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Original paper

Direct visualization of capsaicin and vanillylamine in *Capsicum* fruit with laser fluorometric imaging

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Summary

Capsaicin, the pungent secondary metabolite found in fruit of the genus *Capsicum*, and vanillylamine, a metabolic precursor to capsaicin, are used in the food science, pharmaceutical, medical, and forensic industries; however, a rapid method to evaluate fruit for the presence of these two compounds is lacking. The present research describes the *in situ* visualization of capsaicin and its precursor in *Capsicum* fruit by laser-induced fluorescence imaging and spectrometry. When excited by ultraviolet lasers at 266 nm, capsaicin and vanillylamine have peak fluorescence emissions at 310 nm. The fluorescence spectra of precursors of capsaicin and analogs, i.e., *trans*-caffeic acid, *trans*-ferulic acid, *trans-para*-coumaric acid, vanillyl alcohol, vanillin, vanillic acid, had different peaks than those of capsaicin and vanillylamine. The localization of capsaicin and its immediate precursor, vanillylamine, was imaged with an ultraviolet-sensitive camera after excitation with a laser at 266 nm. Fluorescence images detected at 310 nm showed the localization of capsaicinoid and/or vanillylamine on the surface of placenta and septa of *Capsicum* fruits. No fluorescence specific to capsaicin and vanillylamine was observed in seeds or pericarp. Both bell pepper and sweet pepper also showed 310 nm fluorescence on the placental surface, suggesting the accumulation of vanillylamine in the placenta. Laser-induced imaging shows considerable promise as a suitable technique for rapidly screening *Capsicum* fruit for their capsaicin and vanillylamine contents.

Introduction

Spices are essential for adding color, flavor and variety to our daily meals. In particular, red hot peppers (*C. annuum, C. frutescence, C. chinense, C. pubescens and C. baccatum*) have greatly contributed to our diet along with black pepper[1,2)]. Red hot pepper is rich in vitamin C, provitamin A, and B vitamins[3)]. The pungent principle of red hot pepper, capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) and its analogs, i.e., dihydrocapsaicin (*N*-(4-Hydroxy-3-methoxybenzyl)-8-methylnonanamide), nordihydrocapsaicin (*N*-(4-Hydroxy-3-methoxybenzyl)-7-methyloctanamide), homocapsaicin ((3*E*)-*N*-(4-Hydroxy-3-methoxybenzyl)-9-methyldec-7-enamide) and homodihydrocapsaicin (*N*-(4-Hydroxy-3-methoxybenzyl)-9-methyldecanamide), are not essential micronutrients; however, the pungent compound plays an essential role in enhancing appetite, and stimulating the secretion of gastric juices[4)]. Recent research on capsaicinoids, i.e. capsaicin and its analogs,[5)] has revealed extremely important roles in human neurobiology[5)]. Capsaicinoids raise the heart rate, perspiration, and stimulate the release of endorphins. The thermogenic effect of capsaicinoids in assisting the prevention of a particular metabolic syndrome is well known[6)]; namely, a study reported in 2008[7)] that capsaicin alters energy utilization produced from ATP. Anticancer and apoptotic effects of capsaicin have been reported[8,9)]; however, the co-carcinogenic activity of capsaicin has been reported, as well[10)]. In spite of such a negative report, the importance of hot red pepper as a spice has not been diminished.

A constant supply of high quality *Capsicum* fruits is necessary for the food science industry and the pharmaceutical, medical, and forensic industries[11)]. Continual efforts have been made to breed new and improved *Capsicum* species with higher Scoville heat units, an organoleptic measure of capsacinoid content often determined by a taste panel. Sensory evaluation is time consuming and

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often depends on the sensitivity of individual tasters. For qualitative analysis of capsaicinoids, thin-layer chromatography (TLC) has been the most popular method[12,13]; however, TLC requires extraction, concentration, and then a lengthy chromatographic process including sample application, development in the glass chamber, drying, and finally visualization with an appropriate color reagent such as 2,6-dichlorochinonechloroimide[13,14]. For more precise quantitative analysis of capsaicin and analogues, gas chromatography-mass spectrometry, high performance liquid chromatography, and high-performance liquid chromatography-mass spectrometry have been used[15–17].

