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Spectral Estimation of Chlorophyll for Non-Invasive Assessment in Apple Orchards

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Abstract: The main aim of our research was to develop a methodology of chlorophyll content in the leaves of apple trees non-invasive assessment in apple orchards and its adaptation to Early Gold and Golden Reinders based on spectral characteristics of chlorophyll content in the canopy. In each measurement period, 30 samples were collected from each of the two apple cultivars studied. For spectral data collection of leaf samples, an AvaSpec 2048 spectrometer was used in the wavelength range 400–1000 nm in three replicates. Principal component analysis (PCA) with varimax rotation was used to identify the wavelength with the highest factor weight to identify the chlorophyll-sensitive wavelength. The models were calibrated with 2/3 of the values in the database and validated with the remaining 1/3. The simple linear regression method generated the model for estimating chlorophyll. The coefficient of determination (R^2) was used to compare the strength of the regression models, and the Root Mean Square Error (RMSE), Normalized Root Mean Square Error (NRMSE), Nash–Sutcliffe efficiency (NSE), Mean Absolute Error (MAE) and Mean Bias Error (MBE) functions were used to measure the accuracy of the estimator models. These metrics help to quickly assess how reliable and accurate a model's predictions are. Nine indices were obtained based on the precision values, and CHL_{apple1} performed best ($R^2 = 0.633$, RMSE = 298.28 μg/g, NRMSE = 9.61%, NSE = 0.60 MBE = 84.59, and MAE = 243.39).

Keywords: apple; chlorophyll estimator models; vegetation index

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1. Introduction

Apples have a crucial role in global fruit production and consumption, with around 80 million tons harvested worldwide annually. In 2022, the European Union (EU) 27 collectively generated about 12 million tons of apples. Poland led as the top apple producer in the EU-27 with 4.5 million tons, followed by Italy at 2.1 million tons and France at 1.5 million tons. Hungary's annual apple production in 2022 amounted to 350 thousand tons [1,2]. Indicators of plant nutrition, photosynthetic capacity, growth, and development include leaf chlorophyll content [3,4].

Drought significantly affects the growth and productivity of plants, including apple trees. Understanding the necessity and rationality of addressing drought stress in apple trees is crucial for sustainable agricultural practices, particularly in the context of climate change. Apple trees, like most crops, require sufficient water for photosynthesis, nutrient uptake, and overall growth. Drought stress leads to reduced fruit size, poor quality, and lower yield. This can be economically damaging for apple growers, making it necessary to manage drought conditions to sustain productivity. In many regions, water resources are becoming scarce due to increased agricultural demands, population growth, and changing climate conditions. Efficient water use and the development of drought-tolerant apple varieties are essential to maintaining agricultural viability in these areas. Apple trees, when exposed to drought stress, face physiological changes such as stomatal closure (to reduce water loss), reduced photosynthesis, and altered metabolic processes. This leads to decreased growth and fruit development. Managing drought stress helps to minimize these effects, ensuring better tree health and higher fruit quality. Chlorophyll estimation is particularly beneficial for apple orchards as it provides an accurate indication of the nutrient and health status of plants, allowing farmers to optimize fertilizer and water use through precision agriculture, thereby improving yields and reducing environmental pressures [5,6]. It also strongly absorbs both the blue and red spectra of light and converts sunlight into chemical energy; thus, chlorophyll content is said to directly affect plant growth and yield [7].

The primary benefit of spectral imaging is its capacity to non-invasively capture precise phenotypic data across vast spatial areas and within specific periods. This data can be further analyzed for comprehensive data-driven insights and utilized for making informed technical decisions to enhance productivity [8]. Typically, as levels of stress in plants rise, there is a tendency for chlorophyll content to decrease [9]. Measuring plant photosynthesis and growth relies significantly on leaf chlorophyll content, which serves as a crucial indicator. Nevertheless, classical methods for chlorophyll quantification, including acetone ethanol extraction, spectrophotometry, high-performance liquid chromatography, and detecting leaf pigments, are laborious, destructive, intermittent, and time-consuming [10]. The optical and infrared (IR) reflectivity of a leaf, spanning wavelengths from 400 to 2500 nm, is influenced by a range of biochemical and physical factors. These factors include the presence of chlorophyll and other leaf pigments, nitrogen levels, and water content, as well as the internal leaf structure and surface properties [11].

Plant pigments exhibit absorption characteristics in specific ranges of electromagnetic radiation, notably in the visible (400–700 nm) and near-infrared (NIR, 1300–2500 nm) wavelengths. The NIR experiences minimal absorption by leaf components due to strong reflection from internal leaf structures. Specific reflectance values measured in the red and NIR regions are very important for understanding changes in plant chlorophyll levels, as these regions respond differently to plant chlorophyll content and health. In the red range, plants, especially chlorophyll, strongly absorb light as they derive the energy needed for photosynthesis. In the case of high chlorophyll content, the plant absorbs a high quantity of red light, so the amount of light reflected in this range will be low (low reflectance). In the case of low chlorophyll content, the plant absorbs less red light, so more light is reflected (higher reflectance). Therefore, low reflectance values in the red range usually indicate higher chlorophyll content, while high reflectance indicates lower chlorophyll levels. In the NIR, plants behave differently. Chlorophyll does not absorb this range, so the reflectance here is not related to chlorophyll content but rather to the structure and water content of the plant. Healthy, living plant tissues strongly reflect NIR light, as the structure of these tissues is designed not to allow deep penetration of infrared light, resulting in a high reflectance. Stressed or dead plant tissues, on the other hand, reflect less NIR light, leading to lower reflectance. The difference in reflectance between red and NIR reflectance can be used as a good indicator of the chlorophyll content and condition of the plant. Spectral analyses often use this difference to calculate various indices, such as the Normalized Difference Vegetation Index (NDVI), which indicates healthy green plant cover. The NDVI and similar indices can be used to detect changes in chlorophyll levels, as high NDVI values (large difference between NIR and red reflectance) indicate high chlorophyll levels and healthy plant condition. Low NDVI values (small difference between NIR and red reflectance) indicate low chlorophyll levels or plant stress. Consequently, the NIR reflection coefficient is influenced by enzyme concentrations and leaf structure. These properties enable the utilization of remote observation techniques across visible and NIR wavelengths for monitoring plant physiological conditions [12,13].

One method for estimating chlorophyll content from reflection spectra involves identifying empirical relationships (indices) between reflection coefficients at specific wavelengths. The selection of these wavelengths is a crucial aspect of the method and contributes significantly to accurately estimating chlorophyll levels [14]. To develop more robust estimation models, partial least squares (PLS) and PCA have been used to improve the accuracy of chlorophyll content prediction models [15,16]. The NIR reflectance of parenchyma tissue and cell membranes is caused by the lignin concentration in these tissues, whereas plant chloroplasts absorb light at 450 nm, 680 nm, and 700 nm wavelengths. The reflectance of leaves is determined by the surface area of the cell walls, not by the size of the space between the cells. The evolution of reflectance values depends on factors such as pigment composition, leaf surface quality, and plant health [17]. Chlorophyll shows strong absorption in the 450–670 nm wavelength range and can be measured to analyze the physiological state of the plant. Due to the internal structure of the canopy, a healthy plant reflects 40–50% of incoming light in the 700–1300 nm range. The measured reflectance plays an important role in differentiating between different plants and possible water stress, even if these species appear similar in the visible spectral range [18].

