

Feasibility of Vis/NIR spectroscopy to detect and estimate fungicide residues on intact lettuces

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All relevant data are within the paper and its Supporting Information files.

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Abstract: Pesticides are applied repeatedly to grain, fruit, and vegetable crops for protection against pathogens, pests, and weeds, in short periods of time before harvest. The effective and fast monitoring of chemical residues in agricultural products is important for the assurance of healthy food. This study was accomplished to evaluate the feasibility to detect and estimate the concentration of dithiocarbamate fungicide (mancozeb) residues on intact lettuce leaves based on Vis/NIR spectral reflectance measurements and multivariate data analysis. In the pre-harvest interval, a high initial rate of decline of dithiocarbamate residues was observed between one and seven days after pesticide spraying (decrease of 90.3%), while a slower decline was verified from seventh to fourteenth day (decrease of 8.7%). The usefulness of this spectrometric method has been evidenced by determination of dithiocarbamate residues at concentrations between 0.23 and 10.3 mg CS₂ kg⁻¹, with detection and quantitation limits of 0.49 and 1.41 mg CS₂ kg⁻¹, respectively. Vis/NIR spectral reflectance combined to the partial least square analysis have potential to be applied for estimating dithiocarbamate concentrations on intact lettuce leaves, presenting advantages such as real-time measurements and the possibility to be built into the industrial processing lines.

1. Introduction

Consumers demand grain, fruits, and vegetables with high sensorial and nutritional qualities, but without pesticides. Although the correct use of fungicides does not cause problems of public concern in health and environmental areas, undesirable residues can remain on agricultural products after harvest if inappropriate or abusive treatments are applied without respecting safety recommendations indicated by the specialized agencies and manufacturers (López-Fernández *et al.*, 2013).

In modern agriculture, pesticides are applied repeatedly to grain, fruit, and vegetable crops for protection against pathogens, pests, and weeds, in short periods of time before harvest (Jankowska *et al.*, 2019). The main exposure to pesticides for humans is via food, especially by consumption of agricultural products.

Introduced between 1940 and 1980, dithiocarbamate fungicides still represent an important class of pesticides widely used in agriculture. They are characterized by a broad spectrum of activity against various plant pathogens, low acute mammal toxicity, and low production costs (Crnogorac and Schwack, 2009). However, laboratory studies showed that dithiocarbamates can result in neuropathology, thyroid toxicity, and developmental toxicity to the central nervous system (IPCS, 1993; Caldas *et al.*, 2006).

Mancozeb is an ethylene-bis-dithiocarbamate used as fungicide to protect fruit and vegetable crops from a range of fungal diseases (Pereira *et al.*, 2014) and was considered a multipotent carcinogenic agent in a long-term study (Belpoggi, 2002). This fungicide is registered to 38 food crops, occupy the third place in the ranking of the most commercialized pesticides in Brazil in terms of tones of active ingredient (ANVISA, 2018; IBAMA, 2018).

Caldas *et al.* (2006) reported data obtained from the Program on Pesticide Residue Analysis in Food (PARA), coordinated by the Brazilian National Sanitary Surveillance Agency (ANVISA), about dithiocarbamate residues on 34% of lettuce samples in a total of 297 analyzed, based on vegetable samples collected from 2001 to 2004. In another Brazilian study, accomplished from 2005 to 2015, Jardim *et al.* (2018) showed that 14.6% of 1483 lettuces presented dithiocarbamate residues. López-Fernández *et al.* (2013) verified the presence of mancozeb and other dithiocarbamate residues on 72% of lettuce samples collected at Spain, with some samples containing residues three times higher than the maximum limit (5 mg kg⁻¹).

Various methods have been improved for the determination of dithiocarbamate residues, including gas and/or liquid chromatography, often in conjunction with mass spectrometry (Crnogorac and Schwack, 2009). These current methods requiring sample preparation (destructive), time consuming, and laboratory labor demanding, well-trained personnel and relatively expensive process chemistry. They also produce chemical and sample waste, which adversely affects product traceability by preventing real-time decision-making (Salguero-Chaparro *et al.*, 2013; Steidle Neto *et al.*, 2017). Although more sensitive, these methods are suitable for spot checks.

