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### ► **To cite this version:**

Flora Tavernier, Stefania Savoi, Laurent Torregrosa, Philippe Huguency, Raymonde Baltenweck, et al.. The single-berry metabolomic clock paradigm reveals new stages and metabolic switches during grapevine berry development. *Journal of Experimental Botany*, 2025, 76 (11), pp.3125-3140. <10.1093/jxb/eraf038>. <hal-05011841>

**HAL Id: hal-05011841**

**<https://hal.science/hal-05011841v1>**

Submitted on 19 Aug 2025

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# The single-berry metabolomic clock paradigm reveals new stages and metabolic switches during grapevine berry development

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## Summary

- Asynchronous development of berries causes metabolic chimerism in usual samples. We thus revisited the developmental changes in the metabolome of the *Vitis vinifera* single berries from anthesis to over-ripening.
- A dataset of 9,256 ions obtained by non-targeted ultra-performance liquid chromatography coupled to high-resolution mass spectrometry was submitted to an analysis workflow combining classification and dimension reduction tools, to reveal the dynamics of metabolite composition without phenological *a priori*.
- This approach led to a metabolome-based definition of developmental stages, as well as the clustering of metabolites into 12 specific kinetic patterns. The single berry intrinsic metabolomic clock alleviates constitutive asynchronicity biases in the usual combination of phenological scales and observer clock. Such increase in temporal resolution enabled the identification of metabolite clusters annunciative of the onset of ripening since the herbaceous plateau. In particular, these clusters included transient lipidic changes and the start of ABA accumulation. We also highlighted a cluster of stilbenes that accumulate after sugar loading stops, during fruit shriveling.
- This non-targeted approach enables a more precise and unbiased characterization of grapevine berry development through the metabolomic clock paradigm. The discovery of new metabolic milestones of berry development paves the way towards an unbiased assessment of berry physiological stages.

**Keywords:** *Vitis vinifera* L., fruit development, metabolomics, untargeted metabolites, single berry, phenology

### Word count:

Introduction: 941

Material and methods: 1313

Results: 1983

Discussion: 2219

Total: 6456

## 44 Introduction

45 By providing both seed protection and dissemination to Angiosperms (Seymour et al.,  
46 2013), the apparition of the fruit stands as a pivotal leap in the evolutionary history of plants  
47 and their related animal vectors. Fleshy fruits shift from an immature seed protective organ,  
48 in green stage, to a strongly attractive one during ripening, when mature seeds become  
49 resistant enough to be disseminated. This key transition includes typical metabolic shifts  
50 affecting both primary metabolites serving as major *osmoticum*, from organic acids to  
51 energy-rewarding soluble sugars, and secondary metabolites, from astringent and  
52 antinutritive tannins to attractive anthocyanins and aromas (Gillaspy et al., 1993). Most  
53 fleshy fruits undergo these developmental transformations regardless of their climacteric or  
54 non-climacteric status. However, by contrast with climacteric fruit which rapidly ripen when  
55 triggered by the ethylene hormone following long periods of post-harvest storage, non-  
56 climacteric fruits do not store significant starch reserve during green stage, and are thus  
57 forced to ripen on the plant, in real time with phloem unloading of soluble sugars, making  
58 them particularly sensitive to environmental conditions during this period (Giovannoni, 2004).

59 Among non-climacteric fruits, grapevine (*Vitis vinifera*) is one of the world's most  
60 important, cultivated to produce table grapes, wine, juice and other products. Grape  
61 phenology was described in two main stages, following a double sigmoid growth curve  
62 (Coombe and Hale, 1973; Coombe and McCarthy, 2000). The first one, also called green  
63 stage, starts with intense cell divisions and expansion, resulting from the vacuolar  
64 accumulation of malic and tartaric acids as major *osmoticum* (Ojeda et al., 1999; Terrier and  
65 Romieu, 2001). Condensed tannins are synthesized at the beginning of this stage (Ollé et  
66 al., 2011). These phenolic compounds are important for wine organoleptic qualities (Del-  
67 Castillo-Alonso et al., 2021). The second stage, also called ripening, starts with an abrupt  
68 softening of the berries indicating the sudden induction of sugar accumulation. Such  
69 accumulation involves an accelerated phloem discharge that is accompanied by a significant  
70 respiration of malic acid (Coombe, 1992; Shahood et al., 2020). By the time sugars reach  
71 their peak, berry volume has doubled due to the parallel import of water. The berries then  
72 concentrate all solutes as they shrivel, but no longer import sugars (Castellarin et al., 2007a;  
73 Daviet et al., 2023; Savoi et al., 2021). The transition between green and ripening stages is  
74 commonly called “veraison” and refers to the start of anthocyanidins accumulation,  
75 responsible for the color change in red-skinned grape varieties (Lund and Bohlmann, 2006).  
76 By extension, this also indicates the softening and the initiation of sugar loading, slightly  
77 before color change, as elucidated on single berries (Bigard et al., 2019; Daviet et al., 2023).

78           Research on grape development is widely documented, but the division into distinct  
79 stages varies between studies (Bigard et al., 2019; Castellarin et al., 2007a; Rogiers et al.,  
80 2017; Savoi et al., 2021; Shahood et al., 2020). Stage characterisation relies on the  
81 measurement of the main primary metabolites (such as sugars, malic and tartaric acids),  
82 berry coloration, softness and growth. Omics technologies have enhanced the exhaustivity  
83 of developmental characterization at both the transcriptomic (Cabral et al., 2023; Castellarin  
84 and Di Gaspero, 2007; Cramer et al., 2014; Goes da Silva et al., 2005; Savoi et al., 2021;  
85 Tornielli et al., 2023) and metabolomic (Bonada et al., 2013; Duan et al., 2019; Leng et al.,  
86 2021; Nicolas et al., 2024; Ollé et al., 2011) levels. Furthermore, some studies have adopted  
87 an integrative approach that combines both transcriptomic and metabolomic analyses,  
88 leading to the identification of genes that could be activated at specific developmental stages  
89 and of molecules that could signal or mark stages beyond those defined by growth, primary  
90 metabolism and anthocyanins (Fasoli et al., 2018, 2012). However, most of these traits vary  
91 continuously during development, making the discretization of development beyond the two  
92 main stages somewhat arbitrary.

93           Research on berry development usually focuses on successive sets of mixed  
94 berries in order to smooth their marked heterogeneity at the plot level. Indeed, the berries  
95 exhibit both intra and inter cluster developmental asynchrony, ripening both at different rates  
96 and dates (Daviet et al., 2023; Shahood et al., 2020). Classical grapevine phenological  
97 scales explicitly refers to the median of the population (Coombe, 1995; Lorenz et al., 1995),  
98 but it was only recently pointed out that smoothing asynchronous berries obviously  
99 generates developmental chimera fundamentally incompatible with the identification of  
100 physiological stages as pure metabolic units (Bigard et al., 2019; Shahood et al., 2020).  
101 Single berry sampling and characterization have enabled to revisit the flow of water and  
102 primary metabolites in depth, to gather quantitative and molecular arguments on the origin of  
103 the sugar/acid relationship in grapes, and to shed light on the organization and energetics of  
104 the unloading of sugars in the berry. Only studies on single berries have made it possible to  
105 establish that softening, initiation of sugar storage, growth resumption and coloration  
106 mentioned above occur step by step in this order (Bigard et al., 2019; Savoi et al., 2021;  
107 Shahood et al., 2020), while they rather seemed to occur simultaneously on sets of mixed  
108 berries (Fasoli et al., 2018).

109           Recently, new methods have been developed to analyze developmental processes  
110 using pipelines combining statistical methods to process omics data. For example, the use of  
111 unsupervised learning methods, based on dimension reduction and analysis of distances  
112 between samples, has already made it possible to define a new grape phenology scale  
113 based on transcriptomics (Tornielli et al., 2023). The present study combines multivariate

114 analyses with untargeted metabolomics on single berries to revisit and refine the  
115 phenological stages of grape development. We show that metabolome-wide analyses  
116 provide a better understanding of berry development, identifying new metabolites, markers  
117 of key steps of berry growth and ripening.

## 118 **Material and Methods**

### 119 ***Experimental design***

120 Single berries were sampled by Savoi et al. (2021) in the years 2018 and 2019 on  
121 *Vitis vinifera* cv. Syrah within the experimental vineyard Pierre Galet of Institut Agro  
122 Montpellier (France). Plants established in 2000, were grafted onto SO4 rootstock and  
123 irrigated to avoid severe water deprivation. Throughout the growth cycle, the plants received  
124 regular phytosanitary sprayings to limit fungus diseases.

125 Berries were sampled in accordance with the double sigmoidal growth pattern, as  
126 identified through recurring photographic observations and quantification of sugars and  
127 organic acids (Savoi et al., 2021). This framework delineated eleven temporal waypoints in  
128 the developmental sequence, called « expert stages » for this study and encompassing the  
129 green growth phase (G1, G2, and G3), the green lag period (L4 and L5), the onset of  
130 ripening (characterized by the softening phase, S6 and S7), the ripening phase (R8, R9, and  
131 R10), culminating to the shriveling stage (Sh11) (Savoi et al., 2023). Systematic sampling  
132 was undertaken, with the green stage (from G1 to L4) being sampled in 2019, and ripening  
133 one (from L5 to Sh11) in 2018. The herbaceous plateau (L4 and L5), allowed to interconnect  
134 the two temporal domains.

135 Berries were frozen without pedicel and seeds in liquid nitrogen, before being  
136 crushed using a stainless-steel ball mill (Retsch MM400, Verder Scientific, Inc., Newtown,  
137 US). Subsequently, the 153 frozen berry powders underwent a lyophilization for 72h  
138 (Cryotec pilot freeze-dryer, Cryotec, Lunel, France), before being analyzed by VIS-NIR  
139 reflectance spectroscopy using a LabSpec 2500 with an optical probe (Analytical Spectral  
140 Devices, Inc., Boulder, CO, US). These spectra allowed the curated selection of 125  
141 samples, using the Kennard-Stone algorithm (Kennard and Stone, 1969), to ensure parity in  
142 berry quantities among stages (Table 1).

### 143 ***Metabolomic analyses using ultra high-performance liquid chromatography coupled*** 144 ***to high-resolution mass spectrometry (UHPLC-HRMS)***

145 Metabolites were extracted using 20  $\mu$ L methanol per mg of lyophilized sample,  
146 containing 5  $\mu$ g/mL of chloramphenicol as an internal standard. The extract was sonicated

147 for 10 minutes, before centrifugation at 13000 g at 10°C for 10 minutes. Quality control  
148 sample (QC) was prepared by mixing an equal volume of each sample to evaluate the  
149 stability of the equipments (retention time and area) and the repeatability of metabolites  
150 detection in all of the samples. Supernatants were analyzed as described previously  
151 (Rodrigues et al., 2023), with some modifications. Metabolomic analyses were performed  
152 using a Vanquish Flex binary UHPLC system (Thermo Scientific, Waltham, MA) equipped  
153 with a diode array detector (DAD). Chromatographic separation was performed on a  
154 Nucleodur C18 HTec column (150 × 2 mm, 1.8 µm particle size; Macherey-Nagel, Duren,  
155 Germany) maintained at 30°C. The mobile phase consisted of acetonitrile/formic acid (0.1%,  
156 v/v) (eluant A), and water/formic acid (0.1%, v/v) (eluant B), at a flow rate of 0.25 mL/min.  
157 The gradient elution program was as follows: 0 to 4 min, 80% to 70% B; 4 to 5 min, 70% to  
158 50% B; 5 to 6.5 min, 50% B isocratic; 6.5 to 8.5 min, 0% B; and 8.5 to 10 min, 0% B  
159 isocratic. The injected volume of sample was 1 µL. The UHPLC was coupled to an Exploris  
160 120 Q-Orbitrap MS system (Thermo Scientific, Waltham, MA) operated with a heated  
161 electrospray ionization source in positive and negative ion modes. The key parameters were  
162 as follows: spray voltage, + 3.5 and – 3.5 kV; sheath-gas flow rate, 40 arbitrary units (arb.  
163 unit); auxiliary-gas flow rate, 10 arb. unit; sweep-gas flow rate, 1 arb. unit; capillary  
164 temperature, 320°C; and auxiliary-gas-heater temperature, 300°C. The scan modes were full  
165 MS with a resolution of 60 000 fwhm (at m/z 200) and ddMS2 with a resolution of 60 000  
166 fwhm; the normalized collision energy was 30 V; and the scan range was m/z 85–1200.  
167 Internal mass calibration was operated using EASY-IC internal calibration source allowing  
168 single mass calibration for full mass range. Data acquisition and processing were carried out  
169 with Xcalibur 4.5 and Free Style 1.7 (Thermo Scientific, Waltham, MA), respectively.  
170 Analyses were performed in both positive and negative ionization modes, thereby  
171 constituting dual data sets for each individual sample.

172 UHPLC-HRMS raw data were processed using the Compound Discoverer 3.3  
173 software (Thermo Fisher Scientific, Waltham, MA, USA). Data processing for positive and  
174 negative modes were performed separately. QC samples were used to verify the  
175 repeatability of retention times and signal intensity throughout the data set. Untargeted  
176 metabolomics workflows were used for peaks detection, peaks groupement and alignment,  
177 and fill in missing peak data. Background compounds found in the blank samples, as well as  
178 known environmental contaminants related to plant protection products were filtered out from  
179 the data set. Peaks alignment parameters mainly included mass tolerance and retention time  
180 (RT), which were set at 5 ppm and at 0.1 min, respectively. Peaks detection was performed  
181 using a signal-to-noise ratio (S/N) of 2 and peak intensity thresholds at 10,000. Poorly  
182 repeatable ions were filtered out by keeping those with a peak rating greater or equal to 4 in

183 at least 4 samples and by keeping ions with a coefficient of variation (CV)  $\leq$  30% in all QC  
184 samples. Using these settings, 5123 and 4133 ions were obtained in positive and negative  
185 modes, respectively. Metabolomics data have been deposited to the EMBL-EBI  
186 MetaboLights database (DOI: 10.1093/nar/gkad1045, PMID:37971328) with the identifier  
187 MTBLS10572.

### 188 ***Data analysis and classifications***

189 The high dimensional dataset documenting the evolution of the berry metabolome  
190 across the 11 expert documented phenological stages (MS data) was further processed to  
191 classify metabolites by similarities in their evolution, using a workflow based on several  
192 multivariate analyses (Fig. 1).

193 Each peak surface was normalized by their sum of areas measured throughout berry  
194 development. Berry samples were classified using the K-means algorithm on the scaled  
195 metabolomic matrix to redefine developmental stages, based on variations in metabolite  
196 composition. The statistically relevant cluster number was determined using the average  
197 silhouette method (Govender and Sivakumar, 2020; Rousseeuw, 1987). The scaled  
198 metabolomic matrix was also submitted to a principal component analysis (PCA). The  
199 successive samples, projected onto the first three principal components (PC), were spread  
200 on a series of linear trajectories separated by abrupt directional changes. Linear regressions  
201 were calculated on all possible planes, before selecting the best ones based on their  
202 coefficient of determination. The curvilinear distance was then calculated, following an  
203 orthogonal projection of each sample on the respective lines, and tracing the progression of  
204 samples in the interconnected regressions. Such distance enabled the definition of a  
205 continuous metabolomic clock.

206 Developmentally invariant metabolites were filtered out using the Kruskal-Wallis test  
207 with a false discovery rate of 0.01. Subsequently, the metabolites were clustered according  
208 to their similarities in developmental profiles, using the K-means algorithm and the average  
209 silhouette method. A PCA on the metabolites within each cluster yielded the specific profile  
210 of its representative “eigen-metabolite”. Their developmental patterns were finally plotted  
211 with respect to the newly defined metabolomic clock. Finally, the filtered MS data were  
212 processed with the t-distributed stochastic neighbor embedding (t-SNE) method, yielding a  
213 three-dimensional projection of the metabolites. t-SNE was performed using a perplexity  
214 parameter of 30 over 1,000 iterations. This approach was designed to preserve the local  
215 structures of the data, providing a more accurate and faithful representation than that  
216 obtained by PCA, and to validate the use of the K-means method against the actual  
217 distribution of the data (Hebra et al., 2021; Olivon et al., 2018; Sorkun et al., 2022).

## 218 **Identification of key metabolites**

219 Metabolites presenting the highest correlation with the eigen-metabolite (correlation  
220 > 0.8) were selected for identification in each cluster showing interesting dynamics. Ion and  
221 metabolite annotations were based on expert analysis of molecular formulae, mass spectra  
222 and MS/MS fragmentation patterns in comparison with authentic standards when available  
223 and with data from MassBank (<https://massbank.eu/MassBank/>), mzCloud  
224 (<https://www.mzcloud.org/>) and Chem-Spider (<http://www.chemspider.com/>) using the  
225 Compound Discoverer 3.3 software. Standards for metabolite identification were purchased  
226 from Sigma-Aldrich (Saint-Quentin Fallavier, France) and Extrasynthese (Lyon, France).  
227 Identification confidence levels (ICL) established by Schymanski et al. (2014) were applied  
228 to score identifications.

## 229 **Results**

### 230 ***De novo deciphering of grape developmental stages based on single berry*** 231 ***metabolomics***

232 A total of 9,256 ions were detected in the 125 berry samples. The 11 expert stages  
233 relied on observations of growth stages, accumulation of primary metabolites (sugars,  
234 tartaric and malic acids and potassium) and the softening date. In contrast, the K-means  
235 blind analysis of metabolomic data allowed to distinguish 18 developmental stages (Table 2),  
236 henceforth coined "metabolomic stages". The intrinsic chemical profile of the fruits enabled  
237 to break down expert stages into sub categories, offering a more accurate view of berry  
238 developmental cycle from early to late stages. For example, the expert stage L4, which  
239 brings together transitional samples between the first and second part of the green phase  
240 (Fig. 2a), was subdivided into 5 distinct metabolomic stages (Table 2), while the L5, G1 and  
241 R9 expert stages precisely matched single metabolomic stages (Fig. 2a). Reciprocally,  
242 metabolic stages could also include samples from different expert stages, frequently  
243 contiguous, but not only (Table 2, Fig. S1).

