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HPLC and SFC of carotenoids – scope and limitations

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Abstract - Today HPLC is an indispensable analytical method in carotenoid research. Different systems for the separation of carotenes, hydroxycarotenoids (lutein/zeaxanthin; violaxanthin/neoxanthin), *E/Z* isomers and optical isomers are discussed. Minimum criteria for the proper identification of a carotenoid are postulated. The application of SFC to the separation of carotenoids is reviewed and it is shown that this method is generally of less importance in the carotenoid field.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

General remarks

The first separation of carotenoids by HPLC was reported in 1971 by Stewart et al. (ref. 1). The publication about 'Continuous Flow Separation of Carotenoids by Liquid Chromatography' described the separation of complex mixtures of carotenoids from citrus extracts. This work used precipitated $ZnCO_3$ and MgO in steel columns and the chromatogram was monitored at 440 nm. The results obtained with the 'home-made' apparatus are remarkable, although the separations took several hours.

Since then the development of the field was very rapid and today in almost every publication on carotenoids, HPLC investigations are described. The literature on the topic is therefore vast. In previous Symposia the field of HPLC of carotenoids has been reviewed by Rüedi (ref. 2) and Khachik et al. (ref. 3), respectively. Further information can be taken from recent articles by Craft (ref. 4), Khachik (ref. 5) and Tsukida (ref. 6). In the forthcoming new series 'Carotenoids' (ref. 7) a review with many examples is given and additional worked examples provide further information.

The reason for the rapid development lies in the improved technology of the apparatus and the efficiency of the method. This is illustrated by the number of theoretical plates, which is a measure of the efficiency of the separation (classical liquid chromatography: 200 per metre; HPLC: 50,000; TLC: 1000; HPTLC: 5000). But although these numbers are impressive it is clear that classical column chromatography and TLC will continue to be used, even for difficult separations. An example is the use of $CaCO_3$ as stationary phase in column chromatography. Although $CaCO_3$ has a low capacity, excellent separations have been obtained (ref. 8).

A milestone in analysis by HPLC has been the introduction of the photodiode array detector (PDAD). With computer-assisted PDADs the spectrum of each peak in a chromatogram can be stored and subsequently compared with standard spectra. The most important advantage is that the instrument can provide a contour plot, showing the relationship between absorbance, wavelength and time. This can be used for the detection of impurities in the sample. One disadvantage of PDADs is that they are less sensitive than monochromatic detectors.

Examples of HPLC separations

The separation of some carotenoids is difficult but at the same time of great importance, especially in biological studies: mixtures of α-carotene/β-carotene, or lutein/zeaxanthin and violaxanthin/neoxanthin are examples. Their separations provide a measure of the quality of the separation system.

Separation of carotenes. The resolution of α- and β-carotene depends critically on the stationary phase. In the literature many C_{18}-reversed phase (RP) systems with different mobile phases have been described. Normally end-capped material has been used, but also separations with non-end-capped stationary phases have been described. These separation systems are listed in ref. 4. It seems that silica is less suitable for the separation. Apart from these RP systems, other systems have been reported where the main emphasis was on the separation of α- and β-carotene, especially with $Ca(OH)_2$ (ref. 9). In general $Ca(OH)_2$ gives good resolution but there can be problems with reproducibility and column packing.

Separation of hydroxycarotenoids; especially lutein/zeaxanthin. Lutein and zeaxanthin are extremely common carotenoids in plants and algae. Many systems have been worked out to separate these compounds. In many cases good separation of α- and β-carotene and of neoxanthin and violaxanthin was also achieved under the same conditions. These systems are useful for an 'Overview chromatogram'. If the sole requirement is a good separation of lutein and zeaxanthin, silica as stationary phase has given good results (Fig.1) (ref. 10). Also nitrile-bonded phase has been used successfully (ref. 11). Most separations of lutein and zeaxanthin, however, have been achieved under RP conditions (ref. 7, 12-17).
Most of these systems have used fully end-capped octadecylsilanols. These procedures are not all capable of baseline separation, but are acceptable because of their other advantages. They may be optimized for speed (t_R< 8 min) and can be applied to plant samples such as extracts of green plants or chloroplasts, green algae, fruits or phytoplankton, or to milk and human plasma. Most of the systems use a solvent gradient and/or a flow gradient, but there are systems which are effective under isocratic conditions or a stepwise gradient. A comparative evaluation of these systems is very difficult because of different experimental parameters. Some examples are given in (Figs. 2-4).
Temperature is a frequently neglected factor in chromatography. The influence of temperature on column selectivity appears to be related primarily to the modification of the stationary phase but is also important for the reproducibility. The influence of temperature was investigated for the separation of lutein/zeaxanthin (ref. 18) and a better resolution was obtained at lower temperature (-13°C).
At least for the separation of lutein and zeaxanthin, β-cyclodextrin-bonded stationary phase has given promising results (Fig. 5) (ref. 19).

