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Acylated anthocyanins from sprouts of *Raphanus sativus* cv. Sango: Isolation, structure elucidation and antioxidant activity

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Highlights

- 9 acylated anthocyanins (4 new) were isolated from the sprouts of *R. sativus* cv Sango
- A novel purification strategy based on orthogonal prep-LC is described
- Their structure was elucidated by combined NMR, tandem-MS and UV-Vis spectroscopies
- Their absolute reactivity with peroxy radicals was measured for the first time
- SARs and the role of acylation for antioxidant anthocyanins are discussed on kinetic ground

1 **Acylated anthocyanins from sprouts of *Raphanus sativus* cv. Sango: isolation, structure**
2 **elucidation and antioxidant activity**

3

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22 **Running title:** Antioxidant acylated anthocyanins from Sango

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26

27 **Abstract**

28 Little is known on structure-activity-relationships of antioxidant anthocyanins. *Raphanus sativus* cv
29 Sango sprouts are among the richest sources (270 mg/100 g fresh weight). We isolated from
30 sprouts' juice 9 acylated anthocyanins, including 4 new compounds. All comprise a cyanidin core
31 bearing 3-to-4 glucose units, multiply acylated with malonic and phenolic acids (ferulic and
32 sinapic). All compounds were equally effective in inhibiting the autoxidation of linoleic acid in
33 aqueous micelles, with rate constant for trapping peroxy radicals $k_{inh} = (3.8 \pm 0.7) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ at
34 37°C. In acetonitrile solution k_{inh} varied with acylation, ranging $(0.9\text{-}2.1) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ at 30°C; each
35 molecule trapped a number n of peroxy radicals ranging 4-to-7. Anthocyanins bearing sinapic acid
36 were more effective than those bearing the ferulic moiety. Under identical settings, deacylated
37 cyanin, ferulic and sinapic acids had k_{inh} of 0.4×10^5 , 0.3×10^5 and $1.6 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ respectively, with n
38 ranging 2-to-3. Results show the major role of acylation on antioxidant performance.

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44 **Keywords:** Anthocyanins, Phenolic acids, Radish sprouts, Peroxy radicals, NMR, Mass

45 spectroscopy

46

47

48 **Chemical compounds studied in this article**

49 Cyanidin CID 128861, Cyanin CID 441688, sinapinic acid CID 637775, ferulic acid CID 445858

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52

53 **1. Introduction**

54 Anthocyanins are members of the broad flavonoid family of polyphenolic compounds and are
55 receiving very high interest from the scientific community for their relevant health-promoting
56 potential, related to the antioxidant activity, and for their role as functional food factors in the
57 prevention of chronic disease (Pojer, Mattivi, Johnson, & Stockley, 2013). Their structure
58 comprises a polyhydroxylated flavylum aglycone (the anthocyanidin) bearing one-to-several
59 glycosidic units. In acylated anthocyanins, such glycosidic units are esterified to one-to-several
60 organic acid residues, which often comprise phenolic acids (e.g. coumaric, ferulic, sinapic, etc.),
61 bringing additional phenol-type hydroxyl functions to the whole structure (Figure 1). The
62 polyphenolic structure is key to their antioxidant activity. While this property is generally accepted
63 and studies have illustrated its relevance, *e.g.* in food preservation, mechanisms and structure-
64 activity relationships associated to their antioxidant behaviour are far from being clarified. For
65 example, an anthocyanin extract from rice bran was recently shown to protect emulsified fish oil
66 from oxidation and to outperform tocols in the protection of cholesterol, although no indication was
67 provided with regard of the structure of anthocyanins in the extract (Zhang, Shen, Prinyawiwatkul,
68 King, & Xu, 2013). A very recent study addressed quantitative structure-activity relationships for
69 antioxidant anthocyanins, however acylated derivatives were not investigated (Jing et al., 2014);
70 furthermore, antioxidant performance was assessed by ORAC, a rapid assay that does not study the
71 actual reactivity with autoxidation chain-carrying peroxy radicals, thereby providing only an
72 estimate of the antioxidant potential (Amorati, Foti, & Valgimigli, 2013; Roginsky & Lissi, 2005).
73 Computational studies indicate that, among anthocyanidin cores, cyanidin that bears the catechol
74 function in flavylum B-ring is a privileged structure with regard to its ability to trap peroxy
75 radicals (Guzmán, Santiago, & Sánchez, 2009), while experimental evidence suggests that
76 glycosylation/acylation pattern has an influence on cyanidin-based antioxidants (Stintzing,
77 Stintzing, Carle, Frei, & Wrolstad, 2002), which would reflect in their nutritional health-promoting
78 value.

79 We have recently reported (Matera et al., 2012) that *Raphanus sativus* cv. Sango sprouts are among
80 the richest sources of cyanidin-based anthocyanins (270 mg/100 g fresh weight), paralleling berries
81 and at variance with other brassica vegetables or radish varieties, which commonly display 4-to-25-
82 fold lower content of pigments based on the pelargonidin core (Horbowicz, Kosson, Grzesiuk, &
83 Debski, 2008). Sango sprouts are also exceptional for the profile of cyanidin-based anthocyanins
84 among other cyanidin sources, as we were able to distinguish 70 different structures mainly
85 comprising polyglycosylated and polyacylated derivatives. Aiming at clarifying their healthy
86 potential and at understanding the structure-activity relationships (SAR) for antioxidant acylated
87 anthocyanins on solid kinetic bases, we subjected Sango sprouts juice to a fractionation procedure,
88 using a combination of high-resolution separation techniques. We report here the isolation and
89 structural elucidation of 9 acylated cyanidin-based anthocyanins, 4 of which have never been
90 isolated before. Furthermore we report a preliminary kinetic investigation of their antioxidant
91 activity, affording for the first time absolute rate constants for their reaction with peroxy radicals.
92 The mechanism and SAR of cyanidin-based antioxidants are preliminarily discussed.

93

94 **2. Experimental**

95 *2.1. General procedures*

96 All solvents and chemicals were from Sigma-Aldrich-Fluka (Milan, Italy) unless otherwise noted
97 and were of the highest grade commercially available. Flash chromatography was carried out with
98 an automatic apparatus (Combiflash™ Sg 100c, Isco Inc., Lincoln, NE, USA), equipped with a
99 fraction collector and UV detector. Separations were achieved using C18 column RediSep® Rf
100 Gold (50 g, particle size = 20-40 μm , 2 $\varnothing \times 15$ cm,) under a flow rate of 30 mL/min and monitoring
101 at $\lambda = 280$ nm. The mobile phase consisted of a combination of A (aq 0.5% v/v HCOOH) and B
102 (MeCN + 0.5% v/v HCOOH) with the following programming: t = 0, A (100%); t = 5 min, A
103 (100%); t = 60 min, A–B (60:40 v/v); t = 75 min, A–B (60:40 v/v). Preparative and semipreparative
104 HPLC separations were carried out on HPLC Gilson (Middleton, WI, USA) using a Rheodyne loop

105 of 1000 or 200 μL , respectively. The HPLC pumps were equipped with UV-Vis detector and
106 injector/fraction collector and all the separations were dual monitored at 530 nm and 330 nm. The
107 mobile phase for all HPLC separations consisted of a combination of A (aq 0.12% v/v TFA) and B
108 (MeCN + 0.12% v/v TFA). Preparative chromatographic separations were achieved at room
109 temperature on reverse phase prep-column Synergi Polar-RP (4 μm , 80 \AA , 21.2 \AA \times 250 mm)
110 (Phenomenex), with a flow rate of 20 mL/min following optimal gradient programs. Program 1 : t =
111 0, B (16%); t = 1 min, A (16%); t = 56 min, B (27%); t = 75 min, B (60%). Program 2: t = 0, B
112 (18%); t = 5 min, B (18%); t = 60 min, B (29 %); t = 61 min, B (34 %), t = 65 min, B (34 %). Semi-
113 preparative HPLC separations were obtained on Luna PFP column (5 μm , 100 \AA , 10.0 \AA \times 250
114 mm), (Phenomenex) optimizing flow rate and gradient conditions. Roughly, the flow rate ranged 5-
115 7.5 mL/min and gradient programs ranged t = 0, B (20-15%); t = 5 min, B (20-15%); t = 60 min, B
116 (15-25%). Analytical HPLC was carried out on a LC-ESI-MS system (Thermo Scientific, San Jose,
117 CA, USA) coupled to a PDA detector and equipped with a mass spectrometer LCQ Fleet[®] (Thermo
118 Scientific). Analytical separation of fractions and sub-fractions was carried out at 25°C using
119 reverse phase columns such as Synergi Polar (4 μm , 80 \AA , 4.60 \AA \times 250 mm) (Phenomenex) or
120 Hypersil GOLD PFP column (5 μm , 175 \AA , 4.60 \AA \times 250 mm) (Thermo Scientific) at flow rate of 1
121 mL/min. Optimized gradient programs were in the range: t = 0, B (20-15%); t = 5 min, B (20-15%);
122 t = 60 min, B (15-35%). Low resolution ESI-MS-MSⁿ(+) spectra were obtained by direct infusion
123 on LCQ Fleet[®] mass spectrometer, following the conditions previously described (Matera et al.,
124 2012). High Resolution mass spectra were recorded in a Thermo-Finnigan MAT95 XP instrument.
125 NMR spectra were recorded on a Varian Inova-600 (600 MHz) spectrometer in *d*₆-DMSO/
126 CF₃COOD-*d*₁ (9:1). The structure of pigments was elucidated by investigation of ¹H (600 MHz)
127 and ¹³C (151 MHz) NMR spectra, including 2D-COSY, 2D ¹H-¹³C HSQC and ¹H-¹³C HMBC. The
128 ¹³C spectrum of pigments was completely assigned using the HSQC and HMBC experiments.
129 Chemical shifts are reported to a TMS internal standard (δ) and coupling constant are in Hz.

