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Prediction of Polyphenol Oxidase Activity Using vis-NIR Hyperspectral Imaging on Mushroom (*Agaricus bisporus*) Caps

Running title header: Prediction of PPO using HSI on mushroom caps

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1 **ABSTRACT**

2 Physical stress (i.e. bruising) during harvesting, handling and transportation triggers
3 enzymatic discoloration of mushrooms, a common and detrimental phenomenon largely
4 mediated by polyphenol oxidase (PPO) enzymes. Hyperspectral imaging (HSI) is a non-
5 destructive technique that combines imaging and spectroscopy to obtain information from a
6 sample. The objective of this study was to assess the ability of HSI to predict the activity of
7 PPO on mushroom caps. Hyperspectral images of mushrooms subjected to various damage
8 treatments were taken, followed by enzyme extraction and PPO activity measurement.
9 Principal component regression (PCR) models (each with 3 PCs) built on raw reflectance and
10 multiple scatter corrected (MSC) reflectance data were found to be the best modeling
11 approach. Prediction maps showed that the MSC model allowed for compensation of
12 spectral differences due to sample curvature and surface irregularities. Results reveal the
13 possibility of developing a sensor which could rapidly identify mushrooms with higher
14 likelihood to develop enzymatic browning and hence aid produce management decision
15 makers in the industry.

16

17 **KEYWORDS:** polyphenol oxidase, tyrosinase, mushrooms, *Agaricus bisporus*, vis-NIR
18 hyperspectral imaging.

19

20 INTRODUCTION

21 Button mushrooms (*Agaricus bisporus*) production is a fermentation industry that is able to
22 produce quality protein from cellulose based agricultural by-products (1). White button
23 mushrooms are one of the most important horticultural crops grown in Ireland with more
24 than 60,000 tons produced annually (2). This produce is very sensitive to inappropriate
25 handling and transportation practices, which cause irreversible injuries on the mushrooms
26 and enhance cap discoloration (3).

27 Browning of mushrooms is the major cause of quality loss that accounts for reduction in
28 their market value. Development of browning is the consequence of a series of biochemical
29 reactions in which polyphenol oxidase (PPO) enzymes, naturally present in mushrooms, play
30 an important oxidative role (4, 5). The PPO family includes catechol oxidase and laccase,
31 both of which oxidise diphenols into corresponding quinones (6). Quinones are slightly
32 colored products that undergo further reactions leading to high molecular mass dark
33 pigments called melanins. Brown discoloration is largely confined to the skin tissue of the
34 mushroom, where levels of phenols and polyphenol oxidase are higher than in other parts
35 of the fungi (7). PPO inactivation has been the target of several postharvest treatments
36 including thermal or microwave heating (8), irradiation (9) and addition of inhibitors (10).
37 However, consumer preference for fresh produce makes the management of PPO activity a
38 problem in the production, distribution and retail of fresh mushrooms.

39 Hyperspectral imaging (HSI) is a rapid and non-destructive technology that has recently
40 emerged as a powerful process analytical tool for food analysis (11). Hyperspectral images
41 are composed of hundreds of contiguous wavebands for each spatial position of an object.
42 Consequently, each pixel in a hyperspectral image contains the spectrum of that specific

43 position. Hyperspectral images, known as *hypercubes*, are three-dimensional blocks of data,
44 comprising two spatial and one wavelength dimension. Hypercube classification enables the
45 identification of regions with similar spectral characteristics. Since regions of a sample with
46 similar spectral properties have similar chemical composition, hypercube classification
47 allows for the visualisation of biochemical constituents of an object, as well as their
48 concentration and distribution over the sample. Due to the large size of hypercubes,
49 multivariate analytical tools, such as stepwise multiple linear regression (MLR), principal
50 component regression (PCR) and partial least squares regression (PLSR) are usually
51 employed for hyperspectral data mining and identification of key wavelengths for the
52 development of automated multispectral sensors.

53 Rapid spectroscopic techniques show potential for replacement of slow and/or expensive
54 analytical measurements while retaining sufficient accuracy (12). Recent studies have
55 demonstrated HSI to be a useful technology for the investigation of various mushroom
56 quality related issues, such as deterioration (13), freeze damage detection (14) and blemish
57 characterization (15). Recent advances in the application of HSI to the assessment of safety
58 and quality of other foodstuffs also include contaminant detection (16, 17), defect
59 identification (18-20), constituent analysis (21) and quality evaluation (22-24).

60 So far, hyperspectral imaging has not been employed to study the activity of enzymes in
61 mushrooms. Short wavelength infrared hyperspectral imaging was recently used to predict
62 α -amylase activity at early germination stages in two classes of wheat kernels and R^2 values
63 of 0.54 and 0.73, respectively, were achieved (25). Given that polyphenol oxidases play a
64 key role in the mushroom browning process and that extraction and current activity
65 measurement techniques, such as radiometric, electrometric, chronometric and especially

66 spectrophometric (26), are time consuming (as an example, in this study, 1.5-2 hours were
67 needed to obtain an extract and measure its activity), it would be desirable to have a fast
68 and non-destructive system that could estimate enzyme activity on mushroom caps. The
69 development of a hyperspectral imaging system with the ability to make simultaneous
70 predictions on multiple mushroom caps could enable faster detection of produce likely to
71 lose market value and hence reduce economical losses in the industry.

72 The aim of the present study was to investigate the potential of vis-NIR (445-945 nm)
73 hyperspectral imaging for the prediction of PPO enzyme activity on mushroom caps.

