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OPTIMIZATION OF LUTEIN-ZEAXANTHINE SEPARATION ON HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract

TABACARU, C., C. SOCACIU, G. NEAMTU, 1993, Optimization of lutein-zeaxanthine separation on high performance liquid chromatography. Not. Bot. Hort. Agrobot. Cluj., XXII-XXIII,125-133. Separation of lutein and zeaxanthine which are carotenoidic isomeric pigments on High Performance Liquid Chromatography (HPLC) was investigated. Three different columns were used to separate standard mixtures: a normal phase silica column, a reversed phase ODS 2 column and a non totally endcapped reversed phase ODS 1 column, in order to find the best protocols for the separation of these two isomers.

Key words: lutein, zeaxanthine, separation on HPLC

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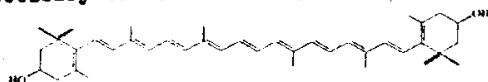
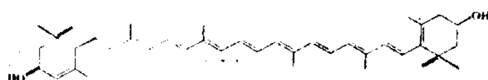
Lutein and zeaxanthine are carotenoids with two hydroxi groups, isomers due to the position of one double bond (Figure 1). Their separation is a very difficult problem but alsa a very important one for carotenoid researchers because they are found in almost total carotenoid plant extract.

Separation with classical chromatography methods (thin layer chr., open columns chr.) of these pigments doesn't give good results. The best method used for their separation seems to be the High Performance Liquid Chromatography (HPLC). In this kind of chromatography, the separation of the mixture is realised on a column with a stationary phase with very fine grains. The eluting solvent is

pushed under pressure in the column by one or two pumps. A detector is also necessary to "see" the separated compounds in the eluate (1).



Zeaxanthine



Lutein

Figure 1. The structure of lutein and zeaxanthine

Depending on the stationary and the mobile phases there are two types of chromatographic separation methods on HPLC; used for carotenoid mixtures:

a. Normal phase separations when the stationary phase is silica or perhaps alumina which are very polar and the mobile phase is nonpolar (hexane, tetrahydrofuran, etc.).

b. Reversed phase separations when the stationary phase is bonded silica which is very non polar and the mobile phase is very polar (water - methanol).

There were many attempts for the separation of these two carotenoids on HPLC and good resolutions were obtained mostly when working with standard mixtures. In complex mixtures a good separation of lutein and zeaxanthine has also to deal with long separation times due to the presence of other carotenoids with a different polarity, so it seems that for these kinds of separations, reversed phase columns fit better.

Good separations were obtained on normal phase sistems using as eluting solvents mixtures of hexane-acetone, hexane-Pr-OH, light petroleum-acetone, isocraticaly or in gradient flows. On these sistems differences of 2 - 3 minutes in the retention times of lutein and zeaxanthine were usualy obtained (2).

The reverse phase separations usualy gave differences of only 1 minute in the retention times of lutein and zeaxanthine but faster separation times, using very different solvent mixtures and running protocols. The separation was in most cases not a base line one (2).

There are also reports of lutein - zeaxanthine separation on non endcapped reverse phase columns. Thayer (3) reports a separation from leaves extract using a mixture of MeOH:MeCN:EtOAc which gave a difference of 2 minutes in retention times and Gilmore (4) from the same matterial using MeCN:MeOH:Tris buffer:Hexane with a lower difference in retention times, on this kind of columns.

Anyway the best separation for lutein and zeaxanthine was obtained until now by Stalcup (5) using carotenoid standards on a cyclodextrin-bonded phase column with various solvents, the difference between the retention times of the two carotenoids beeing of 9 minutes.

This paper presents an investigation into the use of three different columns: a silica normal phase column, a normal octadecylsilanol reversed phase column (ODS 2) and a non totally endcapped reverse phase column (ODS 1) in order to have a good lutein-zeaxanthine separation.