130

131 *2.2. Plant material, preparation and fractionation of the freeze-dried juice*

132 *Raphanus sativus* (L.) cv. Sango seeds were supplied by Suba & Unico (Longiano, FC, Italy) and
133 stored in a dry and dark place at room temperature. Seeds were identified by a lot number and
134 guaranteed by the producer for the quality and the homogeneity of the product. The preparation and
135 fractionation of the freeze-dried juice followed the detailed procedure already described (Matera et
136 al., 2012). Briefly, seven days radish sprouts were squeezed to provide a dark violet juice, which
137 was collected in liquid nitrogen and lyophilised. The obtained dark violet powder (33 g) was
138 washed with petroleum ether (800 mL) and with acetone (800 mL) to remove lipophilic
139 components. The remaining violet residue was suspended with 800 mL of aqueous acid solution
140 (0.5% v/v in TFA). The pink suspension was shaken, sonicated for 5 min, and then centrifuged for
141 15 min at 2100g. This procedure was repeated with fresh aqueous acid solution until complete
142 disappearance of the pink colour in the extract.

143

144 *2.3. Isolation of anthocyanins*

145 The collected aqueous extracts (0.5% v/v in TFA) were subjected to SPE through strong cation-
146 exchange columns (Septra-SCX, Phenomenex, 50 µm, 65 Å, 4 Ø × 15 cm) previously conditioned
147 with H₂O and MeCN. The elution of pigment fraction was carried out with 500 mL solution of
148 MeCN: aq 0.1 M HCl (75:25). MeCN was removed under reduced pressure whereas the remaining
149 water solution was lyophilized to furnish the anthocyanin-rich extract as a purple residue (1.15 g).
150 This extract was further purified by flash chromatography, on C18 column. After MeCN removal
151 under vacuum, the aqueous solutions were freeze-dried to give two enriched anthocyanin fractions
152 (A= 273 mg, B= 260 mg). Fractions A and B were subjected to repeated preparative HPLC to
153 obtain several sub-fractions that were subsequently purified on semipreparative HPLC. Isolation of
154 pure pigments **1-9** (0.5 to 5 mg each) from eluted aqueous fractions was achieved by SPE using a
155 C18 cartridge (Strata C18-U, 55 µm, 70 Å, 1g/6mL) previously activated with MeCN, H₂O and aq.

156 0.12% v/v TFA, eluting with 0.5% v/v TFA in MeOH. Methanol was removed under a gentle
157 stream of nitrogen and the residue was dried overnight in a vacuum desiccator.

158

159 *2.4 Antioxidant activity in aqueous micelles*

160 Antioxidant activity was determined by studying the inhibited autoxidation of 30 mM linoleic acid
161 in Triton-X100 (0.16 M) neutral micelles, in water containing 1% v/v formic acid for pH correction,
162 which was necessary to maintain anthocyanins in the flavylium form. Autoxidation at 37 °C was
163 initiated at constant rate $R_i=6.6\times 10^{-9}$ Ms⁻¹ by addition of 8.3 mM AAPH (2,2'-Azobis(2-
164 amidinopropane)dihydrochloride). *R,R,R*- α -Tocopherol (1-5 μ M) was used as reference antioxidant.
165 Isolated anthocyanins were tested at a final concentration in the range 2-10 μ M, while for
166 anthocyanin fractions similar concentration was calculated on the average molecular weight of 1200
167 amu. The reaction was followed by monitoring the oxygen consumption with a miniaturized Clark-
168 type electrode (Instech, Plymouth Meeting, PA) connected to a dual-channel amplifier Mod 203 and
169 a A/D converter Duo.18 (World Precision Instruments, Sarasota, FL). After thermal equilibration at
170 37 °C of the oxidizable mixture, the appropriate amount of initiator was injected into the cell at the
171 beginning of data collection. After a few minutes, the solution of the antioxidant was injected
172 (Amorati, Pedulli, Cabrini, Zambonin, & Landi, 2006).

173

174 *2.5 Antioxidant activity in organic solution*

175 The absolute reactivity with peroxy radicals was determined from kinetics of oxygen consumption
176 during the inhibited autoxidation of 2.1 M styrene or 1.7 M cumene in MeCN (containing 1% v/v
177 formic acid) at 30 °C (Kumar, Engman et al., 2007; Kumar, Johansson et al., 2007). The reaction
178 was thermally initiated at constant rate (R_i in the range $(2-9)\times 10^{-9}$ Ms⁻¹) by the decomposition of
179 2,2'-azodiisobutyronitrile (AIBN, $(1-5)\times 10^{-2}$ M) and the oxygen consumption was measured in a
180 differential oxygen-uptake apparatus based on a Validyne (Northridge, CA) DP15 pressure
181 transducer, which has been previously described (Lucarini, Pedulli, Valgimigli, Amorati, &

182 Minisci, 2001). *R,R,R*- α -Tocopherol was used as reference antioxidant (Valgimigli, Lucarini,
183 Pedulli, & Ingold, 1997). From the slope of the oxygen consumption during the inhibited period
184 (R_{inh}), k_{inh} values were obtained by using eq. 1 (Amorati, Pedulli, & Valgimigli, 2011), where R_0 is
185 the rate of oxygen consumption in the absence of antioxidants, $2k_t$ is the bimolecular termination
186 rate constant of styrene or cumene (Amorati, Foti et al., 2013), and n is the stoichiometric
187 coefficient of the antioxidant, which was determined experimentally from the length of the inhibited
188 period (τ) by eq. 2. When the inhibited period was not clearly visible, kinetic data were confirmed
189 by fitting the experimental traces with numerical simulations using Gepasi 3.0 software, as
190 previously described (Amorati, Lynett, Valgimigli, & Pratt, 2012).

$$191 \quad (R_0/R_{inh}) - (R_{inh}/R_0) = nk_{inh}[AH]/\sqrt{(2k_tR_i)} \quad (1)$$

$$192 \quad n = \tau R_i/[AH] \quad (2)$$

193

194 **3. Results and Discussion**

195 *3.1 Isolation of Anthocyanins*

196 Preliminary HPLC analysis of the freeze-dried sprouts' juice, obtained by mechanical squeezing,
197 revealed a very complex pattern of anthocyanins distribution, at variance with the relatively simple
198 chromatographic profiles reported in other studies for the mature radishes of different *R. sativus*
199 varieties (Matera et al., 2012). Therefore, we were prompted to set up a suitable isolation procedure
200 based on the combination of SPE and high resolution chromatographic approaches. The whole
201 anthocyanins fraction was first isolated from the freeze-dried juice. The freeze dried matrix was
202 suspended in petroleum ether and acetone for complete removal of lipophilic compounds, then the
203 isolation of anthocyanins was achieved by repeated extractions with aqueous acid solution (0.5%
204 v/v in TFA). The subsequent strong-cation-exchange purification (SCX-SPE) and flash
205 chromatography on C18 were carried out according to a previously optimized protocol (Matera et
206 al., 2012). HPLC-MS analysis of the fraction revealed that the vast majority of components had
207 multi-glycosylated cyanidin core, with variable acylation pattern mainly comprising phenolic acids.

