Two-photon excitation with pico-second fluorescence lifetime imaging to detect nuclear association of flavanols

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17

18 ABSTRACT

19 Two-photon excitation enabled for the first time the observation and measurement of

20 excited state fluorescence lifetimes from three flavanols in solution, which were ~ 1.0 ns

21 for catechin and epicatechin, but <45 ps for epigallocatechin gallate (EGCG). The shorter

2

22lifetime for EGCG is in line with a lower fluorescence quantum yield of 0.003 compared to

23catechin (0.015) and epicatechin (0.018).

24

25*In vivo* experiments with onion cells demonstrated that tryptophan and quercetin, which

26tend to be major contributors of background fluorescence in plant cells, have sufficiently

27low cross sections for two-photon excitation at 630 nm and therefore do not interfere with

28detection of externally added or endogenous flavanols in *Allium cepa* or *Taxus baccata*

29cells. Applying two-photon excitation to flavanols enabled 3-D fluorescence lifetime

30imaging microscopy and showed that added EGCG penetrated the whole nucleus of onion

31cells. Interestingly, EGCG and catechin showed different lifetime behaviour when bound to

32the nucleus: EGCG lifetime increased from <45 to 200 ps, whilst catechin lifetime

33decreased from 1.0 ns to 500 ps. Semi-quantitative measurements revealed that the relative

34ratios of EGCG concentrations in nucleoli associated vesicles : nucleus : cytoplasm were

35*ca.* 100:10:1.

36

37Solution experiments with catechin, epicatechin and histone proteins provided preliminary

38evidence, via the appearance of a second lifetime (τ_2 = 1.9 to 3.1 ns), that both flavanols

39may be interacting with histone proteins. We conclude that there is significant nuclear

40absorption of flavanols. This advanced imaging using two-photon excitation and

41biophysical techniques described here will prove valuable for probing the intracellular

42trafficking and functions of flavanols, such as EGCG, which is the major flavanol of green

43tea.

44

3

45*Keywords*: Fluorescence lifetime imaging microscopy, flavanols, epigallocatechin gallate,

46nuclear association, histone proteins, multiphoton.

47

48**1. Introduction**

49Plants synthesise >4000 different flavonoid compounds, which can be grouped into several

50different subgroups. Flavanols (Fig. 1) are an important subgroup that is widespread in

51plants and plant foods [1]; they are also precursors of condensed tannins, which are the

52fourth largest group of natural plant products after cellulose, hemicellulose, and lignin [2].

53These polyphenolic compounds are attracting considerable interest, because diets rich in

54fruits and vegetables are associated with improved health and a reduction of age-related

55diseases such as cancer, osteoporosis and cardiovascular diseases [3-7]. Flavonoids are

56considered to be 'lifespan essentials' and recent reviews suggest that their antioxidant

57properties alone are unlikely to explain their beneficial effects on human health or their

58functions in plants [8-10].

59

60A consensus is emerging that *in vitro* and *in vivo* experiments need to probe the

61bioavailability of these polyphenols and their molecular targets [3,6,8,10-11]. *In vitro*

62studies have tended to require 10 to 100-fold higher polyphenol concentrations than are

63usually found in mammalian plasma and tissues in order to achieve many of the reported

64medicinal effects [5,12]. However, the existence of high-affinity targets for dietary

65polyphenols might explain their health-promoting effects and in this context it is pertinent

66to examine more closely recent evidence that nuclei from both plant *and* mammalian cells

67acted as sinks for flavanols [13-17]. Although the function of these secondary plant

4

4

68 metabolites requires further elucidation, evidence is emerging that they may be important in

69 cell development. For example, loss of flavanols has been linked to defective pollen

70 development [14]. Different types of flavanol distribution patterns were observed in *Tsuga*

71 *canadensis* at the sub-nuclear level [13] and the authors questioned whether the epigenetic

72 code of histones could affect flavanol-chromatin associations. Moreover, Feucht *et al*. [15]

73 found identical flavanol patterns within different cell lineages in a meristematic plant tissue

74 and suggested that this could be indicative of a synchronized, transcriptional regulation. In

75 addition, nuclear flavanol concentrations clearly depended on the season, i.e. during

76 dormancy they were almost absent but during growth periods relatively high amounts were

77 observed [18]. The fact that flavanols were associated with both interphase and mitotic

78 chromosome states posed the question of whether flavanols might be associated with

79 histones. If this is the case, then this could open a new perspective on genomic regulation.

80

81 This research by Feucht's group made use of the fact that flavanols form a blue

82 condensation product with dimethylaminocinnamaldehyde (DMACA) [19]. The DMACA

83 reagent is, however, a relatively aggressive reagent that requires 0.75 M sulfuric acid for

84 the staining reaction and this could cause some physical damage within the cells. Polster *et*

85 *al*. [18], therefore, tested the existence of nucleus-bound flavanols with a milder technique,

86 i.e. laser microdissection and pressure catapulting (LMPC), which separated intact nuclei

87 from cells and these also stained blue in the subsequent DMACA reaction. Nevertheless,

88 LMPC causes physical rupture of the cytoplasm that surrounds the nuclei and could have

89 given rise to an artificial DMACA reaction. Moreover, histological studies with DMACA

90 cannot distinguish between different flavanols or between flavanol monomers, oligomers or

91polymers [19]. Techniques are therefore required that can establish the sub-cellular

92localisation, and concentrations therein, of flavanols to probe their functionality and

93metabolism in plant and mammalian cells.

94

95Nifli *et al.* [29] recently applied confocal fluorescence microscopy to map the intracellular

96distribution of a major plant flavonol, i.e. quercetin (Fig. 1), which has a UV absorption

97λ_{max} of 372 nm. Quercetin revealed a specific fluorescence (488 nm$_{ex}$/500-540 nm$_{em}$) in the

98cellular environment at physiologically relevant concentrations (<5 µM), which the authors

99attributed to non-covalent binding to cellular components. Intracellular tracing of flavanols

100(λ_{max} ~ 280 nm; Fig. 1 and Fig. S1) by UV-Vis spectroscopy or confocal fluorescence

101microscopy is, however, not possible because plant and mammalian cells contain numerous

102compounds which would interfere with the detection of flavanols by giving background

103fluorescence signals (termed "auto-fluorescence"). Fig. 2 illustrates the photophysical

104processes in a conventional Jablonski diagramme, which depicts one- and two-photon

105excitation and various relaxation pathways that are open to the electronic excited state

106following photon(s) absorption.

107

108In fluorescence life-time imaging microscopy (FLIM) both fluorescence intensities and

109fluorescence lifetimes of specific compounds can be measured at each pixel in the image

110[21,22]. In addition, variations in fluorescence lifetime can provide further image contrast:

111lifetime shifts can serve as sensitive probes for detecting molecular interactions and may

112yield information on a compound's environment, such as pH or oxygen concentration [23-

11325]. Lifetime, τ, is derived from the time-constant of the fluorescence decay (Fig. 2), where

6

114 $\tau = 1/k_{fluorescence}$. FLIM is based on either single- or multi-photon excitation techniques.

115 Multi-photon excitation with femtosecond lasers offers many advantages for biological

116 measurements over more conventional single photon excitation [23,26]:

117 ✓ Excitation with red light that is not directly absorbed by cellular

118   materials.

119 ✓ Reduced cellular toxicity in biological studies.

120 ✓ Reduced photo-bleaching.

121 ✓ Deeper penetration of the near-infrared light into the biological

122   specimen.

123 ✓ Femtolitre volume excitation.

124 ✓ A flexible imaging platform that is capable of resolving several

125   (and related) compounds.

126 ✓ The ability to deliver UV-equivalent photon energies directly beneath UV

127 absorbing materials and molecules.