74

75 **MATERIAL AND METHODS**

76 **Mushroom supply and damage**

77 *Agaricus bisporus* mushrooms (strain Sylvan A15, Sylvan Spawn Ltd., Peterborough, UK)
78 were grown in plastic bags and tunnels in Kinsealy Teagasc Research Centre (Kinsealy, Co.
79 Dublin, Ireland) following common practice in the mushroom industry. Only uniform
80 undamaged closed cap mushrooms from the 1st and 2nd flush with a diameter of 3-5 cm
81 were hand-picked, placed in a metal grid and carefully delivered to the laboratory in
82 purpose-built containers, to minimize mechanical damage during transport. Mushrooms
83 arrived at the laboratory premises within 1 hour after harvesting and were stored overnight
84 at 4°C.

85 Some samples were subjected to vibrational bruising to simulate crop handling and
86 transport. Mushrooms were damaged in batches of 600g (approx) units inside polystyrene
87 plastic boxes. Mechanical damage was induced by using a Gyrotory Shaker Model G2

88 shaking table (New Brunswick scientific Co., Edison, N.J., USA) at 300 rpm amplitude for
89 controlled periods of time. A shaking period of 10 min led to loss of 6 units of lightness (L^*)
90 and color difference (ΔE) of 7.79 in C.I.E. $L^*a^*b^*$ color space. A shaking period of 20 min led
91 to loss of 12 units of L^* and ΔE of 15.57. ΔE defines the magnitude of the total color
92 difference and is expressed by the following equation:

$$93 \quad \Delta E = \sqrt{L_0^* - L^*{}^2 + a_0^* - a^*{}^2 + b_0^* - b^*{}^2}$$

94 where the $_0$ subscript refers to color measurements before shaking and no subscript refers
95 to color measurements after shaking.

96 Mushrooms were placed on polystyrene trays in groups of approx. 10 and over-wrapped
97 with PVC film following common practice in the mushroom industry. The trays were stored
98 under refrigeration (GRAM K400LU, Denmark) for the duration of the experiment.

99 Mushrooms of three damage levels [undamaged (D0), 10 min shaking damage (D10) and 20
100 min shaking damage (D20)] were monitored throughout five time points (days 0, 1, 2, 3 and
101 6 of storage).

102 At each sampling time point during refrigerated storage, one tray of each damage level was
103 randomly selected and removed from storage 15 min prior to testing. Wrapping was
104 removed and all the mushrooms in the packet were scanned with the hyperspectral imaging
105 equipment, then subsequently divided into two groups of five mushrooms for enzyme
106 extraction. This procedure was repeated for each tray. A total number of 549 mushrooms
107 were scanned and 114 extracts were obtained.

108

109 **Image acquisition system**

110 Hyperspectral images were obtained using a pushbroom line-scanning HSI instrument (DV
111 Optics Ltd, Padua, Italy). The instrument comprised a moving table, illumination source (150
112 W halogen lamp source attached to a fiber optic line light positioned parallel to the moving
113 table), mirror, objective lens (16 mm focal length), Specim V10E spectrograph (Spectral
114 Imaging Ltd, Oulu, Finland) operating in the wavelength range of 400-1000 nm
115 (spectroscopic resolution of 5 nm), CCD camera (Basler A312f, effective resolution of 580 ×
116 580 pixels by 12 bits), acquisition software (SpectralScanner, DV Optics, Padua, Italy) and PC.
117 A cylindrical diffuser was placed in front of the fiber optic line light to produce a diffuse light
118 source. In this study, only spectral data within the wavelength range of 445-945 nm were
119 used, as beyond this range the noise level of the camera is high and the signal efficiency of
120 the light source is low.

121 Reflectance calibration

122 Reflectance calibration was carried out prior to mushroom image acquisition in order to
123 account for the background spectral response of the instrument and the “dark” camera
124 response. The bright response (‘W’) was obtained by collecting a hypercube from a uniform
125 white ceramic tile; the dark response (‘dark’) was acquired by turning off the light source,
126 completely covering the lens with its cap and recording the camera response. The corrected
127 reflectance value (‘R’) was calculated from the measured signal (‘I’) on a pixel-by-pixel basis
128 as shown by:

129
$$R_i = \frac{(I_i - dark_i)}{(W_i - dark_i)}$$

130 where i is the pixel index, i.e. $i=1,2,3,\dots,n$ and n is the total number of pixels.

131 **Enzyme Extraction**

132 Mushroom homogenates were prepared in duplicate from each sample tray, as follows:

133 5g of the outer skin of mushroom caps were extracted using a sharp knife, chopped and
134 placed in a Turrax homogenizer (ULTRA-TURRAX T25, Janke & Kunkel IKA Labortechnik,
135 Germany) in a 1:4 (w: v) ratio with 0.5 M phosphate buffer, pH 6.5, containing 50g/L
136 polyvinylpyrrolidone (Sigma-Aldrich, Dublin, Ireland). Homogenization was carried out for 1
137 min at 4°C and 8000 rpm. The homogenate was centrifuged (2K15 Laborzentrifugen, SIGMA,
138 Germany) at 12,000g for 35 min at 4°C. The supernatant was collected by filtration through
139 no. 1 Whatman paper and used as crude enzyme extract. Extracts were kept at 4°C in the
140 dark until spectrophotometric assay (within 2 h).

141 PPO activity was measured spectrophotometrically by a modified method based on those of
142 Galeazzi et al. (27) and Tan and Harris (28). The reaction mixture contained 0.1 mL crude
143 enzyme extract and 2.9 mL substrate solution [0.011 mol/L catechol (Sigma-Aldrich, Dublin,
144 Ireland) as substrate in 0.05 mol/L phosphate buffer, pH 6.5]. The rate of catechol oxidation
145 was followed at 410 nm (UV2 UV/vis Spectrometer, UNICAM, UK) and 25°C and represented
146 against time. The maximum slope of the straight-line section of the activity curve was used
147 to express the enzyme activity (EAU/g of fresh mushroom). A unit of enzyme activity was
148 defined as an increase of 0.001 absorbance units per minute.

