65].

365         The complexity of the matrix, for the different industries interested in melanoidins, is  
366 an analytical challenge and explains our lack of knowledge of these compounds. Melanoidins  
367 are thought to be the cause of the formation of a brown color in food and wastewater samples  
368 after thermal treatment [32]. This brown color formation is coupled with increased UV  
369 absorbance and is largely observed in the liquid phase of thermally treated sludge, which is  
370 also correlated to refractoriness [8,21,66,67]. The brown color can be linked to the decrease of  
371 the soluble protein fraction in samples that may have reacted during Maillard reactions from  
372 78 % to 68 % with an increase in treatment temperature from 135 °C to 190 °C [66]. The non-  
373 biodegradable soluble chemical oxygen demand (COD) and UV absorbance were 2.7 and 1.7  
374 times higher for the thermal treatment of sewage sludge at 165 °C versus 140 °C, linked to the  
375 increase of synthetic melanoidin in solution [21]. Finally, melanoidins exhibited a strong IR  
376 absorption band, notably at 1607  $\text{cm}^{-1}$ , attributed to extensive conjugation, which can be  
377 connected to the identification of UV bands shifting to high wavelengths. These results  
378 indicate that spectrophotometric methods can be used for melanoidin quantification and  
379 wastewater monitoring.

380           The core constant structure of melanoidins is formed by mixture of  $\alpha$ -dicarbonyl  
381 Maillard reaction intermediates, branched with amino compounds. Pyrolysis of the isolated  
382 polymers produced typical Amadori products, such as pyrazines, pyrroles, pyridines and  
383 furans. Longer thermal treatment time and higher temperature are correlated with HMW  
384 melanoidins formation [32]. The molecular weight and nitrogen composition of the  
385 recalcitrant polymers depend on the nature of reactants and of the industry's mixture of raw or  
386 wastewater: > 30 kDa in bread [68], > 10 kDa in coffee [69], but the reaction conditions have  
387 a major influence. In the case of sewage sludge, temperatures involved in some thermal  
388 sludge treatments exceed 120 °C to be energetically efficient [8,12]. The final biological and  
389 physicochemical properties of wastewater samples is correlated with analytical data from  
390 melanoidins. The majority of the color (68 %), DOC (53 %) and UV absorbance at 254 nm  
391 (54 %) were in the analytes fraction > 10 kDa, indicating a significant amount of aromaticity  
392 and conjugated molecular structures in the HMW fractions of hydrolyzed sludge samples  
393 from thermal hydrolysis processes [21]. But simple physicochemical parameters are not  
394 significant for the specific identification of melanoidins, and high levels of soluble COD can  
395 be found in the LMW fraction, below 5 kDa. HMW contributes to refractoriness for classic  
396 biological WWTP treatments [70] and melanoidins are no exception. A major part (65 %) of  
397 the total refractory organic carbon (rTOC) was above 10 kDa and 25 % was under 0.5 kDa for  
398 sewage sludge treated at 208 °C [21,22].

399           Melanoidins contain significant nitrogen quantities and their refractoriness may affect  
400 compliance with discharge standards. DON increases up to 50 % with thermal treatment of  
401 sewage sludge, before anaerobic digestion [21], and up to 24 % after anaerobic digestion [28].  
402 Regarding structural analyses, about 50 % of rDON is found in HMW fractions up to 10 kDa  
403 [22]. It should be pointed out that the literature mainly depicts Maillard reactions as being  
404 responsible for refractoriness generated during thermal treatments since, quantitatively, they

405 are probably the main cause of refractory compound formation [48]. Other sources can still  
 406 be expected such as caramelization reactions [71] or the liberation of cell walls in recalcitrant  
 407 components. Table 4 summarizes all available data, fully presented in Table S1  
 408 (supplementary data), for the structure of compounds synthesized during Maillard reactions,  
 409 as well as the Amadori intermediates that can be identified, depending on the progress of the  
 410 reaction, the conditions and the reactants presents in the wastewater and sludge.

411 Table 4. Chemical species, identified after thermal processes on residual water and sludge.

Thermal process	Temperature (°C)	Identified chemical species	Range of MW (g.mol <sup>-1</sup> )	Reference
HTC	170	Aromatic, Heterocyclic, Pyrazines, Pyrimidines, Aldehydes, Ketones	60 - 282	[23]
HTC	200 - 320	Ketones, Heterocyclic, Carboxyl, Amide, Pyridines, Humic substances, Phenols	94 - > 10 000 (HS)	[34]
THP	172	Alkanes, Esters, Carboxyl, Amide, Pyrrolidines, Pyridines, Benzenoids, Peptides	127 - 572	[72]
HTC	208	Aldehydes, Ketones, Phenols, Heterocyclic, Pyrazines, Pyrimidines	73 - 182	[73]

412

413 Table 4 shows the wide structural variety of Amadori intermediates found during and  
414 after thermal processes from the Maillard reactions. The very different physicochemical  
415 properties (polarity, hydrophobicity, molar mass, volatility, chromophores, and fluorescence  
416 properties) of these compounds will require specific analytical developments to identify and  
417 quantify them all. This structural and functional diversity may also explain why HMW  
418 compounds are not always identified in the studies cited. The techniques used to identify these  
419 recalcitrant compounds may not be suitable for the identification of macromolecules (HS,  
420 melanoidins).

421

## 422 2.3 Physicochemical and spectrophotometric analyses

423 DOM is heterogeneous in nature, chemical functions and properties. The  
424 physicochemical methods that have been developed for wastewater analysis are designed to  
425 identify and quantify certain chemical or species functions, such as proteins, polysaccharides  
426 and charged colloids, and to understand the characteristics of the samples such as  
427 biodegradability, the presence of recalcitrant compounds and the color of the water. The  
428 interest of these physicochemical techniques lies in their short analysis times, the use of  
429 simple equipment and the simple sample preparation and justifies that they are still widely  
430 used today.

431

432

### 433 2.3.1 Dissolved organic carbon (DOC) and nitrogen (DON)

434 DOC is a concentration parameter representative of the total dissolved organic  
435 molecules (NOM, DOM and rDOM). The DOC concentration is determined with a total  
436 organic carbon (TOC) analyzer after sample filtration (0.45 to 0.22  $\mu\text{m}$ ). TOC measurements  
437 provide information on total carbon (TC) and inorganic carbon (IC) concentrations. DOC is



438 calculated by subtracting IC from TC [74]. Biodegradable DOC is the DOC fraction that can  
439 be metabolized by bacteria in wastewater, and recalcitrant species and recalcitrant DOC can  
440 be identified by analyzing the non-biodegradable fractions [75].

441 DON refers to soluble organic compounds retrieved after filtration (0.45 to 0.22  $\mu\text{m}$ ),  
442 that also contain nitrogen. The DON concentration is determined by subtracting the  
443 concentrations of ammonia, nitrate and nitrite from the concentration of total nitrogen. Total  
444 nitrogen concentrations in wastewater samples are determined using various colorimetric  
445 methods such as the persulfate digestion method, nitrates using the dimethylphenol method,  
446 nitrites using the diazotization method and ammonia using the salicylate method [22]. These  
447 methods' yield is less accurate when the inorganic nitrogen concentrations are high. For the  
448 recalcitrant compounds monitored in wastewater, melanoidins are the most intensively studied  
449 complex, and heterogeneous nitrogen-containing Maillard reaction products. A first approach  
450 to rDON and melanoidin quantification can be achieved using DOC/DON measurements and  
451 the calculation of the carbon-on-nitrogen ratio (C/N). This ratio increase with the  
452 concentration of different reducing sugars and glycine [21]. The removal of biodegradable  
453 DOC is of great importance for the water research and water treatment industry and  
454 normalization of rDOM concentrations vs DOC or DON are performed routinely. This field  
455 of research is now able to focus on more specific species, such as specific melanoidins or  
456 Amadori intermediates. In this case, it is necessary to add selective sample preparation  
457 techniques upstream to ensure a more resolute fractionation.

458

### 459 2.3.2 UV/Vis spectrophotometry

460 UV/Vis spectrophotometry is an analytical method widely used for the  
461 characterization of organic matter in all kinds of natural and wastewater. Used since the  
462 1970s, it first served to model the composition of ocean waters and the fate of various

463 dissolved compounds: chlorophyll pigments and NOM. The UV-visible spectrum is studied  
464 for the classification of samples from specific measurements of chlorophyll pigments at 430  
465 nm and continuous analysis between 300 and 700 nm [76]. More broadly, UV/Vis  
466 spectrophotometry provides structural information on the nature of the bonds present in the  
467 sample (chromophores and specific chemical functions,  $sp^2$ -hybridized carbon) and  
468 quantitative information on the concentration of absorbent species in the spectral range  
469 studied (proteins, nucleic acids, HS, etc.). Even if the NOM is represented by species that  
470 have characteristic UV spectra, sample spectra are typically broad without any fractionation.  
471 The comparison of samples is mainly performed by measurements at specific wavelengths  
472 such as 254 nm, 210 nm or 280 nm, and ratios between these values [77]. The resulting  
473 indices give insight into the aromaticity of DOM and its molecular weight can be discussed  
474 [78]. Certain macromolecular DOM show a distinctive ultraviolet absorbance ratio index  
475 (URI) calculated using the  $UVA_{210}/UVA_{254}$  ratio, due to their different compositional  
476 proportions. URI values show the lowest values for HA (1.59, highest aromaticity),  
477 intermediate for FA (1.88, intermediate aromaticity), and highest for proteins (13.5 for BSA,  
478 lowest aromaticity) [79–81]. Specific UV absorbance at 254 nm ( $SUVA_{254}$ ) is a powerful  
479 index measured using the ratio of UV absorbance to DOC, correlated with the NOM species.  
480 For these spectrophotometric measurements, it is necessary to control the turbidity of the  
481 samples by filtration. Some ions in solution (nitrate, nitrite, iron) may have a matrix effect,  
482 depending on their concentration [82].

483         The importance of sample absorbance is linked to biological response and the removal  
484 of the colored fraction from the thermally-treated sludge liquid fraction with resins could  
485 increase biodegradability from 33.2 to 57.8 % [83]. Most of the UV-quenching substances can  
486 be found in HMW substances, which are presumed to be mostly refractory. For access to  
487 these macromolecular species, a size fractionation sample preparation can be coupled to

488 UV/Vis spectrophotometry measurements [21,22,84]. The use of specific wavelengths does  
489 not differentiate the rDOM chemical families, it is then necessary to combine several  
490 physicochemical measures such as COD, DOC or TOC. SUVA<sub>254</sub> is a specific indicator of  
491 aromaticity, which is generally a property of refractory compounds. With a SUVA<sub>254</sub> value  
492 greater than 4, a sample is composed mainly of hydrophobic and aromatic NOM, with  
493 HMWs, whereas a SUVA<sub>254</sub> value lower than 3 suggests a mixture of low molecular weight  
494 hydrophilic NOM [85].

495

### 496 2.3.3 Fluorescence spectroscopy

497 Fluorescence spectroscopy provides useful data on the structure of soluble compounds  
498 in water. Fluorescence in aquatic matrices has been extensively studied and sample  
499 monitoring can be achieved in different areas such as WWTP influents and effluents, textiles  
500 and the food industry. The specific excitation and emission wavelength values and intensity  
501 can be correlated to specific structural functions, such as carboxylic acidity or the aromatic  
502 character of amino acids which comprise macromolecules and recalcitrant compounds. It is a  
503 rapid, sensitive technique requiring simple sample treatment with filtration, then pH  
504 adjustment and small volumes for analysis. The most widely used methods for fluorescence  
505 spectra measurements applied to wastewater matrices are excitation emission matrices  
506 (EEMs) and synchronous fluorescence spectroscopy (SFS) [16]. An EEM is a 3D scan  
507 resulting in a contour plot of emission wavelength as a function of the excitation wavelength  
508 with fluorescence intensity in the third dimension. EEMs can provide specific fingerprint  
509 profiles for fluorescent components comprising wastewaters [86].

510 This specificity makes it possible to assign certain compounds or functions to specific  
511 Ex/Em wavelength couples, or EEM coordinates as presented in Fig. 4.

512

513 Fig. 4. presents the identification of 7 classes of recalcitrant compounds in a wastewater  
514 sample, four corresponding to proteins (B1, B2, T1 and T2), two to HS (A and C) and one  
515 corresponding to melanoidins (M). For wastewater, EEM contour plots are located in two  
516 major groups. The first is made up of protein-like peaks: peak T1 (275-285 / 320-350 nm,  
517 tryptophan-like), T2 (215-237 / 340-381 nm, tryptophan-like), B1 (275-310 / 305-320 nm,  
518 tyrosine-like) and B2 (220-237 / 305-320 nm, tyrosine-like). The second is made up of humic-  
519 like peaks: peak A (230-260 / 400-480 nm, fulvic-like), C (320-360 / 420-460 nm, humic-  
520 like) and M (290-310 / 370-420 nm). Protein-like peaks T1 and T2 tend to be predominant in  
521 urban wastewater due to microbial activity and the accumulation of biodegradable organic  
522 matter [87]. Humic-like peaks (A and C) are usually the most abundant in natural water  
523 samples due to the presence of FA and HA of microbial origin. Indices are calculated from the  
524 EEM spectra: the fluorescence index (FI), that is the ratio of emission intensity at 450/500 nm  
525 measured at an excitation wavelength of 370 nm [88], and the humification index (HIX)  
526 determined as the ratio of fluorescence intensities for two integrated regions of the emissions  
527 scans: Em 435-480 nm divided by Em 300-345 nm, at Ex 254 nm [89]. These indices are used  
528 for comparative studies of effluents, depending on the agricultural, urban, or industrial nature  
529 of the organic matter's origins. Biodegradability predictions are notably made using EEM  
530 analyses performed in one hour instead of the conventional biodegradability tests that can take  
531 between 5 and 30 days [90,91]. Melanoidins formed during thermal sewage sludge processes  
532 have been studied using EEM in the literature and the related Em/Ex couples are given in  
533 Table 5.

534

535 Table 5 – Ex/Em wavelength associated to melanoidins recalcitrant compounds.

Matrix	Peak 1 (nm)	Peak 2 (nm)	Reference
Synthetic melanoidin	360/440	230/440	[21]

Thermally treated sewage sludge	330/410	230/400	[21]
Thermally treated sewage sludge	320/400	280/350	[34]
Thermally treated sewage sludge	360/425		[92]
Thermally treated sewage sludge	320/420		[93]

---

536

537 SFS is performed on water samples to produce 2D graphs obtained by scanning both  
538 the emission and excitation monochromators simultaneously at a constant wavelength  
539 interval. The development of SFS was devoted to the analysis of specific micro-pollutants  
540 such as polycyclic aromatic hydrocarbons [94]. Absorption and excitation mechanisms are  
541 followed by electron relaxation to basic energy levels, with the emission of a photon-termed  
542 fluorescence [95]. The specific excitation/emission wavelengths are characteristic of  
543 particular molecular conformations and peak intensity is proportional to the concentration of  
544 the fluorophore in solution. The precise excitation/emission signature depends on the  
545 chemical structure and composition of wastewater samples, but matrix effects are important,  
546 as well as pH and the temperature of the samples.

547 Compared to UV absorbance, fluorescence is more specific and some functions or  
548 types of compounds can be measured at specific Ex/Em wavelength couples. Fluorescence  
549 sensitivity is increased 10 to 1,000-fold over that of UV absorption spectroscopy. The last  
550 advantage of fluorescence is its potential for discriminating NOM and rDOM, which absorb at  
551 similar wavelengths but emit at specific values [87].

