Non-destructive identification of fake eggs using fluorescence spectral analysis and hyperspectral imaging

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Abstract

In this study, fluorescence hyperspectral imaging (FHSI) was used for the rapid, non-destructive detection of fake, manmade eggs from real eggs. To identify fake eggs, protoporphyrin IX (PpIX)–a natural pigment present in real eggshells–was utilized as the main indicator due to its strong fluorescence emission effect. The fluorescence images of real and fake eggs were acquired using a line-scan-based FHSI system, and their fluorescence features were analyzed based on spectroscopic techniques. To improve the detection performance and accuracy, an optimal waveband combination was investigated with analysis of variance (ANOVA), and its fluorescence ratio images (588/645 nm) were created for visualization of the real eggs between two different egg groups. In addition, real and fake eggs were scanned using a one-waveband (645 nm) handheld fluorescence imager that can perform real-time scanning for on-site applications. Then, the results of the two methods were compared with one another. The outcome clearly shows that the newly developed FHSI system and the fluorescence handheld imager were both able to distinguish real eggs from fake eggs. Consequently, FHSI showed a better performance (clearer images) compared to the fluorescence handheld imager, and the outcome provided valuable information about the feasibility of using FHSI imaging with ANOVA for the discrimination of real and fake eggs.

Key words: analysis of variance, fake egg, fluorescence imaging, hyperspectral imaging, non-destructive evaluation
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Introduction

Food safety is one of the most important scientific disciplines for the prevention of foodborne illnesses, as billions of consumers are still exposed to the risks of unsafe food (Fung et al., 2018). In recent years, the food safety issues posed by artificial (fake) eggs made by humans have received attention in certain countries (Joshi et al., 2020; Soon, 2020; Fearnley, 2021; Kim et al., 2021b). The main aim in producing these fake eggs is that they can be processed at a fraction of the cost of the genuine article, thereby generating an easy profit. Some unconscionable entrepreneurs manufacture these fake eggs using mixtures of several harmful chemicals, as they do not want to wait for hens to lay real eggs.

These fake eggs are fabricated from ingredients that make them look like real eggs, and they are difficult to distinguish from real eggs (Joshi et al., 2020; Gautam et al., 2021). Fake eggs are usually made of sodium alginate for albumen and yolk formation, tartrazine dye for yellow food coloring, calcium chloride for the thin membrane surrounding the albumen, and paraffin wax and gypsum powder (calcium sulfite dehydrate) for the eggshell (Joshi et al., 2020). These chemical constituents of fake eggs can cause serious health problems, including increased blood pressure, poor bone health, congestive heart failure (Zahid et al., 2013), severe allergies (More, 2020), respiratory diseases (More, 2020), nerve and liver diseases (Zahid et al., 2013), and intestinal obstruction, which can lead to abdominal pain, nausea, vomiting, and constipation (MedlinePlus, 2021).

Despite these serious health problems, the research on the detection of fake eggs thus far is insufficient (Joshi et al., 2020). Joshi et al. (2020) investigated Raman spectral features for the rapid, non-destructive detection of the external and internal parameters of fake eggs, using multivariate analysis methods and line-scan-based Raman hyperspectral imaging (1,500 to 3,900 cm\(^{-1}\)). They achieved high accuracy in distinguishing the fake and real eggs. However, the critical disadvantage of the Raman scanning method for the detection of a target material is that it is time-consuming (Qin et al., 2014; Kim et al., 2022) and very hard to apply in the field due to its complex structure and high-power light (laser) source (Behbahani et al., 2021). Thus, developing a non-destructive and rapid detection system for fake eggs is also an emerging issue for field application.

To overcome those problems, protoporphyrin IX (PpIX)–a natural pigment present in real eggshells (Ostertag et al., 2019)–was selected as a key factor for the detection of fake eggs due to its strong fluorescence emission effect within the visible–near-infrared (Vis-NIR) region (Idris Miah, 2001). In one study, the freshness of frozen pork was evaluated by fluorescence hyperspectral imaging (FHSI), and the fluorescence emission peak of the PpIX pigment was detected within the visible–near-infrared spectral region of the frozen pork (Zhuang et al., 2022). Bravo et al. (2017) developed a hyperspectral data processing algorithm for improving hyperspectral imaging acquisition in terms of the sensitivity and contrast of PpIX maps of human brain tumors.