The chlorophyll content of apple tree leaves can be used as an indicator of the health of the plant. Changes in chlorophyll levels can indicate nutrient deficiencies or early stages of disease, allowing early intervention. Determining the optimal chlorophyll level can help to use nutrients (such as nitrogen) more efficiently, reducing the environmental impact of excessive fertilizer application and increasing the potential for sustainable farming. Drought can cause severe stress to plants, affecting chlorophyll levels and reducing soil nutrient availability. A decrease in chlorophyll content can indicate nutrient deficiencies, which can be addressed in time, for example by appropriate fertilization. Drought conditions can favor certain diseases and pests that can affect chlorophyll content. The specific index allows early detection of stress, which can allow appropriate measures (e.g., irrigation or nutrient replenishment) to be applied in time. In times of drought, efficient use of water is particularly important. With chlorophyll content as a strong indicator of plant water requirements, irrigation can be better planned, and water wastage minimized [19,20].

The primary objective of this research is to develop and adapt a non-invasive chlorophyll assessment method in apple orchards based on the spectral characteristics of foliar chlorophyll content in the 400–1000 nm wavelength range. The selected varieties were Early Gold and Golden Reinders, the two least drought-tolerant apple varieties. Based on the hyperspectral reflectance curves of apple leaves, the concept aims to identify the pigment-sensitive wavelengths where chlorophyll content is most significant, thus developing and adapting models to estimate chlorophyll content.

2. Materials and Methods

2.1. Study Site and Sampling Method

The samples analyzed were obtained from apple orchards located at the Horticultural Unit of Pallag, University of Debrecen, Hungary. The research was conducted on a 0.68 ha area of Early Gold and Golden Reinders apple orchards, which have been intensely cultivated for a decade and are equipped with micro-irrigation systems. All trees were grown using M9 rootstock. The choice of these two apple cultivars was based on their known susceptibility to drought and heat stress, as noted by Oaussat and Allam (2017) [21]. The orchard's sandy soil composition and precarious water balance make it especially prone to water and heat stresses, as highlighted by Nagy (2015) [17].

Sampling occurred during the peak drought periods, between 9 and 10 am, weekly from 7 July 2019 to 29 August 2019 (8 weeks). The time of sample collection was not chosen by chance, as this time frame can minimize the effect of diurnal variations, which can have a significant influence on chlorophyll measurements. The chlorophyll content and photosynthetic activity of plants fluctuate during the day, especially in response to light intensity and temperature. Between 9 and 10 in the morning, the sun is already high enough for the photosynthetic system of plants to stabilize after the night rest period. By this time, the dew and morning mist have mostly disappeared, and the sunlight already provides sufficient energy for photosynthesis but is not yet strong enough to cause stress to the plant, as it does in the midday hours. In 2019, the annual average temperature was 11.20 °C (July: 20.46 °C, August: 22.18 °C), the annual average precipitation was 480.4 mm (July: 47.81 mm, August: 31.61 mm), the annual average wind speed was 2.674 m/s (July: 2.353 m/s, August: 2.140 m/s), and the annual average relative humidity was 73.75% (July: 67.15%, August: 69.16%) (Supplementary File S1).

2.2. Measurement of Chlorophyll Content

During the weekly sampling events, 30-30 samples of both apple varieties were collected for a total of 480 samples. The samples were taken from randomly selected trees to ensure sampling homogeneity, and leaf samples from the middle of a branch at a height of 1.2 m were taken to determine the pigment content of the selected trees based on Nemeskéri et al. (2009) [22]. Leaf samples were stored and transported refrigerated at 4 °C and measured in the laboratory within 6 h. To ensure homogeneity, samples were blotted with 1 g of quartz sand and 80% acetone. After extraction, the suspensions were centrifuged in a Hettich ROTOFIX 32A at 3000 rpm for 3 min., and the clear solution then was transferred to a 2.5 mL quartz vial. The absorbance of the solution was measured with a SECOMAN Anthelie Light II at 470 nm, 644 nm, and 663 nm [23]. The chlorophyll content of the samples was determined according to the equation based on Droppa et al. (2003) [24]:

$$\text{Chlorophyll (a + b) } \mu\text{g/g fresh weight} = (20.2 \times A_{644\text{nm}} + 8.02 \times A_{663\text{nm}}) \times V/w \quad (1)$$

2.3. Spectral Data Acquisition of Apple Leaves

The AvaSpec 2048 spectrometer was utilized to capture spectral data from leaf samples within the 400–1000 nm wavelength range, with a precision of 0.6 nm. The 400–1000 nm wavelength range was chosen to measure chlorophyll levels in apple orchards because this spectral range accurately covers the chlorophyll absorption maxima and the NIR range, where plant tissues typically show high reflectance. Chlorophyll molecules, in particular chlorophyll-a and chlorophyll-b, strongly absorb light in the blue (430–450 nm) and red (640–680 nm) regions. The 400–1000 nm range ensures that these critical absorption bands are fully measurable, providing accurate information on chlorophyll levels. Wider wavelength ranges (e.g., 300–2500 nm) include spectral regions that are not relevant for measuring chlorophyll levels, such as the ultraviolet (300–400 nm) and mid-infrared (above 1000 nm). These ranges do not provide valuable data on chlorophyll but increase the measurement noise. The setup includes the spectrometer, an AvaLight-HAL halogen light source, and a patented sampling box designed to conduct measurements in complete darkness, ensuring that readings are unaffected by external light or noise. Since variations in ambient light (such as from LEDs or fluorescent bulbs) can alter reflectance at specific wavelengths, eliminating these background light sources is critical for accurate results. After calibrating the spectrometer using white and dark references, the leaf sample was illuminated, and measurements were taken in triplicate [25]. To further examine the spectral characteristics of the leaf samples, the values of the reflectance curves were divided into four equal interval groups based on chlorophyll content (1800–2700 μg/g, 2700–3100 μg/g, 3100–4000 μg/g and 4000–4600 μg/g). In addition, the relative standard deviation of the reflectance (%) data was also grouped into four groups. In this case, the criterion for grouping was to create groups with increasing chlorophyll ranges in groups, to detect the effect of increasing pigment content on the reflectance variability standard deviation (1800–2700 μg/g, 1800–3100 μg/g, 1800–4000 μg/g, 1800–4600 μg/g). This can reveal pigment-related effects that might otherwise go unnoticed in a broader, ungrouped dataset.

2.4. Statistical Tests and Development of the Estimator Model

Statistical analysis and PCA were carried out using the Statistical Package for the Social Sciences (SPSS 30) software. Chlorophyll-sensitive wavelengths were identified by

PCA with varimax rotation to determine the wavelength with the highest factor weight. PCA, as a statistical method, was used to dimensionalize the reflectance data and examine the relationships between reflectance and chlorophyll data. One of its basic steps is to standardize data to have the same units of measurement or scales. Standardizing the data ensures that all variables have the same importance in the subsequent steps. By using principal component analysis, the new space has fewer dimensions than the original one, while preserving the most important features of the data. The amount of dimension reduction can be controlled by the number of principal components selected. PCA was used to explore the relationships between the reflectance data at different wavelengths and pigment contents, thus helping us to understand which features are important for the model. Varimax rotation is an orthogonal rotation technique that transforms the components generated during principal component analysis so that they remain as independent of each other as possible, and so that each component is concentrated as much as possible on a single original variable. The aim of Varimax rotation is to ensure that the new components are as cleanly related to the original variables as possible so that the information contained in the new components better reflects the structure of the data. The components are rotated in such a way that as many of the original variables as possible are concentrated in each component, while the variables associated with the other components are minimized. The rotation preserves the orthogonality of the components, i.e., no new correlated components are created [26].