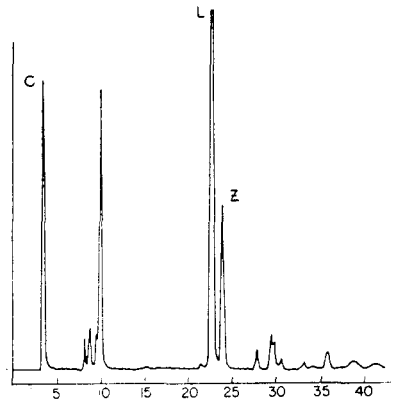

Fig.1. Separation of corn grain extract on silica (stepwise gradient of 2-propanol in hexane) (ref. 10).

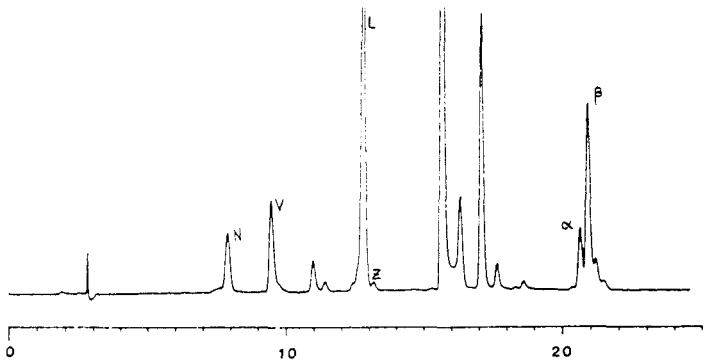

Fig.2. Pigments from chloroplasts of *Phaseolus vulgaris*, separated on reversed phase (C_{18}). Stepwise gradient of ethyl acetate in acetonitrile/water/TEA (90:10:0.1) (ref. 16).

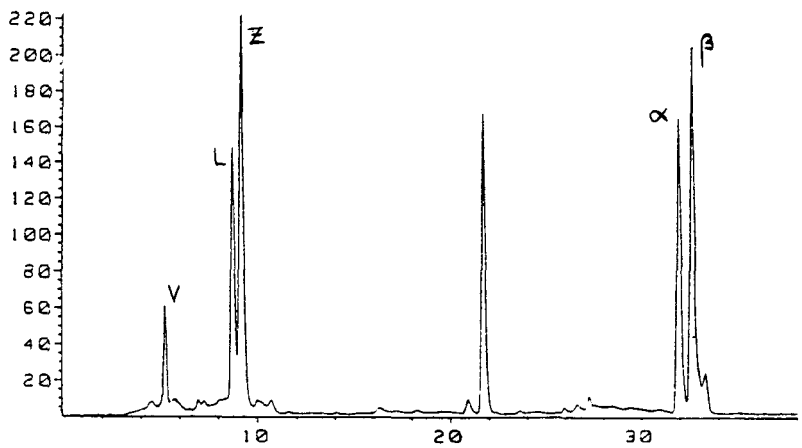

Fig. 3. Separation of carotenoid standards on reversed phase (C_{18}) (gradient of MeOH/CH_2Cl_2/hexane/acetonitrile). (V = violaxanthin; L = lutein; Z = zeaxanthin; α = α-carotene; β = β-carotene) (ref. 12).