128 ✓ An ability to perform time-resolved studies due to the short pulsed light

129 source.

130 In two-photon excitation (2PE) the simultaneous absorption of two lower energy photons

131 mimics the absorption of a single photon of equivalent higher energy (Fig. 2). Thus, 2PE at

132 560 nm mimics UV excitation at 280 nm [23,27]. In FLIM ultrafast lasers providing pulse

133 lengths of the order of 200 femtoseconds (200×10^{-15} s) enable time-resolved

134 measurements, which can detect molecular interactions in solution and cells [23-25,28] and

7

135 can be used to construct fluorescence life-time maps of a compound's distribution within

136 viable cells.

137

138 Here we describe 2PE experiments designed to eliminate any doubts regarding the results

139 from previous histological studies that employed the DMACA staining reagent. Two-

140 photon excitation coupled to 3-D fluorescence lifetime imaging microscopy enabled

141 examination of intact biological tissues and highly localised, non-destructive and selective

142 detection of flavanols. The fluorescence behaviour of three flavanols, catechin, epicatechin

143 and epigallocatechin gallate (EGCG) (Fig. 1), was measured first in model solution systems

144 and then in two natural cell systems, onion epidermis cells and *Taxus* pollen mother cells.

145 These flavanols were chosen because they are widespread in plants and are also

146 bioavailable and bioactive in several *in vitro* and *in vivo* mammalian cell systems

147 [1,6,9,29]. Solution phase studies were first used to optimise and measure spectroscopic

148 shifts and lifetime changes of free flavanols *versus* flavanols bound to DNA or histone

149 proteins at normal physiological pH values. The optimised spectroscopic parameters were

150 then applied to probe the intra-cellular location of externally added flavanols in *Allium*

151 *cepa* cells and of endogenous flavanols in *Taxus baccata* cells. The same plant models had

152 been tested previously with the DMACA staining reagent [14,30].

153

154 **2. Methods and materials**

155 *2.1. Reagents*

156 The following reagents were purchased from Sigma-Aldrich Company Ltd, UK:

157(+)-catechin (98%), (-)-epicatechin (90%), (-)-epigallocatechin gallate (95%; EGCG), tris-

158(hydroxymethyl)amino methane (Tris), K_2HPO_4 (ACS reagent, ($\geq$ 98%), KH_2PO_4 (ACS

159reagent, ($\geq$ 99%), DNA from calf thymus and Histone type II-A. Histone was supplied by

160Roche Diagnostics Ltd, UK. Histone sulphate from calf thymus was purchased from Fluka

161(Sigma-Aldrich Chemie, Steinheim, Germany; Polster *et al.*, 2003). Ethanol (LiChrosolv, $\geq$

16299.9%) was purchased from VWR-Merck, UK.

163

164Tris buffers (0.1 M) were prepared and adjusted to pH 7.0 and 8.0 with HCl and phosphate

165buffers (0.1 M) at pH 5.8, 7.1 and 8.2 were prepared using K_2HPO_4 and KH_2PO_4 as

166described [16].

167

168*2.2. Calculation of relative fluorescence quantum yields of the flavanols*

169Flavanols were dissolved in methanol to yield 0.01 M stock solutions. Subsequent dilutions

170for 20 and 40 µM flavanol concentrations were made with sodium phosphate buffer (pH

1717.4, 0.1 M, 0.05% sodium azide). These were placed in a 10 mm quartz Suprasil

172fluorescence cuvette (Hellma, Germany) and UV-Vis spectra were first recorded from 190

173to 500 nm using a Helios β spectrophotometer (Spectronic Unicam, U.K.). Then

174fluorescence spectra were recorded using a luminescence LS-55 spectrometer (Perkin

175Elmer, U.K.) from 290 to 530 nm with excitation at 295 nm under continuous stirring. The

176excitation and emission slits were both set to 5 nm and scanning speed was 200 nm min^{-1}.

177All experiments were carried out at 37 ºC. The literature reported a quantum yield of 0.12

178for tryptophan (Trp) at 270 nm (website) and we confirmed this for 295 nm. Therefore, the

179quantum yields of flavanols (Flav) were calculated relative to tryptophan using the

9

180 integrated area between 300 and 530 nm under the fluorescence spectra [31-33] according

181 to:

182   Absorption at 295 nm (Trp) * 0.12 * Fluorescence (Flav)

183   Quantum yield$_{(Flav)}$ =   ---

184   Absorption at 295 nm (Flav) * 0.12 * Fluorescence (Trp)

185

186 *2.3. Flavanol solutions*

187 Flavanols were dissolved in ethanol (~10 mM) and prepared fresh on a daily basis. Just

188 before measuring the fluorescence lifetimes, aliquots (10 µL) were removed and diluted

189 with buffer, DNA or histone protein solutions (90 and 40 µL) to obtain flavanol

190 concentrations between 1 and 2 mM.

191

192 DNA (0.6 mg) was dissolved in Tris buffer (pH 8.0; 30 mL) overnight at 4 ^{o}C. Sigma

193 histone (5.9 mg) was dissolved in Tris buffer (pH 7.0 and 8.0; 2.85 mL). Ethanol (98 µL)

194 was added to the pH 8.0 buffer to facilitate dissolution. Histone sulphate (0.8 mg) was

195 dissolved in Tris buffer (pH 7.0 and 8.0) according to Polster *et al.* [16]. The supernatants

196 were used after centrifugation. Roche histone (1.0 mg) was dissolved in Tris buffer (pH 7.0

197 and 8.0; 500 µL) and ethanol (10 µL).

198

199 *2.4. Plant samples*

200 The thin adaxial epidermis from onion (*Allium cepa*) bulb scale was removed, cut into 2

201 cm^2 pieces and incubated with aqueous catechin or EGCG solutions (1 mM; 20 mL) for up

202 to 8 h [30].

203

204Male cones from yew (*Taxus baccata*) were harvested on 5th October 2008. The eight cover

205leaves were removed and the yellow anthers were gently squeezed with tweezers in order to

206release the mother pollen cells. Preliminary experiments revealed that these cells stained

207dark blue with the DMACA reagent (10 mg DMACA dissolved in 1 mL of 0.75 M H_2SO_4)

208[30].

209

210*2.5. Multiphoton microscopy*

211The set up used in this study has been previously described [23]. Briefly, a custom built

212two-photon microscope was constructed using scanning XY galvanometers (GSI Lumonics

213Ltd). A diode-pumped (Verdi V18) titanium sapphire (Mira F900) operating at 700-980 nm

214generated laser light at a wavelength of 585 ± 2 nm and was used for the solution studies

215and at 630 ± 2 nm for the plant cell studies through an optical parametric oscillator (OPO,

216APE-Coherent GmbH, Berlin, Germany) operating at 180 fs pulses at 75 MHz. The pulse

217width was maintained using a femto control unit (APE Coherent GmbH). The laser beam

218was focused to a diffraction-limited spot using a water-immersion ultraviolet corrected

219objective (Nikon VC x60, NA 1.2) and specimens were illuminated at the microscope stage

220of a modified Nikon TE2000-U with UV transmitting optics. Fluorescence emission was

221collected without descanning, bypassing the scanning system, and passed through a 340 ±

22220 nm interference filter (U340, Comar Instruments, Cambridge, UK). Emission

223fluorescence was detected using an external fast microchannel plate photomultiplier tube

224(Hamamatus R3809U-50) and recorded using a Time-Correlated Single Photon Counting

225(TCSPC) PC module SPC830 (Becker and Hickl GmbH, Berlin, Germany). Fluorescence

226 lifetime image microscopy was performed by synchronising the XY galvanometer positions

227 with the fluorescence decay. The X,Y galvanometers were raster scanned at 1 ms or 2 ms

228 per pixel for 128 x 128 or 256 x 256 image size, respectively, giving a 33 sec per image

229 frame. The presented images were three accumulations to allow for enough photon counts

230 per channel for the data analysis.

