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Phenolic distribution in apple epidermal and outer cortex tissue by multispectral deep-UV autofluorescence cryo-imaging

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23 **Abstract**

24 Phenolic compounds in fruit are involved in responses to biotic and abiotic stresses and are
25 responsible for organoleptic properties. To establish the distribution of these secondary
26 metabolites at the tissue and sub-cellular scales, mapping of fluorescence in apple epidermis
27 and outer cortex tissue in cryogenic condition was performed after deep-UV excitation at 275
28 nm. Douce Moën and Guillevic cider apple varieties were sampled and frozen after harvest,
29 after 30 days at 4 °C and after 20 days at room temperature. Image analysis of fluorescence
30 emission images acquired between 300 and 650 nm allowed the assignment of fluorescence
31 signals to phenolic compound families based on reference molecules. Emission attributed to
32 monomeric and/or condensed flavanol was localized in whole tissue with major fluorescence
33 in the cuticle region. Hydroxycinnamic acids were found predominantly in the outer cortex
34 and appeared in the cell wall. Fluorescent pigments were mostly found in the epidermis. The
35 distribution of flavanols in the sub-cuticle and phenolic acids in the outer cortex distinguished
36 apple varieties. Storage conditions had no impact on phenolic distribution. The proposed
37 fluorescent imaging and analysis approach enables studies on phenolic distribution in relation
38 to fruit development, biotic/abiotic stress resistance and quality.

39

40 **Key words:** *Malus domestica*, phenolic compounds, autofluorescence, multispectral imaging,
41 deep-UV synchrotron light, cryo-microscopy.

42 **1. Introduction**

43 Phenolic compounds are common plant secondary metabolites that have been grouped
44 into three classes: flavonoids, phenolic acids and stilbenes [1]. The flavonoids encompass
45 flavanols that are also found as oligomers and polymers (i.e., proanthocyanidins and
46 condensed tannins), anthocyanins and flavonols, while phenolic acids comprise
47 hydroxybenzoic acids and hydroxycinnamic acids (Figure 1). Except for flavanols and
48 hydroxybenzoic acids, which are mainly present in their free form, most phenolic compounds
49 are glycosylated and/or acylated derivatives. Their ultraviolet absorption capacity limits light-
50 induced damage to tissues while they are part of plant defence mechanisms against external
51 biotic stresses [2, 3]. In fleshy fruit, phenolic compounds are known for their antioxidant
52 contribution to foods and organoleptic properties, such as colour, astringency or bitterness [4-
53 6]. Various studies in apple and grape have shown important differences in phenolic content
54 between varieties according to fruit development and tissue [7]. Phenolic compounds are
55 commonly characterized by biochemical techniques on whole fruit, skin, flesh, seed or juice
56 extracts. However, there are few reports on the distribution of specific phenolic compounds at
57 the tissue and sub-cellular scales under *in-vivo-like* conditions. Flavanols and condensed
58 tannins were determined to be localized in the epidermis, parenchyma and seeds by
59 microscopy of fresh or chemically fixed apple [8], grape [9, 10] and other fruits [11-14].
60 Flavanols were localized by DMACA staining (4-dimethylaminocinnamaldehyde) [14-16]
61 while flavonols were highlighted by fluorescence microscopy using the specific dye DPBA
62 (diphenylboric acid-2-aminoethyl ester) [17, 18]. However, chemical preparation damage to
63 fleshy fruit tissue integrity affects the cellular localization of diffusible compounds [19], and
64 dyes are limited by their specificities.

65 To address these issues, mapping phenolic compounds through their autofluorescence
66 characteristics appears to be a good alternative as no specific stain is required.

67 Hydroxycinnamic acids express a blue fluorescence when excited at the UV wavelength of
68 approximately 350 nm [20, 21] while anthocyanins and flavonols are autofluorescent at
69 visible wavelengths [22]. Monomeric and condensed tannin ((+)-catechin, (-)-epi-catechin,
70 procyanidins) fluorescence requires deep-UV wavelength excitation at approximately 280 nm
71 [23] [24]. The use of different bandpass filters to select specific ranges of fluorescence
72 emissions offers the opportunity to localize different compounds within the same sample
73 through a multispectral approach [25]. However, data on the fluorescence emission of specific
74 phenolic compounds are scarce due to the high number of entities that constitute this
75 metabolite family and because access to pure reference phenolic compounds is limited.

76 To avoid artefacts related to small compound diffusion and loss during sample
77 preparation, cryo-fixation, cryo-microtomy and fluorescence cryo-observation of biological
78 samples have seen recent developments. Cryo-confocal observations at high resolutions were
79 introduced with the realization of specific microscope stages and immersion objective lens
80 prototypes [26, 27]. A simpler and readily accessible setup was developed to observe fleshy
81 fruit tissue by cryo-laser scanning confocal microscopy (LSCM) [18]. Among the advantages
82 of cryo-observations is the lower fluorescence bleaching compared to that during room
83 temperature observations and thus the recovery of higher fluorescence intensity [26, 28].

84 Preliminary tests of fresh fruit auto-fluorescence responses after excitation at 375 nm
85 were performed on apple and grape. The latter revealed promising autofluorescence signals,
86 while a lower excitation wavelength was found necessary to assess flavanol localization. The
87 objective of the present work was to map phenolic compounds in cryo-fixed outer tissues of
88 apple. The mapping was performed at a large field of view by multispectral autofluorescence
89 imaging after deep-UV excitation at 275 nm using the setup developed for fleshy fruit cryo-
90 observation. Two cider apple varieties stored in three different conditions were studied to
91 evaluate the genetic and physiological impacts on phenolic compound distribution.