Experimental section

The liquid chromatograph used was equipped with LC-T 414 Kontron Instr. Pumps and consisted of a Waters (Millipore) 991 Model, connected to a programmable photodiode array detector (PDA). Dates

were processed by a NEC/Multisyne 2A terminal attached to the detecto[r].

For the NP-HPLC separations we used a Nucleosil 1oo-5 (Macherey Nagel AG, Switzerland) column (2oo x 4 mm).

For RP-HPLC separations a Spherisorb 5 ODS 2 (Wellington House Hplc Technology, UK) column (25o x 4,6 mm) and a non totally end-capped Spherisorb 5 ODS 1 column (25o x 4,6 mm).

The carotenoid standards: B-carotene, B-cryptoxanthine, lutein and zeaxanthine were provided by Hoffman Ltd., Basel, Switzerland. Stock standard mixture was prepared and stored at -2o°C, protected from light; alliquots of it were dissolved each time in the chromatographic solvent mixture.

Mobile phases consisted of mixtures of - HPLC purity grade - solvents: hexane, acetone, acetonitrile, ethyl acetate, tetrahidrofuran, methyl alcohol, water, filtered and desaerated before use.

The columns were opperated at ambient temperature and flow rat[e] ranged from o,5 - 2 ml/min. The NP column was equilibrated each ti[me] at least 1 hour before each injection with the starting solvent mixture and the RP columns were also equilibrated 15 minutes before each injection and 1 hour after each solvent changing with the starting solvent mixture.

For normal phase separation a carotenoid standard mixture consisting of B-carotene, lutein, zeaxanthine and B-cryptoxanthine was used. For the reversed phase separation a mixture without B-cryptoxanthine was used.

The experiments were carried out in the laboratories of prof. Pfander from the University of Berne, Switzerland.

Results

The best results obtained for lutein and zeaxanthine separation on the three columns mentioned before, using carotenoid standard mixtures are listed in table 1.

Table 1. Difference of lutein and zeaxanthine retention times and elution order for the separation of standard mixtures on different columns (Flow = 1 ml/min., = 45onm)

Nr. crt.	Column type	Solvent mixture	Separation time	t_R	Elution order	Gradient or concentration
1.	NP Silica	Hexan : Acetone	Gradient	o,95	B-carotene Lutein Zeaxanth.	Time(min) Acetone 0 - 8,5 5-12% 8,5-2o,5 12-17% 2o,5-26,5 17% 26,5-3o 17-2o%
2.	RP ODS 2	CH_3CN : EtOAc	Gradient	2,3	Lutein Zeaxanth. B-carotene	Time(min) Acetone o-23 1o% 23-3o 1oo%
3.	RP ODS 1	CH_3CN : EtOAc	Isocratie	1,2	B-carotene Lutein Zeaxanth.	95 : 5
4.	RP ODS 1	CH_3CN : Acetone	Isocratic	2,95	Lutein Zeaxanth. B-carotene	8o : 2o
5.	RP ODS 1	CH_3CN : THF	Isocratic	1,6	Lutein Zeaxanth. B-carotene	98 : 2

The corresponding chromatograms are shown in figures 2, 3 and 4.

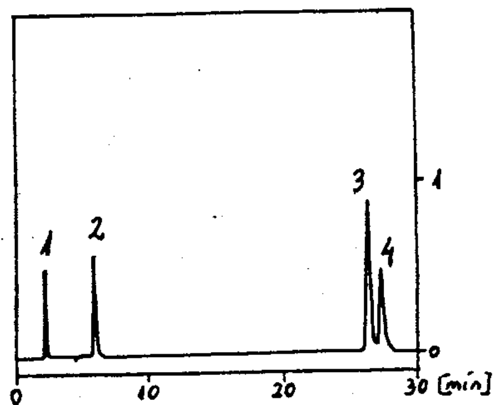


Figure 2. Separation of carotenoid standards on normal phase column. Peak identification: 1 - B-carotene; 2 - B-cryptoxanthine ; 3 - Lutein; 4 - Zeaxanthine

