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Analysing avian eggshell pigments with Raman spectroscopy

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ABSTRACT

Avian eggshells are variable in appearance, including coloration. Here, we demonstrate that Raman spectroscopy can provide accurate diagnostic information about major eggshell constituents, including the pigments biliverdin and protoporphyrin IX. Eggshells pigmented with biliverdin showed a series of pigment-diagnostic Raman peaks under 785 nm excitation. Eggshells pigmented with protoporphyrin IX showed strong emission under 1064 nm and 785 nm excitation, whereas resonance Raman spectra (351 nm excitation) showed a set of protoporphyrin IX informative peaks characteristic of protoporphyrin IX. As representative examples, we identified biliverdin in the olive green eggshells of elegant crested tinamous (*Eudromia elegans*) and in the blue eggshells of extinct upland moa (*Megalapteryx didinus*). This study encourages the wider use of Raman spectroscopy in pigment and coloration research and highlights the value of this technique for non-destructive analyses of museum eggshell specimens.

KEY WORDS: Biliverdin, Chemical analysis, Moa, Non-destructive, Tinamou, Protoporphyrin IX

INTRODUCTION

Studies of avian eggshell colours have revealed important linkages between surface appearance and nesting environment (Kilner, 2006). While digital photography and spectrophotometry are excellent methods for quantifying eggshell appearance, they do not provide robust chemical identification of the pigments that confer coloration (Cassey et al., 2012). Increasingly, studies are examining biliverdin-based eggshell pigmentation because of its putative function as an antioxidant in laying females and its potential to serve as a signal to males (Moreno and Osorno, 2003; see also Reynolds et al., 2009). Protoporphyrin eggshell pigmentation has also been of recent research interest for a variety of adaptive functions (Cassey et al., 2011). To date, the identification and quantification of pigments and their concentrations have required destructive methods (Kennedy and Vevers, 1976; Igic et al., 2010, 2012). This is particularly unfeasible for extensive studies of unique museum specimens, where the majority of eggshell samples are deposited and preserved (Hauber, 2014). However, Raman spectroscopy may provide a reliable, non-destructive method for describing avian eggshell pigments.

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Raman spectroscopy is a vibrational spectroscopic method that is widely used for characterising molecules (Lewis and Edwards, 2001). In addition to being non-destructive, Raman spectroscopy requires no specialised sample preparation and can analyse small regions of samples through microscope objectives (Thomas et al., 2014). A previously unexplored relevance of Raman spectroscopy to avian eggshell studies includes: (1) the chemical identification of pigments and other shell components; and (2) the potential quantification of pigment concentrations. Concentration information would require a careful calibration study in conjunction with a second analytical technique. Here, we focused on the first of these points: the detection and chemical identification of eggshell pigments. Previous analyses with destructive techniques have found that just two pigments, biliverdin and protoporphyrin IX, are largely responsible for the colours of all avian eggshells (Kennedy and Vevers, 1976). Chemically confirming the types of pigment in particular eggshells or mapping the distributions of pigments across an eggshell surface are routine research objectives for which a non-destructive technique would be ideal. Raman spectroscopy easily satisfies this analytical niche and requires relatively few methodological considerations for routine analyses of even rare and irreplaceable materials, including the eggs of extinct bird species (Igic et al., 2010).

RESULTS AND DISCUSSION

Wavelength selection

Raman spectroscopy analyses of blue–green or olive green eggshells with 785 or 1064 nm excitation produced excellent spectra with very low or flat baselines, minor levels of other noise, and distinct Raman peaks (supplementary material Table S1). Analyses of brown eggshells from domestic chicken (*Gallus domesticus*) and wild-type, beige and maculated eggshells from Japanese quail (*Coturnix japonica*) performed with 785 and 1064 nm excitation did not produce useful spectra. The brown eggshell spectra were dominated by intense emission that obscured Raman spectral information.

