crea
Consiglio per la ricerca in agricoltura
e l'analisi dell'economia agraria

2nd MS-Wine Day

# CAROTENOIDS IN WINE GRAPES: HPLC-DAD-ESI-MS$^n$ APPLICATION FOR THEIR COMPREHENSIVE ANALYSIS

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# Introduction

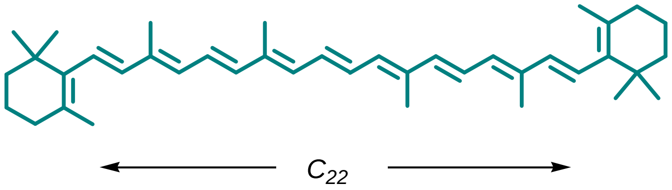
Chemically, carotenoids are C40 tetraterpenoids with a long chromophore of conjugated double bonds, which is responsible for their red to yellow coloration (absorbing from 400 to 550 nm).

They are paricularly unstable being easly oxidized and cis-trans isomerized by light and other physico-chemical agents (i.e. T, acids, ecc.).

They are devided into the hydrocarbon carotenes, such as lycopene and β-carotene or xanthophylls, typified by lutein.

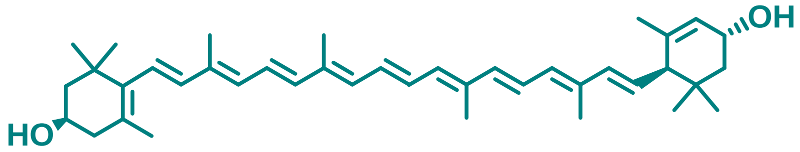
**CAROTENI**

**lycopene**



**β-carotene**

**XANTHOFILLE**

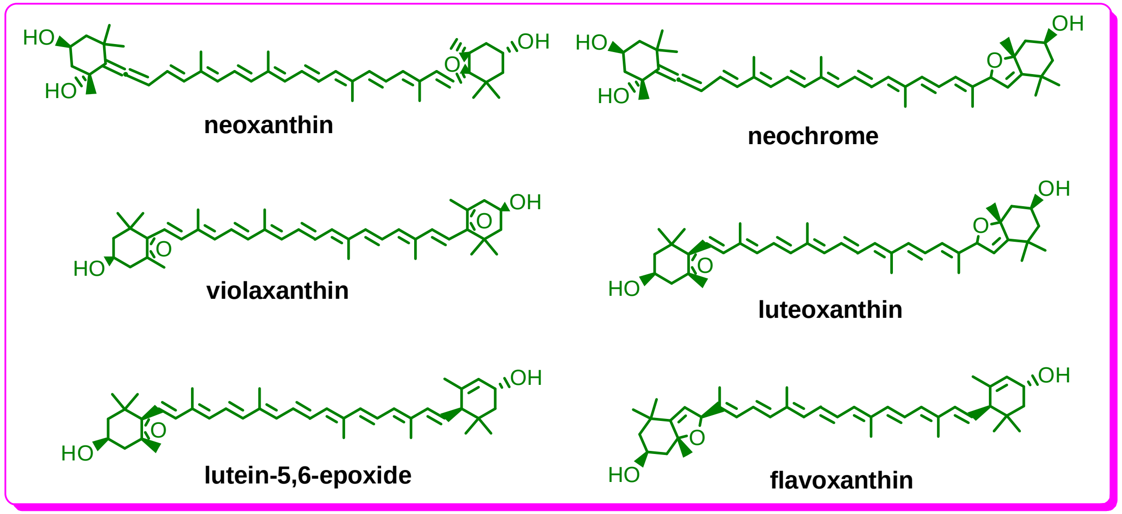


**lutein**

# Introduction

Mature grapes berries contain numerous carotenoid compounds, which have all the corresponding bicyclical structures of carotenoids associated with multiprotein complexes of plant chloroplast membranes that make up the PSI and PSII photosystems.

The most common are β-carotene and lutein, representing nearly 85% of the total, and are accompanied by minor carotenoids such as:

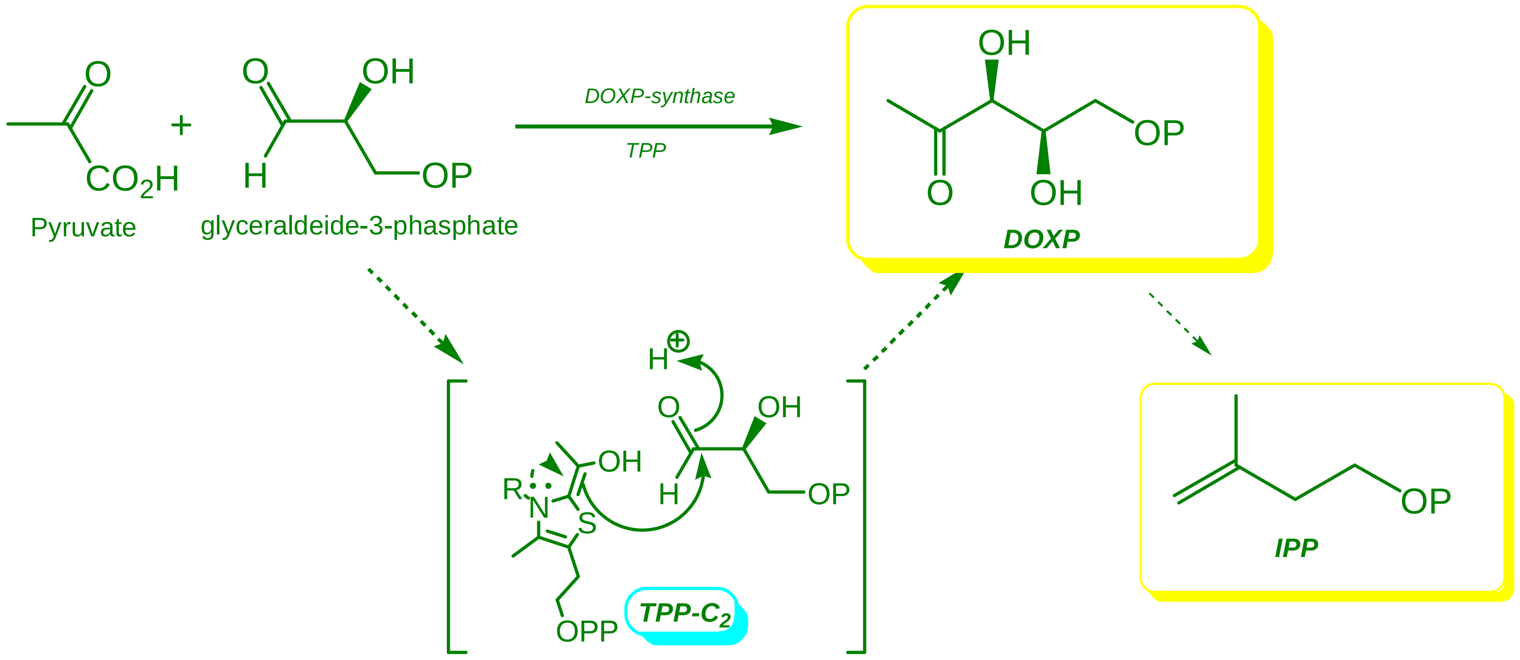


The level of carotenoids in grapes depends on climatic factors, agricultural practices, grape variety and the clone. Carotenoids are mostly found in the skin at levels two to three times higher than in the pulp, and generally their concentration decreases from veraison during grape ripening.

Razungles,A. et al. *Am.J.Enol.Vitic. 39(1),* **1988**, 44-48; Razungles, A. et al. *J.Agric.Food Chem. 44*, **1996**, 3821-3825; Oliveira, C. et al. *J.Agric.Food Chem. 51*, **2003**, 5967-5971.

# Introduction

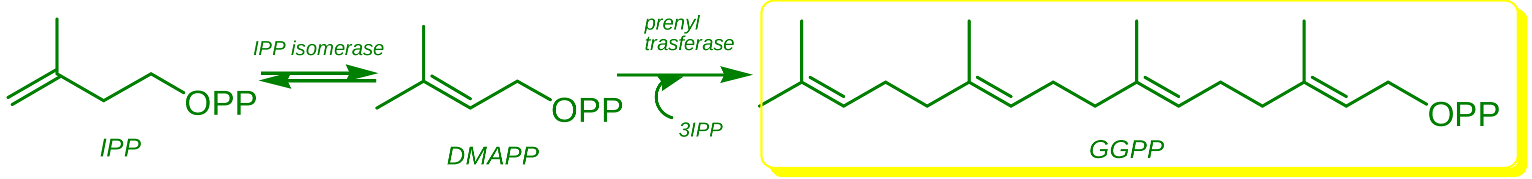
Carotenoids derived from isopentenil diphosphate (IPP) in the plastids, through the 1-deoxi-D-xylulosio-5-phosphate (DOXP) pathway. DOXP is formed by the condensation of pyruvate and glyceraldheide-3-phosphate (glycolitic intermediates).



Bramley, P.M. *J.Exper.Bot. 53(377),* **2002**, 2107-2113.

