ELSEVIER

Journal of Chromatography A, 667 (1994) 361–366

JOURNAL OF CHROMATOGRAPHY A

Short Communication

Thin-layer chromatographic determination of β-carotene, cantaxanthin, lutein, violaxanthin and neoxanthin on Chromarods

A.J. Rosas Romero*,[a], J.C. Herrera[a], E. Martinez De Aparicio[a], E.A. Molina Cuevas[b]

[a]*Departamento de Química, Universidad Simón Bolívar, Apartado 89000, Caracas 1080A, Venezuela*

[b]*Departamento de Matemáticas Puras y Aplicadas, Universidad Simón Bolívar, Apartado 89000, Caracas 1080A, Venezuela*

(First received June 9th, 1993; revised manuscript received November 29th, 1993)

Abstract

The separation and the simultaneous determination of β-carotene, cantaxanthin, lutein, violaxanthin and neoxanthin was accomplished using thin-layer chromatography on Chromarods, flame ionization detection and a two-stage development technique. Data were transformed through an unweighted straight-line regression of the logarithm of peak area ratios on the logarithm of the mass ratios. The determinations are highly reproducible and all statistical estimates are highly significant. The linear concentration range for each compounds is reported. No evidence of degradation of the carotenoids during the analyses was found. Up to ten samples can be analysed simultaneously in less than 2 h.

1. Introduction

Open-column chromatography (OCC) is the traditional method most frequently used to perform the separation of carotenoids [1,2]. It is generally employed for preparative separations and has also received quantitative applications, which includes separation using magnesium oxide followed by photometric determination [3]. Analysis using this method requires a long time and consumes considerable amounts of solvents and chromatographic materials. At the same time it has the disadvantage of not allowing the separation of the individual carotenoids; instead, the elutions are made according to the polarity in the three main groups, carotenes and mono- and dihydroxyxanthophylls.

In the last few years, OCC has been progressively displaced by high-performance liquid chromatography (HPLC) in the analysis of carotenoids [2]. Both normal-phase [2,4] and reversed-phase systems [5–7] can be used for the separation of carotenoids. The optimum conditions for maximum recovery and selectivity, using different C_{18} columns, have been reported [8].

Historically, thin-layer chromatography (TLC) is the method most often used for the separation of carotenoids [1,2]. There are many reports on the applications of silica gel TLC to the separation of this family of compounds [9–14]. TLC separations with magnesium oxide [15] and with a chemically bonded C_{18} stationary phase [16,17]

* Corresponding author.

0021-9673/94/$07.00
SSDI 0021-9673(93)E0017-O

have also been employed in the separation of individuals carotenoids. Recent work [17] pointed out the degradation of β-carotene during multiple-development TLC as a major difficulty for the separation and analysis of these compounds.

The Iatroscan chromatograph combines the capability of TLC with the quantification power of the flame ionization detector, (TLC–FID) [18,19]. Different aspects related to the quantitative application of the technique have been reviewed [20,21]. This paper presents a description of the application of TLC–FID to the determination of the five major carotenoids, β-carotene, lutein, violaxanthin, neoxanthin and cantaxanthin.

2. Experimental

2.1. Apparatus and experimental conditions

The equipment consisted of an Iatroscan TH-10 MK II TLC–FID analyser (Iatron Labs., Tokyo, Japan), Waters (Milford, MA, USA) 745/745B data module integrator and silica gel SIII Chromarods (Iatron Labs.). The Iatroscan was operated under the following conditions: hydrogen flow-rate, 160 ml/min; air flow-rate, 2000 ml/min; and scanning speed, 0.42 cm/s.

2.2. Materials

β-Carotene was obtained from BDH Biochemicals (Poole, UK), lutein, cantaxanthin, violaxanthin and neoxanthin from Hoffmann-La Roche (Basle, Switzerland), methyl tetracosanoate from Analabs (North Haven, CT, USA) and the fatty alcohol cholesterol from Polyscience (Niles, IL, USA). All organic solvents were of the highest purity available and used as received. Light petroleum (b.p. 55–70°C) was used. Sep-Pak silica cartridges (Waters) were used for the purification of oxidized β-carotene solution.