Optimum wavelength selection is a sample-dependent problem and is often dictated by the capacity for molecules to absorb and rapidly emit light (i.e. autofluoresce). Here, the brown eggshells contained at least one constituent that fluoresced under 785 or 1064 nm excitation. These eggshells were partially or solely pigmented with protoporphyrin IX (Table 1), which provides an analytical solution to the emission observed with 1064 and 785 nm excitation through resonance enhancement. When the laser wavelength is matched to a major absorption wavelength of the pigment, the Raman scattering has enhanced intensity and bands are evident (i.e. scattering is resonantly enhanced, producing a resonance Raman spectrum; see Smith and Dent, 2005). Protoporphyrin IX has an absorption maximum at 352 nm (e.g. Ding et al., 2011). Subsequent Raman spectroscopy measurements of the brown eggshells performed with 351 nm excitation produced useful resonance Raman spectra (Fig. 1).

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Table 1. Representative pigment concentrations of avian eggshells

Species	Eggshell colour	Pigment detected with Raman spectroscopy	Pigment concentration (nmol mm^{-2})
Emu *Dromaius novaehollandiae*	Black–green	Biliverdin	Biliverdin, 4.614
Elegant crested tinamou *Eudromia elegans*	Olive green	Biliverdin	Biliverdin, 0.756
Great tinamou *Tinamus major*	Blue–green	Biliverdin	Biliverdin, 0.107
Japanese quail *Coturnix japonica*	Brown, tan and white	Protoporphyrin IX	Protoporphyrin IX, 0.124
Chicken *Gallus gallus*	Brown	Protoporphyrin IX	Protoporphyrin IX, 0.117
Upland moa *Megalapteryx didinus*	Blue	Biliverdin	Protoporphyrin IX, 0.0000021*

N=1–3 fragments were averaged, following the methods of Igic et al. (2010). Pigment concentration data are from mass spectrometry or HPLC.
*Data are from Igic et al. (2010), originally reported as 0.21 pmol cm^{-2}.

In addition, the blue–green eggshell analysed with 351 nm excitation also produced useful spectra (Fig. 1). The choice of wavelength is thus an important experimental consideration for Raman analyses of eggshell pigments. Generally, 351 nm sources are less commonly used in Raman spectroscopy instruments than 785 nm sources.

Identifying biliverdin

Emu (*Dromaius novaehollandiae*), elegant crested tinamou (*Eudromia elegans*) and great tinamou (*Tinamus major*) eggshells were analysed destructively with mass spectrometry and non-destructively with Raman spectroscopy (Tables 1, 2): biliverdin was the only pigment detected in these eggshells with mass spectrometry. Different eggshell fragments were analysed with each technique. A mass spectrum (*m*/*z*) peak at 611.3 and the Raman spectral peaks described below were considered evidence for the presence of biliverdin. Raman spectra from blue–green and olive green eggshells (see Table 1 for colours) had seven spectral peaks that identified the presence of biliverdin: 1619, 1588, 1467, 1295, 1248, 1174 and 970 cm^{-1} (Table 2; supplementary material Table S1). Peak positions varied slightly between eggshell samples and reference synthetic compounds analysed elsewhere (Hu et al., 2000), probably because of differences in instrumentation, spectrometer