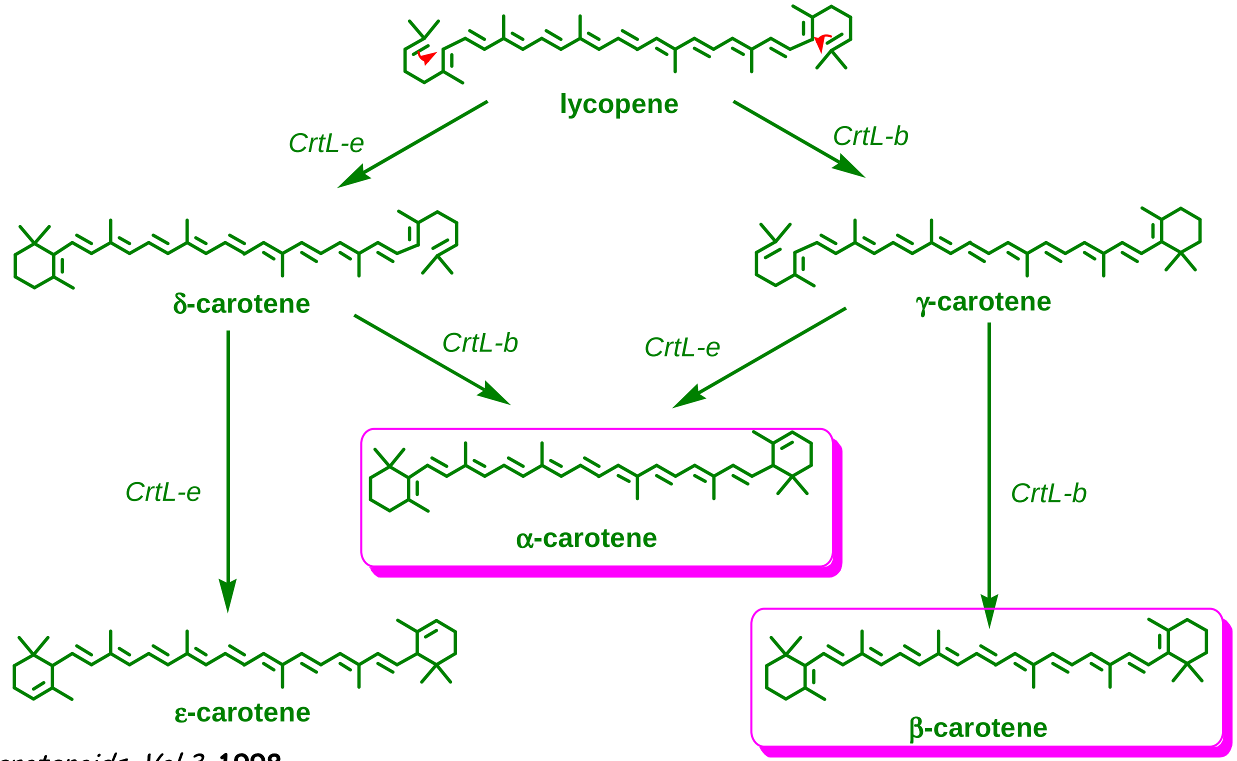
# Introduction

IPP isomerized to DMAPP, then, by its condensation with other 3 IPP, GGPP (the actual precursor of C40 carotenoids) is obtained



Phythoene is formed by the "head-to-head" condensation of two GGPP, then it gives lycopene (acyclic C40) after four successive dehydrogenation steps.

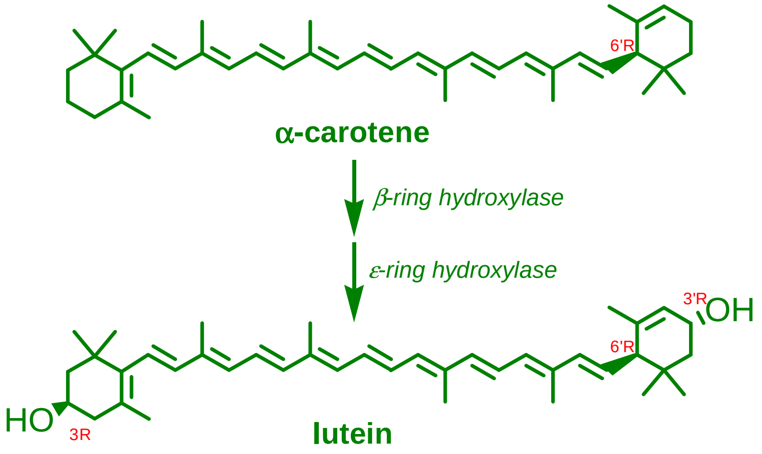
Finally, the cyclization of lycopene gives a series of carotenes with one or two β or ε type rings.



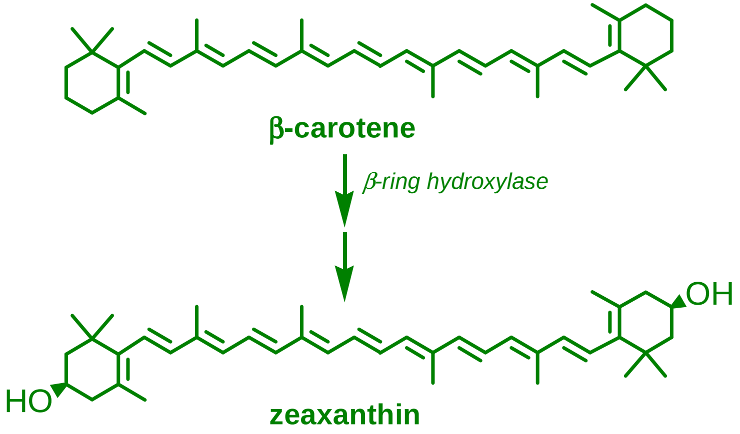
Britton, G. *Overview of carotenoid biosynthesis. In Carotenoids. Vol.3* **1998**.

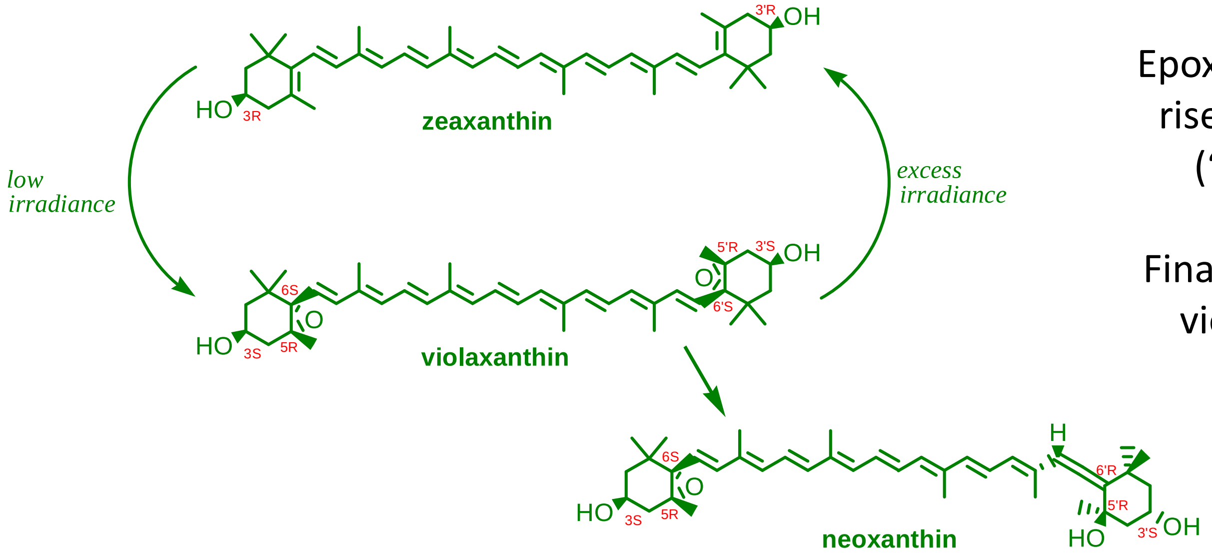
# Introduction

α-carotene totally transorfms into luteina.



β-carotene partially transforms into zeaxanthin by asimmetric hydroxylation.





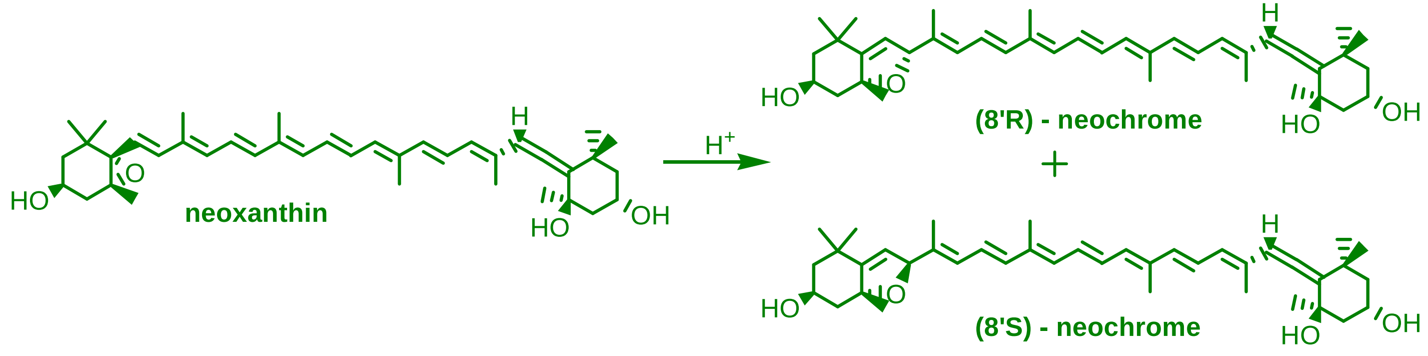
Epoxidation of zeaxanthin and lutein gives rise to violaxanthin and 5,6-epoxylutein ("xanthophyll cycles"), respectively.

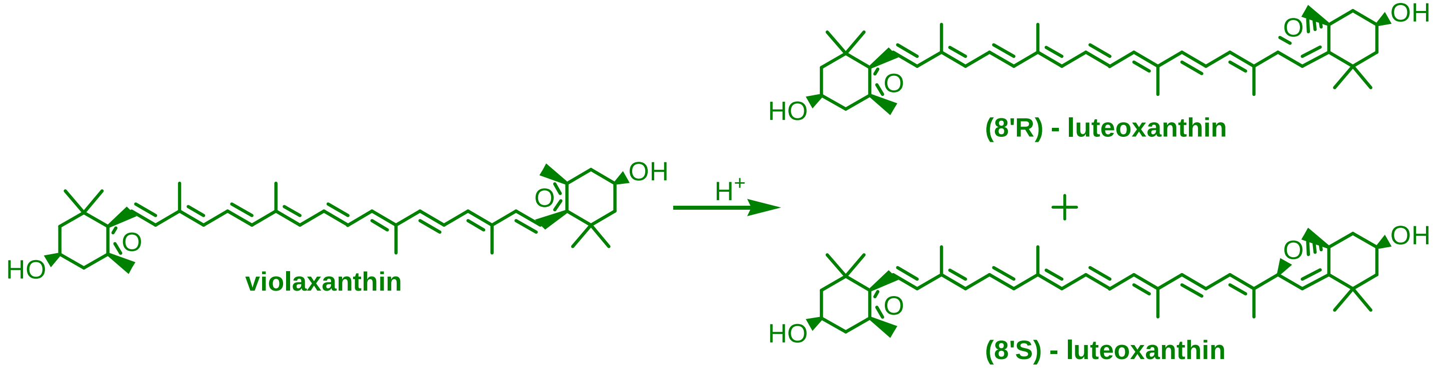
Finally, a stereoselective rearrangment of violaxanthin gives rise to neoxanthin.

