Identifying Initiation and Aging of Hens During the Laying Period by Raman Analysis of Beaks

Shujie Wang¹, Mohan Liu¹, Da Tian², Mu Su², Qiao Li¹, Zhen Li² and Zhenlei Zhou¹

¹College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, Jiangsu 210095, China
²College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing, Jiangsu 210095, China

Running title: Raman Analysis of Hens’ Beaks

Correspondence:
Zhen Li, College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing, Jiangsu, 210095, China. (E-mail: lizhen@njau.edu.cn)
Zhenlei Zhou, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, Jiangsu, 210095, China. (E-mail: zhouzl@njau.edu.cn)
Raman spectroscopy has been widely applied in the analysis of biological tissues. In this study, beak cuticle was studied to investigate its compositional and secondary structural changes during the laying period and aging of laying hens. The analysis revealed markedly increased contents of amide I and amino acids (phenylalanine and tyrosine) within the beak during the intense laying period from 17 to 20 weeks. In addition, α-helical protein was also gradually synthesized in this period. The relative area ratio of 1003/1448 cm$^{-1}$ (assigned to the vibrations of phenylalanine and organic C-H respectively) was confirmed as an excellent indicator for estimating the start of the laying period. This ratio increased from 0.36 to 0.42 from 17 to 20 weeks. The Raman peak at 1156 cm$^{-1}$ was assigned to carotenoids in the beak. The intensities of the 1156 cm$^{-1}$ peak significantly decreased during aging. The area ratio of 1156/1448 cm$^{-1}$ was successfully applied to estimate ages (still within the laying period) of laying hens. This study shows the potential of Raman spectroscopy to quantify ages and laying period of birds.

**Keywords:** age identification, beak, cuticle, laying hens, laying period
Introduction

The beak is a unique feeding organ of all birds. The bone parts of the beak consist of premaxilla and mandible, and the outer surface of the bone is usually covered with cuticle (Speer and Powers, 2016). This cuticle is very thick compared with common epidermis. The major component of the cuticle is keratin, which is a common fibrous protein in hair, feather, and skin of vertebrates and invertebrates. Cells of the cuticle contain free calcium phosphate and orientated crystals of hydroxyapatite (King and McLelland, 1984; Carril et al., 2015). The combination of mineral and keratin in beak is the basis of its stiffness (in contrast to soft tissues) and the subsequent functions.

Accurate determination of the status of the laying period and age is an essential prerequisite in poultry science. However, it is usually difficult to establish an animals’ rate of growth and identify their maturity. Eye lens weight is currently one of the typically accepted methods for age identification in mammals due to the steadily increasing size and mass of the lens during aging (Morris, 1972). This method has been well reviewed and accepted since the 1960s (Friend, 1968). However, there are still limited studies on age determination for birds.

Bone metabolism of layers appears to change during aging and in the laying period (Fleming et al., 1998; Whitehead, 2004; Li et al., 2016). Therefore, the beak might also display corresponding characteristic changes during aging and the laying period for birds. The technique of Raman spectroscopy is sensitive to chemical differences in both secondary structure and amino acid composition of proteins (Church et al., 1997; Kuzuhara et al., 2007). It has been applied in multiple analyses of bone and cuticle of animals (Williams et al., 1994; Li and Pasteris, 2014a; Li et al.,...
Previous biological applications of Raman spectroscopy mainly focused on bone and tooth materials, whose hardness is primarily due to abundant mineral, i.e., bioapatite (Li and Pasteris, 2014b). Raman analysis of chicken bone was able to roughly identify the laying period and aging stages (Li et al., 2016). However, whether the same determinations can be made for the beak, whose hardness is due to keratin and minerals, is unknown. Considering that the beak is easily obtained, its examination could be instructive for the future application of Raman analysis on biological tissues of birds in vivo (Caspers et al., 1998; Caspers et al., 2001; Evans et al., 2005).

