Visible and near infrared spectroscopy as an authentication tool:

Preliminary investigation of the prediction of the ageing time of

beef steaks

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21 **Abstract**

22 Visible-near infrared spectroscopy (Vis-NIRS) has been suggested to have potential for
23 authentication of food products. The aim of the present preliminary study was to assess if this
24 technology can be used to authenticate the ageing time (3, 7, 14 and 21 days *post mortem*) of beef
25 steaks from three different muscles (*M. Longissimus thoracis*, *M. Gluteus medius* and *M.*
26 *Semitendinosus*). Various mathematical pre-treatments were applied to the spectra to correct scattering
27 and overlapping effects, and then partial least squares-discrimination analysis (PLS-DA) procedures
28 applied. The best models were specific for each muscle, and the ability of prediction of ageing time
29 was validated using full (leave-one-out) cross-validation, whereas authentication performance was
30 evaluated using the parameters of sensitivity, specificity and overall correct classification. The results
31 indicate that overall correct classification ranging from 94.2 to 100% was achieved, depending on the
32 muscle. In conclusion, Vis-NIRS technology seems a valid tool for the authentication of ageing time of
33 beef steaks.

34 **Keywords:** partial least square discriminant analysis; meat maturation; fraud; chemometrics; premium
35 steaks.

36

37 1. Introduction

38 During ageing meat undergoes many biochemical changes, the most interesting of which are related
39 to the activity of proteases, which increase palatability (Bidner, Montgomery, Bagley, & McMillin,
40 1985; Dransfield, 1994). Therefore, aged beef products may command a premium because customers
41 are willing to spend more on the expectation of a better eating experience (Feuz, Umberger, Calkins, &
42 Sitz, 2004). For these reasons, both the meat industry and consumers have an urgent need for methods
43 that can ensure that products that are labelled as “aged” for a particular period are genuine (Downey,
44 1996). This might be particularly important for wet aged meat cuts [wet ageing is the storage of meat
45 cuts in chilled vacuum packaging for between 7 to 35 days (Smith et al., 2008; Warren & Kastner,
46 1992)]. It must be stressed that nowadays vacuum package is a well-established procedure for
47 facilitating extended ageing of beef, especially for the hospitality and restaurant industry (Watson,
48 1994), but also becoming common at retail level in many countries. To our knowledge, few methods
49 have been developed to ensure the authenticity of steaks marketed as “aged” and to guard against
50 fraudulent products. Briefly, the state-of-the-art knowledge in the field indicates that the content of
51 hypoxanthine and xanthine may be used in biosensors to predict meat ageing (Yano, Kataho,
52 Watanabe, Nakamura, & Asano, 1995), while Castejón, García-Segura, Escudero, Herrera, & Cambero,
53 (2015) proposed the use of metabolomics in ageing prediction. However, the use of analytical methods
54 to verify ageing of beef is lengthy, complex, destructive and not suitable for application in quality
55 control *per se*. On the other hand, the use of new rapid and non-destructive spectroscopic methods has
56 been examined by Bauer, Scheier, Eberle, & Schmidt (2016) who tried to predict meat tenderness using
57 RAMAN on steaks differing in ageing time. Visible-near infrared reflectance spectroscopy (Vis-NIRS)
58 also seems promising, since it is a non-destructive technique that is quick and relatively easy to use
59 (Prieto, Roehe, Lavín, Batten, & Andrés, 2009). A near infrared (NIR) spectrum is a specific

fingerprint of a sample due to the identifiable absorption of radiation of each organic compound (water, protein and fat) at different wavelengths (from 400 to 2500 nm). As this fingerprint can be modified by biochemical changes taking place in meat during ageing (Damez & Clerjon, 2008; Hildrum, Nilsen, Mielnik, & Næs, 1994) or changes in physical characteristics such as colour (Prieto, Andrés, Giráldez, Mantecón, & Lavín, 2008) measurement of the NIR spectrum may have potential for authenticating the ageing of meat. In fact, successful applications of near infrared reflectance spectroscopy (NIRS) technology include the discrimination of fresh and frozen beef (Downey & Beauchêne, 1997), the identification of different chicken meat muscles (McDevitt, Gavin, Andrés, & Murray, 2005) and the identification of meat from different animal species (Downey, McElhinney, & Fearn, 2000). However, despite the versatility of NIRS technology, there are no studies specifically designated to use NIRS technology as an authentication tool for aged beef steaks in the meat industry. Consequently, the present preliminary study was designed to fill this industry need. Our hypothesis was that NIRS technology is able to detect biochemical changes taking place during ageing and therefore discriminate between steaks aged for different period of time. The aim of this preliminary study was to investigate the reliability and accuracy of Vis-NIRS linked to chemometric analysis to detect the specific fingerprint of beef samples from three different muscles, differing in ageing time, independent of chemical information.

