



**HAL**  
open science

## Multi-excitation hyperspectral autofluorescence imaging for the exploration of biological samples

Madiyeh Ghaffari, Anne Laure Chateigner-Boutin, Fabienne Guillon, Marie  
Francoise Devaux, Hamid Abdollahi, Ludovic Duponchel

### ► To cite this version:

Madiyeh Ghaffari, Anne Laure Chateigner-Boutin, Fabienne Guillon, Marie Francoise Devaux, Hamid Abdollahi, et al.. Multi-excitation hyperspectral autofluorescence imaging for the exploration of biological samples. *Analytica Chimica Acta*, 2019, 1062, pp.47-59. 10.1016/j.aca.2019.03.003 . hal-02620782

**HAL Id: hal-02620782**

**<https://hal.inrae.fr/hal-02620782>**

Submitted on 26 Oct 2021

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

1

2

3 **Multi-excitation hyperspectral autofluorescence imaging for the exploration**  
4 **of biological samples**

5

6 Mahdiyeh Ghaffari<sup>1,2</sup>, Anne-Laure Chateigner-Boutin<sup>3</sup>, Fabienne Guillon<sup>3</sup>, Marie-Françoise Devaux<sup>3</sup>,  
7 Hamid Abdollahi<sup>1</sup>, Ludovic Duponchel<sup>2\*</sup>

8

9 *<sup>1</sup>Department of Chemistry, Institute for Advanced Studies in Basic Sciences (IASBS), Zanjan 45137-*  
10 *66731, Iran.*

11 *<sup>2</sup>Université de Lille, CNRS, UMR 8516 - LASIR - Laboratoire de Spectrochimie Infrarouge*  
12 *et Raman, F-59655 Villeneuve d'Ascq, France.*

13 *<sup>3</sup>INRA – Centre de recherche Angers-Nantes, UR BIA “Biopolymères Interactions Assemblages” 1268,*  
14 *Nantes, France.*

15 *(\*)corresponding author : ludovic.duponchel@univ-lille.fr*

16

17

18

19

20

21

22

23

24

25 **Abstract**

26 Many plant tissues can be observed thanks to autofluorescence of their cell wall components.  
27 Hyperspectral autofluorescence imaging using confocal microscopy is a fast and efficient way of  
28 mapping fluorescent compounds in samples with a high spatial resolution. However a huge  
29 spectral overlap is observed between molecular species. As a consequence, a new data analysis  
30 approach is needed in order to fully exploit the potential of this spectroscopic technique and  
31 extract unbiased chemical information about complex biological samples. The objective of this  
32 work is to evaluate multi-excitation hyperspectral autofluorescence imaging to identify  
33 biological components in wheat grains during their development through their spectral profiles  
34 and corresponding contribution maps using Multivariate Curve Resolution - Alternating Least-  
35 Squares (MCR-ALS), a signal unmixing algorithm under proper constraints. For this purpose  
36 two different scenarios are used: 1) analyzing the total spectral domain of data sets using MCR-  
37 ALS under non negativity constraint in both spectral and spatial modes; 2) analyzing a reduced  
38 spectral domain of data sets using MCR-ALS under non negativity in both modes and trilinearity  
39 constraint in spectral mode. Considering the original instrumental setup and our data analysis  
40 approach, we will demonstrate that extracted contribution maps and spectral profiles of  
41 constituents can provide complementary information used to identify molecules in complex  
42 biological samples.

43

44 **Key words**

45 Multivariate Curve Resolution - Alternating Least-Squares; multi-excitation hyperspectral  
46 images; Autofluorescence; Trilinearity constraint; Wheat grain.

47

48

49

50

## 51        **1. Introduction**

52        Plants are complex organisms with a specialized body plan that requires multilevel  
53 organization (organs, tissues, cell types, subcellular compartments) and highly specialized tissues  
54 with distinct properties. Plant organs are therefore a mosaic of different cell types, with variation  
55 in their structure and composition under genetic and environmental controls. Plant organs are  
56 widely used for food, feed and industrial applications. Studying the tissue composition of organs  
57 is therefore of tremendous interest to understand plant biological functions and to evaluate the  
58 quality of plant tissues for nutritional and industrial applications.

59        Due to the heterogeneous nature of plant tissues and to compare multiple plant samples, there is  
60 a need for imaging techniques that rapidly provide information concerning the chemical  
61 composition of tissues with good spatial resolution. Spectroscopic methods such as Raman, Mid-  
62 infrared or UV-Visible fluorescence are classically used to analyze plant tissue sections.<sup>1-6</sup>  
63 However, to be applicable to plant comparison, spectral imaging techniques must deal with  
64 minimal sample preparation (e.g. avoid tissue dehydration and resin embedding), high spatial  
65 resolution ( $< 3 \mu\text{m}$  per pixel), high acquisition speed and a high field of view while keeping  
66 enough spectral information to ease molecular identification. As a consequence full field  
67 fluorescence imaging systems such as confocal microscopes equipped with spectral detectors is  
68 certainly the only technique meeting such requirements. Taking advantage of the  
69 autofluorescence properties of many plant compounds<sup>2,3,6-9</sup> confocal or multiphoton imaging can  
70 be performed with little tissue preparation and, more important, without labeling.

71        Hyperspectral autofluorescence imaging was considered in this work to follow the evolution of  
72 wheat grain tissues during grain development. For this purpose, we collected confocal  
73 hyperspectral images of wheat grains sections at different stages of development. The wheat  
74 grain comprises several tissues: embryo, endosperm and outer layers. Autofluorescence in cereal  
75 grain is due to several compounds, including pigments such as chlorophyll in green tissues,  
76 carotenoids, anthocyanidin and proanthocyanidin in colored grains.<sup>10-12</sup> In the grain outer layers,  
77 autofluorescence is particularly important in cell walls where it has been linked to the presence  
78 of phenolic compounds such as lignin and hydroxycinnamic acids (ferulic acid, para-coumaric  
79 acid).<sup>13-15</sup> However, the fluorescence properties of major compounds largely overlap in the  
80 spectral domain. For instance, lignin and hydroxycinnamic acids both fluoresce after UV

81 excitation while only lignin is fluorescent after visible excitation.<sup>2,16,17</sup> Chemometric methods  
82 have already been used to overcome this problem and identify tissues in dry mature grain and in  
83 particles from autofluorescence multispectral images.<sup>11, 14-16</sup> However we need to get beyond that  
84 because ambiguity has not been totally lifted. Indeed combining autofluorescence hyperspectral  
85 images obtained by confocal microscopy after different excitations may help to further identify  
86 tissues with overlapping autofluorescence responses or in cases of the co-localization of  
87 components. For each excitation wavelength, one hyperspectral image is acquired. The set of  
88 hyperspectral images forms a multi-excitation hyperspectral image. It can also be considered that  
89 for each pixel in an image, an excitation emission fluorescence matrix is acquired. Thus we can  
90 see that such experiments will impose constraints. First, as emission wavelengths are always  
91 longer than excitation ones, it results in a different spectral domain depending on the excitation  
92 wavelength and a partial excitation–emission matrix. Second, the final multi-excitation  
93 hyperspectral image cannot be strictly considered as a full 3-way data set. Finally, with the  
94 purpose of comparing samples during grain development, several images have to be analyzed  
95 together in a consistent way. As a consequence data sets have to be reorganized and chemometric  
96 methods have to be adapted for this specific analysis.

97 Multivariate Curve Resolution-Alternating Least Squares (MCR-ALS) is one of the signal  
98 unmixing techniques that can provide pure spectra and contribution maps of different  
99 components in the samples.<sup>21</sup> It is an iterative algorithm that solves the bilinear model with no  
100 prior knowledge. This algorithm extracts from the data set pure contributions (concentration and  
101 spectral profiles) of all compounds present in the sample from suitable alternating least-squares  
102 optimization subjected to different constraints. The latter is imposed based on the chemical  
103 knowledge of the studied system.<sup>22-25</sup> When data sets are analyzed by MCR-ALS, the result  
104 might be challenging due to lack of unique solutions, which is an intrinsic characteristic of  
105 bilinear matrix decompositions if incomplete information is available about the system.<sup>26</sup> The  
106 resulting uncertainty may be dramatically large in certain cases, e.g., when extensive profile  
107 overlap occurs in one of the data modes. However, imposing different constraints such as non-  
108 negativity, unimodality, selectivity and trilinearity may drastically decrease the extent of  
109 rotational ambiguity by adding more information to the MCR analysis of the system under  
110 study.<sup>27-31</sup> A Trilinearity constraint can guarantee accurate unique profiles for constituents with  
111 trilinear structure.<sup>32,33</sup> To fulfill the trilinearity condition, the augmented data matrix should

112 contain some similar factors in its sub-matrices, factors which should not be affected by  
113 experimental conditions so that they share a common spectral shape. Only their areas (and  
114 vertical heights) should proportionately change according to the constituent concentration. It is a  
115 very strong constraint that forces the decompositions to give unique solutions under mild  
116 conditions.

117 The objective of this work is to evaluate the method of multi-excitation hyperspectral  
118 imaging by confocal microscopy combined with the exploration of second order data sets with  
119 the MCR-ALS approach to identify different components in the developing wheat grain based on  
120 their autofluorescence properties. Contribution maps and spectral profiles of different  
121 compounds are complementary information which will be extracted from the exploration of this  
122 kind of data sets. For this purpose two different scenarios are used: 1) Analyzing the whole  
123 spectral domain - obtained by merging the data sets of each excitation wavelength - using MCR-  
124 ALS under non negativity constraint in both modes; 2) Analyzing the reduced spectral domain -  
125 common to all excitation wavelength - using MCR-ALS under non negativity in both modes and  
126 trilinearity constraint in spectral mode in order to get second order advantage. The two  
127 approaches will be compared and discussed on the basis of extracted contribution maps and pure  
128 spectral profiles of all components. These results will be also compared with images generated  
129 from the standard method based on emission signal integration.

130

## 131 **2. Material and methods**

### 132 **2.1. Plant materials and growth conditions**

133 Wheat plants (*Triticum aestivum* L. cv. Recital) were grown in containers filled with plain  
134 soil at INRA Clermont-Ferrand (France) under conditions of natural day length and temperature.  
135 Upon flowering plants were transferred to a covered structure allowing to control and monitor  
136 the temperature. Temperature was set at 21°C from 6 a.m. to 9.30 p.m. and 14°C at night. Wheat  
137 grain development is influenced by the time elapsed since flowering, the temperature and the  
138 position of the grain within the spike. Thus its development was monitored using the thermal  
139 time method<sup>34</sup> which is more robust than only considering time when temperature is varying.  
140 The thermal time unit is Celsius degrees days after flowering (°DAF). This measure is the

141 cumulated daily average temperature since flowering. Individual spikes were tagged when the  
142 first flowering event was observed. Grains were harvested at different desired developmental  
143 stages as already described in a previous work.<sup>35</sup> More precisely samples were collected at 150,  
144 270, 350, 460, 560, 630, 790 °DAF and at mature stage.

