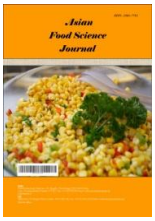

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Non-Invasive Rapid Method to Evaluate Tunisian Virgin Olive Oil Based on color Measurement by Digital Images and Chlorophyll-Carotenoids Content

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Authors' contributions

This work was carried out in collaboration among all authors. Author MF designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors SM and FJ managed the analyses of the study. Author AL supervised the study and contributed to the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Color and pigment analyses are not required by the majority of olive oil marketing standards. However, it is a basic attribute that is highly associated, by most consumers, with the idea of quality. In this study, we aim at providing a fast non-invasive method for routine analysis that can be used on a large scale in the laboratories of olive oil analysis starting from pigment quantification and color range measurements. A selection of 172 virgin olive oil samples obtained in Tunisia between 2018 and 2019 were used for this purpose. Chlorophyll and carotenoid contents were analyzed using a UV spectrophotometer standard method while color range was measured using digital images taken under controlled conditions. All samples showed high significant differences in chlorophyll and carotenoid contents ($p < 0,01$) confirming that the visual selection of the set of samples was satisfactory for this study. Chlorophyll content varied from 3,0 to 28,3 ppm for samples SM137 and SM96 respectively, while carotenoids oscillated between 0,7 and 6,2 ppm for SM138 and SM100 respectively. Principal component analysis using chlorophyll and carotenoids

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contents along with RGB-CYMY color measurements showed a higher significant correlation P<0,05 between pigment contents and Red, Green, Blue and Yellow colors. Bivariate tests suggest that although color and pigments are correlated, color range assessment using digital imaging may represent a more sensitive method to discriminate olive oil according to cultivar, geographical origin, maturation index and year of harvest.

Keywords: Color; olive oil; digital imaging; chlorophyll; carotenoid.

1. INTRODUCTION

Virgin olive oil, one of the main components of the Mediterranean diet, is highly valued around the world for its taste and beneficial nutritional properties. The olive oil nutritional benefits are mainly related to the fatty acid composition due to the high content of oleic acid and also balanced ratio between saturated and polyunsaturated fatty acids [1]. Moreover, olive oil contains considerable concentrations of natural antioxidants and other minor healthy compounds. The color of the olive oil is a parameter that depends on its pigment composition [2], which is responsible for green-to-yellow balance of the olive oil [3]. Pigment analysis is not required by the majority of olive oil marketing standards. However, it is a basic attribute that is highly associated, by most consumers, with the idea of quality [4]. Actually, color is the initial characteristic perceived by consumers since their first contact with the product, and this has a direct influence on their choice, although it does not necessarily influence other organoleptic properties [5]. Thus, olive oil color has been the subject of several studies. For example, in a study starting from two types of olive oil, one of a dark green and the other of a dark yellow, Dekhili et al. [6] demonstrated that Tunisian consumers have appreciated the green color more than French consumers. Another study conducted in Spain showed that the dark yellow color of the Arequipa's olive oil was more appreciated by consumers in the Terragone region of Catalonia, whereas the dark green color was appreciated in Andalusia [7]. In Uruguay, the green and dark green colors are associated with more expensive, richly flavored and tasty oils [8], while the pale yellow color was considered as a sign of poor quality and associated with cheap products having a slighter taste, and are therefore the least acceptable.

The color, ranging from yellowish-green to transparent yellow, is mainly due to the chlorophylls and carotenoids naturally existent in the fruit [9]. However, it varies according to the cultivar, growing techniques and processing methods as well as storage conditions. Unlike all other vegetable oils, olive oil contains a large amount of chlorophyll pigments [9] which are responsible, along with pheophytins, for the typical color of the olive oil [10]. The composition and total pigment content are important in determining the quality, being closely related to the color. The yellow-greenish coloration of virgin olive oil is mainly due to the existence of coloring pigments belonging to the carotenoid and chlorophyll family [11]. According to Giuliani et al [12], chlorophylls *a* and *b* are the pigments responsible for the characteristic green color of olive fruits. It has been reported that their content in natural olive oil varies between 1 and 20 ppm. Chlorophyll is localized mostly in the exocarp, the mesocarp contains significant amounts of phosphoenolpyruvate carboxylase [13]. The difference between chlorophyll *a* and *b* lies in the substituent of the C-7; chlorophyll *a* has a methyl group, while chlorophyll b has a formyl group [14]. Thanks to the numerous conjugated double bonds in their structure, chlorophylls absorb light radiation. It has also been reported that during storage in dark places, some chlorophyll derivatives act as antioxidants. These photosensitizers could show slight antioxidant effects on the oils in the dark, probably by donating hydrogen to break the free radical chain reactions [15]. Chlorophylls are photosensitive compounds able to transmit the energy of light to free oxygen radicals which then react with the unsaturated fatty acids [9]. When oil is extracted from black olives, pheophytin is almost the only pigment that exists in this class [16]. In addition, several studies have shown that the chlorophyll content decreases during the ripening process [17]. Indeed, the chlorophylls degrade and other substances form, such as the anthocyanins which is responsible for the violet or purple color of the fruit [18]. Also, the concentration of carotenoids decreases during maturation progress. Carotenoids are a kind of pigments widely distributed in nature. Thanks to their strong absorption in the visible range (between 320 and 550 nm), they contribute to reduce the photooxidation process and, thus, preserve the