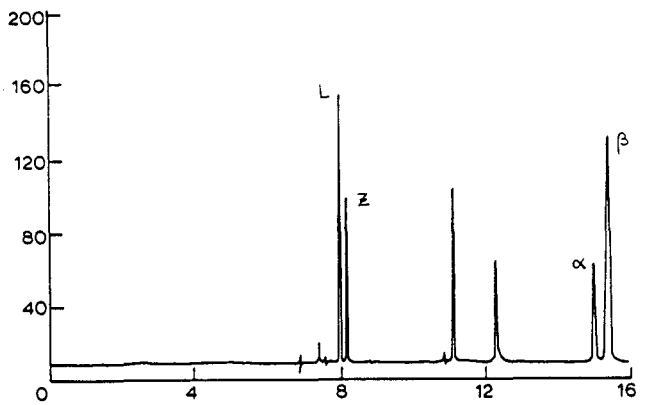

Fig. 4. Separation of carotenoid standards on reversed phase (C_{18}), with a flow gradient (ref. 15).

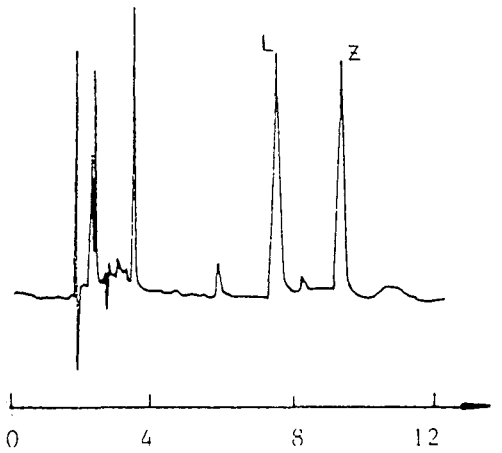

Fig. 5. Separation of lutein and zeaxanthin on β-cyclodextrin (hexane/ethanol 95:5) (ref. 19).

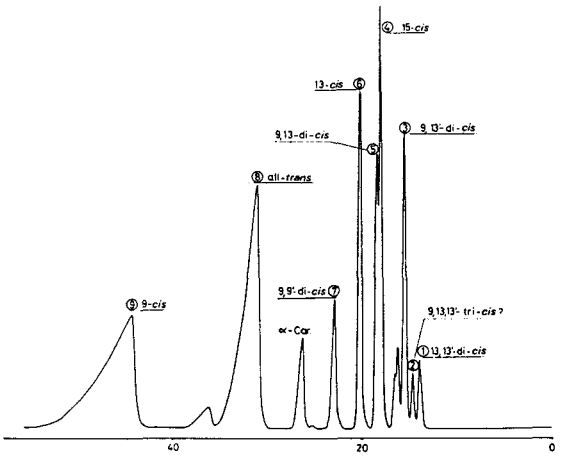

Fig. 6. Separation of *E/Z* isomers of β-carotene on alumina (hexane with controlled content of water) (ref. 21).

Separation of *E/Z* isomers. In recent years a revival of interest in *E/Z* isomers of carotenoids has taken place. It was shown that many *Z* isomers occur naturally and may have important biological activities. Today, many sophisticated HPLC systems for the separation of *E/Z* isomers are known but proper structure elucidation is critical in such work (ref. 20). In general, from a whole extract it is not possible to separate all the individual carotenoids and their *E/Z* isomers in one step. In this case a pre-separation into different fractions *i.e.* all the geometrical isomers of one single carotenoid, followed by a second separation of the isomers, is necessary.

It is often important only to separate directly a restricted number of *E/Z* isomers, which are typical for a particular source. In this case, the method must be applicable for routine analysis which means good reproducibility and high speed. These routine analyses are performed mostly with reversed and/or nitrile phases (ref. 5).

The best separations of large sets of *E/Z* isomers of a given carotenoid have been obtained with columns of $Ca(OH)_2$ or alumina. Both these stationary phases are difficult to use, however, and require more experience than do the chemically bonded phases.

Two systems have been described which will separate more than ten *E/Z* isomers of β-carotene. As stationary phases either $Ca(OH)_2$ or alumina have been used. With alumina as stationary phase and hexane with a controlled content of water as mobile phase 13 isomers were separated (ref. 21) (Fig. 6). The system was very sensitive to temperature changes and water content.

The system with $Ca(OH)_2$ does not need any strict control of temperature or water, but the column has to be packed by the user and furthermore the $Ca(OH)_2$ from most manufacturers does not give efficient separations (ref. 22).

The separation of racemic astaxanthin diacetate into ten *E/Z* isomers has been achieved under normal phase (NP) conditions with LiChrosorb SI60 as stationary phase (ref. 23). The *E/Z* isomers of astaxanthin can also be separated without derivatization on silica, coated with phosphoric acid (ref. 24).

