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Determining chlorophyll and carotenoid content in *Bombyx mori* L. excreta by Near Infrared Spectroscopy

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**Abstract.** For the needs of the experiment excreta age five of silkworms (populations of the monovoltine crossbreds of *Bombyx mori* L. Super1xYeca2, 19x1014 and 1013x1014) raised in spring have been used. Spectral measurement (NIR spectroscopy) of excreta has been made non-destructively by measuring diffuse reflection of samples within the range 900–1700 nm with the spectrometer NIRQuest 512. Afterwards chlorophyll a, chlorophyll b and carotenoids content in these samples has been determined through acetone extraction and determining the absorption of the resulting solution at 440.5, 644 and 662 nm by spectrophotometer Spekol 11. Partial Least Square Regression (PLS) has been used to obtain equations for determining the amount of chlorophyll and carotenoids in excreta based on near-infrared spectral data. The results obtained show that chlorophyll a, chlorophyll b and carotenoids content in silkworm excreta can be successfully determined by non-destructive spectral analysis within the range 900-1700 nm. The obtained correlation coefficients, revealing the relationship between the parameters tested are higher than 0.96. Extremely high accuracy has been reached in determining the content of chlorophyll a and carotenoids. Lower is the accuracy in determining chlorophyll b, which can be accounted for by the fact that chlorophyll b is more unstable and quickly dissociates after extraction.

**Keywords:** silkworms’ excreta, chlorophyll a, chlorophyll b, carotenoids, near-infrared spectroscopy

**Introduction**

Enhancing sericulture efficiency is related to the use of products considered so far to be waste such as silkworm excreta. That would make it possible both to receive additional income from those products not used so far and ecological effect due to reducing the amount of waste generated. Interesting is the application of chlorophyll paste, pectin, phytollum, carotene and other substances extracted from silkworm excreta. They are used in medicine for treatment of various diseases such as hepatitis, pancreatitis, chronic nephritis, gastritis, etc., as well as in the cosmetic industry (Singh and Jayasomu, 2002). Raghavendra et al., 2010, found that partially purified protein from the *Bombyx mori* L. fecal mass with molecular mass 35kDa shows considerable hepaprotection effect in case of liver damage. In recent years the possibility has been studied for chlorophyll and chlorophyll derivatives to be used as antimicrobial, antiviral and antitumor agent or as part of photodynamic therapy.

The efficiency of photodynamic therapy by using chlorophyll derivatives to prevent the development of bladder cancer after surgical treatment was studied by Liu et al., 1998. They found statistically reliable effect on cancer recurrence. Lim et al., 2002 studied chlorophyll derivatives from silkworm excreta in photodynamic antimicrobial chemotherapy. The vesicular stomatitis virus has been used as a model organism. The results obtained show that chlorophyll derivatives can be used as a potential photodynamic antiviral agent which stops the replication of the studied virus. The action of chlorophyllin, a chlorophyll derivative, in concentrations of 25–400 µg/ml, on tumor cells was studied by Chiu et al. (2003, 2005). They found that chlorophyllin reduced the proliferation of tumor cells by 8.2–95.7% after 72 hours of incubation and caused apoptosis of MCF-7 cells.

The objective of this study is to investigate the possibility to determine chlorophyll and carotenoids content in *Bombyx mori* L. excreta through direct non-destructive spectral analysis in the near-infrared range.

**Materials and methods**

The study has been conducted at the Training Experimental Centre of Sericulture section at the Department of Animal Science – Non-ruminants and Other Animals at the Faculty of Agriculture, Trakia University during the spring season of 2012. In the course of rearing during age five of silkworm development excreta from 3 *Bombyx mori* L. crossbreds 19 x 1014, 1013 x 1014 and Super 1x Xeca 2 were collected.

Chlorophyll a, chlorophyll b and carotenoids content have been determined through acetone (100%) extraction and determining the absorption of the resulting solution at 440.5, 644 and 662 nm by spectrophotometer Spekol 11. After recording absorption the amount of chlorophyll and carotenoids in the extract is calculated by the formulae of Holm and Weststein (Tretyakova, 1982):

\[
\text{Chlorophyll a} = 9.784xD_{440} – 0.990xD_{662}
\]

\[
\text{Chlorophyll b} = 21.426xD_{662} – 4.650xD_{440}
\]

\[
\text{Carotenoids} = 4.695xD_{440} – 0.268(x_{\text{Chl. a}} + x_{\text{Chl. b}})
\]

After that their concentration in excreta is determined in units mg.g⁻¹ on the basis of the initial amount and the volume of acetone extract.

Spectral measurement (NIR spectroscopy) of excreta was performed non-destructively by measuring diffuse reflection of
samples on the spectrometer NIRQuest 512.
NIRQuest 512 by Ocean Optics company is a portable scanning spectrophotometer operating in the range 900-1700 nm. It is a new generation of spectrophotometers operating with fiber optics probes and a diode array with 512 pixel as a detector. The spectrophotometer is linked to a computer via USB port and is controlled by the software package SpectraSuite by the Ocean Optics company. The spectral data from NIRQuest are recorded as a text file and then opened in the software Pirouette 4.5 (Infometrix, Inc., USA), which is further used for processing spectral data.