2. Material and Methods

2.1 Slaughter procedure, sampling and scanning of samples

Fourteen Holstein steers, twenty-four months-old and reared in the same production system were slaughtered in a commercial plant by captive bolt stunning followed by exsanguination from the jugular vein, evisceration and hide removal. Then, each half carcass from the same animal followed a different treatment, being there suspended using Achilles or Aitch bone methods to create variability in

tenderness (28 half carcasses in total). After 48 h in the chill at 0°C the *M. Longissimus thoracis* (LT, 10th rib), *M. Gluteus medius* (GM) and *M. Semitendinosus* (ST) muscles were removed, vacuum packed and transported to Teagasc, Food Research Centre Meat Industrial Development Unit, Ashtown, Dublin. At 3 days post-mortem (unaged), 25 mm steaks were cut, wrapped in oxygen-permeable polyvinylchloride film (oxygen permeability of 580 mL m⁻² h⁻¹) and kept at standard temperature (2°C ± 0.5). Additional steaks were cut at 3 days post-mortem, vacuum packed in polyethylene bags and stored in a chill (3°C ± 1°C) until they reached 7, 14 or 21 days of ageing. At each ageing time, and after 1 hour of oxygenation, the steaks were scanned (350-2500 nm at 1 nm intervals, raw spectra) in reflectance mode using a portable ASD Labspec 5000 (ASD Inc., Boulder Colorado, USA) NIR spectrometer fitted with a high-intensity contact probe with a 10 mm spot size and using the Indico Pro program (ASD Inc.). Raw spectra were recorded in duplicate (two different spectra per steak) by removing and replacing the scanning probe head on the meat surface between scans (De Marchi, 2013). Between readings, the probe was cleaned with ethanol and dried with a paper tissue. Finally, the whole set of spectra (672 spectra) collected was exported as JCAMP [log(1/R)] to the Unscrambler X version 10.3 (CAMO ASA, Oslo, Norway) for chemometric analysis (1 steak x 28 half carcasses x 3 muscles x 4 ageing periods x 2 duplicate spectra) .

2.2 Chemometric procedures

The bibliography available for authentication shows a number of different chemometric approaches that can be used to analyse the samples for authenticity. We followed the chemometric procedures described by Osorio et al. (2013) and Woodcock, Downey, & O'Donnell, (2008) modified slightly to fit with the design characteristics of the present study. Briefly, the spectra were examined and clearly incorrect scans were discarded; duplicate spectra were averaged and the beginning (350-399 nm) and end (2390-2500 m) wavelength ranges were avoided due to high distortion in these regions. Principal

106 component analysis (PCA) using full (leave-one-out) cross-validation with an uncertainty test for
107 optimal number of components and a Non-linear Iterative Partial Least Square (NIPALS) algorithm
108 was applied using all the averaged raw spectral data (n=336 spectra) as a general screening of outliers.
109 This procedure allowed the identification of four outliers per muscle (all of them from the same half-
110 carcass) that were discarded before subsequent chemometric analyses.

111 The spectra corresponding to each muscle (n=108 spectra per muscle) were modified by applying
112 different mathematical pre-treatments [standard normal variate (SNV), second derivate Savitzky-Golay
113 with 5 (2D5P), 10 (2D10P) and 20 (2D20P) symmetric kernel points] to correct the scattering and
114 overlapping effects of the signal. Both the raw and pre-treated spectra were analyzed by partial least
115 squares-discrimination analysis (PLS-DA) using full (leave-one-out) cross-validation with an
116 uncertainty test for optimal number of components and the NIPALS algorithm. The analysis was done
117 using the whole dataset (n=108) separately for each muscle (LT, ST and GM, with 108 spectra
118 differing in ageing time), either using the full spectra (400-2400 nm) or only the NIR region (1100-
119 2400 nm).