olive oil quality during storage [19]. In general, carotenoids are the pigments responsible for the yellowish and orange color of fruits and vegetables as a result of the presence of a chromophore in its molecule, which contains conjugated double bonds [20]. There are three main carotenoids whose consumption is very important for the human body: β-carotene, lutein and lycopene, which constitute 80% of the pigment intake [21]. Numerous epidemiological studies have shown that carotenoids capture free radicals and oxygen. High consumption of certain carotenoids decreases the risk of cancer disease [23]. According to Cichelli et al. [3], olive oil contains carotenoid levels ranging from 1 to 100 ppm. Inarejos-García et al. [23], reported that their concentration is rather related to the olive variety, degree of fruit ripeness and oil extraction process. Irrigation has also a strong influence on the increase (or retention) of chlorophyll [24]. It has been noted that oils obtained from olive trees grown in dry conditions show an intense green color, while those obtained from irrigated trees have a yellow color whose intensity tends to decline with the increase of water supplies. Beltrán [25] found that a lack of water in the plant leads to an increase in the concentration of carotenoids in the oils. Numerous studies have shown that there is a slight relationship between the color of the epidermis and the qualitative and quantitative composition of chlorophyll in different varieties of olives during the same period of ripening [20]. The rate of chlorophyll degradation is specific to each variety. Storage conditions (time, temperature, packaging etc.) have also an effect on color beside fatty acid composition and tocopherols [26]. Also, the extraction method plays an important role in reducing the pigment quality [27]. Actually, light accelerates oils photoxidation by reducing the chlorophyll contents during the first three months of storage, which mainly depends on the initial level of these pigments in the oil [28]. It has been found that about 80% of chlorophylls and 40% of carotenoids are lost during the extraction of olive oil [29]. The intervention of new technological procedures to reduce the intensity of virgin olive oil bitterness by thermally treating the olives before the extraction also affects the oil´s color by increasing the content of chlorophylls and carotenoids [30]. Although, at the moment, the profile of chlorophyllic and carotenoid pigments present in an olive oil is not included in the regulated quality standards [31], some regulations have included the color of oils as one of the obligatory quality parameters [32].

The methods used to measure the color of the oil can be broadly classified into two categories; instrumental methods and visual methods. In 1929, Hardy developed a spectrophotometer called "Color Analyzer" to increase measurement accuracy over simple colorimeters which depend on the observer and the illumination of the sample. Six years later, in 1935, Hardy's apparatus evolved; it then allowed drawing a reflectance or transmittance spectrum. Since then, other devices have been developed, which are spectrophotometers (D54A and D54P that emerged in1978-1979, Applied Colors System's Spectro-Sensor (1979) and IBM's 7409 device. The development of software and user interfaces has made color measurements easier. However, since the 1980s, many spectrophotometers have been developed, and the limits of detection and ease of use have changed significantly.

