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CAROTENOID ANALYSIS BY TWO-DIMENSIONAL, TWO-ADSORBENT THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A thin-layer chromatographic analysis for carotenoids has been developed utilizing a two-adsorbent plate and two-dimensional development with different solvents. The method has been applied to the analysis of alfalfa and corn meal extracts and mixtures of these two. Separation of lutein, zeaxanthin, violaxanthin, neoxanthin, and certain of their isomers, plus isolation of the mono-hydroxy carotenoids, individually or as a group, has been accomplished by this procedure.

INTRODUCTION

The thin-layer chromatographic (TLC) method of NELSON AND LIVINGSTON[1] has been extensively used in this laboratory for the analysis of alfalfa processing and storage samples. Carotene, lutein, and three isomers (designated X_1, X_2 and X_3) and violaxanthin and neoxanthin are separated by that method. However, it has been realized that the lutein fraction contained zeaxanthin, and that the X_3 band, located between lutein and carotene, probably consisted of more than one compound[2]. Resolution of the "lutein" and "X_3" bands is therefore desirable. *Cis* isomers of several xanthophylls are undoubtedly frequently present, particularly in processed plant materials. In addition, the violaxanthin and neoxanthin fractions obtained from stored or adversely treated samples frequently contain 5,8-epoxide isomers in addition to the 5,6-epoxides. We sought to develop a method for separating and estimating all of these carotenoid constituents on one TLC plate.

JENSEN AND JENSEN[3] used Kieselguhr G-impregnated circular paper chromatography to obtain satisfactory separation of grass extract carotenoids into carotenes, monohydroxy- and dihydroxy-xanthophyll groups, but apparently further separation (*e.g.*, of *cis–trans* isomers, lutein–zeaxanthin, etc.) required additional chromatography on this paper. Using the same medium, KATAYAMA[4] found lutein and zeaxanthin mixed and subsequently separated them on magnesium oxide–HyfloSupercel-impregnated paper. Two-dimensional chromatography on a Kieselguhr G TLC plate was used by BUNT[5] for analysis of algal pigments, but he found it difficult or impossible to detect minor xanthophyll components when one sample was chromatographed in both dimensions on one plate. STRAIN *et al.*[6] tried Kieselguhr G TLC

plates with unsatisfactory results due to "bleaching" of the pigments. LOSEV[7] described a distinct separation of lutein from zeaxanthin on a thin-layer of magnesium oxide powder plus starch by one-way development with petroleum ether–acetone (3:1). We initially sought to separate lutein from zeaxanthin on a magnesium oxide thin layer, expecting a second-dimensional development on silica gel — lime to accomplish the other desired separations. Kieselguhr G was later used as a filter aid to modify the magnesium oxide adsorbent.

EXPERIMENTAL

A two-adsorbent TLC plate was prepared, consisting of a strip of magnesium oxide–Celite adsorbent along one side, adjoining the Silica Gel G–calcium hydroxide (1:6) layer of NELSON AND LIVINGSTON. Magnesium oxide powder (ACS Reagent grade) and diatomaceous earth (Celite* Analytical Filter-Aid, Johns Manville Co.) (1:4) were mixed dry and 6.25 g of the mixture was ground in a mortar with 15 ml of water. The silica gel–lime slurry was prepared by mixing 30 g of the blended adsorbent powder with 50 ml of water. The thin-layer applicator (Kensington Scientific Co.) with a 250-μ gate was provided with a reservoir divider spaced 3 cm from the guided edge so that the magnesium oxide–Celite and silica gel–lime slurries could be applied simultaneously to the plates, producing a continuous layer. The (five) plates were then dried and stored in the recommended manner[1].

5 μl of a cold-saponified extract of alfalfa or corn meal, concentrated under reduced pressure so that 1 ml represented 2 g of meal, was applied as one spot on the magnesium oxide–Celite strip 2.5 cm from one end. (In some trials, alfalfa and corn meal extracts were combined in order to augment the array of carotenoids.) This spot was developed first up the magnesium oxide–Celite strip with hexane–acetone (7:3) to which ethoxyquin (0.5 g/250 ml solution) had been added. The plate was then dried briefly in the dark and developed in the second direction with 2 % butanol in benzene, containing ethoxyquin. The resulting spots were scraped off by means of a fritted-glass sample collection tube and eluted with hexane–acetone (7:3), adjusting the final volume to 5 ml. Visible absorption spectra were recorded in a 10-cm path-length cell in a Cary 15 spectrophotometer, using the 0.0–0.1 absorbance slidewire when necessary for the determination of peak wavelengths. Certain eluates were also tested for the presence of mono- or di-epoxide carotenoids by treatment with methanolic HCl and subsequent examination for spectral shift[8,9]. On this TLC plate the spots shown in Fig. 1 were isolated repeatedly, but not all were present in every extract chromatographed.