Direct observation of the localized distribution of capsaicin and vanillylamine in *Capsicum* fruit without preliminary treatment will save time and money. By taking advantage of modern imaging technology with an ultraviolet laser and a digital UV camera system, we directly and qualitatively visualized the location of capsaicin and its immediate precursor, vanillylamine, in *Capsicum* fruit.

Materials and Methods

1. Principles of the imaging system

The basic principle of the imaging system used in this study is based on the observation that excitation of capsaicinoids with ultraviolet light at 277 nm gives a fluorescence emission in the UV-B region at 330 nm[18,19]. We assumed that the fluorescence emission of capsaicinoids in the UV-B region must be derived from 8-methyl-6-vanillyl moieties. Therefore, capsaicin and probably the immediate precursor vanillylamine either should yield the same spectral peak. In our system, we introduced a digital UV camera in place of a HPLC detector to present the location of capsaicinoid as images on a display. The imaging system used in this study is composed of a laser light source for UV light irradiation, a UV fluorescence detection camera, and a UV spectrophotometer as shown in Fig. 1.

Individual hardware used in the experiment is as described below:

Laser light source: OPO (optical parametric oscillator) (Hamamatsu Photonics, Japan) for fluorescence spectrophotometric analysis. Nd: YAG laser (Hamamatsu Photonics, Japan) for fluorescence imaging.

Emission fluorescence detector (UV-camera): UV-CCD camera (Hamamatsu Photonics, Japan)

UV spectrophotometer: Multi-channel spectrometer (Hamamatsu Photonics, Japan)

