Application note

Analysis of chlorophyll content in food products through UV-Visible absorption measurements

Introduction

Chlorophyll is a naturally occurring green pigment found in plant materials. The highly conjugated porphyrin ring (Figure 1) included in the overall structure allows for absorption in the red region of the UV-Visible spectrum.[1] As the photons which are able to reach the Earth's surface span the visible to near-infrared spectral range, with a maximum photon flux at ~500 nm,[2] plants containing molecules like chlorophyll are able to absorb a greater percent of incident photons. Through this light-harvesting mechanism, photosynthetic pathways are possible by which energy is provided for the plant.[1,3]

As many commercially available food products contain plant material or are derived from plant-based sources, varying concentrations of chlorophyll can be observed. Leafy greens, such as a spinach and lettuce, will naturally contain some concentration of chlorophyll as evidenced by their green color, however these compounds can also be found in the seeds and extracted oils as well.[4] In some food industries, it can be important to determine the chlorophyll concentrations present in food products. For example, in canola oil, chlorophyll is an undesired contaminant as not only does it alter the color of the oil, but it can participate in unwanted reactions, lowering the overall quality of the oil.[5–7] Under these circumstances the determination of chlorophyll content can be vital.

There are multiple different chlorophyll compounds found in nature, and food products by extension, with varied chemical structures. For example, chlorophyll a and chlorophyll b differ only in a methyl and aldehyde group on one of the pyrrole rings making up the overall porphyrin structure (Figure 1).[1, 3] Additionally, pheophytin a is similar to chlorophyll a, however it does not contain the magnesium counter ion within the porphyrin ring.[8] Chlorophyll a is more ubiquitous than the other forms of chlorophyll, though varying ratios of chlorophyll pigments can be found in many different sources.[1, 3] The small changes in the structure affect the overall conjugation of the molecule, leading to differences in the electronic structure, and thereby the absorption properties of the material. As such, these compounds have different UV-Visible absorption spectra from one another. Consequently, UV-Visible absorption techniques can be a useful non-destructive method for detecting and quantifying different chlorophyll derivatives in a given food product.

Herein, the Thermo Scientific™ Evolution™ Pro UV-Visible Spectrophotometer was used to analyze extracted chlorophyll from commercially available spinach samples, as well as analyze the chlorophyll content present in olive and canola oil samples. The resulting UV-Visible spectra were compared to chlorophyll standards measured on the instrument and further mathematical analyses were performed to determine the concentrations of chlorophyll a and b. For canola oil, the chlorophyll concentration was quantified following procedures outlined by the American Oil Chemists Society (AOCS).[9]

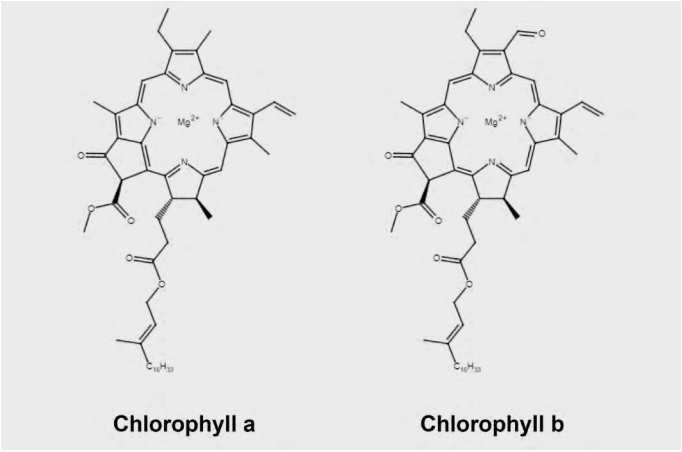

Figure 1: Chemical Structures for Chlorophyll a and Chlorophyll b.

Experimental

Chlorophyll standard preparation

Chlorophyll a and b (Chl a and Chl b, respectively) standards were made separately by dissolving 1.0 mg each in 0.5 mL of 95% ethanol. Each standard was then diluted in both dimethyl sulfoxide (DMSO) and 80% aqueous acetone to result in two solutions with absorption maxima close to 1.0. The final concentration of the Chl a and Chl b standards in both solvents were 13.3 µM (12.0 mg/L) and 5.0 µM (4.6 mg/L), respectively, according to UV-Visible measurements. These concentrations were used to ensure the sample absorbance was below 1.0 at the longest wavelength peak maximum. The standards were made and analyzed with no room lights to prevent photodegradation of the material.

