

Experimental method for reliably establishing the refractive index of buprestid beetle exocuticle

J. A. Noyes*, P. Vukusic and I. R. Hooper

School of Physics, The University of Exeter
Exeter, Devon, EX4 4QL, UK
j.a.noyes@exeter.ac.uk

Abstract: In this study we apply an existing optical characterisation technique to establish reliably the complex refractive indices of layers comprising a natural multilayer reflector in the beetle *Chrysochroa raja*. Its reflector characteristics, ultrastructure and layer thicknesses were established using electron and optical microscopy. We recorded a significant number of wavelength dependent optical data sets from the same regions of sample using both linear polarisations and from a variety of different angles. These optical data sets were modelled simultaneously in order to significantly reduce the degeneracy of the fitting process. For the *C. raja* sample in question, the fitted complex refractive indices of both layer types were determined to be $n=1.68$ $k=0.03$ and $n=1.55$ $k=0.14$.

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1. Introduction

Iridescent beetles have been the subject of many optical studies; their intensely coloured surfaces attracting the interest of both biological and photonics scientists [1,2]. Many different optical effects, such as colour mixing and optical activity, have been observed in a broad range of coleopteran species [3,4]. However, while specific forms of multilayer reflector have been shown responsible for the iridescence in the majority of species studied, a rigorous method for reliably characterising the complex refractive indices of each system has largely not been demonstrated. To this end, we initiated this study.

In general, when modelling the optics of many-layer systems, one of the principle difficulties comprises overcoming the degeneracy between complex refractive indices and layer thicknesses. For example, a wide range of similar physical characteristics can be tuned to produce the same optical reflection spectra [5,6]. Such degeneracy, however, can be significantly reduced by simultaneously considering many different sets of optical data from the same sample so that angle and polarisation are also considered as optical variables, in addition to wavelength and absolute values of reflectivity and transmissivity.

In previous studies, many experimental methods have been used to obtain the reflection spectra of samples. One of the more common methods comprises the use of integrating spheres [7]. These are designed to collect nearly all light that is diffusely reflected from a sample: this diffusely reflected light is directed, via many internal reflections, to an exit aperture where it is delivered to a spectrometer.

Such multiple reflections are problematic, however. They remove the polarisation state of the reflection from the sample. Additionally, components of the multiple diffuse reflections from the internal surfaces of the sphere are directed back at the sample at a range of different angles. These lead to a convolved reflection from the sample comprising components of many different angles. Therefore, recording accurate angle-dependent and polarisation-dependent optical data is extremely challenging using conventional integrating spheres.

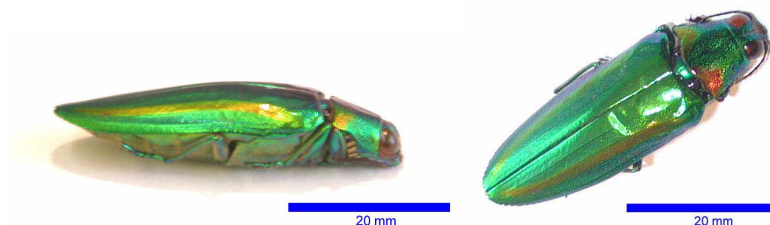


Fig. 1. Image of *C. raja* showing the iridescence in the elytra. In this image the red stripe looks orange due to the angle between the camera, the surface of the elytra and the light source. There is also a blue hue visible at the edges of the elytra.

A range of micro-spectrophotometers has been developed to measure the reflection spectra from natural samples [1]. In principle they comprise a conventional spectrometer that is attached to the column of an optical microscope. These also exhibit limitations; such as the inability to record accurately the absolute values of reflectivity or transmissivity (in terms of the exact percentage of incident light) as well as a very limited independent control of incident and reflected angles. There is, therefore, a clear need for a method to overcome the existing limitations of conventionally used experimental techniques.

In this paper we describe a reliable method for quantifying the complex refractive indices of the component layers of a natural multilayer reflecting system. We describe how we obtain absolute wavelength-dependent optical reflectivity and transmissivity data comprising different incident polarisations and a range of angles of incidence. Then by simultaneously modelling a significant number of independent sets of optical data from the same sample using code based on recursive Fresnel formulae, the degeneracy of the system is significantly reduced and its complex refractive indices are extracted.

