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Article

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**Analysis of oligomer proanthocyanidins in different barley genotypes using HPLC-FLD-
MS and NIR methodologies**

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Abstract

Proanthocyanidins are a class of polyphenols present in many foodstuffs (i.e. tea, cocoa, berries, etc.) that may reduce the risk of several chronic diseases. Barley, with sorghum, rice and wheat, are the only cereals that contain these compounds. Because of that, two barley genotypes, named waxy and non-waxy, were analyzed by NP-HPLC-FLD-MS. Total proanthocyanidins content ranged between 293.2 to 652.6 $\mu\text{g/g}$ of flour. Waxy samples reported the highest content ($p < 0.05$) of proanthocyanidins.

Dimer compounds were the principal proanthocyanidin constituents of barley samples.

Moreover, the possibility to use near infrared (NIR) spectroscopy as a rapid method to discriminate between waxy and non-waxy samples and to predict quantitatively proanthocyanidins in barley samples was evaluated. PLS models were built to predict the proanthocyanidins constituent obtaining determination coefficients (R^2) ranging from 0.92 to 0.97, in test set validation. Because of that, this study highlights that NIR spectroscopy technology with multivariate calibration analysis could be successfully applied as a rapid method to determine proanthocyanidins content in barley.

Keywords: barley, proanthocyanidins, flavan-3-ols, waxy and non-waxy, FT-NIR

27 Introduction

28 Proanthocyanidins, also known as condensed tannins, are a group of phenolic compounds
29 derived from flavan-3-ols. They are oligomers or polymers of flavan-3-ols (epicatechin or
30 catechin) linked through interflavan bonds. B-type proanthocyanidins are linked by C4→C8
31 and/or C4→C6 bonds. A-type proanthocyanidins contain an additional ether bond between
32 C2→O7.¹

33 Proanthocyanidins may have direct effects on the intestinal mucosa and protect it against
34 oxidative stress or the actions of carcinogens. Moreover, their consumption has shown to
35 increase the plasma antioxidant capacity, to have positive effects on vascular function, and to
36 reduce platelet activity in humans.² Several authors reported a series of *in vitro* and *in vivo*
37 studies indicating that proanthocyanidins can act as anticarcinogenic agents through their
38 antioxidant, apoptosis-inducing, immuno-modulating, and/or enzyme modulating properties.
39 Besides, proanthocyanidins could be particularly safe dietary compounds and have effects on
40 epigenetics.³

41 Proanthocyanidins are found in fruits, tree nuts, cereals, legumes, wine and chocolate. Most
42 foods contain exclusively B-type proanthocyanidins, whereas, a small number of foods, such
43 as cranberries, plum and peanuts, contain A-type proanthocyanidins.¹

44 Among cereals, barley, rice, sorghum and wheat are the only ones in which proanthocyanidins
45 have been detected.⁴⁻⁸

46 Sorghum is the first one regarding proanthocyanidin content ranging between 700-1900
47 mg/100g f.w. in whole grain; logically this content would be higher if the bran was
48 considerate.⁴ Wu et al.⁹ reported that proanthocyanidin extract from sorghum bran has a
49 positive effect against the oxidative damage in mice by increasing the activities of dismutase
50 and glutathione peroxidase in liver and serum. Moreover, procyanidins inhibit tumor growth

and metastasis formation by suppression of vascular endothelial growth factor production in vivo.

Compared to sorghum grain, lower content of proanthocyanidins were detected in rice and barley. Recently, Min and co-workers⁸ reported that proanthocyanidin content in rice was about 130 mg /100 g of grain. The content of proanthocyanidins in barley varied between 25 and 250 mg/100 g of grain.^{4, 10-12} Kamimura and Takahashi¹³ demonstrated that procyanidin B-3 from barley can directly promote hair epithelial cell growth *in vitro*, counteract the growth-inhibiting effect caused by TGF- β 1 *in vitro* and stimulate anagen induction *in vivo*.

The analytical technique usually applied to determine the proanthocyanidins in cereal consist of HPLC coupled to DAD, fluorimetric and mass spectrometer detectors^{5,6,14} or MALDI-TOF analysis.¹⁵ However, in the last years the use of IR spectroscopy as non-destructive method has been evaluated.¹⁶⁻¹⁸

Due to the large use of barley in food production, the aim of this work was to investigate the oligomeric proanthocyanidins content in different barley genotypes (waxy and non-waxy) using HPLC-FLD-ESI-MS. Moreover, fourier transform near infrared (FT-NIR) spectroscopy was performed to evaluate the possibility to discriminate between waxy and non-waxy samples and to estimate the proanthocyanidins in barley flours by using a fast and non-disruptive method.

Materials and methods

Samples

Fourteen different varieties of barley grown under the same agronomic conditions in the same experimental field at the DISTAAM, University of Molise (Italy) (41°34'00"N 14°40'00"E) were analyzed.

75 The soil of the site is classified as a fine silty clay loam with 29% silt, 35% clay, 36% sand,
76 1.7% organic matter, total available P (6.1 mg kg⁻¹) and total available K (78.3 mg kg⁻¹), and
77 pH = 7.3.

78 The trials were sown in a three-replication split-plot experimental scheme in October 2013
79 (sowing density was 300 seeds/m²). Four of the barley varieties were waxy genotype (Canada,
80 USA2, USA3 and USA7) and the other ten were non-waxy genotype (Acquarelle, Bombay,
81 Boreale, Margret, Messina, Metis, Naturelle, Otis, Rangoon and Svenja). Harvest took place
82 in June 2014 at grain full ripening stage.

83 Whole grains were milled (particle size 150-250 µm) and the flours were used for the
84 analysis. The resulted flours reported moisture between 11.4 and 12.0%.

85

86 *Chemicals*

87 HPLC-grade acetonitrile and water, methanol, acetone were purchased from Merck KGaA
88 (Darmstadt, Germany). Catechin and procyanidin B2 were from Sigma-Aldrich (St. Louis,
89 MO).

90

91 *Extraction of proanthocyanidins in barley samples*

92 The protocol of Verardo et al.¹¹ was used to isolate the proanthocyanidin fraction. Briefly,
93 barley flour (2 g) was extracted in an ultrasonic bath (20 min) with 20 mL of a solution of
94 acetone/water (4:1 v/v). After centrifugation at 1000g for 10 min, the supernatant was
95 removed, and the extraction was repeated once more. The supernatants were collected,
96 evaporated, and reconstituted in 2 mL of methanol/water (1:1 v/v). The final extracts were
97 filtered through 0.2 µm PTFE syringe filters and stored at -18 °C until the analyses.

