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Furanolysis with menthofuran: A new depolymerization method for analyzing condensed tannins

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1 **ABSTRACT**

2 An improved analytical depolymerization method for characterizing condensed tannins was
3 developed with menthofuran (3,6-dimethyl-4,5,6,7-tetrahydro-1-benzofuran) as the
4 nucleophilic trapping reagent. Herein, menthofuran was compared with routinely used
5 nucleophiles, phloroglucinol and 2-mercaptoethanol. At 30°C and in the presence of 0.1 M
6 HCl, menthofuran displayed the outstanding ability to enable the fast and full
7 depolymerization of procyanidin B2 using only a 1:1 molar ratio of both reactants. In the same
8 conditions, phloroglucinol and 2-mercaptoethanol led to a reaction equilibrium with
9 significantly lower conversion yields. Application to commercial tannin extracts showed that
10 a menthofuran to extract weight ratio of 1 gave the same yields of procyanidin constitutive
11 units as 10-fold higher mol. eq. phloroglucinol and 100-fold 2-mercaptoethanol. Finally,
12 guidelines for implementing the menthofuran depolymerization method are proposed to
13 assess the tannin content and composition of extracts as well as of plant materials without
14 prior extraction.

15

16 **KEYWORDS**

17 Menthofuran, Furan derivatives, Condensed tannins, Depolymerization, Analytical method,
18 Furylated flavonoids, UHPLC-DAD-MS

19 INTRODUCTION

20 Condensed tannins (proanthocyanidins) are polymers of flavan-3-ol units. The constitutive
21 units are covalently linked by interflavan bonds between the phloroglucinol ring (C8 and/or
22 C6 carbon atoms) and the benzylic C4 carbon atom of the extension units, thus resulting in B-
23 type proanthocyanidins (Scheme 1 for carbon numbering).^{1,2} Additional linkages resulting
24 from oxidation processes can lead to A-type proanthocyanidins, or biaryl and biaryl ether
25 linked compounds.² Condensed tannins, along with hydrolyzable tannins and phlorotannins,
26 constitute the polyphenolic secondary metabolite class of tannins.³ These plant polyphenols
27 represent the fourth most abundant organic polymer in the terrestrial biomass and the second
28 one, after lignin, when considering only aromatic polymers.^{4,5}

29 The need to characterize and quantify the condensed tannin fraction from plant or food
30 samples generally meets three objectives. First, characterization of condensed tannins can be
31 used in taxonomy or structure-function relationship studies,⁶ including response to
32 environmental stresses.^{7,8} The second objective is to qualify plant extracts or tannin-rich
33 formulations eventually intended for commercial products, such as cosmetics and dietary
34 supplements, in relation to health benefits including antioxidant activities.⁹⁻¹¹ The third
35 motivation is to develop specialty chemicals or polymer materials from renewable phenolics
36 accordingly to the properties and specificities of the different types of tannins.¹²⁻¹⁴

37 Non-degradative and degradative methods have been developed to characterize the
38 condensed tannin fraction of plant materials. Among the former, colorimetric assays based on
39 redox reactions are not specific to polyphenols and tannins and should be interpreted with
40 caution.¹⁵ Methods based on ¹H NMR¹⁶ and ¹³C NMR,¹⁷ 2D ¹H-¹³C HSQC NMR¹⁸ spectroscopy,
41 as well as on mass spectrometry with electrospray ionization¹⁹ and MALDI-TOF systems,²⁰
42 have been developed with the advances of technologies. These methods provide good

43 information on the nature of constitutive units, types of linkages and degrees of
44 polymerization of tannin structures. The limitations of these methods mainly result from the
45 dispersity of tannin polymers associated with a possible discrimination against highly
46 polymerized structures.²¹

47 On the other hand, degradative methods are based on a chemical depolymerization reaction
48 leading to the release of two kinds of constitutive units: the terminal units, with free C4 carbon
49 atom, and the extension units, where the C4 position is linked to the next unit of the polymeric
50 chains (Scheme 1). The depolymerization products can then be analyzed by chromatography
51 to infer characteristics of the initial tannin structures, including the type and amounts of
52 constitutive units, mean degree of polymerization and galloylation degree. Depolymerization-
53 based methods are currently the most informative methods to characterize condensed
54 tannins. They have recently been shown to give results consistent with NMR and MALDI
55 analyses.²² Recent developments based on proanthocyanidin in-source fragmentation and
56 mass spectrometry analysis also gave similar results in terms of composition and mean degree
57 of polymerization of oligomeric and polymeric tannin fractions as chemical
58 depolymerization.²³ However, some information remains inaccessible to depolymerization-
59 based methods, such as the molecular weight distribution of the tannin fractions or the
60 sequences of constitutive units in the polymeric chains beyond hexamers, owing to their too
61 low content in the samples.²⁴ Moreover, some linkages are much more stable with respect to
62 cleavage, such as interflavan linkages of 5-deoxy condensed tannins found in quebracho and
63 acacia,²⁵ while others are totally resistant, like the A-type patterns²⁶ and biaryl or biaryl ether
64 linkages resulting from oxidation.²⁷

65 Since the first report by Betts et al.,²⁸ different depolymerization-based methods have been
66 developed,^{29–31} compared,^{4,32} and applied to characterize the condensed tannin fraction in

67 plant extracts or food samples.^{21,33,34} Updates and improvements of these methods are still
68 regularly published.³⁵⁻³⁷ The first nucleophiles used to trap the extension units released by the
69 acid-catalyzed depolymerization of condensed tannins were mercaptans, such as thioglycolic
70 acid and benzylmercaptan.^{22,28,34,38} Later works proposed to substitute mercaptans by analogs
71 of the catechin A-ring (e.g., phloroglucinol, 2,4,6-trihydroxytoluene or resorcinol).^{30,39} Ever
72 since, these methods have evolved with analytical techniques and useful optimizations were
73 performed on reaction conditions, solvents and work-up, but no significant breakthrough was
74 achieved regarding the reactants. Indeed, the typical smell of mercaptans has often been an
75 obstacle to their use in the analysis of condensed tannins.³⁰ The toxicity of the chemicals
76 involved in the depolymerization methods is more generally questioned, owing to the fact
77 that the trapping nucleophiles are used in large excess. This especially concerns mercaptans
78 frequently used in the analysis of proanthocyanidins,^{40,41} while phloroglucinol seems to
79 require higher doses to cause adverse effects.⁴²

80 Recently, the possibility to use metalloles (five-membered heterocyclic aromatic compounds)
81 in the depolymerization of condensed tannins to produce biobased chiral ligands or fully
82 biobased aromatic building blocks for applications in specialty chemicals and materials was
83 evidenced by Fu et al.⁴³ with pyrrole derivatives and by Rouméas et al.^{44,45} with furan
84 derivatives. In the framework of our studies on the depolymerization of condensed tannins
85 in the presence of substituted metalloles, preliminary experiments have led us to identify
86 menthofuran (3,6-dimethyl-4,5,6,7-tetrahydro-1-benzofuran), a tri-substituted furan, as both
87 an efficient and commercially available nucleophilic trapping reagent. Menthofuran, a major
88 component of essential oils such as pennyroyal oil, has been the subject of numerous
89 toxicological studies.^{46,47} It is used as a flavoring agent (strong peppermint odor) in the food
90 industry at a concentration up to 1000 ppm (i.e., in the same order of magnitude as in the