Fake eggshells are manufactured using paraffin wax and gypsum powder, which are less sensitive to fluorescence than PpIX; therefore, the latter was selected as the most significant factor that distinguished fake eggs from real eggs. The main principle of fluorescence is absorbing and emitting light between two different frequencies or wavelengths. This occurs when the molecule absorbs the energy of a specific frequency, which can cause an electronic transition from a low-energy state to a high-energy state that finally emits lower-energy light (Kim et al., 2001). This is most striking when a specimen absorbs ultraviolet (UV) light and emits visible light. The spectral intensity and the pattern emitted by the fluorescent substance are different depending on the concentration level and composition of the substance utilized for quantitative analysis (Qin et al., 2017; Lee et al., 2018).
To detect fake eggs, the FHSI technique was used in this study. Hyperspectral imaging is a novel technique that acquires data at a series of narrow and contiguous wavelength bands for each spatial pixel of a captured scene, creating a 3D hyperspectral cube to obtain both spatial and spectral data of multiple samples in a specific area (Kim et al., 2020a; 2021a; Seo et al., 2021). This method can provide both physical and chemical information about the sample, and can also produce simple optical regions, as used by standard color cameras. FHSI is a technique that integrates fluorescence and hyperspectral imaging, providing physical and chemical information for different applications, including diagnostics, remote imaging, and agricultural quality and safety sensing (Kim et al., 2001; ElMasry et al., 2012; Yang et al., 2022).

For the analysis of FHSI data, two FHSI techniques were proposed and compared in this study: The critical fluorescence emission wavelength (FEW) was determined to distinguish real and fake eggs. The fluorescence-based handheld imager (FHID) was utilized to verify the viability of its field application based on the selected essential FEW. To improve the quality and accuracy of FHSI, analysis of variance (ANOVA) was conducted.

ANOVA has been widely used for effective chemometrics in the field of food quality evaluation (Cho et al., 2013; Lee et al., 2017; Mo et al., 2017), since it is capable of producing ratio images for visualization of a target material between two groups (Lohumi et al., 2016; Lee and Shin, 2020). Therefore, ANOVA was performed to find one or two waveband ratios to distinguish between real and fake eggs based on the highest F-values, indicating more statistically significant discrimination.

Several independent studies have been carried out in order to identify the features that affect the quality of various foods and medical products; however, to date, we are unaware of any study yet conducted in terms of providing FHSI information with regard to fake eggs. Although some FHSI-based automated inspection devices have been studied for food quality and safety applications to help on-site inspectors (Feng and Sun, 2012; Huang et al., 2014; Baiano, 2017; Pu et al., 2019; Kim et al., 2020b), to the best of our knowledge, no studies have been conducted for the rapid in situ detection of fake eggs for field applications. Therefore, the objectives of this study were to develop FHSI techniques and to provide fluorescence spectral analysis as a non-destructive evaluation method for the rapid detection of manmade fake eggs.

Materials and Methods

Sample preparation

Real white eggs were purchased from a local grocery store in the Republic of Korea, and the fabrication process of the fake eggs included the following steps: First, warm water and sodium alginate were mixed and stirred evenly to prepare a fine paste, with a white and transparent appearance similar to that of egg albumen. Furthermore, a small portion of this paste was separated to prepare the fake egg yolk by adding tartrazine dye to give it a yolk-like color. The used chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The dye was mixed manually and poured into vials, where it was further mixed by placing the filled vials onto a high-speed vortex mixer. The whole egg was prepared by pouring the yolk paste into the yolk-shaped mold and dipping it into the mixture (formed by mixing calcium chloride and water) to create a membrane layer around the fake egg yolk. Then, the prepared yolk was added to the fake egg albumen paste to form the whole egg. Finally, a fake eggshell was prepared to cover the entire egg, using gypsum powder and paraffin wax. The real
and fabricated eggs are shown in Fig. 1. The entire manufacturing process was conducted at room temperature. All of the real and fabricated white egg samples (total 10) were stored together at 5°C to ensure that the same temperature was maintained for both samples. The numbers of real and fake egg samples used were 30 and 10, respectively, because the used sample images (100 mm × 100 mm) included sufficient egg pixel information. The size of a single pixel was 0.1 mm × 0.1 mm. In addition, since eggs are washed with water before distribution, there were no foreign substances on the eggs’ surface.