552

#### 553 2.3.4 Fourier transform infrared spectroscopy (FT-IR)

554 FT-IR has been applied for NOM quantification and characterization, in wastewater,  
555 for monitoring the sludge, or the composition of WWTP influents. This analytical method is  
556 based on the determination of spectra of the samples in the infrared wavelength range. The

557 results are used to determine specific absorption bands for the identification of chemical  
 558 functions and structures [96]. The samples must be completely dehydrated by heating (105 °C  
 559 for 6 h) or freeze-drying to remove residual water before any measurement. This sample  
 560 preparation protocol is incompatible with labile organic compounds. By analyzing for specific  
 561 standards, sensitive methods are used to compare the intensity of absorption bands and  
 562 specific spectra transformations to obtain a semi-quantitative analysis of NOM [97]. Spectral  
 563 databases were reported for characteristic absorption bands of substances that most occur in  
 564 wastewater [98]. Table 6 summarizes the main chemical structures and functions identified in  
 565 sludge and wastewater, including reactive proteins, sugars, polysaccharides and amines,  
 566 which induce melanoidins during thermal treatment.

567 Table 6. FT-IR Library for analysis of the activated sludge composition, from [97], copyright  
 568 Elsevier.

<b>Wavenumber (cm<sup>-1</sup>)</b>	<b>Shape</b>	<b>Intensity</b>	<b>Bond</b>	<b>Compound(s)</b>	<b>Class</b>
3650 - 3200	b	str	st O-H	Glycerol	Polyalcohol
	b	m		Glucose	Monosaccharide
	b	m		Lactose	Saccharide
	b	m		Sucrose	Saccharide
	b	m		Phenol	Phenols
3300 -2800	b	m	st NH <sub>3</sub> (N <sup>+</sup> -H)	Albumin	Protein
	b	m		Ammonium sulfate	Ammonium
	b	m		Dry meat extract	Peptide

	b	m		Peptone G	Peptide
3000 -2840	s (m)	str	st C-H	Glycerol	Polyalcohol
	s (m)	m		Glucose	Monosaccharide
	s (m)	m		Oleic acid	Fats
	s (m)	w		Sucrose	Saccharide
2900	s	w		Lactose	Saccharide
2360	s	w	st NH <sup>+</sup>	Ammonium sulfate	Ammonium
2075	s	str	C≡N	Potassium cyanide	Cyanides
2045	s	str	SC≡N	Potassium thiocyanate	Thiocyanides
1775	s	w	st C=O	Sodium carbonate	Carbonates
1710	s	str		Oleic acid	Fats
1680	s	str	st C=C	Phenol	Phenols
1655	s	str	st as COO <sup>-</sup>	Albumin	Protein
1650 - 1575	s (m)	str		Dry meat extract	Peptide
1600	s	str		Peptone G	Peptide
1570	s	w	def NH <sub>2</sub>	Ammonium sulfate	Ammonium
1535	s	str	sy NH <sub>3</sub> <sup>+</sup>	Albumin	Protein
	s	str		Peptone G	Peptide
1515	s	str		Dry meat extract	Peptide

1460	s	str	st C=O	Sodium carbonate	Carbonates
	s	str		Acid sodium carbonate	Carbonates
1445 -1600	s	m	def C-H (skelet)	Glucose	Monosaccharide
	s	m		Lactose	Saccharide
	s	m		Sucrose	Saccharide
1440	s	m	def CH <sub>2</sub>	Oleic acid	Fats
1410	s	str	CH <sub>2</sub>	Glycerol	Polyalcohol
1400	s	str	S(=O) <sub>2</sub> (SO <sub>4</sub> )	Ammonium sulfate	Ammonium
1400	s	str	def NH <sub>3</sub>	Dry meat extract	Peptide
1380	s	str	N=O	Sodium nitrate	Nitrate
1270	s	s	st NO <sub>2</sub>	Sodium nitrite	Nitrite
1255	s	m	def NH <sub>3</sub>	Peptone G	Peptide
1245	s	m		Albumin	Protein
1235	s	m	def c=O	Oleic acid	Fats
1135	s	s	st S=O (R-SO- OR)	Sodium sulfate	Sulfate
	s	s		Sodium sulfite	Sulfite
	s	s		Sodium thiosulfate	Thiosulfate
1100-1040	s (m)	s	st C-O	Glycerol	Polyalcohol



1100	s	s	st Si-O	Sodium silicate	Silicate
1100-1000	s (m)	s	st CO	Lactose	Saccharide
	s (m)	s		Sucrose	Saccharide
1075	s	w	S=O	Ammonium sulfate	Ammonium
1015	s	s	C-O (CH <sub>2</sub> -OH)	Glucose	Monosaccharide

*b* broad band, *s* sharp band, *s (m)* multiple sharp band, *str* strong intensity band, *m* medium intensity band, *w* weak intensity band, *st* stretching vibration, *st* as stretching asymmetric vibration, *def* deforming vibration, *sy* symmetrical vibration, *skelet* skeletal vibration.

569

570 The study of activated sludge before and after thermal treatment by FT-IR helps to  
 571 identify the origin of the recalcitrant compounds. The main limitations of the methodology  
 572 are the need for samples dehydration, the impossibility of studying labile compounds, and  
 573 bands overlapping observed for these complex matrices.

574

### 575 3. Advances and trends in chromatographic and NMR approaches

#### 576 3.1 Sample treatment

577 The complexity of the composition of the matrices from surface water, wastewater,  
 578 and samples from the various steps of the thermal treatment of sludge led to the development  
 579 of particle sample treatments. The bulk DOM accumulating in water-soluble compounds is a  
 580 heterogeneous mixture of HS, hydrophilic acids, proteins, lipids, polysaccharides, carboxylic  
 581 acids, amino acids, chemicals and complex aggregates that vary in size, shape, composition  
 582 and physicochemical properties. This complexity requires consideration of the heterogeneity

583 of the samples that induce variance related to the scale and physical properties of the matrices  
584 (colloid concentration, viscosity, depth of sampling) [99]. Extraction and pre-concentration of  
585 all the dissolved organic species are critical to ensuring precise characterization and  
586 quantification. Water matrices can be fractionated using resin adsorbents in six classes:  
587 aquatic HS, hydrophobic acid (HoA), hydrophobic bases (HoB), hydrophobic neutrals (HoN),  
588 hydrophilic acids (HiA), hydrophilic bases (HiB), and hydrophilic neutrals (HiN), as  
589 presented in Fig. 5. HoA are represented by humic and fluvic acids; HoB aromatic amines;  
590 HoN hydrocarbons, pesticides and carbonyl compounds; HiA sugars acids, fatty acids and  
591 hydroxyl acids; HiB proteins and amino sugars, HiN oligo and polysaccharides.

592         After filtration and the elution steps on three cation and anion resin columns, the  
593 distribution of these six classes were quantified by UV absorbance at 285 nm and dissolved  
594 organic carbon (DOC), and varied substantially depending on the kind of wastewater and the  
595 type of treatment. The ratio calculated with UV absorbance vs DOC was also investigated on  
596 each sample fraction and provided recalcitrant compound identification [100]. Amberlite  
597 XAD resins, which are porous, spherical, macroreticular polystyrene polymers, are the most  
598 commonly used sorbents, which can separate NOM into hydrophobic and hydrophilic  
599 fractions [101,102]. Recalcitrant organic matter present in surface water can be determined  
600 after this total DOM sample preparation procedure and is separated into aromatic or aliphatic  
601 species and fractions [103]. The specific fractionation technique has been improved and  
602 adapted to characterize HS in soils and their water-soluble fractions [104] by combining  
603 liquid-liquid extractions (LLE), using several organic solvents and water, as well as size  
604 exclusion preparative chromatography (prep HPSEC) on a Biosep SEC-2000 column. The  
605 fractions obtained, containing specific polar and molar mass species, were then characterized  
606 by several GC/MS and NMR techniques for humeomic structural studies. Prep HPSEC is a  
607 chromatographic method used in water sample preparation to separate NOM molecules

608 according to their size, molecular weight and hydrodynamic volume [105]. The development  
609 of high-performance SEC columns allowed the generalized development of prep HPSEC for  
610 fractionation during sample treatment steps for the study of NOM and recalcitrant compounds  
611 [106]. The use of solid phase extraction (SPE) cartridges prior to reversed-phase analysis,  
612 enabling the NOM to be split using different organic solvents of decreasing polarity. This  
613 technique has been used to isolate nitrosamine precursors, which are present in wastewater.  
614 The use of serial SPE supports improves the resolution of specific toxicity and recalcitrant  
615 compound fractions in water samples [107]. The polarity rapid assessment method (PRAM) is  
616 a development dedicated to the study of NOM according to the polarity of the species. The  
617 protocols consist of sampling different SPE cartridges before and after, using different organic  
618 solvents. The selectivity of the SPE cartridges, as well as their porosity, are decisive for  
619 splitting. PRAM allows elution of the same species at the same steps, without any matrix  
620 variations (pH, ionic force, composition) of the wastewater samples [108]. It is possible to  
621 split NOM and rDOM by membrane filtration, with microfiltration, ultrafiltration, and  
622 nanofiltration processes with different molecular weight cutoffs of 0.5 K, 1 K, 3 K, 10 K, 30  
623 K, and 100 K [109]. Fundamentals studies of the steric and electrostatic interactions between  
624 NOM and membrane materials show that NOM is very complex in structure [79]. The split  
625 process induces molecular volume and charges of the analytes, then pore size and charges of  
626 the membrane. Membrane filtration is very effective for NOM fractionation based on  
627 molecular structure and size, the main obstacle with this method is membrane fouling and the  
628 matrix effect for concentrated samples [110].

629         Field-flow fractionation (FFF) and Flow field-flow fractionation (FIFFF) are used to  
630 separate the NOM constituents by size distribution. In the FFF system, a field is applied to a  
631 water sample, perpendicular to the direction of the flow pumped into a fine canal with a long  
632 length, resulting in the separation of the macromolecular species. The FIFFF system consists

633 of a separation cell with an ultrafiltration membrane on the bottom. The pore size of the  
634 membrane represents the lower-cutoff of the macromolecular analytes. A perpendicular field  
635 is applied to provide retention of the analyte, due to the diffusion variation linked to the size  
636 distribution [111].

637         The pH control of the samples and the concentration of divalent and trivalent metal  
638 ions are necessary to guarantee the DOC physicochemical measurements and fluorescence  
639 spectra [112]. It is often necessary to adjust the pH of all the samples either to that of the  
640 mobile phase used in the analytical steps, or to set it at an acid value, generally between pH =  
641 6 and pH = 4, by HCl additions. Removal of metal ions can be done by adding chelating agent  
642 or cation exchange resin (chelex), then filtration. Molecular complexity affects some  
643 analytical techniques when the detection limit by species is reached and requires  
644 concentrating fractions or samples. The most commonly used concentration techniques are  
645 pressure-reducing evaporation, freeze-drying, and more rarely electrodialysis and reverse  
646 osmosis. Evaporation and freeze-drying techniques require the least amount of samples, but  
647 may lose volatile compounds. Reverse osmosis is the technique that eliminates all insoluble  
648 ions and particles and respect all organic species but requires a large amount of sample.  
649 Electrodialysis eliminates ions and concentrates organic matter, but the cost of use is high  
650 [113]. Sample preparation for highly heterogeneous wastewater matrices, especially after heat  
651 treatment, must take into account changes in viscosity and foam formation. These  
652 mechanisms, characteristic of the amphiphilic macromolecular species, synthesized by  
653 thermal means, can alter the composition of fractions and samples. Since amphiphilic  
654 constituents need to be isolated and determined, it is possible to quantify the foaming  
655 potential of each sample using standardized methods to control homogeneity. The addition of  
656 anti-foam is sometimes necessary [28]. The selection of a sampling method for the analysis of  
657 NOM and rDOM in wastewater before and after treatment, (thermal, oxidation) is dependent

658 on the nature of the samples and the final objectives. Classically, wastewater sampling is  
659 conducted in two directions, depending on the next analytical procedure: grab sampling and  
660 simple filtration or centrifugation for measurements over a short time period (< 5 min) or  
661 integrated sampling (filtration, fractionation, concentration, adjustments) extending over a  
662 longer period of more than one hour.

663

### 664 3.2 Reversed-phase high performance liquid chromatography (RP-HPLC) and 665 size exclusion chromatography (SEC)

666 RP-HPLC can be used for DOM analysis, depending on the hydrophobicity of the  
667 analytes. Typically, an RP-18 stationary phase is used, with an elution gradient mixture of  
668 organic modifier and buffer, to split between 2 and 5 species, depending on the samples. The  
669 pH and ionic strength of the gradient have a significant influence on the hydrophobic analytes  
670 and fraction measurements [114]. RP-HPLC makes it possible to isolate micro-pollutants in  
671 wastewater, but it does not discriminate between macromolecules, proteins and  
672 polysaccharides.

673 Reversed-phase specific retention mechanisms are used for the fractionation of  
674 specific DOM macromolecules based on structural properties, such as the CH<sub>4</sub> vs O ratio.  
675 After optimizing the elution gradient, a correlation can be found between the retention time of  
676 HS, its C/O ratio, and its molar mass. It is also possible to split HS species according to their  
677 degree of unsaturation. The major interest in the use of reversed phases in HS separation is the  
678 possibility of structurally separating different isobaric macromolecular species [115].

679

680 SEC is one of the most widely used chromatographic techniques for the investigation  
681 of wastewater samples. The NOM, DOM and recalcitrant compounds in these samples are  
682 composed of polymorphous and polyfunctional organic. Molecular structures are complex,

683 with a wide range of molecular sizes and functions and the result is a high variance in the  
684 physicochemical properties of the solutes studied, with difficulties in developing  
685 chromatographic methods with direct stationary phases or RP-HPLC, and retention  
686 mechanisms related to hydrophobic or polar interactions.

687 Molecular weight distribution (MWD) and size distribution are specific fundamental  
688 properties that need to be measured for DOM in order to understand the physical and  
689 chemical characteristics and the reactivity of all organic analytes, especially in wastewater.  
690 MWD and size distribution of soluble species in surface water or wastewater are key  
691 structural parameters that have been linked to specific properties such as adsorption, metal  
692 binding [116], hydrophobic organic partitioning and electrostatic effects [81]. MW is a key  
693 factor for the study of recalcitrant compounds generated by DOM reactivity, the study of the  
694 bioreactivity and biodegradation and optimization of the water treatment process. Molecules  
695 with LMW have smaller molecular volume, allowing nanopore adsorption [117].

696 The fastest technique for studying MWD and size distribution is SEC, with the use of  
697 high-performance permeation columns (HP-SEC). The development of SEC methods was  
698 carried out to model the different wastewater treatment processes to obtain a profile of  
699 samples representative of the DOM based on the properties of the species in solution [118].  
700 Four main factors may influence SEC analyses for MWD measurements. The first is related to  
701 DOM reactivity, particularly with the stationary phase and the resulting non-steric  
702 interactions, these interactions can be minimized by optimizing the type of mobile phase,  
703 ionic strength and pH to suppress charge interactions with the use of buffer eluent [119]. The  
704 second is associated with data processing of the SEC chromatograms, with the baseline  
705 definition and peak integration. Due to the complex mixture analysis, peak construction,  
706 baseline recovery and MW cutoff affect the values of average MWs [120]. When the  
707 analytical conditions have been set, the third parameter that will affect the measurements will

708 be the calibration of the SEC chromatogram. Calibration standards of the same molecular type  
709 (similar MW range and charge distribution) as the water sample must be used. When these  
710 standards are not available, as for recalcitrant compounds, the use of macromolecular  
711 mixtures including polydextrans (PDX), proteins, oligosaccharides and blue dextran can be  
712 used with LMW compounds (glycerol, acetone or methanol) for total diffusion determination  
713 [121,122]. Finally, MWD and average MW can be influenced by the detection mode, which  
714 changes the shape of the SEC chromatograms [123].

