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Article

Detection of Food Safety Using Fluorescence Fingerprint

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1. What is a fluorescence fingerprint

Before describing fluorescence fingerprints, first we describe the known phenomenon of fluorescence. Typically, as shown in Fig. 1 (left) fluorescence refers to irradiation of a specimen with light comprising only a certain, specific wavelength component (an excitation wavelength), and in response, generation of light of various wavelengths (a fluorescence spectrum). In common use, the phenomenon of fluorescence produces the white light of a fluorescent lamp when non-visible ultraviolet light irradiates a fluorescent body applied as a coating to the inside of a fluorescent tube, generating a white light with a broad wavelength range. Spectrofluorometry makes use of this type of fluorescence phenomenon to distinguish and quantify various chemical components and is characterized by sensitivity much higher than that of ordinary absorption spectroscopy. In this case, there is one type of stimulus (a specific excitation wavelength) and one corresponding response (a fluorescence spectrum), allowing analysis of one set of stimulus and response. However, the more information that exists, the more potential there is to extract useful information contained therein. Let us then consider increasing the amount of information. To do this, we create multiple stimuli (that is, sequential, scanning irradiation with multiple excitation wavelengths), and if multiple, corresponding responses (fluorescence spectra) are produced, we obtain an enormous amount of three-dimensional information like that shown in Fig. 1 (right). Viewed from above, these three-dimensional data illustrate a pattern resembling a contour map. This pattern is regarded as a complete expression of the unique fluorescence characteristics of the specimen concerned and is called a fluorescence fingerprint or an excitation emission matrix.

In reality, measuring a fluorescence fingerprint involves a great deal of effort. For nearly seven years, we held

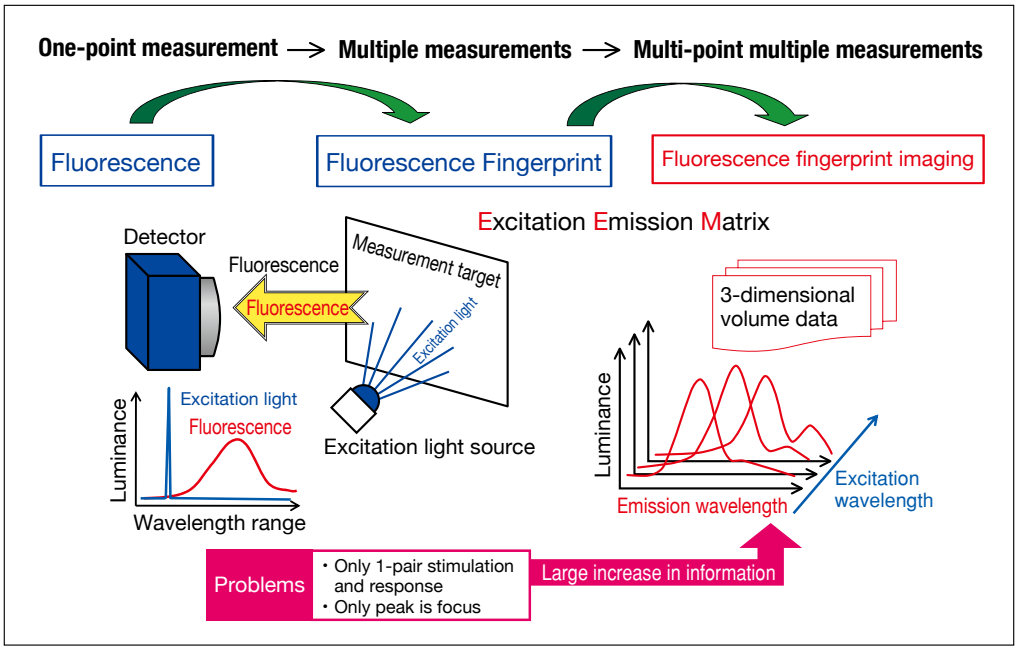

Fig. 1 From fluorescence to fluorescence fingerprint

an interest in this measurement technique, and fortunately we realized that an imported, extremely expensive fluorospectrophotometer in a neighboring laboratory included this measurement function. When we first began to collect data, measurements for a single specimen took 6 hours. Because of scanning on both the excitation side and the fluorescence side, inevitably, data collection proceeded at a plodding and frustrating pace. At a certain point, however, when we heard that Hitachi High Technologies would release the fastest fluorospectrophotometer in the world, we grabbed at this straw and visited their Analysis Center, and there we were shown a working prototype. We were astonished that measurements which had until then taken 6 hours could be completed in a mere 4 minutes, and just as suddenly, the prospect of advances in our research appeared. At present, our own laboratory houses two fluorospectrophotometers (F-7000) in experimental use for various applications in food research fields.

