Research Article

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# Spectrophotometric versus NIR-MIR assessments of cowpea pods for discriminating the impact of freezing

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## Abstract

**BACKGROUND: Freezing represents an important storage method for vegetal foodstuffs, such as cowpea pods, and thus the impact of this process on the chemical composition of these matrices arises as a prominent issue. In this sense, the phytochemical contents in frozen cowpea pods (i.e. at 6 and 9 months) have been compared with fresh cowpea pods material, with the samples being concomitantly assessed by Fourier-transform infrared spectroscopy (FTIR), both mid-infrared (MIR) and near infrared (NIR), aiming to evaluate the potential of these techniques as a rapid tool for the traceability of these matrices.**

**RESULTS: A decrease in phytochemical contents during freezing was observed, allowing the classification of samples according to the freezing period based on such variations. Also, MIR and NIR allowed discrimination of samples: the use of the first derivative demonstrated a better performance for this purpose, whereas the use of the normalized spectra gave the best correlations between the spectra and specific contents. In both cases, NIR displayed the best performance.**

**CONCLUSION: Freezing of cowpea pods leads to a decrease of phytochemical contents, which can be monitored by FTIR spectroscopy, both within the MIR and NIR ranges, whereas the use of this technique, in tandem with chemometrics, constitutes a suitable methodology for the traceability of these matrices.**


**Keywords:** cowpea; immature pods; phenolic composition; radical scavenging capacity; FTIR-spectroscopy; multivariate analysis

## INTRODUCTION

To attain more balanced and healthy diets, it is essential to increase the consumption of plant foods, including legumes, which represent a rather sustainable source of essential and non-essential nutrients and bioactive compounds. In this sense, the chemical composition of legumes depends on a plethora of factors, including pre-harvest and postharvest aspects, with respect to agro-food production, which modulate the benefits associated with their dietary ingestion.[1]

Amongst the legume species currently included in the European agro-food system, cowpea (*Vigna unguiculata* L. Walp) has been highlighted with respect to several agronomic, environmental and economic advantages, in addition to its ability to grow under semi-arid conditions with low input requirements.[2] Additionally, because of the composition of the cowpea aboveground material concerning nutrients and non-nutrients, this crop has been noted as a source of valuable material with respect to providing the nutrients and phytochemical compounds required to prevent several metabolic and cardiovascular disorders.[3,4] Moreover, currently available information supports the interest in this legume species with respect to increasing the ratio of bioactive phytochemicals in the diet.[5]

Therefore, obtaining innovative legume-based foodstuffs, aiming to fulfill the claims of an increasingly demanding food chain, requires that new processing and storage alternatives are taken into account, including the evaluation of their impact on plant material composition.[1,6] However, this fact should not be always considered as a shortcoming because it may eventually promote the development of tools capable of discriminating the effect of storage conditions on the quality and safety of plant products and thus their suitability for tracing foodstuffs along the food chain.[7,8]

Furthermore, the assessment of plant materials with respect to their relevance as potential sources of phenolic compounds is generally conducted by spectrophotometric techniques, which represent a comprehensive approach for determining the interest of these foodstuffs regarding certain healthy attributions. However, these techniques entail a time consuming pre-treatment of samples, which may eventually led to chemical modifications of the matrix to be assessed, and they also involve the use of pollutant reagents and solvents that are harmful for both the operatives and the environment. Therefore, to avoid these constraints, several spectroscopic applications have been implemented in recent

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years that resort to approaches such as Fourier-transform infrared spectroscopy (FTIR), which constitutes a prompt, direct and simple method allowing the preservation of samples, particularly when used in combination with attenuated total reflectance (ATR).[9]

In this regard, the application of FTIR in the study of legumes has increased noticeably during the last decade, mainly to evaluate the composition with respect to crude fiber and dietary fiber, carbohydrates, starch, fatty acids, dry matter, ash, crude protein and essential amino acids, vitamins and minerals, and phenolics,[10–16] whereas some reports based in the application of FTIR have described changes in protein structure during the gastric digestion and how this may affect protein digestibility.[17]

Therefore, the present study aimed to use FTIR spectroscopy, either within the near infrared (NIR) and mid-infrared (MIR) ranges, as associated with chemometrics, to differentiate fresh cowpea pods from those frozen at −18 °C for long periods (6 and 9 months), as well as allow a comparison between both intervals for this purpose. Discrimination was achieved using partial least squares regression (PLS-R) approach to reduce the spectroscopic data to factors, whereas PLS-discriminant analysis (PLS-DA), a supervised method, was subsequently applied to these condensed set of data (factors) to produce the discriminant models. Moreover, PLS-DA was also applied to the spectrophotometric data, aiming to assess their potential for the discrimination, and thus allowing a comparison between these parameters and the former approach. Furthermore, the suitability of this methodology, based on MIR and NIR spectra measurements coupled with PLS-R, for producing quantitative models for the prediction of total phenolics, flavonoids and *ortho*-diphenols content of cowpea pods, was concomitantly assessed.

## MATERIALS AND METHODS

### Solvents and chemicals

Folin–Ciocalteu's reagent, 3,4,5-trihydroxybenzoic acid (gallic acid) and acetic acid, both extra pure (>99%) were purchased from Panreac (Panreac Química SLU, Barcelona, Spain). Sodium nitrate, aluminum chloride and sodium carbonate, all extra pure (>99%), were purchased from Merck (Merck Darmstadt, Germany). The compounds 2,2-azino-bis(3-ethylbenzothiazoline)-6 sulphonic acid ($ABTS^{\bullet+}$), 2,2-diphenyl-1-picrylhidracyl radical ($DPPH^{\bullet}$), Trolox and catechin, all of extra pure grade (>99%), were purchased from Sigma–Aldrich (St Louis, MO, USA). Sodium molybdate (99.5%) was from Chem-Lab (Chem-Lab NV, Zedelgem, Belgium). All other reagents used were of analytical grade. The water used in the experiments was deionized, obtained from a water purification system (Millipore, Bedford, MA, USA).

### Cowpea material, storage conditions and sample preparation

Cowpea (*V. unguiculata* L. Walp.) pods ($n = 18$ per treatment) were harvested at immature stage in Ronfe, Guimarães (elevation: 142 m; latitude: 41.4398993°; longitude: −8.3837558°), at the North-West of Portugal, and immediately chopped after harvest, being obtained from Frescura Sublime (Braga, Portugal). Pieces were frozen in a fluidized bed until the temperature at the center reached −18 °C. Pieces of cowpea pods were analyzed in the fresh form and after 180 and 270 days (6 and 9 months, respectively) of storage at −18 °C. After the freezing period, samples were lyophilized, grown to fine powder and protected from light and humidity until analysis. Control samples of fresh pods were immediately lyophilized, ground to fine powder and protected from humidity until analysis.