244 In parallel, a "metabolomic clock" has been established by applying linear  
245 regressions on PCA-projected sample trajectories, calculating the distances between  
246 samples. The 3D PCA projection, where PC 1 to 3 accounted for respectively 27.9%, 12%  
247 and 5.5% of the dataset variance, allowed the assessment of the distribution of berries and  
248 its comparison with expert stages annotations (Fig. 2a). The dispersion around the 3D curve  
249 decreased from green stage to softening, and became almost non-existent thereafter.  
250 According to this projection, the grape development followed 5 main successive trajectories  
251 delimited by abrupt changes in direction during L4, S6, R8 and end R9. The "early green

252 stage” (spanning G1 to G3 and some L4 samples) and the “herbaceous plateau stage”, also  
253 known as the latent stage (encompassing certain L4 berries and all L5 ones), are distinctly  
254 demarcated on the PC2/3 plane. Notably, PC3 clearly distinguished the L4, L5 and S6  
255 expert stages subsequent to sudden changes in berry composition. This segment of the  
256 PCA plot is sparsely populated, indicating a potential lack of samples from this stealthy  
257 stage. The ripening period itself is subdivided into three trajectories: an “early ripening”  
258 group composed of soft and green berries (S6 S7), however these samples harvested a few  
259 days apart largely overlap. The subsequent trajectory included R8, R9, and one berry from  
260 the Sh11 stage, with a large evolution on PC2. The final trajectory, encompassing R10 and  
261 the remaining Sh11 berries, represented the post phloem arrest stage (Savoi et al., 2021) as  
262 shown in Fig. **2a**. Fig. **2b** shows that expert stages are less resolute than metabolic clock,  
263 particularly for stages G2, G3 and L4. In contrast, expert stages G1, L5, S6, S7, R10 and  
264 Sh11 group berries in a narrower range of metabolomic time. When comparing days after  
265 flowering (DAF) and the metabolomic clock (in Fig. **2c**), a fast evolution can be observed  
266 inside the L4 expert stage and at the end of maturation. The 50 % flowering date is  
267 measured at the scale of the whole cluster and not for each single berry, so it does not take  
268 asynchrony into account. It is also important to note that the two sampling seasons (G1 to L4  
269 for 2019 and L5 to Sh11 for 2018, Table **1**) do not appear to be sharply separated on the  
270 PCA and graphical representations of Fig. **2**. Altogether, these results clearly show that the  
271 metabolomic synchronization of berries outperforms the most severe sorting procedures  
272 based on observer sampling time, relative growth and primary metabolites.

### 273 ***Clustering of the untargeted metabolites using K-means***

274 Clustering single berry metabolites according to their relative amounts in the extracts  
275 of 125 single berries revealed 12 major developmental patterns, labeled A to L. These  
276 profiles showed 3 major trends: clusters of metabolites that decreased along development  
277 (Fig. **3**, profiles A to D), those showing a peak at certain stages (Fig. **4**, profiles E to I) and  
278 finally, clusters with a metabolite content that increased along berry development (Fig. **5**,  
279 profiles J to L).

280 The decreasing profiles A, B and C (Fig. **3**) comprised 161, 405 and 1339  
281 metabolites respectively, that could be efficiently summarized with a single PC explaining  
282 more than 50% of the intra-cluster variance. In contrast, profile D is distinguished by a larger  
283 number of metabolites (1,630), but its PC1 captured a smaller proportion of the total  
284 variance (25%). It suggests that this cluster may include more heterogeneous developmental  
285 profiles, as underlined by the distribution of correlations between individual metabolite  
286 profiles and that of the corresponding eigen-metabolite (Fig. **S2**). The decline of profile A

287 was particularly fast and occurred in the very early green stage, during the G1 and G2 expert  
288 stages. Metabolites in profile B decreased continuously through the green stage, from G1 to  
289 L4. Profile C exhibited a more gradual decrease over time, starting from a peak at the  
290 beginning of the early green stage and declining until the shriveling stage. In contrast, profile  
291 D displayed an unusual pattern with a slight decrease during the entire green phase (from  
292 the early green stage to the herbaceous plateau) followed by a sharper decline starting at  
293 the softening stage and continuing to the shriveling stage.

294 Figure 4 shows the profiles exhibiting accumulation peaks at different times during  
295 berry development. Among these, G and H profiles displayed particularly transient peaks.  
296 These profiles contained 146 and 87 metabolites respectively, and could be efficiently  
297 summarized with a single PC that explained more than 60% of their variation. Interestingly,  
298 the peak in the G profile coincided with the herbaceous plateau phase (L4 and L5),  
299 commonly described as a state of metabolic homeostasis. In contrast, profiles E, F and I  
300 showed a more gradual accumulation dynamic extending over several stages. These profiles  
301 included a higher number of metabolites, 747, 1,296 and 596 respectively, and a lower  
302 percentage of variance explained by their first PC by comparison with profiles G and H (Fig.  
303 4, Fig. S2). Profile E showed significant accumulation during the early green stage, peaking  
304 at the G3 stage and then gradually decreasing. Interestingly, this peak occurred exactly  
305 when profile A reached its minimum. Profile F showed a peak during the herbaceous plateau  
306 stage (expert stages L4 and L5), marking a transition between the early green stage and the  
307 beginning of the ripening. The G profile exhibited a much more abrupt peak compared to  
308 profiles E and F, with particular points reaching their maximum at the beginning of the  
309 herbaceous plateau stage and then decreasing at the beginning of the softening stage,  
310 specifically between stages L4 and S6. The H profile was characterized by an even more  
311 pronounced peak, starting at the end of the herbaceous plateau stage (L5) and reaching its  
312 maximum during the softening stage (S6 and S7), marking the beginning of grape ripening.  
313 At the same time, the I profile also reached its maximum, but with a more gradual increase  
314 and decrease, starting at the end of the early green stage (between G3 and L4) and ending  
315 at the completion of berry development (Sh11).

316 Clusters profiles with an ascending shape starting at the S6 expert stage  
317 encompassed metabolites specifically accumulated during the berry ripening phase (Fig. 5).  
318 Profile J began to accumulate from the start of the softening stage (S6), reaching a peak at  
319 the end of the ripening stage, before decreasing during the shriveling stage. Profile K started  
320 to increase at the end of the softening stage, from expert stage R8, and reached its  
321 maximum at the end of shriveling (expert stage Sh11). Profile J stands out for its large  
322 number of metabolites, with 1,297 compounds, and a PC1 that explains only 31% of their

323 variance. On the other hand, profiles K and L contained a smaller number of metabolites,  
324 827 and 72 respectively, and a larger part of their variability was explained by their first PC.  
325 The L profile in particular included the largest proportion of metabolites with a correlation of  
326 at least 0.8 with the eigen-metabolite (Fig. **S2**). This profile showed a late pattern of  
327 accumulation, after phloem arrest and during shriveling (expert stages R10 and Sh11).

328 Finally, the t-SNE method provides an alternative to PCA for metabolites visualization  
329 (Fig. **6**). The metabolites separated into two main branches on the 3D graph, reflecting their  
330 evolution over time. Early green stage specific patterns (A, B, C and D in shades of blue) are  
331 at the upper end of the t-SNE, while dynamics varying during ripening and shriveling stages  
332 (J, K and L in shades of red) are at the lower left end of the graph. The dynamics showing  
333 accumulation peaks between the early green stage and the ripening stage are in the central  
334 part. Transient dynamics observed in clusters A, E, F, G, H, I, J, and L indicate abrupt  
335 variations at specific points in berry development, facilitating accurate estimation of  
336 developmental stages. In addition, some of these clusters, such as A, H and I, are  
337 particularly distinct and clearly separated from the others.

### 338 ***Metabolite annotation in selected clusters***

339 Among the 12 metabolite clusters, those with the most transient dynamics were  
340 chosen for annotation of their metabolites. Profiles A, C (Fig. **3**), E, F, H, I (Fig. **4**), J, K and L  
341 (Fig. **5**) were therefore selected, focusing on particularly well classified ions, *i.e.* having a  
342 correlation greater or equal to 0.8 with their respective eigen-metabolites. The total number  
343 of selected ions was 2,586, and ranged from 60 to 606 for L and C clusters, respectively.  
344 Expert analysis of MS and MS/MS data in the 10 selected clusters resulted in the annotation  
345 of 483 of the 2,586 ions (Table **S1**), which could be attributed to a total of 117 metabolites,  
346 listed in Table **S2**, together with their molecular families. Thus, 19 % of all selected ions  
347 could be annotated, this proportion ranging from 7 % (cluster E) to 83 % (cluster L) (Fig. **S3**,  
348 Table **S1**).

349 Cluster A was mainly composed of monomers and small polymers of gallate ester  
350 tannins (Fig. **S4**), including confirmed compounds by molecular standard (ICL 1 on the  
351 Schymanski et al. (2014) scale) such as epicatechin gallate (ECG) and epigallocatechin  
352 gallate (EGCG). Although condensed tannins were predominant in cluster C, particularly  
353 procyanidins of types A, B, and C, a few type A procyanidins were also detected in cluster A.  
354 Cluster C also contained various flavonoids, including glycosylated derivatives of chalcone  
355 and kaempferol, as well as their dihydro-derivatives (ICL 4). Additionally, quercetin  
356 glucuronide (ICL 2) was identified in this cluster, along with phenolic acids coupled with  
357 tartaric acid, including caftaric, coutaric, and fertaric acids (ICL 1). Malic and citric acids were

358 detected in cluster F (ICL 1). Cluster H encompassed C<sub>18</sub> and C<sub>20</sub> lipids, notably linolenic  
359 acid (C<sub>18</sub>), confirmed by molecular standard (ICL 1), and its potential derivatives, noted as  
360 ICL 2 and 3. Aspartic acid was identified in cluster I (ICL 1), along with polyol sugars and  
361 hexose (ICL 2). Cluster J included an anthocyanin, cyanidin glucoside (ICL 1), flavonoids  
362 such as derivatives of isorhamnetin and syringetin (ICL 3), the glycosylated form of abscisic  
363 acid (ABA-glucoside), as well as glycosylated and esterified coumaric acid and sugar  
364 polymers (ICL 1 and 2). Cluster K contained several flavonoids, including derivatives of  
365 isorhamnetin and kaempferol (ICL 1 for glycosylated forms and ICL 3 for other derivatives),  
366 as well as anthocyanins like myricetin (ICL 2) and malvidin (ICL 1), and contained ABA-  
367 glucoside (ICL 2) and proline (ICL 1). Given that ABA-glucosides were present in both  
368 clusters J and K, The profile of free ABA was searched for among metabolites with a  
369 correlation lower than 0.8 with the eigen-metabolite. Indeed, the free form of ABA had a  
370 correlation of 0.75 with the eigen-metabolite of cluster I (Table **S1**). Finally, based on the  
371 annotation of the vast majority of its ions (83%), the metabolites in group L belonged almost  
372 exclusively to the stilbene family, including isomeric forms and derivatives of resveratrol and  
373 piceid (ICL 1), as well as viniferin glucoside (ICL 1).

## 374 **Discussion**

375 Present work provides a new perspective on grapevine berry development based  
376 solely on its solute composition to establish an internal metabolomic clock. By employing a  
377 novel strategy that combines single berry sampling, untargeted metabolomics, multivariate  
378 analyses and expert annotation, we provide a much detailed description of the phenological  
379 sequence that undergoes grapevine fruit. This approach revealed potentially important  
380 groups of metabolites in fruit development. Furthermore, the integration of clustering coupled  
381 with t-SNE, applied independently from observer time, led to a more precise and incisive  
382 understanding of the grape ripening process.

### 383 ***Single berry metabolomics reveals an internal clock regulating grape development***

384 The blind statistical analysis of metabolites at single berry level highlighted transient  
385 metabolic shifts, which would have been smoothed by averaging asynchronous berries as  
386 usually carried out (Bigard et al., 2019; Shahood et al., 2020; Zamboni et al., 2010). In  
387 addition, the main solutes that exhibit monotonous changes during green stage or ripening,  
388 such as malic acid and sugars, do not allow these phases to be divided into transient stages.  
389 We have shown that relying only on variations in fruit intrinsic composition led to a more  
390 accurate synchronization of berries than sorting by growth or primary metabolites. The use  
391 of PCA to get insights on sample distribution during fruit development has already revealed a  
392 common "U" shape pattern throughout the literature, but uncontrolled variations in the real

393 age pyramid of berries inside each sample scatters the data and prevents to detect  
394 discontinuities (Dai et al., 2013; Tornielli et al., 2023). Without prior filtering of the data  
395 except invariant metabolites, sub periods in the green and ripening stages were clearly  
396 resolved on the first 3 PCs, being discriminated by very sudden changes in sample direction  
397 vectors, which are necessarily smoothed in average samples. The t-SNE method confirmed  
398 the robustness of using metabolites to characterize grape development, organizing them  
399 within a developmental continuum. This technique also highlighted isolated groups (clusters  
400 A, H, and I) which, although distinct, remained integrated within the developmental flow.

#### 401 ***Profiles are consistent across harvest years***

402 The kinetics of the two sampling years were linked by the lag phase, during which  
403 berry growth pauses and which is widely accepted as quite stable during grape development  
404 (Coombe, 1976; Matthews et al., 1987; Thomas et al., 2008). Examination of the PCA and  
405 representative dynamics revealed a remarkable consistency: the change of vintage did not  
406 seem to affect the evolution of the samples. Indeed, the L5 group was not impacted by the  
407 sampling year and was equidistant from the other stages (L4 and S6). This continuity was  
408 also obvious on representative dynamics, particularly in profiles B, C, D, I, K and L (Fig. **S4**).  
409 These results validated our sampling procedure and underlined a certain robustness over  
410 two vintages.

#### 411 ***Metabolic clusters reveal transient shifts in berry physiology***

412 Blind, dynamic-based classification of grape metabolites resulted in 12 distinct  
413 developmental clusters. Among these, 10 showed relatively transient dynamics, enabling us  
414 to trace the developmental history of grapevine berry and propose a metabolic map, which  
415 highlights some metabolites and pathways as milestones of grape development (Fig. **7**).

416 Catechins monomers are phenolic compounds produced via the flavonoid  
417 biosynthesis pathway, but their polymerisation in condensed tannins remains elusive (Yu et  
418 al., 2023). The conversion of phenylalanine into p-coumaroyl-CoA is considered as the first  
419 step in the phenylpropanoid pathway. The latter is then converted into flavonoids, including  
420 tannins, by chalcone synthase (CHS), or into stilbenes by stilbene synthase (STS) (Flamini  
421 et al., 2013; Rienh et al., 2021, Fig. **7**). Profile A and C showed that the monomers and  
422 small proanthocyanidin (PA) polymers (EC, EGC, etc., from monomers to hexamers), rapidly  
423 decreased after anthesis. This decline coincided precisely with the period when higher  
424 molecular weight PAs began to accumulate, suggesting small polymers might be rate-  
425 limiting intermediaries (Kennedy et al., 2001, 2000; Ollé et al., 2011). Puzzlingly, profile A,  
426 encompassing many galloylated PA derivatives (Fig. **S3**), decreased before the non-

427 galloylated ones in cluster C. Further work is needed to understand if the faster decrease of  
428 galloylated forms reflects (i) an increased in gallate/shikimate competition subsequent to an  
429 acceleration of the PA branch, (ii) an improved NADPH reducing power, or (iii) a sequential  
430 expression of specialized SDH/ isogenes (Tahara et al., 2020).

431 Linolenic acid, found in the H cluster announcing the onset of ripening at softening, is  
432 a precursor of jasmonic acid, a phytohormone involved in various developmental processes  
433 (Singh et al., 2022). It also serves as a precursor to diverse volatile compounds determinant  
434 for fruit aroma and defense mechanisms (Li et al., 2021; Rienth et al., 2021; Schwab et al.,  
435 2008). Its accumulation should mark increased membrane phospholipid turnover  
436 immediately before the onset of ripening, where its peroxidation takes place (Pilati et al.,  
437 2014).

438 The accumulation profiles of metabolites belonging to clusters J, K and L are  
439 consistent with their involvement in successive ripening-associated biological processes.  
440 During ripening, a second growth phase is set on due to a significant accumulation of water  
441 and solutes, mainly glucose and fructose, originating from sucrose translocated by phloem  
442 mass flow (Daviet et al., 2023). When the berries reach their maximum volume, phloem  
443 unloading in berries ceases, leading to a loss of water and volume known as the shriveling  
444 or over-ripening (Griesser et al., 2024; Rogiers et al., 2017; Savoi et al., 2021). The fruits  
445 then undergo constitutive water stress, which influences their metabolic composition.

#### 446 ***ABA as a key player of the ripening process***

447 Cluster I started with the herbaceous plateau (L4) and peaked at the softening  
448 coloring transition (S7/R8). This cluster is marked by the accumulation of abscisic acid (ABA)  
449 in its free form, the level of which began to rise at the end of the herbaceous plateau, before  
450 peaking at the softening stage (Fig. **S5**). ABA is a phytohormone regulating plant growth and  
451 development. It plays multiple roles including tolerance to desiccation (Fujii and Zhu, 2009).  
452 ABA has been shown to promote color changes in non-climacteric fruits like strawberries  
453 (Batista-Silva et al., 2018; Jia et al., 2011; Jiang and Joyce, 2003). Analysis of grapevine  
454 berry development has highlighted ABA as an early indicator (if not a trigger) of veraison and  
455 berry coloration (Pilati et al., 2017), particularly by positively regulating the phenylpropanoid  
456 biosynthesis pathway (Chaves et al., 2010; Giribaldi et al., 2010; Kuhn et al., 2014;  
457 Lacampagne et al., 2010; Sun et al., 2010; Wheeler et al., 2009). This regulatory role of ABA  
458 is also evident in other non-climacteric fruits, such as strawberries (Jia et al., 2011), where  
459 ABA levels continue to increase from the beginning of ripening until the end of fruit  
460 development. In contrast, in grapevine berries, ABA peaks during the initial color change of  
461 the berries but then declines as the ripening phase progresses (Davies et al., 1997; Fortes et

462 al., 2015; Pilati et al., 2017; Villalobos-González et al., 2016). Present single berry results  
463 largely refined these views, showing that ABA starts to accumulate before softening, acting  
464 as a trigger rather than a consequence of ripening, and maintains a high level throughout the  
465 entire phase of active phloem unloading, before shriveling (Fig. **S5**). If ABA belonged to  
466 cluster I, its glucose ester derivative (ABA-GE) accumulated later, and was found in cluster  
467 K. ABA-GE has been considered as a storage form of ABA or a final inactive product of its  
468 catabolism (Zeevaart, 1999). Hydrolysis of ABA-GE by specialized glucosidases has been  
469 shown to lead to a rapid increase in free ABA concentrations in response to osmotic stress  
470 in *Arabidopsis thaliana* (Xu et al., 2012). Moreover, the ABA catabolite phaseic acid was  
471 associated with cluster J (correlation of 0.69), suggesting that active ABA catabolism is  
472 responsible for the rapid decrease of free ABA at the very end of ripening (Fig. **S5**). Cluster J  
473 also contained a large number of sugar-derived ions, consistent with the accumulation profile  
474 of glucose and fructose as major *osmoticum* in ripening berries, starting at S6. To our  
475 knowledge, this is the first evidence that the sugar accumulation process precisely starts at  
476 the peak of ABA concentration (cluster I). ABA has been shown to activate the transcription  
477 of a cascade of enzyme and transporter genes playing key roles in sugar metabolism and  
478 accumulation during berry ripening (Bennett et al., 2023; Pan et al., 2005). Altogether, the  
479 rapid shift of the veraison-associated active ABA pool to inactive ABA forms such as ABA-  
480 GE and phaseic acid suggests that fine temporal tuning of ABA concentration is critical to a  
481 proper coordination of the ripening process.

#### 482 ***Flavonol and proline dynamics during ripening as indicators of constitutive stress***

483 Noticeably, the sequential accumulation of anthocyanins and flavonols such as  
484 kaempferol, quercetin, isorhamnetin and myricetin derivatives, as well as malvidin and  
485 cyanidin derivatives in clusters J and K, reflects the decline of dihydroflavonols, such as  
486 dihydrokaempferol, in cluster C. This developmental shift in flavonol profiles follows the  
487 biosynthetic pathway and aligns with the expression profiles of the genes encoding  
488 dihydroflavonol-4-reductase and late genes of the anthocyanin regulated by MybA1  
489 (Cutanda-Perez et al., 2009; Massonnet et al., 2017).

