JPSA
Japan Poultry Science Association

http://www.jstage.jst.go.jp/browse/jpsa
doi:10.2141/jpsa.0180094

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

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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 using Raman spectroscopy to quantify ages and laying period of birds.

Key words: age identification, beak, cuticle, laying hens, laying period

J. Poult. Sci., 56: 159−165, 2019

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*., 2015; Wang *et al*., 2017). 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 still 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, 2001; Evans *et al*., 2005).

ISA Brown layer hens are one of the most reared layer

Received: August 28, 2018, Accepted: October 9, 2018
Released Online Advance Publication: November 25, 2018
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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 usually reach sexual maturity at approximately 18 weeks of age and their 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 weeks old hens had not start egg laying, while all 20 weeks 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 were cleaned of the palate and extraneous soft tissue in the inner surface using a scalpel (the outer surface was not similarly cleaned to protect the original surface). 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; Themo 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*.,

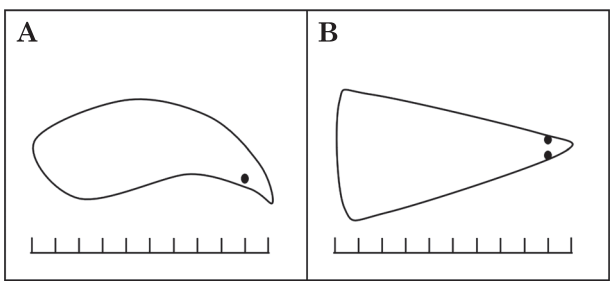

Fig. 1. **Sketch of the regions (black dots) for the Raman analysis on beaks.** Two analysis regions are located on the front one-tenth of each beak sample (the same position on both sides). Panel A is the side view of beak and panel B is the top view of beak.

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).

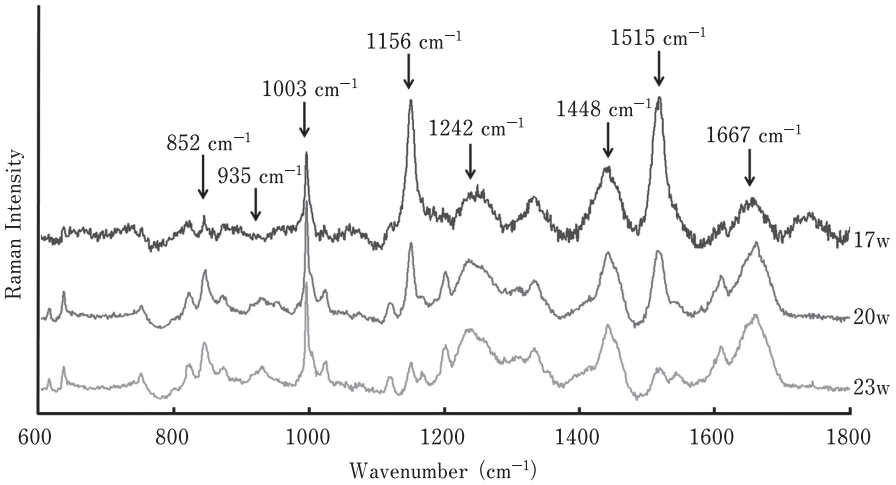

Fig. 2. **Raman spectra in the 600 to 1800 cm^{-1} region of beak samples from 17 to 23 weeks.** The intensity of the 852 cm^{-1} peak and 1667 cm^{-1} peak are both significantly lower at 17 weeks than at 20 and 23 weeks. The intensity of the 1156 cm^{-1} peak decreased dramatically from 17 weeks to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

2007, 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 the 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 the 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 reached 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 α-helical structure at 935 cm^{-1} was observed at 20 and 23 weeks, suggesting that some protein with α-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 rela-