Fig. 1. A photograph of real and manmade (fake) eggs.

Spectral feature extraction

A fluorescence spectrometer is an electromagnetic spectroscope that can detect and analyze fluorescence emission peaks generated by a sample. The spectral resolution was set to a 1 nm interval. Thus, the fluorescence emission data of real eggs and fake eggs were measured with a fluorescence spectrometer (FluoroMate FS-2, Scinco, Seoul, Korea) to analyze the peak emission wavelengths of the PpIX pigment, and their peak relative fluorescence intensities (RFIs) were compared. The measurement of the real eggs and fake eggs was conducted simultaneously in circular cells composed of a non-fluorescent substance. In a fluorescence spectrometer, the light of a specific wavelength band is passed through a solution, transmitting the light towards a filter and into a detector for measurement. The amount of light absorbed by the sample (the excitation spectrum) and the amount of light emitted by the sample (the emission spectrum) can be quantified. For sample measurement, the fluorometer photomultiplier tube’s power and the integration time were 700 V and 20 ms, respectively. An excitation wavelength range of 405 - 700 nm was continuously emitted from a 150 W xenon lamp with 5 nm intervals, while the light emitted from the samples was measured within 1 nm intervals from 420 to 780 nm.

FHSI system

A line-scan-based fluorescence hyperspectral imaging system was utilized to obtain the fluorescence images of the fake and real egg samples. This system is based on a push broom scan method, and comprises seven main components: an electron-multiplying charge-coupled device (EMCCD) (MegaLuca R, Andor Technology, South Windsor, CT, USA); a line-scan imaging spectrograph (VNIR Headwall Photonics, Fitchburg, MA, USA); a lens with a focal length of 25 mm f/1.4, a frame of 1,004 (spatial) × 1,002 (spectral) pixels, and a dynamic data range of about 14 bit with a spectral range from 400 - 1,000 nm (although for this study we used the range from 460 - 800 nm); UV-A LEDs (Model XX-15A, Spectronics Corp., NY,
USA) with a central wavelength of 405 nm, used to illuminate the egg samples; a stepper motor for moving the conveyor unit; data acquisition software; and a display unit. A conceptual diagram and a photograph of this system are presented in Fig. 2a and b, respectively.

As shown in Fig. 2b, to extract line-scan hyperspectral images, all of the egg samples were put in the sample holder and transported to the conveyor unit of the FHSI system at a particular speed (2 mm·sec⁻¹) controlled by a step motor. The motor slid the conveyor unit towards the camera’s field of view (FOV), and the hyperspectral images were obtained simultaneously. The FOV of the hyperspectral camera used was 200 mm (W) × 200 mm (L), and the eggs were placed into a sample holder containing six samples. The distance between the egg samples and the EMCCD camera was set to about 200 mm to cover the spatial range of the camera. The egg samples placed on the moving stage were scanned line by line at a 0.5 mm step to obtain the fluorescence hyperspectral image data. The collected hyperspectral images were saved in a three-dimensional (3D) format, accommodating both spectral and spatial (x- and y-directions) dimensions. The software for data acquisition was developed on Microsoft Visual Basic (version 6.0) using a Windows XP operating system.

![Conceptual diagram of fluorescence hyperspectral imaging system (a), and a photograph of the system used to measure the real and fake egg samples (b).](image)

**Fig. 2.** Conceptual diagram of fluorescence hyperspectral imaging system (a), and a photograph of the system used to measure the real and fake egg samples (b).

**Spectral calibration and region of interest (ROI) selection**

Six selected egg samples (real and fake) were placed in an aluminum holder emitting no fluorescence response, and 60 samples in total were scanned using the developed FHSI system described above. All of the egg samples were scanned line by line with the EMCCD camera, and the collected images were saved in a three-dimensional (3D) format accommodating both the spectral and spatial information. The obtained hyperspectral image cube measured 400 × 400 × 80. Data acquisition was carried out with an exposure time of 0.3 s and at a step size of 0.5 mm, giving a total of 400 line images. As the intensity of the used UV light source was not evenly distributed for the different spatial regions, the deviation of the UV light source for spatial distribution was calibrated by multiplying the coefficients. A calibration process was then conducted to remove the unnecessary camera noise in order to enhance the original fluorescence signal. The calibration process was defined as shown in Equation (1) (Lee et al., 2018):

\[ I_F = (I_R - I_D) \times I_C \]  