Baumes, R.et al. *Anal.Chim.Acta 458,* **2002**, 3-14; Bungard, R.A. et al. *Proc. Nat.Acad.Sci. 96*, **1999**, 1135-1139.

# Introduction

In acidic medium 5,6-epoxyxanthophylls can be subjected to a non-stereoselective rearrangment to form 5,8-epoxyxanthophylls stereoisomers.



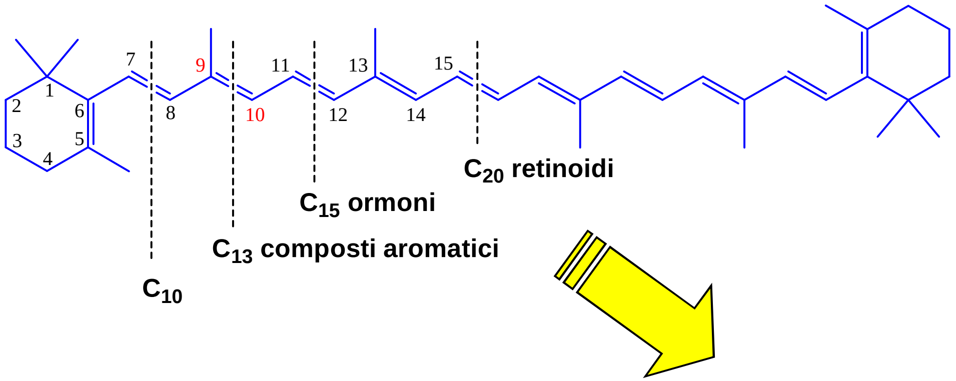


Asai, A.et al. *J. Nutr. 134,* **2004**, 2237-2243.

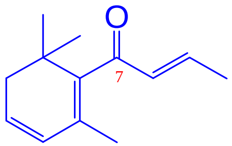
# Introduction

Carotenoids features:

-they works as “energy transfers”
-they protects the photosystems (i.e. $O_2$ quenchers)
-they are antioxidants and provitamine A
-they are varietal aroma precursors.

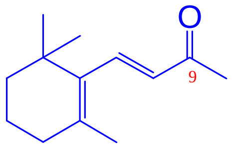


9,10-oxidative cleavage origines the flavors **$C_{13}$-norisoprenoids**, having an higher odor activity value.



β-damascenone

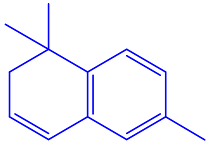
*Serie damascone*



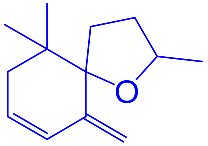
β-ionone

*Serie ionone*

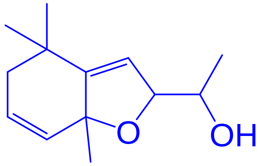
Forme megastigmane ossigenate



TDN
1,1,6-trimetil-1,2-diidronaftalene



Vitispirani

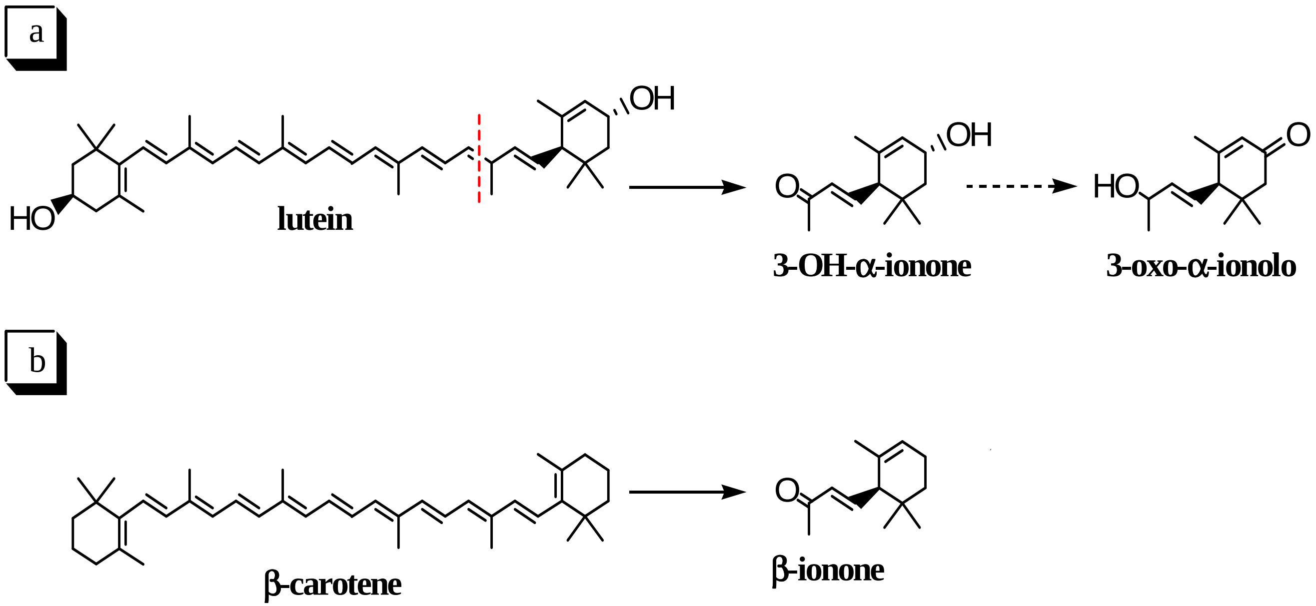


Actinidoli

Forme non megastigmane

# Introduction

The knowledge of the qualitative profile of carotenoids in grapes is very important because it is correlated to the $C_{13}$-norisoprenoids type formed in the wines.



Baumes et al. *Anal. Chim. Acta 458*, **2002**, 3-14

# *Goal*

Norisoprenoids content is correlated to the degradation of carotenoids and, obviously, to their pattern and concentration. Therefore, this information can be useful from a technological point of view, allowing to know the aroma potentiality of grapes.

AIM

Developing a comprehensive analytical method to provide information concerning structural and geometrical isomers of carotenoids that occur in a representative wine grape variety of the Apulian region (Negramaro) by means of HPLC-DAD-ESI-MS$^n$ with a C30 stationary phase.

# M&M: extraction of carotenoids

Hard points in the carotenoids analysis:

- High structural diversity
- Instability to light, heat, $O_2$ and acids (oxidation and *cis/trans* isomerization)

N.B. – add antioxidant and basic agents

- minimize light exposition
- avoid high T
- use high purity solvents without peroxides
- minimize the analysis time

50 g of fresh berries were homogenized in $N_2$ liq. in the presence of magnesium carbonate basic and BHA

↓

The provided sample was spiked with 200 μL S.I. and diluted with 40 mL of water (HPLC grade)

↓

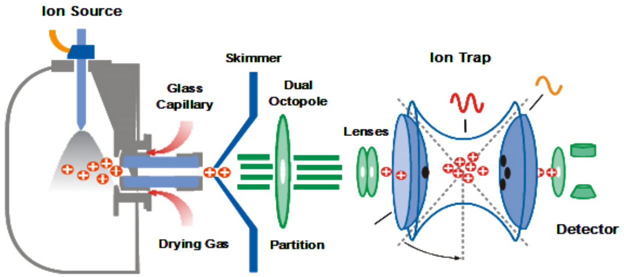
Extraction was done with hexane/ether (1:1, v/v) by agitating the mixture for 30 min (1x40 mL; 2x20 mL)

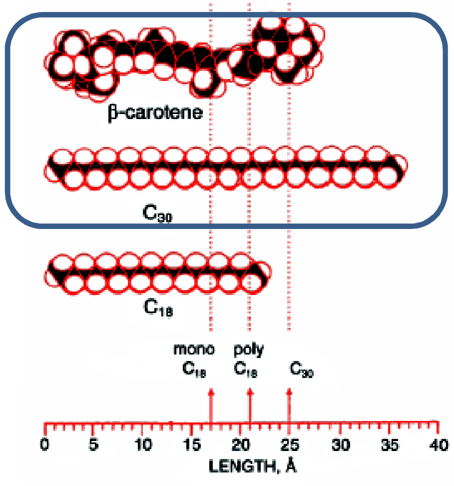
↓

The pooled extract was evaporated to dryness and re-suspended in 2 mL of TBME/hexane (1:1, v/v)

Crupi et al. *J.Mass Spectrom.* **2010**, *45(9)*, 971-980.

# M&M: analysis of carotenoids





Agilent 1100- LC/MSD Trap XCT Plus

*Column.: YMC C30 5μm, 250 mm x 3.0 mm i.d.;*

*Flow Rate: quaternary pump, 0.2mL/min*

*F.M.: A= 0.05% TEA in $H_2O$; B= 0.05% TEA in MeOH; C = 0.05% TEA in TBME.*

*Gradient = 0 min. 40%A-60%B-0%C; 5 min. 20%A-80%B-0%C; 10 min. 4%A-81%B-15%C; 60 min. 4%A-11%B-85%C.*