98

99 *Determination of proanthocyanidin compounds by HPLC-FLD-ESI-MS*

NP-HPLC analysis was performed by an Agilent 1200 Series (Agilent Technologies, Palo Alto, California, USA), equipped with a binary pump delivery system, a degasser, an autosampler, a fluorimetric detector (FLD) and a mass-spectrometer detector (MSD).

A Develosil Diol 100Å column 5 µm, 250 x 4.6 mm ID (Phenomenex, Torrance, CA, USA), was used. All solvents were HPLC-grade and filtered through a 0.45 µm filter disk. The gradient elution was the same reported by Robbins et al.¹⁹ Fluorescence detection was conducted with an excitation wavelength of 230 nm and an emission wavelength of 321 nm.

The injection volume was 5 µL. All the analyses were carried out at 35 °C. Calibration curves of (+)-catechin and procyanidin B2 were arranged from 5-500 and 1-500 µg/mL, respectively, at 6 concentration levels for each compound; correction factors suggested by Robbins and co-workers¹⁹ were used for trimer, tetramer and pentamer compounds.

The HPLC analysis was replicated three times for each extract and calibration point ($n=3$).

Mass spectrometer analyses were carried out using an electrospray ionization (ESI) interface at the following conditions: drying gas flow (N₂), 9.0 L/min; nebulizer pressure, 50 psi; gas drying temperature, 350 °C; capillary voltage, 3500 V; fragmentor voltage and scan range variables. The fragmentor and m/z ranges used for HPLC-ESI/MSD analyses were as follows: 120 V and m/z 50-1000, 140 V and m/z 1000-2000.

FT-NIR spectroscopy

A spectrophotometer Vector 22/N (Bruker Optics, Ettlingen, Germany) in diffuse reflectance mode was used to obtain the NIR spectra. The instrument was equipped with a rotating integrating sphere that allows a wide illumination of the sample and a better reduction of light scattering due to the irregularity of the particle surfaces. NIR spectra were obtained by average of 32 scans of each sample. Three replicates for each individual sample (about 60 g of barley flours) were collected for a total of 42 spectra. Background was defined by acquiring

the spectrum of the quartz support without the sample, in the same instrumental conditions (25 °C). The speed of acquisitions was 10 kHz in a range from 800 nm to 2500 nm.

Statistical analysis

The *Pearson* correlation coefficients ($p < 0.05$) were calculated to define a relation between the proanthocyanidins using Statistica 8.0 software (2007, StatSoft, Tulsa, OK, USA).

Significant differences (at $p < 0.05$) between means of the proanthocyanidins compounds were explored by using analysis of variance (ANOVA) combined with the Tukey's post-hoc test using Statistica 8.0 software. Mean-centred data were subjected to Principal Component Analysis (PCA) to found a possible discrimination between sample as function of barley genotypes (waxy and non-waxy) using The Unscrambler ver. 9.7, (CAMO, Oslo, Norway).

As regard the NIR spectra, to remove the effects of light scattering, NIR spectra were pre-treated with multiplicative scattering correction (MSC). The first part of the spectra, until 1100 nm, was deleted before processing because it contained no useful chemical information, but only instrumental noise. Data were also treated by applying the first derivative (Savitsky–Golay) to the absorbance data. Subsequently, the spectral data were subjected to PCA and Partial Least Square (PLS) regression. The first one was applied as an exploratory analysis in order to define a possible discrimination between sample as function of barley genotypes (waxy and non-waxy), while the second one to predict the proanthocyanidin compounds. The PLS models were validated by using a full cross validation and a test set validation technique. For the first validation, the same data set, characterized by an amount of 42 cases (three replicates for 14 samples), was used to calibrate and validate the model: a sample of total entire dataset is removed one by one from the construction of the model and used to validate. For the test set validation, the dataset was randomly divided into two sub-samples, one to calibrate the system (70% of the entire dataset) and the other (30%) to validate it. Test set

validation was repeated 5 times for each reference values (proanthocyanidin compounds) and the predictions index (determination coefficient, R^2 and Root Mean Square Errors, RMSE) were averaged, since the PLS convergence is influenced by the data set.

All the statistical elaborations were carried out by using The Unscrambler ver. 9.7 (CAMO, Oslo, Norway).

Results and discussion

Proanthocyanidins identification by HPLC-ESI-MS

The method established by Robbins et al.¹⁹ was used to determine the proanthocyanidin oligomers in barley flours and the resulted chromatogram is showed in **Figure 1**.

In order to identify the proanthocyanidins revealed by fluorimetric detection, mass spectrometry was used to confirm the peak identity (**Table 1**). As reported from several authors,^{19, 20} the compounds eluted according to their degree of polymerization; firstly eluting the monomers and then the different oligomers according to their degree of galoylation (for the same degree of polymerization).

Catechin/epicatechin was the first compound that was identified. It provided a molecular ion at 289 m/z ; in addition, a fragment ion at m/z 245 ($[M-H-CO_2]^-$) was also shown.

Several oligomeric procyanidins (PC) and prodelphinidins (PD) were identified; many of them reported the fragmentation pathways for proanthocyanidins such as retro-Diels-Alder (RDA) cleavage and subsequent elimination of a water molecule, interflavanic bond cleavage through the quinone-methine (QM) mechanism, and (c) heterocyclic ring fission (HRF). (5)

A procyanidin dimer with molecular ion at 577 m/z and two principal fragments at 425 and 289 m/z (corresponding to RDA and QM, respectively)²¹ was detected at 17.6 minutes.

Two prodelphinidin dimers were also detected at 20 and 20.2 minutes; they showed a molecular ion at 593 m/z and three fragment ions at 467, 425 and 289 m/z corresponding to HRF, RDA and QM, respectively.⁵

For procyanidin trimer (molecular ion at 865 m/z), fragments at m/z 739, 713 and 289 were obtained as results of HRF, RDA and QM, respectively.

Two single peaks eluting at 30 and 30.2 minutes with m/z 881 and 897 ($[M-H]^-$) were detected. The first one produced three fragment ions at 593, 577 and 303 m/z . This compound was identified as prodelphinidin trimer monogallate; moreover, the presence of fragment ions at 593 m/z (corresponding to GC-C dimer) and 577 m/z (corresponding to C-C dimer) suggested that the peak was the sum of (epi)gallocatechin-(epi)catechin-(epi)catechin and (epi)catechin-(epi)gallocatechin-(epi)catechin trimers.

The other peak at 897 m/z and fragment ions at 771, 729 and 593 m/z was identified as digalloylated prodelphinidin trimer according to Friedrich and co-workers.²²

Two proanthocyanidin tetramers were detected. Procyanidin tetramer with molecular ion at 1153 m/z and a fragment ion at 865 m/z ($[M-H-catechin]^-$) was revealed at 36.7 minutes.