ISA Brown layer hens are one of the most cultured layer strains globally, because of advantages that include high egg production, long peak of egg production, and high feed conversion. Environmental factors greatly influence the sexual maturity of laying hens, especially the availability of light (prolonged illumination can promote sexual maturity in laying hens). To achieve the maximum egg production performance, hens are expected to simultaneously reach sexual maturity and body maturity, and enter the high production period as soon as possible. ISA Brown layer hens reach sexual maturity at approximately 18 weeks of age and peak production occurs at approximately 23 weeks of age. Therefore, it is critical to accurately identify the state of sexual maturity of laying hens in the period from 17 to 23 weeks. Delayed or early maturing laying hens could enter the high production period on time through the adjustment of the period of illumination. Additionally, the high production period lasts up to 20 weeks and the laying rates of ISA Brown layer hens gradually decrease after 50 weeks. Thus, this breed is ideal for studies designed to identify laying period and age.

The aim of this study is to establish a convenient and rapid method to identify the
laying period and age of ISA Brown layers. The changes of secondary structure and amino acid composition of proteins comprising the beak were analyzed by Raman spectroscopy. Both the position and width of representative peaks, in addition to their area ratios, were investigated.

**Materials and Methods**

*Preparation of the beaks of laying hens*

A total of 60 healthy laying hens (ISA Brown layer) were raised for this study. The hens were divided into six groups according to their age (17, 20, 23, 35, 50, and 87 weeks). The hens in each group were similar in weight (see deviations in Table 1). All selected 17 week old hens had not started egg laying, while all 20 week old hens had. The hens were raised in colony layer cages and fed a diet contains 11.55 MJ/kg of metabolizable energy, i.e., 16.50 wt.% crude protein, 3.63 wt.% Calcium, 0.40 wt.% Phosphorus (total P), 0.35 wt.% methionine, and 0.95 (0.85) wt.% lysine. All the hens were healthy and no hormone was applied.

The upper beaks were sawed off completely at the dorsal base (in front of the nostrils) and cleaned of the palate and extraneous soft tissue in the inner surface using a scalpel (the outer surface was not similarly cleaned). The upper beaks were carefully washed with deionized water and air dried for Raman analyses. No additional chemicals were used during the beak preparation.

*Raman microprobe spectroscopy*

Raman microprobe spectroscopy (model DXR532; Thermo Fisher Scientific, Madison, WI, USA) to observe vibrational modes was performed on the outer surface of the beaks using an MSPlan 20× objective (Olympus, Tokyo, Japan). The spectral
region of 600-1800 cm\(^{-1}\) was recorded using a 532 nm laser. The laser power was 10 mW with a 25 µm slit aperture and 30×5 s scans. The peak position was calibrated using a silicon wafer (520.5 cm\(^{-1}\)). As shown in Fig. 1, Raman analysis for each sample was performed on two different regions (with three randomly selected spots for each region, 20 regions total for each group). There was no significant variation around each spot.

All samples were obtained in accordance with relevant guidelines and regulations. All experimental protocols were approved by the College of Veterinary Medicine and Ethical Committee at Nanjing Agricultural University.

**Statistical analysis**

The differences among the groups were determined by one-way analysis of variance (SPSS 22, ANOVA, Tukey). The results are expressed as mean ± standard deviation. The level of statistical significance was set at P<0.05.

**Results**

**Assignments of representative Raman peaks**

Raman spectroscopy was performed on the outer surface of the beaks of layers. The Raman peaks ranged from 600 to 1800 cm\(^{-1}\) and reflected the representative characteristics of the cuticle. Within this limit, identifiable vibrations could be assigned to amino acids (phenylalanine and tyrosine) (Barry et al., 1993; Williams et al., 1994; Iconomidou et al., 2001), amide I and amide III (Barry et al., 1993; Kuzuhara et al., 2007; Kuzuhara, 2013), and carotenoid (C–C and C=C stretching) (Oliveira et al., 2010; Schulz et al., 2010; Mendes-Pinto et al., 2012). In addition, some alkane
vibrations were identified, and included the C–H deformation and C–C skeletal stretching vibration of the α-helix (Williams et al., 1994; Kuzuhara, 2005a).

Raman spectra in the region of 600 to 1800 cm\(^{-1}\) of beak samples from 17 to 23 week old layers are presented in Fig. 2. In the amide I region (1600-1700 cm\(^{-1}\)), the Raman spectrum of beaks exhibited a well-defined peak at 1667 cm\(^{-1}\), which was assigned to the β-sheet and/or random coil forms. The absence of the peak at 1650 cm\(^{-1}\) suggested that the α-helical structure was not favored or its level remained below the detection line. A peak at 1242 cm\(^{-1}\) was assigned to the β-sheet within the amide III range (1230-1320 cm\(^{-1}\)). Moreover, the peak observed at 1448 cm\(^{-1}\) was attributed to the C–H bending. This band displayed no significant change of intensity and was suitable for normalization of peak intensity. The two intense peaks at 1156 cm\(^{-1}\) and 1515 cm\(^{-1}\) are the main characteristic carotenoid peaks (Raman spectra of pure carotenoid standard also has two strong bands at 1156 cm\(^{-1}\) and 1515 cm\(^{-1}\)) (Schulz et al., 2010), and their intensities evidently decreased during aging from 17 to 23 weeks.