715 For a mixture of soluble molecules such as humic and fulvic substances and  
716 recalcitrant compounds, MWD is defined by the number average molecular weight ( $M_n$ ), the  
717 weight-average molecular weight ( $M_w$ ), and polydispersity ( $\rho$ ).  $M_n$  and  $M_w$  were determined  
718 using the following eq. (1) and eq. (2):

719

$$720 \quad M_n = \frac{\sum_{i=1}^n h_i}{\sum_{i=1}^n \frac{h_i}{M_i}} \quad (1)$$

$$721 \quad M_w = \frac{\sum_{i=1}^n h_i M_i}{\sum_{i=1}^n h_i} \quad (2)$$

722

723 where “ $h_i$ ” is the peak height at elution volume “ $T$ ” and  $M_i$  is the MW at elution volume  $i$ . The  
724  $M_w/M_n$  ratio gives  $\rho$ .

725 As said in section 2.2, compounds formed during thermal sewage sludge processes can  
726 be of various MW, from a few Da (ketones, small aromatics, etc.) to 100 kDa (HMW  
727 melanoidins), and each of them is more or less refractory. SEC coupled with organic carbon  
728 detection (OCD) is often developed to study which MW species are degraded or removed  
729 with various treatments such as precipitation, resins and biodegradation [83]. The specific  
730 recalcitrant compounds generated by thermal processes showed variations in  $M_w$  from 2 kDa

731 to 200 kDa depending on the reactive DOM in the wastewater input [124]. But the question  
732 arises as to the limits of measuring polydispersity by measuring the molecular size range for  
733 DOM in general, and for charged electrolytes such as HA or melanoidins. By modelling, it is  
734 possible to show that, due to the fact that a statistical linear segment can be composed of  
735 several ordinary segments, LMW compounds will consist of a limited number of statistical  
736 segments. But for HS, LMW species are not necessarily linear molecules, they can be rather  
737 irregular particles with a significant influence of functional polydispersity at this small scale.  
738 The result is a significant shift between molecular size and molecular weight. The higher the  
739 molecular weight, the lower the drift, but there is an analytical bias in the measurement of the  
740 molar mass of polymer charged with SEC [81].

741 Knowledge of the size distribution of the DOM is helpful in assessing the properties  
742 and reactivity of the analytes. LMW compounds have a lower radius and molecular volume,  
743 allowing them to interact with nanopores, excluding HMW molecules. They also have a  
744 higher diffusion coefficient that leads to faster adsorption kinetics, and are more soluble in  
745 water and can therefore be more bioavailable. Conversely, HMW compounds will be less  
746 soluble in water and will have greater free energy of adsorption. They may generate higher  
747 electrostatic fields and show higher aromaticity, leading to greater adsorption of hydrophobic  
748 compounds [117]. The size distribution of the DOM allows to link the abundance of specific  
749 fractions with their sedimentation property and origin in term of microbial biopolymers [125].  
750 For nitrogen containing compounds, bioavailability is directly related to molar mass.  
751 Degradation kinetics and bioavailability of isolated surface water DON can be linked to  
752 specific molar fractions  $> 1$  kDa and  $< 1$  kDa [126]. To take these mechanisms into account, it  
753 is possible to compare water samples by integrating a greater number chromatographic  
754 descriptors in addition to the size distribution, calculated from the SEC chromatograms. For  
755 HSs, 20 SEC descriptors including distribution coefficients and statistical moments that



756 describe the SEC-elution curve, allow structural discrimination for surface water sample  
757 comparisons [127]. To correlate the size distribution, specifically with carbon and molecular  
758 nitrogen, selective OCD and OND (organic nitrogen detector) detectors can be used.  
759 SEC/OCD/OND is a particular development for surface water and wastewater, applied to the  
760 study of DOM in order to characterize the SEC fractions by specific organic carbon OCD and  
761 organic nitrogen OND detectors [51]. SEC/OCD/OND had already been used to describe  
762 thermally treated sewage sludge [23,72,124]. As this technique separates biopolymers,  
763 hydrophobic dissolved organic carbon, HS, building blocks and LMW acids and neutrals, its  
764 use before and after biodegradation of thermally treated sludge gives information on which  
765 fraction is refractory, but also provides some quantification. Studies showed that almost 80 %  
766 of refractory matter could be found in humic-like substances (20-30 %), hydrophobic  
767 fractions (20-30 %) and LMW neutrals (20-30 %) [23,72].

768         An analysis of SEC retentions is used to develop liquid chromatography at the critical  
769 condition (LCCC). LCCC is a technique that provides easy identification of trace components  
770 in polymer systems, distribution of functionality and distribution of molecular weight of the  
771 polymers in copolymers. The critical condition corresponds to the chromatographic condition  
772 in which Gibbs free-transfer energy for this specific species of the polymer is zero. The term  
773 enthalpy and the term entropy in the Gibbs equation compensate for each other exactly. At the  
774 critical conditions of a specific neutral polymer, variable molecular weight oligomers are  
775 released at the same retention time. However, if a functionalized polymer is injected to the  
776 critical conditions of the neutral lead, a separation is obtained that is entirely due to the results  
777 of the distribution of functionality [128]. By combining LCCC and SEC, it is possible to  
778 improve analytical resolution in the DOM study of surface water, especially for HS, with  
779 LCCC providing information on functional polydispersity and SEC on the size range of each  
780 functional fraction [129].

781

782           Table 7 summarizes the stationary phases and key chromatographic parameters used in  
783 HPLC and SEC to identify the different chemical species of rDOM. The major advantage of  
784 chromatographic techniques is to introduce separation of compounds based on physico-  
785 chemical properties (hydrophobicity, molar mass). The limits of these methods appear at the  
786 validation stage and the absence of standardization in the use of stationary phases, solvents  
787 and buffer solutions.

Table 7. Stationary phase geometry, porosity and particle size, and analytical conditions for DOM and recalcitrant compound analysis in various matrices and industries.

Matrix/Industry	Column, geometry, porosity, particle size	Eluent	Detection	Compounds identified	Reference
Humic acids, soil and surface water, Water treatment	Asahipak GF 310 HQ 2x250 mm, 400 Å, 5 µm (Keystone Scientific)	ACN: water, acetate and phosphate buffer	UV: 200 nm	Humic acids	[129]
Glucose-Lysine model, food	TSK G3000 7.5x600 mm, 250 Å, 5 µm (Tosoh) YMC-Pack ODS-5-A 4.6x250 mm, 120 Å, 5 µm (YMC , GmbH)	0,05 M phosphate buffer, pH 7.2 ACN: water	UV : 280 ; 450 nm PDA	Melanoidin 1 spc. Maillard intermediates, 2 spcs.	[130]
Surface water, Water treatment	Nucleosil 100-3 C18 HD 100x2.1 mm, 100 Å, 3 µm	Water: MeOH (5 mM ammonium acetate) gradient	ESI-HRMS	Phenazone, byproducts	[131]

Instant coffee, food	PL aquagel-OH MIXED 7.5x300 mm, Macroporous, 8 µm (PL Laboratory)	0.1 M NaNO <sub>3</sub>	RI	Melanoidins 6 spcs	[132]
green coffee beans model, food	Hypersil Gold RP C18 100x2.1 mm, 175 Å, 1.9 µm (Thermo Scientific)	Water: ACN (0.1 % formic acid) gradient	PDA-ESI-MS	Chlorogenic acids, Chlorogenic acid–arabinose hybrids	[133]
Surface water model with HS, Water treatment	Gemini C18 50x4.60 mm, 110 Å, 3 µm (Phenomenex)	ACN: water, MeOH: water, modifiers	PDA	Sulfamerazine, byproducts,	[134]
Wastewater	LiChrosorb RP-18 125x4 mm, 100 Å, 5µm	MeOH: water (0.014 M oxalic acid)	UV: 270 nm	Trimethoprim	[135]
Instant coffee, food	Purospher STAR RP-8 EC 150x4.6 mm, 120 Å, 3 µm (Merck)	ACN: water (0.05 mM per-fluorooctanoic acid)	ESI-MS/MS UV: 280 nm	Acrylamide, Hydroxymethyl furfural, Antioxidants, melanoidins (after ultrafiltration)	[136]
Wastewater	Zorbax Bonus RP C18 2.1x150	Water: ACN (0.1%	UV : 285 nm	Dinitrobenzene for DOM	[80]

	mm, 80 Å, 5 µm (Agilent)	formic acid)		sorption characterization	
Surface water	Kinetex XB-C18 2.1x100 mm,	Water: MeOH (0.1%	ESI-MS/MS	Micropollutants, 39 spcs,	[137]
downstream industrial areas and urban WWTPs	100 Å, 1.7 µm (Phenomenex)	formic acid), gradient		correlation with EEMs	
Cocoa beans, Food	Accucore C18 3.0x150 mm, 80 Å, 2.6 µm Thermo Fisher	Water: ACN (0.1% formic acid), gradient	UV: 280, 320 nm ESI-HRMS	Soluble and bound to melanoidins phenolic compounds, 15 spcs	[138]

### 789 3.3 Hyphenated techniques

790 Mass spectrometry associated with GC or LC is generally the preferred technology for  
791 investigating DOM because it has high recognition potential. Hyphenated methods covering  
792 spectroscopic and spectrometric techniques for the analysis of DOM can follow different  
793 strategies: scan targets, suspect objects, and non-targets as presented in Fig. 6, using mass  
794 spectrometry to identify targeted or unknown molecules linked to a specific risk.

795 In the case of DOM, which needs to be comprehensively studied, analytes cannot be  
796 studied with reference to commercial standards. The important structural variability will  
797 require specific developments (sample preparation, fractionation, detection modes) to  
798 establish resolute analytical profiles with the aim of replacing reference standards with  
799 surrogate parameters.

800 The combination of SEC and PDA detection online is the most commonly used  
801 method for DOM spectra analysis.  $SUVA_{254}$  provides robust detection of aromatic structures,  
802 but cannot discriminate different classes, such as HS, proteins or melanoidins. Specific  
803 wavelengths can be analyzed for each chromatographic peak to correlate water quality and  
804 SEC profiles. DOM with LMW ( $< 0.5$  kDa) shows a peak area measured at 210 nm linked to  
805 oxidized nitrogen concentrations and the concentrations of active bacteria in drinking water  
806 [140]. UV spectral analyses in line with SEC are simple and fast to develop for qualitative  
807 comparisons. To further the structural analyses, online fluorescence detection will improve  
808 identification scores. In addition to humic and fulvic-like fluorescent compounds, a large  
809 variety of protein-like compounds can be monitored, linked to MWD by their specific  
810 tyrosine and tryptophan-like fluorescence. Hyphenated SEC/PDA/Fluorescence techniques  
811 are used for the characterization of specific fractions for a complete surface water analysis as  
812 shown in Fig. 7.

813           The specific protein fluorescence properties are related to the fact that of the 20 natural  
814 amino acids, only 3 can emit fluorescence after excitation at a characteristic UV wavelength:  
815 phenylalanine, tyrosine and tryptophan. Since these 3 amino acids are not frequent in natural  
816 proteins, each of them produces a characteristic spectrum [95]. Phenylalanine has the lowest  
817 wavelengths for excitation and emission, often incompatible with the solvents and buffers  
818 used in SEC. Tryptophan has the highest quantum yield, and its fluorescence properties are  
819 influenced by the environment (polarity, pH solvent type). For water and wastewater analysis,  
820 tyrosine-like and tryptophan-like proteins are determined by selecting the specific  
821 excitation/emission wavelengths of tyrosine (220/310 nm) or tryptophan (270/355 nm). Other  
822 biopolymers can emit specific fluorescence, such as HA and FA (330/425 nm and 390/500  
823 nm) [141]. The aromaticity of each SEC fraction can be quantified: The absorbance ratio at  
824 210 nm vs 254 nm provided surrogate quantification of the aromatic character and  
825 hydrophobic/hydrophilic properties of each MW species [142]. From these considerations,  
826 reading Fig. 7 shows that the study of 7 different classes of samples – raw water and 6  
827 samples from various treatments – is possible from the analysis of 6 SEC fractions. The left  
828 column shows the chromatograms obtained for each of the 4 specific Em/Ex pairs of tyrosine-  
829 like and tryptophan-like proteins, as well as UV absorption at 210 and 254 nm. The results  
830 show that the ozonation process studied in the publication produces a poor decreases the  
831 aromaticity of 6 fractions. HA and FA are indeed recalcitrant compounds to this process,  
832 since the areas of the 6 SEC fractions are almost identical before and after treatment, while  
833 some tryptophan like proteins are degraded.

834

### 835 3.4 High-resolution mass spectrometry

836

837 HRMS is used to improve detection resolution for the identification of low  
838 concentrations of each of the soluble species isolated by a chromatographic technique. In  
839 particular, the non-targeted studies presented Fig. 7 will involve basic analyses to identify the  
840 structures. The complexity of the structures entails using different approaches to separate and  
841 extract HRMS data. For the study of LMW compounds, RP18 separation is often sufficient.  
842 The state of charge of the species and possible adducts should be taken into account. The  
843 presence of many mineral ions in solution requires searching for multiple adducts  $[M+H]^+$ ,  
844  $[M+Na]^+$ ,  $[M+K]^+$ ,  $[M+NH_4]^+$ , for monocharged species in ESI+ or  $[M-H]^-$ ,  $[M+Cl]^-$ ,  $[M+Br]^-$   
845 in ESI- mode. Wastewater analysis also shows complex species with divalent cations or more.  
846 In reversed phase, HRMS hyphenation, using a simple aqueous eluent with an acid modifier  
847 (formic acid), can be used to search for only protonated or deprotonated species with a simple  
848 charge state of +1 or -1 [143]. LC-HRMS analysis of monoisotopic signals allows authors to  
849 identify 2409 molecular features that provide probabilities of raw formulas of chemical  
850 species by structural database comparison. The highest mass accuracy allows formulas to be  
851 assigned with an error < 5 ppm. Compared to conventional COD, this method allows  
852 molecular-scale monitoring for MW < 1 kDa. Hydrothermal liquefaction of wastewater from  
853 sewage sludge shows the diversity of DOM, identified by an elemental analysis of HRMS  
854 data [23,63]. Non-targeted HRMS studies allow structural analysis of DOM, with a specific  
855 monitoring of the different chemical species, according to the steps of the treatment process.  
856 More than 2,400 species identified by their empirical formulas, can be tracked to identify  
857 compounds that are recalcitrant to biological treatment processes. The resolution of HRMS  
858 systems, coupled with SEC or RP-HPLC systems, does not allow direct study of compounds  
859 with MW > 1000 Da [143]. In targeted studies, more than 111,000 compounds potentially  
860 present in the wastewater DOM can be investigated [144]. The majority of species found in  
861 wastewater contained C, H, O elements accounting for 38–43 % of species detected in ESI-



862 mode, and 53-83 % of species detected in ESI+ mode. Species containing C, H, O, N, P, S,  
863 and Cl were essentially found in ESI-, those with C, H, O, and N in ESI+. Very few  
864 compounds with P, S, and Cl were detected in ESI+ [145]. Elemental analyses are used to  
865 calculate specific indices to characterize the DOM [146]. It is possible to determine the H/C,  
866 N/C, O/C ratios for all identified species and to graphically represent these ratios in a Van  
867 Krevelen plot, which gives resolution mapping as presented Fig. 8.