2.3. Chromatographic procedure

Chromarods were activated by passing them through the FID flame just before application of the sample. Each rod was spotted with 1 μl of sample solution. After being spotted, the rods were placed in a constant-humidity chamber (65%) for 30 min. This chamber was one of the developing tanks containing 35.8% sulphuric acid. Development was carried out in glass chambers, wrapped in black paper, with three of their internal faces covered with filter-paper. After development in the appropriate mobile phase, the rods were left to dry at room temperature for 5 min and immediately scanned in the TLC–FID system.

A constant volume of developing solvent was used, sufficient to cover the first 3 mm of the rods. Developing solutions were freshly prepared each day. After 20–30 runs the rods were immersed overnight in 10% sulphuric acid, and washed thoroughly with deionized water.

The analysis is carried out by a double development technique. In the first stage, β-carotene, the internal standard and the non-saponifiable neutral lipid fraction were separated in light petroleum–chloroform–acetone (89.5:10:0.5), while the xanthophylls did not move from the injection point. The developing time for this stage was kept constant at 30 min. After development, the rods were taken to the Iatroscan and burnt down to 0.5 cm from the injection point.

In the second stage, the xanthophylls were separated by developing the rods in light petroleum–chloroform–2-propanol (50:40:10) for 45 min and burning the whole length of the rod.

All handling of the samples was carried out under dim, diffuse artificial light.

2.4. Calibration

For each of the carotenoids, chloroform solutions were prepared in concentrations ranging from 0.3 to 10 mg/ml. All solutions contained 4

mg/ml of methyl tetracosanoate as internal standard. Ten different concentrations were analysed simultaneously for each compound in each run. Calibration graphs were constructed by plotting the peak-area ratio (carotenoid/methyl tetracosanoate) against their mass ratio (carotenoid/methyl tetracosanoate). From the calibration graphs, the linear range for each of the analyses and the statistical parameters were calculated.

3. Results and discussion

In addition to improving the reproducibility, it proved easier to keep the developing time constant rather than trying to develop the rods until the solvent had reached a certain, pre-established height. Moreover, this procedure avoided the difficulty of visually localizing the solvent front.

To ensure reproducibility, the rods were matched according to the speed of solvent migration during development. Throughout this study, only those rods which did not present appreciable differences were employed.

In a study on the instability of plant carotenoids during their separation by multiple developments [17], evidence was presented of degradation of the carotenoids through photolytic and/or oxidatives processes. It was found that when β-carotene was subjected to multiple development on silica gel TLC plates, a decrease in the absorbance of the β-carotene peak was observed following each development, together with the formation of a considerably more polar compound that remained at the origin. When β-carotene was spotted on octadecylsilanized silica gel, the same decrease in response was found after each development, without the obvious formation of a new product. The phenomenon was found to be time dependent, particularly the time spent on the dried layer during sample application, solvent evaporation and scanning densitometry.

In view of those results, we performed the same kind of experiments on silica gel SIII Chromarods. Following the procedure explained under Experimental, we spotted, developed and scanned β-carotene on ten Chromarods. We found no decrease in the FID response or the formation of new products after a second development. The same results were obtained when the spotted Chromarods were left in the constant-humidity chamber for 1 h instead of 30 min. The whole procedure was repeated for each of the other compounds under study. Again, no evidence of a decrease in signal or of the formation of new products was found. In another experiment we worked with an intentionally oxidized β-carotene solution, and in this instance we observed a new peak that remained at the injection point. This peak disappeared when the polar compounds were previously removed from the oxidized β-carotene solution in light petroleum by filtration through a Sep-Pak silica cartridge.

We believe that the difference among our results and those reported previously [17] maybe due to differences between the TLC plates and the SIII Chromarods, along with the differences imposed by the scanning techniques employed in each instance.

3.1. Determination of β-carotene

A typical chromatogram of this first stage of the analysis is shown in Fig. 1A. Peaks a and b correspond to β-carotene and the internal standard, respectively.

The neutral lipid fraction appears separate from those two peaks. Fig. 1B depicts the chromatogram of a sample that contained β-carotene, the internal standard and a mixture of fatty alcohols and sterols, which are the types of compounds that would remain after extraction of the carotenoids and removal of the saponifiables from, for example, green leaves [18,19].