The peak at 1003 cm\(^{-1}\) was ascribed to phenylalanine (Phe), which is usually intense and relatively isolated from other peaks. In addition, the skeletal C–C stretch band located at 935 cm\(^{-1}\) was only observed for beaks at 20 and 23 weeks of age (Fig. 2 and 3B), and was assigned to the α-helical backbone. The peak at 852 cm\(^{-1}\) was assigned to tyrosine (Tyr). The intensities of the 852 cm\(^{-1}\) and 935 cm\(^{-1}\) peaks at 17 weeks were significantly lower than those at 20 and 23 weeks (see Fig. 2). However, these two peaks were usually weak compared with the 1003 cm\(^{-1}\) peak.

Estimating laying period by Raman spectroscopy

ISA Brown layers reach sexual maturity between 17 and 20 weeks of age. The changes in beak cuticle during this time are critical to the identification of their following laying period. The intensity and area ratio of Raman peaks can be applied to
semi-quantify the relative contents of the corresponding compounds (Morris and Mandair, 2011; Li and Pasteris, 2014a). Additionally, the normalization (denominator for the ratio) is the precondition for accurate estimation. Normalization based on the C–H band (1448 cm\(^{-1}\)) was first selected as it is isolated (with no interference from neighboring peaks) and apparent with high intensity.

The ratios of the 852/1448 cm\(^{-1}\) and 1667/1448 cm\(^{-1}\) peaks were both significantly increased from 17 to 20 weeks and remained stable from 20 to 23 weeks. Both ratios indicated that the contents of Tyr and amide I increased when layers reached the laying period. However, the 852 cm\(^{-1}\) and 1667 cm\(^{-1}\) peaks were normally overlapped by adjacent peaks and could not be applied to accurately estimate the laying period. The characteristic peak of the \(\alpha\)-helical structure at 935 cm\(^{-1}\) was observed at 20 and 23 weeks, suggesting that some protein with \(\alpha\)-helical structure may be produced at the beginning of the laying period. The change of secondary structure of proteins was an indicator of the laying period. However, the intensity of 935 cm\(^{-1}\) was too weak to be further analyzed when applied it to calculation.

The ratios of the 1003/1448 cm\(^{-1}\) peaks of 17, 20 and 23 weeks were 0.36, 0.42, and 0.40, respectively (Table 2). The ratios significantly increased from 17 to 20 weeks, but were relatively stable from 20 to 23 weeks. In addition, the band attributed to Phe (1003 cm\(^{-1}\) peak, Fig. 2 and 3A) was relatively isolated. Thus, the selection of a relative peak ratio of 1003/1448 cm\(^{-1}\) was reliable for quantifying the changes when layers reached the laying period.

**Estimating age by Raman spectroscopy**

The 935 cm\(^{-1}\) peak became less obvious beginning at 35 weeks (Fig. 4). The ratios of the 1667/1448 cm\(^{-1}\) peaks at 50 weeks were decreased from 1.49 at 35 weeks to 1.36 (Table 2). However, the changes of Raman spectra from 35 to 87 weeks were not
significant and so could not be applied to accurately identify the age of laying hens.

The intensities of Raman spectra of beaks remained generally stable from 35 to 87 weeks (Fig. 4), whereas the changes of Raman peaks during aging between 17 and 23 weeks were remarkable, especially for the 1156 cm\(^{-1}\) and 1515 cm\(^{-1}\) peaks. The measurement of 1156 cm\(^{-1}\) peak area was more accurate, since the peak width of 1156 cm\(^{-1}\) peak was narrower than that of 1515 cm\(^{-1}\). The area ratios of 1156/1448 cm\(^{-1}\) peaks of 17, 20, and 23 weeks were 0.98, 0.53, and 0.15, respectively (Table 2), which markedly decreased from 17 to 23 weeks. The decrease was continuous (Fig. 3D and Table 2) and the ratio was significantly influenced by aging. Therefore, the area ratio of 1156/1448 cm\(^{-1}\) peaks (on the beak) was reasonable to estimate ages from 17 to 23 weeks.