The reasons for Vis/NIR spectroscopy great success as one of the most important and versatile techniques in analytical chemistry include its speediness and easiness to handle and provide molecular specific information for different types of samples in any

physical state, with little or no previous chemical treatment (González *et al.*, 2011). However, one possible drawback is the range of concentration of the target analyte. Pesticide residues tend to have very small concentrations in foods. Despite this, previous studies proved the feasibility of spectroscopy to detect and quantify low concentrations of analytes in fruits and vegetables (Saranwong and Kawano, 2005; González *et al.*, 2011; Acharya *et al.*, 2012). Nevertheless, very few published studies have addressed the use of Vis/NIR spectroscopy for predicting pesticides residues in harvested intact samples. The scientific researches of Sánchez *et al.* (2010), for peppers, and Jamshidi *et al.* (2016), for cucumbers, reported estimates of pesticides without pre-treatment or preparation of samples. This advantage allows that spectroscopy technique can be built into the processing line, enabling large-scale individual analysis and real-time decision-making. Another recent technique for trace level detection of pesticides is the Surface Enhanced Raman Spectroscopy (SERS), that use noble metal nanostructures (e.g. gold) to increase the weak signals from analytes. But, SERS requires spectrometer, laser source, probe, sample holder, and substrates that are more expensive when compared with spectral reflectance equipment.

The present scientific research was carried out to evaluate the feasibility to detect and estimate the concentration of dithiocarbamate fungicide residues on intact lettuce leaves based on Vis/NIR spectral reflectance measurements and multivariate data analysis.

2. Materials and Methods

Lettuce cultivation

Lettuce (*Lactuca sativa* L. cv. Regina) with plain green leaves, was cultivated under organic conditions in a certified farm located at the Capim Branco city, Minas Gerais State, Brazil (19° 34' S latitude, 44° 10' W longitude, and 816 m a.s.l.). According to Köppen classification, the region climate is Cwa (warm temperate - mesothermal), with dry winter and rainy summer.

The lettuce seeds were planted in plastic trays containing organic substrate. Seedling production occurred under a low-density polyethylene cover (Suncover Av Blue 120 µm, Ginegar Polysack, São Paulo, Brazil), which allowed a better irrigation control and was internally coated with a photoselective

shading mesh (ChromatiNet Raschel Red 35%, Ginegar Polysack, São Paulo, Brazil), capable of reducing the percentage of beam solar radiation on the plants, also increasing the fresh mass production.

The vigorous and healthy seedlings were individually transplanted to plastic pots containing a thin gravel layer overlapped by soil mixed with organic compound (cattle manure and vegetable biomass). The crop irrigation was performed by a drip system, controlled by a digital timer. The experiment consisted of 500 lettuce plants. However, 355 plants were effectively used as experimental units and 145 plants were cultivated for boundary effects.

Some days before plants reach the physiological maturation, the lettuces were transported to a greenhouse with the objective of performing the fungicide spraying. The greenhouse is located at the Campus Sete Lagoas of the Federal University of São João del-Rei, which is distant from the organic farm about 22 km. This greenhouse also was covered and coated with the same polyethylene film and photo selective shading mesh described above.

Fungicide spraying

A non-systemic fungicide (mancozeb) was used, which is classified in the alkylenebis group (dithiocarbamate). The active ingredient of this pesticide is a Carbon Disulphide (CS_2) precursor and is registered in the Brazilian Ministry of Agriculture, Livestock, and Food Supplies (MAPA) for application on some crops. However, the dithiocarbamate (mancozeb) is not authorized by the Brazilian National Sanitary Surveillance Agency (ANVISA) for lettuce crop. This fungicide was chosen based on reports of the Brazilian Monitoring Program for Pesticides Residues in Food, developed by ANVISA, that mentioned the indiscriminate use of this pesticide on lettuces and other crops by some Brazilian farmers. Contrarily, this fungicide is authorized for lettuce crop by other agencies and committees, such as the European Food Safety Authority and the FAO Codex Alimentarius.