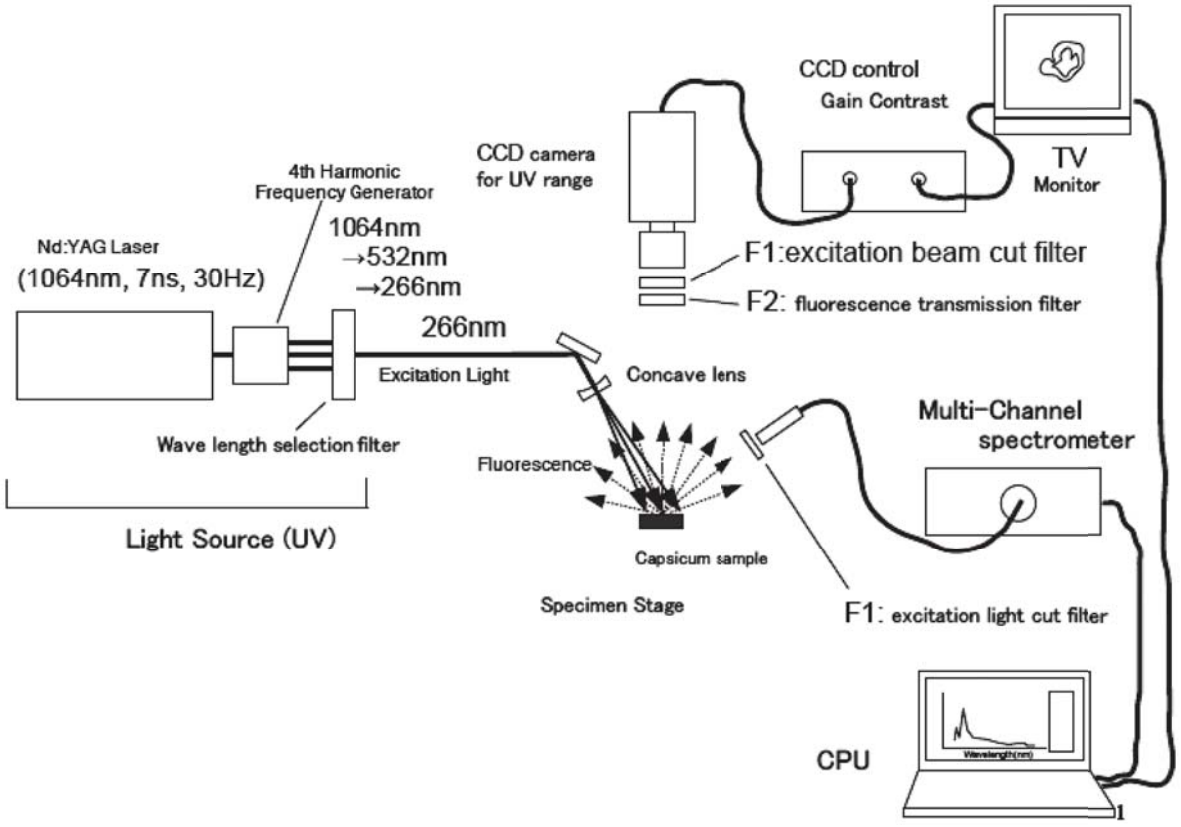

Fig. 1. Schematic outline of the UV laser fluorescence imaging system

The imaging system is composed of four parts: i.e., (A) laser light source section using a Nd: YAG laser (1064 nm, 7 ns, 30 Hz), 4th harmonic frequency radio wave generator, (B) specimen stage, (C) multichannel spectrometer, and (D) fluorescence imaging camera using a CCD camera equipped with CCD control gain contrast.

2. Materials provided for the analysis

Prior to taking *in situ* images of capsaicinoids in *Capsicum* fruits, UV fluorescence spectra of authentic samples of compounds involved in capsaicinoid synthesis and related compounds taken.

Authentic samples were as follows: capsaicin and vanillylamine-HCl salt (reagent grade) were purchased from Sigma-Aldrich®; guaranteed reagent grade precursors of capsaicinoid, L-phenylalanine, L-tyrosine, *trans*-ferulic acid, *trans*-caffeic acid, *p*-coumaric acid, and vanillin, vanillic alcohol, and vanillic acid were purchased from Nacalai Tesque Co.®, Kyoto, Japan. Capsaicin was used as an ethanol solution, and other authentic standards were used as aqueous solutions.

Red hot pepper (*Capsicum annuum var. annuum* cv. *Yatsubusa*) was purchased from a commercial market in Hamamatsu in September 2010. Fresh bell pepper (*Capsicum annuum var. annuum* cv. *Paprika*) and fresh sweet pepper (*Capsicum annuum var. annuum*) were purchased from a commercial market in Hamamatsu in October 2010. Red hot pepper fruits (cv. *Yatsubusa*) were collected immediately before each experiment, and cut into longitudinal or cross sections with a sharp scalpel. For both the bell pepper and sweet pepper, fruits were cut open immediately before fluorometric analysis.

3. Fluoro-spectrophotometric analysis of capsaicin, its precursors and related vanillyl-compounds

Firstly, fluorescence spectra for capsaicin, its precursors and related vanillyl-compounds were taken. Each sample (10~20 micro-liters) was placed on the sample stage, and entirely irradiated by the UV-C (270 nm) beam spot. The fluorescence spectrum of the sample was measured immediately after the sample was placed on the stage.

4. Imaging of capsaicin and vanillylamine in *Capsicum* fruit with a UV-camera

Secondly, after recording the fluorescence spectra of capsaicin, its precursors and vanillyl-compounds, red hot pepper fruits were cut longitudinally or in cross section and placed on the sample stage. The fruits were exposed to UV-C (266 nm) light and the fluorescence images were recorded within a few minutes by the UV camera.

