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Note

Determination of pheophorbide *a*, pyropheophorbide *a* and phytol

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It has been well known in Japan since olden times that ear shell (abalone), one of the favourite Japanese foods, which contains a number of chlorophyll derivatives, causes photosensitivity dermatitis[1]. Etiological studies[1] revealed that pheophorbide *a* and/or pyropheophorbide *a* are the main compounds with photodynamic action. These chlorophyll derivatives are also contained in chlorella food[2–5] and pickles[6], whose yearly production in Japan is said to be more than 890,000 tons[7]. The detailed degradative pathway of chlorophyll to pyropheophorbide *a* has not yet been clarified, but the main photosensitizing agent, pheophorbide *a*, is known to be derived from chlorophyll *a* under the action of chlorophyllase localized in chloroplast[8]. This report is concerned with a concise microanalytical method for the determination of pheophorbide *a*, phytol, counterpart of pheophorbide *a* and estimation of chlorophyllase activity.

Various methods for the analysis of pheophorbide *a* and pyropheophorbide *a* have been published: thin-layer chromatographic (TLC)[2,5,9,10], paper chromatographic[11] and liquid chromatographic[12]. They are, however, too tedious for a routine procedure for foods.

We have devised a more convenient and more sensitive TLC method for the determination of pheophorbide *a* and pyropheophorbide *a*, and a gas chromatographic (GC) method for phytol in chlorophyll-containing foods. These techniques enable us to monitor rapidly the changes in the amounts of toxic pigments during processing and storing of foods.

EXPERIMENTAL

Preparation of pheophorbide a, pyropheophorbide a and phytol

Pure pheophorbide *a* and pyropheophorbide *a* were prepared from chlorella protothecoides and from *Brassica juncea* var. *integrifolia* (Japanese name, Takana) as described by Tamura *et al.*[4] and Amano *et al.*[2], respectively. The structures of pheophorbide *a* and pyropheophorbide *a* were confirmed by converting pheophorbide *a* into the known methylpyropheophorbide *a*[13], which was derived from pyropheophorbide *a* by methylation with diazomethane. Phytol was purchased from Wako.

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Extraction of pheophorbide a and pyropheophorbide a from chlorella and vegetables

The extraction procedure is based on the method reported by Tamura *et al.*[4] with slight modifications. Chlorella (100 mg; in the case of vegetables, 10 g) was mixed with 2 g of quartz powder (200 mesh) in a mortar and ground well with a pestle. The mixture was extracted three times with 20, 10, and 10 ml of 85% (v/v) acetone. Then 20 ml of the aqueous acetone extract (40 ml) was mixed with 40 ml of diethyl ether, and the extract released was washed twice with 40 ml of 3% Na_2SO_4. The ether extract was shaken with 20 ml of 17% HCl, and the acid extract was diluted with 150 ml of 5% Na_2SO_4 and extracted twice with 20 ml of diethyl ether. The ether extract was dried over anhydrous Na_2SO_4 and concentrated to near dryness. The concentrated ether extract was dissolved in 1 ml of diethyl ether, which was used for determination of pheophorbide *a* and pyropheophorbide *a* by high-performance reversed-phase TLC (HPTLC-RP-8, F-254 s. from Merck).

Extraction of phytol from chlorella and vegetables

Half the volume (20 ml) of the above aqueous acetone extract was mixed with 40 ml of 3% Na_2SO_4 and shaken twice with 40 ml of *n*-hexane. The hexane extract was dried over anhydrous Na_2SO_4 and concentrated to 1 ml, which was placed on a column packed with activated charcoal (0.2 g) and alumina (2 g). The column was eluted with 10 ml of *n*-hexane and then with 30 ml of ethyl acetate. The *n*-hexane eluted was discarded and the ethyl acetate fraction was used for gas–liquid chromatography.

Determination of pheophorbide a and pyropheophorbide a

For separation and identification of pheophorbide *a* and pyropheophorbide *a*, high-performance reversed-phase TLC was carried out. Two solvent systems were used: benzene–*n*-hexane–acetic acid (20:10:0.3, v/v) and methanol–acetone–2-propanol–water (70:18:2:10, v/v). Quantitative estimation was made by densitometry at 667 nm (TLC scanner CS-920, Shimadzu).

Determination of phytol

Quantitative estimation of phytol was performed by gas chromatography at 250°C on a Hewlett-Packard Model 5840 A gas chromatograph equipped with a flame ionization detector and an all-glass column packed with 2% OV-101 on Chromosorb W (1.2 m × 3 mm I.D.).

