



HAL
open science

Resolving phytosterols in microalgae using offline two-dimensional reversed phase liquid chromatography x supercritical fluid chromatography coupled to quadrupole time-of-flight mass spectrometry

Magali Batteau, Adriana Ramirez-Romero, Elodie Bouju, Sylvie Nuccio, René de Vaumas, Florian Delrue, Karine Faure

► To cite this version:

Magali Batteau, Adriana Ramirez-Romero, Elodie Bouju, Sylvie Nuccio, René de Vaumas, et al.. Resolving phytosterols in microalgae using offline two-dimensional reversed phase liquid chromatography x supercritical fluid chromatography coupled to quadrupole time-of-flight mass spectrometry. *Analytical Methods*, 2024, 16, pp.2278-2285. 10.1039/D3AY02261G . hal-04493944

HAL Id: hal-04493944

<https://hal.science/hal-04493944v1>

Submitted on 7 Mar 2024

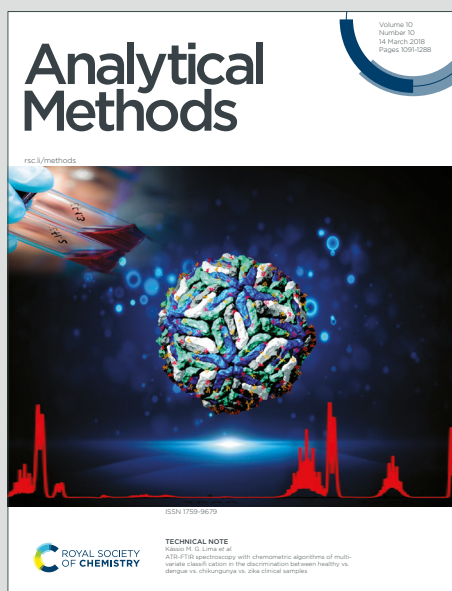
HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Analytical Methods

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: M. Batteau, E. Bouju, A. Ramirez-Romero, S. Nuccio, R. De Vaumas, F. Delrue and K. Faure, *Anal. Methods*, 2024, DOI: 10.1039/D3AY02261G.



This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this Accepted Manuscript with the edited and formatted Advance Article as soon as it is available.

You can find more information about Accepted Manuscripts in the [Information for Authors](#).

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard [Terms & Conditions](#) and the [Ethical guidelines](#) still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.

1 Resolving phytosterols in microalgae using offline two-dimensional reversed 2 phase liquid chromatography × supercritical fluid chromatography coupled 3 to quadrupole time-of-flight mass spectrometry

4
5 Magali Batteau¹, Elodie Bouju², Adriana Ramirez-Romero³, Sylvie Nuccio², René De
6 Vaumas², Florian Delrue³, Karine Faure^{1*}

7
8 ¹Université de Lyon, CNRS, Université Claude Bernard Lyon 1, Institut des Sciences
9 Analytiques, UMR 5280, 5 rue de la Doua, F-69100 VILLEURBANNE, France

10 ²Extrasynthese, Impasse Jacquard, F-69730 GENAY, France

11 ³MicroAlgae Processes Platform, CEA, CEA Tech Région Sud - Provence-Alpes Côte d'Azur,
12 F-13108, Saint Paul lez Durance, France

13 * Corresponding author: Karine Faure, Institut des Sciences Analytiques, 5 rue de la Doua, F-
14 69100 Villeurbanne, France; phone: +33-437423686; e-mail: karine.faure@isa-lyon.fr. ORCID
15 0000-0002-5416-4911

16 **Abstract**

17
18 Sterols are unsaponifiable lipids from plant metabolism that exhibit interesting bioactive
19 properties. Microalgae reveal a major source of specific phytosterols, most of them still not
20 fully characterized. The similarity of sterol structure and the existence of positional isomers
21 make it a great challenge in separation science. A method was developed based on offline two-
22 dimensional (2D) system, reversed phase liquid chromatography (RPLC) × supercritical fluid
23 chromatography (SFC)/quadrupole time-of-flight (Q-ToF) for the identification of sterols in
24 microalgae. Subsequent positive-mode MS/MS was used to confirm the identified phytosterols.
25 The 2D chromatogram revealed a pattern typical from the position of the unsaturations that was
26 confirmed by standard injection, helping in the structural elucidation. The analysis of the

1 27 unsaponifiable fraction of two algae, namely *Scenedesmus obliquus* a freshwater microalgae
2
3 and *Padina pavonica*, a marine macroalgae, highlighted the ability of the method to distinguish
4
5 a large number of sterol isomers.
6
7
8
9
10
11
12
13

View Article Online
DOI: 10.1039/C4AY02261G

14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32

32 **Abbreviations**

33 ¹D, ²D: first and second dimensions, respectively

34 SFC: supercritical fluid chromatography

35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

40

41

42

43

44

I. Introduction

Phytosterols are unsaponifiable lipids resulting from the secondary metabolism of the terpenoid pathways of plants. The main sterols in plants are β -sitosterol, stigmasterol, campesterol, and ergosterol, which present bioactive properties related to anti-obesity, anti-diabetes, antioxidant, and hepatoprotection activity, among others. The presence of one or more double bonds in the B-ring, the position of a double bond in the side chain or the degree of ethylation on the C24 position (see Fig.1 for IUPAC numbering scheme), result in a very large number of phytosterols, with > 250 molecules identified in higher plants so far ¹.

While the main source of these lipids is found in edible oils, a promising source of these components is microalgae ². Indeed, microalgae exhibit the ability to hyperaccumulate lipids in nutrient-depleting conditions. This behavior has led to a commercial use of microalgae for biofuel production ³, a process that is unfortunately not yet economically relevant as compared to petroleum oil. On the other hand, the production of high-value products such as unsaponifiable lipids for nutraceuticals raises a great interest amongst large-scale microalgal producers ⁴. Isolation of the various components of the lipid fraction of microalgae may indeed contribute to the valorization of the entire plant. Analysis of the algae content of free sterols is of primary interest to improve the strain selection and cultivation conditions for the sustainable production of free sterols ⁵. But most research discussing the lipid composition of algae report only the total amount of sterols without any distinction between molecules ⁶. On the other hand, an intensive work has been done on developing analytical methods for the identification of sterols in higher plants. Most studies report the use of gas chromatography coupled to FID or mass spectrometry (GC-MS) after derivatization of the sterols ^{7,8}. However, the high working temperature may cause degradation and hence eliminate the differentiation between conjugated (esterified or glycosylated) and free sterols. Moreover, some co-elution of phytosterols have

69 been evidenced in GC ⁹. Underivatized sterols have been quantitatively analyzed using LC-
70 MS/MS in edible oils ¹⁰ and in cattle feedstuffs ¹¹. However, these works targeted sterols with
71 different molecular masses and did not address the separation of eventual positional isomers.
72 Because the main fragmentation pattern of sterols is obtained by the cleavage of the C-ring¹¹,
73 positional isomers differing by their side chain structure may be confused. Moreover, the risk
74 is high that coeluting sterols may provide the same fragment due to their major structural
75 similarity. For example, campesterol produces fragments at m/z 395 (stigmasterol monitoring
76 ion), and this results in a quantitative bias as baseline separation is not reached for these two
77 sterols using RPLC ⁸. Therefore it seems of primary important to improve the separation of
78 sterols for a confident identification. Multidimensional chromatography, a separation technique
79 which greatly improves peak capacity, has not been reported so far for the separation of algal
80 sterols. In the present work, we wish to explore the ability of off-line LCxSFC to separate free
81 sterols without derivatization, with a special attention to isomers separation.