The solubilization of mancozeb in water was done to provide a sufficient volume for uniform and homogeneous application on lettuces. The dosage prescribed in the Brazilian package leaflet for other green leafy vegetables, such as cabbage, was adopted ($2\text{--}3\text{ kg of fungicide ha}^{-1}$). This dose is recommended to control the mildew (*Peronospora parasítica*) and the pod spot (*Alternaria brassicae*). Excepting for five plants (control units), which were randomly selected in the greenhouse, the application of mancozeb to lettuces was performed using an electric sprayer (droplets with average diameter of $29\text{ }\mu\text{m}$).

The application time and distance from the sprayer nozzle to the plants were standardized.

Similar to the dosage, the pre-harvest interval for consumption of green leafy vegetables informed in the Brazilian package leaflet (14 days) was considered in this study. Samplings for the spectral reflectance measurements and laboratory analyzes were carried out on alternate days during the pre-harvest interval, totaling 7 days and starting one day after the fungicide spraying.

Daily 10 samples were collected, each one weighting more than 500 g and corresponding to five lettuce plants randomly selected. The fresh mass was determined using a precision balance and discarding the roots. Therefore, 50 plants were used per day, totaling 350 plants during the sampling period.

The statistical design was entirely randomized with 7 treatments (alternate days after spraying) and 10 repetitions (samples).

Reflectance measurements

Spectral reflectance was measured by a miniature and hand-held spectrometer (JAZ-EL350, Ocean Optics, Dunedin, USA), coupled to a tungsten-halogen light source, and preconfigured to acquire and store reflectance data from 350 to 1000 nm, with spectral resolution of 1.3 nm. A specific clip probe (SpectroClip-R, Ocean Optics, Dunedin, USA) was used to collect reflected light from the lettuce leaves (Fig. 1). This probe contains an integrating sphere that captures diffuse reflected light more efficiently



Fig. 1 - Hand-held spectrometer, clip probe, and diffuse reflectance standard used to collect reflected light from the lettuce leaves.

than lens-based collection optics. The active illuminated leaf area in the clip probe is 5 mm diameter. Two premium fibers Vis/NIR (600 μm) interconnected the spectrometer and the light source to the clip probe. A diffuse reflectance standard with Spectralon™ was used as a reference to measure spectral reflectance.

After the warm up time of the light source, the reference standard measurements were made before the spectral reflectance measurements on lettuce leaves. Data acquisitions were performed in a temperature-controlled environment with the purpose of avoiding the overheating of the light source and the spectrometer detector due to the extended using time. The reflectance values were calibrated by means of the software (OceanView, Ocean Optics, Dunedin, USA) and expressed as a relative percentage of the reference standard (Xing *et al.*, 2006):

$$R_{\lambda}^{\text{cal}} = [(R_{\lambda}^{\text{leaf}} - R_{\lambda}^{\text{dark}}) / (R_{\lambda}^{\text{ref}} - R_{\lambda}^{\text{dark}})] \times 100 \quad (1)$$

where R_{λ}^{cal} is the calibrated spectral reflectance from the leaves (%), $R_{\lambda}^{\text{leaf}}$ is the spectral reflectance from the leaves (dimensionless), $R_{\lambda}^{\text{dark}}$ is the spectral reflectance considering light absence (dimensionless), and R_{λ}^{ref} is the spectral reflectance from the diffuse reflectance standard (dimensionless).

Three leaves of each plant were randomly selected from the external, middle, and central parts of the lettuce head. Three separate measurements on standardized and equidistant locations of the adaxial surface were performed in each leaf, avoiding its central vein and boundaries. Thus, 3195 spectral signatures were obtained during the sampling period, considering the 350 sprayed plants and the five control lettuces (without dithiocarbamate).

The electronic files containing the spectral signatures were stored in a memory card and later transferred to a notebook for analyzes performed with electronic spreadsheets. During the analyzes, spectral signature averages were obtained for each lettuce sample. After this, data was stored in an external hard disk.