2. Method

Small regions from the elytra of male *C. raja* beetles (Fig. 1) were examined using scanning and transmission electron microscopy (SEM and TEM) and optical spectroscopy.

A Hitachi S-3200N electron microscope was used for the SEM; samples were cold sputtered with 6 nm of gold. TEM analysis was undertaken after fixing samples in 3% glutaraldehyde at 21° C for 2 hours followed by rinsing in sodium cacodylate buffer. Subsequent fixing in 1% osmic acid in buffer for 1 hour was followed by block staining in 2% aqueous uranyl acetate for 1 hour, dehydration through an acetone series (ending in 100% acetone) and embedding in Spurr resin [8]. After microtoming, sample sections were stained with lead citrate and examined using a JEOL 100S TEM instrument.

Experimentally, a 2 mm² region of sample was first illuminated by a UV-Visible-Near IR light source (Ocean Optics DH-200-BAL). The reflectivity was collected and delivered by a 400µm diameter UV-visible fibre optic cable set at 3-4 cm from the sample to a UV-Visible-Near IR spectrometer (Ocean Optics USB2000-UV-VIS). Glan-Thomson polarisers were used to set the polarisation of the incident light. This configuration has a working range between 300 nm and 900 nm.

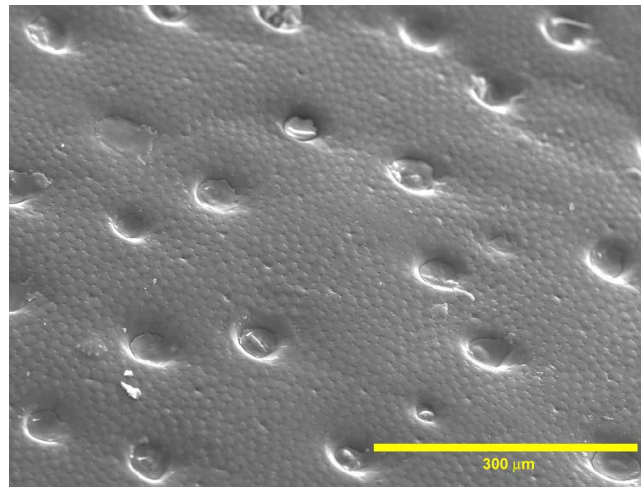


Fig. 2. SEM image of the surface of *C. raja* showing the irregularities in the surface of the cuticle.

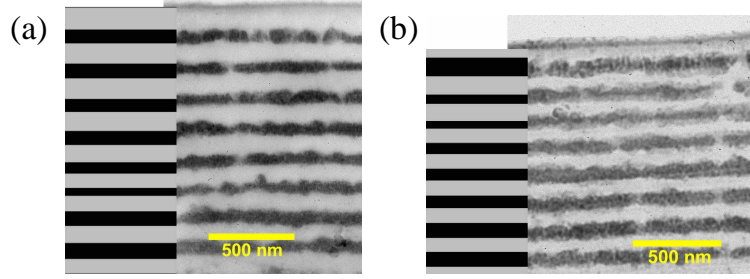


Fig. 3. (a) Image showing the multilayer structure responsible for the iridescent colour in the red region of the elytral wing of *C. raja* displaying both the system obtained from modelling (left) and actual cross-sectional TEM images (right). (b) Same image for the green region.

The *C. raja* elytral samples, like the majority of other natural samples exhibit surface curvature and some roughness. For this reason the light reflected when using either a white light source or a collimated laser light source is spread over a wide angle. To overcome this, we collected wavelength-dependent reflection spectra (i.e. when illuminating the sample with the white light source) by placing the spectrometer fibre centrally within the broad reflection from the sample. Next, a sequence of three lasers (with wavelengths 472nm, 543nm and 632nm) was used to illuminate the same wing region. Each laser was incident through an optical modulator to enable phase sensitive detection and improve signal quality. All of the reflected laser light was collected with a large area lens and focused onto a large area photodiode [9]. Absolute reflection intensities at several different angles and both linear polarisations were determined by ratioing this data against the intensity of the laser without the sample present.