(1)
Where $I_f$ is the calibrated hyperspectral fluorescence image, $I_r$ represents the raw fluorescence image data, $I_o$ represents the dark-current reference image data, and $I_c$ is the coefficient of spatial distribution for the hyperspectral fluorescence imaging system. The dark-current reference image of 0% reflectance was achieved by covering the cap on the used camera lens. A ROI selection step was also used to remove the background from the image and extract the sample spectra. After taking the sample images, a region of interest (ROI) selection step was carried out for a more precise analysis by removing the background from the sample image, and the sample spectra of the ROI could be obtained. Finally, the actual spectral information of the acquired egg images was extracted by conducting the spectral calibration step and ROI selection process. Both the calibration and ROI selection steps were conducted using MATLAB software (2015, MathWorks, Natick, MA, USA).

**ANOVA analysis**

The ultimate objective of this study was to develop a rapid imaging device to detect fake eggs. Therefore, hyperspectral imaging data are not suitable for rapid, portable devices, since high dimensionality and large image data are burdens for real-time processing. To achieve this goal, analysis of variance (ANOVA) was performed to find one or two waveband ratios that could distinguish between real and fake eggs based on the highest F-values, indicating statistically greater discrimination. This is a type of statistical modeling used to compare the differences between group means, along with related factors such as the variation between two groups (Kim et al., 2003). Using the F-distribution function of ANOVA can provide valuable information about the variance of each population (within) and grouping of populations (between) to check whether the variability between and within each population is significantly different. Therefore, F-values of one-way ANOVA were applied in this study, and one or two waveband ratios were calculated to find the best waveband to perform a discrimination analysis between real and fake egg images. Based on the waveband selection, threshold fluorescence values were evaluated in iterative threshold increments of 0.01, and were used to find the highest resulting classification accuracy to distinguish fake eggs from real eggs. All of the selection steps were performed using MATLAB software (MathWorks, Natick, MA, USA). To clarify the image processing steps, spectral extraction, and ANOVA analysis, the workflow used for analyzing the hyperspectral images was as shown in Fig. 3.

**Fig. 3.** Flowchart of hyperspectral image processing and data analysis for fake egg detection. ROI, region of interest; ANOVA, analysis of variance.
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Overall configuration of FHID

After analyzing the spectral features of real and fake eggs and selecting the optimal waveband ratio for the detection of fake egg images, visualization of fake eggs was achieved using the FHID developed by the Environmental Microbiology and Food Safety Laboratory, Beltsville Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture. Its detailed specifications and description are available in our previous report (Lee et al., 2013). This device is an inexpensive fluorescence-based automated inspection instrument designed to visualize fluorescence emissions when excited by violet light. In addition, with its Wi-Fi communication, live inspection images, and real-time video streaming data, the FHID is capable of transporting data to different external monitoring devices, such as laptops, desktop computers, or IOS- and Android-based mobile appliances.

The FHID comprises illumination, power management, image acquisition, signal processing, and wireless transmission components. The main components are high-power UV/Violet LED illumination (405 nm, LZ4, Osram, Germany), a black-and-white CCD camera (1ATMC-NCR, IRIS, Texas, USA), an optical filter (645 nm, Edmund Optics, New Jersey, USA), a 12 V battery, and a remote Wi-Fi transmitter (FPV 803W, SUNKIN Global Trading Company, Guangdong, China). The miniature spectrometer module was controlled by an application development kit provided by the developer. As mentioned above, because the PpIX pigment in real eggshell has been reported to have fluorescence emission characteristics in the Vis–NIR region (about 400 - 700 nm) (Seo et al., 2019), a UV/Violet LED was used for the excitation source.

The UV/Violet LEDs, CCD camera, and optical filters were integrated with a disk-type aluminum plate (150 mm), and the total weight of the FHID was about 1,200 g, making it lightweight enough to be easily handled with one hand. The majority of that weight was contributed by the used battery, the aluminum plate, and the filter changer. For more practical use, the size and weight could be further reduced with improvements to the energy-efficient and low-weight battery, by designing a miniature electric circuit. The external casing of the FHID was fabricated using a 3D printer (Fortus 250mc, Stratasys, Rehovot, Israel). In addition, imaging inspection data could be easily transformed to digital documentation or data-based information. The used FHID is shown in Fig. 4a.