A prodelphinidin tetramer at 38.8 minutes was identified due to its molecular ion at 1457 m/z ; the fragment ion at 728 m/z permits to identify this compound as digalloylated oligomer, according to Lazarus et al.²³

Finally, the compound at 41.0 minutes and 1441 m/z was identified as procyanidin pentamer according to Robbins et al.¹⁹ As far as we are concerned, this is the first time that procyanidin pentamer has been detected in barley samples.

Proanthocyanidins quantification by HPLC-FLD

In order to quantify the proanthocyanidins in barley samples, catechin and procyanidin B2 calibration curves were used. Moreover, the correction factors suggested by Robbins and co-workers¹⁹ were chosen to calculate trimer, tetramer and pentamer contents.

Barley samples exhibited ample variability of total proanthocyanidins content, with a range from 293.2 to 652.6 µg/g of flour (**Table 2**) and waxy and non-waxy genotypes reported statistical differences ($p < 0.05$). In fact, the mean of total oligomeric content in waxy genotypes was about 1.6 times higher than non-waxy samples.

Monomers ranged between 10.9 and 48.3 µg/g of flour in non-waxy barley samples, whereas the content of monomers in waxy barley was significantly higher ($p < 0.05$), ranging from 44.1 to 87.1 µg/g of flour. Generally, these data are in the same order of magnitude of those reported by other authors.^{12,24,25}

Dimer compounds were the principal proanthocyanidin constituents of barley samples. Total dimers content ranged between 116.8 and 214.6 µg/g of flour and these amounts are in agreement with the results reported in literature.^{12,24,25} As seen for monomers, waxy samples showed higher content of dimers (182.9-214.6 µg/g of flours) compared to non-waxy samples (116.8-173.8 µg/g of flour). Procyanidin dimers represented the 35.4-51.2% of total dimers class and waxy samples contained higher content (except for Bombay sample) with an average of 89.6 µg/g of flour. Barley samples contained from 67.8 to 118.6 µg/g of flour of total prodelphinidin dimers, and waxy samples showed the highest content.

Proanthocyanidin trimers ranged between 149.8 and 317.4 µg/g of flour. Significant differences ($p < 0.05$) between waxy and non-waxy samples were found for procyanidin trimer: waxy samples showed an average of 127.4 µg/g of flour; instead non-waxy barley had an average of 61.3 µg/g of flour. Similar amounts were determined by Holtekjølen and co-workers.¹²

220 According to Holtekjølén and co-workers,¹² monogalloylated prodelphinidin trimers ranged
221 between 53.6 and 105.3 µg/g of flour and represented the 55.5-70.0% of total prodelphinidin
222 trimers. Digalloylated prodelphinidin trimers were 31.7-63.7 µg/g of flour. No statistical
223 differences ($p < 0.05$) between waxy and non-waxy samples were noticed for prodelphinidin
224 trimers content.

225 Tetramers constituted the 6.3-10.0% of total proanthocyanidins content and their amounts
226 varied from 20.8 to 55.2 µg/g of flour. Procyanidin tetramers ranged from 6.7 to 18.9 µg/g of
227 flour, whereas digalloylated prodelphinidin was contained in the range of 14.1-36.3 µg/g of
228 flour; no statistical differences ($p < 0.05$) were shown between waxy and non-waxy samples.

229 Pentamer oligomers were represented exclusively by one procyanidin that showed a total
230 content from 4.5 to 22.7 µg/g of flour; waxy samples showed higher content (average 14.3
231 µg/g of flour) compared to non-waxy samples (average 6.6 µg/g of flour).

232 The sum of tetramers and pentamers is in the same order of magnitude of the results reported
233 by Hellström and co-workers.⁶

234 Comparing the data obtained for barley to literature results^{4,8} about other cereals, is evident
235 that sorghum and rice are characterized by a higher content of total proanthocyanidins and a
236 higher degree of polymerization of proanthocyanidins. As reported by several authors^{4,26}
237 sorghum proanthocyanidins were represented by 68% of polymers followed by oligomers
238 from 4 to 10 monomeric units. Similar composition was reported by Min et al.⁸ for rice.

239 Instead, barley showed a low degree of polymerization, from monomers to pentamers, but
240 reported a higher content of dimers and trimers compared to sorghum and rice and higher
241 content of monomers than rice and equal or higher than sorghum.

242 This aspect encourage the use of barley in functional food formulation as cereal source of
243 proanthocyanidins because, as reported by Ou and Gu,¹ proanthocyanidins with a degree of

polymerization over 4 ($DP > 4$) are not absorbable because of their large molecular size and gut barrier.

The *Pearson* correlation matrix (**Table 3**) point out that all the compounds are positively correlated; the correlation between PD trimer II and Monomers or PD dimer is not significant.

The score plot of the PCA performed to discriminate between samples with different barley genotypes is reported in **Figure 2**. The two first principal components accounted for 83% of total variance. PC1, explaining 66% of total variation, is clearly linked to proanthocyanidins composition. A clear separation between waxy (grey) and non-waxy samples (black) was observed. The waxy samples were grouped along the positive values of PC1 (x-axis), while the non-waxy samples were grouped on the negative side of the axis.

The X-loadings plot shows how well a variable is taken into account by the model components and it is used to understand how much each variable contributes to the meaningful variation in the data, and to interpret variable relationships. Further, the comparison between score and loadings plot allows interpreting the relationships and dependence between variable and samples. In this case, the X-loadings plot (**Figure 3**) shows that all waxy samples are characterized by higher values of all proanthocyanidin compounds than non-waxy samples. Particularly, USA3 sample, respect to the other waxy samples, is characterized by a high content of PD trimer II, PD dimer II and PD trimer I and by a low content of monomers, PD dimer I and PC trimer.

Near infrared spectroscopy (NIR)

NIR spectra provided characteristic broad and overlapping peaks between 1100 and 2500 nm. The spectra of all the samples and an example of first derivate are reported in **Figure 4**. All the samples showed the main absorption bands. In particular, the overtones of the C–H bonds of the methylic and methylenic groups between 1170 and 1230 nm, the bands of the O–H

bonds due to water and starch at about 1450 and 1940 nm, and the bands of the N–H bonds at about 1500–1570 and 2050–2070 nm were identified. The absorptions of constituents like starch, cellulose and protein are located at higher wavelengths. The flavonoid constituents can be observed in the regions from 1415 nm to 1512 nm, from 1650 to 1750 nm and from 1955 to 2035 nm.^{16,17,27}

The score plot of the PCA (in the 1100–2500 nm range, with MSC and first derivate treatments), performed to discriminate between waxy and non-waxy samples, is reported in **Figure 5**. A clear separation between waxy and non-waxy samples was observed along the PC1 (89%). The X-loadings plot (**Figure 6**) of the first component shows that maximum variance between spectra was identified from 1850 to 2500 nm, as a consequence the discrimination might be attributed with flavonoid constituents, starch, cellulose and protein.