Dithiocarbamate analytical determination

Samples were quartered, milled in an electric grinder, placed in hermetic packages, and frozen at -30°C in an ultrafreezer for minimizing the degradation and metabolization of the dithiocarbamate.

The analytical determination of the concentration of dithiocarbamate was performed based on the method proposed by Cullen (1964) and improved by Keppel (1971). Mancozeb residues were measured by the spectrophotometric determination of the cupric

complex formed with the CS_2 evolved from the acid decomposition of dithiocarbamate in the presence of stannous chloride as a reducing agent (Caldas *et al.*, 2004). The solution of the complex formed from the reaction between CS_2 and copper (II) acetate monohydrate was measured at 435 nm in UV/Vis spectrophotometer (Cary 50, Varian, Agilent Technologies Inc., USA). At the end of the laboratory analyzes, 70 reference measurements were obtained, corresponding to 10 values for each treatment.

Data analysis

The Partial Least Squares (PLS) multivariate analysis was applied with the purpose of developing a mathematical model capable of predicting the dithiocarbamate concentrations based on lettuce pre-treated spectral signatures. Thus, a response matrix, composed by the dithiocarbamate concentrations obtained by laboratory analytical measurements, was correlated with a spectral matrix, containing the average reflectance measurements. An orthogonal basis of latent variables was constructed one by one in such a way that they were oriented along the directions of maximal covariance between the two original spaces (response and spectral matrices), trying to achieve an optimal prediction for new data (Wold *et al.*, 2001; Anderson, 2009; Cozzolino *et al.*, 2011).

The latent variables were calculated by iterative methods as linear combinations of the original independent variables (spectral reflectances) and the dependent ones (dithiocarbamate concentrations). New variables were found, representing estimates of the latent variables or their rotations. These new variables were called X-scores and were predictors of the response ones. A weight matrix was also calculated so that each of their elements maximized the covariance between response variables and the corresponding latent variable scores. The unexplained part of the predictor variables was represented by the deviations between the measured and predicted responses, which were also calculated and called Y-residuals (Wold, 2001; Lopes and Steidle Neto, 2018).

The detrending pre-treatment was applied to the spectral signatures prior to the model calibration and external validation. The detrending algorithm corrected scatter and simple deformations of the spectra baseline as vertical shift and slope (Barnes *et al.*, 1989; Steidle Neto *et al.*, 2016). According to Moura *et al.* (2016), this was the most effective pre-treatment for removing irrelevant information which could not be handled by the regression technique and principal component analysis.

As recommended by Huang *et al.* (2008), two thirds of the data for each response variable were used as the calibration with cross-validation set, whereas one third of the data as the external validation set. The calibration set was used for developing the model and the external validation set was employed for assessing the calibrated model prediction performance. This procedure was applied for predicting independent data, not related with those included in the calibration with cross-validation set (Agelet and Hurburgh, 2014). During calibration the leave-group-out cross-validation technique was applied to data. This sampling plan also agrees with the suggested by Kramer (1998), who affirmed that the number of data in the calibration set should be more than 10 times the number of variable components in the experiment. In this study the dithiocarbamate residues represented the independent source of significant variation in the data.

The software SPECTOX was specifically developed for this study with the purpose of assisting in the spectral reflectance data processing and the multivariate analysis (calibration and validation), also allowing the performance evaluation of the prediction model for dithiocarbamate concentrations. The SPECTOX was written in Java language, by using the free NetBeans IDE (Apache Software Foundation, Maryland, USA). Additional algorithms of the pre-treatments and multivariate methods were included in the SCILAB software (Scilab Enterprises, Versailles, France).

The optimal number of latent variables was determined as recommended by Jha (2010), considering the minimum value for the root mean square error to avoid over fitting (Eq. 2).

$$RMSE = \sqrt{\frac{\sum (Y_o - Y_p)^2}{n}} \quad (2)$$

Where RMSE is the root mean square error (mg kg^{-1}), Y_o are the values measured by the UV/Vis spectrophotometer (mg kg^{-1}), Y_p are the values predicted by the model (mg kg^{-1}), and n is the number of samples (dimensionless).