145

## 146 **2.2. Multi-excitation hyperspectral autofluorescence imaging**

147 Freshly harvested grain samples were frozen and cut in the equatorial region of the grain  
148 using a cryotome (HM 500 OM, Microm) into 20  $\mu\text{m}$  cross sections. In the case of dry grains,  
149 the embryo was removed and the grains were placed onto moist paper for 24 h at 4°C to facilitate  
150 sectioning. For each grain, serial sections were placed on a slide. This was then mounted in water  
151 and analyzed using a confocal laser-scanning system (A1, Nikon) equipped with a x40 objective  
152 for confocal imaging. The microscope was equipped with a spectral detector unit and provided  
153 three excitation wavelengths: 375 nm (UV), 488 nm (blue) and 561 nm (green). At the chosen  
154 magnification, the field of view was  $317 \times 317 \mu\text{m}$  and images were digitized as matrix of  $512 \times$   
155  $512$  pixels with a pixel size of  $0.62 \times 0.62 \mu\text{m}$ . As a consequence each hyperspectral data cube  
156 contained 262,144 emission spectra. Moreover for each field of view, three hyperspectral images  
157 were recorded by collecting emitted light from 404 to 714 nm for UV excitation, 504 to 744 nm  
158 for blue excitation and 574 to 744 nm for green excitation with a 10 nm step between spectral  
159 variables. A dichroic mirror was used to filter the three excitation wavelengths. For each  
160 development stage, two or three serial sections were imaged. Two regions per section were  
161 analyzed: (1) opposite to the crease and (2) on the side of the grain, called dorsal and side  
162 respectively, with the objective to visualize all the tissues of the wheat grain (Figure 1). In the  
163 case of the youngest grains, one field of view was not always sufficient. In this case, two images  
164 were acquired to observe the whole region. Finally the whole data set contained 40 multi-  
165 excitation hyperspectral autofluorescence images considering different parts of the grain and  
166 development stages; this corresponds to more than 10 million emission spectra in total.

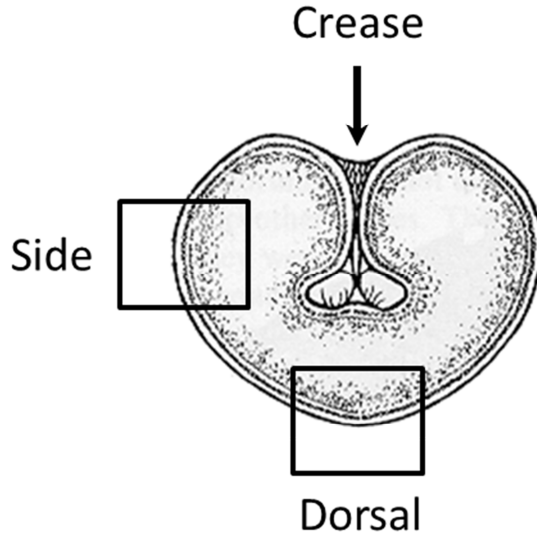
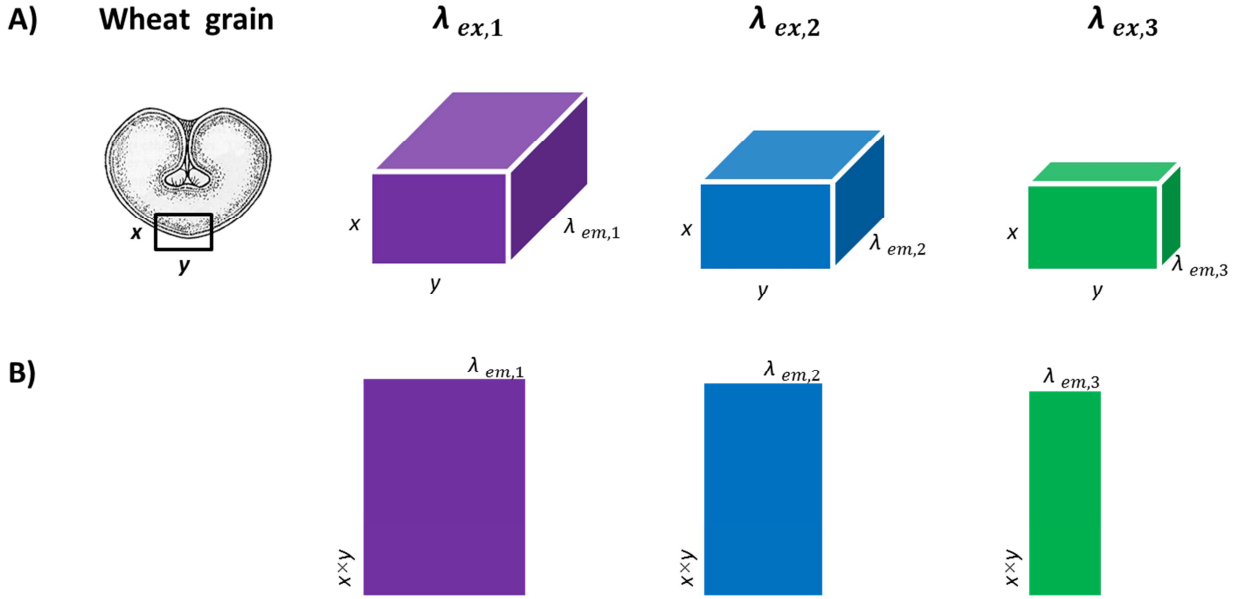


Figure 1. A simple scheme of wheat grain cross section with spectral acquisition areas.

### 2.3.Spectral data arrangement

In this work, multiple excitation wavelengths were used to generate autofluorescence hyperspectral images of the wheat grain; which means one 3D autofluorescence hyperspectral image (two spatial modes  $x$  and  $y$  and one emission mode  $\lambda_{em}$ ) per excitation wavelength denoted  $\lambda_{ex,1}$ ,  $\lambda_{ex,2}$  and  $\lambda_{ex,3}$ , respectively (Figure 2a).  $\lambda_{em,1}$ ,  $\lambda_{em,2}$  and  $\lambda_{em,3}$  are the three emission wavelength ranges corresponding to the three excitation wavelengths. So for these three excitation wavelengths, there are three 3D cubes thus forming together a 4D data set with two spatial modes  $x$  and  $y$ , one excitation mode and one emission mode. Each multi-excitation hyperspectral autofluorescence image corresponds to a 4D image as shown in figure 2a by considering together the purple, blue and green cubes. As a consequence, an excitation emission autofluorescence matrix is acquired for each pixel of the sample surface instead of only one emission spectra as it is usually the case for conventional fluorescence analysis. However, this matrix is only partial because the spectral domains corresponding to each excitation wavelength differs. In order to analyze the acquired data sets by using a curve resolution method like MCR-ALS, a 2D matrix of data is needed. Consequently each 3D autofluorescence hyperspectral image matrix corresponding to each excitation is unfolded to a matrix with  $x \times y$  rows and  $\lambda_{em,i}$  columns ( $i=1, 2$  and  $3$ ) as shown in figure 2b.





187

188 Figure 2. Data arrangements: a) the three 3D blocks (denoted  $\lambda_{ex,1}$   $\lambda_{ex,2}$  and  $\lambda_{ex,3}$ ) are three  
 189 autofluorescence hyperspectral images collected using the three excitation wavelengths. b)  
 190 Unfolded version of the three cubes.

191

192 **2.4. Data analysis – the MCR-ALS approach**

193 The MCR-ALS method is used to decompose simultaneously a hyperspectral image into the  
 194 pure spectra of the image constituents and corresponding contribution maps. MCR-ALS is based  
 195 on a bilinear model which assumes that each observed spectrum is a linear combination of pure  
 196 components spectra present in the system.<sup>36,37</sup> This model can be written in matrix form as:

197  $\mathbf{D} = \mathbf{C}\mathbf{S}^T + \mathbf{E}$  (1)

198 Where,  $\mathbf{D}$  is the unfolded hyperspectral image data set,  $\mathbf{C}$  is the matrix of the relative amounts or  
 199 contributions,  $\mathbf{S}^T$  is the pure spectra matrix and  $\mathbf{E}$  is the matrix associated to non-modeled part of  
 200 data and potentially only noise. Naturally signal intensity in every pixel of the image should not  
 201 be negative and neither should the contributions of the different constituents. It is therefore  
 202 logical to use non-negativity constraint during alternating least squares optimization. The  
 203 spectral matrix  $\mathbf{S}^T$  is also normalized to avoid scale ambiguity. At the end of the ALS  
 204 optimization procedure, the optimized  $\mathbf{C}$  matrix can be refolded, to recover a 2D image

205 (distribution map) of the contributions of every component. To evaluate the quality of extracted  
206 profiles obtained from the MCR-ALS procedure, the percentage of lack of fit (*lof*) and the  
207 percentage of explained variance ( $R^2$ ) are calculated according to the two following equations:

$$208 \quad lof (\%) = 100 \times \sqrt{\frac{\sum_{ij} d_{ij} - \hat{d}_{ij}}{\sum_{ij} d_{ij}^2}} \quad (2)$$

209 where  $d_{ij}$  is the element of the hyperspectral image data set  $\mathbf{D}$ , and  $\hat{d}_{ij}$  is the corresponding  
210 element of this data matrix recalculated by MCR-ALS. This lack of fit value gives a measure of  
211 the fit quality in relative terms with the same units as the measured data, and comparable to the  
212 experimental relative error estimation. For the explained variance,  $R^2$  is calculated as:

$$213 \quad R^2 = 100 \times \left(1 - \frac{\sum_{ij} e_{ij}^2}{\sum_{ij} d_{ij}^2}\right) \quad (3)$$

214 where  $e_{ij}$  are the elements of the  $\mathbf{E}$  matrix. The main advantage of this algorithm is the amount  
215 of information that can be included in the optimization process and the ability of working with  
216 either a single data matrix or multiset data structures i.e. using simultaneously several data cubes.  
217 However it is known that such an approach does not always extract unique solutions if not well  
218 managed. Therefore additional chemical information and well-selected constraints used in the  
219 decomposition process can significantly reduce or even eliminate this uncertainty. One of the  
220 most well-known constraints to ensure uniqueness of extracted solutions in MCR-ALS is  
221 trilinearity. Due to the trilinearity constraint, data arrays should contain some similar factors in  
222 their modes while factors should be independent from experimental conditions.<sup>37</sup> Trilinearity is a  
223 very strong constraint that forces trilinear decompositions to have unique solutions under mild  
224 conditions.<sup>38,39</sup> An important achievement in the analysis of complex data matrices resulted when  
225 Multivariate Curve Resolution methods were applied to several data matrices simultaneously to  
226 the so-called Matrix Augmented - Multivariate Curve Resolution - Alternating Least Squares,  
227 MA-MCR-ALS.<sup>40</sup> Generally speaking, resolution ambiguities and rank deficiency problems in  
228 the analysis of two-way data sets can be reduced significantly if it is possible to analyze data  
229 structures with more information. MA-MCR-ALS, as an extension of MCR-ALS, can be easily  
230 adapted to the trilinear analysis of the three-way data sets generated from data matrices with the  
231 same row or column, taking advantage of their structure. When the trilinearity constraint is used

232 in the MA-MCR-ALS framework, profiles of a component in different data matrices are forced  
233 to have a common shape during each ALS optimization but may differ by a scaling factor. When  
234 this constraint is inserted into the ALS iterative optimization procedure, it forces the shape of the  
235 loading vectors to be the same in all sub-matrices. An interesting aspect of the trilinearity  
236 constraint in the ALS optimization is that its use is optional for each component of the system.