Multilayer optical code, based upon recursive Fresnel formulae, was then used to model simultaneously the multiple experimental data sets. In the model, the reflection and transmission amplitude coefficients were calculated for each interface by using the Fresnel equations

$$\text{s-polarised:} \quad r_{ij} = \frac{k_{zj} - k_{zi}}{k_{zj} + k_{zi}} \quad (1)$$

$$\text{p-polarised:} \quad r_{ij} = \frac{\epsilon_j k_{zi} - \epsilon_i k_{zj}}{\epsilon_j k_{zi} + \epsilon_i k_{zj}} \quad (2)$$

where the subscripts ij refer to the media on either side of the interface, k_{zx} is the normal components of the wavevector of the incident light given by $k_{zx} = k_0 \sqrt{n_x^2 - n_1^2 \sin^2 \theta_1}$ where x is i or j , k_0 is the wavevector of the incident light, n_x is the refractive index of the medium in which the wavevector is being calculated, n_1 is the refractive index of the incident medium of the stack and θ_1 is the angle of incidence of the light on the stack (measured from the normal to the interface of the first interface). The entire structure's reflection coefficient can then be obtained by

$$r_{i,m} = \frac{r_{ij} + r_{i+1,m} e^{2i\beta_i}}{1 + r_{ij} r_{i+1,m} e^{2i\beta_i}} \quad (3)$$

starting at the exit medium where $\beta_i = k_{z,i}d_i$, d_i is the thickness of the i^{th} layer and the subscript m corresponds to the exit medium [5,10]. The reflection intensity can then be calculated by

$$R = rr^* \quad (4)$$

All experimental optical results obtained (i.e. 16 individual data sets comprising 2 polarisations at 4 angles of incidence from 2 sections) were fitted simultaneously to this model using an automated least squares reduction routine. All thicknesses were allowed to vary freely within bounds defined from observations made from a significant range of SEM and TEM images. The starting values for the refractive indices were defined using previously published values for the refractive indices only as an initial guide.

3. Results and discussion

C. raja, a buprestid beetle, has an iridescent green colour over the whole of its body except for a red stripe on each elytral wing (Fig. 1). It displays the normal iridescence associated with conventional multilayers; its peak reflected colour reduces in wavelength as the angle of incidence increases (the peak reflections from the green and red coloured epicuticle become distinctly blue at grazing angles). The underside of a detached elytron, despite exhibiting a low intensity iridescent green, is principally brown in colour since it is dominated by the optical absorption that is attributed to the presence of melanin [6].

The surface of the elytra, when examined under SEM (Fig. 2), exhibits 50 μm diameter concavities at irregular intervals. The biological function of these is largely unknown. Their optical effect is to increase the angular spread of reflected light, over and above that associated with the curvature of the elytra itself.

TEM images (Fig. 3) reveal the 1.5 μm thick multilayer structure at the surface of both the green and red elytral regions (the elytra themselves are approximately 1 mm thick). Despite some variation seen in the TEM sections, both green and red regions comprised approximately 17 layers (sometimes as few as 8 layers, but also as many as 22 layers). For the red region the individual dark contrasted layers were found to range in thickness from 52 nm to 92 nm and the light contrasted layers from 72 nm to 111 nm. For the layers from the green region of wing, the values measured were from 50 nm to 60 nm for the dark contrasted layers and 65 nm to 92 nm for the light contrasted layers. Figure 4 shows a series of optical data sets recorded on both green and red regions at four different angles of incidence using both linear polarisations. This figure also shows the theoretical data returned from fitting all 16 optical data sets simultaneously. This fitting process yielded values for the refractive indices (n) and absorption coefficients (k) for the two layer types present in the cuticle of this beetle. These values were $n=1.68$ and $k=0.03$ for the light contrasted layers and $n=1.55$ and $k=0.14$ for the dark contrasted layers. For this system, the dispersion of the layer materials was taken to be negligible. While this may not be a significant issue for the real component of the refractive index, it may be less insignificant for the imaginary component. Further work is in progress to verify this.

While we believe the method by which we derived the refractive indices of the *C. raja* beetle to be rigorous and our results accurate, it is appropriate here to consider the ways in which conventional values for beetle cuticle refractive indices were originally determined and have been variously used in literature over the last few decades.