231

232 *2.6. Image analysis*

233 Steady state grey scale images (8 bit, up to 256 x 256 pixels) are produced by binning all

234 decay photons as a single channel. Fluorescence lifetime images were obtained for control

235 cells and flavanol-loaded cells by analysing the decay at individual pixels using a single or

236 double exponential curve fitting (SPCImage 2.94 analysis software Becker and Hickl). A

237 thresholding function within the FLIM analysis software ensured that noncorrelating

238 photons leading to background noise arriving at the detector were not included in the

239 analysis. Single point decay analysis was carried out without binning while FLIM was

240 analysed with a maximum of 2 binning.

241

242 **3. Results and discussion**

243 *3.1. Flavanol fluorescence lifetimes in aqueous solutions*

244 It is well known that flavanols oxidise readily in alkaline pH [4], therefore lifetimes were

245 first examined at pH values ranging from 5.8 - 8.2. Fig. 3 shows that the fluorescence

246 lifetime, τ, of catechin (2 mM catechin solution in 0.1 M phosphate buffer) was relatively

247 stable between pH 5.8 and 7.1: τ was 1.0 ns at the start and 0.9 ns after 20 min. However, at

248 pH 8.2 the lifetime changed from 1.0 to 0.7 ns within 20 minutes. When the same

249measurements were conducted under a nitrogen blanket, lifetime reduction was kept to 9%

250over a 30 min period and this agrees with Sang *et al.* [34] who found that flavanols were

251not oxidised under nitrogen. Therefore, all subsequent solution measurements were

252determined immediately after mixing the solutions, i.e. within 30 seconds. Fig. 3 also

253shows that pH *per se* had no effect on catechin lifetimes: τ of catechin was ~1.0 ns at pH

2545.8, 7.1 and 8.2. The reduction in τ values can also not be ascribed to sample concentration

255or the presence of non-interacting or energy transfer products, as the excited state lifetime

256is independent of both of these.

257

258The natural lifetime of catechin in solution in the absence of oxygen is ~1.1 ns (Fig. 3).

259This reduces, via quenching, as expected in the presence of dissolved oxygen (7.6 mg·L^{-1})

260at room temperature and pressure to ~1 ns. It is worth noting that at high oxygen

261concentrations, 30 mM, the quenched lifetime observed will be as expected taking into

262account diffusion control rate. Therefore the subsequent change in lifetime (Fig. 3) (to ~0.7

263ns after 20 min in oxygen) is very likely due to the formation of a deprotonated or oxidised

264product as the OH groups in the B-ring are particularly susceptible to deprotonation and

265therefore oxidation at alkaline pH [35]. It is interesting to note that the reduced lifetime

266fitted well to a single exponential decay, again indicating a single fluorescent molecular

267species is present and favouring the observed decreases in lifetime results from either a

268photoproduct, which also fluoresces, or an oxygen quenched process. Further studies using

269high performance liquid chromatography may help identify these oxidised products.

270

13

271 Importantly, Fig. 4 shows that flavanols had different fluorescent decay curves.

272 Fluorescence lifetimes of catechin and epicatechin were similar (1.0 and 1.1 ns,

273 respectively). However, in the case of EGCG at pH 8.1 (2 mM flavanol solutions in 0.1 M

274 phosphate buffer) the lifetime was found to be within the instrument response function and

275 Fig. 4 shows only the characteristics of the fast micro-channel plate (<45 ps). Utrafast time-

276 resolved Kerr gated fluorescence spectroscopy will be needed to resolve the EGCG lifetime

277 in the future. EGCG differs from catechin and epicatechin by the presence of a galloyl

278 group at C-3 (Fig. 1). The lifetime of the excited state is given by the sum of the different

279 competing relaxation processes, which include fluorescence, non-radiative decay,

280 intersystem crossing and chemical reaction as illustrated in Fig. 2. The shorter lifetime for

281 EGCG is most likely due to the presence of additional phenolic groups, which would be

282 expected to enhance the solvation effects and which in turn would influence the non-

283 radiative decay processes. These extra phenolic groups also enhance its antioxidant

284 properties [36] and this presumably makes it more susceptible to oxidation. Indeed, the

285 fluorescence quantum yield of EGCG is much lower than that of catechin or epicatechin

286 (Table 1) suggesting that the non-radiative rate (k_{IVR}; Fig. 2) dominates in the relaxation of

287 the electronic excited state.

288

289 At pH 8, epicatechin also had a two-component fluorescence decay lifetime (see footnote in

290 Supplementary Table). The exact physical origin of the bi-exponential lifetime is unknown.

291 However, it is not uncommon for fluorophores in complex cellular environments to

292 demonstrate multiple decay times as seen in Table 2. Different decay times represent

293 differing physical influences that the nascent electronic excited states are subjected to and

14

294consequently may lead to differences in the efficiency of the energy loss process and return

295to the ground state. The fact that we see a bi-exponential decay indicates that the flavanols

296find themselves in two differing states and/or two different environments; for example free

297and bound forms (Supplementary Table). Further investigations studying the ultrafast

298dynamics will be needed to help explain these differences and/or whether diastereoisomers

299such as catechin and epicatechin have different fluorescence properties.

300

301*3.2. Fluorescence lifetime imaging microscopy*

302*3.2.1. Control experiments with onion cells*

303The experimental conditions developed above for flavanol solutions were applied initially

304to onion root cells (tissue soaked in water for 5 h; and followed by two-photon excitation at

305585 nm). Lifetime decay curves, at several different points in the cells, could be fitted to a

306single exponential decay giving a τ value between 2.3 and 2.6 ns ($\chi^2 = 1.05$). It is highly

307likely, however, that under these excitation conditions the emission and lifetime values are

308mainly due to auto-fluorescence contributions from tryptophan [23]. In order to avoid

309significant background fluorescence signals from other cellular materials, in particular

310aromatic amino acids, e.g. tryptophan, when using UV excitation at 290 nm (equivalent to

311580 nm 2PE excitation) the 2PE excitation wavelength was shifted to 630 nm, which has

312been shown to give little background interference [23]. Control experiments were then

313carried out without added flavanols, i.e. in the presence of just water, in order to

314substantiate that the fluorescence was due to flavanols. The onion sample without added

315flavanol showed only weak auto-fluorescence and a τ value of 0.8 ns ($\chi^2 = 1.60$) (Fig. 5c)

316confirming that our FLIM measurements were tracking the flavanol presence in cells (see

317Section 3.2.2. below). Whole onions are known for their high quercetin concentration (Fig.

3181) [6], but given the low photon count, we can conclude that neither tryptophan nor

319quercetin interfered with flavanol detection, λ_{em}, at 340± 20 nm if 2PE with λ_{ex} was 630 nm.

320

321*3.2.2. Absorption of flavanols by onion nuclei*

322Fig. 5a and 5b show fluorescence lifetime maps of cells in an onion epidermis, which had

323been soaked in 1 mM aqueous flavanol solutions. Following absorption of catechin or

324EGCG, the fluorescing nuclei and several bright, small spots of ~ 2 to 7 µm were clearly

325visible to a much greater extent than the surrounding cell matrix. Careful analysis of Fig. 5b

326showed a bright spot of 4 µm diameter. It is known that inactive nuclei possess very small

327nucleoli of the order of ~1 µm [37]. The observed spot is too large to be a nucleolus, we

328therefore propose that the bright spot was a clustering of perinucleolar organiser regions

329(NORs) [38]. NORs tend to surround the nucleoli and strongly absorb flavanols [13].