91 method described herein).^{48,49} This led us to evaluate it as a potential new reagent for the
92 analytic depolymerization of condensed tannins. In the present work, menthofuran was
93 compared to phloroglucinol and 2-mercaptoethanol, that are routinely used in standard
94 depolymerization methods.^{30,31}

95

96 **MATERIAL AND METHODS**

97 **Chemicals**

98 The grape seed extract was purchased from Union des Distilleries de la Méditerranée (UDM,
99 France). Pycnogenol, a commercial tannin bark extract from maritime pine (*Pinus pinaster*
100 Aiton subsp. *atlantica* syn. *P. maritima*), was kindly offered by Horphag Research (Geneva,
101 Switzerland). Samples of grape pericarp powder (*Vitis vinifera* L. subsp. *sativa* (DC.) Hegi,
102 cultivar Savagnin), prepared as previously published⁵⁰ were kindly supplied by Dr. Charles
103 Romieu. Bark from Douglas fir (*Pseudotsuga menziesii*), kindly provided by Brassac Industrie
104 sawmill (Brassac, France), was obtained from trees felled in March 2015 on a plot located at
105 43° 33' 39.5'' N, 2° 42' 14.0'' E (altitude 936 m) and debarked in April 2015 under batch
106 reference "chantier Caraman n°UG 47109". The bark was ground by knife milling using a
107 Retsch SM 100 system operating at room temperature at a speed of 1500 rpm with a 2 mm
108 size screen. Menthofuran (3,6-dimethyl-4,5,6,7-tetrahydro-1-benzofuran, ≥95%) and 2-
109 mercaptoethanol (>99%) were purchased from Sigma-Aldrich (France). Phloroglucinol (>99%)
110 was purchased from Merck (France). Procyanidin B2 (≥90%), (-)-epicatechin (≥99%), (+)-
111 catechin (≥99%), (-)-epicatechin-3-*O*-gallate (≥97.5%), (-)-epigallocatechin (≥98%) and
112 (-)-epigallocatechin-3-*O*-gallate (≥98%) were purchased from Extrasynthese (France).

113 **Depolymerization experiments**

114 All depolymerization experiments described in the following sections were performed in three
115 independent replicates.

116 **Depolymerization of procyanidin B2 with 1 molar equivalent of nucleophile.** Owing to the
117 purity of the commercial B2 sample ($\geq 90\%$), a B2 solution was first prepared with approximate
118 concentration. After determination of the concentration by measuring the peak area at
119 280 nm with the UHPLC-DAD-MS system, the B2 solution was then precisely adjusted to 1.05
120 mM by addition of methanol. For each depolymerization kinetics, equal volumes of
121 methanolic solutions of procyanidin B2 (1.05 mM), nucleophile (1.05 mM) and hydrochloric
122 acid (HCl, 0.3 M) were mixed and distributed in 8 vials, which were immediately sealed and
123 incubated at 30°C. Vials were withdrawn at different times and directly analyzed by UHPLC-
124 DAD-MS.

125 **Depolymerization of procyanidins from a grape seed extract with optimized amounts of**
126 **nucleophiles.** Methanolic solutions of phloroglucinol (30 g·L⁻¹; 0.24 M), 2-mercaptoethanol
127 (165 g·L⁻¹; 2.1 M, prepared as a 15:85 v/v 2-mercaptoethanol/methanol mixture) and
128 menthofuran (3 g·L⁻¹; 0.020 M) were prepared. For each depolymerization kinetics, equal
129 volumes of methanolic solutions of grape seed extract (3 g·L⁻¹), nucleophile and HCl (0.3 M)
130 were mixed and distributed in 10 vials, which were immediately sealed and incubated at 30°C.
131 Vials were withdrawn at different times and directly analyzed by UHPLC-DAD-MS.

132 **Characterization of pycnogenol with the menthofuran method.** For each depolymerization
133 kinetics, equal volumes of methanolic solutions of pycnogenol (3 g·L⁻¹), menthofuran (3 g·L⁻¹;
134 0.020 M) and HCl (0.3 M) were mixed and distributed in 3 vials, one for each reaction time,
135 which were immediately sealed and incubated at 30°C. Vials were withdrawn after 90, 120
136 and 150 min and directly analyzed by UHPLC-DAD-MS.

137 **Characterization of pycnogenol with the phloroglucinol method.** The analysis was based on
138 the protocol proposed by Kennedy and Jones.³⁰ Methanolic solutions of pycnogenol (15 g·L⁻¹),
139 ascorbic acid (30 g·L⁻¹; 0.39 M) and HCl (0.3 M) were prepared. For each depolymerization
140 experiment, the pycnogenol solution was used to solubilize phloroglucinol (150 g·L⁻¹; 1.2 M).
141 Immediately after, equal volumes of ascorbic acid, HCl and pycnogenol/phloroglucinol
142 solutions were mixed and the depolymerization solution was incubated at 50°C in a closed
143 flask. The final pycnogenol concentration was thus 5 g·L⁻¹. At 20 min of reaction time, the
144 depolymerization solution was mixed with five volumes of an aqueous solution of sodium
145 acetate (40 mM). The final solution was directly analyzed by UHPLC-DAD-MS.

146 **Characterization of grape pericarp powder with the menthofuran method.** A sample of grape
147 pericarp powder (18 mg) was suspended in 0.6 mL of methanol in a closed flask. Then,
148 methanolic solutions of menthofuran (0.6 mL; 30 g·L⁻¹; 0.24 M) and HCl (0.6 mL; 0.3 M) were
149 added. The closed flask containing the depolymerization solution was incubated at 30°C. At
150 2h of reaction time, a sample was withdrawn, centrifuged 1 min at 3000 x *g*, filtrated and
151 directly analyzed by UHPLC-DAD-MS.

152 **Characterization of grape pericarp powder with the 2-mercaptoethanol method.** The
153 analysis was based on the protocol proposed by Tanaka et al.³¹ A sample of grape pericarp
154 powder (18 mg) was suspended in 0.6 mL of methanol in a closed flask. Then, methanolic
155 solutions of 2-mercaptoethanol (0.6 mL; 165 g·L⁻¹; 2.1 M) and HCl (0.6 mL; 0.3 M) were added.
156 The closed flask containing the depolymerization solution was incubated at 40°C. At 2h of
157 reaction time, a sample was withdrawn, centrifuged 1 min at 3000 x *g*, filtrated and directly
158 analyzed by UHPLC-DAD-MS.

159 **Characterization of the procyanidins from Douglas fir barks with the menthofuran method.**
160 500 mg of Douglas fir barks ground and sieved at 2 mm were suspended in 50 mL methanol

161 (10 g·L⁻¹). Then, menthofuran (94 μL; final concentration 2 g·L⁻¹; 0.013 M) and HCl (417 μL;
162 final concentration 0.1 M) were added and the depolymerization medium was incubated at
163 30°C. At defined time intervals, a sample was withdrawn, centrifuged 1 min at 3000 x *g*,
164 filtrated and directly analyzed by UHPLC-DAD-MS.