490 Cluster K shows a massive, but late accumulation of proline, already known to over-  
491 accumulate when both berry growth and net protein accumulation have ceased (Stines et al.,  
492 1999). The levels of delta-1-pyrroline-5-carboxylate synthetase mRNA and protein, a key  
493 regulatory enzyme in proline biosynthesis, do not seem to be affected by ripening (Deluc et  
494 al., 2009; Stines et al., 1999). Present results revealed that proline didn't start to accumulate  
495 before late ripening (R8), and largely escaped the peak of free ABA, which in addition clearly  
496 vanished after phloem arrest, while proline accumulation persisted. This very poor

497 coincidental timing between ABA and proline in single berries apparently contradicts the  
498 hypothesis that ABA would simply trigger proline biosynthesis in grapevines submitted to  
499 water deficit (Deluc et al., 2009). Finally, most metabolites associated to cluster L, which are  
500 characterized by a sharp accumulation at the very end of the ripening process, have been  
501 identified as stilbenes, including *trans*-resveratrol, *trans*- and *cis*-piceid and viniferins (Table  
502 **S2**). This cluster appeared therefore remarkably homogeneous, as all its annotated  
503 compounds, representing 83% of all ions, were derived from stilbenes (Table **S1**, Fig. **S3**).  
504 Stilbene biosynthesis has been associated with a wide array of biotic and abiotic stresses in  
505 grapevine (Chong et al., 2009). In grapes, stilbene metabolism has been shown to respond  
506 to water deficit, albeit with different responses depending on varieties (Deluc et al., 2011). In  
507 addition, transcriptomic analysis of berries subjected to post-harvest withering has shown a  
508 massive induction of the stilbene synthase gene family (Vannozzi et al., 2012). In the context  
509 of berry development, the present work shows that stilbenes can be considered as metabolic  
510 markers of the water stress associated to the cessation of phloem sap flow and wilting  
511 occurring at over-maturation stage.

#### 512 ***Dealing with complex accumulation profiles***

513 K-means clustering, while useful for grouping data by similarity, may keep complex  
514 metabolic profiles hidden, resulting in misclassification of physiologically important  
515 metabolites. For example, by contrast with proline which also derives from glutamate,  
516 gamma-aminobutyric acid (GABA), the key intermediary of the GABA shunt in plants (Ansari  
517 et al., 2021), was found progressively accumulated during the green phase, followed by a  
518 sudden disappearance preceding softening, and a sharp increase at the end of maturation  
519 (Fig. **S6**). The recent identification of GABA as an inhibitor of the malate channel ALMT1  
520 (Long et al., 2020), may provide a functional link between GABA abrupt decrease and the  
521 trigger of the typical reversal of malate accumulation during the green stage/ripening  
522 transition. Then, GABA being reversibly accumulated in mature berries submitted to O<sub>2</sub>  
523 deprivation (Tesnière et al., 1994), its terminal accumulation is symptomatic of increased  
524 anaerobiosis in the fruit core at this stage (Xiao et al., 2018). Such a particular profile led to  
525 its misclassification in cluster E, albeit with a correlation of 0.62 to the cluster eigen-  
526 metabolite (Fig. **S6**). Similarly, tryptophan, a precursor of the phenylpropanoid pathway (Fig.  
527 **7**), had a distinct profile with a moderated peak at veraison but an overall decreasing trend.  
528 This led to its placement in cluster D with a correlation of 0.64. To address this issue,  
529 increasing the number of clusters in K-means could allow for a finer classification of  
530 metabolites with specific trends. Furthermore, the use of more sophisticated grouping  
531 methodologies, for example by combining a Gaussian mixture model (Polanski et al., 2015)  
532 with the uniform or tree manifold approximation and projection (UMAP, TMAP) dimension

533 reduction methods (Ebbels et al., 2023; Olivon et al., 2018), could allow better adaptation to  
534 these atypical profiles. This potential has already been partially explored in this study using t-  
535 SNE.

## 536 **Conclusion**

537 This study provides a detailed analysis of grapevine berry development through an  
538 untargeted metabolomic profiling, identifying 12 distinct metabolite clusters that map the  
539 fruit's phenological stages. Utilizing single berry sampling and multivariate analyses, we  
540 highlighted abrupt metabolic changes and the key roles of many metabolites. These results  
541 improve our understanding of the grape's internal metabolic clock, offering the possibility of  
542 optimizing the harvesting of berries according to their internal developmental clock, thanks in  
543 particular to high-throughput and non-destructive phenotyping methods, opening the way to  
544 new applications in breeding. Additionally, this research paves the way for studying the  
545 physiology of non-climacteric fruits, allowing for more effective identification of key  
546 developmental regulators, such as the gene networks involved in the onset of ripening. This  
547 enhanced understanding could provide deeper insights into the molecular mechanisms  
548 underlying plant acclimatization to abiotic stresses.

## 549 **Acknowledgments**

550 This work was supported by the Fondation Poupelain, the Agence Nationale de la  
551 Recherche (ANR, G2WAS project, ANR-19-CE20-0024), the Institut National de Recherche  
552 pour l'Agriculture, l'alimentation et l'Environnement (INRAe) Biologie et Amélioration des  
553 Plantes (BAP) department (Métab'EAU project) and the Région Occitanie (Métab'EAU  
554 project). Thanks to the INRAE UMR AGAP for helping with the sampling and to the INRAE  
555 UMR SVQV of Colmar for the metabolomics analysis and to Camille Rustenholz and  
556 Amandine Velt for the metabolomics pipeline.

## 557 **Competing interests**

558 The authors declare no competing interest.

## 559 **Author contributions**

560 F.T. prepared the samples for LCMS analysis, established and implemented the  
561 multivariate analysis workflow, interpreted the results, and drafted the manuscript. S.S.  
562 monitored and collected the grapevine berry samples, prepared the samples, and  
563 contributed to the review and editing of the manuscript. L.T. provided the plant material and

564 contributed to the review and editing of the manuscript. P.H. interpreted the results, and  
565 drafted the manuscript. R.B. supervised the metabolomic analyses, generated raw  
566 metabolomics data, identified the metabolites, interpreted the results, and drafted the  
567 manuscript. V.S. conceived and designed the study, coordinated and supervised the  
568 experiments, interpreted the results, and drafted the manuscript. C.R. monitored and  
569 collected the grapevine berry samples, conceived and designed the study, coordinated and  
570 supervised the experiments, interpreted the results, and drafted the manuscript. All authors  
571 have reviewed and approved the final version of the manuscript for publication.

## 572 **Data availability**

573 Metabolomics data have been deposited to the EMBL-EBI MetaboLights database  
574 (DOI: 10.1093/nar/gkad1045, PMID:37971328) with the identifier MTBLS10572.

## 575 **References**

- 576 Ali, K., Maltese, F., Choi, Y.H., Verpoorte, R., 2010. Metabolic constituents of grapevine and  
577 grape-derived products. *Phytochem. Rev.* 9, 357–378. [https://doi.org/10.1007/s11101-](https://doi.org/10.1007/s11101-009-9158-0)  
578 009-9158-0
- 579 Ansari, M.I., Jalil, S.U., Ansari, S.A., Hasanuzzaman, M., 2021. GABA shunt: a key-player in  
580 mitigation of ROS during stress. *Plant Growth Regul.* 94, 131–149.  
581 <https://doi.org/10.1007/s10725-021-00710-y>
- 582 Batista-Silva, W., Nascimento, V.L., Medeiros, D.B., Nunes-Nesi, A., Ribeiro, D.M., Zsögön,  
583 A., Araújo, W.L., 2018. Modifications in Organic Acid Profiles During Fruit Development  
584 and Ripening: Correlation or Causation? *Front. Plant Sci.* 9.  
585 <https://doi.org/10.3389/fpls.2018.01689>
- 586 Bennett, J., Meiyalaghan, S., Nguyen, H.M., Boldingh, H., Cooney, J., Elborough, C., Araujo,  
587 L.D., Barrell, P., Lin-Wang, K., Plunkett, B.J., 2023. Exogenous abscisic acid and sugar  
588 induce a cascade of ripening events associated with anthocyanin accumulation in  
589 cultured Pinot Noir grape berries. *Front. Plant Sci.* 14, 1324675.
- 590 Bigard, A., Romieu, C., Sire, Y., Veyret, M., Ojeda, H., Torregrosa, L., 2019. The kinetics of  
591 grape ripening revisited through berry density sorting. *OENO One* 16.
- 592 Bonada, M., Sadras, V., Moran, M., Fuentes, S., 2013. Elevated temperature and water  
593 stress accelerate mesocarp cell death and shrivelling, and decouple sensory traits in  
594 Shiraz berries. *Irrig. Sci.* 31, 1317–1331. <https://doi.org/10.1007/s00271-013-0407-z>
- 595 Cabral, I.L., Teixeira, A., Ferrier, M., Lanoue, A., Valente, J., Rogerson, F.S., Alves, F.,  
596 Carvalho, S.M.P., Gerós, H.V., Queiroz, J., 2023. Canopy management through crop  
597 forcing impacts grapevine cv. 'Touriga Nacional' performance, ripening and berry  
598 metabolomics profile. *OENO One* 57, 55–69. [https://doi.org/10.20870/oeno-](https://doi.org/10.20870/oeno-one.2023.57.1.7122)  
599 one.2023.57.1.7122

- 600 Castellarin, S.D., Di Gaspero, G., 2007. Transcriptional control of anthocyanin biosynthetic  
601 genes in extreme phenotypes for berry pigmentation of naturally occurring grapevines.  
602 *BMC Plant Biol.* 7, 46. <https://doi.org/10.1186/1471-2229-7-46>
- 603 Castellarin, S.D., Matthews, M.A., Di Gaspero, G., Gambetta, G.A., 2007a. Water deficits  
604 accelerate ripening and induce changes in gene expression regulating flavonoid  
605 biosynthesis in grape berries. *Planta* 227, 101–112.
- 606 Castellarin, S.D., Pfeiffer, A., Sivilotti, P., Degan, M., Peterlunger, E., Di Gaspero, G., 2007b.  
607 Transcriptional regulation of anthocyanin biosynthesis in ripening fruits of grapevine under  
608 seasonal water deficit. *Plant Cell Environ.* 30, 1381–1399.
- 609 Chaves, M.M., Zarrouk, O., Francisco, R., Costa, J.M., Santos, T., Regalado, A.P.,  
610 Rodrigues, M.L., Lopes, C.M., 2010. Grapevine under deficit irrigation: hints from  
611 physiological and molecular data. *Ann. Bot.* 105, 661–676.  
612 <https://doi.org/10.1093/aob/mcq030>
- 613 Chong, J., Poutaraud, A., Hugueney, P., 2009. Metabolism and roles of stilbenes in plants.  
614 *Plant Sci.* 177, 143–155. <https://doi.org/10.1016/j.plantsci.2009.05.012>
- 615 Coombe, B. g., 1995. Growth Stages of the Grapevine: Adoption of a system for identifying  
616 grapevine growth stages. *Aust. J. Grape Wine Res.* 1, 104–110.  
617 <https://doi.org/10.1111/j.1755-0238.1995.tb00086.x>
- 618 Coombe, B.G., 1992. Research on Development and Ripening of the Grape Berry. *Am. J.*  
619 *Enol. Vitic.* 43, 101–110. <https://doi.org/10.5344/ajev.1992.43.1.101>
- 620 Coombe, B.G., 1976. The Development of Fleshy Fruits. *Annu. Rev. Plant Physiol.* 27, 207–  
621 228. <https://doi.org/10.1146/annurev.pp.27.060176.001231>
- 622 Coombe, B.G., Hale, C.R., 1973. The Hormone Content of Ripening Grape Berries and the  
623 Effects of Growth Substance Treatments. *Plant Physiol.* 51, 629–634.  
624 <https://doi.org/10.1104/pp.51.4.629>
- 625 Coombe, B.G., McCarthy, M.G., 2000. Dynamics of grape berry growth and physiology of  
626 ripening. *Aust. J. Grape Wine Res.* 6, 131–135.
- 627 Cramer, G.R., Ghan, R., Schlauch, K.A., Tillett, R.L., Heymann, H., Ferrarini, A., Delledonne,  
628 M., Zenoni, S., Fasoli, M., Pezzotti, M., 2014. Transcriptomic analysis of the late stages of  
629 grapevine (*Vitis vinifera* cv. Cabernet Sauvignon) berry ripening reveals significant  
630 induction of ethylene signaling and flavor pathways in the skin. *BMC Plant Biol.* 14, 370.  
631 <https://doi.org/10.1186/s12870-014-0370-8>
- 632 Cutanda-Perez, M.-C., Ageorges, A., Gomez, C., Violet, S., Terrier, N., Romieu, C.,  
633 Torregrosa, L., 2009. Ectopic expression of *VlmybA1* in grapevine activates a narrow set  
634 of genes involved in anthocyanin synthesis and transport. *Plant Mol. Biol.* 69, 633–648.  
635 <https://doi.org/10.1007/s11103-008-9446-x>
- 636 Dai, Z.W., Léon, C., Feil, R., Lunn, J.E., Delrot, S., Gomès, E., 2013. Metabolic profiling  
637 reveals coordinated switches in primary carbohydrate metabolism in grape berry (*Vitis*

- 638 *vinifera* L.), a non-climacteric fleshy fruit. *J. Exp. Bot.* 64, 1345–1355.  
639 <https://doi.org/10.1093/jxb/ers396>
- 640 Davies, C., Robinson, S.P., Boss, P.K., 1997. Treatment of Grape Berries, a Nonclimacteric  
641 Fruit with a Synthetic Auxin, Retards Ripening and Alters the Expression of  
642 Developmentally Regulated Genes.
- 643 Daviet, B., Fournier, C., Cabrera-Bosquet, L., Simonneau, T., Cafier, M., Romieu, C., 2023.  
644 Ripening dynamics revisited: an automated method to track the development of  
645 asynchronous berries on time-lapse images. <https://doi.org/10.1101/2023.07.12.548662>
- 646 Del-Castillo-Alonso, M.-Á., Monforte, L., Tomás-Las-Heras, R., Ranieri, A., Castagna, A.,  
647 Martínez-Abaigar, J., Núñez-Olivera, E., 2021. Secondary metabolites and related genes  
648 in *Vitis vinifera* L. cv. Tempranillo grapes as influenced by ultraviolet radiation and berry  
649 development. *Physiol. Plant.* 173, 709–724. <https://doi.org/10.1111/ppl.13483>
- 650 Deluc, L.G., Decendit, A., Papastamoulis, Y., Mérillon, J.-M., Cushman, J.C., Cramer, G.R.,  
651 2011. Water Deficit Increases Stilbene Metabolism in Cabernet Sauvignon Berries. *J.*  
652 *Agric. Food Chem.* 59, 289–297. <https://doi.org/10.1021/jf1024888>
- 653 Deluc, L.G., Quilici, D.R., Decendit, A., Grimplet, J., Wheatley, M.D., Schlauch, K.A.,  
654 Mérillon, J.-M., Cushman, J.C., Cramer, G.R., 2009. Water deficit alters differentially  
655 metabolic pathways affecting important flavor and quality traits in grape berries of  
656 Cabernet Sauvignon and Chardonnay. *BMC Genomics* 10, 212.  
657 <https://doi.org/10.1186/1471-2164-10-212>
- 658 Dong, N.-Q., Lin, H.-X., 2021. Contribution of phenylpropanoid metabolism to plant  
659 development and plant–environment interactions. *J. Integr. Plant Biol.* 63, 180–209.  
660 <https://doi.org/10.1111/jipb.13054>
- 661 Duan, S., Wu, Y., Fu, R., Wang, L., Chen, Y., Xu, W., Zhang, C., Ma, C., Shi, J., Wang, S.,  
662 2019. Comparative Metabolic Profiling of Grape Skin Tissue along Grapevine Berry  
663 Developmental Stages Reveals Systematic Influences of Root Restriction on Skin  
664 Metabolome. *Int. J. Mol. Sci.* 20, 534. <https://doi.org/10.3390/ijms20030534>
- 665 Ebbels, T.M.D., van der Hooft, J.J.J., Chatelaine, H., Broeckling, C., Zamboni, N., Hassoun,  
666 S., Mathé, E.A., 2023. Recent advances in mass spectrometry-based computational  
667 metabolomics. *Curr. Opin. Chem. Biol.* 74, 102288.  
668 <https://doi.org/10.1016/j.cbpa.2023.102288>
- 669 Fasoli, M., Dal Santo, S., Zenoni, S., Tornielli, G.B., Farina, L., Zamboni, A., Porceddu, A.,  
670 Venturini, L., Bicego, M., Murino, V., Ferrarini, A., Delledonne, M., Pezzotti, M., 2012. The  
671 Grapevine Expression Atlas Reveals a Deep Transcriptome Shift Driving the Entire Plant  
672 into a Maturation Program. *Plant Cell* 24, 3489–3505.  
673 <https://doi.org/10.1105/tpc.112.100230>
- 674 Fasoli, M., Richter, C.L., Zenoni, S., Bertini, E., Vitulo, N., Dal Santo, S., Dokoozlian, N.,  
675 Pezzotti, M., Tornielli, G.B., 2018. Timing and Order of the Molecular Events Marking the  
676 Onset of Berry Ripening in Grapevine. *Plant Physiol.* 178, 1187–1206.  
677 <https://doi.org/10.1104/pp.18.00559>