The input parameter for the PCA bands has 1063 variables, including reflectance % values derived from collected leaf samples at each band in the 400–1000 nm range and 1 more variable for the chlorophyll content values measured for each sample. Since there is a negative correlation between reflectance and chlorophyll content in the visible range [27], this component was used where the factor weight of chlorophyll is the closest to −1. Based on the factor weight results of this component, wavelengths will be selected that possess the highest factor weight in the given component, identifying the most sensitive wavelengths. When indexing the least pigment-sensitive wavelengths, it is also important to consider representing backscattering and non-pigment relations [28]. Based on these wavelengths, chlorophyll estimator models will be set after selecting the chlorophyll-sensitive and less-sensitive wavelengths.

In the development of estimator models, two [29] and three-band models [30] were also developed. Using this approach, the absorption of pigments was modeled using two or three narrow spectral bands. In the case of two bands, one sensitive and one less-sensitive wavelength were used to set the index, as described in the NDVI, SR, where the ratio of bands that have the greatest and least impact on biomass was used in indexing, or alternatively using the ratio of the difference and the sum of the two bands, resulting in a normalization of the data. In the three-band models, the first and second bands are maximally sensitive to chlorophyll absorption, but one is also affected by the absorption of other pigments, and the third band has less sensitivity, showing that the effect of chlorophyll pigment is small. Backscattering controls reflectance, as in the case of the Normalized Chlorophyll Index (NCI), Chlorophyll sensitive Ripening Monitoring Index (CRMI), Pigment sensitive Ripening Monitoring Index (PRMI) [31] and REP, mSR_{705}, $mNDVI_{705}$, and MCARI. In general, the above-mentioned indices were set based on the logic that proportionalizes the sum of the pigment-sensitive bands to the non-sensitive ones or vice versa. The new models were validated with the remaining 1/3 of the database data after calibration with 2/3 of the data. A simple linear regression method was used to generate the chlorophyll estimation model using chlorophyll data and the developed index. Therefore, the combination of bands with the highest regression parameter, i.e., R^2, was sought, since Abd-Elrahman et al. (2011) [32] discovered a significant correlation between chlorophyll levels and spectral indices derived from hyperspectral imaging reflectance, especially for the two- and three-band indices. A coefficient of determination (R^2) was used to compare the strength of the regression models.

To measure the accuracy of the estimator models the following calculations were used:

Root Mean Square Error—RMSE:

$$\mathrm{RMSE} = \sqrt{\frac{\sum_{i=1}^{n}(\mathrm{yi} - \acute{y}_{\imath})^2}{n}} \tag{2}$$

Normalized Root Mean Square Error—NRMSE:

$$\mathbf{NRMSE} = \frac{\sqrt{\frac{\sum_{i=1}^{n}(\mathbf{yi} - \acute{y}_{\imath})^2}{n}}}{(\bar{y})} \mathbf{100} \tag{3}$$

a Nash–Sutcliffe efficiency—NSE:

$$\mathbf{NSE} = \mathbf{1} - \frac{\sum_{i=1}^{n}(\acute{y}_{\imath} - \mathbf{yi})^2}{\sum_{i=1}^{n}(\acute{y}_{\imath} - \bar{y})^2} \tag{4}$$

Mean Absolute Error—MAE

$$\mathbf{MAE} = \frac{1}{n}\sum_{i=1}^{N}|\acute{y}_{\imath} - \mathbf{yi}| \tag{5}$$

Mean Bias Error—MBE

$$\mathbf{MBE} = \frac{1}{n}\sum_{i=1}^{N}(\acute{y}_{i} - \mathbf{yi}) \tag{6}$$

where

y_i: estimated value;

$\acute{y}_{\imath}$: measured value;

n: number of samples used for validation.

The RMSE is a commonly used uncertainty measure for estimating absolute errors, while the NRMSE relates the RMSE to the observed range of the variable. The NSE ranges from 1.0 (implying a perfect fit) to $-\infty$. In this case, non-zero efficiency means that the mean of the estimated value would have been a better estimator than the model. MAE was used to measure the number of errors between paired observations expressing the same phenomenon. The MBE was chosen to estimate the average model bias because it directly shows whether the model tends to systematically underestimate or overestimate the measured data, thus providing users with easy-to-understand feedback on the accuracy and possible bias of the model [33].

2.5. Tested Vegetation Indices

In addition to the models developed, existent and practical vegetation indices were calculated for comparison. The Normalized Difference Vegetation Index (NDVI) and the Red Edge Position Index (REP) were observed as the maximum slope point of the plant leaf reflectance spectrum between the red and NIR wavelengths. The Red Edge Normalized Difference Vegetation Index ($NDVI_{705}$) is a slight modification of the traditional NDVI and is used to make use of high spectral resolution reflectance data [34]. $NDVI_{705}$ considers a smaller waveband near the edge of the chlorophyll absorption characteristic (such as 705 nm) rather than in the middle, in contrast to the standard NDVI [35,36]. The $NDVI_{705}$ is more affected by chlorophyll content than the NDVI and has common applications in precision agriculture, forest monitoring, forest fire, and vegetation stress detection [37]. The Modified Red Edge Simple Ratio Index (mSR_{705}) is a modification of the broadband simple ratio (SR). It uses red edge bands and includes a correction for leaf specular reflectance. Applications include precision agriculture, forest monitoring, and vegetation stress detection [38]. The Modified Red Edge Normalized Difference Vegetation Index ($mNDVI_{705}$) is a spectral index used to detect plant health and chlorophyll content, to monitor photosynthetic activity and vegetation condition. It is particularly sensitive to changes in the red edge range, which is ideal for accurately detecting chlorophyll content and plant stress, even in situations that are difficult to detect using the conventional NDVI [39]. The Photochemical Reflectance Index (PRI) is sensitive to changes in carotenoid pigments in living foliage. Carotenoid pigments indicate the efficiency with which photosynthetic light is used, or the amount of carbon dioxide absorbed per unit of energy by the foliage. As such, they are used in studies of vegetation productivity and stress. The PRI measures whether plants respond to stress, making it an effective tool for determining the general health of an ecosystem when combined with satellite data or other types of remote sensing [40]. The Modified Chlorophyll Absorption Ratio Index (MCARI) responds to leaf chlorophyll concentration and soil reflectance. In general, high MCARI values indicate low leaf chlorophyll content and should be interpreted in conjunction with the NDVI or Leaf-Area Index (LAI) [41] (Table 1).

Table 1. Summary of indices tested (near-infrared—NIR).