868         These ratios can be determined for certain combinations of elements: CHO, CHON,  
869 CHONS, including halogen. H/C vs O/C ratios identify the main chemical families found in  
870 wastewater: 1 - lipids, 2 - proteins, 3 - amino sugars, 4 - carbohydrates, 5 - condensed  
871 hydrocarbons, 6 - lignin, and 7 - tannin (Fig. 8). For each chemical family, it is possible to  
872 follow the rDOM species that are always present after a treatment process. Fig. 8 also shows  
873 that recalcitrant compounds are present in all chemical classes, but that some structures in  
874 each class are less recalcitrant, especially when the H/C ratio is close to 2. The double bond  
875 equivalent (DBE) measurements reflect the level of unsaturated compounds in wastewater.  
876 With this classification, it is possible to monitor the specific organic nitrogen in wastewater  
877 effluents and to identify the consumed, produced and recalcitrant compounds at the molecular  
878 level, for a reactivity characterization of the different effluents [147,148]. The reactivity of  
879 DOM species can also be linked to the formation of recalcitrant by-products, such as  
880 nitrosodimethylamine, which is promoted by the presence of species identified by HRMS  
881 with high hydrogen saturation ( $H/C > 1.5$ ) [149]. To model the aromaticity of DOM, it is  
882 possible to calculate a DBE minus oxygen (DBE-O) index in order to discriminate species  
883 with carboxylic function (rich oxygen species) and the ratio between DBE and carbon number  
884 (DBE/C), which gives a more accurate picture of the unsaturated carbonated skeleton of  
885 macromolecules. DBE estimates the unsaturation of DOM for all class of compounds, that is a  
886 major advance compared to  $SUVA_{254}$ , which only applies to UV-active molecules

887 [150,151,152]. Significant progress is made in the comparative study of wastewater, by  
888 identifying the predominant species. In particular, among the raw DOM, HRMS allows to  
889 isolate the characteristic signals of a molecular signature. In the study of a treatment process,  
890 the authors assigned 4  $m/z$  signals 297.1530, 311.1686, 325.1843, 339.1999 which have been  
891 identified as anthropogenic alkyl benzene sulfonates [150,151]. The resolution of HRMS  
892 systems, which has been constantly growing for more than 20 years, enables us to detect  
893 different exact masses for around ten compounds of the same molar mass, with MW < 1000  
894 Da, separated in the same chromatogram peak. Ultra high resolutions are needed to resolve  
895 more eluted compounds with small MW or polymers. For MW < 1000 Da compounds, it is  
896 possible to calculate the Kendrick mass defect (KMD) of each identified species by dividing  
897 the nominal molar mass by the exact mass of a selected molecular fragment ( $\text{CH}_2$ ,  $\text{C}_2\text{H}_4\text{O}$ ,  
898 etc.) [148,146]. Compounds with the same number of heteroatoms, rings and double bonds,  
899 but different numbers of  $\text{CH}_2$  groups, will have identical KMD.  
900 Homologous structure classification is then possible, as shown in Fig 9.

901 Fig. 9 shows the identification of 31 homologs with  $\text{CH}_2$  and 38 with  $\text{C}_2\text{H}_4\text{O}$  in and  
902 out of treatment process, all of them were retained having KMD error < 5 ppm. Each graph  
903 shows the Kendrick mass defect as a function of nominal Kendrick mass for all ions observed.  
904 The homolog species, for example the members of the  $\text{CH}_2$  series, have the same Kendrick  
905 mass defect but different nominal Kendrick mass and are identified along the horizontal lines  
906 on the graph.

907 Monitoring water samples for screening for known compounds and emerging  
908 pollutants uses hyphenated methods with LC or GC and HRMS, with a search for the exact  
909 masses of parent ions, precursors or degradation products identified in commercial or home-  
910 made databases. In this study, 33 pharmaceuticals and 113 of their known transformation  
911 products were identified and quantified, with a limit of quantification in order of  $\text{ng}\cdot\text{L}^{-1}$  in

912 wastewaters. The main advance was obtained by differentiating between isobaric structural  
913 isomers [153]. Recently, the characterization of DOM at the chemical formula level was  
914 achieved by using ion mobility and FT-ICR tandem mass spectrometry. The orthogonal use of  
915 HRMS and MS/MS is capable to isolate species with  $m/z$  difference of 0.036 [154]. For  
916 general DOM studies, the sample preparation is limited to filtration. For certain classes of  
917 analytes, such as emerging contaminants and pharmaceuticals, which are also recalcitrant  
918 compounds, pH adjustments and SPE extraction for concentration and matrix simplification  
919 are required [155]. Multi-residue HRMS analyses and quantification are possible for  
920 wastewater study, with relative standard deviation  $< 20\%$  [156], which allow to develop new  
921 tools for evaluating molecular-scale treatment processes [157]. If the research involves the  
922 identification of enantiomers, enantioselective separations can be developed, particularly in  
923 supercritical mode, with tandem mass spectrometry for wastewater matrices. The interest of  
924 SFC-MS is found in fast gradients ( $< 10$  min). The method shows significant performance in  
925 this wastewater matrix, with a linearity of calibrations in a wide range ( $0.005$  to  $1000\text{ mg. L}^{-1}$   
926 in ESI+ and up to  $8000\text{ mg. L}^{-1}$  for ESI-), a recovery rate  $> 70\%$  and a relative standard  
927 deviation  $< 20\%$  [158]. Some antibiotics, such as macrolides, and in particular azithromycin,  
928 have been identified as recalcitrant compounds after the various stages of wastewater  
929 treatment. Identification was conducted using reverse-phase liquid chromatography with an  
930 ESI-ion trap detector in MS/MS mode [159]. Some UV absorbers used in cosmetics, personal  
931 care products and paints are found in wastewater and are also recalcitrant compounds, such as  
932 benzophenone derivatives, identified by reversed-phase liquid chromatography and tandem  
933 mass spectrometry [160]. For an overall characterization of the refractory organic matter, an  
934 initial ultrafiltration fractionation technique can be combined with a UV absorption or  
935 fluorescence analysis on each fraction, followed by LC/MS and GC/MS analyses. This  
936 identifies volatile compounds by extraction using a non-polar organic solvent and of those

937 which are more polar, and water-soluble. In this case, specific extraction methods are needed,  
938 with long evaporation steps and recovery with compatible solvents [161]. Maillard precursors  
939 and LMW reaction products are usually identified by reversed-phase liquid chromatography  
940 with HRMS. The identification of known reducing sugars and amino acids can be achieved by  
941 elemental analysis, with a high accuracy ( $< 4$  ppm). For the identification of new Maillard  
942 precursors, reaction models with a mixture of different sugars and amino compounds are  
943 prepared. After reaction, the analysis of all the identified compounds with tandem mass  
944 spectrometry can produce identified reaction products by fragmentation [162]. Maillard  
945 reaction products and Amadori compounds, which are formed during the first steps of the  
946 Maillard reaction, could be analysed by reversed-phase liquid chromatography with mass  
947 spectrometry or tandem mass spectrometry, but the main problem is quantification in complex  
948 matrices, given the lack of analytical and isotopic standards [48]. Melanoidins have been  
949 extensively studied in the food industry. By combining SEC for splitting by MW, then  
950 analyzing each fraction in reversed-phase liquid chromatography coupled with mass  
951 spectrometry, it was possible to identify melanoidins with very HMW ( $> 200$  kDa) and to  
952 study the oligosaccharide moieties of each fraction. With this 2D technique, it is possible to  
953 identify the  $m/z$  signal of the hexose unit (162 Da) and the different degrees of  
954 oligosaccharide polymerization (DP), from DP2 ( $m/z$ : 341 Da) to DP4 ( $m/z$ : 665 Da) [163].  
955 Melanoidins are negatively-charged complex polymeric structures of various molecular  
956 weights, determined by various chromatographic and HRMS techniques [32,71]. The analysis  
957 of melanoidin degradation products after hydrolysis, using high-performance anion-exchange  
958 chromatography with pulsed amperometric detection (HPAEC-PAD) or high-performance  
959 thin layer chromatography (HPTLC), provides structural information. By increasing the  
960 degree of polymerization of carbohydrates used as reducing sugars in the Maillard reaction,  
961 the release of monosaccharides increased from about 3 % to 95 %, depending on the

962 carbohydrate [31,164]. The study of SEC fractions of melanoidins by pyrolysis/GC/MS  
963 provides identification of products. The origin of nitrogen-containing polymers can be  
964 assigned to specific Amadori intermediates, as presented in Table S1 (supplementary data).  
965 All accurate masses are understood to exact masses to identify raw formulas of the  
966 intermediates [52]. The characterization of DOM in solution, generated in particular during  
967 thermal treatments, can be achieved by ESI Fourier-transform ion cyclotron resonance mass  
968 spectrometry (ESI-FT-ICR MS), coupled with SEC fractionation [23]. The very high  
969 resolution of these detectors has been widely used to elucidate complex petroleum oil  
970 structures. In the case of wastewater, the DOM can be characterized in a single analysis and  
971 compounds classified according to structural criteria: composition in heteroatoms and notably  
972 nitrogen, DBE, carbon number in the structure and all ratios related to these elementary  
973 analyses. Depending on the species sought, it is possible to search for specific classes, as  
974 shown in Fig. 10, presenting the preheat treatment and post-heat treatment fraction study, with  
975 the production of HMW conjugate species after treatment. It should be noted that this  
976 technique only identifies ionized species by the ESI probe and that species without ionisable  
977 functions will not be identified. Quantification is only possible by using isotopic standards of  
978 the species studied, in the absence of these standards, comparison of samples is done by  
979 analysing relative abundances.

980 Fig. 10 clearly shows the increase in the relative abundance of all classes of nitrogen-  
981 containing compounds after thermal treatment of sludge, observed by an increase in intensity  
982 (red color) on the graph. There is also an increase in the number of carbon and DBE, showing  
983 that higher molecular weight species are generated, and more conjugated (DBE shifted to  
984 higher values).

985 Table 8 summarizes the stationary phases and eluent used in hyphenated methods for  
986 DOM characterization. SEC and reversed-phase columns were largely used for the separation

987 and elemental analysis of LMW and HMW DOM and recalcitrant compoundsUV  
988 spectrometry and fluorescence detection, widely used to characterize rDOM, do not allow  
989 structural identification and, in particular, do not allow the study of specific species such as  
990 nitrogen containing compounds. HRMS is widely used in global DOM studies and molecular-  
991 scale characterization of rDOM. The elemental analysis, possible by HRMS, allows to  
992 identify the molecular structures that are related to refractoriness. Important advances are  
993 possible for targeted and non-targeted monitoring, allowing an optimization of wastewater  
994 treatment processes. But HRMS studies are only possible, for the majority of detectors  
995 currently available, for molecules of MW <1000 Da. This limitation explains the low number  
996 of studies available for melanoidins. For these macromolecules, advances were obtained by  
997 ESI-FT-ICR MS. For this last technique, it is the very high molecular diversity of species that  
998 will limit the interpretation of mass spectra.

999 Table 8. Stationary phase geometry, porosity and particle size, analytical conditions and detection modes used in the different hyphenated  
 1000 methods developed for DOM and recalcitrant compound analysis in various matrices and industries.

Matrix/Industry	Column, geometry, porosity, particle size	Eluent	Detection	Identified compounds	Reference
Humic acids, soil and surface water, water treatment	Asahipak GF 310 HQ 2x250 mm, 400 Å, 5 µm (Keystone Scientific)	ACN: water, acetate and phosphate buffer	UV: 200 nm	Humic acids	[129]
Glucose-Lysine model, food	TSK G3000 7.5x600 mm, 250 Å, 5 µm (Tosoh)	0.05 M phosphate buffer, pH 7.2	UV : 280 ; 450 nm	Melanoidin 1 spc.	[130]
	YMC-Pack ODS-5-A 4.6x250 mm, 120 Å, 5 µm (YMC, GmbH)	ACN: water	PDA	Maillard intermediates, 2 spcs.	
Surface water, water treatment	Nucleosil 100-3 C18 HD 100x2.1 mm, 100 Å, 3 µm	Water: MeOH (5 mM ammonium acetate) gradient	ESI-HRMS	Phenazone, by- products	[131]
Instant coffee, food	PL aquagel-	0.1 M NaNO <sub>3</sub>	RI	Melanoidins 6 spcs	[132]

	OH MIXED 7.5x300 mm, Macroporous, 8 µm (PL Laboratory)				
green coffee bean model, food	Hypersil Gold RP C18 100x2.1 mm, 175 Å, 1.9 µm (Thermo Scientific)	Water: ACN (0.1 % formic acid) gradient	PDA-ESI-MS	Chlorogenic acids, Chlorogenic acid– arabinose hybrids	[133]
Surface water model with HS, water treatment	Gemini C18 50x4.60 mm, 110 Å, 3 µm (Phenomenex)	ACN: water, MeOH: water, modifiers	PDA	Sulfamerazine, by- products	[134]
Wastewater	LiChrosorb RP-18 125x4 mm, 100 Å, 5 µm	MeOH: water (0.014 M oxalic acid)	UV: 270 nm	Trimethoprim	[135]
Instant coffee, food	Purospher STAR RP-8 EC 150x4.6 mm, 120 Å, 3 µm (Merck)	ACN: water (0.05 mM per-fluorooctanoic acid)	ESI-MS/MS UV: 280 nm	Acrylamide, Hydroxymethyl furfural, Antioxidants, melanoidins (after ultrafiltration)	[136]



Wastewater	Zorbax Bonus RP C18 2.1×150 mm, 80 Å, 5 µm (Agilent)	Water: ACN (0.1 % formic acid)	UV: 285 nm	Dinitrobenzene for DOM sorption characterization	[80]
Surface water downstream industrial areas and urban WWTPs	Kinetex XB-C18 2.1x100 mm, 100 Å, 1.7 µm (Phenomenex)	Water: MeOH (0.1 % formic acid), gradient	ESI-MS/MS	Micropollutants, 39 spcs, correlation with EEMs	[137]
Cocoa beans, food	Accucore C18 3.0×150 mm, 80 Å, 2.6 µm Thermo Fisher	Water: ACN (0.1 % formic acid), gradient	UV: 280, 320 nm ESI-HRMS	Soluble and bound to melanoidins phenolic compounds, 15 spcs	[138]
Wastewater	Acquity UPLC BEH C18 2.1x50 mm, 130 Å 1.7 µm Waters SAS	Water: ACN (0.1 % formic acid), gradient	ESI-HRMS	2409 compounds detected, Van Krevelen plots	[143]
Wastewater	Hypersil Gold PFP, 100x2.1 mm, 175 Å, 1.9 µm Thermo Scientific	Water: ACN (0.1 % formic acid), gradient	ESI-HRMS	33 pharmaceuticals and 113 by-products	[153]

### 1001 3.5 Nuclear magnetic resonance spectroscopy (NMR)

1002 NMR approaches are not yet extensively used in the field of complex mixture analysis  
1003 in the environmental sciences and the characterisation of NOM. In 2019, a review [166]  
1004 summarized the main input of NMR over the past twenty years on the characterization of the  
1005 organic matter in water. Both liquid-state and solid-state NMR experiments can be performed  
1006 using dedicated spectrometers and probes [167–170]. NMR spectroscopy and MS, has the  
1007 advantage of providing structural information, based on elemental composition and chemical  
1008 shift, on sample content and also emphasizes the holistic characterization of a sample. NMR  
1009 can be used to observe the nuclei of elements with a nonzero spin quantum number, and  
1010 several active nuclei can be detected, the most common being protons, carbon-13, fluorine-19,  
1011 phosphorus-31 and, to a lesser extent, nitrogen-14 or-15. NMR spectroscopy is highly  
1012 reproducible, robust, and quantitative by nature since the NMR signal response i.e. the  
1013 integrated signal area, is proportional to the number of nuclei contributing to the signal [171].  
1014 The fact that NMR is a non-selective method is an advantage for the analysis of mixtures to  
1015 the extent that all the compounds present in the solution (provided they contain the nucleus  
1016 under investigation) are detected simultaneously in a single run. It is also a non-destructive  
1017 technique that can provide relevant information on the composition of the mixture from the  
1018 analysis of different nuclei and through various NMR experiments. Analyses range from one  
1019 dimensional experiments focusing on  $^1\text{H}$  [172] or, for most studies, on the  $^{13}\text{C}$  nucleus  
1020 [167,169] to homonuclear and heteronuclear 2D experiments like  $^1\text{H}$ - $^1\text{H}$  COSY or  $^1\text{H}$ - $^{13}\text{C}$   
1021 HSQC commonly used in liquid-state NMR [167]. Beyond these common nuclei, it should be  
1022 mentioned that the  $^{15}\text{N}$  nucleus was also successfully investigated for organic matter  
1023 characterization in solid-state NMR with CP-MAS (Cross Polarization Magic-Angle  
1024 Spinning) experiments and DEPT (Distortionless Enhancement by Polarization Transfer)  
1025 sequences in solutions [173,174]. Furthermore, studying the phosphorus nucleus can be useful

1026 to obtain the distribution of different phosphorus compound classes in environmental samples  
1027 [175,176]. For instance,  $^{31}\text{P}$  liquid and solid-state NMR were recently used to understand  
1028 changes in phosphorus speciation after thermal hydrolysis pre-treatment of sludge. The  
1029 analysis demonstrated that orthophosphate was the most abundant phosphate present [177].  
1030 Lastly, other specific NMR approaches based on Low-Field NMR relaxometry techniques for  
1031 probing the mobility of protons have been found useful in characterizing the water present in  
1032 biomass [178,179].