82 SFC technique has been scarcely applied to sterols. The earliest studies used C18 columns ^{12, 13}
83 which resulted in the separation of sterols with the elution order related to the molecular mass,
84 in combination with UV detection, while the most recent used polar stationary phase ^{14, 15},
85 showed promising selectivity, yet without being able to separate isomers. Combining both SFC
86 and RPLC in a two-dimensional separation induces an orthogonality, so increasing the peak
87 capacity of the overall 2D separation.

88 While online LCxSFC is still being investigated, we selected in this work an off-line
89 configuration, since a slower second dimension allows the implementation of MS/MS
90 fragmentation without compromising the acquisition frequency. Thus, each separation
91 dimension has been optimized and the orthogonality between LC and SFC has been
92 demonstrated on a set of 7 sterols standards. After careful optimization of the APCI ionization

1 93 conditions, the off-line LCxSFC-MS/MS method has been applied to two industrially
2
3 94 promising microalgae genera: *Scenedesmus obliquus* a freshwater microalgae and *Padina*
4
5 *pavonica*, a marine macroalgae.
6
7
8

View Article Online
DOI: 10.1039/C4AY02261G

96 II. Experimental

97 1. Chemicals

98 Acetonitrile (ACN) and methanol (MeOH) of LC-MS grade (chromasolv ≥ 99.9 %) were
99 purchased from Honeywell (Germany), n-heptane of HPLC grade (≥ 99 %) was from Carlo-
100 Erba (Italy), ethanol (EtOH) of LiChrosolv grade (≥ 99.9 %) was from Supelco, Merck
101 (Germany), methyl tert-butyl ether (MTBE) of extra pure grade (≥ 99.9 %) was purchased from
102 Acros Organics (USA), Purelab Classic system from Elga (UK) delivered 18.2 M Ω -cm type I
103 ultrapure water. Sodium chloride BioReagent (≥ 99 %) and potassium hydroxide (≥ 90 %) were
104 obtained from Sigma Aldrich (Germany). Pressurized carbon dioxide (CO₂) N45 ($\geq 99,995$ %)
105 was obtained from Air Liquide (France). Reference standards (ergosterol, cholesterol,
106 campesterol, fucosterol, stigmasterol, α -spinasterol and β -sitosterol) had a purity exceeding 95
107 % and were obtained from Sigma Aldrich (Germany). Standard mixtures were prepared
108 between 0.1 and 0.6 mg/mL in ACN/MeOH 3/7 for LC analysis and in MTBE or n-heptane for
109 SFC analysis.

110 2. Microalgae samples

111 The microalgae *Scenedesmus obliquus* UTEX 393 were grown under controlled conditions (25
112 °C, 2 % CO₂, 120 rpm, 14 h day/10 h night) and harvested by Dr. Delrue at CEA. Cell disruption
113 was performed by grinding/freeze-thawing, then algae were dried at 80 °C for 2 hours. The
114 acetone extract of macroalgae *Padina pavonica*, grown in Tahiti, was provided by Laboratoire
115 Texinfine (Lyon, France) company. The saponification of the algae was carried out by mixing
116 5 g of dried microalgae or 5 g of dried macroalgae acetone extract, respectively with 100 mL

1 117 of a methanol KOH solution (150 g/L). This mixture was heated to 40° C for 3 h with magnetic
2
3 118 stirring, then it was subjected to ultrasound for 20 minutes. Liquid-liquid extraction was then
4
5 119 performed by adding 100 mL of water and 100 mL of heptane. The aqueous phase was rinsed
6
7
8 120 3 times with 100 mL of heptane. These organic extracts were combined and evaporated until
9
10 121 dryness to produce unsaponifiable extracts.

122 3. Instruments

123 Agilent Infinity II 1260 LC system was equipped with a quaternary solvent delivery pump
124 (G7111A), an autosampler (G7129A) with a 900 μ L loop, a column oven (G7116A) compatible
125 with temperatures up to 90 °C, a DAD detector (G7117C) with a 1 μ L flow-cell and a fraction
126 collector (G1364F) with a capacity of 120 fractions. The maximum allowable flow rate was 4
127 mL/min and the maximum allowable pressure was 400 bar. Data acquisition was performed
128 using Chemstation software (Agilent Technologies, Waldbronn, Germany). The extra-column
129 variance and dwell volume were determined experimentally and found to be 36 μ L² and 2.66
130 mL, respectively.

131 Agilent Infinity II 1260 SFC system was equipped with a binary solvent delivery pump
132 (G4782A), an Infinity II autosampler (G4767A) with a 100 μ L loop, a column oven (G7116A)
133 compatible with temperatures up to 85 °C, a DAD detector (G7115A) with a 2 μ L flow-cell
134 withstanding a pressure up to 400 bar, a back pressure regulator (BPR) (G4301A), and a make-
135 up pump. The maximum allowable pressure was 400 bar. The dwell volume was measured in
136 quasi-liquid conditions at 600 μ L. Injection conditions were as follows: overfeed volume 4 μ L,
137 feed speed 1000 μ L/min, and heptane as the feed solvent. The instrumental variance due to
138 extra column was estimated at 32 μ L² (liquid conditions). An Agilent 6560A Ion-Mobility
139 Quadrupole Time-of-Flight LC/MS equipped with APCI source was used. The accurate mass
140 of the precursor ion was recorded with a mass tolerance of \pm 2 ppm.