Preparation of chlorophyll extracts from spinach

Samples of crude chlorophyll extracted from spinach were prepared based on procedures described in Porra et. al.[10] A section of a spinach leaf was crushed using a mortar and pestle in the presence of 2.0 mL of 80% acetone. The slurry was then transferred to a sample vial and the mortar and pestle were rinsed with 2.0 mL of 80% acetone three times. The rinses were collected as well in the same sample vial, with a total recovery of 6.0 mL of spinach solution. Following collection, the sample was then centrifuged for 10 min at 2500 RPM using a Thermo Scientific Sorvall™ ST 16 Centrifuge. The solution was then decanted to remove residual white/pale green solid. Two additional extracts were collected following the same procedure. The weight of the spinach leaf section used are described in Table 1.

Spinach extract sample	Weight of spinach (mg)	Concentration (g/L)
1	38.4	6.4
2	46.9	7.8
3	42.3	7.1

Table 1: Sample weight and concentration of each spinach extract sample.

Preparation of oils for chlorophyll analysis

An olive oil and a canola oil sample were chosen to be analyzed for chlorophyll content. Both samples were acquired from a local grocery store and used as received. For experiments in which the extinction coefficient of Chl a was to be determined in canola oil samples, solutions spiked with the Chl a standard (13.3 µM) were prepared. The final concentrations of Chl a in each canola oil sample measured are described in Table 2. The absorbance collected for each canola oil sample spiked with Chl a was corrected by subtracting the absorption spectrum of canola oil without Chl a from the measured spectrum of each canola oil sample spiked with Chl a.

Canola oil sample	Chl a concentration (ppm)
1	11.9
2	4.8
3	2.4
4	1.2
5	0.0

Table 2: Concentration of standard chlorophyll a in canola oil samples.

Instrumentation

The UV-Visible spectra for each sample solution were acquired using the Evolution Pro spectrophotometer. Absorption spectra were collected between 350 nm and 800 nm using a 1.0 nm spectral bandwidth and 2 nm data interval. All samples which were measured in a 1.0 cm pathlength were held in a plastic cuvette. Samples measured using a 5.0 cm pathlength were held in a quartz cuvette.

Results and discussion

Chlorophyll standards

UV-Visible absorption spectra of Chl a and Chl b standard solutions in DMSO and 80% acetone were acquired using the Evolution Pro as described previously. By eye, both solutions appeared green in color. As shown in Figure 2, the spectra for both chlorophyll standards are distinct and include multiple absorption maxima. The prominent absorption bands for Chl a are found at 432 nm and 664 nm in 80% acetone. Chl b has strong absorption peaks at 460 nm and 647 nm in the same solvent (Table 3), in agreement with literature.[10] In porphyrins, these bands are referred to as the B and Q bands, respectively.[11] Chl b includes an aldehyde moiety, replacing one of the methyl groups on a pyrrole ring within the porphyrin structure. This change in chemical structure influences the overall electronic structure of the compound, leading to the difference in observed spectra between Chl a and Chl b.

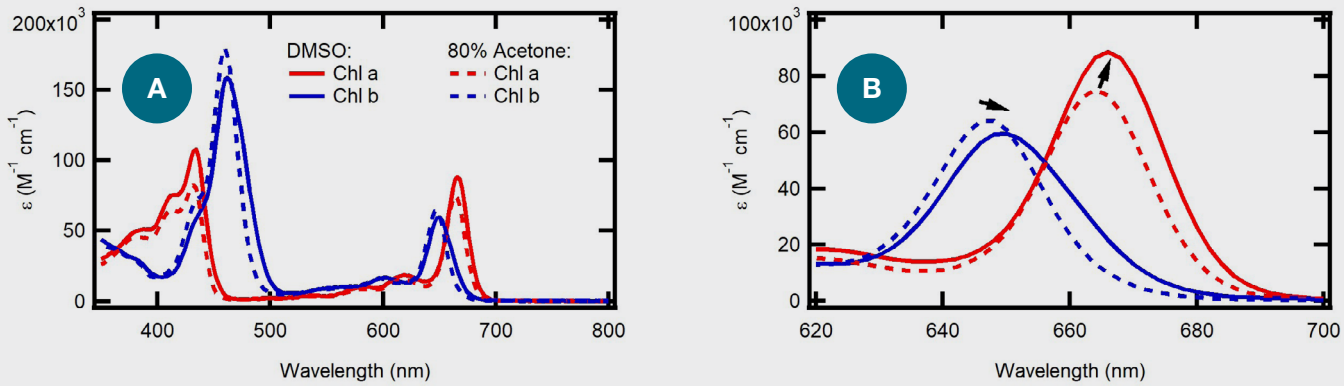

Figure 2: Molar absorptivity of Chl a (red) and Chl b (blue) in DMSO (solid line) and 80% acetone (dashed line) (a) across the full spectrum and (b) between 620 nm and 700 nm. Arrows indicate the spectral shift observed as solvent polarity increases.

