Carotenoid profile determination of bee pollen by Advanced Digital Image Analysis

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ABSTRACT

Bee pollen is a natural matrix widely studied in its nutritional and bioactive compounds, including carotenoids. That composition is usually identified by Rapid Resolution Liquid Chromatography (RRLC) coupled to UV-Vis spectrophotometry, an expensive method that requires complex sample preparation and long analysis time. In this work, a correlation between colorimetric coordinates and carotenoid composition was evaluated. Through Digital Image Analysis (DIA) by DigiEye, the color characteristics were determined, and carotenoids profile was done by RRLC. The correlations were made by multiple linear

regression (MLR). From 12 carotenoids found in the samples, six had a coefficient R^2>0.75 between reference and predict value. Heterogeneous mixtures of bee pollen samples were analyzed, and the suitability of the mathematical models could be corroborated because the relative error for most of the compounds was less than 20%. It has been demonstrated that union of Tristimulus Colorimetry and Image Analysis represent an effective tool to estimate the chemical composition in food industry.

Key words: Image Analysis; Carotenoids; Bee Pollen; Multiple Linear Regression

1. Introduction

Bee pollen is a natural product made by worker bees when they collect nectar and pollen. Floral pollen is mixed with salivary secretions and nectar. Afterward, it is recollected by beekeepers through traps in the hive and since that moment it can be named bee pollen (Fuenmayor B et al., 2014; Kieliszek et al., 2018). It is composed of two protective layers, intine and exine, which protect the interior of the oxidation grain, radiation damage and chemical degradation (Atkin et al., 2011). Exine is composed of various organic and inorganic substances, among which sporopollenin is a very complex polymer that gives chemical resistance to pollen (Kovacik et al., 2009). The structure of sporopolenin has been extensively studied, finding that it presents a variety of substances, such as some types of carotenoids, tocopherols, provitamin A and vitamin D (Domínguez-Valhondo et al., 2011). Among carotenoids, β-carotene, cryptoxanthin, zeaxanthin and lutein were identified in bee pollen (Domínguez-Valhondo et al., 2011; Schulte et al., 2009). In a previous study of Colombian bee pollen, traces of lutein, zeaxanthin, β-carotene and phytoene were identified (Gardana et al., 2018). Carotenoids are important because they are related to different positive effects in cancer, cardiovascular diseases, diabetes, cataracts and others (Kong et

al., 2010; Saini et al., 2015). These compounds are also responsible for color (Saini et al., 2015; Sant'Anna et al., 2013).

Some studies characterized bee pollen color and carotenoid content, and looked for a relation between both parameters (Domínguez-Valhondo et al., 2011; Machado De-Melo et al., 2016; Schulte et al., 2009; Xu et al., 2013). However, the heterogeneity of the pollen samples makes it difficult to measure color by conventional colorimetric techniques. In these cases, Digital Image Analysis (DIA) allows to evaluate the color of each point within a given area, which makes this technique ideal for the analysis of bee pollen (Salazar-González et al., 2018). This technology also allows to develop Chemical Imaging, where it is possible to indicate the presence or concentration in pseudo-color scales of a chemical compound in an image (Rodríguez-Pulido et al., 2017).

Several studies have been made in the last years correlating data obtained by DIA with bioactive compounds in several foods: grapes or tomatoes (Rodríguez-Pulido et al., 2012; Stinco et al., 2013). In a study on tomato products (fresh and processed), the authors could correlate Digital Image Analysis with lycopene isomers content (LYC). When tridimensional character of color is considered and a multivariate analysis is needed, the correlation coefficient increased up to 0.77 (Stinco et al., 2013).

Previous studies showed that it is possible to predict chemical composition by Digital Image Analysis in food products. Then, the aim of this work was to evaluate the possibility to predict carotenoid content from image analysis parameters in Colombian bee pollen. These correlations would help beekeeping chain to value its products with one simple image.

