Identification of UV-Fluorescence Components Associated with and Detection of Surface Damage in Green Pepper (*Capsicum annum* L)

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ABSTRACT

Fluorescence imaging has been used to detect fruit surface damage, but has not yet been applied to vegetables, such as green pepper. In this report, we extract and identify fluorescent components from the exocarp (skin) of green pepper. The fluorescence excitation and emission wavelengths of these extracted compounds were determined using a fluorescence spectrophotometer and identified using nuclear magnetic resonance spectroscopy and mass spectrometry. Red and blue fluorescent components with excitation and emission wavelengths 667 – 685 nm and 400 - 438 nm respectively, were found. In subsequent research, the red fluorescent compounds were targeted, as these compounds have a higher fluorescence intensity, around 97 a.u. Pheophytin *a* is one of these red fluorescent compounds, appearing in the mass spectrum at 871 m/z. Furthermore, when a fluorescence imaging system was set up, with halogen illumination, it was shown that this system could successfully detect surface damage in green pepper.

Keywords

Green pepper, fluorescence, excitation, wavelength, damage, machine vision

1. INTRODUCTION

Inspection for surface damage in green pepper (*Capsicum annum* L), such as scars, abrasion, cuts, bruises, and puncture marks, is an important component of grading and quality control. Not only does this damage lower the quality of the green pepper, but it also reduces shelf life, leading to economic losses. For the fresh market, peppers are initially sorted according to color and the presence of damage; acceptable peppers are then separated out for marketing. While grading bell peppers for color using machine vision can achieve accuracies of up to 96%, the detection of damage is more difficult: achieving accuracies of only around 63% (Shearer and Payne 1990). Finding alternative methods to detect surface damage in green peppers is a worldwide challenge for researchers.

Recently, fluorescence imaging techniques have been developed for characterizing plant tissues, such as leaves, fruit, and vegetables. These techniques have the advantage of being both rapid and non-destructive. Examples of such fluorescence imaging systems include: peel defect detection in citrus (Momin et al. 2013); orange fruit-grading using a machine vision system with a pair of white and UV LED lighting devices and a color CCD camera (Kurita et al., 2009); and rot detect in citrus (Kondo et al., 2009).

Furthermore, chlorophyll fluorescence technology has been used to detect various types of damage in agricultural products. Early postharvest studies used chlorophyll fluorescence techniques to follow the development of chilling injury in banana (*Musa* Group AAA, Subgroup Cavendish); in mango *Mangifera indica* L.) (Smillie et al. 1987); and in cucumber and bell pepper (Tijskens et al. 1994; van Kooten et al. 1994). Moreover, scald development in 'Delicious' apples at harvest was evaluated by DeEll et a1. (1996) using chlorophyll fluorescence. Such an approach for the detection of surface damage in green pepper, though, has yet to be reported.

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There is, however, reason to think that such chlorophyll fluorescent markers are present in green pepper. It has been reported that the primary pigments in green pepper include chlorophylls *a* and *b* (Deli and Molnar, 2002). Two types of chlorophyll exist in higher plants; chlorophyll *a* and the minor components chlorophyll *b* approximately in a ratio of 3:1 (Lichtentaler and Babani 2004). As isolated pigments in an organic solution, both chlorophyll types exhibit typical fluorescence emission spectra with a high maximum in the red region and a shoulder in the far-red, with an emission range near 690 nm in the red region and near 730-740 nm in the far-red region (Da-wen sun, 2009).

Thus, the objectives of this paper are to identify in-vitro fluorescence components in the exocarp of green pepper. Then to focus on identifying the strong red fluorescence compounds in the exocarp of green pepper. By so doing, we will be able to identify appropriate excitation wavelengths for these components in order to construct an efficient light excitation and detection system. Once the light excitation properties are determined, an imaging system will be set in order to validate the detection of surface damage in green pepper based on fluorescence emission characteristics.