92 Fluorescence excitation and emission characteristics of reference phenolic compounds were
93 recorded to help identify fruit tissue phenolics.

94 **2. Materials and methods**

95 *2.1. Materials*

96 *2.1.1. Chemicals*

97 Phenolic compounds used for reference fluorescence spectra were from the laboratory
98 collections of ESA-GRAPPE (Angers, France) and BIA-PRP (Rennes, France). Formic acid
99 was purchased from Sigma-Aldrich (UK) and methanol was purchased from CarloErba
100 (Italy).

101 *2.1.2 Fruits*

102 Apple (*Malus domestica*, Royal Gala) and grape (*Vitis vinifera*, Red Italian) for
103 preliminary fluorescence analysis after 375 nm UV excitation were from a local retail store.
104 These fruits were used without further storage. For the fluorescence mapping following
105 excitation at 275 nm, two cider apple varieties, Douce Moën (*M. domestica*, Douce Moën)
106 and Guillevic (*M. domestica*, Guillevic), were provided by IFPC (Le Rheu, France). Fruits
107 were harvested on October 2017 in orchards at Surzur (Morbihan, France). Nine fruits of each
108 variety were subjected to three different storage conditions: three were immediately sampled,
109 three were stored at 4 °C for 30 days and three were stored at room temperature for 20 days
110 before sampling.

111 *2.2. Methods*

112 *2.2.1. Spectrofluorometry*

113 Phenolic references were dissolved at 2 mg mL⁻¹ in 2 mL of H₂O:MeOH (1:1 in
114 volume) + 1% formic acid (in volume). Quartz cuvettes (10 x 4 mm, 114F-QS, Suprasil

115 HELMA Analytics, France) were filled with 500 μ L of standard solution and observed by
116 spectrofluorimetry (F-4500, HITACHI, Japan). Emission and excitation spectra for each
117 compound were recorded between 200 nm and 600 nm.

118 2.2.2. *Laser scanning confocal microscopy (LSCM)*

119 Cubes of 5 x 5 x 5 mm³ were sampled from the epidermal region of equatorial fruit
120 slices of commercial apple and grape. Sections of 100 μ m thickness were cut from fresh
121 cubes with a vibrating blade microtome (HM 650V, MICROM, France) and immediately
122 observed or dipped in standard buffer solution pH 10 (HI 70010C, NIST standard, HANNA
123 INSTRUMENTS, Hungary) followed by H₂O milliQ wash prior to LSCM observations at
124 room temperature. LSCM (A1 Eclipse Ti inverted microscope, NIKON Inc., Japan) was
125 configured with laser diode excitation at 375 nm. Fluorescence emission was acquired at 20 \times
126 magnification with a resolution of 0.63 μ m pixel⁻¹ and image size of 512 x 512 pixels² (16-
127 bit). Four bandpass emission filters were used to map fluorescence: 400-450 nm, 500-530 nm,
128 560-600 nm and 630-700 nm. These form sets of multispectral images of four channels with
129 spectral controlled excitation and emission parameters.

130 2.2.3. *Synchrotron deep-UV wide-field fluorescence microscopy (DUV)*

131 One 5 x 5 x 5 mm³ cube was randomly sampled from the epidermal region of a cider
132 apple fruit equatorial slice. The cube was frozen in liquid nitrogen-cooled isopentane and
133 stored at -20 °C prior to analysis. Sections (60 μ m thick) were cut from frozen cubes using a
134 cryostat microtome (HM 500 OM, MICROM, France) at -20 °C. The section was placed
135 between quartz lamella (R52-5000, ESCO Optics, USA) glued together by frozen water
136 droplets. Synchrotron UV microscopy was performed at the DISCO (Dichroism, Imaging,
137 mass Spectrometry for Chemistry and biOlogy) beamline at the SOLEIL (Source Optimisée
138 de Lumière à Energie Intermédiaire du LURE (Laboratoire à Utilisation du Rayonnement

139 Electromagnétique)) synchrotron radiation facility [29] (Gif-sur-Yvette, Saint-Aubin, France)
140 on the inverted epi-fluorescence TELEMOS microscope [30]. Observation was realized under
141 cryogenic conditions using a handmade setup composed of a Peltier stage (PE100, LINKAM
142 Scientifics, UK) cooled by dry ice as described in [18]. The wide-field microscope
143 TELEMOS modified by the DISCO beamline staff was a Zeiss Axio Observer Z-1 designed
144 to observe samples after deep-UV (DUV) excitation. The microscope was equipped with a
145 motorized sample plate (MS-2000 XY, Applied Scientific Instrument, USA) to move the
146 sample along the X and Y axes. Sample movement along the Z axis was controlled by the
147 inverted microscope. The setup used the 10× magnification Zeiss Ultrafluar lens (N.A 0.2,
148 WD 7.4) and a sharp 300 nm dichroic mirror “DM” (Omega Optical), which reflected the 280
149 nm excitation and transmitted emission light above 300 nm. Multispectral fluorescence
150 emission was recorded using six different emission bandpass filters: 300-306 nm, 327-353
151 nm, 352-388 nm, 412-438 nm, 420-480 nm and 600-650 nm (Semrock, Rochester, USA). In
152 the text, filters are referred to by their low wavelength limit. A back-illuminated CCD camera
153 (Pixis BUV, Princeton Instrument, USA) recorded the images (1024 x 1024 pixels; 1 x 1 μm^2
154 per pixel; grey level coded in 16 bit). Acquisition times were 12.5 s for the 300 nm filter, 5 s
155 for the 327, 352, 412 and 420 nm filters, and 7.5 s for the 600 nm filter. Acquisition was
156 performed from the sample cuticle to approximately one mm inside the tissue. With three
157 biological repetitions and three different storage modalities, a total of nine images were
158 registered per variety.