The potential of NIR to determine proanthocyanidin oligomers in barley has been evaluated.

Table 4 shows the results of PLS regressions in terms of coefficient of determination (R^2) and Root Mean Square Error (RMSE) for calibration, full cross validation and test set validation. The models were built considering all the spectrum points (from 1100 to 25000 nm) or the only absorption bands related to phenolic and flavonoid constituents (1415–1512, 1650–1750 and 1955–2035 nm). Generally, in full cross and test set validation, the best results were obtained using the NIR regions associated with phenolic and flavonoid constituents. In full cross validation the R^2 values range from 0.94 (PC tetramers) to 0.98 (monomers), while in test set from 0.97 (PC trimer) to 0.92 (PD trimer II). However, the difference between the results obtained using all the spectrum points and bands related to phenolic and flavonoid constituents, are very low.

Because of that, the capability of FT-NIR spectroscopy technology, as rapid and non-destructive method to determine the proanthocyanidin oligomers in barley samples could be hypothesized.

294 In conclusion, the coupling of HPLC with FLD and MS detectors allows the determination of
295 proanthocyanidin oligomers in barley samples. Proanthocyanidins include procyanidins and
296 prodelphinidins, and some of them are galloylated. The lower degree of polymerization of
297 barley proanthocyanidins compared to other cereals encourages the use of barley in food
298 formulation. Moreover, waxy barley genotype showed a higher proanthocyanidin content than
299 non-waxy genotype.

300 In addition, the PLS results obtained by FT-NIR spectroscopy indicate that infrared
301 methodology has a good potential to predict proanthocyanidin compounds in barley samples.
302 This aspect represents an advantage and proposes the use of this fast and non-destructive
303 methodology for industry application. To confirm these preliminary results and the
304 performance of NIR for proanthocyanidin determination in barley flours, the sampling should
305 be improved in future investigations.

306

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Figure caption

Figure 1. HPLC-FLD chromatogram of oligomeric proanthocyanidins in barley sample.

Number of the peaks are the same reported in Table 1.

Figure 2. Score plot obtained by the PCA of the proanthocyanidin compounds determined by HPLC-FLD.

Figure 3. X-loadings of the PCA carried out with proanthocyanidin compounds content.

Figure 4. NIR spectra and first derivate.

Figure 5. Score plot obtained by the PCA of the proanthocyanidin compounds determined by FT-NIR

Figure 6: X-loadings for the PCA carried out with NIR spectra (from 1100 to 2500 nm).

Table 1. HPLC–ESI-MS Data of Proanthocyanidins in Barley Samples

Compound	Rt (min)	[M-H] [−]	Fragments(m/z)
Catechin/epicatechin	7.13	289	245
Procyanidin dimer	17.61	577	425, 289
Prodelphinidin dimer I	20.30	593	467, 425, 289
Prodelphinidin dimer II	21.66	593	467, 425, 289
Procyanidin trimer	27.30	865	739, 713, 289
Prodelphinidin trimer (monogalloylated)	30.00	881	593, 577, 303
Prodelphinidin trimer (digalloylated)	32.87	897	771, 729, 593
Procyanidin tetramer	36.65	1153	865
Prodelphinidin tetramer (digalloylated)	38.81	1457	728
Procyanidin pentamer	41.03	1441	-

Table 2. Proanthocyanidins Content in Barley Samples Obtained by HPLC-FLD (µg/g d.w. flour). Different Letters in the Same Column Means Significantly Different Values ($p < 0.05$).

	Monomers	PC dimer	PD dimer I	PD dimer II	PC trimer	PD trimer I	PD trimer II	PC tetramer	PD tetramer	PC pentamer	Total
No waxy samples											
Acquarelle	32.9±0.1 d	55.0±0.8 b,c	16.1±1.1 a,b	74.7± 3.3c-e	64.7±0.9 c,d	75.5±0.7 b	49.8±0.7 e,f	13.1±0.6 c,d	21.8±0.4 c,d	6.6±0.7 b,c	410.5±9.4 d
Bombay	27.7±0.6 c,d	87.7±0.8 g	16.5±1.4 a,b	69.5±3.2 c-e	65.1±0.0 c,d	92.6±0.8 d	40.2±1.3c,d	12.5±0.2 b,c	23.8±1.2 d,e	6.1±0.7 b	442.0±3.9 e
Boreale	22.5±0.3 b,c	59.0±1.9 c	15.5±0.4 a	62.1±1.4 b,c	43.7±0.1 a,b	79.1±1.4 b	46.8±0.5 d,e	11.3±0.4 b	25.8±0.8 d,e	8.3±0.5 d	374.2±4.1 b,c
Margret	10.9±1.0 a	60.0±2.0 c	15.7±0.3 a	58.3±0.8 b	54.5±1.4 b,c	81.4±2.6 b	44.6±1.3 d,e	10.1±1.1 b	24.1±0.8 d,e	6.7±0.3 b,c	366.4±2.8 b,c
Messina	16.7±0.0 a,b	71.4±2.0 d	19.3±0.3 b,c	69.3±0.3 c-e	66.0±0.1 d,e	92.5±0.1 d	51.3±0.5 f	13.1±0.4 c,d	30.8±1.1 f	8.7±0.1 d	439.2±2.0 e
Metis	48.3±1.0 e	48.8±1.4 a,b	22.4±0.3 d	49.3±1.8 a	71.7±0.7 e	54.2±1.4 a	23.8±0.5 a	8.0±0.2 a	14.6±0.9 a	4.5±0.1 a	345.8±7.9 b
Naturelle	23.5±1.0 b,c	48.9±0.8 a,b	17.1±0.7 a,b	50.7±1.0 a	36.7±1.1 a	54.5±1.4 a	31.7±0.5 a	7.3±0.8 a	16.1±0.3 a,b	6.6±0.1 b,c	293.2±9.4 a
Otis	26.8±0.2 c,d	75.2±3.1 d,e	19.7±0.2 b,c	66.0±1.8 c,d	71.1±0.4 e	89.5±3.2 c	44.3±1.2 d,e	12.7±0.7 b,c	27.2±1.1 e,f	6.6±0.1 b,c	439.2±5.4 e
Svenja	26.3±0.9 c,d	76.2±0.6 d,e	15.8±0.7 a	56.8±0.4 b	85.3±4.4 f	84.7±1.4 b	36.3±0.3 b	12.1±1.2 b	21.2±0.7 c	6.4±0.7 b,c	421.1±5.3 d
Rangoon	17.0±0.2 a,b	48.3±1.5 a	16.7±1.2 a,b	71.2±1.9 c-e	54.2±0.1 b,c	53.6±2.4 a	43.1±0.4 b,c	6.7±0.9 a	14.1±1.2 a	5.71±0.5 b	330.6±9.8 b
Mean	25.27	63.06	17.50	62.80	61.32	75.77	41.19	10.71	21.96	6.63	386.21
CV%	40.9	21.8	13.0	14.0	23.3	21.0	20.6	23.6	25.4	18.0	13.5
Waxy samples											
Canada	87.1±1.6 g	82.7±1.4 f	35.0±0.7 f	75.6±1.1 d,e	137.2± i	86.9±1.9 c	45.2±1.5 d,e	14.1±1.1 d	22.8±0.6 c,d	12.1±0.1 f	598.8±6.1 f,g
Usa2	72.1±0.1 f	97.8±0.1 h	37.6±0.2 f	79.2±0.7 f	118.5±1.1 h	92.9±0.6 d	44.8± 0.5 d,e	13.6±0.3 d	24.9±0.3 d,e	9.6±0.2 e	591.0±5.5 f,g
Usa3	44.1±0.7 e	90.6±1.1 g	21.9±0.1 d	96.7±0.9 g	87.6±1.2 g	105.3±2.5 e	63.7±1.9 g	15.1±1.0 e	29.1±0.6 e,f	12.9±0.4 f	567.0±6.5 f
Usa7	74.1±2.8 f	87.4±2.9 g	30.9±2.0 e	64.6±1.6 c,d	166.5±1.4 l	103.1±2.0 e	48.0±0.9 f	18.9±0.5 f	36.3±0.6 h	22.7±1.1 g	652.6±7.5 g,h
Mean	69.37	89.63	31.38	79.02	127.45	97.04	50.42	15.43	28.26	14.35	602.35
CV%	26.1	7.1	22.0	16.9	26.0	9.0	17.7	15.7	21.0	40.0	6.0