2. A paradigm shift in data processing

In many instances of conventional fluorescence analysis, luminance values were analyzed primarily only for peak information. Certainly if a single, distinctive fluorescence component was the target, this was the reasonable for investigation, but recent sensor technology has improved dramatically, and digital quantification allow capture of not only information from visible intensity light but also various reactions based on minute energy balances. In other words, some information can be ascertained by enlarging micro-fluctuations on a fluorescence spectrum, shoulders, and even plateaus with no fluorescence. Analized data is thus not limited to peaks, and current computer technology excels in uniform handling of all data, coupled with construction of models for skillful extraction of only the information required, i.e., data mining.

Current advances in ICT (information and communications technologies) are also making possible what was previously impossible. In recent times, the progress of digital cameras has made acquisition of high-resolution image possible even with mobile phones. Likewise, people now recognize that image is a collection of small dots called pixels. If these pixels could be used as a detector for fluorescence fingerprints as described, it would also be possible to acquire a fluorescence fingerprint for each pixel. And if the characteristics of individual pixels could be evaluated and replaced by colors, items currently invisible could also be made visible. This is the fluorescence fingerprint imaging technology which we are developing first among the world.

3. Measurement of fluorescence fingerprints

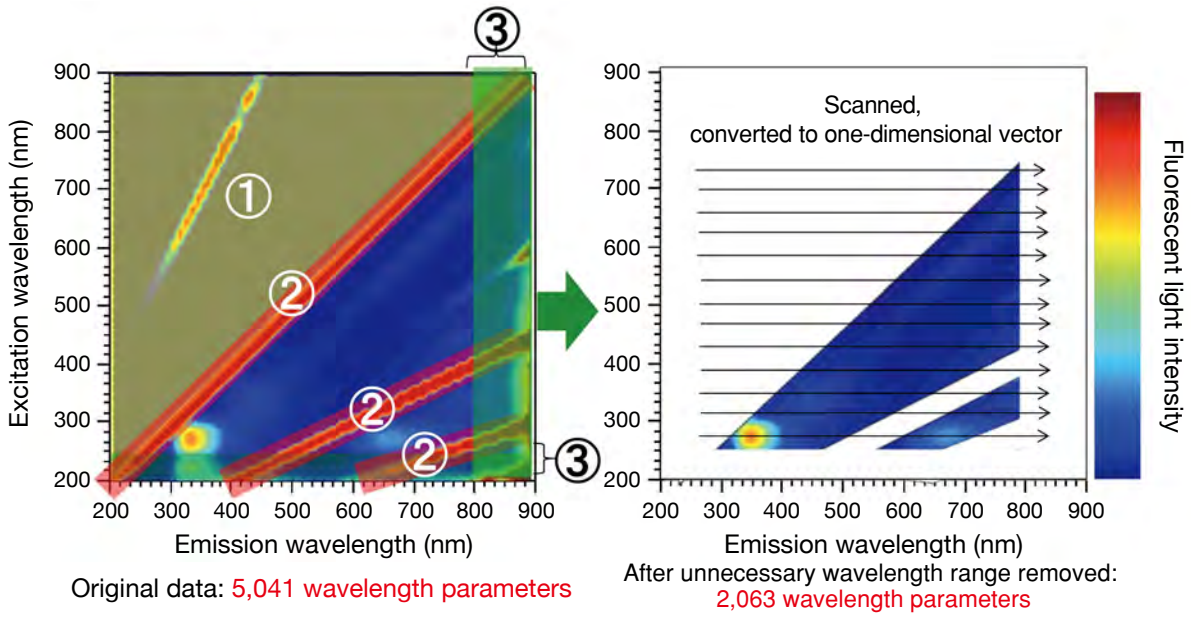

Fig. 2 Preprocessing of fluorescence fingerprint data (removal of unnecessary wavelength range)

