ACTA CHEMICA SCANDINAVICA 13 (1959) 1863–1868

Quantitative Paper Chromatography of Carotenoids

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A highly selective, rapid and simple method for the quantitative, circular chromatography of carotenoids on a commercially available paper with a kieselguhr filler is described. The R_F-values for a series of carotenoids are listed in Tables 1 and 3. The applications of the method are discussed.

The indisputable success of paper chromatography for the separation of complex mixtures of closely related compounds would lead one to expect that this method has been well developed also in carotenoid chemistry. One of the reasons for this not being the case may be the efficiency of column chromatography in the carotenoid field. The few published records of paper chromatography are concerned mainly with the separation of carotenoids from chlorophylls [1-3]. No simple and satisfactory system for separation of carotenoid mixtures on paper seems to have been developed.

Since kieselguhr columns have been used successfully in column chromatography of carotenoids [4], it was expected that paper with a kieselguhr filler might prove useful. The present work led to the development of a highly selective, rapid and simple method for the separation of carotenoid mixtures.

MATERIALS AND METHODS

Schleicher and Schüll filter paper No. 287 (Kieselguhrfilter, kieselguhr content about 20 %) was used throughout this work. The solvents were of analytical grade and used without further purification. The β-carotene, lutein and zeaxanthin were synthetic products obtained commercially. A sample of synthetic cryptoxanthin was kindly supplied by Hoffmann-La Roche & Co., a crystalline sample of chloroxanthin from a green mutant of *Rhodopseudomonas spheroides* by Dr. T. O. M. Nakayama and a crystalline specimen of rhodoxanthin from *Narthecium ossifragum* (L.) Huds, by Dr. A. Stabursvik. Crystalline samples of astaxanthin from lobster shells, lycopene from tomatoes, fucoxanthin from brown seaweeds and violaxanthin from *Viola tricolor* were provided by the Organic Chemistry Laboratories of this University. Crystalline bacterioruberine *a* was isolated from a *Halobacterium* sp. (Strain 1c, from the Department of Biochemistry, this University), whilst spirilloxanthin, P481, OH-P481 and rhodopin were isolated in the crystalline state from *Rhodospirillum rubrum* (Strain S1) and *Rhodopseudomonas palustris* (from dried cells kindly supplied by Dr. C. B. van Niel, Hopkins Marine Station, Pacific Grove, Calif.);

Table 1. R_F-values for a series of carotenoids

Carotenoid	Member of the set	Structure	Conjugated double bonds	Isolated double bonds
β-Carotene	all-*trans*	bicyclic	11	0
Cryptoxanthin	all-*trans*	»	11	0
Zeaxanthin	all-*trans*	»	11	0
Lutein	all-*trans*	»	10	1
Violaxanthin		»	9	0
Astaxanthin	all-*trans*	»	11	0
Rhodoxanthin	all-*trans*	»	12	0
Fucoxanthin	all-*trans*			
γ-Carotene	all-*trans*	monocyclic	11	1
Lycopene	all-*trans*	aliphatic	11	2
P481 [6]	all-*trans*	»	12 [7]	1 [7]
	Neo A [8]	»	12	1
Spirilloxanthin [9]	all-*trans*	»	13	0
	Neo a [8]	»	13	0
	Neo b [8]	»	13	0
Chloroxanthin [10]	all-*trans*	»	9	2
Rhodopin (OH-Lycopene) [11]	all-*trans*	»	11	1
OH-P481 [6]	all-*trans*	»	12 [12]	0 [8]
	Neo A [8]	»	12	0
	Neo B [8]	»	12	0
Bacterioruberine α [14]	Neo U [8]	»	13	0
	all-*trans*	»	13	0
	Neo A [8]	»	13	0
	Neo B [8]	»	13	0

* acetone in pet. eth. b.p. 60—70°C.

γ-carotene was isolated from *Chlorobium thiosulfatophilum* (Strain L1, obtained from Dr. J. Lascelles, Department of Microbiology, University of Oxford).