Table 3. Symmetric Matrix of Pearson Correlation Coefficients Obtained Between the Proanthocyanidin Compounds ($p < 0.05$).

Proanthocyanidins	Monomers	PC dimer	PD dimer I	PD dimer II	PC trimer	PC trimer I	PD trimer II	PC tetramer	PD tetramer	PC pentamer	Total
Monomers	1										
PC dimer	0.61	1									
PD dimer I	0.93	0.64	1								
PD dimer II	0.41	0.66	0.4	1							
PC trimer	0.91	0.69	0.84	0.46	1						
PC trimer I	0.43	0.89	0.41	0.68	0.61	1					
PD trimer II	0.15	0.49	0.16	0.88	0.31	0.69	1				
PC tetramer	0.66	0.66	0.54	0.57	0.8	0.77	0.55	1			
PD tetramer	0.43	0.71	0.41	0.5	0.62	0.9	0.64	0.78	1		
PC pentamer	0.74	0.62	0.65	0.53	0.85	0.69	0.55	0.76	0.8	1	
Total	0.84	0.87	0.8	0.71	0.92	0.83	0.58	0.85	0.78	0.87	1

Table 4: PLS Regression Results

Constituent		NIR regions			
		1100-2500 nm		1415-1512,1650-1750,1955-2035 nm	
		R ²	RMSE	R ²	RMSE
Monomers	C	0.99	2.54	0.99	2.16
	CV	0.97	4.04	0.98	3.58
	TSV	0.94	5.94	0.96	4.84
PC dimer	C	0.98	2.59	0.99	1.86
	CV	0.95	4.14	0.97	3.14
	TSV	0.96	3.53	0.95	3.48
PD dimer I	C	0.98	2.19	0.98	2.02
	CV	0.94	3.50	0.95	2.96
	TSV	0.92	3.91	0.93	3.62
PD dimer II	C	0.97	1.38	0.98	1.06
	CV	0.94	1.93	0.96	1.66
	TSV	0.87	2.38	0.93	1.93
PC trimer	C	0.99	4.12	0.99	4.07
	CV	0.97	6.69	0.97	6.05
	TSV	0.97	6.71	0.97	7.43
PD trimer I	C	0.98	2.31	0.99	1.94
	CV	0.96	3.94	0.97	3.09
	TSV	0.95	3.98	0.95	4.69
PD trimer II	C	0.98	1.56	0.98	1.30
	CV	0.94	2.47	0.96	1.97
	TSV	0.93	2.59	0.92	2.47
PC tetramer	C	0.97	0.81	0.98	0.59
	CV	0.94	1.17	0.94	1.17
	TSV	0.94	1.31	0.94	1.25
PD tetramer	C	0.98	1.02	0.98	0.78
	CV	0.94	1.65	0.96	1.36
	TSV	0.93	1.78	0.94	1.64
PC pentamer	C	0.97	0.65	0.98	0.64
	CV	0.94	1.03	0.96	0.97
	TSV	0.95	1.06	0.95	1.02
Total	C	0.98	15.81	0.99	13.51
	CV	0.96	25.34	0.97	22.71
	TSV	0.96	25.64	0.95	22.00