**Discussion**

The cuticle is common in animal hair, feather, and skin. However, previous studies had been mostly focused on the cuticle of human and animal hair (Hsu et al., 1976; Shishoo and Lundell, 1976; Pande, 1994; Church et al., 1998). Although the cuticle of the beak covers the jawbone of birds, it has not been extensively studied.

Raman spectroscopy has been used in previous studies of keratin fibers (Frushour and Koenig, 1975). The intensity of the Raman peaks (by area) indicates the contents of the corresponding compounds. The high sensitivity of Raman spectroscopy permits the identification of even subtle changes of carbonate substitution for phosphate in bioapatite (Li and Pasteris, 2014a). Normalization based on a specific Raman peak is the precondition for accurate estimation. In previous studies, normalization of Raman spectra of keratin fibers was often carried out based on the C–H band at 1448 cm\(^{-1}\) and
amino I band at 1667 cm$^{-1}$ (Jones et al., 1998; Kuzuhara and Hori, 2003; Kuzuhara, 2005b). In this study, normalization based on the C–H band (1448 cm$^{-1}$) was selected, as the peak is isolated from other peaks and has relatively high intensity.

The area ratio of 1003/1448 cm$^{-1}$ was successful in estimating the start of the laying period of laying hens. The content of Phe and Tyr significantly increased when layers reached the laying period (from 17 to 20 weeks). The results may be due to an increase in the keratin content of the cuticle. In such situations, the keratin filaments probably became more tightly packed to improve the stability of cuticle. Secondly, it is also possible that the Phe and Tyr contents of the cuticle protein were increased during this period. It has been shown that aromatic groups of amino acids are able to stabilize tertiary and local structures of protein (Kemmink et al., 1993; Shimohigashi et al., 1999; Toth et al., 2001). Therefore, the structure of the beak cuticle may become more compact and stable when layers enter their laying period.

The secondary structure of proteins obtained by Raman spectroscopy indicated that the beak cuticle is mainly composed of $\beta$-sheet and/or random coil proteins, which are characteristic of the disordered conformation in proteins. This is consistent with previous findings in cuticle cells were demonstrated to have a high proportion of cystine, proline, serine, and valine residues (Bradbury and Ley, 1972; Bradbury et al., 1973). These are generally considered to be non-helical-forming amino acid residues. It is worth noting that the characteristic peak of the $\alpha$-helical structure at 935 cm$^{-1}$ was observed in the laying period. This suggests that protein with $\alpha$-helical structure may be formed during sexual maturity.

The area ratios of 1156/1448 cm$^{-1}$ were successfully applied in estimating ages of layers (between 17-23 weeks). The decline of area ratios of 1156/1448 cm$^{-1}$ was caused by the reduction of carotenoids in beak during aging. Carotenoids have
important functions in many physiological processes, and are used by many bird species as integumentary colorants (Blount and Surai, 2003; Mendes-Pinto et al., 2012). The carotenoid content of beak in some bird species will change, which can help the birds attract mates (Blount and Surai, 2003; Alonso-Alvarez et al., 2012). In addition, carotenoids are present in egg yolk to protect the embryo from oxidative stress associated with high anabolic turnover (Surai, 2002). Therefore, layers may allocate fewer carotenoids to the body surface, since birds cannot synthesize carotenoids de novo (Mendes-Pinto et al., 2012).

Beaks with abundant keratin did display significant changes during the laying period. In addition, Raman spectroscopy analysis of the beak is a low-cost, quick, and nondestructive technique for identification of the laying period in vivo. The combination of the 1003/1448 cm\(^{-1}\) (to identify the start of the laying period) and 1156/1448 cm\(^{-1}\) (to identity the aging stage within the laying period) ratios permit a better understanding of the laying period of hens. Identification of laying period is very important in the poultry industry, which can improve the efficiency of breeding performance. For example, the accurate identification of sexual maturity of ISA Brown layers from 17 to 23 weeks can allow the birds to enter the high production period on time and maintain a high production period for a longer time. These advantages of nondestructive examination make it reasonable to apply the Raman technique on other animals with beaks, e.g., birds. In particular, the identification of age and laying period can effectively improve the breeding of endangered birds.