*Injection Volume = 10 μl*

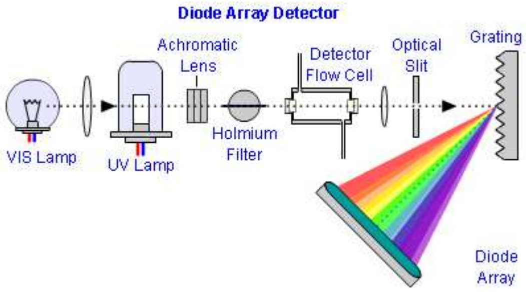
*DAD= 447 nm; 250-650 nm*

*MS: $ESI^+$ range 100-1200 m/z*

*Capillary: 4000 V*

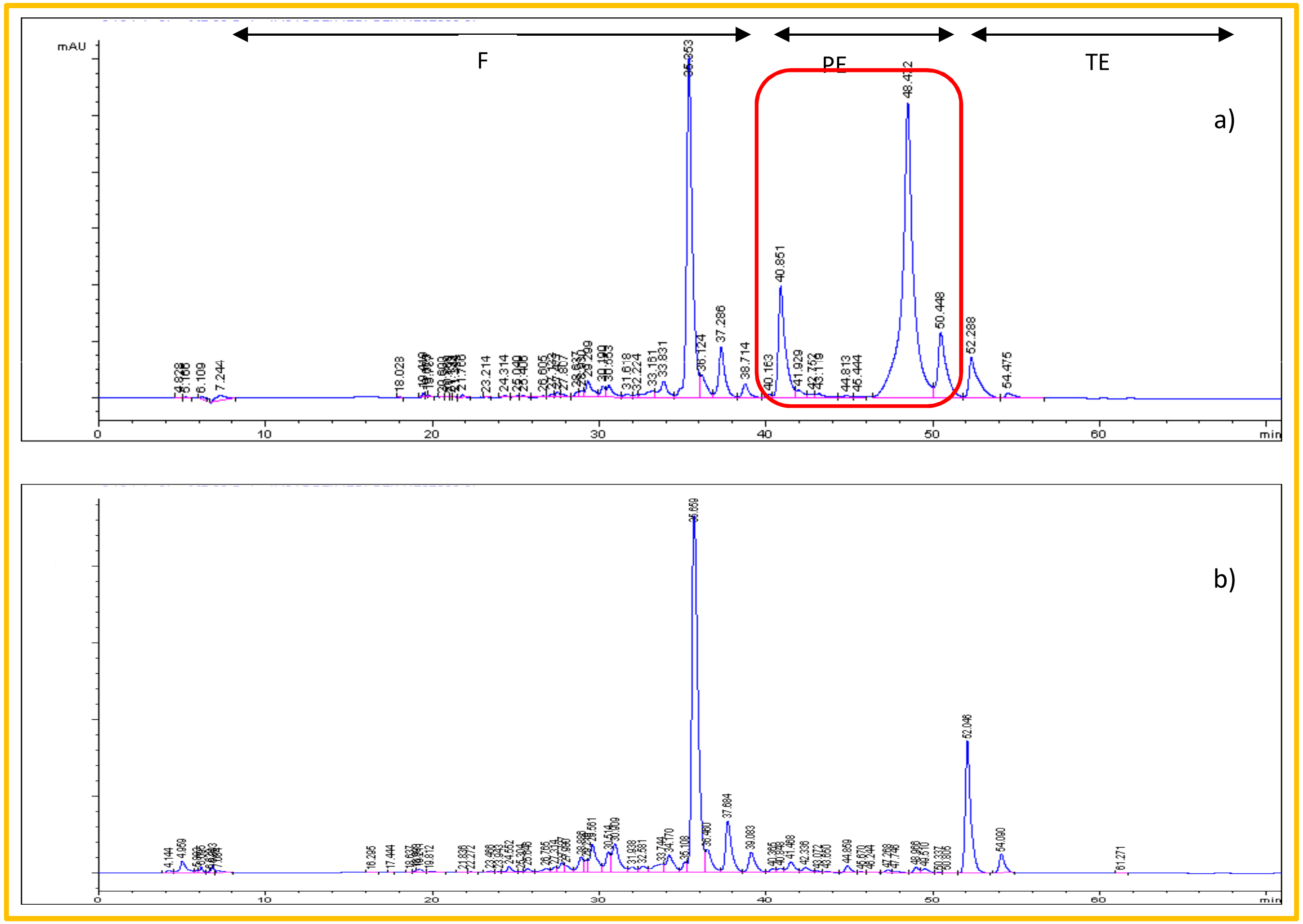
*Skimmer: 40V*

*Nebulizer: 15 psi; 5 L/min*



# R&D: identification of carotenoids

During “fruit ripening” polar xanthophylls may form esters with fatty acids; for this reason, after the sample extraction, traditionally, the second step is the alcaline saponification. In our work this step was avoided.



*HPLC-DAD analysis of carotenoids extracts from black grapes :*

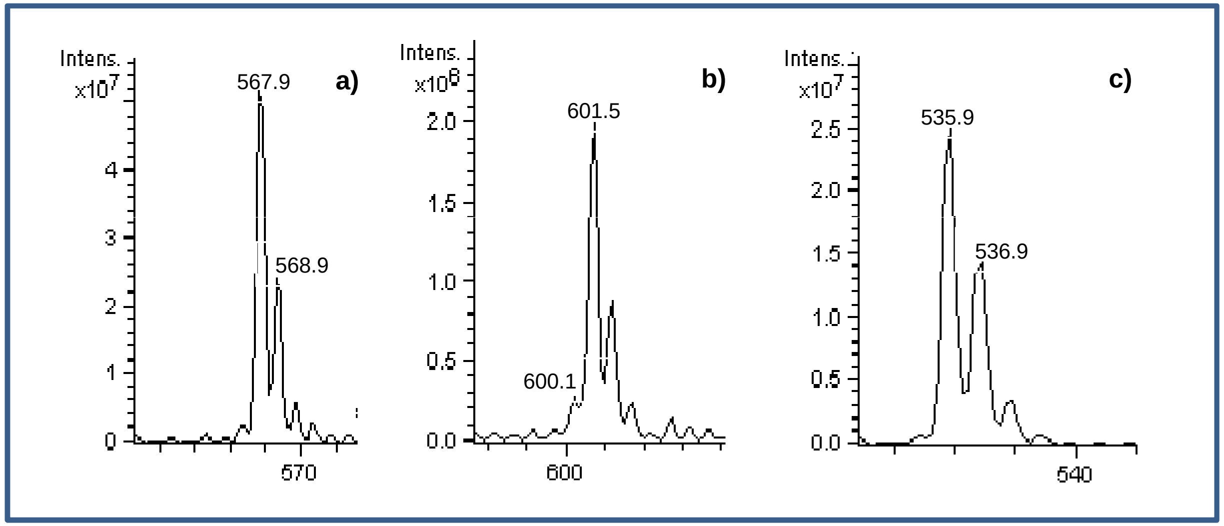
- *a) Metodo 1(Crupi 2010): luteina (35.35 min.), β-carotene (52.29 min.);*
- *b) Metodo 2 (Razungles, A 1987): luteina (35.66 min.), β-carotene (52.05 min.). S.I. β-apo-8’-carotenale (40,85 min.).*

*F = free fraction;*
*PE = partially esterified fraction;*
*TE = total esterified fraction.*

Crupi et al. *J.Mass Spectrom.* **2010**, *45(9),* 971-980.

# R&D: identification of carotenoids

FIA were carried out to determine suitable electrospray parameters and to test mobile phase composition on sample ionization. Because of easy oxidation through loss of an electron, the polyene β-carotene is normally ionized by a radical process, giving $[M]^{\cdot+}$ prevalently. Conversely, xanthophylls could afford both ionization processes, producing molecular and protonated species in ESI-MS.



ESI-MS spectra of a) (*all* E)-lutein in $H_2O$/MeOH/TBME 4:45:51 v/v; b) violaxanthin in $H_2O$/MeOH/TBME 4:61:35 v/v; c) β-carotene in $H_2O$/MeOH/TBME 4:20:76 v/v recorded in flow injection.

Crupi et al. *J.Mass Spectrom.* **2010**, *45(9)*, 971-980.

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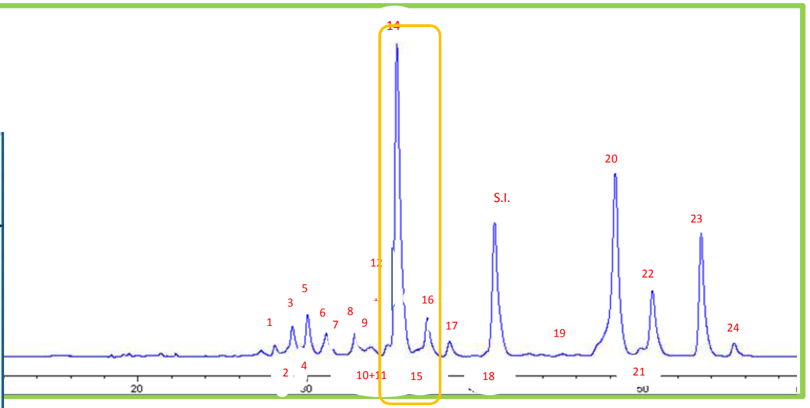
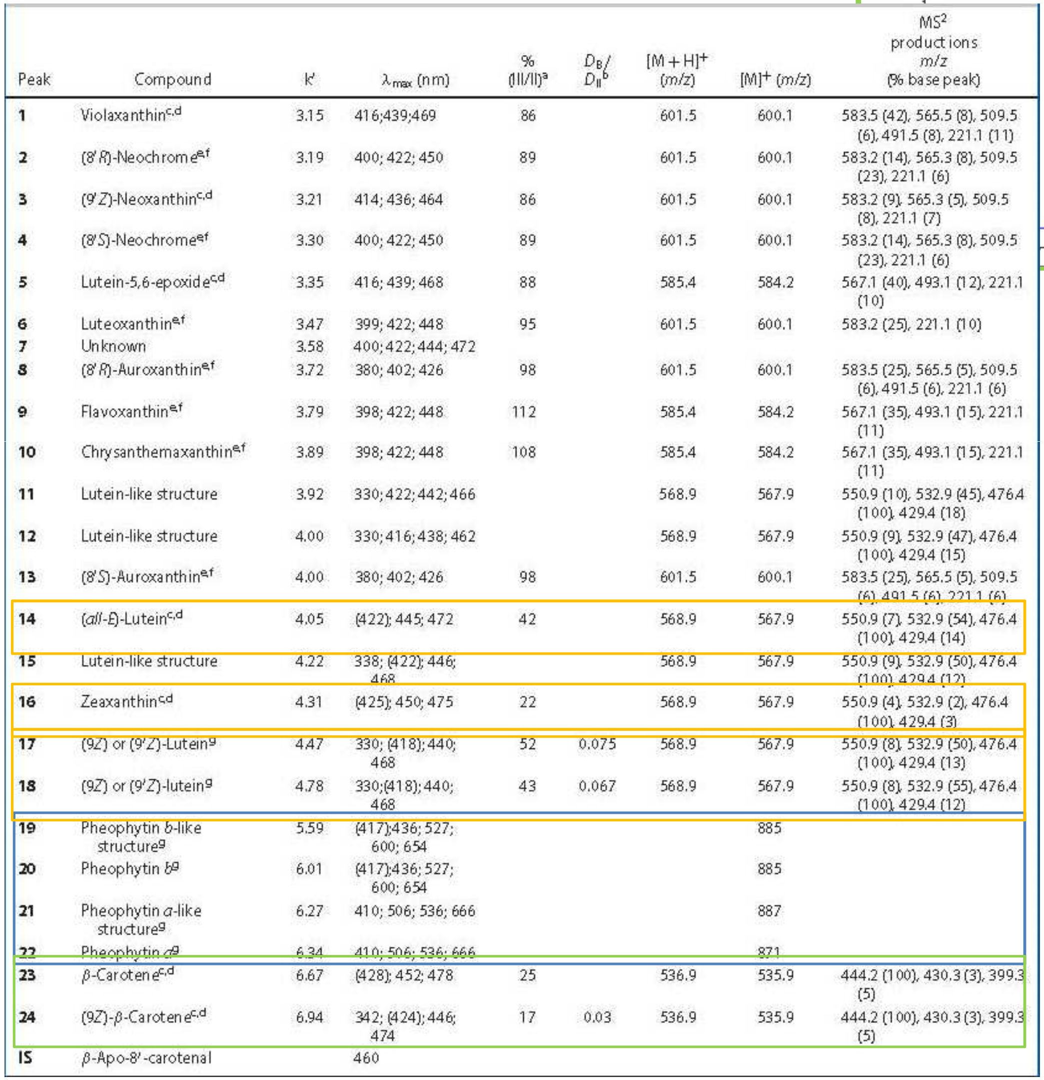
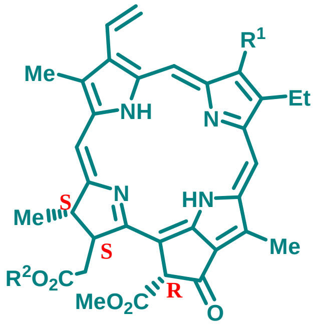
# R&D: carotenoids profile