208 In order to optimize the subsequent fractionation on preparative HPLC, we comparatively tested a
209 number of stationary phases with higher polarity and endowed with enhanced aromatic selectivity,
210 as compared to common C18 columns used in most published protocols. Among the tested
211 stationary phases, the ether-linked phenyl phase (Synergi Polar-RP) endowed with additional polar
212 groups revealed as optimal for our purpose, allowing to separate the majority of acylated
213 anthocyanins with only few co-eluted peaks at analytical scale. After prep-HPLC fractionation,
214 semi-preparative separations were achieved under the orthogonal pentafluorophenyl (PFP) phase,
215 endowed with complementary electronic features. Final purification of isolated anthocyanins
216 required one-to-two additional passages either on PFP-phase or on Synergi Polar-RP.
217 Overall, we were able to isolate in pure form nine acylated anthocyanins, comprising four new
218 compounds (**1-4**) and five pigments (**5-9**) previously isolated from different botanical sources.
219 Structures are summarized in Figure 1.

220 < Figure 1 about here >
221

222 3.2 Structure elucidation of isolated anthocyanins (**1-9**)

223 The structure of all anthocyanins was drafted by a combination of UV-Vis and MSⁿ analyses (Table
224 1), followed by NMR (Tables 2-3) elucidation by ¹H, homonuclear (¹H-¹H) COSY, and
225 heteronuclear (¹H-¹³C) HSQC and HMBC spectra, comparing to the reported data for analogous
226 compounds. Using UV-Vis spectral features λ_{vis} , and λ_{acyl} from HPLC-DAD profiles and the relative
227 absorbivities of **1-9** (See Table S1 in Appendix) it was possible to identify the anthocyanins as
228 either mono- or biosides (Table 1): E_{440}/E_{vis} absorbivity ratio of 29–35% indicates a monoside (as
229 shown by monoglycosilated cyanidin-3-glucoside, $E_{440}/E_{\text{vis}} = 29\%$ (Fossen & Andersen, 1998)),
230 whereas a ratio of 15–24% indicates a bioside, additionally, $E_{\text{acyl}}/E_{\text{vis}}$ absorbivity ratio of 53–69% or
231 98–128% suggests monoacylation or diacylation, respectively (Arapitsas, Sjoeborg, & Turner,
232 2008). All anthocyanins belonged to the cyanidin family bearing the cyanidin 3-sophoroside-5-

233 glucoside as a common structure, variably substituted with malonic, ferulic and sinapic acids.
234 Interestingly, three of the new pigments (**2**, **3** and **4**) bear a diglucoside pattern at the 5 position.
235 The five known compounds (**5-9**) were identified by spectral analyses and comparison with
236 literature data (Idaka et al., 1987; Madhavi, Juthangkoon, Lewen, Berber-Jimenez, & L., 1996;
237 Saito et al., 2008; Suzuki, Nagata, & Terahara, 1997; Tatsuzawa et al., 2010), as detailed in the
238 Appendix.

239 The novel anthocyanin **1** (C₅₄H₅₉O₂₈) (Figure 1) shows absorbance percentage ratios $E_{440}/E_{vis} =$
240 17% and $E_{acyl}/E_{vis} = 105%$ suggesting an anthocyanin bioside biacylated with two aromatic
241 residues. The ESI(+)-MS analysis exhibits the molecular ion at m/z 1155. CID fragmentation of m/z
242 1155 yields the ion at m/z 993 by loss of a glycosyl residue (162 amu) and at m/z 449 by loss of a
243 diglycosyl-feruloylsinapoyl residue (706 = 324+176+206 amu). Fragmentation of m/z 993 in MS³
244 spectrum produced the ion m/z 287 corresponding to the cyanidin aglycone, by loss of the 706 amu
245 residue (Table 1). The anthocyanin **1** was presumed to be based on cyanidin-3-feruloyl-sinapoyl-
246 diglucoside-5-glucoside and was subjected to complete NMR characterization to assign the exact
247 connectivity of glycosyl and acyl moieties using ¹H, COSY, HSQC and HMBC experiments
248 (Tables 2-3 and ¹H spectrum in Appendix). Three signals corresponding to the chemical shifts of
249 acetal groups at the anomeric carbons were detected and assigned to different sugars using
250 previously reported data for similar anthocyanins isolated in *R. sativus* (Otsuki, Matsufuji, Takeda,
251 Toyoda, & Goda, 2002). The most downfield non-aromatic proton's signal belongs to H₁ of Glc A
252 (δ 5.62), followed by those of Glc B (δ 5.10), and Glc C (δ 5.03). The typical diaxial coupling
253 constants ($J = 7.2$ - 8.4 Hz) of anomeric protons of sugar residues, assigned to β -glucopyranoside in
254 ¹H-NMR experiment, together HMBC correlation between H₁ of Glc B (δ 5.10) with C₂ of Glc A (δ
255 78.3) confirmed the typical connectivity of β -1,2 glycosidic linkage of sophorose. Literature data
256 revealed that sophorose is always linked to C₃ position of cyanidin even though very weak HMBC
257 correlation between the most deshielded acetalic signal belonging to H₁ of Glc A (δ 5.62) and
258 cyanidin C₃ (δ 145.9) was observed. Moreover, HMBC correlations exist between H₁ of Glc C (δ

259 5.03) and C₅ of cyanidin (δ 154.6) indicating that Glc C is linked in 5-position of cyanidin. The
260 exact connectivity pattern of sugar residues was obtained by ¹H and COSY experiments and by
261 comparing with literature data (Otsuki et al., 2002). Proton signals from sinapic acid residue (R₃)
262 were observed at δ 7.42, 6.88, 6.40, which correlated with the singlet at δ 3.75, integrating for six
263 protons. Furthermore, HMBC correlation exists between H₂ of Glc B (δ 4.60) and carbon signal of
264 sinapic acid carbonyl (δ 166.3). Two geminal methylene protons H_{6a} and H_{6b} of Glc B (δ 4.25 and
265 4.32) showed a downfield shift indicating that this position is acylated with ferulic acid (R₂) as
266 demonstrated by HMBC correlations with ferulic carbonyl (δ 165.6). The cinnamic residues were
267 elucidated as *trans* based on the large coupling constants ($J = 15-16$ Hz) of the α and β protons of
268 ferulic and sinapic moieties. Moreover, HMBC correlations between proton signals of 3-OCH₃ (δ
269 3.70) and C₃ (δ 147.6) of the feruloyl group proceed through stepwise ³J_{CH} up to the carbonyl (δ
270 165.6) of ferulic group. Similarly, observed HMBC correlations between the 3-OCH₃ and 5-OCH₃
271 proton signal (δ 3.75) up to the carbonyl (δ 166.3) of sinapoyl group allow to discriminate the
272 remaining signals related to each cinnamic moiety by crossing data from HMBC, HSQC and COSY
273 experiments. Therefore, the novel anthocyanin **1** was identified as cyanidin 3-*O*-[2-*O*-(2-*O*-(*E*)-
274 sinapoyl-6-*O*-(*E*)-feruloyl- β -D-glucopyranosyl)- β -D-glucopyranoside]-5-*O*-(β -D-glucopyranoside).
275 The novel anthocyanin **2** (C₆₃H₇₁O₃₆) (Figure 1) shows a percentage ratio E₄₄₀/E_{vis} of 23%
276 suggesting an anthocyanin bioside, while the ratio E_{acyl}/E_{vis} of 98% suggests biacylation with two
277 aromatic residues. The ESI(+)-MS analysis showed m/z 1403 as molecular ion. MS² fragmentation
278 of m/z 1403 revealed the three main ions (m/z 1359, m/z 993 and m/z 697) originating by loss of
279 carbon dioxide (44 amu), malonoyl-diglucoside (410 amu) and the diglycosyl-feruloylsinapoyl (706
280 amu) residues, respectively. MS³ fragmentation of each ion produced the cyanidin aglycone
281 fragment at m/z 287 (Table 1). The anthocyanin **2** was presumed to be based on cyanidin-3-feruloyl-
282 sinapoyl-diglucoside-5-malonoyl-diglucoside and was subjected to full NMR characterization (¹H,
283 COSY, HSQC and HMBC) (Tables 2-3 and ¹H spectrum in Appendix). Four signals corresponding
284 to the chemical shifts of acetalic groups at the anomeric carbons were detected and assigned to