Index Name	Index Formula
NDVI	$\frac{NIR - RED}{NIR + RED}$
REP	$700 + 40 \times \frac{(R_{670} + R_{780})/2 - R_{700}}{R_{740} - R_{700}}$
$NDVI_{705}$	$\frac{R_{750} - R_{705}}{R_{750} + R_{705}}$
mSR_{705}	$\frac{R_{750} - R_{445}}{R_{705} + R_{445}}$
$mNDVI_{705}$	$\frac{R_{750} - R_{705}}{R_{750} + R_{705} - 2R_{445}}$
PRI	$\frac{R_{531} - R_{570}}{R_{531} + R_{570}}$
MCARI	$[(R_{700} - R_{670}) - 0.2 \times (R_{700} - R_{550})] \times (\frac{R_{700}}{R_{670}})$

3. Results

3.1. Apple Leaf Reflectance % Results

Chlorophyll and carotenoids, which are integrated into the thylakoid membrane, are the pigments in control of photosynthesis. The two primary categories of oxygen-containing carotenoids are xanthophylls and carotenes. In the plant, integral proteins of thylakoids are found where chlorophylls and carotenoids are found bound [42]. Furthermore, the development and stress processes in apples can be identified by the ratio of chlorophyll to carotenoids [43]. Linear regression confirmed that there is a strong correlation between the chlorophyll content and carotenoid content measured in this study ($R^2 = 0.758$) (Figure 1). As a result, changes in plant pigments occur simultaneously and interactively.

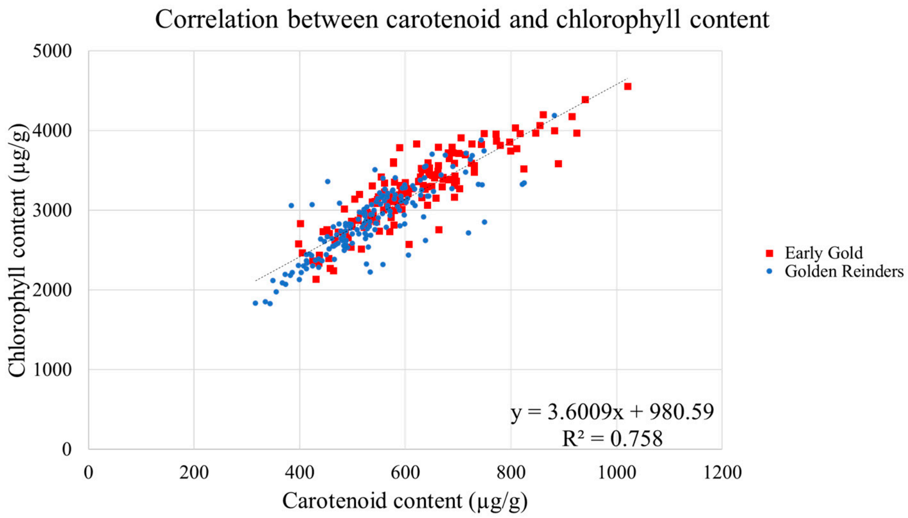

Figure 1. Correlation between carotenoid and chlorophyll content**.**

Chlorophyll content reflectance profiles were evaluated in the 400–1000 nm range. The lowest chlorophyll content was 1828.06 μg/g and the highest chlorophyll content was 4576.08 μg/g considering both varieties (Figure 2). These results reveal that leaves with a high chlorophyll content had lower reflectance values between 8 and 10%, indicating a decreased reflectance with an increase in chlorophyll values, since more chlorophyll captures more light. At low chlorophyll values of 1800–2700 μg/g, a reflectance value of 11–12% was observed. The maximum reflectance of carotenoids was measured in the 520–580 nm wavelength range, which gave a low reflectance value of around 12% at high chlorophyll content. The carotenoid at low chlorophyll content interval values gave a reflectance value of 17–18% (Figure 2). Thus, plant stress can be detected with high reflectance values in the 500–700 nm wavelength range. Depending on the structural properties of the leaf, much of the energy is transmitted and reflected, producing a high NIR curve. The red rim, which is used to determine plant stress and is more inherently connected to pigments, is located between the red and NIR bands during the high rise in reflectance. The red fringe is in the wavelength range between the red and NIR (around 680–750 nm), where the reflectance of plants increases sharply. This steep change indicates the end of the chlorophyll absorption spectrum and a high reflectance in the NIR range, which is closely related to the chlorophyll content and health of the plant. When the plant is under stress (e.g., due to water shortage, nutrient deficiency, or disease), chlorophyll levels decrease and with it the position of the red fringe shifts, often towards lower wavelengths. A shift in the red margin is therefore a sensitive indicator of plant stress, as this change indicates changes in pigment levels and reduced photosynthetic activity. Vegetation indices are derived mainly from reflectance data in the red and NIR bands, numerical measurements that measure biomass or the progress of vegetation state based on the spectral characteristics of vegetation [44].

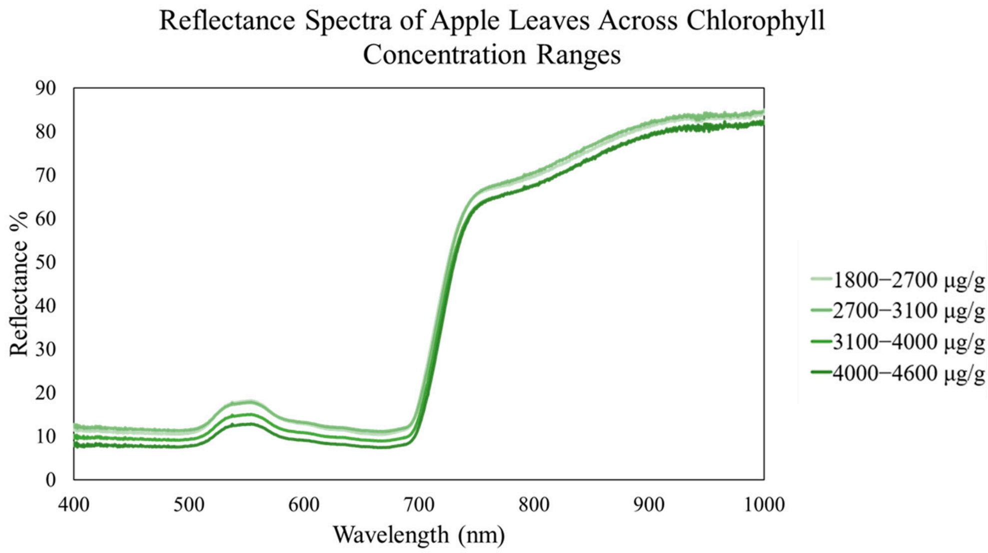

Figure 2. Spectral profile of apple leaves.