In conclusion, the study of the cuticle layer of the beak indicates that the relative peak intensity of Phe (1003/1448 cm\(^{-1}\)) is suitable for quantifying the changes when layers reach the laying period. In addition, the relative peak intensity of 1156 cm\(^{-1}\) is suitable as an evaluation scale for age identification. This study provides a technique
that can be used to determine the laying period and age. Since feathers and scales of the cuticle layer also contain keratin, it allows establishing a complete age identification method combining the analysis of feathers and scales.

Acknowledgements

This work was supported by the National Key R&D Program of China (Project No. 2017YFD0502200), China Postdoctoral Science Foundation (No. 2017M610330), and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

References

Alonso-Alvarez C, Pérez-Rodriguez L, Ferrero ME and De-Blas EG. Adjustment of female reproductive investment according to male carotenoid-based ornamentation in a gallinaceus bird. Behavioral Ecology & Sociobiology, 66: 731-742. 2012.

Barry BW, Williams AC and Edwards HGM. Fourier transform Raman and IR spectra of snake skin. Spectrochimica Acta Part A Molecular Spectroscopy, 49: 801-807. 1993.

Blount JD and Surai PF. Carotenoid modulation of immune function and sexual attractiveness in zebra finches. Science, 300: 125-127. 2003.

Bradbury J and Ley K. The Chemical Composition of Wool XI. Separation and Analysis of Exocuticle and Endocuticle. Australian Journal of Biological Sciences, 25: 1235-1248. 1972.
Bradbury J, Anfinsen C, Edsall J and Richards F. Advances in protein chemistry. Academic Press, 27: 111. 1973.

Carril J, Degrange FJ and Tambussi CP. Jaw myology and bite force of the monk parakeet (Aves, Psittaciformes). Journal of Anatomy, 227: 34-44. 2015.

Caspers PJ, Lucassen GW, Wolthuis R, Bruining HA and Puppels GJ. In vitro and in vivo Raman spectroscopy of human skin. Biospectroscopy, 4: S31-S40. 1998.

Caspers PJ, Bruining HA, Puppels GJ, Lucassen GW and Carter EA. In vivo confocal Raman microspectroscopy of the skin: noninvasive determination of molecular concentration profiles. Journal of Investigative Dermatology, 116: 434-442. 2001.

Church JS, Corino GL and Woodhead AL. The analysis of merino wool cuticle and cortical cells by Fourier transform Raman spectroscopy. Biopolymers, 42: 7-17. 1997.

Church JS, Corino GL and Woodhead AL. The effects of stretching on wool fibres as monitored by FT-Raman spectroscopy. Journal of Molecular Structure, 440: 15-23. 1998.

Evans CL, Potma EO, Puoris’haag M, Côté D, Lin CP and Xie XS. Chemical imaging of tissue in vivo with video-rate coherent anti-Stokes Raman scattering microscopy. Proceedings of the National Academy of Sciences of the United States of America, 102: 16807-16812. 2005.

Fleming RH, McCormack HA and Whitehead CC. Bone structure and strength at different ages in laying hens and effects of dietary particulate limestone, vitamin K and ascorbic acid. British Poultry Science, 39: 434-440. 1998.

Friend M. 1968. The lens technique. Transactions of the North American Wildlife and Natural Resources Conference 33, 279-298. 1968.
Frushour B and Koenig J. Raman spectroscopy of proteins. Advances in Infrared and Raman Spectroscopy, 1: 35-97. 1975.

Hsu S, Moore WH and Krimm S. Vibrational spectrum of the unordered polypeptide chain: a Raman study of feather keratin. Biopolymers, 15: 1513-1528. 1976.

Iconomidou VA, Chryssikos GD, Gionis V, Willis JH and Hamodrakas SJ. "Soft"-cuticle protein secondary structure as revealed by FT-Raman, ATR FT-IR and CD spectroscopy. Insect Biochemistry & Molecular Biology, 31: 877-885. 2001.

Jones DC, Carr CM, Cooke WD and Lewis DM. Investigating the photo-oxidation of wool using FT-Raman and FT-IR spectroscopies. Textile Research Journal Publication of Textile Research Institute Inc & the Textile Foundation, 68: 739-748. 1998.