2. MATERIAL AND METHOD

2.1. Sample preparation, extraction and spectra measurements

Green peppers (*Capsicum annum* L; variety Miogi) were collected from a farmer in Kochi Prefecture, Japan to be used in this experiment. Prior to experimentation, the green peppers were stored at 25 ^{0}C for a day to acclimate to room (laboratory) temperature. One kilogram of green pepper exocarp was taken and mixed with 1000 mL methanol and 2000 mL chloroform, crushed finely using a centrifugal mill, and then soaked for a day to extract the fluorescence components. The chloroform and methanol layer of the extract was filtered (Ø125 mm) and then concentrated using a rotary vacuum evaporator (Evaporator Type N-1, EYELA). One hundred ninety four milligram of extract sample from resulted extract was then purified through SiO_2 column chromatography with solvent (40 mL Hexane:40 mL CHCL3 ; 60 mL Hexane:20 mL CHCL3 ; 80 mL CHCL3 ; 47.5 mL CHCL3:2.5 mL MeOH ; 45 mL CHCL3:5 mL MeOH ; 40 mL CHCL3:10 mL MeOH ; 100 mL MeOH) respectively. Approximately 25 test tube fractions were acquired and then checked under UV-A (black light blue lamp) illumination. Two fluorescence patterns were observed, a blue fluorescence was observed in fraction tubes 15 and 16; and a red fluorescence was observed in fraction tubes 20 through to 23. The active blue and red fluorescence fractions were then concentrated using a rotary evaporator to remove all the solvent. The dried active fractions were then dissolved in chloroform and placed in a measuring cell (quartz fluorometer cell, 4 clear windows, Teflon stopper, with pathlengths 10 mm) for UV and fluorescence spectra measurements. A fluoro-spectrophotometer (F-4500, Hitachi, Ltd., Tokyo, Japan) was used to measure excitation and fluorescence spectra. A UV-VIS-NIR spectrometer (U-4000, Hitachi, Ltd., Tokyo, Japan) was used to measure the UV absorbance spectra. Nuclear Magnetic Resonance (NMR) analysis with deuterated chloroform solvent was performed. The ^{1}H NMR spectra were recorded at 500 MHz frequency and a temperature of 23 ^{0}C, using a Bruker AVANCE III NMR instrument. Mass Spectrometry (MS) was also performed, 1 mg of dried sample was dissolved in 1mL of organic methanol as solvent, 10 µL of solution was mixed with matrix (glycerol). The mixed solution was set on the target MS and measured using (JEOL JMS-700 Mass Spectrometer).

2.2. Fluorescence image acquisition

Fluorescence images were taken from intact green pepper to compare with spectrum information, which were obtained during the identification procedure. The system consisted of a CCD camera (FC1450, Takex, Japan), tunable filter (VariSpec, Cambridge Research & Instrumentation, Inc., USA). The spectrum was measured over the 400-720 nm range with a resolution of 10 nm. The camera with a 8 mm focus lens (iris set at 1.2 opening) was adjusted as follows; gain: 255; offset: 255; and shutter speed: 66.76 ms. Four halogen lamps (12V50W-AKW, Philips, Japan), equipped with wide band C-PL (W) 62 mm filters, were used. The images were obtained at a light intensity of 15.540 lx, at a 5 nm interval over the 400-720 nm range.

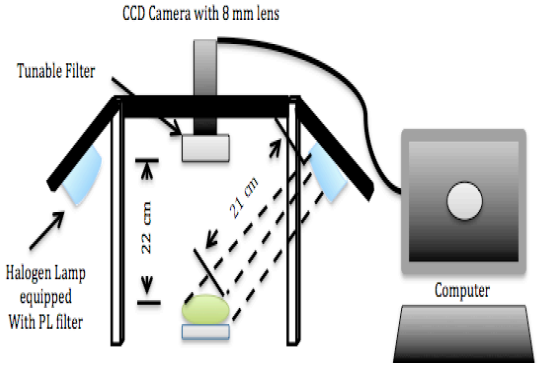

Figure 1a. Image acquisition system (schematic layout picture)

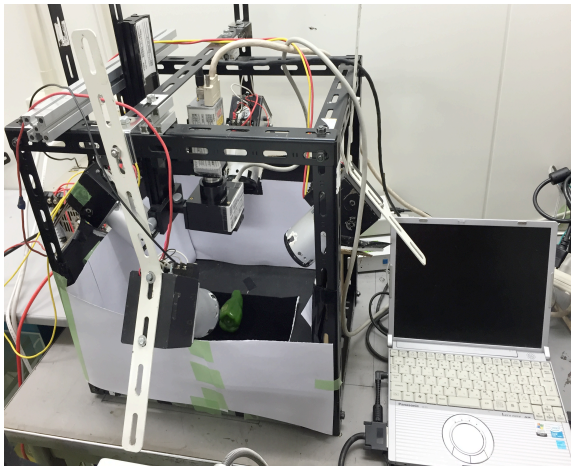

Figure 1b. Real system picture

To detect fluorescence associated with damage, the surface of the green pepper was artificially damaged by knife. The target object was manually placed in the view area of the camera, an image of the target captured and displayed on a computer monitor. The resulting image was processed using programming software MATLAB 2013b (windows platform). The set-up of the image acquisition system is shown in **Figure 1 a to b.**