One of the original and most heavily quoted sources for beetle cuticle refractive indices is by Bernard and Miller [11]. They used a Leitz interference microscope to conduct Mach-Zehnder interference microscopy to obtain an average refractive index of the layered system in the corneas of diptera. This average value was 1.548. By assuming that one of the layers consisted largely of water, they estimated that the refractive index of each alternate layer was 1.4 and 1.73 (for the more and less hydrated layers of cuticle respectively [11]). These values, although referring to dipteran cuticle, appear to have been applied generically to coleopteran cuticle by many recent workers.

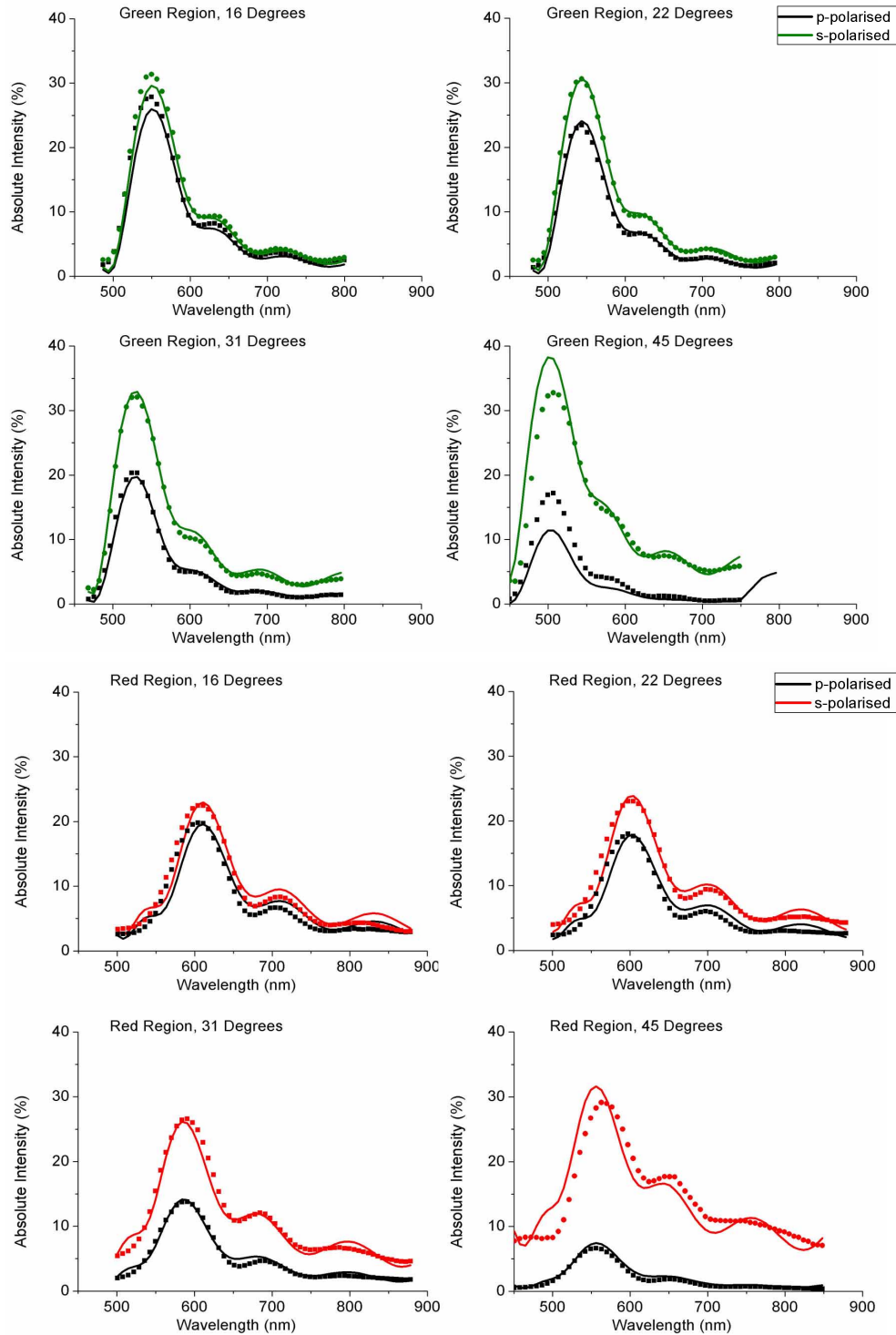


Fig. 4. Comparison of the percentage of absolute reflected intensity of the experimental data (points) and the accompanying simultaneous theoretical modelling (lines) for the 16 data sets covering two regions at four different angles of incidence for p- and s-polarised light

