Studies on the Bio-Pigments and Vitamins

III. The Application of Paper Chromatography to the Separation and Determination of Carotenoid Pigments

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Introduction

Numerous investigations on the separation and determination of naturally occurring carotenoid pigments have been reported. Most of these, however, employ the column chromatographic method and in this method an appreciable amount of sample is needed, and moreover analytical procedures are complicated. If the paper partition chromatography (PPC) may be able to apply for the separation and determination of these pigments, it will be able to reduce an amount of used materials and simplify analytical procedures.

Up to now, few reports appeared on this subject of the carotenoid pigments. For instance, Bauer[1] has reported on the qualitative separation of these pigments and chlorophylls by PPC using various organic solvents-mixture as developing solvent. Recently, Suzuki[2] has reported on PPC for micro-determination of vitamin A and carotene and obtained considerable successful results and however, considerations for the other carotenoids have been removed. Therefore, the authors have attempted the fundamental studies on PPC for the separation and determination of carotene, lycopene and xanthophylls which occur abundantly in plant materials, from the view point of practical use. It is purpose of this paper to show the possibility that adsorbents-impregnated PPC may be a rapid and simple method for the separation and determination of these pigments obtained from plant tissues.

Method and Apparatus

Pigment standards from natural sources were prepared as follows: carotene was extracted from sweet potato tubers (Hayato variety) and lycopene from tomato fruits in the usual manner in this laboratory and they were purified in crystal form as shown in Fig. 1 and 2. Lutein (one of the xanthophylls) was prepared from rice plant-straws, although it could not be obtained in crystal form. Pigment standards thus obtained, contents of which were previously measured by the Beckman type spectrophotometer as described later, were mixed with p-ether (b.p. 40–60°C) and the mixture was used for the qualitative and quantitative analyses in the fundamental studies.

The method employed for the preparation of the adsorbents-impregnated papers is essentially that descrived by Suzuki.[2][3] The following two kinds of papers were prepared in this laboratory: $Ca(OH)_2$- and $MgCO_3$-impregnated paper (Ca-

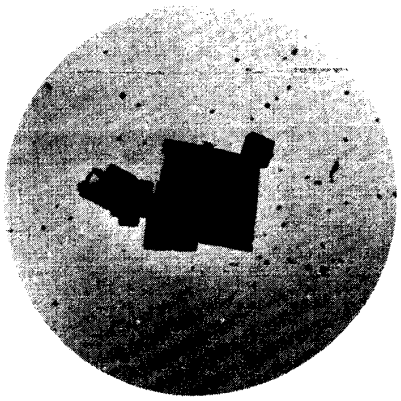

Fig. 1. β-carotene from p-ether

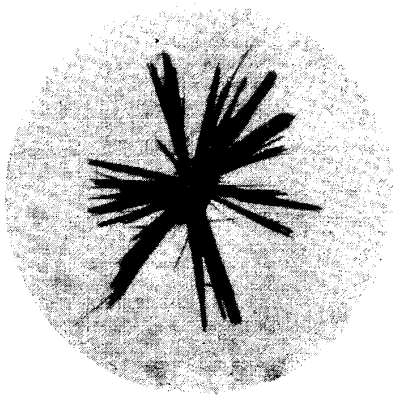

Fig. 2. Lycopene from p-ether

and Mg-paper, respectively). In one instance, the preparation of Mg-paper was carried out as follows: Tōyō No. 50 filter paper was immersed in 10% $MgSO_4$ and then in 10% Na_2CO_3 solution for 10 minutes respectively, followed by washing with water and drying in air oven at 120° C for 10 minutes accurately. Especially it has been recognized by many workers that the drying temperature is the most important factor influencing upon the paper's activity, so that the maintenance of the drying temperature (120° C) was performed with extreme precautions.