- 678 Flamini, R., Mattivi, F., Rosso, M.D., Arapitsas, P., Bavaresco, L., 2013. Advanced  
679 Knowledge of Three Important Classes of Grape Phenolics: Anthocyanins, Stilbenes and  
680 Flavonols. *Int. J. Mol. Sci.* 14, 19651–19669. <https://doi.org/10.3390/ijms141019651>
- 681 Fortes, A.M., Teixeira, R.T., Agudelo-Romero, P., 2015. Complex Interplay of Hormonal  
682 Signals during Grape Berry Ripening. *Molecules* 20, 9326–9343.  
683 <https://doi.org/10.3390/molecules20059326>
- 684 Fujii, H., Zhu, J.-K., 2009. Arabidopsis mutant deficient in 3 abscisic acid-activated protein  
685 kinases reveals critical roles in growth, reproduction, and stress. *Proc. Natl. Acad. Sci.*  
686 106, 8380–8385. <https://doi.org/10.1073/pnas.0903144106>
- 687 Gillaspay, G., Ben-David, H., Gruissem, W., 1993. Fruits: A Developmental Perspective. *Plant*  
688 *Cell* 5, 1439–1451.
- 689 Giovannoni, J.J., 2004. Genetic Regulation of Fruit Development and Ripening. *Plant Cell*  
690 16, S170–S180. <https://doi.org/10.1105/tpc.019158>
- 691 Giribaldi, M., Gény, L., Delrot, S., Schubert, A., 2010. Proteomic analysis of the effects of  
692 ABA treatments on ripening *Vitis vinifera* berries. *J. Exp. Bot.* 61, 2447–2458.  
693 <https://doi.org/10.1093/jxb/erq079>
- 694 Goes da Silva, F., Iandolo, A., Al-Kayal, F., Bohlmann, M.C., Cushman, M.A., Lim, H.,  
695 Ergul, A., Figueroa, R., Kabuloglu, E.K., Osborne, C., Rowe, J., Tattersall, E., Leslie, A.,  
696 Xu, J., Baek, J., Cramer, G.R., Cushman, J.C., Cook, D.R., 2005. Characterizing the  
697 Grape Transcriptome. Analysis of Expressed Sequence Tags from Multiple *Vitis* Species  
698 and Development of a Compendium of Gene Expression during Berry Development.  
699 *Plant Physiol.* 139, 574–597. <https://doi.org/10.1104/pp.105.065748>
- 700 Govender, P., Sivakumar, V., 2020. Application of *k*-means and hierarchical clustering  
701 techniques for analysis of air pollution: A review (1980–2019). *Atmospheric Pollut. Res.*  
702 11, 40–56. <https://doi.org/10.1016/j.apr.2019.09.009>
- 703 Griesser, M., Savoi, S., Bondada, B., Forneck, A., Keller, M., 2024. Berry shrivel in  
704 grapevine: a review considering multiple approaches. *J. Exp. Bot.* 75, 2196–2213.  
705 <https://doi.org/10.1093/jxb/erae001>
- 706 Hebra, T., Elie, N., Poyer, S., Van Elslande, E., Touboul, D., Eparvier, V., 2021.  
707 Dereplication, Annotation, and Characterization of 74 Potential Antimicrobial Metabolites  
708 from *Penicillium Sclerotiorum* Using t-SNE Molecular Networks. *Metabolites* 11, 444.  
709 <https://doi.org/10.3390/metabo11070444>
- 710 Jia, H.-F., Chai, Y.-M., Li, C.-L., Lu, D., Luo, J.-J., Qin, L., Shen, Y.-Y., 2011. Abscisic Acid  
711 Plays an Important Role in the Regulation of Strawberry Fruit Ripening. *Plant Physiol.*  
712 157, 188–199. <https://doi.org/10.1104/pp.111.177311>
- 713 Jiang, Y., Joyce, D.C., 2003. ABA effects on ethylene production, PAL activity, anthocyanin  
714 and phenolic contents of strawberry fruit. *Plant Growth Regul.* 39, 171–174.  
715 <https://doi.org/10.1023/A:1022539901044>

- 716 Kennard, R.W., Stone, L.A., 1969. Computer Aided Design of Experiments. *Technometrics*  
717 11, 137–148. <https://doi.org/10.1080/00401706.1969.10490666>
- 718 Kennedy, J.A., Hayasaka, Y., Vidal, S., Waters, E.J., Jones, G.P., 2001. Composition of  
719 Grape Skin Proanthocyanidins at Different Stages of Berry Development. *J. Agric. Food*  
720 *Chem.* 49, 5348–5355. <https://doi.org/10.1021/jf010758h>
- 721 Kennedy, J.A., Troup, G.J., Pilbrow, J.R., Hutton, D.R., Hewitt, D., Hunter, C.R., Ristic, R.,  
722 Iland, P.G., Jones, G.P., 2000. Development of seed polyphenols in berries from *Vitis*  
723 *vinifera* L. cv. Shiraz. *Aust. J. Grape Wine Res.* 6, 244–254.  
724 <https://doi.org/10.1111/j.1755-0238.2000.tb00185.x>
- 725 Kuhn, N., Guan, L., Dai, Z.W., Wu, B.-H., Lauvergeat, V., Gomès, E., Li, S.-H., Godoy, F.,  
726 Arce-Johnson, P., Delrot, S., 2014. Berry ripening: recently heard through the grapevine.  
727 *J. Exp. Bot.* 65, 4543–4559. <https://doi.org/10.1093/jxb/ert395>
- 728 Lacampagne, S., Gagné, S., Gény, L., 2010. Involvement of Abscisic Acid in Controlling the  
729 Proanthocyanidin Biosynthesis Pathway in Grape Skin: New Elements Regarding the  
730 Regulation of Tannin Composition and Leucoanthocyanidin Reductase (LAR) and  
731 Anthocyanidin Reductase (ANR) Activities and Expression. *J. Plant Growth Regul.* 29,  
732 81–90. <https://doi.org/10.1007/s00344-009-9115-6>
- 733 Leng, F., Duan, S., Song, S., Zhao, L., Xu, W., Zhang, C., Ma, C., Wang, L., Wang, S., 2021.  
734 Comparative Metabolic Profiling of Grape Pulp during the Growth Process Reveals  
735 Systematic Influences under Root Restriction. *Metabolites* 11, 377.  
736 <https://doi.org/10.3390/metabo11060377>
- 737 Li, S., Chen, K., Grierson, D., 2021. Molecular and Hormonal Mechanisms Regulating  
738 Fleshy Fruit Ripening. *Cells* 10, 1136. <https://doi.org/10.3390/cells10051136>
- 739 Long, Y., Tyerman, S.D., Gilliam, M., 2020. Cytosolic GABA inhibits anion transport by  
740 wheat ALMT1. *New Phytol.* 225, 671–678. <https://doi.org/10.1111/nph.16238>
- 741 Lorenz, D. h., Eichhorn, K. w., Bleiholder, H., Klose, R., Meier, U., Weber, E., 1995. Growth  
742 Stages of the Grapevine: Phenological growth stages of the grapevine (*Vitis vinifera* L.  
743 *ssp. vinifera*)—Codes and descriptions according to the extended BBCH scale†. *Aust. J.*  
744 *Grape Wine Res.* 1, 100–103. <https://doi.org/10.1111/j.1755-0238.1995.tb00085.x>
- 745 Lund, S.T., Bohlmann, J., 2006. The Molecular Basis for Wine Grape Quality-A Volatile  
746 Subject. *Science* 311, 804–805. <https://doi.org/10.1126/science.1118962>
- 747 Massonnet, M., Fasoli, M., Tornielli, G.B., Altieri, M., Sandri, M., Zuccolotto, P., Paci, P.,  
748 Gardiman, M., Zenoni, S., Pezzotti, M., 2017. Ripening Transcriptomic Program in Red  
749 and White Grapevine Varieties Correlates with Berry Skin Anthocyanin Accumulation.  
750 *Plant Physiol.* 174, 2376–2396. <https://doi.org/10.1104/pp.17.00311>
- 751 Matthews, M., Cheng, G., Weinbaum, S., 1987. Changes in water potential and dermal  
752 extensibility during grape berry development. *J. Am. Soc. Hortic. Sci.* 112, 314–319.

- 753 N. Clifford, M., B. Jaganath, I., A. Ludwig, I., Crozier, A., 2017. Chlorogenic acids and the  
754 acyl-quinic acids: discovery, biosynthesis, bioavailability and bioactivity. *Nat. Prod. Rep.*  
755 34, 1391–1421. <https://doi.org/10.1039/C7NP00030H>
- 756 Nicolas, S., Bois, B., Billet, K., Romanet, R., Bahut, F., Uhl, J., Schmitt-Kopplin, P.,  
757 Gougeon, R.D., 2024. High-Resolution Mass Spectrometry-Based Metabolomics for  
758 Increased Grape Juice Metabolite Coverage. *Foods* 13, 54.  
759 <https://doi.org/10.3390/foods13010054>
- 760 Ojeda, H., Deloire, A., Carbonneau, A., Ageorges, A., Romieu, C., 1999. Berry development  
761 of grapevines: relations between the growth of berries and their DNA content indicate cell  
762 multiplication and enlargement. *VITIS-J. Grapevine Res.* 38, 145.
- 763 Olivon, F., Elie, N., Grelier, G., Roussi, F., Litaudon, M., Touboul, D., 2018. MetGem  
764 Software for the Generation of Molecular Networks Based on the t-SNE Algorithm. *Anal.*  
765 *Chem.* 90, 13900–13908. <https://doi.org/10.1021/acs.analchem.8b03099>
- 766 Ollé, D., Guiraud, J. I., Souquet, J. m., Terrier, N., Ageorges, A., Cheynier, V., Verries, C.,  
767 2011. Effect of pre- and post-veraison water deficit on proanthocyanidin and anthocyanin  
768 accumulation during Shiraz berry development. *Aust. J. Grape Wine Res.* 17, 90–100.  
769 <https://doi.org/10.1111/j.1755-0238.2010.00121.x>
- 770 Pan, Q.-H., Li, M.-J., Peng, C.-C., Zhang, N., Zou, X., Zou, K.-Q., Wang, X.-L., Yu, X.-C.,  
771 Wang, X.-F., Zhang, D.-P., 2005. Abscisic acid activates acid invertases in developing  
772 grape berry. *Physiol. Plant.* 125, 157–170. <https://doi.org/10.1111/j.1399-3054.2005.00552.x>
- 774 Petrusa, E., Braidot, E., Zancani, M., Peresson, C., Bertolini, A., Patui, S., Vianello, A.,  
775 2013. Plant Flavonoids—Biosynthesis, Transport and Involvement in Stress Responses.  
776 *Int. J. Mol. Sci.* 14, 14950–14973. <https://doi.org/10.3390/ijms140714950>
- 777 Pilati, S., Bagagli, G., Sonogo, P., Moretto, M., Brazzale, D., Castorina, G., Simoni, L.,  
778 Tonelli, C., Guella, G., Engelen, K., Galbiati, M., Moser, C., 2017. Abscisic Acid Is a Major  
779 Regulator of Grape Berry Ripening Onset: New Insights into ABA Signaling Network.  
780 *Front. Plant Sci.* 8. <https://doi.org/10.3389/fpls.2017.01093>
- 781 Pilati, S., Brazzale, D., Guella, G., Milli, A., Ruberti, C., Biasioli, F., Zottini, M., Moser, C.,  
782 2014. The onset of grapevine berry ripening is characterized by ROS accumulation and  
783 lipoxygenase-mediated membrane peroxidation in the skin. *BMC Plant Biol.* 14, 87.  
784 <https://doi.org/10.1186/1471-2229-14-87>
- 785 Polanski, A., Marczyk, M., Pietrowska, M., Widlak, P., Polanska, J., 2015. Signal Partitioning  
786 Algorithm for Highly Efficient Gaussian Mixture Modeling in Mass Spectrometry. *PLOS*  
787 *ONE* 10, e0134256. <https://doi.org/10.1371/journal.pone.0134256>
- 788 Rienth, M., Vigneron, N., Darriet, P., Sweetman, C., Burbidge, C., Bonghi, C., Walker, R.P.,  
789 Famiani, F., Castellarin, S.D., 2021. Grape Berry Secondary Metabolites and Their  
790 Modulation by Abiotic Factors in a Climate Change Scenario—A Review. *Front. Plant Sci.*  
791 12. <https://doi.org/10.3389/fpls.2021.643258>

- 792 Rodrigues, M., Forestan, C., Ravazzolo, L., Huguene, P., Baltenweck, R., Rasori, A.,  
793 Cardillo, V., Carraro, P., Malagoli, M., Brizzolara, S., Quaggiotti, S., Porro, D., Meggio, F.,  
794 Bonghi, C., Battista, F., Ruperti, B., 2023. Metabolic and Molecular Rearrangements of  
795 Sauvignon Blanc (*Vitis vinifera* L.) Berries in Response to Foliar Applications of Specific  
796 Dry Yeast. *Plants* 12, 3423. <https://doi.org/10.3390/plants12193423>
- 797 Rogiers, S.Y., Coetzee, Z.A., Walker, R.R., Deloire, A., Tyerman, S.D., 2017. Potassium in  
798 the Grape (*Vitis vinifera* L.) Berry: Transport and Function. *Front. Plant Sci.* 8.  
799 <https://doi.org/10.3389/fpls.2017.01629>
- 800 Rousseeuw, P.J., 1987. Silhouettes: A graphical aid to the interpretation and validation of  
801 cluster analysis. *J. Comput. Appl. Math.* 20, 53–65. [https://doi.org/10.1016/0377-0427\(87\)90125-7](https://doi.org/10.1016/0377-0427(87)90125-7)
- 803 Savoi, S., Torregrosa, L., Romieu, C., 2023. Single berry development – a new phenotyping  
804 and transcriptomics paradigm. *VITIS - J. Grapevine Res.* 62, 49–55.  
805 <https://doi.org/10.5073/vitis.2023.62.special-issue.49-55>
- 806 Savoi, S., Torregrosa, L., Romieu, C., 2021. Transcripts switched off at the stop of phloem  
807 unloading highlight the energy efficiency of sugar import in the ripening *V. vinifera* fruit.  
808 <https://doi.org/10.1101/2021.01.19.427234>
- 809 Schwab, W., Davidovich-Rikanati, R., Lewinsohn, E., 2008. Biosynthesis of plant-derived  
810 flavor compounds. *Plant J.* 54, 712–732. <https://doi.org/10.1111/j.1365-313X.2008.03446.x>
- 812 Schymanski, E.L., Jeon, J., Gulde, R., Fenner, K., Ruff, M., Singer, H.P., Hollender, J., 2014.  
813 Identifying Small Molecules via High Resolution Mass Spectrometry: Communicating  
814 Confidence. *Environ. Sci. Technol.* 48, 2097–2098. <https://doi.org/10.1021/es5002105>
- 815 Seymour, G.B., Østergaard, L., Chapman, N.H., Knapp, S., Martin, C., 2013. Fruit  
816 Development and Ripening. *Annu. Rev. Plant Biol.* 64, 219–241.  
817 <https://doi.org/10.1146/annurev-arplant-050312-120057>
- 818 Shahood, R., Torregrosa, L., Savoi, S., Romieu, C., 2020. First quantitative assessment of  
819 growth, sugar accumulation and malate breakdown in a single ripening berry. *Oeno One*  
820 54, 1077–1092.
- 821 Singh, P., Arif, Y., Miszczuk, E., Bajguz, A., Hayat, S., 2022. Specific Roles of  
822 Lipoxygenases in Development and Responses to Stress in Plants. *Plants* 11, 979.  
823 <https://doi.org/10.3390/plants11070979>
- 824 Sorkun, M.C., Mullaj, D., Koelman, J.V.A., Er, S., 2022. ChemPlot, a Python library for  
825 chemical space visualization.
- 826 Stines, A.P., Naylor, D.J., Høj, P.B., van Heeswijck, R., 1999. Proline Accumulation in  
827 Developing Grapevine Fruit Occurs Independently of Changes in the Levels of  $\Delta$ 1-  
828 Pyrroline-5-Carboxylate Synthetase mRNA or Protein1. *Plant Physiol.* 120, 923.  
829 <https://doi.org/10.1104/pp.120.3.923>

- 830 Sun, L., Zhang, M., Ren, J., Qi, J., Zhang, G., Leng, P., 2010. Reciprocity between abscisic  
831 acid and ethylene at the onset of berry ripening and after harvest. *BMC Plant Biol.* 10,  
832 257. <https://doi.org/10.1186/1471-2229-10-257>
- 833 Tahara, K., Nishiguchi, M., Funke, E., Miyazawa, S.-I., Miyama, T., Milkowski, C., 2020.  
834 Dehydroquinate dehydratase/shikimate dehydrogenases involved in gallate biosynthesis  
835 of the aluminum-tolerant tree species *Eucalyptus camaldulensis*. *Planta* 253, 3.  
836 <https://doi.org/10.1007/s00425-020-03516-w>
- 837 Tang YuHan, T.Y., Jiang Yao, J.Y., Meng JiaSong, M.J., Tao Jun, T.J., 2018. A brief review  
838 of physiological roles, plant resources, synthesis, purification and oxidative stability of  
839 alpha-linolenic acid.
- 840 Terrier, N., Romieu, C., 2001. Grape Berry Acidity, in: Roubelakis-Angelakis, K.A. (Ed.),  
841 *Molecular Biology & Biotechnology of the Grapevine*. Springer Netherlands, Dordrecht,  
842 pp. 35–57. [https://doi.org/10.1007/978-94-017-2308-4\\_2](https://doi.org/10.1007/978-94-017-2308-4_2)
- 843 Tesnière, C., Romieu, C., Dugelay, I., Nicol, M.Z., Flanzy, C., Robin, J.P., 1994. Partial  
844 recovery of grape energy metabolism upon aeration following anaerobic stress. *J. Exp.*  
845 *Bot.* 45, 145–151. <https://doi.org/10.1093/jxb/45.1.145>
- 846 Thomas, T.R., Shackel, K.A., Matthews, M.A., 2008. Mesocarp cell turgor in *Vitis vinifera* L.  
847 berries throughout development and its relation to firmness, growth, and the onset of  
848 ripening. *Planta* 228, 1067–1076. <https://doi.org/10.1007/s00425-008-0808-z>
- 849 Torielli, G.B., Sandri, M., Fasoli, M., Amato, A., Pezzotti, M., Zuccolotto, P., Zenoni, S.,  
850 2023. A molecular phenology scale of grape berry development. *Hortic. Res.* 10,  
851 uhad048. <https://doi.org/10.1093/hr/uhad048>
- 852 Vannozzi, A., Dry, I.B., Fasoli, M., Zenoni, S., Lucchin, M., 2012. Genome-wide analysis of  
853 the grapevine stilbene synthase multigenic family: genomic organization and expression  
854 profiles upon biotic and abiotic stresses. *BMC Plant Biol.* 12, 130.  
855 <https://doi.org/10.1186/1471-2229-12-130>
- 856 Villalobos-González, L., Peña-Neira, A., Ibáñez, F., Pastenes, C., 2016. Long-term effects of  
857 abscisic acid (ABA) on the grape berry phenylpropanoid pathway: Gene expression and  
858 metabolite content. *Plant Physiol. Biochem.* 105, 213–223.  
859 <https://doi.org/10.1016/j.plaphy.2016.04.012>
- 860 Wheeler, S., Loveys, B., Ford, C., Davies, C., 2009. The relationship between the  
861 expression of abscisic acid biosynthesis genes, accumulation of abscisic acid and the  
862 promotion of *Vitis vinifera* L. berry ripening by abscisic acid. *Aust. J. Grape Wine Res.* 15,  
863 195–204. <https://doi.org/10.1111/j.1755-0238.2008.00045.x>
- 864 Xiao, Z., Rogiers, S.Y., Sadras, V.O., Tyerman, S.D., 2018. Hypoxia in grape berries: the  
865 role of seed respiration and lenticels on the berry pedicel and the possible link to cell  
866 death. *J. Exp. Bot.* 69, 2071–2083. <https://doi.org/10.1093/jxb/ery039>
- 867 Xu, Z.-Y., Lee, K.H., Dong, T., Jeong, J.C., Jin, J.B., Kanno, Y., Kim, D.H., Kim, S.Y., Seo,  
868 M., Bressan, R.A., Yun, D.-J., Hwang, I., 2012. A Vacuolar  $\beta$ -Glucosidase Homolog That  
869 Possesses Glucose-Conjugated Abscisic Acid Hydrolyzing Activity Plays an Important

870 Role in Osmotic Stress Responses in Arabidopsis. *Plant Cell* 24, 2184–2199.  
871 <https://doi.org/10.1105/tpc.112.095935>

872 Yang, B., He, S., Liu, Y., Liu, B., Ju, Y., Kang, D., Sun, X., Fang, Y., 2020. Transcriptomics  
873 integrated with metabolomics reveals the effect of regulated deficit irrigation on  
874 anthocyanin biosynthesis in Cabernet Sauvignon grape berries. *Food Chem.* 314,  
875 126170. <https://doi.org/10.1016/j.foodchem.2020.126170>

876 Yu, K., Song, Y., Lin, J., Dixon, R.A., 2023. The complexities of proanthocyanidin  
877 biosynthesis and its regulation in plants. *Plant Commun.* 4, 100498.  
878 <https://doi.org/10.1016/j.xplc.2022.100498>

879 Zamboni, A., Di Carli, M., Guzzo, F., Stocchero, M., Zenoni, S., Ferrarini, A., Tononi, P.,  
880 Toffali, K., Desiderio, A., Lilley, K.S., Pè, M.E., Benvenuto, E., Delledonne, M., Pezzotti,  
881 M., 2010. Identification of Putative Stage-Specific Grapevine Berry Biomarkers and Omics  
882 Data Integration into Networks. *Plant Physiol.* 154, 1439–1459.  
883 <https://doi.org/10.1104/pp.110.160275>

884 Zeevaart, J.A.D., 1999. Chapter 8 - Abscisic acid metabolism and its regulation, in:  
885 Hooykaas, P.J.J., Hall, M.A., Libbenga, K.R. (Eds.), *New Comprehensive Biochemistry,*  
886 *Biochemistry and Molecular Biology of Plant Hormones.* Elsevier, pp. 189–207.  
887 [https://doi.org/10.1016/S0167-7306\(08\)60488-3](https://doi.org/10.1016/S0167-7306(08)60488-3)

## 888 **Tables and Figures**

### 889 **Figures**

890 **Figure 1. Metabolomic data analysis workflow.** The 11 expert stages were defined before  
891 our study by Savoi et al. (2023). The workflow starts with raw metabolomic data, each of the  
892 9,256 detected ions surfaces is initially scaled by its sum over berry development. The  
893 normalized data underwent a classification of the samples by the K-means algorithm, in  
894 order to identify new metabolomic stages independently from expert stages and observer  
895 time. In parallel, the normalized data were submitted to a principal component analysis  
896 (PCA). Single berry molecular clock was calculated from the curvilinear distance of samples  
897 projected on successive linear regressions, selecting the best principal component (PC)  
898 plane for each one. These newly identified stages and clock were then compared to the  
899 expert stages. 653 invariant ions were then filtered out using a Kruskal-Wallis test with a  
900 false discovery rate of 0.01. The 8,603 remaining ions were then grouped together using K-  
901 means on the metabolites, followed by a PCA for each metabolite cluster, enabling the  
902 representative dynamics (or “eigen-metabolite”) to be shown. Finally, a t-distributed  
903 stochastic neighbor embedding (t-SNE) representation allowed more direct insights on the  
904 data structure.