159 The beamline intensity, which was focused at the centre of the observation field, led to
160 raw images that required corrections to homogenize illumination. In addition, the camera
161 provided a non-null background without illumination. Both inhomogeneous illumination and
162 background corrections were realized according to the following formula:

163

$$I_c = \frac{I - \text{Dark}}{\text{White} - \text{Dark}}$$

164 where I_c is the corrected image; I is the raw image; Dark is the background and White
165 is the flat field

166 For this purpose, nine different "dark" images were recorded without sample and without any
167 illumination, one for each of the six emission filters. The "White" images were acquired using
168 a frame (1.0 x 1.0 cm², AB-0576, Gene Frame, THERMOSCIENTIFIC, UK) displaying a
169 homogeneous autofluorescent signal. Thresholding was also required to define the sample
170 image from the camera noise. Image pretreatments were performed using MATLAB® 2017b.

171 The corrected images were cropped to 300 x 400 μm² size to fit within a pertinent
172 sample image area. To take into account out-of-plane sample sections, several acquisitions
173 were recorded for the same sample area with a maximum of six focal planes. As this method
174 was not confocal imaging, a clear image was reconstructed by combining the different focal
175 plane views with the extended focus software HeliconFocus6® (version 6.8.0) using the
176 pyramidal method.

177 *2.2.3.1. Multispectral analysis of DUV fluorescence images*

178 DUV fluorescence emission was valued based on the grey scale intensity of pixels in
179 cropped images. Principal component analysis was applied separately on each multispectral
180 image, and fluorescence profiles were extracted from selected emission filters.

181 *2.2.3.2. Image Analysis*

182 Individual emission images were unfolded to form one vector [31]. The image vectors
183 were combined into one table per sample containing six columns corresponding to the six
184 filters and n rows corresponding to the n pixels of the image. Principal component analysis
185 (PCA) was applied to the resulting data table, and correlation circles were drawn from the

186 PCA loadings. Principal component images were obtained by refolding scores [31].
187 Component images were filtered to keep 99% of pixels closer to the median value and to
188 remove eventual aberrant pixel values prior to normalization of grey levels between 0 and
189 255. PCA reconstructed colour images were created from components 1, 2 and 3 identified in
190 the blue, red and green channels, respectively.

191 Localization of phenolic compounds in samples was also analysed by computing fluorescence
192 intensity profiles from the cuticle to the inner tissue. This "image distance" analysis was
193 realized using FIJI/ImageJ® software [32] and the mathematical morphology plugin
194 process/erode (3D) [33]. Thresholding was first applied to create a binary mask of the sample.
195 Distance from the cuticle was obtained by the following procedure: one-pixel erosion (pixel
196 size = $1 \times 1 \mu\text{m}^2$) was applied to the sample mask and the resulting binary image was
197 subtracted from the initial sample binary image. This step was iteratively applied until the last
198 image column was reached. The mean profile was smoothed to reduce intensity variations due
199 to the presence of cell lumens with low fluorescence and cell walls with high fluorescence.
200 This was realized by applying local regression (LOESS) with a degree of smoothing of 0.4.
201 Normalization between 0 and 1 was realized on the maximum smoothed mean value to allow
202 profiles comparison between samples. Examples of raw and smoothed profiles are shown for
203 Douce Moën at harvest in Supplementary Figures 1 and 2. The smoothed profiles from the
204 three biological replicates per storage modality were averaged and the mean profile per
205 modality was plotted with its 95% confident interval (Supplementary Figure 3).

206 2.2.3.3. *Statistical analyses*

207 The effect of storage was evaluated by Student's t-tests of the smoothed mean pixel
208 values of three biological replicates per pixel distance. As no significant differences were
209 found between storage modalities, Student's t-test for variety effect was performed on the
210 nine replicates.

211 Principal component analysis, LOESS smoothing and Student's t-tests were performed
212 in R [34].

213 **3. Results**

214 *3.1. Fluorescence properties of reference phenolic compound*

215 Excitation and emission fluorescence spectra were registered from standard phenolic
216 compounds (Supplementary figure 4) and the maximum wavelength responses recorded
217 (Table 1). If associating specific compounds with peculiar emission wavelengths is difficult
218 due to the width of spectra with large overlaps, distinguishing families of compounds, such as
219 flavanols (including condensed tannins), phenolic acids, anthocyanins and flavonols remains
220 possible. For example, phenolic acids, such as p-coumaric acid, ferulic acid and caffeic acid
221 have a maximum emission at approximately 420 nm, while flavanols such as (+) catechin, (-)
222 epi-catechin and procyanidin share maximum emission at approximately 320 nm. In fact, due
223 to their large excitation spectra, most phenolic compounds will fluoresce under excitation at
224 275 nm. Among the tested reference compounds, only phloridzin, rutin and arbutin will not
225 fluoresce after deep-UV excitation.