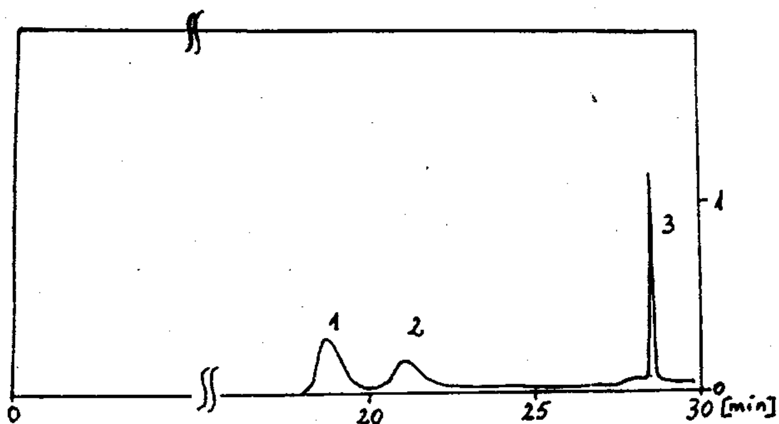


Figure 3. Separation of carotenoid standards on reversed phase ODS-2 column. Peak identification: 1 - Lutein; 2 - Zeaxanthine ; 3 - B-carotene