3. Result and Discussion

3.1. Spectra of extracts

Fluorescence spectra at specific excitation wavelengths are shown in **Figure 2** and **3**. There were two broad peaks in fluorescence, a blue and a red one. The blue fluorescence had an emission range from 400-550 nm, with a maximum peak at 438 nm when excited at 250 nm. The red fluorescence had an emission range from 610-740 nm, with a maximum peak at 685 nm when excited at 667 nm. Blue-green fluorescence in plants mostly originates from the covalently bound blue-green fluorophore ferulic acid present in all plant cell walls; as has been shown by detailed chemical analysis of hydrolyzed cell walls (Harris and Hartley 1976; Lichtenthaler and Schweiger 1998). Blue-green fluorescence is characterized by maximum peaks between 420-430 nm, and a much lower shoulder in the green wavelength region near 520 nm. Our results show a similar pattern. However, it was not confirmed that the blue fluorescence compound of green pepper actually originates from ferulic acid. Further chemical analysis will be necessary to ascertain this.

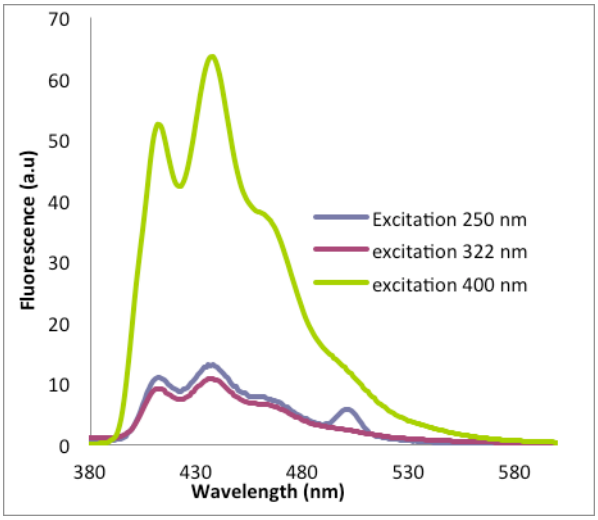

Figure 2. Fluorescence Spectra of Blue Fluorescent

Since the intensity of the red fluorescence was significantly higher than that of blue fluorescence in green pepper: 64 a.u for blue fluorescent and 97 a.u for red fluorescent; we focused on identifying the red fluorescence compound.

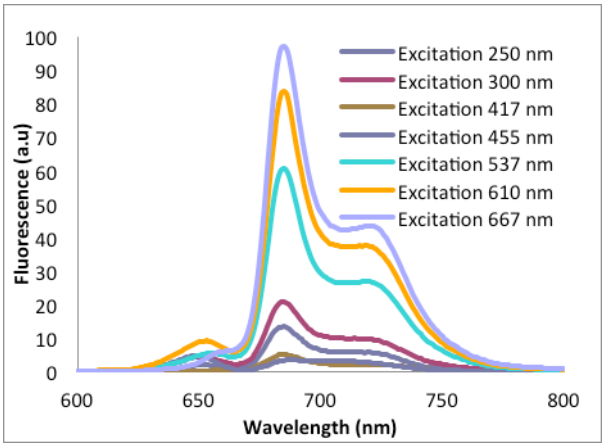

Figure 3. Fluorescence Spectra of Red Fluorescent

3.2. Identification of targeted red fluorescence components

Analysis of fluorescence components was performed using a Nuclear Magnetic Resonance (^{1}HNMR), and Mass Spectroscopy (MS) in order to identify their chemical structures. Results of a NMR and MS spectrum is shown in **Figure 4**. From the NMR spectrum we identified a ^{1}H chemical shift (shown in Figure 4a) of Pheophytin *a* red fluorescence, which is in agreement with what has been reported by Smith et al. (1984). The chemical structure of this is shown in **Figure 5**.

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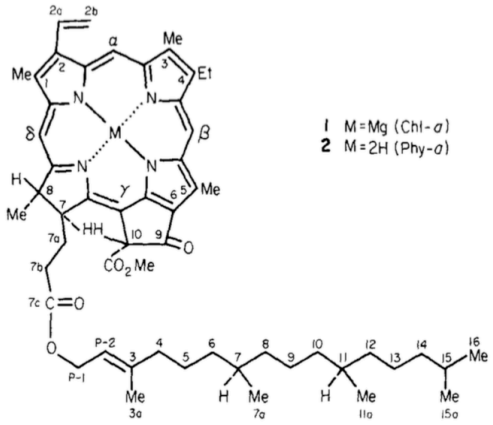