226 *3.2. Preliminary analysis of autofluorescence observed after excitation at 375 nm*

227 In a preliminary study, autofluorescence observations of fleshy fruit were realized with a
228 laboratory confocal laser scanning microscope using a UV diode laser at 375 nm. This test
229 was performed to check the pertinence of autofluorescence analysis on two types of fleshy
230 fruits: Royal Gala apple and Italian Red grape. In all cases, the cuticle presented bright and
231 intense fluorescence as previously observed [35]. Red spots were observed within the cells of
232 both fruit but particularly in grape, while light yellow fluorescence was observed in vacuole-
233 like compartments only in apple. Red fluorescence could correspond to chlorophyll residues
234 while yellow emission could be related to compounds of the flavonol class, such as quercetin

235 derivatives [36] [37]. A weak blue emission was also observed in the cell wall at native pH
236 and was found to increase at pH 10, as reported for ferulic or coumaric acids [20]. These
237 emission variations after UV excitation at 375 nm provided support for the mapping of
238 phenolic compounds using multispectral autofluorescence imaging. However, a lower
239 excitation wavelength was sought to access compounds in the flavanol family.

240 *3.3. Autofluorescence after excitation at 275 nm*

241 The autofluorescence of flavanols in fleshy fruit tissue requires excitation at a low UV
242 wavelength, which is yet inaccessible with a common microscopy laser beam. The
243 synchrotron beamline at DISCO allows for exciting samples in the 200-350 nm range. To
244 limit the diffusion of phenolic compounds, apple tissue was first cryo-fixed prior to cryo-
245 observation of multispectral fluorescence from 300 nm to 650 nm (Figure 3). Outer-cortex
246 parenchyma fluorescence was distinguished from that of epidermal tissue, which
247 encompasses cuticle and sub-cuticular cell layers (Figure 3). Between 327 and 388 nm, sub-
248 cuticular cell layer fluorescence was more intense and disappeared at higher bandpass
249 wavelength filters except, for the 600 nm filter. Intense fluorescent spots were observed inside
250 the cell mainly with 300 and 327 nm filters, but in all cases, cells walls showed a more
251 intense signal than cells.

252 To better distinguish differences in sample fluorescence, principal component analysis
253 was applied to the collection of images obtained with the different bandpass filters. All
254 principal component analyses performed on the three modalities presented similar loadings.
255 For example, the loading and component images are shown in Figure 4 for representative
256 samples of Douce Moën and Guillevic apple varieties at harvest. Fluorescence through 300,
257 327, 352, 412, 420 and 600 nm filters contributed mainly to components 1 and 2 and
258 fluorescence at 300, 327 and 600 nm contributed more weakly to components 3 and 4. Most
259 filters contributed to component 1, which corresponded to the general variations of

260 fluorescence intensity and explained 65.8% and 61.7% of the total variance for Douce Moën
261 and Guillevic, respectively. The component images showed that the highest overall
262 fluorescence intensity was mainly localized in the walls of outer-cortical cells. The second
263 component, expressing the contribution of the 300 and 327 nm filters (positive side) in
264 opposition to the emission filters 412 and 420 nm (negative side), contrasted the sub-cuticular
265 cell layers. Filters 352 and 600 nm were inversely related to this second component for the
266 two varieties. The third component pointed out the cuticle region with a relatively higher
267 fluorescence measured using the 327 nm emission filter (in red) compared to the 300 and 600
268 nm filters (in blue), showing the rest of the sample. The fourth component showed a higher
269 level of noise and a relatively higher fluorescence level associated with the 300 nm filter (in
270 blue) in the epidermal region as well as in the cell walls of sub-cuticular cells and outer
271 cortical cells. Thus, fluorescence distinguished three areas: the cuticle region with a relatively
272 higher fluorescence emission using the 327 nm filter, the sub-cuticular cell layers with the
273 300 nm and 600 nm filters, and the walls of outer-cortical cells with the 352, 412 and 420 nm
274 filters. The three first component images of the representative samples were combined in an
275 RGB image to better visualize the tissue regions according to fluorescence (Figure 4). To
276 objectivize differences observed in fluorescence distribution, normalized intensity profiles for
277 all images of the two varieties at the different storage conditions were measured.

278 Intensity profiles from the cuticular to the outer cortical tissue were obtained for the
279 four bandpass filters discriminating the most apple tissues: 300, 327, 420 and 600 nm. As cell
280 lumen and cell walls showed marked differences in fluorescence intensities, smoothing of
281 each profile was realized by local regression (LOESS) at the expense of fine structures
282 contributing to fluorescence (cells, cell walls, sub-cellular structures). The smoothed and
283 normalized curves allowed comparison of trends in fluorescence variations along the first 400
284 μm under the cuticle. Examples of raw and smoothed profiles are shown for the Douce Moën