237 MCR-ALS optimization always starts with the generation of initial estimates of either  
238 concentration or spectral profiles. In general, the use of chemically meaningful estimates is an  
239 essential factor that can lead to not only a rapid convergence of the extractions but also a  
240 decrease in ambiguity of solutions in some cases. Different methods can be used to find suitable  
241 initial estimates to start the MCR-ALS calculation. The so-called SIMPLISMA based on the  
242 concept of purest variables is usually used to calculate them.<sup>41</sup> However it was not possible to  
243 use it in this work due to the very high number of spectra in the considered multiset analysis. We  
244 have, therefore, decided to use the Kennard–Stone (KS) algorithm in order to generate initial  
245 estimates.<sup>42</sup> This algorithm allows for the selection of spectra from all parts of the data space  
246 even in the case of nonhomogeneous distribution. It starts by finding the two most distant spectra  
247 in the data set using Euclidean distance, and then continues with other points until the selected  
248 rank is reached.

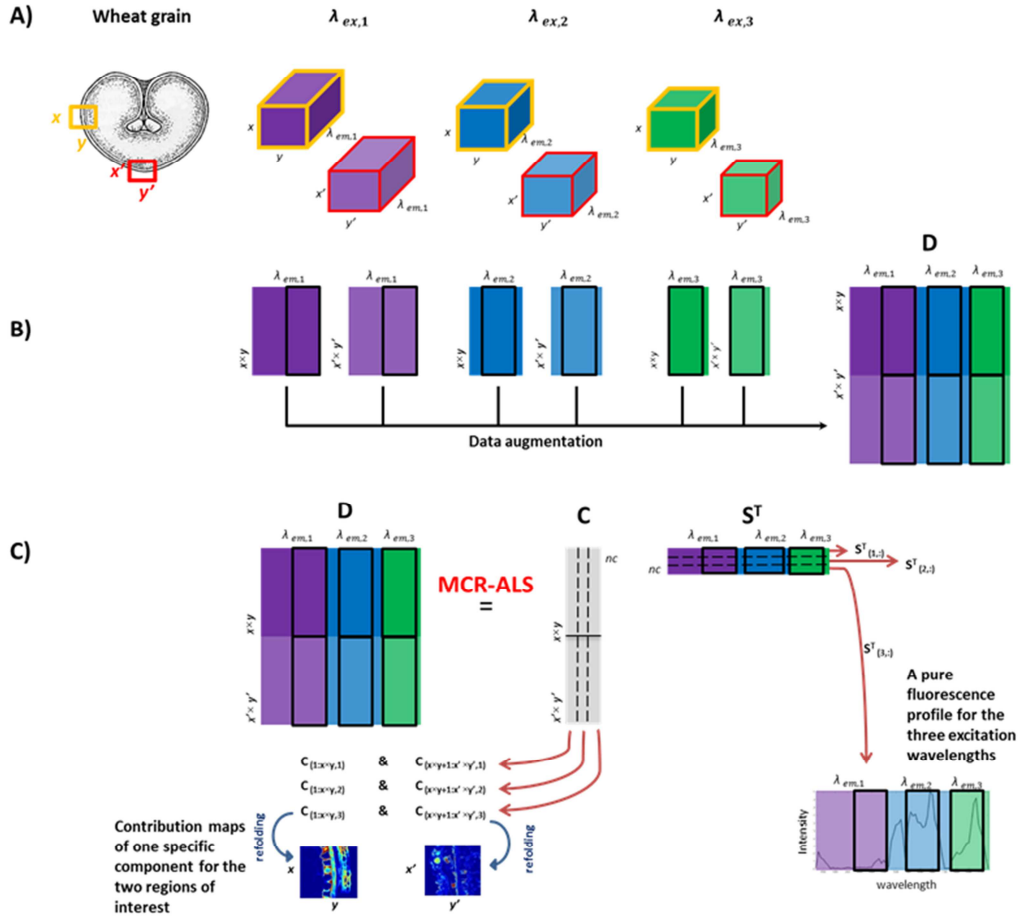
249 The rank of the data set i.e. its total number of independent signals is usually obtained from  
250 Principal Component Analysis (PCA) considering the magnitude of singular values. Singular  
251 values related to chemical contributions are typically large, whereas singular values related to  
252 noise are smaller and similar among themselves. This diagnostic can be complemented by  
253 looking at the emergence of noisy patterns in scores and loadings profiles, typical in noise-  
254 related components. When in doubt, few MCR models with different number of components can  
255 be calculated. The final model is selected as the smallest one providing an optimal model fit and  
256 chemically meaningful resolved profiles.<sup>43</sup> In this work for most of the data sets several MCR  
257 models were calculated and the selected one had minimum residuals and optimal fit. In order to  
258 extract spectral profiles and contribution maps of all active components in the sample, MCR-  
259 ALS approach has been applied following two different scenarios.

260 In a first scenario, an augmented data set **D** was obtained by considering the whole spectral  
261 domain of each data cube and merging the corresponding three 2D unfolded matrices as shown

262 in figure 3b. In this case, MCR-ALS was applied under non-negativity constraint only in order to  
263 extract spectral autofluorescence emission profiles and contribution maps of active components.  
264 As explained in the data collection section, several regions were observed for each grain section  
265 and several serial sections were also observed for each development stage, resulting in several  
266 multisets (in red and orange in figure 3a). In the figure 3b, purple, blue and green cubes  
267 correspond to the three different excitation wavelengths used while light and dark colors  
268 correspond to two sample regions of interest. Then we use column- and row-wise data  
269 augmentation to fuse all matrices and generate a global matrix **D**. Lastly, the augmented data set  
270 is used to extract pure concentration and spectral matrices using MCR-ALS (Figure 3c). To  
271 tackle the large volume of data from the 40 multi-excitation hyperspectral images (i.e. more than  
272 10 million of emission spectra), augmented data sets were built and analyzed separately for each  
273 development stage that still correspond to the multivariate analysis of 1.3 million spectra each.

274

275



276

277 Figure 3. Data arrangements; a) three 3D blocks are three autofluorescence hyperspectral images  
 278 acquired with three different excitation wavelengths:  $\lambda_{ex,1}$ ,  $\lambda_{ex,2}$  and  $\lambda_{ex,3}$  which are shown in  
 279 purple, blue and green, respectively (both dark and light colors are related to orange and red  
 280 regions of interest of the wheat grain). b) Unfolding of cubes and final data augmentation  
 281 generating D matrix. c) The augmented data set is used to extract pure concentration and spectral  
 282 matrices using MCR-ALS. Open black rectangles in the figure represent the common spectral  
 283 range used when the trilinearity constraint is applied.

284

285 A second scenario has been considered in this work because rotational ambiguity (i.e.  
 286 uncertainty of MCR-ALS results) cannot be eliminated in the majority of cases only using the  
 287 non-negativity constraint. Therefore it has been decided to apply additionally the trilinearity  
 288 constraint in order to reach uniqueness of solutions in MCR-ALS, or at least verify if other

289 extractions are obtained in these new conditions. However, to analyze the acquired data set under  
290 the trilinearity constraint, it must have a trilinear structure at least for one mode. In the case of  
291 fluorescence, the shape of the emission spectrum for a given fluorophore is largely independent  
292 from excitation wavelength. Therefore the recorded autofluorescence hyperspectral images using  
293 different excitation wavelengths are potentially suitable for applying trilinearity constraint in  
294 spectral mode. However due to our specific instrumental setup, hyperspectral images have not  
295 exactly the same spectral domain, a necessary condition for applying this new constraint in  
296 MCR-ALS. As a consequence, a reduced spectral domain (574-714 nm) common to the three  
297 unfolded matrices has been considered. This common spectral window is represented by black  
298 open rectangles in Figure 3. In this scenario, we can already see that the application of trilinearity  
299 constraint has paid a price of losing spectral information below 574 nm.

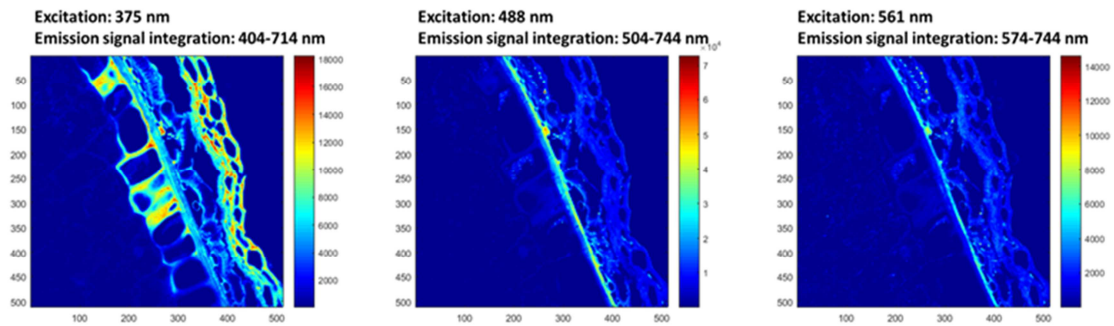
300

### 301 **3. Result and discussions**

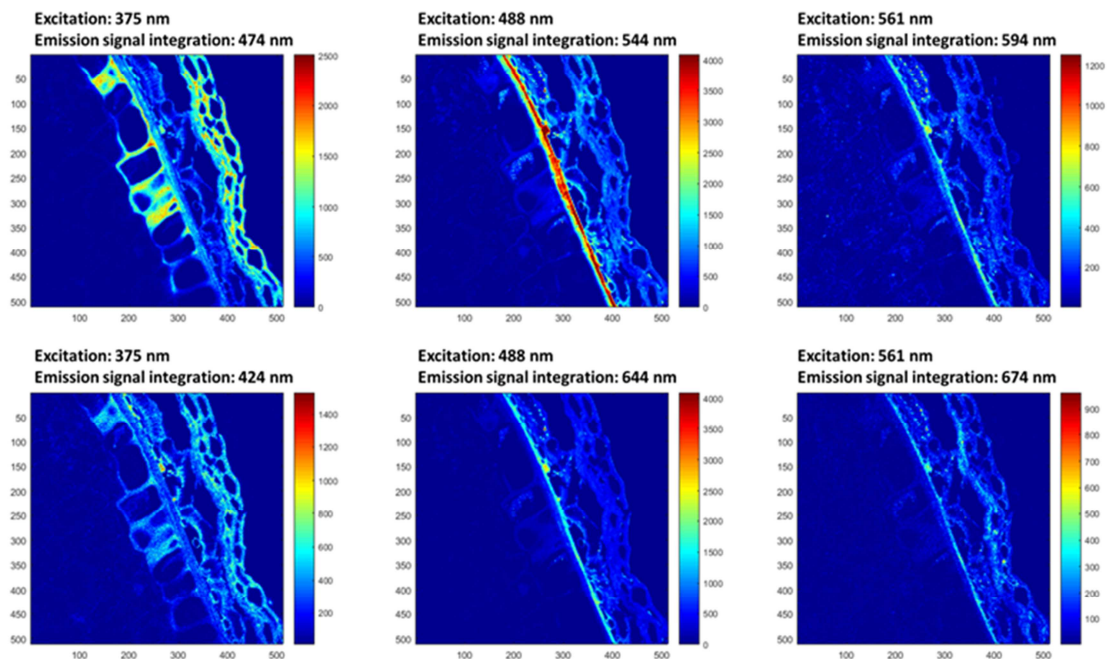
#### 302 *3.1 Autofluorescence image generation with a conventional approach*