1 141 The SFC column was hyphenated to the MS using a “preBPR splitter with a sheath pump”
2
3 142 configuration [16]. The stainless steel tubing kit (50 cm) was provided by Agilent Technologies.
4
5
6 143 Osiris software from Datalys (France) was used to measure retention models in LC and
7
8 144 determine optimized LC gradient conditions. Data acquisition was performed using
9
10 145 Chemstation software (Agilent Technologies) and treated using MassHunter Qualitative 10.0.
11
12 146 The 2D chromatograms were plotted using an in-house developed Matlab program from S.
13
14 147 Heinisch and F. Rouvière. The software MS Finder was used for structure proposition using
15
16 148 MS and MS/MS spectra of unknown compounds ¹⁶.
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

4. Method development

149
150
151 Two LC columns were screened: Kinetex F5 PFP (150 x 4.6 mm, 5 μ m), and Phenomenex Luna
152 Omega C18 (150 x 4.6 mm, 3 μ m). The screening mobile phase was A: ACN, B: IPA, gradient
153 0%- 50% B with a normalized slope of 3%.
154
155 Five SFC columns were also assessed: 2-PIC, DEA, 1-AA and Diol with dimensions 100 x 3.0
156 mm, 1.7 μ m, all from Torus series, Waters (Milford, USA), using a generic gradient elution (5
157 % - 40 % of methanol as the co-solvent, normalized gradient slope of 2.5 %).
158
159 Table 1 summarizes the off-line LCxSFC-MS conditions. A set of 46 fractions were collected
160 from the first dimension, every 0.192 min, providing a fraction volume of 345 μ L. Considering
161 an average peakwidth at 6σ , this sampling rate corresponded to two collected fractions per peak.
162
163 The ¹D eluent was then evaporated and 150 μ L heptane was added to the dried fractions. The
SFC separation was carried out at maximum flow rate allowed for the column, determined to
be 1.2 mL/min when using 35 % co-solvent (generating around 400 bars). The overall analysis
took 4.8 hours in comprehensive mode, i.e. untargeted analysis of the entire sample, and 2.7

164 hours in selective comprehensive mode where only the sterols ¹D elution zone was analyzed in
 165 the second dimension.

View Article Online
 DOI: 10.1039/D3AY02261G

166

167 Table 1 Off-line LCxSFC-MS conditions

	First dimension (¹ D)	Second dimension (² D)	HRMS	
Injection volume	60 µL in 70/30 MeOH/ACN	15 µL in heptane	Mode	APCI+
Injection mode	Loop injection	Feed solvent heptane (volume 4 µL; speed 1000 µL/min)	Mass range	100-1000 <i>m/z</i>
Column parameters	Phenomenex Luna Omega C18 (150 x 4.6 mm, 3 µm)	Torus 1-AA (100 x 3 mm, 1.7 µm)	Acquisition rate	0.67 spectra/s
Temperature	20 °C	40 °C	Transient	12241
BPR	N/A	140 bars	Drying Gas temp	325 °C
Flow rate	1.8 mL/min	1.2 mL/min	Drying gas	12 L/min
Mobile phase A	A : ACN	A : CO ₂	Nebulizer	50 psi
Mobile phase B	B : IPA	B : MeOH	Vaporizer temperature	350 °C
Gradient conditions	10 %-12.8 % B in 5.53 min, 12.8 % for 0.73 min, 12.8 % to 70 % B in 0.73 min, 70 % to 10 % in 0.73 min	5 %-12 % B in 5.44 min, 12 %-35 % B in 0.39 min, 35 %-5 % in 0.39 min	Capillary voltage	1000 V
Sampling time	0.192 min	N/A	Corona	4 µA
Make up solvent	N/A	MeOH; 200 µL/min	Fragmentor voltage	100 V
			Oct 1 RF	750 Vpp

168

169 For MS/MS, the parameters were adapted as follows: mass range 50-1000 *m/z*; acquisition rate
 170 2 spectra/s, transient 4000, collision energy 20 eV; max precursor selection 3. All other
 171 parameters remained the same.

172

173

174

175

176 III. Results and discussion

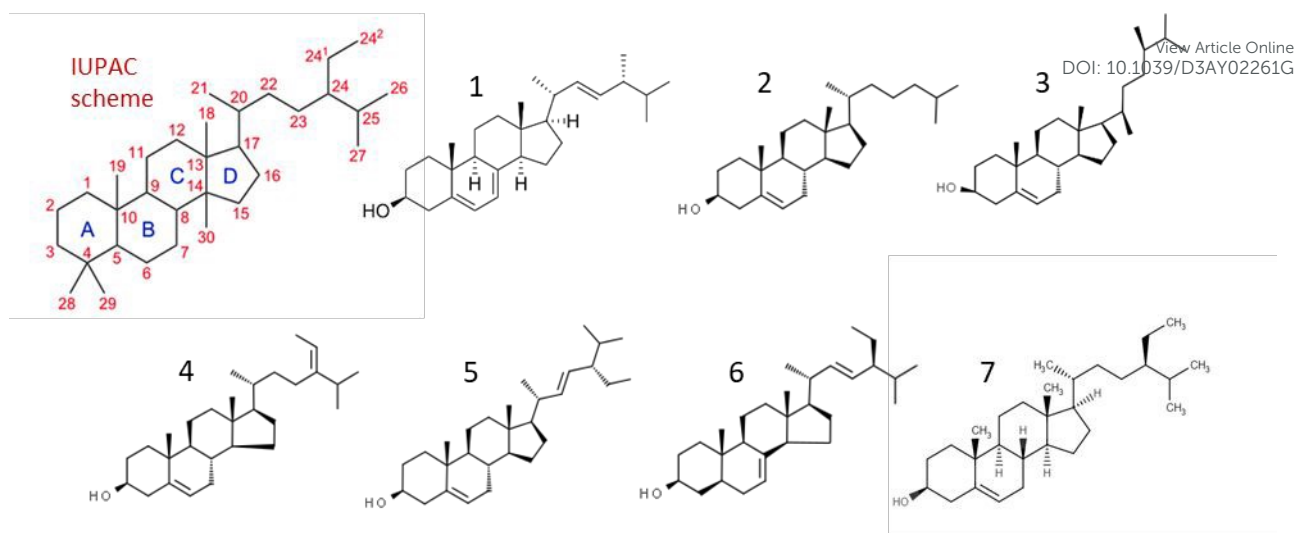
177

178 The LC and SFC chromatographic methods were developed using a representative mixture of
179 seven free sterols that can be found in microalgae. Figure 1 summarizes their name,
180 monoisotopic mass and provides the code that is used throughout the manuscript.

181

182 The seven sterol standards were selected not only due to their occurrence in algae, but mostly
183 to monitor the chromatographic selectivity induced by specific chemical structures (Fig. 1).
184 Hence, cholesterol (2), campesterol (3) and β -sitosterol (7) are structurally-related sterols
185 without any double bond in the side chain, but with increasing alkylation on the C24 position,
186 and they were incorporated in the mixture to monitor methylene selectivity. Stigmasterol (5)
187 and α -spinasterol (6) are positional isomers related to the double-bond position on the B-ring,
188 while ergosterol (1) exhibits a double bond at both position 5 and position 7. Finally, fucosterol
189 (4) and stigmasterol (5) are positional isomers related to the location of the double bond in the
190 side chain, in location C24-C24' and in location C22, respectively.