Kemmink J, Mierlo CPMV, Scheek RM and Creighton TE. Local Structure Due to an Aromatic-Amide Interaction Observed by 1 H-Nuclear Magnetic Resonance Spectroscopy in Peptides Related to the N Terminus of Bovine Pancreatic Trypsin Inhibitor. Journal of Molecular Biology, 230: 312-322. 1993.

King AS and McLelland J. Birds, their structure and function. Bailliere Tindall, 1 St. Annes Road. 1984.

Kuzuhara A and Hori T. Reduction mechanism of tioglycolic acid on keratin fibers using microspectrophotometry and FT-Raman spectroscopy. Polymer, 44: 7963-7970. 2003.

Kuzuhara A. Protein structural changes in keratin fibers induced by chemical modification using 2-iminothiolane hydrochloride: A Raman spectroscopic investigation. Biopolymers, 79: 173-184. 2005a.

Kuzuhara A. Analysis of structural change in keratin fibers resulting from chemical
treatments using Raman spectroscopy. Biopolymers, 77: 335-344. 2005b.

Kuzuhara A, Fujiwara N and Hori T. Analysis of internal structure changes in black human hair keratin fibers with aging using Raman spectroscopy. Biopolymers, 87: 134-140. 2007.

Kuzuhara A. Analysis of internal structure changes in black human hair keratin fibers resulting from bleaching treatments using Raman spectroscopy. Journal of Molecular Structure, 1047: 186-193. 2013.

Li Z and Pasteris JD. Tracing the pathway of compositional changes in bone mineral with age: Preliminary study of bioapatite aging in hypermineralized dolphin's bulla. Biochimica et Biophysica Acta (BBA)-General Subjects, 1840: 2331-2339. 2014a.

Li Z and Pasteris JD. Chemistry of bone mineral, based on the hypermineralized rostrum of the beaked whale Mesoplodon densirostris. American Mineralogist, 99: 645-653. 2014b.

Li Z, Wu S and Ye C. Temperature-related changes of bioapatite based on hypermineralized dolphin's bulla. Journal of Raman Spectroscopy, 46: 964-968. 2015.

Li Z, Li Q, Wang SJ, Zhang L, Qiu JY, Wu Y and Zhou ZL. Rapid increase of carbonate in cortical bones of hens during laying period. Poultry Science, 95: 2889-2894. 2016.

Mendes-Pinto MM, Lafountain AM, Stoddard MC, Prum RO, Frank HA and Robert B. Variation in carotenoid-protein interaction in bird feathers produces novel plumage coloration. Journal of the Royal Society Interface, 9: 3338. 2012.

Morris MD and Mandair GS. Raman assessment of bone quality. Clinical Orthopaedics and Related Research, 469: 2160-2169. 2011.
Morris P. A review of mammalian age determination methods. Mammal Review, 2: 69-104. 1972.

Oliveira VED, Castro HV, Edwards HGM and Oliveira LFCD. Carotenes and carotenoids in natural biological samples: a Raman spectroscopic analysis. Journal of Raman Spectroscopy, 41: 642-650. 2010.

Pande CM. FT-Raman spectroscopy: applications in hair research. Journal of the Society of Cosmetic Chemists, 45: 257-268. 1994.

Shimohigashi Y, Nose T, Yamauchi Y and Maeda I. Design of serine protease inhibitors with conformation restricted by amino acid side-chain-side-chain CH/π interaction. Biopolymers, 51:9-17.1999.

Shishoo R and Lundell M. Investigation of structural changes in wool fibers due to annealing. Journal of Polymer Science Part A: Polymer Chemistry, 14: 2535-2544. 1976.

Speer B and Powers LV. Anatomy and Disorders of the Beak and Oral Cavity of Birds. Veterinary Clinics of North America: Exotic Animal Practice, 19: 707-736. 2016.

Schulz H, Baranska M and Baranski R. Potential of NIR-FT-Raman spectroscopy in natural carotenoid analysis. Biopolymers, 77: 212-221. 2010.

Surai PF. Natural Antioxidants in Avian Nutrition and Reproduction. Nottingham University Press. Nottingham. UK. 2002.

Toth G, Watts C, Murphy R and Lovas S. Significance of aromatic-backbone amide interactions in protein structure. Proteins Structure Function & Bioinformatics, 43: 373-381. 2001.