Figure 2 at left shows an example of a fluorescence fingerprint as measured. In the figure, the X-axis represents fluorescence wavelength, and the Y-axis represents excitation wavelength. A position on the 45° line running along the diagonal means that the wavelength of the excitation light radiated and the wavelength of the emission(fluorescent) light are equal and constitute "scattered light" (the intensity of reflection spectrum can be observed along the Z-axis). According to measurement principles, this scattered light also appears at locations which are integral multiples (2nd order light, 3rd order light...). And because emission light always appears at a longer wavelength than its excitation wavelength (Stokes' law), patterns appearing on the fluorescence wavelength axis side of the 45° diagonal line (in Fig. 2, the area below the diagonal line) are also fluorescence fingerprints.

Consequently, information used in actual data analysis must be stripped of such unneeded information, and only the remaining information can be used as a fluorescence fingerprint. Specifically, what must be eliminated is ① the area of excitation wavelengths larger than fluorescence wavelengths (the area closer to excitation wavelength than the diagonal line), ② 1st order, 2nd order, 3rd order... scattered light, and ③ regions of high noise and low sensitivity. When this preprocessing is performed, the resulting fluorescence fingerprint region is an area like that in Fig. 2 at right. As one example, raw data comprising 5,041 fluorescent light intensity values (Z-axis values) were reduced to 2,063 points. Finally, the remaining data were scanned along the X-axis as discrete data and used in further analysis as one-dimensional vector data.

4. Estimation of mycotoxins in wheat[1)-5)]

Mycotoxins produced by Fusarium fungi in wheat and corn include deoxynivalenol (DON), nivalenol (NIV), and zearalenone (ZEA). Mycotoxin contamination can lead to decreased yield and quality of cereals, and humans and animals consuming contaminated cereals exhibit adverse effects such as vomiting, diarrhea, and headaches, making this a major problem worldwide. On this basis, we investigated simultaneous quantitative estimation of each of these mycotoxins in wheat flour in an attempt to develop a rapid, simple, and non-destructive technology for measuring mycotoxin contamination.

Mycotoxin-contaminated wheat (variety: Hokushin) was harvested from four different test fields with different levels of Fusarium blight (low, moderate, high, severe), and flour milled from these wheat was used as specimens. Chemical analysis values for DON concentration serving as a reference were analiezed by liquid chromatography based on an official analytical method, and analogous reference values for NIV and ZEA were measured by LC/MS/MS due to trace levels resulting in ND (not detected) results by official analytical method. Fluorescence fingerprints were measured by Fluorescence Spectrophotometer (Hitachi F7000) using the flours as it were. The fluorescence fingerprints obtained were subjected to PLS regression analysis to produce quantitative estimates for concentrations of mycotoxin contamination in each case.

Figure 3 shows results for PLS regression analysis of DON, NIV, and ZEA in validation samples. Estimation results based on chemical analysis and fluorescence fingerprinting each shows linearity, and it was possible to estimate concentration for each mycotoxin. We hypothesized that these results reflect minor differences in fluorescence characteristics according to mycotoxin variety. These results suggest that a combination of fluorescence fingerprint measurement and multivariate analysis may have application as a primary screening technique for estimating the concentration of mycotoxin contamination in wheat flour, and for simultaneous quantification of different mycotoxins.