285 different sugars using previously reported data: the acetalic proton's signal shifted at the lowest
286 field (δ 5.60) belongs to the H₁ of Glc A, then signals at higher field belong to Glc B (δ 5.13), and
287 Glc C (δ 5.21), respectively. The doublet of the acetalic proton shifted at the highest field (δ 4.27)
288 belongs to Glc D. The exact connectivity pattern of sugar residues was obtained by ¹H and COSY
289 experiments. The typical diaxial coupling constants ($J = 7.1-7.8$ Hz) of anomeric protons of sugar
290 residues shown in ¹H-NMR experiments, which was assigned to β -glucopyranoside, together with
291 the HMBC correlation between H₂ of Glc A (δ 4.12) with C₁ of Glc B (δ 99.7), confirmed the
292 typical connectivity of β -1,2 glycosidic linkage of sophorose. This is linked to C₃ position of
293 cyanidin even though a very weak HMBC correlation appears between the most deshielded acetalic
294 signal belonging to H₁ of Glc A (δ 5.60) and cyanidin C3 (δ 144.9). Four couples of methylene
295 protons belong to C₆ positions of each glucose as confirmed by HSQC correlations, together with an
296 additional cross peak indicating the methylene of malonic acid (R₄) (δ 3.30). The deshielded signals
297 of geminal protons H_{6a} e H_{6b} of Glc A and Glc C refer to positions acylated with ferulic (R₁) and
298 malonic acid (R₄), respectively, as confirmed by the weak HMBC correlation between ferulic ester
299 carbonyl (δ 166.8) and two geminal protons, H_{6a} e H_{6b}, of Glc A (δ 4.21 and 4.36), together with a
300 correlation between the malonic ester carbonyl (δ 166.7) and two protons, H_{6a} e H_{6b}, of Glc C
301 (δ 4.26 and 4.39). Proton signals from sinapic acid (R₃) were observed at δ 7.43, 6.91, 6.46, which
302 correlated with a singlet (δ 3.78) integrating for six protons. Furthermore, HMBC correlation exists
303 between proton at H₂ of Glc B (δ 4.64) and the carbonyl (δ 166.2) of sinapic acid. The cinnamic
304 residues' configuration was elucidated as *trans* due to the large coupling constants ($J = 16.2$ Hz).
305 By means of HMBC analysis, ³J_{CH} stepwise correlations were observed from olefinic ferulic
306 protons at α and β position (δ 6.24 and 7.33, respectively) up to the proton signal of 3-OCH₃ (δ
307 3.71) and the C₃ (δ 148.4) of the ferulic acid. Similarly, HMBC correlation is observed between the
308 3-OCH₃ and 5-OCH₃ proton signal (δ 3.78) and the carbon signal at the C₃ and C₅ (δ 148.5) of
309 sinapic acid. Crossing data from HMBC, HSQC and COSY allowed distinguishing all signals
310 related to ferulic and sinapic acid. The complete data set for this anthocyanin is very similar to the

311 known compound **5** (See Appendix), except for the signals related to the additional Glc D. Due to
312 the paucity of material analyzed, no HMBC correlations were observed between Glc C and Glc D
313 because the intrinsic difficulty with observing $^3J_{CH}$ in flexible cycles like glycosides even at lower
314 temperature (15 °C). For this reason, in this study we refer to the structure elucidation of a very
315 similar acylated pelargonidin isolated from rhizome of *R. sativus*, based on β -1,4 diglucoside,
316 recently published by Mori and coworkers (Mori, Nakagawa, Maeschima, Niiura, & Yoshida, 2006)
317 due to the identity of the vegetal species. Therefore, the novel anthocyanin **2** was identified as
318 cyanidin 3-*O*-[6-*O*-(*E*)-feruloyl-2-*O*-(2-*O*-(*E*)-sinapoyl- β -D-glucopyranosyl)- β -D-glucopyranoside]-
319 5-*O*-(4-*O*- β -D-glucopyranosyl-6-*O*-malonoyl- β -D-glucopyranoside).

320 The novel anthocyanin **3** (C₆₂H₆₉O₃₅) (Figure 1) shows very similar UV-Vis and MSⁿ properties as
321 anthocyanin **2**. The ESI(+)^{MS} analysis shows *m/z* 1373 as molecular ion suggesting it to be
322 acylated with two ferulic acids (Table 1). The spectral data of both compounds were very similar:
323 the ¹H and ¹³C NMR spectra (Table 2) revealed that **2** and **3** have feruloyl and sinapoyl groups, and
324 two feruloyl groups, respectively. The connectivities of the glycosides, malonic residue and two
325 acyl groups in **2** and **3** were equivalent as confirmed by HMBC analysis and were referred to a
326 similar acylated pelargonidin isolated by Mori and coworkers from *R. sativus* (Mori et al., 2006).
327 Therefore, the novel anthocyanin **3** was identified as cyanidin 3-*O*-[6-*O*-(*E*)-feruloyl-2-*O*-(2-*O*-(*E*)-
328 feruloyl- β -D-glucopyranosyl)- β -D-glucopyranoside]-5-*O*-(4-*O*- β -D-glucopyranosyl-6-*O*-malonoyl-
329 β -D-glucopyranoside) (see complete structure elucidation in Appendix)

330 The novel anthocyanin **4** (C₆₂H₆₉O₃₅) (Figure 1) shows almost identical UV-Vis and MSⁿ properties
331 as anthocyanin **3**. The ESI(+)^{MS} analysis shows *m/z* 1373 as molecular ion suggesting it to be
332 acylated with two ferulic acids (Table 1). The spectral data of anthocyanins **3** and **4** were similar:
333 the ¹H (Table 2) and COSY revealed both compounds share two feruloyl groups. The
334 connectivities of the glycosides, the malonic residue and two ferulic groups in **3** and **4** differentiate
335 only for the acylation site as revealed by the downfield shifted signals of methylene protons of H₆ of
336 Glc B (δ 4.27), which indicate the ferulic acid (R₂) acylation. The ¹H-NMR data set of anthocyanin

337 is very similar to compound **9** (see details in Appendix), except for the signals related to the
338 additional Glc D. In the absence of sufficiently intense ¹³C signals (not listed in table 3) the
339 connectivity between Glc C and Glc D was referred to the work by Mori and coworkers on *R.*
340 *sativus* (Mori et al., 2006). Therefore, the novel anthocyanin **4** was identified as cyanidin 3-*O*-[6-*O*-
341 (*E*)-feruloyl-2-*O*-(6-*O*-(*E*)-feruloyl-β-D-glucopyranosyl)-β-D-glucopyranoside]-5-*O*-(4-*O*-β-D-
342 glucopyranosyl-6-*O*-malonoyl-β-D-glucopyranoside) (see complete structure elucidation in
343 Appendix)

344

345 *3.3 Antioxidant activity of isolated anthocyanins in aqueous micelles*

346 The study of the inhibited autoxidation of an oxidizable substrate under controlled conditions is the
347 golden standard in antioxidant testing, as it challenges the performance of antioxidants in close-to-
348 real model systems and directly evaluates the ability of the antioxidant to trap peroxy radicals,
349 which are responsible for oxidative chain-carrying (Amorati, Foti et al., 2013; Valgimigli et al.,
350 1997; Valgimigli & Pratt, 2012). Therefore, we investigated the ability of isolated anthocyanins to
351 protect linoleic acid, incorporated in aqueous neutral micelles, from autoxidation induced by
352 controlled thermal decomposition of water soluble azo-initiator AAPH at 37°C. Since previous
353 knowledge indicated that the antioxidant behaviour of anthocyanins depends on pH and that the
354 characteristic flavylum form is only prevailing under acidic conditions, we added 1% formic acid
355 in all experiments and verified the presence of the flavylum form by UV-Vis spectrophotometry.
356 Any of the tested pigments afforded good antioxidant protection, significantly reducing the rate of
357 autoxidation already when used at micromolar levels, and the inhibition was proportional to the
358 concentration, as shown in Figure 2A. However the performance was sensibly lower than that
359 offered under similar settings by reference α-tocopherol, which gave almost complete inhibition of
360 oxygen consumption for an inhibited period (τ) that lasted until complete consumption of the
361 antioxidant. Interestingly, the apparent antioxidant performance was identical for any tested
362 anthocyanin, irrespective of the degree and type of acylation/glycosylation. For anthocyanins **1-9**