![Fig. 4. Averaged fluorescence excitation–emission matrix of each dataset for real (a) and fake (b) egg samples.](image-url)
Results and Discussion

Fluorescence characteristics of real and fake eggs

Fluorescence emission information of real and fake eggs was obtained as a three-dimensional mesh plot. It was rotated in a contour plot (Fig. 4) to accentuate the optimal excitation wavelength regions. As mentioned above, when the PpIX pigment is exposed to the excitation wavelength, it emits a different frequency due to the fluorescence effect (Kim et al., 2001). Finally, we acquired the fluorescence spectra data of both types of eggs within the 400 to 700 nm region. The fluorescence emission–excitation matrix plot of real and fake white eggs was created, as shown in Fig. 4. In Fig. 4, the fluorescence UV excitation and emission are represented by the x- and y- axes, respectively. The fluorescence emission waveband and the color bar show the intensity of the fluorescence emission wavelengths generated by the egg samples. The two sample groups represent the real (Fig. 4a) and fake egg samples (Fig. 4b), stored under the same conditions.

As shown in Fig. 4a, the peak emission wavelengths were generally not dependent on the UV excitation wavelengths, and the contour plot can provide an easy means of determining the excitation wavelengths responsible for RFI s. Thus, subsequent PpIX spectra are shown in the form of contour plots. The most dominant fluorescence features were emission peaks in the green and red regions of the spectra at about 450 to 550 nm and 630 to 670 nm, respectively. Moreover, these regions were also created at the 405 nm excitation wavelength. Therefore, 405 nm was selected as an effective excitation wavelength that can detect the presence of PpIX—a natural pigment present in real eggshells. The peak emission wavelengths observed in the red region (630 to 670 nm) were consistent with a different report on PpIX detection (Idrish Miah, 2001; Seo et al., 2019), and they were selected as major spectral information for ANOVA analysis. However, the peak emission wavelengths appearing in the green region (450 to 550 nm) were not considered as appropriate spectral data for hyperspectral imaging of PpIX detection, because they are not typical fluorescence emission peaks generated by PpIX (Kim et al., 2003; Seo et al., 2019). As shown in Fig. 4b, no wavelengths were emitted from the fake eggshells, because fake eggs are usually manufactured using chemicals that are less sensitive to fluorescence than real eggs. As shown in Fig. 4, a clear difference between real and fake eggs was established in the obtained contour plots.

Spectral analysis and FHID scanning

For spectral visualization of real and fake eggs, the average peak emission range (500 to 780 nm) under the excitation of 405 nm was extracted from the EMEX matrix, and the spectra were compared, as shown in Fig. 5, where the x- and y- axes represent the excitation wavelengths and the intensity of the fluorescence emission wavelengths, respectively. Moreover, it was found that the fluorescence intensity of real eggs was much higher than that of the fake eggs within the entire selected emission wavelength region.

The most dominant wavelengths were observed at 635 nm and 670 nm. As cited above, these wavelengths are strongly correlated with PpIX—a precursor of hemoglobin (Idrish Miah, 2001). This phenomenon of fluorescence on the surface of real eggs under violet light is due to a particular molecule that is naturally found in the eggshell. It is a kind of pigment capable of releasing oxygen in the bloodstream and giving a red color to blood. PpIX can be converted into heme—the non-protein component of hemoglobin—by the action of a particular enzyme. This enzyme’s activity is low in the oviducts of some birds, including chickens (Li et al., 2013). Therefore, eggshell fluorescence is caused by the electrons in PpIX molecules, which absorb violet light. This causes the electrons to assume a higher-energy ‘excited state’, which is short-lived. First, some
of the excess energy is lost to the surroundings—for example, as a form of heat energy. Then, the electrons fall back to their original energy levels, getting rid of their excess energy in the form of visible light; this is seen as fluorescence. Furthermore, the amounts of pigment can vary depending on the intensity of the eggshell coloring (Dean et al., 2011). At the same time, there was no information for fake eggs (Fig. 5).

Fig. 5. Representative fluorescence emission spectra of real and fake eggs excited at 405 nm.