Efforts to improve the synthesis of optically active zeaxanthin resulted in the isolation of eleven *Z* isomers separated on silica (Fig. 7) (ref. 25).

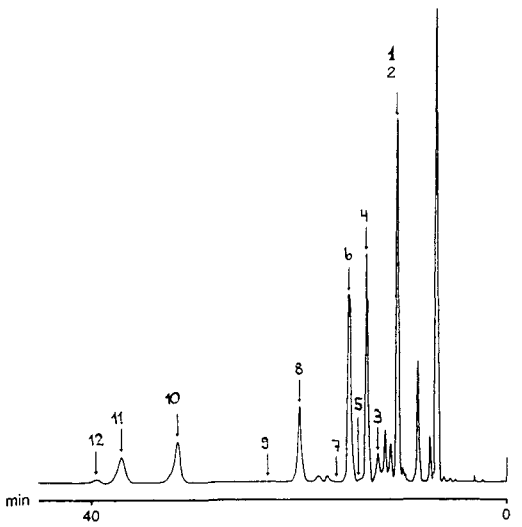

Fig. 7. Separation of *E/Z* isomers of zeaxanthin on silica (1=all-*E*; for the assignment of the other peaks see ref. 25).

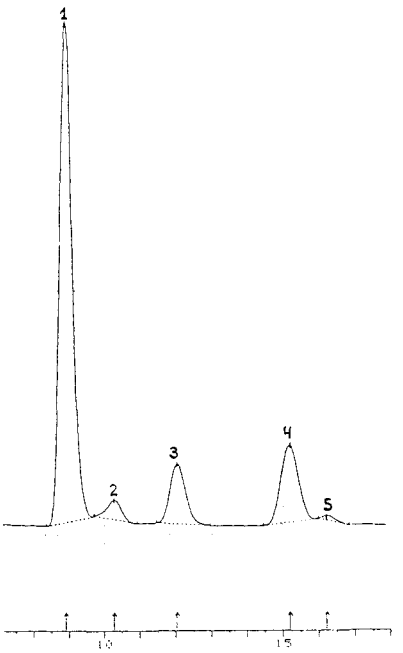

Fig. 8. *E/Z* isomers of bacterioruberin (1=all-*E*; 2=5Z; 3=9Z; 4=13Z; 5=15Z). Separation on ODS-2 (methanol/ethyl acetate/water/ TEA 85.5:9:5.5:1) (ref. 26).

During our investigations of the kinetics and thermodynamics of isomerization of C_{50} carotenoids, we developed a separation system for the major *E/Z* isomers, *e.g.* of bacterioruberin (Fig. 8) (ref. 26). It is a RP system and especially interesting is the elution order of the isomers, which is obviously related to the overall geometry of the molecule. The same order of the isomers was also observed for other carotenoids on RP, *e.g.* β-carotene.

Separation of optical isomers. Out of more than 600 naturally occurring carotenoids about 400 are chiral. In Nature, normally one optical isomer of a carotenoid occurs specifically. In some cases, however, mixures have been observed. Furthermore, in synthesis, the optical purity of the product has to be investigated. For the separation of optical isomers, conversion into diastereoisomers can be applied or a chiral stationary phase can be used. These two principles will be shown in the following examples.
For the HPLC resolution of the optical isomers of zeaxanthin both principles have been used. Zeaxanthin was reacted with (*S*)-α-(1-naphthyl)ethyl isocyanate to give the diastereoisomeric dicarbamates which were separated on a non-chiral stationary phase (ref. 27). On the other hand separation on a chiral phase, Sumipax OA-2000, has been used to prove the natural occurrence of all three stereoisomers (fig. 9) (ref. 28).
Tunaxanthin (ε,ε-carotene-3,3'-diol) is known to occur in fish as several optical isomers. A system, again with a Sumipax OA-2000 column, was developed for the resolution of all ten stereoisomers, and applied to a fish extract (ref. 29).
Astaxanthin is the chiral carotenoid that is most important commercially. The first separation of the three stereoisomers (*RR*, *RS*, *SS*) was achieved following esterification with camphanic acid. On a silica phase, the three all-*E* isomers can be resolved. On a nitrile-bonded phase also some *Z* isomers may be separated from the all-*E* isomers (Fig. 10) (ref. 30). The chiral column approach to resolving optical isomers of astaxanthin was also reported (ref. 31).