165 **Characterization of the procyanidins from Douglas fir barks with the 2-mercaptoethanol**
166 **method.** Douglas fir barks ground and sieved at 2 mm (500 mg) were suspended in 47 mL
167 methanol. Then, 2-mercaptoethanol (2.5 mL; final concentration 5:95 v/v) and HCl (417 μL;
168 final concentration 0.1 M) were added and the depolymerization mixture obtained was
169 incubated at 40°C. At defined time intervals, a sample was withdrawn, centrifuged 1 min at
170 3000 x *g*, filtrated and directly analyzed by UHPLC-DAD-MS.

171 **Preparation, isolation and characterization of epicatechin-menthofuran (EC-MF)**

172 The grape seed extract (20 g) was dissolved in methanol (280 mL). Menthofuran (21.0 mL;
173 0.136 mol) and HCl (4.17 mL of 37% HCl in 200 mL methanol) were added. The reaction was
174 performed for 1h at 30°C under magnetic stirring. The medium was then neutralized with a
175 solution of sodium hydrogenocarbonate (4.2 g) in water (700 mL). Methanol was evaporated
176 under vacuum. The remaining aqueous phase was extracted with ethyl acetate (3 times 500
177 mL). The organic layers were gathered, dried with sodium sulfate, filtrated and evaporated
178 under vacuum. The dark powder obtained (27 g) was triturated and sonicated for 5 min in
179 diethyl ether (3 times 300 mL). The diethyl ether fractions were pooled, dried with sodium
180 sulfate, filtrated and evaporated under vacuum. Remaining traces of menthofuran were
181 eliminated by trituration in petroleum ether (3 times 100 mL). A purple powder was obtained
182 (21 g) containing C, EC, ECG, C-MF, EC-MF and ECG-MF (see the abbreviation section and
183 Scheme 1). A sample (1 g) was purified by flash chromatography on a PF430 system (Interchim,
184 France) equipped with a silica gel column (120 g; granulometry 63-200 μm). The flow rate was

185 set to 40 mL·min⁻¹ and the gradient was: solvent A (CH₂Cl₂), solvent B (CH₂Cl₂–CH₃OH, 90:10,
186 v/v); 0–2 min, 0% B (isocratic); 2–15 min, 0% to 50% B (linear gradient); 15–28 min, 50%–80%
187 B (linear gradient); and 28–45 min, 80% B (isocratic). Fractions were analyzed by UHPLC-DAD-
188 MS before being combined and evaporated under vacuum to yield a pale purple pulverulent
189 solid (80 mg) containing EC-MF (> 90 % purity according to UHPLC analyses). For NMR
190 characterization, EC-MF was dissolved in d₆-DMSO.

191 1D and 2D NMR spectra acquisitions were performed at 25°C with an Avance III HD NMR
192 spectrometer (Bruker, Germany) at 500 MHz for ¹H and 126 MHz for ¹³C. HRMS spectrum was
193 acquired on a MicroTof QII mass spectrometer (Bruker) using the TOF MS ES+ mode, with
194 samples dissolved in MeOH. Spectra are provided as supporting information (Figures S1 to S5).

195 *Epicatchin-(4→5)-menthofuran*. ¹H NMR δ (ppm): 9.12 (1H, s, H₁₀), 9.04 (1H, s, H₁₁), 8.89 (1H,
196 s, H_{8'}), 8.77 (1H, s, H_{7'}), 6.85 (1H, s, H_{2'}), 6.68 (1H, d, *J* = 8.3 Hz, H_{5'}), 6.58 (1H, d, *J* = 8.3 Hz, H_{6'}),
197 5.87 (1H, s, H₈), 5.78 (1H, s, H₆), 5.05 (1H, bs, H₉), 4.77 (1H, s, H₂), 4.05 (1H, s, H₄), 3.78 (1H, bs,
198 H₃), 2.56 (1H, m, H_{7''}), 2.27 (2H, m, H_{4''}), 2.10 (1H, m, H_{7''}), 1.84 (1H, m, H_{6''}), 1.77 (1H, m, H_{5''}),
199 1.67 (3H, s, H_{8''}), 1.28 (1H, m, H_{5''}), 1.03 (3H, d, *J* = 6.6 Hz, H_{9''}). ¹³C NMR δ (ppm): 157.2 (C₇),
200 156.9 (C₅), 156.0 (C_{8a}), 148.3 (C_{2''}), 147.0 (C_{7a''}), 144.7 (C_{3'}), 144.6 (C_{4'}), 130.3 (C_{1'}), 118.0 (C_{3a''}),
201 117.7 (C_{6'}), 114.9 (C_{2'}), 114.8 (C_{5'}), 113.9 (C_{3''}), 98.3 (C_{4a}), 95.2 (C₈), 94.0 (C₆), 75.1 (C₂), 69.5
202 (C₃), 37.9 (C₄), 31.9 (C_{5''}), 30.8 (C_{7''}), 29.2 (C_{6''}), 21.5 (C_{9''}), 19.6 (C_{4''}), 7.8 (C_{8''}).

203 HRMS (ESI): [M+H]⁺ found with *m/z* 439.1751 (calculated for C₂₅H₂₇O₇: 439.1751).

204 **Analytical method (UHPLC-DAD-MS system)**

205 The liquid chromatography system was an Acquity ultra-high-pressure liquid chromatography
206 (UHPLC) equipped with a photodiode array detector (DAD, Waters, Milford, MA). The column
207 (HSS T3, 100 × 2.1 mm, 1.8 mm) contained a Nucleosil 120-3 C18 endcapped phase (Macherey-
208 Nagel, Sweden). The flow rate was 0.55 mL·min⁻¹ and the gradient conditions were as follows,

209 except for experiments with menthofuran: solvent A (H₂O–HCOOH, 99:1, v/v), solvent B
210 (CH₃CN–H₂O–HCOOH, 80:19:1, v/v/v); 0–5 min, 0.1% to 40% B (linear gradient); 5–7 min, 40%
211 to 99% B (linear); 7–8 min, 99% B (isocratic); and 8–9 min, 99% to 0.1% B (linear). For the
212 analyses involving menthofuran: 0–5 min, 0.1 to 60% B (linear gradient); 5–7 min, 60% to 99%
213 B (linear); 7–8 min, 99% B (isocratic); and 8–9 min, 99% to 0.1% B (linear). The Acquity UHPLC
214 system was coupled online with an amaZon X Ion-Trap mass spectrometer (Bruker Daltonics,
215 Germany), with electrospray ionization operating in the positive ion mode. In the source, the
216 nebulizer pressure was 44 psi, the temperature of dry gas was set at 200°C with a flow of
217 12 L·min⁻¹ and the capillary voltage was set at 4 kV. The mass spectra were acquired over a
218 m/z range of 90–1500. The speed of mass spectrum acquisition was set at 8100 m/z s⁻¹.