### Measurement of phenolic composition

Each sample (100 mg) was mixed with 1.5 mL of methanol/distilled water (70:30, v/v). Then, samples were vortexed and phenolic compounds were extracted by agitation at room temperature for 30 min and subsequently centrifuged at 2951 × *g* for 5 min, at 4 °C (Sigma-2-16 K; Sigma, Steinheim, Germany) and the supernatant was collected. This procedure was repeated three times and the final volume was made up to 5.0 mL. Supernatants were filtered through a 0.45-μm polyvinylidene fluoride filter (Millex HV13; Millipore) and stored at 4 °C until analysis. The content in total phenols, flavonoids, and *ortho*-diphenols was determined according to the methodology previously reported by Mena *et al.*[18] adapted to microscale. Reactions were performed in 96-well microplates and measured using a Multiscan FC microplate reader (Thermo-Fisher Scientific, Oporto, Portugal).

For the assessment of cowpea pods regarding the content in total phenolics, 180 μL of Milli-Q water, 12 μL of sample appropriately diluted with MeOH and 13 μL of Folin–Ciocalteu reagent were mixed and vortexed in an eppendorf tube. After exactly 1 min, 45 μL of 200 g $L^{-1}$ sodium carbonate was added, followed by vortexing again, and the mixtures was allowed to rest at room temperature, protected from light, for 120 min. Absorbance was recorded at 750 nm and gallic acid was used as standard. Results were expressed as mg of gallic acid equivalents (GAE) $g^{-1}$ dry weight (mg GAE $g^{-1}$ dw).

For the assessment of the content of flavonoids in fresh cowpea pods, a mixture of 100 μL of distilled water, 10 μL of $NaNO_2$ (50 g $L^{-1}$) and 12 μL of sample properly diluted with MeOH was used. After exactly 5 min, 15 μL of $AlCl_3$ (200 g $L^{-1}$) was added and the mixture was allowed to react for 6 min. Then, 50 μL of NaOH (1 mol $L^{-1}$) and 50 μL of distilled water were added to the mixture. Absorbance was recorded at 510 nm and the flavonoid content was quantified using catechin as standard. The results were expressed as mg of catechin equivalents (CE) $g^{-1}$ dry weight (mg CE $g^{-1}$ dw).

The content of *ortho*-diphenols in fresh cowpea pods samples was determined by adding 40 μL of $Na_2MoO_4$ (50 g $L^{-1}$) and 160 μL of the samples diluted appropriately. Mixtures were vortexed and allowed to stand at room temperature, protected from light, for 15 min. Absorbance was recorded at 375 nm and quantified using gallic acid as standard. The results were expressed as mg GAE $g^{-1}$ dw.

### Radical scavenging capacity

Polyphenolic extracts of samples (fresh and frozen cowpea pods, $n = 18$ per treatment) were centrifuged at 10 000 × *g* (Sigma-2-16 K; Sigma) for 5 min at room temperature. The free radical scavenging activity was determined using the free radicals $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ in accordance with previously described methods.[18] The $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging power was evaluated by measuring the variation in absorbance at 520 and 734 nm after 30 min of reaction, respectively. The results were expressed as μmol of Trolox equivalents (TE) $g^{-1}$ dry weight (μmol TE $g^{-1}$ dw).

### FTIR assessment

For the FTIR assessments, the Infrared spectra were registered in a Thermo Scientific Nicolet iS50 FTIR spectrometer, resorting to an ATR accessory with a diamond crystal for MIR, and to integrating-sphere diffuse reflectance infrared fourier transform spectroscopy (DRIFT) accessory for NIR. The instrument was operated using Omnic, version 9.2.28 (Thermo Fisher Scientific Inc.,

Lisbon, Portugal). The MIR spectra were collected in the interval 400–4000 $cm^{-1}$, with a resolution of 4 $cm^{-1}$, and 64 accumulations were registered for each spectrum. The NIR spectra were collected in the range 4000–10 000 $cm^{-1}$, with a resolution of 8 $cm^{-1}$, and 128 accumulations were registered for each spectrum. All spectra were recorded as the absorption value at each data point, which were registered with a spacing of 0.482 and 3.857 $cm^{-1}$, in MIR and NIR, respectively.

For the collection of each FTIR-ATR (MIR) spectrum, a small quantity of powdered sample was placed on the ATR crystal, with the accessory tip placed on the top, and its infrared spectrum measured. For the collection of each FTIR-DRIFT spectrum (NIR), a quantity of sample that was sufficient to completely cover the window of the accessory was place directly on its top, and the spectrum was measured. In both techniques, a background spectrum was recorded before sampling, to allow subsequent subtraction. In all cases, three spectra were collected for each sample.

### Multivariate and statistical analysis

For the spectral pre-treatments, in MIR, auto-baseline was performed, resorting to fourth-order polynomials. For the multivariate analyses, different spectral treatments were assessed, for both techniques (MIR and NIR); namely, mean normalization, first derivative and first derivative after mean normalization. Either the normalized spectra or the first derivative, as well as both treatments, were considered for the discrimination purpose because these procedures allow spectral artifacts to be overcome, such as any baseline drifts that may remain after the previous spectral treatments, in addition to demonstrating better performance in previous studies with respect to the use of the spectra for statistical purposes.[9,19,20] The use of the second derivative has not been tested because a poor performance has been observed for this approach in previous studies.[20] Additionally, the use of the Savitzky–Golay (SG), simultaneous derivative smoothing algorithm was assessed with resort to second order polynomials and 20-point windows.[19]

Concerning the MIR range, the intervals 400–1900 and 2400–3650 $cm^{-1}$ were considered for the multivariate analyses because these spectral ranges correspond to the fundamental vibrational modes of interest, in addition to some visible overtones in the second interval mentioned. In the first region, < 1900 $cm^{-1}$, bending and stretching modes involving C and O atoms are found, whereas the latter can be coupled to (CH) and (OH) deformations, and, in the second region (2400–3650 $cm^{-1}$), the peaks correspond to (CH) and (OH) stretching modes, with the latter corresponding to a broad peak at higher frequencies. Regarding NIR, all of the interval was used because its peaks always correspond the combination modes or overtones.