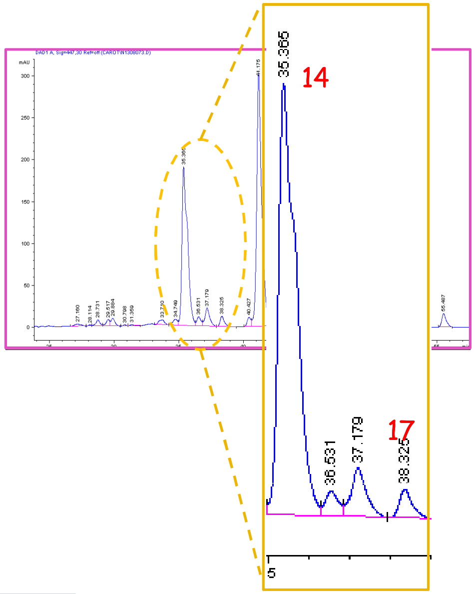
| Peak | Compound | k' | $\lambda_{max}$ (nm) | % (III/II)$^a$ | $D_B/D_{II}$$^b$ | $[M+H]^+$ (m/z) | $[M]^+$ (m/z) | MS$^2$ product ions m/z (% base peak) |
|---|---|---|---|---|---|---|---|---|
| 1 | Violaxanthin$^{c,d}$ | 3.15 | 416;439;469 | 86 | | 601.5 | 600.1 | 583.5 (42), 565.5 (8), 509.5 (6), 491.5 (8), 221.1 (11) |
| 2 | (8′R)-Neochrome$^{e,f}$ | 3.19 | 400; 422; 450 | 89 | | 601.5 | 600.1 | 583.2 (14), 565.3 (8), 509.5 (23), 221.1 (6) |
| 3 | (9′Z)-Neoxanthin$^{c,d}$ | 3.21 | 414; 436; 464 | 86 | | 601.5 | 600.1 | 583.2 (9), 565.3 (5), 509.5 (8), 221.1 (7) |
| 4 | (8′S)-Neochrome$^{e,f}$ | 3.30 | 400; 422; 450 | 89 | | 601.5 | 600.1 | 583.2 (14), 565.3 (8), 509.5 (23), 221.1 (6) |
| 5 | Lutein-5,6-epoxide$^{c,d}$ | 3.35 | 416; 439; 468 | 88 | | 585.4 | 584.2 | 567.1 (40), 493.1 (12), 221.1 (10) |
| 6 | Luteoxanthin$^{e,f}$ | 3.47 | 399; 422; 448 | 95 | | 601.5 | 600.1 | 583.2 (25), 221.1 (10) |
| 7 | Unknown | 3.58 | 400;422;444;472 | | | | | |
| 8 | (8′R)-Auroxanthin$^{e,f}$ | 3.72 | 380; 402; 426 | 98 | | 601.5 | 600.1 | 583.5 (25), 565.5 (5), 509.5 (6), 491.5 (6), 221.1 (6) |
| 9 | Flavoxanthin$^{e,f}$ | 3.79 | 398; 422; 448 | 112 | | 585.4 | 584.2 | 567.1 (35), 493.1 (15), 221.1 (11) |
| 10 | Chrysanthemaxanthin$^{e,f}$ | 3.89 | 398; 422; 448 | 108 | | 585.4 | 584.2 | 567.1 (35), 493.1 (15), 221.1 (11) |
| 11 | Lutein-like structure | 3.92 | 330;422;442;466 | | | 568.9 | 567.9 | 550.9 (10), 532.9 (45), 476.4 (100), 429.4 (18) |
| 12 | Lutein-like structure | 4.00 | 330;416;438;462 | | | 568.9 | 567.9 | 550.9 (9), 532.9 (47), 476.4 (100), 429.4 (15) |
| 13 | (8′S)-Auroxanthin$^{e,f}$ | 4.00 | 380; 402; 426 | 98 | | 601.5 | 600.1 | 583.5 (25), 565.5 (5), 509.5 (6), 491.5 (6), 221.1 (6) |
| 14 | (all-E)-Lutein$^{c,d}$ | 4.05 | (422); 445; 472 | 42 | | 568.9 | 567.9 | 550.9 (7), 532.9 (54), 476.4 (100), 429.4 (14) |
| 15 | Lutein-like structure | 4.22 | 338; (422); 446; 468 | | | 568.9 | 567.9 | 550.9 (9), 532.9 (50), 476.4 (100), 429.4 (12) |
| 16 | Zeaxanthin$^{c,d}$ | 4.31 | (425); 450; 475 | 22 | | 568.9 | 567.9 | 550.9 (4), 532.9 (2), 476.4 (100), 429.4 (3) |
| 17 | (9Z) or (9′Z)-Lutein$^g$ | 4.47 | 330; (418); 440; 468 | 52 | 0.075 | 568.9 | 567.9 | 550.9 (8), 532.9 (50), 476.4 (100), 429.4 (13) |
| 18 | (9Z) or (9′Z)-lutein$^g$ | 4.78 | 330;(418); 440; 468 | 43 | 0.067 | 568.9 | 567.9 | 550.9 (8), 532.9 (55), 476.4 (100), 429.4 (12) |
| 19 | Pheophytin b-like structure$^g$ | 5.59 | (417);436; 527; 600; 654 | | | | 885 | |
| 20 | Pheophytin b$^g$ | 6.01 | (417);436; 527; 600; 654 | | | | 885 | |
| 21 | Pheophytin a-like structure$^g$ | 6.27 | 410; 506; 536; 666 | | | | 887 | |
| 22 | Pheophytin a$^g$ | 6.34 | 410; 506; 536; 666 | | | | 871 | |
| 23 | β-Carotene$^{c,d}$ | 6.67 | (428); 452; 478 | 25 | | 536.9 | 535.9 | 444.2 (100), 430.3 (3), 399.3 (5) |
| 24 | (9Z)-β-Carotene$^{c,d}$ | 6.94 | 342; (424); 446; 474 | 17 | 0.03 | 536.9 | 535.9 | 444.2 (100), 430.3 (3), 399.3 (5) |
| IS | β-Apo-8′-carotenal | | 460 | | | | | |



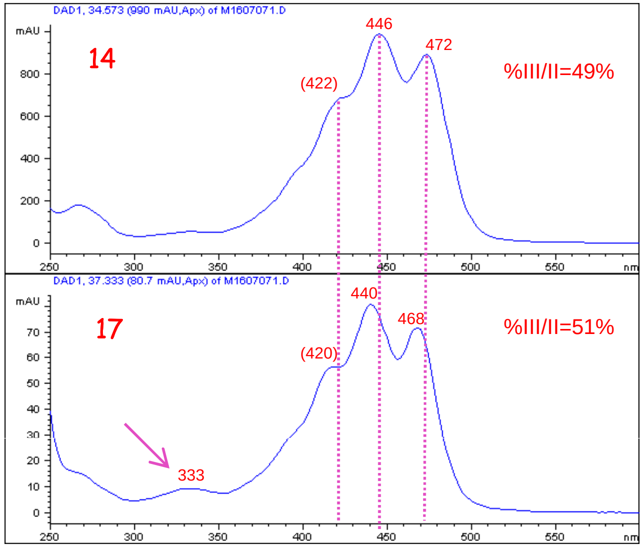
**Pheophytin a** $R^1$= $-CH_3$; $R^2$= phytyl
**Pheophytin b** $R^1$= $-CHO$; $R^2$= phytyl