2. Material and Methods

2.1 Samples

Bee pollen was collected monthly through 2016 by random sampling. The experimental unit was an apiary in the geographic region of the Colombian high Andean forest (2.800 and 3.200 meters above sea level) in Boyacá. Samples used in this work were classified into two sets:

1) Twelve groups: heterogeneous samples were manually classified to obtain pellets with uniform color and from this classification 12 groups were selected (A-M, letter "I" was omitted to avoid mistakes in the analysis). The homogeneity of these groups was probed by botanical, colorimetric and chemical analysis.
2) Sixty-nine heterogeneous samples. The samples obtained were used first in DIA, and then in carotenoids analysis, to achieve correlation between both analyses.

The procedure is also observed in Figure 1.

2.2 Chemicals and standards

Hexane and acetone were of analytical grade (VWR, Seattle, WA, USA). Methanol and methyl tert-butyl ether were of HPLC grade from Merck (Darmstadt, Germany). α-carotene, β-carotene, β-cryptoxanthin, lutein and zeaxanthin were obtained from Sigma-Aldrich (Steinheim, Germany), whereas phytoene was isolated from appropriate sources in accordance to standard procedures (Stinco et al., 2019). α-tocopherol was purchased from Calbiochem (Merck, Darmstadt, Germany).

2.3 Palynological analysis

For the qualitative analysis, samples were acetolyzed according to Erdtman (1969). Pollen pellets were washed with acetic acid, followed by the mixture for acetolysis and centrifuging. The supernatant was decanted, and the sediment was washed with acetic acid and distilled water, followed by centrifuging. One slide of each sample was prepared by adding glycerine and using glycerin jelly and paraffin for permanent preparations. At least

400 pollen grains were counted (Salazar-González et al., 2018). The identification of the pollen types was based on the reference collection of the Melisopalinology Laboratory of LABUN and on the pollen catalogs of various authors (Colinvaux et al., 1999; Roubik and Moreno, 1991; Velásquez, 1999).

2.4 Carotenoids analysis

2.4.1 Extraction

It was made according to Stinco et al. (2019) with some modifications. 50 mg of an homogeneous bee pollen sample were mixed with the extraction solution (hexane:acetone 1:1 v/v). The mixture was vortexed and centrifuged, and the colored fraction was recovered. The procedure was repeated until there was no more color extraction. The organic fractions were evaporated to dryness at a temperature below 30°C. For the saponification step, the residue was dissolved in 500 µL of dichloromethane and treated with 500 µL of KOH (20% w/v in methanol) for 1 hour in darkness at room temperature. Saponified extracts were washed with NaCl (5% w/v) and water until neutral pH. The extracts were concentrated until dryness at a temperature below 30°C. The residue was re-dissolved in 100 µL of ethyl acetate prior to the Rapid Resolution Liquid Chromatography (RRLC) analysis. The extraction was done by triplicate.

2.4.2 Rapid resolution liquid chromatography (RRLC)

It was made according to Stinco et al. (2019). Bee pollen extracts were analyzed by RRLC in an Agilent 1260 system. A YMC C30 column (150 × 4.6mm, 3µm) and a C30 YMC pre-column (10mm × 4mm, 3 µm) (Dinslaken, Germany) were used as stationary phase. As a mobile phase, methanol (A), methyl-ter-butyl-ether (B) and deionized water (C) were used. The selected wavelengths for quantifying were 285 nm for phytoene and 450 nm for the rest of carotenoids and α-tocpherol using the OpenLab ChemStation software.

2.4.3 Identification and quantitative analysis of carotenoids

Carotenoids identification was made by comparing their chromatographic and UV-Vis spectroscopic characteristics with their corresponding carotenoid standards (Sigma-Aldrich, Germany). The quantitative analysis was done by external calibration building calibration curves with aliquots of each carotenoid standard. Results were reported in µg carotenoid/g bee pollen.

2.5 Digital image analysis

The DigiEye® system was used. This device consists of a closed illumination box, specially designed (by VeriVide Ltd., Leicester, UK) to illuminate the samples consistently with two fluorescent tubes that emulate the standard illumination D65, a 10.2-megapixel digital camera Nikon® D80 with Nikkor® 35mm f/2D. The camera was calibrated by means of a calibration chart included in the equipment (DigiTizer, VeriVide, Leicester, UK). Lamps were switched on at least 10 minutes before being used to stabilize them, according to manufacturer conditions (Rodríguez-Pulido et al., 2017).