As carotenes cannot be derivatized, their optical isomers may be separated only by a chiral stationary phase. The separation of the optical isomers of α-carotene (= β,ε-carotene) and those of ε,ε-carotene on β-cyclodextrin was reported at this symposium (ref. 32).

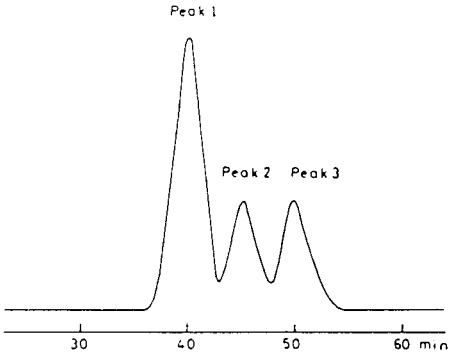

Fig.9. Separation of zeaxanthin stereoisomers on chiral phase (1=3*R*,3'*S* [=meso]; 2=3*R*,3'*R*; 3=3*S*,3'*S*) (ref. 28).

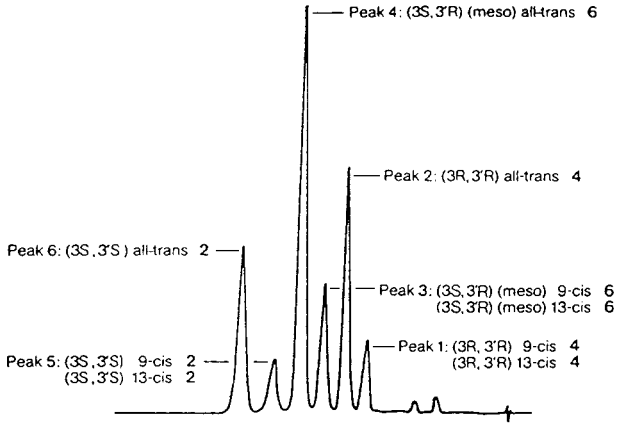

Fig.10. Separation of astaxanthin stereoisomers after derivatization with camphanic acid (nitrile phase; hexane/isopropyl acetate/acetone 76:17:7) (ref. 30).

Some important practical applications

HPLC of carotenoids in human plasma and serum and analysis of carotenoids in food. In both cases it is essential that the investigations can be performed routinely. There is keen interest in quantifying each of the various carotenoids in human plasma because of the potential function of carotenoids as anticarcinogenic agents. Recently Khachik reported methods for detailed separation and quantification of 18 carotenoids as well as Vitamin A in human plasma by HPLC on reversed-phase and silica-based nitrile-bonded columns. In this work the importance of reference samples was emphasized and a list of sources for these compounds given (ref. 5). From the same authors RP HPLC investigations of carotenoids in fruits and vegetables have also been reported (ref. 33).

Identification

The examples have shown that HPLC is a very powerful analytical method. Almost any separation problem can be solved and many trace carotenoids have been detected. With these minor amounts the identification, *i.e.* the structure elucidation, becomes more difficult. These difficulties must not weaken the criteria for structure elucidation.

In principle a chromatogram is reproducible if all the parameters are identical. But experience shows that very often this is not the case. Sometimes it is even difficult to reproduce a chromatographic separation in the same laboratory. Sometimes one can even observe that changes take place during one day. And therefore retention times are not reliable. To postulate a structure based solely on a UV/Vis spectrum and a retention time is *simply not allowed.*

Therefore internal standards and especially reference substances are of utmost importance. For identification the following minimum criteria should be fulfilled:

i) UV/Vis absorption spectrum in agreement with the chromophore suggested.

ii) Chromatographic data in two different systems, preferably R_f (TLC) and t_R (HPLC), including co-chromatography with an authentic sample.

iii) Mass spectrum at least of a quality allowing confirmation of the molecular mass.

To fulfil criterion ii) the two systems must have either different stationary phases or the mobile phases must be significantly different.

For routine analysis these data have to be elaborated at the beginning of the investigation, *i.e.* when working out the separation system for specific problems.