219 **Peak identification and quantification**

220 The peaks from the UV chromatograms (280 nm) were attributed to the corresponding
221 compounds by comparing the associated mass spectra and retention times to those obtained
222 with authentic standards of (+)-C, (-)-EC, (-)-ECG, (-)-EGC and (-)-EGCG. The products resulting
223 from the trapping of extension units by a nucleophile, i.e., (epi)catechin-(4→X)-nucleophile;
224 or (E)C-NU, are not commercially available. Their mass spectra and retention times were
225 determined by depolymerizing procyanidin B2 with the nucleophiles studied, as this reaction
226 yields mainly epicatechin and the targeted EC-NU. The procyanidin depolymerization products
227 obtained with 2-mercaptoethanol have also been characterized by NMR in a previous study.⁵¹
228 Figures 1 and S6 show examples of the UV chromatograms obtained along the kinetic
229 experiments.

230 The molar responses at 280 nm of C, EC, ECG, EGC and EGCG were determined by calibration
231 with the corresponding commercial standards. The molar responses at 280 nm of the (E)C-NU
232 products were assessed by depolymerizing a procyanidin B2 sample with a large excess of

233 nucleophile. As the amount of extension units trapped by the nucleophile (i.e., EC-NU) and EC
234 produced from the terminal units were expected to be equal, the ratio of the corresponding
235 peak area was attributed to the ratio of respective molar responses of the products.^{45,51} For
236 ECG-PG, the molar response relative to EC of 3.7 given by Kennedy and Jones³⁰ was applied.
237 For ECG-MF and ECG-ME, a molar response relative to EC of 3.7 was used based on the
238 coefficient experimentally determined for ECG, assuming that the nucleophile moiety did not
239 alter the molar response of ECG. This assumption was consistent with the molar response of
240 1 relative to EC found for EC-MF and EC-ME. All values, expressed proportionally to EC molar
241 response, are given in supporting information (Table S1).

242

243 **RESULTS AND DISCUSSION**

244 Experiments were first conducted on procyanidin B2 with 1 molar equivalent of nucleophile
245 relatively to B2 (Scheme 2). Then, menthofuran, phloroglucinol and 2-mercaptoethanol were
246 compared in the depolymerization of a grape seed commercial extract to characterize its
247 tannin composition. Each nucleophile was used at the optimized concentration described
248 earlier in standard literature procedures, while the other reaction conditions remained equal.
249 The analytical method using menthofuran was then compared to the Kennedy and Jones³⁰
250 phloroglucinolysis method for the characterization of the tannin composition of another
251 commercial tannin extract, pycnogenol^{9,10} and with mercaptolysis for the direct analysis of a
252 grape pericarp powder and of a Douglas fir bark sample without prior extraction.⁵¹

253 **Depolymerization of procyanidin B2 with one molar equivalent of nucleophile**

254 The B2 dimer was chosen as a model of condensed tannins because its depolymerization
255 conveniently yields only two products: (2R, 3R)-epicatechin as the released terminal unit, and
256 (2R, 3R)-epicatechin-(4→X)-nucleophile as the trapped extension unit, respectively referred

257 to as EC and EC-NU. To examine the difference of reactivity between the nucleophiles
258 quantified by the corresponding amounts of EC-NU produced, the reactions were carried out
259 with a stoichiometric amount of each nucleophile with respect to procyanidin B2. Reactions
260 were performed in methanol in the presence of 0.1 M HCl, as in most of the standard methods.
261 The depolymerization tests were performed at 30°C to limit epimerization at C2 carbon atom
262 of the flavanol unit that may occur following the ring opening in the acidic conditions. The
263 percentages of EC-NU produced and of residual B2 with respect to initial B2 concentration
264 were determined from the depolymerization experiments for the three nucleophiles tested
265 (Table 1). The time required to reach the plateau of maximum EC-NU concentration is also
266 indicated.

267 Menthofuran exhibited the highest efficiency to promote procyanidin B2 depolymerization
268 under the reaction conditions applied. Indeed, an almost full consumption (>98.8%) of the
269 procyanidin B2 dimer was observed in 40 min, with a recovery of 92% of the extension units
270 in the form of EC-MF. Menthofuran purity ($\geq 95\%$) may have limited the calculated EC-MF yield
271 as the nucleophile concentration was adjusted assuming 100% purity. The actual menthofuran
272 to B2 dimer initial molar ratio was thus between 0.95:1 and 1:1. In contrast, the reactions
273 performed with 2-mercaptoethanol (ME) and phloroglucinol (PG), prepared from >99% pure
274 products, reached an equilibrium with much lower proportions of EC-NU (41% of EC-ME and
275 23% of EC-PG, respectively), a lower proportion of terminal units, and a sizeable proportion of
276 remaining B2 (5% with ME and 14% with PG, see also Figure S7).

277 The differences between the initial B2 concentration and final of EC-NU, EC and residual B2
278 concentrations, likely correspond to oligomers that could not be accurately quantified in the
279 UHPLC-DAD-MS analysis, even though dimers like EC-(4→8)-EC-(4→2)-PG and trimers were
280 observed in the chromatograms. Indeed, depolymerization products including EC-NU and EC,

281 as well as procyanidin B2, are competitive nucleophiles that can add onto the cationic site of
282 extension units after cleavage, resulting in a large diversity of products and decreasing the
283 yields in EC and EC-NU. The relative amounts of these oligomers could be assessed based on
284 stoichiometry, from the difference between the amounts expressed in EC equivalents, of
285 products formed (EC-NU + EC) and B2 consumed at the considered reaction time. They were
286 found to account for 39% and 53% of B2 consumption in the reactions with 2-
287 mercaptoethanol and phloroglucinol, respectively, when the proportion of EC-NU reached its
288 maximal value. In the case of menthofuran, they were estimated to account for 9% of B2
289 consumption.

290 The high initial reaction rate observed with phloroglucinol evidences its good reactivity as a
291 trapping reagent, but the only partial depolymerization of the B2 dimer at reaction equilibrium
292 shows that the EC-PG product is also cleaved at a high rate in a reverse reaction. Such an
293 equilibrium between procyanidin B2 and EC-PG was predictable considering the structural
294 similarity of these products. Indeed, they both consist in a phloroglucinol-like ring linked to
295 the EC benzylic carbon at C2, and epicatechin-(4→2)-phloroglucinol also undergoes acid-
296 catalyzed cleavage of the (4→2) bond in the reaction conditions applied. Other equilibria
297 involving the new oligomers occurred at the same time, impacting the equilibrium between
298 procyanidin B2 and EC-PG. The same phenomenon occurred with EC-ME, where the
299 mercaptoethanol moiety can also be substituted in acidic conditions.³² Contrarily, the high
300 yield of conversion of the B2 dimer into depolymerization products obtained with
301 menthofuran in a 1:1 initial molar ratio indicate that EC-MF units were not significantly
302 affected by this reversibility issue (Table 1). Menthofuran thus advantageously solves this
303 concern by displacing strongly and rapidly the depolymerization equilibrium towards EC and
304 EC-MF, even when this nucleophile is used in stoichiometric amount.