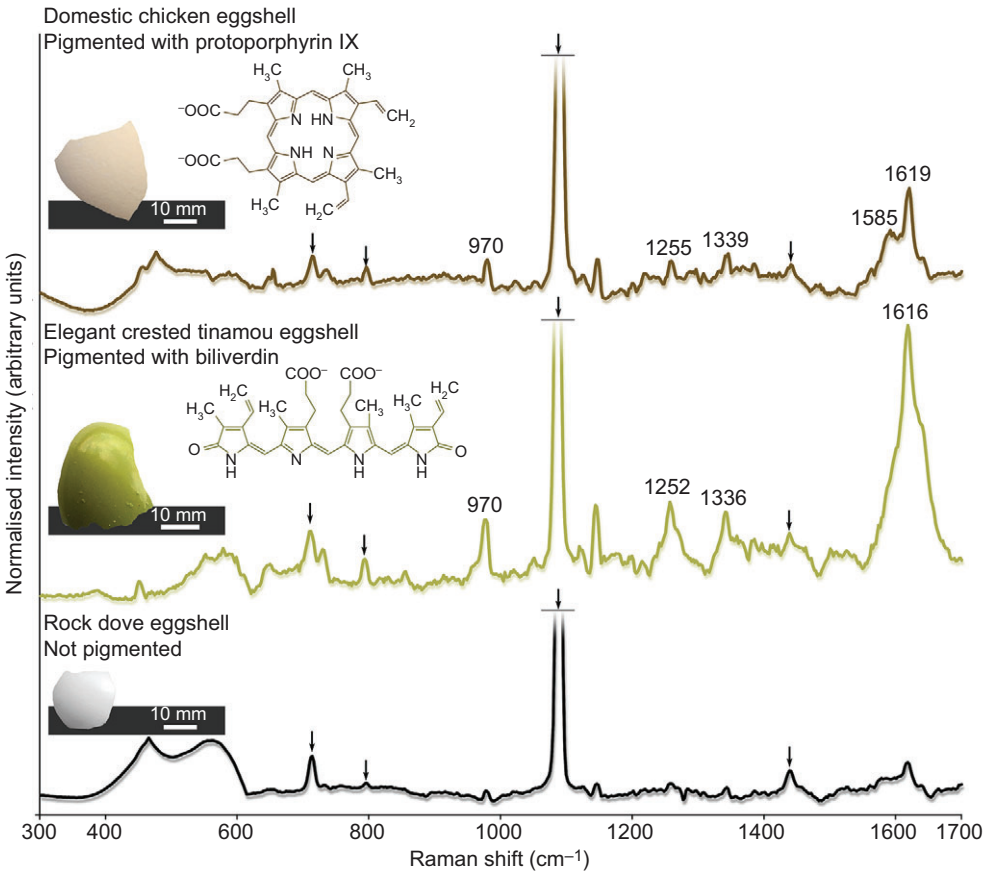

Fig. 1. Resonance Raman spectra from eggshells pigmented with protoporphyrin IX and with biliverdin, compared with a spectrum from an unpigmented eggshell. Spectra were collected using 351 nm excitation. Peaks attributed to pigments are labelled with wavenumber positions. Arrows identify peaks attributed to non-pigment constituents. Spectra have been normalised against the tallest peak in each spectrum (calcite, 1087 cm^{-1}).

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Table 2. Raman spectral peaks identify biliverdin and calcite in eggshells

Constituent	Peak position (cm^{-1})	Vibrational mode	References
Biliverdin	1619	Lactam stretching	Hu et al. (2000)
	1588	C=C within rings	Hu et al. (2000)
	1467	C–C deformation, probably between rings	Hu et al. (2000, 2001)
	1295	C–N stretching mixed with N–H bending	Hu et al. (2000); Aydin (2013)
	1248	Deformation of terminal rings	Hu et al. (2001)
	1174	C–H twisting	Hu et al. (2000)
	970	C–C stretching mixed with C–H rocking	Hu et al. (2000)
Calcite	1748	CO_3^{2-} mixed symmetric and asymmetric stretching	Parker et al. (2010); De La Pierre et al. (2014)
	1436	CO_3^{2-} asymmetric stretching	Parker et al. (2010); De La Pierre et al. (2014)
	1086	CO_3^{2-} symmetric stretching	Parker et al. (2010); De La Pierre et al. (2014)
	712	CO_3^{2-} symmetric deformation	Parker et al. (2010); De La Pierre et al. (2014)
	281	Phonon mode	Parker et al. (2010); De La Pierre et al. (2014)
Protoporphyrin IX	1619	C=C stretching within ethylene side chain	Hu et al. (1996)
	1585	C=C stretching	Hu et al. (1996)
	1339	C=C stretching	Hu et al. (1996)
	1255	C–C deformation	After Hu et al. (2001)
	970	C–C stretching mixed with C–H rocking	After Hu et al. (2000)

calibration or chemical environments (cf. variation in PO_4^{3-} peak positions; Thomas et al., 2011). From previous Raman studies of synthetic biliverdin, we assigned vibrational modes to the seven biliverdin peaks observed in eggshell spectra (Fig. 2, Table 2).