For β-carotene, the working concentration range lies between 0.6 and 5 mg/ml. For concentrations greater than 5 mg/ml, the β-carotene peak broadens and overlaps with the peak of the internal standard, and consequently the detector

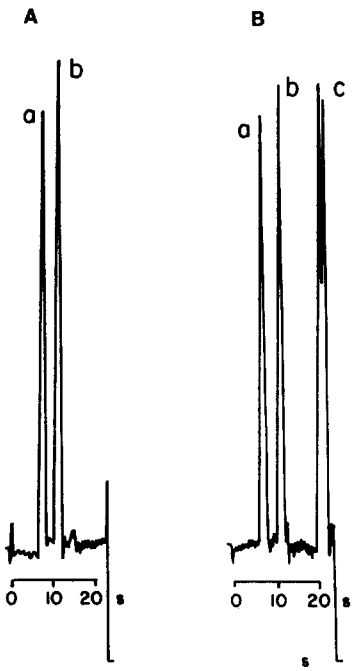

Fig. 1. (A) TLC of β-carotene and the internal standard. For experimental conditions, see text. First development with light petroleum–chloroform–acetone (89.5:10:0.5). β-Carotene (peak a) appears at 7.8 s and the internal standard (peak b) at 11.4 s. (B) TLC of a solution containing β-carotene, internal standard and sterols and fatty alcohols (as an example of non-saponifiable lipids). Developing system, light petroleum–chloroform–acetone (89.5:10:0.5). Non-saponifiable lipids (peak c) appear at 21 s, well resolved from β-carotene (peak a) and the internal standard (peak b).

response is smaller than expected and the slope progressively diminishes.

3.2. Determination of xanthophylls

Fig. 2 shows a typical chromatogram of the separation of the xanthophylls in the second development of the samples. Peaks a, b, c and d correspond to cantaxanthin, lutein, violaxanthin and neoxanthin, respectively.

As in the analysis of β-carotene, outside the working concentration range the peaks broaden and the FID response progressively decreases. This range is 0.3–9 mg/ml for cantaxanthin, 1.2–8 mg/ml for lutein, 0.5–8 mg/ml for violaxanthin and 1–10 mg/ml for neoxanthin.

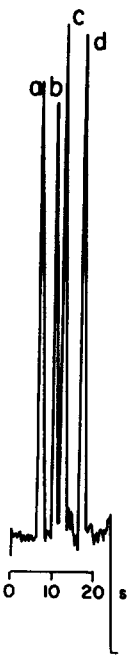

Fig. 2. Chromatographic separation of xanthophylls in light petroleum–chloroform–2-propanol (50:40:10) for 45 min. Peaks: a = cantaxanthin (6 s), b = lutein (9.6 s), c = violaxanthin (12.6 s) d = neoxanthin (16.8 s). For experimental conditions, see text.

3.3. Statistical analysis

The data consisted of several measurements of peak areas at different mass levels, *i.e.*, a repeated measurements analysis. A scatter plot of peak-area ratios against mass ratios revealed a nearly linear relationship, with a slight curvature towards the lower and higher mass ratio levels. A plot of standard deviations against mass ratio showed an increasing relationship. This behaviour of both the trend and the standard deviations, which has been reported previously [22,23], suggests the need for a stabilizing transformation, a weighted analysis or a combination of both.

A simple linear regression was performed in order to make a residual analysis. The residual analysis shows influential observations at the lower and higher mass ratio levels. The normal probability plot emphasizes the need to adjust the analysis for the non-homogeneity of the

variances. A simple straight-line fit is inadequate, giving too much weight to the middle range of the data. A weighted analysis improves the non-homogeneity of the variances but gives too much weight to the lower range of the data. This residual analysis reveals the need to include a second-order term. Therefore, a second-degree polynomial was fitted by weighted least squares; this stabilizes the variance and takes into account the slight curvature in the trend, at the expense of having to deal with an extra parameter.

As a second step, a Box–Cox transformation analysis was performed [24]. This second analysis favours a power transformation of the peak area ratios ($Y^{0.7}$) over the logarithmic transformation. However, an unweighted straight-line regression of the logarithm of peak-area ratios on the logarithm of the mass ratios renders a similar fit, with a simpler model:

$$\log(\text{peak-area ratio}) = a + b \log(\text{mass ratio}) \quad (1)$$

For all the compounds, the residual normal probability plot for eq. 1 does not show important departures from normality, while the residual analysis is satisfactory, showing that the model allows for the slight curvature in the trend, and stabilizes the variances at the same time.

Taking into consideration that the main objective of the analysis is to find a calibration graph for interpolation, we decided in favour of eq. 1. This model renders a simple interpolation procedure without sacrificing either precision or goodness of fit.