Ascending chromatographic technique was used in 3 × 21 cm large sized test-tubes with cork stoppers. The pigments mixture was previously spotted on the 2 × 19 cm impregnated paper strips at the point of 3 cm from the bottom and the strips were dried and suspended in the test-tubes in addition all being placed so that they dipped into the solvent. P-ether (40–60° C) was used as developing solvent (It has been found most suitable in preliminary studies in this laboratory.[4]). In the repeated experiments, it has been found that Rf value on the chromatogram of the individual pigment might be changed by various factors described later, (temperature, volume of solvent, concentration of sample, etc.) and therefore, the developing process has been carried out under a constant condition.

Qualitative identifications of the individual pigment on the paper strips dried after the development were done by comparison of Rf value on the chromatogram with that of a known pigment-standard.

Quantitative analyses for the pigment concentration were carried out as follows: a pigment band on the chromatogram was cut off from the paper strip and eluted with acetone and evaporated in an all-glass still under reduced pressure and a slow stream of CO_2, and the residues thus obtained were dissolved in 5 cc of p-ether and its absorbance was measured with the spectrophotometer. The specific extinction coefficient was calculated in the usual manner.

$$E\,\frac{1\%}{1\,\mathrm{cm}} \text{ at } 450\,\mathrm{m}\mu \text{ in p-ether} = 2430^{(5)}$$

Results and Discussion

1. Qualitative and Quantitative Studies on the Pigment Standards During the period of the repeated developments with p-ether, it has been found that Rf values on the

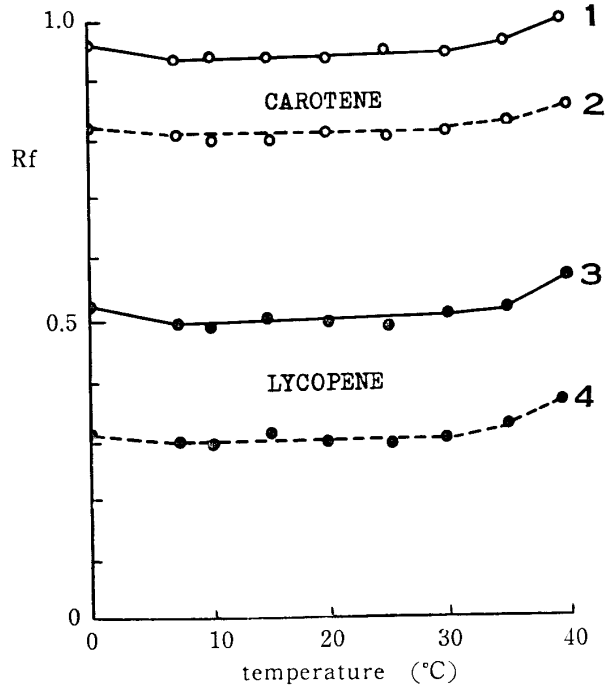

Fig. 3. Effect of the developing temperature on Rf values. One spot contained 0.5 γ of each pigment. P-ether was used as developing solvent. Curve 1 and 3, on Ca-paper; curve 2 and 4, on Mg-paper.

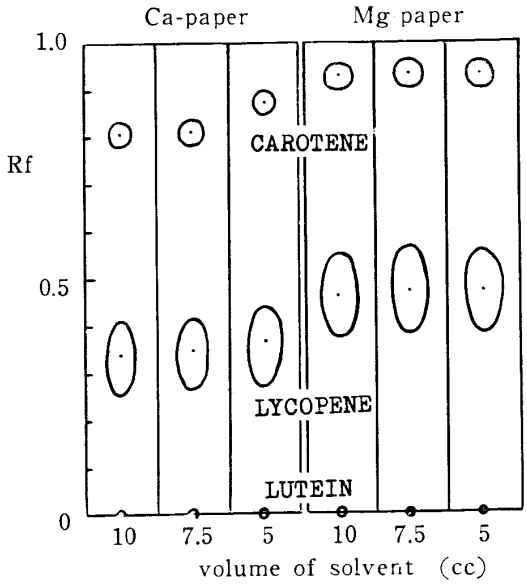

Fig. 4. Effect of the solvent volume on Rf values

chromatograms might be changed by various factors described befor, so that the following experiments have been designed to study on these factors.