For field applications, 635 nm was selected as the key FEW because of its higher intensity and simplicity for field application. The scan results showed a tremendous difference in fluorescence intensity between the two egg groups, as shown in Fig. 6. This demonstrates a high potential for developing a rapid real-time scanning system with the chosen vital FEW. As mentioned previously, compared to Raman imaging techniques (Joshi et al., 2020), the FHID shows more rapid and simple detection performance for field applications. A real-time video distinguishing fake eggs from real eggs is provided in the Supplementary Materials section for better understanding. In this video, the real-time scan results are directly displayed on a smartphone (Galaxy 8 plus, Samsung, Suwon, Korea) with Wi-Fi software (WIFI AVIN 2.1.7, provided by Google Play, 2021).

Fig. 6. Fluorescence-based handheld imager (a), a photograph of the scanning of real and fake egg samples (b), RGB images of real and fake eggs (c), and 645 nm fluorescence images of real and fake eggs obtained via smartphone (d).
ANOVA analysis

To improve the 635 nm fluorescence images, ANOVA was conducted, and the optimal band–ratio combination was used to more effectively distinguish fake eggs from real eggs. Before performing ANOVA, the spectra were obtained by selecting the ROI of both eggs and removing the useless background image from the original fluorescence image. To determine the significant wavebands for the detection of the fake egg samples, ANOVA was carried out for the spectral datasets. It is also helpful to analyze the impact of one or two factors by comparing the means of different samples. Joshi et al. (2020) provided a discrimination analysis between washed and unwashed egg samples using ANOVA, where all of the means were significantly different from one another (Joshi et al., 2019).

The spectral image processing sequence for distinguishing fake eggs from real eggs applied two-waveband ratio imaging, which is more effective for removing noise than single-waveband imaging. The F-values of one-way ANOVA for the fake eggs were calculated for all 340 wavelengths in the 460 to 800 nm region in order to select the best combination of wavebands to distinguish between the two groups. To accomplish this, the spectral information sets were divided into two groups. The calibration set contained 70% of the data, and validation set contained the remaining 30%. To represent the resultant two wavebands, a contour plot of ANOVA based on the calibration groups was constructed, as shown in Fig. 7, delineating the most effective spectral wavelengths for distinguishing between real and fake eggs.

![Contour plot of F-values calculated by the waveband ratio at the excitation of 405 nm.](image)

In Fig. 7, the color bar on the right-hand side of the contour plot shows the increasing intensity of F-values from blue to red. As cited above, the contour image produced by F-values obtained from ANOVA shows that the means of the two groups were significantly different, because a large F-value indicates a more statistically significant mean separation between two groups (Lohumi et al., 2016; Qin et al., 2017). The dependent variable was the ratio intensity of the emitted wavelength, and the independent variable was the excitation wavelength. Therefore, based on these results, the optimal wavebands of the ratio were 588 and 645 nm, with a maximum F-value of 20,000 (white arrow).

These obtained wavebands were directly related to the characteristics of PpIX (Idrish Miah, 2001). Furthermore, classification accuracy for the real and fake egg samples was then calculated for each specific range of band ratio values. To determine the accuracy, the number of classified pixels was compared with the ratio to the threshold value at the
corresponding pixel (Cho et al., 2011). Fig. 8b shows that the classification accuracy for the ratios of 588 and 645 nm obtained the largest F-values, indicating that the average values of the two set of samples at these two wavelengths represent the most visible differences. The wavelength of 645 nm was the absorption peak for the real eggs, while no visible absorption was obtained at 588 nm. Therefore, the obtained threshold value of 0.79, as shown in Fig. 8a, gave the best classification result, while the highest classification accuracy observed (87.19%) was calculated for the ratio R588/645 of real and fake eggs, respectively. The classification accuracy was calculated for each waveband ratio value to determine the optimal threshold value for the discrimination of fake eggs from the real eggs, using calibration (70%) and validation egg samples (30%) (Cho et al., 2013). Thus, the following two wavebands at an excitation of 405 nm should be helpful to classify real and fake egg pixels in the images.

**Fig. 8.** Threshold value (a) and classification accuracy (b) plot as a function of waveband ratio values for real and fake eggs.