For decades, pigment quantification has been typically performed by single-column (one-dimensional) chromatography. However, the complexity of some matrices required the use of better detection performance allowing the emergence of multidimensional chromatography [33]. Comprehensive two-dimensional liquid chromatography (LC×LC) was therefore presented as a valuable method based on a simultaneous double detection principle. This emerging method has been efficient for the quantification of esterified carotenoids [34]. Alternatively, standard C30 LC columns together with a complex matrix series were efficiently employed allowing to reach a better resolution in peak threshold detection when the samples are more complex (25 %) [35]. Currently, the most suitable method for carotenoid analysis is the HPLC with diode array detector or mass spectrometry. The last one can provide a more accurate identification capability based on the combination of molecular weight, fragment pattern, retention time and spectral individual identity. Actually, the slight dissimilarity that may exists between many carotenoid’s UV-vis spectra can be resolved by mass spectrometry thanks to the identification of the compound’s molecular weight, excepting for some carotenoids which have the same features for both. Many researches have been published on the combination of these two detection approaches [36]. Recently, carotenoid identification was performed by other methods based on photoacoustic and photothermal principles starting from a quantity of energy that is absorbed and then converted to heat. This is considered as an important advance because it

makes the sample preparation easier and offers a fast non-destructive method with more practical possibilities compared to conventional approaches [37].

The visual method is the most direct way to assess the color of an olive oil comparing it with color scales. These scales consist of a series of colored solutions, which are quite stable and can be made from readily available dyes. However, it is very important to place the samples to be evaluated in appropriate cuvettes and reference solutions. The samples and references are then compared using a standardized blank solution in diffused light [38,39]. Colored glasses can also be used as reference standards instead of colored solutions. These types of glasses are used in by Lovibond method [40]. In the case of olive oil, the bromothymol blue (BTB) method has been widely used. This method provides a subjective visual comparison between the color of a sample and a two-dimensional scale with 60 fixed solutions and called BTB standards, looking for the solution whose color is closest to the sample. Appropriate transformation equations from the BTB to CIELAB color space [41] have been proposed, taking into consideration that the BTB is an oil-specific color scale, though the current international specifications of the International Commission on Illumination recommend the use of CIELAB (or CIELUV). However, further research has revealed that the BTB method is not highly precise and reliable [42]. A certain lack of temporal stability of the colors has been reported in the 60 BTB standards 2 to 3 months after preparation [43]. A new color scale for virgin olive oils called Uniform Oil Color Scales (UOCS) was proposed in 2004 by Melgosa et al. [44]. The UOCS has the same number of standards as the BTB (i.e. 60) so that the two scales can be meaningfully compared. Nevertheless, UOCS standards are not real solutions, rather theoretical points in a three-dimensional color space. So the UOCS method is not based on subjective visual comparison, as the BTB method. The UOCS method is objective, as it uses a theoretical calculation of the smallest difference between the color of a virgin olive oil sample measured by instrument along with the set of colors specified in the 60 standards of the 'UOCS. It was found that for a large set of 1700 samples of virgin olive oil produced in Spain during four different harvests, the UOCS improved the BTB scale by about two factors [44]. Indeed, several portable color measuring instruments have been developed by some of the authors for different applications related to color assessment [45-47].

Image color analysis method based on the intensity of the three primary colors red, green and blue (RGB) in the color space has been used in several plants species. Grunenfelder et al. [48] used color indices to assess chlorophyll development and greening of fresh potatoes. Some studies have proven that the content of vegetable chlorophyll can be assessed by RGB image analysis. Ku et al. [49] found that R / (R-B) ratio was the best parameter for estimating the chlorophyll content of rye leaves using a digital camera. Suzuki et al. [50] used G / (R + G + B) ratio to estimate the chlorophyll content of broccoli. Also, a study estimating the chlorophyll content of rice by imaging using RGB analysis has been reported [51], it estimated chlorophyll *a* (Chl a), chlorophyll *b* (Chl b) and carotenoids (CAR) from digital imag.es. They have, thus, developed a rapid and non-invasive method for estimating the chlorophyll content of rice using color analysis from images captured from the leaves. The average R, G and B values of the total leaf area were obtained using Adobe Photoshop software.

In the current study, 172 samples of olive oil from different cultivars and various geographical areas of Tunisia were analyzed to adopt an alternative approach of color measurement, based on digital imaging capture under controlled conditions and further treatment of image and color spectrum analysis. The results were statistically compared with a standard analysis of chlorophyll and carotenoid content measured by spectrophotometric method. The aim of this study is to provide a fast non-invasive method for routine analysis that can be used on a large scale in the laboratories of olive oil analysis starting from pigment concentration assessment and color range measurements.

2. MATERIALS AND METHODS

2.1 Vegetal Material

A total of 172 olive oil samples from 60 different accessions collected during the 2018-2019 and 2019-2020 harvests from 8 sites, mainly in the north of Tunisia were analyzed. The oils were obtained in the specialized unit in the Olive Institute in Tunis. The olives were cold extracted by an ABENCOR system starting with 700 g of fruits. The oils were then decanted, filtered and conserved for further analysis.