149 Enzyme activity was measured in triplicate for each mushroom extract and the average
150 value was computed. The standard error (SE) of this method was 350.50 EAU/g of fresh
151 mushroom.

152 **Image processing and data analysis**

153 Data were recorded in reflectance, saved in ENVI header format using the acquisition
154 software and then exported to MATLAB R2007b (The Math Works, Inc. USA).

155 Masking

156 A masking step was carried out to separate the mushroom pixels from the background. The
157 mask was created by thresholding the mushroom image at 940 nm, where a pixel threshold
158 value of 0.2 was used to segment the mushroom from the background. All background
159 regions were set to zero and the non-zero elements of the image were used to extract one
160 mean spectrum for each mushroom.

161 False RGB images

162 False RGB images were obtained by extracting mushroom images at 460 nm (blue), 545 nm
163 (green) and 645 nm (red) and stacking them.

164 Model building

165 One of the main challenges involved in building predictive models with hyperspectral image
166 data is that such images contain a vast amount of spectral data, whilst only one or a few
167 measurements of the variable of interest can be taken for each sample studied. In this
168 particular study, the reference method for enzyme extraction involved using the skin of
169 three to five mushrooms to obtain one single enzyme extract. Consequently, three to five
170 hyperspectral images were to be matched with one single enzyme activity value in
171 regression modeling.

172 When developing regression models with hyperspectral data, it is common practice to
173 extract the mean spectrum of each sample and use it to build a prediction model to
174 estimate an attribute (29). With that approach in mind, two different modeling strategies
175 were used:

176 a) Strategy 1: The first strategy extracted the mean spectrum of each mushroom and
177 assigned the same enzyme activity value to all the mushrooms used in obtaining one
178 particular extract. A training set of $n_{\text{TRAIN}_1}=280$ and a test set of $n_{\text{TEST}_1}=269$ were
179 used for this strategy.

180 b) Strategy 2: The second strategy computed the mean spectra of all the mushrooms
181 used to obtain one enzyme extract and assigned the enzyme activity value of that
182 extract to the resulting spectrum. A training set of $n_{\text{TRAIN}_2}=60$ and a test set of
183 $n_{\text{TEST}_2}=54$ were used for this strategy.

184 The following spectral preprocessing methods were used in order to remove non-chemical
185 biases, such as scattering effects and variations arising from mushroom surface curvature,
186 from the spectral information: standard normal variate (SNV) (30) and multiplicative scatter
187 correction (MSC) (31). MSC aims to reduce the effects of scattering in a set of spectra by
188 performing linear regression on a “target” spectrum. Two different target spectra led to two
189 different MSC methods: a) “set MSC”, where the mean spectrum of each mushroom was
190 corrected using the mean spectrum of the data set as the target spectrum and b) “sample
191 MSC”, where the spectrum of each pixel in a mushroom was corrected using the mean
192 spectrum of that mushroom as the target spectrum. The mean sample MSC corrected
193 spectrum for each mushroom was obtained and used for the model.

194 To improve normality of the distribution of the reference variable, enzyme activity values
195 were transformed into natural logarithmic units and mean centered.

196 Three regression methods were used to build models for enzyme activity prediction:

197 a) Multiple linear regression (MLR): optimal wavelengths for enzyme activity prediction
198 were selected by the “forward” method in best subsets stepwise linear regression
199 using the “leaps” package in R (32). Multicollinearity of predictor variables is
200 problematic for MLR models based directly on spectroscopic values, tending to
201 results in unstable model predictions (33). The variance inflation factor (VIF) is an
202 index commonly used to measure the collinearity between variables in regression
203 models: typically, predictor variables with $VIF > 10$ are considered to be highly
204 correlated. In order to test the predictor wavelengths for multicollinearity, the VIF of
205 each predictor was calculated using the “DAAG” package in R (32).

206 b) Principal component regression (PCR): principal component analysis (PCA) reduces
207 the dimensionality of spectral data by transforming them into principal component
208 scores in order of decreasing variance. The autoscaled matrix of spectral values was
209 transformed into PC space by representing the original data in the directions defined
210 by orthogonal eigenvectors using R (32). PCR models were developed using PC space
211 scores instead of wavelength space values. Analysis of variance (ANOVA) was
212 employed using R (32) to compare models with increasing number of PCs. The
213 decision on the number of PCs to be taken for each model was made based upon
214 ANOVA test results. Only significant components ($p < 0.05$) were included in the
215 model.

216 c) Partial least squares regression (PLSR): this technique is commonly used when
217 predicting a response from many measured variables which may be collinear. PLSR
218 was applied using the “pls” package in R (32). Leave-one-out cross-validation was
219 used on the training set. Performance of the prediction models was evaluated using
220 the root of the mean of the sum of squared differences between predicted and
221 measured enzyme activity values of the training set (RMSECV) and the number of
222 latent variables required (# LV). The optimal number of latent variables for inclusion
223 in the PLSR models was estimated using the method described by Martens et al. (34)

224 The experiment was carried out two times, making two independent mushroom sets: a
225 training set ($n_{\text{TRAIN}_1}=280$ mushrooms and $n_{\text{TRAIN}_2}=60$ extracts) and a test set ($n_{\text{TEST}_1}=269$
226 mushrooms and $n_{\text{TEST}_2}=54$ extract). Overall, 549 mushrooms were used to obtain 114
227 extracts in total. All of the models were built on training sets and then applied to
228 independent test sets of samples. The ratio of percentage deviation (RPD), which is the
229 ratio of the standard deviation of the laboratory measured (reference) data to the root-
230 mean-square of cross-validation ($\text{RPD}_{\text{TRAIN}}$) or root-mean-square error of prediction (RPD_{TEST})
231 (35), was used to assess model performance. Twenty four models were classified in terms of
232 their ability to generalize following criteria outlined by Viscarra Rossel et al. (36), based on
233 which $\text{RPD}_{\text{TEST}} < 1.0$ indicates very poor model/predictions and their use is not recommended;
234 $1.0 < \text{RPD}_{\text{TEST}} < 1.4$ indicates poor model/predictions where only high and low values are
235 distinguishable; $1.4 < \text{RPD}_{\text{TEST}} < 1.8$ indicates fair model/predictions that may be used for
236 assessment and correlation; $1.8 < \text{RPD}_{\text{TEST}} < 2.0$ indicates good models/predictions where
237 quantitative predictions are possible; $2.00 < \text{RPD}_{\text{TEST}} < 2.5$ indicates very good, quantitative
238 model/predictions and $\text{RPD}_{\text{TEST}} > 2.5$ indicates excellent model/predictions.