303 As described in section 2.3, the multi-excitation hyperspectral confocal images acquired form  
304 a set of three 3D blocks. Their analysis and display are therefore not straightforward. The usual  
305 ways to generate a 2D image from a fluorescence imaging data set are the integration of the  
306 signal over the whole spectral domain of emission, the integration around the wavelength of a  
307 maximum emission or a signal extraction at a particular spectral channel corresponding to a  
308 specific emission. Figure 4 shows generated autofluorescence images of the same wheat section  
309 using the three excitation wavelengths 375, 488 and 561 nm. More precisely, images in Figure 4a  
310 have been obtained considering signal integration on the whole emission domain of each  
311 excitation wavelength. Autofluorescent compounds are observed in all tissues corresponding to  
312 the wheat grain outer layers and no autofluorescence is observed into the endosperm (see figure  
313 5 for tissue and cell layers annotation if needed). Moreover the use of different wavelengths of  
314 excitation seems to be a way to highlight different subparts of the section. Indeed, after  
315 excitation at 375 nm, all outer layers fluoresce from the aleurone layer to the epiderm, while the  
316 seed coat is highlighted after excitation at 488 nm. Using excitation at 561 nm, some small points  
317 are also revealed inside cross cells corresponding to pigments.<sup>44</sup>

## A) Global integration



## B) Single wavelength integration



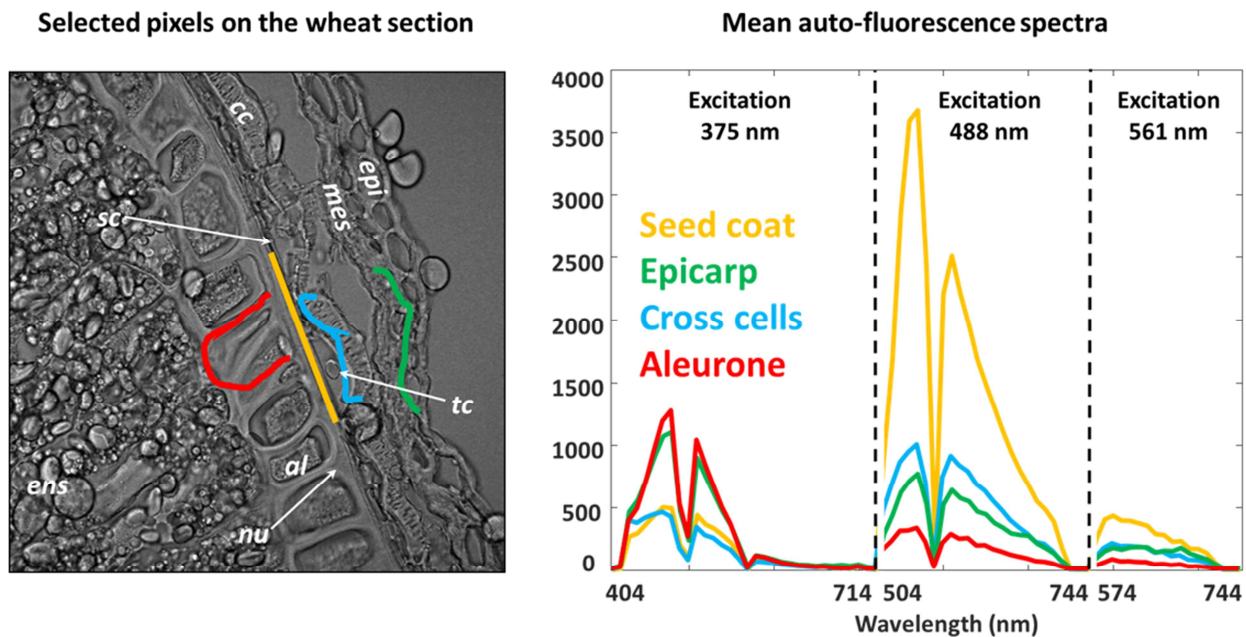
318

319 Figure 4. Autofluorescence images of the same wheat section at different excitation wavelength  
320 A) when emission is integrated over the whole spectral domain B) for emission at given  
321 wavelengths.

322

323 Autofluorescence images in Figure 4b have been generated considering only emission at  
324 given wavelengths. These particular wavelengths have been selected because it was possible to  
325 generate different emission images highlighting different subparts of the wheat section.

326 Variations in intensities in the different images suggest that each cell type shows some specific  
 327 fluorescence spectral response. In autofluorescence imaging, no chemical probes are used and all  
 328 molecules in the sample that are intrinsically fluorescent give emission in different parts of the  
 329 spectral domain. Autofluorescence is often considered as a spurious signal but it is not. It is in  
 330 fact a good opportunity to observe simultaneously different molecular contributions.<sup>15</sup> All  
 331 images shown in figure 4 obviously reveal the presence of different autofluorescent behaviors of  
 332 the different tissues. However even if specific sub-parts of the grain section are highlighted at  
 333 given wavelength of integration, there is no evidence that it corresponds to the contribution of  
 334 only one molecule, simply because of the huge bandwidth of the considered spectroscopy. The  
 335 complexity of the data structure is even more striking when looking at spectra extracted from  
 336 specific areas of the considered wheat section. Figure 5 presents manual selections of pixels in  
 337 cell walls of different grain tissues such as aleurone cells (red line), seed coat (orange line), cross  
 338 cells (blue line), and epicarp (green line) . Corresponding mean spectra of selected pixels are also  
 339 presented in the figure.



340  
 341 Figure 5. Manual selection of pixels in cell walls of different grain tissues (aleurone = red line,  
 342 seed coat = orange line, cross cells = blue line and epicarp = green line) and corresponding mean  
 343 spectra of emission for different excitation wavelengths. Annotations: *epl* = epicarp, *mes* =



344 mesocarp, *cc* = cross cell, *tc* = tube cell, *sc* = seed coat, *nu* = nucellar epidermis, *al* = aleurone  
345 layer and *ens* = endosperm.

346 Although these four specific tissues are known to contain different fluorescent molecules or  
347 different concentration of several ones, the global shape of emission spectra is almost the same,  
348 only relative intensity changes being observed for different excitation wavelengths. In  
349 conclusion, a simple integration approach does not allow us to obtain unbiased information about  
350 the fluorescent molecules present in the grain tissues due to a huge spectral overlap of  
351 fluorescence.<sup>3,6,45</sup> As a consequence, it was decided to apply the signal unmixing approach  
352 MCR-ALS in order to extract pure fluorescence signatures of molecules in the next section of the  
353 paper.

354

### 355 *3.1 Signal unmixing using MCR-ALS using the whole spectral domain*

356 All acquired data sets of each specific stage (more than 1.3 million of emission spectra) were  
357 analyzed together considering a column-wise data augmentation. In other words, we had eight  
358 augmented data sets corresponding to the eight development stages. Each data set has been  
359 analyzed separately using MCR-ALS under proper constraint. The chemical rank for each data  
360 set has been determined using singular value decomposition (SVD).<sup>22</sup> However it was sometimes  
361 necessary to calculate different MCR models with different number of components when SVD  
362 information was not so clear. Considering the principle of parsimony, the final MCR model was  
363 selected as the one with the smallest chemical rank, a good model fit and chemically meaningful  
364 resolved profiles. A mean LOF value of 0.9% and a mean  $R^2$  value of 0.96 were observed for  
365 MCR-ALS models which are good figures of merit considering the signal to noise ratio. As  
366 summarized in table 1, a total of seven components were extracted with MCR-ALS from the  
367 eight development stages when using the whole spectral domain and non-negativity constraint  
368 only (i.e. following the first scenario). Some components were present throughout the whole  
369 grain development and others only at specific stages. This demonstrates the great potential of  
370 such methodology being able to detect various molecular contributions even when the spectral  
371 overlap is maximal. Indeed, on the basis of previous results (figure 4 and 5), it was very difficult  
372 to envisage observing so many spectral contributions on such complex biological samples.

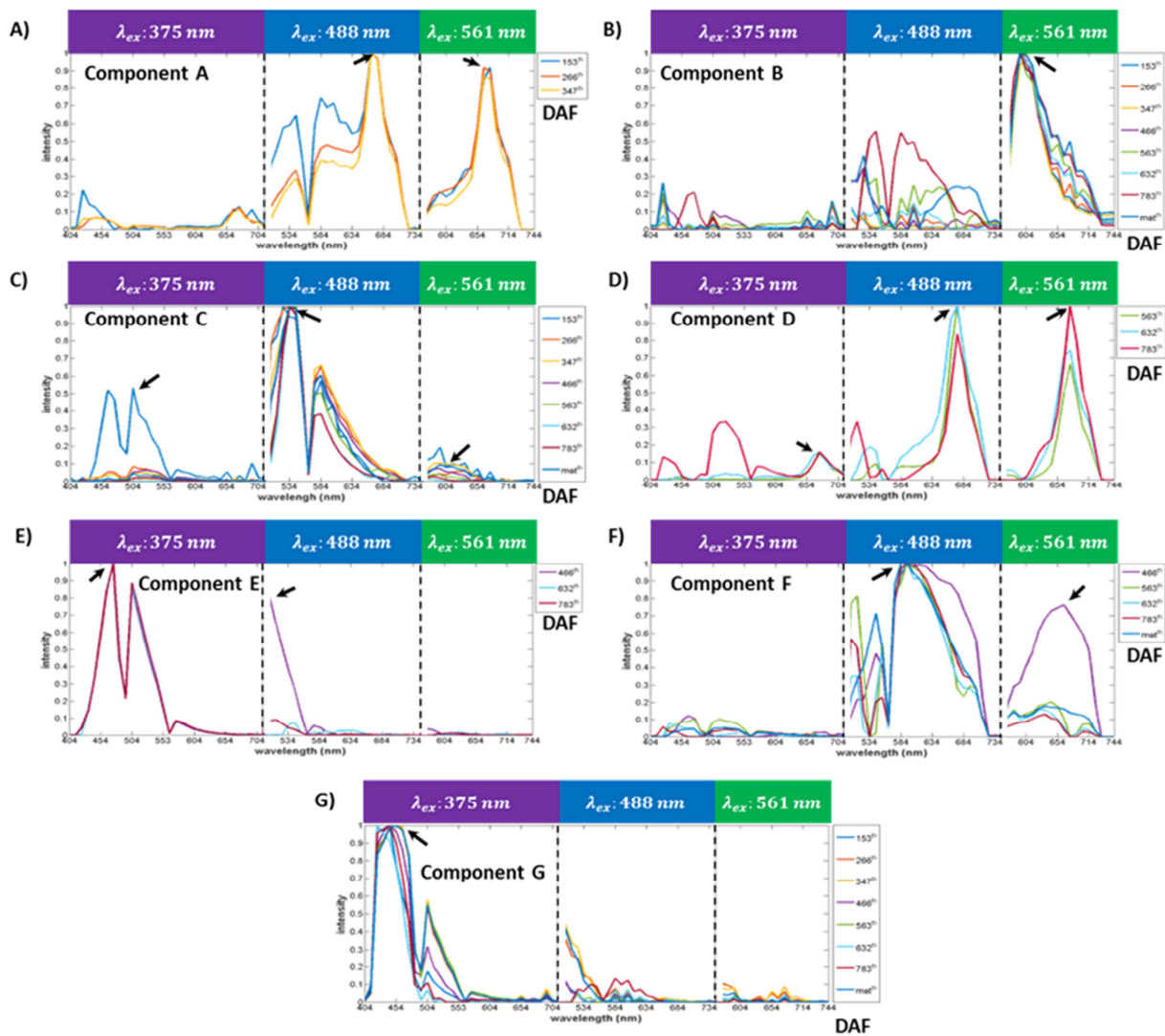
	150	270	350	470	560	630	780	Mature
	°DAF	°DAF	°DAF	°DAF	°DAF	°DAF	°DAF	
<b>Component A</b>	✓	✓	✓					
<b>Component B</b>	✓	✓	✓	✓	✓	✓	✓	✓ <sup>377</sup>
<b>Component C</b>	✓	✓	✓	✓	✓	✓	✓	✓ <sup>378</sup>
<b>Component D</b>					✓	✓	✓	
<b>Component E</b>				✓		✓	✓	
<b>Component F</b>				✓	✓	✓	✓	✓
<b>Component G</b>	✓	✓	✓	✓	✓	✓	✓	✓ <sup>382</sup>