For the full identification of a carotenoid, including *E/Z* geometry, high resolution NMR spectroscopy is indispensable. The structure elucidations in the examples shown previously are all based on NMR spectroscopy. In too many investigations, however, an unknown minor peak has been assigned the structure of a definite *Z* isomer, *e.g.* for lutein, which is unsymmetrical.

Discussion
HPLC is a powerful method, especially when a PDAD can be used. Further development will take place. It can be predicted that the hardware will be improved *e.g.* better sensitivity of the detectors, new stationary phases. But a more sensitive method requires also a skilful operator. HPLC is an experimental method which needs a lot of experience and which has a lot of pitfalls.

SUPERCRITICAL FLUID CHROMATOGRAPHY (SFC)

The technique of SFC goes back to 1962 but, due to technical problems, the method fell into disuse. In the past few years activities in the field have rapidly increased, mainly because new apparatus for SFC is available commercially, developed by different manufacturers.

General Remarks
In general, SFC is very similar to HPLC. The only difference is that in SFC a supercritical fluid is used as mobile phase instead of a liquid as in HPLC. A supercritical fluid can be defined by means of a phase diagram (Fig. 11).

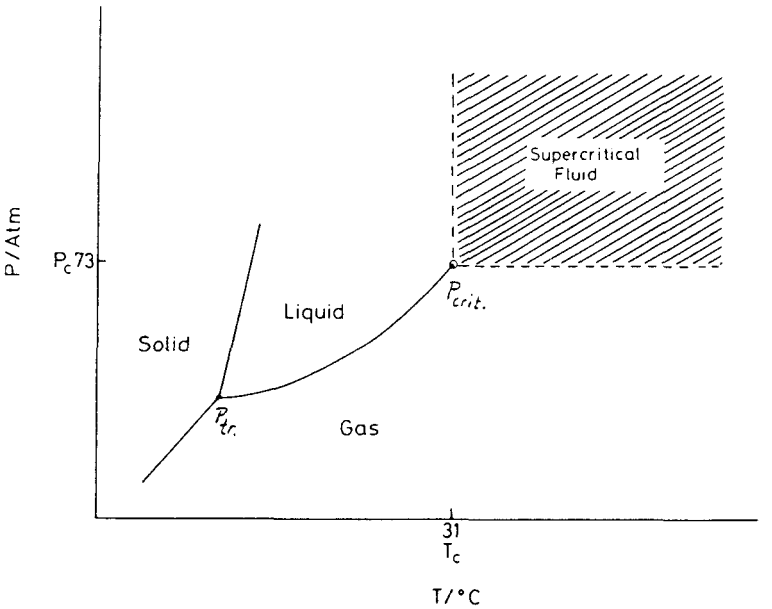

Fig. 11. Phase diagram of carbon dioxide

In the diagram, the areas which are separated by phase borders correspond to the solid, liquid and gas phases. Characteristic points are 1) the triple point P_{tr} where all three phases are in equilibrium and 2) the critical point P_{crit} which is defined by critical temperature t_{crit} and critical pressure p_{crit}. Both points are material constants. Above the critical point is located the 'supercritical area' (hatched area). A supercritical fluid is a unique phase, where liquid cannot be distinguished from gas and the physical properties are intermediate between those two phases. It is obvious that for practical reasons compounds with low critical pressure and temperature are preferred. Generally this is the case for nonpolar substances and that is why supercritical fluids which are normally used are nonpolar. One of the most important properties of the supercritical phases is their ability to dissolve a wide range of compounds, including substances with high molecular mass as well as small molecules. This makes them very interesting as mobile phases in chromatography. The density of supercritical fluids and therefore the solubility and the chromatographic retention of substances can easily be controlled by pressure and temperature.

Instrumentation
As SFC is very similar to HPLC, the instrumentation is very similar to that of HPLC. The mobile phase is transported as a gas or liquid to the oven, where it is heated up to the critical temperature and pumped via the injector through the column and the detector which are adapted from HPLC and GC. Beyond the detector is located a pressure restrictor, which maintains the pressure in the system. At this point some disadvantages of the commercial apparatus have to be mentioned. Apparatus for SFC is expensive compared to that for HPLC. Furthermore, up to now, no detectors have been developed which allow the collection of a fraction. In view of the need for proper identification this is a great disadvantage. Although