Results and Discussion

1. Fluorometric spectra of capsaicin and four of its major precursors are shown in Fig. 2. Capsaicin showed a

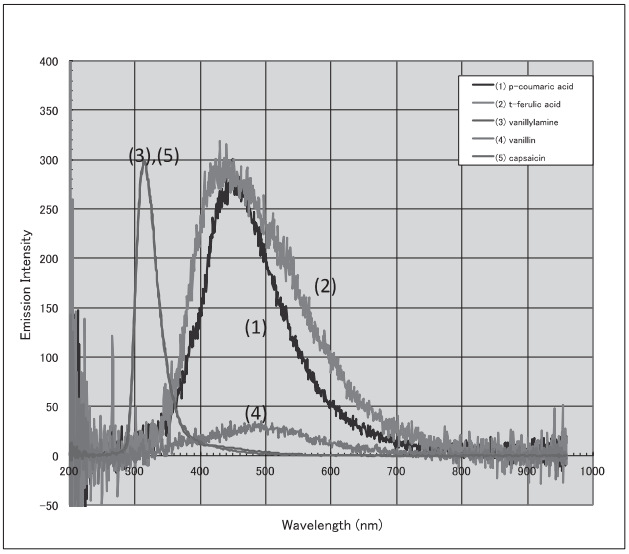

Fig. 2. UV fluorescence spectra of capsaicin and related compounds
UV fluorescence spectra of capsaicin and its precursors.
The excitation wavelength was 266 nm. Spectra for authentic samples of (1) *trans-p*-coumaric acid, (2) ferulic acid, (3) vanillylamine, (4) vanillin and (5) capsaicin were taken.

fluorescence maximum at 310 nm. Capsaicin's immediate precursor, vanillylamine, also showed a fluorescence maximum at the same wavelength. Fluorescence maxima of the three other precursors were at distinctively different wavelength regions. Phenylalanine and *trans*-caffeic acid both showed different fluorescence maxima (data not shown). As we predicted, the fluorescence maxima of capsaicinoids should be attributed to the 8-methyl-*N*-vanillyl moieties, because the fluorescence maximum of vanillylamine (8-methyl-*N*-vanillylamine) completely coincided at 310 nm. On the other hand, those of vanillin, vanillic acid, and vanillyl alcohol did not coincide in spite of similar structures, a result that also strongly indicates the amine moiety is responsible for the fluorescence maximum at 310 nm.

2. Imaging of capsaicin and/or vanillylamine in *Capsicum* fruits

Fig. 3 and Fig. 4 show fluorescence images of hot red pepper, *Capsicum annuum var. annuum* cv. *Yatsubusa* and bell pepper (*Capsicum annuum cv. Paprica*), respectively. The bright white zone observed with red hot pepper corresponds to the placenta and septum tissue, whereas no bright fluorescence was observed for pericarp tissues or seeds. Since capsaicin is known to localize in placental tissues[20–22], our results were in coincidence with our previous reports. HPLC analysis of capsaicin and vanillylamine in the *Capsicum* fruits confirmed the localization of capsaicin and its precursor vanillylamine was localized in the septa and placenta (data not shown).