Hydrolytic activity of chlorophyllase in chorella or vegetables

The sample was mixed with acetone–0.1 *M* phosphate buffer, pH 8.0 (3:7) and incubated for 3 h at 37°C. Extraction and determination of pheophorbide *a*, pyropheophorbide *a* and phytol were performed as described above.

Gas chromatography–mass spectrometry of phytol

Identification of phytol from chlorella was made by gas chromatography–mass spectrometry (GC–MS) on Dimaspec Model 321 GC/MS equipped with an all-glass column (1.2 m × 2 mm I.D.) packed with 2% OV-17 on Gas-Chrom Q (100–120 mesh) at 200°C.

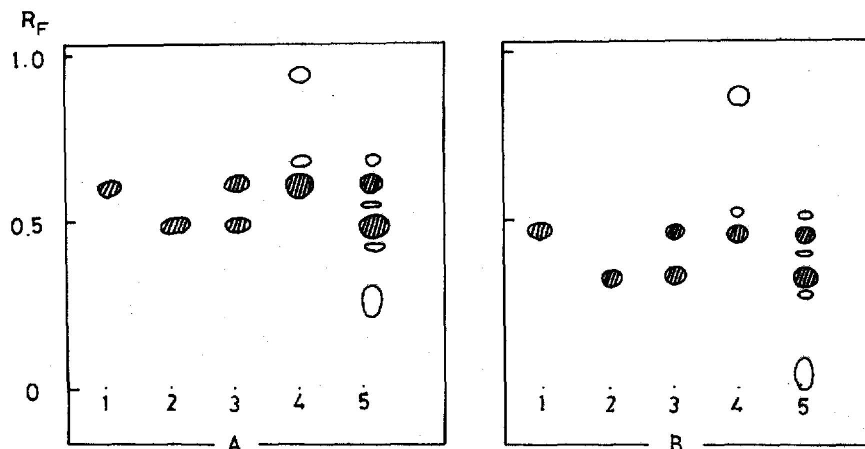

Fig. 1. Thin-layer chromatograms of chlorophyll derivatives. Plate: HPLC-RP-8 F254 s precoated plate (Merck). Eluent: A = benzene–*n*-hexane–acetic acid (20:10:0.3); B = methanol–acetone–2-propanol–water (70:18:2:10). Spots: 1 = standard of pheophorbide *a*; 2 = standard of pyropheophorbide *a*; 3 = 1 + 2; 4 = extract from chlorella; 5 = extract from salted vegetable.

RESULTS AND DISCUSSION

High-performance reversed-phase thin-layer chromatograms of pheophorbide *a*, pyropheophorbide *a* and chlorella extract are shown in Figs. 1 and 2, which show enough resolution for precise quantitative analysis of pheophorbide *a* and pyropheophorbide *a* in samples. The limits of determination for pyropheophorbide *a* and pheophorbide *a* were 10 ng and 20 ng, respectively. Fig. 3 shows the calibration curves for both pigments. From the extraction step to final stage took only 2 h. The amount of phytol in foods was determined by GC (Fig. 4), with a determination limit of 0.001 μg, compared with published values of more than 10 μg[14] and 0.02 μg[15].

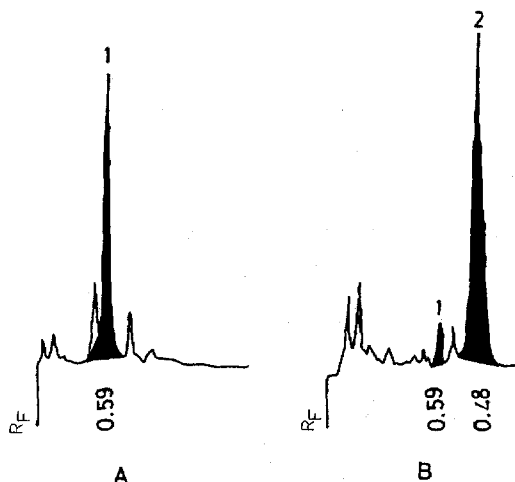

Fig. 2. Densitometric chromatograms of pheophorbide *a* (1) and pyropheophorbide *a* (2) from extract of chlorella (A) and salted vegetable (B) by TLC chromatoscanner (Shimadzu).