2.2 Pigment Quantification

Chlorophyll and carotenoid concentrations were determined according to the method described by Minguez- Mosquera et al. [29] and Bajoub et al. [52] with a few modifications starting with 0,25 g of homogenized oil dissolved in 25 ml of cyclohexane and subsequently mixed. The optical density was then recorded for carotenoid and chlorophyll measurements at a wavelength of 470 nm and 670 nm respectively, using a PG INSTRUMENTS T60 UV-Spectrophotometer and Quartz cuvettes. Pigment concentrations were calculated as following:

Carotenoid content (ppm) = (DO470 × 〖10〗 ^ 6)/(E × 100)

Chlorophyll content (ppm) = (DO670 × 〖10〗 ^ 6)/(E × 100)

With:
DO: absorbance at wavelengths 470 and 670.
E: Extinction coefficient (600 for Chlorophyll and 2000 for Carotenoid).

2.3 Image Capturing

A standardized sample of 10 ml was taken from each olive oil sample and individually dispensed onto an anti-drip micro-film placed under a neutral background. Stable illumination conditions were ensured by using a totally enclosed box equipped with three stabilized 24 W halogen lamps distributed throughout the box in order to avoid light interference. The images were then taken at a fixed distance of 20 cm using a high-resolution NIKON D3200 digital camera coupled with a Sigma 105mm F2.8 DG OS HSM lens. The shooting mode has been kept in manual setting.

2.4 Image Processing

Images were processed by Adobe Photoshop CS6 software, Version 13.0 by automatically removing background color. Two color models were used for assessing the RGB model (red, green and blue) and the CMYK model (cyan, magenta, yellow and black). An average color composition measurement was then directly collected from the software (Fig. 1).

2.5 Statistical Analysis

Statistical analysis was performed using SPSS software (IBM version 20). Correlation coefficients and bivariate tests as well as Principal Component Analysis and correspondent Graphs were performed using this software.

3. RESULTS AND DISCUSSION

3.1 Pigment and Color Analyses

All samples showed high significant differences in chlorophyll and carotenoid contents ($p < 0,01$), confirming that the visual selection of the set of samples was satisfactory for this study. In addition, it was found that chlorophyll and carotenoid contents are positively correlated with each other. Actually, it can be observed from Fig. 2 that the chlorophyll and carotenoid are proportional to the majority of the samples. Indeed, it was widely described by the literature that the two pigment systems are morphologically associated to the cell where they are attached to identical or very similar proteins in the chloroplast lamellae, which explains their binding. In this study, chlorophyll content varied from 3,0 to 28,3 ppm for samples SM137 and SM96 respectively, while carotenoids oscillated between 0,7 and 6,2 ppm for SM138 and SM100 respectively (Fig. 2). This level of variation was also observed by Beatriz Gandul-Rojas et al. [14] in a similar study carried out on olive oil, demonstrating that 80% of the analyzed oils showed a significant correlation ($R^2 = 0,993$) between chlorophyll and carotenoid pigment fractions. In this study all the samples showed a significant correlation of $R^2=0,627$.

Other researchers demonstrated that Spanish olive oils contain from 0,4 to 45 ppm of total chlorophylls depending on the fruit cultivar, fruit maturity, processing system, and storage conditions, but the majority of these compounds were present in the form of degradation products such as pheophytins ([10]; [18]). Fig. 2 showed that the majority of the intersections between chlorophyll content and the color patterns seem to be correlated. Chlorophylls are the main photosynthetic pigments. They are therefore present in almost all photosynthetic organisms and are the source of their green color, since they strongly absorb visible light in the wavelengths corresponding to blue and red and allow a large part of the green light to filter out.

3.2 Principal Component Analysis

Principal component analysis using chlorophyll and carotenoids contents along with RGB-CYMY