905 **Figure 2. Comparative analysis of berry developmental stages: expert classification**  
906 **single berry metabolic clock and days after flowering (DAF), color-coded by expert**  
907 **stages.** (a) 3D principal component analysis (PCA) plot displaying berries colored according  
908 to expert-defined phenological stages (G = green, L = lag, S = softening, R = ripening, Sh =  
909 shriveling). Each color represents a different stage, illustrating the separation and distribution  
910 of berries within the multi-dimensional space defined by the first three principal components  
911 (PC) of the dataset. (b) Scatter plot showing a side-by-side comparison of the metabolomic  
912 clock (y-axis) against the expert-defined stages (x-axis). Data points are color-coded by the  
913 expert stages. The metabolomic clock was determined by calculating the distances between  
914 samples across successive linear regressions of PCA-projected samples trajectories in **a**,  
915 providing a continuous scale that contrasts with the discrete categorization of the expert  
916 stages. (c) Comparison of the metabolomic clock (x-axis) and days after flowering (DAF) (y-  
917 axis) and colored by expert stages.

918 **Figure 3. Representative profiles of metabolite clusters decreasing throughout**  
919 **grapevine berry development.** n indicates the number of metabolites within each cluster.  
920 Each point is a single berry. The y axis represents the first principal component (PC1) from  
921 the cluster principal component analysis (PCA) and the x axis is the metabolomic clock. For  
922 expert stages description see Table 1.

923 **Figure 4. Representative profiles of metabolites that accumulate as a peak at specific**  
924 **stages of the berry development.** n indicates the number of metabolites within each  
925 cluster. Each point is a single berry. The y axis represents the first principal component  
926 (PC1) from the cluster principal component analysis (PCA) and the x axis is the metabolomic  
927 clock. For expert stages description see Table 1.

928 **Figure 5. Clusters representative profiles of metabolites showing an accumulation**  
929 **trend during the ripening phase.** n value indicates the number of metabolites within each  
930 cluster. Each point is a single berry. The y axis represents the first principal component  
931 (PC1) from the cluster principal component analysis (PCA) and the x axis is the metabolomic  
932 clock. For expert stages description see Table 1.

933 **Figure 6. 3D t-distributed stochastic neighbor embedding (t-SNE) representation of**  
934 **the untargeted metabolites.** Each point represents a metabolite, colored by its K-means  
935 cluster with its representative pattern over the metabolomic clock (see details in Fig. 2, 3 and  
936 4). Patterns A through D include metabolites consumed in the early green stage. Between E  
937 and I, there are noticeable peaks in metabolite accumulation, signaling the stepwise  
938 transition from green phase to ripening. Finally, patterns J to L depict the accumulation of

939 metabolites toward the end of the berries' development. In the 3D t-SNE visualization, these  
 940 three cluster groups appear to be organized into three distinct branches.

941 **Figure 7. Biosynthesis pathways of the identified metabolites and their accumulation**  
 942 **profile through the grapevine fruit development.** Each point represents a metabolite,  
 943 colored by its K-means cluster with its representative pattern over the metabolomic clock  
 944 (see details in Fig. **S5**). Metabolites in gray correspond to molecular standards; most of  
 945 these molecules have a correlation with the eigen-metabolite of their cluster of less than 0.8  
 946 (see dynamics in Fig. **S6**). Chalcone, epicatechin, gallocatechin, and epigallocatechin have  
 947 been identified in their derivative, fragment, or polymerized forms. Due to the multiple  
 948 profiles associated with each fragment/derivative/polymer, we have chosen not to display  
 949 them in this figure (see details in Fig. **S5**). (KEGG Pathway database  
 950 (<https://www.genome.jp/kegg/pathway.html>), Ali et al., 2010; Castellarin et al., 2007b; Dong  
 951 and Lin, 2021; Clifford et al., 2017; Petrusa et al., 2013; Tang YuHan et al., 2018; Yang et  
 952 al., 2020).

953

#### 954 **Tables**

955 **Table 1. Repartition of the 125 samples analyzed by UHPLC.**

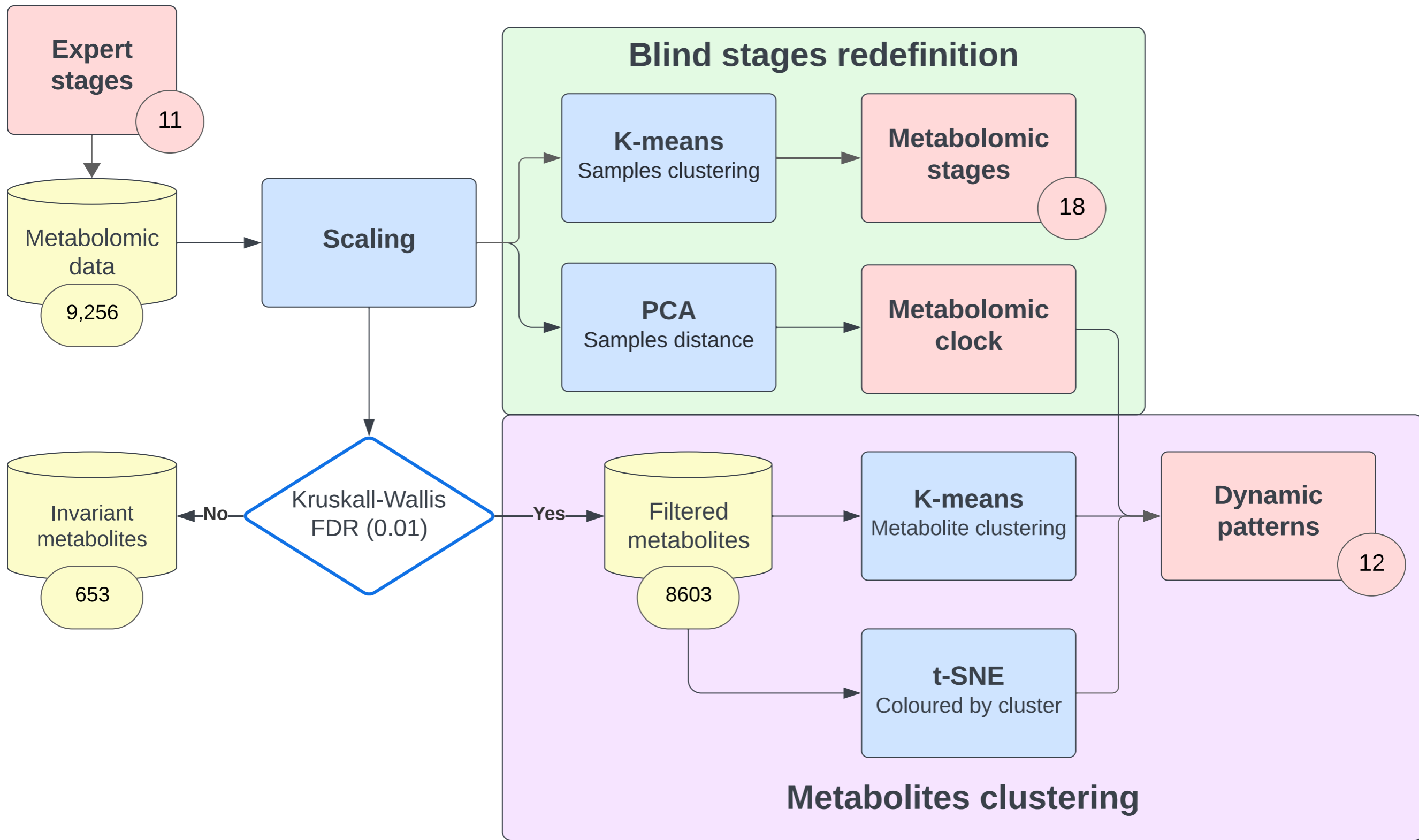
	Green					Ripening					Over-ripening
	Growth			Lag		Softening		Colouring			Shrivelling
<b>Expert stages</b>	G1	G2	G3	L4	L5	S6	S7	R8	R9	R10	Sh11
<b>Year of sampling</b>	2019	2019	2019	2019	2018	2018	2018	2018	2018	2018	2018
<b>Harvested samples</b>	4	26	28	19	8	9	9	11	14	12	13
<b>Sample selected</b>	4	14	14	17	8	9	9	11	14	12	13

956

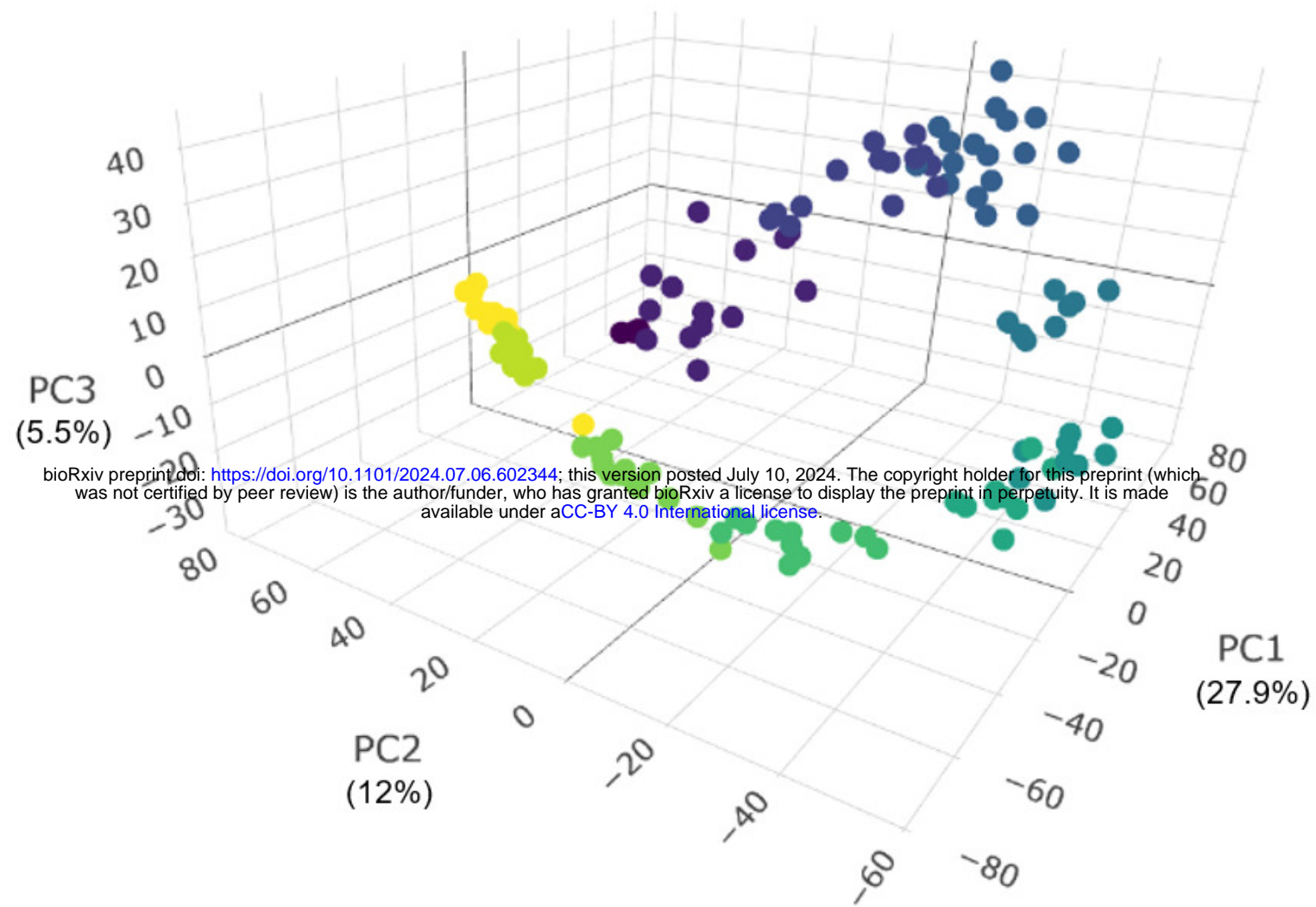
957 **Table 2. Relationship between expert stages and K-means determined metabolomic**  
 958 **stages.** Each cluster represents a stage where berries exhibit similar metabolomic  
 959 characteristics, G = green phase, L = lag phase, S = softening, R = ripening and Sh =  
 960 shriveling.

<b>Expert stages</b>	G1	G2	G3	L4	L5	S6	S7	R8	R9	R10	Sh11
<b>K-means stages</b>	1	1,2,3 ,4	4,5	5,6,7, 8,9	10	11,12 ,13	12,13	14,15	15	16,17, 18	15,17, 18

961



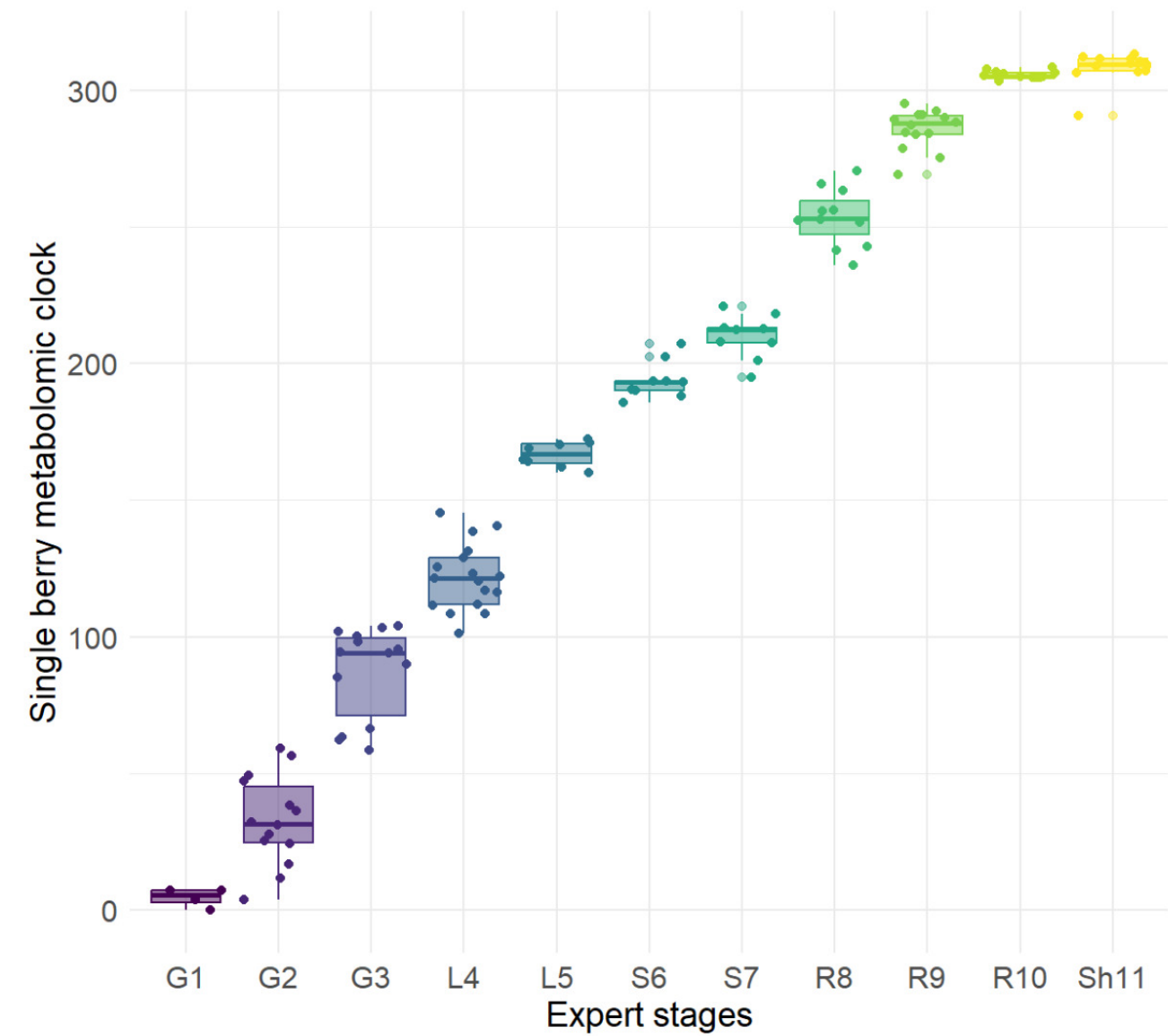
**(a)** PCA representation colored by expert stages



