CHROM. 6042

Thin-layer chromatographic identification of zinc and copper complexes of pheophytins and pheophorbides in mixtures with chlorophylls and derivatives

Chlorophyllides and pheophorbides may be formed in a number of processed green food products as the result of chlorophyllase activity which may take place under the conditions of processing the given plant material. Procedures for such conversion have been described in patent and other literature and have been discussed in recent reports[1,2]. Pheophorbides represent the principal chlorophyll derivatives of cucumbers, okra and probably other green vegetables preserved by brining[3,4].

The intentional and unintentional formation of zinc and/or copper complexes of chlorophyll derivatives during food processing for preservation and during storage after processing have been reported. FISCHBACK AND NEWBURGER[5] indicated the practice of addition of zinc chloride to okra at the time of canning, for color enhancement. SCHANDERL *et al.*[6,7] in spectrometric and spectrophotometric studies concluded that regreening of stored, processed vegetables, sometimes seen in the commercial processing industry, was caused by the formation of complexes with very small amounts of copper and zinc in the food product. The pigments were pheophytin complexes, although the possibility of pheophorbide complex formation was suggested. JONES *et al.*[8] have investigated spectral curve characteristics and metal content of pigment extracts from cucumbers to which solutions of copper and zinc salts were added during brining and pickling. The formation of copper and zinc complexes was observed.

The occurrence of copper pheophorbide in the gut of certain marine life was reported by KENNEDY AND NICHOL[9]. Studies on the formation of copper pheophytin in grass meal suspensions with copper ions in low concentration has suggested an interference of chlorophyll with the copper utilization of grazing cattle, under certain conditions[10].

SWEENY AND MARTIN[11], in a study of chlorophyll analyses of fresh and cooked vegetables, suggested that the formation of zinc complexes in extracts might provide a useful qualitative check on their method. Based on the absorption spectra of copper and zinc complexes of the pheophytins and pheophorbides[12], a quantitative method has been developed for the estimation of the components of pigment mixtures of the chlorophylls, chlorophyllides, pheophytins and pheophorbides and the copper and zinc complexes of the pheophytins and pheophorbides[13]. The study to be reported is a continuation of the thin-layer identification of the chlorophylls and derivatives by reversed phase partition[14].

Zinc and copper complexes of the pheophytins and pheophorbides were prepared by procedures previously described[12]. Thin-layer chromatographic (TLC) techniques were adapted from the study by JONES *et al.*[14]. The separated zones of copper and zinc complexes in thin-layer chromatograms are characteristically blue-green and yellow-green for the *a* and *b* components respectively, when viewed under white light. Under ultraviolet (UV) light the zones of zinc complexes fluoresce reddish to pinkish. Copper complexes are non-fluorescent and appear as black lines or spots when illuminated with UV.

As reported earlier, zinc and copper chelates of pheophytins and pheophorbides are readily allomerized during preparation and storage[12]. Such pigments resemble their chlorophyll and chlorophyllide counterparts by being slightly more strongly adsorbed when allomerized than when not allomerized. As do the chlorophyllides, the zinc and copper pheophorbides tend to separate as enlarged spots and streak-like, elongated zones. It is necessary to add antioxidant to the pigment before applying to Kieselguhr G or Kieselguhr G–Silica Gel G mixture plates to avoid artifact formation, as reported by JONES *et al.*[14]. Presented in Table I are approximate hR_F values for

TABLE I

APPROXIMATE hR_F[a] VALUES OF CHLOROPHYLLS AND DERIVATIVES CHROMATOGRAPHED BY REVERSED-PHASE PARTITION

Layer: Kieselguhr G, impregnated with peanut oil in isooctane (14% v/v); solvent: methanol–acetone–water (20:4:3) saturated with peanut oil; run 10 cm on impregnated area.

Phytylated	hR_F[a]	*Non-phytylated*[a]	hR_F[a]
Chlorophylls and derivatives			
Pheophytin *a*	1	Pheophorbide *a*	34
Pheophytin *b*	1	Pheophorbide *b*	39
Chlorophyll *a*	5	Chlorophyllide *a*	81
Chlorophyll *b*	9	Chlorophyllide *b*	94
Metal complexes			
Copper pheophytin *a*	0	Copper pheophorbide *a*	30
Copper pheophytin *b*	0	Copper pheophorbide *b*	39
Zinc pheophytin *a*	4	Copper pheophorbide *a*-ox[b]	45
Zinc pheophytin *b*	5	Copper pheophorbide *b*-ox[b]	53
Zinc pheophytin *a*-ox[b]	5	Zinc pheophorbide *a*	56
Zinc pheophytin *b*-ox[b]	9	Zinc pheophorbide *b*	60
		Zinc pheophorbide *a*-ox[b]	65
		Zinc pheophorbide *b*-ox[b]	71

[a] $hR_F = 100 \times R_F$ (ref. 15).
[b] Signifies allomerized pigment (phase test negative).

non-allomerized and allomerized pigments. The close similarity of the values for the zinc pheophytins and their chlorophyll counterparts practically precludes identification solely by hR_F values. Identification of the various pigments is based on a comparison of separated zone hR_F values; color under white light and UV; and response to solution acidification during or before chromatography.