1033 To the best of our knowledge, none of the studies reported characterization of  
1034 recalcitrant compounds using NMR after a thermal degradation process. The main recent  
1035 studies involving the characterization and analysis of recalcitrant compounds, however, are  
1036 reported in Table 9. Depending on the nature of the samples, liquid- or solid-state NMR was  
1037 carried out. The presence of recalcitrant compounds in Brazilian urban wastewater, which  
1038 mainly include surfactants and phthalates, was investigated in 2015 [180] and 2017 [181]. In  
1039 these studies, both untargeted-NMR profiling ( $^1\text{H}$  and HSQC), leading to quantitative  
1040 estimation of some pollutants, and structural identification of recalcitrant compounds with  
1041 conventional 1D and 2D NMR experiments were performed. Moreover, in the 2017 study  
1042 [181], multivariate chemometric analyses from quantitative NMR data of the aromatic region  
1043 were used to monitor the degradation of organic matter and treatment performances. In 2014,  
1044 there was a report [182] on the characterization by several mono- and bi-dimensional NMR  
1045 techniques (COSY, TOCSY, DOSY) of a refractory end product of HA subjected to a  
1046 humeomics procedure i.e. previously separated in size fractions by preparative HPSEC  
1047 chromatography. In a study performed in 2019 [183] the application of both solution-state  
1048 (2D  $^1\text{H}$ - $^{13}\text{C}$  HSQC) and solid-state ( $^{13}\text{C}$ -CPMAS) NMR is reported to characterize particulate  
1049 organic matter (POM) in agricultural and food waste digesters collected from anaerobic  
1050 digestion plants in Sweden. In this study, the combination of NMR data and multivariate

1051 chemometrics was used to characterize both the labile POM fraction in solid waste (lipids,  
1052 starch-like carbohydrates) and recalcitrant POM structures (protein-like compounds, lignin,  
1053 polysaccharides). The recording of  $^{13}\text{C}$  NMR spectra with CP-MAS NMR is particularly well  
1054 suited to analyzing soil samples with minimal sample preparation. The method can be used to  
1055 assess the relative proportions of functional groups corresponding to a specific chemical shift  
1056 area i.e. alkyl ( $\delta\text{C}$ : 0-47 ppm), O-alkyl ( $\delta\text{C}$ : 47-90 ppm), anomeric ( $\delta\text{C}$ : 90-108 ppm),  
1057 aromatic ( $\delta\text{C}$ : 108-145 ppm), O,N-substituted aromatic ( $\delta\text{C}$ : 145-167 ppm), and carbonyl  
1058 groups ( $\delta\text{C}$ : 167-220 ppm). A similar approach using CP-MAS was recently used to  
1059 investigate the chemical composition of cultivated and forest soils in subtropical and  
1060 temperate regions [184]. Advanced pulse sequences were used for the characterization of  
1061 recalcitrant aromatic components in soil organic matter in order to study their changes in  
1062 relation to land use and soil management in the Mollisol region of China [185]. The  
1063 composition of recalcitrant humin from estuarine sediments was studied [186] by combining  
1064 solid-state NMR and Comprehensive Multiphase-NMR (CMP-NMR). The advantage of  
1065 CMP-NMR is its use in performing a full range of NMR experiments on the same unaltered  
1066 natural sample, without prior extraction, containing liquid-, gel- and solid-like phases. This  
1067 approach made it possible to conclude that the recalcitrant OM in estuarine sediments is  
1068 mainly composed of proteins. All the NMR experiments reported above and in Table 9 for the  
1069 characterization of recalcitrant compounds demonstrated that NMR can be used to (i) screen  
1070 samples holistically (ii) quantitate samples (iii) determine the structure of recalcitrant species  
1071 after chromatographic purification.

1072

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1076 Table 9: Summary of recent publications using NMR approaches for the investigation of recalcitrant compounds

Matrix	Recalcitrant compounds	NMR experiments	Spectrometer $^1\text{H}$ frequency, probe, temperature, specific procedure or data treatment	Reference
Liquid-state NMR				
Humic acid from volcanic soil	refractory end product of a humic acid	$^1\text{H}$ , $^1\text{H}$ DOSY, COSY, TOCSY, $^1\text{H}$ - $^{13}\text{C}$ HSQC,	- 400 MHz, 5 mm BBI probe, 298 K - water suppression with presaturation or Watergate 3-9-19	[182]
Urban wastewater	linear alkylbenzene sulfonate surfactant homologs	$^1\text{H}$ , COSY, HSQC, HMBC	- 600 MHz, 5 mm $^1\text{H}$ - $^{13}\text{C}/^{15}\text{N}$ TCI cryoprobe, 298 K - zgpcpr pulse sequence for water suppression - quantification with Eretic2 <sup>TM</sup> method	[180]
Urban wastewater	surfactants and phthalates	$^1\text{H}$ , HSQC	- 600 MHz, 5 mm $^1\text{H}$ - $^{13}\text{C}/^{15}\text{N}$ TCI cryoprobe, 298 K - zgpcpr pulse sequence for water suppression - quantification with Eretic2 <sup>TM</sup> method - multivariate analysis: PCA, HCA, PLS-DA	[181]
Agricultural and food waste	cellulose-like and protein-like	$^1\text{H}$ , HSQC	- 600 MHz, 5 mm BBO cryoprobe, 298 K - multivariate analysis: PCA, OPLS-DA	[183]

digesters substances		Solid-state NMR, CP-MAS		
Estuarine	methylene moieties	$^1\text{H}$ , $^{13}\text{C}$ , DOSY,	- 500 MHz, 4 mm prototype CMP MAS $^1\text{H}/^{13}\text{C}/^{19}\text{F}/^2\text{H}$	[186]
sediments	from protein-like substances	TOCSY, NOESY $^{13}\text{C}$ RAMP	probe, 298 K  - 500 MHz, 4 mm BB $^1\text{H}$ -X probe	
Soil organic matter	aromatic components	$^{13}\text{C}$ CP/TOSS,	- 400 MHz, 4 mm double resonance probehead	[185]
Land (Mollisol)		$^{13}\text{C}$ DP/TOSS, $^{13}\text{C}$ DP/MAS	- quantification of specific functional groups - multivariate analysis: PCA,	
Agricultural and food waste digesters	cellulose-like and protein-like substances	$^{13}\text{C}$	- 500 MHz, 4 mm MAS probe with $^1\text{H}$ decoupling - relative C distribution (% of total integrals) - multivariate analysis: PCA	[183]
Soil	carbonyl, phenolic, aromatic compounds	$^{13}\text{C}$	- 400 MHz, 4 mm standard bore probehead, 297 K - statistical analysis: Chi-square, AIC, ANOVA	[184]

1077 As described in section 2.2, melanoidins are refractory end-products of thermal  
1078 treatment applied to sewage sludge. NMR experiments had not yet been described for  
1079 characterization of melanoidins in the environmental field but were applied to follow their  
1080 formation as well as to structural studies [187,188]. In agro-food applications, solution-state  
1081 NMR was recently used to investigate the effect of roasting time on the chemical structure of  
1082 coffee melanoidins [69] and to characterize them after fractionation by SEC in dulce de leche,  
1083 a confectionary dairy product [189]. These studies, along with the bibliography dealing with  
1084 the analysis of recalcitrant compounds with NMR, illustrate the feasibility of characterization  
1085 of melanoidins from sludge samples after thermal treatment, an area that deserves further  
1086 investigation.

1087 In the field of this review, one advantage of NMR is that it requires easy sample  
1088 preparation for the analysis of raw samples. Indeed, solid samples can be analyzed after a  
1089 drying step using CP-MAS NMR. For solution-state NMR, sample preparation requires  
1090 dissolution or dilution in a deuterated solvent, commonly deuterated water for environmental  
1091 samples. The only precaution to take for both solid and liquid samples is, if necessary, the  
1092 removal of paramagnetic mineral species samples, in particular Fe-containing minerals. This  
1093 step can be essential prior to NMR spectroscopy, paramagnetic species would otherwise  
1094 induce signal loss and extensive peak broadening in the NMR spectra. It can be done through  
1095 prior acidic treatment of the sample with HCl [183] or 2% hydrofluoric acid [190] for liquid-  
1096 or solid- state, respectively. However, if NMR is to be applied for the complete structural  
1097 characterization of a recalcitrant compound, a prior isolation with a set of chromatographic  
1098 methods is required. Another key point of NMR is its ability to quantify compounds without  
1099 the need for specific standard compounds, but this advantage should be linked to the main  
1100 drawback of NMR, its intrinsic insensitivity. Currently, a limit of detection on the nanomole  
1101 level can be considered using conventional 5 mm NMR tubes on a 500 MHz solution-state

1102 spectrometer for a pure compound. Environmental samples consist of a complex mixture of  
1103 molecules, and quantification of their components is a real challenge. The quantification of  
1104 organic compounds in wastewater samples at low concentrations (micromolar and, in some  
1105 cases, nanomolar) has been reported, but this result can only be achieved through prior pre-  
1106 concentration by HPLC-(UV/MS)-SPE-NMR with automatic sampling storage [180]. This  
1107 example illustrates that, for the characterization of organic matter and recalcitrant compounds,  
1108 the power of NMR will be increased if the method is used as an orthogonal and  
1109 complementary method with a chromatographic approach. In the coming years, recent  
1110 sensitivity improvements based on higher magnetic fields, better probes, advanced pulse  
1111 sequences and hyperpolarization [191] may significantly reinforce the role of NMR in these  
1112 applications. The combined approach of NMR with the aforementioned chromatographic  
1113 and/or spectroscopic methods offers promising integrated analytical tools whose  
1114 complementarity would be strengthened by the use of chemometric approaches.

1115

#### 1116 **4. Chemometrics**

1117 Since the nature and properties of DOM in wastewater are extremely heterogeneous,  
1118 comparative sample studies involve orthogonal analytical techniques that generate a large  
1119 amount of information. Several steps are needed to process this data, from method validation  
1120 to combined data analysis, to identify known structures, map samples, or quantify similarities  
1121 or differences, seeking to limit the time it takes to process the data. Chemometrics is already  
1122 known as a powerful support tool for analyzing, interpreting and solving environmental issues  
1123 [192,193]. Due to the huge amounts of measurements obtained on miscellaneous collected  
1124 samples, the correct data processing techniques and statistical methods are required but can be  
1125 very tricky and difficult to select [194]. Multivariate statistical data analysis, such as cluster  
1126 analysis (CA) or principal component analysis (PCA), as well as the discriminant analysis



1127 techniques, can be used as emphasized methods to find relevant patterns and trends in various  
1128 analytical datasets. Chemometric approaches are useful for handling and interpreting large  
1129 and complex data matrices and for highlighting the correlations between the variables  
1130 evaluated. Many graphical representations of the results simplify their interpretation by  
1131 reducing and categorizing variables into groups with similar characteristics.

1132

#### 1133 4.1 Cluster analysis (CA)

1134 CA is an unsupervised statistical approach that classifies samples without prior  
1135 knowledge of the dataset. This exploratory method organizes data into clusters by maximizing  
1136 the similarities between samples within each group and by maximizing the dissimilarity  
1137 between groups. Hierarchical cluster analysis (HCA) is the most common method in which  
1138 agglomerative clusters are produced using the distance between samples as a measure of  
1139 similarity [195]. Results are generally illustrated by a dendrogram where similarities of  
1140 structures are expressed by distance depending on the species studied. In water analysis, CAs  
1141 are frequently employed when physicochemical parameters are collected. Among the studies  
1142 reported, we can cite the work by Singh *et al.* [196] in which 31 physicochemical parameters  
1143 were analyzed over 5 years within a stretch of about 12 km from 26 wastewater drains that  
1144 discharged into the Gomti River in India. The clusters identified provide information on  
1145 sewage and were used to group some drains to be diverted and connected to individual  
1146 sewage treatment plants. In another study, twelve geochemical parameters of water from  
1147 South Chennai Lake in India were studied to identify the factors affecting the pre-monsoon  
1148 and post-monsoon chemical composition of the water. The dendrogram analysis gave clusters  
1149 depending of the pollution levels at the stations and was helpful in determining the effects of  
1150 the monsoon on each station [197]. In the last example, 22 physicochemical parameters were

1151 used to evaluate the water quality of the Pisuerga River in Spain and to classify samples based  
1152 on seasonal and temporal criteria [198].

#### 1153 4.2 Principal component analysis (PCA)

1154 PCA is more commonly used than CA for data from the analytical techniques. PCA is  
1155 also an unsupervised multivariate statistical method frequently used in exploratory data  
1156 analysis. PCA simplifies the dataset by reducing the number of dimensions and creating new  
1157 ones. The principal components are a linear combination of the original variables which  
1158 explains most of the variance in the data matrix. The eigenvalues give the variance ratio used  
1159 by each principal component. Depending on the normalized data, mean-centering or  
1160 autoscaling, and the aim of the analysis, covariance or the correlation matrix is considered.  
1161 Score plots (samples) and loading plots (variables) are the graphical representations of PCA  
1162 used to determine the relationships between samples and/or variables [199]. PCA is  
1163 meaningful for water sample analyses for data resulting from spectroscopy applications.  
1164 Indeed, PCA is a powerful tool for analyzing complex matrices and determining the  
1165 relationships and correlations between the variables obtained with the same analytical  
1166 method. The use of a fast, non-destructive, user-friendly method, such as UV-visible, infrared  
1167 and fluorescence spectroscopy, without complex sample preparation, may initially be  
1168 preferred to the new and more sensitive methods of analysis such as GC, LC coupled with  
1169 mass spectrometry or NMR, to explore biological wastewater treatment processes [200], for  
1170 example, or for monitoring wastewater treatment efficiency [192,201]. Of course, PCA is also  
1171 applied to the physicochemical parameters to identify the producers of water pollution in  
1172 rivers [198] and in the air, or by combining these parameters with analytical results. For  
1173 wastewater treatment monitoring, the intensity of specific wavelengths (240, 250, 254, 260,  
1174 275 and 365 nm) is measured in UV-visible spectrometry and used to characterize the quality

1175 assessment [202]. The relationship between soil organic properties and the bands of IR  
1176 spectra from the corresponding HA is established by PCA [203].

1177 Hyphenated methods with LC or GC for the separation part and NMR and mass  
1178 spectrometry as the analyzer, usually require more complex, specific sample preparation (see  
1179 sections 3.2, 3.3 and 3.4) for data recording and also dedicated data treatment before  
1180 multivariate analysis. First, a peak alignment algorithm, such as icoshift [204,205], can be  
1181 applied to synchronize peaks or signals on the chromatograms or NMR spectra due to the  
1182 small variations of retention times or chemical shifts. Second, an automatic binning procedure  
1183 is used to segment all data into small regions preferably containing only one signal, or else  
1184 the selected signals are integrated manually depending on the information that needs to be  
1185 determined [206]. PCA has been carried out to investigate variations in the NMR spectral  
1186 dataset to characterize the labile POM fraction in solid wastes from anaerobic digestion plants  
1187 in Sweden [183], the recalcitrant aromatic components in soil organic matter from  
1188 Heilongjiang Province in China [185] or to monitor the degradation of organic matter from a  
1189 sewage treatment plant in São Carlos-SP in Brazil [181]. Recently, PCA was used to evaluate  
1190 datasets of two chromatographic methods coupled with mass spectrometry of drinking water  
1191 samples, wastewater and river water samples from Warburg, Germany [207].