363 the apparent rate constant for peroxy radical trapping in aqueous micelles, obtained by processing
364 oxygen consumption plots with equation (1), was in the range $k_{inh}=(3.8\pm0.7)\times10^4 \text{ M}^{-1}\text{s}^{-1}$, as
365 compared to the value of $(6.1\pm0.3)\times10^5 \text{ M}^{-1}\text{s}^{-1}$ recorded for α -tocopherol. Indeed, when we tested a
366 semi-purified fraction (fraction 8, see Appendix) containing about 13 main anthocyanins differing
367 for the pattern of acylation/glycosylation, again we obtained the same antioxidant performance.
368 Basic hydrolysis of the fraction (to remove acylating units) did not significantly alter the antioxidant
369 activity (Appendix). This behaviour apparently suggests that the presence of acylating phenolic
370 acids and the actual acylation/glycosylation pattern have little influence on the antioxidant
371 performance, which must come entirely from the cyanidin. This conclusion, however, would
372 conflict with previous experimental evidence (Stintzing et al., 2002), and would be counterintuitive,
373 considering that phenolic acids (the acylating units) have long been considered good antioxidants
374 (Amorati et al., 2006; Foti, Piattelli, Baratta, & Ruberto, 1996; Kikuzaki, Hisamoto, Hirose,
375 Akiyama, & Taniguchi, 2002). A reasonable explanation for the observed behaviour is that the
376 reactivity of water soluble anthocyanins, residing outside the micelle lipid core, with lipid soluble
377 peroxy radicals, formed during autoxidation of linoleic acid inside the lipid core, is mainly
378 regulated by the exchange of antioxidant inside/outside the micelle. Therefore, the protective
379 activity of the antioxidant is limited by its ability to access the micelle, which favours lipid soluble
380 α -tocopherol and disfavours anthocyanins, whose partition is only marginally affected by their
381 acylation pattern. This finding is in line with previous observation that the antioxidant activity of
382 phenolic acids and related compounds in heterogeneous media is influenced by their O/W partition
383 coefficient (Foti et al., 1996; Kikuzaki et al., 2002).

384

385 *3.4. Antioxidant activity in homogenous solution*

386 To gain knowledge on the absolute reactivity of anthocyanins with peroxy radicals and draw
387 structure-activity relationships on reliable kinetic ground, we turned to homogenous organic
388 solution, and studied the inhibited autoxidation of cumene and styrene in MeCN at 30°C. Although

389 they are not biomimetic substrates, cumene and styrene are, by far, the best known model systems
390 in autoxidation studies and provide complementary information (Amorati, Valgimigli, Panzella,
391 Napolitano, & d'Ischia, 2013). Due to limited availability of purified material, we focused our
392 kinetic measurements on anthocyanins **5**, **7**, **8**, **9**, together with their acylating acids, de-acylated
393 cyanin (bearing glucose A and C bound to the cyanidin core) and α -tocopherol as reference
394 antioxidant. As previously discussed, formic acid was added to the solutions to maintain the
395 anthocyanin in the flavylum form.

396 In the controlled autoxidation of cumene (Figure 2B), characterized by a modest oxidizability (the
397 propagation rate constant for oxidative chain is $k_p=0.34 \text{ M}^{-1}\text{s}^{-1}$ at 30 °C, while the corresponding
398 value for linoleic acid is $k_p=62 \text{ M}^{-1}\text{s}^{-1}$ (Valgimigli & Pratt, 2012)), any of the tested anthocyanins
399 gave complete inhibition already at micromolar level, until all the antioxidant was consumed.
400 Although this prevented distinguishing the performance of the different anthocyanins, it allowed the
401 accurate determination of the stoichiometric factor, n , *i.e.* the number of peroxy radicals trapped by
402 one molecule of antioxidant. Common phenolic antioxidants are expected to trap two peroxy
403 radicals per molecule ($n = 2$): this was indeed the case for α -tocopherol, for isolated phenolic acids
404 (ferulic and sinapic) or for their simple esters, as summarized in table 4. Cyanin had $n \sim 3$
405 suggesting that, beside the catechol moiety expected to trap two peroxy radicals (Amorati &
406 Valgimigli, 2012), also the free OH function in 7 position contributes to some extent to the
407 antioxidant activity. Interestingly all tested anthocyanins had much larger stoichiometric factors,
408 ranging from $n \sim 4$ for anthocyanin **8** bearing one ferulic acid residue, to about 6-7 for anthocyanins
409 **5**, **7** and **9** bearing two phenolic acids (sinapic or ferulic). This indicated a nearly additive
410 contribution of the antioxidant behaviour of the different moieties, each phenolic acid trapping
411 approximately two peroxy radicals and the aglycone trapping two-to-three peroxy radicals. Hence,
412 each acylated anthocyanin outperformed α -tocopherol in terms of duration of the antioxidant effect,
413 which largely depended on the degree of acylation.

414 In the autoxidation of more oxidizable styrene ($k_p=41 \text{ M}^{-1}\text{s}^{-1}$, close to linoleic acid (Amorati et al.,
415 2012)), it was instead possible to sharply differentiate the variable antioxidant performance of
416 diverse anthocyanins and their main moieties, as shown in figure 2C. As expected, reference α -
417 tocopherol gave a neat inhibited period, whose length corresponded to $n = 2$, followed by the re-
418 onset of uninhibited oxygen uptake after antioxidant consumption. Acylated anthocyanins had
419 instead a different behaviour: they gave an initial inhibited period of length corresponding to $n \sim$
420 2-4, followed by a retarded period, where the autoxidation was not fully inhibited yet it was
421 significantly slower than in the absence of an antioxidant (Figure 2C).

422 This indicated that different antioxidant sites of the anthocyanin molecule have diverse ability in
423 trapping peroxy radicals (Kumar et al., 2010), although the different sites cannot be distinguished
424 in the autoxidation of cumene, resulting in a unique longer inhibited period. Analysis of the specific
425 portions of oxygen uptake plots by means of equation (1) allowed to obtain the apparent rate
426 constant for peroxy radical trapping of the different active moieties, as summarized in Table 4.
427 Focusing on the first inhibited period, anthocyanins **5** and **7** bearing the sinapic residue gave
428 significantly higher inhibition rate constants ($k_{inh} \sim 2 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$), and comparison with the
429 measured k_{inh} values for sinapic acid and its methyl ester suggests that such residue is the main
430 contributor to their antioxidant performance. Indeed de-acylated cyanin showed only $k_{inh} = 4 \times 10^4$
431 $\text{M}^{-1}\text{s}^{-1}$, identical to the value recorded for ferulic acid or its ethyl ester. Anthocyanins **8** and **9**
432 bearing one and two ferulic acid residues, respectively, had somewhat intermediate antioxidant
433 performance, with k_{inh} ranging $0.9\text{-}1.2 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, thereby suggesting some positive interference
434 between one ferulic acid residue and the cyanidin moiety (Amorati & Valgimigli, 2012). Analysis
435 of the subsequent retarded period in oxygen uptake plots provided a second inhibition rate constant
436 $k_{inh} \sim 3\text{-}4 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ for any acylated anthocyanin, *i.e.* corresponding to the antioxidant
437 performance of the glycosylated cyanidin or one ferulic acid residue.

438 Taken together, data suggest that the most active molecular site is sinapic acid, when present, which
439 is able to trap two peroxy radicals by formal H-atom transfer and to interact with the cyanidin

440 residue, *e.g.* by hydrogen exchange with the catechol moiety. For this reason, an extended duration
441 of inhibition is observed, trapping 3-4 peroxy radicals with maximum efficiency. The mechanistic
442 and thermodynamic basis of analogous synergic interaction occurring intermolecularly in
443 antioxidant mixtures has recently been discussed (Valgimigli et al., 2013). Similarly, a synergic
444 interaction between the ferulic residue and the cyanidin moiety is responsible for the (somewhat
445 less effective) fast antioxidant response of anthocyanins bearing only ferulic acid residues. In all
446 cases additional antioxidant functions are left on the anthocyanin, namely the (second) ferulic acid
447 residue or the unblocked hydroxyl functions on the cyanidin core, which will trap additional
448 peroxy radicals with a lower, yet relevant, rate constant, thereby extending the overall antioxidant
449 performance.

450

451 **4. Conclusions**

452 By means of a novel fractionation approach we have isolated and characterized nine acylated
453 anthocyanins from the sprouts juice of *R. sativus* cv Sango, which share the cyanidin core and bear
454 three-to-four glycosidic residues acylated with malonic, ferulic and sinapic acids. Four new
455 compounds were characterized for the first time. All compounds had similar antioxidant activity in
456 aqueous micelles, while their performance differentiated in homogenous organic solution. Acylation
457 pattern significantly affected their antioxidant performance and pigments bearing a sinapic acid
458 moiety were the most effective, approaching the reactivity of reference α -tocopherol in quenching
459 peroxy radicals. Every tested compound outperformed α -tocopherol in terms of number of peroxy
460 radicals trapped by each antioxidant molecule. This is the first investigation providing absolute
461 peroxy-radical-trapping kinetics of acylated anthocyanins and highlighting the distinctive
462 contribution of the different structural moieties in the antioxidant performance. Hopefully our
463 results will help rationalize their structure-activity relationships. They highlight the importance of
464 acylation/glycosylation pattern of anthocyanins in determining their healthy-nutritional value.