239 Prediction maps

240 The two models whose performance was found to be best were selected and applied to
241 each pixel in the hypercube data of individual mushrooms. This enabled the generation of
242 virtual prediction images for enzyme activity.

243

244 **RESULTS AND DISCUSSION**

245 **Spectra**

246 Average reflectance spectra obtained from the hyperspectral imaging data of undamaged
247 (D0), damaged 10 (D10) and damaged 20 (D20) mushrooms are shown in Figure 1a. The
248 average reflectance of damaged samples was lower than the average reflectance of non-
249 damaged mushrooms over the entire spectral region. Bruising due to mechanical damage
250 was expected to have led to loss of whiteness and lightness (L^*) and therefore lower
251 reflectance values. A remarkable difference in intensity was observed between D0 and D20
252 mushrooms, whereas the intensity of D10 spectra was intermediate between D0 and D20.
253 Broad spectra in the visible-near infrared wavelength range are characteristic of undamaged
254 mushrooms, corresponding to their white appearance (13). The greatest differences in
255 shape between bruised and non-bruised samples arose in the 600-800 nm region, where
256 undamaged mushrooms exhibited broader spectral features than the damaged mushrooms.
257 The spectral differences mentioned above could be related to the formation of brown
258 pigments (14) mainly melanins, which derive from enzyme-catalyzed oxidation products
259 called quinones.

260 **Enzyme activity**

261 The average polyphenol oxidase enzyme activity of each mushroom group is shown in
262 Figure 1b. The higher activity values observed in bruised mushrooms suggest that
263 mechanical damage has an effect on enzyme expression. Considering that physical injuries
264 are one of the factors that lead to mushroom browning (3) and that this phenomenon is
265 mediated by PPO enzymes (37), this result was not unexpected. The difference in PPO
266 activity between D10 and D20 was not significant ($p>0.05$), which could mean that the stress
267 caused by D10 damage level was sufficiently high to bring enzyme expression to its
268 maximum, and further damage did not contribute to further activation of tyrosinase.

269 **Modeling**

270 VIF was greater than 10 for every MLR model built with more than two wavelengths.
271 Therefore, MLR models that used only two wavelengths were considered for further
272 analysis. In the case of PCR models, the inclusion of the third PC was not always significant
273 ($p<0.05$) so 2 and 3 PC models were considered for further sections. For all PLSR models, 2
274 was the optimal number of latent variables to include in the model. Previous studies in the
275 field employed models that performed well using low numbers of wavelengths (13),
276 principal components (14, 38) or PLS latent variables (39).

277 Model performance in terms of RPD is shown in Table 1. RPD_{TRAIN} is a measure of model
278 performance within the model training data set and RPD_{TEST} indicates how the model
279 performed when applied to an independent model testing data set. RPD_{TEST} was considered
280 to be more adequate to assess model performance and further sections of this paper will
281 focus only on RPD_{TEST} values.

282 Models were classified in terms of RPD_{TEST} as follows: $RPD_{TEST} < 1.0$ = “very poor”,
283 $1.0 < RPD_{TEST} < 1.4$ = “poor”; $1.4 < RPD_{TEST} < 1.8$ = “fair”; $1.8 < RPD_{TEST} < 2.0$ = “good”;
284 $2.00 < RPD_{TEST} < 2.5$ = “very good” and $RPD_{TEST} > 2.5$ = “excellent”.

285 Strategy

286 Overall, models with a better generalization ability to predict the independent data set were
287 obtained when strategy 1 was employed. As it can be seen in Table 1, for any preprocessing
288 and chemometric technique combination, the RPD obtained under model strategy 1 (i.e.
289 when the mean spectrum of each mushroom was extracted and the same enzyme activity
290 value was assigned to all the mushrooms used for one extract) was higher than the RPD
291 obtained under model strategy 2 (i.e. when the mean spectra of all the mushrooms used to
292 obtain one enzyme extract was computed and the enzyme activity value of that extract was
293 assigned to the resulting spectrum). In fact, strategy 2 only gave “poor” or “very poor”
294 predictive models, whose RPD_{TEST} ranged from 0.81 to 1.3. This could be because when the
295 mean spectrum was computed for an extract under strategy 2, some features arising from
296 the original spectral variability of the mushrooms within that extract might have been lost.
297 This would result in partial loss of their ability to generalize and decrease in RPD_{TEST} values.

298 Pre-treatment

299 For MLR, raw reflectance spectral data and sample MSC corrected reflectance spectra led to
300 better performance models than SNV or set MSC spectra. The better models were “fair” and
301 the worse ones were “poor” (according to the previously mentioned RPD classification) and
302 therefore discarded. Similar trends were observed in PCR models, where “very good”
303 models were obtained with raw reflectance and sample MSC corrected reflectance spectra

304 (RPD_{TEST}=2.13 with 3 PCs), a “good” model with SNV pre-treated reflectance data
305 (RPD_{TEST}=1.84 with 2 PCs) and a “fair” model with set MSC corrected reflectance spectra
306 (RPD_{TEST}=1.77 with 2 PCs). The number of PCs was lower in the case of SNV and set MSC but
307 adding a third one did not significantly improve model performance or RPD_{TEST}. For PLSR
308 models, all pre-treatments resulted in “poor” models, whose highest RPD_{TEST} was 1.22.