330

331This study demonstrated that FLIM combined with 2PE at 630 nm enabled *in vivo* detection

332of both catechin and EGCG and avoided interference by tryptophan or quercetin, as the

333control showed hardly any fluorescence (Fig. 5c). We have previously shown that there is

334negligible excitation of cellular auto-fluorescence, particular from tryptophan, following

335multiphoton excitation at 630 nm [23]. Although tryptophan may be excited by multi-

336photon treatment at 590 nm, which is equivalent to single photon excitation (1PE) at 295

337nm, this diminishes by a factor of 10 at 630 nm. Furthermore, the excited state lifetime of

338tryptophan (~3 ns) is significantly different to that of the flavanols investigated here. These

339findings, therefore, provided clear and unequivocal evidence for nuclear flavanol

340absorption. Since the excited state lifetime may be influenced by the environment of the

341flavanols, the colour trend seen in the FLIM images (Fig. 5a,b) may be due to slight

342differences in the environment of the absorbed flavanols. A series of z axis images taken

343through a cell revealed that EGCG was detectable throughout the nucleus and not just at the

344surface (Video Clip S1). EGCG appeared to be concentrated in the NORs; relative

345proportion of EGCG photon counts were 1 to 3 (cytoplasm) : 10 (nucleus) : 100 to 150

346(NORs) (*data not shown*).

347

348*3.3. FLIM lifetimes of bound versus free flavanols in solution*

349Fluorescence decay curves of nucleus-bound catechin were best fitted to two components,

350i.e. τ_1 = 0.5 ns (77.5%) and τ_2 = 2.7 ns (22.5%; χ^2 of 1.04) (Table 2). It is unlikely that τ_2

351emanates from tryptophan as the same experiments with EGCG fitted to a single

352component decay with an average τ of 0.25 ± 0.05 ns (Table 2). The increase in EGCG

353lifetime from <0.045 ns in solution (Fig. 4) to 0.25 ns in the nucleus is a reverse of the

354trend seen for catechin, which showed a lifetime of ~ 1 ns in solution and 0.5 ns in the

355nucleus.

356

357The effect of nuclear association generating different lifetimes is given in Table 2 and was

358recorded when Fig. 5 was taken. Taken together, these observations suggest that the two

359flavanols (catechin and EGCG) may differ in their interaction mechanisms with nuclear

360components. The lowering of a lifetime indicates either an enhanced non-radiative decay

361(through for example formation of hydrogen bonds) [31] or possibly self-association which

362has been reported for catechin [39]. A decrease in lifetimes upon cellular absorption has

17

363 also been reported for 5-hydroxytryptophan and was attributed to self-quenching or

364 environmental effects [23]. Further research will be needed to establish whether oxidation

365 during the cellular absorption experiment could have contributed to the shorter catechin

366 lifetime (Fig. 3) and whether oxidation would have increased EGCG fluorescence lifetime.

367 It seems, however, more likely that this contrasting lifetime behaviour is indicative of

368 different interaction mechanisms.

369

370 *3.4. Endogenous flavanols in* Taxus baccata

371 The same 2PE experimental conditions were then applied to pollen mother cells which had

372 been isolated from microspores of male *Taxus baccata* cones. According to Feucht *et al.*

373 [14] late tetrads and early microspores possess endogenous catechin and epicatechin. We

374 observed, however, fluorescence lifetimes, which could be fitted to single component

375 decays with τ of 0.2 ns and which resembled EGCG, rather than catechin or epicatechin

376 (Table 2). Interestingly, the photon count of the signal to noise ratio from endogenous

377 flavanols in the *Taxus* cells was not dis-similar to onion cells soaked in a 1 mM EGCG

378 solution. Younger cones at the tip of the *Taxus baccata* twig yielded twice as many photons

379 compared to slightly more mature cones from further along the twig. This finding agrees

380 with previous observations [14,15] that nuclear DMACA staining for flavanols was most

381 intense during high cell activity, e.g. in mitotic and stem cells. Interestingly, it also

382 coincides with observations of higher EGCG concentrations in foetal than maternal plasma

383 of rats: absorbed catechins were found in the brain, eye, heart, lung, kidney, liver and

384 placenta of fetal organs [40].

385

18

386 *3.5. Flavanol fluorescence lifetimes in the presence of DNA or histone proteins*

387The fact that flavanols bind to the nucleus raises an important question: which nuclear

388components act as the binding sites? Several previous studies demonstrated that DNA

389interacts with planar flavonoids, such as flavonols and anthocyanidins, and depending on

390the experimental conditions, these interactions were either weak or led to intercalation [41].

391However, flavanols are *not* planar and may therefore not be able to intercalate with DNA.

392We, therefore, explored fluorescence lifetime behaviour of flavanols in the presence of

393DNA. Addition of DNA had no effect on catechin or epicatechin lifetimes in aqueous

394solutions (2 mM; pH 8 in Tris buffer; data not shown). This agrees with results from UV-

395Vis spectroscopic titrations which also found that DNA did not interact with catechin or

396EGCG in 0.1 M Tris at pH 7.4 or 8.0 [16].

397

398However, Polster *et al.* [16] reported that histone proteins might be the nuclear targets for

399catechin and EGCG. Using UV-Vis titration experiments, they showed that both flavanols

400bound to histone sulphate and interactions were more pronounced at pH 8.0 than 7.4 in Tris

401buffer. Since these titration experiments required approximately 1 h in total [42], it could be

402argued that this might be sufficient time for oxidative reactions and artefact formation to

403occur especially at higher pH values as determined in Fig. 3. The present fluorescence

404lifetime measurements were, however, made within 30 s of mixing the flavanol and histone

405solutions (note: the fluorescence lifetime experiments were also done in Tris buffer, as the

406UV-Vis titrations revealed that histone sulphate showed a less pronounced interaction with

407catechin in phosphate than Tris buffer [16]).

408

409The lifetimes of catechin and epicatechin in the presence of histone proteins were

410investigated at two concentrations (1.1 and 1.9 mM) and pH (7 and 8) (Supplementary

411Table). Given the short lifetimes recorded and the errors in fitting bi-exponential decays,

412the data for the different flavanols need careful interpretation. At this stage we are unable to

413clearly identify whether or not histones bind to flavanols, which would be expected to be

414shown by a change in lifetime (i.e. due to quenching). From other work it is, however, also

415clear that histone preparations differ in their ability to associate with flavanols [13] and,

416therefore, further fluorescence studies will be needed. Nevertheless, these initial findings

417demonstrate the potential power of studying flavanol–histone interactions by fluorescence

418methods. It is now also evident that much fundamental work is needed for characterising

419how the chemical environment (including pH and oxygen concentration) influence

420fluorescence lifetimes, quantum yields and spectra of flavanols. With regard to pH, the pK_a

421values of catechin, for example, are *ca.* 8.6 and 9.4 [43] and thus at pH 8 three different

422protolyte species exist for catechin: BH_2, BH^-, and B^{2-}. The fluorescence behaviour of each

423of these species will need to be understood and only through such careful measurements

424can these types of fluorescence measurements provide the much needed tool for elucidating

425the interactions between flavanols, histones and DNA.

426

427*3.6. Possible role of nuclear flavanols beyond an antioxidant function*

428Nuclear absorption has been reported not only for flavanols [13] but also for some other

429flavonoids. *Arabidopsis thaliana* nuclei absorb flavonols [44.45], *Drosophila* follicle nuclei

430absorb quercetin [46] and *Flaveria chloraefolia* nuclei absorb sulfonated flavanols [47]. It

431used to be widely accepted that the major function of polyphenols such as flavonoids was

432to protect DNA against UV damage and oxidative stress, but this has now been questioned

433[8,10]. Instead they were shown recently also to affect cell signalling and gene expression

434[48,49]. Flavanols are involved in the transcriptional activation of genes and modulation of

435epigenetic changes [36,50].