Although preliminary our data show that capsaicin

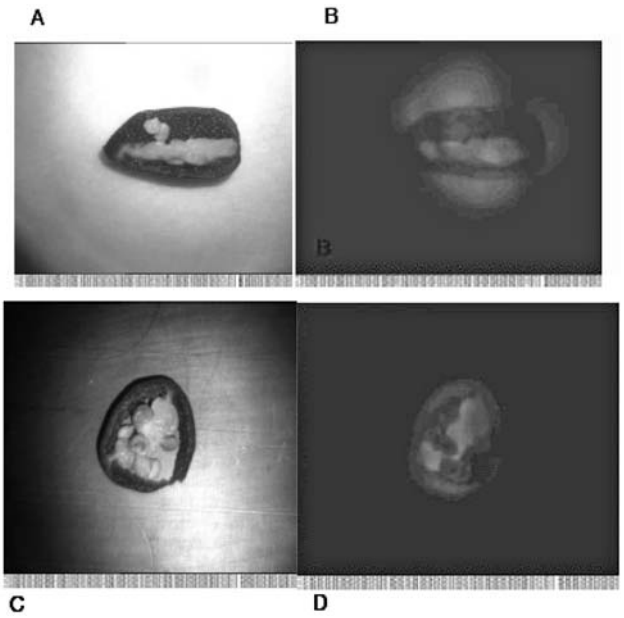

Fig. 3. UV fluorescence image of hot red pepper (*C. annuum var annuum* cv *Yatsubusa*)
(A) Upper left: longitudinal sections of *Capsicum* fruit.
(B) Upper right: UV fluorescence image of the longitudinal section shown in (A).
(C) Lower left: cross section of *Capsicum* fruit.
(D) Lower right: UV fluorescence image of the cross section shown in (C).
Note the faint white fluorescence corresponding to the placenta.

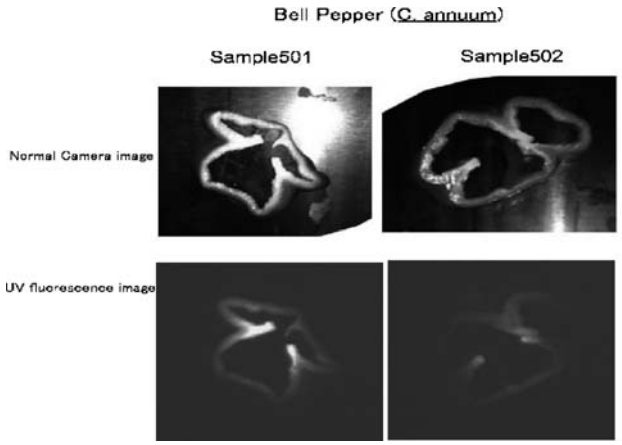

Fig. 4. UV fluorescence images of green bell pepper (Sample 501) and red bell pepper (Sample 502)
Upper left; green bell pepper cross section.
Upper right; red bell pepper cross-section.
Lower left; UV fluorescence image of the sample used for the upper left photograph (Sample 501).
Lower right; UV fluorescence image of the sample used for the upper right photograph (Sample 502).

and its immediate precursor, vanillylamine, could be directly visualized in *Capsicum* fruit; however, the data present in this paper are qualitative. We are now investigating ways to obtain semi-quantitative data. Hopefully, a compact device that enables breeders and producers to monitor capsaicin content on site will be developed as a commercial product in the near future.

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References

1) Kosuge S, Inagaki Y, Okumura Y (1962). Studies on the pungent principles of red pepper. Part VIII. On the chemical constitutions of the pungent principles. Nippon Nogei Kagaku Kaishi (J. Agric. Chem. Soc.), 35: 923–927.
2) Govindarajan VS (1985). Capsicum-production, technology, chemistry, and quality. Part I: history, botany, cultivation, and primary processing. *CRC Crit Rev Food Sci Nutr.* 22: 109~76.
3) Marina, A, Ferreres F. Tomas-Barberan, F.A., Gil MI (2004). Characterization and quantitation of antioxidant constituents of sweet pepper (*Capsicum annuum L.,*). *J. Agric. Food Chem.* 52: 3861–3869.
4) Myers BM, Smith JL, Graham DY (1987). Effect of red pepper and black pepper on the stomach. *Am. J. Gastroenterol.* 82: 211~4.
5) Dray A (1992). Mechanism of action of capsaicin-like molecules on sensory neurons. *Life Sci.* 51: 1759~65.
6) Lejeune MP, Kovacs EM, Westerterp-Plantenga MS (2003). Effect of capsaicin on substrate oxidation and weight maintenance after modest body-weight loss in human subjects. *British J. Nutr.* 90: 651~59.
7) Yasser A. Mahmmoud (2008). Capsaicin Stimulates Uncoupled ATP Hydrolysis by the Sarcoplasmic Reticulum Calcium Pump. J. Biol. Chem. 283: 21418~21426. *Journal of Biological Chemistry* 283 (31): 21418–21426.
8) Mori A, Lehmann S, O'Kelly J et al. (2006). Capsaicin, a component of red peppers, inhibits the growth of androgen-independent, p53 mutant prostate cancer cells. *Cancer Res.* 66: 3222–3229.
9) Ito K, Nakazato T, Yamato K et al. (2004). Induction of apoptosis in leukemic cells by homovanillic acid derivative, capsaicin, through oxidative stress: implication of phosphorylation of p53 at Ser-15 residue by reactive oxygen species. *Cancer Res.* 64: 1071~1078.
10) Hwang MK, Bode AM, Byun S, Song NR, Lee HJ, Lee KM, and Dong Z. (2010). Cocarcinogenic effect of capsaicin involves activation of EGFR signaling but not TRPV1. *Cancer Res.* 70: 6859~69.