Identifying protoporphyrin IX

Domestic chicken, Japanese quail and great tinamou eggshells were analysed with mass spectrometry and resonance Raman spectroscopy (Tables 1, 2). Different eggshell fragments were analysed with each technique. A mass spectrum (*m*/*z*) peak at 591.3 and the Raman spectral peaks described below were considered evidence for protoporphyrin IX. Protoporphyrin IX was the principal pigment detected in the brown chicken eggshell with mass spectrometry, and both protoporphyrin IX and biliverdin were detected in the Japanese quail eggshell (concentrations varied between samples: Table 1). Raman spectra collected from the brown chicken eggshell showed protoporphyrin IX informative peaks at 1619, 1585 (broad), 1339, 1255 and 970 cm^{-1} (supplementary material Table S1; Fig. 1). Spectra from the quail eggshell were similar. Note that a resonance Raman spectrum from a great tinamou eggshell collected with 351 nm excitation had peaks at 1616, 1336, 1252 and 970 cm^{-1} (in contrast with Table 2), which are characteristic of biliverdin. Biliverdin and protoporphyrin IX are structurally similar molecules and have superficially similar Raman spectra (Fig. 1). Pigment composition may be difficult to determine from visual inspection of Raman spectra if biliverdin and protoporphyrin are co-deposited in the same eggshell fragment. Hence, Raman studies that analyse eggshells with 351 nm should use multivariate statistical methods to classify eggshell pigments (e.g. partial least squares discriminant analysis: France et al., 2014) as both pigments may be present.

Identifying other eggshell constituents

Unpigmented ostrich *Struthio camelus* and domesticated rock pigeon *Columba livia* eggshells were analysed with 351 and 785 nm Raman spectroscopy. Spectra from the external surface of the ostrich eggshell contained only peaks attributed to calcite (Parker et al., 2010) (Table 1; supplementary material Table S1). Spectra from the internal surfaces of both the rock pigeon and chicken eggshells provided evidence of calcite and a matrix protein (e.g. collagen or albumin; Frushour and Koenig, 1975). Five spectral peaks attributed to protein were well resolved in analyses of the chicken eggshell (internal), and 17 protein-attributed peaks could be identified in spectra from the rock pigeon eggshell (internal).

Case study 1: colour variation between tinamou species

Raman spectra and mass spectrometry identified biliverdin as the principal pigment in both the blue–green and olive green tinamou eggshells (Table 1, Fig. 2). Biliverdin is well established as the pigment that produces the blue–green coloration in eggshells (Kennedy and Vevers, 1976), but strictly green pigments in avian tissues have proven challenging to identify (Dyck, 1992). Here, we confidently demonstrate that biliverdin can be a green pigment in avian tissues, and show that the dramatically different eggshell colours of olive green in the elegant crested tinamou and blue–green in the great tinamou are produced by the same core pigment (Figs 1, 2). Conjugation of biliverdin with proteins may modify the absorption spectrum and perceived colour.

Case study 2: pigment detection in extinct moa eggshells

The pigment in two blue eggshell fragments from upland moa *Megalapteryx didinus* had previously been identified as biliverdin using destructive techniques (Igic et al., 2010). Here, we also identified biliverdin in upland moa eggshell fragments with non-destructive Raman spectroscopy (Fig. 2). Specimen AV12393, AV13889 and 2012.44.2 had biliverdin-informative peaks around 1629, 1615, 1586, 1470, 1298, 1250, 1173 and 970 cm^{-1} (supplementary material Table S1). Specimen AV9326 was visually lighter than the other moa eggshell specimens. Raman spectra from specimen AV9326 were visually similar to spectra from the other moa fragments, albeit with weaker biliverdin peaks and weak features at 1586, 1470, 1174 and 972 cm^{-1}. Gathering chemical evidence is a valuable step in identifying the paleoecological significance of moa eggshell pigmentation.