384 Table 1. The seven extracted components, A, B, C, D, E, F and G from the eight development  
 385 stages. A tick sign indicates the presence of a component at a particular stage.

386

387 MCR-ALS extracted spectral profiles of all components A-G at each development stages are  
 388 shown in figure 6. Correlation coefficients were used in order to set out representations of the  
 389 same component from the different development stages (i.e. different MCR-ALS models). Each  
 390 panel of a component is divided in three separated parts corresponding to the three excitation  
 391 wavelengths 375, 488 and 561 nm respectively highlighted in purple, blue and green. Figure 7  
 392 presents corresponding extracted contribution maps of these components. If needed, all these  
 393 high resolution images can be retrieved in the supplementary material section in Matlab figure  
 394 format.

395



396

397 Figure 6. Extracted spectral profiles of components A-G. Subparts of each panel correspond with  
 398 the three excitation wavelengths. Arrows indicate selected maxima of emission.

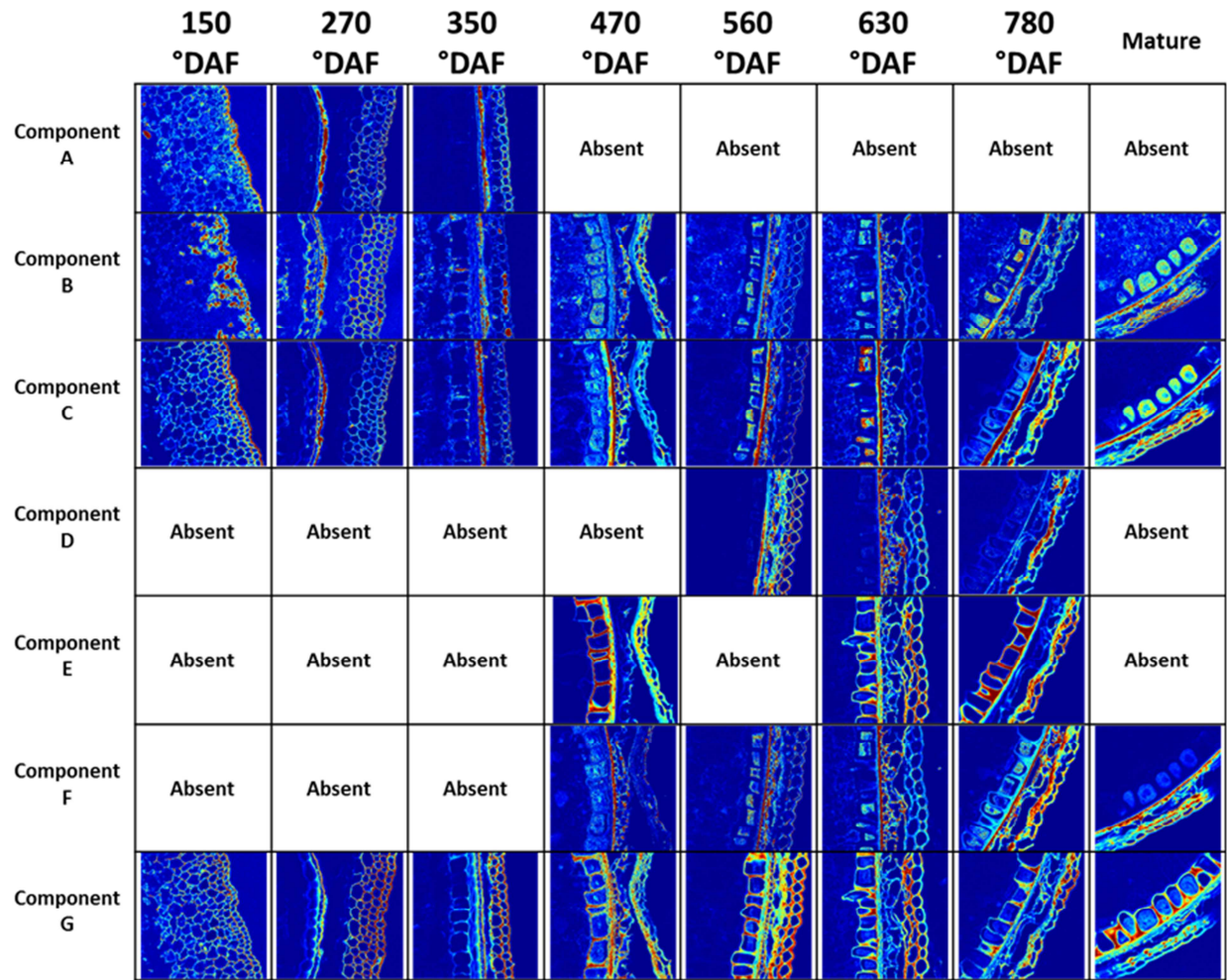
399

400

401

402

403



404

405 Figure 7. Examples of extracted contribution maps of all components in the wheat grain.  
 406 Minimum and maximum values in each contribution maps are represented with cold and hot  
 407 colors respectively.

408

409 From a general point of view, the MCR-ALS approach extracted specific spectral profiles for  
 410 each component. Molecular identification in fluorescence spectroscopy is naturally based on the  
 411 selection of maximum emission wavelengths on pure extracted profiles but our approach also  
 412 allow to us to observe hidden components at particular excitation wavelengths. Moreover  
 413 presence or absence of emission can be used jointly for spectral interpretation. The extracted  
 414 components are examined in more details here.

415 Component A (Figure 6A) is present from 150 °DAF to 350 °DAF (as shown in table 1). Its  
416 maximum of emission is observed at 664 nm after excitations at 488 and 561 nm (black arrows).  
417 Moreover this component exhibits no significant emission when excited at 375 nm. A broad  
418 emission is also observed between 500 and 630 nm with an excitation at 488 nm. Note that the  
419 emission drop observed at 561 nm corresponds to the wavelength excluded by the dichroic  
420 mirror for the three excitation wavelengths. Corresponding contribution maps of component A  
421 are observed on the first row of Figure 7. Component A is mainly found in the chloroplasts of  
422 cross cells, and in the outer cell wall of the epicarp for the samples at 150° and 270 °DAF. At 350  
423 °DAF this component particularly highlights the cross cells and the seed coat. Considering  
424 spectral profile and subcellular locations of this component, it might be assigned to pigments like  
425 flavonoids, carotenoids and chlorophyll.<sup>3,7,46-48</sup>

426 Component B (Figure 6B) is detected from 150 °DAF to mature stage (as shown in table 1).  
427 Its maximum of emission is observed at 604 nm for an excitation at 561 nm. Similar to  
428 component A, this component have no significant emission for an excitation at 375 nm.  
429 Concerning the excitation at 488 nm, a moderate emission is observed in the 500-630 nm  
430 spectral region. Corresponding contribution maps are presented in the second row of figure 7 for  
431 all stages of development. The location of this component depends on the development stage. At  
432 early stages (from 150 to 350 °DAF), it is mainly found inside cells of the epicarp and mesocarp  
433 and also inside cross cells. From 560 to 630 °DAF, it is mainly located inside cross cells and in  
434 the outer cell wall of the seed coat which is covered by a cuticle. From 780 °DAF to the mature  
435 stage, this component is mainly observed in the seed coat, inside aleurone cells and in the cell  
436 walls of the epicarp and mesocarp. The multiple location of the component in all stages makes  
437 the spectral interpretation of this component difficult. However considering the excitation-  
438 emission bands observed and the very similar shape of the component for all stages, this  
439 fluorescence might be attributed to flavonoid pigments.

440 Component C (Figure 6C and 7) is present at all investigated stages. The maximum of  
441 emission is located at 544 nm after excitation at 488 nm. Almost no fluorescence is observed for  
442 excitation at 561 nm and 375 nm. The only exception is a moderate emission observed at 150  
443 °DAF after an excitation at 375 nm. Component C is mainly found in epicarp cell wall at  
444 150°DAF, in the seed coat, the tube cell walls, and the nucellar epidermis from 350 °DAF to 630  
445 °DAF. In addition, it is detected inside aleurone cells and on all pericarp cell walls at the mature

446 stage. The assignment of this fluorescence to only one chemical compound is not possible due to  
447 the different subcellular locations. Moreover the excitation/emission profile does not correspond  
448 to a known autofluorescence compound.<sup>3</sup> It should be stressed that an extracted contribution of  
449 the MCR-ALS approach can potentially contain different molecules which are correlated with  
450 concentration, which may account for the difficulty of its interpretation.

451 Component D (Figure 6D and 7) appears from 560 °DAF to the 780 °DAF. The maximum of  
452 emission is located at 674 nm for excitations at 561 nm and 488 nm as indicated by black arrows.  
453 During these development stages, the compound is present in all pericarp cell walls and in the  
454 seed coat with a gradual decrease over time. Unfortunately considering both spectral behavior  
455 and localization, no attribution of this compound can be proposed at this time.