309 Regression method

310 Under strategy 1, PCR models performed better than MLR or PLSR models for all of the pre-
311 treatments. This happened for both training and test sets. The performance of MLR and
312 PLSR models for the test set was not as good as it was for the training set, but that did not
313 happen for PCR models, where RPD_{TEST} values were higher than RPD_{TRAIN} values.

314 Under model strategy 2, all chemometric methods performed similarly for the training set.
315 For the test set, PCR models performed better than MLR or PLSR but still “poor” predictions
316 (RPD_{TEST}<1.3) were obtained.

317 PCR models developed on raw reflectance and sample MSC corrected reflectance data
318 under model strategy 1 were selected as best models and used in further analysis. The
319 coefficient of determination and root mean-squared error of cross-validation/prediction for
320 these models were: R²_{TRAIN_1}=0.75, RMSECV=0.38 [ln(EAU/g)], R²_{TEST_1}=0.78 and RMSEP=0.30
321 [ln(EAU/g)]. Root mean-squared errors of cross-validation/prediction are frequently used to
322 assess the performance of the regression and low values indicate good models.

323 In Figure 2, enzyme activity values predicted by one of the selected models (model strategy
324 1, PCR, raw reflectance data) are plotted against experimental enzyme activity values, for (a)
325 training and (b) test sets, respectively. The range of measured reference values was wider in

326 the training set than in the test set, where PPO activity levels were, in general terms, lower
327 and confined to a narrower range of values. This scenario is not optimal for model testing
328 but it is common when dealing with horticultural products, whose postharvest behavior is
329 known to be affected by biological variation. Burton (3) reported that mushroom bruisability
330 can vary from crop to crop. A study by Mohapatra et al. (40) observed 30% to 41% variability
331 in enzyme activity measurements and attributed it to batch-to-batch variability. Some
332 vertical scattering can be seen in this figure too, indicating variability in predicted values for
333 mushrooms with similar reference enzymes activities. This would explain the relatively low
334 values of the coefficients of determination obtained ($R^2_{\text{TRAIN}_1}=0.75$ and $R^2_{\text{TEST}_1}=0.78$). The
335 horizontal scattering is mainly attributable to mushroom to mushroom variability.

336 Prediction maps

337 Hyperspectral imaging has the ability to map the spatial distribution of components on a
338 sample. The two selected models (model strategy 1, PCR, non-treated reflectance and
339 sample MSC corrected reflectance) were applied to each pixel in the hypercube data of
340 individual mushrooms and that enabled the generation of virtual prediction images for
341 enzyme activity. In such images, the grayscale intensity is related to the value of the
342 predicted enzyme activity at different regions of the mushroom cap: the lighter the color,
343 the higher the predicted activity value.

344 Figure 3 and Figure 4 show the predicted distribution of enzyme activity over the cap of
345 undamaged (D0) and damaged (D20) mushroom samples, respectively. Each figure shows
346 (a) false RGB images, (b) prediction maps based on the raw reflectance model and (c)
347 prediction maps based on the sample MSC pre-treated reflectance model of four mushroom
348 caps whose skin was processed together to obtain one single enzyme extract. The mean and

349 standard deviation (SD) of the predictions, both in $[\ln(\text{EAU/g})]$, are displayed below each
350 map in (b) and (c). The values below false RGB images correspond to the activity
351 measurement obtained experimentally for each extract, which is the same for all of the
352 mushrooms within each figure.

353 The main difference between the prediction images of D0 and D20 is the grayscale intensity.
354 The dark gray tonality in Figures 3(b) and 3(c) indicates that the models predicted low
355 activity values on D0 mushroom caps. D20 predictions, on the contrary, show much lighter
356 colours in Figures 4(b) and 4(c), which reveal higher predicted values for enzyme activity. At
357 scanning time, damaged mushrooms looked different from undamaged ones and the
358 corresponding extracts exhibited much higher enzyme activity, for which it was expected
359 that the models would generate very different prediction images according to damage level.

360 For all of the mushrooms in Figures 3 and 4, the mean predicted values by raw reflectance
361 and sample MSC corrected reflectance models (displayed under each image in columns (b)
362 and (c)) were very similar. This indicates that both raw reflectance and sample MSC
363 corrected reflectance models performed very similarly in terms of quantitative prediction.
364 This is in agreement with the similarities observed previously in the coefficient of
365 determination and the root-mean-square error of both models. However, the very different
366 appearance of predictions maps in (b) and (c) point out these two models have some
367 dissimilarities too.

368 • In raw reflectance predicted images (Figures 3(b) and 4(b)), the distribution of
369 enzyme activity prediction is uneven throughout the cap. The relatively high
370 standard deviation values under each map reveal this heterogeneity too. As clearly
371 seen in Figure 3(b), the highest predicted values concentrate around the mushroom

372 edges, (i.e the region showing higher level of bruising on false RGB images (Figure
373 3(a)). This could be partly due to increased presence of brown coloured pigments at
374 edge regions, which are derived from PPO-mediated reaction products, but spectral
375 differences related to mushroom curvature might have also affected the
376 performance of the model differently in different regions of the cap. It is difficult to
377 estimate the extent of such phenomena and at this point. The lack of shading effects
378 in Figure 4(b), where predicted values do not show any clear morphological trend,
379 suggest that the effect of sample curvature on the reflectance model may not be
380 observable when the levels of damage and browning are high.