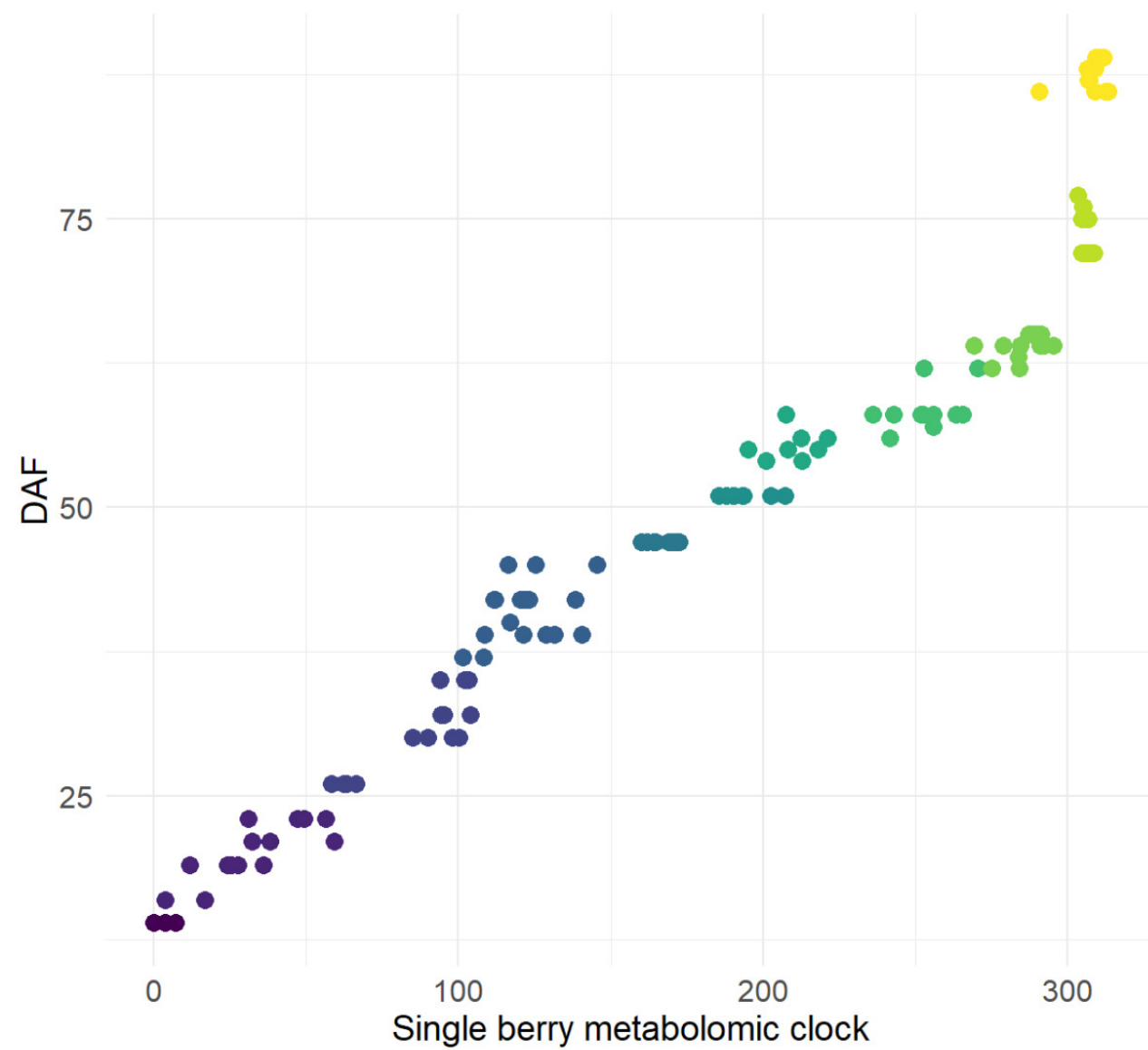
**(b)** Comparison between single berry metabolomic clock and expert stages