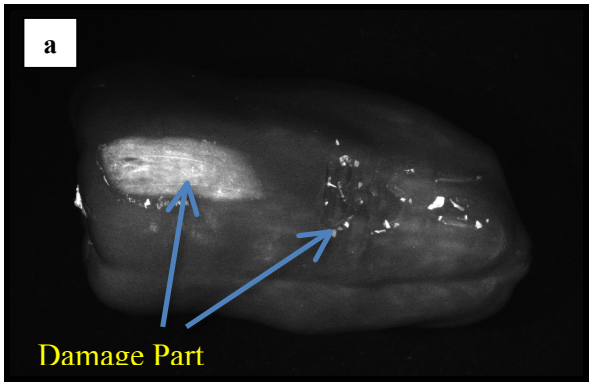
Figure 5. Pheophytin *a*

Further analysis of Pheophytin *a* as the red fluorescence component in green pepper was done by Mass Spectroscopy.

The initial Mass Spectrum of Pheophytin *a* was conducted by Jackson (1979). A removal of Mg-atom from chlorophyll fraction was confirmed by a peak appearing at *m/z* 871, which is in agreement with Lim (2009). This leads to the identification of the molecular ion as Pheophytin *a*. The mass spectrum shows that the two most abundant fragments–ions were observed: at *m/z* 533 corresponding to $(M\text{-}CH_3COOC_{20}H_{39})^+ = (M\text{-}338)^+$; and 593 corresponding to $(M\text{-}C_{20}H_{38})^+ = (M\text{-}278)^+$, a result that is in agreement with Sanja et al (2012).

3.3. Fluorescence Image

A prototype image acquisition system was set-up using a tunable filter set at 685 nm, in accordance with the identified optimal wavelength for red fluorescent emission, to detect damage in green pepper. The resulting image captured by this system is shown in **Figure 6.** By using image segmentation the damaged part can be clearly distinguished.



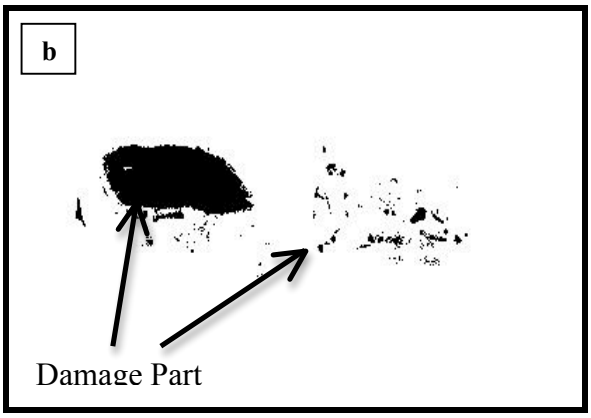


Figure 6. Image resulted from the system, 685 nm wavelength image (a). Processed image threshold minimum 4 and threshold maximum 114 (b).

Thus, it can be seen that a 685 nm wavelength image can successfully identify fluorescence of a different intensity from the damaged part of the green pepper. An explanation for this phenomenon may include reflected light from the surface of the undamaged part of the green pepper, or it could also originate from the red fluorescence of the chlorophyll in green pepper. Further study is necessary to investigate this phenomena.

4. CONCLUSION

We have demonstrated that fluorescence components can be extracted from green pepper, and that these are associated with blue and red fluorescence. As the red fluorescence has a higher emission intensity than that of blue fluorescence, the red fluorescent component was targeted and determined to be Pheophytin *a*, which has fluorescence emission peak at 685 nm (intensity of 97 a.u.) when excited at 667 nm. Furthermore, a prototype fluorescence imaging system based on the above characteristics demonstrated that surface damage on a green pepper can be successfully identified. Thus, damage on a green pepper can be successfully distinguished by fluorescence imaging at 685 nm; a result consistent with red fluorescence emission from the damaged part of the green pepper.