285 variety at harvest in Supplementary Figures 1 and 2. Considering close fruit calibres within
286 varieties, Student's t-test at each distance point per filter showed that storage modalities had
287 no effect on fluorescence profiles (p-value > 0.05; Supplementary Figure 3). Thus, all
288 biological replicates (n= 9) were used to evaluate the effect of apple varieties on fluorescence
289 intensity profiles. Significant differences were particularly noted with the 327 nm and 420 nm
290 filters (Figure 5). For the 327 filter, an inverse gradient was observed between the two
291 varieties with a rupture point at approximately 100 μm from the cuticle. Douce Moën showed
292 higher fluorescence intensity than Guillevic, particularly in the first 10 μm under the cuticle
293 but was lower than that of Guillevic 60 μm away from the cuticle. The 420 nm filter also
294 presented two significantly different areas, first in the epidermis where the Guillevic signal
295 was higher than that of Douce Moën and opposite to the 327 nm filter profile and second in
296 the outer cortex (300 to the 400 μm limit of the images) where Guillevic fluorescence was
297 lower. A maximum was observed at approximately 100 μm for Guillevic, while for Douce
298 Moën, it was at approximately 330 μm from the cuticle. Weak significant differences were
299 observed between Guillevic and Douce Moën in the sub-cuticular cell layers for the 300 nm
300 filter and in the outer cortex for the 600 nm filter. These two profiles also showed similar
301 ruptures at approximately 75-100 μm from the cuticle. The mean smoothed plot profiles
302 (Figure 6) showed good agreement with the fluorescence distribution observed in the
303 representative PCA reconstructed images (Figure 4). The three regions observed may be
304 approximately delimited from these results (Figure 6) for Douce Moën to range from
305 approximately 0 to 50 μm , 50 to 200 μm and 200 μm to 400 μm while for Guillevic, to range
306 from approximately 0 to 60 μm , 60 to 150 μm and 150 to 400 μm . These regions may
307 correspond to the cuticle and the sub-cuticle forming the epidermis and the outer cortex as
308 depicted in Figure 3.

309 The fluorescence emission of reference phenolic compounds allowed us to propose the
310 possible nature of chemicals in the different tissues (Table 1). Fluorescence observed with the
311 300 nm filter was more likely due to proteins as this range of emission is related to the amino-
312 acids tyrosine and phenylalanine [38]. Fluorescence through the 327 nm filter may have
313 corresponded to the tryptophan and most likely to catechins that are predominant in apple
314 fruit tissue. Since these compounds are flavanol monomers and oligomers, by extension such
315 fluorescence would also reveal condensed tannins. Likewise, fluorescence at 420 nm would
316 represent the phenolic acid family, such as hydroxycinnamic acids, while the fluorescence at
317 600 nm would be pigments, such as anthocyanin or chlorophyll. According to these
318 propositions, pigments would be particularly localized in the sub-cuticular cell layers of both
319 varieties, while flavanols including condensed tannins would be present in the epidermis and
320 outer cortex of both varieties, though with higher intensity in the sub-cuticular cell layers of
321 Guillevic. The distribution of phenolic acids differed markedly between the two varieties. In
322 Douce Moën, they were present mainly in the outer cortex, while in Guillevic they were also
323 present in the sub-cuticular cell layers. Storage conditions did not appear to affect these
324 distributions.

325 **4. Discussion**

326 The results showed that multispectral observation of auto-fluorescence provides a
327 convenient alternative for localizing phenolic compound families in fleshy fruit tissue with
328 limited sample preparation and artefact introduction compared to classical resin embedding
329 and staining methods. Adaptation of the cryogenic procedures developed for cryo-LSCM [18]
330 to the wide-field TELEMOS microscope of the DISCO beamline at the SOLEIL synchrotron
331 facility allowed observation of fluorescence from 275 nm, which excited flavanols and
332 condensed tannins together with other phenolic compounds in well-preserved apple tissue
333 with limited metabolite diffusion. The obtained images confirmed the efficiency of the

334 cryogenic process as intracellular content appeared filled, though of lower fluorescence
335 intensity compared to cell walls. Cryo-observation also improved fluorescence intensity [26],
336 likely by reducing fluorescent bleaching due to the temperature dependency of quencher
337 diffusion [28]. This phenomenon was particularly helpful in establishing the qualitative
338 distribution of fluorescent compounds in fruit tissue.

339 Fluorescence tended to indicate phenolic compounds within cell walls. However,
340 most phenolics are localized within cell organelles, vacuoles or the cytoplasm [39, 40] with
341 fewer in cell walls. In apple, phenolic compounds have not been described as part of the cell
342 wall polysaccharide composition [41] [42]. Some authors [43] suggested that tannin
343 accumulation takes place in the vacuole, but their polymerization appeared near the cell walls.
344 As apple fruit cells are highly hydrated, ice crystals formed during cryo-fixation of the water-
345 filled vacuoles [44] may have pushed fluorescent organelles and cytoplasmic materials close
346 to the cell walls. As a consequence, part of the cell wall fluorescence observed with the 10×
347 magnification lens (resolution: 1.24 μm / pixel) may have resulted from nearby intracellular
348 fluorescent compounds/organelles. However, the blue emission observed by LSCM in the cell
349 wall of fresh apple and grape tissue and its increased fluorescence at basic pH supported the
350 presence of hydroxycinnamic acids in cell walls. This observation was realized at higher
351 magnification (20×, resolution of 0.63 μm / pixel) than that of the TELEMOS setup and
352 excluded the potential of ice crystal effects on cell wall fluorescence. Hydroxycinnamic acids
353 have already been reported in apple fruit mostly in vacuoles due to their characterization in
354 the juice [45] [46, 47], but they are also known to be present in plant cell walls [48]. Their
355 low concentration in apple cell walls likely prevented their detection by classic analytical
356 techniques. However, further work is needed to assess the nature of these compounds and
357 whether they are esters of cell wall polysaccharides, as reported for other plants [20] [49, 50].