**ANOVA image acquisition**

Fig. 9 shows the visualization of the image processing sequence for distinguishing real eggs from fake eggs by applying two-waveband ratio imaging. The overall steps of ANOVA analysis and hyperspectral image processing are provided as follows: Fig. 9a shows the RGB image of real and fake egg samples obtained via a conventional digital camera. The RGB image was inserted for better understanding. With the naked eye, it was very difficult to distinguish between the fake and real egg samples. As shown in Fig. 9b, the acquired hyperspectral images were processed by the relative reflectance correction described in Section 2.4. The corrected hyperspectral images contained 340 wavelengths from 460 to 800 nm. The 645 nm image with the greatest difference in intensity between the background and the egg samples (white: real eggs, dark gray: fake eggs) for the two wavebands was selected. Then, spectral data from real and fake egg pixels were extracted from the corrected hyperspectral images by using selected ROIs. The ratio images for 588 and 645 nm (Fig. 9c), as suggested by ANOVA, were produced using the waveband ratio (588/645 nm). The final image, obtained by applying a threshold to the ratio image, showed the distinguishing of real eggs from fake eggs (Fig. 9d). The threshold value was acquired from the average value between the minimum and maximum values of eggs and the background pixel intensity. The final image clearly demonstrates that fake eggs can be removed with background pixels.
Therefore, the chemicals used to fabricate the fake eggshell—i.e., gypsum powder and paraffin wax—were not sensitive to the fluorescence measurement, and all of the pixel information of the fake eggs was removed from the ratio image. Consideration of the acquired spectra and analysis of the spatial aspects of the response images at the chosen waveband ratio (R588/R645 nm) suggests that fluorescence spectral analysis was able to distinguish real eggs from fake eggs.

**Comparison between FHID and ANOVA images**

In Fig. 10, three groups (i.e., RGB image, FHID image, and ANOVA ratio image of real and fake eggs) are compared. Among these groups, the ANOVA ratio image (Fig. 10c) showed the greatest difference. This was created by binary pixels based on 645 nm filtered fluorescence information. Moreover, some of the fluorescence emission appeared around the fake eggs due to the influence of strong emission from the real eggs, but this was negligible because it could be controlled by the excitation light intensity and the determination of a threshold value to remove the useless fluorescence effects.

In this study, the ANOVA algorithm was not integrated with the FHID. For more precise accuracy and clear detection, ANOVA ratio imaging was the best option. However, ANOVA ratio imaging includes another obstacle for field application, because it removes everything except fluorescence emission pixels generated from the eggshell. This is the main reason that ANOVA was not used with the FHID. To apply ANOVA ratio imaging to the FHID, it is necessary to display other objects around the eggs for better vision recognition. Therefore, ANOVA ratio imaging was not always the best alternative for field application. Because of this limitation, the development of image processing techniques that can retain other objects will be required for future study.
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Fig. 10. Comparison of 3 groups: RGB image of real and fake eggs (a), fluorescence-based handheld imager (FHID) image (b), and analysis of variance (ANOVA) ratio image (c).

**Conclusion**

This study shows that the FHSI technique is a novel approach for distinguishing fake eggs from real eggs. Spectral images of fluorescence responses to violet (405 nm) excitation indicate the capability of fluorescence imaging to distinguish real eggs from fake eggs. In terms of the emission wavelength, the key FEW (645 nm) and the chosen ANOVA ratio image (588/645 nm) demonstrated the potential to be used in discrimination analysis for both of the egg samples. For field application, fluorescence imaging using an FHID equipped with the key FEW filter was able to produce a simple, economical, and clear classification. In addition, the ANOVA ratio imaging provided higher accuracy and more clear detection performance than the fluorescence imaging with the FHID.

Based on our results, additional enhancements are required for both detection methods. An image processing algorithm that removes some of the fluorescence effect around the real eggs is required, and the intensity of the useless fluorescence emission should be minimized. In addition, the binarization of the ANOVA ratio image should be controlled within the fake eggs for more intuitive understanding. Thus, the results of this study demonstrate the feasibility of extracting fundamental information, and illustrate the usefulness of fluorescence imaging in distinguishing real eggs from fake eggs. Investing in this technology for inspection would provide benefits that include reductions in the time and cost associated with the early detection and classification of real and fake eggs.

**Conflict of Interests**

No potential conflict of interest relevant to this article was reported.

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