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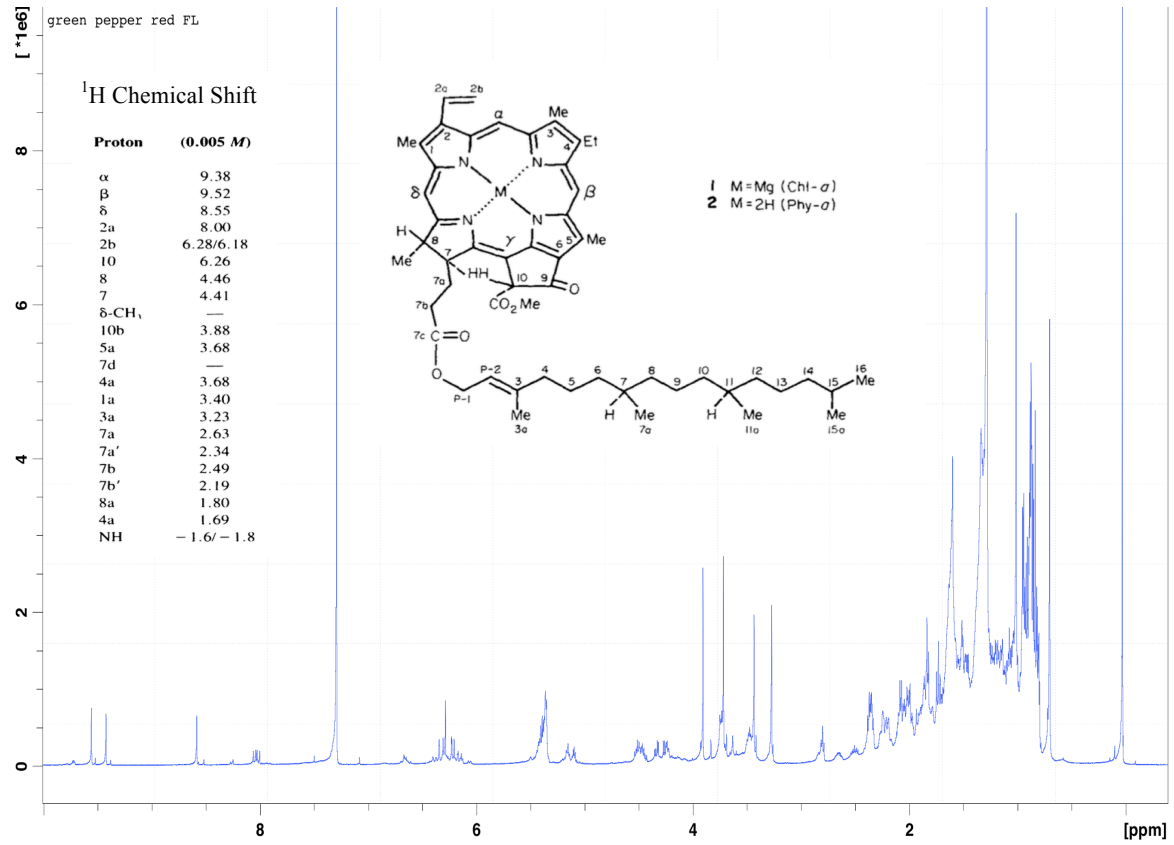

Figure 4 a. Nuclear Magnetic Spectrum (^{1}H NMR) of red Fluorescence

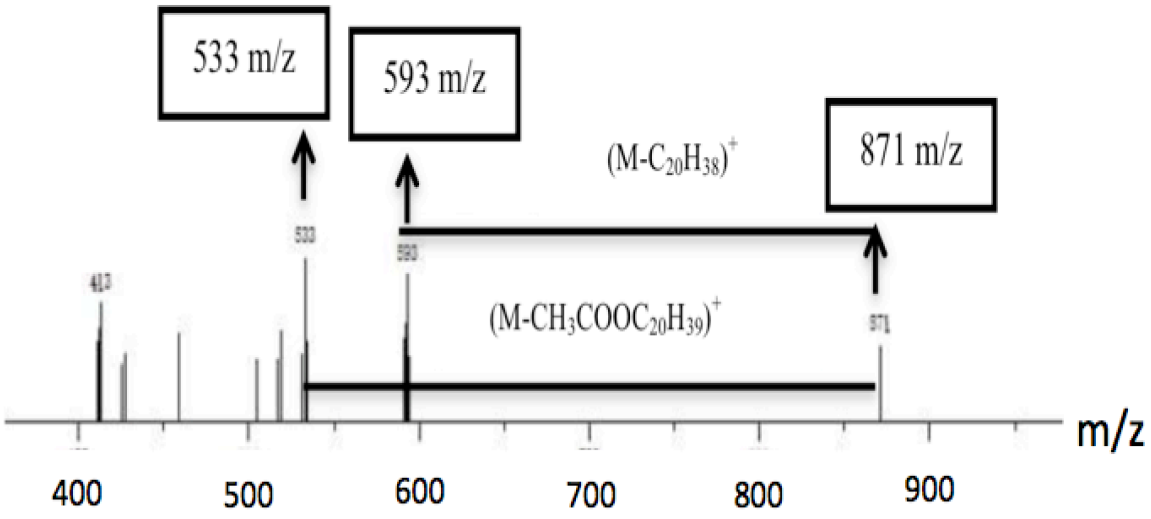

Figure 4b. Mass Spectroscopy spectrum of Red Fluorescence

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