305 **Depolymerization of procyanidins from a grape seed extract with optimized amounts of**
306 **nucleophiles**

307 The promising results shown with menthofuran for the depolymerization of procyanidin B2
308 motivated the development of an analytical method for characterizing more complex
309 proanthocyanidin extracts. Preliminary experiments carried out on a grape seed extract
310 ($1 \text{ g}\cdot\text{L}^{-1}$) showed that a 1:1 (w/w) menthofuran to extract weight ratio was sufficient to achieve
311 maximal depolymerization yield of procyanidins. A comparison with phloroglucinol and
312 2-mercaptoethanol at the optimized ratios reported in literature was done by performing
313 kinetic experiments at 30°C on $1 \text{ g}\cdot\text{L}^{-1}$ of the same grape seed extract in methanol containing
314 0.1 M HCl . In these experiments, 10:1 and 55:1 (w/w) nucleophile to extract ratios were used
315 for phloroglucinol and 2-mercaptoethanol, respectively, corresponding to the 10:1 (w/w)
316 weight ratio defined by Kennedy and Jones³⁰ for phloroglucinol and to the 5% (v/v) volume
317 ratio proposed by Tanaka and coworkers^{31,35} for 2-mercaptoethanol. In the extreme case
318 where the tannin extract would consist only of EC extension monomers (molecular weight
319 $290 \text{ g}\cdot\text{mol}^{-1}$), these weight ratios corresponded to a molar excess of nucleophile of 2, 23 and
320 200 for menthofuran, phloroglucinol and 2-mercaptoethanol, respectively.

321 The depolymerization products were categorized in four types of units, considering on one
322 hand, extension units versus terminal units and on the other hand, galloylated units versus
323 non-galloylated units (Figure 2). For instance, the C-NU and EC-NU concentrations measured
324 were summed to evaluate the amount of non-galloylated extension units. This enabled to infer
325 the average composition in constitutive units of the polymers.

326 The release of non-galloylated units and of galloylated units followed different kinetics. The
327 maximum concentration of non-galloylated units was reached faster (after 120-150 min) than
328 that of galloylated units (after 200-300 min). It can also be noted that the amount of non-

329 galloylated units decreased over time, contrary to galloylated units, indicating higher stability
330 of the latter. These results point to the importance of performing complete kinetic
331 experiments when characterizing tannin composition instead of selecting an arbitrary time,
332 because this optimal time may vary depending on the tannin extract. This is especially
333 important when reactions are slow, which can lead to numerous side-reactions.

334 The concentrations reached at the plateau in the kinetic experiments were used to calculate
335 the weight percentages of the four types of constitutive units obtained with each nucleophile
336 for the grape seed extract (Table 2). In each case, depolymerizable units represented around
337 46% (w/w) of the grape seed extract and consisted of around 25% of extension units, 13% of
338 terminal units, 5% of galloylated extension units and 2% of galloylated terminal units. The
339 results were thus comparable despite the different amounts of nucleophile applied.

340 The analytical depolymerization involving menthofuran as the nucleophilic reagent thus
341 demonstrated the same performance as with 10- and 100-times higher amounts of
342 phloroglucinol and 2-mercaptoethanol, respectively. To our knowledge, this makes
343 menthofuran the only nucleophilic trapping reagent described so far that enables the
344 depolymerization of condensed tannins with maximal yield using a near to quantitative
345 nucleophile to procyanidins molar ratio.

346 **Comparison of furanolysis with standard methods**

347 The high efficiency of menthofuran to trap the extension units released from tannin
348 depolymerization led to evaluate its use in comparison with the phloroglucinolysis developed
349 by Kennedy and Jones³⁰ and with mercaptolysis based on the work of Tanaka et al.³¹

350 Figure 3A compares the results of the analysis of a maritime pine bark extract, commercially
351 available under the name pycnogenol, using a 1:1 (w/w) weight ratio of menthofuran to
352 procyanidin extract and a 10:1 (w/w) weight ratio of phloroglucinol to procyanidin extract as

353 optimized in the standard method. Both reactions were performed in methanol containing
354 0.1 M HCl. Reaction with menthofuran, performed at 30°C for 90 min, resulted in a very similar
355 procyanidin composition profile as phloroglucinolysis (50°C, 20 min), with a good
356 reproducibility and equivalent depolymerization yields, although a 10-fold lower nucleophile
357 to extract weight ratio was used. This represented a non-negligible saving of reactants.

358 The menthofuran method was also compared to mercaptolysis for the characterization of the
359 proanthocyanidin fractions of a grape pericarp powder. These methods were applied directly
360 on the biomass sample (i.e., without prior extraction of tannins), in methanol containing 0.1 M
361 HCl. Furanolysis was performed with a 1:1 weight ratio of menthofuran to grape pericarp
362 powder at 30°C for 2h, while mercaptolysis was performed with a 5.5:1 weight ratio of 2-
363 mercaptoethanol to grape pericarp powder at 40°C for 2h. Chromatograms are given as
364 supporting information (Figures S8 and S9). Both methods showed similar results and good
365 reproducibility (Figure 3B), despite using a 10-fold lower molar amount of nucleophile for
366 furanolysis.

367 The menthofuran method was also applied to the direct analysis of Douglas fir bark powder,
368 without a preliminary extraction step. The depolymerization products reached their maximal
369 concentration after 20h, yielding a procyanidin content of 3.9% (w/w) of biomass dry weight,
370 including $3.2 \pm 0.2\%$ of extension units and $0.7 \pm 0.0\%$ of terminal units. On the same sample,
371 direct mercaptolysis (selected for reference since the standard phloroglucinolysis protocol
372 first proceeds with tannin extraction) gave a similar procyanidin content (3.7% w/w), including
373 $3.0 \pm 0.1\%$ extension units and $0.7 \pm 0.0\%$ terminal units. It should be noted that
374 mercaptolysis was faster (the maximal concentration was reached in 4h) due to the higher
375 temperature (40°C vs. 30°C) and the high nucleophile excess (60-times higher than
376 menthofuran) used in this method. The differences in polarity and solvation of the

377 nucleophiles may also affect the depolymerization kinetics through mass transfer limitations
378 within the solid bark sample.

379 Menthofuran thus displays interesting properties as a trapping reagent for the analytical
380 depolymerization of procyanidins. Its high efficiency allows its use at low concentration,
381 contrary to the large molar excesses required with the classical nucleophiles, and low
382 temperature for a fast conversion of condensed tannins into monomeric units without
383 significant reversal of the reaction. It is a readily available commercial chemical, and its use in
384 near to stoichiometric amounts contributes to save cost and to lower the exposure to
385 reactants. In the same way to the other nucleophiles, adjustments may be required in the
386 protocol according to the sample to be analyzed. Since the amount of procyanidin in the
387 sample is unknown, the quantity of menthofuran needs to be estimated to ensure it is higher
388 than the quantity of extension units. Also, like with the other nucleophiles and methods, the
389 minimum reaction time required to reach the maximum depolymerization yield may vary
390 according to the sample (plant species, organ and physiological status affect tannin
391 concentration and constitutive unit composition) and especially according to its type of
392 preparation (ground raw biomass or more or less purified extracts). The good stability of the
393 depolymerization products in the presence of menthofuran at 30°C enables to use a single,
394 longer than required reaction time for samples of a same type when (recommended)
395 systematic kinetics experiments cannot be performed. As a general guideline, the following
396 conditions may be applied for unknown samples.