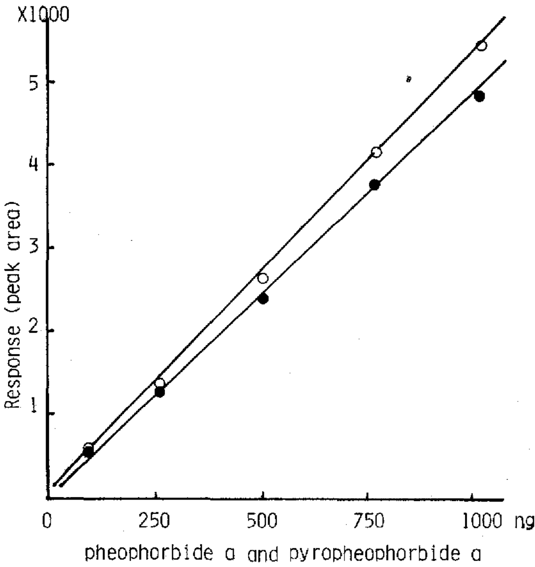

Fig. 3. Densitometric peak area as a function of spotted amount of pheophorbide *a* (●) and pyropheophorbide *a* (○) on HPTLC plate.

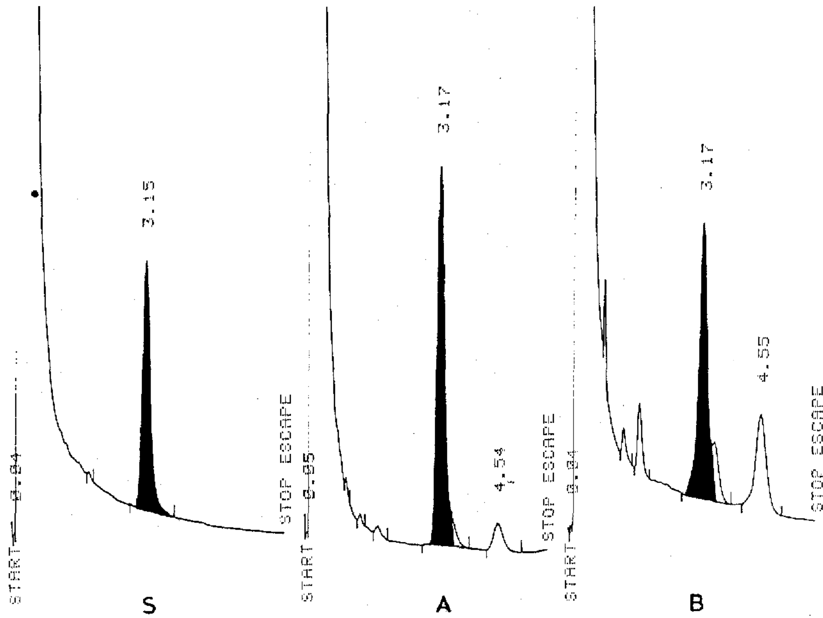

Fig. 4. Gas chromatograms of phytol. S = standard phytol; A = extract from chlorella; B = extract from salted vegetable.

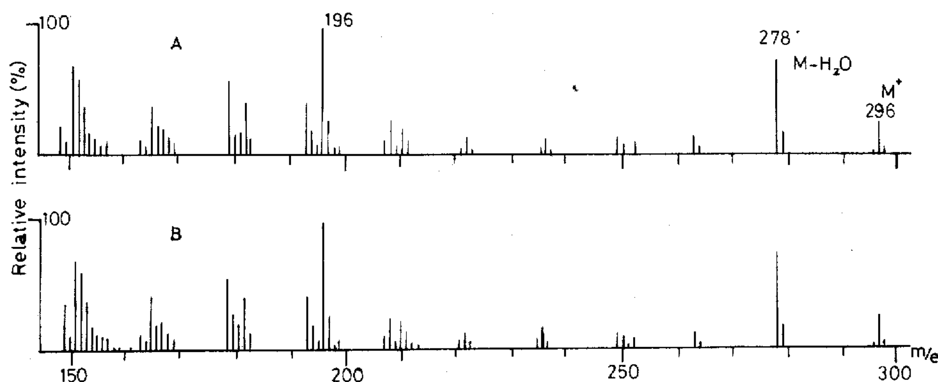

Fig. 5. GC electron impact mass spectrum of phytol. (A) Standard phytol. (B) Phytol from sample extract (chlorella).