465

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470

471 **Appendix. Supplementary data**

472 Supplementary data associated with this article can be found, in the online version, at

473

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- 560
- 561

Figures and Tables Legend

Figure 1. Structures of the anthocyanins isolated from *Raphanus sativus* cv. Sango sprouts.

Figure 2. Oxygen uptake kinetics during the controlled autoxidation of: A) 30 mM linoleic acid in Triton-X100 (0.16 M) neutral micelles at 37°C without inhibitor (a), or inhibited by: anthocyanin **1**, 4.0 μM (b), or 8.0 μM (c); anthocyanin **3**, 4.0 μM (d), or 8.0 μM (e); 3.3 μM α-tocopherol (f); B) 1.7 M cumene in acetonitrile at 30°C without inhibitor (a), or inhibited by: 5.5 μM anthocyanin **8** (b); 4.5 μM anthocyanin **7** (c); 3.9 μM anthocyanin **5** (d). C) 2.1 M styrene in acetonitrile at 30°C without inhibitor (a), or inhibited by: 5.0 μM Ethyl ferulate (b); 6.0 μM α-tocopherol (c); 4.2 μM anthocyanin **7** (d). Dashed lines represent linear regressions of the inhibited and subsequent portion of autoxidation; τ indicates the inhibition time.

Table 1. HR-ESI(+)MS, ESI (+)-MS/MSⁿ and UV-Vis spectroscopic data for anthocyanins isolated from *R. sativus* cv. Sango.

Table 2. ¹H NMR spectroscopic data of anthocyanins isolated from *R. sativus* cv. Sango sprouts in DMSO-*d*₆: TFA-*d*₁ (9:1).

Table 3. ¹³C NMR spectroscopic data of anthocyanins isolated from *R. sativus* cv. Sango sprouts in DMSO-*d*₆: TFA-*d*₁ (9:1). Signals were indirectly detected by HSQC and HMBC analysis.

Table 4. Rate constants and stoichiometric factors for reaction with peroxy radicals of anthocyanins from *R. sativus* cv. Sango sprouts, their acylating phenolic acids and corresponding esters, and reference compounds, determined from the inhibited autoxidation of styrene and cumene in acetonitrile (containing 1% v/v formic acid) at 30°C.

Table 1. HR-ESI(+)-MS, ESI (+)-MS/MSⁿ and UV-Vis spectroscopic data for anthocyanins isolated from *R. sativus* cv. Sango.

ID	Molecular formula	HR-ESI(+) MS found (calculated)	ESI [M] ⁺	MS tandem ^a			UV-Vis spectral properties ^b			
				MS ² ions	MS ³ ions	MS ⁴ ions	$\lambda_{\text{acyl max}}$ [nm]	$\lambda_{\text{vis max}}$ [nm]	Acylation ratio (%) ^c	Glycosylation ratio (%) ^d
1	C ₅₄ H ₅₉ O ₂₈	1155.3199 (1155.3187)	1155	993 (100), 449 (30)	287 (100)		329	535	105	17
2	C ₆₃ H ₇₁ O ₃₆	1403.3725 (1403.3720)	1403	1359 (20), 993 (100), 697 (75)	993 (70), 287 (100)		330	535	98	23
3	C ₆₂ H ₆₉ O ₃₅	1373.3631 (1373.3614)	1373	1329 (25), 963 (100), 697 (90)	697 (100), 653 (10), 287 (70)		329	535	98	18
4	C ₆₂ H ₆₉ O ₃₅	1373.3627 (1373.3614)	1373	1329 (20), 963 (100), 697 (10)	963 (5), 625 (10), 287 (100)	287 (100)	328	534	107	16
5	C ₅₇ H ₆₁ O ₃₁	1241.3184 (1241.3191)	1241	1197 (20), 993 (70), 535 (100)	993 (100), 491 (60),	287 (100)	329	533	99	17
6	C ₄₄ H ₅₁ O ₂₅	979.2720 (979.2714)	979	979 (10) 817 (100) 449 (30) 287	287 (100)		334	527	58	18
7	C ₅₄ H ₅₉ O ₂₈	1155.3175 (1155.3187)	1155	993 (100), 449 (10)	993 (70), 287 (100)		329	533	128	22
8	C ₄₆ H ₅₁ O ₂₇	1035.2619 (1035.2612)	1035	787 (70), 535 (100)	287 (100)		327	522	53	18
9	C ₅₆ H ₅₉ O ₃₀	1211.3098 (1211.3086)	1211	1167 (20), 963 (100), 535 (70)	287 (100)		328	530	110	18

^a Relative intensities of each ion are in parentheses; the most abundant ion (in bold) is selected for subsequent fragmentation.

^b Evaluated by HPLC-DAD on Hypersil GOLD PFP column (5 μm , 175 \AA , 4.6 \times 250 mm) eluting with A (aq 0.12% v/v TFA) and B (MeCN + 0.12% v/v TFA). See gradient program on the text.

^c Acylation ratio = $E_{\text{acyl}}/E_{\text{vis}}$, 59–63% indicates monoacylation, 98–128% indicates diacylation.

^d Glycosylation ratio = E_{440}/E_{vis} , 15–24% indicates bioside, 29–35% indicates monoside.

Table 2. ¹H NMR spectroscopic data of anthocyanins isolated from *R. sativus* cv. *Sango* sprouts in DMSO-*d*₆: TFA-*d*₁ (9:1)^a

	1	2	3	4	5	6	7	8	9
Cyanidin									
4	8.69 s	8.71 s	8.71 s*	8.77 s	8.73 s	8.83 s	8.74 s	8.73 s	8.78
6	7.02 s	6.94 s	6.90 s	7.04 s	6.91 s	6.92 s	6.93 s	6.91 s	6.91 s
8	6.89 s	6.90 s	6.88 s	6.88 s	6.91 s	6.97 s	6.88 s	6.98 s	6.91 s
2'	7.85 d (2.4)	7.89 s	7.87 s	7.97 s	7.88 d (1.8)	7.92 s	7.89 s	7.99 d (2.4)	7.90 s
5'	7.07 d (9.0)	7.10 d (8.4)	7.01 d (8.8)	7.05 d*	7.11 d (9.0)	7.10 d (8.8)	7.11 d (8.4)	7.04 d (8.4)	7.07 m*
6'	8.31 dd (9.0, 1.8)	8.34 d (8.4)	8.30 d (8.8)	8.21 d (8.4)	8.33 dd (9.0, 2.2)	8.34 d (8.8)	8.33 d (8.4)	8.20 dd (8.4, 2.4)	8.23 (8.4)
Glc A									
1	5.62 d (7.2)	5.60 d (6.6)	5.58 d (7.2)	5.58 d (7.2)	5.64 d (7.2)	5.51 d (7.6)	5.66 d (7.2)	5.61 d (7.2)	5.62 d (7.3)
2	4.07 t (7.8)	4.12 d (7.8)	4.10 t (7.6)	4.00 m	4.12 t (7.8)	4.03 t (8.0)	4.12 t (8.2)	4.06 t (8.0)	4.01 t (7.9)
3	3.61 t (8.4)	3.65 m *	3.64 m*	3.85 m*	3.65 m*	3.68 m*	3.64 t (7.8)	3.67 m *	3.70 m*
4	3.60 m*	3.45 m *	3.45 m*	3.88 m*	3.45 m*	3.40 m*	3.49 m*	3.35 m *	3.45 m*
5	3.65 m*	3.93 m *	3.97 m*	3.98 m*	3.94 m*	3.30 m*	3.91 m	3.97 m	3.91 m*
6a	3.86 m*	4.21 m *	4.27 m*	4.21 m*	4.18 dd (12.6, 5.8)	3.40 m*	4.23 dd (11.2, 5.6)	4.28 m	4.20 d (11.9)
6b	3.60 m*	4.36 d (12.0)	4.37 m*	4.30 m*	4.39 m*	3.40 m*	4.34 d (11.2)	4.44 d (10.2)	4.41 d (11.9)
Glc B									
1	5.10 d (7.8)	5.13 d (7.8)	5.12 d (8.0)	4.70 d (7.2)	5.15 d (8.4)	5.13 d (7.6)	5.14 d (8.2)	4.61 d (7.8)	4.78 d (7.7)
2	4.60 t (9.0)	4.64 t (7.8)	4.64 t (8.0)	3.10 m*	4.65 t (8.4)	4.60 t (8.8)	4.67 t (8.5)	2.94 t (8.7)	3.10 m *
3	3.44 m*	3.35 m *	3.35 m*	3.15 m*	3.37 m*	3.70 m*	3.37 t (9.0)	3.06 t (9.0)	3.21 m*
4	3.40 m*	3.14 m *	3.16 m*	3.20 m*	3.16 m*	3.40 m*	3.15 t (9.0)	2.99 t (9.0)	3.35 m*
5	3.35 m*	3.04 m*	3.03 m*	3.08 m*	3.05 t (7.2)	3.30 m*	3.08 t (8.5)	2.67 m	3.61 m *
6a	4.25 m	3.39 m *	3.38 m*	4.27 m*	3.41 m*	3.40 m*	3.60 m*	3.11 m *	3.89 m*
6b	4.32 m	3.63 m *	3.60 m*	4.27 m*	3.65 m*	3.40 m*	3.40 m*	3.15 m *	4.01 m *
Glc C									
1	5.03 d (7.2)	5.21 (6.0)	5.19 d (7.0)	5.07 d (7.6)	5.13 d (7.2)	5.07 d (7.6)	5.06 d (7.5)	5.10 d (7.2)	5.06 d (7.8)