120 The prediction ability of Vis-NIRS using PLS-DA models was calculated specifically for each
121 ageing day (day 3, day 7, day 14 and day 21) and muscle. Accordingly, a dummy Y-variable of 1 was
122 assigned to the target ageing day in each case [4 models were developed with the four different
123 targeting predictions (3, 7, 14 and 21 days of ageing], and 0 for the other days (non-target); thus, if the
124 predicted dummy value was >0.5 , the sample was assigned to the target category, if not it was
125 classified as not from the target category (Osorio et al., 2013; Woodcock et al., 2008). The best pre-
126 treatment in each case was chosen as that providing the best discrimination of samples according to
127 each ageing time when using the lowest number of PLS factors.

Subsequently, the prediction capacity of the best PLS-DA models was evaluated per ageing day according to three parameters; sensitivity (percentage of samples for each ageing time which belongs to the target group and was correctly predicted to belong to the target group), specificity (percentage of samples not belonging to the target group which was correctly rejected by the model) and finally the overall classification of samples correctly identified (both belonging to the target or non-target groups correctly identified), as described by Rezzi, Axelson, Héberger, Reniero, Mariani, & Guillou, (2005) and applied to chemometrics by Osorio, Haughey, Elliott, & Koidis, (2015). The models showing the best results for all these parameters are summarized in Table 2

3. Results and discussion

The use of Vis-NIRS in meat authentication is relatively new, and while some studies have been carried out in this field (Downey & Beauchêne, 1997; McDevitt, Gavin, Andrés, & Murray, 2005; Downey, McElhinney, & Fearn, 2000) there is no literature on its use in ageing time authentication or prediction.

3.1. Prediction ability of selected models for ageing time

Examination of the scores plot of the initial data using PCA for each muscle separately, indicated a rough initial clustering, with samples aged for 3 and 7 days separated from those aged for 14 and 21 days (data do not shown; LT muscle results are available in Moran, Allen & Moloney, 2016). This was probably due to the intrinsic physical and chemical properties caused by ageing in each muscle, which were recorded by the spectra.

The preliminary results using the whole dataset of each muscle (n=108, samples aged during different periods of time for each muscle) indicate that both raw and pre-treated spectra achieved high levels of success, with a maximum of 3/27 target and 10/81 non-target misclassified samples in the

150 discrimination of meat samples according to ageing time (Table 1). However, the best results were
151 achieved with those spectra mathematically pre-treated such as the second derivative of Savitzky-Golay
152 and using the full spectra (400-2400 nm). The second derivative of Savitzky-Golay has been
153 demonstrated to be extremely useful to correct scattering and overlapping over the full spectra, thus
154 increasing the resolution of signals and reducing the baseline shift (Hruschka, 1987). Therefore, the
155 best predictive models for each muscle differed only in the number of symmetric kernel points as
156 follows: second derivative with 20 points for the LT, second derivative with 10 points for the GM and
157 second derivative with 5 points for the ST at all ageing times (Table 1).

158 Moreover, according to Gowen, Downey, Esquerre & O'Donnell (2011) a desirable trait for a model
159 is keeping the number of PLS factors as low as possible in order to avoid overfitting of the prediction
160 equation, and hence failure of authentication with samples not included in the calibration set.
161 Accordingly, the results of the present study indicate that the use of the full spectra was more effective
162 in authenticating the ageing time of the three muscles studied (Table 1), since the number of PLS
163 factors needed in each equation was between 4 and 11, whereas the number increased (5-15 PLS
164 factors) when using only the NIR spectra. The results are in agreement with those reported by other
165 authors, where better performance of the full (Vis-NIRS) rather than only NIR spectra for
166 authentication of bloomed meat was reported (Prieto, López-Campos, Zijlstra, Uttaro, & Aalhus, 2014).
167 The importance of the visible region is probably due to a possible relationship between colour
168 information provided by the Vis range (400-700 nm) and ageing of meat samples (Vitale, Pérez-Juan,
169 Lloret, Arnau & Realini, 2014) as further discussed in section 3.4.