456 Component E (Figure 6E and 7) is present in the cell walls at 470, 630 and 780 °DAF. The  
457 maximum of emission is observed at 474 nm after excitation at 375 nm as indicated by black  
458 arrows. In this case, no significant fluorescence is observed for excitations at 561 nm and 488 nm  
459 except for 460 °DAF where a remaining fluorescence is found in the blue region. For the three  
460 stages, the component is mainly found in the cell walls of the aleurone layer, nucellus epidermis  
461 and those of the mesocarp and epicarp. Considering the cell wall locations and the spectral  
462 signature, this contribution might be attributed to hydroxycinnamic acids like ferulic or para-  
463 coumaric ones, even though hydroxycinnamic acids have been detected in cell walls before 460  
464 °DAF.<sup>47</sup> The fluorescence of these compounds is known to vary for instance with pH, types of  
465 bonds, and different molecular environments.

466 Component F (Figure 6F and 7) is present from 470 °DAF to the mature stage. The shape of  
467 its emission profile is changing between different stages. Consequently, several wavelengths of  
468 maximum emission are observed for this component. In addition there is almost no fluorescence  
469 with an excitation at 375 nm. From 470 °DAF to 630 °DAF, the component is located in the seed  
470 coats, in the cell walls of cross and tube cells and in the outer cell wall of epicarp. It is  
471 noteworthy that both seed coat and epicarp are covered by a cuticle. After 780 °DAF, all pericarp  
472 cell walls show an important amount of this component. Considering both spectral and spatial  
473 information, this contribution could correspond to some lignin and/or cuticle compounds. Indeed  
474 it has already been reported that the autofluorescence of lignins and cuticles was due to multiple  
475 fluorophores.<sup>2,47</sup>

476 Component G (Figure 6G and 7) is present in all investigated stages. The maximum of  
477 emission is observed at 450 nm. Fluorescence of this compound is found in the cell walls of all  
478 outer tissues of the grain at all stages. Only some variations of intensities are observed. For  
479 example, the fluorescence is weaker in the nucellar epidermis compared to the other tissues. It  
480 increases in the cell walls in the aleurone layer from 350 °DAF. Considering the spectral pattern  
481 of this compound and its localization, it can be assigned to hydroxycinnamic acids like ferulic or  
482 para-coumaric ones.<sup>35,49</sup>

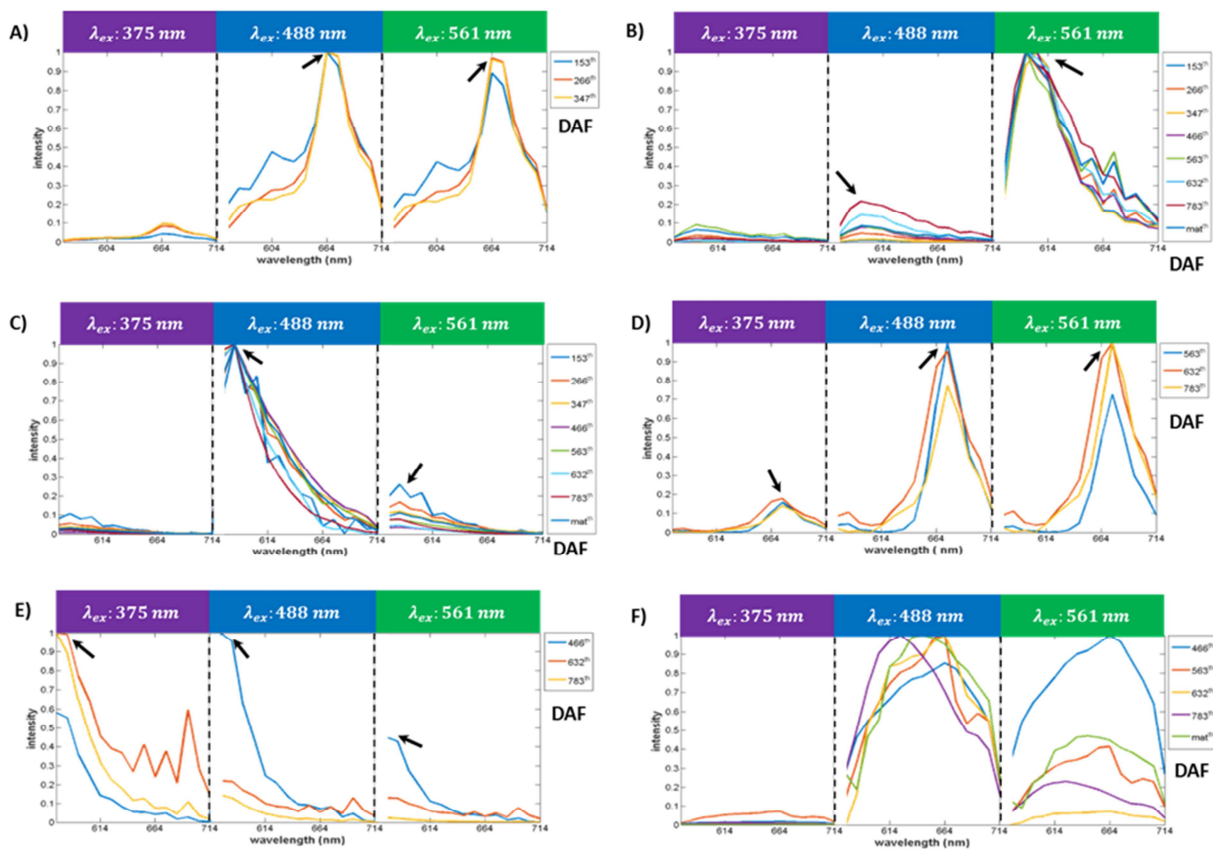
483 Analyzing multi-excitation hyperspectral autofluorescence imaging data sets with the MCR-  
484 ALS approach has therefore a good potential to obtain more information about molecules and  
485 their localization than with conventional data analysis techniques even if all components are not  
486 interpreted at this time. In this first scenario of MCR-ALS optimization, however only non-  
487 negativity constraint has been applied which may give rise to some ambiguities on extracted  
488 spectral profiles and corresponding contribution maps. It is in this sense that the impact of using  
489 the trilinearity constraint in MCR-ALS has to be evaluated in the next section.

490

### 491 *3.1 Signal unmixing using MCR-ALS using trilinearity constraint*

492 Trilinearity constraint can potentially guaranty accuracy of unique extractions from MCR-  
493 ALS. However, due to the trilinearity constraint, the augmented data set should contain some  
494 similar factors in their modes while the factors should be independent from experimental  
495 conditions. Thus profiles of a component in different data matrices are forced to have common  
496 shape during each ALS optimization but may differ by a scaling factor. During the ALS iterative  
497 optimization procedure, the constraint forces the shape of the loading vectors to be the same in  
498 all sub-matrices. In order to analyze the above mentioned data sets using MCR-ALS under  
499 trilinearity constraint, it was necessary to have the same spectral domain of emission. The  
500 spectral region of emission (574-714 nm) common to the three excitations was then considered.  
501 The common part of three recorded data sets were augmented row-wise and analyzed using  
502 MCR-ALS under non-negativity in both modes and trilinearity in spectral mode. Under these  
503 new conditions, only six components have been extracted with MCR-ALS with a mean LOF  
504 value of 4% and a mean  $R^2$  value of 0.99. Their presence and absence were exactly similar to the  
505 ones given in Table 1 except for component G which was absent. In fact, component G was

506 effectively present in grain sections but the reduction of the spectral domain prior MCR in this  
 507 second scenario has led to the loss of specific spectral information. MCR-ALS extracted spectral  
 508 profiles under non-negativity and trilinearity constraints of the six components A-F are shown in  
 509 figure 8. Generally speaking, we can observe that for a given component, extracted spectral  
 510 profiles are more consistent between stages than the ones in Figure 6. Indeed, the trilinearity  
 511 constraint drastically decreases rotational ambiguity of solutions.

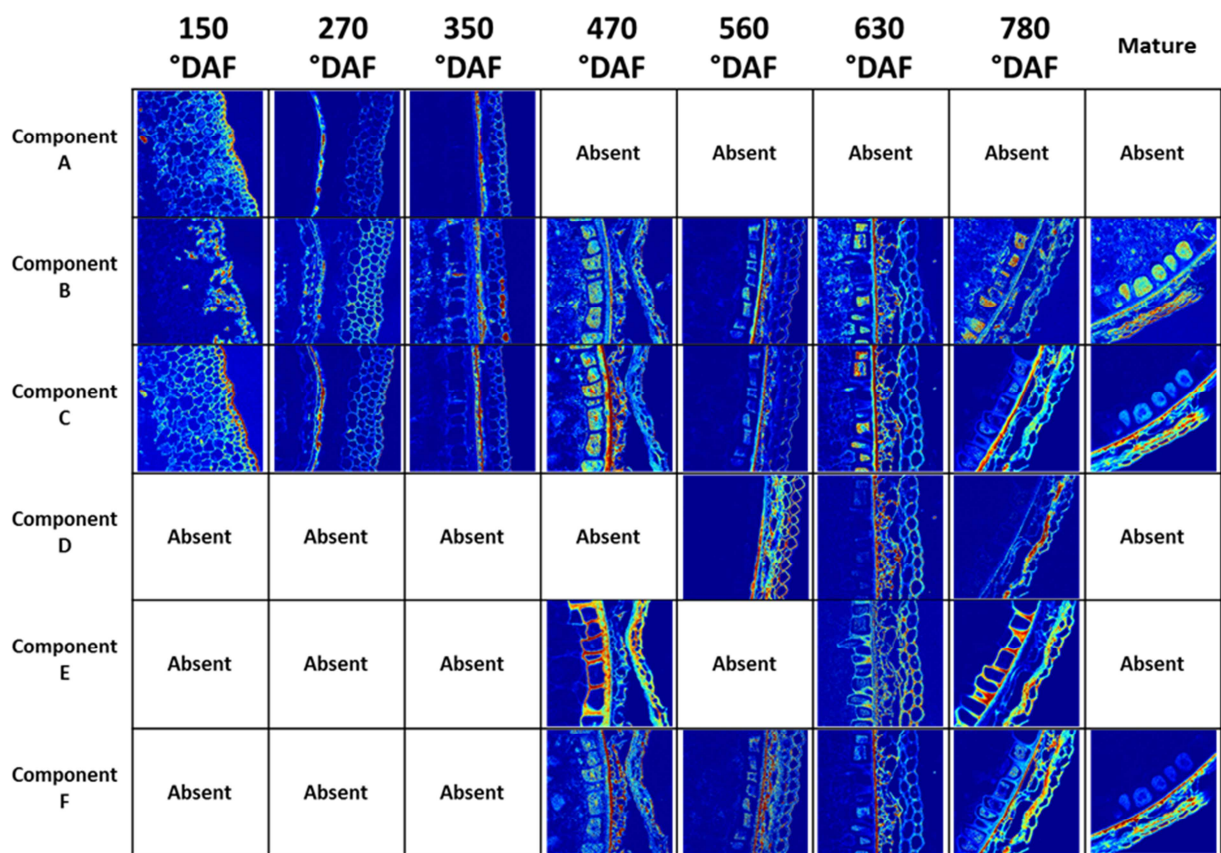


512 Figure 8. Extracted spectral profiles of component A-F when trilinearity and non-negativity  
 513 constraints are applied.  
 514

515  
 516 Moreover maxima of emission of components A, B and D are exactly the same as the ones  
 517 observed in Figure 6. It is slightly different for component C with a maximum of emission now  
 518 observed at 584 nm while it was 544 nm with non-negativity only. However this difference is not  
 519 due to a real spectral shift of this band. Indeed the maximum emission of this component is



520 always centered on the same wavelength but the restricted spectral domain imposed by the use of  
 521 trilinearity constraint only allows us to extract the bottom of this band. We observe the same  
 522 situation for component E with a maximum of emission at 474 nm with the application of non-  
 523 negativity constraint becoming 574 nm with trilinearity. As already pointed out, emission profile  
 524 of component F changes throughout wheat grain development certainly due to different chemical  
 525 environments. Figure 9 shows extracted contribution maps when trilinearity constraint is applied.