At first, effect of the developing temperatures on Rf values on the chromatograms is given in Fig. 3 where Rf values remain unchanged at ordinary room temperatures (10–30° C) and however, are changed at a temperature of more than 30° C. Therefore, it will be suggested that the development must be performed at a temperature of from 10° C to 30° C.

Volume of the developing solvent is also one of the important factors giving an effect to Rf values. Fig. 4 indicates that both carotene- and lycopene-standard have showed somewhat unusual behaviour due to the difference of the solvent volume during the development on Ca-paper and on the other hand, have not on Mg-paper. Lutein remained at the original point in either case. Accordingly, above all in the case of Ca-paper, it must be considered that an accurate volume of the solvent should be taken. In all following experiments, 10 cc of the solvent was always used in this laboratory.

Subsequently a study was carried out on an effect of amounts of sample spotted on Rf value. It is indicated in Fig. 5 that Rf value of both carotene- and lycopene-standard on Ca-paper become gradually higher with increasing over 1.0 γ in quantity and on the other hand in the case of Mg-paper, that of only lycopene become likewise higher over 0.5 γ, although that of carotene was hardly influenced within from 0.1 γ to 5.0 γ. Therefore, it can be said that carotene and lycopene may be separated without any change of Rf value when amounts less than 0.5 γ of sample are used on Ca-paper and less than 0.2 γ on Mg-paper.

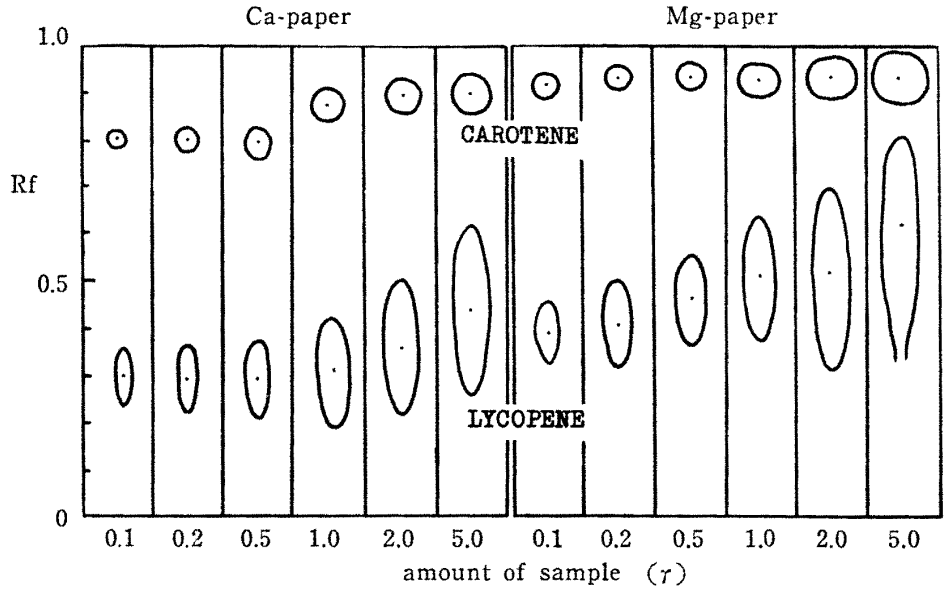

Fig. 5. Effect of the sample amount on Rf values

From the above findings, it was qualitatively confirmed that there are several developing conditions necessary to maintenance of the constant Rf value.

In the next step, the authors have attempted to find a detectable minimum quantity of pigment standard on the paper strip. Detection was done by vision, as these pigments are colored from red to yellow. Table 1 shows its results, in which its amounts are 0.05 γ for carotene and 0.04 γ for lycopene.