397 *For the characterization of tannins in soluble extracts*, the following reaction conditions are
398 proposed as a standard setup: the depolymerization of a 1 g·L⁻¹ tannin extract in methanol in
399 the presence of 0.1 M HCl is carried out with 1 g·L⁻¹ menthofuran at 30°C over 2h, either with
400 end-point analysis or, preferably when possible, following the complete kinetics.

401 *For the direct analysis of raw biomass*, a concentration of 10 g·L⁻¹ of dry biomass sample may
402 allow a good response in LC-DAD(-MS), using 2 g·L⁻¹ menthofuran. Compared to soluble
403 extracts, mass transfer limitations may require a longer reaction time, as shown with the
404 analysis of the bark sample, for which the reaction was complete in 20h. Alternatively,
405 menthofuran concentration can also be increased to speed up the reaction. As the water
406 content of raw biomass samples may affect the efficiency and rate of the depolymerization
407 process, it is advisable to dry such samples to less than 15% water (w/w).

408 At appropriate times, the reaction medium can be directly analyzed by a LC-DAD(-MS) system
409 according to a protocol similar as the one given in Material & Methods. When significant
410 delays are expected between the reaction and the analysis of the reaction products, for
411 example when large series of samples are scheduled in parallel, it is preferred to raise the pH
412 of the reaction medium to pH 4-5 after the reaction is over, in order to avoid side-reactions.

413 On a more general note, the menthofuran method, referred to as furan analysis, demonstrates
414 the potential of furans as nucleophilic trapping reagents in the depolymerization of condensed
415 tannins. In a former work, furan and sylvan, two compounds that can be obtained by
416 conversion of C5 sugars from wood biomass, were indeed proven to be efficient nucleophiles
417 for quantitatively supplying fully biobased building blocks from condensed tannins.^{44,45} The
418 superior efficiency of the menthofuran method needs to be tested against tannin structures
419 known to be more recalcitrant to the usual depolymerization conditions, such as 5-deoxy
420 tannins or A-type proanthocyanidins, using harsher conditions (e.g., higher temperature
421 and/or acid concentration).

422

423 **ABBREVIATIONS USED**

424 Flavanols: B2, procyanidin B2 or B2 dimer; C, catechin; CG, catechin-3-*O*-gallate; EC,
425 epicatechin; ECG, epicatechin-3-*O*-gallate; EGC, epigallocatechin; EGCG, epigallocatechin-3-*O*-
426 gallate. Nucleophiles: ME, mercaptoethanol; MF, menthofuran; PG, phloroglucinol; NU,
427 nucleophile. Flavanol derivatives (representative examples of the numerous combinations,
428 see also Schemes 1 & 2): C-ME, catechin-(4→2S)-mercaptoethanol; EC-MF,
429 epicatechin-(4→5)-menthofuran; ECG-PG, epicatechin-3-*O*-gallate-(4→2)-phloroglucinol;
430 EGCG-NU, epigallocatechin-3-*O*-gallate-(4→X)-nucleophile; (E)CG-NU, (epi)catechin-3-*O*-
431 gallate-(4→X)-nucleophile.

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436 **SUPPORTING INFORMATION**

437 UV chromatograms (280 nm) of the grape seed extract depolymerized with menthofuran
438 throughout the kinetic experiment. Detailed kinetics of the depolymerization tests performed
439 on procyanidin B2 with 1 molar equivalent. UV chromatograms (280 nm) of the products of
440 depolymerization of the grape pericarp powder containing procyanidins and prodelfinidins
441 with menthofuran and 2-mercaptoethanol.

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- 595

596 **FIGURE CAPTIONS**

597 Scheme 1: Depolymerization of procyanidins leading to the release of terminal units and the
598 trapping of extension units by a nucleophile (example of the products obtained with
599 menthofuran).

600

601 Scheme 2 : Other procyanidin derivatives encountered in this study. Abbreviations: C,
602 catechin; EC, epicatechin; CG, catechin-3-*O*-gallate; ECG, epicatechin-3-*O*-gallate; PG,
603 phloroglucinol; ME, mercaptoethanol.

604

605 Figure 1: UV chromatograms (280 nm) of (A) the grape seed extract dissolved in methanol
606 ($1\text{ g}\cdot\text{L}^{-1}$) and (B) the same extract after acid-catalyzed depolymerization with menthofuran
607 ($1\text{ g}\cdot\text{L}^{-1}$; 65 min reaction). **1:** C (291 m/z), **2:** EC (291 m/z), **3:** ECG (443 m/z), **4:** C-MF (439 m/z),
608 **5:** EC-MF (439 m/z), **6:** ECG-MF (591 m/z).

609

610 Figure 2: Depolymerization kinetics of the procyanidins contained in the grape seed extract
611 according to the nucleophile used: menthofuran, phloroglucinol or 2-mercaptoethanol.
612 Extension units (C-NU + EC-NU) are represented by red circles, terminal units (C + EC) by blue
613 squares, galloylated extension units (ECG-NU) by purple triangles and galloylated terminal
614 units (ECG) by green diamonds. Experimental points are means and error bars are standard
615 deviations, calculated from three independent kinetic experiments.

616

617 Figure 3: Comparison of the menthofuran method (A) with a standard phloroglucinol method
618 through the characterization of a pine bark extract (pycnogenol), (B) with mercaptolysis
619 through the characterization of a grape pericarp powder containing procyanidins and

620 prodelphinidins. Mass contents are means and error bars are standard deviations calculated
621 from three independent experiments.

TABLES

Table 1: Procyanidin B2 conversion yields in the presence of 1 molar equivalent of nucleophiles

Nucleophile	Time to reach the plateau	EC-NU production yield at t_{max}	EC production yield at t_{max}	Remaining B2 dimer at t_{max}
	t_{max} (h)	mol% of theoretical maximum ⁱ	mol% of theoretical maximum ⁱ	mol% of initial B2 concentration
Menthofuran	0.7	91.6 ± 1.1	88.1 ± 1.9	1.2 ± 0.0
2-Mercaptoethanol	2.0	40.5 ± 0.4	74.9 ± 2.1	5.4 ± 0.7
Phloroglucinol	0.2	22.5 ± 0.4	65.8 ± 0.7	14.1 ± 0.1

t_{max} : time at which the plateau of maximum EC-NU concentration is reached

ⁱ the theoretical maximum corresponds to the conversion of 1 mol B2 dimer into 1 mol EC plus 1 mol EC-NU.

Values of mean and standard deviation were determined by performing three independent experiments.

Table 2: Composition in procyanidin constitutive units of a grape seed extract according to the nucleophile used for its characterization.