The calibration and cross-validation processes were evaluated by the root mean square errors for calibration (RMSEC) and cross-validation (RMSECV) sets, respectively. Additionally, the predictive capacity of the adjusted model regarding external validation was evaluated by the statistical parameters mean absolute error (MAE), mean bias error (BIAS), coefficient of determination (R^2), and index of agreement (d).

Willmott and Matsuura (2005) pointed out that

MAE (Eq. 3) is unambiguous and the most natural measure of the mean error magnitude. These authors considered that MAE should be used as the basis for all dimensioned evaluations and inter-comparisons of model performance. The BIAS (Eq. 4) represents the average difference between measured and predicted data. Thus, values close to zero indicate low systematic error between the measured and predicted values (high accuracy of the model). Also, negative BIAS values indicate underfitting, while positive BIAS values reveal overfitted predictions (Steidle Neto *et al.*, 2016). The coefficient of determination (Eq. 5) represents the proportion of explained variance of the response variable in the validation set, with results varying from 0 to 1, and the maximum value reflecting a perfect agreement between measured and predicted data (Steidle Neto *et al.*, 2017). Finally, index of agreement (Eq. 6) varies from 0 to 1, where the maximum value reflects a perfect agreement between measured and predicted data. As affirmed by Willmott (1981), this is an important index since it is not a measure of correlation or association in the formal sense but rather a measure of the degree to which the model's predictions are error free.

$$MAE = \frac{\sum |Y_p - Y_o|}{n} \quad (3)$$

$$BIAS = \frac{\sum (Y_o - Y_p)}{n} \quad (4)$$

$$R^2 = \frac{[\sum (Y_p - \bar{Y}_p)(Y_o - \bar{Y}_o)]^2}{\sum (Y_p - \bar{Y}_p)^2 \sum (Y_o - \bar{Y}_o)^2} \quad (5)$$

$$d = 1 - \frac{\sum (Y_p - Y_o)^2}{\sum (|Y_p - \bar{Y}_p| + |Y_o - \bar{Y}_o|)^2} \quad (6)$$

Where MAE is mean absolute error (mg kg^{-1}), BIAS is the mean bias error (mg kg^{-1}), R^2 is the coefficient of determination (dimensionless), and d is index of agreement (dimensionless).

The limit of detection (LOD) and limit of quantitation (LOQ) are frequently used to describe the smallest concentrations of a sample that can be reliably measured by an analytical procedure. The LOD corresponds to the lowest analyte concentration at which detection is feasible, while the LOQ is the lowest concentration at which the analyte can be effectively quantified. Thus, LOQ tends to be equivalent or higher than LOD (Armbruster and Pry, 2008). In this study, the statistical LOD and LOQ determinations were applied (CLSI, 2004; Jeon *et al.*, 2007). That is,

the spectra of five representative blank lettuce samples (containing no dithiocarbamate residues) were measured, following the same procedures used during the calibration and external validation of the PLS model. The mean and standard deviation of the predicted CS₂ concentrations were calculated based on these spectra, which were used as input for the developed model. The LOD and LOQ were equal to the mean of the predicted CS₂ concentrations plus three times or ten times the standard deviation of the mean, respectively. According to CLSI (2004) and Armbruster and Pry (2008), although the blank samples are devoid of analyte, they can produce an analytical signal that might be consistent with a low concentration of dithiocarbamate.

3. Results

Figure 2 presents the degradation curve of dithiocarbamate residues on lettuce leaves during the pre-harvest interval, obtained from average values of laboratory analytical measurements. The estimated half-life of the dithiocarbamate residues was 5-7 days. The rates of decline of dithiocarbamate concentration in two-day intervals were quite variable from first to seventh day after spraying (4.7, 3.2, and 1.4 mg CS₂ kg⁻¹), corresponding to a decrease of 90.3%. After the seventh day, the rates of decline were 0.3 mg CS₂ kg⁻¹, indicating a decrease of 8.7%. Although very small concentration residues persisted at the final of pre-harvest interval, the results indicate that after this period the pesticide metabolization is advanced, assuring reliability for lettuce consumption.