436

437Whilst several dietary flavonols and the green tea flavanol, EGCG, have been implicated in

438interacting and protecting DNA against damage [41,51, 52], the fact that they inhibit DNA

439methyltransferases *in vitro* and *in vivo* at the μmolar to sub-μmolar level is potentially more

440important for their effects on health [5,7,29,48,53]. Moreover, EGCG was also a potent

441inhibitor of histone acetyltransferase [50]. Histone acetylation has previously been shown

442to affect flavanol association [13,16] and is known to alter the chromatin structure, which in

443turn has been linked to the transcriptional activation of genes [15]. Both processes, DNA

444methylation and histone acetylation, are involved in epigenetic changes [54]. Indeed,

445Yamada *et al.* [53] concluded that EGCG may have inhibitory effects on the epigenetic

446changes that occur during carcinogenesis and aging. Whilst we found no evidence for

447interactions between flavanols and DNA, the results presented here in terms of flavanol

448association and penetration through the nucleus do not rule out the possibility that histones

449may be a target for EGCG, catechin and epicatechin. Solution phase ultrafast structure and

450dynamics studies such as time resolved infra-red (IR) or time resolved 2-dimensional IR

451will be needed to probe the origin of the bi-exponential lifetimes, which differed between

452the three flavanols. Such time resolved spectroscopic techniques will indicate the functional

453groups responsible for the fast dynamics that differ amongst these flavanols.

454

455*3.7. Future prospects*

456Given the recent discoveries identifying that flavanols may well be involved in epigenetic 457changes, highly sensitive techniques will be needed to trace their uptake and trafficking at 458the sub-cellular and sub-nuclear level and at physiologically relevant concentrations. Our 459results suggest that not all flavanols will interact via the same molecular mechanism and 460this will require new techniques with sufficient specificity and sensitivity. New 461developments in fluorescence lifetime imaging techniques and ultra-fast spectroscopy, as 462demonstrated here, may hold the key and pave the way for studying their functions and 463synthesis in plant cells. The trafficking, uptake and subcellular localisation of flavanols is 464of acute interest also for current research on tannin synthesis in plants [55]. Unravelling this 465last hurdle of flavonoid biosynthesis, storage and release would facilitate the development 466of new plant varieties with tannin compositions that can offer enhanced biological activities 467for nutrition and health [11].

468Such analytical developments will facilitate new types of biological experiments that can 469test how these compounds, when present in plant foods, can impact on mammalian cells 470and health. Although FLIM is a new technique to both mammalian and plant cell biologists 471alike, its application is growing rapidly [23-26,28], particularly in protein-protein 472interactions that involve energy transfer processes. However, this is the first study to report 473FLIM for other plant components and as the technique becomes more readily available, its 474impact can only grow. This study has shown that relatively small changes in flavanol 475structures (EGCG *versus* catechin or epicatechin; Fig. 1) lead to measurable changes in 476lifetime behaviour in the free and bound states. As plants synthesise several types of 477flavonoids, that vary in oxidation and substitution patterns [1], it is expected that other

22

478flavonoid compounds will be detectable using different combinations of excitation and

479emission wavelengths.

480

481**4. Conclusions**

482In conclusion, 2-photon excitation at 585 and 630 nm has enabled for the first time the

483measurement of fluorescence lifetimes from three flavanols, catechin, epicatechin and

484EGCG, in solution and *in vivo*. Lifetimes ranging from <45 ps to 1 ns in solution have been

485determined. *In vivo* experiments with onion cells demonstrated that tryptophan and

486quercetin have sufficiently low absorbance at 630 nm and this allowed the detection of

487externally added and endogenous flavanols within *Allium cepa* and *Taxus baccata* cells.

488Interestingly, fluorescence decay curves of catechin and EGCG differed markedly both in

489solution and when bound at the nucleus. This fact could be used in the future for selectively

490tracing the different flavanols *in vivo*. Furthermore, this work demonstrates how the

491application of fluorescence lifetime technology may be used to investigate the way

492flavanols interact with individual cellular components. We also conclude that flavanols are

493absorbed by cell nuclei and this provides new research challenges with regard to their

494intracellular functions.

495

496Semi-quantitative measurements revealed that the relative ratios of EGCG concentrations in

497perinucleolar organiser regions : nucleus : cytoplasm were approximately 100:10:1.

498Moreover, 3-D FLIM showed that externally added EGCG penetrated the whole nucleus of

499onion cells and was not just absorbed on the surface. The FLIM technique described here

500proved therefore a significant advance to DMACA staining and is capable of providing

23

501 quantitative biophysical information to probe the intra-cellular functions of flavanols such

502 as EGCG, which is the major flavanol of green tea.

503

504 **Acknowledgements**

508

509 **References**

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598**Legend to Figures**

599

600**Fig. 1:**

601Structures of three flavanols, catechin (**1**), epicatechin (**2**), and epigallocatechin gallate (**3**),

602and one flavonol, quercetin (**4**) (*note*: A, B, C denote the flavonoid rings).

603

604**Fig. 2**:

605The Jablonski diagramme depicting the energy levels of a molecule. S_o represents the

606ground singlet states, S_1, S_2 the excited singlet states; T_1 the triplet excited states. Electronic

607levels are subdivided into vibrational levels (v_1, v_2 … v_n). IC indicates internal conversion,

608$k_{fluorescence}$: rate of fluorescence leading from S_1 ($v_1 = 0$) to S_o (v_1 or v_n), k_{IVR}: intramolecular

609vibrational relaxation, k_{ISC}: rate of intersystem crossing and k_{quench}: rate of reaction with

610other molecules, chemical or energy transfer.

611

612**Fig. 3:**

613Time course of fluorescence lifetimes (ns) of catechin (2 mM) in 0.1 M phosphate buffer in

614air or nitrogen atmospheres at pH 5.8, 7.1 and 8.2 ($\lambda_{ex} = 585$ nm).

615

616**Fig. 4:**

617Fluorescence decay curves of catechin, epicatechin and epigallocatechin gallate (EGCG)

618solutions (2 mM) in 0.1 M phosphate buffer at pH 8.1 ($\lambda_{ex} = 585$ nm).

619

620**Fig. 5:**

28

621 Fluorescence lifetime images (λ_{ex} = 630 nm) of a cell from an onion epidermis soaked in 1

622 mM aqueous solutions of a) catechin (τ_1 = 0.4 ns (82%), τ_2 = 2.6 ns (18%)), b)

623 epigallocatechin gallate (τ = 0.2 ns) and c) control in water without added flavanol. Image

624 (A) shows a steady state image of the total emission lifetimes and image (B) shows the

625 analysed fluorescence excited state map. The distribution of fluorescence lifetimes in (B) is

626 illustrated in image (C), where the vertical axis represents the frequency and the horizontal

627 axis represents lifetime in pico-seconds. (*Note*: control nucleus shows hardly any

628 fluorescence in Fig. 5c).

629

630 **Supporting information**

631 Additional Supporting Information may be found in the online version of this article:

632 **Supplementary Table**: Fluorescence lifetimes (ns) of flavanols in the presence of different

633 histones (Tris buffers, pH 7 and 8). Pre-exponential factors are shown in brackets.

634

635 **Fig. S1.** UV-Vis spectra of catechin, epicatechin and epigallocatechin gallate recorded from

636 200 to 595 nm.

637

638 **Video Clip S1**. 3D stack of multiphoton excited (λ_{ex} = 630 nm) fluorescence image from an

639 onion cell epidermis soaked in a 1mM aqueous solution of epigallocatechin gallate. Images

640 were recorded at 0.5 - 2.0 µm slices.