358 The cuticle was the most fluorescent zone of apple and grape fruit tissues observed by
359 LSCM (Figure 2). The observation of cuticle autofluorescence was already reported in several
360 fruits, including apples and grapes and may be related to high concentrations of proteins and
361 phenolic acids [35] [51]. Though anthocyanins, flavonols, flavanols (including condensed
362 tannins), hydroxycinnamic acids and proteins were essentially reported in the epidermis
363 region [52-54], they were more rarely localized in sub-cuticular cell layers [8, 10, 14]. The
364 high concentration of phenolic compounds in the sub-cuticular cell layers is probably related
365 to defence against pathogens [55] or light damage to tissues. The firmer mechanical resistance
366 of grape and apple skins compared to their flesh [56, 57] may also be associated to the
367 important presence of phenolic compounds in the skin. Indeed, phenolic compounds and, in
368 particular, flavonoids were proposed to increase the rigidity of the cutin matrix in the cuticle
369 of ripe tomato [58]. Comparatively, excitation with DUV at 275 nm revealed a lower cuticle
370 autofluorescence. Although flavonoids and phenolic acids, the main cuticle phenolic
371 compounds, have large excitation and emission spectra (Supplementary Figure 4), 275 nm
372 excitation was not optimal to yield high autofluorescence intensities. Though such a
373 wavelength was beneficial for exciting flavanols and proteins fluorescence, it appears that
374 flavanols are in low concentration - if any- within the cuticle but are present in the first cell
375 layers underneath (Figure 3).

376 The red-skinned Douce Moën and the yellow-skinned Guillevic cider apples were
377 used as model fruits to help distinguish phenolic compound locations by their
378 autofluorescence. Douce Moën is known to be richer in hydroxycinnamic acids and in
379 monomeric tannins, such as (+) catechin, (-) epicatechin and procyanidin with lower tannin
380 degrees of polymerization compared to Guillevic [59]. Anthocyanins were not characterized
381 in the two varieties, but the more colourful Douce Moën skin evidenced higher anthocyanin
382 and/or pigment contents than that of Guillevic. Based on distinct fluorescent emissions of

383 reference compounds after 275 nm excitation, autofluorescence in Douce Moën and Guillevic
384 cryo-sections allowed the detection of pigments in the sub-cuticular cell layers in agreement
385 with the known flavanols, anthocyanins and chlorophyll concentrations in apple skin [60, 61].
386 The main distribution of tannins in the sub-cuticular area together with that of phenolic acids
387 in the outer cortex parenchyma may be related to the ratio reported for procyanidin (PC) and
388 hydroxycinnamic acid (HC) concentrations in the fresh peel (HC/PC: 0.68) and parenchyma
389 (HC/PC: 1.29)[62]. This different distribution was not reported in the literature for grape in
390 which flavanols were mainly localized in the epidermis and in the seeds although
391 hydroxycinnamic acid content was higher in skin than in pulp [16, 54, 63].

392 Changes in phenolic compounds distribution were not observed with storage
393 modalities that were aimed at impacting apple ripening. This result is at odds with studies
394 showing phenolic compound variations during ripening of fruit [7], such as apple [52, 64] and
395 grape [10, 65]. The lack of autofluorescence discrimination between ripening stages may be
396 related to the small tissue area observed. Instead, the present study evidenced a variation in
397 phenolic distribution between apple varieties. This may be related to different tissue
398 thicknesses as for both varieties, different rupture points in phenolic distributions were
399 observed along distance profiles from the cuticle. These ruptures could correspond to
400 different cell layers in the epidermis and the outer cortex. In particular, a rupture was
401 discernible in fluorescence profiles at an approximately 100 μm depth from the skin samples.
402 Apple histology studies have shown changes from smaller cells in the epidermis to larger
403 cells in the outer cortex parenchyma tissue at an approximately 100- μm depth from the
404 surface of mature fruit [66-68]. Thus, differences in phenolic compound distribution between
405 the two varieties could reveal genetically distinct thicknesses of epidermal cell layers as well
406 as different secondary metabolism in these tissues.

407 **5. Conclusion**

408 The cryo-method used here could benefit from further developments in sample
409 preparation and means of cryo-multispectral fluorescence observations. Applying microwaves
410 during cryo-fixation [69] to avoid ice crystal growth combined with the use of confocal
411 microscope stages and lenses for high-resolution cryo-observations [26, 27] is expected to
412 improve the localization of phenolic compounds in the cell and cell wall. Nevertheless,
413 multispectral fluorescence cryo-imaging at 275 nm excitation allowed the distinction of three
414 major classes of phenolic compounds in tissue under *in-vivo-like* conditions without any
415 specific dyes. The detection of hydroxycinnamic acids in apple and grape cell walls requires
416 their identification and linkage in the walls. Coupling multispectral analysis based on LSCM
417 observations in the UV-visible wavelength and synchrotron deep-UV analysis could provide a
418 convenient way to extend the range of accessible autofluorescent phenolic compound
419 distributions in the same sample tissue section. These observations will enable other studies
420 on the mechanical behaviour resulting from the presence of these compounds in fruit tissues
421 in the context of fruit development, quality and processing.

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424 synchrotron SOLEIL. This work was also supported in part by the Food for Tomorrow
425 programme from the Region Pays de la Loire.