HPLC and SFC are quite similar, no modular system has been developed which allows the use of part of the system for both methods.
Both packed columns and open-tubular columns can be used in SFC. The most widely used stationary phases in packed columns are conventional HPLC phases such as silica and ODS. Polysiloxane-based stationary phases are predominant in capillary SFC.
Most substances which are liquid or gaseous at room temperature can be turned into supercritical fluids, but the critical temperature and pressure should not be too high. The supercritical phase most often used is CO_2, followed by pentane and hexane. The main advantages of CO_2 are the low critical temperature and pressure, the non-toxicity, compatibility with most detectors, availability, relatively low price and non-flammability. The polarity of nonpolar eluents and therefore the selectivity of chromatographic separations can be increased by adding polar modifiers; alcohols are used most commonly. The most popular eluent system is CO_2 with methanol as a modifier.

Examples
One of the first applications of SFC for carotenoids was published in 1968 when the separation of α- and β-carotene was reported (Fig. 12) (ref. 34). As mobile phase CO_2 was used and the separation was carried out at 40°C. Recently systematic investigations have been carried out in which the effect of different parameters on the separation of α-and β-carotene was investigated (Fig. 13) (ref. 35).

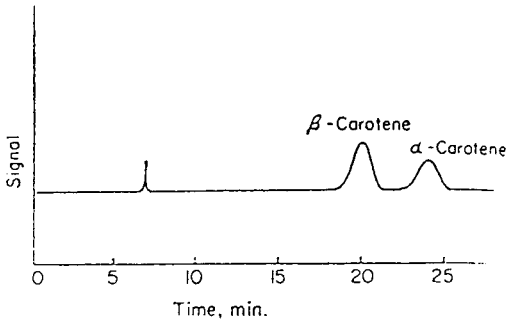

Fig.12. Separation of α-and β-carotene (column: 1.5 m x 0.21 cm ID; CO_2; 500 bar; 40°C (ref. 34).

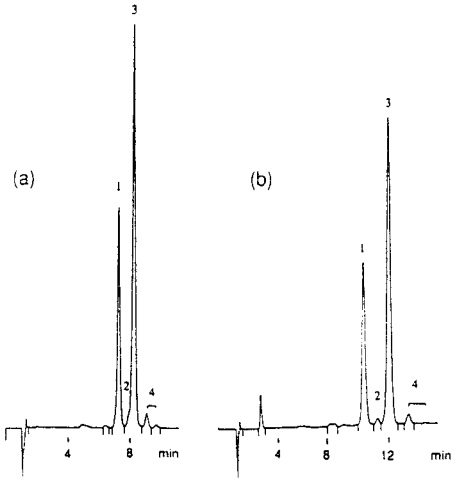

Fig.13. Chromatogram of a carrot extract in CO_2/MeOH: a) 95:5 v/v, 200 bar; b) 85:5, 500 bar (1=(all-*E*)-, 2=*Z* isomers of α-carotene; 3=(all-*E*)-, 4=*Z* isomers of β-carotene) (ref. 35).

The best results were obtained at a temperature of 22-25°C, which represents a subcritical mobile phase.
In another study carotenoids with a wider range of polarity, namely β-carotene, echinenone, canthaxanthin, astacene and fucoxanthin, have been separated: An open tubular column with crosslinked carbowax was used as stationary phase and CO_2 as mobile phase (ref. 36). The pressure was increased from 120 to 300 bar within 30 min. A critical feature in these separations was the applied temperature of 60-80°C, which might cause decomposition and/or *E/Z* isomerization.

Discussion
The efficiency, or the number of theoretical plates achieved in the experiments is about the same for SFC and HPLC. As yet, SFC has not been developed extensively in the field of the carotenoids and, therefore, at the moment, it is not the method of choice. However, supercritical fluids such as CO_2 are an alternative to common solvents for the extraction of carotenoids. This method, supercritical fluid extraction (SFE) is today widely used for extractions on an industrial scale *e.g.* for the decaffeination of coffee. Maybe a coupling of SFE and SFC would be promising for carotenoids.

SUMMARY

Analytical chemistry and separation work may look less attractive and less fancy than other research fields. However, one should not forget that isolation is the basis for structure elucidation, and structure elucidation is the basis for any research in the field of carotenoids. HPLC is a mature method which is used routinely whereas SFC has not yet made an important contribution.

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