191



Code	Name	Monoisotopic mass (Da)	¹ t _R (min)	² t _R (min)
1	ergosterol	396.3392	4.96	3.86
2	cholesterol	386.3549	6.30	3.20
3	campesterol	400.3705	7.06	3.27
4	fucosterol	412.3705	6.11	3.49
5	stigmasterol	412.3705	7.27	3.47
6	α-spinasterol	412.3705	7.19	3.90
7	β-sitosterol	414.3862	8.21	3.32

192

193 **Fig. 1** Chemical structures of selected sterols, names, monoisotopic mass, and retention times

194 IV. Development of the first dimension

195 Due to the low polarity of the solutes, a non-aqueous reversed phase mechanism was selected
 196 as first dimension. Two different columns were screened, pentafluorophenyl (PFP) and C18,
 197 (Supplementary Information Fig. S1). The performance of the PFP column was fairly poor
 198 when using a non-aqueous gradient, with a low retention but also very low efficiency (30 %
 199 lower than in aqueous phase). A gradient performed in water/ACN conditions on that column
 200 showed that the selectivity between positional isomers 4/5/6 was interesting, but tailing peaks
 201 deteriorated the overall sterol separation. Noticeably, the selectivity of the positional isomers
 202 was different when using aqueous or non-aqueous mobile phase with PFP column, with the
 203 order 4/6/5 in the first case and 4/5/6 in the latest. The C18 column provided good peak shapes,
 204 and was able to distinguish isomers differing from the double bond position on the side chain,

1 205 i.e. fucosterol (4) and stigmasterol (5) but could not resolve isomers resulting from the position
2 206 of the double bond in the B-ring, i.e. stigmasterol (5) vs. α -spinasterol (6). Because isopropanol
3 207 has a high viscosity, an attempt to increase the column temperature was performed, but while
4 208 the mass transfer and so the peak width were improved, the resolution between critical pairs
5 209 decreased (Fig. S2). The best compromise between resolution and instrumental stability was to
6 210 set up the temperature to 20 °C. Gradient conditions (initial composition and normalized
7 211 gradient slope) were optimized using Osiris software which, from a limited number of
8 212 experiments (generally 3 to 4), determines the conditions providing maximal resolution on
9 213 critical pairs. The average slope of the retention model on the seven sterols was measured thanks
10 214 to isocratic experiments, at 1.71 ± 0.7 . The injection process was also optimized. The sterols
11 215 exhibited higher solubility in alcohols while the use of MeOH or isopropanol as diluent limited
12 216 the injection volume due to peak broadening (increased elution strength). The best compromise
13 217 was found by diluting the samples at 25 mg/mL in 70/30 MeOH/ACN. With this diluent, a
14 218 maximal volume of 60 μ L could be injected while keeping 80% of the chromatographic peak
15 219 capacity.

220

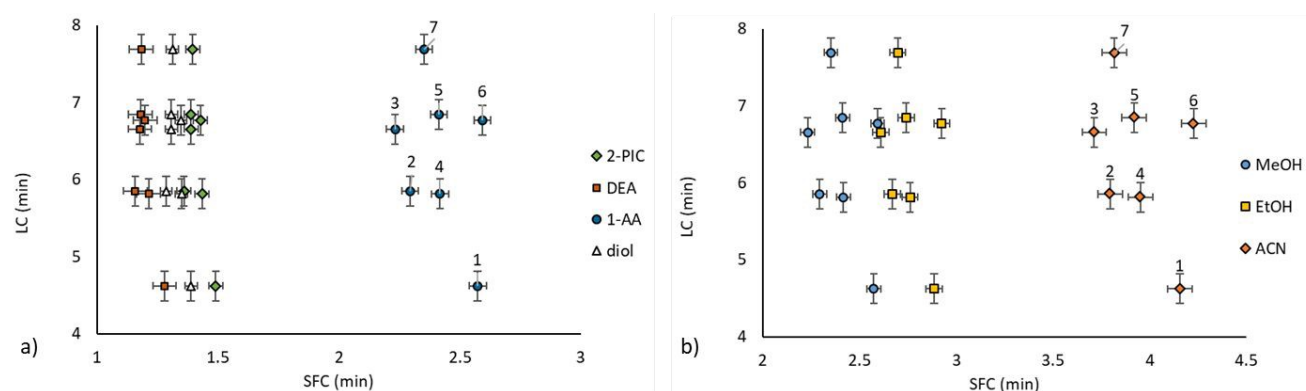
221 V. Development of the second dimension

222 The choice of SFC stationary phase and co-solvent was guided by the difference in selectivity
223 it could provide in regards to LC separation. Thus the retention times of sterol standards were
224 plotted against their retention time in the previously developed LC (Fig. 2a). While the retention
225 order was similar on the four stationary phases tested, the selectivity and the retention were
226 both higher when using 1-aminoanthracene. For example, isomers 5 and 6 exhibited a resolution
227 of 3.8 on 1-AA vs. around 1.0 on the other columns (see Fig. S3 for individual chromatograms),
228 which resulted from the strong ability of this stationary phase to generate π - π interactions, as
229 opposed to the other SFC columns tested¹⁷. The C18 LC column exhibited strong interactions
230 related to McGowan volume, allowing the separation according to the carbon number on the
231 side chain (homologs 2/3/7), but also distinguishing positional isomers based on the position of
232 the double bond in the side chain (isomers 4/5). On the other hand, the 1-AA SFC column
233 showed ability to generate π - π interactions which enabled the separation of sterols that coeluted
234 in LC (compounds 2/4), but most interestingly resolved the isomers based on the position of
235 the double bond in the B ring (isomers 5/6). While increasing the overall retention, ethanol and

236 ACN led to larger peaks (Fig. 2b), thus decreasing the resolution. MeOH was preferred as co-
 237 solvent.

View Article Online
 DOI: 10.1039/D3AY02261G

238



239

240 **Fig. 2** 2D-maps representing the LC retention times and the SFC retention times of sterols for
 241 a) four representative stationary phases using MeOH as co-solvent and b) three different
 242 cosolvents, using 1-AA as stationary phase. Gradient 5 % - 40 % in 5.53 min, 1.2 mL/min.
 243 Column geometry 3 x 100 mm; 1.7 μm. The bars represent the average baseline peak width
 244 (6σ)

245

246 Once again, the SFC conditions were optimized taking into account the critical pairs that may
 247 not be fully resolved by the LC dimensions. Despite a very narrow elution range, it appeared
 248 that a 0.5 % gradient steepness performed better than an isocratic elution mode (Fig. S4). As
 249 expected, increasing the gradient slope led to a decrease in resolution (experimentally measured
 250 using peak width at half-height).