381 • However, it is interesting to note that all of 3(b) and 4(b) figures reveal the ability of
382 this model to point out the regions that look “different” in false RGB images. The
383 model captures the spectral variability arising from surface bruises/marks (e.g.
384 confined regions which show browner colour in false RGB images) and reflects it
385 onto the prediction maps. For undamaged mushrooms, Figure 3(b) exhibits lighter
386 grayscale tonality (indicating higher predicted value) on the small regions that show
387 signs of bruising in Figure 3(a). Similarly, for damaged mushrooms, Figure 4(b)
388 presents darker color (indicating lower predicted value) on those regions where
389 browning had yet not developed in Figure 4(a).

390 • Sample MSC corrected reflectance predicted images, on the other hand, appear
391 smoother than raw reflectance predictions. All the pixels within one sample MSC
392 corrected reflectance prediction image have similar predicted values and therefore
393 the grayscale intensity is very uniform and the SD values are low. The MSC correction
394 estimates the relation of the scatter of each pixel with respect to the target
395 spectrum (in this case, the mean spectrum of all the pixels) (31). Thus, a similar level

396 of scatter is obtained for all spectra and the effect that the morphology of the
397 sample (i.e. mushroom curvature) could have on the model is diminished too.

398 Figure 5 shows the enzyme activity prediction of imaginary lines drawn through the centre
399 of each mushroom cap, shown in red in Figure 5(a). Figure 5(b) shows how the raw
400 reflectance model predicted the pixel values on those lines; the pixels that form the line are
401 represented in the X axes, while the predicted enzyme activity values are shown in the Y
402 axes. The line in Figure 5(c) corresponds to the prediction of the sample MSC corrected
403 reflectance model. For an undamaged mushroom (see top row), the curved shape of the
404 prediction line in (b) indicates that pixels from the centre and edge regions of the cap were
405 predicted differently; the activity was low in the central region of the mushroom and
406 increased gradually towards the edges. This is in agreement what was observed in Figure
407 3(b) and could be because the enzyme activity distribution was not uniform along the
408 mushroom cap surface or because this model is not able to deal with spectral differences
409 arising from mushroom cap surface curvature. The line in (c), predicted by the sample MSC
410 corrected reflectance model, is much flatter than the one in (b), which indicates that
411 predictions along the imaginary line were more homogeneous and suggests enzyme activity
412 was equally distributed over the mushroom cap. Despite the fact that both models
413 predicted similar mean activity values (9.91 [ln(EAU/g)] and 9.94 [ln(EAU/g)], respectively),
414 differences in pixel distribution suggest that the ability of each model to overcome spectral
415 variability due to sample morphology is different. For damaged mushrooms (see bottom
416 row), the line predicted by the reflectance model (b) was uneven but, as opposed to what
417 was observed in the undamaged mushroom, it did not have a clear curved shape. In this
418 case, the variation of predicted enzyme activity values across the imaginary line could be

419 related to the level of damage/browning, whereas the relationship between predicted
420 values and pixel position/surface curvature was not as clear as for undamaged mushrooms.
421 The line in (c) was flatter than in (b), as observed for undamaged mushrooms. Raw
422 reflectance and and sample MSC corrected reflectance models predicted almost identical
423 mean enzyme activity values (10.36 [ln(EAU/g)] and 10.37 [ln(EAU/g)], respectively) and
424 their distributions across pixel line was more similar than in the case of undamaged
425 mushrooms.

426 The ability of a HSI system to predict PPO activity on mushroom caps was assessed in this
427 study. PPO activity prediction maps of were generated to gain understanding of (a) the
428 distribution of the enzyme activity over the mushroom cap and (b) the effect of sample MSC
429 pre-treatment on the predictive ability of the model. Results reveal some potential of vis-
430 NIR hyperspectral imaging as a tool to estimate the activity of enzymes responsible for
431 mushroom browning. The mushroom industry could benefit from such a tool for rapid
432 identification of mushrooms of reduced marketability.

FIGURES

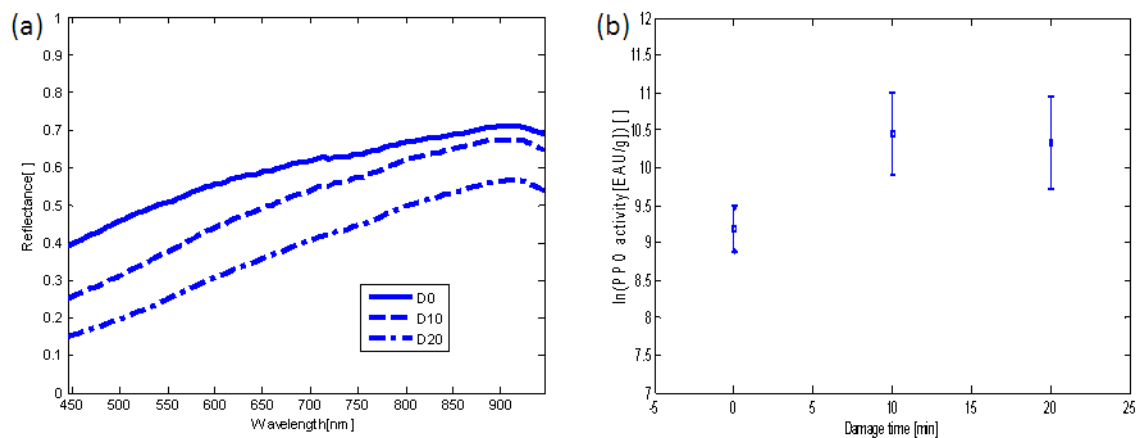


Figure 1 (a) Average raw reflectance spectra for mushroom at different damage levels. (b) Average \pm standard deviation of polyphenol oxidase activity as a function of damage level.