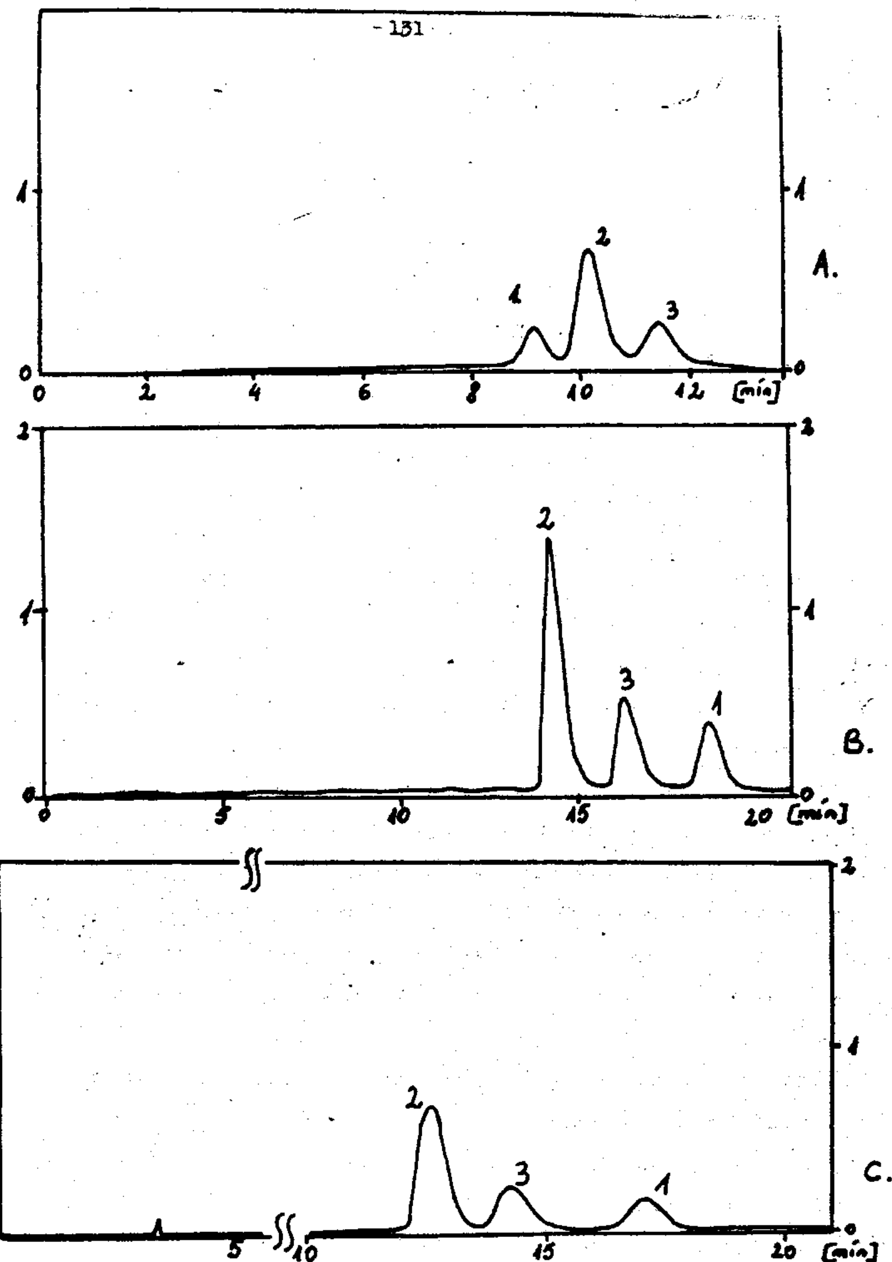


FIGURE 4 Separation of carotenoid standards on reverse phase, ODS 1 column.
Peak identification: 1, B - Carotene : 2, Lutein : 3,Zeaxanthine.
A. Solvent mixture: CH_3CN and EtOAc:B. Solvent mixture: CH_3CN and Acetone: C. Solvent mixture: CH_3CN and THF.

Conclusions

1. The separation on normal phase column has a relatively long separation time (3o minutes) but it has also the advantage of a long difference between the retention times of B-carotene and lutein which offers the possibility of a good separation of other carotenoids with intermediate polarity from natural extracts.

2. The separation of the two isomeric carotenoids on reversed ph[illegible] is better than on normal phase and non-aqueous solver [illegible]ixtures gave better differences in their retention times.

3. Separation of lutein and zeaxanthine is very good on non tot[illegible] endcapped column, ODS 1, even on isocratic conditions but it has the disadvantage of very close peaks for a big difference in polarity [illegible] (zeaxanthine and B-carotene) so we think it should be chosen only i[illegible] we need just a lutein/zeaxanthine separation and not for a complex total extract.

4. The elution order on the same column (ODS 1) was different [illegible] we used different solvents. When acetonitrile and ethyl acetate we[illegible] used, B-carotene eluted first as on normal phase silica column and when acetonitrile and THF or acetonitrile and acetone were used, B-carotene eluted last, as on reverse phase ODS 2 column. We can [illegible] that the ODS 1 column worked as an intermediate normal phase - re[illegible] phase column.

5. It is interesting the fact that on every column we used, [illegible] eluted before zeaxanthine, even if the normal expected order of [illegible] (thinking of polarities) on reverse phase column should place ze[illegible] thine before lutein.

6. The resolution between lutein and zeaxanthine in all sy[illegible] can be improved by working with a lower flow but that has the d[illegible] tage of long separation times.

7. Both normal phase and reversed phase (ODS 2) separation systems on HPLC are suitable for the separation of complex carotenoid mixtures.

In conclusion, we propose good normal phase and reverse phase separation systems on HPLC for lutein - zeaxanthine separation from complex natural extracts.

Rezumat

TABACARU, C., C. SOCACIU, G. NEAMTU, 1993, Optimizarea separării luteinei de zeaxantină prin cromatografie lichidă de înaltă performanţă. Not. Bot. Hort. Agrobot. Cluj. XXII-XXIII, 125-133. În această lucrare se urmăreşte separarea celor două carotenoide izomere care sînt foarte răspîndite în mediul vegetal, cu ajutorul cromatografiei lichide de înaltă performanţă (HPLC). În acest scop s-au folosit trei coloane umplute cu material adsorbant diferit şi s-au determinat concentraţiile optime ale amestecurilor eluate de solvenţi, necesare pentru o separare optimă

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