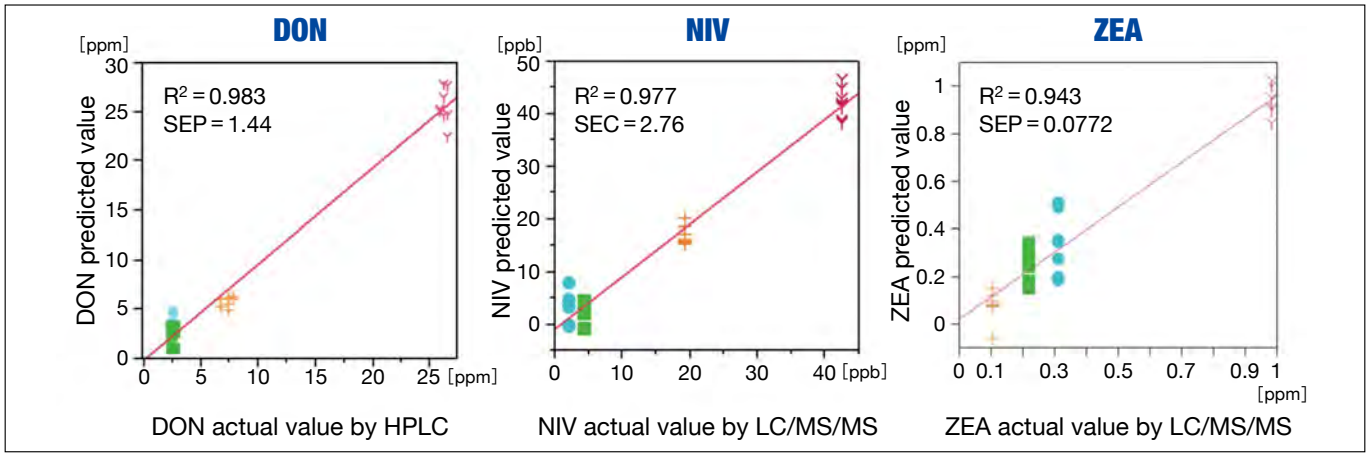

Fig. 3 Simultaneous estimation of 3 mycotoxins (validation results)

5. Estimation of aflatoxins in nutmeg[6)-8)]

Aflatoxins are a type of mycotoxin produced by Aspergillus flavus and other fungi populating a region from the tropics to the subtropics. They are known as the most potent carcinogenic substances in natural products. A regulatory standard for aflatoxin B1 (10 ppb) has been established in Japan (note: changed to regulation of total AF as of 10/2011), and HPLC using a multifunction column and chemical analysis such as LC/MS are used as official analytical methods. However, these techniques require complex preprocessing and practiced operation of instruments, and it is expected to develop a simple and rapid screening technique that is practicable at spice production sites and in the distribution stage. We therefore investigated a technique for simple and rapid detection of aflatoxin B1 in liquid nutmeg extract by fluorescence fingerprinting, without complex preprocessing.

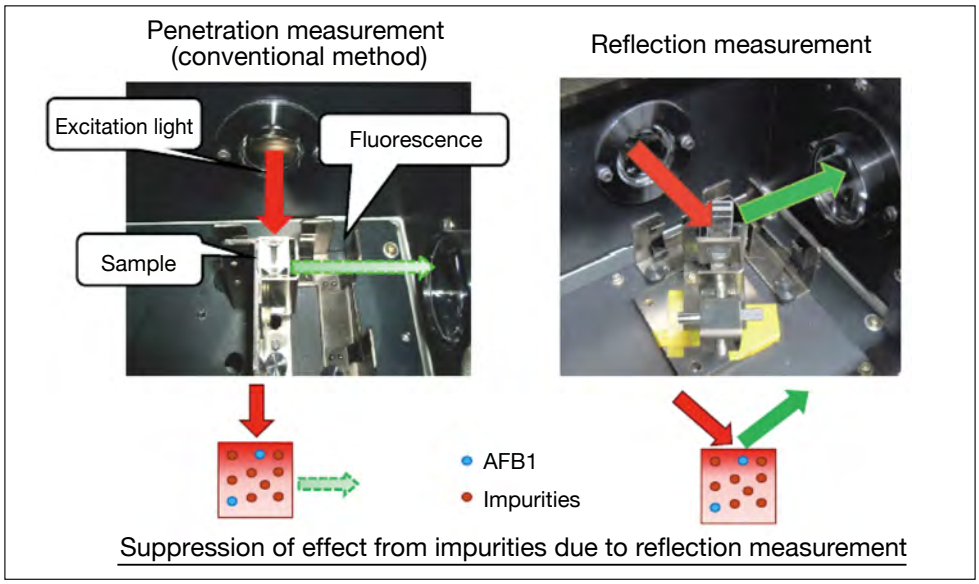

Fig. 4 Simultaneous estimation of 3 mycotoxins (validation results)