Expert stages

- G1
- G2
- G3
- L4
- L5
- S6
- S7
- R8
- R9
- R10
- Sh11

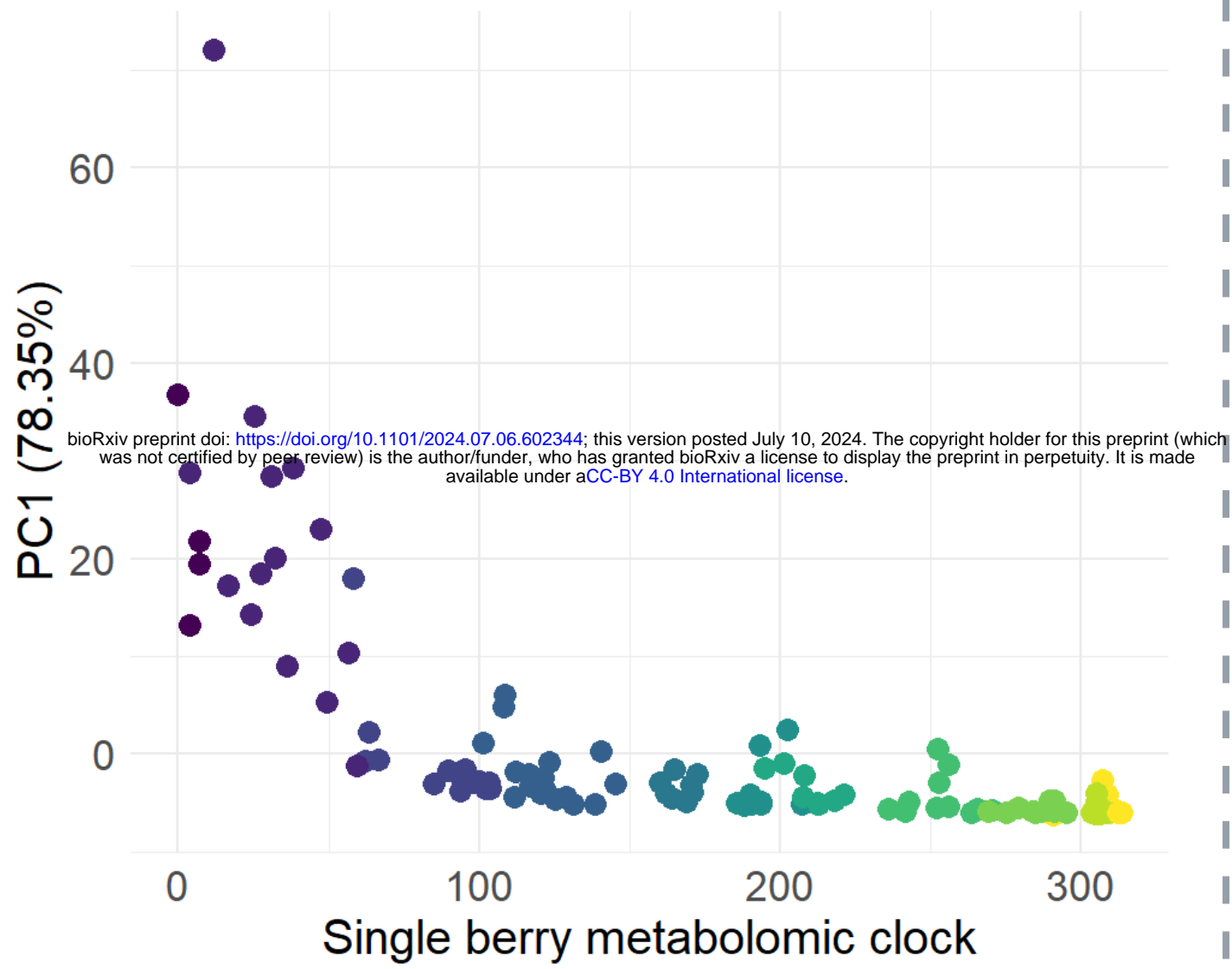


**(c)** Comparison between single berry metabolomic clock and DAF



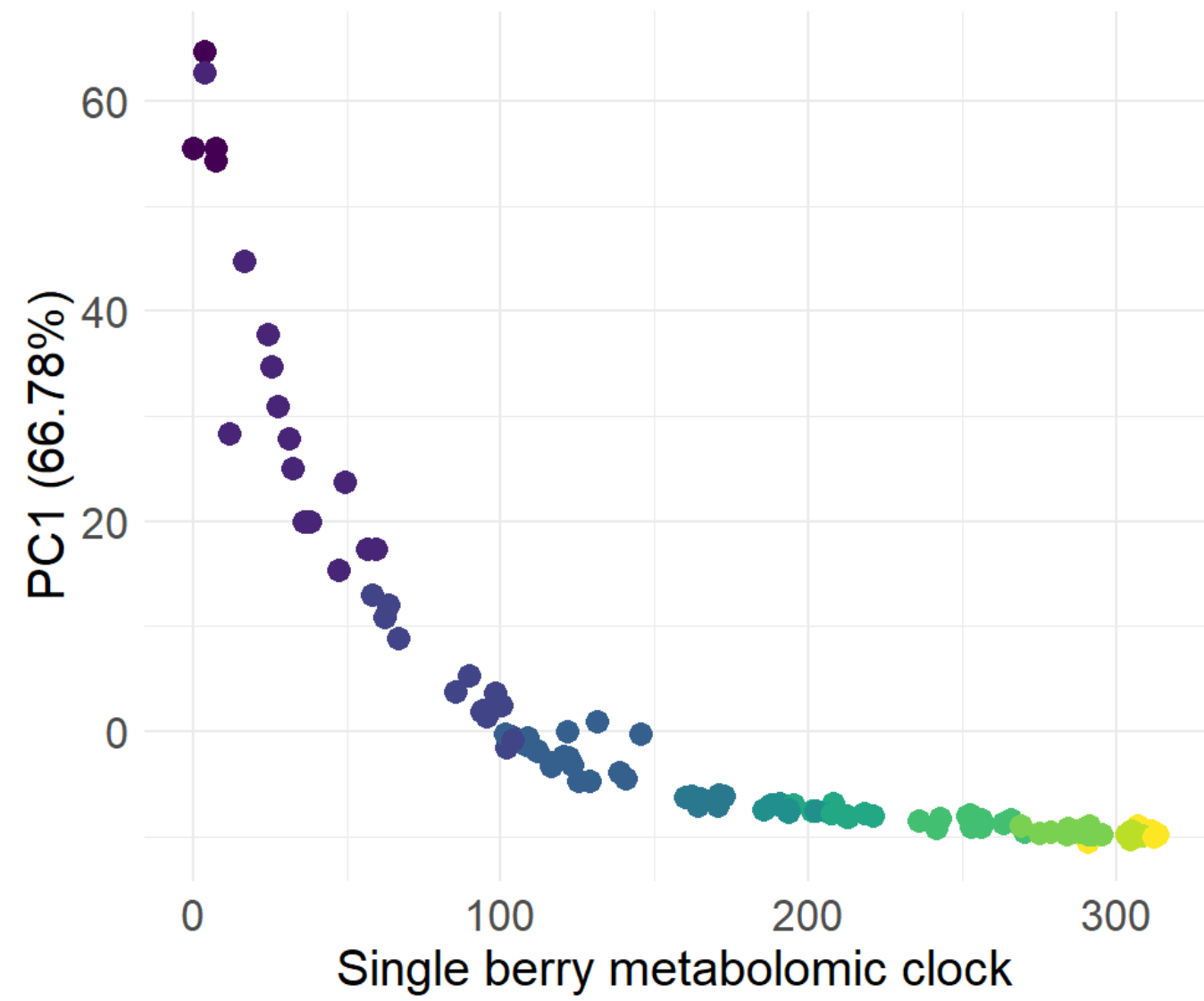
### Profile A

n = 162



### Profile B

n = 405

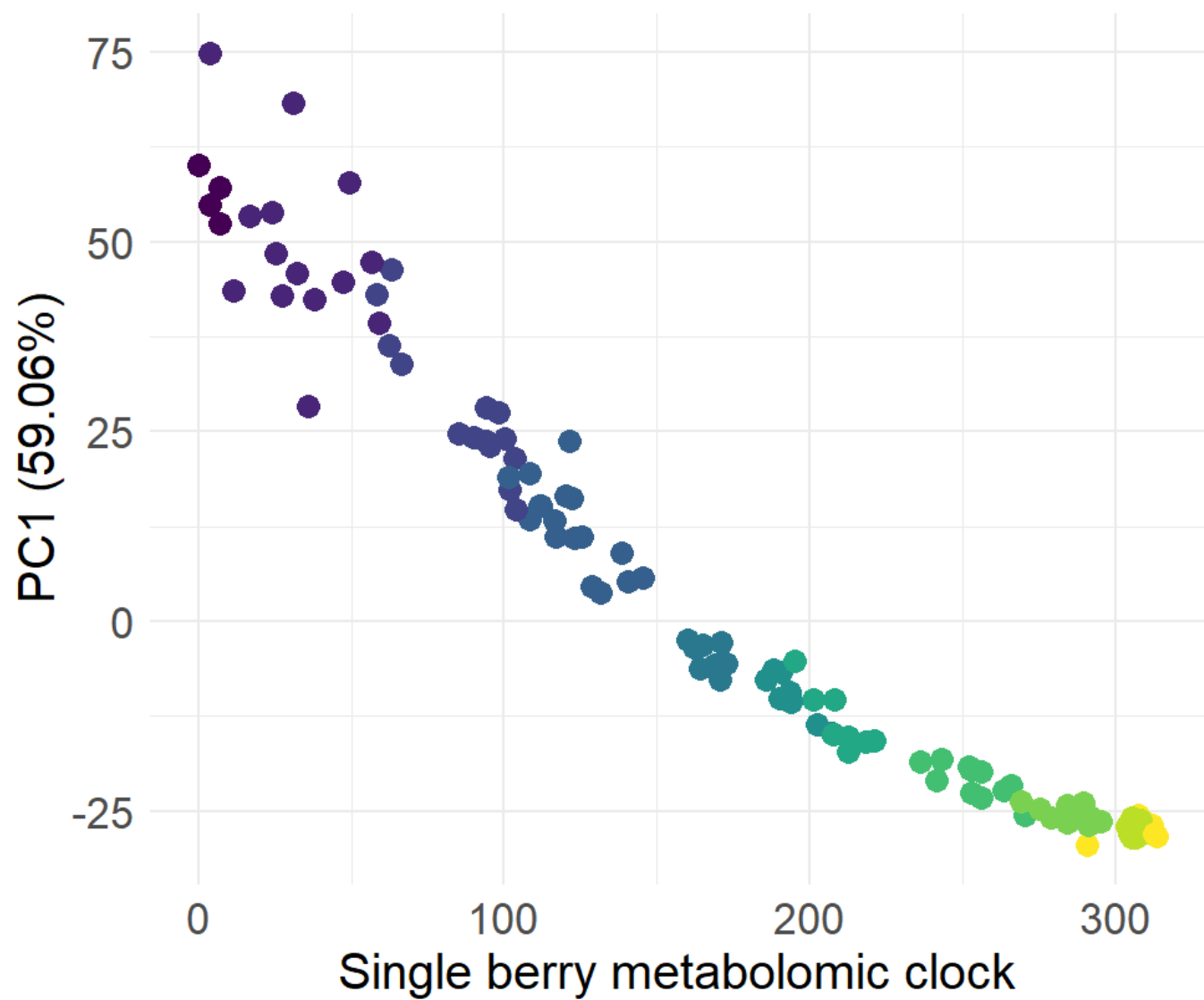


Expert stages

- G1
- G2
- G3
- L4
- L5
- S6
- S7
- R8
- R9
- R10
- Sh11

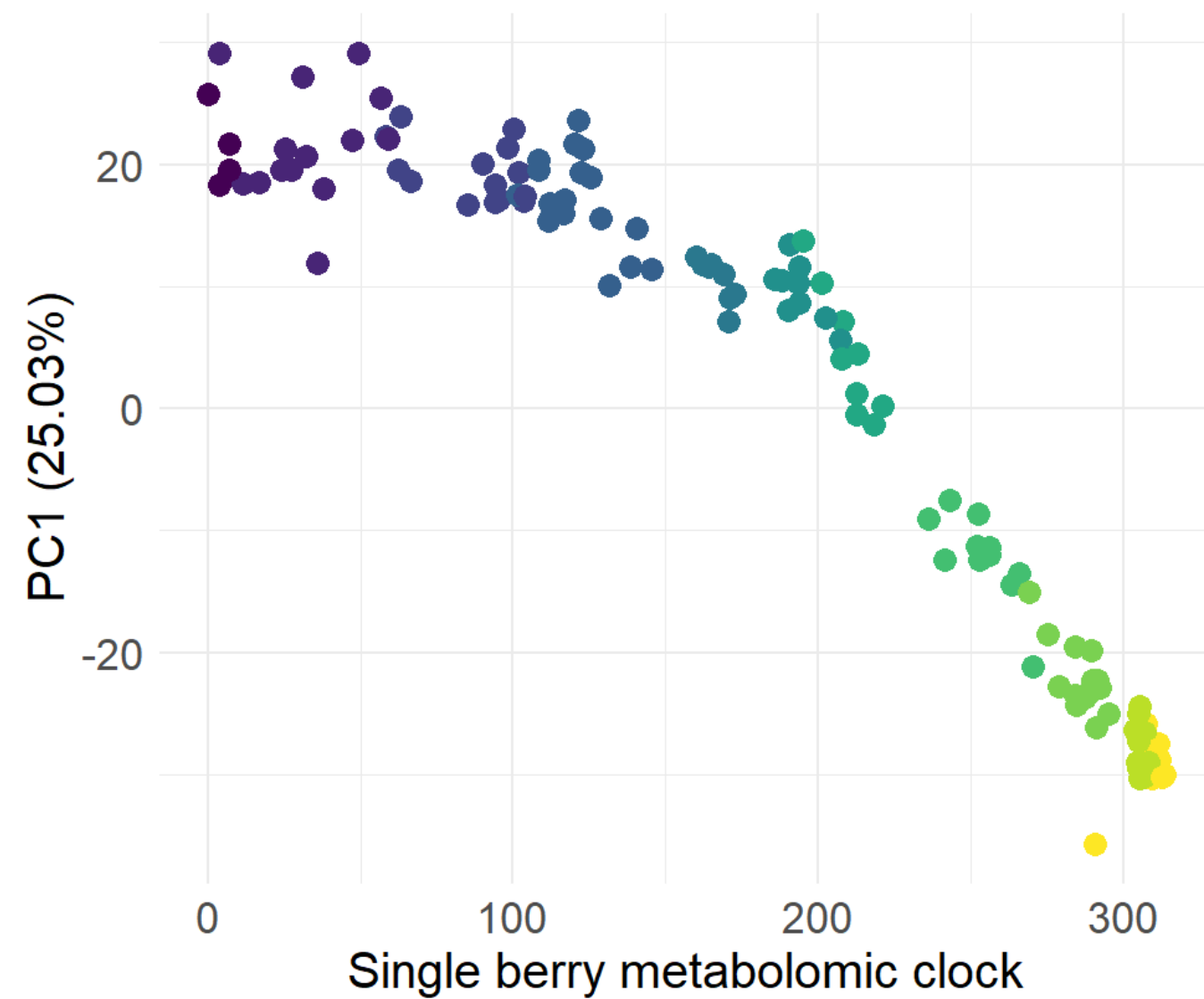
### Profile C

n = 1344

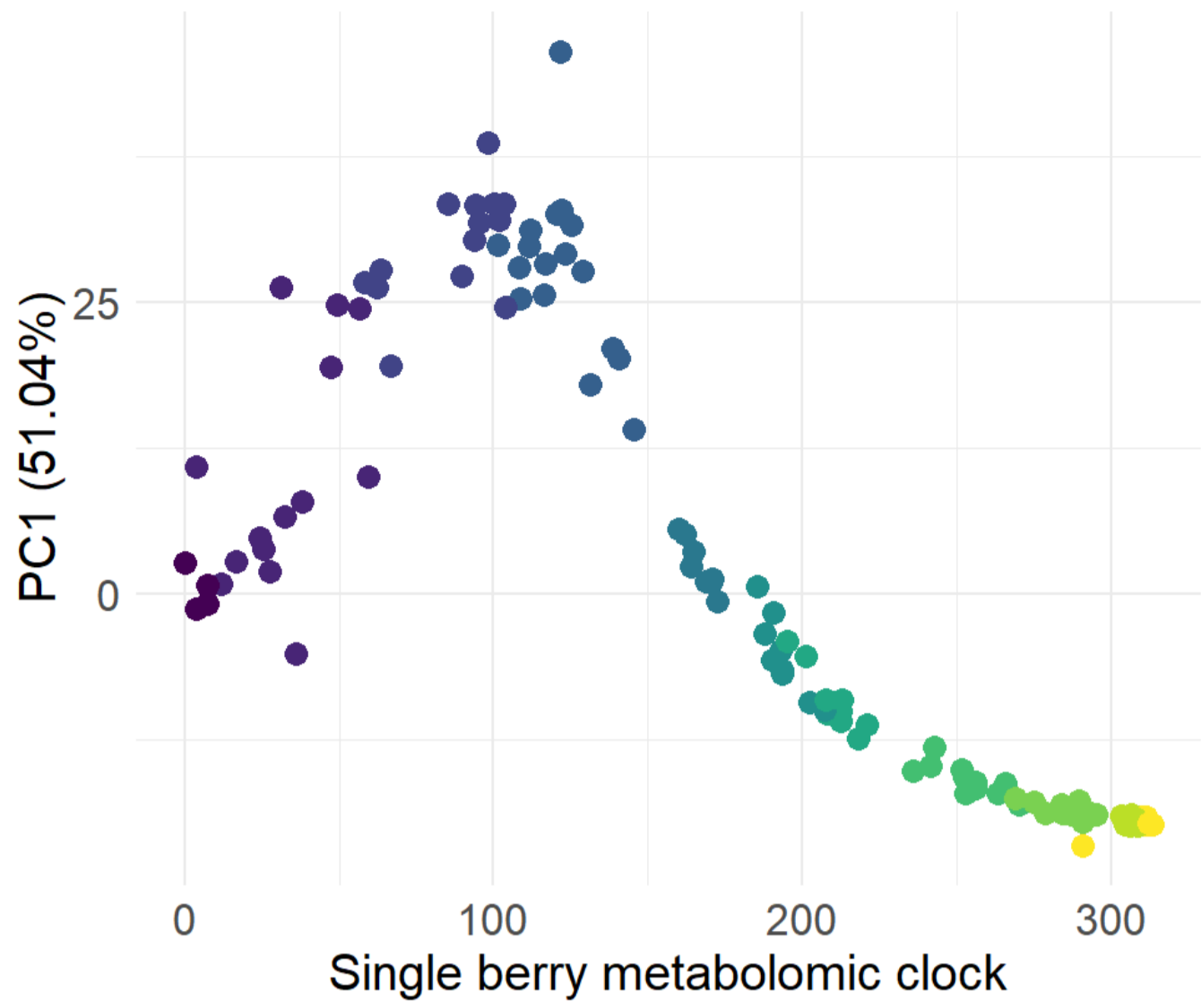


### Profile D

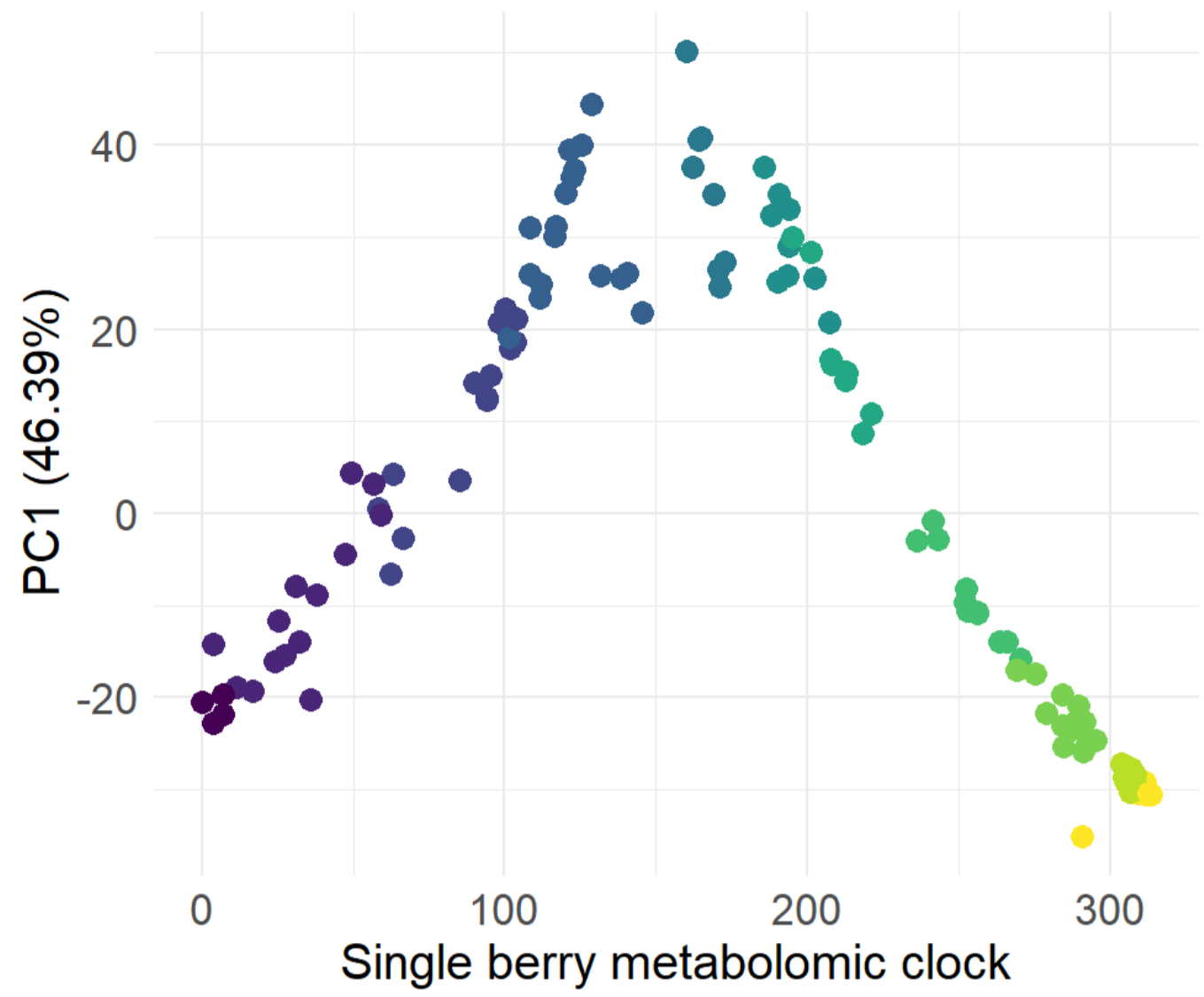
n = 1626



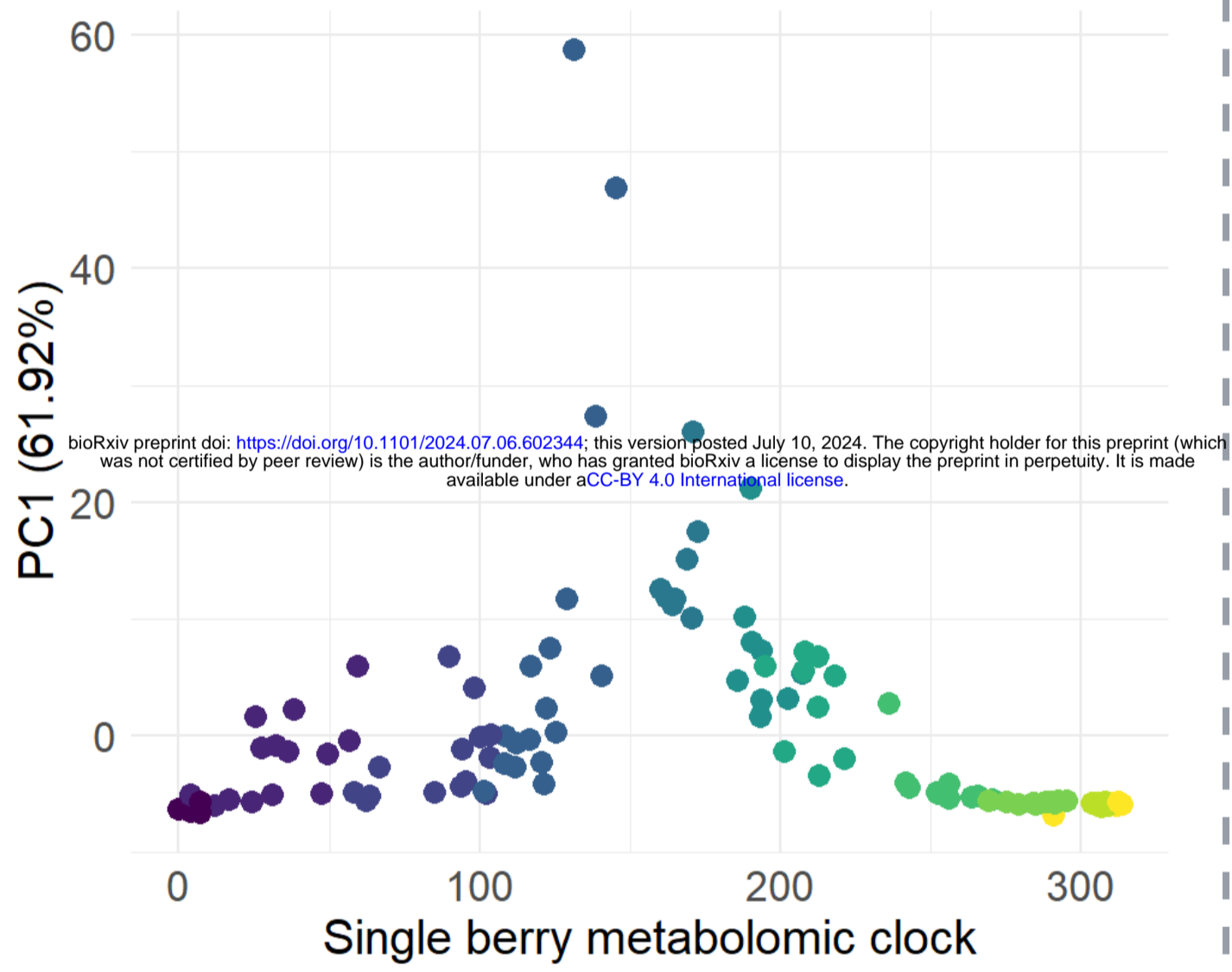
Profile E  
n = 750



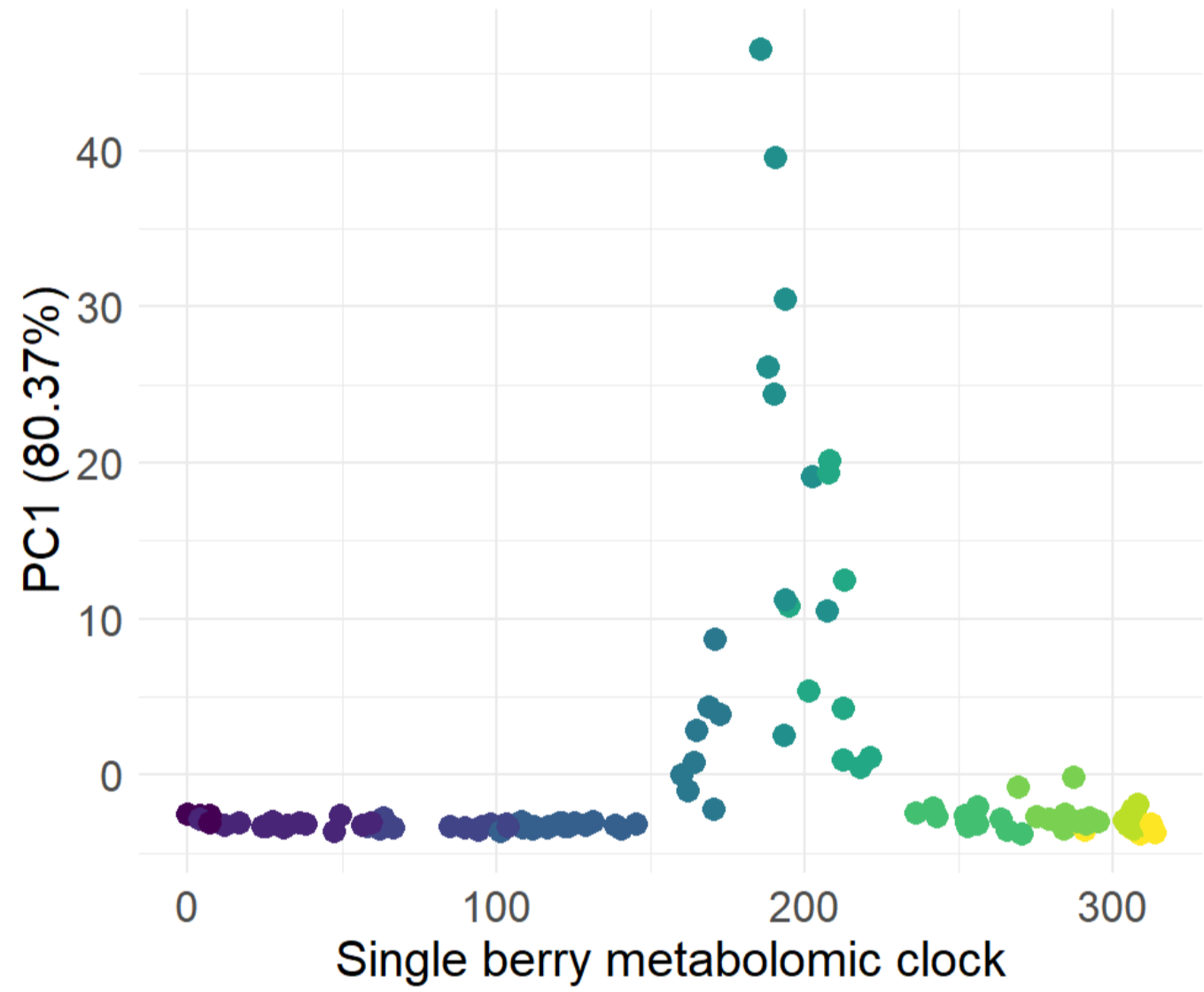