Table 1 summarizes the results of the regression analysis of eq. 1 for each of the compounds studied. As can be seen, all determinations are precise and accurate. For all the compounds studied the detector response increases significantly with increasing mass ratio, *i.e.*, the observed values for the slope are significantly different from zero (for $p < 0.05$). Table 1 shows that eq. 1 provides an excellent goodness of fit, as indicated by the values of the coefficient of determination, R^2, which measures the proportion of total variation explained by the model [25].

Table 1
Linear regression analysis for the calibration graphs, using the logarithm of peak-area ratios on the logarithm of mass ratios

Compound	Slope	Intercept	R^2
β-Carotene	1.40 ± 0.02	−0.26 ± 0.02	98.41
Cantaxanthin	1.56 ± 0.02	0.07 ± 0.02	99.16
Lutein	1.98 ± 0.02	−0.88 ± 0.02	99.40
Violaxanthin	2.15 ± 0.02	0.57 ± 0.02	99.08
Neoxanthin	0.98 ± 0.01	0.589 ± 0.007	99.37

Results are expressed as the mean ± standard error. Each mass was measured ten times. All values are statistically significant at $p < 0.05$.

4. Acknowledgements

These studies were supported by a grant from the Decanato de Investigaciones y Desarrollo of the Universidad Simón Bolívar (Venezuela).

5. References

[1] B.H. Chen, S.H. Yang and L.H. Han, *J. Chromatogr.*, 543 (1991) 147.
[2] R.F. Taylor, *Adv. Chromatogr.*, 22 (1983) 157.
[3] W. Horwitz (Editor), *Official Methods of Analysis of the Association of Official Analytical Chemists*, AOAC, Washington, DC, 13rd ed., 1980, p. 738.
[4] S.H. Rhodes, A.G. Netting and B.V. Milborrow, *J. Chromatogr.*, 442 (1988) 412.
[5] G. Britton, *Nat. Prod. Rep.*, 8 (1991) 223.
[6] F. Granado, B. Olmedilla, I. Blanco and E. Rojas-Hidalgo, *J. Liq. Chromatogr.*, 14 (1991) 2457.
[7] F.W. Quackenbush, *J. Liq. Chromatogr.*, 10 (1987) 643.
[8] K.S. Epler, L.C. Sander, R.G. Ziegler, S.A. Wise and N.E. Craft, *J. Chromatogr.*, 595 (1992) 89.
[9] G. Britton, R.K. Singh, T.W. Goodwin and A. Ben-Aziz, *Phytochemistry*, 14 (1975) 2427.
[10] P.M. Bramley and B.H. Davis, *Phytochemistry*, 14 (1975) 463.
[11] G. Britton and T.W. Goodwin, *Phytochemistry*, 14 (1975) 2530.
[12] U. Leuenberger and I. Steward, *Phytochemistry*, 15 (1976) 227.
[13] K. Iryama, M. Yoshiura, M. Shiraki, S. Yano and S. Saito, *Anal. Biochem.*, 106 (1980) 322.
[14] M.N. Merzlyak, V.A. Kovrizhnih and Y.N. Kaurov, *J. Chromatogr.*, 262 (1983) 331.
[15] R. Sadowski and W. Wojcik, *J. Chromatogr.*, 262 (1983) 455.

[16] M. Isaksen and G.W. Francis, *J. Chromatogr.*, 355 (1986) 358.
[17] M.J. Cikalo, S.K. Poole and C.F. Poole, *J. Planar Chromatogr.*, 5 (1992) 200.
[18] J.K.G. Kramer, R.C. Fouchard and E.R. Farnworth, *J. Chromatogr.*, 198 (1980) 74.
[19] R. Thomas Crane, S.C. Goheen, E.C. Larkin and G. Ananda, *Lipids*, 18 (1983) 74.
[20] R.G. Ackman, C.A. McLeod and A.K. Banerjee, *J. Planar Chromatogr.*, 3 (1990) 450.
[21] R.G. Ackman, *Methods Enzymol.*, 72 (1981) 205.
[22] C.C. Parrish and R.G. Ackman, *Lipids*, 20 (1985) 521.
[23] C.C. Parrish, *Can. J. Fish. Aquat. Sci.*, 44 (1987) 722.
[24] A.C. Atkinson, *Plots, Transformations and Regression*, Oxford University Press, Oxford, 1985, pp. 85–90.
[25] N.R. Drapper and H. Smith, *Applied Regression Analysis*, Wiley, New York, 1966, p. 26.