251 The off-line configuration allowed the treatment of the LC fractions to remove the ^1D mobile
 252 phase, especially the protic solvent that generated deleterious injection effect on polar SFC
 253 stationary phase¹⁸. While MtBE and heptane are usually described as ideal injection solvent in
 254 SFC, the dilution of the fractions in heptane allowed to inject higher amounts on the column
 255 without peak broadening (Fig. S5). The injection volume was determined by monitoring the
 256 residual peak capacity when injecting increasing amount of the algae extract. For *Scenedesmus*
 257 *obliquus*, a 15 μL injection volume was found acceptable with less than 70 % peak capacity
 258 loss (Fig. S6). The same volume was injected for *Padina pavonica* and no peak broadening was
 259 observed.

VI. Optimisation of SFC-MS hyphenation

Because the eluent is composed of CO₂ mostly, precipitation in the transfer line to MS can occur after depressurization. To avoid this risk, a preBPR splitter with a sheath pump configuration [16] was set up. It consisted in the addition of a make-up flow after the split between BPR line and MS transfer line. The nature of the make-up has a direct impact on the MS response and should be adapted to the ionization process¹⁹. The evaluation of the efficiency of the ionization mode (ESI versus APCI) confirmed the observation made in LC-MS^{20,21} that a better response was obtained in APCI positive mode, which formed [M+H - H₂O]⁺ ions. The addition of a make-up in SFC-MS allows a fine tuning of the additives. Two alcohols and four additives were evaluated. The trend was similar to all sterol solutes, despite different extents (Fig. 3). Methanol improved the response compared to ethanol. This may result from its lower proton affinity (754.3 kJ/mol vs. 776.4 kJ/mol for ethanol²²), favoring the charge transfer to the solute during ionization process.

Additives were already found to have an effect on APCI, despite a gas phase ionization process²³. For sterols, the effect was rather negative (Fig. 3), with the addition of ammonium fluoride being the less deleterious and ammonia the worst, whatever the solute. The addition of small amounts of water resulted in 50-60 % loss of signal, which came as a surprise given that aqueous NH₄F was the best additives in SFC-APCI-MS when steroids were analyzed²⁴.

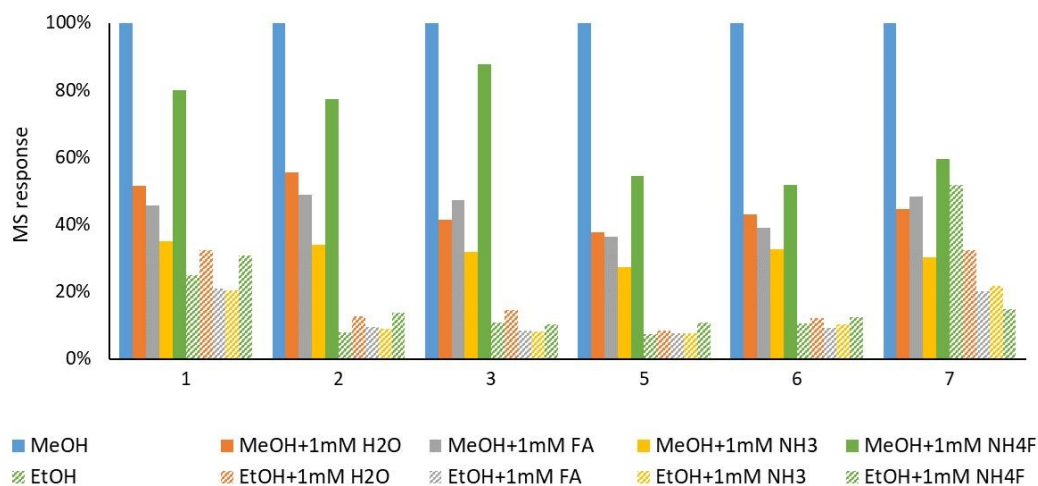


Fig. 3 APCI-MS response for various make-up solvents (full mark: methanol and hatched mark: ethanol) without additive or with additives. Solute number as coded in Table 2. 0.2 mL/min.

Downloaded from https://pubs.rsc.org on 05/07/2024 11:20:20 AM by University of Cambridge

283 APCI+ SIM mode, capillary voltage 3.0 kV, Corona current 4 μ A, fragm. 80 V. The peak
284 intensity was normalized with the intensity obtained using MeOH as make-up
285

View Article Online
DOI: 10.1039/D3AY02261G

286 **VII. Off-line LCxSFC-MS/MS of *Scenedesmus obliquus* unsaponifiable** 287 **fraction and appearance of a chromatographic pattern**

288 The LCxSFC method allowed the resolution of the seven standards (Fig. S7). Table 2 reports
289 the mean ¹D and ²D retention times for each sterol standard. The intermediate precision of the
290 method was evaluated by analyzing the mixture on three different days. The standard deviation
291 of ¹t_R was 0.02 min (intra-day) and 0.05 min (inter-days). The standard deviation of ²t_R was 0.9s
292 (intra-day) and 1.33s (inter-days). The detection window was defined as ¹t_r \pm 0.19 min
293 (corresponding to the LC modulation) and ²t_r \pm 4 s (corresponding to 3 σ).

294 The method was then applied to the unsaponifiable fraction of *Scenedesmus obliquus* freshwater
295 microalgae. The base peak chromatogram (Fig. 4a) highlighted the large orthogonality and peak
296 capacity provided by the method with over 67.5 % of the separation space occupied. The sterols
297 were retained in both dimensions (dashed area in Fig. 4a) which greatly limited co-elution with
298 non-sterol compounds, avoiding potential ion suppression in the MS source. The targeted
299 sterols were identified using the two retention times and associated detection window (Fig. 4b).
300 Moreover, the off-line configuration allowed the coupling of SFC with MS/MS. The identity
301 of the targeted sterols were confirmed by the MS/MS fragmentation pattern of the sterols. Most
302 fragments are generated from the cleavage of the C-ring. More information on fragmentation
303 pattern of sterols can be found in Mo et al. ¹⁰ and Munger et al. ²⁵. MS1 and MS2 spectra were
304 collected for each standard (data available in supplementary information Fig. S8), confirming
305 that isomers with modification on the side chain generate different fragments, while isomers in
306 the B-ring exhibited the same fragmentation. Interestingly, the non-target analysis (BPC, Fig.
307 4b) showed a chromatographic correlation between isomers. Isomers with the same exact mass
308 but different fragmentation patterns aligned with a nearly coelution in the SFC dimension. This
309 was the case of standards 4 (fucosterol) and 5 (stigmasterol) differing only by their side chain.
310 Following this pattern, it was easy to detect compound 2b, isomer of compound 2 (cholesterol),
311 for which databases did not provide any suspected structure. On the other hand, a trend appeared
312 on horizontal lines, with isomers co-eluting in the LC dimensions, such as pairs 7/7b, 5/6, 3/3b,
313 8a/8b. All these pairs of isomers exhibited the exact same fragmentation pattern (5 identical
314 major ions in MS2) and the selectivity between the pairs was constant (0.4 min corresponding