Determining the standard deviation (SD) is key to analyzing the relationships between chlorophyll content and wavelength, as it helps to understand the variability and reliability of the data. The SD shows exactly how far the measured values deviate from the mean value, providing insight into the dispersion of the results, which is essential for detailed studies of photosynthetic systems and pigment behavior. The standard deviation of chlorophyll content indicates the variation in chlorophyll concentration between different samples. The efficiency of photosynthesis is closely related to chlorophyll content, so changes in chlorophyll quantity can also affect the response of photosynthetic systems. If the chlorophyll content varies significantly between samples, the absorbance values at different wavelengths may also show a larger variation. The relative standard deviation values of the reflectance (%) of the chlorophyll concentration values were divided into four groups based on chlorophyll content to further study the spectral characteristics of the leaf samples (1800–2700 µg/g, 1800–3100 µg/g, 1800–4000 µg/g, and 1800–4600 µg/g). Low standard deviation (up to 520 nm ± 30 nm) was observed in the low chlorophyll groups (Figure 3). The reflectance scatter increased with chlorophyll content. Therefore, this range may be suitable for plant maturity studies and for determining the specific wavelengths at which the prediction accuracy will be highest. The peak of the standard deviation is prominent due to the absorption characteristics of chlorophyll measured in this wavelength range, being sensitive at 670 nm for high chlorophyll content. It is observed that the standard deviation of reflectance values calculated in the wavelength ranges of 550 nm, 670 nm, and 700 nm is pigment-sensitive. This sensitivity decreases with increasing carotenoid content due to an increase in absorbance. Thus, this spectral characteristic disappears with increasing carotenoid content. This is confirmed by Nagy et al. (2016) [31] and Zur et al. (2000) [45], who investigated the variation in apple and maple leaf pigment content and came to similar conclusions.

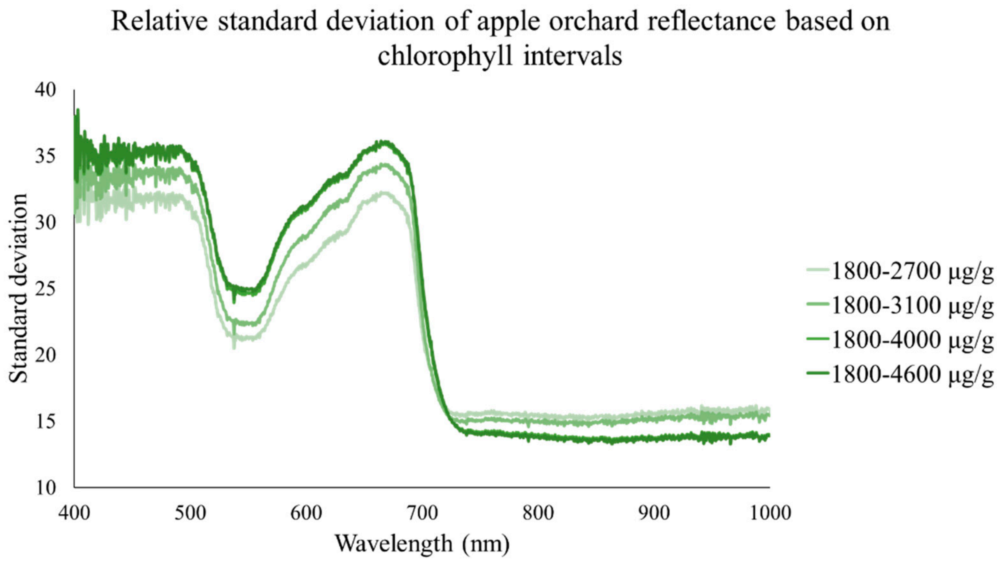

Figure 3. Relative standard deviation of apple leaf reflectance.

3.2. Development of Apple Chlorophyll Estimation Models

3.2.1. Calibration of the New Apple Chlorophyll Estimation Models

Principal component analysis produces five principal component results that capture the variance of the data. The first component explains 84.601% of the total variance, which means that it captures most of the information in the dataset. Component 2 adds a further 11.558%, bringing the cumulative explained variance to 96.159%. Component 3 adds 2.069%, resulting in a cumulative total explained variance of 98.228%. Component 4 increases the explanation by 1.092%, reaching 99.320%. Component 5 explains only 0.211%, resulting in a cumulative variance of 99.530%. Since Component 1 captures most of the variance (84.601%) and Component 2 increases this to 96.159%, these two components alone are sufficient to represent the bulk of the data structure (Table 2). This would significantly reduce the complexity from five dimensions to only two without a large loss of information. To test each variable of the measured sample, we used the Kaiser–Meyer–Olkin (KMO) test. The KMO was 0.83, indicating that the PCA is in the middle and demonstrating that the sampling is adequate [46].

Table 2. Results of the principal component analysis.

Component	% of Variance	Cumulative%
1	84.601	84.601
2	11.558	96.159
3	2.069	98.228
4	1.092	99.320
5	0.211	99.530

Figure 4 shows the PCA factor weight values that are most relevant as a function of chlorophyll content for developing the estimator models. Based on the first component factor weights, the two largest scatterings of reflectance are observed in the 556 and 710 nm wavelength range.

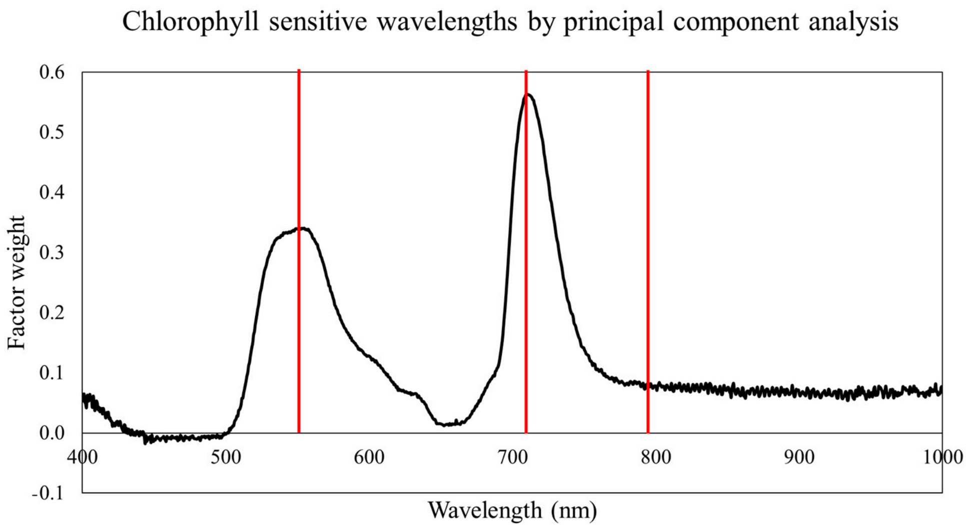

Figure 4. Factor analysis of spectral values of apple orchard**.**

To analyze the bands' importance in pigment sensitivity, a PC1-PC2 plot was also presented. Since the total variable number is 1063, the interpretation of the PCA matrix (PC1 × PC2) plot of variables should be very overcrowded; therefore, we put only the 3 band + chlorophyll on the plot. Based on the PC1–PC2 plot, the wavelength vector at 800 nm is moderately correlated with PC2, as it is mainly located along the *y*-axis. Meanwhile, for the chlorophyll content, the 556 nm and 710 nm wavelengths contribute to a lesser extent to PC2 and are more associated with PC1. However, the 556 nm wavelength is associated to a lesser extent with the first principal component, as its vector is short and tends to point along the first component. The directional vector of 710 nm is longer and is located along the first component, indicating a strong coupling. The chlorophyll vector points almost entirely towards the negative region of the *x*-axis, indicating that the chlorophyll content is more related to PC1, but shows a weaker correlation. The negative direction arises since increasing chlorophyll content results in decreasing reflectance at 556 and 710 nm [31], while reflectance at 800 nm is less dominant for biomass or the relationship with chlorophyll (Figure 5).