The PLS-R approach was applied to develop models not only for discriminating the freezing time, but also for predicting the parameters evaluated through the Infrared spectra (either MIR or NIR).[21] Accordingly, the experimental values were considered as dependent variables, whereas the absorption at each wave number represented the independent variables, thus correlating the spectra with the contents being assessed. The models were calibrated by the regression of the mean spectra registered for each sample versus the mean values observed for the same samples concerning each parameter assessed. This procedure was performed in parallel for the distinct data sets obtained from the different pre-treatments applied. The optimal number of factors for each multivariate regression was determined through cross-validation (CV) methodology, which was applied with resort to the leave-one-out (LOO) approach. The quality of the models was evaluated through linear regressions of the experimental versus predicted values (for all spectra), as well as through the predicted residual error sum of squares (PRESS) errors observed.[21]

To obtain the classification models concerning the distinct freezing timings, PLS-R was applied to the data, setting the distinct samples as dependent variable, aiming to reduce data to factors that were suitable to be used for prediction because these were obtained without *a priori* training with the classification of samples. The factors extracted were used in PLS-DA, which is a supervised statistical method used to find a linear combination of structures, characterizing or separating classes of observations.[22] Different numbers of factors have been tested for each discrimination model.

Concerning the use of the phytochemical contents of samples for discrimination purposes, the values obtained were used in the PLS-DA, with each distinct assay representing an independent variable. For the quality assessment of the PLS-DA classification models, the CV-LOO approach was developed.

All of the multivariate statistical analyzes were performed using OriginPro, version 9.1 (OriginLab, Northampton, MA, USA). All measurements in each sample ($n = 18$) were performed in triplicate and values are expressed as the median ± SD. The results were subjected to an analysis of variance and a multiple range test (Tukey's test), whereas any possible correlation between the distinct parameters evaluated was assessed with a Pearson test, using SPSS, version 21.0 (IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered statistically significant.

## RESULTS AND DISCUSSION

Freezing represents one of the most extensively used preservation alternatives developed for the conservation of raw plant materials, which are perishable and frequently affected, because they represent seasonal productions. This procedure has been promoted as a result of its capacity to slow the deterioration rate and to maintain fresh-like characteristics with a minimal loss of valuable compounds, including antioxidants, during extended storage periods.[23] However, to the best of our knowledge, there is currently a general lack of studies dealing with the impact of the freezing process on the phytochemical quality of cowpea pods, as well as the development of new time and resource saving analytical alternatives that are suitable for implementation by the agro-food industry with respect to quality monitoring during freezing.

### Effect of freezing on total phenols, flavonoids and *ortho*-diphenols

The polyphenolic status of fresh cowpea pods and its evolution during the freezing process is shown in Fig. 1. The analysis of fresh cowpea pods, concerning the content in total phenols, demonstrated concentrations ranging from 18.97 to 25.50 mg GAE $g^{-1}$ dw (Fig. 1A). However, these values must be interpreted with caution because Folin–Ciocalteau reagent can react not only with phenolics, but also with a variety of non-phenolic reducing compounds, including tertiary aliphatic amines, amino acids (tryptophan), hydroxylamine, hydrazine, certain purines, and other organic and inorganic reducing agents, leading to an overestimation of the phenolic contents.[24]

Furthermore, these concentrations cannot be compared with previous studies because of a lack of data on this plant material

  

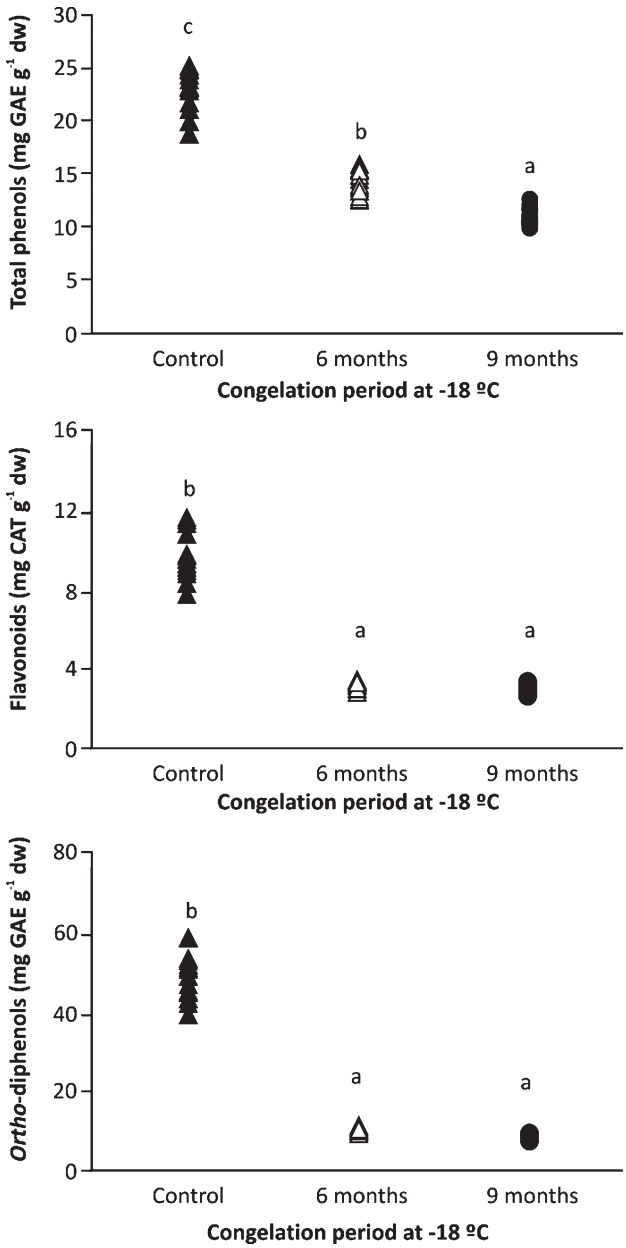


**Figure 1.** Content in total phenols, flavonoids and *ortho*-diphenols of fresh cowpea (*V. unguiculata* L. Walp) pods and plant material frozen at −18 °C for 6 and 9 months. Different lowercase letters for each sample indicate significant differences at $P < 0.05$ ($n = 18$ per treatment).