Table 1. Decision of the detectable minimum quantity of pigment standards on the chromatogram

pigment	amounts contained in one spot (γ)	finding by vision
carotene	0.06	+
	0.05	+
	0.04	±
	0.03	−
lycopene	0.04	+
	0.03	±
	0.02	−

But, the following problems must be previously considered before studying on the application of this impregnated PPC to the separation and determination of the naturally occurring carotenoid pigments: namely, those of a determinable minimum quantity and a degree of recovery of sample spotted on the paper strip remain. For this purpose, such experiments as shown in Table 2 and 3 have been studied. Table 2 shows the numerical values that carotene standard solution showing 0.496 of the absorbance has been diluted by 2, 5, 10, 20 and 40 times with p-ether and its absorbance has been measured in each diluted solution. According to these results, it may be observed that the carotene standard solution can be estimated in concentration of more than 10.2 γ % almost without errors. No experiment has been done with lycopene and in the usual case, however, the contents of lycopene are calculated as β-carotene. Therefore, the results shown in Table 2 can be equally applicable to lycopene.

Table 2. Decision of the determinable minimum quantity for carotene standard solution

dilution	concentration (γ %)	absorbance		ratio (%)
		E_0	E	$E/E_0 \times 100$
1	204	0.496	0.496	100
2	102	0.248	0.249	100
5	41	0.099	0.102	103
10	20.4	0.050	0.050	100
20	10.2	0.025	0.026	104
40	5.1	0.012	0.019	153

E_0 : theoretical
E : experimental

Table 3. Recovery of pigment standards by the adsorbents-impregnated paper chromatography

pigment	amounts in one spot (γ)	E_0	Ca-paper		Mg-paper	
			E	$E/E_0 \times 100$ (%)	E	$E/E_0 \times 100$ (%)
carotene	0.56	0.028	0.026	92.4	0.027	96.4
	1.12	0.055	0.052	94.5	0.056	98.2
	2.24	0.109	0.104	95.4	0.108	99.1
	3.36	0.165	0.157	95.1	0.160	97.0
	4.48	0.218	0.203	94.5	0.226	103.6
lycopene	0.52	0.025	0.023	96.0	0.024	96.0
	1.01	0.049	0.046	93.8	0.049	100.0
	2.02	0.098	0.093	96.0	0.092	94.0
	3.58	0.174	0.164	94.0	0.168	96.5
	5.48	0.266	0.252	94.8	0.253	95.1

Experiments were designed to find a degree of recovery of pigment spotted on the paper. The results are shown in Table 3, in which the degree of recovery amounts to about 95 per cent in all cases and especially, that of carotene about 98 per cent on Mg-paper. Therefore, Ca- as well as Mg-paper is a satisfactory impregnated paper for this work.

2. Attempts for the Separation and Determination of Naturally Occurring Carotenoid Pigments. On the basis of the fundamental investigations above mentioned, the application of the adsorbents impregnated PPC to the separation and determination of carotenoid pigments from various plant materials has been attempted. The method and apparatus are the same as described before. The materials used for this work are as follows: tomato, *Citrus grandis*, carrot, spinach, sweet potato tuber and its leaf.