526  
 527 Figure 9. Extracted contribution maps of all components in the wheat grain when trilinearity and  
 528 non-negativity constraints are applied. Minimum and maximum values in each contribution maps  
 529 are represented with cold and hot colors respectively.

530  
 531 Compared with Figure 7, we can see that the localization of all components for all stages are  
 532 almost the same as it was with the first scenario, except for component G which is naturally

533 absent when trilinearity is considered. However a deeper analysis (particularly by zooming in on  
534 concentration maps) highlights better contrasted images when trilinearity constraint is applied.  
535 Indeed in these new conditions, rotational ambiguity is decreased and purer concentration  
536 profiles can be extracted. In other words, more zones with absence of components are observed.  
537 Less ambiguity in spectral profiles allows us to generate less biased images of the biological  
538 samples. From a data analysis perspective, it is obvious that applying the trilinearity constraint is  
539 a good way to extract better spectral profiles and corresponding contribution maps, component G  
540 being absent only because of our specific instrumental setup.

541

#### 542 **4. Conclusion**

543 This work demonstrates the great potential of multi-excitation hyperspectral autofluorescence  
544 imaging for the exploration of complex biological samples. In the present work, fluorescence  
545 properties were followed after UV and visible excitations in wheat grain outer layer during  
546 development. Our instrumental setup allowed us to obtain fluorescence information from this  
547 large spectral domain with a high spatial resolution without any labelling of the samples with  
548 additional fluorophores. There is a great potential in such spectral imaging techniques for  
549 comparing set of samples by statistical approaches. A set of 40 images containing more than 10  
550 million emission spectra was acquired to analyze wheat grains for eight development stages and  
551 one of the challenges of the work was to define a strategy of data analysis for multiset  
552 hyperspectral images. Having understood the limits of simple integration approaches on such  
553 spectral data sets, a signal unmixing technique has been evaluated. Two implementations of the  
554 MCR-ALS approach were proposed to extract pure component spectra and contribution maps  
555 according to development stages. In the first case, the full spectral range was taken into account  
556 while in the second one, trilinearity constraint was applied on the common spectral emission  
557 range of the three excitation wavelengths. In both cases, augmented data sets were obtained by  
558 merging the repetition of images of a given development stage. Pure components could be  
559 compared for the two MCR-ALS implementations except for the component found in the  
560 specific emission ranges of the UV excitation. Four to six pure components were detected  
561 depending on the stages. Components were assigned to hydroxycinnamic acids, lignin and  
562 cuticle compounds. Pigments compound were also highlighted and found in several tissues of the

563 wheat grain. Some compounds were difficult to interpret requiring additional investigation of the  
564 natural fluorophores encountered in plants. To the best of our knowledge, it is the first time  
565 MCR-ALS with trilinearity constraint is used to analyze simultaneously multiple hyperspectral  
566 autofluorescence data sets and extract pure spectral profiles and corresponding contributions  
567 maps of different molecules present in the sample. Despite the natural spectral overlap of  
568 chemical species and the complexity of biological samples, it is possible to unmix signals and  
569 extract more information about pure components with this original concept. With these results,  
570 we are convinced that the combination of multi-excitation hyperspectral autofluorescence  
571 imaging and MCR-ALS approach represent a real breaking point in the analysis of complex  
572 biological samples.

573

#### 574 **Acknowledgments**

575 This work has been supported by Campus France (PHC Gundishapur N°40890RA).

576

577

- 579 (1) Dokken, K. M.; Davis, L. C.; Marinkovic, N. S. Use of Infrared Microspectroscopy in Plant  
580 Growth and Development. *Applied Spectroscopy Reviews* **2005**, *40* (4), 301–326.  
581 <https://doi.org/10.1080/05704920500230898>.
- 582 (2) Donaldson, L.; Radotić, K.; Kalauzi, A.; Djikanović, D.; Jeremić, M. Quantification of  
583 Compression Wood Severity in Tracheids of *Pinus Radiata* D. Don Using Confocal  
584 Fluorescence Imaging and Spectral Deconvolution. *Journal of Structural Biology* **2010**, *169*  
585 (1), 106–115. <https://doi.org/10.1016/j.jsb.2009.09.006>.
- 586 (3) García-Plazaola, J. I.; Fernández-Marín, B.; Duke, S. O.; Hernández, A.; López-Arbeloa,  
587 F.; Becerril, J. M. Autofluorescence: Biological Functions and Technical Applications.  
588 *Plant Science* **2015**, *236*, 136–145. <https://doi.org/10.1016/j.plantsci.2015.03.010>.
- 589 (4) Gierlinger, N.; Schwanninger, M. The Potential of Raman Microscopy and Raman Imaging  
590 in Plant Research. *Spectroscopy* **2007**, *21* (2), 69–89. <https://doi.org/10.1155/2007/498206>.
- 591 (5) Gierlinger, N. Revealing Changes in Molecular Composition of Plant Cell Walls on the  
592 Micron-Level by Raman Mapping and Vertex Component Analysis (VCA). *Frontiers in*  
593 *Plant Science* **2014**, *5*, 306. <https://doi.org/10.3389/fpls.2014.00306>.
- 594 (6) Talamond, P.; Verdeil, J.-L.; Conéjéro, G. Secondary Metabolite Localization by  
595 Autofluorescence in Living Plant Cells. *Molecules* **2015**, *20* (3), 5024–5037.  
596 <https://doi.org/10.3390/molecules20035024>.
- 597 (7) Gillbro, T.; Cogdell, R. J. Carotenoid Fluorescence. *Chemical Physics Letters* **1989**, *158*  
598 (3–4), 312–316. [https://doi.org/10.1016/0009-2614\(89\)87342-7](https://doi.org/10.1016/0009-2614(89)87342-7).
- 599 (8) Hutzler, P.; Fischbach, R.; Heller, W.; Jungblut, T. P.; Reuber, S.; Schmitz, R.; Veit, M.  
600 Tissue Localization of Phenolic Compounds in Plants by Confocal Laser Scanning  
601 Microscopy. *Journal of Experimental Botany* **1998**, *49* (323), 953–965.
- 602 (9) Conéjéro, G.; Noirot, M.; Talamond, P.; Verdeil, J.-L. Spectral Analysis Combined with  
603 Advanced Linear Unmixing Allows for Histolocalization of Phenolics in Leaves of Coffee  
604 Trees. *Frontiers in Plant Science* **2014**, *5*. <https://doi.org/10.3389/fpls.2014.00039>.
- 605 (10) Knievel, D. C.; Abdel-Aal, E.-S. M.; Rabalski, I.; Nakamura, T.; Hucl, P. Grain Color  
606 Development and the Inheritance of High Anthocyanin Blue Aleurone and Purple Pericarp  
607 in Spring Wheat (*Triticum Aestivum* L.). *Journal of Cereal Science* **2009**, *50* (1), 113–120.  
608 <https://doi.org/10.1016/j.jcs.2009.03.007>.
- 609 (11) Adom, K. K.; Sorrells, M. E.; Liu, R. H. Phytochemicals and Antioxidant Activity of  
610 Milled Fractions of Different Wheat Varieties. *Journal of Agricultural and Food Chemistry*  
611 **2005**, *53* (6), 2297–2306. <https://doi.org/10.1021/jf048456d>.
- 612 (12) Furukawa, T.; Maekawa, M.; Oki, T.; Suda, I.; Iida, S.; Shimada, H.; Takamura, I.;  
613 Kadowaki, K. The Rc and Rd Genes Are Involved in Proanthocyanidin Synthesis in Rice  
614 Pericarp: Rc and Rd Genes for Red Rice Seed. *The Plant Journal* **2006**, *49* (1), 91–102.  
615 <https://doi.org/10.1111/j.1365-313X.2006.02958.x>.
- 616 (13) Fulcher, R. G.; O'Brien, T. P.; Lee, J. W. Studie on the Aleurone Layer 1. Conventional  
617 and Fluorescence Microscopy of the Cell Wall with Emphasis on Phenol-Carbohydrates  
618 Complexes in Wheat. *Australian Journal of Biological Sciences* **1972**, *25*, 23–34.
- 619 (14) Beaugrand, J.; Crônier, D.; Thiebeau, P.; Schreiber, L.; Debeire, P.; Chabbert, B. Structure,  
620 Chemical Composition, and Xylanase Degradation of External Layers Isolated from  
621 Developing Wheat Grain. *Journal of Agricultural and Food Chemistry* **2004**, *52*, 7108–  
622 7117. <https://doi.org/10.1021/jf049529w>.