C: calibration, CV: Cross Validation, TSV: Test set validation

Figure 1

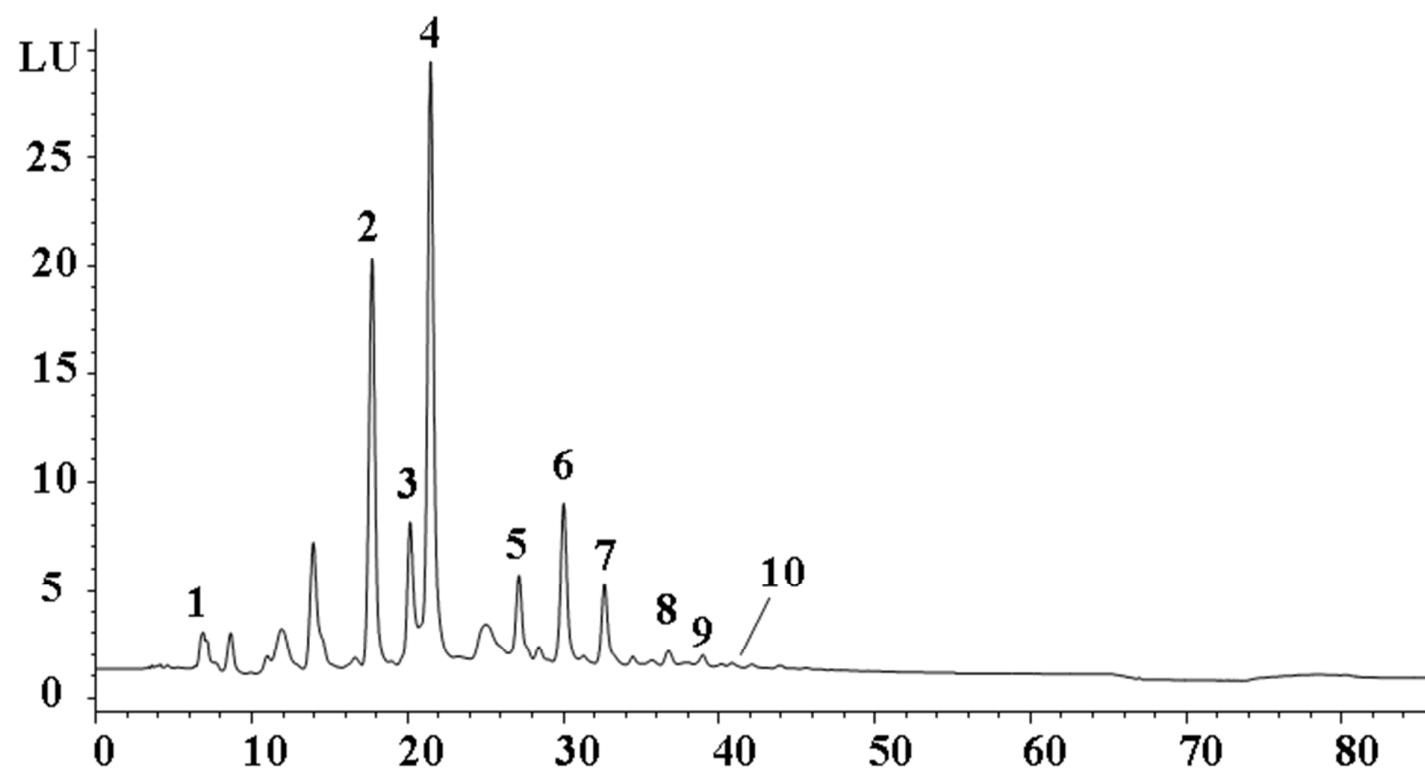


Figure 2