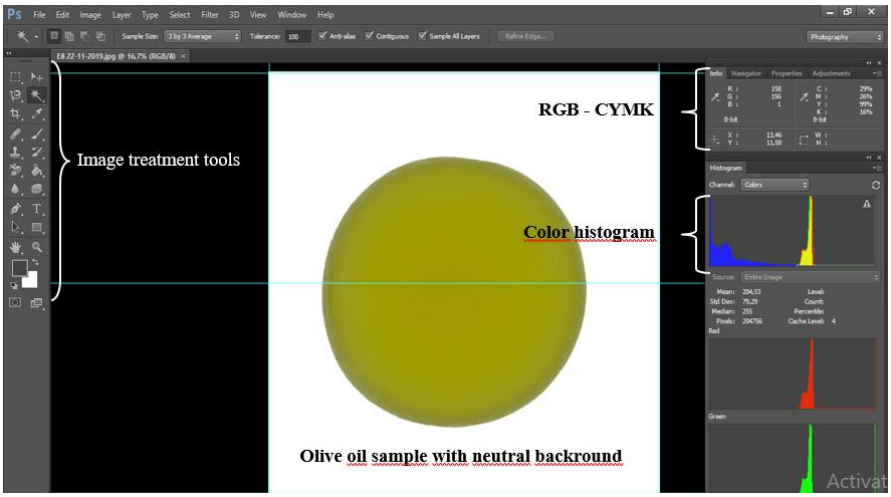

Fig. 1. Image traitment and assesment using Photoshop CS6 software, Version 13.0

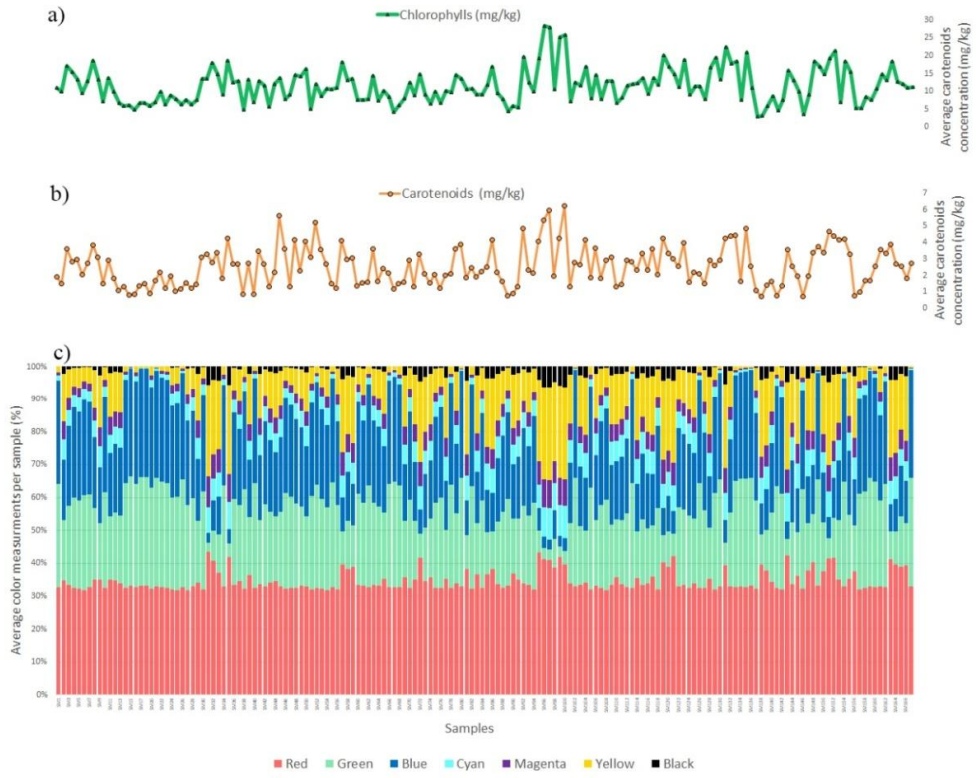

Fig 2. Pigment concentrations compared to color range percentage obtained by digital imaging under controlled conditions. a) Chlorophyll concentrations (mg/kg) b) Carotenoid concentrations (mg/kg) c) RGB CMYB color range percentage

colors measurements showed a higher significant correlation P<0,05 between chlorophyll and carotenoid content from one side, and four color measurement from the other side. These colors are Red, Green, Blue and Yellow. The correlation was negative with Red, Green and Blue while it was positive with Yellow. Kaiser-Meyer-Olkin and Barlett's Test showed that performing a principal component analysis is substantial for the set of samples and showed a

measure of sampling adequacy ranging 0,59. The largest amounts of variance that have been explained by principal component analysis correspond to Yellow, Green and Red colors respectively. The two component solutions explained 86,5% of variance. The component matrix showed that Yellow, Red and Green colors are the primary variables that explain the first component (because they are related to each other), while chlorophylls and carotenoids mainly explain the second component.