1192

#### 1193 4.3 Partial Least Squares (PLS) and PLS discriminant analysis (PLS-DA)

1194 PLS is a multivariate statistical method and has some similarities with PCA. PLS is  
1195 useful for modeling a relationship between a set of dependent variables (Y) and a set of  
1196 independent variables (X). PLS regression attempts to maximize the covariance between  
1197 matrices X and Y and the model generates latent variables in X that will predict the latent  
1198 variables in Y [208]. This linear regression is known as PLS1 if it deals with only one Y

1199 response and PLS2 for several Y responses. A special form of PLS is PLS discriminant  
1200 analysis (PLS-DA), wherein Y is an indicator, a value 0 or 1 for example, depending which  
1201 class the object is a member of [209]. The score plot shows clustering, while the loading plot  
1202 indicates the discriminating variables between groups. A cross-validation method or separate  
1203 test sets are used to estimate the optimal number of latent variables and to guard against  
1204 overfitting. For multivariate calibration, PLS is also widely used [210]. PLS has been applied  
1205 to explore the possible relationship between the IR spectrum and SOC or the humification  
1206 index based on HA absorbance at 465 nm [203]. Predictive models including PLS1 and PLS2  
1207 were developed to find spectral wavelengths in UV-Vis spectra dataset in correlation with  
1208 water quality parameters such as DOM and TOC. This approach was intended to predict the  
1209 concentration of several water components and parameters and to be used to monitor the  
1210 performances of wastewater treatment plants [202]. Spectral wavelengths were selected based  
1211 on variable importance in the projection scores obtained using the model [211]. PLS has also  
1212 been applied for the estimation of the characteristics of urban wastewater [212] and for fault  
1213 detection in the wastewater treatment process [213]. In addition to wastewater quality  
1214 parameters, the permeate of a membrane bioreactors has been successfully modelled by PLS  
1215 on 2D-fluorescence dataset [201]. The discriminant version of PLS is a powerful statistical  
1216 tool and has been employed to find seasonal variations in the composition of sewage [196]  
1217 and to investigate which micropollutant signals in LC-HRMS were the most important in the  
1218 discrimination for complex water matrices [207].

1219         The interest of chemical information extraction by data-driven means in the design of  
1220 physicochemical indices related to the properties of DOM leads to the specific development  
1221 of descriptors dedicated to the study of the biodegradability of DOM and to the study of  
1222 recalcitrant compounds. To characterize DOM heterogeneity related to aromaticity, SEC-  
1223 generated MWs can be combined with fluorescence spectra collected between 300 nm and

1224 480 nm (Em) for an excitation wavelength (Ex) set at 254 nm. By calculating all humification  
1225 indices (HIX) for each emission spectrum (HIX<sub>i</sub>), it is possible to calculate HIX-based MW  
1226 values (MW<sub>HIX</sub>) from the SEC chromatogram for each separated species. Water samples from  
1227 different treatments can be compared based on the MW<sub>HIX</sub> to identify refractory species and  
1228 to study the evolution of aromaticity and biodegradability [193]. All of these approaches have  
1229 also been applied to large microscopy datasets to monitor activated sludge in wastewater  
1230 treatment processes [214]. Fig. 11 summarize the different chemometric methods used for  
1231 DOM and recalcitrant compound characterization.

1232 Fig. 11 shows a dendrogram derived from a hierarchical CA for DOM samples (A)  
1233 showing a link between marine samples, with a correlation of > 0.5 for offshore or estuary  
1234 samples, and a correlation of > 0.1 between the 2 types of sampling. However, there is no link  
1235 for samples taken in the river upstream of the estuary. To identify these links, formulas  
1236 developed from 2,143 compounds from a library developed by the authors were searched in  
1237 each sample. Then the O/C and H/C ratios and various indices (DBE, etc.) were calculated to  
1238 characterize each sample. The hierarchical CA was constructed from these indices until the  
1239 correlation coefficients were obtained in the matrix. To identify the key parameters, a PCA  
1240 analysis was performed (B), showing that nearly 60 % of the variance is explained by PC1  
1241 (58.3 %) related to the aromaticity of the compounds present in the water, calculated from the  
1242 DBE and DBE/C ratios. Other chemometric analyses provide a classification of compounds  
1243 by chemical families (C), using van Krevelen diagrams representing the H/C vs O/C ratios for  
1244 each analyte. It is possible to identify each recalcitrant compound by comparing the graphs  
1245 before and after a degradation process. All analytes are identified in their chemical family  
1246 based on HRMS analyses. By determining the HIX indices for each peak identified in SEC, it  
1247 is possible to represent an HIX chromatogram to identify the most condensed species present

1248 in surface water samples, represented in the figure by the highest peaks. These most  
1249 condensed species show significant refractory properties.

1250

## 1251 **5. Conclusions**

1252 The identification, characterization and quantification of the different chemical species  
1253 constituting DOM and rDOM in wastewater, will implement simple physicochemical or  
1254 complex hyphenated techniques, depending on the objective. The value of physicochemical  
1255 methods (DOC, DON, UV, IR) is found in the actual standardization and normalization and  
1256 the simple sample preparation needed. But these methods are limited in the search for  
1257 characterizations which will require a specific fractionation step (molar mass, charge,  
1258 polarity) and a sensitive and resolute detection technique. The sample preparation step can  
1259 also constitute a first level of splitting of this complex DOM. It is essentially ion exchange  
1260 resins and membrane filtration techniques that are most used to split DOM and study  
1261 recalcitrant macromolecular compounds after removal of low molecular weight fractions. For  
1262 these techniques, no automation is envisaged and no standardization studies are available. Yet  
1263 solute/membrane interactions are an important negative point to be studied for robustness. It  
1264 appears that the sample preparation step is more oriented towards the analytical method that  
1265 follows than towards a normalization of the treatment to guarantee the extraction of all the  
1266 species present for identification and quantification. It therefore seems essential to develop  
1267 studies from standards, such as melanoidins synthesized under specific conditions, or  
1268 commercial HA and FA, to define the limits of each method of sample preparation. New  
1269 developments appear in FFF and FIFFF and provide repeatable, robust fractionation.  
1270 Miniaturization of this stage will certainly be a challenge to make it universally usable in  
1271 routine.

1272           Recalcitrant identification can be achieved with specific physicochemical ratio such as  
1273 SUVA<sub>254</sub>, URI and DOC/DON ratios that provide information and quantify rDOM. Compared  
1274 to UV absorbance, fluorescence is therefore more specific and sensitive for discrimination of  
1275 recalcitrant compounds, but the limited number of transitions and the absence of specific data  
1276 for particular chemical functions require the introduction of another analytical dimension  
1277 complementary to fluorescence, such as FT-IR or a separation technique. FT-IR is widely  
1278 used for sludge monitoring, since the sample must be completely dehydrated before analysis,  
1279 which may limit the scope of application. Significant advances in the characterization of  
1280 DOM and notably in the identification of rDOM and all families of recalcitrant compounds  
1281 have been achieved using separation methods. The SEC technique is essentially the most used  
1282 in the study of NOM, DOM and rDOM. Its main interest lies in the classification of analytes  
1283 according to molecular weight distribution, which represents a constant property in the very  
1284 large variability of the DOM. The most commonly used stationary phases are the 8 or 5 µm  
1285 permeation columns. The main defect of the SEC technique is the existence of secondary  
1286 interactions between analytes and stationary phases. It is then necessary to use perfectly  
1287 characterized eluents with defined pH and concentration buffers to be optimized for the best  
1288 resolution. The result is a lack of normalization for this technique, with each solution  
1289 appearing to have better resolution under a particular condition. In addition, the standards  
1290 available to calibrate SEC columns do not always correspond to the compounds studied, such  
1291 as melanoidins, which do not have standardization. Since MWD is influenced by the mode of  
1292 detection, the nature of the solutes, the buffer used and the range of separation of the  
1293 stationary phase, it is sometimes necessary to combine the SEC with several spectroscopic  
1294 methods, in order to develop hyphenated techniques suitable for the desired structural study.

1295           The fastest and cheapest advances in hyphenated techniques have been achieved with  
1296 SEC/PDA/Fluorescence, which provides all the advantages of each element of the analytical

1297 chain in a single injection. It is thus possible to measure the main characteristic indices of  
1298 NOM, DOM and rDOM for each chromatograph peak. Relative quantitative analysis is also  
1299 possible. If the structural study is to be further developed, the use of HRMS is needed. We  
1300 note an attempt to replace separation techniques with the use of very high-resolution MS (FT-  
1301 ICR MS), which allows elementary analyses on small or HMW molecules, without  
1302 degradation and without prior separation. The use of hyphenated HRMS methods, however,  
1303 allows the use of more conventional HRMS detectors, with much success. HRMS techniques  
1304 provide a global analysis (elementary, H/C ratio, O/C, N/C, DBE), online. For homologous  
1305 compounds, it is also possible to differentiate species. In the literature, major advances in  
1306 HRMS are found for small molecules with a MW < 1000 Da. Very few studies are available  
1307 for native melanoids and macromolecules of rDOM. Yet the structural study of these  
1308 molecules is the key to understanding refractory properties. Only high-precision frequency-  
1309 derived data (NMR, HRMS and FT-ICR MS ) can provide sufficient resolution to resolve the  
1310 nonrepetitive complex structure of rDOM [216]. When using HRMS, the low  
1311 chromatographic resolution of SEC was advantageous for spectra measurements of low and  
1312 high molecular mass. A correlation between SEC calibration and elemental composition can  
1313 provide information on the aromaticity and heteroatom composition of some moieties [159].  
1314 On the other hand, the mass spectra are complex to analyze, and it is necessary to study the  
1315 positive and negative ESI spectra. Depending on the species and adducts with the different  
1316 anions and cations present in the wastewater, a very large number of signals will be generated  
1317 for the same species. The matrix effect is strong, which limits quantitative analyses, especially  
1318 since isotopic standards are non-existent for macromolecular rDOM. If there are some new  
1319 developments in HPTLC, for example, or in Pyr-GC/MS, in libraries under construction –  
1320 notably for the study of Amadori compounds – no 2D study has been developed to date for  
1321 the analysis of rDOM and melanoidins. It is, however, possible to develop SEC separation



1322 with a second specific dimension based on hydrophobic interactions, as performed to separate  
1323 specific proteins from gum arabic with amphiphilic properties [217]. New LCCC  
1324 developments provide a superior response to conventional SEC chromatography,  
1325 complementing a comparison on functional polydispersity of samples. Future 2D  
1326 developments will be necessary, combining orthogonal retention mechanisms to connect with  
1327 specific rDOM properties, to increase separation power before analysis using hyphenated  
1328 techniques, to improve the quantitative response, to decrease the matrix effect, and to study  
1329 various interactions (steric, hydrophobic, electrostatic). The "omics" studies modeled on  
1330 humeomics, developed for the study of NOM, have not yet been developed for the  
1331 characterization of recalcitrant compounds. While there are constant structures in HSs that  
1332 provide validation of the methods developed, there is still no consensus around recalcitrant  
1333 compounds and melanoidins from sludge and wastewater. It is essential to improve our  
1334 structural knowledge of these compounds, as currently developed for the food industry, in  
1335 order to propose new developments based on the study of reaction mechanisms for the  
1336 synthesis and degradation of all rDOM present in wastewater. This new development will  
1337 include advances in proteomics, applied to the study of rDOM, the aim being to understand  
1338 the mechanisms related to refractory properties.

1339         For the structural determination of recalcitrant compounds, NMR could be considered  
1340 as a method of choice. Nevertheless, the potential of NMR approaches in the area of this  
1341 review has yet to be fully tapped. Indeed, the intrinsic insensitivity of NMR is not a limitation  
1342 in all environmental applications, while its inherently quantitative nature and its holistic  
1343 approach are its main strengths. Moreover, current and future NMR developments may  
1344 enhance the technique's potential. Dissolution dynamic nuclear polarization is a promising  
1345 approach, a versatile method for enhancing the sensitivity of NMR by boosting signal  
1346 intensities by four to five orders of magnitude [218]. All of the techniques described in this

1347 review can be combined, leading to huge amounts of data to be processed and analyzed. The  
1348 implementation of chemometric tools, such as CA, PCA and PLS multivariate approaches are  
1349 essential for comprehensive data analysis. Finally, by combining all the developments  
1350 described in each analytical field, linking a property to a set of physicochemical descriptors or  
1351 to specific structural data, future developments will open up a new field in the mechanistic  
1352 study of rDOM for theoretical knowledge and water treatment industry applications.