The PLS model for dithiocarbamate concentration on lettuces was more precise and accurate when detrending pre-treatment was applied to spectral signatures, compared with predictions obtained from spectra without pre-treatment or treated with other methods (centering, standardization, first and second derivatives). Despite NIR bands were more sensible,

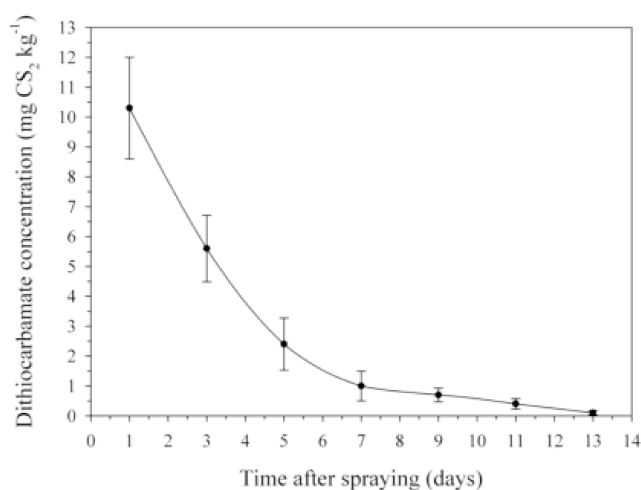


Fig. 2 - Dithiocarbamate concentration decay (mg CS₂ kg⁻¹) on lettuce leaves at seven intervals after mancozeb spraying. Vertical bars represent the standard error of the average values.

all wavelengths (350-1000 nm) presented potential to explain the dithiocarbamate concentration residues on lettuce leaves from spectral reflectance, contributing to the good performance of the prediction PLS model.

Table 1 shows the statistical results for the dithiocarbamate concentration model, considering the calibration with cross-validation and external validation datasets. The data processing showed that the use of more than four latent variables for dithiocarbamate concentrations resulted in an over-fitting, characterized by a slight divergence in the downward trend of the RMSE, which continued to decrease for calibration (RMSEC), but almost established for cross-validation (RMSECV).

The proposed PLS model was satisfactory since presented low RMSEC, RMSECV, RMSE, MAE, and BIAS when compared to the range values (Table 1). Additionally, the performance of the model for the external validation presented high index of agreement (0.94), reflecting a good accuracy for independent predictions of the dithiocarbamate concentra-

Table 1 - Statistical parameters of calibration with cross-validation and external validation processes for the estimation model of dithiocarbamate concentration on lettuces

	Calibration		External validation
Number of latent variables	4	R ² (dimensionless)	0.87
RMSEC (mg CS ₂ kg ⁻¹)	1.86	RMSE (mg CS ₂ kg ⁻¹)	1.41
RMSECV (mg CS ₂ kg ⁻¹)	2.74	MAE (mg CS ₂ kg ⁻¹)	1.24
Range (mg CS ₂ kg ⁻¹)	0.23-10.3	BIAS (mg CS ₂ kg ⁻¹)	-0.37
		d (dimensionless)	0.94

tions on lettuces. The negative BIAS value indicated a majority tendency of underfitting predictions by the model, mainly from 2.3 mg CS₂ kg⁻¹ (Fig. 3). Also, low BIAS value represented small systematic error (Table 1).

Regarding the differences among statistics presented in Table 1, the spectral measurements and predictions of external validation may deviate from the calibration as they originate from different sample sets. As mentioned by Liu and Ying (2005), in this way, the ability of the calibration model to withstand unknown variability is assessed.

The correlations between values determined by the reference analytical method and those predicted by the external validation of the PLS model are presented in figure 3. Prediction performance resulted in good agreement between reference and estimated values, with R² of 0.87 (Table 1), indicating that 87% of the measured values were accurately represented by the model.