Differential conversion of magnesium-containing and zinc-containing derivatives can be accomplished on thin-layer plates by the use of an acidified developing solution of suitable composition. In Fig. 1 evidence is presented of the identification of zinc complexes by this procedure. The chromatogram shown is a composite in which the segment numbered 1 was the lower portion of a chromatogram on a plate developed with a solution of methanol–acetone–water (20:4:2) and segment numbered 2 was the lower portion of a chromatogram on a plate developed with methanol–acetone–saturated aqueous oxalic acid (20:4:2). For this demonstration, the developing solutions were made slightly less polar than that used in obtaining the values listed in Table I (20:4:3), in order to favor greater separation of the chlorophylls and the zinc pheophytins.

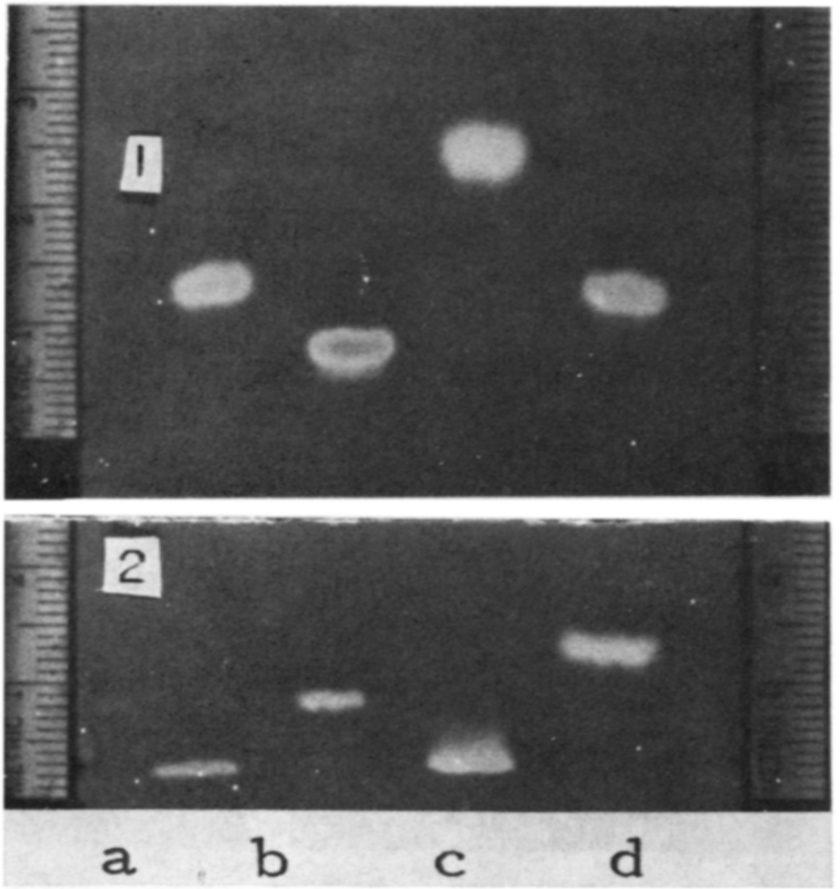

Fig. 1. Thin-layer chromatogram of chlorophylls and zinc pheophytins by reversed-phase partition. Layer: Kieselguhr G, impregnated with peanut oil in isooctane (14% v/v); solvent: segment 1—methanol–acetone–water (20:4:2) saturated with peanut oil, run 10 cm on impregnated area; segment 2—methanol–acetone–saturated aqueous oxalic acid (20:4:2) saturated with peanut oil, run 10 cm on impregnated area; pigment: segment 1—(a) chlorophyll *a*, (b) zinc pheophytin *a*, (c) chlorophyll *b*; (d) zinc pheophytin *b*. segment 2—(a) pheophytin *a*, (b) zinc pheophytin *a*, (c) pheophytin *b*, (d) zinc pheophytin *b*, Application on impregnated area; visualization: UV 365; recording: at 10 min after development, Polacolor 108, copied in black and white.

Segment 2 of Fig. 1 demonstrates a distinguishing characteristic of the zinc complexes. The zinc pheophytins were not converted to free pheophytins during chromatography with the acidified solvent; the chlorophylls were. Not shown in Fig. 1, but observed, was the conversion of chlorophyllides to free pheophorbides and the non-conversion of the zinc pheophorbides to the free pheophorbides by the acidified solvent (20:4:2). Verification of the presence of the zinc complexes was accomplished by acidification of a diethyl ether solution of the pigments with 12 *N* hydrochloric acid (0.05 ml/25 ml). The conversion took place during a 1 h period, after which the acid was washed out with water. The solution was again chromatographed yielding only a chromatogram of free pheophytin(s) and/or free pheophorbide(s).

The copper complexes may be readily identified. Under white light chromatographed *a* or *b* zones are characteristically blue-green or yellow-green, respectively, while under UV these zones are black (non-fluorescent). Copper complexes are much more stable than zinc complexes in acidified solutions and are not converted by such solutions into fluorescent copper-free components.

Study supported in part by Research Grant FD-0078 from the Food and Drug Administration D.H.E.W.

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First received November 16th 1971; revised manuscript received March 14th, 1972