641

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643 supporting materials supplied by the authors. Any queries (other than missing material)

644 should be directed to the corresponding author for the article.

645
646

647**Table 1**

648Fluorescence quantum yields of catechin, epicatechin and epigallocatechin gallate (EGCG)

649in methanol at 37 °C.

650

Compound	Quantum yield[a]
Catechin	0.018
Epicatechin	0.015
EGCG	0.003

651

652[a] Estimated accuracy = ±16%

653

Table 2

655Fluorescence lifetimes (ns) and pre-exponential factors (A_1 and A_2) of externally added

656flavanols, which were absorbed by onion epidermis, and endogenous flavanols in *Taxus*

657*baccata* male cones (± standard deviations).

658

Sample	τ_1 (ns)	A_1 %	τ_2 (ns)	A_2 %
Onion epidermis:				
control in water	0.84 [a]	100		
Onion epidermis:				
+ catechin	0.5 ± 0.04	77.5 ± 6.92	2.7 ± 0.19	22.5 ± 6.92
+ epigallocatechin gallate	0.25 ± 0.05	99.2 ± 1.13		
(EGCG)				
Taxus baccata male cones:				
in water	0.2 ± 0.03	93.4 ± 6.46	0.5 ± 0.2	6.6 ± 6.46

659

660[a] The control had a very low photon count in the absence of externally added flavanols and

661the data were quite noisy (see Fig. 5c), therefore it was not possible to obtain a standard
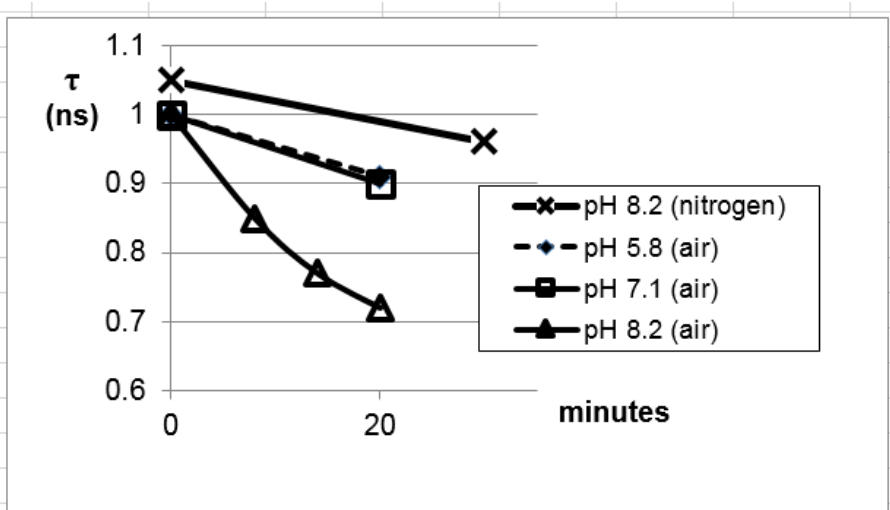
662deviation of the background lifetime.

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664

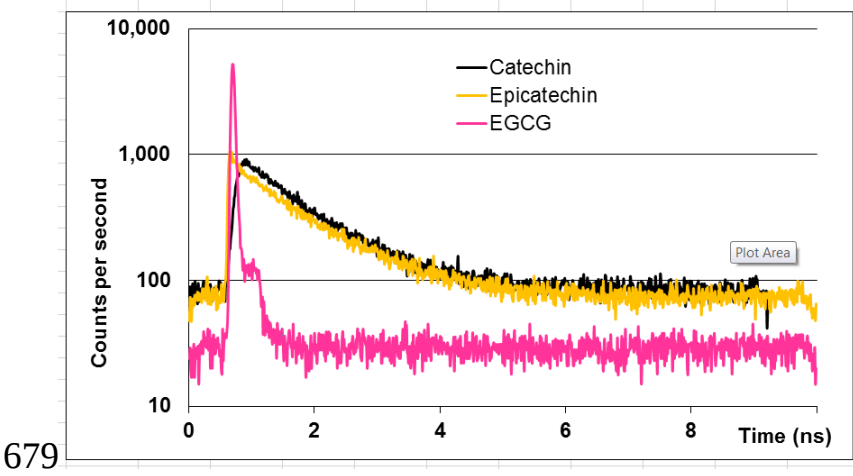665**Figure 1**:
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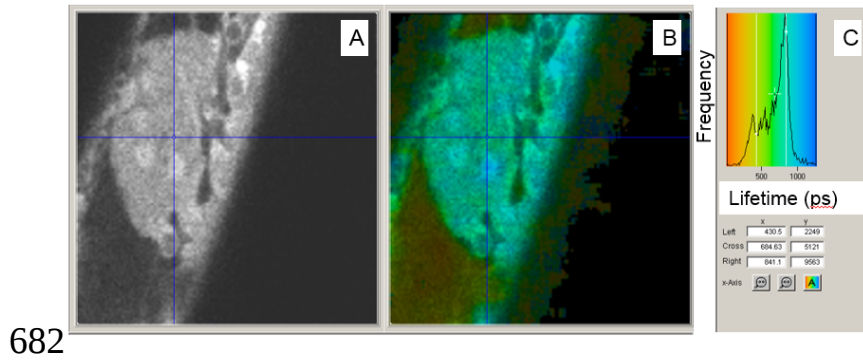
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670 Fig 2

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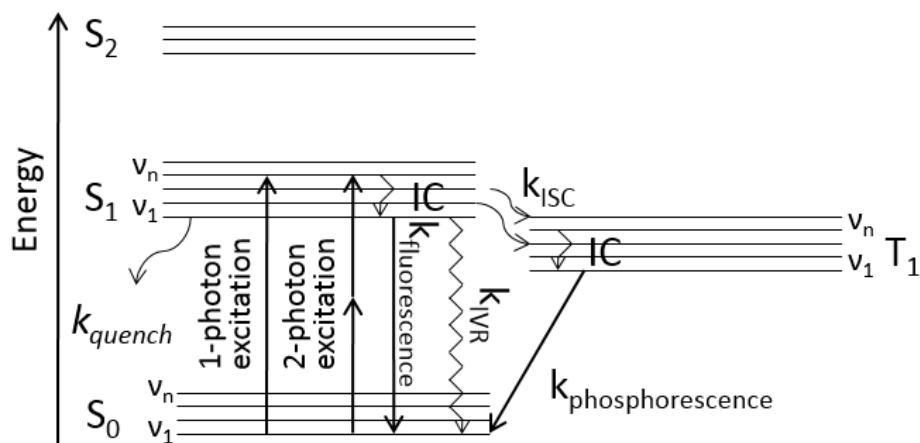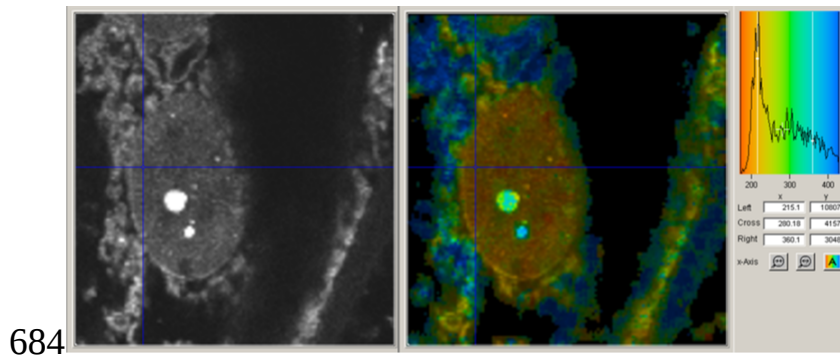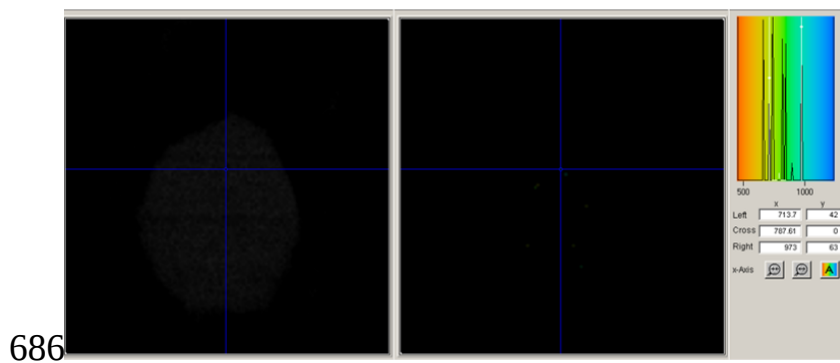