Images were analyzed by an algorithm that sequentially opens the image, identifies bee pollen pellets from de background, select bee pollen pixels with its colorimetric coordinates and save all this information with sample label. For obtaining appearance parameters and color information, the software DigiFood® was used (Rodríguez-Pulido et al., 2012). Because the sample background was a plain white surface, the segmentation process started with a classification based on a k-means algorithm applied to the CIELAB coordinates of each pixel. Then, only the pellets having an area in the range the mean plus or minus three times its standard deviation were collected. This way, possible small particles on the surface were discarded. Finally, an erosion was applied using a disk with five pixel of

diameter as kernel. The regions selected by this criterion were used for the remaining processes.

2.6 Statistical analysis

Matlab (The MathWorks Inc., Natik, USA) was used for image segmentation, extraction and tabulation of colorimetric data, algorithm programming for obtaining chemical imaging, and process automatization. Statistica 8.0 (StatSoft Inc., Tulsa, USA) was used for applying multivariate statistic (multiple linear regression) to create mathematical models for prediction.

3. Results and Discussion

Groups A-M were selected to create models that correlate colorimetric characteristics and chemical composition, thus it was necessary that they were homogeneous. This was probed by pollen, color and carotenoid profile analysis, but only the relation between the chemistry of the groups and their optical properties were used to generate the models.

Then, heterogeneous samples (the way that there find in nature) were used to probe the feasibility of the models obtained previously. To accomplish that, the color and chemical composition of these samples were obtained by the aforementioned methods and compared with the results obtained by the models.

Taking this into account, the results were divided into two parts: first, a characterization of the A-M groups in botanic, color and carotenoid composition; and second, the generation of mathematical models for the prediction of carotenoid composition through colorimetric coordinates and for probing their efficiency.

3.1 Sample characterization of groups A-M

Since the objective of this work was to establish the relationships between the appearance and composition of pollen, the first step was a calibration. For this purpose, it was

necessary to have homogeneous samples, so that all the points of the image had the same composition, this was corroborated with the analysis previously mentioned.

When the colorimetric homogeneity of groups (A-M) was probed, palynological and carotenoid determination were made. Figure 2 shows pictures of the pollen pellets used, and different colorimetric and morphological parameters are observed.

3.1.1 Palynological analysis

In order to verify the suitability of this separation, the results of floral species were analyzed palynologically, as shown in Table 1.

In previous studies of bee pollen from the Colombian high Andean forest, different species were found: *Acalypha diversifolia*¸ Brassicaceae (*Brassica* vs. *Raphanus*), *Cecropia peltata*, *Eucalyptus globulus*, *Gaiadendron punctatum*, *Hypochaeris radicata*, *Muehlenbeckia tamnifolia*, *Rubus* sp, *Trifolium pratense*, *T. repens*, *Viburnum* sp. and *Weinmannia* sp. (Chamorro-García et al., 2013; Chamorro et al., 2017). The results obtained for pure color groups are similar, except for *Acalypha diversifolia, Cecropia peltata, Muehlenbeckia tamnifolia, Rubus* sp. and *Weinmannia* sp. These species are concordant with the flora of the region, which is mainly composed of clovers, dandelion, forage turnip and eucalyptus.

These differences occur because the botanical origin is linked to the geographical position, thus in each location there are different floral species that depend on the climatic conditions, soil, solar radiation and nutrients. This happens even in the same country (Domínguez-Valhondo et al., 2011; Raphaella et al., 2017; Soares de Arruda et al., 2013).

The high quantity of floral species presented in bee pollen samples is due to the high floral diversity of the Colombian high Andean forest. However, in all the groups, the main specie

content was superior to 58%. According to Louveaux et al. (1978), a pollen type could be considered as predominant when it has a content superior to 45%. In this case, all the main species in each group are predominant pollen, which probed the homogeneity of the groups A-M.