170 [INSERT TABLE 1, NEAR HERE, PLEASE]

171 3.2. Authentication performance of selected models for each muscle

172 The final models selected for each muscle are presented in Table 2. These models were those
173 providing the best classification of meat samples according to ageing time, that is to say, those
174 developed using the full spectra and 2D20p (LT), 2D10p (GM) and 2D5p (ST). In general, the models
175 were quite robust, taking into account the low number of samples analyzed. The specificity of the
176 models was particularly good for ST and GM muscles (100%), while the sensitivity was slightly poorer
177 for LD and ST muscles (96.3-100% for both LT and ST). The LT aged for 3 and 7 days was the most
178 difficult muscle to classify using Vis-NIRS technology. This may be related to the higher initial
179 tenderness in LT muscle (Belew, Brooks, McKenna & Savell, 2003), making the ageing differences
180 less important than in the other muscles and reducing the variability between initial days for this trait,
181 thus decreasing the prediction ability of the equations. Nevertheless the prediction of ageing time for
182 LT was also good, with sensitivity ranging from 96.36-100% and specificity from 98.8 to 100%
183 depending on the ageing day.

184 [INSERT TABLE 2 NEAR HERE, PLEASE]

185 3.3. Muscle biochemistry under-pinning the prediction

186 [INSERT FIGURE 1 NEAR HERE, PLEASE]

187 According to the results of the present study, the information collected by the full Vis-NIRS spectra
188 of meat samples (which is probably related to the biochemical, structural or physicochemical properties
189 of each sample) allowed an accurate discrimination of beef steaks according to their ageing time. In
190 this sense, the detailed observation of the spectra helped to understand the reasons behind the success
191 of this technology for our purpose, which is especially relevant when there is a lack of reference data,
192 such as happened in the present study (Downey, 1996). As an example, we present in Figure 1 the
193 mean spectra of LT muscle for each ageing day plotted as $\log(1/R)$ and 2D20p. Even though several

194 particularities were observed among muscles, the general graphics obtained for GM and ST muscles
195 were similar to those obtained for LT, so they are not included.

196 The visible area [400-700 nm, Figure 1(a) 1/R and second derivative]) is an important region of the
197 spectrum related with colour properties of meat samples, and hence the presence of different meat
198 pigments such as oxymyoglobin or metmyoglobin. The importance of this region in discrimination of
199 shorter ageing times (3-7 days) was highlighted by larger loading weights (data not shown) of this
200 region in these two models (3 and 7 days ageing for LT). In agreement with this statement, the analysis
201 of the raw collected from LT meat samples with the shortest ageing times (3-7 days) showed clear
202 differences compared with the spectra of LT samples with the longest ageing times (14-21 days)
203 [Figures 1 (a) 1/R and second derivative]. This is mainly due to the higher absorbance at 545 nm and
204 575 nm wavelengths for LT samples with the shortest ageing times (3-7 days), whereas the tendency
205 was the opposite around 410 nm and 635 nm. These bands have been previously described by Lawrie
206 (1998) and Liu et al. (2003) who found four main broad bands related to meat pigments at
207 approximately 430, 545, 575, and 635 nm. The absorbance at 545 and 575 nm has been related to the
208 presence of oxymyoglobin species whereas the absorbance at 410 nm and 635 nm has been related to
209 the presence of myoglobin degradation derivatives (Liu & Chen, 2000; Liu et al., 2003).