The chromatographic separations were carried out on circular papers at room temperature in a Petri dish or a desiccator using the technique of Rutter [5]. The solvent system consisted of petroleum ether (b.p. 60—70°C) alone or mixed with various amounts of acetone. For the separation of chloroplast pigments of brown algae petroleum ether with 0.5—2 % *iso*propanol was found favourable. The carotenoid mixtures were dissolved either in acetone or in acetone-petroleum ether mixtures. An aliquot containing 10—100 μg of carotenoids was applied in portions to the centre of the paper by means of a capillary such that the diameter of the spot did not exceed 1 cm. After the application of each portion the solvent was evaporated at room temperature in a stream of pure nitrogen. When the whole aliquot had been put on, a small amount of acetone was applied to the paper on the wick just outside the edge of the spot. This caused the carotenoids to form a narrow ring shaped band surrounding the centre of the paper and was found to result in more regular zones. The separation took 15 min with a 12.5 cm and 20 min with an 18 cm diameter paper. For determination of R_F-values the papers were dried in a stream of warm air, the zones marked with a pencil and the position of the solvent front was determined in UV light (bluish fluorescence). Otherwise the chromatograms were not dried before elution of the pigments in order to avoid oxidation of the carotenoids. The coloured zones containing the carotenoids were cut out immediately after separation, tightly packed in small glass tubes with one end drawn out to a capillary and eluted with acetone, whereafter the extracts were made up to a known volume with acetone and the absorption spectrum determined in a Zeiss PMQ2 spectrophotometer. When separating compounds with low R_F-values the solvent was allowed to evaporate from the rim of the paper which extended outside the petri dish.

on Schleicher & Schüll No. 287 paper.

Functional groups	Character of OH-groups	R_F-value				
		0 %	2 %	5 %	10 %	20 % *
		0.95	0.98	0.98	0.98	
1 OH	secondary	0.29	0.62	0.81	0.91	
2 OH	secondary		0.09	0.30	0.59	0.87
2 OH	secondary			0.39	0.72	0.91
2 OH, 2–O–	secondary			0.18	0.44	0.83
2 OH, 2 C=O	secondary				0.57	0.85
2 C=O				0.10	0.27	0.72
>(2 OH, 2 C=O)					0.40	0.81
–		0.68				
–		0.53	0.86			
1 OCH_3[7]			0.35	0.60		
1 »			0.50	0.75		
2 OCH_3			0.18	0.40	0.76	
2 »			0.31	0.54		
2 »			0.48	0.73		
1 (?) OH	tertiary	0.13	0.46	0.73	0.90	
1 OH	tertiary			0.39	0.75	
1 OH[6], 1 OCH_3[13]	tertiary[8]				0.54	
1 » 1 »	»				0.78	
1 » 1 »	»				0.94	
2 (?) OH	tertiary[8]				0	0.36
»	»				0.02	0.44
»	»				0.06	0.51
»	»				0.10	0.57

APPLICATIONS

In the present work the method has been applied mainly to the separation of carotenoids from brown seaweeds and bacteria. The R_F-values obtained for a series of carotenoids are presented in Table 1.

1. Grouping and separation of carotenoids. Quantitative separation of bicyclic carotenes, mono-hydroxy-xanthophylls and dihydroxy-xanthophylls was accomplished as exemplified by the separation of the series β-carotene, cryptoxanthin and zeaxanthin. These groups could be further separated into their individual components in the cases investigated.

Within the group of aliphatic, bacterial carotenoids studied a similarly satisfactory separation of the different all-*trans* compounds was obtained.

2. Separation of stereoisomeric sets. Separation of *cis-trans* isomers belonging to the same set could easily be obtained as seen from Table 1.

3. Recoveries and quantitative determinations. Recoveries obtained by this method were determined by applying known amounts of pure carotenoids to the paper. After the chromatographic development the amount of carotenoid eluted was measured in the spectrophotometer. The results are presented in Table 2.

Table 2. Pigment recovery after chromatography.

Compound	Amount in μg	
	Applied	Recovered
β-Carotene	14.3	14.3
	7.2	7.1
Zeaxanthin	3.9	3.8
	7.8	7.8
	7.8	7.6

The recoveries obtained both with β-carotene and with zeaxanthin were between 97 and 100 %.

The carotene content of dried grass and of seaweed meal was determined by means of paper chromatography. Details of the procedure are to be published[15]. Using the paper chromatographic method, the values obtained for the carotene content were somewhat higher than those obtained with the conventional procedure. This may be a result of the higher over-all recoveries of the new method.