3.1.2 Optical properties analysis

The morphological and color characteristics of the groups A-M were obtained by DigiEye® and are shown in Table 2. Significant differences are observed in all parameters of the groups A-M. As expected according to Figure 2, for caliber and area the differences between the same groups are equal for both parameters. For colorimetric coordinates, only b* and C^*_{ab} have the same patron.

A and D have the highest values of L*; while G, K and M are the darkest. Chroma (C^*_{ab}) and hue (h_{ab}) are the parameters usually used to identify the color. With increasing h_{ab}, the hue changes from red (0) to yellow (90); however, it is important to analyze the information together. G should be the yellowest group due to its high value of h_{ab}, but Figure 1 shows that it is not the case. This can be explained by the C^*_{ab} value, since its lowest value indicates the most achromatic group.

D, E and F are the groups with the most vivid color. E is for orange hue (less h_{ab} value for the three groups) and the others for yellow hue. H, K and M have hue values lower than E, so, they are the most orange. The low L* values of K and M make those groups the darkest of the orange ones.

Regarding MCDM (Mean Color Differences from the Mean), the group with more heterogeneity is L because of its higher MCDM value compared to the other groups. C, F, H and K present mean values and there are no significant differences between them.

3.1.3 Carotenoid composition

Carotenoid profile of groups A-M are presented in Table 3. α-tocopherol and ten carotenoids were identified in most of the bee pollen samples: phytoene, two lutein isomers, lutein, two anteraxanthin isomers, zeaxanthin, zeinoxanthin, β-cryptoxanthin and β-carotene.

A great variability is observed. These differences are due to the botanical and geographical origin, as well as multiple factors that cause diversity of floral species (Sarungallo et al., 2015; Soares de Arruda et al., 2013). Carotenoids are secondary metabolites produced by plants, thus that diversity causes several differences between the contents of each compound (Machado De-Melo et al., 2016; Saini et al., 2015).

β-carotene (4.7-17.5 ppm), cryptoxanthin (4.3-9.5 ppm), zeaxanthin (0.20-7.9 ppm) and lutein (0.81-5.73 ppm) are the main carotenoids identified in bee pollen (Domínguez-Valhondo et al., 2011; Gardana et al., 2018; Schulte et al., 2009). The other carotenoids in Table 3 are being reported in bee pollen for the first time. It can be observed that Colombian bee pollen has substantially higher quantity of β-cryptoxanthin, zeaxanthin and lutein with respect to North American pollen (Schulte et al., 2009). However, the content of β-carotene was lower than the reported (Schulte et al., 2009). In case of the Colombian bee pollen, solar radiation through the year and the amount of flora existing in the region are two important factors that affect carotenoid composition. Plant exposition to high temperature with high light intensity generates an increase in carotenoids (Sarungallo et al., 2015). For this reason, carotenoid profiles and composition were different among countries.

It can be observed that major carotenoids are xanthophylls: zeinoxanthin, zeaxanthin and lutein isomers. Lutein and zeaxanthin are macular carotenoids, meaning they are important

in ocular health, because they reduce the risk of age related macular degeneration (Kim et al., 2016; Song et al., 2016). β -carotene and β-cryptoxanthin are provitamin A carotenoids, because they have at least one unmodified β-ionone ring in their structure (Saini et al., 2015). The high zeaxanthin content in samples, along with the quantity of lutein isomers and high β-cryptoxanthin found in this study allow us to catalogue Colombian bee pollen as a good source of these compounds, which adds value to this natural product. These results are similar to those of a previous study of Colombian bee pollen, where the authors identified traces of lutein, zeaxanthin, β-carotene and phytoene (Gardana et al., 2018).

3.2 Carotenoid prediction by image analysis parameters

Color of pixels from groups A-M was stored and the concentration of each carotenoid identified was assigned to each pixel. To these groups, Multiple Linear Regression (MLR) was applied to predict carotenoid content from the parameters obtained by DIA. Comparing the values found with RRLC analysis with those predicted, α-tocopherol, both lutein isomers, anteraxanthin isomer 1, zeaxanthin and β-cryptoxanthin have R^2 coefficients greater than 0.75. Models for all compounds were done, but only those with coefficients above that value are presented.