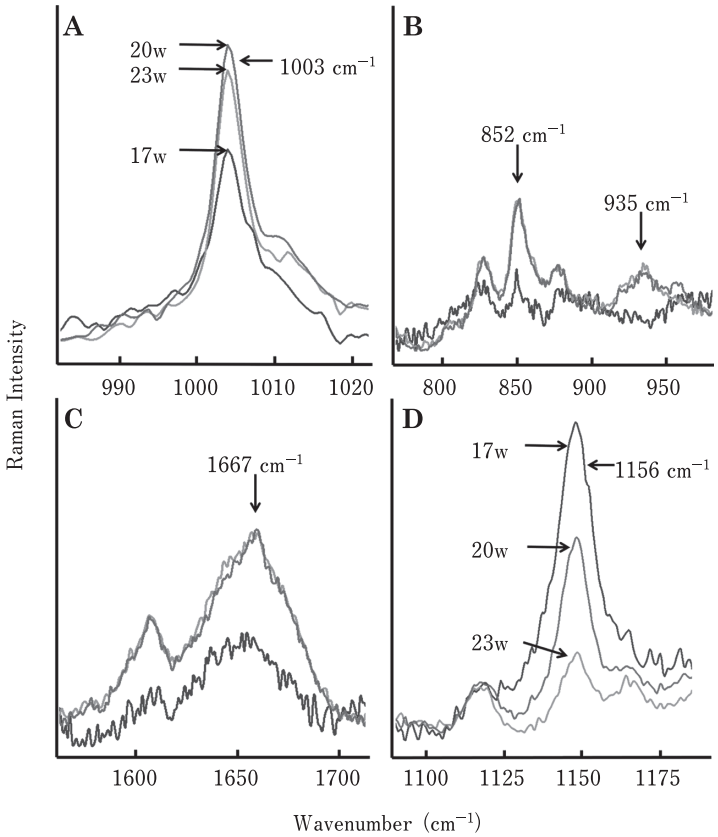

Fig. 3. **Raman spectra for estimating laying period and ages.** Panels A and C show that the intensity of 1003 cm^{-1} peak and 1667 cm^{-1} peak are both significantly lower at 17 weeks than 20 and 23 weeks. Panel B shows that the intensity of 852 cm^{-1} peak at 17 weeks is significantly lower than those at 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).

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	$0.03^{a}\pm0.00$	—	$0.36^{a}\pm0.01$	$0.98^{a}\pm0.02$	$0.83^{a}\pm0.05$
20 weeks	$0.23^{b}\pm0.02$	0.30 ± 0.01	$0.42^{b}\pm0.02$	$0.53^{b}\pm0.01$	$1.43^{b}\pm0.01$
23 weeks	$0.24^{b}\pm0.00$	0.29 ± 0.01	$0.40^{ab}\pm0.01$	$0.15^{c}\pm0.01$	$1.47^{b}\pm0.02$
35 weeks	$0.23^{b}\pm0.01$	—	$0.43^{b}\pm0.03$	$0.12^{c}\pm0.03$	$1.49^{b}\pm0.06$
50 weeks	$0.24^{b}\pm0.02$	—	$0.40^{ab}\pm0.04$	$0.13^{c}\pm0.01$	$1.36^{b}\pm0.02$
87 weeks	$0.22^{b}\pm0.03$	—	$0.38^{ab}\pm0.01$	$0.12^{c}\pm0.03$	$1.33^{b}\pm0.04$

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.

tively 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 Ages 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 at 50 weeks (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.

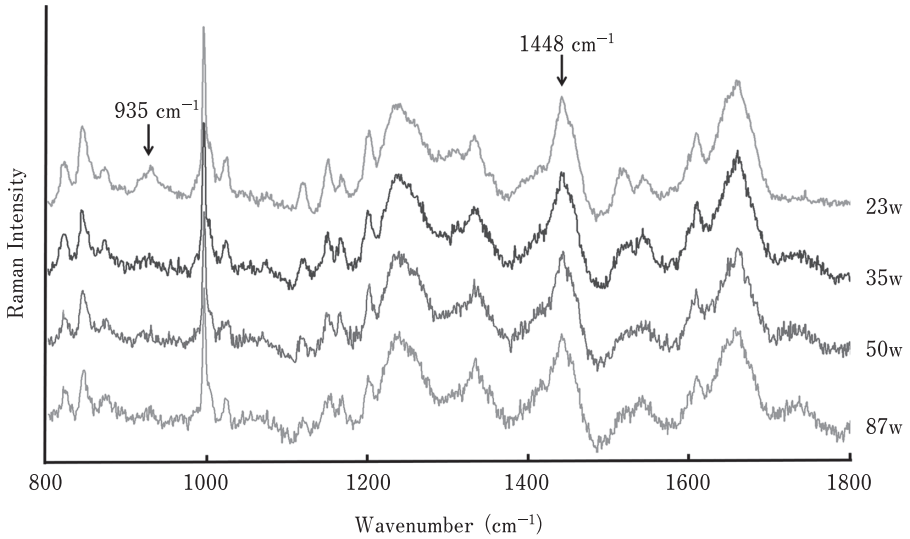

Fig. 4. **Raman spectra in the 800 to 1800 cm^{-1} region of beak samples from 23 to 87 weeks.** Raman spectra remained generally stable after 23 weeks, and the 935 cm^{-1} peak became less obvious beginning at 35 weeks (All spectra were normalized to the intensity of the 1448 cm^{-1} peak).

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 amide 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 β-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 α-helical structure at 935 cm^{-1} was observed in the laying period. This suggests that protein with α-helical structure may be formed during sexual maturity.

The area ratios of 1156/1448 cm^{-1} were successfully ap-

plied 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 also 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.

Acknowledgments

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).

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