Although in some trials with this plate neoxanthin and violaxanthin were isolated free of their 5,8-epoxide isomers, often there was a serious degree of 5,6 → 5,8-epoxide isomerization, apparently occurring after the development of the plate. A TLC separation carried out on a magnesium oxide layer in two dimensions had earlier demonstrated that neoxanthin and violaxanthin were more stable on this medium. Therefore, two-adsorbent plates were prepared having a 3-cm-wide Silica Gel G–lime strip adjoining a magnesium oxide–Celite layer. It was again necessary

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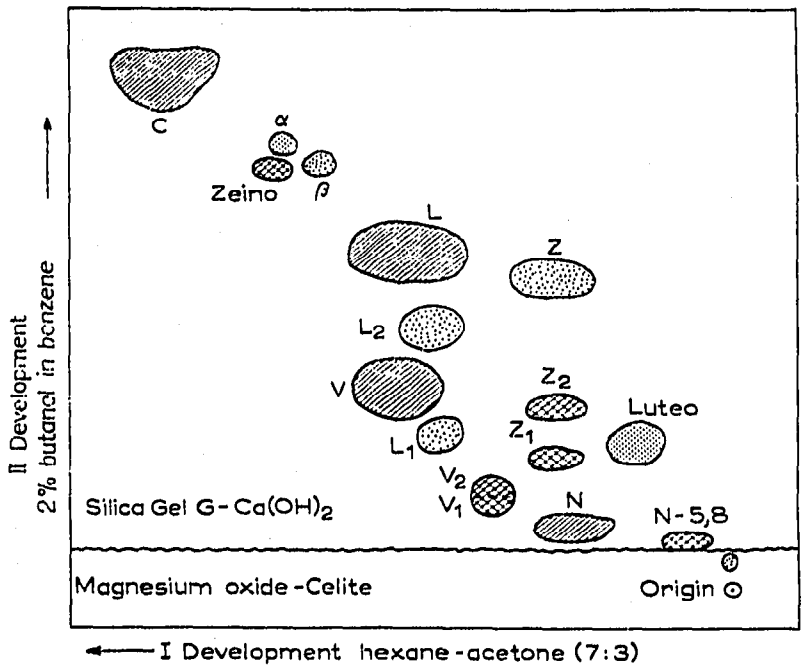

Fig. 1. Two-dimensional, two-adsorbent TLC of a carotenoid mixture on a magnesium oxide–Celite + Silica Gel G–$Ca(OH)_2$ plate. C = Carotenes, α = α-cryptoxanthin, β = β-cryptoxanthin, Zeino = zeinoxanthin, L = lutein, Z = zeaxanthin, L_1 and L_2 = lutein *cis* isomers (corresponding to "neo-A" or "V" and "neo-B" or "U", respectively), V = violaxanthin, V_1 and V_2 = violaxanthin *cis* isomers, Z_1 and Z_2 = zeaxanthin *cis* isomers, Luteo = luteoxanthin, N = neoxanthin, N-5,8 = neoxanthin 5,8-epoxide.

to grind the magnesium oxide–Celite slurry in a mortar because of particles which caused streaking of the adsorbent layer. This step was eliminated by substituting Kieselguhr G (E. Merck, according to Stahl, for TLC) for Celite, with satisfactory results.

The two-adsorbent plates recommended were therefore prepared as follows.

Silica gel–lime slurry

6 g of Silica Gel G–calcium hydroxide (1:6) were stirred with 9 ml of water for 1 min.

Magnesium oxide–Kieselguhr slurry

4 g of magnesium oxide powder sieved through a No. 60 (U.S. Series equivalent) screen were mixed dry with 16 g of Kieselguhr G in a beaker; 35 ml of water were added and the slurry was mixed for 1 min, until smooth and homogeneous.