The comparison of parameters (LOD, LOQ, and range) associated to different methods of determination of dithiocarbamates on lettuces are showed in

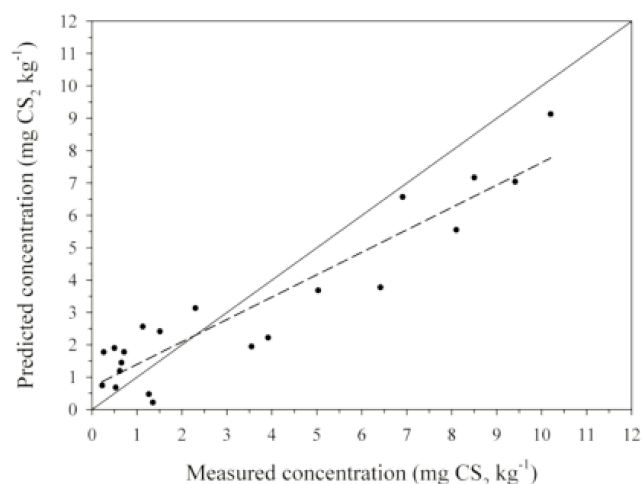


Fig. 3 - External validation of the PLS model for estimating dithiocarbamate concentrations (mg CS₂ kg⁻¹) on lettuce leaves.

Table 2. The LOD and LOQ values verified in this study were higher than those obtained with the gas or liquid chromatography, as well as the spectrophotometric method.

The usefulness of the methodology presented in this study has been evidenced by the determination of dithiocarbamate residues on lettuce samples at concentrations between 0.23 and 10.3 mg CS₂ kg⁻¹. This range can be considered appropriated when the results of previous studies are used as reference (Table 2). Further, values between these lower and upper limits are sufficient to measure a wide range of dithiocarbamate concentrations on lettuces.

4. Discussion and Conclusions

Past studies showed dithiocarbamate degradation profiles for lettuces similar to those found in this study. Yip *et al.* (1971) found that the dithiocarbamate concentration (maneb) from a single spray on lettuces declined from 45 mg kg⁻¹ initially to about 5 mg kg⁻¹ after 15 days (decrease of 89%). On the other hand, Hughes and Tate (1982) monitored mancozeb residues on lettuces and verified a reduction of 90 mg kg⁻¹ after a 14-day interval (decrease of 72%). These authors also reported a high initial rate of decline in dithiocarbamate concentration during the first seven days after spraying, confirming the high degradation of analyte.

Despite of the dithiocarbamates with active ingredient based on mancozeb are not authorized by ANVISA for lettuces in Brazil, the European Food Safety Authority (EFSA) allows the use of this pesticide on lettuce crop in the countries that integrate the European Union, considering a maximum residue limit of 5 mg kg⁻¹ (EFSA, 2013). In addition to the dietary risk, the edafoclimatic differences and dietary patterns of the countries justify the distinct positions between ANVISA and EFSA. Based on the results, lettuces presented dithiocarbamate concentrations

Table 2 - Limit of detection, limit of quantitation, and range (expressed in mg CS₂ kg⁻¹) for different methods of determination of dithiocarbamates on lettuces

Method	Limit of detection	Limit of quantitation	Range	Reference
Gas chromatography	0.004	0.013	0.04-5.0	Česnik and Gregorčič (2006)
Liquid chromatography	0.04	0.11	0.50-9.3	López-Fernández <i>et al.</i> (2012)
Gas chromatography	0.02	0.05	0.04-1.0	Pizzutti <i>et al.</i> (2017)
Spectrophotometric	0.28	0.40	0.20-4.5	Pizzutti <i>et al.</i> (2017)
Spectrometric	0.49	1.41	0.23-10.3	Present study

lower than the maximum residue limit established by EFSA from the fourth day after spraying.

According to Lopes and Steidle Neto (2018) detrending, which was the most appropriated spectral pre-treatment in this study, is usually used to remove specific data offsets that are not related to the chemical or physical properties of interest for the chemometric modeling. Sánchez *et al.* (2010) also developed PLS models for predicting pesticide residues on intact peppers using near-infrared reflectance spectroscopy, applying detrending method for scatter correction in data, and obtaining good results. Steidle Neto *et al.* (2016) affirmed that detrending was the best spectra pre-treatment when predicting chlorophyll content in lettuces, helping to remove non-linear trends in spectroscopic data, and consequently correcting scatter.