Principal component analysis performed on the six color's measurements has generated two factors that explain 98,5% of variance. The first factor, which is mainly composed by Green, Red, Blue and Yellow colors explains 82,1% of variance. This factor was further considered as an attitudinal factor. A second attitudinal factor was obtained using chlorophyll and carotenoid concentrations. Statistical analysis showed that the correlation is significantly high between these two resulting factors.

Bivariate test performed on the attitudinal factor resulting from pigment measurements and all registered variables (geographical origin of the samples, cultivar's name, year of harvest, maturation index and growing conditions) didn't showed a significant correlation, except for the year of harvest variable. The same result was observed when chlorophyll and carotenoid were considered independently. However, this correlation is highly significant when we use the attitudinal factor resulting from color range measurements for all the variables except one of them; the growing conditions variable. This suggest that although color and pigments are correlated, color range assessment using digital imaging may represent a more sensitive method to discriminate olive oil according to cultivar, geographical origin, maturation index and year of harvest.

Fig. 3 summarizes the observation of the resulting dispersion of the sample according to its geographical origin using the two resulting attitudinal factors from pigment and color measurements combined with maturity index of the fruits.

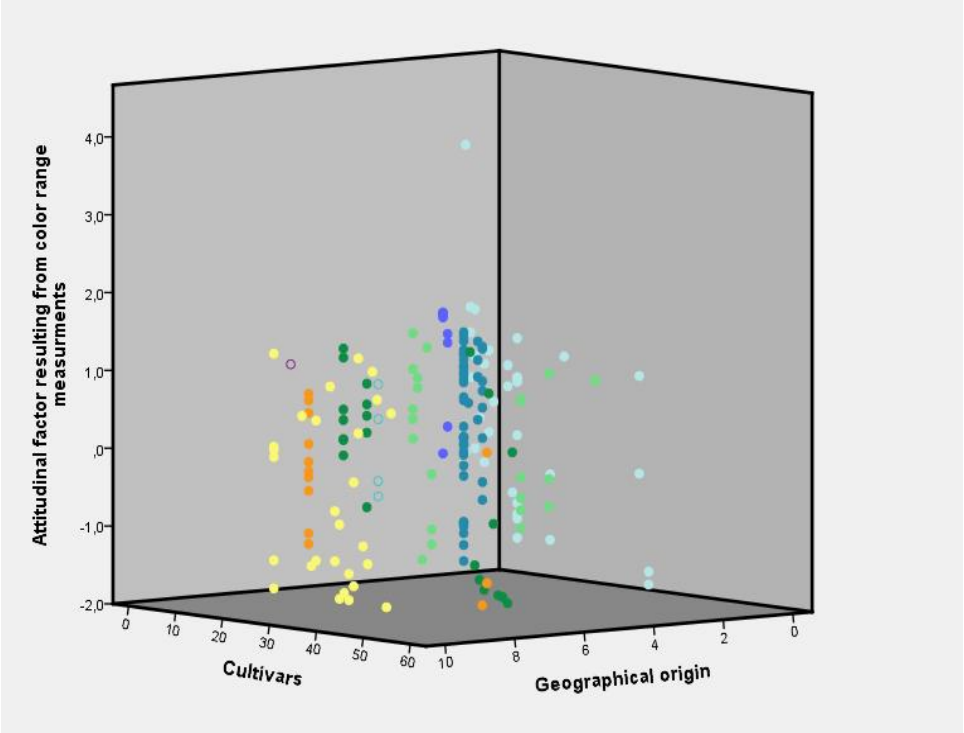

Fig. 3. Samples distribution by cultivar and geographical origin using the attitudinal factor obtained through color range measurements. Each color of the points represents a single geographical origin

4. CONCLUSION

All samples showed high significant differences in chlorophyll and carotenoid contents ($p < 0,01$) confirming that the visual selection of the set of samples was satisfactory for this study. Chlorophyll content varied from 3,0 to 28,3 ppm for samples SM137 and SM96 respectively, while carotenoids oscillated between 0,7 and 6,2 ppm for SM138 and SM100 respectively. Principal component analysis using chlorophyll and carotenoids contents along with RGB-CYMY color measurements showed a higher significant correlation $P<0,05$ between pigment concentrations and Red, Green, Blue and Yellow. Bivariate tests suggest that although color and pigments are correlated, color range assessment using digital imaging may represent a more sensitive method to discriminate olive oil according to cultivar, geographical origin, maturation index and year of harvest.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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