With the spreader arranged as described above, both slurries were poured into it at once and the (five) plates were spread promptly. The plates were dried for 30 min at 110° and cooled to room temperature before use.

To use this plate a 10-μl sample spot was placed on the silica gel–lime strip 2.5 cm from one end and 2.5 cm from the outer edge. The spot was first developed (dimension I) up the silica gel–lime strip with 2% butanol in benzene, containing ethoxyquin; development time was approximately 1 h 20 min. The plate was then dried (1–2 min under a stream of nitrogen, protected from direct light), turned 90° and developed up the magnesium oxide–Kieselguhr layer (dimension II) in hexane–acetone(7:3), containing ethoxyquin. This required about 40 min. The resulting spots

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TABLE I

DESCRIPTION OF INDIVIDUAL CAROTENOIDS COMPRISING THE MIXTURE USED FOR EVALUATION OF TLC METHODS

Carotenoid	*Source*	*Isolation procedure*	$\lambda_{max.}$ *in hexane–acetone*		
Carotenes	Dehydrated alfalfa	Column chromatography, silica gel[a]	475	448	(425)[b]
Monohydroxy xanthophyll	Dehydrated alfalfa	Column chromatography, silica gel[c]	470.5	443	411
Lutein	Lutein preparation	Crystallized from acetone	474	445	421
Zeaxanthin	Zeaxanthin preparation	Crystallization	476.5	449.5	(426)
Violaxanthin	Freeze-dried alfalfa	Column chromatography, silica gel	469	439	418
Luteoxanthin	Dehydrated alfalfa	Column chromatography, silica gel	447.5	422	400[d]
Auroxanthin	Stored violaxanthin solution	Column chromatography, MgO[e]	425.5	401	379.5
Neoxanthin	Dehydrated alfalfa	Column chromatography, silica gel	465	436	414
Neoxanthin 5,8-epoxide	Dehydrated alfalfa	Column chromatography, silica gel	447.5	421	400[f]

[a] Silica Gel G–HyfloSupercel(1:1).

[b] Parentheses indicate spectral shoulder rather than peak.

[c] Monohydroxy xanthophylls eluted as one band, by the procedure of QUACKENBUSH *et al.*[10].

[d] $\lambda_{max.}$ at 447.5 "shifted" to 425 mμ upon HCl–CH_3OH treatment.

[e] Seasorb 43 magnesium oxide–HyfloSupercel(1:1).

[f] No shift of the 447.5 mμ peak to 425 mμ upon HCl–CH_3OH treatment.

were outlined and a photostatic copy of the plate made, as a record; the carotenoids were then promptly eluted and their visible absorption spectra recorded as described above.

For evaluation trials of this method a mixture of known carotenoids, individually described in Table I, was prepared.

One-dimensional TLC of these carotenoids, individually spotted on a silica gel–lime plate, yielded more than one discrete spot each for monohydroxy xanthophyll, lutein, zeaxanthin, violaxanthin, luteoxanthin and neoxanthin 5,8-epoxide. Lutein, violaxanthin and zeaxanthin each presented two fainter spots, well-separated and at lower R_F values than the all-*trans* spots. One-dimensional TLC of the mixture, however, resolved only four spots at lower R_F values than the all-*trans* lutein spot. After preliminary two-dimensional TLC trials with this mixture, it was supplemented with *cis* isomerized lutein and violaxanthin solutions, prepared by treatment with iodine in direct sunlight for 20–25 min (ref. 11). (The partial *cis* isomerization effected was indicated by spectral absorption peak shifts as follows: lutein, 474, 446, 422.5 → 472, 443.5, 416.5; violaxanthin, 469.5, 440, 416.5 → 467, 437, 414.) Zeaxanthin *cis*-isomer content was increased by adding zeaxanthin derived from corn gluten extract to the mixture.

This carotenoid mixture, chromatographed in two dimensions on the two-adsorbent plate just described, typically produced a display of spots as shown in Fig. 2.

The absorption maxima of the carotenoids thus isolated are presented in Table II. Literature values are also presented, for comparison.

Freeze-dried and heat-dehydrated alfalfa extracts were also analyzed by this TLC method, with satisfactory results.