Wang SJ, Zhang PH, Kong XF, Xie SD, Li Q, Li Z and Zhou ZL. Delicate changes of bioapatite mineral in pig femur with addition of dietary xylooligosaccharide:
Evidences from Raman spectroscopy and ICP. Animal Science Journal, 88: 1820-1826. 2017.

Whitehead CC. Overview of bone biology in the egg-laying hen. Poultry Science, 83: 193-199. 2004.

Williams A, Edwards H and Barry B. Raman spectra of human keratotic biopolymers: skin, callus, hair and nail. Journal of Raman spectroscopy, 25: 95-98. 1994.

Fig. 1. Sketch of the regions (black dots) for the Raman analysis on beaks. Two analysis regions are located on the front one-tenth of each beak sample (the same position on both sides). Panel A is the side view of beak and panel B is the top view of beak.

Fig. 2. Raman spectra in the 600 to 1800 cm\(^{-1}\) region of beak samples from 17 to 23 weeks. The intensity of the 852 cm\(^{-1}\) peak and 1667 cm\(^{-1}\) peak are both significantly lower at 17 weeks than at 20 and 23 weeks. The intensity of the 1156 cm\(^{-1}\) peak decreased dramatically from 17 weeks to 23 weeks (All spectra were normalized to the intensity of the 1448 cm\(^{-1}\) peak).

Fig. 3. Raman spectra for estimating laying period and ages. Panels A and C show that the intensity of 1003 cm\(^{-1}\) peak and 1667 cm\(^{-1}\) peak are both significantly lower at 17 weeks than 20 and 23 weeks. Panel B shows that the intensity of 852 cm\(^{-1}\) peak at 17 weeks is significantly lower than 20 and 23 weeks, and the 935 cm\(^{-1}\) peak is absence at 17 weeks. Panel D shows that the intensity of 1156 cm\(^{-1}\) peak decreased sharply from 17 weeks to 23 weeks (All spectra were normalized to the intensity of the 1448 cm\(^{-1}\) peak).
peak).

Fig. 4. Raman spectra in the 800 to 1800 cm\(^{-1}\) region of beak samples from 23 to 87 weeks. Raman spectra remained generally stable after 23 weeks, and the 935 cm\(^{-1}\) peak became less obvious beginning at 35 weeks (All spectra were normalized to the intensity of the 1448 cm\(^{-1}\) peak).
Table 1. The weights of laying hens at six different ages

| Age, weeks | 17         | 20         | 23         | 35         | 50         | 87         |
|------------|------------|------------|------------|------------|------------|------------|
| Weight, g  | 1.32±0.03  | 1.46±0.08  | 1.54±0.07  | 1.78±0.07  | 2.00±0.05  | 2.08±0.08  |

The weight is the total weight of the laying hen before execution. Values are expressed as mean ± standard deviation (N=10).

Table 2. The relative peak intensities (by area) which are estimated based on calibration

| Ratios     | 852/1448 cm\(^{-1}\) | 935/1448 cm\(^{-1}\) | 1003/1448 cm\(^{-1}\) | 1156/1448 cm\(^{-1}\) | 1667/1448 cm\(^{-1}\) |
|------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| 17 weeks   | 0.03±0.00             | —                     | 0.36±0.01             | 0.98±0.02             | 0.83±0.05             |
| 20 weeks   | 0.23±0.02             | 0.30±0.01             | 0.42±0.02             | 0.53±0.01             | 1.43±0.01             |
| 23 weeks   | 0.24±0.00             | 0.29±0.01             | 0.40±0.01             | 0.15±0.01             | 1.47±0.02             |
| 35 weeks   | 0.23±0.00             | —                     | 0.43±0.03             | 0.12±0.03             | 1.49±0.06             |
| 50 weeks   | 0.24±0.02             | —                     | 0.40±0.04             | 0.13±0.01             | 1.36±0.02             |
| 87 weeks   | 0.22±0.03             | —                     | 0.38±0.01             | 0.12±0.03             | 1.33±0.04             |

The 1448 cm\(^{-1}\) band (C-H bending) was selected for normalization of peak intensity. Values are expressed with mean ± standard deviation (N = 60) in each group. No common superscripts (a, b and c) within the column of each classification are significantly (P < 0.05) different. (-) = under detection.
Fig. 1.
Fig. 3.