When Chl a and Chl b are analyzed in DMSO, a solvent with a higher polarity, small spectral changes can be observed, consistent with literature.[12] Firstly, for both Chl a and Chl b the peak maxima are red-shifted (bathochromic) by ~ 2.0 nm when in a solvent of a higher polarity (Figure 2b and Table 3). This solvent-dependent shift is referred to as solvatochromism and arises as a result of differences in the solvation of the ground state or lowest-lying excited state of a molecule, thereby changing the electronic structure. A red-shifted absorption spectrum with increasing solvent polarity is referred to as positive solvatochromism, implying the molecule in its excited state is more polar than when in the ground state and therefore better stabilized.[13] The stabilization of the excited state inherently lowers the excited state energy to a greater degree than the ground state, resulting in a smaller energy difference between ground and excited states, corresponding to an absorption peak at longer wavelengths.

Chlorophyl standard	Wavelength maxima (nm)		Spectral shift
	80% acetone	DMSO	
Chl a	432	434	2.0
	664	665	1.0
Chl b	460	462	2.0
	647	649	2.0

Table 3: Wavelength maxima observed for Chl a and Chl b in 80% acetone and DMSO and spectral shift associated with changes in solvent environment.

Secondly, the molar absorptivity/extinction coefficient (ε) of Chl a and Chl b are also influenced by the solvent used. With increasing solvent polarity, ε increases for Chl a, and decreases for Chl b. ε reflects the transition probability between the ground and excited states. A higher ε correlates to a higher probability that electrons will be promoted to the excited state. This implies the transition probability is higher for Chl a when in a more polar solvent, like DMSO. For Chl b, the transition probability is lower when the solvent polarity increases. According to Beer's law, concentration (c) is proportional to ε in a given pathlength (l) as shown in equation 1.

$$A = cel \quad (1)$$

As both the location of peak maxima and ε are affected by the solvent environment, it is important to be careful to ensure the correct ε is used when quantifying chlorophyll samples through Beer's law.

Chlorophyll analysis—spinach samples

As described previously, chlorophyll can be found in a majority of food samples, including spinach leaves. Chlorophyll detection and quantification from spinach leaves has been performed previously and has shown to yield fairly high concentrations of chlorophyll within spinach.[14] Consequently, spinach was chosen as a model system to demonstrate the ability to analyze chlorophyll content in food samples through UV-Visible spectroscopy. Extracts from spinach leaves were collected through the procedure outlined previously and analyzed using the Evolution Pro spectrophotometer.

Figure 3a includes the UV-Visible spectrum of the extracted chlorophyll sample from spinach. Strong absorption maxima were found at 665 nm and 432 nm, similar to the absorption spectrum of Chl a. However, the extracted chlorophyll spectrum does not exactly match the spectrum of Chl a, implying there are additional absorbers present. According to literature, extracted spinach samples also contain some amount of chlorophyll b, among other chlorophyll like pigments (ex: carotenoids).[14]

The absorption spectrum of a solution containing multiple components can be represented as a linear combination of the absorption of each component present (eqn. 2).

$$A_T = A_1 + A_2 + \cdots + A_n \quad (2)$$

As a result, a linear combination of the Chl a and Chl b spectra can be used to fit the extracted chlorophyll spectrum, and consequently determine the concentrations of each component. For the spinach extract samples, the collected UV-Visible spectra were fit to equation 3,

$$A_{spinach}(\lambda) = \left(b * A_{Chl\,a}(\lambda)\right) + \left(c * A_{Chl\,b}(\lambda)\right) \quad (3)$$

where $A_{spinach}(\lambda)$ is the absorption collected for the spinach extracts as a function of wavelength, $A_{chl\,a}(\lambda)$ is the absorption measured for the 12.0 mg/L Chl a standard as a function of wavelength, $A_{chl\,b}(\lambda)$ is the absorption measured for the 4.6 mg/L Chl b standard as a function of wavelength, and b and c were fitting parameters. Parameters b and c were allowed to vary until the fit spectrum was optimized between 600 and 700 nm. Figure 3a includes the resulting fit function for spinach extract solution 1 (Table 1).