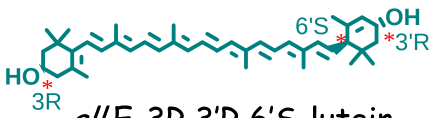
Zeaxanthin α = 1,06

# R&D: carotenoids profile

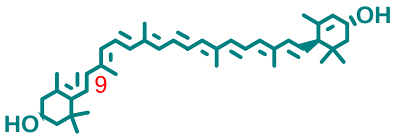


**UV-vis**



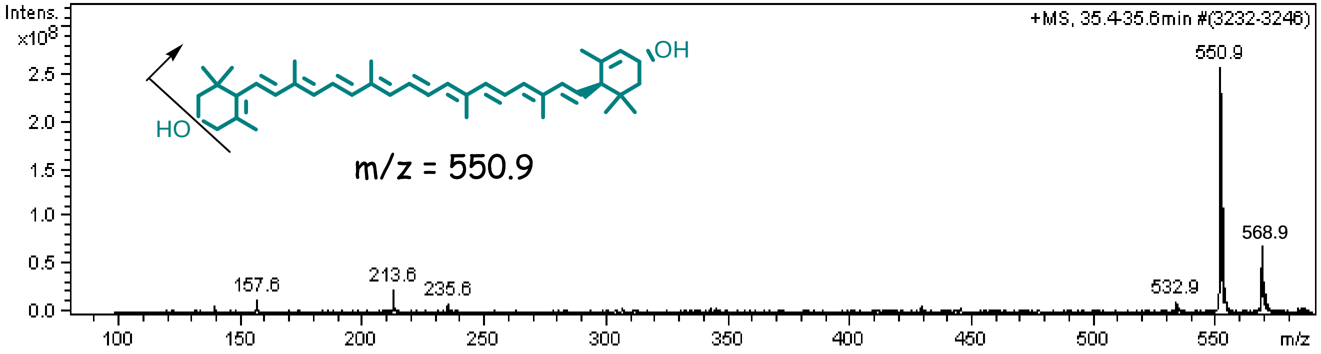


*all* E-3R,3'R,6'S-lutein



9Z o 9'Z-lutein

**MS**

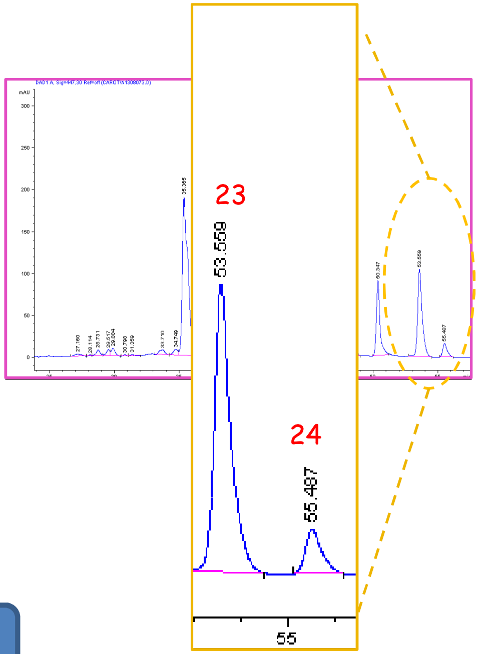


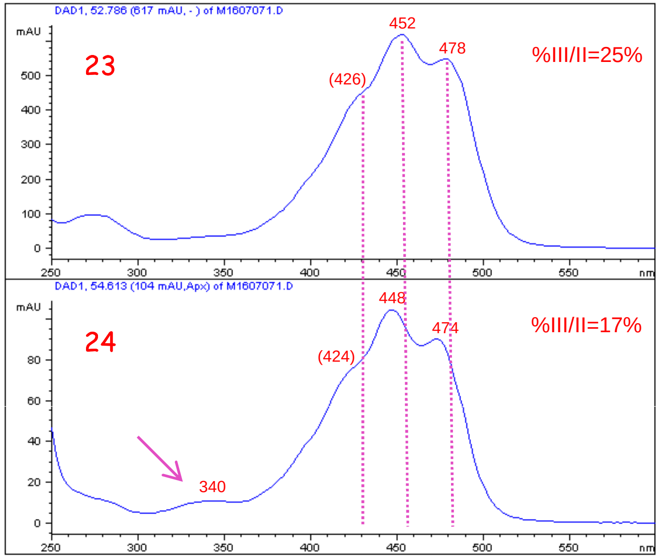
m/z = 550.9

$D_B/D_{II}$ = 0,075

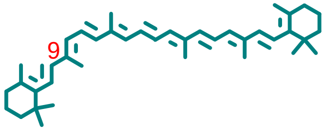
# R&D: carotenoids profile

**UV-vis**



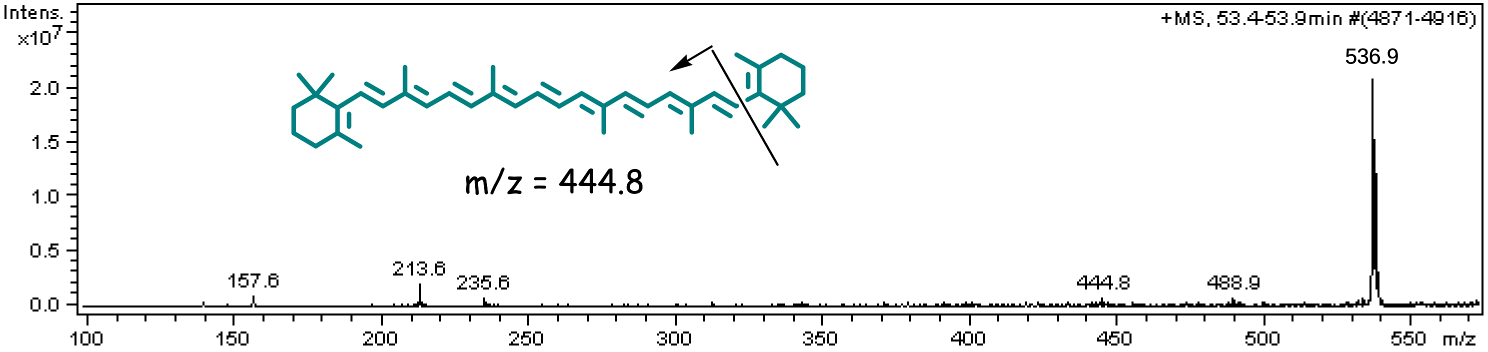


*all* E-β-carotene



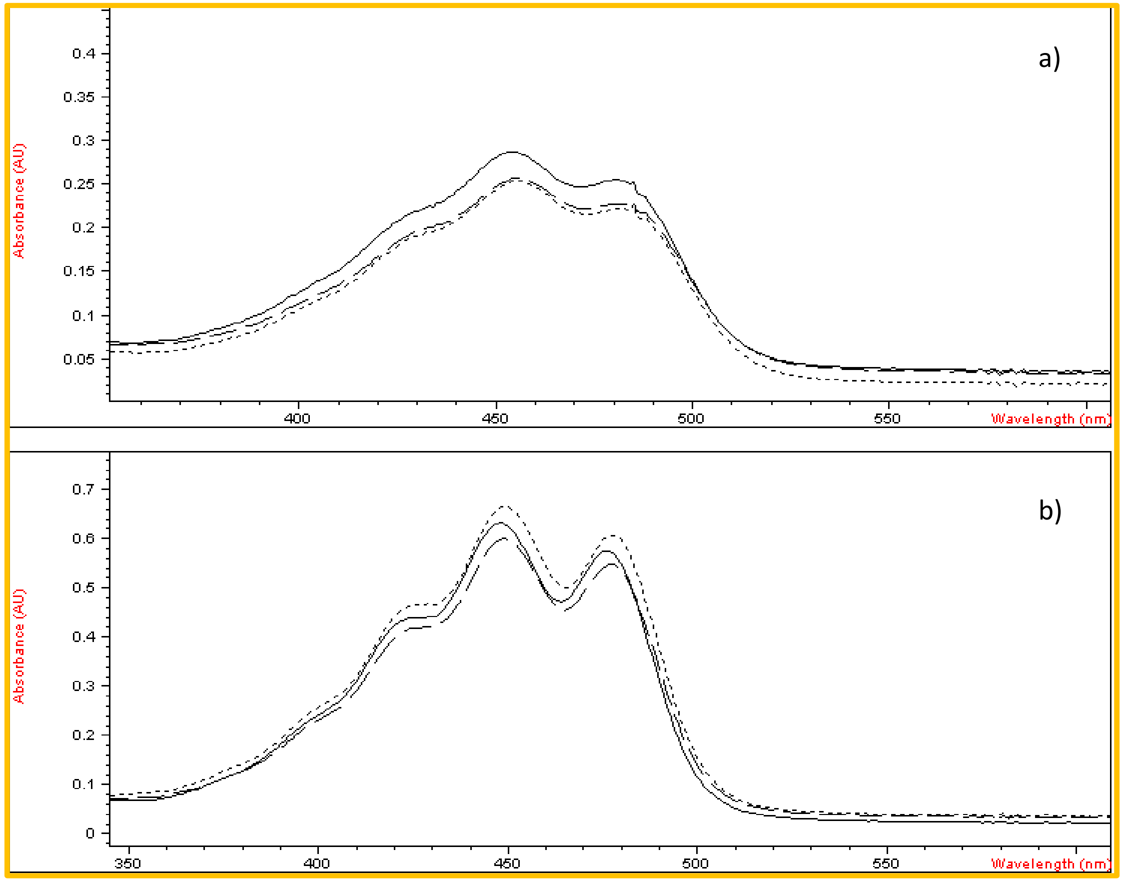
9Z -β-carotene

**MS**



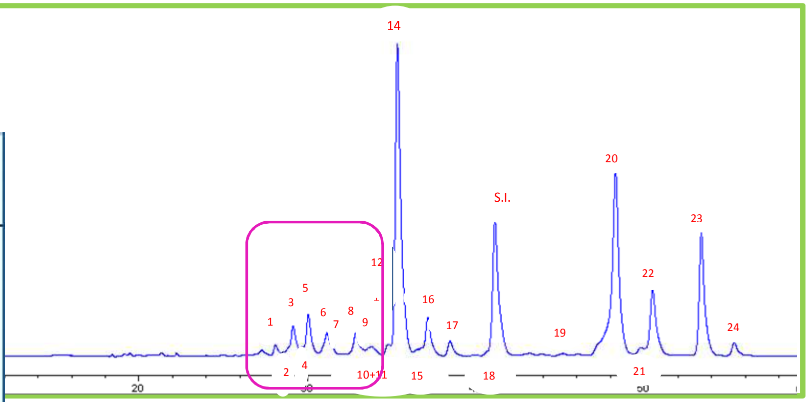
# R&D: carotenoids profile

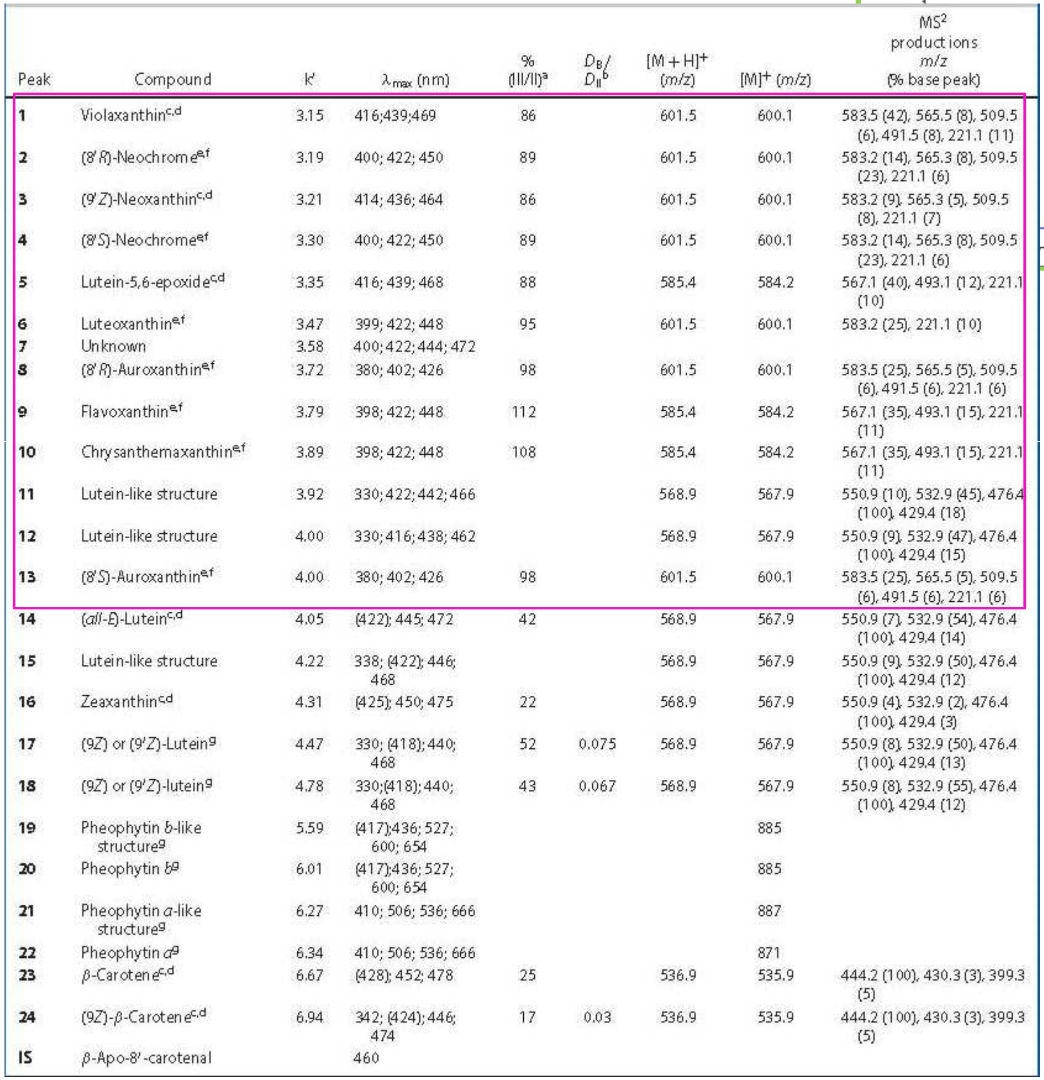
The identified cis-isomers, (9Z)-β-carotene and (9 o 9’Z)-lutein, cannot be artifact compounds.



*UV-vis spectra of β-carotene a) and lutein b) in presence of tartaric acid (15 g/L).*

# R&D: carotenoids profile





| Peak | Compound | k′ | λ$_{max}$ (nm) | % (III/II)$^a$ | $D_B/D_{II}$$^b$ | [M + H]$^+$ (m/z) | [M]$^+$ (m/z) | MS$^2$ product ions m/z (% base peak) |
|---|---|---|---|---|---|---|---|---|
| **1** | Violaxanthin$^{c,d}$ | 3.15 | 416;439;469 | 86 | | 601.5 | 600.1 | 583.5 (42), 565.5 (8), 509.5 (6), 491.5 (8), 221.1 (11) |
| **2** | (8′R)-Neochrome$^{e,f}$ | 3.19 | 400; 422; 450 | 89 | | 601.5 | 600.1 | 583.2 (14), 565.3 (8), 509.5 (23), 221.1 (6) |
| **3** | (9′Z)-Neoxanthin$^{c,d}$ | 3.21 | 414; 436; 464 | 86 | | 601.5 | 600.1 | 583.2 (9), 565.3 (5), 509.5 (8), 221.1 (7) |
| **4** | (8′S)-Neochrome$^{e,f}$ | 3.30 | 400; 422; 450 | 89 | | 601.5 | 600.1 | 583.2 (14), 565.3 (8), 509.5 (23), 221.1 (6) |
| **5** | Lutein-5,6-epoxide$^{c,d}$ | 3.35 | 416; 439; 468 | 88 | | 585.4 | 584.2 | 567.1 (40), 493.1 (12), 221.1 (10) |
| **6** | Luteoxanthin$^{e,f}$ | 3.47 | 399; 422; 448 | 95 | | 601.5 | 600.1 | 583.2 (25), 221.1 (10) |
| **7** | Unknown | 3.58 | 400;422;444; 472 | | | | | |
| **8** | (8′R)-Auroxanthin$^{e,f}$ | 3.72 | 380; 402; 426 | 98 | | 601.5 | 600.1 | 583.5 (25), 565.5 (5), 509.5 (6), 491.5 (6), 221.1 (6) |