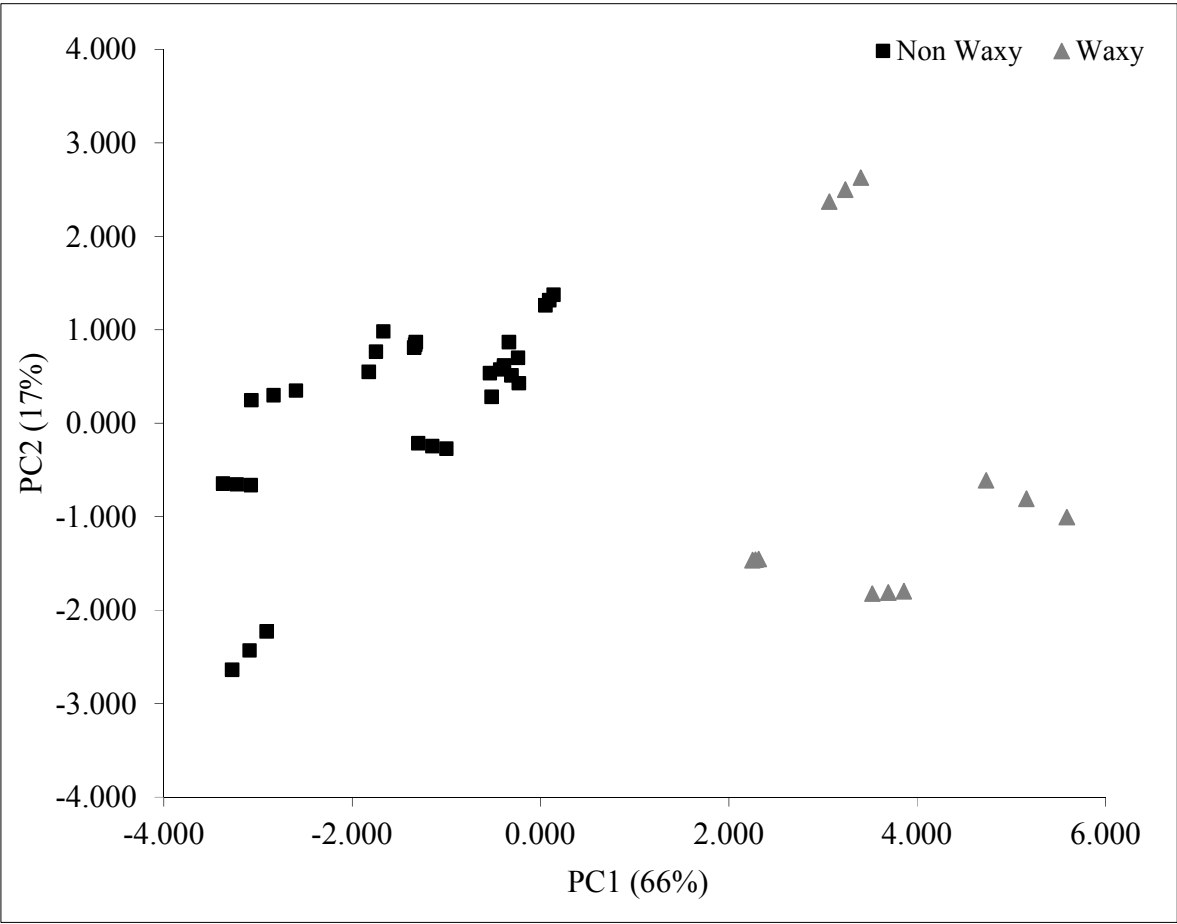


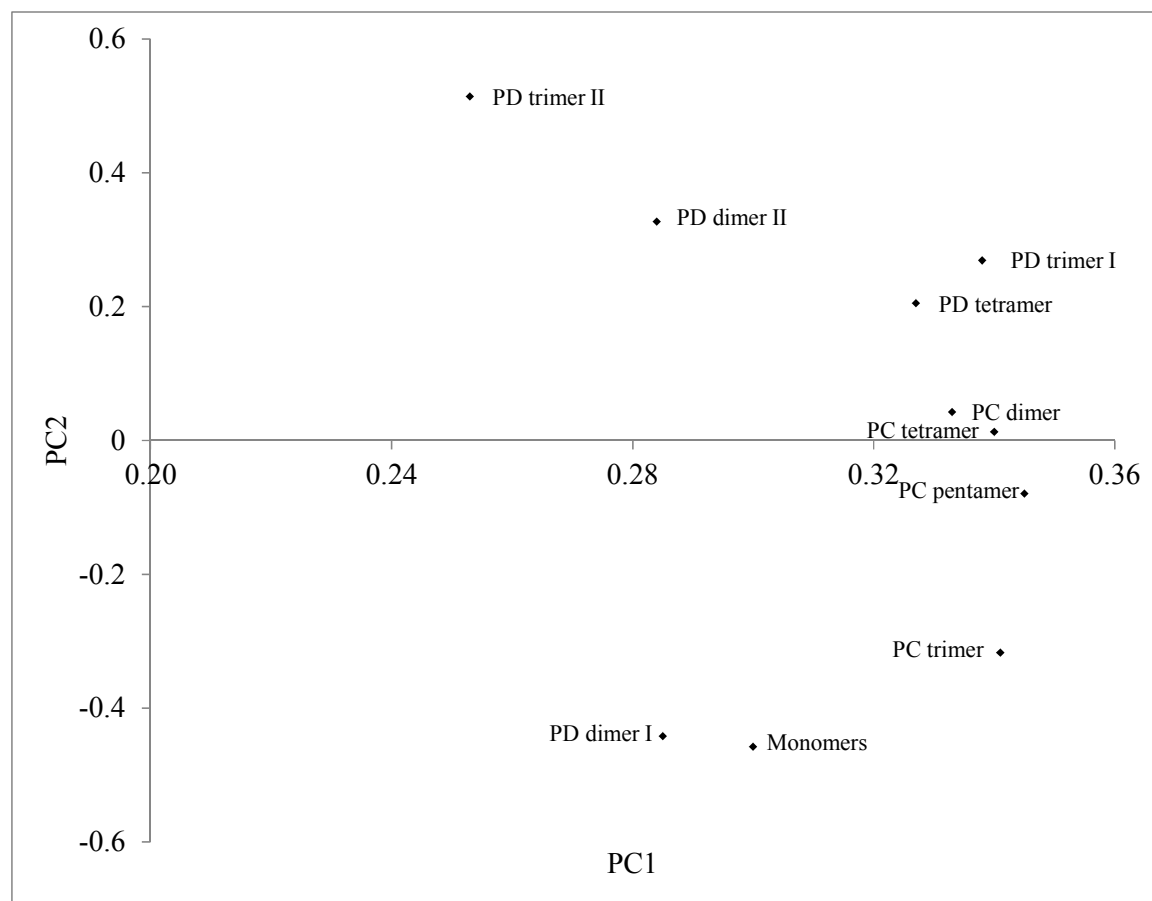
Figure 3

Figure 4

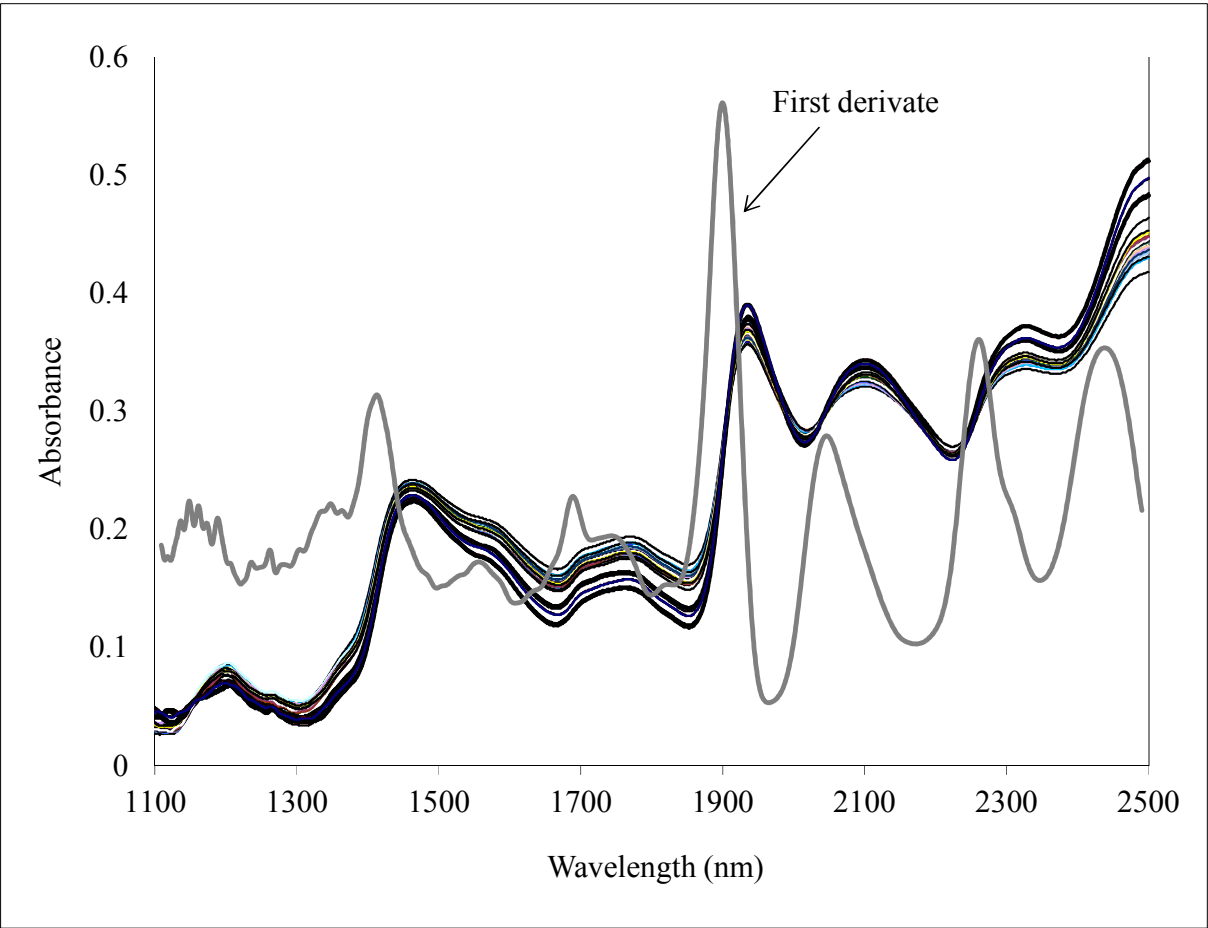


Figure 5