in the literature, even though, regarding additional cowpea products, the phenolic composition of cowpea pods surpassed the concentration described in cowpea seeds and seedlings (3.30 and 3.70 mg GAE $g^{-1}$ dw, respectively),[25] which presented a noticeably lower concentration with respect to the content in cowpea seed coats (41.50–60.10 mg tannic acid equivalents $g^{-1}$ dw).[26] The differences found could be attributable not only to the distinct composition of the diverse plant materials, but also to the relative amounts obtained when evaluating concentrations resorting to different standards (gallic acid versus tannic acid). Finally, the concentration of total phenols in cowpea pods appeared to be up to 52.3% higher compared to the composition of defatted cowpea flour,[27] which could be a result of the effect of the defatting process, with a visible impact on the total phenolic composition.[28]

With respect to flavonoids, the concentration observed in fresh cowpea pods was 9.98 mg CAT $g^{-1}$ dw, on average (ranging from 7.96 to 11.91 mg CAT $g^{-1}$ dw) (Fig. 1B). Once again, even though no previous data are available in the literature, concerning the flavonoid content of cowpea pods, the concentration of compounds within this phenolic class was in the range reported for defatted cowpea flour (7.24–12.16 mg $g^{-1}$ dw).[27] Nevertheless, interestingly, additional investigations presented much lower values concerning cowpea seeds (0.03–0.21 mg $g^{-1}$ dw) than those observed in the present study regarding cowpea pods, although, in this case, determinations were performed by chromatographic analysis, which lowers the unspecific quantification of other compounds as flavonoids.[29] Moreover, when analyzing the content in *ortho*-diphenols of fresh cowpea pods, values ranging from 40.20 to 60.28 mg GAE $g^{-1}$ dw were found (Fig. 1C).

As a consequence of the freezing process (6 and 9 months at −18 °C), a decrease in the concentration of total phenols (by 38.0% and 52.2%, on average, respectively), flavonoids (by 76.8% and 79.2%, on average, respectively) and *ortho*-diphenols (by 79.3% and 82.2%, on average, respectively) was observed relative to the fresh material (Fig. 1). This decrease has been widely reported concerning traditionally consumed legumes, as accompanied by changes in radical scavenging activity, in variable proportions that can reach up to 70.0%.[30] This decrease could be a result of polymerization and/or decomposition reactions affecting aromatic ring structures according to Granito *et al.*[31]

### Effect of freezing on DPPH• and ABTS•+ scavenging power

The antioxidant capacity of cowpea pods measured by the DPPH assay (Fig. 2A) ranged from 19.15 to 264.49 μmol TE $g^{-1}$ dw, whereas the radical scavenging activity against ABTS•+ (Fig. 2B) ranged from 9.93 to 24.98 μmol TE $g^{-1}$ dw. The initial antioxidant activity of the raw material under evaluation, prior to the freezing procedure, was similar to the values available in the literature for diverse cowpea materials (28–282 μmol TE $g^{-1}$ dw),[25,29] even though no reports concerning the radical scavenging capacity of cowpea pods are available to date. The wide variability concerning the radical scavenging capacity, with respect to the values found in the literature, is a result of the evaluation of distinct cultivars, as well as the assessment of plant materials on radical scavenging capacity, as undertaken by different methods focused on the determination of the antioxidant capacity of compounds with distinct chemical properties. In this sense, a comparison with previously evaluated edible cowpea materials suggested the potential benefits derived from the cowpea pods consumption as a source of antioxidant compounds.

The changes observed on the radical scavenging capacity of cowpea pods, as a consequence of the freezing process, demonstrated a decrease proportional to the changes observed in the concentration of total phenols, flavonoids and *ortho*-diphenols (Fig. 2). Thus, the DPPH and ABTS-based antioxidant activities decreased significantly ($P < 0.05$) by 60.8% and 47.7%, on average, respectively, in cowpea pods frozen for 6 months and by 89.0% and 54.1%, on average, respectively, in cowpea pods frozen for 9 months (Fig. 2). Moreover, the correlations between the distinct parameters have been assessed, indicating that all of the variables are significantly correlated ($P < 0.05$). This shows that all of the distinct components assessed are equally degraded throughout the freezing period, thus indicating the generalized impact of freezing on all of the distinct phytochemical contents.

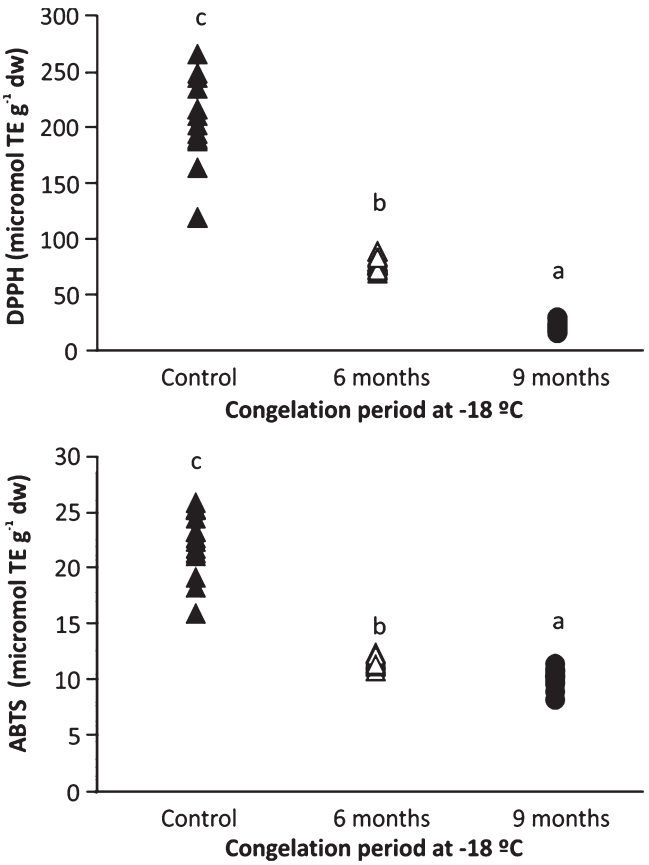


**Figure 2.** DPPH and ABTS radical scavenging activity of phenolic extracts of fresh cowpea (*V. unguiculata* L. Walp) pods and plant material frozen at −18 °C for 6 and 9 months. Different lowercase letters for each sample indicate significant differences at $P < 0.05$ ($n = 18$ per treatment).