| **9** | Flavoxanthin$^{e,f}$ | 3.79 | 398; 422; 448 | 112 | | 585.4 | 584.2 | 567.1 (35), 493.1 (15), 221.1 (11) |
| **10** | Chrysanthemaxanthin$^{e,f}$ | 3.89 | 398; 422; 448 | 108 | | 585.4 | 584.2 | 567.1 (35), 493.1 (15), 221.1 (11) |
| **11** | Lutein-like structure | 3.92 | 330;422;442;466 | | | 568.9 | 567.9 | 550.9 (10), 532.9 (45), 476.4 (100), 429.4 (18) |
| **12** | Lutein-like structure | 4.00 | 330;416;438; 462 | | | 568.9 | 567.9 | 550.9 (9), 532.9 (47), 476.4 (100), 429.4 (15) |
| **13** | (8′S)-Auroxanthin$^{e,f}$ | 4.00 | 380; 402; 426 | 98 | | 601.5 | 600.1 | 583.5 (25), 565.5 (5), 509.5 (6), 491.5 (6), 221.1 (6) |
| **14** | (all-E)-Lutein$^{c,d}$ | 4.05 | (422); 445; 472 | 42 | | 568.9 | 567.9 | 550.9 (7), 532.9 (54), 476.4 (100), 429.4 (14) |
| **15** | Lutein-like structure | 4.22 | 338; (422); 446; 468 | | | 568.9 | 567.9 | 550.9 (9), 532.9 (50), 476.4 (100), 429.4 (12) |
| **16** | Zeaxanthin$^{c,d}$ | 4.31 | (425); 450; 475 | 22 | | 568.9 | 567.9 | 550.9 (4), 532.9 (2), 476.4 (100), 429.4 (3) |
| **17** | (9Z) or (9′Z)-Lutein$^g$ | 4.47 | 330; (418); 440; 468 | 52 | 0.075 | 568.9 | 567.9 | 550.9 (8), 532.9 (50), 476.4 (100), 429.4 (13) |
| **18** | (9Z) or (9′Z)-lutein$^g$ | 4.78 | 330;(418); 440; 468 | 43 | 0.067 | 568.9 | 567.9 | 550.9 (8), 532.9 (55), 476.4 (100), 429.4 (12) |
| **19** | Pheophytin b-like structure$^g$ | 5.59 | (417);436; 527; 600; 654 | | | | 885 | |
| **20** | Pheophytin b$^g$ | 6.01 | (417);436; 527; 600; 654 | | | | 885 | |
| **21** | Pheophytin a-like structure$^g$ | 6.27 | 410; 506; 536; 666 | | | | 887 | |
| **22** | Pheophytin a$^g$ | 6.34 | 410; 506; 536; 666 | | | | 871 | |
| **23** | β-Carotene$^{c,d}$ | 6.67 | (428); 452; 478 | 25 | | 536.9 | 535.9 | 444.2 (100), 430.3 (3), 399.3 (5) |
| **24** | (9Z)-β-Carotene$^{c,d}$ | 6.94 | 342; (424); 446; 474 | 17 | 0.03 | 536.9 | 535.9 | 444.2 (100), 430.3 (3), 399.3 (5) |
| **IS** | β-Apo-8′-carotenal | | 460 | | | | | |

The mass spectra of 5,6-epoxyxanthophylls were very similar to that of 5,8-epoxyxanthophylls, both with the same molecular ions and protonated molecules, and the same fragmentation profile.