A liquid nutmeg extract was prepared by pulverizing nutmeg in which no aflatoxins had been detected, performing shaking extraction with a methanol solution (methanol: water = 8:2) and collecting the extracted residue by centrifuge. An aflatoxin B1 standard reagent (methanol: water = 8:2) was added to the liquid extract, and concentration was adjusted to create a pseudo-contamination of 0.0-4.0 ppb (23 concentration stages). An aflatoxin B1 standard solution and the pseudo-contamination specimen, without preprocessing or further modification, were then sampled for fluorescence fingerprint measurement. To investigate the measurement parameters of fluorescence fingerprinting, we experimented with penetration measurement and reflection measurement (Fig. 4).

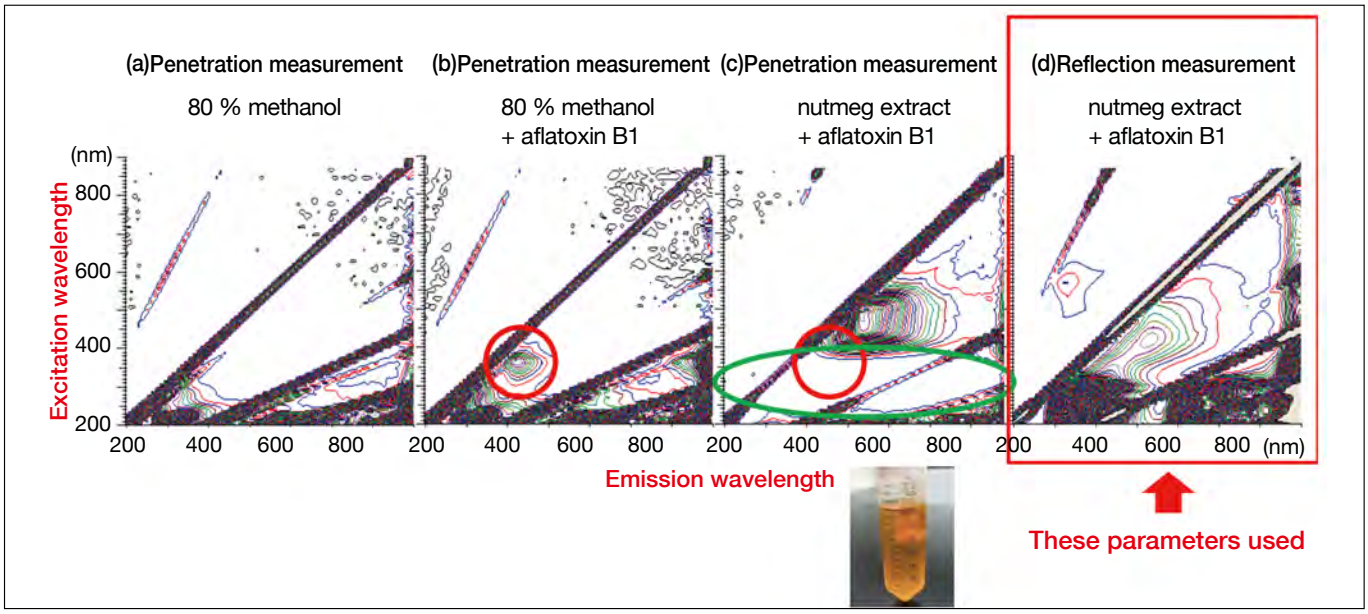

Fig. 5 Fluorescence fingerprint measurement of aflatoxin B1

Figure 5 shows the results. In Fig. 5(a), with a sample of transparent methanol alone, nearly no fluorescence information was observed. When aflatoxin B1 was added to this sample, the characteristic fluorescence peak of aflatoxins was observed. However, when aflatoxin B1 was added to the liquid nutmeg extract in lieu of methanol, the coloring of the liquid nutmeg extract caused absorption of the excitation light, and as illustrated in the lower portion of Fig. 4, excitation light was unable to reach the interior of a cuvette cell in the transmission method, and in effect, fluorescence information could no longer be obtained. In the reflection method, however, the fluorescence phenomenon was produced only at the surface of the cuvette cell, and as Fig. 5(d) shows, we found that a fluorescence fingerprint including aflatoxin information was obtained. In other words, the use of a reflection method in fluorescence fingerprint measurement allowed measurement of aflatoxins contained in a pseudo-contamination sample without a complex preprocessing step. Application of PLS regression analysis to the fluorescence fingerprint data showed that the estimated values demonstrated high linearity to the actual added concentration of aflatoxin B1 (Fig. 6). These results suggested that low, ppb-levels of aflatoxin B1 concentration in liquid nutmeg extract can be estimated simply and easily through the use of fluorescence fingerprint.

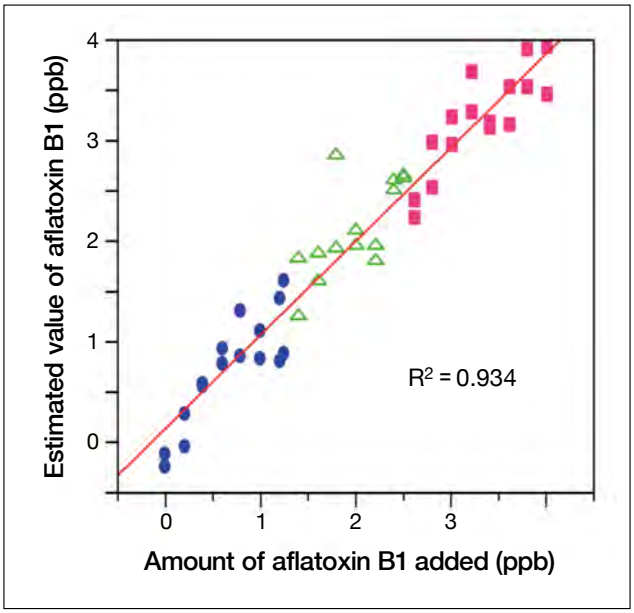