The number of latent variables considered adequate for predicting dithiocarbamate concentrations on lettuce leaves in this study agree with Cozzolino *et al.* (2011), who affirmed that if more than optimum number of latent variables is used, the solution can become over-fitted and the model will be very dependent on the dataset, giving poor predictions. Otherwise, using less than the optimum number of latent variables will cause under-fitting and the model will not be accurate enough to capture the variability in the data. This result also agrees with other researchers who applied spectroscopy and PLS models to non-destructively predict of pesticide concentrations. Jamshidi *et al.* (2016) showed that 5-7 latent variables were required for PLS models when predicting diazinon residues on cucumbers.

Although the proposed model tended to underestimate the pesticide concentrations, slight overestimates were verified for low dithiocarbamate concentrations, evidenced in this study until the fifth day after spraying.

The method based on spectral reflectance provided sufficient sensibility for detecting and quantifying concentrations lower than the maximum residue limit allowed by the European Food Safety Authority for mancozeb-based dithiocarbamates on lettuces (5 mg kg⁻¹). Makino *et al.* (2009) used spectral reflectance for detection and quantification of chlorpyrifos residues on apple surface, also demonstrating the feasibility of spectroscopy for estimating low concentrations of pesticide residues in fruits.

According to Acharya *et al.* (2012), the spectral features can be assigned to overtone and combination bands of various C-H and N-H bonds within these molecules. Considering that the chemical structure of

mancozeb has 2 NH and 2 CH₂, and that the NH bond position is unhindered within the chemical structure, there is a high likelihood it will produce a sharp and strong absorption band, which should improve detection of mancozeb.

The mean spectral signatures of intact lettuce leaves with absence and presence of fungicide presented differences mainly in NIR region, with fungicide-contaminated samples resulting in greater absorbance than that in the fungicide-free lettuce leaves. According to that reported by Sánchez *et al.* (2010) and Jamshidi *et al.* (2016), increase of the absorbance in NIR region after 900 nm could be due to the C-H absorption.

Based on the results, it can be said that the Vis/NIR spectroscopy combined to the multivariate data analysis have potential to be applied as an alternative method to estimate dithiocarbamate concentrations on lettuce leaves. However, the success and widespread adoption of this method also depends of suitable measurement practices. It is important that measurements are performed using a spectrometer with high spectral resolution, after the time required to warm-up the light source, and after adequate spectrometer calibration (proper sampling of reference standard). Additionally, important factors to achieve good results include the standardization of the measurement points in the samples, the homogeneity of the target area, and the positioning of samples on a black non-reflective panel with the purpose of prevent the light reflection going through the leaf. Another essential aspect is related to the incidence angle of the light bunch, which is emitted by the light source over the sample and directly influences the light reflection by sample (Steidle Neto *et al.*, 2017). Although the effects of the abovementioned individual error sources may appear small, their combined presence may result in measurements with significant errors, influencing the spectral reflectance independently of dithiocarbamate concentrations, as well as, the model predictions.

Vis/NIR spectral reflectance measurements combined to the partial least square analysis showed to be feasible and effective as a promising method for estimating concentration of dithiocarbamate fungicide residues on intact lettuce leaves. The developed PLS model allowed the detection and quantification of the dithiocarbamate without any preparation and/or processing of lettuce samples. This method offers advantages over traditional laboratory methods, such as real-time measurements and the possibility to be built into the industrial processing lines,

enabling large-scale individual analysis in 100% of the lettuces.

The spectral behavior of other vegetable species certainly will differ from that of lettuce, as well as, different dithiocarbamate types (thiram, metiram, propineb, zineb, ziram, and maneb) tend to cause variations in the estimating models. Future researches will be performed in order of evaluating these effects when detecting and quantifying fungicide residues in vegetable crops. The results of these new studies will complement the findings of the present work, making this a more wide-ranging method.

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