2	3.47 m	3.57 m *	3.56 m*	3.59 m*	3.52 m*	3.55 m*	3.50 m*	3.50 t (8.4)	3.51 m*
3	3.32 m*	3.46 m *	3.46 m*	3.52 m*	3.38 m*	3.70 m*	3.35 t (9.2)	3.33 m *	3.35 m*
4	3.11 t (7.2)	3.52 m *	3.41 m*	3.40 m*	3.19 m*	3.40 m*	3.22 t (9.6)	3.17 t (9.0)	3.20 m*
5	3.40 m*	3.99 m *	3.90 m*	3.30 m*	3.73 m*	3.30 m*	3.48 m*	3.70 m *	3.69 m*
6a	3.17 m*	4.26 m *	4.20 m *	3.70 m*	3.88 dd (12.0, 6.6)	3.40 m*	3.54 m*	4.35 d (10.8)	3.96 m *
6b	3.03 m*	4.39 d (12.0)	4.35 m *	3.70 m*	4.36 m*	3.40 m*	3.74 d (12.0)	3.88 m	4.35 d (11.9)

Glc D (R₅)

1		4.27 d (7.1)	4.26 d (7.6)	4.26 d (7.8)
2		3.00 t (7.1)	3.00 m*	3.58 m*
3		3.18 m *	3.16 m*	3.52 m*
4		3.06 m *	3.09 m*	3.40 m*
5		3.27 m *	3.25 m*	3.35 m*
6a		3.43 m *	3.41 m*	3.70 m*
6b		3.71 m *	3.69 m*	3.70 m*

Feruloyl (R₁)

2		6.97 s	7.16 s	7.00 s	6.97 s	7.05 s	6.98 s	7.07 s
5		6.70 d (7.2)	6.75 d (8.4)	6.88 d (7.2)	6.71 d (9.0)	6.76 d (8.2)	6.69 d (8.4)	6.72 d (8.4)
6		6.90 m *	6.99 d (8.0)	6.70 d (7.8)	6.87 d (8.4)	6.95 d (8.0)	6.88 d (7.8)	6.89 m*
α		6.24 d (15.6)	6.37 d (15.6)	6.23 d (16.2)	6.24 d (16.2)	6.24 d (15.9)	6.25 d (16.2)	6.10 d (15.9)
β		7.33 d (16.2)	7.45 d (15.6)	7.32 d (15.6)	7.32 d (16.2)	7.33 d (15.7)	7.34 d (15.6)	7.19 d (15.9)
3-OCH ₃		3.71 s	3.77	3.74 s	3.69 s	3.74 s	3.68 s	3.75 s

Feruloyl (R₂)

2	6.86 m*			7.04 s				7.07 s
5	6.72 d (7.8)			6.72 d (7.8)				6.71 d (8.0)
6	6.91 d (8.4)			6.84 d (7.8)				6.87 m*
α	6.22 d (15.6)			6.08 d (15.6)				6.24 d (15.7)

β	7.30 d (15.6)			7.17 d (15.0)				7.35 d (15.5)
3-OCH ₃	3.70 s			3.71 s				3.71 s
Sinapoyl or feruloyl (R ₃)								
2	6.88 s	6.91 s	6.95 s	6.91 s	6.87 s	6.96 s		
5			6.67 d (8.4)					
6	6.88 s	6.91 s	6.87 d*	6.91 s	6.87 s	6.96 s		
α	6.40 d (15.6)	6.46 d (15.6)	6.24 d (16.0)	6.44 d (16.2)	6.40 d (16.0)	6.46 d (15.7)		
β	7.42 d (15.6)	7.43 d (16.2)	7.30 d (15.6)	7.46 d (16.2)	7.42 d (16.0)	7.47 d (16.4)		
3-OCH ₃	3.75 s	3.78 s	3.69	3.78 s	3.76 s	3.79 s		
5-OCH ₃	3.75 s	3.78 s		3.78 s	3.76 s	3.79 s		
Malonoyl (R ₄)								
CH ₂		3.30 s *	3.27 s	3.30 s	3.34 s		3.33 s	3.37 s

^a Values in parentheses indicate coupling constants (*J* in Hz)

* Overlapped signals

Table 3. ^{13}C NMR spectroscopic data of anthocyanins isolated from *R. sativus* cv. *Sango* sprouts in $\text{DMSO-}d_6$: $\text{TFA-}d_1$ (9:1) . Signals were indirectly detected by HSQC and HMBC analysis.

	1	2	3	5	7	8
Aglycon						
2	167.7	162.7	162.5	162.5	162.6	162.6
3	145.9	144.9	144.9	144.6	144.7	144.9
4	135.5	130.7	131.9	131.7	132.0	131.3
4 a	112.5	111.7	111.8	111.6	111.5	111.9
5	154.6	155.0	155.1	155.1	155.2	155.1
6	112.3	104.5	104.8	104.4	104.7	104.7
7	168.5	167.8	167.8	167.3	167.8	167.5
8	96.5	96.1	94.6	96.0	96.2	96.2
8 a	155.4	155.7	155.6	155.1	155.2	155.0
1'	119.5	120.0	120.1	119.9	120.1	119.7
2'	117.8	116.8	117.2	117.1	117.2	117.5
3'	147.5	147.2	147.3	146.7	146.9	146.6
4'	155.1	155.8	155.3	155.6	155.7	155.4
5'	117.6	117.2	117.3	116.8	117.0	116.9
6'	129.2	129.2	128.8	128.6	128.7	127.7
Glc A						
1	99.4	96.8	98.4	98.8	98.8	99.1
2	78.3	77.2	77.3	77.5	77.5	80.8
3	76.0	76.3	76.4	76.5	76.3	76.0
4	69.8	70.1	70.1	70.2	70.1	75.8
5	74.5	73.3	72.7	73.7	73.8	73.7
6	62.1	63.5	64.1	63.2	62.7	63.1
Glc B						
1	100.4	99.7	99.3	99.6	99.9	103.6
2	74.8	73.8	74.3	74.1	74.0	74.5

3	78.5	79.9	75.1	74.8	74.7	76.0
4	71.5	70.4	71.1	70.7	70.8	69.5
5	74.5	77.5	77.8	77.5	77.7	76.7
6	62.0	61.3	61.5	61.3	61.0	60.6

Glc C

1	102.5	101.2	101.4	101.5	102.2	101.5
2	74.2	72.8	74.6	73.0	73.4	72.9
3	76.9	74.8	79.7	75.8	76.2	76.5
4	70.5	70.1	70.2	69.6	69.9	69.8
5	78.1	72.1	74.2	74.3	77.8	74.1
6	62.1	62.5	63.4	64.2	61.0	64.1

Glc D (R₅)

1		103.4	103.8			
2		73.5	73.5			
3		76.3	70.4			
4		70.1	70.0			
5		76.6	77.3			
6		61.1	61.6			

Feruloyl (R₁)

1		126.0	126.2	125.8	125.7	125.7
2		111.6	111.4	111.7	111.8	111.8
3		148.4	148.4	148.0	148.3	148.0
4		147.9	148.1	149.7	149.3	149.6
5		115.6	115.9	115.6	115.8	115.5
6		123.0	123.4	123.1	123.2	122.9
α		114.2	115.3	114.0	115.7	113.9
β		149.5	145.6	145.6	145.7	145.4
3-OCH ₃		55.8	56.1	55.7	55.9	55.6
CO		166.8	166.7	166.2	166.9	166.9

Feruloyl (R₂)

1	125.2
2	116.5
3	147.6
4	149.5
5	116.0
6	123.8
α	114.6
β	146.2
3-OCH ₃	55.6
CO	165.6