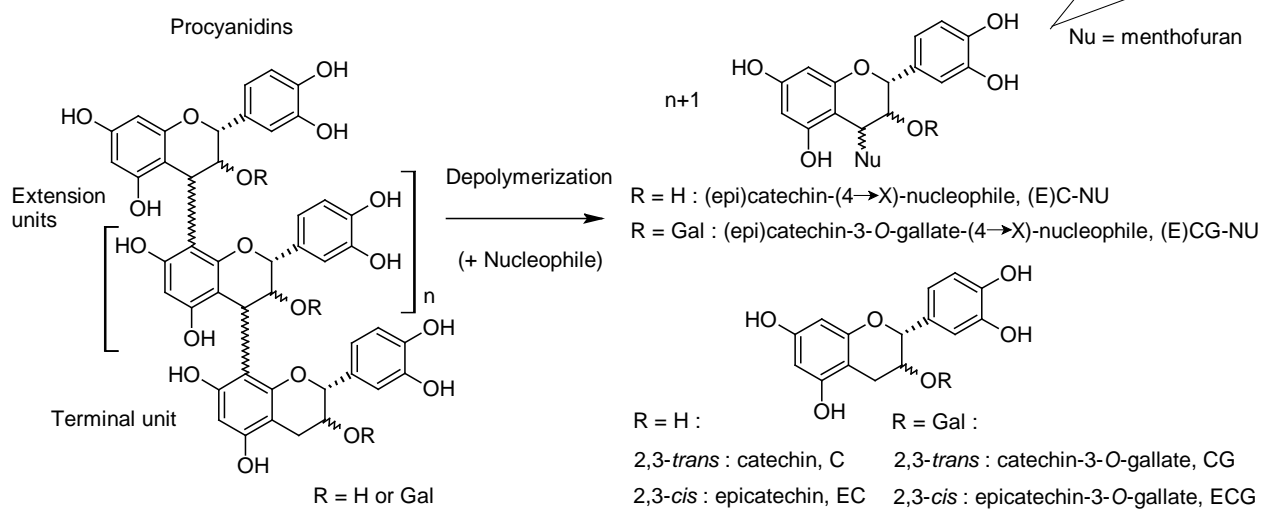
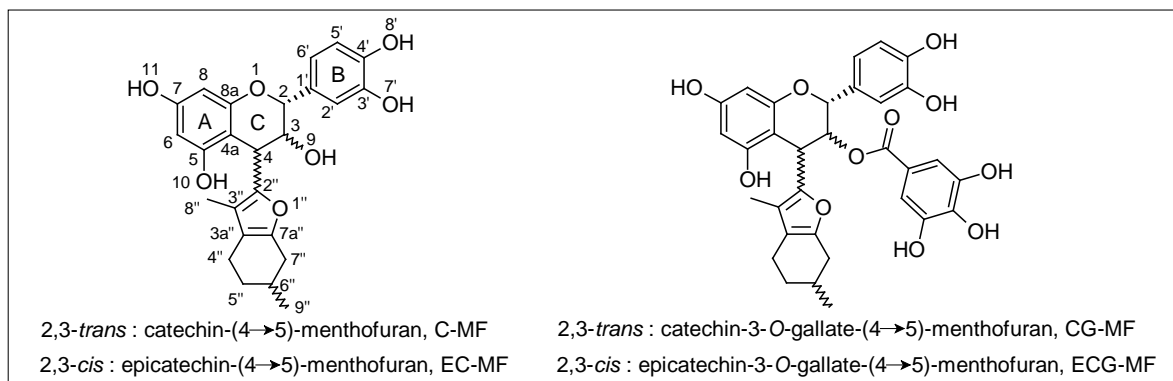
Nucleophile	Extension Units ^a	Terminal Units ^b	Galloylated Extension Units ^c	Galloylated Terminal Units ^d
	% (w/w) ⁱ	% (w/w) ⁱ	% (w/w) ⁱⁱ	% (w/w) ⁱ
Menthofuran	25.9 ± 0.4	13.1 ± 0.3	5.2 ± 0.1	1.6 ± 0.0
2-Mercaptoethanol	25.0 ± 0.3	12.9 ± 0.2	5.1 ± 0.1	1.6 ± 0.0
Phloroglucinol	25.6 ± 0.3	12.5 ± 0.2	5.2 ± 0.2	1.6 ± 0.0

Compositions calculated from the plateau of maximum concentration of ^a: C-NU + EC-NU, ^b: C + EC, ^c: ECG-NU, ^d: ECG

^{i,ii} Mean values and standard deviations were calculated for each nucleophile from three independent kinetic experiments using concentrations at (i) three reaction times (9 experimental points) and (ii) two reaction times (6 experimental points).

FIGURE GRAPHICS

Scheme 1



Scheme 2

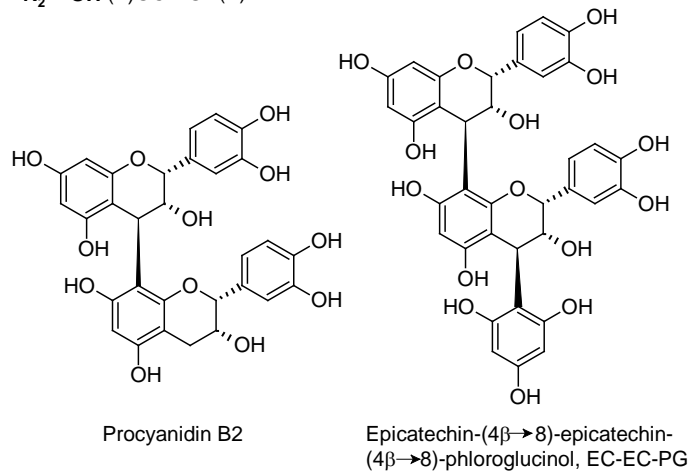
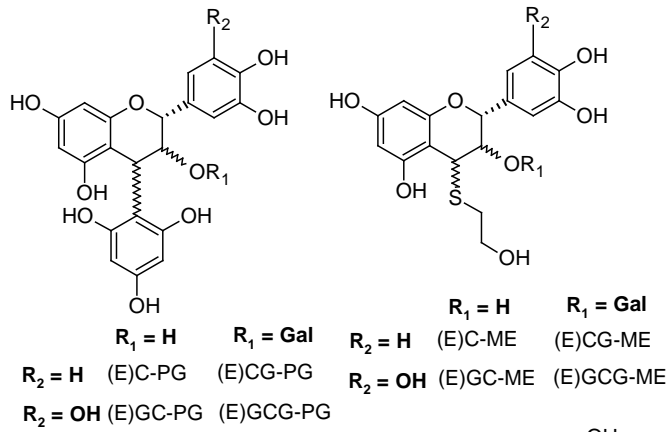