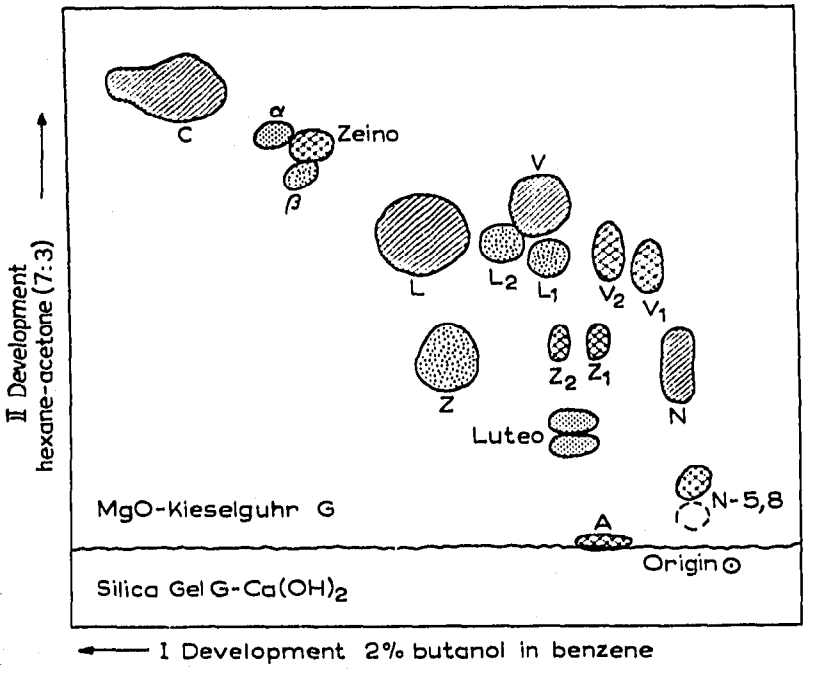

Fig. 2. Two-dimensional, two-adsorbent TLC of a carotenoid mixture on a Silica Gel G–$Ca(OH)_2$ + MgO–Kieselguhr G plate. A = Auroxanthin; all other abbreviations as in the legend to Fig. 1.

TABLE II

VISIBLE ABSORPTION MAXIMA OF CAROTENOIDS, (mμ)

Isolated by two-dimensional TLC of carotenoid mixture	*Hexane–acetone (7:3)*			*Literature values and solvent*							
Carotene, total	475	449	[a]								
α-Carotene				475	445	420	Hexane[12]	475	446	422	Hexane[13]
β-Carotene				477	450	425	Hexane[12]	478	451	426	Petroleum ether[14,15]
Monohydroxy carotenoids											
α-Cryptoxanthin	471	446	[a]	475	446	421	Hexane[13]	474	445	420	Petroleum ether[14]
Zeinoxanthin	472	446.5	[a]	474	445	422	Hexane[16]				
β-Cryptoxanthin	476	451	425	478	451	427	Petroleum ether[14]	480	452		Hexane[17,18]
Lutein											
trans	474	445	421	474	446	423	Ethanol[20]	476	446.5	423	Ethanol[19]
cis (neo B, "L_2")[b]	468	440	415	469	442	418	Ethanol[20]	472	443		Petroleum ether[19]
cis (neo A, "L_1")[b]	466	439	415	467	440	417	Ethanol[20]	471	442		Petroleum ether[12]
Zeaxanthin											
trans	478	451	429	478	452	428	Ethanol[20]	480	452		Petroleum ether[21]
cis (neo B, "Z_2")				474	446	425	Ethanol[20]				
cis (neo A, "Z_1")				472	444	425	Ethanol[20]				
Violaxanthin											
trans	470	440	417.5	471	440	417	Ethanol[20]	470	439	416	Methanol–diethyl ether[22]
cis (neo B, "V_2")	465	436	414	466	437	414	Ethanol[20]				
cis (neo A, "V_1")	464	435	413	463	435	411	Ethanol[20]				
Luteoxanthin	448	421	395	449	422	399	Methanol–diethyl ether[22]				
Auroxanthin	426	[a]	[a]	426	401	380	Methanol–diethyl ether[22]				
Neoxanthin	465	436	[a]	465	436	413	Methanol–diethyl ether[22]	466	437	415	Hexane[23]
								466	437		Ethanol[24]
Neoxanthin 5,8-epoxide	447	419	[a]	448	421	398	Methanol–diethyl ether[22]	448	422	400	Ethanol[25]

[a] $\lambda_{max.}$ not determined because of absorption due to ethoxyquin.
[b] Neo A ≅ "neo V"; neo B ≅ "neo U".