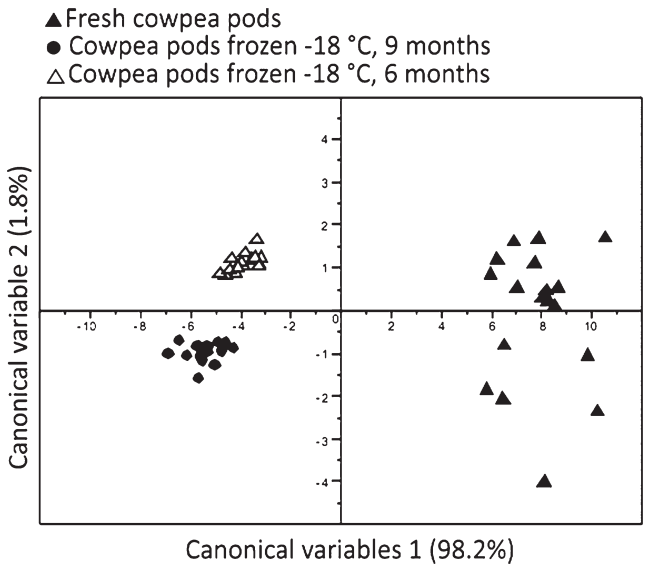


**Figure 3.** Scores plot for the discriminant canonical variables, extracted from PLS-DA, of fresh and frozen cowpea pod samples for the two freezing storage lengths developed with data from spectrophotometric determinations of the content in total phenolics, flavonoids and *ortho*-diphenols and the DPPH and ABTS radical scavenging capacity.

### Fresh and frozen pods discrimination resorting to spectrophotometric determinations

Regarding the spectrophotometric determinations undertaken, as observed previously, significant differences were observed between fresh and frozen pods, as well as regarding samples with distinct freezing timings with respect to both phytochemical content and antiradical activity (Figs 1 and 2). Therefore, the values observed for the parameters assessed were subjected to discriminant analysis (PLS-DA), aiming to evaluate their suitability to differentiate freezing timings.

As shown in Fig. 3, the two canonical variables (Can. Var.) allowed clear differentiation not only between fresh and frozen cowpea pods, but also samples with 6 and 9 months of freezing storage. Can. Var. 1 discriminated fresh and frozen pods, whereas Can. Var. 2 discriminated the samples according to the freezing period (Fig. 3). These two Can. Var. described 100.0% of the variability between data concerning the differences between the three distinct groups for classification, whereas Can. Var 1 (98.2% of variability) separates the fresh pods for positive scores, to the right side of the plot, and Can. Var. 2 (1.8%) discriminates samples with 6 and 9 months of freezing, to positive and negative scores, respectively (Fig. 3).

Concerning differentiation between fresh and frozen pods mirrored by the weight of Can. Var. 1, the contents in *ortho*-diphenols, as well as the radical scavenging activity (as measured by the ABTS assay), comprised those quantities with the greatest influence on this differentiation (see Supporting information, Table S1). By contrast, with respect to periods, DPPH scavenging power showed the greatest influence, whereas significant differences can be observed for this parameter between samples with 6 or 9 months of freezing (Figs 2 and 3; see also Supporting information, Table S1). Moreover, flavonoid contents also displayed a notable preponderancy for this discrimination, although the lack of significant differences between samples with 6 and 9 months of freezing for this parameter dramatically reduced the weight of these compounds for the classification of freezing time (Fig. 1; see also Supporting information, Tables S1).

Finally, discriminant analysis allowed the correct classification of all of samples in the LOO procedure undertaken to validate the PLS-DA, where each sample was excluded from the data set and classified, resorting to the other samples, with this procedure being repeated until every sample was excluded, at least once, from the data set. Therefore, the parameters assessed through these colorimetric assays allow classification of samples regarding freezing time, when discriminant approaches are applied. Nevertheless, these methods are rather laborious for routine use and require the use of harmful solvents, although they have the advantage of screening the specific contents of samples.

### Supervised cowpea pods freezing monitoring using FTIR spectra data

One of the main aims of the present study is not only the evaluation of spectroscopic means to monitor the freezing time of the pods (and its impact), but also a comparison between the NIR and MIR intervals (corresponding to distinct experimental set-ups) for this purpose. Accordingly, FTIR spectra have been collected for the samples in both intervals, each one reflecting the compositional changes that can occur during the storage process. Figure 4(A) and (D) depicts both spectral intervals. In the MIR range (400–4000 $cm^{-1}$), the infrared peaks corresponded to fundamental vibrational modes, which can be directly related to specific functional groups, thus allowing their correlation with the composition of the samples.[13]