11) Reilly CA, Droudh DJ, and Yost G (2001). Quantitative analysis of capsaicinoids in fresh peppers, oleoresin *Capsicum* and pepper spray. *J Forensic Sci*. 46: 502~509.
12) Müller-Stock A, Joshi RK, Buchi J. (1973). Thin-layer and column chromatographic separation of capsaicinoids: study of the constituents of *Capsicum*. *J. Chromatogr*. 13: 577~9.
13) Spanyár P, Blazovich M. (1969). A thin-layer chromatographic method for the determination of capsaicin in ground paprika. *Analyst*. 94: 1084–89.
14) Todd P Jr, Bensinger M, Biftu T. (1975). TLC screening techniques for the qualitative determination of natural and synthetic capsaicinoids. *J Chromatogr Sci*. 13: 577–9.
15) Iwai K, Suzuki T, Suzuki T, Kobashi M. (1976). Quantitative microanalysis of capsaicin, dihydrocapsaicin and nordihyrocapsaicin using mass fragmentography. *J Chromatogr*. 123(1): 119–28.
16) Iwai K, Suzuki T, Fujiwake H, Oka S. (1979). Simultaneous microdetermination of capsaicin and its four analogues by using high-performance liquid chromatography and gas chromatography-mass spectrometry. *J Chromatogr*. 172: 303–11.
17) Suzuki, T. and Iwai, K. (1982). Constituents of Red Pepper Species: Chemistry, Biochemistry, Pharmacology and Food Science of the Pungent Principle of Capsicum Species. *The Alkaloids*, 23: 228–299 [A. Brossi ed.] Academic Press, New York, NY
18) Saria A, Lembeck F, Skofitsch G (1981). Determination of capsaicin in tissues and separation of capsaicin analogues by high-performance liquid chromatography. *J. Chromatogr*., 208: 41–6.
19) Kozukue N, Han J-S, Kozukue E, Lee S-J, Kim J-A, Lee K-R, Levin CE, Friedman M. (2005). Analysis of eight capsaicinoids in peppers and pepper-containing foods by high-performance liquid chromatography and liquid chromatography-mass spectrometry. *J. Agric. Food Chem*. 53: 9172–81.
20) Iwai, K., Suzuki, T., Fujiwake, H. (1979). Formation and accumulation of capsaicin and Its analogues in fruits of *Capsicum annuum var. annuum* cv. Karayatsubusa at different growth stages after flowering. *Agric. Biol. Chem*. 43: 2493~2498.
21) Suzuki, T., Fujiwake, H., Iwai, K. (1980). Intracellular localization of capsaicin and its analogues, capsaicinoid, in *Capsicum* fruit. 1. Microscopic investigation on the structure of the placenta of *Capsicum annuum var. annuum* cv. Karayatsubusa. *Plant & Cell Physiol*. 21: 839~853.
22) Fujiwake, H., Suzuki, T., Iwai, K. (1980). Inrtracellular localization of capsaicin and its analogues in *Capsicum* fruit. [2]. The vacuole as the intracellular accumulation site of capsaicinoid in the protoplast of *Capsicum* fruit. *Plant & Cell Physiol*. 21: 1023~1030.