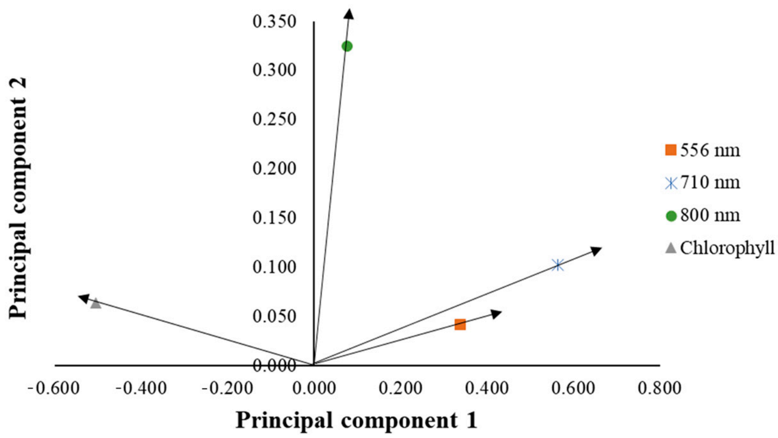

Figure 5. Two-dimensional PCA matrix (PC1 × PC2) for the used wavelength bands**.**

Two minima in the factor weight were observed, of which the 800 nm range was used together with the 556 and 710 nm ranges to construct the chlorophyll estimation indices. Correlation studies were carried out between reflectance and chlorophyll concentration.

The results of the correlation studies show that reflectance values in the 556 nm and 710 nm wavelength range correlate with chlorophyll values (Figure 6). In contrast, the 800 nm wavelength range does not correlate with the observed chlorophyll values. These observed results are also in agreement with our PCA results from the factor analysis.

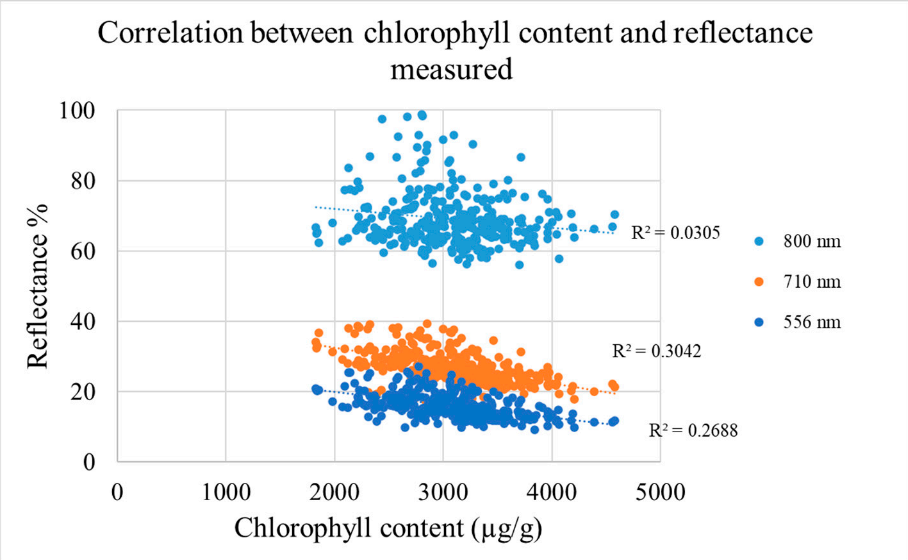

Figure 6. Correlation between chlorophyll content and reflectance measured.

Based on the principal component analysis, nine new indexes for estimating apple chlorophyll content were created, which were abbreviated as $CHL_{1–9}$ (Table 3). In indexing, the authors followed the principle of two and three-band indexing [47], which is about to use the maximally and least sensitive wavelength for indexing.

Table 3. Generated chlorophyll indices.

	Index	R^2
CHL_{apple1}	$(R_{800} - R_{710})/(R_{800} + R_{710})$	0.560
CHL_{apple2}	$(R_{800} - R_{710})/R_{556}$	0.500
CHL_{apple3}	$(R_{800} - R_{556})/R_{710}$	0.560
CHL_{apple4}	R_{710}/R_{800}	0.550
CHL_{apple5}	R_{800}/R_{710}	0.566
CHL_{apple6}	$(R_{710} + R_{556})/R_{800}$	0.540
CHL_{apple7}	$(R_{800} + R_{556})/R_{710}$	0.533
CHL_{apple8}	$R_{710}/(R_{556} + R_{800})$	0.523
CHL_{apple9}	$R_{800}/(R_{710} + R_{556})$	0.547

3.2.2. Validation of the New Apple Chlorophyll Estimation Models

The validation results suggest that, in most cases, new models perform better than conventional ones (Supplementary File S2). The CHL_{apple1} operating in the 400–1000 nm wavelength range had NSE = 0.60 MBE = 84.59 µg/g, and MAE = 243.39 µg/g (Figure 7). The chlorophyll values ranged from 2084.01 to 4045.38 µg/g, with a mean-variance of 3017.81 ± 394.80 µg/g. The CHL_{apple2} showed NSE = 0.59, MBE = 62.59 µg/g, and MAE = 246.94 µg/g. Estimated chlorophyll values ranged from 2371.95 to 4040.12 µg/g, with an average standard deviation of 3039.81 ± 355.60 µg/g. Using the CHL_{apple3} NSE = 0.60, MBE = 91.48 µg/g, and MAE = 244.61 µg/g were obtained. Estimated chlorophyll values ranged from 2212.83 to 4218.36 µg/g, with an average value of 3010.92 ± 395.61 µg/g. The CHL_{apple4} model had NSE = 0.59 MBE = 85.32 µg/g and MAE = 244.10 µg/g. The chlorophyll values ranged from 2023.84 to 3966.20 µg/g, with a mean chlorophyll value and standard deviation of 3017.08 ± 392.62 μg/g. For CHL_{apple5}, the NSE = 0.59 MBE = 81.44 μg/g and MAE = 244.92 μg/g. Estimated chlorophyll values ranged from 2231.94 to 4268.86 μg/g, with a mean chlorophyll value and standard deviation of 3020.96 ± 395.86 μg/g. For CHL_{apple6}, the values NSE = 0.58, MBE = 100.83 μg/g, and MAE = 248.37 μg/g were obtained. Estimated chlorophyll values ranged from 1995.69 to 3898.97 μg/g, with a mean chlorophyll value and standard deviation of 3001.56 ± 395.32 μg/g. CHL_{apple7} had NSE = 0.55, MBE = 69.09 μg/g, and MAE = 251.53 μg/g. Estimated chlorophyll values ranged from 2289.43 to 4243.55 μg/g, with a mean chlorophyll value and standard deviation of 3033.31 ± 382.07 μg/g. CHL_{apple8} had NSE = 0.56, MBE = 71.0 μg/g, and MAE = 246.44 μg/g. Estimated chlorophyll values ranged from 2161.85 to 3986.06 μg/g, with a mean chlorophyll value and standard deviation of 3031.38 ± 374.02 μg/g. In CHL_{apple9}, the NSE = 0.69, MBE = 94.79 μg/g, and MAE = 246.06 μg/g. Estimated chlorophyll values ranged from 2251.67 to 4151.26 μg/g, with a mean chlorophyll value and standard deviation of 3007.60 ± 384.04 μg/g (Figure 7).