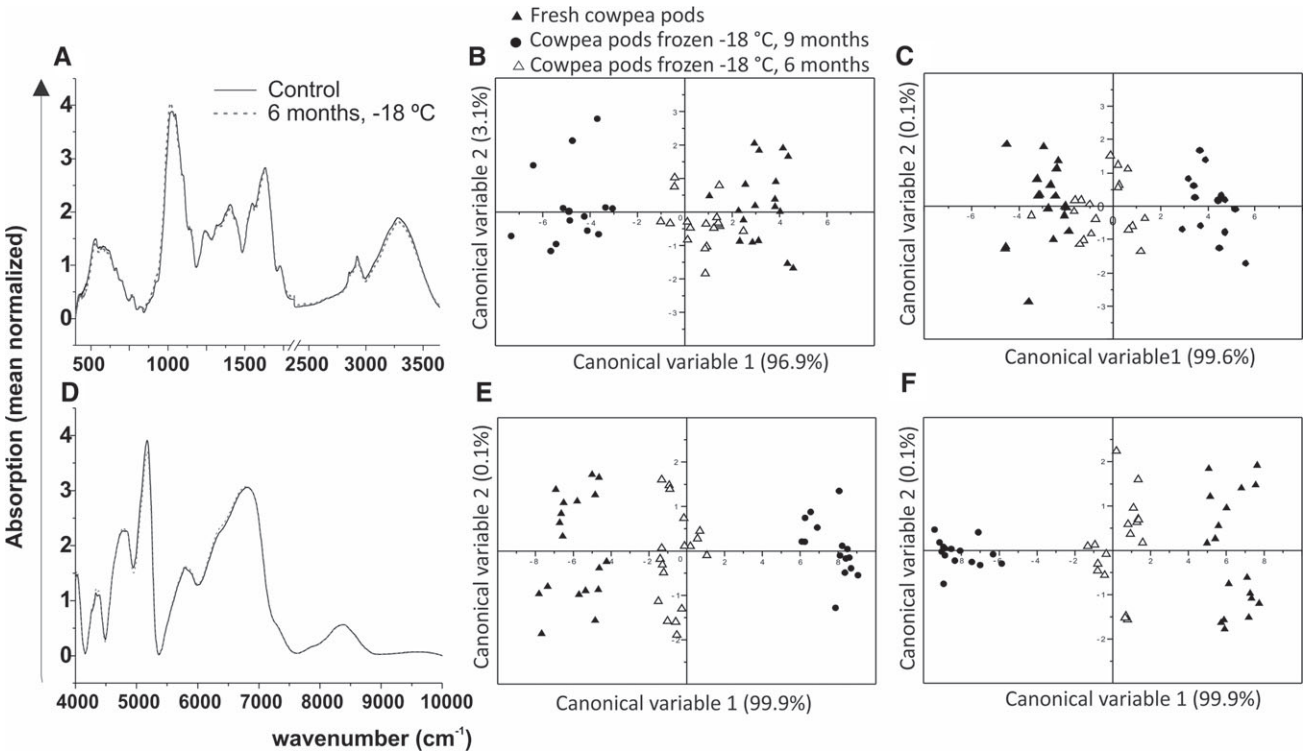


**Figure 4.** Plots of the scores for the discriminant canonical variables 1 and 2 retrieved from MIR-ATR and NIR-DRIFT data. For the cowpea pods: (B) scores for the PLS-DA undertaken resorting to the normalized MIR-ATR spectra and (C) resorting to first derivative after normalization of the MIR-ATR spectra; as well as (E) scores for the PLS-DA undertaken resorting to the normalized NIR-DRIFT spectra and (F) resorting to first derivative after normalization of the NIR-DRIFT spectra. Respresentative FTIR spectra are included within the MIR range (A) and the NIR range (B).

Indeed, in the intervals selected (400–1900 and 2400–3650 $cm^{-1}$), the difference concerning the most intense peak, at approximately 1050 $cm^{-1}$, corresponding to (C-O) stretching modes, arises as the most noticeable one (Fig. 4A), whereas this bond is mandatory in all polyphenols (Ph-O). Moreover, in the following region, 1150–1500 $cm^{-1}$, vibrational modes displaying contributions from both (C-O) stretching and in-plane bending ((CH and (OH)) modes were found, until 1300 $cm^{-1}$, whereas, between 1300 and 1500 $cm^{-1}$, the vibrational pattern was dominated by bending ((CH) and (OH)) modes. In the 1500–1800 $cm^{-1}$ interval, one strong peak was observed, at approximately 1620 $cm^{-1}$, comprising contributions from (C = O) and (C = C) stretching modes, whereas the third peak from this group, at 1730 $cm^{-1}$, was a result of (C = O) stretching modes, arising from distinct chemical systems, including several polyphenols. In the high frequency region (2400–3650 $cm^{-1}$), very weak spectral features can be observed between 2400 and 2800 $cm^{-1}$, corresponding to overtones (transitions to higher vibrational states), whereas the peaks corresponding to fundamentals were found in the intervals 2800–3000 $cm^{-1}$; namely, two sharp peaks (corresponding to (CH) stretching modes), and 3000–3650 $cm^{-1}$, where the broad peak assigned to the (OH) stretching modes was placed, with both features (CH) and (OH) being present in all polyphenolic systems (Fig. 4A).[20,22]

In NIR (4000–10 000 $cm^{-1}$), the spectral features observed corresponded to combination modes, or fundamental overtones, with their assignment to specific functional groups being rather infeasible. Nevertheless, this latter interval has been successfully used for several chemometric approaches, normally resorting to its full spectral window and eventually presenting better performance, with respect to MIR, as a result of the greater stability of radiation in this interval and the larger quantity of sample used, and as explored by the greater penetration depth of this radiation, thus, increasing the reproducibility of this technique.[32]

In Table 1, the percentages of erroneous classifications for each discriminant analysis (comprising both MIR and NIR, and the distinct spectral treatments), resorting to different numbers of factors, are presented, up to a maximum of 15 factors. Because, in some cases, the first factor extracted is sufficient to fully discriminate the samples, leading to the extraction of a single Can. Var., the plots presented always refer to the scores of two Can. Vars. obtained from a discriminant analysis undertaken with the first two factors extracted in each case, aiming to allow a graphical comparison (Fig. 4).

Concerning MIR, the best performance can be assigned to the application of the first derivative after normalization (NDer), with the first three factors extracted allowing full discrimination of the samples, leading to classification errors of 0.0% (Table 1). In this case, these three factors explained more than 95.0% of variability between samples, whereas the first factor by itself explained 76.4%. In all the other cases, in MIR, this factor explained less than 50.0%, with an exception made for the use of the normalized spectra (52.7%). Unexpectedly, this treatment (referred to as 'Spectra') displayed the worst performance, with 12 factors being necessary to fully discriminate the samples, at the cost of a severe over-fitting, as shown by the bouncing error observed when more than three factors are used, whereas when resorting to the first two factors, an error percentage as low as 3.7% was observed (Table 1).

With respect to the use of the NIR interval for monitoring the cowpea pods, when Table 1 is analyzed, a better overall

**Table 1.** Percentage of erroneous classifications in CV (PLS-DA), for each number of factors used, concerning the distinct spectral treatments

| | ATR (MIR) | | | | DRIFT (NIR) | | | |
|---|---|---|---|---|---|---|---|---|
| Factors[c] | Spectra[b] | Der | SG | NDer | Spectra | Der | SG | NDer |
| 1 | 17.4 [a] | 32.2 | 32.2 | 7.4 | 0.0 | 1.9 | 1.9 | 0.0 |
| 2 | 3.7 | 11.1 | 11.1 | 1.9 | 0.0 | 0.0 | 0.0 | 0.0 |
| 3 | 3.7 | 1.9 | 1.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 4 | 5.6 | 1.9 | 1.9 | 1.9 | 0.0 | 0.0 | 0.0 | 0.0 |
| 5 | 5.6 | 0.0 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 6 | 3.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 7 | 3.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.9 |
| 8 | 3.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 9 | 3.7 | 1.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 10 | 1.9 | 1.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 11 | 1.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 12 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 13 | 1.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 14 | 1.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 15 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Optimal number of factors | 2 | 3 | 3 | 3 | 1 | 2 | 2 | 1 |
| Factor 1[d] | 52.7[e] | 47.9 | 45.2 | 76.4 | 86.8 | 81.4 | 76.9 | 86.7 |
| Factor 2 | 27.4 | 34.0 | 37.0 | 11.0 | 4.8 | 8.4 | 11.8 | 6.6 |
| Factor 3 | 1.7 | 10.5 | 8.5 | 8.0 | 0.7 | 6.0 | 4.8 | 4.5 |
| Factor 4 | 2.2 | 4.0 | 5.2 | 2.3 | 2.4 | 2.8 | 3.3 | 1.1 |
| Factor 5 | 5.1 | 2.0 | 2.3 | 0.8 | 1.0 | 0.9 | 1.3 | 0.6 |