Figure 1

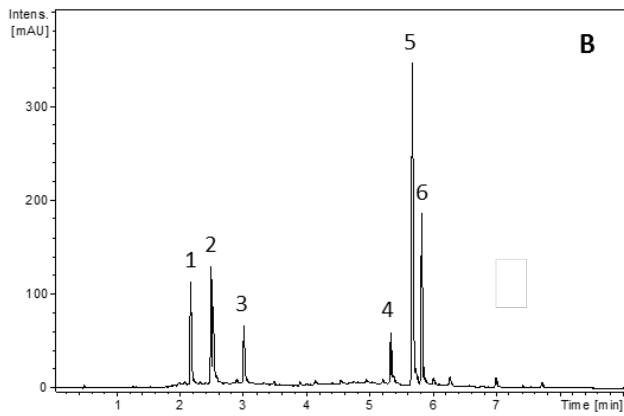
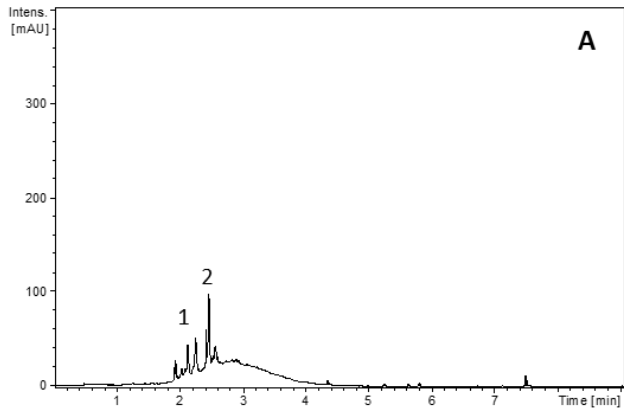
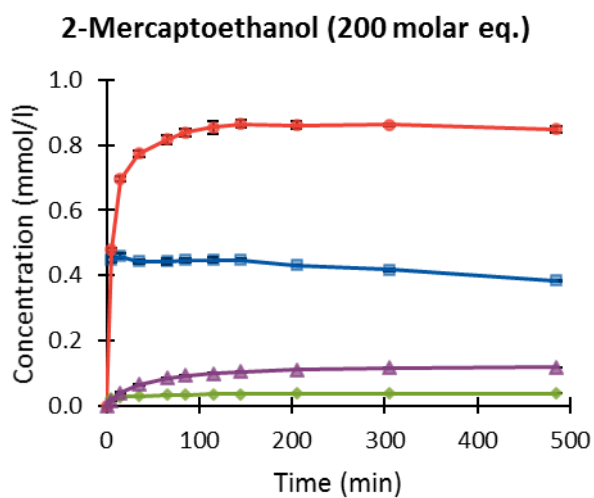
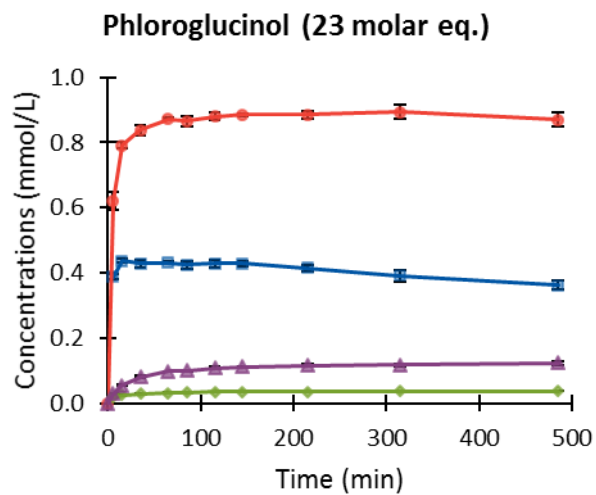
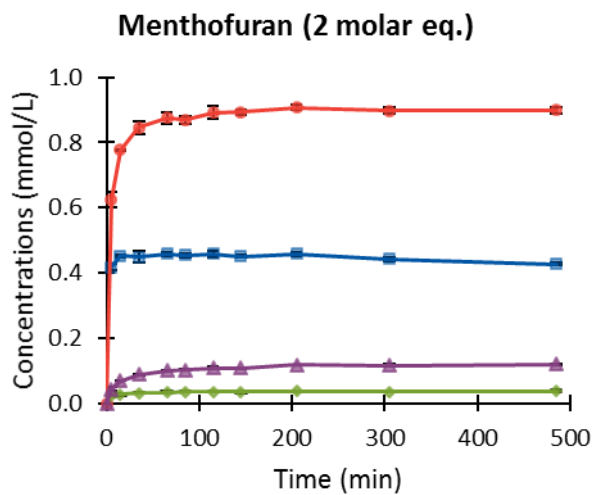


Figure 2



- Extension units
- Terminal units
- Galloylated extension units
- Galloylated terminal units

Figure 3

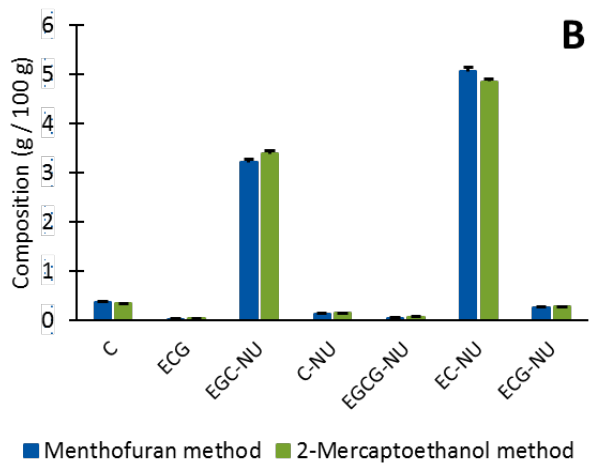
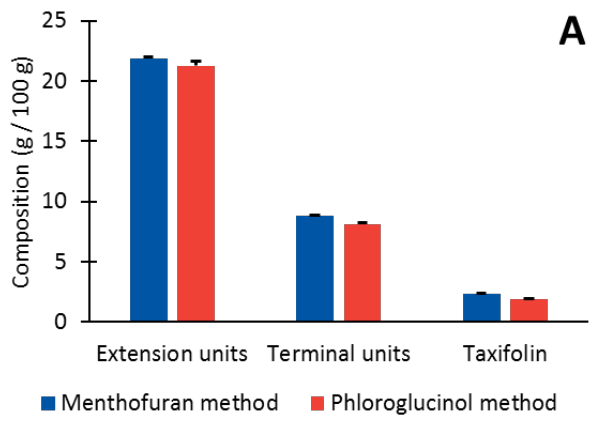
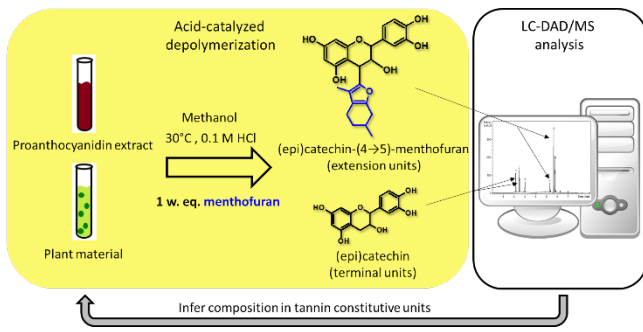


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