DISCUSSION

By the method here described the carotene(s) always produced one irregular spot, well-isolated; no effort was made to further resolve it. The monohydroxy xanthophylls could be separated into three components, two α-type and one β-type. Lutein, zeaxanthin, neoxanthin, neoxanthin 5,8-epoxide, luteoxanthin, auroxanthin, and the zeaxanthin and violaxanthin *cis* isomers, when present, were well isolated. The positions of the lutein *cis* isomer spots were somewhat variable, being found seemingly rotated about the violaxanthin spot, from positions below it (lower R_F value in dimension II) to positions between violaxanthin and lutein. This may be due to small variation in relative migration during the first and the second development in different trials. The spectral $\lambda_{max.}$ and lack of epoxide-type shift with HCl treatment easily distinguishes the lutein isomers from all-*trans* violaxanthin or its *cis* isomers, which display the characteristic peak of auroxanthin at approximately 425 mμ after HCl treatment.

Luteoxanthin and neoxanthin 5,8-epoxide, whose absorption spectra are nearly identical, were well separated on the plate and their distinction was readily confirmed by HCl isomerization of luteoxanthin to auroxanthin. Both luteoxanthin and neoxanthin 5,8-epoxide were frequently found as pairs of spots, slightly separated in dimension II. CURL AND BAILEY[22] also found these by countercurrent distribution analysis. The paired compounds usually differ in visible spectral $\lambda_{max.}$ by only 1 or 2 mμ.

The auroxanthin spot is typically quite compressed, located at the juncture of the two adsorbents, and greenish yellow in color.

Dehydrated alfalfa extracts, analyzed by this procedure, showed all the isolated spots of the carotenoid mixture TLC plate; however, the *cis* isomers of violaxanthin and zeaxanthin, and two of the three monohydroxy xanthophylls were frequently present in such low amounts that, although discernible on the plate, their visible spectra did not show well defined peaks whose $\lambda_{max.}$ could be determined. The freeze-dried alfalfa TLC display of spots was simpler, revealing only one lutein *cis* isomer, no *cis* isomers of violaxanthin or zeaxanthin, and one monohydroxy xanthophyll spot. Its central $\lambda_{max.}$ was approximately 449 mμ, whereas the dehydrated alfalfa monohydroxy had $\lambda_{max.}$ of 470.5 and 444.5. Corn meal extracts consistently produced a group of monohydroxy spots, usually three: "α" was spectrally α-cryptoxanthin-like, "Zeino" was zeinoxanthin-like and "β" was β-cryptoxanthin-like.

Several types of separation are demonstrated by this procedure and the conclusions may be drawn that adsorbability is increased: (1) in both media, with increasing number of hydroxyls; (2) in dimension I, by epoxide groups; (3) in dimension II, by 5,8-epoxide groups; (4) in dimension II, by increased length of conjugated double bond system (α- *vs.* β-carotenoids); (5) in dimension I, by certain *trans* → *cis* isomerizations.

The commercial dehydration process[26,27] or other heat treatment[28–30] of forages is known to produce *cis* and furanoid isomers of carotenoids in varying amounts. Storage of dehydrated forages also results in such changes[26,31] which are deleterious to the product. Precise determination of these isomerizations is therefore required if detailed studies of these treatments are to be made. It is recognized that these conversions could also occur during TLC analysis, and the cautions expressed by STRAIN

et al.[25] regarding use of siliceous adsorbents for carotenoid chromatography are well advised. We found, however, as they did, that if the epoxide xanthophylls are promptly eluted following TLC separation on a plate such as here recommended, they can be recovered with little detectable isomerization. The addition of ethoxyquin to the developing solvents[1] results in masking of the absorption spectra of the eluted pigments in the shorter λ region of the visible spectrum. However, all the carotenoids may be adequately identified and the inclusion of ethoxyquin for antioxidant protection is recommended.

The method here presented is more time-consuming than one-dimensional TLC but permits the separation, on one plate, of the various monohydroxy carotenoids, of lutein from zeaxanthin, of *cis* and *trans* isomers and of isomeric epoxide xanthophyls. With appropriate equipment and procedure it can be carried out quantitatively.

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