[a] Number of factors used for the discriminant analyses.
[b] Distinct treatments applied to the spectral data set: spectra, mean normalized spectra; Der, application of first derivative; SG, simultaneous smoothing and derivative resorting to the Savitzky–Golay algorithm; NDer, application of first derivative after mean normalization.
[c] Percentage of erroneous classifications in the CV procedure of the discriminant analyses developed resorting to the corresponding number of factors.
[d] Factors extracted from the PLS-R procedure to reduce the data set.
[e] Percentage of variability explained by each factor.
Variances explained by the first five factors extracted from the PLS-R procedure are included.

performance is immediately observed with respect to MIR. For example, when the normalized spectra or first derivative after normalization data sets were used, the first factor extracted fully discriminated all of the samples, whereas, for the other treatments ('Der' and 'SG', corresponding to the use of the spectra without normalization and the SG algorithm, respectively), the first two factors extracted were necessary to fully discriminate the samples (Table 1). Furthermore, in this case, independent of the treatment applied, the first factor always explained more than 80.0% of variability, with an exception made for 'SG', where this factor corresponded to 76.9% of variability, probably as a result of a loss of information caused by the 'smoothing' process. Hence, as observed for MIR, in this case, 'NDer' apppears to be the best treatment for this monitoring process, whereas only the MIR interval subjected to normalization failed to completely discriminate the samples (Table 1).

Even though, in some cases, namely in NIR, one factor is sufficent to fully discriminate the samples according to the freezing time, a discriminant analysis has been conducted, for all the cases presented, resorting to the first two factors, aiming to allow the vizualization of a direct comparison between the performances of the distinct approaches used. From the analysis of the plots of the discriminant Can. Vars. extracted (Fig. 4B, C, E, F), it can be seen immediately that the samples with 6 months of freezing tended to be found within the center of the plots, whereas the fresh samples and those presenting 9 months of freezing were found some distance apart, to the opposite sides of the plot, according to Can. Var. 1. Moreover, similar to the observation made for the factors extracted from the PLS-R, Can. Var. 1 explains almost completely (99.9%) the variability between samples, regarding their discrimination, whereas, for MIR, the variability described by the first Can. Var. is somewhat lower. This fact is reflected by the projection of the sample scores, with the discrimination resorting to NIR completely separating the groups of distinct samples only through Can. Var. 1 (Fig. 4B, ), whereas, in MIR, some of the samples were found within clusters of samples with distinct characteristics, as a result of the scores displayed for Can. Var. 1, which occurred mainly when the normalized spectra was used for discrimination (Fig. 4B, C). Finally, regarding the scores of the samples, the plots corresponding to 'Spectra' and 'NDeriv', in NIR, present very similar distances between groups, concerning Can. Var. 1 (Fig. 4E, F).

Because the discrimination resorting to the spectra reflects the compositional differences between samples, the correlations between the spectral features and the contents assessed (total phenolics, flavonoids, and *ortho*-diphenols) were also evaluated, as well as the correlation with radical scavenging activity (DPPH and ABTS). These results are presented in Table 2.

Concerning the use of MIR for this purpose, the data set 'Spectra' showed the best performance for all contents, with an exception made for 'total phenolics', for which 'NDer' achieved the best results. In addition, for flavonoids and *ortho*-diphenols, the use of

**Table 2.** Partial least square regression multivariate calibrations for the quantification of total phenols, flavonoids, *ortho*-diphenols, and DPPH and ABTS radicals scavenging capacity, resorting to the optimal number of factors for each spectral treatment

| | Total phenolics | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | ATR (MIR) | | | | DRIFT (NIR) | | | |
| Multivariate regression parameters | Spectra[a] | Der | SG | NDer | Spectra | Der | SG | NDer |
| $r^2$ cal.[b] | 0.94 | 0.85 | 0.84 | 0.99 | 0.82 | 0.81 | 0.76 | 0.76 |
| $r^2$ ext.[c] | 0.76 | 0.82 | 0.75 | 0.94 | 0.67 | 0.83 | 0.78 | 0.76 |
| Number of factors[d] | 3 | 2 | 2 | 8 | 1 | 2 | 2 | 1 |
| PRESS[e] | 0.5500 | 0.7061 | 0.6664 | 0.4831 | 0.6604 | 0.6735 | 0.6751 | 0.6004 |
| | Flavonoids | | | | | | | |
| | ATR (MIR) | | | | DRIFT (NIR) | | | |
| Multivariate regression parameters | Spectra | Der | SG | NDer | Spectra | Der | SG | NDer |
| $r^2$ cal. | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 |
| $r^2$ ext. | 0.71 | 0.92 | 0.87 | 0.85 | 0.98 | 0.98 | 0.97 | 0.99 |
| Number of factors | 8 | 8 | 8 | 4 | 8 | 9 | 14 | 4 |
| PRESS | 0.3788 | 0.4831 | 0.4488 | 0.4848 | 0.3170 | 0.3666 | 0.3708 | 0.4983 |
| | *Ortho*-diphenol | | | | | | | |
| | ATR (MIR) | | | | DRIFT (NIR) | | | |
| Multivariate regression parameters | Spectra | Der | SG | NDer | Spectra | Der | SG | NDer |
| $r^2$ cal. | 0.99 | 0.99 | 0.99 | 0.99 | 1.00 | 0.99 | 0.99 | 0.99 |
| $r^2$ ext. | 0.71 | 0.91 | 0.83 | 0.89 | 0.98 | 0.96 | 0.96 | 0.99 |
| Number of factors | 11 | 11 | 7 | 4 | 13 | 6 | 11 | 4 |
| PRESS | 0.4680 | 0.6402 | 0.5754 | 0.6617 | 0.3400 | 0.3956 | 0.3729 | 0.5998 |
| | DPPH | | | | | | | |
| | ATR (MIR) | | | | DRIFT (NIR) | | | |
| Multivariate regression parameters | Spectra | Der | SG | NDer | Spectra | Der | SG | NDer |
| $r^2$ cal. | 0.98 | 0.86 | 0.93 | 0.73 | 0.99 | 0.95 | 0.93 | 0.97 |
| $r^2$ ext. | 0.87 | 0.84 | 0.83 | 0.59 | 0.99 | 0.96 | 0.95 | 0.96 |
| Number of factors | 4 | 2 | 4 | 1 | 7 | 3 | 3 | 2 |
| PRESS | 0.2564 | 0.6683 | 0.6029 | 0.6719 | 0.3238 | 0.4820 | 0.4753 | 0.3566 |
| | ABTS | | | | | | | |
| | ATR (MIR) | | | | DRIFT (NIR) | | | |
| Multivariate regression parameters | Spectra | Der | SG | NDer | Spectra | Der | SG | NDer |
| $r^2$ cal. | 0.99 | 0.99 | 0.99 | 0.99 | 0.97 | 0.98 | 0.97 | 0.82 |
| $r^2$ ext. | 0.88 | 0.93 | 0.89 | 0.93 | 0.96 | 0.95 | 0.94 | 0.82 |
| Number of factors | 11 | 5 | 5 | 4 | 6 | 3 | 4 | 1 |
| PRESS | 0.3614 | 0.4947 | 0.4296 | 0.5447 | 0.5998 | 0.4404 | 0.4559 | 0.5278 |