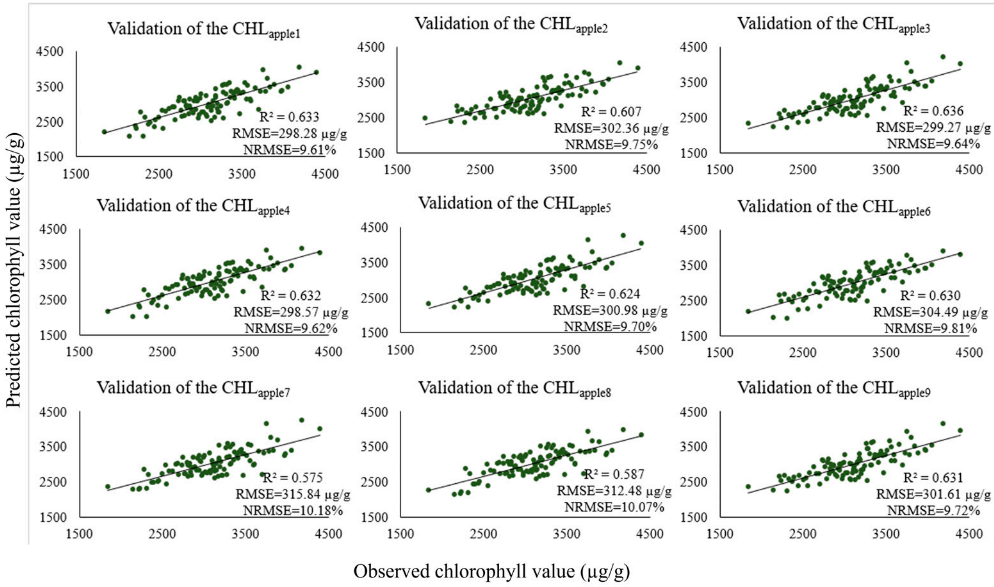

Figure 7. Accuracy of estimator models based on validation of chlorophyll models.

In addition to the models developed, existing vegetation indices and those used in practice were calculated for comparison. For the NDVI, R^2 = 0.206 (NRMSE = 13.72%) and R^2 = 0.373 (NRMSE = 12.11%) were obtained for the REP index, as the maximum slope point of the plant leaf reflectance spectrum between the red and NIR wavelengths (Figure 8). The $NDVI_{705}$ was determined using an NRMSE of 9.69% and R^2 = 0.623. In the mSR_{705}, R^2 = 0.172 and NRMSE = 13.99% were obtained. The $mNDVI_{705}$ was estimated with R^2 = 0.356 (NRMSE = 12.29%), and the PRI was calculated with R^2 = 0.055 (NRMSE = 15.00%). The MCARI was determined for R^2 = 0.233 and 13.36% NRMSE. In all cases, the tested indices gave lower R^2 and higher NRMSE values compared to the established indices (Table 2, Figure 8).

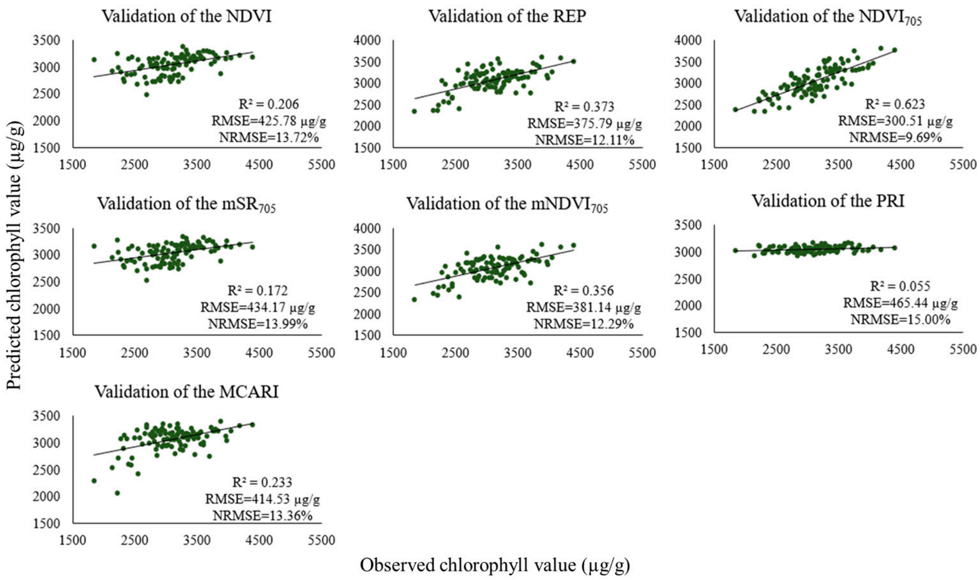

Figure 8. Chlorophyll estimators for indices used in practice**.**

Based on the precision values, nine indices were obtained, with CHL_{apple1} performing the best ($R^2 = 0.633$, RMSE = 298.28 µg/g, NRMSE = 9.61%, NSE = 0.60 MBE = 84.59, and MAE = 243.39). The developed model was 42% more accurate compared to the NDVI, 26% more accurate than the REP index, 0.8% more accurate than $NDVI_{705}$, 45% more accurate than mSR_{705}, 27% more accurate than $mNDVI_{705}$, 56% more accurate than PRI, and 39% more accurate than MCARI.

4. Discussion

The drought tolerance of plants is closely related to photosynthetic pigment status [48]. The anatomical structure of drought-tolerant plants is different, with denser leaf blades and higher stomatal density. The chlorophyll content varied from 3870 to 4920 µg/g in apple cultivars of different ecological and geographical origins: Idared, Erli Mac, Dayton (USA), Prikubanskoe, Rassvet, and Fortuna (Russia) during drought [49]. However, during the dry summer period, precipitation deficiency was not uniformly reflected in the photosynthetic activity of apple cultivars in Kiseleva's study [50]. In their results, chlorophyll content distributions (1995.69 and 4268.86 µg/g) were like the present study (1828.06–4576.08 µg/g), although different apple cultivars were surveyed. Nemeskéri et al. (2010) [51] found that the relative chlorophyll value (SPAD) of apple leaves on the eastern side of the trees was lower than the SPAD measured on the western and southern sides at the onset of the prolonged drought. Stomatal density was noticeably higher, and leaf mass was also higher than in previous periods. In addition, chlorophyll content ranged from 1599.03 to 3518.12 µg/g in Early Gold and Golden Reinders apple cultivars with hail net cover [23]. In addition to drought, soil properties also influenced the results of the present study, as acidic sandy soils are typical in the Pallag area. The characteristics of sandy soils can affect plant chlorophyll levels in many ways, and soil quality and properties play a crucial role in nutrient supply, water uptake, and overall plant health. Sandy soils are generally low in organic matter and nutrients. They can be particularly low in nitrogen, potassium, and magnesium, all of which are key elements for chlorophyll production and photosynthesis. Nitrogen is the essential building block of the chlorophyll molecule. If the soil is deficient in nitrogen, the plant cannot produce enough chlorophyll, which can lead to yellowing. Magnesium is the central atom of chlorophyll, so its deficiency also negatively affects chlorophyll levels. Sandy soils have a very low water-holding capacity, which means that they permeate water quickly, so plants often do not get enough water, which can lead to water stress. Sandy soils have a poor cation exchange capacity, making it difficult to fix nutrients. As a result, plants do not have a constant supply of the nutrients they need, which inhibits chlorophyll production. The pH of sandy soils can vary but is often more acidic due to their low organic matter content. An acidic environment can inhibit nutrient uptake, making plants less efficient at absorbing the nutrients needed for chlorophyll synthesis. If the pH is too low or too high, the availability of certain nutrients such as iron, manganese, and magnesium can be reduced, which can also cause chlorophyll deficiency. Sandy soils heat up and cool down faster than other soil types, which can stress plants, especially if the root zone temperature is too high or too low [52].