1353

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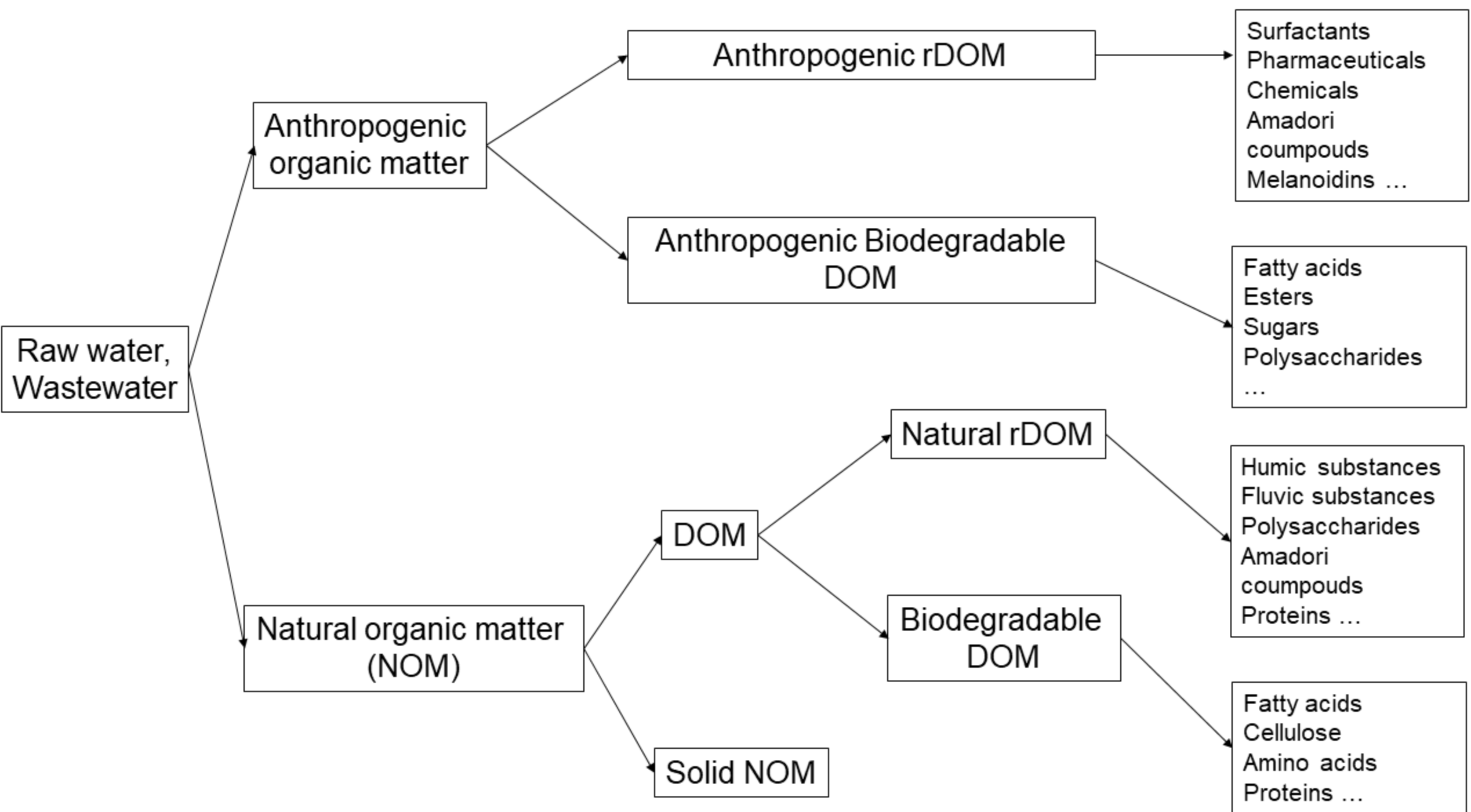
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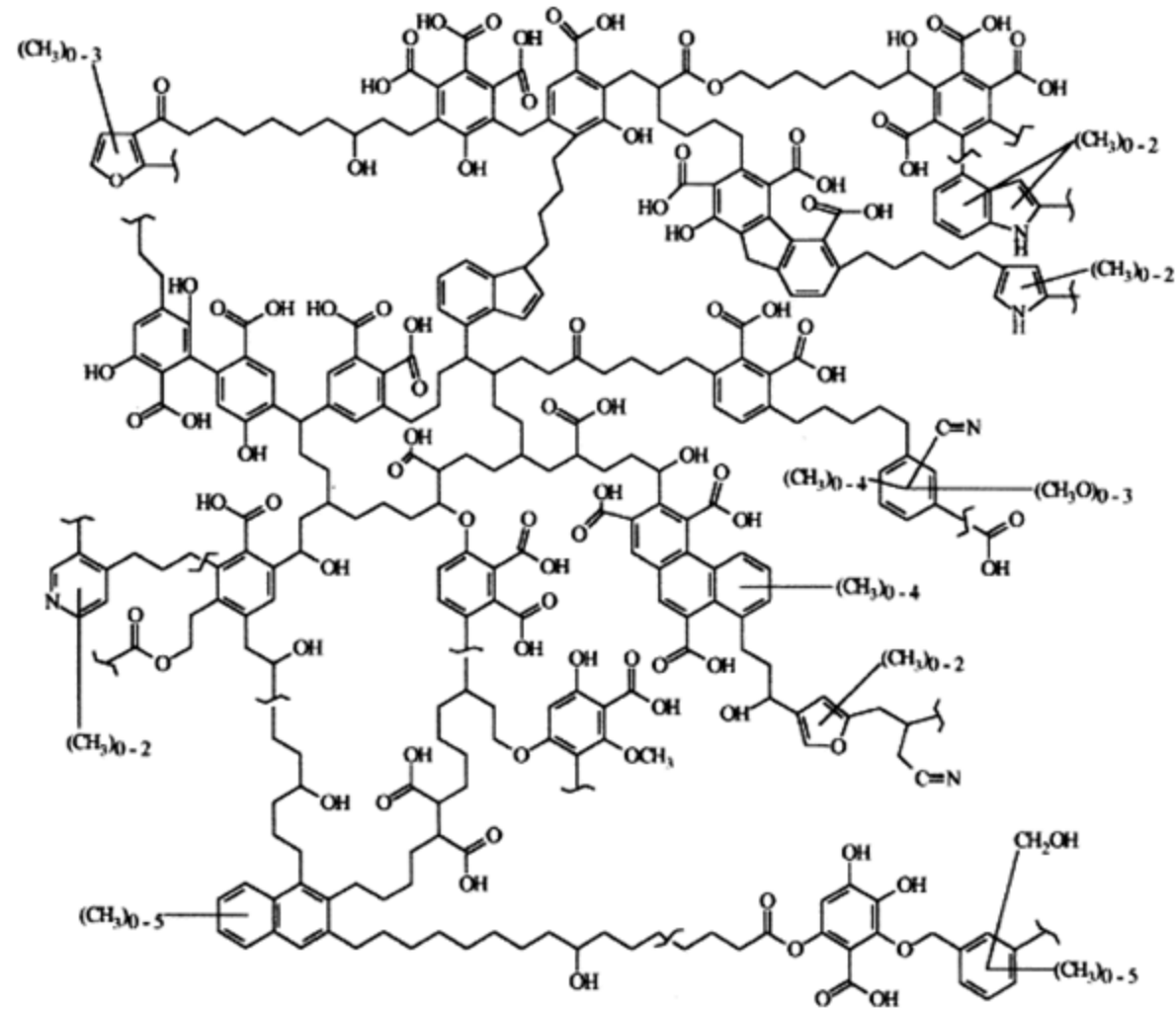
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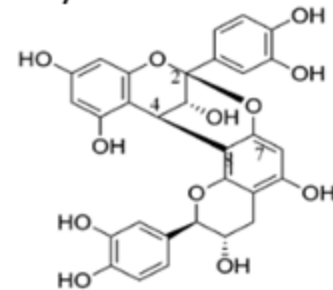
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2129 dissolution dynamic nuclear polarization in physical, chemical and biological contexts,  
2130 *Journal of Magnetic Resonance.* 305 (2019) 41–50.  
2131 <https://doi.org/10.1016/j.jmr.2019.06.001>.  
2132



a) Humic substances



b) Phenol



Proanthocyanidin A1, epicatechin-(2β→7,4 β→8)-catechin

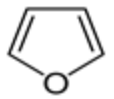
c) Maillard compounds



Pyrazine

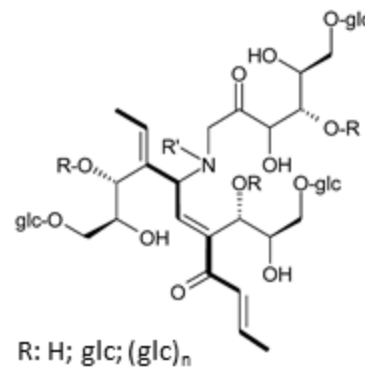


pyridine

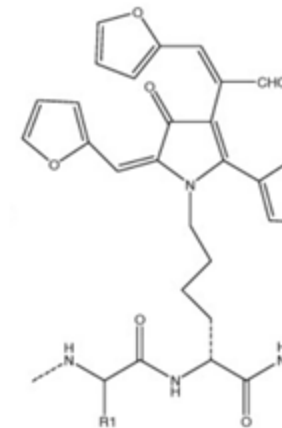


Furan

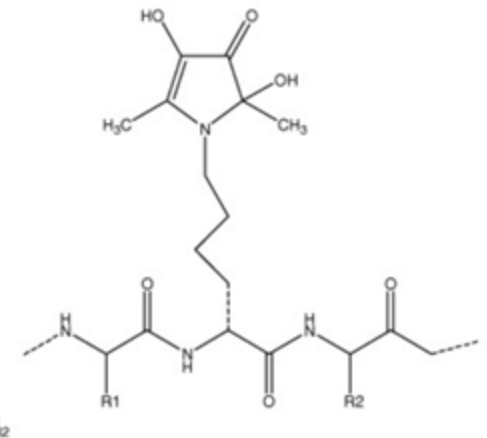
d) Melanoidins



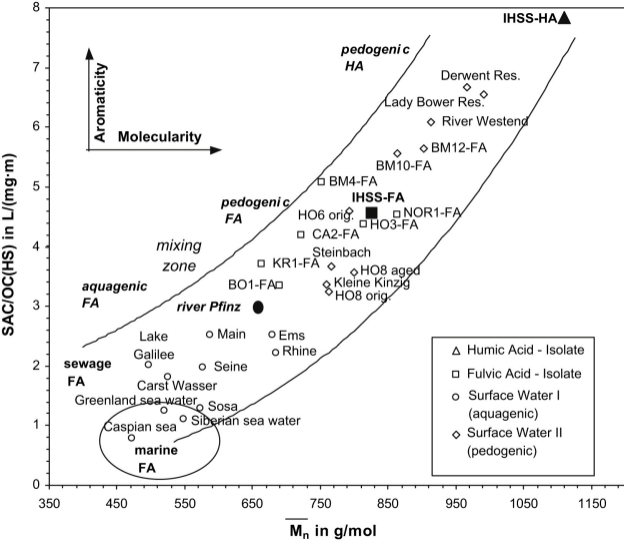
Melanoidin structure formed from 3-deoxyhexosuloses and ARP