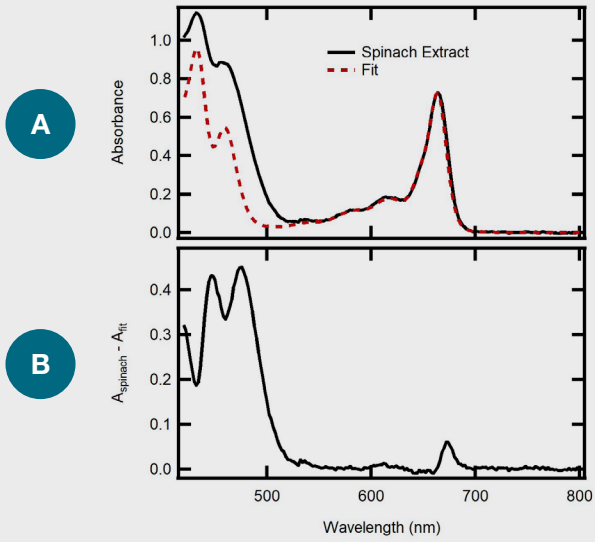

Figure 3: (a) UV-Visible spectrum of the spinach extract solution 1 (black) and the fit comprised of a linear combination of the chlorophyll a and b spectra (red). (b) Difference spectrum calculated by subtracting the fit from the measured UV-Visible spectrum. The solvent for the spinach extract, as well as the chlorophyll a and b samples used to construct the fit, was 80% acetone.

From the fits, the average concentration of Chl a and Chl b was determined and is reported in Table 4. As each sample measured had a varying amount of spinach used in the extraction procedure, the results were normalized to reflect the mass of chlorophyll a or b present per mass of spinach (Table 4). According to literature, the concentration of the Chl a and Chl b components can also be estimated through equations 4 and 5, respectively,

$$[Chl\ a] = (12.25 * A_{664}) - (2.55 * A_{647}) \quad (4)$$

$$[Chl\ b] = (20.31 * A_{647}) - (4.91 * A_{664}) \quad (5)$$

where *[Chl a]* and *[Chl b]* are the concentrations of Chl a and Chl b, respectively, in µg/L, A_{664} is the absorbance at 664 nm, and A_{647} is the absorbance at 647 nm.[10] These equations take into account contributions from both Chl a and Chl b at the analysis wavelengths to avoid overestimation of the true concentration of each respective chlorophyll pigment present. As described previously, *ε* for chlorophyll is solvent dependent. Consequently, the values used in equations 4 and 5 are only applicable for samples dispersed in 80% acetone. The equations above were used to determine the concentrations of Chl a and Chl b in the spinach extract samples and returned similar values as were determined through fitting (Table 4). These equations can be automated using the Insight Pro Software (Figure 4) to limit the amount of post-measurement analysis required.

While the absorption in the 600 nm – 700 nm spectral region of the spinach extract closely matches the fit function, the collected absorption at shorter wavelengths do not match well. This result implies, as expected, the presence of additional components in the extracted samples. To better ascertain the identity of these additional absorbers, a difference spectrum was collected by subtracting the fit function from the measured UV-Vis spectrum. As absorption is a linear combination of all absorbers present (eqn. 2), the removal of the absorption due to Chl a and Chl b from the measured spectrum can be used to estimate the absorption spectrum of the remaining absorptive compounds.

Figure 3b includes the difference spectrum obtained and notably has narrow bands centered at 448 nm, and 474 nm. Carotenoids, such as β-carotene and lutein, are known to absorb between 400 nm and 500 nm,[14] with maxima close to 450 nm and 470 nm.[15, 16] The exact identification of the carotenoids included is out of the scope this study, however these results indicate that other carotenoids were extracted from the spinach leaves, as can be expected.