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674 Fig 3

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678Fig 4

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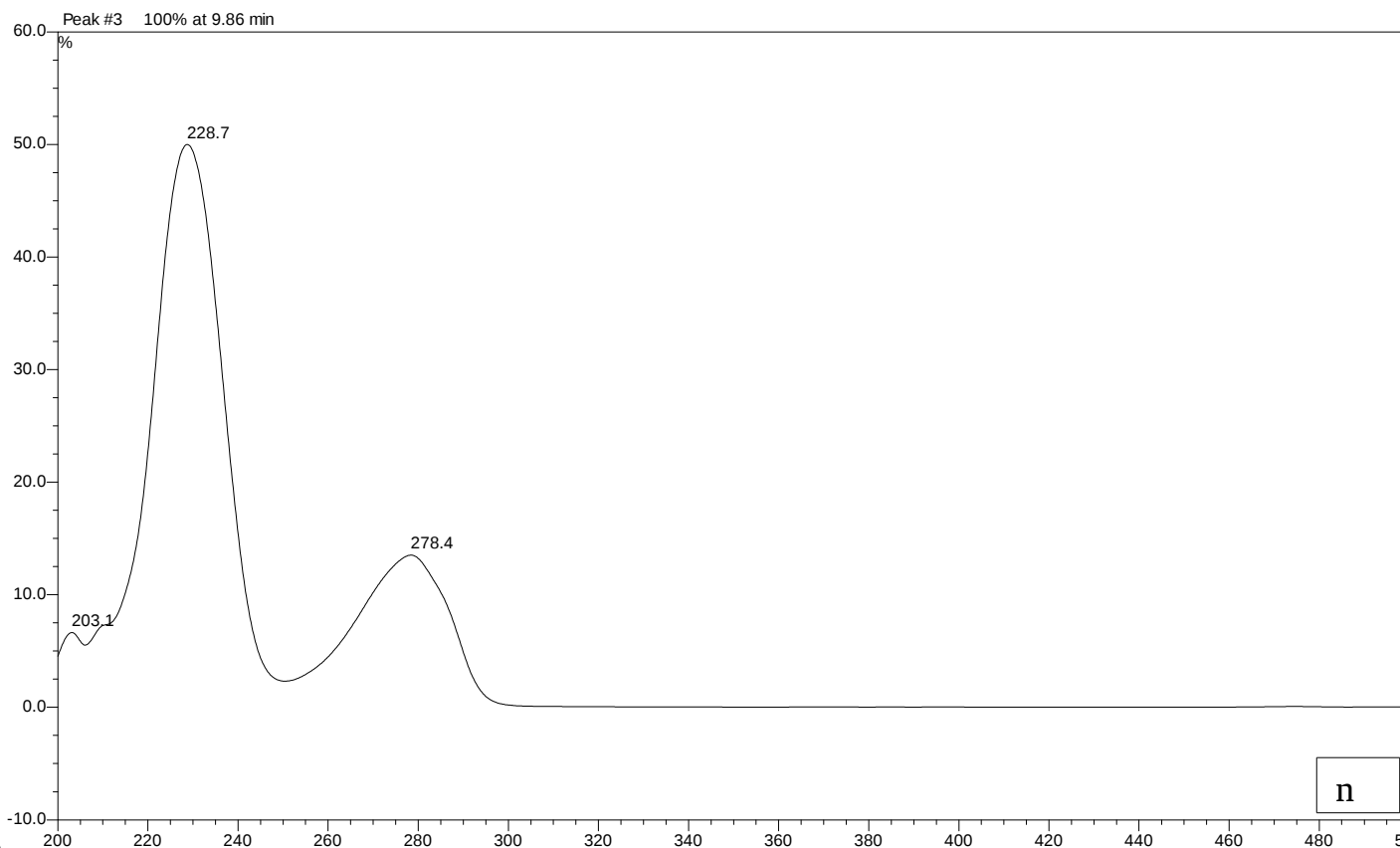680