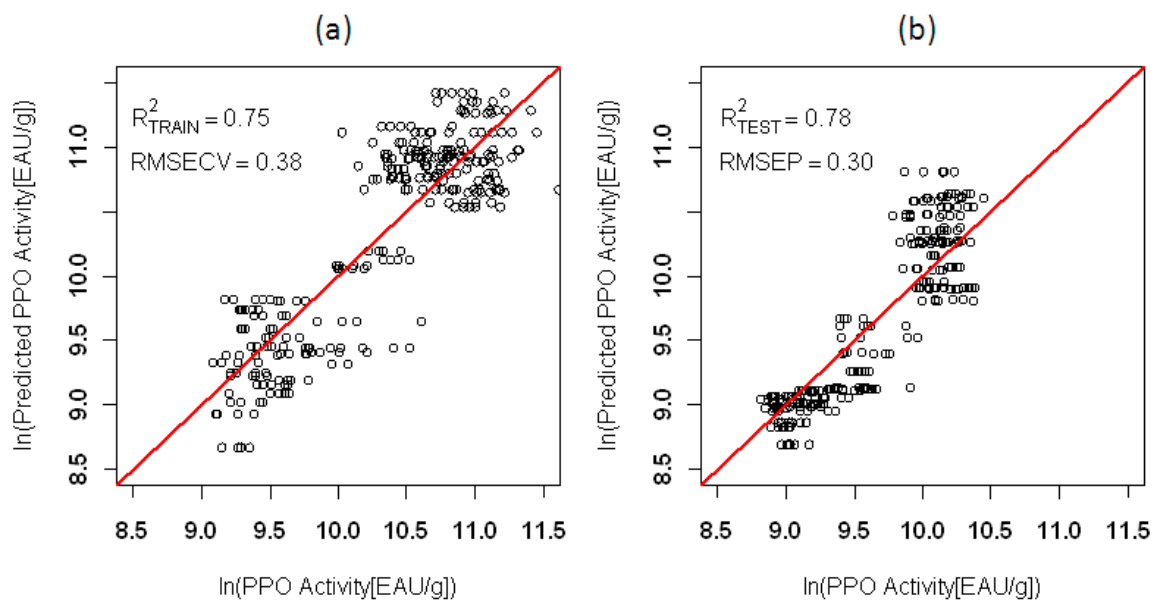


Figure 2 Predicted PPO activity as a function of actual PPO activity for 3 PC PCR model applied to training (left) and test (right) raw data sets under model strategy 1.

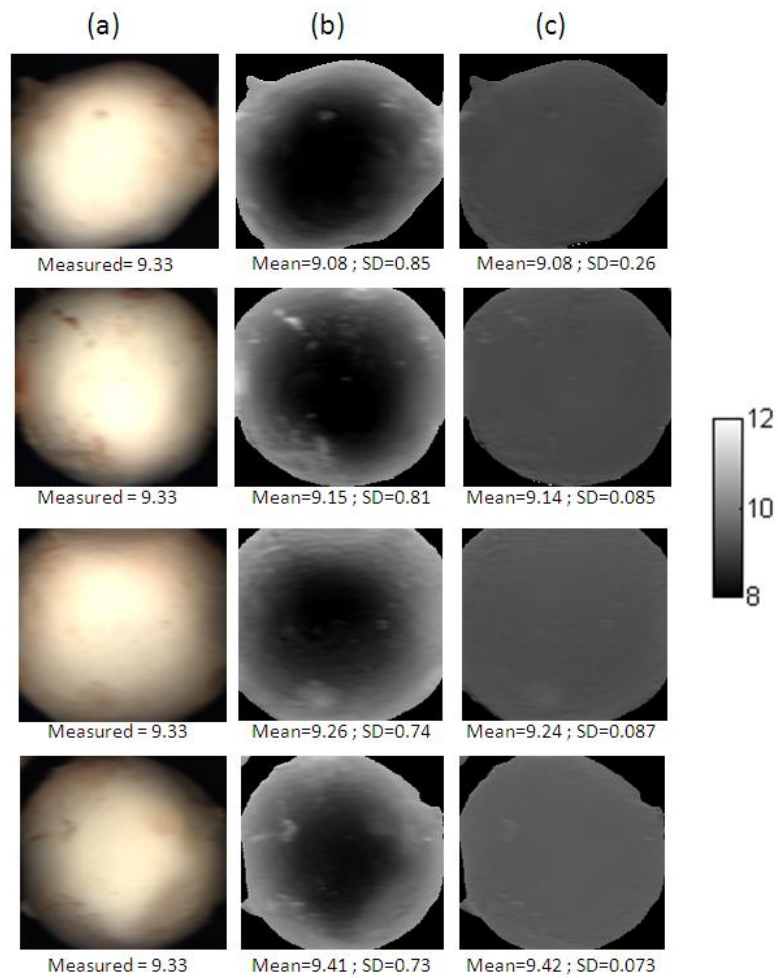


Figure 3 Undamaged mushroom caps, where (a) false RGB image, (b) prediction maps by raw reflectance model and (c) prediction maps by sample MSC corrected reflectance model.