Fig. 6 Estimation of aflatoxin B1 concentration by PLS regression analysis (circles, triangles, and squares indicate 3 concentration stages, added for convenience)

6. Potential for fluorescence fingerprint

The concept of fluorescence fingerprint, also known as an excitation emission matrix, is by no means new. Nonetheless, actual measurement in a comparatively short time and without considerable effort has only become possible in the past four to five years. The recent advancement in computer and information science enables accurate quantitative determination and sample discrimination based on huge amount of data.Addition to the example described here, this technique has been successful in applications such as determining the origin of mangoes,[9] estimating the blended composition of soba flour and wheat flour in dry soba noodles,[10),11)] discriminating varieties and grades of cereals,[12)] and estimating aerobic bacterial counts on meat for consumption.[13)] Additionally, we are utilizing the advantages of this technique in research on fluorescence fingerprint imaging that extends fluorescence fingerprint measurement from points to planes, examples of which include visualization of the gluten and starch distribution in bread dough,[14-16)] and visualization of bacterial count distribution on the surface of meat for consumption.[17)] Now and in the future, a number of developments hold promise for basic technologies useful to society: improvement of fluorospectrophotometers for greater ease of use; development of compact, rapid scanning devices usable on site; development of hardware such as fluorescence fingerprint imaging devices; and food industry advances such as development of new applications, and progress in discriminating and quantifying various substances and components.

References:

1) Fujita K., Tsuta M., and Sugiyama J., Detection of Deoxynivalenol in Water Solution by Using Excitation-Emission Matrix, *Journal of the Japanese Society for Food Science and Technology* 55, 177-182, 2008

2) Fujita K., Tsuta M., Kokawa and Sugiyama J., Detection of deoxynivalenol using fluorescence excitation-emission matrix, *Food and Bioprocess Technol.*, 3, 922-927(2010)

3) Fujita K., Tsuta M., Sugiyama J., Kushiro M., and Shibata M., Non-destructive Measurement of Deoxynivalenol in Wheat Flour Using Fluorescence Fingerprinting, *Journal of the Japanese Society for Food Science and Technology*, 58, 375-381(2011)