681Fig 5a

682

683Fig 5 b

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685Fig 5c

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688

690**Figure S1:** UV-Vis spectra of catechin, epicatechin and epigallocatechin
691gallate recorded from 200 to 595 nm.
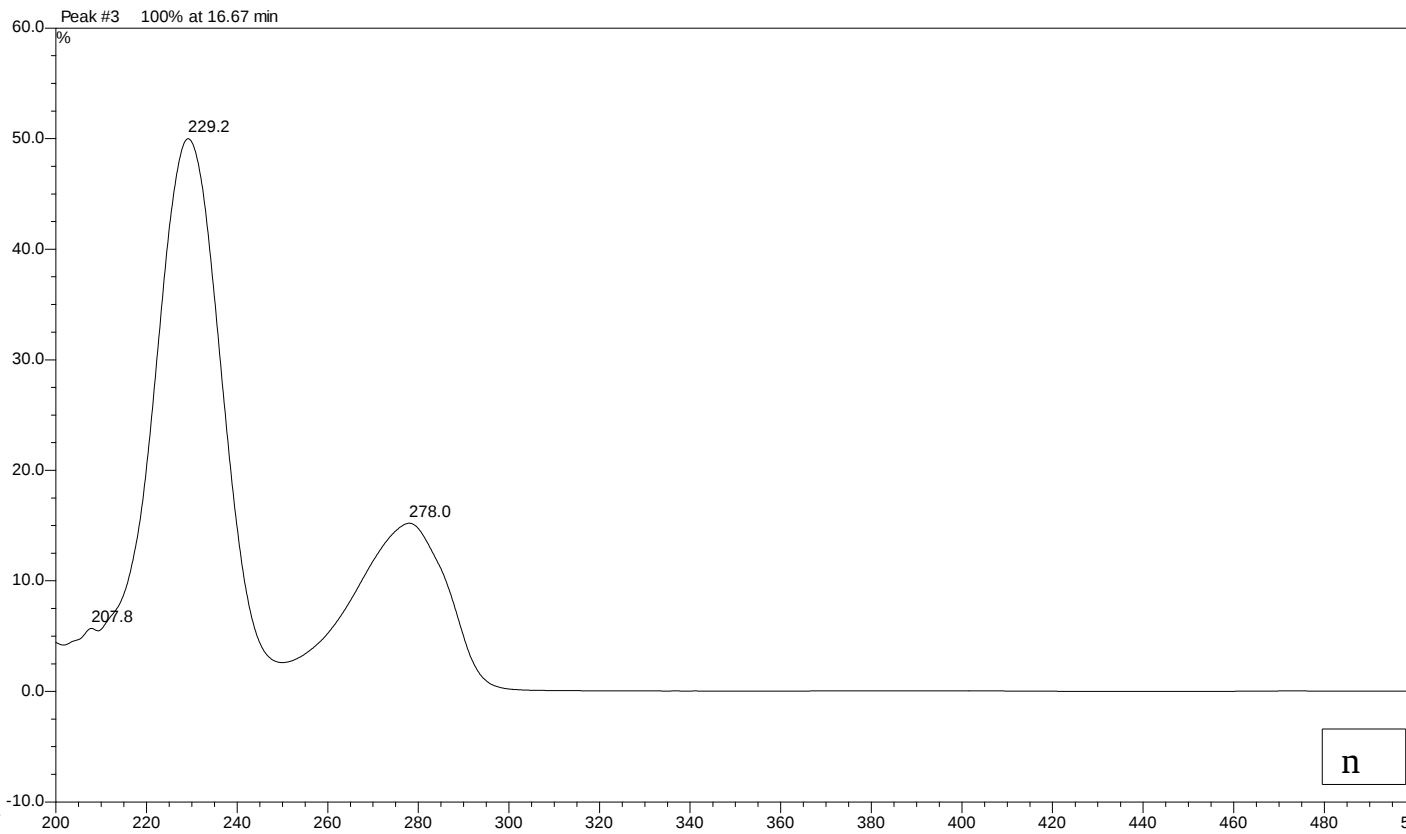692
693**Catechin**
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705**Epicatechin**
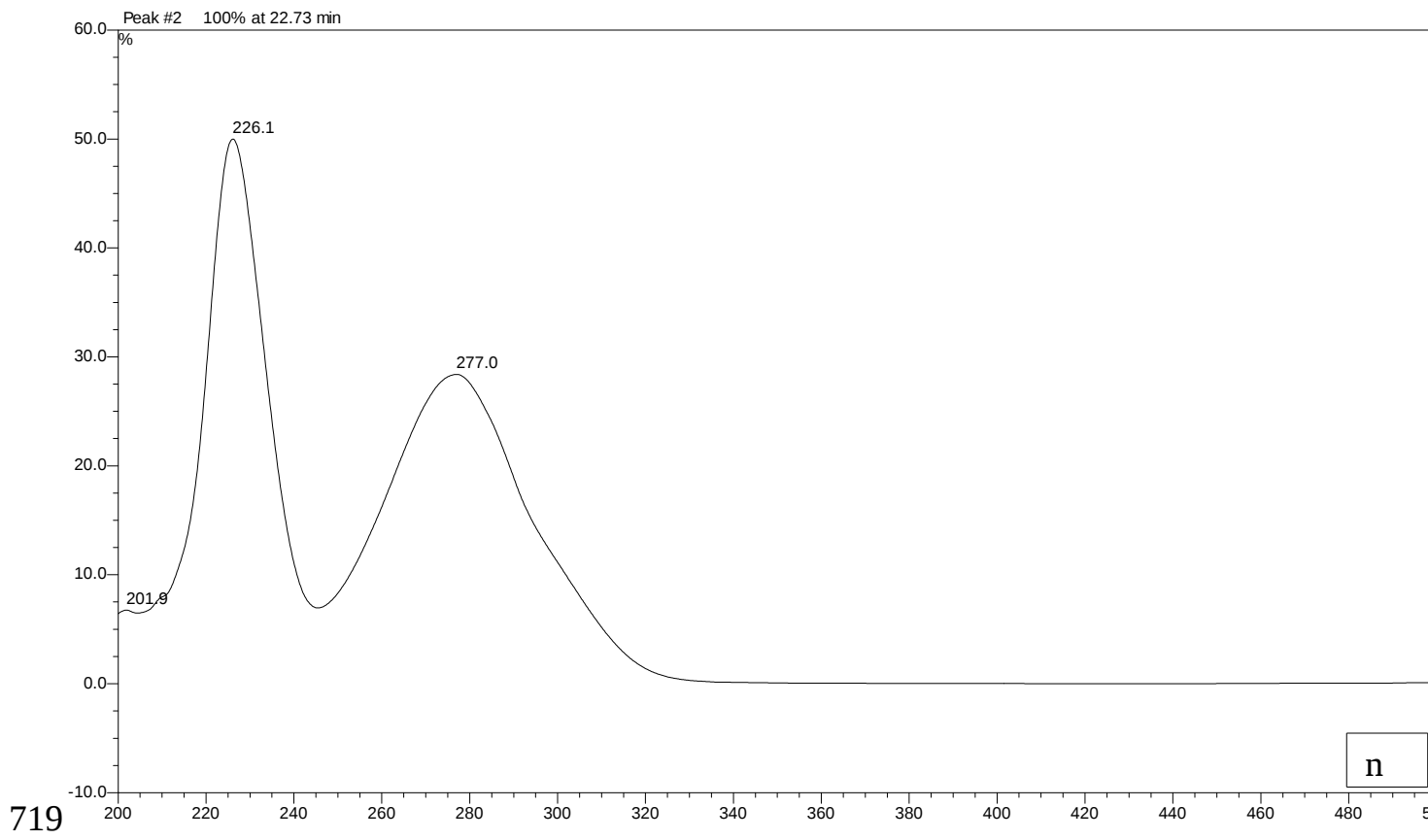706

Peak #3   100% at 16.67 min

[UV spectrum with peaks labeled 207.8, 229.2, and 278.0; y-axis %, x-axis from 200 to 500 nm]

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717**Epicatechin gallate**

718

Peak #2   100% at 22.73 min

719

720Supplementary **Table**

721Fluorescence lifetimes (ns) of flavanols in the presence of different histones (Tris buffers, pH 7 and 8). Pre-exponential factors

722are shown in brackets.

	1 mM Flavanol				2 mM Flavanol			
	pH 7		pH 8		pH 7		pH 8 [b]	
	τ_1 [a]	τ_2	τ_1	τ_2	τ_1	τ_2	τ_1	τ_2
(+)-Catechin [b]			0.9		1.1			
+ Sigma histone	0.9 (75%)	1.9 (25%)	(92%)	2.5 (8%)	(82%)	1.7 (18%)	1.1 (80%)	1.9 (20%)
+ Histone sulphate	1.1 (95%)	4.0 (5%)	0.9 (75%)	2.0 (25%)	1.1 (88%)	2.1 (12%)	1.1 (88%)	2.0 (12%)
+ Roche histone	1.0 (96%)	3.5 (4%)	0.9 (80%)	2.7 (20%)	0.8 (86%)	2.5 (14%)	1.1 (93%)	3.2 (7%)
(-)-Epicatechin [b]			0.9		1.1			
+ Sigma histone	0.8 (75%)	2.0 (25%)	0.9 (90%)	2.5 (10%)	1.1 (85%)	1.8 (15%)	1.0 (86%)	2.0 (14%)
+ Histone sulphate	nd[c]	nd	nd	nd	1.1 (87%)	1.9 (13%)	1.1 (84%)... 1.0 (84%)	1.9 (16%)
+ Roche histone	1.0 (92%)	2.8 (10%)	0.9 (80%)	2.3 (20%)	1.1 (90%)	3.1 (10%)	1.0 (90%)	2.7 (10%)
Average	0.9	2.4	0.9	2.3	1.1	2.3	1.1	2.4

723[a] Experimental error is ±50 ps; [b] For comparison, lifetime measurements in 0.1 M phosphate buffer at pH 8.1 gave τ = 1.0 ns for

724catechin and τ_1 = 1.1 (72%) and τ_2 = 0.1 ns (28%) for epicatechin (note: Due to the poor signal-to-noise at longer times the errors

725are significantly larger for the second lifetime (τ_2) and the pre-exponential factors of less than 10% may be due to a fluorescence

726contribution from impurities); [c] nd = not determined.