315 to a delta in composition at elution of 0.5 % MeOH). Because stigmasterol (5) and α -spinasterol
 316 (6) differed only on the position of the double-bond in the B-ring (Δ^5 for stigmasterol and Δ^7
 317 for α -spinasterol), we suggested that SFC dimension was able to distinguish this difference,
 318 while LC-MS/MS was blind on that aspect. To confirm such hypothesis, 2 rare sterols with a
 319 Δ^7 structure were injected: fungisterol, an isomer of campesterol (3) and schottenol, an isomer
 320 of β -sitosterol (7). Their retention times in both dimensions and MS1/MS2 spectra were found
 321 to be the same as peaks 3b and 7b, respectively, which ascertained both their presence in the
 322 *Scenedesmus obliquus* algae and the chromatographic pattern observed.

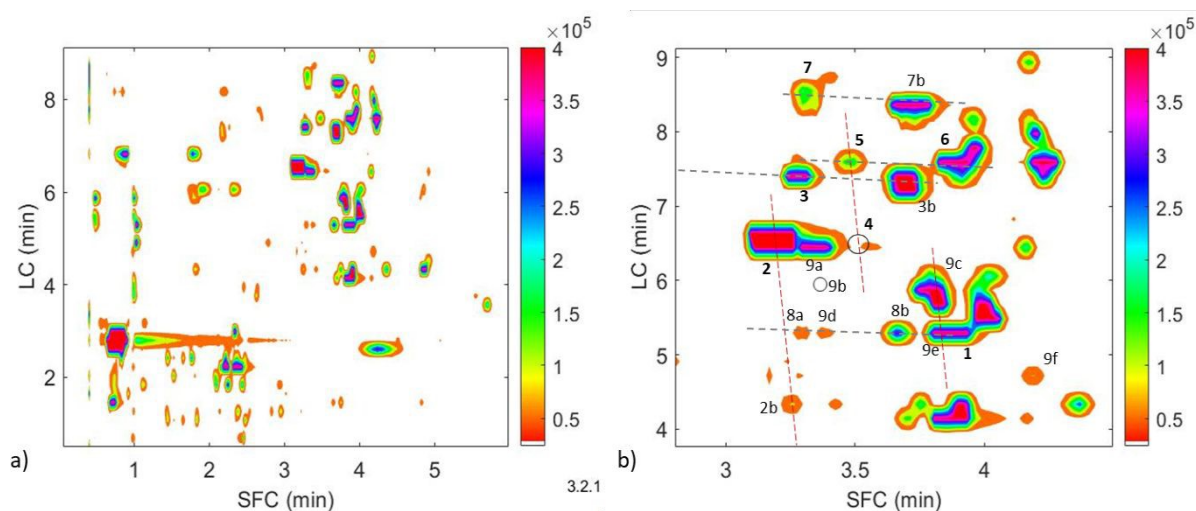


Fig. 4 Off-line LCxSFC-HRMS of *Scenedesmus obliquus* unsaponifiable extract. a) Base peak chromatogram with the elution area of sterols indicated. b) zoom on the sterol elution zone. Conditions in Table 2. Compounds 1 to 7 as in Table 1. Peaks 8 and 9 are unknown sterol isomers. Isomers with the same fragmentation pattern aligned on horizontal grey dashed lines, while isomers with different MS2 fragments aligned on vertical red dashed lines. Peaks described with a letter are isomers of the sterol bearing the same number.

This pattern can also be of use to reduce the number of candidates in the process of unknown sterol identification. During the untargeted analysis, two isomers noted 8a and 8b were detected (m/z 367.3367). They coeluted in LC dimension and exhibited the exact same MS/MS fragmentation pattern. According to the previous observations, it was hypothesized that they share the same side chain structure. Requesting MS Finder, three hits were provided, namely the structures (Δ^5 , Δ^7), (Δ^5 , Δ^{24}) and (Δ^7 , Δ^{24}). Based on SFC selectivity, we proposed that the least retained compound is a Δ^5 -sterol and the most retained a Δ^7 -sterol. Based on LC coelution, that they share the same side-chain structure and so that over the three suggested

1 340 candidates, one of the isomeric candidate structures could be deemed to be unlikely. Peak 8a
2 341 would be 5- α -cholesta-5,24-dien-3 β -ol, also known as desmosterol and peak 8b would
3 342 be its isomer 5- α -cholesta-7,24-dien-3 β -ol. Of course, the confidence in the identity
4 343 would require isolation before NMR analysis or injection of a commercial standard that
5 344 unfortunately is not available.

6 345 The same chromatographic pattern was also found with isomers bearing the m/z 381.3516,
7 346 noted 9. Extracting the corresponding EIC (Fig. S9) showed that 5 isomers were present in the
8 347 pattern (same B-ring or same side chain) and one was much more retained in SFC dimension.
9 348 One of the Δ^5 -sterol could correspond to brassicasterol or chalinasterol, two marine sterols, but
10 349 the number of potential structures suggested by MS Finder (over 34 candidates) was too large
11 350 to make any assumption.

351

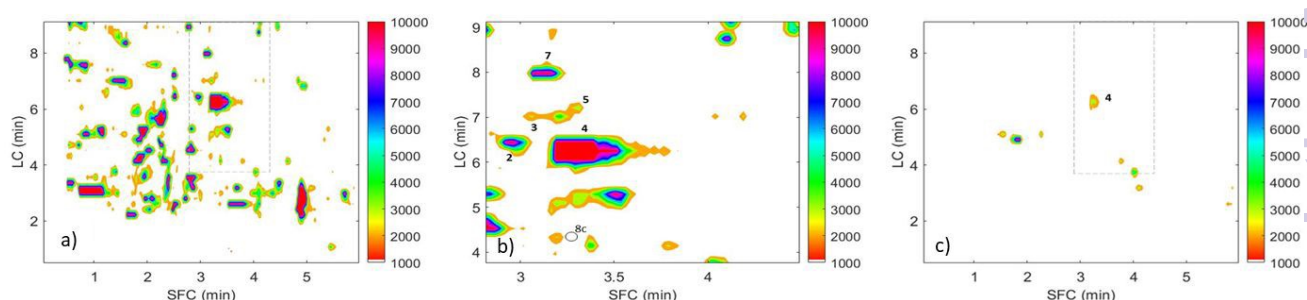
352

353 VIII. Off-line LCxSFC-MS/MS of marine algae *Padina pavonica*

354

355 *Padina pavonica* is a marine brown macroalgae cultivated in warm seawaters. A recent study
356 has shown that the acetone extract of *Padina pavonica* exhibits anti-aggregator biological
357 activities that may potentially be of interest in the protection of mitochondrial membrane, a
358 mechanism involved in neurodegenerative diseases ²⁶. The chromatographic profile of its
359 unsaponifiable fraction exhibited a wide variety of molecules (Fig. 5a), but a lower number of
360 free sterols compared to UTEX sample.