Plant pigments can be determined using a variety of narrow-band spectral methods, from simple colorimetric techniques to radiative transfer techniques. Gitelson et al. (2006) [30] found the spectral wavebands in the red fringe (700–750 nm), red (630–690 nm), and green band (500–580 nm) particularly useful for estimating chlorophyll concentration. In the green light range, there is an obvious reflection peak at 600–700 nm. Leaves absorb red light for photosynthesis and form a low reflection region, which is also observed in the results of the present study. In their study, Wang et al. (2015) [47] considered the sensitive wavelengths at 554 nm and 713 nm, as well as the first derivative value of the sensitive wavelengths at 530 nm, 581 nm, 697 nm, and 734 nm for estimating the chlorophyll content of the Red Fuji apple. Kira et al. (2015) [48] and Zhao et al. (2019) [49] reported that wavelengths near 550 nm and 707 nm were the most sensitive for chlorophyll content in chestnut and mangrove trees, respectively. In the present study, PCA identified five principal components with the highest variances in reflectance at 556 nm and 710 nm, which were used to construct chlorophyll estimation indices, along with the 800 nm range. Reflectance values at 556 nm and 710 nm showed strong correlations with chlorophyll values, while the 800 nm range did not correlate well. Plant stress can be detected by high reflectance values in the 500–700 nm range. Based on the structural properties of the leaf, most of the energy is transmitted and reflected, creating a high NIR curve. The red edge, which is located between the red and NIR bands during a strong increase in reflectance, is used to detect plant stress and is more closely related to pigments [53]. The wavelength of the inflection point was determined using the first, second, or amplitude derivative in most of the early red-edge research, and this was then connected with the amount of chlorophyll [50]. A study by Nagy et al. (2016) [23] found that spectral data show that the 678 nm wavelength is sensitive to low chlorophyll content, and therefore the red (678 nm) range is suitable for assessing ripening and maturity. Although the reflectance measured at 678 nm ± 30 nm showed higher variability at high chlorophyll content, the reflectance measured at 700 nm can be used to monitor early ripening and changes in pigment content. Based on these results, new spectral indicators for monitoring maturation were established. This confirms the sensitivity of the 710 nm wavelength used in the present study in accurately constructing estimator models.

Therefore, vegetation indices are usually derived primarily from red-boundary bands using red and NIR band reflectance data, numerical measurements that measure biomass or vegetation state progress based on the spectral characteristics of vegetation [54]. Three of these indices, the NDVI, $NDVI_{705}$, and mSR_{705}, were used to estimate chlorophyll concentration in plant leaves in this study. However, only the $NDVI_{705}$ showed considerable accuracy in chlorophyll content estimation in the apple canopy. Gitelson et al. (2006) [30] made a significant advance by developing a three-band model for estimating multiple pigment contents. Using this semi-analytical approach, the absorption of pigments was modeled using three narrow spectral bands: in the first band (R_1), the reflectance is maximally sensitive to the absorption of the pigment of interest, but is also affected by the absorption of other pigments and the variability of backscattering; in the second band (R_2), these effects are eliminated, where other pigments absorb the light but the effect of the pigment of interest is small; and in the third band (R_3), backscattering controls the total reflectance. Three-band indexing was used to estimate other parameters, i.e., total nitrogen content, with success in different crops proving that three-band indices outperform two-band indices. Certainly, there are several plant stress and pigment monitoring indices available, and REP, the mNDVI$_{705}$, and MCARI were chosen to be tested in this study, showing moderate but still slightly better pigment estimation accuracy for the apple canopy compared to the NDVI, mSR$_{705}$, and PRI. In addition, using PCA-selected pigment-sensitive wavelengths (R$_{556}$, R$_{710}$, and R$_{800}$), nine new apple canopies with specific two- and three-band indices were developed and tested to the rapid non-invasive estimation of chlorophyll content. The models showed good estimation performances, where the NRMSE was between 9.61 and 10.18%. There are several other statistical methods available to develop a non-invasive estimation method of stress parameters of plants [31,55]. Guo et al. (2014) [56] determined the chlorophyll content of foliage in an apple orchard using visible/NIR spectroscopy and partial least squares (PLSR) and genetic algorithms. The results of their final model were $R^2 = 0.91$, which gave more accurate results compared to the present study, which could be the results of a different modeling approach. However, using PLSR Wang et al. (2015) [47] estimated the chlorophyll content of young apple tree leaves, resulting in 0.589–0.621 R^2 values in correspondence with our results (0.575–0.633). Nagy et al. (2024) [33] developed chlorophyll estimator models from maize leaf analysis using three-band applications (R$_{516}$, R$_{551}$, and R$_{763}$). The R^2 of the developed models ranged from 0.518 to 0.641. These multivariate statistical methods can be applied by reducing the variance of the data to fewer wavelengths and bands during the grid arrangement of high-dimensional correlated variables. In this way, testing other statistical methods to determine which bands contain the most relevant information can improve further modeling and analysis results in the future.

The current results are based on active sensor technology that performs spectral measurements under standard lighting conditions. This could contribute to the development of portable instruments that enable rapid, non-destructive chlorophyll measurements by measuring canopy reflectance at specific wavelengths using a best-performance machine learning method. Our method requires a sensor head that can filter out the effects of ambient background lighting and uses an active light source. However, standard lighting conditions can also be a limitation if measurements are made using passive sensor technology. Given the potential for field application, further sensitivity studies using an open sensor head are needed to explore the effects of environmental variability and ground conditions on reflectance and the models developed. It is also important to consider that over-reliance on specialized tools such as hyperspectral sensor models may limit the wider applicability of the method in regions where these resources are not available.

5. Conclusions

In conclusion, this research has successfully developed a methodology for non-invasive assessment in apple orchards, specifically tailored to Early Gold and Golden Reinders. By sampling and spectral analysis of canopy chlorophyll content, significant progress has been made in the rapid, non-destructive determination and quantification of chlorophyll content in apple trees. The results of the research on apple leaf reflectance give a significant fresh understanding of the relationships between reflectance properties and leaf pigment content, as well as the development and validation of models for estimating chlorophyll. Statistical techniques such as PCA with varimax rotation were used to identify the wavelengths most sensitive to chlorophyll content, thus establishing their estimation models. The robustness of the models was evaluated using various accuracy values, including R^2, RMSE, NRMSE, NSE, MAE, and MBE, providing an overall assessment of their accuracy. The calibration and validation of the models demonstrated the effectiveness of three bands models in estimating chlorophyll content, resulting in the CHL$_{apple1}$ being the best performing model out of the nine indices. By providing growers with reliable tools to assess chlorophyll content, our methodology offers the potential to optimize cultural practices, enhance plant resilience, and improve crop production.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10121266/s1, Figure S1: Meteorological data in the study area during the experimental period; Table S1: All the values of calculated prediction accuracy indices.

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