4. Separation of chloroplast pigments. Minor variations in the composition of the solvent system allowed a ready separation of the chloroplast pigments from various sources as shown in Table 3.

DISCUSSION

The efficiency of the Schleicher and Schüll filter paper No. 287 for the separation of carotenoids must be ascribed to its content of kieselguhr, since ordinary filter paper gave very poor separations. Used in circular chromatography the kieselguhr paper allowed a ready separation of carotenoid mixtures into carotenes, mono-hydroxy- and dihydroxy-xanthophylls. These groups could be separated further into their components provided they were more strongly adsorbed than β-carotene.

Table 3. Separation of chloroplast pigments.

Extract of		R_F-values	
		1.5 % *iso*propanol *	10 % acetone *
Ascophyllum nodosum	β-carotene	0.96	
	lutein	0.80	
	violaxanthin	0.36	
	fucoxanthin	0.33	
	chlorophyll a	0.55	
Dried grass	carotene		0.98
	lutein		0.73
	zeaxanthin		0.60
	chlorophyll a		0.41
	chlorophyll b		0.21

* in petroleum ether b.p. 60–70°C.

Probably the best demonstration of the efficiency of the method was the ready separation of *cis-trans* isomers of carotenoids belonging to the same stereoisomeric set. The method allowed quantitative studies of different *cis-trans* isomerization processes to be carried out much more easily, with greater accuracy and with much smaller amounts of pigments than are required for column chromatography. A mixture containing approximately 50 μg of carotenoid could be separated quantitatively into the main stereoisomers which were eluted and examined in the spectrophotometer. The rapidity of the method is particularly valuable for studies of carotenoids which isomerize readily or are otherwise very labile. Thus, it was possible to carry out a stereochemical investigation of bacterioruberine α[8] which was sterically too labile to allow exact *cis-trans* isomerization studies by means of column chromatography.

With slightly modified solvent systems the method gave satisfactory separation of the chloroplast pigments of both algae and grass extracts, and allowed quantitative determination of the major components.

When care was taken, the R_F-values of the compounds were reproducible within ± 0.01 units. The solvent mixtures should be used only once when determining R_F-values; the reason for this is probably that the paper removes relatively more of the acetone than of the petroleum ether. Evaporation of the solvent from the Petri dish during the chromatographic run must be avoided. Large amounts of fatty material in the extracts may influence the R_F-values of the pigments considerably. In such cases saponification or some other purification process should precede the chromatographic separation in order to obtain the characteristic R_F-values.

It may be seen from Table 1 by comparing the R_F-values of the bacterial carotenoids with those of carotenoids from other sources that the R_F-values were not influenced merely by the number of functional groups and the number of double bonds. The nature of the skeleton (aliphatic or cyclic) and the character of the hydroxyl groups (secondary or tertiary) obviously have a definite effect on the R_F-value. For example OH-P481 is more strongly adsorbed to the paper than zeaxanthin and lutein; the reverse is true for adsorption on alumina columns. Bacterioruberine α chromatographs very similarly to lutein on alumina, but is held more strongly than lutein on paper. Also for some of the other pigments investigated the order of adsorbtion on paper differed from that obtained with other adsorbents. The method thus offers an additional tool for identification of chloroplast pigments.

The recovery of pigments extracted from the paper was found to be quantitative, and the eluted pigments showed absorption spectra in visible light comparable with the best recorded in the literature. Quantitative determination of the main components of several complex carotenoid mixtures were successfully performed by means of the above method.

Paper chromatography is particularly useful for purity tests of small samples, for following chemical and biochemical transformations and for investigations in which only very small amounts of material are available. The fact that only small amounts of material are required, and that the method gives a high resolving power, makes it a very good test for identification of carotenoids when combined with co-chromatography of known substances.

Acknowledgements. We are indebted to Mr. Ian Dundas of the Department of Biochemistry for the cultivation of *Chlorobium thiosulfatophilum* and the *Halobacterium* sp., and to Professor N. A. Sørensen, Organic Chemistry Laboratories, for critically reading the manuscript. One of us (SLJ) gratefully acknowledges a maintenance grant from *Norges Tekniske Høgskole.*

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Received July 20, 1959.