210 The NIR region (1100-2400 nm) of the full spectrum may also provide valuable information for
211 discrimination purposes, such as changes in water distribution and hence water holding capacity
212 (Pearce, Rosenvold, Andersen, & Hopkins, 2011). For example, in the present study, two wavelengths
213 usually related to the second (1450 nm) and first overtones of O-H bonds (1940 nm) (Prieto et al.,
214 2009), showed higher absorbance values in LT meat samples aged for 14 and 21 days when compared
215 to those aged for 3 and 7 days. Accordingly, the loading weights (data not shown) of these wavelengths
216 were more important in the models developed to discriminate between samples aged for 3 and 7 days

217 when compared to those developed for samples aged for 14 and 21 days. This fact also can be observed
218 in the differences of the second derivative spectra [Figure 1(c) second derivative] at these wavelengths.
219 Other important area described by is the absorbance at 980 and 1027, our results indicate clear
220 differences between ageing days at this wavelength [Figure 1(b) second derivative] in agreement with
221 Liu et al. (2003) who highlighted this area as essential to discriminate meat samples with different
222 ageing times.

223 Moreover, the absorbance at wavelengths corresponding to the first overtone of the C-H stretching
224 bonds (1620-1780 nm) and C-H combination bands (1300-1400 nm) was higher for samples aged for
225 21 and 14 days (LT) compared with samples aged for 3 and 7 days [Figure 1(c) 1/R and second
226 derivative]. Moreover, the second derivative spectra also highlighted large differences in the
227 absorbance at 2200-2400 nm (also related to the absorbance of C-H bonds) of LT samples aged
228 differently [Figure 1(c) second derivative]. This evidence, together with the high loading weight (data
229 not shown) of these wavelengths in the models selected, indicate that these regions were also very
230 important for discrimination of LT samples according to ageing time. In this sense, Liu & Chen (2000)
231 stated that an increased vibration (and hence, absorbance) of C-H bonds is associated with an
232 improvement of meat tenderization due to ageing. Moreover, Cozzolino, Murray, Chree, & Scaife
233 (2005) found that the absorbance at 2200-2400 nm was modified by the oxidation and hydrolytic
234 degradation of fats. In support of this, Gorraiz, Beriain, Chasco & Insausti (2002) found that the
235 concentration of fatty acid degradation products in meat increased with ageing time, which was related
236 to the increase in the free fatty acid levels as a result of the lipolytic enzyme activity (Hood & Allen,
237 1971).

238 Finally, the best models to predict different ageing time reflected variations in the wavelengths
239 related with the absorbance of N-H bonds [e.g., 1460-1570 nm and 2000-2180 nm; (Prieto et al., 2009)]

independently of the ageing day. This is logical, since during ageing, the action of proteases such as μ -calpains provokes changes in the myofibril structure by degradation of Z-disks and desmin, thus modifying physicochemical characteristics of meat (Koohmaraie, 1994; Wu & Smith, 1987). Nishimura, Liu, Hattori & Takahashi (1998) also reported collagen degradation during the ageing of meat.

3.4 Practical application

The results described in this paper are preliminary and more robust models should be developed using a larger sample set to confirm the real potential of this technology with meat obtained from different breeds, other muscles, or animals differing in age or sex. The use of a bigger set of samples will also permit the use of independent validation sets, which is the best way to validate a model when there are enough samples to do so (Martens & Dardenne, 1998; Braga-Neto & Dougherty, 2004). However, based on the good predictability of the models developed in the present experiment, the practical application of Vis-NIRS technology for authentication of the ageing process of meat seems to be possible for the retailing industry. It is likely that industry would be interested in the authentication of expensive cuts such as striploin, cube roll or tenderloin (*M. Longissimus lumborum*, LT, and *M. Psoas major* respectively). Having this strategy in mind, the use of independent models for each cut/muscle will allow the industry to obtain better results for the authentication of those premium cuts. Other considerations such as applying four predictive models (one for each ageing time) in just one sample will only take a couple of minutes once standardized, and will contribute to a guarantee of the authenticity of the ageing time.

The excellent results obtained using only the NIR region of the spectra are also encouraging, and may allow the industry to reduce the cost of the device without impacting on the results obtained in the authentication of ageing process, specifically in the case of those steaks presented to the consumer as

vacuum or skin vacuum package. It will be of interest to test the ability of Vis-NIRS technology to predict the ageing time of non-bloomed steaks to confirm if this technology can be used on un-opened vacuum or skin vacuum packaged steaks.

4. Conclusions

According to the results of the present study, Vis-NIRS technology allows the ageing time of premium beef steaks to be authenticated. However, further studies are required to generate more robust models broadening the range of variability of samples included in order to fully develop the potential of this procedure for application in the retail industry.