426 **Appendix A: Supplementary data**

427 Supplementary data associated with this article can be found, in the online version.

428

429

430 **Legend to Figures**

431 **Figure 1:** Generic phenolic compound structures. -R may correspond to -H, -OH or -OCH₃
432 and -X may correspond to -H in monomeric form or other flavanol units in polymeric form
433 (Vermerris and Nicholson 2006).

434 **Figure 2:** Local retail store fresh apple and grape autofluorescence at natural pH and at
435 pH=10.1 after excitation at 375 nm. The fluorescence emissions recorded are 400-450 nm
436 (blue), 500-530 nm (green), 560-600 nm (yellow), and 630-700 nm (red). C: cuticle, CW: cell
437 wall, Chl: chlorophyll residues, Flv: flavonols. Scale bar: 20 μm.

438 **Figure 3:** Frozen apple autofluorescence for one replicate of Douce Moën and Guillevic at
439 harvest after excitation at 275 nm. Tissue regions are drawn on the right image resulting from
440 a combination of whole Guillevic filter emission images. C: cuticle, CW: cell wall, Scale bar:
441 40 μm.

442 **Figure 4:** Principal component analysis on the six emission filter images for one replicate of
443 Douce Moën (blue character and arrows) and Guillevic (black character and arrows) at
444 harvest and the PCA images of the four components. On the component images, blue and red
445 pixels represent negative and positive contributions of the emission filters to the component.
446 RGB reconstructed images from PCA components were built from dimension 1 (blue),
447 dimension 2 (red) and dimension 3 (green). C: cuticle, CW: cell wall

448 **Figure 5:** Smoothed (LOESS method: $\alpha = 0.4$) and normalized average plot profiles of Douce
449 Moën (DM) and Guillevic (GU) signal acquisition for emission filters 300 nm, 327 nm, 420
450 nm and 600 nm after excitation at 275 nm. The dotted lines represent the 95% confidence
451 interval. The coloured areas correspond to significant differences between DM and GU with a
452 p-value < 0.01 by Student's test (n = 9).

453 **Figure 6:** Superposed smoothed (LOESS method: $\alpha = 0.4$) and normalized average plot
454 profiles of Douce Moën and Guillevic signal acquisition for emission filters 300 nm (grey

455 line), 327 nm (green line), 420 nm (blue line) and 600 nm (red line) after excitation at 275
456 nm. The profiles were plotted along the sample section distance from the outer cuticle to 400
457 μm deep inside the tissue.

458

459 **Table 1:** Fluorescence excitation/emission maxima of phenolic compounds registered by
 460 spectrofluorometry: regular case: literature, **bold case:** this study.

Compounds	$\lambda_{\text{max excitation}}$ (nm)	$\lambda_{\text{max emission}}$ (nm)	Compounds	$\lambda_{\text{max excitation}}$ (nm)	$\lambda_{\text{max emission}}$ (nm)
Emission range (<350 nm)			Emission range (400-500nm)		
<i>Protein group</i>			<i>Phenolic acids group</i>		
Phenylalanin[70]	258	285	Ferulic acid	310/370	420
Tyrosin [70]	276	302	p-Coumaric acid	350	420
Emission range (300-400 nm)			Caffeic acid	270/360	440
<i>Flavanols group</i>			Chlorogenic acid	360	460
(-) Epicatechin	290	320	<i>Others compounds</i>		
(+) Catechin	290	320	Kaempferol[21]	268	422
Procyanidin B1	285	320	Rutin	300	460
Procyanidin B2	295	320	Emission range (500-600nm)		
Epigallocatechin	275	320	<i>Flavanols group</i>		
<i>Others compounds</i>			Anthocyanins[22]	300/410	360/420/530
Tryptophan [22]	280	357	Cyanidin hydrate	278	550
2,5-Dihydroxybenzaldehyde[21]	278	360	Quercetin-3-O-glucoside	260/410	510
Syringic acid	310	360	Quercetin	420	520
Vanillic acid[21]	278	360	Quercetin galactoside	295	600
Gallic acid	320	370	<i>Others compounds</i>		
Myricetin[21]	268	370	Arbutin	420	530
Resveratrol	310/360	390	Phloridzin	250/330	540
			Chlorophyll/chloroplast[37]	360	680

461

462

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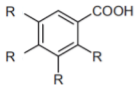
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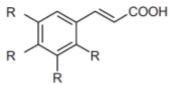
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Phenolics acids

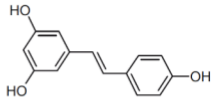


Hydroxybenzoic acids



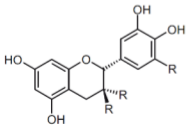
Hydroxycinnamic acids

Stilbenes

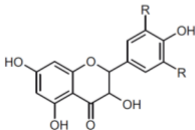


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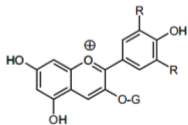
Flavonoids



Flavanols



Flavonols

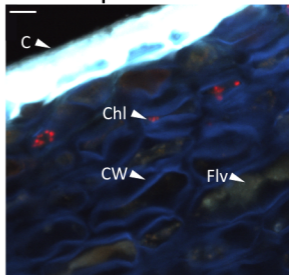
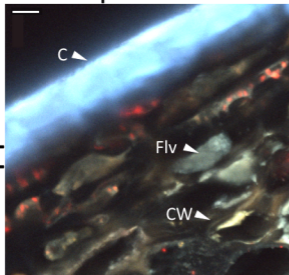