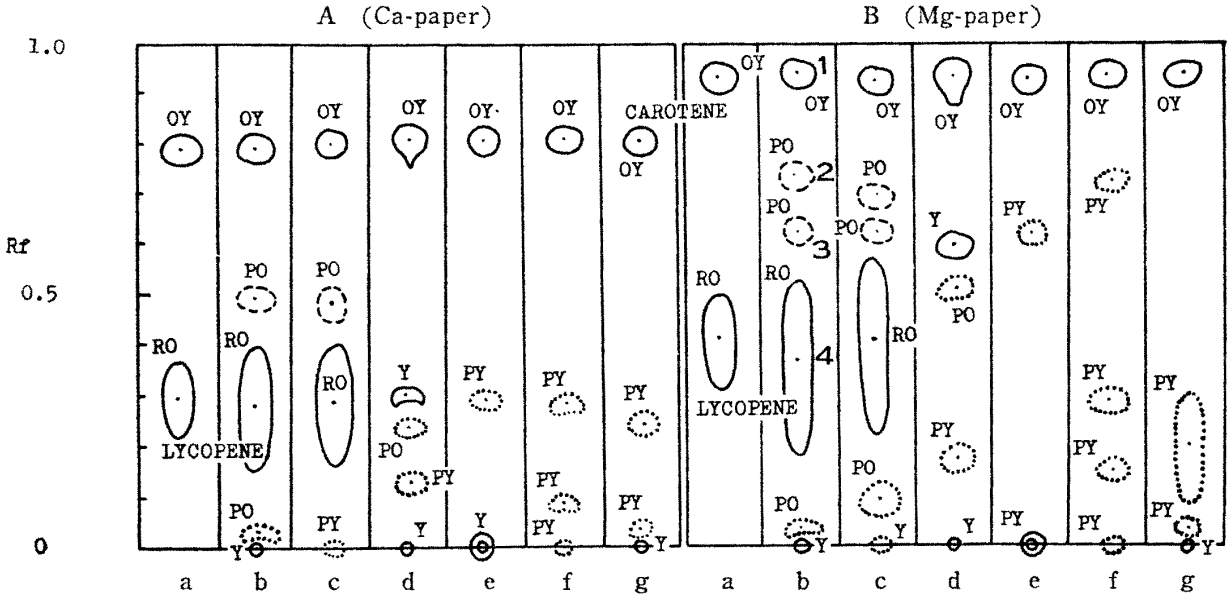

Fig. 6. Chromatograms of carotenoid pigments from natural materials by the adsorbents-impregnated paper chromatography

Materials: a, control. b, tomato. c, *Citrus grandis* d, carrot. e, spinach. f, sweet potato tuber. g, sweet potato leaf.

Symbols of color: O, orange. Y, yellow. R, red. P, pale.

Fig. 6 shows the paper chromatograms of pigments from the aforementioned plant tissues. In this figure, it is shown that the chromatograms of Ca-paper are different from those of Mg-paper. For instance, five spots were recognized on the former in the case of the tomato's pigments (Fig. 6Ab) and on the other hand, six spots on the latter (Fig. 6Bb). Similar results were also obtained in the case of the other plant materials.

There seemed to be two possible explanations for the above findings: (a) It may be due to an excellent separation power of Mg-paper. (b) During the period of development, a kind of pigment (especially lycopene) might be isomerized, though great care was excercised to avoid isomerization, because it is well known that this pigment isomerizes in solution very rapidly.[5] Attempts were made to elucidate the question for these possibilities. When the pale orange spot showing 0.5 in Rf value on the chromatograms of Ca-paper (Fig. 6Ab) was cut off from the strip and eluted with acetone and then applied to rechromatography on Mg-paper, this spot was separated into the same two spots (0.63 and 0.74 in Rf value) as shown in Fig. 6Bb. This finding suggests that Mg-paper is more excellent in separation power than Ca-paper. Moreover, this suggestion could be confirmed by the fact that there has been never such behaviour when pure lycopene standard was developed on Mg-paper. Therefore, Mg-paper has been found most reliable characteristic for this work. And furthermore, the partial identification and the content measurement of the separated pigments were done. Identification was accomplished by comparison of Rf value of unknown pigment with that of the control (Fig. 6) and at the same time absorption measurements were made with the spectrophotometer as the needs of the case demand. One illustration is presented in Table 4