361



362

363 **Fig. 5** Off-line LCxSFC-HRMS of *Padina pavonica* unsaponifiable extract. a) Base peak
364 chromatogram with the elution area of sterols indicated in dashed line. b) Zoom on the sterol
365 elution zone. c) Extracted ion chromatogram of m/z 411.36295. Conditions in Table 2.

366

367

368

369

370

1 366 Compound numbering as in Table 1. The position of peak 8c is indicated, but only visible when
2 367 extracting the corresponding EIC (available in supplementary material Fig S10). View Article Online
DOI: 10.1039/D3AY02261G

3 368
4
5
6 369 Cholesterol (2), fucosterol (4) and β -sitosterol (7) were the main sterols detected (Fig. 5b), with
7
8 370 a predominance of fucosterol as reported in all *Phaeophyta phylum*²⁷. Reported by Kaniyas et
9
10 371 al in 1992, campesterol (3) and stigmasterol (5) were present in low amount in the extract²⁸.
11 372 The presence of β -sitosterol (7) was not reported so far, maybe because most prior analyses
12 373 were carried out using GC-MS, where β -sitosterol and fucosterol often coeluted²⁹. Kamenarska
13 374 et al.³⁰ also reported in *Padina pavonica* the presence of two rare sterols with raw formulae
14 375 $C_{27}H_{44}O$, one being 22E-cholesta-5,22-dien-3ol (a.k.a. 22E-dehydrocholesterol) and the other
15 376 one being either 22Z-cholesta-5,22-dien-3ol (a.k.a. 22Z-dehydrocholesterol) or 27-nor-24-
16 377 methyl-cholesta-5,22-dien-3ol (i.e. ocellasterol). The two latest could not be distinguished by
17 378 GC-MS, since they had the same GC retention and mass spectra. In the present work, the main
18 379 ion of these molecules (m/z 367.3367) was found in the macroalgae extract, with only one peak
19 380 (noted 8c in Fig.5b) and a very low response, only visible on Extracted Ion Chromatogram (Fig
20 381 S10). This peak eluted in SFC as a Δ^5 -sterol, isomer of peaks 8a and 8b observed in
21 382 *Scenedesmus obliquus* sample, but the peak detected here exhibited a different LC retention and
22 383 fragmentation pattern. In the present work, we could not distinguish Z/E conformers, and so the
23 384 two conformers of 22-dehydrocholesterol may co-elute. On the other hand, ocellasterol, a
24 385 positional isomer having a branched side chain, should elute at a different time and have a
25 386 different MS/MS fragmentation pattern. Unfortunately, no standards are available and
26 387 fragmentation patterns lack in the databases to help the identification. In the EIC chromatogram
27 388 of m/z 367.3367 (Fig S10), only one peak was visible, clearly stating that the compounds 22-
28 389 dehydrocholesterol and ocellasterol do not co-exist in the sample. So either our sample
29 390 contained only one unknown isomer or the mixture of the conformers 22-dehydrosterol. The
30 391 example shows that GC-MS and LCxSFC-MS could bring complementary information on
31 392 isomer suspected structures.

32 393
33 394 The analysis of *Padina pavonica* unsaponifiable extract method also revealed the presence of
34 395 isobars of free sterols outside the selected elution zone. For example, ion at m/z 411.36295
35 396 exhibited numerous peaks, as shown in Fig. 5c, including the minor ion designated $[M + H -$
36 397 $2H]^+$ of fucosterol (4)²⁰. This could reflect the presence of conjugated sterols that were left
37 398 intact during the saponification process but fragmented in the APCI source. Steryl glycosides

399 were suspected from the lower LC retention. Indeed, the sugar moiety of sterol glycoside is not
400 hydrolysed upon alkaline conditions³¹ and leads to a lower retention than free sterol in RPLC
401³². The formation of aglycone ions from SG [SG-Glc+H]⁺ is a result of glycosidic bond cleavage
402 during in-source fragmentation. The MS spectra of sterol glycosides usually correspond to
403 [Aglycone+H-H₂O]⁺, and [Aglycone+H]⁺, which was the case in Fig. 5c. Lower and higher
404 retention of suspected conjugated sterols in the SFC retention may come from the presence of
405 intact sterol glycosides or sterol glycosides released from acylated sterol glycosides. These
406 hypotheses have to be confirmed by standard injection but may open opportunities to detect
407 simultaneously free sterols and conjugated sterols.

IX. Conclusion

411 Algae represent a source of high-value phytosterols that is yet to be exploited. Identification of
412 known sterols with high level of confidence, but also discovery of unknown sterols specific to
413 lower plants requires a powerful analytical technique able to decipher high structural similarity.
414 The methods involving one chromatographic technique coupled with high-resolution mass
415 spectrometry may confuse positional isomers that are numerous in such samples. To tackle this
416 isomeric issue, to confirm the identity of targeted sterols with the highest confidence possible
417 and to enable discovery of new sterols, an LCxSFC-MS/MS method was considered. Using a
418 mixture of seven sterols, each chromatographic dimension was optimized taking into account
419 the orthogonality of the combination, then the SFC-APCI hyphenation was optimized.
420 The developed method was able to provide an annotation of sterols with 4 dimensions: two
421 retention times (¹tr ± 0.19 min, ²tr ± 4 s), a parent ion (*m/z* ± 2 ppm) and a fragmentation pattern
422 (5 MS² ions). Moreover a structural information could be hypothesized according to the
423 unknown sterol position towards a known sterol isomer, whether the isomeric difference is
424 located in the side-chain (separated by LC) or in the B-ring (resolved by SFC).
425 The analysis of an extract from *Scenedesmus obliquus* showed the presence of the seven
426 targeted sterols, three isomers of the targeted sterols and many unidentified sterols among which
427 isomers have been separated. The analysis of an extract of *Padina pavonica* exhibited little
428 variety among free sterols, mainly fucosterol, with cholesterol and β-sitosterol in lower
429 amounts. Interestingly, the presence of peaks with the same MS/MS fragmentation pattern at
430 much lower retention times raised suspicion on the presence of conjugated sterols in the extract.