TABLE I

CHANGES IN THE AMOUNT OF CHLOROPHYLL DERIVATIVES BY CHLOROPHYLLASE DURING INCUBATION FOR 3 h

Results are expressed as mean ± S.D. (n = 3).

Sample	*Content of pheophorbide a, pyropheophorbide a and phytol (mg/100 g)*					
	Before incubation			*After incubation*		
	Pheophorbide a	*Pyropheophorbide a*	*Phytol*	*Pheophorbide a*	*Pyropheophorbide a*	*Phytol*
Chlorella 1	75.1 ± 6.5	—	35.0 ± 3.1	421.5 ± 35.8	—	315.0 ± 27.5
Chlorella 2	70.5 ± 5.8	—	35.6 ± 2.7	293.8 ± 12.0	—	210.3 ± 10.0
Chlorella 3	61.0 ± 4.5	—	26.9 + 2.0	153.8 + 5.8	—	73.0 ± 8.5
Chlorella 4	190.1 ± 10.3	—	100.0 ± 9.0	227.0 ± 15.5	—	155.0 ± 8.0
Chlorella 5	47.4 ± 2.0	—	25.3 ± 1.0	51.0 ± 3.0	—	25.5 ± 2.0
Vegetable 1*	7.5 ± 0.5	—	3.8 ± 0.1	10.3 ± 0.4	2.0 ± 0.1	6.4 ± 0.2
Vegetable 2**	7.0 ± 0.3	—	3.6 ± 0.1	6.6 ± 0.2	5.7 ± 0.2	6.0 ± 0.3

* Vegetable 1 = *Brassica* s.p. Nozawana.
** Vegetable 2 = *Brassica pekinensis* var. *Hiroshima*.

The structural confirmation of phytol in samples was made by GC–MS (Fig. 5). The spectrum indicates a molecular weight of 296 for phytol and is identical with that reported by Lilijenberg and Odham[15].

This technique may also be applicable to the determination of chlorophyllase activity. Table I shows the amount of chlorophyll derivatives in chlorella and their changes after incubation for 3 h. In fresh chorella, phytol content (M.W. 296.5) was about half of the pheophorbide *a* content (M.W. 593.2) and consequently, the molar ratio was about 1:1. But in some incubated chlorella, it was about 3:2, which may be due to further decomposition of pheophorbide *a* during incubation. This indicates that chlorophyllase activity can be determined more precisely by determining phytol than by determining pheophorbide *a*.

REFERENCES

1 Y. Hashimoto and J. Tsutsumi, *J. Food Hyg. Soc. Jap.*, 4 (1963) 185–191.
2 R. Amano, K. Ike and M. Uchiyama, *Food Sanitation Res.*, 28 (1978) 739–745.
3 Y. Komai, K. Onuki, H. Yamagishi and T. Shiratori, *Food Sanitation Res.*, 4 (1978) 747–752.
4 Y. Tamura, S. Nishigaki, T. Maki and Y. Shimamura, *Food Sanitation Res.*, 4 (1978) 753–759.
5 K. Miki, O. Tajima, E. Matsuura, K. Yamada and T. Fukimbara, *Nippon Nogeikagaku Kaishi*, 54 (1980) 721–726.
6 K. Yamada and N. Nakamura, *J. Jap. Soc. Food Natur.*, 25 (1972) 466–471.
7 T. Ogawa, *Food Sanitation Res.*, 31 (1981) 437–446.
8 G. Krossing, *Biochemistry*, 305 (1940) 359.
9 R. J. Daley, C. B. J. Gray and S. R. Brown, *J. Chromatogr.*, 76 (1973) 175–183.
10 S. W. Jeffrey, *Biochim. Biophys. Acta*, 162 (1968) 271–285.
11 Y. Chiba, I. Aiga, M. Idemori, Y. Satoh, K. Matsushita and T. Sasa, *Plant Cell Physiol.*, 8 (1967) 623–635.
12 S. J. Schwartz, S. L. Woo and H. von Elbe, *J. Agr. Food Chem.*, 29 (1981) 533.
13 F. C. Pennington, H. H. Strin, W. A. Sevec and J. J. Katz, *J. Amer. Chem. Soc.*, 86 (1964) 1418.
14 R. K. Ellsworth and H. J. Perkins, *Anal. Biochem.*, 17 (1966) 521.
15 C. Lilijenberg and G. Odham, *Physiol. Planta.*, 22 (1969) 686.