Chlorophyll analysis—oil samples

Chlorophyll can also be found in various vegetable oils, including olive and canola oil. For olive oil, chlorophyll and related derivatives gives rise to the green color of the oil.[17–19] As shown in Figure 5, an extra virgin olive oil sample has an absorption spectrum with absorption maxima at 420 nm, 456 nm, 484 nm, and 670 nm and is in agreement with literature.[17,19] For olive oil, the absorption is not mostly from Chl a or b, but is instead primarily from pheophytin derivatives, chlorophyll molecules without Mg^{+}.[19, 20] Canola oil however, does not have a similar absorption spectrum when measured in the same pathlength (Figure 5), implying the oil analyzed in this experiment was processed to ensure the removal of chlorophyll.

Unlike with olive oil, the presence of chlorophyll in canola oil can indicate a lower quality product as it can prevent hydrogenation and promote oxidation when exposed to room or sunlight.[5–7] Additionally, chlorophyll imparts a green color to the product, which is not desired. Processes exist by which chlorophyll can be removed in products like canola oil,[6] however the amount of chlorophyll present post refinement still must be determined in these products for quality purposes. AOCS has developed a spectrophotometric method for determining chlorophyll content in refined oils (Method Cc 13d – 55) by measuring the absorption spectrum of refined oils in a 5 cm pathlength cuvette.[9]

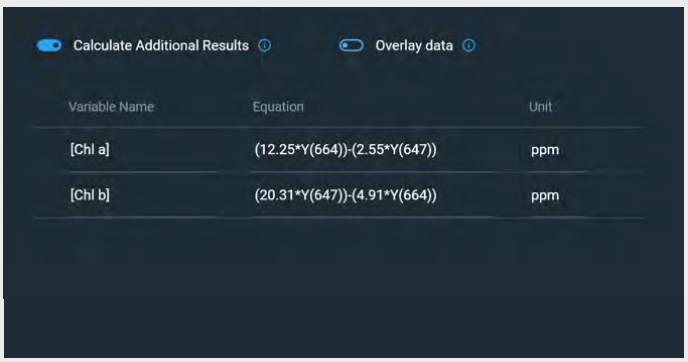

Figure 4: Insight Pro custom calculations

		Average extracted chlorophyll concentration	
	Units	*[Chl a]*	*[Chl b]*
From fit	mg/L	8.1 ± 0.7	2.7 ± 0.3
	g chlorophyll / kg spinach	1.1 ± 0.1	0.36 ± 0.03
From equations*	mg/L	7.9 ± 0.7	3.3 ± 0.4
	g chlorophyll / kg spinach	1.1 ± 0.1	0.46 ± 0.05

* Equations found in Porra et. al[10]

Table 4: Average Chlorophyll a and b concentrations determined through fitting procedures and literature equations.

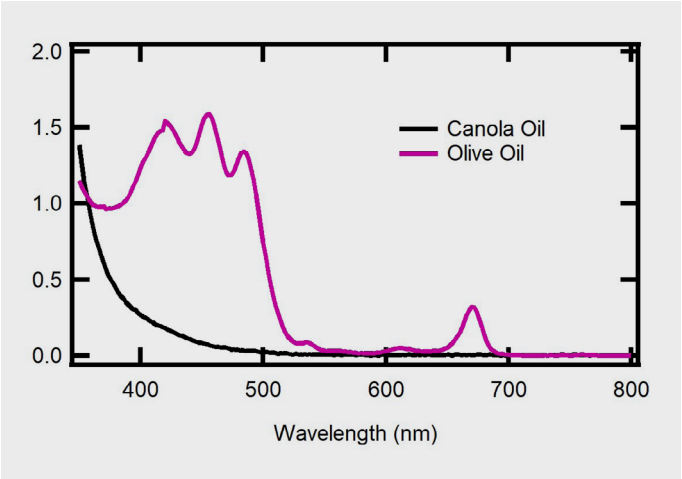

Figure 5: UV-Visible spectra of canola oil (black) and olive oil (pink) measured in a 1.0 cm cuvette.