Table 4 presents the variables for prediction. The best results are R^2: 0.89 for lutein isomer 1, R^2: 0.88 for lutein isomer 2 and R^2: 0.87 for anteraxanthin isomer 1, zeaxanthin and zeinoxanthin and $RMSE_{CV}$ were 18.90, 19.76, 2.68, 34.66 and 142.69 µg carotenoid/g bee pollen, respectively.

Evaluating the correlation coefficients obtained in this work, they are similar to those reported by the literature for relationships of color-composition in food products (Pace et al., 2013; Stinco et al., 2013). In a study made in different types of carrots (external and internal parts), the authors found good relations between color parameters obtain by

computer vision with total phenol content and antioxidant activity. Fitted equations were found using multiple linear regressions, and the predicted values obtained are well correlated with the measurements when external and internal parts data are used (Pace et al., 2013).

Regarding to carotenoids, Stinco et al. (2013) made a study in tomato products (fresh and processed) to correlate Digital Image Analysis and lycopene isomers content (LYC). The researchers found significant correlations when total samples (fresh and processed) and only fresh ones were used, and simple regressions between LYC and color parameters were made. However, it is necessary to consider tridimensional character of color, so, all the colorimetric coordinates must be considered together and a multivariate analysis is needed. When L*, a* and b* and all the samples were used, the coefficients increased up to 0.77. They were able to propose equations from colorimetric parameters for a rapid determination of lycopene in fresh fruits.

For the present study, linear equations were obtained for each carotenoid from MLR. Those equations have the coefficients for colorimetric variables and an independent term, that allows obtaining the concentration of each carotenoid when applied to image data. The model obtained was included in the Matlab algorithm, which sequentially opens each bee pollen image, segments it and calculates the compound concentration of each pixel. The algorithm also creates a pseudo-color image with carotenoid prediction and calculates the average concentration of that compound for the whole image (Figure 3). As shown in Figure 3, the algorithm was only applied in bee pollen pellets, which allows corroborating the correct segmentation process. The figure depicts examples of the algorithm applied to different groups images for some compounds.

By applying the algorithm to heterogeneous samples, the suitability of the mathematical models could be corroborated. These samples were used only for this purpose and were not included in the generation of models.

The value measured by RRLC and the one predicted by image analysis are shown in Figure 4 for one of the samples. Each image belongs to a carotenoid. In the left part, the image is showed as the camera acquires it. The right part contains the concentration of an analyte in a color scale. Since the samples are heterogeneous, there is a considerable deviation in the measurements calculated for each pixel. Therefore, the predicted value is obtained from the average of pixels that belong to the sample.

4. Conclusions

A methodology was developed for the estimation of individual carotenoids from digital images in bee pollen samples. For α-tocopherol, both lutein isomers, anteraxanthin isomer 1, zeaxanthin and β-cryptoxanthin, high correlations were achieved between the estimated values and those measured by reference methods. This methodology is a great advance for the rapid identification of carotenoids in bee pollen samples; however, other optical techniques, such as infrared spectra and harvest regions, could be used to improve the correlation in more compounds. Even though it is not a substitute for conventional chemical analysis, this methodology is an alternative for a carotenoid identification in a simple and less expensive way.

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FIGURE CAPTIONS

Figure 1. Experimental design of analyses for groups (A to M) and samples

Figure 2. Pellets from groups A to M.

Figure 3. Groups before and after of applying the algorithm. a) group E, zeaxanthin and b) group L, β-cryptoxanthin. All units are expressed in µg/g.

Figure 4. Heterogeneous sample before and after of applying the algorithm. a) original image. b) α-tocopherol (Reference: 65.99 / Predicted: 63.36). c) β-cryptoxanthin (R: 16.78 / P: 14.90). d) Lutein isomer 1 (R: 127.94 / P: 126.85). e) Lutein isomer 2 (R: 135.83 / P: 132.50). f) Anteraxanthin isomer 1 (R: 16.22 / P: 16.73). g) Zeaxanthin (R: 261.23 / P: 242.88). All units are expressed in µg/g.