Anthocyanidins

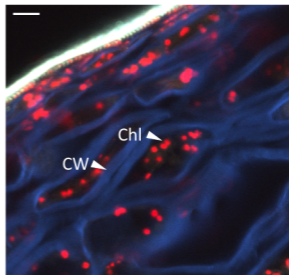
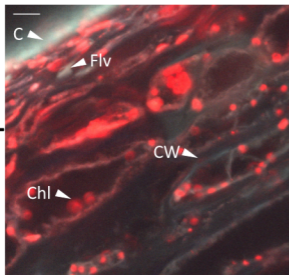
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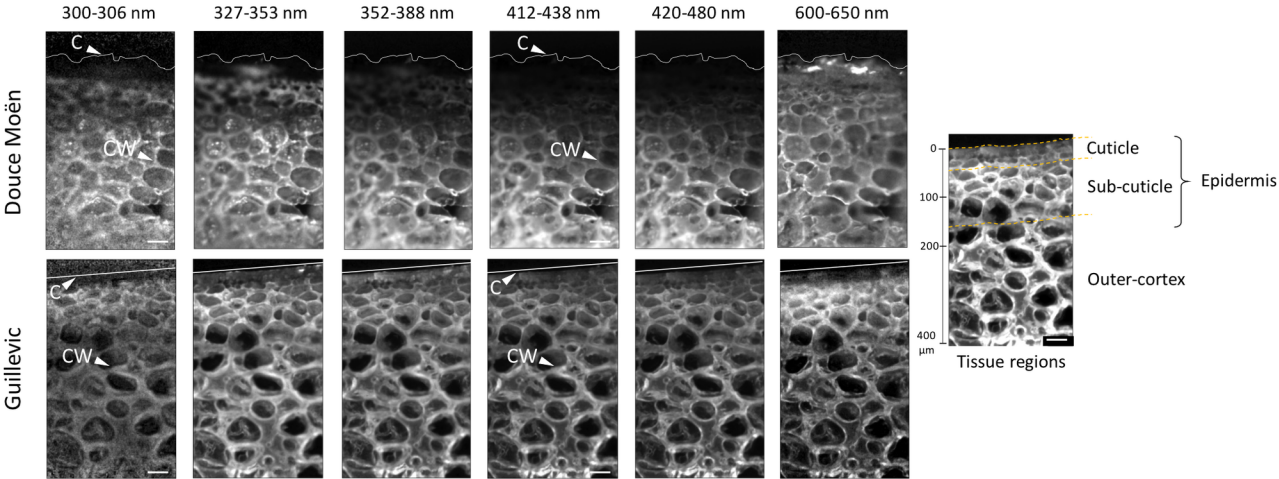
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Apple



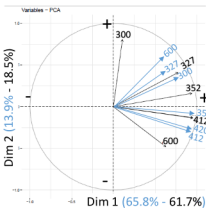
Grape



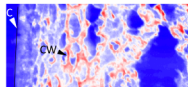
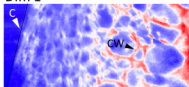


Douce Moën

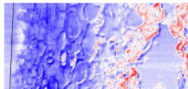
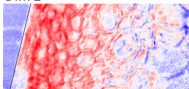
Guillevic



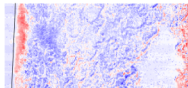
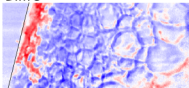
Dim 1



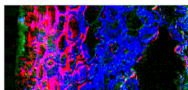
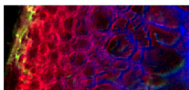
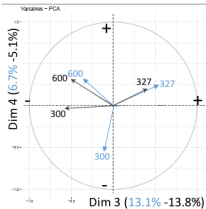
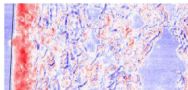
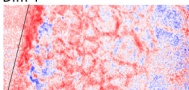
Dim 2



Dim 3

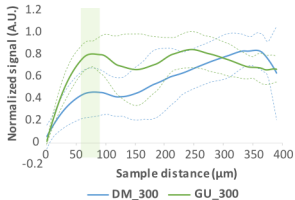
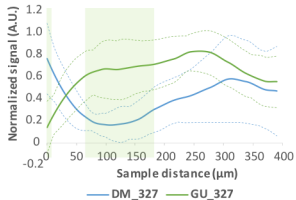
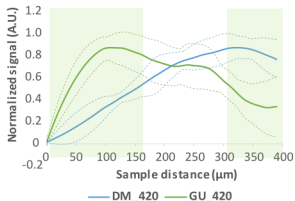
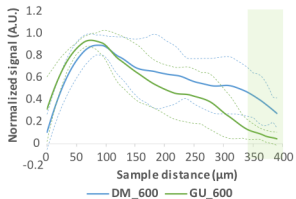


Dim 4

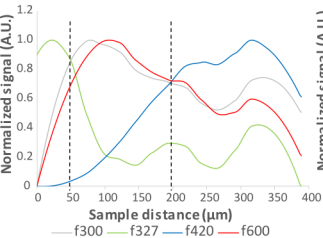


0 50 100 200 300 400
 μm

0 50 100 200 300 400
 μm

DM vs GU: filter 300 nm**DM vs GU: filter 327 nm****DM vs GU: filter 420 nm****DM vs GU: filter 600 nm**

Douce Moën



Guillevic