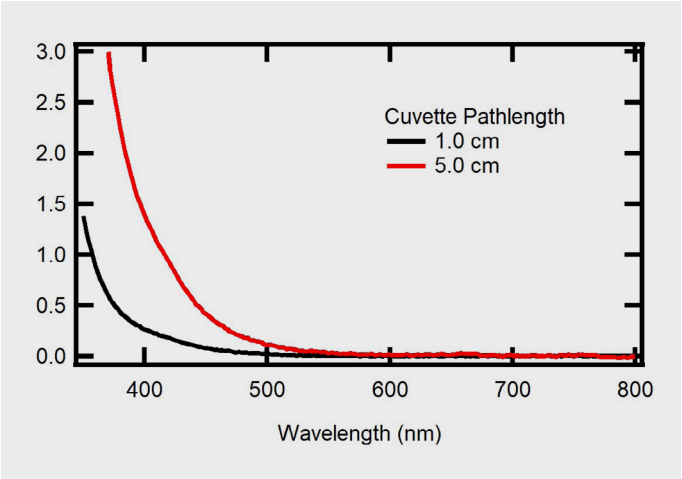

Figure 6: UV-Visible absorption spectra of canola oil measured in a 1.0 cm (black) and 5.0 cm (red) path.

Herein, the spectrum of a canola oil sample was measured using a 5 cm quartz cuvette to quantify the remaining chlorophyll content according to this method. As shown in Figure 6, no significant features in the red spectral region are observed, as expected. To confirm there is minimal chlorophyll present in the canola oil sample, a modified version of AOCS method Cc 15d – 55 was followed. This procedure utilizes the principles of Beer's law (eqn 1) in which the concentration can be determined if the absorption, extinction coefficient and pathlength are known. As ε is dependent on solvent, as described earlier, the extinction coefficient for Chl a in canola oil needed to be determined experimentally.

Four separate canola oil samples were made and each spiked with varying concentrations of Chl a standard. Chl a was used as this is the most common form of chlorophyll present in plant-based products.[1, 3] The red absorption maximum was found to be 666 nm for each sample measured. As shown in Table 5, the average ε determined at 666 nm was found to be 0.11 ± 0.01 ppm^{-1} cm^{-1}.

Chl a concentration (ppm)	A_{666}	ε_{666} (ppm^{-1} cm^{-1})
11.9	1.30	0.11
4.76	0.54	0.11
2.38	0.28	0.12
1.19	0.10	0.09
Average	—	0.11 ± 0.01

Table 5: Absorbance and calculated extinction coefficients for Chl a in canola oil. Samples were measured in a 1.0 cm pathlength cuvette.

Now that ε is known, a modified version of the calculation included in AOCS method Cc 13d – 55 can be used to quantify the chlorophyll content.[9] As the absorption maximum was shifted from 670 nm, the analysis wavelength outlined in AOCS, to 666 nm, equation 6 was used instead,

$$[Chl] = \frac{A_{666} - \left(\frac{A_{706} - A_{626}}{2}\right)}{\varepsilon_{666} l} \quad (6)$$

where *[Chl]* is the total chlorophyll concentration in ppm, A_{666}, A_{706} and A_{626} are the measured absorption at 666 nm, 706 nm and 626 nm, respectively, ε_{666} is the extinction coefficient at 666 nm (AOCS refers to it as the "factor"), and *l* is the pathlength used. Using this equation, the concentration was estimated to be 0.01 ppm. This value is low enough, that the concentration of Chl a can be considered negligible. As this sample was sourced from a supermarket, the chlorophyll content is expected to be low as a minimal amount of chlorophyll would be present post refinement in commercially available canola oils.

Conclusions

UV-Visible absorption methods are inherently useful for quantification due to the linear relation between absorption and concentration unique to the analyte of interest, as well as the non-destructive nature of the technique. Herein the Evolution Pro UV-Visible Spectrophotometer was used to analyze food samples for chlorophyll content. Spectrophotometric methods allow for not only the analysis of chlorophyll concentrations, but differentiations between varying chlorophyll derivatives, including Chl a and Chl b. Through calculations and fitting procedures, the concentration of Chl a and Chl b in spinach extracts were determined, demonstrating multiple different methods available for this analysis. In oil samples, UV-Visible absorption measurements were used to identify the presence of chlorophyll derivatives in commercially available olive oil samples. These derivatives give the characteristic green/yellow hue to the oil. Conversely this technique was able to confirm the lack of chlorophyll content following AOCS procedures within canola oil, an oil in which chlorophyll is not expected to be present.