[a] Distinct treatments applied to the spectral data set: spectra, mean normalized spectra; Der, application of first derivative; SG, simultaneous smoothing and derivative resorting to the Savitzky–Golay algorithm; NDer, application of first derivative after mean normalization.
[b] Regression coefficient for the comparison between experimental values, and values predicted for the training set in the CV procedure (values predicted for each sample when excluded from the training set).
[c] Regression coefficient for the comparison between experimental values, and values predicted for the distinct spectral replicates.
[d] Optimal number of factors, retrieved from the CV procedure, for each determination.
[e] PRESS error retrieved from the CV procedure (mean error of all rounds).

'NDer' and 'SG', respectively, led to higher $r^2$ values (0.85 and 0.83) with respect to the prediction of these parameters for the external set of spectra (all the replicates registered) compared to the 'Spectra' treatment (0.71 and 0.71), whereas the latter approach led to smaller PRESS errors. Nonetheless, because the PRESS was calculated based on the values predicted for the samples excluded in the LOO procedure, thus allowing an evaluation of the ability to analyze samples outside the calibration set, the feasibility of using the data set 'Spectra', leading to the lowest PRESS in most cases, has to be taken into account (Table 2).

For NIR, the use of the data set 'Spectra' led to the best results in three cases (flavonoids, *ortho*-diphenols and DPPH), whereas, regarding these parameters, the PRESS errors observed were lower than the values achieved when MIR was used for the prediction (Table 2). Furthermore, for the two remaining analytical parameters evaluated (total phenolics and ABTS), the best regression in NIR was obtained resorting to 'NDer' and 'Der', respectively, whereas, in both cases, the PRESS errors observed were larger than those observed for MIR for the prediction of these parameters. Unexpectedly, the application of NIR displayed the best results for all the parameters (concerning all quality items), whenever the use of 'Spectra' displayed the best performance, whereas, for total phenolics, where 'NDer' provided the optimal results in both intervals, the regression resorting to MIR appeared to be superior in all aspects. This was also true for the prediction of ABTS. Accordingly, the best models for each interval displayed very similar performances, even though the use of 'Spectra', presenting the best performance in MIR, led to a smaller PRESS error with respect to NIR, for which 'Der' displayed the best performance (Table 2).

In both intervals, when the regression is undertaken with resort to the data set 'Spectra', more factors tended to be extracted for the optimal result (as selected through the CV procedure) compared to the other data treatments, whereas the use of 'NDer' leads to the extraction of fewer factors to describe the data set. Indeed, one reason for using the first derivative, instead of the 'raw' spectra, besides overcoming spectral artifacts and baseline drifts, is the possibility of reducing the variability of the data set with fewer factors, thus avoiding the occurrence of over-fitting in the prediction models. By contrast, in the present study, the use of the spectra and the consequent larger numbers of factors led to the best results for the prediction of the phytochemical contents. Nonetheless, for the discrimination purposes, the use of the first derivative has performed better in MIR, and both treatments have displayed similar performances, when the NIR interval is used to discriminate the cowpea pods according to the distinct freezing timings.

Therefore, although the contents screened resorting to the colorimetric methods also allowed discrimination of samples according to the freezing time, it has been shown that both the NIR and MIR intervals allow this classification to be performed, whereas the latter methods (spectroscopic) are more suitable for use in real-time monitoring *in situ* because no sample preparation is required, and no solvents are used, thus potentially allowing use outside the laboratory environment. Moreover, NIR equipment does not represent such a large investment because it is presently widespread and in use by several food industries for distinct purposes, whereas this spectroscopic approach displayed the best performance with respect to the traceability of freezing time in the present study.

Finally, it is worth noting that, even though the suitability of this approach has been demonstrated, further steps are still necessary to allow this procedure to be used routinely for the traceability of these frozen matrices. Accordingly, the registration of spectra from larger sets of samples will be necessary, which would hopefully include samples from distinct geographical locations, harvested at different seasons in the same maturation stage. Also, the achievement of such a set of spectra, besides encompassing distinct variables in the classification models, would also allow validation of the classification models, using samples for assessment as an external set (e.g. one-third of the samples) that would be excluded from the calibration step.

## CONCLUSIONS

The phytochemical content in frozen cowpea pods decreases during the storage period, whereas such variations can be monitored by spectroscopic approaches, namely FTIR, which has been shown to represent an appropriate tool for the traceability of these frozen foodstuffs during extended storage periods.

The use of both MIR and NIR intervals has been shown to be suitable for the classification of samples, with respect to freezing times, as well as for the evaluation of the impact of the storage period on their phytochemical content. Concerning the classification of samples, the NIR interval has performed better, mainly when the spectra or the first derivative after normalization are used, whereas, for the evaluation of phytochemical contents, the use of the spectra, within the NIR interval, generally achieved the best performance, presenting lower PRESS errors for the regressions. Nonetheless, concerning the parameters for which the best results were obtained with the first derivatives, the best performances were observed for the MIR interval.

Therefore, the FTIR approach, both within the MIR and NIR intervals, followed by distinct treatment of the data for use in tandem with multivariate approaches (PLS-R and PLS-DA), represents a suitable and powerful methodology to be used for the traceability of frozen vegetal products, with the differences between spectra reflecting the compositional differences between the pods with respect to phytochemical contents. Finally, even if the parameters assessed colorimetrically allow classification of the samples, according to the freezing time, the spectroscopic-based tools comprise a simpler method, showing sufficient potential for their use in the assessment of the contents of these food matrices.

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