- 623 (15) Corcel, M.; Devaux, M.-F.; Guillon, F.; Barron, C. Comparison of UV and Visible  
624 Autofluorescence of Wheat Grain Tissues in Macroscopic Images of Cross-Sections and  
625 Particles. *Computers and Electronics in Agriculture* **2016**, *127*, 281–288.  
626 <https://doi.org/10.1016/j.compag.2016.06.016>.
- 627 (16) Saadi, A.; Lempereur, I.; Sharonov, S.; Autran, J. C.; Manfait, M. Spatial Distribution of  
628 Phenolic Materials in Durum Wheat Grain as Probed by Confocal Fluorescence Spectral  
629 Imaging. *Journal of Cereal Science* **1998**, *28* (2), 107–114.  
630 <https://doi.org/10.1006/jcrs.1998.0195>.
- 631 (17) Harris, P. J.; Trethewey, J. A. K. The Distribution of Ester-Linked Ferulic Acid in the Cell  
632 Walls of Angiosperms. *Phytochemistry Reviews* **2010**, *9* (1), 19–33.  
633 <https://doi.org/10.1007/s11101-009-9146-4>.
- 634 (18) Jensen, S.; Munck, L.; Martens, H. The Botanical Constituents of Wheat and Wheat Milling  
635 Fractions. I. Quantification by Autofluorescence. *Cereal Chemistry* **1982**, *59*, 477–484.
- 636 (19) Baldwin, P. M.; Bertrand, D.; Novales, B.; Bouchet, B.; Collobert, G.; Gallant, D. J.  
637 Chemometric Labeling of Cereal Tissues in Multichannel Fluorescence Microscopy Images  
638 Using Discriminant Analysis. *Analytical Chemistry* **1997**, *69* (21), 4339–4348.  
639 <https://doi.org/10.1021/ac970145x>.
- 640 (20) Courcoux, P. Simultaneous Decomposition of Multivariate Images Using Three-Way Data  
641 Analysis Application to the Comparison of Cereal Grains by Confocal Laser Scanning  
642 Microscopy. *Chemometrics and Intelligent Laboratory Systems* **2002**, *62*, 103–113.
- 643 (21) Piqueras, S.; Duponchel, L.; Tauler, R.; de Juan, A. Monitoring Polymorphic  
644 Transformations by Using in Situ Raman Hyperspectral Imaging and Image Multiset  
645 Analysis. *Analytica Chimica Acta* **2014**, *819*, 15–25.  
646 <https://doi.org/10.1016/j.aca.2014.02.027>.
- 647 (22) Zhang, X.; Tauler, R. Application of Multivariate Curve Resolution Alternating Least  
648 Squares (MCR-ALS) to Remote Sensing Hyperspectral Imaging. *Analytica Chimica Acta*  
649 **2013**, *762*, 25–38. <https://doi.org/10.1016/j.aca.2012.11.043>.
- 650 (23) Felten, J.; Hall, H.; Jaumot, J.; Tauler, R.; de Juan, A.; Gorzsás, A. Vibrational  
651 Spectroscopic Image Analysis of Biological Material Using Multivariate Curve Resolution–  
652 Alternating Least Squares (MCR-ALS). *Nature Protocols* **2015**, *10* (2), 217–240.  
653 <https://doi.org/10.1038/nprot.2015.008>.
- 654 (24) Piqueras, S.; Duponchel, L.; Tauler, R.; de Juan, A. Resolution and Segmentation of  
655 Hyperspectral Biomedical Images by Multivariate Curve Resolution-Alternating Least  
656 Squares. *Analytica Chimica Acta* **2011**, *705* (1–2), 182–192.  
657 <https://doi.org/10.1016/j.aca.2011.05.020>.
- 658 (25) Amigo, J. M.; Ravn, C. Direct Quantification and Distribution Assessment of Major and  
659 Minor Components in Pharmaceutical Tablets by NIR-Chemical Imaging. *European*  
660 *Journal of Pharmaceutical Sciences* **2009**, *37* (2), 76–82.  
661 <https://doi.org/10.1016/j.ejps.2009.01.001>.
- 662 (26) Golshan, A.; Abdollahi, H.; Beyramysoltan, S.; Maeder, M.; Neymeyr, K.; Rajkó, R.;  
663 Sawall, M.; Tauler, R. A Review of Recent Methods for the Determination of Ranges of  
664 Feasible Solutions Resulting from Soft Modelling Analyses of Multivariate Data. *Analytica*  
665 *Chimica Acta* **2016**, *911*, 1–13. <https://doi.org/10.1016/j.aca.2016.01.011>.
- 666 (27) Rajkó, R.; Abdollahi, H.; Beyramysoltan, S.; Omidikia, N. Definition and Detection of  
667 Data-Based Uniqueness in Evaluating Bilinear (Two-Way) Chemical Measurements.  
668 *Analytica Chimica Acta* **2015**, *855*, 21–33. <https://doi.org/10.1016/j.aca.2014.12.017>.

- 669 (28) Omidikia, N.; Beyramysoltan, S.; Mohammad Jafari, J.; Tavakkoli, E.; Akbari Lakeh, M.;  
670 Alinaghi, M.; Ghaffari, M.; Khodadadi Karimvand, S.; Rajkó, R.; Abdollahi, H. Closure  
671 Constraint in Multivariate Curve Resolution: Closure Constraint in MCR-ALS. *Journal of*  
672 *Chemometrics* **2017**, e2975. <https://doi.org/10.1002/cem.2975>.
- 673 (29) Beyramysoltan, S.; Abdollahi, H.; Rajkó, R. Newer Developments on Self-Modeling Curve  
674 Resolution Implementing Equality and Unimodality Constraints. *Analytica Chimica Acta*  
675 **2014**, 827, 1–14. <https://doi.org/10.1016/j.aca.2014.03.019>.
- 676 (30) Beyramysoltan, S.; Rajkó, R.; Abdollahi, H. Investigation of the Equality Constraint Effect  
677 on the Reduction of the Rotational Ambiguity in Three-Component System Using a Novel  
678 Grid Search Method. *Analytica Chimica Acta* **2013**, 791, 25–35.  
679 <https://doi.org/10.1016/j.aca.2013.06.043>.
- 680 (31) Ghaffari, M.; Abdollahi, H. Duality Based Interpretation of Uniqueness in the Trilinear  
681 Decompositions. *Chemometrics and Intelligent Laboratory Systems* **2018**, 177, 17–25.  
682 <https://doi.org/10.1016/j.chemolab.2018.04.007>.
- 683 (32) de Juan, A.; Rutan, S. C.; Tauler, R.; Massart, D. L. Comparison between the Direct  
684 Trilinear Decomposition and the Multivariate Curve Resolution-Alternating Least Squares  
685 Methods for the Resolution of Three-Way Data Sets. *Chemometrics and Intelligent*  
686 *Laboratory Systems* **1998**, 40 (1), 19–32. [https://doi.org/10.1016/S0169-7439\(98\)00003-3](https://doi.org/10.1016/S0169-7439(98)00003-3).
- 687 (33) de Juan, A.; Jaumot, J.; Tauler, R. Multivariate Curve Resolution (MCR). Solving the  
688 Mixture Analysis Problem. *Anal. Methods* **2014**, 6 (14), 4964–4976.  
689 <https://doi.org/10.1039/C4AY00571F>.
- 690 (34) Saiyed, I. M.; Bullock, P. R.; Sapirstein, H. D.; Finlay, G. J.; Jarvis, C. K. Thermal Time  
691 Models for Estimating Wheat Phenological Development and Weather-Based Relationships  
692 to Wheat Quality. *Canadian Journal of Plant Science* **2009**, 89 (3), 429–439.  
693 <https://doi.org/10.4141/CJPS07114>.
- 694 (35) Chateigner-Boutin, A.-L.; Lapiere, C.; Alvarado, C.; Yoshinaga, A.; Barron, C.; Bouchet,  
695 B.; Bakan, B.; Saulnier, L.; Devaux, M.-F.; Girousse, C.; et al. Ferulate and Lignin Cross-  
696 Links Increase in Cell Walls of Wheat Grain Outer Layers during Late Development. *Plant*  
697 *Science* **2018**, 276, 199–207. <https://doi.org/10.1016/j.plantsci.2018.08.022>.
- 698 (36) Tauler, R. Multivariate Curve Resolution Applied to Second Order Data. *Chemometrics*  
699 *and Intelligent Laboratory Systems* **1995**, 30 (1), 133–146. [https://doi.org/10.1016/0169-7439\(95\)00047-X](https://doi.org/10.1016/0169-7439(95)00047-X).
- 700 (37) Tauler, R.; Smilde, A.; Kowalski, B. Selectivity, Local Rank, Three-Way Data Analysis  
701 and Ambiguity in Multivariate Curve Resolution. *Journal of Chemometrics* **1995**, 9 (1), 31–  
702 58. <https://doi.org/10.1002/cem.1180090105>.
- 703 (38) Cattell, R. B. Parallel Proportional Profiles and Other Principles for Determining the  
704 Choice of Factors by Rotation. *Psychometrika* **1944**, 9 (4), 267–283.
- 705 (39) Stegeman, A.; Sidiropoulos, N. D. On Kruskal's Uniqueness Condition for the  
706 Candecomp/Parafac Decomposition. *Linear Algebra and its Applications* **2007**, 420 (2–3),  
707 540–552. <https://doi.org/10.1016/j.laa.2006.08.010>.
- 708 (40) Culzoni, M. J.; Goicoechea, H. C.; Ibañez, G. A.; Lozano, V. A.; Marsili, N. R.; Olivieri, A.  
709 C.; Pagani, A. P. Second-Order Advantage from Kinetic-Spectroscopic Data Matrices in the  
710 Presence of Extreme Spectral Overlapping. *Analytica Chimica Acta* **2008**, 614 (1), 46–57.  
711 <https://doi.org/10.1016/j.aca.2008.03.013>.
- 712

- 713 (41) Windig, W.; Stephenson, D. A. Self-Modeling Mixture Analysis of Second-Derivative  
714 near-Infrared Spectral Data Using the SIMPLISMA Approach. *Analytical Chemistry* **1992**,  
715 *64* (22), 2735–2742. <https://doi.org/10.1021/ac00046a015>.
- 716 (42) Kennard, R. W.; Stone, L. A. Computer Aided Design of Experiments. *Technometrics*  
717 **1969**, *11* (1), 137–148. <https://doi.org/10.1080/00401706.1969.10490666>.
- 718 (43) de Juan, A.; Tauler, R. Factor Analysis of Hyphenated Chromatographic Data. *Journal of*  
719 *Chromatography A* **2007**, *1158* (1–2), 184–195.  
720 <https://doi.org/10.1016/j.chroma.2007.05.045>.
- 721 (44) Lachman, J.; Martinek, P.; Kotíková, Z.; Orsák, M.; Šulc, M. Genetics and Chemistry of  
722 Pigments in Wheat Grain – A Review. *Journal of Cereal Science* **2017**, *74*, 145–154.  
723 <https://doi.org/10.1016/j.jcs.2017.02.007>.
- 724 (45) Donaldson, L. Softwood and Hardwood Lignin Fluorescence Spectra of Wood Cell Walls  
725 in Different Mounting Media. *IWA Journal* **2013**, *34* (1), 3–19.  
726 <https://doi.org/10.1163/22941932-00000002>.
- 727 (46) Morrison, I. N. Ultrastructure of the Cuticular Membranes of the Developing Wheat Grain.  
728 *Revue canadienne de botanique* **1975**, *53* (18), 2077–2087.
- 729 (47) Fernández, S.; Osorio, S.; Heredia, A. Monitoring and Visualising Plant Cuticles by  
730 Confocal Laser Scanning Microscopy. *Plant Physiology and Biochemistry* **1999**, *37* (10),  
731 789–794. [https://doi.org/10.1016/S0981-9428\(00\)86692-9](https://doi.org/10.1016/S0981-9428(00)86692-9).
- 732 (48) Baker, E. Phenolic Constituents of Tomato Fruit Cuticles. *Phytochemistry* **1980**, *19*, 1415–  
733 1419.
- 734 (49) Antoine, C.; Peyron, S.; Mabilie, F.; Lapierre, C.; Bouchet, B.; Abecassis, J.; Rouau, X.  
735 Individual Contribution of Grain Outer Layers and Their Cell Wall Structure to the  
736 Mechanical Properties of Wheat Bran. *Journal of Agricultural and Food Chemistry* **2003**,  
737 *51* (7), 2026–2033. <https://doi.org/10.1021/jf0261598>.
- 738