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Table 1. Potential of PLS-DA models applied to the full dataset (n=108 spectra of each muscle, 27 per ageing time) using (leave-one-out) cross validation to discriminate steaks aged for different times with only the NIR (NIR, 1100-2400 nm) or the full spectra (All, 400-2400 nm).

	Ageing day	<i>M. Longissimus thoracis</i>						<i>M. Gluteus medius</i>						<i>M. Semitendinosus</i>					
		Target		Non-Target		n		Target		Non-Target		n		Target		Non-Target		n	
		All	NIR	All	NIR	All	NIR	All	NIR	All	NIR	All	NIR	All	NIR	All	NIR	All	NIR
Raw	3	25	24	81	81	8	9	26	26	81	81	7	7	27	26	81	81	9	11
	7	27	27	80	80	10	10	27	27	81	81	8	8	27	27	81	81	13	11
	14	26	27	81	81	9	8	26	26	81	81	11	11	26	27	80	81	12	15
	21	27	27	80	80	9	9	27	27	81	81	12	12	27	27	81	81	15	15
SNV	3	26	26	81	78	7	12	26	26	81	80	6	6	27	26	81	81	7	11
	7	27	27	80	81	12	10	27	27	81	79	10	9	27	27	81	81	12	11
	14	27	27	81	81	11	10	26	26	81	81	10	8	27	27	81	81	11	15
	21	27	27	81	81	9	11	27	25	81	78	8	8	27	27	81	81	15	15
2D 5p	3	26	27	81	80	5	3	27	27	81	81	6	2	27	26	81	81	7	10
	7	27	27	80	80	5	2	27	25	81	80	5	2	27	27	81	81	5	11
	14	23	21	80	76	6	2	27	25	81	71	4	2	27	27	81	81	6	15
	21	27	18	77	74	6	1	27	13	80	74	5	1	27	27	81	81	9	15
2D 10p	3	26	26	81	81	4	6	27	26	81	81	5	7	27	27	81	81	5	7
	7	27	27	80	80	4	6	27	27	81	81	4	9	27	27	81	81	4	7
	14	26	25	81	81	10	9	27	27	81	80	3	8	27	27	81	81	6	7
	21	27	27	81	80	10	15	27	27	81	80	5	11	27	27	81	81	15	15
2D 20p	3	26	26	81	81	5	5	27	26	81	81	7	9	27	27	81	81	5	6
	7	27	27	80	80	4	6	27	27	81	81	4	7	27	27	81	81	4	9
	14	27	27	81	81	9	10	27	27	81	80	4	8	27	27	81	81	4	9
	21	27	27	81	81	11	9	27	27	81	79	9	15	27	27	81	81	15	15

PLS-DA: Partial least square discrimination analysis; Raw: log (1/R); SNV: standard normal variate; 2D5P, 2D10P, 2D20P: second derivate Savitzky-Golay with 5, 10 or 20 points symmetric kernel points respectively; Target: number of target samples correctly classified; Non-Target: number of non-target samples correctly classified. n: number of PLS factors in the model.

Table 2. Potential of the best PLS-DA models separately developed for *M. Longissimus thoracis* (LT), *M. Gluteus medius* (GM) and *M. Semitendinosus* (ST) using the full spectra (400-200 nm) as independent variables.

Pre-treatment	Ageing	All variables			
		Sensitivity	Specificity	Overall	n
2D20p	LT				
	3	96.3	100	99.1	5
	7	100	98.8	99.1	56
	14	100	100	100	10
2D10ps	GM				
	3	100	100	100	5
	7	100	100	100	4
	14	100	100	100	3
2D5p	ST				
	3	96.3	100	99.1	7
	7	100	100	100	5
	14	100	100	100	6
2D20p	GM				
	3	100	100	100	5
	7	100	100	100	4
	14	100	100	100	3

PLS-DA: Partial least square discrimination analysis; 2D5P, 2D10P, 2D20P: second derivate Savitzky-Golay with 5, 10 or 20 points symmetric kernel points respectively. Overall: overall percentage of samples correctly classified. n: number of PLS factors in the model.