References

1. Björn, L.O.; Papageorgiou, G.C.; Blankship, R.E.; Govindjee, A Viewpoint: Why Chlorophyll a?, *Photosynth. Res.*, **2009**, 99, 85 – 98.
2. Chen, M.; Blankenship, R.E., Expanding the Solar Spectrum Used by Photosynthesis, *Trends Plant Sci.*, **2011**, 16, 427 – 431.
3. Miazek, K.; Ledakowicz, S., Chlorophyll Extraction from Leaves, Needles and Microalgae: A Kinetic Approach, *Int. J. Agric. Biol.*, **2013**, 6, 107 – 115.
4. Daun, J.K, Spectrophotometric Analysis of Chlorophyll Pigments in Canola and Rapeseed Oils, *Lipid Technol.*, **2012**, 24, 134 – 136.
5. Endo, Y.; Thorsteinson, C.T.; Daun, J.K., Characterization of Chlorophyll Pigments Present in Canola Seed, Meal and Oil, *JAOCS*, **1992**, 69, 564 – 568.
6. Mag, T.K.; Canola Oil Processing in Canada, *JAOCS*, **1983**, 60, 380 – 384.
7. Daun, J.K.; Thorsteinson, C.T., Determination of Chlorophyll Pigments in Crude and Degummed Canola Oils by HPLC and Spectrophotometry, *JAOCS*, **1989**, 66, 1124 – 1128.
8. Hsu, C.-Y.; Chao, P.-Y.; Hu, S.-P.; Yang, C.-M., The Antioxidant and Free Radical Scavenging Activities of Chlorophylls and Pheophytins, *Food Sci. Nutr.*, **2013**, 4, 1 – 8.
9. American Oil Chemists Society. Official Method Cc 13d-55 Chlorophyll Pigments in Refined and Bleached Oils. In: Official Methods and Recommended Practices of the AOCS, 7th edition. Urbana, IL: AOCS.
10. Porra, R.J.; Thompson, W.A.; Kriedemann, P.E., Determination of Accurate Extinction Coefficients and Simultaneous Equations for Assaying Chlorophylls a and b Extracted with Four Different Solvents: Verification of the Concentration of Chlorophyll Standards by Atomic Absorption Spectroscopy, *Biochim. Biophys. Acta. Bioenerg. BBA-Bioenergetics*, **1989**, 975, 384 – 394.
11. Hashimoto, T.; Chow, Y.-K.; Nakano, H., Hirao, K., Theoretical Study of the Q and B Bands of Free-Base, Magnesium, and Zinc Porphyrins, and their Derivatives, *J. Phys. Chem. A*, **1999**, 103, 1894 – 1904.
12. Croce, R.; Cinque, G.; Holzwarth, A.R.; Bassi, R., The Soret Absorption Properties of Carotenoids and Chlorophylls in Antenna Complexes of Higher Plants.
13. Nigam, S.; Rutan, S., Principles and Applications of Solvatochromism, *Appl. Spectrosc.*, **2001**, 55, 362A – 370A.
14. Khalyfa, A.; Kermasha; S., Alli, I., Extraction, Purification, and Characterization of Chlorophylls from Spinach Leaves, *J. Agric. Food Chem.*, **1992**, 40, 215 – 220.
15. Domenici, V.; Ancora, D.; Cifelli, M.; Serani, A.; Veracini, C.A.; Zandomeneghi, M., Extraction of Pigment Information from Near-UV Vis Absorption Spectra of Extra Virgin Olive Oils, *J. Agric. Food Chem.*, **2014**, 62, 9317 – 9325.
16. Scott, K.J., Detection and Measurement of Carotenoids by UV/VIS Spectrophotometry, *Curr. Protoc. Food Anal. Chem.*, 1, **2001**, F2 – 2.
17. Alves, F.C.G.B.S.; Coqueiro, A.; Março, P.H.; Valderrama, P., Evaluation of Olive Oils from the Mediterranean Region by UV-Vis Spectroscopy and Independent Comonent Analysis, *Food Chem.*, **2019**, 273, 124 – 129.
18. Gandul-Rojas, B.; Cepero, M.R.-L.; Minguez-Mosquera, M.I., Use of Chlorophyll and Carotenoid Pigments Composition to Determine Authenticity of Virgin Olive Oil, *JAOCS*, **2000**, 77, 853 – 858.
19. Minguez-Mosquera, M.I.; Rejano-Navarro, L.; Gandul-Rojas, B.; Sánchez-Gómez, A.H.; Garrido-Fernandez, J., Color-Pigment Correlation in Virgin Olive Oil, *JAOCS*, **1991**, 68, 332 – 336.
20. Niewiadomski, H.; Bratkowska, I.; Mossakowska, E., Content of Chlorophylls and Carotenes in Rapeseed Oil, *JAOCS*, **1965**, 42, 731 – 734.