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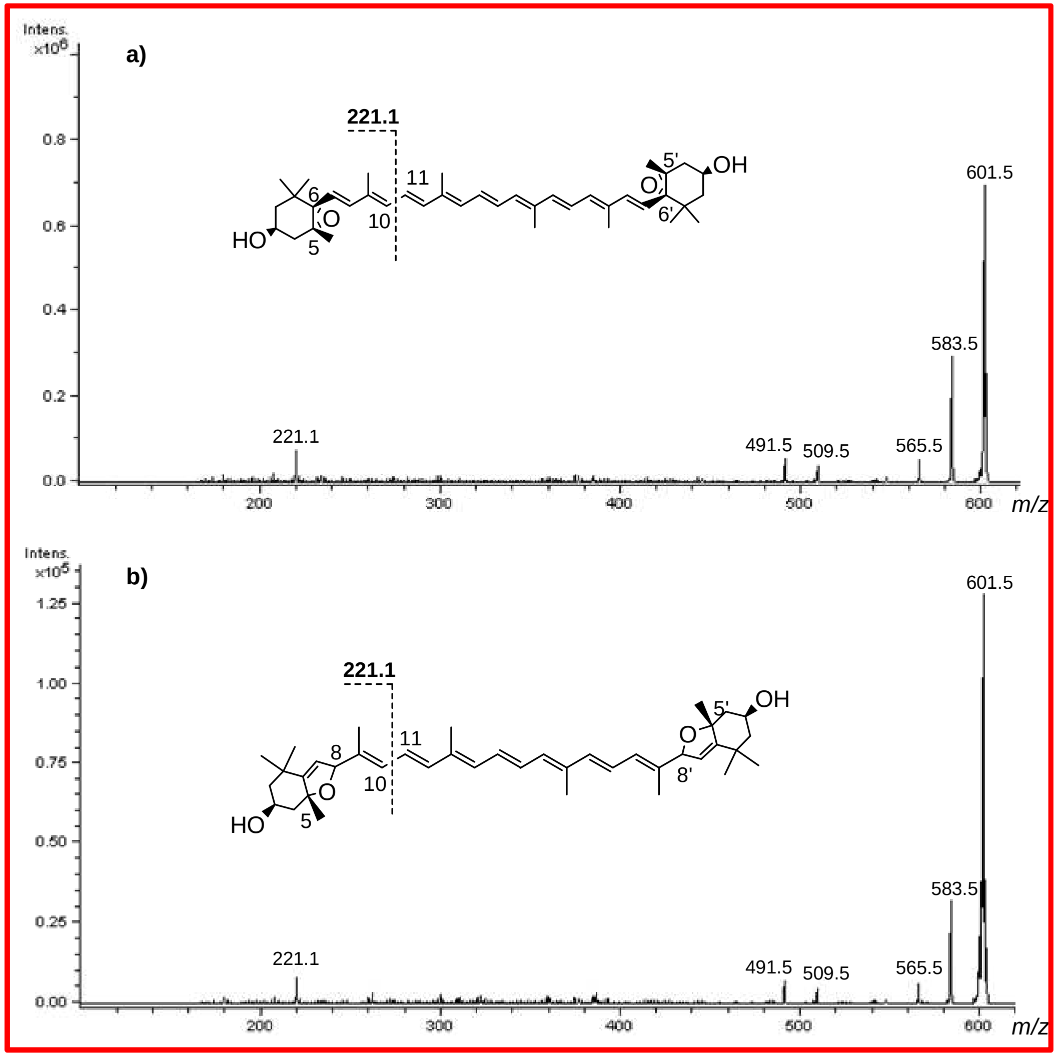
In particular, the regioisomers, violaxanthin and auroxanthins, which are symmetrical epoxy xanthophylls having a 3-OH-5,6-epoxy and 3-OH-5,8-epoxy end groups, respectively, on both sides of the polyene chain, showed almost the same product ions patterns.



CID $MS^2$ spectrum of $[M+H]^+$ of (a) violaxanthin and (b) auroxanthin.

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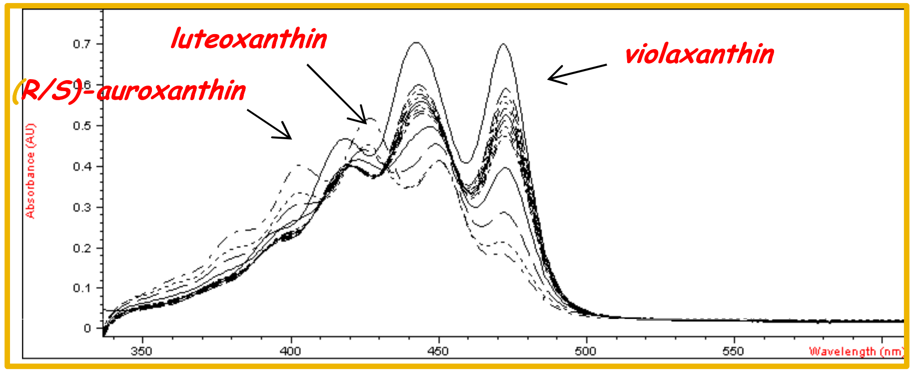
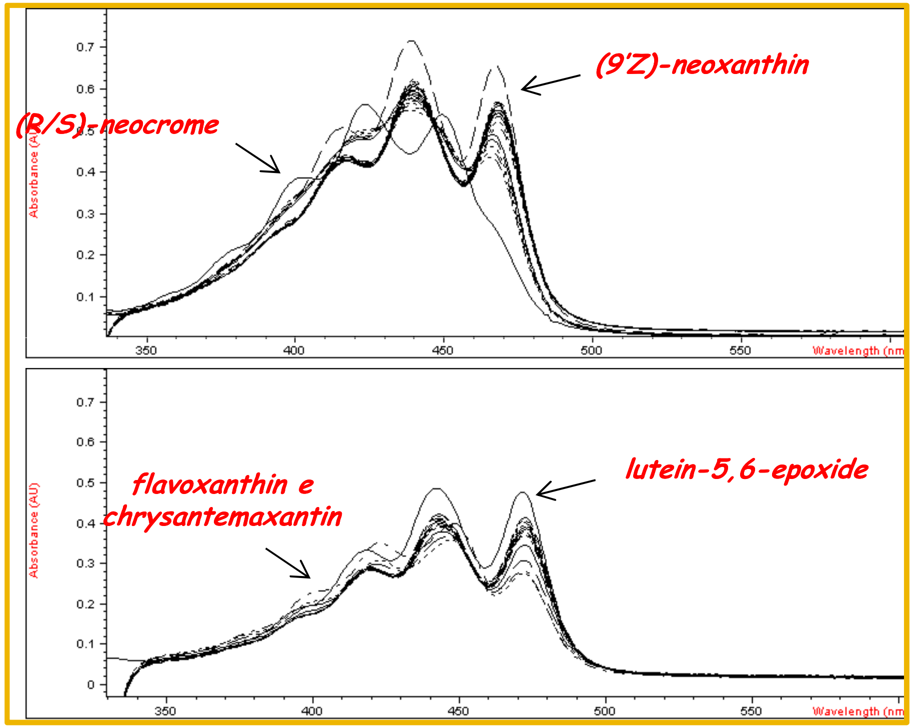
UV-vis spectra were used to confirm the difference between 5,6- and 5,8-epoxixanthophylls regioisomeric compounds.





Because of the presence of one 5,8-furanoid group, the (R/S)-neochrome, luteoxanthin, flavoxanthin, and chrysanthemaxanthin showed hypsochromic shifts of 15-20 nm as compared to the relative 5,6-epoxyxanthophylls.

On the contrary, the presence of two 5,8-furanoid groups in the (R/S)-auroxanthin provoked higher hypsochromic shifts of 40 nm as compared to violaxanthin.

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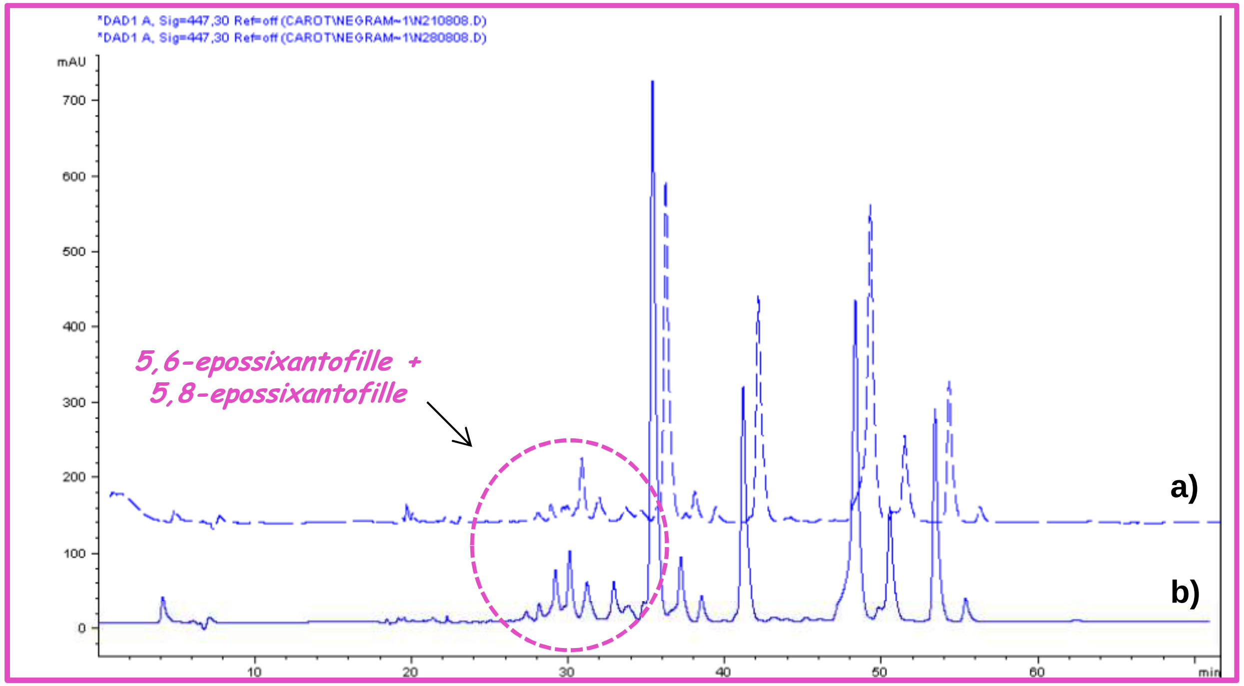
Are they extraction artefact?

a) Grape extract analysis (pH=3,3).
b) Grape extract analysis with $(MgCO_3)*4Mg(OH)_2*5H_2O$ (pH=6,8).

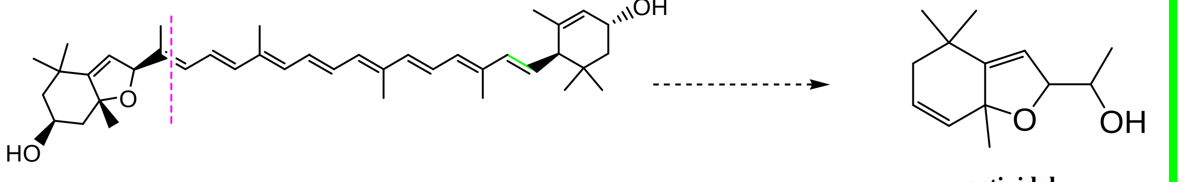


Evidently, 5,6- → 5,8-epoxyxanthophylls isomerization already happens in the grape cells.
Even though, the extraction medium might vary their relative ratio.

# Conclusions

- Over 20 compounds, between regio- and geometric isomers of carotenoids and chlorophyll-derived, were identified by means of comprehensive HPLC-DAD-ESI-MS$^n$ analysis.

- The chromatographic and UV-Vis analyses showed that 5,8-epossixanthophylls could be partially “artifact compounds” of the extraction medium, but they are already present in the grape skin cells.
Moreover, aroma analysis confirmed the presence of actinidols, derived from 5,8-epossixanthophylls, in wine.



actinidol