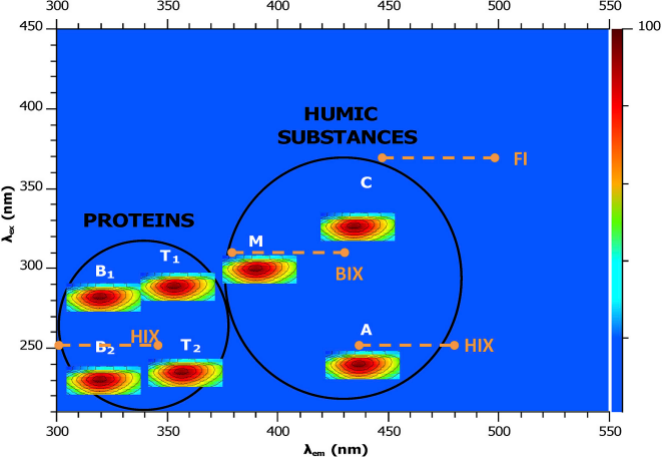


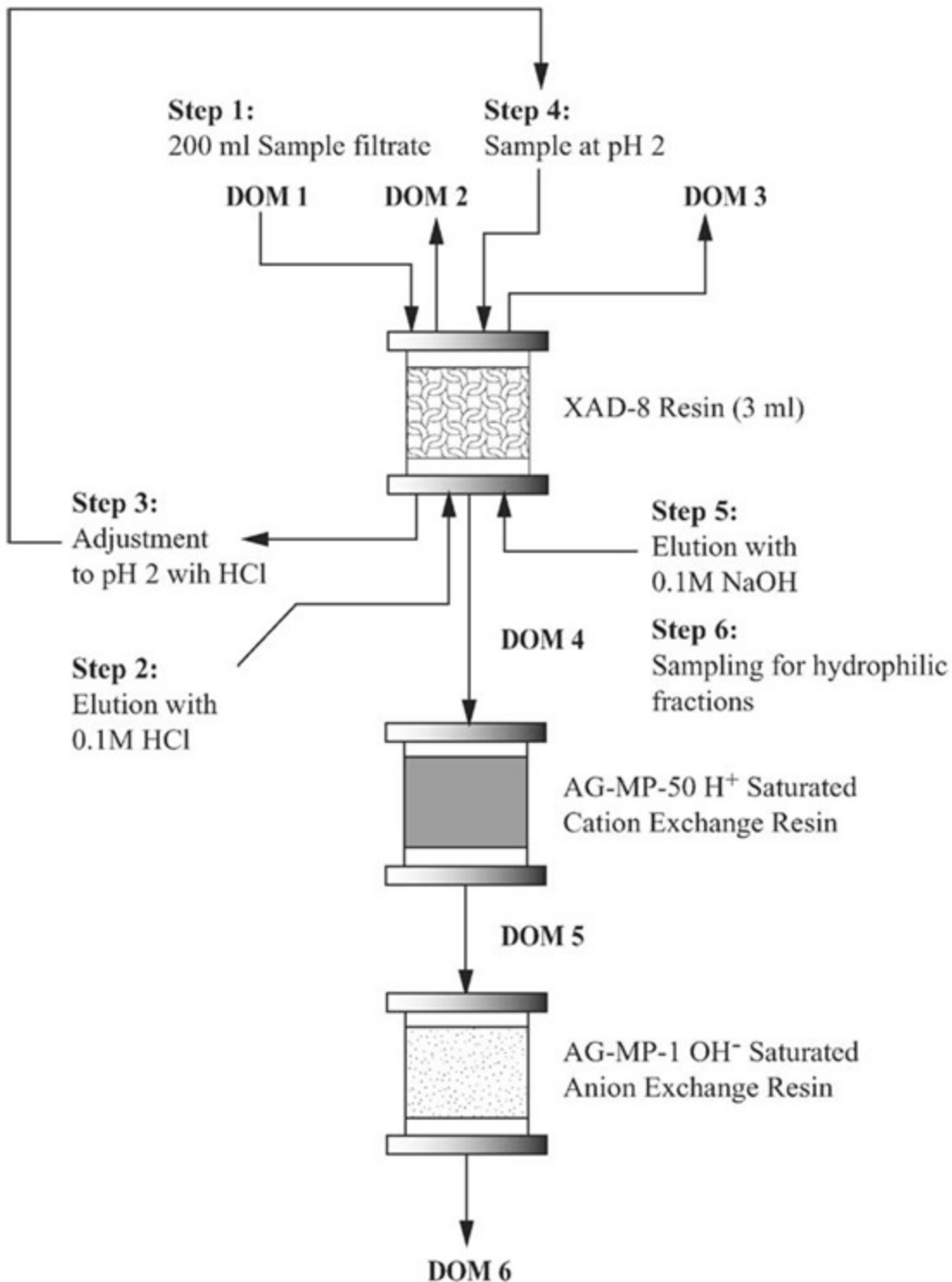
Trifuryl-pyrrolinone

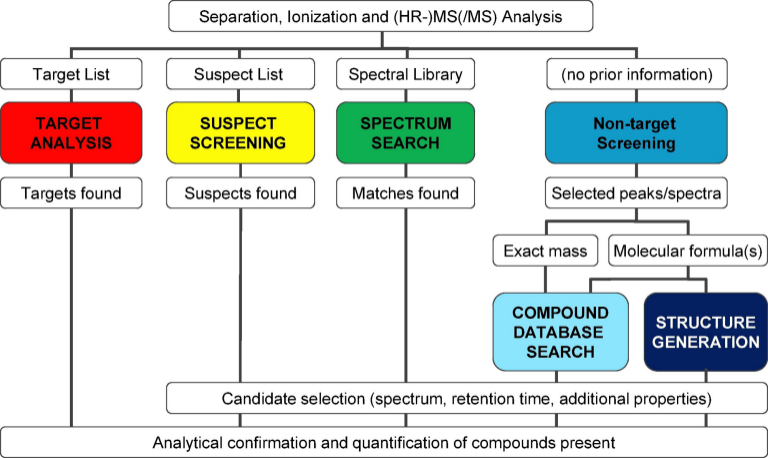


3) Pronyl-protein

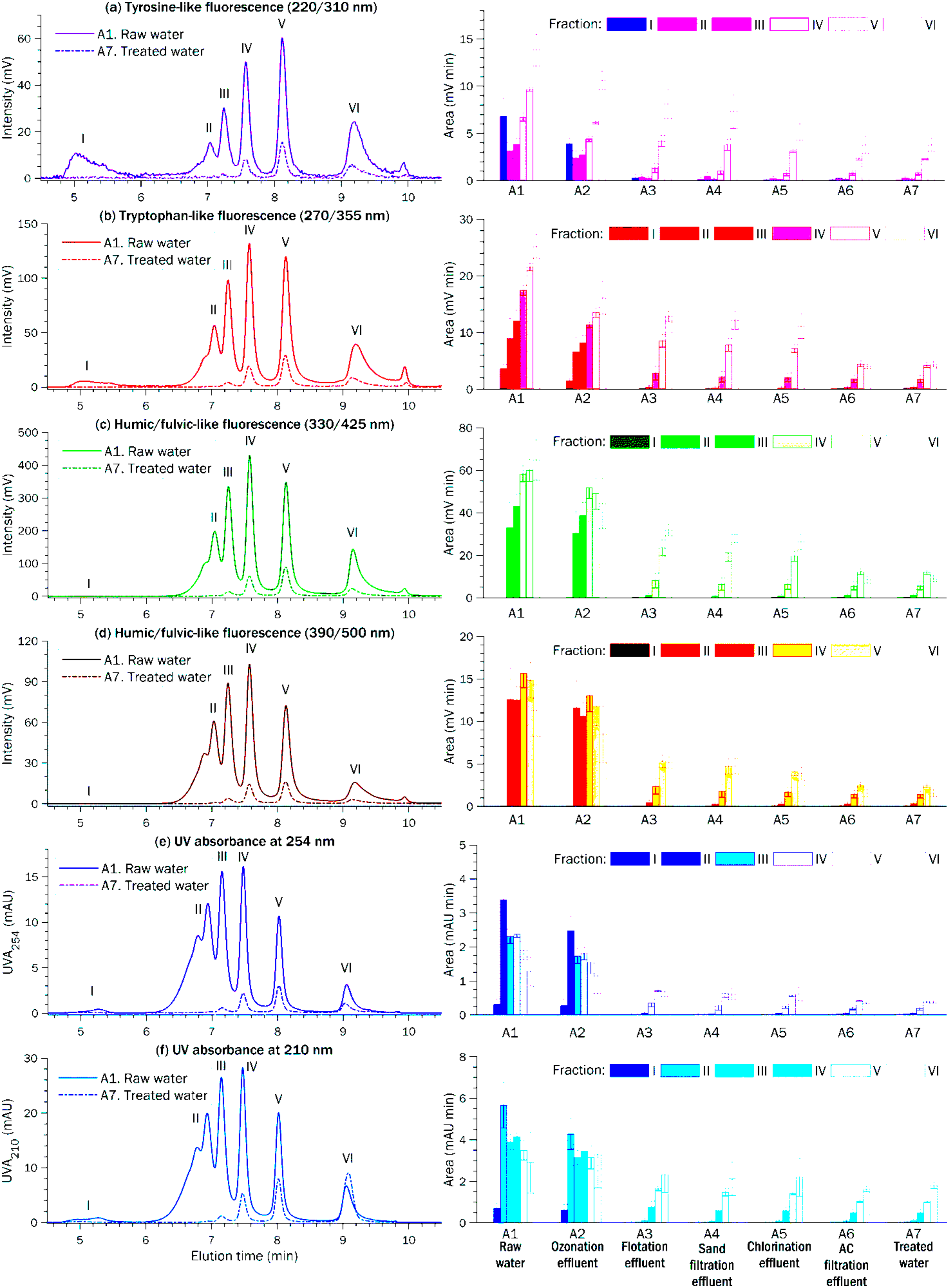


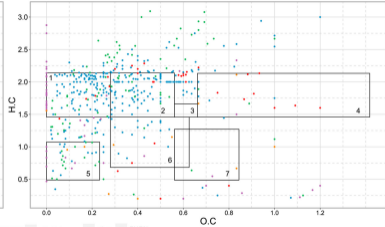
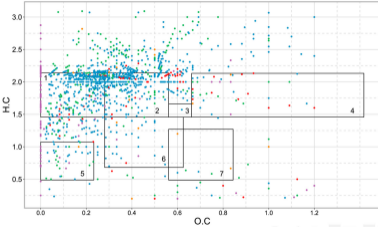






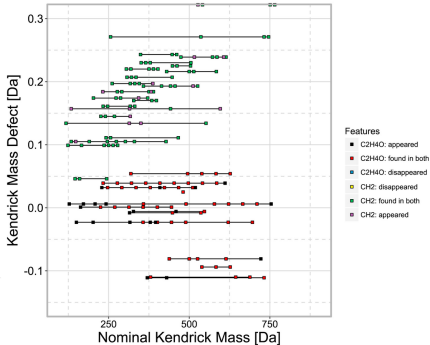
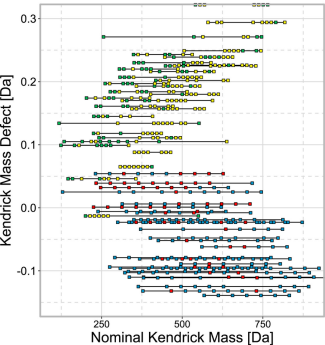




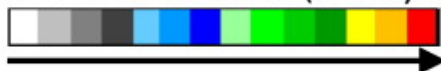


Type of matter

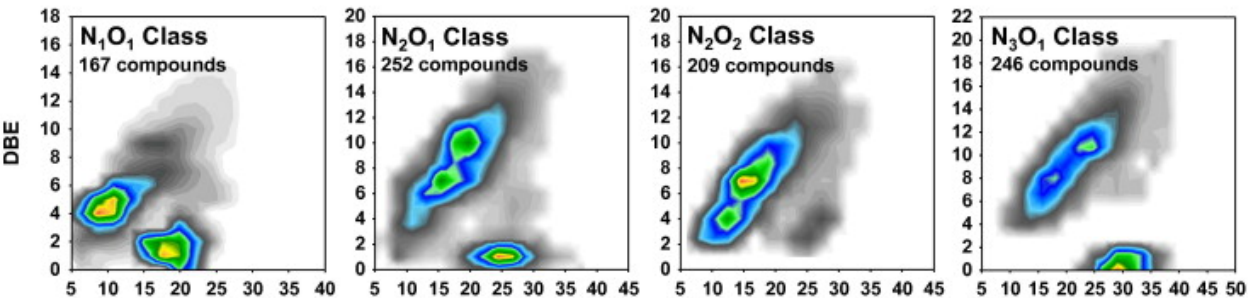
- CHO
- CHONS
- with Halogens
- other
- CHON



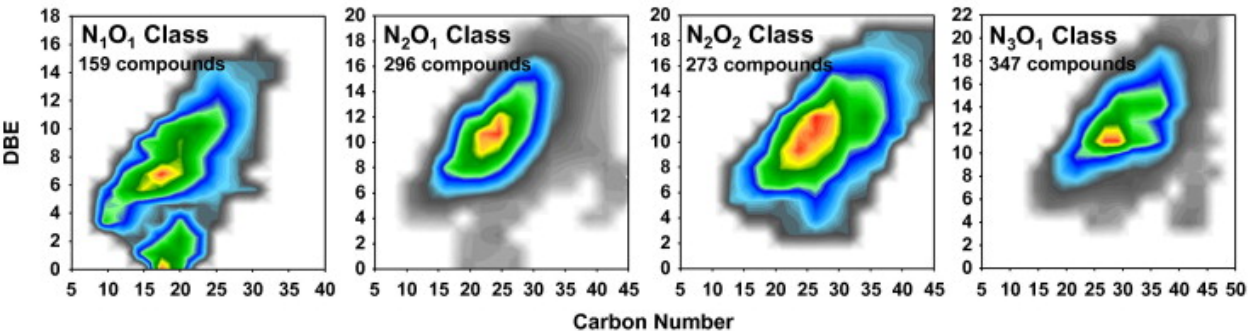
Relative Abundance (% total)

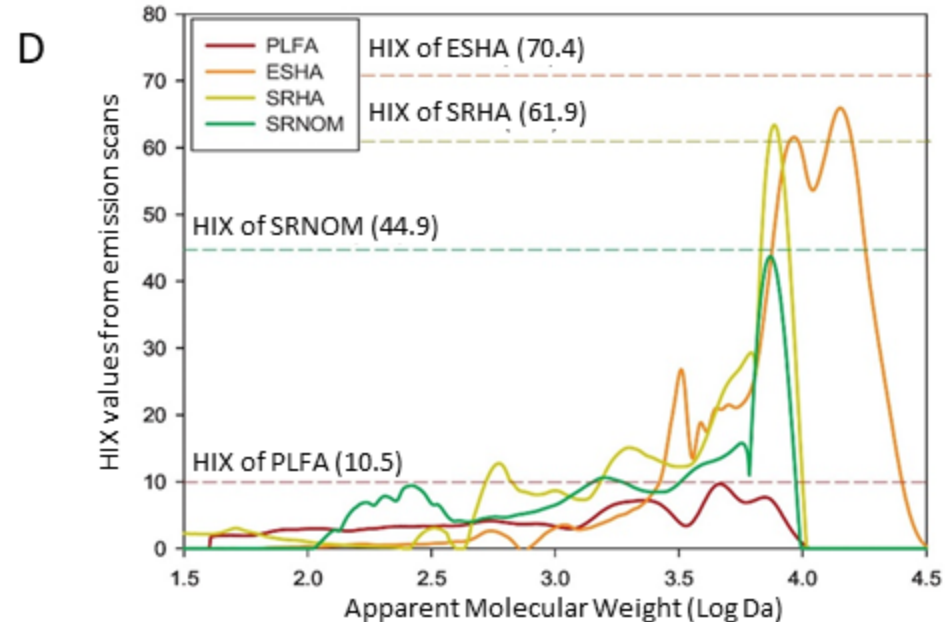
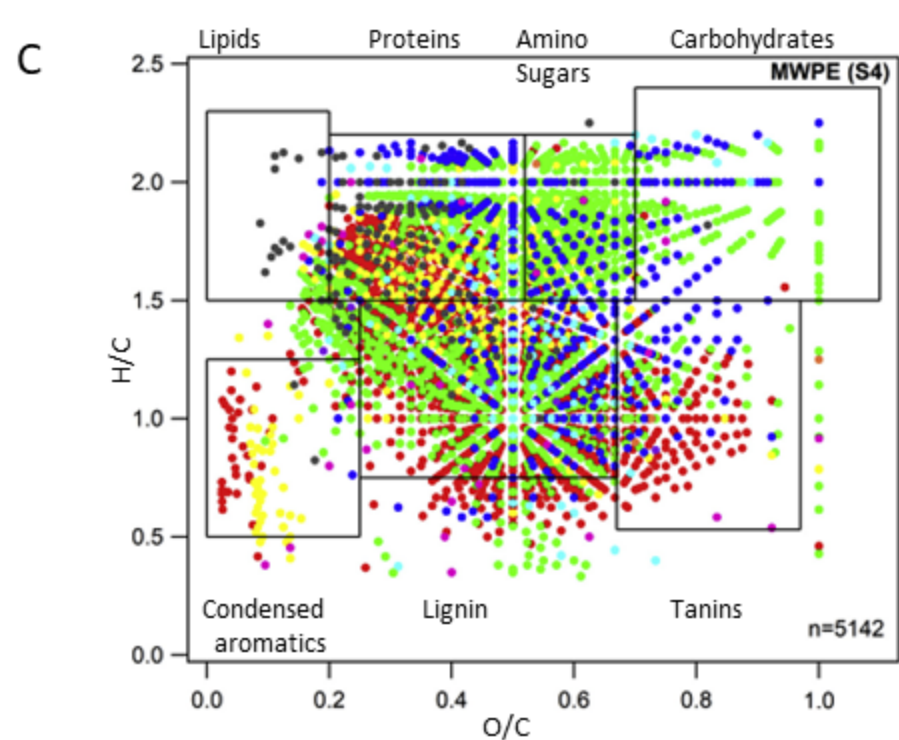
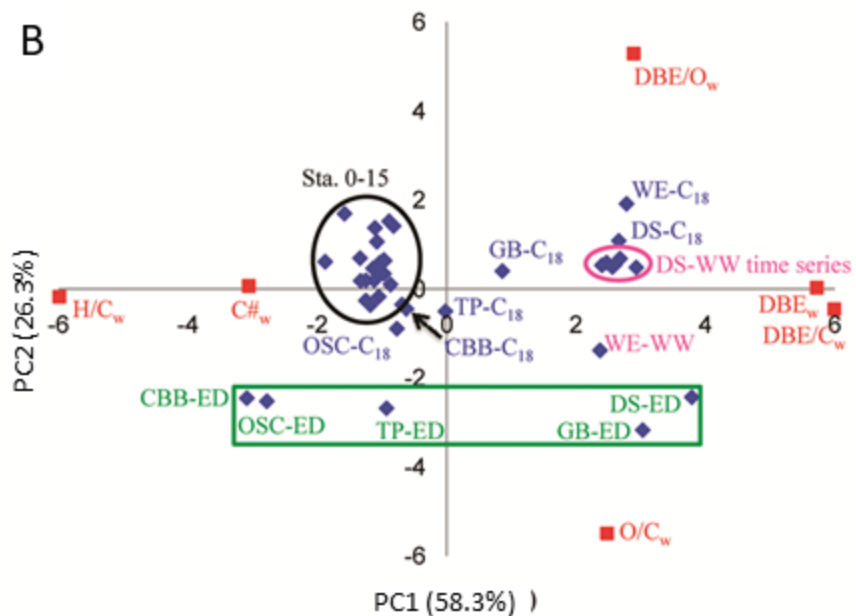
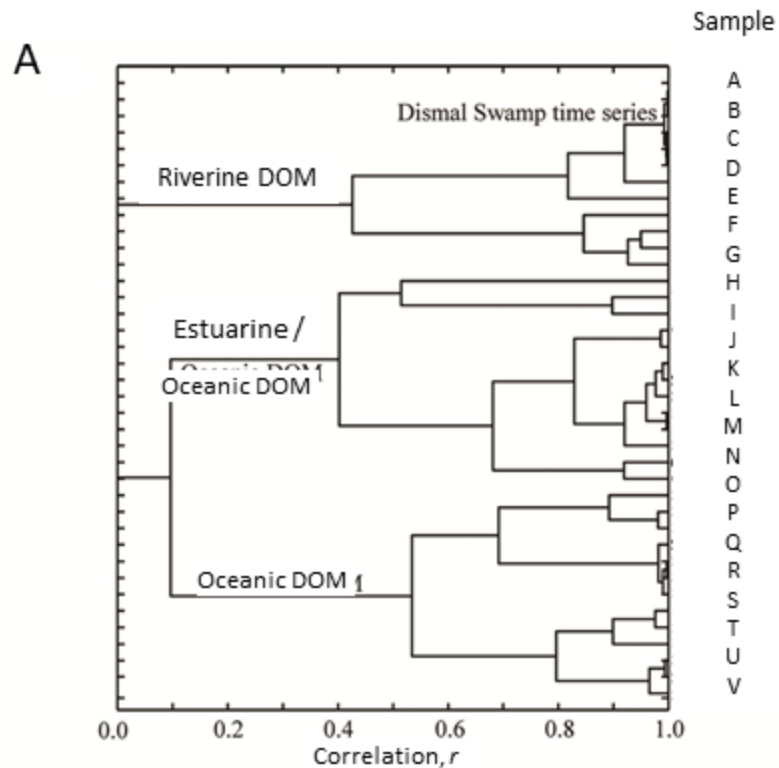


Aqueous Fraction



HTL Bio-Oil





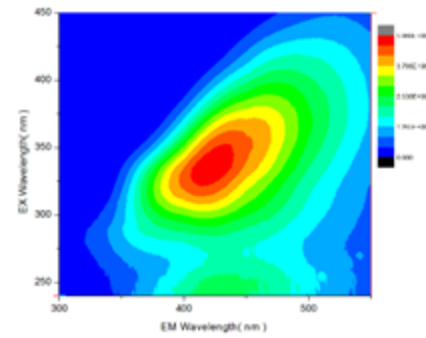
Wastewater sample

Analytical and Chemometrics

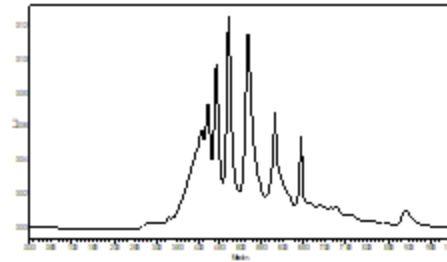
Chemical structure



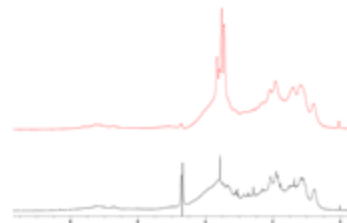
Fluo



SEC



NMR



MS