Conclusions and applications

Raman spectroscopy can provide qualitative information about the chemical constituents that collectively are responsible for avian eggshell composition and appearance. This non-destructive method will allow studies on rare or irreplaceable specimens such as museum collections and extinct taxa. Raman spectroscopy could help us to understand the adaptive significance of eggshell pigments in particular bird groups (Moreno and Osorno, 2003; Gosler et al., 2005; Ishikawa et al., 2010; Martínez-Padilla et al., 2010; Fargallo et al., 2014).

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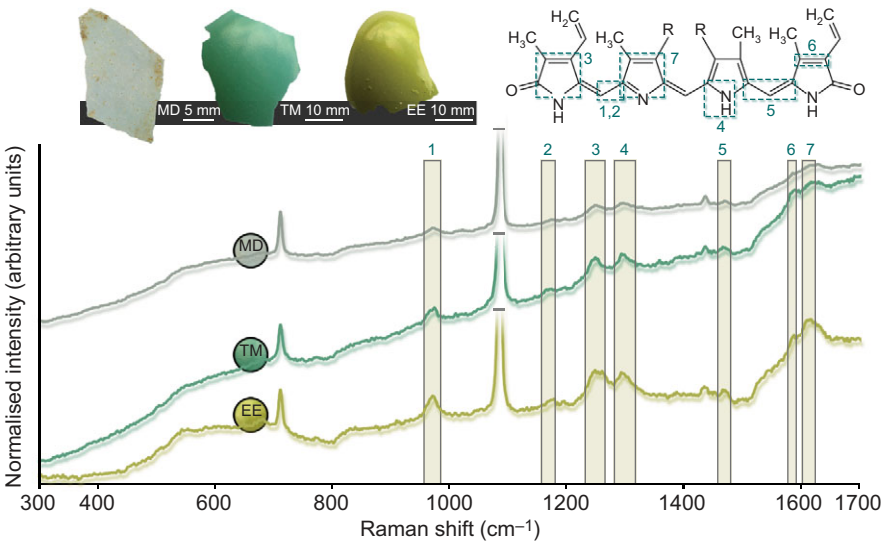

Fig. 2. Raman spectra from eggshells showing peaks attributed to biliverdin. Spectra were collected with 785 nm excitation. Eggshells are from extinct upland moa (*Megalapteryx didinus*, MD), extant elegant crested tinamou (*Eudromia elegans*, EE) and great tinamou (*Tinamus major*, TM). Peak assignments after Hu et al. (2000, 2001) and Aydin (2013). Spectra have been normalised against the tallest peak in each spectrum (calcite, 1087 cm^{-1}). Biliverdin structure is shown top right.

MATERIALS AND METHODS

Raman spectroscopy: samples, instrumentation and analysis

Eggshell fragments from six modern species were analysed with Raman spectroscopy: emu [*Dromaius novaehollandiae* (Latham 1790); one fragment], ostrich (*Struthio camelus* Linnaeus 1758; one fragment), elegant crested tinamou [*Eudromia elegans* (Saint-Hilaire 1832); three fragments], great tinamou [*Tinamus major* (Gmelin 1789); three fragments], domestic chicken (*Gallus gallus* Linnaeus 1758; two fragments), Japanese quail (*Coturnix japonica* Temminck and Schlegel 1849; one fragment) and rock pigeon (*Columba livia* Gmelin 1789; one fragment). Four fragments from extinct upland moa *Megalapteryx didinus* (Owen 1883) were also analysed: moa eggshell fragments are probably more than 1000 years old (inferred from Worthy, 1997). Eggshell fragments were acquired by D.B.T. from aviculturists in New Zealand, or by M.E.H. from a commercial breeder and the Bronx Zoo (NY, USA) and were cleaned in distilled water and sent to D.B.T. in New Zealand following the Ornamental Products of Animal Origin Import Health Standard (Ministry of Primary Industries, New Zealand). Upland moa eggshell fragments were borrowed from Canterbury Museum (Christchurch, New Zealand; Loan OL2014.9, specimens AV9326, AV12393, AV16978 and 2012.44.2).