431 It would be of interest to check that the method could be extended to conjugated sterols if
432 hydrolysis was avoided during sample preparation.
433 Identification of high-value sterols could be an important step in the increase value of algae in
434 a biorefinery context and this analytical method, providing important selectivity toward the
435 similar chemical structures, could be of great help.

View Article Online
DOI: 10.1039/D3AY02261G

436

437

438 **Statements and Declarations**

439 Partial financial support was received from Extrasynthese company (Genay, France).

440 The authors have no competing interests to declare that are relevant to the content of this article.

441

- 442 1. R.A. Moreau, *et al.*, *Prog. Lipid Res.*, 2018. 70: 35-61. DOI: <https://doi.org/10.1016/j.plipres.2018.04.001>.
- 443 2. X. Luo, *et al.*, *Mar. Drugs*, 2015. 13(7): 4231-4254. DOI: 10.3390/md13074231.
- 444 3. G. Wang and T. Wang, *Journal of the American Oil Chemists' Society*, 2012. 89(1): 135-143. DOI: doi:10.1007/s11746-011-1879-8.
- 445 4. K.N. Gangadhar, *et al.*, *Open Chem.*, 2016. 14(1): 299-307. DOI: 10.1515/chem-2016-0029.
- 446 5. A. Randhir, *et al.*, *Algal Research*, 2020. 46: 101772. DOI: <https://doi.org/10.1016/j.algal.2019.101772>.
- 447 6. L. Yao, *et al.*, *Journal of Agricultural and Food Chemistry*, 2015. 63(6): 1773-1787. DOI: 10.1021/jf5050603.
- 448 7. H. Kiani, *et al.*, *Frontiers in Energy Research*, 2022. 10. DOI: 10.3389/fenrg.2022.904014.
- 449 8. G. Gachumi and A. El-Aneed, *Journal of Agricultural and Food Chemistry*, 2017. 65(47): 10141-10156. DOI:
450 10.1021/acs.jafc.7b03785.
- 451 9. M.A. Ziemann dos Santos, *et al.*, *Austin Chromatography* 2014. 1(5): 1021.
- 452 10. S. Mo, *et al.*, *Lipids*, 2013. 48(9): 949-956. DOI: 10.1007/s11745-013-3813-3.
- 453 11. G. Simonetti, *et al.*, *Food Chemistry*, 2021. 340: 127926 DOI: <https://doi.org/10.1016/j.foodchem.2020.127926>.
- 454 12. N.L. Qi, *et al.*, *Journal of Food and Nutrition Research*, 2019. 58(4): 363-369.
- 455 13. J.M. Snyder, *et al.*, *Journal of the American Oil Chemists' Society*, 1999. 76(6): 717-721. DOI: <https://doi.org/10.1007/s11746-999-0165-5>.
- 456 14. J.-J. Zhang, *et al.*, *Journal of Agricultural and Food Chemistry*, 2023. 71(43): 16371-16380. DOI: 10.1021/acs.jafc.3c05857.
- 457 15. J.B.S.K. Jayantha, *et al.*, *Journal of Chromatography B*, 2023. 1225: 123737. DOI: <https://doi.org/10.1016/j.jchromb.2023.123737>.
- 458 16. A. Vaniya, *et al.*, *Phytochemistry Letters*, 2017. 21: 306-312. DOI: <https://doi.org/10.1016/j.phytol.2016.12.008>.
- 459 17. C. West, *et al.*, *Journal of Chromatography A*, 2016. 1440: 212-228 DOI: 10.1016/j.chroma.2016.02.052.
- 460 18. M. Batteau and K. Faure, *Journal of Chromatography A*, 2022. 1673: 463056. DOI: <https://doi.org/10.1016/j.chroma.2022.463056>.
- 461 19. K. Plachká, *et al.*, *Analytical Chemistry*, 2022. 94(11): 4841-4849. DOI: 10.1021/acs.analchem.2c00154.
- 462 20. G. Gachumi, *et al.*, *Molecules*, 2021. 26(5). DOI: 10.3390/molecules26051402.
- 463 21. B. Cañabate-Díaz, *et al.*, *Food Chemistry*, 2007. 102(3): 593-598. DOI: <https://doi.org/10.1016/j.foodchem.2006.05.038>.
- 464 22. E.P.L. Hunter and S.G. Lias, *Journal of Physical and Chemical Reference Data*, 1998. 27(3): 413-656. DOI: 10.1063/1.556018.
- 465 23. L. Akbal and G. Hopfgartner, *Analytical & Bioanalytical Chemistry*, 2020. 412(25): 6667-6677. DOI: 10.1007/s00216-020-02715-4.
- 466 24. M.K. Parr, *et al.*, *Journal of Chromatography B*, 2018. 1091: 67-78. DOI: <https://doi.org/10.1016/j.jchromb.2018.05.017>.
- 467 25. L.H. Münger, *et al.*, *Frontiers in Chemistry*, 2018. 6(342). DOI: 10.3389/fchem.2018.00342.
- 468 26. M. Caruana, *et al.*, *Molecules*, 2021. 26(5). DOI: 10.3390/molecules26051444.
- 469 27. G. Lopes, *et al.*, *Journal of Phycology*, 2011. 47(5): 1210-1218. DOI: <https://doi.org/10.1111/j.1529-8817.2011.01028.x>.
- 470 28. G.D. Kanas, *et al.*, *Fresenius' Journal of Analytical Chemistry*, 1992. 344(7): 334-339. DOI: 10.1007/BF00321843.

19

1 473 29. R.M. dos Santos MAZ, de Pereira CMP, Freitag RA and de Bairros AV. , *Austin Chromatogr.* , 2014. 1(5): 4.
2 474 30. Z. Kamenarska, *et al.*, 2002. 45(4): 339-345.DOI: doi:10.1515/BOT.2002.034.
3 475 31. J. Toivo, *et al.*, *Food Chemistry*, 2000. 68(2): 239-245.DOI: [https://doi.org/10.1016/S0308-8146\(99\)00201-0](https://doi.org/10.1016/S0308-8146(99)00201-0). DOI: 10.1039/D3AY02261G
4 476 32. R. Rozenberg, *et al.*, *Journal of Cereal Science*, 2003. 38(2): 189-197.DOI: [https://doi.org/10.1016/S0733-5210\(03\)00022-5](https://doi.org/10.1016/S0733-5210(03)00022-5).
5
6
7 477
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

View Article Online
DOI: 10.1039/D3AY02261G

Analytical Methods Accepted Manuscript