4) Fujita K., Sugiyama J., Tsuta M., Kozawa T., Shibata M., Yoshimura M., Kokawa M., and Kushiro M., Nondestructive Method for Detection of Mycotoxins in Wheat, *Agricultural Information Research*, 21, 11-19 (2012)

5) Sugiyama J., Tsuta M., Tomita K., and Yoshimura M., METHOD FOR DETECTING HAZARD, DEVICE FOR DETECTING HAZARD, AND PROGRAM, Patent Abstracts of Japan,Publication number: 2012−177606 (Date of publication of application: 2012. 09 .13)

6) Fujita K. Sugiyama J., Tsuta M., Shibata M., Kokawa M., Onda H. and Sagawa T. Detection of aflatoxins B1, B2, G1 and G2 in nutmeg extract using fluorescence fingerprint, *J. Food Sci. Technol. Res.*, (in press)

7) Sugiyama J., Tsuta M., Tomita K., and Sagawa T., Method for Detecting Aflatoxin, Device for Detecting Aflatoxin, and Program, Patent Abstracts of Japan,Publication number:2012-177607 (Date of publication of application:2012. 09 .13)

8) Fujita K., Sugiyama J., Tsuta M., Shibata M., Kokawa M., Onda H., and Sagawa T.: “Detection of aflatoxins B1, B2, G1 and G2 in nutmeg extract using fluorescence fingerprint,” *Food Science and Technology Research*, (in press)

9) Nakamura Y., Fujita K., Sugiyama J., Tsuta M., Shibata M., Yoshimura M., Kokawa M., Nabetani H., and Araki T., Discrimination of the Geographic Origin of Mangoes Using Fluorescence Fingerprint, *Journal of the Japanese Society for Food Science and Technology*, 59,387−393(2012)

10) Sugiyama T., Fujita K., Tsuta M., Sugiyama J., Shibata M., Kokawa M., Araki T., Nabetani H., and Sagara Y., Prediction for Mixture Rate of Buckwheat Flour Against Wheat Flour Using Excitation-emission Matrix (EEM), *Journal of the Japanese Society for Food Science and Technology*, 57, 238-242, 2010

11) Shibata M., Fujita K., Sugiyama J., Tsuta M., Kokawa M., Mori Y. and Sakabe H., Predicting the buckwheat flour ratio for commercial dried buckwheat noodles based on the fluorescence fingerprint, *Biosci. Biotechnol and Biochem.*, 75, 1312-1316 (2011)

12) Tsuta M., Sugiyama J., Maeda T., and Endo S., Method and Apparatus for Discriminating Grain Flour, Patent number: 5274288 (2013. 5 .24)

13) Oto N., Oshita S., Makino,Y. Kawagoe Y., Sugiyama J. and Yoshimura M., Non-destructive evaluation of ATP content and plate count on pork meat surface by fluorescence spectroscopy, *Meat Sci.,* 93, 579-585(2012)

14) Kokawa M, Fujita K., Sugiyama J., Tsuta M., Shibata M., Araki T. and Nabetani H., Visualization of gluten and starch distributions in dough by fluorescence fingerprint imaging, *Biosci. Biotechnol. Biochem.*, 75, 2112-2118 (2011)

15) Kokawa M., Fujita K., Sugiyama J., Tsuta M., Shibata M., Araki T. and Nabetani H., Quantification of the distributions of gluten, starch and air bubbles in dough at different mixing stages by fluorescence fingerprint imaging, *J. Cereal Sci.*, 55,15-21 (2011)

16) Kokawa M., Sugiyama J. Tsuta M. Yoshimura M., Fujita K. Shibata M., Araki T. and Nabetani H., Development of a quantitative visualization technique for gluten in dough using fluorescence fingerprint imaging, *Food and Bioprocess Technol.*, 5, (2012)

17) Nishino K., Nakamura K., Tsuta M., Yoshimura M., Sugiyama J., and Nakauchi S., Optimization of excitation emission band-pass filter for visualization of viable bacteria distribution on the surface of pork meat, Optics Express, 21(10)(2013) DOI:10.1364/OE.21.012579