Table 4. Absorption maxima of some carotenoid pigments found in tomato fruit by the adsorbents-impregnated paper chromatography

identified pigment	pigment* No.	absorption maxima (mμ)	
		p-ether (60–70° C)	$CHCl_3$
β-carotene	1	478, 450, 425 (478, 448, 420)	492, 464 (497, 466)
neo-lycopene	2	495, 465, 441, 362 (499.5, 468, 439)	
neo-lycopene	3	497, 466, 441, 362	
lycopene	4	503, 473, 445, 363 (506, 475.5, 447)	514, 482, 454 (517, 480, 453)

* Refer to Fig. 6Bb.
Figures in ( ) show those of literature.[7]

and Fig. 7. Pigment 1 and 4 are identified as β-carotene and lycopene respectively, by their spectral absorption data (Table 4) and relative position on the paper strip. Pigment 2 and 3 are likely considered to be *neo*-lycopene, an isomer of lycopene which has been found previously in this laboratory[6] and then a degree of isomerization might be larger in pigment 2 than in pigment 3, but more experience would be probably required to confirm its speculation.

Next problem remains on the contents of the pigments which can be determined spectrophometrically as mentioned before. The determination by the usual column chromatographic method (A.O.A.C. method[8]) was paralleled for the purpose of the comparison with the adsorbents-impregnated PPC. The results are shown in Table 5 where a considerable good agreement between values obtained by the PPC- and A. O. A. C.-method was found, with one marked exception, from the practical use standpoints. The exception is

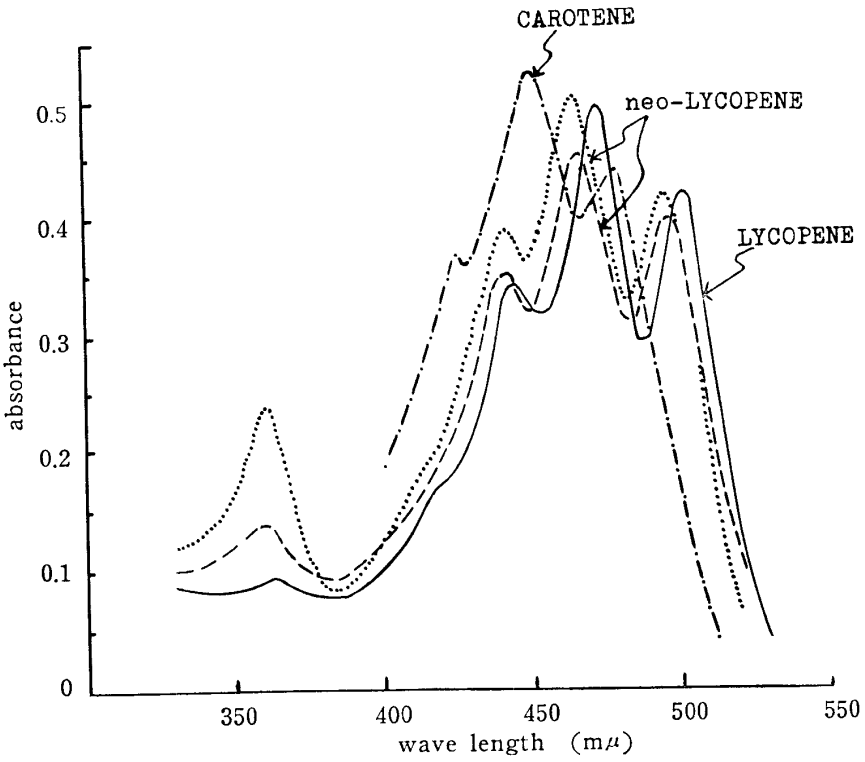

Fig. 7. Absorption curves of some carotenoid pigments found in tomato fruit

Table 5. Comparison of between two methods for the pigment contents obtained from various materials