Sinapoyl or
feruloyl (R₃)

1	125.5	124.9	124.8	125.0	124.9
2	107.1	106.3	112.0	106.7	106.4
3	147.9	148.5	148.4	148.4	138.6
4	107.1	139.9	140.2	145.5	148.5
5	147.9	148.5	116.2	148.4	138.6
6	106.5	106.3	123.1	106.7	106.4
α	116.3	115.5	114.2	115.6	115.8
β	145.8	145.8	146.0	145.2	145.4
3-OCH ₃	56.1	56.0	56.1	56.2	56.4
5-OCH ₃	56.1	56.0		56.2	56.4
CO	166.3	166.2	166.3	166.2	166.2

Malonoyl (R₄)

1		166.7	167.7	167.0		167.0
2		41.1	41.4	41.2		41.1
3		168.3	167.7	168.3		168.3

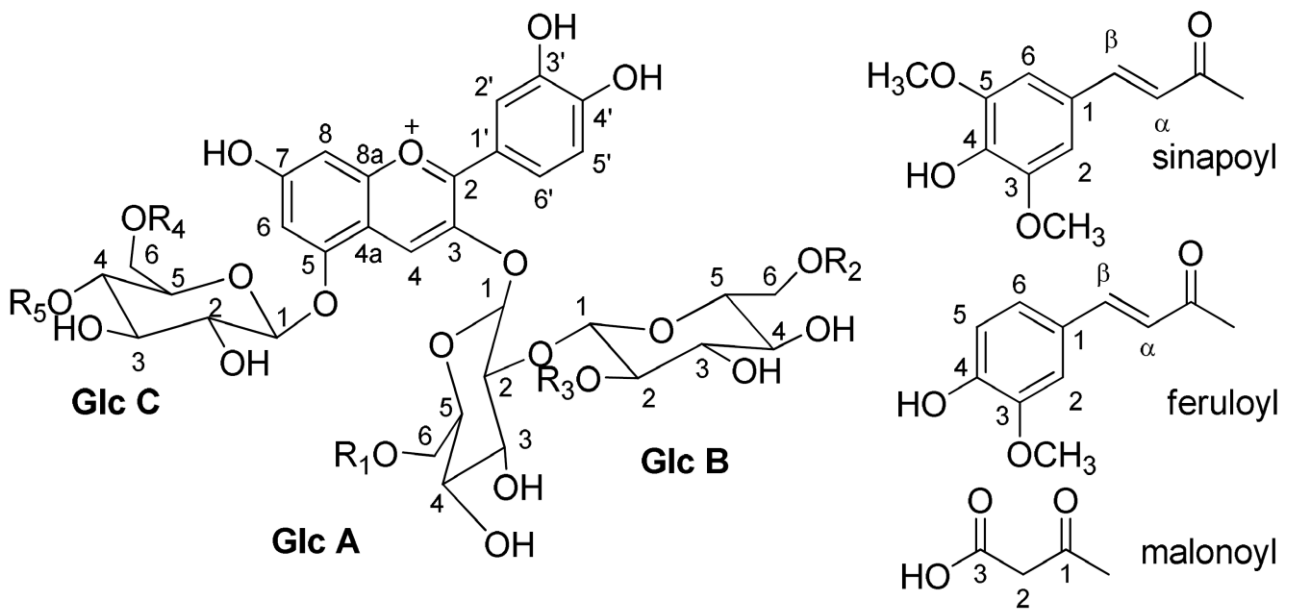
Table 4. Rate constants and stoichiometric factors for reaction with peroxy radicals of anthocyanins from *R. sativus* cv. *Sango* sprouts, their acylating phenolic acids and corresponding esters, and reference compounds, determined from the inhibited autoxidation of styrene and cumene in acetonitrile (containing 1% v/v formic acid) at 30°C.

id	R ₁	R ₂	R ₃	R ₄	10 ⁻⁵ • k _{inh} (in styrene)		n	
					Inhibited ^a	Retarded ^a	(styrene) ^b	(cumene) ^b
5	feruloyl	H	sinapoyl	malonoyl	2.0±0.2	0.40±0.10	3.5±0.4	6.8±0.6
7	feruloyl	H	sinapoyl	H	1.9±0.2	0.35±0.05	3.7±0.2	6.3±0.5
8	feruloyl	H	H	malonoyl	0.9±0.2	0.34±0.06	1.8±0.2	4.0±0.5
9	feruloyl	feruloyl	H	malonoyl	1.2±0.2	0.34±0.07	2.2±0.1	5.7±0.6
Methyl Sinapate					1.8±0.4		1.9±0.3	
Sinapic acid					1.6±0.2		2.1±0.2	
Ethyl Ferulate						0.4±0.1		(2.0) ^c
Ferulic acid						0.3±0.1		2.0±0.2
Cyanin						0.4±0.2		3.0±0.5
Tocopherol					6.0±1.0		2.0±0.1	

^a Determined, respectively, from the slope of inhibited period and retarded period in the autoxidation of styrene. Refer to text and figure 2.

^b Stoichiometric factor measured in the autoxidation of the substrate indicated in parenthesis

^c Not determined. In parenthesis is indicated the expected value based on the measured value of ferulic acid.



ID	R ₁	R ₂	R ₃	R ₄	R ₅
1	H	feruloyl	sinapoyl	H	H
2	feruloyl	H	sinapoyl	malonoyl	β-glucose
3	feruloyl	H	feruloyl	malonoyl	β-glucose
4	feruloyl	feruloyl	H	malonoyl	β-glucose
5	feruloyl	H	sinapoyl	malonoyl	H
6	H	H	sinapoyl	H	H
7	feruloyl	H	sinapoyl	H	H
8	feruloyl	H	H	malonoyl	H
9	feruloyl	feruloyl	H	malonoyl	H

Figure 2. Structures of the anthocyanins isolated from *Raphanus sativus* cv. Sango sprouts.

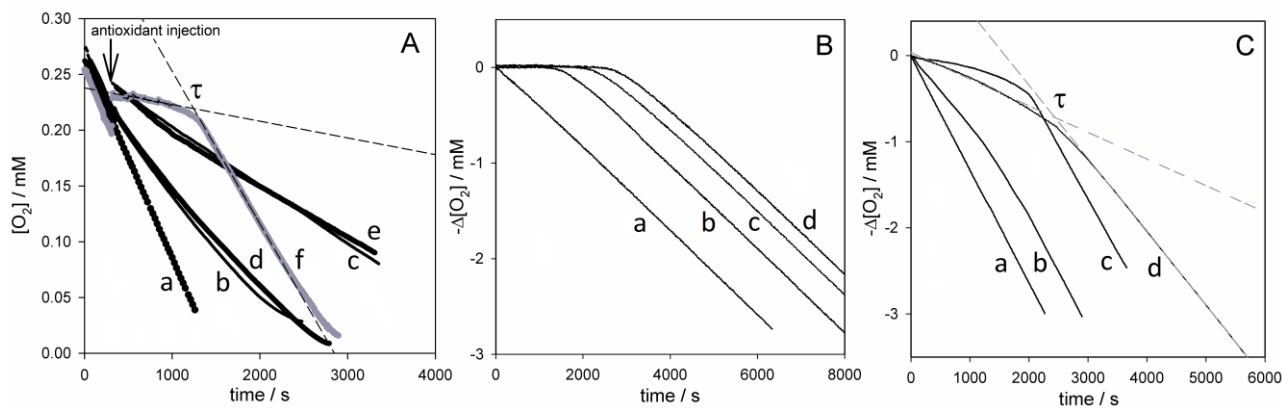


Figure 2. Oxygen uptake kinetics during the controlled autoxidation of: A) 30 mM linoleic acid in Triton-X100 (0.16 M) neutral micelles at 37°C without inhibitor (a), or inhibited by: anthocyanin **1**, 4.0 μM (b), or 8.0 μM (c); anthocyanin **3**, 4.0 μM (d), or 8.0 μM (e); 3.3 μM α -tocopherol (f); B) 1.7 M cumene in acetonitrile at 30°C without inhibitor (a), or inhibited by: 5.5 μM anthocyanin **8** (b); 4.5 μM anthocyanin **7** (c); 3.9 μM anthocyanin **5** (d). C) 2.1 M styrene in acetonitrile at 30°C without inhibitor (a), or inhibited by: 5.0 μM Ethyl ferulate (b); 6.0 μM α -tocopherol (c); 4.2 μM anthocyanin **7** (d). Dashed lines represent linear regressions of the inhibited and subsequent portion of autoxidation; τ indicates the inhibition time.