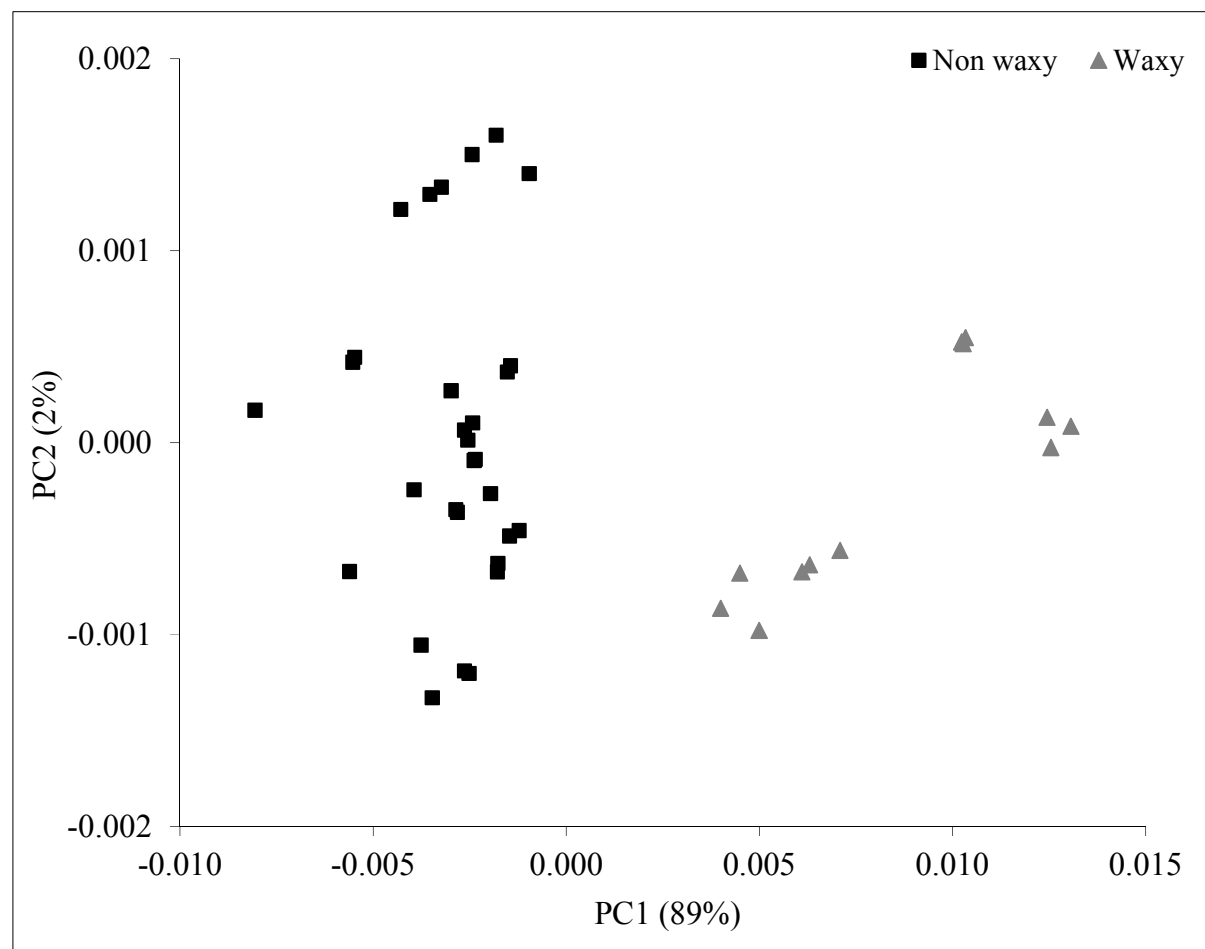


Figure 6

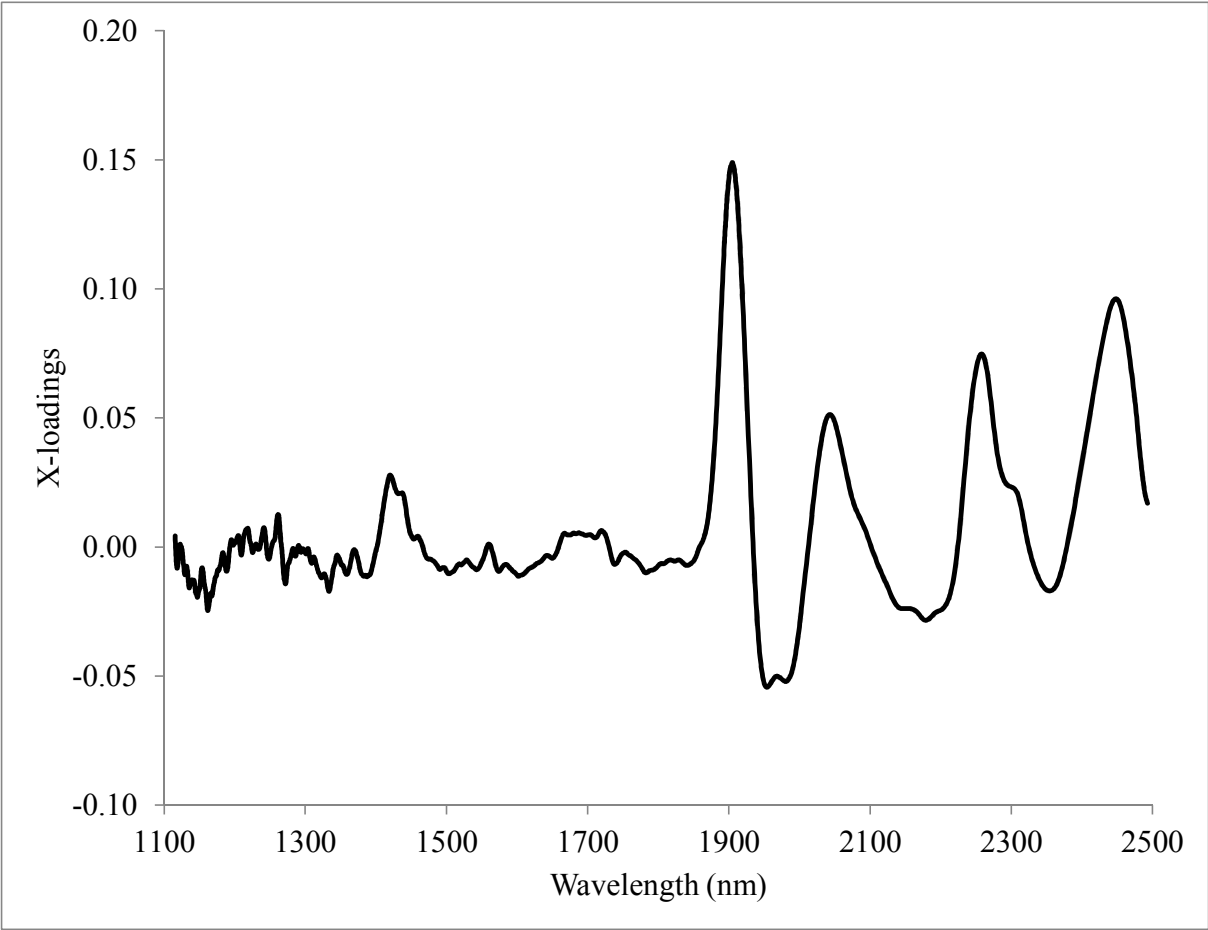


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