In another widely referenced source, Neville and Caveney [4] commented that Bernard and Miller's values were "so far outside the range of refractive indices ever measured for insect cuticles (1.5 and 1.6) that they should be carefully checked". However, Caveney later conducted experiments on the cuticle of an iridescent scarab beetle and determined the average refractive index of its elytral multilayer using a Baker interference microscope [12]. He showed that, in the presence of uric acid, estimates of values for beetle cuticle refractive index of around 1.7, were not unrealistic [13].

Mossakowski was one of the first workers to avoid the calculation of the average refractive index of a multilayer system [1] and subsequently used this average value to estimate the refractive indices of its individual layers. For the multilayer system in the iridescent beetle *C. vittata*, he obtained layer thicknesses from TEM images and optical data from a Zeiss microspectrophotometer. He then derived values for the refractive indices of the layers by comparing the wavelength of maximum reflectance in his experimental data to that obtained using the optical theory described by Land [14]. Although his approach was rigorous for its time, a lack of computing power and a limited number of optical data sets (he only used one angle of incidence and one polarisation) led to rather inconclusive results.

Schultz and Rankin returned to a measurement of the average refractive index of the cuticle of a tiger beetle [15]. They employed the Becke line test for this. They measured layer thicknesses from TEM images and used a well known theoretical treatment [16] to estimate values for the layers' individual refractive indices of 1.5 and 2.0 [15].

More recently, Parker echoed some of Neville's earlier words: he stated "there is a general lack of information on refractive indices of materials involved in beetle reflectors" [6]. In his paper, Parker uses the matrix method published by Macleod [17] to calculate the reflectance of a green and a gold beetle using the values given by Bernard and Miller [11] i.e. 1.4 for the low index layer and 1.73 for the high index layer. He also notes that an alternative value for the high index could also be 1.684 as used by Caveney [13], and by Neville [18] or 1.56 as suggested by Land [14].

Hariyama [2] investigated the multilayer system in the beetle *Plateumaris sericea*. He modelled the greyscale contrast of digitised TEM images of multilayer sections. This layer contrast exhibited a distinct oscillation that they inferred as representing a variation in refractive index between two specific values. These two values, 1.7 for the high index layers and 1.4 for the low index layers, were taken from Bernard and Miller [11] and were used to generate predicted reflectivities that were compared to data collected experimentally.

It is clear that many of the values for beetle refractive index, which have been used to characterise optically the individual layers in beetle reflector systems, are not based on completely rigorous measurement techniques: most are based on the measurement of an average of two different values followed by an estimate of each individual value. These estimated values have then been used by different workers for many years. The technique we describe in this paper, and which is very widely used in other fields of optical characterisation [19,20,21], is a significant improvement on the conventional methods used by earlier workers. By directly measuring absolute wavelength-dependent reflectivity, at many angles and under both linear polarisations (and not using an integrating sphere which corrupts the angle dependence of reflectivity data, and which has been shown to err away from absolute signal measurement), the technique provides a large number of sufficiently accurate optical data sets with which to significantly lift the degeneracy associated with optical fitting. In this way, the complex refractive indices of individual layers are accessible.

4. Conclusions

In this paper we described a complete method for producing wavelength-dependent optical reflectivity data using both linear polarisations and a range of angles of incidence. By using a combination of a broad band white light source and a range of laser sources we ensured that the reflection spectra recorded from the samples were calibrated absolutely, i.e. correspond to an accurate value between 0% and 100%. By simultaneously modelling 16 independent sets of these optical data using code based on recursive Fresnel formulae, the degeneracy of

solutions to the fitting was significantly overcome and complex refractive indices of the layers in the system's reflector were extracted.