Raman spectra were collected using a Fourier-transform (FT) Raman spectrometer, a Raman microscope and a resonance Raman instrument. FT Raman analyses were performed using an Equinox 55 interferometer bench with a FRA 106/S FT-Raman module (Bruker Optik, Ettlingen, Germany), a Compass 1064-500 N laser (1064 nm Nd:YAG; Coherent Inc., Santa Clara, CA, USA) and a liquid nitrogen-cooled D418-T Ge detector. Laser power was set to 120 mW and analyses were performed with a 'downward-looking' objective (300 µm diameter spot). Data were recorded from 0 to 3500 cm^{-1} at 4 cm^{-1} resolution, with up to 256 scans.

Raman analyses were also performed using a Senterra Raman microscope (Bruker Optik, Ettlingen, Germany), with a 785 nm laser set to 100 mW. Data were collected across three spectral windows that together spanned 65–3500 cm^{-1} at a spectral resolution of 3–5 cm^{-1}, and each analysis was the co-addition of up to 20 spectra accumulated at up to 24 s exposure each. Analyses were performed using a 20× objective that had a numerical aperture of 0.4 (Olympus Corporation, Shinjuku-ku, Tokyo, Japan), giving an analytical spot size of approximately 2 µm diameter. Data collection was controlled by OPUS 6.5 software (Bruker Optik).

Resonance Raman scattering (351 nm) was generated with a continuous-wave krypton-ion laser (Innova I-302, Coherent Inc.). The incident beam and the collection lens were in a back-scattering arrangement. Scattered light was collected and collimated, and then focused through a notch filter (Kaiser Optical Systems, Ann Arbor, MI, USA) and a quartz wedge (Spex, HORIBA Scientific, Kyoto, Japan) onto the 100 µm entrance slit of a spectrograph (Acton Research SpectraPro 500i, Princeton Instruments, Acton, MI, USA). This was dispersed by a 1200 grooves mm^{-1} ruled diffraction grating (blaze wavelength 500 nm) and detected by a liquid nitrogen-cooled back-illuminated Spec-10:100B CCD controlled by an ST-133 controller and WinSpec/32 version 2.5.8.1 software (Roper Scientific, Princeton Instruments, Trenton, NJ, USA).

Specialised sample preparation was not required. We used fragments ranging from several millimetres to several centimetres in length. Fragments were placed on a motorised stage (FT and microscope instruments) or fixed in the path of the laser (resonance Raman). For the FT and microscope instruments, the region chosen for analysis was positioned into the field of view (as monitored from live video). Data from all three systems were analysed in OPUS 5.5. Peaks in each spectrum were isolated and subsequently modelled using a 50:50 Gaussian:Lorentzian curve (a baseline curve was modelled in parallel to remove the effect of fluorescence from the curve shape). Peak positions and intensities were extracted from each modelled curve.

Mass spectrometry: samples, instrumentation and data analysis

Mass spectrometry data were compared with observations from Raman spectroscopy. Sulphuric acid extraction of eggshell pigments and subsequent mass spectrometry were performed on eggshell fragments from domestic chicken, emu, elegant crested tinamou, great tinamou and Japanese quail, following protocols described in Igic et al. (2010). Eggshell samples were acquired as above, but were not the same eggshells used for Raman spectroscopy.

Biliverdin and protoporphyrin were extracted from eggshells using acidified methanol, which converted the pigments to biliverdin dimethylester and protoporphyrin dimethylester, respectively. The methylesterified pigments were then extracted into chloroform, and quantified by UV-Vis absorption spectroscopy. Additional mass spectrometric analyses on a micrOTOF-Q2 ESI-MS instrument (Bruker Daltonik, Bremen, Germany) were performed as further confirmation of the presence of each pigment. Ions at *m/z* 611.3 ($C_{35}H_{39}N_4O_6$) and 591.3 ($C_{36}H_{39}N_4O_4$) were used to fingerprint biliverdin dimethyl ester and protoporphyrin dimethyl ester, respectively.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

D.B.T. collected specimens and data, and wrote the manuscript. M.E.H. collected specimens, developed the methodology and wrote the manuscript. D.H. wrote the manuscript. G.I.N.W. collected data and wrote the manuscript. S.F. collected data and wrote the manuscript. K.C.G. collected data and wrote the manuscript.

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Supplementary material
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