Partial Least Square Regression (PLS) is used for quantitative analysis. With this method spectral data and the values of the necessary quantitative parameter are processed simultaneously and new factors are calculated, the first factor describing maximum part of variations in spectral and quantitative data, the second factor describing maximum part of the remaining variations and so on. In this way through several such factors we can describe both the spectral and quantitative information about the samples. Additionally, for improving the accuracy of determination the orthogonal signal a correction method is used. With this method we define which parts of the available spectral information correlate with the defined parameter and which do not. The spectral information of those wavelengths that are irrelevant for determining the necessary parameter is removed prior to applying the PLS regression. In this way models with lesser number of factors and smaller error of determination could be obtained.

### Results and discussion

The results obtained about concentration of chlorophyll a, chlorophyll b, the sum total of chlorophyll a+b and carotenoids in the analyzed excreta from the three crossbreds obtained through acetone extraction, spectrometric measurement of the extract and calculation by the equations described are presented in Table 1. Variations have been observed in the chlorophyll and carotenoids content in the samples analyzed and the variations in the carotenoids content are less than the variations in the chlorophyll content.

The spectral data obtained from measurement of the excreta, transformed as second derivative, are presented on Figure 1. Differences can be seen in the absorption of the various wavelengths and differences in the absorption of the same wavelength among samples, which is the basis for quantitative determination of the content of various components in excreta.

The statistical parameters of the calibration equations obtained for determining the concentration of chlorophyll a, chlorophyll b, the sum total of chlorophyll a+b and carotenoids in the analyzed excreta based on their spectral data are presented in Table 2. The parameter SEC is the standard error in the calibration equation and SECV is an error in cross validation, which is an estimation of what error could be obtained when analyzing unknown samples. Rcv and Rcal is a multiple correlation coefficient in cross validation and calibration. A very good accuracy in determining the studied parameters has been obtained. A graphic illustration of the results is presented on Figures 2, 3 and 4. For all parameters the obtained correlation coefficients of the calibration equations are higher than 0.966 both in calibration and in the cross check. When comparing the results it can be seen that the lowest is the accuracy in

### Table 1. Range, mean values and standard deviation of the values of chlorophyll a, chlorophyll b, the sum total of chlorophyll a+b and carotenoids in the analyzed excreta

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Chlorophyll a</th>
<th>Chlorophyll b</th>
<th>Chlorophyll a+b</th>
<th>Carotenoids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.636</td>
<td>0.566</td>
<td>1.201</td>
<td>0.304</td>
</tr>
<tr>
<td>Min</td>
<td>0.260</td>
<td>0.195</td>
<td>0.454</td>
<td>0.103</td>
</tr>
<tr>
<td>Max</td>
<td>1.217</td>
<td>1.1831</td>
<td>2.376</td>
<td>0.728</td>
</tr>
<tr>
<td>SD</td>
<td>0.288</td>
<td>0.304</td>
<td>0.585</td>
<td>0.163</td>
</tr>
</tbody>
</table>

### Table 2. The statistical parameters of the calibration equations obtained for determining the concentration of chlorophyll a, chlorophyll b, the sum total of chlorophyll a+b and carotenoids in the analyzed excreta based on their spectral data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PLS factor</th>
<th>SECV</th>
<th>r_cvl</th>
<th>SEC</th>
<th>r_cvl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyll a</td>
<td>4</td>
<td>0.009</td>
<td>0.998</td>
<td>0.007</td>
<td>0.999</td>
</tr>
<tr>
<td>Chlorophyll b</td>
<td>10</td>
<td>0.044</td>
<td>0.967</td>
<td>0.022</td>
<td>0.993</td>
</tr>
<tr>
<td>Chlorophyll a+b</td>
<td>8</td>
<td>0.029</td>
<td>0.996</td>
<td>0.015</td>
<td>0.999</td>
</tr>
<tr>
<td>Carotenoids</td>
<td>3</td>
<td>0.004</td>
<td>0.999</td>
<td>0.003</td>
<td>0.999</td>
</tr>
</tbody>
</table>

![Figure 1. Spectral data of excreta samples transformed as second derivative](image1.png)

![Figure 2. Correlation between the actual values of chlorophyll a and the ones determined on the basis of the spectral data in *Bombyx mori* L. excreta samples](image2.png)
determining the chlorophyll b content. That can be accounted for by the fact that chlorophyll b is more unstable and quickly dissociates after extraction. When analysing the results no dependence of the accuracy of determining the studied parameters on the silkworm crossbreds from which the excreta have been taken, is found. That could allow the arriving at general calibration equations for each crossbred.

Figure 5 presents a diagram of the parameter „correlation spectrum” in determining chlorophyll and carotenoids in excreta samples. The diagram shows that there is significant correlation between the spectral data of certain wavelengths, reaching 0.8, and the chlorophyll and carotenoids content. There are also some differences between the spectral information significant for determining the measured components. Lower is the correlation between the spectral data and the chlorophyll b content which results in lower accuracy of determining.

**Conclusion**

The data obtained in this study show that chlorophyll a, chlorophyll b and carotenoids content in the excreta of silkworms from 3 crossbreds – 19 x 1014, 1013 x 1014 and Super 1x Xeca 2 can be successfully determined by direct spectral analysis within the range 900–1700 nm. The obtained correlation coefficients showing the relation between the spectral data and the content of the studied parameters are greater than 0.96.

Extremely high accuracy of determining has been obtained in determining the chlorophyll a and carotenoids content. Lower is the accuracy in determining chlorophyll b, which can be accounted for
by the fact that chlorophyll b is more unstable and quickly dissociates after extraction.

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