Profile F  
n = 1293



Profile G  
n = 152



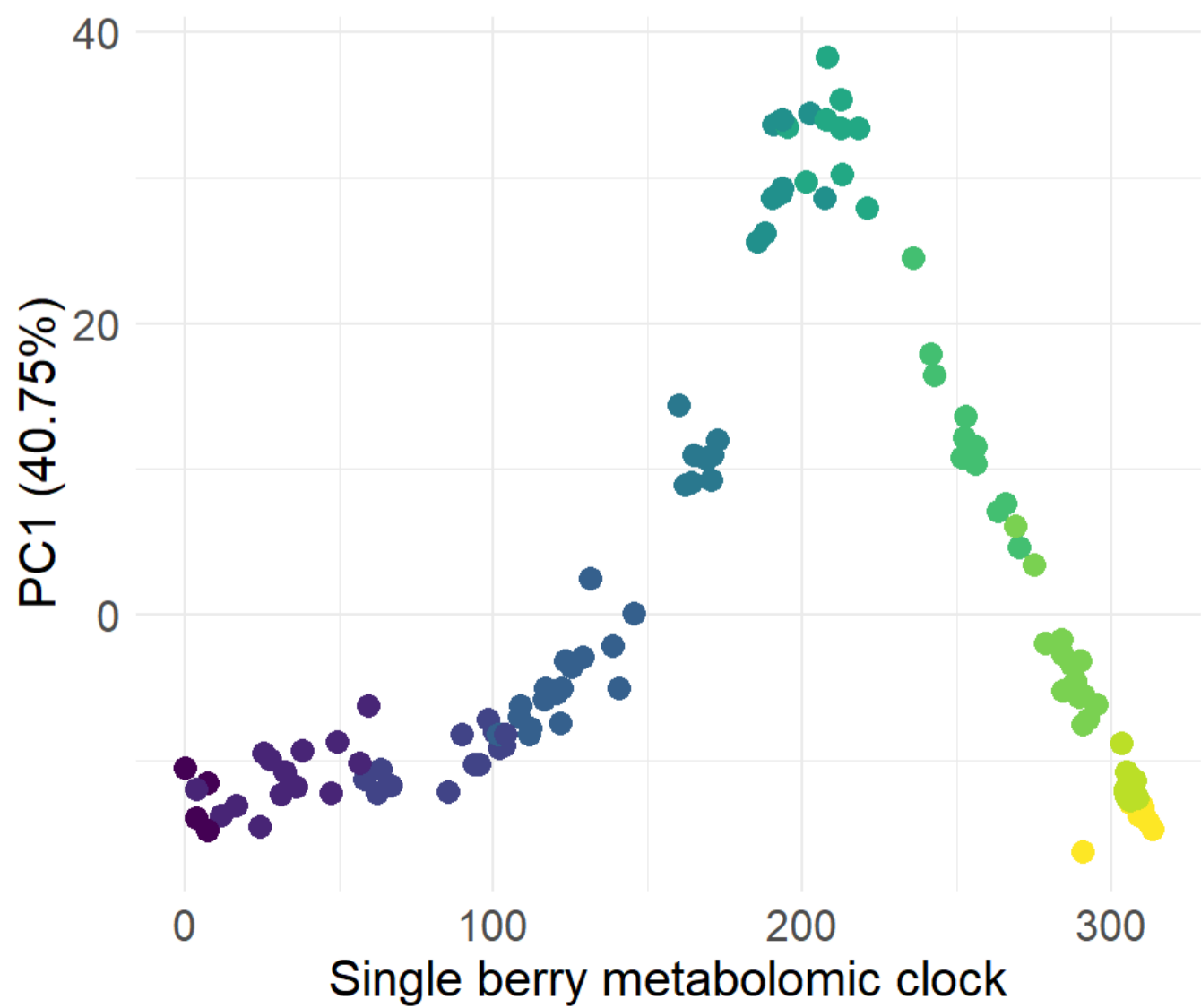
Profile H  
n = 87



Expert stages

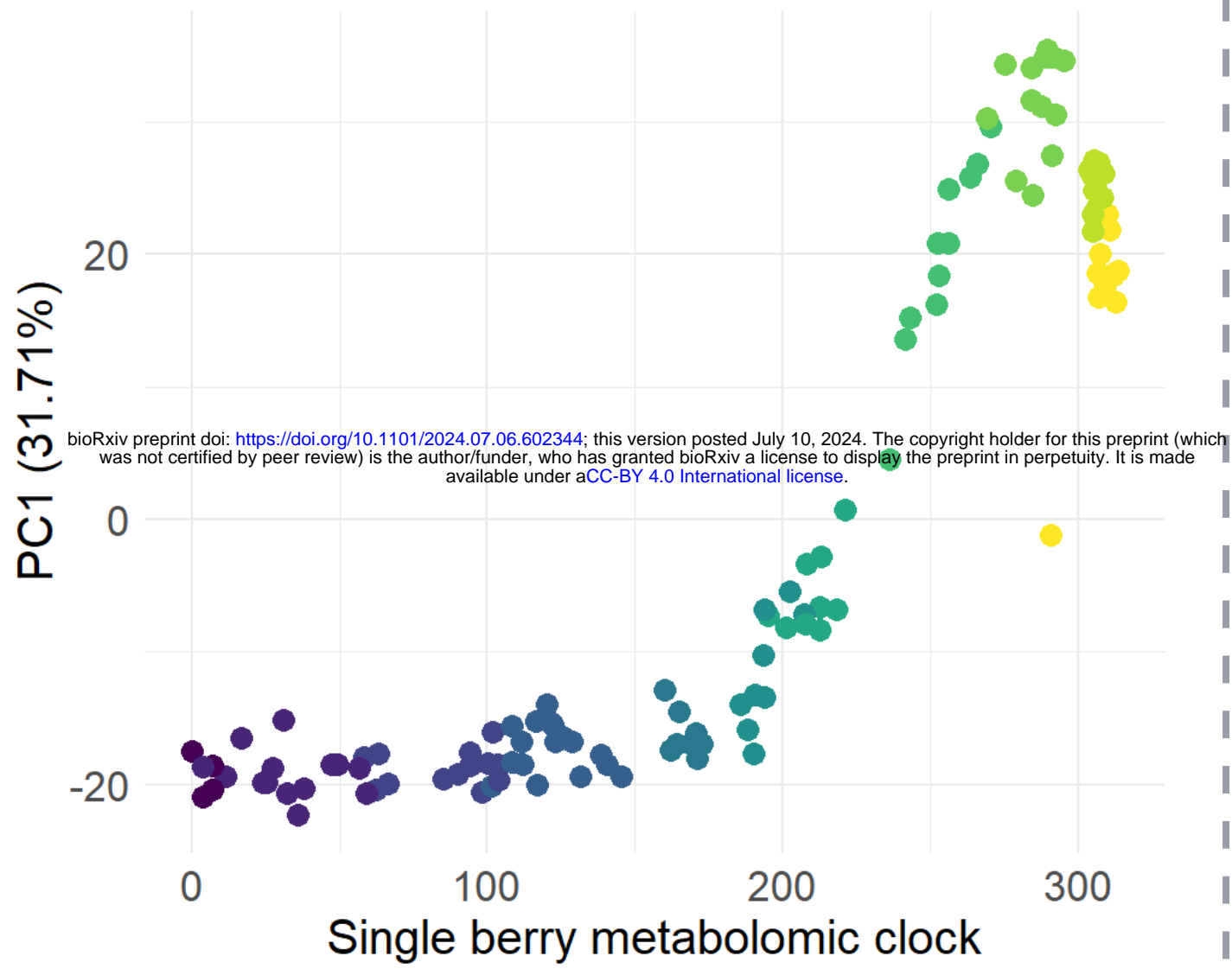
- G1
- G2
- G3
- L4
- L5
- S6
- S7
- R8
- R9
- R10
- Sh11

Profile I  
n = 584



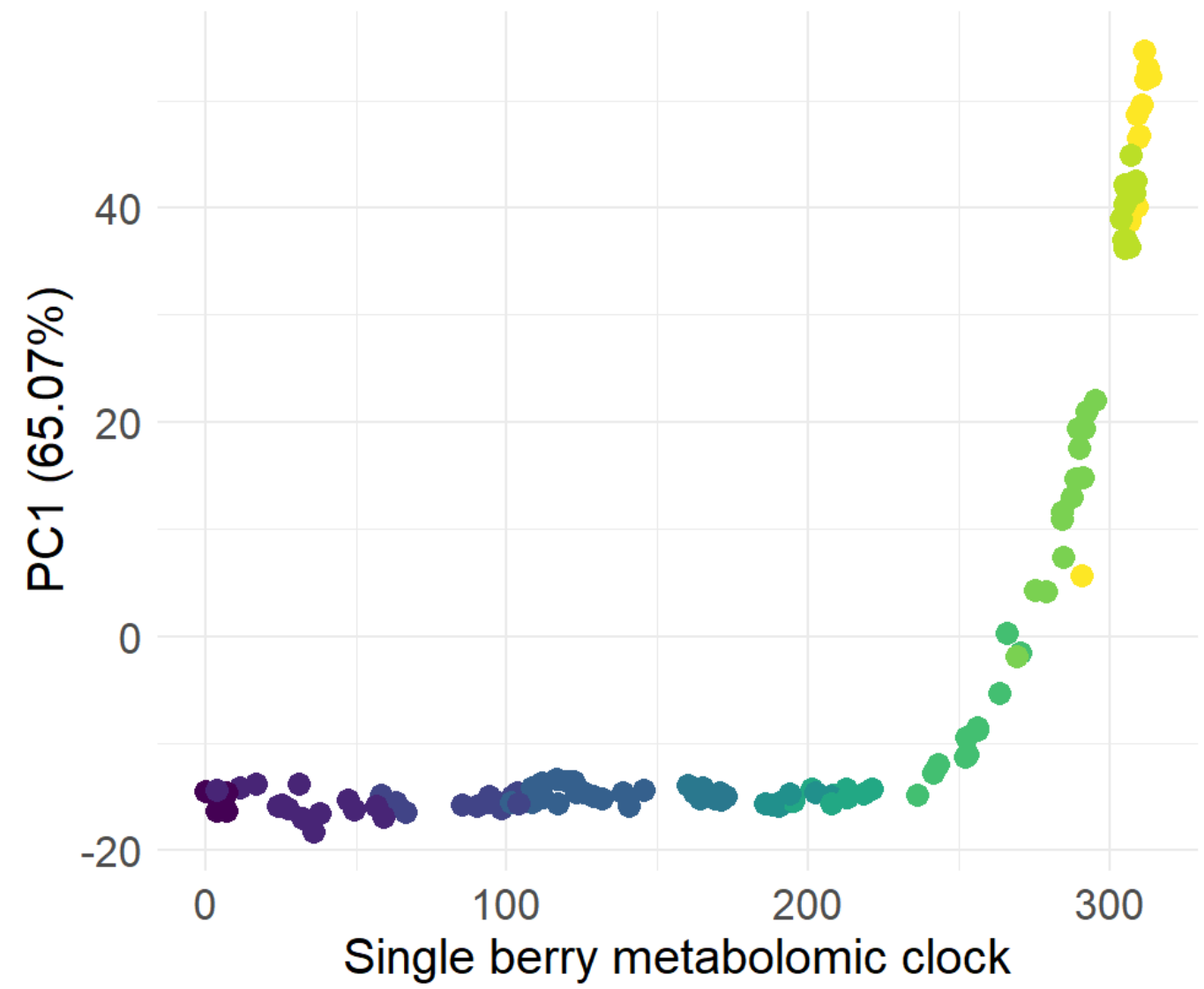
### Profile J

n = 1300



### Profile K

n = 828

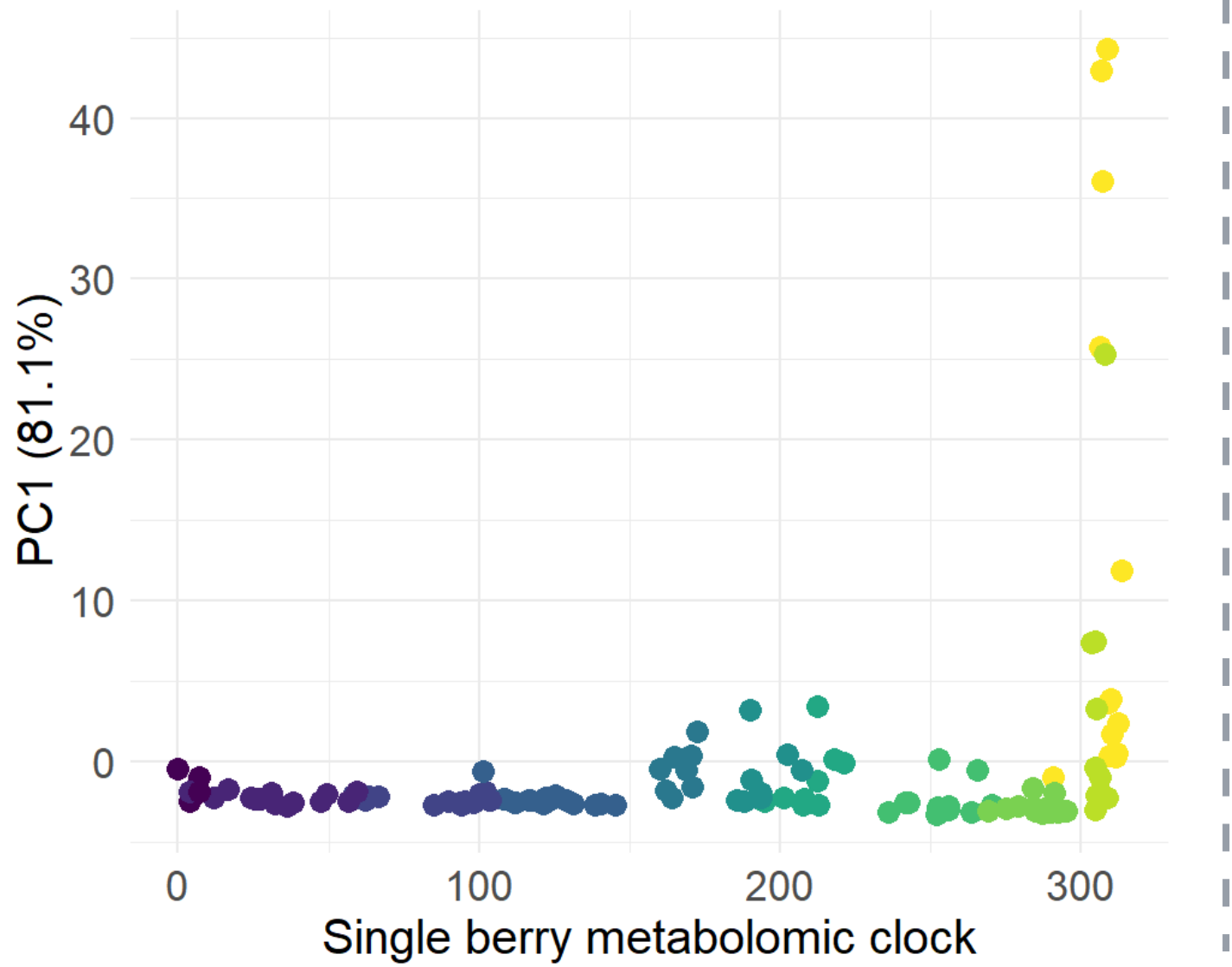


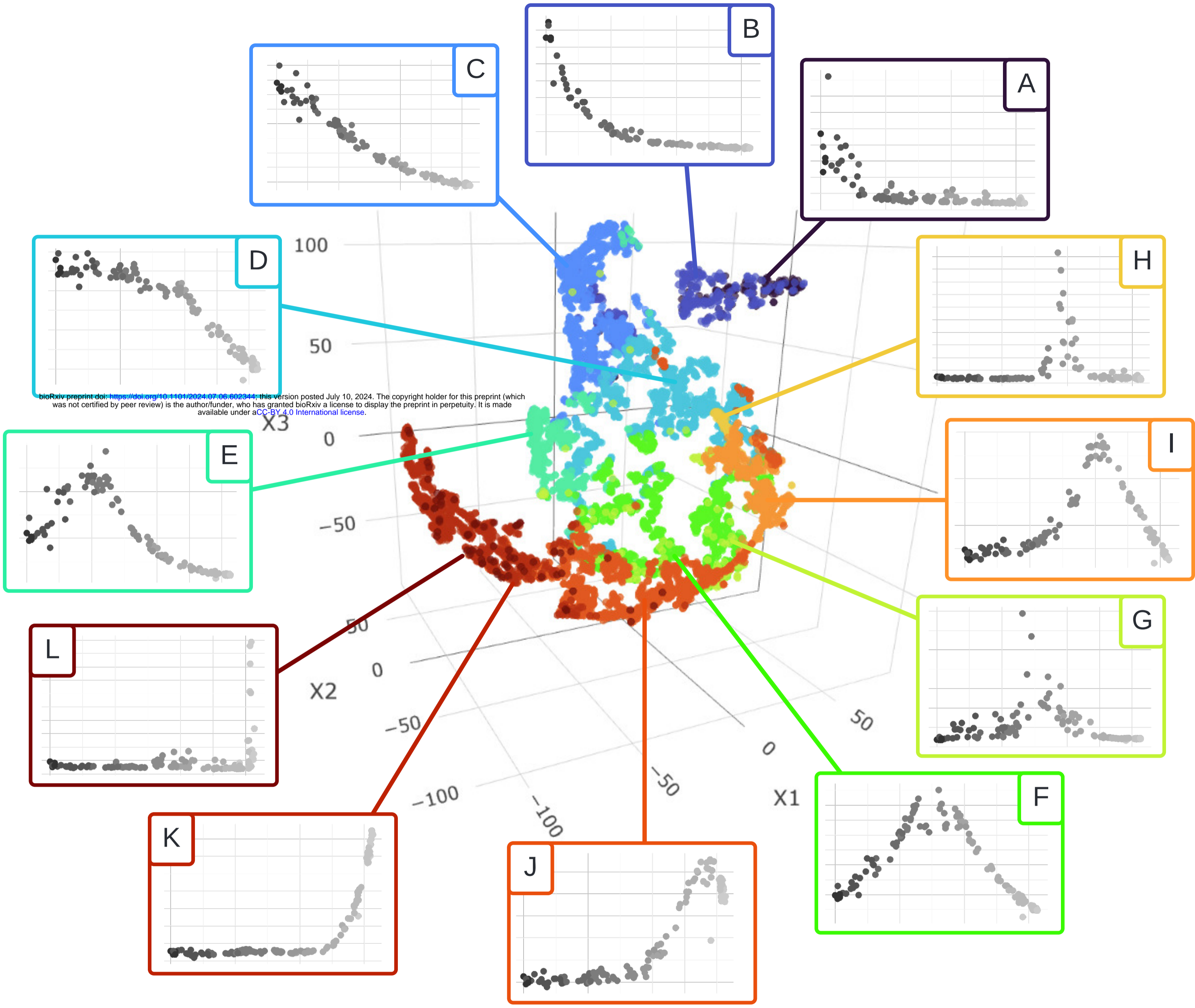
Expert stages

- G1
- G2
- G3
- L4
- L5
- S6
- S7
- R8
- R9
- R10
- Sh11

### Profile L

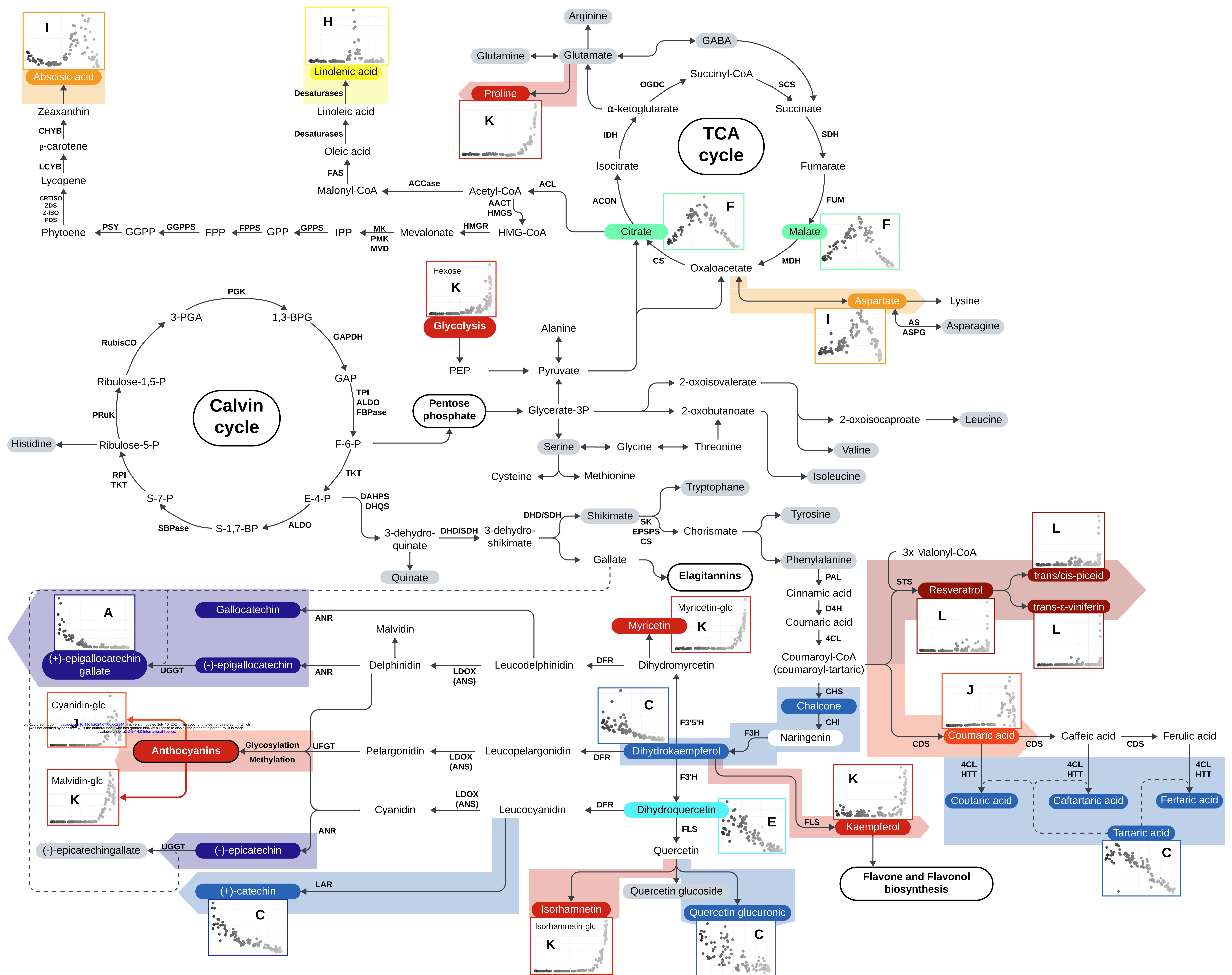
n = 72





Clusters of metabolites

● A ● B ● C ● D ● E ● F ● G ● H ● I ● J ● K ● L



Clusters of metabolites