pig-ment	sample	contents (γ %)			ratios (%)	
		A.O.A.C. (A)	Ca-paper (B)	Mg-paper (C)	$\frac{(B)}{(A)}\times 100$	$\frac{(C)}{(A)}\times 100$
carotene	sweet potatoes					
	Kagoshima					
	7-1057···	12,243	12,071	11,831	98 6	96.6
	7-1061···	13,745	13,306	13,580	97.3	98.8
	Hayato ·····	1,914	1,881	1,975	98.2	103.0
	carrot·····	12,840	12,757	13,087	99.4	102.0
	spinach ·····	3,523	3,251	3,539	92.2	100.5
	tomato ·····	753	741	724	98.4	96.2
	Citrus grandis ·····	85	88	82	103.3	96.0
lycopene	tomato 1 ·····	2,812	2,112	2,147	75.1	76.4
	tomato 2 ·····	2,454	1,881	1,878	76.6	76.5
	Citrus grandis ·····	297	258	248	86.6	84.0

observed on tomato fruits, in the case of which there seemed to be the following possible explanation for disagreements between values by the two methods. Briefly, lycopene content obtained by A.O.A.C. method presents that of lycopene itself plus *neo*-lycopenes as discussed later and on the other hand, by the adsorbents-impregnated PPC, that of only lycopene. This finding was further confirmed by the fact that, when a single layer belonging to lycopene on the column chromatograms was eluted with the solvent and was rechromatographied by the adsorbents-impregnated PPC, it showed the same chromatograms as shown in Fig 6Ab and 6Bb.

The results thus obtained are chiefly concerned about the qualitative and the quantitative analyses for three carotenoid pigments (carotene, lycopene and xanthophylls). Therefore, further examinations would be required for studies on the other carotenoid pigments and the separation of xanthophylls from each other.

Summary

It is well known that there are numerous publications concerning the column chromatography for the separation and determination of naturally occurring carotenoid pigments, but few reports appeared on the application of the paper partition chromatography for this subject.

The present report was undertaken to study fundamentally on these possibilities by means of the adsorbents-impregnated paper partition chromatographic techniques.

The experiments may be briefly summarized as follows:

(1) Adsorbents-impregnated papers (Ca- and Mg-paper) were prepared for these purposes and it was confirmed that they are of equally excellent properties in the preliminary studies using pigment standards (carotene, lycopene and lutein).

(2) During the period of the repeated experiments, it was recognized that Rf values on the chromatograms may be changed, and therefore various factors influencing upon Rf values were studied.

(3) The qualitative and the quantitative analyses were performed for the purpose of the separation and identification of carotenoid pigments; both a detectable and a determinable minimum quantity of pigments were decided on the standard mixture. The former was more than 0.05 γ for carotene and more than 0.04 γ for lycopene and the latter more than 10.2 γ per cent. Still more, a degree of recovery of the pigment was studied and that of both carotene and lycopene amounted to about 95 per cent.

(4) On the basis of the fundamental studies, the application of this method to the separation and determination of the carotenoid pigments from the following plant materials has been made: tomato, *Citrus grandis*, carrot, spinach, sweet potato tuber and its leaf. The results obtained are as follows: (a) The lycopene contents obtained by PPC are less than those by A.O.A.C. method. The possible explanation for this problem is described in detail. (b) It has been proved that this method is applicable to the practical use.

References

1) Bauer, L.: *Naturwissenschaften*, **39**, 88 (1952)
2) Suzuki, T.: *Vitamin (Japan)*, **13**, 212 (1957)
3) Suzuki, T.: *J. Agr. Sci. Tōkyo Agr. Univ.*, **3**, 17 (1956)
4) Yamamoto, Y.: *unpublished*
5) Peterson, W. J., Hughes, J. S. and Freeman, H. F.: *Ind. & Eng. Chem. Anal. Ed.*, **9**. 71 (1937)
6) Yamamoto, Y. and Tomita, Y.: *J. Agr. Chem. Soc., (Japan)* **31**, 650 (1957)
7) Karrer, P.: *Carotenoids*, P. 120, P. 124, P. 135 (1950)
8) A.O.A. C. *7th ed.*, P. 770 (1950)