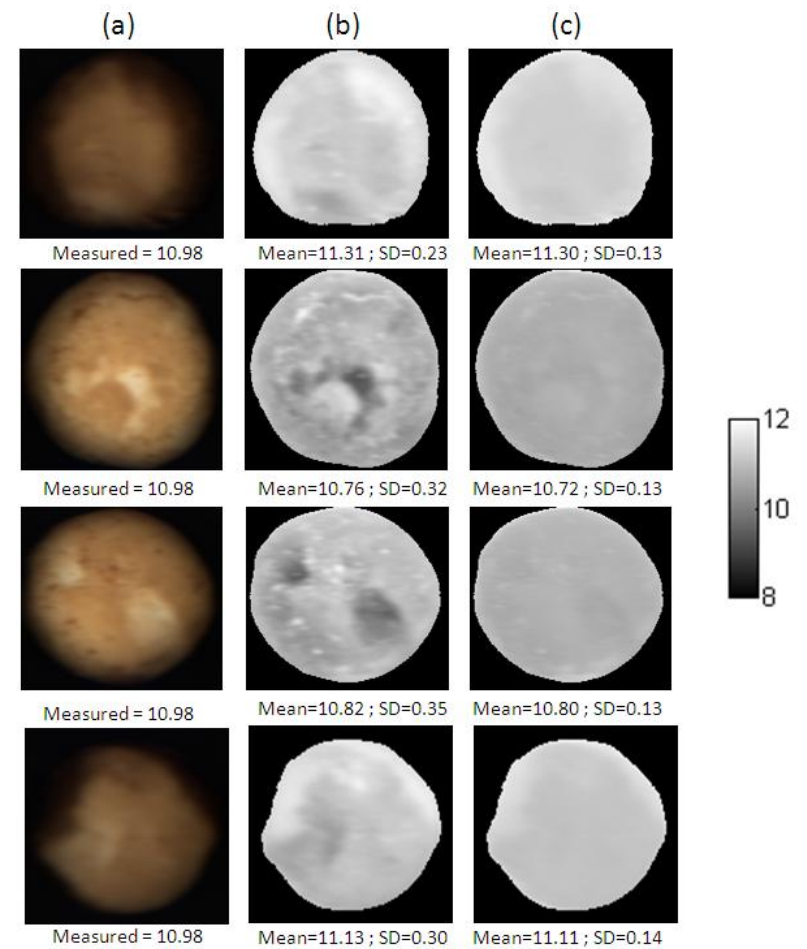


Figure 4 Damaged mushroom caps, where (a) false RGB images, (b) prediction maps by raw reflectance model and (c) prediction maps by sample MSC corrected reflectance model.

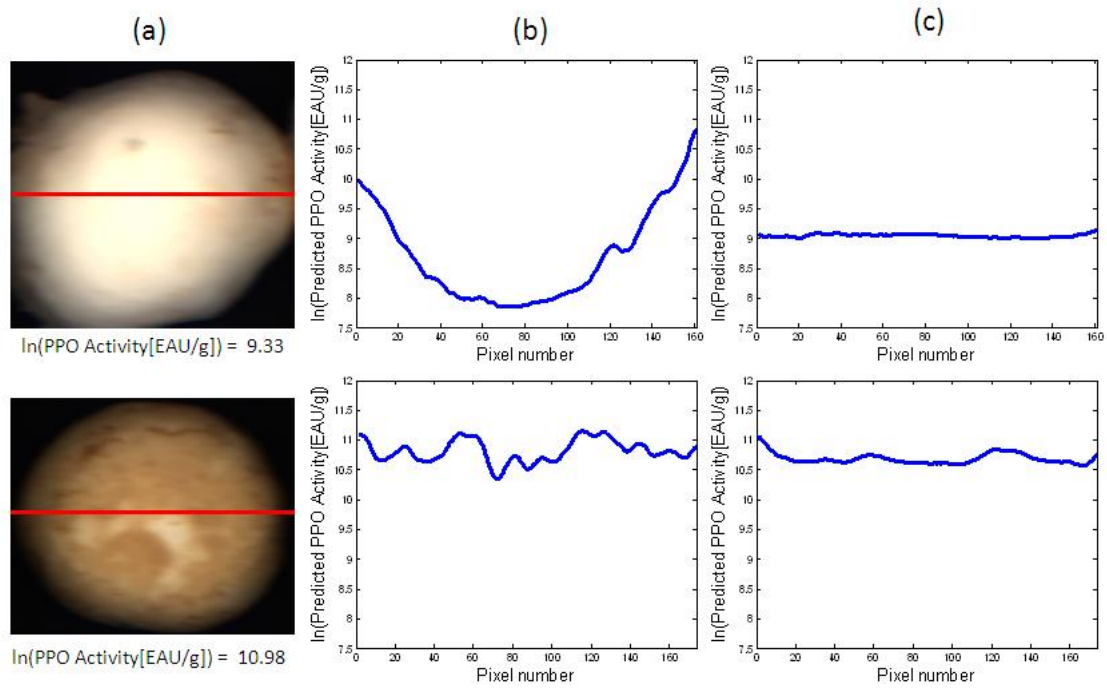


Figure 5 (a) Imaginary line drawn through the centre of false RGB images of undamaged (top row) and damaged (bottom row) mushroom caps and their corresponding predictions by (b) raw reflectance model and (c) sample MSC corrected reflectance model.

TABLES

Table 1 Ratio percentage deviation (RPD) for different model strategies, spectral pre-treatments and chemometric methods. MLR: multiple linear regression; PCR: principal component regression; PLSR: partial least squares regression; SNV: standard normal variate; MSC: multiple scatter correction; # PCs: number of principal components; # LVs: number of latent variables.

Strategy	Pre-treatment	MLR			PCR			PLSR		
		λ (nm)	RPD _{TRAIN}	RPD _{TEST}	# PCs	RPD _{TRAIN}	RPD _{TEST}	# LVs	RPD _{TRAIN}	RPD _{TEST}
1*	None	450, 945	1.87	1.47	3	2.01	2.13	2	1.95	1.16
	SNV	835, 560	1.02	1.06	2	1.71	1.84	2	1.63	1.22
	Set MSC	835, 545	1.52	1.14	2	1.65	1.77	2	1.62	1.20
	Sample MSC	465, 945	1.91	1.43	3	2.01	2.13	2	1.95	1.14
2*	None	470, 945	1.28	1.16	2	1.27	1.30	2	1.25	0.97
	SNV	450, 465	1.22	1.07	1	1.17	1.20	2	1.17	0.85
	Set MSC	450, 575	1.15	0.89	1	1.17	1.16	2	1.17	0.81
	Sample MSC	495, 945	1.35	1.22	2	1.35	1.27	2	1.33	1.22

*as described in *Model building* subsection of *Materials and Methods* section.

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